INVERTASE ACTIVITY IN RELATION TO PHYSIOLOGY AND BIOCHEMISTRY OF THE PINEAPPLE PLANT

THESIS SUBMITTED

TO THE

UNIVERSITY OF CALICUT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE

FACULTY OF SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled **Invertase Activity in Relation to Physiology and Biochemistry of the Pineapple Plant** submitted by Sri. S. Unnikrishnan in part fulfilment for the award of the Ph.D. degree of the University of Calicut is a bonafide record of the research work carried out by him under my supervision and guidance. No part of the present work has formed the basis for the award of any other degree or diploma previously.

Dr. S. NANDAKUMAR

Dedicated to

My Father (late)

and

My Mother

DECLARATION

I hereby declare that the thesis entitled **Invertase Activity in Relation to Physiology and Biochemistry of the Pineapple Plant** submitted by me for the award of Doctor of Philosophy of this University is an original research work carried out by me in the Department of Botany, Calicut University. No part of this work has formed the basis of the award of any other degree or diploma.

S. UNNIKRISHNAN

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ACKNOWLEDGEMENTS

With great pleasure the author records his deep indebtedness and gratitude to Dr. S. Nandakumar, Professor of biochemistry, Department of Botany, University of Calicut, for his invaluable guidance and supervision of the present investigation. The author is highly thankful to Dr. K. Unnikrishnan, Professor and Head of the Department of Botany, University of Calicut, for providing the facilities needed for the work. The help and assistance rendered by Professor B. K. Nayar and Professor K. S. Manilal, former Heads of the Department of Botany, are also thankfully acknowledged.

The author is deeply indebted to Prof. P. S. Krishnan, F. N. A., the erstwhile Emeritus Professor of Biochemistry, University of Calicut, for his interest and constant encouragement throughout the period of research and his invaluable help in the preparation of the dissertation.

The author is thankful to Dr. E. Nabeeza Salim, Lecturer in Botany, University of Calicut, for the helps extended by her throughout the period of research.

The author is also grateful to Dr. Zachariah Varghese, Prof. of Biostatistics, Department of Zoology, University of Calicut, for suggesting the method of statistical analysis of the data.

The author records his sincere thanks to all research scholars of the Department of Botany, particularly of the Biochemistry laboratory, for their help rendered during the various stages of the present work. The co-operation extended by the research scholars, especially in the Department of Zoology, Chemistry, Physics and Life Sciences, University of Calicut, is remembered with gratitude.

The awarding of the Junior Research Fellowship and a Senior Research Fellowship to the author by the University of Calicut during the course of investigation is thankfully acknowledged.

The author records his sincere thanks to the Kerala Agricultural University, Trichur, Kerala, for providing authentic pineapple propagules as planting material for cultivation and raising of research plants for the investigation.

The author thanks all the members of the staff of the Department of Botany, Zoology, Chemistry, Life Sciences and especially the Instrumentation Centre, University of Calicut, for their help, directly or indirectly, during the hours of need.

The author is thankful to the University of Kerala, for the permission granted to him for the continuance of research as a part-time scholar in the University of Calicut and for providing the necessary leave for study purposes, which enabled the author to the successful completion of the pending experiments.

Thanks are due to Dr. Susan Abraham, Professor and Head of the Department of Botany, department of Kerala, for the timely help rendered by her which enabled the author for the early submission of the thesis.

The help rendered by Dr. P. N. Unni, Scientist, CWRDMM, Calicut, Kerala, is also thankfully remembered.

The author records his thanks to the Rubber Research Institute of India for providing the necessary laboratory facilities, and especially to Ms. Meenakumari, T., Research Fellow of the Division of Botany, extended to the author, in times of need.

Finally the author thanks his brother Sri. S. Venugopal and sister Ms. Sreedevi, for their understanding and whole hearted co-operation which enabled the early completion of the investigation.

S. UNNIKRISHNAN

CONTENTS

GENESIS

Importance of the pineapple plant and distinctive characteristics

The author's studies on invertase were on the pineapple plant (*Ananas comosus* (L.) Merr.). The pineapple fruit is one of the important commercial fruits in the world.In recent years, India has entered the world market as an exporter of the fruit and its products.In India, the Kerala state occupies a special position in the fruit production.

The pineapple plant (family Bromeliaceae) is monocarpic in the strict sense and has xerophytic characteristics. In Nature, it is probably polycarpic, since ratoon growth permits indefinite growth and flowering.

The horticultural varieties of the pineapple plant, with parthenocarpic fruit, are propagated vegetatively, employing axillary outgrowths (or crowns). In respect of propagation in this manner, the pineapple propagule is analogous to the vegetative, cuttings (Jackson, 1986), but differs by possessing not only a number of leaves, but also a few roots, some emerged, ("aerial roots") but mostly as buds embedded in the tissues. The process of rooting ,without exogenously supplied auxin, takes a few weeks, and offers considerable scope for a critical study of the biochemistry of rooting and the interaction among the five known growth factor groups; it offers, also, an ideal system to explore any biochemical changes attendant on the transformation of the aerial-to the terrestrial root.

The leaves of the pineapple plant are characterized by a distinct achlorophyllous region at the base separated from the chlorophyllous region by a diffusely pigmented region.The green part contains water accumulating cells, which, probably, play a role during the stress imposed by the rooting process. The leaves are a rich source of the pyrophosphate-specific phosphofructokinase, in addition to the conventional ATP-linked enzyme (Carnal and Black, 1983). They have the Crassulacean type of metabolism, but with soluble neutral sugars as the storage products, in the place of starch, in the light (Carnal and Black, 1989). An outstanding feature of the leaves is the virtual nonexistence of senescence, suggesting that ethylene is not produced in sufficient amounts, or that ethylene action is antagonized by kinetins and auxins.

A remarkable, and probably unique, characteristic of the transition of the vegetative-to the reproductive shoot apex is the ease with which it can be induced ("forced") by exogenous ethylene (or ethylene-generating chemicals) and the fact that this induction can be brought about also in chronologically, and probably also physiologically immature plants. Apparently, receptor sites and acceptor molecules are always available. The mechanism of flower induction by exogenously applied ethylene is not clear; ethylene functions as the senescence-inducing hormone in plants in general.Does the supplied ethylene elicit its autocatalytic augmentation by activation of ACC (1-amino cyclopropane-1-carboxylic acid) synthase, the rate limiting enzyme in the pathway of ethylene biogenesis? In this connection, it has to be borne in mind that the pineapple fruit is one of the better known non-climacteric fruits and that absence of autocatalysis is the most important criterion distinguishing

non-climacteric from climacteric fruits. It would be of interest to examine any enhanced generation of free radicals such as superoxide and hydroxyl, which can convert ACC to ethylene. The forcing of flowering in pineapple can be effected also by auxin. Does auxin function by promoting ACC synthase activity and the enzyme (system) forming ethylene from ACC?

Among all plant species, the pineapple plant is outstanding in the reprogramming, under normal conditions, of the reproductive-to the vegetative apex, a reversion which it shares with a few other plants (Atherton, 1987). In the ultimate analysis, the reversion is genetically determined, but the immediate agency for the triggering action is apparently a hormone or a hormonal balance. The pineapple plant offers an ideal research material to explore the reverse transition from the reproductive to the vegetative state.

The non-climacteric nature of pineapple fruit suggests that autocatalytic formation of ACC does not occur in the fruit tissues, possibly by a block in ACC-synthase. This is rather unexpected, because the vegetative shoot apex is responsive to autocatalysis through exogenous ethylene. Recent studies indicate that ripening and senescence are genetically distinct stages in fruit development.There is reason to believe that on biochemical grounds, senescence in pineapple fruit is distinct from ripening.

INTRODUCTION

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INVERTASE

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INVERTASE

Literature

Invertase (ß-fructosidase; ß-D-fructofuranoside fructohydrolase, E C 3.2.1.26) in higher plants has been reviewed by Gottschalk (1958), ap Rees (1974, 1984), Avigad (1982), Duffus and Duffus (1984), Dey and Del Campillo (1984), Hawker (1985) and Copeland (1990). Aspects of invertase have been dealt with by Pontis (1977). An early review on yeast and *Neurospora* invertases is that by Lampen (1971).

Reaction(s) catalysed

Invertase catalyses the hydrolysis of sucrose into glucose and fructose:

Sucrose + H_2O -----> glucose + fructose

$\Delta G'$ = -27.6 kJ at pH 7.0.

The large change in free energy and the dominance of the equilibrium position by water rules out invertase as a means of significant synthesis of sucrose in plants. The enzyme has been shown also to effect fructosyl transfer in varying degrees (Cairns, 1987).

Sucrose cannot, by itself, directly participate in respiratory metabolism; invertase by its action gives rise to hexose, which, apart from functioning as the respiratory substrate, is the central source material for practically all cellular components.

Occurrence of invertase

The enzyme is wide-spread in the vascular plants; it occurs in both photosynthetic and non-photosynthetic tissues. Different tissues of a plant vary widely in their invertase activity. In a given tissue, marked changes in activity may occur depending on the physiological status of the tissue.The changes so occurring provide valuable insight into the physiological role played by the enzyme.

Forms of invertase

Invertase activity is not due to a single protein.It is customary to distinguish between two main types:

a. those with pH optima in the range 3.5 to 5.1, acid invertases

and

b. those with optima in the range pH 7.0 to 7.8 alkaline (neutral) invertases.

The distribution of the two types is closely related to the intracellular pH and extracellular pH, considered in a following section.

Another distinction is between:

a. soluble invertase, whose activity is readily extracted on cell rupture in aqueous media and

b. insoluble or extracellular invertase, whose activity resides in the cell wall fraction on differential centrifugation of aqueous homogenates.

The wall-associated type is an acid invertase. Wall association of enzymes and their solubilization are being considered in a later section.

As will be evident from subsequent sections, acid invertase occurring intracellularly and extracellularly can each be made up of a group of enzymes.

A. BIOCHEMISTRY

Localization

To understand the physiological significance of an enzyme, it is necessary to know the localization of:

i. the enzyme and

ii. its substrate (ap Rees, 1987).

i. Enzyme

The characteristically low pH optimum for acid invertase suggests that it operates in the

(a) vacuole, or (b) the free space, or (c) in both.

a. The isolation of vacuoles presents considerable difficulties in view of the fragility of the tonoplast. With standardization of the technique of enriching the vacuolar fraction/isolation of vacuoles, by mechanical means from cells or from protoplasts, it has been possible to demonstrate that acid invertase is located in vacuoles, in the storage tissue. Leigh, ap Rees, Fuller and Banfield (1979) utilized the fact that the red pigment betanin of the storage roots of beet root is confined to the vacuoles to isolate these organelles using a mechanical technique. Vacuoles were isolated from (a) mature tissue which contained large amounts of sucrose and little detectable acid invertase and (b) thin slices washed by continuous circulation in aerated water over three days when there was a marked increase in acid invertase activity and decrease in sucrose content. It was found that in beet root most of the sucrose and much of the acid invertase are in the vacuoles. Determinations in washed slices demonstrated an inverse relationship between sucrose content and acid invertase activity.

That in sucrose storing tissues, vacuolar invertase activity is either inversely proportional to the sucrose content, or virtually absent was concluded also by Thom, Maretzki and Komer (1982), using sugarcane suspension culture cells.

Acid invertase was 83 per cent vacuolar in preparations from tobacco suspension culture (Boller and Kende, 1979).

Techniques of isolation of vacuoles have been worked out, enabling studies of the biochemical composition of the contents. Isla, Leal, Vattuone and Sampietro (1992) prepared protoplasts from potato by digesting macerated slices with

commercial cellulases. The digest was filtered through gauze and the filtrate centrifuged at 270 x g for 5 min to sediment the protoplasts. Vacuoles were obtained by subjecting the protoplasts to osmotic shock and sedimenting in low gravity fields, which eliminated contamination with other organelles. The vacuolar preparation, obtained in yield of 61% of protoplasts, was homogeneous, as revealed by vital staining with neutral red and brilliant cresyl blue. Freedom from contamination with extravacuolar cell components was shown by the absence of marker enzymes specific for such material.Also, lysates from the preparation contained the major part of cellular ß-galactosidase (87%) and acid phosphatases (85%), considered as vacuolar markers. The soluble acid invertase of potato tuber was recovered in 82% yield from this preparation, indicating that it is a vacuolar enzyme.

Somewhat opposite findings have been made by a few workers. In a recent study on the vacuolar localization of (sugars and) sucrose transforming enzymes, Echeverria and Valich (1988) found that in protoplasts from Valencia orange juice sacs, acid and neutral invertases were exclusively located in the cytosol fraction, no activity being found in the isolated vacuolar fraction. Sucrose in this tissue was entirely vacuolar. The authors considered that the acid invertase in the cytosol was nonfunctional in view of the unfavourable pH.

b. There is substantial evidence that in many plant tissues, some of the acid invertase is located in the free space. it is often found that appreciable proportions of acid invertase in plant tissues are recovered in the cell-wall fraction on differential centrifugation of homogenates, suggesting cell wall

location of the enzyme. In sugarcane, the properties of the cell wall enzyme differ from those of the soluble enzyme.It has also been established that in sugarcane internode and some other tissues, the apoplastic uptake of sucrose is preceded by extracellular hydrolysis.

In addition to localization study by biochemical methods, histochemical method has been employed for general aspects of invertase localization (Krishnan, Blanchette and Oikita, 1985). Using a coupled glucose oxidase and nitrotoluene tertrazolium reaction, the deposition of insoluble formazan product indicated invertase activity.By this method, free-hand sections of dark-grown wheat coleoptiles were shown to contain both cell wall and cytoplasmic invertase. Furthermore, higher levels of invertase activity were found to be associated with the vascular bundles compared to the bulk parenchyma cells, supporting the role of the enzyme in sucrose translocation and utilization.

In conclusion, it may be stated that acid invertase has, in general, a dual location in plant tissues and that the relative proportion in the vacuole and free space varies with tissue and the state of its differentiation.

ii Substrate (Sugars)

Sucrose

The vacuolar localization of sucrose in sugar beet was referred to (Leigh *et al.*, 1979). Sucrose is exclusively located in the vacuoles in sugarcane suspension

culture cells (Thom *et al.*, 1982) and in citrus fruit protoplasts (Echeverria *et al.*, 1992) and Valencia orange protoplasts (Echeverria and Valich, 1988).

Hexoses

In Valencia orange juice sacs, about 75% of each of glucose and fructose was present in the vacuole, the remaining 25% being located in the cytosol (Echeverria and Valich, 1988). This distribution differs from that of apple fruit flesh and that of tulipa leaf, in which 100% of both hexoses is found within the vacuole (Wagner, 1979; Yamaki, 1982). However, in *Heppeastrum* petals, the percentage of glucose and fructose in the vacuoles was found to be 81 to 85% respectively (Wagner, 1979). It is interesting to note that in tulipa petals, 100% of the glucose was found to be in the vacuole, but only 50% of the fructose was within the same compartment (Wagner, 1979).

The standardization of the preparation of homogeneous, uncontaminated vacuoles from potato tuber protoplasts by osmotic shock enabled Isla, *et al.*, 1992) to study also the localization of sugars. (Mannitol was employed in place of sucrose as the medium for the preparation of protoplasts). The sugars were composed of sucrose, glucose and fructose. Sucrose was the most abundant component, followed by fructose whose concentration was about a third that of the disaccharide, but twice that of glucose. The major part of the protoplast sucrose, 65% was in the vacuole; the content of glucose and fructose was substantial, being 42% and 40% respectively of the protoplast content. The vacuolar content and the proportion of the sugars were not altered when vacuoles were incubated for 30 min at 4°C; this indicated that:

(a) no loss of sugars occurred during vacuole's isolation and (b) sucrose was not hydrolysed during the isolation of vacuoles (which the authors assumed to be due to product inhibition of invertase).

There is reason no believe that substantial amounts of sugars are present also in the apoplast (Avigad, 1982; Getz, 1991).

The occurrence together of invertase and hexose (into vacuoles and apoplast) has considerable significance in the light of a possible regulation of the enzyme by its reaction products.

Intracellular pH

The importance of intracellular pH in cell activity was reviewed by Kurkdjian and Guern (1989). Large transmembrane pH differences are built across membranes such as the tonoplast (typically, pH at the cytoplasmic side 7.5, pH at the vacuolar side 5.0) and the thylakoid membrane in the light (typically, pH inside the thylakoid 4.0, pH in the stroma 8.0). The cytoplasmic (cytosolic) pH is maintained at relatively constant values close to neutrality (range pH 6.7 to 7.9), despite the existence of pH-perturbing processes. The situation is quite different for vacuoles which generally have a rather variable pH in the range 4.0 to 6.0 with some remarkable exceptions as low as 1.0 in some plant species. The maintenance of cytoplasmic pH is understood, but little is known about the *in vivo* regulation of the vacuolar pH. The wide variation in the vacuolar pH suggests that, contrary to the requirement for

a well-controlled homeostasis of the proton concentration in the cytoplasm, plant cells can sustain large variations in their vacuolar pH.

In keeping with the marked difference in pH between the vacuoles and the cytosol, intracellular acid invertase is localized in the vacuole and the neutral invertase in the cytosol.

The vacuole of CAM plants undergoes dramatic pH shifts, from about pH 6.0 in the light to about 3.5 in the dark, that is, acidification by nearly 3 pH units, owing to the accumulation of malic acid and protons in the vacuole (Luttge and Ball, 1979; Luttge, Smith, Marigo and Osmond, 1981). The transport of L-Malate^{2-} is accompanied by 2 H^+ and is coupled to the activity of the tonoplast H^+-ATPase.

Acid invertase has, in general, fairly sharp pH optimum at pH 5. Vacuolar acid invertase in CAM plants can, therefore, be expected to manifest considerable variations in endogenous activity, depending on the prevailing pH, other factors remaining constant. Vacuolar acid invertase in the leaves of the pineapple plant is, thus, subtly regulated by the ambient pH.

Non-enzymatic hydrolysis, by the acidity in the vacuole, cannot be expected to play a significant role in sucrose metabolism in CAM plants, as it does in mature acid lime fruits, in whose vacuoles the pH value is as low as 2.1 (Echeverria, *et al.*, 1992).

Extracellular pH

In tissues in which the uptake of sugars is in the form of hexoses, extracellular invertase plays an essential role, by hydrolysing the phloem-transported sucrose. This aspect is being considered in a later section. The pH and ionic conditions in the apoplast have been reviewed by Grignon and Sentenac (1991). The relative volume of the apoplast is very small, 5% or less of the tissue volume of aerial organs and of root cortexes. Apoplastic pH has been variously reported, ranging from 4 to 7, but more often from 5.0 to 6.5. Apoplastic pH is higher for dicotyledons than for monocotyledons and higher for angiosperms than for gymnosperms.

In support of the physiological significance of a pH optimum as low as 3.1 for the cell wall invertase of beet root, Getz (1991) referred to reported finding of cell wall pH of 3 to 4 using an apoplastic fluorescent tracer.

Cell wall proteins

The topic of cell wall proteins has been reviewed by Cassab and Varner (1988). Cell walls are comprised of cellulose, hemicelluloses, pectic compounds, lignin, suberin, proteins and water. Primary cell walls, the first to be formed by the cell, from dicotyledonous plants contain 5-10% protein. The protein component of cell wall may be either structural or enzymic. The best characterized structural protein is extensin, which is a hydroxyproline-rich glycoprotein, covalently bound and which is believed to play a role in cell wall architecture. A number of enzymes

are associated with the cell walls-peroxidases and hydrolases, inclusive of invertase; however, we cannot yet distinguish enzymes in the wall or from those in the wall space. The "lytic compartment" of cells is made up of the cell wall space besides the vacuoles (Matile, 1975).

Many, but not all, of the cell wall enzymes have a dual localization, being present also intracellularly, in particular, in the vacuoles.Cell wall enzymes presumably play a role in modification of substrates in muro and in defence mechanisms.Invertase was for long considered particularly important in plants such as sugarcane and maize, in which substantial sugar movement occurred through the cell wall space and was accompanied by the action of extracellular invertase.Recent evidence, however, indicates that although much hydrolysis is often observed, invertase activity may not be essential for sucrose uptake into either sugarcane stem or maize kernel (Duke, McCarty and Koch, 1991).

The solubilization of cell wall enzymes is being considered separately. These enzymes, usually glycoproteins, can be extracted from the wall to varying degrees.Of all glycoproteins, peroxidase is the most studied.It has to be borne in mined that changes in the conformation of wall enzymes are likely to occur on solubilization.At low ionic strength, wall-bound acid phosphatase shows negative cooperativity, while in free solution it shows Michaelis-Menten kinetics (Grignon and Sentenac, 1991).

It has been proposed that extension is initially present as a salt-extractable glycoprotein, but is slowly insolubilized in the cell wall by a covalent link (Cassab and Varner, 1988). Reports exist that cellwall associated invertase occurs in both

salt-extractable and EDTA-extractable forms in same tissues (Ranwala, Suematsu and Masuda, 1992). It is not clear whether in these instances distinct invertases exist in the cell wall, or the same invertase occurs in the process of change in mode of attachment to the wall.

Release of wall-bound invertase

The activity manifested by a fine dispersion of well-washed cell-wall fraction may be taken as representing the maximum potential activity of the cell wall.With this as the base value, the activity solubilized by a particular treatment can be calculated as the percentage of activity extracted. Enzyme bound by ionic interaction can be solubilized by treatment with high molarity salt solution (1 M NaCl); the enzyme more firmly bound by chelation can be extracted with chelating agent such as EDTA.

It was already pointed out that a wall-associated enzyme may undergo change in kinetic properties on solubilization as a result of conformational changes. Also, that the distinction between ionic linkage and covalent linkage may not be rigorous, since extensin attachment may be ionic initially, but, later, changes to covalent linkage.

Purification of acid invertase

Soluble enzyme

Typical examples will be described.

1. Potato tuber

Potato tuber, a typical storage tissue, would have been expected to contain the alkaline form of invertase,but the acid form is prominent.

The presence of invertase in potatoes was first reported over 80 years ago,but it was only in recent times that purification could be achieved. The most satisfactory method is probably that of Bracho and Whitaker (1990 b). The starting material was the tuber which had high invertase activity and low inhibitor activity and which had been stored in the cold for 3 to 4 weeks, a step which resulted in about 3-fold enrichment with respect to enzyme activity.

The purification schedule is summarized below:

Fraction	Specific activity units/mg protein	Recovery of activity (%)	Purification (-fold)
Crude Extract	0.042	(100)	(1.0)
(a) Con A-Sepharose 4B	0.46	110	11.0
(b) DEAE-Sephadex A-50-120	8.10	69	193
(c) G-150 Sephadex	8.8	51	210
(d) DEAE-Sepahadex A-50-120	65.5	29	1560

The following may be noted:

1. The crude extract, the starting material, was obtained after centrifugal removal of the cell wall fraction. Since cell wall fractions of plant tissues retain only a comparatively small proportion of the total protein in

homogenates, the protein in extract may be taken as a rough estimate of the tissue protein.

2. Affinity chromatography with Concanavalin-A, which binds mannose, effected only 11-fold enrichment. The recovery of activity was 10% in excess of the starting material, suggesting release of inhibitor from the enzyme inhibitor complex present in the crude extract.

3. DEAE-Sephadex A-50-120 chromatography was carried out twice, the second being after G-150 Sephadex filtration as an intermediate step.

4. The second DEAE-Sephadex A-50-120 chromatography yielded the enzyme as a homogeneous protein as indicated by SDS-PAGE and by regular PAGE. The enzyme was 1,560-fold purified, with 29% recovery. SDS-PAGE of the purified enzyme gave a single band, with an estimated *Mr* of 30,000 on the basis of mobility relative to that of standard calibration protein. The native *Mr* was estimated to be about 60,000 by Sepahadex G-150 chromatography. This indicated that potato invertase was composed of two identical molecular weight polypeptide chains.

Burch, Davies, Cuthbert, Machray, Hedley and Waugh (1992) reported purification of soluble acid invertase from tuber and leaf of potato. Following precipitation with ammonium sulphate 30 to 80% saturation, the enzyme was subjected to Phenyl-Sepharose treatment, Concanavalin-A Sepharose chromatography and methyl mannoside elution and metal-chelating Sepharose (for tuber only

to remove patalin, the major storage protein) and Sephadex G-75 gel filtration. A *Mr* of 60,000 was obtained for each invertase on gel filtration. Under denaturing conditions (SDS-PAGE), the purified invertase was found to be associated in each case with a 58,000 *Mr* polypeptide. These results indicated that the purified invertase was associated in each case with a 58,000 *Mr* polypeptide. These results contradict the finding of Bracho and Whitaker (1990 b) that potato invertase is a dimer of two 30,000 subunits.

Soybean hypocotyl acid invertase activity, with peak of activity at 5.0, was concentrated in the 50-70% (particularly 60-70%) range of ammonium sulfate saturation (Chen and Black, 1992).

Wheat

Krishnan, *et al.*, (1985) purified soluble acid invertase from wheat coleoptile 4 days seedlings to near homogeneity. The overall purification was 175-fold, with a recovery of about 26% from the starting material, which was an ammonium sulfate (75% saturation) precipitated protein fraction, from tissue extract in buffer (31,000 x g, 20 min, supernatant from 3,000 x g, 20 min, supernatant of homogenate).A major step in the purification schedule was the utilization of Concanavalin A-Sepharose affinity chromatography, following DEAE-Cellulose chromatography, which step alone resulted in about 100-fold purification. The elution from the concanavalin A-Sepharose column was with 2-methylmannoside. This step was followed by filtration on a Sephacryl S-300 column, standardized with the standard proteins of known molecular weight.Analysis of the peak eluted from this column

gave a specific activity of 1,305 units/mg protein. Molecular weight was 158,000. Analysis by SDS-PAGE indicated a major polypeptide band at 53,000, indicating that wheat coleoptile invertase was a multimeric protein, possibly a homotrimer.

Leaf

(1) Potato

The purification of potato leaf by Burch *et al.*, (1992) was reported along with that of the tuber.

(2) Citrus

The citrus leaf enzyme was reported by Schaffer, (1986).

Cactus fruit

The purification of invertase from *Opuntia ficus-indica* fruits (Ouelhazi, Ghrir, Diep Le and Lederer, 1992) was of special interest, representing as it does a CAM tissue. The enzyme assay was carried out at 60°C, for 5 min. The starting material for purification was the fruit juice which was centrifuged at 14,000 x g for 15 min to give the crude extract. This was subjected to the DEAE-Sephadex A 50 batch adsorption, DEAE-Sephadex A 50 column chromatography and Concanavalin-A Sepharose affinity chromatography. A 100-fold purification was achieved, with 15% yield and with specific activity of 1,210 units mg^{-1} protein. SDS-PAGE resolved one major diffuse band at 54,000. This band exhibited invertase activity in native gel electrophoresis, indicating that it is a monomeric protein. The enzyme was optimally

active at pH 4.5. Tests with various α-galactosides and α-and ß-glucosides indicated that it has absolute specificity toward sucrose. At concentration of 1 mM, Na^{+} and NH_4^{+} had no effect. Pyridoxal (10 mM), a general inhibitor of plant invertases (Prado, Vattuone, Fleischmacher and Sampietro, 1985; Pressey, 1968; Pressey and Avants, 1980), was without effect on the cactus enzyme. EDTA (10 mM) and Mg^{2+} (1 mM) stimulated activity. The enzyme was particularly sensitive to Hg^{2+}, which in 1 mM concentration brought about the total inhibition of the enzyme.

The temperature optimum of the cactus enzyme sets it out as markedly different from all other enzymes, inclusive of the pineapple plant enzyme.

Specific activity of acid invertase

Bracho and Whitaker (1990 b) have pointed out that wide ranges of specific activity are found for higher plant invertase from different sources and even isoenzymes from the same organism,as evident from the following comparative specific activities (units/mg protein); invertases from microbial sources are also given, for comparative purposes.

Radish seedlings	: 25 (Faye, Berjonneau and Rollin, 1981).
Lily pollen	: 50 (Singh and Knox, 1984).
Castor bean	: 166 (Prado, *et al.*, 1985).
Wheat coleoptile	: 1,305 (Krishnan, *et al.*, 1985)
Potato	: 65.5 (Bracho and Whitaker, 1990 b).
Neurospora	: 1,820 (Metzenbery, 1963)

Yeast : 4,000 (Neumann and Lampen, 1967).

Such an order of variation is unusual for an enzyme. Presumably, the amino acid composition and sequence are not the same in acid invertase from different sources. This raises the question whether variations in the enzyme protein occur during ontogeny of the plant and whether the enzyme in different organs of a given plant exhibit variation in composition among themselves.

Insoluble

Wheat coleoptile also contains a cell wall-bound acid invertase (Krishnan, *et al.*, 1985) but in a much smaller proportion of the total activity than the soluble enzyme. The wall enzyme could be extracted with high molarity salt solution. This enzyme form was partially purified (DEAE-Cellulose column chromatography), but details were not reported.

The isolation of wall-bound invertase in homogeneous form was reported by Lauriere,Lauriere, Sturm, Faye and Chrispeels (1988) using suspension cultured carrot cells (8 days after transfer), in which 98% of the total activity was in the cell wall extract and only 2% in the supernatant fraction. This distribution may be contrasted with that of the seedlings (7 days old) in which 85% to 90% was in the supernatant fraction and only 10 to 15% in the cell wall extract. The high specific activities of the acid invertase in cell wall extract from the cultured cells and of the soluble invertase in seedling was evident from the figures reported by the authors. For cultured cells, the specific activity (n Kat mg^{-1} protein) was 100 for the wall

extract and 0.5 for the supernatant; for the seedling, the specific activity of the supernatant was 6.5, while for the cell wall extract it was 1.8.

The soluble ß-fructosidase from carrot seedlings (7 days old) was enriched, but could not be purified to homogeneity. The seedlings were ground with fortified acetate buffer. Following centrifugation at 1,000 x g for 5 min, the supernatant, which contained 85 to 90% of the total activity, was precipitated with 40 to 70% saturation of ammonium sulfate. Chromatography of the protein fraction on a carboxymethyl-cellulose column and elution led to the separation of activity in two major peaks, I and II, establishing that the soluble enzyme itself is made up of at least two forms.

It may be pointed out that carrot seedlings and suspension cultured cells did not contain the neutral form of the enzyme. In this respect they differ from carrot roots, which possess both acid and neutral forms of the enzyme (Richardo and ap Rees, 1970).

Lauriere, *et al.*, (1988) did not employ affinity chromatography with Concanavalin-A. However, they pointed out that all forms of carrot invertase (acid) bind to Concanavalin-A-Sepharose, from which they could be eluted with 250 mM α-methylmannoside; but the yield was relatively low.

The ß-fructosidases were examined after SDS-PAGE and immunoblotting. The purified enzyme preparation was used for production of antibodies after deglycosylation. Immunoblotting with antibodies against deglycosylated cell wall ß-

fructosidase showed that there were three molecular forms of ß-fructosidase. The largest form, 63,000, was present in the wall extract of cultured cells and seedlings. The soluble ß-fructosidase of seedlings was made up of *Mr* 58,000 and 52,000, the latter being more abundant. The soluble enzyme in cultured cells had *Mr* of 58,000.

Antibodies raised against the cell wall ß-fructosidase, purified to homogeneity and deglycosylated,cross-reacted with the two soluble enzymes.

Glycan moiety

Treatment of purified cell wall ß-fructosidase from carrot cells with endoglycosidase H (endo-ß-N-acetylglucosaminidase H) resulted in the removal of one high mannose glycan. The *Mr* was reduced from 63,000 to 61,000. Treatment with trifluoromethane sulphonic acid, for total deglycosylation, leading to a *Mr* of 58,000, indicated the presence of 2 complex glycans per molecule of ß-fructosidase. Thus, the enzyme has one high mannose glycan and two complex glycans (which contain fucose). These results were confirmed by HPLC analysis of (^{3}H) Glc NAc- and (^{3}H) fucose-labelled glycopeptides obtained on trypsin digestion of radioactively-labelled enzyme.

Kinetics

a. *Km* value

The *Km* value for sucrose of the acid invertase varies from 2 to 13 mM.

b. Catalytic sites

Acid invertases, in general, are most stable and have maximum activity around pH 5.0. From the pH- activity curve of purified potato invertase, it can be seen that the pH values for half maximum activity are 3.7 and 5.9 (Bracho and Whitaker, 1990 b). This is consistent with the participation of carboxyl and imidazole groups in catalysis. In addition, a carboxy group may also participate in binding.

c. k cat

K cat, moles of sucrose hydrolysed per min per mole of enzyme was 2,940 for date invertase and 1,125 for the potato enzyme (Bracho and Whitaker, 1990 b).

Chemistry

A. Amino acid composition

Lauriere *et al.* (1988) determined the amino acid composition of the pure ß-fructosidase from cell wall of suspension cultured carrot cells. The most remarkable feature of the amino acid composition was the high glycine content (18.6 mol %), with another 13.6 mol % in the glycyl-like residues of Ala and Ser, a common feature of one of the two groups of cell wall-associated proteins (the other group being characterized by high hydroxyproline content).

A subtle difference between the amino acid sequence in insoluble and soluble invertase may be expected, because the polypeptide part of the enzyme carries the

signal for targeting enzyme from Golgi bodies to extracellular or vacuolar compartments in cells.

The amino acid composition of cactus fruit soluble acid enzyme, purified to near homogeneity (Quelhazi *et al.* 1992) showed no similarity to the composition of the insoluble enzyme of carrot (Lauriere *et al.* 1988) The cactus enzyme protein resembled the acid invertase from cultured tobacco cells and yeast enzyme Quelhazi *et al.* (1992) also reported the end group analysis of the enzyme protein.

Where soluble and insoluble enzymes occur together, the more basic protein is usually found in cell wall association (Faye *et al.* 1986).

B.Glycosyl group

This will be considered separately.

Glycoprotein nature of invertase

Although the material presented in this part relates to acid invertase, the author of this dissertation has included a survey also of alkaline invertase in view of the close relationship between the two activities.

Reviewing the biochemistry of invertase in 1974, ap Rees stated that the lack of purified preparations for analysis rendered it uncertain whether the enzyme is associated with carbohydrate in the manner of the external invertase of yeast. Acid invertase from diverse sources,inclusive of monocot and CAM plant has since been

purified (reviewed elsewhere in this dissertation) and their analysis has shown that invertase protein is indeed glycosylated.

The occurrence of carbohydrate grouping is shown by:

1. Staining of the enzyme of SDS-PAGE gels by periodic acid-Schiff's reagent.

2. Phenol-sulphuric acid colorimetry. Assayed by the phenol-sulphuric acid reaction, using sucrose as the standard, potato invertase had a carbohydrate content of 10.9% by weight (Bracho and Whitaker, 1990 b). Date invertase had 8.2% carbohydrate (Al-Bakir and Whitaker, 1978).

A number of enzymes occur glycosylated (Battey, Dickinson and Hetherington, 1993) and it is interesting to speculate whether the availability of carbohydrate could become limiting for the elaboration of enzyme.

It may be pointed out in this connection that the proteinaceous inhibitor of invertase in potato does not contain carbohydrate, as shown by the absence of staining reaction and by not reacting with phenol-sulphuric acid (Bracho and Whitaker, 1990 *b*).

3. Enzymatic/chemical removal of carbohydrate grouping. The study by Lauriere, *et al.*, (1988) was referred to elsewhere. The mannose-containing polysaccharide moiety constituted 3.2% of the invertase, the fucose-containing moiety made up 4.9% of the invertase. Together, the glycosyl grouping accounted for 7.9% of the acid invertase.

4. Binding to lectin

Concanavalin A is a lectin that binds specifically to α-D-mannose- or α-D-glucose-terminal residues of the carbohydrate chains. Methyl α-D-mannopyranoside elutes the glycoprotein from lectin-Sepharose column. Wheat germ agglutinin, another lectin, has affinity for N-acetyl-ß-D-glucosaminyl residues of sugar chains and N-acetyl-ß-D-glucosamine oligomers. N-Acetyl-D-glucosamine elutes the glycoprotein from lectin-Sepharose column.

Concanavalin A-Sepharose affinity chromatography has been a crucial step in the purification of a number of acid invertases. Chen and Black (1992) reported that soybean hypocotyl acid invertase binds to Concanavalin A, but not to wheat germ agglutinin.

The glycosylation status of alkaline invertase is uncertain, largely because pure enzyme preparations have not been available for tests, until very recently. It has been generally assumed that alkaline invertase also is a glycoprotein. Dey (1986) reported that the alkaline invertase of mung bean seeds and seedlings was also bound by Sepharose-Concanavalin-column. Invertase activities in crude extracts of both dormant seeds (predominantly alkaline activity) and 192 h germinated seeds (predominantly acid activity) were found to be totally adsorbed onto Concanavalin A-Sepharose columns; the activities were eluted with methyl mannoside. The alkaline/acid activity ratio in the eluate fractions was comparable with that of the corresponding crude extracts. Contradicting the assumption of earlier workers, Stommel and Simon (1990) reported that enzyme from suspension-cultured carrot

cells did not bind to Concanavalin A, which suggested that the enzyme was without the glycosyl grouping characteristic of acid invertases. Chen and Black (1992) concluded that the electrophoretically homogeneous preparation of alkaline invertase which they isolated from soybean hypocotyl was non-glycostylated, since it did not stain with periodic acid-Schiff's reagent and did not bind to Concanavalin A and wheat germ agglutinin columns, even though an acid invertase purified from the same source responded positively to the staining test and Concanavalin - A binding.

In view of the unexpected nature of the findings by Chen and Black (1992) and their total contradiction of the findings of Dey (1986) and other workers, it is necessary to have independent confirmation by analysis of alkaline invertase purified from other sources. A possibility that the carbohydrate moiety in the alkaline invertase was lost during the enzyme processing was not considered by Chen and Black (1992). Staining reaction would have meaning only when carried out on homogeneous enzyme preparation, but affinity chromatography could be carried out even with intermediate stages of purification and even in the presence of contamination by acid enzyme.

Chen and Black (1992) hypothesized that glycosylation of acid invertase would be necessary for its transport across either the tonoplast or the plasma membrane, which is consistent with the location of acid invertase in the cell wall, apoplast and vacuole; non-glycosylation of alkaline invertase would be consistent with its putative location in the cytosol fraction. However, studies by a number of

authors show that in the higher places it is the polypeptide part, and not the glycosyl moiety, that is responsible for targeting.

Sorting of proteins to the vacuoles of plant cells

Despite its large volume,the fluid-filled vacuole of a typical plant cell contains only 1-10% of the cellular protein (Boller and Wiemken, 1986). The topic of sorting of proteins in the secretory system and the targeting of proteins to the vacuole in plant cells has been reviewed by Chrispeels (1991), Vitale and Chrispeels (1992) Nakamura and Matsuoka (1993) and Faye, Fitchette-Laire, Gomord, Chekkafi, Delaunay and Driouich, 1993).

Proteins destined for the vacuoles, or for the cell surface, are synthesized by polysomes bound to the endoplasmic reticulum (ER), and inserted co-translationally into the lumen of the ER. The signal peptide that allows co-translational crossing of the ER membrane is removed co-translationally in the lumen of the ER. It is then transported to the Golgi complex. Upon exit from the Golgi complex, different transport vesicles direct ("sort out") the protein to the cell surface or the inner compartments. The "secretory pathway" sorts a large number of soluble proteins that either are secreted or accumulated in vacuoles. Secretion is a bulk-flow process that requires no information beyond the presence of a signal peptide necessary to enter the ER. Vacuolar targeting information, which determines that the protein is directed to the vacuoles instead of being secreted, is not contained in the glycans as it is in animal cells. Rather, the targeting information is in the polypeptide

domains as it is in yeast cells. Sorting of vacuolar proteins from secretion appears to operate through mechanisms that are unique to plant cells.

Passage through the Golgi complex

Many vacuolar proteins are glycoproteins and the glycans are often modified as the proteins pass through the Golgi complex. Secreted as well as vacuolar glycoproteins have high mannose as well as complex Asn-linked glycans, resulting from a series of enzymatic reactions in the Golgi complex. The structure: "Glc NAc_2 Man_3 $GlcNAc_2$" is the key intermediate from which all the complex chains can be generated. The most common complex chains found on glycoproteins are very similar in vacuolar as well as secretory proteins. Also, antibodies against the complex chains of carrot cell wall invertase, a secretory protein, recognize the complex chains of vacuolar proteins.These observations indicate that vacuolar and secretory proteins follow the same route in the Golgi complex. Sorting for the different destinations is, therefore, likely to occur when, or after, these proteins exit from the compartment.

Biological role of glycosylation

The precise role of the carbohydrate moiety of invertase is not understood. (Faye *et al.* 1993). However, the carbohydrate component is not necessary for the enzyme activity or for the stability of the enzyme (Faye, *et al.*, 1981; Faye, *et al.*, 1986). As will be evident from the following section, alkaline invertase does not appear to be glycosylated (Chen and Black, 1992).

Proteinaceous inhibitor of invertase

Occurrence

The first evidence for an endogenous proteinaceous inhibitor of invertase in potatoes was obtained by Schwimmer, Makower and Rorem (1961) from kinetic studies on the enzyme in crude extracts. Pressey (1966, 1967) carried out detailed studies on the proteinaceous inhibitor of potato invertase. The inhibitor was purified to homogeneity and characterized; the key step in purification was adsorption on alumina C_8. A partial purification of potato invertase and its proteinaceous inhibitor was reported by Anderson and Ewing (1978). Invertase inhibitors have been found also in red beet and sugar beet (Pressey, 1968) and sweet potato (Pressey, 1968, Matsushita and Uritani, 1976), and maize endosperm (Janes and Nelson, 1971 b). The invertase inhibitor was characterized by Matsushita and Uritani, 1974.

These higher plant invertase inhibitors were all held to be soluble proteins. Their inhibitor activity seemed to be limited to acid invertases.

The most comprehensive studies on the proteinaceous inhibitor of invertase in potato tuber are those by Bracho and Whitaker (1990 *a*, *b*); a brief review is included in the following account.

Molecular weight

SDS-PAGE of potato invertase and inhibitor gave single band each, with *Mr* respectively of 30,000 and 17,000, on the basis of mobilities relative to those of

standard calibration proteins. The native molecular weights of invertase and inhibitor were 60,000 and 17,000, respectively, by Sephadex G-150 chromatography. These results indicated that invertase was composed of two identical molecular weight peptide chains and the inhibitor was composed of one.

The molecular weight of inhibitor, 17,000 was identical with that reported by Pressey (1966).

Acid invertase inhibitor from other plants have similar molecular weight.

Sweet potato	: 22,900 (Pressey, 1968) 19,500 (Matsushita and Uritani, 1976).
Red beet	: 17,800 (Pressey, 1968)
Sugar beet	: 18,000 (Pressey, 1968)

The inhibitor gave a negative test for carbohydrate.

Kinetics

The inhibitor and invertase form an inactive complex. Complex formation required 1 day to reach completion at 2°C and 1 h at 37°C.The kinetics of this inhibition was distinctive in some respects. The binding of the inhibitor to the invertase protein was a slow two-step process (Bracho and Whitaker, 1990 *a*, *b*). Initial interaction was rapid, relatively weak and readily reversible; this was followed by isomerization to a higher complex. The rate of inhibitor binding could be reduced by increasing the ionic strength and by the use of citrate in the buffered extraction

media (Janes and Nelson, 1971 b). In addition, bubbling N_2 through the tissue extract destroyed unbound inhibitor, preventing any further inactivation (Pressey, 1967; Reuveni, Lerner and Poljakoff-Mayber, 1985). Electrophoretic separation of the inhibitor protein from enzyme protein was proposed by Echeverria, *et al.*, (1992).

The optimal pH for inhibitor binding was 4.8, which is similar to the optimum pH for catalytic activity of invertase. The inhibitor activity would thus be limited to acid invertases.

Modulation of invertase by reaction products

There is increasing evidence from *in vitro* studies that acid invertase from many sources is inhibited by the reaction products, glucose and fructose and it has been suggested that hexose plays a regulatory role for invertase activity *in vivo.*

Soluble invertase

The sugarcane leaf acid invertase was competitively inhibited by fructose, Ki 32 mM, and noncompetitively by glucose, Ki 37 mM (Sampietro, Vattuone and Prado, 1980). Isla, Vattuone and Sampietro (1991) conducted detailed studies on the kinetics of invertase inhibition by fructose and glucose. The enzyme preparation used was purified from potato tuber. PAGE revealed a single activity band of invertase,but the preparation was not homogeneous, being contaminated by minor protein bands. Glucose was a classical noncompetitive inhibitor (Ki 1020 mM) whose inhibitory action was not suppressed by proteins. The inhibition by fructose fitted with a case of competitive inhibition by two interacting sites on the invertase (Ki 180 mM, α Ki

330 mM). A plot of the activity of the enzyme against fructose concentration gave a sigmoidal curve. The inhibition by fructose was not suppressed by proteins, as occurs with invertases inhibited in a single way by their reaction products. Thus, fructose could be an important effector, in vivo, of this enzyme. Since about 40% of the cellular fructose in potato tube was in the vacuoles, the hexose would be able to regulate the acid invertase localized in the vacuole. The hydrolysis of vacuole-localized sucrose would depend upon the use of fructose in metabolic reactions.

Invertase from *R. cummunis* and *I. majus* are non-regulatory.

Burch, *et al.*, (1992) studied product inhibition of purified tuber and leaf enzyme of potato. Glucose and fructose both inhibited leaf and tuber invertase activity. The kinetics of inhibition was different for glucose and fructose for a given enzyme; the kinetics differed also between the tuber and leaf enzyme. Product inhibition by fructose was linear mixed for the leaf enzyme and competitive for the tuber enzyme. Glucose inhibition was uncompetitive for the leaf enzyme and non-competitive for the tuber enzyme. The findings for the tuber enzyme agreed with those for fructose inhibition of potato enzyme by Isla, *et al.*, (1991).

The modulation by fructose, as proposed by Isla, *et al.*, (1991), assumes significance in determining the cleavage pathway of sucrose *in vivo*. The action of sucrose synthase generates fructose; an accumulation of fructose would inhibit invertase and promote channelling of sucrose cleavage by the synthase pathway. It has to be borne in mind that sucrose synthase is cytosolic, contrasting with the vacuolar location of invertase.

A regulation by fructose is dependent on its concentration.Avigad (1982) pointed out that free fructose is barely detectable in intact healthy leaves; in storage tissues,roots, developing seeds and in exudates, the level of fructose is often higher. As a metabolic salvage mechanism, any fructose taken into the cytoplasm may rapidly be phosphorylated by hexokinase or fructokinase. The regulation of invertase activity by reaction products assumes added significance in pineapple tissue because of the pronounced diurnal variation in sugar constituents (Carnal and Black, 1989).

Insoluble invertase

Experimenting with invertase from mature red beet (the hypocotyl turned into storage organ), Getz (1991) claimed to have found for the first time a regulatory cell-wall bound invertase from a non-green tissue. The cell wall fraction from "aged" tissue was extracted with high ionic NaCl and the enzyme concentrated by 75% saturation with ammonium sulphate.Using this admittedly crude preparation, Getz (1991) found that the activity was inhibited by the reaction products. Inhibition by α-D-glucose was competitive, with Ki of 12 mM. Inhibition by ß-D(-) fructose was noncompetitive with Ki of 29.5 mM. In the estimated physiological range of apoplastic sucrose concentrations, upto 35 mM, fructose inhibition did not appear to have a physiological significance. Glucose, with lower Ki,might play a role.

Alkaline Invertase

In an early study with crude enzyme preparation, Matsushita and Uritani (1974) found slight inhibition of alkaline invertase of sweet potato by glucose and glucose 6-phosphate.

Alkaline (neutral) invertase

(ß-fructosidase; ß-fructofuranoside frutohydrolase, E C 3.2.1.27).

Distinctiveness

ap Rees (1974) cited the following as evidence of the distinctiveness of alkaline invertase from acid invertase, apart from pH optimum:

1. The two activities are separable. The alkaline enzyme is concentrated in the 20-40% saturation fraction of ammonium sulphate and the acid enzyme in the 60-70% fraction.This separation by conventional salt saturation technique has been confirmed by modern methods such as column chromatography (Schaffer, 1986).

2. The two activities vary independently during development.This was investigated in sugarcane storage tissue and in the roots of carrot and peas.

3. The two have different properties. In general, alkaline invertases has higher K*m* value than acid invertase for sucrose.

In recent times, a number of other differences have been observed. A particularly important difference is the non-glycosylated nature of alkaline invertase, isolated from soybean hypocotyl, an observation which needs confirmation by analysis of purified preparations, from other sources (Chen and Black, 1992). A clinching evidence for the separate identity of alkaline invertase is its immunological distinctiveness from acid invertase from the same tissue.

Localization

ap Rees (1974) cited the following as evidence, essentially circumstantial and negative, for localization of invertase in the cytosol fraction:

1. The pH optimum of the enzyme is close to the pH of cytoplasm.

2. During differentiation of the cells in pea roots, the ratio of alkaline invertase activity to readily extractable protein remains constant. This appears to be a specious argument; there is no theoretical basis for cytoplasmic alkaline invertase activity to vary with total soluble protein in the supernatant fraction.

3. Alkaline invertase activity is always recovered exclusively in the soluble fraction of homogenates on differential centrifugation.

It is necessary to point out that too much emphasis cannot be placed on the finding of the cytoplasmic location of an enzyme. There is reason to believe that in the cytoplasm itself, sub-localization might occur. Enzymes of the glycolytic pathway and gluconeogenetic pathway are both cytoplasmic, but there must be some

mechanisms to permit glycolytic reactions to proceed independently of gluconeogenetic reactions, and *vice versa* as otherwise futile reactions might occur. Sucrose is essentially compartmented in the vacuole, ~~but some sucrose is essentially compartmented in the vacuole,~~ but some sucrose will always be present in cytoplasm along with alkaline invertase. There must be a mechanism to keep the two separate when sucrose breakdown is not needed. Similarly, in the vacuoles, sucrose, regulatory acid invertase and inhibitory fructose/glucose might be subtly compartmented, depending upon the metabolic status of the tissue.

It will be noted that contrasting with the dual spatial localization of acid invertase, alkaline invertase has unitary localization. Also contrasting with the occurrence of possible isoforms of acid invertase both intracellularly and extracellularly, alkaline invertase appears to exist in only one form.

Purification

Alkaline invertase was enriched 2-3 fold from germinating *Phaseolus vulgaris* seeds (cited by Chen and Black, 1992), carrot roots (Ricardo and ap-Rees, 1970) and cultured carrot root cells (Stommel and Simon, 1990). A higher order of enrichment, 39 to 135-fold, was achieved from fresh sweet potato roots (Matsushita and Uritani, 1974), mature sugar beet roots (Masuda, Takahashi and Sugawara, 1987) and infected soybean nodules (cited by Chen and Black, 1992), with specific activities of 2.4, 2.5 and 10.4 μ mol mg^{-1} protein min^{-1}, respectively. Chen and Black (1992) reported,for the first time, the purification of alkaline invertase to electrophoretic

homogeneity, with specific activity of 48 units mg^{-1} protein min^{-1}, the starting material being sprouting soybean. The following is an account:

Step	Specific Activity (U mg^{-1} protein)	Purification (-fold)	Yield (%)
Crude extract	0.49	(1)	(100)
20-40% Ammonium sulfate precipitate	1.10	2.2	53
DEAE-cellulose chromatography	3.20	6.5	33
Green 19 dye chromatography	19.00	40	26
Second Green 19 dye chromatography	38.00	79	17
Blue dye chromatography	48.00	98	11

Note may be made of the fact that affinity chromatography on Concanavalin A-Sepharose column was not a step in the purification of the alkaline invertase. The fold purification (98) and yield (11%) data were not quantitative because of overlapping of acid and alkaline activities in the initial steps. The final purified alkaline invertase had a specific activity of 48 units mg^{-1} protein. The specific activity was higher than that of soybean nodule. It may be pointed out that the specific activity of the alkaline enzyme was much lower than that of acid invertase isolated from a number of tissues.

Molecular weight

Using Sephadex G-200 chromatography, the molecular weight of the native alkaline invertase was found to be 240 kDa. A single band was obtained also in denaturing SDS-PAGE, with an estimated subunit *Mr* of 58 kDa. These data showed that alkaline invertase of soybean hypocotyl is a homotetramer. The *Mr* of native alkaline invertase from plants generally ranges from 60 to 240 kDa (Copeland, 1990).The native *Mr* of alkaline invertase from sugar beet root was 280 kDa and from the leaves of *C. sinensis* was 200 kDa. The subunit molecular mass of other alkaline invertases has not been reported. Western blots of alkaline invertase from a number of plants with the soybean alkaline invertase antibody showed that there was only one type of subunit and that the subunits *Mr* was quite uniform among different plants.

Kinetic properties

a. pH optimum

The pH optimum for plant alkaline invertases lies in the range from 7.0 to 7.8 (Avigad, 1982). The pH optimum for enzyme from soybean hypocotyl (Chen and Black, 1992) was between 7.0 and 7.6 for the purified preparation; it was the same for the 20-40% ammonium sulfate fraction. Schaffer (1986) found that the alkaline invertase from *Citrus* mature leaves was optimally active at pH 6.8.

b. Substrate saturation curve

Tested with the purified enzyme, the activity response to increasing sucrose concentration fitted Michaelis Menten kinetics, with no inhibition even at sucrose concentration as high as 700 mM. (Chen and Black, 1992) The apparent *Km* value for sucrose was about 10mM Alkaline invertase from plants have K*m* values for sucrose in the range 10 to 65 (Avigad, 1982; Cepeland, (1990).Crude preparations of alkaline invertase however, showed biphasic kinetics with sucrose. Chen and Black (1992) concluded that the biphasic saturation curves reported by Matsushita and Uritani (1974) for sweet potato and Masuda, Takahashi and Sugawara (1988) for mature sugar beet root were due to the use of crude enzyme preparations.

c. Substrate specificity

On the basis of the findings with acid invertase,it has been believed that sucrose is a typical substrate for the ß- fructofuranosidase activity of the alkaline enzyme. Schaffer (1986) found the acid invertase of young *Citrus* leaves, separated from alkaline invertase by Sephadex G-200 filtration, acted on raffinose (gal-fru-glu) but not on melezitose (glu-fru-glu). The alkaline enzyme from mature leaves, separated from acid invertase by Sephadex G-200 filtration, acted on both raffinose and melezitose. However, the pH optimum for melezitose hydrolysis was 5, suggesting that melezitose hydrolysis activity was due to contamination with an α-glucosidase.

The homogeneous enzyme isolated from soybean radicle (Chen and Black, 1992) was found to be specific for sucrose, exhibiting no release of hexose from raffinose, maltose, lactose or cellobiose.

Inhibitors

Like acid invertase from several plants, the soybean alkaline enzyme (Chen and Black, 1992) was totally inhibited by 10 µM $HgCl_2$ (*Ki* value 2 µ M). This suggests that a reduced sulfhydryl group is essential for the activities of both enzymes.

Pyridoxal is a general inhibitor of plant invertases (Pressey, 1968; Pressey and Avants, 1980). The *O. ficus-indica* fruit enzyme, however, was not affected by pyridoxal (Ouelhazi, *et al.*, 1992). The soybean enzyme was inhibited by 50µM pyridoxal phosphate (*ki* value 5 µM) (Chen and Black, 1992). The physiological significance of this inhibition is not known.

Tris is a powerful inhibitor of alkaline invertase but not acid invertase (Kato and Kubota, 1978), Schaffer (1986) found that 10 mM Tris, while completely inhibiting alkaline invertase of *Citrus* at pH 7, was without significant effect on the acid activity (6% inhibition at pH 5). The enzyme from soybean hypocotyl (Chen and Black, 1992) was strongly inhibited by 10mM Tris (*Ki* value 4 mM). The mechanism of this reaction is not known.

Isoforms

During enzyme fractionation from soybean hypocotyl, Chen and Black (1992) did not obtain any evidence for multiple forms of alkaline invertase, unlike as in the case of acid invertase from some plant sources (Ranwala, *et al.*, 1992). Isoenzymicity of an enzyme is a mechanism of regulation of its activity. Presumably, the alkaline activity is not endowed with this characteristic.

B. PHYSIOLOGY

Alternative pathway of sucrose cleavage:sucrose synthase

Sucrose synthase catalyses the following reaction.

$$\text{UDP-glucose (or ADP-glucose)} + \text{D-fructose} \rightleftharpoons \text{Sucrose} + \text{UDP (or ADP)}$$

The highlights of the reaction are:

a. the ready reversibility, permitting the generation of UDPG from sucrose and conserving the free energy of linkage in the disaccharide

b. the wide specificity towards the nucleoside base and

c. the location in the soluble phase of the cytoplasm in photosynthetic and non-photosynthetic tissue.

Sucrose biosynthesis:sucrose phosphate synthase and sucrose synthase

Sucrose can be synthesized by the catalytic action of:

a. sucrose synthase, localized in the cytoplasm and

b. sucrose phosphate synthase, also localized in the cytoplasm, followed by sucrose phosphate phosphatase.

Researches along three lines-thermodynamic considerations, labelling studies and enzyme activity determinations-have provided compelling evidence that sucrose synthesis in plants is mediated through the action, predominantly, if not entirely, of sucrose phosphate synthase. This is true not only of photosynthetic cells, but also non-photosynthetic cells. The discovery of the regulation of the enzyme by phosphokinase action (Fallon and Trewavas, 1993; Mackintosh and Mackintosh, 1993). lends further support to the role played by sucrose phosphate synthase.

It follows that the role of sucrose synthase is essentially that of sucrose cleavage.

The synthesis of sucrose takes place in the cytoplasm utilizing triose phosphate moving out of chloroplast. Sucrose is made in the cytoplasm also in gluconeogenic tissues.

Significance of dual pathways of sucrose cleavage

Sucrose is utilized by plants:

1. for generation of hexoses, which enter the glycolytic, pentose phosphate and finally, the Krebs cycle pathways of intermediary metabolism.

Sucrose is utilized by plants:

2. also for polysaccharide synthesis, both storage and structural.

As a general rule, utilization of sucrose in intermediary metabolism,inclusive of respiration, is initiated by invertase action(acid and alkaline); conversion to polysaccharide is via sucrose synthase action. Whereas sucrose synthase pathway conserves much of the energy of the glycosidic bond of sucrose,utilization by the invertase pathway leads to the loss of all this energy.

In addition, there is reason to believe that sucrose is involved in morphogenetic reactions, in which a hormonal mechanism has sometimes been postulated (ap Rees, 1974). The morphogenetic effect may be elicited by provision of respiratory substrate, or transient storage form of carbohydrate, by the action of invertase or/and sucrose synthase. In case sucrose effect is hormonal, the promotor (inducer) has to be eliminated from the site once morphogenesis has been set in motion. This can be brought about by invertase or sucrose synthase; their role would then be an indirect one.

Conversion to polysaccharide

Structural polysaccharide are elaborated mainly via UDP-glucose. Starch, the principal storage carbohydrate, is made via ADP-glucose. Fructosans, storage carbohydrates, do not seem to utilize the sugar nucleotide pathway. Both UDP-glucose and ADP-glucose could be made directly from sucrose by sucrose synthase action

$$\text{Sucrose} + \begin{matrix}\text{UDP} \\ \text{ADP (?)}\end{matrix} \rightleftharpoons \begin{matrix}\text{UDP - glucose} \\ \text{ADP - glucose (?)}\end{matrix} + \text{fructose}$$

The fructose liberated in the process can give rise to UDP-glucose/ADP-glucose after initial kinase action

$$\text{Fructose} + \text{ATP} \xrightarrow[\text{hexokinase/fructokinase}]{} \text{fructose 6-phosphate} + \text{ADP}$$

$$\text{Fructose 6-phosphate} \rightleftharpoons \underset{\text{Phosphohexoisomerase}}{\text{glucose 6-phosphate}}$$

$$\text{Glucose 6-phosphate} \rightleftharpoons \underset{\text{phosphoglucomutase}}{\text{Glucose 1-phosphate}}$$

$$\text{Glucose 1-phosphate} + \begin{matrix}\text{UTP} \\ \text{ATP}\end{matrix} \underset{\text{pyrophosphorylase}}{\rightleftharpoons} \begin{matrix}\text{UDP-glucose} \\ \text{ADP-glucose}\end{matrix} + \text{PPi}$$

$$\text{PPi} + H_2O \xrightarrow[\text{pyrophosphatase}]{} 2\text{Pi}$$

Among the various lines of evidence for the involvement of sucrose synthase in polysaccharide synthesis is the fact that tissues known to be particularly active in converting sucrose to polysaccharide (developing endosperm of maize; developing cotyledons of broad bean, seed of paddy; stele of root of pea) often have a high

maximum catalytic activity of sucrose synthase, contrasting with tissues which are known to synthesize sucrose (shoot of pea; leaves of spinach; leaves of broad bean; scutellum of germinating maize) and which have high sucrose phosphate synthase activity. This correlation has been demonstrated not only for the specific activity, but the actual amount of the enzyme.In developing kernels of maize, sucrose synthase is one of the major proteins, accounting for 2.8% of the total protein.

A significant finding in support of sucrose synthase involvement is the fact that the cells of pea roots receive their carbon for polysaccharide synthesis as sucrose via the symplasm; the transport system, thus, delivers sucrose directly to sucrose synthase.

The most telling evidence for the view that conversion of sucrose to polysaccharide proceeds mainly via sucrose synthase is from tissues where the invertase is very low, while sucrose synthase activity is high, as exemplified by developing cotyledons of broad bean and developing endosperm of maize (Hawker, 1971) and developing grains of barley (Baxter and Duffus, 1973).

Invertase and sucrose synthase activities in tissues

Tissue	Enzyme activity, specific,			Units of activity
	Sucrose synthase	Invertase		
		acid	alkaline	
A. Polysaccharide synthesising tissues				
Developing cotyledons of broad bean	260	3	70	nmol sucrose min^{-1} g^{-1} fresh weight

Developing endosperm of maize	683 9500	170	170	n mol sucrose min^{-1} g^{-1} fresh weight nmol sucrose min^{-1} mg^{-1} protein
Developing grains of braley: 8 days after anthesis	20	15	15	nmol sucrose min^{-1} per grain
28 days after anthesis	620	15	15	nmol sucrose min^{-1} per grain
B. Rapidly developing and respiring tissues				
1) Sugarcane internode Immature (14)		1310	3	nmol sucrose $min^{-1}g^{-1}$ fresh weight
Maturing (25)		12	15	do
Mature (35)		3	90	do
2) Caroot Roots				
20 days old (0.5)		366	9	nmol sucrose min^{-1} g^{-1} fresh weight
40 days old (3.4)		139	12	
55 days old (15.8)		81	46	
Maturing (23)		44	81	
Mature (30)		12	32	
3) Sugar beet roots				
9 days old		500	..	nmol sucrose min^{-1} mg^{-1} protein
12 days old		375		
22 days old [30]		..		
28 days old		10		
42 days old [250]		4		
58 days old [320]		2		
C. Distribution of activity in roots of 5-day-old pea seedlings				

(a) Aprical, 6 mm	19	100	20	nmol sucrose min^{-1} mg^{-1} Protein
(b) Stele, 6-24 mm from apex	34	11	26	
(c) Cortex 6-24 nm from apex	17	91	19	

( ) = Sucrose content, mg g^{-1} fresh weight
[ ] = Sucrose content, mg g^{-1} dry weight

The following are noteworthy:

In the developing cotyledons of broad bean,alkaline invertase is 23-fold as active as acid invertase. This is contrary to the hypothesis that alkaline invertase is characteristically much less active than acid invertase in developing tissues. In developing endosperm of maize, the two invertases have the same maximum specific activity. In developing grain of barley also, acid and alkaline invertase have the same activity.

It may be pointed out, in passing, that several aspects of the transformation of sucrose to polysaccharide are yet to be understood. Among these are (a) the intracellular location of the relevant enzymes and (b) the formation of ADPG needed for starch synthesis.

Provision of respiratory substrate: role of acid invertase

The data reported in the above table, show that rapidly respiring and developing sugarcane internode, carrot root and sugar beet root have high acid invertase activity, which greatly exceeds sucrose synthase activity. High acid invertase activities correlate closely the growth also of epicotyl of lentil and pea, petals of

morning glory, and roots of pea. Most of the acid invertase is probably intracellular. The data reported in the table suggest that in rapidly respiring tissues, conversion of sucrose to the respiratory substrate is mediated essentially by invertase.

Physiological role of alkaline invertase

As the tissues cited develop and sucrose storage takes place, acid invertase progressively falls to very low levels. Both in sugarcane internode and carrot root, alkaline invertase activity, very low in the immature tissue, progressively increases (with an exception) and manifests several fold the acid invertase activity in the mature tissue. This suggests that alkaline invertase, cytosol-located, could take over from acid invertase the role of providing respiratory substrate from sucrose in the mature tissue.

Relationship between sucrose storage, intracellular acid invertase and alkaline invertase

The data reported in the above table, show that sucrose storage in maturing tissue occurs simultaneously with lowering of acid invertase. Sucrose storage is in the vacuole, acid invertase also is located in vacuole. High activity of vacuolar acid invertase ensures that sucrose stored in, or entering, the vacuole is rapidly converted to hexoses. These move into the cytoplasm where the glycolytic enzymes are localized. Following oxidation in the mitochondria, the chemical energy conserved in the form of ATP is used for growth purposes. As growth declines, there is less demand for respiratory substrate, acid invertase drops and sucrose storage occurs in

vacuoles. The basal need for respiration of the mature tissue is met by alkaline invertase. Sucrose moving out of vacuole into cytoplasm will be acted on by alkaline invertase.

Neither pathway is mutually exclusive

On the basis of the occurrence of enzymes, without spatial relationship, it is possible that the invertase pathway could also provide sugar nucleotides for polysaccharide synthesis.The hexoses formed by hydrolysis could follow the pathway indicated for transformation of fructose set free when sucrose synthase acts on sucrose. Similarly, the sucrose synthase pathway, which results in free fructose which can enter the respiratory pathway, can divert the glycosidically linked glucose moiety also into respiratory metabolism by the following pathway (Feingold and Avigasd, 1980):

ADP-glucose + Pi ----------->ADP + glucose 1-phosphate
ADPG phosphorylase

UDP-glucose ----------> UMP + glucose 1-phosphate
UDPG phosphodiesterase

Regional differences in the activity of sucrose metabolizing enzymes in root tissue

The above hypothesis finds support in the distribution of enzymes and metabolic activity in the roots of 5-day-old seedling of pea. Acid invertase activity was very high in the apex and cortex; labelling studies showed that these two tissues had high rate of respiration.In the stele, where acid invertase was much lower, and

sucrose comparatively high, much less of the labelled sucrose was respired and there was a corresponding increase in the proportion stored.

Sugar-Transforming Enzymes in Relation to Sink-Source Transformation in Leaves

With a view to assessing whether sugar transforming enzymes play a key role in the growth of developing leaves, enzymes acting on sucrose, sucrosyl galactoses and polyols have been analysed for their levels of activity (Turgeon, 1989). Particular attention has been devoted to sucrose transforming enzymes, invertases and sucrose synthase.

A. Invertase

a. Invertase activity correlated to growth

In *Phaseolus vulgaris*, a readily soluble acid invertase is most active during the early stages of leaf growth (Morris and Arthur, 1984). The high activity of the enzyme correlates well with the hexose/sucrose ratio at the same period of development.

A similar correlation of acid invertase (soluble) activity and growth is found in developing leaves of *Citrus* (Schaffer, 1986; ———————————.

Schaffer, Sagee, Goldschmidt and Goren, 1987). Of special interest to the present author is the finding that in *Citrus*, invertase has an acid pH optimum in the early stages, but, later, a neutral invertase is more prominent.

In *Lolium*, also, there exists a correlation of acid invertase activity and growth of leaves (Polock and Lloyd, 1977).

Unlike in *Phaseolus* and *Citrus*, the acid invertase of *Lolium*, is also in the insoluble fraction, suggesting that it is extracellular, that is, cell wall-associated.

A role for a cell wall-associated enzyme could be of physiological significance only if the uptake of sucrose by the cell is by the apoplastic route. The preponderance of evidence indicates that phloem unloading in sink leaves is symplastic and, therefore, imported sucrose would not be exposed to the action of wall-associated enzyme.By following the fate of asymmetrically labelled sucrose in sugar beet, it has been found that there is no extracellular hydrolysis of sucrose during accumulation in sink leaves (Giaquinta, 1977). It was concluded that sucrose enters intact the vacuoles of mesophyll cells in sink leaves and is hydrolysed there by soluble acid invertase to hexoses, which traverse the tonoplast back into the cytoplasm, in response to demand (Giaquinta, 1978).

No correlation between invertase activity and growth rate

Contrasting with the correlation of invertase activity and growth in *Phaseolus*, *Citrus* and *Lolium*, the activities of acid invertase in source and sink leaves are approximately the same in sugar beet (Giaquinta, 1978).

As in sugar beet, the amount of invertase (acid or alkaline) is not correlated with development in the leaves of a variety of other species belonging to C_3 and C_4

pathways, nor is there a gradient from lamina base to tip (Claussen, Loveys and Hawker, 1985).

Invertase activity actually increases with declining growth in tomato (Manning and Maw, 1975).

Other lines of evidence

Studies in which invertase activity, in comparison with sucrose synthase activity, was determined as a gradient from lamina base to tip, using (^{14}C) fluorosucrose as a substrate, did not provide support for the concept that invertase activity is characteristic of growing leaves.

In a comparative study of the utilization of sucrose and mannitol in *Celery*, Fellman and Loescher (1987) found that, contrasting with mannitol, sucrose was degraded by both immature leaf and fully grown leaf.

The above brief review will show that no unequivocal conclusion can be drawn as to the role of invertase in hydrolysis of imported sucrose and, especially, in controlling the rate of import. Apart from contradictory data on the relation between invertase activity and growth rate of leaves, considerable uncertainty in interpretation is introduced on account of the facts that:

a. the enzyme occurs in multiple forms

b. invertase is often not a rate limiting enzyme; the reported

levels in leaves are actually higher than that needed for complete sucrose hydrolysis.

c. the timing of changes in substrate and enzyme levels often do not coincide and

d. sucrose metabolism in growing tissues can follow pathway other than hydrolysis to the constituent hexoses.

All the above facts have considerable relevance to the findings of the present author on pineapple tissues.

B. Sucrose synthase

Claussen, *et al.*, (1985), analysing growing, mature and old leaves of a number of C_3 and C_4 plants, found that sucrose synthase activity declined considerably as leaves aged. As already pointed out, invertase activity was not correlated with leaf development in these plants. The authors found also that there existed a lamina base-to-tip gradient, indicating that changes in activity follow the same basipetal pattern as the sink-source transition (Turgeon, 1989). There was no such base-to-tip gradient in acid or alkaline invertase activity.

Schaffer, *et al.*, (1987) observed that sucrose synthase activity declined in *Citrus* leaves with development (-as was the case with invertase-).

In contrast to the above two reports, Giaquinta (1978) found little difference in sucrose synthase activity between source and sink leaves in sugar beet (-as was found also for invertase activity-).

Davis, Fellman and Loescher (1988) observed that in *Celery*, the highest sucrose synthase activity was actually in mature leaves.

Schmalstig and Hitz (1987) determined the relative contribution of sucrose synthase and invertase in leaves by employing as substrate (^{14}C) fluorosucrose, which is cleaved at very different rates by the two enzymes. By providing labelled control sucrose and the gluco-derivative as substrates to source leaf and monitoring incorporation into sink leaves, it was found that all cleavage of sucrose by very young leaves was by sucrose synthase, but the contribution of invertase to metabolism increased to about one-half by the end of the sink period.

To summarize, no positive conclusion could be drawn as to the role of sucrose synthase in sucrose cleavage in developing leaves.

Neither invertase, nor sucrose synthase, seems to be truly regulatory (with apparent exceptions), in marked contrast to sucrose phosphate synthase which is subject to both coarse and fine control (Battey *et al.* (1993).

Metabolic role of the multiple forms of invertase

The role of the multiple forms of invertase in the metabolism of sucrose is not entirely clear.

Present extracellularly, the role of the (acid) invertase is to hydrolyse apoplastic sucrose prior to uptake by the cell. Present intracellularly, distinctive roles of the multiple forms of invertase acid and alkaline forms can only be conjectured, especially in those few cases where the acid form also is cytosolic.

Patterns of enzyme distribution in tissues, particularly in fruits, which provide a clue to the metabolic role are:

a. Sink tissues accumulating hexose sugars often have high levels of acid invertase (Yelle, Chetelat, Doaris, DeVerna and Bennett, 1991; Hubbard, Pharr and Huber, 1990; Hubbard and Pharr, 1991). Results obtained with strawberry fruit support this concept (Ranwala, *et al.*, 1992). Sucrose was barely detectable in the immature green fruit; it appeared in the mature, just turning red stage and thereafter increased steadily throughout development to the over-ripe stage. Soluble invertase which increased steadily from the immature to the red-ripe state decreased markedly in the over-ripe state, when sucrose was maximum. The inference was drawn that soluble acid invertase, by catalyzing the cleavage of sucrose imported into fruit regulated the levels of sucrose and the hexoses.

b. Sink tissues accumulating sucrose predominantly are characterized by the decline of acid invertase activity concomitant with sucrose accumulation (Yelle, *et al.*, 1991; Kato and Kubota, 1978; Yamaki and Ishikawa, 1986; Lingle and Dunlop, 1987; Schaffer, Alani and Fogelman, 1987; Mc Collum, Huber and Cantliffe, 1988, Hubbard, Huber and Pharr, 1989; Ranwala, *et al.*,

1991). Studying post-ripening strawberry fruits, Ranwala, Suematsu and Masuda (1992) found a decrease in acid invertase while sucrose concentration increased.

c. High activities of alkaline (neutral) invertase have been reported in sucrose storing stages of several sink tissues (Kato and Kubota, 1978; Hatch and Glasziou, 1963; Masuda, *et al.*, 1987; Ricardo and ap Rees, 1970).

Of interest was the finding by Ranwala, *et al.*, (1992) that both types of wall-bound invertase decreased continuously with fruit development, almost disappearing in the over-ripe fruit. This was in contrast to the soluble acid invertase which increased continuously up to the red-ripe stage, to drop in the over-ripe fruit. The turnover of intracellular protein is well known, but there is no information about the turnover of wall-bound enzyme protein.

MATERIALS AND METHODS

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CULTIVATION OF PLANT

Selection of variety and propagule

The cultivation of horticultural varieties of pineapple is by vegetative means. The 'Kew' variety is cultivated extensively due to the excellent qualities of the fruit. Hapas dominate over shoots and suckers in 'Kew'; slips production is rare. The use of crowns was avoided since these were not available in sufficient numbers at a time. Plants for the present research were raised from hapas. Hapas start forming when fruit development takes place and continues after harvesting of the mature fruit. Ratoon crop was avoided, since the chronology of physiological and biochemical events may not correspond with those of crop growth.

Although the origins of slip/hapa/shoot/ sucker are different morphologically, the ontogenetic changes undergone by the shoot apex may be expected to be qualitatively the same irrespective of the propagule employed for raising the crop growth. The findings with 'Kew' cultivar may be expected to be qualitatively applicable to the other pineapple varieties, inclusive of those insensitive, or differently sensitive, to photoperiodism and those which produce seeds.

Planting

Authentic propagules were procured from the Kerala Agricultural University, Trichur, and supplemented with the material raised in the campus. The propagules used for planting were of about 3 months age; uniform sized propagules were selected. There was no obligatory period of storage of propagule before planting.

The hapas were planted, adopting the package of practices recommended by the Kerala Agricultural University, in two to three rows and the plants were placed 12 to 15 inches apart both ways. The bed was ploughed well before planting and manured heavily with dried cattle-dung. In order to expose the root buds in the stem and to facilitate root penetration in the soil, a few of the lower leaves were stripped off before planting. Any dried roots at the base of the propagules were also discarded. Mulching was done, after planting, with green leaves applied heavily on the bed. Apart from soil improvement in the long run, mulching minimized weed growth and served to retain soil moisture. Planting was done during the rainy season (June-August). During the hot season, the plants were watered once in a week with hose pipe. The plants were observed every day and care was taken to protect from any pests.

Selection of stages

The studies pertained to selected stages in the growth and development, the ontogeny, of the pineapple plant;

Stage I

The mature propagule collected fresh from mother plant.

Stages II A and II B

Representative of the terrestrial rooting of the propagule, that is, the transition from the semi-autotrophic to the fully autotrophic stage, were studied one month, (II A), and two months (II B), after planting.

Stage III A and III B

The mature vegetative plant, studied 12 months and again at 14 months after planting, when it was "ripe to flower", leading to the next stage:

Stage IV

The transition from the mature vegetative to the reproductive state, under natural environmental conditions (Vince-Prue, Thomas and Cockshull, 1984). This, in turn, led on to the next stage.

Stage V

The transformation of the evoked shoot apex to the organogenetic state.

Stage VI

Immediate post-organogenic stage: inflorescence development.

Stage VII

Advanced post-organogenic stage:early fruit development.

Stage IV was in all probability a rapid change (2 to 3 days) (Collins, 1968). Stage V also was apparently a quick event (Kerns, Collins and Kim, 1936). The subsequent Stages VI and VII, spanned several days.

The identification of the transition stage and the following stages was based on morphological considerations, rather than chronology, since the plants were flowering under environmental conditions, instead of being forced by chemical/hormonal treatment. The plants were in the "transition" state when the first kinked bract appeared (Kerns, Collins and Kim, 1936). This examination was not possible *in situ*; the stem top had to be excised and defoliated. In actual practice, a large number of plants had to be sacrificed in the search for the transitional apex.

The organogenic stage was determined following Evans (1975), Vince-Prue (1975, 1983) and Bernier (1980, 1988).

Collections in Stages VI and VII were based on the height of the inflorescence-differentiating shoot tip.

The sample collection schedule and the distinguishing morphological features are set out in Chart I.

Chart I
Sample collection and distinguishing physiological and morphological features

Stage	Physiological status	Morphological features
I	Heterotrophic Propagule	Leaves 40-55 Roots (aerial) 5-60 mm long; root hairs not prominent; roots numbered 20-40
IIA	Pre-autotrophic, (rooting) One month	Leaves 40-55 Roots 5-100 mm long; root hairs present; roots numbered 30-50
IIB	Two months	Leaves 40-50 Roots 5-300 mm long; distinct elongation of the existent roots; root hairs abundant; roots numbered 50-70
IIIA	Autotrophic Vegetative 12 months, mature	Leaves 70-80
IIIB	14 months 'ripe to flower'	Leaves 70-80
IV	Reproductive Transition	Leaves 70-80 Sudden expansion of the apical dome; first kinked bract
V	Organogenic	Leaves 70-80 One to three kinked bracts; Stem apex projected 0.5-1 cm vertically
VI	Post-organogenic, immediate; Inflorescence	Leaves 70-80; no reddish tinge yet; Rows of kinked bracts in rosette form; Stem apex projected 1-2 cm vertically

VII	Post-organogenic, advanced; early fruit	Leaves 70-80; the top most rows of leaves totally devoid of pigment; leaves below this region characteristically pigmented reddish at the junction between the achlorophyllous and the chlorophyllous part; leaves subjacent to the pigmented leaves, which were collected for analysis, were curved backward and were rigid at the curvature. Rows of bracts in rosette form; Stem apex projected 6-10 cm vertically; A swollen tip, (resembling a small fruit), on the top of the peduncle; eyes similar to those found in fruit.

Emphasis on selected stages

Of the above 7 stages (9 to be more precise), arbitrarily selected, but integrally inter-related, the emphasis was on the transition to flowering in the monocot and, to an extent , on the rooting of the propagule.

The terrestrial rooting study represented an original approach, very little biochemical work having been carried out on adventitious root formation during vegetative propagation (Jackson, 1986).

Sampling of tissues

Twenty four to 48 h prior to sampling, the beds were thoroughly watered and the leaf canopy sprinkled with water. Sample collection was restricted to a definite time of the day, so as to minimize any possible influence of diurnal variations in the analysis. This was fixed in the interval 7.30 to 9.30 a.m., more often 8 to 9a.m. At each of the stages, selected parts of the stem and the 'D' leaves were separately

analysed. With the exception of the propagule, and Stages II A and II B ('upper segment'), one plant each yielded sufficient stem tissue for analyses. The 'D' leaves from a single plant yielded sufficient tissue for analyses in all stages.

Preliminary operations

The whole plant was uprooted from the soil with the aid, when necessary, of a crow-bar. The plants were washed under the tap to free from gross extraneous matter and taken to the nearby laboratory. Here, the stem base with roots was cleaved away. The stem was defoliated in the sequence of leaf origin, starting at the base and plucking off the leaves one by one. The stem was thoroughly washed, first under the tap and then with distilled water, since pockets of soil and manure are common at leaf bases. Because of their turgid nature, the sessile leaves broke off at the base leaving varying amounts of achlorophyllous tissue ringing a major part of the stem. With experience, the residual leaf material adherent to the stem could be kept at a minimum. The leaf base still adherent to the stem was shaven away delicately using a sharp stainless steel blade, without damaging the stem. In rooting studies, in addition to the residual leaf base, the emerged roots were also cut way. For this purpose, a sharp knife was first used for gross removal and then a stainless steel blade to slice away the root stumps flush with the stem. It was considered advisable not to scoop out the root material embedded within the cortex, since this would have damaged the surrounding tissue. Invertase level is known to be influenced by wounding (Matsushita and Uritani, 1974).The stem was washed again with distilled water and surface dried. The leaves selected for analyses were similarly

washed with tap water followed by distilled water and surface dried. The propagule did not require such elaborate cleaning.

A. Stem

Heterotrophic

Stage I (propagule)

The defoliated stem was cut transversely into three segments, somewhat arbitrarily; (a) the upper, (b) the middle and (c) the lower segment.

The hapa stem measured 7.5 to 9.5 cm in length and gradually curved and tapered from the middle part (b) toward the base (c) where the propagule was attached to the mother plant. An interesting feature, which does not seem to have been reported in literature, was the presence of localized greenish patches around the middle part (b) and lower part (c), scattered all around the stem, and which were found, on dissection, to extend deep into the cortex. The pigmentation was more concentrated at the region of leaf attachment to the stem . The upper part of the stem (a) was completely devoid of the green patches. Roots, 20 to 40 in number and ranging from 0.5 to 6 cm in length, emerged from the middle part (b), as well as from the upper regions of the lower segment (c). In rare cases, roots pierced the leaves at the basal region. The reddish pigmentation present in the chlorophyllous part in some of the leaves at mature stages (page 69) was apparently absent in the leaves of the propagules. The region of attachment of the hapa/shoot to the leaf axil of parent plant was woody and roots did not emerge from this region. Prior to

sectioning of the stem for analysis, this woody region was sliced away. There was gradual disintegration of the woody region after planting of propagules in the soil . This region was sliced away, prior to sampling also in Stages II A and II B.

a. Upper: About 1.5 to 2 cm length of stem, sliced at the commencement of the bulge, constituted the upper part; the diameter at the base was about 1.5 to 2 cm. This consisted of, besides the shoot apex proper, quantitatively a minute fraction of the total tissue mass, the tissue below in various levels of differentiation. The cut region exposed adventitious root buds, prominent by their brown pigmentation against a white background, encircling the pith in two or three rows, along the periphery of the stele. The tissue was tender and the preparation of cell-free suspensions from these samples was much easier than from all the other tissues, except achlorophyllous leaf part.

b. Middle: The middle portion was about 2cm in length. As already pointed out, this region had patches of green pigmentation in the propagule stage, but these were absent in Stages II A and II B. It was nearly cylindrical with a diameter of 2 to 2.5 cm. The upper region contained the root buds and the lower region also the emerged roots. The root portion passing through the cortex in b was in general not as thick and leathery as in c. However, a few such thick brown roots were seen in this region also. The tissue in b was somewhat leathery.

c. Lower: The rest of the stem, 4 to 5 cm in length and 2 to 2.5 cm in diameter above and 1 to 1.5 cm in diameter below, at the point of attachment to the mother plant which in Stages II A and II B was below ground, was characterized by brown

pigmentation in the rooting stages. The slight bend, characteristic of the basal portion of the propagule, was prominent in Stage II B.

Pre-autotrophic

Stage II A (1 month old)

a. Upper: The processing was as in the case of the propagule.

b. Middle: The dimensions remained practically unaltered. The tissue was somewhat leathery; hence much effort had to be expended during homogenization. This segment was completely above the ground.

c. Lower: The dimensions remained more or less unaltered. The segment c was completely below the ground level. The tissue in this segment was quite brown and highly leathery and homogenization was difficult.

Stage II B (2 months old)

a. Upper: There was no material alteration from the previous Stage (II A).

b. Middle: The processing was as in stage II A.

c. Lower: The dimensions were not materially altered. Homogenization of the tissue was very difficult. The bend seen in hapas before detaching from the mother plant persisted in this stage.

Autotrophic

For the studies on the other stages of growth, the stem tissue was collected only from the upper part.

In 12 months and 14 months old plants and also in the transition stage, there was no visible projection of the apical dome.

Mature vegetative:

Stage III A (12 months) and III B (14 months 'ripe to flower')

A 1.5 to 2 cm length of the stem tip was excised; the diameter at the base was 2.5 to 3.5 cm, which remained unaltered in the following stages.

Reproductive

Stage IV (transition)

In Stage IV also, the apical dome did not project out. The sample excised had the same dimensions as above.

Stage V (organogenic)

In the next stage (Stage V), when the apex elongated 0.5 to 1.0cm, a 2 to 3.5cm length of the stem tip was excised, inclusive of the projecting inflorescence. The diameter at the base was 2.5 to 3.5cm.

Stage VI

Post-organogenic, immediate (inflorescence)

From plants belonging to stage VI, when the stem tip elongated 1 to 2cm, 2.5 to 3 cm length of the stem including the projected inflorescence was excised. The diameter at the base was 2.5 to 3.5cm.

Stage VII

Post-organogenic, advanced (early fruit)

In stage VII, when the tip projected 6 to 10 cm, the entire length, with 1 to 1.5 cm of the subjacent stem, was excised. The diameter at the base was 2.5 to 3.5cm. The inflorescence at this stage was covered with soft bracts. These were carefully sliced away with a blade, without damage to the tissue underneath.

B. Leaf

Selection of leaves

The considerations underlying the selection of leaves for analysis are set out below.

The leaves selected by the present author were not exactly identical with the D-leaf whorl or semi-mature leaf whorl as described by earlier workers (Su, 1969; Bartholomew and Kadzimin, 1977). The whorl of 5-6 leaves selected did not include the longest leaves on the plant. As already explained, the author was guided by the

proximity of the selected leaf to the shoot apex and considerations of source and sink. A rather empirical technique which the author adopted in earlier stages, on the advice of Kerala Agricultural University, was the force necessary to pluck the leaves from the plants. These leaves offer the least resistance to pulling out from the plant. By this criterion, the leaves would correspond to the "immature" leaf referred to by Su (1969). It will be evident that, though these leaves were not exactly identical with D-leaves referred to by Bartholomew and Kadzimin (1977), they were immediately adjacent to the D-leaves. By pooling as many as 5 to 6 consecutive leaves from a single plant, the author has ensured that some at least of the leaves would have the optimum interaction with the shoot apex. Incidentally, the Kerala Agricultural University used the term D-leaves to refer to these leaves.

The lamina was cut transversely at the junction where the purely achlorophyllous part terminated and again at the junction where the uniformly chlorophyllous part commenced and each sampled.

In the 5 to 6 leaves selected for analysis, the achlorophyllous region in the lowermost leaf constituted 7 to 10% and the chlorophyllous region 78 to 84% of the total leaf area, whereas in the uppermost leaf, the achlorophyllous region constituted 16 to 20% and the chlorophyllous region 62 to 68%.

(i) achlorophyllous (non-chlorophyllous) region

This segment contained very little fibre and could be homogenized readily; it was sliced into small pieces and thoroughly mixed.

(ii) chlorophyllous region

The extreme tip region which contained spines and narrowed down to a sharp point was rejected. Four to 6 strips were cut out by transverse and oblique section over the entire length, avoiding the reddish pigmented region if and when present. The strips were then sliced into very small pieces and randomized.

(iii) intervening diffuse region (sub-chlorophyllous region)

The intervening segment, with diffuse green pigmentation, was normally discarded, but for pH-activity delineation, this tissue also was used. It was sliced transversely into an upper and a lower part, of nearly equal dimensions. Each part was comminuted, randomized and samples weighed out for pH-enzyme activity profile and protein determination.

c. Root

This constituted a set of experiments separate from the ontogenetic studies outlined earlier.

Experiments were carried out using root tip tissue also, in plants belonging to a few selected growth stages. These included heterotrophic (Stage I) and pre-autotrophic (Stages II A II B) and also mature reproductive stage (Stage V). The experiments were intended to give an idea about the function, if any, of invertase in roots in relation to the rooting process in the pineapple plant.

The major constraint was the non-availability of sufficient root tip tissue from a single plant unlike in ontogenetic studies. Thus, several plants had to be sacrificed for each analysis.

Sample size

Stem and leaf

The stem tissue samples collected from a single plant were comminuted and randomized. Two 5 g portions of the various tissues (except in the case of stem segment (upper) of propagule and Stages II A and II B, where tissue was sufficient for only one 5 g sample) were weighed out, wrapped in polythene sheet and stored at -18°C, for use in enzyme activity and protein determination. A 2 to 5g sample each was used for dry weight determination.

Root

After preliminary operations, (described earlier), root tip samples about 10mm long, which had not yet acquired the brown colour, and the root buds just emerging from the stem, were dissected out and pooled. A minimum of 6 plants had to be used in Stage I for a single analysis. Similarly, 3-4 plants had top be used in Stage II A and II B and 2-3 plants in Stage V. About 0.6 to 1.0 g of tissue could thus be collected for a single analysis.

Invertase activity determination

pH-activity relationship

Before proceeding with the detailed analyses of invertase activities in various tissues, the pH optima of the enzyme had to be separately determined. As a first step, this was carried out using the supernatant fraction (soluble form of the enzyme) of tissue homogenates.

In the following description, typical of the general procedure adopted, the tissues used were from plants 12-14 months of age, that is, fully mature, but still in the vegetative state.

Homogenization

A basal medium consisting of 10mM phosphate buffer, pH 7.0 and 1mM 2-mercaptoethanol was first employed for homogenization of various tissues. The protocol of these experiments was as follows:

The tissue (5g) was mixed with acid-washed sand as abrasive and ground in the cold with about 5ml of the basal medium, in a prechilled mortar with pestle, kept surrounded with broken ice. More of chilled medium was added from time to time after the coarse grinding was over. The volume of the fine slurry, in which not only the cell walls but also all the membraneous structures were ruptured (as far as practicable), was made to 25 ml (20% w/v) with medium. Such preparations will hereafter be referred to as homogenates, though the term homogenate as originally

proposed had a very restricted connotation. Twenty ml of the homogenate was centrifuged in the cold at 2000 x g for 10 min in an International Refrigerated Centrifuge, model PR-6, and the supernatant collected. The residue was suspended in about 20 ml of the homogenizing medium and the volume noted. The activity of invertase, if any, in the residual fraction was not tested at this stage. However, they were separately carried out as reported in a later section. When the pH optima of invertase in tissues at different ontogenetic stages were studied (page 93), the procedure outlined above was essentially adopted. However, when root tissues were analysed 4% (w/v) homogenates were prepared. Invertase activity was determined both in gel filtrate and in residue. Assay was carried out in the pH range 3.6 to 8.0 using 0.3ml of the enzyme fraction. Other assay procedures were as described earlier.

Protein was estimated in the homogenate, residue, supernatant and gel filtrate.

Sephadex G-25 gel filtration

Preliminary studies (data not reported) showed that invertase cannot be accurately estimated in homogenates/ supernatants of tissue extracts because of the high background absorbance mainly due to endogenous reducing sugars. Dialysis using viscose bags was time-consuming and also led to undue dilution.Also, there was the risk of the slow inactivation of invertase by proteinaceous inhibitor (Bracho and Whitaker, 1990 a). The method of choice for eliminating/reducing the background absorbance was gel filtration. The technique aimed at group separation

to yield a fraction containing all the proteins and other high molecular substances, excluded from the endogenous low molecular substances.

The gel employed was Sephadex G-25 (coarse). The method of gel treatment, column preparation, and loading was after Fischer, (1969). The procedure adopted for gel filtration of supernatants is given in some detail below.

The dry gel beads were first allowed to swell in distilled water overnight and the fine particles removed by swirling and decantation of the supernatant. The gel was now equilibrated with 10mM phosphate buffer pH 7.0, overnight and was packed into a glass column of 2.5 cm inner diameter. The height of the gel bed was generally about 16cm giving a total bed volume of 80 ml. A filter paper disc was carefully placed over the top surface of the gel bed. The column was then transferred to a refrigerator and allowed to cool overnight.

The medium above the filter paper disc was drained by operating the stopcock till the fluid level was in line with the filter paper disc. Ten ml of the supernatant was carefully layered without disturbing the gel surface. An equal volume of the liquid was drained away through the stopcock at a flow rate of 2ml/min. The column was now flushed with fresh equilibrating medium. Since Blue Dextran was not used for the determination of void volume of the column, 30% of the total bed volume was taken as the void volume of the Sephadex G-25. Four 5 ml fractions were collected (resulting in a two fold dilution of the original supernatant) at a flow rate of 2ml/min. after the void volume.

The preparation of the bed and equilibrations were carried out at room temperature, but the sample loading and collection of the protein fraction were always inside a refrigerator. After completion of the extraction of the protein fractions, the column was washed with several bed volumes of distilled water to remove the small molecular weight substances (from the sample) retained in the column. Subsequent equilibrations were carried out by passing 2 bed volume of the medium through the column. A column packed once could be used several times during the course of the experiments, thus avoiding the repeated packing each time. When the column was not in use for several days, the gel was taken out of the column and suspended in 0.02% (w/v) sodium azide (to prevent bacterial contamination) and stored in the refrigerator till needed.

Special advantage in the use of gel filtrate as enzyme source

Invertase assays in crude enzyme preparations from various tissues necessitate deproteinization prior to copper reduction with Somogyi's reagent. By the use of a gel filtrate of enzyme extract, one circumvented the deproteinization step, by adding the alkaline copper sulphate reagent directly (Jaynes and Nelson, 1971 a), inactivating enzyme; this effected considerable saving of time when a number of analyses had to be carried out simultaneously. Also, the sensitivity of the assay could be enhanced 3-fold as the whole volume of the assay system was used for colour development.

Enzyme assay

Immediately after the collection of gel filtrates, the activity determinations were carried out. (It was found from separate experiments that almost 50% of initial activity was lost on keeping the gel filtrates for 12 hours, even in the cold).

The assay system was patterned after Hatch and Glasziou, (1963) and consisted of 0.5ml of 200mM buffer (acetate buffer in the pH range 3.6 to 5.6 and phosphate buffer 5.7 to 8.0), 0.2 ml of the gel filtrate and water to 0.8ml. The tubes were then transferred to a thermostatic water bath maintained at 30°C. The reaction was started by the addition of 0.2ml of 250 mM sucrose (substrate) in the experimental tubes. At the end of 30 minutes incubation, the reaction was stopped with one ml of alkaline copper sulphate reagent (Somogyi, 1952). Following this substrate was added to the control tubes also and mixed well.

A modified procedure was adopted for the assay of the enzyme in the residual fractions. This was necessary as the cell debris would otherwise interfere with the colorimetric measurements, if the reaction were to be terminated with alkaline copper sulphate reagent and colour developed directly in the assay tubes. The method is as follows:

The assay was carried out as mentioned earlier in the pH range 3.6 to 5.6 (acetate buffer) and 5.7 to 8.0 (phosphate buffer). After incubation for 30 minutes, the reaction was terminated with 1.0ml of $Ba(OH)_2$ followed by $ZnSO_4$ (Somogyi,

1952). Following mixing and centrifugation, 1.0 ml aliquots of the supernatants were used for colour development.

Colour development

The tubes were heated in boiling water for 10 minutes to complete reduction. After cooling the tubes under running tap water, one ml of arsenomolybdate (chromogenic reagent, Nelson, 1944) solution was added, mixed and the colour developed.

After appropriate dilution with distilled water, the colour intensities were measured at 540 nm in a spectrophotometer or in a colorimeter using green filter (filter No.54). A reagent blank was used for adjusting 100% transmittance before readings were taken. Glucose was used as standard.

Extractability of invertase

As was mentioned in the *Introduction,* both soluble and cell wall associated/insoluble forms of invertases have been reported in many plant tissues. It was, therefore, felt necessary to determine the extent of extractability of the invertase under varying extraction conditions so as to arrive at a suitable homogenizing medium for the detailed ontogenetic studies envisaged. Since the pH optima determinations carried out earlier were by using the gel filtrates of supernatants, the possibility of the presence of insoluble/cell wall associated enzyme in the residue had to be separately tested.

The following experiments were therefore carried out with different tissues, using basal medium, basal medium supplemented with Triton x-100, and also employing repeated extractions of the residual fraction.

The following is the protocol of a typical experiment using chlorophyllous leaf tissue obtained from 12 month old plants.

The effect of successive grinding and Triton X-100 on invertase extraction

Triton X-100 is a non-ionic surface active agent used with remarkable success with animal tissues in the release and solubilization of lysosomal enzymes and other particle-associated and latent enzymes. The reagent has found application also in plant tissues.

The following experiments were first carried out using frozen samples of chlorophyllous leaf parts. Acid washed sand was used as abrasive during extraction. The gel filtration and assays followed soon after all the extractions were complete. The first extract (after centrifugation) was gel filtered; subsequent extracts were directly assayed as most of the reducing sugars passed on to the first extract itself. The assay was at pH 4.8 (the optimum pH as determined earlier). The residue upon completion of extraction, was suspended in an appropriate volume of the basal medium and analysed for invertase activity in the pH range from 3.6 to 8.0.

The basal medium, as described earlier (Homogenization, page 71) was used as the control. The extraction with the basal medium was carried out 4 times and each extract was separately collected and assayed, along with the residual fraction.

When the effect of Triton X-100 was tested, it was incorporated in the medium at a final concentration of 0.5% (v/v) for the entire extraction steps; or it was incorporated in the successive extraction of the residue left after the initial extraction with the basal medium. The residue in every case was suspended in the basal medium only.

Observations using the chlorophyllous leaf part, showed only marginal effect on Triton X-100 treatment on enzyme extractability. Most of the enzyme could be solubilized in the first extraction with the basal medium itself. Hence, Triton X-100 incorporation was not resorted to with the other tissues (stem and achlorophyllous leaf). Repeated extractions were carried out with the basal medium. Another reason for the deletion of Triton X-100 in the medium was the interference of Triton X-100 during colour development in invertase assays.

Since the well washed cell wall fraction was devoid of invertase activity, extractions with high salt concentration and ethylenediaminetetraacetate (EDTA), as outlined in the *Introduction*, was not carried out.

Lack of solubility of protein in the chlorophyllous leaf tissue

An unexpected observation during the invertase activity and protein determination in the chlorophyllous leaf tissue was the failure to detect protein in the supernatant and gel filtrate there from by Lowry *et al.* (1951) method. As all other tissues analyzed gave estimable quantity of protein in the gel filtrates, the question arose as to the reason for this lack of detectable levels of protein in the

chlorophyllous leaf tissue supernatant/gel filtrate. Analysis of the homogenate and residual fraction, revealed that the two fractions had nearly equal quantities of protein. This indicated that very little protein got solubilized under the homogenizing conditions using the basal medium. Nevertheless, the gel filtrates had high invertase activity, but the residue was devoid of any.

Pineapple is a CAM plant and hence one should expect high acidity in the leaf, particularly in the morning, when the samples were collected. During tissue disruption,the acidity could have resulted in rendering the bulk of proteins insoluble. The pH of the homogenate was tested with a pH meter and was found to be around 5.0. Whether protein solubilization could be enhanced by neutralizing the acid during tissue homogenization, was to be tested. In order to accomplish this, tissue acidity was first determined by titration using aqueous extracts. Solid sodium bicarbonate in molar quantity equivalent to the acid content of the tissue was used to exactly neutralize the acidity in the tissue during homogenization. This was done by weighing the exact quantity of $NaHCO_3$ and adding it along with tissue sample and acid washed sand during homogenization in the basal medium. The resulting pH of the homogenate was found to be 6.6 to 6.8. For comparison, homogenization in the basal medium (control) was simultaneously carried out using another sample of the same pooled tissue.

Determination of invertase activity in the gel filtrate, and protein in the homogenate, residue, supernatant and gel filtrate showed that while invertase activity could be fully recovered in the gel filtrate of supernatant, protein failed to get

extracted to any detectable quantity colorimetrically, in both the $NaHCO_3$ treated and control tissues.

Freezing and thawing

The emphasis of the present investigations was on ontogenetic studies and samples had to be collected when the appropriate stage was reached. In the reproductive stages, the gap between successive stages was short and analyses of samples collected of a previous stage were never complete by the time the next stage was ready. Often, the number of tissue samples collected on a single day was as many as 36. Therefore, the samples had to be preserved in the frozen condition, till the analyses were complete. In order to see whether this freezing for several days and subsequent thawing of the samples during homogenization, affected enzyme activities, separate experiments were performed as follows.

Six samples (5 g each) of the pooled chlorophyllous leaf tissue from 3 plants (12 months old) were weighed out. One sample was analyzed for invertase activity immediately; the others were stored in a deep freeze at -18°C. After 2 days, all the stored samples were transferred to a refrigerator maintained at about 5°C and allowed to thaw. One sample was analysed (single cycle of freezing and thawing). The remaining samples were returned to the deep freeze. After two weeks, the remaining samples were allowed to thaw, a third sample was analysed (two cycles) and the remaining samples returned to the deep freeze. The other two samples were similarly analysed at the end respectively of 4(3 cycles) and 6 (4 cycles) weeks.

Optimization of assay conditions

In order to have reliable and reproducible values on enzyme activity measurements of tissues at ontogenetically different growth stages, it is necessary to optimize the various factors which influence the enzyme velocity. These factors include:

i. the relationship between reaction velocity and enzyme concentration

ii. enzyme velocity–substrate concentration relationship

iii. relation between incubation period and enzyme activity

and

iv. relation between incubation temperature and reaction velocity.

Each of these was separately determined at the predetermined pH optima for the respective tissues.

i. Relationship between reaction velocity and enzyme concentration

The assay system was as already determined. The final substrate concentration was 50mM. Different volumes of the gel filtrates equivalent to a 10% (w/v) homogenate was used as enzyme, except in chlorophyllous leaf tissue, where the gel filtrate used was 2%. The tissues analyzed were (a) stem tissue (all the three segments–upper, middle and lower) (b) achlorophyllous and (c) chlorophyllous leaf parts in rooting studies and (d) stem tip tissue (upper segment only) and (e) achlorophyllous and (f) chlorophyllous leaf parts in the mature vegetative stages.

ii. Enzyme activity–substrate concentration relationship

The effect of increasing the substrate concentration was tested in the range 5 to 60mM sucrose, except in the case of achlorophyllous leaf tissue, where the maximum concentration tested was 200 mM. Assay conditions and tissues analyzed were as described above.

iii. Incubation period and enzyme activity

This was tested in stem tip, achlorophyllous, and chlorophyllous leaf tissue, at the mature vegetative stage; predetermined optima with respect to pH, enzyme concentration and substrate concentration were employed. Other assay conditions were as already reported.

iv. Incubation temperature and reaction velocity

This was tested only in stem tip tissue of mature vegetative plant. The temperature tested were 30, 35, 40, and 50°C. Incubation period was 30 minutes and enzyme and substrate concentration were as optimized previously.

Validity of standardized assay conditions

Comparison of quantitative levels of enzyme activities among different tissues and at different stages of growth become valid only if measurements are made under optimal conditions. These conditions differed from tissue to tissue. Therefore, there was no uniformly common assay system that could be employed. Each tissue was

analyzed employing separately standardized assay conditions as described above. The results are expected to be optimum and comparable among different tissues.

Estimation of protein

General

Unlike in animal tissues, protein estimation in plant tissues in general, and crude enzyme preparations therefrom, presents many difficulties. Pineapple is not a phenolics-rich plant, nevertheless, protein estimation in its tissues necessitated special precautions. Working with tissue homogenates and suspensions of residual material, the cellular debris added to the difficulty by greatly adding to the bulk of the protein precipitate with trichloroacetic acid.

Precipitation

Appropriate aliquots of homogenate, supernatant on centrifugation, its gel filtrate and residue suspension, in the range 0.50 to 2.0 ml and contained in 15 ml centrifuge tubes with tapering bottom, were mixed with 100% (w/v) trichloroacetic acid to 5% (w/v) final concentration and the mixture left in the cold for flocculation. The stock solution of precipitant was prepared in the high concentration so that the volume of the acidified sample could be kept a minimum. This was of special advantage in the case of gel filtrates, whose protein concentration was low. Chilled in an ice bath, the flocculation was complete in about 30 min. The mixture was centrifuged for 10 min and the supernatant decanted off.

Elimination of interfering materials

The subsequent treatment of protein precipitate was after Khanna *et al.*(1969) aimed at eliminating/minimizing starch and pigments and any phenolics. The volumes in which reagents were employed in washing were based on the bulk of the protein residue and were kept a minimum, sometimes a few drops only, since protein was sightly soluble in the various reagents.

The protein precipitate was first washed with 2% trichloroacetic acid (0.20 to 0.50ml) and centrifuged. The resulting residue was then washed once with 1% trichloroacetic acid (0.1 to 0.3 ml) and spun down.

The above residue was kept suspended in 10% (w/v) perchloric acid (0.3 to 0.5ml) for 10min. (Since the starch content in pineapple tissues was not high, with the prominent exception of segment *b* of the stem in the rooting stage, the 30% and 15% perchloric acid washing steps were avoided.) After 10 min. the suspension was centrifuged and the supernatant decanted away. The residue was next washed once with 5% perchloric acid (0.1 to 0.3ml).

The residue was then washed with anhydrous acetone twice (0.3 to 0.5ml in the first stage and 0.1 to 0.3ml in the second stage,followed by 80% acetone (v/v) twice (0.3 to 0.5 ml initially and 0.1 to 0.3 ml subsequently) for protein from chlorophyllous tissue and the lower part of stem tissue of rooting stages. In the case of other tissues, the precipitate was washed only once each.

Dissolution

The residue was suspended in 5.0 ml of 0.1 N NaOH and heated for 5 min with mixing in a bath of boiling water. In the case of protein precipitates from homogenate and residue, centrifugation was necessary to get a clear solution, while for protein from supernatant from homogenate and gel filtrate, centrifugation was not needed, since the protein got dissolved readily and the solution was clear.

The resulting protein solution had a brownish colour when working with homogenate, or residual fraction, of chlorophyllous leaf and the lower segment of stem tissue in rooting studies, in particular, Stage II B. Colourless solutions were obtained from all parts of the stem tissue of the propagule. In Stage II A, only the lower part of stem gave rise to pigmented solutions, but the colour was less intense than that of corresponding fraction from Stage II B. In the two months old plants, the middle portion of the stem also gave rise to coloured protein solution, but these solutions were less brown than the corresponding fraction from the lower part of stem. The other preparations were essentially free of pigment. The protein fractions from gel filtrates were always colourless, even when derived from the lower part of the stem in Stages II A and II B.

Colorimetry

The method adopted was that of Lowry *et al.* (1951). Aliquots (0.10ml in the case of protein from whole homogenate and residue and 0.50 ml in the case of protein from supernatant and gel filtrate) were made to 1.0 ml with water and 5.0ml

alkaline copper sulphate reagent was added. At the end of 10 min at room temperature, 0.5ml of Folin Ciocalteau phenol reagent was added. Colour intensities at 660 nm were measured after 30 to 60 min in a spectrophotometer, set to 100% transmittance with a blank containing the two reagents and 1.0 ml water.

Bovine serum albumen was used as standard. A stock solution containing 1 mg of the protein per ml was prepared (25.0 mg in 25.0 ml solution) and stored in the cold; this solution could be used for about 2 weeks. A working standard was prepared each time by diluting 1.0ml to 10.0ml, that is, 0.10mg/ml. One ml aliquots were used directly for colour development, without trichloroacetic acid precipitation. The author has preferred to have a standard colour prepared each time, instead of relying on a standard graph relating protein to optical density.

Determination of dry solids

Since valuable insight into the physiology of the plant was provided by relating not only the protein but also the enzyme activity to dry solids content in the tissues, dry weight determinations assumed considerable importance. The principle consisted in rapid heating of tissue at a high temperature for a short period to inactivate enzymes, followed by continuous heating to constant weight at a temperature sufficiently low to prevent loss of non-volatile tissue constituents.

As already pointed out, comminuted tissues were separately pooled and randomized and a number of samples quickly weighted out. While the samples for enzyme activity (and protein) determination were transferred to a deep-freeze, a

sample was directly used for dry weight determination. It was transferred to an oven maintained at 100°C. The period of heating at this temperature depended upon the nature of the tissue. The more tough tissues such as chlorophyllous leaf and the lower and middle parts of stem in States I, II A and II B were heated for an hour, while the tender upper part of stem tip tissue was heated for 45 min. The more delicate achlorophyllous leaf tissue was heated for only 30 min. The samples were then transferred to an oven maintained at 60°C until constant weight was attained .Prior to weighing, the samples were transferred to a desiccator and allowed to attain room temperature.

Expression of data

Data on total enzyme activity and total protein are expressed both on the basis of fresh tissue and dry tissue equivalent.

Unit of activity

One unit of invertase activity was defined as the amount of enzyme which hydrolysed 1 μ mole of sucrose (one half the reducing equivalent at the end of assay) in 20 min at 30°C, under the optimal conditions of assay.

Specific activity

Specific activity was defined as units of activity per mg protein in the enzyme preparation. In the case of stem tissue and achlorophyllous leaf tissue, the protein was that determined in the gel filtrate. In the case of the chlorophyllous leaf part,

protein could not be determined in the gel filtrate, or the extract prior to gel filtration; the activity was expressed in relation to the protein in the homogenate. For purposes of comparison, the activity in the stem tissue and achlorophyllous leaf tissue was calculated also on the basis of protein in the respective homogenates.

Replications

The ontogeny studies were carried out with 6 samples of each tissue at every growth stage.

Statistical evaluation

All values are expressed as mean ± S E. Statistical significance was tested using the students 't' test. A '*p*' value of greater than 0.05 was taken as not significant.

Chemicals

All reagents and solutions were prepared from analytical grade chemicals.

RESULTS

RESULTS

Part A. **Biochemistry**: Preliminary studies

Part B. **Physiology**

RESULTS

Part A: Biochemistry: Preliminary Studies

pH optima of invertase

The rationale behind the separate determination of the optimum pH in each tissue was already mentioned. The results obtained are graphically represented in Fig 1 to 4.

Heterotrophic and pre-autotrophic stages

a. Stem segments

Only acid invertase with a pH optimum of around 4.8 was present in the stem, irrespective of the segment in the heterotrophic and pre-autotrophic stages. A sharp pH optimum was observed in the case of the lower segment (**c**). There was a tendency of broadening of the pH optimum commencing with the middle segment (**b**) and accentuating in the upper segment (**a**) (pH ranged from 3.6 to 5.6, that is, by as much as 2 pH units).

[Fig. 1 A]

b. Leaf

i. achlorophyllous

Both acid and neutral invertase were present in this tissue. There was a sharp peak for acid invertase between 4.8 and 5.2. The activity declined sharply thereafter to a minimum value at pH 6.5. A second peak of invertase activity (neutral) was seen in the tissue, after pH 7.0, the activity decreasing above pH 7.3. The acid invertase was more prominent than the neutral invertase.

[Fig. 1 B]

ii. Chlorophyllous

Only the acid form was present with a sharp pH optimum at 4.8. There was rapid decrease thereafter with total loss of activity at pH 8.0.

[Fig. 1 B]

iii. intervening diffuse regions (sub-chlorophyllous)

The tissues resembled the achlorophyllous tissue in the pH profile, in that both activities were present.

a. Lower part

The acid invertase had a sharp peak at pH 4.6. The activity fell sharply at pH 6.0 and rose to register a second peak at pH 6.6.

[Fig. 2 B]

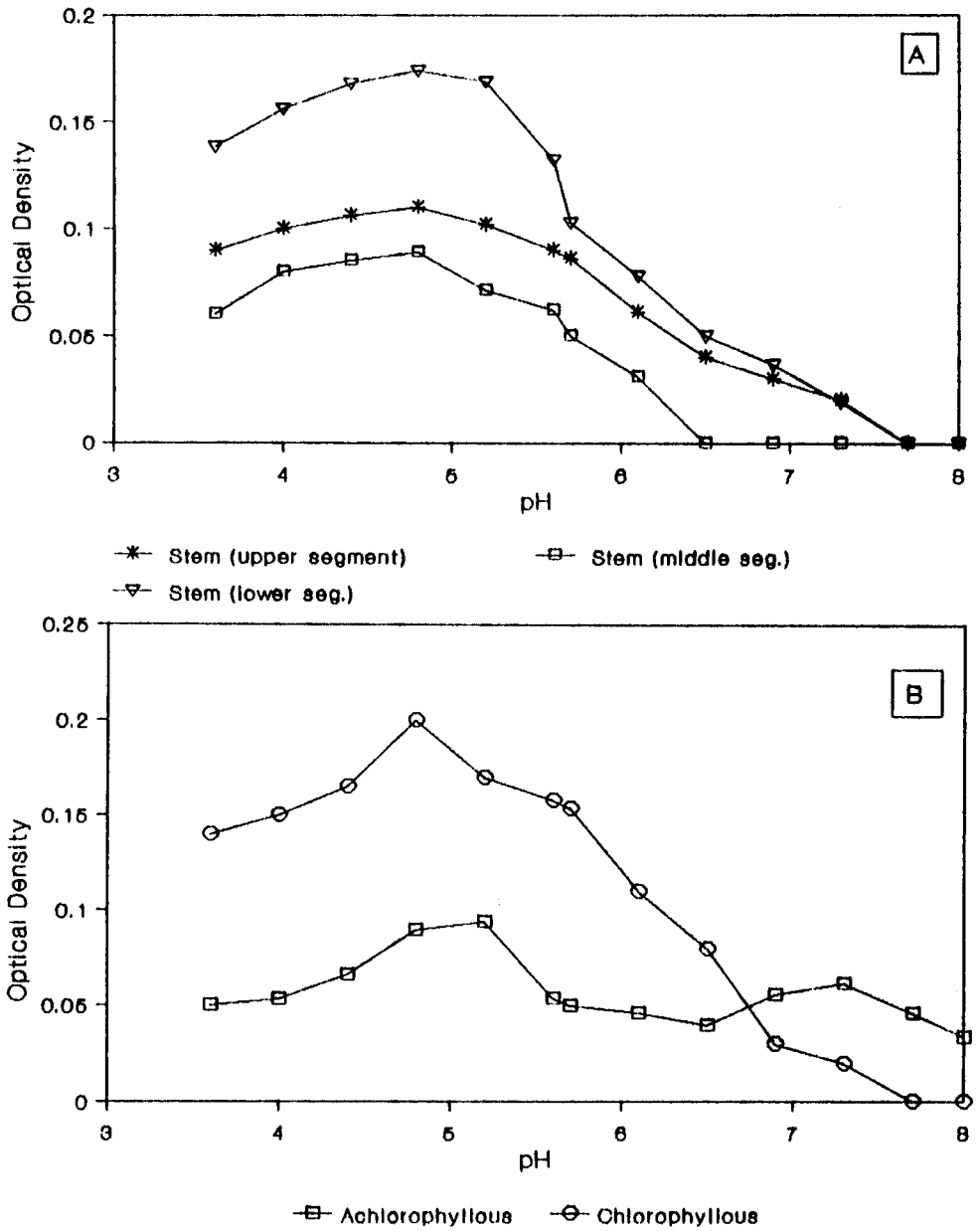


Fig. 1 pH optimum of invertase in different tissues of the pineapple plant: Stage I

A: Stem segments. B: Leaf tissue

b. Upper part

An identical pattern of pH-activity profile, with acid invertase activity peaking at pH 4.6 and neutral at pH 7.0, was observed in the upper part as in the lower part. However, in relation to the acid invertase activity, the neutral activity was less prominent in the upper part than in the lower part. In other words, while the lower part tended to resemble the achlorophyllous leaf region, the upper part tended to resemble the chlorophyllous leaf part.

[Fig. 2 B]

c. Root

(i) Stage I

No invertase activity could be demonstrated in the root tips at this stage.

(ii) Stage II A

Both acid and neutral invertases were present, with pH optimum at 4.6 and 7.0, respectively. The profile stood out prominently in that the neutral invertase was more active than the acid invertase, though only marginally.

[Fig. 2A]

(iii) Stage II B

A similar profile as in Stage II A with peaks at 4.6 and 7.0 was observed. However, the neutral invertase was twice as active as the acid invertase.

The root tip tissue in Stage II, thus, resembled the achlorophyllous leaf tissue and the intervening region of the leaf in the pH activity profile.

[Fig. 2 A]

Autotrophic

a. Stem tip (upper)

In marked contrast to the heterotrophic and pre-autotrophic stages, only the neutral form of invertase was present, with a sharp peak at around 7.0.

[Fig. 3 A]

b. Leaf

i. Achlorophyllous

Similar to the heterotrophic and pre-autotrophic stages of the tissue, the autotrophic stage showed both the acid and the neutral forms of the enzyme with pH optimum in the range 4.8 to 5.2 and 7.0 to 7.3 respectively. The latter peak was marginally lower than the former.

[Fig. 3 A]

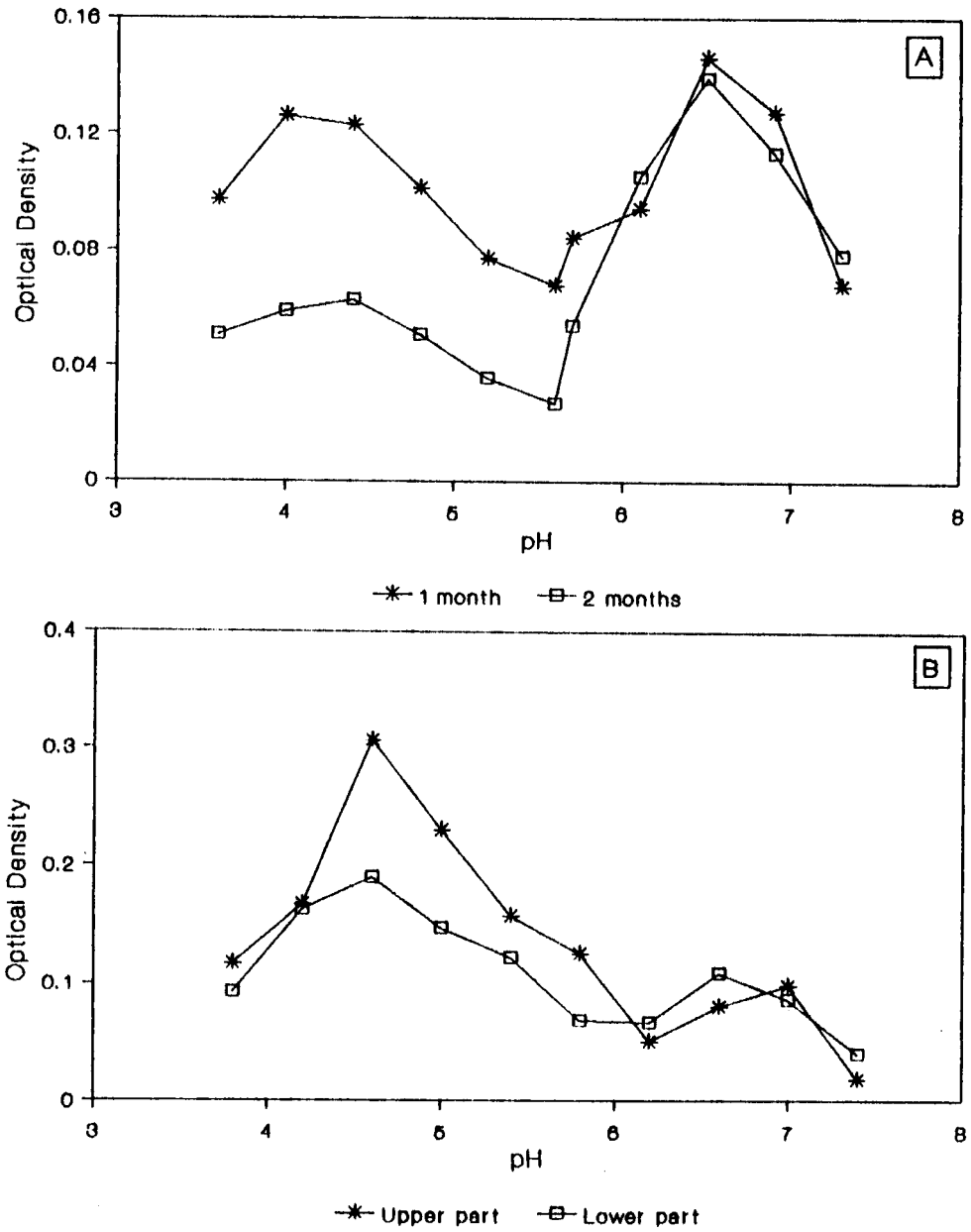


Fig. 2: pH optimum of invertase in different tissues of the pineapple plant: Stage I

A: Root tip tissue B: Leaf-intervening diffuse region (-Subchlorophyllous-)

ii. Chlorophyllous

Only the acid form was present with a sharp peak at pH 4.8. The activity diminished steeply and disappeared completely at pH 7.0 and thereafter. The profile was comparable to that obtained in the heterotrophic and pre-autotrophic stages.

[Fig. 3 A]

c. Root

The sample tested was from Stage V of development of the plant. The pH-activity profile was very similar to that obtained in root tip tissue of Stage II A, with peak for acid invertase between 4.6 and 5.0 and for neutral invertase at pH 7.0. The latter enzyme was marginally more active than the former.

[Fig. 3 B]

Choice of assay pH

The pH optimum obtained for various tissues are tabulated and shown in Table 1.

From the above chart, it is clear that in most tissues, acid invertase was optimally active at pH 4.8. Even when a sharp pH optimum at pH 4.8 was not obtained in some tissues, the activity obtained at ± 0.4 pH units on either side of 4.8 showed only marginal difference. Therefore, 4.8 was fixed as the pH optimum for acid invertase assays irrespective of the tissue or stage. Neutral invertase in most

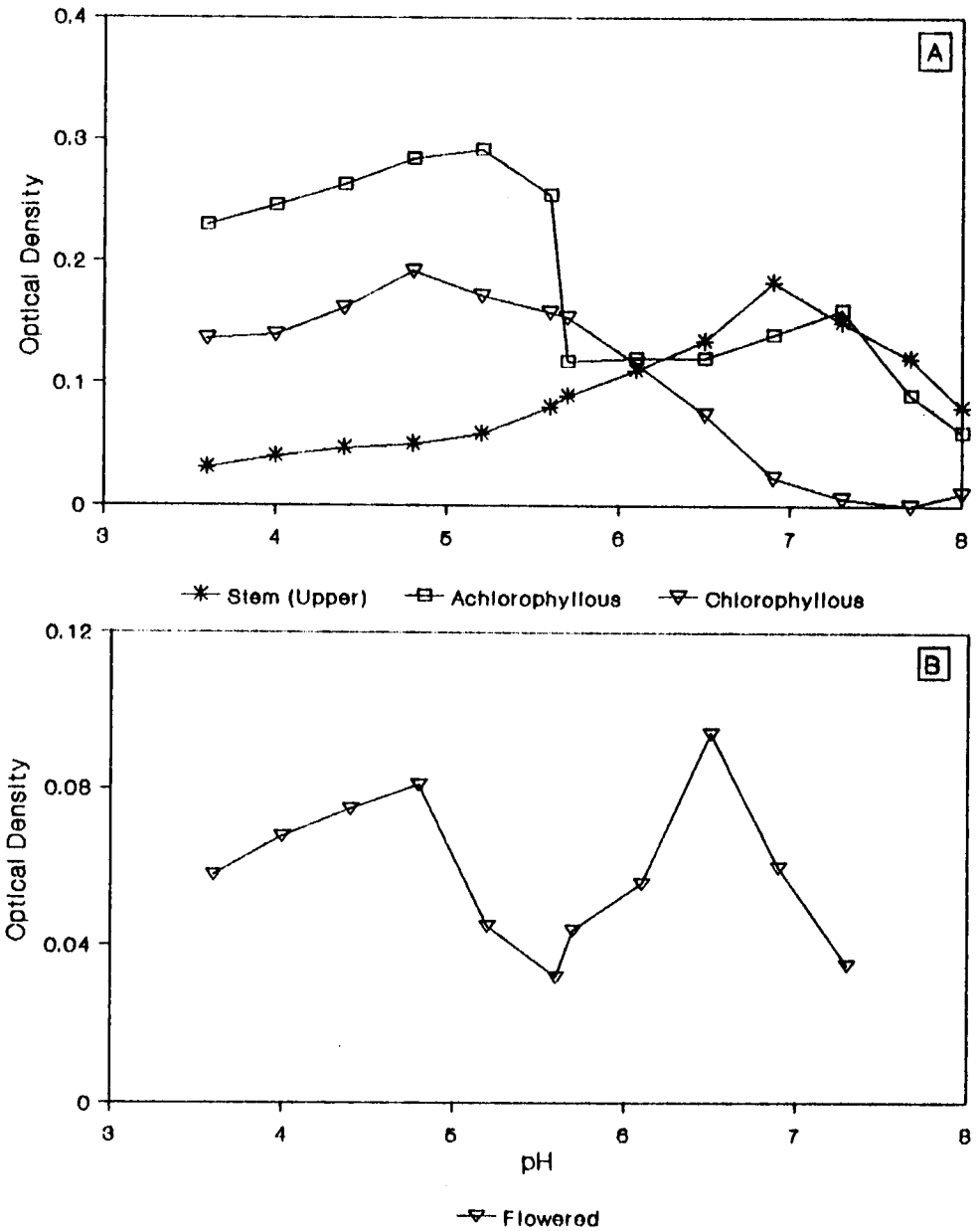


Fig. 3: pH optimum of invertase in different tissues of the pineapple plant: Autotrophic

A: Stem tip & Leaf tissues B: Root tip tissue

Table 1
pH optima of acid and neutral invertases in the tissues during ontogeny of the pineapple plant

Stage and tissue		pH optima	
		Acid	Neutral
Heterotrophic & Pre-autotrophic			
A. Stem	Upper	3.6 to 5.6	absent
	Middle	4.0 to 5.2	absent
	Lower	4.4 to 5.2	absent
B. Leaf			
(i) Achlorophyllous		4.8 to 5.2	6.9 to 7.3
(ii) Chlorophyllous		4.8	absent
(iii) Intervening			
(a) Lower		4.6	6.6
(b) Upper		4.6	7.0
C. Root			
(i) Stage I		absent	absent
(ii) Stage IIA		4.2 to 4.6	7.0
(iii) Stage IIB		4.2 to 5.0	7.0
Autotrophic			
A. Stem tip (3 months)		absent	6.6 to 7.0
(12 months)		absent	7.0
B. Leaf			
(i) Achlorophyllous		4.8 to 5.2	6.9 to 7.3
(ii) Chlorophyllous		4.8	absent
C. Root (Stage V)		4.2 to 5.0	7.0

The assays were carried out in acetate buffer in the range pH 3.6 to 5.6 and phosphate buffer in the range pH 5.7 to 8.0. For tissues in root tip and intervening region of leaf, citrate-phosphate buffer was used in the range 3.8 to 8.0. Other procedures were as reported in *Materials and Methods*.

tissues showed an optimum around pH 7.0. The slight differences in the pH value, ± 0.4 unit on either side of 7.0 did not markedly affect the enzyme activity. The choice of pH 7.0 as the optimum was therefore, justifiable.

p*Ka* of acetate buffer is 4.7 and that of phosphate buffer is 6.9. Thus, assays of acid invertase at pH 4.8 and neutral at pH 7.0 are expected to be under conditions for efficient buffering.

Shift from acid-to neutral form of invertase during development

All tissues of the pineapple plant manifested invertase activity, with the prominent exception of the tip of the aerial root. A salient feature of pineapple tissues was the wide distribution of acid invertase. The prominent exception was the stem tip of the autotrophic plant, which contained only the neutral form. Neutral invertase was present also in the other nonchlorophyllous tissues, the achlorophyllous leaf part and the tip of the terrestrial root, in which two tissues, the acid form coexisted with the neutral form. The intervening diffuse regions of leaf tissue resembled the achlorophyllous part in this respect.

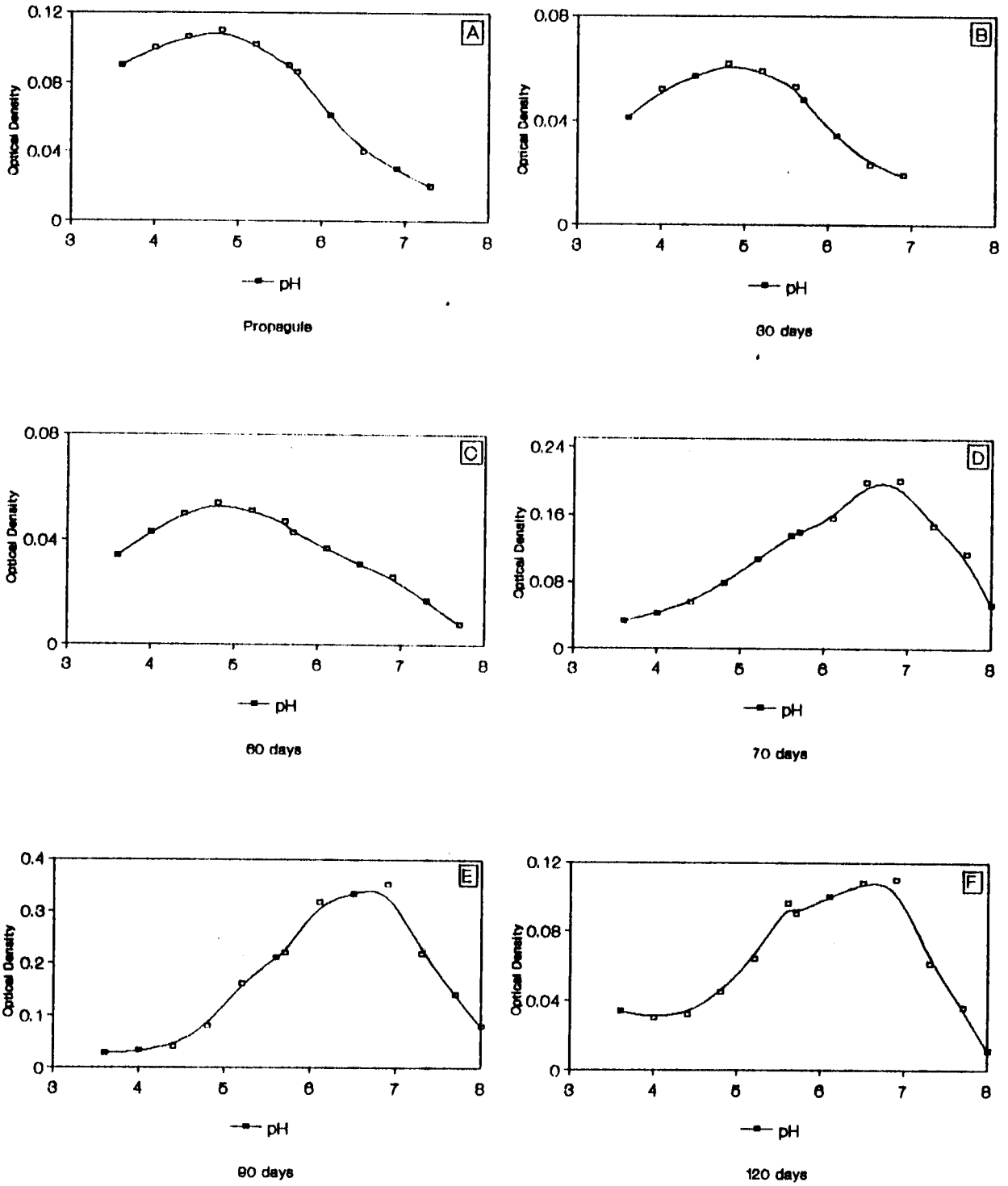
The shift from the acid activity of the shoot tip to the neutral form when passing from Stage II B (two months after planting) to Stage III A (12 months after planting) was unexpected and warranted further investigations.

In order to determine the stage during development (heterotrophic to autotrophic) in which the shift from acid to neutral form took place, separate experiments were planned. At intervals of 2 months, samples of stem tip were

analyzed for the pH-activity relationship and compared with that of the propagule. It was found that while propagule and the two months old plant exhibited only the acid form of the enzyme, at 4 months and thereafter, the neutral form alone was present. This indicated that the shift from acid to neutral form occurred between the second and the fourth month.To precisely identify the exact chronological age of the plant at which the transition took place, experiments were repeated at shorter intervals starting with the two months old plant, that is, 60 days, 70 days, 90 days and 120 days. It was found that by about the 70th day, or so, the acid invertase disappeared and was replaced by neutral form. Only the neutral form was present in subsequent stages. This observation indicated that the shift from one form to the other form of the enzyme was a relatively rapid process and might have occurred in a few days' time.

The exact physiological significance of the above observation in relation to the role of invertase in the pineapple plant is not certain. Also, the molecular biology underlying this remarkable phenomenon was not clear, but merits further researches.

[Fig. 4]



Shift from acid – to neutral form of invertase in the stem tip during the ontogeny of the pineapple plant

Fig. 4

Extractability of invertases

Chlorophyllous leaf tissue

The results are recorded in Table 2.

A. Basal medium as extractant (control)

Supernatant

On extraction with basal medium and analysis separately in each extract, it was seen that 80% of the enzyme activity passed on to the first supernatant, 12.9% in the second, 6.5% in the third and 1% in the fourth supernatant. The residue was devoid of activity.

B. Triton X-100 supplemented medium as extractant

a. Two extractions

When the tissue was extracted twice, using the basal medium supplemented with Triton X-100, the initial extract yielded 91% and the second the remaining activity.

b.Three extractions

Using the Triton X-100 supplemented medium, when three extractions were carried out, and each analyzed, 96% activity passed into the initial extract, 2.8% into the second and 1.3% into the third extract.

c. Triton X-100–supplemented medium used after basal medium

When the initial extractions were carried out in the basal medium and the residue re-extracted twice with Triton X-100 supplemented medium, it was found that the activity in the combined first and second extract was almost 90%; and the third extract had 3.4% and the combined fourth and fifth extract 2.9% activity.

Residue

No invertase activity was observed in any of the above residual fractions left after the completion of extraction, at any of the pH values tested in the range 3.6 to 8.0.

On the basis of the results from the foregoing experiments, it was concluded that a single extraction with the basal medium is a satisfactory step and Triton X-100 supplementation has very little advantage. Achlorophyllous and stem tip tissues were, therefore, analysed for extractability studies in the basal medium only.

Results showed that over 80 to 90% of the enzyme (both acid and neutral) could be recovered in the first extract itself (data not shown) with no detectable activity in the residual fractions over the pH range 3.6 to 8.0.

Routine extraction procedure for enzyme assays

During ontogenetic studies, spread over 15 months, a large number of samples had to be analyzed. As many as 4 homogenizations were carried out on a single day, prior to centrifugation and gel filtration. Repeated extraction was time

Table 2
Effect of successive grinding operations and Triton X-100 supplementation on acid invertase extraction from chlorophyllous part of the pineapple leaf

Expt. No.	Extraction Medium	Extract Number and Fraction	Activity, units/g fresh tissue	Percentage recovery in extracts/ residue
1	A. Basal	Supernatant I	17.20	79.6
	-do-	Supernatant II	2.80	12.9
	-do-	Supernatant III	1.40	6.5
		Supernatant IV	0.22	1.0
		Residue	nil	nil
2	B. Supplemented	Supernatant I	24.38	91
		Supernatant II	2.36	9
		Residue	nil	nil
	Supplemented	Supernatant I	17.95	96.0
		Supernatant II	0.52	2.8
		Supernatant III	0.24	1.3
3.	C. Basal	Supernatant I	18.5	89.7
		Supernatant II		
		Supernatant III	1.3	6.6
	Supplemented	Supernatant IV	0.76	3.7
		Supernatant V		
		Residue	nil	nil

The basal medium was made up of 10 mM phosphate buffer pH 7.0 and 1 mM 2-mercaptoethanol. Triton X-100 was supplemented in final concentration of 0.5% v/v. Other details were as outlined in the *Materials and Methods*. The percentage of recoveries were calcualted on the basis of the sum of activities recovered in the different extracts. The above experiment was duplicated and the results given represent a typical experiment.

consuming and did not offer any added advantage as most of the enzyme could be recovered in the first extract itself. Therefore, activity of the enzyme in this fraction was taken as representative of the whole tissue equivalent.

The effect of freezing and thawing

The effect of freezing (and thawing) on invertase activity in the chlorophyllous leaf tissue was tested and the results are given in the table below.

Effect of freezing and thawing on invertase activity from chlorophyllous leaf part

Extracted activity, units per g fresh tissue equivalent				
Number of cycles of freezing and thawing				
Control (Fresh sample)	1	2	3	4
18.2	17.6	18.0	17.2	18.5

The experiment was spread over 6 weeks. Freezing was at -18°C. Other details were as reported in *Materials and Methods*.

The results obtained showed that:

1. Fresh tissue had the same activity as tissue frozen for 2 days. This indicated that the full activity of the enzyme was expressed even without the aid of endogenous cell rupture by freezing and thawing. Additional cycles of freezing and thawing did not alter the extractable activity.

2. Based on this findings, *likely to be applicable also to the other tissues*, the regular assays could be carried out with tissues in the fresh condition. In actual practice, it was preferred to analyze after storage frozen for a few days, since only 2 to 4 samples could be analyzed on the same day; also, the grinding operation was easier.

Optimization of assay condition

Relationship between reaction velocity and enzyme concentration

A. Stem tissue

The three segments of the stem in the rooting stages and the stem tip in the mature vegetative stage (12 months) were tested separately for reaction velocity-enzyme concentration relationship. Using a 10% equivalent of gel filtrate, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 ml aliquots were used in the assay system. The activity was found to be nearly linear upto 0.2ml of the enzyme used in the assays. A typical plot using Stage III A tissue as the enzyme source is given in Fig. 5 A

[Fig. 5 A]

In regular assays, irrespective of the stage of growth, 0.20ml aliquot of 10% equivalent of gel filtrate was employed. Under these conditions measurable activities, within the sensitivity of the colorimetric procedure, could be obtained.

B. Leaf

i. Achlorophyllous

Analyses were carried out at pH 4.8 and 7.0 using 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 ml of 10% gel filtrates of the tissue extracts at Stage I and Stage III A plants. The enzyme activity at either pH value was proportional upto 0.3ml of the enzyme.

[Fig. 5 B]

The sensitive range was between 0.05ml to 0.15 ml in the case of acid invertase and between 0.10 to 0.20ml in the case of neutral invertase. In routine assays, 0.10ml of 10% equivalent of gel filtrate was used for acid invertase and 0.20ml of enzyme for neutral invertase activity.

ii. Chlorophyllous

The chlorophyllous leaf tissue had very high invertase activity as revealed from preliminary experiments. Therefore, the original gel filtrate (10% equivalent) was diluted 5 times (2% equivalent) with the basal medium. The determinations were at pH 4.8 using 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 ml aliquots. The enzyme activity increased proportionately upto 0.30 ml, the maximum amount tested.

The colorimetric readings were in the sensitive range when enzyme aliquot in the assay system was between 0.10 and 0.20ml. In regular assays, 0.20ml enzyme preparation was employed.

[Fig. 5 C]

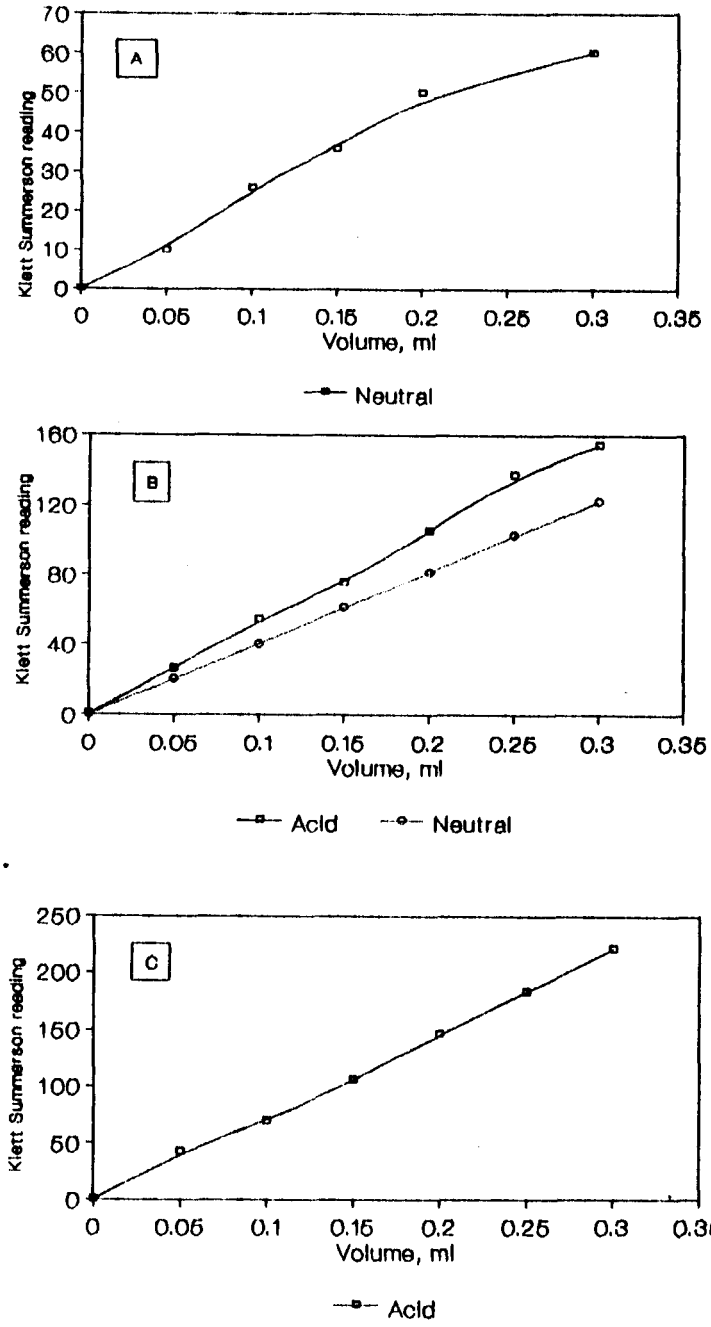


Fig. 5: Relationship between reaction velocity and enzyme concentration

A: Stem tip (Stage IIIA); B: Achlorophyllous (Stage IIIA) C: Chlorophyllous (Stage IIIA)

Overview

From the shape of the various curves, the following conclusions seemed to be justified:

1. Since there was no lag in the exhibition of activity, that is, activity was manifested from the lowest amount tested, the reagents used in assay system were free of toxic impurities.

2. Since the reaction velocity-enzyme concentration yielded a straight line relationship with increasing enzyme concentration, with the possible exception of the neutral activity in the stem tip in the autotrophic stage, the presence of a reversible inhibitor of the enzyme can be excluded.

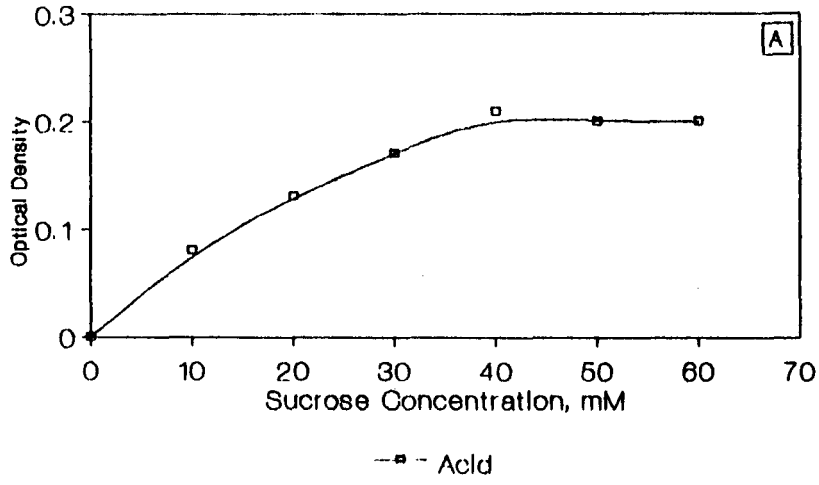
Enzyme activity-substrate concentration relationship

A. Stem

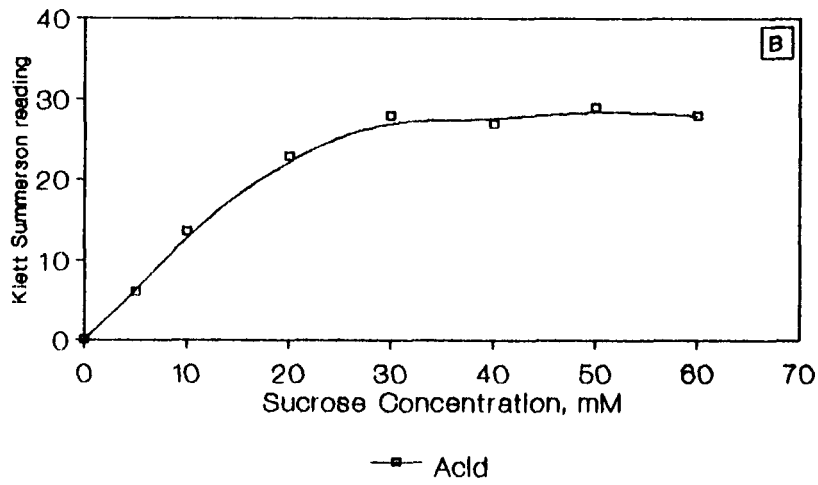
The invertase activity was determined in assay systems containing sucrose in the range 5 m*M* to 60 m*M* for all the three segments (-upper, middle and lower) at the rooting stages. A typical Michaelis-Menten relationship was obtained. Saturation was reached around 40 m*M* to 60m*M* sucrose. The stem tip at the mature vegetative stage resembled the three stem segments in the substrate saturation profile.

[Figs. 6 A, 6 B, 7 A and 9 A]

In the routine assays the substrate concentration was kept at 50m*M*.

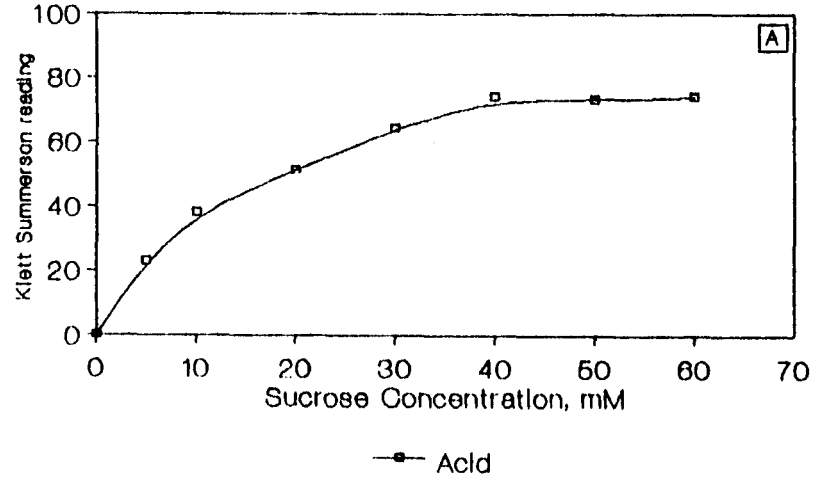


Propagule: Stem (upper segment)

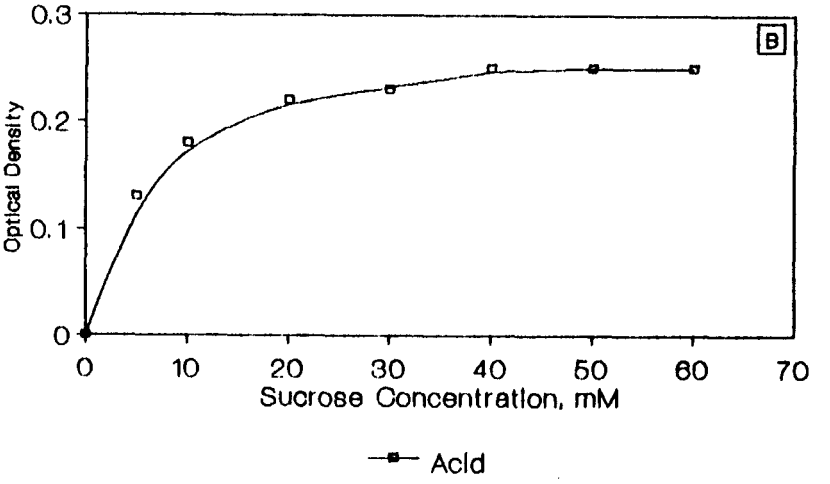


Propagule: Stem (middle segment)

Fig. 6: Substrate saturation curve of Invertase



Propagule: Stem (lower segment)



Propagule: Chlorophyllous

Fig. 7: Substrate saturation curve of Invertase

The K*m* values calculated from the substrate saturation curves gave 15, 12, 10, and 10 m*M* for the upper, middle and lower segments of stem (at the rooting stages) and the stem tip at the mature vegetative stage (12 months) respectively.

B. Leaf

i. Achlorophyllous

Acid invertase

A typical Michaelis-Menten kinetics was observed with substrate concentration in the range 5 to 200m*M*. Saturation was attained at a concentration of 120 mM sucrose with no further change in velocity at higher concentration. For assays, the substrate concentration was kept at 150 m*M*.

The K*m* value as calculated from substrate saturation curve was 20 m*M*.

[Figs. 8 A and 10 A]

Neutral

Substrate saturation was tested in the range 5 to 100 m*M*. Michaelis-Menten curve showed that substrate saturation was reached at 70 m*M*. Thereafter the activity remained constant. In routine assays 75 m*M* sucrose was employed.

The K*m* value as calculated from the substrate saturation curve was about 10m*M*.

[Figs. 8 B and 10 B]

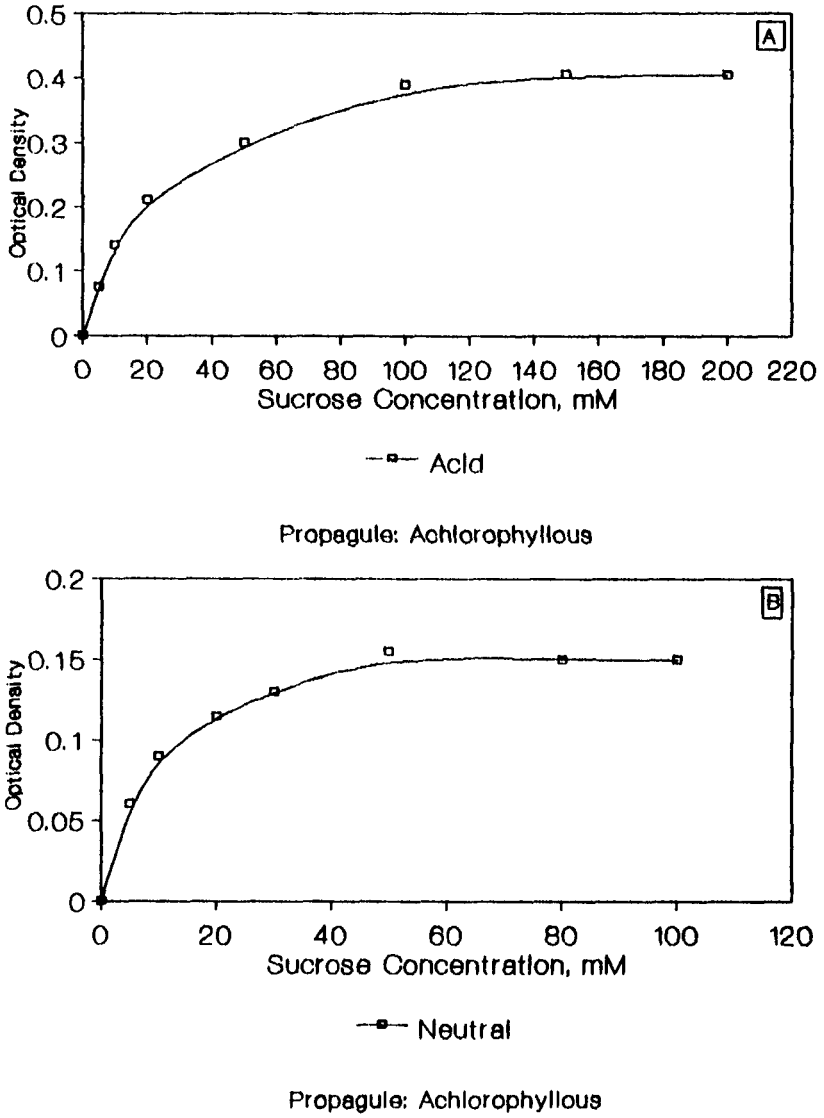


Fig. 8: Substrate saturation curve of Invertase

ii. Chlorophyllous

Tested in the range 5 to 60 m*M* sucrose, a typical Michaelis-Menten behaviour was observed with saturation attaining at 40 m*M*. Thereafter, the activity remained constant. In routine assays, sucrose concentration was kept at 50 m*M*.

The K*m* value calculated from the substrate saturation curve was 5 m*M*.

[Figs. 7 B and 9 B]

Comparison of K*m* values

Michaelis-Menten constant (K*m*) relates to the affinity of an enzyme towards its substrate. A comparison of various K*m* values obtained for the various tissues in the pineapple plant showed that the chlorophyllous acid invertase has the highest affinity with the lowest K*m* value of 5 m*M*. The stem tissues (upper, middle and lower) gave near identical values in the range 10 to 15 m*M* which was double that for the chlorophyllous acid invertase. Of the acid invertases, the achlorophyllous invertase showed the highest K*m* (therefore, the least affinity) of 20 m*M*. This was double that of the stem acid invertase.

Neutral invertase in the stem tip had the same K*m* value as that in the achlorophyllous region (10 m*M* each). This value was comparable with the K*m* for the acid invertase in the stem.

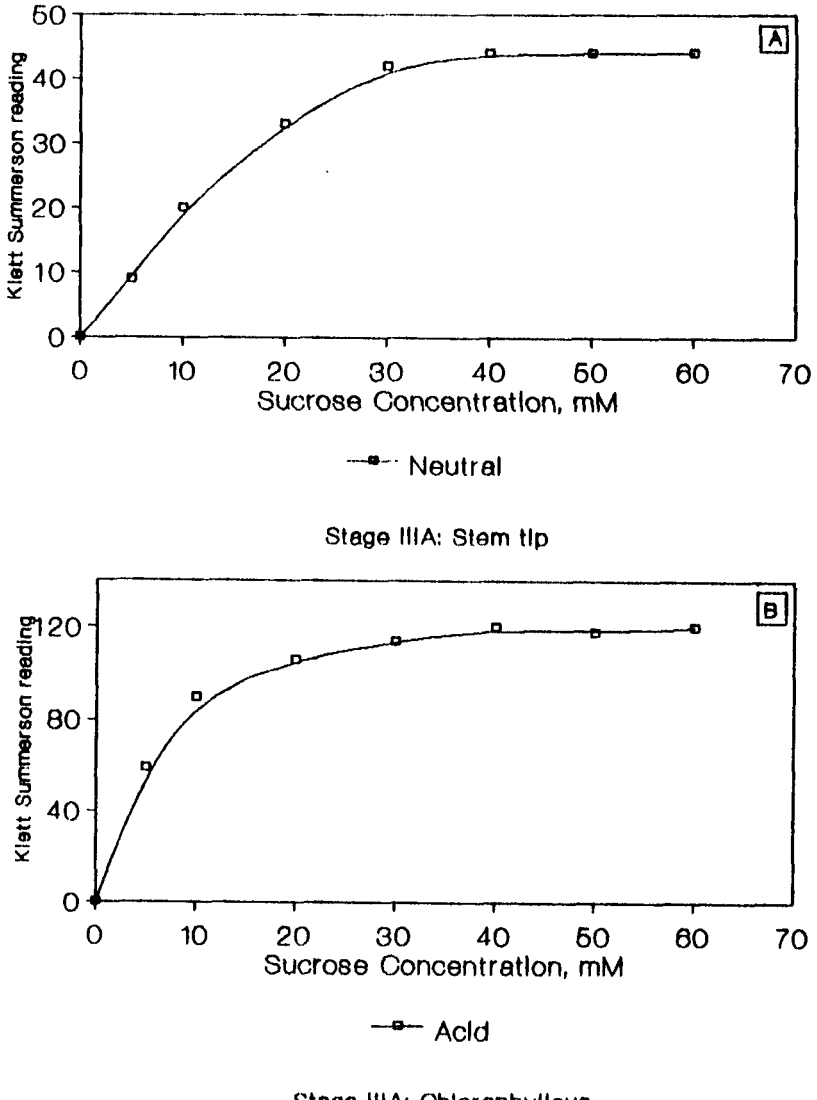
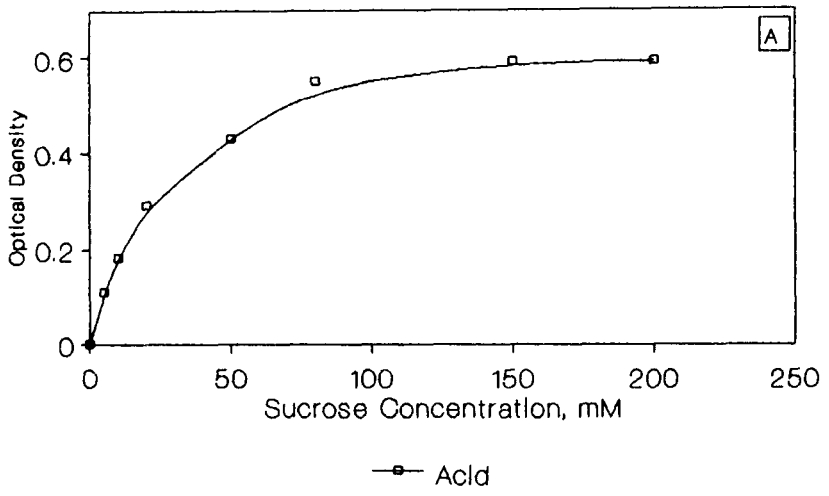
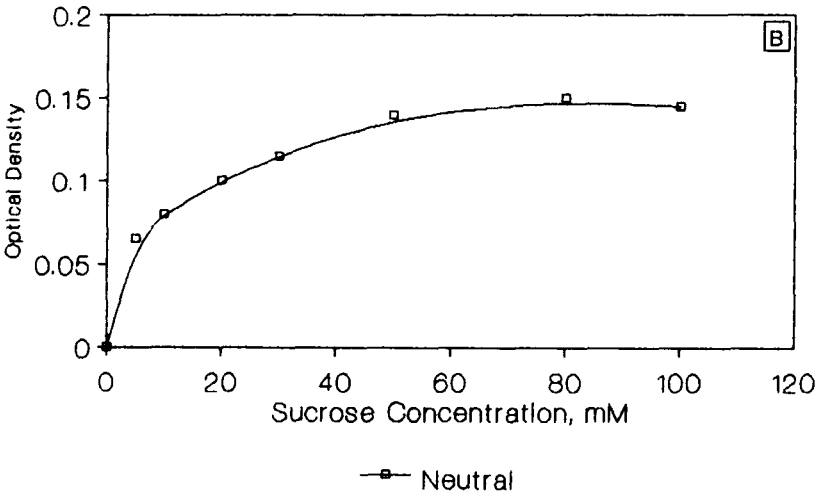


Fig. 9: Substrate saturation curve of Invertase



Stage IIIA: Achlorophyllous



Stage IIIA: Achlorophyllous

Fig. 10: Substrate saturation curve of invertase

When the acid - and the neutral invertases were present together in achlorophyllous tissue, the affinity of the enzyme for sucrose was greater in the neutral form. That this has physiological significance is a distinct possibility.

Whether the acid invertase of different tissues belong to the same enzymic protein or whether they are isoenzymic forms is not certain. However, the wide variation in the K*m* value between the chlorophyllous and achlorophyllous acid invertases may point to distinctly different enzymic proteins.

Incubation period and enzyme activity

Employing the enzyme preparations (gel-filtrates) from the various tissues, in the proportionality range and substrate concentration at saturating levels the assay system was incubated for 10, 20, 30, and 45 min. A straight-line relationship was obtained for all preparations over the entire range of time, showing that the activity was directly proportional to the incubation period (Fig. 11 A). The shorter the reaction time, the greater the possibility that the initial rate of reaction of an enzyme is being determined. In regular assays, the incubation period was held at 20 min rather than 10 min since absorbance readings under this condition fell in the most sensitive range of the instrument.

[Fig. 11 A]

Incubation temperature and enzyme activity

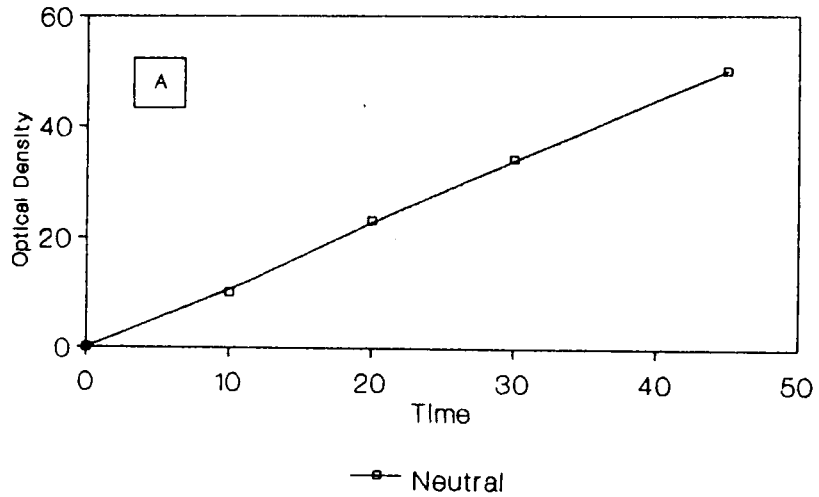
Using the enzyme preparation from Stage III A stem tip, the assay was carried out, at 30, 35, 40, and 50°C, with incubation period of 30 min. The peak of activity was at 35°C, with 30 minutes incubation. The invertase activity was found to be markedly reduced above 50°C. The regular assays were conducted at 30°C, which during the greater part of the year was only slightly higher than the ambient temperature so that the thermostatic mechanism of water bath functioned most efficiently.

[Fig. 11 B]

The large majority of enzymes are heat-sensitive. Apart from enzymes of thermophilic organisms a few enzymes from higher tissues are heat-stable. One of the best examples is pancreatic ribonuclease. The temperature sensitivity of the pineapple enzyme was somewhat unexpected. The pineapple plant has properties of a xerophyte and its fleshy tissues may be expected to attain high temperatures in the sun. Again, invertase is a glycoprotein (*c.f.* Strumeyer and Malin, 1970) the carbohydrate molety of an enzyme usually bestows thermostability.

Stability of enzyme preparation

On storage of the gel filtrate of an extract of stem tip of mature vegetative plant (Stage III A) inside a refrigerator, the (neutral) invertase activity was observed to decrease continuously. There was about 50% decrease in activity after 2 days storage. Results reported earlier had shown that, in association with tissue matter,



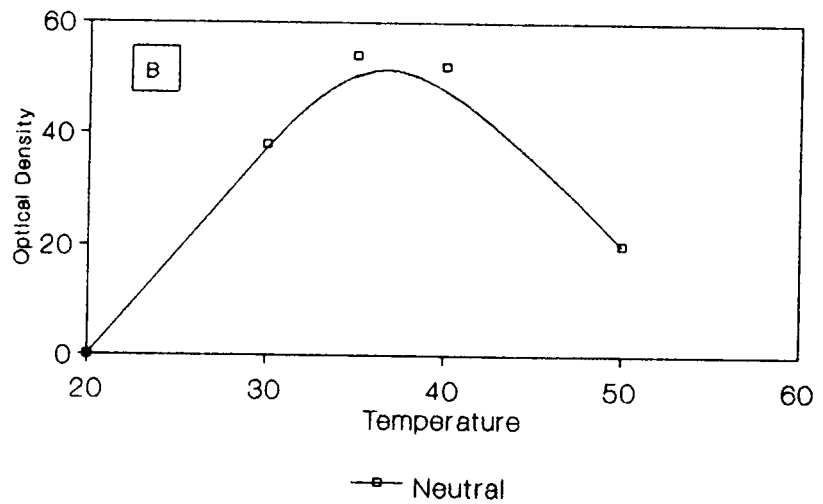


Fig. 11: Proportionality with respect to time, and temperature

A: Time proportionality; B: Temperature proportionality

invertase was highly stable in the frozen state and that extracts in buffers were stable for several hours when maintained in the cold. It was possible that gel filtration was associated with the removal of endogenous stabilizing factor(s) of low molecular weight. The dilution resulting on gel filtration might also be a contributory factor to storage sensitivity.

Part B : Physiology

Ontogenetic changes in physiological and biochemical parameters in the pineapple plant - Parameters considered

The parameters considered were:

1. dry solids content (-moisture status-)
2. protein content

   and
3. invertase activity

1. Dry solids content (-moisture status-)

[Figs. 12 and 13]

The data are presented in Tables 3 A, 3 B, 4 and 8.

Ontogenetic changes could be interpreted along two lines; (A) stage-specific, in which the events occurring in the stem and leaves are considered together at every

stage and (B) tissue-specific, in which the changes in a given tissue are followed over the entire ontogeny.

A. Stage-specific

State I: The percentage dry weight in the upper stem segment was 10.98. The middle and lower portions had 10.6% ($P<0.01$) and 63% higher values respectively ($P<0.01$).

The lowest value of 6.21% (the minimum among all tissues of all stages) was observed in the achlorophyllous leaf part in this stage. The chlorophyllous tissue had almost double the value, as the achlorophyllous ($P<0.01$).

Stage II A: In the stem, the upper segment registered the minimum value. There was a 45% higher value ($P<0.01$) in the middle portion. This dry weight was the highest for any tissue at any stage of ontogeny. The lower portion had 23% less ($P<0.01$) dry weight than the middle.

The achlorophyllous region had 42% lower dry matter content ($P < 0.01$) than the upper stem segment. The chlorophyllous region had 38% higher dry weight than the achlorophyllous ($P < 0.01$).

Stage II B: As in Stage II A the minimum value was in the upper segment. The middle segment had 34% ($P < 0.01$) more dry weight. In this respect Stage II A and II B were nearly identical. The lower segment in Stage II B had only marginally lower value (N.S.) than the middle segment at this stage. Achlorophyllous region

registered the minimum dry weight among different tissues in this stage also. The chlorophyllous tissue had 86% more dry weight than the achlorophyllous ($P < 0.01$).

Stage III A: Stem tip had 12.2% dry matter. As in every stage, Stage III A also had the minimum dry matter in the achlorophyllous region among the different tissues. The dry matter was more than double in the chlorophyllous than in the achlorophyllous ($P < 0.01$).

From Stage II B to Stage VI, a more or less similar pattern in terms of dry weight as in III A was observed. However, in the VIIth Stage, the achlorophyllous registered a higher value which was the highest (8.94%) among the achlorophyllous tissues.

Tissue - specific

Stem tip: The upper segment of stem registered the minimum value at Stage I. An increase of 61% ($P<0.01$) occurred at Stage II A. The value was the maximum among all stages for the stem tip. There was a marginal decrease of 8.5% ($P < 0.01$) in Stage II B. There was a further decrease of 25% ($P<0.01$) in Stage III A. The value was nearly the same in Stage III B. At Stage IV, a marginal increase of 10% ($P < 0.01$) was observed. The values remained nearly constant in Stage V and VI. A drop of 16% ($P<0.01$) occurred at Stage VII. The dry weight at this stage was almost the same as in Stage I.

Stem (middle) The minimum value was in Stage I with more than doubling at Stage II A ($P < 0.01$) Stage II B registered a 15% ($P < 0.01$) reduction from the preceding stage.

Stem (lower) There was a gradual increase in percentage dry weight from Stage I to Stage II B. The increases were 10% (N.S.) from Stage I to II A and 5% from Stage II A to II B (N.S.).

Achlorophyllous: The lowest value was in Stage I. A significant increase of 64% ($P<0.01$) occurred in Stage II A, with a reduction of 26% ($P<0.01$) in Stage II B, and a further reduction of 12% ($P<0.01$) in Stage III A. The values remained nearly constant upto Stage VI. At Stage VII, 25% ($P<0.01$) increase in percentage dry weight from the preceding stage occurred, to reach the maximum value for this tissue at any stage.

Chlorophyllous: As in other tissues, chlorophyllous region had the minimum dry weight at Stage I. An increase of 15% at Stage II A was significant ($P<0.01$). Only marginal fluctuations occurred thereafter in dry weight percentage upto Stage VII.

Overview

The pineapple in common with many other bromeliaceous plants, has water storage tissues, which is the basis for designating the plant as a 'succulent' or xerophyte. The non-chlorophyllous leaf part, in spite of its higher water content, does not appear to have water storage cells. The water-storage tissue is prominent in the chlorophyllous region of the leaf; in the fully turgid pineapple, this occupies

approximately half the leaf cross section in the median portion of the central region (Krauss, 1949). In the sub-chlorophyllous region, the tissue assumes water storage function as the chlorophyllous region is approached.

An increase in dry solids (-moisture loss-) could be an artefact of desiccation or as a result of active redistribution or an active accumulation of dry matter. As mentioned earlier, the plants were profusely watered on the previous day of plant collection. Desiccation due to environmental factors such as temperature, humidity etc. may not have appreciably influenced the water status in the tissues. However, in Stage II A and to a lesser extent in Stage II B partial desiccation due to internal water redistribution might have occurred as a result of inadequate development of soil roots for water absorption. The marked increase in dry solids in the stem regions in Stages II A and II B may be a result of this water redistribution. In the mature vegetative and the reproductive stages the increase in dry weight is more likely a result of active accumulation of metabolites as the plants are fully autotrophic by this time.

II. PROTEIN CONTENT

The word "content" is used here in the general sense. The various tissues were not weighed whole, but only sampled for analyses. A more appropriate term is probably "concentration", that is, content per unit weight of tissue.

Unless otherwise specified, the interpretation of the protein content will be on the basis of the dry weight of tissue.

[Figs. 13, 14 and 15]

[Tables 3 A, 3 B, 4 and 8]

The results are put under three categories/headings - pre-autotrophic; mature, vegetative; and reproductive - so that the changes can be correlated with the physiological state of the plant. Wherever necessary, tissue-specific and stage-specific comparisons have been made.

Pre-autotrophic (semi-autotrophic) stages:

Propagule and its rooting

Stage I

Stem: Protein content was lowest in the upper part of the stem, rather unexpectedly; it increased 19% in the middle part ($P<0.01$) and this level was practically maintained in the lower part, although this tissue was harder.

Leaf

Achlorophyllous: The protein content in the achlorophyllous leaf part, the maximum in this tissue at any stage, was over 2-fold ($P<0.01$) as high as in the various segments of the stem.

Chlorophyllous: The protein content in the green leaf was only marginally higher (5.8%; N.S.) than in the achlorophyllous leaf part unlike in most other stages when protein content was much higher in the green tissue.

Stage II A

Stem: The pattern of protein distribution in the stem was substantially altered following planting of propagule. Protein decreased 10% in the upper part ($P < 0.01$) and 16.4% in the middle part ($P<0.01$). In marked contrast, protein content doubled in the lower portion ($P<0.01$), which was unexpected because this tissue tended to turn "woody". The lowest protein content was in the upper part, as in the propagule, but this was only 10% less than in the middle part ($P<0.01$). The protein content in the lower part was 2.7-fold that in the upper part ($P<0.01$) and 2.4-fold that in the middle part ($P<0.01$). Protein in the upper part was the lowest at any stage of stem ontogeny. As already pointed out, the upper part in Stage II A had more dry solids than the corresponding part in any other stage.

Leaf

Achlorophyllous: Protein content in the achlorophyllous leaf part, per g fresh weight, decreased 22.4% following propagule planting ($P<0.01$). Since dry solids content increased in this tissue, the decrease in protein content was particularly marked when expressed per g dry weight (52.7% $P<0.01$). The protein content in the achlorophyllous leaf was 40.8% and 27% higher than the content respectively in the upper and middle part of the stem ($P<0.01$ and $P<0.01$ respectively), but it was 48% lower than in the lower stem part ($P<0.01$).

That a change occurred in protein per g dry weight in Stage II A in relation to Stage I and that the magnitude of this change was so high argued against a

concept that the dry solids increase (reported above) was an artefact of as yet inadequate water uptake from soil environment. It appeared as if protein was in a labile form in the achlorophyllous leaf part, readily mobilizable to meet the needs of transition of the plant from the semi-autotrophic to the fully autotrophic state. It was possible also that the decrease in protein was likely to be a stress response to desiccation consequent on transplanting.

Chlorophyllous: Protein was found to have decreased also in the green leaf, but the decrease was only 20.4% ($P<0.01$); it was now 78.1% higher than in the achlorophyllous part ($P<0.01$).

Protein content, thus, varied independently in the achlorophyllous and chlorophyllous leaf parts; it was pointed out earlier that dry solids also varied independently in the green - and non-green portion of the leaf, during the growth of the plant.

Stage II B

Stem: Protein content increased in relation to the one month old stem, in particular in the lower segment (27%, $P<0.01$). The distribution pattern was, however, the same as in Stage II A, in that the maximum was in the lower part and the minimum in the upper part. The lower part had over 3-fold the protein content of the upper part and 2.7-fold that in the middle part.

Leaf:

Achlorophyllous: Protein increased 50% in comparison to Stage II A ($P<0.01$), which was more prominent than the increase in any part of the stem. As in Stage II A, protein in the achlorophyllous leaf was higher than in the upper and middle stem (respectively 88%, $P<0.01$, and 63%, $P<0.01$), and less than in the lower part 39%, $P<0.01$).

Chlorophyllous In the 2 months old plant, there was 66% increase in protein ($P<0.01$), an increase which coincided with the 50% increase in the achlorophyllous part. The highest protein content in the chlorophyllous part in any stage was recorded in Stage II B.

Autotrophic stages

A. Vegetative: Stages III A and III B

Stem tip: In Stage III A. (12 months old) the protein content in stem tip increased 25% in relation to the top segment of the stem of the 2 months old plant (II B) ($P<0.01$).

In the 14 months old plant (III B), there was 15% decrease in the stem tip ($P<0.01$). The protein content in Stage III B represented the lowest among the autotrophic stages, though it was higher than in all the pre-autotrophic stages. It was of interest to note that, on fresh weight basis, protein content in stem tissue was the

lowest in Stage II B among all the developmental stages, barring the propagule; this was in keeping with the near constancy of dry solids in Stages III A and III B.

The stem tip (rather, the apex), with reduced protein content, was now fully poised to respond to signal from leaf and transform into the reproductive state.

Leaf

Achlorophyllous: Protein content from Stage II B decreased 29% ($P<0.01$) in Stage III A, contrasting with the increase occurring in the stem tip. It was now only marginally higher in the achlorophyllous leaf than in the stem tip (6.0%, N.S.).

The protein content was further reduced, by 10% ($P<0.02$) in the 14 months old plant; the content was now the lowest at any vegetative stage in the achlorophyllous leaf. Protein in the achlorophyllous leaf was 12% higher ($P<0.05$) than in the stem tip. The plant readying to flower had lowered protein both in the stem tip and the achlorophyllous part of the leaves attached to the stem tip.

Chlorophyllous: In Stage III A, along with the decrease in the achlorophyllous leaf part, there was a marked drop (31%, $P<0.01$) in protein content in the green part. The level of protein remained unaltered in the 14 months, contrasting with the decrease which occurred both in the stem tip and in the achlorophyllous leaf. Protein in the green leaf was 92% ($P<0.01$) and 112.0% ($P<0.01$) higher than in the achlorophyllous leaf, respectively in Stages III A and III B.

Thus, a lowered level of protein in the achlorophyllous part and a low level maintained in the chlorophyllous part, seemed to characterize the leaf undergoing the induction reactions. This was followed by a sharp decrease in the chlorophyllous leaf part, but a sharp increase in the achlorophyllous part, when the transition actually occurred in the apex.

B. Reproductive stages

Stage IV: Transition stage

Stem tip: The protein content, per g dry weight, increased 28% ($P<0.01$). Since the dry solids increased at this stage, the increase in protein was more marked when expressed per unit fresh weight of tissue (41%, $P<0.01$).

An effect observed on analyzing a 5 g portion of the stem tip might be a diluted form of the event occurring specifically at the apex proper; it may also be a primary event in the subjacent tissue, modulating the response by the apex.

Leaf: Pronounced changes in protein content seemed to characterize the leaf parts immediately following the induction reactions.

Achlorophyllous: Protein content increased by 24% ($P<0.01$), coinciding with the increase in the stem tip. Protein in the achlorophyllous leaf continued to be higher than in the stem tip, but the increase was marginal (8%, N.S.).

Chlorophyllous: Contrasting with the increase in the achlorophyllous leaf part, there was 29% decrease in protein content in the green tissue ($P<0.01$).

Stage V: Organogenesis

Stem tip: There was a small increase in the protein content (7.4%, N.S).

Leaf

Achlorophyllous: Protein decreased by 42% ($P<0.01$) to reach the lowest value in the achlorophyllous leaf tissue at any growth stage. Protein in the tissue was lowest also on fresh weight basis.

Protein in the achlorophyllous leaf was 42% ($P<0.01$) lower than in the stem tip. The organogenetic stage marked the commencement of a lowered level of protein in the achlorophyllous leaf in relation to the stem tip. This decrease occurred at a time when there was increased protein in stem tip, though marginally.

It appeared that protein was mobilized from the achlorophyllous leaf part to meet the needs of organogenesis at the shoot apex. A depletion of protein in the achlorophyllous leaf was observed also in Stage II A.

Chlorophyllous: The protein content increased in Stage V (20%, $P<0.01$), contrasting with the marked decrease in the achlorophyllous part. There was apparently a partial restoration of protein lost during Stage IV. This level represented the highest protein content among all reproductive stages, but was only about one-half the peak content attained in the 2-months old plant. Protein in the green leaf was about 1.5-fold higher than in the achlorophyllous leaf ($P<0.01$).

Distribution between the achlorophyllous and chlorophyllous leaf parts was often one of magnitude, but the direction of changes sometimes altered. The increase in the green leaf in Stage V occurred when there was a marked reduction in protein content in the achlorophyllous leaf part. Independent changes in chlorophyllus and non-chlorophyllous leaf parts were noticed also in dry solids content.

The total dry solids content was preferentially labile in the achlorophyllous leaf part, as pointed out above, but there was no indication of a preferential lability with respect to protein in either leaf part, the content in both altering during the growth of the plant. The variation was in the same direction in Stage II A, II B, and III A, but in opposing directions in Stage IV, V and VI. In Stage III B, protein content remained unaltered in the chlorophyllous part, but decreased in the achlorophyllous part. In Stage VII the variation was in the same direction.

C. Post-organogenic stages: Stages VI and VII

Stem tip: Protein content was practically unchanged in Stage VI. A marginal increase occurred in Stage VII.

Leaf

Achlorophyllous: Protein increased in the achlorophyllous leaf in Stage VI and Stage VII, with partial restoration to the level in Stage IV. The increase was only 14% in Stage VI ($P<0.05$). In Stage VII, protein increased 34% on dry weight basis ($P<0.01$). Since there was an increase also in dry solids percentage, the increase in protein per

unit fresh weight of tissue was highly prominent (67%, P<0.01). Protein was 34% (P<0.01) and 17% (P<0.01) lower than in the stem tip respectively in Stages VI and VII.

Chlorophyllous: Protein decreased continuously in the green leaf in the post-organogenic stages, contrasting with the increases in the achlorophyllous leaf part and the stem tip. In Stage VI, the decrease was 48% (P<0.01), when protein in the green leaf exceeded that in the achlorophyllous leaf by 12% (P<0.05). The increase in Stage VII was 32% (P<0.01). Protein was now higher in the chlorophyllous than in the achlorophyllous leaf, though marginally (13%, P<0.01). Unlike in all the other stages, the protein content of the green leaf was lower than that of the stem tip (6%, NS).

Apparently, storage proteins (Fraction-I protein is one) were being degraded by endogenous proteolysis to meet the requirements of the developing fruit.

C. Root tip

The difficulties encountered in the collection and analysis of root tissue have been mentioned in *Materials and Methods.* Analyses were carried out in the propagule (Stage I) and one month- (Stage II A) and two months old plants (Stage II B), representing the pre-autotrophic stages; for comparison, analyses were also carried out in one of the mature stages, that is, Stage V. Dry weight determinations were not carried out and hence the values are reported on a fresh tissue basis.

This results are represented in Table 9.

The content was lowest in the propagule. There was only marginal increase in Stage II A (7%) and a further increase of 57 % in stage II B. The highest 'content' was in Stage V, which was only 12 % higher than that in Stage II B, but was 87 % higher than that in the propagule.

In the propagule, the protein content was much higher in the root tip (6 to 200%) than in the three stem segments. A more or less similar relationship was seen with the achlorophyllous tissue (100 % higher). However, the chlorophyllous tissue had nearly the same protein content as the root tip, on a fresh weight basis.

In Stage II A, the protein content remained higher in the root tip (125% and 40%) in relation to the upper segment and the middle segment respectively. However, the value was lower (25%) in the root than in the lower stem segment at this stage. The achlorophyllous tissue had considerably lower (64%) protein content than the root tip tissue. Protein was marginally higher (13 %) in the root tip than in the chlorophyllous tissue on a fresh weight basis.

In Stage II B, a similar pattern as in Stage II A was discernible in the protein content, but the magnitude of changes were more prominent. The root tip had 244% and 123% higher protein than the upper and middle segments respectively. In contrast, protein content was 13 % lower in the root tip than in the lower stem segment.

The achlorophyllous leaf region in Stage II B had only a fourth of the protein as in the root tip. The chlorophyllous leaf tissue had only marginally lower protein content (6%) than the root tip tissue.

In Stage V, the root tip tissue had 223% higher protein content than the stem tip. In comparison to the achlorophyllous leaf region, the root tip had 9.6-fold higher protein content which was very prominent than the preceding stages. In the chlorophyllous tissue, the protein content was only 46 % as that of the root tip.

On a fresh weight basis, root tip had consistently higher protein value than the stem tip, achlorophyllous or chlorophyllous tissues, the exception being the lowest segments in Stage II A and II B. As a generalization, one can say that the lower segment and root tip resembled in protein content in the pre-autotrophic stage, with progressive increase in protein content in both. The high levels of protein in the root tip may reflect high metabolic activities inclusive of protein biosynthesis. Whether the high values obtained for protein was an artefact due to the pigmentation in the tissue homogenate resulting in interference in the colorimetric determination of proteins in homogenates (as mentioned in an earlier section) is not certain.

Protein extractability

The solubility (extractability) pattern of protein in the root tissue in the basal medium was comparable to that of the stem tissue, particularly middle segments in Stages I, II A and II B (Table 3 A). Only about 24 to 30 % of the protein in the

root tissue homogenates passed on to the supernatant in Stage I to II B. By Stage V, the extractability was considerably reduced to about 15%.

In pineapple plant, roots originate from the middle and lower segments of the stem. That the two tissues (root and stem (middle and lower segments) share common protein solubility characteristics is an interesting observation worth investigation.

Protein solubilization

Since the residue on centrifugation of homogenate was not re-extracted with medium (to avoid dilution of the extract which was to be gel filtered, vide *Materials and Methods)*, protein in the residue would be a slightly higher value; but, by packing the residue hard on centrifugation and carefully draining away the supernatant, the wetting of the residual fraction and the sides of the centrifuge tube was kept to a minimum. The sum of the protein in the residual fraction and the supernatant gave the value for a reconstituted homogenate; this value did not always tally exactly with the value determined directly on the homogenate. The percentage recovery reported in the extract/residue was calculated in relation to the total protein directly determined in the homogenate. Protein solubilization data were compiled as part of invertase activity studies using fresh/frozen tissue samples and hence percentage recoveries are calculated on fresh weight basis.

The results are recorded in Table 5.

Stem

Propagule: The highest solubilization of protein was from the upper part; there was progressive decrease in the middle and lower part.

Stage II A: The pattern of solubilization of protein was the same as from the propagule; however, the decrease in the extraction from the middle and lower part was steep. The extract from the lower segment contained as little as 8% of protein. The sum of the recovery of protein in the separated fractions from the lower part of stem was only 86%.

Stage II B: The same pattern of protein solubilization was observed in Stage II B. Only 5.3% of protein was recovered in the extract from the lower part of the stem. In this case also, about 10% protein could not be accounted for, in the reconstituted value for the homogenate.

The reason for the low solubilization of protein from the lower part of stem in Stage II A and II B may be because of the fibrous nature of the tissue. Interference in estimation by the pigment in the protein solution from the homogenate and residual fraction (*Materials and Methods*) was a possibility.

Stage III A to VII: Protein recovered in the extract of stem tip ranged between 30 and 45%.

Leaves

Achlorophyllous: Among the tissues studied, the highest solubilization of protein was from the achlorophyllous leaf; the extraction from the propagule tissue was particularly prominent. In all the post-transitional stages, the protein solubilization was less than in the vegetative stages, and the transitional stage.

The higher solubilization of protein from the achlorophyllous leaf part may be associated with the higher moisture content of the tissue (page 109). Ice crystals formed during freezing of tissue samples promote cell rupture. At the same time, it was possible that protein in the achlorophyllous part of the leaf had distinctive solubility characteristics; there might be a higher proportion of albumins in this tissue.

Chlorophyllous: Although protein content was the highest, on a homogenate basis, in the chlorophyllous region among the tissues examined, trichloroacetic acid failed to yield a precipitate when the gel filtrate, or the extract itself, was acidified. It is likely that the bulk of the proteins in the chlorophyllous tissue is in a nonextractable form in the basal medium. Invertase is, however, mostly soluble under these conditions. Practically the entire protein in the homogenate was found in the residue on centrifugation.

pH of homogenates

The homogenate of the tissues prepared in the neutral basal medium had pH values in the ranges:

stem upper segment:	pH 6.6
stem middle part:	pH 6.6
stem lower part:	pH 6.6
achlorophyllous:	pH 7.1
chlorophyllous:	pH 5.0 to 5.2

The reason for the lack of solubilization of protein from the green leaf was not clear. Maximum acidity in Crassulacean tissues develops at night. The pH of the homogenates of the chlorophyllous leaf part was in the range 5.0 to 5.2, contrasted with pH values around neutrality for homogenates of stem tissue and of achlorophyllous leaf tissue. It was possible that the acidity developing during homogenization of green leaf denatured the proteins, rendering them insoluble. However, experiments in which the tissue acidity was neutralized with $NaHCO_3$ during grinding (*Materials and Methods*) again failed to extract proteins. Thus, the raising of the pH of homogenate to neutrality appears to have little influence on protein solubility. Whether the chlorophyllous tissue is characterized by proteins which are mostly insoluble in the low ionic strength of the medium is not certain, but is a possibility.

As will be pointed out elsewhere, acid invertase was highly active in the gel filtrate of extract from green leaves. A fortuitous outcome of the absence of appreciable protein in the extract was the several-fold enrichment of the invertase in green leaves by the single step of grinding the tissue with weakly buffered neutral medium.

At the same time, and by the same reasoning, it was possible that invertase in green leaf was in part denatured along with the total denaturation of other proteins and that a part of the enzyme was inactivated and rendered insoluble during homogenization. This would mean that the values obtained for invertase activity in green leaves were low. It was possible, also, that a neutral form of invertase was originally present in the green leaf, as in the achlorophyllous leaf part, but that, unlike the acid invertase in the tissue, it suffered total inactivation during the homogenization process. This possibility was discounted since no neutral invertase could be demonstrated in gel filtrates of extracts or the residual fractions when $NaHCO_3$ was used to neutralize the acidity during tissue homogenization (*Materials and Methods*).

III Invertase Activity

It is necessary to touch upon a few points before presentation of the data and their interpretation:

1. In the case of the chlorophyllous leaf part: (a) protein in the extract was apparently too low a quantity to be precipitated by trichloroacetic acid (Table 8), although the extract had comparatively high invertase activity (b) the homogenate of the tissue in weakly buffered neutral medium (successfully used with other tissues to achieve homogenates with near-neutral pH), had a low pH which in all probability resulted in denaturation and precipitation of non-invertase protein. Furthermore, the residual fraction resulting on

centrifugation of homogenate was devoid of invertase activity, even after treatment with Triton X-100 (vide an earlier section).

2. Invertase activity determination was in gel filtrates of the extracts of the various tissues. In the case of stem tissue and achlorophyllous leaf part, the specific activity was calculated, in the conventional manner, in relation to the protein in the gel filtrate. Since this was not possible in the chlorophyllous leaf part, an analogous value for the green tissue was calculated in relation to protein in the homogenate. For purpose of comparison, such values have been calculated also for the stem tissue and achlorophyllous leaf part and reported in the table, though not discussed. These values enabled the calculation of enzyme "enrichment" in the extracts (analysed as gel filtrates).

3. The specific activity was a reliable value when reported on the basis of protein in gel filtrates, since this estimation was accurate. But, when related to the protein in tissue homogenates, the specific activity value was subject to any errors in the determination of protein in the particular tissue. As pointed out under *Materials and Methods*, the protein solutions used for the final estimation were devoid of extraneous colour only in the case of the stem tip and achlorophyllous leaf part. Error in the estimation of protein in tissue will affect also the calculation of enrichment of activity; this will be evident while considering middle and lower stem segments in Stages I and II A and II B.

Data on invertase activities in various tissues are given in Tables 6 A, 6B, 7, and 8 and Figs. 16, 17, 18, 19, 20 and 21.

Pre-autotrophic stages: Stages I, II A and II B

[Fig. 16]

[Table 6 A]

Stem: Invertase acid total activity in the upper segment of the stem of the propagule was the highest among any tissue in the pre-autotrophic stages. The one month (II A) - and two months (II B) old plants had 43% ($P<0.01$) and 34% ($P<0.01$) activities as the propagule. Specific activity showed a similar pattern.

In the propagule, invertase total activity in the middle segment of the stem was nearly the same as in the upper portion. The total activity registered a marked drop in the lower part (49%, $p<0.01$); however, the specific activity of the enzyme remained unaltered.

In Stage II A, the total activity in the middle segment of stem was only marginally lower than in the top part; but this activity was markedly lower (57%, $P<0.01$) than that of the corresponding part in the propagule. The specific activity in the middle segment was 28% ($P<0.01$) higher than the top segment. Enzyme activity in the lower segment of stem in Stage II A was of interest. The total activity was 68% higher than in the upper segment ($P<0.01$), in spite of the fact that protein extractability was only about a fifth; also, it was considerably higher 43%, ($P<0.01$),

than in the corresponding part in the propagule. The specific activity increased 144% in relation to the middle part ($P<0.01$), attaining the second highest value for any stem sample in the pre-autotrophic stages. Enzyme enrichment in the lower part of stem was about 13-fold, as against 4-fold in the middle part.

Invertase total activity in stem top in Stage II B was reduced from the level in Stage II A by 21% ($P<0.05$) the activity in the middle was 41% ($P<0.01$) lower. The lower segment showed only a marginal increase from Stage II A. The total activity in the middle part of stem in Stage II B was only 71% that in the upper part, ($P<0.01$) although protein solubilization was only 6% less (Table). The specific activity of the enzyme, the lowest level in stem part in any pre-autotrophic stage showed an identical decrease. In the lower segment of stem, the total activity was 2.3-fold higher than that in the middle part ($P<0.01$) and the specific activity was over 5-fold higher although protein extraction in the lower part was only 5.3%,a fifth of that from the middle part. This was in support of increased invertase formation in the lower part of stem also in Stage II B. The enrichment of activity was as high as 20-fold.

The reliability of the data for the specific activity on gel filtrate protein basis, but plausible flaw in the values for enrichment, were pointed out earlier.

Leaf

Achlorophyllous

Acid: Acid invertase total activity in the achlorophyllous leaf part was higher than that in the stem tissues in all pre-autotrophic stages. The enrichment of enzyme in the extracts was 2-to 2.5-fold.

The total activity decreased 50% in Stage II A ($P<0.01$) parallel with the decrease in protein content; the specific activity increased 34% ($P<0.01$). This suggested a preferential retention of invertase protein in this stage, a physiological need; it could also be an artefact of preferential solubilization of invertase protein during tissue grinding in the buffered medium. Total activity increased 46% ($P<0.01$) in Stage II B, corresponding to a 50% increase in protein; the specific activity remained unaltered.

There was hardly any relation between the changes in (acid) invertase activity in the stem segments and the achlorophyllous leaf part.

Neutral: Neutral invertase was always less active than acid invertase in the achlorophyllous leaf tissue, expressed as total activity or specific activity. Neutral invertase, both total and specific, in the achlorophyllous leaf tissue was more active than the acid activity in stem parts in the pre-autotrophic plant. The enzyme was enriched 2-to 2.5-fold in the various extracts.

Total neutral invertase activity decreased over 50% ($P<0.01$) in Stage II A; the specific activity increased 30% ($P<0.01$) signifying preferential retention or solubilization of invertase protein as mentioned earlier. The total activity increased 77% in Stage II B ($P<0.01$); the specific activity increased 23% ($P<0.01$).

The pattern of activity changes in the achlorophyllous leaf appeared to be more or less the same in acid and neutral invertase, whether calculated per g dry tissue or mg protein in gel filtrate. However, for a given type of enzyme, there was not only quantitative but qualitative variation in activity per g dry tissue and per mg protein, because of change in protein content. Thus, total activity of the acid/neutral enzyme decreased in Stage II A, but the activity per mg protein in tissue increased.

Chlorophyllous

Invertase (acid) was always more active in the green leaf than in the achlorophyllous leaf (-and in the stem segments of the pre-autotrophic plant-). Since protein could not be estimated in extracts, "enrichment" could not be quantified but it was likely to be of a higher order than in the achlorophyllous leaf and the stem tissue.

Acid invertase total activity decreased 18% ($P<0.01$) in Stage II A and then increased 12% ($P<0.01$) in Stage II B, reaching the peak of activity at any stage. The decrease in invertase activity in the green leaf in Stage II A was similar to a decrease in the acid invertase in the achlorophyllous leaf.

The specific activity remained nearly the same in Stage I and in Stage II A, but, decreased 33% in Stage II B ($P<0.01$). Protein content had decreased 20% in Stage II A and then increased 66% in Stage II B to reach the maximum value in any stage for the chlorophyllous part. It appeared as if invertase protein was preferentially retained in the green leaf, as in the achlorophyllous leaf part, in Stage II A.

Autotrophic stages

Mature, vegetative: Stages III A and III B

Stem tip

Propagules started emerging when the plant was mature.It was of interest to note that the mother plant with neutral invertase activity in the stem tip had the acid form of activity in the stem tip of its offshoot.

Neutral invertase activity in the stem tip in all autotrophic stages was higher than the acid invertase activity in the top segment of the stem in the pre-autotrophic stages.

Neutral invertase, calculated as total activity or specific activity was equally active in the stem tip of the 12-and 14 months old plants.

Leaf

Achlorophyllous

Acid: Acid invertase total activity in the achlorophyllous leaf was 32% higher ($P<0.01$) in the 12 months old plant than in the 2 months old plant, registering nearly the maximum at any growth stage. The activity decreased 17% ($P<0.01$) in the 14 months old plant. In Stage IV, the activity increased only marginally. But a lowering of 48% was registered in Stage V. The level was maintained in Stage VI. The specific activity nearly doubled in the 12 months old plant than in the 2 months old plant, reaching the maximum; the specific activity decreased 24% ($P<0.01$) in the 14 months old plant from the 12 months plant.

The specific activity of the enzyme was maintained at a high level during the autotrophic growth of the plant, except in Stage VII. A decrease of 30.8% was noted in Stage VII at which the value was the lowest in any stage.

Neutral: Neutral invertase (both total and specific) was, at all stages, less active than acid invertase in the achlorophyllous leaf, as already pointed out. The ratio acid to neutral was highest in Stage II A (1:5.3) and lowest in Stage VII (1:2.4). Neutral invertase total activity in the achlorophyllous leaf was more active than neutral invertase in the stem tip in the vegetative stage, but, in the reproductive stages, the two activities were equal in one stage (Stage IV) and the activity in the achlorophyllous leaf was less than that in the stem tip in another (Stage VII).

Neutral invertase total activity in the achlorophyllous leaf was 42% less active in the 12 months old plant than in the 2 months old plant ($P<0.01$); the activity remained unchanged in the 14 months old plant. Neutral invertase specific activity was 15% lower in the 12 months old plants than in the 2 months old plants ($P<0.01$); the specific activity decreased further (11%, $p<0.05$) in the 14 months old plant.

The decrease in neutral invertase activity both total and specific, in the 12 months old plant contrasted with the increase in the acid activity. As will be shown later, a quantitative difference in response by the two activities was manifested in Stage VII.

Chlorophyllous

In the chlorophyllous leaf part, acid invertase total activity was 15% lower in the 12 months old plant than in the 2 months old plant ($P<0.01$); the activity increased marginally (9%, $P<0.05$) in the 14-months old plant. The specific activity in the 12 months old plant was 23% ($P<0.01$) higher than in the 2 months old plant, with a further increase of 11% in the 14 months old plant ($P<0.02$).

Reproductive state

Stage IV: Transition stage

Stem tip

Neutral invertase total activity in the stem tip was enhanced 54% ($P<0.01$) on transition of the mature vegetative plant to the reproductive state, attaining the peak

of activity in stem tip at any growth stage. The specific activity increased 22% ($P<0.01$), also registering a peak. There was no distinctive enrichment of the enzyme in the extract, contrasting with the apparent enrichment which occurred in the lower stem segment in Stages II A and II *b*.

The (neutral) invertase total activity in the stem tip was now nearly the same as that in the achlorophyllous leaf. Such a relationship did not hold good in specific activity changes; the specific activity was 24% ($P<0.01$) higher in the stem tip than in the achlorophyllous leaf in Stage IV and the two were nearly the same in Stage VII. In subsequent Stages V,and VI, also the (neutral) invertase activity in the stem tip was nearly the same as the neutral invertase in the achlorophyllous leaf, but in Stage VII neutral invertase was higher (37%, $P<0.01$) in stem tip than in the achlorophyllous leaf.

The increase in specific activity of the enzyme in Stage IV suggested preferential synthesis or preferential stabilization of invertase. Since there was an increase also in total protein, considerable amounts of invertase must have been synthesized. That the increase in invertase activity was an integral metabolic feature associated with the transitional phase of the shoot apex received support from the finding that the activity reverted to the original level in the succeeding stage (organogenesis), as would be pointed out below.

(1) The endogenous activity of an enzyme can be regulated by (a) the turnover of the protein (equilibrium between synthesis and degradation and (b) inhibitors. Naturally occurring inhibitors of invertase are known, but the present data

do not permit any conclusion whether or not inhibitors are involved in the regulation of the activity.

(2) Evocation reactions are distinctive of the shoot apex. However, the biochemical reactions in the group of cells in the apex proper in transition are likely to be influenced by reactions occurring in the subjacent partly differentiated and fully differentiated tissues. Such a relationship is likely also in the organogenic and subsequent stages.

Leaf

Achlorophyllous

Acid: The acid invertase total activity in the achlorophyllous leaf was nearly unchanged,although the protein content increased 24%. The specific activity of the enzyme decreased negligibly by 8% (N.S.). It appeared that acid invertase in the achlorophyllous leaf did not have a special role in the evocative reactions at the shoot apex; this feature appeared to be shared by neutral invertase in the tissue, as will be shown below.

Neutral: The neutral invertase total activity was enhanced 20% in the achlorophyllous leaf ($P<0.01$) .The increase in specific activity in the gel filtrate was essentially of the same magnitude as was to be expected from equivalence between the increase in protein and enzyme activity per g dry weight. It appeared that unlike in stem tip, there was no preferential synthesis, or conservation, of invertase protein in the transition stage.

Chlorophyllous

Acid invertase total activity in the chlorophyllous leaf was essentially unaltered on transition of the shoot apex from the vegetative to the reproductive state. However, the specific activity increased 29% ($P<0.01$), which considered in conjunction with 30% protein decrease, was an indication of conservation of the enzyme.

A perceptible response by invertase to the transformation of the plant from the vegetative to the reproductive state was confined to the stem tip and did not extend to the 'D' leaves, in particular the achlorophyllous part.

Organogenic stage

Stage V:

Stem tip

When the shoot apex passed into the organogenic state, there was 32% ($P<0.01$) decrease in the neutral invertase total activity in the stem tip. The specific activity decreased 28% ($P<0.01$). The level of total activity was the same and the specific activity only slightly lower (12%, $P<0.01$) than that in the pre-evocation stage (Stage III B), an unequivocal evidence in support of the above conclusion that increased invertase activity was a specific manifestation of the evocation at the shoot apex.

Leaf

Achlorophyllous

Acid: Acid invertase total activity decreased 32% ($P<0.01$) in the achlorophyllous leaf part. It will be recalled that the enzyme had remained unaltered on transition from the vegetative to the reproductive state. The invertase specific activity showed 44% increase ($P<0.01$), attaining the same high level as in Stage III A. There had been a marked decrease in the protein content (42%) and a small decrease in the extractability of protein and its recovery in the gel filtrate (Table No. 5) No clear picture emerged from these disparate findings, but it was possible that invertase protein was preferentially conserved.

Neutral: Neutral invertase total activity decreased 35%, ($P<0.01$). The specific activity increased 18%, ($P<0.01$), registering the highest for any stage.

The response of the leaf enzyme to organogenesis at the shoot apex was the same for the acid-and neutral form.

Chlorophyllous

Acid invertase total activity in the chlorophyllous leaf tissue decreased 10% ($P<0.01$) in the organogenic state; the specific activity decreased 24% ($P<0.01$). The stem tissue manifested a definite response by its invertase activity in Stage IV and Stage V. Stage IV and V *per se*, did not reveal a marked change in invertase activity in the leaf.

A possible role for leaf invertase in reactions centered round evocation in shoot apex in Stage IV seemed to be confined to the achlorophyllous part.

Post-organogenic:stages VI and VII

Stem tip

Neutral invertase total activity and specific activity in stem tip decreased 8% (N.S.) and 13% ($P<0.01$) respectively in Stage VI. In Stage VII, the former increased 22% ($P<0.01$) and the latter decreased marginally (N.S.).

Leaf

Achlorophyllous

Acid: Acid invertase total activity in the achlorophyllous leaf remained unchanged in Stage VI but decreased 48% in Stage VII ($P<0.01$) (although there was 34% increase in protein content), to attain the lowest value at any stage of development. The specific activity decreased marginally in Stage VI (14%, $P<0.01$), but markedly in Stage VII (63%, $P<0.01$), also to reach the lowest level at any stage.

Although there was an increase in total protein in Stage VII, invertase activity, both total and specific, decreased. The increase in protein in Stage VII was with respect to constituents other than invertase; that is, there was preferential destruction of the enzyme.

Neutral: The pattern of neutral invertase total activity in the achlorophyllous leaf tissue was the same as for acid invertase, with no change occurring in Stage VI. There was marginal decrease (9%, $P<0.05$) in Stage VII, to attain the lowest level at any stage; at which level the activity fell 27% below that in the stem tip ($P<0.01$). The specific activity decreased continuously, 11% in Stage VI ($P<0.01$) and 37% in Stage VII ($P<0.01$) to attain a value lower than in all other stages.

There was general resemblance between the response by the acid and neutral form of invertase in the achlorophyllous leaf in the post-organogenic stages. As in the case of the acid form, there was probably preferential destruction of neutral invertase in Stage VII, but this was not as pronounced as for acid invertase.

Chlorophyllous

Acid invertase total activity in the chlorophyllous leaf decreased 16% in Stage VI ($P<0.01$) and increased marginally in Stage VII (9%, $P<0.01$) . The enzyme specific activity increased 61% ($P<0.01$) in Stage VI, but decreased 18% ($P<0.01$) in Stage VII.

Since the protein content of the green leaf decreased 48% in Stage VI and the specific activity of the enzyme increased, it appeared that invertase was conserved in Stage VI. However, with an increase in protein in stage no corresponding increase in activity was observed in Stage VII, indicating that while invertase is conserved other proteins are synthesized. Stage VII witnessed diametrically opposite response by invertase in the achlorophyllous and chlorophyllous parts of the leaf.

Root tip

Total activities are expressed on a fresh weight basis; specific activity was on the gel filtrate basis, unless otherwise specified.

[Table 9]

No invertase activity, either acid or neutral could be detected in the propagule. Both activities appeared from stage II A.

Acid versus neutral

In the stages selected for the study, neutral activity was more active than the acid invertase - 19%, 134% and 18% more - in Stages II A, II B and V respectively. This was in striking contrast to the two enzymes in the achlorophyllous leaf tissue throughout the ontogenetic stages, where acid invertase was more active than the neutral invertase (See Table 7) on a fresh tissue basis.

Acid invertase

The value was the maximum in Stage II A than in any of the other stages studied. In stage II B there was a decrease of about 50% with a further decrease of 13% in Stage V. The specific activity which was the highest in Stage II A decreased by 64% in Stage II B but increased 25% in Stage V (although the total protein increased only marginally).

Acid invertase in the root tip tissue was 6.14, 4.46 and 3.26 - fold as active as the enzyme in upper, middle and lower segments respectively in Stage II A, on a fresh weight basis. The enzyme was 92% as active as the achlorophyllous tissue enzyme, but was only a fifth as active as the chlorophyllous leaf enzyme in Stage II A.

The specific activity of the root tip acid invertase was 3.4, 2.4 and 0.4-fold higher (on a gel filtrate basis) than that of the upper, middle and lower segments of the stem, respectively, in Stage II A. In contrast, the achlorophyllous region had 59% and the chlorophyllous region 4.7-fold higher specific activity than the root tip acid invertase.

In Stage II B, the enzyme activity was 3.4, 3.8 and 0.46 fold higher than in the upper, middle and lower stem segments. However, it was 56% and 91% lower than the achlorophyllous and chlorophyllous tissues respectively.

The specific activity of the root tip enzyme in Stage II B, was 74% and 135% higher than that of the upper and middle stem segments, respectively in Stage II B. In contrast, the enzymes had 63% lower specific activity in comparison to the lower stem segment. The achlorophyllous leaf had 3.4-fold higher specific activity than the root invertase. The chlorophyllous tissue had 11.5-fold higher specific activity than the root tip on a homogenate basis.

In Stage V, acid invertase was more active (85%) in the achlorophyllous region and highly active (over 8-fold higher) in the chlorophyllous region than the

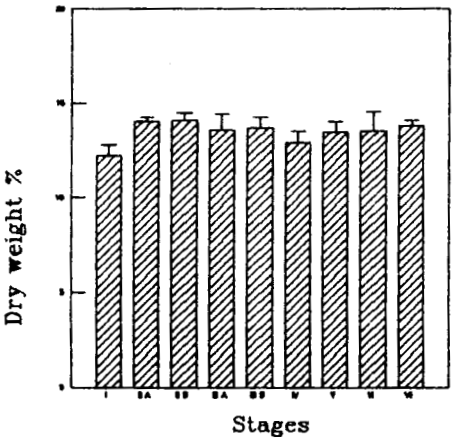
root tip enzyme on a fresh tissue basis. The specific activity of the enzyme was 6-fold higher (on gel filtrate basis) in the achlorophyllous tissue and 18-fold higher (homogenate basis) in the chlorophyllous tissue.

Neutral invertase

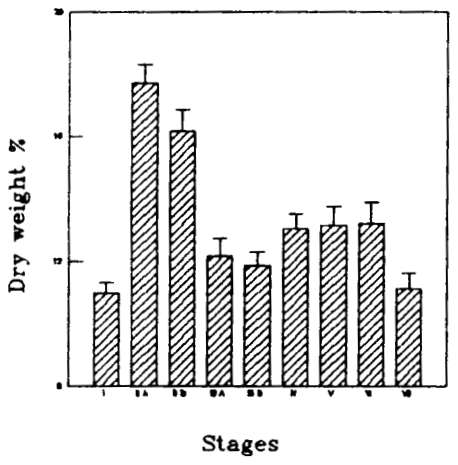
The activity remained unchanged in Stage II B, but was reduced to less than a half in Stage V. The specific activity decreased by 31% at Stage II B, although the total activity did not change. A decrease (33%) in specific activity in Stage V was paralleled by a decrease in total activity.

The total activity of the neutral invertase, on a fresh tissue basis, was 32% higher in the root tip than in the stem tip, at Stage V. The activity was 215% and 142% and 182% higher in the root than in the achlorophyllous tissue in stages II A, II B and V respectively.

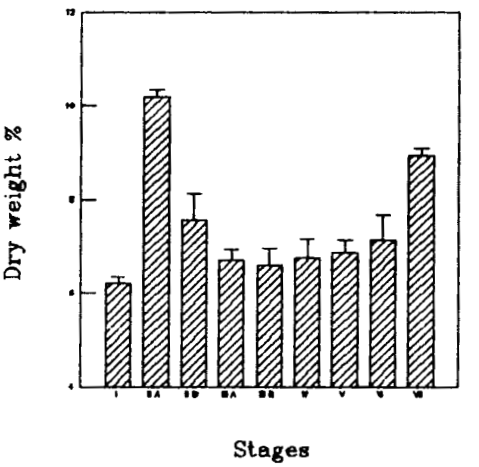
The specific activity of the enzyme in the root was the same as that of the stem tip, but the achlorophyllous enzyme had 32% higher specific activity, in Stage V.



Chlorophyllous leaf tissue



Stem tip tissue

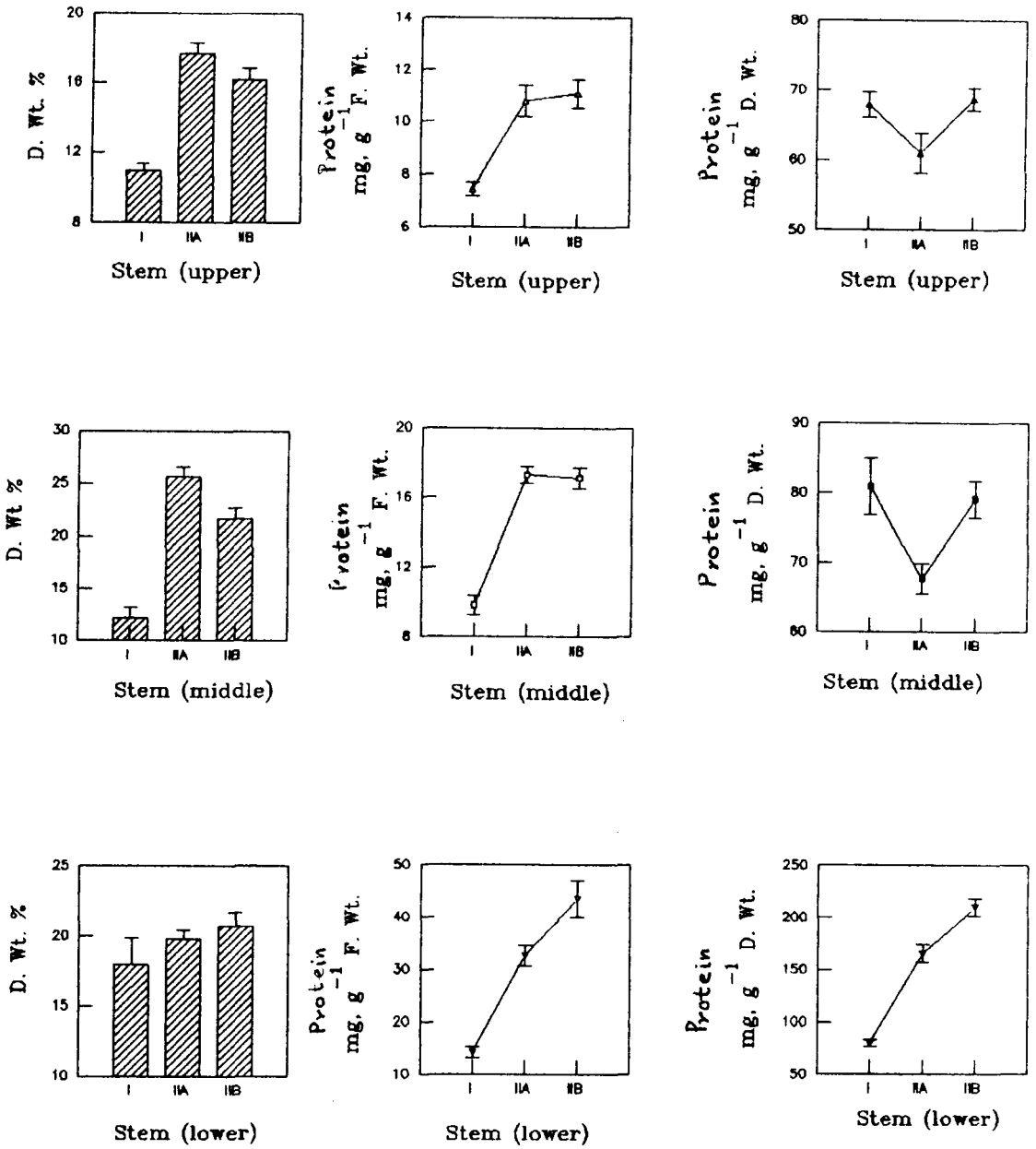


Achlorophyllous leaf tissue

Changes in dry solid content during the ontogeny of the pineapple plant.

Data are expressed as mean ± SD of six replicates. Determinations are as described in Materials and Methods. Error bar denotes the standard deviation.

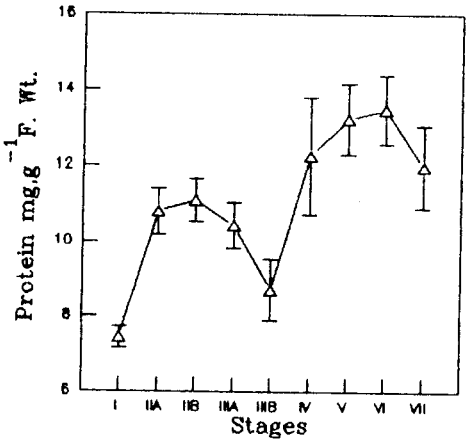
Fig. 12



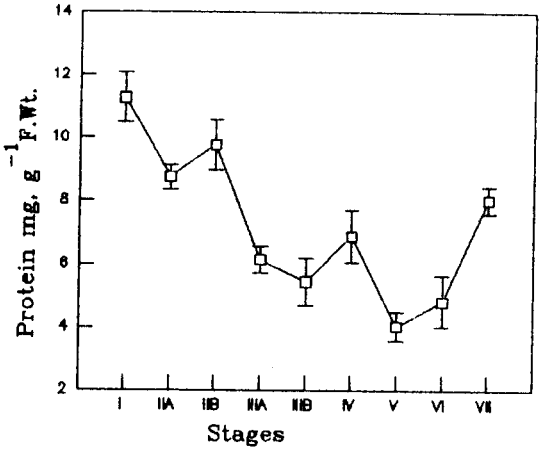
Changes in dry solid and protein contents in the stem segments during the pre-autotrophic stages of the pineapple plant

Data are expressed as mean ± SD of six replicates. Determinations of dry solid (bar) and protein contents are as described in materials and methods. Protein values are expressed both on fresh tissue basis (mg,g^{-1} F. Wt; ( ) and dry tissue basis (mg, g^{-1} D.Wt;)

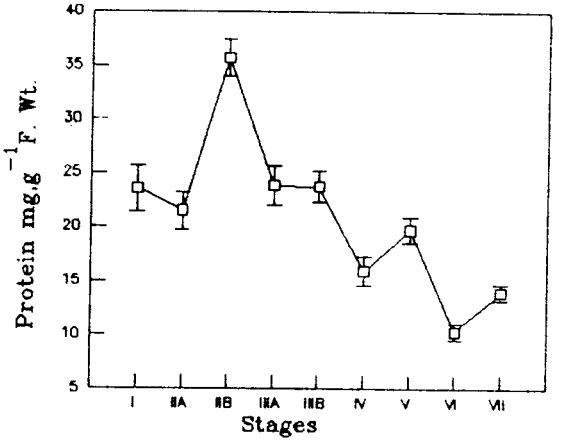
Fig. 13



Stem tip



Achlorophyllous leaf

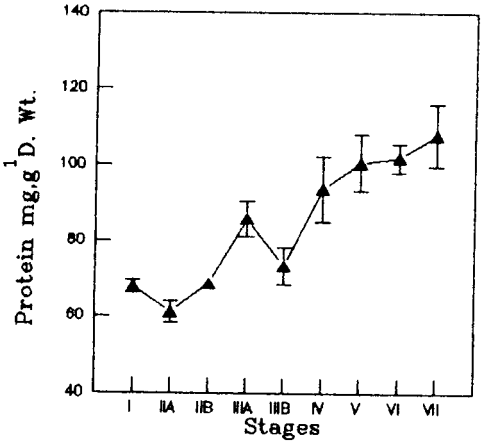


Chlorophyllous leaf

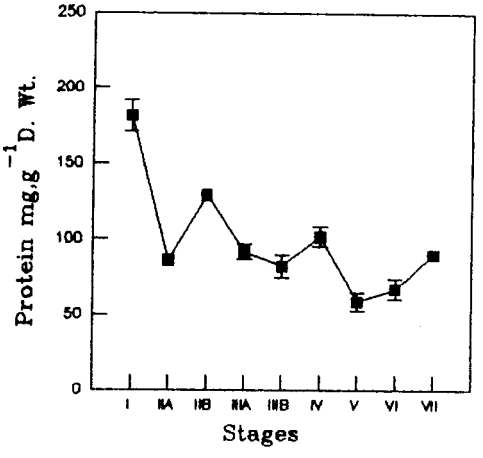
Changes in protein content during the ontogeny of the pineapple plant.

Data are expressed as mean ± SD of six replicates. Determinations of protein are as described in materials and methods. Protein values are expressed on mg/g fresh tissue basis (mg.g^{-1}F.Wt.)

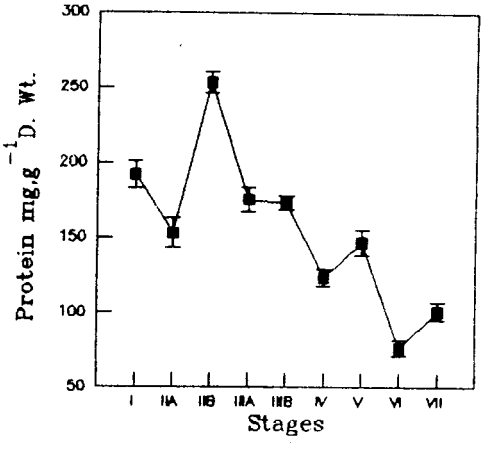
Fig. 14



Stem tip tissue



Achlorophyllous leaf tissue

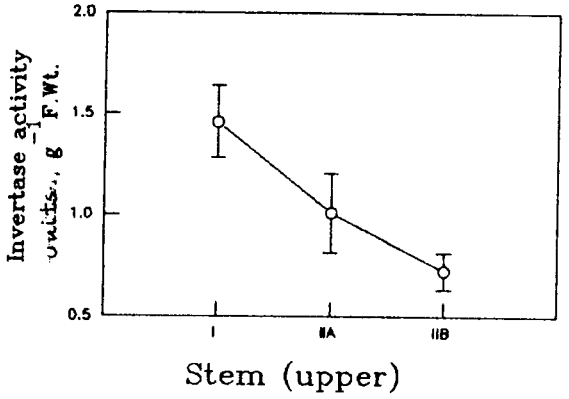


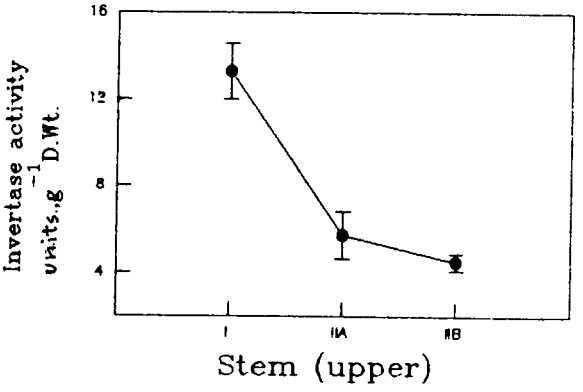
Chlorophyllous leaf tissue

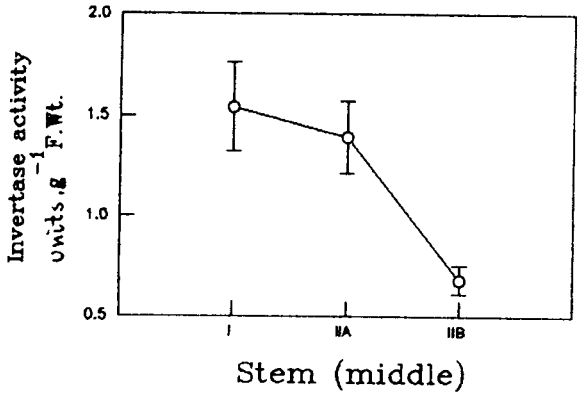
Changes in protein content during the ontogeny of the pineapple plant

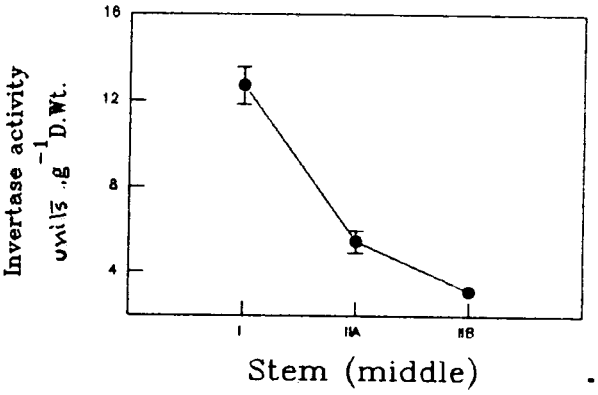
Data are expressed as mean ± SD of six replicates. Determinations of protein as are as desribe in materials and methods. Protein values are expressed on mg/g dry tissue basis (mg.g^{-1}D.Wt)

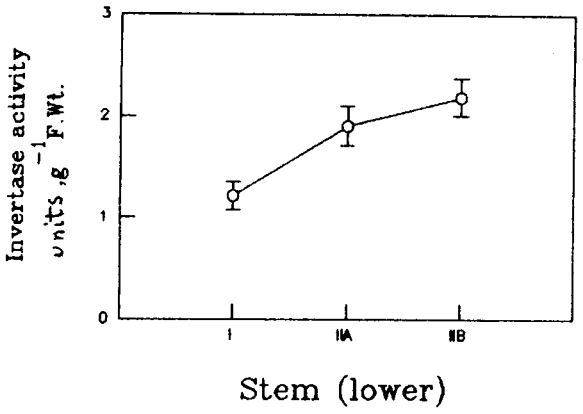
Fig. 15

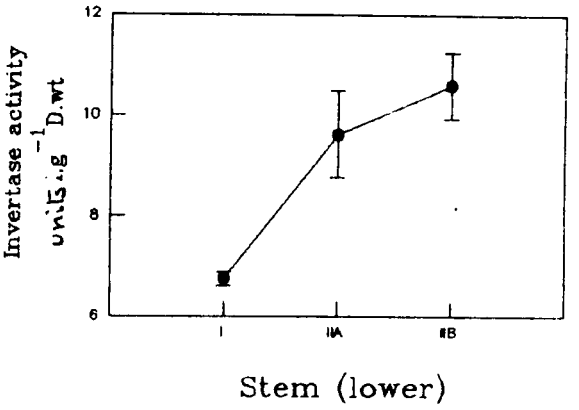








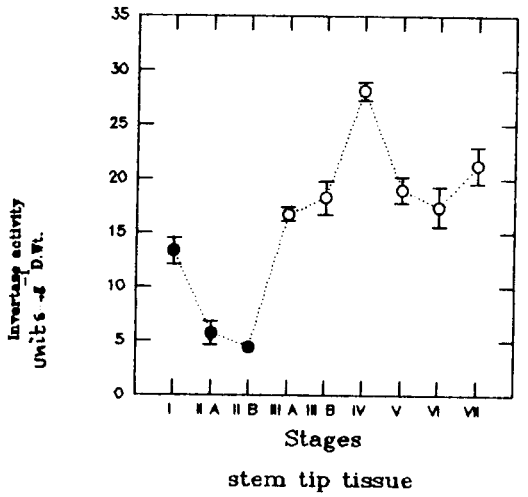




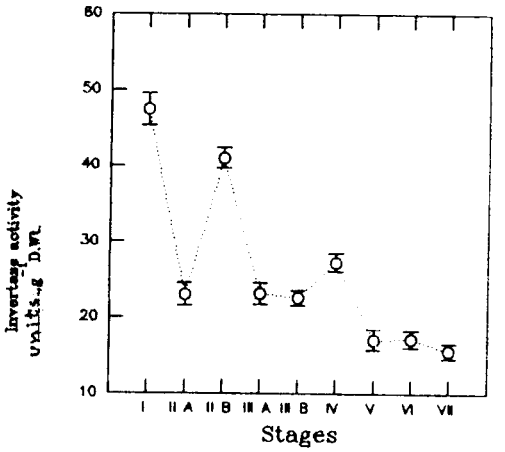
Changes in invertase activity in stem segments during the pre-autotrophic stages of the pineapple plant.

Values are expressed as the mean ± SD of six replicates. Assay conditions are as described in materials and methods. Data are expressed both on fresh tissue basis [units.g⁻¹ F.Wt. ( )] and dry tissue basis [units.g⁻¹ D.Wt. ( )]. Error bar denotes the standard deviation.

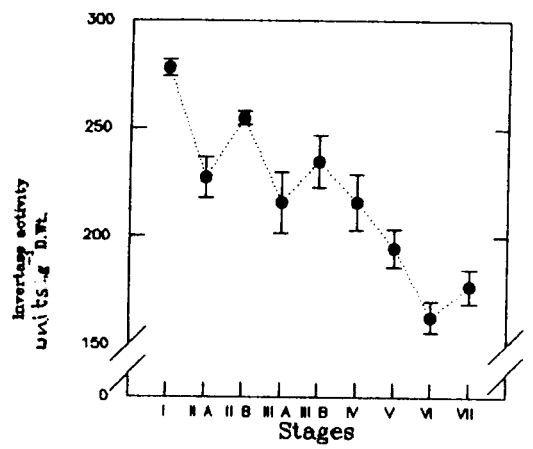
Fig. 16



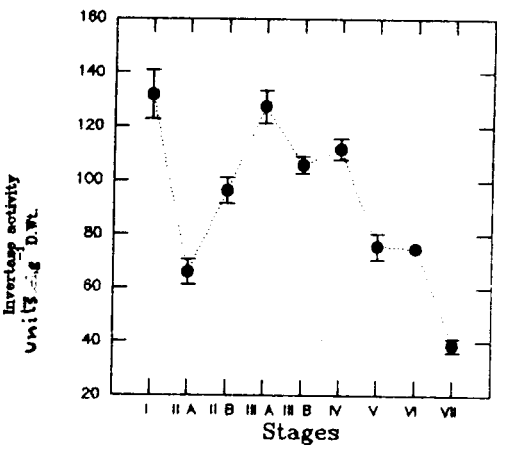
stem tip tissue



Achlorophyllous leaf tissue



Chlorophyllous leaf tissue



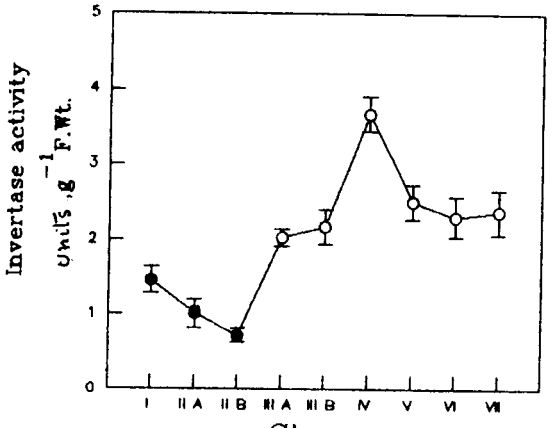
Achlorophyllous leaf tissue

Changes in invertase activity during the ontogeny of the pineapple plant.

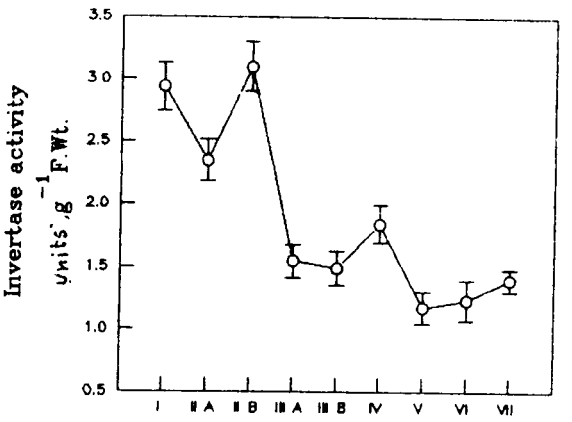
Values are the mean ± SD of six replicates. Assay conditions are as reported in materials and methods.

- ● Acid invertase
- ○ Neutral invertase

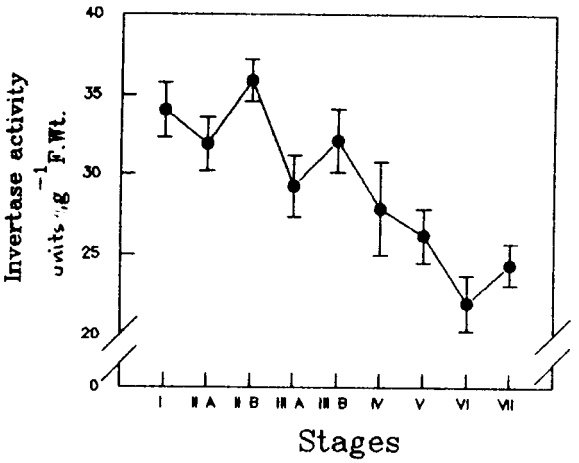
Fig. 17



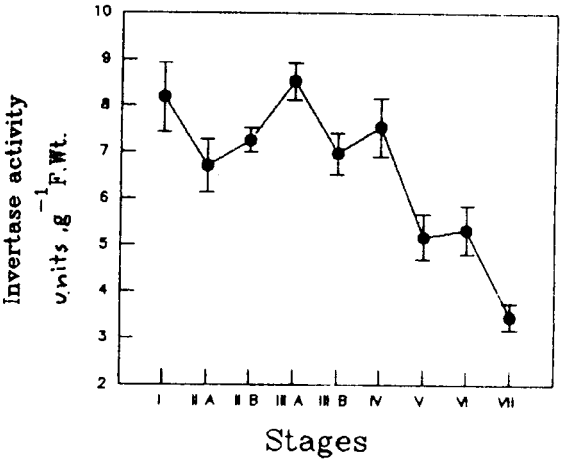
Stem tip tissue



Achlorophyllous leaf tissue



Chlorophyllous leaf tissue



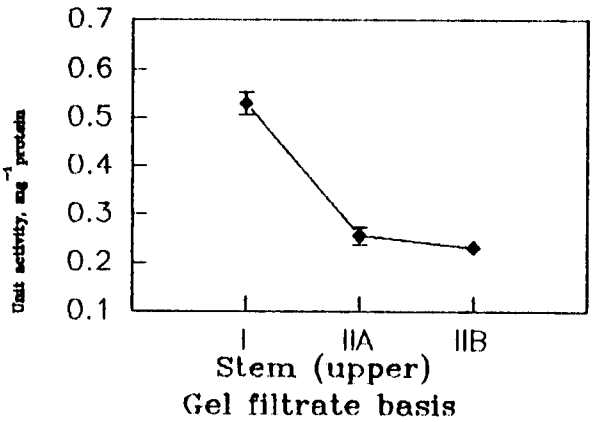
Achlorophyllous leaf tissue

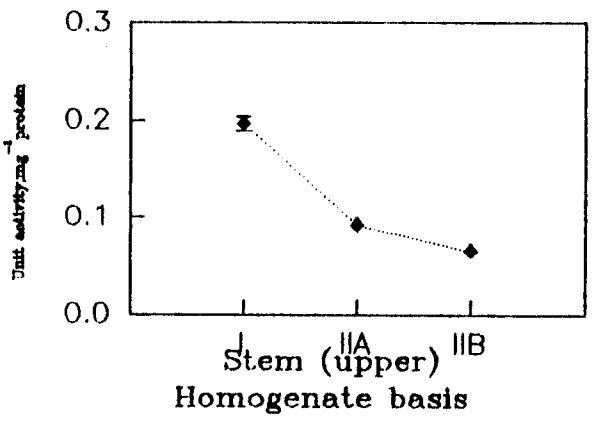
Changes in invertase activity during the ontogeny of the pineapple plant.

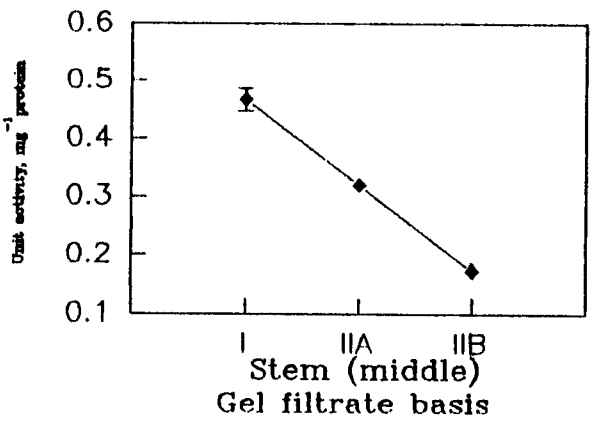
Values are expressed as the mean ± SD of six replicates. Assay conditions are as described in materials and methods.

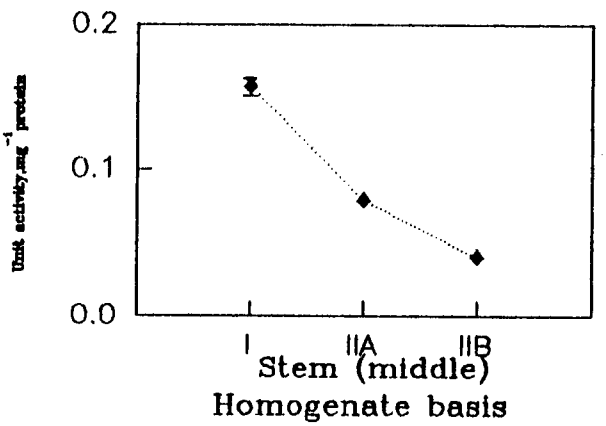
- ● Acid invertase
- □ Neutral invertase

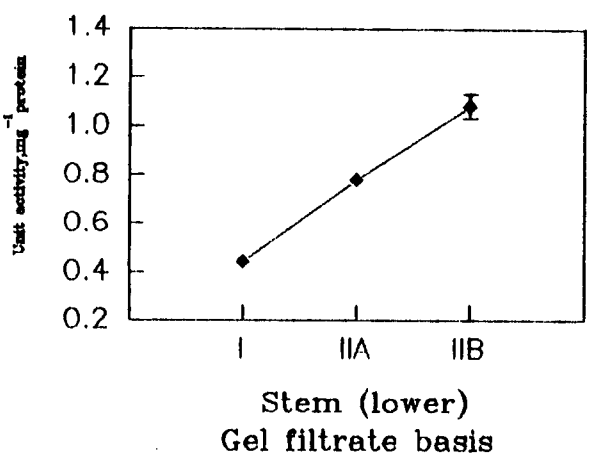
Fig. 18

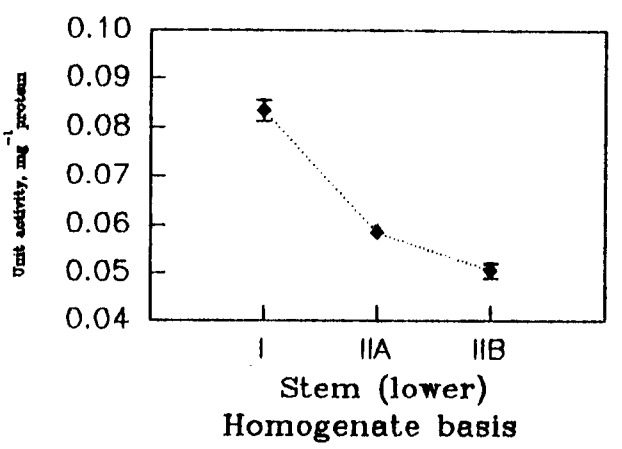








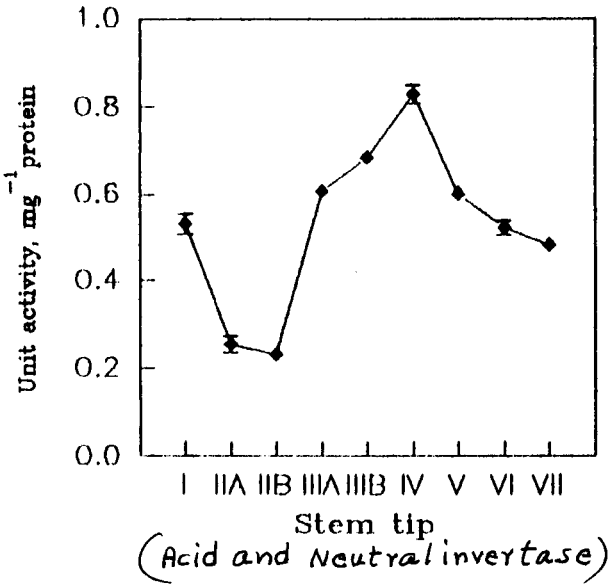


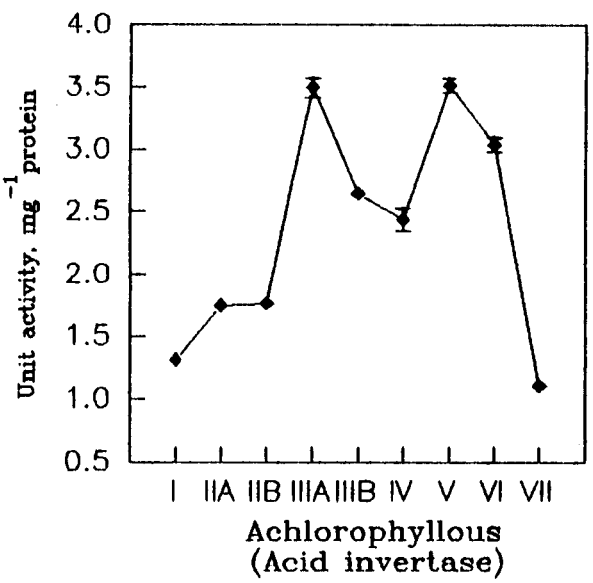


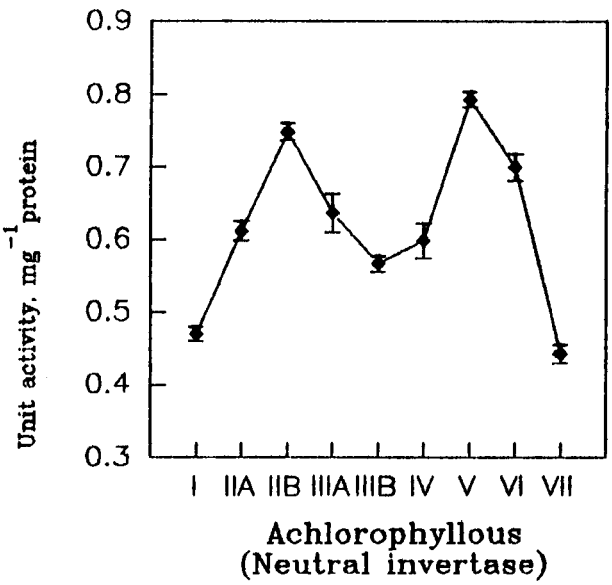
Changes in invertase specific activity in the stem segments during the pre-autotrophic stages of the pineapple plant.

Values are the mean ±SD of six replicates. Assay conditions are as described in materials and methods. Data are expressed both on the basis of protein in gel filtrate and in tissue homogeneate .

Fig. 19



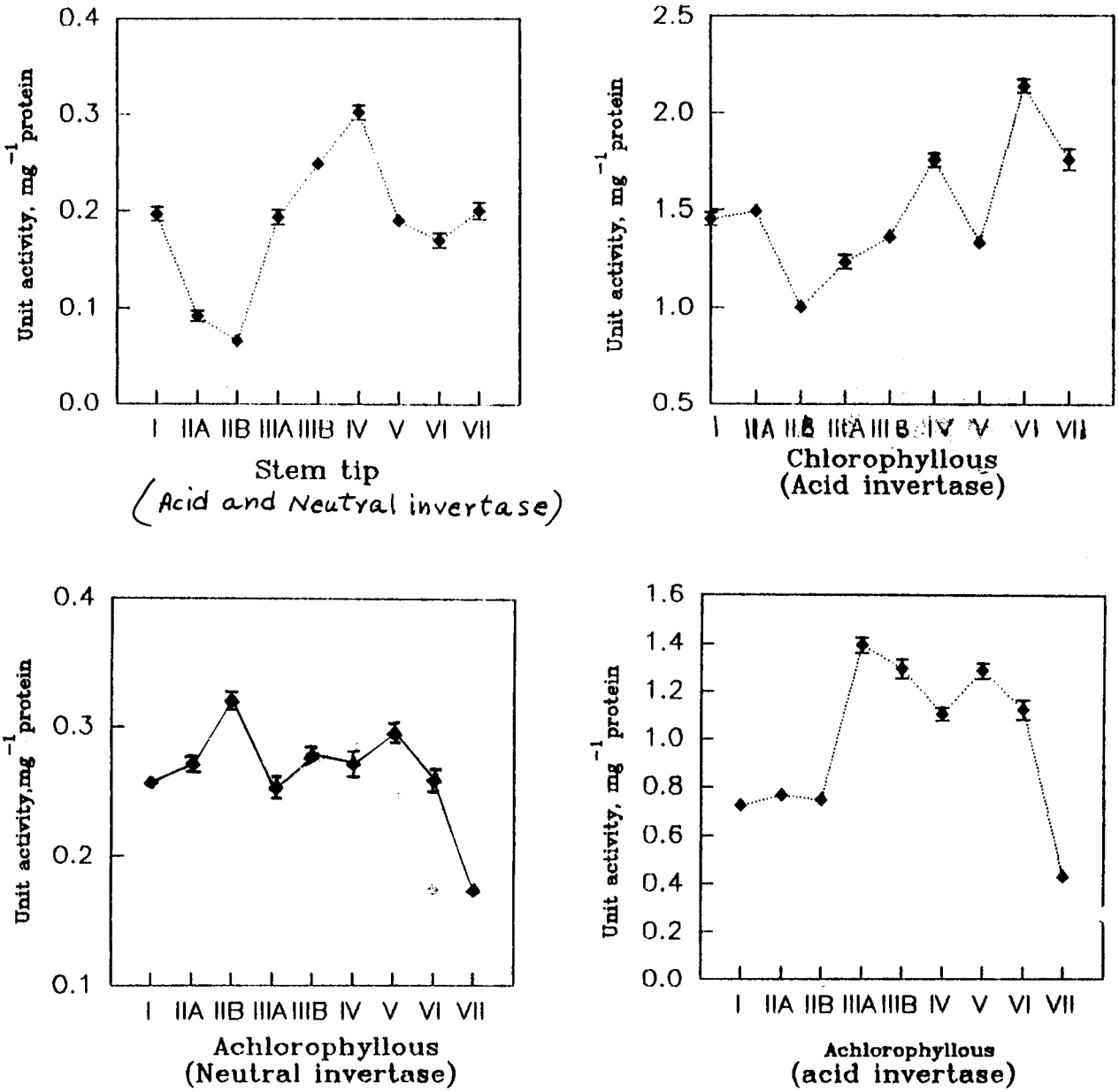




Changes in invertase specific activity, calculated on the basis of protein value in gel filtrate, during the ontogeny of the pineapple plant.

Values are the mean ± SD of six replicates. Assay conditions are as reported in materials and methods.

Fig. 20



Changes in invertase specific activity calculated on the basis of protein value in tissue homogenate, during the ontogeny of the pineapple plant.

Values are the mean ± SD of six replicates. Assay conditions are as described in materials and methods.

Fig. 21

Table: 3 A

Changes in dry solid and protein contents during pre-autotrophic stages of the pineapple plant: stem segments

Stage	Stem Tissue	Dry weight (%)	Protein, mg/g tissue equivalent							
			Homogenate		Residue		Supernatant		Gel-filtrate	
			Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
I	Upper (Stem tip)	10.98 ± 0.14	7.44 ± 0.12	67.8 ± 0.73	4.23 ± 0.07	38.6 ± 0.9	3.09 ± 0.05	28.2 ± 0.49	2.77 ± 0.028	25.2 ± 0.23
	Middle	12.14 ± 0.42	9.8 ± 0.23	80.9 ± 1.63	6.7 ± 0.05	55.5 ± 1.73	3.76 ± 0.05	31.1 ± 0.8	3.3 ± 0.06	27.2 ± 0.48
	Lower	17.95 ± 0.78	14.2 ± 0.45	79.5 ± 1.33	12.32 ± 0.7	68.6 ± 2.57	3.03 ± 0.03	16.98 ± 0.58	2.75 ± 0.04	15.4 ± 0.58
II_a	Upper	17.70 ± 0.24	10.79 ± 0.25	60.95 ± 1.16	6.3 ± 0.19	35.5 ± 0.83	4.25 ± 0.09	24 ± 0.38	3.99 ± 0.1	22.6 ± 0.54
	Middle	25.60 ± 0.38	17.3 ± 0.19	67.6 ± 0.91	12.9 ± 0.17	50.3 ± 0.27	5.05 ± 0.11	19.7 ± 0.21	4.37 ± 0.15	17 ± 0.35
	Lower	19.77 ± 0.26	32.7 ± 0.84	165.1 ± 3.49	25.4 ± 0.37	128.8 ± 1.81	2.72 ± 0.11	13.7 ± 0.51	2.45 ± 0.095	12.4 ± 0.47
II_b	Upper	16.20 ± 0.29	11.08 ± 0.23	68.6 ± 0.68	7.37 ± 0.21	45.6 ± 0.68	3.64 ± 0.09	22.6 ± 0.64	3.15 ± 0.08	19.5 ± 0.63
	Middle	21.70 ± 0.43	17.1 ± 0.24	79.1 ± 1.1	12.8 ± 0.27	59.2 ± 2.2	4.6 ± 0.06	21.2 ± 0.42	4 ± 0.1	18.4 ± 0.33
	Lower	20.70 ± 0.39	43.6 ± 1.42	210.1 ± 3.5	36.9 ± 1.31	178.1 ± 4.2	2.32 ± 0.12	11.2 ± 0.52	2.05 ± 0.11	9.87 ± 0.45

Date are expressed as mean ± SE of six replicates. Determinations of dry solid and protein contents are as described in materials and methods. Protein values are expressed both on fresh tissue and dry tissue basis.

Table: 3 B

Changes in dry solid and protein contents during the ontogeny of the pineapple plant: stem

Stage	Tissue	Dry weight (%)	Protein, mg/g tissue equivalent							
			Homogenate		Residue		Supernatant		Gel-filtrate	
			Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
I	upper (stem tip)	10.98 ±0.14	7.44 ±0.12	67.8 ±0.73	4.23 ± 0.07	38.6 ± 0.90	3.09 ± 0.05	28.2 ± 0.49	2.77 ± 0.028	25.2 ± 0.023
II_a	upper (stem tip)	17.7 ± 0.24	10.79 ± 0.25	60.95 ± 1.16	6.3 ± 0.19	35.5 ± 0.83	4.25 ± 0.09	24 ± 0.38	3.99 ± 0.1	22.6 ± 0.54
II_b	upper (stem tip)	16.2 ± 0.29	11.08 ± 0.23	68.6 ± 0.68	7.37 ± 0.21	45.6 ± 0.68	3.64 ± 0.09	22.6 ± 0.64	3.15 ± 0.08	19.5 ± 0.63
III_a	stem 'tip'	12.2 ± 0.24	10.42 ± 0.24	85.8 ± 1.92	6.77 ± 0.28	55.8 ± 2.5	3.69 ± 0.11	30.3 ± 0.63	3.36 ± 0.1	27.6 ± 0.59
III_b	stem 'tip'	11.87 ± 0.18	8.72 ± 0.34	73.4 ± 2.01	5.5 ± 0.21	46.3 ± 1.52	3.57 ± 0.21	30 ± 1.53	3.18 ± 0.19	26.8 ± 1.32
IV	stem 'tip'	13.04 ± 0.2	12.3 ± 0.63	93.8 ± 3.52	7.68 ± 0.42	58.8 ± 2.53	4.89 ± 0.25	37.4 ± 1.48	4.45 ± 0.21	34 ± 1.16
V	stem 'tip'	13.15 ± 0.24	13.2 ± 0.38	100.7 ± 3.01	8.9 ± 0.34	67.7 ± 2.53	4.8 ± 0.18	36.5 ± 1.02	4.2 ± 0.13	31.7 ± 0.71
VI	stem 'tip'	13.23 ± 0.27	13.5 ± 0.37	102 ± 1.53	8.9 ± 0.27	67.5 ± 1.27	4.9 ± 0.19	36.9 ± 0.95	4.41 ± 0.15	33.3 ± 0.81
VII	stem 'tip'	11.1 ± 0.22	11.98 ± 0.44	107.9 ± 3.37	7.15 ± 0.32	64.6 ± 3.38	5.5 ± 0.31	49.1 ± 1.96	4.95 ± 0.27	44.5 ± 1.72

Data are expressed as mean ± SE of six replicates. Determinations of dry solid and protein contents are as described in materials and methods. Protein values are expressed both on fresh tissue and dry tissue basis.

Table: 4

Changes in dry solid and protein contents during the ontogeny of the pineapple plant: Achlorophyllous leaf part

Stage	Dry weight (%)	Protein, mg/g tissue equivalent							
		Homogenate		Residue		Supernatant		Gel-filtrate	
		Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
I	6.21 ± 0.06	11.28 ± 0.32	181.6 ± 4.26	4.67 ± 0.19	75.1 ± 2.53	7.04 ± 0.21	113.3 ± 2.89	6.25 ± 0.16	100.7 ± 1.91
II_a	10.19 ± 0.07	8.75 ± 0.17	85.9 ± 1.14	4.8 ± 0.1	47.1 ± 0.74	4.42 ± 0.07	43.4 ± 0.49	3.85 ± 0.08	37.8 ± 0.54
II_b	7.58 ± 0.23	9.77 ± 0.32	128.9 ± 1.58	5.37 ± 0.12	71 ± 1.44	4.5 ± 0.08	59.5 ± 1.48	4.13 ± 0.08	54.7 ± 1.2
III_a	6.7 ± 0.1	6.13 ± 0.18	91.5 ± 2.09	3.93 ± 0.15	58.6 ± 2.06	2.75 ± 0.08	41.1 ± 1.17	2.45 ± 0.07	36.5 ± 1.17
III_b	6.6 ± 0.14	5.43 ± 0.3	82.1 ± 3.04	2.66 ± 0.24	40 ± 3	2.92 ± 0.11	44.2 ± 0.95	2.64 ± 0.07	40. ± 0.61
IV	6.75 ± 0.17	6.9 ± 0.34	101.7 ± 2.79	3.69 ± 0.24	54.4 ± 2.36	3.5 ± 0.08	51.9 ± 1.1	3.1 ± 0.04	46. ± 1.38
V	6.86 ± 0.11	4.03 ± 0.19	58.8 ± 2.31	2.55 ± 0.11	37.2 ± 1.36	1.62 ± 0.09	23.6 ± 1	1.48 ± 0.07	21.5 ± 0.74
VI	7.13 ± 0.23	4.82 ± 0.33	67.2 ± 2.72	2.79 ± 0.28	38.7 ± 2.78	2.01 ± 0.08	28.1 ± 0.48	1.76 ± 0.06	24.7 ± 0.4
VII	8.94 ± 0.06	8.03 ± 0.17	89.8 ± 1.37	4.58 ± 0.11	51.2 ± 1.16	3.56 ± 0.07	39.8 ± 0.65	3.13 ± 0.06	35 ± 0.64

Data are expressed as mean ± SE of six replicates. Determinations of dry solid and protein content are as described in materials and methods. Protein values are expressed both on fresh tissue and dry tissue basis.

Table 5
Solubilization of Protein

Stage	Protein Recovery, %						
	Stem tissue			Achlorophyllous leaf			Chloro-phyllous leaf
	Residue	Extract	Gel Filtrate	Residue	Extract	Gel filtrate	Residue
Stem Up	56.9	41.6	37.2 (89.6)				
I Mi	68.4	38.4	33.7 (87.8)	41.4	62.4	55.4 (88.8)	100.4
Low	86.8	21.3	19.4 (90.8)				
Stem Up	58.4	39.4	37.0 (93.9)				
IIA Mi	74.6	29.2	25.3 (86.5)	55.0	46.1	42.3 (91.8)	99.1
Low	77.7	8.3	7.5 (90.1)				
Stem Up	66.5	32.9	28.4 (86.5)				
IIB Mi	74.9	26.9	23.4 (87.0)	55.0	46.1	42.3 (91.8)	99.4
Low	84.6	5.3	4.7 (88.4)				
Stem tip IIIA	64.9	35.4	32.2 (91.1)	64.1	44.9	40.0 (89.1)	100.0
Stem tip IIIB	63.1	40.9	36.5 (89.1)	49.0	53.8	48.6 (90.4)	99.6

(Table-5 contd.)

Stem tip IV	62.4	39.8	36.2 (90.7)	53.5	50.7	44.9 (88.6)	100.0
Stem tip V	67.4	36.3	31.8 (87.5)	63.3	40.2	36.7 (91.4)	99.5
Stem tip VI	65.9	36.3	32.7 (90.0)	57.9	41.7	36.5 (87.6)	100.0
Stem tip VII	59.7	45.9	41.3 (90.0)	57.0	44.3	39.0 (87.9)	96.2

The recovery of protein in the extract and the residue on centrifugation was calculated as a percentage of protein in homogenate. The recovery in the gel filtrate was calculated as a percentage of protein in the tissue and, in parenthesis, as a percentage of protein in extract. Extracts of chlorophyllous leaf tissue did not yield a precipitate with trichloroacetic acid. The percentages have been calculated on the basis of the protein data being reported elsewhere (Tables 3A, 3B, 4 and 8).

Table 6 A

Changes in invertase activity during the pre autotrophic stages of the pineapple plant: stem segments

Stage	Tissue	Dry weight %	Invertase activity, units/ g tissue				Protein, mg/g tissue				Specific activity, activity, per mg protein			
			Acid		Neutral		Homogenate		Gel filtrate		Acid		Neutral	
			Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	H	Gel	H	Gel
I	Upper	10.98 ± 0.14	1.46 ± 0.07	13.3 ± 0.52			7.44 ± 0.12	67.8 ± 0.73	2.77 ± 0.028	25.2 ± 0.23	0.197 ±0.007	0.53 ± 0.024		
	Middle	12.14 ± 0.42	1.55 ± 0.09	12.7 ± 0.35			9.8 ± 0.23	80.9 ± 1.63	3.3 ± 0.06	27.2 ± 0.48	0.16 ± 0.006	0.47 ± 0.019		
	Lower	17.95 ± 0.78	1.21 ± 0.05	6.75 ± 0.057			14.2 ± 0.45	79.5 ± 1.33	2.75 ± 0.04	15.4 ± 0.58	0.08 ± 0.002	0.44 ± 0.017		
II_a	Upper	17.7 ± 0.25	1.01 ± 0.08	5.71 ± 0.45			10.79 ± 0.25	61 ± 1.16	3.99 ± 0.1	22.6 ± 0.54	0.09 ± 0.006	0.26 ± 0.018		
	Middle	25.6 ± 0.38	1.39 ± 0.07	5.43 ± 0.21			17.3 ± 0.19	67.6 ± 0.91	4.37 ± 0.15	17 ± 0.35	0.08 ± 0.004	0.32 ± 0.009		
	Lower	19.77 ± 0.26	1.9 ± 0.08	9.62 ± 0.35			32.7 ± 0.84	165.1 ± 3.49	2.45 ± 0.095	12.4 ± 0.47	0.058 ± 0.001	0.78 ± 0.02		
II_b	Upper	16.2 ± 0.29	0.72 ± 0.04	4.5 ± 0.16			11.08 ± 0.23	68.6 ± 0.68	3.15 ± 0.08	19.5 ± 0.63	0.065 ± 0.002	0.23 ± 0.01		
	Middle	21.7 ± 0.43	0.68 ± 0.029	3.2 ± 0.12			17.1 ± 0.24	79.1 ± 1.1	4 ± 0.1	18.4 ± 0.33	0.04 ± 0.001	0.17 ± 0.006		
	Lower	20.7 ± 0.39	2.20 ± 0.076	10.6 ± 0.27			43.6 ± 1.42	210.1 ± 3.5	2.05 ± 0.11	9.87 ± 0.45	0.051 ± 0.002	1.08 ± 0.05		

Data are expressed as mean ± SE of six replicates. Assay conditions are as described in materials and methods. Invertase activity is expressed both on fresh tissue and dry tissue basis. Specific activity is expressed on the basis of protein value in gel filtrate as well as in tissue homogenate.

Table 6 B

Changes in invertase activity during the ontogeny of the pineapple plant: stem

Stage	Tissue	Dry weight %	Invertase activity, units/g tissue				Protein, mg/g tissue equivalent				Sp. activity, units per mg protein			
			Acid		Neutral		Homogenate		Gel filtrate		Acid		Neutral	
			Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	H.	Gel	H.	Gel
I	Stem (up)	10.98 ±0.14	1.46 ±0.07	13.3 ±0.52			7.44 ±0.12	67.8 ±0.73	2.77 ±0.028	25.2 ±0.23	0.197 ±0.007	0.53 ±0.024		
II_a	Stem (up)	17.7 ±0.24	1.01 ±0.08	5.71 ±0.45			10.79 ±0.25	61 ±1.16	3.99 ±0.1	22.6 ±0.54	0.092 ±0.006	0.26 ±0.018		
II_b	Stem (up)	16.2 ±0.29	0.72 ±0.04	4.5 ±0.16			11.08 ±0.23	68.6 ±0.68	3.15 ±0.08	19.5 ±0.63	0.065 ±0.002	0.23 ±0.01		
III_a	Stem (tip)	12.2 ±0.24			2.03 ±0.05	16.7 ±0.26	10.4 ±0.24	86 ±1.92	3.36 ±0.1	28 ±0.59			0.19 ±0.007	0.61 ±0.008
III_b	Stem (tip)	11.87 ±0.18			2.17 ±0.09	18.2 ±0.62	8.72 ±0.34	73.4 ±2.01	3.18 ±0.19	26.8 ±1.32			0.25 ±0.0048	0.68 ±0.01
IV	Stem (tip)	13.04 ±0.2			3.67 ±0.1	28.1 ±0.34	12.3 ±0.63	93.8 ±3.52	4.45 ±0.21	34 ±1.16			0.3 ±0.007	0.83 ±0.02
V	Stem (tip)	13.15 ±0.24			2.5 ±0.09	19 ±0.49	13.2 ±0.38	100.7 ±3.01	4.2 ±0.13	31.7 ±0.71			0.19 ±0.004	0.6 ±0.009
VI	Stem (tip)	13.23 ±0.27			2.31 ±0.11	17.4 ±0.74	13.5 ±0.37	102 ±1.53	4.41 ±0.15	33.3 ±0.81			0.17 ±0.007	0.52 ±0.016
VII	Stem (tip)	11.1 ±0.22			2.37 ±0.12	21.3 ±0.69	11.98 ±0.44	107.9 ±3.38	4.95 ±0.27	44.5 ±1.72			0.2 ±0.008	0.48 ±0.008

Data are expressed as mean ± SE of six replicates. Assay conditions are as described in materials and methods. Invertase activity is expressed both on fresh tissue and dry tissue basis. Specific activity is expressed on the basis of protein in gel filtrate as well as in tissue homogenate.

Table 7

Changes in invertase activity during the ontogeny of the pineapple plant: Achlorophyllous leaf part

Stage	Dry weight %	Invertase activity, units/g tissue				Protein, mg/g tissue equivalent				Sp. activity, unit/mg protein			
		Acid		Neutral		Homogenate		Gel filtrate		Acid		Neutral	
		Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	H.	Gel	H.	Gel
I	6.21 ±0.06	8.19 ±0.3	131.8 ±3.64	2.94 ±0.08	47.4 ±0.87	11.28 ±0.32	181.6 ±4.26	6.25 ±0.16	100.7 ±1.91	0.73 ±0.02	1.31 ±0.03	0.26 ±0.006	0.47 ±0.01
II_a	10.19 ±0.07	6.72 ±0.23	65.9 ±1.92	2.35 ±0.07	23.1 ±0.59	8.75 ±0.17	85.9 ±1.14	3.85 ±0.08	37.8 ±0.54	0.77 ±0.016	1.75 ±0.034	0.27 ±0.006	0.61 ±0.014
II_b	7.58 ±0.23	7.27 ±0.11	96.2 ±1.97	3.1 ±0.08	41 ±0.56	9.77 ±0.32	128.9 ±1.58	4.13 ±0.08	54.7 ±1.2	0.75 ±0.015	1.76 ±0.023	0.32 ±0.005	0.75 ±0.012
III_a	6.7 ±0.1	8.53 ±0.16	127.3 ±2.53	1.55 ±0.053	23.9 ±0.59	6.13 ±0.18	91.5 ±2.09	2.45 ±0.07	36.5 ±1.17	1.39 ±0.03	3.5 ±0.078	0.25 ±0.008	0.64 ±0.027
III_b	6.6 ±0.14	6.98 ±0.19	105.6 ±1.26	1.49 ±0.06	22 ±0.41	5.43 ±0.3	82.1 ±3.04	2.64 ±0.07	40 ±0.61	1.3 ±0.04	2.65 ±0.044	0.28 ±0.006	0.57 ±0.011
IIV	6.75 ±0.17	7.55 ±0.26	111.7 ±1.61	1.85 ±0.059	27.3 ±0.48	6.88 ±0.34	101.7 ±2.79	3.1 ±0.038	46 ±1.38	1.1 ±0.027	2.44 ±0.091	0.27 ±0.01	0.60 ±0.024
V	6.86 ±0.11	5.19 ±0.2	75.5 ±2.06	1.17 ±0.05	17.1 ±0.55	4.03 ±0.19	58.8 ±2.31	1.48 ±0.066	21.5 ±0.74	1.29 ±0.03	3.52 ±0.058	0.29 ±0.006	0.79 ±0.011
VI	7.13 ±0.23	5.34 ±0.21	74.8 ±0.83	1.24 ±0.067	17.2 ±0.47	4.82 ±0.33	67.2 ±2.72	1.76 ±0.059	24.7 ±0.4	1.12 ±0.041	3.04 ±0.059	0.26 ±0.008	0.7 ±0.018
VII	8.94 ±0.06	3.47 ±0.11	38.8 ±1.09	1.4 ±0.037	15.6 ±0.39	8.03 ±0.168	89.8 ±1.37	3.13 ±0.056	35 ±0.64	0.43 ±0.009	1.11 ±0.027	0.18 ±0.003	0.44 ±0.012

Values are the mean ± SE of six replicates. Assay conditions are as reported in materials and methods. Invertase activity is expressed both on fresh tissue and dry tissue basis. Specific activity is expressed on the basis of protein value in gel filtrate and also in tissue homogenate.

Table 8

Changes in invertase activity during the ontogeny of the pineapple plant: chlorophyllous leaf part

Stages		Dry weight %	Invertase activity, unit/g tissue		Protein, mg/g tissue equivalent				Specific activity on homogenate basis
			Acid		Homogenate		Residue		
			Fresh	Dry	Fresh	Dry	Fresh	Dry	
I	Propagule	12.23±0.23	34.1±0.7	278.5±1.5	23.5±0.86	192.2±3.76	23.6±0.92	214.4±5.94	1.45±0.032
II_a	1 month	14.02±0.1	31.9±0.68	227.4±3.8	21.5±0.71	153±4.02	21.3±0.64	151.5±3.63	1.49±0.029
II_b	2 months	14.08±0.16	35.9±0.54	254.9±1.33	35.7±0.72	253.4±2.98	35.5±0.83	252.4±3.57	1±0.014
III_a	12 months	13.58±0.34	29.3±0.78	215.9±5.75	23.8±0.75	175.4±3.42	23.8±0.67	175.3±3.27	1.23±0.037
III_b	14 months	13.68±0.23	32.1±0.81	234.8±4.9	23.7±0.59	173.3±1.93	23.8±0.61	174±2.08	1.36±0.023
IV	Transition	12.9±0.26	27.9±1.2	216±5.21	15.9±0.56	123.3±2.26	15.9±0.55	122.9±2.31	1.75±0.037
V	Flowering 0.5 to 1 cm	13.46±0.24	26.2±0.69	194.8±3.6	19.7±0.48	146.6±3.5	19.6±0.43	146±3.46	1.33±0.17
VI	1 to 2 cm	13.5±0.43	22±0.7	163.1±2.95	10.3±0.3	75.5±2.12	10.3±0.3	76.3±2.22	2.14±0.035
VII	6 to 10 cm	13.8±0.11	24.4±0.52	177±3.22	13.9±0.32	101.1±2.47	13.79±0.33	100±2.54	1.76±0.053

Values are the mean ± SE of six replicates. Assay conditions are as described in materials and methods. Determination of dry solid and protein contents are as described in materials and methods. Invertase activity is expressed both on fresh tissue and dry tissue basis. Specific activity is expressed only on the basis of protein value in homogenate.

Table 9

Changes in invertase activity during the ontogeny of the pineapple plant: root tip

Stage	Protein, mg/g fresh tissue				Invertase activity units/g tissue		Specific activity			
	Homogenate	Residue	Supernatant	Gel filtrate	Acid	neutral	Acid		Neutral	
							Homogenate	Gel filtrate	Homogenate	Gel filtrate
I	22.8	17.6 (77.2%)	5.7 (25%)	[79%] 4.5 (19.7%)	Nil	Nil	..	..	..	..
II A	24.3	15.2 (62.5%)	7.3 (30%)	[81%] 5.9 (24.3%)	6.2	7.4	0.26	1.05	0.3	1.25
II B	38.1	32.9 (86.4%)	9.1 (23.9%)	[90%] 8.2 (21.5%)	3.2	7.5	0.08	0.39	0.2	0.91
V	42.6	39 (91.5%)	6.2 (14.6%)	[85%] 5.3 (12.4%)	2.8	3.3	0.07	0.53	0.08	0.62

The values reported for the total protein are the mean of 3 to 4 experiments.

The percentage recovery of protein (the values in parentheses) in the residue and the extract are calculated on the basis of homogenate.

The values in square brackets above represent the recovery in the gel filtrate from the supernatant.

DISCUSSION

DISCUSSION

DISCUSSION

Invertase is a regulatory enzyme ?

Sucrose is the principal transport form of the photosynthetic assimilation. Also, it is a storage product in some tissues. This storage is either comparatively permanent or transient. In the first category are sugarcane internode and sugar beet root. Instances of transient storage, of relevance in the present study are:

a. in the rapidly developing shoot tip, which includes besides the apex, the partially differentiated tissue;

b. a spurt in sucrose accumulation in the shoot tip at the time of evocation;

c. sucrose accumulation in the leaf during the induction process, leading to evocation of shoot apex;

d. the lower regions of the stem of the pineapple propagule during rooting and terrestrial establishment;

e. the tip of the developing root, which includes the apex;

f. the diurnal accumulation of sucrose in the pineapple leaf as a storage product for malate formation in the night.

Invertase occupies a strategic position in the metabolism of sucrose. It is one of the two enzymes acting on sucrose, the other being sucrose synthase. Of the two, invertase is the only enzyme to have a single reaction pathway, namely, hydrolytic degradation; sucrose synthase can readily function also in the reverse direction. As such, it is to be expected that its activity could be modulated *in vivo*.

Among possible modulation mechanisms are:

1. Ontogenetic variation. This, a process spread over the life cycle of the monocarpic plant, is a critical theme of this dissertation.

2. Diurnal variation in enzyme activity. Such a variation was reported by Glasziou, Waldron and Bull (1966), but a detailed study does not seem to have been conducted.

3. Regulation by the acidity developing in the vacuole.

The activity of a regulatory enzyme can have both "fine control" and "coarse control." In fine control, the activity is modulated by effector molecules, or end products; this is a rapid process. The ATP-mediated phosphofructokinase reaction is an example. In coarse control, which takes a somewhat longer time, the amount of the enzyme is altered. A well-studied and widely prevalent control is by post-translational phosphorylation of the enzyme protein by protein kinase in the presence of ATP and Mg^{2+}, yielding active/inactive enzyme and reversal by dephosphorylation by a phosphatase yielding inactive/active enzyme. The original discovery of this mechanism (for glycogen phosphorylase in liver) earned the 1992 Nobel prize in

medicine by the American Biochemists Krebs and Fischer. Sucrose phosphate synthase is regulated by this mechanism (Battey, *et al.*, 1993).

There is evidence to show that acid invertase activity can be regulated by phytohormones (Morris and Arthur, 1984).

The significance of the occurrence of a proteinaceous inhibitor of invertase will be considered in a following section.

A survey of literature does not indicate that a critical study has been conducted to screen effector substances (low molecular, organic) for invertase activity modulation, with the possible exception of pyridoxal phosphate.

Nor have various end products (derived from glucose/fructose) been tested for possible regulatory action, excepting a report on the inhibition of invertase by glucose 6-phosphate. However, the reaction products of invertase action, fructose and glucose, exert inhibitory action.

Induction of enzyme

Induction of enzymes has been well studied in microorganisms. However, recent studies establish that the phenomenon of induction plays a significant role in the regulation of plant enzymes also (Wray, 1992). The accumulation of sucrose in the shoot apex at the time of evocation can be thought of as inducing invertase activity (Pryke and Bernier, 1978). A well known phenomenon is the several-fold

accumulation of invertase on storage of potato tuber in the cold for a few weeks (ap Rees, 1981; Hawker, 1985).

Invertase has been shown to accumulate in considerable concentration on ageing of tissues (Bacon, MacDonald and Knight, 1965; Getz, 1991). This accumulation is characteristic not only of the soluble form but also of the insoluble form.

Is invertase a rate-limiting enzyme?

Closely related to the regulatory role of an enzyme is the concept of its being a rate limiting enzyme or not. The literature survey shows that in the large majority of cases invertase is present in ample excess in tissues. However, there are occasional reports that invertase can be a rate limiting enzyme (Ho, 1988).

The question of rate of rate-limitation assume considerable importance in the author's studies, in which the increase in invertase activity on evocation is totally non-commensurate with the increase in sucrose.

Assay for enzyme activity

a. use of homogenates and crude extracts

Unlike many other enzymes, acid invertase is a rugged enzyme, withstanding adverse conditions likely to develop during tissue disruption. The enzyme activity determined in conventionally prepared homogenates and crude extracts may, therefore, be taken as representative of the activity *in vivo*. This is in contrast to the

sensitive nature of sucrose synthase and sucrose phosphate synthase, whose activities as determined in homogenates and crude extracts may be under-estimates. The validity of the use of whole extracts enables ready assessment of any ontogenetic variations in invertase activity and comparison of enzyme activities among different tissues at a given growth stage.

At the same time, there are reports that purified enzyme fractions, when dilute, are unstable (Bracho and Whitaker, 1990 b). Rapid ultrafiltration to reduce the volume and incorporation of BSA during assay are recommended.

b. Medium for cell disruption

Water may be used as the medium for cell disruption when the cell wall fraction, with comparatively high invertase activity, is to be isolated for enzyme extraction (Lauriere, *et al.*, 1988). Most often a buffer is employed, as in the case of plant enzymes in general, often fortified with supplements, particularly to combat the action of phenolics. The incorporation of special supplements during invertase assay to minimize the action of endogenous proteinaceous inhibitor has been referred to in another context. When soluble invertase and wall-associated invertase have to be extracted together, O.1 M. sodium chloride has been used (Day, 1986). Invertase is, probably a sulfhydryl protein and, as such, a sulfhydryl agent such as 2-mercaptoethanol is advantageously employed, not only during cell disruption, but also during processing and in the assay system.

It may be pointed out that Tris buffer, in common use for many enzymes, cannot be used for invertase assay because of its inhibiting activity, as will be pointed out elsewhere, under neutral/alkaline invertase.

c. Assay method

The Somogyi method as modified by Nelson (1944), remains a very sensitive and precise procedure to determine invertase activity by estimating the total reducing sugar liberated from sucrose. The high molar extinction coefficient, 3.50×10^4 $M^{-1}cm^{-1}$ at 660 nm, permits the use of very low amounts of enzyme and short incubation periods in the assays. Invertase is completely inactivated upon addition of the Cu^{2+} reagent, thereby stopping further enzyme activity. Centrifugal clarification may be necessary when one is dealing with enzyme preparations containing interfering material.

By determining the activity by assaying glucose liberated by glucose oxidase method, any transfructosylation reaction catalyzed by the enzyme is corrected. When the enzyme activity has to be evaluated by fructose liberation, fructokinase or fructose dehydrogenase reaction can be used as coupling system.

d. Control

In tests on enzyme activity-temperature relationship, the activity at higher temperatures also have to be included. Invertase assays in some tissues are carried out at high incubation temperatures, as for example, 60°C which is the optimum for cactus tissues. While delineating the pH-activity profile of invertase in tissues in

general, one starts with buffer of pH 3. As a matter of fact, examples are known of invertase activity with optimum in the neighbourhood of pH 3. In view of the extreme sensitivity of sucrose to low pH and to high temperature at acid pH, it is essential to have an appropriate control wherein sucrose is exposed to the same conditions as in the experimental tube, but without enzyme.

Overlapping of acid-and neutral (alkaline) invertase activity

Unlike in the case of acid phosphatase and alkaline phosphatase, the optimum-pH difference between acid invertase (around pH 5 in the majority of cases) and neutral invertase (whose optimum is sometimes at pH 6.8) may not be marked in crude extracts of tissues and partially purified enzyme preparations. An acid invertase with much higher activity than neutral invertase in a given tissue may affect the determination of the activity of the latter and *vice-versa*. Hatch and Glasziou (1963) reported that acid invertase is present in immature sugarcane storage tissue and is replaced by an alkaline invertase in mature storage tissue. Carrot root tissue showed two distinct peaks for invertase activity, one in the acidic and the other in the alkaline range (Ricardo and ap Rees, 1970).

Analyzing invertase activity in the developing leaf of *Lolium temulentum*, a monocot, Pollock and Lloyd (1977) concluded that no neutral invertase was present at any stage; the activity present at pH 7.4 was considered to be residual acid invertase activity.

In *citrus*, there was a peak (pH 4.5) only in the acid range in young developing (1 month old) leaf, while in the mature (4 months old) leaves, there was a peak (6.8) only in the neutral range (Schaffer, 1986). The acid invertase was over 5-fold as active as the neutral invertase, on fresh weight basis. The activity at pH 7 of both tissue was similar. Sephadex G-200 gel filtration of leaf extract showed that in developing leaf, there was a broad peak of activity at pH 5, but this was accompanied by an additional larger molecular weight fraction with activity at pH 7; similarly, the mature leaf contained a small proportion of lower molecular weight acid invertase along with the major proportion of neutral invertase. It was concluded that acid invertase predominated in the young leaf and neutral invertase predominated in the mature leaf, without either being present exclusively although the pH-activity profile showed a single peak in either case.

The overlapping of activities was particularly marked in germinating mung bean (*Vigna radiata*) seed. Dey (1986) reported the pH-activity profile, from pH 3 to 8, of the resting seed and at definite stages of germination over an eight day period, numerical data were also presented for acid and alkaline activity, without specifying the pH at which these activities were assayed. The resting seed contained high alkaline activity, with a peak around pH 7, but with activity demonstrable also at pH 3, and evaluated as such (acid invertase), though without a peak in the acid range. The alkaline activity decreased markedly, retaining the peak, at the end of 24 h of germination; the acid activity increased again without a peak in the acid range and was quantified. Analyzed at the end of 72 h of germination, the peak in the alkaline range was no longer demonstrable, but the activity at pH 7, a considerably

reduced value, was evaluated as alkaline activity. An optimum in acid range was first demonstrable in 72 h; the acid invertase activity in this stage was almost twice the alkaline activity. The alkaline activity decreased further in 96 h and 192 h, with a semblance of a peak in the region; the acid activity, with a peak in the region, increased and at the end of 192 h, the activity at pH around 3 was only slightly less than the alkaline activity around pH 7 in the resting seed. The profile figure presented by Dey (1986) showed that at about pH 6 the activity tended to be constant over the entire experimental period.

Chen and Black (1992) pointed out that it was difficult to assess whether acid invertase (pH optimum 5.0) or alkaline invertase (pH optimum 7.0), constituting 70% and 30% respectively of the total activity, was being estimated in crude extracts of soybean hypocotyls at a given pH because of overlapping of activities.

It will be evident from the above, that considerable uncertainty can arise when invertase activity in crude extracts is categorized as acid-and alkaline-particularly so without a demonstrable peak. The present author has evaluated the pH-activity profile at every stage of development and reserved categorization only when a peak was observed in the acid or alkaline range or both. At the same time, the occurrence of a small proportion of neutral activity along with high acid activity in the stem tip of propagule and rooting stage and a small proportion of acid invertase along with high neutral activity in the shoot tip of the autotrophic plant could not be excluded. Also, the green leaf, with very high acid activity, might have contained a small proportion of neutral activity

Granting that summation of activities could take place when the two activities are present together, the peak in the alkaline range would be elevated by the residual activity from a highly active acid enzyme.

Incidentally, were an acid invertase to be present partly in the cytoplasm, it could exert its catalytic effect with an efficiency comparable to that of the neutral invertase present in low concentration and with its known weaker affinity for sucrose.

No method seems to have been worked out to selectively inhibit one type of activity in a mixture and assay the other. Plausible techniques are: (a) the incorporation of Tris buffer which effects total inhibition of the alkaline activity with only slight inhibition of the acid activity (Hawker, 1985) (b) the incorporation of the proteinaceous inhibitor from potato, which apparently inhibits only the acid enzyme (Bracho and Whitaker, 1990 *b*). Another approach is a preliminary separation of the two activities. This is possible by chromatography on Concanavalin A-Sepharose column, based on the presence of lectin-sensitive glycosyl group only in the acid enzyme. Another separation method is by fractionation with ammonium sulfate. Studies with bean embryo, young carrot root, mature sugar beet root and young corn root and stem and soybean hypocotyl have shown that alkaline invertase precipitates on 20 to 40% saturation with ammonium sulfate, while acid invertase appears in the 50 to 70% saturation fraction (Chen and Black, 1992). However, other workers have found that ammonium sulfate is an unsatisfactory precipitant for invertase.

Physiological significance of proteinaceous inhibitor

The proteinaceous inhibitor has been implicated in the regulation of invertase activity in plant tissues. A direct experimental proof is non-existent. However, it is not clear how the inhibition can be considered to be regulatory, since regulation in its true sense connotes a reversible reaction.

The endogenous inhibitor of potato has been implicated also as playing a role in the increase in invertase activity resulting from cold treatment, wounding or transferring of callus into liquid medium (Bracho and Whitaker, 1990 *a*).

The present author has not made a detailed study into a possible occurrence of a proteinaceous inhibitor of invertase in pineapple tissue. However, the fact that the plot of enzyme activity against period of incubation up to 1h, using chlorophyllous leaf as enzyme source, was a straight line was proof for the non-existence of a free inhibitor; in case an inhibitor was present, the plot would have yielded a curve, with increasing curvature with increasing period of incubation.

It is doubtful whether Pressey's inhibitor is specific for invertase. The inhibitor has been shown to be an agglutinin. Potatoes are known to contain an agglutinin, which also is an invertase inhibitor, qualifying as a second endogenous proteinaceous inhibitor of invertase in potato. Lectins behave as general "effectors" of glycosidases, which are glycoproteins and which complex with lectins.

It has also to be borne in mind that not all tissues contain Pressey's inhibitor. The inhibitor has been reported to be absent in sugarcane stem tissue, sugarcane leaf

sheath and papaya fruits (Isla, *et al.*, 1991). A clinching evidence against a physiological role for the inhibitor in regulating soluble invertase in potato appears to be the finding by Isla, *et al.*, (1992), that, contrary to the assumed vacuolar/cytosol location of the inhibitor, the inhibitor was located in the digested cell wall fraction resulting on preparation of protoplasts from potato tuber by digestion with carbohydrases. It might be thought that this location was an artefact resulting on processing of the tissue, but this was unlikely since intact protoplasts and vacuoles prepared therefrom did not contain invertase inhibitor when tested in ruptured preparations. The known agglutinin also was located in the digested cell wall fractions. Isla, *et al.*, (1991) concluded that the compartmentation of the inhibitor in a location non-contiguous, with two membranes separating with the vacuoles which contain the acid invertase renders unlikely a regulatory role for the inhibitor. A cell wall-associated (acid) invertase could, however, be acted on by the inhibitor. The present author has been unable to find any report on wall-associated invertases in potato. Confirmation of the finding of wall-association of inhibitor is necessary with other plant sources known to contain inhibitor.

Absence of wall-associated invertase in pineapple tissues

Plant tissues which have been carefully analyzed contain a form of invertase associated with cell wall fraction, apparently reflecting an *in vivo* occurrence. Such an activity is essential in tissues in which the cellular uptake is in the form of hexoses derived from the phloem unloaded sucrose. In the present investigation,

invertase activity could not be demonstrated in any of the tissues studied. The following reasons appear plausible:

1. Only a small proportion of the total invertase was in the insoluble form and it escaped detection in an assay system which necessitated the use of barium hydroxide and Zinc sulfate to clarify the incubated system. Whereas 98% of the total invertase activity was wall associated in cultured carrot cells, only about 10% was insoluble in carrot seedlings (Lauriere, *et al.*, 1988).

2. Wall-associated invertase was confined to the cells close to the phloem tissue and this tissue constituted a small proportion of the total cellular mass. Such a likelihood was envisaged by Getz (1991) in beet root hypocotyl.

3. The wall-associated invertase was in ionic linkage with the cell wall and as such extractable with high ionic salt solution (1 to 2M sodium chloride, phosphate, etc.,) without the need for disrupting covalent linkage, as by the use of EDTA. Dey (1986) showed that 0.1M sodium chloride was adequate for extracting the entire invertase activity from resting and germinating mung bean seeds, no further activity being extracted when 0.1M was followed by 0.5M sodium chloride. The extractant used by the present author was 0.01M with respect to potassium phosphate. Crassulacean tissues may be expected to contain high endogenous concentration of ions. Together, sufficient ionic strength was available to solubilize the wall enzyme on tissue disruption.

4. It must be mentioned, at the same time that the inability to detect wall associated enzyme was due to an actual absence of the enzyme. Canesugar internode has long been considered as the typical case of a tissue requiring hydrolysis of sucrose prior to uptake. Even in this tissue recent evidence casts doubt on this concept. It is possible that in the pineapple tissues examined, all of which were meristematic, or only partly differentiated, the uptake of sucrose was totally symplastic. In leaves of *Lolium temulentum*, particulate invertase constituted a high proportion of total invertase activity in mature leaves, but in the early stages of leaf development invertase activity was totally soluble (Pollock and Lloyd, 1977). Schaffer (1986) observed that insoluble invertase was absent not only in the young but also the mature leaves.

A word in explanation of the use of Triton-X 100 in assay system to test for invertase activity in the cell wall fraction (from green leaves). One of the likely causes for the dramatic increase in "ageing" of tissues is the occurrence of invertase in vesicles (Getz, 1991). Such vesicle-contained enzyme may normally be "dormant" and can be exposed by treatment with a detergent, as in the standard practice in assaying lysosomal enzymes in liver tissue.

Distribution of enzyme forms

In sugarcane internode (Hatch, *et al.*, 1963), carrot roots (Ricardo and ap Rees, 1970), sugarcane leaf sheaths (Sampietro, *et al.*, 1980) and onion bulbs (Lercari, 1982) invertase occurs in both acid and neutral forms. In wheat coleoptile

(Krishnan, *et al.*, 1985) and strawberry fruit (Ranwala, *et al.*, 1992) only the acid form is present. Acid invertase has both an extracellular and vacuolar location in many plants. In carrot seedlings, 85 to 90% of the total activity was in the supernatant fraction of homogenate and 10 to 15% with the cell wall fraction. On the other hand, in suspension cultured carrot cells 98% of the total activity was in the cell wall and only 2% in the supernatant fraction (Lauriere, *et al.*, 1988). The cell wall of mature leaves of *Nicotiana tabaccum* contains invertase. Potato invertase in its soluble acid form has been studied by a number of workers Bracho and Whitaker, 1990 *b*) but there does not seem to be any authentic report of alkaline invertase and cell wall associated acid invertase in the tissue.

In general, cultured cells and tissues exhibit high cell wall- associated acid invertase activity.

As will be evident from the preceding pages, invertase may not occur in only one form in a tissue. A particular form may be predominant in the tissue, but other forms may coexist, though in quantitatively minor amounts.

The pineapple plant has distinctive features. Invertase activity was assessed in (a) the shoot tip (b) the diagnostic leaf ('D' leaf) and (c)the root tip in selected stages of the ontogeny of the plant.

Invertase was active in all tissues of the pineapple plant analysed, with an exception. Only the soluble form was demonstrable and this was composed of the acid enzyme and neutral (alkaline) invertase. The enzyme occurred singly in the

acidic- or neutral form or the two together. The tissue exception was the aerial root of propagule in which neither acidic nor neutral invertase was demonstrable.

Three types of transition of activity were encountered, one in the stem, another in the leaf and the third in the root tip. In the heterotrophic (the developed axillary shoot, serving as propagule) and pre-autotrophic stages (following transplanting of propagule, about two months period) all the three stem segments studied (upper (stem tip), middle and lower) contained only acid invertase. On the other hand, the stem tip of the pineapple in the autotrophic stages, both in the vegetative and reproductive stages, showed only neutral invertase activity.

In limited studies conducted in this direction, the transition was demonstrable in the shoot tip, by about 70 days following planting. However, no sample of shoot tip could be identified wherein the two forms were present side by side and where the neutral form dominated over the acid form. Also to be noted was the fact that the stem tip of the autotrophic plant which continued to be a utilization sink contained the alkaline form of the enzyme whereas the acid form of the enzyme is normally found in this type of tissue. It is possible that the inability to demonstrate the presence of the two enzymes together was due to overlapping of the activity, as discussed in the preceding section.

The second type of enzyme activity transition was built into the tissues of the leaf which was composed of an achlorophyllous-(wraping round the stem) and chlorophyllous part, with an intervening diffused regions. The achlorophyllous leaf part had both acid and neutral invertase. The chlorophyllous part had only the acid

form. The intervening diffuse regions(-sub-chlorophyllous) resembled the achlorophyllous leaf tissue in that both acidic and neutral activities were present. However, the relative activities of the two forms changed acropetally in the intervening diffuse regions. The lower part of the intervening diffuse region had a sharp peak at pH 4.6 which decreased and then rose to register a second peak at pH 6.6. In the region contiguous to the chlorophyllous leaf part, the activity at acid pH was very dominant and the activity in the alkaline region touched a minimum.

The third type of transition in activity was manifested in the root tip. No invertase activity could be demonstrated in the root tip in the heterotrophic stage, that is, when still aerial. In one month and two months old plant, the root tip tissue contained both acid and neutral invertases with pH optimum between 4.6 and 5.0 for acidic form and 7.0 for neutral form. This pattern of activity in the root tip tissue continued during the further development of the plant. Thus, the root tip tissue in the organogenetic stage showed the pH activity profile similar to that obtained in one month old plant with optimum activity for acid invertase between 4.6 and 5.0 and for neutral invertase at pH 7.0.

Induction, evocation and morphogenesis

A wealth of literature exists on the flowering process. The most recent review is that by Bernier (1988).

Day length is most effectively perceived by young expanded leaves. It is believed that the leaf transmits a promoter to the shoot apex, leading to evocation

of the latter. In the present author's study, the "D" leaves were assumed to discharge this function.

The events occurring in the shoot apex that commit it to flower formation are called evocation. Evocation is characterized by marked histological and morphological changes. One of the most conspicuous events of the floral transition is on increase in the rate of cell division. No distinct cell lineages are involved, but floral transition obligatorily involves the activation of the central zone. The start of evocation is difficult to determine accurately. Evocation is essentially a molecular and cellular process whose completion triggers the changes at the higher levels of organization, irreversibly committing the meristem to flower formation.

Gene expression is highly regulated during flower development. Increased synthesis in all RNA fractions is among the earliest events occurring in transitional meristem. It has not so far been possible to identify the mRNAs that may change at evocation. Immunological and electrophoretic analysis has established that protein complement of transitional apices is altered before flower primordia are initiated. Most of these changes are quantitative, but there are a few possible qualitative changes.

Among other molecular changes are rises in sucrose and ATP levels, invertase activity, mitochondrion number and energy charge. There is evidence to show that the increase in sucrose is unrelated to photosynthetic input, but is critically involved in evocation. The morphogenetic role of sucrose, referred to in the Introduction, assumes considerable significance in the light of this observation. In some plants,

early increases in the activity of the enzymes of the pentose phosphate pathway have been found.

Pryke and Bernier (1979) found 58% increase in invertase activity on fresh weight basis in the evoked shoot apex of *Sinapis alba*.

The present study shows about 60% increase in the (neutral) invertase activity on fresh weight basis (about 54% increase on dry weight basis)which is unexpectedly low compared to the 7-fold increase in sucrose reported by Madhusudanan and Nandakumar (1983). It has to be emphasized that the sampling of tissues both stem tip and leaf by the present author differed from that by Madhusudanan and Nandakumar; the present author collected about 2cm length of stem tip, contrasting with the 5mm shoot tip by Madhusudanan and Nandakumar. This enhancement was abolished in the next stage. In this context, two important factors have to be borne in mind; a. there is no evidence to suggest that invertase activity is rate-limiting in the stem tip of the pineapple plant b. sucrose cleavage can be brought about also by sucrose synthase, acting independently of invertase. The study by Duke, *et al.*, (1991) shows that under certain physiological conditions, sucrose cleavage is effected wholly by sucrose synthase.

In studies by earlier workers, and as is to be expected, the invertase in the shoot tip is the acid type (Pryke and Bernier, 1978). Unexpectedly, but interestingly and probably significantly, it is the neutral form of invertase that seems to be operational in pineapple shoot. In this context, the observation that the affinity of neutral (alkaline) invertase of the shoot tip is comparable to that of the acid

invertase of the shoot tip in propagule and rooting stages, assumes significance. The affinity for sucrose is, if any, enhanced in the stem tip during development of the pineapple plant starting from the propagule, although the acid form was present in the latter and the neutral form in the developing plant.

Ideally, the biochemical studies on shoot have to be carried out on the apical meristem. This is difficult in practice and many workers have employed apices bearing leaf primordia and young internodes. In justification, it may be pointed out that it is not the current photosynthesis alone that is involved, but that storage material may also be brought into the metabolic picture. There is reason to believe that the shoot tip sample selected by the present author is constituted of a comparatively high proportion of meristematic tissues. Shoot development is a manifestly complex phenomenon (Torrey, 1967). It involves the total activity of the shoot apex, including the initiation of leaf primordia at the apex, the formation of new cell populations which perpetuate the cell layers formed at the apex, the differentiation of the primary tissues, including the vascular tissues of the stem, and, all the while, the constant elongation of cells below the apex, giving growth in height. In addition, new shoot apices are initiated in the axils of leaves, buds which may be completely suppressed in their development or begin elongation and leaf formation too, producing a much branched shoot system. Two general types of shoot development can be distinguished:

a. the long-shoot type, in which internodal elongation occurs regularly and often extensively,

and

b. the short shoot type, in which internodal elongation is almost absent and leaves are inserted one close upon the other along the axis.

In short shoot type plants, leaves are produced in succession at the apex as in long-shoot types, but the internodes fail to elongate and the leaves remain close together in a rosette arrangement. It is this type of stem which occurs in the pineapple plant. The stem tip of the pineapple plant can, therefore, be expected to contain a comparatively high proportion of meristematic tissues.

The splitting of vacuoles at the apex is an early ultrastructural change in some plants. This assumes significance in the light of the localization of acid invertase in vacuoles.

Are acid invertase and alkaline invertase distinct proteins?

A decrease in invertase activity with optimum in the acid range with growth of tissue and the appearance of, or increase in, activity in the alkaline range in the mature tissue (constituting the basis of the roles postulated for the two forms of enzyme) could, *a priori*, be interpreted as a transformation of the acid- into the alkaline enzyme. Similarly, germination profile of enzyme activity could be thought of as transformation of the alkaline invertase protein in the resting seed into the acid invertase protein in seedling.

There is ample evidence that acid invertase from diverse sources is a glycoprotein. The detailed study by Chen and Black (1992) showed that electrophoretically homogeneous alkaline invertase from soybean hypocotyl, the first such preparation from a plant source, is not glycosylated. An earlier study by Stommel and Simon (1990) also was indicative of the absence of glycosylation in alkaline invertase, the enzyme being obtained from suspension-cultured carrot cells. A change from acid-to alkaline invertase and *vice versa* could be thought of as being brought about by deglycosylation. Such a post-translational modification of invertase would appear to be analogous to the post-translational phosphorylation-dephosphorylation of some enzyme proteins and nucleotidylation-denucleotidylation of some other enzymes (Battey, *et al.*, 1993). However, there is an integral difference between these processes. One of the proteins resulting from phosphorylation-dephosphorylation and adenylylation-deadenylylation (nucleotidylation-denucleotidylation) is enzymatically inactive. In the case of invertase, both the products are enzymatically active. Of course, the glycosyl-free form of the enzyme differs in location, pH optimum and affinity towards sucrose. Also, there is no evidence for the reversal of the reaction, glycosylation of deglycosylated protein; in the forward direction, glycosylation is effected in the Golgi body. That deglycosylations of a protein can occur *in vivo* has been established in the case of a lectin. Concanavalin A, the major constituent of the jack-bean seed, is glycosylated at its active site for the major part of its maturation in the Golgi complex, but in the final stage of its synthesis, a glycanase removes this side group and "activates" the molecule (Bowles, 1993).

That the above postulated mode of transformation of the acid-into the alkaline enzyme activity by deglycosylation could not be operative in some tissues, at any rate, follows from two lines of evidence:

1. Chen and Black (1992) employed eletrophoretically homogeneous alkaline invertase and acid invertase (the latter with glycosyl group intact) isolated from soybean hypocotyl to raise rabbit polyclonal antibodies. The soybean alkaline invertase antibody was found to be specific for the alkaline invertase and to cross-react with partially purified alkaline invertase from other plants. Neither the purified soybean alkaline invertase nor any of the crude alkaline invertase from a number of other plant sources cross reacted with the soybean acid invertase antibody. (This may be contrasted with the cross reactivity of the antibody from cell wall acid invertase with soluble acid invertase (Lauriere, *et al.*, 1988)). The immunological distinction between the alkaline and acid invertase of soybean hypocotyl suggested that the two enzymes are encoded by distinct nuclear genes.

2. Dey (1986) observed that dormant mung bean seeds have high alkaline invertase activity. During germination, the alkaline activity rapidly decreased and was replaced by increasing acid invertase activity. Cycloheximide prevented the appearance of the acid activity during germination. The author concluded that acid invertase was formed by *de novo* synthesis of protein. This study cannot be considered to be unequivocal:cycloheximide is a general translational inhibitor. The germination process itself was inhibited by use of

cycloheximide. Also, the high alkaline activity initially present decreased markedly in the treated seeds, which the author attributed to endogenous degradation of the protein by preexisting proteases.

The present author feels that it has not been definitely proven that alkaline invertase and acid invertase from all plant sources are not interconvertible.

Are the different forms of (acid) invertase coded by a single gene or multiple genes?

I. Comparison of properties of invertase multiple forms

Studies by Pollock and Lloyd (1977) and Lauriere *et al.*, (1988) showed the occurrence of plural forms of soluble invertase, respectively in *Lolium temulentum* and carrot seedling. Ranwala *et al.*, (1992) showed two types of wall-bound invertase in strawberry fruit. Isoelectric focussing, probably the most sensitive method to detect isoenzymes, showed the presence of a number of isoenzymes in cell wall fraction of radish seedling (Faye *et al.*, 1986).

An account of the differences, if any, in the properties of the isoforms follows.

A. Soluble invertase and wall-bound invertase and forms of wall-bound invertase

Wheat coleoptile

Krishnan *et al.*, (1985) found that the solubilized cell wall enzyme of wheat coleoptile, which was only partially pure, and the highly purified cytoplasmic soluble

enzyme (-which represented the major part of the total enzyme activity-), both acidic, differed in pH optimum for activity (cell wall enzyme pH 4.5 and cytoplasmic enzyme pH 5.5), chromatographic behaviour on DEAE-cellulose, thermal stability, K*m* value and response to light treatment of tissue.

Strawberry fruit

Ranwala *et al.*, (1992) found that the soluble invertase, which accounted for most of the total invertase activity, and the two wall-bound invertases, NaCl-released fraction and EDTA-released fraction, all tested as crude preparations, had somewhat similar properties with regard to:

optimum pH: (range 4.2 to 5.2; soluble invertase, pH 4.6, the NaCl-released cell wall fraction, pH 5.0 and the EDTA-released cell wall enzyme, pH 4.2.)

Optimum temperature: (40 to 50°C; soluble invertase 50°C, the NaCl-released wall enzyme, 40°C and the EDTA-released cell wall enzyme, 50°C)

K*m* value for sucrose: (3.5 to 4.4 mM; 3.5 for soluble enzyme, 3.7 for NaCl-released enzyme and 4.4 for EDTA-released enzyme)

substrate specificity: (raffinose and stachyose were also acted upon; respective activities towards raffinose and stachyose, as a percentage of the activity toward sucrose, were: 25% and 15% for soluble enzyme, 26% and 13% for NaCl-released fraction and 23% and 15% for EDTA-released fraction).

Findings common to the above two tissues are: (a) the soluble enzyme represented the major part of total invertase activity of tissue and (b) the wall-bound enzyme had a lower pH optimum than the soluble enzyme.

In all these properties, the strawberry enzyme resembled the acid invertases reported in other tissues. However, they exhibited different degrees of inhibition of activity by metal ions, Ag^{+}, Hg^{2+}, and Mn^{2+}.The complete inhibition of the (NaCl released wall-bound)strawberry fruit invertase by 1.7mM Mn^{2+} distinguishes it from other acid invertases. That differences existed between the two forms of wall-bound invertase was shown by the fact that while 1.7 mM $MnCl_2$ led to total inhibition of NaCl-released enzyme, the EDTA-released enzyme was inhibited only 35%.

There, thus, appeared to be "two types" of wall-bound invertase in strawberry fruit based on difference in degree of inhibition by $MnCl_2$.

It may be pointed out that while the wall-bound enzyme fractions were completely precipitated by 80% saturation with ammonium sulfate, soluble invertase was precipitated only 40% in 80% saturation and required 90-100% saturation with ammonium sulfate for total precipitation.

Of interest was the finding by Ranwala *et al.*, (1992) that both types of wall-bound invertase decreased continuously with fruit development, almost disappearing in the over-ripe fruit. This was in contrast to the soluble acid invertase which increased continuously upto the red-ripe stage, to drop in the over-ripe fruit. The

turnover of intracellular protein is well known, but there is very little information about the turnover of wall-bound enzyme protein.

Carrot

Acid invertase isolated in homogeneous form from the cell wall fraction of suspension cultured carrot cells and in an enriched form from the cytoplasm of 7 days old carrot seedlings (Lauriere, *et al.*, 1988) differed in the following respects:

1. The soluble enzyme (-a mixture of two molecular forms-) had its pH optimum around 5.6 and the cell wall-associated enzyme around 4.6, as determined in seedlings.

2. The cell wall enzyme (not only of seedling, but also the cultured cells) had *M r* of 63,000, while the soluble enzyme was made up of two isoforms, separable by chromatography, with *M r* of 58,000 and 52,000 (-the latter being the more abundant form -); only the *M r* 58, 000 form was present in the cultured cell.

Comparison of soluble invertase from different organs of the same plant

Employing the purified enzyme preparations from potato tuber and mature fully expanded leaves from the top of the potato plant, Burch *et al.*, (1992) found that in terms of:

pH optimum (5.0 for the two enzymes)

ability to hydrolyze lactose (10–11% of the rate of sucrose for either enzyme)

and

M r (58,000, monomeric, for each enzyme)

The invertase from tubers and leaves appeared to be similar. However, in:

K*m* value for sucrose (7.9 for tuber enzyme and 2.4 for leaf enzyme) reactivity towards raffinose and trehalose (tuber enzyme acts on both, leaf enzyme does not act on either)

and

product inhibition characteristics (the inhibition by fructose was linear mixed for the leaf enzyme and competitive for the tuber enzyme; glucose inhibition uncompetitive for the leaf enzyme and non-competitive for the tuber enzyme)

The two invertases differ from each other.

B. Soluble acid invertase and alkaline (neutral)invertase

This was considered separately.

A detailed study of the properties of invertase occurring in different tissues of the pineapple plant was outside the scope of the author's investigation. However, some conclusions can be drawn from the limited studies carried out.

pH optimum

A sharp acid pH optimum, 4.8, was obtained only in the chlorophyllous part of the leaf. In all other tissues, the optimum acid activity was spread over a range. The choice of pH optimum 4.8 in standard assay of all tissues is explained in the Results Section.

K*m* value

The comparatively wide range in the K*m* value for sucrose among the acid invertases from different tissues, suggestive of the existence of isoforms of invertase, was emphasized in the results section.

A remarkable finding emerging from the present study was the comparable K*m* value for sucrose of the neutral enzyme occurring in the stem tip tissue and the acid enzyme occurring in the achlorophyllous stem tissues in the propagule and its rooting stages. Also remarkable was the finding that in the pineapple plant, the K*m* value of the neutral invertase in different tissues was lower than the K*m* value of acid invertase in one particular tissue, namely, the achlorophyllous leaf tissue. It is generally held that the neutral invertases have a higher K*m* value than the acid invertases (Avigad, 1982).

B. *In vivo* and *in vitro* transformations'

Multiple forms of soluble invertase were reported in wheat leaves by Roberts (1975) and an increase in the proportion of the high molecular weight form

occurred when cold-hardy cultivar was grown at low temperatures. In studies by Pollock and Lloyd (1977) on developing leaves of *Lolium temulentum*, fractionation of soluble invertase by gel filtration showed the appearance of a high molecular weight form at the time when the insoluble activity was rising .This transient form could represent the insoluble form prior to incorporation into the cell wall matrix.

In *Utrica diocia*, Fahrendorf and Beck (1990) reported that limited proteolysis of the soluble 56,000 *M r* leaf invertase resulted in a range of forms on gel filtration which were both larger and smaller than the undegraded enzyme.

Yelle, *et al.*, (1991) found that the highly purified proportion of acid invertase from tomato fruits contained three polypeptides of 52, 30 and 22 KDa. The authors concluded that the smaller polypeptides were products of degradation of 52 KDa polypeptide because the N-terminal amino acid sequences of the 52 KDa and 30 KDa polypeptides were identical.

Arai, *et al.*, (1991) could not obtain any evidence for secondary formation of invertase. Acid invertase from hypocotyl of etiolated seedlings was separated into two fractions of A and B by chromatography on Hydroxylapatite. Acid invertase fraction B consisted of two polypeptides 30 KDa and 38 KDa, but that in fraction A was 70 KDa in size. Immunochemical reaction and Concanavalin A binding reaction showed that acid invertase in hypocotyls of mung bean seed is actually present in two forms: a monomer 70 KDa and a heterodimer of 30KDa and 38KDa polypeptides. Monomer was not converted into the heterodimer during incubation

of crude extract and was present together with the heterodimer in very young hypocotyls.

As already pointed out, wide ranges of specific activity are found for higher plant invertase from different sources and even isoenzymes from the same organism.

Molecular weight (*M r*) for invertase varies from 48,500 for the radish seedlings (Faye, *et al.*, 1981) to 450,000 for the *Lolium* pollen (Singh and Knox, 1984). Many purified invertase have *M r* in the range 50-60,000. The invertases are either monomeric or polymeric. In radish seedlings it is a monomer whereas in *Lolium* pollen it is an oligomer. Prado, *et al.*, (1985) reported that in Castor bean *Mr* 77,800 is a heptomer composed of identical *M r* subunits (*M r* 11,000). Al-Bakir and Whitaker (1978) reported that date invertase, *M r* 130,000 is composed of two identical subunits, *M r* of 70,000. Bracho and Whitaker (1990 b) reported that potato invertase had *M r* of 60,000 and that it was a dimer of 30,000 subunits. The dimeric nature of potato invertase could not, however, be confirmed by Burch, *et al.*, (1992) who found that SDS-PAGE gave a *M r* of 58,000.

An exception to the usual molecular range is the enzymes from sugar beet seedlings (28,000).

All the above mentioned polymeric forms are composed of identical subunits. Arai, *et al.*, (1991) reported for the first time the presence of a heterodimeric form made up of 50 KDa and 30 KDa.

When soluble and insoluble invertase occur together, it is usually found that insoluble enzyme has a higher molecular weight than the soluble enzyme (Faye, *et al.*, 1986; Lauriere, *et al.*, 1988).The insoluble enzyme in suspension cultured carrot cells had a M_r of 63,000 whereas the soluble form which occurred in two isoforms had M_r of 58,000 and 52,000.

The glycosylated nature of invertase may lead to a lack of precision (over estimation) in estimating the molecular weight. Ovomucoid, a glycoprotein, with a M_r of 27,000, when subjected to Sephadex Gel filtration, revealed a M_r of 38,000 to 45,000 (Whitaker, 1963).

Three types of yeast invertase molecules have been identified:

(a) a high molecular weight, glycosylated external enzyme located at the periplasmic space of the cell; (b) a non- or poorly glycosylated intracellular enzyme and (c) an invertase species with an intermediate level of glycosylation associated with "cell membrane" structure. The various invertase species in yeast are all related since they represent different stages of the pathway leading to the synthesis of a single glycoprotein molecule (Avigad, 1982).

Involvement of a single gene or multiple genes

In the case of yeast, the different forms of invertase are products of a single gene (Perlman, Halvorson and Cannon, 1982). The soluble and the cell wall-associated forms of ß-fructosidase in radish seedlings exhibit high antigenic similarities (Faye, *et al.*, 1984). The different forms of carrot ß-fructosidase are

antigenically related, as tested with immune serum specific for deglycosylated cell wall ß-fructosidase from carrot suspension cells (Lauriere *et al.*, 1988). It is not certain whether the different forms of ß-fructosidase in higher plants are products of a single gene, as observed in yeast invertase. The study by Chen and Black (1992) suggesting that alkaline invertase coding is by a distinctive gene, was already referred to.

Chitinase and ß-1, 3-glucanase exist in multiple forms in higher plants, grouped into two classes, basic and acidic, with different subcellular location. The basic forms are generally vacuolar, while the acidic forms are usually secreted into the extracellular space (Van Den Buleke, *et al.*, 1989; Keefe, *et al.*, 1990). For each enzyme, the two forms are products of similar, but distinct, genes.

Deficiency/absence of invertase in aerial roots

Many plants have two root systems that differ in origin (Fahn, 1989).One is the *primary root system*, whose origin can be traced back to the radicle developed during embryogenesis. The other is an *adventitious root system*, which arises on parts of the plant not originating from the embryonic root, that is, from parts of the shoot. Adventitious roots usually initiate endogenously from tissue within the parent plant, though a few cases of exogenous origin are known. Roots which arise on the primary root are the *lateral roots*, referred to sometimes also as *secondary roots*. Lateral roots are sometimes also called adventitious; the term *adventive* can be applied to them to distinguish from roots of shoot origin (Barlow, 1986).

Adventitious rooting is the basis of vegetative propagation of plants, mostly from cuttings (Jackson, 1986). Thus, adventitious rooting is involved in the vegetative propagation of the horticultural varieties of the pineapple plant.

Presumably, the future of development of each root type is determined at its inception, or at a very early stage of growth. The apices once formed and determined continue to produce tissues of the appropriate type. Anatomical changes occur when aerial roots of *Ficus benghalensis* enter the soil and get converted into terrestrial roots. These changes are presumably the result of a redetermination of the meristem causing it to produce a new pattern of tissue differentiation(Barlow, 1986).Whether the enzyme profile is influenced on transformation of the aerial to the terrestrial root is not known.

Carbohydrates have most often been considered to be the principal source of energy and carbon skeletons during rooting (Haissig, 1986). Many studies have been carried out on carbohydrate–rooting relations involving either total soluble carbohydrate, or total non–structural carbohydrate. Reducing sugars and sucrose, but not starch levels, differed between the upper (non–rooting) and basal (rooting) stems of jackpine seedling cuttings during propagation.

The inhibiting activity of invertase inhibitors of higher plants seems to be limited to acid invertases (Bracho and Whitaker, 1990 b). This observation has considerable relevance to the present author's studies on root invertase. The inability to demonstrate an enzyme activity in a particular tissue could possibly be due to inactivation by an inhibitor during homogenization and processing. That such an

artefact did not result in the present study was indicated by results of assay by the mixed homogenate technique, which gave no indication of the presence of residual free inhibitor in the extracts of aerial roots. Also, the action of inhibitor on the enzyme is a slow process, taking several hours for completion at low temperature. Invertase activity in the terrestrial roots of the pineapple plant was distinctive in that the alkaline activity was higher than the acid activity; the two activities coexisted also in the achlorophyllous leaf part and intervening diffuse region, but the acid activity dominated over the neutral activity. The root tip of the aerial roots failed to exhibit not only acid but also alkaline activity, which latter is not susceptible to inhibition by the inhibitor. The conclusion may be drawn that the absence/deficiency of invertase activity is an *in vivo* feature of the aerial root. Sucrose generally is believed to enter root tips without traversing the extracellular space (ap Rees, 1984), so that it is not obligatory that it be hydrolyzed before uptake into cell. In the absence of invertase, sucrose utilization by the tissue could theoretically be initiated only via action of sucrose synthase.

A factor which might be contributing to the inability to demonstrate invertase activity in the tip of aerial root could be the cellular composition of the tissue. In the 5-day-old pea seedling root, the acid invertase activity in the apex region is much higher in the cortex than in the stele. In case, the root of the propagule contained a much higher proportion of cells corresponding to stele, in comparison with cortex, the homogenate derived from the composite tissue would manifest a low activity which could be missed.

That invertase could be totally dispensed with under certain physiological states has recently become evident. Duke, *et al.*, (1991) reported organ-specific invertase deficiency affecting only the primary root system in the Oh 43 inbred maize; substantial invertase activity was evident, in extracts of the other tissues. This deficiency was evident also in the lateral roots arising from the primary root. In contrast, morphologically identical lateral roots from the adventitious root system had normal invertase levels. Ontogenetic origin of root tissue appeared to be a determinant of invertase activity in maize. Adventitious roots (including the seminals) arise above the scutellar node and are, therefore, of shoot origin. The genetic potential for invertase activity was present. The lack of activities might have resulted from altered regulation of gene expression. The study with Oh inbred maize showed invertase activity was not essential for maize root growth. Sucrose synthase was active in extracts from all root apices and could mediate the sucrose degradation in primary root tips of Oh 43. Sucrose synthase activity was not assayed in the aerial root of the pineapple plant; it is possible that this enzyme was active in the tissue and was able to cleave translocated sucrose.

Silva and Ricardo (1992) have drawn attention to the fact that sucrose may have also important roles in the morphogenetic processes in plant cells. Wetmore and Rier (1963) and Jeffs and Northcote (1967) showed that sucrose induces vascular differentiation in callus and tissue culture. Other aspects of callus metabolism and growth are also influenced by sucrose concentration. (Edelman and Hanson, 1971; Parr, Edelman and Hawker, 1976). In experiments involving the *in vitro* promotion of flowering of tissues or apical buds, the importance of sugar levels

in the culture medium has been demonstrated (Hendrickson, 1954; Deltour, 1970). Whether the primary role of sucrose is as source of energy/storage carbohydrate, or as a morphogenetic effect, invertase/sucrose synthase would be involved as a step.

Influence of light

In terminally differentiated tissue, invertase levels (radish cotyledons) are influenced by light (Zouaghi, 1976). In radish hypocotyls, the levels of cell wall-bound invertase activity are enhanced by light treatment (Zouaghi, Klein-Eude and Rollin, 1979; Zouaghi and Rollin, 1976). It was proposed that light via phytochrome induced a transfer of the radish enzyme from the cytosol to the cell wall. In studies by Krishnan, *et al.*, (1985), it was found that the levels of cell wall-bound invertase are enhanced by light treatment. Phytochrome is probably involved.

The soluble-and the wall-associated invertases of wheat differed in their response to light treatment, as tested in coleoptile and root of light-grown and dark-grown 4 days seedling. Cell wall-enzyme increased 60-90% in light-grown seedling both in the coleoptile and roots. Soluble enzymes were slightly less in the light-grown both in the coleoptile and root. Thus, illumination had a pronounced influence on the distribution of the cell-wall-bound and soluble forms and lesser effect on the total invertase activity in these tissues.

Faye, et al., (1986) reported that when 36-hour-old dark-grown radish seedlings were transferred to far-red light, there was a decrease in cytoplasmic ß-fructosidase and an increase in cell wall-ß-fructosidase compared to the dark

controls. Growth of radish seedlings in the presence of tunicamycin resulted in a partial inhibition of ß-fructosidase glycosylation but nonglycosylated ß-fructosidase still accumulated in the cell wall under far-red light.

The studies on illumination effect have bearing on the present author's studies on: (a) achlorophyllous leaf versus chlorophyllous leaf and (b) possibly also in aerial root versus terrestrial root.

Slow, but progressive transformation of the achlorophyllous-to the chlorophyllous leaf part

Conifer seedlings can synthesize chlorophyll in the dark. Many algae, as for example, the brown-and blue green algae, also have the ability to elaborate chlorophyll in the dark. However, the large majority of the higher plants require light for the chlorophyll synthesis. Light is necessary not only for the synthesis of chlorophyll, but also for the elaboration of the internal structures of the chloroplast.

As the pineapple leaf develops and increases in length, the acropetal region of the part intervening between the achlorophyllous and the chlorophyllous regions gets progressively exposed to light and greens. The intervening region, as also the achlorophyllous part, get narrower and narrower, until minima are attained in the fully mature leaf. Ordinarily, the chloroplast develops directly from the proplastid, which is abundant in meristematic tissue. Since the achlorophyllous leaf part is not wholly meristematic, chloroplasts in this tissue can be considered to originate from plastids rather than directly from the proplastids. It appears likely that etioplasts are

present in the achlorophyllous region and that these develop into chloroplasts under the influence of light. Etioplasts are usually a transitory stage; they are formed when the development of proplastids into chloroplasts is interrupted by lack of light. When etiolated tissue is exposed to light, the etioplasts rapidly develop into chloroplasts and the prolamellar bodies are transformed into the thylakoid membrane system.

Studies with etiolated seedlings, which contain small amounts of protochlorophyllide-holochrome, have shown that on exposure to short flashes (as short as 10^{-4}s), interspersed with 10-15 min. dark periods, considerable chlorophyll can accumulate (Goodwin and Mercer, 1983). After a lag period of about 2h, the rate of protochlorophyllide formation and its conversion into chlorophyllide are increased by at least 10-times. This is associated with the rapid formation of the lamellar structure.

An alternative precursor of chloroplast is the chromoplast. In Nature, chromoplasts, though capable of developing directly from the proplastids, are formed from chloroplasts. Chromoplasts have most of the structural features of the chloroplasts; the photosynthetic membrane system has, however, been replaced by structures rich in carotenoids. The reverse process, the transformation of the chromoplast to the chloroplast, has been observed in carrots and oranges. It should, however, be pointed out that there is no indication of the occurrence of chromoplasts, characteristically pigmented yellow, orange and red due to carotenoids, in the achlorophyllous leaf region.

Yet another possibility is that the chloroplast is formed from an amyloplast. Amyloplasts are usually formed as intermediate stages in the development of etioplasts and chloroplasts. Even the apparently stable amyloplast of potato tuber will develop a green thylakoid system if illuminated.

Studies on the effect of light on etiolated tissue have centred on the transformation to chloroplast. However, it may be remembered that invertase is not known to be associated with chloroplasts but is present in the vacuoles and cell wall.

Non-enzymic hydrolysis of sucrose

It is generally held that substrates are metabolized *in vivo* by enzymatic pathways. A few reactions are, however, known which are non-enzymic. The extreme sensitivity of the hexoses linkage in sucrose to acidity (Goodwin and Mercer, 1983) renders sucrose readily susceptible to non-enzymic hydrolysis in vacuoles which accumulate considerable organic acids. Vacuolar acid hydrolysis of sucrose has been postulated as the only *in vivo* mechanism for sucrose breakdown in mature acid lime juice vesicle cells (Echeverria, *et al.*, 1992). This non-enzymatic system replaces sucrose degradative enzymes present during earlier stages of ontogeny. The existence of vacuolar acid hydrolysis is dependent on three conditions operating concurrently:

1. the absence in vacuoles of enzyme with sucrose degradative activity-acid invertase and sucrose synthase

2. a vacuolar pH of *ca* 2.1

3. sucrose compartmentation in the vacuoles

The first two conditions are highly uncommon in plant cells, but their operation was demonstrated in acid lime juice vesicle cells. It is, however, not clear how sucrose and such high acidity could coexist in a compartment.

The diurnal variation in vacuolar acidity in the pineapple plant (pH 3.5 to 6.0) and other CAM plants operates against acid hydrolysis as a means of sucrose degradation.

SUMMARY

SUMMARY

The ontogenetic changes in: (A) dry solids, (B) total protein and (C) invertase activity in stem and leaves were followed during growth of the pineapple plant from propagule to the early stage fruit, through the 'ripe to flower' vegetative state and the transition state. The results obtained have been interpreted in particular relation to the biochemical and physiological events during rooting of the propagule and the transition to flowering state. *Inter alia,* the data permitted the assessment of the distinctive reactions in the achlorophyllous and the chlorophyllous portions of the same leaf.

A. Dry solids

1. Dry solids content varied with growth stage in all the plant parts- the stem tip and the achlorophyllous- and the chlorophyllous parts of the "D" leaf. However, the variation was a minimum in the green leaf, which appeared to maintain a fair degree of homeostasis in moisture content after an initial increase (Stage II A) from the level in the propagule (Stage I).

A month after planting of propagule (Stage I), there was a marked increase in the dry solids percentage in plant parts. This was particularly prominent in the achlorophyllous leaf part and stem. In the stem, the increase was greatest in the middle segment. At the end of 2 months following planting (Stage II B), by which time the terrestrial roots appeared to have established themselves, there was

reduction in dry solids in the stem and achlorophyllous leaf. The increase which occurred during rooting could be an artefact of desiccation undergone by the propagule, or a redistribution of water, or even an active accumulation of dry solids.

3. With the transition from the vegetative (Stage III B) to the reproductive state (Stage IV), there was a small increase in the dry solids percentage in the stem tip and a tendency for increase in dry solids in the achlorophyllous leaf, but a decrease in the chlorophyllous leaf. The increase registered in the stem tip was irreversible, there being constancy in the dry weight percentage in the subsequent stages (Stages V and VI). (This contrasted with invertase activity, which after registering a pronounced increase in Stage IV reverted to the former level (Stage III B) in Stage V).

4. Dry solids decreased in the stem tip in the advanced post-organogenetic stage (Stage VII), presumably indicative of the early stage of development of the multiple fruit. Simultaneously, dry solids increased in the achlorophyllous leaf, suggesting diversion of water to the fruit, or an active accumulation of dry solids to meet the anticipated demands of the developing fruit.

B. Total protein

I. On dry weight basis

1. There were marked changes in the protein content (concentration), during development in all the tissues analyzed : stem tip and the achlorophyllous and

chlorophyllous parts of the "D" leaf. This contrasted with the dry solids percentage picture, in which the green leaf manifested comparative homeostasis.

2. One month following propagule planting, simultaneously with increase in dry weight percentage, protein decreased in all tissues (in stem only the top segment taken into consideration), particularly prominently in the achlorophyllous leaf.

3. Two months following planting, when the plant apparently turned autotrophic, protein increased in all tissues. The level was restored to the propagule level in the stem tip, was enhanced to a higher level in the chlorophyllous leaf, but remained lower in the achlorophyllous leaf. The level of protein obtaining in the propagule was never restored in the achlorophyllous leaf at any growth stage.

4. During the development of terrestrial roots, protein remained fairly constant in the top segment of stem. But, there was a marked increase in the apparent protein content in the lower segment of the stem.

5. In the autotrophic stages of growth, protein in stem varied independently of that in the achlorophyllous leaf. In the leaf, protein in the achlorophyllous part varied independently of that in the chlorophyllous part.

6. When the plant was ready to flower, ("ripe-to-flower" stage), under wholly environmental conditions, protein level was lowered in the stem tip. In the leaf, in which induction reactions were apparently in progress, there was a lowering of protein level in the achlorophyllous part and maintenance of low level in the chlorophyllous part.

7. When the transition from the vegetative to the reproductive state took place, protein increased in the stem tip and continued to rise, gradually, in the subsequent stages.

During the transition, protein increased also in the achlorophyllous leaf, but in the succeeding stage (organogenesis) there was a pronounced decline. In the green leaf, there occurred a marked decrease in protein at the time of transition; in the following stage there was a partial restoration.

8. In the post-organogenesis stages, protein increased continuously in the achlorophyllous region. In contrast, protein declined in the chlorophyllous region, signifying degradation, for utilization of the products elsewhere in the plant.

II. On fresh weight basis

1. The increase in apparent protein in the lower segment of stem during propagule rooting was prominent. Protein decreased in the two parts of the leaf at the end of one month, but increased particularly in the chlorophyllous part in the 2-months old plant.

2. Protein was at a low level in the three tissues in the fully mature plant ready to flower.

3. On transition, protein increased in the stem tip and in the achlorophyllous leaf, but decreased in the chlorophyllous part.

4. In the post-organogenetic stages, protein declined in the green region and increased in the achlorophyllous region.

C. Invertase

Forms of invertase

Barring the tip of the "aerial" root of the propagule, invertase was active in all tissues of the pineapple plant and at all growth stages tested. The enzyme occurred in both 'acid form' (pH optimum around 4.8) and 'neutral form' (pH optimum around 7.0). Detailed tests with the chlorophyllous leaf tissue, employing buffered aqueous media for cell disruption, revealed that the enzyme was entirely in the soluble form, no activity being demonstrable in a suspension of the cell wall fraction. The other tissues examined also gave no indication of cell wall-associated enzyme.

Distribution of activity

(a) Stem tip

Only the acid form of invertase was active in the stem tip of mature propagule and during the two months period required for developing root system and the establishment of the plant as autotroph (pre-autotrophic stage). But, only the neutral form of invertase was active in the stem tip in the autotrophic stages of the plant, both in the vegetative (-tested in plants upto 'ripe to flower' stage) and reproductive state (commencing with evocation of the apex). In separate

experiments, it was found that this transition from the acid- to the neutral form of invertase occurred by about 70 days following planting of the propagule.

(b) Leaf

In the subterminal leaves, ("D"-leaf group), throughout the life cycle of the plant, the non-chlorophyllous part contained both forms of invertase activity, while the chlorophyllous part contained only the acid form. The intervening region of the leaf showed a pattern of activity approximating to that of the contiguous green/non-green tissue.

(c) Root

The roots emerging from the propagule did not manifest invertase activity while still aerial, that is, attached to the mother plant. But, the tip of the terrestrial root contained both acid- and neutral form of the enzyme, analyzed at the end of one month, when it was establishing itself, and at the end of two months, when it had presumably established itself as an autotroph. The same pattern of activity was observed also in the root tip of the mature plant. Assays carried out by mixing of the inactive preparations of the aerial root tip and the root tip of the autotrophic plant showed that a free inhibitor was not present in the aerial root.

Quantification of activity

Invertase activity was followed during the ontogeny of the pineapple plant, by analysing Sephadex G-25 gel filtrates from tissue extracts in buffered media. The

assay for activity was not only at the predetermined pH optimum, but also at the atypical pH (- an acid invertase at neutral pH and a neutral invertase at acid pH-). The ratio of the two activities calculated at succeeding stages permitted detection of any preferentially altered activity at the atypical pH, though a peak was not manifested because of the carry over effect of the dominant form of activity (overlapping of activity). However, no clear evidence was obtained for such a masking of an activity. Dry solids in the tissues (root tip was an exception) and their total protein content at every stage permitted calculation of enzyme activity on tissue dry weight basis and protein basis, in addition to fresh weight basis. Protein determination was both in homogenates (interference not ruled out in green leaf, root tip and lower regions of the stem of propagule, one month- and two months old plant) and in Sephadex G-25 filtrates (except in green leaves, where protein concentration was too low to be assayed). The activity based on the latter value was taken as the 'specific activity' of the partially enriched fraction.

(a) Acid versus neutral form

In the pre-autotrophic stage, acid invertase was more active in the achlorophyllous leaf tissue than in the stem tip. Acid invertase in the chlorophyllous leaf part was several fold more active than in the achlorophyllous region in the pre-autotrophic stage, as also at all other growth stages. The activity in the chlorophyllous part remained the same whether the gel filtrate was from a homogenate allowed to develop high acidity during its preparation by using the same buffered medium as for other tissues or from homogenate in a fortified medium

designed to yield a neutral preparation, by the addition of bicarbonate equivalent to the predetermined acidity.

In the autotrophic stage, neutral invertase in the stem tip was more active than the acid invertase in the pre-autotrophic stages.

A comparison of the acid- and neutral invertase activity in the root tip showed that the neutral form was more active than the acid form, particularly in the two months old plant. This contrasted with the pattern of activity in the achlorophyllous leaf tissue.

(b) Ontogeny of the pineapple plant

There were quantitative variations in the activity of the enzyme forms in the stem tip and "D"leaves during plant growth, superimposed on two qualitative phenomena : (a) the shift from the acid form of enzyme in the stem tip (of the propagule and its rooting stage) to the neutral form (in the rooted vegetative plant and in plants in the reproductive state) and (b) the appearance *de novo* of enzyme activity in the tip of the root on terrestrial establishment. These quantitative variations appeared to be related to the physiological status of the pineapple plant. In particular, the transition of the mature vegetative plant to the reproductive state was associated with a distinctive increase in invertase activity in the stem tip. However, the order of this increase (54%) was not commensurate with that reported for sucrose (7-fold) in the evoked stem tip.

Rooting of propagule

During rooting of the pineapple propagule in garden soil, spread over a two-months period, changes occurred in invertase activity along the stem and in the leaves. While the "D" leaves were used in enzyme analysis in every case, the determination of enzyme activity in the stem was separately in three segments: the upper (stem tip), the middle and the lower, the latter two being the site of origin of roots. Enzyme extraction from the lower parts of the stem proved to be difficult, because of their woody nature and the embedding of root buds. However, the increase in invertase activity in the lower segment of stem was particularly prominent.

Enzyme kinetics

an attempt was made to evaluate, on the basis of kinetic properties, whether or not : (a) the same acid invertase occurred in the achlorophyllous and chlorophyllous parts of the "D" leaves and also in the stem tip in the pre-autotrophic stage and (b) the same neutral enzyme occurred in the achlorophyllous leaf part and the stem tip in the autotrophic stage (both vegetative and reproductive). A comparison was made also between the neutral enzyme and acid enzyme.

K*m* value

A typical Michaelis-Menten relationship was obtained between the substrate concentration and enzyme activity. Of the acid invertases, the lowest K*m* value of 5 mM was observed in the chlorophyllous leaf tissue whereas the highest value (20 mM) was in the achlorophyllous leaf tissue. The transformation of achlorophyllous-

to chlorophyllous leaf part was associated not only with the suppression of the neutral form and an intensification of the acid form of invertase activity, but also by an increase in affinity of the acid enzyme to sucrose. The acid invertase of the stem tissues (upper (stem tip), middle and lower segments) gave near identical values in the range 10 to 15 mM which was 2 to 3- fold that for the chlorophyllous acid invertase.

Neutral invertase in the stem tip had the same K*m* value as that in the achlorophyllous region (10 mM each). This value was comparable with the K*m* for the acid invertase in the stem tissues. In other words, the transformation of the acid invertase to the neutral invertase in the stem tip was attended by an increase, if any, in the affinity of the enzyme for sucrose.

Relevance of biochemical findings

The relevance of the biochemical findings has been discussed in relation to the physiology of the pineapple plant, inclusive of the rooting process, the asexual mode of reproduction, the induction process in the leaf, the evocation in the shoot apex and the reversion of the reproductive to the vegetative state.

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