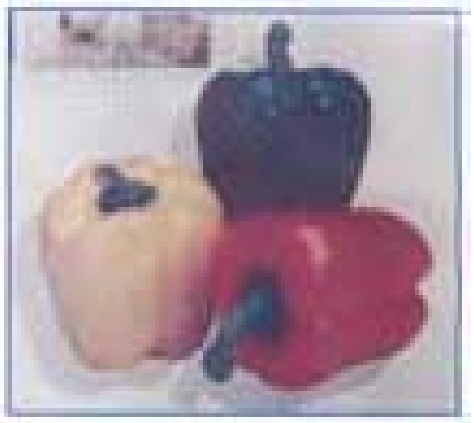
CHARACTERIZATION OF β-HEXOSAMINIDASE FROM BELL CAPSICUM (Capsicum annuum var variata): IMPLICATION IN FRUIT RIPENING

A THESIS Submitted to the

UNIVERSITY OF MYSORE



For the award of degree

Doctor of Philosophy

In

BIOCHEMISTRY

By

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OCTOBER 2002

DECLARATION

I hereby declare that the thesis entitled “CHARACTERIZATION OF β-HEXOSAMINIDASE FROM BELL CAPSICUM (Capsicum annuum var variata); IMPLICATION IN FRUIT RIPENING" submitted to the UNIVERSITY OF MYSORE for the award of the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY, is the result of research work carried out by me initially under the guidance of late Dr. T. N. Prabha, and subsequently under Dr. K. SRINIVASAN, Scientist, Department of Biochemistry and Nutrition, C.F.T.R.I., Mysore -570 013, India during the period of 1999 - 2002. I further declare that the research data presented in this thesis have not been submitted elsewhere for the award of any other degree/fellowship.

(JAGADEESH, B. H)

Date: October 2002
Place: Mysore.

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CERTIFICATE

This is to certify that the thesis entitled "CHARACTERIZATION OF β-HEXOSAMINIDASE FROM BELL CAPSICUM (Capsicum annuum var variata); IMPLICATION IN FRUIT RIPENING" submitted by Mr. JAGADEESH, B.H., for the award of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY to the UNIVERSITY OF MYSORE is the result of research work carried out by him in the Department of Biochemistry and Nutrition, initially under the guidance of late Dr. T. N. Prabha, and subsequently under my guidance during the period of 1999 -2002.

(K. SRINIVASAN)

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J AGADEESH, B.H.

Dedicated to

Beloved parents &

Teachers ……

CONTENTS

LIST OF ABBREVIATIONS

μl	:	Micro liter
μM	:	Micro molar
Ca	:	Approximate
Cm	:	Centimeter
Con-A	:	Concanavalin-A
dMNJ	:	Deoxymannojirimicin
g	:	Gram
Gal	:	Galactopyranoside
GalNAc	:	N-acetyl galactosaminopyranoside
Glc	:	Glucopyranoside
GlcNAc	:	N-acetyl glucosaminopyranoside
kDa	:	Kilo Dalton
L	:	Liter
M	:	Molar
Man	:	Mannopyranoside
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
N	:	Normality
nm	:	Nano meter
O.D.	:	Optical density
PAGE	:	Polyacrilamide gel electrophoresis
p-NP	:	Para nitrophenyl
SOS	:	Sodium dodecyl sulfate
Sec	:	Second
SW	:	Swansonine
Tris	:	Tri (hydroxymethyl)-aminomethane
v / v	:	Volume / volume
w / w	:	Weight / weight

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INTRODUCTION

Fruits are edible products of higher plants. These constitute a commercially significant and nutritionally indispensable food commodity. Nutritionally, fruits are important source of energy, dietary fiber, vitamins and minerals. Due to their nutritional, medicinal and organoleptic properties, they form most interesting and highly popular commodities. Thus, there is an ever-increasing demand for them throughout the world.

India is a prime producer of fruits and vegetables with an annual production crossing over 108MT (Fruits: 42MT and Vegetables: 66MT). Production of fruits and vegetables have been increased to a considerable extent, primarily, due to the development of new production techniques, but the impact is lessened because of huge losses that take place between harvesting and consumption. The loss is estimated to be nearly 40% or more amounting to Rs. 30,000 million in our country per year. The major reasons for this loss are:

* Highly perishable nature of fruits owing to high moisture content (70- 95%), very high rates of respiration leading to generation of heat and easily damageable soft texture.
* Lack of proper handling process.
* Insufficient storage and transportation facilities.
* Losses caused by external agents like bacteria, fungi and pests.
* Losses caused by some endogenous factors like senescence and sprouting.

APPROACHES TO MINIMIZE HEAVY LOSS

All fruits are highly perishable in nature owing to high water content and soft texture. Constant attempts are made to evolve better ways of preserving their freshness and extending their shelf life. Following are some approaches meant to improve the fruit shelf life:

1. Post harvest techniques: Post harvest technologies like cold storage, controlled atmosphere, modified atmosphere, antimicrobial treatment, wax coating, etc.,
2. Pre-harvest technique: By genetically manipulating the ripening process.

1.1 FRUITS STUDIED IN THIS INVESTIGATION

1.1.1 Bell capsicum (Capsicum annuum var variata)

Capsicums are the second most important crop among the Solanaceous fruits. From the point of nutrients, capsicums are far superior to both tomato and egg plant in vitamin A and C content. The world production of capsicums and chilies is around 6,671 x 10^3 MT grown in 890 x 10^3 ha.

Bell capsicum is the most widely cultivated among capsicum species; depending on the cultivars it may be pungent or non-pungent. Other species; C. baccatum, C. chinense, and C. pubescens are cultivated in parts of South America. Capsicums are perennials in the tropics and annuals in temperate zones. Capsicum plants have been domesticated and used as a condiment in

many parts of world. Capsicum grows well in warm climates and can tolerate extreme hot weather. Maximum fruit set of capsicum occurs at constant temperature of 16-32°C, but fails when temperatures are below 16°C and above 32°C.

1.1.2 Tomato (Lycopersicon esculentum Mill)

Tomato is an annual shrubby plant in temperate zones and a short- lived perennial in the tropics. The world tomato production is 49,201 x 10^3 MT grown in an area of 2,414 x 10^3 ha. Fruits are round, lobed or pear shaped depending on the genetic makeup of the cultivars. Poor fruit set occurs when day temperature exceeds 38°C for 5-8 days before anthesis, and 1-3 days after anthesis. This is due to destruction of pollen, egg cell or embryo after pollination. At 10°C or below, a large percentage of flower abortion take place.

The optimum temperature for fruit ripening is 18-24°C, at temperature below 13°C, fruit ripens poorly and slowly, and below 10°C, chilling occurs and fruit may not ripen at all. If the temperature exceeds 32°C during the storage of harvested mature green fruits, formation of red color is inhibited and fruits remain yellowish when they are ripe. Mature green tomatoes should be stored at 13-18°C and 85-90% humidity. Ripening of harvested tomatoes is more rapid at temperatures >18°C. If stored at temperatures 30°C, the ripe fruits will assume orange to yellow in color. Red ripe fruit should be stored at 3-4°c; at high temperatures, the fruit continues to ripen with simultaneous rapid deterioration in quality. Ripe tomatoes are rich in Vitamin A and C, the contents of which increase as the fruit develops on the vine but decreases when mature green fruits are allowed to ripe off the vine.

1.2 FRUIT RIPENING:

Fruit development and ripening are processes unique to plant species. The development and maturation of fruit tissue represents the final phase of floral development typically proceeding and signaled by successful fertilization, Although much is known about hormonal and physiological signals that trigger fruit development, molecular regulatory signal for maturation and ripening still remains unknown.

In flowering plants, the seeds are enclosed by a structure known as the fruit. The development of fruit in most species depends on successful fertilization and thus proceeds with the development of enclosed seeds. For fleshy fruits like tomato, capsicum, banana, cucurbits and apples, an initial phase of cell division is followed by a phase of massive cell expansion. This cell expansion phase of fruit leads to fruit enlargement and maturation. Following the cell expansion, fruit ripening occurs (1).

From the standpoint of agriculture, ripening confers both positive and negative attributes to the resulting commodity. The positive attributes are desirable flavor, color and texture, while negative characters are fruit softening followed by pathogen attack and mechanical damage during handling or marketing.

Fruit ripening is a dynamic and closely regulated developmental process unique to plants and is genetically controlled (2). Although most fruits display modifications in color, texture, flavor, aroma and pathogen susceptibility during maturation, two major classification of ripening fruit, climacteric and non-climacteric, have been utilized to distinguish fruits on the basis of respiration and ethylene biosynthesis rates. Climacteric fruits like tomato, cucurbits, capsicum, avocado, banana, peaches, plums and apples are distinguished from non-climacteric fruits, such as citrus, strawberry and grapes, by there increased respiration and ethylene biosynthesis rates during ripening (3). Although non-climacteric fruits such as citrus, may respond to ethylene (de-pigmentation), ethylene is not required for fruit ripening of this species (4). The major physiological and biochemical changes that occur in fruit ripening are: 1) increased ethylene biosynthesis; 2) changes in gene expression; 3) increased respiration (climacteric fruits); 4) loss of photosynthetic enzymes and degradation of chlorophyll; 5) synthesis of color pigments like lycopene, carotenoids, etc., 6) changes in organic acid metabolism; 7) textural softening by increased activities of polysaccharide hydrolyzing enzymes like polygalacturonase, pectin methyl esterase, cellulase, arabananase, etc., and 8) increased susceptibity to pathogen attack.

In nearly all fruits there is a disorganization of chloroplasts during ripening (5), which in some cases precedes their reorganization into organized chromoplasts (6,7), In apple, during the onset of ripening, the chloroplast lamellae breaks down and constituents of the membrane, viz" lipids and protein undergo hydrolysis (8), There is evidence from tracer studies that enhanced RNA and protein biosynthesis is associated with the climacteric peak in apple (9), avocado (10, 11) and pear (12). Studies have revealed that the inhibitors of RNA and protein synthesis inhibit the processes associated with fruit ripening, such as development of ethylene synthesizing machinery (13), breakdown of chlorophyll and softening of the tissue (12,14). It has been shown in pears that during ripening, there is a shift in the pattern of proteins within the tissue (15) and that, during this period there is an enhanced incorporation of amino acids into specific enzyme proteins (12). Higher incorporation of amino acids particularly into malic enzyme has been shown during ripening of pome fruits (16,17,18). These studies strongly suggest that the synthesis of new enzyme proteins is the dominant feature during climacteric ripening, where they may have a secondary role in ripening process.

The protein constituents of fruit, although occurring in low concentrations, are of primary importance not only as components of nuclear and cytoplasmic structures, but also as enzymes involved in the metabolism during growth, development, maturation and post-harvest life of the fruit. The major interest in fruit proteins lies in relation to their involvement in various physiological aspects of the fruit, including respiration, enzyme activities during ripening, as well as the undesirable senescence.

Reducing sugars often increase steadily throughout growth and maturation in climacteric fruits, e.g., tomato (19), passion fruit (20) and in non-climacteric fruit, e.g., citrus (21) and grape (22).

1.2.1 Carbohydrates:

In most of the fruits, carbohydrates serve as the storage material. Part of carbohydrate is present as cellulose and pectin substances in the cell walls. Starch which is present in almost all fruits, is known to disappear during the ripening of fruits like mango (23), banana (24) and apple (25), leading to building up of sugars. Some fruits like plum (26) contain negligible amount of starch, whereas, fruits like apple contain up to 3% (25). Starch content of mango (23) and banana (24) is high.

Pectins together with cellulose serve as an integral part of the fruit cell wall. Structure of pectins can be correlated with the texture of fruits. Pectins from mango (27,28) and guava (29) have been characterized. During the ripening of fruits, the protopectin fraction decreases with a simultaneous increase in soluble pectin fraction (30-33), thus leading to softening of the fruit tissue.

Sugars are responsible for the sweetness of fruits. The most commonly occurring sugars in fruits are, glucose, fructose and sucrose. Sugars are known to increase during ripening of fruits like banana (24), mango (34,35) and tomatoes (36). Accumulation of sugars in climacteric fruits following the harvest is accompanied by a corresponding decrease in starch content (37).

Apart from glucose, fructose and sucrose, fruits also contain minor sugars like galactose (38), arabinose (39) xylose (40) lactose (38), maltose (38,41), raffinose (22) and stachyose (22). Avocado fruit (42) has been extensively studied as a source of longer chain sugars. Sugar acids, which occur in fruits, are: galacturonic (42), gluconic (43), galacturonic (43) and mucic (44) acids. Fruits also contain sugar alcohols like sorbitol (26) and myoinositol (45). Sugar phosphates, active intermediates of sugar metabolism are present at low concentration in fruits. Glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and fructose-1, 6-diphosphate (FOP) in apple (46) and banana (47) have been reported. During the climacteric phase of banana (47), FOP content is known to increase to a greater extent than G-6-P and F-6-P, where as apple showed (46) no such increase during ripenlng.

1.2.2 Lipids

Fruits in general, contain less amounts of lipid with very few exceptions like avocado and olive, which are good sources of lipids. During ripening, the lipid composition changes. Decrease in unsaturated fatty acids with a slight increase in saturated fatty acids has been observed in the peel and pulp, during ripening of banana (48). Galactolipids and phosphatidyl glycerol have been shown to decrease in the apple pulp during ripening (49). Fatty acid composition of lipids has been shown to remain unchanged during ripening of avocado (33). Percentage of linoleic and oleic acids decrease with a corresponding increase in phospholipid content during ripening of tomatoes (50). The major fatty acids here are palmitic, stearic, oleic, linoleic and linolenic acids.

1.2.3 Proteins and nucleic acids

Fruits are relatively low in protein. But they are of importance in maintaining the cellular organization and as enzymes involved in ripening. Protein content of fruits increase during ripening (51,52). Increased protein synthesis including synthesis of some enzyme protein (12,53) during ripening, has been shown in avocado (54), pear (12), apple (9), banana (15) and tomato (55) by incorporation of labeled amino acids. Fruit ripening and ethylene synthesis are inhibited when protein synthesis was blocked by treatment with cycloheximide at the early climacteric stages (12,54,15). Ripening phase is preceded by RNA synthesis (11,12). Total RNA content is known to increase during the climacteric period (56).

1.2.4 Amino acids

Fruits contain considerable amounts of free amino acids, the exact role of which in the ripening process is not completely understood. Apart from protein amino acids, fruits also contain other non-protein amino acids like y-amino butyric acid (57,58), pipecolic acid (59), citrulline (60), 4-hydroxymethyl proline (61-63) and amines such as aspargine (57,64) and glutamine (64). Few amino acids, however, occur in relatively large amounts in a particular fruit. Proline and aspartic acids are the major amino acids of oranges (57) and Eureka lemon (65) respectively. Alanine and arginine are predominant amino acids of grapes (66). Aspargine is present at a higher concentration in apricot (67). Dates have been reported to be the richest natural source of 5-hydroxy pipecolic acid (68). There are few reports regarding the changes in amino acids during ripening of fruits. Total free amino acids increase during ripening of grape berries (69), arginine and proline being the major amino acids of the ripe fruit. Valine and leucine, the precursors of volatiles, have been reported to increase in ripening banana (70). Increase in glutamic and aspartic acid concentrations during the ripening of tomatoes is accompanied by a decrease in y-amino butyric acid (71).

1.2.5 Non-volatile organic acids

Along with the acids of TCA cycle, other organic acids such as oxalic, tartaric, lactic, quinic, shikimic etc. are also present in fruits. Though fruits contain several organic acids most of them are in traces, some of them are present in large amounts and usually one acid predominates. For example, malic acid in apple (72), citric acid in mango (34) and citrus fruits (73) like orange, grape fruit, lemon and lime, and tartaric acid in grapes (74). The organic acids in fruits are stored in vacuoles. Generally the organic acid content decreases during fruit ripening as in apple (72) and mango (34). There are exceptions to this like in banana (75), wherein; the total acidity increases during ripening due to the accumulation of malic and citric acids. Despite the decrease in total organic acid content, increase in some minor acids like malic acid in mango (76) and citric acid in apple (72) during ripening have been reported. Similarly, in tomato, although titratable acidity decreases during ripening, increase in citric and malic acids have been reported (36). Pyruvic, ∝-ketoglutaric and oxaloacetic acids increase during the ripening of banana (77) whereas, in mango, ∝-ketoglutaric and pyruvic acids increase during the climacteric and then decrease during the post-climacteric period (35).

1.2.6 Enzymes

Changes in the number of enzyme systems, which reflect physical and chemical transformations that occur in fruit during ripening, are well documented in literature. Cellulase, polygalacturonase, pectin methylesterase and β-glucosidase involved in softening of the tissue generally increase during ripening of fruits. Increase in cellulase activity in tomato (78), peaches (79) and avocado (80) has been reported. Pectinolytic enzymes such as polygalacturonase in tomato (81) and avocado (82), pectin methylesterase in tomato (81), banana (83), mango (84) and orange (85) increase during ripening. Involvement of β-glycosidase in softening of tomato (86) and apple (87) has been indicated.

Amylase, a starch-degrading enzyme, increases during ripening of fruits thus leading to the accumulation of sugar. Increased activities of amylase in pears (88) and mango (84) have been reported. Enzymes involved in sucrose synthesis such as sucrose synthetase, sucrose phosphate synthetase and sucrose phosphatase increase during the development of grape berries with a simultaneous increase in sugars (89). Invertase a sucrose-hydrolyzing enzyme has been reported to increase in ripening grapes (90) and mango (84). Glycolytic enzymes have been studied in detail in banana. Increase in phosphofructokinase during ripening of banana paralleled climacteric rise in respiration (35,47). Hexokinase and phosphoglucomutase activities in banana (91) increase during ripening, indicating enhanced glycolysis as the fruit ripened. Operation of pentose phosphate pathway in fruits is evident in pear (92), tomato (93) and apple (94). Glucose-6-phosphate dehydrogenase has been reported in cherry (95), grapes (90) and avocado (96). Enzymatic oxidation of sugars to their respective aldonic acids has been demonstrated in oranges (97). During ripening of banana (98) and apple (94,99), contribution of pentose phosphate pathway decreases with a simultaneous increase in glycolysis, as revealed by studies with enzymes of pentose phosphate pathway and specifically labeled glucose. In mango (34), however, increased activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase have been reported.

Enzymes connected with synthesis and degradation of organic acids has been studied in fruits. Citrate synthetase activity has been demonstrated in citrus fruits such as lemons (100,101), oranges (101), grape fruit (90) and mango (102), which are known to accumulate citric acid. The role of malic enzyme in the metabolism of malic acid in ripening apple (103), mango (34,35), grapes (90) and cherries (95) has been indicated. Pyruvate decarboxylase increases during the ripening of apple (103) and banana (98).

Enzyme systems involved in fatty acid synthesis have been studied in avocado (104 -106). Lipase activity increases during the ripening of avocado (8), with a parallel increase in fatty acids. Increase in lipoxidase activity during the ripening of apple (107) has been shown to be associated with ethylene biosynthesis. There are a number of reports on catalases and peroxidase in fruits (108). Role of peroxidase (109) and polyphenol oxidase (110,111) in ethylene biosynthesis has been evidenced. Peroxidase activity in banana is known to increase during climacteric and decrease during post-climacteric perio.d (112). A three-fold increase in catalase and peroxidase activities in pre-climacteric mango slices has been observed, when treated with ethylene. Acid phosphatase involved in carotenoid biosynthesis is known to increase in ripening banana (113) and mango (114).

There are only few reports regarding the enzymes connected with amino acid metabolism. Glutamic acid decardoxylase activity has been detected in orange and lemon (115), which has been reported to decrease during the ripening of tomato (71).

1.2.7 Polypenols

Phenolic compounds are widely distributed in plants and in some fruits they are important in determining color and flavor. Phenolics have been assigned a role in the disease resistance of plants (116). Polyphenols present in fruits are mainly cinnamic acid derivatives, monomeric and polymeric flavans, others being anthocyanins and flavonol glycosides. Chlorogenic acid is the most important cinnamic acid derivative found in fruits. Polyphenols are known to change during ripening of fruits (117,118). Astringency, which is due to dimers of flavans and other condensed fruit phenolics decrease, as their condensation increase (119). Flavanoids such as naringin and neohesperidine impart bitterness to grape fruit (120).

1.2.8 Pigments

The chief pigments of fruits can be classified into (i) carotenoids (yellow to orange red) (ii) chlorophyll (green) (iii) anthoxanthine (yellow) and (iv) anthocyanins (red, blue and purple). Most of the pigments occur in plastids and they develop as the fruit ripens. Changes in the color during fruit ripening are due to both degradative and synthetic processes. In the ripening banana, green color disappears due to the breakdown of chlorophyll and there is no change in the total yellow pigments in tomato (121), the change in color is due to chlorophyll breakdown and formation of carotenoid pigments. Synthesis of carotene (122), lycopene and anthocyanin (123) during the later stages of ripening has been reported. Increase in carotene (124) and mevalonic acid (125) has been observed during ripening of mango. Pigments and flavor components have been isolated from orange oils (126). Involvement of phytochromes in carotenoid biosynthesis in ripening tomato has been demonstrated (127). Increased biosynthesis of carotene in peaches and apricots during ripening has been reported (128).

1.2.9 Aroma of fruits

Aroma of a fruit depends on the nature and quantity of components, and the intensity of aroma of each component. Most of the compounds responsible for fruit aroma are volatiles. Fruit volatiles include esters, aldehydes, ketones, hydrocarbons, etc. Development of banana flavor is paralleled by an increase in the concentration of volatiles (129). The organoleptically significant compounds are, however, usually present in small amounts and they are characteristic of each fruit. They are hexanal and ethyl 2-methyl butyrate in apple (120), eugenol and related compounds in banana (120), linalool and geraniol in Muscat grapes (130). The characteristic aroma of citrus fruits (120) is ascribed to the relatively high boiling, sparingly water soluble oil which is a mixture of terpenes and other hydrocarbons. Though limonene constitutes 80-90% of most citrus oils, oxygenated terpenes representing about 5% of the oil provide the typical aroma.

1.2.10 Role of ethylene in fruit ripening

The earliest mention dates back to 1910, where, Cousins recommended to the Jamaican Agricultural Department that oranges should not be stored with bananas on ships because some emanations from the oranges cause bananas to ripen prematurely. This was the first report saying that fruits release a gas that stimulate ripening, but it was not until 1934 that Gane proved that ethylene is synthesized by plants and is responsible for faster ripening. In many fruits, little ethylene is produced until just the respiratory climacteric signaling the onset of ripening. Ripening is stimulated when the content of this gas in the intercellular air space rises dramatically (131). In non-climacteric fruits, ethylene seems to play no role in natural ripening, although it is used commercially to de-green oranges and lemons (132).

Yang and his colleagues elucidated the pathway of ethylene biosynthesis (133). Fig.1.1 shows the pathway for ethylene biosynthesis.

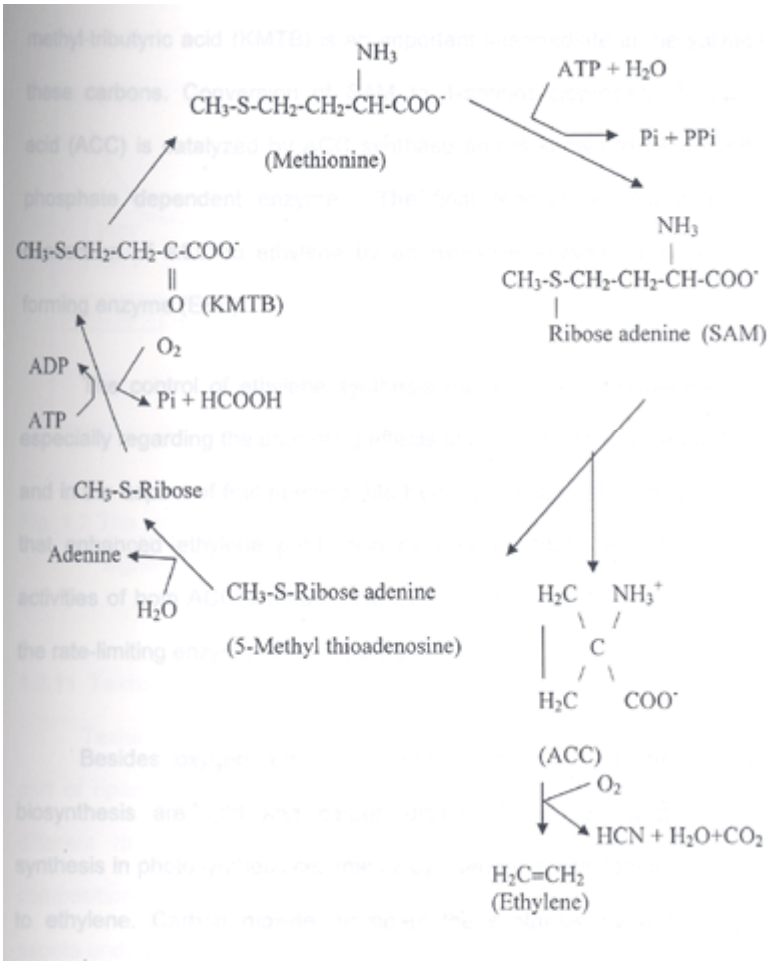


Fig. 1.1 Ethylene biosynthetic pathway (133).

S-adenosyl methionine (SAM) is formed from Adenosine triphosphate and methionine in an ATP dependent process and is the precursor in ethylene biosynthesis. Evidence indicates that four carbon atoms of ribose unit of

SAM are salvaged and reappear in methionine. An intermediate a-keto-y-methyl-tributyric acid (KMTB) is an important intermediate in the salvage of these carbons. Conversion of SAM to 1-aminocyclopropane-2-carboxylic
acid (ACC) is catalyzed by ACC synthase and is known to be a pyridoxal phosphate dependent enzyme. The final reaction in this pathway is conversion of ACC to ethylene by an oxidative enzyme called ethylene- forming enzyme (EFE).

The control of ethylene synthesis has received considerable study, especially regarding the promoting effects of auxin, wounding, drought stress and in the aspect of fruit ripening. Mc Keon and Yang (134) has pointed out that enhanced ethylene production by ripening fruits requires increased activities of both ACC synthase and EFE and in some fruits EFE becomes the rate-limiting enzyme in ethylene biosynthesis.

Besides oxygen, other environmental factors that affect ethylene biosynthesis are light and carbon dioxide. Light inhibits the ethylene synthesis in photosynthetic cell mainly by interfering with conversion of ACC
to ethylene. Carbon dioxide promotes the synthesis by enhancing the conversion of ACC to ethylene (135). Fig. 2 shows the model for interaction among developmental, hormonal and light signaling systems that impact ripening (136).

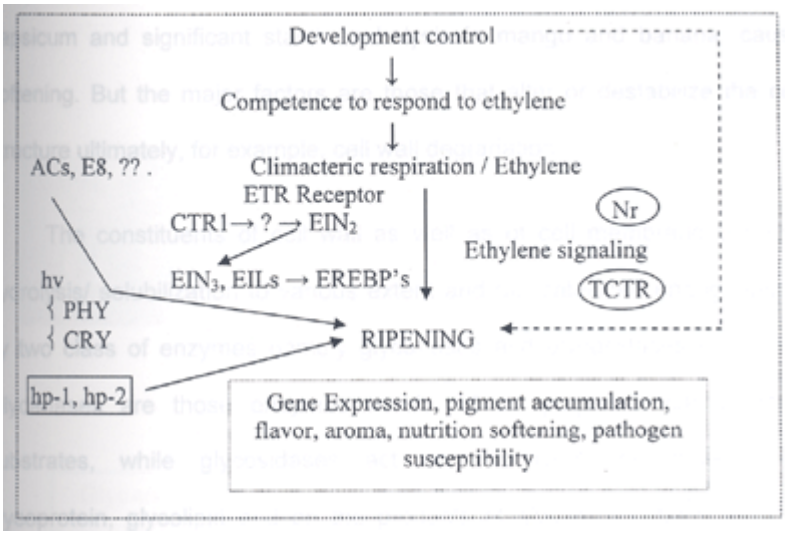


Fig. 1.2 The model for interaction among developmental, hormonal and light signaling systems that impact ripening (136).

1.2.11 Textural softening

Textural softening is a major event in fruit ripening and is an integral part of ripening phenomenon in almost all fruits. Different fruits soften at different rates and to varying degrees depending on their inherent composition and nature. Fruits like mango, banana, avocado, papaya and sapota undergo extensive dramatic textural change from stone hard stage to very soft texture; but fruits like, apple, lemon and orange do not exhibit dramatic softening in texture though they undergo some textural modification during ripening. This reflects that several mechanisms operate to cause

softening. Loss of moisture resulting in loss of turgor pressure in bell capsicum and significant starch hydrolysis in mango and banana, cause softening. But the major factors are those that alter or destabilize the cell structure ultimately, for example, cell wall degradation.

The constituents of cell wall as well as of cell membrane undergo hydrolysis/ solubilization to various extent and are catalyzed endogenously by two class of enzymes namely glycanases and glycosidases (Table 1).

Glycanases are those enzymes which act on polymeric carbohydrate substrates, while glycosidases act on oligosaccharide moieties of glycoprotein, glycolipid and on the products of glycanases itself. Fig. 1.3 gives an over view of fruit ripening. The above figure clearly depicts the involvement of these carbohydrate hydrolases in fruit textural softening.

1.3 CARBOHYDRATE HYDROLASES

Changes in enzyme systems, which participate in physical and chemical transformations during fruit ripening, are well documented. Most of these enzymes are constitutive and are present in low levels throughout fruit development. But during ripening, generally all carbohydrate hydrolases show increased activity with climacteric peak.

1.3.1 Glycanases

In the context of fruit ripening, tomato polygalacturonase (PG) has been the most widely studied cell wall hydrolase. This is due to initial observation

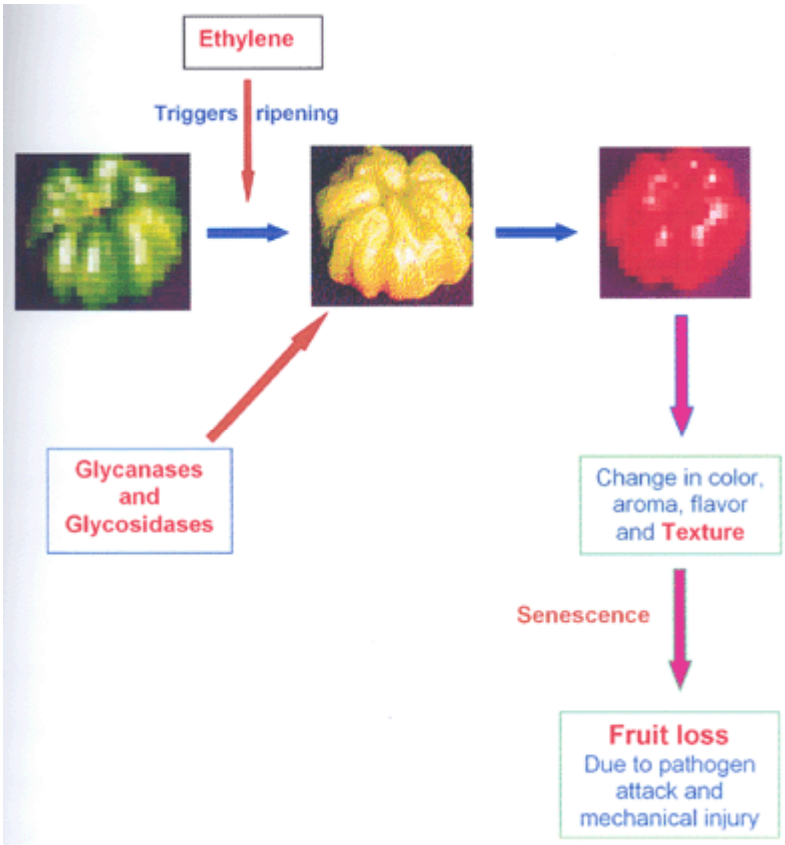


Fig. 1.3 An over view of fruit ripening phenomenon in climacteric fruits.

of a high-level extractable endo-PG activity that increases in parallel with the ripening process (137). Gene isolation and subsequent functional characterization of tomato PG in transgenic plants indicated that PG activity alone is not sufficient to significantly impact texture (138 -140); thus, it is likely to function in concert with additional factors.

Pectin Methyl Esterase (PM E) has been tested for a function in cell wall metabolism, in addition to PG. PME shows activity through out the fruit development and may increase accessibility of PG to its substrate. Antisense repression of tomato fruit PME resulted in decreased pectin degradation, but consistent with PG repression, it did not alter additional ripening characteristics, including softening (141). Two tomato hemicellulases showed differential expression in ripening fruit and are designated as CEL1 and CEL2. When these were suppressed via antisense did not show any observable impact on fruit ripening and softening (142).

Repression of additional ripening related cell wall metabolism enzymes, such as members of the β-galactosidase gene family (143), in additional to adding up of cell wall metabolism antisense genes through the crosses of available transgenic lines, may shed additional light on the genetic regulation of this complicated metabolic process.

Most definitive results concerning ripening related modification have emerged from the analysis of tomato expansins. Expansins are cell wall proteins associated with numerous tissues and developmental stages undergoing changes in size and shape (144).

Table 1.1: Enzymes involved in textural softening during fruit ripening.

GLYCANASES	GLYCOSIDASE
1. Polygalacturonase	β-and ∝-hexoasaminidase
2. Pectin methyl esterase	β-and ∝-mannosidase
3. Cellulase	β-and ∝-glycosidase
4. Amylase	β-and ∝-glucosidase
5. Galactanase	β-and ∝-fucosidase
6. Mannanase	β-and ∝-xylosidase
7. Xylanase	
8. Rhamnogalactanase	
9. Arabananase	
10. Xyloglucanase	

Tomato and strawberry expansin genes up-regulated during fruit ripening and repression of a fruit ripening specific expansin (EXP1) in tomato resulted in reduced softening (145,146). Over-expression of expansin gene (EXP1) via CaMV35 promoter, resulted in an enhanced softening,

including softening of matured green fruit (147). These results shows that, although the activity of fruit cell hydrolases may well be important for in vivo textural modifications associated with ripening.

1.3.2 Glycosidases

β-Hexosaminidase (EC 3.2.1.30) is a type of glycosidase acting on oligosaccharides, aminoglycans, glycoproteins and glycolipids containing N-acetyl glucosamine or N-acetyl galactosamine residues in β-linkage and the end products are free N-glycans. ∝-Mannosidase (EC 3.2.1.24) acts on oligo-saccharides, glycoproteins and glycolipids containing mannose residue in ∝-confirmation.

Plant N-glycans, an integral part of N-glycoproteins, are classified into two types based on the presence (complex type) or absence (β-mannosidic type) of a P172 linked xylosyl residue in addition to mannosyl and N-acetyl glucosamine residues. Research on N-glycoprotein has included structural analysis, biosynthesis and activity, and role of N-glycans in glycoprotein secretion (148). Free plant N-glycans are known to exhibit biological activity. For example, 1) Man_3(Xyl)GlcNAc(Fuc)GlcNAc, a xylomannoside is known to act as growth factor at nanomolar concentration during early development of Linum usitatissium (149). 2) The N-glycans Man_3(Xyl)GlcNAc(Fuc)GlcNAc and MansGlcNAc are shown to stimulate tomato fruit ripening (150). Thus, N-glycans might be considered as a new class of oligosaccharins that affect plant metabolism (151,152). In humans, free N-glycans are associated with enzyme deficiencies, resulting in physiological disorders (153,154).

In addition to the biological activity of N-glycans in tomato, other reports suggest the importance of N-glycoconjugates in tomato fruit metabolism (155). Blocking of N-glycosylation with tunimycin delayed fruit ripening, which suggested that N-glycoproteins might be important in the ripening process (155).

1.4 β-HEXOSAMINIDASE

1.4.1 β-Hexosaminidase from animal systems

β-Hexosaminidase has been observed for the first time and studied in commercial preparations of emulsin (156,157). Later it was found that β-glycosides of the two amino sugars N-acetyl-D-glucosamine and N-acetyl-D-galactosamine are cleaved in the lysosomes by the enzyme (158 -160), which was therefore called β-hexosaminidase or simply hexosaminidase. The lysosomal location of this enzyme was demonstrated by subcellular fractionation (161,162).

In 1968, Rabinson and Stirling separated the hexosaminidase of human spleen into two isoenzymes, an acid form A and basic form B, which was then confirmed by isoelectric focusing (163). Hexosaminidase A consists of two different subunits, α- and β-, where as B isoenzyme is a homomer of two β-subunits. In most of the human tissues, the β-subunit is cleaved proteolytically in the lysosome into non-identical fragments of similar size (164), therefore the subunit composition of hexosaminidase A and B is frequently denoted as $\alpha\beta_a\beta_b$ and$(\alpha\beta_a\beta_{b)2}$, respectively. Two enzymic

variants of an inherited disease known as 'GM2 Gangliosidosis' are caused by defects of hexosaminidase subunits. In a variant B of this disorder (Taysach's disease) hexosaminidase A is deficient (165,166), due to defect of the subunits towards glycolipid substrates under physiological condition. The residual hexosaminidase found in the tissue of these patients is hexosaminidase 8 (165,167) is a dimer of a-subunit (168,169). Other hexosaminidase isoenzymes described are those with intermediate isoelectric point and electrophoretic mobility and designated as hexoseaminidase-1 (170,171), and the P form that is elevated in the serum of pregnant woman (172). Hexosaminidase I and P appear to be differently glycosylated forms of hexosaminidase B (173,174).

Purification and characterization of hexosaminidase has been reported in some higher organisms, viz., prawn liver (175), rat spleen and epididymis (176,177), midgut gland of scallop (178), bovine kidney and brain (179,180) and chicken brain (181).

The molecular weight of intact hexosaminidase A and B is reported to be in the range of 100 kDa to 140 kDa (182,183,169). For hexosaminidase S, values of 130 kDa (169) and 154 kDa (184) are reported. The isoelectric point of hexosaminidase A is around pH: 5.0, depending upon the source of enzyme, where as hexosaminidase B have a pi of 7.0 to 7.5 (159,165,185,186). For hexosaminidase 8, the pi is 4.2 (169). Hexosaminidase B is more stable against thermal denaturation than the A enzyme (169,185,186). At pH 4.4, hexosaminidase A is comparatively stable up to 55°C, whereas hexosaminidase B is inactivated with a half life of 10min at 50°C (169,186) and of 3min at 55°C(186).

Hexosaminidases, as most of other lysosomal proteins, are glycoproteins, with variable proportion of phosphorylated and unphophorylated high mannose and complex-type oligosaccharide chains (164,187). The kinetic constants of hexosaminidase A and B were generally found to be identical. The pH optimum for the both isoenzymes with glucosaminides as well as galactosaminides of p-nitrophenol and of 4-methylumbelliferme (4-Mu-GlcNAc) was 4.4 (183,185,186, 188,189). In contrast, hexosaminidase B had pH optimum of 4.8 -5.0 (169,184).

The Michaelis constant for 4-Mu-GlcNAc is found to be 0.5-1 mM for all the three isoenzymes, viz., A, Band S (169,183,186,189). Slightly lower Km values (0.3-0.95 mM) were found with the p-nitrophenyl substrates for these hexosaminidase isoenzymes (182,190). Hexosaminidase A and B are completely inhibited by the free amino sugars N-acetyl-D-glucosamine (K, 4-9 mM) and N-acetyl-D-galactosamine (K1 : 0.5-0.7mM), as well as by acetate (K1 : 3-8 mM) (182). Irreversible inactivation of hexosaminidases was found with Hg2+ (190,191) and Ag2+ (182,191).

Tews et al. (192) have proposed 3-dimensional structures for N-acetyl β-D-hexosaminidase from bacteria to study the catalytic mechanism and look into the basis for human Tay-Sachs disease (Fig.1.4).

Koga et al (175) have purified the prawn liver hexosaminidase by ammonium sulfate fractionation followed by chromatography on Sephadex G-100, hydroxylapatite, DEAE-Cellulose and Cellulofine GLC-2000-m. The purified

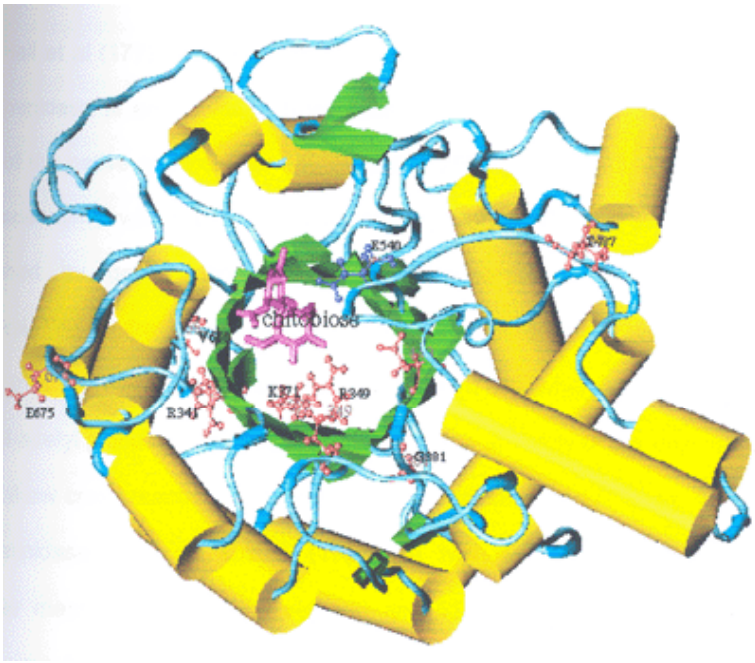


Fig. 1.4 3D structure of β-hexosaminidase from bacteria. Reproduced from Tews et al, 1996(192)

enzyme has an apparent molecular weight of 64 kDa and 110kDa on SDS-PAGE and gel filtration, respectively. The optimum temperature and optimum pH were 50°C and 5.0-5.5, respectively. The enzyme ws found to be stable at pH 4 to 11 and below 55°C. The enzyme had a Km of 0.055mM for GlcNAc3, 0.044mM for GlcNAc4 and 0.045 mM for GlcNAc5 at 25°C.

The enzyme is said to be an exo-type hydrolytic enzyme involved in chitin degradation acting preferably on shorter substrates.

Hall et al (177) have reported the purification and characterization of hexosaminidase A and B forms from adult rat epididymis. The molecular weight of the native enzyme was approximately 250 kOa and 223 kOa for A and 8 isoenzyme, with a subunit molecular weight of 63 kOa and 56 kOa as determined by gel filtration and gel electrophoresis under reduced conditions. The neutral hexosaminidase purified from rat brain (193) had less activity towards N-acetyl galctosamine substrates (0.5%) when compared to that of N-acetyl glucosamine substrates. Further, the antibody of this enzyme did not show cross reactivity with acid hexosaminidase A and B forms. The hexosaminidase from rat spleen (176) had two peptides of apparent molecular mass of 54kO (alpha subunit) and 51 kO (beta subunit). This enzyme was found to be active on O-GlcNAc bearing glycoproteins in the nucleoplasmic and cytoplasmic compartments of cells.

Sakai et al (178) have purified p-hexosaminidase from midgut of scallop by making acetone-dried powder. The purified enzyme was observed to be homogeneous on SOS-PAGE and had a molecular weight of 56 kDa. The enzyme hydrolyzed both p-GlcNAc and p-GaINAc; with an optimum

pH of 3.7, optimum temperature of 45°C and Km of 0.24 mM for p-GlcNAc as substrate. The enzyme was stable around the pH 3.5-5.5.

1.4.2 β-Hexosaminidase in microbial systems

In microbes, purification and characterization of hexosaminidase is reported in bacteria (194 - 196) and fungi (175,197-199). Koga et al (175) have studied the β-N-acetylhexosaminidase from Tricoderma harzianum. The enzyme was purified 13.2-fold to homogeneity by ultracentrifugation and sequential chromatography on SP- Toyopearl and Sephacryl S-200. The molecular weight was e$timated to be about 150 kDa by gel filtration. The pH and temperature optima were 4.0 -5.5 and 50°C, respectively. The enzyme hydrolyzed N-acetylchitooligosaccharides at non-reducing ends releasing GlcNAc monomer. The enzyme showed strict substrate specificity to the sugar chains in complex carbohydrates, hydrolyzing only the linkage of GlcNAc-β(1-3)Gal, but not hydrolyzing the other linkages such as GlcNAc β(1-3)Gal and GlcNAc-β (1-3)Man.

Sakai et al (178) have purified and characterized a thermostable β-N-acetyl hexosaminidase from Bacillus stearothermophilus. The enzyme was a single polypeptide with a molecular weight of 74kDa and the N-terminal sequence was WDKVGVTDLIISLNIPEADAVWGMTLQLQLQALHLY. The enzyme specifically hydrolyzed N-acetyl chitooligosaccharides and p-nitrophenyl derivatives. The enzyme hydrolyzed p-nitrophenyl-β-N-acetylgalactosaminide with 26% that of β-N-acetylglucosaminide. The enzyme exhibited 15 to 20% activity in the reaction mixture containing 80% organic solvents and maintained 91 % of its activity after exposure to 8M urea. The optimum and stable pH was around 6.5. Fe^{2+}, Zn^{2+} and Ca^{2+} activated the enzyme, while Hg^{2+} was inhibitory. N-acetyl-D-glucosaminide inhibited the enzyme competitively, whereas N-acetyl-D-glucosaminide did not. In contrast, D-glucosamine and D-galactosamine activated it.

Keyhani and Roseman (194) have isolated, characterized and cloned the periplasmic β-N-acetylglucosaminidase from Vibrio furnissii. The cloned β-N-acetylglucos-aminidase gene (exol) encoded a 69.37 kDa protein having 611 amino acids and displayed significant homologies to the ∝- and β-chains of human hexosaminidase despite their marked differences in substrate specificities and pH optima. .

Del Rio and Berkeley (196) have separated hexosaminidase from growth medium into two fractions (peak A and B) on DEAE-Sephadex and CM-Sephadex. The peak A was purified 1800-fold and was homogeneous on PAGE. The enzyme was most stable at pH 8.0 and had a molecular mass of about 90 kDa. The enzyme was specific for substrates with non-reducing N-acetyl muramic acid end groups and attacked several synthetic and natural substrates.

Sakamoto et al (197) have reported a novel endo-β-N-acetylglucosaminidase that specifically acts on plant glycoproteins. The molecular mass was 89 kDa. The enzyme was stable at pH 5.5-7.0, up to 30°C and showed highest activity at pH 6.0. The enzyme prefers the xylose containing sugar

chains over hybrid type and complex type sugar chains. Moreover, the enzyme releases sugar chains from native horseradish peroxidase and stem bromelain, which are known to be glycoproteins.

Geimba et al (198) have reported the purification and characterization of β-N-acetylglucosaminidase from the phytopathogenic fungus Bipolaris sorokiniana. The purification was about 70-fold, with an yield of 41 %, the activity measured with p-GlcNAc as substrate. The enzyme had a pH and temperature optima of 4.5 and 55°C, respectively. The molecular weight of the enzyme was found to be 120 kOa by gel filtration chromatography and about 55 kDa by SDS-PAGE. The enzyme was also active with p-nitrophenyl-chitobioside and p-N-acetylgalactosaminide as substrates.

Tao et al., (199) reported the purification and characterization of β-N-acetylhexosaminidase from Aspergillus tamarii. The ratio of β-N-acetyl glucose-aminidase (β-GlcNAcase) and β-N-acetyl galactosaminidase (β-GalNAcase) was 2.5 and remained constant throughout the purification. The Molecular weight (M_r) estimated was 140 kDa with concentration gradient PAGE and subunit M_r was 72kOa as determined by SOS-PAGE. The pl was 4.2 and the optimum pH was 5.5-6.5 and 5.0-6.0 for β-GlcNAcase and β-GalNAcase respectively with stable pH range of 5.5-8.3 for both. The optimum temperature was 60°C and the residual activity was 52.7 % after exposure at 50°C for 8h. The activity was slightly activated by Mn^{2+} or Fe^{2+}, while inhibited strongly by Hg^{2+} and slightly by Ag^{2+}, CU^{2+}, Pb^{2+}, Cd^{2+} or Zn^{2+}.

1.4.3 β-Hexosaminidase in plant system

Bouquelet and Spik have reported four forms of β-hexosaminidase from germinating seeds of fenugreek (Trigonella foenumgraecum) and they demonstrated that these isoenzymes originated from different parts of the germinated seeds. They designated these forms as isoenzyme forms -I, -II, - III and IV and studied their properties (200,201).

The molecular weight of four molecular forms of hexosaminidase is found to be 84, 72, 180 and 150 kDa for isoenzymes -I, -II, -III and –IV respectively. Reduced conditions of electrophoresis yielded bands having molecular weight of 28, 28, 30 and 30 kDa for the respective isoforms (201). This suggested that the four isofoms might be polymers formed by association between three or six subunits of identical molecular weight. Immunological studies revealed common antigenic determinants between the forms I and II, located in the cotyledon and endosperm, and between isoforms III and IV located predominantly in the embryo. The isoelectric pH (pI) are found to be 6.78, 6.3, 4.9 and 4.65 for isoforms I to IV respectively. The divalent cat ion Fe^{2+} was known to increase the activity of form I, while depressed the activity of other forms and 5JlM concentration of $HgCl_2$ / p-chloromercurybenzoate did not affect the activity of form I, while inhibited other forms. The Km value was 0.04, 0.62, 0.087 and 0.37 mM for p-GlcNAc and 0.15, 0.55, 0.09 and 0.04 mM for p-GaiNAc as substrate for isoforms I to IV respectively.

Posci et al (202) have purified and characterized hexosaminidase B from germinating Lupin seeds and also studied the mechanism of inhibition of the same with some 2-acetoimide-2-deoxyaldono (1→4) lactones.

According to Posci et a/., (202) the hexosaminidase B from germinating Lupin seeds has a molecular weight of 69 kDa as determined by SDS-PAGE and 62.5 kDa as observed by gel filtration. This enzyme had neutral isoelectric point (pl:7.05) and found to have activity on p-nitrophenyl-2-acetamido-2 -deoxy-β-D-glucosaminide and p-nitrophenyl-2 -acetamido-2-deoxy-β-D-galactosaminide substrates, but lacks N,N-diacetylchitobiase activity. They also reported that some 2-acetoimido-2-deoxyaldono (174) ctones inhibit the hexosaminidase except the one having D-arabinose sidue (203).

1.4.4 Physiological role of β-Hexosaminidase

- Deficiency of the enzyme in higher organisms causes Tay-Sachs and Sandhoffs disease (204,205)
- Plays a role in the metabolism of glycoproteins, glycolipids and glycosamino-glycans in plant, microbial and animal systems (203,206,207).
- Staphylococcal endo-β-N-acetylglucosaminidase inhibits response of human lymphocytes to mitogens and interferes with the production of antibodies in mice (208).
- Involved in the proliferation of bovine airway smooth muscle by activating p44/42 mitogen activated protein kinase and protein kinase C (209).
- Egg cortical granule N-acetylglucosaminidase is required for the mouse zona to block polyspermy (210).
- Sperm requires β-N-acetylhexosaminidase to penetrate through the egg zona pellucid a (211,212).
- Bacillus cereus spore coat associated hexosaminidase plays an important role in spore germination (213)

1.5 ∝-MANNOSIDASE

Early work on -mannosidase was in almond emulsin. There has been a revival of interest in this enzyme from all sources, and an extensive survey of plant material was made by Levvy and McAllen in 1962 (214). In 1956, it was unexpectedly observed that ∝-mannosidase is ubiquitous in mammalian tissues (215). Levels of ∝-mannosidase have been measured in human blood (216), in cow's milk (217), in semen from a number of different species (218) and in different parts of the alimentary tract (219). Table-2 presents the ∝-mannosidase activity in mammalian tissues.

It has been known for a long time that ∝-D-mannosidase occurs in plant seeds (214,220,221). Table 3 presents enzyme activity values in certain plant seeds.

Purification and characterization of ∝-D-mannosidase has also been reported in helminthes (222), insects (223), porcine (224), hen oviduct (225), Persian cats (226) and bovine kidney (227).

Table1.2 ∝-Mannosidase activity of certain mammalian tissues

Tissue	Rat	Mouse	Pig	Rabbit
Epididymis	150,000	16,000	15,000	------
Liver	6,400	5,400	3,000	4,200
Kidney	3,800	7,300	4,600	3,000
Spleen	1,800	4,700	1,800	------
Pancreas	5800	------	1100	520
Smallintestine	6,200	1,100	1,700	4,400
Prostate	2, 500	320	------	------

Units: µg of p-nitrophenolliberated per gm of moist tissue in 1 h at 37°C (Reproduced from Snaith and Levvy, 1973: Ref.228)

Table 1.3 ∝-D-Mannosidase activity of certain plant seeds

Material	Activity
Jack-bean meal	750,000
Almond emulsin	60,000
French bean (Phaseolus vulgaris)	61,000
Lettuce	40,000
Lucerne	25,000
Rye grass	8,000

Units: µg of p-nitrophenol liberated per gm of seeds in 1 h at 37°C (Reproduced from Snaith and Levvy, 1973: Ref.228)

1.5.1 ∝-Mannosidase in animal systems

Kawar et al. (223) have cloned and characterized an insect cell cONA encoding a class II ∝-mannosidase with amino acid sequence and biochemical similarities to mammalian Golgi ∝-mannosidase-il. The enzyme was cobalt-dependent and could hydrolyze Man5GlcNAC2 to Man3GlcNAc2, but not GlcNAcMan5GlcNAc2. This enzyme is not lysosomal because it was not active at acidic pH.

Jin et al. (224) have purified ∝-mannosidase by DEAE-Sephacel, Red-Amicon and Superdex 200 column chromatography, and studied the properties of the major ∝-mannosidase in laminal fluid of porcine epididymis. The purified enzyme consisted of 63 and 51 kOa subunits in equimolar amounts. It cleaved ∝1-2 linked mannosyl residues and to a lesser extent cleaved ∝1-3 and ∝1-6 linked mannosyl residues in the high-mannose oligosaccharides. The optimum pH to hydrolyse oligosaccharide was in the acidic pH range (3.5 to 4.0). Total a-mannosidase activity in the porcine epididymal fluid increased from proximal to distal epididymis. At least two kinds of ∝-mannosidase were present in porcine epididymal fluid.

Yamashiro et al. (225) purified and characterized neutral ∝-mannosidase from hen oviduct. The molecular mass of the enzyme was 480 kDa on gel filtration, and 100 kDa on SOS-PAGE with 2-mercaptoethanol indicating that it was composed of four subunits. The activated enzyme hydrolyzed both p-nitrophenyl-∝-D-mannopyranoside and high mannose type sugar chains. The enzyme was activated 24-fold on pre-incubation with Co^{2+}, the activation with other metal ions, like Mn^{2+}, Ca^{2+}, Fe^{2+} and Sr^{2+}, was less than 5-fold, while Zn^{2+}, CU^{2+}, and Hg^{2+} inhibited the enzyme. The optimum pH for both the enzyme activity and activation with Co^{2+} was around 7.0.

Berg et al. (226) have purified and characterized recombinant human lysosomal ∝-mannosidase. The enzyme was secreted as an active homodimer of a 130 kDa precursor that was proteolyzed into two polypeptides of 55 and 72 kDa during the subsequent purification. The immunoaffinity purified enzyme, which mainly consisted of the 130 kDa precursor, displayed specific activity and kinetics similar to those of the processed form.

Tollersrud et al. (227) have purified ∝-mannosidase and characterized its gene to determine the two mutations that caused a-mannosidosis in bovine kidney. The gene was organized in 24 exons that spanned 16kb and its corresponding cDNA contained an open reading frame of 2997bp beginning from a putative ATG start codon. The deduced amino acid sequence contained a signal peptide of 50 amino acids adjacent to protein sequence of 949 amino acids that was cleaved into five polypeptides in the mature enzyme; starting with the peptide derived from the N-terminal part of the precursor, their molecular weight were 35/38 (peptide a), 11/13 (peptide b), 22 (peptide c), 38 (peptide d) and 13/15kDa (peptide e). Variation in the degree of N-glycosylation accounts for molecular mass heterogeneities of peptides a, band e. peptides a, band c were disuiphide-linked. A T961→C (Phe321→Leu) transition was identified in the cDNA of ∝-mannosidosis affected Angus cattle and G662→A (Arg221→His) transition in Galloway cattle.

Berg et al. (229) have purified lysosomal ∝-mannosidase and determined its cDNA sequence and identified the mutation causing ∝-mannosidosis. The active enzyme consisted of three polypeptides, with molecular masses of 72, 41 and 12 kDa, joined by non-covalent forces. The enzyme is synthesized as a single precursor chain with a signal peptide of 50 amino acids followed by a polypeptide chain of 957 amino acids, which is cleaved into three polypeptides of the mature enzyme. The deduced amino acid sequence was 81.1 and 83 % identical with those of human and bovine lysosomal ∝-mannosidases respectively. A 4bp deletion was identified in a ∝-mannosidosis affected Persian cat by DNA sequencing of reverse transcriptase PCR products. The deletion resulted in a frame shift from codon 583 and premature termination at codon 645.

1.5.2 ∝-Mannosidase in microbial systems

In microbes, purification and characterization of ∝-D-mannosidase has been reported in fungal cells (230,231), in bacteria (232) and in baculovirus infected Spodoptera frugiperda (233). Vazquez-Reyna et al. (230) have purified and characterized two soluble a-mannosidase (E-I and E-II) from Candida albicans by a three step procedure consisting of size exclusion and ion exchange chromatographies on Sepharose CL4B and Monoq columns, respectively and preparative native electrophoresis. The isoenzymes E-I and E-II moved as monomeric polypeptides of 54.3 and 93.3 kDa respectively in SOS-PAGE. Purified enzymes known to act on both p-nitrophenyl-∝-D-mannopyranoside and 4-methylumbelliferyl-∝-D-manno-pyranoside (4-MU-Man) as substrates with optimum pH of 6.0 and optimum temperature of 42°C in 50 mM MES- Tris buffer. The apparent Km values for hydrolysis of p-nitrophenyl-∝-O-mannopyranoside and 4-methylumbelliferyl-∝-D-manno-pyranoside by E-I were 2.3 mM and 0.83 j.1M, respectively. Corresponding values for E-II were 1.86mM and 0.25µM. Swansonine (SW) and deoxymannojirimicin (dMNJ) strongly inhibited the hydrolysis of 4-MU-Man by both the enzymes. On the other hand hydrolysis of pnp-∝-O-mannopyranoside was not affected by both the inhibitors. Mn^{2+} and Ca^{2+} slightly stimulated the activity of E-II at 0.5-2mM concentration, while Mg^{2+} was slightly inhibitory for both the enzymes. E-I and E-II preferentially cleaved ∝1-6 and ∝1-3 linkages, respectively.

Gaikwad et al (231) have purified and characterized ∝-mannosidase from Aspergillus sp. ∝-Mannosidase has been purified to homogeneity by preparative PAGE. The native enzyme had a molecular mass of 412 kDa and consisted of six identical subunits of molecular mass 69.5 kDa. The enzyme is acidic (pi: 4.5) and a glycoprotein with a carbohydrate content of 3.8%. The temperature and pH optima of the enzyme were in the range of 50-55°C and 6.0-6.5, respectively. At pH 6.0, the enzyme is stable for 30min 5acC. The Km and Vmax for p-nitrophenyl-∝-D-mannopyranoside were 83µM and 0.2µmoll min per mg of the enzyme respectively. The enzyme was strongly inhibited by 1 mM Hg^{2+} and Cu^{2+} and partially by 30mM glucose and mannose. The enzyme hydrolyzed Man(∝1-3)Man at a very high rate allowed by Man(∝1-2)Man, while the rate of hydrolysis was very low for an(∝1-6)Man. The rate of hydrolysis for high mannose oligosaccharide $(Man)_6$ was higher than that for $(Man)_9$ and yeast mannan was not at all hydrolyzed.

Rivera-Marrero et al. (232) have cloned and expressed ∝-mannosidase gene in Mycobacterium tuberculii. Mannosidase activity was optimum at pH 6.5, and was not inhibited by dMNJ, but mildly inhibited by SW and stimulated two-fold by EDTA. Gene bank BLAST analysis for sequences homologous to eukaryotic a-mannosidase revealed a 3.6 kb putative gene (Rv0648) in Mb cosmid SCY20H 1 0 with strong homology (48%) to the rat ER/cytosolic ∝-mannosidase and containing signal sequence of class 2 ∝-mannosidase. Gene Rv0648 was cloned and expressed in E. coli. Expression of ∝-Man-pET in E. coli cells resulted in a 8-fold increase in ∝-mannosidase activity towards 4-MU-Man, upon IPTG induction. Partial purification of the histidine-tagged Mtb mannosidase by metal chelation affinity chromatography, and analysis by SDS-PAGE, showed a protein with the

molecular mass of 137.5 kDa. The enzyme showed activity towards synthetic aryl-man nose substrates.

Ren et al. (233) have purified ∝-mannosidase and studied its properties from Golgi-like membranes of baculovirus infected Spodoptera frugiperda. The enzyme was purified to apparent homogeneity by using a combination of steps including DEAE-Cellulose, hydroxylapatite, Con-A Sepharose and gel filtration chromatography. The molecular mass of this purified protein was approx. 120 kDa by SOS-PAGE under reducing conditions and approx. 240 kDa under non-reducing conditions. Enzyme acted on GlcNAc-Mans-GlcNAc-GlcNAc (under both reducing and non-reducing conditions) and on p-nitrophenyl-∝-D-mannopyranoside. Metal ions were not required for enzyme activity with any of the substrates, but Cu^{2+} was strongly inhibitory. The activity of the enzyme was inhibited at low concentration of SW, but much higher concentration of dMNJ was required to achieve inhibition.

1.5.3 ∝-Mannosidase in plant systems

Even though the ∝-mannosidase was first reported from plant system, purification and characterization were attempted only in last two decades. Purification and characterization of this enzyme was first reported in jack bean meal (228,234) and later from Canavalia seeds (235), Indian lablab beans (236), Capsicum (237) and tomato (238).

Einhoff and Rudiger (235) have studied the chemical and kinetic properties of ∝-mannosidase from Canavalia seeds. The enzyme was composed of two pairs of subunits having molecular mass of 44 and 66 kDa, which form a tetramer of 220 kDa. The larger subunit was glycosylated and both the subunits had similar amino acid composition. The larger subunit contained surplus of alanine, aspartic acid / asparagine, histidine, phenylalanine and tyrosine, while the smaller one had surplus of glutamic acid / glutamine, serine and threonine. The enzyme was inhibited by mannose and stimulated the proliferation of B-lymphocytes from nude mice.

Tulasi and Nadimpalli (236) have purified ∝-mannosidase to homogeneity from Indian lab lab beans. Purified ∝-mannosidase had an apparent molecular weight of 195±kDa with 4.5% carbohydrate. On SDS-PAGE under reduced conditions, the enzyme dissociated into two major bands corresponding to M_r 66 and M_r 44. The antibody of jack bean ∝-mannosidase cross-reacted with the enzyme from the lablab beans.

Priya Sethu and Prabha (237) have reported ∝-mannosidase from Capsicum annuum. The enzyme showed a constant increase in activity with the progress in fruit softening and was purified to homogeneity by precipitation with ammonium sulfate followed by gel filtration on Sephadex G-100, DEAE-Sephadex A-50 and HPLC gel filtration on GF-250. The purified enzyme had a native and subunit molecular weight of 43 kDa and 23 kDa respectively. The pH and temperature optima were

5.7 and 50°C, respectively. The enzyme was thermally stable up to 60°C for 15min. the Km for p-nitrophenyl -∝-D-mannopyranoside was 0.7mM.

Subsequently, Suvarnalatha and Prabha (238) have reported two ∝-mannosidase isoforms from tomato, which were purified by chromatography on DEAE-Sephadex A-50 and Sephadex G-100. Both the enzyme forms had an acidic pH optima (4.5) and were thermally stable at 65°C up to 15min. isoform showed a broad temperature optima (55 to 65°C), whereas isoform II had an optimal activity at 65°C. The Km values for p-nitrophenyl-∝-D-mannopyranoside were 1.11 and 1.05mM, respectively. Isoform I was inhibited by Hg^{2+} (1mM) whereas isoform II was inhibited by Cu^{2+} (0.1mM) and Hg^{2+} (1 mM). Purified isoform II had a SOS Mr of ca. 38 kDa.

1.5.4 Physiological role of ∝-mannosidase

- Deficiency causes lysosomal storage disease a-mannosidosis (239)
- Accelerates endoplasmic associated glycoprotein degradation (240,241).
- ∝-Mannosidase plays potential role in sperm-egg interaction (242)

SCOPE OF PRESENT INVESTIGATION

Capsicums are the second most important crop among the Solanaceous fruits. Capsicums are an excellent source of vitamin A and C. Bell capsicum is generally an expensive vegetable in India and its high cost is mainly due to high perishability resulting in short life. The post-harvest losses are mainly material loss, which include loss of weight, color, bruise, decay, etc., at various stages from harvest to consumption. The expected shelf life of capsicum depends upon post-harvest handling and storage environment. To date cold storage (~7-90C) is the only known technique economically feasible for short-term storage of fresh capsicums. In India, the prevailing tropical climate and lack of adequate cold storage facilities shorten the life of capsicum to only 6-8 days at ambient temperature (27±4°C). Hence there is a need to extend the shelf life of capsicum at ambient temperatures to extend the market season and create distant markets. In this connection, a thorough understanding of the regulation of fruit ripening is necessary. Hence, the factors that bring about textural softening during fruit ripening are considered for this investigation. Glycosidases (β-hexosaminidase and ∝-mannosidase) -the carbohydrate hydrolases involved in fruit softening during ripening were specifically studied in this investigation in the context of fruit ripening.

The major objectives of this investigation are:

1. To study the enzyme activity profiles of β-hexosaminidase and ∝-mannosidase during fruit development and ripening in bell capsicum and compare the same with that of tomato, which belongs to the same family Solanaceae and considered a model system for scientific studies in higher plants.
2. Purification and characterization of β-hexosaminidase from bell capsicum as well as from tomato (for comparison) in order to understand its implication in fruit ripening.

CHAPTER – 2: MATERIALS AND METHODS

2.1 INSTRUMENTS

1. UV-Vis double beam spectrophotometer, Shimadzu, UV-160A, Kyoto, Japan.
2. pH meter, Control Dynamics, Hyderabad, India.
3. Water bath with temperature control, Instruments and Equipments (I) Pvt. Ltd., Bombay, India.
4. Centrifuges: Hermie Z 320K, Germany; Plastocrafts, Bombay, India; Sorwal RC8, USA.
5. Lyophilizer: Virtis Freeze Mobile, Japan.
6. Fraction collector, LKB Bomma, USA.
7. Electrophoresis unit: Pharmacia Mini slab gel electrophoresis model SE280-10A-75, Hoffer Pharmacia Biotech Inc. CA, USA.
8. Remi vortex mixer, India

2.2 CHEMICALS

Acrylamide, bis-acrylamide, ammonium persulfate, glycine, bromophenol blue, bovine serum albumin, DEAE-Cellulose (0.86 meq/ml), nitrocellulose tubing (MW cut off. 12 kDa), pnp-β-N-acetylglucosamino pyranoside, pnp-β-N-acetyl galactosaminopyranoside, pnp-β-glucopyranoside, pnp-β-galacto pyranoside, pnp-α-glucopyranoside, pnp-α-galactopyranoside, pnp-α-mannopyranoside, polyvinylpyrrolidone (PVP) Sodium azide, NNNN- Tetramethyl ethylenediamine (TEMED) and Tris were procured from Sigma Chemical Co., St. Louis, USA. Trifluoroacetic acid was from Lanchester, UK.

DEAE-Sephadex A 50, Sephadex G-25, Sephadex G-200, and SDS-Molecular weight markers (14.2-94 kDa) were from Pharmacia Fine Chemicals, Uppasala, Sweden. Sugar standards -glucose, galactose, mannose, inositol, arabinose, fucose, rhamnose and xylose were procured from ICN pharmaceuticals Inc. Life Science Group, Cleveland, USA. Acetone, methanol, silver nitrate, sodium thiosulfate and acetonitrile were procured from E-Merck, Darmstadt, Germany.

All other chemicals used in this investigation were of analytical reagent (AR) grade procured from either Qualigen Fine Chemicals, Mumbai, India, or SO Fine Chemicals Ltd., Bolsor, India, or Ranbaxy Laboratories Ltd, SAS Nagar, Punjab, India.

2.3 ANALYTICAL METHODS

2.3.1. Total carbohydrates

Estimation of total carbohydrates was done by the phenol-sulfuric acid method (243) using glucose as standard. 1.0 MI appropriately diluted sample solution was treated with 0.5 ml phenol reagent (5% Phenol (v/v) in water) followed by 5.0 ml con. H_2SO_4 and immediately mixed. The reaction mixture was placed in a water bath at 25°C for 30 min and absorbance was read at 490nm. The absorbance was compared with that of standard glucose (0-80µg) similarly treated.

2.3.2 Reducing sugar

Reducing sugar estimation was carried out by the modified method of Schales (244). To 1.0 ml sample or standard containing 10 -100µg of glucosamine, 2ml of alkaline ferricyanide reagent (0.25gm potassium ferricyanide in 500ml of 0.5M sodium carbonate) was added and kept over a boiling water bath for 15 min. The reaction mixture was cooled to room temperature and absorption was measured at 420nm.

2.3.3 Protein

Estimation of protein in the aqueous extracts of acetone dried powders from the fruits were done by employing Folin-Ciocalteau method, dye binding method and by measurement of absorption at 280 nm.

2.3.3.1 Folin-Ciocalteau Method (245)

The protein was estimated by the method of Lowry et al (1951).

Reagents:

Solution A: 0.1 N Sodium hydroxide

Solution B: 98ml of 4% Sodium Carbonate + 1 ml of 2% Copper Sulfate + 1 ml of 2% Sodium Potassium Tartarate.

Solution C: 1 N Folin-Ciocalteau reagent

To 0.2 ml of sample, 0.2 ml of solution A was added followed by 1ml of solution B and mixed well and incubated at room temperature for 10min. 0.1ml of solution C was added and mixed well in a vortex mixer. The absorbance was read at 670nm after 30min. Bovine serum albumin (0-50µg) was used as reference protein.

2.3.3.2 Dye binding method (246)

Dye reagent was prepared by dissolving Coomassie brilliant blue G-f 250 (100mg) in 50ml of 95% ethanol and made up to 1 L with 3% phosphoric acid. The reagent was filtered through Whatman No 1 filter paper and stored in a brown bottle.

To 0.5 ml sample solutions 0.5ml of dye reagent was added and mixed. The absorbance was read at 595 nm after 10min of incubation within next 40 min. The absorbance was compared with the one obtained with reference Bovine serum albumin (0-50 µg)

2.3.3.3 A_{280} method

Protein content of samples was also determined by measuring absorption of an appropriately diluted sample at 280 nm and comparing with reference Bovine serum albumin (100-1000 µg/ml).

2.3.4 β-Hexosaminidase activity assay (237)

The enzyme activity was determined by incubating for 15 min at 37°C, a reaction mixture consisting of 100 mM sodium acetate buffer (pH: 5.0), 1.25 mM pnp-β-D-N-acetyl glucosamino pyranoside (or pnp-β-D-N-acetyl galactosamino pyranoside), and a suitable aliquot of the enzyme sample. The activity was determined by measuring the liberated p-nitrophenol at 405 nm after addition of 500mM sodium bicarbonate to the reaction mixture. One unit of the enzyme is defined as the amount of enzyme required to liberate one µmol of p-nitrophenol per min. The specific activity was expressed as enzyme units per mg protein.

2.3.5 α-Mannosidase activity assay (237)

The enzyme activity was determined by incubating for 15min at 37°C, a reaction mixture consisting of 100 mM sodium acetate buffer (pH: 5.0), 1.25 mM pnp-α-D-mannopyranoside, and a suitable aliquot of the enzyme sample. The activity was determined by measuring the liberated p-I nitrophenol at 405 nm after addition of 500 mM sodium bicarbonate to the reaction mixture.

2.3.6 α-Galactosidase activity assay (237)

The enzyme activity was determined by incubating for 15 min at 37°C, a reaction mixture consisting of 100 mM sodium acetate buffer (pH: 5.0), 1.25 mM pnp-α-D-galactopyranoside, and a suitable aliquot of the enzyme sample. The activity was determined by measuring the liberated p-nitrophenol at 405 nm after addition of 500 mM sodium bicarbonate to the reaction mixture.

2.4 PLANT MATERIAL

Bell capsicum: Fruits of bell pepper (Capsicum annuum var. variata) at different stages were freshly harvested from a local farm near Srirangapatna (Mysore district). They were washed thoroughly with water and rinsed with distilled water, wiped dry and used for experiments. The four different developmental stages were selected based on size / weight and days (d) after fruit set. 1, 8d (2.5-3.5g); 2, 16d (8.5-9.5g); 3, 24d (60-62g) and 4, 32d (65-67g). The four stages of ripening chosen were based on the days after harvest. I). mature dark green (3 d), II) light yellow/light green (7 d), III) orange yellow/orange red (11 d), IV) red ripe stage (21 d). Acetone dried powders were prepared from the respective samples using 3 volumes of acetone and used for the experiments. The mature green fruits were kept at ambient temperature for normal ripening process. Post-climacteric bell capsicum served as the source of enzyme for purification and characterization.

Tomato: Freshly harvested tomato (Lycopersicon esculentum) fruits were collected from a local farm. The four different developmental stages were selected based on size / weight and days (d) after fruit set, thus: stage 1, 7d (2.5 -3.5 g); 2, 14d (8.5 -9.5g); 3, 21d (60 -62g) and 4, 28d (65 -67g). The four stages of ripening chosen were based on the days after harvest, thus, stage 5, Mature dark green (Od); 6, Light green (5d); 7, Orange (10d) and 8, Red ripe stage (15d). Acetone dried powders were prepared from these various samples of tomato fruits using 3 volumes of acetone.

2.5 ISOLATION AND PURIFICATION OF β- HEXOSAMINIDASE

The enzyme was extracted from acetone powders of bell capsicum and tomato fruits by soaking in different buffer systems viz., potassium phosphate buffer (pH 6.6) containing 0.5M sodium chloride and 0.2% PVP, sodium acetate buffer (pH: 6.6) containing 0.1% triton x-100 and 0.2% and sodium acetate buffer (pH: 6.6) with 0.2% PVP. Sodium acetate buffer (pH: 6.6) was used for isolation and purification of β-hexosaminidase and α-mannosidase from the respective enzyme sources. All extractions were performed at 4°c unless otherwise mentioned. In a separate set, the enzyme was also extracted using sodium acetate buffer with 0.1% triton x 100 and 0.2%PVP for interaction studies.

2.5.1 Purification of β-hexosaminidase:

The β-hexosaminidase was extracted from acetone dried powders of bell capsicum and tomato with (3x) sodium acetate buffer, pH 6.6 containing 0.2% PVP and kept overnight at 4oC with constant stirring. The enzyme extracts were pooled, filtered through two layers of nylon cloth, centrifuged at 7000 rpm for 15 min and dialyzed in a cellulosenitrate membrane with ca 12 kDa cut off. The dialysate was recentrifuged and the supernatant considered as crude enzyme extract and used for purification. This extract was subjected to stepwise ammonium sulfate precipitation (0-35% and 35-70% saturation). The precipitate rich in β-hexosaminidase and α-mannosidase was dissolved in a limited amount of water (10 ml) and extensively dialyzed against water (5x). The dialyzed fraction was subjected to ion exchange chromatography on DEAE-Sephadex A-50 followed by gel filtration chromatography on Sephadex G-200.

2.5.2 CHROMATOGRAPHIC METHODS

2.5.2.1 Ion exchange chromatography:

DEAE-Sephadex A-50 gel matrix was soaked overnight in water containing 0.05 mM NaCl. The swollen gel was activated by treating with 0.1 M sodium hydroxide and the pH was brought to neutral by washing with water. This was followed by 0.1M HCl treatment and bringing the pH to neutral as above. The gel matrix was equilibrated with 25 mM sodium acetate buffer, pH 6.6. The matrix thus equilibrated was packed on to a glass column (3.0 Gm x 35 Gm). The bed volume of the gel was about 80 ml.

The dialyzed and concentrated protein solution was applied on to the column with a flow rate of 0.7ml/min and eluted stepwise with two-bed volumes of buffer containing 0.05-0.25M NaCl. 6 MI fractions were collected using a fraction collector, and subjected to protein and enzyme activity determinations. Distinctly separable fractions were designated as isoform-l, -II and -III based on elution profile and enzyme rich fractions were pooled, dialyzed and suitably concentrated.

2.5.2.2 Gel Filtration chromatography

Sephadex G-200 gel matrix (8 gm) was allowed to swell overnight in ca. 400 ml water. The matrix was washed several times with water to remove fine particles. Then the matrix was equilibrated with 50mM sodium acetate buffer (pH: 6.6) containing 0.2 M NaGI. The gel matrix was loaded on to a

glass column (110 cm x 1.5 cm) and the flow rate was maintained at 0.25 ml / min. The bed volume was 150 ml with a void volume of 53 mi. The post-ion exchange chromatography protein fractions concentrated to 1 ml were applied individually on to this column and eluted with the same buffer. 4 MI fractions were collected using a fraction collector and analyzed for protein and enzyme activity. Protein and carbohydrate content was estimated by standard methods. Enzyme rich fractions were pooled, dialyzed and concentrated.

2.6 PURIFICATION OF α-MANNOSIDASE

For isolation and purification of α-mannosidase from bell capsicum and tomato fruits, the procedure was the same as described for β-hexosaminidase (p.50) except that Con A affinity chromatography was employed after gel permeation chromatography.

2.6.1 Con A Sepharose chromatography

Con A (2ml) was pre-equilibrated with Tris-HCl buffer, pH 6.8 (20ml) containing 150mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.2% NaN_3. After applying the protein sample, the column was washed with 10-bed volumes of buffer and eluted with the same buffer containing 100mM and 300mM methyl-α-D-mannopyranoside. The protein and enzyme activity were monitored in specific fractions.

2.7 ELECTROPHORETIC METHODS

2.7.1 Polyacrylamide gel electrophoresis

Vertical slab gel electrophoresis was carried out in a Pharmacia Mighty small electrophoresis unit at room temperature as well as at 4°C.

2.7.1.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous slab gel electrophoresis under reduced and non-reduced conditions was carried out according to Laemmli (247).

Reagents:

A. Acrylamide solution (30 %): 30g of acrylamide and 0.8g of bis-acrylamide in 100ml and filtered through Whatman No.1 filter paper
B. Resolving gel buffer (pH 8.8): 18.15g of Tris in ca. 50 ml water; pH adjusted to 8.8 with 1 N HCI. Volume made upto 100 ml and filtered.
C. Stacking gel buffer (pH 6.8): 6.15g of Tris in ca. 50ml water and pH adjusted to 6.8 with 1 N HCI. Volume made upto 100ml and filtered.
D. Ammonium persulfate solution: 10 % w/v, in water.
E. SOS solution: 10 % w/v, in water.
F. Electrode reservoir buffer (pH 8.3): 14.4 g glycine, 3.0 g Tris and 19 of SOS in 1000 ml.
G. Sample buffer (2x): 19 of SOS, 2 ml glycerol, 2 ml bromophenol (0.1 % w/v in water), 1.25 ml of 1 M Tris-HCI, pH 6.8, 2 ml of β-mercaptoethanol made up to 10 mi. When this solution is diluted to single strength, sample buffer will contain 5% w/v SDS, 10% v/v glycerol, 10% v/v β-mercaptoethanol and 0.0825 M Tris-HCl, pH 6.8

Running gel (10%) was prepated by mixing 2.0 ml of solution A, 1.5 ml of solution B and 2.4 ml of water. The mixture was degassed and 30 µl each of solution D and E were added. Finally 20 µl of TEMED was added and mixed well. The contents were poured between the notched and rectangular glass plates separated by 0.75 mm Teflon spacers. Gels were allowed to polymerize for 8-10 h at room temperature.

Stacking gel (5%) was prepared by mixing 0.35 ml of solution A, 0.5 ml of solution B and 1.17 ml of water. The mixture was degassed and 15µl each of solution D and E were added. Finally, 10 µl of TEMED was added and mixed well. The solution thus prepated was poured about the polymerized resolving gel. The gel thus preapted has a dimension of 10 cm x 12 cm x 0.075 cm.

Samples were prepared by dissolving a suitable amount of protein in 25 µl of solution G (1x) without β-mercaptoethanol and centrifuged at 8000 rpm before loading on to the gels. Gels were run at 120 V (8-16 mA) for 90 min. A few marker proteins were also co-electrophoressed to serve as reference molecular weight.

2.7.1.2 Native SDS gel electrophoresis (Native SDS-PAGE)

The discontinous slab gel electrophoresis under non-reduced conditions was carried out according to Laemmli (247). The resolving and stacking gels were prepared as above for non-denatured SDS-PAGE. Samples were prepated by dissolving a suitable amount of protein in 25 µl of solution G (1x) without β-mercaptoethanol and centrifuged at 8000 rpm before loading on to the gels. Gels were run at 120 V(8-16 mA) for 90 min.

2.7.1.3 Glycol chitosan incorporated PAGE

The constituents of the gels were similar to that of SOS-PAGE except that it had 0.03 % glycol chitin and did not contain SDS.

2.6.1.4 Acid polyacrylamide gel electrophoresis (248)

The discontinuous slab gel electrophoresis was carried out according to Raisfeild et al.

Reagents:

A) Resolving gel solution (30%): 30g of acrylamide and 0.8g of bis-acrylamide in 100ml; Filtered through Whatman No.1 filter paper.

B) Stacking gel solution (10%): 10g of acrylamide and 2.5g of bis-acrylamide dissolved in 100ml; Filtered.

C) Separating gel buffer: 12.8 ml of glacial acetic acid in about 30m l of distilled water, 1.0 ml of TEMEO and pH adjusted to 4.0 with 1 M KOH and made up to 100 ml with water.

D) Stacking gel buffer: 4.3ml of glacial acetic acid in about 30ml of water, 0.46ml of TEMEO and pH adjusted to 5.0 with 1 M KOH and made up to 100ml with water.

E) Riboflavin solution: 4mg / 100ml water.

F) Electrode reservoir buffer 14.2g of β-alanine dissoived in about 800ml of water, pH adjusted to 4.0 with acetic acid and made up to 1000ml with water.

G) Sample buffer: 20mg of methyl green, 5ml glycerol and made up to 10ml.

Running gel (10 %) was prepared by mixing 4.0 ml of solution A, 1.5 ml of solution C, 1.5 ml of solution E and 5.0 ml of water. The contents were poured between the notched and rectangular glass plates separated by 1.0 mm Teflon spacers. Gels were allowed to polymerize for 2-3 h in presence of 100W incandescent lamp at room temperature.

Stacking gel (5 %) was prepared by mixing 2.0 ml of solution 8, 1.0 ml of solution 0, 1.0 ml of solution E and 4.0 ml of water. The solution thus prepared was poured above the polymerized resolving gel and exposed to light as above. The gel thus prepared had a dimension of 10 cm x 12 cm x 0.075 cm.

Samples were prepared by dissolving a suitable amount of protein in 25 µl of solution G and centrifuged at 8000 rpm before loading on to the gels. Electrophoresis was carried out at 120 V (8-16mA) for 90 min with reverse polarity, i.e., electrophoresis directed towards negative electrode.

2.7.1.4 Chitosan incorporated PAGE

The gel composition was similar to that of acid-PAGE except that the resolving gel also contained 0.03 % chitosan. Electrophoretic details were similar to the above.

2.7.2 STAINING

2.7.2.1 Coomassie Brilliant blue staining

After the electrophoresis, the gels were immersed in 0.1 % Coomassie Brilliant R-250 solution containing 30% methanol and 11 % acetic acid for about 15 -30 min with constant shaking. The gels were destained with a solution containing 15% methanol and 7% acetic acid.

2.7.2.2 Silver staining (249)

Parallel sets of gels were also stained with silver stain for protein using the following reagents.
Reagents:

- Fixer solution: 30 ml methanol and 7 ml acetic acid in 100 ml water.
- Solution A: 50 ml of methanol in water.
- Solution B: 20 mg sodium thiosulfate in 100ml of water.
- Solution C: 200 mg of silver nitrate and 75 µl of formaldehyde solution in 100 ml water.
- Solution D: 6 g of sodium carbonate, 40 µl of formaldehyde solution and 250 µl of solution Bin 100 ml water.

Immediately after the electrophoresis, the gel was fixed in 100ml fixer solution for 12h. The gel was washed thrice with solution A for 15 min. The gel was further treated with solution B for 1 min and

washed thrice with water for 5 min. The gel was transferred to clean glass tray containing solution C and shaken well for 15 min. The excess silver nitrate stain was removed by washing the gel thrice with water. The gel was immersed in solution D and shaken well till the bands appeared. Washing the gel in water and soaking in fixer solution arrested the staining.

2.7.2.3 Glycoprotein staining (250)

After the electrophoresis, the gels were immersed in 5 % phosphotungstic acid in 2NHCI for 120 min at room temperature with gentle shaking. Further, gels were transferred to 50 ml of fixer solution containing 7% methanol and 14% acetic acid for 60 min with shaking and repeated till all SOS leaches out. The glyco- part of proteins was oxidizing by incubating the gel in 1 % periodic acid (in 7 % trichloroacetic acid) for 60 min. The excess periodic acid was removed by repeated washing of the gel with 0.5 % sodium metabisulfite solution (in 0.1 N HCI) at room temperature. Initially, the gel will stain with the iodine formed upon the reaction of periodic acid with reducing agent. This amber color will fade soon after formation of iodide in approx. 60 min. Thus treated gels were immersed in Schiffs reagent and placed at 4°C till the clear pink color bands appeared.

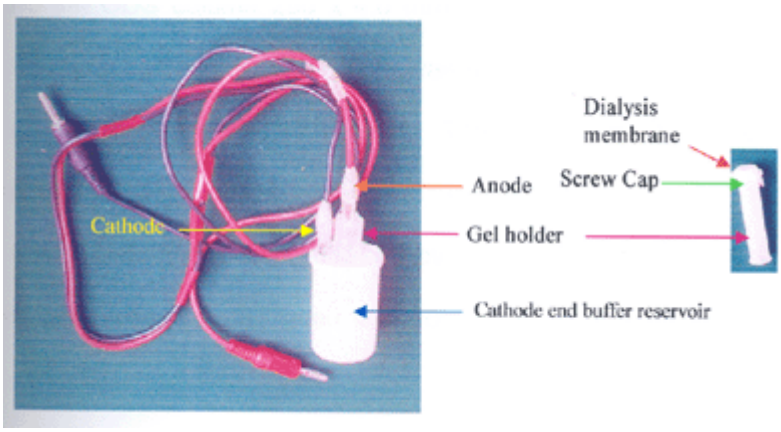
2.7.2.4 Chitosanase and Chitinase staining

After the electrophoresis, the gels were incubated at 37°C for 8h in citrate buffer, pH 6.0. The gels were then stained with 0.1 % Congo Red, followed by immersing in 5 % acetic acid (to enhance the contrast of the gel). Activity bands were observed as clear or reddish areas against bluish background.

2.7.3 Electro elution

This instrument was designed to elute protein from the gels after gel electrophoresis employing the principle of that of electrophoresis. The instrument consisted of a cylindrical gel holder 5cm long, 1 cm inner diameter and a cap that holds dialysis membrane, 30 ml capacity cylindrical butter tank and platinum tipped electrodes.

Protein band of interest was taken out from the gel, located by activity staining or protein staining after gel electrophoresis (SOS-PAGE or Native- PAGE). The gel piece containing interested protein band was placed in the as gel holder fitted with the dialysis membrane and having 5 ml of 2X Tris-HCI 18 Ifiw -buffer, pH 8.8. This was fixed to the lower butter tank having 30 ml of above buffer as shown in the Fig. 2.1. The anode (+) was connected to the gel holder while cathode (-) was connected to the lower butter tank. The DC voltage of 125 -150 V was applied for 45 min under refrigerated condition. The elution was monitored at 280nm and dialyzed against double distilled water.



Fig, 2.1: Protein elution device.

2.8 REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This was carried out to evaluate the purity of β-hexosaminidase isoforms of bell capsicum. Solvent system:

- Solvent A: 0.1 % Trifluoroacetic acid (TFA) in triple distilled and degassed water.
- Solvent B: 0.1 % TFA and 70 % acetonitrile in triple distilled and degassed water.

The triple distilled, 0.2 µ Millipore filtered and degassed water was used throughout the study unless otherwise mentioned. The desalted and lyophilized purified enzyme was dissolved in 0.1 % TFA and analyzed on RP-HPLC, Shimadzu LC-3A. 25 µl (~20 µg) of protein was injected into C-18 (Shimadzu 5 µm ODS column 250 x 4.6 mm) previously equilibrated with solvent A. The gradient run for separation was done by 0% of solvent B to 100 % in 45 min at a flow rate of 1 ml/min. The elution was monitored at 230nm and 0.06 AUFs. At the end of the gradient, the concentration of solvent B was 100 % for 10 min, followed by 0% solvent B in the next 10 min. The column was re-equilibrated with solvents before loading the next sample.

2.9 CIRCULAR DICHROISM

The CD measurements were made on Jasco 180 automated recording spectropolarimeter. The instrument was continuously purged with pure nitrogen gas before and during experiment. Slits were programmed to yield 10A band width at each wavelength. The chart speed, wavelength expansion and the time constant of the instrument were set so as to obtain the best signal to noise ratio and reproducibility of the spectra. The scans were repeated thrice for reproducibility. The spectra for 195 to 260 nm were recorded. The preotein confirmations were done using Yang.jwr software (251).

2.10 ENZYME PROPERTIES

2.10.1 Effect of pH on enzyme activity

The effect of pH on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate was examined at 37°C over a wide range of pH in 100 mM of each buffer (KCI -HCI buffer, 2.0 -3.0; sodium acetate buffer, 3.0 -6.0 and phosphate buffer, 6.0 -7.0). Suitably diluted purified enzyme was added to the reaction mixture containing different buffers of defined pH, 1.25 mM p-nitrophenyl-β-D-N- acetyl glucosaminopyranos- de. Incubation was carried out for 15 min at 37°C and the reaction was stopped with the addition 20 µI of 500 mM sodium bicarbonate to the reaction mixture. The color intensity of the liberated p-nitrophenol was measured at 405 nm.

2.10.2 Effect of temperature on enzyme activity and stability

The effect of temperature on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate was examined at optimum pH of the respective isoforms by carrying out the enzyme reaction at different temperatures (27- 77°C). The purified enzyme was incubated at varying temperatures in a reaction mixture containing 100mM sodium acetate buffer, pH 5.6 for 15 min. The reaction was stopped by the addition 20 µI of 500 mM sodium bicarbonate to the reaction mixture. The color intensity of the liberated p-nitrophenol was .measured at 405 nm.

2.10.3 K_m and V_{max}

The kinetic parameters were determined by incubating the suitably diluted purified enzyme with 100mM sodium acetate buffer (optimum pH of the respective enzymes) and various substrate concentrations from 1 mM to 20mM of p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside. The reaction mixture was incubated for 15 min at optimum temperatures of the respective isoforms. The reaction was stopped by the addition 20 µI of 500 mM sodium bicarbonate to the reaction mixture. The color intensity of the liberated p- nrtrophenol was measured at 405 nm.

2.10.4 Effect of divalent metal ions and EDTA on enzyme activity

The effect of metal ions (CU^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) and EDTA on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside was determined by the amount p- nitrophenol released. The reaction mixture consisted of suitably diluted purified enzyme, 100mM sodium acetate buffer of optimum pH of respective enzymes, 1 mM divalent metal ions and EDTA, and 5mM substrate. The mixture was incubated at optimum temperature for 15 min. The reaction was stopped by the addition 20 µI of 500 mM sodium bicarbonate to the reaction mixture. The color intensity of the liberated p-nitrophenol was measured at 405 nm.

2.11 ANTIBODY FOR α-MANNOSIDASE

Antibody for α-mannosidase was raised in rabbit employing the standard procedure. The purified α-mannosidase enzyme from bell capsicum in 100 mM sodium phosphate buffer, pH 7.2 containing 0.85 % NaGI was used as the antigen. Rabbit was immunized with a primary dose and three booster

doses of -300µg each. The rabbit was administered with the antigen by i.m. Injection along with equal volume of Freud's complete adjuvant at 15-day intervals. The antiserum was separated from the blood 2 days after the fourth injection and subjected to ammonium sulfate fractionation (60%) for immunoglobulin precipitation (252). The precipitated immunoglobulin fraction was dissolved in 100 mM phosphate buffer-saline, pH 7.2 and dialyzed against the same buffer and stored as 100 µl aliquots in eppendorf tubes at -20°C pending their use. Cross reactivity of the antibody was confirmed both by Ouchterlony double diffusion method and antigen- antibody precipitin method (253).

2.12 WESTERN BLOT

After SDS-PAGE, proteins from the polyacrylamide gel were transferred on to nitrocellulose membrane using the method of Towbin et al (254). Initially Whatman No.3 filter paper (16 No.) and nitrocellulose membranes were cut to the size of electrophoretic gel. About 8 filter papers were placed on cathode terminal side of semi dry electrotranfer unit after wetting in transfer buffer, pH 8.3 (48 mM Tris, 39 mM glycine, 20 % methanol and 0.037 % SOS) and air bubbles were removed by gently rolling a glass rod on the filter paper pad. The gel was placed on this, followed by nitrocellulose membrane and the air bubbles were removed as before. The other 8 wetted filter papers were placed one after the other on the nitrocellulose membrane and air bubble if any, was removed as before. The upper lid (anode) of the transfer unit was placed carefully on it and electro transfer of protein from gel to nitrocellulose was carried out at 0.8 mA/cm2 for 45 min. Similarly tissue blot was done to see the cross reactivity of ∝-mannosidase antibody with tissue prints at different stages of fruit ripening in bell capsicum and tomato.

After the transfer, the nitrocellulose membrane was carefully taken out and rinsed twice with TBST buffer, pH 7.4 (20 mM Tris, 0.9 % NaCl, 0.1 % Tween-20 and the pH adjusted to 7.4 with Conc. HCl). The nitrocellulose membrane was incubated with 20 ml 2 % 88A for 60 min at room temperature followed by washing (x3) for 10 min in TSBT buffer. The membrane was incubated with ∝-mannosidase antibody (1: 5000 in T8ST buffer) at room temperature with constant shaking for 60 min, followed by washing (x3) with T88T buffer for 10 min. The membrane was incubated in 20 ml solution of secondary antibody tagged with alkaline phosphatase (1: 10,000) for 60 min with constant shaking. Repeated washings with T88T buffer removed unbound secondary antibody. For the development of color bands, thus treated membranes were immersed in 20 ml solution containing alkaline phosphatase buffer, pH 9.5 (100 mM Tris, 100 mM NaCl, 5 mM bn MgCl2 and the pH adjusted with HCl), 66 µl of N8T (25 mg in 500 µl of 70 % dimethyl formamide) and 33 µl of BCIP (25 mg in 500 µl dimethyl formamide) till the clear band appeared. Further development of bands was arrested by 19 immersing the membrane in 50 ml solution containing 200 µl of 500 mM EDT A, 0.9 % NaCl, pH 8.0 and the membrane was dried.

CHAPTER – III

GLYCOSIDASES PROFILE DURING FRUIT DEVELOPMENT AND RIPENING

INTRODUCTION

Glycosidases are a class of carbohydrate hydrolases that act on short chain oligosaccharides, which may be present in glycoproteins, and on carbohydrate heteromers or homomers, unlike glycanases, which act only on longer chain carbohydrate polymers. Glycosidases, such as β-hexosaminidase and ∝-mannosidase are implicated in signal transduction by way of deglycosilation in crucial biochemical events both in animal and microbial systems (197,201,255). β-Hexosaminidase acts on glycoproteins and glycolipids containing N-acetyl-β-D-glucosaminopyranoside residues in their oligosaccharide moiety, while ∝-mannosidase acts on oligosaccharides containing ∝-D-mannopyranoside. Like, β-Hexosaminidase, ∝-mannosidase also accelerates the glycoprotein and glycolipid degradation (240) and plays a potential role in sperm-egg interaction (242). The deficiency of ∝- mannosidase in animals causes ∝-mannosidosis leading to the accumulation of un-degraded glycoproteins having ∝-mannoside residues in their oligosaccharide part (239).

In animal and microbial systems, both β-hexosaminidase and ∝- mannosidase are shown to be glycoproteins containing N-acetyl-β-D- glucosamine, D-glucosamine and ∝-D-mannoside residues in their oligosaccharide chain (256,198). In plants, jack bean ∝-mannosidase is a glycoprotein containing at least one mannosylated oligosaccharide. In the native enzyme, the oligosaccharide is sterically masked from the interaction with either β-hexosaminidase H or Con-A. Denaturation into subunits permits β-hexosaminidase hydrolysis and removal of oligosaccharide from the glycoprotein enzyme, ∝-mannosidase. The complete removal of the oligosaccharide, however requires joint action of both β-hexosaminidase and ∝-mannosidase.

Free N-glycans are the hydrolysis products of N-glycoproteins released by the action of either β-hexosaminidase or ∝-mannosidase or both, Free N-glycans are shown to exhibit biological activity. For example, the xylomannoside, Man_3(Xyl)GlcNAc(Fuc)GlcNAc is described as a growth factor during the early development of Linum usitatissium (149), the N-glycans Man_3(Xyl)GlcNAc(Fuc)GlcNAc and MansGlcNAc are shown to stimulate tomato fruit ripening (150) and N-glycans prepared from a yeast elicitor extract are found to be suppressors of corresponding glycopeptide elicitors of the stress responses in tomato cells (257). In addition to the biological activities of N-glycans in tomato, there are other reports suggesting the importance of N-glycoconjugates in tomato fruit metabolism. Blocking N-glycosylation with tunicamycin delays fruit ripening, which suggests that N-glycoproteins may be important in the ripening process (258). In humans, free N-glycans are associated with enzyme deficiencies, resulting in physiological disorders (153,154).

In an attempt to determine the potential elements that stimulate the release of N-glycans from N-glycoproteins, we have examined in the current study the profile of β-hexosaminidase and ∝-mannosidase activity at various stages of fruit development and ripening, as they are involved in this process. The interrelation between these two enzymes, both being glycoproteins, has also been studied in their native form as well as in their forms separated into subunits, assuming that the two enzymes become substrate for one another.

A. β-HEXOSAMINIDASE AND ∝-MANNOSIDASE ENZYME ACTIVITIES DURING FRUIT DEVELOPMENT AND RIPENING IN BELL CAPSICUM

Summary: β-Hexosaminidase and ∝-mannosidase are glycosidases that play a major role in fruit softening during ripening. The activity of β- hexosaminidase in bell capsicum increased slightly during fruit development, while it increased significantly during the ripening phase. On the other hand, a-mannosidase showed a prominent activity during fruit development compared to its activity during fruit ripening. β-Hexosaminidase activity was always found to be higher than that of ∝-mannosidase in this fruit system. The specific activities of these two glycosidases in bell capsicum fruits were higher after dialysis, suggesting the presence of dialyzable type of inhibitors for these two enzymes especially during ripening. Ripe fruit showed the highest activity of β-hexosaminidase among different parts of the bell capsicum plant. ∝-Mannosidase from shoot tip showed the highest activity followed by flower, leaf stalk and ripe fruit. Protein profile of the fruit during development stage was different from that during ripening phase showing a sharp demarcation between development and ripening. Also, some of the ripening specific proteins appeared to be glycoproteins. Further, carbohydrate to protein ratio in the aqueous extract obtained from acetone dried powder at different stages of fruit development and ripening was highest at climacteric. A comparison of the ion exchange chromatography profile of β-hexosaminidase and ∝-mannosidase isoforms during development and ripening also indicated ripening specificity of β-hexosaminidase. The CD spectrum analysis of the native form and the subunit form of β-hexosaminidase isoform-I revealed similarity in the secondary structure of the native and subunit form of β-hexosaminidase and difference in the same between subunits of β-hexos-aminidase and ∝-mannosidase.

RESULTS

Activity of various glycosidases in different fruits

Table 3.1 depicts the activities of a few important glycosidases in different fruit systems at their climacteric stage. In bell capsicum, β-hexosaminidase showed maximum activity of 43.6 unit at climacteric stage followed by ∝-galactosidase (28.1 unit) and ∝-mannosidase (20.7 unit), while the activity of β-galactosidase, ∝-glucosidase and β-glucosidase were relatively very less. Tomato showed highest activity for β-hexosaminidase (45.1 unit) followed by ∝-galactosidase (32.5), ∝-mannosidase (27.7) and β-galactosidase (6.7). Banana showed highest activity for ∝-mannosidase (20.7 unit) followed by β-hexosaminidase, while mango showed a little activity of ∝-mannosidase and

negligible activities of other glycosidases screened. Papaya had almost equal activities of ∝-mannosidase and β-galactosidase, followed by ∝- galactosidase and ∝-mannosidase.

Tissue distribution of some important glycosidases in bell capsicum plant

The activity of β-hexosaminidase, ∝-mannosidase, ∝-galactosidase and β-glucosidase enzymes were measured in different parts of the bell capsicum plant collected at different stages of fruit development (Table-3.2). Interestingly, shoot tip and flowers had a significantly higher activity of β-hexosaminidase and ∝-mannosidase enzymes compared to the young fruit, i.e., prior to fruit development. Ripe fruit showed highest activity of hexosaminidase followed by shoot tip and flower. ∝-Mannosidase activity was found highest in shoot tip, followed by flower, leaf stalk and ripe fruit. The highest activity of ∝-galactosidase was noticed in shoot tip followed by leaf stalk, stem, flower and fruit stalk. The activity of β-glucosidase was insignificant in all the tissues tested.

Activity profiles of β-hexosaminidase and ∝-mannosidase in bell capsicum during fruit development and ripening

Fig. 3.1 shows the enzyme activity profiles in bell capsicum at different stages of fruit development and ripening. β-Hexosaminidase activity increased slightly during fruit development, while during ripening there was a more prominent further Increase (Fig. 3.1A). The Increase in enzyme activity was as much as 3-fold by the end of ripening. Activity of ∝-mannosidase on the other hand, increased prominently during fruit development, but did not increase significantly any further during ripening (Fig. 3.1 B). At any point of time, the enzyme activity of (β-hexosaminidase was always found higher than that of ∝-mannosidase in bell capsicum.

Fig. 3.2 presents a comparison of the specific activity of (β-hexosaminidase and ∝-mannosidase enzymes of the dialyzed and undialyzed aqueous extracts of acetone dried powders of bell capsicum. Significantly higher specific activity of (β-hexosaminidase and ∝-mannosidase was observed in dialyzed extracts from ripening fruits as compared to the activity prior to dialysis.

Protein profile of bell capsicum during fruit development and ripening

Fig. 3.3 (A & B) shows the PAGE profile of proteins in bell capsicum during fruit development and ripening. It is clear from the picture that the protein profile during fruit development is different from that of fruit ripening showing a sharp demarcation between development and ripening. Native 80S-PAGE (Fig. 3.3A), revealed only two prominent proteins in k developmental stage (i.e., Band No.1 and 7). Protein corresponding to band No.10, though present at all the stages of fruit development and ripening, it was very prominent.at climacteric stage of ripening. It is striking that band No.7 that prominently increased during fruit development, almost disappeared during subsequent ripening. Proteins corresponding to bands 1- 6 and 8-10 were generally observed to intensify during ripening progression.

Table: 3.1 Activities of important glycosidases in different fruit systems at climacteric stage.

	Bell capsicum	Tomato	Banana	Mango	Papaya
β-Hexosaminidase	43.6	45.1	4.27	1.19	20.7
∝-Mannosidase	20.7	27.8	20.7	5.97	10.3
∝-Galactosidase	28.1	32.6	1.88	1.70	14.3
β-Galactosidase	12.1	17.8	14.7	1.28	23.8
∝-Glucosidase	0.27	0.59	0.96	0.41	0.59
β-Glucosidase	2.71	6.71	2.06	0.73	6.48

Values are enzyme units per g of acetone-dried powder.

Table 3.2; total and specific activity of β-hexosaminidase, ∝-mannosidase, ∝-galactosidase and β-glucosidase in different tissues of bell capsicum

	β-hexosaminidase		∝-manosidase		∝-galactosidase		β-Glucosidase	
Tissue	Total Activity*	Specific Activity♣	Total Activity*	Specific Activity♣	Total activity*	Specific Activity♣	Total activity*	Specific Activity♣
Flower	38.6	0.91	42.2	1.28	11.3	0.34	5.69	0.17
Stem	27.0	1.08	11.2	0.46	12.7	0.52	2.64	0.11
Young furit	20.1	0.73	10.7	0.39	---	---	---	---
Climacteric	49.1	2.70	19.3	1.05	---	---	---	---
Young leaf	22.1	0.51	18.4	0.61	5.21	0.17	3.96	0.13
Old leaf	27.4	0.62	12.0	0.43	4.26	0.15	3.10	0.11
Leaf stalk	25.9	1.06	20.7	0.80	14.8	0.57	3.70	0.14
Fruit stalk	20.6	1.86	2.90	0.14	11.0	0.56	5.15	0.25
Shoot tip	41.6	0.80	47.7	1.27	15.5	0.41	4.68	0.12
Root tip	15.4	0.21	2.26	0.15	0.98	0.06	1.52	0.10

* Total activity per g acetone powder

♣ enzyme activity per mg protein

In denatured SDS-PAGE (Fig. 3.3B), protein bands No.1 and 4, which were not present initially during developmental stage, appeared only in ripening stage. Bands 2 & 3 were very prominent during ripening, and were maximum at post-climacteric stage. Protein bands No.5→9 were faint during fruit development, became more prominent during ripening with maximal level towards the end of ripening. Protein band No.9 in fact corresponds to the enzyme β-hexosaminidase (Isoform-II).

β-Hexosaminidase and ∝-mannosidase isoforms in developmental and ripening stages

β-Hexosaminidase and ∝-mannosidase enzymes resolved into three isoforms each upon subjecting the aqueous extracts of acetone dried powder at the second stage of fruit development and at climacteric stage of bell capsicum to ion exchange chromatography on DEAE Sephadex A-50. These were designated as isoform -I, -II and -III each, based on their protein elution pattern. Fig. 3.4 &

3.5 and Table-3.3 depicts the ion exchange chromatographic profiles of β-hexosaminidase and ∝-mannosidase isoforms in developmental and ripening stage. The specific activity of β-hexosaminidase and ∝-mannosidase isoforms increased significantly from developmental to ripening stage. The specific activity of β-hexosaminidase isoforms was generally higher than those of corresponding ∝-mannosidase isoforms. The carbohydrate / protein ratio in these glycoprotein enzymes was 0.39 for isoform-lll, which was higher compared to other isoforms at

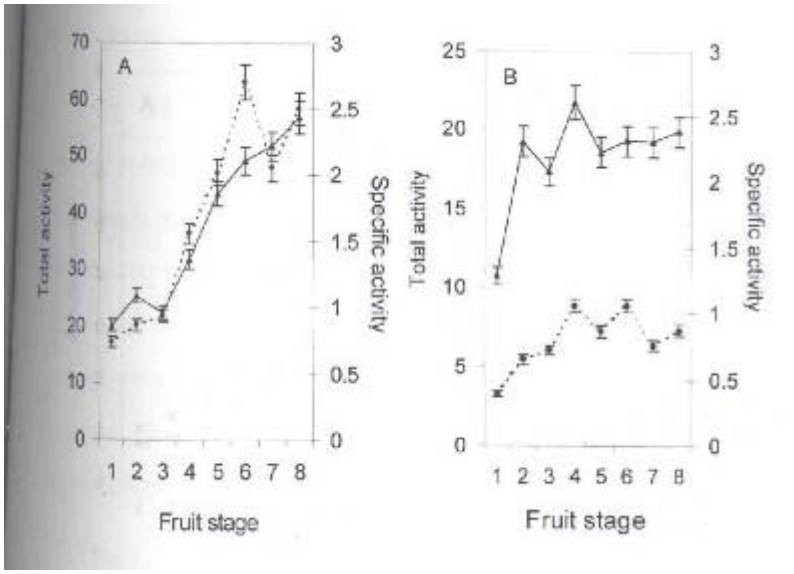


Fig. 3.1 Total and specific activity of β-hexosaminidase (A) and ∝-manno- sidase (8) in bell capsicum at different stages of fruit development and ripening.

1→4: Development stage; 5→8: Ripening stage

(... ▲ ...) Total activity and (-•-) specific activity

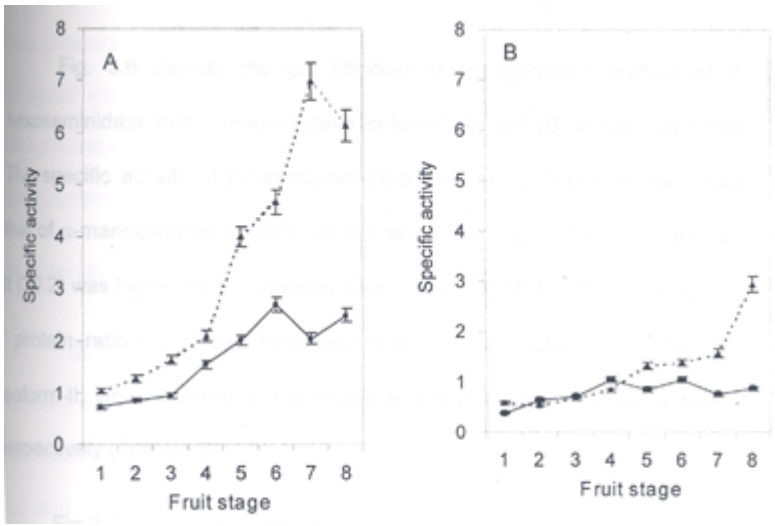


Fig. 3.2 Specific activity of β-hexosaminidase (A) and ∝-mannosidase (B) of bell capsicum at different stages of fruit development and ripening as affected by dialysis.

1→4: Development stages and 5→8: Ripening stages.

(... ▲ ...) specific activity after dialysis

(-●-) specific activity before dialysis.

developmental stage. This ratio was 0.37 for isoform-11 at ripening stage, which was higher compared to other isoforms at this stage of the fruit.

Fig. 3.6 depicts the gel filtration chromatographic profiles of β-hexosaminidase and ∝-mannosidase isoform-I (A) and (B) at ripening stage. The specific activity of β-hexosaminidase isoform I (4.09) was lower than that of ∝-mannosidase isoform I (6.08), while the β-hexosaminidase isoform I (30.2) was higher than a-mannosidase isoform II (15.4). The carbohydrate I protein ratio for β-hexosaminidase was 0.29 for Isoform-I and 0.22 for isoform-II, while 0.95 for ∝-mannosidase isoform-I and 0.37 for isoform-II respectively (Table-3.3).

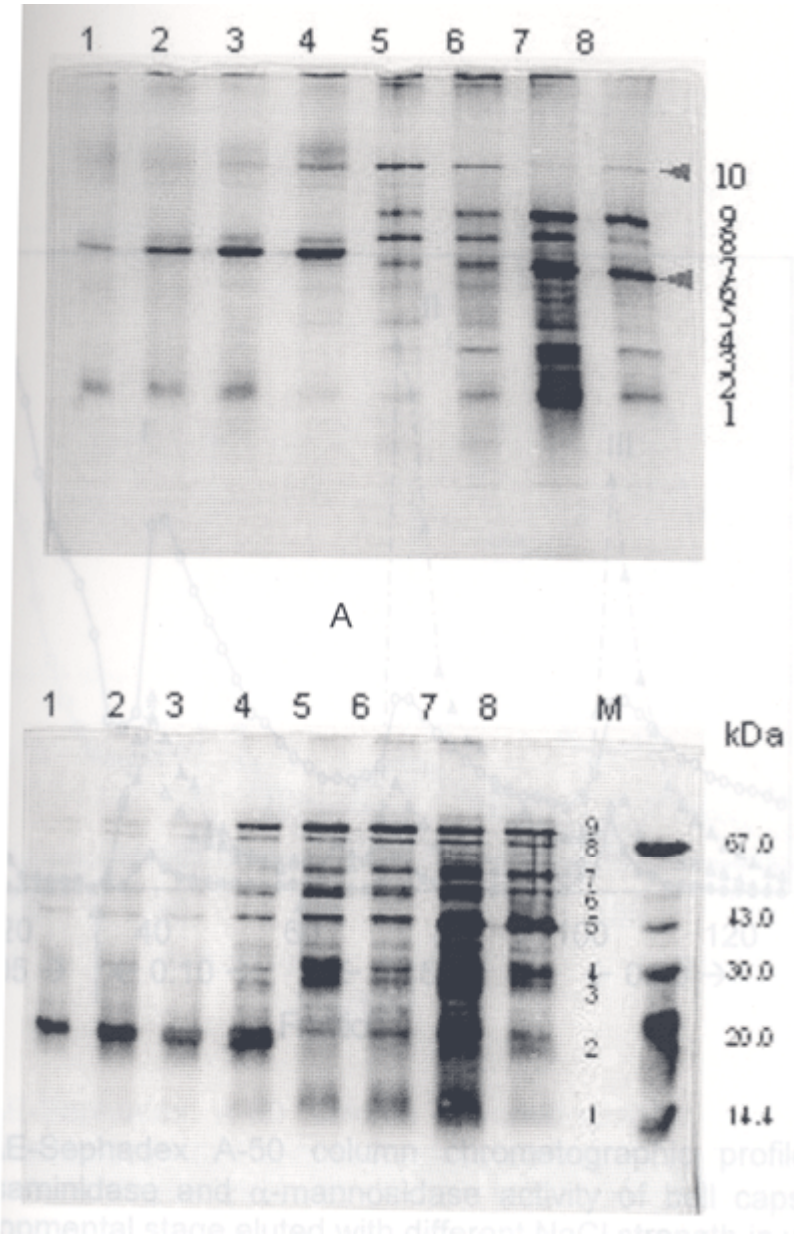
Fig.3.7 shows the ion exchange chromatographic profiles of β-hexosaminidase and ∝-mannosidase isoforms at climacteric stage eluted with sodium acetate buffer, pH 5.6 containing different NaCI strengths. At this acidic condition, β-hexosaminidase and ∝-mannosidase could be separated from one another on DEAE-Sephadex A-50 chromatography. The elution profile of the two enzymes thus differed completely form that obtained with buffer-less NaCI elutants. β-Hexosaminidase isoform-I was not detected under this acidic chromatographic condition.

Glycoprotein patterns in bell capsicum during development and ripening

Fig. 3.8 & 3.9 shows the glycoprotein patterns at different stages of fruit development and ripening in bell capsicum. Glycoprotein bands developed only during ripening stages, while they were very faint in developmental stages (Fig. 6a). When the ratio of carbohydrate to protein was determined in the samples prior to using them for gel electrophoresis (dialyzed extensively and 70% acetone precipitated), it was observed that carbohydrate to protein ratio increased during progression of the fruit from developmental to ripening stages (Fig. 3.9).

Circular dichroism analysis

Fig. 3.10 shows the CD spectrum of the native β-hexosaminidase isofrom-I and its separated subunits having both β-hexosaminidase and ∝-mannosidase activities. The native enzyme showed two negative peaks at 208 and 217nm and a positive peak at 195 nm. The isoform-1 was thus presumed to have 24.8 % ∝-helix, 33.2 % β-pleated, 11 % β-turn and 31% random coil structure. The CD spectra of separated subunit having β-hexosaminidase activity revealed almost similar secondary structure for the enzyme protein, namely, 23.3 % ∝-helix, 37.5 % β-pleated, 10 % β-turn and 29 % random coil. The CD spectra of the separated subunit having ∝-mannosidase revealed one negative peak at 205 nm and one positive peak at 195 nm, suggesting 71 % β-pleated, 11.2 % β-turn and 17.7 % random coil conformation.



Fig, 3.3 Native SDS-PAGE (A) and denatured SDS-PAGE (B) protein profiles of bell capsicum at different stages of fruit development and ripening.

Lane 1, 8-d; lane 2, 16-d; lane 3, 24-d; lane 4, 32-d; lane 5, dark green; lane 6, light green; lane 7, orange green; lane 8, red ripe and lane M, molecular weight markers.

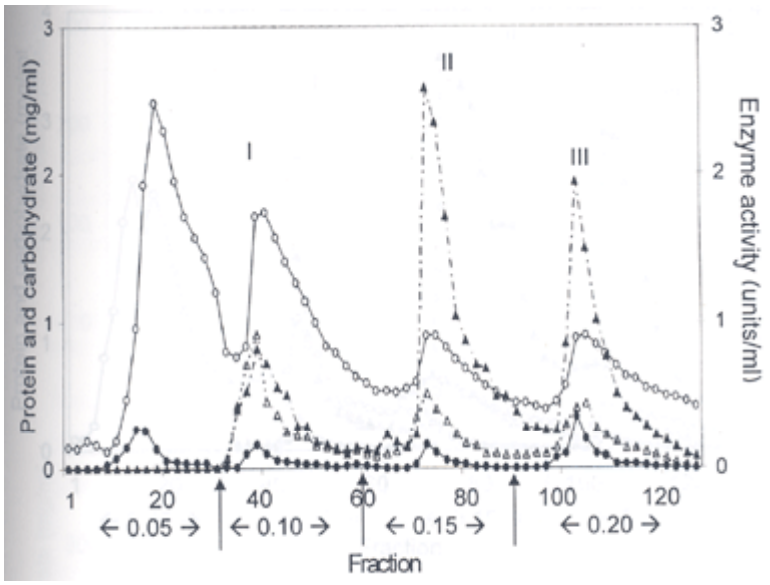


Fig. 3.4 DEAE-Sephadex A-50 column chromatographic profile of β-hexosaminidase and ∝-mannosidase activity of bell capsicum at developmental stage eluted with different NaCl strength in water.

(--△--) ∝-Mannosidase, (-▲-) β-hexosaminidase, (-o-) Protein and (-•-) Carbohydrate.

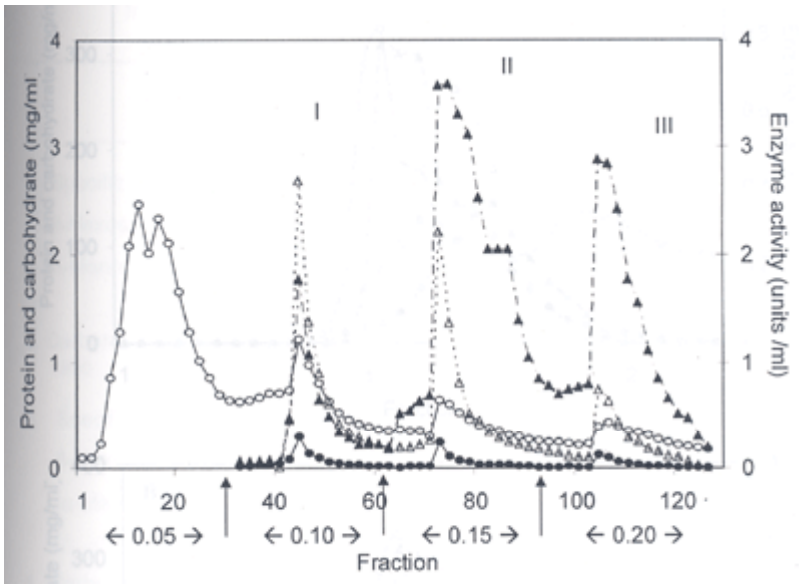


Fig. 3.5 DEAE-Sephadex A-50 column chromatographic profile of β-hexosaminidase and ∝-mannosidase activity of bell capsicum at climacteric stage eluted with different NaCl strength in water

(--△--) ∝-Mannosidase, (-▲-) β-Hexosaminidase,

(-o-) Protein and (-•-) Carbohydrate

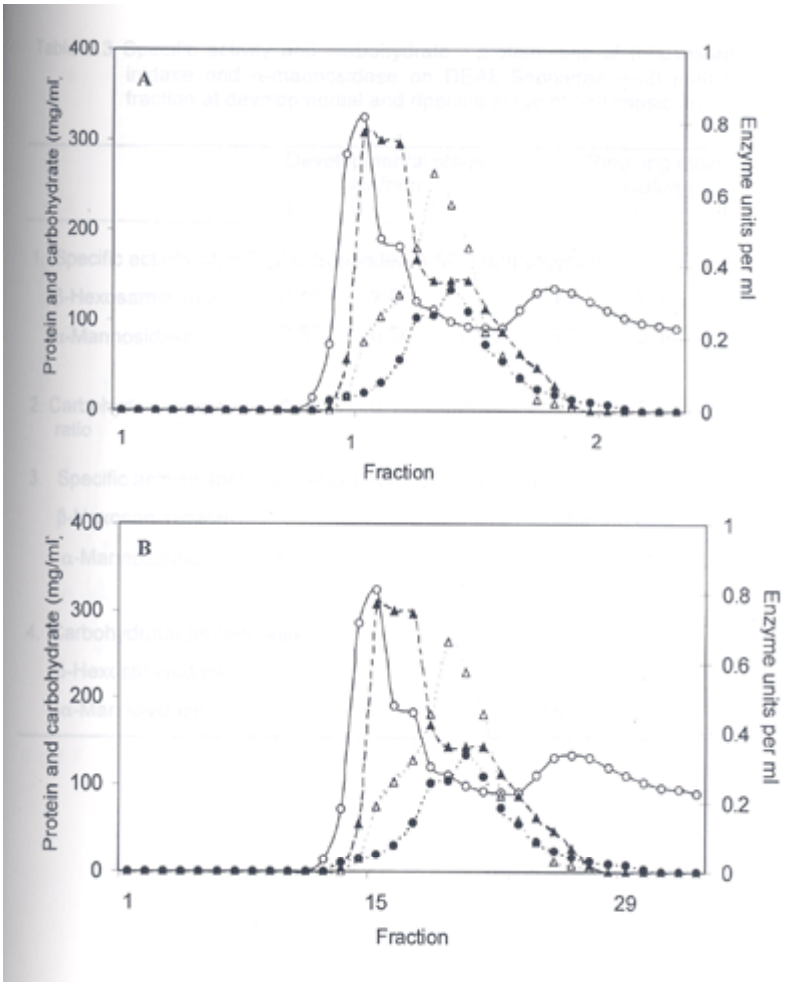


Fig.3.6 Sephadex G-200 column chromatography of β-hexosaminidase isoform-I (A) and isoform-II (B) and ∝-mannosidase activity of bell capsicum at post-climacteric stage
(--△--)∝-mannosidase, (-▲-) β-hexosaminidase, (-o-) Protein and (-•-) Carbohydrate

Table 3.3 Specific activity and carbohydrate / protein ratio of β-hexosam-inidase and ∝-mannosidase on DEAE-Sephadex A-50 purified fraction at developmental and ripening stage of bell capsicum.

	Developmental stage Isoform			Ripening stage Isoform		
	I	II	III	I	II	III
1. Specific activity after dEAE-sephadex A-50 chromatography						
β-Hexosaminidase	0.48	2.85	2.18	1.47	5.60	7.62
∝-mannosidase	0.53	0.56	0.46	2.23	3.44	1.98
2. Carbohydrate / protein ration	0.09	0.56	0.46	2.23	3.44	1.98
3. Specific activity after dEAE-sephadex A-200 chromatography						
β-Hexosaminidase	---	---	---	4.09	30.2	---

∝-mannosidase	---	---	---	6.08	15.4	---
4. Carbohydrate / protein ration						
β-Hexosaminidase	---	---	---	0.29	0.22	---
∝-mannosidase	---	---	---	0.95	0.37	---

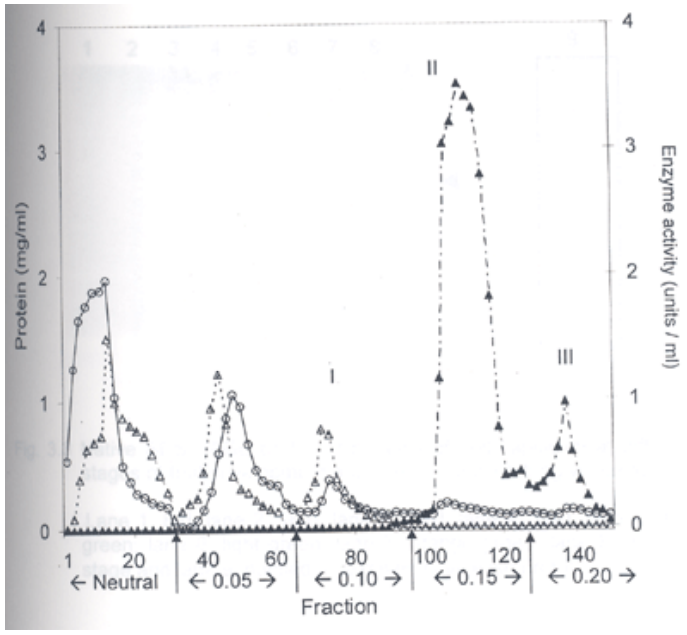


Fig. 3.7 DEAE-Sephadex A-50 column chromatographic profile of β-hexosaminidase and ∝-mannosidase activity of bell capsicum at climacteric stage eluted with 50mM sodium acetate buffer 9ph; 5.6) containing different NaCl stregth

(--△--) ∝- Mannosidase, (-- ▲--) β-Hexosaminidase and (--o--) Protein

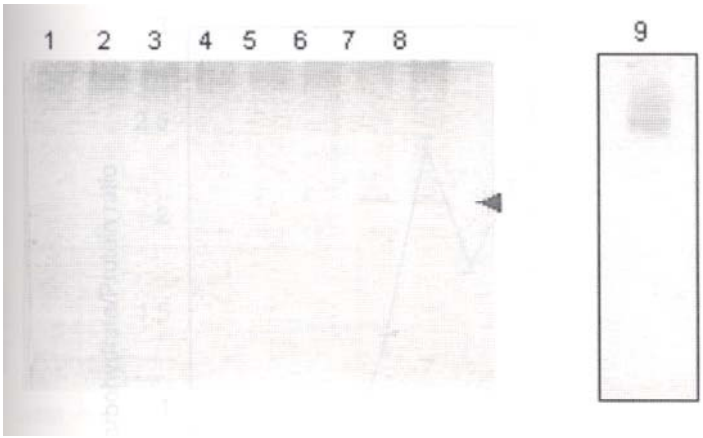


Fig. 3.8 Native SDS-PAGE profile *of* proteins *of* bell capsicum at different stages *of* fruit development and ripening stained for glycoprotein.

Lane 1, 8-d; lane 2, 16-d; lane 3, 24-d; lane 4, 32-d; lane 5, dark green; lane 6, light green; lane 7, orange green; lane 8, red ripe stage and lane 9, purified β-hexosaminidase isoform-II

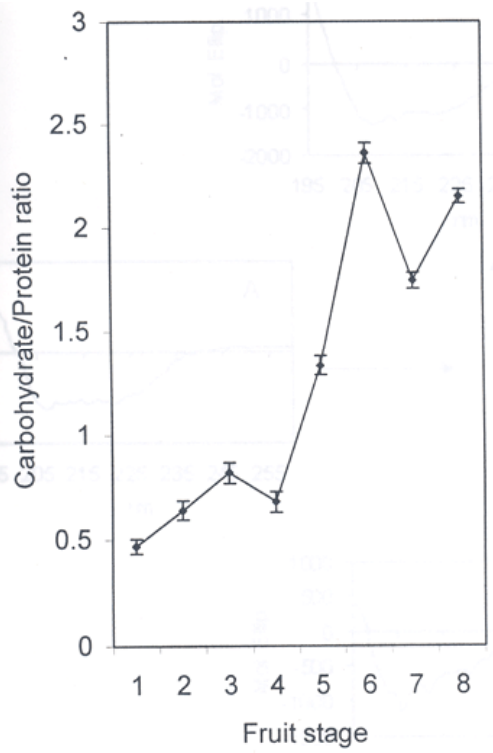


Fig. 3.9 Carbohydrate to protein ratio in bell capsicum at different stages of fruit development and ripening.

1→4: Developmental stages 5→8: Ripening stages

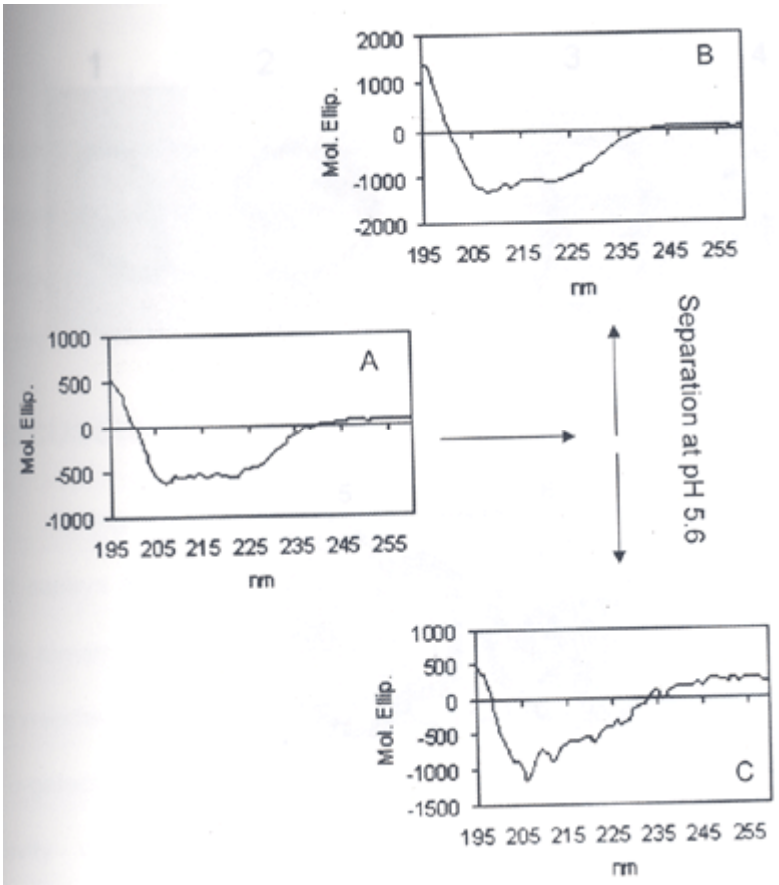


Fig. 3.10 CD spectrum (195-260nm) of purified intact β-hexosaminidase isoform-1 (A), separated β-hexosaminidase isoform-1 (B) and ∝-mannosidase (C) from the same under acidic condition (pH: 5.6).

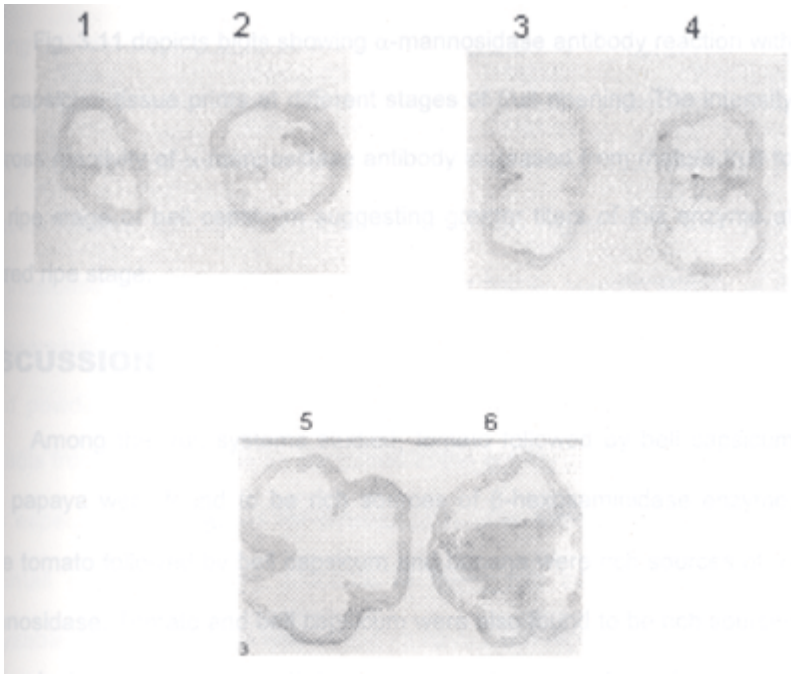


Fig. 3.11 Tissue blots of bell capsicum at different stages of fruit ripening.
1. Dark green stage; 2. Light green stage; 3. Yellow green stage;
4. Climacteric stage; 5. Ripe stage and 6. Red ripe stage.

Tissue blotting and cross reactivity with ∝-mannosidase antibody

Fig. 3.11 depicts blots showing ∝-mannosidase antibody reaction with bell capsicum tissue prints at different stages of fruit ripening. The intensity of cross reactivity of ∝-mannosidase antibody increased from mature fruit to red ripe stage of bell capsicum suggesting greater titers of this enzyme at the red ripe stage.

DISCUSSION

Among the fruit systems studied, tomato followed by bell capsicum and papaya were found to be rich sources β-hexosaminidase enzyme, while tomato followed by bell capsicum and banana were rich sources of ∝-mannosidase. Tomato and bell capsicum were also found to be rich sources of ∝-galactosidase enzyme. Bell capsicum and tomato showed maximum activity of β-hexosaminidase followed by ∝-galactosidase and ∝-mannosidase. The profile of different glycosidases in bell capsicum and tomato seems to be similar as they belong to same family - *Solanaceae.* Banana showed higher activity of ∝-mannosidase compared to mango and papaya. The activity of ∝-mannosidase in bell capsicum and banana are found to be equal but lesser than that found in tomato. Papaya showed higher activities of β-galactosidase and β-hexosaminidase compared to mango and banana. Hence, bell capsicum and tomato, being rich sources of β-hexosaminidase and ∝-mannosidase enzymes, were selected for a detailed study of these enzymes.

The activity of β-hexosaminidase in bell capsicum increased slightly during fruit development, while it increased significantly during the ripening phase. On the other hand, ∝-mannosidase showed a prominent activity during fruit development compared to its activity during fruit ripening. β-Hexosaminidase activity was found to be always higher than that of ∝- mannosidase in this fruit system. Significantly higher specific activity of β-hexosaminidase and ∝-mannosidase in the aqueous extracts of acetone dried powders obtained from bell capsicum fruit was observed in dialyzed extracts from ripening fruits as compared to the activity prior to dialysis. This was especially conspicuous for β-hexosaminidase, indicating the presence of small molecular weight inhibitors of these two enzymes, which are dialyzable. Such dialyzable type inhibitors have been reported for other post- climacteric fruits -mango and apple (260).

The activity of few glycosidase enzymes was measured in different parts of the bell capsicum plant collected at different stages of fruit development. Interestingly, flowers had a significantly higher activity of β-hexosaminidase and ∝-mannosidase compared to the young fruit, i.e., prior to fruit development. Ripe fruit showed highest activity of hexosaminidase followed by shoot tip and flower. ∝-Mannosidase activity was found highest in shoot tip, followed by flower, leaf stalk and ripe fruit. The highest ∝-galactosidase activity was noticed in shoot tip followed by leaf stalk, stem, flower and fruit

stalk, while the activity of β-glucosidase was significantly less in all the tissues compared to other glycosidases tested. β-Hexosaminidase activity in leaves was higher in older ones than in young leaf indicating increased enzyme activity towards senescence, which is similar to the effect of ripening phenomenon. Unlike β-hexosaminidase, ∝-mannosidase activity in fruit stalk, fruit and older leaves was relatively lower compared to shoot tip, flower and young fruits. This suggests that this enzyme activity is present more in actively growing *I* proliferative tissues compared to senescent ones.

β-Hexosaminidase and ∝-mannosidase enzymes of the aqueous extract from acetone dried powder of fruits at the second stage of development and climacteric stage of bell capsicum resolved into three isoforms each on subjecting to ion exchange chromatography on DEAE Sephadex A-50. These were designated as isoform-I, -II and -III based on their protein elution pattern. The specific activity of β-hexosaminidase and ∝-mannosidase isoforms increased significantly from developmental stage to ripening stage. The specific activity of β-hexosaminidase isoforms was generally higher than those of corresponding a-mannosidase isoforms. The carbohydrate *I* protein ratio for these glycoprotein enzymes varied from one isoform to other from 2nd stage of development to climacteric stage and during further purification. Carbohydrate *I* protein ratio of ∝-mannosidase isoform was higher compared to β-hexosaminidase isoforms. The specific activities of β-hexosaminidase and ∝-mannosidase isoforms suggest that isoform II of both are quite stable and have higher specific activity compared to isoform I. It must be noted here that pure carbohydrate polymers decrease during ripening due to higher rate of degradation by various carbohydrate hydrolases (which are active in ripening stage). The higher carbohydrate to protein ratio in fruits evidenced at Post-climacteric stage must hence be due to active synthesis of lysosomal enzymes, which are essentially glycoproteins. This is a common phenomenon in fruits and has also been reported in bell capsicum (259)

A significantly increased activity of β-hexosaminidase and appearance of specific proteins in bell capsicum during ripening process is thus clear from this investigation. Further, a few glycoproteins appear to develop only during ripening. The current observation also suggested that both β-hexosaminidase and ∝-mannosidase are specific to the ripening stage of the fruit. It has been reported that free N-glycans are released from polypeptide chain of glycoproteins by β-hexosaminidase or N-glycanase tomato (150,261). N-Glycans act as a new class of signaling molecules in tomato and cultured cells. Kimura et al. (262) has further reported that such to bioactive free N-glycans Occur in ripening tomato fruits and leaves and are generally absent in stems and roots of tomato plants. In bell capsicum too, the β-hexosaminidase and ∝-mannosidase may release free N-glycans from N-glycoproteins upon their action and stimulate the ripening process.

β-Hexosaminidase in animal and microbial systems (256,198) and ∝- mannosidase in jack bean (234) are reported to be glycoproteins. In bell capsicum, the native β-hexosamrnrdase Isoform-I was separated into two enzymes showing β-hexosaminidase and ∝-mannosidase activity. These enzymes showed differences in their secondary confirmation among them, while the separated β-

hexosaminidase and the native β-hexosaminidase were similar in their secondary structure. Thus, β-hexosaminidase isoform-I seems to behave like jack bean ∝-mannosidase as reported by Bowles et al, (234), where the oligosaccharide moiety of ∝-mannosidase is presumably masked by β-hexosaminidase. Such a close interrelation between β-hexosaminidase and ∝-mannosidase might exist even in this bell capsicum system.

The β-hexosaminidase isoform-II seems to be a glycoprotein in bell capsicum. β-Hexosaminidase and β-mannosidase enzymes may act on each other when they are separated from one another under specific in vivo conditions. The tissue prints of bell capsicum with ∝-mannosidase antibody staining revealed the ripening specificity of these enzymes in this system.

B. β-HEXOSAMINIDASE, ∝- MANNOSIDASE AND ∝- GALACTOSIDA- SIDASE ENZYME ACTIVITIES DURING FRUIT DEVELOPMENT AND RIPENING OF TOMATO

Summary: The activity of β-hexosaminidase and ∝-galactosidase decreased initially during fruit development but later increased significantly during fruit ripening in tomato. On the other hand, activity of ∝-mannosidase, which increased during fruit development, again steadily increased during ripening after an initial decrease. The specific activity of β-hexosaminidase and ∝- mannosidase in tomato were lower in dialyzed aqueous extract than that of un-dialyzed one. On the other hand ∝-galactosidase showed higher specific activity in dialyzed samples. Protein profiles on SDS-PAGE showed differences in the presence of protein bands between fruit developmental and ripening stages. The carbohydrate / protein ratio increased from fruit developmental to ripening stage with highest value at climacteric stage. All the three ∝-mannosidase isoforms in tomato were found to have a common subunit having a molecular mass of 55 kDa, while isoform-II and -III were found to have an additional subunit of molecular mass of 36 kOa. Antibody raised against bell capsicum ∝-mannosidase also cross-reacted with the tomato ∝-mannosidase. The intensity of cross-reaction increased towards ripening stage of the fruit from development stage, showing ripening specificity of this enzyme in tomato fruits.

RESULTS

Activity profiles of β-hexosaminidase, α-mannosidase and α-galactosidase in tomato during fruit development and ripening

Fig. 3.12 shows the enzyme activity profile at different stages of fruit, development and ripening in tomato. The activity of β-hexosaminidase and α-galactosidase decreased initially during fruit development but later increased significantly during fruit ripening with a peak at light green stage of the fruit. On the other hand, activity of α-mannosidase increased during fruit development with peak activity at 2nd and 3rd stage, following which it decreased and again steadily increased towards ripening with a climacteric peak. The specific activity of β-hexosaminidase increased steadily towards ripening with a peak at light green stage, while that of α-galactosidase had a similar pattern but had

peak at the dark green stage. The specific activity of α-mannosidase increased during fruit development with a peak at 3rd stage, further decreased and increased to a maximum during ripening with climacteric peak.

Fig. 3.13 compares specific activity of β-hexosaminidase, ∝-mannosidase and ∝-galactosidase in the dialysed and undialysed aqueous extracts of acetone dried powders obtained from tomato fruit. The specific activity of β-hexosaminidase and α-mannosidase in tomato were lower in dialyzed aqueous extract than that of undialysed one. On the other hand ∝-galactosidase showed higher specific activity in dialyzed samples.

Protein profile of tomato during fruit development and ripening

Fig. 3.14 (a & b) shows the PAGE profile of proteins in tomato during fruit development and ripening. It is clear from the picture that the protein profile did not vary much from the stage of fruit development to fruit ripening. In Fig. 3.14a (Native SDS-PAGE), the protein bands 1, 5 and 7 appeared only during fruit ripening and were not initially present during fruit development. The protein band No.2 was found to be prominent both in fruit development and ripening and was present throughout. The protein band *No.14* seemed to decrease from development to ripening stage.

Protein profile in denatured SDS-PAGE is shown in Fig. 3.14b. Protein band No.1 and 3, which were not present initially during developmental stage, appeared only in ripening stage. Band No.2 was very prominent during fruit development, but diminished during ripening. Protein band *No.11* in fact corresponds to the enzyme ∝-mannosidase (Isoform-I).

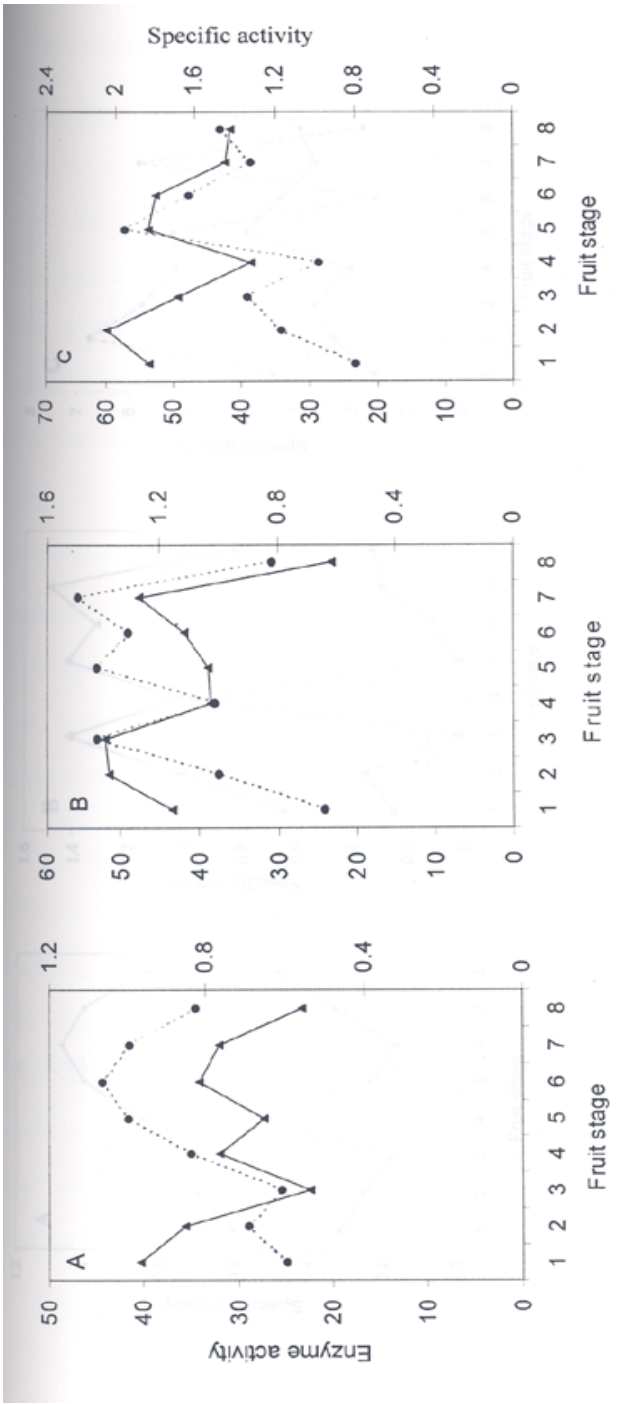


Fig.3.12 total and specific activity of β-hexosaminidase (A), ∝-mannosidase (B) and ∝-galactosidase (C) from tomato at different stages of fruit development and ripening

1→4: development stages and 5→8: ripening stages (--▲--) Total activity and (…•…) Specific activity

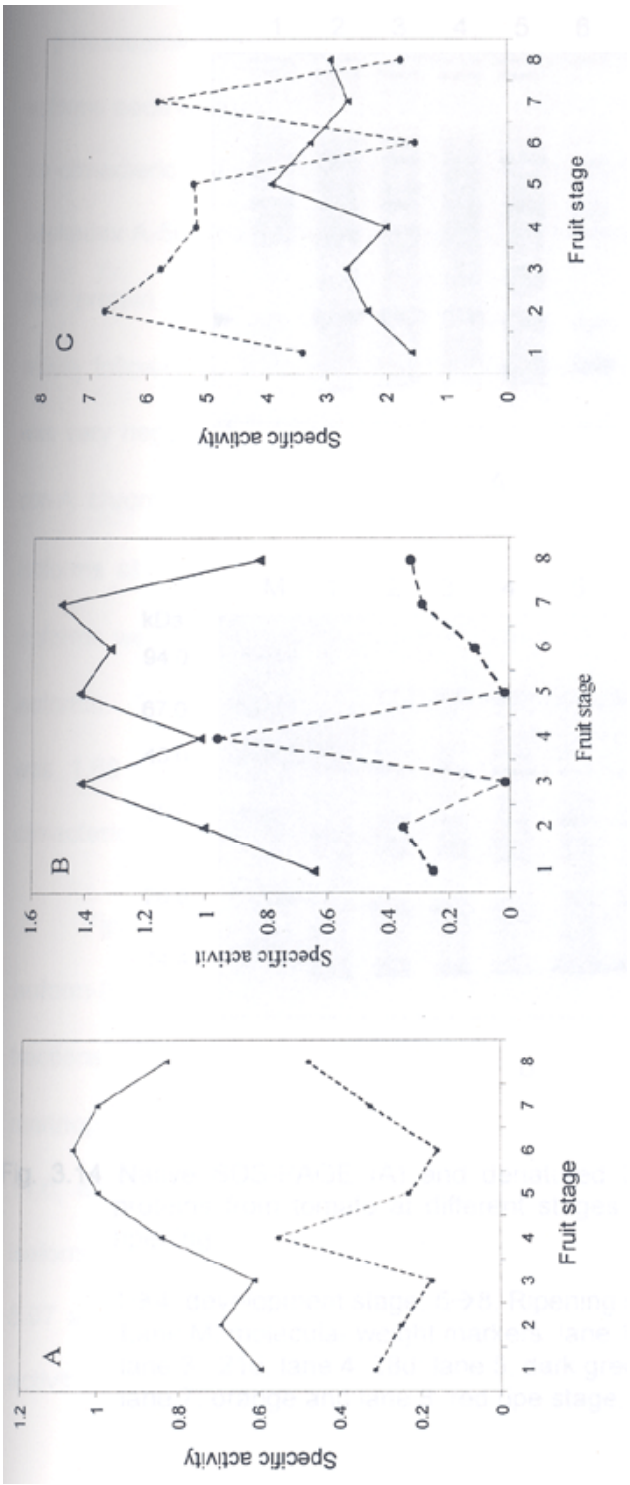


Fig. 3.13: Specific activity of β-hexosaminidase (A), ∝-mannosidase (B) and ∝-galactosidase (C) activity in tomato at different stages of fruit development and ripening as affected by dialysis

1→4: development stages and 5→8: ripening stage

(--▲--) Total activity before dialysis and (…•…) Specific activity after dialysis

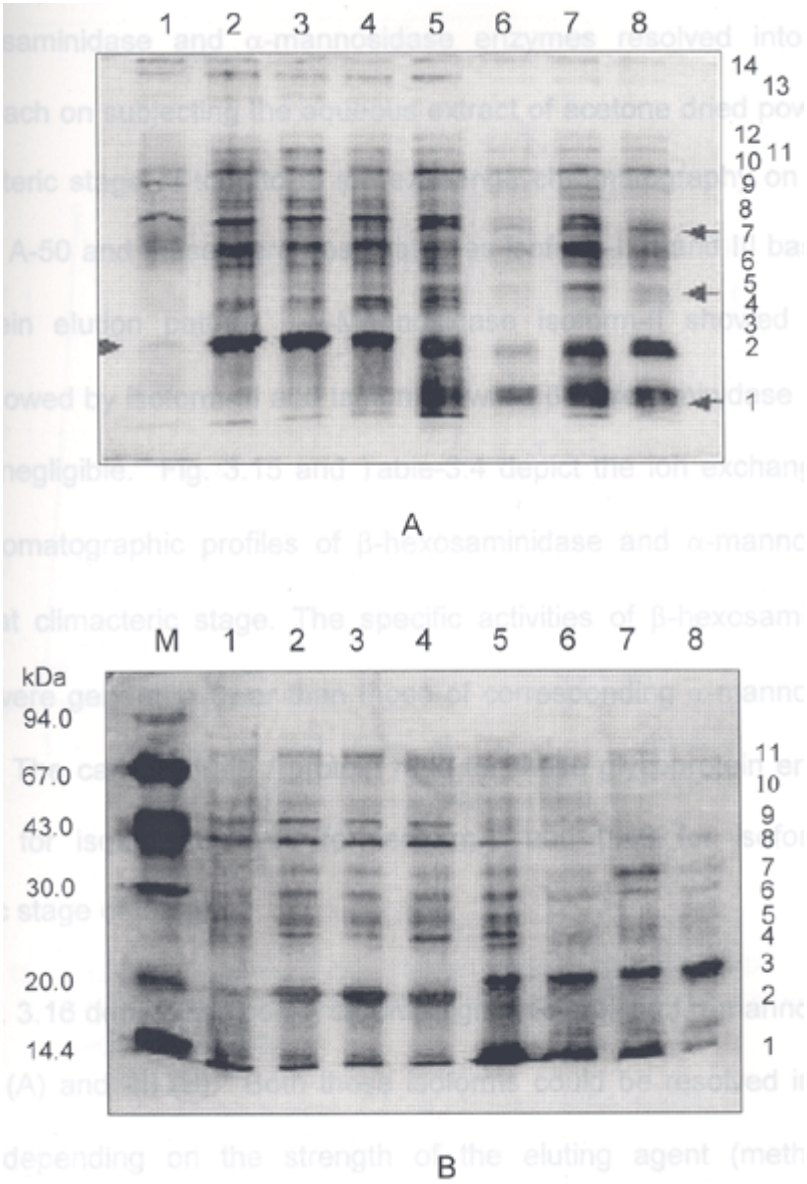


Fig 3.14 Native SDS-PAGE (A) and denatured SDS-PAGE (B) profile of proteins from tomato at different stages of fruit development and ripening

1→4: development stage; 5→8: Ripening stage Lane M, molecular weight markers; lane 1, 7d; lane 2, 14d;

lane 3, 21d; lane 4, 28d; lane 5, dark green; lane 6, light green; lane 7, orange and lane 8, red ripe stage.

β-Hexosaminidase and ∝-mannosidase isoforms

β-Hexosaminidase and ∝-mannosidase enzymes resolved into three isoforms each on subjecting the aqueous extract of acetone dried powder at the climacteric stage of tomato to ion exchange chromatography on DEAE Sephadex A-50 and these were designated as isoform-I, II and III based on their protein elution pattern. ∝-Mannosidase isoform-II showed higher activity followed by isoform-III and Isoform-I, while β-hexosaminidase activity was very negligible. Fig. 3.15 and Table-3.4

depict the ion exchange and con-A chromatographic profiles of β-hexosaminidase and ∝-mannosidase isoforms at climacteric stage. The specific activities of β-hexosaminidase isoforms were generally lower than those of corresponding ∝-mannosidase isoforms. The carbohydrate *I* protein ratio for these glycoprotein enzymes was 1.68 for isoform-lll, 0.69 for isoform-11 and 0.43 for isoform-I at climacteric stage of tomato.

Fig. 3.16 depicts the con-A chromatographic profile of ∝-mannosidase isoform-II (A) and -III (B). Both these isoforms could be resolved into two fractions depending on the strength of the eluting agent (methyl-∝-D- mannopyranoside). The specific activity of isoform-II eluted at 100 mM was 11.6, and higher compared to 300 mM eluted fraction (5.61). While the isoform-III eluted with 100 mM eluting agent showed a specific activity of 8.97 and the one eluted with 300 mM of the eluting agent had a specific activity of 1.42 (Table 3.4).

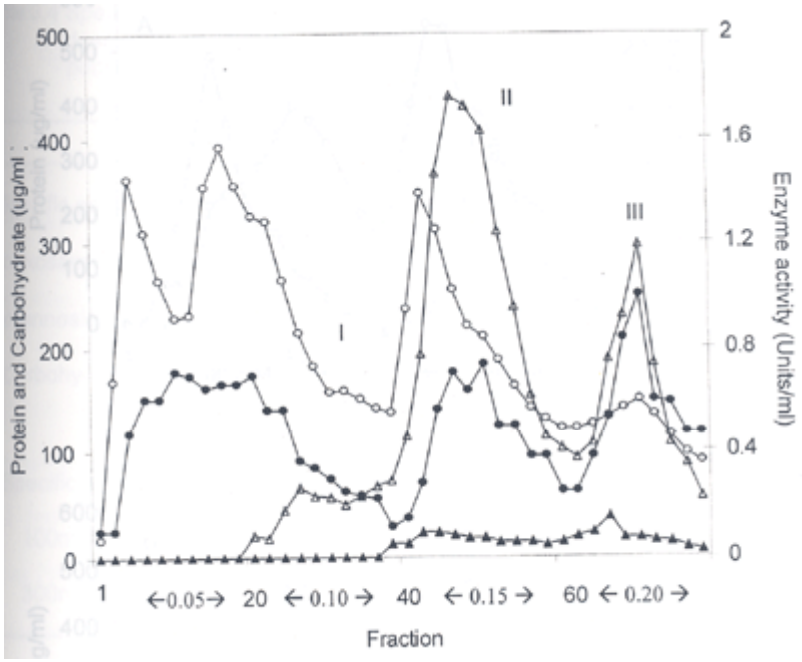


Fig.3.15 DEAE-Sephadex A-50 column chromatographic profile of β-hexosaminidase and ∝-mannosidase at tomato climacteric stage of ripening.

(--△--) ∝-mannosidase, (-▲-) β-hexosaminidase, (-o-) Protein and (-•-) Carbohydrate.

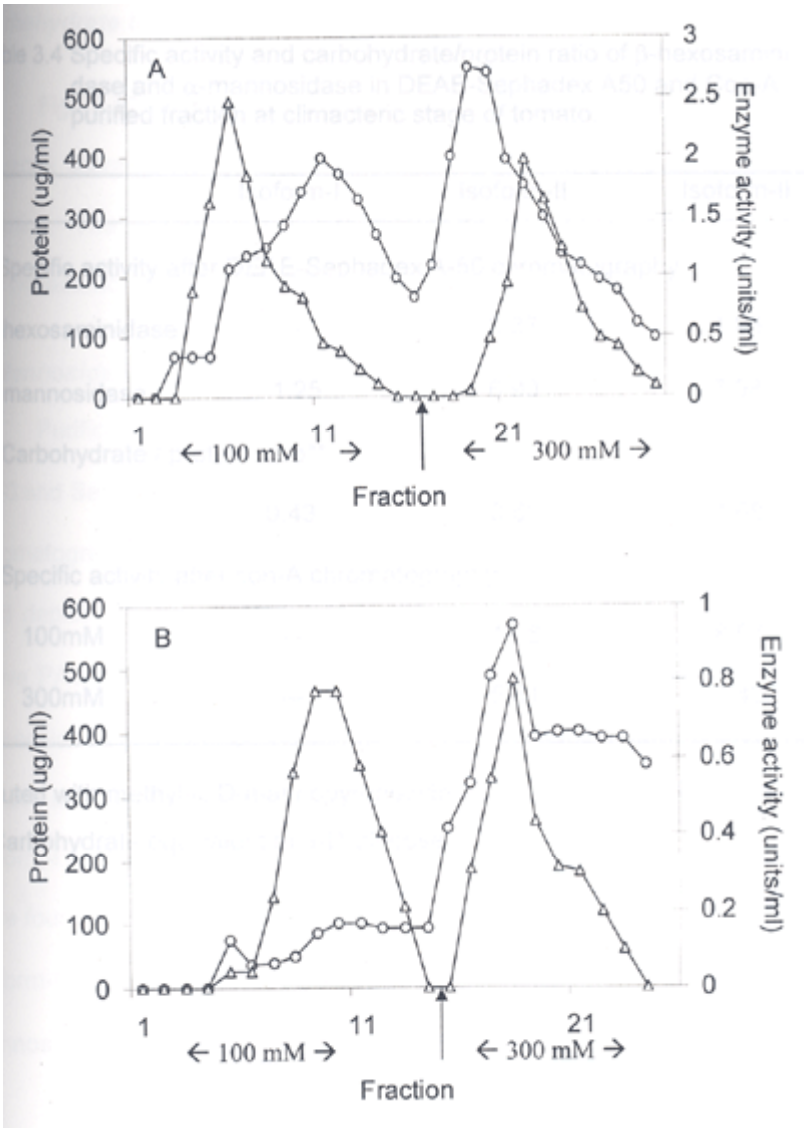


Fig. 3.16 Con -A column chromatographic profile of ∝-mannosidase isoform-II (A) and -III (8) from tomato at climacteric stage of fruit.

(--△--) ∝-Mannosidase: (-o-) Protein

Table 3.4 Specific activity and carbohydrate/protein ratio of β-hexosaminidase and ∝-mannosidase in DEAE-Sephadex A50 and Con-A purified fraction at climacteric stage of tomato.

	Isoform-I	Isoform-II	Isoform-II
1. Specific activity after DEAE-Sephadex A-50 chromatography			
β-hexosaminidase	---	0.37	1.06
∝-mannosidase	1.25	6.90	7.98
2. Carbohydrate / protein ratio**			
	0.43	0.69	1.68
3. Specific activity after con-A chromatography			
100 mM	---	11.6	8.97
300mM	---	5.61	1.42

* Eluted with methyl-∝-D-mannopyronoside.

** Carbohydrate equivalent to ∝-D-glucose.

Carbohydrate to protein ratio

Fig. 3.17 shows the carbohydrate *I* protein ratio of the dialyzed aqueous extracts from the fruit at various stages of development and ripening. The highest carbohydrate to protein ratio (1.85) was observed at the climacteric stage of fruit ripening among different stages.

∝-.Mannosidase purification

Purification of α-mannosidase was carried out using DEAE-Sephadex A-50 and Sephadex G-200 column chromatography followed by either con-A chromatography or electro elution after preparative gel electrophoresis. Fig. 3.18 depicts the native SDS-PAGE (A&B), denatured SDS-PAGE (C) and native PAGE (0) of post-gel permeation (A&B) and electro eluted (B&C) ∝-mannosidase isoforms from tomato. Native isoform-II and -III were shown to have molecular mass greater than 94 kDa on native SDS-PAGE, while the isoform-I had a molecular mass of ca. 55 kDa (Fig. 3.18A). All the isoforms were found to have a common subunit having molecular mass of 55 kDa and isoform-II and III had an additional 36 kDa subunit. All the three ∝-mannosidase isoforms were found to be glycoproteins (Fig. 3.18D).

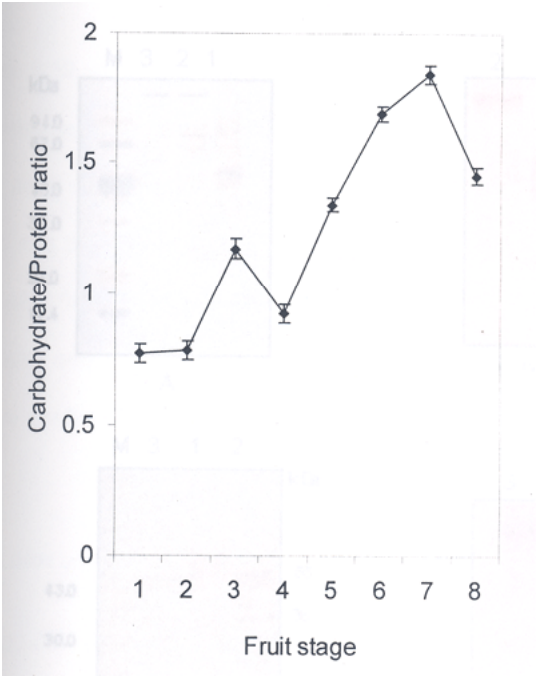


Fig. 3.17 Carbohydrate to protein ratio of dialyzed aqueous extracts from tomato at different stages of fruit development and ripening.

1→4: Development stage; 5→8: Ripening stage.

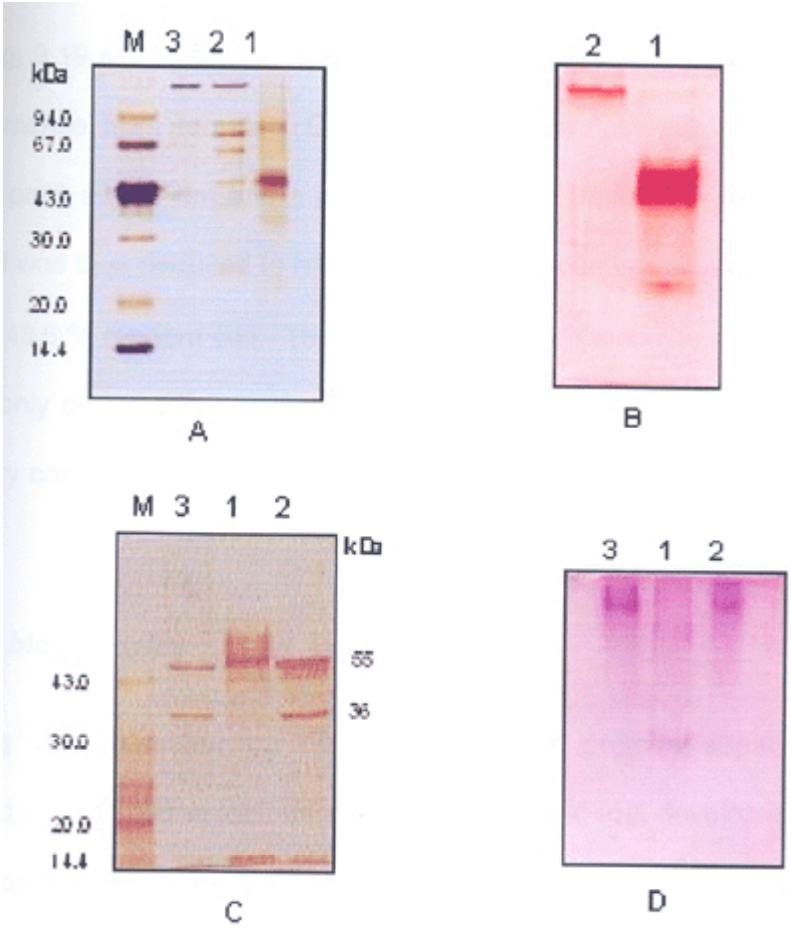


Fig. 3.18 Native SDS-PAGE (A & B), denature SDS-PAGE (C) and native PAGE of post-gel permation (A & D), electroeluted (B & C) ∝-mannosidase isoforms from tomato.
Gels A, B & C is silver stained and D is glycoprotein stain.
Lane M, molecular weight markers; lane 1, isoform-I; lane 2, isoform-II and lane 3, isofrom-III.

Circular dichroism

Fig. 3.19 shows the CD spectrum for con-A eluted and electro eluted ∝-mannosidase from tomato. Con-A eluted ∝-mannosidase showed one negative peak at 197 nm and a positive peak at 210 nm. The con-A eluted isoform-II was thus deduced to have the secondary confirmation of 51.4 % β-turn and 48.6 % random coil. The electro eluted ∝-mannosidase (isoform-II) showed only one positive peak at 197 nm and thus presumed to have the secondary confirmation of 10.1 % ∝-helix, 73.4 % β- pleated and 16.5 % β-turn.

Western blot analysis

Fig. 3.20 presents the Western blot of the proteins separated on denatured SDS-PAGE at various stages of flower and fruit development and. different parts of the plant and cross-reacted with ∝-mannosidase antibody. The aqueous extract from different stages of flower development as well as those of different parts of the plant did not show any cross reactivity to ∝- mannosidase antibody (Fig. 3.20A & B). The aqueous extracts from different stages of fruit development showed cross reactivity with ∝- mannosidase antibody and the intensity of the reaction increased from mature dark green stage to red ripe stage. Tissue blotting study also supported this observation (Fig. 3.21).

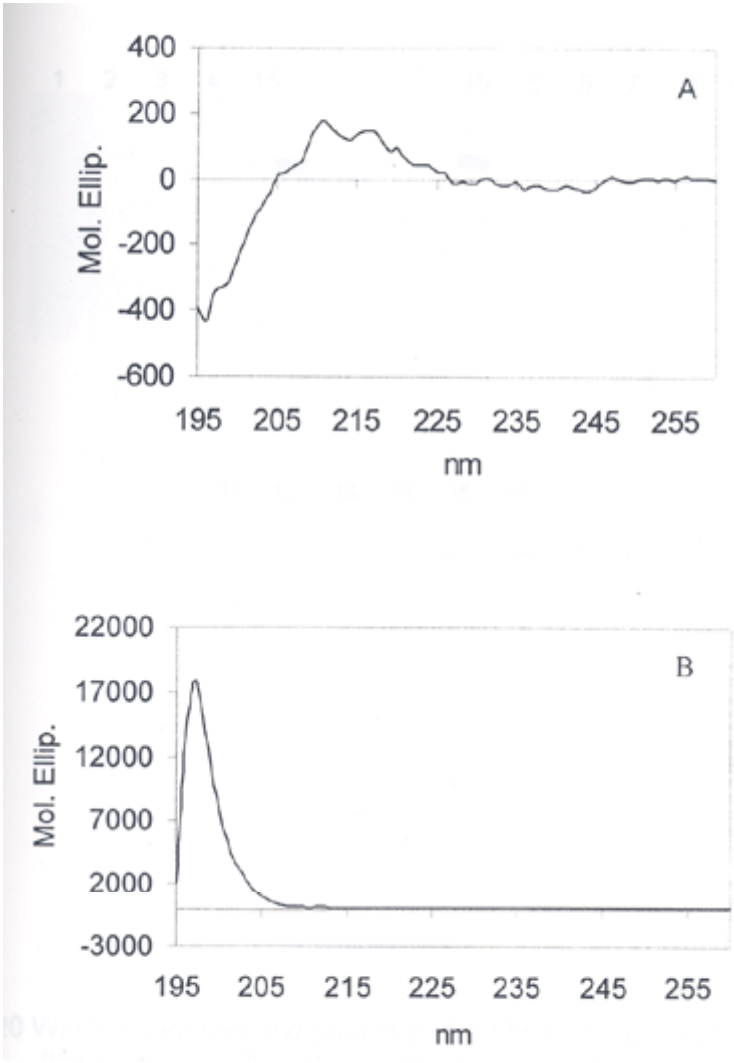


Fig. 3.19 CD spectrum of Con A eluted ∝-mannosidase (A) and electroeluted ∝-manosidase (B) from tomato at climacteric stage.

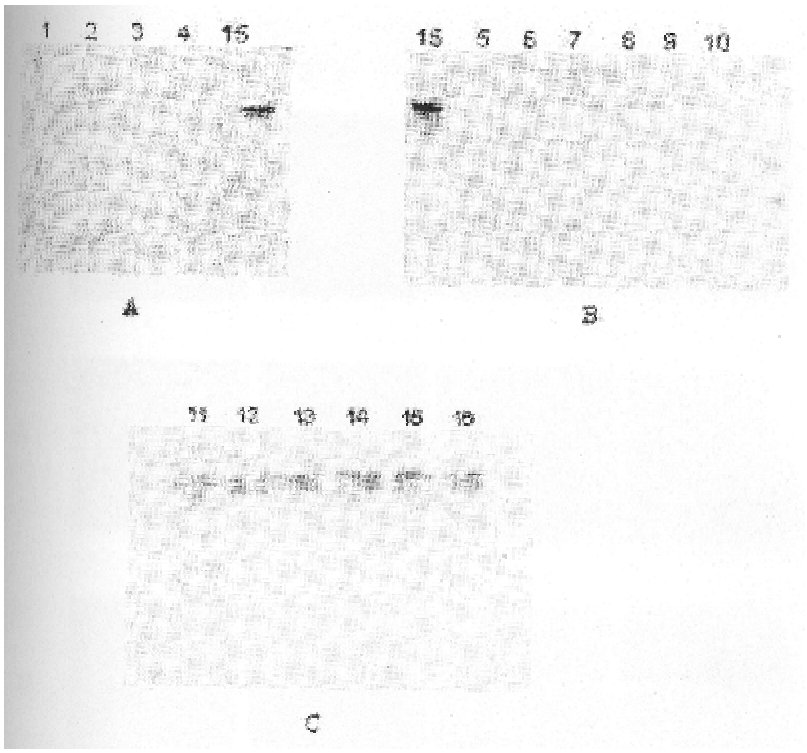


Fig. 3.20 Western blot showing protein profile of tomato at different stages of flower (A), in different tissues (B) and at different stages of fruit ripening (C).
Lane 1, at flower set; lane 2, 5-d after flower set; lane 3, 10-d after flower set, lane 4, 15-d after flower set; lane 5, stem; lane 6, flower; lane 7, root; lane 8, leaves; lane 9, stalk; lane 10, seeds; lane 11, dark green; lane 12, light green; lane 13, yellow green; lane 14, climacteric; lane 15, ripe and lane 16, red ripe stage.

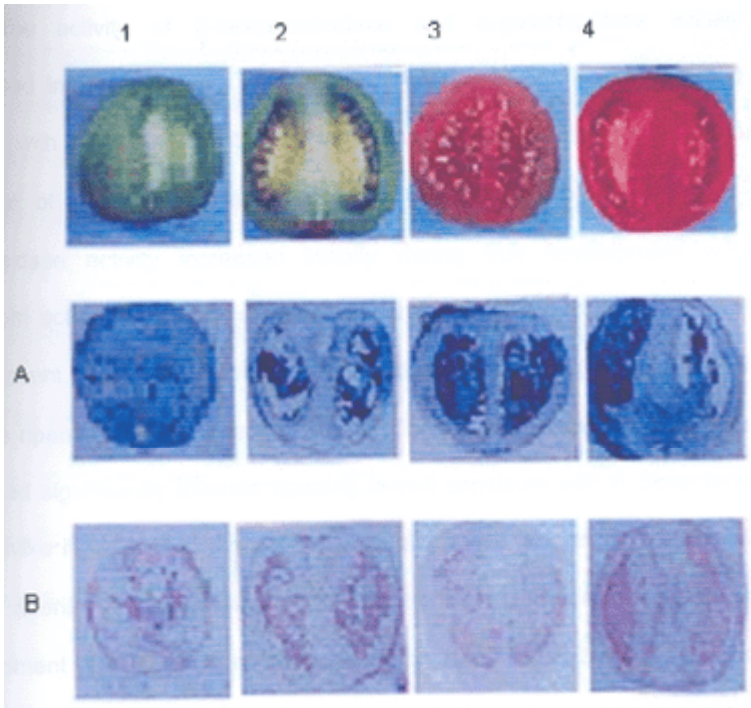


Fig. 3.21 Tissue prints of tomato of different stages of ripening on nitro- cellulose membrane stained for protein (A) and a-mannosidase (B)

Lane 1, dark green stage; lane 2, light green stage; lane 3, ripe I stage and lane 4, red ripe stage.

DISCUSSION

The activity of β-hexosaminidase and ∝-galactosidase initially decreased in tomato during fruit development but later increased during ripening with maximum activity of β-hexosaminidase at the light green stage and that of ∝-galactosidase at the dark green stage of ripening. ∝-Mannosidase activity increased initially during fruit development with maximum activity at the 3rd stage and decreased towards the end of fruit development. Nevertheless, ∝-mannosidase activity again increased towards ripening with a climacteric peak. The activity of β-hexosaminidase increased significantly towards ripening in bell capsicum with a climacteric peak unlike its activity in tomato, where there was a progressive decrease during ripening. The activity of ∝-mannosidase increased during fruit development of bell capsicum with maximum activity at the 3rd stage similar to that of tomato. The activity of the same in bell capsicum remained more or less constant throughout the ripening stage, while in tomato, the activity of ∝-mannosidase, which had fallen at the end of fruit development increased further during ripening to a maximum at climacteric.

The specific activity of all the three glycosidases, viz, β-hexosaminidase, ∝-mannosidase and ∝-galactosidase increased from fruit development to fruit ripening with maximum values corresponding to that of total activities. The specific activity of β-hexosaminidase and ∝-mannosidase enzymes were found to be higher in the dialyzed extracts from acetone dry powders of bell capsicum compared to the activity prior to dialysis, suggesting the presence of small molecular weight inhibitors for these two enzymes especially during ripening. On the other hand, these enzyme activities in tomato were lower in the dialyzed extracts compared to the undialysed ones suggesting the essentiality of one or more small molecular weight activators. The specific activity of ∝-galctosidase was found to be higher in dialyzed samples compared to non-dialyzed ones, similar to β-hexosaminidase and ∝-mannosidase of bell capsicum.

The specific activity of ∝-mannosidase isoforms was higher than that of corresponding β-hexosaminidase in tomato. It has to be noted here that the specific activity of β-hexosaminidase is higher than that of ∝- mannosidase in bell capsicum. β-Hexosaminidase isoform-I, which was eluted with 0.1 M NaCl on ion-exchange chromatography in bell capsicum, could not be observed under similar circumstances in the case of tomato. The carbohydrate / protein ratio was highest for ∝-mannosidase isoform-III of tomato compared to any other isoforms and also the isoforms from that of bell capsicum. Con-A column chromatography has further separated the two isoforms of ∝-mannosidase into two fractions.

The molecular mass data of ∝-mannosidase isoforms derived from SDS-PAGE profiles have revealed that all the three ∝-mannosidase isoforms have a subunit of 55 kDa, while isoform-II and -III

have an additional subunit having molecular mass of 36 kDa. ∝-Mannosidase of cowpea is reported to have a subunit molecular mass of 66 and 44 kDa on SDS-PAGE and has a native molecular mass of 195 kOa (236). ∝-Mannosidase of Capsicum annuum has been reported to have a native molecular mass of 43 kDa and subunit molecular mass of 23 kDa (237).

CD spectrum of Con-A eluted ∝-mannosidase isoform-II and electro-eluted ∝-mannosidase isoform-I revealed the difference in their secondary confirmation, these altered confirmations might have occurred during the course of elution from the preparative gel but did not affect the enzyme activity. This suggests that ∝-mannosidase is active even when it has different secondary confirmations. The antibody raised against bell capsicum ∝-mannosidase cross-reacted with aqueous protein extract of tomato at different stages of fruit ripening but did not react with the aqueous extract from different stages of flower development and from other different tissues of the plant. This suggests that ∝-mannosidase from bell capsicum and tomato has same antigenic properties. The increasing reactivity of ∝-mannosidase antibody towards ripening in tomato revealed the increase in activity of this enzyme towards ripening with maximum intensity at red ripe stage. The reactivity of ∝-mannosidase antibody with tissue prints also confirms this observation. Hence these observations suggest the ripening specificity of ∝-mannosidase in tomato.

Purification and Properties of β-hekosaminidase

INTRODUCTION

β-Hexosaminidase is an important carbohydrate hydrolase classified as glycosidase and it catalyzes the release of free N-glycans from polymeric *I* oligomeric substrates containing hexosamine residues. The enzyme has been studied in several animal and microbial systems, where it is implicated in important physiological events such as, signal transduction, cell division and cell integrity (197,255,263). β-Hexosaminidase occupies a crucial position in signal transduction via deglycosylation of glycoproteins and glycolipids, thus enabling them for complete hydrolysis and further use of the hydrolysis products for synthetic purposes. Any impaired activity of β-hexosaminidase leads to severe physiological impairment resulting in Tay- sach's and Sandhoffs diseases in human beings (204,205) due to accumulation of gangliosides in neurons and in brain. Other important physiological roles have been attributed to β-hexosaminidase in different systems. In mice, β-hexosaminidase has been implied in egg fertilization, where, the enzyme of egg cortical granule blocks polyspermy (210), while that of sperm is required for facilitating the penetration of the sperm into egg (211). In microbial system, it plays an important role in the germination of *Bacillus cereus* spores (213). In humans and mice infected with *Staphylococcus,* β-hexosaminidase of the bacteria inhibits the response of human lympocytes to mitogens and thus interferes with the antibody production (208). The enzyme is also reported to be involved in host-pathogen interactions involving microbe-plant systems (264,255)

β-Hexosaminidase enzyme has been studied in microbial and animal systems, where amino sugars are commonly present (197,263). In higher plant systems, β-hexosaminidase activity has been reported in *Phaseo/us vulgaris* (221) *and Canava/ia ensiformis* (265). The purification, characterization and existence of different molecular forms of this enzyme are reported only in fenugreek seeds (201), Lupinus letteus L seeds (202), cabbage (266), and *Ginkgo biloba* seeds (262).

It has been reported that free N-glycans are released from polypeptide chain of glycoproteins by β-hexosaminidase or N-glycanase in tomato fruits (150,261). N-glycans act as a new class of signaling molecules in tomato and cultured cells (150,261). Kimura et al., (262) have further reported that such bioactive free N-glycans occur in ripening tomato fruits and leaves but are generally absent in stems and roots of the plant. The purification of β-hexosaminidase, its characterization and the role of this enzyme in fruit ripening are not reported in tomato or any other fruit system. Studies in this direction are likely to shed light on this newly identified class of signal molecules and their ways of promoting the fruit-ripening phenomenon. Fruit softening during fruit ripening is an undesirable phenomenon as it directly dictates the shelf life and quality of the ripe fruit. Hence the role of β-hexosaminidase in fruit softening may suggest an alternative to slow down or to arrest the softening through the conventional ethylene biosynthesis pathway.

A. PURIFICATION OF β-HEXOSAMINIDASE FROM BELL CAPSICUM (Capsicum annuum var variata) AND CHARACTERIZATION OF THE ENZYME PROPERTIES

Summary: β-Hexosaminidase from bell capsicum *(Capsicum annuum var. variata)* was resolved into three isoforms upon ion exchange chromatography with step wise gradient (0.10M, 0.15M and 0.20M NaCl) having an abundance of 38, 47 and 15 per cent for isoforms I, II and III respectively. Isoform I and II were further purified on gel permeation chromatography. The pH optimum for these two isoforms was around 5. Isoform II exhibited a higher thermal stability. Hg^{2+} and Zn^{2+} inhibited both the isoforms, but isoform I showed a much higher inhibition by Cu^{2+} also. The Km for isoform I and II with *pnp*-β-*D-N-acetyl* glucosamine pyranoside was 3.00 and 1.75 mM respectively. Isoform II turned out to be a monomer on SDS-PAGE with a relative molecular mass of 85kD. This isoform (the major) appeared to be electrophoretically homogeneous. The molecular masses were 178 and 83.5 kDa for isofrom-I and –II on Sephadex G-200 respectively.

RESULTS

Purification of β-hexosaminidase from bell capsicum

The purification profile of β-hexosaminidase enzyme at various stages of purification is detailed in Table 4.1. The purification profile of the same on DEAE-Sephadex A-50 followed by Sephadex G-200 is depicted in Figure 4.2 and 4.3, respectively. Ion exchange chromatography resolved the β-hexosaminidase activity into three distinct fractions elutable with 0.10M, 0.15M and 0.20M NaCl with a relative abundance of 38, 47 and 15% respectively. Based on the elution profile, they were designated as isoform I, II and III. Further studies were conducted with isoforms I and II, after gel permeation chromatography. The purity of isoforms I and II increased by 9 - and 10-fold respectively with a recovery of 10 and 11 %.

Enzymatic properties of p-hexosaminidase from bell capsicum pH optimum

The effect of pH on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate was examined at 37°C over a range of 2.0 -7.0 (Fig. 4.4). The isoform I and II of the enzyme exhibited maximum activity at pH 4.6 and 5.0 respectively.

Temperature optimum

The effect of temperature on the activity of β-hexosaminidase isoforms is depicted in Fig. 4.5. The effect of temperature on the activity of β-hexos-aminidase isoforms with p-nitrophenyl-β-D-N-acetylglucosamino pyranoside as substrate was examined at the optimum pH of the respective isoforms by carrying out the enzyme reaction at different temperatures (27- 77°C). The isoform I and II of the enzyme showed maximum activity at temperature 37-47°C and 47°C respectively.

Thermal stability

The thermal stability of the β-hexosaminidase isoforms is shown in Fig. 4.6. The thermal stability of the β-hexosaminidase isoforms is examined by incubating the enzymes at varying temperatures (35 -67°C) for 15 min. β-Hexosaminidase II retained 100% of its activity at 57°C for 15 min, while β-hexosaminidase I retained 71 % of the activity under the same condition.

Kinetic constants

The kinetic parameters (Fig. 4.7) were determined by incubating the suitably diluted purified enzyme with 100mM sodium acetate buffer (at the optimum pH of the respective enzymes) and various substrate concentrations from 1-20 mM of p-nitrophenyl-β-D-N-acetyl glucosamino pyranoside. The Km for pnp-β-D-N-acetyl glucosamine pyranoside was 3.00 mM and 1.75 mM for isoforms I and II respectively.

HPLC analysis

Fig. 4.7 depicts the HPLC profile of post-ion exchange and post-gel permeation chromatography purified β-hexosaminidase isoform-1 and postgel permeation chromatography of purified β-hexosaminidase isoform-II from bell capsicum. Isoform-I had a retention time of 16.4 & 17.6 min, while that of isoform-II had 16.8 min on reverse phase HPLC.

Table 4.1 Purification profile of β-hexosaminidase from bell capsicum

Purification steps	Total protein (mg)	Total activity* (EU)	Specific activity (EU/mg protein)	Purification fold	Recovery (%)
1. Crude extraction	256.0	268.9	1.05	1.00	100
2. Ammonium sulfate precipitation (35-70%)					
	136.4	174.5	1.27	1.20	64.9
3. DEAE-Cellulose Chromatography					
Isoform I	14.3	53.5	3.75	3.57	19.9
Isoform II	15.2	43.9	2.88	2.74	16.3
Isoform III	8.0	17.7	2.21	2.10	6.58
4. Gel Permeation Chromatography					
Isoform I	3.01	30.5	10.1	9.62	11.3
Isoform II	2.85	26.6	9.31	8.87	9.87

* 1EU is equivalent to 1μmol p-nitrophenol released min^{-1}

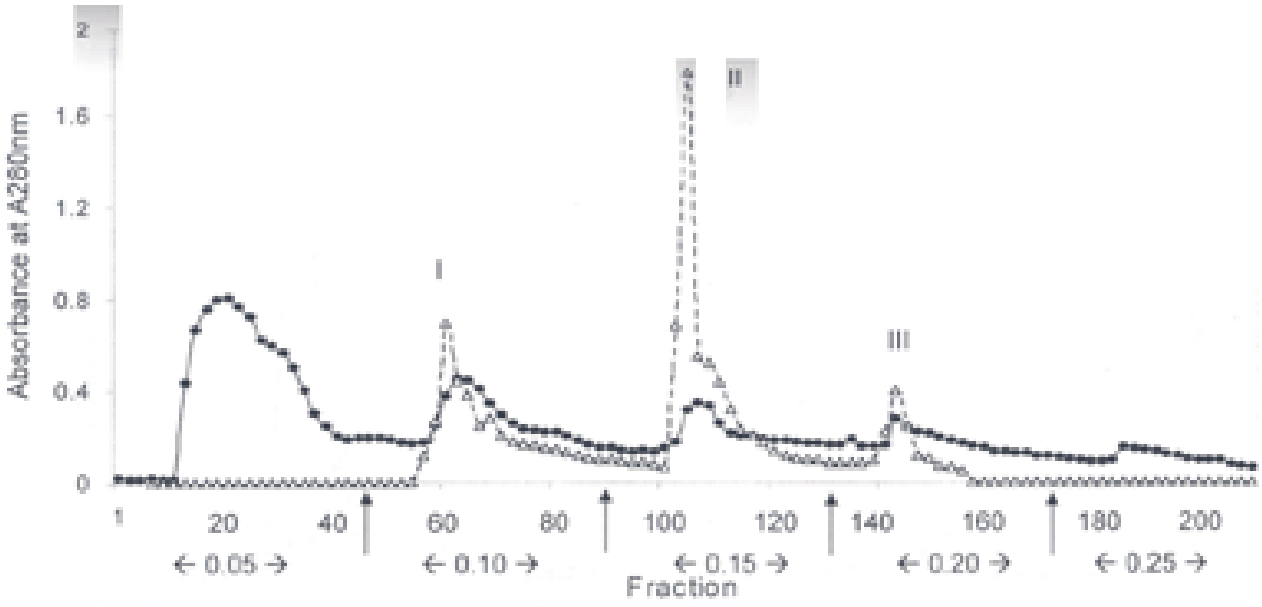


Fig. 4.1 Elution profiles of β-hexosaminidase isoforms from bell capsicum jon DEAE-cellulose.
Column equilibrated with double distilled water and eluted with increasing concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25M NaCl; pHJ 6.8, flow rate 0.66ml min^{-1}. Isoform I, -II and –III eluted with 0.10, 0.15 and 0.20M NaCl, (--•--) Absorbance and (--△--) Enzyme activity.

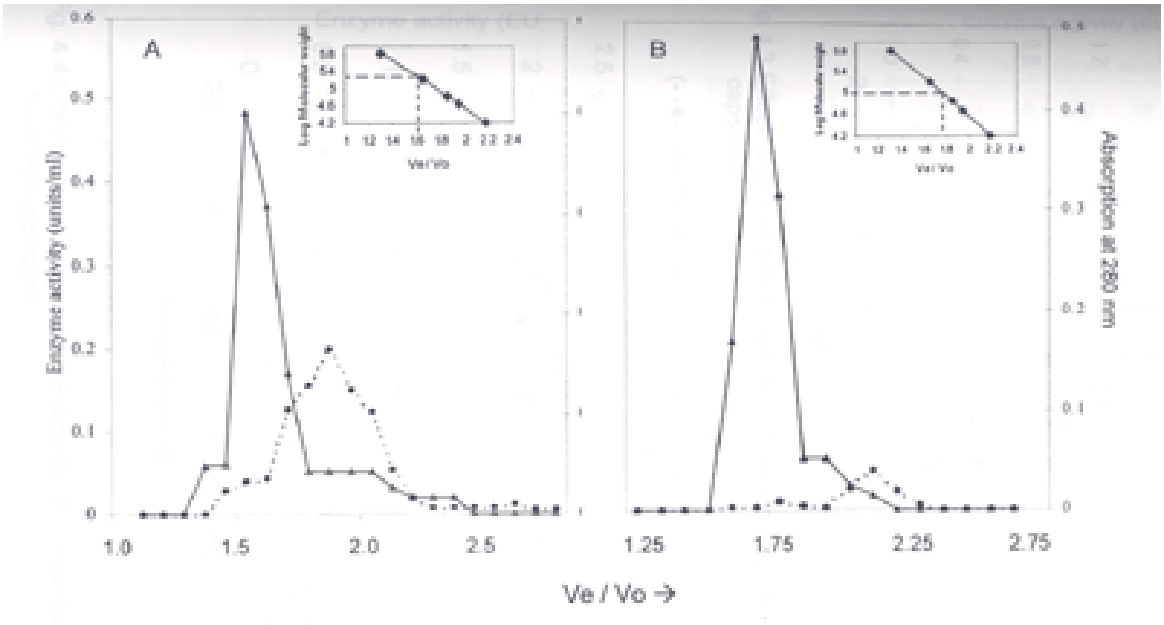
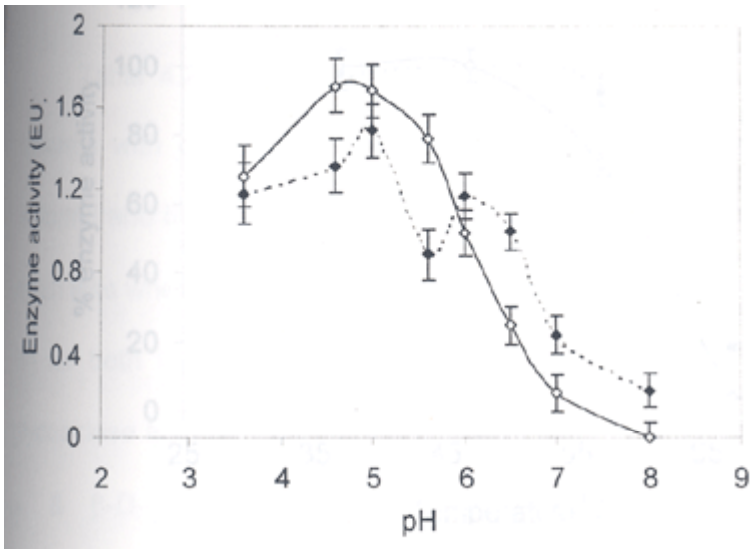
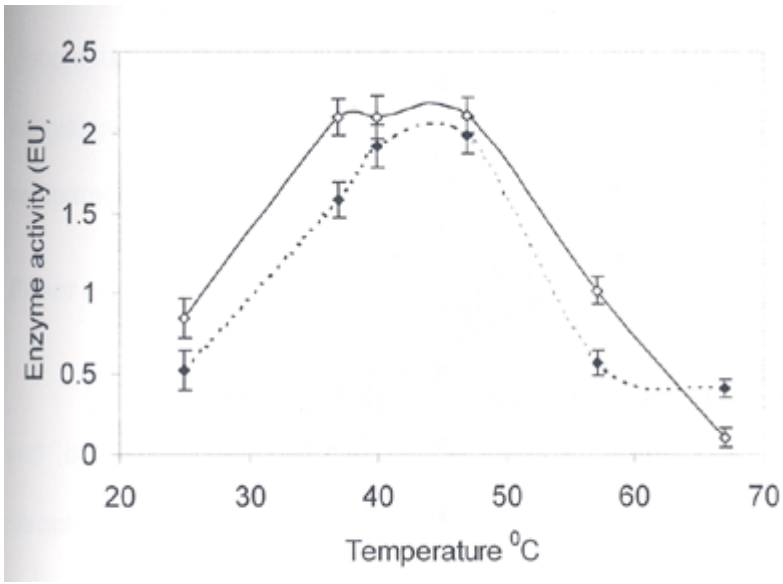


Fig. 4.2 Elution profiles of β-hexosaminidase isoform-I (A) and isoform-II (B) from bell capsicum on Sephadex G-200.
(--•--) Absorbance and (--△--) Enzyme activity.



Fig, 4.3 Effect of pH on the activity of β-hexosaminidase isoforms from bell capsicum.
(--♦--) isoform I and (-◊-) Isoform II



Fig, 4.4 Effect of temperature on the activity of β-hexosaminidase isoforms from bell capsicum
(--♦--) isoform I and (-◊-) Isoform II

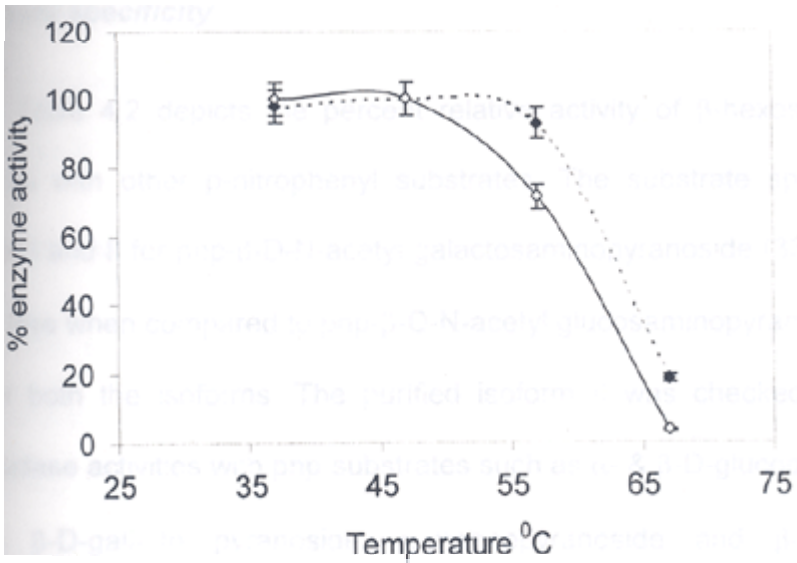


Fig.4.5 Effect of temperature on the stability of β-hexosaminidase isoforms.

(--♦--) isoform I and (-◊-) Isoform II

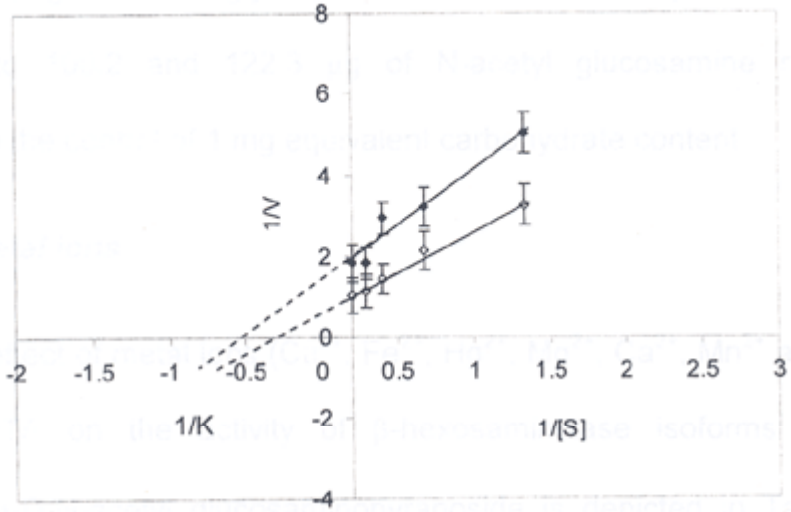


Fig. 4.6 Effect of substrate concentration on the activity of β-hexosaminidase isoforms from bell capsicum.

(--♦--) isoform I and (-◊-) Isoform II

Substrate specificity

Table 4.2 depicts the percent relative activity of β-hexosaminidase isoforms with other p-nitrophenyl substrates. The substrate specificity of isoform I and II for pnp-β-D-N-acetyl galactosaminopyranoside (33.5 %) was much less when compared to pnp-β-D-N-acetyl glucosaminopyranoside (100 %) for both the isoforms. The purified isoform II was checked for other glycosidase activities with pnp-substrates such as ∝- & β-D-glucopyranoside, ∝- & β-D-galacto pyranoside, ∝-mannopyranoside and β-D-N-acetyl galactosaminopyranoside. This enzyme did not show any significant activity with these substrates except for β-D-N-acetyl galactosaminopyranoside. β-Hexosaminidase isoform-II showed activity with natural substrates such as glycoprotein (Fig. 4.9)

and glycolipid isolated from bell capsicum and was equivalent to 100.2 and 122.3 µg of N-acetyl glucosamine reduction compared to the control of 1 mg equivalent carbohydrate content.

Effect of metal ions

The effect of metal ions (CU^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) and of EDTA on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside is depicted in Table 4.3. Among the metal ions tested for inhibitory action, Hg^{2+} and Zn^{2+} showed higher inhibition, and the percent inhibition being more for isoform I compared to isoform II. Also, CU^{2+} showed a high inhibition for isoform I. At 1mM concentration of Hg^{2+}, CU^{2+} and Zn^{2+}, the enzyme showed 3, 9 and 14% residual activity over control for isoform-I, while it was 5, 78 and 38% for isoform II. EOTA at 1mM concentration exhibited 40% more activity for isoform II.

Electrophoretic profile

The electrophoretic profiles (native and denatured SDS-PAGE) for crude and post-ion exchange chromatographic fractions (a & b) are depicted in Fig. 4.8. The purified enzyme (isoform II) on non-denatured SDS-PAGE was homogeneous. (Fig. 4.8c). This isoform showed a single band on SDS-PAGE with a relative molecular mass of 85 kD (Fig. 4.8d) and appeared to be a monomer. Purified p-hexosaminidase was able to act on natural glycoprotein isolated from the neutral fraction of DEAE-Sephadex A-50 chromatography in bell capsicum (Fig. 4.9). All isoforms under acidic conditions exhibited chitinase activity (Fig. 4.10)

Table 4.2 Activity of β-hexosaminidase isoforms with various substrates

Substrates	Reflecting enzymes activity	β-hexosaminidase	
		Isoform – I	Isoform – II
1. pnp-β-D-N-acetyl glucosaminopyranoside	β-hexosaminidase	100	100
2. pnp-β-D-N-acetyl galactosaminopyranoside		33.3	33.5
3. pnp-∝-D-mannopyranoside	∝-Mannosidase	6.3	5.7
4. pnp-∝-D-glucopyranoside	∝-Glucosidase	5	5
5. pnp-β-D-glucopyranoside	β-Glucosidase	5	10
6. pnp-∝-D-galactopyranoside	∝-Galactosidase	8	40
7. pnp-β-D-galactopyranoside	β-Galactosidase	10	9

Value represents activity expressed as relative % activity obtained with pnp-β-D-N-acetyl glucosamino pyranoside

Table 4.3 Effect of divalent metal ions on β-hexosaminidase activity.

	Activity retained (%) ± S.D

Inhibitor (1mM)	Activity retained (%) ± S.D	
Control	Isoform I 100.0 ±0.19	Isoform II 100.0 ± 0.30
CU2+	9.0±0.34	78.0±0.51
Fe2+	90.2 ± 0.54	119.4 ± 0.63
Hg2+	2.8±0.30	5.2±0.31
Mg2+	109.5 ± 0.48	67.3 ± 0.40
Ca2+	110.8 ± 0.86	84.0 ± 0.36
Mn2+	86.6 ± 0.81	101.4 ± 0.61
Zn2+	14.6± 0.33	38.7 ± 0.25
EDTA	115.2 ± 0.88	139.9 ± 0.81

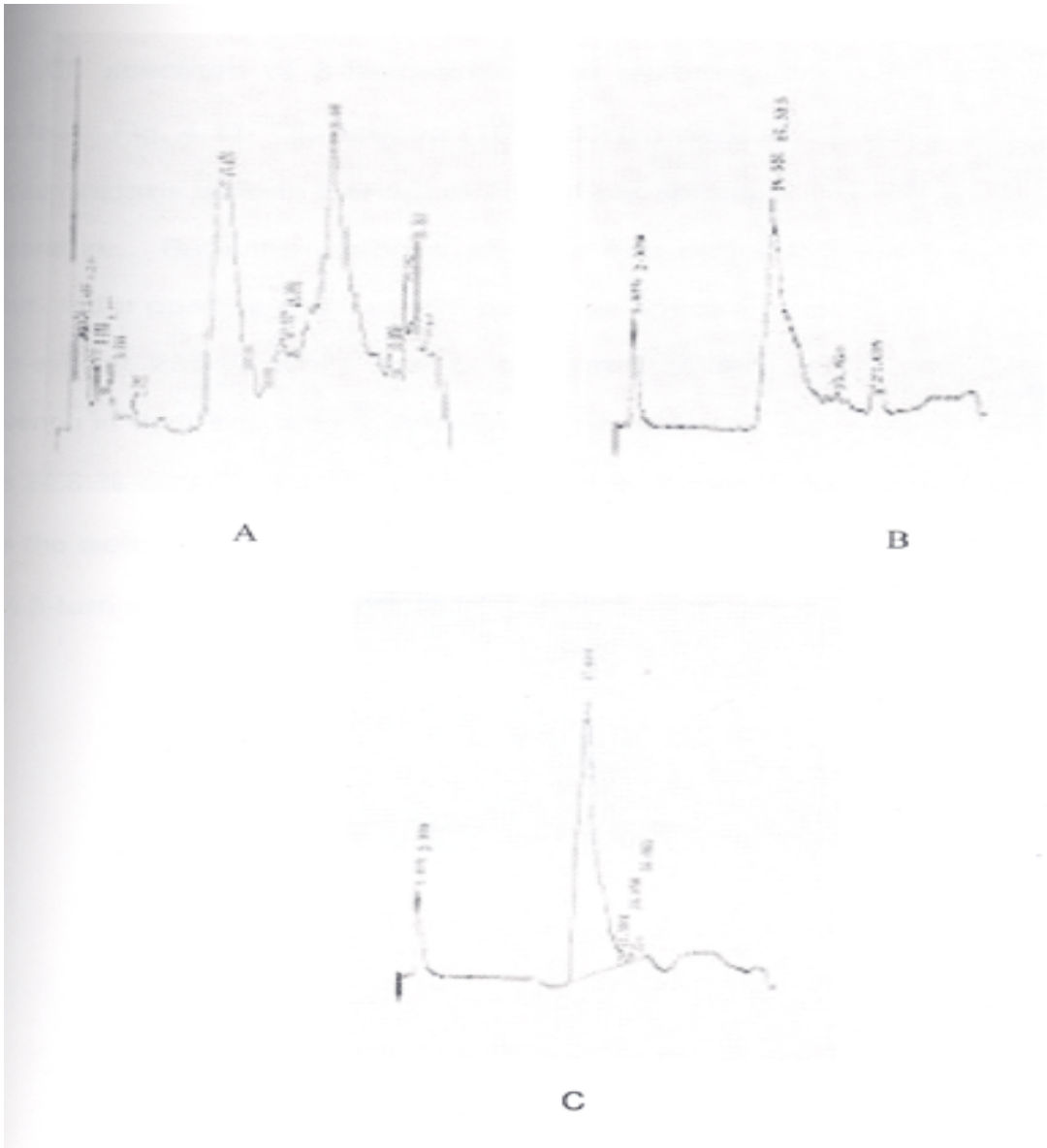


Fig. 4.7 HPLC profiles of β-hexosaminidase isoforms from bell capsicum.

A) Post ion exchange isoform II, B) Post gel permeation isoform II and C) Post gel permeation isoform I

Circular dichroism analysis

CD spectrum of β-hexosaminidase isoforms from bell capsicum is depicted in Fig. 4.11. CD spectra (195-260nm) were recorded for purified β-hexosaminidase isoform I and II in 25mM phosphate buffer, pH 6.0 at room temperature. Both the isoforms showed two negative peaks at 208 and 217nm and a positive peak at 195 nm. The spectra of isoform-I intersected the x-axis at 237nm while that of isoform-II at 241 nm indicating a slight difference in their secondary structure. The isoform-I was thus presumed to have 24.8 % a-helix, 33.2 % β-pleated, 11 % β-turn and 31 % random coil, while the isoform-II was deduced to contain 24 % ∝-helix, 41.8 % β-pleated, 6.4 % β-turn and 28 % random coil.

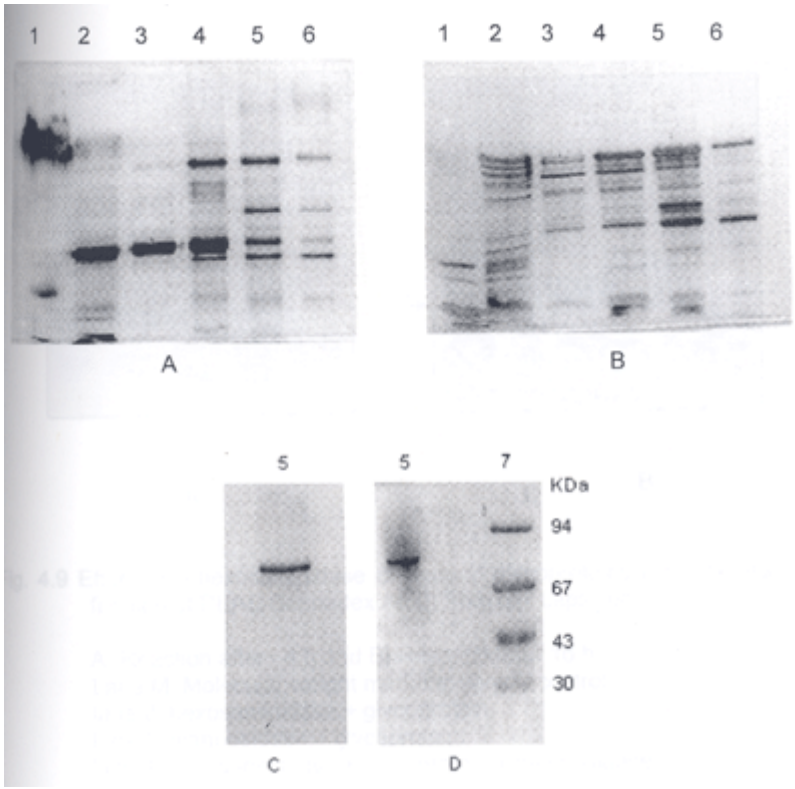


Fig. 4.8 Native (A & G) and SDS-PAGE (B & D) of β-hexosaminidase isoforms from bell capsicum.

Lane 1, crude; 2, unbound fraction; 3, O.O5M NaGI eluted; 4, Isoform I; 5, Isoform II; 6, Isoform III, and 7, molecular mass standards

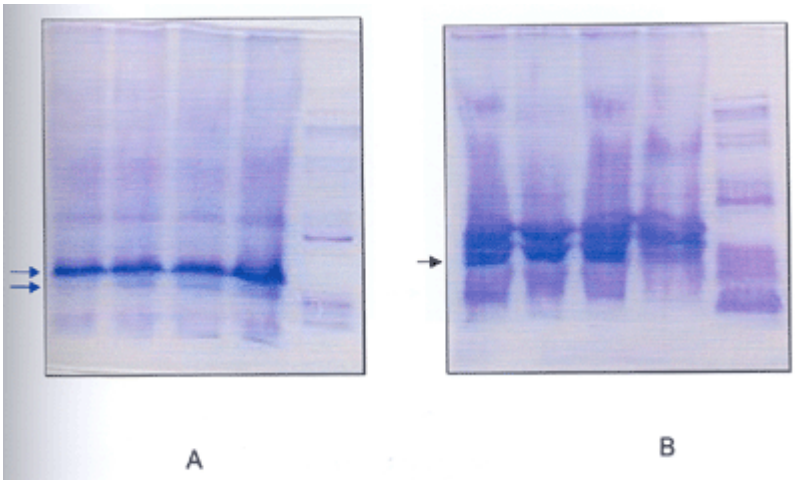


Fig. 4.9 Effect of β-hexosaminidase on natural glycoproteins (from neutral fraction of DEAE-Sephadex A-50) from bell capsicum.

A) Reaction after 4 h and B) Reaction after 16 h.

Lane M, Molecular weight markers; lane1, control; lane 2, hexosaminidase + glycoprotein; lane 3, mannosidase + glycoprotein; lane 4, hexosaminidase + glycoprotein + mannosidase

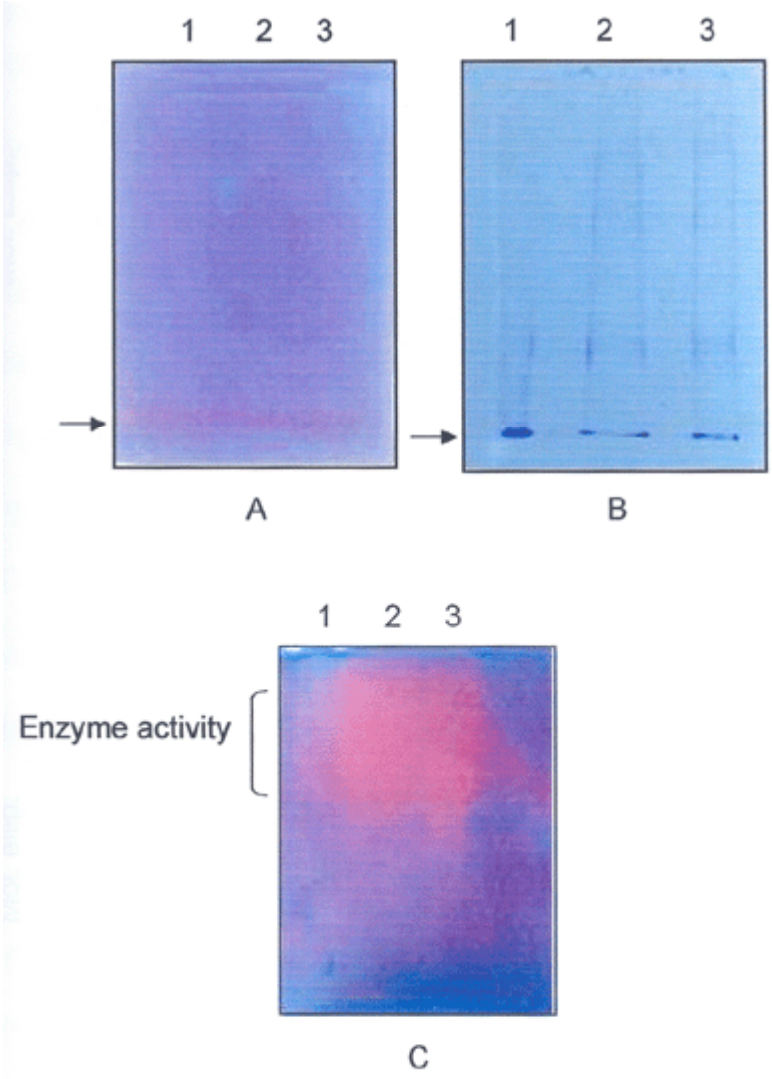


Fig. 4.10 Substrate incorporated acidic (A & B) and basic (C) gel electrophoresis for acivity of β-hexosaminidase isoforms on PAGE.

A) Chitosan incorporated acidic gel, B) Protein stained acid gel and C) glycol-chitin incorporated basic gel.

Lane 1, β-hexosaminidase isoform-I; lane 2, β-hexosaminidase .isoform-II and lane 3, β-hexosaminidase isoform-III

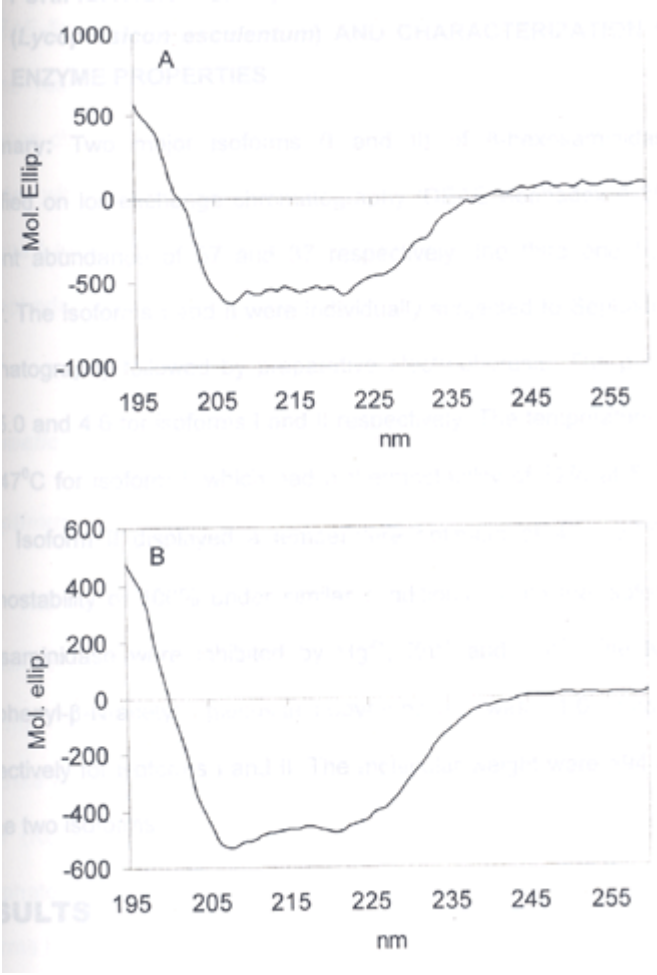


Fig.4.11 CD spectrum of β-hexosaminidase isoform-I (A) and isoform – II (B) from bell capsicum

B. PURIFICATION OF β-HEXOSAMINIDASE FROM TOMATO *(*Lycopersicon esculentum*)* AND CHARACTERIZATION OF THE ENZYME PROPERTIES

Summary: Two major isoforms (I and II) of β-hexosaminidase were identified on ion-exchange chromatography (DEAE Sephadex A-50) with a percent abundance of 57 and 37 respectively, the third one being very minor. The isoforms I and II were individually subjected to Sephadex G-200 chromatography followed by preparative electrophoresis. The pH optimum was 5.0 and 4.6 for isoforms I and II respectively. The temperature optimum was 47°C for isoform I, which had a thermostability of 72% at 57°C for 15 min. Isoform II displayed a temperature optimum of 47 -57°C, with a thermostability of 100% under similar conditions. Both the isoforms of β- hexosaminidase were inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+}. The Km for p-nitrophenyl-β-N-acetyl glucosaminopyranoside was 1.6 and 1.1 mM respectively for isoforms I and II. The molecular weight were >94 & 64 kDa for the two isoforms.

RESULTS

Purification β-hexosaminidase from tomato

β-Hexosaminidase of tomato at climacteric stage was purified by ion- exchange chromatography on DEAE Sephadex A-50, followed by gel permeation chromatography on Sephadex G-200. The specific activity of the enzyme increased by almost 8-fold during these purification steps with a percent recovery of 41 % (Table 4.3). The purification profile of the enzyme on DEAE-Sephadex-A50 is depicted in Fig. 4.12. The β-hexosaminidase enzyme resolved into three distinct isoforms (I, II and III) by this ion- exchange chromatography upon stepwise elution with 0.05 -0.2M sodium chloride, with relative abundance of 57, 35 and 8% respectively. The isoforms I and II were further subjected to gel permeation chromatography on Sephadex G-200 and the elution profile is presented in Fig.4.13A and Enzymatic properties of β-hexosaminidase from tomato

PH optimum

The effect of pH on β-hexosaminidase activity for isoform I and II is depicted in Fig. 4.14. The effect of pH on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate was examined at 37°C over a wide range of pH in 100mM of each buffer (KCI -HCI buffer, 2.0 -3.0; sodium acetate buffer, 3.0 -6.0 and phosphate buffer, 6.0 -7.0). The activity was maximum at 5.0 and 4.6 for isoforms I and II respectively.

Temperature optimum

The effect of temperature on the activity of β-hexosaminidase isoforms is shown in Fig. 4.15. The effect of temperature on the activity of β-hexosamini-dase isoforms with p-nitrophenyl-β-D-N-acetylglucosamino-

Table 4.4: Purification profile of β-hexosaminidase from tomato

Purification	Total protein	Total	Specific	Purification	Recovery

Steps	(mg)	activity* (EU)	activity (EU/mg protein)	fold	(%)
1. Crude extraction	146.6	368.9	2.52	1.00	100
2. DEAE-Sephadex A-50 Chromatography					
Isofrom I	24.7	153.7	6.23	2.47	41.7
Isoform II	3.0	20.4	6.70	2.65	05.5
Isoform III	13.2	95.3	7.22	2.86	25.8
3. Sephadex G-200 chromatography					
Isoform I	12.0	91.8	7.63	3.03	24.7
Isoform II	4.4	61.4	9.31	5.50	16.6

* 1EU is equivaluent to 1 μmol p-nitrophenol released min^{-1}

* Per 10 g acetone dried powder

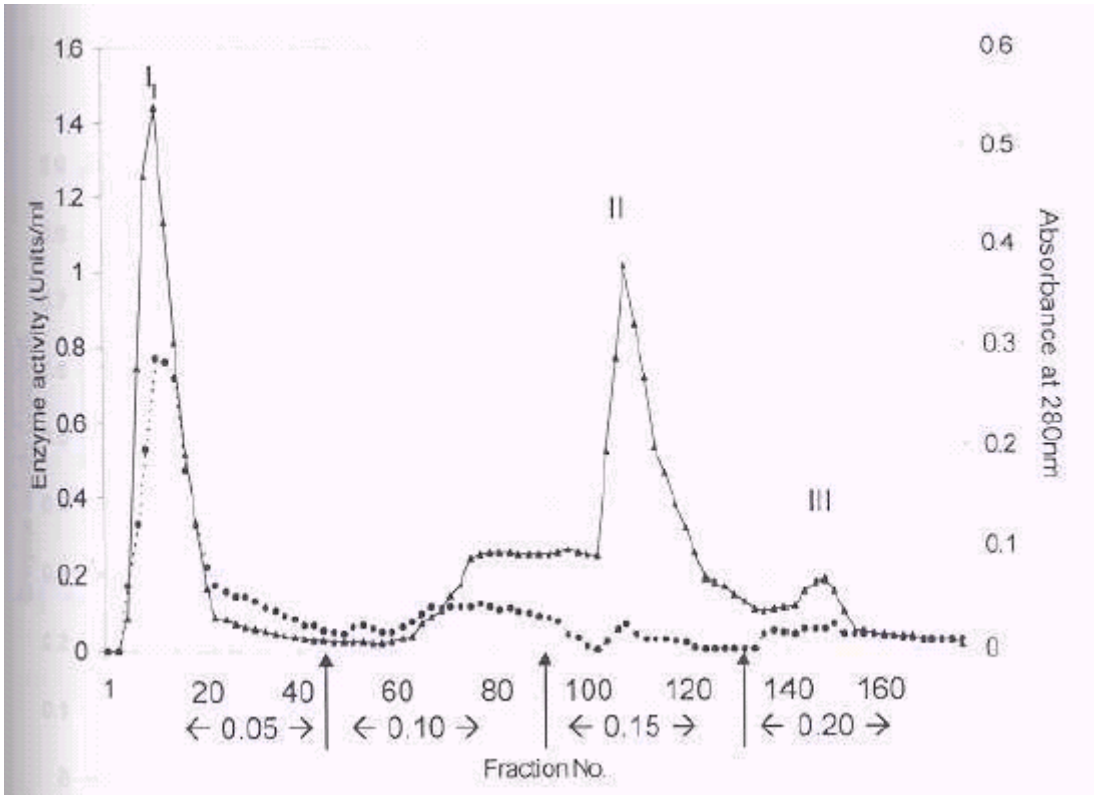


Fig. 4.12 Elution profiles of β-hexosaminidase isoforms from tomato on DEAE-Sephadex A-50. Golumn equilibrated with double distilled water and eluted with increasing concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25M NaCI; pH6.8, flow rate 0.66ml min^{-1}. Isoform I, -II and -III eluted with 0.10, 0.15 and 0.20M NaCI, respectively. Arrows indicate the change of NaCI gradient.

(--•--) Absorbance and (-△-) Enzyme activity

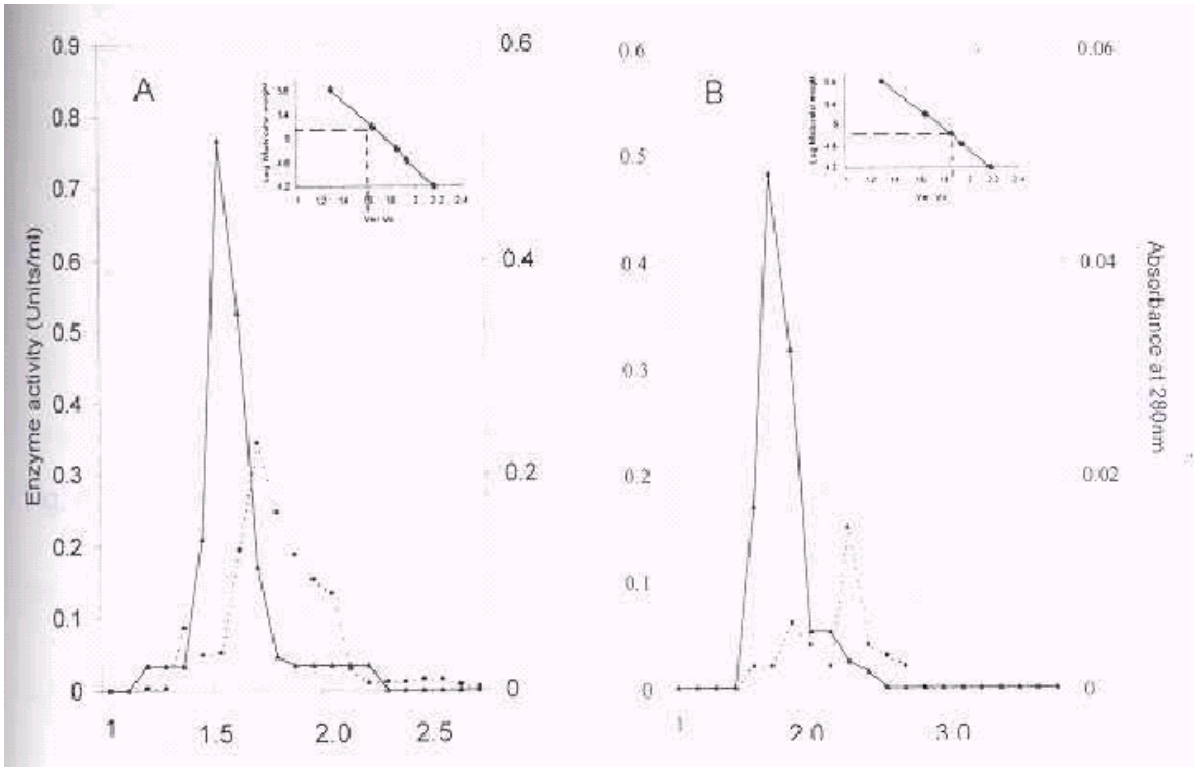


Fig. 4.13 Elution profiles *of* β-hexosaminidase isoform-I (A) and isoform-II (8) from bell capsicum on Sephadex G-200.

(--•--) Absorbance and (-△-) Enzyme activity, Temperatur

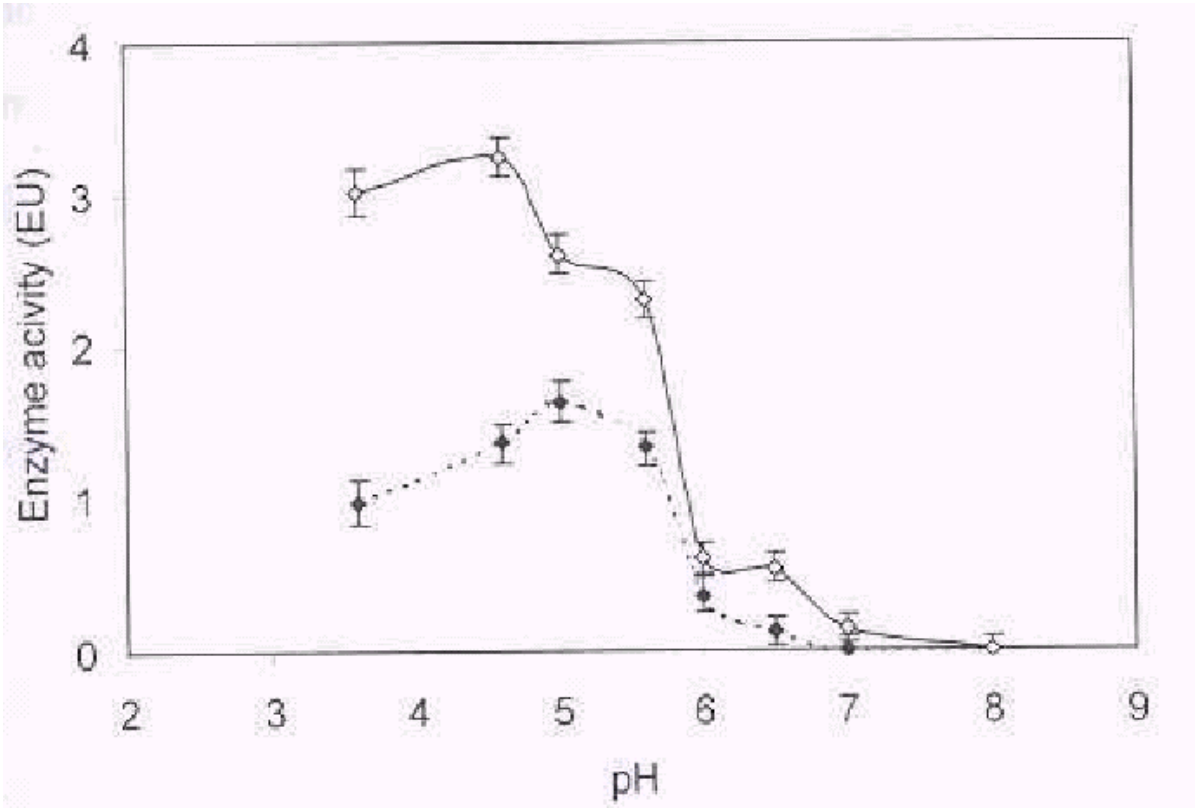


Fig. 4,14 Effect of pH on the activity of β-hexosaminidase isoforms from tomato.

(--♦--) isoform-1 and (-◊-) Isoform-II

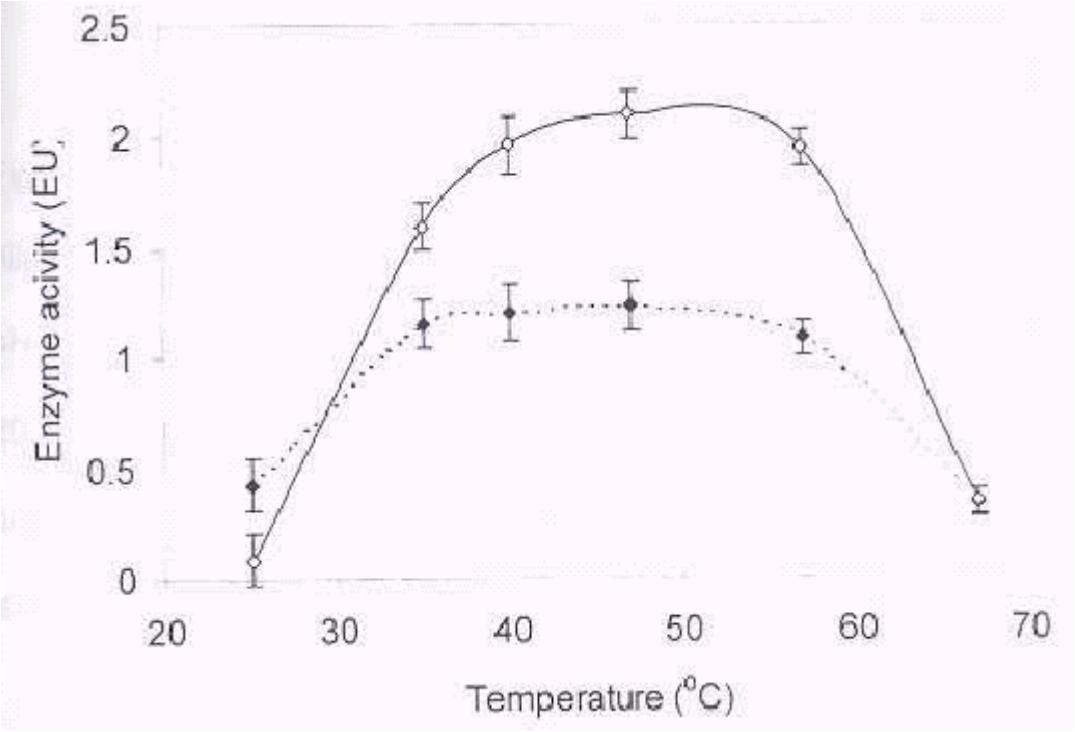


Fig.4. 15 Effect of temperatures on the activity of p-hexosaminidase isoforms from tomato.

pyranoside as substrate was examined at the optimum pH of the respective isoforms by carrying out the enzyme reaction at different temperatures (27- 77°C). The optimum temperature was found to be 47°C for isoform-1 and 47- 57°C for isoform-II.

Thermal stability

The thermal stability of the β-hexosaminidase isoforms is shown in Fig. 4.16. The thermal stability of the β-hexosaminidase isoforms was examined by incubating the enzymes at varying temperatures (35-670C) for 15 min. β-Hexosaminidase-II retained 100% of its activity, while β-hexosaminidase-I retained 71 % of the activity under the same condition.

Kinetic constants

The Lineweaver-Burk plots of β-hexosaminidase isoforms is shown in Fig. 4.17. The kinetic parameters were determined by incubating the suitably diluted purified enzyme with 100mM sodium acetate buffer (at the optimum pH of the respective isoforms) and various substrate concentrations from 1- 20mM of p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside. The Km for pnp-β-D-N-acetyl glucosaminopyranoside was 1.6 mM and 1.1 mM for isoforms I and II respectively.

Substrate specificity

Table 4.4 depicts the percent relative activity of β-hexosaminidase isoforms with other p-nitrophenyl substrates. The substrate specificity of isoform I and II for pnp-β-D-N-acetyl galactosaminopyranoside (34.7 % and 35.7 %) was much less when compared to pnp-β-0-N-acetyl glucosaminopyranoside (100 %). The purified isoform II was checked for other glycosidase activities

with pnp-substrates such as ∝- & β-D-glucopyranoside, ∝-& β-D-galactopyranoside, ∝-mannopyranoside and β-D- N-acetyl galactosaminopyranoside. This enzyme did not show any significant activity with these substrates except for β-D-N-acetyl galactosaminopyranoside and isoform-II for ∝-mannopyranoside (112.6%).

Effect of metal ions

The effect of metal ions (CU^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) and EDT A on the activity of β-hexosaminidase isoforms with p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside is depicted in Table 4.5. Among the various metal ions tested for inhibitory action, divalent metal ions were generally inhibitory to these isoforms of β-hexosaminidase. At 1 mM level, Cu^{2+} and Hg^{2+} showed nearly 100% inhibition of both the isoforms of β-hexosaminidase. Zn^{2+} showed nearly 95% inhibition for isoform I and 82% inhibition for isoform II. Fe^{2+} showed 35% inhibition of isoform I and about 9% inhibition of isoform II. Mn2+ inhibited isoform I by 5% while it was 15% for isoform II. Mg^{2+} and Ca^{2+} did not appear to inhibit p-hexosaminidase activity. EDTA (a metal chelator) showed slight activation of isoform I.

Electrophoretic profiles

The electrophoretic profiles (native and denatured SDS-PAGE) for crude, post-ion exchange and post-gel permeation chromatographic fractions are depicted in Fig. 4.18 & 4.19. The molecular weights were >94 & 64 kDa for the isoform I and II, respectively. All isoforms under acidic conditions exhibited chitinase activity (Figure 4.20).

Circular dichroism analysis

CD spectrum of β-hexosaminidase isoforms from bell capsicum is shown in Fig. 4.21. CD spectra (195-260nm) were recorded for purified β-hexosaminidase isoform I and II in 25mM phosphate buffer, pH 6.0 at room temperature. β-hexosaminidase isoform I showed two negative peaks at 197 and 203 nm and a positive peak at 195 nm, while the isoform II have one negative peak at 200 nm and a positive peak at 197 nm. The spectra of isoform I intersected the x-axis at 196 nm while that of isoform II at 200nm indicating a slight difference in their secondary structure. The isoform-1 is thus deduced to contain 1 % ∝-helix, 68.5 % β-pleated and 30.5 % random coil, while the isoform II was deduced to have 2 % ∝-helix, 74.8 % β-pleated and 23.2 % random coil.

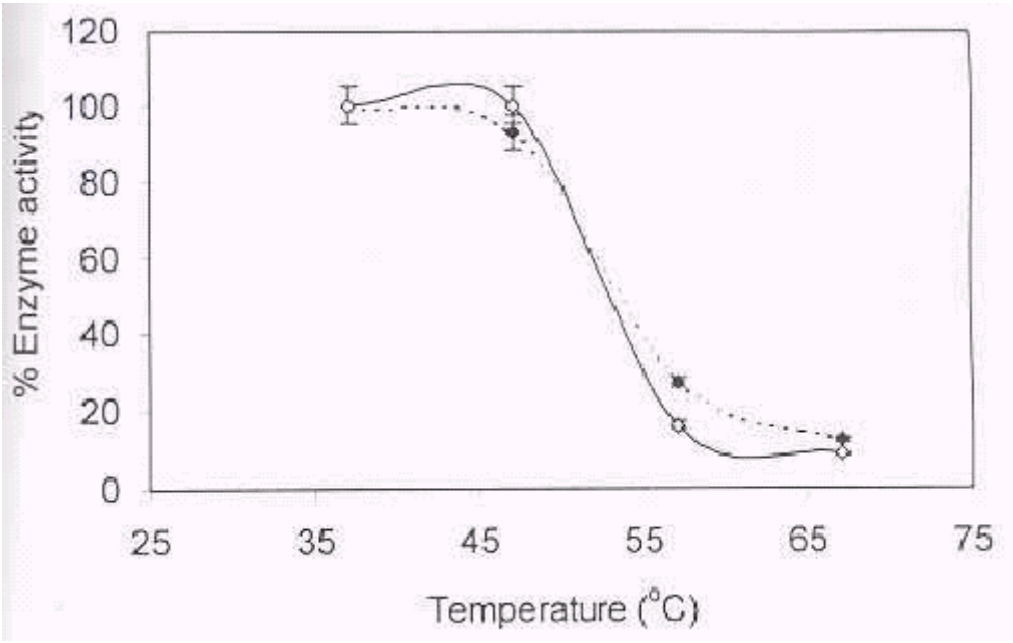


Fig. 4.16 Effect of different temperatures on stability of tomato β-hexosa- minidase isoforms.

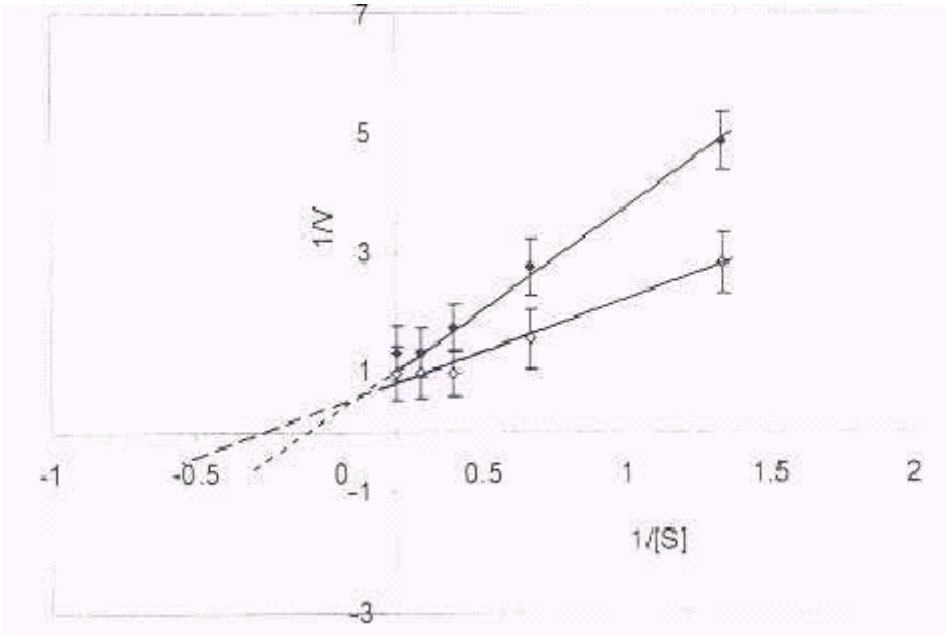


Fig. 4.17 Effect of substrate concentration on activity of β-hexosaminidase isoforms from bell capsicum.

(--♦--) isoform I and (-◊-) Isoform II

Table 4.5 Activity of β-hexosaminidase isoforms with various substrates

Substrates	Reflecting enzyme activity	β-hexosaminidase	
		Isoform I	Isoform II
1. pnp-β-D-N-acetyl glucosaminopyranoside	β-hexosaminidase	100	100
2. pnp-β-D-N-acetyl galactosaminopyranoside		34.7	35.7

3. pnp-∝-D-mannopyranoside	∝-Mannosidase	0.5	112.6
4. pnp-∝-D-glucopyranosode	∝-Glucosidase	4	8
5. pnp-β-D-glucopyranoside	β-Glucosidase	1.7	17.8
6. pnp-∝-D-galactopyranoside	∝-Galactosidase	8	41
7. pnp-β-D-galactopyranoside	β-Galactosidase	5.2	9.9

Table 4.6 Effect of metal ions on (3-hexosaminidase activity.

Metal ion (1mM)	Activity retained (%) ± S. D	
	Isoform I	Isoform II
Control	100±0.19	100.0±0.30
Cu2+	01.5±0.12	1.7±0.0
Fe2+	65.3±21	91.3±15
Hg2+	2.0 ± 0.30	2.0±0.32
Mg2+	103.3±23	97.3±25
Ca2+	106.1 ± 0.30	87.7± 0.17
Mn2+	94.7±0.13	84.3±0.13
Zn2+	04.5 ±0.18	18.0±0.22
EDTA	143.0±0.32	100.0±0.12

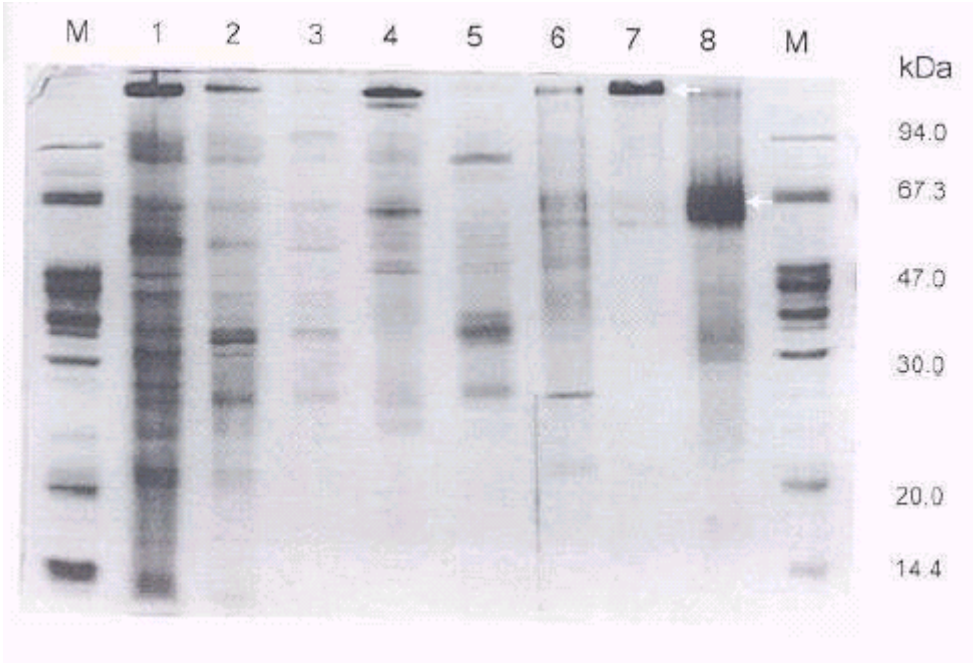


Fig. 4.18 Native SDS-PAGE profile of proteins from tomato fruit at different steps of purification. Lane 1, crude; lane 2, dialyzed; lane 3, neutral; lane 4, isoform-I; lane 5, 0.10M NaGI eluted; lane 6, isoform II (lane 4-6 are from IEG); lane 7, isoform I; lane 8, isoform II (lane 7 and 8 are from GPG) and lane M, molecular weight markers.

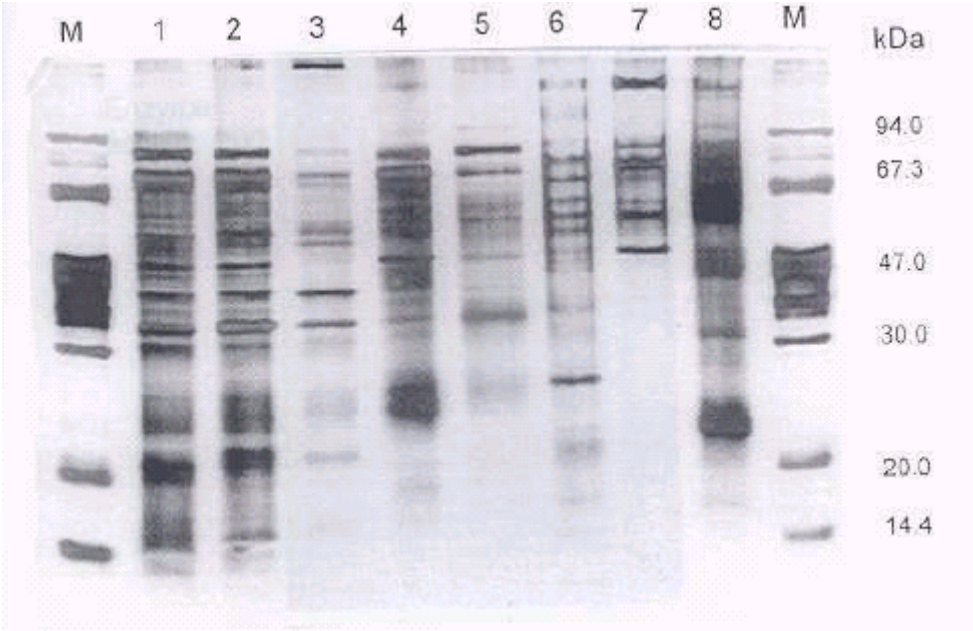


Fig. 4.19 Denatured SDS-PAGE profile of proteins from tomato fruit at different steps of purification. Lane 1, crude; lane 2, dialyzed; lane 3, neutral; lane 4, isoform-I; lane 5, 0.10M NaGI eluted; lane 6, isoform II (lane 4-6 are from IEG); lane 7, isoform I; lane 8, isoform II (lane 7 and 8 are from GPG) and lane M, molecular weight markers.

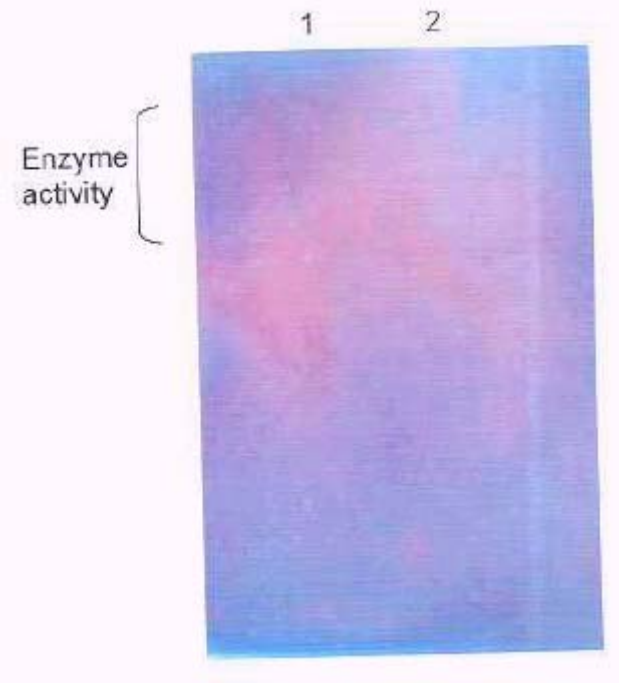


Fig. 4.20 Glycol chitin incorporated acidic gel electrophoresis for acivity of β-hexosaminidase isoforms on PAGE.

Lane 1, Isoform I and and 2, Isoform II

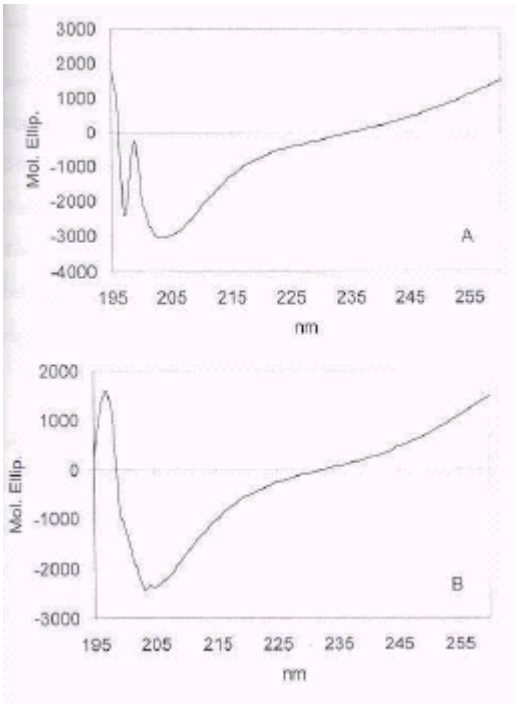


Fig.4.21 CD spectrum of hexosaminidase isoform I (A) and isoform II (B) from tomato

DISCUSSION

Though the enzyme β-hexosaminidase is shown to be important in animal and microbial systems, its importance in higher plant systems and its significance in fruit ripening have not received any attention. By the fact that β-hexosaminidase showed a much higher activity with a climacteric

peak during ripening in several ripening fruits, it was thought important to study this enzyme and view its properties in the light of 'fruit ripening'. Similar to bell capsicum, two major isoforms of β-hexosaminidase were recognized in ripening tomato also.

The present study has revealed that the properties of β-hexosaminidase viz., optimum pH, optimum temperature, thermal stability and inhibition by metal ions do not vary much between their sources -tomato and bell pepper suggesting that there is considerable similarity in their properties. The only deviation is that while β-hexosaminidase isoform I of tomato is resolved with 0.05M NaCl with an abundance of 57%, the same was resolved with 0.10M in bell capsicum with an abundance of 38%. The β-hexosaminidase purified from cabbage has an optimum pH of 4.0 (266), which are close to that of isoform II from both the system studied. The isoform II from bell capsicum has an optimum temperature range of 37-47°C, while that of tomato has 47 -57°C, suggesting that tomato β-hexosaminidase isoform II is active over a wide temperature range and is almost near to the temperature optimum of cabbage β-hexosaminidase (266) but higher than that of *Ginkgo biloba* β- hexosaminidase i.e., 35°C (262).

The Km value for p-nitrophenyl-β-N-Acetyl glucosaminopyranoside was generally higher for the β-hexosaminidase isoforms of bell capsicum than for the corresponding enzyme isoforms of tomato, indicating higher affinity of the enzyme for this substrate in the later fruit. The cabbage β-hexosaminidase has been reported to have a Km of 0.94 mM for p-nitrophenyl-β-N-Acetyl glucosaminopyranoside (266) suggesting that the later has more affinity towards the substrate than those of bell capsicum and tomato. The inhibition by the metal ion Cu^{2+} is 100% for isoform II of tomato, as revealed in the present study, while the inhibition was only 22% for isoform II of bell capsicum.

The native molecular mass for β-hexosaminidase isoform I and II of bell capsicum are 178 and 83.5 kDa, while the same for tomato isoenzymes are 190.5 and 64 kDa respectively (as revealed by Sephadex G-200 column chromatography). These isoforms on native SDS-PAGE revealed molecular mass of 85 kOa for isoform II from bell capsicum and >94 and 64 kDa for isoform I and II from tomato respectively. The β-hexosaminidase purified from lettuce has been reported to have a molecular mass of 69 kDa on SDS-PAGE and 62.5 on Bio-gel column (203), while that from cabbage has 150 kDa on Sephacryl S-200 and three subunits of molecular mass 64, 57 and 51 kDa on SDS-PAGE (266) and Ginkgo biloba p-hexosaminidase has 63 kDa on SOS-PAGE and 62 kDa on Hiprep S-200 column (262)

Both the β-hexosaminidase isoforms from bell capsicum and tomato fruits have exhibited glycanase activity at pH 4.0, wherein they are able to hydrolyze glycol chitin and chitosan on the gel. This suggests that at this pH all the isoforms of bell capsicum undergo partial hydrolysis (proteolytic cleavage) to a molecular size of <12 kDa which exhibit N-glycanase activity.

Circular dichroism spectrum of β-hexosaminidase from bell capsicum and tomato has revealed that the enzyme from the two fruit systems differ from one another in their secondary structure and also slightly among the isoforms within the same fruit system. Bell capsicum β-hexosaminidase isoforms have ∝-helix, β-pleated and random coil in their secondary confirmation compared .to very high β-pleated (70 %) and random coil (25 %) nature of tomato β-hexosaminidase. This suggests that the enzyme having different types of secondary confirmation may still catalyse the same type of reaction, but with a difference in substrate affinity.

β-Hexosaminidase generates free N-glycans from glycoproteins or any hetero-oligomers *I* polymers containing hexosamine residues. Presence of free N-glycans is reported in ripe tomato with a presumable role as signal molecules (267). Whether such types of oligo- or polymeric fractions (glycoproteins or otherwise) are present in the *in vivo* situation of the fruit remains to be established. ∝-Mannosidase is a glycoprotein enzyme containing N-acetylglucosamine and mannose residues, and is a glycosidase implicated in fruit ripening. Mannosidase enzyme from jack bean is reported *to* be a glycoprotein containing N-glycan residues (234). Isoforms *of* a-mannosidase from both capsicum and tomato are also found to be glycoproteins in our current study. Hence, it is possible that ∝-mannosidase as a glycoprotein could itself be a susceptible substrate for hexosaminidase. This is inferred from a somewhat close interrelationship between the activities *of* β-hexosaminidase and ∝-mannosidase enzymes in bell capsicum and tomato during ripening.

Chapter V

Summary

Two glycosidases viz, β-hexosaminidase and ∝-mannosidase - the carbohydrate hydrolases involved in fruit softening during ripening were specifically studied in this investigation in the context of fruit ripening with the following objectives. a) To examine the enzyme activity profiles of β-hexosaminidase and ∝-mannosidase during development and ripening stages in bell capsicum and compare the same with that of tomato, which belongs to the same family Solanaceae and considered a model system for scientific studies in higher plants, and b) Purification of β-hexosaminidase from bell capsicum as well as from tomato (for comparison) and study of the enzyme properties in order to understand its implication in fruit ripening.

1. Bell capsicum and tomato were found to be richest source of glycosidases such as β-hexosaminidase, ∝-galactosidase and ∝-mannosidase compared to banana, mango and papaya fruits at climacteric stage.
2. Highest activity of β-hexosaminidase in bell capsicum plant was noticed in ripe fruit followed by shoot tip and flower, while highest ∝-mannosidase activity was noticed in shoot tip followed by flower, leaf stalk and ripe fruit.

3. In bell capsicum, β-hexosaminidase activity increased slightly during fruit development, while during ripening there was a further prominent increase. In tomato β-hexosaminidase activity decreased during fruit development and increased slightly during ripening; but had less activity than during fruit development. Increase in β-hexosaminidase was as much as 3-fold by the end of the ripening in bell capsicum compared to that of tomato, where it decreased to half at the end.

4 ∝-Mannosidase activity increased prominently during fruit development and did not increase any further, but remained almost constant during ripening in bell capsicum. In tomato, ∝-mannosidase showed a prominent activity both during fruit development and ripening. Increase in a-mannosidase was almost 2-fold in bell capsicum compared to that in tomato, where the activity was slightly lower at the beginning than at the end.

5 ∝-Galactosidase activity was found to be significant in tomato along with β-hexosaminidase and ∝-mannosidase.

6. The specific activity of β-hexosaminidase and a-mannosidase enzymes were found to be higher in the dialyzed extracts of bell capsicum compared to the activity prior to dialysis, suggesting the presence of small molecular weight inhibitors for these two enzymes especially during ripening. On the other hand, these enzyme activities in tomato were lower in the dialyzed extracts compared to the undialysed ones suggesting the essentiality of one or more small molecular weight activators.

7. Protein profiles on native SDS-PAGE and denatured SDS-PAGE during fruit development and ripening showed differences in protein bands as well as their intensity in both the fruit systems.

8. β-Hexosaminidase and a-mannosidase resolved into three isoforms each on subjecting the aqueous extracts of acetone dried powder of 2nd stage of fruit development and climacteric stage of bell capsicum to DEAE-Sephadex A-50 chromatography.

9. The specific activity of β-hexosaminidase was generally higher than those of corresponding ∝-mannosidase isoforms in bell capsicum. While in tomato the specific activity of ∝-mannosidase was higher than those of corresponding β-hexosaminidase isoforms at climacteric stage.

10. Carbohydrate / protein ratio in both bell capsicum and tomato fruits was maximum at climacteric stage. β-Hexosaminidase isoform-II from bell capsicum and all the ∝-mannosidase isoforms appeared to be glycoproteins with differences in their carbohydrate / protein ratio.

11. Separation of native β-hexosaminidase isoform-I at pH: 5.6 from bell capsicum yielded two fractions having the activity of both β- hexosaminidase and ∝-mannosidase. Thus separated fractions showed the difference in their secondary confirmation and the separated β-hexosaminidase sub-fractions had the same conformation as that of the native form.

12. All the three ∝-mannosidase isoforms had the common subunit having molecular mass 55 kDa, while ∝-mannosidase isoform-II and - III had an additional subunit having molecular mass of 36 kDa compared to ∝-mannosidase isoform-I.

13. Electro eluted ∝-mannosidase isoform-I and -II from tomato retained their enzyme activities but showed differences in their secondary structure when they were compared with that of Con-A purified one. The results suggested that electro elution after preparative electrophoresis can be used to purify ∝-mannosidase isoforms.
14. Western blot analysis of aqueous extracts from various tomato tissues and various stages of flower and fruit development revealed the ripening specificity of a-mannosidase in this system.
15. The molecular mass for β-hexosaminidase isoforms were found to be 178 kDa for isoform-I and 83.5 kDa for isoform-II on Sephadex G-200 chromatography, while isoform-II appeared to have a molecular weight of 85 kDa on SDS-PAGE. The molecular mass of β- hexosaminidase from bell capsicum were found to be 190.5 for isoform-I and 64 kDa for isoform-II on gel permeation chromatography, while on native SDS-PAGE revealed >94 kDa and 64 kDa for isoform-I and -II respectively.
16. β-Hexosaminidase was isolated and purified from both bell capsicum and tomato at climacteric stage of the fruit. ∝-Mannosidase from tomato was also purified and its cross-reactivity with the antibody for bell capsicum enzyme was examined.
17. The pH optimum for β-hexosaminidase isoform-I and -II were 5.0 and 4.7 respectively in both bell capsicum and tomato.
18. The temperature optimum was 37°C and 37 -47°C for β-hexosaminidase isoform-I and -II respectively in bell capsicum, while corresponding isoforms from tomato had an optimum temperature of 47°C and 47-57°C.
19. β-Hexosaminidase isoform-1 and -II from both the fruit systems had the temperature stability of 72 % and 100 % at 57°C for 15 min.
20. β-Hexosaminidase isoform-I and -II had a Km of 3.0 and 1.75 mM towards p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside respectively in bell capsicum, while the β-hexosaminidase isoform-I and -II from tomato had a Km of 1.6 and 1.1 mM towards the same substrate.
21. At 1 mM concentration, Hg^{2+}, Cu^{2+} and Zn^{2+} showed 3, 9 and 14 % residual activity over control for β-hexosaminidase isoform-I, while it was 5, 78 and 38 % for isoform-II respectively in bell capsicum. At same concentrations, β-hexosaminidase isoform-1 and -II did not show any residual activity in presence of Hg^{2+} and CU^{2+}, while Zn^{2+} showed nearly 5 % residual activity for isoform-1 and 18 % for isoform-II in tomato.
22. Metal chelator, EDTA was found to increase the activity of β-hexosaminidase isoform-I by more than 40% over the control in both the system.
23. β-Hexosaminidase isoforms showed almost 35 % activity towards p-nitrophenyl-β-D-N-acetylgalactosaminopyranoside compared to p-nitro phenyl-β-D-N-acetylglucosaminopyranoside in both systems.
24. β-Hexosaminidase and a-mannosidase enzyme activities of bell capsicum could be separated on DEAE-Sephadex A-50 ion-exchange column using acidic elution conditions (pH 5.6).

25. Circular dichroism analysis of purified β-hexosaminidase isoforms from bell capsicum revealed differences in their secondary confirmation with corresponding isoforms from tomato.

26. The results of this investigation suggested a possible new pathway, where fruit softening may be controlled to improve its shelf life and post-harvest qualities. The present investigation evidences the possible role of glycosidase in the fruit ripening as their peak activity coincides with climacteric stage of the fruit and their specificity towards tissues. β-Hexosaminidase was found be involved in the ripening of bell capsicum, while a-mannosidase is required for development of the bell capsicum fruit.

27. Further investigation involving gene isolation, characterization and development of transgenic plants, particularly for p-hexosaminidase and a-mannosidase in bell capsicum and tomato is necessitated to get clues regarding the direct involvement of these two enzymes in fruit ripening.

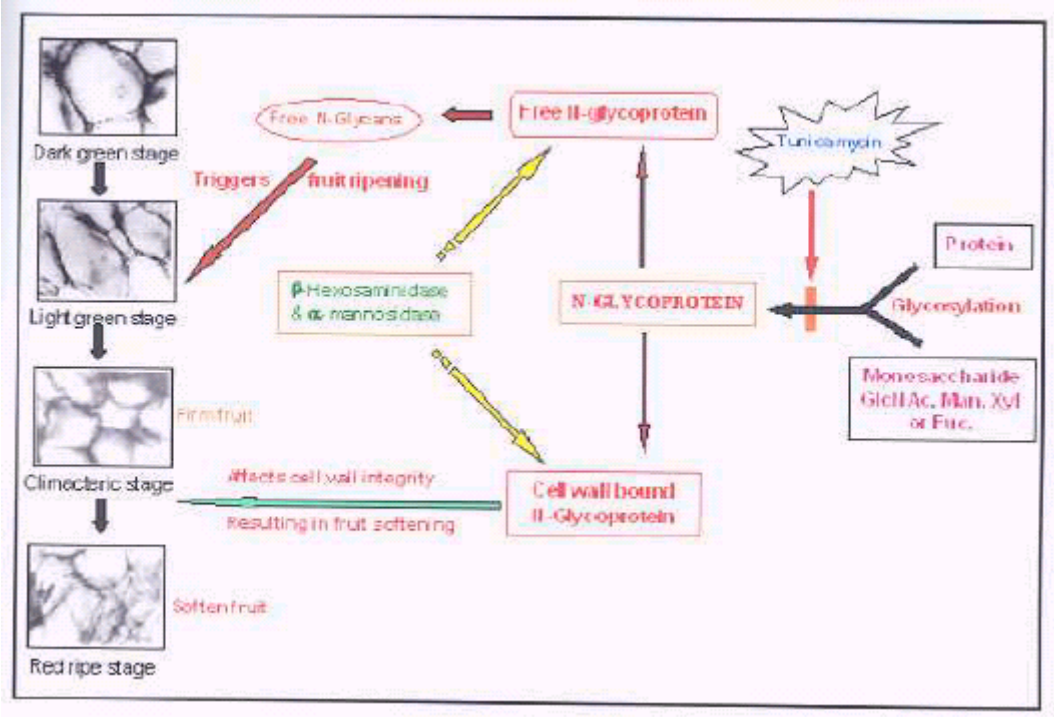


Fig. 5.1 Possible role of glycosidases in fruit ripening and fruit softening.

28. The role of two glycosidases -β-hexosaminidase and ∝-mannosidase in fruit ripening and softening with respect to bell capsicum is summarized in Fig.5.1. This investigation has evidenced significant increase in the activities of β-hexosaminidase in bell capsicum and of ∝-mannosidase in tomato during fruit ripening with a climacteric peak. These enzymes are known to be involved in deglycosylation of glycoproteins resulting in release of free N-glycans. It has been reported that free N-glycans (and hence N-glycoproteins) have a role in fruit ripening in tomato where tunicamycin application to mature green fruit prevented both ripening and softening of the fruit. From the present investigation, it is inferred that β-hexosaminidase and ∝- mannosidase play an important role in fruit ripening phenomena in bell capsicum by acting on N-glycoproteins and releasing free N-glycans such as

Man_3(Xyl)GlcNAc(Fuc)Glc-NAc and Man_5GlcNAc. Further, free N-glycans released by β-hexosamini-dase and ∝-mannosidase enzymes might trigger the ripening of fruits by increasing the ethylene biosynthesis, or via an alternative pathway independent of ethylene biosynthesis. Glycosidases β-hexosaminidase and ∝-mannosidase also act on cell wall/membrane bound N-glycoproteins to release N-glycans. Proteins devoid of oligosaccharide moiety will assume altered configuration which directly affects cell wall/membrane chemistry of the fruit. Thus changed cell wall/membrane structure affects the cell integrity leading to cell burst or collapse resulting in fruit softening.

APPENDIX

Beneficial Influence of heat treatment in improving the shelf life of bell capsicum fruits by manipulation of the activities of β-hexosaminidase, ∝-mannosidase and ∝-galactosidase

Summary: Glycosidases -β-hexosaminidase, ∝-mannosidase and ∝-galactosidase are likely to play an important role in fruit softening during ripening of fruits. Heat treatment of bell capsicum at dark green stage of the fruit decreased the enzyme activity of all these three enzymes. But heat treatment of the fruit at light green and climacteric stage resulted in either increased enzyme activities or did not affect the same. SDS-PAGE protein profile revealed a heat stress specific protein of m.w. approx. 34 kDa. The carbohydrate / protein ratio was lesser in bell capsicum heat treated at dark green stage compared to its untreated control. Higher carbohydrate to protein ratio was noticed in bell capsicum heat treated at light green stage and climacteric stage over their control. Higher water loss was noticed in bell capsicum heat treated at dark green stage, light green stage and climacteric stage over their respective controls after 72 h storage at ambient temperature following heat treatment. Heat treatment at dark green stage increased the shelf life of the bell capsicum by an average of 9 days. The fruits heat treated at dark green stage retained the luster even after 6 days (144 h) while those of other stages last the luster. The data suggested that heat treatment at the dark green stage of bell capsicum could probably be applied to improve the shelf life of these fruits.

INTRODUCTION

Capsicums are an important vegetable crop among the *Solanaceous* fruits. Capsicums are an excellent source of vitamin A and C. Bell capsicum is generally an expensive vegetable in India and its high cost is mainly due to high perishability resulting in short life. The post-harvest losses are mainly material loss, which include loss of weight, color, bruise, decay, etc., at various stages from harvest to consumption. The expected shelf life of bell capsicum depends upon post-harvest handling and storage environment. As of now, cold storage (~7-90C) is the only known technique economically feasible for short-term storage of fresh capsicums. In India, the prevailing tropical climate and lack of adequate cold storage facilities shorten the life of capsicum to only 6-8 days at ambient temperature (27±4°C). Hence there is a need to extend the shelf life of capsicum at ambient temperatures to extend the market season and create distant markets.

Glycosidases β-hexosaminidase, ∝-mannosidase and ∝-galactosidase - the carbohydrate hydrolases are understood to be intricately involved in fruit softening of bell caupsicum during ripening. In this study, activities of these glycosidases were examined 74 h after a brief exposure of the fruits at different stages to heat and the activity profiles were compared with those of untreated fruits. Percent water loss and carbohydrate / protein ratio were also determined in these fruit samples.

MATERIALS AND METHODS

Bell capsicum, at different stages of fruit ripening were collected from local market and were exposed to heat at 100°C for exactly 10 min (in a hot air oven). Acetone dried powders were prepared from the heat exposed fruit samples with 3 volumes of chilled acetone after three days of storage at ambient temperature.

Extraction: Acetone dried powders of respective samples (1g) were soaked in sodium acetate buffer, pH 6.8 containing 0.2 % polyvenylpyrrolidone and 0,05% triton x-100 at 4°C with constant stirring for 16 h. The extracts were passed through three layers of cheese cloth and centrifuged at 10,000 rpm for about 15 min at 4°C. Supernatant was dialyzed against double distilled water and concentrated with anhydrous polyethylene glycol.

Electrophoresis: Denatured SDS-PAGE was carried out according to Laemmli and stained for proteins with silver stain (Perro et al). Protein was estimated by modified Lowry method and total carbohydrate in the protein extract was estimated by the method of Dubois et al.

Enzyme activity determinations: The reaction mixture consisted of 1,25 mM p-nitrophenyl-β-D-N-acetyl glucosaminopyranoside, p-nitrophenyl-∝-D-manno-pyranoside or p-nitrophenyl-∝-D-galactopyranoside, 100mM sodium acetate buffer (pH: 5.0) and suitable aliquot of the enzyme. Incubation was carried out for 15 min at 37°C and the reaction stopped with the addition of 500 mM sodium bicarbonate to the reaction mixture. The color intensity of the liberated p-nitrophenol was measured at 405 nm. One unit of the enzyme is defined as the amount of enzyme required to liberate one µmol of p- nitrophenol per min.

RESULTS

Activities of glycosidases: Fig.1 depicts the effect of heat stress applied on bell capsicum at different stages of fruit ripening on the activity of its p- hexosaminidase. Heat treatment of bell capsicum at dark green stage of the fruit decreased this enzyme activity (total activity by 42% and specific activity by 20%). The enzyme activity was higher than the control in all other stages of fruit ripening.

Fig. 2 depicts the influence of heat treatment given to bell capsicum at different stages of fruit ripening on its a-mannosidase activity. Heat treated bell capsicum at dark green stage exhibited significantly lesser activity of ∝-mannosidase compared to its control. ∝-Mannosidase activity (total) was more or less unaffected in the fruits heat exposed at light green stage. ∝-Mannosidase activity in heat treated fruits at climacteric stage was slightly higher than the corresponding control.

Fig. 3 depicts the influence of heat treatment of bell capsicum at different stages of fruit ripening on its a-galactosidase activity. α- Galactosidase activity (total) was found to be lesser in heat treated bell capsicum at dark green stage and light green stage compared to untreated fruits. The enzyme activity was unaffected by heat stress at climacteric stage.

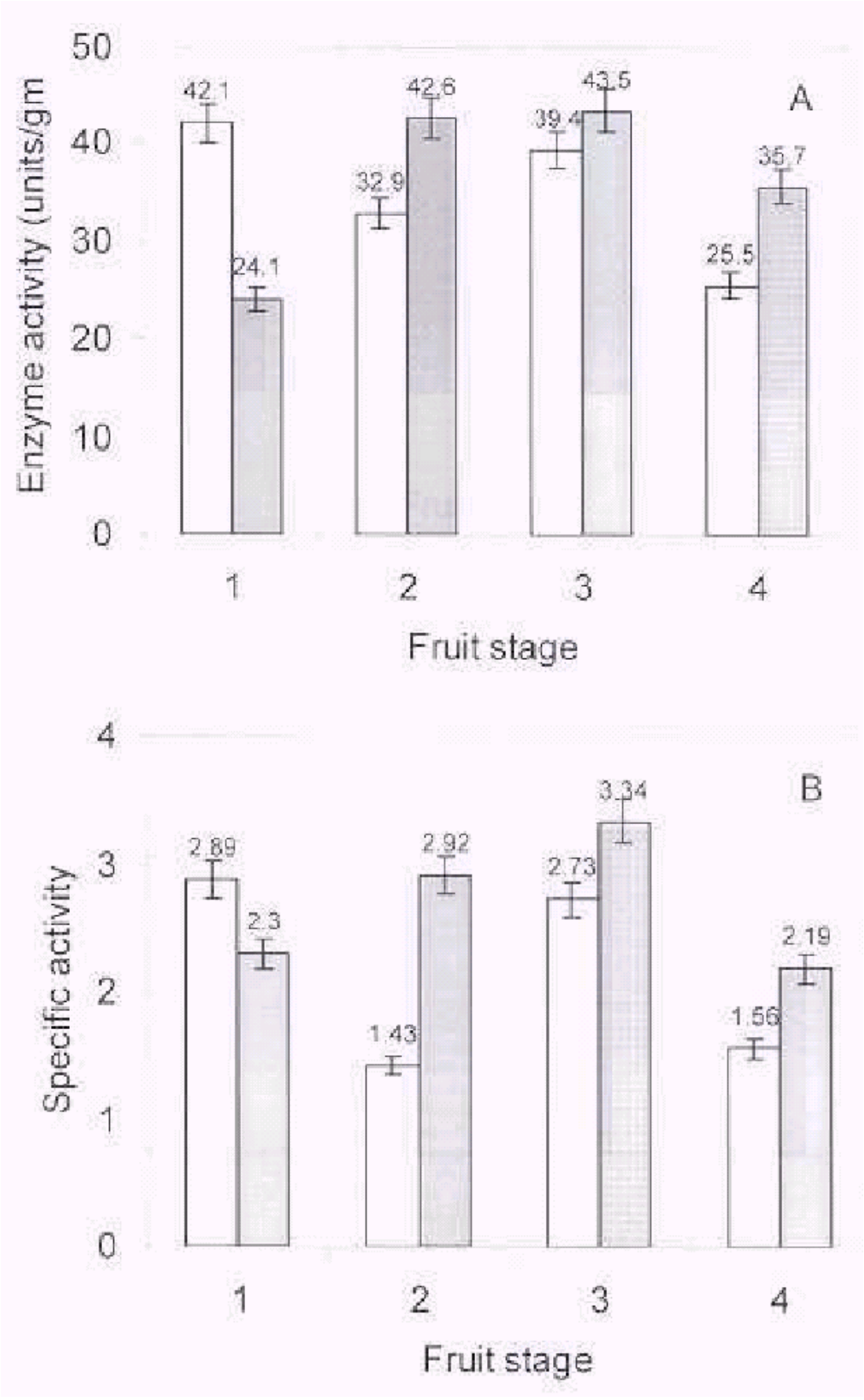


Fig. 1: Activity (A) and specific activity (B) of β-hexosaminidase enzyme from bell capsicum.

□: Control and ■: Heat-treated

1, Dark green stage: 2**,** Light green stage: 3, climacteric stage and

4, Red ripe stage.

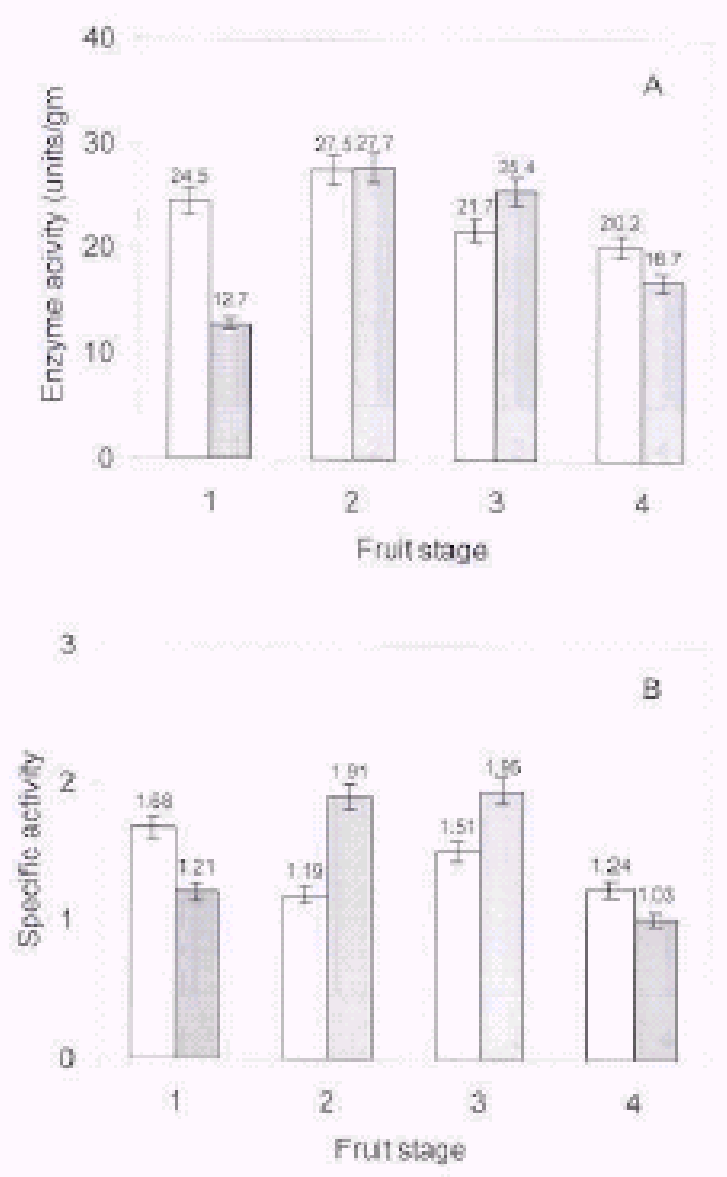


Fig. 2: Activity (A) and specific activity (B) of ∝-mannosidase enzyme from bell capsicum.

□: Control and ■: Heat-treated

1, Dark green stage: 2**,** Light green stage: 3, climacteric stage and

4, Red ripe stage.

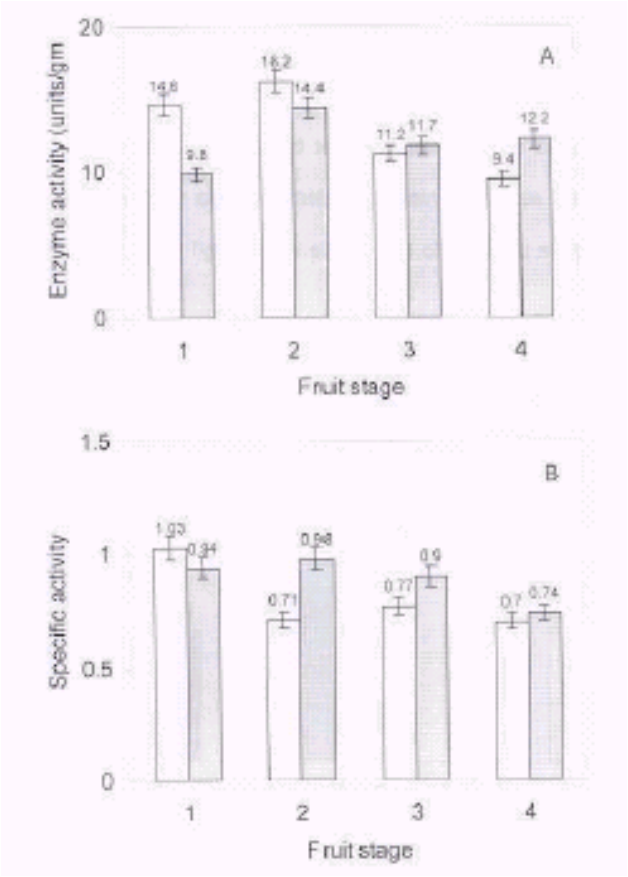


Fig. 3: Activity (A) and specific activity (B) of ∝-galactosidase enzyme from bell capsicum.

□: Control and ■: Heat-treated

1, Dark green stage: 2**,** Light green stage: 3, climacteric stage and

4, Red ripe stage.

Carbohydrate *I* Protein ratio: Fig. 4 depicts the influence of heat treatment of bell capsicum at different stages of fruit ripening on carbohydrate / protein ratio of aqueous enzyme extracts. The carbohydrate / protein ratio was lesser in bell capsicum heat treated at dark green stage compared to its untreated control. Higher carbohydrate to protein ratio was noticed in bell capsicum heat treated at light green stage and climacteric stage over their

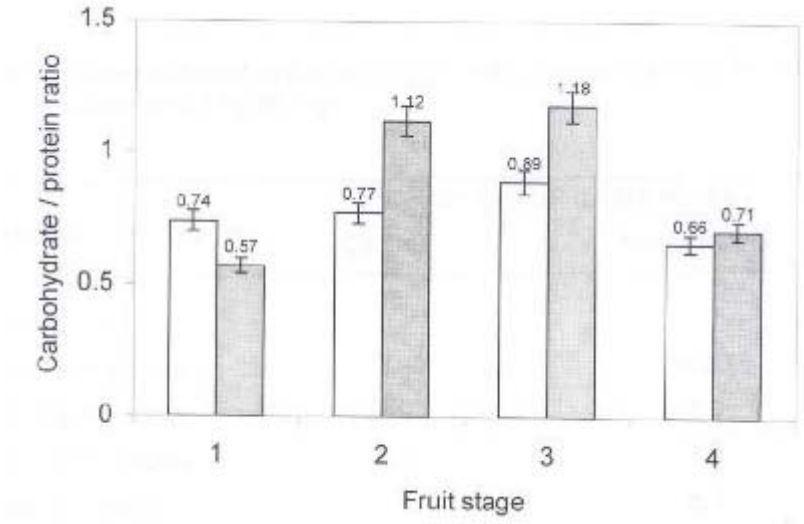


Fig. 4: Carbohydrate / protein ratio of aqueous extracts from bell capsicum.

□: Control and ■: Heat-treated

1, Dark green stage: 2, Light green stage: 3, climacteric stage and 4, Red ripe stage.

Water loss: Table 1 presents the influence of heat treatment of bell capsicum at different stages of ripening on its water content. Higher water loss was noticed in bell capsicum heat treated at dark green stage, light green stage and climacteric stage over their respective controls after 72 h storage at ambient temperature following heat treatment. Water loss at red ripe stage remained more or less the same for both heat-treated bell capsicum and its untreated control. Water loss from the fruit was almost equal in all the stages at the end of 144 h.

Table 1: influence of heat stress on percent water loss per gram of fresh bell capsicum during storage.

Fruit stage	% Water loss per g fresh weight	
	Control	Heat treated
Stored for 72 h		
1. Dark green stage	6.69	8.20
2. Light green stage	6.93	7.9
3. Climacteric stage	6.34	7.5
4. Red ripe stage	5.45	5.0
Stored for 144 h		
1. Dark green stage	4.05	4.5
2. Light green stage	4.22	4.73
3. Climacteric stage	3.91	3.4
4. Red ripe stage	3.61	2.05

Protein profile: Fig. 5 depicts the SDS-PAGE protein profile of heat treated and control unheated bell capsicum at different stages of fruit ripening. A protein of approx. m.w. 34 kOa was observed in heat treated bell capsicum in 50 % acetone precipitated protein samples.

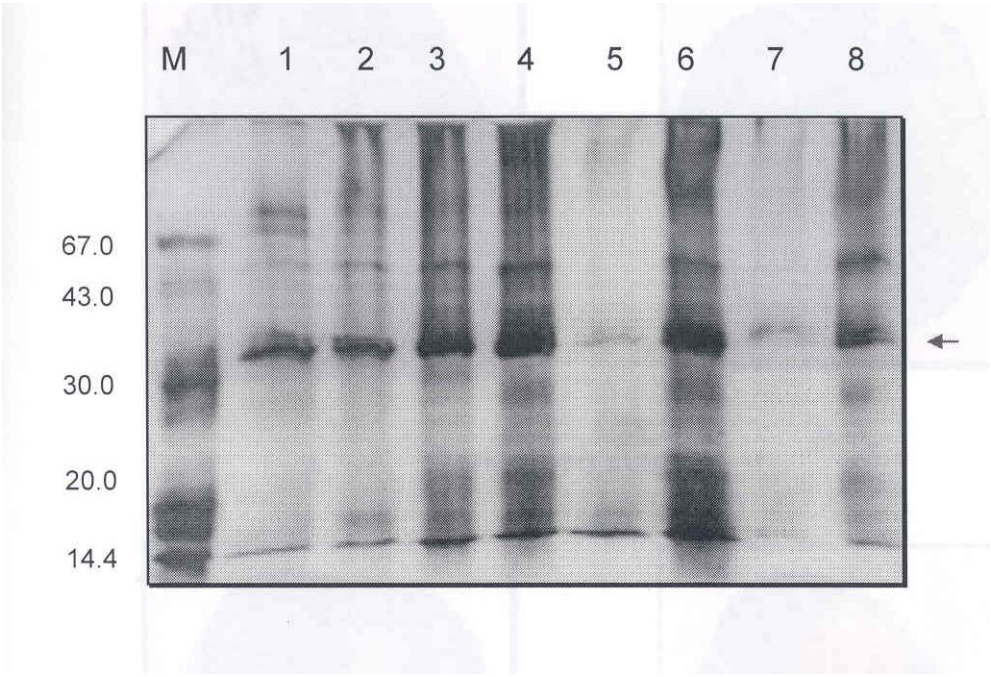


Fig. 5: Denatured SDS-PAGE profile of bell capsicum at different stages of fruit development. Lane: M, Molecular weight markers; 1, dark green control; 2 dark green heat treated; 3, light green control; 4, light green heat treated; 5, climacteric control; 6, climacteric heat treated; 7, red ripe control and 8, red ripe heat treated.

Shelf life studies: Shelf life of the bell capsicum was assessed based on its appearance, color and luster. Fig. 6 depicts the appearance of heat treated and control bell capsicum fruits at dark green stage and climacteric stage after 72 h of storage at room temperature following the heat treatment.

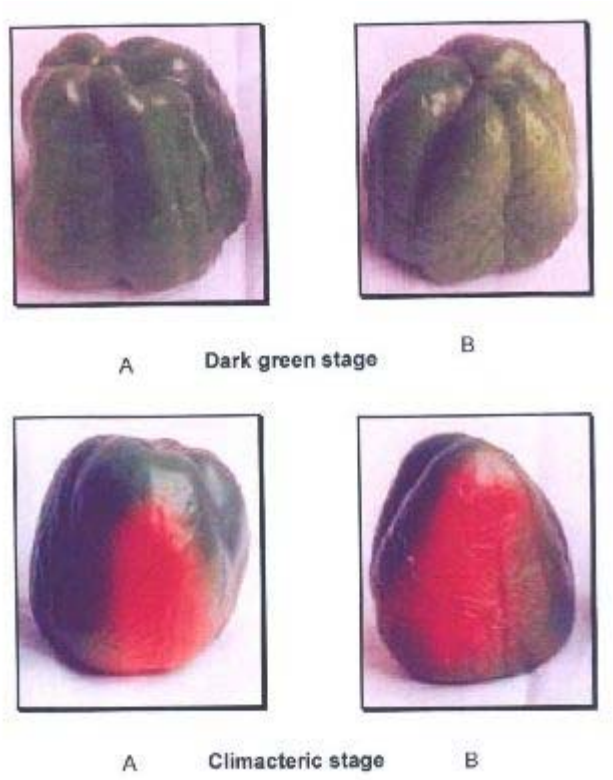


Fig. 6: Appearance of bell capsicum 3 days after heat treatment at dark green / climacteric stage.
A: Heat treated; B: Control

Bell capsicum heat treated at all stages of ripening except red ripe stage retained the luster, but shriveled to some extent compared to the fruits heat treated at dark green stage. The fruits heat treated at dark green stage retained the luster even after 6 days (144 h) while those of other stages last the luster.

Fig. 7 shows the appearance of bell capsicum heat treated at dark green stage after 9 days of storage at room temperature. Bell capsicum fruits heat treated at dark green stage (Fig. 7 A) not only retained its appearance but also appeared to be free from pathogen attack in spite of mechanical damage.

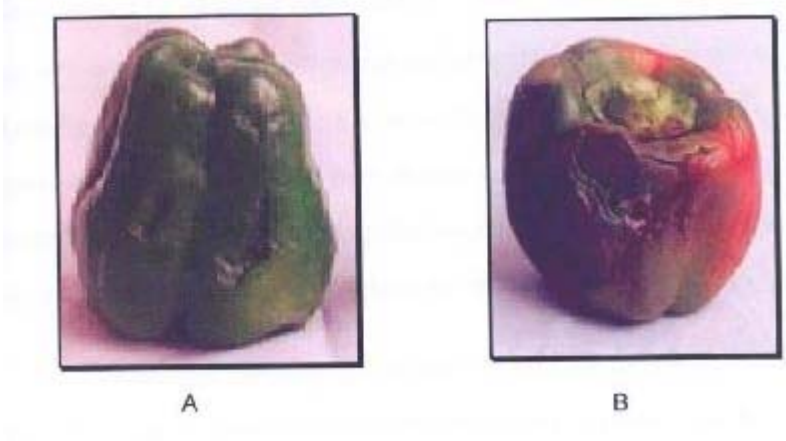


Fig. 7: Appearance of bell capsicum 9 days after heat treatment at dark green stage.
A: Heat treated; B: Control

DISCUSSION

Heat treatment of bell capsicum at dark green stage has resulted in a significant decrease in the activity of all the three-glycosidase enzymes, viz., β-hexosaminidase, ∝-mannosidase and ∝-galactosidase. But heat treatment at later stages viz., light green stage, climacteric stage and red ripe stage increased the activity of β-hexosaminidase. Such a reduced enzyme activity after heat stress has been reported for polygalacturonase enzyme protein and activity and the mRNA at temperature 32°C and above in tomato (Kagan-Zur et al 1995). In this system, the exogenous ethylene treatment did not reverse heat stress induced inhibition of polygalacturonase gene expression. The poly-galacturonase gene expression was gradually and irreversibly shut off during heat stress (for 24 h). Since β-hexosaminidase, ∝-mannosidase and ∝-galactosidase are understood to have a positive role in fruit softening during ripening, a reduction in these enzyme activities may prove advantageous from the point of view of shelf life of the fruit. Such a beneficial reduction in enzyme activity has been achieved in bell capsicum by a brief exposure of the fruits to heat at the dark green stage.

The reduced activity of glycosidases in heat treated bell capsicum at dark green stage could have resulted from its switching over to senescence phase, where gene expressions along with other biochemical changes alters fruit biochemistry, hence heat stress along with its changing gene expression might have brought down the expression of enzyme or the heat stress might have altered the enzyme activity.

The increased or unaltered enzyme activity of these glycosidases in bell capsicum heat treated at later stages might be due to the already switching on of the ripening gene and subsequent expression of the proteins responsible for fruit ripening. This is evident from the increased specific activity and increased carbohydrate / protein ratio at these later stages.

Bell capsicum heat treated at dark green stage was found to have lost higher amount of water (g % of fresh weight) compared to its control and this water loss was higher than the fruits heat treated at rest of the stages. The percent water loss was almost similar in both heat treated and control bell capsicum after 72 h to 144 h of storage.

The shelf life of the heat treated bell capsicum at dark green stage was 9 days on an average compared to bell capsicum heat treated at light green stage, which was 6 days, while heat treatment at climacteric and red ripe stage did not have significant effect on the shelf life. Heat treatment at dark green stage also prevented the spoilage of fruit due to pathogen attack following mechanical damage. This observation that a brief heat exposure at dark green stage of bell capsicum extends its shelf life by 9 days assumes importance from the point of view of a possible application of this strategy to enhance the shelf life of these perishable fruits.

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