Role of Gibberellic Acid and Calcium Chloride in Ripening Related Biochemical Changes in Guava (*Psidium guajava* L.) Fruit

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2016

CERTIFICATE – I

This is to certify that this thesis entitled, **"Role of Gibberellic Acid and Calcium Chloride in Ripening Related Biochemical Changes in Guava (*Psidium guajava* L.) Fruit**" submitted for the degree of **Doctor of Philosophy** in the subject of **Biochemistry** to the **Chaudhary Charan Singh Haryana Agricultural University, Hisar,** is a bonafide research work carried out by **Ms. Reena Devi** under my supervision and guidance and that no part of this thesis has been submitted for any other degree.

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ABBREVIATIONS

µM	:	Micromolar
µmol	:	Micromole(s)
2, 6-DCPIP	:	2, 6-Dichlorophenol indophenol
ACC	:	1-aminocyclopropane-1-carboxylate
ADF	:	Acid detergent fibres
APS	:	Ammonium persulphate
APX	:	Ascorbate peroxidase
BSA	:	Bovine serum albumin
Ca	:	Calcium
$CaCl_2$	:	Calcium chloride
CAT	:	Catalase
CD	:	Critical difference
cm	:	Centimeter
Eq	:	Equilvalent weight
d	:	Day/days
DOS	:	Days of storage
DTNB	:	Dithiobis-2-nitrobenzoic acid
EDTA	:	Ethylene diamine tetra acetic acid
f.wt.	:	Fresh weight
Fig.	:	Figure
g	:	Gram
GA_3	:	Gibberellic acid
GR	:	Glutathione reductase
h	:	Hour
ha	:	Hactare
IG	:	Immature green
kDa	:	Kilodalton
kg	:	kilogram
L/l	:	Litre
LOX	:	Lipoxygenase
M	:	Molar
MG	:	Mature green
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
mm	:	Millimeter
M_r	:	Molecular weight
mRNA	:	Messenger RNA
MT	:	Metric ton

NADPH	:	Nicotinamide adenine dinucleotide phosphate reduced
NBT	:	Nitroblue tetrazolium
NDF	:	Neutral Detergent Fibres
nmol	:	Nanomole(s)
^{o}C	:	Degree Celsius
OR	:	Overripe
PAGE	:	Polyacrylamide gel electrophoresis
PG	:	Polygalacturonase
pI	:	Isoelectric point
PLW	:	Physiological loss in weight
PME	:	Pectin methylesterase
POX	:	Peroxidase
PUFA	:	Polyunsaturated fatty acids
PVP	:	Polyvinyl pyrrolidone
R	:	Ripe
R_m	:	Relative mobility
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rpm	:	Revolution per minute
s	:	Second
SDS	:	Sodium dodecyl sulphate
SOD	:	Superoxide dismutase
T	:	Turning
TA	:	Titratable acidity
TCA	:	Trichloro acetic acid
TEMED	:	N,N,N',N'–Tetra ethyl methylene diamine
Tim.	:	Time
Temp.	:	Temperature
Tris	:	Tris (hydroxymethyl) amino methane
Tris-HCl	:	Tris (hydroxymethyl) amino methane – hydrochloride
TSS	:	Total soluble solids
Vol.	:	Volume
vs	:	versus
Wt.	:	Weight

CHAPTER-I

INTRODUCTION

Guava (*Psidium guajava* L.), also known as "apple of the tropics", is one of the most delicious fruits popular among both the rich and the poor because of its high nutritive value, availability at moderate price, a pleasant aroma and good flavor. In India, it is 5th most widely grown fruit, occupying an area of 2.2 lakh ha, with annual production of 22.7 lakh MT (Anonymous, 2010). In Haryana, area under guava cultivation is 7.8 thousand ha with an annual production of 55.8 thousand MT (Anonymous, 2010).

Guava is a climacteric fruit which ripens rapidly after harvest, and loses its texture and quality in 3-4 days at ambient temperature. It contains high percentage of their fresh weight as water and consequently exhibit relatively high metabolic activity which continues post-harvest and makes it highly perishable commodity. Its soft skin makes it susceptible to bruising and mechanical injury due to which it cannot be stored for more than a week even during winter season. During peak harvesting time, when there is a glut of its fruits in the local market, huge post-harvest losses are observed. To avoid glut and to reduce per cent losses in guava, it becomes desirable to evolve technologies for prolonging its keeping quality through delaying the softening process so as to improve the opportunity of its transportation to distant market. Development of practical solutions to these post-harvest problems requires detailed understanding of biochemistry and molecular biology of fruit ripening process, which is a genetically programmed and highly coordinated physiological event of organ transformation from unripe to ripe stage.

Fruit ripening is a stressful process and is considered to be a functionally modified protracted form of senescence. It includes a series of biochemical, physiological and structural changes such as synthesis and degradation of pigments, conversion of starch to sugars, production of volatiles, increase in ethylene production and hydrolysis of cell wall components which are associated with the changes in firmness and texture of a fruit to yield an attractive edible fruit with optimum blend of colour, taste, aroma and texture (Biale *et al.* 1975; Brady, 1987). During ripening, fruit softening occurs due to enzymatic hydrolysis of cell wall polysaccharides like cellulose, hemicellulose, calcium pectate, polyuronides and glycoproteins (Ali *et al.* 2004; Missang *et al.* 2004; Rosli *et al.* 2004), and membrane deterioration occurs due to over accumulation of reactive oxygen species (ROS) leading to cellular decompartmentation and loss of tissue structure (Ahn *et al.* 2002; Reddy and Srivastava, 2003; Mondal *et al.* 2004).

Plants, however, possess an impressive array of defense mechanisms against oxidative stress including the enzymatic and non-enzymatic antioxidant systems (Foyer and

Noctor, 2000; Shewfelt and del Rosario, 2000; Apel and Hirt, 2004). The antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Allen, 1995), while the non-enzymatic antioxidants include water soluble (ascorbate, glutathione, phenolic compounds and flavonoids) and lipid soluble (α-tocopherol, β–carotene, lycopene) metabolites (Anderson *et al.* 1995; Rao *et al.* 1998). Superoxide dismutases (SODs), the metalloenzymes, are believed to play a crucial role in antioxidant defense (Alscher *et al.* 2002) because they catalyze the dismutation of superoxide radical ($^{\cdot}O_2^-$) to hydrogen peroxide (H_2O_2) while removal of H_2O_2 is taken care of by CAT and/or POX and enzymes of ascorbate-glutathione pathways (Asada, 1994). Inspite of such an efficient scavenging system, accumulation of ROS occurs due to stimulation by various environmental, mechanical and physical stresses (Legendre *et al.* 1993) such as exposure to drought (Selote and Khanna, 2006), heavy metals (Valko *et al.* 2006), high salt concentration (Hasegawa *et al.* 2000; Chawla *et al.* 2013) and biotic factors such as invasion of various pathogens (Bolwell *et al.* 2002; Perez and Brown, 2014). In addition, ROS production has also been reported to be stimulated during plant senescence (Prochazkova *et al.* 2001; Lee *et al.* 2012; Woo *et al.* 2013) and during ripening and storage of fruits (Hodges *et al.* 2001; Jimenez-Bermudez *et al.* 2002; Ram, 2007; Mondal *et al.* 2009; Kumar *et al.* 2011).

Just like any other development process, fruit ripening is also probably under the control of plant growth regulators (McGlasson *et al.* 1978; Bruinsma, 1983). Ethylene is routinely found to be involved in ripening (Lelievre *et al.* 1997) and hence is referred to as the "ripening hormone". Efforts have been made to increase the shelf-life of fruits using antisense RNA technology to inactivate the expression of ripening related proteins such as ethylene biosynthetic enzymes *viz*., ACC synthase and ACC oxidase (Gomez *et al.* 2009; Johnston *et al.* 2009) and cell wall degrading enzymes *viz.*, polygalactouronase (PG) and pectin methylesterase (PME) (Jimenez-Bermudez *et al.* 2002). Silencing of specific genes in transgenic plants has made it possible to genetically modify fruit ripening process to achieve the desired objective to delay ripening and prevent losses of fruits during their storage and transportation. By the use of these modern molecular techniques, only a limited success has been achieved in the direction of preventing fruit loss during storage and transportation. Moreover, this involves the development of socially controversial genetically engineered fruit crops. In order to achieve a big breakthrough and in order to develop a technology acceptable to general public, the problem needs to be tackled from different angles. Therefore, exogenous application of chemicals is, still being used to retard the physiological changes of the produce and to increase their shelf-life.

Different methods like use of growth regulators, hot water treatment, low temperature storage, irradiation, etc., can be employed to enhance the shelf-life of various fruits (Verma

and Joshi, 2002). The use of certain chemicals or plant growth hormones was also employed to delay ripening, to reduce losses and to improve and maintain the color and quality by slowing down the metabolic activities of the fruit (Huang and Jiang, 2012). These chemicals are reported to arrest the growth and spread of micro organisms by reducing the shriveling which ultimately leads to an increased shelf-life and maintain the marketability of the fruit for a longer period.

Gibberellins are a group of growth substances, known to retard ripening and acts as antisenescent agent during storage. Gibberellic acid (GA_3) affects the degradation of complex carbohydrates as well as synthesis of sucrose and thereby delayed ripening in papaya (Rajkumar *et al.* 2005), banana (Osman and Abu-Goukh, 2008; Alfonso and Johnny, 2010), tomato (Pila *et al.* 2010) and sapota (Yadav *et al.* 2013) fruits. GA_3 treatment decreased the tissue permeability and thereby reduced physiological loss in weight and decay per cent in tomato (Pila *et al.* 2010; Kumar *et al.* 2005; Choudhary and Dhruve, 2014). Calcium (Ca) delays the process of ripening, more particularly softening and hence, increases the shelf-life by altering intercellular and extracellular processes (Chrardonnet *et al.* 2003). Calcium ion forms cross-links between pairs of negatively charged homogalacturonans (Picchioni *et al.* 1998), thus improves rigidity of cell walls and obstructs enzymes such as PG from reaching their active sites, there by retarding tissue softening and ultimately delaying ripening (Marzouk and Kassem, 2011). The activities of PG and PME enzymes of fresh-cut dragon fruit were decreased when treated with $CaCl_2$ (Chuni *et al.* 2010). Treatment of strawberry fruits with $CaCl_2$ at the concentration of 0.05 % has resulted in an increased shelf-life (Asrey and Jain, 2005). Decreased electrolyte leakage by calcium application has been reported to increase antioxidative enzymes activities (Mortazavi *et al.* 2007). Calcium was has been found to maintain the freshness of various fruits e.g. honeydew (Saftner *et al.* 2003), strawberries (Aguayo *et al.* 2006) and mango (Souza *et al.* 2006). This makes it imperative to study the biochemistry and molecular biology of fruit ripening and to investigate the role of GA_3 and $CaCl_2$ in ripening related changes in guava where practically no such information is available.

Cell wall disassembly in ripening fruit is a complex process involving the dismantling of multiple polysaccharide networks by a diverse group of wall modifying proteins including PG, PME and pectate lyase (PL) (Nishiyama *et al.* 2007). Loss of fruit firmness has been correlated with decrease in pectin substances (Rosli *et al.* 2004) which is intimately related to PG, a pectin depolymerising enzyme. PG gene was the first gene to be cloned for studying textural regulation in tomato and the transformed tomato with PG antisense gene resulted in improved fruit with firmer texture and extended shelf-life (Hadfield and Bennett, 1998). This gave remarkable clues regarding the role of PG in fruit cell wall metabolism. Despite similar catalytic properties, PGs differ from fruit to fruit, thus reducing the per cent homology of PG

genes. PG has been purified and characterized from a number of fruits including banana, Jamaica cherry and mango (Pathak *et al.* 2000; Gayathri *et al.* 2007; Prasanna *et al.* 2006; Gayathri and Nair, 2014). However, not much information is available on the characteristics of polygalacturonase from guava fruit.

Keeping these viewpoints in forefront, the present study was proposed to provide base-line information regarding the biochemical changes in guava fruit during ripening and to find out the suitable post-harvest treatment for improving the storage life of guava fruits with the following objectives:

1. To study the biochemical changes during ripening of guava fruit
2. To study the effect of GA_3 and $CaCl_2$ on the quality with regard to biochemical characteristics of guava fruit
3. To purify and characterize polygalacturonase for its physicochemical and kinetic properties

CHAPTER-II

REVIEW OF LITERATURE

Fruit ripening is a highly co-ordinated, genetically programmed, irreversible and metabolically active process involving a series of physiochemical, biochemical and organoleptic changes which influence appearance, texture, flavor and aroma of fruit so that it can achieve an edible final quality for consumption (Giovannoni, 2001, 2004; Bouzayen *et al.* 2010; Gapper *et al.* 2013). These changes during ripening and post ripening take place very quickly in guava. Because of excessive textural softening, fully ripe guava fruits bruise easily and are highly perishable which leads to mechanical injuries, high incidence of rotting caused by fungi, shriveling of the fruit and malformation (Jain *et al.* 2003). It depreciates the post-harvest quality of guava and makes its handling, storage, transport and marketing troublesome (Kader, 2002). To regulate the supply of quality fruits for longer period in domestic and distant markets, per cent losses in guava can be reduced by enhancing the shelf-life. Calcium contributes to improve the turgidity of cell wall and retards tissue softening and hence, delays fruit ripening (Goncalves, 2000). Similarly application of gibberellic acid has been reported to delay senescence in fruits (Kumar *et al.* 2005; Pila *et al.* 2010). Practically no information is available on the role of GA_3 and $CaCl_2$ in ripening related changes in guava. The literature pertaining to the subject, "Role of gibberellic acid and calcium chloride in ripening related biochemical changes in guava (*Psidium guajava* L.) fruit" has been reviewed under the following heads:

2.1 Physico-chemical and biochemical changes in fruits during ripening

2.2 Effect of gibberellic acid and calcium chloride treatments on ripening related changes in fruits

2.3 Purification and characterization of polygalacturonase enzyme

2.1 Physico-chemical and biochemical changes in fruits during ripening

2.1.1 Physico-chemical changes

Firmness of the fruit is one of the indices for determining the maturity of the fruits. In fruits, firmness is mainly due to pectin polymers (Fertonani, 2006), can be bonded to ions, mainly Ca^{2+}, which maintains adjacent chains bonded among themselves (Taiz and Zeiger, 2004). Abu-Goukh and Bashir (2003) and Hind *et al.* (2003) reported decrease in fruit firmness during ripening of guava. The decline in firmness was about 8 fold from hard mature green to final soft ripen stage.

The total soluble solids (TSS) which generally contain sugar, mineral and acids, give a reliable index to judge the proper stage of maturity. Hedge and Chharia (2004) and Tosun *et al.* (2008) reported that TSS increased significantly throughout development and ripening in

guava and blackberries respectively. Similarly, Bashir and Abu-Goukh (2003) reported that TSS increased 1.2 fold during ripening in guava. Conversion of starch and other insoluble carbohydrates into soluble sugars was reported to be accountable reason for initial increase in TSS of fruits (Singh *et al.* 1981) whereas slight decline at the later stages was due to utilization of soluble solids and sugars in respiratory processes.

Titratable acidity (TA) gives a measure of the amount of acid present in a fruit. The organic acids contributing to acidity in guava are mainly citric, malic, glycolic, tartaric and lactic acids (Hui, 2006). Organic acids usually decline during ripening as they are respired or converted to sugars. Titratable acidity increased during development, but was less in ripe blackberry fruits (Tosun *et al.* 2008). However, Damodaran *et al.* (2001) have reported that acidity declined throughout the period of ripening in sapota *cv.* Cricket Ball. Similar results were observed by Nag *et al.* (2011) in guava.

2.1.2 Biochemical changes

2.1.2.1 Sugars, cell wall components and their hydrolyzing enzymes

The sugars present in fruits impart sweetness, which influence the taste and flavor. Tosun *et al.* (2008) recorded increase in total sugars during the ripening period in blackberries. The increase in sugar with the fruit growth might be due to conversion of certain cell wall material such as pectin and hemicelluloses into reducing substance during ripening (Kumar, 2008). The increase in total sugars might be due to the conversion of starch into simple sugar and later on reduction due to utilization of sugar in the process of respiration.

The level of the cell wall material decreases with the enhancement of ripening (Sharma and Siddiqui, 2004) thus causing structural changes in pectin, hemicellulose and cellulose which are assumed to be responsible for the dissolution of the middle lamella and disruption of the primary cell wall during ripening-related loss of firmness (Crookes and Grierson, 1983; Seymour *et al.* 1990). Yadav *et al.* (2012) reported that cellulose and pectin contents decreased during ripening in ber fruit. Jain *et al.* (2001) reported that the cell wall constituents *viz.* cellulose, hemicelluloses and lignin decreased up to ripe (R) stage, while the pectin content decreased throughout up to overripe (OR) stage in guava. In strawberry fruits, hemicellulose and cellulose decreased during ripening (Rosli *et al.* 2004; Sharma and Siddiqui, 2004). However, no change in cellulosic fractions was observed during ripening of stone fruits (Jain *et al.* 2003). Decrease in total pectin has been also reported during ripening of fruits (Jain *et al.* 2003; Ali *et al.* 2004).

The decrease in cell wall components during ripening is intimately related to the activity of cell wall degrading enzymes (Lunn *et al.* 2013). Pectin methyl esterase is responsible for the de-esterification of pectin before it is depolymerized by polygalacturonase, which degrades demethylated rather than methylated pectin. Therefore, PME may play an important role in determining the extent to which pectin is accessible to degradation by PG.

Polygalacturonase and cellulase activities have been reported to increase progressively during ripening with a high correlation between the increase in enzyme activity and fruit softening and pectin esterase (PE) followed the climacteric pattern of respiration in guava (Abu-Goukh and Bashir, 2003) and tomato (Ali and Abu-Goukh, 2005). Jain *et al.* (2001) observed that PG and cellulase exhibited progressive increase in activity throughout ripening, while PME activity increased up to T stage and then decreased up to OR stage in guava. Yadav *et al.* (2012) reported 20 and 10, 8.4 and 5.7 and 5.5 and 4.4 fold increase in PME, PG and cellulase activities, respectively during ripening of two varieties of ber fruits differing in their shelf lives, *viz.* Umran and Ilaichi. The basal level of activities of all these enzymes was higher at all the stages of ripening in Illaichi variety, having short shelf-life, as compared to Umran, with long shelf-life. Similar observations were made earlier in tomato (Barka *et al.* 2000), apple (Goulao *et al.* 2007) and guava (Carvalho *et al.* 2009). Increase in PG activity during ripening has also been reported in guava (Mondal, 2005) and ber fruit (Yadav *et al.* 2012). Changes in cell wall polysaccharides during ripening of bush butter fruit is related with increase in PG activity (Missang *et al.* 2004).

2.1.2.2 Lipid peroxidation and oxidative stress

Fruit ripening is a stressful process, considered to be a functionally modified protracted form of senescence. Through various biological redox reactions, a number of ROS *viz.* superoxide radical ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), alkoxyl radical ($RO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), nitric oxide radical ($NO^{\cdot}$) are generated as by-products of cellular metabolism (Masaki *et al.* 1999; Terman *et al.* 2006). Accumulation of ROS leads to fruit maturation and aging (Esterhazy *et al.* 2008). Particularly, superoxides and hydroxyl radicals are strong oxidizing species among the ROS that can rapidly attack all types of biomolecules, leading to irreparable metabolic dysfunction and cell death (Halliwell and Gutteridge, 1989; Chaudhuri *et al.* 2012). The hydroxyl radical reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids (Chaudhuri *et al.* 2012). It has been reported that fruits like saskatoon (Rogiers *et al.* 1998), tomato (Mondal *et al.* 2004) and orange fruit (Huang *et al.* 2007) accumulate ROS during ripening. It was documented that fruits with better ability to scavenge ROS may have higher shelf-life (Mondal *et al.* 2009).

Oxidative stress can be best assessed by the extent of lipid peroxidation catalyzed by lipoxygenase (LOX) which plays a central role in membrane deterioration by peroxidizing free polyunsaturated fatty acids. Membrane deterioration due to increased LOX activity has been reported to be responsible for loss of tissue structure during fruit ripening (Mondal *et al.* 2004; Liu *et al.* 2008). Indices of oxidative stress *viz*. LOX activity, malondialdehyde value and H_2O_2 content increased throughout during ripening in guava (Mondal *et al.* 2009) and ber (Kumar *et al.* 2011) fruits. Kumar *et al.* (2011) observed higher values of these parameters in

Kaithali variety of ber (short shelf-life) as compared to Umran variety (long shelf-life), at almost all stages of fruit ripening which suggested that membrane deterioration and hence loss of membrane integrity and tissue structure is much faster in soft variety than in the firm one.

2.1.2.3 Antioxidative enzymes and metabolites

Plants, however, possess an impressive array of defense mechanisms against oxidative stress including the enzymatic and non-enzymatic antioxidant systems (Foyer and Noctor 2000; Shewfelt and Del Rosario, 2000; Apel and Hirt, 2004). Antioxidant defense systems work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS (Gill, 2010).

Superoxide dismutases, a metalloprotein, catalyzing the dismutation of superoxide radicals to H_2O_2 and molecular oxygen, is considered to be a key antioxidant in aerobic cells (Fridovich, 1986). SOD activity has been reported to decrease in orange (Huang *et al.* 2007). Maximum SOD activity was observed at turning pink stage of tomato and color turning stage of ber (Thakur and Pandey, 1999; Kumar *et al.* 2011). However, SOD activity increased upto ripe stage followed by a decline in tomato fruits (Ahn *et al.* 2002). Reddy and Srivastava (2003) and Mondal *et al.* (2004) observed that the activity of SOD passed through a minimum level at mature green and breaker stage of ripening in mango and tomato, respectively. During storage, SOD activity decreased in tomato fruit (Mondal *et al.* 2003), while its activity increased in pear fruit (Pinto *et al.* 2001).

Catalase is one of the primary enzymatic defenses against oxidative stress induced by senescence (Zimmermann *et al.* 2006) and most of its activity is associated with peroxisomes where it removes the H_2O_2 formed during photorespiration (Foyer and Mullineaux, 1994). Mondal *et al.* (2009) observed that CAT activity increased up to turning (T) stage of guava and decreased thereafter. However, CAT activity increased continuously during ripening of tomato (Andrews *et al.* 2004; Mondal *et al.* 2004) and ber fruits (Kumar *et al.* 2011) whereas, in saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007), it has been reported to decrease continuously. Decline in catalase activity has been reported during storage of pear (Pinto *et al.* 2001) and tomato (Mondal *et al.* 2003) fruits.

Peroxidase is involved in the scavenging of H_2O_2 that is not removed by CAT (Willekens *et al.* 1997). The literature is conflicting regarding the profile of POX activity during ripening of fruits. A continuous decrease in POX activity has been reported during ripening in tomato (Mondal *et al.* 2004), guava (Ram, 2007) and ber (Kumar *et al.* 2011). On the contrary, an increase in POX activity during ripening of tomato fruits has been observed by Thakur and Pandey (1999). Ahn *et al.* (2002) demonstrated highest POX activity at orange stage and lowest at red stage in tomato. Mango fruit, however, exhibited highest POX activity during mature green stage, which significantly declined as the ripening proceeded (Reddy and Srivastava, 2003).

Ascorbate peroxidase, widely distributed antioxidant enzyme which reduces H_2O_2 to water using ascorbate as the electron donor. Activity of APX increased up to T stage and decreased thereafter in papaya (Silva *et al.* 1990), tomato (Andrews *et al.* 2004) and guava (Mondal *et al.* 2009). Similiarly, APX activity increased during initial stages but thereafter decreased during ripening in tomato (Mondal *et al.* 2004), guava (Ram, 2007) and ber fruits (Kumar *et al.* 2011). However, a continuous decrease in APX activity has been reported during ripening of orange (Huang *et al.* 2007). Decline in APX activity at overripe (OR) stage has been reported to result in the inefficiency of antioxidant system to scavenge ROS, causing oxidative stress and lead to the accumulation of endogenous H_2O_2, membrane deterioration and loss of tissue structure during fruit ripening (Hossain *et al.* 2006). The decrease in APX activity at OR stage may be either due to substrate limitation or the enzyme inactivation.

Glutathione reductase is the principal soluble antioxidant enzyme which has a major role in maintaining the intracellular glutathione pool in reduced state (McKersie and Leshem, 1994). Activity of GR increased during initial stages but thereafter decreased during ripening in tomato (Andrews *et al.* 2004; Mondal *et al.* 2004), in ber fruits (Kumar *et al.* 2011) and in guava (Mondal *et al.* 2009). Contrarily, continuous increase in GR activity was observed during ripening of saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007). In ber, Kumar *et al.* (2011) observed that antioxidative enzymes exhibited comparatively higher activity in Umran, a long shelf-life variety as compared to Kaithali, a short shelf-life variety. Lower activities of antioxidative enzymes associated with higher oxidative stress in Kaithali, suggest the accumulation of ROS to be responsible for comparatively higher membrane deterioration and loss of tissue structure in Kaithali.

Glutathione is the major low molecular weight thiol compound which acts as reductant to protect thiol groups on enzymes, regenerates ascorbate and reacts with singlet oxygen and hydroxyl radical (Arora *et al.* 2002). Mondal *et al.* (2009) reported that guava fruits had maximum content of total, reduced and oxidized glutathione at the MG stage and reduced glutathione was more than the oxidized one at all the stages of ripening inguava. Tomato and saskatoon fruit also responded to the increase in oxidative stress by increasing reduced and oxidized glutathione during development (Andrews *et al.* 2004).

Ascorbate acts as important antioxidant in removal of H_2O_2 and can scavenge $^1O_2$, $^{\cdot}OH$ and $^{\cdot}O_2^-$ radicals. It also acts as electron donor for APX (Hernandez *et al.* 2004). During ripening, a concomitant increase in ascorbic acid and APX activity from immature green (IG) to turning (T) stage and thereafter, a decline was observed in apple (Joshi *et al.* 2004), tomato (Mondal *et al.* 2004), guava (Mondal *et al.* 2009) and ber fruit (Kumar *et al.* 2011). Increase in ascorbic acid content as the fruit matures has been attributed to the breakdown of starch to glucose which was used in the biosynthesis of ascorbic acid (Lim *et al.* 2006). During storage, decrease in ascorbic acid was observed by Leong and Shui (2002) that could be due to

conversion of ascorbic acid to dehydroascorbic acid by action of ascorbic acid oxidase (Singh *et al.* 2005).

β-carotene, a fat soluble vitamin, is a potent antioxidant and singlet oxygen quencher (Rao and Rao, 2007). Kumar *et al.* (2011) revealed that during ripening there was a continuous decrease in β-carotene in ber from IG to OR stage. These findings are contrary to the results reported in mandarin (Lallan and Godara, 2005) and capsicum (Ha *et al.* 2007), where carotenoid content has been reported to increase during ripening.

Several phytochemicals, such as flavonoids, phenolic acids, amino acids, ascorbic acid, tocopherols and pigments, might contribute to the total antioxidant activity (Chu *et al.* 2000). It has been reported that ascorbic acid and anthocyanins are the major antioxidant components in strawberries (Hannum, 2004) whereas, according to Lim *et al.* (2006), total phenol content and ascorbic acid content in guava contributed to total antioxidant capacity.

2.1.2.4 Ethylene and ACC oxidase

Ethylene induces a mature, non growing tissue to rapidly differentiate into a new state-to switch from non-ripening to ripening. That eventually led the scientists to consider this growth regulator as the 'ripening hormone'. It affects the transcription and translation of many ripening-related genes (Giovannoni, 2001; Hiwasa *et al.* 2003).

In climacteric fruits, ripening is accompanied by a peak in respiration and a concomitant burst of ethylene whereas in non-climacteric fruits, respiration shows no dramatic change and ethylene production remains at a very low level (Alexander and Grierson, 2002). Ladaniya (2007) reported that ethylene plays an important role in changing fruit color, flavor, chemical composition and texture of citrus fruit. Exogenous application of ethylene exerts a positive response on respiration and promotes ripening in citrus fruit although they produce very low levels of ethylene throughout development.

The increase in ethylene production that is induced by fruit ripening is accompanied by increase in the activities of both ACC synthase and ACC oxidase (Yang and Hoffman, 1984; Mathooko, 1996). ACC oxidase activity has been reported to reach its maximum during the ripened stage of mango fruit (Nair *et al.* 2004). Gomez *et al.* (2009) isolated papaya ACC oxidase gene using PCR and transformed embriogenic cells to drive the expression of the PCR fragment in sense orientation. Fruits were evaluated and observed a sharp reduction in ethylene and CO_2 production, whereas softening and color development of the peel were also altered. Overall, transgenic fruits showed a delay in ripening rate. A reduction in mRNA level for ACC oxidase in transgenic fruit was clearly detectable by northern blot.

2.2 Effect of gibberellic acid and calcium chloride treatments on ripening related changes in fruits

Gibberellins are known to hinder ripening and senescence of fruits (Ben-Arie *et al.* 1995). The mechanism by which GA_3 retards ripening has not been clearly elucidated, but it may be supposed to act at the gene level, or through modifying the effect of other hormones.

These growth regulators affect the degradation of complex carbohydrates as well as synthesis of sucrose, ethylene production and there by delayed ripening (Rossetto *et al.* 2003). Similar results have been reported in papaya (Rajkumar *et al.* 2005), tomato (Pila *et al.* 2010), banana (Alfonso and Johnny, 2010) and sapota fruit (Yadav *et al.* 2013).

Physiological loss in weight (PLW) of fruit has been reported to increase significantly with the increase in storage period due to the increase in respiration and water evaporation during storage in mango (Jawandha *et al.* 2012). The GA_3 treatment decreased tissue permeability and thereby reduced PLW in tomato (Choudhary and Dhruve, 2014) which might be due to its anti-senescent action (Kumar *et al.* 2005; Pila *et al.* 2010; Choudhary and Dhruve, 2014). During storage GA_3 had effectively reduced PLW in mango (Kumar, 1998), guava (Tamil and Bal, 2005) and papaya (Rajkumar *et al.* 2005). Dipping of plum fruits in solution of GA_3 (40 ppm) significantly reduced the PLW and retained firmness for longer period during storage (Mahajan *et al.* 2008). GA_3 treatment might slow down the process of ripening by retarding the pre climacteric respiration rate and subsequently ethylene production (Jagadeesha *et al.* 2015).

Hiwale and Singh (2003) found that guava treated with 200 ppm of GA_3 showed higher firmness as compared to control. Similar results were observed by Tamil and Bal (2005) on treating guava *cv.* Sardar, with 25 ppm. Sapota fruits treated with 100 ppm GA_3 and 100 ppm Kinetin retained its firmness (Yadav *et al.* 2013). Similar results were experienced by Mahajan and Dhatt (2004) in Asian pear, Rajkumar *et al.* (2005) in papaya and Alfonso and Johnny (2010) in banana. The combined application of phenyl urea and 100 ppm GA_3 retained firmness for longer time in banana during storage (Huang *et al.* 2014).

Increase in TSS for few days in storage followed by gradual decrease till end of storage was observed in guava (Sharma and Dashora, 2001; Abu-Goukh and Bashir, 2003; Mahajan *et al.* 2004), ber (Kannan and Thirumaran, 2003; Singh *et al.* 2007), mandrin (Bhardwaj *et al.* 2005), sapota fruit (Pawar *et al.* 2011). Guava fruits treated with GA_3 and kinetin showed reduced TSS during storage as compared to control (Hiwale and Singh, 2003; Tamil and Bal, 2005; Mahajan *et al.* 2011). The slower respiration slows down the biosynthetic processes, resulting in lower TSS due to the slower change from carbohydrates to sugars in tomato (Pila *et al.* 2010; Choudhary and Dhruve, 2014). However, the sapota fruits treated with 100 ppm GA_3 showed increase in TSS of up to 10 days during storage (Yadav *et al.* 2013). Similarly, papaya fruits treated with 100 ppm GA_3 maintained higher TSS during storage (Rajkumar *et al.* 2005). The increase in TSS might be due to slow conversion of insoluble sugars into soluble forms and least utilization of organic acids in respiration.

During storage, decrease in fruit acidity was observed which might be due to conversion of acids into salts and sugars by the enzymes particularly invertase (Hiwale and

Singh, 2003; Tamil and Bal, 2005; Mahajan *et al.* 2011). In sapota, minimum decrease in acidity was observed in fruits treated with 200 ppm GA_3 (Yadav *et al.* 2013). However, papaya fruits treated with 100 ppm GA_3 maintained higher acidity value up to 9^{th} day of storage (Rajkumar *et al.* 2005). Similarly, titratable acidity in GA_3 treated tomato was more as compared to the control (Kumar *et al.* 2005; Pila *et al.* 2010; Choudhary and Dhruve, 2014).

Bhardwaj *et al.* (2005) found that ascorbic acid content of mandarin reduced during storage period. The decrease in ascorbic acid during storage was due to oxidative destruction of ascorbic acid by ascorbic acid oxidase. Ascorbic acid could be retained up to some extent by using 200 ppm GA_3 in guava (Hiwale and Singh, 2003) and in sapota (Kadu and Gajipara, 2009) and by using 50 ppm GA_3 in custard apple (Patel *et al.* 2011). In banana, 50 mg GA_3 showed maximum retention of ascorbic acid during storage (Huang *et al.* 2014). This might be due to the respiration of fruits or oxidation of ascorbic acid content was reduced by GA_3 (Kher and Bhat, 2005; Kumar *et al.* 2005; Pila *et al.* 2010; Choudhary and Dhruve, 2014). Using GA_3 (50 mg), the antioxidant activity was maintained in banana (Alfonso and Johnny, 2010; Huang *et al.* 2014) and in broccoli (Huang and Jiang, 2012) during storage. The GA_3 treatment also resulted in lowering the activities of cell wall degrading enzymes such as PME, PG and cellulase as compared to the untreated tomato fruits (Choudhary and Dhruve, 2014).

Calcium delays the process of ripening, more particularly softeningand hence, increases the shelf-life by altering intercellular and extracellular processes (Chrardonnet *et al.* 2003). Calcium is involved in the formation of calcium pectate in which it forms intermolecular bridges between pectin molecules (Luna-Guzman and Barrett, 2000) and hence, it increases rigidity of the middle lamella and cell walls, leading to increased resistance to PG, PME and β-Gal activities (Mignani *et al.* 1995). It also contributes to firmness by stabilizing the cell membrane and reducing tissue water loss, thereby increasing cell turgor pressure (Mignani *et al.* 1995; Picchioni *et al.* 1998). Ferguson (1984) reported that calcium directly influenced membrane lipid peroxidation by lowering the concentration of ROS during ripening of apple. Increase in tissue calcium content by post-harvest calcium treatment maintained storage quality of whole fruit including strawberry (Hernandez-Munoz *et al.* 2008), peach (Manganaris *et al.* 2007) and fresh-cut produce (Saftner *et al.* 2003; Silveira *et al.* 2011).

Calcium treatment effectively maintained fruit firmness compared to non-treated guava fruit (Kumar *et al.* 2011). Ishaq *et al.* (2009) reported that 3 % $CaCl_2$ dips improved firmness in apricot fruits. On treating fruits with $CaCl_2$ (1 %), TSS of fruit juices found to be increased might be due to slow utilization of starch or sugars in respiration (Mirdehghan and Ghotbi, 2014). Post-harvest calcium treatment enhanced ascorbic acid content of cornelian cherry (Aghdama *et al.* 2013). Mujtaba *et al.* (2014) reported that 2 % $CaCl_2$ was more effective in maintaining titratable acidity as compared to 1 % and 3 % $CaCl_2$. Similarly,

higher values for total phenolic content, ascorbic acid and total antioxidant content were also achieved for the 2 % $CaCl_2$ as compared to 1 % and 3 % $CaCl_2$. Aghdama *et al.* (2013) demonstrated that a post-harvest $CaCl_2$ treatment also enhanced antioxidant capacity of cornelian cherry fruit by effectively maintaining higher total phenols, total flavonoids and anthocyanin contents. Similarly, post-harvest $CaCl_2$ treatment also maintained higher total phenols and total ascorbic content (Ramezanian *et al.* 2010) in pomegranate fruit that could be due to stimulating the phenylpropanoid pathway by increasing the PAL activity (Jacobo-Velazquez *et al.* 2011; Aghdama *et al.* 2013).

Calcium can delay the onset of the ethylene climacteric period and climacteric peak (Ben-Arie *et al.* 1995). High calcium concentrations resulted in decreased ethylene production and electrolyte leakage (Hewajulige *et al.* 2003). Shirzadeh *et al.* (2011) recorded minimum ethylene production in 4 % calcium treated apple fruits while, maximum ethylene was observed in untreated fruits. Ben-Arie *et al.* (1995) reported that calcium delayed the onset of the climacteric rise in respiration and ethylene evolution.

Due to possible role of calcium in protecting the pectic backbone from the enzymes, calcium was found to maintain the freshness of various types of cut fruits e.g. honeydew (Saftner *et al.* 2003), strawberries (Aguayo *et al.* 2006) and mango (Souza *et al.* 2006). The activities of PG and PME enzymes of fresh-cut dragon fruit were decreased when treated with $CaCl_2$ as observed by Chuni *et al.* (2010). Decreased electrolyte leakage by calcium application has been reported to increase antioxidative enzymes activities, the cell wall integrity and stability (Mortazavi *et al.* 2007). The combination of hot air and $CaCl_2$ increased SOD and CAT activities in comparison to untreated apple fruits and higher activities of these enzymes lead to decrease of H_2O_2 (Rabie *et al.* 2011). Shirzadeh *et al.* (2011) recorded more CAT activity in 2 % calcium, as compared to the control. SOD activity was higher in $CaCl_2$ treated fruits as reported by Schrmitz-Eiberger *et al.* (2002).

2.3 Purification and characterization of polygalacturonase enzyme

Polygalacturonases fall into the group of enzymes termed as polysaccharide lyases or polysaccharide eliminases. PG catalyzes the hydrolytic cleavage of α (1→4) galacturonan linkages of pectin which are the main constituents of middle lamella and primary cell wall of plant cells. Need of studying PG is pertinent as this is one of the major enzymes involved in pectin catabolism in fruits during ripening. Softening in mango and strawberry has been reported to be accompanied by a decline in pectin (Roe and Bruemner, 1981; Rosli *et al.* 2004). Furthermore, this decline in alkaline soluble pectin was found to be correlated with the loss of firmness of the mango fruit and was also closely correlated with the increase in PG activity during fruit ripening.

Three multiple forms of polygalacturonase namely PGI, PGII and PGIII were isolated, purified and characterized from ripe mango fruit by Singh and Dwivedi (2008).

Native molecular weights of PGI, PGII and PGIII were found to be 120, 105 and 65 kDa, respectively. On SDS–PAGE analysis, PGI was found to be a homodimer of subunit size 60 kDa each while those of PGII and PGIII were found to be heterodimers of 70, 35 and 38, 27 kDa subunit size each, respectively. Three isoforms of PG differed with respect to the effect of pH, metals, reducing agents and their susceptibility towards heat. PGI and PGIII exhibited inhibition at high substrate concentration while PGII did not. Km for polygalacturonic acid was found to be 0.02 % for PGI. PGIII was purified from banana fruit pulp by Pathak *et al.* (2000). The molecular weight of the native enzyme was found to be 90±10 kDa with a subunit molecular weight of 29±2 kDa. The enzyme exhibited optimum activity at pH 4.3 and temperature 40°C. A unique property of the enzyme was the requirement of thiol groups for the enzyme activity. The enzyme was inhibited by p-CMB and activated by 2-ME and DTT. The inhibition of p-CMB could be reversed by DTT. The Km of the enzyme was 0.15 % for polygalacturonic acid.

CHAPTER-II

MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Fruits

The present investigations were carried out on two cultivars of guava (*Psidium guajava* L.) fruit *viz*. L-49 (shelf-life 7-8 days) and Hisar Surkha (shelf-life 4-5 days) procured from the Horticulture Farm, CCS Haryana Agricultural University, Hisar.

3.1.2 Chemicals and reagents

All the chemicals and biochemicals used during the present course of investigations were of analytical grade and purchased from Sigma Chemical Company, (USA), E. Merck (Bombay), Himedia Laboratories Limited (Bombay) and Sisco Research Laboratories Pvt. Ltd., (Bombay).

3.2 METHODS

3.2.1 Sampling

Guava fruits of cultivars L-49 and Hisar Surkha were harvested at immature green (IG), mature green (MG) and turning (T) stages of ripening on the basis of visual observations of size, firmness, liquefaction and pigmentation. To get ripe (R) and overripe (OR) stage, fruits harvested at turning (T) stage were wrapped in newspaper and kept for 2 and 4 days (Hisar Surkha) and 4 and 6 days (L-49), respectively at room temperature. The samples were analyzed for various ripening related parameters. All the data was recorded in six replicates.

3.2.2 Post-harvest treatment of guava with GA_3 and $CaCl_2$

Fruits of both the guava cultivars were harvested at mature green stage. To optimize concentration and time of GA_3 and $CaCl_2$ treatments for increasing shelf-life of guava, fruits free of any visible defects and approximately of same size, were treated with different concentrations of GA_3 *viz.* 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm and of $CaCl_2$ *viz.* 0.5 %, 1 %, 2 %, 3 %, 4 %, 5 % for 3, 5and 10 min. The fruits were then taken out, extra solution wiped off, air dried and were analyzed for TSS and Firmness. Best time of treatment (5 min) and two concentrations for GA_3 (100 ppm and 150 ppm) and $CaCl_2$ (1 % and 2 %) treatment, were selected on the basis of TSS and firmness. For the storage study of guava, fruits of both cultivars (MG stage) were treated with the selected concentrations of GA_3 and $CaCl_2$ for 5 min and then stored at room temperature. Samples were taken at two day interval until complete decay and were analyzed for the ripening related parameters. All the observations were taken in triplicates.

3.3 Physico-chemical parameters

3.3.1 Physiological loss in weight

Weight of freshly harvested fruits was recorded at 0 day of storage and termed as initial weight. On each day of observation, the stored fruit was again weighed and termed as final weight on that particular day of observation. The per cent loss in weight (PLW) on each sampling date was calculated using the following formula:

$$\text{PLW (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

The PLW at each of the storage period was commuted with that of the preceding storage period to have total PLW on that day of storage.

3.3.2 Firmness

Flesh firmness was measured by hand held fruit pressure tester, using cylindrical plunger of 8 mm diameter and firmness scale of 13 kg/sq.cm. The firmness was measured from each side of the equatorial region of the fruit. Firmness of six fruits per treatment was measured and expressed in kg/cm^2.

3.3.3 Total soluble solids

Pulp of three randomly selected fruits per treatment was crushed for extracting juice. TSS of juice was measured with the help of hand refractometer (0-32ºbrix) and expressed as per cent soluble solids.

3.3.4 Titratable acidity

Total acids were estimated by titration against 0.1N sodium hydroxide (Ranganna, 2003). Five grams of fruit pulp was macerated in 5 ml of distilled water. Its volume was made to 100 ml, shaken and filtered through Whatman No. 4 filter paper. An aliquot of 20 ml was titrated against 0.1 N NaOH using 1 % phenolphthalein as an indicator. Appearance of pink colour was observed. From the volume of alkali used, acidity was calculated and expressed as g citric acid /100 g fruit pulp.

$$\text{Acidity (\%)} = \frac{\text{Titre vol. (ml)} \times \text{Normality of alkali} \times \text{Eq. wt. of acid} \times \text{Vol. made (ml)} \times 100}{\text{Vol. of aliquot (ml)} \times \text{Wt. or volume of sample (g or ml)} \times 1000}$$

Eq. wt. of citric acid = 64.04

3.4 Sugars, cell wall components and their hydrolyzing enzymes

3.4.1 Sugars

3.4.1.1 Total Sugars

Total sugars were estimated by the modified method of Dubois *et al.* (1956). One ml properly diluted sugar extract was mixed with 2 ml of 2 % phenol solution, followed by 5 ml

concentrated H_2SO_4. Acid was added in such a way that it directly poured on the solution. The tubes were shaken and kept for 30 min and then absorbance of solution read at 490 nm. Concentration of total sugars was calculated from the standard curve of glucose (20-100 µg) prepared simultaneously.

3.4.1.2 Reducing sugars

Reducing sugars were determined by the method of Nelson (1944) as modified by Somogyi (1952).

Reagents

Copper reagent A

Anhydrous Na_2CO_3	25 g
Rochelle salt (sodium potassium tartrate)	25 g
Sodium bicarbonate	25 g
Anhydrous sodium sulphate	200 g
Volume	1L

Copper reagent B

$CuSO_4$. $5H_2O$	15g
Conc. H_2SO_4	1-2 drops
Volume	100 ml

Alkaline copper reagent

A: B:: 25:1 (prepared fresh before use)

Arsenomolybdate reagent

Ammonium molybdate	25 g
Conc. H_2SO_4	21 ml
Sodium hydrogen arsenate	3 g
Total volume	500 ml

Preparation of arsenomolybdate reagent

Ammonium molybdate (25 g) was dissolved in 450 ml distilled water and 21 ml conc. H_2SO_4 was added to it. Then 3.0 g disodium hydrogen arsenate was dissolved in 25 ml distilled water and added to the acidified molybdate solution with constant stirring. The volume was made to 500 ml with distilled water. Solution was kept in an incubator at 37°C for 24 h. This reagent was stored in glass stopped brown bottle.

Procedure

Suitable aliquot of the extract was taken in a 25 ml graduated test tube. One ml alkaline copper reagent was added, mixed well and heated for 20 min in the boiling water bath. Then tubes were cooled and 1 ml arsenomolybdate reagent was added, mixed thoroughly and diluted to 25 ml. A stable blue colour quickly appeared, absorbance of which

was read at 520 nm on spectrophotometer. Concentration of reducing sugars was calculated from the standard curve of glucose (10-100 µg)) prepared simultaneously.

3.4.1.3 Non–reducing sugars

The concentration of non-reducing sugars was calculated by subtracting the reducing sugars from the total sugars.

3.4.2 Cell wall components

Cell wall components i.e. neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose, lignin and cellulose were estimated by the method of Van Soest (1967) modified by (Pradhan and Bhatia, 1986).

3.4.2.1 Neutral detergent fibre

NDF extraction reagent

Sodium lauryl sulphate	10.00 g
EDTA	18.61 g
Borax (sodium borate decahydrate)	6.81 g
Disodium hydrogen phosphate (anhydrous)	4.56 g
Ethylene glycol monoethyl ether	10 ml
Volume	1L

NDF was determined by refluxing 1.0 g sample with 100 ml NDF extraction reagent for 1 h. The solution was filtered through sterilized glass crucibles and the residue was washed first with hot distilled water and then with acetone, dried overnight at 100°C and weighed. The residue represented NDF content and was expressed as per cent.

$$\text{NDF (\%)} = \frac{\text{Residue weight}}{\text{Sample weight}} \times 100$$

3.4.2.2 Acid detergent fibre

ADF extraction reagent

Cetrimide	5 g
Sulphuric acid (conc.)	28 ml
Volume	1L

ADF was determined by refluxing 1 g sample with 100 ml ADF extraction reagent for 1h. The solution was filtered, washed, dried overnight at 100°C and weighed. It was calculated as

$$\text{ADF (\%)} = \frac{\text{Residue weight}}{\text{Sample weight}} \times 100$$

3.4.2.3 Cellulose

Residue left after the extraction of ADF was taken in the sintered crucibles and washed twice by stirring with 72 % H_2SO_4. Acid filled crucibles were kept for 3 h and after that acid was removed. Contents were made acid free by washing with hot water. Crucibles were dried

overnight at 100°C and weighed. The loss in weight from original amount of acid detergent fibre corresponded to cellulose content which was expressed as per cent.

Weight of acid detergent fibre –

$$\text{Cellulose (\%)} = \frac{\text{Weight of residue after } H_2SO_4 \text{ treatment}}{\text{Weight of sample}} \times 100$$

3.4.2.4 Lignin

The residue left after the extraction of cellulose was ashed at 550°C for 3 h in a muffle furnace then cooled in a dessicator and weighed. Weight loss upon ignition represented the lignin content.

$$\text{Lignin (\%)} = \frac{\text{Weight of residue after } H_2SO_4 \text{ treatment} - \text{Ash content}}{\text{Weight of sample}} \times 100$$

3.4.2.5 Silica

Silica content was calculated by subtracting the original weight of crucibles from the weight of crucibles after ashing in muffle furnace. Results were expressed as per cent dry weight basis.

$$\text{Silica (\%)} = \frac{\text{Ash content}}{\text{Weight of sample}} \times 100$$

3.4.2.6 Hemicellulose

Hemicellulose content in the sample was calculated by the difference between NDF and ADF.

3.4.2.7 Pectin

Pectin was extracted by the method of Ahmed and Labavitch (1977).

Extraction

To 1 g guava fruit, 25 ml 72 % H_2SO_4 (w/v) was added. The mixture was stirred for 30 min and made to 100 ml with distilled water. It was then filtered through Whatman No. 1 filter paper. Pectin was determined by estimating uronic acid content by the method of Blumekrantz and Asboe-Hansen (1972).

Reagents

0.15 % m-hydroxy diphenyl solution in 0.5 % NaOH

Borax solution:

Sodium tetraborate	476 mg
Conc. H_2SO_4	100 ml

It was dissolved by keeping it in hot water bath for 10 min.

An aliquot (0.2 ml) was mixed with 2 ml borax solution and kept for 5 min. After shaking vigorously, it was incubated in boiling water bath for 10 min and cooled. After cooling 20 µl m-hydroxy diphenyl solution was added and the contents were shaken vigorously till pink colour developed. The volume was made to 5 ml with conc. H_2SO_4 and

the absorbance read at 520 nm on spectrophotometer. The amount of uronic acid was calculated from a calibration curve prepared by using D-galacturonic acid (10-50 µg) as standard.

3.4. 3 Hydrolytic enzymes

3.4.3.1 Pectin methylesterase (EC 3.1.1.11)

Pectin methylesterase was extracted and assayed by the method of Hagerman and Austin (1986).

Extraction

Fresh fruit tissue (10 g) was homogenized chloride in a pre-chilled pestle and mortar with 50 ml chilled 0.1 M Tris-HCl buffer (pH 7.5), containing 10 % NaCl. Homogenate was centrifuged at 10,000 x g for 30 min. The supernatant represented the enzyme extract.

Assay

The reaction mixture contained 100 µl enzyme extract, 2.5 ml 0.5 % (w/v) apple pectin in buffer (2 mM Tris-HCl, pH 7.5) and 0.4 ml 0.01 % (w/v) bromothymol blue in the same buffer. The absorbance at 620 nm was measured immediately and after 30 min. The difference in absorbance between 0 and 30 min was the measure of PME activity. Calculation of the activity was carried out against a standard curve of galacturonic acid (50 to 500 µg) prepared under the same assay condition and the enzyme activity expressed as mg galacturonic acid (carboxy group equivalent) released for 30 min g^{-1} f.wt.. One enzyme unit was expressed as the amount of enzyme required to release 1 mg of galacturonic acid/30min.

3.4.3.2 Polygalacturonase (EC 3.2.1.15)

Extraction

Polygalacturonase was extracted according to the method of Singh and Singh (1993). Fruit sample (1.0 g) was extracted in 0.1 M sodium acetate buffer (pH 5.2) containing 0.02 M sodium metabisulphite and 10 % (w/v) sodium chloride in a pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. Supernatant obtained was dialysed against 0.01 M sodium acetate buffer (pH 5.2) for 4 h by changing buffer every h.

Assay

The enzyme was assayed according to the method of Ahmed and Labavitch (1980). The assay mixture (1 ml) contained 0.2 ml enzyme extract, 0.2 ml cold sodium acetate buffer (0.1 M, pH 5.2), 0.5 ml 0.3 % (w/v) polygalacturonic acid and 50 µl containing 125 µg each of chloramphenicol and cycloheximide. The mixture was incubated at 37°C for 20 h. Reaction was terminated by heating the tubes in a boiling water bath for 10 min and reducing sugars were estimated by the method described under the 3.4.1.2 using galacturonic acid as standard (20-100 µg).

One enzyme unit was defined as the amount of enzyme required to release 1 mg of galacturonic acid/20 h at 37°C.

3.4.3.3 Cellulase (EC 3.2.1.4)

Extraction and assay system were the same as for polygalacturonase except that 0.5 % (w/v) sodium salt of carboxymethyl cellulose was used as substrate instead of polygalacturonic acid. The reaction was started by the addition of 0.5 ml substrate solution and was terminated by heating the tubes in a boiling water bath for 10 min. Reducing sugars were estimated by the method described under the 3.3.8 using glucose (20-100 µg) as the standard.

One enzyme unit was expressed as the amount of enzyme required to release 1 mg glucose released/20 h at 37°C.

3.5 Lipid peroxidation, oxidative stress and antioxidative system

3.5.1 Lipid peroxidation and oxidative stress

3.5.1.1 Lipoxygenase (EC 1.13.11.12)

Lipoxygenase was extracted as explained under the 3.5.1.1. The enzyme activity was determined spectrophotometrically at 234 nm by the method of Catherine *et al.* (1998).

Assay

The reaction mixture (3 ml) contained 15 µl 30 mM linoleic acid solution in methanol, 2.785 ml 0.1 M phosphate buffer (pH 6.8) and 200 µl enzyme extract. The reaction was started by the addition of enzyme extract. Increase in absorbance was measured at room temperature for 2 min. LOX activity was measured by monitoring the formation of conjugated dienes from linoleic acid (extinction coefficient 2.74×10^4 M^{-1} cm^{-1}) (Chen and Whitaker, 1986). One unit of LOX was defined as amount of enzyme required to produce 1 µmol of conjugated dienes min^{-1}.

3.5.1.2 Hydrogen peroxide

Extraction

One g tissue was macerated in 5 ml chilled 0.8 N $HClO_4$ and centrifuged at 10,000 × g for 25 min. The clear supernatant was decanted carefully and was used for the estimation of H_2O_2 and superoxide radical.

Estimation

H_2O_2 was estimated by the method of Sinha (1972). To 0.4 ml extract, 0.6 ml 0.1 M phosphate buffer (pH 7.0) and 3 ml mixture of 5 % (w/v) potassium dichromate and glacial acetic acid (1:3, v/v) was added. The mixture was heated for 10 min in a boiling water bath. Color of solution changed to green due to the formation of chromic acetate. After cooling, absorbance was recorded at 570 nm against the reagent blank without sample extract. The quantity of H_2O_2 was determined from the standard curve of H_2O_2 (10-160 µmoles).

3.5.1.3 Superoxide radical

Superoxide radical was measured by monitoring the nitrite formation from hydroxylamine following the method of Elstner and Heupel (1976).

The extract (0.75 ml) was incubated with 2.1 ml 65 mM of sodium phosphate buffer (pH 7.8) and 0.14 ml 0.1 mM hydroxylamine hydrochloride for 20 min at room temperature followed by addition of 0.75 ml each of 17 mM sulfanilamide and 7 mM N-1 naphthyl ethylenediamine dihydrochloride (NED). The reaction mixture was again incubated at room temperature for 20 min. The absorbance of colored complex thus formed was read at 540 nm against blank which was prepared similarly except that fruit extract was replaced with water. Amount of NO_2^- formed which corresponded to $^{\cdot}O_2^-$ production, was calculated from standard curve of NO_2^- (10-100 nmol) prepared by the above procedure.

3.5.2 Antioxidative systems

3.5.2.1 Antioxidative enzymes

The extraction medium for lipoxygenase, superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase consisted of 0.1 M potassium phosphate buffer (pH 7.5) containing 5 % (w/v) PVP, 1 mM EDTA, 10 mM β-mercaptoethanol. The extraction buffer for peroxidase, however, consisted of 0.01M phosphate buffer (pH 7.0) containing 4 % (w/v) PVP. The enzymes were extracted by macerating 4 g tissue in chilled pestle and mortar in the presence of 5 ml 0.1 M phosphate buffer (pH 7.5) and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was carefully decanted and used as enzyme extract.

3.5.2.1.1 Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium, adopting the method of Giannopolities and Ries (1977). The reaction mixture (3 ml) contained 2.1 ml 50 mM phosphate buffer (pH 7.8), 0.2 ml 14 mM L-methionine, 0.2 ml 10 µM nitroblue tetrazolium, 0.2 ml 3 µM riboflavin, 0.2 ml 0.1 mM EDTA and 0.1 ml enzyme extract. Riboflavin was added in the end. The tubes were properly shaken and placed 30 cm below light source consisting of two 15 W-fluorescent lamps (Phillips, India). The reaction was started by switching on the light and was terminated after 40 min of incubation by switching off the light. After terminating the reaction, the tubes were covered with black cloth to protect them from light. A non-irradiated reaction mixture that did not develop color served as the blank. The reaction mixture without enzyme extract developed maximum color and its absorbance decreased with increasing volume of extract. The absorbance was recorded at 560 nm. One enzyme unit was defined as the amount of enzyme which could cause 50 % inhibition of the photochemical reaction.

3.5.2.1.2 Catalase (EC 1.11.1.6)

Catalase activity was determined by the procedure of Sinha (1972). The reaction mixture (1.0 ml) consisted of 0.5 ml 0.2 M phosphate buffer (pH 7.0), 0.4 ml 0.2 M hydrogen peroxide and 0.1 ml enzyme extract. After incubating at 37°C for 3 min, the reaction was terminated by adding 3 ml mixture of 5 % (w/v) potassium dichromate and glacial acetic acid (1:3 v/v) to the reaction mixture. The tubes were heated in boiling water bath for 10 min. A

control was run under similar conditions where enzyme extract was added after stopping the reaction. After cooling the tubes, absorbance of test and control was measured at 570 nm. The amount of residual H_2O_2 in the reaction mixture was determined by subtracting the absorbance of test samples from that of control. One CAT unit was defined as amount of enzyme required to catalyze the oxidation of 1 µmol H_2O_2 min^{-1} under assay conditions.

3.5.2.1.3 Peroxidase (EC 1.11.1.7)

The enzyme activity was estimated by the method of Shannon *et al.* (1966). The reaction mixture (2.75 ml) contained 2.5 ml 50 mM phosphate buffer (pH 6.5), 0.1 ml 0.5 % hydrogen peroxide, 0.1 ml 0.2 % O-dianisidine and 0.05 ml enzyme extract. The reaction was initiated by the addition of 0.1 ml of H_2O_2. The assay mixture without H_2O_2 served as blank. Change in absorbance was followed at 430 nm for 3 min. One unit of peroxidase was defined as amount of enzyme required to cause change in O.D. of 1unit min^{-1}.

3.5.2.1.4 Ascorbate peroxidase (EC 1.11.1.11)

The enzyme activity was determined following the oxidation of ascorbic acid (Nakano and Asada, 1981). The reaction mixture (2.7 ml) contained 2.25 ml 100 mM phosphate buffer (pH 7.0), 0.2 ml 0.5 mM ascorbate, 0.2 ml 0.1 mM H_2O_2 and 0.05 ml enzyme extract. The reaction was initiated by the addition of H_2O_2. The decrease in absorbance at 290 nm was recorded spectrophotometrically which corresponded to oxidation of ascorbic acid. The enzyme activity was calculated using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbic acid. One enzyme unit was defined as amount of enzyme required to oxidize 1 nmol of ascorbate min^{-1}.

3.5.2.1.5 Glutathione reductase (EC 1.6.4.2)

Method of Halliwell and Foyer (1978) was followed for measuring GR activity. The reaction mixture consisted of 2.7 ml 0.1 M phosphate buffer (pH 7.5), 0.1 ml 5 mM oxidized glutathione (GSSG), 0.1 ml 3.5 mM NADPH and 0.1 ml enzyme extract in final volume of 3 ml. The decrease in absorbance at 340 nm due to oxidation of NADPH was monitored. Non-enzymatic oxidation of NADPH was recorded and subtracted from it. An extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH was used to calculate the amount of NADPH oxidized which corresponded to GR activity. One enzyme unit was defined as amount of enzyme required to oxidize 1 µmol of NADPH oxidized min^{-1}.

3.5.2.2 ROS scavenging metabolites

3.5.2.2.1 Ascorbic acid

Extraction

Tissue (1 g) was homogenized in 5 ml 5 % (w/v) metaphosphoric acid in glacial acetic acid and homogenate was centrifuged at 10,000 x g for 25 min. The supernatant thus obtained was used for the estimation of ascorbic acid content.

Estimation

Ascorbic acid content was estimated by the method of Roe (1964) which is based on the reduction of 2, 6-dichlorophenol indophenol (2, 6-DCPIP) by ascorbic acid. An aliquot (0.5 ml) was titrated with 2, 6-DCPIP (0.01 %) reagent until a pink end point was reached which persisted for 30 sec. The quantity of ascorbic acid was calculated by comparing the amount of 2, 6-DCPIP reagent used for unknown sample with that used with known quantities of ascorbic acid (5-40 µg).

3.5.2.2.2 Glutathione

Glutathione was extracted as explained under the 3.5.1.2.

Estimation

Glutathione was estimated by the method of Griffith (1980). The reaction mixture (2.5 ml) consisted of 2.1 ml 125 mM phosphate buffer (pH 7.5) containing 6.3 mM EDTA, 0.1 ml 2.5 mM NADPH, 0.1 ml 6 mM 5,5'-dithiobis (2-nitrobenzoic acid) and 0.5 units glutathione reductase enzyme and 0.1 ml supernatant neutralized with sodium bicarbonate. Increase in absorbance was recorded at 412 nm for 3 min. Glutathione content was estimated from the standard curve prepared with 1-50 µmol GSH.

3.5.2.2.3 β-carotene

β-Carotene was estimated by the method of AOAC (2000).

Reagent

Water saturated n-butanol: n-butanol and water were mixed in ratio of 6:2 (v/v) and shaken vigorously. Then kept to stand till it separated into two phases; the upper clear layer was water saturated n-butanol.

Procedure

Ten gram fresh fruit sample was dispersed in 50 ml water-saturated n-butanol to make a homogenous suspension. Shaken gently and kept to stand overnight (16 h) at room temperature in dark. The suspension was shaken again and filtered through Whatman filter paper No. 1. The volume of filtrate was made to 100 ml and the absorbance of the clear filtrate was measured at 440 nm in Spectrophotometer using saturated n-butanol as a blank. The amount of β-carotene was calculated from the standard calibration curve of β-carotene (0.5-5.0 µg).

3.5.2.2.4 Antioxidant activity (% scavenging of DPPH)

Antioxidant activity was measured using stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, as per the method described by Shimada *et al.* (1992).

Preparation of dye

Twenty five mg DPPH dye was dissolved in 10 ml methanol with vigorous shaking. One ml of this solution was diluted to 100 ml.

Extraction

Five hundred mg of fruit pulp was macerated in 10 ml methanol and centrifuged at 4,000 rpm for 15 min. Supernatant decanted and volume was made 10 ml with methanol. The supernatant was further diluted 10 times with methanol.

Procedure

Three ml dye (diluted) was mixed with 0.5 ml diluted supernatant and incubated for 20 min. The absorbance was read at 517 nm on spectrophotometer. Dye mixed with 0.5 ml methanol was used as blank. Antioxidant activity was expressed as per cent scavenging of DPPH and it was calculated using the following formula:

$$\text{Scavenging activity (\%)} = [(Ao - A_1)/Ao] \times 100\ \%$$

Where A is absorbance

Where Ao = Absorbance of blank

A_1 = Absorbance of sample

3.6 Ethylene evolution and ACC oxidase

3.6.1 Ethylene evolution

Three fresh fruits (pre-weighed) were placed in glass jar fitted with rubber septum. The jars were sealed with silicone rubber stoppers and incubated for 4 h at room temperature. Gas sample (1ml) was removed from the jars with the help of syringe and injected into a Thermo scientific trace GC 600 gas liquid chromatograph equipped with a Porapak – N column having flame-ionization detector. The temperature of the oven was fixed at 80°C and detector and injector was fixed at 110°C and the flow rate each of N_2, H_2 and O_2 was kept at 1 kg/cm^2. Ethylene identification was based on the retention time compared with standard C_2H_4 (purity 99.9%). Content of ethylene evolved was calculated (Hardy *et al.* 1968) and results expressed as nmol C_2H_4 produced h^{-1} g^{-1} f.wt.

3.6.2 ACC oxidase

ACC oxidase activity was determined according to the method followed by Moya-Leon and John (1994).

Extraction

Tissue (4 g) was homogenized with 5 ml extraction buffer consisting of 0.1 M Tricine (pH 7.5), 10 % (v/v) glycerol, 2.5 % (w/v) polyvinyl polypyrrolidone (PVPP), 0.04 g ml^{-1} polyethylene glycol 4000 (PEG), 2 mM dithiothreitol (DTT). Homogenate was centrifuged at 10,000 × g for 30 min and supernatant was taken as enzyme extract.

Assay

ACC oxidase activity was assayed by measuring the ethylene produced (Ververidis and John, 1991). The reaction mixture (3 ml) in test tubes provided with rubber septum caps contained 0.1 M Tricine (pH 7.5) containing 10 % (v/v) glycerol, 1 mM ACC, 0.02 mM $FeSO_4$, 5 mM sodium ascorbate, 1 mM dithiothreitol, 20 mM sodium bicarbonate and enzyme

extract. The reaction was initiated by the addition of 0.5 ml enzyme extract. The tubes were capped tightly and incubated at 30°C for 2 h. One ml gas sample was withdrawal with the help of a syringe from the headspace and was injected in gas chromatograph for C_2H_4 determination. One enzyme unit was defined as amount of enzyme required to produce 1 nmol of C_2H_4 per h.

3.7 Purification and characterization of polygalacturonase from guava fruit

Fresh fruits of L-49 cultivar were used for purification of the enzyme polygalacturonase. All procedure during the extraction and purification of the enzyme were carried out at 0-4°C.

3.7.1 Preparation of crude extract

Crude extract was prepared as explained in 3.4.3.2.

3.7.2 Ammonium sulphate fractionation

To the crude extract obtained after centrifugation, solid $(NH_4)_2SO_4$ was added slowly with constant stirring so as to bring the saturation to 30 per cent. The stirring was continued for another few minutes and the solution was kept in a refrigerator for five hs for complete precipitation of proteins. Then the solution was centrifuged at 10,000 x g for 20 min. The precipitates were discarded as it had negligible activity of polygalacturonase. The resultant supernatant was brought to 70 per cent saturation with solid $(NH_4)_2SO_4$. The precipitates were collected by centrifugation as before and dissolved in dissolved in 5 ml 0.1M sodium acetate buffer (pH 5.2). The resulting solution was dialyzed against the same buffer for 24 h. Every 4 h the dialysis buffer was changed. The dialyzed $(NH_4)_2SO_4$ fraction (15 ml) was concentrated against solid sucrose and used for gel filtration chromatography.

3.7.3 Sephadex G-100 column chromatography

Ten grams Sephadex G-100 was suspended in 300 ml 0.1 N NaCl solution and was kept to swell by keeping at room temperature for 8 h with intermittent shaking. Remaining NaCl solution was decanted and then the gel was suspended in 0.1M sodium acetate buffer (pH 5.2) to prepare final volume to 300 ml. The suspension was poured into a LKB glass column of dimensions 90 cm x 1.5 cm and allowed to settle by gravity. After complete sedimentation of Sephadex in the column, the effective length of column was 50 cm. The column was equilibrated with 0.1M sodium acetate buffer (pH 5.2). The void volume was calculated by passing blue dextran (2 mg/ml) through the column. After passing two bed volumes of same buffer, the column was calibrated with standard molecular weight markers (2 mg/ml each) *viz.* cytochrome-C (12.4 kDa), β-lactoglobulin (18.0 kDa), carbonic anhydrase (29.0 kDa), pepsin (34.7 kDa), ovalbumin (45.0 kDa), albumin bovine (66.0 kDa) and alcohol dehdyrogenase (150.0 kDa) and then made protein free by running about 4 bed volumes of the same buffer. The enzyme preparation obtained after $(NH_4)_2 SO_4$ fractionation was concentrated, loaded onto the the top of the Sephadex bed and eluted with 0.1 M sodium acetate buffer (pH 5.2). The fractions of 3 ml each were collected at a flow rate of 12 ml/h. These fractions were analyzed for protein

content (A_{280}) and enzyme activity. Fractions showing the enzyme activity were pooled and concentrated against solid sucrose. The concentrated fraction was then further purified by ion exchange chromatography on CM-cellulose.

3.7.4 Ion-exchange chromatography

Fifteen grams CM-cellulose powder was suspended in 300 ml 1 N HCl for half an h with intermittent gentle stirring so that fibres do not break. It was then filtered through Whatman No. 1 filter paper followed by washing with distilled water until the pH reached 4.0-4.5. It was then suspended in 300 ml 1 N NaOH for half an hour to make it alkaline, immediately filtered through Whatman No. 1 filter paper and washed free of alkali with distilled water till pH 8.0-8.5 was attained. The cation exchanger was again transferred in 1 N HCl for half an hour so as to make it acidic, filtered and washed with distilled water till pH 7.0.

This preparation was packed in a LKB glass column of 60 cm × 3 cm. After complete sedimentation of the gel, the effective length of column was 30 cm. The column was equilibrated with 0.1 M sodium acetate buffer (pH 5.2). The enzyme preparation obtained after gel filtration was concentrated, loaded onto the column and eluted first with 0.1 M sodium acetate buffer (pH 5.2) and then with a linear gradient of 0.1–0.5 M KCl in the same buffer at a flow rate of 30 ml/h. The fractions of 3 ml each were collected and analyzed for protein (280 nm) and enzyme activity. The fractions with high enzyme activity were pooled together, concentrated against sucrose and stored at 4°C and further used for investigating various characteristics of the enzyme.

3.7.5 Protein Estimation

Protein content in crude extract and the enzyme preparations at various stages of purification was quantified by the method of Lowry *et al.* (1951).

Reagents

A: 2 % Na_2CO_3 in 0.1 N NaOH

B: 1 % solution of $CuSO_4.5H_2O$ in distilled water.

C: 2 % sodium potassium tartarate.

D: Prepared fresh before use by mixing reagents B and C in 1:1 ratio.

E: Prepared by adding 1 ml of reagent D to 50 ml of reagent A.

F: 1 N Folin-Ciocalteau reagent

Procedure

To 1 ml protein sample, 5 ml reagent E was added, mixed well and kept at room temperature for 10 min. Then 0.5 ml reagent F was added and the contents were mixed immediately on a vortex mixer. After 30 min colour intensity was read at 660 nm on Spectrophotometer.

The equivalent amount of protein was calculated from a standard curve prepared by using bovine serum albumin (20-200 µg).

3.7.6 Enzyme assay

The enzyme activity was measured as mentioned under 3.4.3.2.

3.7.7 Polyacrylamide gel electrophoresis (PAGE)

Purity of the enzyme preparation was checked by native-PAGE (10 % gel) using anionic system of Davis (1964). To determine the molecular weight and subunit composition of the enzyme, denaturating SDS-PAGE was performed by using method of Laemmili (1970).

Reagents

(A) Acrylamide-bis-acrylamide solution

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Volume	100 ml

The solution was filtered through Whatman No. 1 filter paper and stored at 4ºC in brown bottle.

(B) N, N, N', N' tetramethylethylenediamine (TEMED)

Used as supplied.

(C) Ammonium persulphate solution (1.5 %)

Ammonium persulphate	0.075g
Volume	5 ml

The solution was prepared fresh

(D) Stacking gel buffer

Tris	6.0 g
1M HCl	48 ml
pH	6.8
Volume	100 ml

(E) Resolving gel buffer

Tris	36.3g
1 M HCl	48 ml
pH	8.8
Volume	100 ml

Reagents D and E were filtered through Whatman No.1 filter paper and stored at 4^0C.

(F) Reservoir Buffer

Tris	3.0 g
Glycine	14.4 g
pH	8.3
Volume	1L

For SDS-PAGE, 1.0 g SDS was added to the above buffer.

(G) SDS solution (10 %)

SDS	1.0 g
Volume	10 ml

(H) 2X sample buffer

1 M Tris-HCl (pH 6.8)	1.25 ml
SDS	0.4 g
β-mercaptoethanol	1.0 ml
20 % glycerol	2.0 ml
1 % bromophenol blue	0.4 ml
Volume	10 ml

(I) Molecular weight markers

Alcohol dehydrogenase	150.0 kDa
Albumin bovine	66.0 kDa
Ovalbumin	45.0 kDa
Pepsin	34.7 kDa
Carbonic anhydrase	29.0 kDa
β-lactoglobulin	18.0 kDa
Cytochrome-C	12.4 kDa

Two mg each of the above mentioned molecular weight markers was dissolved in 1 ml of 0.125 M Tris-HCl buffer (pH 6.8).

(J) Staining solution

Coomassie Brilliant Blue	1.25 g
Methanol	200 ml
Glacial acetic acid	35 ml
Volume	500 ml

The reagent was stored in tightly capped bottle at room temperature and was stable for several months.

(K) Destaining solution

Acetic acid	75 ml
Methanol	50 ml
Volume	1 L

Sample preparation

(a) Native PAGE

One ml enzyme sample each from crude extract, $(NH_4)_2 SO_4$ fractionation, Sephadex G-100 elution and CM-cellulose elution was taken in separate tubes. To each tube, 0.2 ml

glycerol (20 %) and 0.05 ml bromophenol blue (1 %) were added and contents mixed thoroughly.

(b) SDS-PAGE

Enzyme preparations and standard molecular weight marker proteins were mixed with equal volume of 2X sample buffer, boiled for 5 min and then cooled.

Gel composition

(a) Composition of gel solution for native PAGE

Stock solutions	**Stacking gel (ml) 3.75 %**	**Resolving gel (ml) 10 %**
Acrylamide bis acrylamide	1.25	10.0
Stacking gel buffer (pH 6.8)	2.5	-
Resolving gel buffer (pH 8.8)	-	3.75
1.5 % APS	0.75	1.5
TEMED	0.015	0.025
Distilled water	5.485	14.725

(b) Composition of gel solution for SDS PAGE

Stock solutions	**Stacking gel (ml) 3.75 %**	**Resolving gel (ml) 10 %**
Acrylamide-bis-acrylamide	1.25	10.0
Stacking gel buffer (pH 6.8)	2.5	-
Resolving gel buffer (pH 8.8)	-	3.75
10 % SDS	0.1	0.3
1.5 % APS	0.75	1.5
TEMED	0.015	0.025
Distilled water	5.385	14.425

Procedure

Electrophoresis was done using slab gel apparatus (M/s Atto, Japan). The glass plates were soaked in chromic acid, rinsed with distilled water and dried. The plates were wiped with an ethanol swab, air dried and the gel casting assembly was assembled. Sides of the plates were sealed by tygon tubing and were clamped. Resolving gel of 10 % acrylamide was prepared by mixing all the components as described in above Table. TEMED and APS were added in the end. Solution was then immediately poured into the space between plates. A layer of water was then gently overlaid using a syringe so as to obtain a smooth top of the gel. Polymerization of the gel took about 30 min and was indicated by a sharp interface between water and gel. Water layered over the gel was removed using filter paper strips. Then, the stacking gel solution was prepared by mixing reagents as described in above Table, was poured and immediately the comb was inserted with care so that no air bubble was trapped. It was allowed to polymerize for 15 min. After polymerization, the comb and tygon tubing were removed and gel casting assembly was fixed to the electrophoresis apparatus. Sample wells were rinsed with the reservoir bufferand enough reservoir buffer (appx. 800 ml) was added so as to make contact with electrodes.

Samples containing about 100 µg protein were loaded in separate wells and electrophoresis was carried out at a constant current of 10 mA for first 30 min followed by 30 mA constant current till the tracking dye reached one cm away from the lower end of the gel. At this stage, electrophoresis was terminated. Gel was removed from glass plates and stained overnight with staining solution. The excess stain was removed by diffusion in destaining solution. After complete destaining, gel was transferred to 7 % acetic acid and photographed.

3.7.8 Characterization of polygalacturonase

3.7.8.1 Determination of molecular weight of the purified enzyme

Molecular weight of the purified enzyme was estimated through Sephadex G-100 column as described under 3.7.3 and plotting log molecular weight *vs.* elution volume.

3.7.8.2 Determination of subunit molecular weight of the purified enzyme

Subunit molecular weight was determined by carrying out polyacrylamide gel electrophoresis (10 % gel) in the presence of sodium dodecyl sulphate as described under 3.7.7. Standard proteins of known molecular weight *viz*. alcohol dehydrogenase (150.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), pepsin (34.7 kDa), carbonic anhydrase (29.0 kDa) and cytochrome-C (12.4 kDa) were run along with the enzyme protein and their mobility relative to the tracking dye (R_m) was calculated.

Calculation of R_m values

Rm values for different bands were calculated by the following relationship:

$$R_m = \frac{\text{Distance travelled by protein band}}{\text{Distance travelled by tracking dye}}$$

The molecular weight of the enzyme was calculated by calibration curve made by plotting log M_r vs R_m value of standard molecular weight marker proteins.

3.7.8.3 Determination of optimum pH for activity and stability:

The optimum pH value for polygalacturonase was determined by assaying enzyme activity (3.4.3.2) using assay buffer of pH value ranging from 3.0-8.0. For maintaining pH ranging from 3.0 to 6.0, 0.1 M sodium acetate buffer was used and for pH ranging from 6.1 to 8.0, 0.1 M sodium phosphate buffer was used. When enzyme assay was carried out in buffer of pH 5.0, maximum activity was obtained so for further characterization assay buffer of pH 5.0 was used and other assay conditions remain same as described under 3.4.3.2.

For determining optimum pH for stability, 100 µl of the purified enzyme was preincubated with 100 µl each of appropriate buffer of different pH value i.e. 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 6.8, 7.0, 7.5 and 8.0 separately for 2 h at ambient temperature. The enzyme activity was measured as mentioned under 3.4.3.2.

3.7.8.4 Determination of optimum temperature and thermal stability

The optimum temperature for the enzyme was determined by measuring the enzyme activity at temperature ranging from 15°C to 70°C. Except the enzyme all constituents of the reaction mixture were maintained at appropriate temperature in a water bath before starting the reaction. Enzyme activity was then determined as outlined in 3.4.3.2.

Thermostability of the purified enzyme was tested by measuring the residual activity after incubating 100 µl of the enzyme for 15, 30, 45 min at temperature ranging from 25 to 80ºC in a water bath.

3.7.8.5 Determination of K_m value

Activity of the purified preparation was measured using polygalacturonic acid as the substrate at final concentrations varying from 0.05 to 0.5 %. The K_m values were determined by using the Lineweaver-Burk reciprocal plot method.

3.7.8.6 Effect of metal ions

The effect of monovalent ions Na^+, K^+, divalent ions Ca^{2+}, Mn^{2+}, Mg^{2+}, $Zn^{2+}$and trivalent ions Fe^{3+} were determined by carrying out enzyme assay (3.4.3.2.) at different concentrations of these metal ions (1, 2, 5 and 10 mM). Chloride salts of all the metals were used for the experiment. Residual activity was calculated taking activity of control as 100 %.

3.7.8.7 Effect of organic solutes, chelating agent and reducing agents

Activity of the purified preparation was measured in the presence of 10 mM EDTA, 2 mM each of β mercaptoethanol, DTT and cysteine. To study effect of organic solutes, enzyme assay (3.4.3.2.) was carried out in presence of 1 M of glucose, maltose, sucrose and glycine.

3.8 Statistical analysis

Estimation of all the chemical/biochemical parameters was done in triplicates. Values presented, therefore, are mean of at least three determinations. The data were statistically analyzed for calculating CD using software ‘Statistical Package for Agriculture Scientists’, OPSTAT.

CHAPTER-IV

RESULTS

During ripening, a number of biochemical changes including synthesis and degradation of pigments, conversion of starch to sugars, production of volatilesand hydrolysis of cell wall components and enhanced rate of respiration are associated with the changes in firmness and texture of a fruit to yield an edible fruit with optimum blend of color, taste, aroma and texture (Biale *et al.* 1975). The present investigations were undertaken to study the role of gibberellic acid and calcium chloride in ripening related biochemical changes in fruits of two guava (*Psidium guajava* L.) cultivars differing in their shelf-life *viz*. L-49 (shelf-life 7-8 days) and Hisar Surkha (shelf-life 4-5 days). The results obtained during the present investigations are presented as below:

4.1 Physico-chemical and biochemical changes in guava fruit during ripening

4.1.1 Physico-chemical changes

Fruit ripening is a highly coordinated process of organ transformation from unripe to ripe stage. These changes can be monitored by studying fruit firmness, total soluble solids and titratable acidity. Fig 4.1 depicts the changes in firmness of guava fruit with the advancement of ripening. Fruit firmness was maximum at IG stage in both the cultivars and decreased continuously during ripening attaining the minimum value at the OR stage. In L-49, the firmness decreased from 15.31 kg/cm^2 at IG stage to 5.91 kg/cm^2 at OR stage, while in Hisar surkha, the corresponding values were 11.48 and 3.91 kg/cm^2 with about 2.5 to 3 fold decrease in both the cultivars from hard immature green to soft overripe stage. The longer shelf-life variety, L-49 had significantly higher fruit firmness as compared to Hisar Surkha throughout the ripening process.

Total soluble solids increased during ripening of guava fruits from 8.85 and 11.25 °Brix at IG stage to 11.92 and 14.63 °Brix at R stage and thereafter, a decline was observed at OR stage to 11.10 and 13.85 °Brix in L-49 and Hisar Surkha, respectively (Fig. 4.2). As is clear from the results, short lived variety, Hisar surkha had more TSS content as compared to L-49 at all the stages of ripening.

As depicted in Fig. 4.3, titratable acidity attained its maximum value at mature green stage followed by a decline with the advancement of ripening. Both the cultivars clinched highest titratable acidity at MG stage i.e. 0.503 per cent in L-49 and 0.442 per cent in Hisar Surkha, which decreased gradually to the respective values of 0.329 and 0.216 per cent at OR stage. L-49 had significantly higher titratable acidity than Hisar Surkha throughout the ripening period. Decrease in titratable acidity was more in Hisar Surkha as compared to L-49.

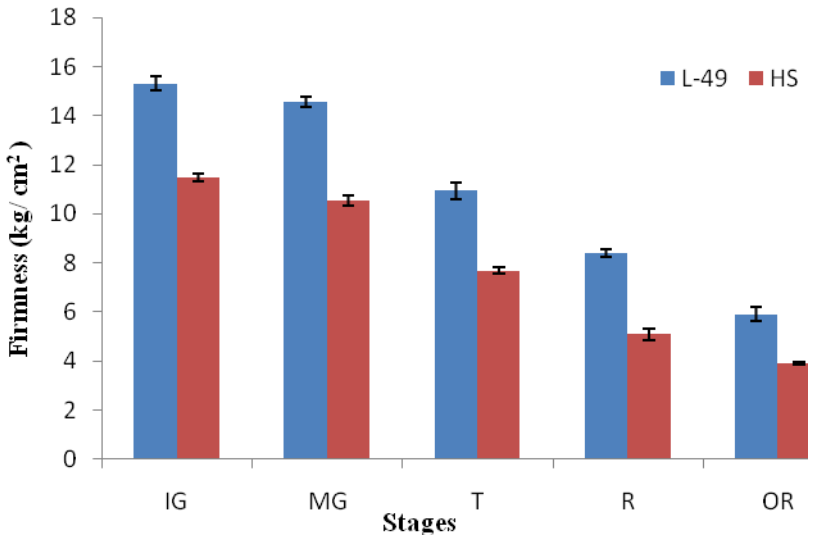

Fig. 4.1 Firmness of guava fruit at different stages of ripening

Two factorial analysis of variance (P≤0.05)
a: 0.271; b: 0.429; (axb): 0.607, a= varieties; b= stages

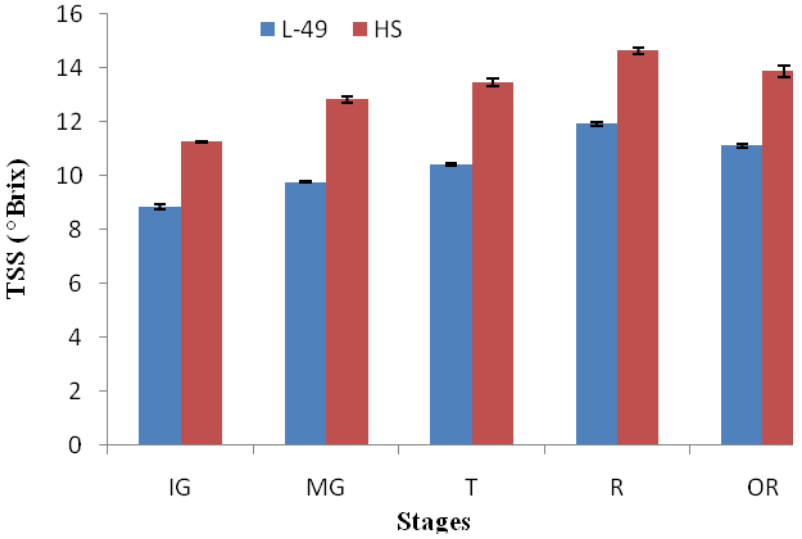

Fig. 4.2 Total soluble solids in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.137; b: 0.217; (axb): 0.307, a= varieties; b= stages

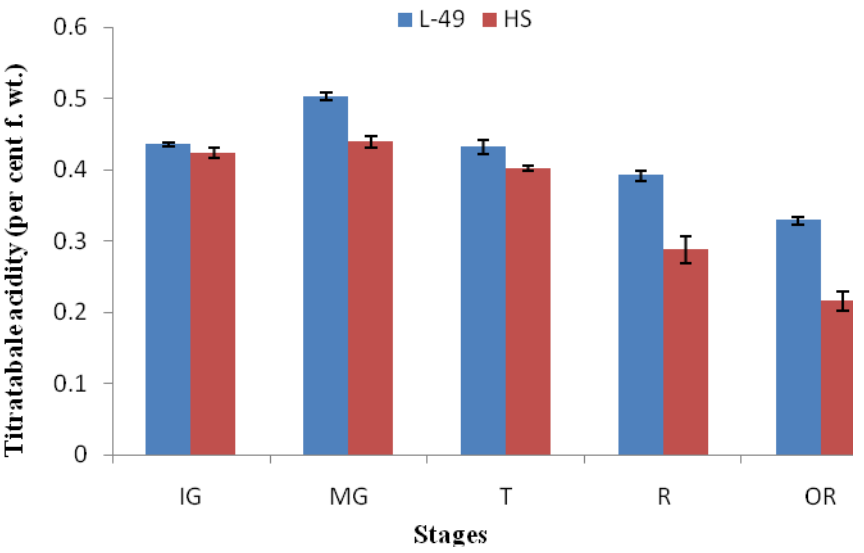

Fig. 4.3 Titratable acidity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.012; b: 0.02; (axb): 0.028, a= varieties; b= stages

4.1.2 Biochemical changes in guava fruit during ripening

4.1.2.1 Sugars, cell wall components and their hydrolyzing enzymes

The sugars present in fruits impart sweetness, which influence the taste and flavor. Total sugars (Fig. 4.4 A), reducing (Fig. 4.4 B) and non-reducing (Fig. 4.4 C) sugars increased significantly upto R stage followed by a decline at OR stage in both the cultivars. Total sugars increased 2 fold from a minimum of 11.86 per cent (L-49) and 14.52 per cent (Hisar Surkha) at IG stage to a maximum of 23.90 per cent (L-49) and 27.25 per cent (Hisar Surkha) at R stage of ripening**.** Thereafter, it decreased at OR stage to 22.41 and 26.52 per cent, in L-49 and Hisar Surkha respectively. Reducing sugars also followed the similar trend. Minimum reducing sugar content of 7.03 and 9.42 per cent in IG fruits increased to 12.36 and 14.02 per cent at R stage in *cvs.* L-49 and Hisar Surkha, respectively, thereafter it decreased. Non-reducing sugars increased from IG stage to ripe stage from 4.83 and 5.09 per cent to 11.54 and 13.23 per cent in *cvs.* L-49 and Hisar Surkha, respectively. L-49, the longer shelf-life variety exhibited higher content of total sugars, reducing sugars and non-reducing sugars than the shorter shelf-life variety Hisar Surkha at all the stages of ripening.

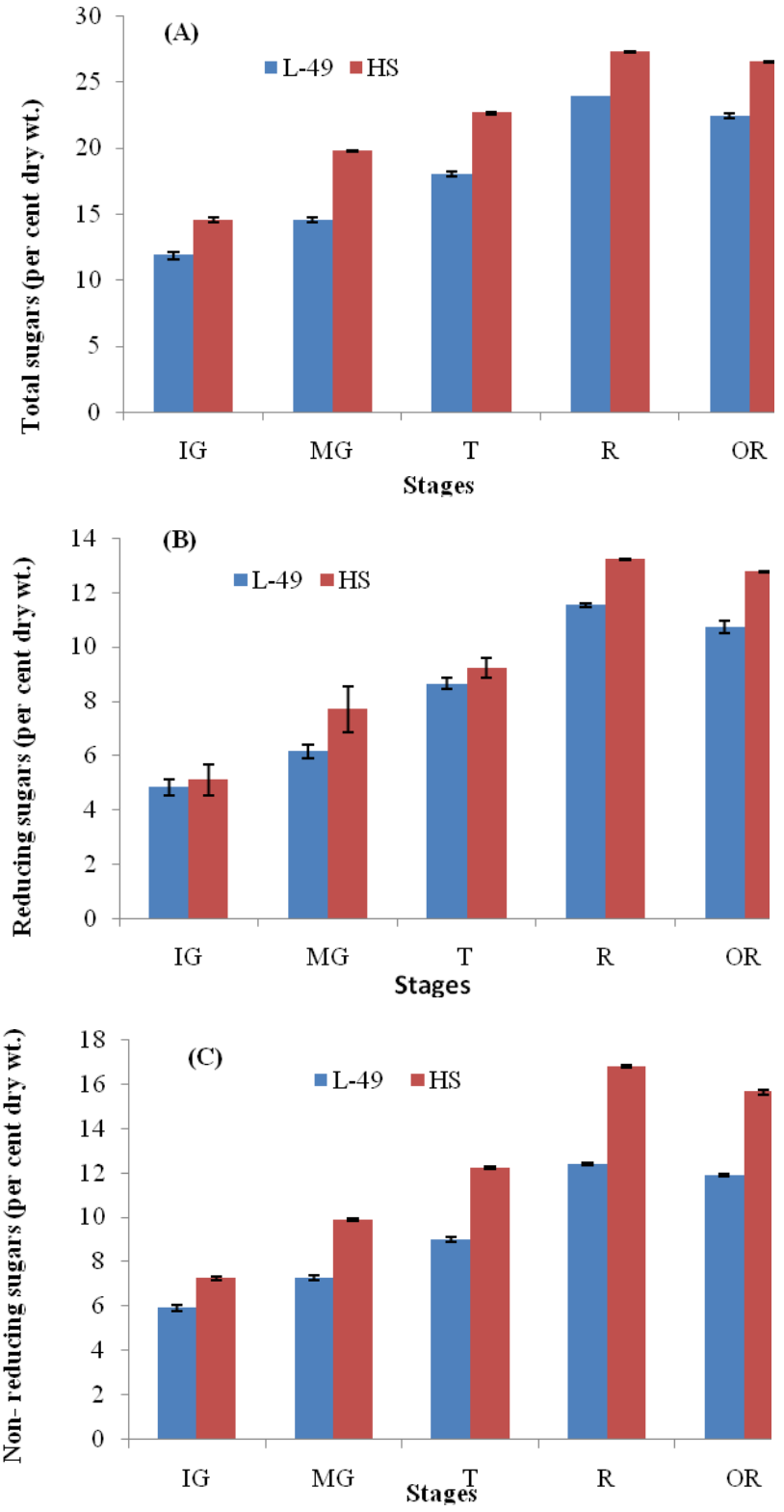

Fig. 4.4 Total (A), reducing (B) and non-reducing (C) sugars in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
(A) = a: 0.207; b: 0.327; (axb): 0.463, (B)= a: 0.449; b: 0.711; (axb): 1.01, (C)= a: 0.492; b: 0.777; (axb): 1.10, a= varieties; b= stages

The cell wall of fleshy fruits undergoes a number of structural and compositional changes as the fruits soften during ripening (Brady, 1987). These changes involve the solubilization of pectins as characterized by increased depolymerization of various pectin classes (Cheng and Huber, 1996; Rose *et al.* 1998), modification of hemicelluloses (Maclachlan and Brady, 1994) and alterations in cellulose microfibrils (Carpita and Gibeaut, 1993). Since the primary cell wall is a highly complex structure, it would be likely that a concerted effort of a variety of enzymes might be required to affect the disassembly of cell wall components and fruit softening (Giovannoni *et al.* 1992). Therefore, loss of fruit firmness during ripening of fruits like guava can be best assessed by studying the changes in cell wall components and their hydrolyzing enzymes during the ripening process.

As depicted in Fig. 4.5, NDF content increased from IG to MG stage followed by a decline with the advancement of ripening in both the cultivars. Maximum NDF content of 52.90 and 46.50 per cent in MG fruits decreased to 31.80 and 34.50 per cent in OR fruits of *cvs*. L-49 and Hisar Surkha, respectively.

ADF content in the two cultivars also showed similar pattern as followed by NDF (Fig. 4.6), having maximum value of 44.35 and 38.40 per cent at MG stage which declined continuously with the advancement of ripening to a minimum value of 31.55 and 28.95 per cent at OR stage in *cvs*. L-49 and Hisar Surkha, respectively. The firm cultivar, L-49, showed higher content of NDF and ADF than the soft variety Hisar Surkha at all the stages of ripening.

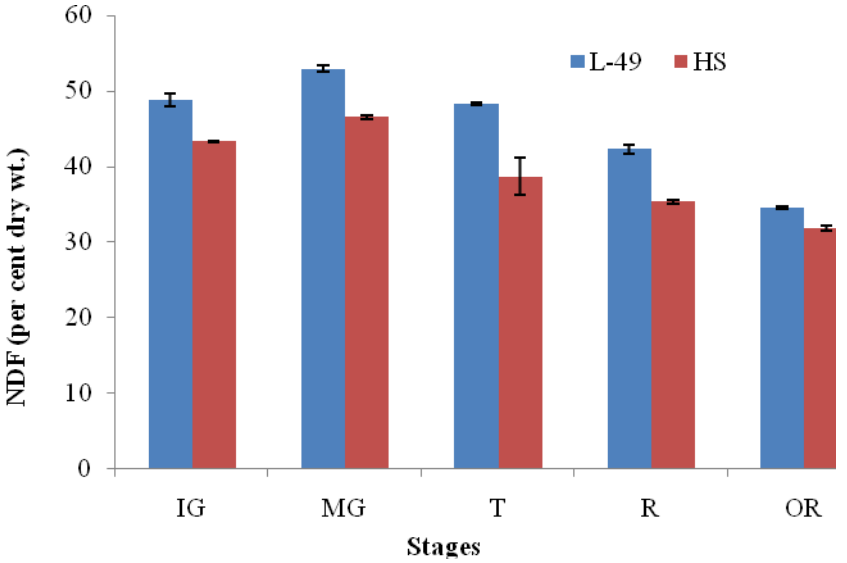

Fig. 4.5 NDF content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 1.16; b: 1.83; (axb): 2.59, a= varieties; b= stages

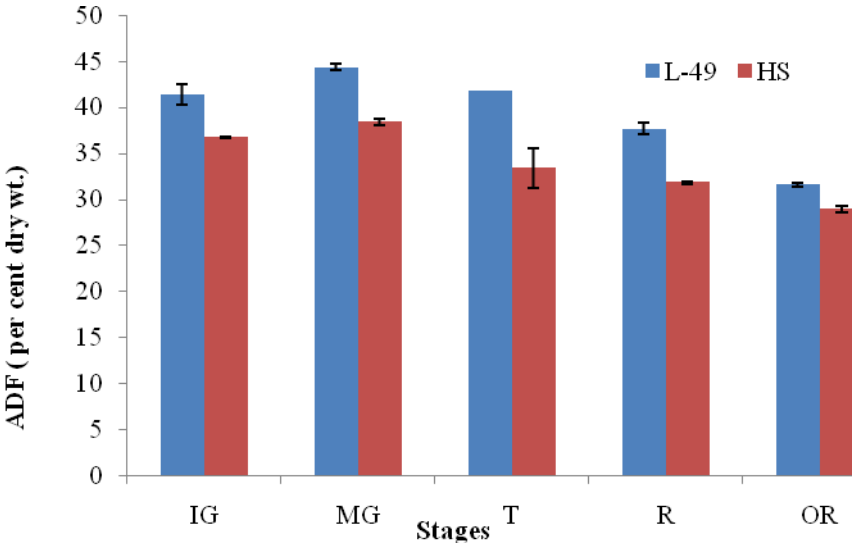

Fig. 4.6 ADF content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 1.094; b: 1.729; (axb): 2.446, a= varieties; b= stages

As is evident from the data, hemicellulose content (Fig. 4.7) also attained its maximum value of 8.55 and 8.10 per cent in MG fruits and then decreased throughout ripening reaching a minimum value of 2.96 and 2.85 per cent in OR fruits of *cvs*. L-49 and Hisar Surkha, respectively.

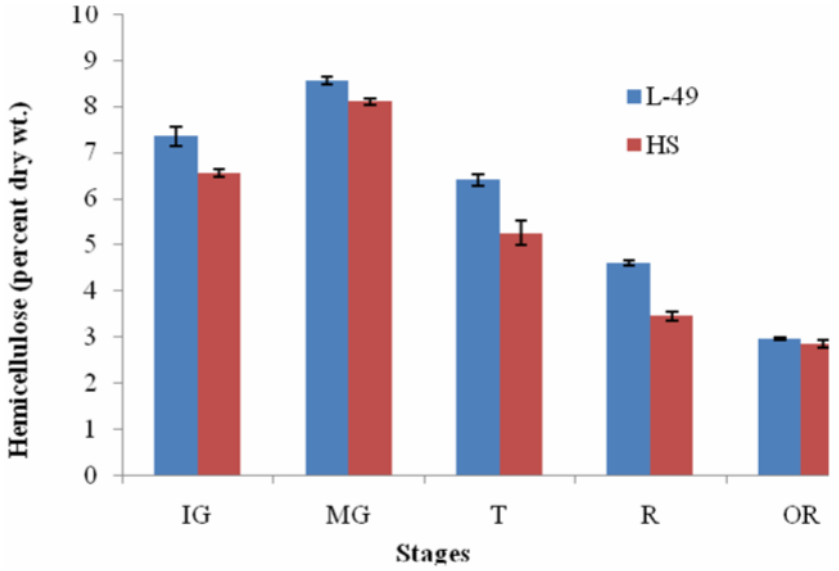

Fig.4.7 Hemicellulose content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.168; b: 0.265; (axb): 0.375, a= varieties; b= stages

Similar to that of NDF, ADF and hemicellulose, cellulose (Fig. 4.8) lignin (Fig. 4.9) and silica (Fig. 4.10) content was also maximum at MG stage and thereafter, decreased

throughout ripening attaining a minimum value at OR stage. Maximum cellulose content of 28.55 and 27.20 per cent at MG stage decreased to 20.55 and 17.4 per cent at OR stage of *cvs.* L-49 and Hisar Surkha, respectively. Similarly maximum lignin content of 15.26 per cent and minimum of 12.30 per cent was observed in L-49 whereas it was 13.18 and 11.33 per cent in Hisar Surkha at MG and OR stage, respectively. Among all the cell wall components, silica was found to be present in least concentration and ranged from 0.34 (OR) to 0.67 per cent in L-49 and from 0.22 (OR) to 0.57 per cent (MG) in Hisar Surkha.

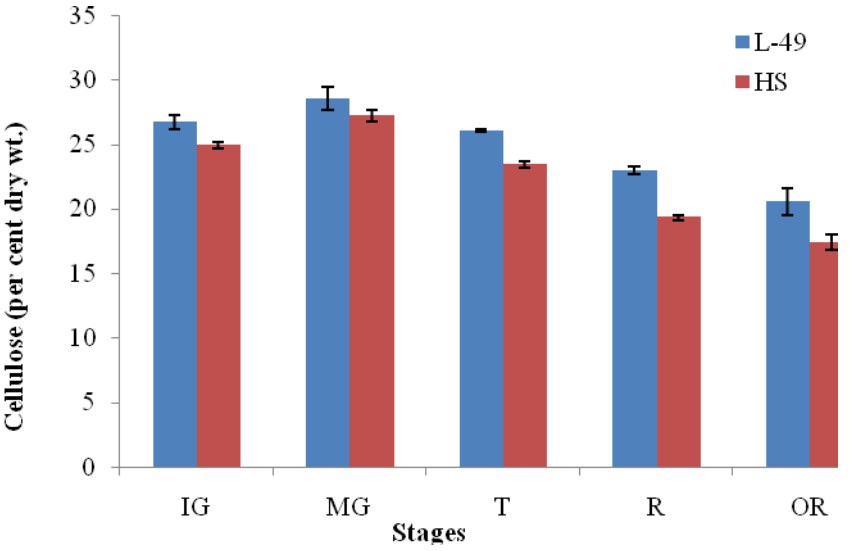

Fig. 4.8 Cellulose content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.72; b: 1.14; (axb): NS, a= varieties; b= stages

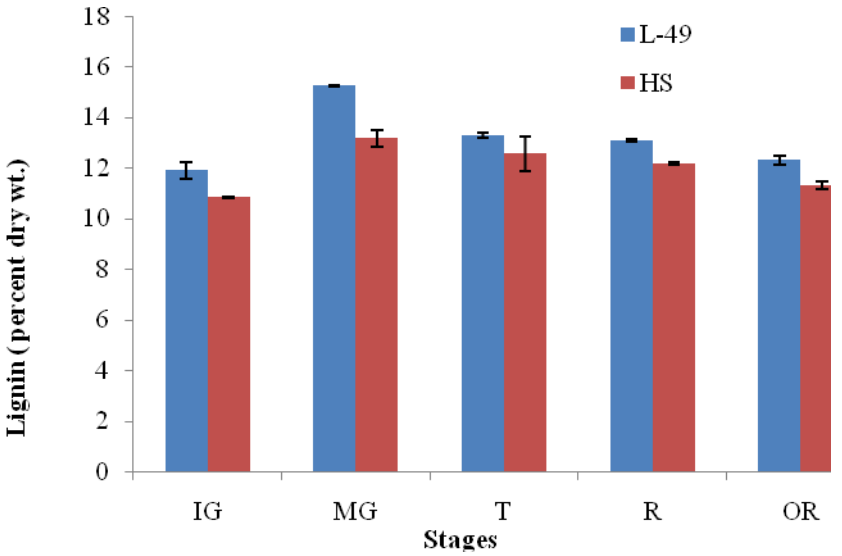

Fig. 4.9 Lignin content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.361; b: 0.571; (axb): NS, a= varieties; b= stages

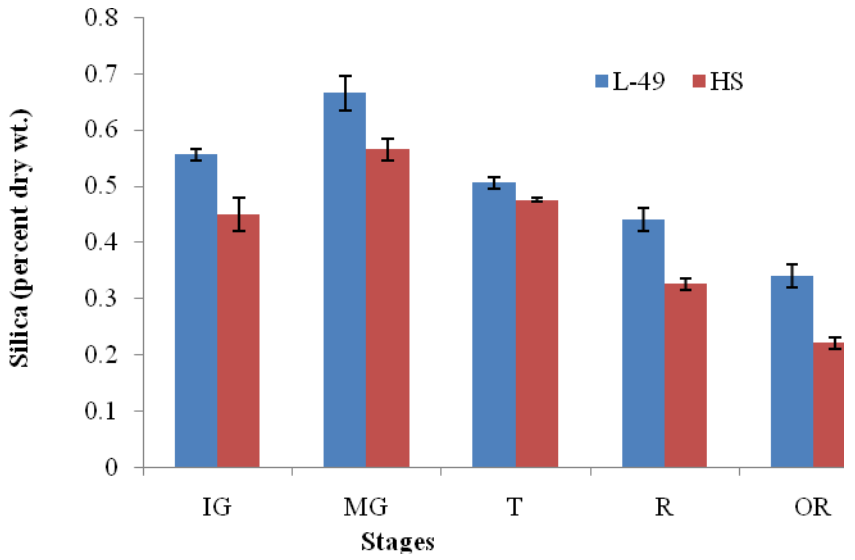

Fig. 4.10 Silica content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.024; b: 0.038; (axb): NS, a= varieties; b= stages

Changes in pectin content are closely related to the changes observed in the other cell wall components (Fig. 4.11). Highest pectin content of 3.19 and 2.57 per cent observed at MG stage decreased to minimum values of 1.34 and 1.15 per cent during OR stage in L-49 and Hisar Surkha, respectively.

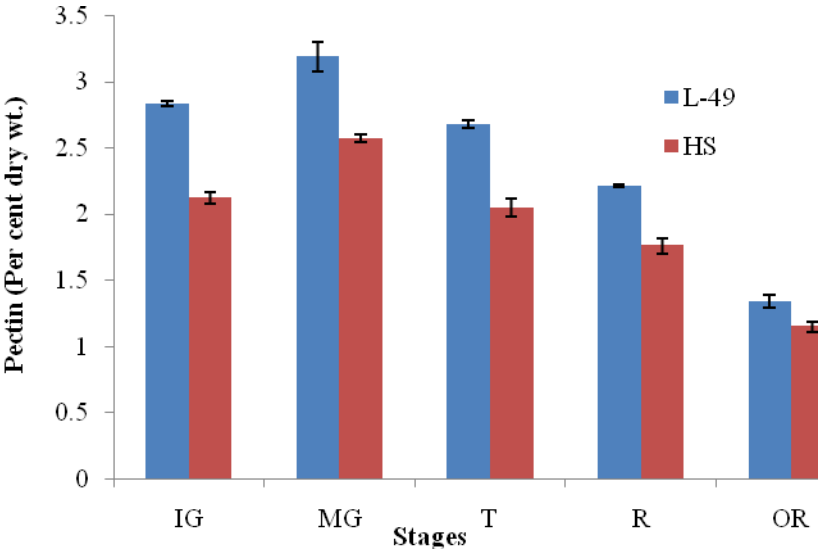

Fig. 4.11 Pectin content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.071; b: 0.113; (axb): 0.159, a= varieties; b= stages

At all the stages of ripening, L-49 had higher content of cell wall components *viz.* cellulose, hemicelluloses, lignin, silica and pectin than Hisar Surkha. The decrement in the

cell wall components during ripening may be due to hydrolysis of cell wall components leading to softening of fruits.

PME activity increased upto T stage followed by a decrease upto OR stage in both the cultivars (Fig. 4.12). At IG stage, PME activity was 2.31 and 4.12 units in L-49 and Hisar Surkha, respectively. The corresponding values at T stage were 8.92 and 15.43 and 4.35 and 8.39 units at OR stage. As is clear from the data, the variety Hisar Surkha had approximately 1.5 fold higher PME activity than L-49 at all the stages of ripening.

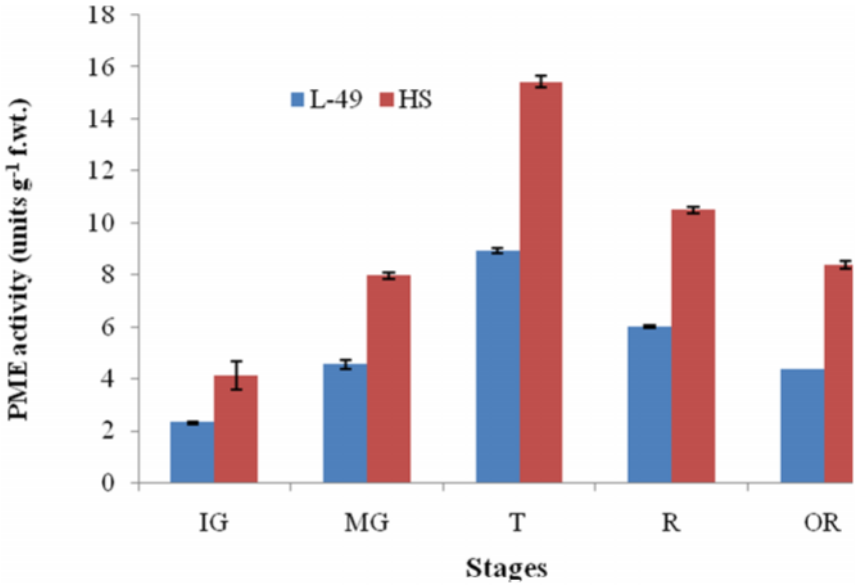

Fig. 4.12 Pectn methylesterase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.272; b: 0.430; (axb): 0.608, a= varieties; b= stages

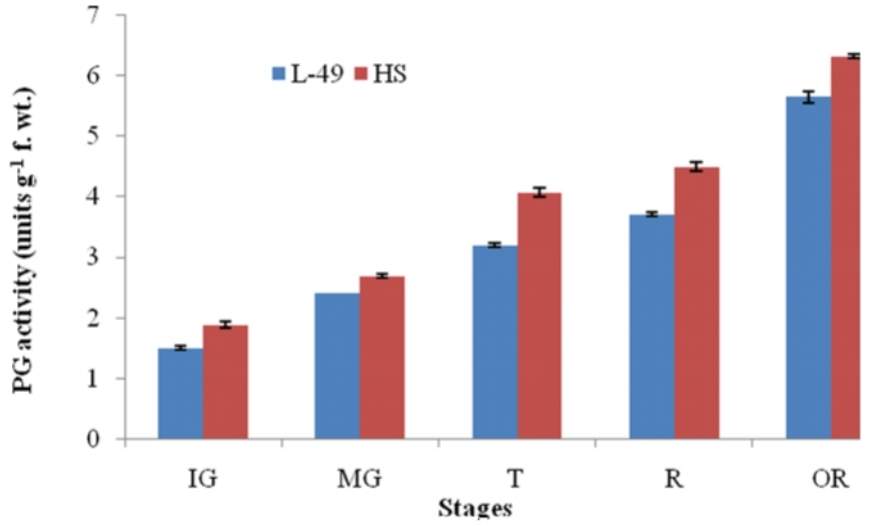

Fig. 4.13 Polygalacturonase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.086; b: 0.136; (axb): 0.192, a= varieties; b= stages

However, activities of other cell wall hydrolyzing enzymes *viz.* polygalacturonase (PG) (Fig. 4.13) and cellulase (Fig. 4.14) increased linearly throughout ripening, attaining their maximum value at OR stage. Though both the varieties had similar pattern, but they exhibited significant differences in their activities, with higher activities in Hisar Surkha throughout ripening. PG and cellulase activities were very low initially (1.51 and 1.89 units; 1.14 and 1.64 units in L-49 and Hisar Surkha for PG and cellulase, respectively) and increased significantly during ripening reaching upto 5.64 and 6.31 units; 5.32 and 6.14 units at OR stage.

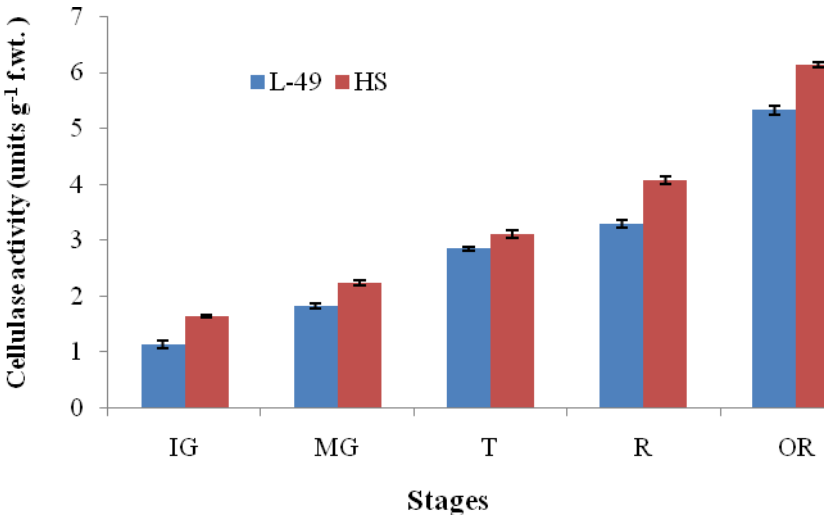

Fig. 4.14 Cellulase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.076; b: 0.119; (axb): 0.169, a= varieties; b= stages

Table 4.1: Pearson correlation matrix between fruit firmness, different cell wall components and their hydrolyzing enzymes

	Firmness	Cellulose	Hemicellulose	Pectin	Cellulase	PME	PG
Firmness	1.000						
Cellulose	0.930**	1.000					
Hemicellulose	0.913**	0.968**	1.000				
Pectin	0.916**	0.946**	0.926**	1.000			
Cellulase	-0.915**	-0.902**	-0.897**	-0.882**	1.000		
PME	-0.447NS	-0.254NS	-0.214NS	-0.187NS	0.220NS	1.000	
PG	-0.923**	-0.886**	-0.888**	-0.864**	0.991**	0.312NS	1.000

As depicted in Table 4.1, cell wall components (cellulose, hemicelluloses and pectin) were positively correlated with the fruit firmness. Cellulase (-0.915**) and PG (-0.923**) were negatively correlated with fruit firmness while no significant correlation was present between PME and fruit firmness. A negative correlation was present between cellulose and cellulase (-0.902**), hemicellulose and cellulase (-0.897**) and pectin and PG (-0.864**).

4.1.2.2 Lipid peroxidation, oxidative stress and antioxidative system

Fruit ripening is characterized by membrane disruption leading to cellular decompartmentation and loss of tissue structure (Paliyath and Droillard, 1992, Ferrie *et al.* 1994) and higher production of ROS (Del Rio *et al.* 1998) and gradual loss in the ability of scavenging enzymes to neutralize the free radicals (Kanazawa *et al.* 2000). Since dysfunction of antioxidative system is believed to be involved in the progression of ripening (Del Rio *et al.* 1998), it was thought worthwhile to study the indices of reactive oxygen species and the antioxidaitve enzymes in guava fruit during ripening.

4.1.2.2.1 Lipid peroxidation and oxidative stress

Changes in LOX activity during ripening of L-49 and Hisar Surkha fruits presented in Fig. 4.15. The activity was lowest at the IG stage in both the cultivars (321.7 and 425.7 µmols of conjugated dienes produced min^{-1} g^{-1} f.wt. in L-49 and Hisar Surkha, respectively) and increased significantly (about 2.5 to 3 fold) reaching the maximum values of 1008.4 and 1122.5 µmol conjugated dienes produced min^{-1} g^{-1} f.wt., respectively at OR stage. The two varieties exhibited significant variations in the LOX activity and the soft cultivar Hisar Surkha had higher LOX activity than the firm cultivar L-49 throughout the ripening period.

Superoxide radicals (Fig. 4.16) are reactive oxygen species and toxic for cell macromolecules. In both the cultivars, superoxide radicals were minimum at IG stage (100.1 and 115.7 nmols g^{-1} f.wt. in L-49 and Hisar Surkha, respectively), increased significantly (about 1.5 fold) throughout the ripening period and were maximum at OR stage (163.8 and 211.6 nmols g^{-1} f.wt. in L-49 and Hisar Surkha, respectively). Here again, Hisar Surkha, the cultivar with short shelf-life had significantly higher value for superoxide radicals than L-49 throughout ripening period.

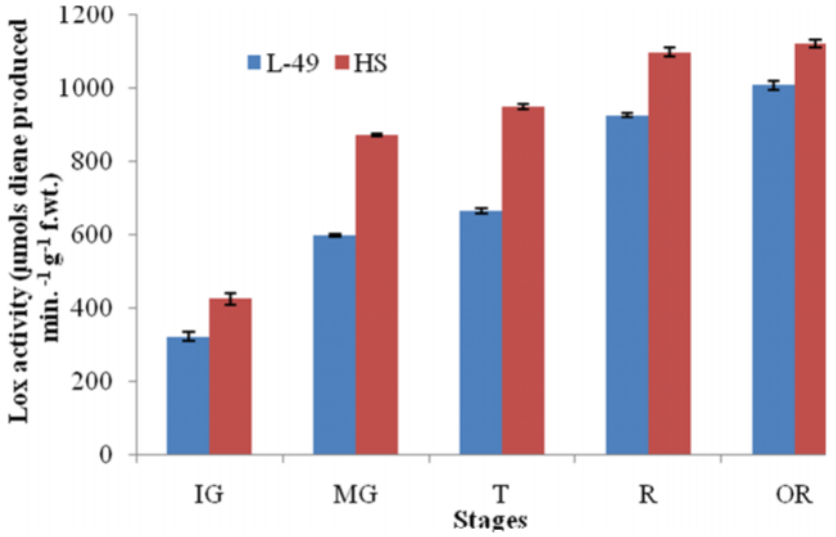

Fig. 4.15 Lipoxygenase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 12.84; b: 20.31; (axb): 28.72, a= varieties; b= stages

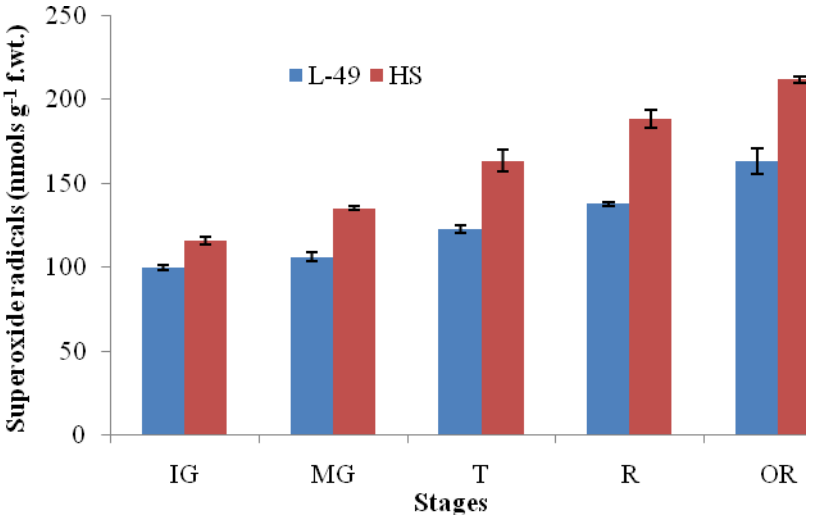

Fig. 4.16 Superoxide radicals in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 5.26; b: 8.32; (axb): 11.76, a= varieties; b= stages

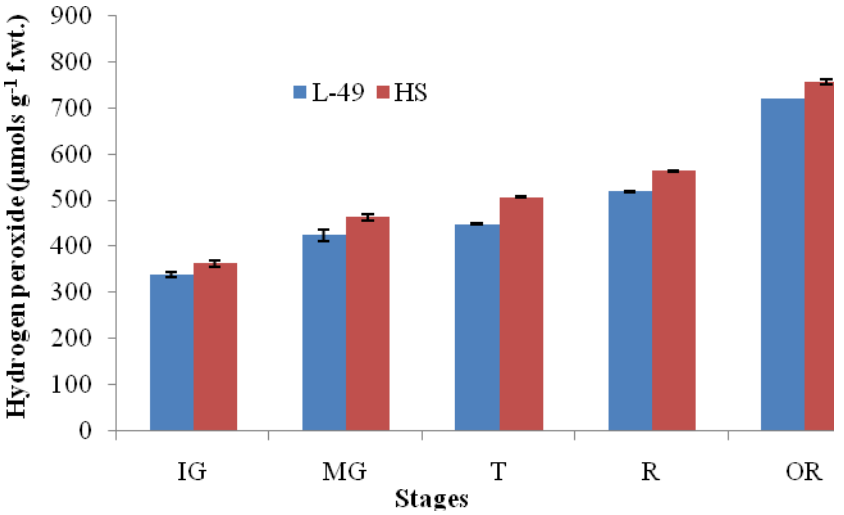

Fig. 4.17 Hydrogen peroxide in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 7.61; b: 12.03; (axb): NS, a= varieties; b= stages

Fig. 4.17 presents the results on H_2O_2 content. Data reveal that H_2O_2 content also exhibited a pattern similar to that exhibited by LOX activity and superoxide radicals, increasing progressively from IG stage (339.4 and 362.6 µmols g^{-1} f.wt. in L-49 and Hisar Surkha, respectively) to OR stage (719 and 757 µmol g^{-1} f.wt. in L-49 and Hisar Surkha, respectively). The soft variety, Hisar Surkha had higher H_2O_2 content than L-49 at all the stages of fruit ripening.

4.1.2.2.2 Antioxidative enzymes and metabolites

Fig. 4.18 depicts the changes in SOD activity in two cultivars of guava fruit during ripening. The activity increased significantly and progressively from 140.78 and 127.45 unit g^{-1} f.wt. at IG stage to 183.85 and 170.69 unit g^{-1} f.wt. at T stage in L-49 and Hisar Surkha,

respectively, followed by a continuous decrease upto OR stage. In general, L-49 exhibited higher activity of SOD as compared to Hisar Surkha at all the stages of fruit ripening

Activity profile of CAT during ripening of two cultivars of guava (Fig. 4.19) followed a pattern similar to that followed by SOD. The activity increased tremendously from 421.3 and 392 units g^{-1} f.wt. at IG stage to 1281.6 and 1101.9 units g^{-1} f.wt. at T stage respectively in L-49 and Hisar Surkha. Thereafter, it decreased gradually to 975.1 (L-49) and 710.6 (Hisar surkha) units g^{-1} f.wt. at OR stage. At all the stages of fruit ripening, similar to SOD, catalase activity was higher in L-49 as compared to Hisar Surkha.

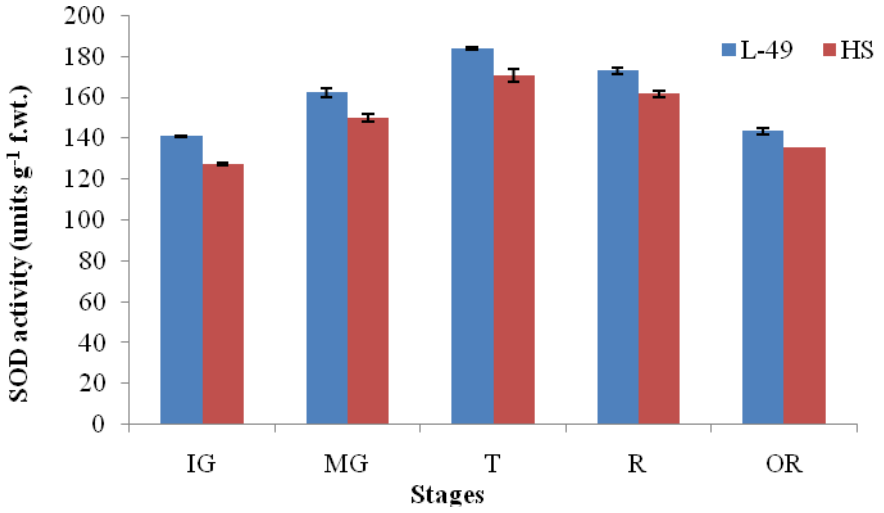

Fig. 4.18 Superoxide dismutase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 2.103; b: 3.325; (axb): NS, a= varieties; b= stages

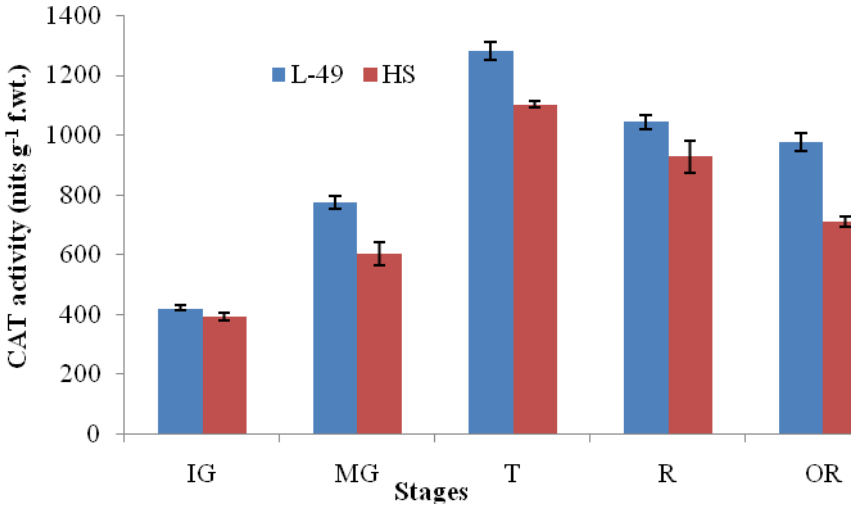

Fig. 4.19 Catalase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 46.10; b: 32.89; (axb): NS, a= varieties; b= stages

Similar to the pattern exhibited by SOD and CAT, APX activity (Fig. 4.20) also increased from IG stage (596.23 and 451.06 units g^{-1} f.wt.) to T stage (1091 and 1012.6 units g^{-1} f.wt.**)** and thereafter declined to 675.08 and 578.64 units g^{-1} f.wt. at OR stage in L-49 and Hisar Surkha, respectively. The differences in enzyme activity of two cultivars at each stage were significant and the variety L-49 had higher activity than Hisar Surkha at all the stages of fruit ripening.

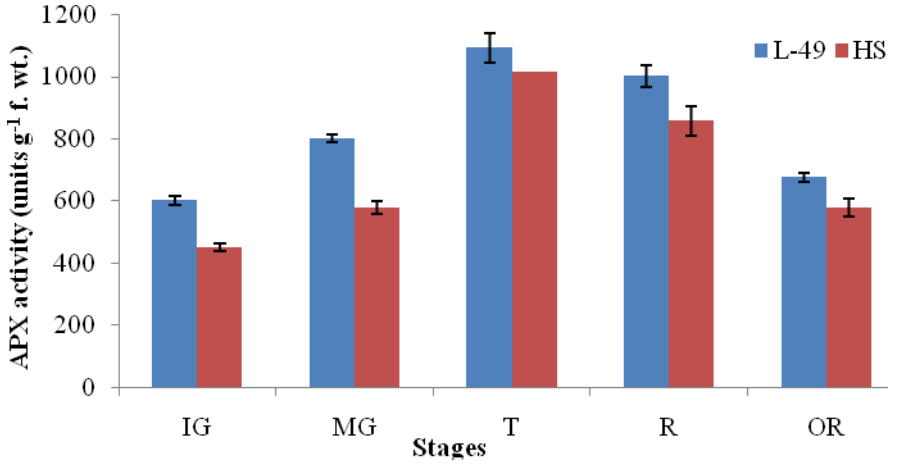

Fig. 4.20 Ascorbate peroxidase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 34.18; b: 54.04; (axb): 76.43, a= varieties; b= stages

Glutathione reductase (Fig. 4.21) also followed a pattern similar to that followed by the other ROS scavenging enzymes except that maximum activity was observed at the MG stage (6.41 and 4.09 units g^{-1} f.wt. in L-49 and Hisar Surkha, respectively) which declined continuously and reached a corresponding value of 1.84 and 1.15 units g^{-1} f.wt. at OR stage. L-49 exhibited significantly higher GR activity than Hisar Surkha at all the ripening stages.

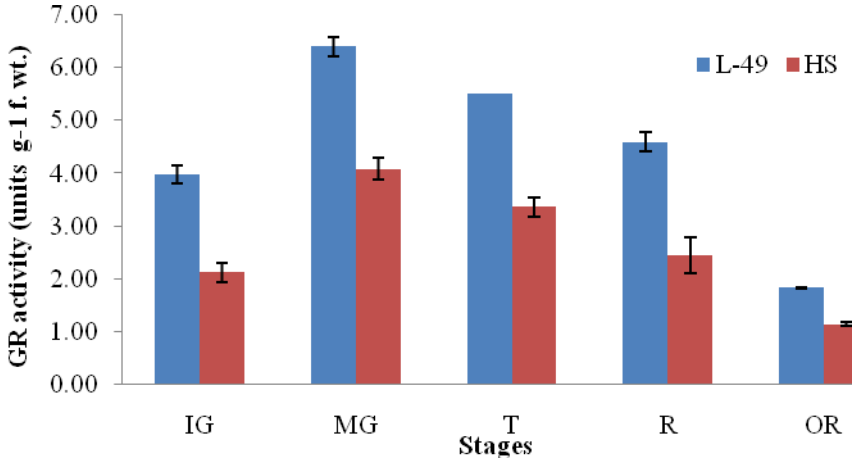

Fig. 4.21 Glutathione reductase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.240; b: 0.379; (axb): 0.537, a= varieties; b= stages

Activity profile of peroxidase (POX) during ripening of two cultivars of guava (Fig. 4.22) reveals that it followed a pattern similar to that followed by GR. The enzyme exhibited progressive increase in activity from 109.50 and 84.72 units g^{-1} f.wt. at IG stage to 154.25 and 144.85 units g^{-1} f.wt. at T stage, followed by a decline in activity to 96.04 and 74.94 units g^{-1} f.wt. at OR stage in L-49 and Hisar Surkha, respectively.

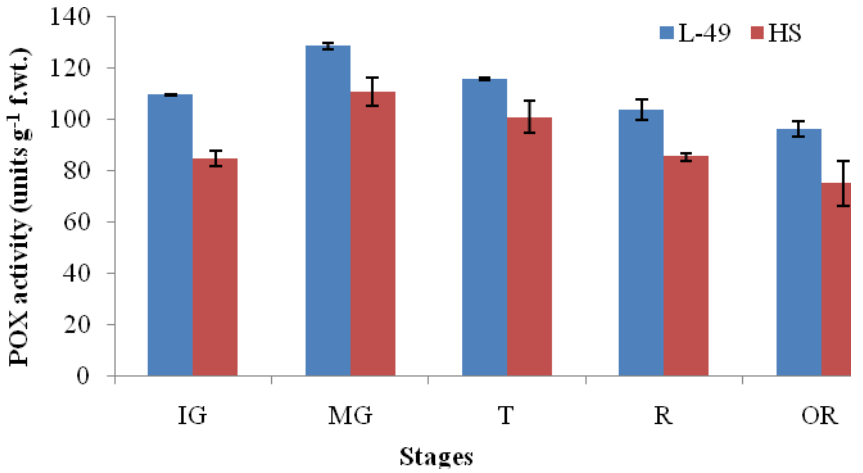

Fig. 4.22 Peroxidase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 5.46; b: 8.64; (axb): NS, a= varieties; b= stages

Ascorbic acid is an important ROS scavenging metabolite. Results presented in Fig. 4.23, reveal that ascorbic acid content increased from IG to T stage from 42.56 and 54.88 mg/100g f.wt. to 161.31 and 198.24 mg/100g f.wt. in L-49 and Hisar Surkha, respectively thereafter, declined progressively to 94.08 and 114.24 mg/100g f.wt. at OR stage in L-49 and Hisar Surkha, respectively. Throughout ripening Hisar Surkha, the variety with short shelf-life had higher ascorbic acid content than L-49.

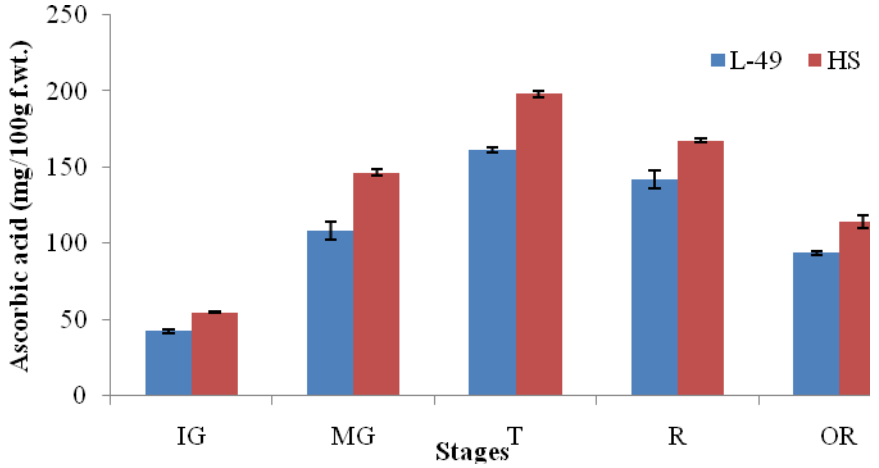

Fig. 4.23 Ascorbic acid content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 4.43; b: 7.00; (axb): 9.90, a= varieties; b= stages

Total glutathione content (Fig. 4.24) was found to be maximum at MG stage in both the guava cultivars being 512.70 and 460.67 µmols g^{-1} f.wt. for L-49 and Hisar Surkha, respectively. Thereafter, total glutathione content decreased, reaching minimum at OR stage i.e. 228.63 and 174.96 for L-49 and Hisar Surkha, respectively. L-49, the variety with long shelf-life had higher total glutathione content than Hisar Surkha throughout ripening.

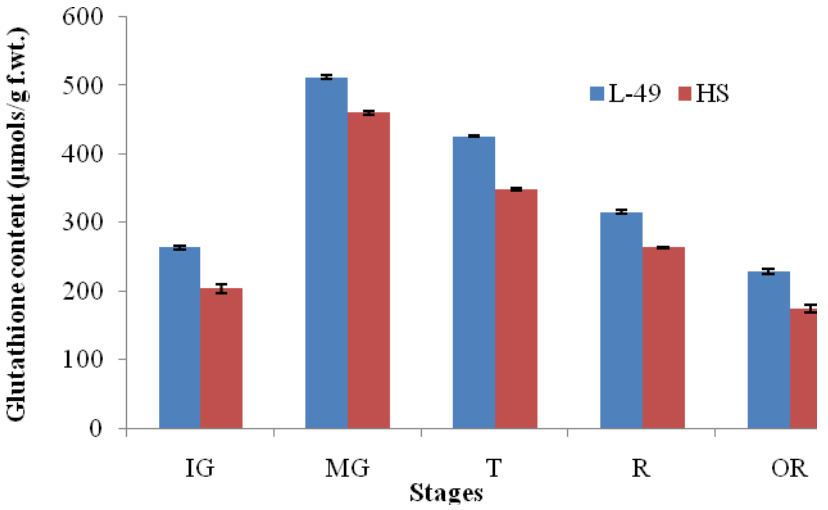

Fig. 4.24 Glutathione content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 4.75; b: 7.51; (axb): 10.62, a= varieties; b= stages

Fig. 4.25 reveals that the maximum β-carotene content of 466.19 and 409.39 µg/100g f.wt. at IG stage in L-49 and Hisar Surkha, respectively, decreased gradually towards ripening and reached a minimum of 184.71 and 103.93 µg/100g f.wt. at OR stage. Hisar Surkha, the variety with short shelf-life had lower β-carotene content than L-49 throughout ripening.

Both the varieties showed highest antioxidant activity at MG stage i.e. 47.27 per cent in L-49 and 76.65 per cent in Hisar Surkha, which decreased gradually to the respective values of 31.78 and 41.75 per cent at OR stage. Hisar Surkha, (short shelf-life) showed significantly higher antioxidant activity than L-49 throughout the ripening period.

4.1.2.3 Ethylene and ACC oxidase

In climacteric fruits, ripening is accompanied by a peak in respiration and a concomitant burst of ethylene. It induces a mature, non growing tissue to rapidly differentiate into a new state-to switch from non-ripening to ripening by affecting the transcription and translation of many ripening-related genes (Hiwasa *et al.* 2003; Giovannoni, 2001). The increase in ethylene production is accompanied by increase in the activity of ACC oxidase (Yang and Hoffman, 1984; Mathooko, 1996).

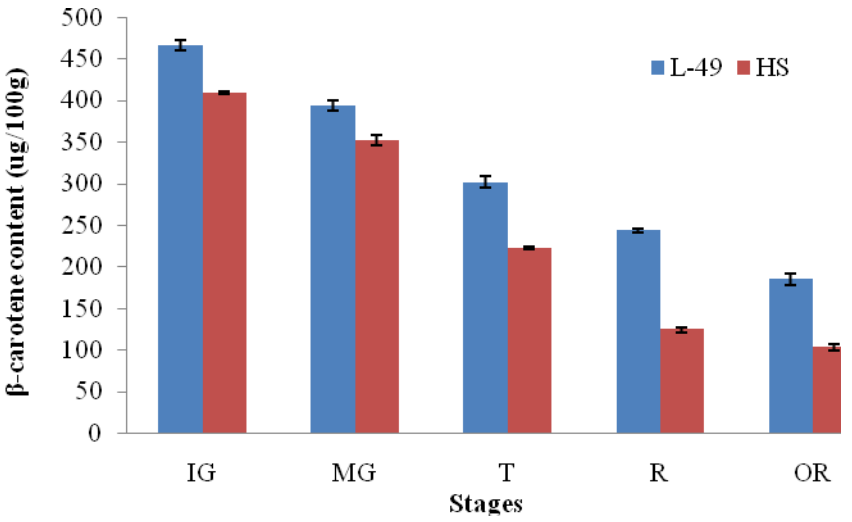

Fig. 4.25 β-carotene content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)

a: 6.77; b: 10.71; (axb): 15.14, a= varieties; b= stages

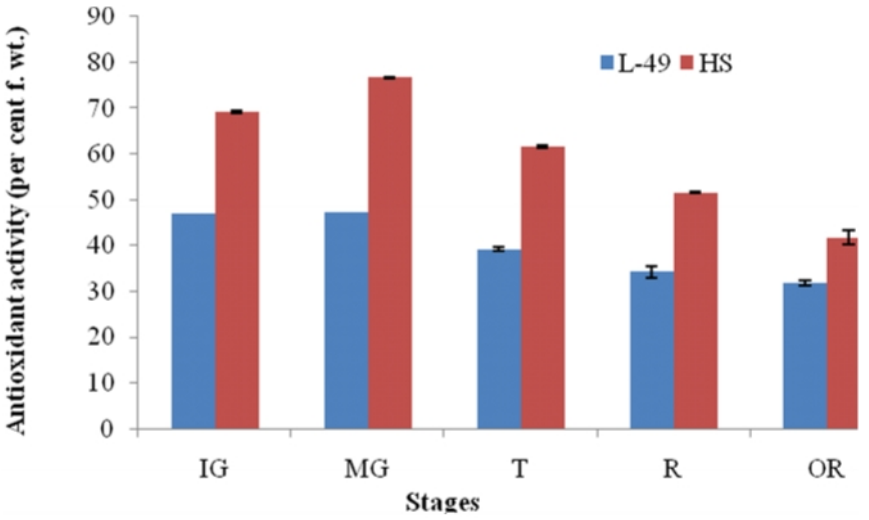

Fig. 4.26 Antioxidant activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.88; b: 1.34; (axb): 1.97, a= varieties; b= stages

Results on ethylene evolution in the two cultivars of guava during fruit development reveals maximum ethylene evolution at turning stage which coincided with the eating ripeness (Fig 4.27). The rate of ethylene evolution increased by more than 4 fold from IG 5.93 and 7.58 nmol h^{-1} g^{-1} f.wt. in L-49 and Hisar Surkha, respectively) to T stage (25.02 and 32.96 nmol h^{-1} g^{-1} f.wt. in L-49 and Hisar Surkha, respectively) of fruit ripening. Significantly higher rates of ethylene evolution were recorded for soft (Hisar Surkha) compared to firm cultivar (L-49).

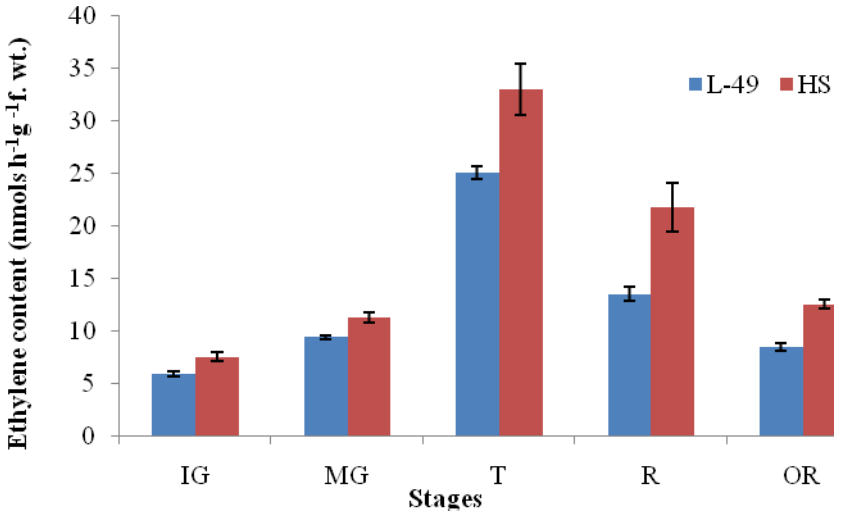

Fig. 4.27 Ethylene content in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 0.869; b: 1.374; (axb): 1.943, a= varieties; b= stages

Data on the activity profile of ACC oxidase (Fig. 4.28) depict that activity increased substantially from 13.97 and 19.48 units g^{-1} f.wt. at IG stage to 50.45 and 67.10 units g^{-1} f.wt. at T stage in L-49 and Hisar Surkha, respectively. During further ripening process, the activity got reduced at OR stage to 31.89 and 37.20 units g^{-1} f.wt., respectively in L-49 and Hisar Surkha. ACC oxidase activity was significantly higher in Hisar Surkha than in L-49 at all the stages of fruit ripening.

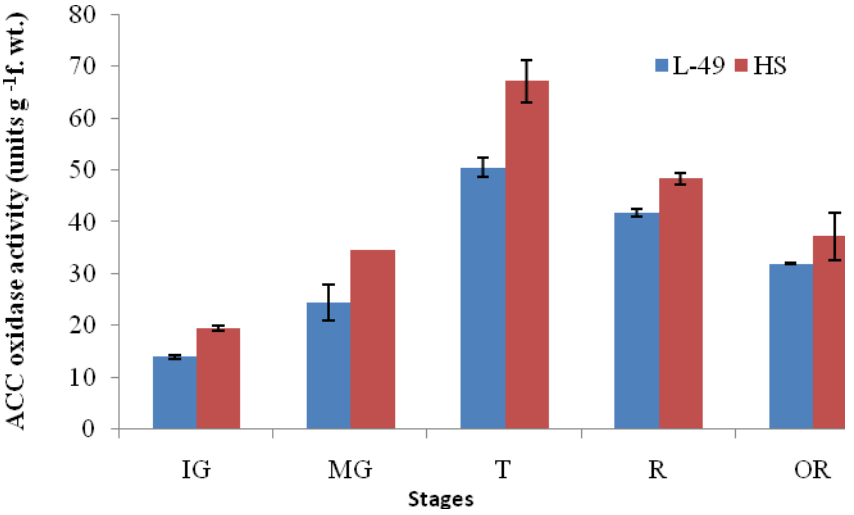

Fig . 4.28 ACC oxidase activity in guava fruit at different stages of ripening

Two factorial analysis of variance (P ≤0.05)
a: 1.800; b: 2.846; (axb): 4.025, a= varieties; b= stages

4.2 Effect of exogenous application of GA_3 and $CaCl_2$ on physico-chemical and biochemical changes in guava fruit

4.2.1 Effect of different concentrations of GA_3 and $CaCl_2$ on fruit firmness and total soluble solids

To optimize concentration and time of GA_3 and $CaCl_2$ treatments for increasing shelf-life of guava, MG fruits of both the cultivars, free of any visible defects and approximately of same size, were treated with different concentrations of $CaCl_2$ *viz.* 0.5 %, 1 %, 2 %, 3 %, 4 %, 5 % and GA_3 *viz.* 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm for 3, 5 and 10 min. The fruits were then taken out, extra solution wiped off, air dried and were analyzed for TSS and Firmness.

Table 4.2: Effect of dose and time of $CaCl_2$ treatment on fruit firmness in guava at mature green stage

Duration of treatment	Firmness (kg/cm^2)								
	Variety	Control	$CaCl_2$ concentration (%)						Mean
			0.5	1	2	3	4	5	
3 min	HS	9.29	9.65	10.62	10.09	10.35	9.82	10.02	**9.98**
5 min	HS	9.06	10.22	10.44	10.62	10.26	10.12	10.27	**10.14**
10 min	HS	9.07	9.74	9.91	10.27	10.08	9.8	10.33	**9.89**
Mean		**9.14**	**9.87**	**10.32**	**10.33**	**10.23**	**9.91**	**10.21**	
3 min	L-49	13.04	13.87	14.15	14.19	13.96	13.66	13.59	**13.78**
5 min	L-49	13.04	14.13	14.51	14.48	14.25	13.17	13.86	**13.92**
10 min	L-49	13.03	13.59	14.25	14.13	13.78	13.97	13.75	**13.79**
Mean		**13.04**	**13.86**	**14.30**	**14.27**	**14.00**	**13.6**	**13.73**	

CD at 5 % level	For HS			For L-49		
	a	-	0.719	a	-	0.665
	b	-	NS	b	-	NS
	Interaction (ab)	-	NS	Interaction (ab)	-	NS

a=Treatment, b=Time

As depicted in Table 4.2 and 4.3 all the treated fruits were more firm as compared to control fruits. Among all the concentrations tested, 1 and 2 % $CaCl_2$ were most effective in maintaining fruit firmness as compared to other treatments as well as control in both the cultivars (Table 4.2). Among the different GA_3 concentrations tested 50 and 100 ppm were most effective treatments in maintaining fruit firmness in Hisar Surkha while 100 and 150 ppm were most effective treatments in L-49 (Table 4.3). The treatment of GA_3 and $CaCl_2$ for 5 min resulted in higher fruit firmness as compared to 3 and 10 min treatments but the effect was not statistically significant.

Table 4.3: Effect of dose and time of GA_3 treatment on fruit firmness in guava at mature green stage

Duration of treatment	Firmness (kg/cm^2)							
	Variety	Control	GA_3 concentration (ppm)					Mean
			25	50	75	100	125	150	
3 min	HS	9.29	9.91	9.83	9.65	9.32	9.37	9.42	**9.55**
5 min	HS	9.06	9.43	10.26	10.16	10.33	10.13	10.21	**9.99**
10 min	HS	9.07	9.43	10.09	9.65	10.08	9.56	9.71	**9.69**
Mean		**9.14**	**9.59**	**10.06**	**9.82**	**9.91**	**9.69**	**9.78**	
3 min	L-49	13.04	13.25	13.86	13.35	13.96	14.03	14.01	**13.64**
5 min	L-49	13.04	13.58	13. 79	13.51	14.21	13.87	14.15	**13.73**
10 min	L-49	13.03	13.33	13.68	13.17	14.13	13.52	13.79	**13.52**
Mean		**13.04**	**13.39**	**13.77**	**13.34**	**14.10**	**13.81**	**13.98**	

CD at 5 % level	For HS			For L-49		
	a	-	0.487	a	-	0.476
	b	-	NS	b	-	NS
	Interaction (ab)	-	NS	Interaction (ab)	-	NS
	a=Treatment, b=Time					

Similarly, all the different concentrations of GA_3 and $CaCl_2$ treatments significantly decreased TSS content as depicted in Table 4.4 and 4.5 but $CaCl_2$ at 1 and 2 % had more pronounced effect in lowering TSS content as compared to other concentrations (Table 4.4).

Table 4.4: Effect of dose and time of $CaCl_2$ treatment on total soluble solids in guava at mature green stage

Duration of treatment	TSS (%)								
	Variety	Control	$CaCl_2$ concentration (%)						Mean
			0.5	1	2	3	4	5	
3 min	HS	13.65	12.88	12.57	12.35	12.25	12.9	12.65	**12.75**
5min	HS	13.65	12.94	11.94	12.01	12.50	12.35	12.75	**12.59**
10min	HS	13.95	12.73	12.25	12.38	12.5	12.53	12.49	**12.69**
Mean		**13.75**	**12.85**	**12.25**	**12.25**	**12.42**	**12.59**	**12.63**	
3 min	L-49	10.43	9.5	9.07	9.25	9.51	10.24	10.6	**9.80**
5min	L-49	10.43	9.95	8.96	9.77	9.32	9.8	9.18	**9.63**
10min	L-49	10.85	9.85	9.28	9.35	9.76	10	9.81	**9.84**
Mean		**10.57**	**9.77**	**9.10**	**9.46**	**9.53**	**10.01**	**9.86**	

CD at 5 % level	For HS			For L-49		
	a	-	0.458	a	-	0.577
	b	-	NS	b	-	NS
	Interaction (ab)	-	NS	Interaction (ab)	-	NS
	a=Treatment, b=Time					

Among GA_3 treatments, 100 and 150 ppm of GA_3 were found to be most effective in lowering TSS content in both the cultivars (Table 4.5). GA_3 and $CaCl_2$ treatments for 5 min resulted in lower TSS as compared to 3 and 10 min treatments in both the cultivars but effect of different time of treatments was not statistically significant.

Table 4.5: Effect of dose and time of GA_3 treatment on total soluble solids in guava at mature green stage

Duration of treatment	TSS (%)								
	Variety	Control	GA_3 concentration (ppm)						Mean
			25	50	75	100	125	150	
3 min	HS	13.65	12.73	12.82	12.47	12.35	12.50	12.4	**12.55**
5 min	HS	13.65	12.50	12.85	12.1	12.34	12.49	12.25	**12.42**
10 min	HS	13.95	12.50	12.64	12.65	12.24	12.87	12.47	**12.56**
Mean		**13.75**	**12.58**	**12.77**	**12.41**	**12.31**	**12.62**	**12.37**	
3 min	L-49	10.43	10.00	9.90	9.50	9.22	10.00	9.75	**9.73**
5 min	L-49	10.43	9.50	9.90	9.50	9.25	9.80	9.25	**9.53**
10 min	L-49	10.85	9.83	9.75	10.2	9.41	9.83	9.69	**9.79**
Mean		**10.57**	**9.78**	**9.85**	**9.73**	**9.29**	**9.88**	**9.53**	

CD at 5 % level	For HS			For L-49		
	a	-	0.457	a	-	0.414
	b	-	NS	b	-	NS
	Interaction (ab)	-	NS	Interaction (ab)	-	NS
	a=Treatment, b=Time					

On the basis of these results two concentrations of $CaCl_2$ selected for storage study were 1 and 2 % and of GA_3 were 100 and 150 ppm, No significant differences were observed w.r.t. different time of treatment but 5 min treatment was selected for storage study as it had comparatively better effect on fruit firmness and TSS content in both the cultivars.

Fruits of guava *cvs.* L-49 and Hisar Surkha were harvested at MG stage, free of any visible defects approximately of same size were dipped in two concentrations (100 and 150 ppm) each of GA_3 acid and $CaCl_2$ (1 and 2 %). After 5 min the fruits were taken out, extra solution wiped off, air dried and stored at room temperature. Samples were taken at two day interval until complete decay and analyzed for various ripening related physico-chemical and biochemical changes. The variety Hisar Surkha got deteriorated very early and could not be sampled after 6 days of storage while L-49 could be stored upto 8 days. The results obtained are presented as below:

4.2.2 Effect of exogenous application of GA_3 and $CaCl_2$ on physico-chemical changes during storage

Results presented in Table 4.6 demonstrate that PLW increased progressively as the period of storage increased, in both the cultivars. The PLW in Hisar Surkha exceeded 10 % threshold by 6th day of storage whereas L-49 exceeded the 10 % threshold by 8th day of storage in all the treated as well as control fruits. During storage, PLW was more in Hisar Surkha, hence it deteriorate rapidly. Pretreatment of guava fruits with GA_3 and $CaCl_2$ differentially affected the PLW during storage. All the treated fruits had lower PLW as compared to control fruits in both the cultivars but $CaCl_2$ treatment was more effective as compared to GA_3 in reducing PLW. Effect was also concentration dependent in L-49, because lower concentrations showed more pronounced effect in reducing PLW. $CaCl_2$ at 1 % reduced PLW from 5.27 and 4.99 % to 4.36 and 3.92 % in L-49 and Hisar Surkha, respectively. However, both the concentrations of GA_3 and $CaCl_2$ had similar effect on PLW in Hisar Surkha.

Table 4.6: Effect of GA_3 and $CaCl_2$ treatment on physiological loss in weight in guava fruits during storage

DOS	Physiological loss in weight (PLW) (%, w/w)											
	L-49						Hisar Surkha					
		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	0	0	0	0	0	**0**	0	0	0	0	0	**0**
2	1.74	1.58	1.62	1.25	1.25	**1.49**	2.33	2.16	2.15	1.39	1.54	**1.91**
4	3.34	2.97	3.12	2.19	2.55	**2.83**	5.96	5.17	5.33	4.23	4.45	**5.03**
6	8.98	8.04	8.26	7.14	7.57	**7.99**	11.69	11.2	11.46	10.07	10.45	**10.97**
8	12.30	11.86	12.09	11.2	11.64	**11.82**						
Mean	**5.27**	**4.89**	**5.02**	**4.36**	**4.60**		**4.99**	**4.63**	**4.74**	**3.92**	**4.11**	**5.27**

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.098	P	→	0.255	a	→	0.134			
Q	→	0.087	Q	→	0.197	b	→	0.084			
PQ	→	0.195	PQ	→	NS	c	→	0.103			
						ab	→	0.189			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.231	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.146	b	→	Variety
						abc	→	NS	c	→	Days of storage

Firmness of guava fruit decreased progressively with the increasing storage period (Table 4.7) from 13.92 to 5.69 kg/cm^2 in L-49 after 8 days of storage and from 10.03 to 3.54 kg/cm^2 in Hisar Surkha after 6 days of storage. All the treatments resulted in more retention of firmness as compared to control fruits in both the cultivars except 150 ppm GA_3 treatment which had no significant effect on fruit firmness in Hisar Surkha. Maximum retention of firmness was observed in 1 % $CaCl_2$ treated fruits i.e. 10.57 kg/cm^2 (L-49) and 7.62 kg/cm^2

(Hisar Surkha). Firmness was higher in L-49 as compared to Hisar Surkha in all treated as well as control fruits, reaching a value of 5.69 kg/cm^2 at 8th day of L-49 storage which was higher than the value 5.33 kg/cm^2 even at 4th day of Hisar Surkha storage under control conditions.

Table 4.7: Effect of GA_3 and $CaCl_2$treatment on firmness in guava fruits during storage

	Firmness (kg/cm^2)											
	L-49						Hisar Surkha					
DOS	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	13.92	13.92	13.92	13.92	13.92	**13.92**	10.03	10.03	10.03	10.03	10.03	**10.03**
2	10.78	11.52	11.15	12.72	12.15	**11.66**	7.89	8.46	8.14	9.22	9.04	**8.55**
4	8.89	9.36	9.08	10.69	10.73	**9.75**	5.33	5.73	5.46	6.37	6.1	**5.79**
6	7.53	7.95	7.81	8.79	8.38	**8.09**	3.54	3.85	3.62	4.86	4.21	**4.02**
8	5.69	6.02	5.87	6.74	6.29	**6.12**						
Mean	**9.36**	**9.75**	**9.57**	**10.57**	**10.29**		**6.69**	**7.02**	**6.81**	**7.62**	**7.35**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.138	P	→	0.242	a	→	0.146			
Q	→	0.138	Q	→	0.217	b	→	0.092			
PQ	→	0.308	PQ	→	0.484	c	→	0.130			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.291	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.184	b	→	Variety
						abc	→	NS	c	→	Days of storage

There was a progressive increase in TSS content of guava fruits with increasing storage period (Table 4.8) from 9.54 °Brix at 0 day to 11.96 °Brix at 6th day of storage in L-49 and from 11.99 °Brix at 0 day to 14.51 °Brix at 4th day of storage in Hisar Surkha. Thereafter, a decline was observed at 8th day of storage in L-49 (11.61 °Brix) and at 6th day of storage in Hisar Surkha (13.59 °Brix). All the treatments resulted in significantly lower TSS content than control fruits in both the cultivars but the effect of $CaCl_2$ treatment was more pronounced than GA_3 treatment. Effect of $CaCl_2$ treatment was also concentration dependent as lower TSS content was recorded in guava fruits treated with 1 % $CaCl_2$ (10.15 % in L-49 and 12.44 % in Hisar Surkha) as compared to 2 % $CaCl_2$ (10.28 % in L-49 and 12.64 % in Hisar Surkha). However, both the tested concentrations of GA_3 had similar effect on TSS content in both the cultivars. Hisar Surkha had higher TSS content as compared to L-49 in all treated as well as control fruits.

Critical perusal of the data indicates that titratable acidity decreased linearly with the increasing period of storage from 0.528 % to 0.316 % in L-49 upto 8 days of storage and from 0.466 % to 0.207 % in Hisar Surkha upto 6 days of storage period (Table 4.9). All the treatments resulted in significantly higher titratable acidity with respect to control in both the varieties. Maximum acidity was found in calcium chloride (2 %) while its lowest value

recorded in control. It increased acidity from 0.418 to 0.434 % in L-49 and from 0.353 to 0.366 % in Hisar Surkha. The firm variety, L-49 had significantly higher acidity than the soft one, Hisar Surkha, throughout the storage period and under all the storage treatments.

Table 4.8: Effect of GA_3 and $CaCl_2$ treatments on total soluble solids in guava fruits during storage

	TSS (°Brix)											
	L-49						Hisar Surkha					
DOS	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	9.54	9.54	9.54	9.54	9.54	**9.54**	11.99	11.99	11.99	11.99	11.99	**11.99**
2	10.37	10.14	10.25	9.78	9.95	**10.09**	13.14	13.02	13.01	12.15	12.31	**12.73**
4	11.13	11.13	10.98	10.16	10.48	**10.78**	14.51	14.34	14.31	13.48	13.66	**14.06**
6	11.96	11.65	11.74	10.77	10.85	**11.39**	13.59	13.1	13.3	12.14	12.58	**12.94**
8	11.61	11.28	11.44	10.48	10.57	**11.08**						
Mean	**10.92**	**10.75**	**10.79**	**10.15**	**10.28**		**13.31**	**13.11**	**13.15**	**12.44**	**12.64**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.068	P	→	0.124	a	→	0.071			
Q	→	0.068	Q	→	0.111	b	→	0.045			
PQ	→	0.151	PQ	→	0.248	c	→	0.063			
						ab	→	0.100			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.142	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.090	b	→	Variety
						abc	→	0.201	c	→	Days of storage

Table 4.9: Effect of GA_3 and $CaCl_2$ treatments on titratable acidity in guava fruits during storage

	Titratable acidity (%)											
	L-49						Hisar Surkha					
DOS	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	0.528	0.528	0.528	0.528	0.528	**0.528**	0.466	0.466	0.466	0.466	0.466	**0.466**
2	0.440	0.446	0.443	0.452	0.458	**0.448**	0.411	0.418	0.420	0.423	0.431	**0.421**
4	0.423	0.429	0.426	0.444	0.439	**0.432**	0.326	0.332	0.328	0.334	0.341	**0.332**
6	0.383	0.387	0.386	0.401	0.404	**0.392**	0.207	0.214	0.209	0.219	0.224	**0.215**
8	0.316	0.319	0.317	0.336	0.34	**0.326**						
Mean	**0.418**	**0.422**	**0.420**	**0.432**	**0.434**		**0.353**	**0.358**	**0.356**	**0.361**	**0.366**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.001	P	→	0.002	a	→	0.001			
Q	→	0.001	Q	→	0.002	b	→	0.001			
PQ	→	0.003	PQ	→	0.004	c	→	0.001			
						ab	→	0.002			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.002	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.002	b	→	Variety
						abc	→	0.003	c	→	Days of storage

4.2.3 Effect of GA_3 and $CaCl_2$ treatments on biochemical changes during storage

4.2.3.1 Cell wall degrading enzymes

Effect of GA_3 and $CaCl_2$ application on PME activity in two cultivars of guava fruits is presented in Table 4.10. The PME activity increased from 4.55 units g^{-1} f.wt. at 0 day to 9.22 units g^{-1} f.wt. at 4th day of storage in L-49 and 7.63 units g^{-1} f.wt. at 0 day to 15.55 units g^{-1} f.wt. at 2nd day of storage in Hisar Surkha and decreased gradually thereafter in both the cultivars.

The variety L-49 (long shelf–life), had significantly lower PME activity than Hisar Surkha (short shelf-life) at all the stages of storage. All the treatments of GA_3 and $CaCl_2$ significantly inhibited PME activity. The effect of $CaCl_2$ treatment was more pronounced than GA_3. The effect was also found to be concentration dependent as the lower concentration of each treatment was found more effective in inhibiting PME activity in both the cultivars.

Table 4.10: Effect of GA_3 and $CaCl_2$ treatments on pectin methylesterase activity in guava fruits during storage

	Pectin methylesterase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
DOS		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	4.55	4.55	4.55	4.55	4.55	**4.55**	7.63	7.63	7.63	7.63	7.63	**7.63**
2	6.05	5.47	5.61	5.12	5.31	**5.51**	15.55	14.65	14.89	13.23	13.79	**14.42**
4	9.22	8.72	8.89	8.15	8.4	**8.68**	10.40	9.93	10.2	8.63	8.69	**9.57**
6	7.71	6.94	7.25	6.23	6.37	**6.90**	9.68	9.20	9.42	8.29	8.43	**9.00**
8	5.56	5.22	5.29	4.79	4.92	**5.16**						
Mean	**6.62**	**6.18**	**6.32**	**5.77**	**5.91**		**10.82**	**10.35**	**10.53**	**9.45**	**9.64**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.167	P	→	0.095	a	→	0.098			
Q	→	0.167	Q	→	0.085	b	→	0.062			
PQ	→	0.374	PQ	→	0.190	c	→	0.087			
						ab	→	0.138			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.195	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.123	b	→	Variety
						abc	→	0.276	c	→	Days of storage

There was a progressive increase in PG activity in guava fruits throughout the storage period upto 8th day (6.08 unit g^{-1} f.wt.) in L-49 and upto 6th day (6.87 unit g^{-1} f.wt.) in Hisar Surkha (Table 4.11). Hisar Surkha had higher PG activity as compared to L-49 in control as well as in treated fruits, attaining a value of 7.13 units g^{-1} f.wt. on 6th day of storage which was even higher than 6.08 units g^{-1} f.wt., the value attained by L-49 on 8th day of storage under control conditions. All the treatments significantly inhibited PG activity. The effect of $CaCl_2$ treatment was more pronounced than GA_3 and 1 % $CaCl_2$ concentration was found to be most effective in inhibiting PG activity. It resulted in decrement in the PG activity from 4.18

to 3.48 unit g^{-1} f.wt. in L-49 and from 4.87 to 4.35 unit g^{-1} f.wt. in Hisar Surkha. However, both the concentration of GA_3 inhibited PG activity to similar extent.

Table 4.11: Effect of GA_3 and $CaCl_2$ treatments on polygalacturonase activity in guava fruits during storage

DOS	Polygalacturonase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	2.40	2.40	2.40	2.40	2.40	**2.40**	2.61	2.61	2.61	2.61	2.61	**2.61**
2	3.35	3.08	3.14	2.55	2.69	**2.96**	4.28	4.07	4.02	3.78	3.91	**4.01**
4	3.88	3.68	3.71	2.93	3.08	**3.46**	5.46	5.09	5.22	4.75	4.87	**5.08**
6	5.19	5.02	5.03	4.29	4.49	**4.80**	7.13	6.87	6.95	6.24	6.38	**6.71**
8	6.08	5.66	5.72	5.25	5.18	**5.58**						
Mean	**4.18**	**3.97**	**4.00**	**3.48**	**3.57**		**4.87**	**4.66**	**4.7**	**4.35**	**4.44**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.077	P	→	0.078	a	→	0.054			
Q	→	0.077	Q	→	0.070	b	→	0.034			
PQ	→	0.172	PQ	→	0.155	c	→	0.048			
						ab	→	0.076			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.108	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.068	b	→	Variety
						abc	→	0.152	c	→	Days of storage

Similar to PG activity, cellulase activity increased continuously from 2.15 units g^{-1} f.wt. at 0 day to 8.13 units g^{-1} f.wt. at 8th day of storage in L-49 and 2.73 units g^{-1} f.wt. at 0 day to 10.91units g^{-1} f.wt. at 4th day of storage in Hisar Surkha (Table 4.12). Soft variety Hisar Surkha had higher cellulase activity as compared to firm variety L-49 in control as well as in treated fruits, attaining a value of 8.79 units g^{-1} f.wt. just on the 4th day of storage which was even higher than the value (8.13 units g^{-1} f.wt.) attained by L-49 on 8th day of storage under control conditions. Pretreatments of fruits resulted in decrease in cellulase activities at all the stages of storage in both the cultivars. $CaCl_2$ treatment showed more pronounced effect on cellulase activity in both the cultivars as compared to GA_3 treatment. The lower concentration of each treatment was found to be more effective in inhibiting cellulase activity.

4.2.3.2 Lipid peroxidation and oxidative stress

Treatment of fruits with GA_3 and $CaCl_2$ resulted in reduction in lipoxygenase (LOX) activity as affected by different concentrations of GA_3 and $CaCl_2$ in both cultivars of guava during storage (Table 4.13). The firm variety, L-49 had significantly lower LOX activity than the soft one, Hisar Surkha throughout the storage period and under all the storage treatments.

Table 4.12: Effect of GA_3 and $CaCl_2$ treatments on cellulase activity in guava fruits during storage

DOS	Cellulase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	2.15	2.15	2.15	2.15	2.15	**2.15**	2.73	2.73	2.73	2.73	2.73	**2.73**
2	3.08	2.87	2.94	2.66	3.54	**3.02**	5.68	5.55	5.61	5.06	5.36	**5.45**
4	4.15	3.92	4.02	3.72	3.58	**3.88**	8.79	8.41	8.64	7.91	8.14	**8.38**
6	6.84	6.56	6.74	6.38	6.07	**6.52**	10.91	10.53	10.78	9.66	10.23	**10.42**
8	8.13	7.87	8.02	7.39	7.24	**7.73**						
Mean	**4.87**	**4.67**	**4.77**	**4.46**	**4.516**		**7.03**	**6.81**	**6.94**	**6.34**	**6.62**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.033	P	→	0.038	a	→	0.026			
Q	→	0.033	Q	→	0.034	b	→	0.017			
PQ	→	0.074	PQ	→	0.076	c	→	0.023			
						ab	→	0.037			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.053	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.033	b	→	Variety
						abc	→	0.074	c	→	Days of storage

Table 4.13: Effect of GA_3 and $CaCl_2$ treatments on lipoxygenase activity in guava fruits during storage

DOS	Lipoxygenase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	601.5	601.5	601.5	601.5	601.5	**601.5**	876.5	876.5	876.5	876.5	876.5	**876.5**
2	712.5	705.7	710.4	645.9	662.7	**687.4**	965.7	957.7	961.5	841.6	890.3	**923.4**
4	856.9	851.4	851.8	762.6	784.9	**821.5**	1092.5	1084.2	1089.5	925.8	976.2	**1033.7**
6	929.6	918.3	921.4	838.4	867.7	**895.1**	1203.5	1192.5	1196.6	1067.4	1113.9	**1154.8**
8	1143.9	1132.6	1137.5	996.3	1042.1	**1090.5**						
Mean	**848.9**	**841.9**	**844.4**	**768.9**	**791.8**		**1034.5**	**1027.7**	**1031.0**	**927.8**	**964.2**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	7.635	P	→	8.859	a	→	6.126			
Q	→	7.635	Q	→	7.924	b	→	3.874			
PQ	→	17.072	PQ	→	17.718	c	→	5.479			
						ab	→	8.663			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	12.252	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	7.749	b	→	Variety
						abc	→	17.326	c	→	Days of storage

LOX activity increased drastically during storage with Hisar Surkha attaining a value of 1203.50 units g^{-1} f.wt. just on the 6th day of storage which was even higher than 1143.9 units g^{-1} f.wt., the value attained by L-49 on 8th day of storage under control conditions. Both

the concentrations of $CaCl_2$ decreased LOX activity significantly whereas, GA_3 had no significant effect on LOX activity. The effect was also found to be concentration dependent as the lower concentration of $CaCl_2$ (1 %) exhibited significantly higher reduction in LOX activity. It decreased LOX activity from 848.9 and 1034.5 unit g^{-1} f.wt. to 768.9 and 927.8 unit g^{-1} f.wt. in L-49 and Hisar Surkha, respectively.

Superoxide radicals increased continuously during storage in both the cultivars from 96.49 to 178.69 nmols g^{-1} f.wt. in L-49 at 8^{th} day of storage and from 127.13 to 197.57 nmols g^{-1} f.wt in Hisar Surkha at 6^{th} day of storage under control conditions (Table 4.14). Hisar Surkha had higher superoxide radical content as compared to L-49 in treated as well as in control fruits. Pretreatment of fruits though resulted in reduction in superoxide radical content, maximum effect was observed in 1 % $CaCl_2$ treated fruits. It resulted in reduction in superoxide radical content from 135.72 to 123.39 nmols g^{-1} f.wt. in L-49 and 159.95 to 151.41 nmols g^{-1} f.wt. in hisar Surkha during storage period.

Table 4.14: Effect of GA_3 and $CaCl_2$ treatments on superoxide radical content in guava fruits during storage

	Superoxide radicals (nmols g^{-1} f.wt.)											
	L-49						**Hisar Surkha**					
DOS	**GA_3 (ppm)**			**$CaCl_2$ (%)**			**GA_3 (ppm)**			**$CaCl_2$ (%)**		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	96.49	96.49	96.49	96.49	96.49	**96.49**	127.13	127.13	127.13	127.13	127.13	**127.13**
2	118.23	113.00	115.32	100.86	102.81	**110.04**	140.61	137.52	139.25	132.35	131.63	**136.27**
4	132.69	128.31	132.71	119.16	122.69	**127.11**	174.50	170.03	172.36	161.82	163.76	**168.49**
6	152.49	149.07	150.75	139.35	142.09	**146.75**	197.57	193.48	195.05	184.34	186.32	**191.35**
8	178.69	171.89	174.42	161.07	164.37	**170.09**						
Mean	**135.72**	**131.75**	**133.94**	**123.39**	**125.69**		**159.95**	**157.04**	**158.45**	**151.41**	**152.21**	**155.81**

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4^{th} sampling					
P	→	0.495	P	→	0.593	a	→	0.054			
Q	→	0.495	Q	→	0.530	b	→	0.034			
PQ	→	1.107	PQ	→	1.185	c	→	0.048			
						ab	→	0.076			
P	→	Treatment of $CaCl_2$ and GA_3				ac	→	0.108	a	→	Treatment of $CaCl_2$ and GA_3
Q	→	Days after storage				bc	→	0.068	b	→	Variety
						abc	→	0.152	c	→	Days after storage

Table 4.15 depicts the effect of GA_3 and $CaCl_2$ treatments on H_2O_2 content in guava fruits during storage. During storage, hydrogen peroxide increased from minimum value of 426.1 and 469.7 µmols g^{-1} f.wt. respectively, in L-49 and Hisar surkha at 0 days of storage to a maximum of 782.9 and 743.0 µmols g^{-1} f.wt. 8^{th} and 6^{th} day of storage under control conditions.

All the treated fruits showed lower H_2O_2 content as compared to the control fruits in both the cultivars. As compared to GA_3, pre-treatment with $CaCl_2$ was more effective in

reducing H_2O_2 content throughout the duration of storage and 1 % $CaCl_2$ (553.0 µmols g^{-1} f.wt.) could reduce H_2O_2 content more effectively than 2 % $CaCl_2$ (559.9 µmols g^{-1} f.wt.) in L-49. However, both the concentrations of $CaCl_2$ had similar effect in reducing H_2O_2 content in Hisar Surkha. Similiarly, the GA_3 effect was found to be independent of the concentration and both concentrations were equally effective in reducing H_2O_2 in both the cultivars. Hisar Surkha generated higher H_2O_2 content as compared to L-49 throughout the storage period under control conditions and in all the treatments.

Table 4.15: Effect of GA_3 and $CaCl_2$ treatments on hydrogen peroxide content in guava fruits during storage

	Hydrogen peroxide (µmols g^{-1} f.wt.)											
	L-49						Hisar Surkha					
DOS	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	426.1	426.1	426.1	426.1	426.1	**426.1**	469.7	469.7	469.7	469.7	469.7	**469.7**
2	548.0	525.9	532.0	476.5	448.3	**506.2**	599.1	581.7	587.5	548.1	561.3	**575.5**
4	617.8	600.0	593.0	543.9	568.2	**584.6**	682.0	662.1	670.8	634.5	627.8	**662.3**
6	692.4	660.2	674.1	625.1	643.8	**659.1**	743.0	727.5	733.6	681.6	695.0	**716.1**
8	782.9	750.0	762.2	693.5	712.9	**740.3**						
Mean	**613.4**	**592.5**	**597.5**	**553.0**	**559.9**		**623.4**	**610.2**	**615.4**	**583.5**	**588.5**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 6th sampling					
P	→	5.016	P	→	7.343	a	→	4.365			
Q	→	5.016	Q	→	6.567	b	→	2.761			
PQ	→	11.216	PQ	→	14.685	c	→	3.904			
						ab	→	6.173			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	8.730	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	5.521	b	→	Variety
						abc	→	12.346	c	→	Days of storage

4.2.3.3 Antioxidative enzymes and metabolites

Results presented in Table 4.16 demonstrate that SOD activity was maximum on 2nd day of storage attaining value of 172.21 unit g^{-1} f.wt. in L-49 and 167.12 unit g^{-1} f.wt. in Hisar Surkha and thereafter it decreased gradually upto 140.10 and 152.95 unit g^{-1} f.wt. respectively. The activity was always higher in Hisar Surkha throughout storage of treated as well as control fruits. All the treated fruits showed higher SOD activity as compared to the control fruits in both the cultivars. $CaCl_2$ was significantly more effective than GA_3 in enhancing SOD activity. The effect was also found to be concentration dependent as the fruits treated with lower concentration of GA_3 and $CaCl_2$ exhibited significantly higher SOD activity

Table 4.16: Effect of GA_3 and $CaCl_2$ treatments on superoxide dismutase activity in guava fruits during storage

DOS	Superoxide dismutase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	158.88	158.88	158.88	158.88	158.88	**158.88**	152.95	152.95	152.95	152.95	152.95	**152.95**
2	172.21	175.10	173.80	181.85	178.58	**176.31**	167.12	171.90	170.71	180.36	175.88	**173.19**
4	154.84	157.77	157.77	162.04	158.69	**158.22**	151.25	155.42	153.33	158.88	158.53	**155.48**
6	147.48	151.01	149.29	156.39	154.20	**151.67**	126.51	128.33	127.63	133.69	132.76	**129.78**
8	140.10	142.59	140.08	146.35	144.43	**142.71**						
Mean	**154.70**	**157.07**	**155.96**	**161.102**	**158.96**		**149.46**	**152.15**	**151.155**	**156.47**	**155.03**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.453	P	→	0.508	a	→	0.337			
Q	→	0.453	Q	→	0.455	b	→	0.213			
PQ	→	1.013	PQ	→	1.017	c	→	0.301			
						ab	→	0.477			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.674	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.426	b	→	Variety
						abc	→	0.953	c	→	Days of storage

Catalase reached to its maximum activity on 2nd of Hisar Surkha storage and 4th day of L-49 storage after which it gradually decreased (Table 4.17). L-49 had significantly higher catalase activity than Hisar Surkha and even on 8th day of storage, L-49 exhibited higher activity (924.2 units g^{-1} f.wt.) than that of Hisar Surkha even on the 6th day of storage (665.9 units g^{-1} f.wt) under control conditions. All the treated fruits showed higher catalase activity as compared to the control fruits in both the cultivars. $CaCl_2$ was significantly more effective than GA_3 in stimulating catalase activity. The effect was also found to be concentration dependent as catalase activity increased significantly with lower concentration of $CaCl_2$ and GA_3 in both the cultivars and at all the stages of storage periods.

As is clear from the data in Table 4.18, both the cultivars had maximum APX activity on 2nd day of storage after which there was continuous decline in activity. Under all storage conditions, L-49 had higher APX activity than Hisar Surkha and even on 8th day of storage, the activity in L-49 (626.9 unit g^{-1} f.wt.) was much higher than in Hisar Surkha on 6th day of storage (542.3 unit g^{-1} f.wt.) under control conditions. $CaCl_2$ treated fruits had significantly higher APX activity as compared to control in both the cultivars. Both concentrations of $CaCl_2$ had similar effect on APX activity in L-49 whereas in Hisar Surkha, 2 % $CaCl_2$ was more effective in stimulating the APX activity (785.2 unit g^{-1} f.wt.). However, GA_3 had non-significant effect on APX activity in L-49 but increased APX activity significantly in Hisar Surkha.

Table 4.17: Effect of GA_3 and $CaCl_2$ treatments on catalase activity in guava fruits during storage

DOS	Catalase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	680.5	680.5	680.5	680.5	680.5	**680.5**	545.5	545.5	545.5	545.5	545.5	**545.5**
2	1139.6	1167.5	1154.6	1193.7	1175.4	**1163.9**	1089.6	1126	1108.1	1178.3	1143.7	**1125.5**
4	1327.0	1362.3	1343.2	1394.2	1376.7	**1356.7**	841.9	871.2	850.2	922.6	890.8	**871.5**
6	1020.1	1045.7	1035.7	1071.1	1050.5	**1043.2**	665.9	679.4	675.8	721.6	687.2	**685.7**
8	924.2	935	931.6	960.8	952.1	**937.9**						
Mean	**1018.3**	**1038.2**	**1029.1**	**1060.1**	**1047.0**		**785.7**	**805.5**	**794.9**	**842.0**	**816.8**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	5.519	P	→	16.002	a	→	8.467			
Q	→	5.519	Q	→	14.312	b	→	5.355			
PQ	→	12.342	PQ	→	32.004	c	→	7.573			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	16.935	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	10.710	b	→	Variety
						abc	→	NS	c	→	Days of storage

Table 4.18: Effect of GA_3 and $CaCl_2$ treatments on ascorbate peroxidase activity in guava fruits during storage

DOS	Ascorbate peroxidase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	804.7	804.7	804.7	804.7	804.7	804.7	580.3	580.3	580.3	580.3	580.3	580.3
2	1101.9	1106.9	1106.2	1113.4	1119.5	**1109.6**	970.0	971.7	971.2	980.8	985.3	**975.8**
4	986.3	989.8	988.8	998.2	1003.2	**993.3**	795.6	803.0	800.3	806.3	810.9	**803.2**
6	712.4	716.4	714.5	726.5	730.8	**720.1**	542.3	545.3	544.4	549.5	554.2	**547.1**
8	626.9	629.1	628.2	636.2	638.2	**631.7**						
Mean	**772.4**	**775.4**	**774.4**	**781.8**	**785.2**		**628.9**	**631.9**	**630.9**	**636.1**	**639.5**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	8.587	P	→	1.987	a	→	1.356			
Q	→	8.587	Q	→	1.777	b	→	0.858			
PQ	→	NS	PQ	→	3.973	c	→	1.213			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	2.713	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	1.716	b	→	Variety
						abc	→	NS	c	→	Days of storage

Total ascorbate content (Table 4.19) was 110.52 and 154.6 mg/100g f.wt. in L-49 and Hisar Surkha respectively, at 0 day of storage which increased drastically to 182.6 and 243.0 mg/100g f.wt. respectively, upto 2nd day of storage. Thereafter, it decreased gradually

throughout storage period in both the cultivars. All the treatments resulted in significantly higher ascorbic acid content than control. $CaCl_2$ treatment was more effective in maintaining ascorbate content as compared to GA_3 treatment and the fruits treated with 1 % $CaCl_2$ exhibited significantly higher ascorbic acid content in both the cultivars i.e. 143.8 (L-49) and 204.4 mg/100g f.wt. (Hisar Surkha). Among the two GA_3 concentrations, 100 ppm had more pronounced effect in maintaining ascorbate content in L-49 (137.0 mg/100g f.wt.) as compared to 150 ppm (135.8 mg/100g f.wt.). However, no significant differences were observed in Hisar Surkha w. r. t. different concentrations of GA_3. Hisar Surkha had higher ascorbic content than L-49 throughout the storage period and under all the storage treatments.

Table 4.19: Effect of GA_3 and $CaCl_2$ treatments on ascorbic acid content in guava fruits during storage

DOS	Ascorbic acid content (mg/100g f.wt.)											
	L-49						Hisar Surkha					
		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	110.5	110.5	110.5	110.5	110.5	**110.5**	154.6	154.6	154.6	154.6	154.6	**154.6**
2	182.6	188.2	184.8	194.8	191.5	**188.4**	243.0	247.5	245.7	255.4	258.0	**249.9**
4	171.4	175.8	174.7	187.0	182.5	**178.3**	209.4	215.1	213.9	224.0	218.4	**216.2**
6	126.6	129.9	128.8	137.8	135.5	**131.7**	169.2	173.6	172.5	183.7	178.1	**175.4**
8	99.7	106.1	105.4	114.2	110.9	**107.2**						
Mean	**133.1**	**137.0**	**135.8**	**143.8**	**141.1**		**194.1**	**197.7**	**196.7**	**204.4**	**202.3**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	1.134	P	→	1.558	a	→	0.991			
Q	→	1.134	Q	→	1.393	b	→	0.627			
PQ	→	2.536	PQ	→	3.116	c	→	0.886			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	1.981	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	1.253	b	→	Variety
						abc	→	NS	c	→	Days of storage

Results presented in Table 4.20 indicate that GR activity decreased continuously throughout storage from 6.23 to 1.53 unit g^{-1} f.wt. in L-49 and 3.59 to 0.93 unit g^{-1} f.wt. in Hisar Surkha. The soft variety had lower enzyme activity than the firm one in both treated as well as control fruits. Hisar Surkha had enzyme activity of 1.47 unit g^{-1} f.wt. on 4^{th} day of storage which was even lower than L-49 exhibited on 8^{th} day of storage i.e. 1.53 unit g^{-1} f.wt. under control conditions. Different treatments did not have any significant effect on GR activity in Hisar Surkha. But $CaCl_2$ was observed to be significantly effective in enhancing GR activity in L-49 and both the $CaCl_2$ concentrations were equally effective, resulted in enhancement of the activity from 7.22 to 4.07 unit g^{-1} f.wt. by 1 % and 4.08 unit g^{-1} f.wt. by 2 % $CaCl_2$.

Table 4.20: Effect of GA_3 and $CaCl_2$ treatments on glutathione reductase activity in guava fruits during storage

DOS	Glutathione reductase activity (unit g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	6.23	6.23	6.23	6.23	6.23	**6.23**	**3.59**	3.59	3.59	3.59	3.59	**3.59**
2	5.23	5.22	5.24	5.28	5.30	**5.25**	**2.99**	3.00	3.00	3.04	3.02	**3.01**
4	4.29	4.30	4.30	4.34	4.36	**4.32**	**1.47**	1.47	1.48	1.50	1.49	**1.48**
6	2.89	2.91	2.91	2.95	2.97	**2.92**	**0.92**	0.93	0.93	0.95	0.94	**0.93**
8	1.53	1.54	1.53	1.55	1.55	**1.54**	**0.00**					
Mean	**4.03**	**4.04**	**4.04**	**4.07**	**4.08**	**4.05**	**1.79**	**2.25**	**2.25**	**2.27**	**2.26**	**2.25**

CD at 5 % level

For L-49			For HS			For L-49/HS upto 6th sampling					
P	→	0.034	P	→	NS	a	→	NS			
Q	→	0.034	Q	→	0.042	b	→	0.020			
PQ	→	NS	PQ	→	NS	c	→	0.028			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	NS	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.039	b	→	Variety
						abc	→	NS	c	→	Days of storage

Glutathione content decreased progressively throughout storage period in both the cultivars. The content decreased from 510.3 to 215.8 nmols g^{-1} f.wt. from 0 to 8th day of storage in L-49 while in Hisar Surkha decrement was from 464.9 (0 day) to 189.3 (6th day) nmols g^{-1} f.wt. (Table 4.21).

Table 4.21: Effect of GA_3 and $CaCl_2$ treatments on glutathione content in guava fruits during storage

DOS	Glutathione content (nmol g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	510.3	510.3	510.3	510.3	510.3	**510.3**	464.9	464.9	464.9	464.9	464.9	**464.9**
2	435.8	439.9	438.2	448.1	449.5	**443.7**	332.2	334.3	333.2	338.5	335.6	**336.2**
4	389.5	391.8	391.8	395.7	397.3	**394.2**	237.6	239.3	238.3	240.5	244.7	**240.3**
6	320.1	323.3	321.9	326.3	321.6	**324.7**	189.3	191.1	190.3	193.2	191.4	**192.1**
8	215.8	218.1	216.9	219.2	219.0	**218.6**						
Mean	**374.3**	**376.7**	**375.8**	**379.9**	**379.5**		**306.0**	**307.4**	**306.7**	**309.3**	**309.1**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	7.038	P	→	4.138	a	→	4.682			
Q	→	7.038	Q	→	3.701	b	→	2.961			
PQ	→	NS	PQ	→	NS	c	→	4.188			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	NS	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	5.922	b	→	Variety
						abc	→	NS	c	→	Days after storage

Higher glutathione content was observed in L-49 as compared to Hisar Surkha in control as well as treated fruits. Treatment of fruits with GA_3 (100 and 150 ppm) and $CaCl_2$ (1 % and 2 %) had no significant effect on glutathione content in both the cultivars.

Peroxidase activity in two guava cultivars increased upto 2nd day of storage and after that it gradually decreased in both the cultivars (Table 4.22). L-49 always exhibited higher POX activity than Hisar Surkha at all the stages of storage and even on 8th day of storage, L-49 had higher activity (74.11 units g^{-1} f.wt.) than Hisar Surkha had on the 6th day (66.97 units g^{-1} f.wt.) under control conditions. All the treated fruits showed higher POX activity as compared to the control fruits in both the cultivars. $CaCl_2$ was significantly more effective than GA_3 in stimulating POX activity. The effect was also found to be concentration dependent as POX activity increased significantly with lower concentration of $CaCl_2$ and GA_3 in both the cultivars and at all the storage periods.

Table 4.22: Effect of GA_3 and $CaCl_2$ treatments on peroxidase activity in guava fruits during storage

	Peroxidase activity (units g^{-1} f.wt.)											
	L-49						**Hisar Surkha**					
DOS		**GA_3 (ppm)**		**$CaCl_2$ (%)**				**GA_3 (ppm)**		**$CaCl_2$ (%)**		
	Control	**100**	**150**	**1**	**2**	**Mean**	**Control**	**100**	**150**	**1**	**2**	**Mean**
0	120.12	120.12	120.12	120.12	120.12	**120.12**	109.50	109.50	109.50	109.50	109.50	**109.50**
2	129.76	133.77	130.94	140.75	137.99	**134.64**	136.22	148.89	141.54	156.61	156.48	**147.95**
4	118.03	127.51	123.69	133.09	131.78	**126.82**	92.69	97.05	94.33	112.16	109.80	**101.21**
6	89.87	95.07	91.64	104.75	98.99	**96.06**	66.97	73.40	69.85	83.59	79.69	**74.70**
8	74.11	77.43	75.42	88.99	85.17	**80.22**						
Mean	**106.4**	**110.8**	**108.4**	**117.5**	**114.8**	**111.6**	**101.3**	**107.2**	**103.8**	**115.5**	**113.9**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	1.510	P	→	0.433	a	→	0.942			
Q	→	1.510	Q	→	0.387	b	→	0.595			
PQ	→	3.377	PQ	→	0.865	c	→	0.842			
						ab	→	1.332			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	1.883	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	1.191	b	→	Variety
						abc	→	2.663	c	→	Days of storage

The β-carotene content decreased progressively from 465.6 ug/100g f.wt. at 0 day to 143.9 ug/100g f.wt. at 8th day of storage in L-49 and from 370.6 ug/100g f.wt. at 0 day to 126.9 ug/100g f.wt. at 6th day of storage in Hisar Surkha (Table 4.23). However, application of both GA_3 and $CaCl_2$ could slow down the rate of degradation of β-carotene significantly in both the variety but GA_3 had more pronounced effect. The effect of GA_3 was also concentration dependent as 150 ppm was the more effective than 100 ppm treatment in maintaining β-carotene content in both the variety. Among the two concentrations of $CaCl_2$

tested, 1 % was more effective in L-49 (287.7 ug/100g f.wt.) but both concentrations (1 and 2%) were equally effective in Hisar Surkha.

Table 4.23: Effect of GA_3 and $CaCl_2$ treatments β-carotene content in guava fruits during storage

DOS	β-carotene content (ug/100g f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	465.6	465.6	465.6	465.6	465.6	**465.6**	370.6	370.6	370.6	370.6	370.6	**370.6**
2	340.1	365.7	370.2	352.4	346.1	**354.9**	310	357.5	366.4	323.2	323.1	**336.0**
4	237.7	274.7	297.5	250.5	245.6	**261.2**	228.6	256.4	264.9	237	238.3	**245.0**
6	192.1	244.4	251.9	214.8	207.9	**222.2**	126.9	151.3	152.8	139.6	136.8	**141.5**
8	143.9	186.4	205.3	155.2	154.3	**169.0**						
Mean	**275.9**	**307.4**	**318.1**	**287.7**	**283.9**		**259.03**	**283.9**	**288.7**	**267.6**	**267.2**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	6.39	P	→	5.84	a	→	4.48			
Q	→	6.39	Q	→	5.22	b	→	2.832			
PQ	→	14.29	PQ	→	11.68	c	→	4.005			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	8.955	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	5.664	b	→	Variety
						abc	→	12.67	c	→	Days of storage

The antioxidant activity of guava fruits of both the cultivars decreased progressively throughout storage period (Table 4.24). Hisar Surkha had higher antioxidant activity as compared to L-49, reaching 42.79 % at 6th day of storage which was even higher than the value observed at 4th day of storage of L-49 i.e. 40.46 %. All the treatments resulted in significant higher antioxidant activity than control. The effect of $CaCl_2$ treatments was more pronounced than GA_3 in maintaining antioxidant activity. Effect was also concentration dependent because lower concentrations of both GA_3 and $CaCl_2$ treatments were more effective in maintaining antioxidant activity in both the cultivars. Maximum antioxidant activity was observed in 1 % $CaCl_2$ pretreatment i.e. 42.92 % in L-49 and 61.45 % in Hisar Surkha.

4.2.3.4 Ethylene and ACC oxidase activity

As is clear from the data, in soft variety, Hisar Surkha, ethylene production was maximum at 2nd day of storage while in firm variety L-49, maximum ethylene production was observed at 4th day of storage (Table 4.25). The corresponding values were 45.08 and 32.87 nmol h^{-1} g^{-1} f.wt., demonstrating that the soft variety had significantly higher rate of ethylene production as compared to the firm one. The GA_3 treatment did not have any significant effect on ethylene evolution, while $CaCl_2$ significantly reduced ethylene evolution in both the cultivars. The effect of 2 % $CaCl_2$ treatment was more pronounced than 1 % $CaCl_2$ that

reduced ethylene content from 19.66 and 28.82 nmol h^{-1} g^{-1} f.wt. to 17.03 and 26.57 nmol h^{-1} g^{-1} f.wt. in L-49 and Hisar Surkha respectively.

Table 4.24: Effect of GA_3 and $CaCl_2$ treatments on antioxidant activity in guava fruits during storage

	Antioxidant activity (% scavenging of DPPH)											
	L-49						Hisar Surkha					
DOS		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	47.16	47.16	47.16	47.16	47.16	**47.16**	70.09	70.09	70.09	70.09	70.09	**70.09**
2	44.71	45.87	45.22	46.92	46.18	**45.78**	62.97	64.82	63.38	68.36	67.16	**65.34**
4	40.46	43.21	41.18	44.37	43.76	**42.59**	50.24	52.36	51.16	57.95	56.63	**53.67**
6	36.62	36.96	37.05	39.84	39.22	**37.94**	42.79	44.51	43.19	49.39	47.93	**45.56**
8	31.54	32.28	32.29	36.3	35.42	**33.57**						
Mean	**40.09**	**41.09**	**40.58**	**42.92**	**42.35**		**56.52**	**57.95**	**56.96**	**61.45**	**60.45**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.373	P	→	0.237	a	→	0.248			
Q	→	0.373	Q	→	0.212	b	→	0.157			
PQ	→	0.834	PQ	→	0.474	c	→	0.222			
						ab	→	0.351			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.496	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.314	b	→	Variety
						abc	→	0.702	c	→	Days of storage

Table 4.25: Effect of GA_3 and $CaCl_2$ treatments on ethylene evolution in guava fruits during storage

	Ethylene content (nmol h^{-1} g^{-1} f.wt.)											
	L-49						Hisar Surkha					
DOS		GA_3 (ppm)		$CaCl_2$ (%)				GA_3 (ppm)		$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	9.12	9.12	9.12	9.12	9.12	**9.12**	11.36	11.36	11.36	11.36	11.36	**11.36**
2	18.27	18.22	18.25	17.29	16.21	**17.65**	45.08	44.87	45.01	44.13	43.2	**44.46**
4	32.87	32.78	32.83	30.63	25.7	**30.96**	36.54	36.52	36.5	34.59	32.08	**35.25**
6	25.68	25.63	25.65	23.64	21.79	**24.48**	22.30	22.28	22.23	21.03	19.62	**21.49**
8	12.36	12.34	12.35	12.29	12.33	**12.33**						
Mean	**19.66**	**19.622**	**19.64**	**18.59**	**17.03**		**28.82**	**28.76**	**28.78**	**27.78**	**26.57**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	0.802	P	→	0.084	a	→	0.497			
Q	→	0.802	Q	→	0.075	b	→	0.314			
PQ	→	1.794	PQ	→	0.168	c	→	0.444			
						ab	→	0.703			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	0.994	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.628	b	→	Variety
						abc	→	NS	c	→	Days of storage

Data in Table 4.26 represent ACC oxidase activity during storage of guava fruits. It followed a pattern similar to the one followed by ethylene evolution. It increased throughout storage in Hisar Surkha, attaining its maximum at 2nd day of storage (58.91 units g^{-1} f.wt.), while in case of L-49, it reached maximum at 4th day of storage (52.02 units g^{-1} f.wt) and then decreased under control conditions. Pretreatment of fruits had no significant effect on ACC oxidase activity. Here again, Hisar Surkha, a softer variety exhibited higher activity of ACC oxidase throughout storage period and under all the storage conditions. A positive correlation was observed between ethylene and ACC oxidase (0.949^{**}).

Table 4.26: Effect of GA_3 and $CaCl_2$ treatments on ACC oxidase activity in guava fruits during storage

DOS	ACC oxidase activity (units g^{-1} f.wt.)											
	L-49						Hisar Surkha					
	GA_3 (ppm)			$CaCl_2$ (%)			GA_3 (ppm)			$CaCl_2$ (%)		
	Control	100	150	1	2	Mean	Control	100	150	1	2	Mean
0	26.05	26.05	26.05	26.05	26.05	**26.05**	35.5	35.5	35.5	35.5	35.5	**35.5**
2	34.40	34.32	34.42	34.23	34.15	**34.28**	58.91	58.85	58.89	58.76	58.77	**58.84**
4	52.02	51.77	51.85	51.76	51.72	**51.82**	49.25	49.19	49.23	49.18	49.17	**49.20**
6	45.05	45.06	44.92	44.89	44.82	**44.92**	41.32	41.26	41.32	41.29	41.35	**41.31**
8	39.29	39.2	39.24	39.2	39.16	**39.22**						
Mean	**39.36**	**39.28**	**39.29**	**39.23**	**39.18**		**46.25**	**46.20**	**46.24**	**46.18**	**46.19**	

CD at 5 % level

For L-49			For HS			For L-49/HS upto 4th sampling					
P	→	NS	P	→	NS	a	→	NS			
Q	→	0.745	Q	→	0.104	b	→	0.291			
PQ	→	NS	PQ	→	NS	c	→	0.412			
						ab	→	NS			
P	→	Treatment of GA_3 and $CaCl_2$				ac	→	NS	a	→	Treatment of GA_3 and $CaCl_2$
Q	→	Days of storage				bc	→	0.582	b	→	Variety
						abc	→	NS	c	→	Days of storage

4.3 Purification and characterization of polygalacturonase

4.3.1 Purification of polygalacturonase

Unless otherwise stated, all steps of enzyme purification were carried out at 0-4°C. PG was partially purified from guava (*Psidium guajava* L.) fruits *cv*. L-49 using conventional techniques of protein purification *viz*. ammonium sulphate fractionation, gel filtration through Sephadex G-100 and ion-exchange chromatography using CM-cellulose as per the details given in 3.7. The enzyme was purified about 88 fold with 35 per cent recovery. A summary of the results showing stepwise purification of the enzyme has been presented in Table 4.27.

Crude extract contained 95.65 mg protein and showed enzyme activity equivalent to 147.67 units (Table 4.27). The total magnitude of activity with 1.54 unit/mg protein was taken as 100 per cent recovery for further calculations. Enzyme from the crude extract was precipitated between 30 to 70 per cent concentration of $(NH_4)_2SO_4$ at 4ºC with continuous

stirring. The precipitates obtained were centrifuged at 10,000×g for 20 mins, dissolved in 0.1M sodium acetate buffer (pH 5.2) and dialyzed against the same buffer for 24 hrs. The dialysate obtained was found to contain enzyme with specific activity of 3.34. This step resulted in about 2.17 fold purification with around 67 per cent recovery.

Table 4.27: Summary of purification of polygalacturonase from guava (*Psidium guajava* L.) fruit

Sr. No.	Fraction	Volume (ml)	Total activity (Units)	Protein (mg)	Specific activity (Units/ mg protein)	Fold purification	Recovery (%)
1	Crude extract	200	147.67	95.65	1.54	-	100
2	$(NH_4)_2SO_4$(30-70 % saturation)	12	98.76	29.53	3.34	2.17	67
3	Sephadex G-100	33	74.76	1.14	65.58	42.58	51
4	CM-cellulose	27	51.59	0.38	135.76	88.16	35

Unit = mg galacturonic acid released /20h

Partial purification of the enzyme was achieved by loading the dialyzed $(NH_4)_2SO_4$ fraction (obtained as above) on to a Sephadex G-100 column pre-equilibrated with sodium acetate buffer (pH 5.2) and pre-calibrated with molecular weight marker proteins as described in Section 3.7 . Sample was eluted with 0.1 M sodium acetate buffer (pH 5.2). Fractions of 3 ml each were collected and tested for protein and enzyme activity. Elution profile of the enzyme is depicted in Fig. 4.29. A single sharp peak of PG activity overlapping the protein peak was observed as shown in Fig. 4.29. PG was eluted after the major protein peak in 11 fractions (30-40) with 43 fold purification and 51 per cent recovery. Rest of the fractions showed enzyme activity in traces. The enzymatically active fractions were pooled (33 ml) and concentrated using solid sucrose and used for further purification by cation exchange chromatography. The results presented in table Table 4.27.

The concentrated fraction from Sephadex G-100 column was loaded on to a CM-cellulose column that had been activated, pre-washed and equilibrated with 0.1 M sodium acetate buffer (pH 5.2) and eluted with 0-0.5 M linear gradient of KCl in sodium acetate buffer. Elution profile on CM-cellulose column is presented in Fig. 4.30. Fractions of 3 ml each were collected and tested from protein and enzyme activity. Bound PG was eluted with increasing ionic strength from 0-0.5 M KCl gradient. Most of the PG activity was recovered when eluted with 0.2 M KCl. Final preparation of PG was purified about 88 fold with approximately 35 per cent recovery. The enzymatically active fractions were pooled (27 ml) and concentrated using solid sucrose and used for further characterization of enzyme.

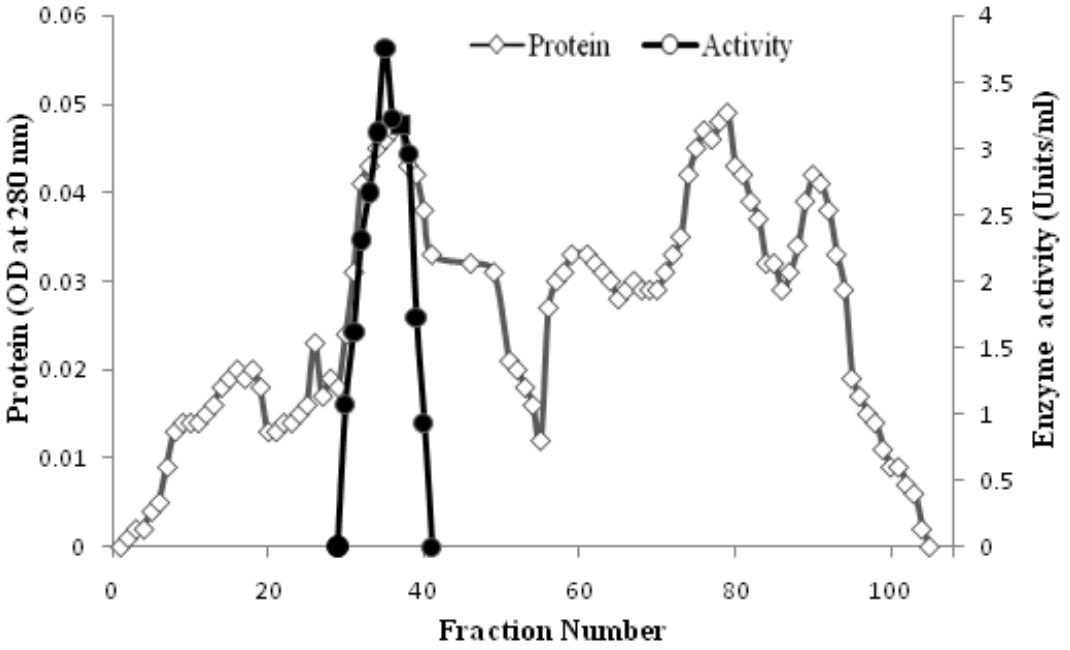

Fig. 4.29: Elution profile of polygalacturonase on Sephadex G-100 column

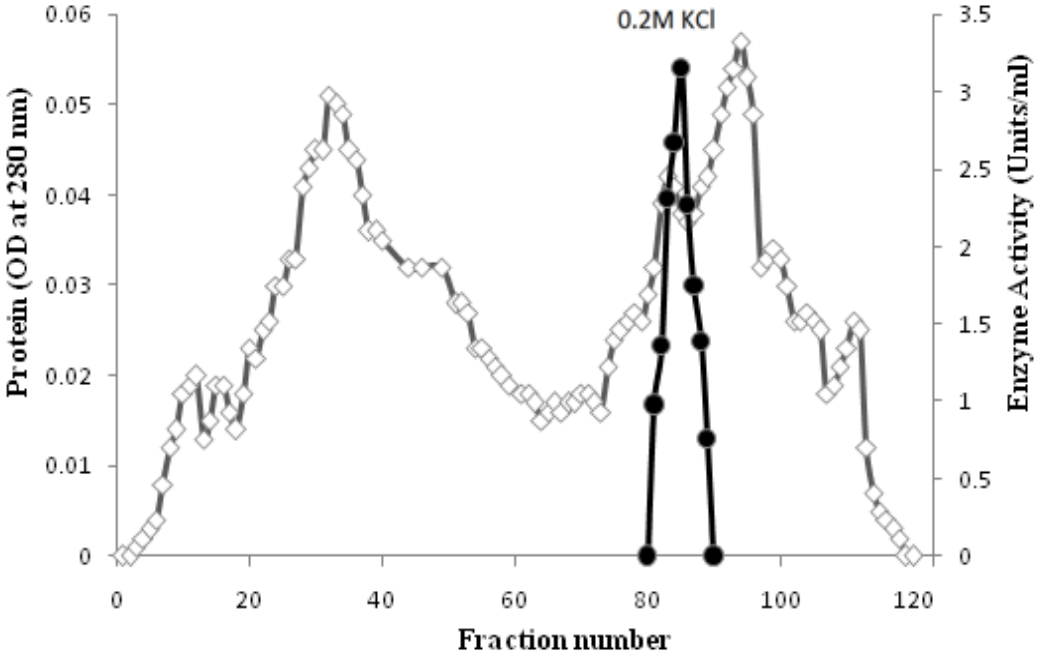

Fig. 4.30: Elution profile of polygalacturonase on CM-cellulose column

The purity of enzyme preparation obtained after ion exchange chromatography on CM-cellulose was judged by performing native-PAGE. Single protein band was visualized on native-PAGE by coomassie brilliant blue staining (Plate 4.1)**.**

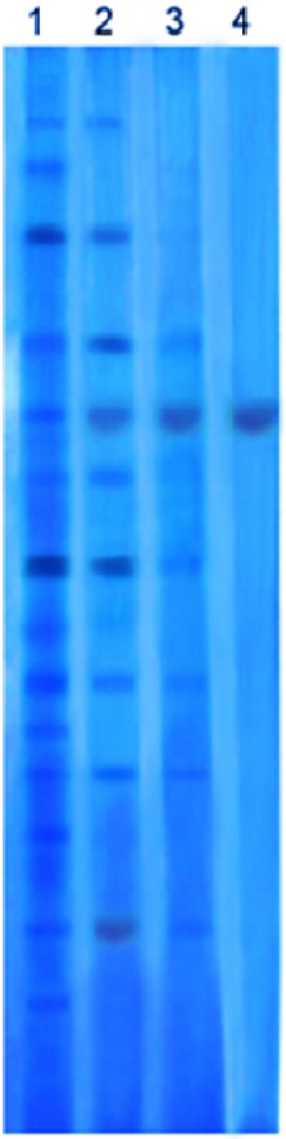

Plate. 4.1

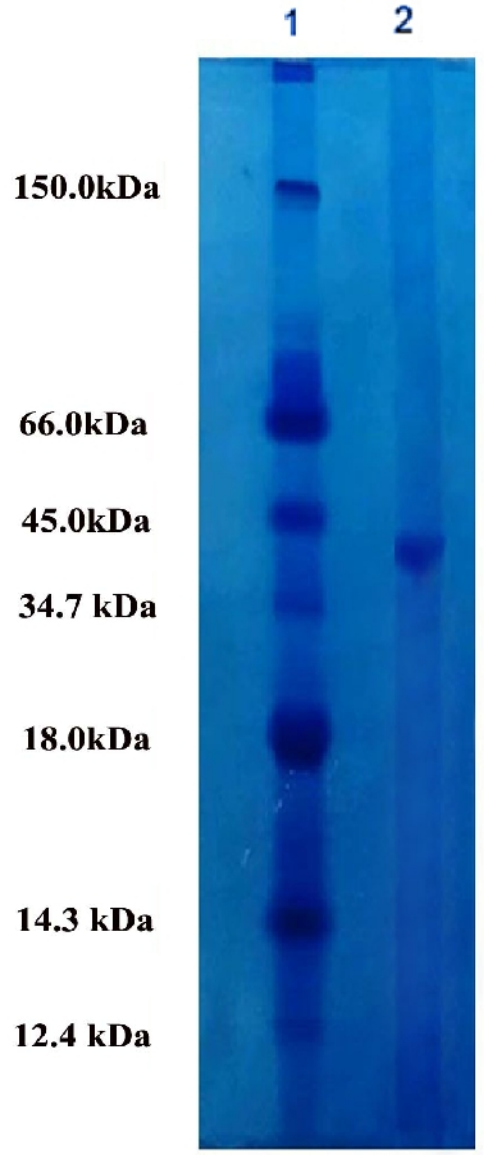

Plate. 4.2

Plate. 4.1: Native-PAGE pattern of polygalacturonase from guava fruit

Lane 1: Crude extract
Lane 2: $(NH_4)_2SO_4$ fraction
Lane 3: Sephadex G-100 fraction
Lane 4: CM-cellulose fraction

Plate 4.2: SDS-PAGE pattern of purified polygalacturonase from guava fruit

Lane 1: Standard Markers: alcohol dehydrogenase (150 kDa), Bovine serum albumin (66 kDa), Ovalbumin (45.0 kDa), Pepsin (34.7 kDa), Carbonic anhydrase (29.0 kDa), β-lactoglobulin (18 kDa) and Cytochrome-C (12.4 kDa)
Lane 2: Purified enzyme after CM-cellulose chromatography

4.3.2 Characterization of purified polygalacturonase

The final enzyme preparation after ion exchange chromatography was used for investigating various characteristics of enzyme protein.

4.3.2.1 Subunits and molecular weight determination

The molecular weight of partially purified polygalacturonase was determined by gel filtration and SDS-PAGE. The purified enzyme was chromatographed on Sephadex G-100 column, which was calibrated with standard proteins. The molecular weight of the enzyme as calculated from the plot of elution volume versus log of molecular weight and was 80 kDa (Fig. 4.31). The purified enzyme showed a single protein band on SDS-PAGE by coomassie brilliant blue staining (Plate 4.2, lane 2) and the molecular weight, calculated from the plot of R_m value versus log of molecular weight and was 40 kDa. It suggests that the enzyme from *Psidium guajava* fruit is composed of two identical subunits i.e. the enzyme is a homodimer (Fig. 4.32).

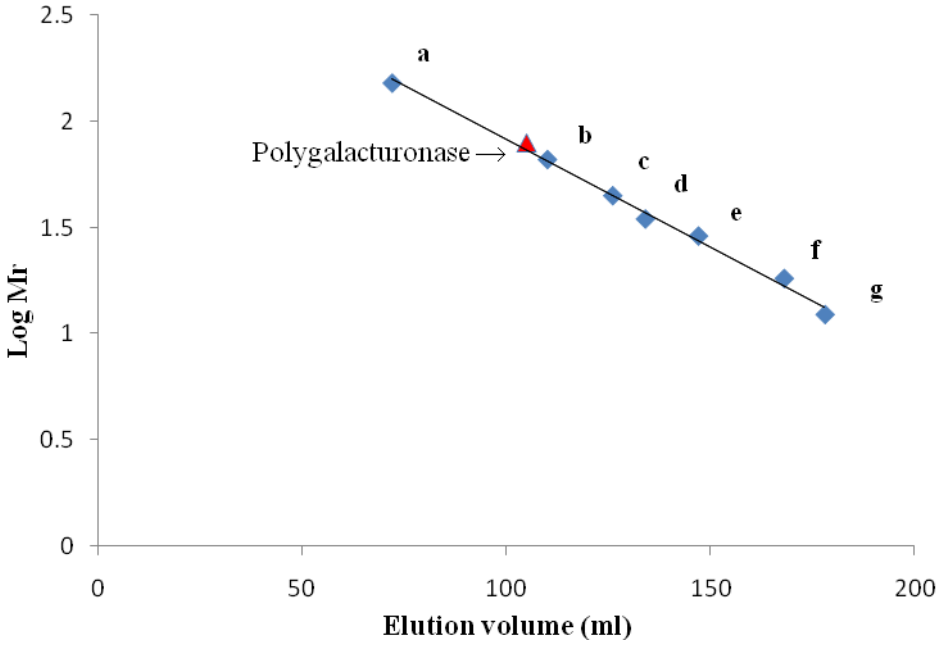

Fig. 4.31 Determination of molecular weight of purified polygalacturonase using gel filtration through Sephadex G-100

a= Alcohol dehydrogenase (150 kDa), b= Albumin bovine (66 kDa), c= Ovalbumin (45 kDa), d= Pepsin (34.7 kDa), e= Carbonic anhydrase (29 kDa), f= β-lactoglobulin (18 kDa), g= Cytochrome-c (12.4 kDa)

4.3.2.2 pH optima for activity and stability

Activity of PG was assayed using assay buffers of varied pH values by buffering the reaction mixture (3.7.8.3) with different pH. For maintaining pH ranging from 3.0 to 6.0, sodium acetate buffer (0.1M) was used and for pH ranging from 6.1 to 8.0, 0.1 M sodium phosphate buffer was used. PG activity increased gradually from 3.68 units/ml at pH 3.0 to 5.65 units/ml at pH 5.0. The activity started decreasing thereafter with gradual increase in pH

till 8.0 of assay buffer (Fig. 4.33). Thus optimum pH for obtaining maximum activity was 5.0. Appreciable activity was observed in the range of pH 4–7.

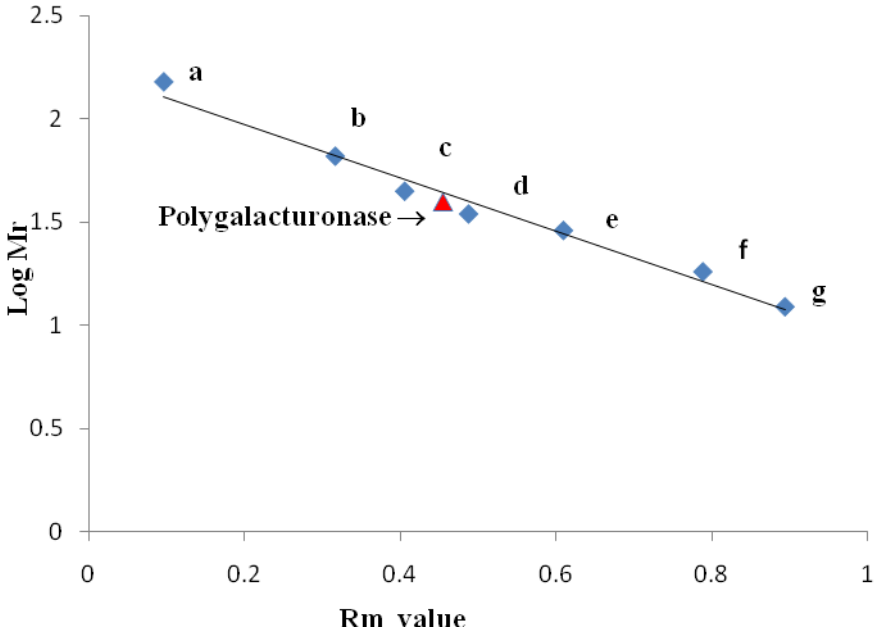

Fig. 4.32: Determination of molecular weight of purified polygalacturonase through SDS-PAGE

a= Alcohol dehydrogenase (150 kDa), b= Albumin bovine (66 kDa), c= Ovalbumin (45 kDa), d= Pepsin (34.7 kDa), e= Carbonic anhydrase (29 kDa), f= β-lactoglobulin (18 kDa), g= Cytochrome-c (12.4 kDa)

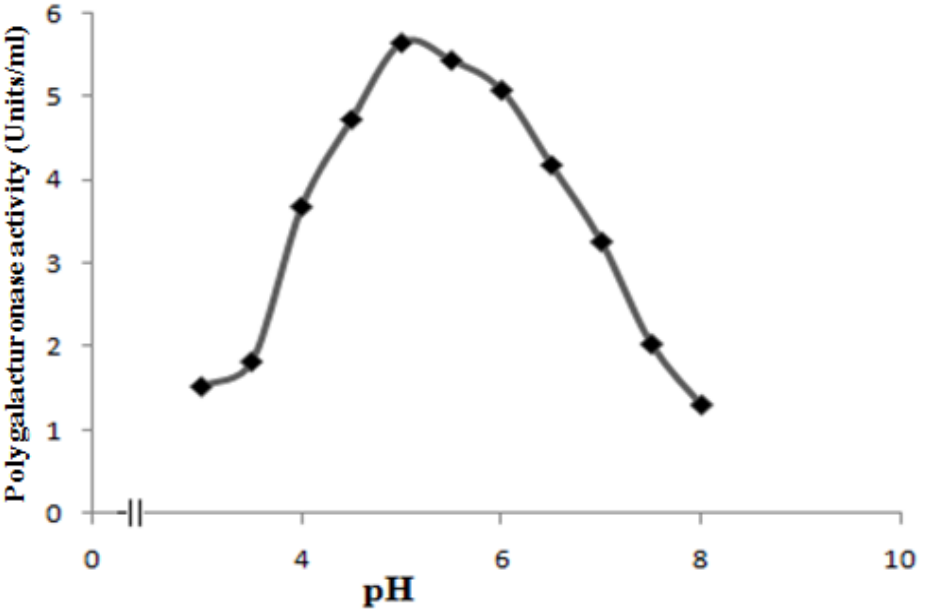

Fig. 4.33: Effect of pH on the activity of purified polygalacturonase

The stability of polygalacturonase was evaluated after incubating the enzyme with buffers of different pH for 2 h. Activity of PG was 4.23 units/ml when the enzyme was preincubated with acetate buffer of pH 4.0 which increased sharply when the pH of the

incubation buffer was raised to 5.2 and attained a value of 6.08 units/ml. Increasing pH beyond 5.2 had deleterious effect on the level of activity of PG. It first decreases gradually with the increase in pH upto 7.0 and thereafter a drastic decrease was observed. At pH 8.0 the enzyme lost more than 74 per cent of its activity detected at pH 5.2. The results demonstrated that purified polygalacturonase was stable over a range of pH 4.0 to 7.0 with highest stability at pH 5.2 (Fig.4.34).

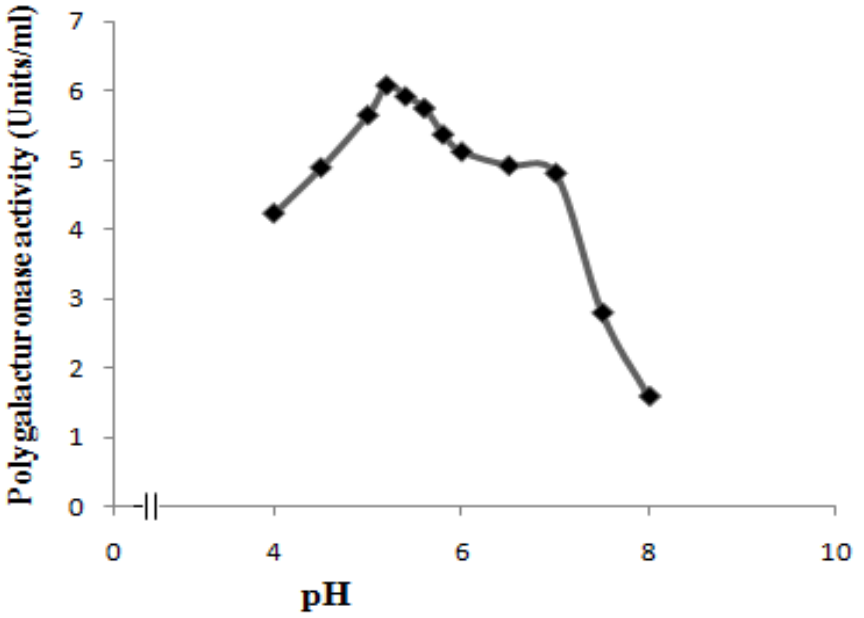

Fig. 4.34: Effect of pH on the stability of purified polygalacturonase

4.3.2.3 Temperature optima for activity and thermostability

The effect of temperature on guava polygalacturonase activity was investigated at specified temperature ranging between 15°C and 70°C (Fig. 4.35). The enzyme activity increased with increasing temperature and reached maximum at 35°C (6.17 units/ml) and then started decreasing gradually with rise in temperature. At 70°C almost 84 per cent of the activity was lost. The optimum temperature for PG activity of guava was 35°C after which there was a decline in the enzyme activity. This clearly showed that higher temperatures affected the activity of the enzyme.

Preincubation of enzyme at 50, 60 and 70ºC for varying time resulted in gradual loss of activity and 45 mins of preincubation resulted in 22, 40 and 54 per cent loss in activity, respectively. However, at 80ºC the enzyme activity decreased drastically and enzyme was completely inactive after 45 minutes of incubation while after 15 and 30 minutes of pre incubation, the enzyme retained its 50 and 28 per cent of its activity, respectively.

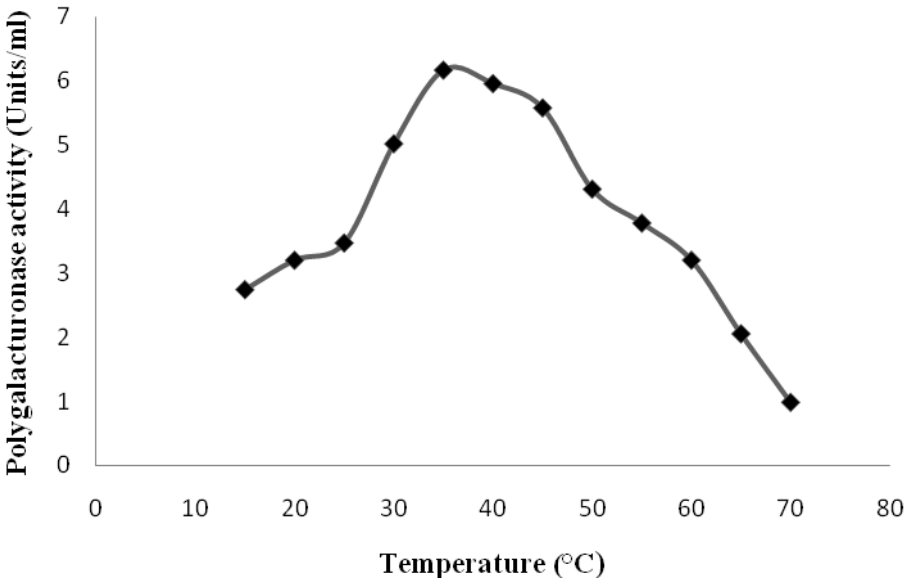

Fig. 4.35: Effect of temperature on the activity of purified polygalacturonase

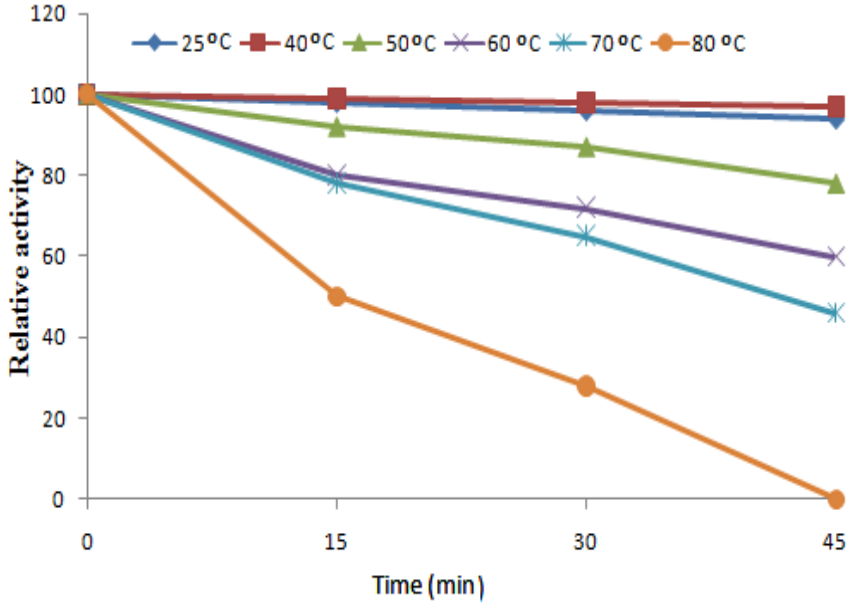

Fig. 4.36: Effect of temperature on the stability of purified polygalacturonase

4.3.2.4 Effect of substrate concentration and K_m value

The enzyme showed a typical hyperbolic response with increasing concentrations of substrate (0.05 to 0.5 per cent polygalacturonic acid) in an otherwise standard assay mixture (Fig. 4.37) revealing that it followed Michaelis-Menten kinetics. The enzyme activity increased with increasing concentrations of polygalacturonic acid until the concentration was raised at 0.3 per cent and further increase in concentration of polygalacturonic acid had no effect on the initial velocity i.e. activity remained almost constant.

The K_m value was calculated from the Lineweaver-Burk plot and was found to be 0.14 per cent (Fig. 4.38) for polygalacturonic acid.

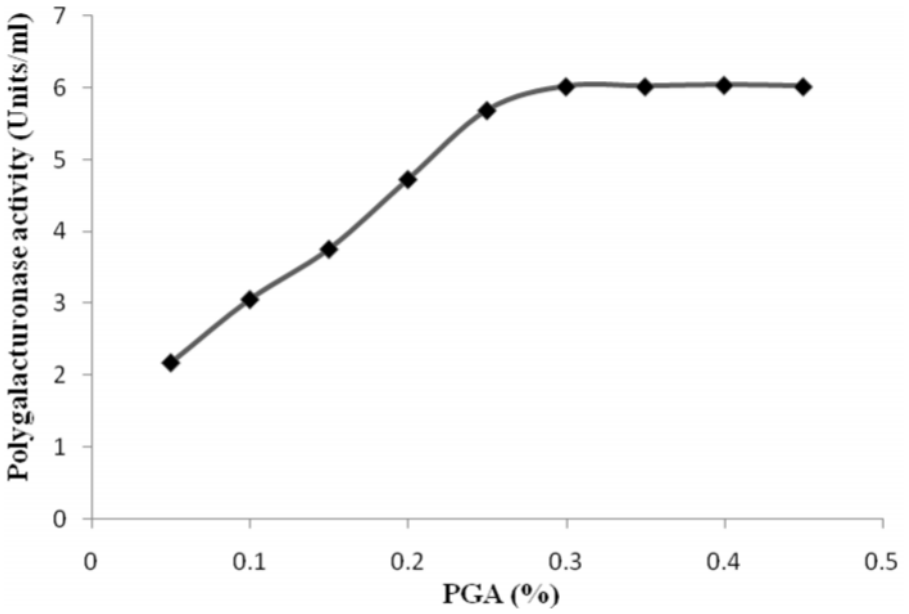

Fig. 4.37: Effect of polygalacturonic acid concentration on the activity of purified polygalacturonase

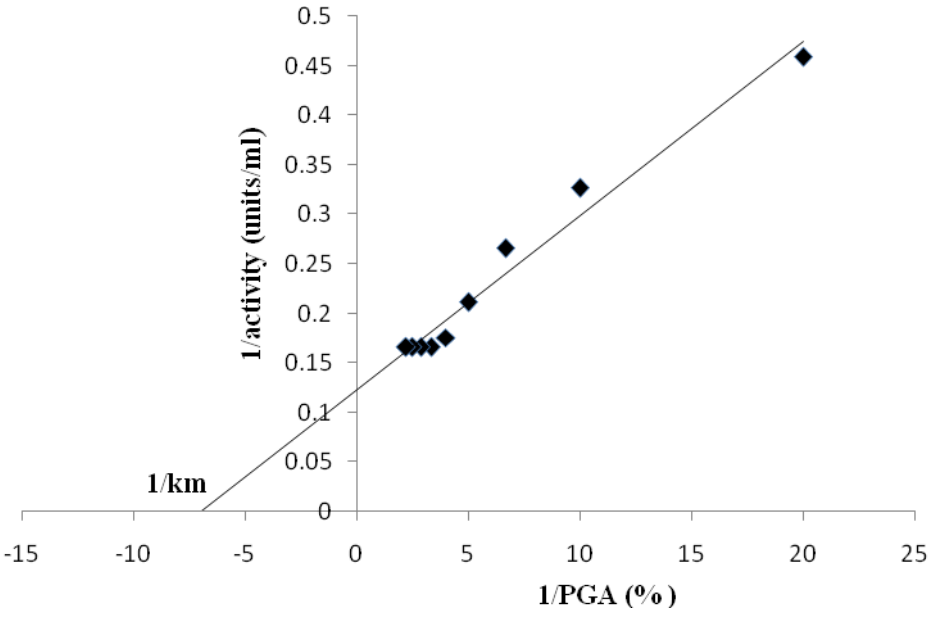

Fig. 4.38: Lineweaver-Burk plot showing K_m value for polygalacturonase as a function of polygalacturonic acid concentration

4.3.2.5 Effect of metal ions

To further characterize polygalacturonase, effect of various metal ions was studied by measuring the activity under standard assay conditions in their presence at concentration ranging from 1 to 10 mM (Table 4.28). K^+ and Fe^{3+} stimulated the enzyme activity at all the tested concentrations. Trivalent Fe^{+3} was most potent activator of PG. The maximum PG activity occurred at concentration 5 mM of K^+ and at 10 mM of Fe^{3+}. Both enhanced the activity by more than 1.5 fold. However, only higher concentrations of Na^+ (5 and 10 mM) accounted for stimulation of PG activity by 20 and 25 per cent respectively. Presence of Ca^{2+} and Zn^{2+} in the assay mixture showed the inhibitory effect at all the tested concentrations whereas Mg^{2+} did not influence activity of enzyme at lower concentrations (1 and 2 mM) to an appreciable extent but it had inhibitory effect at higher concentrations.

Table 4.28: Effect of various metal ions on the activity of purified polygalacturonase

Metal ion	Enzyme activity (% of control)			
	1 mM	2 mM	5 mM	10 mM
Fe^{3+}	103	120	145	160
Ca^{2+}	80	75	60	53
Mg^{2+}	100	98	83	77
K^+	107	114	152	129
Na^+	100	103	120	125
Zn^{2+}	98	85	72	72

* Activity in control was: 6.2 ±0.04 units/ml

4.3.2.6 Effect of organic solutes, chelating and reducing agents

To study the response of PG to various organic solutes, glycine, glycerol, glucose, maltose and sucrose at 1 M concentration each, were included in the otherwise standard assay mixture. The results (Table 4.29) clearly demonstrate that all organic solutes tested were inhibitory to varying degrees of effectiveness. The relative order of increasing effectiveness as indicated by relative activity (per cent of control) was glycine, glucose, maltose and sucrose. The metal building reagent EDTA inhibited enzyme activity at 10 mM concentration as depicted in Table 4.29. The effect of different reducing agents on polygalacturonase activity was determined by measuring the activity under standard assay conditions in presence of various reducing agents at specified concentrations (Table 4.29). All reducing agents namely β-mercaptoethanol, DTT and cysteine activated PG. Amongst all tested reducing agents, DTT was most potent activator of PG followed by cysteine and β-ME.

Table 4.29: Effect of organic solutes, chelating agent and reducing agents on activity of purified polygalacturonase

S.No.	Substance	Concentration (M)	Relative Activity (%)
l	Control	-	100
2	Glucose	1M	78
3	Maltose	1M	72
4	Sucrose	1M	56
5	Glycine	1M	95
6	EDTA	10 mM	87
7	β-ME	2 mM	128
8	DTT	2 mM	216
9	cysteine	2 mM	207

* Activity in control was: 6.2 ±0.04 units/ml

CHAPTER-V

DISCUSSION

Being part of a balanced diet, fruits play a vital role in human nutrition by supplying the necessary growth-regulating factors essential for maintaining normal health. However, they in general contain a very high percentage of water and exhibit a relatively high rate of metabolic activity, which makes them highly perishable commodities with short shelf-life. In fruits, various physiological activities going on even after their harvest result in various physico-chemical and biochemical changes. Hence, it is imperative to study the various changes occurring in the fruits during ripening and after harvest during storage period. It can help us to know that for how long the fruit can be fit for consumption and how the use of various chemicals can be helpful to enhance the shelf-life of the fruits. The present investigations were carried out on the topic entitled "Role of gibberellic acid and calcium chloride on ripening related biochemical changes in guava (*Psidium guajava* L.) fruit". The aim was to observe the effect of different treatments on shelf-life of guava fruits. The results recorded during the present investigations are discussed in this chapter under the following sub-headings:

5.1 Physico-chemical and biochemical changes in guava fruits during ripening

5.1.1 Physico-chemical changes

Firmness of fruits is an important quality attribute from the horticulture prospective. The rate of loss of firmness may influence not only fruit quality but also the storage life. In the present study, the firmness of fruits decreased continuously during the ripening process (Fig. 4.1). The longer shelf-life variety, L-49 had higher fruit firmness than Hisar Surkha throughout the ripening process. Abu-Goukh and Bashir (2003) and Hind *et al.* (2003) also reported decrease in fruit firmness during ripening of guava. Similarly, Ullah *et al.* (2013) observed a declining trend in fruit firmness in peach as fruit ripening period progressed. Softening, which accompanies fruit ripening, is presumably due to change in cell wall polysaccharides caused by hydrolytic enzyme activity (Giovannoni *et al.* 1992; Rose and Bennett, 1999; Payasi *et al.* 2009).

The total soluble solids which generally contain sugar, mineral and acids, is a reliable index to judge the proper stage of maturity. A progressive increase in TSS was observed during ripening of guava fruits from IG stage to R stage and thereafter a decline was observed at OR stage (Fig. 4.2). Hisar Surkha, the variety with short shelf-life had significantly higher TSS content as compared to L-49 at all the stages of ripening. These observations are in agreement with those reported earlier in sapota and guava fruit (Pawar *et al.* 2011; Mahajan *et*

al. 2004). Conversion of starch and other insoluble carbohydrates into soluble sugars was reported to be accountable reason for initial increase in TSS content of fruits and thus causing changes in the flavor (Singh *et al.* 1981) whereas slight decline at the later stages was attributed to utilization of soluble solids in respiratory processes. However, Tosun *et al.* (2008) and Hedge and Chharia (2004) reported that TSS increased significantly throughout development and ripening in blackberries and guava respectively. Similarly, Bashir and Abu-Goukh (2003) reported that TSS increased 1.2-fold during guava ripening.

Titratable acidity is directly related to the concentration of organic acids present in the fruit, which is an important parameter in maintaining the quality of fruits (Shirzadeh *et al.* 2011). The variety with long shelf-life, L-49 had significantly higher titratable acidity than Hisar Surkha throughout the ripening period. Both the varieties had maximum titratable acidity at MG stage, which decreased gradually with the advancement of ripening period (Fig. 4.3). The increase in titratable acidity from IG to MG stage may be due to the formation of organic acids during maturation and also associated with high concentration of undissociated organic acids stored in the vacuole. As the ripening progressed, these organic acids declined due to their utilization in respiration process and conversion into sugar thus, leading to decrease in TA (Tosun *et al.* 2008). The results are in accordance with the earlier reported results on guava (Selvaraj *et al.* 1998; Hedge and Charria, 2004). Decrease in acidity during ripening has also been reported in other fruits such as sapota (Damodaran *et al.* 2001) and ber (Kannan and Thirumaran, 2003). However, Damodaran *et al.* (2001) and Nag *et al.* (2011) reported that acidity declined throughout the period of ripening in sapota and guava respectively.

5.1.2 Biochemical changes

5.1.2.1 Sugars, cell wall components and their hydrolyzing enzymes

It is evident from Fig 4.4 (A , B and C) that during the ripening process total, reducing and non reducing sugars of the guava fruits increased continuously upto R stage followed by a decline at OR stage in both the cultivars. The two varieties exhibited significant variations in the sugar content with the short shelf-life variety, Hisar Surkha having higher sugar content than L-49 (long shelf-life variety) at all the stages of ripening. These results are in accordance with the findings of Pawar *et al.* (2011) in sapota who reported significant increase at different stages of ripening with a slight decline at over ripe stage. Increase in sugar content during ripening was also reported in ber (Baviskar *et al.* 1995; Goel and Siddiqui, 1999; Praduman, 2010), banana (Shivashankar, 2002) and blackberries (Tosun *et al.* 2008). Increase in sugars may be attributed to conversion of polysaccharides into monosaccharides (sugars) (Kumar, 2008), hydrolysis of starch into sugars by α-amylase activity (Kohar, 2003) and of acids into sugars (Pool *et al.* 1972). A decline in sugar content at OR stage is predictable as these are the primary substrate for respiration. Degradation of

certain cell wall components such as pectin and hemicellulose may also contribute to increase in reducing sugars (Jain *et al.* 2001).

The level of the cell wall material decreases with the enhancement of ripening (Sharma and Siddiqui, 2004) thus, causing structural changes in pectin, hemicellulose and cellulose which are assumed to be responsible for the dissolution of the middle lamella and disruption of the primary cell wall during ripening-related loss of firmness (Seymour *et al.* 1990; Carpita and Gibeaut, 1993; Maclachlan and Brady, 1994; Cheng and Huber, 1996; Rose *et al.* 1998). These changes include not only solubilisation and depolymerisation of the polysaccharides but also rearrangements of their associations (Rose *et al.* 1998). Since the primary cell wall is a highly complex structure, it would be likely that a concerted effort of a variety of enzymes might be required to affect the disassembly of cell wall components and fruit softening (Giovannoni *et al.* 1992). Therefore, loss of firmness during ripening of fruits like guava can be best assessed by studying the changes in cell wall components and their hydrolyzing enzymes during the ripening period.

Critical perusal of the data on cell wall components (Fig. 4.5-4.11) reveals that during guava fruit ripening, these components were sequentially modified and the levels of the wall materials decreased with the advancement of ripening in both the cultivars. These changes continued during ripening and over ripening causing the softness of the fruit and loss of tissue structure at OR stage. According to this, two distinct phases of ripening could be identified. The first phase (upto MG stage), during which fruit became physiologically mature was characterized by the synthesis and accumulation of cell wall components, whereas in the next ripening phase, when the fruit became attractive for consumption, cell wall components were degraded leading to loss of fruit firmness. Hisar Surkha, a soft variety had lower cell wall contents at all the stages of ripening as compared to the long shelf-life variety, L-49. The present results are in agreement with those reported in mango (Mitcham and McDonald, 1992), plantain (Kojima *et al.* 1994), ber (Sharma and Siddiqui, 2004, Yadav *et al.* 2012), strawberry (Rosli *et al.* 2004), bush-butter fruit (Missang *et al.* 2004) and guava (Jain *et al.* 2001) fruits. Cell wall components (cellulose, hemicelluloses and pectin) were found to be positively correlated with the fruit firmness (Table 4.1). Loss of fruit firmness during ripening has been correlated with decrease in pectin substances in strawberry (Rosli *et al.* 2004). From the results obtained during present investigations, it could be argued that at later stages of fruit ripening, hydrolysis of cell wall components occurs, resulting in softening of fruits.

Modifications in cell wall during ripening are considered to be involved in the coordination and interdependence of a range of hydrolytic enzymes including pectin PME, PG and cellulase. Activities of these enzymes determine the texture (juiciness, crispness) of fruit during ripening (Payasi *et al.* 2009; Lunn *et al.* 2013). Pectin methylesterase is responsible for the deestrification of pectin before it is depolymerized by polygalaturonase.

PME activity increased upto T stage and decreased thereafter upto OR stage in both the cultivars (Fig. 4.14). Results reported for mango (Abu-Sarra and Abu-Goukh, 1992) and guava (Jain *et al.* 2001; Mondal, 2005) corroborate our findings. However, no change in PME activity was observed during ripening of pear (Ahmed and Labavitch, 1980). However, a continuous increase in PME activity throughtout the ripening process was reported by Barka *et al.* (2000) in tomato, Goulao *et al.* (2007) in apple, Carvalho *et al.* (2009) in guava and Yadav *et al.* (2012) in ber fruit. Data on PG (Fig. 4.12) and cellulase (Fig. 4.13) revealed that activities of cell wall degrading enzymes increased throughout the ripening process. These results are in agreement with the findings in tomato (Barka *et al.* 2000; Ali and Abu-Goukh, 2005), ber (Sharma and Siddiqui, 2004; Jawanda *et al.* 2009; Yadav *et al.* 2012), apple (Goulao *et al.* 2007), guava (Jain *et al.* 2001; Abu- Goukh and Bashir, 2003; Mondal, 2005; Carvalho *et al.* 2009) and peach (Ullah *et al.* 2013). Cellulase (-0.915**) and PG (-0.923**) were negatively correlated with fruit firmness while no significant correlation was present between PME and fruit firmness (Table 4.1). A negative correlation was present between cellulose and cellulase (-0.902**), hemicellulose and cellulase (-0.897**) and pectin and PG (-0.864**). A positive correlation between the appearance of PG and initiation of softening has also been reported in a number of fruits like mango (El-Zoghbi, 1994), carambola fruit (Chin *et al.* 1999) and ber (Yadav *et al.* 2012). However, it has been reported that degradation of cell wall polysaccharides during ripening of bush butter fruit was related with increase in PG activity while cellulases and hemicellulases were not involved in fruit softening (Missang *et al.* 2004).

The basal level of activities of all these enzymes was higher at all the stages of ripening in Hisar Surkha variety, having short shelf-life, as compared to L-49, with long shelf-life. Higher activities of cell wall degrading enzymes in Hisar Surkha may be one of the factors for faster cell wall hydrolysis during ripening. Similar observations were also reported earlier in ber (Yadav *et al.* 2012), higher activities of these enzymes in Illiachi variety as compared to Umran, confirming its shorter shelf-life due to higher cell wall degradation throughout ripening.

5.1.2.2 Lipid peroxidation and oxidative stress

Oxidative stress can be best assessed by the extent of lipid peroxidation catalyzed by lipoxygenase which plays a central role in membrane deterioration by peroxidizing free polyunsaturated fatty acids. The free radicals produced during ripening induce the degradation of phospholipids to polyunsaturated fatty acid (PUFA) which are then peroxidised by lipoxygenase. Progressive increase in activity of LOX with very high increment at later (R and OR) stages of ripening (Fig. 4.15) suggested that lipid hydroperoxides from higher LOX activity at later stages of ripening contribute to an increase in cellular oxidative status thereby causing disruption of the membrane integrity of the fruit which in turn leads to softening of

the fruit. Membrane deterioration due to increased LOX activity has been reported to be responsible for loss of tissue structure during ripening in saskatoon fruit (Rogiers *et al.*1998), tomato (Mondal *et al.* 2004) and ber (Kumar *et al.* 2011), guava (Mondal *et al.* 2009).

Fruit ripening is a stressful process and is considered to be a functionally modified protracted form of senescence. Therefore, mechanism of membrane deterioration and loss of tissue structure during ripening seems to be similar to that characterized in true senescing systems involving the accumulation of reactive oxygen species (ROS). Production of superoxide radicals and H_2O_2 content (Fig. 4.16 and 4.17) followed a pattern similar to that exhibited by LOX. These were minimum at IG stage, increased continuously throughout the ripening period and reached maximum at OR stage in both the cultivars. Hydrogen peroxide is relatively stable and important indicator of oxidative stress has been reported to increase exponentially with increasing stress in mangrove leaves (Cheeseman, 2006). During ripening, an increase in H_2O_2 content has been reported in ber (Kumar *et al*. 2011), saskatoon (Rogiers *et al.* 1998), guava (Ram, 2007; Mondal *et al.* 2009) and tomato (Jimenez-Bermudez *et al.* 2002). However, the results are in contradiction to those reported in tomato fruits (Mondal *et al.* 2004) where H_2O_2 content was found to be maximum at IG stage and decreased gradually as the fruit progressed towards ripening. Production of ROS such as superoxide anion radical and hydrogen peroxide production occurs primarily as by-products of cellular metabolism in the mitochondria (Masaki *et al.* 1999; Terman *et al.* 2006) and is considered as an important cause of cellular oxidation and consequent membrane lipid peroxidation, linking to fruit maturation and aging (Esterhazy *et al.* 2008).

The parallel increase in LOX activity, superoxide radicals and H_2O_2 content during ripening indicates that production of ROS increased during ripening thereby disrupting the membrane integrity of the fruit and in turn causing softening of the fruit. Higher values for LOX activity, superoxide radical content and H_2O_2 in Hisar Surkha, a short lived variety suggest that membrane deterioration causing loss of membrane integrity and tissue structure is much faster in soft variety than in firm one.

5.1.2.3 Antioxidative enzymes and metabolites

Under normal conditions, ROS produced during metabolism are efficiently neutralized by various free radical scavengers non-enzymatically (e.g., ascorbate, glutathione) and/or catabolized enzymatically (e.g., SOD, POX, CAT, APX and GR). Oxidative stress results when there is a shift in the equilibrium between free radical production and consumption through these pathways to favour production (Purvis *et al.* 1995).

Perusal of the data (Fig. 4.18) indicates that SOD activity increased from IG stage to T stage and decreased thereafter as the ripening process progressed to R and OR stages in both the varieties. In general, L-49 variety exhibited higher SOD activity at all the stages of fruit ripening, thus dismutating $O^{\cdot}{}_2^{-}$ significantly. A positive correlation was present between

superoxide radical content and hydrogen peroxides suggesting that hydrogen peroxides were also increasing when superoxides were increasing (0.871^{**}). These observations are in agreement with those reported earlier in tomato (Ahn *et al.* 2002), guava (Mondal *et al.* 2009) and ber (Kumar *et al.* 2011) fruits. However, Reddy and Srivastava (2003) and Mondal *et al.* (2004) observed highest SOD activity at IG stage in tomato and mango fruits, respectively. The decrease in SOD activity during ripening of fruits has been also observed in saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007). Superoxide dismutase removes superoxide and hence decreases the risk of hydroxyl radical formation from superoxide via the metal-catalysed Haber–Weiss-type reaction (Arora *et al.* 2002). In transgenic (SOD^{+}) tobacco plants, enhancement of active oxygen-scavenging system leads to increased oxidative stress protection (Gupta *et al.* 1993). Due to a substantial increase in respiration of guava, a climacteric fruit, oxy free radical production probably increased at later stages of development.

Catalase activity increased from IG stage to T stage and thereafter decreased throughout the ripening process in both the varieties (Fig. 4.19). A similar initial increase and then decrease in the CAT activity was reported in mango (Masia, 1998), saskatoon (Rogiers *et al.* 1998) and guava (Mondal *et al.* 2009). The loss in CAT activity observed at later stages of ripening may account for the accumulation of H_2O_2. While comparing the two varieties, it has been observed that L-49 had higher catalase activity which might be reason for its long shelf-life than Hisar Surkha. However, continuous increment in CAT activity during ripening in ber (Kumar *et al.* 2011) and tomato (Andrews *et al.* 2004; Mondal *et al.* 2004) and progressive decline in CAT activity has been reported in saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007).

Similar to the pattern exhibited by SOD and CAT, the APX activity (Fig. 4.20) also increased from IG stage to T stage and thereafter declined with the further advancement of ripening in both the cultivars. Long shelf-life variety, L-49 had significantly higher activity than Hisar Surkha at all the stages of fruit ripening. Present results are in agreement with those reported in guava (Mondal *et al.* 2009), papaya (Silva *et al.* 1990), tomato (Andrews *et al.* 2004). Similiarly, APX activity increased during initial stages but thereafter decreased during ripening in tomato (Mondal *et al.* 2004), guava (Ram, 2007) and ber fruits (Kumar *et al.* 2011). However, a continuous decrease in APX activity has been reported during ripening of orange (Huang *et al.* 2007). Decline in APX activity at OR stage results in inefficiency of the antioxidant system to scavenge ROS causing oxidative stress. Such a decrease in APX activity for initiating senescence process has also been reported in gladiolus petals (Hossain *et al.* 2006).

Both the varieties clinched highest GR and POX activity at MG stage which decreased gradually with the enhancement of fruit ripening (Fig. 4.21 and 4.22). L-49

exhibited significantly higher enzyme activity than Hisar Surkha throughout the ripening period. Similar results were observed for GR activity in ripening tomato (Andrews *et al.* 2004; Mondal *et al.* 2004), guava (Ram, 2007; Mondal *et al.* 2009) and ber (Kumar *et al.* 2011) fruits. Contrarily, continuous increase in GR activity was observed during ripening of saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007). Contrarily, continuous increase in GR activity was observed during ripening of saskatoon (Rogiers *et al.* 1998) and orange (Huang *et al.* 2007). Similarly, Mango fruit had highest POX activity at MG stage, which significantly declined as the ripening proceeded (Reddy and Srivastava, 2003). However, a continuous decrease in POX activity has been reported during ripening in tomato (Mondal *et al.* 2004) and ber (Kumar *et al.* 2011) whereas in mango (Mattoo *et al.* 1968). POX has been reported to exhibit a continuous increase in activity during ripening. Glutathione reductase has a major role in maintaining the intracellular glutathione pool in reduced state (McKersie and Leshem, 1994) and peroxidase is involved in the scavenging of H_2O_2 that is not removed by CAT (Willekens *et al.* 1997). An increase in the activities of these antioxidative enzymes during initial stages of fruit ripening further suggests that increased production of lipid hydroperoxides and other ROS during ripening eventually induce higher activities of these enzymes but not upto later stages. Pastori and Trippi (1992) observed correlation between the oxidative stress resistance and activity of GR and suggested that oxidative stress caused by paraquat or H_2O_2 could stimulate GR de novo synthesis, probably at the level of translation by preexisting mRNA. The decrease in antioxidative enzymes activity at later ripening stages may be either due to substrate being limited or the enzyme being inactivated.

The down regulation of these enzymes has been suggested to be responsible for accumulation of endogenous H_2O_2, membrane deterioration and loss of tissue structure during fruit ripening. In general, lower activities of antioxidative enzymes associated with higher oxidative stress in Hisar Surkha, suggest the accumulation of ROS to be responsible for membrane deterioration and loss of tissue structure in fruits and hence short shelf-life of this variety of guava. Similar results were observed by Kumar *et al.* (2011) in ber fruit.

Ascorbate acts as an important antioxidant in removal of ROS. Data on ascorbic acid reveal that ascorbic acid increased from IG stage to T stage followed by a continuous decrement upto OR stage (Fig. 4.23). Hisar Surkha (short shelf-life variety) had higher ascorbic acid content than L-49 (long shelf-life variety). This may probably be due to higher APX activity in L-49 which utilizes ascorbic acid as the substrate. These results are in accordance with those reported in tomato fruit during ripening (Mondal *et al.* 2004) which exhibited increase in ascorbic acid content up to T stage followed by a decline at ripe stage. It has also been reported that the short shelf-life variety had higher ascorbic acid content than the one with longer shelf-life (Mondal *et al.* 2004). Increase in ascorbic acid content upto T

stage has been attributed to the breakdown of starch to glucose which was used in the biosynthesis of ascorbic acid (Lim *et al.* 2006). The decline at later stages of ripening may be either due to oxidation of ascorbic acid or its utilization by APX. An initial increase and then decrease in ascorbic acid during ripening has also been reported in apple (Joshi *et al.* 2004), tomato (Mondal *et al.* 2004), orange (Huang *et al.* 2007) and ber (Kumar *et al.* 2009). However, a continuous increase in ascorbic acid has been reported in guava (Hedge and Chharia, 2004) and a continuous decline has been reported in cherry laurel (ElBulk *et al.* 1997; Kadioglu and Yavree, 1998).

Glutathione, a low molecular weight thiol compound, was found to be maximum at MG stage and decreased with the progression of ripening to OR stage, in both the guava cultivars (Fig. 4.24). Thereafter, the contents of total glutathione decreased. Results are in accordance with those reported in ber (Kumar *et al.* 2011), saskatoon (Rogiers *et al.* 1998), orange (Huang *et al.* 2007) and guava (Ram, 2007; Mondal *et al.* 2009) fruits during ripening. However, Jimenez-Bermudez *et al.* (2002) reported a continuous increase in glutathione content during tomato fruit ripening, whereas, it has been reported to decrease in watermelon during ageing (Hsu and Sung, 1997). Tomato and saskatoon fruit also responded to the increase in oxidative stress by increasing reduced and oxidized glutathione content during development (Andrews *et al.* 2004). Because of its role in ascorbate reduction, glutathione has been reported to protect membrane by maintaining α-tocopherol and zeaxanthin in the reduced state (Xiang *et al.* 2001). APX and GR were positively correlated with their substrate ascorbate (0.731^{*}) and glutathione (0.881^{**}) respectively, suggesting that enzymes activities were also increasing with the increase in availability of their substrate.

There was a continuous decrease in β-carotene from IG stage to OR stage in both the cultivars of guava fruits during ripening (Fig. 4.25). Similar results were observed by Kumar *et al.* (2011) in ber fruit. However, present findings are contrary to the results reported in mandarin (Lallan and Godara, 2005) and capsicum (Ha *et al.* 2007), where carotenoid content has been reported to increase during ripening.

Both the varieties exhibited highest antioxidant activity at MG stage, which decreased gradually upto OR stage (Fig. 4.26). Hisar Surkha, a short shelf-life variety showed significantly higher antioxidant activity than L-49 throughout the ripening period. Phenolic and vitamin C are the major contributors to the antioxidant activity of guava fruits, while the contribution of carotenoid is negligible. Findings of the present investigations are in agreement to those of Lim *et al.* (2006) who also reported that the greater DPPH scavenging activity of guava fruit at the unripe stage might be associated to its higher total phenol content levels rather than vitamin C content. However, Mauricio *et al.* (2012) observed that antioxidant activity increased during the ripening process up to the climacteric maximum and decreased during senescence. The decline in scavenging capacity during maturation might be

linked to decrease in the concentration of total phenolicsand rapid consumption of anthocyanins and compositional changes as a result of fruit development.

Perusal of data on change in oxidative stress taken in conjunction with the results on antioxidative system in both the guava cultivars during ripening, it could be suggested that although ripening of guava fruit entails a multitude of physico-chemical changes, it is accompanied by a progressive increase in oxidative stress. It appears that a decline in the ROS scavenging ability and the associated increase in oxidative stress may be mediating the various physico-chemical and biochemical changes occurring during ripening of guava fruit. Higher activities of antioxidative enzymes with more contents of antioxidant metabolites in L-49, a long shelf-life variety, could help to scavenge ROS and combat oxidative stress better in this variety. The low oxidative stress and higher antioxidant system in L-49 might be one of the contributing factors to its long shelf-life as compared to Hisar Surkha variety. Also, it has been suggested for saskatoon (Rogiers *et al.* 1998) and tomato (Mondal *et al.* 2004) fruits.

5.1.2.4 Ethylene and ACC oxidase

Ethylene is known as 'ripening hormone' and it affects the transcription and translation of many ripening-related genes (Giovannoni, 2001; Hiwasa *et al.* 2003). Ethylene evolution increased from IG to T stage (Fig. 4.27) and the abrupt increase at this stage coincided with the eating ripeness. Significantly higher rates of ethylene evolution were recorded for Hisar Surkha as compared to firm cultivar, L-49 and this could be correlated with reduced firmness and short shelf-life of the fruit. Similar results have been reported in peach (Tonutti *et al.* 1991), raspberry (Iannetta *et al.* 1999), mango (Reddy and Srivastava, 2001) and apple (Abbasi *et al.* 2010). Ladaniya (2007) reported that ethylene plays an important role in changing fruit color, flavor, chemical composition and texture of citrus fruit.

Data on the activity profile of ACC oxidase depicted that activity increased substantially from IG stage to T stage in both the cultivars (Fig. 4.28). During the later stages of ripening, the activity decreased upto OR stage. ACC oxidase exhibited a pattern similar to that exhibited by ethylene evolution and its activity was more in Hisar Surkha than in L-49 at all the stages of fruit ripening. The results obtained during present investigations showed parallelism with those reported by Reddy and Srivastava (2001) in mango fruit. However, ACC oxidase activity has been reported to reach its maximum during the ripened stage of mango fruit (Nair *et al.* 2004). A positive correlation was observed between ethylene and ACC oxidase (0.949^{**}). ACC oxidase is thought to be membrane bound (Yang and Hoffman, 1984) and has been reported to require membrane integrity for its synthesis (Hoffman and Yang, 1982). The increased oxidative stress at later stages of fruit ripening evidenced by high LOX activity (Fig. 4.15), high superoxide radical content (Fig. 4.16) and high H_2O_2 content (Fig. 4.17) might have caused membrane deterioration resulting in loss of ACC oxidase activity and thus ethylene production.

5.2 Effect of post-harvest treatment of guava with GA_3 and $CaCl_2$ on physico-chemical and biochemical parameters during storage

5.2.1 Physico-chemical parameters

The physiological loss in weight of the guava fruits increased with increase in period of storage in both the cultivars (Table 4.6). The increase in PLW during storage has been also reported in sapota (Gautam and Chundawat, 1990), guava (Abu-Goukh and Bashir, 2003) and aonla (Nayak *et al.* 2011). The PLW during storage was mainly due to evaporation of water and loss of metabolites during respiration (Shafiee *et al.* 2010; Jawandha *et al.* 2012). The PLW in Hisar Surkha exceeded 10 % threshold by 6^{th} day of storage whereas L-49 exceeded the 10 % threshold by 8^{th} day of storage in all the treated as well as control fruits. During storage, PLW was more in Hisar Surkha, hence it deteriorated rapidly. The fruits stored under control conditions exhibited higher PLW as compared to the treated fruits. This may be due to rapid shrinkage and wilting caused by water loss and due to higher rate of respiration and ethylene production. Gibberellic acid decreased the tissue permeability there by reducing the rate of water loss leading to delayed fruit ripening and hence reduced physiological loss in weight (Wills *et al.* 1998; Kumar *et al.* 2005; Pila *et al.* 2010; Choudhary and Dhruve, 2014). It might slow down the process of ripening by retarding the pre-climacteric respiration rate (Jagadeesha *et al.* 2015). Sudha *et al.* (2007) postulated that the reduction of weight loss in the fruits treated with GA_3 might be due to its anti-senescent action as well as its role in the chemical changes of fruit components, which may retain more water against the force of evaporation (Kumar *et al.* 2011). Similar results of GA_3 treatment have been also reported by other workers in mango (Kumar, 1998), guava (Tamil and Bal, 2005), papaya (Rajkumar *et al.* 2005), plum (Mahajan *et al.* 2008) and tomato (Choudhary and Dhruve, 2014). The physiological loss in weight was significantly diminished by post-harvest $CaCl_2$ treatments in tomato and sapota fruits (Pila *et al.* 2010; Tsomu and Patel, 2014). These results are in accordance with that of Lester and Grusak, (1999), who reported that calcium application was effective in terms of membrane permeability and integrity maintenance by binding to the polar head group of the phospholipids. Hence, the lower loss of phospholipids and proteins and reduced ion leakage which could be responsible for the lower weight loss in calcium treated plum.

Softening of fruits is associated with dissolution of the middle lamallae with turnover in the composition, structure and linkages between polysaccharides (Vicente *et al.* 2007). In the present investigations, the firmness of fruits decreased progressively with increasing storage period in both the cultivars (Tables 4.7). All the treatments resulted in significant retention of firmness for longer period during storage with respect to control fruits except 150 ppm GA_3 treatment which showed no significant difference with respect to control fruits in Hisar Surkha. The retention of firmness by GA_3 treatments in guava has also been reported by

other workers (Hiwale and Singh, 2003; Kher and Bhat, 2005; Tamil and Bal, 2005). Similar results were observed in peach, sapota fruit, Asian pear, papaya, banana and citrus fruits (Romero *et al.* 2000; Damodran *et al.* 2001; Mahajan and Dhatt, 2004; Rajkumar *et al.* 2005; Ritenour *et al.* 2005; Alfonso and Johnny, 2010; Yadav *et al.* 2013; Huang *et al.* 2014). Similarly, calcium treatment maintained firmness effectively in strawberry and guava fruit during storage as observed by Chen *et al.* (2011) and Kumar *et al.* (2011). Also, the present results are in agreement with the report of Benavides *et al.* (2002) and Casero *et al.* (2004) in apple and Ishaq *et al.* (2009) in apricot fruits that suggested post-harvest application of calcium decreased softening and maintained fruit firmness during storage. The retention of firmness in calcium treated fruits might be due to its accumulation in the cell walls leading to facilitation in the cross linking of the pectic polymers which increases wall strength and cell cohesion (White and Broadley, 2003). It has also been reported to stabilize pectin–protein complexes in the middle lamallae thereby acting as an intermolecular binding factor (Dey and Brinson, 1984), enhancing the cell membrane integrity (Lester and Grusak, 1999) and reducing tissue water loss thereby increasing cell turgor pressure (Mignani *et al.* 1995; Picchioni *et al.* 1998). According to John (1987), addition of calcium improves rigidity of cell walls and obstructs enzymes such as polygalcturonase from reaching their active sites, there by retarding tissue softening. Hence, the maintenance of higher firmness by application of GA_3 and calcium may be due to the role of these compounds in maintaining cellular organization and regulating enzyme activities (Horvitz *et al.* 2003).

There was a progressive increase in total soluble solids (TSS) of guava fruits with increase in storage period followed by a decline at the end of storage (Table 4.8). These results are in agreement with those reported earlier in guava (Sharma and Dashora, 2001; Abu-Goukh and Bashir, 2003; Mahajan *et al.* 2004), ber (Kannan and Thirumaran, 2003; Singh *et al.* 2007), mandrin (Bhardwaj *et al.* 2005) and sapota (Pawar *et al.* 2011). Increase in TSS during storage may be due to the breakdown of complex polymers into simple substances by hydrolytic enzymes which might be further metabolized during respiration and thus the level got decreased during subsequent storage (Hussain *et al*, 2008; Akhtar *et al*, 2010; Kumar *et al.* 2011). All the treatments resulted in significant reduction in TSS with respect to control fruits in both the cultivars. It could be probably because of delayed ripening, reduced respiration rate, lowered hydrolytic enzymes and metabolic activities (Balakrishnan, 1998). These results are in agreement with the earlier reports in guava (Hiwale and Singh, 2003; Tamil and Bal, 2005; Mahajan *et al.* 2011) and tomato (Pila *et al.* 2010; Choudhary and Dhruve, 2014) fruits treated with GA_3. Also, the $CaCl_2$ (3 %) dip resulted in delaying the increase in TSS during storage in peach fruits (Sohail *et al.* 2015). Contrarily, $CaCl_2$ treatment has been reported to increase TSS content of pomegranate fruits (Mirdehghan and Ghotbi, 2014).

A progressive decline in titratable acidity was observed in guava fruits during storage (Table 4.9), which might be due to conversion of acids into salts and sugars by the enzymes particularly invertase (Hiwale and Singh, 2003; Tamil and Bal, 2005; Mahajan *et al.* 2011). Results are in agreement with those of Cheour *et al.* (1991) and Mahmud *et al.* (2008) who also observed decrease in TA during storage of strawberry and papaya, respectively. High acidity in pulp is a desirable character as it provides better storage quality. All the treatments during storage resulted in significantly higher acidity content with respect to control. It could probably be due to delay in physiological ageing and alteration in metabolism (Mitra *et al.* 1996). Similar results with GA_3 has been reported in guava (Singh, 1988), tomato (Kumar *et al.* 2005; Pila *et al.* 2010; Choudhary and Dhruve, 2014), papaya (Rajkumar *et al.* 2005), litchi (Pila *et al,* 2010) and sapota (Yadav *et al.* 2013). Similarly, post-harvest application of calcium chloride has been reported to maintain acidity content of strawberry (Amini and Habibi, 2015). Results are in accordance with those reported by Mujtaba *et al.* (2014) that 2% $CaCl_2$ was more effective in maintaining titratable acidity as compared to other concentrations of $CaCl_2$ (1 and 3 %) and control. However, Manganaris *et al.* (2005) reported that post-harvest $CaCl_2$ dips did not affect TA in peaches during storage. This effect of GA_3 and $CaCl_2$ might be due to the decrease in cellular metabolic activities such as respiration thereby, preventing loss of organic acids and hence, decreasing loss of acidity.

5.2.2 Biochemical parameters

5.2.2.1 Cell wall degrading enzymes

PG has been reported to play a central role in the softening process. Other enzymes suggested to be involved in fruit softening include PME (Zhou *et al.* 2000), cellulase (Abu-Goukh and Bashir, 2003), xyloglucanase (Redgwell and Fry, 1993) and mannase (Pressey, 1989). Hence, fruit softening is a highly complex process and involves the coordinated and inter dependent activity of a range of cell wall modifying proteins. The results obtained during present investigations (Table 4.10, 4.11 and 4.12) clearly demonstrate that although activity of PME increased only initially, PG and cellulase exhibited continuous increase throughout storage of guava fruits indicate that softening of fruit during storage is the function of modification of cell wall components catalyzed by various cell wall degrading enzymes (Giovannoni *et al.* 1992). The higher activities of cell wall degrading enzymes in Hisar Surkha (short-shelf-life) than in L-49 (long-shelf-life) as observed during present investigations are in parallelism with those reported earlier in raspberry (Iannetta *et al.* 1999), guava (Mondal, 2005) and ber (Praduman, 2010) fruits, suggesting faster degradation of cell wall components in soft variety than in the variety with long shelf-life. The observed changes in cell wall degrading enzymes are supported by the already reported alterations in the activities of these enzymes in apple (Siddiqui *et al.* 2004) and ber (Praduman, 2010) fruits

during storage. Missang *et al.* (2004) however, reported PG to increase but PME to remain constant throughout the storage period of bush butter fruit.

Although activities of cell wall degrading enzymes (PG, PME and cellulase) got reduced by pretreatment of guava fruits with different concentrations of GA_3 and $CaCl_2$ but 1 % $CaCl_2$ proved to be the most effective treatment. Studies on the effect of different plant growth regulators on PG activity indicated that treatment of GA_3 inhibited enzyme activity in strawberry fruit (Villarreal *et al.* 2009). Majumder and Majumder (2001) observed the effect of gibberellin on pectic substances and their degrading enzymes in developing fruits of Cape gooseberry and found that treatment with gibberellin was highly effective in reducing its activity. GA_3 treatment has been reported to decline PME activity during cold storage of ber fruits (Randhawa *et al.* 2009). Choudhary and Dhruve, (2014) observed that the GA_3 treatment resulted in lowering the activities of cell wall degrading enzymes such as PME, PG and cellulase as compared to the untreated tomato fruits. GA_3 significantly suppressed the cellulase activity in ber (Jawandha *et al.* 2009). The activities of PG and PME enzymes of fresh-cut dragon fruit decreased when treated with 7.5 g l^{-1} of $CaCl_2$ (Chuni *et al.* 2010). $CaCl_2$ controlled ripening process by reducing PG activity, might be the reason for increase shelf-life in $CaCl_2$ treated tomato fruits (Srividya, 2014). Calcium has been reported to delay the process of ripening, more particularly softeningand hence, increases the shelf-life by altering intercellular and extracellular processes at ambient temperature (Chrardonnet *et al.* 2003). Firming and resistance to softening resulting from addition of calcium, have been attributed to the formation of calcium pectate in which calcium ions form intermolecular bridges between pectin molecules by interaction with free carboxyl groups of pectic acid polymers (Luna-Guzman and Barrett, 2000). The enhanced rigidity of the middle lamella and cell walls could lead to increased resistance to PG, PME and β-Gal activities (Mignani *et al.* 1995; Lara *et al.* 2004). Calcium improves rigidity of cell wall and obstructs enzymes such as polygalacturonase from reaching their active sites, thereby retarding tissue softening and delaying ripening (John, 1987; Marzouk and Kassem, 2011). Reported results indicate that non-degradation of cell wall components caused by the hydrolytic enzymes during storage is due to effective maintenance of cell wall integrity by both GA_3 and $CaCl_2$ (Yiwei *et al.* 2008).

5.2.2.2 Lipid peroxidation and oxidative stress

The deterioration of fruits during storage may be due to increased accumulation of ROS either because of their over production or because of the inability of the antioxidative system to scavenge them. These ROS are known to damage membranes by lipid peroxidation by reacting with unsaturated fatty acid (Jimenez-Bermudez *et al.* 2002). Loss of membrane integrity by lipid peroxidation is the major characteristics of deterioration (Thompson, 1988) which is evident from progressive ultra structural deterioration of the cell and increased leakage of solutes (Pauls and Thompson, 1984). During present investigations, progressive

increase in LOX activity with more pronounced increase in soft variety (Hisar Surkha) than in firm one (L-49) was observed during storage of guava fruits (Table 4.13). Continuous increase in LOX activity during storage was also observed in pear (Pinto *et al.* 2001), tomato (Mondal *et al.* 2006), guava (Ram, 2007) and ber (Kumar *et al.* 2011) fruits.

Both the concentrations of $CaCl_2$ decreased LOX activity significantly whereas, GA_3 had no significant effect on LOX activity. The protection of cell membrane integrity by Ca during senescence has been explained by the ability to binding to membrane phospholipids and, in this way, to stabilize the membrane (Thompson, 1988), thus making it less prone to degradation by lipolytic enzymes (Cheour *et al.* 1992). It has been reported that calcium plays an important role in delaying fruit senescence through delaying membrane lipid catabolic processes during post-harvest storage of apple fruit (Picchioni *et al.* 1998). In addition to reducing lipid peroxidation, calcium has been also reported to maintain membrane integrity by regulating the expression and synthesis of proteins and enzymes (Poovaiah and Reddy, 1993) and slowing down catabolism of total phospholipids and delaying an increase in the total free sterol to total phospholipid ratio (Lester and Grusak, 1999). Concomitant with our results, calcium treatment decreased LOX activity in sweet cherry fruit and tomato (Wang *et al.* 2014; Cheour and Souiden, 2015).

Among the various postulates concerned with the initiation of lipid peroxidation, the involvement of free radicals has attracted considerable attention (Dhindsa *et al.* 1981). Activated oxygen species such as $^{\cdot}O^{-}_2$ and H_2O_2 react with proteins, lipids and nucleic acids (Elstner, 1991) cause inactivation of enzymes and initiate lipid peroxidation (Asada and Takahashi, 1987; Halliwell, 1987). Continuous increase in superoxide radicals and hydrogen peroxide content was observed during the storage of fruits (Table 4.14 and 4.15). Hisar Surkha (short shelf-life variety) had higher values for these ROS as compared to L-49 (long shelf-life variety). The increase in H_2O_2 content during storage has earlier been reported in spinach (Hodges *et al.* 2001) and tomato (Mondal *et al.* 2006), guava (Ram, 2007) and ber (Kumar *et al.* 2011). Pretreatment of fruits with GA_3 and $CaCl_2$, though declined both superoxide radicals and hydrogen peroxide content at all the stages of storage, $CaCl_2$ was the most effective in both the cultivars. The combination of hot air and $CaCl_2$ lead to decrease in H_2O_2 in apple fruits (Rabie *et al.* 2011).

Pretreatment of guava fruits with GA_3 and $CaCl_2$ not only resulted in reduced LOX activity but also reduced superoxide radical and H_2O_2 content, suggesting that these treatments have the ability to decrease lipid peroxidation and scavenge free radicals. Higher values of LOX activity, superoxide radical content and H_2O_2 in Hisar Surkha suggest that membrane deterioration causing a loss of membrane integrity and tissue structures is much faster in soft variety, Hisar Surkha than in the firm one.

5.2.2.3 Antioxidative enzymes and metabolites

Plant cells are known to possess antioxidative defense systems to provide protection against the deleterious effects of ROS that these reactive species into less-toxic products (Lim *et al.* 2006; Gonzalez-Aguilar *et al.* 2010). POX, CAT and SOD are important oxyradical detoxification enzymes in plant tissue. In response to stress, plant normally increase the activity of these enzymes (Xu *et al.* 2009). As is evident from the results obtained during present investigations, soft variety had lower activity of ROS scavenging enzymes *viz.* SOD (Table 4.16), CAT (Table 4.17), APX (Table 4.18), GR (Table 4.20) and POX (Table 4.22), throughout storage and thus high rate of membrane deterioration as compared to the firm one. These activities increased initially but declined during later period of storage, suggesting that accumulation of ROS is favored at later stages of storage. Initial increase in enzyme activities may be due to induction caused by ROS and decrease during later storage period may be either due to limitation of substrate or inactivation of the enzyme. Results are in accordance with those of Spychalla and Desborough (1990) reported that SOD activity increased initially and then decreased in potato tubers during storage. Mondal *et al.* (2006) and Ram (2007) observed SOD activity decreased during storage of tomato and guava fruits, respectively. Contrarily, Pinto *et al.* (2001) and Dama (2007) observed an increase in SOD activity during storage of pear and mushrooms. Decline in CAT activity has been also reported during storage of pear (Pinto *et al.* 2001) and tomato (Mondal *et al.* 2003) fruits. The loss in CAT activity observed in present investigations during storage of fruits may account for the accumulation of H_2O_2. The results are supported by those of Mondal *et al.* (2006) who also reported decrease in CAT activity during storage of tomato fruits which further decreased with increase in storage temperature. An initial increase and then decrease in APX activity has been reported in potato tubers during storage (Dipierro and Leonardis, 1997). Decrease in APX activity during storage was also observed by Mondal *et al.* (2006) in tomato and by Ram (2007) in guava. Present results observed for GR activity are in conformity to those of Mondal *et al.* (2006) and Ram (2007) in tomato and guava respectively, where a decrease in GR activity during storage was observed. These findings explain that dysfunction of antioxidative system might be involved in the progression of fruit spoilage during storage as has been reported earlier (Andrews *et al.* 2000; Mondal *et al.* 2003; Kumar *et al.* 2011).

In general, pretreatment of fruits with GA_3 and $CaCl_2$ resulted in higher antioxidative enzymes activities as compared to the control fruits. $CaCl_2$ was more effective in stimulating the enzymes activity as compared to GA_3 treatments. These results are in agreement with that of Schrmitz-Eiberger *et al.* (2002) who reported higher SOD activity in $CaCl_2$ treated fruits. Similarly, combination of hot air and $CaCl_2$ increased SOD and CAT activity in comparison to untreated apple fruits (Rabie *et al.* 2011). Shirzadeh *et al.* (2011) also recorded higher CAT activity in 2 % calcium treated apple fruits as compared to the control fruits. Calcium chloride treatment could effectively retain the activity of POX in tomato fruit during the storage and

similar findings were reported by Ruoyi *et al.* (2005) in *Prunus persica* fruit. Calcium chloride was effective in the maintenance of POX and SOD in banana (Srivastava and Dwivedi, 2000). Higher activity of antioxidant enzymes may delay senescence in fruits by scavenging ROS.

The results revealed a marked increase in ascorbic acid content upto two days and thereafter, a significant reduction was observed during storage (Table 4.19). The initial increase was perhaps due to availability of fruit sugar, a precursor of ascorbic acid synthesis, during this period and therefore synthesis of L-ascorbic acid from fruit sugar continued. The loss in ascorbic acid during prolonged storage of fruits seems to be due to the activation of enzymes like ascorbic acid oxidase, peroxidase and catalase which oxidized L-ascorbic acid into dehydroascorbic acid (Sharma *et al.* 1992; Singh *et al.* 2005). A continuous decrease in ascorbic acid content has been reported in guava fruits (Kumar *et al.* 2003) during storage. During storage, decrease in ascorbic acid was also observed by Leong and Shui (2002), in number of fruits. Commercial freezing and storage of okra, potatoes, green beans, broccoli, spinach and peas also resulted in decreased ascorbic acid content (Tosun and Yucecan, 2008). In the present investigations, more retention of ascorbic acid was observed in GA_3 and $CaCl_2$ treated fruits as compared to control during storage. Similar results with growth regulators have been observed in mango (Singh *et al.* 1995). In tomato, GA_3 enhanced retention of ascorbic acid could be due to lowering of respiration or reducing oxidation of ascorbic acid content (Kher and Bhat, 2005). Higher ascorbic acid content in these treatments might have been due to the inhibitory effect of GA_3 on the oxidative enzymes and hence the rate of degradation of ascorbic acid slowed down. Calcium has been reported to delay the rapid oxidation of ascorbic acid (Veltman *et al.* 2000) causing an increase in fruit ascorbic acid content by calcium application in the present study. Results are in agreement with those reported in jujube (Al-Obeed, 2012) and peaches (Ruoyi *et al.* 2005). Similar increase in ascorbic acid content as a result of calcium application was found by Singh *et al.* (2007).

Glutathione and β-carotene content decreased progressively throughout storage period (Table 4.21 and 4.23). Higher glutathione content was observed in L-49 but β-carotene content was higher in Hisar Surkha. Treatment of fruits with GA_3 had no overall effect on glutathione content of fruits. However, application of both GA_3 and $CaCl_2$ could slow down the rate of degradation of β-carotene significantly in both the variety during storage but GA_3 had more pronounced effect on β-carotene in both the cultivars. Similar results were obtained by Choudhary and Dhruve (2014) in tomato where GA_3 treated fruits showed higher β-carotene content as compared to control during storage. Effect of GA_3 on β-carotene content seems to be mainly due to its effect on color development, although other aspects of ripening processes are also affected (Srividya *et al.* 2014). Mujtaba *et al.* (2014) reported that 2 %

calcium chloride was effective in maintaining β-carotene in all the treated tomato fruits as compared to control during storage.

Several phytochemicals, such as flavonoids, phenolic acids, amino acids, ascorbic acid, tocopherols and pigments might contribute to the total antioxidant activity of fruits (Cardner *et al.* 2000). In the present investigations, the antioxidant activity of guava fruits decreased progressively throughout the storage period (Table 4.24). Treatments of GA_3 and $CaCl_2$ could prevent loss in antioxidant activity in both the cultivars and loss was minimum in fruits treated with 1 % $CaCl_2$ thus retaining maximum antioxidant activity. Similar observations with GA_3 have also been reported by other workers in guava (Hiwale and Singh, 2003; Mahajan *et al.* 2011), papaya (Rajkumar *et al.* 2005), banana (Alfonso and Johnny, 2010; Huang *et al.* 2014) and broccoli (Huang and Jiang, 2012) during storage. Aghdama *et al.* (2013) demonstrated that a post-harvest $CaCl_2$ treatment also enhanced antioxidant capacity of cornelian cherry fruit by effectively maintaining higher total phenols, total flavonoids and anthocyanin contents. Similarly, post-harvest $CaCl_2$ treatment maintained the nutritional quality of pomegranate fruit with higher total phenols and total ascorbic content (Ramezanian *et al.* 2010). It has been postulated that enhanced antioxidant activity due to enhancement of total phenols and total ascorbic content in fruit by calcium treatment may be due to stimulating the phenylpropanoid pathway by increasing the PAL activity (Jacobo-Velazquez *et al.* 2011; Aghdama *et al.* 2013).

Pretreatment of guava fruits with $CaCl_2$ before storage, decreased lipoxygenase activity (Table 4.13), superoxide radical content (Table 4.14), hydrogen peroxide content (Table 4.15) and increased the activities of SOD (Table 4.16), CAT (Table 4.17), APX (Table 4.18), GR (Table 4.20) and POX (Table 4.22) significantly, suggesting that $CaCl_2$ have the ability to decrease lipid peroxidation and scavenge free radicals. Decreased electrolyte leakage by calcium application has been reported to increase antioxidative enzymes activities, the cell wall integrity and stability (Mortazavi *et al.* 2007).

5.2.2.4 Ethylene production and ACC oxidase activity

Rate of ethylene production in fresh fruits and vegetables is considered good index for the determination of storage life. In soft variety, Hisar Surkha, ethylene production was maximum on 2^{nd} day of storage while in firm variety L-49, maximum ethylene production was observed on 4^{th} day of storage (Table 4.25). ACC oxidase activity during storage of guava fruits followed a pattern similar to the one followed by ethylene evolution (Table 4.26). As it is clear from the data, ethylene evolution and ACC oxidase activity are correlated with fruit spoilage. The soft variety which deteriorates early had higher rate of ethylene evolution than the firm one. A similar increase in ethylene evolution and ACC oxidase activity in mango fruit during storage has been reported by Nair *et al.* (2004). The GA_3 treatment did not show any significant effect on ethylene evolution while $CaCl_2$ significantly inhibited ethylene

evolution. Similarly, calcium treatment resulted in decreased ethylene production in apple, tomato and pineapple (Hewajulige *et al.* 2003; Senevirathna and Daundasekera, 2010; Shirzadeh *et al.* 2011). Effects of calcium are presumably mediated by reduced ethylene production and respiration rate (Hewajulige *et al.* 2003; Recasens *et al.* 2004). Njoroge *et al.* (1998) observed that calmodulin was involved in the calcium inhibited ethylene biosynthesis. Gibberellin had no significant effect on ethylene production in pea seedlings but the actions of gibberellin and ethylene are mutually antagonistic in their effects on growth and development of tissues (Fuchs and Lieberman, 1968). In contrary, it has been previously reported that GA_3 treatment might slow down the process of ripening by retarding the pre climacteric respiration rate and subsequently ethylene production (Gholami *et al.* 2010; Siddqui *et al.* 2013; Jagadeesha *et al.* 2015). Pretreatment of fruits with different chemicals had no significant effect on ACC oxidase activity and hence, it suggested that calcium may be preventing ethylene production, by means of inhibiting ACC availability for ACO (Wang *et al.* 2005).

5.3 Partial purification and characterization of polygalacturonase

Fruit softening is an important aspect of ripening process in fleshy fruits and is caused by the cumulative action of a group of cell wall-modifying enzymes. Polygalacturonase is the key enzyme involved in the fruit softening process in guava fruit. An increase in PG activity, protein content and mRNA levels has been reported during ripening in several climacteric fruits (Hadfield and Bennett, 1998). This study reports the purification and characterization of polygalacturonase enzyme from a naturally delayed ripened (long shelf-life) guava cultivar (L-49).

5.3.1 Purification of polygalacturonase

Specific activity in crude extract prepared in 0.1M sodium acetate buffer (pH 5.2) was 1.54 units/mg protein and 3.34 units/mg protein in ammonium sulphate fraction (30 –70 %) with a recovery of 67 % (Table 4.27). Thus, only 33 % of the activity was lost during this step of purification. The percentage ammonium sulphate saturation obtained is comparable to that reported for polygalacturonase from ripe avocado fruit mesocarp (Wakabayashia and Donald, 2001).

Purification of the ammonium sulphate concentrated fraction by molecular exclusion chromatography through Sephadex G-100 resulted in recovery of 51 % of activity. The active pooled fraction was enriched 43 fold from the initial extract (Fig. 4.29). The pooled and concentrated fraction from Sephadex G-100 column was loaded on CM-cellulose column for further purification. The enzyme got eluted with increasing ionic strength from 0-0.5M KCl gradient in sodium acetate buffer, as a single peak between fractions 81 to 89 and was enriched 88 fold (Fig. 4.30). This clearly demonstrates that the enzyme protein carried a net positive charge under the experimental conditions. The guava PG resembled the mango PGI (Singh and Dwivedi, 2008) in this respect. They recovered PGI activity in CM-cellulose

bound fraction during purification of PG from mango where unadsorbed PGI from DEAE cellulose column was subjected to CM cellulose chromatography and eluted with 500 mM NaCl. The final enzyme preparation in the present investigations had specific activity of 135.76 units/mg protein and the enzyme was purified about 88 fold with 35 per cent recovery. The purification factor obtained was comparable to that reported for PG-III from banana where Sephadex G-150 chromatography led to a 41-fold purification which was further enriched 78 fold as a single peak on Sephacryl S-200 chromatography (Pathak *et al.* 2000).

Crude extract and enzyme preparation at different steps were analyzed by native-PAGE. The purified fraction showed only one band detected with coomassie brilliant blue (Plate 4.1) confirming that the enzyme was purified to near homogeneity. Elution profile on Sephadex G-100 and CM-cellulose column also showed a single peak (Fig. 4.29 and 4.30).

5.3.2 Characterization of purified polygalacturonase

5.3.2.1 Subunits and molecular weight

Molecular weight of purified polygalacturonase, as determined from gel filtration through Sephadex G-100, was 80 kDa (Fig. 4.31). However, the purified enzyme showed a single protein band on SDS-PAGE by coomassie brilliant blue staining (Plate 4.2, lane 2) and the molecular weight calculated from the plot of R_m value versus log of molecular weight was 40 kDa (Fig. 4.32) indicating it to be a homodimer. These results are in agreement with those of Singh and Dwivedi (2008) who isolated and purified three isoforms of PG from mango fruit *cv.* Dashehariand all the isoforms were found to be dimers on SDS-PAGE. Similar molecular weight and subunit composition has been reported for PGI of Dashehari mango but the subunit structure elucidation of other isoforms of Dashehari mango i.e. PGII and PGIII, revealed to be heterodimers. Similarly, PGIII was purified and characterized by Gayathri *et al.* (2007) from Jamaica cherry (*Muntingia calabura* L.) that showed molecular weight of 85 kD, by size exclusion chromatography and two dissimilar bands of 62 and 21 kD as heterogenous subunits on SDS-PAGE. Also, the gel filteration chromatography of the purified enzyme of banana indicated Mr of 90±10 kDa and the Mr of subunit was 29±2 kDa (Pathak *et al.* 2000). Present study reports only one form of PG from guava fruitsand this was confirmed by the single peak obtained at 280 nm both in ion exchange and gel filtration chromatography. Similarly, only one form of PG was purified from banana ripened fruits *cv.* Kadali, which showed a single band on native PAGE (99 kDa) and two subunits on SDS-PAGE (58.78 and 26 kDa) (Gayathri and Nair, 2014). However, PG isoforms of avocado, tomato and mango were reported to be monomeric (Prasanna *et al.* 2006; Pressey and Avants, 1973; Wakabayashi and Huber, 2001).

5.3.2.2 pH optima for activity and stability

PG showed maximum activity at pH 5.0 that decreased with further increase in pH (Fig. 4.33). These results are in accordance with those of Singh and Dwivedi (2008) in

Dashehari mango where PG isoforms exhibited pH optima 5.0 (PGI) and 6.0 (PGII and PGIII). Guava PG exhibited pH profile similar to those of peach, pear, strawberry (pH optima of 5.5, Nogata *et al.* 1993; Pressey and Avants, 1973; Pressey and Avants, 1976) and avocado (pH optima of 6.0, Wakabayashi and Huber, 2001). However, three isoforms of PG from banana exhibited lower pH optima i.e. 3.3, 3.7 and 4.3 (Pathak and Sanwal, 1998) than PG of guava. Similiarly, all the three isoforms of Alphonso mango exhibited pH optima at more acidic zone namely, 3.2, 3.6 and 3.9 (Prasanna *et al.* 2006). The moderately acidic nature of the guava PG in this study corroborated the influence of pH on PG activity. The purified enzyme preparation was stable over a range of pH 4.0 to 7.0 with highest stability at pH 5.2 (Fig 4.34). The pH stability of guava PG is almost similar to that of the PGI purified from mango *cv*. Alphonso, which was stable over a wide pH range 4–7.5 unlike PG II and III, which were stable at pH 4 and 5, respectively (Prasanna *et al.* 2006).

5.3.2.3 Temperature optima for activity and thermostability

The enzyme activity increased with increasing temperature and showed maximum activity at 35°C after which there was a decline in the enzyme activity (Fig.4.35). Usually, the loss of activity is severe at higher temperatures, which may be due to the unfolding processes caused by elevated temperature that damage the active site. However, at low temperature, substrate/product diffusion rates may decrease that resulted in lower enzyme activity. These results are in accordance with earlier reports on mango PG where temperature optimum for Zebda variety was reported to be between 30 and 35 °C (Labib *et al.* 1995). PG isoforms from Dashehari mango differed with respect to temperature optima (Singh and Dwivedi, 2008) as PG I and PGII exhibited temperature optima of 37 °C while PGIII of 40 °C. Alphonso mango PG showed temperature optima of 37 °C for PGI and PGIII while 42 °C for PGII (Prasanna *et al.* 2006). However, banana PG and 3 isoforms of mango PG exhibited maximum activity at 40°C (Pathak *et al.* 2000; Prasanna *et al.* 2006; Gayathri and Nair, 2014).

The enzyme purified during the present investigation was stable upto 40 °C over a preincubation period of 45 min and underwent gradual inactivation with increasing temperature (Fig. 4.36). Enzyme lost 40 and 54 per cent activity at 60 and 70 °C respectively and completely inactive at 80ºC after 45 mins preincubation. Results are in accordance with that of mango PGIII, retaining 60 and 50 per cent activity at 60 and 70 °C within 5 min, respectively. Tomato PGI is reported to be inactivated at 90 ºC and PGII at 65 ºC over 5 min incubation (Rodrigo *et al.* 2006). Tomato PGI and PGII lost their 50 per cent activity at 78, 57 °C and 100 per cent at 95 and 65 °C, respectively (Pressey and Avants, 1973). Gayathri *et al.* (2007) purified enzyme from Jamaica cherry that had an optimum temperature of 40°C and was relatively stable at 50°C (60 per cent activity) and 60°C (50 per cent activity).

5.3.2.4 Effect of substrate concentration and $K_m$value

Guava PG followed typical Michaelis-Menten kinetics with Km values of 0.14 % for polygalacturonic acid (PGA) (Fig. 4.37 and 4.38). Similarly, hyperbolic saturation curves for the substrate have been reported for this enzyme from banana but three PG isoforms of banana exhibited higher Km values for polygalacturonic acid (PGA) namely 0.22, 0.14 and 0.15 % (Pathak and Sanwal, 1998; Pathak *et al.* 2000). The substrate saturation curve for PG-3 of Jamaica cherry was also hyperbolic and Lineweaver - Burk's plot indicated Km value of 0.27 % for PGA. (Gayathri *et al.* 2007). However, Zimbabwean fruit, Dashehari mango and Alphonso mango, PG showed showed Km for PGA in lower range (0.02–0.025 % PGA) (Muchuweti *et al.* 2005; Prasanna *et al.* 2006; Singh and Dwivedi, 2008). PG from mango pulp *cv.* Alphonso had Km for pectic acid in the range 0.22–0.25 mg ml^{-1} (Prasanna *et al.* 2006). Km reported for the polygalacturonase from four Zimbabwean wild fruits (*Uapaca kirkiana, Zizphus mauritiana, Tamarindus indica* and *Berchemia discolor* fruits) was in the range 0.115–0.152 mg ml^{-1} (Muchuweti *et al.* 2005). The Km value (0.14 %) of the protein from guava fruits indicated the low substrate affinity of PG to its natural substrate PGA and this may be the reason for the low activity of the enzyme in this delayed ripened cultivar.

5.3.2.5 Effect of metal ions on enzyme activity

Data presented in Table 4.28 showed that the PG activity was differentially affected by various metal ions. K^+ and Fe^{3+} stimulated the enzyme activity whereas presence of divalent metal ions Ca^{2+} and Zn^{2+} showed inhibitory effect at all the tested concentrations in the assay mixture but Mg^{2+} is inhibitory at higher concentrations. Similarly, Mg^{2+} weakly inhibited the enzyme of Jamaica cherry (Gayathri *et al.* 2007).

The inhibitory effect of the divalent cations was probably a result of physical interactions with the pectin and not a direct effect on the enzyme (Walter, 1991). Similar inhibiting effect of divalent ions was observed in Jamaica cherry (Gayathri *et al.* 2007). Calcium ions may enhance the gelling of the pectin molecules, thereby producing interlinking and making the substrate inaccessible to the enzyme (Muchuweti *et al.* 2005). Trivalent Fe^{3+} was most potent activator of PG which is in accordance with PG from banana and Dashehari mango (Singh and Dwivedi, 2008; Gayathri and Nair, 2014). Only higher concentrations of Na^+ enhanced the activity. Similarly, PG was activated by the monovalent ions (Na^+) in Zimbabwean fruit (Muchuweti *et al.* 2005). Banana and tomato PG are reported to be activated by monovalent ions like Na^+ and K^+ (Pathak and Sanwal, 1998; Pressey and Avants, 1973). The monovalent cations, Na^+ and K^+ showed slight activation of the enzyme activity of Jamaica cherry (Gayathri *et al.* 2007) and this may be due to the activation of the enzyme substrate complex. These ions presumably bind to the enzyme, inducing conformational orientation favouring substrate binding. However, Dashehari mango PG activity was slightly inhibited or negligibly affected by the monovalent metal ions (Singh and Dwivedi, 2008).

However, peach polygalacturonase showed inhibition by monovalent ions (Pressey and Avants, 1973) as observed for Dashehari mango PG. In contrary, Alphonso mango isoforms and banana fruit were reported to be inhibited by Fe^{3+} (Pathak and Sanwal, 1998; Prasanna *et al.* 2006). Divalent Ca^{2+} activated PGI and PGII while inhibited PGIII of Dashehari mango. Contrary to the presented results, activation of PG by Ca^{2+} is reported for banana as well as Alphonso mango (Pathak and Sanwal, 1998; Prasanna *et al.* 2006).

5.3.2.6 Effect of organic solutes, chelating and reducing agents on polygalacturonase activity

The response of PG to various organic solutes (Table 4.29) clearly demonstrated that all organic solutes tested were inhibitory to varying degrees of effectiveness. The relative order of increasing effectiveness as indicated by relative activity was glycine, glucose, maltose and sucrose. The order corresponds directly to molecular weight, suggesting that differences may have resulted from differences in the concentration of the solute. Higher the concentration of the solute, higher was the inhibition. High sugar concentrations may result in lowered water activity, which is less suitable for enzymatic action (Chang *et al.* 1965). Inhibition caused by these organic solutes is probably the result of several factors, including an environment of lowered water activity, the polyol-pectin interaction and perhaps, some degree of interference of the enzyme active site.

The metal building reagent like EDTA can inactivate enzyme either by removing the metal ions from the enzyme forming coordination complex, or by binding enzyme as a ligand (Schmid, 1979). Similarly, mango PG isoforms were found to be inhibited by EDTA. The inhibition of guava PG by EDTA was also similar to that of cucumber, strawberry, mango and banana PGs (Pressey and Avants, 1975; Nogata *et al.* 1993; Prasanna *et al.* 2006; Singh and Dwivedi, 2008; Gayathri and Nair, 2014). In contrary, EDTA activated PG of banana (Pathak and Sanwal, 1998).

All reducing agents namely β-ME, DTT and cysteine activated PG, DTT being the most potent while β-ME least amongst these (Table 4.29). Enhanced activity of the enzymes in presence of these agents suggests that the reduction of thiol groups improves their performances. Similar report of PG activation by reducing agents is available from banana fruit and mango (Pathak *et al.* 2000; Singh and Dwivedi, 2008). Pathak *et al.* (2000) also observed increase in PG activity on incorporation of sulfhydryl agents (DTT, β-ME) that lends support to the need of sulfhydryl groups for the functioning of the enzyme in banana.

CHAPTER-VI

SUMMARY AND CONCLUSION

The present investigations were carried out with the objectives to study biochemistry of fruit ripening process in guava (*psidium guajava* l.) and to study role of gibberellic acid and calcium chloride on ripening related biochemical changes in guava fruit for enhancing its shelf-life. For storage studies, fruits harvested at MG stage were stored at room temperature and analyzed at two day interval until complete deterioration. Enzyme polygalacturonase was purified from ripening guava fruits of var. L-49 harvested and characterized for physico-chemical, kinetic and regulatory properties. The results are summarized as follow:

To achieve the first objective, guava fruits *cvs*. L-49 (shelf-life 7-8 days) and Hisar Surkha (shelf-life 4-5 days) at immature green (IG), mature green (MG), turning (T), ripe (R) and overripe (OR) stages were analyzed for various physico-chemical characteristics, sugars, cell wall components and their hydrolyzing enzymes; ROS indices and their scavenging enzymes and metabolites; ethylene and ACC oxidase enzyme. It was observed that TSS and sugars (total, reducing and non reducing) increased while fruit firmness and acidity decreased during ripening. Different cell wall components, *viz*. NDF, ADF, hemicellulose, cellulose, lignin and pectin were maximum in the MG fruits and their contents decreased during the later stages of fruit ripening. PME showed its maximum activity at the turning stage while PG and cellulase exhibited continuous increase during ripening. This suggests that during ripening, cell wall components are being synthesized and degraded simultaneously favouring synthesis during early ripening period and degradation during later stages of ripening. LOX activity, superoxide radicals and H_2O_2 content increased throughout ripening. Various ROS scavenging enzymes *viz.* SOD, CAT, POX, APX and GR exhibited an increase in their activities initially, but later on during ripening, a significant decline was observed in the activities of these enzymes. ROS scavenging metabolites like ascorbate, glutathione and antioxidant activity also exhibited the pattern followed by ROS scavenging enzymes whereas β-carotene showed continuous decrease throughout ripening suggesting that a gradual loss in the ability of ROS scavenging system is responsible for the over accumulation of reactive oxygen species at later stages of fruit ripening causing membrane deterioration and loss of tissue structure. Ethylene evolution and its biosynthetic enzyme ACC oxidase were observed to be substantially high at the turning stage and drastically low at the later stages of fruit ripening indicating the climacteric nature of the guava fruit. Fruits from variety L-49, in general, had higher amount of cell wall components and lower activities of cell wall degrading enzymes, lower LOX activity, superoxide radicals and H_2O_2 content while higher

activities of ROS scavenging enzymes and metabolites and lower rate of ethylene production than the soft variety-Hisar Surkha at all the stages of fruit ripening.

To optimize concentration and time of GA_3 and $CaCl_2$ treatments for increasing shelf-life of guava, MG fruits of both the cultivars were treated with different concentrations of GA3 (25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm) and $CaCl_2$ (0.5 %, 1 %, 2 %, 3 %, 4 % and 5 %) for 3, 5 and 10 min. Then fruits were analyzed for TSS and firmness. On the basis of these two parameters, best time of treatment (5 min) and two concentrations each of GA_3 (100 ppm and 150 ppm) and $CaCl_2$ (1 % and 2 %) treatments, were selected for further studies. To study the effect of gibberellic acid and calcium chloride on various ripening related biochemical changes during storage, fruits of both cultivars (at MG stage) were treated with the selected concentrations of GA_3 and $CaCl_2$ for 5 min. Then the fruits were stored at room temperature and samples taken at 2 day interval until complete decay and analyzed for various ripening related parameters. The soft variety Hisar Surkha deteriorated very fast and could not be sampled after six days while the firm variety L-49 could be stored upto eight days.

There was a progressive increase in PLW and TSS content of guava fruits with increasing storage period while fruit firmness and titratable acidity exhibited continuous decrease throughout storage. The soft variety had higher PLW, TSS but lower fruit firmness and titratable acidity than the firm one at all the storage periods. $CaCl_2$ and GA_3 treatments decreased PLW, TSS and retained fruit firmness and acidity significantly during storage. PME activity increased during early storage but later on during storage its activity decreased while PG and cellulase exhibited continuous increase in their activities throughout storage. The soft variety (Hisar Surkha) had higher activities of the cell wall degrading enzymes than the firm one at all the storage periods. GA_3 and $CaCl_2$ treatments decreased cell wall degrading enzyme activity.

Superoxide radicals, H_2O_2 content and LOX activity increased significantly during storage while the activities of ROS scavenging enzymes were invariably low at the later stages during storage. A gradual loss in the activity of ROS scavenging enzymes seems to be the major cause of membrane deterioration during storage. GA_3 and $CaCl_2$ in general decreased the activity of LOX and increased the activities of ROS scavenging enzymes suggesting that these treatments have the ability to decrease lipid peroxidation and scavenge free radical. ROS scavenging metabolite, ascorbic acid increased upto 2^{nd} day of storage and thereafter decreased in both the guava cultivars. However, antioxidative activity, glutathione and β-carotene content decreased continuously throughout storage period. The antioxidative metabolites were more in L-49 while antioxidative activity was higher in Hisar Surkha throughout storage. The treatment of GA_3 and $CaCl_2$ significantly increased antioxidative activity, ascorbate and β-carotene content but no significant effect on glutathione was observed during storage. Similarly ethylene evolution and the activity of its biosynthetic

enzyme ACC oxidase increased during storage but later during storage their values declined. $CaCl_2$ however, had no significant effect on ACC oxidase activity while ethylene evolution was decreased slightly by higher concentrations of $CaCl_2$ whereas GA_3 was non-inhibitory.

Based on the results obtained in the present investigations, it can be concluded that in both the cultivars of guava fruits, among all the treatments of GA_3 and $CaCl_2$, 1% $CaCl_2$ was most effective treatment in delaying the softening process during ripening, hence can be used for enhancing the shelf life of guava fruits.

Polygalacturonase was purified from fruits of L-49 to 88 fold with 35 per cent recovery employing the conventional techniques of protein purification such as $(NH_4)_2SO_4$ fractionation, gel filtration through Sephadex G-100 and ion exchange chromatography on CM cellulose. On Native-PAGE, the final enzyme preparation gave one major band indicating that the enzyme has been purified to near homogeneity. Molecular weight of the enzyme protein as determined by gel filtration was 80 kDa. On SDS-PAGE, the enzyme gave a single band of 40 kDa indicating the enzyme to be a homodimer. The enzyme showed Michaelis Menten kinetics and gave a typical hyperbolic response with increasing concentrations of polygalacturonic acid. The Km value as determined by double reciprocal plot was found to be 0.14 %. The purified enzyme was found to be thermostable as it retained about 95 to 100 per cent activity when preheated at 40ºC for 45 min. K^+, Na^+ and Fe^{3+} ions were found to be stimulatory and the maximum enzyme activity was observed at 5 mM of K^+ and and at 10 mM of Fe^{3+}. However, divalent ions Ca^{2+}, Zn^{2+} and Mg^{2+} had inhibitory effect.

Various low molecular weight polyols were found to be inhibitory to varying degrees of effectiveness and the inhibitory effect was concentration dependent. The metal binding reagent EDTA can inactivate enzyme either by removing the metal ions from the enzyme forming coordination complex, or by binding inside enzyme as a ligand. All reducing agents namely β-ME, DTT and cysteine activated PG, DTT being the most potent while β-ME least amongst these. Enhanced activity of the enzymes in presence of these agents suggests that the reduction of thiol groups improves their performances that lends support to the need of sulfhydryl groups for the functioning of the enzyme.

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Appendix I: Changes in firmness, total soluble solids, titrable acidity in guava fruit during ripening

Stages	Firmness (kg/cm^2)		Total soluble solids (°Brix)		Titrable Acidity (%)	
	L-49	HS	L-49	HS	L-49	HS
IG	15.31±0.26	11.48±0.15	8.85±0.09	11.25±0.03	0.436±0.00	0.424±0.01
MG	14.54±0.18	10.55±0.09	9.75±0.03	12.80±0.12	0.503±0.01	0.442±0.04
T	10.94±0.33	7.67±0.13	10.40±0.06	13.45±0.14	0.432±0.01	0.397±0.00
R	8.40±0.17	5.09±0.24	11.92±0.07	14.63±0.12	0.392±0.01	0.288±0.02
OR	5.91±0.27	3.91±0.06	11.10±0.06	13.85±0.20	0.329±0.01	0.216±0.01
CD (P ≤0.05)	a: 0.271 b: 0.429 (axb): 0.607		a: 0.137 b: 0.217 (axb): 0.307		a: 0.012 b: 0.020 (axb): 0.028	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix II: Changes in total sugars, reducing sugars and non reducing sugars in guava fruits during ripening

Stages	Total Sugars (per cent dry wt.)		Reducing Sugars (per cent dry wt.)		Non reducing Sugars (per cent dry wt.)	
	L-49	HS	L-49	HS	L-49	HS
IG	11.86±0.28	14.52±0.19	7.03±0.19	9.42±0.37	4.83±0.09	5.09±0.56
MG	14.52±0.18	19.76±0.07	8.37±0.08	12.06±0.93	6.15±0.26	7.70±0.86
T	18.02±0.22	22.65±0.08	9.38±0.02	13.43±0.29	8.64±0.20	9.22±0.37
R	23.9±0.01	27.25±0.03	12.36±0.06	14.02±0.06	11.54±0.06	13.23±0.03
OR	22.41±0.19	26.52±0.06	11.68±0.07	13.76±0.50	10.73±0.25	12.76±0.01
CD (P ≤0.05)	a: 0.207 b: 0.327 (axb): 0.463		a: 0.449 b: 0.711 (axb): 1.01		a: 0.492 b: 0.777 (axb): 1.10	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix III: Cell wall components (per cent dry weight) in guava fruits at different stages of ripening

Stages	NDF		ADF		Hemicellulose		Cellulose		Lignin		Silica		Pectin	
	L-49	HS	L-49	HS	L-49	HS	L-49	HS	L-49	HS	L-49	HS	L-49	HS
IG	48.75± 0.89	43.30± 0.12	41.4± 1.09	36.70± 0.06	7.35± 0.20	6.55± 0.09	26.75± 0.55	24.95± 0.26	11.90± 0.35	10.85± 0.03	0.56± 0.01	0.45± 0.03	2.83± 0.02	2.12± 0.04
MG	52.90± 0.40	46.50± 0.23	44.35± 0.32	38.40± 0.29	8.55± 0.09	8.10± 0.06	28.55± 0.89	27.20± 0.46	15.26± 0.03	13.18± 0.32	0.67± 0.03	0.57± 0.02	3.19± 0.11	2.57± 0.03
T	48.20± 0.17	38.65± 2.45	41.80± 0.06	33.40± 2.19	6.40± 0.12	5.25± 0.26	26.05± 0.09	23.45± 0.26	13.28± 0.10	12.55± 0.68	0.51± 0.01	0.48± 0.00	2.68± 0.03	2.05± 0.07
R	42.25± 0.55	35.30± 0.23	37.65± 0.61	31.85± 0.14	4.60± 0.06	3.45± 0.09	23.00± 0.29	19.35± 0.20	13.09± 0.06	12.18± 0.072	0.44± 0.02	0.33± 0.01	2.21± 0.01	1.76± 0.06
OR	34.50± 0.23	31.80± 0.29	31.55± 0.20	28.95± 0.38	2.96± 0.03	2.85± 0.09	20.55± 1.01	17.4± 0.58	12.30± 0.16	11.33± 0.14	0.34± 0.02	0.22± 0.01	1.34± 0.05	1.15± 0.04
CD (P ≤0.05)	a: 1.16 b: 1.83 (axb): 2.59		a: 1.094 b: 1.729 (axb): 2.446		a: 0.168 b: 0.265 (axb): 0.375		a: 0.72 b: 1.14 (axb): NS		a: 0.361 b: 0.571 (axb): NS		a: 0.024 b: 0.038 (axb): NS		a: 0.071 b: 0.113 (axb): 0.159	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix IV: Changes in cell wall degrading enzymes in guava fruits during ripening

Stages	PME (units g^{-1} f.wt)		PG (units g^{-1} f.wt)		Cellulase (units g^{-1} f.wt)	
	L-49	HS	L-49	HS	L-49	HS
IG	2.31±0.05	4.12±0.53	1.51±0.03	1.89±0.05	1.14±0.07	1.64±0.02
MG	4.56±0.17	7.96±0.11	2.41±0.04	2.69±0.04	1.82±0.04	2.24±0.05
T	8.92±0.08	15.43±0.22	3.20±0.03	4.07±0.08	2.85±0.04	3.11±0.06
R	6.01±0.05	10.49±0.12	3.71±0.09	4.49±0.07	3.29±0.06	4.08±0.07
OR	4.35±0.02	8.39±0.14	5.64±0.11	6.31±0.03	5.32±0.07	6.14±0.05
CD (P ≤0.05)	a: 0.272 b: 0.430 (axb): 0.608		a: 0.086 b: 0.136 (axb): 0.192		a: 0.076 b: 0.119 (axb): 0.169	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix V: Changes in H_2O_2, superoxide radicals and lipoxygenase activity in guava fruits during ripening

Stages	Lipoxygenase (units g^{-1} f.wt.)		Superoxide radicals (nmols g^{-1} f.wt.)		H_2O_2 (µmol g^{-1} f.wt.)	
	L-49	HS	L-49	HS	L-49	HS
IG	321.66±11.8	425.64±15.79	100.06±1.59	115.74±2.03	339.38±5.96	362.59±7.45
MG	598.29±2.37	873.34±3.43	106.08±2.82	135.24±1.34	424.22±12.18	462.66±7.31
T	665.52±7.32	948.77±6.65	122.83±2.06	163.33±6.41	448.15±1.59	505.79±1.35
R	925.73±5.33	1098.62±12.98	137.62±1.05	188.16±5.46	518.89±1.35	562.97±0.81
OR	1008.36±12.14	1121.52±9.27	163.14±7.68	211.60±2.28	719.07±0.54	756.72±5.33
CD (P ≤0.05)	a: 12.84 b: 20.31 (axb): 28.72		a: 5.26 b: 8.32 (axb): 11.76		a: 7.61 b: 12.03 (axb): 11.76	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix VI: Activities of ROS scavenging enzymes in guava fruits at different stages of ripening

Stages	SOD (units g^{-1} f.wt.)		CAT (units g^{-1} f.wt.)		APX (units g^{-1} f.wt.)		GR (units g^{-1} f.wt.)		POX (units g^{-1} f.wt.)	
	L-49	**HS**	**L-49**	**HS**	**L-49**	**HS**	**L-49**	**HS**	**L-49**	**HS**
IG	140.78± 0.06	127.45± 0.53	421.26± 8.87	391.98± 12.9	596.23± 4.78	451.06± 3.05	3.98± 0.17	2.13± 0.18	109.50± 0.29	84.72± 2.98
MG	162.23± 2.01	149.78± 1.95	775.95± 21.16	601.6± 39.03	813.40± 5.61	572.29± 7.66	6.41± 0.18	4.09± 0.20	128.38± 1.15	110.60± 5.49
T	183.85± 0.82	170.69± 2.88	1281.55± 30.01	1101.95± 11.40	1091.01± 48.72	1012.62± 0.007	5.51± 0.00	3.37± 0.17	154.25± 0.94	144.85± 2.32
R	172.85± 1.65	161.58± 1.63	1043.28± 23.28	927.30± 53.35	1000.57± 34.8	855.91± 48.72	4.59± 0.18	2.45± 0.35	115.63± 0.29	100.75± 6.28
OR	143.59± 1.62	135.13± 0.00	975.05± 29.42	710.64± 16.56	675.08± 13.92	578.64± 27.84	1.84± 0.02	1.15± 0.04	96.04± 2.91	74.94± 8.62
CD ($P \leq 0.05$)	a: 2.103 b: 3.325 (axb): N.S		a: 46.10 b: 32.89 (axb): N.S		a: 34.18 b: 54.04 (axb): 76.43		a: 0.240 b: 0.379 (axb): 0.537		a: 5.46 b: 8.64 (axb): NS	

Two factorial analysis of variance ($P \leq 0.05$) a= varieties; b= stages

Appendix VII: Ascorbic acid content, antioxidant activity, β-Carotene and glutathione content in guava fruits at different stages of ripening

Stages	Ascorbic acid (mg/100g f.wt.)		Glutathione (µmols g^{-1} f.wt)		β-Carotene content (µg/100g f.wt.)		Antioxidant Activity (%)	
	L-49	HS	L-49	HS	L-49	HS	L-49	HS
IG	42.56±1.29	54.88±0.65	263.57±3.52	203.60±6.32	466.19±6.74	409.39±1.88	46.88±0.014	69.10±0.23
MG	108.64±5.82	146.72±1.94	512.70±2.84	460.67±2.66	393.53±6.08	352.23±6.89	47.27±0.09	76.65±0.26
T	161.31±1.94	198.24±1.94	426.37±0.98	349.05 ±1.56	301.68±6.59	222.19±1.29	39.13±0.42	61.70±0.29
R	142.24±5.82	167.68±1.48	315.76±3.35	263.77±1.38	243.26±2.81	124.35±3.12	34.23±1.18	51.55±0.14
OR	94.08±1.29	114.24±3.88	228.63±3.41	174.96±5.68	184.71±6.96	103.93±3.90	31.78±0.48	41.75±1.53
CD (P ≤0.05)	a: 4.43 b: 7.00 (axb): 9.90		a: 4.75 b: 7.51 (axb): 10.62		a: 6.77 b: 10.71 (axb): 15.14		a: 0.88 b: 1.34 (axb): 1.97	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

Appendix VIII: Changes in ethylene and ACC oxidase activity in guava fruits during ripening

Stages	Ethylene (nmol h^{-1} g^{-1} f.wt.)		ACC Oxidase (units g^{-1} f.wt)	
	L-49	HS	L-49	HS
IG	5.93±0.19	7.58±0.41	13.97±0.32	19.48±0.52
MG	9.26±0.18	11.32±0.48	24.50±3.50	34.60±0.40
T	25.02±0.61	32.96±2.42	50.45±1.85	67.10±4.10
R	13.53±0.68	21.80±2.32	41.75±0.75	48.41±1.11
OR	8.49±0.39	12.56±0.41	31.89±0.11	37.20±4.50
CD (P ≤0.05)	a: 0.869 b: 1.374 (axb): 1.943		a: 1.800 b: 2.846 (axb): 4.025	

Two factorial analysis of variance (P ≤0.05) a= varieties; b= stages

ABSTRACT

Title of Thesis	:	Role of Gibberellic Acid and Calcium Chloride in Ripening Related Biochemical Changes in Guava (*Psidium guajava* L.) Fruit
Full Name of the Degree Holder	:	**REENA DEVI**
Admission Number	:	2011BS01D
Title of the Degree	:	**Doctor of Philosophy**
Name of Discipline	:	Biochemistry
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During present investigations, guava fruits of cultivars L-49 and Hisar Surkha, harvested at IG, MG, T, R and OR stage were analyzed for various physico-chemical and biochemical parameters. TSS and sugars (total, reducing and non reducing) increased while fruit firmness and acidity decreased during ripening. Content of cell wall components *viz.* NDF, ADF, hemicellulose, cellulose, lignin, pectin and silica was maximum at MG stage and decreased thereafter. PME was maximally active at turning stage while PG and cellulase increased continuously. ROS indices *viz.* LOX activity, superoxide radical and H_2O_2 content increased progressively while the activities of ROS scavenging enzymes *viz.* SOD, CAT, POX, APX and GR and the content of ROS scavenging metabolites *viz.* ascorbate and glutathione increased initially but later on during ripening, a significant decline was observed. However, β-carotene content decreased throughout ripening. Ethylene evolution and ACC oxidase activity were maximum at turning stage. Pre-treatment of fruits with GA_3 and $CaCl_2$ caused significant inhibition of cell wall degrading enzymes, activation of ROS scavenging enzymes and reduction in ethylene evolution during storage. ACC oxidase and glutathione content were however, not affected by treatments. PG, purified 88 fold with 35 per cent recovery, was thermostable and a homodimer with Mr of 80 kDa. It exhibited Michaelis Menten kinetics with Km value of 0.14%. K^+, Na^+ and Fe^{3+} ions were found to be stimulatory. However, divalent ions Ca^{2+}, Zn^{2+} and Mg^{2+} had inhibitory effect. The metal binding reagent EDTA and various low molecular weight polyols were found to be inhibitory. All reducing agents namely β-ME, DTT and cysteine activated PG.

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List of publications:

1. **Reena Devi** and Veena Jain (2015). Compositional and hydrolytic enzymatic changes in guava (*Psidium guajava* L.) fruit during ripening. In *National Symposium on Germplasm to Genes: Harnessing Biotechnology for Food Security and Health*, being held on August 9-11, 2015 at National Agricultural Science Center, Pusa, New Delhi, India
2. **Reena Devi** and Veena Jain (2014). Ripening related changes in antioxidative enzymatic system of guava (*Psidium guajava* L.) fruit. In *National Seminar on "Reorientation of Agricultural Research to Ensure National Food Security***"** being held on January 6-7, 2014 at Chaudhary Charan Singh, Haryana Agricultural University, Hisar.

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Reena Devi