I. INTRODUCTION

Fruits represent the commercially valuable and nutritionally indispensable food commodity. They are edible seed vessels or receptacles developed from a mature fertilized ovary. They are highly specialized organs in higher plants offering a great variety of aesthetic qualities with their complex or delicate aroma, pleasant taste, exotic colours, succulence, flavour and texture. They play a vital role in human nutrition, by supplying the necessary growth factors essential for maintaining normal health. Nutritionally, they are known for their high energy, roughage value, minerals, vitamins (B-complex, C and K), β-carotene (pro-vitamin A) and phenolics (antioxidants) (Prasanna et al., 2007). Based on their respiratory pattern and ethylene biosynthesis during ripening, harvested fruits can be further classified as climacteric and non-climacteric type. Climacteric fruits, harvested at full maturity, can be ripened off the parent plant. The respiration rate and ethylene formation though minimal at maturity, raise dramatically to a climacteric peak, at the onset of ripening, after which it declines. Non-climacteric fruits are not capable of continuing their ripening process, once they are detached from the parent plant. These fruits produce a very small quantity of endogenous ethylene, and do not respond to external ethylene treatment. They show a comparatively low profile and a gradual decline in their respiration pattern and ethylene production, throughout the ripening process (Gamage and Rehman, 1999).
1.1 Fruit ripening

Fruit ripening is a highly co-ordinated, genetically programmed and an irreversible phenomenon involving a series of physiological, biochemical and organoleptic changes that lead to the development of a soft edible ripe fruit with desirable quality attributes. It includes a large number of biochemical changes such as increased respiration, biosynthesis of carotenoids, anthocyanins, essential oils and flavor and aroma components, increased activity of cell wall degrading enzymes and increase in ethylene production (Brady, 1987).

The colour change during fruit ripening is due to the degradation of chlorophyll and dismantling of photosynthetic apparatus and synthesis of different types of anthocyanins and their accumulation in vacuoles, and accumulation of carotenoids such as β-carotenes, xanthophylls esters, xanthophylls and lycopene (Tucker and Grierson, 1987; Lizada, 1993). The increase in flavor and aroma during the ripening is mainly due to the production of volatile compounds such as ocimene and myrcene (Lizada, 1993). The sweetness in ripened fruits is the result of increased gluconeogenesis, hydrolysis of polysaccharides, especially starch and, decreased acidity and accumulation of sugars and organic acids resulting in an excellent sugar/acid blend (Lizada, 1993; Grierson et al., 1981). Metabolic changes during fruit ripening include increase in biosynthesis and evolution of the ripening hormone, ethylene (Yang and Hoffman, 1984).
1.2 Textural changes during ripening

Textural change is the major event in fruit softening and is the integral part of ripening. Fruit texture is influenced by various factors like structural integrity of the primary cell wall and the middle lamella, accumulation of storage polysaccharides and the turgor pressure generated within the cells by osmosis (Jackman and Stanley, 1995). Change in turgor pressure, and degradation of cell wall polysaccharides and starch determine the extent of fruit softening (Brady, 1987; Tucker and Grierson, 1987). Starch is the bulk polysaccharide present in some fruits (mango and banana), and its enzymatic hydrolysis results in pronounced loosening of cell structure and sweetness development. The process of textural softening is of commercial importance as it directly dictates the fruit shelf life and quality (Tucker, 1993). This should be considered to avoid mechanical damage during harvesting and transportation. The textural properties of fruit play a very significant role in the consumer acceptability. The textural qualities of the fruits are attributed to its inherent cell wall composition.

1.3 Plant cell wall

All plant cells are surrounded by a cell wall consisting of polysaccharides as the most abundant component, proteins and sometimes lignin. The cell walls have several very important functions in the plant: they allow the plant to stand up right, they give the cells form, they mediate interactions between cells and they provide a barrier against attack from herbivores and disease agents. The cell wall
consists of polymers that are deposited outside the cell but they are not a dead and inert substance. In contrast, the walls are plastic and polysaccharides are turned over and reformed continuously in the primary wall (Gibeaut and Carpita, 1991; Inouhe et al., 1997). Hence, cells are able to expand their length to more than hundred times the initial length although they are encased in a wall of strong cellulose microfibrils and matrix polysaccharides.

The polysaccharides of cell wall are usually divided into cellulose, hemicelluloses and pectin. Polysaccharides from different sources vary in their chemical, biological, physico-chemical, structural and functional characteristics. They are capable of regulating gene expression and host–defense mechanism by the generation of elicitor-active oligogalacturonide fragments from the cell wall (Ridley et al., 2001). The primary cell wall is a highly complex structure because of this it would be likely that a concentrated effort of a variety of enzymes might be required to effect cell wall carbohydrate disassembly and fruit softening. In this regard two major group of wall degrading enzymes can be identified namely pectinases and hemicellulases. The enzyme pectinase may comprise various isoforms of polygalacturonase, pectin esterase, β-galactosidase etc. The hemicellulase may consists of enzymes such as endo-(1,4) β-glucanases, xyloglucanases and xyloglucan endotransglycosylases. Numerous studies have done on this enzymes but their precise role in fruit ripening remain unclear. Conceivably all fruits contain the respective enzyme classes but the relative
abundance and types of isoforms present, and the timing of expression of relevant genes or gene families during ripening may vary with the species or cultivars.

### 1.4 Pectins

Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactopyranosyluronic acid (Galp A) residues. These are characterized by relatively high extractability using acid or chelators and a high content of galactosyluronic acid. Hemicelluloses and pectins together constitute the matrix in which cellulose microfibrils are embedded. The interactions between different polysaccharides ensure the strong yet dynamic and flexible properties of the cell wall (Fig.1).

![Diagram of Pectin Network](cse.naro.affrc.go.jp)
Various pectic polysaccharides can be detected in the cell wall including homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan 1 (RG1) and rhamnogalacturonan II (RGII). The ratio between HG, XGA, RG1 and RG II is also variable, but typically HG is most abundant polysaccharide constituting about 65% of the pectin, while RG 1 constitutes 20% - 35% (Mohnen, 2008). XGA and RG II are minor components, each constituting less than 10% (Zandleven et al., 2007; Mohnen, 2008). The different pectic polysaccharides are not separate molecules but covalently linked domains.

Homogalacturonan (HG) is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Galp A) residues in which some of the carboxyl groups are methyl esterified. Depending on the plant source, HGs may be partially O-acetylated at C-3 or C-2. Xylogalacturonan (XGA), contain β-D-xylosyl residues attached to C-3 of the back bone (Kikuchi et al., 1996) are present in the walls of reproductive plant tissues (e.g. apple, carrot, cotton and pine). Apiogalacturonans are present in the walls of some aquatic monocotyledons (e.g. Lemna and Zostera), contain β-D-apiofuranosyl (Apif) residues attached to C-2 and C-3 of the back bone either as a single Apif residue or as the disaccharide β-D-Apif-(1\rightarrow3') – β-D-Apif – (1\rightarrow) (Cheng and Kindel, 1997).

Rhamnogalacturonan 1 (RG1) is a family of pectic polysaccharides that contain a back bone of the repeating disaccharide [\rightarrow4) –α-D-Galp A – (1\rightarrow2) –α-L –Rhap – (1\rightarrow]. The rhamnosyl (Rhap) residues can carry side chains of neutral
and acidic sugars positioned at the C-4. The degree of substitution of RG 1 varies depending on plant species, tissue type and extraction method. Typically between 20-80% of the Rha residues are substituted at the C-4 (O’Neill et al., 1990). The Gal A residues can be acetylated at the C-2 and/or at the C-3 (Ishii, 1997a). There is no clear evidence for methylation of the Gal A residues in the RG 1 backbone as no pure RG 1 with methylated Gal A residues has been isolated, though RG 1 enriched cell wall fraction from flax contained methyl-ester (Rihouey et al., 1995). The GalA in RG 1 can furthermore have β-D-Glc A attached to the C-3 (Renard et al., 1999).

Rhamnogalacturonan II (RG II) is composed of 12 different sugars including the rare 3-deoxy-D-manno-octulosinic acid, 3-deoxy-D-lyxo-2-heptulosaric acid and Apiose. RG II consists of four chains named A-D residing on a GalA back bone resembling the HG. This back bone is at least eight residues long (Whitcombe et al., 1995).

1.5 Functions of pectins

The primary role of cell wall is to give physical strength to the plant and to provide a barrier against the outside environment. The main role of pectin is to participate in these two functions together with the other polymers. Especially HG and RG II are well known to be involved in strengthening the wall. The mechanical properties of HG and RG II have been reviewed in earlier reports (Ryden et al., 2003; Caffall and Mohnen, 2009). Plant pathogens cause degradation of pectin,
and oligogalacturonides (α-1,4- linked oligomers of Gal U A) are well established to be part of a signaling cascade that senses wall degradation upon pathogen attack (Ridley et al., 2001; Kohorn et al., 2009).

1.6 Pectin degrading enzymes

Pectolytic enzymes are found in plants, fungi and bacteria. These enzymes are responsible for the degradation of pectin and pectic substances in plant cell walls. They act on plant tissues, especially on the main polyuronide chains of pectins and eventually cause cell lysis. Pectin degrading enzymes are classified, based on their mode of action on pectin and pectic substances into Polygalacturonase (PG), Pectin Methylesterase (PME) and Pectate Lyase (PL) (Chauhan et al., 2001; Wong, 1995).

1.6.1 Polygalacturonase (PG)

PG is the primary enzyme playing a key role in pectin dissolution. This hydrolytic enzyme, acts on pectic acid (Polygalacturonic acid, PGA) and hydrolyses the α-1,4- glycosidic bonds between the galacturonic acid residues in galacturonans. PGs are classified into exo-PG (exo-poly (1,4-α-D-galacturonide) galacturonohydrolase, EC 3.2.1.67) and endo-PG (endo-poly (1,4-α-D galacturonide) glycanohydrolase, EC 3.2.1.15). The former catalyses the hydrolysis of the glycosidic bonds between the de-esterified galacturonans from the non-reducing end, which results in the release of galacturonic acid as the major reaction product. The rate of hydrolysis depends on the degree of polymerization.
and it increases with increase in the molecular size of the substrate and it interrupts at the branching point (Pressey and Avants, 1975). Exo-PG action causes a large increase in the formation of reducing groups and show decrease in viscosity. The exo-PG does not remove the terminal galacturonic acid residue from the long polygalacturonan chain and hence it has not much effect on pectin solubility (Pressey and Avants, 1978). Thus this enzyme is not involved in ripening, as pectate degradation does not occur. Exo-PG in tomato was found to elicit ethylene production, which in turn trigger the ripening process (Baldwin and Pressey, 1990). But endo-PG depolymerises pectic acid randomly, resulting in a rapid decrease in viscosity and therefore an involvement in the ripening process. The rate of hydrolysis decreases with decrease in the length of the chain. Fruits like apple, freestone peach and persimmon possess only exo-PG, while other fruits such as avocado, clingstone peach, lemon, mango and kiwi contain only endo-PG (Lang and Dornenburg, 2000). Cucumber, papaya, passion fruit, peach, pear, strawberry and tomato contain both exo- and endo- PGs (Lang and Dornenburg, 2000). The extent and rate of textural softening during ripening is directly related to PG composition. There is extensive softening occurs if endo- or both endo- and exo-PG are present and softening is minimum if only exo-PG is present (Bartley, 1978; Huber, 1984). There is a correlation between the appearance of PG and dissolution of middle lamella and primary cell wall during ripening (Crookes and Grierson, 1983). PG is the major enzyme responsible for dissolution of middle lamella during fruit ripening (Jackman and Stanley, 1995; Voragen et al., 1995).
1.6.2 Pectin methylesterase (PME)

Pectin methylesterase (EC 3.1.1.11), de-esterifies polyuronides by removing methyl groups from the C-6 position of galacturonic acid residues of high molecular weight pectin. Demethylation of pectin to their free carboxyl groups changes the pH and charge in the cell wall, allows the aggregation of polyuronides into a calcium–linked gel structure, and makes the polyuronides susceptible to degradation by PG (Pressey and Avants, 1982; Carpita and Gibeaut, 1993). PME activity is a key control point for both the assembly and disassembly of the pectic network. The action of PMEs can promote the formation of supra molecular pectin gels. However, the degree and pattern of methyl esterification is very important in regulating the cleavage of homogalacturonan (HG) by pectinolytic enzymes. In addition to PME action regulating the occurrence of de-esterified HG regions that can be cross-linked by calcium, the occurrence of such sites can be regulated by enzymes that can cleave the HG backbone. These enzymes have the capacity to degrade potential sites of HG chain interaction and also have the capacity to generate oligosaccharide fragments that have physiological functions (Dumville and Fry, 2000).

In tomato, PME is found as a small gene family representing at least four genes, some of which are highly homologous (Ray et al., 1988; Harriman et al., 1991; Gaffe et al., 1997). Three of the genes are present in the genome as a tandem repeat (Hall et al., 1994; Turner et al., 1996). PME protein is present in most tissues of the plant and exists in multiple isoforms (Pressey and Avants, 1972;
Tucker et al., 1982; Warrilow et al., 1994) and these enzymes are synthesized as pre proteins of 540-580 amino acids (Gaffe et al., 1997). The activity of PME increases as mature green tomatoes pass through different colour stages to become full red. Ripe fruits are rich in hydrolase enzymes, while unripe fruits are enriched with PME. The activity of PME was found to decrease (Prabha et al., 2000; Abu-Sarra and Abu-Goukh, 1992), or increase (Selvaraj and Kumar, 1989) or remain constant (Ashraf et al., 1981) during fruit ripening. Several PME isoforms have been identified in tomato (Tucker et al., 1982). Antisense suppression of PME mRNA abundance and activity by 90% in tomato (Gaffe et al., 1994; Tieman et al., 1992) has little effect on fruit firmness.

1.6.3 Pectate Lyase (PL)

Pectate lyases catalyse the cleavage of de-esterified or esterified galacturionate units by a trans β-elimination of hydrogen from the C-4 and C-5 positions of galacturonic acid. Exo-PL (exo-poly1,4 α-galacturonide lyase, EC 4.2.2.9) acts from the non-reducing end, where as Endo-PL (endo–poly 1,4 α-D galacturonide lyase, EC 4.2.2.2) acts randomly on de-esterified galacturonans (Prasanna et al., 2007).

1.6.4 Cellulase

Cellulase hydrolyzes the β (1-4) link between adjacent glucose residues. These enzymes are unable to degrade the native galactans and its natural substrate remains unknown. These enzymes can be considered as true β-galactanases and
could be implicated in wall degradation. Cellulase often referred to as EGase (Endo (1→4) β-D glucanase) hydrolyzes internal linkages of (1→4) β-D-linked glucan chains. EGase activity has been found in fruits of all species (Brummell et al., 1994). EGases Cel 1 and Cel 2 showed increase in mRNA coincident with ripening (Lashbrook et al., 1994). β-1,4-glucanases (EC 3.2.1.4) are highly expressed in fruits and play major role in fruit ripening of strawberry (Harpster et al., 1998; Llop-Tous et al., 1999) and peach (Trainotti et al., 1997).

### 1.6.5 Xylanase

Xyloglucans, heteroxylans and xylans are structural components of primary and secondary cell walls of angiosperms and endoxylanase activity was described in several plants (Miller et al., 1987; Paull and Chen, 1983). Xylanaes (EC 3.2.1.8) catalyze the hydrolysis of β-1,4-xylan. β-1,4-D-endo-xylanase and β-1,4-D-exo-xylanase are reported as cell wall degrading enzymes from fruits including banana (Prabha and Bhagyalakshmi, 1998).

### 1.6.6 Invertase

Invertase (EC 3.2.1.26) catalyzes the hydrolysis of sucrose into hexoses (glucose + fructose). Major enzymes playing a role in the synthesis of sucrose of fruits during ripening are sucrose phosphate synthase (SPS, EC 2.4.1.14) and sucrose synthase (SuSy, EC 2.4.1.13) (Fils-Lycaon et al., 2011).
1.7 Ethylene and fruit ripening

Ethylene plays an important role in many aspects of plant growth, development and senescence. Endogenous production of ethylene increases during certain stages of growth and development, such as seed germination, fruit ripening and leaf and flower senescence and abscission and also in response to drought, flooding, physical wounding, pathogen infection and chemical inducers (Yang and Hoffmann, 1984). Fruits are classified based on their pattern of respiration and ethylene production during maturation and ripening (Biale and Young, 1981). Fruits with enhanced ethylene production and increased respiration rate at the onset of ripening are referred to climacteric and include tomato, apple, peach and banana, whereas fruits do not produce elevated levels of ethylene are known as non climacteric and include citrus, grape and strawberry. Two systems of ethylene production have been identified in plants. System 1 functions during normal growth and development and during stress responses; whereas system 2 operates during floral senescence and fruit ripening. System1 is autoinhibitory, such that exogenous ethylene inhibits synthesis and inhibitors of ethylene action can stimulate ethylene production. In contrast, system 2 is stimulated by ethylene and is therefore autocatalytic, and inhibitors of ethylene action inhibit ethylene production (Barry and Giovannoni, 2007). The ethylene biosynthesis pathway has now been completely elucidated due to the advanced techniques of biochemical analysis (Yang and Hoffmann, 1984; Kende, 1993).
The first step of this metabolic pathway involves the conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), catalyzed by the enzyme ACC synthase (ACCS). The second step catalysed by ACC oxidase (ACCO), consists in the conversion of ACC to ethylene, CO\textsubscript{2} and HCN. Alternatively, ACC can be malonylated, producing N-malonyl transferase (NMT), reducing substrate availability for ACCO. In some specific cases, ethylene regulates its own production (autocatalytic biosynthesis) inducing de novo synthesis of ACCS and ACCO (Yang and Hoffmann, 1984). The genes encoding these two enzymes belong to multigene families and are cloned and characterized in several plant species (Kende, 1993). In tomato plant, at least six genes encode ACC synthase (Lincoln et al., 1993; Olson et al., 1991; Rottmann et al., 1991); ACC oxidase is encoded by three (Barry et al., 1996). These genes have been isolated and structurally characterized with different expressions in various tissues at different stages of development and in response to specific stimuli which induce ethylene biosynthesis.

Fig. 2 Ethylene Biosynthesis and Fruit Ripening Process (Bapat et al., 2010)
1.8 Ethylene receptors and signaling pathways in fruit ripening

During climacteric fruit ripening, the burst of autocatalytic ethylene co-ordinates and accelerates the ripening process. The exact mechanism of ethylene signal transduction are not yet fully understood, however the seedlings that show disruption of normal triple response phenotype have given valuable insights into ethylene perception and signaling.

Ethylene receptors have homology to bacterial two component receptors, which consist of a sensor protein and a separate response regulator protein that function together, allowing bacteria to respond to different environmental conditions (Chang and Stewart, 1998). All ethylene receptors have a sensor domain that can be subdivided into a transmembrane domain and a GAF domain (found in cGMP phosphodiesterases, adenylate cyclases and Fh 1 a transcription factors), a histidine kinase domain and a response domain. The GAF domain binds cyclic nucleotides in a number of bacterial proteins, and the chromophore in the plant photoreceptor phytochrome (Aravind and Ponting, 1997), however the function of this domain in ethylene receptor is unknown. The binding of ethylene to the receptor is mediated by a copper co-factor (Rodriguez et al., 1999).

CTR1 is a protein kinase with homology to the Raf family of mitogen activated protein kinase (MAP3K) and it is identified as the next component of the signal transduction pathway (Kieber et al., 1993). The N-terminus of CTR1 interacts with two ethylene receptors, ETR1 and ERS1 (Clark et al., 1998) and the
current model of ethylene action suggest that, in the absence of the hormone, receptors signal to the negative regulator CTR1 and the response pathway is blocked. Binding of ethylene by the receptors releases the negative regulator allowing ethylene responses to occur (Bleecker et al., 1998). Ethylene promotes MAPK activity suggesting that both negative and positive ethylene signal transduction may be mediated through MAP kinase cascades (Novikova et al., 2000).

Ethylene has a major role in fleshy fruit ripening (Bapat et al., 2010) (Fig.2) and this ripening leads to change in texture, aroma and colour of the fruit. Post harvest lives of the fruits determine the quality of each fruit and is varied according to the type of fruit, softening process, perishability etc.

1.9 Relevance of the work

Bananas are the most valuable fruits in terms of international trade and nutritional quality. These fruits are best sources of fiber, vitamin and minerals. It contain vitamin B6 (pyridoxine), vitamin C and β-carotenes. Bananas are also the rich sources of potassium, an essential mineral for maintaining normal blood pressure and heart function. Since the average banana contains 467 mg of potassium and 1mg of sodium, banana a day may help to prevent high blood pressure and protect against atherosclerosis. These fruits are an exceptionally rich source of fructo oligosaccharide, a compound called a prebiotic because it nourishes probiotic (friendly) bacteria in the colon. These beneficial bacteria
produce vitamins and digestive enzymes that improve our ability to absorb nutrients and compounds that protect us against unfriendly microorganisms. Green bananas contain indigestible (to humans) short chain fatty acids (SCFAs) that are a favorite food of the cells that make up the lining of the intestines. When these cells are well-nourished and healthy, body’s ability to absorb nutrients such as calcium can increase dramatically. Some banana cultivars are also rich in provitamin A carotenoids, which have been shown to protect against chronic disease, including certain cancers, cardiovascular disease and diabetes. Bananas with more golden flesh contain most carotenoids. Bananas also contain high amount of antioxidant phenolic compounds.

India is the largest producer of bananas with 30% of world output, but our exporting potential is very negligible in global market. This is mainly due to the short shelf life of the fruits. Excessive softening is the main factor limiting the fruit shelf life and storage. The post harvest deterioration of fruit crop is determined by the rate of softening of the fruit, which influences shelf life, wastage, infection by pathogens and thus limits transportation and storage. All of this directly affects the economic value of the fruit. Disassembly of the fruit cell wall is largely responsible for softening and textural changes during ripening.

Ripening imparts desirable flavour and colour, but the changes in the fruit firmness enhance its susceptibility to the attack of pathogens and in later stages of ripening it leads to the undesirable texture. These aspects are the major cause for fruit loss in the post harvest period and have a significant commercial and
economical importance. Fruit firmness and texture also affect the integrity of processed fruits. From the customer’s point of view, texture is the principal quality attribute for acceptance in the market. In fleshy fruits, textural quality is generally more important than aromatic properties.

Textural changes in fruits involve the loss in turgor pressure, degradation and other physiological changes in the composition of membranes, degradation of starch and modifications in the cell wall structure and dynamics. The disassembly of the cell wall structural network involves the concerted and synergistic action of several different enzymatic activities, where one family of cell wall modifying enzymes may mediate the activity of another, resulting in ordered cell wall modifications. Cell wall degradation bring about by the action of various cell wall hydrolyzing enzymes like Polygalacturonase (PG), Pectin Methylesterase (PME), Pectate Lyases (PLs) and other cell wall hydrolases. The activity of these enzymes are triggered by the phytohormone ethylene. This gaseous trigger “ethylene” is found in all climacteric fruits including banana and causes the ripening process.

Kerala has a wide source of banana cultivars due to its peculiar agroclimatic nature. All these cultivars are unique in their fruit firmness, fruit colour, taste, size and shape. The present work was carried out in eight cultivars from Kerala, each differ in its nutritional quality, fruit firmness and peel thickness. Protein domains are the 3-dimensional packing of amino acids, which play important role in the activity of proteins. A number of protein domains involved in ripening process in banana and among these various domains glycosyl hydrolase
family play significant role in cell wall metabolism. Glycosyl hydrolase family includes proteins such as polygalacturonase, β-1,3-glucanase, β-galactosidase etc. Information on the exact role of these proteins especially β-1,3-glucanase was very limited. Hence in this study the focus was to understand the role of glycosyl hydrolase enzymes in softening and their biochemical and molecular characterization. The main objectives of the present study were as follows…

1. Biochemical analysis of banana fruits at three stages of fruit ripening (early ripened, ripened and late ripened).
2. Extraction and assay of fruit ripening enzymes (PG, PME, cellulase, xylanase and invertase).
3. Purification and characterization of enzymes belongs to glycosyl hydrolase family from cultivars showing minimum and maximum activities.
4. Expression analysis of putative PG gene in these two cultivars during natural and post ethylene treated ripening.
5. Sequencing and structural modelling of purified proteins.