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# Cell Wall Metabolism of Two Varieties of Ber (*Ziziphus mauritiana* Lam.) Fruit During Ripening

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**Summary**

Two ber varieties differing in their shelf lives (Umran, 8 to 9 and Illaichi, 4 to 5 days) were analyzed for cell wall components, cell wall degrading enzymes and their isoenzyme profile at immature green, mature green, turning colour, ripe and overripe stages of ripening. Cellulose and pectin contents decreased during ripening in both varieties. This decrease was accompanied by a corresponding increase in pectin methylesterase, polygalacturonase and cellulase. The increase in the activity of pectin methylesterase was about 20- and 10--fold, that of polygalacturonase about 8.4- and 5.7-fold, and of cellulase 5.5- and 4.4-fold in Umran and Illaichi, respectively. 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. This was further confirmed by the intensity of isoenzyme bands of these enzymes. The isoenzyme profile also revealed that two isoenzymes of each, pectin methylesterase and polygalacturonase, and one of cellulase could be considered as isoenzymes responsible for the softening of cell wall during ripening and could be potential targets for manipulation to delay softening during ripening.

*Key words*: cellulase, cellulose, isoenzymes, pectin, pectin methylesterase, polygalacturonase

## Introduction

Fruits constitute a commercially important and nutritionally indispensable food commodity. 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. Moreover, developing countries including India lack the efficient system of transportation and storage of these horticultural crops and suffer from the postharvest losses of about 30 % of total production, amounting to a monetary loss of 8880 million US dollars per annum (*1*). Ber, a tropical fruit tree species belonging to the family Rhamnaceae, is very popular among consumers due to its high nutritive value and comparatively lower market price. It is a hard tree, which can thrive well under adverse climatic conditions. However, due to perishable nature and poor shelf life of the fruit, high postharvest losses are the major constraints in developing ber fruit industry. Development of practical solutions to these postharvest 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. During ripening, many biochemical changes including synthesis and degradation of pigments, conversion of starch to sugars, production of volatiles and hydrolysis of cell wall compo-

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nents 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 (*2*). Fruit softening caused by enzymatic hydrolysis of cell wall polysaccharides like cellulose, hemicellulose, pectins and glycoproteins (*3–6*) is characterized by alterations in cellulose microfibrils (*7*), modification of hemicelluloses (*8*) and increased depolymerization of various pectin classes (*9,10*). The cell wall-degrading enzymes such as pectin methylesterase (PME), polygalacturonase (PG) and cellulose belong to multigene families and their multiple forms have been reported in a number of fruits, including lemon (*11*), peach (*12*), kiwi fruit (*13*), marsh white grapefruit (*14*) and sweet cherry (*15*). However, the information on isoenzyme profile of these enzymes during ripening of ber fruit is lacking. We report here the changes in the cell wall components, their hydrolyzing enzymes and isoenzyme profile during ripening of two varieties of ber fruit differing in their shelf lives. Efforts have been made to correlate these changes with the shelf life of the fruit. The information can be exploited to formulate possible strategies to downregulate the appearance of ripening-related isoenzyme and to enhance the shelf life of ber fruit. This can go a long way in avoiding the glut formation during the season.

## Materials and Methods

### *Fruits*

Two varieties of ber (*Ziziphus mauritiana* Lam.) fruit, *viz*. Umran (shelf life 8 to 9 days) and Illaichi (shelf life 4 to 5 days) were harvested from ten-year-old trees at Horticulture Farm, CCS Haryana Agricultural University, Hisar, Haryana, India. The harvested fruits free of visible defects were washed thoroughly in sterilized distilled water and air dried. Based on the visual observations of colour, firmness and liquefaction, the fruits were categorized as immature green (IG), mature green (MG), turning colour (TC), ripe (R) and overripe (OR).

### *Chemicals*

All chemicals and biochemicals were of analytical grade. Trizma® base, D-galacturonic acid, polygalacturonic acid and carboxymethyl cellulose were obtained from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Gel staining dyes (Ruthenium Red, Congo Red and Methylene Blue) were purchased from E. Merck (Mumbai, India), cycloheximide and chloramphenicol from HiMedia Laboratries Pvt. Ltd. (Mumbai, India), and sodium metabisulphite and apple pectin from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

### *Estimation of cell wall components*

#### Cellulose

Cellulose was determined according to the method of Van Soest and Wine (*16*) modified by Pradhan and Bhatia (*17*). Acid detergent fibre (ADF) was first removed by refluxing 1.0 g of sample with 100 mL of ADF extraction reagent containing 0.5 g of ceramide and 2.8 mL of concentrated sulphuric acid for 1 h. Residue left after the removal of ADF was taken in a sintered crucible and washed twice by stirring with 72 % (by mass per volume) $H_2SO_4$. Acid-filled crucible was kept for 3 h and after that the acid was removed. Contents were made acid free by washing with hot water. Crucible was dried overnight at 100 °C and weighed. The loss in mass corresponded to cellulose content.

#### Pectin

Pectin was extracted at (100±2) °C by the method of Rayah Ahmed and Labavitch (*18*). To 1.0 g of ber fruit, 25 mL of 72 % (by mass per volume) $H_2SO_4$ were 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 using the method of Blumenkrantz and Asboe-Hansen (*19*).

Suitable aliquot (0.2 mL) was mixed with 2 mL of borax solution (476 mg of sodium tetraborate in 100 mL of concentrated $H_2SO_4$) and kept for 5 min. After shaking vigorously, it was incubated in boiling water bath for 10 min and cooled. A volume of 20 μL of metahydroxydiphenyl solution (1.5 g/L in 12.5 mM NaOH) was added and the contents were shaken vigorously until pink colour developed. The volume was made to 5 mL with concentrated $H_2SO_4$ and the absorbance was read at 520 nm. The amount of uronic acid was calculated from a calibration curve prepared by using D-galacturonic acid (10–50 μg) as standard.

### *Cell wall-degrading enzymes*

#### Pectin methylesterase

Pectin methylesterase (EC 3.1.1.11) was extracted and assayed by the method of Hagerman and Austin (*20*). Fruit tissue (10 g) was homogenized with 50 mL of chilled 0.1 M Tris-HCl buffer (pH=7.5) containing 100 g/L of NaCl. Homogenate was extracted on ice for 1 h with slow and constant stirring before centrifugation at 10 000×*g* for 30 min. The supernatant represented the enzyme extract. The reaction mixture contained 100 μL of enzyme extract, 2.5 mL of apple pectin concentration of 5 g/L in the buffer (2 mM Tris-HCl, pH=7.5) and 0.4 mL of Bromothymol Blue concentration of 0.1 g/L in the same buffer. The change in the absorbance at 620 nm for 30 min was converted to galacturonic acid from the standard curve (50 to 500 μg) prepared under the same assay conditions. The enzyme activity was expressed as nkat per g of fresh mass of the fruit.

#### Polygalacturonase

Polygalacturonase (EC 3.2.1.15) was extracted according to the method of Singh and Singh (*21*). Fruit sample (1.0 g) was extracted in 0.1 M sodium acetate buffer (pH=5.2) containing 0.02 M sodium metabisulphite and 100 g/L of sodium chloride in a prechilled pestle and mortar. The homogenate was centrifuged at 10 000×*g* for 30 min at 4 °C. The obtained supernatant was dialysed against 0.01 M sodium acetate buffer (pH=5.2) for 4 h by changing the buffer every hour. The enzyme was assayed according to the method of Rayah Ahmed and Labavitch (*18*). The assay mixture (1 mL) contained 0.2 mL of enzyme extract, 0.2 mL of sodium acetate buffer (0.1 M, pH=5.2), 0.5 mL of polygalacturonic acid (3 g/L) and 50 μL of 125 μg of each 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 of Nelson (*22*) as modified by Somogyi (*23*) using galacturonic acid (20–100 μg) as the standard. The enzyme activity was expressed as nkat per g of fresh mass of the fruit.

Cellulase

Cellulase (EC 3.2.1.4) extraction and assay system were the same as for polygalacturonase except that carboxymethyl cellulose sodium salt (5.0 g/L) was used as substrate instead of polygalacturonic acid. The reaction was started by the addition of 0.5 mL of substrate solution and was terminated by heating the tubes in a boiling water bath for 10 min. Reducing sugars were estimated by the method of Nelson (*22*) as modified by Somogyi (*23*) using glucose (20–100 μg) as the standard. The enzyme activity was expressed as nkat per g of fresh mass of the fruit.

### *Isoenzymes*

A volume of 10 mL of each protein sample (extracted either in Tris HCl or in sodium acetate buffer) was filled in scintillation vials and lyophilized for 96 h at –28 °C with vacuum pump temperature of (–78±2) °C. The samples lost more than 90 % moisture by sublimation and became concentrated enough for isoenzyme studies. To 400 μL of lyophilized samples, 15 μL of Bromophenol Blue (5.0 g/L) was added as tracking dye and 85 μL of 40 % (by volume) glycerol. The contents were mixed thoroughly and used for native PAGE. Electrophoresis was carried out on 10 % polyacrylamide gels at a constant current of 10 mA for the first 30 min followed by the current of 20 mA until the tracking dye reached one cm away from the lower end of the gel. Immediately after electrophoretic run, the gasket was removed and the glass plates were separated with the help of a spatula. Gels were stained with specific staining solutions as described below for each enzyme.

Pectin methylesterase

PME isoenzymes were detected by the method of Mwenje and Ride (*24*). Pectin was incorporated at 0.5 % (by mass per volume) into resolving gel. After electrophoresis, first the gel was incubated for 10 min at 5 ºC in 10 mM $CaCl_2$ followed by incubation for 40 min at room temperature in 20 mM Tris-HCl (pH=8.5) containing 10 mM $CaCl_2$. The gel was stained overnight in 0.03 % (by mass per volume) Ruthenium Red. Excess Ruthenium Red was removed using at least three changes of distilled water. Red coloured bands appeared against white background.

Polygalacturonase

PG isoenzymes were detected by the method of Reisfeld *et al.* (*25*). After electrophoresis, gels were equilibrated twice for 15 min in 0.2 M NaCl and 0.1 M sodium acetate buffer (pH=4.0) at 37 °C. The gels were then incubated for 30 to 60 min at 37 °C in 1.0 % (by mass per volume) polygalacturonic acid and 0.2 M NaCl (pH=4.0). The gel was briefly rinsed with water before staining for 10 min in 0.5 % (by mass per volume) Methylene Blue and destaining in water. White coloured bands appeared against blue background.

Cellulase

The protein sample was mixed with protein loading dye and then incubated at 68 °C for 1 h. These samples were loaded onto a 10 % polyacrylamide gel containing 0.1 g/L of carboxymethyl cellulose (CMC) which was then subjected to electrophoresis (*25*). The gel was then gently shaken in 50 mM phosphate buffer (pH=6.8) containing 25 % isopropanol for 30 min. It was transferred to 50 mM phosphate buffer (pH=6.8) and shaken for 30 min. The buffer was removed, and the gel was incubated for 20 min at 37 °C. This gel was stained with 1 % Congo Red solution (10 g/L) for 5 min and destained with 1 M NaCl solution. White coloured bands appeared against brown background.

## Results and Discussion

### *Enzyme and cell wall components*

Cellulose content decreased as the ripening progressed in both cultivars. Maximum cellulose content per dry matter of 196.67 and 158.33 g/kg at IG stage decreased to 94.67 and 82.33 g/kg at OR stage in Umran and Illaichi cultivars, respectively (Fig. 1).

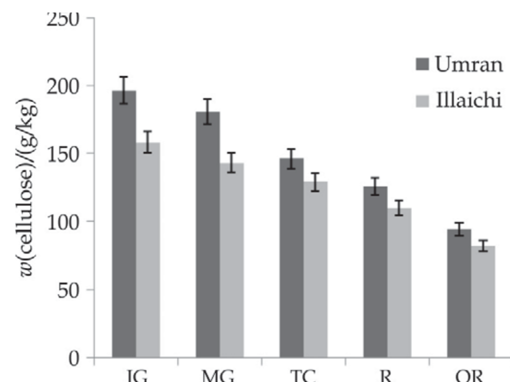


**Fig. 1.** Cellulose content per dry mass of ber fruit during ripening IG=immature green, MG=mature green, TC=turning colour, R=ripe, OR=overripe

Pectin content (Fig. 2) also showed continuous decrease from IG to OR stage. The highest pectin content

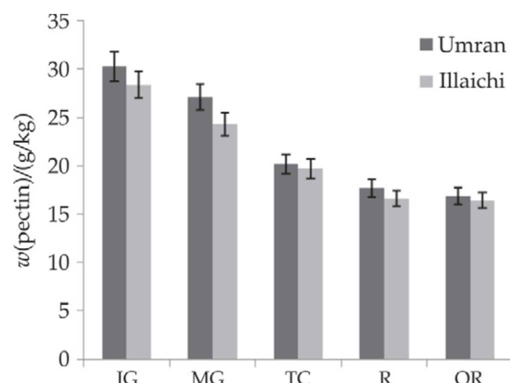


**Fig. 2.** Pectin content per dry mass of ber fruit during ripening IG=immature green, MG=mature green, TC=turning colour, R=ripe, OR=overripe

per dry matter of 30.27 and 28.40 g/kg observed at IG stage reached minimum values of 16.90 and 16.37 g/kg at OR stage in Umran and Illaichi, respectively. As it is clear from the data, there is a higher content of cell wall constituents in long shelf life variety Umran than in short shelf life variety Illaichi at all stages of ripening.

Critical perusal of the data on cell wall components revealed that during ber fruit ripening, these components were sequentially modified and the levels of the wall material decreased with the enhancement of ripening in both cultivars. These changes continued during ripening and overripening, causing the softness of the fruit and loss of tissue structure at OR stage. Undoubtedly, Illaichi had lower content of cell wall constituents at all stages of ripening than Umran. However, the differences were more prominent at early stages of fruit ripening, indicating that the basal level of cell wall constituents was much higher in Umran than in Illaichi. These results very amply demonstrated the degradation of cell wall components during ripening as had been reported earlier in ripening ber (*26*) and guava (*27*) fruits. However, ripening of apple (*28*) and banana (*29*) was not associated with any changes in cellulose content.

Contrary to cell wall components, the activities of cell wall-degrading enzymes increased linearly during ripening, reaching the maximum value at OR stage. Fig. 3 depicts the activity of pectin methylesterase (PME) showing a continuous increase from 3.57 nkat/g at IG stage to 67.13 nkat/g at OR stage in Umran, while in Illaichi, the corresponding values were 7.72 and 74.65. Here again, the basal level of PME was quite different in the two cultivars (Illaichi had almost more than double PME activity than Umran) at IG stage and the difference narrowed down at OR stage. Pectin methylesterase is responsible for the deestrification of pectin before it is depolymerized by polygalaturonase (*30*). Similar observations were made earlier in tomato (*31*), apple (*32*) and guava (*33*). However, the role of PME in fruit softening has been questioned since the absence of softening has been reported in Nor (non-ripening) and Nr (never ripe) mutants of tomato which display PME activity similar to that of normally ripening genotypes (*34*).

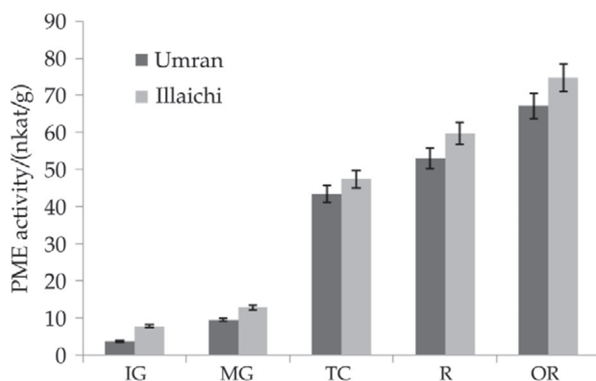


**Fig. 3.** PME activity per fresh mass of ber fruit during ripening IG=immature green, MG=mature green, TC=turning colour, R=ripe, OR=overripe

Similar to PME, polygalacturonase (PG) (Fig. 4) and cellulase (Fig. 5) also exhibited continuous increase throughout ripening, attaining their maximum values at OR stage.

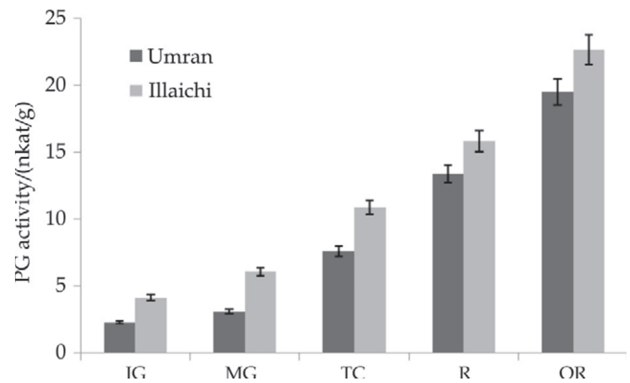


**Fig. 4**. PG activity per fresh mass of ber fruit during ripening IG=immature green, MG=mature green, TC=turning colour, R=ripe, OR=overripe

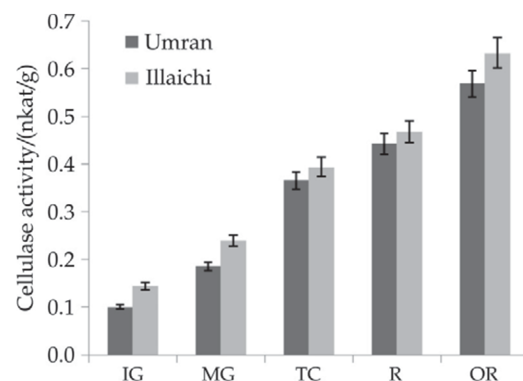


**Fig. 5.** Cellulase activity per fresh mass of ber fruit during ripening IG=immature green, MG=mature green, TC=turning colour, R=ripe, OR=overripe

PG and cellulase activities were very low at IG stage (2.23 and 0.10 nkat/g, and 4.15 and 0.144 nkat/g in Umran and Illaichi, respectively) and enhanced to 7.62 and 0.36 nkat/g, 10.90 and 0.39 nkat/g at TC stage, and 19.48 and 0.57 nkat/g, and 22.67 and 0.63 nkat/g at OR stage. Although both varieties had similar pattern, they exhibited significant differences in their activities, with higher activities in Illiachi, confirming its shorter shelf life due to higher cell wall degradation throughout ripening. A correlation between the increases in PG activity and softening has been reported in carambola fruit (*35*). Increase in PG activity during ripening has also been reported in ber (*26*) and guava (*36*) fruits. Cellulase is considered to be responsible for the hydrolysis of cellulose fibrils of the cell wall causing its softening. The results observed during the present investigations are in agreement with the findings of Jawanda *et al.* (*37*) in ber fruits.

### *Isoenzyme analysis*

Isoenzyme profile of cell wall-degrading enzymes is presented in Fig. 6. Fig. 6a shows that no PME isoenzyme was present at IG stage in both varieties. This corresponded to very low activity of the enzyme at this stage. One isoenzyme of pectin methylesterase (PME I) common to both varieties appeared at MG stage and remained active throughout ripening. However, other two variants of PME (PME II and PME III) were expressed at

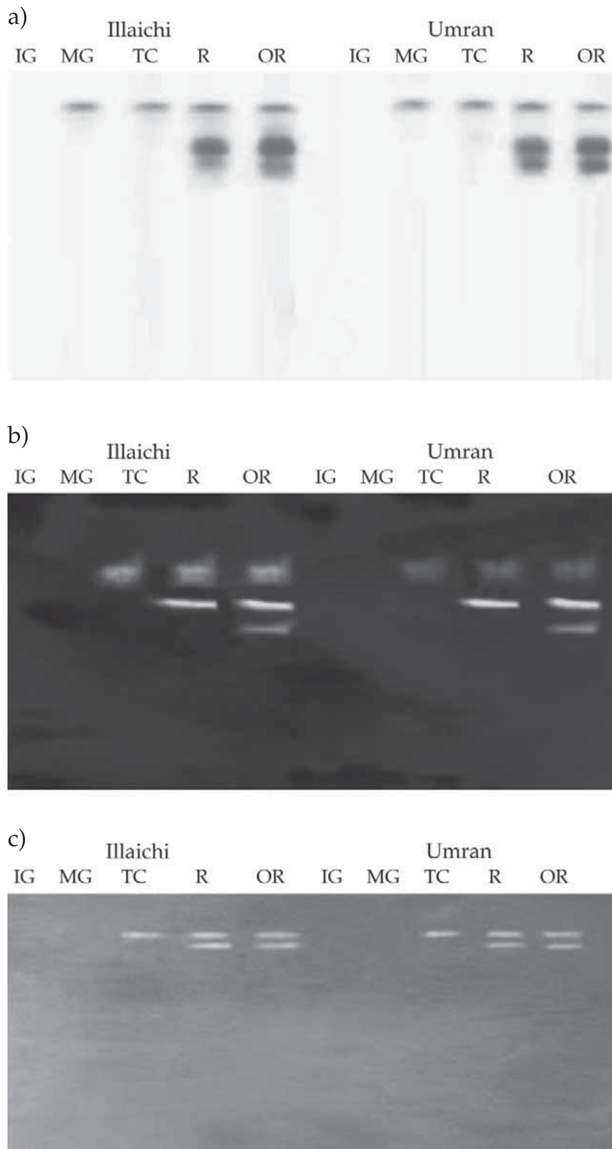


**Fig. 6.** Isoenzyme pattern of cell-wall-degrading enzymes: a) PME, b) PG and c) cellulase

R stage in both varieties and remained active until the OR stage. This could account for the dramatic increase in the enzyme activity during these stages. Similarly, the isoenzyme pattern of polygalacturonase depicted in Fig. 6b indicated that in both varieties PG I, PG II and PG III appeared at TC, R and OR stages, respectively. The absence of any isoenzyme at IG and MG stages again corresponded to very low enzyme activities at these stages. Increase in the number of isoenzymes during ripening was in agreement with the increase in enzyme activity. Isoenzyme profile of cellulase (Fig. 6c) showed the absence of any isoenzyme at IG and MG stages of both varieties; one band appeared at TC stage, while anther band appeared at R stage in both cultivars. The appearance of isoenzymes of cell wall-degrading enzymes during ripening might account for the observed increase in enzyme activity.

The results on isoenzyme pattern observed in ber fruits during ripening in the present investigations could not be compared because there are no published reports in literature. However, multiple forms of PME have been reported in a number of other fruits such as lemon (*11*), peach (*12*) and sweet cherry (*15*). Three isoforms, one neutral (PME1n) and two acidic (PME2a and PME3a) were observed in normal and hyperhydric leaves of carnation plant by Saher *et al.* (*38*). However, Coetzee *et al.* (*39*) reported four different bands of PME (PME1–PME4) and eight different bands of PG (PG1-PG8) after cluster analysis of the *Armillaria* isolates. Stanley and Brown (*40*) observed that PG produced by *Collectrichum musae* in liquid culture on banana pectic substances as the sole carbon source resolved into five distinct isoenzymes. Igual *et al.* (*41*) observed two bands of cellulase from *Casuarina* (Cel2 and UGLO20603) and one band from *Frankia* Hrl1 isolates.

Isoenzyme pattern of cell wall-degrading enzymes (PME, PG and cellulase) clearly revealed that both Umran and Illaichi had three bands of each PG and PME, while only two bands of cellulase at OR stage. A direct correlation was observed between the enzyme activities and the appearance of isoenzyme bands of cell wall-degrading enzymes. Critical perusal of the isoenzyme pattern of the cell wall-degrading enzymes demonstrated that two isoenzymes of each PME and PG, and one of cellulase which were not present during early ripening stages but appeared only during later stages could be considered as ripening-related proteins, and thus can be potential targets for manipulation by modern molecular tools to delay the ripening process and to enhance the shelf life of ber fruits during storage.

## Conclusions

Ripening in ber fruits can be correlated with the increase in cell wall-degrading enzymes and the consequent decrease in cell wall constituents like pectin and cellulose, which is more pronounced in short shelf life variety, Illaichi compared to Umran. PME, PG and cellulase isoenzymes that are not present during early ripening stages but appeared only in later stages could be considered as ripening-related isoenzymes.

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