Chapter - 4

The role of softening enzymes in the internal breakdown of Alphonso mango.
1. Introduction

Ripening of the fruit is characterized by softening of the fruit flesh. Softening in fleshy fruits is primarily due to cell wall modification. The rate of softening of the fruit increases by increased deesterification of cell wall pectins, followed by degradation of solubilized pectin. Also during the fruit ripening there will be loss of fruit firmness due to the liberation of polyuronides. A key step in the ripening process could be the loss of pectic substances in the middle lamellae and cell walls, which results in loss of cell wall integrity. Earlier workers reported a direct correlation between the activity of fruit softening enzymes and ethylene production. Ethylene stimulates the ripening process by inducing the increased activities of hydrolytic enzymes associated with the fruit cell wall degradation. Therefore the ripening of fruit is considered to be a process that includes a number of concomitant processes including ethylene production, respiration, softening of cell walls, the loss of pectic substances, increased protein synthesis and increased hydrolytic enzyme activity. Often it is difficult to separate these processes.

Since limited information is available on mango cell walls and the softening process during ripening and also there are considerable differences between cultivars. Therefore here in the present study the three important fruit softening enzymes namely pectin methyl esterase, cellulase and polygalactouronase were estimated in all the 6 different fruit tissues as they play a key role in the mango fruit ripening. Pectin methyl esterase is responsible for the deesterification of galactosyl uronate methyl esters of cell wall pectins to their free carboxyl groups. Polygalactouronase enzyme is responsible for the solubilisation of cell wall pectins. Cellulase may degrade the hemicellulosic fraction of the fruit cell wall and here the sugars released due to the enzyme activity is estimated spectrophotometrically by DNS method. Pectin methyl esterase activity was estimated spectrophotometrically using the dye Bromothymol blue.
2. Materials and methods

Estimation of the activities of the fruit softening enzymes

2.1 Polygalactouronase,
2.2 Cellulase,
2.3 Pectin methyl esterase.

2.1 Estimation of Polygalactouronase activity: Assay was done as per the methods by Selvaraj and Kumar(1989).

Chemicals required:-

1) 0.2M Sodium chloride solution pH - 6.5:- 2.922gm of sodium chloride was dissolved in 250ml of distilled water and pH was adjusted to 6.5.

2) 0.5 % Sodium Polypectate (substrate):- 500mg of sodium polypectate was dissolved in 100ml of 0.2ml of sodium chloride buffer pH - 6.5.

3) DNS reagent (Dinitro salicylic acid reagent):- 4gm of DNS, 8gm of phenol, 2gm of sodium sulphite and 800gm of Rochelle salt were dissolved in 2 Litres of 2 % of sodium hydroxide solution and further diluted to 4 Litres with distilled water.

Enzyme extraction method:- 0.5gm of acetone powder was kept in cold for overnight incubation in 10ml of 0.2M sodium chloride buffer pH - 6.5 that was homogenized using a chilled mortar and pestle and filtered. Then the homogenate was centrifuged at 8000g for 10 minutes. Extraction and subsequent steps were carried out in cold condition. The resulting supernatent was assayed for polygalactouronase activity.

Enzyme assay method:- The assay mixture contained 1.0ml of crude enzyme extract, 2.0ml of sodium chloride buffer pH - 6.5 and 0.5ml of sodium polypectate solution. An
enzyme solution previously heated in boiling water for 5 min was used as a blank. The reaction mixture was shaken well and incubated at 37°C for 1 - 2 hours. The reaction was stopped by adding saturated lead acetate solution and centrifuged. Excess lead was separated out by using solid sodium carbonate and centrifuged again. To the resulting supernatent solution 3ml of DNS reagent was added. All the tubes were kept in boiling water bath for 5 minutes and cooled in running tap water. Then 20ml of distilled water was added for dilution and mixed well. Then the absorbance of the solution was read at 540nm against the blank solution.

2.2 Cellulase activity:- Assay was done as per the methods by Hinton.D.M and Pressey.R (1974).

Chemicals required:-

1) 0.05M Sodium phosphate buffer pH - 7.0:-
   a) 0.2M monobasic sodium phosphate solution – 15.6gm dissolved in 500ml of distilled water.
   b) 0.2M dibasic sodium phosphate solution – 17.7gm dissolved in 500ml of distilled water.
39ml of a and 61ml of b, mixed, diluted to 200ml and pH was adjusted to 7.0

2) 0.5 % carboxymethyl cellulose solution (CMC):- 500mg of CMC was dissolved in 100ml of distilled water.

3) DNS reagent (Dinitro salicylic acid reagent):- 4gm of DNS, 8gm of phenol, 2gm of sodium sulphite and 800gm of Rochelle salt were dissolved in 2 Litres of 2 % of sodium hydroxide solution and further diluted to 4 Litres with distilled water.

Enzyme extraction method:- 0.5gm of acetone powder was kept in cold for overnight incubation in 10ml of 0.05M sodium phosphate buffer pH - 7.0. That was homogenized using a chilled mortar and pestle, filtered and centrifuged at 2000rpm for 20 minutes. The
supernatent was used for enzyme assay. Extraction and subsequent steps were carried out in cold condition.

**Enzyme assay method:** The assay mixture contained 1.0ml of enzyme extract, 3.0ml of sodium phosphate buffer pH-7.0 and 0.5ml of carboxymethyl cellulose. Heat denatured enzyme solution was used for the blank test. The assay mixture was shaken well and incubated for 2 hours at 37°C. The reaction was stopped by adding saturated lead acetate solution and centrifuged. Excess lead was separated out by using solid sodium carbonate and centrifuged again. To the resulting supernatent solution 3ml of DNS reagent was added. Then the tubes were kept in boiling water bath for 5 minutes and cooled in running tap water. 20ml of distilled water was added for dilution and absorbance of the solution was read at 540nm against the blank solution.

2.3 Estimation of pectin methyl esterase activity:- Assay was done as per the methods by Selvaraj et al., (1989)

**Chemicals required:**

1) 8.8 % Sodium chloride buffer pH - 7.5: - 8.8gm of Sodium chloride was dissolved in 100ml of distilled water and pH was adjusted to 7.5.

2) 0.5 % Pectin solution: - 500mg of Pectin was dissolved in 100ml of distilled water and the pH was adjusted to 7.5.

3) 0.01 % Bromothymol blue in 0.003M Potassium Phosphate buffer pH - 7.5: - 10mg of bromothymol blue was dissolved in 100ml of potassium phosphate buffer pH - 7.5

**Enzyme extraction method:** 0.5gm of acetone powder was kept in cold for overnight incubation in 10ml of 8.8 % sodium chloride buffer pH - 7.5. That was homogenized using a chilled mortar and pestle, filtered and centrifuged at 10,000rpm for 10 - 15
minutes. Extraction and subsequent steps were carried out at 4°C. The pH of the supernatent solution was adjusted to 7.5 accurately and was used for enzymatic assay.

**Enzyme assay method:** To start with the enzyme assay pH of all the solutions were adjusted to 7.5. This method is pH sensitive as the dye changes its color with the change of pH. The assay mixture contained 1.0ml of enzyme extract (pH was adjusted to 7.5), 0.7ml of sodium chloride buffer pH - 7.5 and 0.5ml of the dye (pH - 7.5). A substrate blank was run without the enzyme extract but with substrate solution and an enzyme blank was run using heat denatured enzyme solution and without adding the substrate solution. A blank solution was prepared by adding dye and buffer solution. The enzymatic reaction was started by adding the substrate solution then immediately the reaction mixture was shaken well and change of optical density was read at 620nm against the blank solutions.

**Statistical analysis:** Analysis of variations (ANOVA) of data was done using CRD (completely randomized design) method. Critical difference (CD) values were calculated at $P = 0.05$ wherever ANOVA was found significant.
3. **Results**

3.1 **Polygalacturonase activity**:- The polygalacturonase activity was significantly different at 5% significant level ($P = 0.05$) between the different tissues. The enzyme activity was one and half times more in the completely ripe healthy tissue (9.5967 U) in comparison with the spongy tissue (5.8011 U). PG activity was also slightly more in the induced spongy tissue which is about 6.5720 U when compared to the natural spongy tissue (5.8011 U). Healthy tissue surrounding spongy tissue showed slightly less activity (8.5662 U) than the completely ripe healthy tissue (9.5967 U). Enzyme activity was maximum in the $3/4^{th}$ ripe tissue (12.5341 U) and was minimum in the mature unripe tissue (3.6822 U).

As with the specific activity mature unripe tissue showed maximum activity of 1.6014 U/mg protein, which is followed by $3/4^{th}$ ripe tissue (0.8006 U/mg protein), induced spongy tissue (0.6544 U/mg protein) and spongy tissue (0.5136 U/mg protein). Healthy tissue surrounding spongy tissue showed slightly more of specific activity (0.4638 U/mg protein) than the completely ripe healthy tissue (0.4302 U/mg protein).

3.2 **Pectin methyl esterase activity**:- According to the analysed data pectin methyl esterase activity was significantly different at 5% significant level between all the six different fruit tissues. The enzyme activity was 2 times more in the completely ripe healthy tissue (21.4800 U) in comparison with the spongy tissue (10.2214 U). Induced spongy tissue showed slightly more activity (11.7228 U) than the natural spongy tissue (10.2214 U). Healthy tissue surrounding spongy tissue showed little less activity (18.7417 U) when compared to the completely ripe healthy tissue (21.4800 U). The enzyme activity increased with the progress of ripening process from mature unripe tissue, to $3/4^{th}$ ripe tissue and to completely ripe tissue with the enzyme activities of 8.8200 U, 13.0213 U and 21.4800 U respectively.

Regarding the specific activity $3/4^{th}$ ripe tissue showed maximum activity of 3.1722 U/mg protein. However the specific activity was more in the spongy tissue
(2.2246 U/ mg protein) when compared to the completely ripen healthy tissue (1.7841 U/ mg protein) and healthy tissue surrounding spongy tissue (1.7625 U/mg protein). Mature unripen tissue and induced spongy tissue showed the specific activities of 2.1841 U/mg protein and 2.0548 U/ mg protein respectively.

3.3 Cellulase activity:- As per the analysis of variation cellulase activity was also significantly different at $P = 0.05$ between all the six different tissues. The enzyme activity increased with the process of ripening i.e. from mature unripen stage to the $3/4^{th}$ ripen stage. Mature unripen tissue showed an activity of 7.1801 U and $3/4^{th}$ ripen tissue showed 14.7410 U. Here also there was a comparable difference between the activities of spongy tissue (8.4621 U) and completely ripen healthy tissue (12.3654). Even the induced spongy tissue showed slightly more activity of 9.7200 U where as the natural spongy tissue showed 8.4621 U of enzyme activity. Cellulase activity was slightly less in the healthy tissue surrounding spongy tissue (11.5432 U) in comparision with the completely ripen healthy tissue (12.3654 U).

When the specific activities of different fruit tissues were studied. Mature unripen tissue showed maximum activity of 1.0840 U/mg protein respectively. There was a slight difference in the activities of spongy tissue (0.4476 U/mg protein) and completely ripen healthy tissue (0.4630 U/mg protein). However the $3/4^{th}$ ripen tissue showed more specific activity of 0.7256 U/mg protein as comparable with the healthy tissue surrounding spongy tissue (0.4750 U/mg protein) and completely ripen healthy tissue (0.4630 U/mg protein). Specific activity was more in the induced spongy tissue (0.6738 U/mg protein) as compared to the natural spongy tissue (0.4476 U/mg protein).

The activities of Polygalactouronase, Cellulase and Pectinmethyl esterase are presented in Table - 2 and figure - 3 and 4.
Table – 2. The activities of Polygalactouronase, Pectin methyl esterase and Cellulase enzymes.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Polygalactouronase</th>
<th>Pectin methyl esterase</th>
<th>Cellulase</th>
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<tbody>
<tr>
<td></td>
<td>mg glucose/ hr/100gm fw</td>
<td>mg glucose/ mg protein</td>
<td>µgm PGA/ min/ gm FW</td>
</tr>
<tr>
<td>MU</td>
<td>3.6822</td>
<td>1.6014</td>
<td>8.8200</td>
</tr>
<tr>
<td>3/4thR</td>
<td>12.5341</td>
<td>0.8006</td>
<td>13.0213</td>
</tr>
<tr>
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<td>21.4800</td>
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<td>18.7417</td>
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<td>10.2214</td>
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</table>


The activities of polygalactouronase, pectin methyl esterase and cellulase enzymes were significantly different at 5% significant level (P = 0.05) between the six different tissues.

Figure – 3. Activity of Polygalactouronase enzyme in the six different tissues.

Each bar in the graph represents enzyme activity/ gm FW.

Figure 4. The activities of Pectinmethyl esterase and Cellulase enzymes.

4. DISCUSSION

Ripening of the mango fruit is characterized by softening of the fruit flesh and softening has been brought about by the hydrolysis of various cell wall components, pectin being one of them. Little is known about cell wall modification during mango ripening in tissues damaged with internal breakdown. In the present study the activities of the three softening enzymes namely polygalacturonase (PG), cellulase and pectin methyl esterase were estimated.

There is a good correlation between loss of firmness and increase of PG activity in mango. Elizabeth et.al.,(1992) reported that the polygalacturonase activity is involved in the extensive softening of ‘Keitt’ and ‘Tommy Atkins’ mangoes which increased with ripening. Similar report has been given by Lazan et.al.,(1986). According to which there was increased PG activity but decreased cellulase activity during ripening of Harumanis mango. Here in the present research study both polygalacturonase activity and cellulase activity increased rapidly from mature unripen stage to the 3/4\textsuperscript{th} ripen stage and then decreases as the fruit ripens completely. Both the enzyme activity is almost same in the completely ripen healthy tissue and healthy tissue surrounding spongy tissue. At the 3/4\textsuperscript{th} ripen stage polygalacturonase and cellulase enzymes showed maximum activity. This is because the ethylene production attained a maximum peak at the 3/4\textsuperscript{th} ripen stage and this ethylene triggered the fruit softening process by activating the cell wall depolymerizing enzymes activity.

Here according to the experimental results, Pectin methyl esterase (PME) activity increased with the onset of ripening process from mature unripen stage, 3/4\textsuperscript{th} ripen stage to the completely ripen stage. The activity was maximum in the completely ripen tissue. Reports on the changes in pectinesterase activity during ripening are often contradictory. For instance Ashraf et.al.,(1981) reported that during ripening the PME activity has been shown to remain constant in some Pakistani mango varieties during storage ripening. But Subramanyam et.al.,(1976) reported maximum activity of pectin methyl esterase in mango fruit prior to the onset of the climacteric maximum and the decreased activity
when the fruit ripens. According to Roe and Bruemmer (1981) cellulase level in unripe mango fruit was generally low and increased dramatically during ripening. For instance Teresa et al.,(1992) reported that the rapid softening of Kiwi fruit can be induced by ethylene treatment which increases the pectinesterase activity. According to them the rate of softening of the fruit increases by increased deesterification of cell wall pectins followed by degradation of solubilized pectin.

Also Pesis et al.,(1978), Marcel and Young (1979) found a direct correlation between the softening enzymes activity, fruit softening, respiration and ethylene production in avocado fruits. As per the present study the spongy tissue showed relatively lesser activity of PME, cellulase and PG enzymes when compared to the healthy tissue. Similar findings has been reported by Selvaraj et al.,(2000) and Lima et al.,(2000). Selvaraj et al.,(2000) reported the lower activity of pectin methyl esterase in the spongy tissue in comparision with the healthy ripen tissue. Lima et al.,(2000) reported that the affected spongy tissue in Tommy Atkins mango showed lower concentrations of soluble pectin and lower activity of pectin methyl esterase and polygalactouronase activity and Chitarra et al.,(1999) also reported that the fruit with internal breakdown had higher cell wall polyuronides content than the healthy ones due to inhibition of pectin hydrolases activity in Tommy Atkins mango fruits.

But Shantha Krishnamurthy (1981) reported that the affected pulp showed increased pectin esterase activity as compared to the healthy pulp. The above facts suggest that the affected spongy tissue is less ripe when compared to the healthy ripen tissue, but this may be the result of internal breakdown and not a causal factor. When the specific activities of all the three enzymes namely cellulase, PME and PG were compared the completely ripen healthy tissue showed very less specific activity this is because the protein content increases about 5 fold during ripening. And when both the enzyme activity and protein concentration is more the specific activity of the enzyme becomes less. In the spongy tissue and mature unripen tissue the protein concentration is relatively very less when compared to their enzyme activity and hence they show more of specific activity. However from the above facts and results it could be concluded that in the
spongy tissue due to the disturbance in the ethylene production pathway lesser ethylene was produced which in turn failed to activate the softening enzymes as a results most of the tissues in the affected fruit remained less ripened. And the spongy tissue formation is supposed to occur just at the initiation of ripening process since the activities of softening enzymes in the spongy tissue was in between the mature unripen stage to the 3/4 th ripen stage.
5. **References.**


