Chapter 4: Cell wall degrading enzymes during ripening of *H. polyrhizus*

4.1 Introduction

One of the major changes that occur during fruit ripening is fruit softening. However, the softening of fruits during ripening results in damages during transportation and increases susceptibility towards pathogenic infections (Ariel *et al.,* 2007). In order to identify proper fruit handling and optimization of fruit quality, it is important to understand the process of softening that occurs during ripening of *H. polyrhizus*.

This phenomenon is largely the result of disassembly of cell wall, the cellular structure that provides the rigidity and is responsible for intercellular adhesion (Seymour and Gross, 1996). Biochemical studies of cell wall changes during fruit ripening indicate that structural changes in pectin, hemicellulose and cellulose are responsible for the alteration of the cell wall structure (Brummel, 2006). The main enzymes that are responsible for the disassembly of the cell wall during ripening are cell wall hydrolases such as pectin methylesterase (PME), polygalacturonase (PG), pectate lyase (PL) and cellulase (Ariel *et al.,* 2007).

PME as a ubiquitous plant enzyme responsible to catalyse the pectin de-esterification methyl esters of pectin (Draye *et al.,* 2008). Previous studies have shown that the activity of PME varies depending on the fruit sample being climacteric or non climacteric; decreases (El-Zoghbi, 1994), increases (Aina *et al.,* 1993) or remain constant (Ahmed and Labavitch,, 1980) during fruit ripening. During fruit ripening, PG is mainly responsible in dissolution of the middle lamella (Jackman and Stanley, 1995). Exo PG breaks down pectin by hydrolysing the α- 1,4-glycosidic bonds between the

galacturonic acid residues in galacturonans from the non-reducing end, which results in the release of galacturonic acid as the major reaction product (Prasanna *et al.,* 2007). PL, otherwise known as pectate transeliminases, catalyses the eliminative cleavage of de-esterified pectin, which is a major component of the primary cell walls of many higher plants (Carpita *et al.,* 1993). Payasi and Sanwal, (2003) reported increased PL activity in bananas during ripening while Goulao *et al.,* (2007) stated PL activity was maintained during ripening of apples.

Another enzyme that also contributes to fruit softening is cellulase. It hydrolyses the 1, 4-β-d linkages between unsubstituted linear glucans, and thus cellulose and xyloglucan within plant cell walls are potential substrates (Rose *et al.,* 2003). Cellulase has never been shown to hydrolyse cellulose that has a semi-crystalline structure and insoluble but found against CMC which is a highly modified soluble glucan. However, the role of cellulase in fruit softening is still uncertain (Abu-Goukh and Bashir, 2003).

Thus, the objective this experiment was to investigate the activity of these cell wall enzymes during the ripening of *H. polyrhizus* in which little work has been done relatively.

4.2 Material and methods

4.2.1 Plant materials

Refer to Chapter 3 subtopic 3.2.1(page 30 - 31)

4.2.2 Protein extraction

The following method for protein extraction buffer is according to Kanellis *et al.,*

(1991) and modified by Chandran (1998)

4.2.2.1 Preparation of protein extraction buffer (stock solution 50ml)

50 mM Tris HCL pH 7.5 *(2.5ml)*
0.5 M NaCl *(1.461g)*
10 mM 2-mercaptoethanol *(0.0349ml)*
1 mM DTT *(0.0385g)*
10 uM leupeptin *(0.05ml)*
1 mM EDTA *(0.0146g)*
10 % glycerol *(5ml)*
0.5 % triton X-100 *(0.25ml)*

The final volume was made up to 50 ml with 42.16 ml of distilled water.

4.2.2.2 Preparation of sample, extraction of protein

Frozen pulp tissue (3.2.1 page 31) was ground into fine powder using a mortar and pestle with liquid nitrogen. Total protein was extracted by adding 6g of pulp sample powder to 8 ml of protein extraction buffer. The mixture was vortexed thoroughly, then placed on ice for 10 minutes and centrifuged at 25,000 x g for 30 minutes at 4°C in a Sorvall RC5C refrigerated centrifuge. Upon centrifugation, pellet was discarded and the supernatant was collected by filtering through one layer of miracloth. Extracted samples were used immediately for analysis.

4.2.2.3 Preparation of reagents for protein estimation

Bradford Reagent

20 mg of Coomassie Blue G250 was dissolved in 10 ml of 95% ethanol. 20 ml of 85 % phosphoric acid was added into the solution. Final volume was made up to 200 ml. The solution was filtered using Whatman filter paper and the solution was stored at 4˚C (Bradford MM, 1976)

Standard protein solution, Bovine serum albumin (1mg/ml BSA)

100 mg of BSA was dissolved in 80 ml of distilled water. Final volume was made up to 100ml.

Standard curve for protein

The BSA standard was shown on table below. Colour development was initiated with the addition of 5 ml of Bradford reagent into each BSA standard solution.

Table 4.1: Preparation of standard curve for Protein

Tubes	0	1	2	3	4	5	6	7	8
SDW(ul)	100	90	80	70	60	50	30	20	0
BSA (ul)	0	10	20	30	40	50	70	80	100
BSA (ug)	0	10	20	30	40	50	70	80	100

4.2.2.4 Preparation of samples and estimation of protein

25ul of extracted samples and 75ul of distilled water were added into a test tube. Then 5 ml of Bradford solution was added into the solution. The mixture was vortexed and incubated at room temperature. Absorbance at 595 nm was read and recorded using a spectrophotometer (Ultra Spec II). Protein content in the sample was estimated using BSA as a standard.

4.2.3 Pectin methylesterase (PME)

The following method was adapted from Lohani *et al.,* (2004) and the activity was calculated using a standard curve of galacturonic acid drawn as described by Hagerman and Austin, (1986)

4.2.3.1 Preparation of Reagents

Sodium hydroxide NaOH (2M)
8 g of NaOH was dissolved in 50ml distilled water. Final volume was made up to 100ml.

1% (w/v) Pectin solution (pH 7.5)
1 g of pectin was dissolved in 80ml of water. The solution was heated and stirred at 40˚C using a hot plate. The solution was let to cool and pH was adjusted to 7.5 using NaOH. Final volume was made up to 100ml. The solution was stored at 4˚C

Potassium dihydrogen phosphate KH_2PO_4 (0.003M) (pH 7.5)
Reagent I
0.04g of KH_2PO_4 was dissolved in 90ml distilled water and pH was adjusted to 7.5 using phosphoric acid. Final volume was made up to 100ml.

Reagent II
0.05g of K_2HPO_4 was dissolved in 90 ml distilled water and pH was adjusted to 7.5 using phosphoric acid. Final volume was made up to 100ml. Finally 16.6ml of Reagent I was added into 83.4 ml of Reagent II.

0.01% (w/v) Bromothymol blue
1ml of 0.1% bromothymol blue was added into 9 ml of phosphate buffer.

Sodium chloride NaCl (0.15M)
0.17g of NaCl was dissolved in 15 ml distilled water. Final volume was made up to final volume of 20 ml.

Galacturonic acid $C_6H_{10}O_7$ (1mM)
0.02 g of galacturonic acid was dissolved into 80 ml of distilled water. Final volume was made up to final volume of 100ml.

4.2.3.2 Standard curve for PME enzyme

2 ml of pectin solution and 0.15 ml of bromothymol blue were added into a test tube. Then galacturonic acid and distilled water were added as shown on table below. Absorbance at 620 nm was read using a spectrophotometer (Ultra Spec II) and readings were recorded immediately after 3 minutes.

Table 4.2: Preparation of standard curve for PME enzyme

Tube	1	2	3	4	5	6	7	8
ddH_2O	0.85	0.82	0.79	0.76	0.73	0.70	0.67	0.64
Galacturonic acid (ml)	0.00	0.03	0.06	0.09	0.12	0.15	0.18	0.21
Galacturonic acid (ug)	0	6	12	18	24	30	36	42

4.2.3.3Preparation of samples and PME assay

pH of the extracted sample was adjusted to pH 7.5 with NaOH. 2 ml of pectin solution, 0.2 ml of NaCl, 0.15 ml of 0.01% Bromothymol blue, 0.45 ml distilled water and 0.2 ml extracted sample were added into a test tube. Test tube was gently shaked. Absorbance at 620 nm was read using a spectrophotometer and readings were recorded immediately and after 3 minutes. A solution containing ddH_2O was used as blank for the PME assay. Activities of enzyme were expressed as specific activity (per unit protein basis).

4.2.4 Polygalacturonase (PG)

Polygalaturonase activity was assayed as described by Pathak and Sanwal (1998).

4.2.4.1 Preparation of reagents

<u>Sodium acetate NaAc (0.2M)</u>
0.16 g of NaAc was dissolved in 8 ml of distilled water and pH was adjusted to 4.5 with acetic acid. Final volume was made up to final volume of 10 ml

<u>Sodium chloride (0.2M)</u>
0.4 g of 5M NaCl was dissolved in 9 ml of distilled water. Final volume was made up to 10ml

<u>1% Polygalacturonic acid (PGA)</u>
0.3 g of PGA was dissolved in 25 ml of distilled water and pH was adjusted to 4.5 with acetic acid. Final volume was made up to final volume of 30 ml

Dinitrosalicylic acid (DNS) solution stock (70 ml)
The following were added into 60ml of distilled water
0.63% of dinitrosalicylic acid (0.441g),
0.5% phenol (0.35ml),
0.5% sodium bisulfite (0.35 g)
2.14% NaOH (1.498 g)
Final volume was made up to 70 ml

40% Rochelle salt (stock solution 50ml)
20 g of Rochelle salt was dissolved in 40 ml distilled water. Final volume was made up to final volume of 50 ml.

5mM Galacturonic acid (stock solution 100ml)
0.11g of Galacturonic acid was dissolved in 90 ml of distilled water. Final volume was made up to final volume of 100ml

4.2.4.2 Standard curve for PG enzyme

1ml DNS was added into a test tube then extracted sample and distilled water were added as shown in Table below. The solution was placed in a boiling water bath for 5 minutes. Then 0.4 ml of Rochelle salt was added into the solution and cooled under running water. Absorbance at 540 nm was read using a spectrophotometer (Ultra Spec II) and readings were recorded.

Table 4.3: Preparation of standard curve for PG enzyme

Tube	1	2	3	4	5	6	7	8	9
ddH_2O	1	0.976	0.952	0.928	0.904	0.880	0.856	0.832	0.808
Galacturonic acid (ml)	0	0.024	0.048	0.072	0.096	0.120	0.144	0.168	0.192
Galacturonic acid (ug)	0	0.03	0.05	0.08	0.10	0.13	0.15	0.18	0.20

4.2.4.3Preparation of samples and PG assay

0.2 ml of NaAc, 0.1ml NaCl and 0.4 extracted samples were added into test tube. Then 0.3 ml of 1% PGA was added into the solution. The solution was incubated at 37˚C oven for one hour. 1 ml DNS was added to stop enzyme reaction. The solution was placed in a boiling water bath for 5 minutes. Subsequently the solution was taken out and 0.4ml Rochelle salt was added into the solution. The solution was cooled under running water. Absorbance at 540 nm was read using a spectrophotometer (Ultra Spec

II) and readings were recorded. A solution containing boiled enzymes (denatured enzyme) was used as blank for the PG assay. Activities of enzyme were expressed as specific activity (per unit protein basis).

4.2.5 Pectate lyase (PL)

Pectate lyase was assayed as described by Moran *et al.,* (1968) with modification by Lohani *et al.,* (2004)

4.2.5.1Preparation of Reagents

4mM sodium acetate NaAc (stock solution 50ml)
0.016g of NaAc was dissolved in 45 ml of distilled water. Final volume was made up to 50ml

1% polygalacturonic acid (PGA) (stock solution 30ml)
0.3g of PGA was dissolved in 25ml of distilled water and pH was adjusted to 4.5 with acetic acid.Final volume was made up to 30ml.

Calcium chloride 4mM ($CaCl_2$) (stock solution 10ml)
0.059g of $CaCl_2$ was dissolved in 9ml of distilled water. Final volume was made up to 10m.l

4.2.5.2 Preparation of sample and PL assay

1.8 ml of 4mM NaAc, 0.9 ml of 1 % PGA, 0.3 ml of extracted sample and 0.3 ml of $CaCl_2$ were added into a test tube. The test tube was incubated at 37˚C oven for 30 minutes and then placed in a boiling water bath for 2 minutes to stop enzyme reaction. Absorbance at 235 nm was read using UV spectrophotometer (Ultra Spec II) and readings were recorded. Blank used for PL assay was boiled enzyme (denatured enzyme).

($CaCl_2$ was added to compensate the Ca2+ ion that was chelated through EDTA in the extraction buffer)

As for PL enzyme activity determination the following calculation were carried out:

$$\text{PL activity (unit/ml)} = \frac{\text{OD (sample) x V (reaction)}}{[\text{Coefficient factor x t x V (sample)}]}$$

V (sample) = volume of sample
t = incubation time (minutes)
Coefficient factor = 4.6
1 unit of enzyme = 1umol of aldehyde PGA/min

1 unit = 1umol/min/ml

4.2.6 Cellulase

Cellulase was assayed according to Dong *et al.,* 1992.

4.2.6.1 Preparation of reagents

Glucose standard solution (1mg/1ml) (stock solution 25 ml)
0.025 g of glucose was dissolved in 20 ml of distilled water. Final volume was made up to 25 ml

Dinitrosalicylic acid (DNS) solution stock (70 ml)
The following were added into 60 ml of distilled water
0.63% of dinitrosalicylic acid (0.441g),
0.5% phenol (0.35 ml),
0.5% sodium bisulfite (0.35 g)
2.14% NaOH (1.498 g)
Final volume was made up to 70 ml

40% Rochelle salt (stock solution 50ml)
20 g of Rochelle salt was dissolved in 40 ml distilled water. Final volume was made up to 50 ml.

4.2.6.2 Standard curve for cellulase enzyme

1ml DNS was added into a test tube then extracted sample and distilled water were added as shown on Table below. The solution was placed in a boiling water bath for 5 minutes. Then 0.4 ml of Rochelle salt was added into the solution and cooled under

running water. Absorbance at 540 nm was read using a spectrophotometer (Ultra Spec II) and readings were recorded.

Table 4.4: Preparation of standard curve for Cellulase enzyme

Tube	1	2	3	4	5	6	7	8
ddH_2O (ul)	1000	900	800	600	500	400	200	0
Glucose (ul)	0	100	200	400	500	600	800	1000
Glucose (ug)	0	100	200	400	500	600	800	1000
Glucose (mg)	0	0.1	0.2	0.4	0.5	0.6	0.8	1.0

4.2.6.3Preparation of samples and cellulase assay

50 mg of cellulose (CMC) and 4 ml extracted sample were added in a Pyrex test tube and incubated at (35˚C, 390 rpm, and 16 hours) in a shaking incubator. After incubation, solution was placed into a boiling water bath for 10 minutes. DNS assay was carried out for reducing sugar content using glucose as standard. 1 ml of DNS solution to 1 ml extracted sample was added into a test tube. Test tube was placed into a boiling water bath for 5 minutes. 0.4 ml of Rochelle salt was added into the solution. The solution was cooled under running water. Absorbance at 540 nm was read and readings recorded. A solution containing boiled enzymes (denatured enzyme) was used as blank for the cellulase assay. Activities of enzyme were expressed as specific activity (per unit protein basis).

4.3 Results

4.3.1 Protein extraction

The changes in protein content of *H. polyrhizus* harvested at three different harvesting maturities during storage are shown in Figure 4.1. An increase of protein content was observed for all stages of maturity throughout the duration of experiment. For D 0 fruit the protein content was from 41.52 mg/g on day of harvest and it increased to 65.10 mg/g on day 13. As for D 1 fruit also showed a similar trend in which the amount of protein content was 27.90 mg/g on day of harvest and it increased to 71.86 mg/g on day 13. An increase of protein content was also detected in D 2 fruit from 34.21 mg/g protein content to 64.02 mg/g on day 13. However, the amount of protein in D 1 and D 2 fruits were similar and on day 11-13.

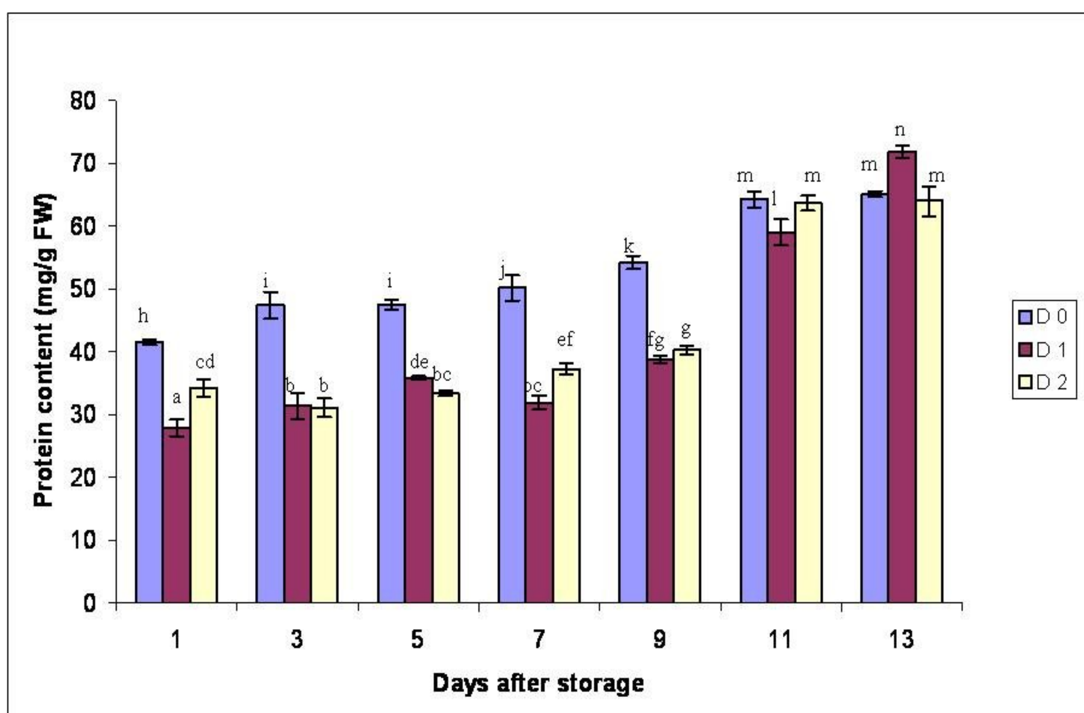

Figure 4.1: Protein content of *H. polyrhizus* harvested at three different harvesting maturities at room temperature

4.3.2 Pectin methylesterase (PME) activity

From Fig 4.2 it is observed that PME activity of D 0 fruit was at least 2 times higher than D 1 fruit and 13 times that in D 2 fruit on day of harvest. However, the PME activity for D 1 and D 2 fruits were 2 to 3 times higher on day 13 compared to D 0 fruits. Over a period of 13 days, PME activity declined for D 0. However, D 1 and D 2 fruits had an increase of PME activity after day 5 onwards and then remained to the initial value at harvest.

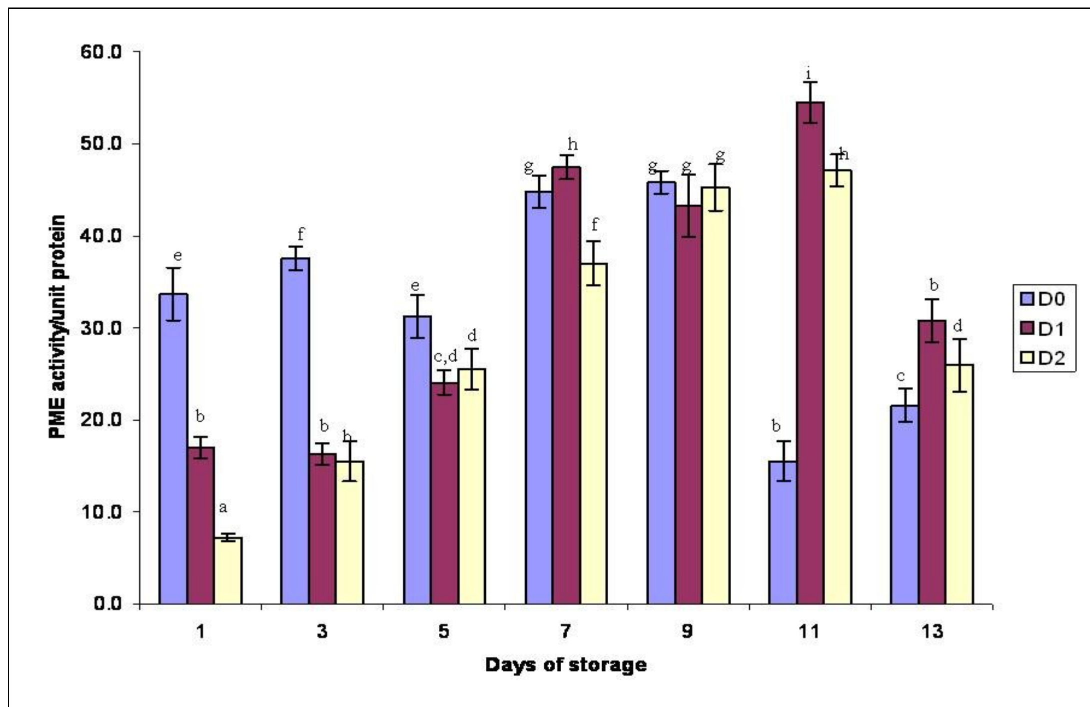

Figure 4.2: Pectin methylesterase activity in *H. polyrhizus* harvested at three different harvesting maturities at room temperature

4.3.3 Polygalacturonase (PG) activity

PG activities are defined as the amount of enzyme required to liberate nmol of galacturonic acid per min under the conditions of the enzyme assay (Miller, 1951). PG activity remained low during the first 5 days and the activity was generally higher in D 0 fruit (Fig 4.3). The activity increased for D 0, D 1 and D 2 fruits from day 5 onwards. The activity of PG increased up to 200 unit protein^{-1} for D 0 fruit compared to 150 unit protein^{-1} for D 1 and D 2 fruits on day 7. However, PG activity in D 1 and D 2 fruits remained at about 150 unit protein^{-1} but for D 0 fruit the PG activity remained till day 13.

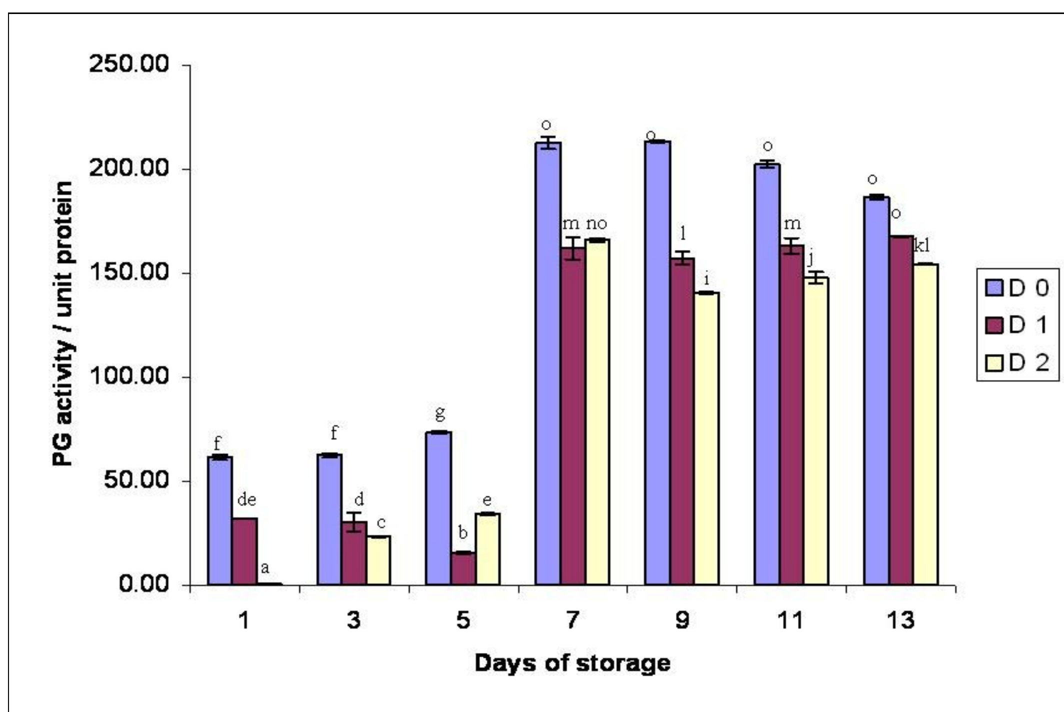

Figure 4.3: Polygalacturonase activity *H. polyrhizus* harvested at three different harvesting maturities at room temperature

4.3.4 Pectate lyase (PL) activity

According to Moran *et al*., (1968) 1 unit (U) of PL activity is defined as the amount of enzyme required to liberate 1 nmol of aldehyde groups from PGA per minute under the conditions of enzyme assay. The activity of pectate lyase activity was within a small range of 0.139 U/ml to 0.216 U/ml for all stages of fruits. For the D 0 fruit, the activity of pectate lyase was within the range as opposed to those harvested earlier in which the activity was not consistent. Activity in D1 fruit tend to maintained till day 9 and increased there after till day 13. The overall activity of pectate lyase was higher in D 1 and D 2 fruits compared to D 0 fruit.

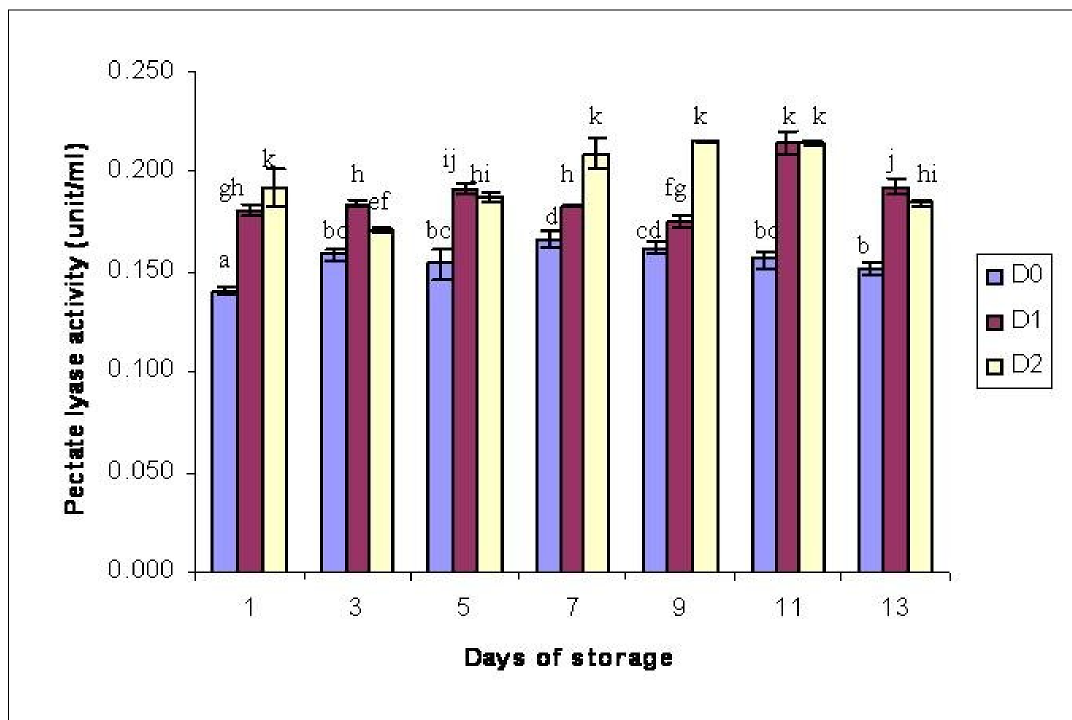

Figure 4.4: Pectate lyase activity in *H. polyrhizus* harvested at three different harvesting maturities at room temperature.

4.3.5 Cellulase activity

Cellulase activity was measured on amount of glucose produced in µg. The cellulase activity was within the range of 0.21 µg to 0.39 µg for all stages of fruits. The cellulose activity for D1 and D2 fruits were similar till day 5. The cellulase activity pattern was not considerably different for both D 1 and D 2 fruits from day 3 onwards till the end of the experiment. However, the activity for all D0, D1 and D2 fruits were on par at the end of the experiment.

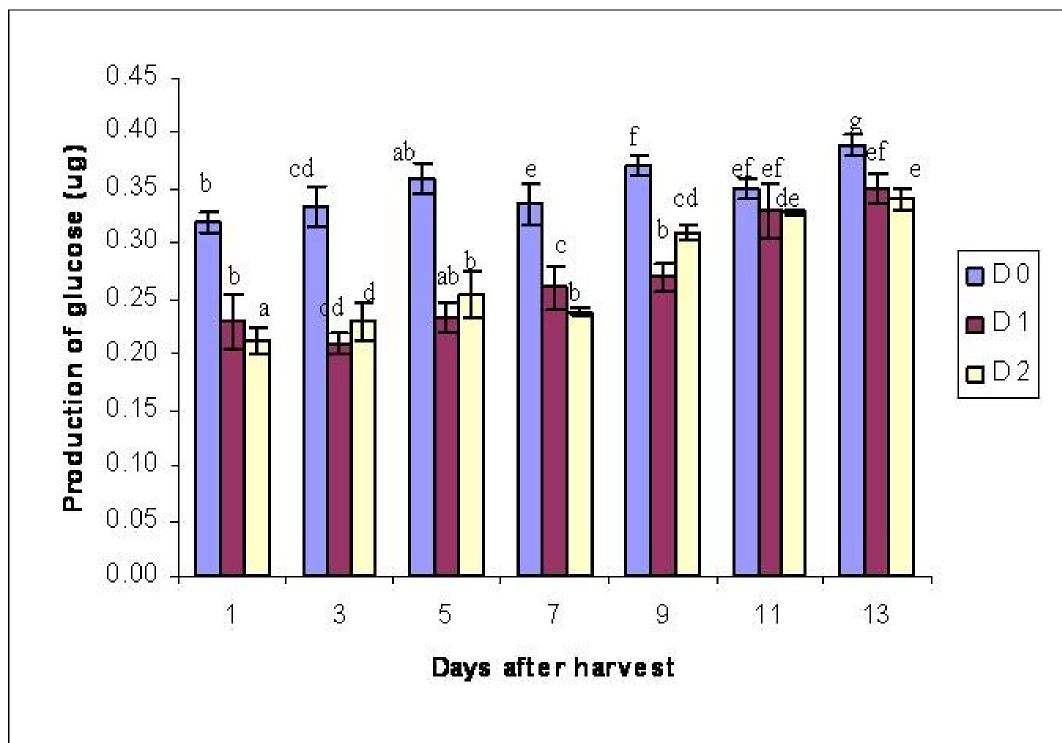

Figure 4.5: Cellulase activity in *H. polyrhizus* harvested at three different harvesting maturities at room temperature.

4.4 Discussion

In this study, total extractable protein for different harvest maturity showed increased level. This is probably indicating that particular proteins were being synthesized for senescence as such increases protease activity (Graeme and Erin, 1995) or production of enzyme that is related to fruit softening that functions at the later stage of the experiment which in turn result in further reduction in firmness. Furthermore, the protein production during storage could be due to, besides actual increase in protein content, better extractability of the proteins from the increasingly softer fruit that results in release of cell wall bound proteins.

A decline in firmness is closely related with textural changes during ripening of a fruit. These two phenomena are referred as fruit softening that has been widely studied (Brownleader *et al.,* 1999; Wakabayashi, 2000). The loss of firmness has been typically associated with dissolution of the middle lamella, depolymerisation, and solubilisation of cell wall polysaccharides and, in some cases, wall swelling (Brummell and Harpster, 2001).

Along with the above events, pectins seem to be the most common components of the primary cell wall and middle lamella contributing to the fruit texture. Therefore, tissue softening is attributed to enzymatic degradation and solubilisation of the protopectin (Sakai *et al.,* 1993). Furthermore, pectins are known as the main substances involved in providing mechanical strength of the primary cell wall (Sirisomboon *et al.,* 2000). Their degradation during ripening seems to be responsible for tissue softening, as reported for a number of fruits including guava (Abu-Goukh and Bashir, 2003), pear (Liu *et al.,* 2008), prickly pear also known as cactus pear (Armando *et al.,* 2002) and many more.

Besides pectic enzymes, cellulase has also been brought into picture as cell wall modifying enzyme (Percival Zhang *et al.,* 2006).

Thus textural changes during ripening are generally accepted as a composite effect of the action of cell wall hydrolases such as PME, PG, PL and cellulase (Zainon *et al.,* 2004). Although different modifications may occur to its different extents but pectin solubilisation is considered to be the main feature of pectin modification (Goulao *et al.,* 2007).

In this study, the result obtained shows that the changes of cell wall enzyme activities after harvest were quite different between the maturity stages. As the time after harvest progressed, D 0 fruit showed reduced level of activity of PME compared to those harvested earlier which showed increased levels of PME activity. This correlates well with firmness of the fruit in which at the day of harvest D 1 and D 2 fruits were firmer than D 0 fruit suggesting PME activity already functioning in D 0 fruit. The decrease in activity of PME after harvest to over ripe stage for D 0 fruit in this work was also observed in other species during ripening such as in tomato (Gaffe *et al.,* 1994). It has been suggested that pectin methylesterase removes the methyl groups of the wall galacturonans to enhance their depolymerisation by both endo- and exopolygalacturonase (Huber, 1983).

Eventually, PG activity increased progressively for all fruits. From this study, the PG activity of D 0 fruit was high at the beginning of the experiment compared to D 1 and D 2 fruits indicating fruit softening was taking place in D 0 fruit on day of harvest. As in those harvested earlier, the PG activity increased to a level close to D 0 fruit following days after harvest. Simultaneously, pulp firmness reduction of D 1 and D 2 fruits was reported as same as D 0 fruit. PG cleaves the α 1-4 galaturonosyl linkages in

demethylated pectin while PME that catalyses the deesterification of galatosyl urinate methyl esters of pectin of their free carboxyl groups. Consequently, increase in PG activity is correlated with the production of substrate by PME that facilitates PG activity on pectin (Lazan *et al.,* 2004). Furthermore, previous study by Imsabai *et al.,* (2002) had revealed the complementary role between PG and PME. Besides providing substrate, PME also noted to modify pH and cation exchange properties of the cell wall in order to assist other enzymes besides PG (Micheli, 2001). This was observed only for D 0 fruit as opposed to the D 1 and D 2 fruits. Armando *et al.,* (2002) also stated that a high PG activity might make up for a relatively low PME in prickly pear. The PME activity seemed to be up till day 9 before declining even though the PG activity already increased during day 7 onwards. This could be explained regarding the role of PME which assist in the other enzymes activity for fruit softening.

PL enzymes activity in D 0 fruit were almost constant although the activity was low in which declined firmness was reported. Meanwhile D 1 and D 2 fruits showed increased level of PL activity indicating function of the enzyme in reducing firmness. PL liberates galacturonate residue from pectate and catalyses α- 1, 4 galaturonan linkages by β-elimination reaction in which it reacts with carbon 4 and 5 providing double bond (Payasi and Sanwal, 2003). Hence, PG and PL catalyse the cleavage of pectin backbones (Bermudez *et al.,* 2002) that leads to fruit softening. The detection of increase activity in early stages of development and ripening of apples (Goulao *et al.,* 2007) was similar to the activity of PL observed in *H. polyrhizus* for those harvested at early maturity in this study.

Increased activities of cellulase during ripening was previously reported in several fruits such as tomato (Maclachan and Brady„ 1994), strawberry (Abeles *et al.,* 1990) and guava (Abu-Goukh and Bashir, 2003) that suggests a role for cellulase in fruit ripening. As in this study, the activity of cellulase increased for D 1 and D 2 fruits with higher activity in D 0 fruit probably due to maturity level as mentioned earlier in which the enzymes are already activated. Nevertheless, cellulase activity is seen to be not consistent in non climacteric fruits according to many studies. Likewise, there was no detection of cellulase activity during ripening of cherry (Barrett and Gonzalez, 1994) and also in grapes (Nunan *et al.,* 2001) although this enzyme exhibited enhanced activity during ripening of strawberry (Trainotti *et al.,* 1999) and also in pepper (Ferrarese *et al.,* 1995). Therefore, the role of cellulase in fruit softening is still uncertain (Percival Zhang *et al.,* 2006). Furthermore Talia *et al.,* (2005) also found no clear relationship between fruit softening and the activity of this enzyme.

In summary, the observed activity of the cell-wall degrading enzymes in this fruit suggests that none of the hydrolytic enzymes acts alone to bring about cell wall structural changes, as similarly reported by Barka *et al.,* (2000) for tomato softening. The changes in cell wall degrading enzymes correlate to the loss of pulp firmness of *H. polyrhizus* in these findings. Apparently, cell wall enzymes may function collectively over time and space eventually modifying cell wall modification during ripening. However, the rate and extent at which the activity of cell wall degrading enzymes proceeds would depend on the structure of the primary wall.