Kasetsart J. (Nat. Sci.) 43 : 267 - 274 (2009)

Polygalacturonase and Pectate Lyase Activity During Ripening of Kluay Hom Thong Fruit

Sukuntaros Tadakittisarn*, Molnapat Songpim and Pilanee Vaithanomsat

ABSTARCT

The research focused on the change of enzymes associated with the maturation of Kluay Hom Thong bananas [*Musa acuminate* (AAA group) 'Gros Michel']. Polygalacturonase (PG) and pectate lyase (PL) enzymes from the Kluay Hom Thong (ripening Stages 2-8) were extracted and partially purified by ammonium sulfate fractionation. The results showed an increase in PG activity from 3.0 ± 0.11unit/g fresh banana in the 2nd Stage to 4.89 ± 0.39 unit/g fresh banana in the 6th Stage . Furthermore, the PG activity decreased slightly to approximately 4.33± 0.49 unit/g fresh banana as the ripening stage increased (the 8th Stage). PL enzyme activity also depended on the ripening stage of the banana. When the banana was riper, the PL activity gradually increased from 8.73 ± 0.23 unit/g fresh banana in the 2nd Stage to 35.37 ± 1.05 unit/g fresh banana in the 8th Stage. When compared to the commercial pectinase enzyme from *Aspergillus aculentus* (Pectinex Ultra SP-L, Novozymes A/S, Denmark), the enzymes obtained from the banana demonstrated much lower activity. The PG and PL activities from the commercial pectinase were 7966.46 and 1709 unit/ml, respectively.

Key words: extraction, polygalacturonase, pectate lyase, banana

INTRODUCTION

The banana is a well-known economic crop among Thai people, as it has more applications than merely as food. It has been planted and grows well in all parts of Thailand. Exports of bananas from Thailand rank third in Asia (FAO, 2006). As ripening progresses, respiration of the fruit increases and ethylene is needed to stimulate full ripening and therefore, ethylene must be used to force ripening. During ripening, the banana will in turn produce its own ethylene and change starch to sugar. The color of the peel also changes, the pulp becomes softer and the fruit is more aromatic. Kluey Hom Thong or Gros Michel is not usually exported as it naturally ripens easily. To delay or extend ripening, the fruit is usually transported in a refrigerated container or a solution of potassium permanganate is added to boxes of bananas to delay ripening.

The ripening of bananas, or other fruit for that matter, is not only due to ethylene, but also involves changes in the pulp, (which becomes softer) or in the peel color of the fruit. Observations of the pulp cells of the fresh fruit through the microscope revealed that there were changes in the cell wall during the ripening process (Smith, 1989). In other fruit, the most distinguishing change is in the pectic polysaccharide cell walls, which become more soluble with reduced

Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: aapsrt@ku.ac.th

molecular size. Changes in the pectin structure indicated that a pectin-degrading enzyme may have been active in these cell walls (Brummell and Harpster, 2001).

Pectinolytic enzymes are widespread in plant fungi and bacteria. Industrially, they are useful enzymes for extraction, clarification and liquefaction of fruit juices and wines, retting of plant fibers and de-clogging pulps. They act on plant tissues, especially pectins, causing cell lysis. Pectins are the main component of the middle lamella and primary cell wall of plant cells. The most apparent change during ripening in many fruits is a decrease in the pectin molecular weight and an increase in polyuronides, which correlate with increased activity of polygalacturonase (PG) (Crookes and Grierson, 1983) and pectate lyase (PL) (Marin-Rodriguez *et al*., 2003).

PG, EC 3.2.2.15, both endo-acting and exo-acting enzymes in ripe bananas and other fruit, have been found to cause a softening of fruit as well (Markovic *et al*., 1975). PG catalyzes the hydrolytic cleavage of α (1→4) galacturonan linkage of pectin. PG is found not only in bananas, but also in other fruits, such as strawberries, tomatoes, peaches, avocados, apples and pears. Pectinase is found at the highest levels in ripe bananas and least or not at all in raw fruit (Markovic *et al*., 1975). Dominquez-Pulgjaner *et al*. (1992) observed a dramatic increase in two polypeptides, 28 and 42 kDa, during ripening of banana fruit and suggested that the 42 kDa polypeptide could be PG-related proteins.

PL, EC 4.2.2.2 are enzymes responsible for catalyzing the cleavage of α(1→4) galacturonan linkages of pectate. Nonetheless, this reaction is different from PG in that the β-elimination reaction generates double bonds between carbon atoms C4 and C5, producing 4,5-unsaturated oligogalacturonates at the non-reducing end of the cut pectin chain. The present belief is that PL is released when the plant is attacked by disease, as in tomatoes (Marin-Rodriguez *et al*., 2002). It is not possible to measure the activity of PL, but PL-like genes were found in ripe tomatoes. Subsequently, Marin-Rodriguez *et al*. (2002) was able to measure activity in bananas for the first time.

The objective of this research was to determine the optimum period of ripeness to extract pectinase from Kluay Hom Thong for use as a database in considering the potential use of pectinase in different industries. At a high enough level, the enzyme in bananas can be extracted.

MATERIALS AND METHODS

Preparation of banana sample

Uniformly mature bananas, of the variety Kluay Hom Thong [*Musa acuminata* (AAA group) 'Gros Michel'], were purchased from a local market. They were dipped in ethephon solution (0.1%v/v) and kept at 25-28°C to further ripening. Bananas from the same hand were used to represent bananas at varying stages of ripeness according to CSIRO standards (CSIRO, 1972) where:

Stage 1 = 100% green peel, hard fruit, no indications of ripeness

Stage 2 = 95% green peel, 5% yellow peel emerging

Stage 3 = 70% green peel, 30% yellow peel emerging

Stage 4 = 30% green peel, 70% yellow peel

Stage 5 = 95% yellow peel, 5% green at the tip

Stage 6 = 100% the whole fruit yellow

Stage 7 = Yellow peel with brown spots appearing, characteristic aroma distinct.

Stage 8 = Yellow peel with more brown spots over ripe, pulp softens and pungent aroma.

Upon reaching the desired ripeness, the fruit were sampled by peeling off the skin and the mid section was selected. The central core with seeds was discarded. The sample was dipped in

liquid nitrogen, packed in a polyethylene bag and placed it in the freezer at -70°C to await the next step.

Enzymatic concentration and partial purification

This process was adapted from Pathak *et al*. (2000) and Payasi *et al*. (2006), with the steps in the extraction of the enzyme shown in Figure 1. All stages were performed at a controlled temperature of 4-6°C. Thirty grams of banana pulp stored at -70°C was homogenized in a blender together with an extracting buffer consisting of 0.02M sodium phosphate buffer pH 7.0, 0.02M EDTA (Ethylenediamine tetracetic acid), 1% Triton X-100, 0.02M L-cysteine HCl and 1 mM PMSF (phenyl methyl sulfonyl fluoride). The proportion of solution to banana pulp was 5:1. The contents were twice blended at maximum speed for 2 min. The solution was then centrifuged at 20,000 x g for 30 min to separate out the solids. The crude enzyme extract was obtained. The solution was precipitated with ammonium sulphate, which was slowly added to the solution

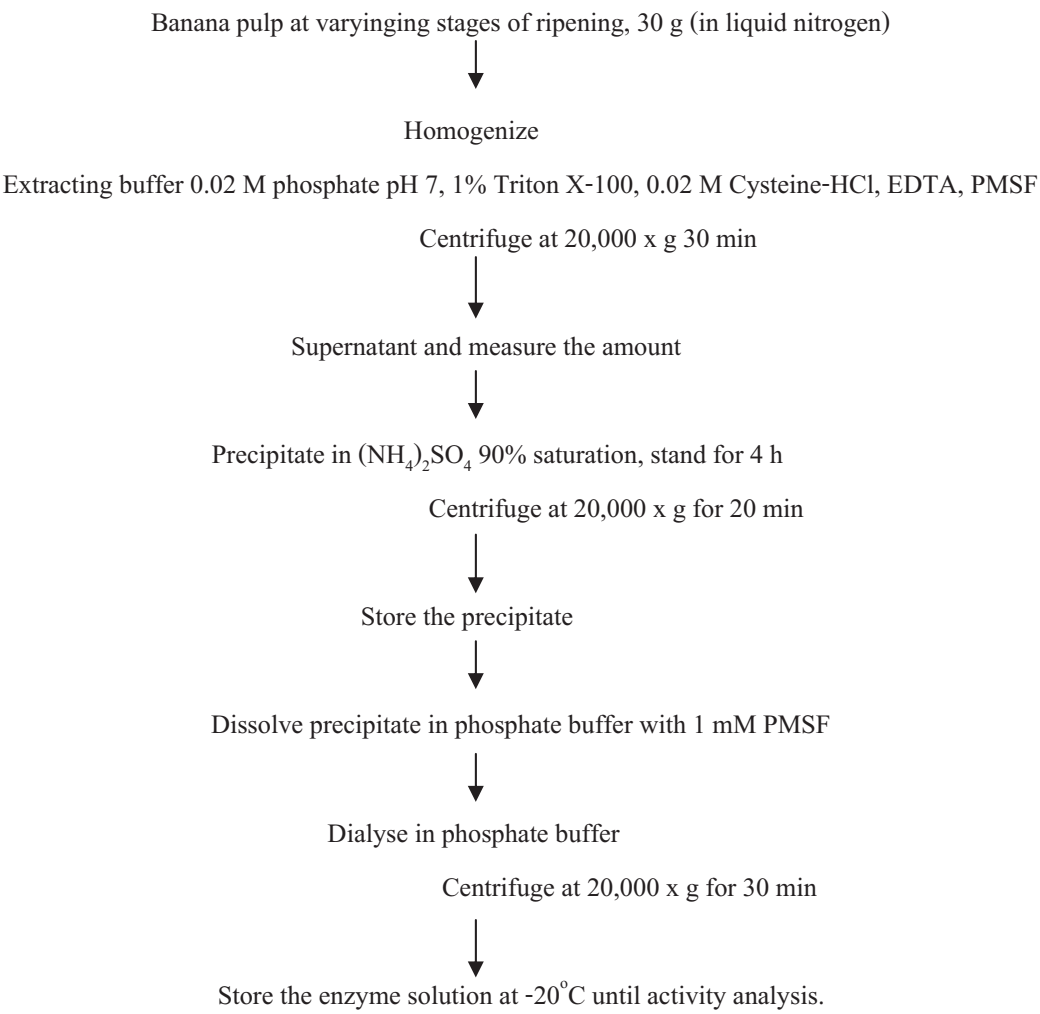

Figure 1 Steps in the extraction of enzymes from bananas. All steps carried out at 4-6°C. (Adapted from Pathak *et al*., 2000 and Payasi *et al*., 2006).

and constantly stirred until 90% saturation. The solution was then stored in a cold room (4°C) for 4 h, after which it was centrifuged again to separate out the enzyme precipitate, which was dissolved in 0.02 M sodium phosphate buffer (pH 7), into which 1 mM PMSF had been added. The enzyme solution was dialysed overnight to separate out the salt. The enzyme solution from dialysis was centrifuged at 15,000 x g for 15 min. The clear supernatant of partially purified enzyme was analyzed for enzyme activity and protein.

Assay of polygalacturonase activity

This process was adapted from Pathak and Sanwal (1998). The analysis involved measuring reducing sugar by the DNS method (Miller, 1959). The assay medium reagents were 0.2 M acetate buffer, pH 4.5 to the amount 0.2 ml, and 1% polygalacturonic acid in 0.05 M acetate buffer solution pH 4.5 to the amount 0.3 ml. One ml of enzyme solution and distilled water was added. The reaction started by adding the enzyme, and it was then left for 30 min at 37°C, after which the reaction was stopped by adding DNS. The solution was then boiled in water for 5 min, after which it was diluted and absorbance measured at a wavelength of 520 nm, using galacturonic acid (0 – 1 mg/ml) as the standard solution.

One unit of polygalacturonase was defined by the catalyzation of the hydrolytic cleavage to form 1 nM of galacturonic acid in 1 s under standard conditions.

Assay of pectate lyase activity

This process was adapted from Silva *et al.* (1993). The extracted pectatelyase activity was measured through an assay medium consisting of 0.3% polygalacturonic acid dissolved in 20 mM sodium acetate buffer at a pH of 4.5 mixed with the 1.5 ml prepared enzyme at an appropriate concentration. The extract was placed in a test tube, shaken well to achieve thorough mixing and incubated in a water bath at 37°C for 30 min. The enzymatic reaction was stopped by boiling in a water bath for 2 min. Measurement of the the resultant reaction indicated 4 5 unsaturated uronides at a wavelength of 235 nm. The controlling solution was denatured by boiling in a water bath for 10 min prior to reaction.

One unit of pectate lyase was defined by the catalyzation of the cleavage of polygalacturonic acid with increasing absorbance at a wavelength of 235 nm equivalent to 0.2 within 10 min under standard conditions.

Protein determination

The amount of protein was determined by Lowry's method (Lowry *et al.*, 1951) through the use of Bovine serum albumin as the standard protein.

RESULTS AND DISCUSSION

Following the extraction of crude enzymes from bananas at various stages of ripeness, using an extraction process with a sodium phosphate buffer and centrifuging at 20,000xg for 30 min, it was found that the determination of the enzymes was not possible due to excessive amount of sugars. Enzymatic activity is usually measured by the resulting reducing sugar, which in this experiment was excessive and distorted the results. Table 1 shows that the reducing sugar significantly increased ($p \leq 0.05$) as ripening proceeded. Additionally, when the enzyme amount was remeasured, the results were highly variable and thus have not been included in this paper. Marin-Rodriguez *et al.* (2003) found that in crude enzymes there were high levels of carbohydrate and phenols making enzymes unstable and so it was difficult to measure enzymatic activity (Dominquez-Pulgjaner *et al.*, 1992). Thus enzyme must be extracted by the addition of a protease inhibitor and partial purification using ammonium sulphate precipitation and dialysis. This procedure will separate out the sugars and phenolic

compounds from the enzyme solution, whose activity can then be determined.

Results in Table 1 show the ripening stages of bananas over time (days) and the significant increase in reducing sugar ($p \leq 0.05$). When the bananas ripened further (yellow and fully ripe at Stage 6), the activity of PG increased from 2.58-3.0 units/g fresh pulp up to 3.93-4.89 units/g fresh pulp, However, beyond this stage, the activity of PG decreased slightly to 3.69-4.44 units/g fresh pulp at Stage 8. Both bunches of bananas showed similar results (Figure 1), with a protein increase with increased ripeness (Table 1). The results corresponded to a study conducted on enzymes in ripe fruit by Ali *et al.*(2004), who found that bananas (*Musa acuminata,* cv. Mas,

Table 1 Changes in reducing sugar, polygalacturonase (PG) activities and protein from Hom Thong banana fruit at various stages of ripening.

Stages	Days after ripening	Bunch No. 1			Bunch No. 2		
		Reducing sugar (mg/g)	PG (U/g)	Protein (mg/g FW)	Reducing sugar (mg/g)	PG (U/g)	Protein (mg/g FW)
2	1	2.52 ± 0.00 ^{a}	3.0 ± 0.11 ^{a}	3.27 ± 0.04	3.60 ± 1.70 ^{a}	2.58 ± 0.41 ^{a}	3.42 ± 0.11
3	2	28.80 ± 9.44 ^{ab}	3.81 ± 0.20 ^{ab}	5.92 ± 0.50	25.69 ± 3.65 ^{a}	2.66 ± 0.40 ^{a}	6.37 ± 0.11
4	2.5	36.66 ± 6.39 ^{ab}	3.95 ± 0.35 ^{bc}	6.07 ± 0.49	35.75 ± 11.18 ^{a}	3.21 ± 0.49 ^{b}	7.58 ± 0.11
5	3	41.60 ± 8.72 ^{ab}	4.58 ± 0.36 ^{bc}	8.18 ± 0.94	46.06 ± 8.67 ^{a}	3.43 ± 0.15 ^{b}	7.57 ± 0.11
6	4	103.68 ± 5.01 ^{c}	4.89 ± 0.39 ^{c}	8.18 ± 0.38	75.98 ± 4.56 ^{a}	3.93 ± 1.71 ^{c}	8.01 ± 0.11
7	4.5	115.70 ± 31.62 ^{c}	4.44 ± 0.68 ^{bc}	8.91 ± 0.29	77.57 ± 13.21 ^{a}	3.69 ± 0.43 ^{bc}	8.70 ± 0.11
8	5	118.5 ± 0.00 ^{c}	4.33 ± 0.49 ^{bc}	9.01 ± 0.09	117.75 ± 16.22 ^{a}	3.22 ± 0.13 ^{b}	9.46 ± 0.11

Values are mean ± standard deviation from two experiments.

Means in each column that are followed by the same letter are not significantly different at $p \leq 0.05$ by Duncan's multiple range test (DMRT).

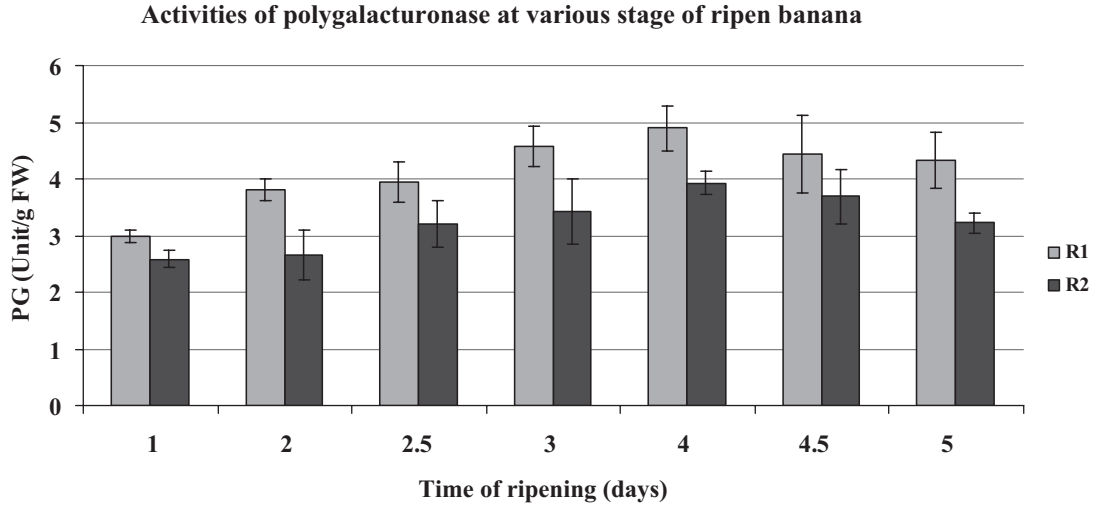

Figure 1 Polygalacturonase (PG) in banana pulp at ripening Stages 2 to 8, from 2 bunches (R1 and R2). Vertical bars are averages with standard deviation. One unit of PG is defined by the amount of catalyzation of the hydrolytic cleavage to form 1 nM of galacturonic acid in 1 sec under standard conditions.

AA) that were mature and dark green had enzymatic activity at PG 2.7 ± 0.7 nkatal/g fresh pulp but activity rose to 9.5 ± 1.7 nkatal/g fresh pulp, an increase of 252%. Asif *et al*. (2005) found that PG increased on the third day, similar to forced ripening, where it was found that the PL level increased, but after that activity decreased. Pathak and Sanwal (1998) studied PG in bananas (*Musa acuminata* cv. Hari Chhal) and found PG by the second day with increasing activity, but that it decreased when the fruit became too ripe. This result was similar to that of tomatoes because respiration is low when the fruit is green. In mangoes, PG increased as the ripening progressed. In guava, PG decreased with a reduction in the firmness of the fruit (Abu-Goukh and Bashir, 2003). Similar to mangoes, the activity of PG in the pulp maximized in the post-climacteric period; thereafter it dropped (Abu-Sarra and Abu-Goukh, 1992; Prasanna *et al*., 2006).

From Table 2, it can be seen that activity of pectate lyase (PL) significantly increased ($p \leq 0.05$) as ripening proceeded, from 8.73-9.59 unit/g fresh banana at Stage 2 to a final peak of 21.92-35.37 unit/g fresh banana at Stage 8. Both bunches showed slight differences as indicated in Figure 2. The results corresponded with studies on tropical fruits (Marin-Rodriguez *et al*., 2003). No reports were found on the determination of PL in ripe tropical fruit. However, studies with bananas (*Musa* AAA Group, cv. Grand Nain) did not find any PL. PL inhibitor may be found in the skin, but in the pulp, the amount of PL corresponded to the ripening stage. Asif *et al*. (2005) reported that the level of PL increased on the third day after forced ripening, similar to PG, but thereafter the level did not drop off.

The extraction of enzymes from banana pulp indicated PG activity of 3.93-4.89 unit/g fresh banana when at Stage 6 of ripeness, where the whole fruit was yellow having 5 units/ml. For comparison, commercial pectinase (Pectinex Ultra SP-L, Novozymes A/S, Denmark) is prepared from *Aspergillus aculentus* and used in the food industry for fruit juice processing to reduce viscosity. It contains different pectinolytic and cellulolytic enzymes PG, PL and Polymethylesterase (PME) and other activities, such as β-glucosidase chitinase and transgalactosidase. Pectinex Ultra SP-L has PG activity at 7,966.46 units/ml, which is more than 1,600 times higher than the enzyme extract from bananas. To obtain a higher activity, the enzyme extract must be purified but only a tripling, from 1.16 units/ml to 3.87 units/ml, has been achieved (Pathak and Sanwal, 1998). When the activity of PL at Stage 7 of ripening was

Table 2 Changes in reducing sugar, pectate lyase (PL) activities and protein from Hom Thong banana fruit at various stages of ripening.

Stages	Days after ripening	Bunch No. 1			Bunch No. 2		
		Reducing sugar (mg/g)	PL (U/g)	Protein (mg/g FW)	Reducing sugar (mg/g)	PL (U/g)	Protein (mg/g FW)
2	1	2.52 ± 0.00 ^{a}	8.73 ± 0.23 ^{a}	3.27 ± 0.04	3.60 ± 1.70 ^{a}	9.59 ± 0.09 ^{a}	3.42 ± 0.11
3	2	28.80 ± 0.44 ^{ab}	11.84 ± 0.99 ^{ab}	5.92 ± 0.50	25.69 ± 3.65 ^{a}	15.23 ± 0.37 ^{b}	6.37 ± 0.11
4	2.5	36.66 ± 6.39 ^{ab}	16.32 ± 4.43 ^{b}	6.07 ± 0.49	35.75 ± 11.18 ^{a}	20.73 ± 0.56 ^{de}	7.58 ± 0.11
5	3	41.60 ± 0.72 ^{ab}	25.57 ± 3.96 ^{c}	8.18 ± 0.94	46.06 ± 8.67 ^{a}	18.47 ± 0.18 ^{c}	7.57 ± 0.11
6	4	103.68 ± 5.01 ^{c}	25.06 ± 1.31 ^{c}	8.18 ± 0.38	75.98 ± 4.56 ^{a}	19.45 ± 0.24 ^{cd}	8.01 ± 0.11
7	4.5	115.70 ± 1.62 ^{c}	27.72 ± 0.31 ^{c}	8.91 ± 0.29	77.57 ± 13.21 ^{a}	23.09 ± 0.14 ^{bc}	8.70 ± 0.11
8	5	118.5 ± 0.00 ^{c}	35.37 ± 1.05 ^{d}	9.01 ± 0.09	117.75 ± 16.22 ^{a}	21.93 ± 0.05 ^{b}	9.46 ± 0.11

Values are mean ± standard deviation from two experiments.

Means in each column that are followed by the same letter are not significantly different at $p \leq 0.05$ by DMRT.

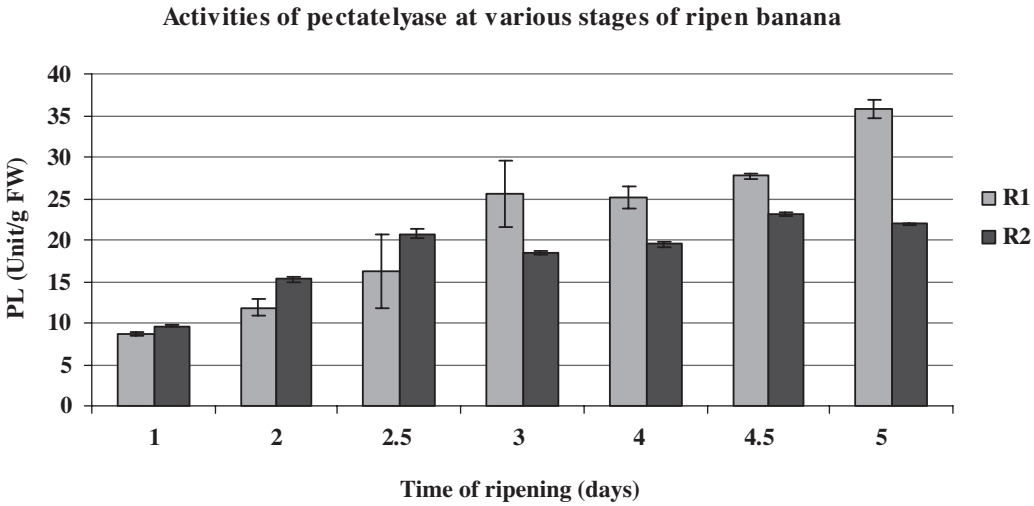

Figure 2 Pectate lyase in banana pulp at ripening Stages 2 to 8, from two bunches (R1 and R2). Vertical bars are averages with standard deviation. One unit of PL is defined by the amount of catalyzation of the cleavage of polygalacturonic acid with an increase in absorbance at a wavelength of 235 nm that is equivalent to 0.2 within 10 min under standard conditions.

checked, it was found to have 21.93-35.37 units/g fresh fruit or 20 units/ml of the crude enzyme solution. In comparison, commercial pectinase has 1,709 units/ml of pectinase.

CONCLUSION

The increase in PG and PL activity during banana ripening confirmed the belief that they were directly connected with the ripening of bananas. Activity of banana PG was maximized when fully ripened and reduced slightly when ripening progressed further. PL activity continued to increase according to the stage of ripeness. Enzymes extracted from bananas were present in very low amounts low when compared with the commercially available enzymes (Pectinex Ultra SP-L, Novozymes A/S, Denmark) used in the fruit juice industry, whose PG activity (7966.46 unit/ml) was over 1,000 times higher than the enzymes in bananas and 50 times higher than PL activity (1,709 units.ml). On the other hand, the extraction procedures, as well as the reagents used, may have influenced the yield but in any case would not be comparable to the yield from the biological process. Studies on emzymatic ripening, as well as softening, of fruit would be useful where it is necessary to prolong the fruit quality postharvest. Such studies may include the influence of a reduced enzymatic activity level in the modified-packing atmosphere or during forced ripening to extend the shelf life of fruit.

ACKNOWLEDGEMENT

This work was financially supported by the Kasetsart University Research and Development Institute (KURDI).

LITERATURE CITED

Abu-Goukh, A. B. A. and H. A. Bashir. 2003. Changes in pectic enzymes and cellulase activity during guava fruit ripening. **Food Chem**. 83: 213-218.

Abu-Sarra, A. F. and A. B. A. Abu-Goukh. 1992. Changes in pectinesterase, polygalacturonase and cellulase activity during mango fruit ripening. **J. Hort. Sci.** 3(2): 32-48.

Ali, Z.M., L. H. Chin and H. Lazan. 2004. A comparative study on wall degrading enzymes, pectin modifications and softening during ripening of selected tropical fruits. **Plant Sci.** 167: 317-327.

Asif, M.H. and P. Nath. 2005. Expression of multiple forms of polygalacturonase gene during pipening in banana fruit. **Plant Physio. Biochem**. 43: 177-184.

Brummell, D.A. and M.H. Harpster. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. **Plant Mol. Biol**. 47: 311-340.

CSIRO. 1972. Division of food research circular: Banana ripening guide. Cited Silayoi, B. 2002. **Banana**. Population Publication. Ltd., Bangkok. 290pp.

Crookes,P.R. and D. Grierson. 1983. Ultrastructure of tomato fruit ripening and the role of polygalacturonase isozymes in cell wall degradation. **Plant Physio** . 72: 1088-1093.

Dominiguez-Puigjaner, E., M. Vendrell and M.D. Ludevid, 1992. Differential protein accumulation in banana fruit during ripening. **Plant Physiol.** 98: 157-162.

FAO. 2006. **Banana statistics 2005**. Available Souce: http://www.faostat.fao.org/faostat/collections, May 12, 2007.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Ranrall. 1951. Protein measurement with Folin phenol reagent. **J. Biol Chem.** 193: 265-275.

Marin-Rodriguez, M.C., D.L. Smith, K. Manning, J. Orchard and G.B. Seymour. 2003. Pectate lyase gene expression and enzyme activity in ripening banana fruit. **Plant Molec. Biol**. 51: 851-857.

Marin-Rodriguez, M.C., J. Orchard and G. B. Seymour. 2002. Pectate lyases, cell wall degradation and fruit softening. **J. of Exp. Bot.** 53: 2115-2119.

Markovic O., K. Heinrichova and B. Lenkey. 1975. Pectolytic enzymes from banana. **Collect Czech Chem Commun.** 40:769-774.

Miller, G.L. 1959. Use of diditrosalicylic acid reagent for determination of reducing sugar. **Anal. Chem.** 31: 426-428.

Pathak, N. and G.G. Sanwal. 1998. Multiple forms of polygalacturonase from banana fruits. **Phytochem**. 48 (2): 249-255.

Pathak, N., S. Mishra and G.G. Sanwal. 2000. Purification and characterization of polygalacturonase from banana fruits. **Phytochem**. 54: 147-152.

Payasi, A., P.C. Misra and G.G. Sanwal. 2006. Purification and characterization of pectate lyase from banana (*Musa acuminota*) fruits. **Phytochem**. 67: 861-869.

Prasanna, V., T.N. Prabha and R.N. Tharanathan. 2006. Multiple forms of polygalacturonase form mango (*Mangifera indica* L. cv *Alphonso*) fruit. **Food Chem**. 95: 30-36.

Silva, D.O., M.M. Attwood and D.W. Tempest. 1993. Partial purification and properties of pectin lyase from *Penicillium expansum*. **World J. Microbiol. Technol**. 9: 574-578.

Smith, N. J. S. 1989. **Texture and Biochemical Changes during Ripening of Bananas**. Ph.D. Thesis, University of Notthingham, UK.