รายงานวิจัยฉบับสมบูรณ์

Biochemical and Molecular approaches to modify the ripening process in mango fruit.

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สารบัญ

บทสรุปของผู้บริหาร

การดัดแปรกระบวนการสุกของมะม่วงโดยการใช้เทคนิคทางชีวเคมี และพันธุวิศวกรรมเพื่อพัฒนาคุณภาพของผลมะม่วง

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ความสำคัญของปัญหา

มะม่วงจัดเป็นผลไม้ส่งออกที่สำคัญของประเทศไทย ปริมาณการส่งออกในลักษณะผลสด 8522 ตัน (2540) มีมูลค่าถึง 4 ล้านเหรียญสหรัฐ แต่เป็นเพียง 0.75% ของผลผลิตมวลรวมของมะม่วงทั้งประเทศ ทั้งนี้เนื่องจากปัญหาของการจัดการหลังการเก็บเกี่ยว และมีช่วงระยะเวลาการสุกที่สั้น จึงส่งผลทำให้

เกิดการเสียหายเป็นจำนวนมาก นอกจากนี้การเก็บมะม่วงที่แก่ไม่เต็มที่เพื่อยืดอายุการเก็บรักษานั้น มักจะส่งผลต่อคุณภาพของมะม่วงเมื่อกระตุ้นให้สุกโดย ethylene เช่น มีลักษณะการสุกที่ผิดปกติ

ความพยายามในการปรับปรุงให้มะม่วงมีคุณสมบัติที่มีช่วงระยะเวลาการสุกที่นานขึ้นนั้นจะเป็น หนทางหนึ่งในการช่วยส่งเสริมการส่งออกได้โดยตรง อีกทั้ง ยังช่วยส่งเสริมอุตสาหกรรมแปรรูปมะม่วงในทางอ้อมด้วย

วัตถุประสงค์

มุ่งศึกษากลไกการสุกของมะม่วง ทั้งในระดับชีวเคมี และโมเลกุล เพื่อที่จะเป็นหนทางในการควบคุมหรือดัดแปรกระบวนการสุก โดยเฉพาะอย่างยิ่งการนิ่ม (Softening) ของผลมะม่วง โดยใช้เทคนิคพื้นฐานทางชีวเคมี และเทคนิคทางพันธุวิศวกรรม

วิธีการดำเนินการ

การดำเนินการแบ่งออกเป็น4ส่วนคือ

- I. ศึกษาการเปลี่ยนแปลงของการแสดงออกของยีนส์ในระหว่าง การสุกของมะม่วง
- II ศึกษาการเปลี่ยนแปลงของเอนไซม์ที่เกี่ยวข้องกับการนิ่มของมะม่วง และศึกษาการเปลี่ยนแปลงของผนังเซลของมะม่วงในระหว่างการสุก
- III สืบค้นหา ยีนส์ ที่มีความจำเพาะต่อการนิ่มของมะม่วง
- IV การสร้าง cDNA library

ผลการดำเนินงาน

I ศึกษาการเปลี่ยนแปลงของการแสดงออกของยีนส์ในระหว่าง การสุกของมะม่วง

- พบการเพิ่มขึ้นทั้งชนิด และปริมาณของโปรตีนบางชนิดใน ระหว่างการสุกของมะม่วง
- พบการเพิ่มขึ้นของ total RNA และ mRNA บาง ชนิดในระหว่างการสุก
- พบการสังเคราะห์โปรตีนใหม่ในระหว่างการสุก ของมะม่วงโดยการใช้เทคนิค *in vitro* translation
- J.Biochem. Mol. Biol & Phys., 3, 75-79 (1999)

II ศึกษาการเปลี่ยนแปลงของเอนไซม์ที่เกี่ยวข้องกับการนิ่มของมะม่วง และศึกษาการเปลี่ยนแปลงของผนังเซล ของมะม่วงในระหว่างการสุก

- พบความสัมพันธ์ของการเพิ่มของปริมาณเอนไซม์ที่เกี่ยวข้องกับการสลายผนังเซลล์ของมะม่วงในระหว่างการสุก (exo-polygalacturonase b-galactosidase และ pectin methyl esterase)
- พบความสัมพันธ์ของการเพิ่มของเอนไซม์ที่เกี่ยวข้องกับการสลายแป้งในมะม่วงในระหว่างการสุก (a-amylase)
- พบมีการเปลี่ยนแปลงของผนังเซลล์ของเนื้อมะม่วงในระหว่างการสุก โดยการศึกษาทางเคมีวิเคราะห์ และการใช้กล้องจุลทรรศน์กำลังขยายสูง (Scanning eletron microscopy)
- ทำการแยกเอนไซม์ที่เกี่ยวข้องกับการสลายผนังเซลล์ของมะม่วงให้บริสุทธิ์ ศึกษาคุณสมบัติของเอนไซม์นั้น เพื่อเป็นหนทางในการควบคุมเอนไซม์ดังกล่าว ทั้งในเชิงชีวเคมี และพันธุวิศวกรรม
- 1. Acta Hort.,509, 171-175 (2000)
- 2. Science Asia (2002) Accepted

III. สืบค้นหายีนส์ที่มีความจำเพาะต่อการนิ่มของมะม่วง

เทคนิค	Primer	mRNA	ผลการวิเคราะห์
RT-PCR	endo PG	400-890	มีความคล้ายคลึงกับ endo-PG ของ Kiwifruit (811 bps)
	PE	300-630	มีความคล้ายคลึงกับ PE ขององุ่น (355 bps)
			มีความคล้ายคลึงกับ aquaporin ของมะเขือเทศ (300bps)
	exo PG	300-1500	มีความคล้ายคลึงกับ hygromycin phosphotransferase
			ของ Arabidopsis (900 bps)
Differential	universal	1000-1500	กำลังทำการวิเคราะห์

- พบความสัมพันธ์ระหว่างการแสดงออกของยีนส์ PE, endo PG และ b-galactosidase กับขบวนการสุกของมะม่วง
- และงานบางส่วนตีพิมพ์ในวารสาร
- 1. J. Biochem. Mol. Biol & Biophys. 3, 75-79, 1999
- 2. Acta Hort 509, 153-158, 2000

IV. การสร้าง cDNA library

- ชิ้นส่วนของ cDNA ที่ได้จากการสืบค้นถูกนำมาต่อเข้ากับ DNA พาหะ และเก็บไว้ในรูปของ cDNA library ใน plasmid และ phage vector เพื่อการใช้งานและศึกษาในรายละเอียดต่อไป

ประเด็นที่เกี่ยวข้องกับการวิจัย

- การสืบค้นและแยกยีนส์ที่เกี่ยวข้องกับกระบวนการสุกของมะม่วง โดยเฉพาะที่เกี่ยวข้องกับกระบวนการนิ่มที่แยกได้ และเก็บไว้ใน cDNA library นั้นจะเป็นประโยชน์อย่างยิ่งต่อการนำมาใช้ช่วยในการปรับปรุงพันธุ์มะม่วง ทั้งวิธีมาตรฐาน (conventional) เช่น การใช้ ช่วยตรวจสอบหาสายพันธุ์ที่มีการแสดงออกของยีนส์เป้าหมาย เพื่อใช้เป็นตัวคัดเลือกสายพันธุ์ หรือวิธีพันธุวิศวกรรมโดยการทำการดัดต่อยีนส์เพื่อสร้างสายพันธุ์ใหม่ที่มีคุณสมบัติที่เก็บรักษาได้นาน และทนต่อการขนส่ง ซึ่งจะเอื้อประโยชน์ต่ออุตสาหกรรมการส่งออกของมะม่วงในอนาคต

Executive Summary

Project Title : Biochemical and molecular approaches to modify the ripening process in mango fruit (RD G2/017/2540)

Investigators : Porntip Chaimanee, Oranart Suntornwat ,Linda Bungaruang , Nuansamorn Lertwikool and Periya Sriroud
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Fruit ripening is a genetically programmed process that involves coordinated changes in fruit firmness, sweetness, acidity, aroma and pigmentation. From an economic standpoint, textural change is the most crucial of all, as it directly affects the shelf-life of the fruit and its keeping quality. The importance of understanding the softening process and its implications are emphasised on carbohydrate solubilization in fruit ripening . Modification of fruit ripening by suppressing expression of specific genes has been extensively studied and demonstrated in tomato . Mango is an economically important crop of many tropical countries including Thailand. Despite the potential , there are several problems associated with the marketing of mango fruits especially fruit softening during ripening, leading to short shelf-life and susceptibility to mechanical damage and invasion by fungal and bacterial pathogens . Recently, we examined the biochemistry and molecular biology of softening in the ripening mango fruit, with the long term objective of finding way to slow the rate of softening and to increase the potential for storage and export of the fruit.

Objectives :

1.Identification of ripening-response proteins in mango especially enzymes involved in softening.
2.Isolation and characterization of the softeninhg related genes and gene proteins.
3.Construction of ripening related cDNA libraries from mango.

Methodology :

The experiments were divided into four parts :

Part I. Study the changes in gene expression during mango fruit ripening.
Part II.Study the changes in softening enzymes and changes in cell wall structure.
Part III.Identification of the specific gene(s) related to mango fruit softening.
Part IV.Partial construction of cDNA library.

Results :

Part I. Study the changes in gene expression during mango fruit ripening

Changes in gene expression during ripening in mango fruit were investigated. Total soluble proteins were extracted from mango at various stages of ripening and analysed by one- and two-dimensional gel electrophoresis. The considerable increase of 64, 42,39 and 26 kDa polypeptides were observed. Along with the protein extraction, the total RNA were extracted and poly (A+) RNAs were purified from various stages of mango fruits.The poly (A+) RNAs were subjected to further analysis by in vitro translation. Analysis of the translated products by SDS gels electrophoresis and autoradiography demonstrated the accumulation of 67, 61 and 48 kDa polypeptides. The results indicate that ripening in mango is associated with the expression of some specific genes (1).

Part II. Study the changes in softening enzymes and change in cell wall structure.

A comprehensive picture of changes in carbohydrates, carbohydrate hydrolases, cell wall structure and texture of mango fruit during ripening were described. The starch and pectin fractions considerably decreased in ripe mango pulp. The significant decrease in galactose level in cell wall implied the degradation of neutral pectic component during ripening, The activities of various carbohydrate hydrolases: exo-polygalacturonase, pectinmethylesterase, β-galactosidase and α-amylase were clearly increased. The electron microscopic study was performed to monitor the changes in cell wall structure and also the amount of starch granules through the process of ripening (2).

Exopolygalacturonase and β-galactosidase were purified and characterized on SDS-PAGE. Exopolygalacturonase was associated with two polypeptide chains with the relative molecular weight of 66 and 58 kDa (3) whileβ-galactosidase activities were of 62 and 37 kDa (4).

Fractionated proteins were transfered onto PVDF membrane . The interesting polypeptide was excised and N-terminal amino acid sequence information can be obtained from the protein-bound PVDF membrane by automatic gas phase protein sequencer at BIOSERVICE UNIT, Mahidol university. Unfortunately, N-terminal sequence were not obtained due to technical problem.

Part III. Identification of the specific gene(s) related to mango fruit softening

The specific genes related to the process of mango fruit softening especially the genes of cell wall hydrolases enzymes were investigated. The total RNAs were isolated from different stages of mango fruits during the ripening process . The poly (A+) RNAs were then purified from total RNA by commercial purification kit (PROMEGA and QIAGEN). The poly (A+) RNAs (mixed from different stages) were then subjected to RT-PCR using specific primers for PG and PE enzymes. Gel fractionation of the RT-PCR products obtained from the reaction using PG-specific primmers showed a series of band ranging from 490-890 bps. The cDNA band of approximately 890 bps were eluted from the gel ligated into plasmid and analysed. The 811-bps

fragment is related by sequence similarity with c-terminal of a number of plant polygalacturonase with the nearest match to kiwi fruit endo-PG (5). The products from the reaction using PE-specific primers showed a series of bands ranging from 300- 630 bps. Two bands of approximately 300 and 355 bps were similarly analysed. The sequence of the 300 -bps fragment is related to aquaporin (transmembrane protein) of tomato , while the 355-bps fragment is closely related to pectin methyl esterase enzymes from various sources with the nearest match to grape . The PE , endo-PG and aquaporin fragments were kept as cDNA library and the PE one was used successfully as probes to follow the expression of these genes during the ripening process in mango as previously reported (1). Northern blot analysis of total RNA from differnt stages of ripening using PE probes showed the increase in PE gene expression during ripening as previously reported, while the endo-PG probes barely detected by hybridization (6).

RT-PCR was also performed using mRNA mixture from different stages of ripening and exo-PG specific primer (EXO 1F and MAN1 REV). Gel fractionation of the RT-PCR product showed a series of band from 300-1500 bps . Three bands of approximately 400, 900 and 1000 bps were similarly analysed. The one with about 1000 bps is related to hygromycin phosphotransferase of Arabidopsis thaliana . The other two fragments were kept as cDNA library for further study and will be sent to Central Equipment Laboratory , Mahidol University for sequence analysis.

mRNA differential display was performed . The first strands of cDNAs were synthesized from the poly (A+) mRNAs from different stages of ripening using commercial cDNA synthesis kit with universal primers (ZAP-Express Gold Cloning Kit, STRATAGENE). Differences in banding patterns between unripe, halfripe and ripe of differential display reactions were identified and the distinct bands in the range of 100-1500 bps were excised and eluted from the gels. The Dna fragments were then ligated into the plasmids. Some of them were kept as cDNA library for further study. Some clones will also be sent for sequence analysis at BIOSERVICE UNIT, Mahidol University .

Dot blot analysis of total RNA from different stages of ripening were performed using our PE probe and mango b-galactosidase probe kindly provided by Dr. G A Tucker showed the increase in the expression of both genes during ripening.

Part IV. Partial construction of cDNA library

All of the cDNAs obtained from the RT-PCR and differential display experiments were ligated into the vector and cloned. These clones include PE, endo-PG aquaporin and other fragments that still need to be properly identified.

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Changes in Gene Expression during Mango Fruit Ripening

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Changes in gene expression during ripening in mango fruit were studied. The total soluble proteins were extracted from mango at various stages of ripening and then analysed by one- and two-dimensional gel electrophoresis. A considerable increase of 64, 42, 39 and 26 kDa polypeptides was observed. In a similar manner, the total RNA and poly(A)$^+$ mRNA were isolated and subjected to *in vitro* translation. The amount of total RNA increased during the ripening process especially from unripe to ripe stage. Analysis of the translated products by SDS gels electrophoresis and autoradiography has showed the accumulation of 67, 61 and 48 kDa polypeptides. The results obtained from our experiments indicated that the ripening process in mango is associated with the expression of specific genes.

Keywords: *Mangifera indica*, Mango, Fruit ripening, Gene expression

INTRODUCTION

Fruit ripening is a genetically programmed process that involves coordinated changes in fruit firmness, sweetness, acidity, aroma and pigmentation. These changes are caused by alterations of enzyme activities, which is thought to be determined by changes in specific gene expression. Recently, the molecular analysis of tomato fruit ripening has enabled us to understand the mechanisms that regulate the ripening-induced genes in this system [1]. However, with the exception of avocado [2], other economically important crops have received much less attention in this regard. Extreme diversity in growth, physiology and storage characteristics exists across the range of fruit types, and this diversity may be reflected at the molecular level.

Mangos (*Mangifera indica*, L.) are climacteric fruits, with their ripening associated with an upsurge in the rate of respiration and ethylene production. Mango suffers from short storage life due to its rapid rate of ripening. During mango ripening there is an increase in the activity of cell wall hydrolysing enzymes such as polygalacturonase [3] and β-galactosidase [4] which can be delayed by cold storage at 10°C [5].

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The aim of the present study was to determine the role of gene expression during the ripening process of mango fruits. The changes in fruit mRNAs and protein population during the process were monitored. It was anticipated that such studies would provide a basis for future investigation especially in the modification of the ripening process and storage behaviour of this crop.

MATERIALS AND METHODS

Plant Material

Mango fruits (*Mangifera indica*, L., cv. Nam-Dorkmai) of 16 weeks old were harvested from commercial orchards in Nakorn Pathom (Thailand). The fruits were immediately shipped back to the laboratory and allowed to ripe at 25°C for 8 days. Once the fruits were ripe, they were peeled, sliced and then frozen in liquid nitrogen and stored at −80°C until needed. Some fruits were sliced and frozen on arrival at the laboratory, these were referred to as unripe fruit. The mesocarp tissue was used in the experiments.

Protein Extraction

Proteins were extracted by homogenisation of the frozen mango pulp in Laemmli buffer [6], containing 10.5% Ficoll, 2.5% SDS, 200 mM DTT in 63 mM Tris buffer pH 6.8 in 1:1 ratio. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was then analysed directly by gel electrophoresis.

Extraction of Total RNA

Total RNA was extracted from frozen mango pulps following the method described by Lopez and Gomez-Lim [7].

Poly(A)$^+$mRNA Preparation and *in vitro* Protein Synthesis

Poly(A)$^+$mRNA was purified from total RNA by using Oligotex^{TM} mRNA purification kit (QIAGEN). The purification of poly(A)$^+$mRNA was performed by the procedures described by the manufacturer. The poly(A)$^+$mRNA was translated in the 25 μl assay wheat germ lysate system according to the procedure described by Promega using L-^{35}S Methionine with Brome Mosaic Virus (BMV) RNA as a positive control. The reactions were optimised for Mg^{2+} and K^+. Incorporated radioactivity was determined according to Mans and Novelli [8].

Gel Electrophoresis

Protein samples obtained from the direct extraction and from *in vitro* translation were electrophoresed on the 0.75 mm thick of 12% acrylamide SDS slab gels [6]. Two-dimensional gel electrophoresis was also performed using the method described by O'Farrell [9]. The proteins were precipitated with 4 volumes of ice-cold acetone, washed with the same solution, and resuspended in the loading buffer for two-dimensional gels. After separation by electrophoresis the gels were stained with Coomassie Blue R, dried and exposed to an Bio Max MR film at −80°C.

The integrity of total RNA from mango was tested by formamide/formaldehyde denaturation and agarose gel electrophoresis as described by Fourney *et al.* [10] and visualised by etidium bromide staining.

RESULTS

Protein Extraction

The SDS-PAGE analysis of the extracted proteins from the mango mesocarp tissue at various stage of ripening revealed different pattern between ripe and unripe mango. The 64, 42, 39 and 26 kDa polypeptides seemed to increase during ripening, while the 62 kDa decreased (Fig. 1). When the same extracts were analysed by two-dimensional gels (Fig. 2), 36 and 26 kDa polypeptides with acidic pI were observed in ripening fruit.

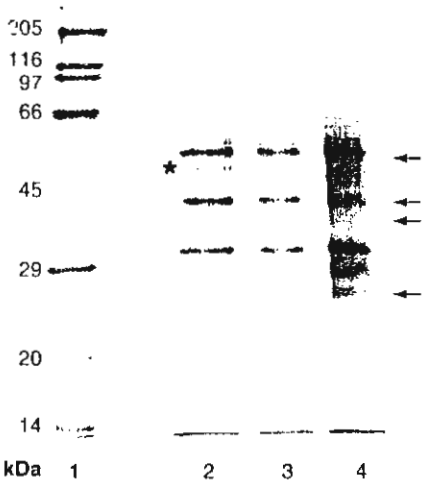

FIGURE 1 Total proteins extracted from mango fruits at various stage of ripening. Proteins were extracted from mango mesocarp and separated on 12% SDS-PAGE. Arrows indicate proteins appearing during ripening and an asterisk indicates protein disappearing during ripening. Lane 1: Molecular weight marker, lane 2: Unripe fruit, lane 3: Half ripe fruit, lane 4: Fully ripe fruit.

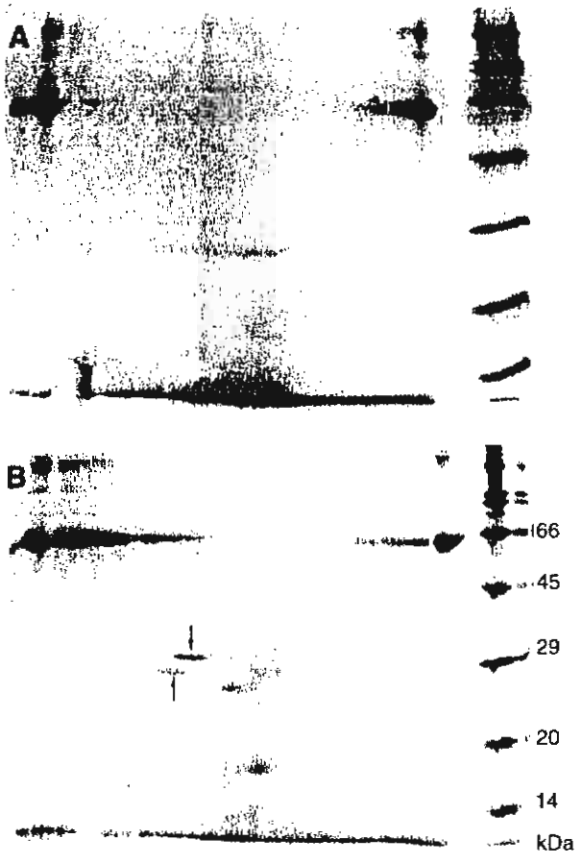

FIGURE 2 Two dimensional gel electrophoresis of total proteins extracted from mango fruit during ripening. Proteins were extracted from mango mesocarp at two stages; unripe (A) and ripe (B) and separated by 2-D gel electrophoresis. The arrows indicate the product appearing during ripening. Numbers on the right-side indicate the molecular weight of the protein markers.

Isolation of Total RNA from Mango Mesocarp

Total RNA obtained from mesocarp by Lopez and Gomez-Lim's method [7] were of good quality and quantity. This method consistently gave good yield (more than 10 μg/g fresh weight) and the A260 : A280 ratio was always higher than 1.85. The amount of total RNA from different stages of ripening increased significantly when it started to ripe as shown in Table I. From the etidium bromide stained gel, four discrete bands corresponding to the 27 and 17S of cytoplasmic rRNA and 26 and 16S of chloroplastic rRNA were obtained (data not shown).

Purification of Poly(A)$^+$ mRNA from Total RNA

The poly(A)$^+$mRNAs were purified using the Oligotex^{TM} mRNA kit (QIAGEN). The yield obtained ranged from 0.10% to 0.42% (Table I). Some of the poly(A)$^+$mRNA were used successfully for *in vitro* translation.

In Vitro Translation

The mRNA from mango mesocarp stimulated the *in vitro* translation in wheat germ system (Promega) about two-fold over the background.

The labelled translation products after one-dimensional SDS-PAGE and autoradiograph were shown (Fig. 3). Significant differences were observed in the patterns of proteins from unripe and ripe mango fruit mRNAs. Proteins of molecular weight 67, 61 and 48 kDa were found to increase while that of 34 kDa was found to decrease.

TABLE I Amount of total RNA and poly(A)$^+$mRNA from mango fruits

Stage of development	(μg/g) fresh weight		(%) poly(A)$^+$mRNA
	total RNA	poly(A)$^+$mRNA	
Unripe (day 1)	37.13	0.037	0.10
Half ripe (day 3)	41.90	0.148	0.30
Fully ripe (day 7)	52.46	0.222	0.42

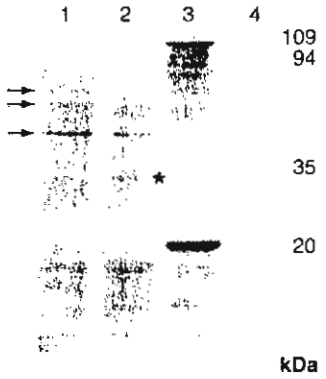

FIGURE 3 Autoradiogram of SDS-PAGE of ^{35}S Methionine polypeptides translated *in vitro* from mango mesocarp poly(A)$^+$RNAs. Equal counts (5×10^6cpm) were applied to each lane. Arrows indicate proteins appearing during ripening and an asterisk indicates proteins disappearing during ripening. Lane 1, added poly(A)$^+$RNAs of ripe fruit; lane 2, added poly(A)$^+$RNAs of unripe fruit; lane 3, Brome Mosaic Virus (BMV) RNA as positive control; lane 4, no added poly(A)$^+$RNAs.

DISCUSSION

The yield of the total RNA (approximate 40–50 μg/g fresh weight) (Table I) was similar to those reported in ripe tomato [11] and mango [7]. The level of total RNA increased significantly during the early stage of ripening (Table I). The levels of poly(A)$^+$mRNAs shown in this study were consistent with the increase of total RNA during mango ripening.

Analyses of *in vitro* translation products indicated that there were major differences in the mRNA population of unripe and ripe mango. The identities of these proteins are still unknown, but there were some similarities in the sizes of the proteins present during ripening to those proteins reported to increase during ripening in mango such as polygalacturonase [3]. In addition to the changes in translation products, there were also changes in the quantities of at least four proteins during the ripening process. Generally, the polypeptide patterns from one-dimensional gel electrophoresis of the *in vitro* translation products cannot readily be compared with those from total protein extracts due to the *in vivo* post-translational processing. However, it implied that changes in the level of these mRNA might be reflected at the protein level.

This report constitutes our first finding in a series of studies on the biochemistry and molecular biology of mango fruit ripening. Further studies are in progress to construct a cDNA library from ripe fruit. A number of homologous cDNA probes will also be constructed to determine whether detectable changes in protein synthesis reflect the changes in transcription and translation.

Acknowledgements

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EXO-POLYGALACTURONASE FROM RIPE MANGO (*Mangifera indica* Linn cv. Nam Dok Mai)

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Keywords: polygalacturonase, ripening process, mango

Abstract

Polygalacturonase (PG) is the most studied cell wall hydrolases. Both exo-PG (EC 3.2.1.67) and endo-PG (EC 3.2.1.15) were detected during the ripening process of mango. The increase in exo-PG was highly correlated with ripening whereas endo-PG activity was constant. Exo-PG was extracted from ripe mango mesocarp tissue. The enzyme has the relative molecular weight of 66 k and 58 k as detected by SDS-polyacrylamide gel electrophoresis. In Northern blot analysis, there was barely detectable signal with the PG probe.

1. Introduction

In many fruits ripening is associated with textural changes that are thought to be due to the disassembly of the primary cell wall (Huber, 1983; Brady, 1987). These modification are at least partly regulated by the expression of gene that encode the cell wall modifying enzymes (Fischer and Bennett, 1991). Pectins are the major class of cell wall polysaccharide which are degraded by both solubilization and depolymerization. In most fruits, endo-polygalacturonase (EC 3.2.1.15) was the key enzymes in ripening associated with pectin degradation. In contrast to tomato, in which exo-polygalacturonase (EC 3.2.1.67) activity was found only in small green fruit and its level remained constant during ripening (Pressey, 1987), exo-PG activity in mango were ripening regulated (Lazan *et al.*, 1986; Chaimanee, 1992). The apparent lack of endo-PG in mango is inconsistent with the observation that a rather extensive depolymerization of cell wall pectins occurs during ripening of the fruit. However, in some fruits, most notably apples (Bartley, 1981) and ripe clingstone peaches (Downs *et al.*, 1992) only exo-PG activity was detected. This paper described the identification, partial purification and some properties of the exo-PG.

2. Material and methods

2.1. Plant meterial

Mango fruits (*Mangifera indica* L. cv. Nam Dok Mai) of 16 weeks old were harvested from commercial orchards in Nakhon Pathom (Thailand). The fruits were immediately transported to the laboratory and allowed to ripen at 25°C for 8 days. Once the fruits were ripe, the skin and seed of the fruits were removed and the mesocarp was diced and stored at –80°C until used. Some fruits were kept frozen upon arrival and was referred to as unripe fruit.

2.2. Extraction and fractionation

Frozen mango mesocarp tissue of 500 g (from each ripening stage) was ground in an ultracentrifugal mill (Retsch, Germany) under liquid nitrogen. The powder was extracted with 1 volume of 100 mM sodium lactate, 200 mM NaCl, 1% polyvinylpyrro-

Proc. Sixth Int'l Mango Symp.
Eds. S. Subhadrabandhu - A. Pichakum
Acta Hort. 509, ISHS 2000

idone, 20 mM β-mercaptoethanol pH 4.0. The extract was stirred at 4°C for 2 hrs and centrifuged 27,000 g for 40 mins at 4°C and the supernatant was filtered through 4 layers of miracloth (Calbiochem). The filtrate was then diluted with 1 volume of 100 mM lactate buffer pH 4.0 with 1 mM DTT. At this stage the extract was referred to as "crude extract".

The crude exdtract was passed through the S-Sepharose FF (Pharmacia LKB Biotechnology) ion-exchange column (2.5 × 30 cm) equilibrated with 50 mM lactate, 100 mM NaCl, pH 4.0. The column was then washed with equilibration buffer containing 1 mM DTT, until zero absorbance at 280 nm was achieved. Protein was eluted with a linear salt gradient from 100 mM to 1 M NaCl. Fractions were collected and those containing activities were pooled and concentrated by Centricon-30 (Amicon).

The concentrated sample was loaded ont^ the Hydroxyapatite column (1.5 × 10 cm) equilibrated with 50 mM lactate (pH 4.0) containing 100 mM NaCl. The column was washed with the equilibration buffer containing 1 mM DTT and fractions were collected. Fractions containing activity were separately pooled and then concentrated by Centricon-30.

2.3. Enzyme activity

Polygalacturonase activity was assayed by measuring the formation of reducing groups by 2-cyanoacetamide method (Gross,1982). The reaction mixture contained 0.1 ml of 1% PGA, 0.8 ml 50 mM lactate, 50 mM NaCl pH 4.0 with an appropriate amount of enzyme preparation in a total volume of 1ml. The reaction was continued for 16 h at 40°C and terminated by the addition of 2.8 ml cold borate buffer, pH 9.0, reducing groups were measured by reaction with 1.2 ml of 1.5% 2-cyanoacetamide. Activity is expressed as the increase in reducing groups as nmole/min. Fractions containing exo-PG activity were distinguished from those containing endo-PG activity by the stimulation of exo-PG activity in response to 0.4 mM calcium in the incubation medium.

2.4. Gel electrophoresis

Electrophoresis of protein was carried out in denaturing polyacrylamide gel (0.75 mm thick, 12% gel) as described by Laemmli (1970).

3. Results

3.1. Extraction and purification of polygalacturonase

Purification of exo-PG was carried using the fully ripe stage fruits as the enzyme source. Two peaks of exo-PG activity were obtained by ion exchange chromatography on S-Sepharose column (Figure 1a). These two activities were designed exoPG 1 and exoPG 2 in the order of their elution from this column. ExoPG 1 was further purified by Hydroxyapatite (Figure 1b). Table 1 shows a summary of the purification procedure.

3.2. Change in polygalacturonase activity during fruit ripening

The activities, expressed in terms of tissue fresh weight, of exoPG was correlated with ripening whereas endoPG was constant (Table 2).

3.3. Molecular weight

The molecular weight of exoPG 1 was estimated by SDS-PAGE to be 66,000 and 58,000 kDA (Figure 2).

4. Discussion

Two forms of exopolygalacturonase were present in mango fruits and the activity of both exoPG ezymes were correlated with ripening (Table 2). The exoPG 1 was purified to a stage where only two polypeptides at 66 kDa and 58 kDa were detectable by SDS-PAGE (Figure 2). The 66 kDa protein was corresponded to exoPG isolated in peach (Downs *et al.*, 1992) and 58 kDa was similar to the estimate of the size of the native

enzyme in apple (Bartley, 1978). Addition evidence for the low level of endoPG in mango was provided by the data from Northern blot analysis. mRNA isolated from ripe mango fruit was barely detectable to mango endoPG probe. (Chaimanee and Suntornwat, unpublished data). The role of exoPG in fruit softening was not clear. However, it may be involved in cleavage of linkage between pectin polysaccharide and the protein rather than in depolymerization of pectin.

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Table 1 Purification of exoPG from fully ripe mango

Fraction	Protein mg/ml	Volume ml	Activity unit*/ml	Specific activity unit/mg protein	Purification fold
Crude	3.23	900	1.53	0.47	1.0
S-Sepharose					
ExoPG 1	0.33	75	0.59	1.79	3.8
ExoPG 2	0.35	70	0.44	1.26	2.7
Hydroxyapatite of exoPG 1					
Peak	0.11	10.4	0.7	6.36	13.5

* 1 unit = nmole/min

Table 2 Changes in exoPG and endoPG activities in mango fruit during ripening

Stage	Weight (g)	exoPG (nmole min^{-1} g^{-1})	endoPG
Mature green	90	0.11	0.08
Half ripe	95	2.56	0.10
Fully ripe	100	4.31	0.06

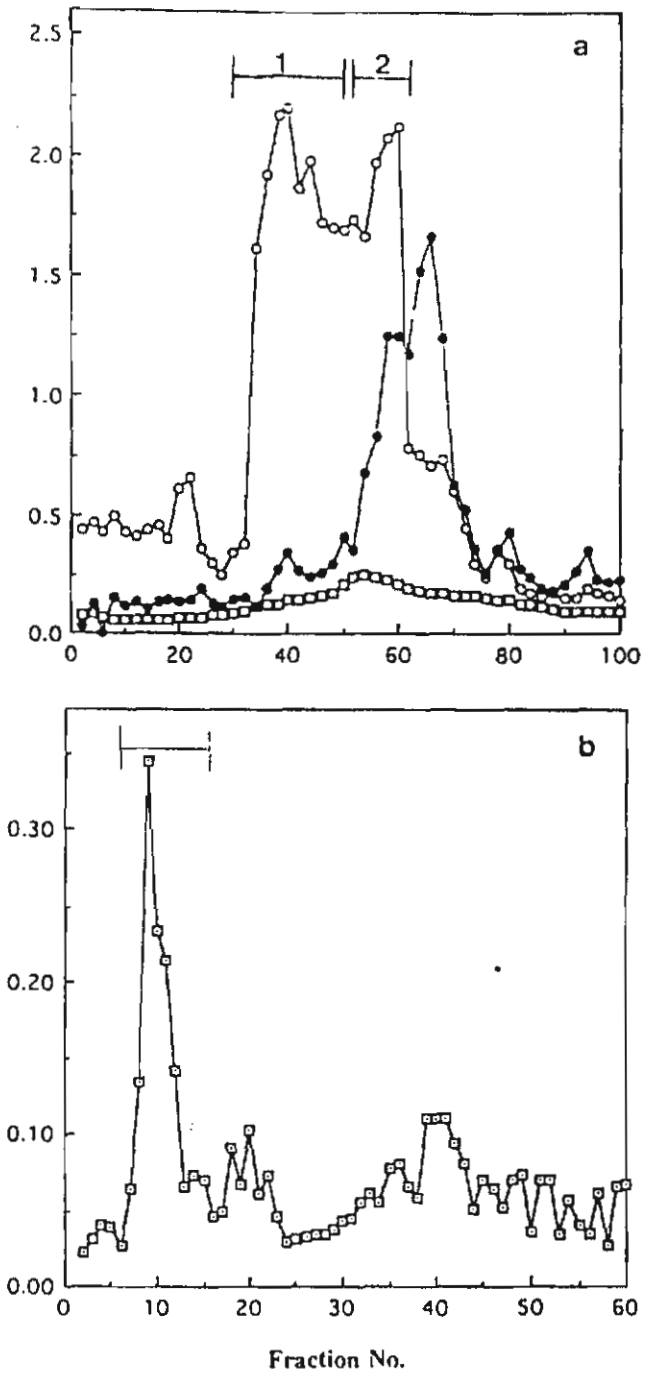

Figure 1 Separation of polygalacturonase from fully ripe mango on a S-Sepharose column (a), and separation of exoPG I from S-Sepharose column on Hydroxyapatite column (b). The fractionation procedure was described in Materials and methods. Enzyme activity was measured as absorbance at 276 nm. (—○—) exoPG activity (—●—) endoPG activity and (—□—) protein as absorbance at 280 nm.

174

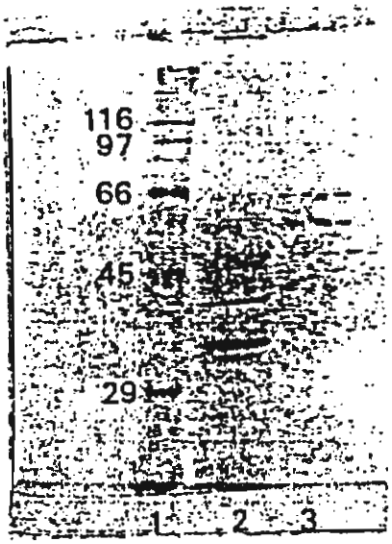

Figure 2 SDS-PAGE of protein samples from fully ripe mango. Protein was fractionate according to methods. (a) Molecular weight marker was loaded in lane 1; tomato PG in lane 2 and pool peak from Hydroxyapatite column in lane 3.

CLONING AND CHARACTERIZATION OF A PUTATIVE ENDO-POLYGALACTURONASE cDNA FROM RIPENING MANGO (*Mangifera indica* Linn cv. Nam Dok Mai)

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Keywords: Mango, hybridization, ripening gene

Abstract

The exo-polygalacturonase enzyme (EC 3.2.1.67) hydrolyses the terminal α (1-4) link between adjacent demethylated galacturonic acid residues while endo-polygalacturonase (EC 3.2.1.15) hydrolyses the same linkage but randomly. In general, polygalacturonase (PG) tends to be absent or barely detectable in green fruit, its acitivty appears only with the onset of ripening and increases dramatically during ripening. Both endo and exo-PG were found in mango fruit with only the exo-PG increasing significantly during ripening.

A cDNA for mango PG was made in order to study the expression of its ripening specific gene. Purified mRNA from ripening mango was used as a template for RT-PCR. Endo and exo-PG sequence from various sources obtained from EMBL data base were aligned. Conserved regions of the sequence were identified and used to design primer for PCR. PCR product were fractionated on an agarose gel and bands with appropriate sizes were eluted and ligated into a plasmid vector. One of the cDNA isolated had a size of 811 bp and encoded a 161 amino acids open reading frames, the deduced sequence of which was related by sequence similarly to plant PGs. A near match was to the endo-PG of kiwi fruit (acession No. P35336) which was 51% similar at the identity level and 67% similar when conserved substitution were allowed. This PG cDNA was used as a probe for hybridisation analysis of fruit RNA preparations.

1. Introduction

Mango is potentially on economically important export crop of many tropical countries including Thailand. Despite the potential, there are several problems associated with the marketing of mango fruit. Mangos soften very quickly and extensively. Softening in fleshly fruits is associated with extensive solubilization and degradation of cell wall polysaccharide which results from the action of a number of cell wall hydrolytic enzymes including PG (Mitcham and McDonald, 1992; Atkinson, 1994). In general, endo-polygalacturonase (PG) activity were found in many fruits, including peaches (Lee *et al.*, 1990), pears (Pressey and Avants, 1976) and kiwi fruit (Wegrzyn and MacRae, 1992). Both endo and exo-PG have been detected in apple (Bartley, 1978). In mango, Roe and Bruemmer (1981) have found a good correlation between loss of firmness and PG activity in "Keitt" mango. An increase of exo-PG (3.2.1.67) during mango fruit ripening were reported in "Harumanis" and "Malgoa" mango (Lazan *et al.*, 1986) and "Nam Dok Mai" mango (Chaimanee, 1992). These reports imply the involvement of the PG enzymes in the softening process of mango.

A number of PG genes and cDNA sequences from plants have been described (Greison *et al.*, 1986; Sheehy *et al.*, 1987; Lee *et al.*, 1990; Atkinson and Gardner, 1993; Atkinson, 1994). The amino acid sequence comparison of these genes and cDNA reveals

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Eds. S. Subhadrabandhu - A. Pichakum
Acta Hort. 509, ISHS 2000

a high level of conservation in certain regions of these sequences.

In this paper both endo and exo-PG sequence conservation were exploited to design primers for PCR to obtain a mango PG cDNA that can be used to study the expression of its ripening specific gene(s).

2. Material and methods

2.1. Plant material

Mango fruit (*Mangifera indica* L. cv. Nam Dok Mai) obtained from wholesale market in Nakhon Pathom and from a mango orchard in Darwin were shipped to the laboratory within 24 hours after harvesting. Fruits are allowed to ripen at ambient temperature. For storage, the skin and seed of mango fruits were removed and the mesocarp was diced and frozen in liquid nitrogen and stored at –80°C until used.

2.2. RNA preparation

Total RNA was extracted from mango mesocarp by the method described by Lopez and Gomez-Lim (1992). The frozen mango mesocarp was homogenized in a lysis buffer, which consists of 2% SDS, 1% β-mercaptoethanol, 50 mM EDTA and 150 mM Tris–borate pH 7.5. The homogenate was quickly mixed with 0.25 volume of absolute ethanol and 0.9 volume of 5 M potassium acetate. Mixing was continued for 1 minutes followed by a chloroform extraction and centrifugation at 20,000 g for 10 minutes. The recovered aqueous phase was extracted once with phenol and once with chloroform. RNA was then precipitated with LiCl (3 M final concentration) at –20°C overnight. The RNA was collected by centrifugation at 20,000g for 90 minutes at 4°C and dissolved in sterile distilled water at a concentration of 5-10 µg/ml and stored at –8°C. Poly $(A)^+$ MRNA was purified from total RNA by using polyAY tract™ (Promega Corp.)

2.3. Oligonucleotide sequence and design

Two oligonucleotides for using as 5'PCR primers, were synthesised based on conserved sequences of both endo and exo-PG from a number of plant species listed on the EMBL Data Base. The two 5'primers were - Man #1 (CCI AAY ACI GAY GGI ATH CA and Man #2 (TGY GGI CCI GGI CAY GGI AT) where I = inosine (A/T/G/C), H = A/C/T, and Y = C/T. The 3'end of the reaction was initiated on the poly $(A)^+$ region of the RNA using RACE primers as described by Frohman *et al.* (1988).

2.4. First-strand cDNA synthesis and PCR

First–strand cDNA synthesis and PCR reaction are based on the method of Frohman *et al.* (1988). The template for the first strand reaction was 1.5 µg of poly $(A)^+$RNA from mango mesocarp. The reaction was carried out for 1 hr in 20 µL of 1X RT buffer with 1 mH deoxynucleotide triphosphates and 40 unit of RNasin (Promega) and 24 unit of AMV RTase (Promega) primed by 100 pmol of oligonucleotide 5'--GACTCGAGTCGAGACATCGA$(T)_{17}$-3' (Frohman *et al.*, 1988).

PCR reaction was carried out in a volume of 25 µl with 1XPCR buffer, 1.8 mM $MgCl_2$, 0.4 mM deoxyribonucleotide triphosphate 10 mM primer, 2 µl of the cDNA synthesis reaction and 7.5 units of Taq polymerase (Perkin Elmer Cetus). Reaction was cycled 2 times at 94°C for 2 minutes, 37°C for 2 minutes, 72°C for 3 minutes and 25 times at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

PCR products were analysed by gel eletrophoresis and bands of interest ligated into T-tailed (Marchuk *et al.*, 1991) pBluescript KS vector (Stratagene) and sequenced by the Dideoxynucleotide method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical). The sequence of a 811-bp fragment so obtained from PCR had some similarity to a number of plants endo-PGs especially that of kiwi fruit.

The encoded amino acid sequence of the mango cDNA was compared with sequences on the SwissProt data base by GAP analysis, and the phylogenetic tree by PILEUP (Genetics Computer Group : Program Manual for the Wisconsin Package,

Version 8, Madison, Wisconsin)

2.5. Northern analysis

RNA was fractionated on formamide/formaldehyde denaturing gels (Fourney *et al.*, 1988) with 10 µg of total RNA from each sample loaded. The fractionated RNA was transferred to a Zeta probe membrane (Bio-Rad) as described by the manufacturer. Filter were probed with ^{32}P-labeled cDNA (811 bp fragment).

3. Results

Gel fractination of the PCR products obtained from the reaction using the Man #2 primer showed a series of bands ranging from 895-490 bps. One band of approximately 890 bp was eluted from the gel, ligated into the plasmid and analysed, The fragment with an actual size of 811 bp contained an incomplete open reading frame encoding 161 amino acids (Figure 1) which is related by sequence similarity, with the carboxyl terminal region of a number of plant polygalacturonase. The nearest matches at the amino acid level were found with avocado (*Persea americana*) PG (accession Q 02096) which was 53% similar at the identity level and 68% similar when conserved substitutions were allowed, and to kiwi fruit (*Actinidia deliciosa*) endo-PG (accession P35336) with 51% and 67% identities (Figure 1).

Northern analysis of the total RNA from different stages of ripening with the 811-bp cDNA fragment as probe barely detected any hybridization (Figure 2).

4. Discussion

Alteration in cell wall structure are thought to be related with the textural changes that occur during fruit ripening. In mango, softening was reported to be associated with the decline in the amount of both water and alkaline soluble pectin (Muda *et al.*, 1995). These evidence implied the involvement of pectolytic enzymes such as polygalacturonase (PG), pectin methylesterase (PE) or β-galactosidase.

Here we have described the isolated from ripening mango of a cDNA encoding part of a putative PG enzyme. The amino acid sequence encoded by the mango cNDA has similarity with PG enzymes from other fruit plants, one of the enzyme from kiwi fruit having been identified as the endo-form. Using the mango PG cDNA as a hybridisation probe we detected only trace amounts of endo-PG mRNA in RNA from mango tissues at various stages of ripening and did not detect any ripening associated increase in the mRNA. The endo-PG enzyme has been detected at a constant low activity in the ripening mango fruit while the exo-form of the enzyme increase in activity as ripening progresses (Chaimanee, 1992). On the basis of these combined observation it is likely that the isolated cDNA encodes an endo-form of the PG enzyme but formal proof awaits more rigorous examination.

Phylogenetic comparison of the amino acid sequence encoded by the mango PG cDNA with other PG sequences listed on the SwissProt data base showed the mango peptide to be included in a group of fruit PGs, with closest similarity to PG sequences from avocado, kiwi fruit, apple and tomato and the woody perenial peach slightly more distant (Figure 2). Isolation of the complete mango cDNA will give a more complete picture of its evolutionary groupings.

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Man #2 primer →→

TGTGGGCCGGGGCATGGGATAAGCATTGGAAGCTTGGGAAAATACAACTCATGTTCACAAATTCAAGATGTA 72
C G P G H G I S I G S L G K Y N S C S Q I Q D V

ACGGTTGATGGAGCATTTATTTCTAACACTGAGAATGGGTTGCGAATTAAAACATGGCAGGGAGGTAGTGGC 144
T V D G A F I S N T E N G L R I K T W Q G G S G

TTTGCCGCTGACATTAAGTTCCGGAATGTGTTGATGGAAAACGTATCAAATCCAATTATAATAGACCAATAC 216
F A A D I K F R N V L M E N V S N P I I I D Q Y

TATTGTGATTCTTCGACACCATGTGCAAACCAGACTTCGGCTGTTAAGATGGATAACATATCCTTTGAGCAC 288
Y C D S S T P C A N Q T S A V K M D N I S F E H

ATTAAAGGAACTTCAGCGACAAAGGATGCTATAATATTTGCATGCAGCAATGACGTACCATGTGAAGGCTTA 360
I K G T S A T K D A I I F A C S N D V P C E G L

TATCTTAAAGACATTCAGCTTGTGTCACAATCTTCGGGGGTCACAAGTTCATTCTGTTGGAACGCTTGTGGG 432
Y L K D I Q L V S Q S S G V T S S F C W N A C G

TCGAGTGCGGGTTTAGTTTCCCCCCTGCTTGCTTCACATGCACTGATGGCTTAATTCAACAGTATGT?TCAT 504
S S A G L V S P L L A S H A L M A *

CTAG?TACGCCTTAACCA?TGAAGGCTTTATCCAACAGAATGTCTCATTTAACTATGCTTTTCAATCCTTTT 576

GAAGAGAGATCAGTCTTTTTTAATATGTGCCACGCCCTTCTAGTGCAGAAGTTACAGAAATTATGACTTGAG 648

CAATTCACATTGTTCTGCATTTGTTTTTTCAGCTCACCAATGAGAATATTGAGTAGGATTGTATAATTATTG 720

TTGTACATGGTTCTAAACTATTTTACACGAATCAAGTTTCATCTGAGAATTATAGTGTTATTCCATTTCTTT 792

TAAAAAAAAAAAAAAAAA

Figure 1 Nucleotide and amino acid sequences of the mango PG cDNA.

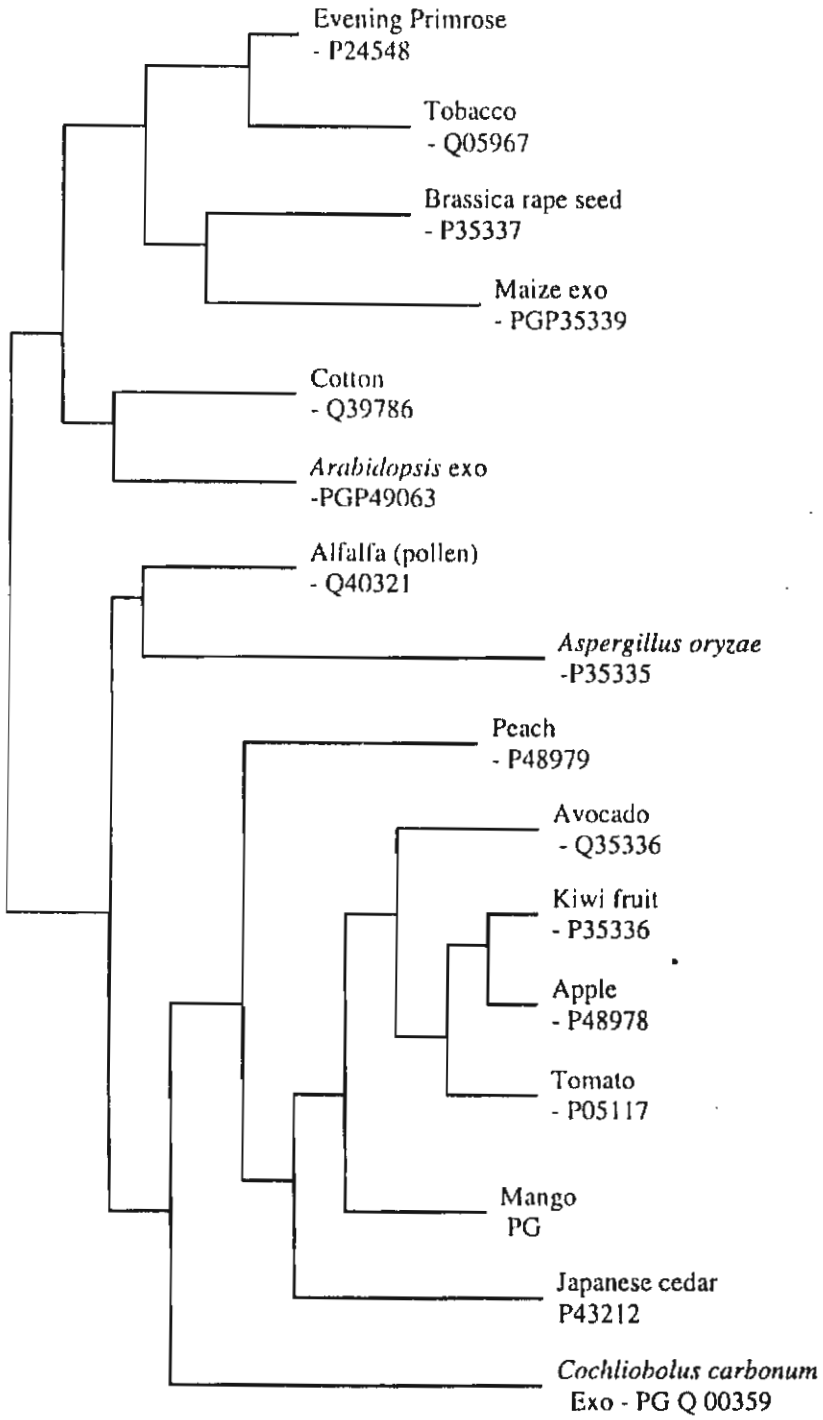

Figure 2 Phylogenic relationship between the mango amino acid sequence and sequences of PG enzymes from a number of other sources.

Changes in Cell Wall Carbohydrates and Hydrolase Activities during Mango (*Mangifera indica* L. cv. Namdorkmai) Fruit Ripening

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ABSTRACT Changes in cell wall carbohydrates, cell wall hydrolases activities, cell wall structure and texture of mango(*Mangifera indica* L. cv. Namdorkmai) fruit during ripening were presented. The starch and pectin fractions decreased considerably in ripe mango pulp. There were significant changes in the level of several cell wall associated sugars and sugar acid during ripening. The activities of exo-polygalacturonase, β-galactosidase and α-amylase were significantly increased while pectin methylesterase was slightly decreased. An electron microscopic (SEM) study confirmed the change in cell wall structure and starch degradation through the process of ripening.

KEYWORDS : Namdorkmai mango ripening , starch, cell wall, hydrolases, AIS, PAW

INTRODUCTION

Mango is potentially on economically important export crop of many tropical countries including Thailand. There are dozens of varieties of mango, varying in fragrance and flavours. Mangos can be divided into two broad categories : those that are eaten green (unripe) and dessert mangos enjoyed for their sweetness(ripe). Despite the potential, there are several problems associated with the marketing of mango fruits due to the short ripening period and shelf life.

Fruit ripening is characterized by a number of biochemical and developmental processes that result in changes of color, texture, flavour and aroma.[1] Cells present in fruit pulp contain primary cell walls. The constituents of the primary cell wall can be classified into various types of polymeric molecules: pectic substance (~35%), cellulose (~30%), hemicellulose (~25%) and protein (10%). Prior to ripening, the fruit has rigid ordered and well- defined cellular structure , where as soft and diffused cell walls exist in ripe fruits.The content and structural feature of the cell wall polysaccharides vary among species, developmental stage and differentiation of the

plant cell and tissue.[2,3] Many fruits including mango soften during ripening and this is a major quality that often dictates shelf life. Fruit softening could arise from one of the three mechanisms : loss of turgor (non-physiological process associated with postharvest dehydration), degradation of starch or breakdown of the fruit cell wall. However, textural changes during the ripening of many fruits are thought to be the result of the modification of cell wall carbohydrates by the action of cell wall associated enzymes. [4] Enzymes capable of altering cell wall texture can be listed as follows. Polygalacturonase is responsible for degrading (1->4) linked galacturonic acid residues. [3,5] Two forms of polygalacturonase were reported as exo-polygalacturonase(EC 3.2..1.67) and endo-polygalacturonase(EC 3.2.1.15) enzymes .[6] Pectin methylesterase (PME, EC 3.2.1.1)) involves the demethylation of galacturonic acid which may be a prerequisite to the action of polygalacturonase (PG). β-galactosidase(EC 3.2.1.23) plays a role in the degradation of (1->4) linked galactopyranose residues which constitute the neutral sugar side-chain polymers of pectin. Cellulase or β-glucanase (EC 3.2.1.4) hydrolyzes the (1-->4) linkage between adjacent glucose units. [4,5]

Starch, the principal food reserved polysaccharide, accumulates in the mature green fruits and is rapidly lost during ripening and completely disappears in the ripe fruit. Three starch degrading enzymes; α-and β-amylase and starch phosphorylase have been identified in many plants.[7,8] Starch content and acidity are also important factors attributing to eating quality.

Since the carbohydrate changes during ripening are commercially important in connection with marketing and storage, our present study focuses on gathering the chemical and biochemical data along with the electron microscopic study on carbohydrate changes during the ripening process in mango which will lead to the understanding of the molecular mechanism of tissue softening.

MATERIALS AND METHODS

Plant materials : Mango fruits (*Mangifera indica* L.cv. Namdorkmai) of 16 weeks old were harvested from commercial orchards in Nakorn Pathom, central Thailand. The fruits were immediately shipped to the laboratory and allowed to ripen at 25°C for 8 days. The set of 10 fruits were collected on the day of arrival (unripe), the fourth (half ripe) and the eighth day (ripe) after arrival and referred to as unripe, half ripe and ripe mangos respectively. The process of fruit pulp collection includes peeling , slicing and then freezing in liquid nitrogen and stored at -80°C until needed.

Carbohydrate Analysis : The mango mesocarp from different stages of ripening was used to prepare the **acetone insoluble solid (AIS)** or **cell wall fraction** which was then treated with **phenol: acetic acid:water (PAW)** to inactivate endogenous enzymes as described by Seymour *et al.*[9] Reducing and total sugars were estimated by the Nelson-Somogyi [10] and Phenol-sulfuric [11] methods respectively. The starch content in PAW-treated acetone insoluble solid was determined by digestion with heat stable bacterial amylase and amyloglucosidase followed by glucose content determination using glucose oxidase as described by the manufacturer (Boeringer Manheim). The starch from PAW-treated acetone insoluble solid was removed by α-amylase and pullulanase treatment, the fraction obtained from this treatment is referrred to as **non-starch acetone insoluble solid**. The neutral sugars of non-starch acetone insoluble solid were released by modified Seaman hydrolysis [12], converted to alditol derivatives and analysed by gas liquid chromatography(GLC). [13]

Uronic acids of the non-starch acetone insoluble solid were separately determined using sulfuric acid hydrolysis [14] followed by the colorimetric method as described by Blumenkrantz and Ashboe-Hansen. [15]

Enzyme Extraction and Assay : Crude extracts of mango mesocarp were prepared in triplicate from different stages of ripening. The extraction and assay methods of exo-polygalacturonase (EC 3.2.1.67), pectin methylesterase (EC 3.1.1.11), β-galactosidase (EC 3.2.1.23) and α-amylase (EC 3.2.1.1) were as previously described. [16, 17, 18, 19] Protein contents were determined by Lowry's method. [20]

Scanning Electron Microscopy : The PAW-treated acetone insoluble solids from different stages of ripening were mounted and coated with gold as standard procedures of CamScan Maxim 2040S scanning electron microscope. The conditions were BEI, EHT= 25.9 kV and WD= 31.4 mm.

RESULTS AND DISCUSSION

Cell wall composition and structure

One of the most significant quality aspects of mango for consumers is firmness as it represents ripeness. The firmness of the mango fruits was estimated by non

destructive measurement according to sensoric scales. The set of four to five mangos were gently squeezed at the "cheeks" by fingertips and thumb. The firmness is rated as 0= very firm (unripe) , 1 = first sensible change in firmness , 2 = clear sensible change toward softening but still very firm, 3= medium soft (half ripe), 4 = soft and 5 = very soft (ripe). [21].The firmness of Namdorkmai mango abruptly declined over the 8-day ripening period and the skin color turned from green to different shades of yellow similar to what has been reported earlier in other cultivars.[4]Total acetone insoluble solids (AIS) were prepared from unripe, half ripe and ripe stages of mango and treated with phenol - acetic acid-water to remove endogenous enzyme activity. The **PAW-treated AIS** are mainly composed of cell wall polysaccharides and starch , the amount of these fractions are highest in unripe mangos and drop sharply when they continue into the ripe stage. The starch content from the PAW-treated AIS from Namdorkmai mango (Table 1) was quite high compared to more than 20 % in Carabao [22, 23] and 11 % in Tommy Atkins [24]. However , there was a decreasing trend in the amount of starch in mango fruits from different cultivars during the ripening process. The breakdown of starch into glucose , fructose or sucrose is a characteristic ripening event in many fruits including mango and this is an important factor that contributes to the sweet taste of the ripe fruit[4, 25, 26]. Starch hydrolysis in the ripening mango has been reported to be associated with the amylase activity which exhibits the properties of both α- (EC 3.2.1.1) and β-(EC 3.2.1.2) amylases.[7] The loss of starch content in mango was evident in the chloroplast where starch granules become progressively smaller as the ripening proceeds and completely disappear in the ripe fruit.[27,28] . A similar episode was also found in the cell wall material of Namdorkmai mango both structurally and enzymatically. (Figure 1 and Table 3) The PAW-treated AIS from unripe mango was characterized with rigid and well-defined structure with lots of starch granules scattered over the polysaccharide network. As mango fruits advanced to the half ripe stage the rigidity and integrity of the structure declined. A large number of starch granules were partially broken. The cell wall structure from the ripe stage appeared collapsed into convex fold structure and the starch granules were barely seen. This microscopic result confirmed both the cell wall and starch degradation during the ripening process.(Figure 1)

The textural changes during ripening are thought to be primarily due to the changes in the cell wall composition. The sugar composition of non-starch AIS was determined using gas liquid chromatography and spectroscopy as shown in Table 2.

The result shows a significant decrease in galactose, glucose and uronic acid while a decreasing trend in arabinose, xylose, rhamnose and mannose was also observed during ripening. The relatively high content of galactose, arabinose and uronic acids suggested that the main non-cellulosic polysaccharides of the cell wall were mainly pectin in nature since the structural composition of pectic polysaccharides can be diverse containing rhamnogalacturonan I and II, arabinans, homogalacturonans, galactans and arabinogalactans. These polymers may be covalently joined to each other and to some phenolics, cellulose and proteins within the primary cell wall. [29, 30, 31]. The result from the cell wall composition of Namdorkmai mango confirmed that solubilization of the pectic polysaccharide of the primary cell wall was also the main event in the mango softening as previously reported in other fruits. [32] Since starch has been depleted from the cell wall material, the loss in glucose content of the cell wall material should imply the degradation of cellulosic polymer of the mango cell wall during ripening.

Cell wall hydrolases

The activities of the cell wall and starch hydrolysing enzymes from different stages of Namdorkmai mango are presented in Table 3. Among the cell wall degrading enzymes the activity of exo-polygalacturonase and β-galactosidase increased up to about seven fold during ripening while that of pectin methylesterase slightly declined. The changing profile of these three enzymes in Namdorkmai mango was very similar to that reported in Kitchner, Dr.Knight [33] and Harumanis mango. [5] In many tropical fruits including mango the polygalacturonase activity is low compared with tomato counterpart and mango enzyme appear to occur predominantly as exo-acting enzyme [5, 34] which is also true in Namdorkmai mango. [35] Pectin methylesterase activity can either decline, remain constant or increase depending on the fruit or extraction procedures used. [4] The role of this enzyme in softening is not clear, however, its action for the deesterification of pectin may be a prerequisite to the action of polygalacturonase. [3] Although the activities of cell wall hydrolases varied considerably among different cultivars, our results are consistent with the possibility that exo-polygalacturonase and β-galactosidase are the two important enzymes in mango fruit softening process. Because of the very high starch content in Namdorkmai mango, the possibility of starch degradation as another factor influencing the softening of mango fruit should be taken into account.

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Table 1 The amount and starch content of cell wall material (PAW-treated acetone insoluble solid) from Namdorkmai mango at different stages of fruit ripening. Values are mean ± SD : n = 3

	Stage of ripening		
	unripe	half ripe	ripe
PAW-treated acetone insoluble solid (mg/ g fresh weight)	127.40 ±1.52	63.20 ± 0.74	23.20 ± 0.39
Starch content (mg/g fresh weight)	37.20 ± 0.73	6.5 ± 0.41	1.15 ± 0.06
Starch content (% fresh weight)	29.20	10.28	4.96

Table 2 Neutral sugars and uronic acid composition of non-strach acetone insoluble solid at different stages of fruit ripening.
Values are mean ± SD : n = 3

Sugar	Amount (mg/g fresh weight)		
	unripe	half ripe	ripe
Rhamnose	0.28 ± 0.04	0.18 ±0.03	0.08 ±0.01
Arabinose	2.78 ± 0.06	2.06 ± 0.05	0.98 ±0.04
Xylose	1.40 ± 0.12	0.82 ± 0.03	0.38 ± 0.05
Mannose	0.34 ± 0.05	0.20 ± 0.04	0.10 ± 0.01
Galactose	3.97 ± 0.56	1.50 ± 0.13	0.78 ± 0.06
Glucose	7.92 ± 0.38	4.92 ± 0.32	2.48 ± 0.10
Uronic acid	8.35 ± 0.54	4.45 ± 0.22	3.31 ± 0.09

Table 3 Changes in cell wall hydrolase and amylase activities at various stages of fruit ripening. Values are means ± SD: n = 3

Enzymes	Total activity (nmoles product $min^{-1}g$ fresh $weight^{-1}$)		
	unripe	half ripe	ripe
Exo-polygalacturonase	1.7 ± 0.28	3.5 ±1.30	13.8 ± 0.40
β-Galactosidase	14.8 ± 1.90	29.3 ± 8.30	111.0 ± 15.3
Pectinmethylesterase	137.7 ± 23.5	106.8 ± 9.40	92.6 ± 4.40
α-Amylase	890.0 ± 65.0	1818.0 ± 13.5	2806.0 ± 57.0

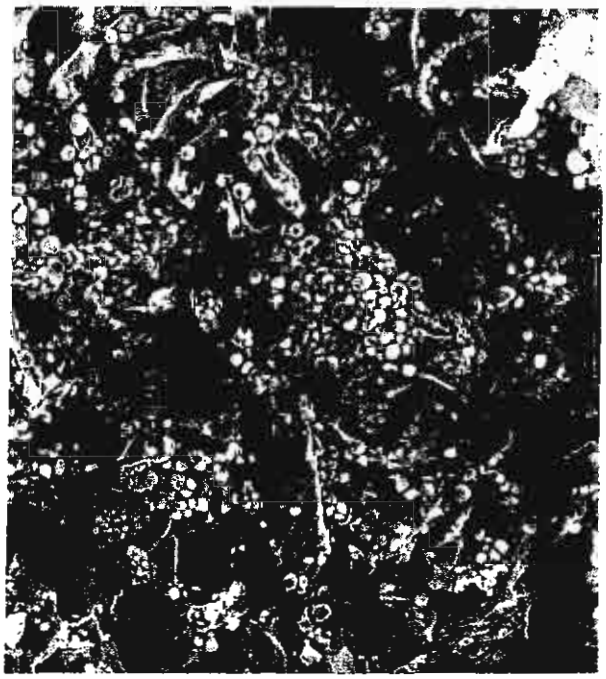
Figure 1 The scanning electron micrographs of the cell wall fractions obtained from three different stages of mango fruit ripening.(200X)

a) unripe stage: The cell wall material from this stage had a rigid, well defined structure with lots of starch granules.

b) half ripe stage: The rigidity and integrity of the cell wall declined, starch granules were partially broken.

c) ripe stage: The wall appeared collapsed into convex fold structure and the starch granules were barely seen.

a



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Biochemical and molecular approaches to modify the ripening process in mango fruit

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Mr. Periya Srijoud

Biochemical and Molecular Approaches to Modify a Ripening Process in Mango Fruit. (I) Softening process.

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Abstract

The biochemical and molecular biological changes during the ripening of mango fruits were investigated. The change in cell wall structure and composition of mango during ripening were detected. The starch and pectin fractions decreased considerably in ripe mango pulp.There were significant changes in the level of several cell wall associated sugars and sugar acids during ripening. The activities of exo-polygalacturonase, β-galactosidase and α-amylase were significantly increased while pectin methylesterase were slightly decreased. The electron microscopic studies confirmed the change in cell wall structure and starch degradation through the process of ripening. The amount of total RNA increased when mango fruit advanced into the ripe stages. The mRNA purified from total RNA was then used for cDNA library construction. Some of the ripening-specific genes were characrterized and found to encode part of the pectinmethyesterase, endopolygalacturonase and aquaporin. Dot blot and Northern Blot analysis of total RNA from different stages of ripening with mango specific probes confirmed the expression of pectinmethylesterase and β-galactosidase gene through out the ripening process. Analysis of translated products of mRNaA from ripe mango demonstrated the accumulation of three distinct proteins of 48, 61 and 67 kDa.

Introduction

Fruit ripening is a genetically programmed process that involves coordinated changes in fruit firmness, sweetness, acidity, aroma and pigmentation. From an economic standpoint, textural change is the most crucial of all, as it directly affects the shelf-life of the fruit and its keeping quality. The importance of understanding the softening process and its implications are emphasised on carbohydrate solubilization in fruit ripening (1). Modification of fruit ripening by suppressing expression of specific genes has been extensively studied and demonstrated in tomato (2). Mango is an economically important crop of many tropical countries

including Thailand. Despite the potential , there are several problems associated with the marketing of mango fruits especially fruit softening during ripening, leading to short shelf-life and susceptibility to mechanical damage and invasion by fungal and bacterial pathogens . Recently, we examined the biochemistry and molecular biology of softening in the ripening mango fruit, with the long term objective of finding the way to slow the rate of softening and to increase the potential for storage and export of this fruit.

Methods

The experiments were divided into four parts :

Part I. Study the changes in softening enzymes , cell wall structure and composition.

Part II. Identification of the specific gene(s) related to mango fruit softening and partilal construction of mango cDNA library

Part III Change in gene expression during mango fruit ripening

Part IV Purification of some softening enzymes

Results

Part I Study the changes in softening enzymes, cell wall structure and composition

One of the most significant quality aspects of mango for cosumers is its texture . The firmness of Namdorkmai mango abruptly declined over the 8-day period and the skin color turned from green and to different shades of yellow similar to what has been reported earlier in other cultivars. (3) The texural changes or softening during ripening are thought to be primarily due to the changes in the cell wall. Among the cell wall degrading enzymes the activity of exo-polygalacturonase and β-galactosidase increased up to about seven folds during ripening while that of pectin methylesterase slightly declined. The changing profile of these three enzymes in Namdorkmai mango was very similar to that reported in Kitchner, Dr. Knight (4) and Harumanis mango. (5) In many tropical fruits including mango the polygalacturonase activity is low compared with tomato counterpart and mango enzyme appear to occurs predominantly as exo-acting enzymes (5,6) which is also true in Namdorkmai mango. (7) Pectin methylesterase activity can either decline , remain constant or increase depending on the fruit or extraction procedures used. (8) The role of this enzyme in softening is not clear , however its action for the deesterification of pectin may be a prerequisite to the action of polygalacturonase. (9) Alhough the

acitivities of cell wall hydrolases varied considerably among different cultivars, our results are consistent with the possibilty that exo-polygalacturonase and β-galactosidase are the two important enzymes in mango fruit softening process.

Total acetone insoluble solid (AIS) were prepared from unripe , halfripe and ripe stages of mango and treated with phenol-acetic acid-water to remove endogenous enzyme activities. The PAW-treated AIS are mainly composed of cell wall polysaccharides and starch, the amount of these fractions are highest in unripe mango and drop sharply when they continue into the ripe satge. The starch content in the PAW-treated AIS from Namdorkmai mango was quite high compared to more than 20 % in Carabao (3,10) and 11% in Tommy Atkins (11). However, there was a decreasing trend in the amount of starch of mango fruits from different cultivars. The break down of starch into glucose, fructose or sucrose is a characteristic ripening events in many fruits including mango and this was an important factor that contribute to the sweet taste of the ripe fruit. (8, 12.13) Starch hydrolysis in the ripening mango has been reported to be associated with the amylase activity which exhibits the properties of both α-(EC 3.2.1.1) and β-(EC 3.2.1.2) amylases. (14) The loss of starch content in mango was evident in the chloroplast where starch granules become progressively smaller as the ripening proceeds and completely disappears in the ripe fruits. (15) A similar episode was also found in the cell wall material of Namdorkmai mango both structurally and enzymatically.(16)

The texural changes during ripening are thought to be primarily due to the changes in the cell wall composition. The sugar composition of non-starch AIS was determined using gas liquid chromatography and spectroscopy . The result shows a significant decrease in galactose, glucose and uronic acid while a decreasing trend in arabinose, xylose, rhamnose and mannose was also observed during ripening . The relatively high content of galactose , arabinose and uronic acids suggested that the main non-cellulosic polysaccharide of the cell wall was mainly pectin in nature since the structural composition of pectin polysaccharide can be diverse containing rhamnogalacturonan I and II, arabinans, homogalacturonans, galactans and arabinogalactans. The result from the cell wall composition of Namdorkmai mango confirmed the solubilization of the pectic polysaccharide of the primary cell wall as previously reported in other fruits.(17)

Part II. Identification of the specific genes (s) related to mango fruit softening

The specific genes related to the process of mango fruit softening especially the genes of cell wall hydrolases enzymes were investigated. The total RNAs were isolated from different stages of mango fruits during the ripening process . The poly (A^+) RNAs were then

purified from total RNA by commercial purification kit (PROMEGA and QIAGEN). The poly (A^+) RNAs (mixed from different stages) were then subjected to **RT-PCR using specific primers for endo-polygalacturonase and pectin methylesterase** enzymes. Gel fractionation of the RT-PCR products obtained from the reaction using endo-polygalacturonase specific primer showed a series of band ranging from 895-490 bps. One band of approximately 890 bps was eluted from the gel ligated into plasmid and analysed. The fragment with an actual size of 811 bps contained an incomplete open reading frame encoding 161 amino acids which is related by sequence similarity is related by sequence similarity with the carboxyl terminal region of a number of plant polygalacturonases with the nearest match to kiwi fruit endopolygalacturonase (18). The products from the reactions using pectin methylesterase - specific primers showed a series of bands ranging from 630-300 bps. Two bands of approximately 300 and 355 bps were similarly analysed. The sequence of the 300-bps fragment is related to aquaporin (transmembrane protein) of tomato , while the 355-bps fragment is closely related to pectin methylesterase enzymes from various sources with the nearest match to grape (Figure 1). The pectin methylesterase, endo-polygalacturonase and aquaporin fragments were kept as cDNA library and the pectin methylesterase one was successfully used as probes to follow the expression of these genes during the ripening process in mango as previously reported (19). Northern blot analysis of total RNA from different stages of ripening using pectin methyleserase probes confirmed the increase in pectin methylesterase gene expression during ripening as previously reported, while the endo-polygalacturonase probes barely detected by hybridization (18)

RT-PCR was also performed using mRNA mixture from different stages of ripening and **exo-polygalacturonase specific primer** (EXO$^{\bullet}$ 1F and MAN1 REV). Gel fractionation of the RT-PCR product showed a series of band from 300-1500 bps (Figure 2a). Three bands of approximately 400, 900 and 1000 bps were similarly analysed. The one with about 1000 bps is related to hygromycin phosphotransferase of *Arabidopsis thaliana* . The other two fragments were kept as cDNA library for further study and will be sent to Central Equipment Laboratory , Mahidol University for sequence analysis.

mRNA differential display was performed as described by Liang and Pardee (20) with some modification. The first strands of cDNAs were synthesized from the poly (A^+) mRNAs from different stages of ripening using commercial cDNA synthesis kit with **universal primers** (ZAP-Express Gold Cloning Kit, STRATAGENE). Differences in banding patterns between unripe, halfripe and ripe of differential display reactions were identified and the distinct bands in the range of 100-1500 bps were excised and eluted from the gels (Figure 2b). The DNA

fragments were then ligated into the plasmids. Some of them were kept as cDNA library for further study. Some clones will also be sent for sequence analysis at Central Equipment Laboratory , Mahidol University .

Dot blot analysis of total RNA from different stages of ripening were performed using our pectin methylesterase-specific probe and mango β-galactosidase probe kindly provided by Dr.G. A.Tucker showed the increase in the expression of both genes during ripening.(Figure 3).

Part III Changes in gene expression and protein pattern during mango fruit ripening

Changes in gene expression and protein pattern during mango fruit ripening were investigated.Total soluble proteins were extracted from mango at various stages of ripening and analysed by one- and two-dimensional gel electrophoresis. From the protein pattern on gel electrophoresis , the polypeptides with the molecular weight of 26, 39,42 and 64 kDa were found to increase considerably during the ripening process. Along with the direct protein extraction, the protein product obtained from in vitro translation was also analysed. The total RNA were isolated and poly (A^+) RNAs were purified from various stages of mango fruits. Then the poly (A^+) RNAs were subjected to *in vitro* translation . The results from these experiments indicated that ripening in mango is associated with the expression of some specific genes. (21)

Part IV. Purification of some softening enzymes

Exo-polygalacturonase and β-galactosidase were purified and characterized on SDS PAGE. Exo-polygalacturonase activities were associated with two polypeptides with the relative molecular weight of 58 and 66 kDa (22) while β-galactosidase activities were of 37 and 62 kDa. (23)

Fractionated proteins were transfered onto PVDF membrane . The interesting polypeptide was excised and N-terminal amino acid sequence information can be obtained from the protein-bound PVDF membrane by automatic gas phase protein sequencer at BIOSERVICE UNIT. Unfortunately, N-terminal sequence was not obtained due to technical problem.

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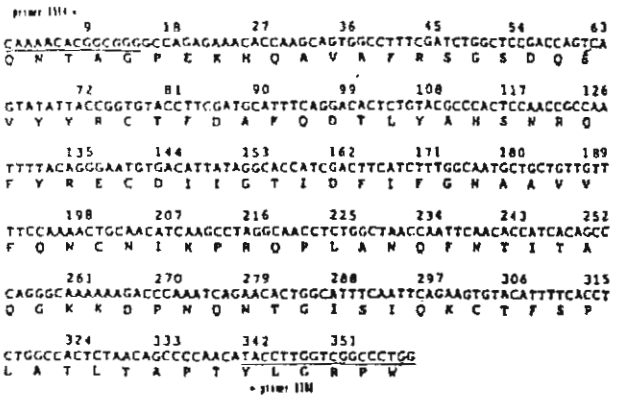
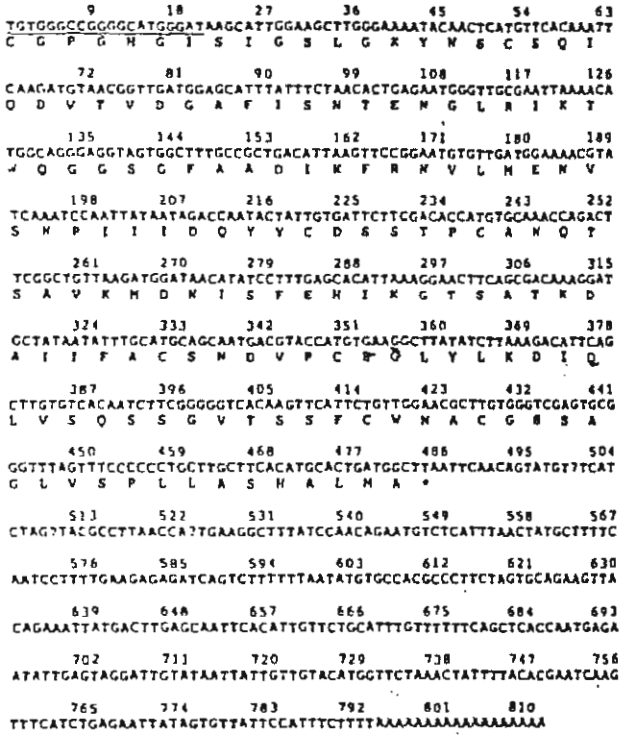
Mango pectin methylesterase (PE)



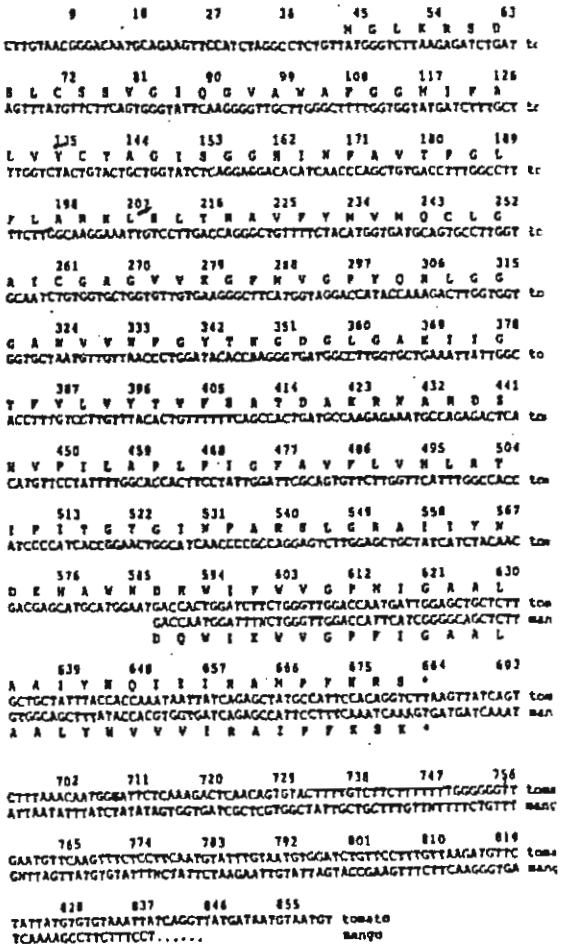
a

Mango endo-polygalacturonase (endo-PG)



b

Mango aqaporin in comparison with tomato cDN



c

Figure 1 Nucleotide and amino acids sequences of the mango cDNA
a) Mango pectin methylesterase (PE)
b) Mango endo-polygalacturonase (endo-PG)
c) Mango aquaporin in comparison with tomato cDNA

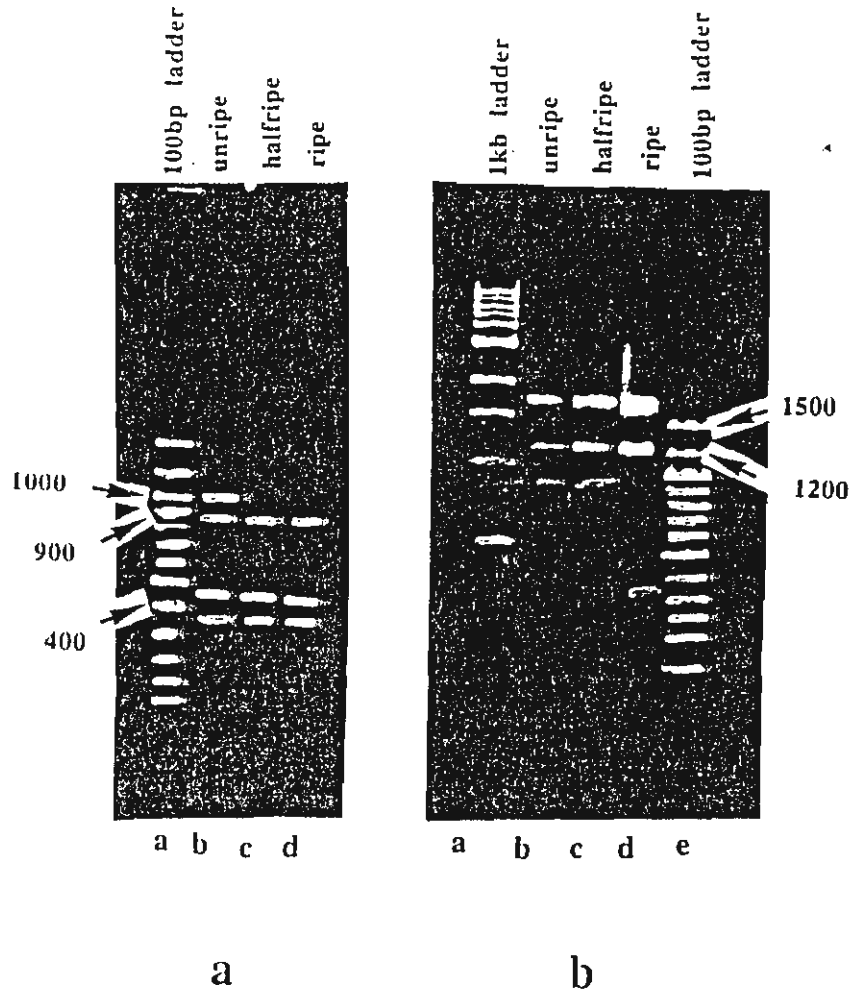


Figure 2 cDNAs from RT-PCR using specific primers and cDNAs from genes differentially expressed during mango fruit ripening.
a) cDNAs with the size ranging from 300-1500 bps from RT-PCR using exo-PG specific primers
b) cDNAs from Differential display experiment using universal primers showing the bands differentially expressed during ripening

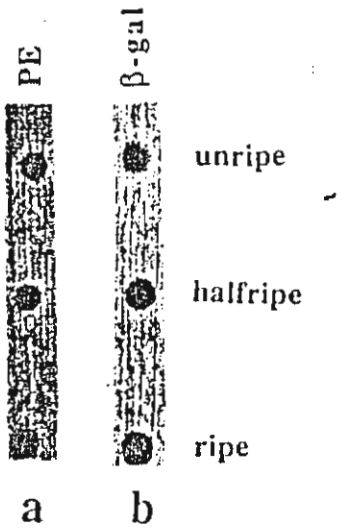


Figure 3 RNA dot blot analysis showing the presence of the pectin methylesterase (a) and β-galactosidase (b) transcripts in total RNA extracted from different stages of mango fruit ripening