ยีนแอลฟากลูโคซิเดสของผึ้งโพรง *Apis cerana* ในประเทศไทย: ลำดับเบสบางส่วนและเอนไซม์แอกทิวิตี

นางสาวประไพพิศ ศรีมาวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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α-GLUCOSIDASE GENE OF *Apis cerana* IN THAILAND: PARTIAL DNA SEQUENCES AND ENZYME ACTIVITY

Miss Prapaipit Srimawong

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ประไพพิศ ศรีมาวงษ์: ยีนแอลฟากลูโคซิเดสของผึ้งโพรง *Apis cerana* ในประเทศไทย: ลำดับเบสบางส่วนและเอนไซม์แอกทิวิตี (α-GLUCOSIDASE GENE OF *Apis cerana* IN THAILAND: PARTIAL DNA SEQUENCES AND ENZYME ACTIVITY)
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ทำการตรวจวัดแอกทิวิตีของเอนไซม์แอลฟากลูโคซิเดส (α -G)ในแต่ละช่วงอายุของผึ้งโพรงในวรรณะผึ้งงานทำการเก็บตัวอย่างผึ้งตามอายุที่ต้องการ โดยทำการแต้มสีตั้งแต่ผึ้งที่ออกจากเซลล์จนถึงตัวเต็มวัยที่มีอายุ 29 วัน แต่ละช่วงมีระยะการเก็บห่างกัน 3 วัน ทำการเก็บตัวอย่างตั้งแต่เดือนกุมภาพันธ์ มีนาคม และเมษายน พ.ศ. 2546 พบว่า ตัวอย่างที่เก็บในเดือนกุมภาพันธ์มีแอกทิวิตีจำเพาะของ α - G ที่ต่ำสุด ซึ่งต่างกับแอกทิวิตีจำเพาะของตัวอย่างที่เก็บในเดือนมีนาคมและเมษายน โดยตัวอย่างในช่วง 2 เดือนหลังมีค่าสูงและใกล้เคียงกัน อย่างไรก็ตามแอกทิวิตีจำเพาะของตัวอย่างจากทั้ง 3 เดือน มีแนวโน้มที่เหมือนกัน คือ ผึ้งที่ออกจากเซลล์จนถึงผึ้งที่มีอายุ 15 วัน จะมีแอกทิวิตีต่ำ และจะสูงขึ้นในผึ้งอายุ 18 วัน จนถึง 24 วัน ต่อจากนั้นจะมีแอกทิวิตีที่ต่ำลงอีกครั้ง สภาวะที่เหมาะสมต่อการทำงานของเอนไซม์ α - G ในสารสกัดอย่างหยาบ คือ pH 5.0 บ่มปฏิกิริยาที่ 45°C เป็นเวลา 30 นาที ทั้งนี้ความเข้มข้นของซูโครสที่เลือกใช้ คือ 50 mM ผลจากการหามวลโมเลกุลโดยวิธี SDS-PAGE มีค่าประมาณ 96 กิโลดาลตัน และพบการแสดงออกของ α - G โดยวิธี Native-PAGE ในผึ้งที่มีอายุ 18 วัน ขึ้นไป จากการตรวจสอบปริมาณ cDNA ของ α - G พบว่า ผึ้งที่ออกหาอาหารมีปริมาณสูงที่สุด นอกจากนี้ยังได้ทำการวัดขนาดของ acini โดยใช้ Scanning Electron Microscope (SEM) พบว่า ผึ้งอายุ 3 วัน ถึง 6 วัน จะมีขนาดของ acini เพิ่มขึ้น และจะมีขนาดเล็กลงเมื่อผึ้งมีอายุ 18 วัน ลำดับเบสของ cDNA ของ α - G ของต่อมไฮโปฟาริงค์ที่ได้จากเทคนิค RT-PCR สามารถใช้แยกความแตกต่างและสร้างสายสัมพันธ์ทางวิวัฒนาการได้ด้วยโปรแกรม Maximum parsimony, UPGMA และ Neighbor joining โดยแบ่งออกเป็น 2 กลุ่ม คือ 1) กลุ่มตัวอย่างจากประเทศไทยรวมทั้งจากเกาะสมุย และ 2) ตัวอย่างจากกรุงเทพที่ต่างออกไปจากกลุ่มแรก ทั้งนี้สาย cDNA ของ α - G ที่ได้ใน *A. cerana* มีความสัมพันธ์อย่างใกล้ชิดกับสาย cDNA ของยีนดังกล่าวใน *A. mellifera*

ภาควิชา……-……...ลายมือชื่อนิสิต……...
สาขาวิชา…….เทคโนโลยีชีวภาพ…….ลายมือชื่ออาจารย์ที่ปรึกษา……
ปีการศึกษา ……..2546…….ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……-……

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PRAPAIPIT SRIMAWONG: -GLUCOSIDASE GENE OF *Apis cerana* IN THAILAND: PARTIAL DNA SEQUENCES AND ENZYME ACTIVITY. THESIS ADVISOR: ASST. PROF. CHANPEN CHANCHAO, Ph. D. 116 pp. ISBN 974-12-3561-8

Enzyme activity of α-glucosidase (α-G) from worker bees, *A. cerana*, at different stages was assayed. Emerged bees were marked with painting color and counted as 0 day. Bees at desired ages were collected during 0-29 days with the time interval of 3 days. The sample collection had been performed from February to April 2003. Low activity was obtained from samples of all stages collected in February but not from those collected in March and April. Considering the activity profile from those 3 groups, they are similar. Briefly, the low activity was found in emerged bees. Then, the activity got increased in 18-day and 24-day workers. Older bees show decreasing activity of -G. The optimum condition for α-G activity in the crude extract was at pH 5.0, at temperature of 45^{o}C, and at incubation of 30 min. The concentration of sucrose at 50 mM was used. The molecular mass was estimated to be 96 kDa by SDS–PAGE and renaturation. In addition, the expression of α-G from 18-day and 29-day worker bees could be detected by Native–PAGE. The expression level of *α-G* cDNA was high in foragers. According to the measurement of acini, the size was increased from 3-day to 6-day worker bees but was decreased in 18-day worker bees. The *α-G* cDNA sequences (357 bp) isolated from hypopharyngeal glands were obtained by RT-PCR. Two groups were observed when maximum parsimony, UPGMA, and Neighbor joining programs were used. The first group contains all samples collected in Thailand including those from Samui Island. The other group contained samples collected in Bangkok only. The obtained cDNA sequence shows closely relationship to the cDNA in *A. mellifera*.

Department……..…-…….Student's signature…….……..
Field of study……Biotechnology…....Advisor's signature…….……..
Academic year……2003…….Co-advisor's signature……-…….

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ABBREVIATIONS

α-G	Alpha glucosidase
bp	Base pair
^{o}C	Degree celcius
g	Gram
h	Hour
HPGs	Hypopharyngeal glands
kDa	Kilodalton
LM	Light microscope
µl	Microlitre
µg	Microgram
min	Minute
M	Molar
mM	Millimolar
µM	Micromolar
MW	Molecular weight
ml	Millilitre
nm	Nanometre
rpm	Revolution per minute
R_f	Relative mobility
U	Unit (s)
w/v	weight by volume
v/v	volume by volume

CHAPTER I

INTRODUCTION

Biology of honey bees

Honey bees, genus *Apis*, are highly evolved and behaviorally complexing insects. *Apis* species can be classified into groups due to the difference of morphology and behavior. They are classified into three lineages.

- The first lineage is the cavity-nesting bee, *A. mellifera*, *A. cerana*, and *A. koschevnikovi*.
- The second lineage is the dwarf bee, *A. florea* and *A. andreniformis*.
- The last lineage is the giant bee, *A. dorsata* (Smith, 1991).

In a colony, highly eusocial insects as honey bees are divided into 3 castes which are a female queen, female workers, and male drones (Fig. 1.1). A queen and workers are heterozygous (diploid, 2n=32). They came from fertilized eggs. In contrast, a drone was grown from an unfertilized egg so it is haploid (Wongsiri, 1989). In a colony, the queen is the mother of all members including drones, workers, and daughter queens. It can lay eggs and release pheromone to control workers to be sterile. Most of population in a colony are workers those are responsible for all tasks such as brood rearing, wax secretion and comb building, food handling and storing, pollen or nectar foraging, and the community defense. The division of labor in honey bee colonies is based on worker ages. The workers change jobs when their age changes. A form of behavioral development is called “age polyethism” (Huang *et al*., 1991).

Juvenile hormone (JH) is involved in the control of age polyethism in adult worker honey bees. JH was first purified by Huber and Mathison (1976). Huang *et al*. (1991) reported similarities and differences in parameters of JH biosynthesis between

nurse bees and foragers. Rates of JH biosynthesis were low in newly emerged bees, 7-9 day old nurse bees, and 14-15 day old bees collected from the nest periphery, but very high in foragers. JH is very low in winter bees (found in December and January) but not in summer bees. Furthermore, it was reported that haemolymph-protein contents and vitellogenin were very high in winter bees (Fluri *et al*., 1982)

During the warm season (about April-August), the colony consists mainly of summer bees. Their lives are short and lasts only about 30 days. In the first 20 days, they act as hive bees by cleaning cells, rearing larvae, building combs, putting food reserves in the cells, and guarding the hive entrance. In the cold season (about November-February), mainly long live bees are in a hive. They survive in this period by forming a winter cluster. No brood is reared at this time. After February, there are more workers beginning to rear broods and forage pollen and nectar. During February to April, the number of winter bees diminishes and the population of summer bees increases. During this period also, the two categories cannot be distinguished morphologically. Based on physiological characteristics, there are some clear differences not only between summer and winter bees, but also between hive and field bees (Rutz *et al*., 1976).

Apis cerana and population genetics in Thailand

The closest relative of *Apis mellifera* L., western bees, is *A. cerana*. This species is native to southern and eastern Asia. In general, *A. cerana* is gentle, deligent, industrious, and managed easily. These include their docile and industrious nature, their being less prone to attacks of wasps, and a high level of resistance to nosema disease and the parasitic Asian mites, *Varroa jacobsonii* and *Tropilaelaps clarae* which plague *A. mellifera* (Verma, 1992). The hive bees, *A. mellifera* and *A. cerana* do not show any major differences in nest construction. Both species can nest several parallel combs in cavities (Fig. 1.2). The combs are built under the ceiling of the cavity and attached to the cavity's walls (Koeniger, 1995).

Taxonomy identification of *A. cerana* is below (Wongsiri, 1989):

Kingdom Metazoa

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super-family Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *cerana*

Furthermore, based on morphology and geographic distribution, *A. cerana* has been classified into 4 subspecies.

1) Northern subspecies, *A. cerana cerana,* from Afghanistan, Pakistan, north India, China, and north Viet Nam

2) Southern subspecies, *A. c. indica,* from south India, Sri Lanka, Bangladesh, Burma, Malaysia, Thailand, Indonesia, and the Philippines

3) Japanese subspecies, *A. c. japonica*

4) Himalayan subspecies, *A. c. himalaya* (Ruttner, 1988).

In recent years, Hepbern *et al*. (2001) used morphometric information to group *A. cerana* population into 8 subspecies. The four more subspecies are

1) *A. cerana skorikovi,* from Tibet

2) *A. c. abaensis,* from central China

3) *A. c. hainanensis,* from Hainan island

4) *A. c. philippina,* from the Philippines.

In Thailand, based on the multivariant statistical analysis (Canonical analysis) of morphometric characteristics, *A. cerana* was divided into 3 groups as followed:

1) Northern latitude bees, from Chiang Rai – Phetchaburi

2) Southern latitude bees, from Chumporn – Song Kla

3) Samui Island bees

In contrast, if Clustering analysis is used, *A. cerana* is divided into two groups: Northern latitude bees and Southern latitude bees including Samui Island bees (Limbipichai, 1990).

Genetic variability and population structure of Thai honeybees, *A. cerana,* was investigated by using PCR–RFLP. Some regions of mitochondrial genome were used (Songrum, 1997). Then, *A. cerana* could be genetically divided into 3 groups. They are

1) northern group (north, northeast, and central); 2) southern group, and 3) Samui Island group. Furthermore, Sihanuntavong (1999) also genetically allocated *A. cerana* into 3 groups. Both (Songrum, 1997 and Sihanuntavong, 1997) share the same grouping of *A. cerana* population in Thailand.

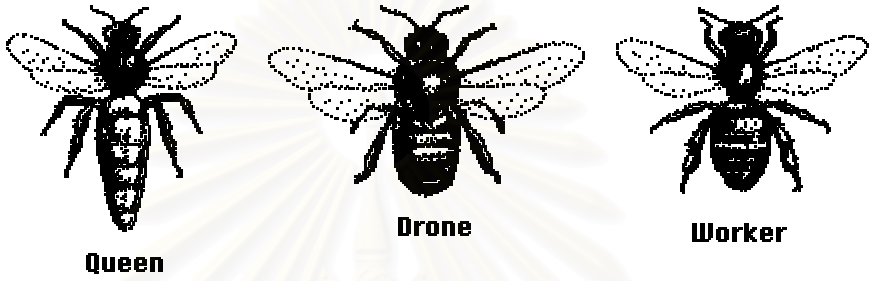
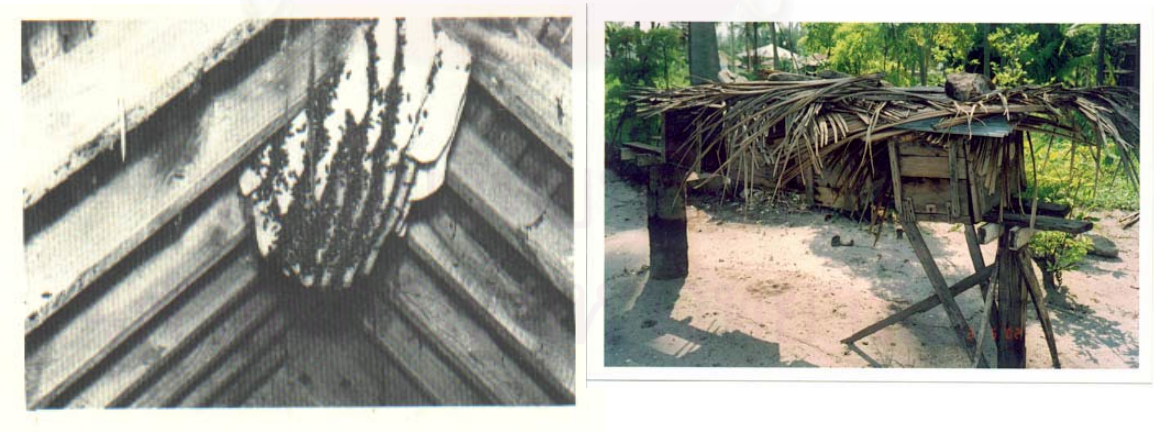


Figure 1.1 Three castes of bees in a colony.



A **B**

Figure 1.2 *A. cerana* in nature (A) and in managed hives (B).

Hypopharyngeal glands

Hypopharyngeal glands (HPGs) of worker bees is a paired long tuberous organ connected to many acini (secretory glands). Each acinus is composed of approximately one dozen of secretory cells. HPGs are located in the head of honeybees as shown in Fig. 1.3. In emerged bees, the acini look plump and creamy. HPGs synthesize and secrete a substance rich in protein, that is fed to all larvae for the first 3 days. Only larvae in queen cells receive brood food until they pupate. At this period, worker bees become nurse bees and are responsible for feeding broods (Dade, 1994). Later, HPGs change to produce digestive enzymes, including α-glucosidase (α-G), invertase, etc (Brouwers, 1982). At this stage, worker bees will become foragers.

Due to protein purification from HPGs of *A. mellifera*, three major proteins with molecular masses of 50, 56, and 64 kDa were restrictively found in nurse bees, whereas a major 70-kDa protein was specifically found in foragers. Immunoblotting analysis against 50, 56, and 64 kDa proteins confirmed that they were only detected in HPGs of nurse bees and also existent in royal jelly (RJ). It suggested that these proteins were synthesized in HPGs of nurse bees and secreted as constituents of RJ. In addition, a 70 kDa protein was purified and immunoblotted, this protein was positively detected in HPGs of foragers only. Subsequently, the 70 kDa protein was characterized and identified as α-G (Kubo *et al*., 1996).

In *A. mellifera*, the mRNA for the 56 kDa protein and α-G were detected, cloned, and characterized. The deduced amino acid sequence of 650 residues revealed 41.9% identity to maltase of mosquito (*Aedes aegypti*) and 42.2%, 46.3%, and 46.2% of maltase 1, 2, and 3 of fruit fly (*Drosophila melonogaster*), respectively (Ohashi *et al*, 1996).

Ohashi *et al*. (1997) used HPGs to study differential expression of protein in accordance with a change in behavior. They also reported that there were two kinds of sucrases (α-G) in HPGs. One of the mentioned sucrase might be invertase found in honey. The 56 kDa one was a glycoprotein which its amino acid sequence shared 63.2% and 56.9% identities to proteins encoded by cDNA for RJ-related proteins, pRJP57-1 and pRJP57-2. The 64 kDa protein encoding cDNA was identical to pRJP57-1. The gene for the 64 kDa protein/pRJP57-1 was expressed specifically in the nurse-bee gland, whereas that for the 56 kDa protein was expressed in both nurse bee and forager bee glands.

Recently, proteins at molecular masses of 57 and 85 kDa were also purified from HPGs of *A. mellifera* foragers and determined by SDS-PAGE. These proteins were already characterized as amylase and glucose oxidase, respectively (Ohashi *et al*, 1999). They are members of the sequence-related family of α-glycohydrolases (family 13) containing many important digestive enzymes (Yao *et al*., 2003).

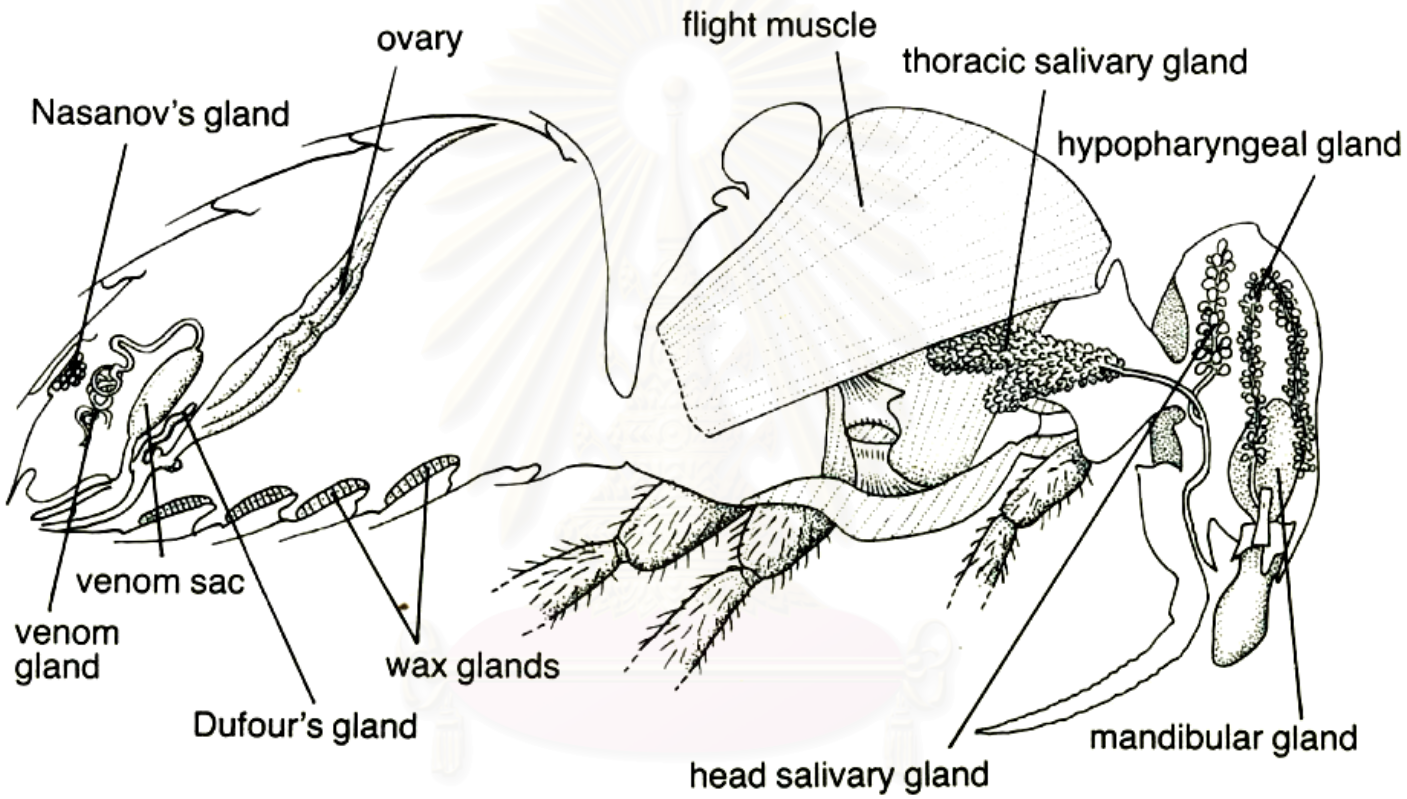


Figure 1.3 Longitudinal section of worker bees showing various glands (O'Toole and Raw, 1991).

Honey

Honey is one of the important products obtained from bees. The demands of honey also increase for food, phamaceutical use, cosmetics, etc. Honey is a supersaturated solution containing sugars such as fructose, glucose, and maltose with traces of sucrose, glucose oxidase, hydrogen peroxide, phenolics, flavonoids, terpenes, etc. The composition sugar make honey hygroscopic (moisture absorbing) and viscous. Furthermore, sugar concentration and other factors including low pH, hydrogen peroxide, and flavonoids, phenolics, and terpenes make honey antimicrobial or prevent microbial growth (Schmidt, 1997).

Nectar secreted by flowers is bee's natural source of carbohydrates. Nectar consists of 1) sugar solution, mainly sucrose (cane sugar, beet sugar, etc.), at various concentrations and 2) traces of essential oils which contribute their distinctive flavors to honey. Honey bees possess enzymes necessary to digest large molecule sugars such as sucrose and maltose to be simpler sugars such as glucose and fructose. Simple sugar can be absorbed through intestine walls without further alteration. Honey is the product of inversion of nectar. It is concentrated by evaporation of water in nectar so the composition of final product is variable. It may contain 80 percent of sugars, mainly glucose and fructose, and up to 18 percent of water (Dade, 1994).

Purification and characterization of α-glucosidase in honey bees

Alpha-glucosidases (α-G) is widely studied in various insects. Its other common names are invertase, sucrase, and maltase. This enzyme is α-D-glucoside glucohydrolase and belongs to EC 3.2.1.20. Like sucrase from mammalian intestinal, α-G is not β fructofuranosidase which is β-D-fructofuranoside fructohydrolase and belongs to EC 3.2.1.26. However, honey is still composed of small amount of β-fructofuranosidase which probably comes from nectar (Takewaki, 1979). Insect α-G are characterized and described in Table 1.1 (Terra and Ferreira, 1994). Alpha–G catalyzes the hydrolysis of terminal, non–reducing α-1, 4-linked glucose residues from aryl-glucosides, disaccharides or oligosaccharides.

Huber and Mathison (1976) purified α-G from whole bodies of honey bees. The *A. mellifer*α-G, Mr 82,000, is found in the head and abdomen of honey bees and its properties are similar to that of α-G found in honey. Thus, this enzyme is probably synthesized in HPGs and converts nectar sucrose into glucose and fructose in the crop. The pH optimum of each honey bee sucrases was different from each other according to the substrates as sucrose (pH 5.5) and p-nitrophenyl-α-glucoside (pH 6.5).

Alpha-G catalyzes α-glucosyl linkage in non-reducing terminal of various substrates. The enzyme was purified from various sources. For example, three forms of α-G I, II, and III were purified from the whole body extracts of adult flies, *Drosophila melanogaster* at yields of 2.1, 5.3, and 6.7%, respectively. Both α-G II and III can hydrolyze sucrose and p-nitrophenyl-α-D-glucoside (PNPG) but α-G I is capable of hydrolyzing PNPG much less extent than sucrose. For sucrose as substrate, the optimum pH for α-G I, II, and III were at 6.0, 5.0, and 6.0, respectively (Tanimura *et al*., 1979).

Order			purification				K_m	Tris *Ki*	
Species (Family)	**Stage**	**Source**	**degree**	**M_r**	**p*I***	**pH**	**(mM)**	**(mM)**	**Reference**
Hemiptera									
Rhodnius prolixus (Reduviidae)	Nymph	G, M	Crude	140,000 (U)	-	4.5	3.6 (NPG)	24	Terra *et al*. (1988a)
Dysdercus perucianus (Pyrrhocoridae)	Adult	M, G	Homogeneous	138,000 (SP)	-	5.0	26 (Mal)	0.85	Silva and Terra (1994b)
Coleoptera									
Pheropsophus aequinoctialis (Carabidae)	Adult	G, S	Crude	120,000 (U)	4.7	5.5	3.0 (Mal)	0.61	Ferreira and Terra (1989)
Tenebrio molitor (Tenebrionidae)	Larva	G, S	Crude	60,000 (U)	3.6	5.3	3.7 (Mal)	-	Terra *et al*. (1985)
Pyrearinus termitilluminans (Elateridae)	Larva	G, S	Crude	79,000 (U)	5.2	6.0	3.5 (NPG)	-	Colepicolo-Neto *et al* (1986)
Sitophilus zea mais (Curculionidae)	Adult	G, S	Partial	130,000 (U)	4.9	5.5	13 (Mal)	-	Baker (1991a)
Hymenotera									
Apis mellifera (Apidae)	Adult	WB, S	Homogeneous	82,000 (F)	6.5	6.5	4 (NPG)	-	Huber and Mathison (1976)
Apis mellifera (Apidae)	Adult	WB, S	Homogeneous	100,000 (F)	7.3	6.5	0.5 NPG)	-	Huber (1975)
Scaptotrigona bipunctata (Apidae)	Larva	G, S	Crude	75,000 (U)	-	5.0	-	-	Schumaker *et al*. (1993)
Scaptotrigona bipunctata (Apidae)	Larva	G, S	Crude	110,000 (U)	-	5.0	-	-	Schumaker *et al*. (1993)
Diptera									
Aedes aegypti (Culicidae)	Adult	SG, S	Crude	68,000 (U)	-	6.0	-	-	Marinotti and James (1990)
Trichosia pubescenc (Sciaridae)	Larva	G, M	Crude	95,000 (U)	-	5.5	6.2 (NPG)	8.9	Espinoza-Fuenyes and Terra (1986)
Trichosia pubescenc (Sciaridae)	Adult	G, M	Crude	156,000 (U)	-	5.8	2.3 (NPG)	0.2	Espinoza-Fuenyes and Terra (1986)
Drosophila melanogaster (Drosophilidae)	Adult	WB, S	Homogeneous	56,000 (U)	-	5.0	8.9 (Suc)	-	Tanimura *et al.* (1979)
Drosophila melanogaster (Drosophilidae)	Adult	WB, S	Homogeneous	200,000 (F)	-	6.0	13 (Suc)	-	Tanimura *et al.* (1979)
Musca domestica (Muscidae)	Larva	G, S	Partial	72,700 (U)	-	6.3	5.2 (Mal)	4	Terra and Jordao (1989)
Musca domestica (Muscidae)	Larva	G, S	Partial	330,000 (U)	-	6.1	4.6 (Mal)	0.7	Terra and Jordao (1989)
Musca domestica (Muscidae)	Larva	G, M	Partial	240,000 (U)	-	6.6	1.0 (Mal)	-	Terra and Jordao (1989)
Lepidoptera									
Thaumetopoea pityocampa (Notodontidae)	Larva	WB, S	Homogeneous	190,000 (F)	3.7	6.0	0.5 (NPG)	-	Praviel-Sosa *et al*. (1986)
Erinnyis ellos (Sphingidae)	Larva	G, S	Crude	134,000 (U)	-	5.8	1.4 (NPG)	-	Santos and Terra (1986b)
Spodoptera frugiperda (Noctuidae)	Larva	G, S	Crude	70,000 (F)	7.2	5.0	-	-	Ferreira *et al.* (1994a)

The source of enzmes was the soluble (S) or membrane (M) fraction of homogenates from midgut (G), salivary glands (SG) or whole bodies (WB), pH optima were determined using maltose (mal), NP Glu (NPG) or sucrose (Suc).

Table 1.1. Properties of α-G isolated from insect guts**.**

Takewaki *et al*. (1980) reported the purification and some properties of both α-G (I and II) from adult honey bees. Two kinds of the enzymes could be chromatographically separated according to their solubility in ammonium sulfate. The molecular mass of α-G I and II were estimated to be approximately 98 and 76 kDa, respectively, by SDS disc electrophoresis. Their pH optimum were 5.0. Both α-G readily hydrolyzed phenyl-α-glucoside, sucrose and maltose.

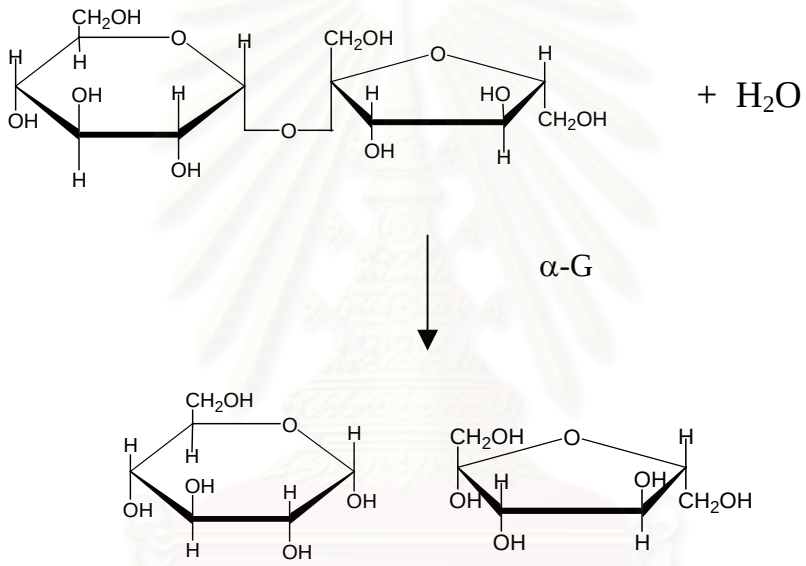
Both α-G I and II were monomeric glycoproteins containing 25% and 15% carbohydrate, respectively. They showed allosteric properties for the cleavages of several kinds of substrates. The former indicated the negative kinetic cooperativity for sucrose, maltose, and p-nitrophenyl α-glucoside. It also indicated the positive kinetic cooperativity for turanose and maltodextrin. In contrast, the latter indicated only the positive kinetic cooperativity for sucrose, turanose, kojibiose, and soluble starch (Nishimoto, 2001).

For substrate specificity, α-G is known to be greatly different to enzyme sources. Takewaki *et al*. (1993) studied substrate specificity of α-G II that can hydrolyse glucose-1-phosphate. Two enzymes (α-G I and II) are difference in molecular mass, pH-stability, and sugar content. Their substrate specificities also differ greatly. They found α-G I in the ventriculus (midgut), α-G II in the ventriculus and hemolymph, and α-G III in the HPGs. That is involved in the aging of honey by hydrolyzing sucrose.

Later, Nishimoto *et al*. (2001) reported the purification and substrate specificity of α-G III from honey bees. Using maltose as substrate, the molecular mass was estimated to be approximately 68 kDa and the optimum pH was 5.5. Alpha-G III was different in substrate specificity from honey bee α-G I and II. The enzyme was

characterized by the ability to rapidly hydrolyze sucrose, phenyl α-glucoside, maltose, and maltotriose (Nishimoto *et al*., 2001).

Alpha-G specifically hydrolyzes α-glucosidic linkages, preferably α-1,4-linkages. It can attack α-1,6 linkages slowly while it is able to attack -glucosidic linkages (Bergmeyer and GraB1, 1983). Alternatively, α-G can hydrolyse sucrose into glucose and frutose as follow:



As mentioned above, sucrose was used as substrate for α-G in crude extract (Sasagawa *et al*., 1989 and Kubo *et al*., 1996). Furthermore, 3,6-Dinitrophthalic acid is used for determination of glucose. Using small amount of samples, 3,6-Dinitrophthalic acid can provide a sensitive and stable color reaction with reducing sugar after heat in sodium carbonate solution in the presence of sodium thiosulfate (Momose *et al*., 1960). The red color in wine originated from the product of glucose in sodium thiosulfate

(Momose and Inaba, 1961). This basic is widely applied to microdetection of reducing sugar. The reagent solution is easily prepared, easy to use and stable for a long time (Momose and Nakamura, 1963)

The objective of this research is to study α-G in *A. cerana* which is native to Thailand. It is involved in the morphology of HPGs, optimum condition, and enzyme activity in crude extract. The profile of enzyme activity at different stages of worker bees was performed. The expression levels of α-G at different stages of honey bees were presented. The activity is high in foragers. In addition, partial cDNA sequences of *α-G* (357 bp) from various provinces in Thailand were indicated. The obtained results may involve in the duty of *A. cerana* workers in a hive. At present, divisions of labor has been extensively studied in *A. mellifera* but not *A. cerana*. In the future, α-G from *A. cerana* will be purified and characterized.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Automatic micropipette P10, P20, P200, and P1,000, Gilson, France
- Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
- Centrifuge, model: universal 32R, Hettich zentrifugen GmbH & Co. KG, Germany
- Cuvette, model: 1.5 ml Semi-micro cuvette, Brand, Germany
- Electronic U. V. transilluminator, Ultra lum Inc., USA
- Electrophoresis, model: Mupid, Advance Co., Ltd., Japan
- Incubator, Germany
- Incubator waterbath, model: 3575-1, Lab-line instruments Inc., USA
- Maxima ultra pure water, model: Maxima UF, ELGA, England
- Microincubator, model: M-36, Taitec, Japan
- Microwave, model: Sharp carousel R7456, Sharp, Thailand
- Minishaker, model: MS1, IKA Works Inc., USA
- Optima water purifier, model: Eigastat optima 60, ELGA, England
- PCR, model: Hybaid omnigene, OmniGene HBTR3cm hybaid Ltd., UK
- Polaroid, model: Direct screen instant camera DS 34 H-34, Peca products, UK
- Power supply, EC 570-90 LVD CE, E-C Apparatus corporation, USA
- pH meter, Cyberscan 500, Eutech cybernetics, Singapore
- Waterbath, model: 20 H, Gallenkamp, Germany

- SDS-Electrophotometry, mini VE, Hoefer, Pharmacia biotech, USA
- Spectrophotometer, model: Ultraspec II, LKB biochrom, England
- Stereomicroscope, Olympus optical Co. Ltd., Japan

2.2 Chemicals

- 3,6-Dinitrophthalic acid, $C_8H_4N_2O_8.C_5H_5N$, F. W. = 335.2, Sigma, Germany
- 2-Mercaptoethanol, BDH laboratory supplies, England
- 2,3,5-Triphenyltetrazolium chloride, $C_{19}H_{15}ClN_4$, Mr = 334.81, Fluka biochemica, Switzerland
- Access RT-PCR system (catalog# A1250), Promega, USA
- 100 % Acetic acid, CH_3COOH, M. W. = 60.05, Merck, Germany
- Acrylamide, M. W.= 71.08, Promega, USA
- Ammonium peroxydisulfate (APS), $(NH_4)_2S_2O_8$, M. W. = 249, BDH laboratory supplies, England
- Bovine serum albumin (BSA), Fraction V, pH 7.0, Serva feinbiochemica GmbH & Co., USA
- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M. W. = 670, BDH laboratory supplies, England
- Coomassie brilliant blue G-250, $C_{47}H_{48}N_3O_7S_2Na$, M. W = 854, BDH laboratory supplies, England
- Coomassie brilliant blue R-250, $C_{45}H_{44}N_3O_7S_2Na$, M. W = 826, Serva feinbiochemica GmbH & Co., USA
- Calcium chloride dihydrate, $CaCl_2.2H_2O$, M. W. = 147, Merck, Germany

- Ethylene diamine tetra-acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M. W. = 292.2, Serva feinbiochemica GmbH & Co., USA
- 95% (v/v) Ethanol, CH_3CH_2OH, M. W. = 46, Thailand
- 37% (v/v) Formaldehyde, CH_2O, M. W. = 30, Thailand
- 99.5% (v/v) Glycerol, $C_3H_8O_3$, M. W. = 92.10, BDH laboratory supplies, England
- Glycine, NH_2CH_2COOH, M. W. = 75.07, BDH laboratory supplies, England
- Hydrochloric acid fuming 37% (v/v), HCl, Merck, Germany
- Leupeptin, $C_{20}H_{38}N_6O_4$.HCl, F. W. = 463.0, Sigma, Germany
- Methanol, CH_3OH, M. W. = 32.04, Merck, Germany
- N, N'-methylene-bis-Acrylamide (Acrylamide/Bis), Sigma, USA
- Octylphenol-polyethyleneglycol ether (Triton X-100), Serva feinbiochemica GmbH & Co., USA
- PCR purification kit (catalog# 28104), Qiagen, Germany
- Pepstatin A, $C_{34}H_{63}N_5O_9$, F. W. = 685.9, Sigma, Germany
- Phenylmethylsulfonylfluoride (PMSF), $C_7H_7FO_2S$, F. W. = 174.2, Sigma, Germany
- 85% (v/v) Phosphoric acid, H_3PO_4, M. W. = 98, Mallinckrodt, USA
- Potassium carbonate, K_2CO_3, F. W. = 138.2, Sigma, Germany
- Sodium acetate, $CH_3COONa.3H_2O$, M. W. = 136.09, M&B Ltd., England
- Sodium dodecyl sulfate, BDH laboratory supplies, England
- Sodium chloride, NaCl, M. W. = 58.4, Merck, Germany
- Sodium hydroxide, NaOH, M. W. = 40, Merck, Germany
- Sodium thiosulfate, $Na_2S_2O_3.5H_2O$, F. W. = 248.2, Sigma, Germany

- SV Total RNA isolation system (catalog# Z3100), Promega, USA
- Sucrose, $C_{12}H_{22}O_{11}$, M. W. = 342.30, Merck, Germany
- TEMED, Promega, USA
- Tris-(hydroxymethyl)-aminomethane, $NH_2C(CH_2OH)_3$, M. W. = 121.4, Pharmacia biotech, USA

2.3 Sample collection

One colony of *Apis cerana* honey bees was taken from Nakhon Sri Thammarat province and maintained at Nonthaburi province for enzyme assay. All samples were collected during February - April, 2003. Newly emerged workers were marked on their thorax by using paint markers (waterproof) and introduced into a free-flying colony (Fig. 2.1 and 2.2, respectively). The colony was fed with sugar candy everyday. Thereafter, marked bees were collected for 29 days with the time interval of 3 days and were immediately stored at –20 ^{o}C until use.

For RNA extraction, nurse bees were collected while they were feeding broods. Forager bees were collected when they returned to the colony after foraging for nectar and pollen. Foragers from 14 colonies were collected from various provinces in Thailand: one colony each from Bangkok, Rayong, Udornthani, Chumporn, Trang, Phuket, Pa-ngan Island, Nakhon Sri Thammarat, Pang-nga, and 5 colonies from different location on Samui Island (Fig. 2.3). The colonies were collected from nature or apiaries (Table 2.1). Samples were frozen in either liquid nitrogen or dry ice during field trips. Then, they were stored at –80^{o}C in laboratory.

A: Nurse bees (9 - 12 days).

They are in different color and are feeding a queen and broods.

B: Forager bees (18 days). They are in green and are at a hive entrance.

Figure 2.1 The duty of bees in a colony depends on age.

Table 2.1. Foragers from 14 colonies were collected from nature or apiaries.

Sampling area	nature	apiaries
North, Northeast, and Middle		
Rayong (RY)	✓	
Udornthani (UD)	✓	
Bangkok (BK)		✓
South and Phuket		
Chumporn (CH)		✓
Phuket (PH)		✓
Pang-nga (PN)	✓	
Trang (TR)	✓	
Nakhon Sri Thammarat (NA)		✓
Samui Island (SA)		
Location 1 (SA1)		✓
Location 2 (SA2)		✓
Location 3 (SA3)		✓
Location 4 (SA6)	✓	
Location 5 (SA7)		✓
Pa-ngan Island (PA)		✓

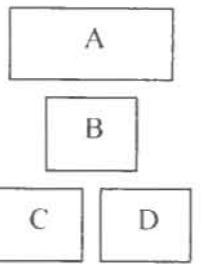
Figure 2.2 The sampling colonies of *A. cerana* locating in various places.

A: a hive from Pa-ngan Island

B: a hive from Phuket

C: a natural hive from Rayong

D: a hive from Samui Island

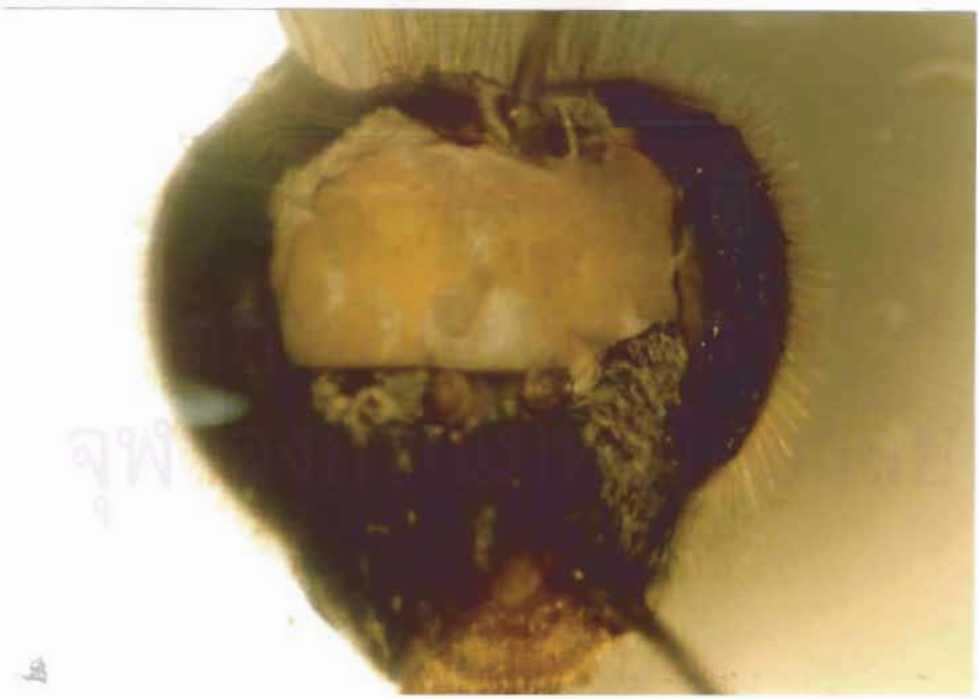


2.4 Preparation of hypopharyngeal glands

Heads of all bees were dissected under a binocular microscope. A point of a knife was used to cut through wall of the mask, around the edges of the compound eyes. The mask then was lifted off. The hypopharyngeal glands (HPGs) would be visible as shown in figure 2.3.

For enzyme assay, a HPGs were dissected out, stored in buffer insect saline (20 glands/500 µl) (Appendix A) and mixed with 1 mM phenylmethylsulfonyl fluoride, 0.1 µg/ml pepstatin, and 100 µg/ml leupeptin. The glands were homogenized and centrifuged 2 times at 4°C, 700 × g for 10 min. The supernatant was stored at –20°C.

For RNA isolation, HPGs were dissected out of each head and stored at –20°C (10 glands/reaction).



2.5 Morphological characterization and measurement of HPGs

The morphology of HPGs from nurse bee and forager bee was examined by Scanning Electron Microscopy (SEM) (JSM-35CF). The HPGs were stored in 70% alcohol. Then, the samples were washed in a sonicator bath for 5 min, 3 times, and treated in 100% alcohol. Later, the HPGs were coated with gold particles(JFC-1100) and photographed under SEM.

For measurement of HPGs, the HPGs were dissected out of each bee and stored in 0.85% (w/v) NaCl solution. The shortest diameter (a) and the longest diameter (b) (μm) of acini were measured by using light microscope and an ocular scale. The area of HPGs was evaluted as S (μm^2) = $\pi \times a/2 \times b/2$ (Sasagawa *et al.*, 1989).

2.6 Activity assay of α - glucosidase (α - G) in HPGs of *A. cerana*

2.6.1 Enzyme assay

Alpha–glucosidase (α-G) activity was determined by measuring glucose liberated from sucrose. The method was modified from Momose's method (Kubo *et al.*, 1996). According to 2.4, the supernatant containing α-G (2-10 μl) was incubated with 20 μl of 10 mM phosphate buffer containing 0.1 M sucrose pH 5.0 at 30^0C for 10 min. Then, the sample was boiled for 3 min to stop a reaction. Fifty μl of 0.3% (w/v) 3,6-dinitrophthalic acid and 50 μl of alkaline solution [5% (w/v) sodium thiosulfate and 25% (w/v) potassium carbonate] were added to the sample. Then, the mixture was boiled for 10 min and was filled up to 1 ml by dd-H_2O. The absorbance of the mixture was measured at 450 nm. One unit is defined as an enzyme activity which can hydrolyze 1 μM of sucrose per minute under the assay conditions. All measurements were conducted in duplication, and the mean value was calculated for the analysis.

2.6.2 Optimum conditions for α-G activity

2.6.2.1 Optimum pH of α-G in crude extract

The reaction mixture was prepared similar to that described in 2.6.1 except pH of a reaction. Ten mM Briton-Robinson buffer solution (Nishimoto *et al*, 2001; Appendix A) at various pHs ranging between 3.0–7.5 were used instead of phosphate buffer. The mixture was conducted in duplication or triplication. The absorbance of the mixture was measured at 450 nm.

2.6.2.2 Optimum temperature of α-G in crude extract

The reaction mixture was prepared similar to that described in 2.6.1. Alpha-G was incubated at various temperatures (20, 30, 35, 40, 45, 50, 55, 60, and 70°C) for 10 min. Due to the results of 2.6.2.1, 10 mM sodium acetate buffer at pH 5.0 was used instead of 10 mM Briton-Robinson buffer solution. The mixture was conducted in duplication and triplication. The absorbance of the mixture was measured at 450 nm.

2.6.2.3 Selective concentration of substrate in crude extract

The reaction mixture was prepared as mentioned in 2.6.1 but the concentration of sucrose was varied from 10, 20, 30, 40, 50, 60, and 70 mM, respectively. The mixture was incubated at 45°C with 10 mM sodium acetate buffer (pH 5.0) according to the results obtained from experimnt 2.6.2.1 and 2.6.2.2. The mixture was conducted in duplication and triplication. The absorbance of the mixture was measured at 450 nm.

2.6.2.4 Optimum incubation time of α-G in crude extract

The reaction mixture was the same prepared as described in 2.6.1 and the sucrose concentration was 50 mM according to the result of 2.6.2.3. The mixture was incubated at 45°C with 10 mM sodium acetate buffer (pH 5.0). The incubation time for α-G was varied from 10, 20, 30, 40, 60, and 90 min, respectively. The mixture was

conducted in duplication and triplication. The absorbance of the mixture was measured at 450 nm.

2.6.3 Specific activity of α-G in crude extract

A standard series of glucose solution were prepared in 5 different concentrations which were 0.02, 0.05, 0.08, 0.11, and 0.14 μM, respectively. The reaction mixture was prepared as described in 2.6.1. Fifty μl of 0.3% (w/v) 3,6-dinitrophthalic acid and 50 μl of alkaline solution were added to the mixture. Then, the mixture was boiled for 10 min and was filled up to 1 ml by H_2O. The O.D. of the mixture was measured at 450 nm. The α -G activity was calculated by the standard curve of glucose. The α -G activity was determined from the absorbance difference between a blank and the reaction mixture.

Protein concentration was determined by the Coomassie blue method (Bradford, 1976). A standard curve was established by using bovine serum albumin (BSA) at various concentrations from 2, 4, 6, 8, and 10 μg/μl, respectively (Appendix C). One hundred ml of sample were mixed with 1 ml of protein reagent (Appendix A) and left for 5 min before recording the absorbance at 595 nm. Due to Bollag *et al.* (1996), the color is fully developed after 5 min. The specific activity of α-G was reported as units/mg protein.

2.6.4 Polyacrylamide gel electrophoresis of crude extract

An electrophoresis of both non-denaturing and denaturing gels was performed by using a discontinuous buffer system.

2.6.4.1 Non-denaturing Polyacrylamide Gel Electrophoresis (Native-PAGE)

Four and 12% polyacrylamide were used for a stacking gel and a separating gel, respectively. Tris-glycine, pH 7.8, was used as electrode buffer (Appendix B). The sample in native buffer (Appendix B) was kept on ice until loading. After electrophoresis at 100 V, α-G activity was stained as described in 2.6.4.4.

2.6.4.2 Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE)

For discontinuous SDS-PAGE, a 4% polyacrylamide stacking gel (pH 6.8) with a 12% polyacrylamide separating gel (pH 8.8) was placed in electrophotometry. Samples in sample buffer (Appendix B) were denatured by heating for 5 min. Broad Range Protein marker (Promega) was used as a standard marker. A constant voltage of 100 V was applied at room temperature. The gel was silver stained as described in 2.6.4.3.

2.6.4.3 Silver staining for protein

When electrophoresis is completed, the gel was placed in fix a solution [50% (v/v) methanol and 10% (v/v) acetic acid] about 1 h at RT. Then, the gel was transferred into 5% (v/v) methanol for 10 min and 160 mM HNO_3 for 10 min. The gel was washed with dd-H_2O and soaked by shaking for 10 min in 0.1% (w/v) silver nitrate. The gel was briefly rinsed by milli-Q H_2O for 2-3 sec before developing the gel in a cool developing solution [3% (w/v) sodium carbonate and 50 µl of 37% (v/v) formaldehyde]. The gel was developed until protein bands were visible. Then, the developing reaction was stopped by soaking the gel with 1 M citric acid for 5 min. Later, the gel was rinsed with dd-H_2O and dried by a cellophane paper at RT.

2.6.4.4 **Activity staining for α-G** [modified from Tanimura *et al.* (1979)]

After electrophoresis of Native-PAGE, the gel was incubated in 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0) at 45^0C for 30 min. The gel was washed by dd-H_2O, then, boiled in freshly prepared solution of 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH for 3 min. Alpha-G activity on the gel was appeared in red. For SDS-PAGE, the gel was soaked in 1.0% (v/v) Triton X-100 at RT with gentle shaking for 2 h. Finally, the gel was incubated with 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0).

2.7 Partial cDNA sequence and phylogenetic tree.

2.7.1 Primer design

The primers of α-G in *A. cerana* for RT-PCR were from parts of cDNA sequences of α-G in *A. mellifera* (Ohashi *et al.*, 1996; Appendix E). The primers were synthesized by Bioservice unit, Thailand. The primer sequences are as followed:

α - G: Forward primer (FW): 5'-TCGAC TTCTA GTTGG TAGCA TGAAG G-3'

Reverse primer (R): 5'-CCTTT CTCAT GTGCA GCACT GACTA G-3'

In addition, control primers were designed from 28S RNA in *A. mellifera* and Elongation factor (EF) genes in *A. cerana*. The primer sequences are as followed:

28S RNA: FW : 5'-AAAGA TCGAA TGGGG ATATT C-3'

R : 5'-CACCG GGTCC GTACC TCC-3'

EF: FW : 5'-TCGCT TTTAC TCTTG GTGTG A-3'

R : 5'-AAACT CCCAA CATAT TATCT CCA-3'

2.7.2 Isolation of total RNA from HPGs of *A. cerana*

Followed from 2.4, HPGs were homogenized with liquid N_2 in a mortar. The grided tissue was transferred to a tube containing 175 µl SV RNA lysis buffer. Then, the protocol was followed by the SV total RNA isolation kit (Promega, catalog# Z3100). Briefly, the mixture was mixed by inversion. Three hundreds and 50 µl of RNA dilution buffer (blue) was added and mixed by inverting 3-4x. The mixture was incubated at 70°C for 3 min. The mixture was then centrifuged at 12,000-14,000 x g for 10 min. The supernatant was transferred to a new tube. Two hundred µl of 95% ethanol were added and mixed by pipeting 3-4x. Later, the mixture was transferred to spin column assembly and centrifuged at 12,000-14,000 x *g* for 1 min. Flow thru in a collection tube was discarded. Six hundreds µl of SV RNA wash solution were added to the previous spin column assembly. Again, it was centrifuged at 12,000-14,000 x g for 1 min. The DNase incubation mix was prepared by 40 µl yellow core buffer, 5 µl of 0.09 M $MnCl_2$, and 5 µl of DNase I enzyme per sample. Then, the DNase incubation mix was transferred to the membrane inside the spin basket. The mixture was incubated at 20-25°C for 15 min. Two hundred µl of SV DNase stop solution were added to the spin basket and centrifuged at 12,000-14,000 xg for 1 min. Then, 600 µl of SV RNA wash solution were added and centrifuged at 12,000-14,000 xg for 1 min. Also, 250 µl of SV RNA wash solution were added again and centrifuged at 12,000-14,000 xg for 2 min. The spin basket from the collection tube was transferred to the elution tube. Later, 100 µl of nuclease-free H_2O were added to the membrane and centrifuged at 12,000-14,000 xg for 1 min. Finally, the spin basket was removed and discarded. The purified RNA was stored at -20°C.

The extracted total RNA was electrophoresed on 0.8% (w/v) agarose gel in 1XTBE buffer (0.05 M Tris-HCl, 0.05 M Boric acid, and 0.65 mM EDTA) as running buffer. The electrophoresis was operated at 100 V for 45 min. *Lambda Hin*d III DNA was used as standard DNA marker. Loading sample was composed of 5 µl of total RNA and 1 µl of loading dye [5X DNA BlueRun™ which contains 150 mM EDTA, 25 mM Tris-HCl (pH 7), 25% (v/v) glycerol, and 0.05% (w/v) bromophenol blue]. After electrophoresis, the gel was stained with ethidium bromide (EtBr) solution and destained in dd-H_2O water. Then, the RNA bands would be visible under UV light and photographed.

2.7.3 RT-PCR amplification

The reaction mixture of RT-PCR was prepared by 1X AMV/*Tfl* reaction buffer, 0.2 mM dNTP mix, 0.4 mM of each α-G primer (FW and R), 1 mM $MgSO_4$, 0.1 U of AMV reverse transcriptase and *Tfl* DNA polymerase, and 2 µl of total RNA. Final volume was quantitated to be 25 µl by nuclease-free H_2O. The RT-PCR cycling profile was modified from Ohashi *et al.* (1996).

The first strand cDNA was synthesized at 48°C for 45 min. Then, the PCR amplification was as followed: 1 cycle of denaturation at 94°C for 2 min; 20 cycles of denaturation at 94°C for 30 sec, of annealing at 50°C for 30 sec, and of extension at 72°C for 45 sec. At last, the final extension was at 68°C for 7 min.

As a control, 28S RNA and EF primers were used as RNA reference markers. The PCR amplification was at the same condition as of α-G primers.

When PCR amplification completed, loading sample was prepared by 5 µl of PCR product and 1 µl of loading dye. The electrophoresis was performed on 1.5%

agarose gel at 100 V for 45 min. One hundred bp DNA ladder was used as DNA standard marker. The gel was then EtBr stained and U.V. transilluminated.

2.7.4 Examination of expression levels of α-G in *A. cerana*

The mRNAs of emerged bees, nurse bees, worker bees of 6 days old, and forager bees were examined. The reaction mixtures of RT-PCR and PCR amplification were the same as described in 2.7.3. except the volume of total RNA. Two hundreds ng of each total RNA were used per reaction. The yield of total RNA and PCR products were determined by spectrophotometry at 260 nm. One absorbance unit (A_{260}) equals to 40 μg of single stranded RNA/ml and 50 μg of double stranded DNA/ml, respectively. The 28S and EF primers of honeybees were used as a control for total RNA preparation and RT-PCR reaction.

2.7.5 Purification of PCR product for sequencing

PCR product was purified by following the protocol of the QIAquick PCR purification kit (Qiagen, catalog# 28104). Five hundreds μl of Buffer PB were added to 100 μl PCR reaction. The QIAquick spin column was placed into a provided 2-ml collection tube. The sample was transferred to the QIAquick spin column and centrifuged for 30-60 sec. Then, flow thru was discarded and the QIAquick column was put back into the same tube. The column was washed by 0.75 ml buffer PE and centrifuged for 30-60 sec. Then, the effluent was discarded. The column was centrifuged again at maximum speed for 1 min. The QIAquick column was placed into a clean 1.5 ml tube. The column was filled by 50 μl milli Q H_2O and spun for 1 min. The purified cDNA was stored at –20°C. PCR product was checked by 1.5% (w/v) agarose gel as described in 2.7.2 before sequenced at Bioservice unit, Thailand.

Forward and reverse primers of *α-G* were used to sequence the products from Bangkok and Samui3. Only forward primer was used to sequence the rest of 12 RT-PCR products.

2.7.6 Sequence alignment and phylogenetic analyses

The nucleotide and deduced amino acid sequences were aligned by using Clustal W program. The cDNA sequences were aligned and compared to the sequences of maltase in fruit fly (*Drosophila melanogaster*), *α-G* in mosquito (*Aedes aegypti*), and *α–G* in *Culex pipiens*. Those cDNA sequences were from GenBank. Genetic relatedness between α-G in *A. cerana* and *A. mellifera* (accession number: D79208 in GenBank) was performed by phylogenetic tree construction using PAUP (version 4.0b). The reliability of the tree was tested by bootstrap for 1000 replicates. Data from sequences were used to reconstruct a phylogeny with maximum parsimony. Genetic distances were computed by neighbor-joining method and UPGMA.

CHAPTER III
RESULTS

3.1 Morphological characteristics and measurement of HPGs

The samples were collected according to their duties in a colony. Nurse bees were collected when they were feeding broods. Forager bees were collected when they returned to the colony after foraging for nectar and pollen. The HPGs were photographed by SEM. The acini of HPGs in nurse bees were well developed and swolen as shown in Fig. 3.1. In contrast, those in forager bees were shrunks and covered with membrane as shown in Fig. 3.2.

The acini of HPGs at developmental size were used for enzyme assay. The acini from the age-defined bees were measured by a light microscope as shown in Fig. 3.5–3.15. The shortest diameter decreased on day-12 and increased again on day-15 (Fig. 3.3). Thereafter, both of the shortest and longest diameters decreased until the day of 29th. The size of HPGs rapidly increased during day-3 to day –9 and became largest on day-15 as shown in Fig. 3.4. The size of most of acini on day-0 to day-9 were similar to each other, as shown in Fig. 3.5 – 3.8). In contradiction, the size of acini on day-12 to day-15 were changeable (Fig. 3.9 and 3.10).

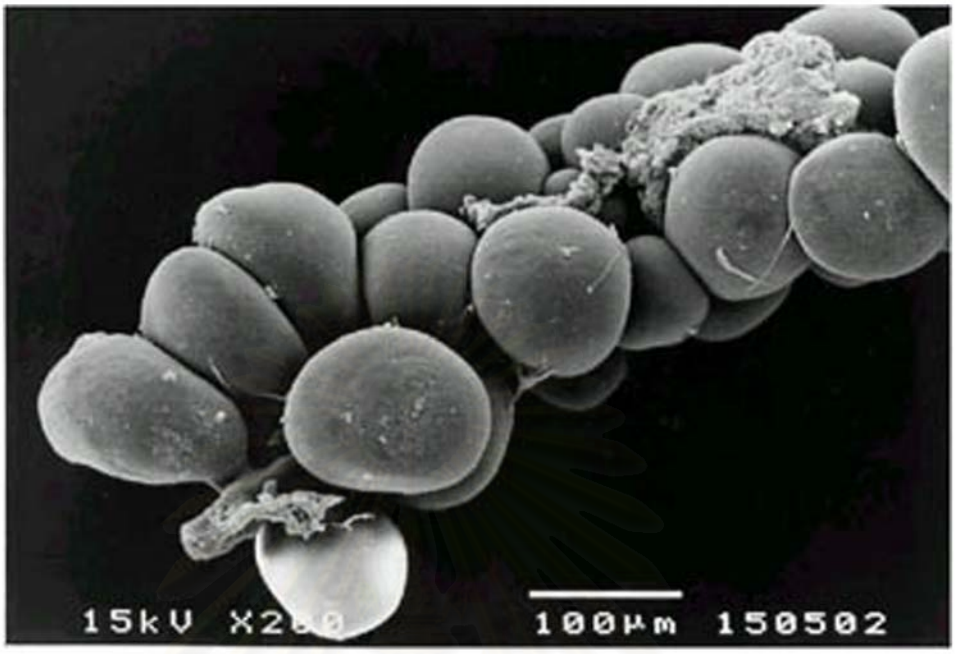


Figure 3.1 SEM of HPGs in nurse bees (*A. cerana*).

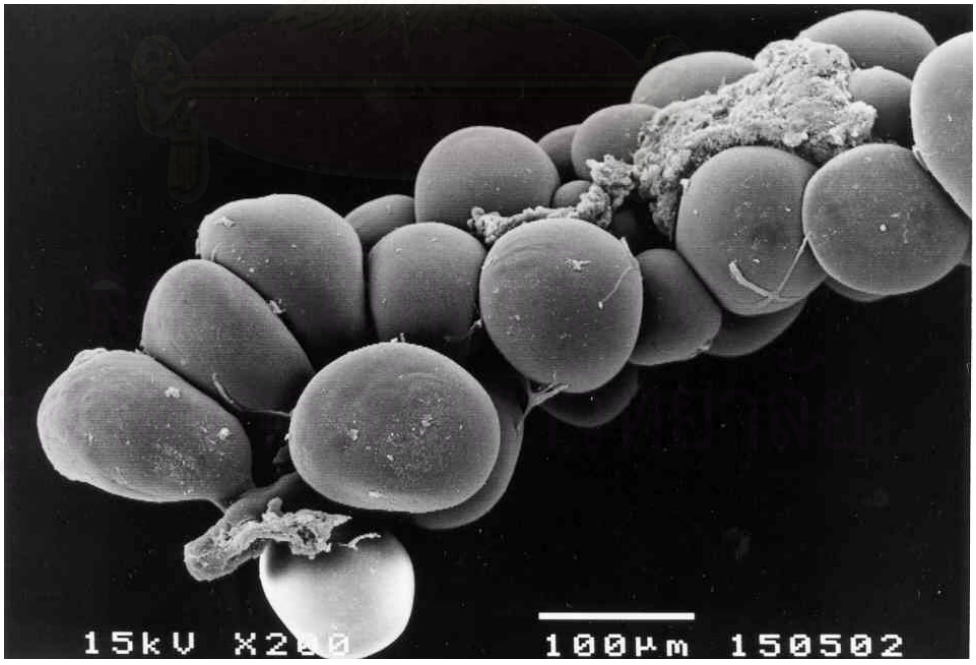


Figure 3.2 SEM of HPGs in forager bees (*A. cerana*).

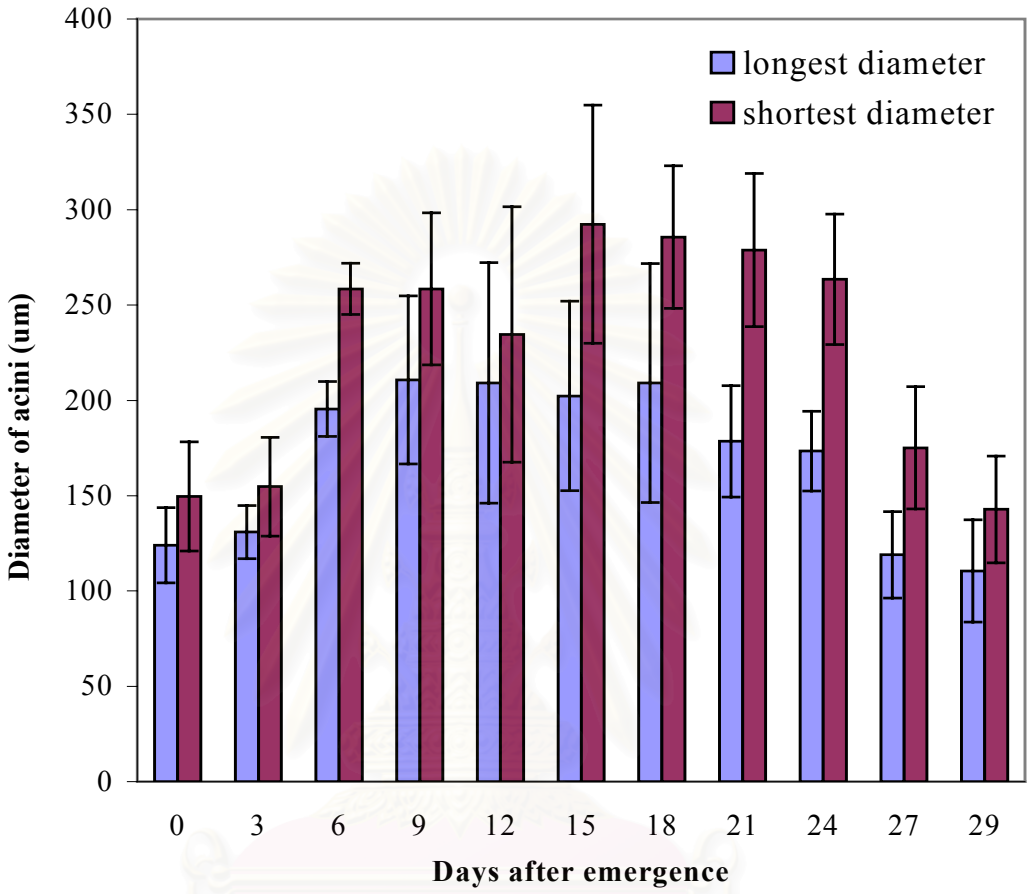


Figure 3.3 The change of acinus sizes in worker bees at various ages. Ten acini from one bee at each stage were measured.

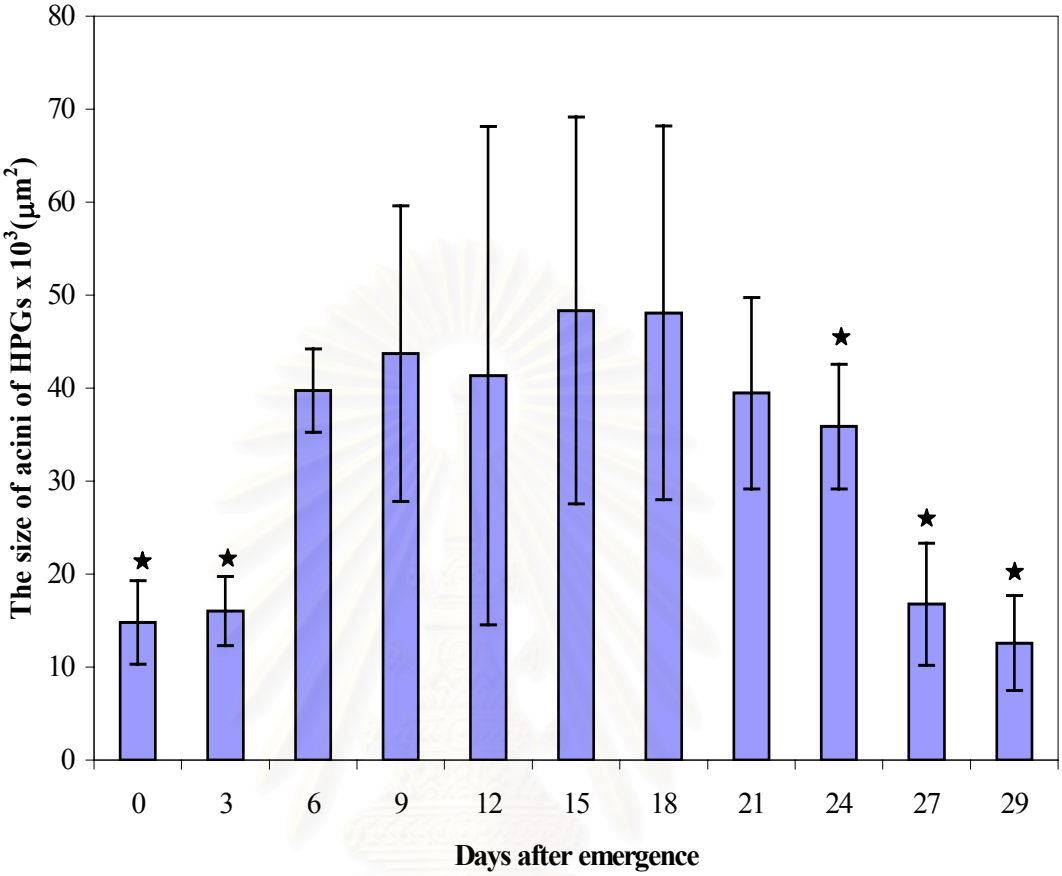


Figure 3.4 The sizes of acini in worker bees at various ages. Ten acini from one bee at each stage were measured. Asterisks indicate the significance of mean difference at the 0.05 level when the size on day-15 was used as the standard.

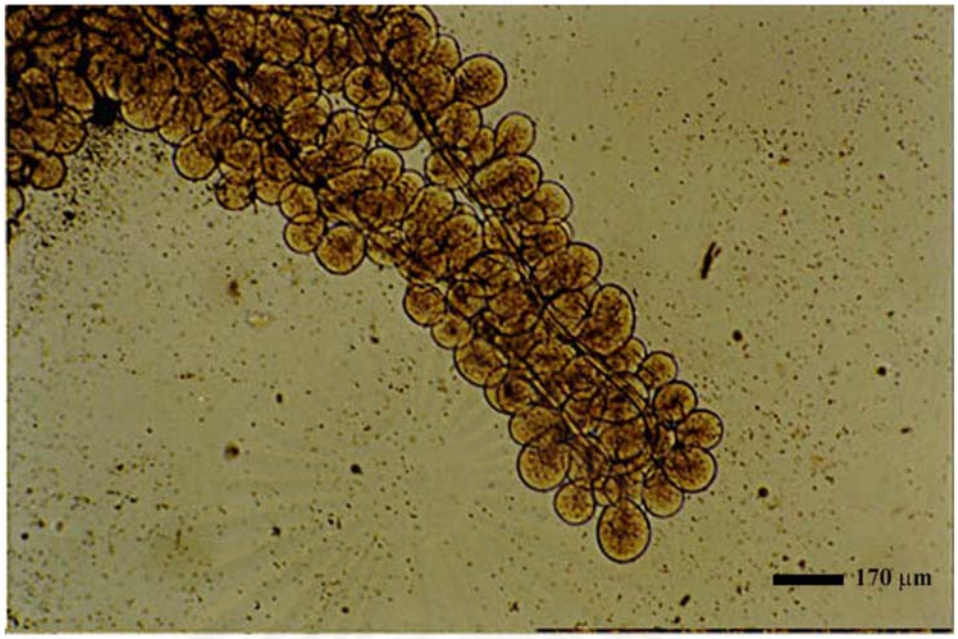


Figure 3.5 The HPGs of emerged bees were photographed with a light microscope (LM) at the 4× magnification of objective lens.

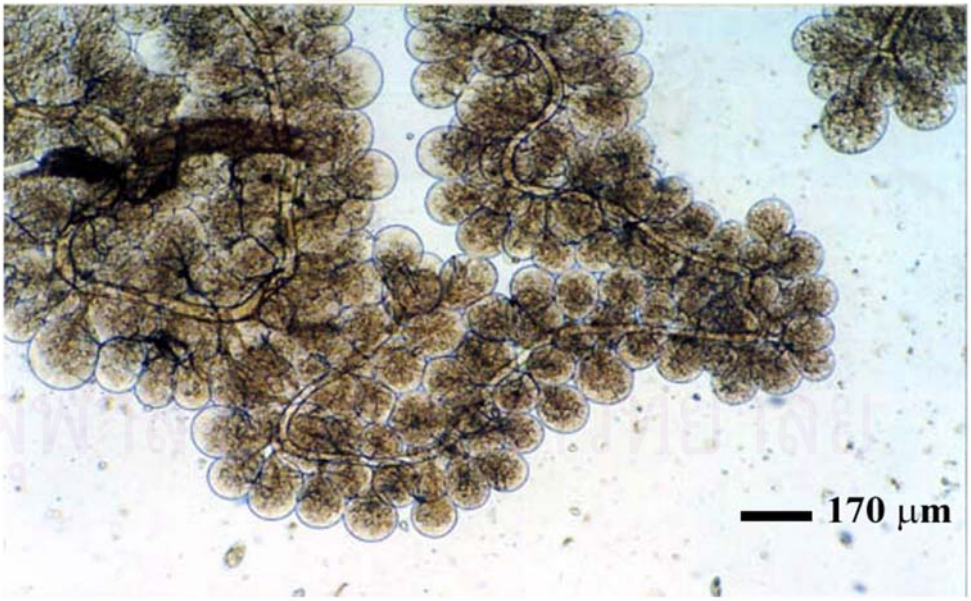


Figure 3.6 The HPGs of worker bees on day-3 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

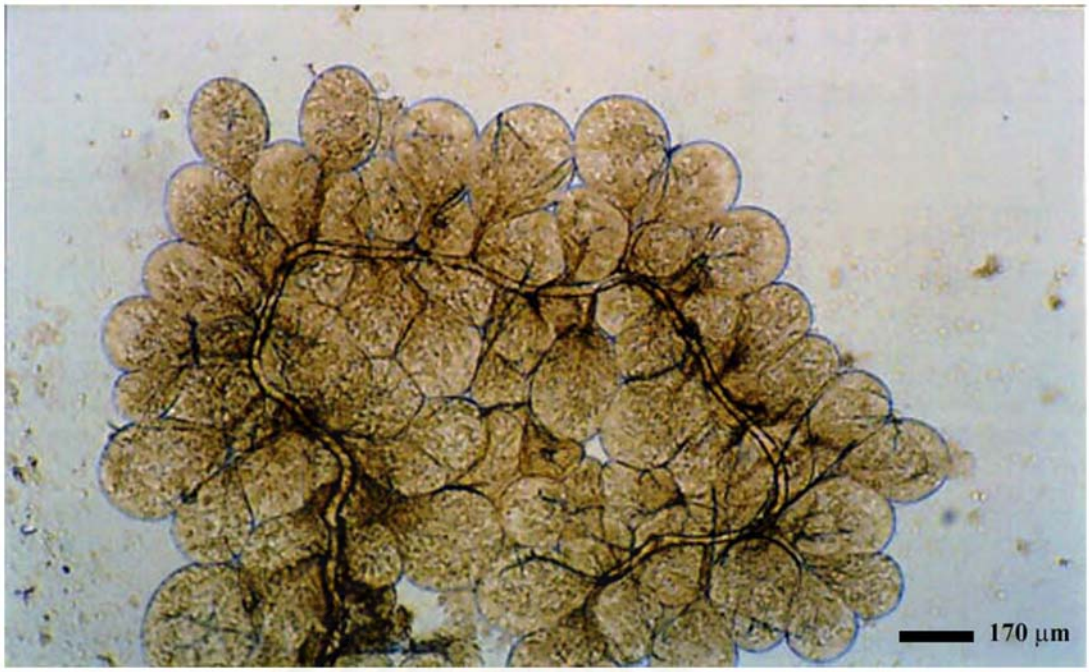


Figure 3.7 The HPGs of worker bees on day-6 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

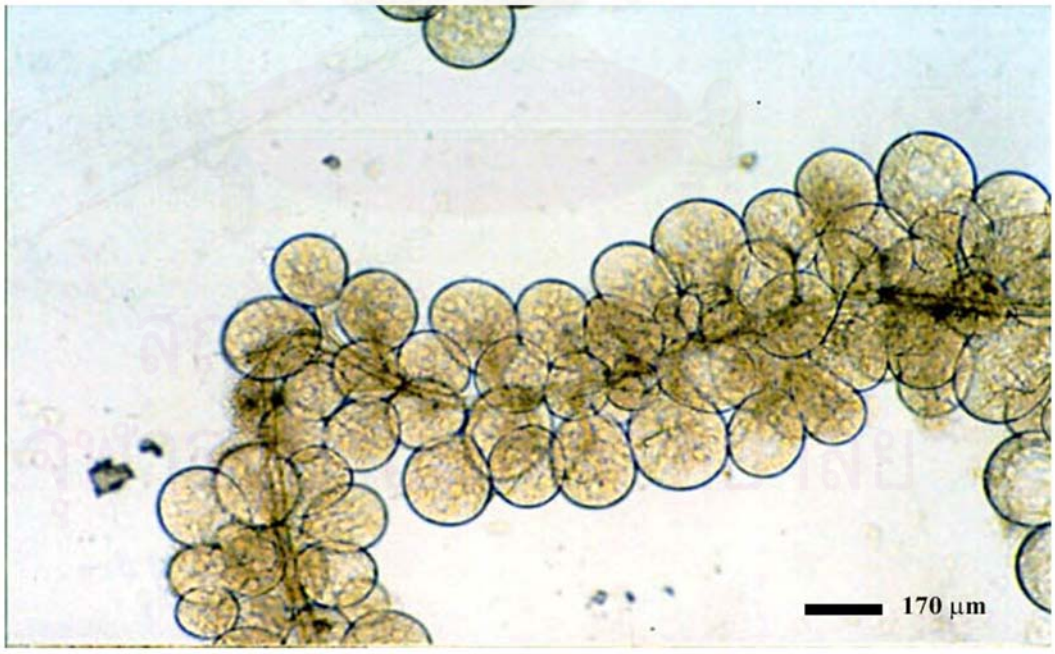


Figure 3.8 The HPGs of worker bees on day-9 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

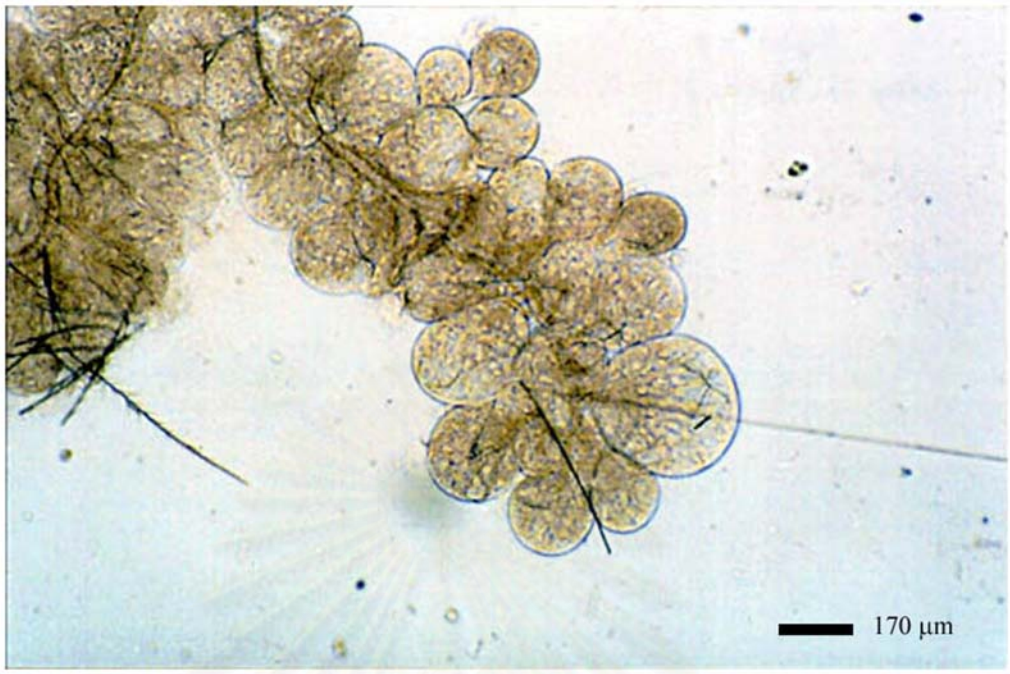


Figure 3.9 The HPGs of worker bees on day-12 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

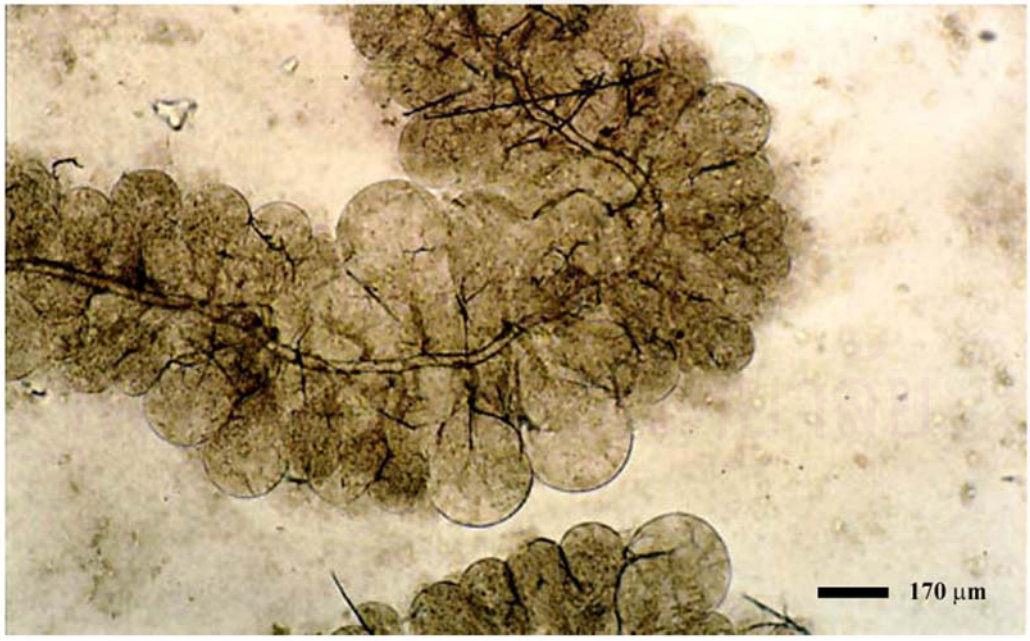


Figure 3.10 The HPGs of worker bees on day-15 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

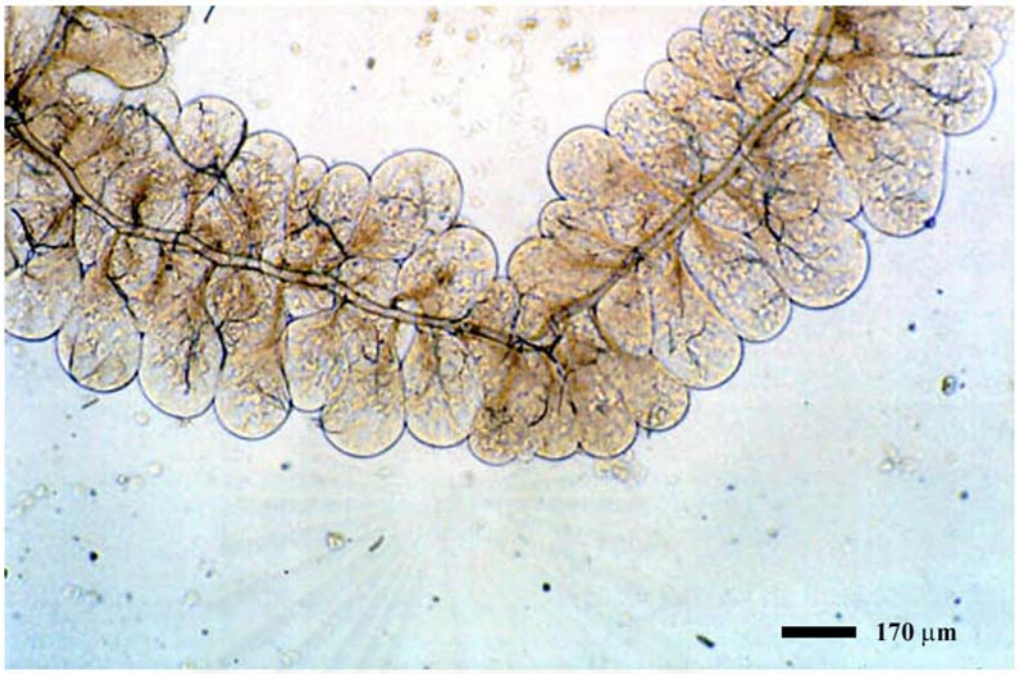


Figure 3.11 The HPGs of worker bees on day-18 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

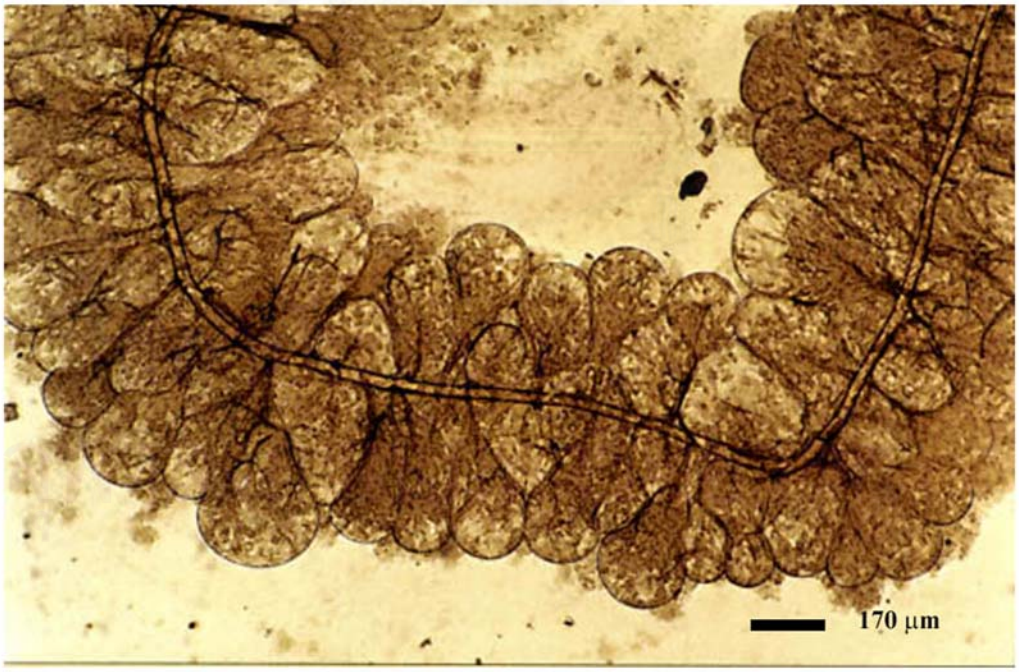


Figure 3.12 The HPGs of worker bees on day-21 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

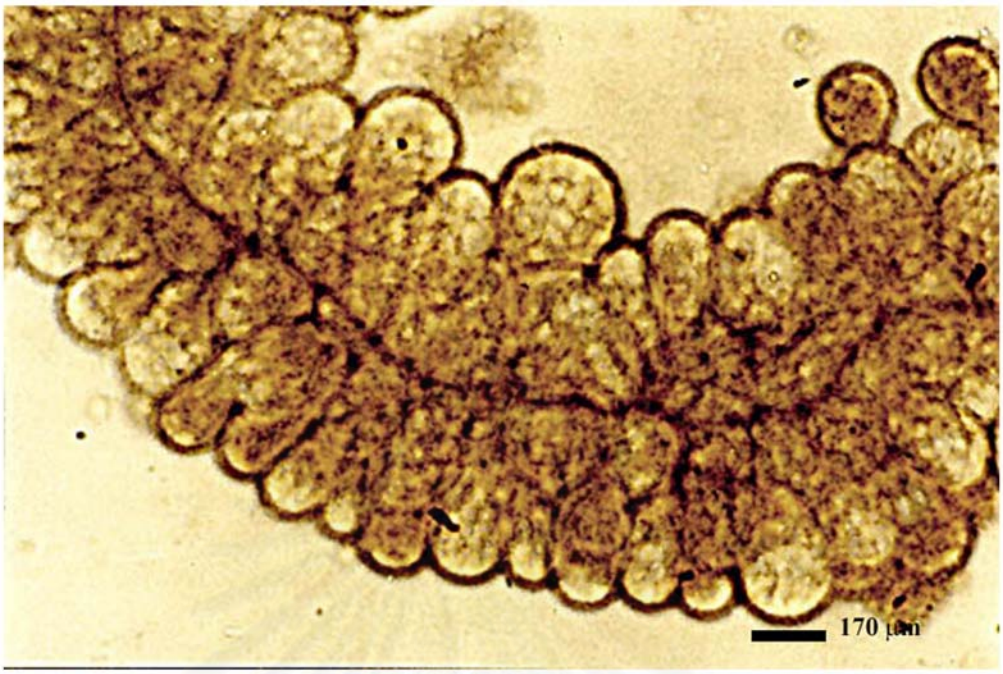


Figure 3.13 The HPGs of worker bees on day-24 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

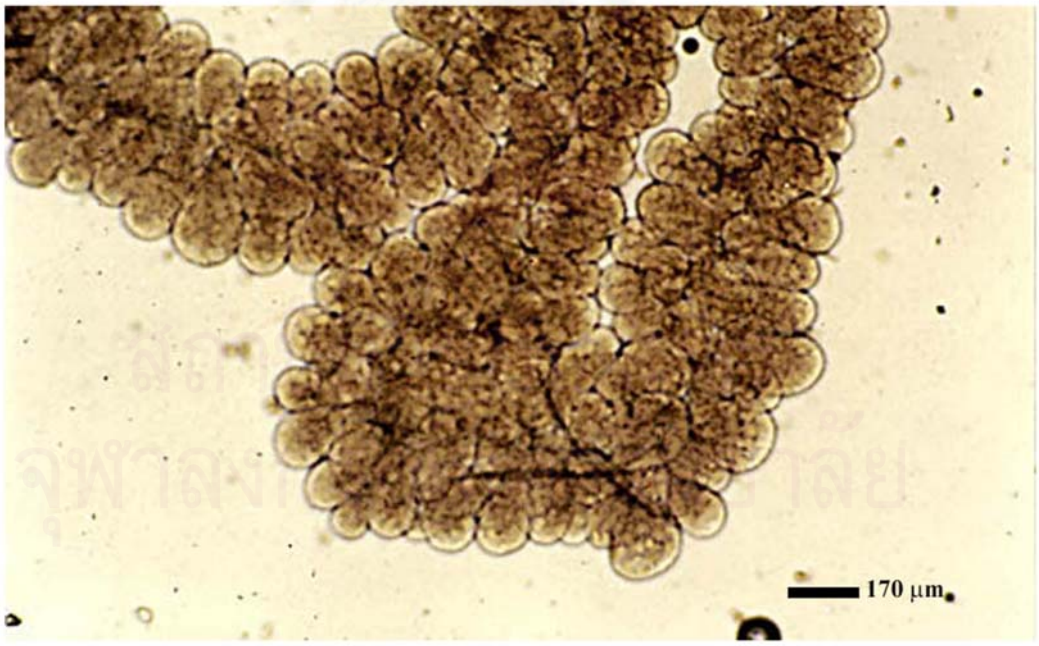


Figure 3.14 The HPGs of worker bees on day-27 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

Figure 3.15 The HPGs of worker bees on day-29 were photographed with a light microscope (LM) at the 4× magnification of objective lens.

3.2 Activity assay of α–G in HPGs of *A. cerana*

3.2.1 Optimum pH of α–G in crude extract

The effect of pH for α–G activity was determined by using crude extract of HPGs. Sucrose was used as the substrate. Briton-Robinson buffer solution was used as the buffer to adjust pH. As shown in Table 3.1 and Fig. 3.16, the highest specific activity was at pH 5.0. This pH was then used to determining optimum temperature of α–G activity in acetate buffer.

3.2.2 Optimum temperature of α–G in crude extract

Acetate buffer (pH 5.0) containing 0.1 M sucrose was used as the substrate buffer in this experiment. The activity was fluctuated between 35–55^{o}C, after that it was decreased. Nevertheless, the highest specific activity was at 45^{o}C as shown in Table 3.2 and Fig. 3.17. This temperature was further used to determining selective concentration of sucrose as substrate in crude extract.

3.2.3 Selective concentration of substrate in crude extract

The results of optimum pH (pH 5.0) and optimum temperature (45^{o}C) for α –G activity in crude extract were used for this experiment. The mixture was prepared but in various concentrations of sucrose from 10, 20, 30, 40, 50, 60, and 70 mM. The reaction was continued for 10 min. The highest specific activity was in a reaction containing 50 mM sucrose (Table 3.3 and Fig. 3.18). Thus, 50 mM sucrose in acetate buffer at pH 5.0 was used for next experiments.

3.2.4 Optimum incubation time of α-G in crude extract

The effect of incubation time was studied. The reaction mixture in acetate buffer (pH 5.0) containing 50 mM sucrose was incubated at 45^{o}C. The incubation time was varied from 10, 20, 30, 40, 60, and 90 min, respectively. The highest specific activity was obtained from the incubation time of 30 min (Table 3.4 and Fig. 3.19). However,

the specific activity at 30 min was also high. Thus, the incubation of 30 min was used for other related experiments.

3.2.5 Alpha–G activity in crude extract of developmental stages of worker bees

The α–G activity was examined from crude extract of HPGs at various ages from 0 day to 29 days. The samples had been collected from Febuary to April, 2003. The activity of α–G was computed by comparing to the standard curve of glucose. The specific activity was mentioned as units/mg protein (U/mg protein). The total protein of crude extract in 1 gland in crude extract are shown in Table 3.5. Amount of total protein were different in each developmental stage of worker bees and in each month. The lowest amount of protein were from emerged bees. The total protein at worker bees on day-0 to day-12 increased rapidly. At worker bees on day-15 to day-29, the amount of protein is fluctuated. As shown in Table 3.6 and Fig. 3.20, the specific activity of α–G collected in Febuary increased very slowly from day-0 (emerged bee) to day-24. It decreased very slowly on day-27 and increased slowly again on day-29. For the samples collected in March, the specific activity was highest on day-24 while for those collected in April, the highest specific activity was on day-27. On the other hand, specific activity of α–G of HPGs in forager bees of *A. mellifera* was 14.19 U/mg protein.

Table 3.1 The table indicates the specific activity of α -G in crude extract at various pHs.

pH	Specific activity (U/mg protein)		
	Experiment 1	**Experiment 2**	**Experiment 3**
3.0	0.148	0.076	0.351
3.5	0.122	0.328	0.114
4.0	0.140	0.328	0.305
4.5	2.572	1.090	0.757
5.0	5.143	2.070	1.014
5.5	2.951	1.523	0.676
6.0	2.970	0.490	0.926
6.5	0.223	0.809	0.290
7.0	0.090	0.200	0.133
7.5	0.144	0.238	0.014

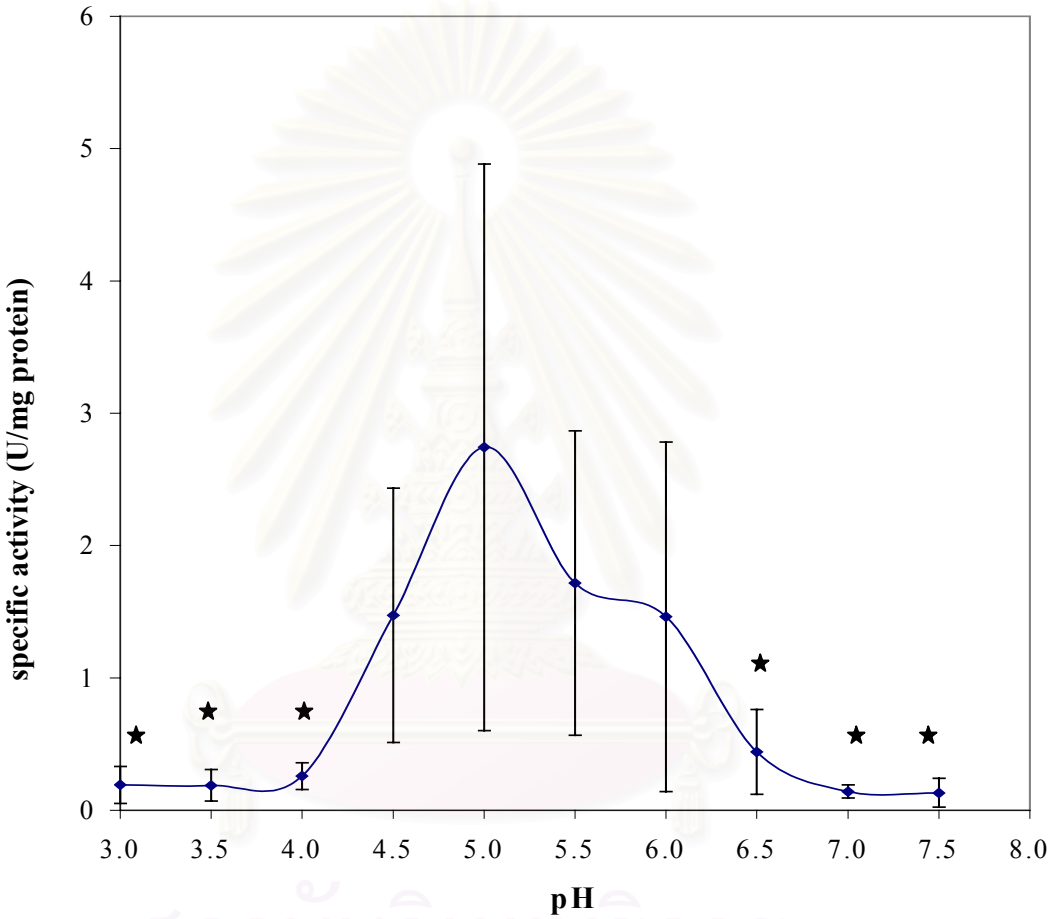


Figure 3.16 The optimum pH of α –G of HPGs in *A. cerana.* Briton-Robinson buffer at various pHs ranging between 3.0-7.5 was used. The optimum pH was 5.0. Asterisks indicate the significance of mean difference at the 0.05 level when specific activity at pH 5.0 was used as the standard.

Table 3.2 The table indicates the specific activity of α -G in crude extract at various temperatures.

Temperature (^{o}C)	Specific activity (U/mg protein)		
	Experiment 1	**Experiment 2**	**Experiment 3**
20	0.638	11.314	4.782
30	5.861	12.253	5.084
35	8.533	21.936	11.245
40	6.100	17.687	13.830
45	13.098	32.312	14.649
50	9.170	25.494	9.823
55	9.589	23.122	13.701
60	1.184	8.745	3.662
70	0.646	1.280	0.963

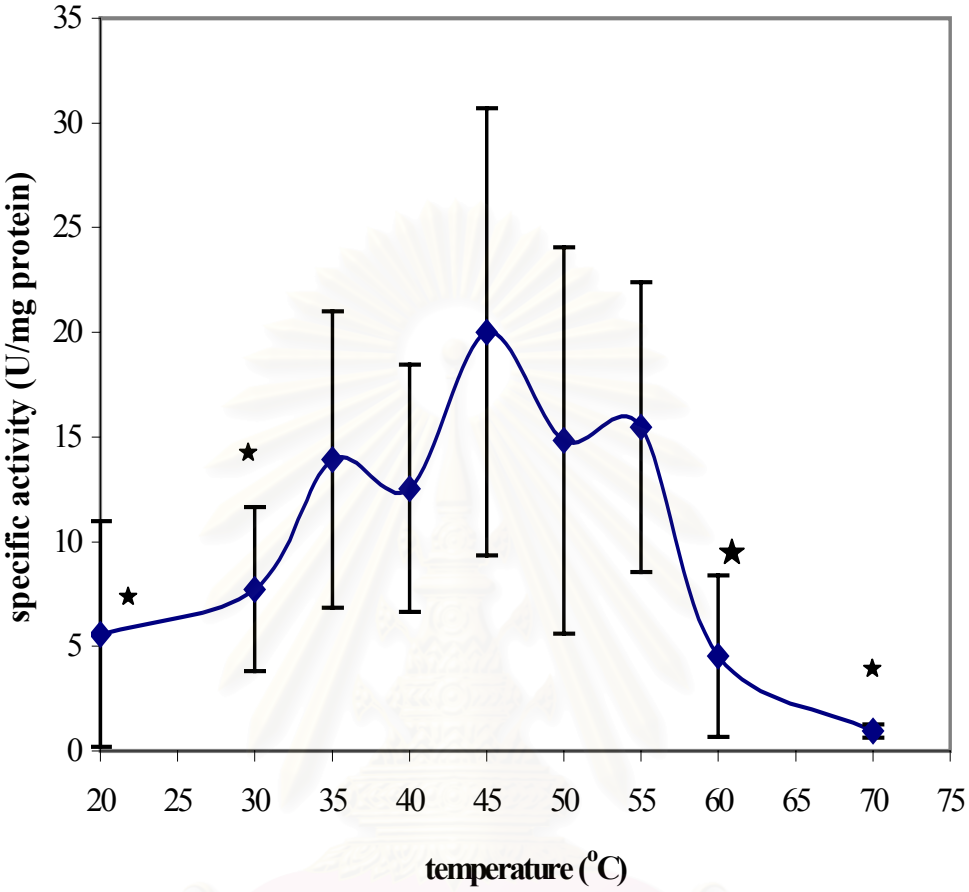


Figure 3.17 The optimum temperature of α –G of HPGs in *A. cerana.* The reaction mixture in acetate buffer (pH 5.0) containing 0.1 M sucrose was incubated at various temperatures ranging between 20, 30, 35, 40, 45, 50, 55, 60, and 70°C for 10 min. The optimum temperature was 45°C. Asterisks indicate the significance of mean difference at the 0.05 level when specific activity at 45 °C was used as the standard.

Table 3.3 The table indicates the specific activity of α -G in crude extract at various concentrations of sucrose.

Concentration of sucrose (mM)	Specific activity (U/mg protein)		
	Experiment 1	Experiment 2	Experiment 3
10	0.626	1.052	0.738
20	1.237	1.389	0.288
30	1.296	0.863	1.123
40	5.449	1.346	0.894
50	8.563	15.037	16.857
50	8.563	15.037	16.857
60	6.895	2.601	9.142
70	6.505	8.004	8.989

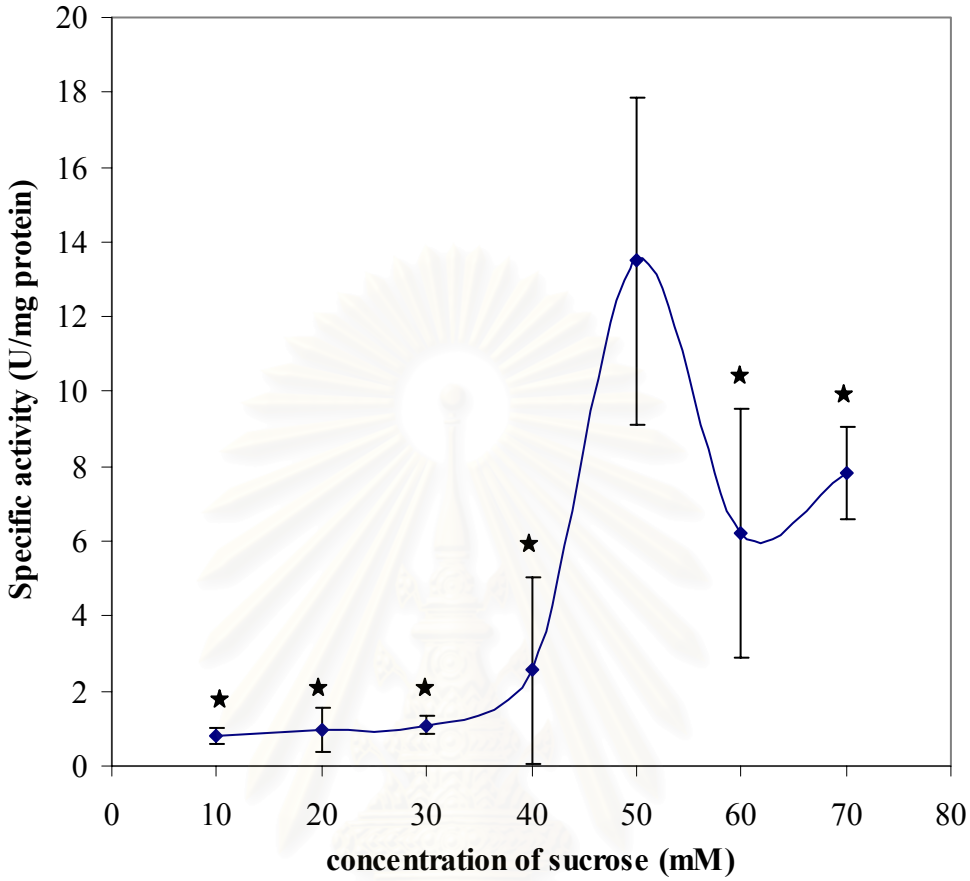


Figure 3.18 The optimum concentration of sucrose as substrate. The reaction mixture was incubated with sucrose at various concentrations of 10, 20, 30, 40, 50, 60, 70 mM, respectively. The optimum concentration of sucrose was 50 mM. Asterisks indicate the significance of mean difference at the 0.05 level when specific activity at 50mM was used as the standard.

Table 3.4 The table indicates the specific activity of α -G in crude extract at various incubation times.

Incubation time (min)	Specific activity (U/mg protein)		
	Experiment 1	Experiment 2	Experiment 3
10	2.936	6.818	6.167
20	2.820	7.215	5.360
30	3.131	9.365	6.945
40	1.009	4.525	2.656
60	1.186	3.071	0.567
90	0.698	1.853	0.554

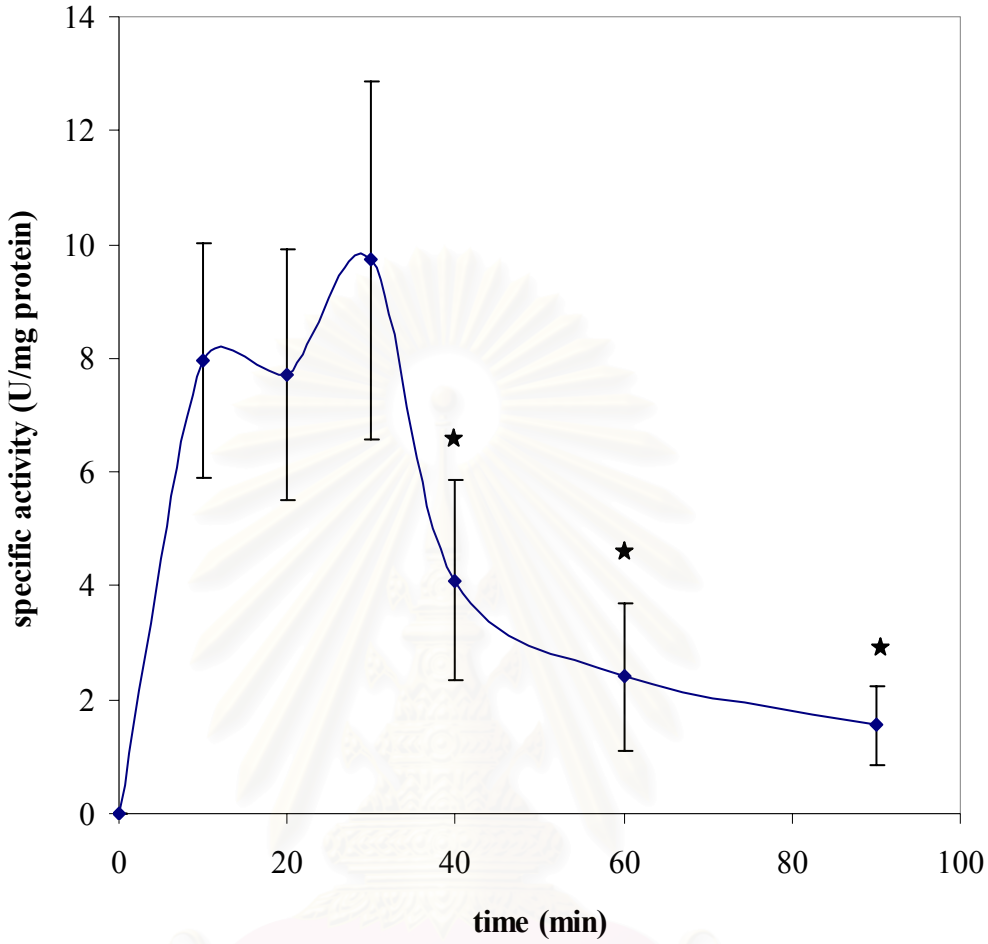


Figure 3.19 The optimum incubation time of α –G of HPGs in *A. cerana*. The reaction mixture was incubated for 10, 20, 30, 40, 60, and 90 min, respectively. The optimum incubation time was 30 min. Asterisks indicate the significance of mean difference at the 0.05 level when specific activity at 30 min was used as the standard.

Table 3.5 The table indicates the total protein (mg) in 1 gland in crude extract of α – G from worker bees at developmental stages.

Days after emergence	Total protein (mg) in 1 gland sample		
	February, 2003	**March, 2003**	**April, 2003**
0	2.666	1.814	2.073
3	29.480	14.793	14.474
6	38.530	34.609	34.689
9	73.477	57.416	47.348
12	75.605	92.404	97.089
15	44.892	18.620	28.788
18	54.435	33.692	16.846
21	69.780	48.545	34.191
24	44.131	6.738	23.365
27	76.501	115.331	6.180
29	20.296	17.564	12.540

Table 3.6 The table indicates the specific activity of α –G from worker bees at developmental stages.

Days after emergence	Specific activity (U/mg protein)		
	February, 2003	**March, 2003**	**April, 2003**
0	0.0426	0.0292	0
3	0.0524	0.0435	0.0120
6	0.0654	0.0284	0.0203
9	0.0229	0.0438	0.6399
12	0.0340	0.3115	0.7412
15	0.1471	1.6517	0.5131
18	0.4787	4.3283	15.4020
21	0.5276	4.0574	5.6223
24	1.3183	18.6623	7.2626
27	0.3030	1.3711	15.7819
29	2.3254	0.3159	8.0801

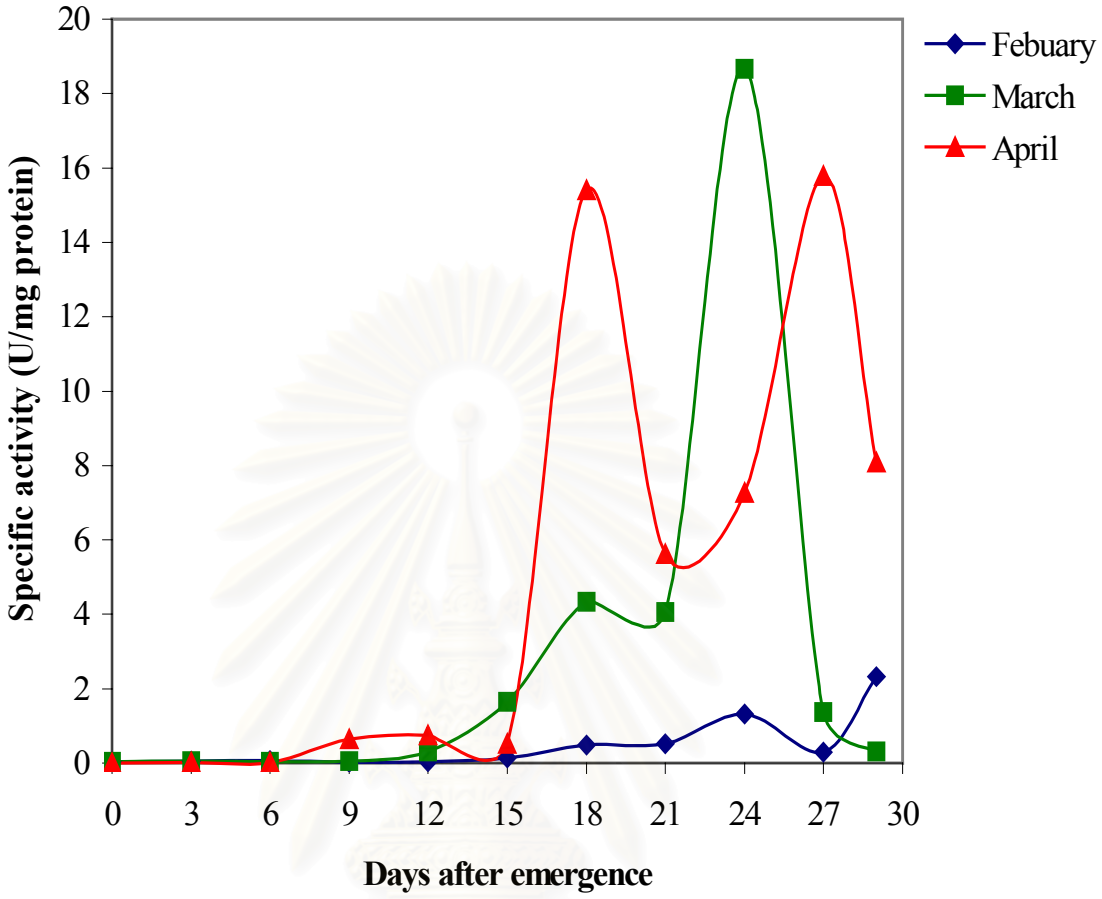


Figure 3.20 The specific activity of α -G of worker bees at developmental stages. The samples for each graph line were collected at different time (February to April, 2003).

3.3 Polyacrylamide Gel Electrophoresis (PAGE) of α–G

3.3.1 Non-denaturing Polyacrylamide Gel Electrophoresis (Native-PAGE)

The crude extract from worker bees at different stages (0, 6, 12, 18, 24, and 29 days) were analysed. The activity of α–G on native-PAGE was detected by 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH for 3 min. As shown in figure 3.21, the activity on the gel was appeared in red. One activity band was visible on the lane containing crude extract of forager bees. In addition, an activity band on the lane containing crude extract of 18-day, 24-day, and 29-day old workers is more intensed than those of 6-day, and 12-day old workers (Fig. 3.22).

3.3.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The crude extract from 6-day old workers and forager bees were used. Many protein bands were detected by silver staining [Fig. 3.23 (B)]. Before activity staining, the gel was treated with 0.1% (v/v) Triton X-100 for about 3 h. Only single activity band was visible on SDS-PAGE [Fig. 3.23 (A)]. The molecular mass of denatured α–G of 6-day old workers and foragers was compared by activity staining. The calibration curve of molecular mass was constructed (Fig. 3.24). The molecular mass of α–G in *A. cerana* was estimated to be about 96 kDa.

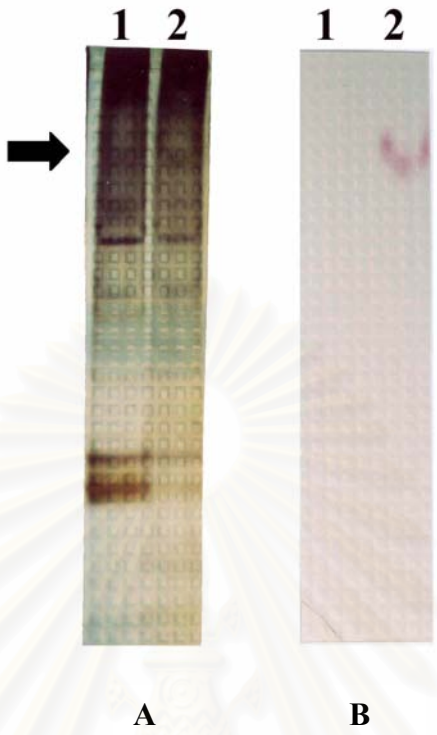


Figure 3.21 Native-PAGE of α–G in crude extract of *A. cerana.*

A: Silver staining

Lane 1: 6-day old worker bee (8 μg)

Lane 2: forager bee (8 μg)

B: Activity staining by 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH

Lane 1: 6-day old worker bee (30 μg)

Lane 2: forager bee (8 μg)

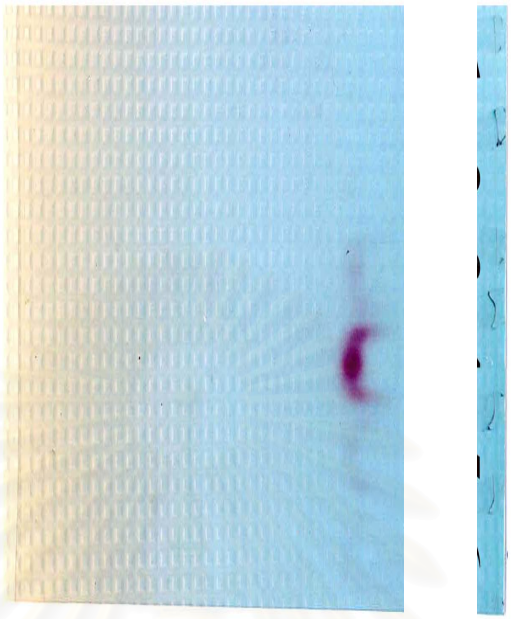


Figure 3.22 Native-PAGE of α–G in crude extract of *A. cerana* at developmental stages. The activity was stained with 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH.

Lane 1: 6-day old worker

Lane 2: 12-day old worker

Lane 3: 18-day old worker

Lane 4: 24-day old worker

Lane 5: 29-day old worker

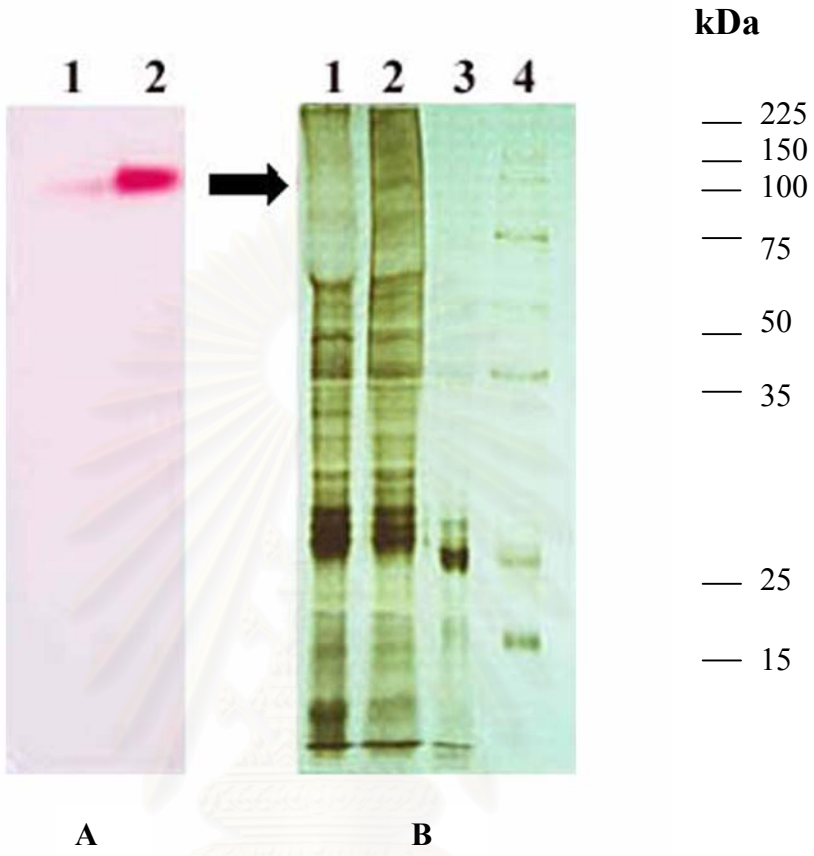


Figure 3.23 SDS-PAGE of α–G in crude extract of *A. cerana.*

A: activity staining by 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH

Lane 1: 6-day old worker (8 μg)

Lane 2: forager bee (8 μg)

B: Silver staining

Lane 1: 6-day old worker (8 μg)

Lane 2: forager bee (8 μg)

Lane 3: commercial α–G from Bakers Yeast

Lane 4: Broad Range Protein marker

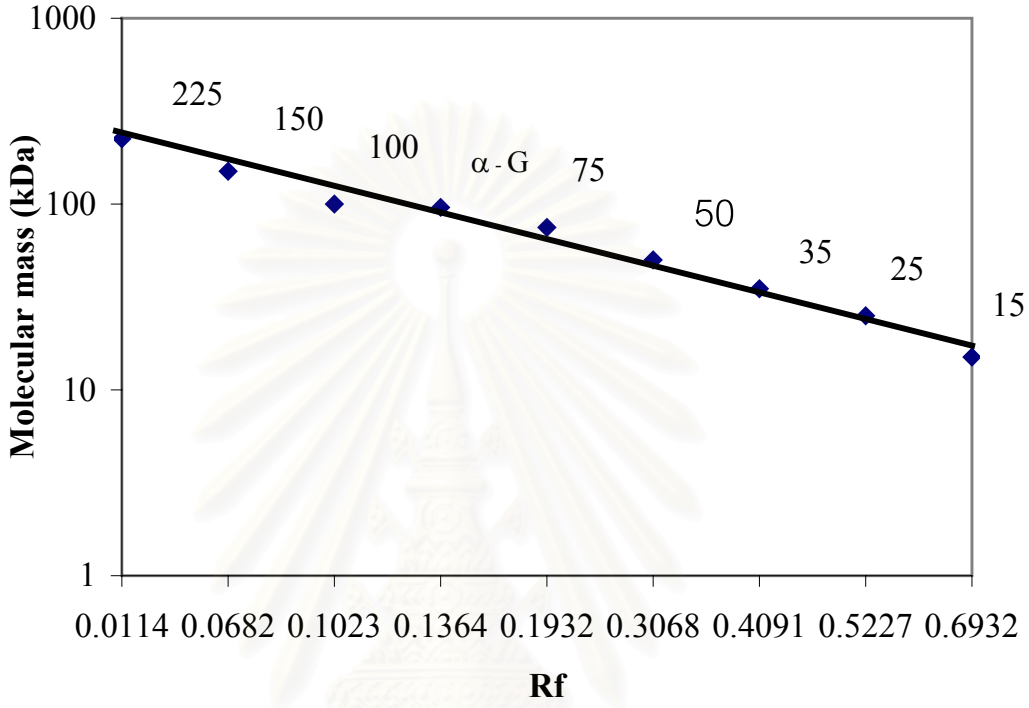


Figure 3.24 Molecular mass of calibration curve of Board Range Protein marker by SDS-PAGE.

3.4 The expression level of *α-G* in *A. cerana*

The expression of *α-G* mRNA in HPGs was examined by RT-PCR experiment. Total RNA was obtained from emerged bees, 6-day old worker bees, nurse bees, and forager bees. Quality of total RNA was determined by 0.8% (w/v) agarose gel. After electrophoresis, 18S and 28S rRNA were visible (Fig. 3.25). The concentrations of total RNA and cDNA were determined by spectrophotometry at 260 nm and calculated as followed (Table 3.2):

Total RNA = A260 × 40 μg/ml

cDNA = A260 × 50 μg/ml

As shown in Fig. 3.26, the expression of *α-G* was not detectable in emerged bees while the expression in worker bees was less than that in nurse bee. The expression of *α-G* in forager was high.

As control, 28S RNA in *A. mellifera* and elongation factor genes in *A. cerana* was successfully amplified (Fig. 3.27). The sizes of 350 bp and 100 bp RT-PCR products amplified by 28S RNA and EF primers were obtained, respectively.

Table 3.7 Concentrations of total RNA and cDNA from emerged bees, worker bees, nurse bees, and forager bees.

Sample	Concentration of total RNA (□g/ml)	Concentration of cDNA (□g/ml)
Emerged bee (1)	79.2	15
Emerged bee (2)	124.8	15
Emerged bee (3)	205.6	45
6-day old worker bee (1)	409.6	200
6-day old worker bee (2)	305.6	160
6-day old worker bee (3)	349.6	175
Nurse bee (1)	784	175
Nurse bee (2)	742.4	275
Nurse bee (3)	671.2	250
Forager bee (1)	251.2	220
Forager bee (2)	57.6	265
Forager bee (3)	82.4	160

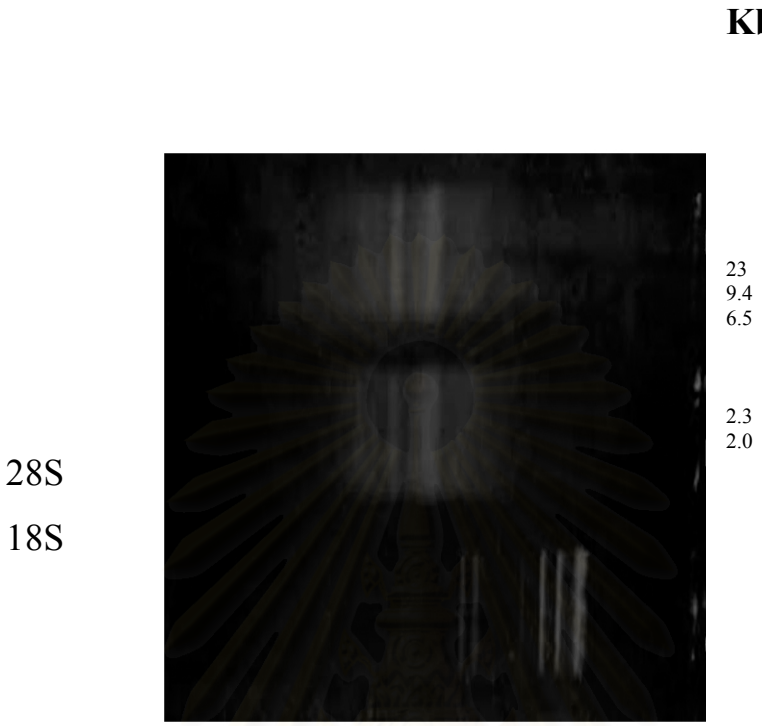


Figure 3.25 Total RNA extracted from HPGs was electrophoresed on 0.8% (w/v) agarose gel.

Lane 1: total RNA of sample from Phuket

Lane 2: total RNA of sample from Nakhon Sri Thammarat

Lane 3: λ *Hin*dIII marker

Figure 3.26 The RT-PCR product of *α-G* from samples at each developmental stage.

Lane 1: 100 bp ladder marker

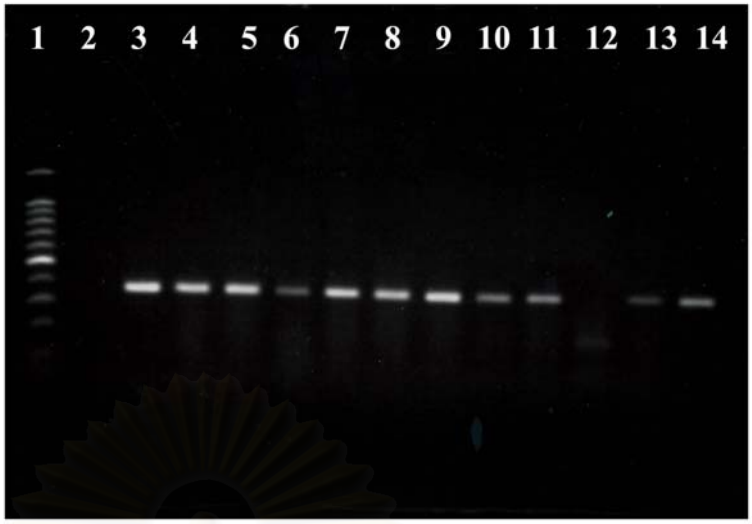
Lane 2: Negative control

Lane 3–5: Emerged bees

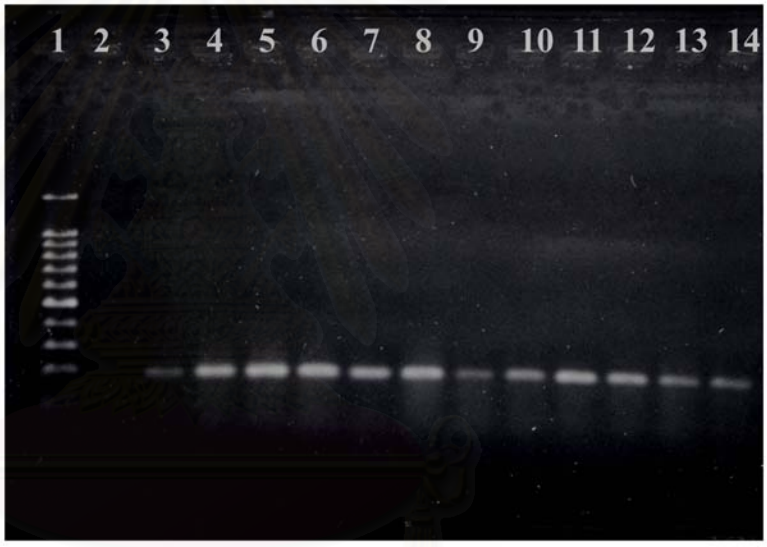
Lane 6–8: 6-day old worker bees

Lane 9–11: Nurse bees

Lane 12–14: Forager bees



A: 28S



B: EF

Figure 3.27 As control, 28S RNA and EF primers were used for RT-PCR. The same RNA samples for RT-PCR of *α-G* were used for this experiment.

Lane 1: 100 bp ladder marker

Lane 2: Negative control

Lane 3–5: Emerged bees

Lane 6–8: 6-day old worker bees

Lane 9–11: Nurse bees

Lane 12–14: Forager bees

3.5 Partial cDNA sequence and phylogenetic tree

Forager bees were obtained from 14 colonies. Each colony was collected from Bangkok, Rayong, Udornthani, Chumporn, Trang, Phuket, Pa-ngan Island, Nakhon Sri Thammarat, and Pang-nga. In addition, 5 colonies were from Samui Island. Total RNA was extracted as mentioned in Materials and Methods. Quality of total RNA was assayed as in Fig. 3.27. After RT-PCR amplification, the PCR products of 357 bp were visible on a 1.5% (w/v) agarose gel as shown in Fig. 3.28. Partial cDNA sequences from foragers of 14 colonies were obtained by Bioservice unit, Thailand (Appendix F).

In Fig. 3.29, the alignment of the nucleotide of 14 samples together with *α–G* cDNA of *A. mellifera* from GenBank are present. A total of 12 base substutions (9 transitions and 3 transversions) were observed. The consensus sequences are obtained. There are a few bases changed in the sequences. When *α–G* cDNA of *A. cerana* is compared to that cDNA from other organisms. The *α-G* sequence of *A. cerana* is partially similar to the maltase sequence in fruit fly (*Drosophila melanogaster*) at 5%, to *α-G* cDNA in mosquito (*Aedes aegypti*) at 39-47%, and to *α-G* cDNA in *Culex pipiens* at 40%, respectively, as shown in Fig. 3.30. Furthermore, the deduced amino acid sequences of α–G were aligned (Fig. 3.31). Two positions (position 52 and 95) of amino acid sequences of *A. cerana* (14 samples) and *A. mellifera* were different. Glutamic acid and isoleucine in *A.mellifera* sequence were changed to be glutamine and leucine in *A.cerana* sequence, respectively.

Aligned sequences were imported into the phylogenetic analysis program PAUP (version 4.0b). The *α–G* cDNA sequence of *A. mellifera* (accession number: D79208 in GenBank) was used as an outgroup. One tree was founded from each program when maximum parsimony, UPGMA, and neighbor-joining program were used for the analysis. The maximum parsimony analysis phylogram and the strict concensus tree are shown in Fig. 3.32 and 3.33, respectively. The consistency index (CI), retention

index (RI) and rescaled consistency index (RC) were the same value,1.0. The patterns of genetic distance from maximum parsimony, UPGMA, and neighbor-joining programs are indicated as shown in Fig. 3.34. The trees of each program share similar characters. Thirteen cDNA sequences of *A. cerana* can be classified into one group while cDNA sequence from Bangkok is separated to be other group. The sequence of cDNA in *A. cerana* is distinguishly seperated from the sequence of *A. mellifera*. The maximum parsimony analysis phylogram of deduced amino acid sequences of α–G were shown in Fig. 3.35. Fourteen deduced amino acid of *A. cerana* were classified into single group.

Figure 3.28 The RT-PCR product of *α–G* of foragers.

Lane 1: 100 bp ladder

Lane 2: *α–G* PCR product of the sample from Udornthani

Lane 3: *α–G* PCR product of the sample from Bangkok

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Samui3          -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Samui7          -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Rayong          -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Pang-nga        -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Samui6          -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Phuket          -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Nak             --ATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Pa-ngan         -GAT-ATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Samui2          CGAT-ATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Samui1          CGATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Udornthani      CGATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Chumporn        --GTAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Trang           --GTAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Bangkok         -GATAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
A.mellifera     --GTAATCGT ATTTTGCCTT ATGGCATTGT CCATTGTGGA CGCAGCATGG
Consensus          * ***** ********** ********** ********** **********
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Samui3          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Samui7          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Rayong          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Pang-nga        AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Samui6          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Phuket          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Nak             AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Pa-ngan         AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Samui2          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Samui1          AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Udornthani      AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Chumporn        AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Trang           AAGCCGCTCC CTAAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Bangkok         AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
A.mellifera     AAGCCGCTCC CTGAAAACTT GAAGGAGGAC TTGATCGTGT ATCAGGTCTA
Consensus       ********** ** ******* ********** ********** **********
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Samui3          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Samui7          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Rayong          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Pang-nga        CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Samui6          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Phuket          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Nak             CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Pa-ngan         CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Samui2          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Samui1          CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Udornthani      CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Chumporn        CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Trang           CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Bangkok         CCCAAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
A.mellifera     CCCGAGAAGC TTCAAGGATA GCAATGGAGA TGGTATTGGT GATATCGAAG
Consensus       *** ****** ********** ********** ********** **********
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Figure 3.29 The multiple alignment of the nucleotide sequences of *α–G* from various provinces and that of *A. mellifera*. Asterisks indicate the consensus bases.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
Samui3          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Samui7          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Rayong          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Pang-nga        GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Samui6          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Phuket          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Nak             GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Pa-ngan         GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Samui2          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Samui1          GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Udornthani      GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Chumporn        GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Trang           GTATTAAACA AAAATTGGAC CATTTTCTCG AAATGGGCGT CGATATGTTT
Bangkok         GTATTAAACA AAAATTGGAT CATTTTCTCG AAATGGGCGT CGATATGTTT
A.mellifera     GTATTAAAGA AAAATTGGAT CATTTTCTCG AAATGGGGGT CGACATGTTT
Consensus       ******** * *********  ********** ******* ** *** ******
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Samui3          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Samui7          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Rayong          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Pang-nga        TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Samui6          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Phuket          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Nak             TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Pa-ngan         TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Samui2          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Samui1          TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Udornthani      TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Chumporn        TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Trang           TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
Bangkok         TGGTTATCTC CTATTTATCC AAGTCCTATG GTCGATTTTG GTTATGACAT
A.mellifera     TGGTTATCCC CTATTTATCC AAGCCCTATG GTCGATTTTG GTTACGACAT
Consensus       ******** * ********** *** ****** ********** **** *****
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
Samui3          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Samui7          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Rayong          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Pang-nga        TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Samui6          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Phuket          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Nak             TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAAACTTAA
Pa-ngan         TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Samui2          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Samui1          TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Udornthani      TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Chumporn        TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Trang           TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
Bangkok         TTCGAATTAC ACCGATGTTC ATCCCATATT TGGCACCTTA TCAGACTTAG
A.mellifera     TTCGAATTAC ACCGACGTTC ATCCCATATT TGGCACCATA TCAGACTTAG
Consensus       ********** ***** **** ********** ******* ** *** *****
```

Figure 3.29 (continued)

```
                ....|

Samui3          A-AAC
Samui7          ATAAC
Rayong          ATAAC
Pang-nga        ATAAC
Samui6          ATAAC
Phuket          ATAAC
Nak             ATAAC
Pa-ngan         ATAAC
Samui2          ATAAC
Samui1          ATAAC
Udornthani      ATAAC
Chumporn        ATAAC
Trang           ATAAC
Bangkok         ATAAC
A.mellifera     ATAAT
Consensus       * **
```

Figure 3.29 (continued)

```
Phuket                 --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Samui3                 --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Pang-nga               --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Samui7                 --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Samui6                 --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Nak                    ---------------------ATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 38
Rayong                 --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Bangkok                --------------------GATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Pa-ngan                --------------------GATA-TCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 38
Samui2                 --------------------CGATA-TCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 39
Samui1                 --------------------CGATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 40
Udornthani             --------------------CGATAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 40
Chumporn               ---------------------GTAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 38
Trang                  ---------------------GTAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 38
A.mellifera            ---------------------GTAATCGTATTTTGCCTTATGGCAT-TGTCCATTGTGGA 38
C. pipens              ------------------------------------------------------------
A.aegypti              ------------ATCGGAACAGGCAGAATGAAGATCTTTGTTCCAC-TTCTAAGCTTCCT 47
Drosophila maltase     ------------------------------------------------------------

Phuket                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Samui3                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Pang-nga               CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Samui7                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Samui6                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Nak                    CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 95
Rayong                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Bangkok                CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Pa-ngan                CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 95
Samui2                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 96
Samui1                 CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 97
Udornthani             CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 97
Chumporn               CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 95
Trang                  CGCAGCATGGAAGCCGCTCCCTAAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 95
A.mellifera            CGCAGCATGGAAGCCGCTCCCTGAAAACTTGAAGGAGGACTTGATCGTGTATCAGGT--- 95
C. pipens              -----------AACCGGACGCCAAGGACTGGTATCAGCACGCGACGTTCTACCAGAT--- 46
A.aegypti              CCTAGCAGGACTAACCACCGGGTTGGACTGGTGGGAACATGGAAACTTCTACCAAGT--- 104
Drosophila             --------CAATGAGTTCTGGCAGGAGAATATTGGCGACTTT-ACGGTGGACAAGGCCAG 51
                                                                        *      *

Phuket                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Samui3                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Pang-nga               -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Samui7                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Samui6                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Nak                    -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 143
Rayong                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Bangkok                -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Pa-ngan                -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 143
Samui2                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 144
Samui1                 -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 145
Udornthani             -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 145
Chumporn               -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 143
Trang                  -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 143
A.mellifera            -CTACCCA--AGAAGCTTCAAGGATAG---CAATGGAG------ATGGTATTGGTGATAT 143
C. pipens              -CTACCCG--AGGTCGTTTTTGGACAG---CAACGGCG------ATGGGATAGGGGATTT 94
A.aegypti              -TTACCCA--AGATCCTTCAAGGACTC---CGACGGCG------ACGGTATCGGGGATCT 152
Drosophila             GTTTCCCA------CCCTCAAGGACAC---CATCGATG-----TGCTGCACAGGCGGGGA 97
                        * * *         ***     *   *  *       *
```

Figure 3.30 The multiple alignment of the nucleotide sequences of *α–G* from various provinces, *A. mellifera, Drosophila melanogaster*, and mosquitoes (*Culex pipens* and *Aedes aegypti*). Asterisks indicate the consensus bases.

```
Phuket          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Samui3          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Pang-nga        CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Samui7          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Samui6          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Nak             CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 202
Rayong          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Bangkok         CGAAGGTATTAAACAAAAATTGGATCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Pa-ngan         CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 202
Samui2          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 203
Samui1          CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 204
Udornthani      CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 204
Chumporn        CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 202
Trang           CGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATAT-GTTTTGGT 202
A.mellifera     CGAAGGTATTAAAGAAAAATTGGATCATTTTCTCGAAATGGGGGTCGACAT-GTTTTGGT 202
C.pipens        GGCGGGGATCACCTCCAAGATGAAGTACCTGGCGGATATTGGGATTGACGC-GACGTGGT 153
A.aegypti       GGACGGAGTCACCGAAAAGCTGAAATATCTGAAAGACATCGGCATGGACGG-AGTTTGGT 211
Drosophila      T-TCAAGGTGGTCCTGACCATCCAACCGTTCATCAGCACGGACAGTGCCAA---CTTCAA 153
                                 *  *    *                          *

Phuket          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Samui3          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Pang-nga        TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Samui7          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Samui6          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Nak             TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 259
Rayong          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Bangkok         TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Pa-ngan         TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 259
Samui2          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 260
Samui1          TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 261
Udornthani      TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 261
Chumporn        TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 259
Trang           TATCTCCTATTTATCCAA-GTC--CTATGGTCGATTTTGGTTATGACATTTCGAATTACA 259
A.mellifera     TATCCCCTATTTATCCAA-GCC--CTATGGTCGATTTTGGTTACGACATTTCGAATTACA 259
C. pipens       TGAGCCC--GCCGTTCAA-GTCACCGCTGAAGGACTTTGGGTACGATGTGTCGGATTTCT 210
A.aegypti       TGTCACCGATTTTCTCTT-CTC--CGATGGCTGATTTTGGCTATGACATCTCGAACTTCC 268
Drosophila      GGATGCAGTGAAACGCAA-GCTGCTGATCTACGAACGCCACTCGGAG----CGGAGTATT 208
                          *              *    *          *     **   *

Phuket          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Samui3          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGA-AAC---------------- 303
Pang-nga        CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Samui7          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Samui6          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Nak             CCGATGTTCATCCCATATTTGGCACCTTATCAAACTTAAATAAC---------------- 303
Rayong          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Bangkok         CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Pa-ngan         CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 303
Samui2          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 304
Samui1          CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 305
Udornthani      CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 305
Chumporn        CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 303
Trang           CCGATGTTCATCCCATATTTGGCACCTTATCAGACTTAGATAAC---------------- 303
A.mellifera     CCGACGTTCATCCCATATTTGGCACCATATCAGACTTAGATAAT---------------- 303
C.pipens        ACGCTATCCAGCCGGAGTACGGGAACTTGACGGATTTTGACAAGTTGGTGGAGGAAGCGC 270
A.aegypti       GAGAGATTCAAACGGAATACGGGGATCTAGATGCTTTCCAGCGGTTGTCCGATAAGTGTA 328
Drosophila      CC--CGCCCTGACCAGGTACAAGAGTAGCTCCAGCGCCGGCGTTCTGGACATAACCAACA 266
                        *  *
```

Figure 3.30 (continued)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Samui3          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Samui7          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Rayong          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Pang-nga        IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Samui6          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Phuket          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Nak             IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Pa-ngan         IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Samui2          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Samui1          IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Udornthani      IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Chumporn        IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Trang           IVFCLMALSI VDAAWKPLPK NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Bangkok         IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
A.mellifera     IVFCLMALSI VDAAWKPLPE NLKEDLIVYQ VYPRSFKDSN GDGIGDIEGI
Consensus       ********** *********: ********** ********** **********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
Samui3          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLET
Samui7          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Rayong          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Pang-nga        KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Samui6          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Phuket          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Nak             KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSNLNN
Pa-ngan         KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Samui2          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Samui1          KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Udornthani      KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Chumporn        KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Trang           KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
Bangkok         KQKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTLSDLDN
A.mellifera     KEKLDHFLEM GVDMFWLSPI YPSPMVDFGY DISNYTDVHP IFGTISDLDN
Consensus       *:******** ********** ********** ********** ****:*:*:.
```

Figure 3.31 The multiple alignment of amino acid sequences deduced from the partial cDNA sequence of *α–G* in *A. cerana* and that in *A. mellifera*. Asterisks indicate the consensus amino acids.

	A. mellifera	Samui3	Samui7	Rayong	Pang -nga	Samui6	Phuket	Nak	Pa-ngan	Samui2	Samui1	Udorn thani	Chumporn	Trang	Bangkok
A. mellifera	-	0.03401	0.03401	0.03401	0.03401	0.03401	0.03401	0.3741	0.03401	0.03401	0.03401	0.03401	0.03401	0.03741	0.03061
Samui3	10	-	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Samui7	10	0	-	0.00000	0.00000	0.00000	0.00000	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Rayong	10	0	0	-	0.00000	0.00000	0.00000	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Pang -nga	10	0	0	0	-	0.00000	0.00000	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Samui6	10	0	0	0	0	-	0.00000	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Phuket	10	0	0	0	0	0	-	0.00340	0.00000	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Nak	11	1	1	1	1	1	1	-	0.00340	0.00340	0.00340	0.00340	0.00340	0.00680	0.00680
Pa-ngan	10	0	0	0	0	0	0	1	-	0.00000	0.00000	0.00000	0.00000	0.00340	0.00340
Samui2	10	0	0	0	0	0	0	1	0	-	0.00000	0.00000	0.00000	0.00340	0.00340
Samui1	10	0	0	0	0	0	0	1	0	0	-	0.00000	0.00000	0.00340	0.00340
Udornthani	10	0	0	0	0	0	0	1	0	0	0	-	0.00000	0.00340	0.00340
Chumporn	10	0	0	0	0	0	0	1	0	0	0	0	-	0.00340	0.00340
Trang	11	1	1	1	1	1	1	2	1	1	1	1	1	-	0.00680
Bangkok	9	1	1	1	1	1	1	2	1	1	1	1	1	2	-

Table 3.8 Estimation of genetic distances among 15 samples obtained from α–*G* cDNA sequences in *A. cerana* and *A. mellifera*.

Above numerics = Total character differences; Below numerics = Mean character differences

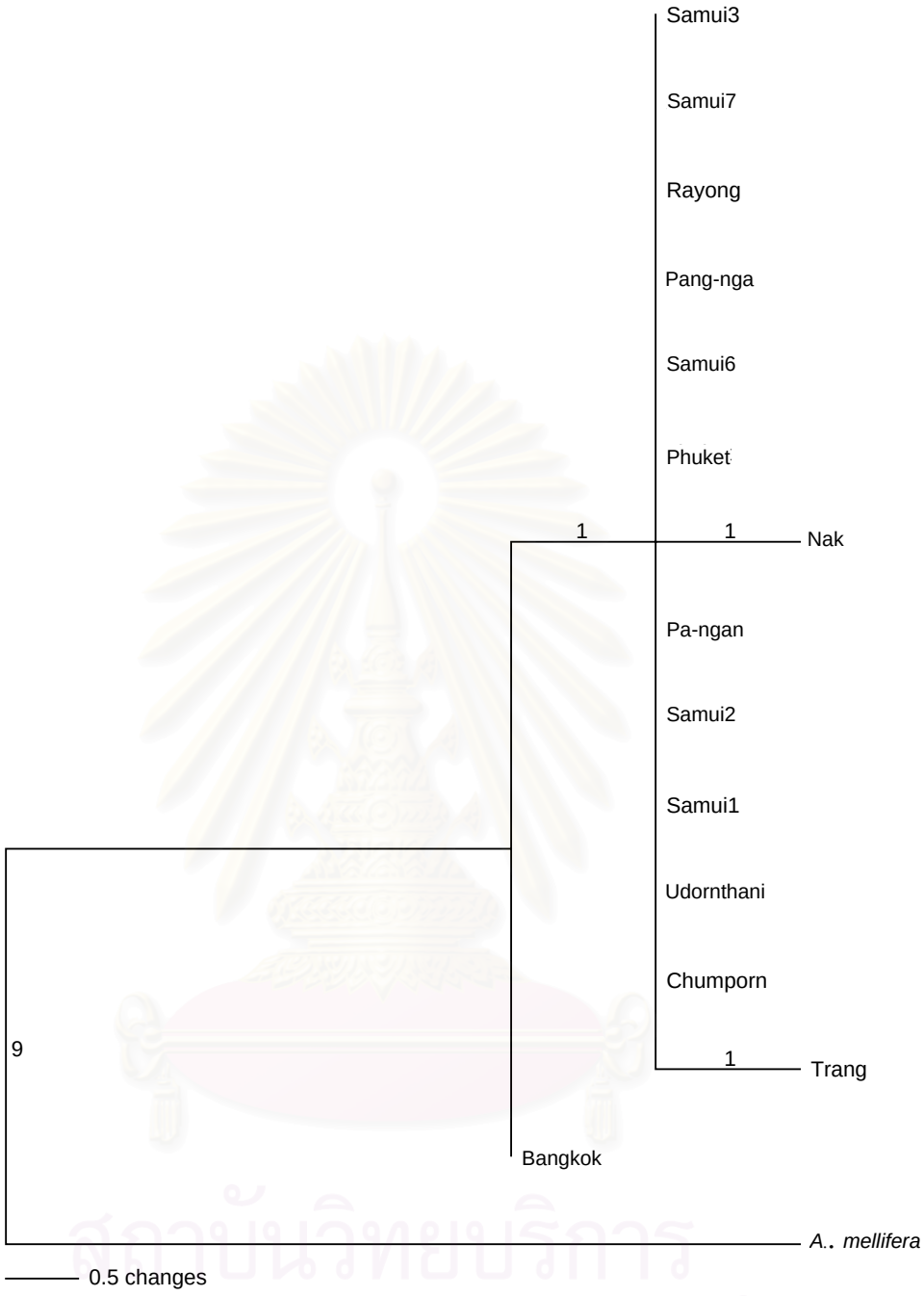

Figure 3.32 The parsimony phylogram of partial cDNA sequences of *α–G* from various provinces. The number on each branch indicates base differences.

Figure 3.33 The strict consensus tree derived from 15 parsimony trees of 305 bp of *α–G* in *A. cerana.* The number on each branch indicates the percentage of bootstrap replicates out of 1000 which supports the branch.

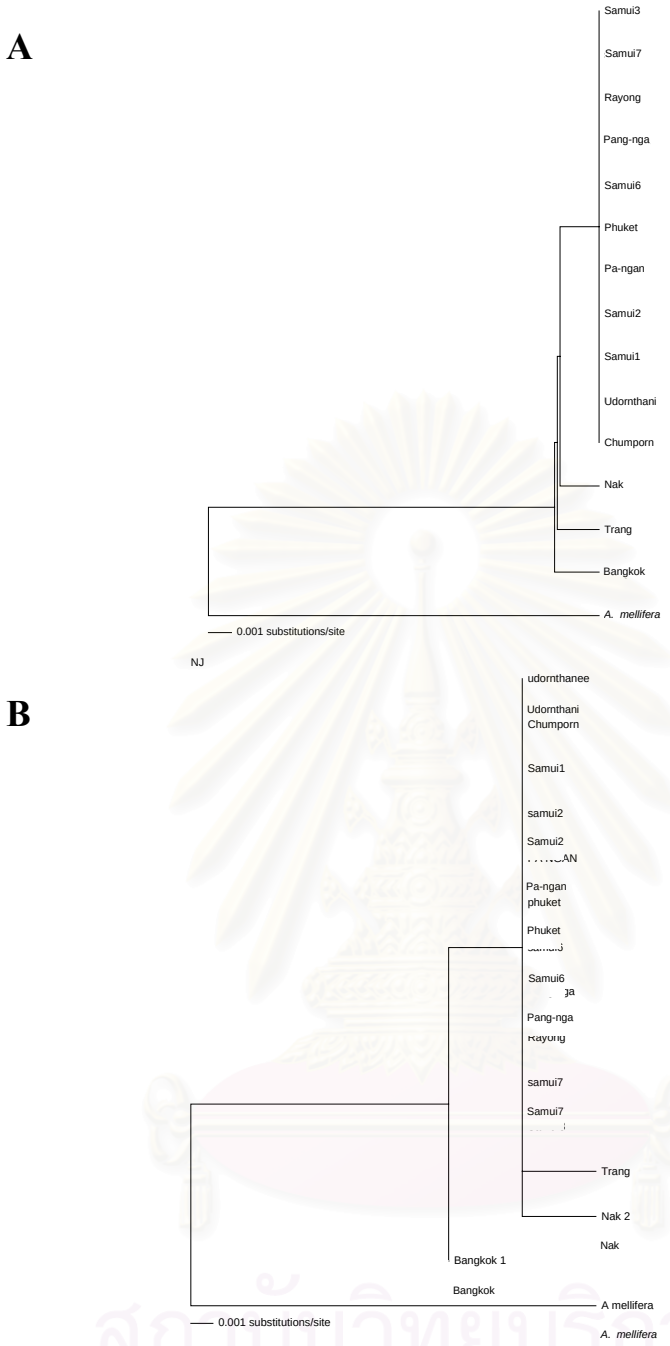

Figure 3.34 Phylogenetic trees illustrating the genetic relationship among partial cDNA of *α–G* from various provinces by UPGMA (A) and neighbor-joining (B). The cDNA sequence of *A. mellifera* was used as an outgroup.

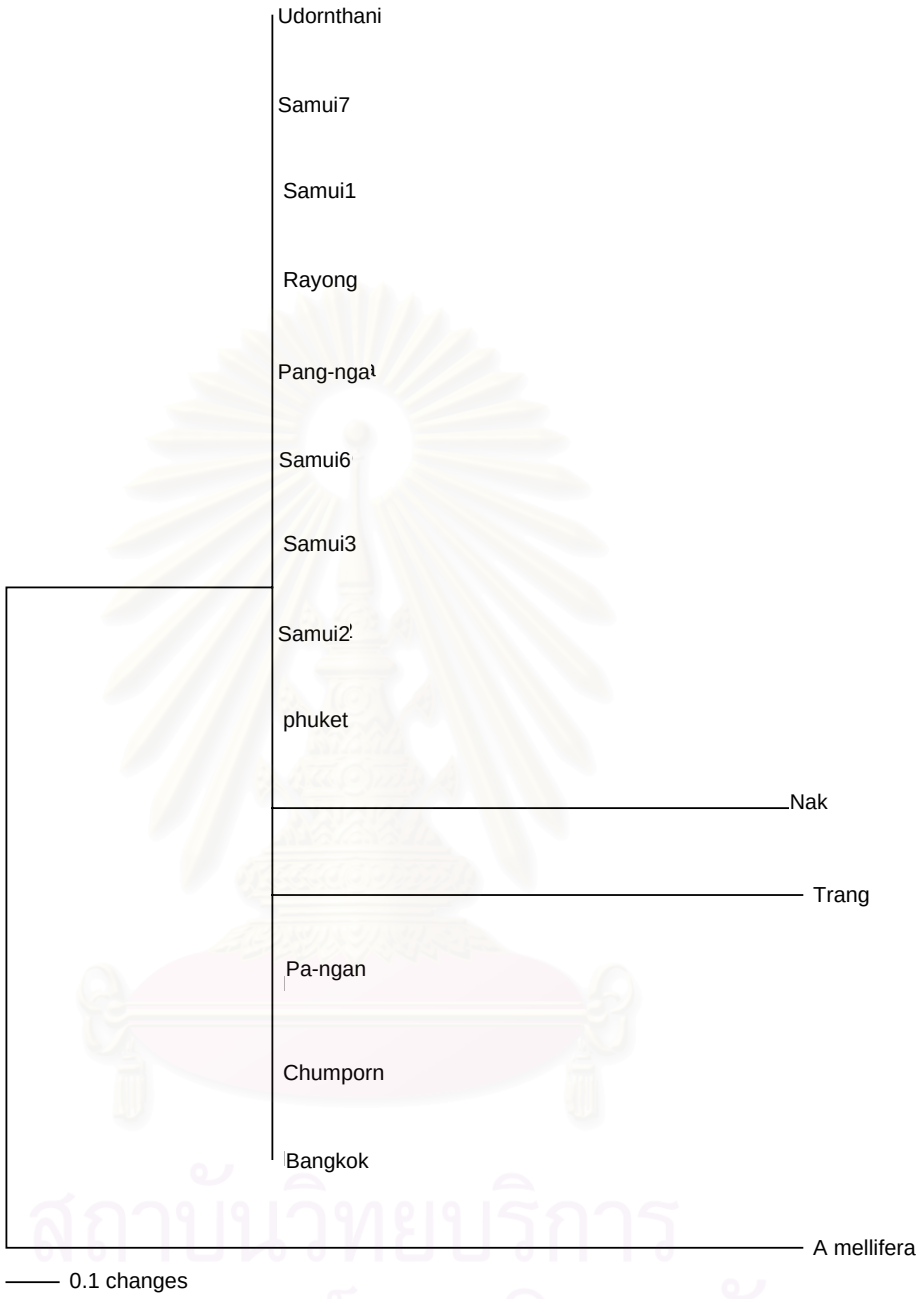

Figure 3.35 The parsimony phylogram of deduced amino acid sequences of *α*–G from various provinces.

CHAPTER IV
DISCUSSION

4.1 Sample collections

Marking bees is a very important method to collect bees at specfically desired age. In this research, worker bees at various ages were needed, so emerged bees were marked and counted as 0 day. There are some problematic concerns, for example, new born bees could die easily. Furthermore, a marked bee may be loosen because of its natural enemies, life spand ending, cleaning behavior, external factors, etc. Since the temperature has an effect on the longevity of bees, the age control of collecting samples during February to April may be hard (Fluri *et al*., 1982). This period was chosen because it is a season of flower blooming in Thailand. It provides a convenience and easiness to manage a colony since there are plenty of food sources. Sometimes, bees were fed with sugar and water in order to provide abundant food. At this point, a problem of bee absconding and migration could be avoided.

In addition, healthy bees are very important for an experiment. A colony needs to be checked everyday to confirm the situation.

4.2 Morphological characteristics of HPGs

The HPGs were photographed by SEM. The glands in nurse bees were well developed while they were shrunk in forager bees. The size of the glands should involve in the duty of nurse bees because bees at this stage have to feed royal jelly (RJ) to broods and a queen. The glands are known to be responsible for synthesizing RJ. Considering the diameter of the acini, it increases during the first few days, but it turns to decrease after the 15^{th} day. The result was the same as the measurement of the

diameters and the sizes of acini. They increased from day-3 to day-15 and decreased after day-18. The size of acini on day-12 and day-15 were changable. This may imply to the task change of worker bees in a colony. Sometimes, worker bees do foraging, then, switch to rear broods (or vice versa). The data are the same as those found in *A. mellifera* (Sasagawa *et al.*, 1989). They reported that treatment of methoprene (a JH analogue) can affect the size and degree of HPGs development in the different ways. For example, stimulation is from lower dosages while inhibition is from higher dosages. However, the size of HPGs depends on tasks of workers at that time which is up to the colony demand or environmental factors.

4.3 Activity assay of α-G in crude extract of *A. cerana*

The crude enzyme was extracted by buffer insect saline which is used to preserve protein (Kubo *et al*., 1996; Ohashi *et al*., 1996 and 1997). The optimum pH is 5.0 when sucrose is used as substrate. The result is the same as α-G II of *D. melanogaster* and α-G I and II of *A. mellifera* when p-nitrophenyl-□-glucoside is used as substrate. In *A. mellifera* and *D. melanogaeter,* the optimum pH of purified α-G I, II, and III were 5.0 - 6.5 but different substrates were used (Huber and Mathison, 1976; Tanimura *et al*., 1979; Takewaki *et al*., 1980; Nishimoto *et al*., 2001). The optimum pH of honey bee sucrase is different from others according to substrate types (Huber and Mathison, 1976). The optimum pH is also dependent on substrate concentrations. However, crude enzyme in this study may contain other enzymes which can interfere pH optimum of α-G. The temperature optimum of the crude enzyme was 45^{o}C, which is not greatly different from α-G III. The stability of α-G III activity is up to 40^{o}C but is lost completely by incubation at 60^{o}C for 15 min (Nishimoto *et al*., 2001).

The α-G activity in crude extract was measured by the hydrolysis of sucrose as substrate. In this research, Momose's method was chosen for enzyme assay because 3, 6-Dinitrophthalic acid can provide a very sensitive color in a reaction with a reducing sugar. Also, it is successfully used in the microdetection, approximate estimation, and determination of sugar. When the reagent dissolved in aqueous alkaline solution is heated with a small amount of reducing sugar, a deep wine-red color appears in a few minutes (Momose and Inaba, 1961; Momose and Nakamura, 1963).

The α-G activities from samples in February, 2003 are low. This may imply that the cold weather affect labor division. That may stop the behavior of foraging. Also, although there are flower blooming in this month, there may be less food sources than in other 2 months. Alternatively, bees may spend most of their time in a hive. In March, the activities are higher than those in February and similar to the amount of activities in April. During March and April, the weather is warm. So foragers prefer to collect pollen and nectar. In addition, the flowers in Thailand are blooming so there are plenty of pollen and nectar for bees to collect. But in the winter season, bees are mainly in a hive (Fluri, 1982). The result of activity is consistent to the incorporation activity from HPGs that summer bees showed highly synthetic activity. The winter bees showed reduced activity (Brouwers, 1982). Although the activities of α-G during these 3 months are not closely similar, the pattern of specific activities flows in the same way. Briefly, the α-G activities of emerged bee of 15-day bees are low, while the α-G activities of 18-day bees to 29-day bees get increased.

4.4 Molecular mass of α-G in crude extract of *A. cerana*

The crude extract from HPGs in *A. cerana* was studied. Total protein in crude extract of 0-day to 15-day worker bees were high while of others were low. Although

total protein obtained from 18-day to 29-day worker bees were low, the α-G specific activity became very high. The latter period coincides with the behavior of foraging of worker bees. The α-G activity was studied by Native-PAGE and SDS-PAGE. Later, the activity was stained by using sucrose as substrate. For Native-PAGE, the α-G activity was high from samples on day–18 to day–29. This data is similar to the result obtained from enzyme assay. Native-PAGE is not a method for determining native molecular mass because protein separation do not depend on molecular mass. This method allows native proteins to be separated by the difference of charge density. The buffer in the gel can maintain the quality of protein. Thus, the enzyme activity can be assayed by staining, especially after electrophoresis. For activity stain in SDS-PAGE, the activity in forager was higher than that in worker bee at day-6. Only one protein band was on an activity gel. The molecular weight of this protein band was estimated to be 96 kDa. The result was different from □-G I, II and III in *A. mellifera* (98, 76 and 68 kDa, respectively) (Takewaki *et al*., 1980; Nishimoto *et al*., 2001). In the future, α-G from HPGs of *A. cerana* will be purified.

4.5 The expression level of *α-G* in *A. cerana*

After total RNA isolation, the quality of RNA was determined. The first check was by electrophoresis. Ribosomal RNA of 18s and 28s have to be visible on an agarose gel. Normally, the largest amount in total RNA is rRNA. If degraded rRNA is observed, this indicates that mRNA (lowest amount in total RNA) should be degraded already. The second check is by measuring the optical density at 260 and 280 nm. The purity of RNA was indicated by the ratio of O. D. 260/280 which it should be 1.8. The third check is by detecting housekeeping mRNA which have to be found in all stages of development. Here, the 28s RNA and Elongation factor (EF) genes were used as

control. RT-PCR products of both genes (350 bp and 100 bp, respectively) were obtained in all samples. It means that the mRNA quality is good enough. If there is no band or less intensed bands of *α-G* amplification, it presents the exact amount of *α-G* at that time. The concentration of total RNA and cDNA of *α-G* was determined as described in Materials and Methods. After RT-PCR amplification, the cDNA product in foragers was higher than that in nurse bees and worker bees younger than 6 days old. The result of RT-PCR pattern is the same as the data obtained from the enzyme assay by the hydrolysis of 3,6-Dinitrophthalic acid. Forager bees have more high specific activity and cDNA product than worker bees at day-6 and nurse bees. According to the obtained expression profile of *α-G,* it indicates that *α-G* is partially expressed in nurse bees and fully expressed in foragers. This result of expression is the same as in *A. mellifera* (Ohashi *et al*., 1996 and 1997).

4.6 Partial cDNA sequence and phylogenetic tree

The partial cDNA sequences of *α-G* in *A. cerana* collected from many provinces in Thailand were obtained. This is to confirm that there are conserved regions in *α-G* and sampling locations have no effect on the coding sequence of *α-G*. In the future, if the full length of *α-G* sequence and the function of α-G enzyme are performed, there will be no problem on locations of sampling. Then, *α-G* cDNA sequence in *A. cerana* obtained from one area can be the best representative to compare to *α-G* cDNA sequences from other organisms. That will provide the best relationship of *α-G* among species.

Based on the results, the cDNA and deduced amino acid sequences of α-G were aligned as shown in Fig 3.29, 3.30 and 3.31, respectively. The genetic relationship of all obtained sequences were studied by maximum parsimony and the genetic distance

was analyzed by UPGMA and neighbor-joining. The result showed that all cDNA sequences are obviously the same, except the cDNA sequence from Bangkok. This might be from the result of mutation. When the partial *α-G* cDNA sequence in *A. cerana* and that in *A. mellifera* were compared, there are 9 transitions and 3 transversions. Comparing between the cDNA sequence of Bangkok and the rest to the cDNA sequences of *α-G* in *A. mellifera*, it is interesting that there is a closer similarity between the cDNA sequence obtained in Bangkok and that in *A. mellifera* from GenBank.

From this research, nurse bees with completely filled acini showed reduced α -G activity. In forager bees, acini are shrunk but α -G activity increases. The season have effect on α -G activity. The partial cDNA sequence of *α -G* in *A. cerana* from Thailand were obtained. In the future, α -G will be purified and may apply to the sweetened industry or food additives.

CHAPTER V
CONCLUSIONS

1) When samples from all stages were needed, samples at defined age were collected according.to the marking with painting color. When nurse bees and forager bees were required, they were collected according to their responsibility mainly. It is because the duty in a colony always depends on the need of the colony first.
2) Considering the morphology of HPGs by SEM, HPGs from nurse bees are larger and more swollen than those from foragers.
3) Considering the HPGs of workers at various ages, the size and diameter of acini were increasing during day-3 to day-9, were the largest on day-15, and were decreasing after day-18.
4) For analysis of α-G in crude extract, HPGs from foragers were used. The optimum pH, temperature, and incubation time for α-G activity were at 5.0, at 45^{o}C, and for 30 min, respectively. The proper concentration of sucrose for α-G activity was 50 mM.
5) The specific activity of α-G in crude extract at developmental stages were high in forager bees (18-29 days) but low in emerged bees (0 day) and nurse bees (6-15 days).
6) After SDS-PAGE and protein renaturation, the positive band of α-G was visible. Its molecular mass is about 96 kDa. The result is coincided to the data obtained from activity staining.

7) Furthermore, it is obvious that, from activity staining, the activity of α-G was visible in foragers, especially day-24 workers. The result coincides to the RT-PCR experiment that will be mentioned in (9).

8) In order to amplify mRNA of *α-G* in *A. cerana*, conserved regions of *α-G* in *A. mellifera* were used to design for RT-PCR primers. The 357 bp PCR product was obtained and sequenced as concluded in (10).

9) According to the profile of RT-PCR products, the most intensed band was obtained from the amplification of forager's mRNA.

10) The partial cDNA sequences of *α-G* in *A. cerana* were obtained from foragers collected from various provinces, Bangkok, Rayong, Udornthani, Chumporn, Trang, Phuket, Pa-ngan Island, Nakhon Sri Thammarat, Pang-nga, and Samui Island.

11) According to construction of the phylogenetic tree, *α-G* from *A. cerana* collected from 13 samples of Thailand were classified into one group. Also, the cDNA sequence is clearly different from the sequence of *A. mellifera*. Among *α-G* of *A. cerana,* the sequence from Bangkok is separated from others but it is most closely to the sequence in *A. mellifera*.

12) In addition, partial *α–G* cDNA of *A. cerana* is compared to that cDNA from other organisms. The partial *α--G* sequence of *A. cerana* is similar to the maltase sequence in fruit fly (*Drosophila melanogaster*) at 5%, to *α–G* cDNA in mosquito (*Aedes aegypti*) at 39-47%, and to *α–G* cDNA in *Culex pipiens* at 40%, respectively.

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APPENDICES

Appendix A: Preparation of solutions

1. Buffer insect saline

1) 0.1 M Tris (10 mM) 10 ml

2) 1.3 M NaCl (130 mM) 10 ml

3) 0.05 M KCl (5 mM) 10 ml

4) 0.01 M $CaCl_2$ (1 mM) 10 ml

Adjusted pH to be 7.4 by 1 M HCl and adjusted volume to be 100 ml by dd-H_2O.

2. Briton-Robinson buffer

1) 1 M Acetic acid (10mM) 1 ml

2) 1 M Phosphoric acid (10 mM) 1 ml

3) 0.1 M Boric acid (10 mM) 10 ml

Adjusted pH to be 3.0–7.5 by 0.2 M NaOH and adjusted volume to be 100 ml by dd-H_2O.

3. Bradford solution

1) 95% Ethanol (5% v/v) 50 ml

2) 85% Phosphoric acid (8.5% v/v) 100 ml

3) Coomassie Brilliant blue G-250 (0.01% w/v) 0.1 g

Adjusted volume to be 1,000 ml by dd-H_2O and filtered before use.

Appendix B: Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

1) 30% Acrylamide and 0.8% bis-acrylamide 100 ml

Acrylamide 29.2 g

N, N'–methylene-bis-acrylamide 0.8 g

Adjusted volume to be 100 ml by d-H_2O.

2) 1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminometane 18.17 g

Adjusted pH to be 8.8 by 1 M HCl and adjusted volume to be 100 ml by d-H_2O.

3) 0.5 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminometane 6.06 g

Adjusted pH to be 6.8 by 1 M HCl and adjusted volume to be 100 ml by d-H_2O.

4) 1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminometane 12.1 g

Adjusted pH to be 6.8 by 1 M HCl and adjusted volume to be 100 ml by d-H_2O.

2. Non–denaturing PAGE

1) 12% Separating gel

30% Acrylamide solution (12%) 6.0 ml

1 M Tris-HCl, pH 8.8 (0.375 M) 5.6 ml

dd-H_2O 3.3 ml

10% $(NH_4)_2S_2O_8$ (0.05%) 75 µl

TEMED (0.05%) 7.5µl

2) 4.0% Stacking gel

30% Acrylamide solution (4%)	0.80 ml
0.5 M Tris-HCl, pH 6.8 (0.125M)	0.75 ml
dd-H_2O	4.4 ml
10% $(NH_4)_2S_2O_8$ (0.05%)	30 µl
TEMED (0.1%)	6 µl

3) Sample buffer (5X loading dye)

1 M Tris-HCl, pH 6.8 (0.312 M)	3.1 ml
Glycerol (50% v/v)	5.0 ml
1% Bromophenol blue	0.5 g
dd-H_2O	1.4 ml

One part of sample buffers was added to four parts of sample.

4) Electrophoresis buffer (25 mM Tris and 192 mM glycine)

Tris (hydroxymethyl)-aminometane	3.0 g
Glycine	14.4 g

Dissolved in d-H_2O to be 1 litre (final pH should be 7.3).

3. SDS-PAGE

1) 12% Separating gel

30% Acrylamide solution (12%)	6 ml
1 M Tris-HCl, pH 8.8 (0.375 M)	5.6 ml
10% (w/v) SDS	150 µl
dd-H_2O	3.16 ml
10% $(NH_4)_2S_2O_8$ (0.05%)	75 µl
TEMED (0.05%)	7.5 µl

2) 4.0% Stacking gel

30% Acrylamide solution (4%)	0.80 ml
0.5 M Tris-HCl, pH 6.8 (0.125M)	0.75 ml
10% (w/v) SDS	60 µl
dd-H_2O	4.3 ml
10% $(NH_4)_2S_2O_8$ (0.05%)	30 µl
TEMED (0.1%)	6 µl

3) Sample buffer (5X loading dye)

1 M Tris-HCl, pH 6.8 (0.312 M)	0.6 ml
Glycerol (50% v/v)	5.0 ml
10% (w/v) SDS	2.0 ml
2-Mercaptoethanol	0.5 ml
1% Bromophenol blue	0.5 g
dd-H_2O	0.9 ml

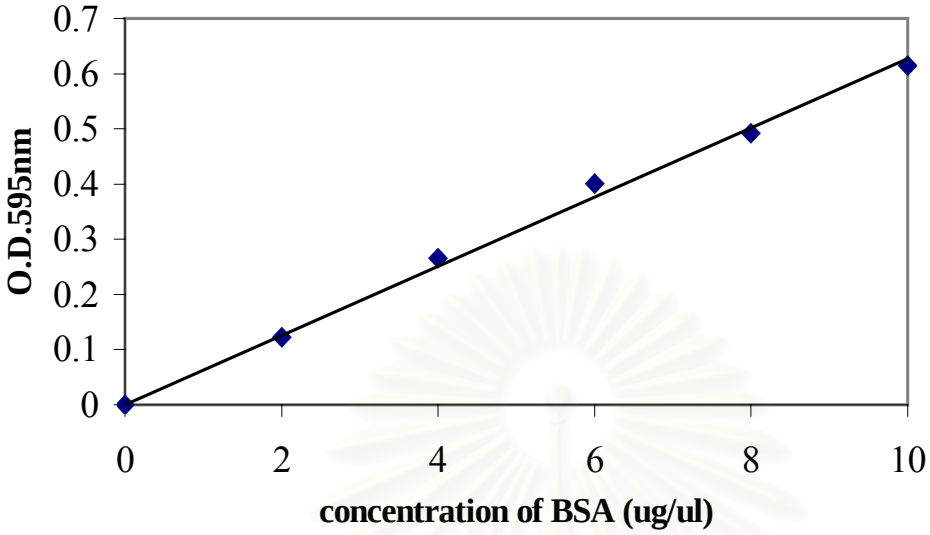
One part of sample buffer was added to four parts of sample. The mixture was heated for 5 min in boiling H_2O before loading to the gel.

4) Electrophoresis buffer (25 mM Tris and 192 mM glycine)

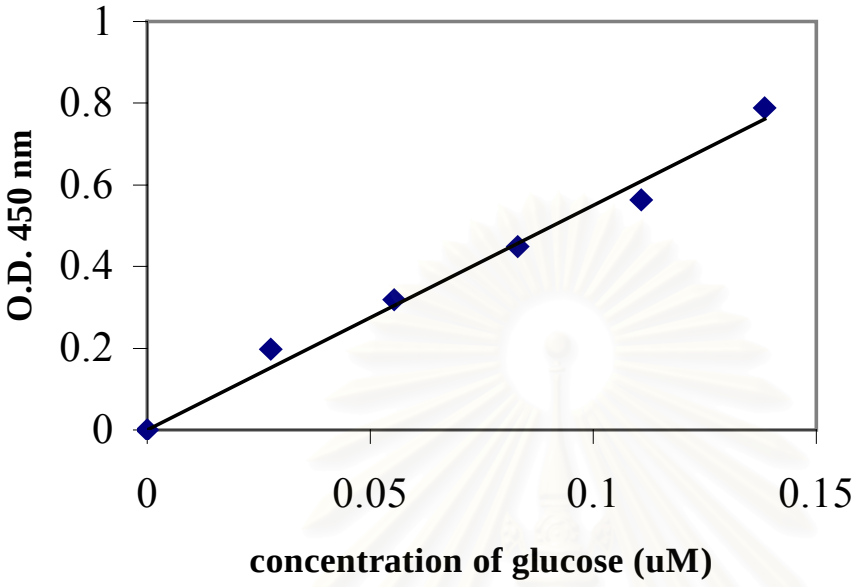
Tris (hydroxymethyl)-aminometane	3.0 g
Glycine	14.4 g
SDS	1.2 g

Adjusted volume to be 1 litre by d-H_2O and adjusted pH to be approximately 7.3.

Appendix C: Standard curve for protein determination by Bradford's method



Appendix D: Standard curve for glucose determination



Appendix E: Amino acid sequence of α *-G* in *A. mellifera* (accession number: D79208 in GenBank)

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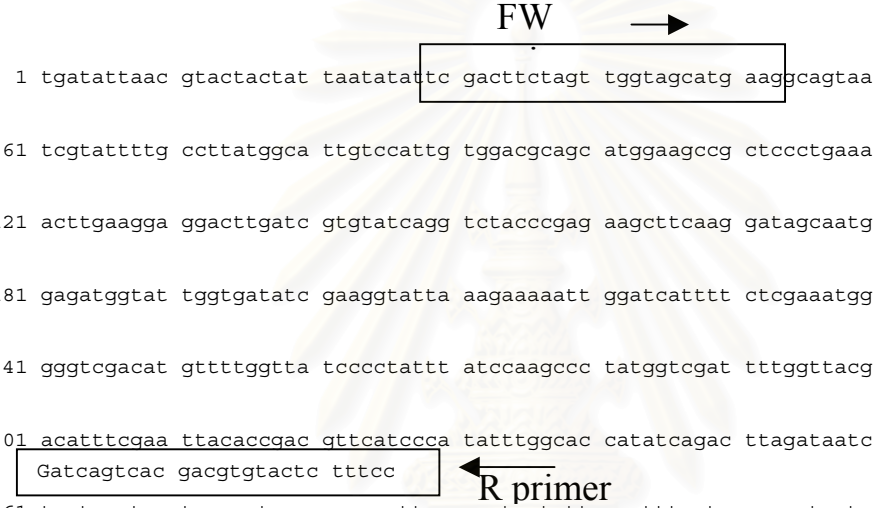


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Appendix F: The DNA sequencing profiles of *α -G*

A: The DNA sequencing profile of *α-G* from Bangkok (FW)

B: The DNA sequencing profile of *α-G* from Bangkok (R)

C: The DNA sequencing profile of *α-G* from Chumporn (FW)

D: The DNA sequencing profile of *α-G* from Nakhon Sri Thammarat (FW)

E: The DNA sequencing profile of *α-G* from Pa-ngan Island (FW)

F: The DNA sequencing profile of *α-G* from Phuket (FW)

G: The DNA sequencing profile of *α-G* from Pang-nga (FW)

H: The DNA sequencing profile of *α-G* from Rayong (FW)

I: The DNA sequencing profile of *α-G* from Samui Island 1 (FW)

J: The DNA sequencing profile of *α-G* from Samui Island 2 (FW)

K: The DNA sequencing profile of *α-G* from Samui Island 3 (FW)

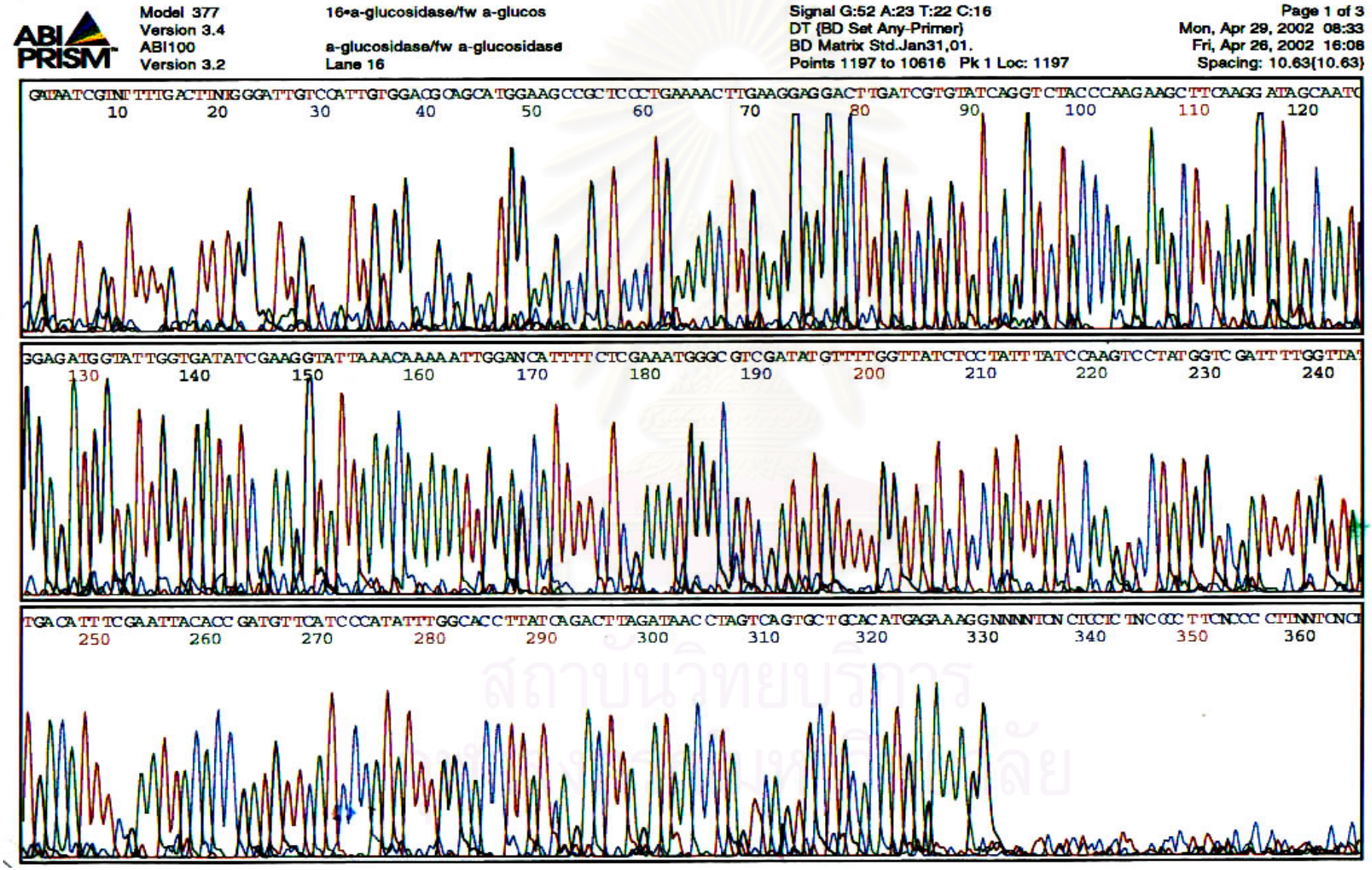
L: The DNA sequencing profile of *α-G* from Samui Island 3 (R)

M: The DNA sequencing profile of *α-G* from Samui Island 6 (FW)

N: The DNA sequencing profile of *α-G* from Samui Island 7 (FW)

O: The DNA sequencing profile of *α-G* from Trang (FW)

P: The DNA sequencing profile of *α-G* from Udornthani (FW)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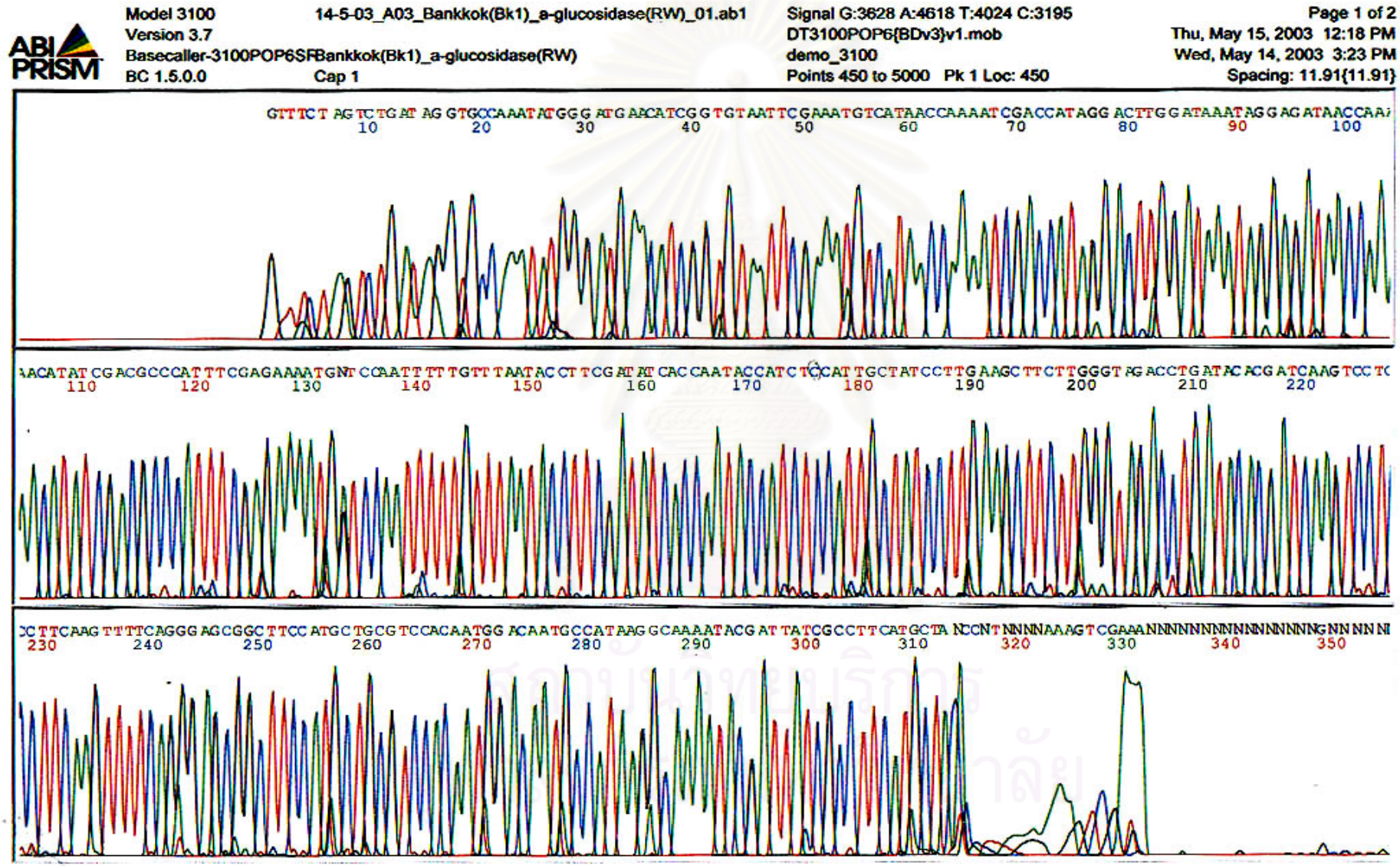
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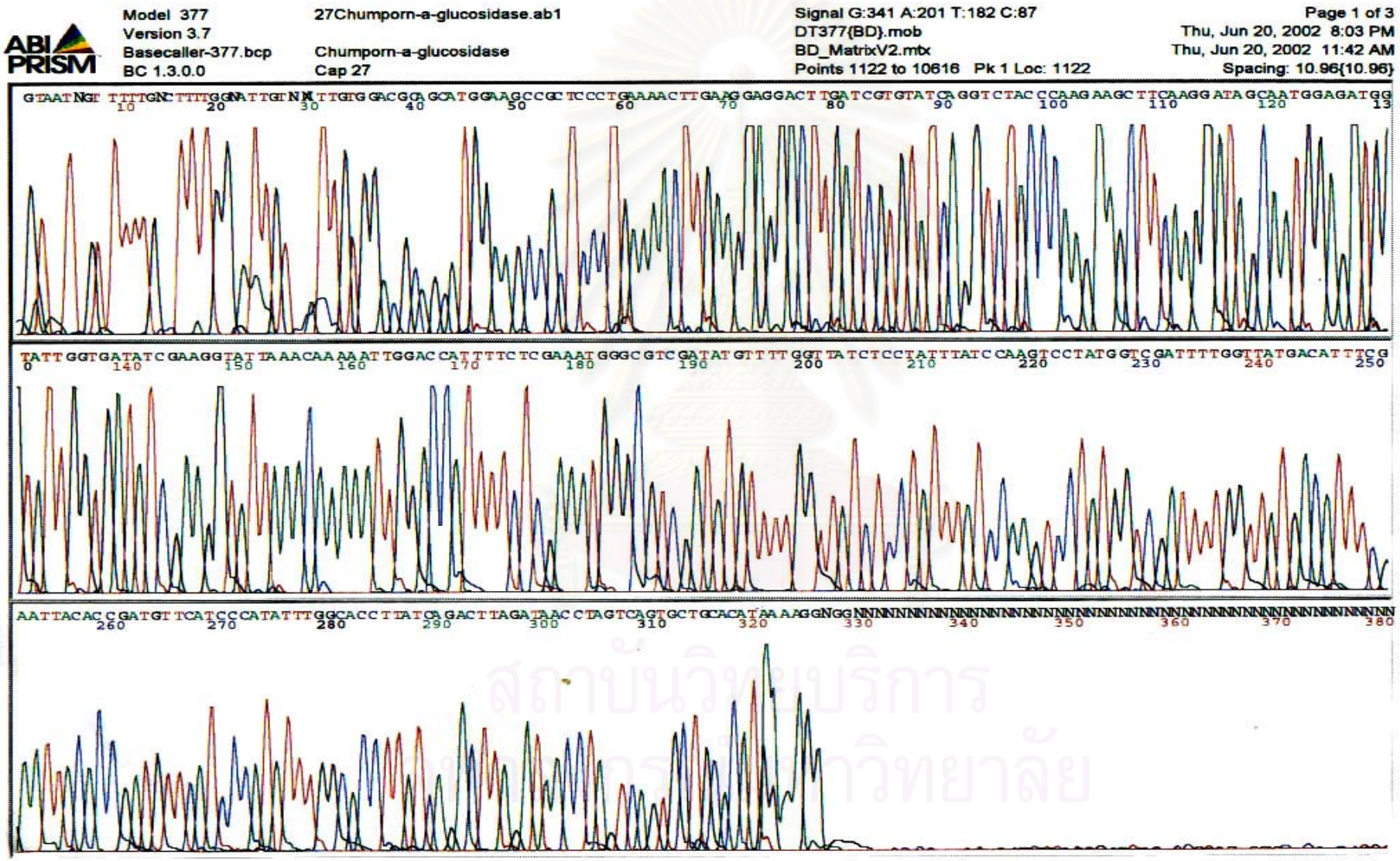
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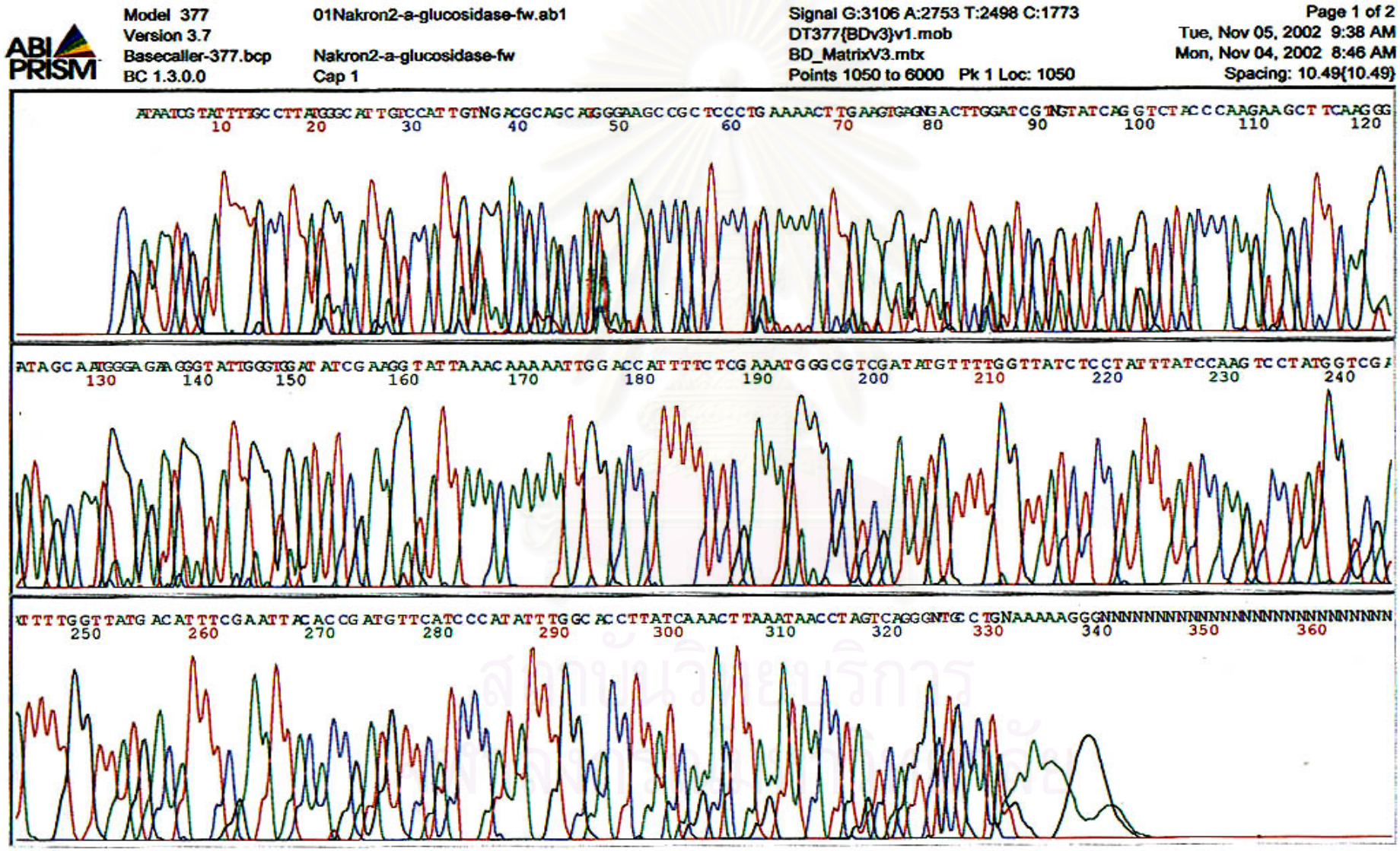
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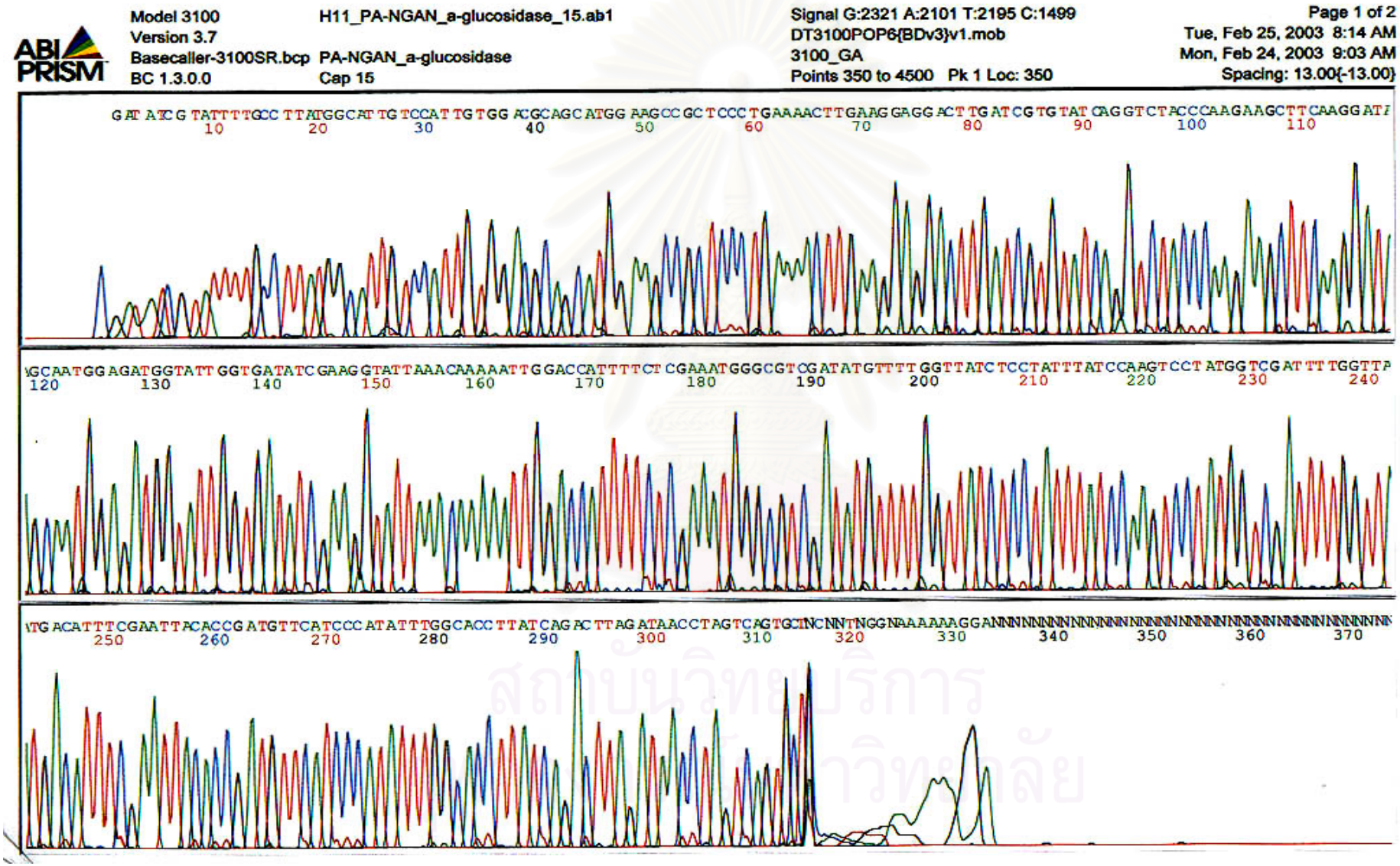
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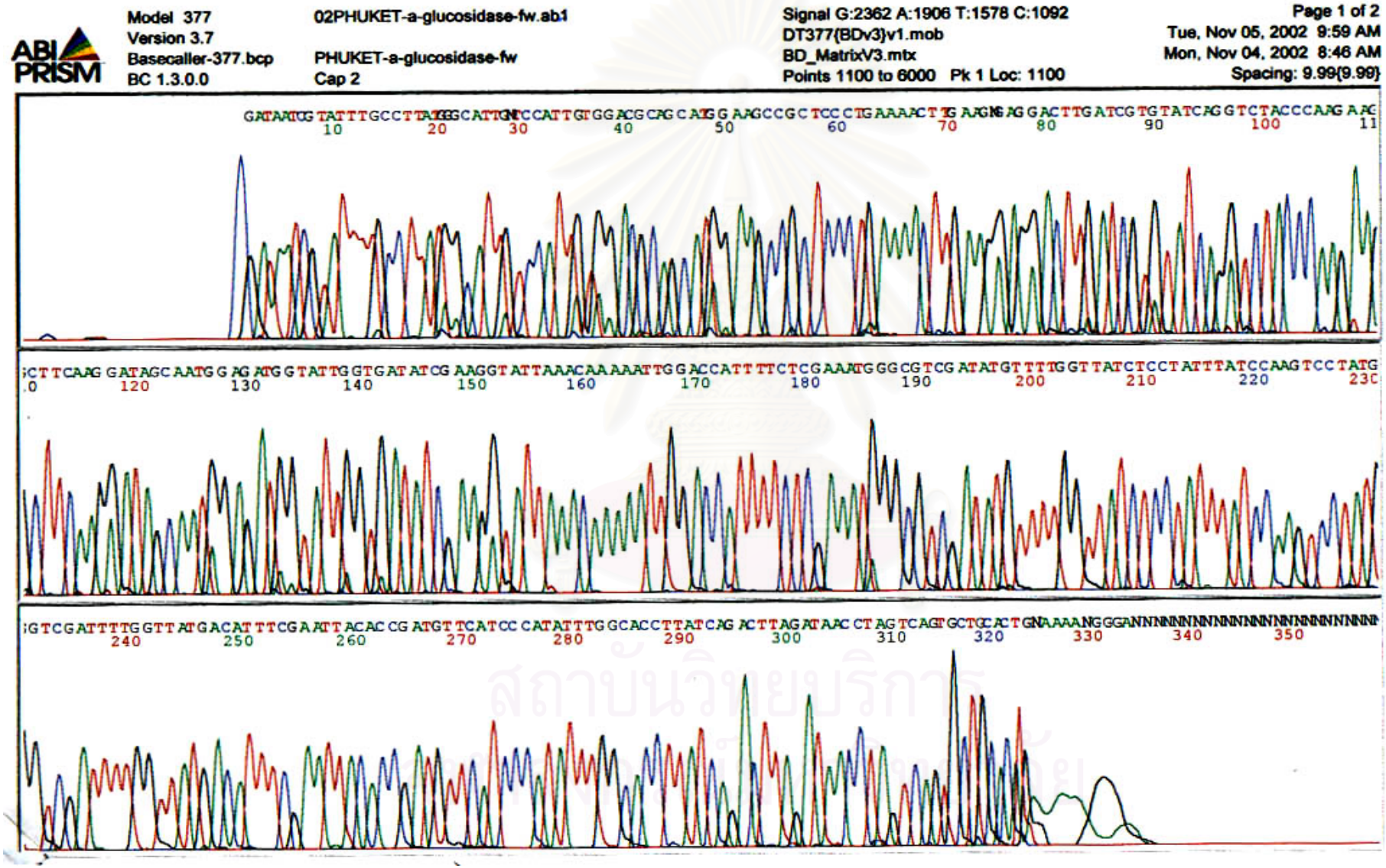
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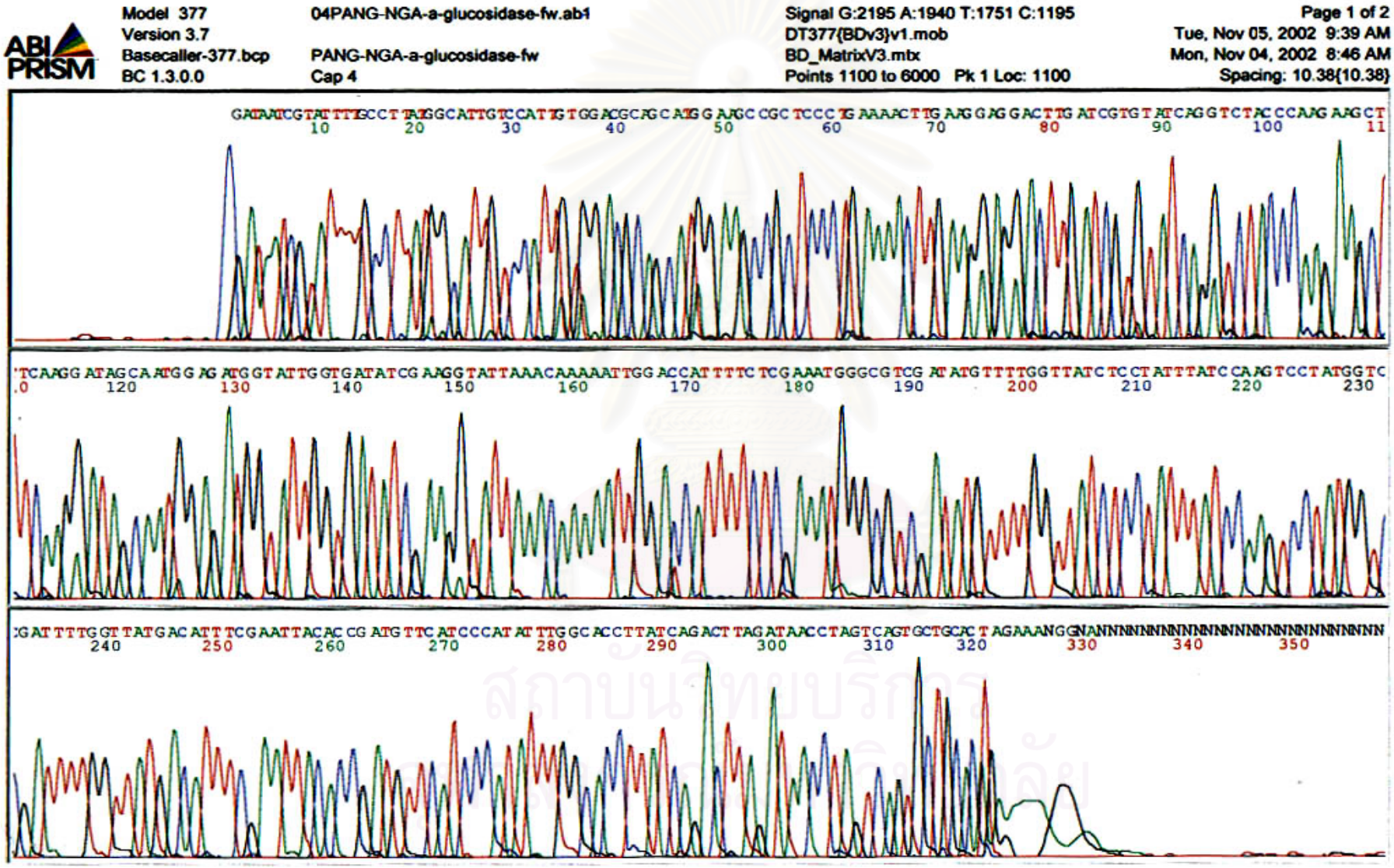
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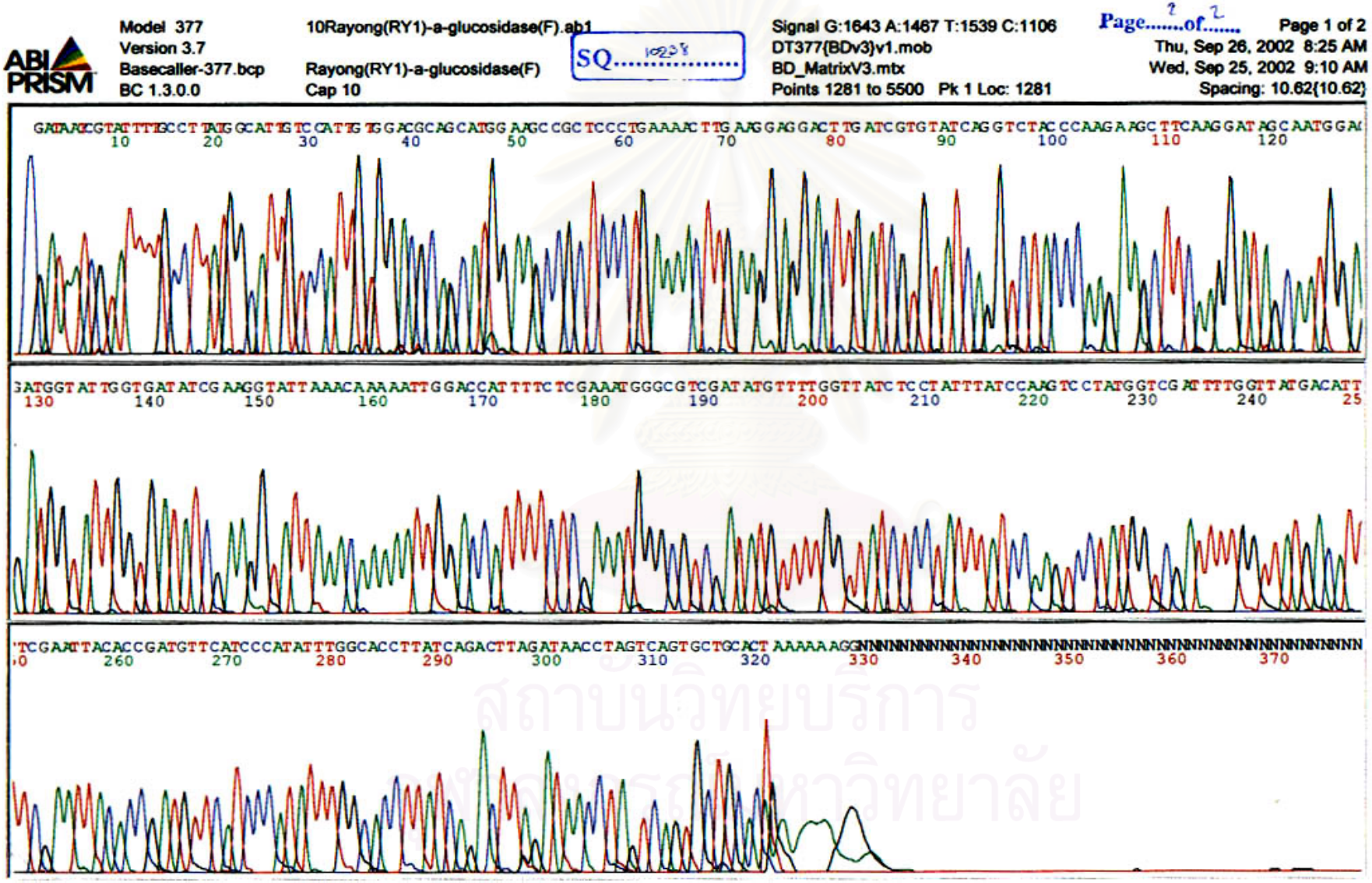
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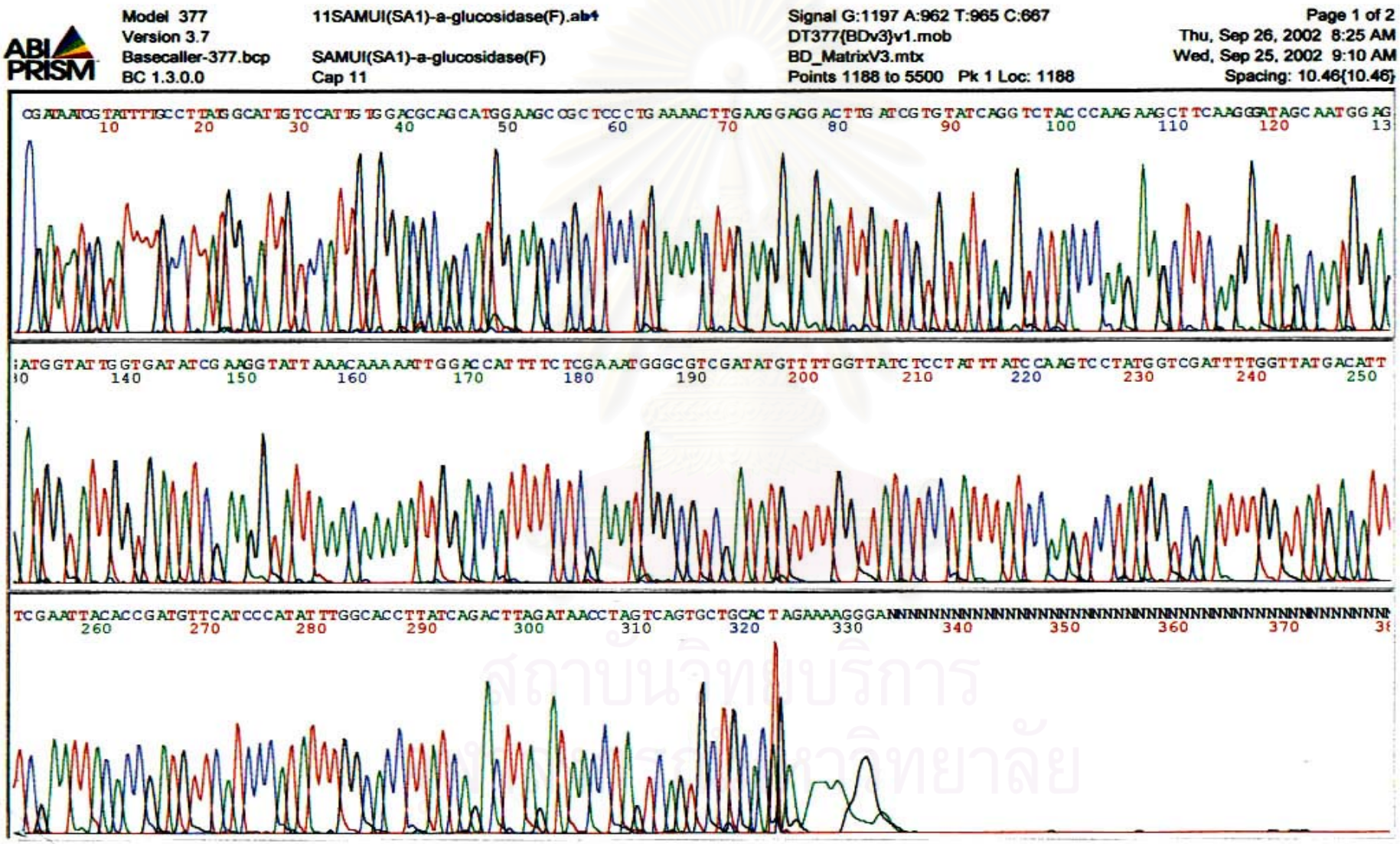
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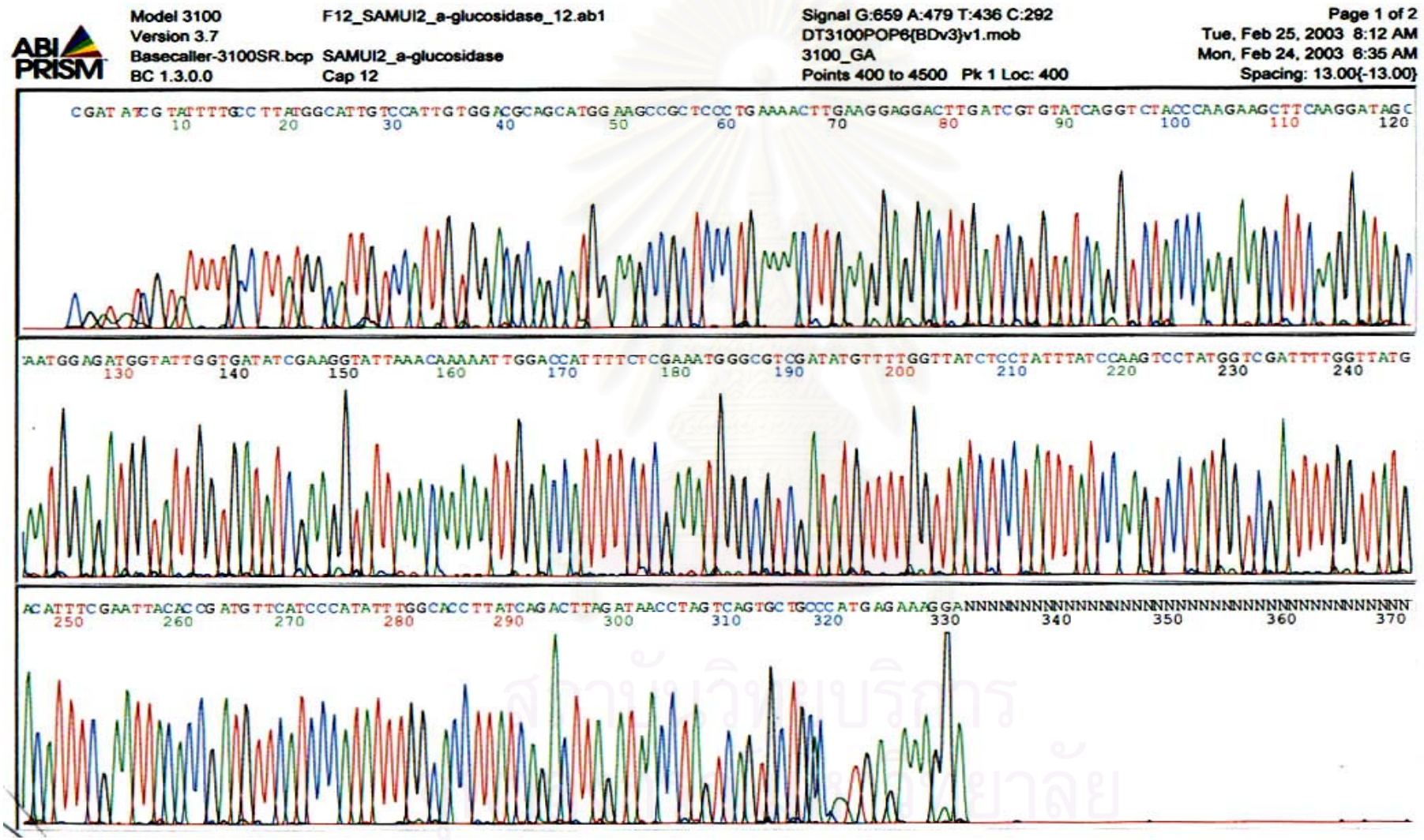
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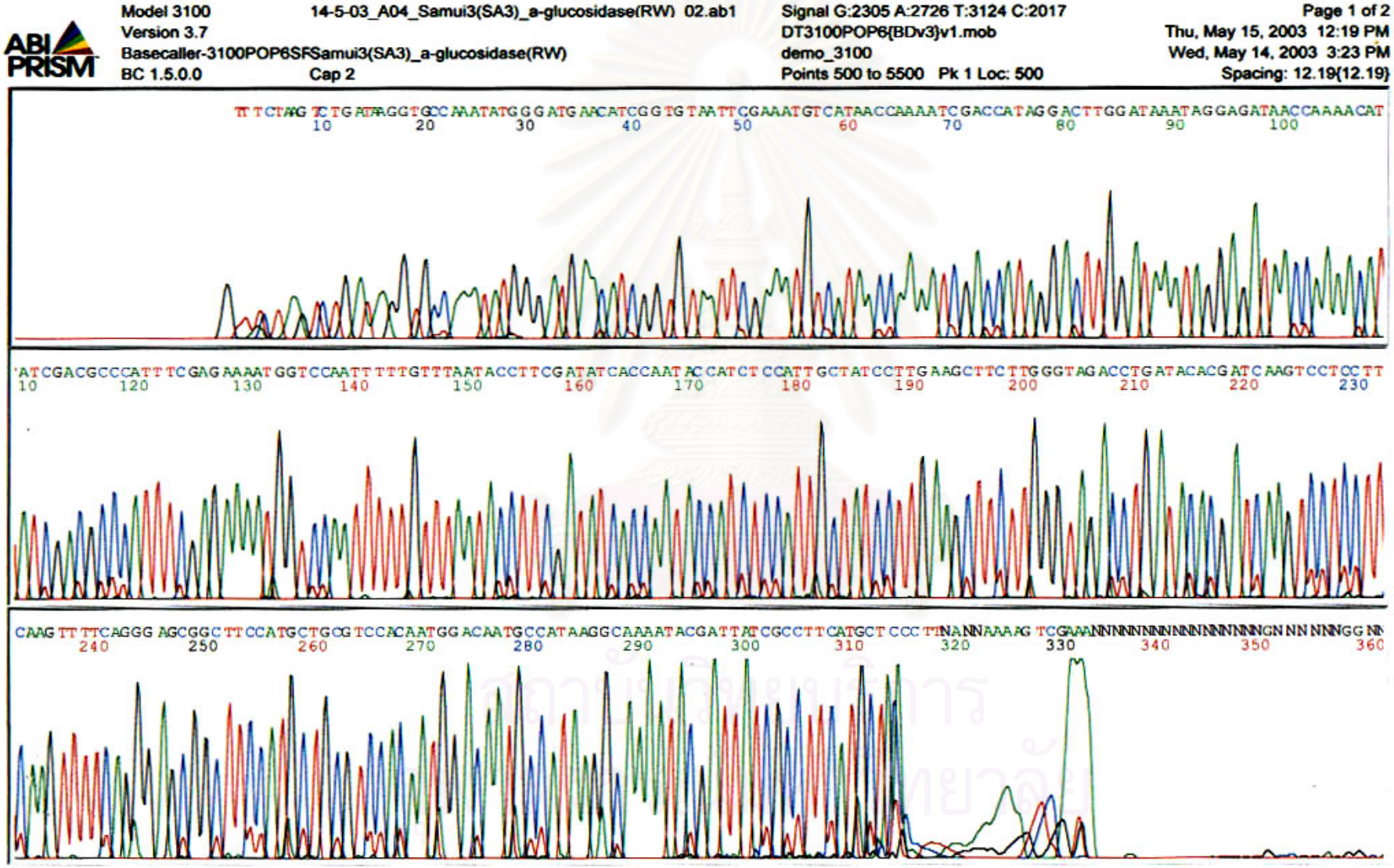
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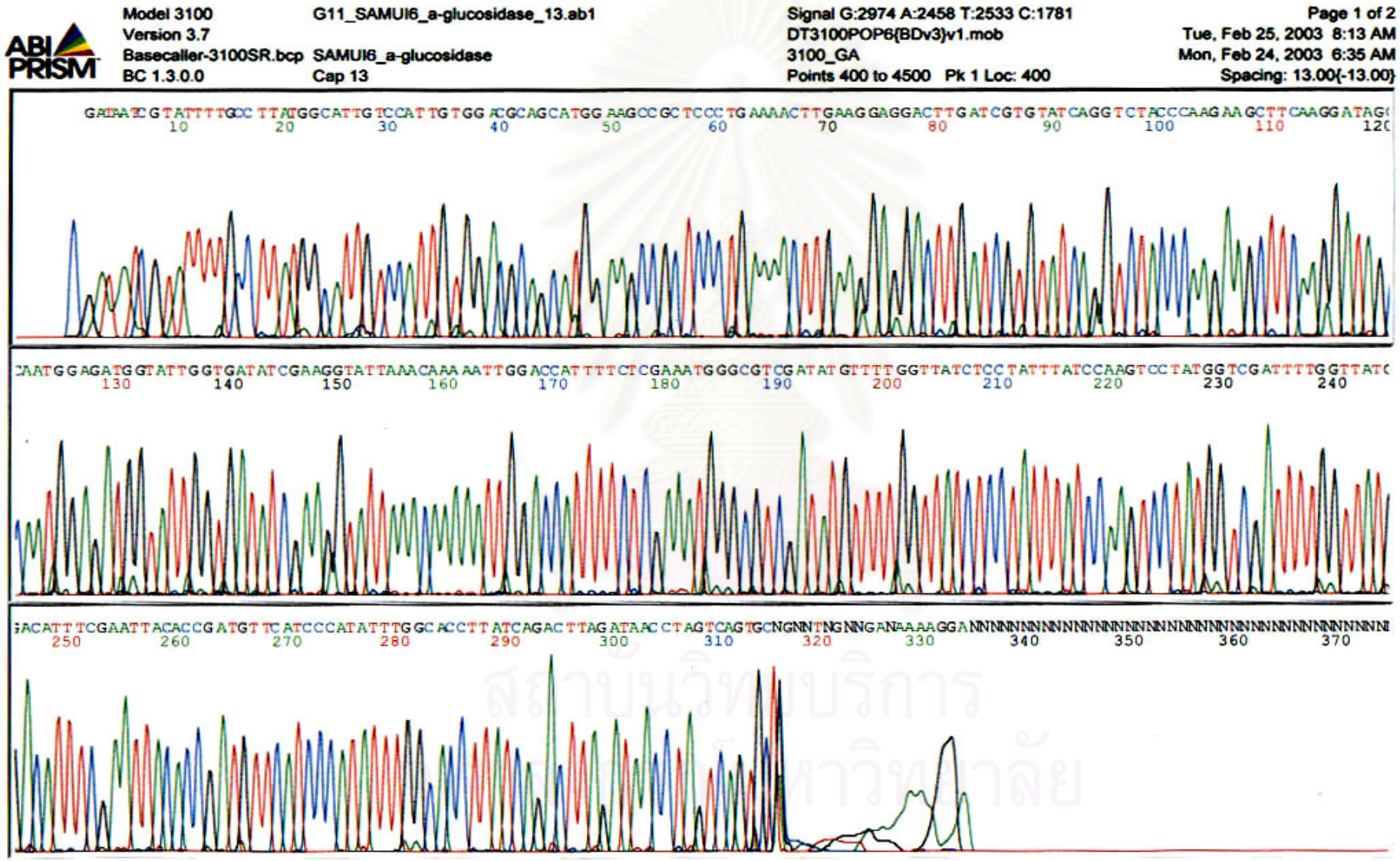
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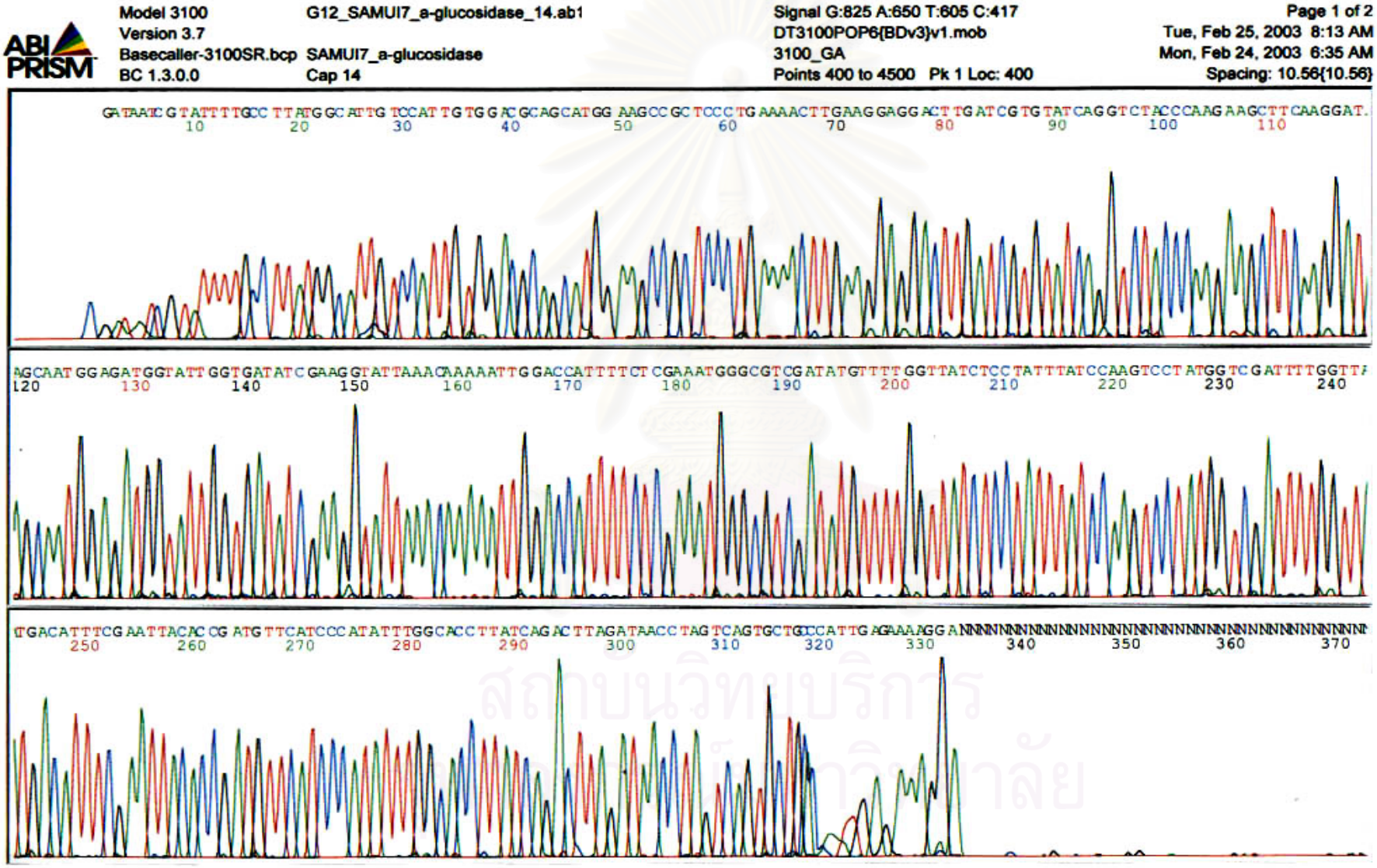
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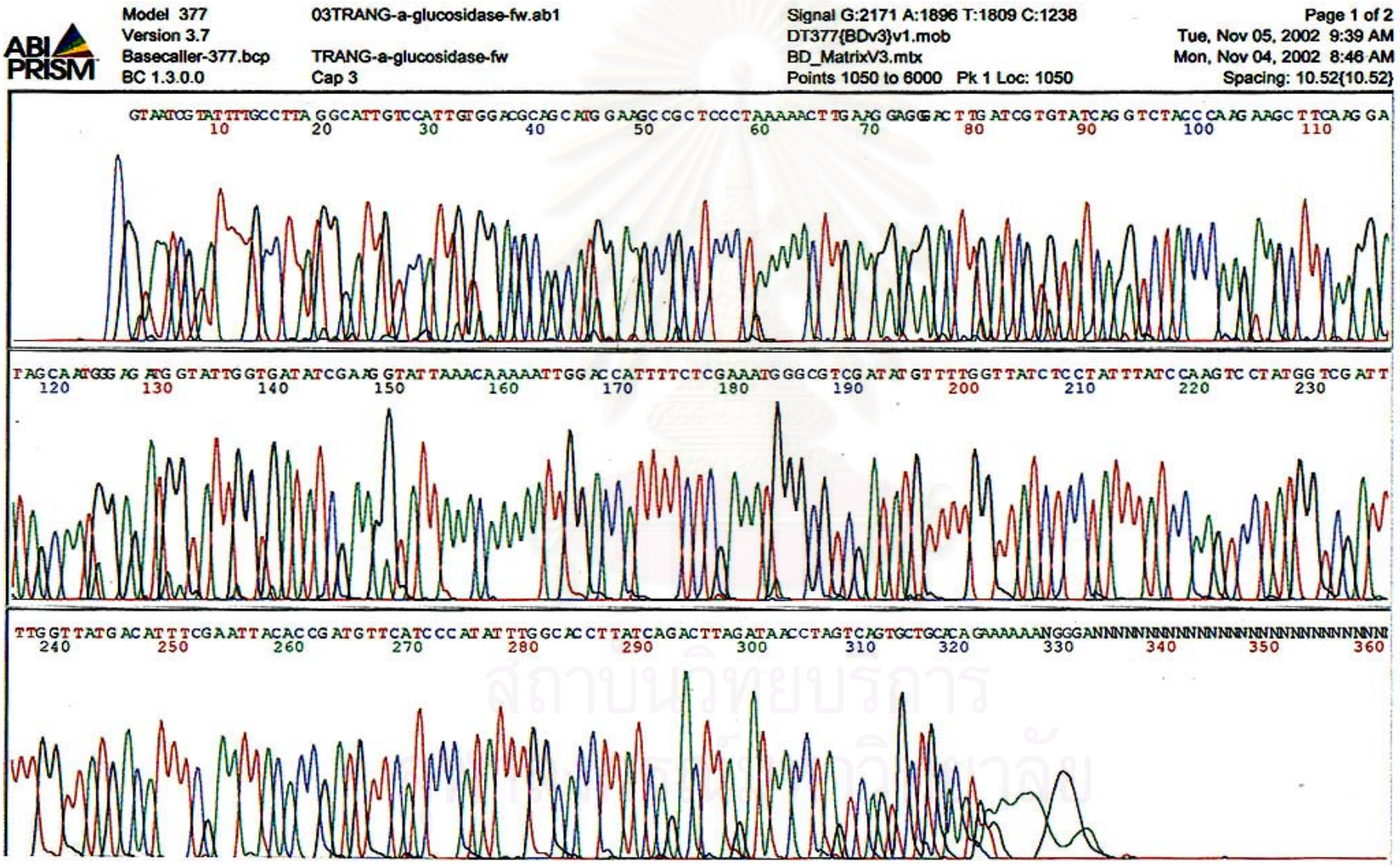
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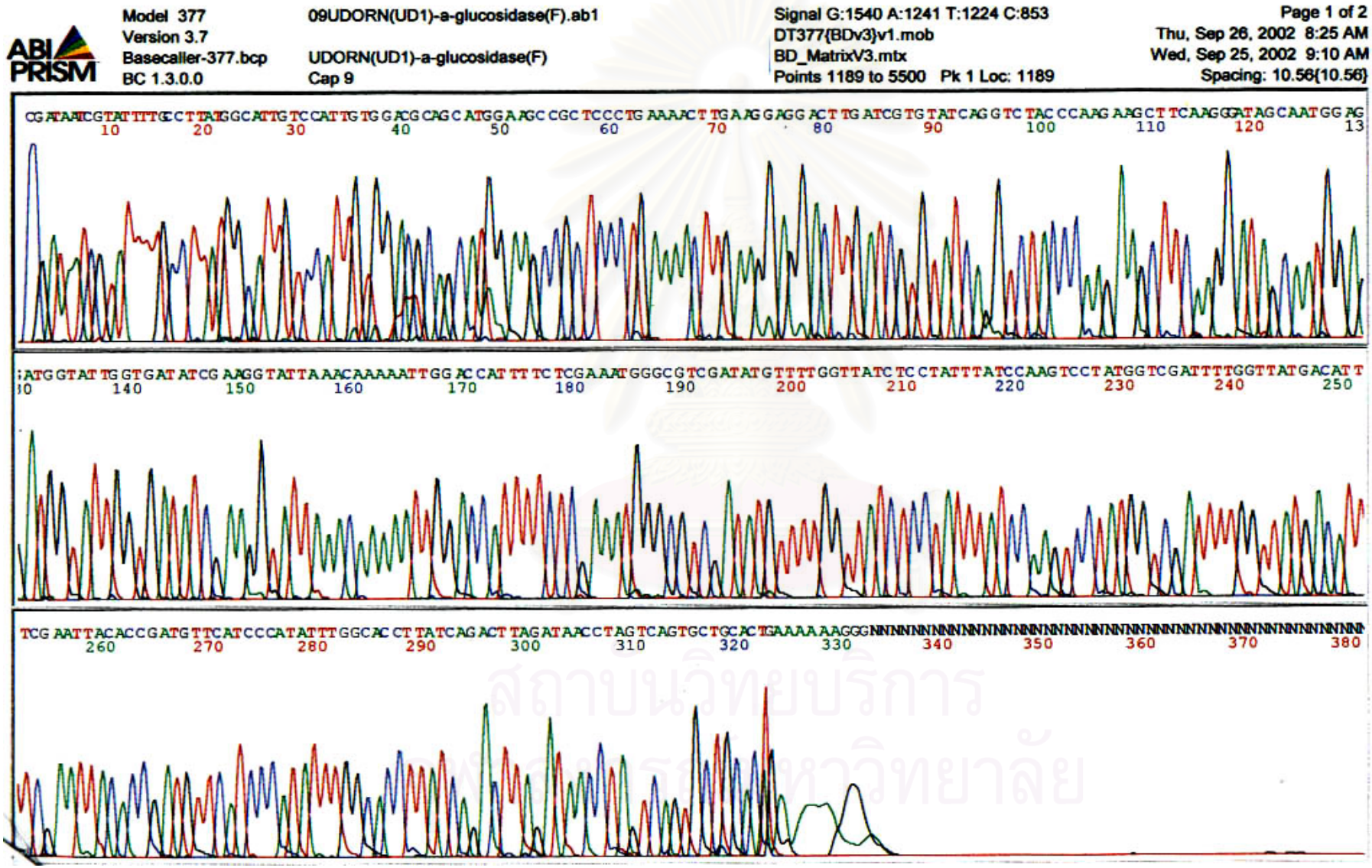
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d

BIOGRAPHY

Miss Prapaipit Srimawong was born on July 13rd , 1978 in Saraburi. She graduated with the Bachelor Degree of Science in Department of Biotechnology, Faculty of Science and Technology, Thammasat University in 2000. Then, she has been a graduate student in the Master's Degree in Biotechnology program, Faculty of Science, Chulalongkorn University since 2000.