ลักษณะสมบัติของ cDNA แอลฟา – กลูโคซิเดสของผึ้งโพรง *Apis cerana*

นางสาวสุวิสา พิลาล้ำ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CHARACTERIZATION OF *Apis cerana* ALPHA-GLUCOSIDASE cDNA

Miss Suwisa Pilalam

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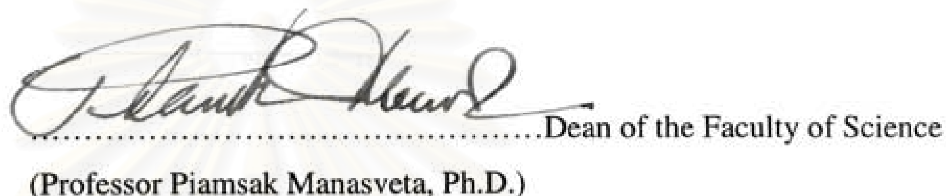

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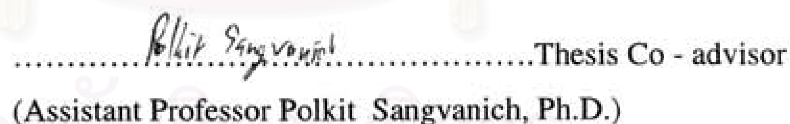

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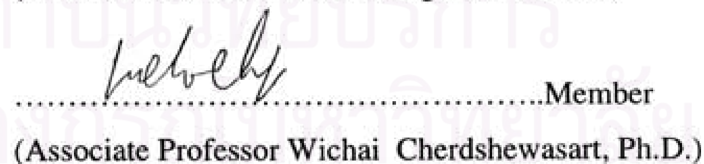

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สถาบันวิทยบริการ
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สุวิสา พิลาล้ำ: ลักษณะสมบัติของ cDNA แอลฟา – กลูโคซิเดสของผึ้งโพรง *Apis cerana* (CHARACTERIZATION OF *Apis cerana* ALPHA - GLUCOSIDASE cDNA) อ. ที่ปรึกษา: ผศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ. ที่ปรึกษาร่วม: ผศ. ดร. พลกฤษณ์ แสงวณิช, 106 หน้า. ISBN 974-14-2322-5

ผึ้งโพรงจัดเป็นผึ้งพื้นเมืองชนิดหนึ่งของไทย สามารถนำมาเลี้ยงในฟาร์มได้เช่นเดียวกับผึ้งพันธุ์ น้ำผึ้งและผลิตภัณฑ์ของผึ้งต่างๆ เช่น นมผึ้ง ไขผึ้ง พรอพอลิส เป็นต้น ซึ่งเป็นที่นิยมของผู้บริโภค แอลฟากลูโคซิเดส (เอจี) เป็นเอนไซม์ในกลุ่ม E.C. 3.2.1.20 ที่ย่อยสลายพันธะ 1–4 แอลฟากลูโคซิดิคของน้ำตาลซูโครสได้เป็นกลูโคสและฟรุคโตส เอจีเกี่ยวข้องกับการสร้างน้ำผึ้ง พบว่าต่อมไฮโปฟาริงค์ที่อยู่บริเวณหัวของผึ้งออกหาอาหารเป็นแหล่งของเอจี ออกแบบไพร์เมอร์ต่างๆ จากบริเวณอนุรักษ์ของยีนนี้ในผึ้งพันธุ์ด้วยเทคนิคอาร์ที พีซีอาร์ ภายใต้สภาวะที่เหมาะสมของเทคนิค สามารถได้สาย cDNA ที่ขนาดความยาว 1,740 คู่เบส เปรียบเทียบความเหมือนของเอจีกับสิ่งมีชีวิตอื่นๆ พบว่าคล้ายกับยีนนี้ในผึ้งพันธุ์สูงถึง 96 เปอร์เซ็นต์ (เปรียบเทียบในระดับความยาวที่ 1,740 คู่เบส) คล้ายกับมอลเตส 1 ในผึ้งพันธุ์ถึง 53.44 เปอร์เซ็นต์ (เปรียบเทียบในระดับความยาวที่ 1,570 คู่เบส) และคล้ายกับมอลเตส ใน *Culicoides sonorensis* ถึง 48.75 เปอร์เซ็นต์ (เปรียบเทียบในระดับความยาวที่ 1,600 คู่เบส) เมื่อสร้างสายสัมพันธ์ทางวิวัฒนาการของลำดับกรดอะมิโนโดยใช้โปรแกรม UPGMA และ Neighbor joining ของกรดอะมิโน พบว่าเอจีจากผึ้งโพรงก็มีความสัมพันธ์ใกล้เคียงกับเอจีของผึ้งพันธุ์มากที่สุด ต่อจากนั้นทำสกัดบริสุทธิ์เอนไซม์เอจีและศึกษาคุณสมบัติ เริ่มสกัดอย่างหยาบ (ผึ้งงานประมาณ 430 กรัม) วัดแอคติวิทีจำเพาะได้ 0.696 ยูนิตต่อมิลลิกรัมโปรตีน แล้วตกตะกอนด้วยแอมโมเนียมซัลเฟต (ความเข้มข้นอิ่มตัว 95 เปอร์เซ็นต์) วัดแอคติวิทีจำเพาะได้ 0.235 ยูนิตต่อมิลลิกรัมโปรตีน สกัดบริสุทธิ์ด้วย DEAE – cellulose วัดแอคติวิทีจำเพาะได้ 2.171 ยูนิตต่อมิลลิกรัมโปรตีน ด้วย CM – cellose วัดแอคติวิทีจำเพาะได้ 0.154 ยูนิตต่อมิลลิกรัมโปรตีน และ Superdex 200 วัดแอคติวิทีจำเพาะได้ 1.804 ยูนิตต่อมิลลิกรัมโปรตีน ค่าความบริสุทธิ์ของแอคติวิทีจำเพาะเพิ่มจาก 0.34 เท่าเป็น 3.11 เท่า 0.22 เท่า และ 2.59 เท่าตามลำดับ สภาวะที่เหมาะสมต่อการทำงานของเอจีบริสุทธิ์หลังสกัดด้วย Superdex 200 คือที่ พีเอช 5.0 บ่มปฏิกิริยาที่ 50 องศาเซลเซียส เป็นเวลา 50 นาที โดยที่ความเข้มข้นของซูโครส คือ 60 มิลลิโมลาร์ พบว่ามวลโมเลกุลของเอจี มีค่าประมาณ 68 กิโลดาลตัน นอกจากนี้นำเอจีที่บริสุทธิ์มาย่อยด้วยเอนไซม์ทริปซิน (ทั้งจากการย่อยในเจลและในสารละลาย) แล้ววิเคราะห์ด้วยเครื่องแมสสเปกโทรมิเตอร์แบบ Matrix assisted laser desorption ionization/ time (MALDI/ TOF) มวลของเปบไทด์ที่ได้จากการวิเคราะห์เข้าคู่กับเปบไทด์ของเอจีจากผึ้งพันธุ์ (เข้าคู่อย่างน้อย 4 เปบไทด์ คิดเป็น 4 เปอร์เซ็นต์ และเข้าคู่อย่างน้อย 18 เปบไทด์ คิดเป็น 19 เปอร์เซ็นต์)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชา.................เทคโนโลยีชีวภาพ......................ลายมือชื่อนิสิต........สุวิสา พิลาล้ำ........

ปีการศึกษา.....................2548.............................ลายมือชื่ออาจารย์ที่ปรึกษา.....จันทร์เพ็ญ จันทร์เจ้า.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.......................................

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SUWISA PILALAM: CHARACTERIZATION OF *Apis cerana* ALPHA - GLUCOSIDASE cDNA. THESIS ADVISOR: ASST. PROF. CHANPEN CHANCHAO, Ph. D., THESIS CO – ADVISOR: ASST. PROF. POLKIT SANGVANICH, Ph. D.,106 pp. ISBN: 974-14-2322-5.

Apis cerana is one of native honeybees in Thailand. It can be as well managed in an apiary as *A. mellifera*. Its honey and other products such as royal jelly, wax, propolis, etc. are popular among consumers. Alpha - glucosidase (AG) as in E.C. 3.2.1.20 is an enzyme that specifically hydrolyses 1, 4 – linked – alpha - glucosidic bond in sucrose to be fructose and glucose. It involves in honey production. Hypopharyngeal glands (HPGs) located in a head of worker bees were used as AG sources. Primers for RT - PCR were designed from conserved regions of *AG* in *A. mellifera*. Under the optimum condition, the cDNA sequence of *AG* (1,740 bp in length) was obtained and compared to that cDNA from other organisms. It is partially similar to the *AG* in *A. mellifera* at 96% (1,740 bp comparison), to maltase in *A. mellifera* at 53.44% (1,570 bp comparison), and to maltase in *Culicoides sonorensis* at 48.75% (1,600 bp comparison). Due to UPGMA and Neighbor joining program, phylogenetic trees of amino acid support that AG of *A. cerana* was very similar to AG of *A. mellifera*. Later, AG of *A. cerana* was purified and characterized. Worker bees (430 g) were homogenized to be crude (0.696 u/ mg), precipitated with 95% ammonium sulfate (0.235 u/ mg), and purified by DEAE - cellulose (2.171 u/ mg), CM - cellulose (0.154 u/ mg), and Superdex 200 chromatography (1.804 u/ mg). Specific activity increased to 0.34, 3.11, 0.22, and 2.59 fold, respectively. The optimum conditions of purified AG after Superdex 200 were at pH 5.0, at temperature of 50°C, and at incubation time of 50 min. The concentration of sucrose at 60 mM was used. MW of AG was estimated to be approximately 68 kDa. Furthermore, purified AG (both by in – gel and in – solution digestions of trypsin) was analysed by MALDI – TOF MS. The mass spectra confirmed the obtained amino acid sequence of AG (at least 4 matching masses with 4% coverage and at least 18 matching masses with 19% coverage).

สถาบันวิทยบริการ
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LIST OF ABBREVIATIONS

2-D electrophoresis	Two-dimentional electrophoresis
ACN	Acetronitrile
APS	Ammonium persulfate
AG	Alpha-glucosidase
Bis	*N,N'*-methylenebisacrylamide
Bp	Base pair
^{o}C	Degree celcius
C	Crosslinking factor [%]
cDNA	Chromosomal DNA
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
DTT	Dithiothreitol
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	Hour
HPGs	Hypopharyngeal glands
IEF	Isoelectric focusing
IPG	Immobilized pH gradients
kDa	Kilodalton
LWM	Low molecular weight
μl	Microlitre
μg	Microgram
MALDI	Matrix Assisted Laser Desorption Ionization
min	Minute
M	Molar
mA	Milliampere
mg	Milligram
μM	Micromolar
MW	Molecular weight
ml	Millilitre

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mM	Millimolar
mm	Millimetre
mRNA	messenger RNA
nm	Nanometre
m/z	Mass per charge
O.D.	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
pI	Isoelectric point
rpm	Revolution per minute
R_f	Relative mobility
RT	room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide-gel electrophoresis
T	Total acrylamide concertration [%]
TEMED	N,N,N',N'-tetramethylethylenediamine
Tof	Time of flight
Tris	Tris(hydroxymethyl)-aminoethane
U	Unit (s)
UV	Ultra violet spectroscopy
Volt	Volt
W	Watt
w/v	weight by volume
v/v	volume by volume

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CHAPTER I

INTRODUCTION

Honeybees in Genus *Apis* are widely distributed throughout the old world but they are not native to the new world. The distribution of honeybees is related to the relationship between biogeography and variation (Ruttner, 1988). At present, genus *Apis* contains ten recognized species. Honeybees can be grouped into 3 major clusters; giant bees (*A. dorsata*, *A. binghami*, and *A. laboriosa*), dwarf bees (*A. andreniformis* and *A. florea*), and cavity – nesting bees (*A. mellifera, A. cerana*, *A. koschevnikovi*, *A. nuluensis*, and *A. nigrocincta*) (Arias and Sheppard, 2005). Five species of honeybees; *A. cerana*, *A. florea*, *A. andreniformis, A. dorsata*, and *A. koschevnikovi* are native to Southeast Asia, a centre of biodiversity (Wongsiri *et al*., 2000). In Thailand, *A. cerana* is one of the native honeybee species and the most valuable for beekeeping. It has been considered as a vital component of natural ecosystem. Its nests are composed of multiple parallel combs and situated in sheltered cavities. It can be well - managed in beekeeping industry as same as *A. mellifera,* the imported European honeybees but it does not provide honey yield as high as *A. mellifera* (Uthaisang *et al*., 1994). In contrast, *A. cerana* is equivalent to *A. mellifera* in comb building, dancing, and nesting behaviors. Being native to the region, *A. cerana* is better adapted to the local environment, to its coevolved flowering plants, and to survive without supplementary feeding and medication (Joshi *et al*., 2002).

Present research is involved in molecular biology level. Lots of reports were performed by using *A. mellifera* as a model so we are interested in using our native species in Thailand, *A. cerana*, *A. florea*, *A. dorsata*, and *A. andreniformis* as models instead. In this research, we have paid attention to *A. cerana* since it is the most economic to the country.

Objectives of this research

We are interested in purifying and in characterizing AG in *A. cerana indica*. It is involved in obtaining the cDNA and amino acid sequences of AG. Homology and phylogenetic trees were observed in order to find the relationship of this gene among other organisms. In addition, the optimum conditions of AG were determined.

Outcomes and benefits

The purified AG and optimum condition of AG may apply to sweetened industry or food additives, industry of apiary, and the breeding improvement of *A. cerana* in Thailand.

1.1 Honey bees (*Apis cerana*)

A. cerana is one of native honeybee species to Thailand and many countries in Asia (Uthaisang *et al*., 1994). It is classified into Order Hymenoptera and Family Apidae. *A. cerana indica* is named after the origin of its location where is India (Ruttner, 1988). It always builds a nest containing a number of parallel combs in hidden places but also builds an open nest (Wongsiri, 1989). Classification of *A. cerana* is as below (Wongsiri, 1989).

Kingdom Metazoa
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Super - family Apoidea
Family Apidae
Sub - family Apinae
Genus *Apis*
Species *Apis cerana*

Honeybees change their physiology and neural functioning during behavioral development. Thus, as one of social insects, they are divided into 3 levels, queen, drones, and workers. There is only one queen in a hive. Workers are sterile because of queen pheromone (Wongsiri, 1989).

(A)

(B)

Figure. 1.1. A hive of *Apis cerana* (A) and (B).

1.2 Bee products

Honeybees are very important in agriculture, industry, medicine, and environment because of their products: pollen, honey, beewax, propolis, royal jelly (RJ), and venom (Mizrahi and Lensky, 1996). Honeybees gather their food, pollen, and nectar from flowers. Pollen provides bees with the building blocks of life: amino acids, vitamins, minerals, and lipids (fats and their derivatives). Nectar provides energy in the form of simple sugars which are processed by the bees and stored in the hive as honey (Hunt, 1991). Honey is a supersaturated solution of sugars, mainly fructose, glucose, and maltose - like sugars with traces of sucrose, glucose oxidase, hydrogen peroxide, phenolics, flavonoids, terpenes, etc. Depending on species of *Apis*, the amount of fructose was significantly higher in *A. dorsata* honey and in *A. cerana* honey than in *A. mellifera* honey (Joshi *et al*., 2000). It may be assumed that sugar is one of the most important stimuli for

honeybees. It was reported that responsiveness of foraging behavior was correlated to the difference in sucrose (Scheiner *et al*., 2004). Furthermore, other products explain the amazing honeybee success. For example, beewax is used as a pliable, stable, and moisture – proof material which is used to construct their nest. Propolis is an outstandingly good caulking in sealing a nest cavity and is also one of the best antimicrobial agents. Also, RJ plays an important role in honeybee nutrition. Most abundant proteins found in RJ are good to feed larva. RJ can control the development of young larvae and is responsible for the high reproductive ability of queen (Ohashi *et al*., 1997). In addition, venom provides an advantage of a formidable defense that is capable of stopping or deterring predators (Wongsiri, 1989; Mizrahi and Lensky, 1996).

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CHAPTER II

LITERATURE REVIEWS

2.1 Hypopharyngeal glands

Hypopharyngeal glands (HPGs) of worker bees are paired long tuberous organs connected to many acini or secretory glands (Fig.2.1). Each acinus is composed of approximately one dozen of secretory cells (Srimawong, 2003). These glands synthesize proteins depending on the age of honeybees. While it is young as nurse bee, HPGs will synthesize major royal jelly proteins. When it gets older to be a forager, HPGs will change to synthesize alpha - glucosidase (AG) instead. Ohashi *et al*. (2000) reported that a change in age – dependent roles was one of the most characteristic features of *A. mellifera* society. This is involved in the production of protein in HPGs.

In HPGs of nurse bees, jelly protein precursors or bee – milk proteins were derived in *A. mellifera* (Hanes and Simuth, 1992). They were purified by DEAE – cellulose chromatography. The MWs of MRJPs are 57 kDa and 55 kDa, respectively. The proteins have been identified with isoelectric points (IEF) in a range of pH 4.5 – 5.0. They found that the MRJPs were solely the dominant proteins in nurse bee heads. Later, Klaudiny *et al*. (1994) determined the nucleotide sequences of cDNA and deduced amino acid of MRJPs.

Later, MRJPs were obtained from HPGs in *A. cerana* (Srisuparbh *et al*., 2003 and Imjongrak *et al*., 2005). The purification of MRJPs of HPGs in *A. cerana* was performed by using Q – Sepharose and Sephadex G – 200 chromatographies. Immunoblotting analysis using affinity – purified antibody against 50 kDa of brood food protein shows the specificity of synthesized protein in HPGs (Kubo *et al*., 1996). It supported that the brood food proteins (royal jelly) were synthesized in the HPGs of nurse bees only.

In 2005, Santos *et al*. partially identified the protein complement of the secretion from HPGs of nurse bees in *A. mellifera* by using a combination of 2 - D gel and by peptide sequencing by MALDI – PSD/ MS. Due to a result of 2 - D gel, there were 61 different polypeptides composed in HPGs. Those proteins would be secreted into royal jelly.

HPGs in forager bees change their function to synthesize enzymes, including invertase, used in the production of honey from nectar. Alpha - glucosidase (AG) belongs to E.C. 3.2.1.20 (alpha – D - glucoside glucohydrolase) is a group of typical exo - type carbohydrases which hydrolyze alpha - glucosidic linkages in the non – reducing terminal of substrate (Chiba, 1997). It is synthesized in HPGs and is able to convert nectar sucrose into glucose. It is found to be a relatively nonspecific alpha - glucosidase and is shown to have trans - glucosidase activity (Huber and Mathison, 1976). In addition, AG precursor (EC 3.2.1.20) is maltase in *A. mellifera* and *Drosophila spp.* (Vieira *et al.,* 1997). Nevertheless, AG is widely discovered in various microorganisms, mammals, plants, and insects. By in plant and animal tussues, AG contributed the metabolisms of starch and glycogen, supplying the glucose utilized for energy production (Chiba, 1997). The proteins directly involved with the carbohydrates and energetic metabolisms were: AG, glucose oxidase and alpha amylase, whose are members of the same family of enzymes, catalyzing the hydrolysis of the glucosidic linkages of starch; alcohol dehydrogenease and aldehyde dehydrogenase, whose are constituents of the energetic metabolism (Santos *et al*., 2005).

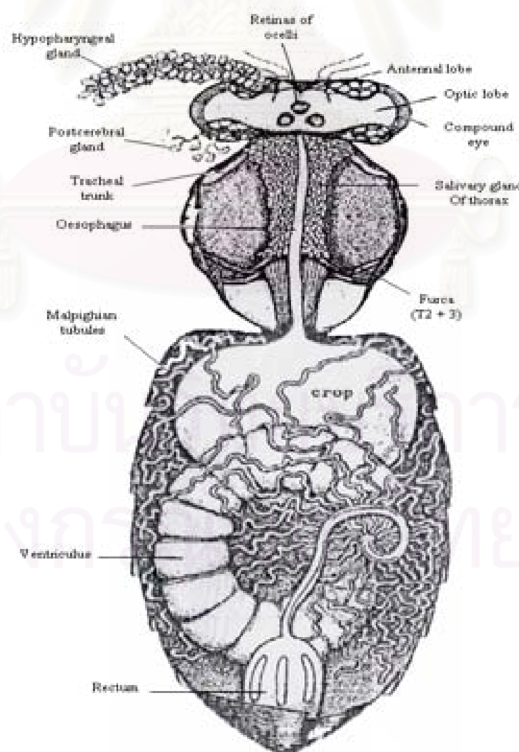

Figure 2.1. Dissection of worker honeybee. The picture was modified from [Plate 8 and 9 in “Anatomy and Dissection of the Honeybee”, ed. Date (1977)].

2.2 Purification and characterization of AG

Huber and Mathison (1979) purified for the first time two kinds of sucrase (or AG) from adult honeybees to homogeneity; AG is often called sucrase or invertase because of the sucrose – hydrolyzing property.

Chiba *et al*. (1983) determined anomeric forms of sugar produced by carbohydrases by gas – liquid chromatography (GLC). Thirteen kinds of alpha – glucosidase (AG), including AG from honey bee and acid AG from pig's liver, were assayed. Both AG and glucoamylase were typical hydrolases which catalyze exo – type hydrolysis liberating glucose from the non - reducing ends of substrates.

AG is composed of AGI, AGII, and AGIII. Locations of enzymes are also different. AGI is in the ventriculus to digest sugar, AGII is in the hemolymph to hydrolyze sugar, and AGIII is in HPGs to produce honey (Chiba *et al*., 1983; Kimura *et al*., 2000, and Kubota *et al*., 2004). In 1980, Takewaki *et al.* reported the purification procedures and some properties of both AG I and AGII in worker bees of *A. mellifera* by salting – out chromatography and ammonium sulfate precipitation. They reported that AGI was purified by chromatography on CM – cellulose and on Sephadex G – 100. AGII was purified by DEAE – cellulose, CM – cellulose, and Bio – Gel P – 150. The MW of both AG was estimated to be approximately 98 and 76 kDa, respectively. Both AG enzymes have optimum pH at 5.0 and can hydrolyze phenyl – alpha – glucoside, sucrose, and maltose as substrates.

Later, Nishimoto *et al.* (2001) reported the purification and substrate specificity of AGIII from *A. mellifera* body. It was purified by chromatographies on salting – out, DEAE – sepharose CL – 6B, Bio – Gel P – 150, and CM – Toyopearl 650M. By using maltose as a substrate, the MW of AGI, AGII, and AGIII were estimated to be approximately 98, 76, and 68 kDa, respectively. Optimum pHs were 5.0, 5.0, and 5.5, respectively. In addition, their substrate specificities were also different.

In 2004, Kubota *et al*. purify AG from honey of *A. mellifera* by using salting – out chromatography, CM – cellulose, Bio – Gel P – 150, and DEAE – Sepharose CL – 6B on chromatographies. For substrate specificity of honey AG, several kinds of substrates were used. It has an ability to hydrolyze sucrose, ρ – nitrophenyl α – glucoside, and maltotriose as same as AGIII. The effects of pH and temperature on the activity of honey AG were examined by using maltose as a substrate. The result presents that pH optimum is 5.5 which is different from AGI and II (pH 5.0). The enzyme is stable up to 40°C, but loses its activity completely by incubation at 60°C for 15 min. The MW is estimated to be

approximately 68 kDa which is the same MW as AGIII. The characteristics of AG purified from honey are the same as AGIII reported in Nishimoto *et al*. (2001).

Ohashi *et al*. (1997 and 1999) isolated the complete cDNA sequence coding for *AG* in *A. mellifera.* In addition, Ohashi *et al*. (1999) reported that purified amylase and glucose oxidase were highly obtained in HPGs of forager bees (*A. mellifera*). Furthermore, they found out that differential expression of those genes was related to the age – dependent role change of the worker bee. *AG* is expressed specifically in HPGs of forager bee (Ohashi *et al.,* 1996 and 1999).

In 2005, Wongchawalit *et al*. reported that there are AGI and AGII in *A. cerana japonica*. This species is native to Japan only. They were isolated by salting – out, ion – exchange, gel – filtration and hydrophobic chromatographies. The MWs were estimated to be 82 kDa (AGI) and 76 kDa (AGII) on SDS – PAGE. The internal peptide sequences of AGI and AGII were analyzed by in – gel digestion and by MALDI – TOF MS. The peptide mass spectra support the cDNA sequences of AGI and AGII.

Srimawong (2003) reported specific activity and optimum conditions of AG in crude in *A. cerana indica* which is native to Thailand. The MW of AG is estimated to be 96 kDa by SDS - PAGE and renaturation condition. Expression level of AG was performed by RT – PCR. The result indicates that AG is highly expressed in forager bees.

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CHAPTER III

MATERIALS AND METHODS

3.1 Equipment

- Akta^{TM} prime system, Amersham pharmacia biotech, Sweden
- Amicon ultra - 4 centrifugal filter devices, Millipore corporation, USA
- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Pipette; P10, P20, P200, and P1,000, Gilson, France
- Blender, model: Cunina HR 1791/ 6, Philips, Indonesia
- Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
- Centrifuge, model: universal 32R, Hettich zentrifugen GmbH & Co. Kg, Germany
- Centrifuge, model: KR - 20000T; rotor: RA - 6, Kubota, Germany
- Cooling, model: F33, Julabo labortechnik GmbH & Co., Germany
- Cuvette (Quartz), type: 18/ Q/ 10, Starna, Optiglass Ltd., UK
- Freeze dryer, Labconco, USA
- Hybridizer, model: HybriLinker HL - 2000, UVP laboratory products, USA
- Snakeskin® pleated dialysis tubing, 3,500 MWCO, Pierce Chemical Co., USA
- Immobiline drystrip kit, Amersham biosciences, Sweden
- Microincubator, model: M - 36, Taitec corporation, Japan
- Microwave, model: Sharp carousel R7456, Sharp, Thailand
- Minishaker, model: MS1, Ika Works Inc., USA
- Orbital shaker, model: KS130B, Ika – Werke GmbH & Co., Germany
- Plastic cuvettes, model: 1.5 Semi - micro cuvette, Brand, Germany
- PCR, model: GeneAmp® PCR system 2400, Applied biosystems, Singapore
- Pipette tip; 10 µl, 200 µl, and 1,000 µl, Sorenson, USA
- Polaroid camera, model: Direct screen instant camera DS 34 H - 34, Peca products, UK
- Polaroid film, Fuji film, Japan
- Poly acrylamide gel electrophoresis model, model: AE - 6530 mPAGE, Atto corporation, Japan
- Power supply, EC 570 - 90 LVD CE, E - C Apparatus Corporation, USA

- pH meter, Denver instrument, model: 215, Denver instrument Co. Ltd., Taiwan
- Microtiter plate reader, model: Specord S 100B, Analytik jena, Germany
- Sonicator, model: BHA - 1000, Branson, USA
- Speed vacuum centrifuge, Heto - holten, Denmark
- Stereomicroscope, Olympus optical Co. Ltd., Japan
- Hotplate, Schott, Germany
- Refrigerator centrifuge beckman coulter avanti – J - 30I, Kokusan H - 103N, Germany
- Vortex mixer, Vortex - genie 2, Scientific industries, USA
- Waterbath, model: WB 22, Memmert, Germany
- Water vacuum pump, Velp scientifica, Europe
- Analytical balance, model: AB204 - S, Mettler - toledo, Switzerland

3.2 Chemicals

- 2 - Mercaptoethanol, BDH laboratory supplies, UK
- 2, 3, 5 - Triphenyltetrazolium chloride, $C_{19}H_{15}ClN_4$, M. W. = 334.81, Fluka biochemical, Switzerland
- 3, 6 - Dinitrophthalic acid, $C_8H_4N_2O_8.C_5H_5N$, F. W. = 335.2, Sigma, Germany
- Access RT - PCR system (catalog# A1250), Promega, USA
- Acetic acid, CH_3COOH, M. W. = 60.05, Merck, Germany
- Acrylamide, M. W. = 71.08, Promega, USA
- Agarose, Research organics, USA
- Ammonium persulfate, M. W. = 228.20, Promega, USA
- Ammonium peroxydisulfate (APS), $(NH_4)_2S_2O_8$, M. W. = 249, BDH laboratory supplies, UK
- Ammonium sulfate $(NH_4)_2SO_4$, M. W. = 132.15, Merck, Germany
- Barium chloride 2 - hydrate, $BaCl_2.2H_2O$, M. W. = 244.27, UniVar, Ajax Chemical
- Bovine serum albumin (BSA), Fraction V, pH 7.0, Serva feinbiochemica GmbH & Co., USA
- Broad range protein MW marker, Merck, Germany
- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M. W. = 670, BDH laboratory supplies, UK
- Calcium chloride dihydrate, $CaCl_2.2H_2O$, M. W. = 147, Merck, Germany
- Citric monohydrate, F. W. = 210.14, Carlo erba, Italy

- Coomassie brilliant blue G - 250, $C_{47}H_{48}N_3O_7S_2Na$, M. W. = 854, BDH laboratory supplies, UK and Sigma, Germany
- Coomassie brilliant blue R - 250, $C_{45}H_{44}N_3O_7S_2Na$, M. W. = 826, Serva feinbiochemica GmbH & Co., USA
- CM cellulose fibrous, Sigma, USA
- DEAE - cellulose fast flow fibrous, Sigma, USA
- Di - sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), M. W. = 141.96, AnalaR® BDH, UK
- Double distilled water, GFL glass water sills, Germany
- 95% (v/v) Ethanol (C_2H_5OH), M. W. = 46, Thailand
- 37% (v/v) Formaldehyde, CH_2O, M. W. = 30, Thailand
- Glycerol, Asia pacific specialty chemicals, Ltd., Australia
- Glycine, NH_2CH_2COOH, M. W. = 75.07, Fisher, USA
- Hydrochloric acid fuming 37% (v/v), HCl, Merck, Germany
- Immobiline ™ drystrip (pH 3 - 10), 7 cm, (catalog# 17 – 6001 - 11), Amersham biosciences, Sweden
- Leupeptin, $C_{20}H_{38}N_6O_4$.HCl, F. W. = 463, Sigma, Germany
- Liquid N_2, Thai industrial gases public Co. Ltd., Thailand
- Methanol, CH_3OH, M. W. = 32.04, Merck, Germany
- *N, N'* – methylene – bis - acrylamide, F. W. = 154.17, Sigma, USA
- Octylphenol - polyethyleneglycol ether (Triton X - 100), Serva feinbiochemica GmbH & Co., USA
- Oligo (dT) 15 primer, Promega, USA
- Ortho - phosphoric acid (85% H_3PO_4), M. W. = 97.995, Carlo erba, Italy
- PCR purification kit (catalog# 28140), Qiagen, Germany
- Pepstatin A, $C_{34}H_{63}N_5O_9$, F. W. = 685.9, Sigma, Germany
- Phenylmethylsulfonyl fluoride (PMSF), $C_7H_7FO_2S$, F. W. = 174.2, Sigma, Germany
- Potassium carbonate, K_2CO_3, F. W. = 138.2, Merck, Germany
- Potassium hydroxide, KOH, M. W. = 56.109, Carlo erba, Italy
- RNase away, Molecular bioproducts Inc., USA
- Silver nitrate, 99.8% $AgNO_3$, M. W. = 169.87, Nacalai tesque, Japan
- Sodium acetate, $CH_3COONa.3H_2O$, M. W. = 136.09, Merck, Germany
- Sodium carbonate, $NaCO_3$, M. W. = 105.99, Merck, Germany

- Sodium chloride, NaCl, M. W. = 58.4, Merck, Germany
- Sodium citrate, $C_6H_5Na_3O_7.H_2O$, M. W. = 294.10, Merck, Germany
- Sodium dihydrogen orthophosphate, NaH_2PO_4. $2H_2O$, M. W. = 137.99, BDH laboratory supplies, UK
- Sodium dodecyl sulfate, $C_{12}H_{25}O_4S_1Na$, M. W. = 288.38, BDH laboratory supplies, UK
- Sodium hydroxide, NaOH, M. W. = 40, Merck, Germany
- Sodium thiosulfate, $Na_2S_2O_3.5H_2O$, F. W. = 248.2, Sigma, Germany
- Sucrose, $C_{12}H_{22}O_{11}$, M. W. = 342.30, Merck, Germany
- Superdex 200 prep grade, Amersham biosciences, Sweden
- SV total RNA isolation system (catalog# Z3100), Promega, USA
- TEMED (N, N, N', N'- tetramethylenediamine), Amersham biosciences, Sweden
- Tris (hydroxyl methyl) - aminomethane, $NH_2C(CH_2OH)_3$, M. W. = 121.14, Promega, USA
- Trypsin, Sigma, Germany

3.3 Beekeeping

For RNA isolation: Colonies of *Apis cerana* were brought from Ban Bangkhuntak, Muang district, Samut Songkram province, and were moved to the Department of Biology, Faculty of Science, Chulalongkorn University for maintenance and sampling. Bees are independent to forage food outside their hives. Forager bees were collected after they returned to a hive.

For alpha - glucosidase purifiction: *A. cerana* was purchased from the same area as mentioned above. Bees were frozen in dry ice during the trip. In the laboratory, they were stored at -20°C for 1 - 2 days.

3.4 Isolation of total RNA

Hypopharyngeal glands (HPGs) were dissected under a binocular microscope. Ten HPGs were used for one reaction. The method was due to a protocol of an SV total RNA isolation kit (catalog# Z3100, Promega). Briefly, HPGs from *A. cerana* were homogenized with liquid N_2 in a motar. The grinded tissue were mixed by 175 µl of SV RNA lysis buffer. Three hundred and fifty µl of RNA dilution buffer was added and mixed by inverting 3 - 4×. The mixture was incubated at 70°C for 3 min. The mixture was then centrifuged at 13,000 rpm for 10 min. The supernatant was mixed by 200 µl of 95%

ethanol. Later, the mixture was transferred to a spin column assembly and centrifuged at 13,000 rpm for 1 min. Flow through (FT) in a collection tube was discarded. Six hundred µl of SV RNA wash solution were added to the previous spin column assembly. Again, it was centrifuged at 13,000 rpm for 1 min. The DNase incubation mix was prepared by 40 µl of yellow core buffer, 5 µl of 90 mM $MnCl_2$, 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 RT for 15 min. Two hundred µl of SV DNase stop solution were added to the spin basket and centrifuged at 13,000 rpm for 1 min. Then, 600 µl of SV RNA wash solution were added and centrifuged at 13,000 rpm for 1 min. Also, 250 µl of SV RNA wash solution were added again and centrifuged at 13,000 rpm 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 13,000 rpm for 1 min. Total RNA was aliquoted and stored at -20°C until use.

3.5 Native agarose gel electrophoresis

An agarose gel of 1.2% (w/v) was prepared with 1× TBE (50 mM Tris – HCl, 50 mM boric acid, and 0.65 mM EDTA). RNA or DNA sample was mixed by 1× loading dye [5X loading dye: 25 mM Tris – HCl (pH 7), 0.05% bromophenol blue, 150 mM EDTA, and 25% glycerol] and loaded onto the gel. The electrophoresis was performed by 1× TBE as running buffer at 100 V for 50 min. Then, a gel was stained by Ethidium bromide (EtBr) for 3 min and destained by d - H_2O for 20 min.

3.6 Formaldehyde gel electrophoresis

RNA sample was mixed by 1× formaldehyde loading dye (95% formamide, 18 mM EDTA, 0.025% xylene cyanol, 0.25% (v/v) glycerol, and 0.025% bromophenol blue). The sample was incubated at 65°C for 15 min. After incubation, the sample was quick spun and cool on ice. For gel preparation, 1% (w/v) agarose was melt in 1× MOPS buffer (40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA). After being cool, it was mixed by 1.2% formaldehyde. The gel was covered by 1× MOPS buffer. The electrophoresis was run at 50 V for 50 min. Then, a gel was stained by EtBr for 1 h and destained for 20 min.

3.7 Primer design

Primers for RT - PCR were from conservative parts of cDNA sequences of *AG* in *A. mellifera* (accession# D79208). RT - PCR primers are located as Appendix C. Primer

sequences are: forward primer (FW1), 5' – TCGA CTTC TAGT TGGT AGCA TGAA GG -3', reverse primer (R1), 5' – CTAG TCAG TGCT GCAC ATGA GAAA GG – 3', FW2, 5' - GCTT ATCG AGGC ATAC ACGA - 3', R3, 5' - CGCC GCTT CAAA GAAT AGAC - 3', FW3, 5' - ACGA GGAA CAAA TCGT GGAT – 3'; and oligo dT primer. Primers of 28S RNA gene in *A. mellifera* and elongation factor (EF) gene in *A. cerana* were used as control. Primer sequences of 28S RNA are 5' - AAAG ATCG AATG GGGA TATT C – 3' as forward and 5' - CACC GGGT CCGT ACCT CC - 3' as reverse while the primer sequences of EF are 5'- TCGC TTTA CTCT TGGT GTGA – 3' as forward and 5' - AAAC TCCC AACA TATT ATCT CCA – 3' as reverse primers.

3.8 RT - PCR amplification

A reaction mixture of RT - PCR was prepared by 1x AMV/ *Tfl* reaction buffer, 0.2 mM dNTP mix, 0.4 mM of each primer (FW and R), 1 mM $MgSO_4$, 0.1 u of AMV reverse transcriptase and *Tfl* DNA polymerase, and 200 ng total RNA. The RT - PCR cycling profile was modified from Ohashi *et al*. (1996) and Srimawong (2003). 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; 30 cycles of denaturation at 94°C for 30 sec, of annealing at 50°C for 30 sec, and of extension at 72°C for 2 min. At last, the final extension was at 68°C for 7 min. As control, 28S RNA and EF primers were used as RNA reference markers. The conditions were the same as the condition of *AG* primers. The product was detected by 1.5% agarose gel electrophoresis.

3.9 Purification of RT - PCR product for cDNA sequencing

A PCR reaction was purified by QIAquick PCR purification kit (catalog# 28104, Promega). Fifty µl (1× vol) of PCR sample were mixed by 250 µl (5× vol) of Buffer PB. Then, the QIAquick spin column was placed into a provided 2 ml collection tube. The sample was applied to the QIAquick column and centrifuged at 13,000 rpm for 1 min. Flow-through (FT) was discarded. The QIAquick column was placed back into the same tube. The QIAquick column was washed by 750 µl of Buffer PB and centrifuged at 13,000 rpm for 1 min. Then, the FT was discarded again. The QIAquick column was moved to a 1.5 ml tube. The QIAquick column was eluted by 50 µl milli Q H_2O and spun at 13,000 rpm for 1 min. The purified cDNA was stored at -20°C.

3.10 Sequencing of cDNA

A cDNA sequence of alpha glucosidase (*AG*) in *A. cerana* was obtained by amplified RT-PCR. Then, the purified product was sent to Bioservice unit, Thailand for DNA sequencing. Ten µM of forward, 10 µM of reverse , or 10 µM of oligo dT primers were used for an analysis.

3.11 Alignment of cDNA sequences and phylogenetic analyses

The nucleotide and deduced amino acid sequences were aligned by Clustal X program. The cDNA sequences of *AG* were aligned and compared to the sequences of *AG* in *A. mellifera* (accession# D79208), maltase 1 in *Apis mellifera* (XM_393379), *Drosophila melanogaster* CG1493 - PA (NM_135678), glucan 1, 6 – alpha - glucosidase putative in *Enterococcus faecalis* (GI: 29343377), sucrose specific enzyme II of the PTS (*ScrA*) and dextran glucosidase (*dexB*) genes in *Lactobacillus sakei* (AF401046), and *Culicoides sonorensis* clone CsMAL1 maltase (AY603565). Phylogenetic trees of *AG* among these organisms were made by using PAUP (version 4.0b). The reliability of the tree was tested by bootstrap for 1,000 replicates. Data from sequences were used to reconstruct a phylogeny with parsimony analysis phylogram. Genetic distances were computed by neighbor - joining and UPGMA.

3.12 Crude extract of hypopharyngeal glands (HPGs) and honey crops

Forager bees were collected and dissected under a binocular microscope. The pairs of HPGs and a honey crop were dissected out of each bee and stored in buffer insect saline (20 glands/ 500 µl) mixing with 1 mM phenylmethylsulfonyl Fluoride, 0.1 µg/ml pepstatin, and 100 µg/ml leupeptin. They were homogenized and centrifuged 2× at 7,000 rpm, 4°C for 10 min. The supernatant as crude extract was stored at -20°C.

3.13 Protein assay

3.13.1 Bradford assay

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 0, 5, 10, 15, 20, 25, and 30 µg/µl, respectively (Appendix B). The volume of protein was adjusted by dd - H_2O to be 100 µl. Then, it was mixed by 1 ml of Bradford working buffer (Appendix A). One hundred µl of sample protein were mixed

with 1 ml of Bradford working buffer. The mixture was incubated at RT for 5 min before recording the absorbance at 595 nm.

3.13.2 Absorbance at 280 nm (A_{280})

Protein was diluted.

Concentration of protein (mg/ ml) = $A_{280} \times$ dilution

Total protein (mg) = concentration of protein × total volume

3.14 AG Purification

3.14.1 Ammonium sulfate precipitation

Frozen honey bees (430 g) were mechanically minced with a small amount of 30 mM sodium phosphate buffer (pH 6.3) in a porcelain motar. Homogenate was mixed by 30 mM sodium phosphate buffer (pH 6.3) in a final volume of 3,000 ml. After being stirred overnight at 4^oC, the suspension was centrifuged at 10,000× g for 15 min, and 2,000 ml of the crude extract was obtained. While being stirred at 4^oC, ammonium sulfate was slowly added to be 0 - 95% saturation. After centrifugation at 10,000× g for 25 min, the precipitate was dissolved in 350 ml of 30 mM sodium phosphate buffer (pH 6.3) and dialyzed by dialysis bag at MWCO 3500 against the same buffer at 4^oC overnight. After centrifugation at 10,000× g for 10 min, the supernatant was kept at 4^oC before chromatography.

3.14.2 Chromatography

3.14.2.1 Ion exchange chromatography

(1) Anion exchange chromatography (DEAE cellulose)

Supernatant (150 mg protein in 30 ml) mentioned in 3.14.1 was subjected to a DEAE cellulose column. The chromatography was performed by using Akta prime system comprising a pump, a UV detector, and a fraction collector (Amersham pharmacia biotech). At the beginning, a DEAE - cellulose column (1.6 × 17 cm) equilibrated by 30 mM sodium phosphate buffer (pH 6.3) was used. Bound materials were eluted by a linear gradient of 0 to 1 M NaCl at flow rate of 1.5 ml/ min and maintained at 4^oC. Protein

absorption was monitored at 280 nm. Fractions were collected and assayed for AG activity due to Momose's method.

(2) Cation exchange chromatography (CM - cellulose)

A chromatography was performed by using Akta prime system as described in 3.14.2.1 (1). Pooled active fractions (unbound fractions) from a DEAE - cellulose column (20 ml) were subjected to a CM - cellulose column (1.6 × 17 cm) equilibrated by 20 mM sodium acetate buffer (pH 4.7). Bound materials were eluted by a linear gradient of 0 to 1 M NaCl at flow rate of 0.5 ml/ min and maintained at 4°C. Protein absorption was monitored at 280 nm. Fractions were collected and assayed for AG activity due to Momose's method.

3.14.2.2 Gel filtration chromatography

Superdex – 200 gel filtration chromatography

A chromatography was performed by using Akta prime system as described in 3.14.2.1 (1). Pooled active fractions from a DEAE - cellulose column (5 ml) was subjected to a superdex - 200 gel filtration column (1.6 × 52.5 cm) equilibrated by 30 mM sodium phosphate buffer (pH 6.3) at flow rate of 0.5 ml/ min and maintained at 4°C. The fractions were collected and then kept at 4°C.

3.15 Activity assay

Alpha - glucosidase activity was determined by using Momose's method (Kubo *et al*., 1996). Supernatant (10 μl) was mixed with 20 μl of 10 mM phosphate buffer (pH 5.0) containing 0.1 M sucrose. The reaction was incubated at 30°C for 10 min and boiled for 3 min. After that, the reaction was terminated by 50 μl of 0.3% (w/v) 3, 6 – dinitrophthalic acid and 50 μl alkaline solution [5% (w/v) sodium thiosulfate and 25% (w/v) potassium carbonate] and boiled for 10 min. The activity was measured at the absorbance of 450 nm. One unit of enzyme activity was defined as the amount of enzyme which can hydrolyze 1 μmole of sucrose/ min under the condition described above.

3.16 SDS - polyacrylamide gel electrophoresis

An SDS - polyacrylamide gel (8 × 9 cm size; 1 mm thick; and 10 wells) was prepared as a discontinuous gel. A 12% separating gel was prepared by 30% bis - acrylamide solution, 1 M Tris - HCl buffer (pH 8.8), 10% (w/v) SDS, dd - H_2O, 10% fresh

ammonium persulfate (APS), and 0.05% TEMED. A 4% stacking gel was mixed by the composition of 30% bis - acrylamide, 0.5 M Tris - HCl (pH 6.8), 10% (w/v) SDS, 10% fresh APS, and 0.1% of TEMED. For sample preparation, 20 µg of crude were mixed with 1× loading dye [For 5× loading dye: 1 M Tris - HCl (pH 6.8), 50% (v/v) glycerol, 10% (w/v) SDS, 2 - mercaptoethanol, and 1% bromophenol blue], boiled for 5 min, and cool in ice. The protein MW marker (molecular mass range of 10 to 225 kDa) was also loaded. The electrophoresis was performed by 1× electrode buffer [25 mM Tris (hydroxylmethyl) - aminometane, 192 mM glycine, and 0.1% (w/v) SDS] and carried out at 100 V until the dye front reached the bottom of the gel. Then, it was coomassie blue stain.

3.17 Coomassie blue stain for SDS - PAGE

After electrophoresis, the gel was incubated in coomassie blue stain solution [50% (v/v) methanol, 10% (v/v) acetic acid, and 1.25 mg/ ml coomassie brilliant blue R - 250] and gently shaken for 25 min or until deeply stained. Then, the gel was washed several times in destain solution [10% (v/v) methanol, 10% (v/v) acetic acid, and 1.25 mg/ ml coomassie brilliant blue R-250] until the background was clear. Finally, the gel was preserved in 10% glycerol and sealed in cellophane.

3.18 Activity stain

After electrophoresis, the gel was soaked in 1% Triton X - 100 at RT with gentle shaking for 2 h. Then, the gel was washed by d - H_2O for 2 - 3×. Then, it was incubated in 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0) at 45^oC for 30 min. The gel was washed by d - H_2O for 2 - 3×. Then, the gel was boiled in freshly prepared solution of 0.1% (w/v) triphenyltetrazolium chloride in 0.5 N NaOH until a reddish band of AG activity on the gel was appeared.

3.19 Two - dimensional electrophoresis

3.19.1 Isoelectric focusing (IEF) and SDS - PAGE

Two - dimensional (2 - D) electrophoresis was performed as described in a manual of 2 - D electrophoresis using immobilized pH gradients (Berkelman and Stenstedt, 1998). For the first - dimension IEF, the AG solution (2mg protein in 100 µl) was mixed by the lysis buffer [8 M urea, 4% 3 - (3 - cholamidopropyl) dimethylammonio – 1 - propane sulfonate (CHAPS), and 2% immobilized pH gradients (IPG) buffer (pH 3 - 10)]. Then, it was mixed by rehydration solution [8 M urea, 2% CHAPS, 2% IPG buffer (pH 3-10), 0.2%

DTT, and 0.002% bromophenol blue]. The mixture was applied on 7 cm IPG strips (pH 3 - 10). The IPG strip was positioned on the immobiline drystrip reswelling tray and allowed to rehydrate at RT overnight. Then, the strip was transferred to the immobiline drystrip aligner. The IEF electrophoresis was carried out at 20°C on the Multiphor II electrophoresis unit with immobiline drystrip kit (Amersham pharmacia biotech). The running condition of IEF was as followed by 3 phases: 200 Voltages (V) for 1 min, 30 Vh (Voltage hour); 3,500 V for 1.5 h, 2,800 Vh; and 3,500 V for up to 4,500 Vh. After IEF, the IPG strip was equilibrated in the equilibration solution [50 mM Tris - HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and 0.002% bromophenol blue] for 15 min. Later, it was equilibrated in equilibration solution containing 2.5% iodoacetamide instead of 1% DTT for 15 min. Each gel strip was embedded on top of the SDS - polyacrylamide gel and covered by 0.5% agarose. For the second - dimension IEF, SDS - PAGE was carried out on a 12.5% *T* + 2.6% *C* SDS - polyacrylamide gel (8 × 9 cm and 1 mm thick) run at 280 V. An initial current was 10 mA/ gel for 15 min, then, it changed to be 20 mA/ gel until the bromophenol blue dye front reached the bottom of gel. The 12.5% *T* + 2.6% *C* SDS - polyacrylamide gel was prepared from the polymerization of the gel solution [4.17 ml of 30% *T* + 3.6% *C* acrylamide bis solution, 2.5 ml of 1.5 M Tris - HCl (pH 8.8), 0.1 ml of 10% (w/v) SDS, 3.18 ml of d - H_2O, 50 µl of 10% APS, and 3.3 µl of TEMED]. The LMW calibration kit was used as standard MW protein marker.

3.19.2 Coomassie blue stain for 2 - D analytical gel

The colloidal staining was used. After electrophoresis, the gel was fixed by 12% (w/v) TCA with gentle shaking for 1 h. After that, the gel was stained overnight in 100 ml of staining solution [0.1% (w/v) coomassie G - 250 in 2% H_3PO_4 and 10% (w/v) ammonium sulfate]. While shaking, 25 ml of methanol was slowly added. The gel was washed in 0.1 M Tris - H_3PO_4 buffer (pH 6.5) for 3 min. Then, it was rinsed in 25% (v/v) methanol. Finally, the gel was preserved in 20% (w/v) ammonium sulfate.

3.20 Optimum conditions

3.20.1 Optimum pH

A reaction mixture was prepared as described in 3.15 except pH of a reaction. Ten mM of Briton - Robinson buffer solution (Appendix A) were modified from Nishimoto *et al.* (2001) at various pHs ranging between 3.0 - 7.5. The assay was conducted in triplication. The absorbance of the mixture was measured at 450 nm.

3.20.2 Optimum temperature

A reaction mixture was prepared as described in 3.15 but 10 mM sodium acetate buffer (pH 5.0) was used instead of 10 mM Briton - Robinson buffer. The reaction mixture was incubated at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C, respectively) for 10 min. The assay was conducted in triplication. The absorbance of the mixture was measured at 450 nm.

3.20.3 Selective concentration of substrate

A reaction mixture in 10 mM sodium acetate buffer (pH 5.0) was prepared as described in 3.15 but the concentration of sucrose was varied from 10, 20, 30, 40, 50, 60, 70, 80, and 90 mM, respectively. The mixture was incubated at 55°C for 10 min. The assay was conducted in triplication. The absorbance of the mixture was measured at 450 nm.

3.20.4 Optimum incubation time

A reaction mixture in 10 mM acetate buffer (pH 5.0) containing 60 mM sucrose was incubated at 55°C. The incubation time was varied from 10, 20, 30, 40, 50, 60, and 70 min, respectively. The reaction mixture was prepared as described in 3.15. The assay was conducted in triplication. The absorbance of the mixture was measured at 450 nm.

3.21 Protein Identification

3.21.1 In - gel digestion

An in - gel digestion protocol had been modified from a method described in Chapter 6: the preparation of protein digestion for Mass Spectrometric sequencing, a book of protein sequencing and identification using Tandem Mass Spectrometry. An interesting protein band was manually excised from an SDS polyacrylamide gel. It was washed 2× by 100 - 500 µl of milli Q H_2O for 15 min each. Then, it was washed 3 - 4× by 100 µl of 50% ACN/ 0.1 ammonium bicarbonate for 10-15 min each or until the gel became white. The gel was dried by using a speed vacuum centrifuge. Dried gel pieces were incubated in 100 µl of 10 mM DTT/ 0.1 M ammonium bicarbonate/ 1 mM EDTA at 60°C for 45 min. Then, the excess DTT solution was removed. The sample was shaken in the dark by 100 µl of 100 mM Iodoacetamide (IAA)/ 0.1 M Ammonium bicarbonate at RT for 30 min. Later, IAA solution was removed. It was washed 3 - 4× by 150 - 200 µl of 50% ACN/ 0.05 M Tris-HCl (pH 8.5) for 5 min each and dried. It was rehydrated in 180 µl of digestion buffer

and 20 µl of trypsin solution (Appendix A) and incubated at 37°C overnight. One hundred µl of supernatant was saved separately (Part I). The rest of 100 µl of trypsin reaction (Part II) was stopped by adding 100 µl of 2% TFA and incubated at 60°C for 30 min. The supernatant was collected and transferred to a 0.6 ml tube. The peptides were subsequently extracted 3×. The first extraction step, it was incubated in 30 µl of digestion buffer at 60°C for 30 min. After that, it was sonicated for 5 min and centrifuged at 7,000 rpm for 1 min. Supernatant (Sup I) was saved. The next step, it was added with 15 µl of 50% ACN and 15 µl of digestion buffer and incubated at 30°C for 10 min. Later, it was sonicated for 5 min and centrifuged at 7,000 rpm for 1 min. Supernatant (Sup II) was also saved. The final step, 15 µl of 5% formic acid in ACN and 15 µl of digestion buffer was added. The mixture was incubated at 30°C for 10 min and sonicated for 5 min. Then, the supernatant (Sup III) was collected. Trypsin reaction (Part I) was combined to Sup I, Sup II, and Sup III. The volume of combined mixture was reduced to be 20 µl by using a speed vacuum centrifuge. The mixture was kept at -20°C until use.

3.21.2 In - solution digestion

The purified AG solution (1 part) was mixed by 1 mg/ ml trypsin (50 parts). It was incubated at 37°C overnight. The trypsin reaction was stopped by adding 5 µl of concentrated formic acid and dried (not completely dried) by a speed vacuum centrifuge. It was incubated in 2% TFA at 60°C for 30 min. The supernatant was collected, desalted, and kept at -20°C until use.

3.22 Nucleotide sequence determination

Protein in positive fractions from Superdex - 200 chromatography (after DEAE - cellulose chromatography) was desalted by using reusable reversed - phase cartridge (Protein trap, Michrom bioresource). The desalted protein was dried by using a freeze dryer. Then, it was dissolved in 50% ACN/ 0.1% TFA. Mass spectra of protein and peptide were acquired by using a MALDI/ TOF Mass Spectrometry operating in linear and reflectron modes. Peptide mass mapping obtained from each digested protein was searched against protein database via the MASCOT program (www.matrixscience.com). The searching parameters were trypsin enzyme, two missed trypsin cleavages, +1 Da mass accuracy, and doublet charged peptides.

CHAPTER IV

RESULTS

4.1 Alpha glucosidase cDNA sequence

Total RNA was extracted from hypopharyngeal glands of forager bees and determined by 1.2% (w/ v) agarose gel and formaldehyde gel. After electrophoresis, 18S and 28S rRNA were visible on agarose gel (Fig. 4.1 A) while only 28S rRNA was observed on formaldehyde gel (Fig. 4.1 B).

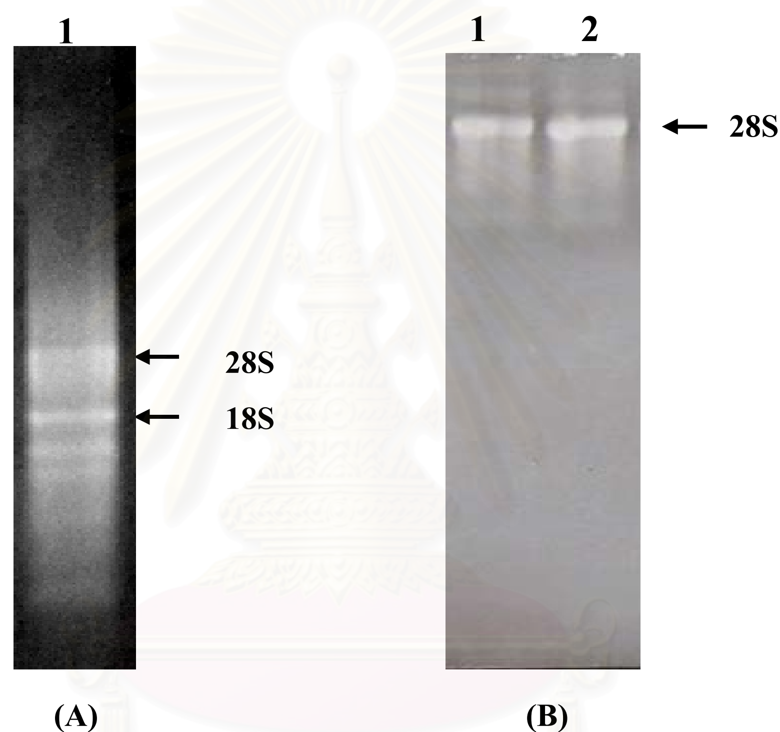

Figure 4.1. Total RNA extracted from HPGs of forager bees was electrophoresed on 1.2% (w/v) agarose gel (A) and formaldehyde gel (B).

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4.2 Partial cDNA sequence

According to RT - PCR amplification, all primers worked at the same condition as mentioned in Materials and Methods. The PCR product of 850 bp was obtained from FW2 and R3 primers (Fig. 4.2A). The product of 750 bp was amplified by FW2 and oligo dT primers (Fig. 4.2B). Also, the product of 220 bp was amplified from FW3 and R3 primers (Fig. 4.2C). Those PCR products were sequenced by Bioservice unit, Thailand (Appendix I).

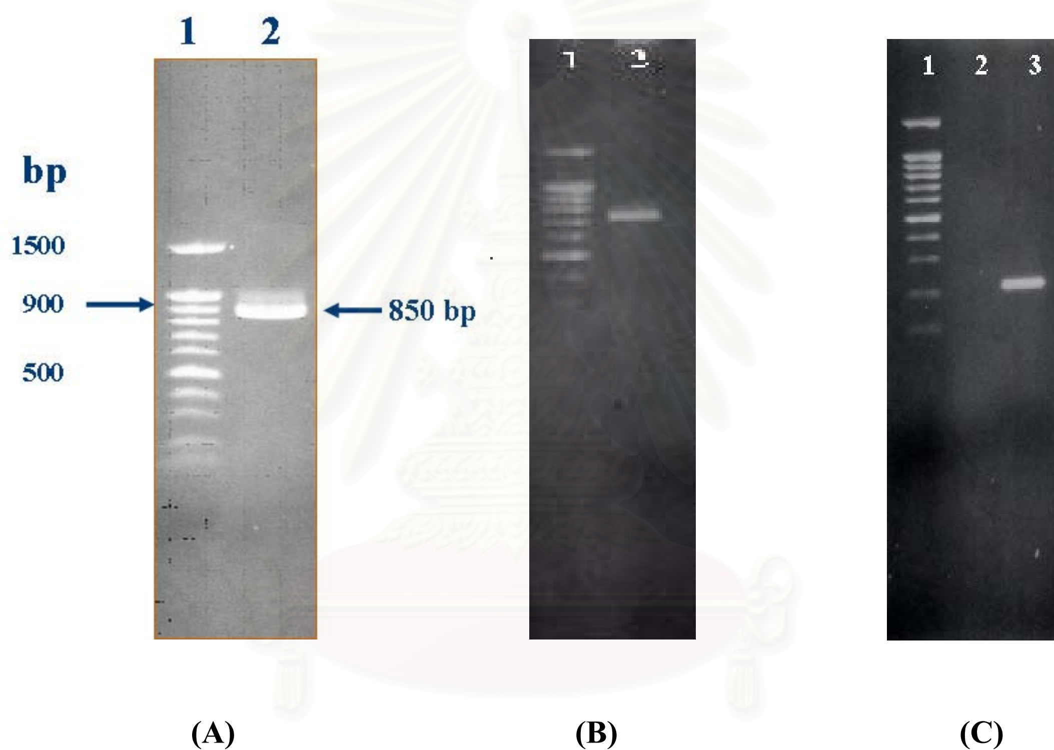

Figure 4.2. The RT - PCR product of AG from HPGs. Lanes 1 in all figures contained 100 bp ladder marker. Lane 2 contained PCR product amplified by FW2 and R3 primers (A) and PCR product amplified by FW2 and oligo dT primers (B). Lane 3 contained PCR product amplified by FW3 and R3 primers (C).

Control experiments were performed by using primers specific to 28S RNA and Elongation factor (EF) genes. The sizes of 350 bp and 100 bp RT - PCR products were obtained, respectively (Fig. 4.3).

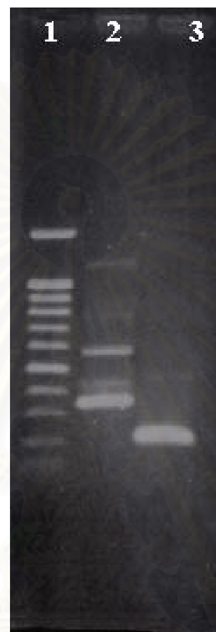

Figure 4.3. Control primers for RT - PCR amplification.

Lane 1: 100 bp ladder marker

Lane 2: product amplified by 28S rRNA primers

Lane 3: product amplified by Elongation factor (EF) primers

The sequences of all above RT - PCR products were shown in Fig. 4.4. The obtained cDNA length of *AG* is 1,740 bp. In addition, the derived amino acid sequence (567 amino acids) is obtained and represented in Fig. 4.5.

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```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50         60
ATGAAGGCGA TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC AGCATGGAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        70         80         90        100        110        120
CCGCTCCCTG AAAACTTGAA GGAGGACTTG ATCGTGTATC AGGTCTACCC AAGAAGCTTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       130        140        150        160        170        180
AAGGATAGCA ATGGAGATGG TATTGGTGAT ATCGAAGGTA TTAAACAAAA ATTGGACCAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       190        200        210        220        230        240
TTTCTCGAAA TGGGCGTCGA TATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       250        260        270        280        290        300
GATTTTGGTT ATGACATTTC GAATTACACC GATGTTCATC CCATATTTGG CACCTTATCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       310        320        330        340        350        360
GACTTAGATA ACTTAGTTAA TGCTGCACAT GAGAAGGGAC TGAAGATAAT CTTGGATTTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       370        380        390        400        410        420
GTTCCGAATC ATACATCTGA TCAACATGAA TGGTTCCAGC TGAGTTTGAA AAACATTGAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       430        440        450        460        470        480
CCTTATAACA ACTATTATAT TTGGCATCCA GGAAAAATTG TAAATGGTAA ACGTGTTCCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       490        500        510        520        530        540
CCAACTAATT GGGTAGGCGT ATTTGGTGGA TCAGCTTGGT CATGGCGAGA AGAACGACAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       550        560        570        580        590        600
GCATATTATC TGCATCAATT TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       610        620        630        640        650        660
GTACTAGATG ATATGCAAAA CGTTCTCAGA TTCTGGCTGA GAAGAGGACT CGATGGTTTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       670        680        690        700        710        720
AGAGTAGATG CTTTGCCTTA CATTTGCGAG GACATGCGAT TCTTAGACGA ACCCCTATCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       730        740        750        760        770        780
GGTGAAACAA ATGATCCCAA TAAAACCGAG TACACTCTCA AGATCTACAC TCACGATATC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       790        800        810        820        830        840
CCAGAAACCT ACAATATAGT TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       850        860        870        880        890        900
AAACACATGC TTATCGAGGC ATACACGAAT TTATCGATGA CGATGAAATA TTACGATTAC
```

Figure 4.4. The cDNA sequence of alpha – glucosidase obtained by RT – PCR.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       910       920       930       940       950       960
GGAGCAGATT TTCCCTTTAA TTTTGCATTC ATCAAGAATG TCTCTAAGGA TTCAAATTCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       970       980       990      1000      1010      1020
TCAGACTTCA AGAAATTGGT CGATAATTGG ATGATATACA TGCCAGCAGA TGGTATTCCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1030      1040      1050      1060      1070      1080
AACTGGGTGC CCGGAAATCA CGATCAATTG AGATTGGTGT CGAGATTTGG AGAGGAGAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1090      1100      1110      1120      1130      1140
GCCCGTATGA TCACCGCGAT GTCGCTTTTG CTGCCAGGTG TTGCCGTGAA TTACTACGGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1150      1160      1170      1180      1190      1200
GATGAAATTG GTATGTCGGA TACTTATATC TCGTGGGAGG ACACGCAGGA TCCACAGGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1210      1220      1230      1240      1250      1260
TGCGGTGCCG GCAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG AACGCCATTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1270      1280      1290      1300      1310      1320
CAATGGGACG ACTCAGTTTC TGCTGGATTT TCCTCAAGCT CTGATACCTG GCTTCGTGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1330      1340      1350      1360      1370      1380
AACGAAAATT ACAAGACTAT CAATTTAGCT GCTGAAAAGA AGGACAAGAA CTCGTTCTTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1390      1400      1410      1420      1430      1440
AATATGTTCA AGAAATTTGC AATGCTGAAA AAATCGCCAC ACTTTAAAGA GGCCAATTTA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1450      1460      1470      1480      1490      1500
AATACGAGGA TGCTGAACGA CAGTGTTTTC GCATTCTCTA GGGAAACCGA AGAAAATGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1510      1520      1530      1540      1550      1560
TCTCTTTACG CAATATTGAA CTTCTCGAAC GAGGAACAAA TCGTGGACTT GAAAGCGTTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1570      1580      1590      1600      1610      1620
AATAACGTGC CGAAAAAATT GAATATGTTT TACACCATTT TTAACTCTGA TATAAAGTCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1630      1640      1650      1660      1670      1680
ATCTCCAACA ATGAACAAAT AAAAGTTTCT GCTTTAGGAT TTTTGATCTT AATTTCTCAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1690      1700      1710      1720      1730      1740
GATGCTAAAT TTGGAAATTT TTAATATCTC CCTGAATATG TCTATTCTTT GAAGCGGCGA
```

Figure 4.4. (continued)

```
---------+---------+---------+---------+---------+
MKAVIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD  50
IEGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS 100
DLDNLVNAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP 150
GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV 200
VLDDMQNVLR FWLRRGLDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE 250
YTLKIYTHDI PETYNIVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY 300
GADFPFNFAF IKNVSRDSNS SDFKKLVDNW MTYMPPSGIP NWVPADHDQL 350
RLVSRFGEEK ARMITAMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG 400
CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYKTINLA 450
AEKKDKNSFF NMFKKFAMLK KSPYFKEANL NTRMLNDSVF AFSRETEENG 500
SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YTIFNSDIKS ISNNEQIKVS 550
ALGFFLLISQ DAKFGNF                                     567
```

Figure 4.5. The amino acid sequence of alpha – glucosidase deduced from the cDNA sequence. The double underline amino acid sequences were different from amino acid of *AG* in *A. mellifera* recorded in Genbank.

4.3 Sequence homology and phylogenetic trees

The cDNA sequences alignment were compared with the sequences of *AG* in *A. mellifera* (D79208), maltase 1 in *A. mellifera* (XM_393379), *Drosophila melanogaster* CG1493 _ PA (NM_135678), sucrose specific enzyme II of the PTS (*Scr*A) and dextran glucosidase (*dexB*) genes in *Lactobacillus sakei* (AF401046), and *Culicoides sonorensis* clone CsMAL1 maltase (AY603565). As shown in Fig. 4.6, the multiple alignments were compared for homology. The deduced amino acid sequences of *AG* were aligned with other organisms (Fig. 4.7). The consensus sequences are obtained.

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Am.AG.nuc      ATGAAGGCAG TAATCGTATT TTGCCTT--A TGGCATTGTC CATTGTGGAC
```

```
AC.AG          ATGAAGGCGA TAATCGTATT TTGCCTT--A TGGCATTGTC CATTGTGGAC
Cs.maltase     ATTTAAAAAA TTAACA-ATT TTACTATCAA TTGCATGTTC TGTATTGGCA
Dm.CG.nuc      ATTTTGAGTG TGGGCCTAGT AGGCAT---A TTGGCCCATA AGCACCAGTC
Am.maltase1    ATGAAGAGCC TCGTCGTGGT CGTACTTCTG CT-CGCGGTC GGCCTTGGCG
Ls.ScrA.nu     ACGTTAGGTG CAGTCGGTAT GGGGATT-TT TGGCCTGGGT TATTCAGCAA
Clustal Co     *              *    *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                       60         70         80         90        100
Am.AG.nuc      GCAGCATGGA -AGCCGCTCC CTGAAA-ACT TGAAGGAGGA CTTGAT-CGT
AC.AG          GCAGCATGGA -AGCCGCTCC CTGAAA-ACT TGAAGGAGGA CTTGAT-CGT
Cs.Maltase     GCA-CCTGAA -GGTGCACGT GAAAAA-GAT TGGTGGGAAA TTGGAAACTT
Dm.CG.nuc      AAA----GGA -GCTGGATGC GAAATATAAT TGGTGGCAGC ACGAGGTCTT
Am.maltase1    CCG------- --GCCAAAAC AACAAG-GGT TGGTGGAAGA ACGCGATCTT
Ls.ScrA.nu     TTGTCTTAAC TGGCTTACAT CAAAGCTTCC CGGCAATTGA AACGACACTT
Clustal Co                              *        *                * *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                      110        120        130        140        150
Am.AG.nuc      GTATCAGGTC TACCCGA--- -GAAGCTTCA AGGATAGCAA TGGAGATGGT
AC.AG          GTATCAGGTC TACCCAA--- -GAAGCTTCA AGGATAGCAA TGGAGATGGT
Cs.Maltase     TTATCAAGTC TATCCAC--- -GAAGTTTCA TGGATTCTGA TGGCGATGGT
Dm.CG.nuc      CTATCAGATC TATCCGA--- -GATCCTTTC AGGACAGCAA TGGTGATGGT
Am.maltase1    CTATCAGGTA TATCCCC--- -GCAGTTTCA TGGATTCCAA TAGTGATGGC
Ls.ScrA.nu     TTGGCAGATA TTGCCAAAAC TGGTGGATCG TTTATTTTTC CCGTTGCAGC
Clustal Co      *  **  *  *  **       *     *      *         *     *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                      160        170        180        190        200
Am.AG.nuc      ATTGGTGATA TCGAAGGTAT TAAAGAAAAA TTGGATCAT- TTTCTCGAAA
AC.AG          ATTGGTGATA TCGAAGGTAT TAAACAAAAA TTGGACCAT- TTTCTCGAAA
Cs.Maltase     GTTGGCGATT TGAAAGGAAT TTCAGAAAAA GTCGGTTAT- TTAAAGGAAA
Dm.CG.nuc      ATTGGTGATC TTCAAGGTAT TACTTCTAGG CTACAGTAC- TTCAAGGATA
Am.maltase1    ATCGGGGATT TAAAAGGTAT TAAGGATAAG CTTTCACAC- TTCATCGAAT
Ls.ScrA.nu     GATGGCAAAT ATTGCTCAAG GGGCTGCAAC TTTCGCTGTA TTCTTCGTTA
Clustal Co        **  *           *         *    *         **    *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                      210        220        230        240        250
Am.AG.nuc      TGGGGGTCGA CATGTTTTGG TTATCCCCTA TTTATCCAAG CCCTATGGTC
AC.AG          TGGGCGTCGA TATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC
Cs.Maltase     TCGGCATGGA TGGTGTTTGG CTTTCACCGA TTTTTGATTC ACCTATGGCA
Dm.CG.nuc      CGGGCATCAC GTCCGTATGG TTGAGTCCCA TTTATGAGTC ACCAATGGTA
Am.maltase1    CTGGAATAAC AGCGATATGG TTATCACCAA TTAATCGAAG TCCTATGGTA
Ls.ScrA.nu     CTAAGAATAA ACAACAAAAG TCATTAACGA CTTCTGCTGG GATTTCTGC-
Clustal Co                         *        * *  *  *             *
```

Figure 4.6. The multiple alignment of the nucleotide sequences of *AG* in *A. cerana* and that in other organisms. '*' Residues in that column are identical in all sequences in the alignment.

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                      260        270        280        290        300
Am.AG.nuc      GATTTTGGTT ACGACATTTC GAATTACACC GACGTTCATC CCATATTTGG
```

```
AC.AG        GATTTTGGTT ATGACATTTC GAATTACACC GATGTTCATC CCATATTTGG
Cs.Maltase   GATTTTGGTT ATGACATTTC AAATTTCACC AAAGTCTTCC CTCAATTCGG
Dm.CG.nuc    GACTTTGGAT ACGATATATC TAACTATACA AATATACAGC CGGAATATGG
Am.maltase1  GATTTTGGAT ACGATATATC TGACTTTAAA GATGTAGATC CAATATTTGG
Ls.ScrA.nu   GATGTTGGGA ATTACTGAAC CAGCATTATT TGGGGTTAAT TTAAAATTGA
Clustal Co   **  ****   *  *     *        *                  *   *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    310        320        330        340        350
Am.AG.nuc    CACCATATCA GACTTAGATA ATCTAGTCAG TGCTGCACAT GAGAAAGGAT
AC.AG        CACCTTATCA GACTTAGATA ACTTAGTTAA TGCTGCACAT GAGAAGGGAC
Cs.Maltase   AGACTTGTCT TCAATTGATG AACTTGTAGC GGAATTCAAT AAAAAAGATA
Dm.CG.nuc    CACCCTTGAG GACTTTGACG CCTTGATAGC CAAGGCCAAT GAACTGGGCG
Am.maltase1  TACTATAAAA GATCTTGAAG ATCTCACTGC AGAAGCGAAG AAACAGAATT
Ls.ScrA.nu   AGTTTC--CA TTCTTTATTG GTTTAATTGC ATCAGGAATC TCATCGTTTA
Clustal Co                  *         *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    360        370        380        390        400
Am.AG.nuc    TGA--AGATA ATCTTGGATT TCGTCCCGAA TCATACATCT GATCAACACG
AC.AG        TGA--AGATA ATCTTGGATT TCGTTCCGAA TCATACATCT GATCAACATG
Cs.Maltase   TGA--AACTC ATTCTGGACT TTGTTCCAAA TCATACAAGT GACCAATGTG
Dm.CG.nuc    TGA--AAGTT ATTTTGGACT TTGTTCCCAA TCACAGCTCA AATAAGCATC
Am.maltase1  TAA--AGGTT ATTCTAGATC TTGTCCCTAA TCATACTTCT GATCAACATA
Ls.ScrA.nu   TTATTGGTTT ATTACATGTT TTATCAGTAT CAATGGGACC TGCAGGAATT
Clustal Co   * *     *  **         *  *    *    *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    410        420        430        440        450
Am.AG.nuc    AATGG-TTCC AG-------- --TTGAGTTT GA-----AAA --ACATTGAA
AC.AG        AATGG-TTCC AG-------- --CTGAGTTT GA-----AAA --ACATTGAA
Cs.Maltase   AGTGG-TTCA AAA------- AATCAATTCA GC-----GTG --ATCCTGAG
Dm.CG.nuc    CCTGG-TTCA TAA------- AGTCAGTAGC CC-----GAG --AGCCAGGG
Am.maltase1  AATGG-TTCC AAATGAGTAT AAATAATACT AATAATAATA --ATACTAAT
Ls.ScrA.nu   ATTGGGTTTA TTGCGATTGC ACCTAAGAGC ATCCCTAGTT TTATGATGGG
Clustal Co     *** **                                      *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    460        470        480        490        500
Am.AG.nuc    CCTTATAACA ACTATTACAT TTGGC---AT CCAGGAAA-- --AATTGTAA
AC.AG        CCTTATAACA ACTATTATAT TTGGC---AT CCAGGAAA-- --AATTGTAA
Cs.Maltase   ---TACAATG ATTACTATAT TTGGC---AT CCGGGTAAGC CAAATCCTGA
Dm.CG.nuc    ---TACGAGG ATTTCTATGT GTGGG---AG GATGGTATT- --CTCCTGGA
Am.maltase1  AAATATAAAG ATTATTACAT ATGGGTTGAT CCTGTCAAAG ACGATAAAGG
Ls.ScrA.nu   AGCTATTATT AGTTTCGTAA TTGCCTTTGT GGGGACATAC TTATACGGTA
Clustal Co      **  *   * *         **           *  *
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Figure 4.6. (continued)

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             ....|....| ....|....| ....|....| ....|....| ....|....|
                    510        520        530        540        550
```

```
Am.AG.nuc     --AT---GGC AAACGTGTTC CACCAACTAA TTGGGTAGGC GTGTTTGGTG
AC.AG         --AT---GGT AAACGTGTTC CACCAACTAA TTGGGTAGGC GTATTTGGTG
Cs.Maltase    TGGT---GGT CGAAATTTAC CCCCAACTAA TTGGGTAAGT GCCTTCAGAA
Dm.CG.nuc     GAAC---GGA ACTCGTGTGC CGCCCAACAA TTGGCTGTCG GTGTTCTCCG
Am.maltase1   AAATCCAATT AAAGACAAAT ATCCTAATAA TTGGCTTAGT GTATTCAATG
Ls.ScrA.nu    AAAAGGCAAT GAAGACAACT GAAGAAGAAA TAATCAATGA AGCACCAGCT
Clustal Co                               *  ** *

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     560        570        580        590        600
Am.AG.nuc     GATCAGCTTG GTCGTGGCGG GAAGAACGAC AGGCATATTA TCTGCATCAA
AC.AG         GATCAGCTTG GTCATGGCGA GAAGAACGAC AGGCATATTA TCTGCATCAA
Cs.Maltase    GTAGTGCCTG GGAATGGAAC GAAGAACGTG GCGAATATTA TTTACATCAA
Dm.CG.nuc     GATCCGCTTG GATGTGGAAC GATGAGAGGC AGCAGTACTA TCTCAGGCAG
Am.maltase1   GTACAGGATG GACTTTCCAC GAGGGTAGGA AACAATTTTA TTTCCATCAA
Ls.ScrA.nu    ACCCCAGA-A GTAGTGGAGA GATTACAAGA TGAAAAGATT AGTGCACCAG
Clustal Co               *   *      **                 *    *    **

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     610        620        630        640        650
Am.AG.nuc     TTTGCACCAG AACAACCAGA TCTAAATTAC TA--TAATCC AGTT---GTA
AC.AG         TTTGCACCAG AACAACCAGA TCTAAATTAC TA--TAATCC AGTT---GTA
Cs.Maltase    TTTTTGGCAC AACAACCCGA TTTGAATTAC CG--CAATCC AAAA---GTG
Dm.CG.nuc     TTCACTTATG GACAACCCGA TTTGAACTAC CG--AAATCC CGCC---GTG
Am.maltase1   TTTTATAAGC AACAACCAGA CTTGAACTAC AG--AAACTC GGAT---GTG
Ls.ScrA.nu    TTACCGGACG AATTGTTGAC TTAGCATCAG TACCTGATCC AGTTTTTGCA
Clustal Co    **          *              *  *        *  *        *

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     660        670        680        690        700
Am.AG.nuc     CTGGATGATA TGC-AAAATG TTCTCAGATT CTGGC--TGA GAAGGGGATT
AC.AG         CTAGATGATA TGC-AAAACG TTCTCAGATT CTGGC--TGA GAAGAGGACT
Cs.Maltase    GTTGAAACAA TGA-AAAACG TTTTAAGATT CTGGC--TTA GCAAAGGTAT
Dm.CG.nuc     ATTAAGGCCA TGG-ATGATG TGATGCTCTT CTGGC--TAA ACAAGGGTAT
Am.maltase1   AGAGAAGAGA TGA-AGAATA TAATGAAATT TTGGT--TGG ATAAAGGAAT
Ls.ScrA.nu    AGTGAAGCAA TGGGAAAAGG CATTGCGATT ATGCCAACTT CTCAGGATGT
Clustal Co        *    * **  *  *      *    **  **             *   *

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     710        720        730        740        750
Am.AG.nuc     TGATGGTTTC AGAGTAGATG CTCTGCCTTA CATTTGCGAA GACATGCG--
AC.AG         CGATGGTTTC AGAGTAGATG CTTTGCCTTA CATTTGCGAG GACATGCG--
Cs.Maltase    CAATGGATTC AGAATTGATG CGGTACCATA TTTGTTTGAA GTGGGACC--
Dm.CG.nuc     TGCCGGCTTC CGCATCGATG CCATTATATA TATTTACGAG GATGCTCA--
Am.maltase1   CGATGGATTC CGCATAGATG CTGTACCACA TTTATTCGAA AGCGCTAACA
Ls.ScrA.nu    ACTTGCACCA GTTACCGGTG TGATAACAAT TGCGGCTAAT ACTGGTCACG
Clustal Co        *            * **    *               *
```

Figure 4.6. (continued)

```
              ....|....| ....|....| ....|....| ....|....| ....|....|
                     760        770        780        790        800
```

```
Am.AG.nuc       -ATTCTTAGA CGAACCTCTA TCAGGT--GA AACAAATGAT CCCAATAAAA
AC.AG           -ATTCTTAGA CGAACCCCTA TCTGGT--GA AACAAATGAT CCCAATAAAA
Cs.Maltase      -AGATGCGAA TGGAAATTAT CCAGAT--GA AATTGA-AAC CCATGCATGC
Dm.CG.nuc       -ACTGAGGGA TGAGCCTCCG AGTGGC--AC TACCGATGAT CCAAATAA--
Am.maltase1     TATCGTTAGA TGAACCACCT TTGGGT--AA AAATCTCAAC TTAAGTCT--
Ls.ScrA.nu      CATACGGGAT AAAATCGGAT GATGGTGCAG AAGTGCTAAT TCATATTGGT
Clustal Co       *                       *        *      *


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
Am.AG.nuc       CCGAGTACAC T--CTCAAGA TC-------- ------TACA CTCACGATAT
AC.AG           CCGAGTACAC T--CTCAAGA TC-------- ------TACA CTCACGATAT
Cs.Maltase      TCAGATCCTT TATCTCAATG TTACTTGTAT CACGATTACA CTCAAAACAG
Dm.CG.nuc       TGAGGCCTAC TTGAGCCACA TC-------- ------TATA CCAGAAATCA
Am.maltase1     CCACGCTTCT TTAAATCACA CT-------- ------TTAA CGAAAGATCA
Ls.ScrA.nu      TTAGATACAG TTAATTTAAA TGGT------ -ATAGGTTTT GAAAAGATTG
Clustal Co                 *      *                    *          *


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
Am.AG.nuc       CCC--AGAAA CCTACAATGT AGTTCGCA-- AATTTAGAGA TGTGTTAGAC
AC.AG           CCC--AGAAA CCTACAATAT AGTTCGCA-- AATTTAGAGA TGTGTTAGAC
Cs.Maltase      GCC--TGAAA CTTTTGAAAT GGTCACGG-- AATGGAGAGC GACTTTGGAG
Dm.CG.nuc       GCC--TGAGG ATTACGGTCT ACTTCAGC-- ATTGGCGGCA ACTTCTGGAT
Am.maltase1     ACC--CGAGA CTTACGAATT GGTAAAAG-- AATGGCGAGA TTTTGTGGAC
Ls.ScrA.nu      TCCAACAGGG ACAACATGTT AGCGAAGGCG ATTTATTAGG TCATTTTGAT
Clustal Co       **                 *            * *             * **


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
Am.AG.nuc       GAATTCCCGC AACCAAA--- -------ACA CATGCTT--- -----ATCGA
AC.AG           GAATTCCCGC AACCAAA--- -------ACA CATGCTT--- -----ATCGA
Cs.Maltase      GAATTTAAAC AAAAGAATGG AGGACCAACA AGAGTTTTAA TG---GTAGA
Dm.CG.nuc       AATTATACAG CTAACCACGA TGGGCCATTG AGGATAATGA TG---ACCGA
Am.maltase1     AACTATGCAG AAGAAAATAA GCGGGATGAA ATAGTACTTT TG---ACAGA
Ls.ScrA.nu      ATTGATAAGA TTAAACAAGC CGGGCTAACA CCGCTAACAA TGACTATTGT
Clustal Co                       *                                  *


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
Am.AG.nuc       GGCATACACG AA----TTTA TCGATGACGA TGAAATATTA CGATTA----
AC.AG           GGCATACACG AA----TTTA TCGATGACGA TGAAATATTA CGATTA----
Cs.Maltase      AGCTTATGCT CC----ATTA ACAAAAGTAA TTCAAATTTA TGGTCAAAAT
Dm.CG.nuc       GGGTTATGCT TC----GGTG TCGCAACTAA TGGAATACTA TGAAGATTCG
Am.maltase1     GGCGTATTCT TC----TTTA GAGAACACTC TCAAATATTA CGAAGT----
Ls.ScrA.nu      GACGAATACA GCGGGATATG CACAAGTTGA TCCGCTTTTA ACAGTCGACA
Clustal Co           *  *          *              *       **
```

Figure 4.6. (continued)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1010       1020       1030       1040       1050
```

```
Am.AG.nuc      ---------- -CGGAGCAGA TTTTCCCTTC AATTTTGCAT TCATCAAGA-
AC.AG          ---------- -CGGAGCAGA TTTTCCCTTT AATTTTGCAT TCATCAAGA-
Cs.Maltase     GGACAGCTAA ATGGAGCTCA AATTCCATTT AATTTCGAGT TCTTGAATA-
Dm.CG.nuc      AATGGTGTAC AGGGCCCCCA GTTTCCCTTC AACTTTGACT TCATCACCG-
Am.maltase1    ---------- -TGGTTCAAA TGTTCCCTTC AATTTTAAAT TTATAACAG-
Ls.ScrA.nu     AGGCTGCTAT GCAAGGCGAA GAAATTATTC AATTACACGC TAAAAAGGAT
Clustal Co                      *  *       **   ** *       *    *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                     1060       1070       1080       1090       1100
Am.AG.nuc      -ATGTTTCTA GGGATTCAAA TTCATCAGAC TTCAAAAAA- --TTGGTCGA
AC.AG          -ATGTCTCTA AGGATTCAAA TTCATCAGAC TTCAAGAAA- --TTGGTCGA
Cs.Maltase     -ATTTGGGAG CCGTAAGTAA TGCTCGTGAT TTCAAAGAC- --GTAATTGA
Dm.CG.nuc      -AACTGAATG CCAATTCGAC AGCTGCGGAC TTTGTCTTC- --TATATCTC
Am.maltase1    -ATGCAAATT CATCTTCCAC GCCAGAACAA TTTAAAGTA- --ATTATAGA
Ls.ScrA.nu     TAAGGGGTAG TTACATGCAA ACTAACTGGT GGCAAAATGC AGTATTTTAT
Clustal Co      *                 *                              *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                     1110       1120       1130       1140       1150
Am.AG.nuc      TAATTGGATG ACGTACATGC CACCAAGTGG TATTCCTAAC TGGGTGCCCG
AC.AG          TAATTGGATG ATATACATGC CAGCAGATGG TATTCCTAAC TGGGTGCCCG
Cs.Maltase     CAATTATCTC AGCACAATCC CAGAAGGAGC AACACCAAAT TGGGTTCAAG
Dm.CG.nuc      CAGGTGGCTC ATCTATATGC CACATGGTCA TGTGGCCAAC TGGGTGATGG
Am.maltase1    CAATTGGATA AAAGGAACGC CCCAAAATAA TGTTCCAAAT TGGGTGATGG
Ls.ScrA.nu     CAAGTCTATC CAAGAAGT-T TTCAAGATAG TAATGGAGAT GGAATTGGTG
Clustal Co      *  *   *                                *   *  *    *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                     1160       1170       1180       1190       1200
Am.AG.nuc      GAAATCACGA TC-------- AATTGAGATT GGTGTCGAGA TTTGGAGAGG
AC.AG          GAAATCACGA TC-------- AATTGAGATT GGTGTCGAGA TTTGGAGAGG
Cs.Maltase     GAAATCACGA TC-------- AACATCGATC AGCATCACGA CTCGGTCCAC
Dm.CG.nuc      GAAATCACGA CA-------- ATCCTCGAGT GGCATCACGA TTCGGTGAGA
Am.maltase1    GAAACCATGA TC-------- GAGTTCGTGT CGGTACACGT TATCCTGGTA
Ls.ScrA.nu     ATATTCAAGG TATTATTCAA AGATTAGATT ACCTAGCTGA TCTGGGTGTA
Clustal Co       *  ** *                   *            *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                     1210       1220       1230       1240       1250
Am.AG.nuc      AGAAGGCCCG TATGATCACC ACGATGTCGC TTTTGC--TG CCAGGTGTTG
AC.AG          AGAAGGCCCG TATGATCACC GCGATGTCGC TTTTGC--TG CCAGGTGTTG
Cs.Maltase     AAAAAGCTGA TGCAGTTAAT ATGTTACTTC AAGTTC--TT CCCGGAGCTG
Dm.CG.nuc      AATCTGTGGA CGCCATGAAT ATGCTGCTGA TGACAT--TG CCAGGAATTG
Am.maltase1    GGGCGGATCA CATGATAA-- -TGTTGGAGA TGATTT--TG CCTGGAGTCG
Ls.ScrA.nu     AATGCAATTT GGCTATCACC AGTTTATCAA TCCCCTAATG TTGATAATGG
Clustal Co                     * *       *              *           *
```

Figure 4.6. (continued)

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                     1260       1270       1280       1290       1300
```

```
Am.AG.nuc      CCGTGAATTA CTA--CGGTG ATGAAATTGG TATGTCGGAT ---ACTTATA
AC.AG          CCGTGAATTA CTA--CGGTG ATGAAATTGG TATGTCGGAT ---ACTTATA
Cs.Maltase     CAGTCACTTA TTA--TGGTG AAGAACTTGC AATGGAAGAC ---GTTTTCG
Dm.CG.nuc      GTATTACTTA TAA--TGGCG AGGAGTTGGG CATGACTGAC TACAGGGACA
Am.maltase1    CGGTCACGTA TTA--TGGAG AAGAAATCGG TATGGTGGAT ----------
Ls.ScrA.nu     CTATGATATT TCAGATTATC AGGCAATTAA TCCGGAATAT GGTTCTATGG
Clustal Co        * *  *    *        * *   *       *    *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                       1310       1320       1330       1340       1350
Am.AG.nuc      TC-TCGTGGG AGGATACGCA GGATCCGCAG GGATGCGGCG CCGGTAAAGA
AC.AG          TC-TCGTGGG AGGACACGCA GGATCCACAG GGATGCGGTG CCGGCAAAGA
Cs.Maltase     TT-CCATGGT CTCGTACTGT CGATCCACAA GCATGTACAA CAGATCCAAA
Dm.CG.nuc      TC-AGCTGGA GCGATACGGT GGATCAGCCC GCTTGTGAGG CTGGAATCGA
Am.maltase1    ---------A ACACTACGAT ATATAAATAT G-ATGTACG- -------TGA
Ls.ScrA.nu     TGGATATGGA GCAGTTAATT GAAGCGGCGA AGATTCGTAA --GATTAAAA
Clustal Co                             *           *                *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                       1360       1370       1380       1390       1400
Am.AG.nuc      AAACTATCAA ACGATGTCGA GAGATCCCGC GAGA---ACG CCATTCCAAT
AC.AG          AAACTATCAA ACGATGTCGA GAGATCCCGC GAGA---ACG CCATTCCAAT
Cs.Maltase     TATTTTCCAT GCCAAGTCAC GTGATCCCGC AAGA---ACA CCCATGATTT
Dm.CG.nuc      CAACTACAAG ACGATTTCTA GAGATCCTGA GCGA---ACT CCCATGCAAT
Am.maltase1    TGGTTGTCGT ACACCATTCC AA-----TGG GATA---ACT CCATTAATGC
Ls.ScrA.nu     TTGTTATGGA CTTAGTTGTT AATCATACAA GTGACCAACA TCCATGGTTT
Clustal Co         *            *                  *   **   *  *


               ....|....| ....|....| ....|....| ....|....| ....|....|
                       1410       1420       1430       1440       1450
Am.AG.nuc      GGGACGACTC AGTTTCTGCT GGATTTTCCT CAA-GCTCTA AT---ACCTG
AC.AG          GGGACGACTC AGTTTCTGCT GGATTTTCCT CAA-GCTCTG AT---ACCTG
Cs.Maltase     GGACTTCACA AAAAAACGCA GGATTTT--- CAA-GTTCAA ATTACACATG
Dm.CG.nuc      GGAGTAGTGA TGTGAATGCA GGATTCTCCT C----CGCCG ATCGCACTTG
Am.maltase1    AGGCTTTAGT AAAATCGCTG AAAATTT--- ----GCTTGA AAAG-AATTG
Ls.ScrA.nu     TTAGAAGCAC GAAAATCAAA AGATAATCCG TATCGTGATT TTTATATTTG
Clustal Co                             *   *                    *  **


               ....|....| ....|....| ....|....| ....|....| ....|....|
                       1460       1470       1480       1490       1500
Am.AG.nuc      GCTT--CGTG TCAACGAAAA TTACAAGACT G-TCAATCT- -AGCTGCTGA
AC.AG          GCTT--CGTG TCAACGAAAA TTACAAGACT A-TCAATTT- -AGCTGCTGA
Cs.Maltase     GCTT--CCAA CTGGACCAGA TTATCGCAAA A-ATAATGTT GAAGTGCAGC
Dm.CG.nuc      GTTG--CCTG TCAATCCGAA TTATAAGGAA C-TTAATCTT CGGAATCAGC
Am.maltase1    GCTA--CCTG TTCATACATC GTACAAAAGT GGACTAAATT TGGAGCAAGA
Ls.ScrA.nu     GCGAGACCCT GCAACCGATG GTAGTGTTCC GAATGATTTA CAAAGTAATT
Clustal Co     *     *                **             *  *
```

Figure 4.6. (continued)

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550
```

```
Am.AG.nuc    AAAGAAGGAC AA---GAACT CGTTCTTCAA TATGTTCAAG AAATTTGCGT
AC.AG        AAAGAAGGAC AA---GAACT CGTTCTTCAA TATGTTCAAG AAATTTGCAA
Cs.Maltase   GTAGTCAGAG AG---GCAGT CA--CTTGAA TATCTTTAAA AAGTTGACTC
Dm.CG.nuc    AGCAGGCGAG GC---GAAGT CA--TTACAA GATCTATCAG TCCCTTCTGA
Am.maltase1  GAAAAAAGAT AG---TATTT CTCATTATCA TCTTTATACC AACTTGACCG
Ls.ScrA.nu   TTAAAGGATC AGCTTGGGCG TTTGATGCGG TTACTGGGCA ATATTATTTA
Clustal Co                              *         *          *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1560       1570       1580       1590       1600
Am.AG.nuc    CGCT------ -GAAAAAATC GCCATACTTT AAAG-AGGCC AATTTAAA--
AC.AG        TGCT------ -GAAAAAATC GCCACACTTT AAAG-AGGCC AATTTAAA--
Cs.Maltase   AACT------ -TCGTAAG-C AAGACATTTT GATGTATGGC ACTTATGA--
Dm.CG.nuc    AGCT------ -CAGACAACT GCCA-GTTCT GAAGAACGGA TCCTTTGT--
Am.maltase1  CTTT------ -AAGAAAG-A GAGATGTGTT GAAAAAAGGA AACTTTAC--
Ls.ScrA.nu   CATTTTTATG CGAAAGAACA ACCGGATTTA AATTGGCAAA ATCCTAAAGT
Clustal Co      *             *                *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1610       1620       1630       1640       1650
Am.AG.nuc    TACGAGGATG CTGAAC--GA CAATGTTTTC GCATTCTCTA GG-GAAACCG
AC.AG        TACGAGGATG CTGAAC--GA CAGTGTTTTC GCATTCTCTA GG-GAAACCG
Cs.Maltase   TAGTTACTTG GCAAAT--GA TGACGTTTTG GTGATTAAAC GT-GAAATTG
Dm.CG.nuc    TCCAGAAGTG GTTAAT--CG CAGGGTCTTC GCTTTCAAGC GA-GAACTGA
Am.maltase1  TATAGAAATT TTAAAC--AA AACTGTTCTG GCTGTCGTGC GACAAAGCGA
Ls.ScrA.nu   TAGAGAAGCT GTCTACCAGA TGATGACTTG GTGGCTTCAA AAAGGGATTG
Clustal Co   *              *          *   *  *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1660       1670       1680       1690       1700
Am.AG.nuc    --------AA GATAATGGAT CTCTTTACGC AATATTGAAC TTCTCGAACG
AC.AG        --------AA GAAAATGGAT CTCTTTACGC AATATTGAAC TTCTCGAACG
Cs.Maltase   --------AG AATAATCGAA CTTTGATTGC TGTCCTTAAC TTGGGTTTCA
Dm.CG.nuc    --------AG AACGAGCACA CTCTGCTGAC CATTGTGAAC GTGAGCAACC
Am.maltase1  --------AG AAGAAGCGGT ATCTCTTT-- --TGATCAAC TTCTCTAAAA
Ls.ScrA.nu   GTGGTTTTAG GATGGACGTT ATTGATTTG- -ATAGGGAAG GAACCTGACC
Clustal Co           *   *          *           *    **

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1710       1720       1730       1740       1750
Am.AG.nuc    AGGAACAAAT CGTGGATTTG AAAGCGTT-C AATAACGTGC CGAAA-----
AC.AG        AGGAACAAAT CGTGGACTTG AAAGCGTT-T AATAACGTGC CGAAA-----
Cs.Maltase   CTGAACAAGT CGTCAATTTG AATTTAAA-T GACCGAGATT GGAAAGTTCC
Dm.CG.nuc    GCACTGAACT GGTTGACATC GCGGACTT-T ATAGAACAGC CCAATCGATT
Am.maltase1  ATAATACTAT CGTGGATATA TCAAAGTT-G GTGAACAAAA GAAAT-----
Ls.ScrA.nu   GCAAAATTAA GGAAAACGGA CCGCAATTAC ATGCGTATCT TCAAGAGATG
Clustal Co               *   *                             **
```

Figure 4.6. (continued)

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1760       1770       1780       1790       1800
```

```
Am.AG.nuc     AAATTGAATA TG-TTTTACA ACAATTT--- -TAACT-CTG ATAT-----A
AC.AG         AAATTGAATA TG-TTTTACA CCATTTT--- -TAACT-CTG ATAT-----A
Cs.Maltase    AGAGAGAATG GA-AGTTGCA ACAGCTTCAG TTAACG-CAG GAATGTTCGA
Dm.CG.nuc     GAGTGTCCTT GT-GGCGGGA GTGGACTCG- -CAACA-CCG G-GTGGGGGA
Am.maltase1   AATGCTAAAA TT-TACACAA GCAGCGT--- -AAACT-CCA ATTTGACAGT
Ls.ScrA.nu    AACGCAAGGG TACTTTCACA GTATGATGTA GTAACGGTTG GAGAGACATG
Clustal Co                         *       *      ***

              ....|....| ....|....| ....|....| ....|....| ....|....|
                    1810       1820       1830       1840       1850
Am.AG.nuc     AAGTCCATCT CCAACAATGA AC-AAGTAAA AGTTTCTGCT ------TTAG
AC.AG         AAGTCCATCT CCAACAATGA AC-AAATAAA AGTTTCTGCT ------TTAG
Cs.Maltase    GAGACAACCC GTTGTGACAA GTGAAGTCTA CGTATCAGCT GG--CGTTGG
Dm.CG.nuc     TCGACTTAAG GCCGAGACAA TT-GAATTGG CGCCCAACGA GG--GATTAG
Am.maltase1   AAATCAAACT GTAAATCCAG TGGCTATCAA TATTCCTGGA GATACATCTA
Ls.ScrA.nu    GGGGGCAACA CCCGAAATTG GCCAGATGTA CAGTAATCCT AATCGCCACG
Clustal Co                                 *

              ....|....| ....|....| ....|....| ....|....| ....|....|
                    1860       1870       1880       1890       1900
Am.AG.nuc     GATTTTTCAT C-TTAATTTC TCAAGATG-- CTAAATTT-- ---------G
AC.AG         GATTTTTGAT C-TTAATTTC TCAAGATG-- CTAAATTT-- ---------G
Cs.Maltase    AGTTGTTCTC G-ATTATCAA GTAGGGCG-- TCAAATTCCC GAACCAAGAG
Dm.CG.nuc     --TTATTCAG C-TGAATAAG CGAAAGTA-- A--------- ----------
Am.maltase1   TAATTGTAGA T-TCATCCAC TTCAGGCG-- CTACTATAGT CAATTATTCA
Ls.ScrA.nu    AACTATCGAT GATCTTTCAA TTTGAACAAA TTAATTTAGA TAAACAATCA
Clustal Co       *

              ....|....| ....|....| ....|....| ....|....| ....|....|
                    1910       1920       1930       1940       1950
Am.AG.nuc     GAAACTTTTA ATTTCTTCCT GAATATGTC- TATTCTTTGA AGCGGCGA..
AC.AG         GAAATTTTTA ATATCTCCCT GAATATGTC- TATTCTTTGA AGCGGCGA..
Cs.Maltase    GTGACGATCC AGGACTATAC GAATAAGAAA TATTCCTCAA ATCTTTGA..
Dm.CG.nuc     ---------- ---------- ---------- ---------- --------..
Am.maltase1   ATCATGATTT TCTTATCCGC AGTGTTCATA TCTTTTTTCC AACGGTAA..
Ls.ScrA.nu    GGGATGACTC GCTGGGATTT AAAACCACTT ATTCCAGCAG AGTTACAT..
Clustal Co
```

Figure 4.6. (Continued)

```
              ....|....| ....|....| ....|....| ....|....| ....|....|
                      10         20         30         40         50
A.c.AG        MKAIIVFCLM ALSIVDAAWK PLPEN----L KEDLIVYQVY PRSFKDSNGD
```

```
A.m.AG          MKAVIVFCLM ALSIVDAAWK PLPEN----L KEDLIVYQVY PRSFKDSNGD
Cs.             FKKLTILLSI ACSVLAAPEG AREKD----W WEIGNFYQVY PRSFMDSDGD
Dm.             VKIAFILSVG LVGILAHKHQ SKELDAKYNW WQHEVFYQIY PRSFQDSNGD
A.m.maltas      MKSLVVVVLL LAVGLGAGQN --NKG----W WKNAIFYQVY PRSFMDSNSD
Ls.             -FVPLIPALT AGGLLMAINN VLTGQG---L FGAQSIVQMF P---------
                                                            *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
A.c.AG          GIGDIEGIKQ KLDHFLEMGV DMFWLSPIYP SPMVDFGYDI SNYTDVHPIF
A.m.AG          GIGDIEGIKE KLDHFLEMGV DMFWLSPIYP SPMVDFGYDI SNYTDVHPIF
Cs.             GVGDLKGISE KVGYLKEIGM DGVWLSPIFD SPMADFGYDI SNFTKVFPQF
Dm.             GIGDLQGITS RLQYFKDTGI TSVWLSPIYE SPMVDFGYDI SNYTNIQPEY
A.m.maltas      GIGDLKGIKD KLSHFIESGI TAIWLSPINR SPMVDFGYDI SDFKDVDPIF
Ls.             ---QWKGFAE IVNMMSSAPF TFLP------ ---ILIAFSA TKRFGGNPYL

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
A.c.AG          GTLSDLDNLV NAAHEKGLKI ILDFVPNHTS DQHEWFQLSL K-----NIEP
A.m.AG          GTISDLDNLV SAAHEKGLKI ILDFVPNHTS DQHEWFQLSL K-----NIEP
Cs.             GDLSSIDELV AEFNKKDMKL ILDFVPNHTS DQCEWFKKSI Q-----RDPE
Dm.             GTLEDFDALI AKANELGVKV ILDFVPNHSS NKHPWFIKSV A-----REPG
A.m.maltas      GTIKDLEDLT AEAKKQNLKV ILDLVPNHTS DQHKWFQMSI NNTNNNNTNK
Ls.             GAAAGMMLVM PNLVN-GYGV AESIATGHMT ---YWHVFGL N----IAQAG
                *                            *       *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
A.c.AG          YNNYYIWHPG -KIVNGKR-- -VPPTNWVGV FGGSAWSWRE ERQAYYLHQF
A.m.AG          YNNYYIWHPG -KIVNGKR-- -VPPTNWVGV FGGSAWSWRE ERQAYYLHQF
Cs.             YNDYYIWHPG KPNPDGGRN- -LPPTNWVSA FRSSAWEWNE ERGEYYLHQF
Dm.             YEDFYVWEDG ILLENGTR-- -VPPNNWLSV FSGSAWMWND ERQQYYLRQF
A.m.maltas      YKDYYIWVDP VKDDKGNPIK DKYPNNWLSV FNGTGWTFHE GRKQFYFHQF
Ls.             YQGQVIPVIG VAFILANLE- -KFFHKHLND AVDFTFTPML SIIITGFLTF
                *                                                    *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
A.c.AG          APEQPDLNYY NPVVLDDMQN VLRFWLRRGL DGFRVDALPY ICED-----M
A.m.AG          APEQPDLNYY NPVVLDDMQN VLRFWLRRGF DGFRVDALPY ICED-----M
Cs.             LAQQPDLNYR NPKVVETMKN VLRFWLSKGI NGFRIDAVPY LFEVGPDANG
Dm.             TYGQPDLNYR NPAVIKAMDD VMLFWLNKGI AGFRIDAIIY IYED-----A
A.m.maltas      YKQQPDLNYR NSDVREEMKN IMKFWLDKGI DGFRIDAVPH LFES----AN
Ls.             TLVGPALRIV SNGVTDSLVW AYQTLGAVGM GIFGLGYSAI VLTG------
```

Figure 4.7. The multiple alignment of amino acid sequences deduced from cDNA sequence of *AG* in *A. cerana* and that in other organisms. ‘*’ Residues in that column are identical in all sequences in the alignment.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
A.c.AG          RFLDEPLSGE TNDPNKTEYT LKIYTHDIPE TYNIVRKFRD VLDEFPQ---
```

```
A.m.AG        RFLDEPLSGE TNDPNKTEYT LKIYTHDIPE TYNVVRKFRD VLDEFPQ---
Cs.           NYPDEIETHA CSDPLSQCYL YHDYTQNRPE TFEMVTEWRA TLEEFKQKNG
Dm.           QLRDEPPSGT TDDPNNEAYL SHIYTRNQPE DYGLLQHWRQ LLDNYTANHD
A.m.maltas    ISLDEPPLGK NLNLSLHASL NHTLTKDQPE TYELVKEWRD FVDNYAEENK
Ls.           -LHQSFPAIE TTLLADIAKT GGSFIFPVAA MANIAQGAAT FAVFFVTKNK


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     310        320        330        340        350
A.c.AG        --PKHMLIEA YTNLSMTMKY YD-----YGA DFPFNFAFIK NVSKDSNSSD
A.m.AG        --PKHMLIEA YTNLSMTMKY YD-----YGA DFPFNFAFIK NVSRDSNSSD
Cs.           GPTRVLMVEA YAPLTKVIQI YGQNGQLNGA QIPFNFEFLN NLGAVSNARD
Dm.           GPLRIMMTEG YASVSQLMEY YEDSNGVQGP QFPFNFDFIT ELNANSTAAD
A.m.maltas    RDEIVLLTEA YSSLENTLKY YE-----VGS NVPFNFKFIT DANSSSTPEQ
Ls.           QQKSLTTSAG ISAMLGITEP AL-----FGV NLKLKFPFFI GLIASGISSF

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     360        370        380        390        400
A.c.AG        FKKLVDNWMI YMPADGIPNW VPGNHDQLRL VSRFGEEKAR MITAMSLLLP
A.m.AG        FKKLVDNWMT YMPPSGIPNW VPGNHDQLRL VSRFGEEKAR MITTMSLLLP
Cs.           FKDVIDNYLS TIPEGATPNW VQGNHDQHRS ASRLGPQKAD AVNMLLQVLP
Dm.           FVFYISRWLI YMPHGHVANW VMGNHDNPRV ASRFGEKSVD AMNMLLMTLP
A.m.maltas    FKVIIDNWIK GTPQNNVPNW VMGNHDRVRV GTRY-PGRAD HMIMLEMILP
Ls.           IIGLLHVLSV SMGPAGIIGF IAIAPKS--- -----IPSFM MGAIISFVIA

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     410        420        430        440        450
A.c.AG        GVAVNYYGDE IGMSDT-YIS WEDTQDPQGC GAGKENYQTM SRDPARTPFQ
A.m.AG        GVAVNYYGDE IGMSDT-YIS WEDTQDPQGC GAGKENYQTM SRDPARTPFQ
Cs.           GAAVTYYGEE LAMEDV-FVP WSRTVDPQAC TTDPNIFHAK SRDPARTPMI
Dm.           GIGITYNGEE LGMTDYRDIS WSDTVDQPAC EAGIDNYKTI SRDPERTPMQ
A.m.maltas    GVAVTYYGEE IGMVDN---- ---------- ---TTIYKYD VRDGCRTPFQ
Ls.           FVGTYLYGKK AMKTTEEEII NEAPATPEVV ERLQDEKISA PVTG--RIVD

              ....|....| ....|....| ....|....| ....|....| ....|....|
                     460        470        480        490        500
A.c.AG        WDDSVSAGFS SSSD-----T WLRVNENYKT -INLAAEKKD KNSFFNMFKK
A.m.AG        WDDSVSAGFS SSSN-----T WLRVNENYKT -VNLAAEKKD KNSFFNMFKK
Cs.           WTSQKNAGFS SSNY-----T WLPTGPDYRK -NNVEVQRSQ RGSHLNIFKK
Dm.           WSSDVNAGFS SADR-----T WLPVNPNYKE -LNLRNQQQA RRSHYKIYQS
A.m.maltas    WDNSINAGFS KIAENLLEKN WLPVHTSYKS GLNLEQEKKD SISHYHLYTN
Ls.           LASVPDPVFA SEAMG----K GIAIMPTSQD VLAPVTGVIT IAANTGHAYG
                       *
```

Figure 4.7. (continued)

```
              ....|....| ....|....| ....|....| ....|....| ....|....|
                     510        520        530        540        550
```

```
A.c.AG       FAMLKKSPHF KEANLNTRML NDSVFAFSRE TEENGSLYAI LNFS-NEEQI
A.m.AG       FASLKKSPYF KEANLNTRML NDNVFAFSRE TEDNGSLYAI LNFS-NEEQI
Cs.          LTQLRKQDIL MYGTYDSYLA NDDVLVIKRE IENNRTLIAV LNLG-FTEQV
Dm.          LLKLRQLPVL KNGSFVPEVV NRRVFAFKRE LKNEHTLLTI VNVS-NRTEL
A.m.maltas   LTALRKRDVL KKGNFTIEIL NKTVLAVVRQ SEEE-AVSLL INFS-KNNTI
Ls.          IKSDDGAEVL IHIGLDTVNL NG--IGFEKI VQQGQHVSEG DLLG--HFDI
                                  *

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   560        570        580        590        600
A.c.AG       VDLKAFNN-- -----VPKKL NMFYTIFNSD IKS---ISNN EQIKVSALGF
A.m.AG       VDLKAFNN-- -----VPKKL NMFYNNFNSD IKS---ISNN EQVKVSALGF
Cs.          VNLNLNDRDW K----VPERM EVATASVNAG MFERQPVVTS EVYVSAGVGV
Dm.          VDIADFIEQ- ------PNRL SVLVAGVDSQ HRVGDRLKAE TIELAPNEGL
A.m.maltas   VDISKLVNK- ------RNNA KIYTSSVNSN LTVNQTVNPV AINIPGDTSI
Ls.          DKIKQAG--- ------LTPL TMTIVTNTAG YAQVDPLLTV DKAAMQGEEI

             ....|....| ...
                   610
A.c.AG       LILISQDAKF GNF
A.m.AG       FILISQDAKF GNF
Cs.          VLDYQVGRQI PEP
Dm.          VIQLNKRK-- ---
A.m.maltas   IVDSSTSGAT IVN
Ls.          IQLHAKKD-- ---
```

Figure 4.7. (continued)

Aligned sequences were imported into a phylogenetic analysis program, PAUP (version 4.0b). One tree was founded from each program when UPGMA and Neighbor - joining programs were used for the analysis. As shown in Figs. 4.8 and 4.9, phylogenetic trees from deduced amino acid were constructed. A pattern of genetic distance was observed from both Figs. 4.8 and 4.9. The obtained trees from each program share similar characters. AG amino acid sequence of *A. cerana* is mostly closed to AG amino acid sequence of *A. mellifera*.

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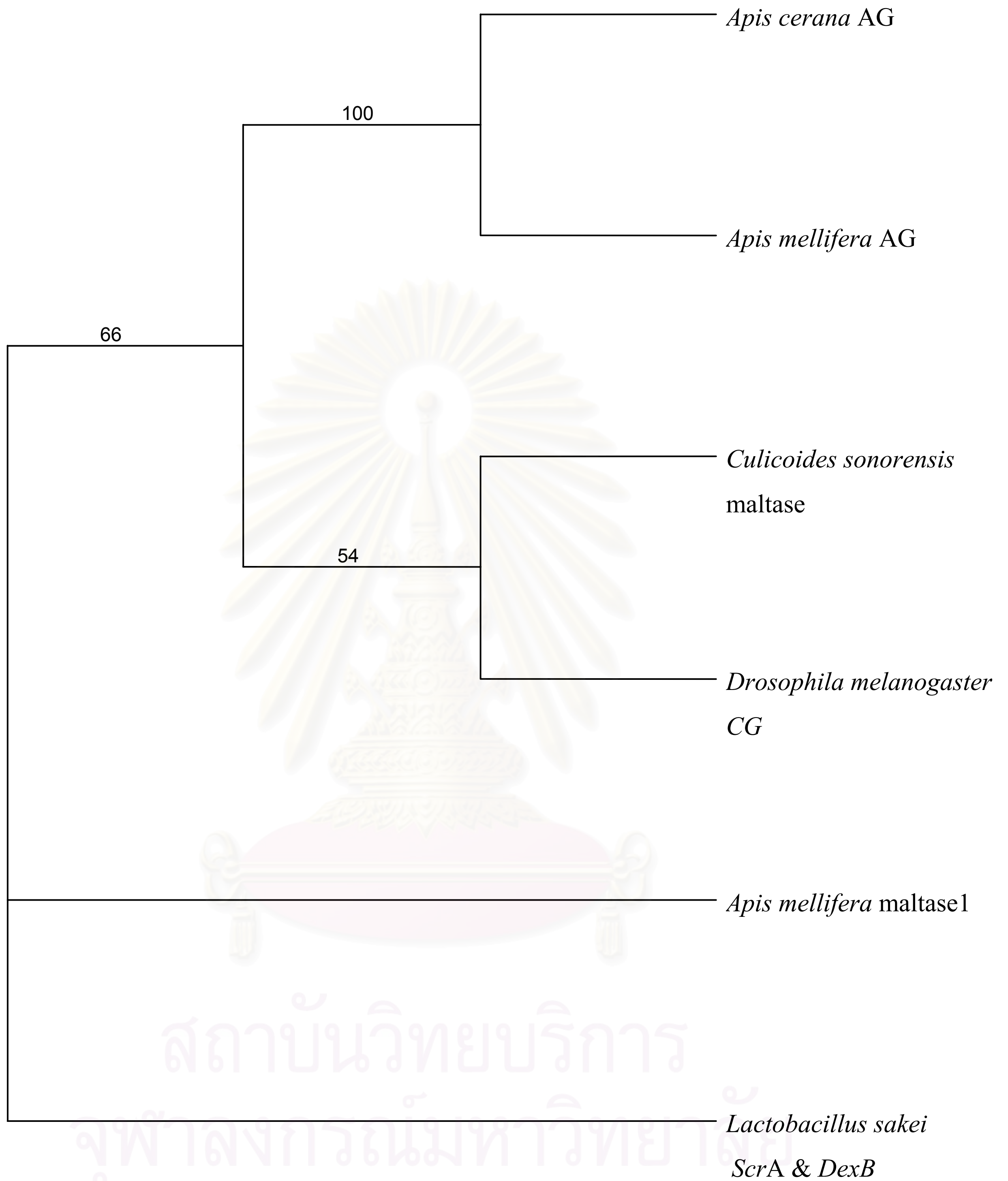

Figure 4.8. Phylogenetic trees illustrating the genetic relationship among amino acid sequences of various species by Neighbor - joining. Numbers above branches indicate bootstrap support percentage over 50% in 1000 replicates. Amino acid sequences of *AG* from *A. mellifera* maltase 1 and *Lactobacillus sakei* were used as outgroups.

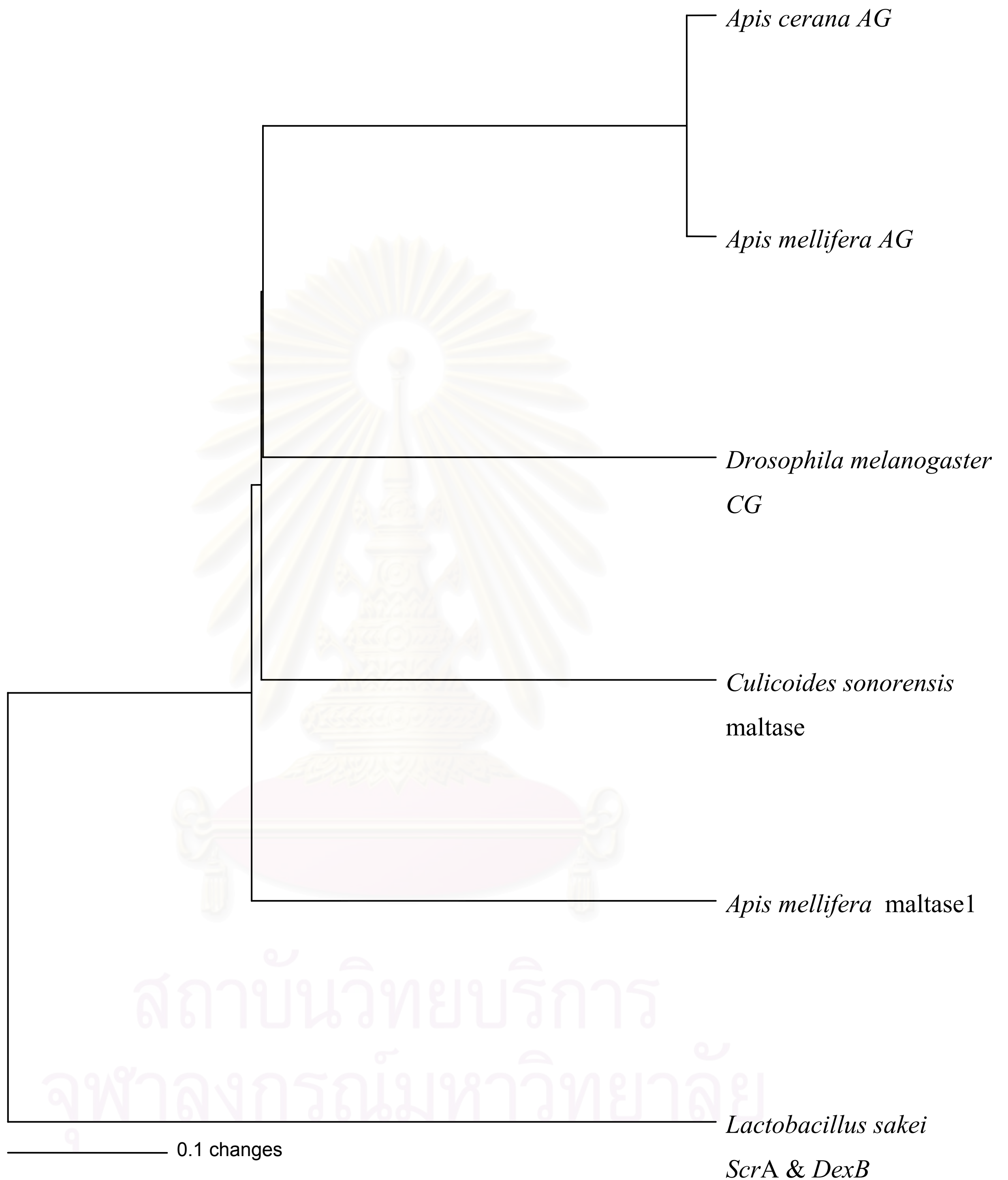

Figure 4.9. UPGMA tree of the genetic relationship among amino acid sequences of various species. *Lactobacillus sakei* was used as an outgroup.

4.4 Denaturation and renaturation of AG

4.4.1. SDS - polyacrylamide gel electrophoresis of crude extract

Crude extracts of hypopharyngeal glands (HPGs) and honey crops were quantitated for protein by Bradford's assay. Due to standard curve of BSA, higher protein content (1.03 g/ g) was obtained from honey crops (table 4.1).

Table 4.1. Protein content in crude of HPGs and honey crop.

Protein source	Amount (g/ g)
HPGs	< 0.33
Honey crop	1.03

Later, crude proteins of HPGs and honey crops were separated by SDS – PAGE and stained by coomassie brilliant blue (CBB). Different patterns of protein in crude extract were visible on SDS - PAGE as in Fig. 4.10. Honey crop crude shows widely MW ranging from 10 to 225 kDa while HPGs crude has two major proteins with approximate MW of 50 and 75 kDa.

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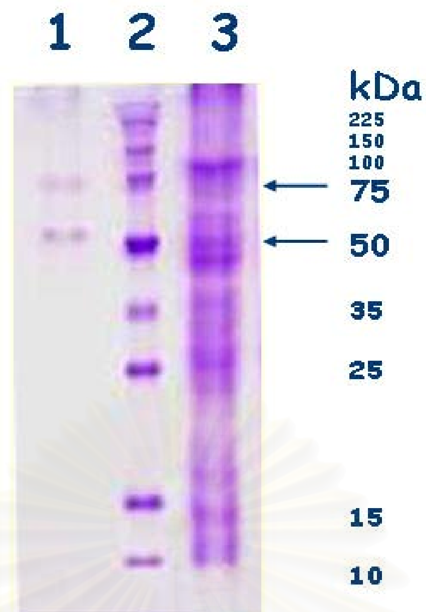

Figure 4.10. SDS - PAGE of HPGs and honey crop.

Lane 1: crude extract of HPGs (50 µg)

Lane 2: Broad range protein marker

Lane 3: crude extract of honey crop (50 µg)

Then, the CBB gel was renatured and stained for AG activity. Only single activity band was appeared in both HPGs and honey crop (Fig. 4.11).

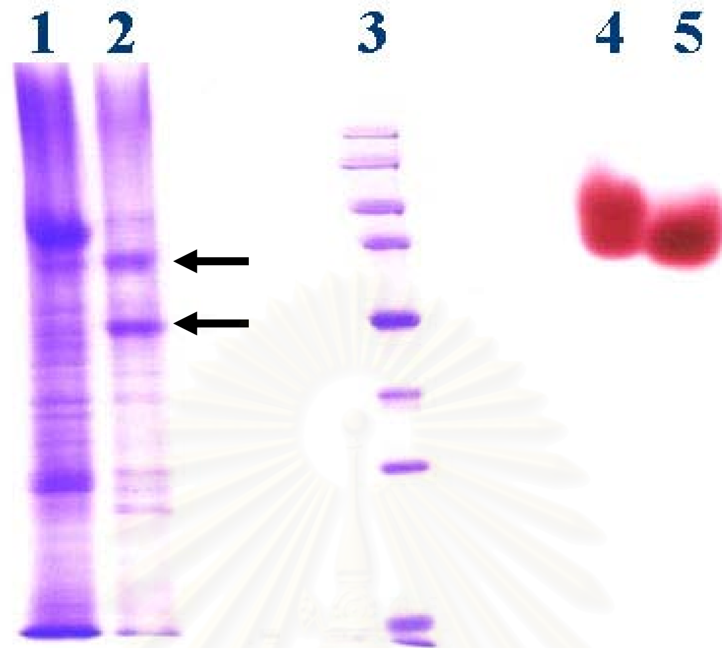

Figure 4.11. Renaturation of AG from HPGs and honey crops. Arrows indicate two major subunit bands.

Lane 1: crude of honey crop (50 µg; CBB gel)

Lane 2: crude of HPGs (50 µg; CBB gel)

Lane 3: protein marker

Lane 4: renatured crude of HPGs (50 µg)

Lane 5: renatured crude of honey crops (50 µg)

4.5 Ammonium sulfate precipitation

Crude extract of honey bees (*A. cerana*) was slowly added by solid ammonium sulfate (AS) to be 0 - 30, 30 - 40, 40 - 50, 50 - 60, 60 - 70, 70 - 80, and 80 - 95% saturation, respectively (Appendix F). Later, the suspension was dialyzed by dialysis bag at MWCO 3500. As shown in Fig. 4.12 and table 4.2, the specific activity of AG was high in unprecipitated suspension (0.87 u/ mg). Low specific activity was assayed from suspension with low concentration of AS. The specific activity got increasing when the concentration of AS was increased. Saturation of AS (95%) was used for purification since it provided the highest specific activity.

Table 4.2. Specific activity of AG after various AS saturation.

% Ammonium sulfate saturation	Specific activity (u/ mg)
Not precipitated	0.87
0-30	0.17
30-40	0.15
40-50	0.21
50-60	0.28
60-70	0.46
70-80	1.23
80-95	1.36

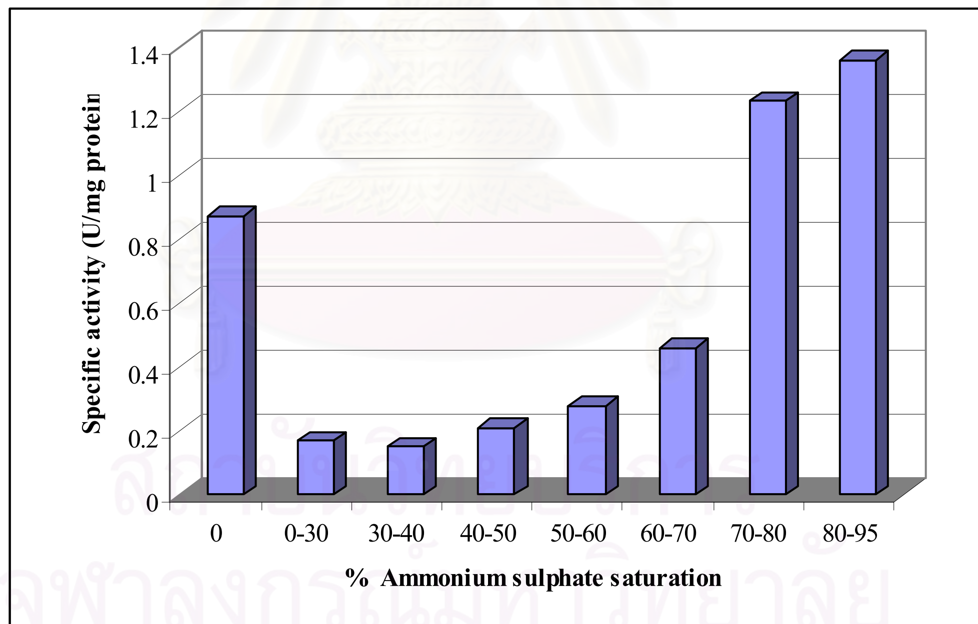

Figure 4.12. Specific activity of AG precipitated by a stepwise increase of AS concentration.

Precipitated crude was separated by SDS – PAGE. Less protein bands were observed than in unprecipitated crude. Protein band of 100 kDa was observed in

unprecipitated crude only while protein band of 75 kDa was observed in all samples (Fig. 4.13.).

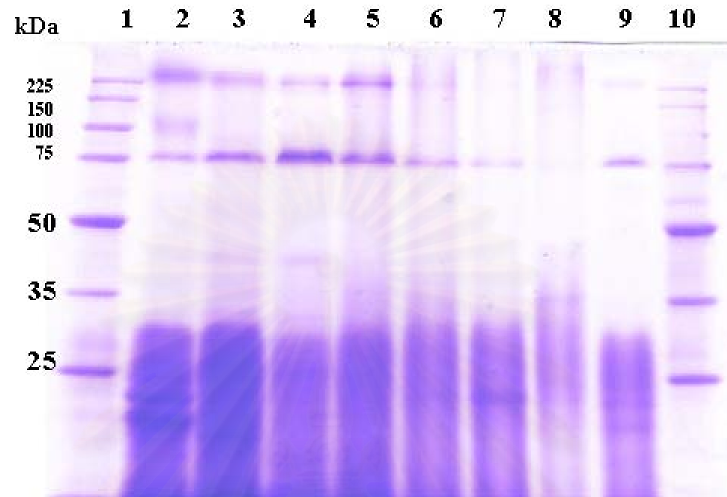

Figure 4.13. SDS - PAGE of protein (50 µg/ lane) saturated by various concentrations of AS.

Lane 1: Protein marker — Lane 2: unprecipitated protein

Lane 3: 0 - 30% AS saturation — Lane 4: 30 - 40% AS saturation

Lane 5: 40 - 50% AS saturation — Lane 6: 50 - 60% AS saturation

Lane 7: 60 - 70% AS saturation — Lane 8: 70 - 80% AS saturation

Lane 9: 80 - 95% AS saturation — Lane 10: Protein marker

4.6 Purification of AG

4.6.1 Ion Exchange Chromatography

Crude protein of honey bees (*A. cerana*) was applied to DEAE – cellulose column (1.6 × 17 cm) as mentioned in Materials and Methods. After elution and AG assay, it showed that activity in Peak I (fractions no. 2 - 12) was not bounded to DEAE - cellulose column. While being eluted by gradient of 0 - 1 M NaCl, activity from Peak II (fractions no. 28 - 33) was eluted at 0.13 M NaCl (Fig. 4.14).

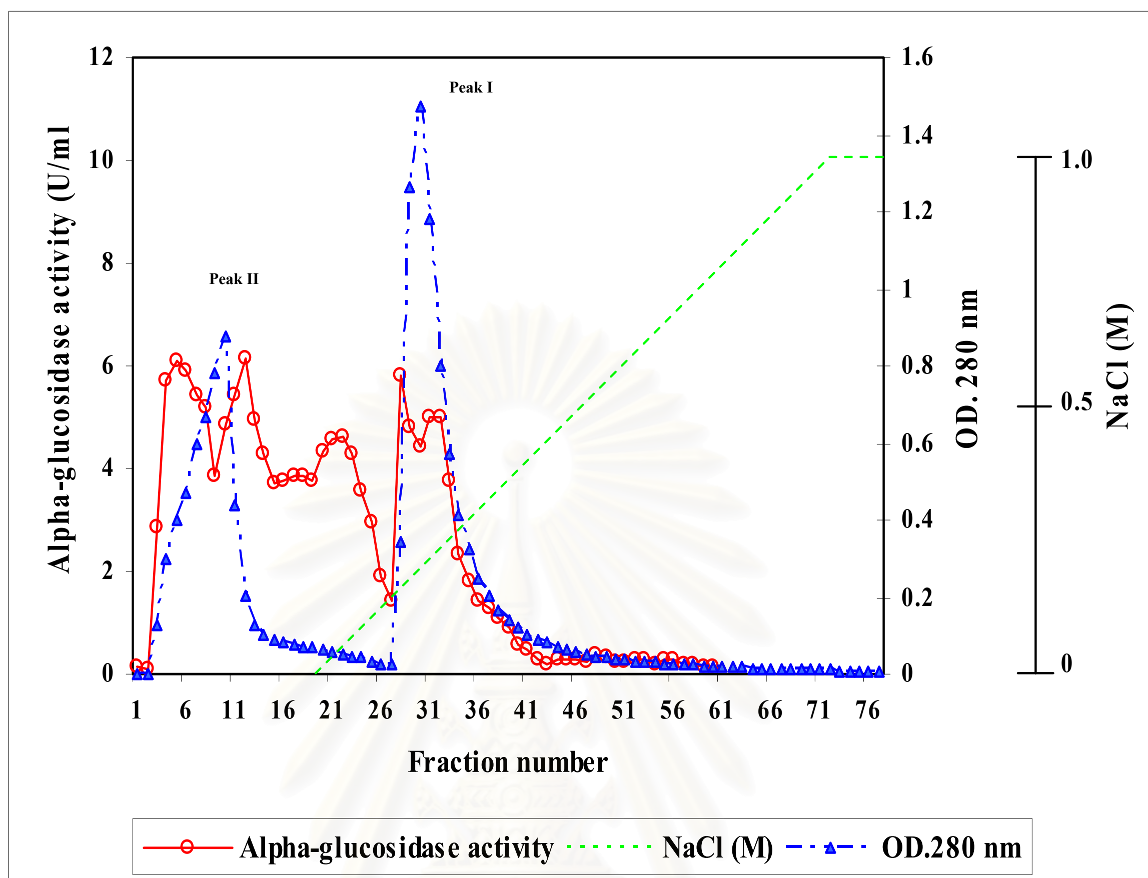

Figure 4.14. Purification profile of AG on DEAE - cellulose. Equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 1 M NaCl; flow rate 60 ml/ h; fraction size 10 ml/ fraction.

Furthermore, active and unbound fractions (fractions no. 2 - 12) from DEAE - cellulose column (20 ml) were subjected to CM - cellulose column (1.6 × 17 cm) equilibrated by 20 mM sodium acetate buffer (pH 4.7). Bound materials were eluted by a linear gradient of 0 to 1 M NaCl at flow rate of 0.5 ml/ min and maintained at 4°C. Two main peaks of protein were eluted from CM - cellulose column but there was no activity peak as shown in Fig. 4.15 and the specific activity of 0.154 u/ mg was collected (table 4.3c).

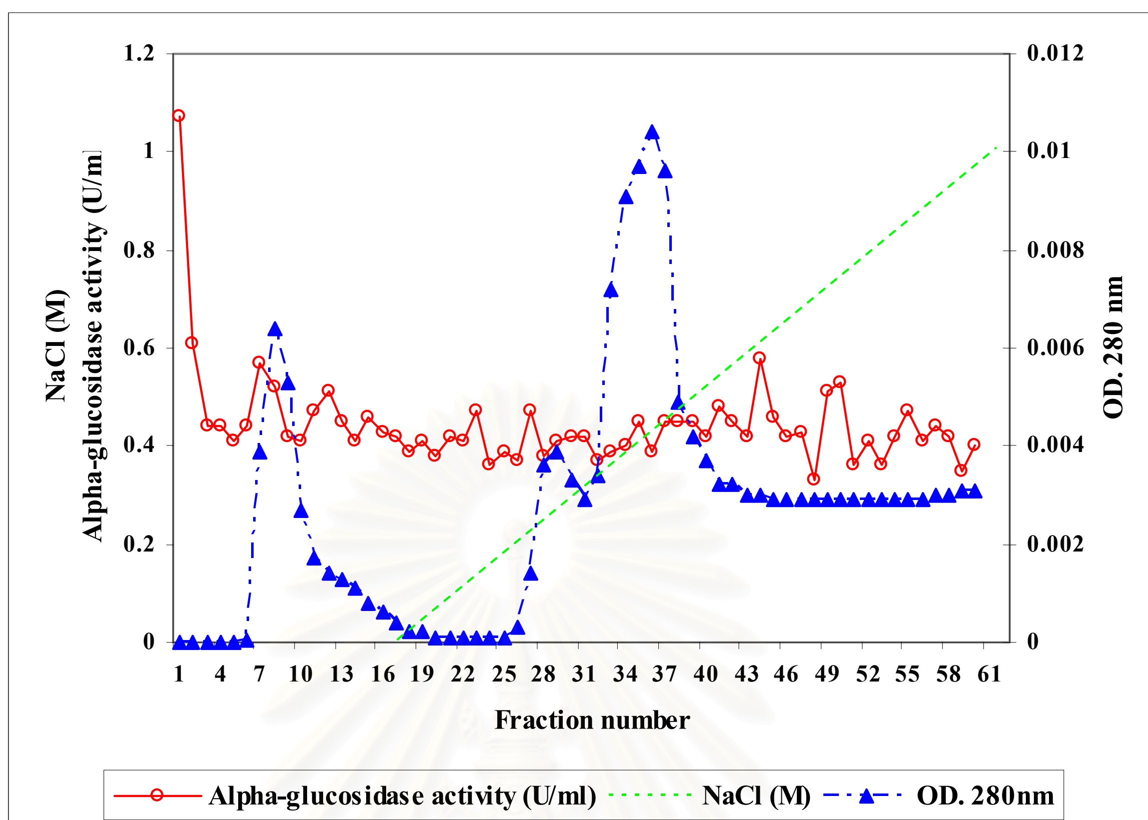

Figure 4.15. Purification profile of AG on CM - cellulose. Equilibrium, 100 mM sodium acetate buffer (pH 4.7); elution, 1 M NaCl; flow rate 30 ml/ h; fraction size 5 ml/ fraction.

4.6.2 Gel filtration chromatography

Unbound (peak I) and bound peaks (peak II) from Fig. 4.14 were concentrated and injected onto a gel filtration column (Superdex 200). Considering Fig. 4.16, AG activity was eluted after the protein peak. Highest peak containing the specific activity of 1.804 u/ mg was collected. The purification fold was calculated to be 2.59 (table 4.3a). Due to Fig. 4.17, AG activity was discovered after the protein peak. The highest peak (fractions no. 17 – 22) contained the specific activity of 1.032 u/ mg. Also, the purification fold was 1.48 as shown in table 4.3b.

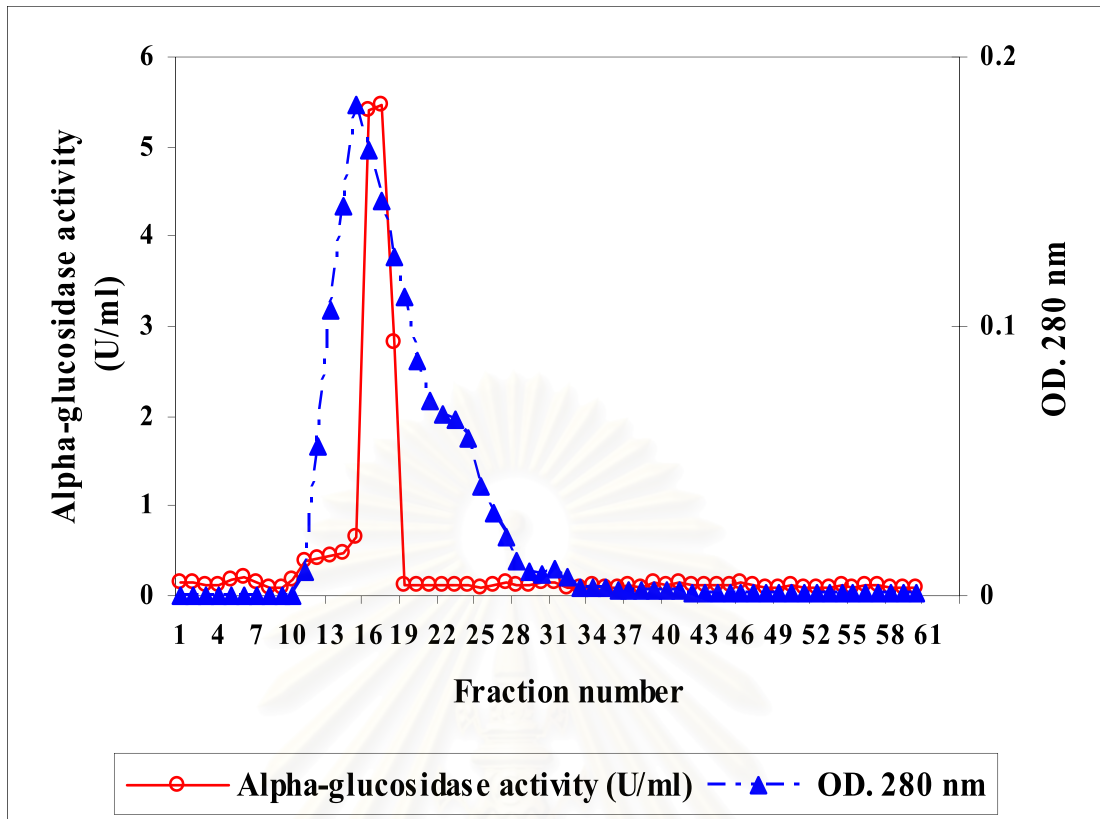

Figure 4.16. Purification profile of AG (pooled bound fractions activity) on gel filtration Sephadex 200 column. Equilibration and elution, 30 mM sodium phosphate buffer (pH 6.3); flow rate 30 ml/ h; fraction size 5 ml/ fraction.

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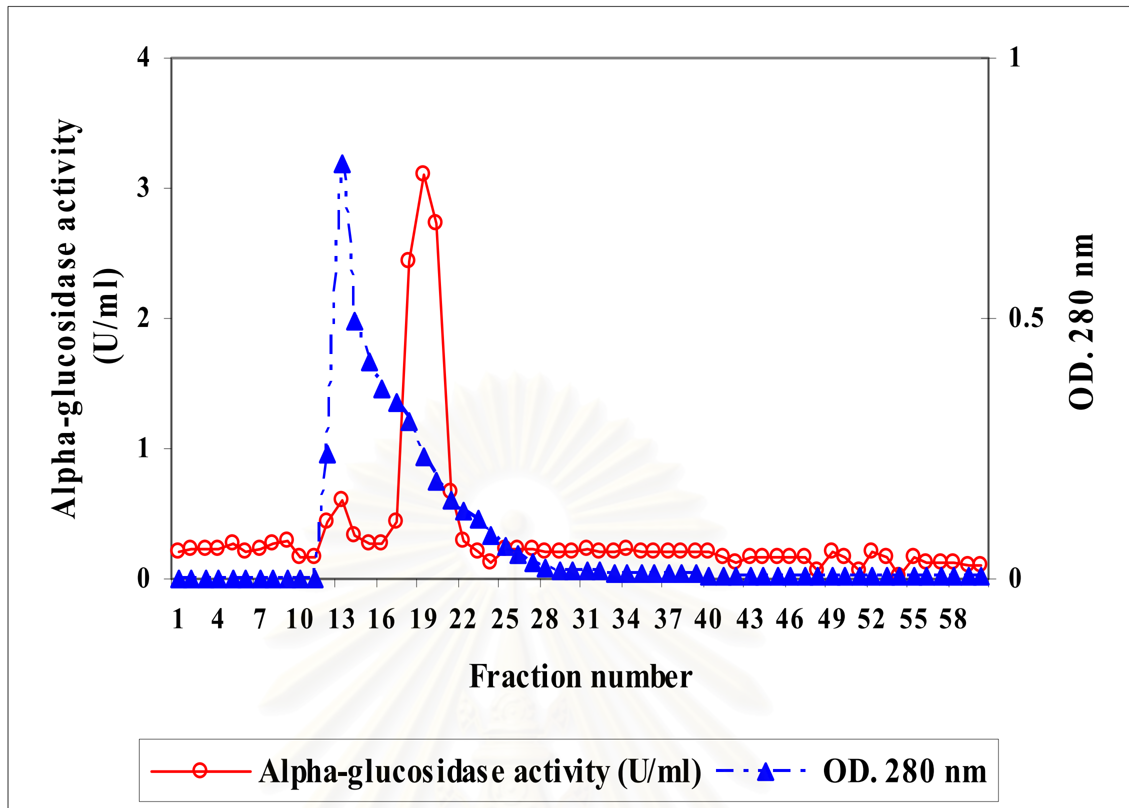

Figure 4.17. Purification profile of AG (pooled unbound fractions activity) on gel filtration Sephadex 200 column. Equilibration and elution, 30 mM sodium phosphate buffer (pH 6.3); flow rate 30 ml/ h; fraction size 5 ml/ fraction.

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Table 4.3a. Summary of purification procedures of AG.

Procedure	Total protein (mg)[a]	Total activity (unit)[b]	Specific activity (unit/ mg)	Yield (%)	Purification (fold)
Crude protein[c]	5951.2	4145.2	0.696	100	1
95% sat. $(NH_4)_2SO_4$	1302	306	0.235	7.382	0.34
DEAE cellulose (bound)	137.5	298.5	2.171	7.201	3.11
Superdex 200 (bound)	75.9	136.92	1.804	3.303	2.59

Table 4.3b.

Procedure	Total protein (mg)[a]	Total activity (unit)[b]	Specific activity (unit/ mg)	Yield (%)	Purification (fold)
DEAE cellulose (unbound)	123.9	131.4	1.061	3.169	1.52
Superdex 200 (unbound)	93	96	1.032	2.315	1.48

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Table 4.3c.

Procedure	Total protein (mg)[a]	Total activity (unit)[b]	Specific activity (unit/ mg)	Yield (%)	Purification (fold)
DEAE cellulose (unbound)	123.9	131.4	1.061	3.169	1.52
CM cellulose (unbound)	77.7	12	0.154	0.289	0.22

[a]This was calculated on the basis of the fact that $E^{1\%}_{1cm}$ at 280 nm.

[b]Alpha - glucosidase activity

[c]This corresponded to the amount of protein from 430 g of honey bees.

In order to avoid the loss of AG activity during AS precipitation, crude extract (150 mg) was directly applied to DEAE - cellulose and CM - cellulose columns. The result of purification was presented in Figs. 4.18 and 4.19.

Due to fig. 4.18, specific activity (0.757 u/ mg) in fractions no. 6 - 11 was determined. The purification fold was 1.21. Also, in Fig. 4.19, specific activity (0.53 u/ mg) in fractions no. 7 – 12 from CM - cellulose was observed but purification fold was 0.85. The result was in table 4.4.

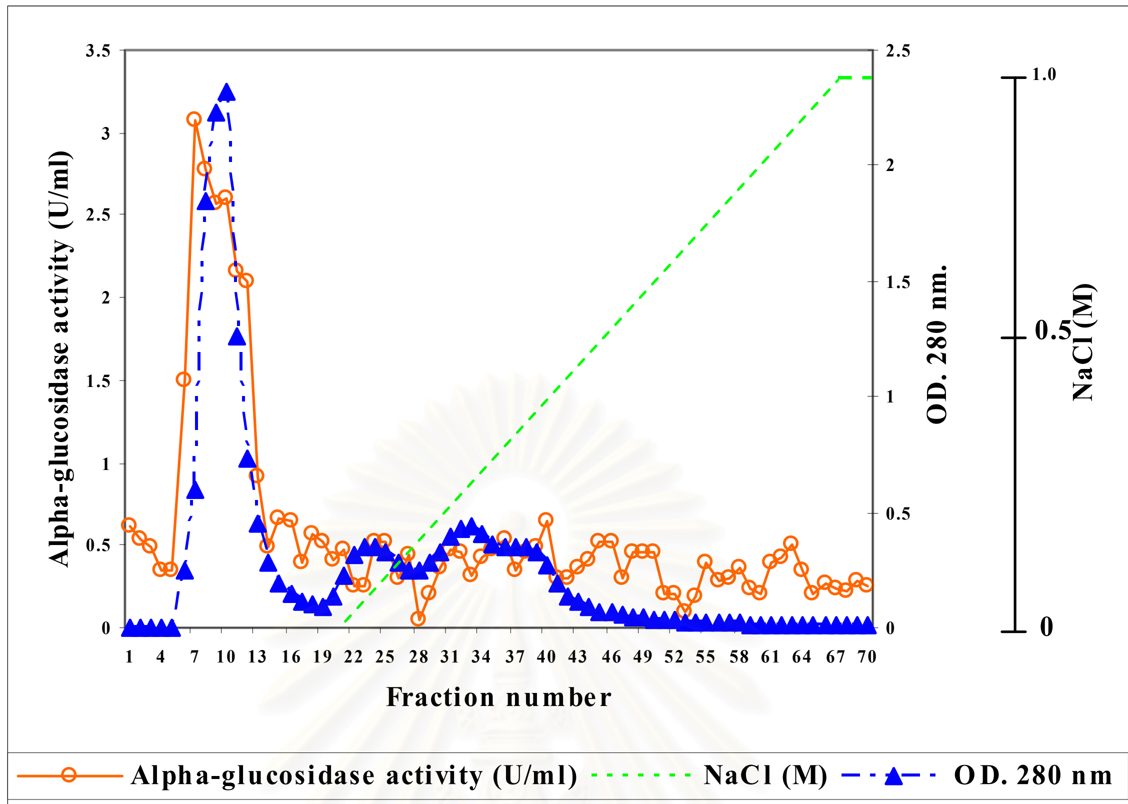

Figure 4.18. Chromatography of AG on DEAE – cellulose. Equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 1 M NaCl; flow rate 60 ml/ h; fraction size 10 ml/ fraction.

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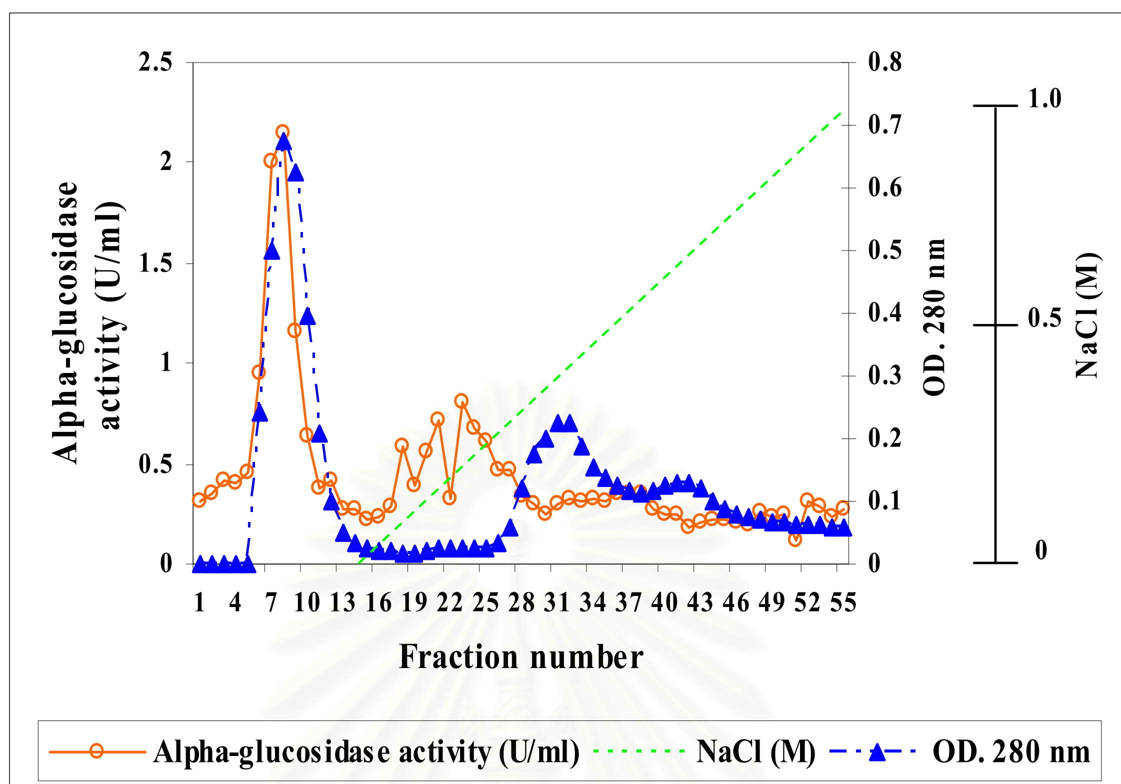

Figure 4.19. Chromatography of AG on CM - cellulose. Equilibrium, 20 mM sodium acetate buffer (pH 4.7); elution, 1 M NaCl; flow rate 30 ml/ h; fraction size 5 ml/ fraction.

Table 4.4. Purification procedures of unprecipitated crude.

Procedure	Total protein (mg)[a]	Total activity (unit)[b]	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude protein[c]	1044	654.9	0.627	100	1
DEAE - cellulose	139.2	105.4	0.757	16.09	1.21
CM - cellulose (unbound)	65	34.5	0.530	5.27	0.85

[a]This was calculated on the basis of the fact that $E^{1\%}_{1cm}$ at 280 nm.

[b]Alpha - glucosidase activity

[c]This corresponded to the amount of protein from 100 g of honey bees.

4.7 SDS - PAGE of purified AG

Purity of AG (from precipitated crude of 95% AS) was checked by SDS - PAGE as shown in Figs. 4. 20 (A), (B), and 4.22. The R_f and mass weight (MW) was plotted (Figs. 4.21 and 4.23). The molecular mass of AG was preliminarily estimated to be 68 kDa. Activity staining was performed in order to determine the AG (Figs. 4.24 and 4.25). After SDS - PAGE, the first part of the gel was CBB stained. The copied gel was renatured in Triton X - 100 and activity stained as mentioned in Materials and Methods. The activity band of MW between 50 – 75 kDa was clearly visible.

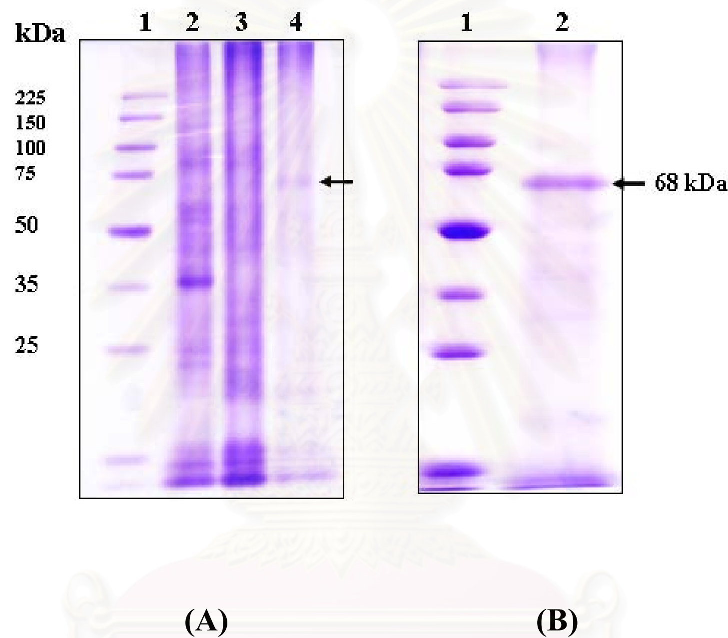

Figure 4.20. SDS - PAGE and CBB.

A:

Lane 1: broad range protein marker

Lane 2: crude protein (100 μg)

Lane 3: 95% saturated $(NH_4)_2SO_4$ precipitate (50 μg)

Lane 4: pooled active fraction of DEAE - cellulose (30 μg)

B:

Lane 1: broad range protein marker

Lane 2: pooled active fractions of Superdex 200 (50 μg)

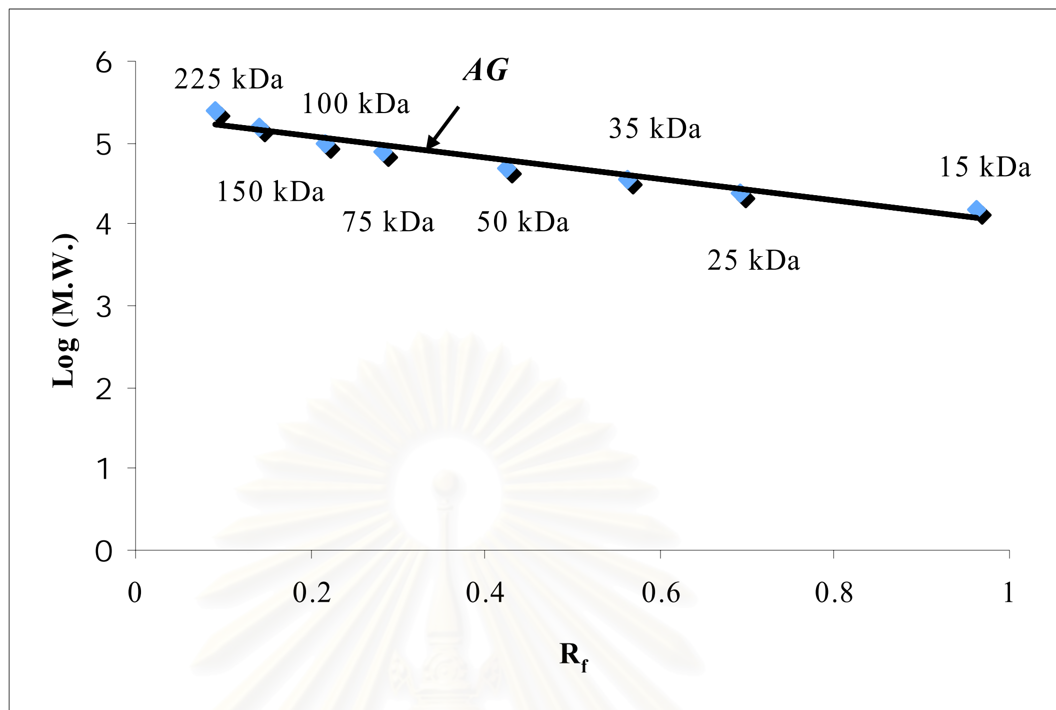

Figure 4.21. Relationship between Log and R_f of standard MW of broad range protein marker.

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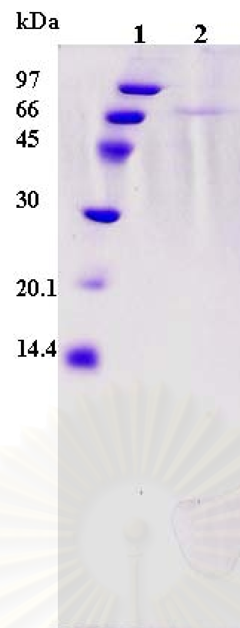

Figure 4.22. SDS - PAGE and CBB.

Lane 1: low molecular weight (LMW) marker

Lane 2: pooled active fractions of Superdex 200 (20 μg)

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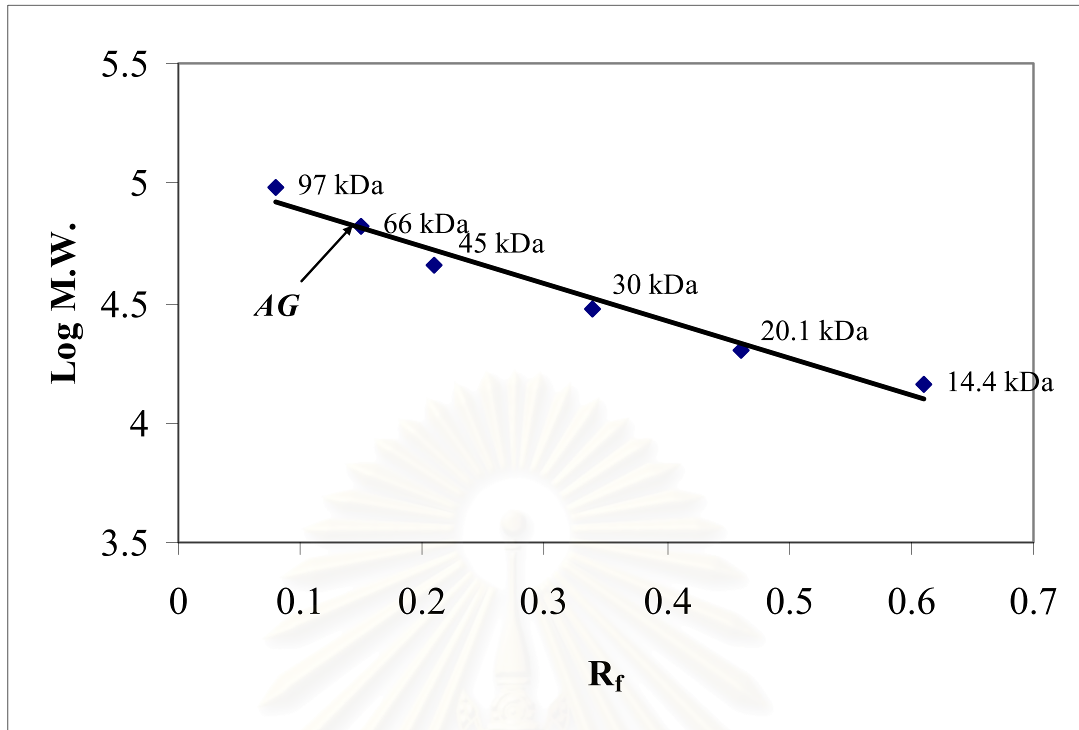


Figure 4.23. Relationship between Log and R_f of low molecular weight (LMW). The LMW standard containing phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and alpha - lactalbumin (14.4 kDa).

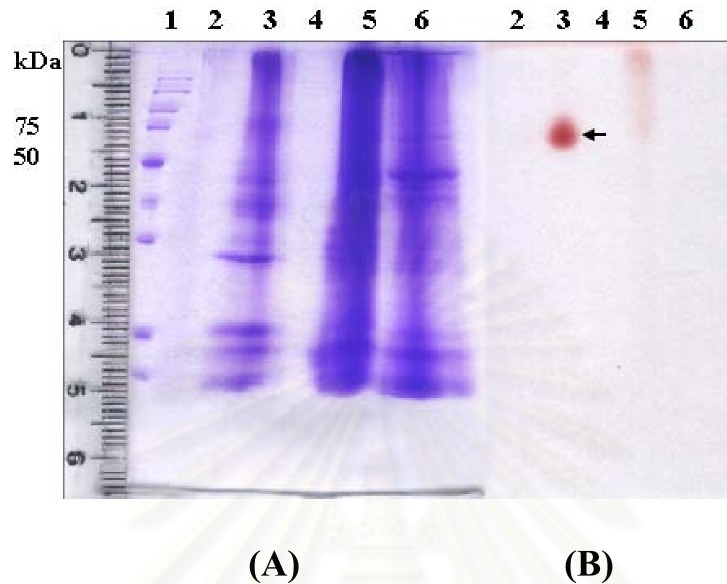


Figure 4.24. CBB (A) and activity stain (B) of SDS polyacrylamide (12.5%). An arrow indicates an activity band of AG after renaturation.

Lane 1: broad range protein marker

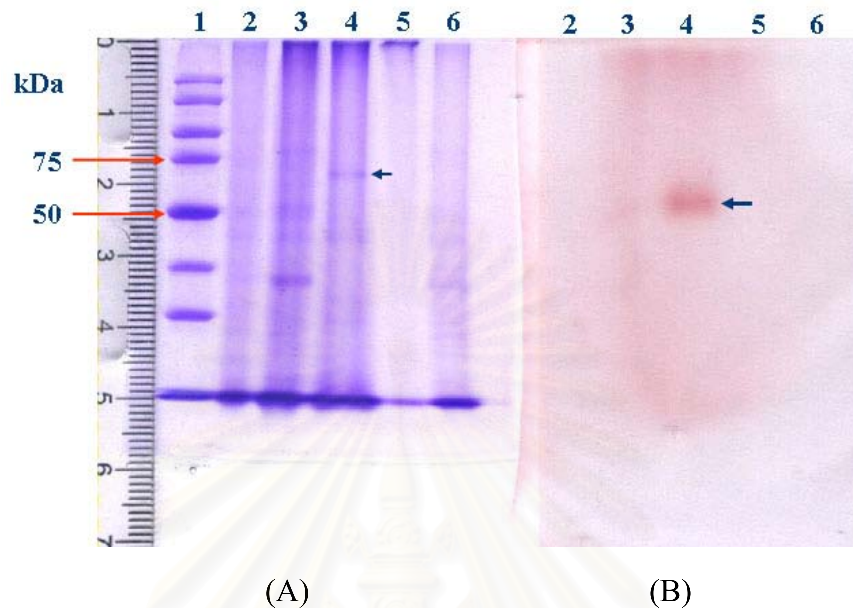
Lane 2: pooled active fractions from Superdex 200 (10 µg); bound peak from DEAE - cellulose

Lane 3: pooled active fractions from Superdex 200 (50 µg); unbound peak from DEAE - cellulose

Lane 4: pooled active fractions from CM - cellulose (10 µg); unbound peak from DEAE - cellulose

Lane 5: pooled active fractions from DEAE - cellulose (120 µg) from unprecipitate

Lane 6: represents CM-cellulose (80 µg) from unprecipitate



(A) (B)

Figure 4.25. CBB (A) and activity stain (B) of SDS polyacrylamide (10%). Arrows indicate AG bands in both conditions.

Lane 1: broad range protein marker

Lane 2: crude protein (20 μg)

Lane 3: precipitate with 95% AS saturation (50 μg)

Lane 4: pooled active fractions from unbound peak of DEAE - cellulose (30 μg)

Lane 5: pooled active fractions from Superdex 200 (fractions no. 12 – 16); unbound peak from DEAE – cellulose (10 μg)

Lane 6: pooled active fractions from Superdex 200 (fractions no. 17 – 23); unbound peak from DEAE – cellulose (20 μg)

4.8 Optimum conditions of purified AG

4.8.1 Optimum pH

The effect of pH on AG activity was determined by using purified AG. Sucrose was used as the substrate. Briton-Robinson buffer solution was used as the buffer to adjust pH. As shown in Fig. 4. 26, the highest specific activity was at pH 5.0. This pH was then used for other conditions.

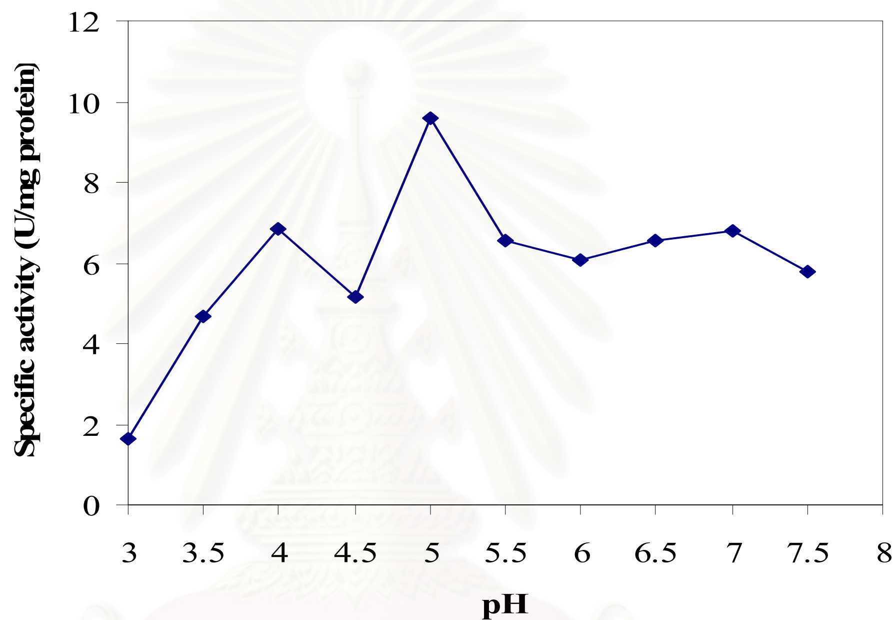


Figure 4.26. The optimum pH of purified AG. Briton - Robinson buffer at various pHs ranging between 3.0 - 7.5 was used. The optimum pH was 5.0.

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4.8.2 Optimum temperature

The result of optimum pH (pH 5.0) for AG activity was used for this experiment. Acetate buffer (pH 5.0) containing 0.1 M sucrose was used as the substrate buffer. The highest specific activity was at 50°C as shown in Fig. 4.27. This temperature was further used for selective concentration of substrate.

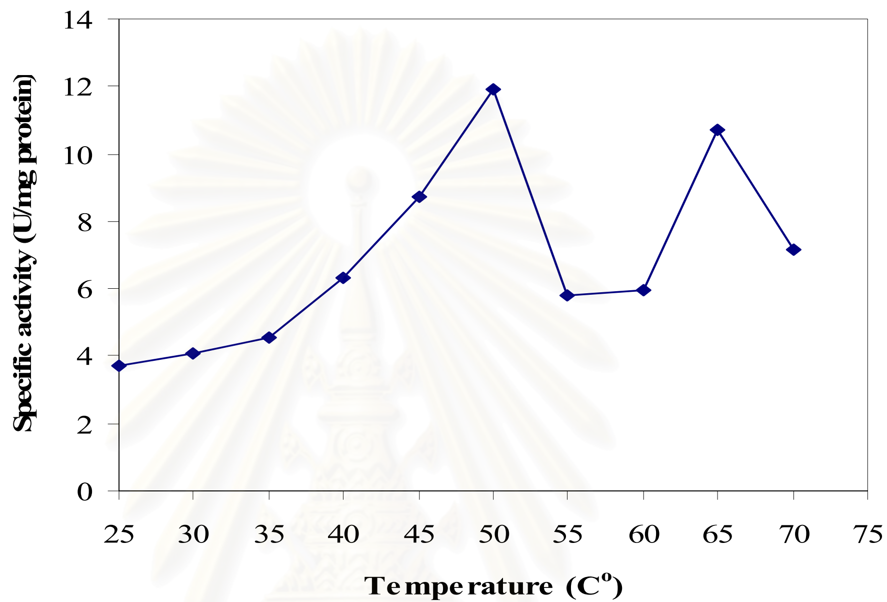

Figure 4.27. The optimum temperature of purified AG. The reaction mixture in acetate buffer (pH 5.0) containing 0.1 M sucrose was incubated at various temperatures ranging between 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C for 10 min. The optimum temperature was 50°C.

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4.8.3 Selective concentration of substrate

The results of optimum pH (pH 5.0) and optimum temperature (50°C) were used for this experiment. The mixture was prepared 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 60 mM sucrose (Fig. 4.28).

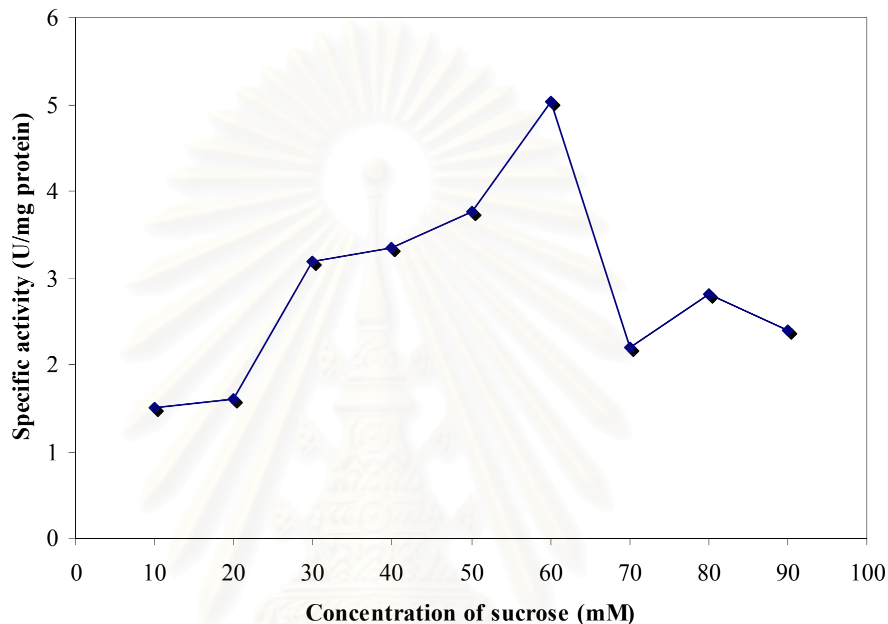

Figure 4.28. The optimum concentration of sucrose as substrate. The reaction mixture was incubated with sucrose at various concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM, respectively. The optimum concentration of sucrose was 60 mM.

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4.8.4 Optimum incubation time

The effect of incubation time was studied. The reaction mixture in acetate buffer (pH 5.0) containing 60 mM sucrose was incubated at 50°C. The incubation time was varied from 10, 20, 30, 40, 50, 60, and 70 min, respectively. The highest specific activity was obtained from the incubation time of 50 min (Fig. 4.29).

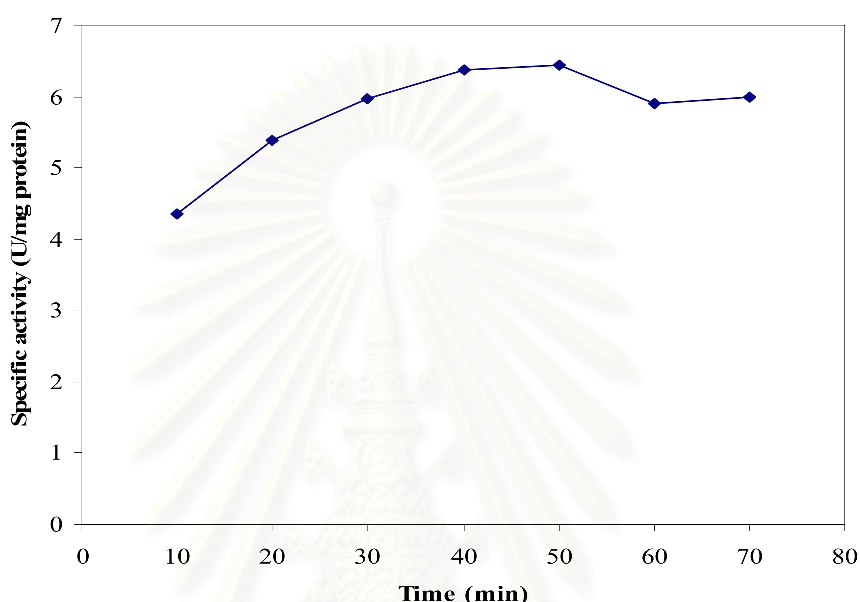

Figure 4.29. The optimum incubation time of purified AG. The reaction mixture was incubated for 10, 20, 30, 40, 50, 60, and 70 min, respectively. The optimum incubation time was 50 min.

4.9 Protein identification

A two - dimensional (2 – D) gel of AG with the different immobilized pH gradients (IPG; pH 3 – 10) was demonstrated in Fig. 4.30. A 2 – D gel was spotted by many types of protein with various molecular mass ranging between 14.4 – 97 kDa. There were more protein spots in CBB - stained gel (12.5% *T*, 2.6% *C*, and pH 3 – 10). Most of protein spots are grouped in the bottom region, which range between pH 4.0 – 8.0. The circle protein spot in Fig. 4.30 is interested, it is molecular mass ranging between 66 – 97 kDa and ranging of pH 6 – 7. Some of protein spots are grouped in right region, which is the basidic (hight pI) region. These can be presumed that most of proteins from *A. cerana* are

basidic proteins. The narrow pI range (pH 6 – 8) gel image reveals the protein much clearer than a wide pI range (pH 4 – 9) gel.

The protein bands of interest [Figs. 4.20B (lane 2) and 4.25A (lane 4)] were manually excised and in – gel digested with trypsin. In contrast, for in – solution digestion with trypsin, fraction no. 17 of Superdex 200 (bound peak from DEAE – cellulose) was used. The tryptic fragments from both digestions were used for protein identification via a peptide mass mapping technique. The amino acid of AG from *A. mellifera* digested by trypsin was simulated by the MassLynx software of BioLynx, Protein/ peptide editor (Appendix G). Therefore, both in – gel and in – solution digestions were used for the peptide mass mapping technique. MALDI – TOF mass spectra of tryptic fragments are shown in Appendix H. The significant peaks in mass spectra were shown in table 4.5 and Fig. 3.31.

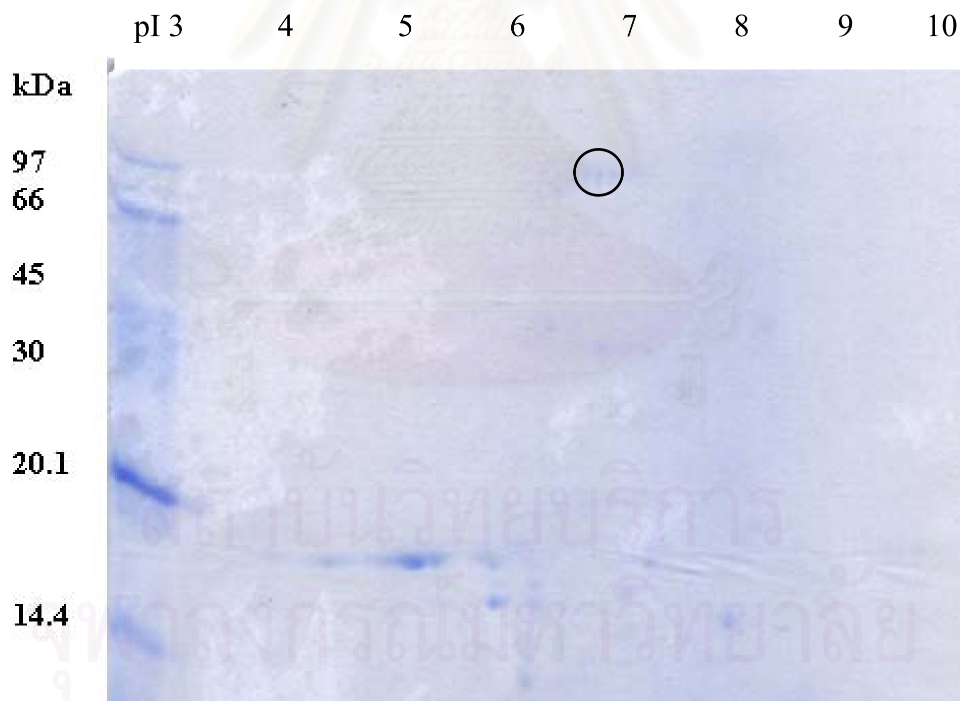

Figure 4.30. A 2 – D gel of AG by CBB – stained gel (12.5% *T*, 2.6% *C*, and pH 3 – 10).

Table 4.5. Peptide mass (Da) from MALDI – TOF analysis of trypsin – treated AG of *A. cerana* compared to tryptic fragments of AG in *A. mellifera,* +1 Da mass accuracy.

Observed Mass* (m/ z)			Sequences of tryptic fragments
A. mellifera [Mass (Da)]	*A. cerana* [Mass (Da)]		
	In – gel	In – solution	
530.32	350.97	530.59	IVNGK
609.29		608.36	FGEEK
641.33	641.87		SPYFK
686.43	686.56	686.31	IVNGKR
693.43		693.36	FASLKK
698.33		698.9	GFDGFR
754.40		754.20	TEYTLK
766.37		766.09	VNENYK
777.45		777.41	FWLRR
789.43		789.27	AFNNVPK
817.42		816.68	EANLNTR
836.43		836.38	FGEEKAR
845.47	845.71		TVNLAAEK
973.57		973.95	TVNLAAEKK
1028.45		1027.59	DSNSSDFKK
1034.48		1033.59	NSFFNMFK
1064.57		1064.25	LVSRFGEEK
1467.67		1468.27	ENYQTMSRDPAR
1756.93		1757.53	EDLIVYQVYPRSFK

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1 MKAVIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD

51 IEGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS

101 DLDNLVSAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP

151 GK**IVNGKR**VP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV

201 VLDDMQNVLR FWLRRGFDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE

251 YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY

301 GADFPFNFAF IKNVSRDSNS SDFKKLVDNW MTYMPPSGIP NWVPGNHDQL

351 RLVSRFGEEK ARMITTMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG

401 CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYK**TVNLA**

451 **AEK**KDKNSFF NMFKK**FASLK** K**SPYFK**EANL NTRMLNDNVF AFSRETEDNG

501 SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YNNFNSDIKS ISNNEQVKVS

551 ALGFFILISQ DAKFGNF

Figure 4.31. From NCBI blast search, it indicates an amino acid sequence of JC4714 alpha – glucosidase (EC. 3.2.1.20) – honeybee by using the mascot search. The underline amino acid sequences are derived from in – solution digestion. For in – gel digestion, matched peptides are shown in bold.

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CHAPTER V

DISCUSSION

5.1 The cDNA sequence of *AG* in *A. cerana*

Due to expression profile of alpha – glucosidase (*AG*), *AG* is highly expressed in foragers (Srimawong, 2003). Total RNA was isolated from hypopharyngeal glands (HPGs) of forager bees. HPGs were obtained by dissection of bee heads under stereomicroscope. Foragers were collected while they returned back to a hive. It is because the role of bees can change if necessary. HPGs seem to exist in two distinct differentiation states susceptible to age – dependent changes. Nurse bees take care of brood by producing royal jelly while forager bees forage nectar and process it into honey (Ohashi *et al*., 1997). Since RNA is unstable and degraded rapidly, the quality of RNA is checked by 2 types of electrophoresis, native agarose and formaldehyde gels. Two ribosomal RNA bands of 18S and 28S or at least one band of 28S rRNA have to be visible because 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 purity of RNA was indicated by the ratio of O.D. 260/ 280 which should be 2.0. It could be assumed that the nucleic acid concentration is negligible (Bollag *et al*., 1996). After electrophoresis, 18S and 28S rRNA bands were visible on agarose gel (Fig.4.1A) while only 28S rRNA band was observed on formaldehyde gel (Fig. 4.1B). Shaper band was observed on a formaldehyde gel since secondary structure of RNA is inhibited.

For RT – PCR, various primers were designed from the *AG* sequence in *A. mellifera* (D79208) as in Appendix C. Under the optimum condition, RT – PCR products at expected sizes were obtained (Fig. 4.2). According to functional primers, it can be preliminary assumed that there is a closed relationship of *AG* in *A. cerana* and *A. mellifera*.

5.2 Partial cDNA sequence and phylogenetic trees

The *AG* cDNA (1,740 bp) and deduced amino acid (567 amino acids) sequences were obtained (Figs. 4.4 and 4.5, respectively). The homology of 96% was estimated when it is compared to the *AG* sequence of *A. mellifera*. When the sequence is compared to *AG* cDNA sequence from other organisms, the *AG* sequence of *A. cerana* is partially similar to the sequences of maltase 1 in *A. mellifera*, maltase in *D. melanogaster* (Vieir *et*

al., 1997). This data coincides to the discussion in 4.1 that *A. mellifera* and *A. cerana* are closed.

Considering phylogenetic trees constructed by UPGMA and NJ (PAUP 4.0b), dextran glucosidase (*dexB*) of *Lactobacillus sakei* was used as an outgroup. *L. sakei* is the only selected organism that is a microorganism while other selected species are insects. To investigate a support for node estimation, bootstrap analysis with 1000 replicates was undertaken in PAUP 4.0b (heuristic search). Confidence probabilities were calculated for branch lengths of the neighbor – joining tree based on standard error test (Arias *et al.,* 2005). The result of both phylogenetic trees still confirms the result that *A. mellifera* and *A. cerana* are closed (Figs 4.8 and 4.9).

5.3 Denaturation and renaturation of AG

Crude from HPGs and honey crop was seperated by SDS – PAGE and renatured by Triton X – 100 in order to determine the activity. Although, many major protein bands were observed on SDS – gel but only one positive band is detected on an activity gel (Fig. 4.11). It indicates that there are AG in both HPGs and honey crop. Considering the graph of Rf value and log MW of protein marker, MW of 2 common protein bands in HPGs and honey crop were estimated approximately to be 50 and 75 kDa (Fig. 4.10). Due to Figs. 4.10 and 4.11, MW of AG may be assumed to be a little bit above 75 kDa. Protein at the MW of 50 and 75 kDa were also detected in crude in *A. mellifera*. These proteins may be common proteins found in HPGs of forager bees. However, a major 70 kDa protein synthesized specifically in HPGs of forager bees was identified as AG (Kubo *et al*., 1996). This 70 kDa band was also kept in mind that it might be our AG in *A. cerana* but in 2003, Srimawong proposed that AG in HPGs should have molecular mass at 96 kDa. Alternatively, if we consider AGI, AGII, and AGIII in *A. mellifera* (98, 76, and 68 kDa, respectively), AGI and AGII might be our targets (Nishimoto *et al*., 2001 and Kubota *et al*., 2004).

5.4 AG purification

In order to get rid off small protein, crude was precipitated by various ammonium sulfate (AS) saturation. As shown in table 4.2 and Fig. 4.12, highest specific activity was obtained in precipitate of 80 - 95% AS (1.36 u/ mg). The more AS saturation, the higher specific activity of AG. Surprisingly, unprecipitate contains high specific activity of AG (0.87 u/ mg). The data indicate that suitable AS saturation is required to optimize the

specific activity. Otherwise AS may inhibit the activity. Considering Fig. 4.13, more small proteins were observed in unprecipitate. In addition, common protein of 75 kDa was observed in all samples. This may interpret that AS can not harm it unlike the 100 kDa protein.

Dialyzed suspension was subjected onto DEAE – cellulose which will absorb cation exchanger (-) (Jenson and Ryden, 1998). After elution, there are 2 peaks of unbound (peak I) and bound peaks (peak II) as in Fig. 4.14. It is possible that AG can not bind to the DEAE – cellulose well enough. That may be because 30 mM sodium phosphate buffer (pH 6.3) may interfere negative charge of protein. Unbound fraction was transferred to CM – cellulose. Both unbound and bound peaks are still observed although higher bound peak was visible. All fractions contain AG activity at the similar amount (Fig. 4.15). It is possible that charges of protein change so much from being in 30 mM sodium phosphate buffer (pH 6.3) to be 100 mM sodium acetate buffer (pH 4.7). It would have been better if AG had been in 100 mM sodium acetate buffer (pH 4.7) before passed through CM – cellulose.

It is very satisfying to separate bound peak (after DEAE – cellulose) on Superdex 200 (gel filtration). The only bound peak (fraction no. 17) has a highest activity (1.804 u/ mg) as in Fig. 4.16. Also, the only band of AG is appeared on SDS – PAGE and CBB stain (Fig. 4.20B). Due to the graph of Rf value and log MW of protein marker, the expected MW of AG is approximately 68 kDa. The MW of expected AG is coincided to AGIII reported in Nishimoto *et al*. (2001) and JBGII in Wongchawalit *et al*. (2005). From the above data, it is sufficient to conclude that we obtained purified AG in fraction no. 17 of Superdex 200.

Since unbound peak after DEAE – cellulose contain AG activity, it may be because we loaded too much protein through the column. Thus, we loaded this unbound peak to Sepahadex 200 as well. The almost similar result to Fig. 4.16 was obtained in Fig. 4.17 but it is odd that the activity peak came out after a protein peak although high AG activity (1.032 u/ mg) was assayed.

Considering table 4.2 and Fig. 4.12, it is interesting to determine whether AS and dialysis have an effect on AG activity or not. Thus, we loaded unprecipitate (0.627 u/ mg) directly to DEAE – cellulose and directly to CM – cellulose. From DEAE – cellulose, only one unbound peak was obtained as in Fig. 4.18. The similar activity (0.757 u/ mg) was obtained. It can be concluded that protein can not bind to the column at all. Thus, unprecipitate crude is not suitable for the procedure. The same result was also obtained by

loading unprecipitate directly to CM – cellulose. Although one unbound and one bound peaks were assayed, high activity (0.530 u/ mg) was in unbound peak (Fig. 4.19). Considering results in Figs. 4.18 and 4.19, it represents that unprecipitate was not good for go on purification. It is possible that there are too much protein in unprecipitate and they interfere the purification procedure.

5.5 Optimum conditions of AG

Momose's method was chosen for AG assay. Also, 3, 6 – dinitrophthalic acid was used as a synthetic substrate because it can provide a very sensitive color in a reaction with a reducing sugar (Kubo *et al.,* 1996). The reagent was dissolved in alkaline solution and 3, 6 – dinitrophthalic acid solution. After being heat, a small amount of reducing sugar will provide a deep reddish – wine color within a few min (Momose and Inaba, 1961). Various conditions had been tried and three replications of all experiments were performed. The average data were used for analysis. The optimum pH of purified AG is 5.0 (Fig. 4.26). It is as same as optimum pH of AG in crude (Srimawong, 2003). Comparing optimum temperature, it is higher in purified AG (50°C) than AG in crude (45°C) as in Fig. 4.27. The optimum incubation time is also different in both sources. The optimum incubation time is 50 min in purified AG while it is only 30 min in crude AG (Fig. 4.29). Moreover, in order to determine a selective concentration of substrate, sucrose was used as a representative substrate (Sasagawa *et al*., 1989 and Kubo *et al*., 1996). The result presents that the best selective concentration of sucrose for purified AG is 60 mM but the best one for crude AG is 50 mM (Fig. 4.28). It may interpret that there are more than one type of AG in crude including many enzymes those can perform the same activity as AG. That makes the optimum temperature, incubation time, and selective concentration of sucrose lower than those in purified AG.

5.6 Protein identification

In order to identify proteins by 1 - D or 2 - D gels and trypsin digestion, it is required that a sample must be concentrated and free from salt or detergent. Otherwise high quality mass spectrum may not be addressed (Sheer *et al*., 1997). In Fig. 4.30, on 2 – D gel and CBB stain, there are many spots in MW ranging between 14 kDa and 66 kDa. Light spots may be involved in low protein amount. If the gel had been silver stained, there might have been more visible protein spots on the gel. The protein dot in a circular

mark was interesting because its molecular mass ranging between 66 – 97 kDa and ranging of pH 6 – 7 is coincided to the activity band from Fig. 4.10. It might be our AG. Later, the AG candidates were excised and peptide analysed by MALDI – TOF MS. The AG candidates are the protein bands in Figs. 4.20B (lane 2) and 4.25A (lane 4) and fraction no. 17 of Superdex 200 (bound peak from DEAE – cellulose). The MALDI – TOF was performed by the database searching via the MASCOT program (www.matrixscience.com). The search parameters including +1 Da mass tolerance, 1 missed cleavage, modifications, trypsin enzyme, and NCBI database were selected. Peptides and peptides profiles were compared to those in other organisms. A total of mass matching ion could be confidently assigned to peptides predicted from the *A. mellifera* database. The search results showed reasonable matching protein in Table 4.5. Obtained lower peaks can't show the search results (monoisotopic masses [M + H]+ in agreement, Appendix H). It might be because of too low protein amount to detect by MS. That is why there are only 4 matching masses with 4% coverage from in – gel digestion while there are up to 18 matching masses with 19% coverage from in – solution digestion. Although the amount of protein is very low but it is still enough to be analysed. At present, this technique is very popular to analyse peptide. Pontoh and Low (2001) used MALDI – TOF to analyse beta – glucosidase from HPGs of *A. mellifera*.

In summary, the cDNA of AG from HPGs of forager bees (*A. cerana*) was partially identified by RT – PCR, peptide sequencing and deduced amino, a protein engine identification tool applied to the honeybee genome. While, AG purified were partially identified by using gel filtration, peptide sequencing by MALDI – TOF and compared to these AG already identified in the proteome complement of the honeybee (Santos *et al*., 2005).

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CHAPTER VI

CONCLUSIONS

1) Various primers of alpha – glucosidase (*AG*) for RT – PCR were designed from *AG* in *A. mellifera.* Under the optimum condition, the length of 1,740 bp was obtained. The similarity of the sequence to that in *A. mellifera* is 96%, to maltase 1 in *A. mellifera* at 53.44%, to maltase in *Culicoides sonorensis* at 48.75%, and to *CG* in *Drosophila melanogaster* at 53.66%.

2) The deduced amino acid of AG (567 amino acid) in *A. cerana* was obtained.

3) Due to phylogenetic trees of amino acid sequence by UPGMA and NJ, it supports the data of blast. It can be summarized that *A. cerana* AG is mostly similar to *A. mellifera* AG.

4) Crude protein was extracted and determined by Bradford's assay (Bradford, 1976). There is less than 0.33 g/ g protein in hypopharyngeal glands (HPGs) but there is 1.03 g/ g protein in honey crop. Due to SDS – PAGE, different patterns of crude protein from both sources were obtained. Protein at MW of about 75 kDa was found in both sources while protein at MW of 50 kDa was found only in HPGs crude. After renaturation of AG in crude, a positive band at the MW of 75 kDa was visible.

5) The AG activity (by Momose's method) was assayed from honeybee worker (430 g). They were homogenized to be crude (0.696 u/ mg), precipitated with 95% ammonium sulfate (0.235 u/ mg), and purified by DEAE - cellulose (2.171 u/ mg), CM - cellulose (0.154 u/ mg), and Superdex 200 gel filtration chromatographies (1.804 u/ mg). The activity fold was 0.34, 3.11, 0.22, and 2.59, respectively.

6) According to positive fractions of Superdex 200, mass weight of AG was clarified to be 68 kDa.

7) The optimum pH, temperature, and incubation time for AG activity were at 5.0, at 50°C, and for 50 min, respectively. The proper concentration of sucrose for AG activity was 60 mM.

8) Unprecipitated crude of *A. cerana* was separated by 2 – D electrophoresis. Pattern was obtained in a range of pH 4.0 – 8.0.

9) AG was analyzed by in – gel and in – solution digestions (or trypsin digestion) and peptide analysed by MALDI/ TOF MS. The peptide masses showed that there are at least 4 matching masses with 4% coverage matched to AG in NCBI blast search (score of 37). Furthermore, there are at least 18 matching masses with 19% coverage matched to AG in NCBI blast search (score of 153). The peptide sequence is corresponded to the amino acid sequence of AG in *A. mellifera*.

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APPENDICES

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Appendix A: Preparation of solutions

A. 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.

B. Briton – Robinson buffer

1) 1 M Acetic acid (10 mM)	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.

C. Bradford solution

1. Bradford stock solution

95% EtOH	100 ml
88% H_3PO_4 (phosphoric acid)	200 ml
Serva Blue G	350 mg

Stable indefinitety at RT

2. Bradford Working buffer

distilled water	85 ml
95% EtOH	3 ml
88% H_3PO_4 (phosphoric acid)	6 ml
Bradford stock solution	6 ml
Total	100 ml

Filter solution through at 0.45 μm filter. Store at RT.

3. BSA stock solution

BSA	0.01 g
Distilled water	1 ml

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D. 10% SDS

SDS (10% w/v)	5.0 g
dd - H_2O	to 50 ml

Filter solution through a 0.45 µm filter. Store at RT.

E. 10% ammonium persulfate (APS)

APS (10% w/v)	0.05 g
dd - H_2O	to 500 µl

Fresh ammonium persulfate prepared just prior to use.

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Preparation for polyacrylamide gel electrophoresis

A. 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.

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B. Single-percentage gel recipes (followed in principle method; Amersham biosciences)

Final Gel Concentration	10%	12.5%
30% Acrlamide/Bis	3.33 ml	4.17 ml
Gel buffer*	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
dd.H_2O	4.02 ml	3.18 ml
10% APS	50 µl	50 µl
TEMED	3.3 µl	3.3 µl
Total volume	10 ml	10 ml

* Separating gel buffer – 1 M Tris-HCl, pH 8.8 or stacking gel buffer – 0.5 M Tris-HCl, pH 6.8

SDS-PAGE followed from Srimawong (2003)

1) 12% Separting 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% APS	75 µl
TEMED (0.05%)	7.5 µl

2) 4% Stacking gel

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

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3) Sample buffer (5X loading dye)

1 M Tris-HCl, pH 6.8 (0.312M)	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	0.1 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 water 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

Adjust volume to be 1 litre by d.H_2O and adjusted pH to be approximately 8.3

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Preparation for In-gel digestion

A. 50% ACN/0.1 M NH_4HCO_3

0.2 M NH_4HCO_3 (M.W. 79.06)	1.581 g in 100 ml of milli Q water
0.1 M NH_4HCO_3 in 50% ACN	
0.2 M NH_4HCO_3	50 ml
50% ACN	50 ml

B. 10 mM DTT/ 0.1 M NH_4HCO_3/ 1 mM EDTA

0.1 M NH_4HCO_3	10 ml
10 mM DTT (M.W. 154.84)	15.482 mg
1 mM EDTA (M.W. 292)	2.92 mg

C. 100 mM IAA/0.1 M NH_4HCO_3 (freshly)

0.1 M NH_4HCO_3	1 ml
100 mM IAA (M.W. 185)	18.5 mg

D. 0.05 M Tris-HCl buffer, pH 8.5 / 50% CAN

0.1 M Tris	1.2114 g

added 100 ml milli Q water and adjusted to be pH 8.5 with 1 N HCl.

0.1 M Tris-HCl buffer, pH 8.5	50 ml
50% ACN	50 ml

E. Extraction buffer (digestion buffer)

trypsin solution (10 µl of trypsin in 1% acetic acid and 90 µl of trypsin buffer containing 50 µl of 0.1 M Tris-HCl, pH 8.5, 1 µl of 100 mM $CaCl_2$, 10 µl of ACN, and 39 µl of distilled water)

F. Trypsin solution

Component	Amount
Trypsin	1 mg
1% CH_3COOH	1 ml

G. 2% TFA

Component	Amount
TFA	0.1 ml
dd – H_2O	4.9 ml

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Phosphate (Sodium) buffer Chart

1. Stock solution A

    2 M monobasic sodium phosphate, monohydrate ($NaH_2PO_4.H_2O$ = 137.99)

$NaH_2PO_4.H_2O$	276 g
dd – H_2O	1 L

2. Stock solution B

    2 M dibasic sodium phosphate (Na_2HPO_4 = 141.96)

Na_2HPO_4	284 g
dd – H_2O	1 L

Mixing an appropriate volume (ml) of A and B as shown in the table below and diluting to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

A	**B**	**pH**
92.0	8.0	0.8
90.0	10.0	5.9
87.7	**12.3**	**6.0**
85.5	15.0	6.1
81.5	19.5	6.2
77.5	**22.5**	**6.3**
73.5	26.5	6.4
68.5	**31.5**	**6.5**
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	**61.0**	**7.0**

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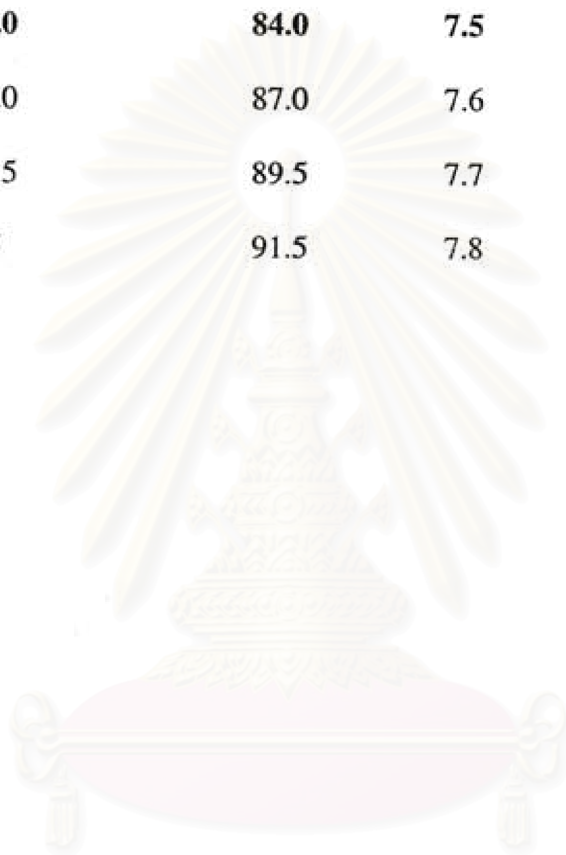
A	B	pH
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	**84.0**	**7.5**
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8



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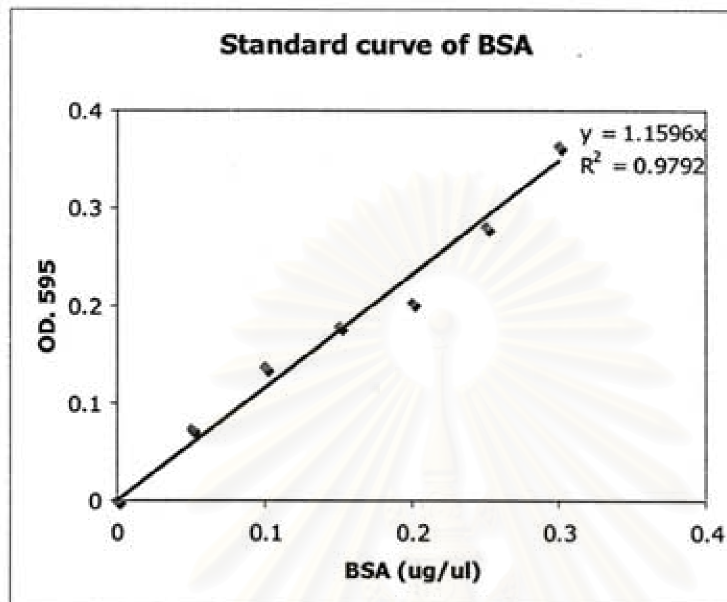
Appendix B: Standard curve of BSA (Bovine Serum Albumin)



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Appendix C: Nucleotide sequence of AG in *A. mellifera*
(accession number: D79208 in GenBank)

FW1 primer

```
   1 tgatattaac gtactactat taatatatTC GACTTCTAGT TGGTAGCATG AAGGcagtaa
  61 tcgtattttg ccttatggca ttgtccattg tggacgcagc atggaagccg ctccctgaaa
 121 acttgaagga ggacttgatc gtgtatcagg tctacccgag aagcttcaag gatagcaatg
 181 gagatggtat tggtgatatc gaaggtatta aagaaaaatt ggatcatttt ctcgaaatgg
 241 gggtcgacat gttttggtta tcccctattt atccaagccc tatggtcgat tttggttacg
 301 acatttcgaa ttacaccgac gttcatccca tatttggcac catatcagac ttagataatC
```

R1 primer

```
 361 TAGTCAGTGC TGCACATGAG AAAGGattga agataatctt ggatttcgtc ccgaatcata
 421 catctgatca acacgaatgg ttccagttga gtttgaaaaa cattgaacct tataacaact
 481 attacatttg gcatccagga aaaattgtaa atggcaaacg tgttccacca actaattggg
 541 taggcgtgtt tggtggatca gcttggtcgt ggcgggaaga acgacaggca tattatctgc
 601 atcaatttgc accagaacaa ccagatctaa attactataa tccagttgta ctggatgata
 661 tgcaaaatgt tctcagattc tggctgagaa ggggatttga tggtttcaga gtagatgctc
 721 tgccttacat ttgcgaagac atgcgattct tagacgaacc tctatcaggt gaaacaaatg
 781 atcccaataa aaccgagtac actctcaaga tctacactca cgatatccca gaaacctaca
 841 atgtagttcg caaatttaga gatgtgttag acgaattccc gcaaccaaaa cacatGCTTA
```

FW2 primer

```
 901 TCGAGGCATA CACGAattta tcgatgacga tgaaatatta cgattacgga gcagattttc
 961 ccttcaattt tgcattcatc aagaatgttt ctagggattc aaattcatca gacttcaaaa
```

R2 primer

```
1021 aattggtcga taattggatG ACGTACATGC CACCAAGTGg tattcctaac tgggtgcccg
1081 gaaatcacga tcaattgaga ttggtgtcga gatttggaga ggagaaggcc cgtatgatca
1141 ccacgatgtc gcttttgctg ccaggtgttg ccgtgaatta ctacggtgat gaaattggta
1201 tgtcggatac ttatatctcg tgggaggata cgcaggatcc gcagggatgc ggcgccggta
1261 aagaaaacta tcaaacgatg tcgagagatc ccgcgagaac gccattccaa tgggacgact
1321 cagtttctgc tggattttcc tcaagctcta atacctggct tcgtgtcaac gaaaattaca
1381 agactgtcaa tctagctgct gaaaagaagg acaagaactc gttcttcaat atgttcaaga
1441 aatttgcgtc gctgaaaaaa tcgccatact ttaaagaggc caatttaaat acgaggatgc
1501 tgaacgacaa tgttttcgca ttctctaggg aaaccgaaga taatggatct ctttacgcaa
```

FW3 primer

```
1561 tattgaactt ctcgaACGAG GAACAAATCG TGGATttgaa agcgttcaat aacgtgccga
1621 aaaaattgaa tatgttttac aacaatttta actctgatat aaagtccatc tccaacaatg
1681 aacaagtaaa agtttctgct ttaggatttt tcatcttaat ttctcaagat gctaaatttg
```

R3 primer

```
1741 gaaactttta atttcttcct gaatatGTCT ATTCTTTGAA GCGGCGaaag gaaacatata
1801 tcgttaaaat ctctctatat tattatatat atatatatgt attagctaat aaattttaaa
1861 tattttgaaa cgtaaaaaaa aaaaaaaaaa aa
```

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Appendix D: The table of quantities of ammonium sulfate per 100 ml of solution required to reach given degree of saturation at $0^{\circ}C$.

Initial concentration of ammonium sulfate (% saturation at 0°C)	Final concentration of ammonium sulfate (% saturation at 0°C)																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	solid ammonium sulfate to add to 100 ml of solution																
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
55								0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
65										0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.2	6.6	10.1	13.7	17.4
80													0	3.3	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7.0
95																0	3.5
100																	0

Note: The pH of the solution may decrease significantly on addition of ammonium sulfate.

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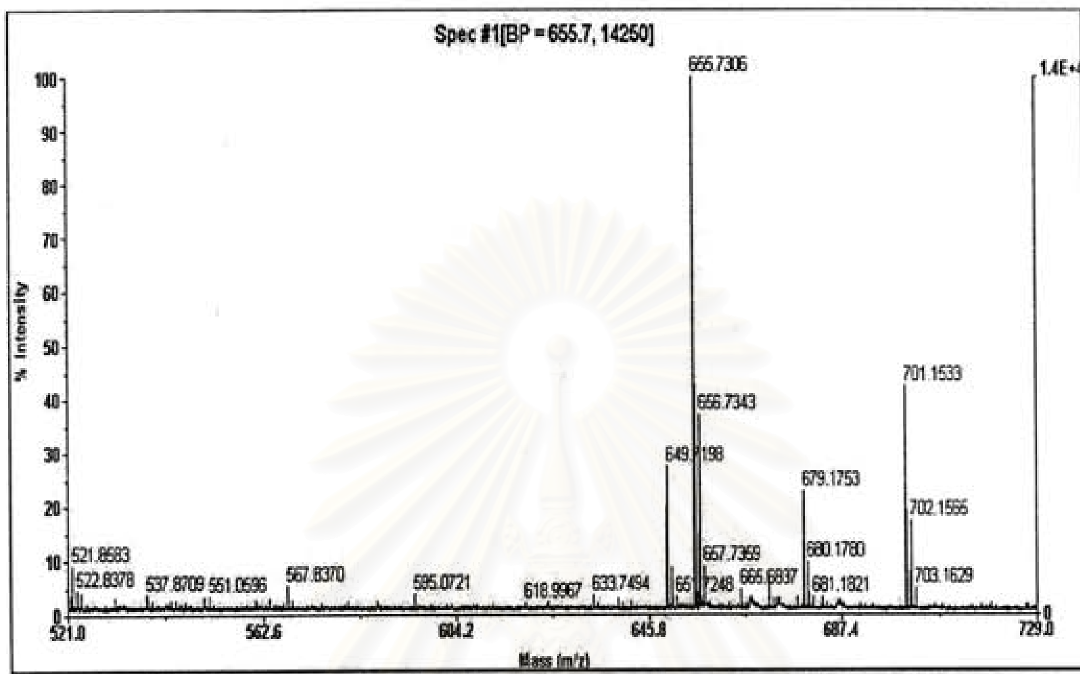
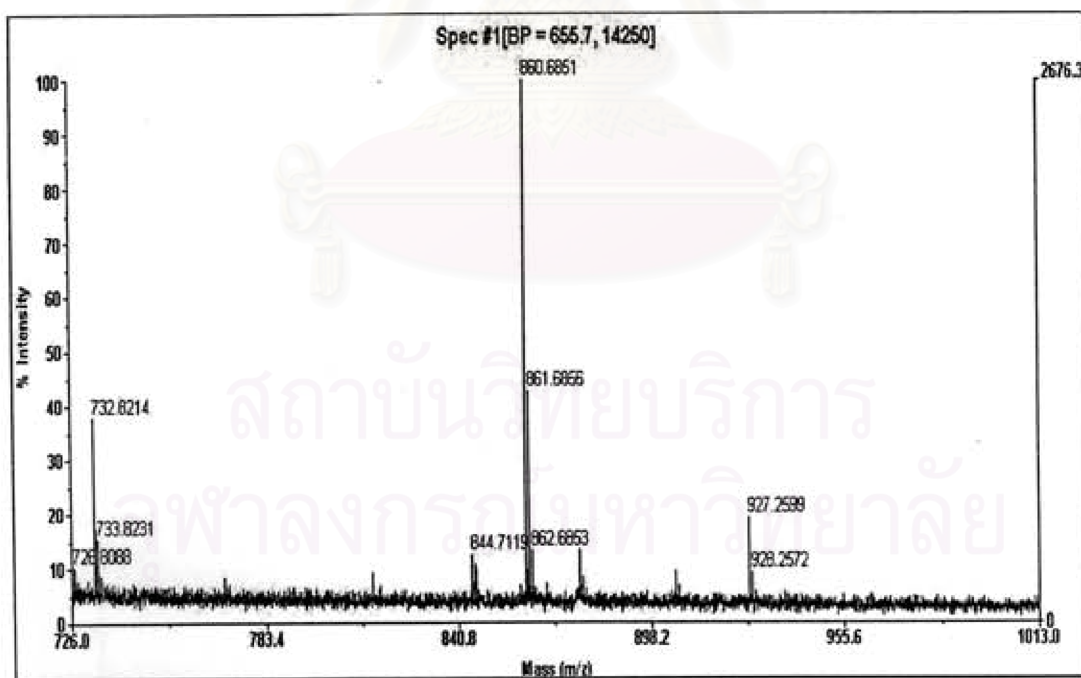
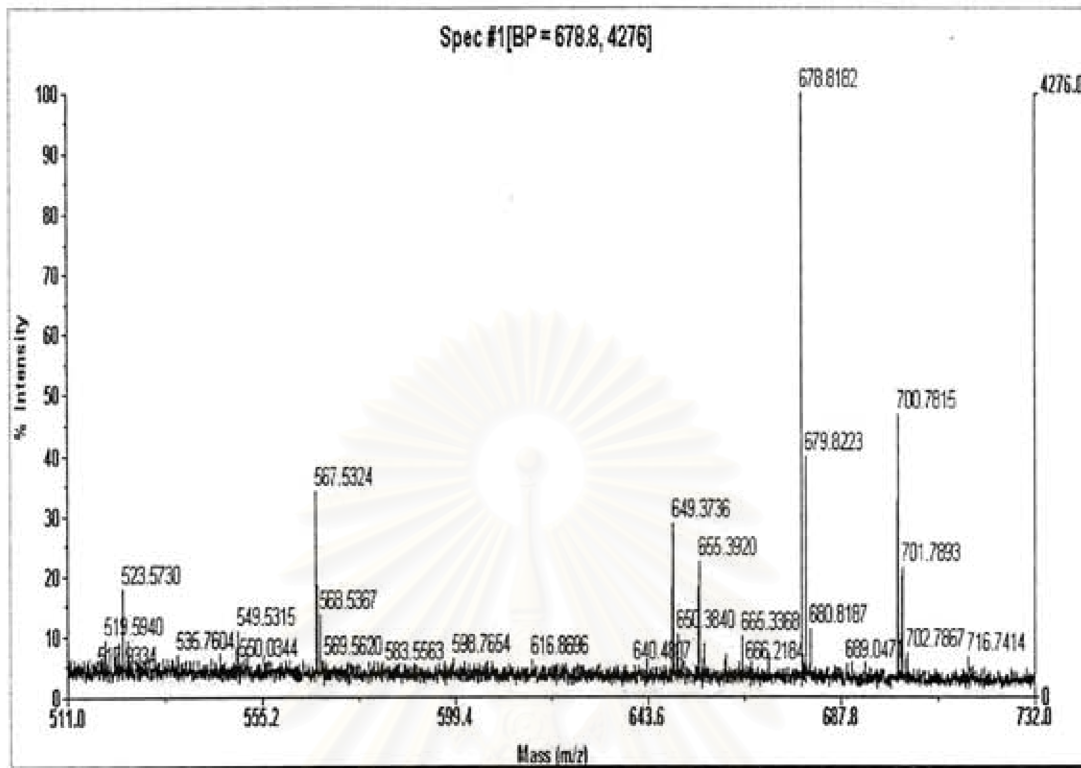
Appendix E: MALDI – TOF mass spectra of tryptic fragments

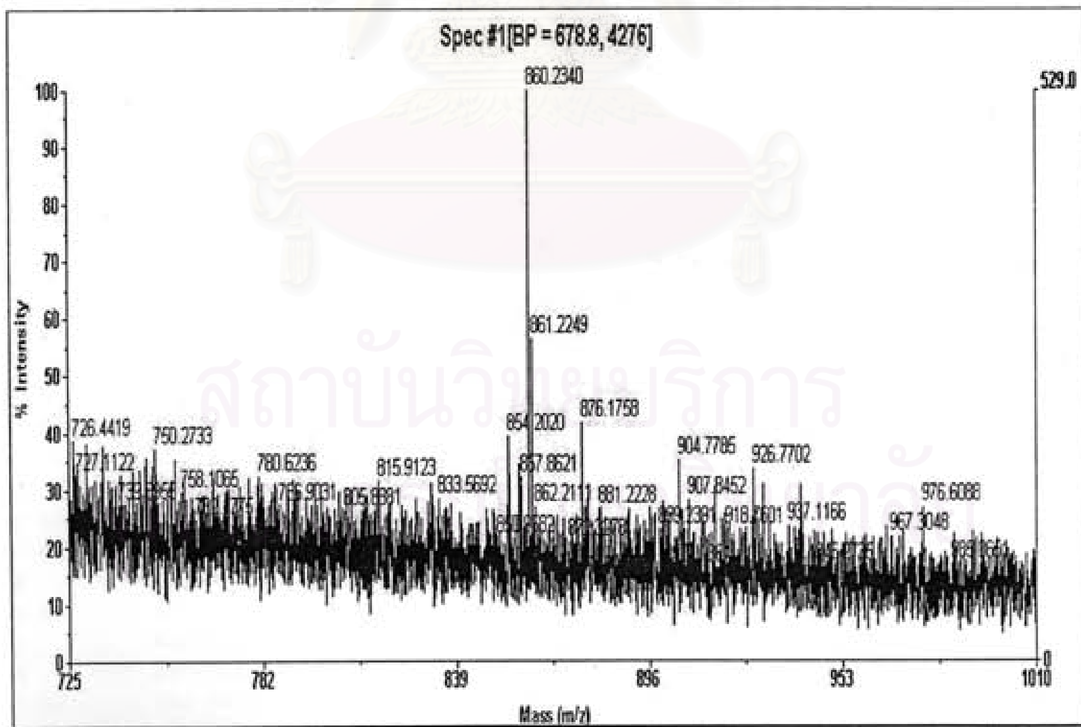
A) Mass spectra of tryptic fragments from in – gel digestion.





B) Mass spectra of tryptic fragments from in – solution digestion





Appendix F: The stimulation of tryptic fragments.

Alpha Glucosidase (Q17058) Mw.65565 Da
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T23	269-269	(R)K(F)	146.11	147.11	74.06	49.71
T46	471-471	(K)K(S)	146.11	147.11	74.06	49.71
■T41	454-454	(K)K(D)	146.11	147.11	74.06	49.71
T52	526-526	(K)K(L)	146.11	147.11	74.06	49.71
T44	465-465	(K)K(F)	146.11	147.11	74.06	49.71
T12	158-158	(K)R(V)	174.11	175.12	88.06	59.05
T17	215-215	(R)R(G)	174.11	175.12	88.06	59.05
T34	361-362	(K)AR(M)	245.15	246.16	123.58	82.72
T42	455-456	(K)DK(N)	261.13	262.14	131.57	88.05
T6	56-57	(K)EK(L)	275.15	276.16	138.58	92.72
T1	1-2	(-)MK(A)	277.15	278.15	139.58	93.39
T8	113-115	(K)GLK(I)	316.21	317.22	159.11	106.41
T24	270-271	(K)FR(D)	321.18	322.19	161.60	108.07
T4	39-41	(R)SFK(D)	380.21	381.21	191.11	127.74
T41-42	454-456	(K)KDK(N)	389.23	390.24	195.62	130.75
T14	177-179	(R)EER(Q)	432.20	433.20	217.11	145.07
T23-24	269-271	(R)KFR(D)	449.28	450.28	225.65	150.77
T37	414-417	(R)DPAR(T)	457.23	458.24	229.62	153.42
T32	352-355	(R)LVSR(F)	473.30	474.30	237.66	158.77
T28	313-316	(K)NVSR(D)	474.26	475.26	238.14	159.09
T56	564-567	(K)FGNF(-)	483.21	484.22	242.61	162.08
T11	153-157	(K)IVNGK(R)	529.32	530.33	265.67	177.45
T45	466-470	(K)FASLK(K)	564.33	565.33	283.17	189.12
T33	356-360	(R)FGEEK(A)	608.28	609.29	305.15	203.77
T16	211-214	(R)FWLR(R)	620.34	621.35	311.18	207.79
T47	472-476	(K)SPYFK(E)	640.32	641.33	321.17	214.45
T11-12	153-158	(K)IVNGKR(V)	685.42	686.43	343.72	229.48
T45-46	466-471	(K)FASLKK(S)	692.42	693.43	347.22	231.82
T44-45	465-470	(K)KFASLK(K)	692.42	693.43	347.22	231.82
T18	216-221	(R)GFDGFR(V)	697.32	698.33	349.67	233.45
T21	249-254	(K)TEYTLK(I)	753.39	754.40	377.70	252.14
T39	440-445	(R)VNENYK(T)	765.37	766.37	383.69	256.13
T46-47	471-476	(K)KSPYFK(E)	768.42	769.42	385.22	257.15
T16-17	211-215	(R)FWLRR(G)	776.44	777.45	389.23	259.82
T51	519-525	(K)AFNNVPK(K)	788.42	789.43	395.22	263.81
T48	477-483	(K)EANLNTR(M)	816.41	817.42	409.21	273.14
T33-34	356-362	(R)FGEEKAR(M)	835.42	836.43	418.72	279.48
T40	446-453	(K)TVNLAAEK(K)	844.47	845.47	423.24	282.50
T17-18	215-221	(R)RGFDGFR(V)	853.42	854.43	427.72	285.48
T29	317-324	(R)DSNSSDFK(K)	898.37	899.37	450.19	300.46
T51-52	519-526	(K)AFNNVPKK(L)	916.51	917.52	459.26	306.51
T40-41	446-454	(K)TVNLAAEKK(D)	972.56	973.57	487.29	325.19
T54	540-548	(K)SISNNEQVK(V)	1017.51	1018.52	509.76	340.18
T29-30	317-325	(R)DSNSSDFKK(L)	1026.46	1027.47	514.24	343.16
T36	406-413	(K)ENYQTMSR(D)	1027.44	1028.45	514.73	343.49
T43	457-464	(K)NSFFNMFK(K)	1033.47	1034.48	517.74	345.50
T32-33	352-360	(R)LVSRFGEEK(A)	1063.57	1064.57	532.79	355.53
T43-44	457-465	(K)NSFFNMFKK(F)	1161.56	1162.57	581.79	388.20
T25	272-281	(R)DVLDEFPQPK(H)	1186.59	1187.59	594.30	396.54
T42-43	455-464	(K)DKNSFFNMFK(K)	1276.59	1277.60	639.30	426.54
T49	484-494	(R)MLNDNVFAFSR(E)	1312.62	1313.63	657.32	438.55
T28-29	313-324	(K)NVSRDSNSSDFK(K)	1354.61	1355.62	678.31	452.54
T5	42-55	(K)DSNGDGIGDIEGIK(E)	1388.64	1389.65	695.33	463.89
T3	28-38	(K)EDLIVYQVYPR(S)	1393.72	1394.73	697.87	465.58

T19	222-233	**(R)VDALPYICEDMR(F)**	1423.65	1424.66	712.83	475.56
T47-48	472-483	**(K)SPYFKEANLNTR(M)**	1438.72	1439.73	720.37	480.58
T36-37	406-417	**(K)ENYQTMSRDPAR(T)**	1466.66	1467.67	734.34	489.89
T24-25	270-281	**(K)FRDVLDEFPQPK(H)**	1489.76	1490.76	745.89	497.59
T39-40	440-453	**(R)VNENYKTVNLAAEK(K)**	1591.82	1592.83	796.92	531.61
T55	549-563	**(K)VSALGFFILISQDAK(F)**	1607.89	1608.90	804.95	536.97
T53	527-539	**(K)LNMFYNNFNSDIK(S)**	1618.74	1619.75	810.38	540.59
T5-6	42-57	**(K)DSNGDGIGDIEGIKEK(L)**	1645.78	1646.79	823.90	549.60
T20	234-248	**(R)FLDEPLSGETNDPNK(T)**	1674.77	1675.78	838.39	559.27
T22	255-268	**(K)IYTHDIPETYNVVR(K)**	1718.86	1719.87	860.44	573.96
T52-53	526-539	**(K)KLNMFYNNFNSDIK(S)**	1746.84	1747.85	874.43	583.29
T4-5	39-55	**(R)SFKDSNGDGIGDIEGIK (E)**	1750.84	1751.85	876.43	584.62
T3-4	28-41	**(K)EDLIVYQVYPRSFK(D)**	1755.92	1756.93	878.97	586.31
T26	282-296	**(K)HMLIEAYTNLSMTMK(Y)**	1781.85	1782.86	891.93	594.96
T22-23	255-269	**(K)IYTHDIPETYNVVRK(F)**	1846.96	1847.97	924.49	616.66
T10	138-152	**(K)NIEPYNNYYIWHPGK(I)**	1906.90	1907.91	954.46	636.64
T27	297-312	**(K)YYDYGADFPFNFAFIK(N)**	1976.90	1977.91	989.46	659.97
T13	159-176	**(R)VPPTNWVGVFGGSAWSW R(E)**	2001.98	2002.99	1002.00	668.34
T55-56	549-567	**(K)VSALGFFILISQDAKFG NF(-)**	2073.09	2074.10	1037.55	692.04
T18-19	216-233	**(R)GFDGFRVDALPYICEDM R(F)**	2102.96	2103.96	1052.49	701.99
T48-49	477-494	**(K)EANLNTRMLNDNVFAFS R(E)**	2111.02	2112.03	1056.52	704.68
T12-13	158-176	**(K)RVPPTNWVGVFGGSAWS WR(E)**	2158.09	2159.09	1080.05	720.37
T20-21	234-254	**(R)FLDEPLSGETNDPNKTE YTLK(I)**	2410.15	2411.16	1206.08	804.39
T13-14	159-179	**(R)VPPTNWVGVFGGSAWSW REER(Q)**	2416.17	2417.18	1209.09	806.40
T10-11	138-157	**(K)NIEPYNNYYIWHPGKIV NGK(R)**	2418.21	2419.22	1210.11	807.08
T27-28	297-316	**(K)YYDYGADFPFNFAFIKN VSR(D)**	2433.14	2434.15	1217.58	812.06
T21-22	249-268	**(K)TEYTLKIYTHDIPETYN VVR(K)**	2454.24	2455.25	1228.13	819.09
T38	418-439	**(R)TPFQWDDSVSAGFSSSS NTWLR(V)**	2474.11	2475.12	1238.06	825.71
T54-55	540-563	**(K)SISNNEQVKVSALGFFI LISQDAK(F)**	2607.39	2608.40	1304.70	870.14
T53-54	527-548	**(K)LNMFYNNFNSDIKSISN NEQVK(V)**	2618.24	2619.25	1310.13	873.76
T9	116-137	**(K)IILDFVPNHTSDQHEWF QLSLK(N)**	2666.35	2667.36	1334.18	889.79
T50	495-518	**(R)ETEDNGSLYAILNFSNE EQIVDLK(A)**	2740.31	2741.32	1371.16	914.44
T2	3-27	**(K)AVIVFCLMALSIVDAAW KPLPENLK(E)**	2740.51	2741.51	1371.26	914.51
T37-38	414-439	**(R)DPARTPFQWDDSVSAGF SSSSNTWLR(V)**	2913.33	2914.34	1457.67	972.12
T25-26	272-296	**(R)DVLDEFPQPKHMLIEAY TNLSMTMK(Y)**	2950.43	2951.44	1476.22	984.48
T8-9	113-137	**(K)GLKIILDFVPNHTSDQH EWFQLSLK(N)**	2964.55	2965.56	1483.28	989.19
T1-2	1-27	**(-)MKAVIVFCLMALSIVDA AWKPLPENLK(E)**	2999.64	3000.65	1500.83	1000.89
T1-2	1-27	**(-)MKAVIVFCLMALSIVDA**	2999.64	3000.65	1500.93	1000.99

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		AWKPLPENLK(E)				
T31	326-351	**(K)LVDNWMTYMPPSGIPNWVPGNHDQLR(L)**	3036.44	3037.45	1519.23	1013.15
T19-20	222-248	**(R)VDALPYICEDMRFLDEPLSGETNDPNK(T)**	3080.41	3081.42	1541.21	1027.81
T30-31	325-351	**(K)KLVDNWMTYMPPSGIPNWVPGNHDQLR(L)**	3164.53	3165.54	1583.27	1055.85
T38-39	418-445	**(R)TPFQWDDSVSAGFSSSSNTWLRVNENYK(T)**	3221.47	3222.48	1611.74	1074.83
T31-32	326-355	**(K)LVDNWMTYMPPSGIPNWVPGNHDQLRLVSR(F)**	3491.72	3492.73	1746.87	1164.92
T50-51	495-525	**(R)ETEDNGSLYAILNFSNEEQIVDLKAFNNVPK(K)**	3512.83	3513.84	1757.42	1171.95
T26-27	282-312	**(K)HMLIEAYTNLSMTMKYYDYGADFPFNFAFIK(N)**	3743.34	3744.35	1872.68	1248.79
T15	180-210	**(R)QAYYLHQFAPEQPDLNYYNPVVLDDMQNVLR(F)**	3755.18	3756.19	1878.60	1252.74
T49-50	484-518	**(R)MLNDNVFAFSRETEDNGSLYAILNFSNEEQIVDLK(A)**	4037.43	4038.44	2019.72	1346.82
T2-3	3-38	**(K)AVIVFCLMALSIVDAAWKPLPENLKEDLIVYQVYPR(S)**	4118.96	4119.97	2060.49	1374.00
T14-15	177-210	**(R)EERQAYYLHQFAPEQPDLNYYNPVVLDDMQNVLR(F)**	4169.60	4170.61	2085.81	1390.88
T15-16	180-214	**(R)QAYYLHQFAPEQPDLNYYNPVVLDDMQNVLRFWLR(R)**	4357.92	4358.93	2179.97	1453.65
T9-10	116-152	**(K)IILDFVPNHTSDQHEWFQLSLKNIEPYNNYYIWHPGK(I)**	4558.09	4559.10	2280.05	1520.37
T35	363-405	**(R)MITTMSLLLPGVAVNYYGDEIGMSDTYISWEDTQDPQGCGAGK(E)**	4629.20	4630.21	2315.61	1544.08
T34-35	361-405	**(K)ARMITTMSLLLPGVAVNYYGDEIGMSDTYISWEDTQDPQGCGAGK(E)**	4856.47	4857.48	2429.24	1619.83
T35-36	363-413	**(R)MITTMSLLLPGVAVNYYGDEIGMSDTYISWEDTQDPQGCGAGKENYQTMSR(D)**	5639.30	5640.31	2820.66	1880.77
T7	58-112	**(K)LDHFLEMGVDMFWLSPIYPSPMVDFGYDISNYTDVHPIFGTISDLDNLVSAAHEK(G)**	6262.06	6263.07	3132.04	2088.36
T6-7	56-112	**(K)EKLDHFLEMGVDMFWLSPIYPSPMVDFGYDISNYTDVHPIFGTISDLDNLVSAAHEK(G)**	6519.35	6520.36	3260.68	2174.13
T7-8	58-115	**(K)LDHFLEMGVDMFWLSPIYPSPMVDFGYDISNYTDVHPIFGTISDLDNLVSAAHEKGLK(I)**	6560.45	6561.46	3281.23	2187.82

Appendix G: The DNA sequencing profiles of *AG*

A: The DNA sequencing profile of *AG* from FW2

B: The DNA sequencing profile of *AG* from R3

C: The DNA sequencing profile of *AG* from FW2/ FW2

D: The DNA sequencing profile of *AG* from FW3/ FW3

E: The DNA sequencing profile of *AG* from FW2/ oligo dT

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Model 3100
Version 3.7
Basecaller-3100APOP6SAG(AC)_FW2(a-G)
BC 1.5.0.0

10_6_04_F11_AG(AC)_FW2(a-G)_11.ab1
Cap 11

SQ 22706

Signal G:422 T:750 A:1132 C:276
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250

Page 2 of 3 Page 1 of 3
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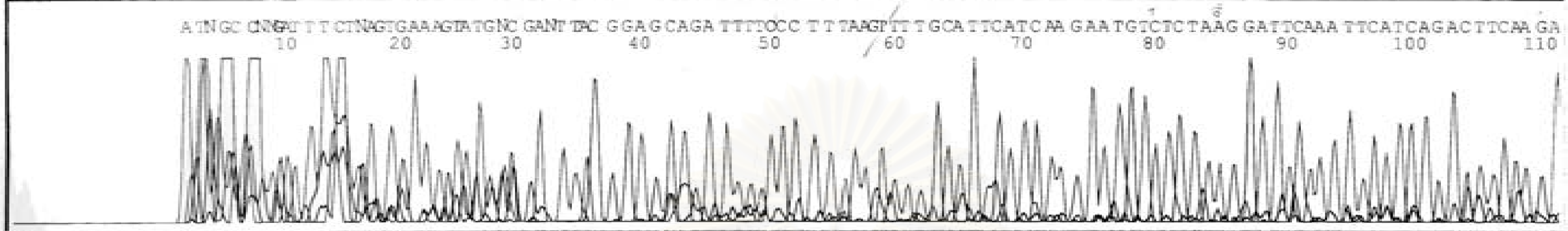
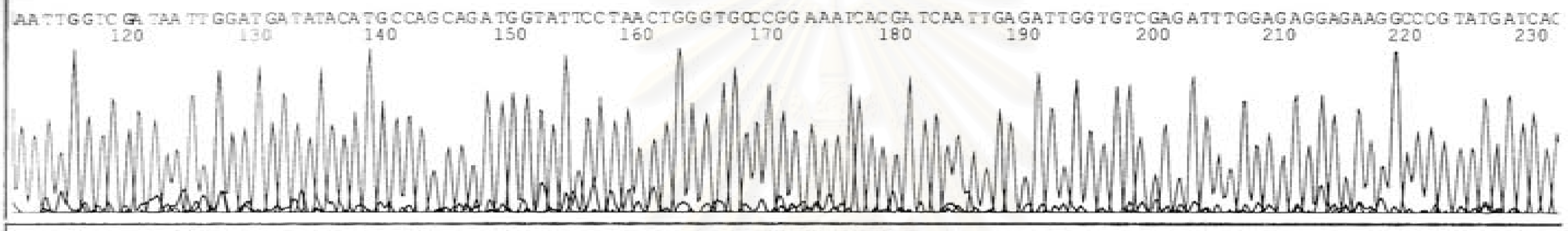
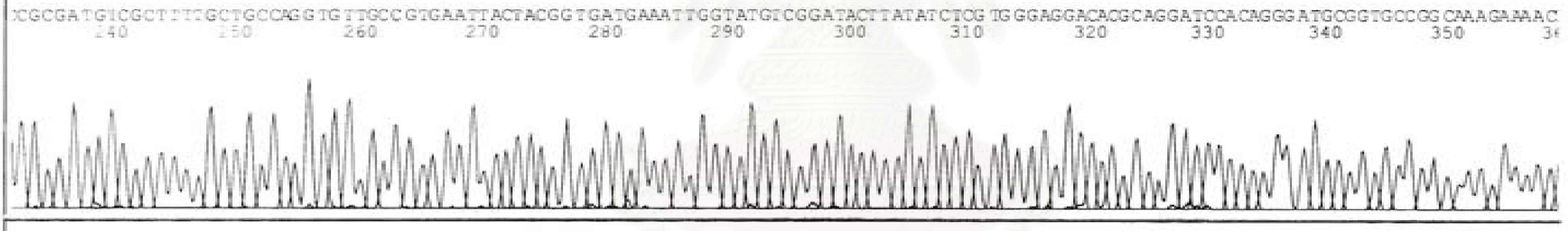
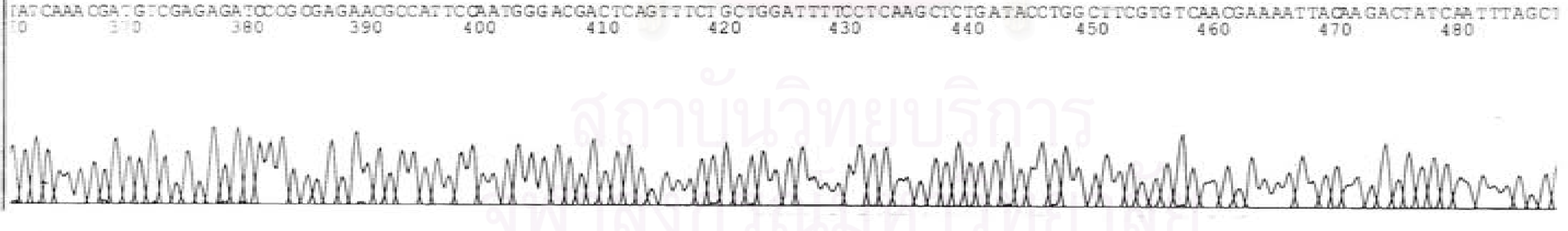
Thu, Jun 10, 2004 7:16 PM
Spacing: 11.38{11.38}









A (1)

ABI PRISM

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Version 3.7 | SQ 22706 | DT3100POP6{ET}50cm.mob | Fri, Jun 11, 2004 8:39 AM
Basecaller-3100APOP6SAG(AC)_FW2(a-G) | demo_3100 | Thu, Jun 10, 2004 7:16 PM
BC 1.5.0.0 | Cap 11 | Points 250 to 10106 Pk 1 Loc: 250 | Spacing: 11.38{11.38}

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490 500 510 520 530 540 550 560 570 580 590 600 610

CTCTAGG GAAACCGAAG AAAATGGATCTCTTTACGC AATATTGAA CTTCTCGAACGAGGAAC AAA TCGTGGACTTGA AAGCGTTT AAT ANCGTG CCGA A AAA T TGAATATGT T TACACCA TTT T
620 630 640 650 660 670 680 690 700 710 720 730

A CTCTGATATAA GT C ATCT CAACA TG AAC AA T AAAGT T CTGCTT AGGA TTTTGATC TA TTTCTC AGATGCT AAT TGGAA TT TAATATCT C NGA T T TNTNTNNANNNG GGG GN NNN NN NNN N
740 750 760 770 780 790 800 810 820 830 840 850

NNNN NN NNN NNN NN NNNNN NNNNN NNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNN NNNNN NNN NN NNN NN NNN
860 870 880 890 900 910 920 930 940 950 960 970

A (2)

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ABI PRISM

Version 3.7
Basecaller-3100APOP6SAG(AC)_RW3
BC 1.5.0.0 Cap 11

SQ 23180

DT3100POP6{ET}50cm.mob
demo_3100
Points 300 to 10106 Pk 1 Loc: 300

Tue, Jun 22, 2004 12:18 PM
Spacing: 11.38{11.38}

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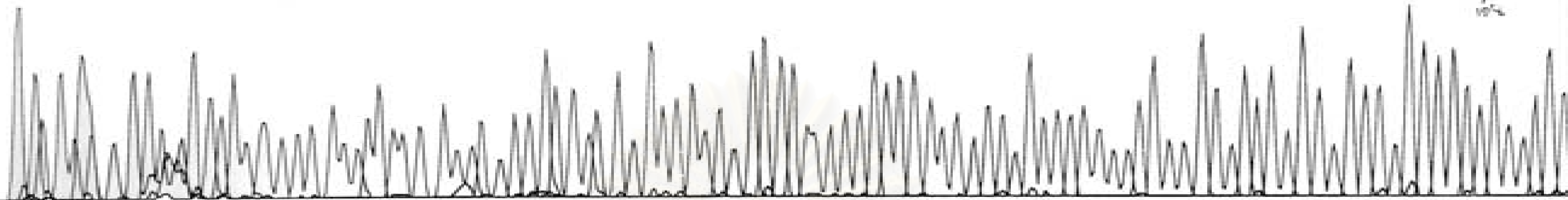

TCAGAG TTAAA ATTGTT GTAAAACA TATTCAA TTTTTTC GGCACG TTATTAAA CGCT TTCAAG TCCACNA TTTGTTCCTCG TTC GAG AA GTTCAA TATTGCG TAAA GAGATCCATT TTCTTCG
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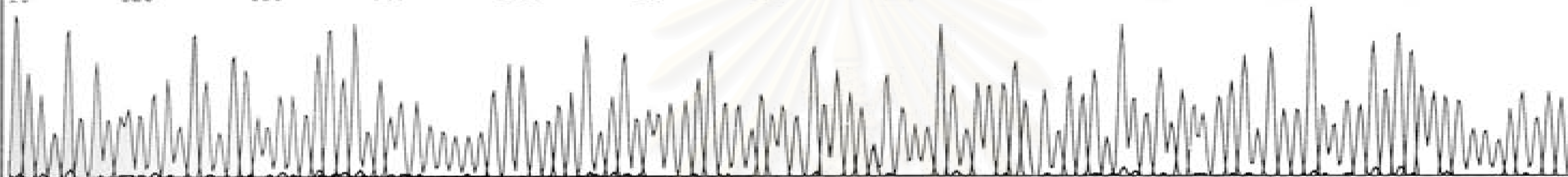

G TTTCCCTAG AGAATGCGAAAACACTG TCGTTCAGCATCCTCGNTATTTAAATTG GCCTCTTTAAAG TGTGGCGATTTTTTCAGCAT TGCAAATTTCTTGAACATATTGAA GAACGAG TTCTTG TCC
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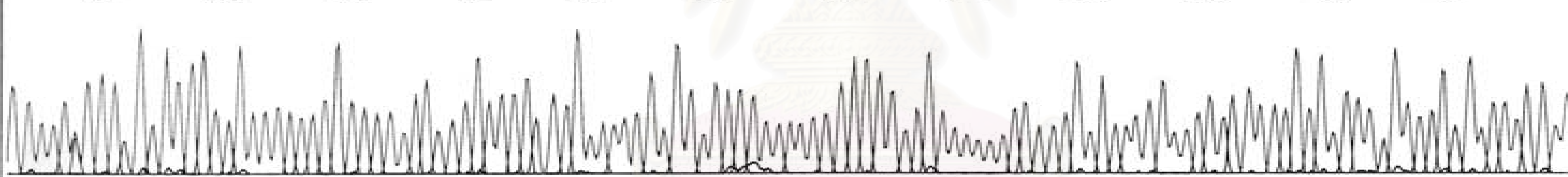

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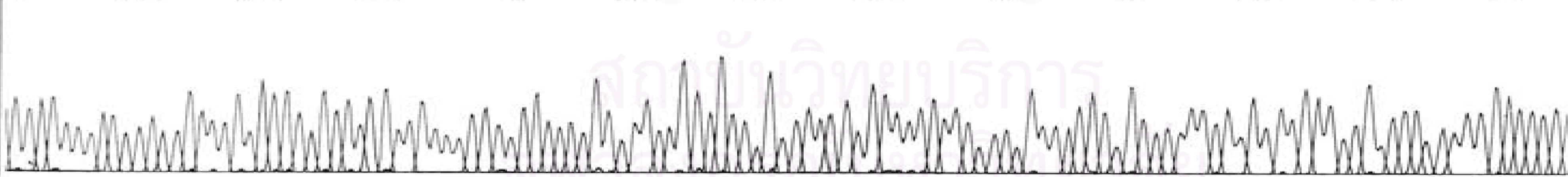

B (1)

ABI PRISM

Model 3100 | 21-06-041_F09_AG(AC)_RW3_11.ab1
Version 3.7
Basecaller-3100APOP6SAG(AC)_RW3
BC 1.5.0.0 | Cap 11

SQ 23180

Signal G:241 T:717 A:890 C:240
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demo_3100
Points 300 to 10106 Pk 1 Loc: 300

Tue, Jun 22, 2004 2:49 PM
Tue, Jun 22, 2004 12:18 PM
Spacing: 11.38{11.38}

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GACATCGTTTGATAGTTTTCTTTGCCGG CACCGCATCCCTG TG GATCCTG CG TGTCCTCCCACG AG ATATAAGTATCCGACATACCAA TTTCATCACCG TAG TAATTCACGG CAA CACCTGG CAG C
  490       500       510       520       530       540       550       560       570       580       590       600       610

AAAAGCGA CATC GCGGTGATCATACGGG CCTTCTCCTCTCCAAATCTCGA CA CCAATCTCAATTGATCGTGATTT CCGG GCACCCAG TTAGGAATACCATCTGCTGGCATGTATGTCATCCAATTATCGACCA
  620       630       640       650       660       670       680       690       700       710       720       730       740

ATT CCTGGAAGTCTGATGAATTGAATCCTAGAGACATTCTG ATGAATGCAAATNAAGG NAAACTGCNCGTATCGTAAATTCACGNCTCGATANTC GTC CCCCNAAAAAAAANNNNNNNNNNNNNNNNNNNNNNNNNNNN
  750       760       770       780       790       800       810       820       830       840       850       860       870       880

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
  890       900       910       920       930       940       950       960       970       980       990       1000      1010
```

B (2)

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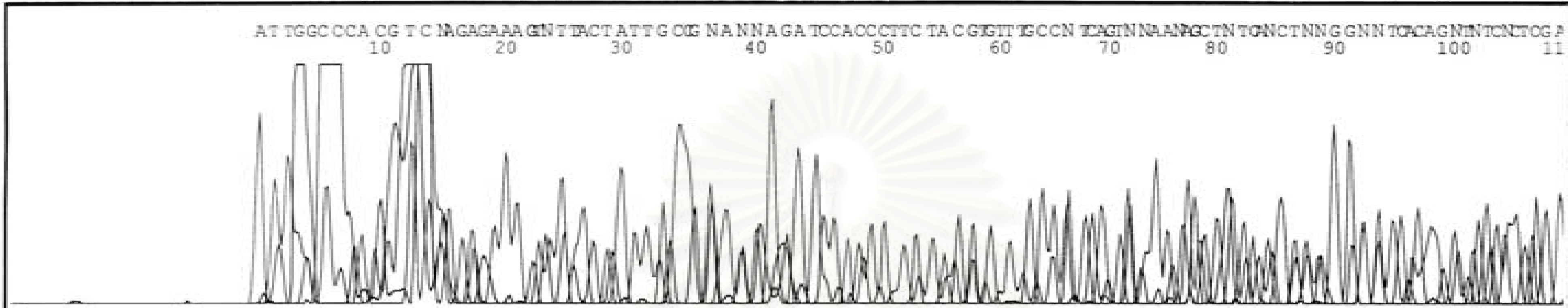
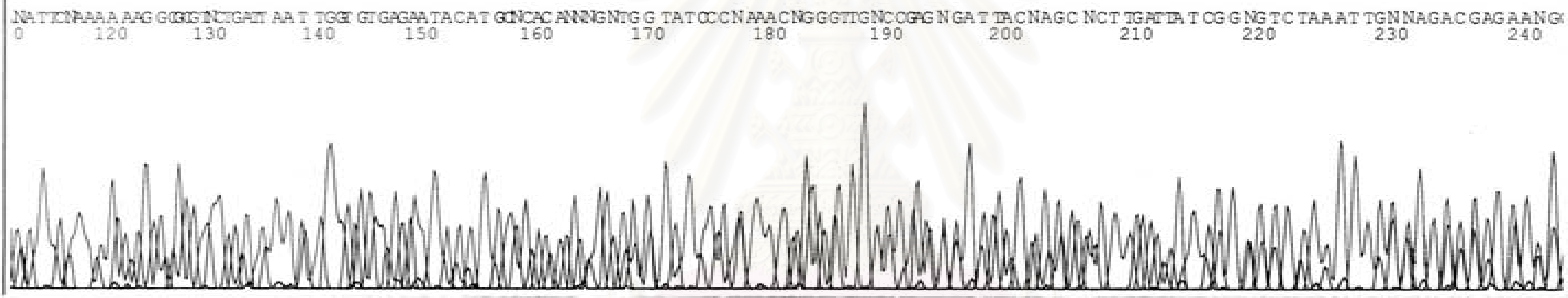
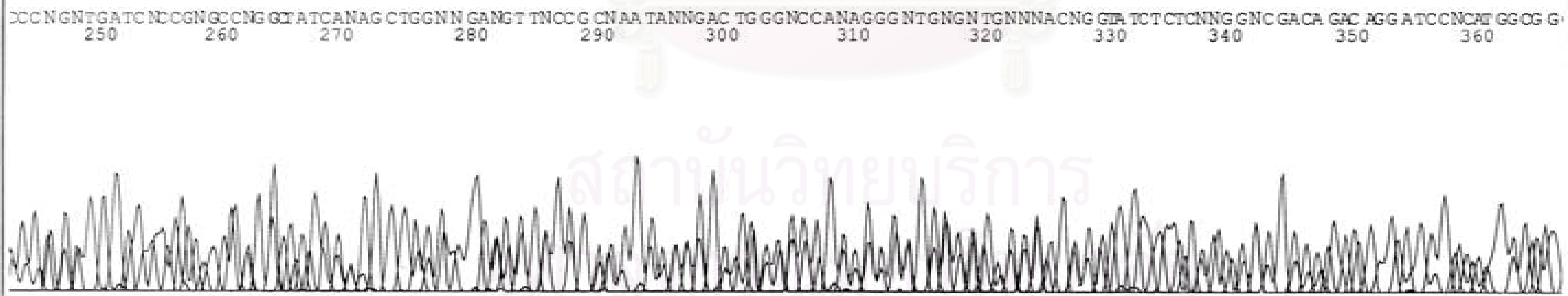
Model 3100 | 1-09-04A_H07_AG(AC)-FW2_FW2_15.ab1 | Signal G:348 T:694 A:991 C:297 | Page 1 of 4
Version 3.7 | DT3100POP6{ET}50cm.mob | Thu, Sep 02, 2004 8:59 AM
Basecaller-3100APOP6SAG(AC)-FW2_FW2 | SQ.....15667..... | demo_3100 | Thu, Sep 02, 2004 2:24 AM
BC 1.5.0.0 | Cap 15 | Points 300 to 10106 Pk 1 Loc: 300 | Spacing: 11.01{11.01}

Page...2...of...3...





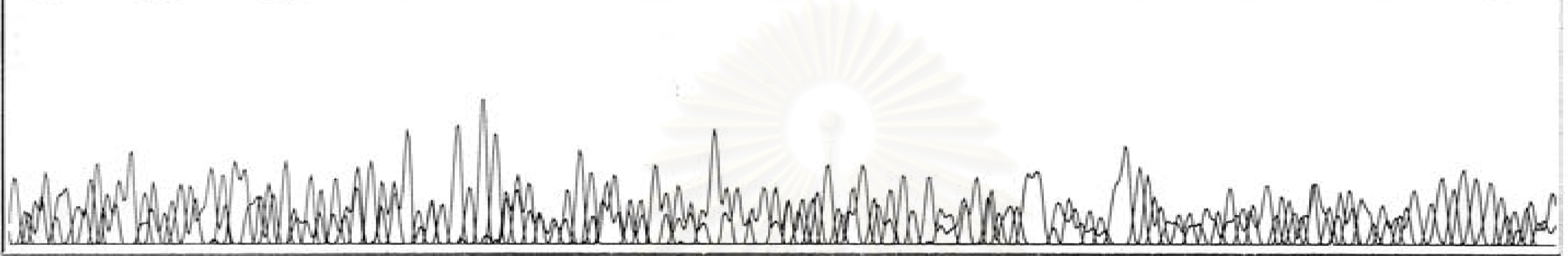


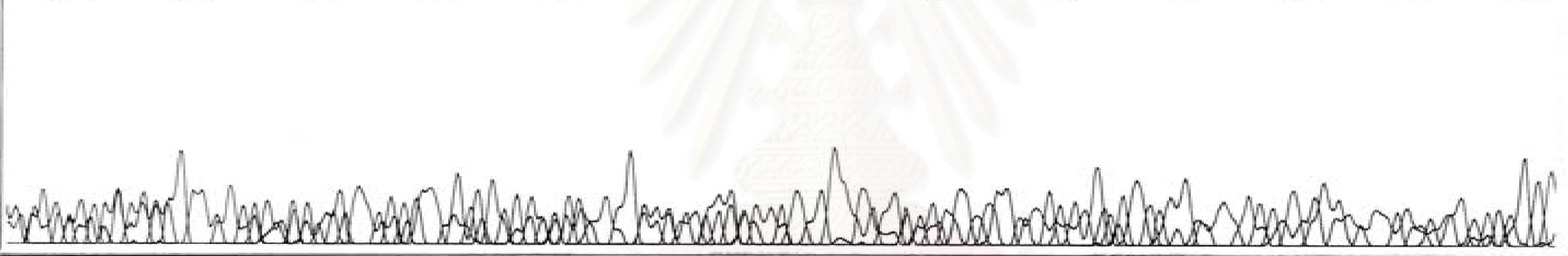
C (1)

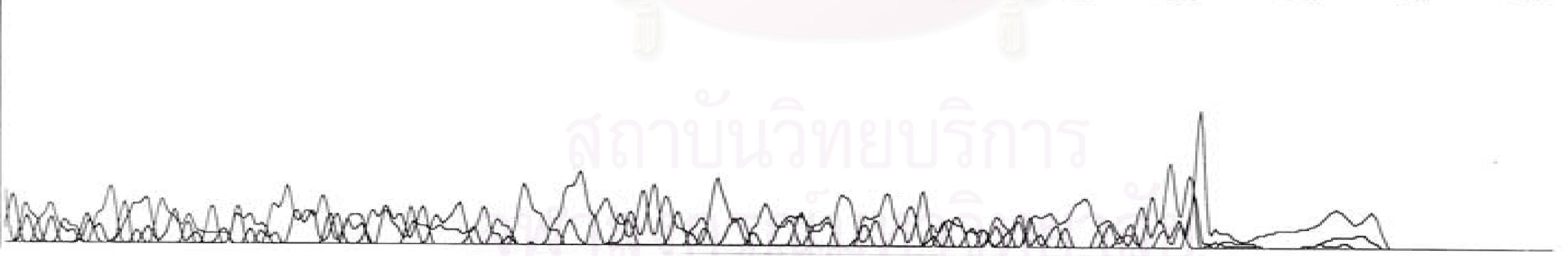
Model 3100 | 1-09-04A_H07_AG(AC)-FW2_FW2_15.ab1 | Signal G:348 T:694 A:991 C:297 | Page 2 of 4
Version 3.7 | DT3100POP6{ET}50cm.mob | Thu, Sep 02, 2004 8:59 AM
Basecaller-3100APOP6SAG(AC)-FW2_FW2 | demo_3100 | Thu, Sep 02, 2004 2:24 AM
BC 1.5.0.0 | Cap 15 | Points 300 to 10106 Pk 1 Loc: 300 | Spacing: 11.01{11.01}

SQ...25667.......

Page...3...of...3...







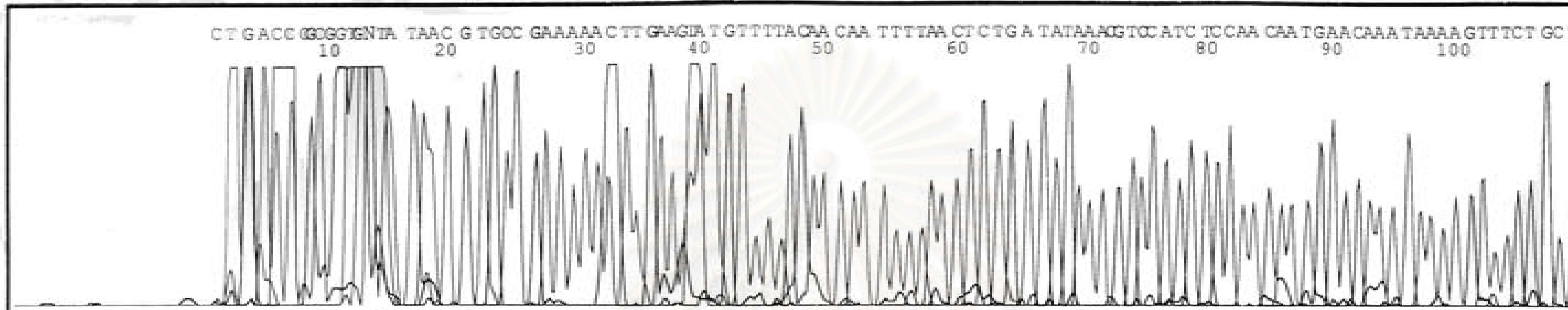
C (2)

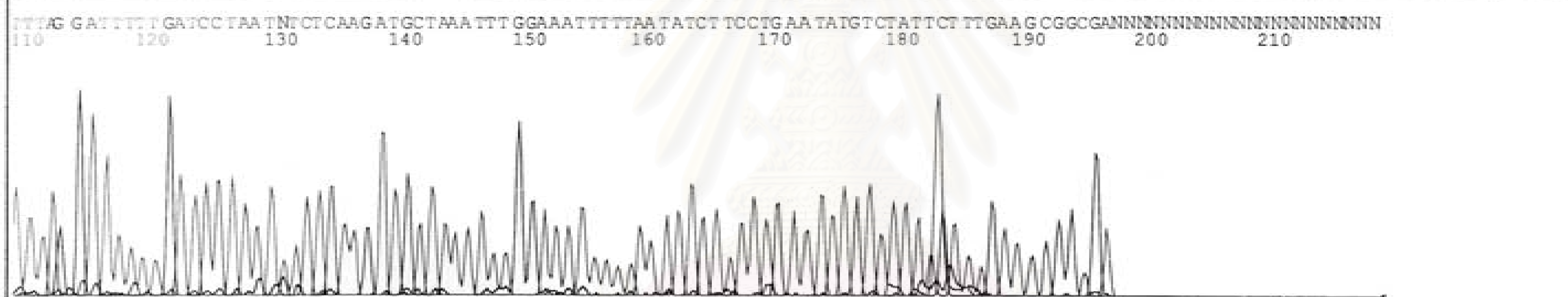
Model 3100 21-09-04A_H01_AG(AC)FW3_FW3_15.ab1
Version 3.7
Basecaller-3100APOP6SAG(AC)FW3_FW3
BC 1.5.0.0 Cap 15

SQ. 26607

Signal G:174 T:544 A:636 C:216
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 3000 Pk 1 Loc: 250

Wed, Sep 22, 2004 8:49 AM
Tue, Sep 21, 2004 7:49 PM
Spacing: 11.77{11.77}





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Model 3100
Version 3.7
Basecaller-3100APOP6SAG(AC)FW2_OligodT
BC 1.5.0.0

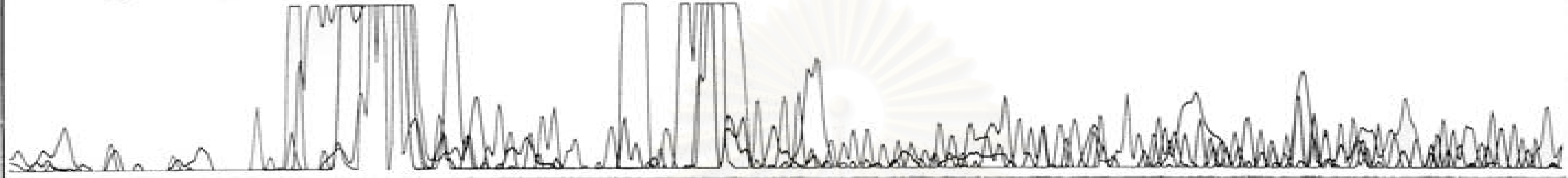
21-09-04A_G02_AG(AC)FW2_OligodT_14.ab1
Cap 14

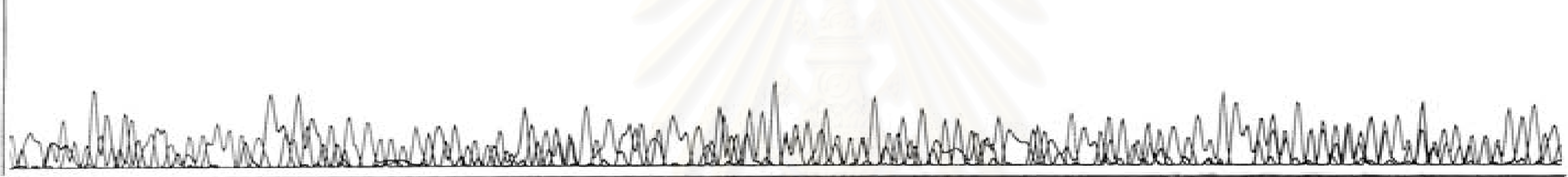
SQ. 26606

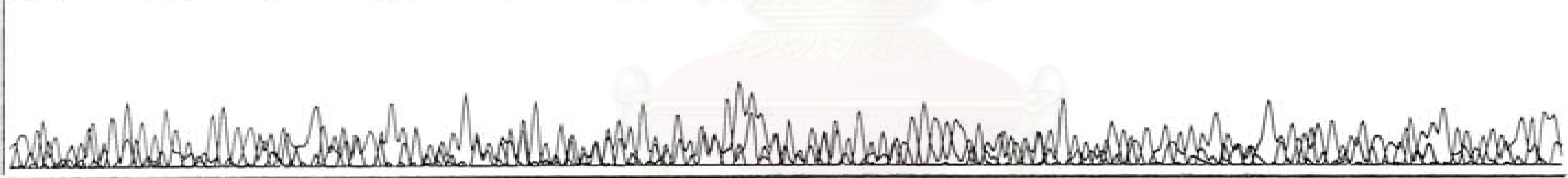
Signal G:56 T:103 A:76 C:83
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250

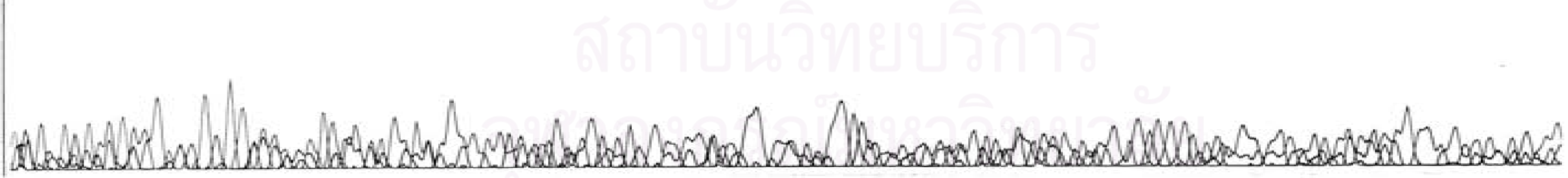
Page 2 of 2

Wed, Sep 22, 2004 8:43 AM
Tue, Sep 21, 2004 7:49 PM
Spacing: 15.52{15.52}









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BIOGRAPHY

Miss Suwisa Pilalam was born on August 17th, 1978 in Sakaew. She graduated with the Bachelor Degree of Science in Department of Biology, Facultly of Science, Burapha University in 2001. Then, she has been a graduate student in the Master's Degree in Biotechnology program, Faculty of Science, Chulalongkorn University since 2003.

Research publications:

Pilalam, S., Chanchao, C. and Wongsiri, S. (2004) Alpha glucosidase in *Apis cerana.* Abstract. The 30th Congress on Science and Technology of Thailand, Bangkok, Thailand. 79.

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Pilalam, S., Chanchao, C., Sangvanich, P. and Wongsiri, S. (2005) Purification of alpha glucosidase in *Apis cerana.* Abstract. The 10th Biological Sciences Graduate Congress, Singapore. 43.

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Pilalam, S., Padoongsupalai, R., Chanchao, C., Sangvanich, P., Wongsiri, S., and Svasti, J. (2006) Purification and characterization of alpha glucosidase in *Apis cerana*. International Union for the study of social insects, Washington, D.C., U.S.A. Abstract (accepted).