การทำให้บริสุทธิ์ ลักษณะสมบัติและการแสดงออกของยีนแอลฟากลูโคซิเดสของผึ้งหลวง *Apis dorsata*

นางสาวมัลลิกา กิลาโส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PURIFICATION, CHARACTERIZATION AND GENE EXPRESSION OF α-GLUCOSIDASE OF GIANT HONEY BEE *Apis dorsata*

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	PURIFICATION, CHARACTERIZATION AND GENE EXPRESSION OF α - GLUCOSIDASE OF GIANT HONEY BEE *Apis dorsata*
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มัลลิกา กิลาโส: การทำให้บริสุทธิ์ ลักษณะสมบัติและการแสดงออกของยีนแอลฟากลูโคซิเดสของผึ้งหลวง *Apis dorsata* (PURIFICATION, CHARACTERIZATION AND GENE EXPRESSION OF α - GLUCOSIDASE OF GIANT HONEY BEE *Apis dorsata*) อาจารย์ที่ปรึกษา: รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อาจารย์ที่ปรึกษาร่วม: รศ. ดร. พลกฤษณ์ แสงวณิช, 100 หน้า.

เอปิส ดอร์ซาตา (ผึ้งหลวง) ซึ่งเป็นผึ้งพื้นเมืองของประเทศไทยจัดเป็นผึ้งขนาดใหญ่ที่สุด น้ำผึ้งของผึ้งหลวงเป็นที่นิยมมากที่สุดต่อผู้บริโภค แอลฟากลูโคซิเดส (เอจี) ซึ่งถูกจัดอยู่ในอีซี 3.2.1.20 เป็นเอนไซม์ที่เกี่ยวข้องโดยตรงในการผลิตน้ำผึ้ง เอจีไฮโดรไลซ์พันธะ 1–2 แอลฟากลูโคซิดิกในน้ำตาลซูโครสของน้ำหวานให้เป็นน้ำตาลโมเลกุลเดี่ยวคือฟรุกโตสและกลูโคสอยู่ในน้ำผึ้ง เพื่อศึกษาระดับการแสดงออกของยีนเอจีชนิดที่ 3 ในผึ้งหลวง อาร์เอ็นเอทั้งหมดถูกสกัดจากไข่ ตัวอ่อน ดักแด้ และผึ้งออกหาอาหาร โดยเทคนิคอาร์ทีพีซีอาร์ การแสดงออกของยีนเอจีชนิดที่ 3 สูงสุดในผึ้งออกหาอาหาร (114.24 นก.) สูงในดักแด้ (107.1 นก.) ต่ำในไข่ (71.4 นก.) และต่ำที่สุดในตัวอ่อน (47.6 นก.) ความยาวเต็มสายของบริเวณโออาร์เอฟของลำดับซีดีเอ็นเอคือ 1,704 คู่เบสและถูกแปลงกลับเป็นลำดับกรดอะมิโนได้ที่ความยาว 567 เรสิดิวซ์ โดยการทำบลาสต์เอ็นและบลาสต์พี พบว่ามีความเหมือนสูงสุดกับลำดับซีดีเอ็นเอและลำดับกรดอะมิโนของเอจีในเอปิส เมลลิเฟอร์รา (ผึ้งพันธุ์) มากที่สุดถึง 96 เปอร์เซ็นต์ สายสัมพันธ์ทางวิวัฒนาการของลำดับกรดอะมิโนของเอจีถูกสร้างโดยยูพีจีเอ็มเอและเอ็นเจ พบว่าผึ้งหลวงมีสายวิวัฒนาการเป็นของตัวเอง เอจีจากผึ้งหลวงในระยะออกหาอาหาร (500 กรัม) ถูกนำมาสกัดบริสุทธิ์ สารสกัดอย่างหยาบของเอนไซม์ (0.01 ยูนิตต่อมก.) ถูกตกตะกอนด้วยแอมโมเนียมซัลเฟตที่ความเข้มข้นอิ่มตัว 95 เปอร์เซ็นต์ หลังตกตะกอน พบว่ามีแอกทิวิตีจำเพาะทั้งในส่วนตะกอน (1.70 ยูนิตต่อมก.) และส่วนใส (1.34 ยูนิตต่อมก.) หลังจากคอลัมน์ดีอีเออีเซลลูโลส ได้แอกทิวิตีจำเพาะ 0.004 และ 3.95 ยูนิตต่อมก. จากส่วนตะกอนและส่วนใสตามลำดับ สำหรับส่วนตะกอน รวมอันบาวน์และบาวด์แฟรกชันที่มีแอกทิวิตีไปสกัดบริสุทธิ์ต่อด้วยคอลัมน์ซูเปอร์เด็กซ์ 200 ได้แอกทิวิตีจำเพาะ 0.09 ยูนิตต่อมก. ในส่วนใส นำอันบาวด์และบาวด์แฟรกชันที่มีแอกทิวิตีไปสกัดบริสุทธิ์ด้วยคอลัมน์ซูเปอร์เด็กซ์ 75 ได้แอกทิวิตีจำเพาะ 0.11 และ 0.26 ยูนิตต่อมก.ตามลำดับ ตรวจหาเอจีที่บริสุทธิ์บางส่วนด้วยเทคนิคเอสดีเอส เพจ ภาวะที่เหมาะสมต่อการทำงานของเอจีที่บริสุทธิ์บางส่วนคือที่พีเอช 6.0 อุณหภูมิในการบ่มที่ 35 องศาเซลเซียส ที่ความเข้มข้นของมอลโทสเท่ากับ 50 มิลลิโมลาร์และระยะเวลาในการบ่มที่ 30 นาที

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต มัลลิกา กิลาโส

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MANLIKA KILASO: PURIFICATION, CHARACTERIZATION AND GENE EXPRESSION OF α-GLUCOSIDASE OF GIANT HONEY BEE *Apis dorsata*. THESIS ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., THESIS CO–ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 100 pp.

Apis dorsata (Giant honey bee) which is native to Thailand is the largest bee. Its honey is the most popular for consumers. α–glucosidase (AG) classified in E.C. 3.2.1.20 is an enzyme directly involved in honey processing. It hydrolyses 1, 2–α-glucosidic bond in sucrose of nectar to be monosaccharide, fructose and glucose, in honey. For the expression level of *AG III* in *A. dorsata*, total RNA was extracted from eggs, larvae, pupae, and foragers. By RT-PCR, the expression of *AG III* was the highest in foragers (114.24 ng), high in pupae (107.1 ng), low in eggs (71.4 ng), and the lowest in larvae (47.6 ng). The full length of ORF of cDNA was 1,704 bp and was deduced to be 567 amino acids. By BLASTn and BLASTp, the most similarity was found to the sequences of AG III in *A. mellifera* at 96%. Phylogenetic trees of amino acid sequence of AG were constructed by UPGMA and NJ. It showed its own evolutionary lineage of *A. dorsata*. AG from *A. dorsata* foragers (500 g) was purified. Crude enzyme (0.01 U/mg) was precipitated by ammonium sulfate at 95% saturation. After precipitation, specific activity of AG was found in both pellet (1.70 U/mg) and supernatant (1.34 U/mg). After DEAE cellulose column, specific activity of 0.004 and 3.95 U/mg were obtained from pellet and supernatant, respectively. For pellet, pooled positive unbound and bound fractions were further purified by Superdex 200 column. Specific activity of 0.09 U/mg was recovered. For supernatant, positive unbound and bound fractions were separately purified by Superdex 75 column. Specific activity of 0.11 and 0.26 U/mg were obtained, respectively. Partial purified AG was determined by SDS PAGE. The optimal conditions of partial purified AG were at pH 6.0, incubation temperature of 35°C, maltose concentration of 50 mM, and incubation time of 30 min.

Field of study Biotechnology Student's signature Manlika Kilaso
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LIST OF ABBREVIATIONS

2DE	Two–dimensional electrophoresis
AG	α-glucosidase
AG	α-glucosidase gene
AS	Ammonium sulfate
bp	Base pair
°C	Degree Celsius
kDa	Kilodalton
LC/ MS	Liquid Chromatography Mass Spectrometry
g	Gram
h	Hour
HPG	Hypopharyngeal gland
MALDI – TOF MS	Matrix Assisted Laser Desorption Ionization/ Time of Flight Mass Spectrometry
µg	Microgram
µl	Microlitre
µM	Micromolar
mg	Milligram
ml	Millilitre
mM	Millimolar
min	Minute
M	Molar
MW	Molecular Weight
nm	Nanometre
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

R_f	Relative mobility
rpm	Revolution per minute
RT	Room temperature
s	Second
Tris	Tris (hydroxymethyl)–aminoethane
UV	Ultraviolet
U	Unit
V	Volt
w/ v	Weight by volume
v/ v	Volume by volume

CHAPTER I

INTRODUCTION AND LITERATURE REVIEWS

1.1 Diversity of honey bee

Honey bees belonging to genus *Apis* are classified as highly eusocial insects since there is a caste division within a hive. They provide honey as a main product so that leads to a name of “honey bee”. Honey is a natural product produced from nectar of flowers or honey dew by honey bees. The composition of honey depends on the floral source, season, and environmental conditions. Sweet taste of honey is derived from the sugar content of monosaccharide. Fructose and glucose were the main components. The minor constituents are consisted of phenolic compounds, minerals, proteins, amino acids, certain enzymes (glucose oxidase and catalase), and vitamins. With the antimicrobial properties, honey is widely used in traditional medicine since the ancient time. In addition, the antioxidant property of honey by phenolic compounds provides benefits on human health (Siriwat Wongsiri, 1989; Taormina *et al*., 2001; Gheldof *et al*., 2002; P´erez *et al*., 2007).

Considering the morphology, honey bees contain typical characters such as pollen baskets locating on hind legs, honey crop, bee venom gland, sting, etc. In Thailand, there are five species of honey bees. Four native species are *A. andreniformis* Smith, 1858, *A. florea* Fabricius, 1787, *A. cerana* Fabricius, 1793, and *A. dorsata* Fabricius, 1793 while one imported species is *A. mellifera* Linnaeus, 1758. In other regions, four species are distributed. *A. laboriosa* Smith, 1877 is found in mountainous regions, particularly the Himalayas while *A. koschevnikovi* Enderlein,

1906 is widely distributed through rainforests of peninsular Malaysia and Borneo. In addition, *A. nuluensis* Tingek, Koeniger and Koeniger, 1996 is mainly located in Crocker range of Borneo and *A. nigrocincta* Smith, 1861 is found in the islands of Sulawesi (Indonesia), Sagihe (Indonesia), and Mindanao (the Philippines) (Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006).

1.2 Life cycle of honey bee

Development of honey bee is known to be complete metamorphosis which is consisted of egg, larva, pupa, and adults (Snodgrass, 1925; Siriwat Wongsiri, 1989). An egg is long, oval, and white. It is attached to the bottom of a cell (Fig. 1.1A). Later, it will hatch at around 76 hours (3 days) to be a larva. The form of larva is worm – like (Fig. 1.1B). It still stays in an open cell. If it develops into a worker, it will be fed bee milk by nurse bees for 3 days. If that larva develops to be a queen instead, it will be fed bee milk continuously and much longer. During the growth, a larva needs to molt 5 times. Roughly, a larva stage takes 4 – 7 days. On the fifth day of larval development, protein transporters, protein receptors, protein nutrients, and immunological proteins correlating to the age increased detectably (Chan and Forster, 2008). After it is fully grown up, it will seal its cell by wax. Later, it will spin fiber to make a cocoon and become pupa (Fig. 1.1C). A lot of change, both in external and internal structures, can be observed during metamorphosis. Also, the appearance of its body looks alike an adult bee. The period of pupa stage is 8 – 15 days. Next, an adult bee bites a cell cap and comes out of the cell during the 15th – 24th day (Fig. 1.1D). The duration in each stage of development is various due to the castes. In general, the life cycle of honey bee (from egg to adult) is between 15 – 24 days (Snodgrass, 1925; Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006).

(A) **(B)** **(C)** **(D)**

Figure 1.1. Developmental stages of honey bee are indicated as egg (A), larva (B), pupa (C), and adult (D).

1.3 Biology of *Apis dorsata*

Apis dorsata is one of native species of honey bee in Thailand (Figure 1.2). Since it is the largest honey bee, its common name is “giant honey bee”. Its body and forewings are about 17 mm and 12 – 15 mm in length, respectively. A yellow abdomen with black stripes is typical in this species. Also, *A. dorsata* is known to be the most aggressive bee among honey bees. A nest of *A. dorsata* is the largest with a diameter of 0.5 – 1 m. It is generally built as a single comb on a branch of tall trees, cliff, or a crossbeam of housings. *A. dorsata* is commonly distributed in all countries in Southeast Asia and along to the South of China, Myanmar, and Sri Lanka but it is restricted to the West of India (Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006).

A. dorsata is classified as below (Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006):

Kingdom Metazoa

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super – family Apoidea

Family Apidae

Sub family Apinae

Genus *Apis*

Subgenus *Megapis*

Species *Apis dorsata*

Figure 1.2. *Apis dorsata* forager.

As mentioned above, honey bees are highly eusocial insects. In one colony, bees are classified into 3 castes which are a female queen, male drones, and female workers. A queen (2n) is the only female which can fertilize and lay eggs. It can release queen pheromone to suppress the ovary development of female workers and to attract drones. About its morphology, it has short wings comparing to a length of its body. Drones (n) are developed from unfertilized eggs. They play a role in mating only. Considering its morphometry, a body of drones is the widest. Workers (2n) perform all activities in a colony such as nursing, guarding, nest-defensing, foraging, but not mating and laying eggs. Some organs are modified to fit the task. For example, honey crop is modified to be able to collect nectar of flowers. Although a queen and workers are female and contain the same genome, the difference is started to be determined at a larval stage. Specific genes are differentially activated. For example, an insulin receptor gene was expressed only in a queen larva, but not worker larvae (Wheeler *et al*., 2006). In worker bees, the division of labors depended on the age after eclosion (Ribbands, 1964; Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006). Young workers less than 14 days (nurse bees) produce and secret bee milk protein which is fed to larvae while older ones (foragers) forage nectar and process it into honey. The role change of worker bees was related to the size and function of hypopharyngeal glands (HPG) and mandibular glands (MG) locating in a head (Kubo *et al*., 1996).

1.4 Mandibular glands

Mandibular glands (MG) are large glands locating on the mandible which was the upper mouth part of honey bees. Not only they produce bee milk protein or royal jelly to rear their broods, but they also synthesize pheromones such as alarm pheromone, foraging pheromone in worker, queen pheromone in a queen, etc. The queen pheromone had many important functions in a hive. For example, it could suppress the development of worker's ovary, attract drones, and provide queen recognition (Siriwat Wongsiri, 1989; Oldroyd and Siriwat Wongsiri, 2006).

Due to proteomic analysis, proteins synthesized by MG are known to indicate the different function between a queen and workers. In *A. mellifera*, three proteins [aldehyde dehydrogenase 1 (ALDH 1), medium – chain acyl – CoA dehydrogenase (MAD), and electron transfer flavoprotein α (ETF α)], were specially found in queen's MG while fatty acid synthase (FAS) was selectively found in worker's MG Furthermore, the different function between a queen and workers was also supported at the transcriptional level. Due to quantitative reverse transcription polymerase chain reaction (qRT - PCR), the expression of *ALDH 1* was regulated in a queen while the expression of *FAS* was regulated in workers (Hasegawa *et al*., 2009).

1.5 Hypopharyngeal glands

Hypopharyngeal glands (HPG) are long paired food glands locating in a head of worker bees. HPG consist of many acini linked to a central duct (Figure 1.3).

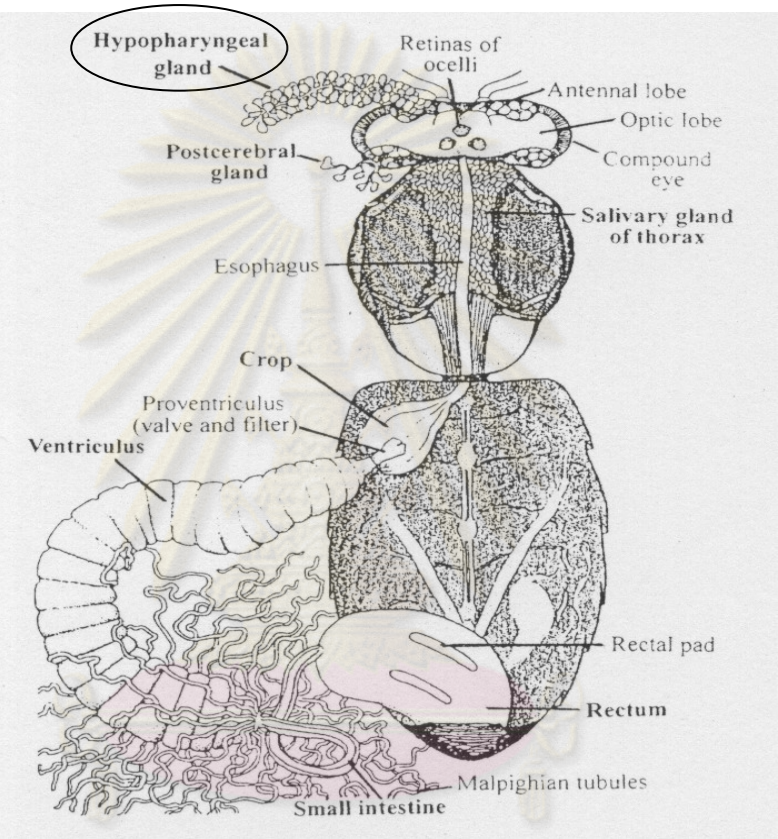


Figure 1.3. Dissection of worker bee (modified from Kubota *et al.*, 2004)**.**

The size of HPG is related to the task of worker bee. In nurse bee (0 – 15 days), these glands are well developed and increase to the full size at day 15. In nurse bees, they produce bee milk protein to feed larva while in forager bees (15 – 29 days), HPG are shrunk and changed to produce α-glucosidase (AG), a digestive enzyme. This enzyme can hydrolyse sucrose in collected nectar and process it to be honey. That is why the expression of *AG*, by RT – PCR, was detectable from a 6 days old

worker bee to a forager. Also, the expression of this gene was the highest in a forager (Chanchao *et al*., 2006).

Four major proteins in HPG were purified and analysed by immunoblotting. Three major proteins (50, 56, and 64 kDa) were synthesized selectively in a nurse bee's HPG so they were identified as bee milk proteins. A major 70 kDa protein was synthesized only in a forager bee's HPG so it was identified as AG. Thus, HPG seem to have two working states by synthesizing different major proteins with the age-dependent role change in worker bees (Kubo *et al*. 1996). Next, two genes encoding 56 and 64 kDa bee milk were analysed by northern analysis. The mRNA for 64 kDa protein was expressed specifically in HPG of nurse bees whereas the mRNA for 56 kDa protein was detected in both HPG of nurse and forager bees. Also, it was interesting that *AG* was detected only in HPG of forager bees according to the same method (Ohashi *et al*., 1997).

In addition, the function of HPG was related to *glucose oxidase* and α-*amylase* involving in carbohydrate metabolism. By northern analysis, the expression of both genes was only found in HPG of forager bees. This coincided to the expression of *AG* (Ohashi *et al*., 1999). Considering the role of these three enzymes, they belonged to the same family of enzyme which could hydrolyze glucosidic linkages of carbohydrate. In addition, alcohol dehydrogenase and aldehyde dehydrogenase which were enzymes for energetic metabolism were also synthesized by HPG (Santos *et al*., 2005).

1.6 α-glucosidase (AG)

α-glucosidase (E.C. 3.2.1.20, α – D – glucoside glucohydrolase) was a group of typical exo-type carbohydrases those hydrolysed α – 1, 2 – glucosidic linkage from the non-reducing terminal of a substrate to produce α-glucose (Chiba, 1997).

AG was classified into two groups by substrate specificities. The first group could be found in insects, brewer's yeasts (*Saccharomyces cerevisiae*), and bacteria. (Takewaki *et al*., 1980; Matsusaka *et al*., 1977; Suzuki *et al*., 1979). The enzyme in this group had high activity on heterogenous substrate such as sucrose and aryl α - glucosides so it was called to be a real α-glucosidase due to an ability to recognize α-glucosyl structure. The second group could be found in animals and plants (Chiba *et al*., 1976; Murata *et al*., 1979). The enzyme in the latter group had high activity on maltooligosaccharide so it was called to be maltase due to an ability to recognize maltosyl structure (Kimura, 2000).

In honey bee, there are three isoforms of AG which are AG I, AG II, and AG III. All AG were purified by various chromatographies. Briefly, proteins were separated by their physical and chemical characteristics. Protein mixture was applied through a column that contained matrix. The interaction between protein and matrix depended on specific characteristic of individual protein. Then, different proteins migrated through a column at different rates. In general, ion exchange, gel filtration, and affinity chromatographies had been widely used.

Ion exchange chromatography was useful at the initial purification step since it provided rapid flow rate and the capacity for high volume of crude extract. This method was directly involved in the net charge of protein. The charge of resin in a column was necessary to be opposite to the net charge of protein so the binding could occur. Injected protein with the same charge of matrix would not bind to the matrix

and was firstly released from the column. The rest with opposite charge could adsorb to the matrix and was eluted by gradually increasing the salt concentration. Therefore, the different protein was separated by different strength of binding to the matrix (Bollag *et al*., 1996).

Gel filtration chromatography was used to separate proteins based on their size. Large protein firstly came out from the column. Then, it was followed by small protein (Bollag *et al*., 1996).

Affinity chromatography was used to separate protein by a specific affinity between a protein and a ligand group attached to the matrix. The ligand group could be biologically specific to target protein such as substrate, peptide, antibody, nucleic acid, etc. When specific protein was bound to a ligand, the others would be moved out from the column. To release adsorbed protein, a more specific eluant would be used (Bollag *et al*., 1996). According to the mentioned well developed chromatographies, it brought to a success in purifying three AG isoforms.

In *A. mellifera*, AG I and AG II were purified by salting - out chromatography with 60 – 80% and 30 – 50% saturation of ammonium sulfate, respectively (Takewaki *et al*., 1980). AG I was further purified by CM-cellulose and sephadex G-100 columns while AG II was further purified by non – adsorbed active fraction on DEAE-cellulose, CM-cellulose, and Bio-Gel P-150 columns. Later, AG III was purified from an adsorbed active fraction after DEAE–cellulose of AG II and was further purified by DEAE-sepharose CL-6B, Bio-Gel P-150, and CM-Toyopearl 650M columns (Nishimoto *et al*., 2001).

After purification, some properties of all isoforms were compared to each other. Although all enzymes were monomeric, they were different in molecular weight (MW). The MW of AG I, AG II, and AG III were estimated to be

approximately 98, 76, and 68 kDa, respectively (Takewaki *et al*., 1980; Nishimoto *et al*., 2001). Three kinds of enzymes were glycoprotein containing about 25%, 15% and 7.4% of carbohydrate, respectively (Takewaki *et al*., 1980; Nishimoto *et al*., 2001). The optimal pH of AG I and AG II was 5.0 while that of AG III was 5.5 (Takewaki *et al*., 1980; Nishimoto *et al*., 2001). Three kinds of AG were also different in substrate specificity. AG I could not hydrolyse isomaltose and soluble starch but AG II could highly hydrolyse isomaltose and slowly hydrolyse soluble starch. Meantime, AG III has a little activity to isomaltose and low activity to soluble starch. All isoforms could hydrolyse sucrose, maltose, and phenyl α-glucoside but they could not hydrolyse α-1, 1-glucosidic linkage in trehalose (Takewaki *et al*., 1980; Takewaki *et al*., 1993; Nishimoto *et al*., 2001).

AG I and AG II were allosteric enzymes. AG I showed the negative kinetic cooperativity for sucrose, maltose, and *p* – nitrophenyl α – glucoside. In addition, it showed the positive kinetic cooperativity for turanose and maltodextrin. AG II revealed only the positive kinetic cooperativity for sucrose, turanose, kojibiose, and soluble starch (Kimura *et al*., 1990; Takewaki *et al*., 1993). In contrast, AG III did not indicate any allosteric properties (Nishimoto *et al*., 2001).

Considering kinetic investigation of substrate specificity, AG III was featured by the ability to more rapidly hydrolyse sucrose, phenyl α-glucoside, maltose, and maltotriose with high value of K_m than AG I and AG II. In addition, maltotriose could be hydrolysed over 3 times as fast as maltose (Nishimoto *et al*., 2001).

Furthermore, the difference among AG isoforms could be found in different organs of honey bee. In order to determine the localization of three AG isoforms in honey bee organs, the immunological method with ouchterlony double diffusion tests were performed. The precipitation reaction of antiserum against AG I, AG II, and AG

III could be observed. AG I was localized in ventriculus while AG II was localized in ventriculus and haemolymph. AG III was synthesized in hypopharyngeal gland and was transferred to honey crop. AG III played a role in changing nectar to be honey by breaking sucrose in nectar to be glucose and fructose in honey production. That led to the finding of AG III activity in honey.

In 2004, Kubota *et al*. could purify AG III from *A. mellifera* honey by salting–out chromatography, CM–cellulose, Bio–Gel P–150, and DEAE–sepharose CL–6B columns. The purification procedure of honey AG was almost similar to that of honey bee AG III. AG from both sources shared a lot in common. For example, both were monomeric and had the molecular weight at approximately 68 kDa. Furthermore, both were glycoprotein with the same carbohydrate content at about 7.4%. Moreover, both shared the identical N–terminal amino acid sequences, the same optimal pH at 5.5, and the same range of pH stability (5.0–10). Both could be stable up to 40ºC. Both could specifically hydrolyse sucrose, *p*–nitrophenyl α–glucoside, and maltotriose but could slowly hydrolyse soluble starch and isomaltose. Unlike honey bee AG III, unusual kinetics such as allosteric properties was not presented in honey AG. (Nishimoto *et al*., 2001; Kubota *et al*., 2004). Due to the data mentioned above, it was strongly supported that honey AG was honey bee AG III that was originally transferred from hypopharyngeal gland to honey crop.

AG has been widely studied in microorganisms (Ezeji and Bahl, 2006; Yamamoto *et al.*, 2004), plants (Sugimoto *et al*., 1995; Frandsen *et al.*, 2000), and animals including insect (Pratviel-Sosa *et al*., 1986), honey bee (Nishimoto *et al*., 2007). In insect, AG was mostly used to digest carbohydrate in food. It could be found in salivary glands and a digestive tract (Katagiri, 1979; Morinotti and James, 1990).

For example, Pratviel-Sosa *et al*. (1986) purified AG from larvae of butterfly *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) by chromatofocusing and hydroxyapatite chromatography. Some properties of purified AG were determined. The optimum pH was 6.0. The K_m value for *p*–nitrophenyl–α–D–glucoside, sucrose, and maltose was 0.5, 2.9, and 0.87 mM, respectively. Also, the enzyme could be stable up to 40ºC.

Moreover, AG from midgut of American cockroaches (*Periplaneta americana*) was purified by DEAE-cellulose column and affinity chromatography. The enzyme was characterized. It showed that the K_m value was 5 mM, the optimum pH was at 5.8, and it was stable up to 50ºC. The isoelectric point at pH 3.54 was also determined (Katagiri *et al*., 1979).

In 1990, Morinotti and James characterized AG involving in sugar digestion from adult salivary glands of vector mosquito *Aedes aegypti*. The MW was estimated to be 68 kDa and the optimum pH for sucrose hydrolysis was at pH 6. In both adult males and females, the maximum level of AG activity was found in salivary glands when they were at the second day after emergence.

Mehrabadi *et al*. (2009) studied the anatomy and digestive enzymes of midgut in *Eurygaster integriceps* Putton (Hemiptera: Scutelleridae). It was an important pest for wheat and barley in Europe and Africa. It was reported that a midgut of this insect was divided into four sections: the 1^{st} (v1), the 2^{nd} (v2), the 3^{rd} (v3), and the 4^{th} (v4) section. Digestive enzymes including α – amylase, AG, and β–glucosidase were presented in all four regions of midgut. The highest enzyme activity was in v3. α–amylase provided the optimal pH at 6.5 while AG and β–glucosidase showed the optimal pH at 5.

In microorganisms, AG was produced for glucose production. That was why it was generally applied into starch industry and other carbohydrate processing. Until now, there have been many researches on purifying native enzymes from microorganisms in order to find new sources of enzymes.

For example, in *Geobacillus thermodenitrificans* HRO10, AG which could hydrolyse starch into glucose was purified by PBE^{TM} 94 chromatofocusing and Superdex 200 columns. The MW of AG (45 kDa) was observed on SDS PAGE. The purified AG was optimal at the pH range of 6.5 – 7.5 and at the temperature of 55ºC (Ezeji and Bahl, 2006).

Yamamoto *et al.* (2004) successfully purified AG from *Acremonium implicatum* in the culture broth by DEAE–Toyopearl 650 M, Butyl–Toyopearl 650 M, and Toyopearl HW – 55 S. The MW of enzyme was estimated to be 440 kDa in tetrameric form and 103 kDa in monomeric form. The optimal pH was at 7. The optimal temperature was at 50ºC and was stable up to 40ºC.

As mentioned above, AG III is directly involved in honey processing. Furthermore, honey of *A. dorsata* is the most popular among Thai consumers due to the typical taste and smell. Characterization and properties of this enzyme in *A. dorsata* became very interesting for us. In addition, a research on *A. dorsata* AG has never been reported yet. There were three main parts in this research. The first one was to observe the expression level of *AG* in *A. dorsata* at various developmental stages by RT – PCR. The second one was to obtain the full length of open reading frame (ORF) of the cDNA. Also, phylogenetic trees of predicted amino acid sequences of AG among *Apis* species were constructed by UPGMA and NJ. Furthermore, for the third purpose, we attempted to partially purify AG III by DEAE–cellulose and Superdex 200 columns. The optimal conditions of partially purified AG

were also assayed. The outcome and benefit from this research was to know some properties of AG III in *A. dorsata* in order to compare to the AG from different sources. It may lead to obtain the new source of enzyme. Moreover, the obtained full length of ORF of cDNA sequence will be used in recombinant protein production for industrial application such as food and sweetener industry.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

- Access RT-PCR system (Cat# A1250), Promega, USA
- 100% Acetic acid, CH_3COOH, M.W. = 60.05, Merck, Germany
- Acetonitrile (ACN), CH_3CN, M.W. = 41.05, Merck, Germany
- Acrylamide, M.W. = 71.08, Promega, USA
- Ammonium hydrogen carbonate, NH_4HCO_3, M.W. = 79.06, Unilab, Australia
- Ammonium peroxydisulfate, [$(NH_4)_2S_2O_8$; APS], M.W. = 249, BDH laboratory supplies, England
- Boric acid, H_3BO_3, M.W. = 61.83 , Merck , Germany
- Bovine Serum Albumin(BSA), fraction V, pH 7.0, Serva feinbiochemica GmbH & Co., USA
- Calcium chloride dehydrate, $CaCl_2.2H_2O$, M.W. = 147, Merck, Germany
- Coomassie® Brilliant Blue G250, Fluka Analytical, UK
- Diethylaminoethyl-cellulose (DEAE-cellulose), Sigma, UK
- Di-sodium hydrogen orthophosphate anhydrous, Na_2HPO_4, M.W. = 141.96, AnalaR®, England
- Dithiothreitol (DTT), $C_4H_{10}O_2S_2$, M.W. = 154.25, USB, Canada
- D-maltose, $C_{12}H_{22}O_{11}.H_2O$, M.W. = 360.32, Labchem, Australia
- Ethylene Diamine Tetra Acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M.W. = 292.2,

Serva feinbiochemica GmbH & Co., USA

- 95% (v/v) Ethanol, CH_3CH_2OH, M.W. = 46, Thailand
- 37% (v/v) Formaldehyde, CH_2O, M.W. = 30, Thailand
- 98/100% Formic acid, HCOOH, 46.03, Fisher Scientific, UK
- Glucose CII (Cat# 432-90913), Wako, Japan
- Glycine, NH_2CH_2COOH, M.W. = 75.07, BDH laboratory supplies, England
- Hydrochloric acid fuming 37% (v/v), HCl, Merck, Germany
- Iodoacetoamide (IAA), C_2H_4INO, M.W. = 184.96, Fluka, USA
- 3-[N-morpholino] propanesulphonic acid (MOPS), $C_7H_{15}NO_4S$, M.W. = 209.27, Bio Basic Inc.
- Methanol, CH_3OH, M.W. = 32.04, Merck, Germany
- N, N'- methylene-bis-acrylamide (Acrylamide/bis), Sigma, USA
- Octylphenol-polyethyleneglycol ether (Triton X-100), Serva feinbiochemica GmbH & Co., USA
- 85% (v/v) Phosphoric acid, H_3PO_4, M.W. = 98, Mallinckrodt, USA
- QIAquick® gel extraction kit (Cat# 28704),Qiagen, Germany
- QIAquick® PCR purification kit (Cat# 28104), Qiagen, Germany
- Sodium acetate trihydrate, $CH_3COONa.3H_2O$, M.W. = 136.08, Merck, Germany
- Sodium chloride, NaCl, M.W. = 58.4, Merck, Germany
- Sodium dihydrogen orthophosphate 1-hydrate, $NaH_2PO_4.H_2O$, M.W. = 137.99, AnalaR®, England
- Sodium Dodecyl Sulfate (SDS), $C_{12}H_{25}SO_4Na$, M.W. = 288.38, BDH laboratory supplies, England
- Sodium hydroxide, NaOH, M.W. = 40, Merck, Germany

- Superdex^{TM} 75 prep grade, GE Healthcare, Sweden
- Superdex 200 prep grade, Amersham Bioscience, USA
- Tetramethylethyldiamine (TEMED), Promega, USA
- Trifluoroacetic acid (TFA), $C_2HF_3O_2$, M.W. = 114, BBH
- (Hydroxymethyl) aminomethane (Tris), $NH_2C(CH_2OH)_3$, M.W. = 121.1, Research Organics, USA
- Trypsin (EC 3.4.21.4), Sigma, Germany

2.2 Equipments and instruments

- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Autoclave, model: Tomy SX-700, Meditop Co., Ltd., Japan
- Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
- Centrifuge, model: Sorvall® pico, Kendro laboratory products, Germany
- Centrifuge/ vortex: Combi-spin FVL-2400, Biosan, USA
- Centrifuge, model: universal 32R, Hettich zentrifugen GmbH & Co., Germany
- Column, model: XK 16/20, Amersham Bioscience, USA
- Column, model: XK 16/70, Amersham Bioscience, USA
- Cuvette, model: 0.7-0.8 ml, Starna, England
- Dialysis bag, regenerated cellulose tubular membrane, model: Cellu Sep® T4, Membrane Filtration Products, Inc., USA
- Electronic U.V. transilluminator, Ultra lum Inc., USA
- Electrophoresis, model: AE- 6450 Dual mini slab kit, Atto, Japan
- Electrophoresis, model: Hoefer mini VE, Amersham Bioscience, USA
- Fraction collector, Akta prime, Amersham Pharmacia Biotech, USA

- Hot plate stirrer, Schott, Germany
- Incubator, Germany
- Microincubator, model: M-36, Taitec, Japan
- Microplate reader, model: Sunrise remote/ touch screen, Tecan, Austria
- Microwave, model: Sharp Carousel R7456, Sharp, Thailand
- Optima water purifier, model: Eigastat optima 60, Elga, England
- PCR, model: Gene Amp PCR system 2400, Applied Biosystem, Singapore
- PCR, model: Gene Amp PCR system 9700, Applied Biosystem, Singapore
- Polaroid, model: Direct screen instant camera DS 34 H-34, Peca products, UK
- Power supply, EC 570-90 LVD CE, E-C Apparatus corporation, USA
- Power supply, EPS 3501 XL, Amersham Bioscience, USA
- Power supply, EPS 301, Amersham Bioscience, USA
- pH meter, model: 215, Denver instrument, USA
- Recirculating water vacuum pump, Velp® scientifica, Europe
- Shaker 35, Labnet, USA
- Shaker Ika® KS 130 basic, GmbH & Co., Germany
- Sonicator, Branson, France
- Spectrophotometer, model: Ultraspec II, LKB biochrom, England
- Ultrafiltration devices, Vivaspin 20, GE Healthcare, UK
- Vacuum centrifuge, Heto, Maxi dry plus
- Vortex, Scientific industries, Inc., USA
- Vortex mixer: KMC-1300V: Vision scientific Co. Ltd., Korea
- Waterbath: Memmert schwabach, Germany

2.3 Sample collection

Apis dorsata bees were collected from a garden in Samut Songkram province. Eggs, larvae, and pupae were picked up directly from cells in a hive. In contrast, foragers were caught by a net. All of collected bees were kept at -20 ºC until use.

2.4 RNA extraction

2.4.1 The first method

The method of RNA extraction was performed by following the protocol of RNeasy® Mini Kit (Cat# 74104, Qiagen). Eggs, larvae, pupae, and heads of foragers (30 mg each) were homogenized by liquid nitrogen in a motar and a pestle. Then, 600 µl of buffer RLT was added to the lysate and mixed. It was centrifuged at 13,000 rpm, RT, for 2-3 min. The supernatant was carefully saved. One volume of 70% ethanol was added and mixed immediately. Sample (700 µl) was transferred into an RNeasy spin column which was placed in a 2 ml collection tube. The lid was closed gently. It was centrifuged at ≥ 10,000 rpm, RT, for 15 s. The flow through was discarded. Next, the spin column membrane was washed by 700 µl of buffer RW1. It was centrifuged at ≥ 10,000 rpm, RT, for 15 s. The flow through was discarded. Then, 500 µl of buffer RPE was added to the spin column and was centrifuged at ≥ 10,000 rpm, RT, for 15 s in order to wash the spin column membrane again. The flow through was discarded. The equal volume of buffer RPE was repeatedly added to the spin column. It was later centrifuged at ≥ 10,000 rpm, RT, for 2 min. In order to confirm the complete removal of buffer RPE, the column was re-centrifuged at ≥ 10,000 rpm, RT, for 1 min. The spin column was placed into a new 1.5 ml collection tube. RNase-free water (30 µl) was directly added to the spin column membrane. Finally, it was centrifuged at 10,000 rpm, RT, for 1 min to elute RNA. The extracted total RNA was stored at -20 ºC.

2.4.2 The second method

RNA was extracted by Trizol reagent (Cat# 15596-026, Invitrogen life technologies). Five heads of foragers (70 mg) or four larvae (960 mg) was homogenized in liquid nitrogen. Then, Trizol reagent was added to the lysate at the ratio of 1 ml of Trizol reagent to 50-100 mg of tissue. The mixture was centrifuged at 12,000 rpm, 4 ºC, for 10 min. The clear homogenate was saved and was incubated at RT for 5 min. After that, 0.2 ml of CH_3Cl per 1 ml of Trizol was added. It was vigorously shaken for 15 s. It was further incubated at RT for 2-3 min. It was centrifuged at ≤ 12,000 rpm, 2-8 ºC, for 15 min. RNA in the aqueous phase was saved and was precipitated by isopropanol at the ratio of 0.5 ml of isopropanol per 1 ml of Trizol. The mixture was incubated at RT for 10 min. It was centrifuged at ≤ 12,000 rpm, 4 ºC, for 10 min. The RNA pellet was washed by 75% ethanol at the ratio of 1 ml of 75% ethanol per 1 ml of Trizol and was vortexed. The mixture was centrifuged at 7,500 rpm, 4 ºC, for 5 min. Then, it was air dry. Finally, RNA was dissolved in 30 µl of RNase-free water. It was incubated at 55-60 ºC for 10 min and was stored at -20 ºC.

2.5 Formaldehyde/agarose gel electrophoresis

It was prepared by mixing 1.2% (w/v) agarose with 1x MOPS buffer. The mixture was melted at above 50 °C. After being cool, formaldehyde was added and mixed (appendix A). Total RNA was mixed by 1x formaldehyde loading dye, incubated at 65 °C for 3-5 min, chilled on ice, and loaded onto a formaldehyde/agarose gel. The electrophoresis was performed by 1x MOPS buffer as an electrode buffer at 5–7 V/cm for at least 30 min. The gel was stained by 10 µg/ml

EtBr for 10 min and was destained by dd-H_2O for 15 min. The 18S and 28S rRNA bands were observed under U.V. light.

2.6 Quantitative measurement of RNA

Extracted RNA was properly diluted by d-H_2O. The optical density (O.D.) of 260 and 280 nm was measured. The ratio of $O.D._{260}/O.D._{280}$ was used to determine the purity of extracted RNA. The good purity should be in the range of 1.8-2.0. The concentration of RNA was calculated from the $O.D._{260}$ due to a formula below:

Concentration of RNA (ng/µl) = $O.D._{260}$ x dilution factor x 40*

*40 is defined as one absorbance unit ($O.D._{260}$) that equals to 40 µg of single-stranded RNA in solution (ml).

2.7 Primer design

Forward and reverse primers for RT-PCR amplification of *α-glucosidase* (*AG*) in *A. dorsata* were designed from the conserved regions of the cDNA sequence of *AG III* in *A. mellifera* (GenBank, accession# NM001011608) (Ohashi *et al*., 1996). Three primer pairs were synthesized by Biodesign, Thailand. In addition, primers designed from the *28S rRNA* gene in *A. mellifera* (GenBank, accession# AB126808) and from the *elongation factor-1 alpha* (*EF-1α*) gene in *A. mellifera* (GenBank, accession# NM001014993) were used as control primers. All of those mentioned primers were shown in Table 2.1.

Table 2.1. Designed primers for RT-PCR amplification of *AG*.

Name of primers	Sequences of forward primers (5' → 3')	Sequences of reverse primers (5' → 3')	Expected size of product (bp)
AG (F1 and R1)	TTCGA CTTCT AGTTG GTAGC ATGAA GG	CCTTT CTCAT GTGCA GCACT GACTA G	350
AG (F1 and R2)	TTCGA CTTCT AGTTG GTAGC ATGAA GG	CACTT GGTGG CATGT ACGTC	1,000
AG (F2 and R3)	GCTTA TCGAG GCATA CACGA	CGCCG CTTCA AAGAA TAGAC	850
AG (F3 and R3)	ACGAG GAACA AATCG TGGAC	CGCCG CTTCA AAGAA TAGAC	200
28S rDNA	AAAGA TCGAA TGGGG AGATT C	CACCA GGTCC GTGCC TCC	358
EF-1α	TCGCT TTTAC TCTTG GTGTG A	AAACT TCCAA CATAT TATCT CCA	198

2.8 RT-PCR amplification

It was performed by using access RT-PCR system kit (Cat# A1250, Promega). The reaction mixture was 25 µl of final volume. It was comprised of 1x avian myeloblastosis virus (AMV)/*Tfl* reaction buffer, 0.4 µM of each F and R primers, 1 mM of $MgSO_4$, 0.2 mM of dNTP, 2.5 U of AMV reverse transcriptase and *Tfl* DNA polymerase, and 200 ng of RNA template. The optimum condition of RT-PCR was as follows: 1 cycle of 48 ^oC for 45 min and 94 ^oC for 2 min; 30 cycles of denaturation at 94 ^oC for 30 sec, of annealing at 42 ^oC for 30 sec, and of extension at 68 ^oC for 1 min; and finally, 1 cycle of final extension at 68 ^oC for 7 min. For *28S rRNA* and *EF-1α* amplification, the condition was the same as the condition for *AG* amplification except the annealing step was 45 ^oC for 30 sec and was 40 ^oC for 1 min, respectively. RT-PCR products were examined by 1.2% agarose gel electrophoresis, 10 µg/ ml EtBr stain, and U.V. visualization. An expected band was excised, purified, and sent to the genome institute, BIOTEC, Thailand for nucleotide sequencing.

2.9 Expression pattern of *AG*

Total RNA extracted from eggs, larvae, pupae, and foragers of *A. dorsata* (200 ng) was used as a template. RT-PCR was performed by using a pair of F1 and R1 primers. The product of 350 bp was expected as the detail in Table 2.1. In addition, the negative control was performed by using no RNA template in a reaction. After that, RT-PCR amplicon was detected by 1.2% (w/v) agarose gel electrophoresis and 10 µg/ml EtBr staining.

2.10 Agarose gel electrophoresis

DNA and cDNA was resolved by 1.2% (w/v) agarose gel in 1x TBE buffer (appendix A). DNA was mixed with 1x loading dye (SibEnzyme or appendix A). Then, the mixture was loaded onto the gel. It was electrophoresed in 1x TBE buffer as an electrode buffer (appendix A) at 100 V for 50 min. After that, the gel was stained by 10 µg/ml EtBr for 5 min and was destained by dd-H_2O for 30 min. A DNA band was visibly detected under U.V. light.

2.11 PCR product purification

2.11.1 In solution purification

RT-PCR product was purified by using QIAquick® PCR purification kit (Cat# 28104, Qiagen). RT-PCR product was firstly mixed by buffer PBI (5x volume). The yellow mixture was applied to a QIAquick spin column which was already placed in a 2 ml collection tube. It was centrifuged at 10,000 rpm, RT, for 30-60 s. The flow through was discarded. Buffer PE (750 µl) was added to the quick column. It was also centrifuged at 10,000 rpm, RT, for 30-60 s. After the flow through was discarded, the column was centrifuged for an additional 1 min. After that, the quick column was placed in a clean microcentrifuge tube (1.5 ml). For DNA elution, buffer EB (20 µl) was added to the center of the quick membrane. After the column was incubated at RT for 1 min, it was centrifuged at 10,000 rpm, RT, for 1 min. The product was kept at -20 ºC until use.

2.11.2 In gel purification

An expected PCR product was excised from an agarose gel and weighed. Then, it was purified by using QIAquick® gel extraction kit (Cat# 28704, Qiagen). Buffer QG (3x volume) was added to the sample (100 mg). The mixture was

incubated at 50 ºC for 10 min and vortexed for every 2-3 min. After the gel was completely dissolved, the yellow mixture was obtained. Then, 1x volume of isopropanol was added and mixed. The sample was applied to the quick column that was placed in a collection tube (2 ml). It was centrifuged at 10,000 rpm, RT, for 1 min. The flow through was discarded. Buffer QG (500 µl) was added to the quick column. It was centrifuged at 10,000 rpm, RT, for 1 min. Buffer PE (750 µl) was added to the column and was centrifuged at 10,000 rpm, RT, for 1 min again. The flow through was discarded. The column was centrifuged at 10,000 rpm, RT, for an additional 1 min. Next, the quick column was placed in a clean microcentrifuge tube (1.5 ml). Buffer EB (20 µl) was added to the center of the quick membrane for DNA elution. After the column was incubated at RT for 1 min, it was centrifuged at 10,000 rpm, RT, for 1 min. The eluted DNA was kept at -20 ºC until use.

2.12 Sequence alignment and phylogenetic construction

Purified DNA was sent to the Genome institute, BIOTEC, Thailand for direct sequencing. The obtained nucleotide sequence was blasted by www.ncbi.nlm.nih.gov. The nucleotide and deduced amino acid sequences were aligned by Bioedit. Then, a phylogenetic tree was constructed by UPGMA and NJ programs.

2.13 Crude extract

Crude extract was prepared according to Takewaki *et al.* (1980). Foragers (500 g) were grinded in sodium phosphate buffer (pH 6.3) (appendix A). The homogenate was adjusted to be 3,000 ml of final volume with the same buffer. The mixture was stirred at 4 ºC for overnight. Then, the suspension was centrifuged at 8,370 rpm (10,000x g), 4 ºC, for 15 min. The supernatant (about 2,300 ml) was

obtained. After that, it was optimally precipitated by slowly adding ammonium sulfate while it was stirring at 4 ºC for overnight. Then, the sample was centrifuged at 8,370 rpm (10,000x g), 4 ºC, for 25 min. The rest of supernatant (2,600 ml) was kept and was stored at 4 ºC until assay and chromatography purification. The sediment was dissolved in 350 ml of the same buffer.

2.14 Optimal concentration of ammonium sulfate

Ammonium sulfate (AS) at 0-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, and 80-95% was added to the supernatant (in 2.13) in order to determine the optimal saturation for protein precipitation.

2.15 Dialysis

Either pellet or supernatant (from 2.13) was dialysed by a dialysis bag (MWCO at 12,000-14,000 Da, flat width: 45 mm, and vol/cm: 6.42 ml) in dd-H_2O at 4ºC for overnight. After dialysis, the sample was centrifuged at 5,000 rpm, RT, for 5 min. The supernatant was kept at 4 ºC before chromatography.

2.16 Chromatography

2.16.1 Pellet after AS to chromatography

2.16.1.1 Diethylaminoethyl-cellulose chromatography (DEAE-cellulose column)

Precipitated crude extract (1,051.25 mg protein in 25 ml) was applied to a DEAE-cellulose column (1.5 x 11.5 cm) in 30 mM sodium phosphate buffer (pH 6.3) as a mobile phase at a flow rate of 1 ml/min. Bound protein was eluted from the column by a stepwise at 0.2, 0.5 and 1 M NaCl. A fraction size was collected at 10 ml

of the volume. The protein absorption was monitored at 280 nm.

2.16.1.2 Gel filtration chromatography (Superdex 200 column)

Positive fractions from DEAE column were pooled for a gel filtration, Superdex 200. The solution (66.67 mg protein) was injected into the column (1.5 x 41 cm) that was equilibrated and was eluted by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3). The flow rate of the column was 0.5 ml/min and the fraction size was collected at 10 ml of the volume.

2.16.2 Supernatant after AS to chromatography

2.16.2.1 DEAE-cellulose chromatography

The supernatant after dialysis (86 mg protein in 5 ml) was injected into a DEAE column (1.6 x 20 cm) in 30 mM sodium phosphate buffer (pH 6.3) as a mobile phase at a flow rate of 2 ml/min. Bound protein was eluted from the column by a stepwise at 0.25, 0.50, 0.75, and 1 M NaCl. The fraction size was collected at 10 ml of the volume.

2.16.2.2 Gel filtration chromatography (Superdex 75 column)

After DEAE, unbound fraction #2 (0.38 mg protein), unbound fraction #3 (2.45 mg protein), and bound fraction #14 (0.94 mg protein) were separately injected into a chromatography of gel filtration, Superdex 75 (1.6 x 60 cm). The column was pre-equilibrated and eluted by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3). The flow rate was 0.5 ml/min and the fraction size was collected at 10 ml of the volume.

2.17 Protein determination

2.17.1 Bradford assay

The concentration of protein was determined by Bradford method (Bradford, 1976). Bovine serum albumin (BSA) at 0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 µg/µl was used as standard protein. Each standard protein (20µl) was transferred to a well of a microtiter plate and was mixed by Bradford solution (200µl, appendix A). The mixture was incubated at RT for 5 min. Later, it was measured at the absorbance of 595 nm. The concentration of standard protein was plotted as an X axis while the optical density at 595 nm was plotted as a Y axis. The correlation of two mentioned values was estimated. Later, the concentration of an interesting protein could be calculated from a standard curve.

2.17.2 Absorbance measurement at 280 nm

Protein concentration was alternatively determined by the absorbance at 280 nm. The concentration could be estimated from the formula below:

Concentration of protein (mg/ml) = $(O.D._{280})$ x dilution factor

2.18 Enzyme activity of AG

A mixture was prepared from 20 µl of 50 mM sodium acetate (pH 5.1) containing 0.05% Triton X-100 and 20 µl of 0.5% maltose as a pre-mixture. It was pre-incubated at 37 ºC for 3 min. Then, the mixture was added with 10 µl of diluted enzyme (1x, 10x, and 100x) and was incubated at 37 ºC for 10 min. After that, 100 µl of 2 M Tris-HCl (pH 7.0) was added in order to stop a reaction. Next, the reaction was mixed by 20 µl of glucostat reagent (Cat# 432-90913, Wako, Japan) for a colorimetric assay. The mixture was further incubated at 37 ºC for 1 h. Then, the absorbance was measured at 505 nm. One unit of enzyme is defined as an activity of enzyme that can hydrolyse 1 µM of maltose (substrate) per minute under the optimal condition.

2.19 Protein precipitation by trichloro acetic acid (TCA)

Protein in a fraction from chromatography was concentrated before separating on SDS PAGE. TCA was added to the sample to provide a 10% (v/v) of final concentration. Then, the solution was vortexed and was incubated on ice for 30 min. It was centrifuged at 9,000 rpm, 4 ºC, for 15 min. The supernatant was discarded and the pellet was resuspended in 100 µl of cold acetone preserved at -20 ºC. The pellet was vortexed and was centrifuged at 9,000 rpm, 4 ºC, for 15 min. The pellet was resuspended again in 100 µl of cold acetone and was centrifuged at 9,000 rpm, 4ºC, for 15 min. Finally, the pellet was air dried and was dissolved in 15 µl of electrode buffer.

2.20 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Protein was resolved via the discontinuous SDS PAGE containing 8% (v/v) separating gel and 4% (v/v) stacking gel (appendix A). Protein was mixed by an electrode buffer (appendix A) and 1x protein loading dye (appendix A). The mixture was heated at 70 ºC for 1 min and was cool on ice. After loading, electrophoresis was performed by using an electrode buffer (appendix A) at 100 V until the font dye moved to the bottom of a gel. After electrophoresis, the gel was stained by coomassie blue staining solution (appendix A) for 20 min and was de-stained by de-staining solution (appendix A) until the background was clear.

2.21 Tryptic digestion

A desired band of protein was cut from an SDS PAGE gel and was placed in a microcentrifuge tube (2 ml size). The piece of gel was washed by dd-H_2O for 3x and the waste was discarded. The gel was cut into small pieces. The sample was added

with 50% acetonitrile (ACN)/0.1 M NH_4HCO_3 in 50 µl of the volume, was vortexed, and was incubated at 30 ºC, 20 min, for 2x. Next, it was dried by speed vacuum for 5 min. The dried gel was swollen in 50 µl of 0.1 M NH_4HCO_3/10 mM dithiothreitol (DTT)/1 mM EDTA and was incubated at 60 ºC for 45 min. After the solution was discarded, it was added with 50 µl of 100 mM iodoacetoamide (IAA)/0.1 M NH_4HCO_3 and was incubated in the dark at RT for 30 min. The solution was removed and the gel was washed by 50 µl of 50% ACN/0.05 M Tris-HCl (pH 8.5), 15 min for 3x. The gel was dried by speed vacuum. Thirty µl of digestion buffer containing trypsin buffer and trypsin solution at the ratio of 9: 1 (appendix A) was added to the gel. It was incubated at 37 ºC for overnight. The reaction was stopped by transferring the solution into a new tube (labeled as a "*" tube). The gel was further added by 25 µl of 2% trifluoroacetic acid (TFA) and was incubated at 60 ºC for 30 min. The solution was transferred to the "*" tube. The gel was next mixed by 40 µl of 0.05 M Tris-HCl (pH 8.5)/1 mM $CaCl_2$, was incubated at 30 ºC for 10 min, and was sonicated for 5 min. The gel was added by 40 µl of 100% ACN, was incubated at 30ºC for 10 min, and was sonicated for 5 min. The solution was moved to the "*" tube. The gel was added by 40 µl of 5% formic acid/100% ACN, was incubated at 30 ºC for 10 min, and was sonicated for 5 min. Next, the solution was transferred to the "*" tube. It was dried by speed vacuum and was examined by MALDI-TOF MS at Department of Chemistry, Faculty of Science, Chulalongkorn University.

2.22 Optimal conditions of AG activity

After gel filtration column (Superdex 200), the most positive fraction was used for the following experiments.

2.22.1 Optimal pH

A procedure was according to the assay for AG in 2.18 except the pre-mixture. It was prepared by mixing 20 µl of 10 mM Briton-Robinson buffer (appendix A) with 20 µl of 0.5% maltose. The pH of a reaction at 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8 was adjusted for this purpose. Triplication of experiments was performed.

2.22.2 Optimal temperature

A reaction mixture was performed by using the obtained AG. A procedure was followed by the assay for AG in 2.18 except the incubation temperature. It was varied from 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 ºC. Triplication of experiments was performed.

2.22.3 Optimal concentration of maltose (substrate)

A reaction mixture was performed by using the obtained AG. A procedure was followed by the assay for AG in 2.18 except the concentration of maltose. It was adjusted to be 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM. Triplication of experiments was performed.

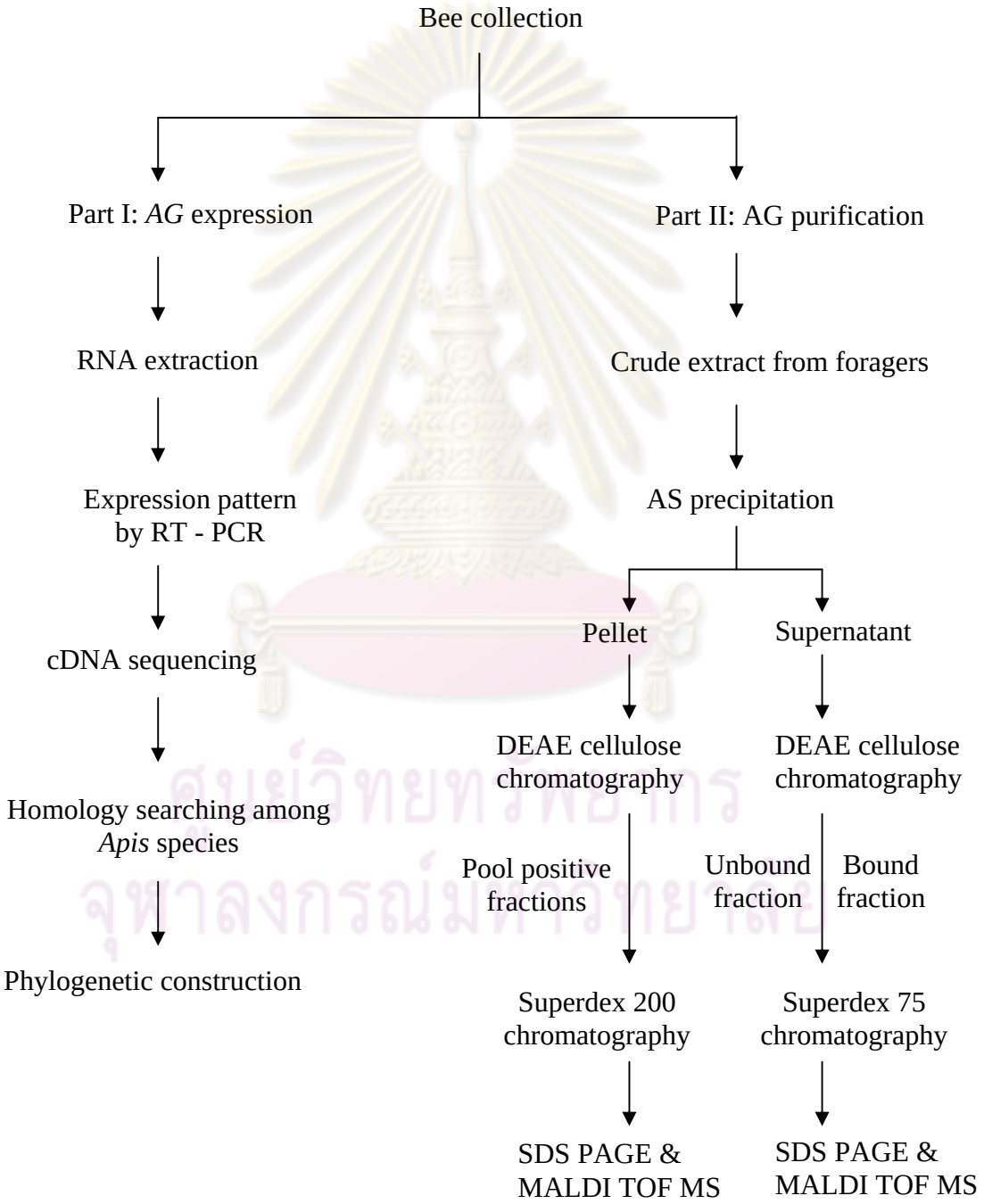
2.22.4 Optimal incubation time

A reaction mixture was performed by using the obtained AG. A procedure was followed by the assay for AG in 2.18 except the incubation time. It was varied from 0, 10, 20, 30, 40, 60, and 120 min. Triplication of experiments was performed.

CHAPTER III

RESULTS

Overview of experiment



3.1 The expression pattern of *α-glucosidase* (*AG*) in *Apis dorsata*

The quality of extracted RNA from eggs, larvae, pupae, and foragers of *A. dorsata* was examined by either 1.2% (w/v) agarose gel for quick determination or 1.2% (w/v) formaldehyde/agarose gel electrophoresis. After EtBr staining, the sharp bands of 18S and 28S rRNA were visible on 1.2% (w/v) agarose gel (Figure 3.1A). Furthermore, the smear of mRNA could be observed on the gel. Also, the 18S rRNA band was found on 1.2% (w/v) formaldehyde gel (Figure 3.1B).

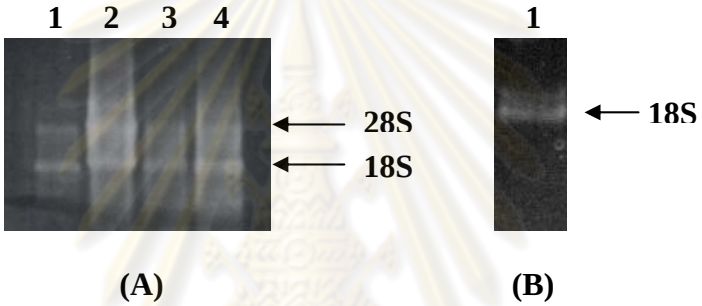


Figure 3.1. Total RNA extracted from *A. dorsata* at various stages on 1.2% (w/v) agarose gel (A) and 1.2% (w/v) formaldehyde/agarose gel (B). Lane 1(A)-4(A) contained total RNA extracted from eggs, larvae, pupae, and foragers, respectively, while lane 1(B) contained total RNA extracted from foragers.

Total RNA (200 ng) from each stage was amplified by F1 and R1 primers under the optimal condition mentioned in Materials and Methods. After electrophoresis, the expression profile of *AG* was obtained (Figure 3.2). A reaction containing no total RNA was used as a negative control. Furthermore, control experiments were performed by amplifying portions of *28S rDNA* and *EF-1α* housekeeping genes with the expected products of 358 and 198 bp, respectively. The

results presented that the amplified RT-PCR products could be obtained from samples at all developmental stages (Figures 3.3A and 3.3B). The quantity of amplified *AG* products was estimated by comparing the intensity of the amplified *AG* product band to the intensity of 100 DNA ladder marker bands. The comparable concentration of *AG* from each stage was reported by the ratio of estimated concentration of the amplified *AG* product to the amplified *EF-1α* product (Table 3.1). Considering Figures 3.2, 3.3, and Table 3.1, the result showed that the highest expression was in foragers (22.8 ng/μl). There was little detectable amplified *AG* product from eggs and pupae (14.3 and 21.4 ng/μl, respectively).

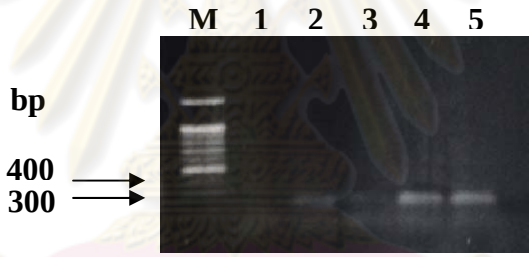


Figure 3.2. The expression pattern of *AG* in *A. dorsata*. Lane M contained 100 bp DNA ladder marker and lane 1 contained a negative control reaction. Lanes 2-5 contained the amplified *AG* product from eggs, larvae, pupae, and foragers, respectively.

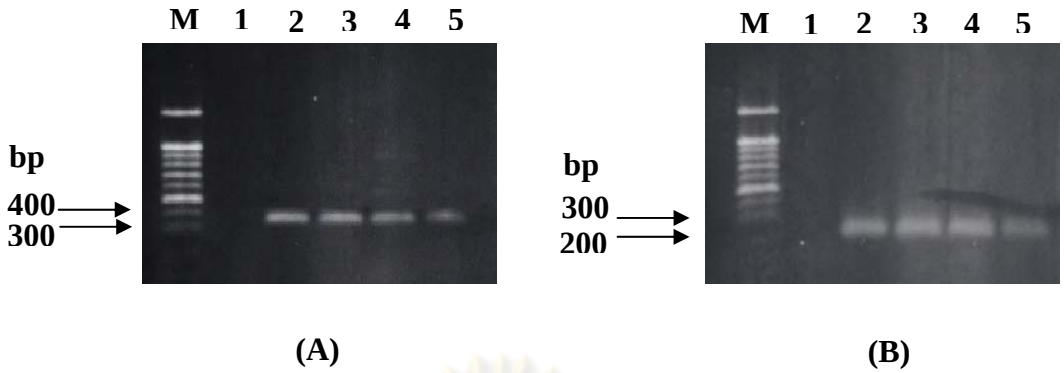


Figure 3.3. Control experiment by using primers for *28S rRNA* (A) and *EF-1α* (B) amplification. Lane M contained 100 bp DNA ladder marker and lane 1 contained a negative control reaction. Lanes 2-5 contained RT-PCR product from eggs, larvae, pupae, and foragers.

Table 3.1 The concentration ratio of RT-PCR product between *AG* and *EF-1α*.

Stage	Concentration of DNA product (ng/μl)	*AG*/*EF-1α*
Eggs	14.3	0.84
Larvae	9.5	0.58
Pupae	21.4	1.31
Foragers	22.8	2.13

3.2 Analysis of full length cDNA

In order to obtain the full length cDNA sequence of *AG*, RT-PCR was performed by using total RNA from foragers as a template and various pairs of primers as in Table 3.1. The conditions of RT-PCR from those primer pairs were the same as mentioned in Materials and Methods. The RT-PCR products were obtained from FW1/R1 primers in the size of 350 bp (Fig. 3.4A), FW1/R2 primers in the size of 900 bp (Fig. 3.4B), FW2/R3 primers in the size of 850 bp (Fig. 3.4C), and FW3/R3 primers in the size of 200 bp (Fig. 3.4D).

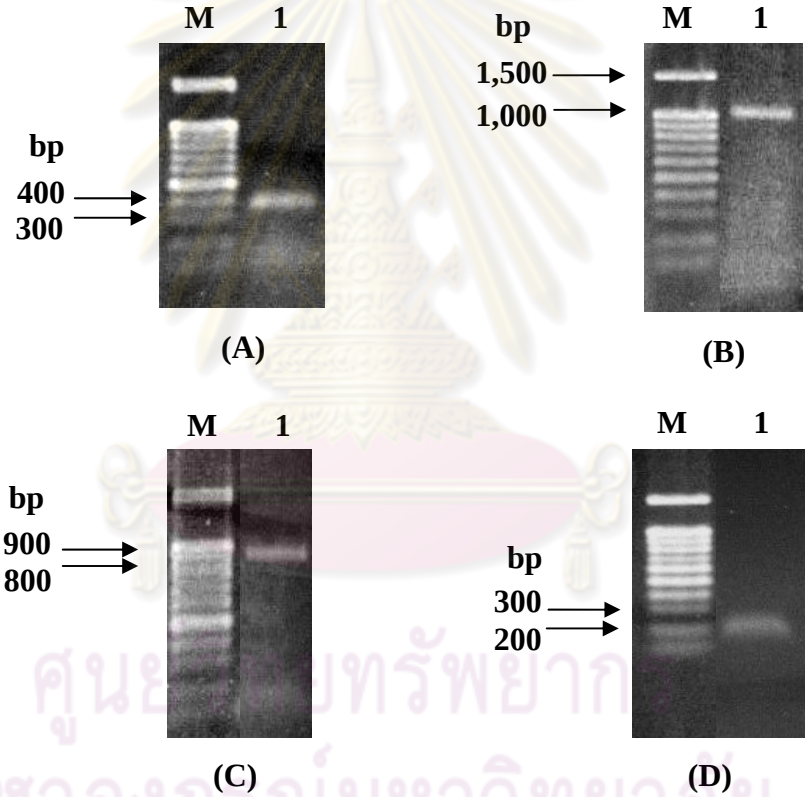


Figure 3.4. Amplified RT-PCR products from various pairs of primers. In all Figs. A-D, lane M contained 100 bp DNA ladder marker while lanes 1(A)-1(D) contained the product of 350 bp from FW1/R1 primers, the product of 900 bp from FW1/R2 primers, the product of 850 bp from FW2/R3 primers, and the product of 200 bp from FW3/R3 primers.

The RT-PCR products of *AG* were purified and sent to Genome institute, BIOTEC, Thailand for nucleotide sequencing. The start codon of the open reading frame was obtained since the FW1 primer was designed to encompass the open reading frame of the template gene. The full length of cDNA sequence at 1,704 bp was finally obtained and was recorded in GenBank as an accession number of GU224269.1. Later, the cDNA sequence was translated into an amino acid sequence by the program of Bioedit. The similarity of cDNA sequences was compared to the cDNA sequences of *AG* in *A. mellifera* (accession# NM_001011608.1), in *A. cerana indica* (accession# EF441271.1), in *A. cerana japonica* (accession# FJ889442.1), and in *A. florea* (accession# EF586680.1). In addition, the similarity of amino acid sequences was compared to the amino acid sequences of AG in *A. mellifera* (accession# NP_001011608.1), in *A. c. indica* (accession# ABO27432.1), in *A. c. japonica* (accession# ACQ45697.1), and in *A. florea* (accession# ABQ57387.1). Then, the obtained cDNA sequences and deduced amino acid sequence were aligned by Clustal X and Bioedit program (Figs. 3.5 and 3.6).

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Mellifera       ATGAAGGCAG TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC
Cerana ind      ATGAAGGCGA TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC
Cerana japo     ATGAGGGCGA TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC
Florea          ATGAAGGCGG TAATCGTGTT TTGCCTTATG GCATTGTCCA TTGTGGACGC
Dorsata         ATGAAGGCGG TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Mellifera       AGCATGGAAG CCGCTCCCTG AAAACTTGAA GGAGGACTTG ATCGTGTATC
Cerana ind      AGCATGGAAG CCGCTCCCTG AAAACTTGAA GGAGGACTTG ATCGTGTATC
Cerana jap      AGCATGGAAG CCGCTCCCTG AAAACTTGAA GGAGGGCTTG ATCGTGTATC
Florea          AGCATGGAAG CCACTCCCTG AAAACTTGAA GGAGGACTTG ATCGTGTATC
Dorsata         AGCATGGAAG CCGCTCCCTG AAAACTTGAA GGAGGACTTG GTCGTGTATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Mellifera       AGGTCTACCC GAGAAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT
Cerana ind      AGGTCTACCC AAGAAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT
Cerana jap      AGGTCTACCC AAGAAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT
Florea          AGGTTTACCC GAGGAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT
Dorsata         AGGTCTACCC GAGGAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
Mellifera       ATCGAAGGTA TTAAAGAAAA ATTGGATCAT TTTCTCGAAA TGGGGGTCGA
Cerana ind      ATCGAAGGTA TTAAACAAAA ATTGGACCAT TTTCTCGAAA TGGGCGTCGA
Cerana jap      ATCGAAGGTA TTAAACAAAA ATTGGACCAT TTTCTCGAAA TGGGCGTCGA
Florea          ATCATAGGTA TTAAAGAAAA ATTGGATCAT TTTCTCGAAA TGGGCGTCGA
Dorsata         ATCGAAGGTA TTAAACAAAA ATTGGATCAT TTTCTCGAAA TGGGCGTCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Mellifera       CATGTTTTGG TTATCCCCTA TTTATCCAAG CCCTATGGTC GATTTTGGTT
Cerana ind      TATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC GATTTTGGTT
Cerana jap      TATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC GATTTTGGTT
Florea          CATGTTTTGG TTATCCCCTA TTTATCCAAG CCCTATGGTC GATTTTGGTT
Dorsata         CATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC GATTTTGGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
Mellifera       ACGACATTTC GAATTACACC GACGTTCATC CCATATTTGG CACCATATCA
Cerana ind      ATGACATTTC GAATTACACC GATGTTCATC CCATATTTGG CACCTTATCA
Cerana jap      ATGACATTTC GAATTACACC GATGTTCATC CCATATTTGG CACCTTATCA
Florea          ACGACATTTC CAATTACACC GACGTTCATC CCATATTTGG CACCATATCA
Dorsata         ACGATATTTC GAATTACACC GACGTTCATC CCATATTTGG CACCATATCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
Mellifera       GACTTAGATA ATCTAGTCAG TGCTGCACAT GAGAAAGGAT TGAAGATAAT
Cerana ind      GACTTAGATA ACTTAGTTAA TGCTGCACAT GAGAAGGGAC TGAAGATAAT
Cerana jap      GACTTAGATA ACTTAGTTAA TGCTGCACAT GAGAAGGGAC TGAAGATAAT
Florea          GATTTAGATG ACCTAGTCAG TGCTGCACAT GAGAAAGGAC TGAAGATAAT
Dorsata         GACTTAGATA ACCTAGTTAA TGCTGCACAT GAGAAAGGAC TGAAGATAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
Mellifera       CTTGGATTTC GTCCCGAATC ATACATCTGA TCAACACGAA TGGTTCCAGT
Cerana ind      CTTGGATTTC GTTCCGAATC ATACATCTGA TCAACATGAA TGGTTCCAGC
Cerana jap      CTTGGATTTC GTTCCAAATC ATACATCTGA TCAACATGAA TGGTTCCAGC
Florea          CTTGGATTTC GTTCCGAATC ATACATCTGA TCAACACAAA TGGTTCCAGT
Dorsata         CTTGGATTTC GTTCCGAATC ATACATCTGA TCAACACGAA TGGTTCCAGT
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Figure 3.5. (Continued)

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
Mellifera       TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTACAT TTGGCATCCA
Cerana ind      TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTATAT TTGGCATCCA
Cerana jap      TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTATAT TTGGTATCCA
Florea          TGAGTTTGAA AAACGTTGAA CCTTATAACA ACTATTATAT TTGGCATCCA
Dorsata         TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTACAT TTGGCATCCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
Mellifera       GGAAAAATTG TAAATGGCAA ACGTGTTCCA CCAACTAATT GGGTAGGCGT
Cerana ind      GGAAAAATTG TAAATGGTAA ACGTGTTCCA CCAACTAATT GGGTAGGCGT
Cerana jap      GGAAAAATTG TAAATGGCAA ACGTGTTCCA CCAACTAATT GGGTAGGCGT
Florea          GGAAAAATTG TAAATGGTAA ACGTGTTCCA CCAAATAATT GGGTAGGCGT
Dorsata         GGAAAAATTG TAAATGGTAA ACGTGTTCCA CCAACTAATT GGGTAGGCGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
Mellifera       GTTTGGTGGA TCAGCTTGGT CGTGGCGGGA AGAACGACAG GCATATTATC
Cerana ind      ATTTGGTGGA TCAGCTTGGT CATGGCGAGA AGAACGACAG GCATATTATC
Cerana jap      ATTTGGTGGA TCAGCTTGGT CATGGCGAGA AGAACGACAG GCATATTATC
Florea          ATTTGGTGGA TCAGCTTGGT CATGGCGGGA AGAACGACAG GCATATTATC
Dorsata         ATTTGGTGGA TCAGCTTGGT CGTGGCGGGA AGAACGACAG GCATATTATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
Mellifera       TGCATCAATT TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT
Cerana ind      TGCATCAATT TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT
Cerana jap      TGCATCAATT TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT
Florea          TGCACCAATT TGCACCAGAA CAACCAGATT TAAATTACTA TAATCCAGCT
Dorsata         TGCATCAATT TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
Mellifera       GTACTGGATG ATATGCAAAA TGTTCTCAGA TTCTGGCTGA GAAGGGGATT
Cerana ind      GTACTAGATG ATATGCAAAA CGTTCTCAGA TTCTGGCTGA GAAGAGGACT
Cerana jap      GTACTAGATG ATATGCAAAA CGTTCTCAGA TTCTGGCTGA GAAGAGGACT
Florea          GTACTGGATG AAATGCAAAA CGTTCTTAGA TTCTGGTTGA AGAGAGGACT
Dorsata         GTACTGGATA ATATGCAAAA CGTTCTCAGA TTTTGGCTGA GGAGAGGACT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
Mellifera       TGATGGTTTC AGAGTAGATG CTCTGCCTTA CATTTGCGAA GACATGCGAT
Cerana ind      CGATGGTTTC AGAGTAGATG CTTTGCCTTA CATTTGCGAG GACATGCGAT
Cerana jap      CGATGGTTTC AGAGTAGATG CTTTGCCTTA CATTTGCGAG GACATGCGAT
Florea          TGATGGTTTC AGAGTAGATG CTCTGCCTTA CATTTGCGAA GATATGCGAT
Dorsata         TGATGGTTTC AGAGTAGATG CTCTGCCTTA CATTTGCGAA GACATGCGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
Mellifera       TCTTAGACGA ACCTCTATCA GGTGAAACAA ATGATCCCAA TAAAACCGAG
Cerana ind      TCTTAGACGA ACCCCTATCT GGTGAAACAA ATGATCCCAA TAAAACCGAG
Cerana jap      TCTTAGACGA ACCCCTATCT GGTGAAACAA ATGATCCCAA TAAAACCGAG
Florea          TCTTAGACGA ACCCCTATCA GGTGAAACAA ATGATCCCAA CAAAACTGAG
Dorsata         TCTTAGACGA ACCCCTATCA GGTGAAACAA ATGATCCCAA TAAAACCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
Mellifera       TACACTCTCA AGATCTACAC TCACGATATC CCAGAAACCT ACAATGTAGT
Cerana ind      TACACTCTCA AGATCTACAC TCACGATATC CCAGAAACCT ACAATATAGT
Cerana jap      TACACTCTCA AGAACTACAC TCACGATATC CCAGAAACCT ACAATATAGT
Florea          TACACTCTCA AGATCTACAC TCACGATATC CCAGAAACCT ACAATGTAGT
Dorsata         TACACTCTCA AAATCTACAC TCACGATATC CCGGAAACCT ACAACGTAGT
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Figure 3.5. (Continued)

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
Mellifera       TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC
Cerana ind      TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC
Cerana jap      TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC
Florea          TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC
Dorsata         TCGCAAATTC AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
Mellifera       TTATCGAGGC ATACACGAAT TTATCGATGA CGATGAAATA TTACGATTAC
Cerana ind      TTATCGAGGC ATACACGAAT TTATCGATGA CGATGAAATA TTACGATTAC
Cerana jap      TTATCGAGGC ATACACGAAT TTATCGATGA CGATGAAATA TTACGATTAC
Florea          TTATCGAGGC ATACACGAAT TTGTCCATGA CGATGAAATA TTACGATTAC
Dorsata         TTATCGAGGC ATACACGAAT TTATCGATGA CGATGAAATA TTACGATTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
Mellifera       GGAGCAGATT TTCCCTTCAA TTTTGCATTC ATCAAGAATG TTTCTAGGGA
Cerana ind      GGAGCAGATT TTCCCTTTAA TTTTGCATTC ATCAAGAATG TCTCTAAGGA
Cerana jap      GGAGCAGATT TTCCCTTTAA TTTTGCATTC ATCAAGAATG TCTCTAAGGA
Florea          GGAGCAGATT TTCCCTTCAA TTTTGCATTC ATCAAGAACG TCTCTAGGAA
Dorsata         GGAGCAGATT TTCCCTTCAA TTTTGCATTC ATCAAGAATG TCTCTAAGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
Mellifera       TTCAAATTCA TCAGACTTCA AAAAATTGGT CGATAATTGG ATGACGTACA
Cerana ind      TTCAAATTCA TCAGACTTCA AGAAATTGGT CGATAATTGG ATGATATACA
Cerana jap      TTCAAATTCA TCAGACTTCA AGAAATTGAT CGATAATTGG ATGATATACA
Florea          TTCAAATTCA TCAGACTTCA AAAAATTGGT CGATAATTGG ATGACGTACA
Dorsata         TTCAAATTCA TCAGACTTCA AGAAATTGGT CGATAATTGG ATGACGTACA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
Mellifera       TGCCACCAAG TGGTATTCCT AACTGGGTGC CCGGAAATCA CGATCAATTG
Cerana ind      TGCCAGCAGA TGGTATTCCT AACTGGGTGC CCGGAAATCA CGATCAATTG
Cerana jap      TGCCAGCAGA TGGTATTCCT AACTGGGTGC CCGGAAATCA CGATCAATTG
Florea          TGCCACCAAN TGGTATTCCT AACTGGGTGC CCGGAAACCA NGACCNATTG
Dorsata         TGCCAGCAAA TGGTATTCCT AACTGGGTGC CCGGAAATCA CGATCAATTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1060       1070       1080       1090       1100
Mellifera       AGATTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGA TCACCACGAT
Cerana ind      AGATTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGA TCACCGCGAT
Cerana jap      AGTTTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGT TCACCGCGAT
Florea          AGATNGGTGT CGAGATTTGG AGAAGAGAAG GGCCGTATGA TCACCACGAT
Dorsata         AGATTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGA TCACCACGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1110       1120       1130       1140       1150
Mellifera       GTCGCTTTTG CTGCCAGGTG TTGCCGTGAA TTACTACGGT GATGAAATTG
Cerana ind      GTCGCTTTTG CTGCCAGGTG TTGCCGTGAA TTACTACGGT GATGAAATTG
Cerana jap      GTCGCTTTTG CTGCCAGGTG TTGCCGTGAA TTACTACGGT GATGAAATTG
Florea          GTCGCTTTTG CTGCCAGGTG TTTCCGTGAA TTATTACGGT GATGAAATTG
Dorsata         GTCGCTTTTG CTGCCAGGTG TTGCCGTGAA TTACTACGGT GATGAAATTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1160       1170       1180       1190       1200
Mellifera       GTATGTCGGA TACTTATATC TCGTGGGAGG ATACGCAGGA TCCGCAGGGA
Cerana ind      GTATGTCGGA TACTTATATC TCGTGGGAGG ACACGCAGGA TCCACAGGGA
Cerana jap      GTATGTCGGA TACTTATATC TCGTGGGAGG ACACGCAGGA TCCACAGGGA
Florea          GTATGTCGGA TACTTATATC TCGTGGGAGG ACACGCAGGA TCCACAGGGA
Dorsata         GTATGTCGGA TACTTATATC TCGTGGGAGG ATACGCAGGA TCCACAGGGA
```

Figure 3.5. (Continued)

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1210       1220       1230       1240       1250
Mellifera       TGCGGCGCCG GTAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG
Cerana ind      TGCGGTGCCG GCAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG
Cerana jap      TGCGGTGCCG GCAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG
Florea          TGCGGTGCCG GTAAAGAAAA CTATCAAACA ATGTCGAGAG ATCCCGCGAG
Dorsata         TGCGGCGCCG GTAAAGAAAA TTATCAAGAG ATGTCGAGAG ATCCCGCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1260       1270       1280       1290       1300
Mellifera       AACGCCATTC CAATGGGACG ACTCAGTTTC TGCTGGATTT TCCTCAAGCT
Cerana ind      AACGCCATTC CAATGGGACG ACTCAGTTTC TGCTGGATTT TCCTCAAGCT
Cerana jap      AACTCCATTC CAATGGGACG ACTCAGTTTC TGCTGGATTT TCCTCAAGCT
Florea          AACGCCATTC CAATGGGACG ACTCACTTTC TGCTGGATTT TCCTCAAGCT
Dorsata         AACGCCATTC CAATGGGACG ACTCAGTTTC TGCTGGATTT TCCTCAAGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1310       1320       1330       1340       1350
Mellifera       CTAATACCTG GCTTCGTGTC AACGAAAATT ACAAGACTGT CAATCTAGCT
Cerana ind      CTGATACCTG GCTTCGTGTC AACGAAAATT ACAAGACTAT CAATTTAGCT
Cerana jap      CTGATACCTG GCTTCGTGTC AACGAAAATT ACAAGACTAT CAATTTAGCT
Florea          CTAATACGTG GCTTCGTGTC AATGAAAATT ACAAGACTGT CAATCTAGCT
Dorsata         CTAATACTTG GCTTCGTGTC AACGAAAATT ACAAGACTGT CAATCTAGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1360       1370       1380       1390       1400
Mellifera       GCTGAAAAGA AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTTGC
Cerana ind      GCTGAAAAGA AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTTGC
Cerana jap      GCTGAAAAGA AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTTGC
Florea          GCTGAAAAGA AGGACAAGAA CTCGTTCTTC AATATGTACA AGAAATTCGC
Dorsata         GCTGAAAAGA AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1410       1420       1430       1440       1450
Mellifera       GTCGCTGAAA AAATCGCCAT ACTTTAAAGA GGCCAATTTA AATACGAGGA
Cerana ind      AATGCTGAAA AAATCGCCAC ACTTTAAAGA GGCCAATTTA AATACGAGGA
Cerana jap      AATGCTGAAA AAATCGCCAC ACTTTAAAGA GGCCAATTTA AATACGAGGA
Florea          GTTGCTGAAA AAATCGCCAT ATTTTAAAGA GGCCAATTTA AGTACGAGGA
Dorsata         GTCGTTGAAA AAATCGCCAT ACTTTAAAGA GGCCAATTTA AATACAAGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1460       1470       1480       1490       1500
Mellifera       TGCTGAACGA CAATGTTTTC GCATTCTCTA GGGAAACCGA AGATAATGGA
Cerana ind      TGCTGAACGA CAGTGTTTTC GCATTCTCTA GGGAAACCGA AGAAAATGGA
Cerana jap      TGCTGAACGA CAGTGTTTTC GCATTCTCTA GGGAAACCGA AGAAAATGGA
Florea          TGCTGAACGA CAATGTTTTC GCNTTCTCTA GGGAAACCGA AGACAATGGA
Dorsata         TGCTGAACGA CAATGTTTTC GCTTTCTCTA GGGAAACCGA ACACAATGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550
Mellifera       TCTCTTTACG CAATATTGAA CTTCTCGAAC GAGGAACAAA TCGTGGATTT
Cerana ind      TCTCTTTACG CAATATTGAA CTTCTCGAAC GAGGAACAAA TCGTGGACTT
Cerana jap      TCTCTTTACG CAATATTGAA CTTCTCGAAC GAGGAACAAA TTGTGGACTT
Florea          TCTCTTTACG TAATAATGAA CTTCTCGAAC GAGGAACAAA TCGTGGATTT
Dorsata         TCTATTTATG TAATAATGAA CTTCTCGAAC GAGGAACAAA TCGTGGACTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1560       1570       1580       1590       1600
Mellifera       GAAAGCGTTC AATAACGTGC CGAAAAAATT GAATATGTTT TACAACAATT
Cerana ind      GAAAGCGTTT AATAACGTGC CGAAAAAATT GAATATGTTT TACACCATTT
Cerana jap      GAAAGCATTT AATAACGTGC CGAAAAAATT GAATATGTTT TACAACAATT
Florea          GAAAGCGTTT GATCACGTGC CGAAGAGATT GAATATGTTT TACAACAATT
Dorsata         GGAAGCGTTT AATAACGTGC CGAAAAAATT GAATATGTTT TATAACAATT
```

Figure 3.5. (Continued)

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1610       1620       1630       1640       1650
Mellifera       TTAACTCTGA TATAAAGTCC ATCTCCAACA ATGAACAAGT AAAAGTTTCT
Cerana ind      TTAACTCTGA TATAAAGTCC ATCTCCAACA ATGAACAAAT AAAAGTTTCT
Cerana jap      TTAACTCTGA TATAAAGTCC ATCTCCAACA ATGAACAAAT AAAAGTTTCT
Florea          TTAACTCTGA TATAAAATCC ATCTCCAACA ACGAGAAAAT AAAAGTTCCT
Dorsata         TTAACTCTGA TGTACAGTCC ATCTCCAACA ACGAGCAAGT AAAAGTTCCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1660       1670       1680       1690       1700
Mellifera       GCTTTAGGAT TTTTCATCTT AATTTCTCAA GATGCTAAAT TTGGAAACTT
Cerana ind      GCTTTAGGAT TTTTGATCTT AATTTCTCAA GATGCTAAAT TTGGAAATTT
Cerana jap      GCTTTAGGAT TTTTGATCCT AATTTCTCAA GATGCTAAAT TTGGAAATTT
Florea          GCTTTAAGAT TTNTAATCTT AATCTCTCAA GATGCTAAAT TTGAAAACAT
Dorsata         GCTTTAGGAT TTTTAGTCTT AATCTCTCAA GATGCTAAAT TTGGAAACTT

                ....

Mellifera       TTAA
Cerana ind      TTAA
Cerana jap      TTAA
Florea          TTAA
Dorsata         TTAA
```

Figure 3.5. The multiple alignment of *AG* cDNA sequences from *A. dorsata* and from another *Apis* species (*A. mellifera*, *A. c. indica*, *A. c. japonica*, and *A. florea*). The gray shadow indicated different base residues within the aligned sequences.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Mellifera       MKAVIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD
Cerana_ind      MKAIIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD
Cerana_jap      MRAIIVFCLM ALSIVDAAWK PLPENLKEGL IVYQVYPRSF KDSNGDGIGD
Florea          MKAVIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD
Dorsata         MKAVIVFCLM ALSIVDAAWK PLPENLKEDL VVYQVYPRSF KDSNGDGIGD


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Mellifera       IEGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS
Cerana_ind      IEGIKQKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTLS
Cerana_jap      IEGIKQKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTLS
Florea          IIGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS
Dorsata         IEGIKQKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Mellifera       DLDNLVSAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP
Cerana_ind      DLDNLVNAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP
Cerana_jap      DLDNLVNAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWYP
Florea          DLDDLVSAAH EKGLKIILDF VPNHTSDQHK WFQLSLKNVE PYNNYYIWHP
Dorsata         DLDNLVNAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
Mellifera       GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV
Cerana_ind      GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV
Cerana_jap      GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV
Florea          GKIVNGKRVP PNNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPA
Dorsata         GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Mellifera       VLDDMQNVLR FWLRRGFDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE
Cerana_ind      VLDDMQNVLR FWLRRGLDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE
Cerana_jap      VLDDMQNVLR FWLRRGLDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE
Florea          VLDEMQNVLR FWLKRGLDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE
Dorsata         VLDNMQNVLR FWLRRGLDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
Mellifera       YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY
Cerana_ind      YTLKIYTHDI PETYNIVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY
Cerana_jap      YTLKNYTHDI PETYNIVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY
Florea          YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY
Dorsata         YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
Mellifera       GADFPFNFAF IKNVSRDSNS SDFKKLVDNW MTYMPPSGIP NWVPGNHDQL
Cerana_ind      GADFPFNFAF IKNVSKDSNS SDFKKLVDNW MIYMPADGIP NWVPGNHDQL
Cerana_jap      GADFPFNFAF IKNVSKDSNS SDFKKLIDNW MIYMPADGIP NWVPGNHDQL
Florea          GADFPFNFAF IKNVSRNSNS SDFKKLVDNW MTYMPPXGIP NWVPGNXDXL
Dorsata         GADFPFNFAF IKNVSKNSNS SDFKKLVDNW MTYMPANGIP NWVPGNHDQL


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
Mellifera       RLVSRFGEEK ARMITTMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG
Cerana_ind      RLVSRFGEEK ARMITAMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG
Cerana_jap      SLVSRFGEEK ARMFTAMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG
Florea          RXVSRFGEEK GRMITTMSLL LPGVSVNYYG DEIGMSDTYI SWEDTQDPQG
Dorsata         RLVSRFGEEK ARMITTMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG
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Figure 3.6. (Continued)

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
Mellifera       CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYKTVNLA
Cerana_ind      CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSDTWLRV NENYKTINLA
Cerana_jap      CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSDTWLRV NENYKTINLA
Florea          CGAGKENYQT MSRDPARTPF QWDDSLSAGF SSSSNTWLRV NENYKTVNLA
Dorsata         CGAGKENYQE MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYKTVNLA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
Mellifera       AEKKDKNSFF NMFKKFASLK KSPYFKEANL NTRMLNDNVF AFSRETEDNG
Cerana_ind      AEKKDKNSFF NMFKKFAMLK KSPHFKEANL NTRMLNDSVF AFSRETEENG
Cerana_jap      AEKKDKNSFF NMFKKFAMLK KSPHFKEANL NTRMLNDSVF AFSRETEENG
Florea          AEKKDKNSFF NMYKKFALLK KSPYFKEANL STRMLNDNVF AFSRETEDNG
Dorsata         AEKKDKNSFF NMFKKFASLK KSPYFKEANL NTRMLNDNVF AFSRETEHNG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
Mellifera       SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YNNFNSDIKS ISNNEQVKVS
Cerana_ind      SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YTIFNSDIKS ISNNEQIKVS
Cerana_jap      SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YNNFNSDIKS ISNNEQIKVS
Florea          SLYVIMNFSN EEQIVDLKAF DHVPKRLNMF YNNFNSDIKS ISNNEKIKVP
Dorsata         SIYVIMNFSN EEQIVDLEAF NNVPKKLNMF YNNFNSDVQS ISNNEQVKVP

                ....|....| ....|..
                       560
Mellifera       ALGFFILISQ DAKFGNF
Cerana_ind      ALGFLILISQ DAKFGNF
Cerana_jap      ALGFLILISQ DAKFGNF
Florea          ALRFXILISQ DAKFENI
Dorsata         ALGFLVLISQ DAKFGNF
```

Figure 3.6. The multiple alignment of AG deduced amino acid sequence from *A. dorsata* and from another *Apis* species (*A. mellifera*, *A. c. indica*, *A. c. japonica*, and *A. florea*). The gray shadow indicated different amino acid residues within the aligned species.

The obtained *AG* (1,704 bp) cDNA sequence and the predicted amino acid sequence were blastn and blastp to the GenBank recorded sequences for homology searching. Considering Table 3.2, the similarity percentage between the cDNA sequences from *A. dorsata* to those from *A. mellifera*, from *A. c. indica*, from *A. c. japonica*, and from *A. florea* was at 96%, 95%, 94%, and 94%, respectively. Furthermore, due to Table 3.3, the similarity percentage between the deduced amino

acid sequences from *A. dorsata* to those from *A. mellifera*, from *A. c. indica*, from *A. c. japonica*, and from *A. florea* was at 96%, 95%, 94%, and 93%, respectively.

Table 3.2. The similarity of *AG* cDNA sequences among *Apis* spp.

Apis spp.	*A. dorsata*	*A. mellifera*	*A. c. indica*	*A. c. japonica*	*A. florea*
A. dorsata	-	96%	95%	94%	94%
A. mellifera	96%	-	96%	95%	95%
A. c. indica	95%	96%	-	99%	93%
A. c. japonica	94%	95%	99%	-	93%
A. florea	94%	95%	93%	93%	-

Table 3.3. The similarity of AG deduced amino acid sequences among *Apis* spp.

Apis spp.	*A. dorsata*	*A. mellifera*	*A. c. indica*	*A. c. japonica*	*A. florea*
A. dorsata	-	96%	95%	94%	93%
A. mellifera	96%	-	96%	95%	94%
A. c. indica	95%	96%	-	98%	92%
A. c. japonica	94%	95%	98%	-	91%
A. florea	93%	94%	92%	91%	-

A phylogenetic tree of AG deduced amino acids was constructed by using Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Neighbor-joining (NJ) methods via the program of Phylogenetic Analysis Using Parsimony methods* (PAUP* version 4.0b) (Swofford, 2000). Both trees shared similar characters. The bootstrap analysis with over than 50% in 1,000 replicates indicated the branch support in trees. The AG amino acid sequence from *Drosophila melanogaster* (accession# CG14934) was used as an out-group in bootstrap and NJ methods. A phylogenetic tree by UPGMA method showed that the AG sequence from *A. dorsata* and those from *A. c. indica,* from *A. c. japonica* and from *A. mellifera* were closely similar to 100 of bootstrap value (Fig. 3.7). In addition, a phylogenetic tree by NJ method (Fig. 3.8) showed two major clades. Clade I was comprised of the amino acid sequences of *A. c. indica, A. c. japonica*, *A. mellifera, A. dorsata,* and *A. florea* while Clade II was comprised of the amino acid sequences of *D. melanogaster*.

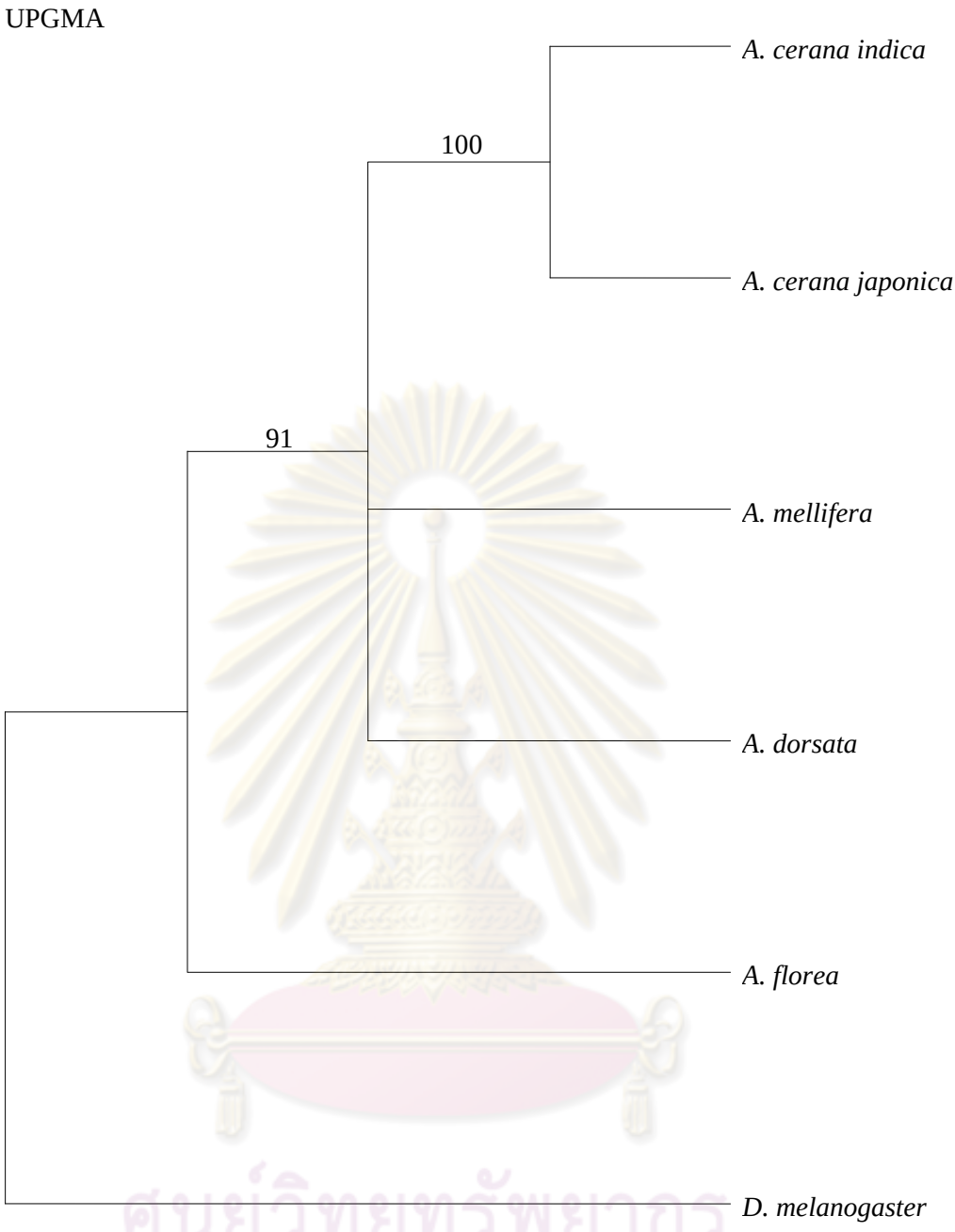


Figure 3.7. A phylogenetic tree of AG deduced amino acid sequences among *Apis* spp. by UPGMA method. The numbers indicated a value of bootstrap showing a branch support.

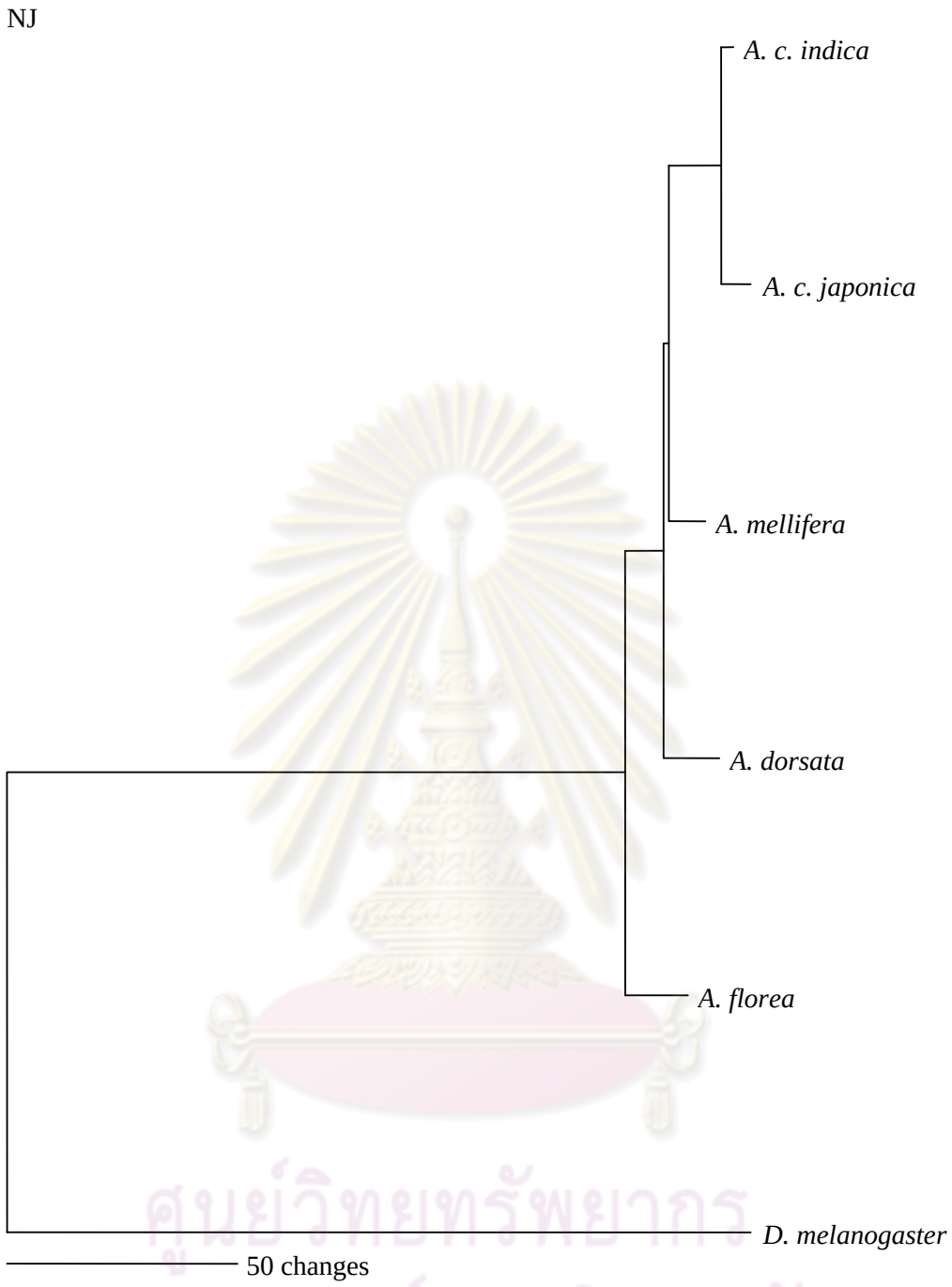


Figure 3.8. A phylogenetic tree of AG deduced amino acid sequences among *Apis* spp. by NJ method. The length of each branch presented the relationship between species.

3.3 Protein precipitation by ammonium sulfate

Different ranges of the percentages of ammonium sulfate (AS) was added to the crude protein to determine the optimal range for protein precipitation. Due to Fig. 3.9, the highest specific activity (0.025 U/mg) was found in precipitation of 80-95% AS while the lowest specific activity (0.004 U/mg) was obtained from precipitation of 30-40% AS. Crude protein without AS precipitation provided 0.009 U/mg of specific activity.

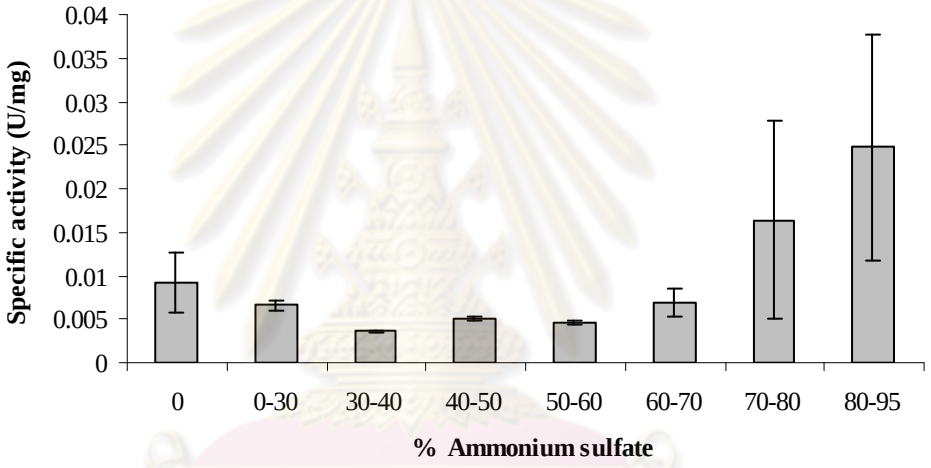


Figure 3.9. Specific activity of AG from the precipitation by different ranges of ammonium sulfate.

3.4 Protein purification

3.4.1 Purification of pellet after precipitation

3.4.1.1 DEAE-cellulose column (Anion exchange chromatography)

After precipitation by 95% AS, the pellet was dialysed and was applied to DEAE-cellulose column equilibrated by 30 mM sodium phosphate buffer (pH 6.3). The column was eluted by stepwise of 0.2, 0.5, and 1 M NaCl. AG activity was detected in both unbound (fractions# 3-6) and bound (fractions# 16-19, 22, and 25) peaks as in Figure 3.10. Then positive fractions were pooled and were freeze dried.

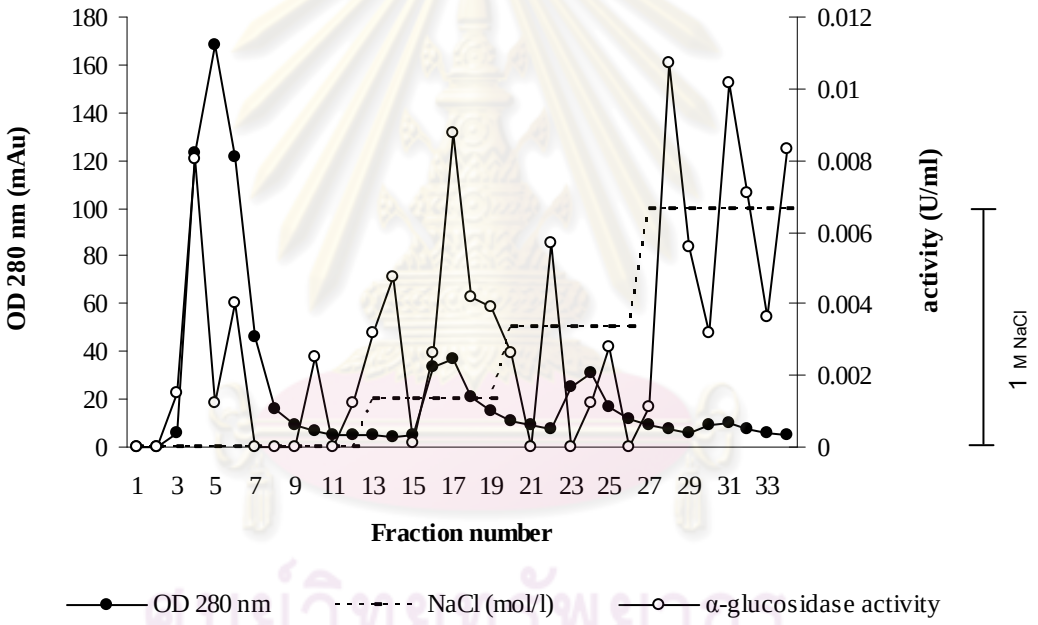


Figure 3.10. Purification of AG in dialysed pellet by DEAE-cellulose column. Protein sample, 1,051.25 mg; column, 1.5 x 11.5 cm; equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 20%, 50%, and 100% of 1 M NaCl; flow rate, 60 ml/h; fraction size, 10 ml.

3.4.1.2 Superdex 200 column (Gel filtration chromatography)

After freeze drying, protein (from Fig. 3.10) was injected into a Superdex 200 column equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3) as shown in Figure 3.11. The fractions containing high AG activity (fractions# 6, 7, 9, 10, 11, and 12) were concentrated to contain protein at the amount of 100 µg. Then, it was run by SDS PAGE (Figs. 3.12A and 3.12B). The expected bands were cut to analyse by MALDI TOF MS.

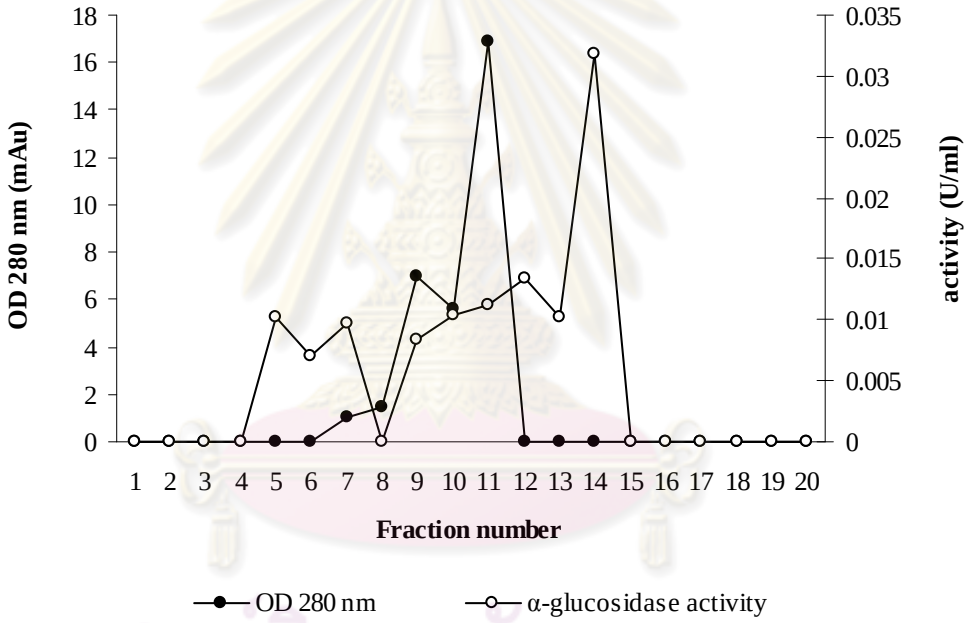


Figure 3.11. Purification of AG by Superdex 200 column. Protein sample, 67 mg; column, 1.5 x 40 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/h; fraction size, 10 ml.

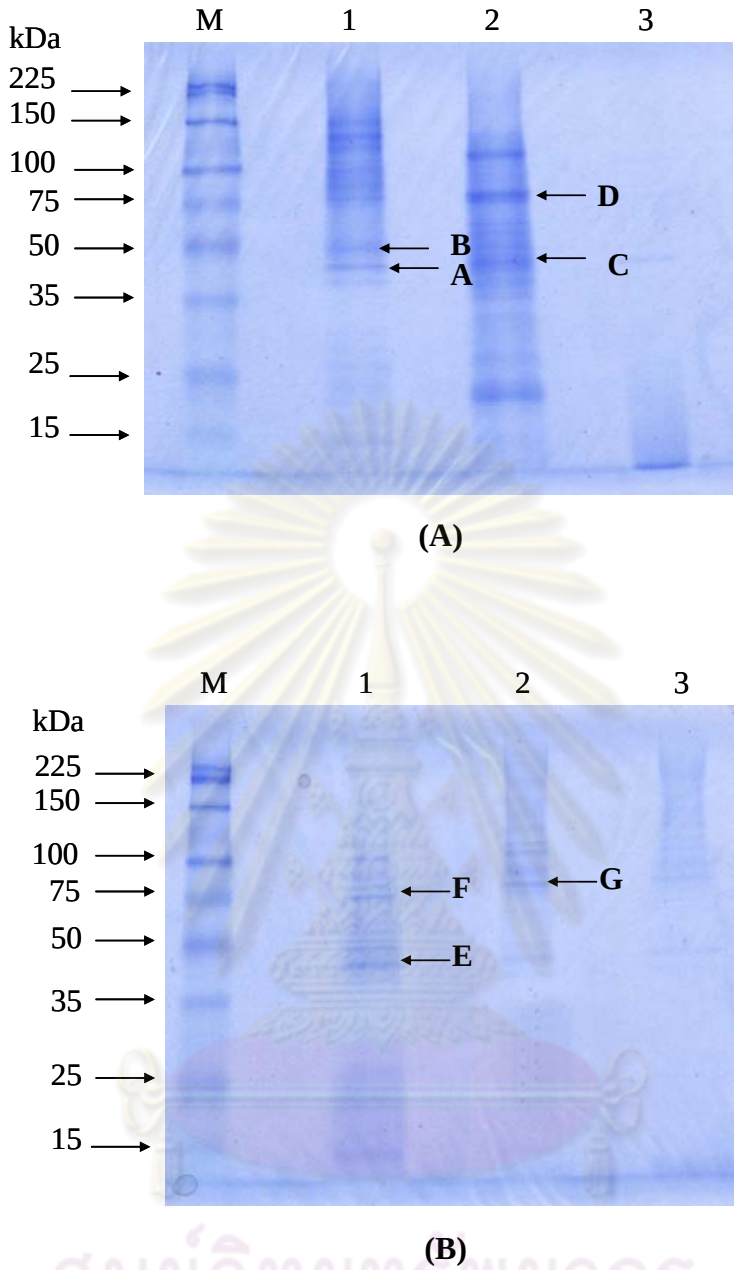


Figure 3.12. Coomassie blue stained SDS PAGE of fractions after Superdex 200 column. In both Figs., lane M contained broad range protein marker. In Fig. A, lanes 1-3 contained protein (100 µg) from fractions# 6, 7, and 9, respectively. In Fig. B, lanes 1-3 contained protein (100 µg) from fractions# 10, 11, and 12, respectively. Arrows indicated cut bands.

3.4.2 Purification of supernatant after precipitation

3.4.2.1 DEAE-cellulose column (Anion exchange chromatography)

Supernatant after precipitation by 95% AS was dialysed and was frozen dried. Then, it was subjected into a DEAE column equilibrated by 30 mM sodium phosphate buffer (pH 6.3). The column was eluted by stepwise gradient of 0.25, 0.5, 0.75, and 1 M NaCl. The AG activity was found in both unbound (fractions# 2-4) and bound (fractions# 13-15 and 22-24) peaks as shown in Figure 3.13.

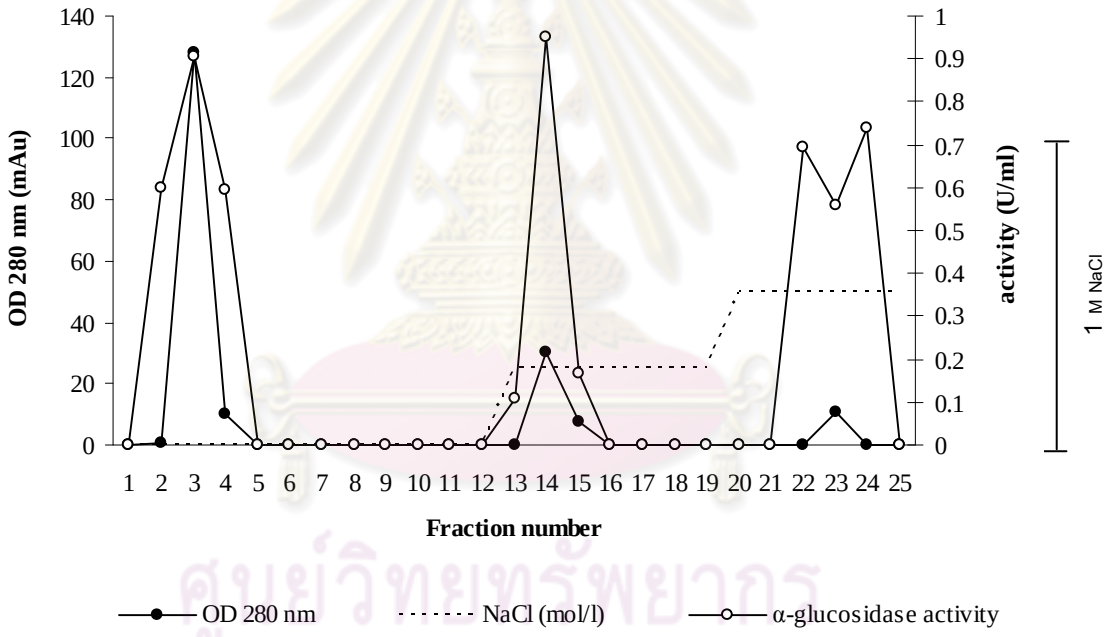


Figure 3.13. Purification of AG in supernatant by DEAE-cellulose column. Protein sample, 86 mg; column, 1.6 x 20 cm; equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 25%, 50%, 75%, and 100% of 1 M NaCl; flow rate, 60 ml/h; fraction size, 10 ml.

3.4.2.2 Superdex 75 column (Gel filtration chromatography)

The concentrated unbound fraction# 2 (after DEAE column, Fig. 3.13) was subjected into Superdex 75 column equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3) as in Figure 3.14. The high activity was obtained from fraction# 10 (0.013 U/ml), fraction# 11 (0.012 U/ml), fraction# 13 (0.11 U/ml) and fraction# 14 (0.013 U/ml). The fraction with high AG activity was examined by SDS PAGE (Fig. 3.15).

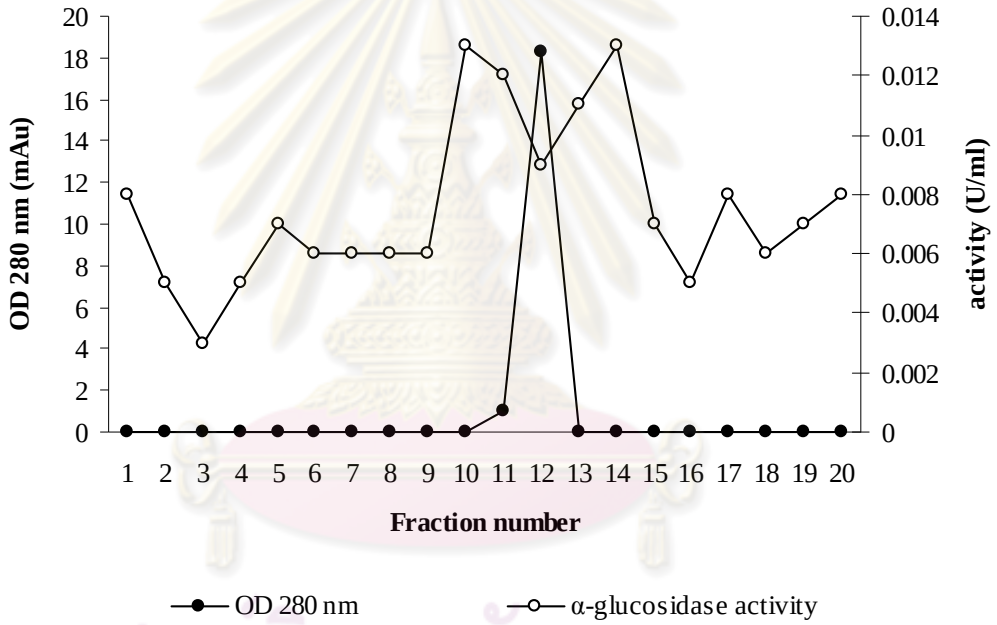


Figure 3.14. Purification of AG by Superdex 75 column. Positive unbound peak (fraction# 2) after DEAE-cellulose column in Fig. 3.13, 0.38 mg protein; column, 1.6 x 60 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/h; fraction size, 10 ml.

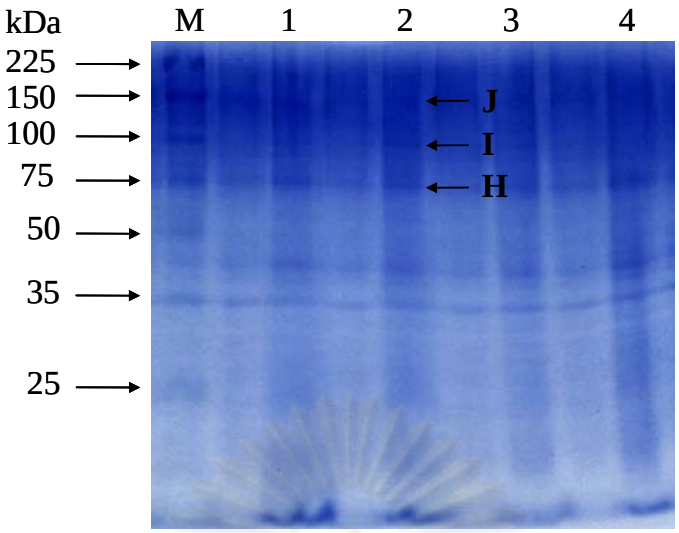


Figure 3.15. Coomassie blue stained SDS PAGE of positive fractions after Superdex 75 column (from Fig. 3.14). Lane M contained broad range protein marker while lanes 1-4 contained 50 µg protein from fractions# 10, 11, 13, and 14, respectively. Arrows presented cut bands.

The concentrated unbound fraction# 3 (after DEAE column, Fig. 3.13) was subjected into Superdex 75 column equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3) as in Figure 3.16. The high activity was obtained from fraction# 3 (0.015 U/ml), fraction# 11 (0.013 U/ml), fraction# 12 (0.015 U/ml), and fraction# 14 (0.014 U/ml). The positive fractions were examined by SDS PAGE (Fig. 3.17).

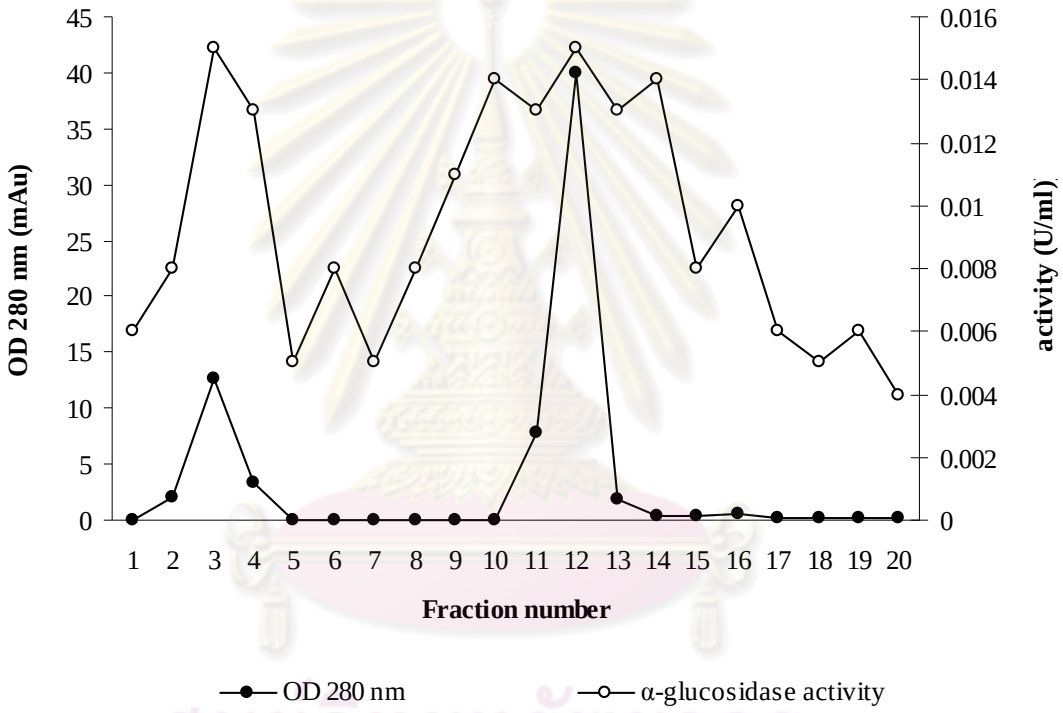


Figure 3.16. Purification of AG by Superdex 75 column. Positive unbound peak after DEAE-cellulose column (fraction# 3) in Fig. 3.13, 2.45 mg protein; column, 1.6 x 60 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/h; fraction size, 10 ml**.**

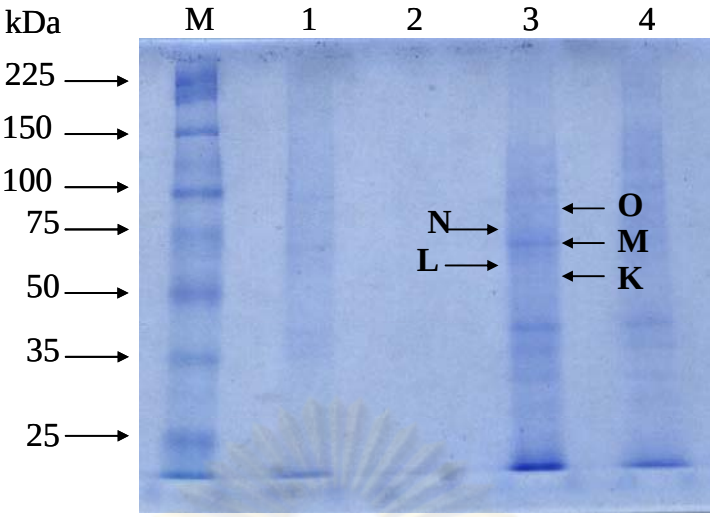


Figure 3.17. Coomassie blue stained SDS PAGE of positive fractions after Superdex 75 column (from Fig. 3.16). Lane M contained broad range protein marker while lanes 1-4 contained 50 µg protein from fractions# 3, 11, 12, and 14, respectively. Arrows indicated cut bands.

The concentrated bound fraction# 14 (from DEAE-cellulose column, Fig. 3.13) was subjected into Superdex 75 column equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3) as in Figure 3.18. The highest activity was from fraction# 13 (0.045 U/ml). The fraction with high AG activity was examined by SDS PAGE (Fig. 3.19A). Also, fraction# 13 after Superdex 75 column (Fig. 3.18) was concentrated by ultrafiltration devices, Vivaspin 20. Protein was precipitated by TCA. Then, the sample was separated on SDS PAGE (Fig. 3.19 B).

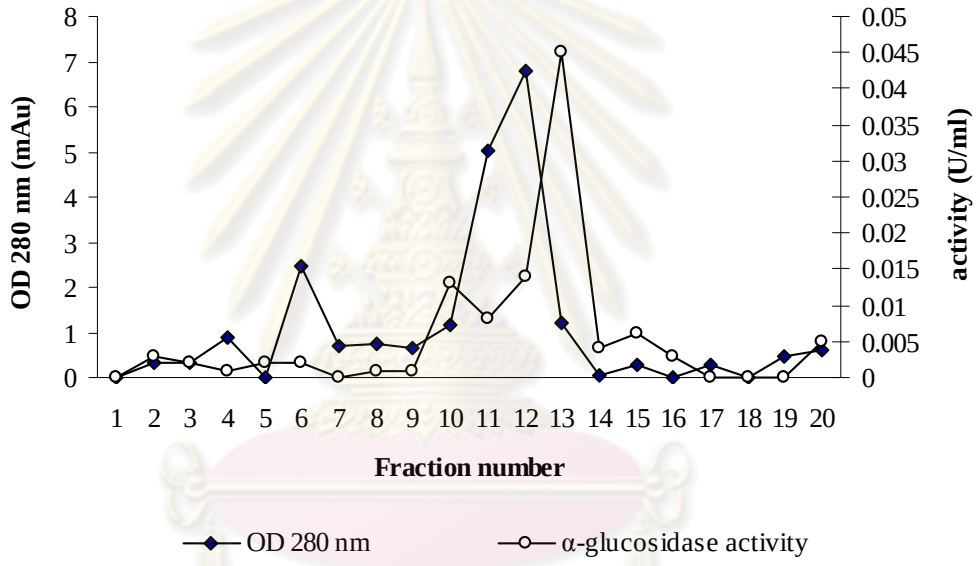


Figure 3.18. Purification of AG by Superdex 75 column. Positive bound peak from DEAE-cellulose column (fraction# 14) in Fig. 3.13, 0.94 mg protein; column, 1.6 x 60 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/h; fraction size, 10 ml**.**

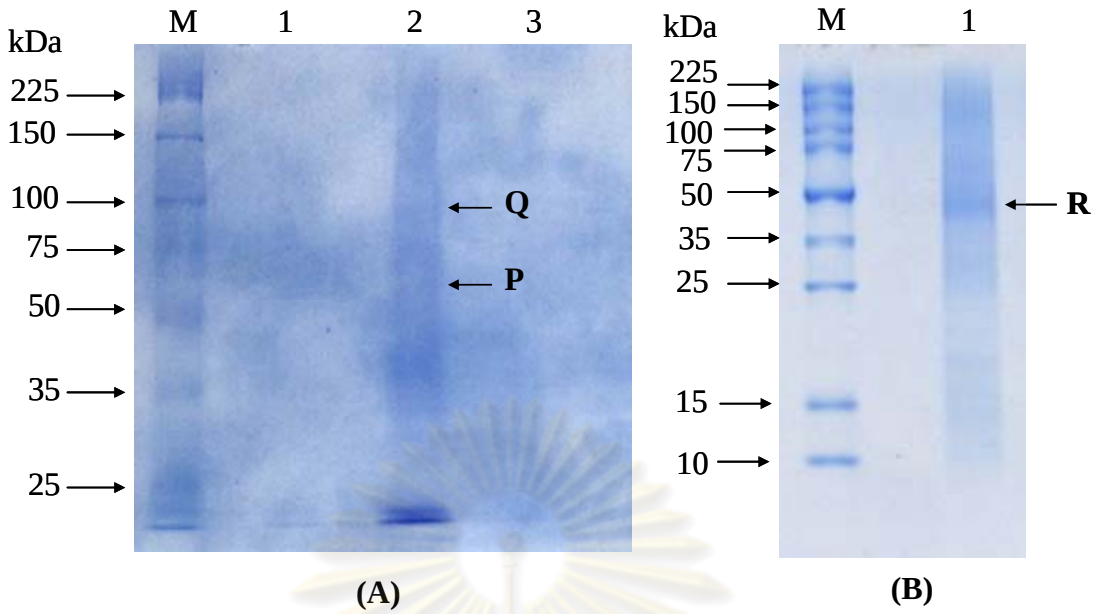
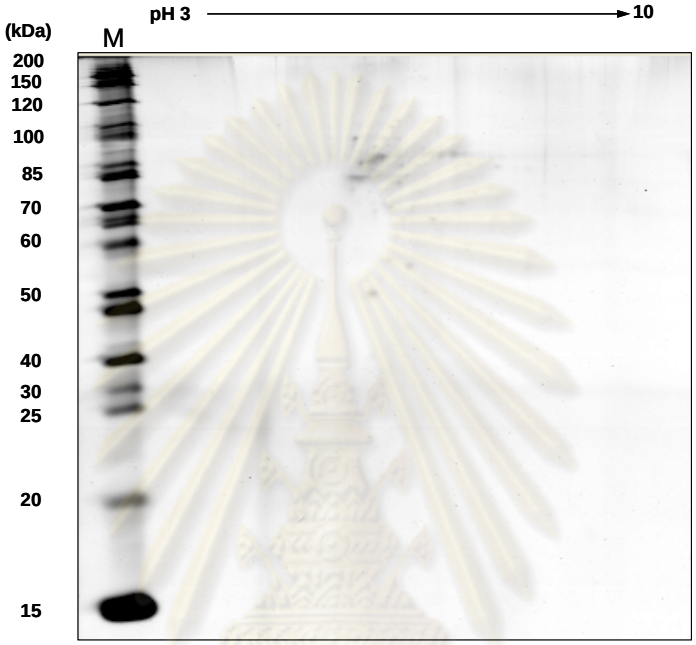
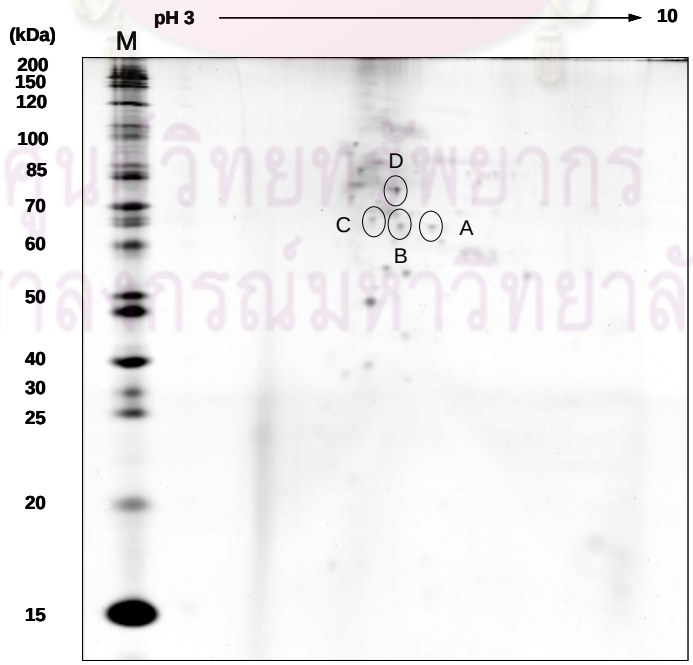


Figure 3.19. Coomassie blue stained SDS PAGE of positive fractions after Superdex 75 column. In both Figs., lane M contained broad range protein marker. In Fig. A, lanes 1-3 contained 50 µg protein from fractions# 12, 10, and 13 (from Fig. 3.18), respectively. In Fig. B, lane 1 contained concentrated sample (0.175 mg protein) from fraction# 13 (from Fig. 3.18). Arrows indicated cut bands.

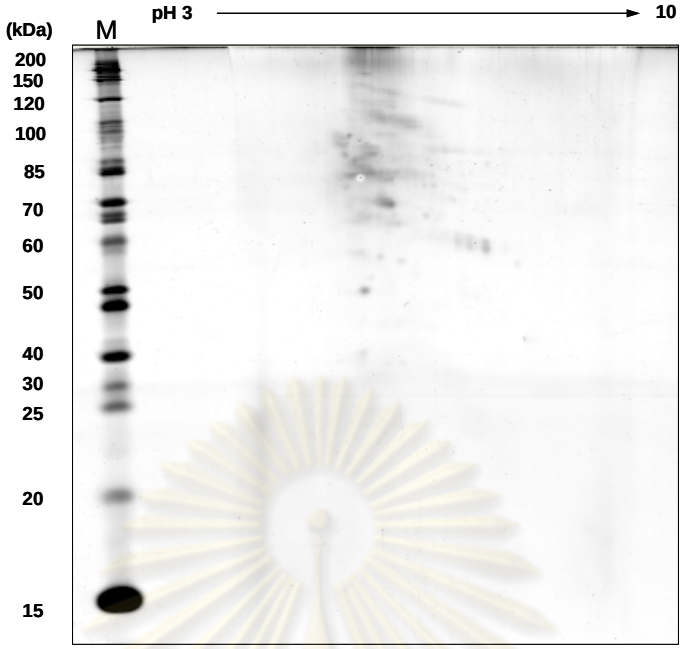
In addition, those fractions containing high AG activity after Superdex 75 column (from Fig. 3.18) were analysed by two-dimensional gel electrophoresis (2DE) by the Genome institute, BIOTEC, Thailand. The result was presented as in Fig. 3.20. Circled spots were cut for MALDI TOF MS.



(A)



(B)



(C)

Figure 3.20. Two-dimensional gel electrophoresis of fractions# 10 (Fig. A), 12 (Fig. B), and 13 (Fig. C) from Superdex 75 column (from Fig. 3.18).

All steps of AG purification could be summarized in Table 3.4. Specific activity of AG in crude protein (0.01 U/mg) was lower than AS precipitated pellet (1.70 U/mg). After precipitation, specific activity of AG was found in both pellet and supernatant. Although specific activity of AG in pellet (1.70 U/mg) seemed to be higher than that in supernatant (1.34 U/mg), it was not significantly different. Pellet and supernatant were firstly purified by DEAE-cellulose column. Both unbound and bound peaks from DEAE-cellulose column were assayed for AG activity. Then, the pellet was further purified by Superdex 200 and the supernatant was further purified by Superdex 75 column. After the second purification by gel filtration chromatography, the specific activity of AG was lost in pellet (0.09 U/mg). After

being purified by two chromatographies, higher specific activity of AG was recovered from supernatant (3.95 and 0.26 U/mg), respectively.

Table 3.4 Summary of the purification procedure for AG.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Pellet					
- Crude protein	51,000	500	0.01	100	1
- 95% AS	306.6	521.5	1.70	104.3	170
- DEAE-cellulose	11.28	0.05	0.004	0.01	0.4
- Superdex 200	0.355	0.032	0.09	0.006	9
Supernatant					
- Crude	97,251	2,217.6	0.02	100	1
- 95% AS	2,327	3,120	1.34	140.7	67
- DEAE-cellulose	1.52	6	3.95	0.3	197.5
- Superdex 75					
- unbound DEAE peak	1.33	0.14	0.11	0.006	5.5
- bound DEAE peak	1.75	0.45	0.26	0.02	13

Considering the *AG* cDNA sequence (1,704 bp) from *A. dorsata*, it could be predicted that the amino acid sequence should be in the length of 567 amino acids. The molecular weight (MW) was estimated to be 62 kDa. Therefore, protein bands appeared on SDS-PAGE in the range of 50-75 kDa were expected. Arrows were used to mark a location of expected bands. The relationship between R_f value and log MW of protein marker was plotted to determine MW of a target protein. According to Figs. 3.21 and 3.22, MW of bands A and B from fraction# 6 and band E from fraction# 10 after Superdex 200 column (from Figs. 3.12A and 3.12B) were calculated to be 54, 61 and 51 kDa, respectively. Meantime, due to Fig. 3.23, MW of bands K, L, and M from fraction#12 after Superdex 75 column (from Fig. 3.17) was calculated to be 51, 57, and 74 kDa, respectively. MW of the concentrated sample R from fraction# 13 after Superdex 75 column (from Fig. 3.19B) was calculated to be 58 kDa (Fig. 3.24). In addition, in Fig. 3.20B, spots A, B, C, and D of 2DE from fraction# 12 after Superdex 75 column (bound peak after DEAE-cellulose column) were located in the expected range and were clearly separated from others. All of those bands and spots were excised and were tryptic digested for peptide mass analysis by either MALDI TOF MS or LC/MS at Department of Chemistry, Faculty of Science, Chulalongkorn University.

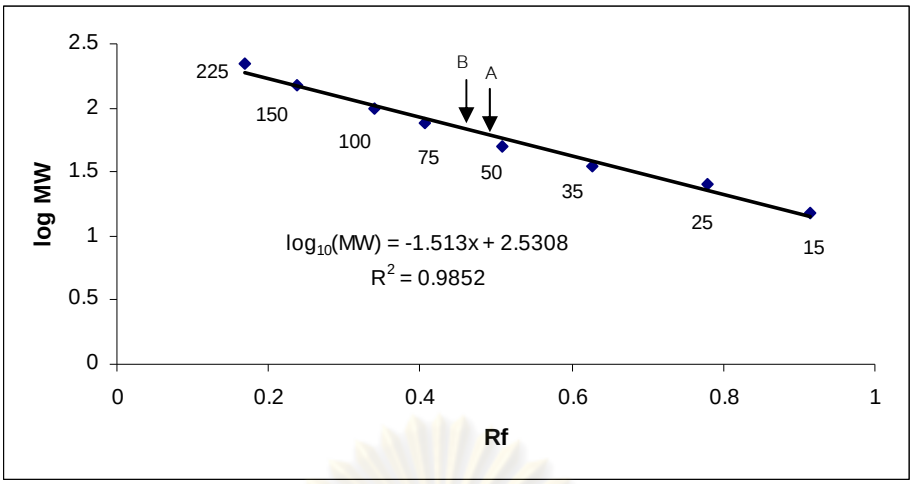


Figure 3.21. The relationship between R_f value and log MW of broad range protein marker. MW of bands A and B from fraction# 6 after Superdex 200 column (from Figs. 3.12A and 3.12B) was estimated.

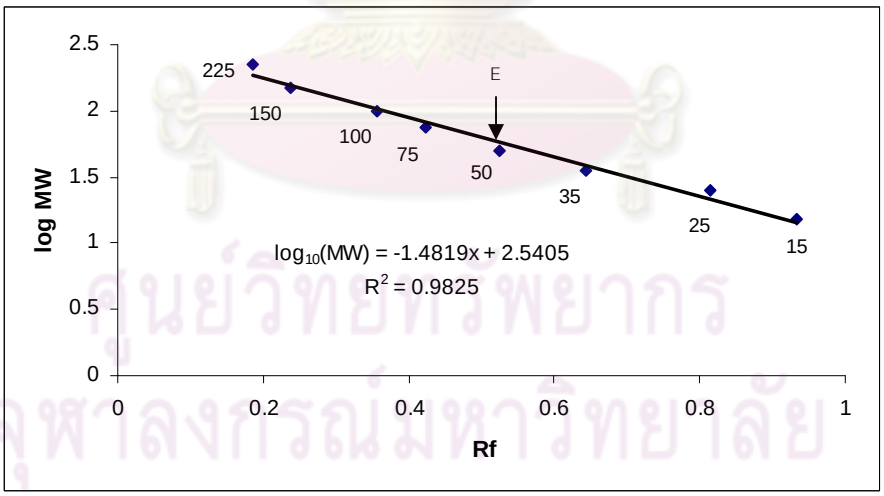


Figure 3.22. The relationship between R_f value and log MW of broad range protein marker. MW of Ag from Fig. 3.12B was estimated. MW of band E from fraction# 10 after Superdex 200 column (from Figs. 3.12A and 3.12B) was estimated.

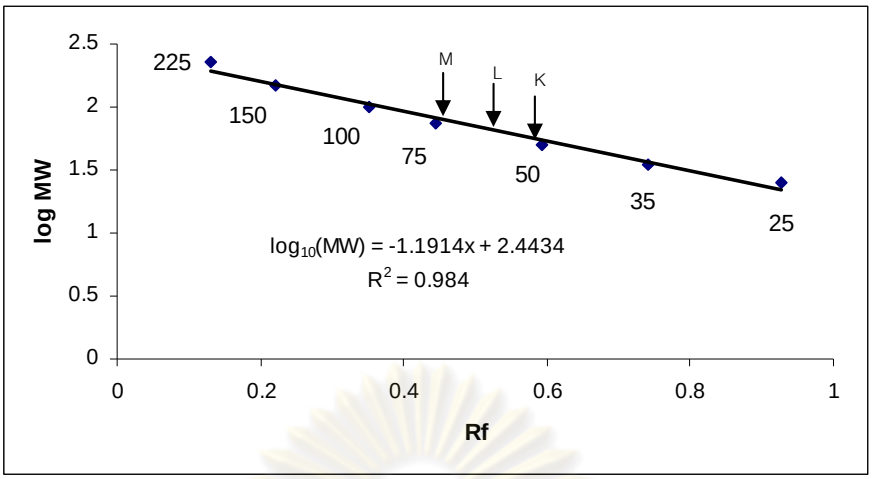


Figure 3.23. The relationship between R_f value and log MW of broad range protein marker. MW of bands K, L, and M from fraction#12 after Superdex 75 column (from Fig. 3.17) was calculated.

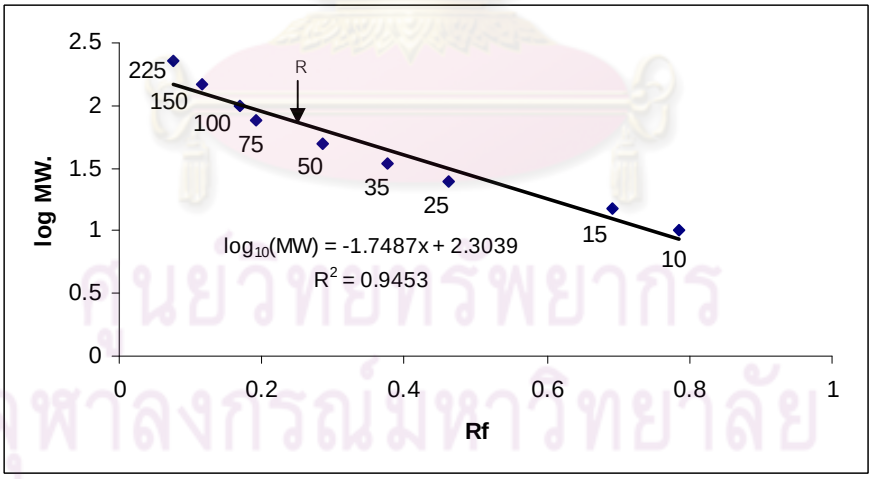


Figure 3.24. The relationship between R_f value and log MW of broad range protein marker. MW of band R from fraction# 13 after Superdex 75 column (from Fig. 3.19B) was estimated.

3.5 Searching for peptide mass mapping by MALDI TOF MS or LC/MS

After AG was purified by two chromatographies (DEAE-cellulose and Superdex 200 or 75 columns), protein was separated by SDS PAGE and 2DE. The expected location of target protein was excised in order to determine peptide mass by Matrix Assisted Laser Desorption Ionization/Time of Flight Mass Spectrometry (MALDI TOF MS) and Liquid Chromatography Mass Spectrometry (LC/MS). Then, the obtained fragment peptide mass was searched against NCBI database via MASCOT program (www.matrixscience.com). However, no peptide mass spectrum of sample was matched to that of AG, there were some peptide mass spectrums which were mostly matched to a novel protein similar to vertebrate dynein, cytoplasmic, light intermediate polypeptide 2 (DNCLI2) in *Danio rerio* (zebrafish) (gi|94733315) . The score was 60 (not greater than 75) which was not acceptable to be significantly similar ($p \leq 0.05$). In Figure 3.25, it showed that the peptide was matched with 25% coverage to DNCLI2.

1 TTGRNTLLSV STNVNNSTSE SQNPEEEDGQ NLWSSILSEV STRSRSKLPS

51 GKNVVVMGEV GSGKTTLVAK LQGVEEYMKG R**GLEYLYFNV HDDDIDDQSR**

101 CNAWVLDGDL YHKGLQKFAI SLENLEDSLI LFVVDLSRPW LALDSLQKWG

151 SVVRDFVDKL R**VPPETMREL EHR**LTKQFQE YVEPGSDLDA VPQRRNPESD

201 EESVLLPLGE NTLTHNLGLP IVVVCTKCDA ISTLEKEHDY KDEHLDFIQS

251 HIRRFCLQYG AALLYTSMKE NKNLDLLYKY LVHRLYGFPF NSPAQVVEKD

301 SVFIPSGWDN EK**KIAILHEN FQMVKAEDSF EDVIVKPPVR** K**FVHEKEVQA**

351 **EDDQVFLVKL QSLLSKQPPV TAGRPVDPTN RAPTGSPR**TT NRSAAANVAN

401 VMPMQSGGQT SEGVLANFFN SLLTKKAGSP GPGGQPAGGG SNTPGTVRKS

451 GSKLGLTDVQ AELDRISNK**S DLDSSAPNAT TPPAENDK**S

Figure 3.25. Matching of determined peptide masses to an amino acid sequence of DNCLI2. Matched peptides were shown in bold letters.

3.6 Optimal conditions of AG activity

The most positive fraction after Superdex 75 column was used to determine the optimal conditions for AG activity. Four parameters were measured for this purpose. They consisted of pH, temperature, concentration of maltose (substrate), and incubation time. Each parameter was performed in three replications. Then, the obtained data was plotted in a graph. The optimal pH was 6.0 (Fig. 3.26). The optimal temperature was 35ºC (Fig. 3.27). The optimal concentration of substrate was 50 mM maltose (Fig. 3.28) and the optimal incubation time was 30 min (Fig. 3.29).

3.6.1 Optimal pH

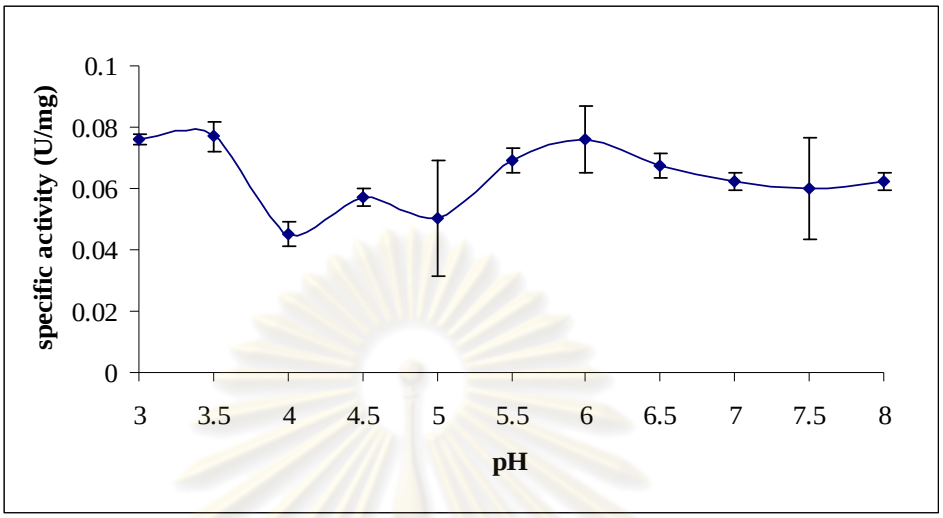


Figure 3.26. The optimal pH of AG in *A. dorsata* by using Briton-Robinson buffer at various pH ranged between 3.0-8.0.

3.6.2 Optimal temperature

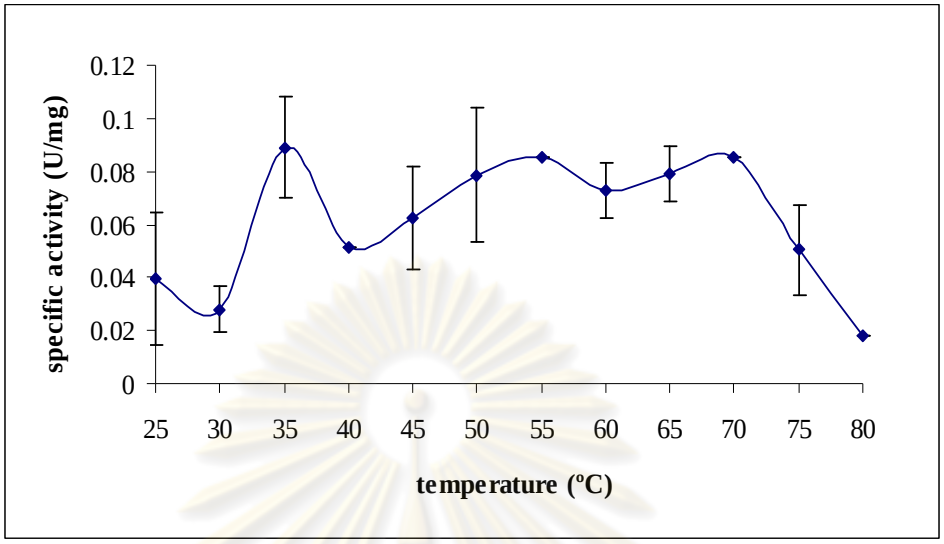


Figure 3.27. The optimal temperature of AG in *A. dorsata*. A reaction mixture (sodium acetate buffer containing 0.05% Triton X-100, 0.5% maltose, and AG) was incubated at different temperatures from 25-80°C with the interval of 5°C for 10 min.

3.6.3 Optimal concentration of substrate

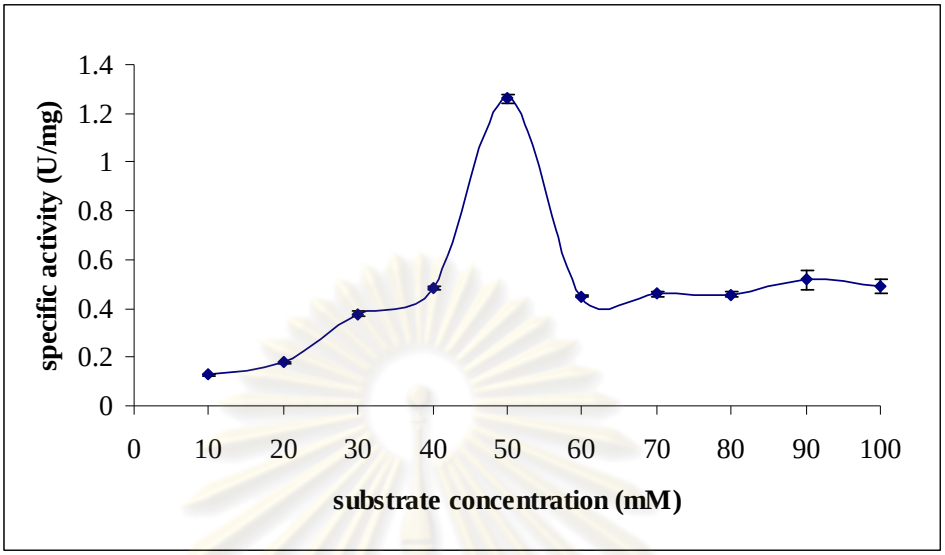


Figure 3.28. The optimal maltose concentration of AG in *A. dorsata*. A reaction mixture contained maltose at different concentrations varying from 10-100 mM with the interval of 10.

3.6.4 Optimal incubation time

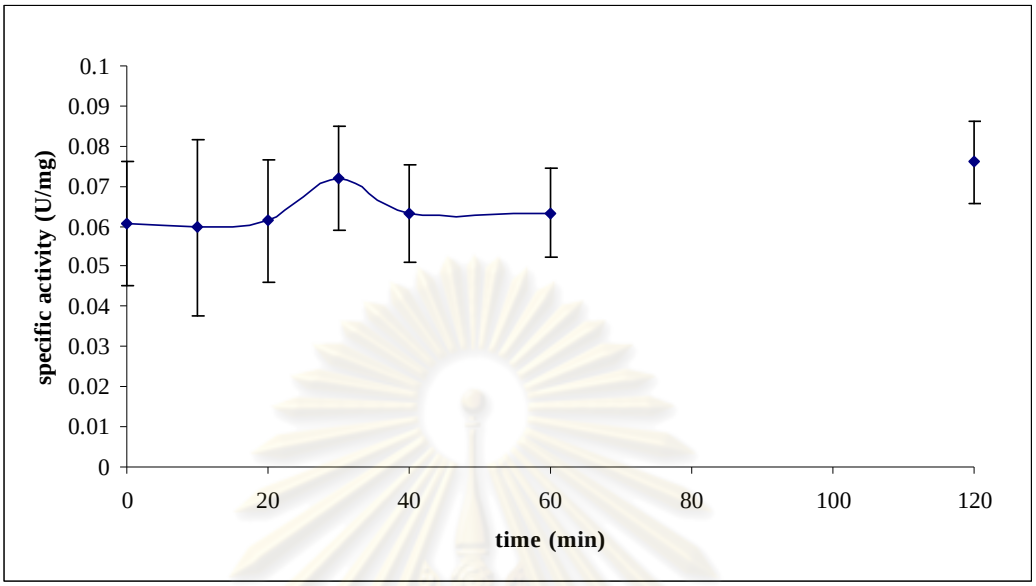


Figure 3.29. The optimal incubation time of AG in *A. dorsata*. A reaction mixture was incubated at 37ºC for 0, 10, 20, 30, 40, 60, and 120 min.

CHAPTER IV

DISCUSSIONS

The α-glucosidase (AG) is the key enzyme in providing monosaccharides such as glucose, fructose which is useful in food industry. Main sources of AG came from microorganisms (Kang *et al.*, 2009), plants (Takahashi *et al*., 1969), fungi (Da Silva *et al*., 2009), and other insects (Fonseca *et al*., 2010). In addition, industrial AG was popularly come from recombinant technology (Chi *et al*., 2008). Since *Apis* spp. which is a eukaryote involves directly in the synthesis of honey which main components are monosaccharides, they are interesting to be new sources of AG. A lot of researches on AG in *A. mellifera*, a western species, were reported (Ohashi *et al*., 1996) but not on native *Apis* spp. in Thailand. In our previous study, we reported AG in *A. cerana* and *A. florea* (Chanchao *et al*., 2007; 2008) so in this research we attempted to report AG in *A. dorsata* due to the most popularly consumed honey.

In order to determine the expression pattern of *AG* in developmental stages of *A. dorsata*, total RNA was extracted from eggs, larvae, pupae, and foragers. The purity of isolated RNA was checked by the ratio of the absorbance at 260 nm and 280 nm. The good ratio should be in the range of 1.8-2.0. The obtained ratio of RNA in this research indicated the good quality of RNA (data not shown). Then, the quality of total RNA was further examined by 1.2% (w/v) agarose and formaldehyde gel electrophoresis. After ethidium bromide staining, the two bands of 28S and 18S rRNA should be observed (Figure 3.1A) or at least one band of 18S rRNA could be noticed (Figure 3.1B). Since the amount of 18S rRNA was much more than the other, the

intensity of 18S rRNA was about twice to the other (Ausubel *et al*., 1997). If either 1 band or 2 bands of rRNA was visible, it implied that the extracted was acceptable in quality. The quality of RNA was lastly checked by RT-PCR technique in control experiments. The control experiments were performed by using primers designed from *28S rRNA* and *EF-1α* genes those were housekeeping genes. They normally express all the time and in all tissue types. Since the expected RT-PCR products of *28S rRNA* (358 bp) and *EF-1α* (198 bp) could be obtained from all samples (Figures 3.3 A and B), it indicated the good RNA preparation and no RNA degradation.

After the success in honeybee (*A. mellifera*) genome, it was very useful for us to design RT-PCR primers (The honeybee genome sequencing consortium, 2006). As expected, by RT-PCR, the highest expression level of *AG III* in *A. dorsata* was in foragers (Figure 3.2 and Table 3.1). The obtained result was the same as in other *Apis* spp. Alternatively, it could be implied that the mode of expression of the honeybee *AG III* was quite unique in that it was activated specifically in the foragers. This presented the relationship between the function of this enzyme and the task of foraging. Younger bees do not synthesize this enzyme but they synthesize royal jelly protein instead. That leads the younger bees stay in the hive and perform the food feeding behavior (Kubo *et al*., 1996). The result coincided to Ohashi *et al*. in 1996 which reported that the gene was expressed specifically in the hypopharyngeal glands of foragers in *A. mellifera* based on RT - PCR. Interestingly, if a bee switches its duties which obviously depend on the hive command, it may be possible that the regulation of *AG III* expression depend on the duty change. In this study, we tried to avoid the conflict of flexible duty which is dependent on the hive demand, not age only (Kubo *et al*., 1996). That is why four stages of eggs (oval shape), larvae (worm – like form), pupae (immature bees living in sealed cells), and foragers were selected

due to the great difference in morphology. Especially in forager stage, bees were collected while they foraged for nectars on flowers, not caught directly from the hive. The rest of samples (eggs, larvae, and pupae) were directly picked up from the honeycomb.

Considering the obtained expression pattern of *AG* in detail (Figure 3.2 and Table 3.1), it showed that the *AG* level was the highest in foragers (22.8 ng/µl), high in pupae (21.4 ng/µl), low in eggs (14.3 ng/µl), and the lowest in larvae (9.5 ng/µl), respectively. Although the tendency of expression gradually increased from eggs to foragers (Chanchao *et al*., 2006; 2007), the overall *AG* expression pattern in *A. dorsata* was different. The lowest expression to the highest expression was found from larvae, eggs, pupae, and foragers. Since the high expression of this gene in eggs was noticed, this might imply high synthesis of food provisions as yolk. Then, it can supply the development of honeybee embryo which needs to highly metabolize carbohydrate substances for uptaking (Borror *et al*., 1954; Müller, 1997). This may explain why *A. dorsata* body size is the largest among another species. It is like endosperm in a plant seed that is used for food supply in germination (Rost *et al*., 2006). Therefore, AG which is a hydrolytic enzyme is contained in a plant seed in order to degrade the storage starch like AG I and AG II in sugar – beet seed (Yamasaki and Suzuki, 1980), AG II in millet seed and germinating millet seed (Yamasaki *et al*., 1996; 2005), and AG II in rice seed (Nakai *et al*., 2007). In addition, food collecting stem or root such as potato tuber, banana rhizome, taro corm, etc. has AG activity in order to hydrolyse the storage starch for food uptaking for plant growth (Taylor *et al*., 1998). These may explain the high expression of *AG* in eggs of *A. dorsata*. The lowest expression in larvae may be because they are fed bee milk by nurse bees. They need not to breakdown any collected food. Considering the

development process in this stage, not much differentiation occurs, except size increase, so little energy is required. When a bee embryo reaches a pupa stage or transformation stage, the *AG* expression level became high but noticeable less than that in forager. That may because of great differentiation and growth. Organogenesis and anatomy are obviously and rapidly changed. Thus, high metabolism rate of food should occur. This should lead to the high activity of AG.

According to the obtained expression profile of *AG* in *A. dorsata* (Figure 3.2) and the amount of amplified *AG* product shown in table 3.1, we collected bees at this stage for purification source and RNA source in order to obtain the full length of cDNA. As mentioned in Table 2.1, for RT-PCR, various primers were designed, including F1 and R1 primers which were designed to encompass the start and stop codons, respectively. At last, the full length ORF of cDNA at 1,704 bp was obtained, was recorded in GenBank as an accession number of GU224269, and was deduced to be an amino acid sequence (Figure 3.6). Considering the cDNA length of *AG III* among *Apis* spp., it is likely that the length is about 1.7 kb. Its homology to the same gene in *A. mellifera* (NM_001011608) was at 96 % while its homology to the same gene in *A. florea* (accession # EF586680) was at 94 %. Furthermore, its homology to the same gene in *A. cerana indica* (accession # EF441271) was at 95 % and in *A. cerana japonica* (accession # FJ889442) was at 94 %. The high percentage of similarity in sequence indicated that we really obtained *AG III* homologue from *A. dorsata.*

Considering phylogenetic trees constructed by UPGMA and NJ, the relationship of *AG* among *Apis* spp. was presented (Figures 3.7 and 3.8). The trees showed that the *AG* sequence in *A. c. indica* was mostly close to that in *A. c. japonica*. The branches of *A. mellifera, A. dorsata,* and *A. florea* sequences were separately

diversed. It indicated that the latter had its own evolutionary lineage. Especially in *A. florea*, the branch was obviously separated from the rest. At present, a phylogenetic relationship of *Apis* spp. has been widely observed. For example, *A. florea* and *A. andreniformis* were on the farthest basal clades distinguishing from other *Apis* spp. The branches of two giant species, *A. dorsata* and *A. laboriosa,* were on the basal clades diversed from the rest, too. Interestingly, the obtained phylogenetic relationship coincided to the habitat. *A. dorsata* and *A. laboriosa* build open-hives while *A. cerana*, *A. nigrocincta,* and *A. nuluensis* build cavity- or close-hives. In addition, a phylogeny supports the idea that open-hive building honeybee is an ancestor of *Apis* spp. (Oldroyd and Wongsiri, 2006).

Following the purification processes of AG in *A. c. indica*, DEAE – cellulose and Sephadex 200 columns were used but, unfortunately, partial purified AG could be obtained. The obtained result was similar to that in *A. florea* (Chanchao *et al*., 2007). Even though the partial purified AG was obtained, the highest specific activity (3.95 U/mg; table 3.4) could be reached. In order to achieve the purified AG, more types of columns were required. Based on the successful purification in AG III in *A. mellifera*, salting – out chromatography, DEAE – cellulose, DEAE – Sepharose CL – 6B, Bio – Gel P – 150, and CM – Toyopearl 650M were used, respectively (Nishimoto *et al*., 2001). In addition, in *A. mellifera*, not only native AG III required more columns, but recombinant AG III also required more columns which were salting – out chromatography, CM – Sepharose CL – 6B, Bio – Gel P – 100, DEAE – Sepharose CL – 6B, and Butyl – Toyopearl 650M, respectively (Nishimoto *et al*., 2007).

Due to figure 3.19B, roughly, the MW of AG is about 50 kDa. Considering AG III, in this research, the predicted MW of *A. dorsata* AG III was 62 kDa while 68 kDa was reported in *A. mellifera* and *A. c. indica* (Nishimoto *et al*., 2001; Chanchao *et*

al., 2008). However, 73 kDa was reported in *A. florea* (Chanchao *et al*., 2007). In addition, this was found in AG II MW as well. The 60 kDa was reported in sugar – beet seeds (Yamasaki and Suzuki, 1980). The 61 kDa was found in cicada (*Quesada gigas*) which was a pest for coffee plantation (Fonseca *et al*., 2010) and the 66 kDa was found in snail (*Archachatina ventricosa*) (Soro *et al*., 2007).

After purifying by 2 columns, an amino acid sequence of AG had been tried by 3 methods, MALDI – TOF MS, LC/MS, and 2DE. Unfortunately, we could not obtain the amino acid sequence. This might be explained that our target protein was rapidly degraded. High yield loss could be considerable. This problem must cause from lacking of a protease inhibitor. Furthermore, the preparation of starting sample should be improved. Only hypopharyngeal glands or at least just heads, should be used instead in order to reduce the proportion of unwanted proteins in the starting homogenate, including other AG.

Although the amino acid sequence could not be reached, the activity of AG could be detected (Table 3.4). It could be determined that the optimal pH of AG III was in the range of 5.0 - 6.5. The optimal pH was 6.0 in *A. dorsata* (for AG III), 5.0 in *A. c. indica* (AG III) (Chanchao *et al*., 2008), 5.5 in *A. mellifera* (AG III) (Nishimoto *et al*., 2001), and 5.0 in *A. c. japonica* (AG I) (Wongchawalit *et al*., 2006). Like recombinant AG, the optimal pH was 5.3 in *A. mellifera* (AG II) (Nishimoto *et al*., 2007) and 5.7 in *A. mellifera* (AG III) (Nishimoto *et al*., 2007). Also, the optimal pH of AG in other organisms was in this range. For example, the optimal pH was 5.5 in snail (*Archachatina ventricosa*) (AG II) (Soro *et al*., 2007), 6.0 in cicada (*Quesada gigas*) (AG II) (Fonseca *et al*., 2010), 6.2 in bacteria (*Thermus thermophilus*) (Zdziebło and Synowiecki, 2002).

The optimal temperature of AG in *A. dordata* was at 35ºC which was close to AG in yeast (*Candida albicans*) (37ºC) (Torre – Bouscoulet *et al*., 2004) and in snail (*Archachatina ventricosa*) (45ºC) (Soro *et al*., 2007).

From all mentioned above, the outcome from this research was that we could obtain the full length of ORF of *AG III* in *A. dorsata*. This will lead to the research on recombinant technology. Also, in the future, we plan to obtain the 5' and 3' untranslated regions of the gene. More parameters of kinetics may be reported later.

CHAPTER V

CONCLUSIONS

1. The expression of α-glucosidase III (*AG III*) gene in *A. dorsata* is the highest in foragers (114.24 ng), high in pupae (107.1 ng), low in eggs (71.4 ng), and the lowest in larvae (47.6 ng), respectively.

2. The full length of ORF of cDNA at 1,704 bp was obtained from various pairs of primers and was deduced to be 567 amino acids.

3. The cDNA and amino acid sequences of the gene in *A. dorsata* were mostly similar to that in *A. mellifera* (96% similarity).

4. By using amino acid sequences, phylogenetic trees constructed by UPGMA and NJ confirmed the close relationship between *A. dorsata* and *A. mellifera*.

5. The precipitation by 95% saturation of ammonium sulfate (AS) was found to be the optimum.

6. After AS precipitation, supernatant purified by DEAE-cellulose and Superdex 200 columns (3.95 and 0.26 U/mg) provided higher specific activity than pellet (0.004 and 0.09 U/mg).

7. The optimal conditions of partially purified AG were 6.0 for pH, 35ºC for temperature, 50 mM maltose for substrate concentration, and 30 min for incubation time.

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APPENDICES

APPENDIX A

Preparation for electrophoresis

1. Formaldehyde/agarose gel electrophoresis

1) 10x MOPS buffer

400 mM MOPS

100 mM Sodium acetate

10 mM EDTA

Adjust pH to be 7 and volume to be 1,000 ml by dd-H_2O, store at 4^oC in the dark, without autoclavation

2) 1.2% (w/v) Formaldehyde agarose gel

Agarose

1x MOPS buffer

12.3 M Formaldehyde(37%)

Heat to melt agarose. After being warm, add formaldehyde, and swhirl to mix. Cover a solidify gel with 1x MOPS buffer.

3) 5x RNA loading buffer

0.16% (v/v) saturated aqueous bromophenol blue solution†

4 mM EDTA, pH 8.0

12.3 M 37% formaldehyde

20% (v/v) glycerol

30.84% (v/v) formamide

4x MOPS buffer

Adjust volume to be 1 ml by RNase-free H_2O. It could be stable up to approximately 3 months at 4°C.

2. Agarose gel electrophoresis

1) 10x TBE buffer

0.89 M Tris base

0.89 M Boric acid

0.5 M EDTA (pH 8)

Adjust volume to be 1,000 ml by dd-H_2O, autoclave for 20 min, and diluted to 1x TBE for using.

2) 0.8% (w/v) agarose gel

Agarose	0.4 g
1x TBE	50 ml

Heat to melt agarose, transfer to a tray, let it solidify, and cover a gel with 1x TBE buffer.

3) 6x DNA loading dye

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol FF

30% (v/v) Glycerol

3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

1) 8% (w/v) Separating gel

8% (v/v) Acrylamide solution

0.375 M Tris–HCl (pH 8.8)

0.1% (w/v) SDS

0.05% (w/v) $(NH_4)_2S_2O_8$

0.05% (v/v) TEMED

2) 4% (w/v) Stacking gel

4% (w/v) Acrylamide solution

0.125 M Tris–HCl (pH 6.8)

0.1% (w/v) SDS

0.05% (w/v) $(NH_4)_2S_2O_8$

0.05% (v/v) TEMED

3. Electrophoresis buffer

25 mM Tris

192 mM Glycine

Adjust pH to be 8.3 and volume to be 1,000 ml by dd – H_2O

4) 6x Blue/orange loading dye

0.03% (w/v) Bromophenol blue

0.03% (w/v) Xylene cyanol FF

0.4% (w/v) Orange G

15% (w/v) Ficoll® 400

10 mM Tris – HCl (pH 7.5)

50 mM EDTA (pH 8)

5) Staining solution

30% (v/v) Methanol

10% (v/v) Acetic acid

0.1 % (w/v) Coomassie Brilliant Blue G – 250

Adjust volume to be 500 ml by dd – H_2O, stored in the dark at RT.

6) Destaining solution

30% (v/v) Methanol

10% (v/v) Acetic acid

Adjust volume to be 1,000 ml by dd – H_2O

Preparation for protein purification

1. Sodium phosphate buffer (pH 6.3)

1) Stock solution A

2 M monobasic sodium phosphate, monohydrate

($NaH_2PO_4.H_2O$, M.W. = 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, M.W. = 141.96)

Na_2HPO_4	284 g
dd-H_2O	1 L

Adjust the an approximate volume (ml) of stock A and B to make pH 6.3

Stock A	Stock B	pH
77.5	22.5	6.3

2. Bradford assay

1) Bradford stock solution

95% Ethanol	100 ml
85% Phosphoric acid	200 ml
Serva Blue G	350 mg

2) Bradford working buffer

95% Ethanol	15 ml
85% Phosphoric acid	30 ml
Bradford stock solution	30 ml

Adjust volume to be 500 ml by dd-H_2O, filter before using, and store at RT in light protected glass bottle.

Preparation for tryptic digestion in gel

1. Stock reagents

Acetonitrile (ACN)	10 ml
0.2 M NH_4HCO_3	10 ml
10 mM EDTA	10 ml
0.1 M Tris-HCl (pH 8.5)	10 ml
2% (v/v) Trifluoroacetic acid (TFA)	10 ml
10 mM $CaCl_2$	10 ml
10% (v/v) Formic acid	10 ml
10% (v/v) Acetic acid	10 ml
100 mM Dithiothreitol (DTT)(fresh preparation)	100 µl
200 mM Iodoacetoamide (IAA) (fresh preparation)	500 µl

2. Working solution

1) 50% (v/v) Acetonitrile (ACN)/0.1 M NH_4HCO_3

100% (v/v) Acetonitrile (ACN)	200 µl
0.2 M NH_4HCO_3	200 µl

2) 0.1 M NH_4HCO_3/10 mM Dithiothreitol (DTT)/1 mM EDTA

0.2 M NH_4HCO_3	200 µl
100 mM Dithiothreitol (DTT)(fresh preparation)	40 µl
10 mM EDTA	40 µl
dd–H_2O	120 µl

3) 100 mM Iodoacetoamide (IAA)/0.1 M NH_4HCO_3

200 mM Iodoacetoamide (IAA) (fresh preparation)	200 µl
0.2 M NH_4HCO_3	200 µl

4) 50% (v/v) ACN/0.05 M Tris-HCl (pH 8.5)

100% (v/v) Acetonitrile (ACN)	200 µl
0.1 M Tris-HCl (pH 8.5)	200 µl

5) 0.05 M Tris-HCl (pH 8.5)/1 mM $CaCl_2$

0.1 M Tris-HCl (pH 8.5)	150 µl
10 mM $CaCl_2$	30 µl
dd–H_2O	120 µl

6) 5% (v/v) Formic acid/ACN

10% (v/v) Formic acid	150 µl
Acetonitrile (ACN)	150 µl

3. Digestion buffer (trypsin buffer and trypsin solution at the ratio of 9: 1)

1) Trypsin buffer (270 µl)

0.1 M Tris-HCl (pH 8.5)	150 µl
100% (v/v) ACN	30 µl
10 mM $CaCl_2$	30 µl
dd-H_2O	60 µl

2) Trypsin solution (30 µl)

0.2 µg/µl Trypsin	15 µl
10% (v/v) Acetic acid	3 µl
dd-H_2O	12 µl

Mix trypsin buffer (270 µl) to trypsin solution (30 µl).

Preparation for optimal pH

Briton – Robinson buffer

1 M Acetic acid (10 mM)	0.2 ml
1 M Phosphoric acid (10 mM)	0.2 ml
0.1 M Boric acid (10 mM)	2 ml

Adjust pH to 3 – 7.5 by 0.2 M NaOH and adjust volume to be 20 ml by dd–H_2O.

BIOGRAPHY

Miss Manlika Kilaso was born on September 25th, 1984 in Chainat province. She obtained a B.S. degree in Biology from Department of Biology, Faculty of Science, Chulalongkorn University in 2004. Then, she has been an M.S. student in Biotechnology Program, Faculty of Science, Chulalongkorn University since 2007.

Publications:

Kilaso, M., Chanchao, C., and Sangvanich, P. (2009) Purification of alpha-glucosidase in *Apis dorsata*. The 14th National Graduate Research Conference, King Mongkut's University of Technology North Bangkok, Thailand. Number B018, p. 1-6.

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