CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Automatic micropipette P10, P20, P100, P200, and P1,000, Gilson, France
- Centrifugal filter devices, Amicon® ultra – 4, Millipore, France
- 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. Kg., Germany
- Column, model: XK 16/ 20, Amersham Bioscience, USA
- Column, model: XK 16/ 70, Amersham Bioscience, USA
- Cooler, model: F33, Jolabo, Germany
- Cuvette, model: 0.7 – 0.8 ml, Starna, England
- Cuvette, model: 1.5 ml Semi – micro cuvette, Brand, Germany
- Dialyse, pleated dialysis tubing, SnakeSkin, Pierce, 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
- Freeze dry system, Frezone® 6, Labconco, USA
- Hot plate stirrer, Schott, Germany
- Immobiline drystrip reswelling tray, Amersham Bioscience, USA
- Incubator, Germany
- IPG strip pH 3 – 10, length 7 cm, Amersham Bioscience, USA
- Maxima ultra pure water, model: Maxima UF, Elga, England
- Microincubator, model: M – 36, Taitec, Japan
- Microplate reader, model: Sunrise remote/ touch screen, Tecan, Austria

- Microwave, model: Sharp carousel R7456, Sharp, Thailand
- Multiphor II electrophoresis unit with immobiline drystrip kit
- 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
- Safety cabinet, Augusta
- Shaker 35, Labnet, USA
- Shaker Ika® KS 130 basic, GmbH & Co. Kg, Germany
- Sonicator, Branson, France
- Spectrophotometer, model: Ultraspec II, LKB biochrom, England
- Vacuum centrifuge, Heto, Maxi dry plus
- Vortex, Scientific industries, Inc., USA
- Vortex mixer: KMC – 1300V: Vision scientific Co. Ltd., Korea
- Waterbath: Memmert schwabach, Germany

3.2 Chemicals

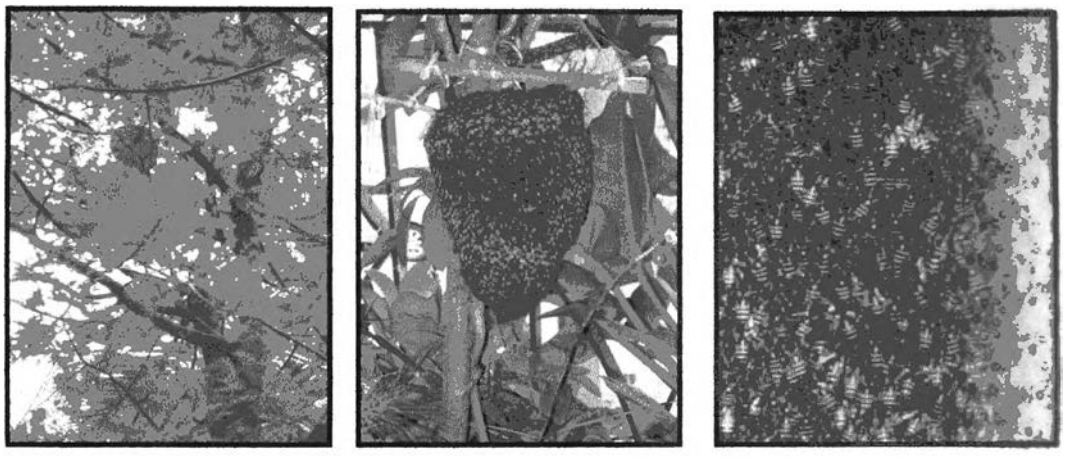
- 3, 6 - Dinitrophthalic acid, $C_8H_4N_2O_8.C_5H_5N$, F.W. = 335.2, Sigma, Germany
- 2 - Mercaptoethanol, BDH laboratory supplies, England
- 2, 3, 5 - Triphenyltetrazolium Chloride, $C_{19}H_{15}ClN_4$, Mr = 334.81, Fluka biochemical, Switzerland
- Access RT – PCR system (catalog# A1250), Promega, USA
- 100% Acetic acid, CH_3COOH, M.W. = 60.05, Merck, Germany
- Acrylamide, M.W. = 71.08, Promega, USA

- Ammonium peroxydisulfate (APS), $(NH_4)_2S_2O_8$, M.W. = 249, BDH laboratory supplies, England
- Barium chloride, $BaCl_2.2H_2O$, M.W. = 244.27, Ajex finechem, Australia
- Bovine serum albumin (BSA), fraction V, pH 7.0, Serva feinbiochemica GmbH & Co., USA
- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M.W. = 670, BDH laboratory supplies, England
- Calcium chloride dehydrate, $CaCl_2.2H_2O$, M.W. = 147, Merck, Germany
- Citric acid, F.W. = 210.14, Carlo erba reagenti, Italy
- CM – cellulose, Sigma, UK
- Coomassie brilliant blue G - 250, $C_{47}H_{48}N_3O_7S_2Na$, M.W. = 854, BDH laboratory supplies, England
- Coomassie brilliant blue R - 250, $C_{45}H_{44}N_3O_7S_2Na$, M.W. = 826, Serva feinbiochemica GmbH & Co., USA
- DEAE – cellulose, Sigma, UK
- Drystip cover fluid, Amersham Bioscience, USA
- Ethylene diamine tetra - acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M.W. = 292.2, Serva feinbiochemica GmbH & Co., USA
- 95% (v/v) Ethanol, CH_3CH_2OH, M.W. = 46, Thailand
- 37% (v/v) Formaldehyde, CH_2O, M.W. = 30, Thailand
- 99.5% (v/v) Glycerol, $C_3H_8O_3$, M.W. = 92.10, BDH laboratory supplies, England
- Glycine, NH_2CH_2COOH, M.W. = 75.07, BDH laboratory supplies, England
- Hydrochloric acid fuming 37% (v/v), HCl, Merck, Germany
- Leupeptin, $C_{20}H_{38}N_6O_4.HCl$, F.W. = 463.0, Sigma, Germany
- Methanol, CH_3OH, M.W. = 32.04, Merck, Germany
- N, N' – methylene – bis – Acrylamide (Acrylamide/ bis), Sigma, USA
- Octylphenol - polyethyleneglycol ether (Triton X – 100), Serva feinbiochemica GmbH & Co., USA
- PCR purification kit (catalog# 28104), Qiagen, Germany
- Pepstatin A, $C_{34}H_{63}N_5O_9$, F.W. = 685.9, Sigma, Germany
- Phenylmethylsulfonylfluoride (PMSF), $C_7H_7FO_2S$, F.W. = 174.2, Sigma, Germany

- 85% (v/v) Phosphoric acid, H_3PO_4, M.W. = 98, Mallinckrodt, USA
- Potassium carbonate, K_2CO_3, F.W. = 138.2, Sigma, Germany
- Sephadex® G – 150, Pharmacia, Sweden
- Sodium acetate, $CH_3COONa.3H_2O$, M.W. = 136.09, M & B Ltd., England
- Sodium chloride, NaCl, M.W. = 58.4, Merck, Germany
- Sodium dihydrogen orthophosphate 1 – hydrate, $NaH_2PO_4.H_2O$, M.W. = 137.99, England
- Sodium dodecyl sulfate, BDH laboratory supplies, England
- Sodium hydrogen orthophosphate, Na_2HPO_4, M.W. = 141.96, England
- Sodium hydroxide, NaOH, M.W. = 40, Merck, Germany
- Sodium thiosulfate, $Na_2S_2O_3.5H_2O$, F.W. = 248.2, Sigma, Germany
- Superdex 200 prep grade, Amersham Bioscience, USA
- SV Total RNA isolation system (catalog# Z3100), Promega, USA
- TEMED, Promega, USA
- Tris - (hydroxymethyl) – aminomethane, $NH_2C(CH_2OH)_3$, M.W. = 121.4, Pharmacia biotech, USA

3.3 Beekeeping

Apis florea colonies were purchased from Mae Klong, Samut Songkram province and maintained at Department of Biology, Faculty of Science, Chulalongkorn University for sampling. Eggs were picked up from cells. Nurse bees were collected when they fed a larva while forager bees were collected when they foraged the nectar of a flower. Crude extract for chromatography, honeybees were directly collected from a beehive. Honeybees were anesthesized on ice before use.



(A) (B) (C)

Figure 3.1 *A. florea* colony; (A) a colony on a tamarind tree (*Tamarindus indica* L.); (B) a colony for sampling; and (C) honeybee in a colony.

3.4 Isolation of total RNA

Total RNA was isolated from eggs, heads of nurse bees, and heads of forager bees by SV Total RNA isolation system (catalog# Z3100, Promega). The sample was homogenized in liquid N_2 and mixed by 175 µl of RNA lysis buffer. Then, it was diluted by 350 µl of RNA dilution buffer. The lysate was mixed by inverting 3 – 4x and incubated at 70°C for 3 min. Then, the mixture was centrifuged at 12,000 – 14,000x g for 10 min. The cleared lysate was mixed with 200 µl of 95% ethanol. After that, the mixture was transferred to a spin column assembly. It was centrifuged at 12,000 – 14,000x g for 1 min. Flow – through (FT) was discarded. Six hundred µl of SV RNA wash solution was added to the spin column. It was centrifuged at 12,000 – 14,000x g for 1 min. DNase incubation mix (40 µl of yellow core buffer, 5 µl of 0.09 M $MnCl_2$, and 0.5 u of DNase I) was directly dropped onto the membrane. It was incubated at RT for 15 min. After incubation, 200 µl of DNase stop solution was added. Then, it was centrifuged at 12,000 – 14,000x g for 1 min. Six hundred µl of RNA wash solution were added on the membrane. It was centrifuged at 12,000 – 14,000x g for 1 min. It was washed again by 250 µl of RNA wash solution and was centrifuged at maximum speed for 2 min. The spin basket from the collection tube was transferred to an elution tube. Thirty µl of nuclease – free H_2O was added to the membrane. It was centrifuged at 12,000 – 14,000x g for 1 min. The elution containing purified RNA was stored at – 20 °C.

3.5 Agarose gel electrophoresis

3.5.1 Native agarose gel

Agarose gel (1.2%) was prepared in 1x TBE buffer (Appendix A). Total RNA was mixed by 1x loading dye [5x DNA BlueRunTM contains 150 mM EDTA, 25 mM Tris – HCl (pH 7), 25% (v/v) glycerol, and 0.05 (w/v) bromophenol blue]. Electrophoresis was performed by 1x TBE as electrode buffer at 100 V for 50 min. After electrophoresis, the agarose was stained by ethidium bromide (EtBr) and destained with dd – H_2O. RNA was detected under UV light.

3.5.2 Formaldehyde gel

For gel preparation, agarose (1%) was melted in 1x MOPS (Appendix A). After being cool, it was mixed by 1.2% formaldehyde. Total RNA was mixed by 1x formaldehyde loading dye. The mixture was heated at 65°C for 10 min and chilled on ice for 3 min. Then, the electrophoresis by 1x MOPS as a running buffer was performed at 50 V for 50 min. After that, the gel was stained in EtBr for 1 h and destained with dd – H_2O. RNA was detected under UV light.

3.6 Primer design

Primers for RT – PCR of *AG* in *A. florea* were designed from a cDNA sequence of *AG* in *A. mellifera* (Ohashi *et al.*, 1996). Primers were synthesized by Bioservice unit, Thailand. Due to Figure 3.2, forward primers were FW1: 5' - TTCGA CTTCT AGTTG GTAGC ATGAA GG - 3'; FW2: 5'- GCTTA TCGAG GCATA CACGA - 3'; and FW3: 5' - ACGAG GAACA AATCG TGGAC - 3'. Reverse primers were R1: 5' - CCTTT CTCAT GTGCA GCACT GACTA G - 3'; R2: 5' - CACTT GGTGG CATGT ACGTC - 3'; and R3: 5' - CGCCG CTTCA AAGAA TAGAC - 3'. In addition, control primers were designed from *28S RNA* in *A. mellifera* and Elongation factor gene (*EF*) in *A. cerana*. The *28S RNA* primers were FW: 5' - AAAGA TCGAA TGGGG ATATT C - 3' and R: 5' - CACCG GGTCC GTACC TCC - 3'. The *EF* primers were FW: 5' - TCGCT TTTAC TCTTG GTGTG A - 3' and R: 5' - AAACT CCCAA CATAT TATCT CCA - 3'.

FW1 primer

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   1 tgatattaac gtactactat taatatatTC GACTTCTAGT TGGTAGCATG AAGGcagtaa
  61 tcgtattttg ccttatggca ttgtccattg tggacgcagc atggaagccg ctccctgaaa
 121 acttgaagga ggacttgatc gtgtatcagg tctacccgag aagcttcaag gatagcaatg
 181 gagatggtat tggtgatatc gaaggtatta aagaaaaatt ggatcatttt ctcgaaatgg
 241 gggtcgacat gttttggtta tcccctattt atccaagccc tatggtcgat tttggttacg
 301 acatttcgaa ttacaccgac gttcatccca tatttggcac catatcagac ttagataatC
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R1 primer

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 361 TAGTCAGTGC TGCACATGAG AAAGGattga agataatctt ggatttcgtc ccgaatcata
 421 catctgatca acacgaatgg ttccagttga gtttgaaaaa cattgaacct tataacaact
 481 attacatttg gcatccagga aaaattgtaa atggcaaacg tgttccacca actaattggg
 541 taggcgtgtt tggtggatca gcttggtcgt ggcgggaaga acgacaggca tattatctgc
 601 atcaatttgc accagaacaa ccagatctaa attactataa tccagttgta ctggatgata
 661 tgcaaaatgt tctcagattc tggctgacaa ggggatttga tggtttcaga gtagatgctc
 721 tgccttacat ttgcgaagac atgcgattct tagacgaacc tctatcaggt gaaacaaatg
 781 atcccaataa aaccgagtac actctcaaga tctacactca cgatatccca gaaacctaca
 841 atgtagttcg caaatttaga gatgtgttag acgaattccc gcaaccaaaa cacatGCTTA
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FW2 primer

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 901 TCGAGGCATA CACGAattta tcgatgacga tgaaatatta cgattacgga gcagattttc
 961 ccttcaattt tgcattcatc aagaatgttt ctagggattc aaattcatca gacttcaaaa
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R2 primer

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

FW3 primer

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

R3 primer

```
1741 gaaactttta atttcttcct gaatatGTCT ATTCTTTGAA GCGGCGaaag gaaacatata
1801 tcgttaaaat ctctctatat tattatatat atatatatgt attagctaat aaattttaaa
1861 tattttgaaa cgtaaaaaaa aaaaaaaaaa aa
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Figure 3.2 Location of primers for RT - PCR.

3.7 RT – PCR amplification

RT – PCR was performed by using access RT – PCR system kit (catalog# A1250, Promega). The reaction mixture (25 µl final volume) was comprised of 1x AMV/ *Tfl* reaction buffer, 0.2 mM dNTP mix, 0.4 µM FW primer, 0.4 µM R primer, 1 mM $MgSO_4$, 0.1 u of AMV reverse transcriptase and *Tfl* DNA polymerase, and 200 ng RNA template. RT – PCR was performed under the optimum condition as followed: 1 cycle of 48°C for 45 min and 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, of annealing at 42°C for 30 sec, and of extension at 68°C for 2 min; and 1 cycle of final extension at 68°C for 7 min. The conditions for primers of *28SRNA* in *A. mellifera* and Elongation factor (*EF*) genes in *A. cerana* were as same as the condition mentioned above. After PCR amplification, PCR product was examined on 1.5% agarose gel electrophoresis and EtBr stained.

3.8 Purification of PCR product

3.8.1 Purification from solution

Purification was performed according to QIAquick PCR purification kit (catalog# 28104, Qiagen). PCR sample (1x vol) was mixed by buffer PB (5x vol). The mixture was applied to QIAquick spin column and was centrifuged at 13,000x g for 30 – 60 s. Flow – through (FT) was discarded and the column was washed by 0.75 ml of buffer PE. Then, it was centrifuged at 13,000x g for 30 – 60 s, 2x. Also, FT was discarded. Fifty µl of buffer EB was added to the center of the membrane. It was incubated at RT for 1 min and was centrifuged at 13,000x g for 1 min. An elution containing purified DNA was kept at – 20°C until use.

3.8.2 Purification from gel

Purification was performed according to QIAquick gel extraction kit (catalog# 28704, Qiagen). The target band on agarose gel was excised and weighed. Gel slice (1x vol) was added by buffer QG (3x vol). The mixture was incubated at 50°C until completely dissolved. Completely dissolved gel was mixed by isopropanol (1x vol). Then, the mixture was applied to the QIAquick spin column and was centrifuged at 13,000x g for 1 min. Flow – though (FT) was discarded. The QIAquick column was added by 0.5 ml buffer QG and was centrifuged at 13,000x g for 1 min. The QIAquick column was added by 0.75 ml buffer PE and the column was incubated at RT for 2 - 5 min before centrifugation. Buffer EB (50 µl) was transferred to the center of the membrane. After incubation at RT for 1 min, it was centrifuged at 13,000x g for 1 min. An elution containing purified DNA was kept at -20°C until use.

3.9 Sequence alignment and phylogenetic analyses

The purified DNA was sequenced at Bioservice unit, Thailand. The obtained nucleotide sequence was blasted by www.ncbi.nlm.nih.gov. The nucleotide and deduced amino sequences were aligned by Clustal W. Finally, a phylogenetic tree was made by UPGMA and NJ programs.

3.10 Quantitative measurement of nucleotides

3.10.1 RNA

The yield of obtained total RNA may be determined spectrophotometrically at 260 nm, where 1 absorbance unit (OD_{260}) equals 40 µg of single – stranded RNA/ ml. The purity may also be estimated by spectrophotometry from the relative absorbances at 260 and 280 nm. The expected range of OD_{260}/ OD_{280} ratios for RNA will be 1.8 - 2.0.

3.10.2 DNA

The yield of DNA was measured by spectrophotometry at 260 nm, where 1 absorbance unit (OD_{260}) equals 50 µg of DNA/ ml. The purity may also be estimated by spectrophotometry from the relative absorbances at 260 and 280 nm. The expected range of OD_{260}/ OD_{280} ratios for DNA will be 1.6 – 1.8.

3.11 Expression profile of AG in life cycle

Total RNA (200 ng) of egg, nurse bee, and forager bee were isolated and were amplified by RT – PCR using FW1/ R1 primers. The product of 350 bp was expected. Then, the product was loaded on 1.5% agarose gel for comparison.

3.12 Crude extract for SDS - PAGE

Ten heads and 10 honey crops were homogenized in buffer insect saline [10 mM Tris – HCl (pH 7.4) containing 130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$] containing 1 mM phenylmethylsulfonyl fluride, 0.1 µg/ ml pepstatin, and 100 µg/ ml leupeptin. The homogenates were centrifuged 2x at 700x g for 10 min and the supernatant was stored at – 20°C.

3.13 Crude extract for chromatography

The extraction of crude protein was prepared according to Takewaki *et al.* (1980). Honeybee workers (500 g) were minced in sodium phosphate buffer (pH 6.3). Final volume of homogenate was adjusted to be 3,000 ml by the same buffer. It was stirred at 4°C overnight. The suspensions were centrifuged at 10,000x g, 4°C for 15 min. After that the supernatant of about 2,000 ml was obtained. While stirring at 4°C overnight, solid ammonium sulfate was slowly added to the supernatant until up to 95% saturation. The solution was centrifuged at 10,000x g, at 4°C for 25 min and drained. The sediment was dissolved in 350 ml of the same buffer.

3.14 Protein precipitation by ammonium sulfate

Extraction of crude protein was performed according to 3.13 except the precipitation step. Ammonium sulfate at 0 – 30%, 30 – 40%, 40 – 50%, 50 – 60%, 60 – 70%, 70 – 80%, and 80 – 95% was added (Appendix E).

3.15 Dialysis

Before chromatography, the sample was dialysed by dialysis bag MWCO 10,000 in sodium phosphate buffer and filter to protect large particle interfere column.

3.16 Chromatography

3.16.1 Anion exchange (DEAE – cellulose)

Precipitated and unprecipitated crude extract (300 mg protein of each) was applied to a DEAE – cellulose column (1.6 x 13 cm) employing a mobile phase of 30 mM sodium phosphate buffer (pH 6.3) at a flow rate of 1 ml/ min. The bounded protein was eluted from the column with a linear gradient of NaCl from 0 to 1 M. The fraction size was collected at the volume of 10 ml. The protein absorption was monitored at 280 nm.

3.16.2 Gel filtration (Superdex 200)

Fractions of bounded peak from DEAE – cellulose were pooled for a gel filtration, Superdex 200. The solution, approximately 10 mg protein, was injected into the column (1.6 x 51 and 1.6 x 38 cm) equilibrated by 30 mM sodium phosphate buffer containing 100 M NaCl (pH 6.3). The column was eluted at flow rate of 0.5 ml/ min. The fraction size was collected at the volume of 10 ml. Fractions of unbound peak was performed as same as the above condition.

3.16.3 Gel filtration (Sephadex G – 150)

Fractions of unbounded peak from DEAE – cellulose were separated on a gel filtration, Sephadex G – 150. The solution, approximately 10 mg protein, was applied to the column (1.5 x 87 cm) equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3). The column was eluted at flow rate of 0.25 ml/ min. The fraction size was collected at the volume of 3 ml.

3.16.4 Cation exchange (CM – cellulose)

Precipitated crude protein and unbound peak from DEAE – cellulose was separated on CM – cellulose. The solution, approximately 300 mg protein, was applied to the column (1.6 x 13 cm) employing a mobile phase of 20 mM sodium

acetate buffer (pH 4.7) at a flow rate of 1 ml/ min. Bound protein was eluted from the column by a linear gradient of NaCl from 0 to 1 M. The fraction size was collected at the volume of 10 ml. The protein absorption was monitored at 280 *nm*.

3.17 Protein assay

3.17.1 Bradford assay

Protein concentration was determined by Bradford method. A standard curve was established by using bovine serum albumin at concentration of 0, 5, 10, 15, 20, 25, and 30 µg. The standard solution (100 µl) was mixed by Bradford solution (Appendix B) (1,000 µl). The mixture was incubated at RT for 5 min. Two hundred µl was transferred to a microtiter plate. The protein absorption was monitored at 595 nm.

3.17.2 Absorbancy at 280

Protein was diluted by dd – H_2O.

Concentration of protein (mg/ ml) = (OD_{280} nm) x dilution factor

3.18 Enzyme assay

AG activity was determined by measuring glucose liberated from sucrose (Momose's method). Supernatant containing AG was incubated with 20 µl of 10 mM phosphate buffer containing 0.1 M sucrose (pH 5.0) at 30°C for 10 min. Then, the sample was boiled for 3 min in order to stop a reaction. Fifty µl of 0.3% (w/v) 3, 6 - Dinitrophthalic acid and 50 µl of alkaline solution [5% (w/v) sodium thiosulfate and 25% (w/v) Potassium carbonate] were added to the sample. Then, the mixture was boiled for 10 min and was filled up to 1 ml by dd – H_2O. The absorbance of the mixture was measured at 450 nm. One unit is defined as an enzyme activity that can hydrolyze 1 µM of sucrose per minute under the assay condition.

$$\text{Specific activity (U/ mg)} = \frac{\text{enzyme activity (U/ ml)}}{\text{protein concentration (mg/ ml)}}$$

3.19 Polyacrylamide gel electrophoresis

Protein was separated on discontinuous gel contain 12% separating gel and 4% stacking gel (Appendix C). The sample was mixed by 5x loading dye (Appendix C), heat for 5 min, and cool on ice. The electrophoresis was performed by

1x electrode buffer (Appendix C) at 100 V until the dye front reached the bottom of the gel. After electrophoresis, the gel was coomassie blue stained.

3.20 Renatured SDS - PAGE

After SDS-PAGE, the gel was incubated in 1.0% (v/ v) Triton X – 100 with gently shaking at RT for 2 h. Then, the gel was stained to determine activity.

3.21 Coomassie brilliant blue stain (CBB)

After electrophoresis, an SDS polyacrylamide gel was incubated in 1.25% (w/ v) CBB for 30 min. Then, the gel was destained in destaining solution (Appendix C) until the background was clear. Finally, the destained gel was preserved in 10% glycerol or wrapped in cellophane.

3.22 Activity stain

After renaturation of an SDS polyacrylamide gel, the gel was incubated in 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0) at 45°C for 30 min. Then, the incubated gel was rinsed by dd – H_2O and boiled in 0.5 N NaOH containing 0.1% (w/v) triphenyltetrazolium chloride for 3 min. A red band indicating the AG activity was detected.

3.23 Two - dimensional electrophoresis of honeybee

Crude protein was separated by a procedure in a manual of 2 – D electrophoresis using immobilized pH gradients. Protein was mixed by lysis buffer [8 M urea, 4% 3 – [3 – (Cholamidopropyl) dimethylammonio] – 1 – propanesulfonate, 2% immobilized pH gradient buffer (pH 3 – 10)]. Then, the solution was mixed by rehydration solution [8 M urea, 2% CHAPS, 2% IPG buffer (pH 3 – 10), 0.2% DTT, and 0.002% bromophenol blue]. After that, the mixture was applied onto the immobiline drystrip reswelling tray. The IPG strip (pH 3 – 10, length 7 cm) was rehydrated into the solution and overlaid by drystrip cover fluid. The tray was allowed to rehydrate at RT overnight. The Multiphor II electrophoresis unit with immobiline drystrip kit was prepared for the first – dimension isoelectric focusing (IEF) at RT. The running condition of IEF was as follow: phase 1, 200 V for 1 min; phase 2, 3500 V for 1.5 h; phase 3, 3500 V for up to 3.7 kVh. After IEF, the IPG strip was prepared for second – dimension SDS – PAGE. It was equilibrated in

equilibration solution [50 mM Tris – HCl (pH 8.8), 6 M urea, 30% (v/ v) glycerol, 2% SDS, 1% DTT, and 0.002% bromophenol blue) for 15 min. Later, it was equilibrated in the equilibration solution that was replaced DTT by IAA for 15 min. The equilibrated IPG strip was transferred to Hoefer mini VE vertical electrophoresis system. The SDS – PAGE was carried out on a 12.5% separating gel and 4% stacking gel (8 x 9 cm, 1 mm thick) at 280 V, an initial current 10 mA/ gel for 15 min and 20 mA/ gel until the dye front far from the bottom of the gel about 1 mm. Low molecular weight calibration kit was used as standard molecular weight marker protein.

3.24 Protein detection for 2 - D gel

Coomassie blue stain was used for 2 – D gel detection but a procedure was different from 2.22. An analytical gel was fixed by fixation solution (Appendix D) for 1 h. The fixed gel was washed by d - H_2O with shaking for 10 min, 3x. The washed gel was stained overnight by the mixture (4 : 1) of dye stock solution (Appendix D) and methanol. The gel was destained by warm H_2O (45 – 55°C) until the background was clear.

3.25 Protein digestion

A protein band was cut out of an SDS polyacrylamide gel and washed 2x by dd – H_2O. The gel piece was washed 2x by 100 µl of 50% ACN/ 0.1 M NH_4HCO_3 at 30°C for 20 min each and dried by using a speed vacuum centrifuge. Dried gel was swollen in 100 µl of 10 mM DTT/ 0.1 M NH_4HCO_3/ 1 mM EDTA and incubated at 60°C for 45 min. The sample was incubated in 100 µl of 100 mM iodoacetamide (IAA)/ 0.1 M NH_4HCO_3 in the dark at RT for 30 min. The gel piece was washed 2x by 100 µl of 50% ACN/ 0.05 M Tris – HCl (pH 8.5) for 5 min each and dried. The dried gel was mixed by 100 µl trypsin solution [10 µl trypsin in 1% CH_3COOH and 90 µl of trypsin buffer containing 50 µl of 0.1 M Tris – HCl (pH 8.5), 1 µl of 100 mM $CaCl_2$, 10 µl of ACN, and 39 µl of H_2O) and incubated at 37°C overnight. The reaction was stopped by adding 10 µl of 2% TFA at 50°C for 30 min. The peptides were extracted 3x by 20 µl of 0.05 M Tris-HCl/ 1 mM $CaCl_2$, 20 µl of 50% ACN/ 25 mM Tris – HCl/ 0.5 mM $CaCl_2$, and 20 µl of 2.5% formic acid/ 50% ACN. Each extraction was incubated at 30°C for 10 min and sonicated for 5 min. Supernatant from all steps was pooled and dried by using speed vacuum

from all steps was pooled and dried by using speed vacuum centrifuge. The digested gel was analysed by MALDI – TOF at Bioservice unit, Thailand.

3.26 Optimum conditions for AG activity

Pooled positive fractions of Superdex 200 (after unbounded peak of DEAE – cellulose) were used to study optimum condition.

3.26.1 Optimum pH

A reaction mixture was prepared as same as a procedure in 2.18 but it was incubated in 20 µl of 10 mM Briton – Robinson buffer (Appendix B) containing 0.1 M sucrose. The pH of the reaction was adjusted to be 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5. The absorbance of the mixture was measured at 450 nm.

3.26.2 Optimum temperature

The purified AG was mixed by 20 µl of 10 mM sodium acetate buffer (pH 5; 2.26.1). The mixture was prepared as same as procedure in 2.18 but the incubation temperature was adjusted to 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, and 80°C. The absorbance of the mixture was measured at 450 nm.

3.26.3 Selective concentration of substrate

The purified AG was mixed by 20 µl of 10 mM sodium acetate buffer (pH 5; 2.26.1) and incubated at 55°C (2.25.2). The mixture was prepared as same as procedure in 2.18 but the concentration of sucrose as substrate was varied from 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM. The absorbance of the mixture was measured at 450 nm.

3.26.4 Optimum incubation time

The purified AG was mixed by 20 µl of 10 mM sodium acetate buffer (pH 5; 2.26.1) containing 80 mM sucrose (2.26.3) and incubated at 55°C (2.26.2). The mixture was prepared as same as a procedure in 2.18 but an incubation time was varied from 10, 20, 30, 40, 60, and 90 min. The absorbance of the mixture was measured at 450 nm.