CHAPTER III

MATERIALS AND METHODS

2.1 Equipment

- Autoclave, Model: Conbraco, Conbraco Ind. Inc., USA
- Automatic micropipette P10, P20, P200 and P1,000, Gilson, France
- Balance, Model: Sartorius BP 610, Germany
- Balance, Model: Precisa 240A, Precisa balance CE95, Germany
- Centrifuge, Model: Universal 30 RF CFC free, Hettich Zentrifugen, Germany
- Centrifuge, Model: Centrifuge 5410, Eppendorf, Germany
- Cuvette, Model: 1.5 ml Semi-micro cuvette, Brand, Germany
- Electrophoresis, Model: Mupid, Advance Co., Ltd., Japan
- Incubator, Memmert, Germany
- Incubator waterbath, Model: 3575-1, Lab-Line instruments Inc., USA
- Light microscope, BH2, Olympus Optical Co. Ltd., Japan
- Maxima ultra pure water, Model: Maxima UF, ELGA, England
- Microincubator, Model: M-36, Taitec, Japan
- Microwave, Model: Sharp Carousel R7456, Sharp, Thailand
- Microtome, Model: Rotary Microtome AO, American optical, USA
- Minishaker, Model: MS1, IKA Works Inc., USA
- Optima water purifier, Model: Elgastat optima 60, ELGA, England
- Olympus exposure control unit AD system, Olympus Optical Co. Ltd., Japan
- PCR, Model: Hybaid OmniGene, OmniGene HBTR3cm Hybaid limited, UK

- Polaroid, Model: Direct screen instant camera DS 34 H-34, Peca products, UK
- Power supply, AC-DC Adapter 5A, Henry, Electric Cooperation, Thailand.
- pH meter, Cyberscan 500, Eutech Cybernetics, Singapore
- Waterbath, Model: 20 H, Gallenkamp, Germany
- SDS-Electrophoresis, Max Fill model: V10-Set, Scie-Plas, UK
- Spectrophotometry, Model: UltrospecII, LKB Biochrom, England
- Stereomicroscope, Olympus, Olympus Optical Co. Ltd., Japan

2.2 Chemicals

- 5-bromo-4-chloro-3-indolyl phosphate disodium salts hydrate (BCIP), $C_8H_4BrClNO_4PNa_2$, M. W. = 370.4 Aldrich Chemical Co., Germany
- 2, 2'-Di-p-nitrophenyl-5-5'-diphenyl 3, 3'-[3, 3'–dimethoxy-4, 4'-diphenylene] ditetrazolium chloride (Nitro Blue Tetrazolium, NBT), $C_{40}H_{30}Cl_2N_{10}O_6$, Sigma, USA
- 2-mercaptoethanol, BDH Laboratory Supplies, Poole, England
- 100% Acetic acid, CH_3COOH, M. W. = 60.05, Merck, Germany
- Ammonium peroxydisulphate (APS), $(NH_4)_2S_2O_8$, M. W. = 249, BDH Laboratory Supplies, Poole, England
- Bovine Serum Albumin (BSA) Fraction V pH 7.0, Serva Feinbiochemica GmbH&Co., USA
- Bromophenol blue $C_{19}H_{10}Br_4O_5S$, M. W. = 670, BDH Laboratory Supplies, Poole, England
- Coomassie Brilliant blue G-250, $C_{47}H_{48}N_3O_7S_2Na$, M. W. = 854, BDH Laboratory Supplies, Poole, England
- Coomassie Brilliant blue R-250, $C_{45}H_{44}N_3O_7S_2Na$, M. W. = 826, Serva Feinbiochemica GmbH&Co., USA
- Calcium chloride dihydrate, $CaCl_2.2H_2O$, M. W. = 147, Merck, Germany

- Diethanolamine, $C_4H_{11}NO_2$, M. W. = 105.1, Sigma, USA
- Disodium hydrogen phosphate anhydrous, Na_2HPO_2, M. W. = 142, Merck, Germany
- Ethylene diamine tetra-acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M. W. = 292.2, Serva Feinbiochemica GmbH&Co., USA
- 95% Ethyl alcohol, CH_3CH_2OH, M. W. = 46, Thailand
- 47% Formaldehyde, CH_2O, M. W. = 30, Thailand
- 99.5% Glycerol, $C_3H_8O_3$, M. W. = 92.10, BDH Laboratory Supplies, Poole, England
- Glycine, NH_2CH_2COOH, M. W. = 75.07, BDH Laboratory Supplies, Poole, England
- Hydrochloric acid fuming 37%, HCl, Merck, Germany
- Magnesium Chloride hexahydrate, $MgCl_2.6H_2O$, M. W. = 203.3, Fluka chemika, Switzerland
- Methanol, CH_3OH, M. W. = 32.04, Merck, Germany
- n-Butanol, $C_4H_{10}O$, M. W. = 74.12, Merck, Germany
- N, N'-methylene-bis-Acrylamide (Acrylamide/Bis), Sigma, USA
- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), $C_8H_{18}N_2O_4S$, M. W. = 238.3, Serva Feinbiochemica GmbH&Co., USA
- Octylphenol-polyethyleneglycol ether (Triton X-100), Serva Feinbiochemica, USA
- Para-nitrophenyl phosphate tablets disodium (PNPP), Sigma, USA
- Para-nitrophenyl standard, Sigma, USA
- Paraplast, Palaplast plus tissue embedding medium, Oxford Labware, USA
- 85% Phosphoric acid, H_3PO_4, M. W. = 98, Mallinckrodt, USA
- Permount, SP15-500 Flammable liquid, Histological mounting medium, Fisher Scientific, USA

- QIAquick gel extraction kit (Catalog # 28704), QIAGEN, Germany
- QIAamp DNA Mini Kit (Catalog # 51304), QIAGEN, Germany
- Saponin, Fluka biochemika, Switzerland
- Sodium dodecyl sulphate, BDH Laboratory Supplies, Poole, England
- Sodium chloride, NaCl, M. W. = 58.4, Merck, Germany
- Sodium hydroxide, NaOH, M. W. = 40, Merck, Germany
- TEMED, Promega, USA
- Tris-(Hydroxymetyl)-aminomethane, $NH_2C(CH_2OH)_3$, M. W. = 121.14, Pharmacia Biotech, USA

2.3 Sample collection

Three healthy colonies of honeybee, *A. mellifera*, were taken from Chantaburi province and were maintained for sampling at Chulalongkorn University. Each colony contains one single queen that is allowed to lay eggs in empty cells. Before sample collection, an empty comb was added into each colony. All colonies were fed with pollen and nectar everyday. In an empty comb provided for a queen to lay eggs, cells were marked with painting color. Eggs at 48 h were collected as the first stage. Sample collection was continued at the interval of every 48 h until 432 h. Developmental stages of collection were egg, larva, pupa, and before emerging adult (Table 3). For cytochemical study, 50 individuals from egg stage (1^{st}), 50 individuals from the first larva stage (2^{nd}), and 30 individuals from 48 h larva stage (3^{rd}) were preferable. In other stages, only 10 individuals were collected. For histochemical study, three individuals from various stages, egg to before emerging adult were collected. Amount of samples for histochemical study was 3 samples. Samples from this collection were used for all experiments except forager bees were used for DNA isolation.

Stage collection	Stage of *A. mellifera* development	Time (h)
1st	The egg at 48 h	0
2nd	The first larva stage	48
3rd	The next 48 h larva stage	96
4th	The next 96 h larva stage	144
5th	The next 144 h larva stage	192
6th	The pre-pupa stage	240
7th	The white eye pupa stage	288
8th	The pink eye pupa stage	336
9th	The brown eye pre-adult stage	384
10th	The emerging adult stage	432

Table 2. Sample collections were named as representative stages of development in *A. mellifera.*

Figure 11. Three colonies of *A. mellifera* were maintained at Chulalongkorn University.

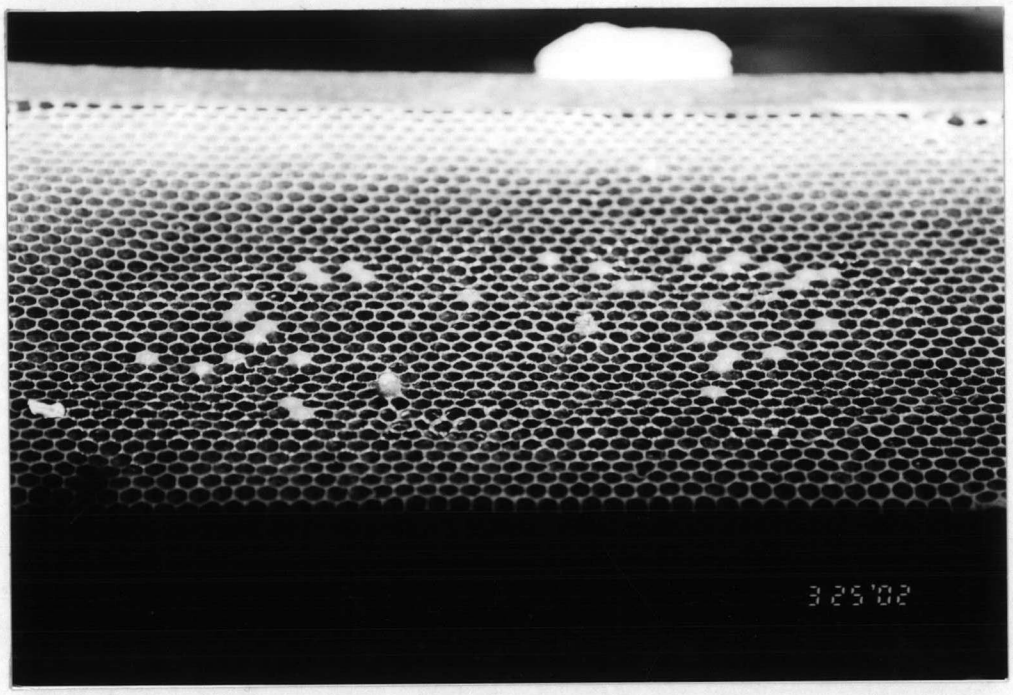

Figure 12. Cells were marked with paint color.

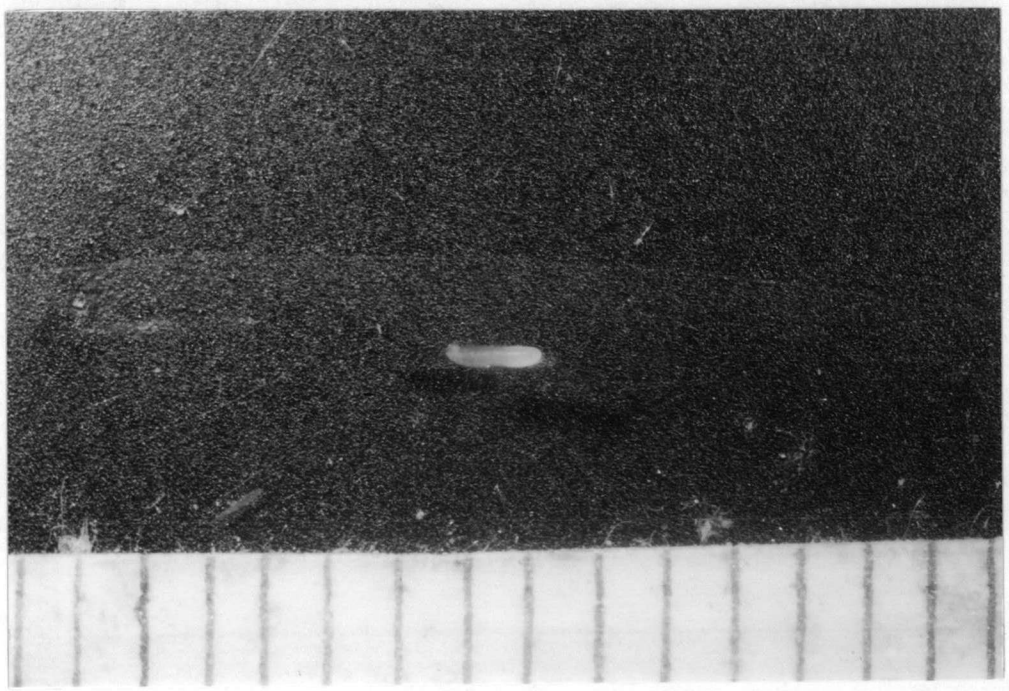

Figure 13. The first collection was the egg at 48 h (1^{st}).

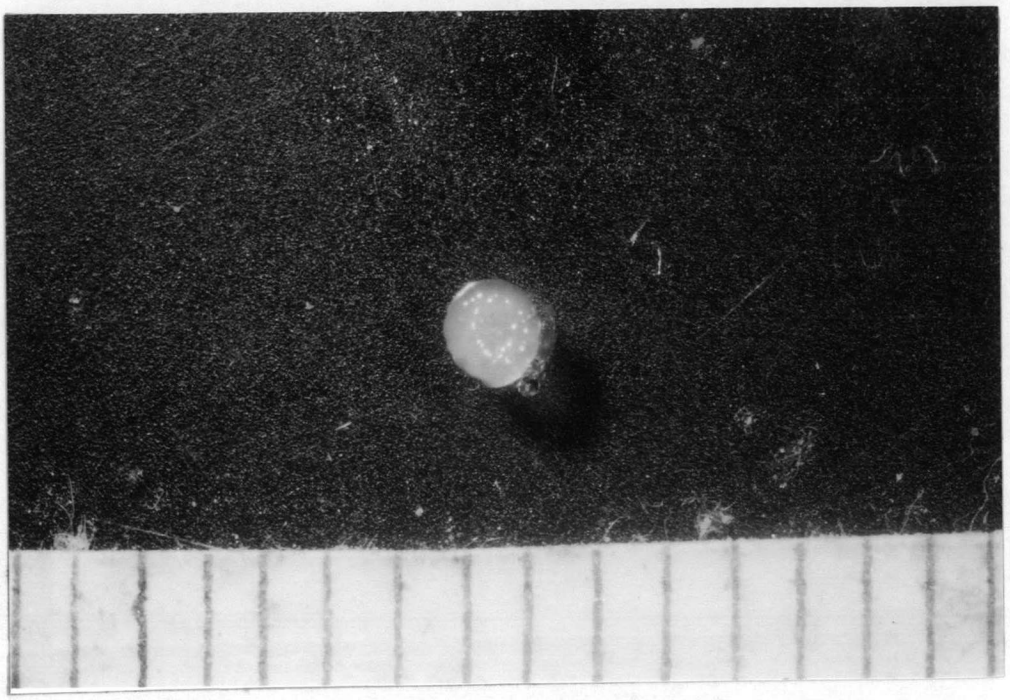

Figure 14. The second collection was the first larva (2^{nd}).

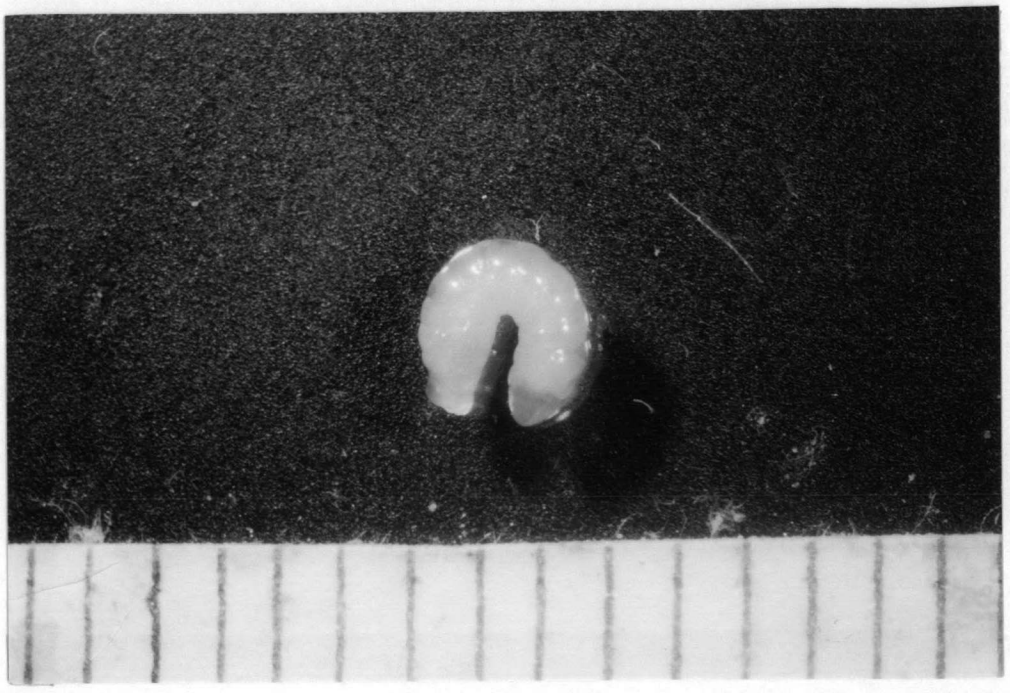

Figure 15. The third collection was the next 48 h larva stage (3^{rd}).

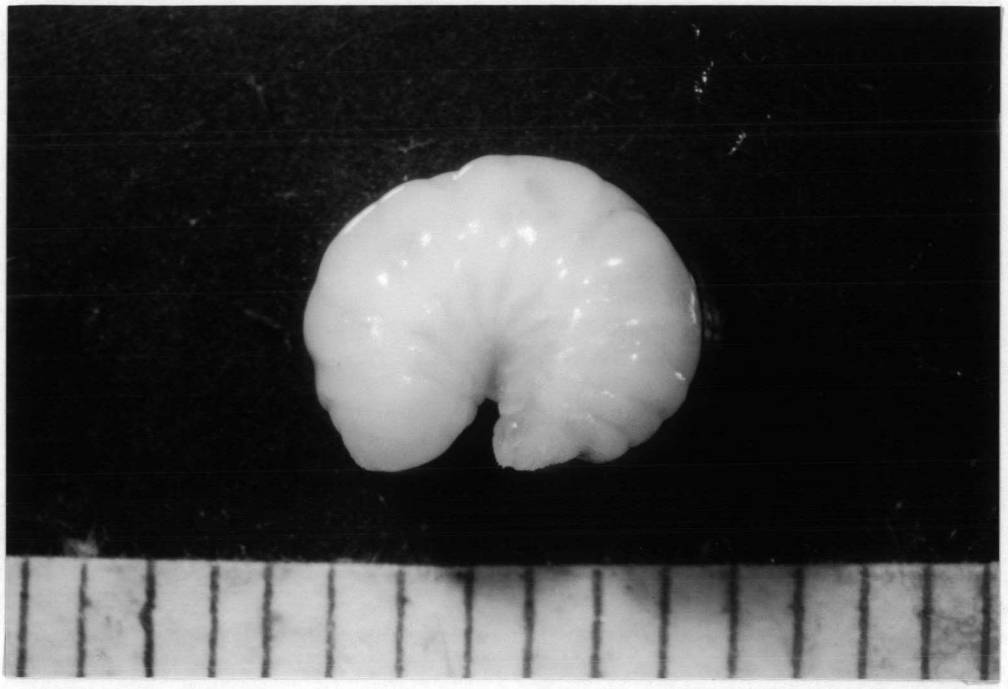

Figure 16. The forth collection was the next 96 h larva stage (4^{th}).

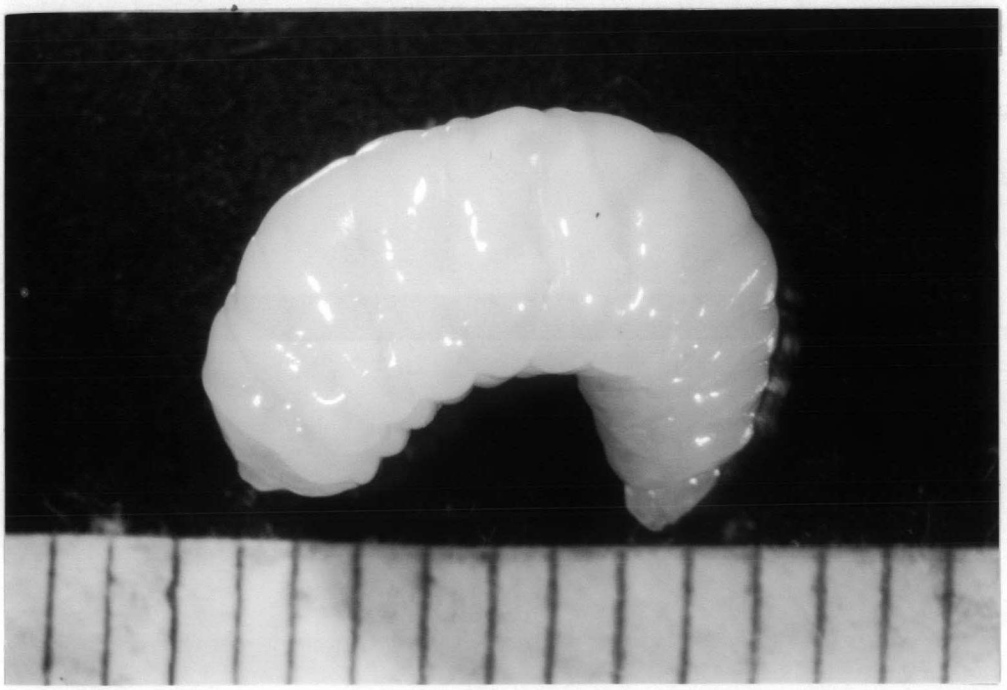

Figure 17. The fifth collection was the next 144 h larva stage (5^{th}).

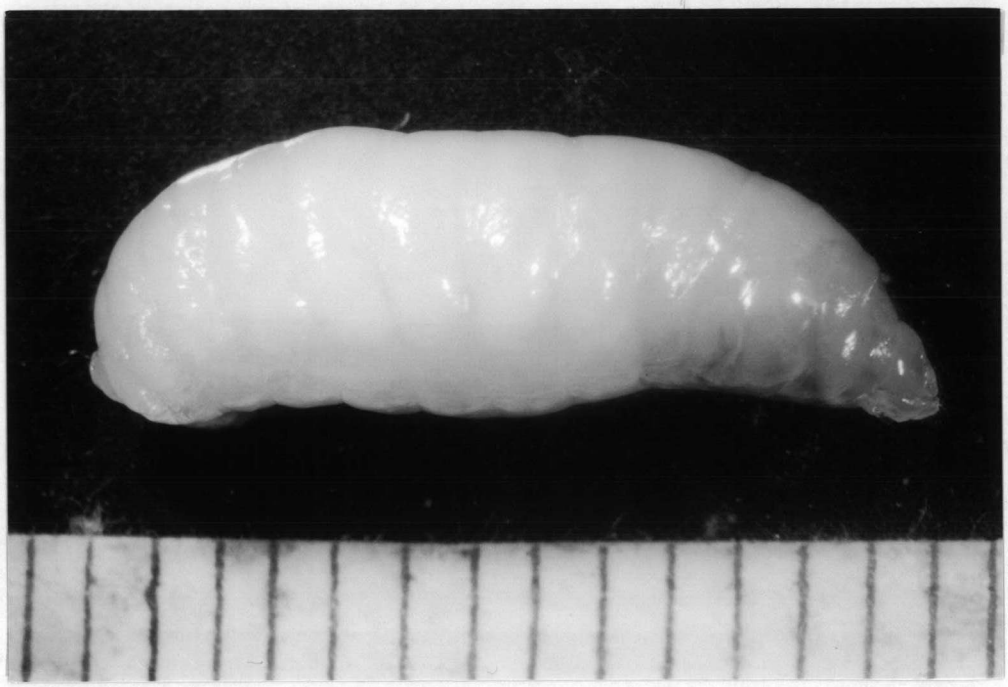

Figure 18. The sixth collection was the prepupa stage (6^{th}).

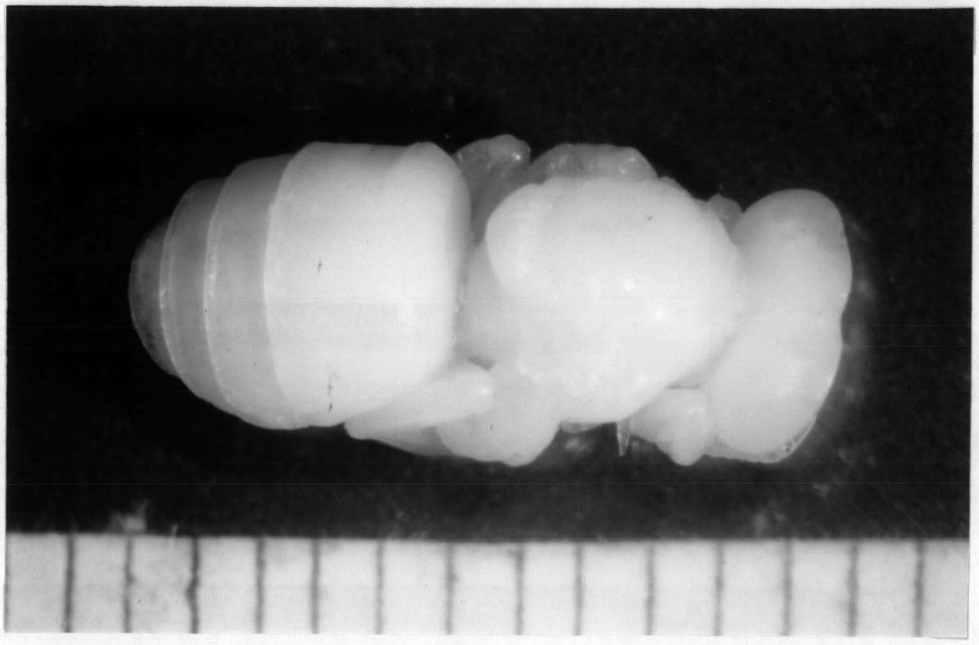

Figure 19. The seventh collection was the white eye pupa stage (7^{th}).

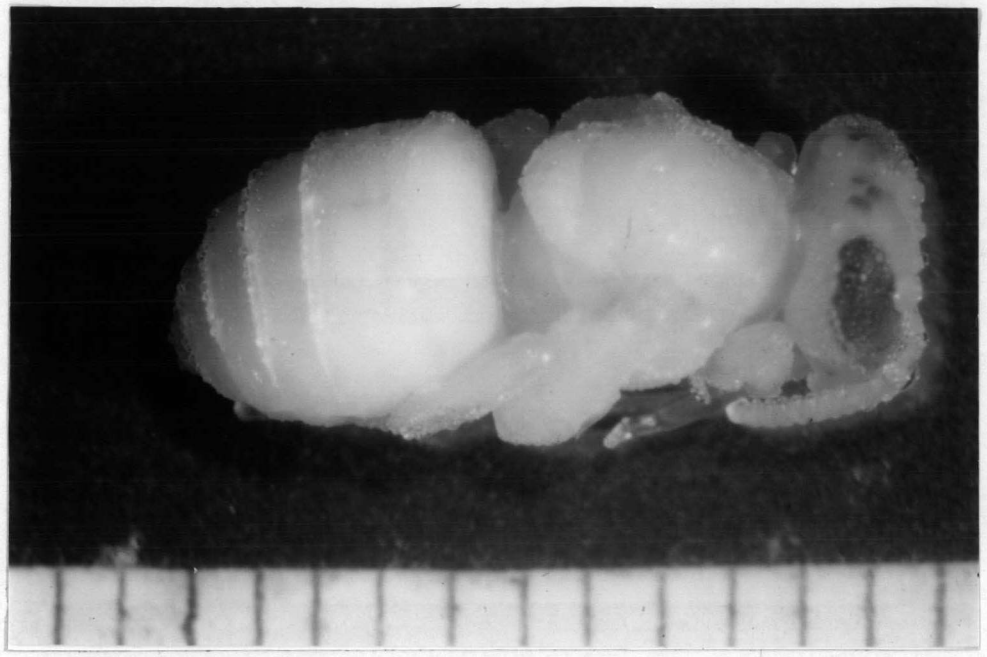

Figure 20. The eighth collection was the pink eye pupa stage (8^{th}).

Figure 21. The ninth collection was the brown eye pupa stage (9^{th}).

Figure 22. The tenth collection was the emerging stage (10^{th}).

2.4 Measurement of AP activity in each developmental stage of *A. mellifera*

2.4.1 Enzyme localization

AP activity in whole mount of all developmental stages was determined before paraffin section. The sample was fixed in 3.7% (v/v) formaldehyde in AP buffer (0.1 M Hepes, 5 mM $MgCl_2$, and 1 mM $CaCl_2$) for 1 h, then, rinsed with AP buffer for over 30 min. The sample was incubated in 0.1% (w/v) saponin containing AP buffer 10 min and rinsed in AP buffer for over 10 min at 3 times. After that, the sample was incubated in 1 ml of 175 μg/ml BCIP and 350 μg/ml NBT in 0.1 M Diethanolamine containing 5 mM $MgCl_2$ at 50°C for over 3 h. The reaction was stopped by AP buffer containing 10 mM Ethylenediamine tetraacetic acid (EDTA). Background staining was reduced by incubation of sample in 100% methanol for three to five min. Then, the sample was rehydrated in phosphate

buffer saline (PBS: 20 mM Na_2HPO_4 and 150 mM NaCl), 50% (v/v), 70% (v/v), and 90% (v/v) glycerol in PBS for 10 min in each step (Change *et al.*, 1993).

Next step, the sample was dehydrated. For activity detection, the whole mount was soaked in 70% and 90% ethanol for 6 h, 2 times, 95% ethanol for 12 h, n-butanol for 1 h, xylene for 1 h, xylene and molten wax 1 for 30 min, molten wax 1 for 30 min, molten wax 2 for 1 h to overnight. Then, the sample was embeded in paraplast. The sample was sectioned at 10 μm by serial microtome. Each section was sealed on a glass slide by 0.1% egg albumin and air dry for 48-72 h. The paraplast was washed by xylene for 10-15 min. The slide was mounted by permount. AP activity in tissue was observed by a light microscope.

2.4.2 Preparation of crude extract of *A. mellifera*

The samples from each stage were weighed and extracted by deionized distilled water (ddH_2O) (Schneiderman *et al.*, 1966). The ratio for extraction was 0.1 g sample per 1,000 μl ddH_2O. The samples were cut into small pieces by a pair of scissors on ice in a microcentrifuge tube. Then, the samples were centrifuged three times at 4°C, 3,000 rpm, for 10 min. Supernatant was transferred to a new microcentrifuge tube. After that, the supernatant was repeatly centrifuged another two times. Finally, the crude extract was kept at –20°C.

2.4.3 Electrophoresis of crude extract.

2.4.3.1 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

The separating gel was prepared 12% Acrylamide/Bis, 0.375 M Tris-HCl at pH 8.8, 0.1% (v/v) Triton X-100, 0.05% Ammonium Persulphate and 0.05% TEMED. The preparation of stacking gel at 4% Acrylamide/Bis was the same as separating gel preparation except 0.125 M Tris-HCl at pH 6.8 was used instead of 0.375 M Tris-HCl at pH 8.8 and 0.1% TEMED was used instead of 0.05% TEMED. Crude extract of 20 μg from each developmental stage was mixed with 5 μl of 5X Native-PAGE loading buffer (0.3125 M Tris-HCl pH at 6.8, 50% glycerine, 10% Triton X-100, and 0.25% Bromophenol blue), quick spun at 4°C

for 2 min, then, loaded onto the gel. The electrode buffer contained 0.2 M glycerine pH 8.3, 25 mM Tris-HCl, and 0.1% (w/v) Triton X-100. The electrophoresis was performed at 80 V for 3 h. The activity staining of gel was stained with 10 ml of 350 μg/ml of 2, 2'-Di-p-nitrophenyl-5-5'-diphenyl 3, 3'-[3, 3'-dimethoxy-4, 4'-diphenylene] ditetrazolium chloride (NBT), 175 μg/ml of 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) in 0.1 M Diethanolamine containing 5 mM $MgCl_2$ at pH 10.5, and 50 ml of 0.1 M Tris-HCl pH 7.0 for longer than 3 h. Gel was kept in cellophane paper.

2.4.3.2 SDS Polyacrylamide Electrophoresis (SDS-PAGE)

SDS-PAGE was prepared as the same as Native PAGE except using 0.1% (w/v) of Sodium dodecyl sulphate (SDS) instead of 0.1% (v/v) of Triton X-100. Before loading, a sample was denatured at room temperature, 50, 70, and 100°C. In order to test tolerate heating and SDS (Verhaert *et al.*, 1990). In addition, to find out subunits of protein in each developmental stage, both a sample was denatured by 0.1% (w/v) of SDS, boiled at 100°C and, mixed with 5x SDS PAGE loading dye (0.3125 M Tris-HCl pH at 6.8, 50% glycerine, 10% SDS, and 0.25% Bromophenol blue). The electrode running buffer contained 0.2 M glycerine at pH 8.3, 25 mM Tris-HCl, and 0.1% (w/v) SDS. After electrophoresis at 80 V for 3 h, the gel was stained by Coomassie blue (solution I) for 30 min, destained by 10% (v/v) methanol containing 10% (v/v) acetic acid (solution II) for overnight, and soaked in 10% (v/v) glycerol (solution III). Gels were kept in cellophane paper and photographed.

2.4.4 Hydrolysis of AP in crude extract

The optimum pH, temperature, and amount of substrate of AP were determined. Para-nitrophenol phosphate (PNPP) used as a substrate. The product of this reaction was paranitrophenol (PNP) that gave yellow color in basic condition. PNPP substrate was prepared to be 1 mg/ml in 0.1 M Diethanolamine containing 5 mM $MgCl_2$ at pH 10.5.

2.4.4.1 Selective concentration of substrate and incubation time for AP in crude extract

The reaction mixture was prepared as the concentration of PNPP was varied from 0.15, 0.30, 0.60, 0.90 and 1.2 μM, respectively. The mixture was incubated at RT for 1 h. The optimum amount of PNPP was obtained from the graph of Michaelis-Menton. In order to determine the optimum incubation time, the reaction mixture was incubated at various period of 15 min to 3 h at the interval time of 15 min.

2.4.4.2 Optimum pH of AP in crude extract

Crude extract was incubated in 0.1 M Tris-HCl at pH 6.5 to 9.5. Then, the reaction mixture was prepared by 1,000 μl of 0.1 M Tris-HCl, PNPP substrate was concentrated at 0.3 μM, 100 μl of crude extract, and ddH_2O to quantitate total volume to be 2 ml. The mixture was incubated at RT for 1 h and measured the absorbance at 405 nm. After that, pH of the reaction mixture was determined pH again. The graph was plotted by X-axis indicating various final pH and Y-axis indicating the activity of AP in crude extract. Peak of the graph was the optimum pH of AP in crude extract of *A. mellifera*.

2.4.4.3 Optimum temperature of AP in crude extact

The reaction mixture was prepared as described in 2.4.4.2. The reaction was incubated for 1 h at different temperatures (35, 40, 45, 50, 55, 60, 65, 70, and 75°C, respectively). Then, the absorbance was measured at 405 nm. The graph was plotted by X-axis indicating various temperature and the Y-axis indicating the activity of AP in crude extract. The peak of the graph was the optimum temperature of AP in crude extract.

2.4.4.4 Specific activity of AP in crude extract

Amount of total protein was determined by Bradford assay. This method was the proportional binding of the dye coomassie to protein that was measured by spectrophotometry at 595 nm. The total protein concentration in crude extract was

determined by comparison to that of series of protein standard know to reproducibly exhibit a linear absorbance profile in the assay. Bovine Serum Albumin (BSA) was widely used for protein standard (Bollag and Edelstein, 1991). The amount of BSA was used at 0, 1, 3, 5, 7, and 10 μg/μl, respectively, mixed with 1,000 μl of Bradford solution and added ddH_2O to quantitate in total volume to be 2 ml. The reaction mixture of protein was immediately determined at 595 nm. The linear relationship graph of standard BSA, $y = ax + b$, was plotted according to calculated amounts of total protein in crude extraction.

Amount of protein (mg) = O.D. at 595 nm/slope of the standard BSA graph

Standard graph of Paranitrophenol (PNP) was plotted according to the relationship of absorbance at 405 nm and different concentrations of the substrate which were 0, 0.02, 0.03, 0.05, and 0.07 μM. The reaction mixture was prepared by 1,000 μl of 0.1 M Tris-HCl at pH 7.0, 0.3 μM of PNPP, 30 μl of crude extract, and ddH_2O to quantitate total volume to be 2 ml. The mixture was incubated at 50°C for 1 h and measured at 405 nm. Then, the absorbance at 405 nm of crude extract was compared to the PNP graph to interprete the enzyme activity. The AP activity in each developmental stage was obtained from a standard curve of PNP

$$\textbf{The AP activity (μM /min)} = \frac{\textbf{O.D. at 405 nm / slope of the standard PNP graph}}{\textbf{time}}$$

After that, specific activity was calculated from the AP activity divided by amount of total protein (mg).

2.5 Partial DNA sequence and homology

2.5.1 Primer design

Primers of AP were designed from the sequence of AP in *D. melanogaster* (Yang *et al.*, 2000) and data of amino acid sequence in GenBank

(www.ncbi.nlm.nih.gov). The conserved region from amino acid sequences of *AP* in non-specific tissue and specific tissue from several organisms were determined. Amino acid sequences of VDPSSCTATA and VEGGRID were selected as representative areas. The characterization of both primers were checked by Oligo program that was used for designing primers. Later, the require primers were synthesized by Bioservice unit (BSU).

2.5.2 Total DNA extraction and PCR amplification

Total DNA was extracted by QIAamp DNA Mini Kit. Twenty five mg of thoraxes was cut by a pair of scissors in 360 µl of buffer ATL. The tissue was cut into small pieces to decrease lysis time. The lysed tissue was mixed with 20 µl of Proteinase K, mixed by vortex, incubated, and shaked at 56°C for 3 h minimum or until the tissue was completely lysed. The sample was quick spun. Then, the sample was mixed by 200 µl of buffer AL, vortexed for 15 sec, and incubated at 70°C for 10 min. After quick spun, the sample was mixed by absolute ethanol, vortexed for 15 sec, and quick spun. The mixture was transferred to a QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The column was removed to a clean 2 ml collection tube while the supernatant was discarded. The buffer AW1 of 500 µl was added to the QIAamp spin column that would be later centrifuged at 8,000 rpm for 1 min. The column was put on top of a clean 2 ml collection tube. The buffer AW2 of 500 µl was added in the QIAamp spin column. Then, it was centrifuged at 14,000 rpm for 3 min. The column was again moved to a clean 2 ml collection tube and the filtrate was discarded. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 100 µl of buffer AE was transferred. The column was incubated at RT for 3 min and centrifuged at 8,000 rpm for 1 min (QIAGEN). After that, a quantity and quality of total DNA was checked by 1.0% agarose gel electrophoresis. Loading sample was mixed between 5 µl of total DNA and 2 µl of loading dye (5X DNA BlueRun™: 150 mM EDTA, 25 mM Tris-HCl (pH 7.0), 2.5% glycerol, 0.05%

bromophenol blue) in total volume of 7 μl. And 5 μl of λ/HindIII was marker. The running buffer was 1X TBE contained 0.05 M Tris-HCl, 0.05 M Boric acid, and 0.65 mM EDTA . The electrophoresis was performed at 80 V for 45 min. Then, the gel was stained with 10 mg/ml ethidiumbromide (EtBr) and destained in ddH_2O. PCR product was visible under UV light and photographed.

Reaction of PCR amplification was prepared by 1X PCR buffer, 0.1 mM dNTPs mixed, 0.4 μM AP1 and AP2 primers, 1 unit of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 4 μl of total DNA, and quantitate total volume to be 25 μl by ddH_2O. Then, the reaction was laid on top by mineral oil and performed by Hybaid OmniGene. The program of amplification would be the pre-step at 94°C for 2.5 min and 35 cycles for amplification at 94°C of denaturing for 1 min, 57°C of annealing for 1 min, and 72°C of extension for 3 min and the last extension at 72°C for 10 min again. Five μl of PCR product mixed with 1X loading dye in total volume of 7 μl was loaded onto each well of 1.5% agarose gel. The electrophoresis was performed as described before.

2.5.3 Preparation of PCR product sequencing

2.5.3.1 PCR product purification

The PCR product was purified by QIAquick gel extraction kit. PCR product fragment was cut out of the agarose gel by a clean and sharp razor blade. The gel slice was weighed. After three volume of buffer QG was added to one volume of the gel slice, the reaction was incubated at 50°C for 10 min and mixed by vortexing every 2 to 3 min during incubation until completely dissolved. It was indicated by the yellow color of the mixture. One volume of isopropanol was mixed to the sample and transferred to a QIAquick spin column in 2 ml collection tube. The column containing the reaction was quick spun for 1 min. After that, the flow-through was discarded and the column was placed back in the same collection tube. Buffer QG of 0.5 ml was added in the column. Then, the column was quick spun for 1 min. The column was washed by 0.75 ml buffer PE and

quick spun for 1 min. The flow-through was then discarded. The column was additionally quick spun for other 1 min at 13,000 rpm and transferred into a clean 1.5 ml microcentrifuge. DNA was eluted by adding 50 µl buffer EB to the column which would be centrifuged at maximum speed for 1 min (QIAGEN).

2.5.3.2 TOPO TA cloning from PCR product

The quantity and purity of purified PCR product was determined by spectrophotometry at the absorbance of 260 and 280 nm. The ligation reaction was prepared by 5 µl of PCR product and 1 µl of TOPO vector (Appendix III). The reaction was mixed gently and incubated at RT for 15 min. After that, if the next step was not immediately performed, the reaction can be kept at –20°C overnight. Before transformation, 10 µl of E. coli stain DH5α was thawed on ice. Five hundred µl of SOC and 4 plates of LB (1.0% Tryptone, 0.5% yeast extract, and 1.0% NaCl, at pH 7.0) with 100 µg/ml Amplicillin were warmed at RT. Three µl of ligation reaction was transferred to 10 µl of DH5α cell, incubated on ice for 30 min, heated shock at 42°C for 45 sec, and on ice for 2 min. The mixture was transferred to 250 µl SOC (2.0% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 20 mM glucose, pH 7.0) in a 4 ml falcon tube and shaken at 37°C, 200 rpm for 45 min. Each LB plate with 100 µg/ml Ampicillin was spreaded by 25 µl of 100 mM IPTG and 25 µl of 40 mg/ml X-gal. After 45 min, 125 µl of the culture was spreaded onto the LB plate mentioned above. And the plate was incubated at 37°C for overnight. The white and blue colonies would be visible. The white colony was picked up by a clean toothpick and transferred to a 4 ml falcon tube containing 1.5 ml LB and 100 µg/ml Ampicillin. The culture was shaken at 200 rpm at 37°C for overnight. Then, plasmid preparation was performed.

2.5.4 Plasmid preparation

The protocol was followed by QIAprep spin miniprep kit (QIAGEN). The overnight culture was transferred to a 1.5 ml eppendorf tube and spun at 13,000

rpm, 4°C for 30 sec. The supernatant was drained. Two hundred and fifty µl of buffer P1 was added and mixed. Then, 250 µl of buffer P2 was added. The mixture was gently mixed by inversion (4-6x). Three hundred and fifty µl of buffer N3 was added and inverted to mix immediately and gently (4-6x). The mixture was spun at 12,000 rpm at RT for 10 min. Then, the supernatant was transferred to a QIAprep column by pipetting. The column was centrifuged at 12,000 rpm, RT for 1 min. The flow-through (FT) was discarded. The column was washed by adding 750 µl buffer PE and spun at 12,000 rpm at RT for 1 min. Later, the FT was discarded. The column was re-spun again to remove the rest of wash buffer. The column was placed to a new 1.5 eppendorf tube. Fifty µl of milliQ H_2O was added to the center of the column. After the column was incubated at RT for 1 min, it was spun at 12,000 rpm, RT for 1 min. The elution was kept and measured spectrometrically at 260 and 280 nm.

2.5.5 Preparation of sequencing reaction

The reaction was prepared according to the protocol of all PE Applied Biosystems thermal cyclers. Five hundred ng of double-stranded DNA were used as template. Eight µl of terminator ready reaction mix was added to the sample. Then, it was followed by 3.2 pM of M13 Forward primer or M13 Reverse primer. The deionized water was added to the reaction to quantitate total volume to be 20 µl. The reaction was mixed well and spun briefly. The reaction tube was placed in a thermal cycler. The program was performed for 25 cycles as followed: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Then, the sample was stored at 4° C until the purification of extension products was performed.

2.5.6 Purification of extension product for sequencing

The method was followed by the protocol of Centri-sep columns (Princeton separations, Inc.). Briefly, the column was tapped shortly to allow dry gel to move to the bottom part of the column. Eight hundred µl of milliQ H_2O was added to the column and tapped to get rid of air bubbles. The column was incubated at RT

for 2 h. Then, a cap and a stopper of the column was removed. The column was removed to a 2 ml wash tube to drain milliQ H_2O by gravity. The FT was discarded. Later, the column was spun at 2,500 rpm at RT for 2 min. The FT was discarded again. The 20 µl of sample from 2.6.5 was loaded onto the center of the column. Then, the column was placed in a 1.5 ml eppendorf tube. It was spun at 2,500 rpm at RT for 2 min. Then, the column was discarded. The elution was dry by speed vacuum for 20-30 min. The solution (3 µl) of sequence dry and formamide at the ratio of 1:5 was added to the sample. The mixture was then heat at 95°C for 2 min and chilled in ice. The mixture tube was wrapped with aluminum foil to avoid the light exposure. Then, the sequencing was performed automatically by ABI prism 377.

2.5.7 Sequence analysis

The nucleotide sequences of AP in *A. mellifera* were aligned with nucleotide sequences of AP in other organisms recorded in database, National Center for Biotechnology Information (GenBank) (www.ncbi.nim.nih.gov), European Bioinformatics Institute (EMBL) (www.ebi.ac.uk), and Institute Nation la de Recherche Agronomicnomique (INRA) (www.prodes.toulouse.inra.fr). The nucleotide sequences were converted to amino acid sequences by the Protein Machine of EMBL database. Both of nucleotide and amino acid sequences were aligned with Clastalw at EMBL database and MultAlin program at INRA database in order to determine the similarity.