CHAPTER V

DISCUSSION

Sample collection of *A. mellifera* in each developmental stage was very important in order to obtain the exact AP activity in crude extract and in tissue. It depends on the starting sample that was egg (1^{st}). Samples from each stage were at 48 h in the interval. An empty comb was inserted in a colony in order that a queen would lay new eggs in it. After that, sample (1^{st}) were collected and cells of age known egg was marked with painting color. The color might be cleaned by worker bees so a colony must be checked everyday. A colony maintained at Chulalongkorn University between January to June 2001 was fed with pollen and nectar because there was a few food during that period. The number of collected sample until egg to emerging adult was ten times. In order to assay AP activity, fresh samples were used but the sample was fixed for histochemical technique. Healthy samples were considered for these techniques because AP activity was high in death cells or infected cells than in normal cells (Gregorc and Bowen, 1997). Numbers of sample collection in each developmental stage was different. The weight of samples at 1^{st}, 2^{nd}, and 3^{rd} stages were lighter than the weight of other stages. All of samples would be pooled and weighed before extraction. To confirm profile of AP activity in each developmental stage, samples from three colonies were used (Figure 84 and 86).

In this research, a histochemical technique of AP in *A. mellifera* tissue in each developmental stage was adapted from many standard histochemical procedures (Verhaert *et al.*, 1990; Chang *et al.* 1993). Then, the sample was undergone paraffin section with serial microtome. This adapted method was not expensive, convenient, and easily applied to characterize AP that could tolerate heat. As known, some steps of paraffin wax were performed at high temperature. The technique could be used for samples at egg, larva, and prepupa stages more

than at pupa and emerging adult. It was because sample in both stages contain high cutin composed cuticle so the chemical could not pass into the cell. This might cause a problem of AP detection. In several organisms, AP was present in multi forms and was varied in different organs (McComb *et al.*, 1979; Voet and Voet, 1995). At 48 h egg, the AP activity was found at the position of cleavage cells and at membrane. It might indicate that AP starts to be localized at the develop position of *A. mellifera* embryo.

AP activity was mostly localized at integument; epidermis, old cuticle, and new cuticle at all stages. The AP could be more easily detected in larva than in pupa because the cuticle in the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} stages was soft. The substrate could pass through cells easily. The AP activity in tissue could be observed after whole mount section.

AP activity in larva tissue was found as free AP in epithelial cell (Change *et al.*, 1993). The activity in the $2^{nd}$and 3^{rd} stages was higher than in other larva stages. The activity was found at peritrophic membrane and free granule of gut at all stages. AP was very abundant in gut wall of insects but its functions was not known (Wigglesworth, 1979). In epithelial cells of 48 h of *A. mellifera* larva, AP activity was found free in cytosol and in brush border of the salivary gland cells. AP activity was found in larva tissue nor in larva haemocoel of any ages. AP activity seems to become enhanced in developing and differentiating columnar cells and basal plasma membrane (Gregorc and Bowen, 1997). AP should involve in developing and differentiating of epithelial cell in gut of *A. mellifera* larva. Furthermore, AP was an important component in peritrophic membrane that would synthesize energy for gut activity.

AP activity was also found in epithelial cell in limb bud that would be developed to legs. It was found at plasma membrane, around nucleus, free granule in cell as well. The report of the epithelial-AP (e-AP) of grasshopper suggested that it might function in epithelial morphogenesis. It expresses near the tips of

limb buds (Chang *et al.*, 1993). In insect, epithelial changes might involve both in cell shape and cell rearrangements (Condic *et al.*, 1991). Extracellular enzyme, like e-AP, could mediate cell adhesion of epithelial junctions or could contribute to a reduction of epithelial adhesion which persists rearrangement. The expression of e-AP in grasshopper limb buds was consistent with function in the interaction of epithelial cells with cells or the extracellular matrix that occur during cell movement and rearrangements in limb morphogenesis (Chang *et al.*, 1993). From this data, characterization of AP in limb buds of *A. mellifera* was seemed to be that way. AP activity was found in numerous free granule in epithelial cells of limb buds. The AP activity was higher at the 2^{nd} or 3^{rd} stages than in other larva stages due to the process of differentiation. The AP activity was found in fatty body cell and free cell in haemocoel. The activity in young larva was higher than in old larva because cells in younger larva might need to store food for next developmental steps. Larva of insect was an active stage so it produces and consumes a lot of energy at the same time. That might be possible that it stores lot of fat and glycogen. The fatty body was the most important site of intermediate metabolism in insects. It was not clear that AP in many insect tissue was an intermediate chemical for metabolism. It might suggest that AP might form part of the system of enzymes those concern in liberating the silk protein forming a complex with nucleic acids (Wigglesworth, 1979). The AP activity was found in tracheae of *A. mellifera* at all stages. Tracheae were invagination of the cuticle that branched everywhere among the tissue. The histochemical structure of tracheae was the same essential as that of the body surface from which they were derived. They consist of a matrix of discrete epithelial cells. The cuticle was thrown into folds and the cuticle lining of tracheae has the same composition as that covering the surface. The lining membrane was excessively delicate, and was freely permeable to water (Wigglesworth, 1979).

The AP activity was found in prepupa at 6th stage at integument tissue such as cuticle and was found at optic cells. In pupa at 7th, 8th, and 9th stages, the body was divided into three parts already. The AP activity was found in head at cuticle covering compound eye, especially at optic lobe such as corneal lens, matrix cell of cornea, reticula cell, and around plasma membrane of nerve cells. Furthermore, the AP activity was found at mouthpart, especially at tube part of proboscis. The AP was found in thorax at cuticle, muscle cells, and in epithelial cell of legs. The high activity was found in abdomen, especially at the last segment. The AP activity was high at midgut, hindgut, and malpighian tubule. The data might be not enough because a section was not as complete as in larva stage. In emerging adult (10th), a section was not complete either because of a hard cuticle. Selective organs in abdomen would be used to provide more data. AP activity might involve in metabolism in several cells that could generate energy and might function at plasma membrane. Furthermore, it should be important in development. The technique works well in egg to larva but not in samples containing hard cuticles. Getting rid of cutin before performing this technique was required.

About localization of AP activity, in tissue the colormetric assay between NBT/BCIP substrate and AP should be relible although NBT and BCIP could be positive for other enzymes those can hydrolyse phosphodiester bond. From control of whole mount, AP activity in tissue and section was not able to change the color of NBT/BCIP. It could confirm the localization of the AP activity in tissue. Data of control were not shown in this thesis.

About partial sequence of *AP* gene, the nucleotide and amino acid sequences were compared to those in several organisms such as bacteria (*E. coli*), human that has four multi families, Bovine, *G. gallus*, and insects those were *B. mori* and *D. melanogaster.* This experiment was based on data of Yang *et al.* (2000). Primers were designed from conserved regions in amino acid sequences of those organisms. The 429 bp PCR product was obtained under the optimum

condition of PCR amplification. To compare homology of nucleotide and amino acid sequences to other non-specific *AP* sequences in those mentioned organisms, the nucleotide was similar to *D. melanogaster* at 39.2%, to *B. mori* at 39.7% , to *Gullus gallus* at 36.6%, to Bovine at 36.9%, and to *Homo sapiens* at 36.7% (Figure 79). The obtained nucleotide sequence was converted to an amino acid sequence of 139 amino acids in *A. mellifera*. The similarity of AP amino acid sequence was at 14.3% of *D. melanogaster*, at 24.3% to *B. mori*, at 28.6 % to *G. gallus*, at 19.8% to Bovine, and at 27.6% to *H. sapiens* (Figure 78). Partial nucleotide or amino acid sequences were used to construct a phylogenetic tree. Partial non-specific *AP* of *A. mellifera* was closed to *AP* in *B. mori*, *D. melanogaster*, and *G. gallus*, respectively. In the future, a full length nucleotide sequence of AP in *A.mellifera* would be obtained. That would provide more data in a phylogenetic tree construction of *AP*. The phylogenetic tree at figure 79 was similar to non-specific *AP* in several organisms that showed at Yang *et al.*, 2000.

The AP activity of *A. mellifera* in each developmental stage extracted by ddH_2O was used for crude extraction. Double distilled ddH_2O was used because it is the main component in cell and was neutral (Schneiderman, 1966). AP was mostly activated in basic condition so, in this experiment, Tris-HCl and diethanolamine were used to provide the basic condition (Chang *et al.*, 1993). The proper pH of AP in crude extract was 9.5-9.6 that was found in other organisms: bacteria, mammal, and other insects such as grasshopper, American cockroach, *Drosophila sp.*, etc (Schneiderman, 1966; McComb *et al.*, 1979; Chang *et al.*, 1993; Verhaert *et al.*, 1990; Yang *et al.*, 2000). AP could be still activated at high temperature. About AP in American cockroach, it was reported that the AP reaction was not completely inactivated at 60°C for 2 min but it was completely inactivated at 100°C for 2 min (Verthaert *et al.*, 1990). AP in *A. mellifera* was more heat stable than AP in American cockroach because it was highly activated at 50°C. From the result of PAGE studying, AP could endure to heating. AP

reaction was still active at 70°C for 5 min but it would be completely denatured at 100°C for 5 min. From the results, AP in crude extract of *A. mellifera* was more highly heat stable than AP in brain of American cockroach.

Amount of total protein in crude extract was almost similar in each developmental stage (Figure 85). The amount of total protein at 1st, 6th, and 10th stages were lower than other stages because protein was used in differentiation or morphogenesis. From the result of SDS-PAGE, bands of protein patterns in each development stage were different in egg, larva, pupa, and emerging adult. Both results indicated the difference of the amount of total protein and subunits of protein in each stage of development.

The AP activity profile in crude extract was measured by the hydrolysis of PNPP as substrate. The peak of AP activity in crude extract was different at all stages. The highest activity was at the 4th stage (Figure 86). The peak of activity in larva was higher than in prepupa because of the cuticle formation. The constant and low AP activity was observed at 7th, 8th, and 9th stages of pupa. Also, pupa from those stages consumed little energy. They just stay still in a cell. At an emerging adult stage, AP activity begins to increase because a bee at this stage begins to do some duties outside a brood cell. According to this, it might suggest that AP would be related to phosphodiester bond breaking. That would synthesize energy for cell activity. AP in *A. mellifera* development might be highly activated in order to produce energy in cell. The AP activity in larva and emerging adult was higher than in pupa. A possible relationship of AP was like some ATPase but unlike inositol phosphate hydrolysing enzymes. It was because ATPase were likely to be more abundantly present in investigated cells as well (Verhaert *et al.*, 1990).

Furthermore, the AP activity was studied by Native PAGE and SDS PAGE stained with NBT/BCIP as substrate (Figure 87). Trend of the AP activity was similar to the AP activity profile in crude extract. A mass weight of AP in *A. mellifera* was ~ 150 kDa which was higher than that in American cockroach

that was 120-130 kDa (Verhaert *et al.*, 1990). It was reported that the mass weight of mammalian AP varies between 100 and 200 kDa (McComb *et al.*, 1979). From the result of denaturation temperature, it was found that the AP activity still remains at 30°C and 50°C. In contrast, the AP activity was completely denatured at 100°C for 5 min. The condition of denaturation in *A. mellifera* is longer than in *P. americana* (Verhaert *et al.*, 1990). It might be concluded that AP in *A. mellifera* was highly tolerate to temperature like AP in other organisms. Due to SDS PAGE, it was found that 0.1% (w/v) of SDS could not denature protein completely, so the crude extract was heat at 100°C. After activity stained with NBT/BCIP, the unboiled crude extract on SDS PAGE was the same positive as the crude extract on Native PAGE. In American cockroach, AP in crude extract was treated with 2.5% SDS and the activity pattern was still similar to that in Native PAGE (Verhaert *et al.*, 1990). It could be assumed that AP in *A. mellifera* would be completely denatured by high temperature and high concentration of SDS.