CHAPTER II

LITERATURE REVIEWS

2.1 Biology of honeybee

Apis florea, dwarf honey bees, is one of native species in Thailand. The distribution area is generally confined to warm climates. *A. florea* is the honeybee of the lowland of South Asia. In some regions, there is only *A. florae,* not other species. Its main habitat is Pakistan, India, Sri Lanka, Thailand, Indochina, Malaysia, parts of Indonesia, and Palawan (Fig. 2.1). An ecological niche in a stratum is dense bushes and small trees. This biotope requires small body size, small nests, and special adaptations for survival. The body size of *A. florea* is about 10 mm in length. It looks reddish, especially the first abdomen in an old worker. A nest of *A. florea* consists of a single comb. Upper part of the comb is for honey storage. Lower parts are pollen storage cells, worker - brood cells, and drone - brood cells, in order. The topmost part of the comb is a horizontal surface which bees use as a dance floor (Ruttner, 1988).

A. florea is one of nine species in the world. In Thailand, 5 species ranking from smallest size to largest size are *A. andreniformisl* Smith, 1858; *A. florea* Fabricius, 1787; *A. cerana* Fabricius, 1793; *A. mellifera* Linnaeus, 1758; and *A. dorsata* Fabricius, 1793. Four undiscovered species are *A. laboriosa* Smith, 1871; *A. nuluensis* Tingek, Koeniger and Koeniger, 1996; *A. koschevnikovi* Buttel - Reepen, 1906; *A. nigrocincta* Smith, 1861 (Wongsiri *et al.*, 2000).

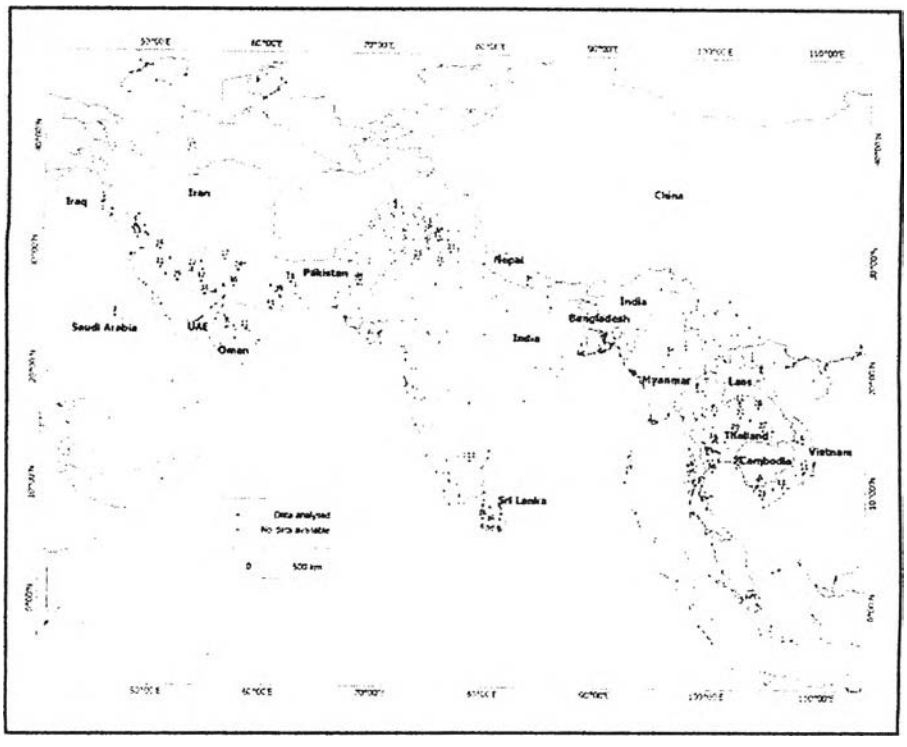

Figure 2.1 Distribution of *A. florea* (Hepburn *et al.*, 2005).

Taxonomy identification of *A. florea* (modified from Wongsiri, 1989):

Kingdom Metazoa

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super - family Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *Apis florea*

Honeybee is a highly eusocial insect and its colony is composed of a queen, drones, and workers (Fig. 2.2). Bees in different divisions have their own distinct phenotype (Wongsiri, 1989).

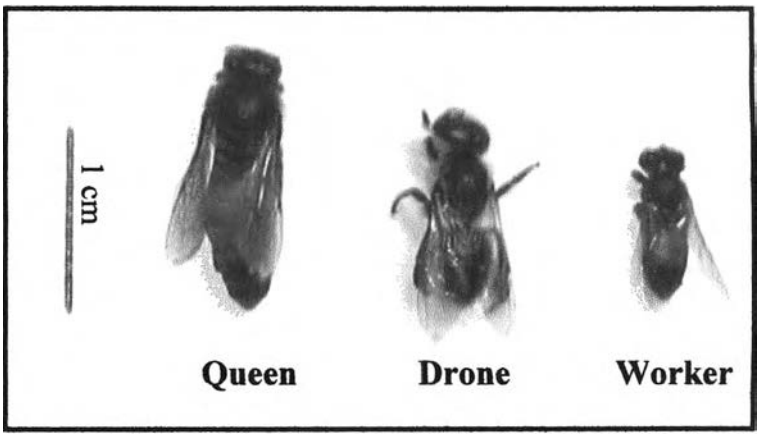

Figure 2.2 Phenotypes of *A. florea* in each caste.

Queen (2n) is the only fertile female in a hive so the important and only job of the queen is to lay eggs. The queen basically keeps workers uninterested in reproduction on their own by secreting a pheromone. Drones (n) are developed by parthenogenesis. Drones, like a queen, lack the body parts to effectively harvest nectar or pollen to feed themselves. Drones also lack any kind of stings. They are designed for mating only. Workers (2n) are completely sterile and lack fundamental reproductive equipment. Workers perform almost tasks in a colony except egg laying. A task performed by an individual worker can be changed and depended on age after eclosion (age polyethism). Young workers (nurse bees) take care of their broods by synthesizing and secreting royal jelly (bee milk) whereas older workers (foragers) forage nectar and process it into honey. In parallel to this age - dependent role change, physiological changes occur in certain organs of a worker for example in hypopharyngeal glands (Kubo *et al.*, 1996).

2.2 Hypopharyngeal glands

Hypopharyngeal glands (HPGs) or food glands are paired acinous glands located in a head of worker bee as shown in Fig.2.3. In emerged bees, the acini look plump and creamy. HPGs synthesize and secrete a substance rich in protein that is fed to larvae, drones, and a queen. HPGs are developed during pupa and early adult stages. Secretion may start a few days after a young worker bee has consumed pollen and continue for several days or weeks. At this stage, HPGs are completely developed, and a worker will be involved in feeding larvae. When a worker becomes a forager, the glands will retrogress (Brouwers, 1982) and will produce digestive enzymes, including AG and invertase (Dade, 1994). The HPGs are believed to synthesize bee milk and are well developed in young bees. In older bee, they get shrunk. In addition, hydrolysis activity of sucrose in nectar to be glucose and fructose is detected (Kubo *et al.*, 1996).

2.3 Honey crop

Forager bee collects nectar in a honey crop. Collected nectar is composed of mostly sucrose. Then, digestive enzymes, AG, are added. Sucrose will be converted to be glucose and fructose while a forager is returning to its hive. In a hive, the bee empties its crop into a cell or the nectar is transferred to another worker that takes it to a honeycomb for evaporation to make honey (Winston, 1987).

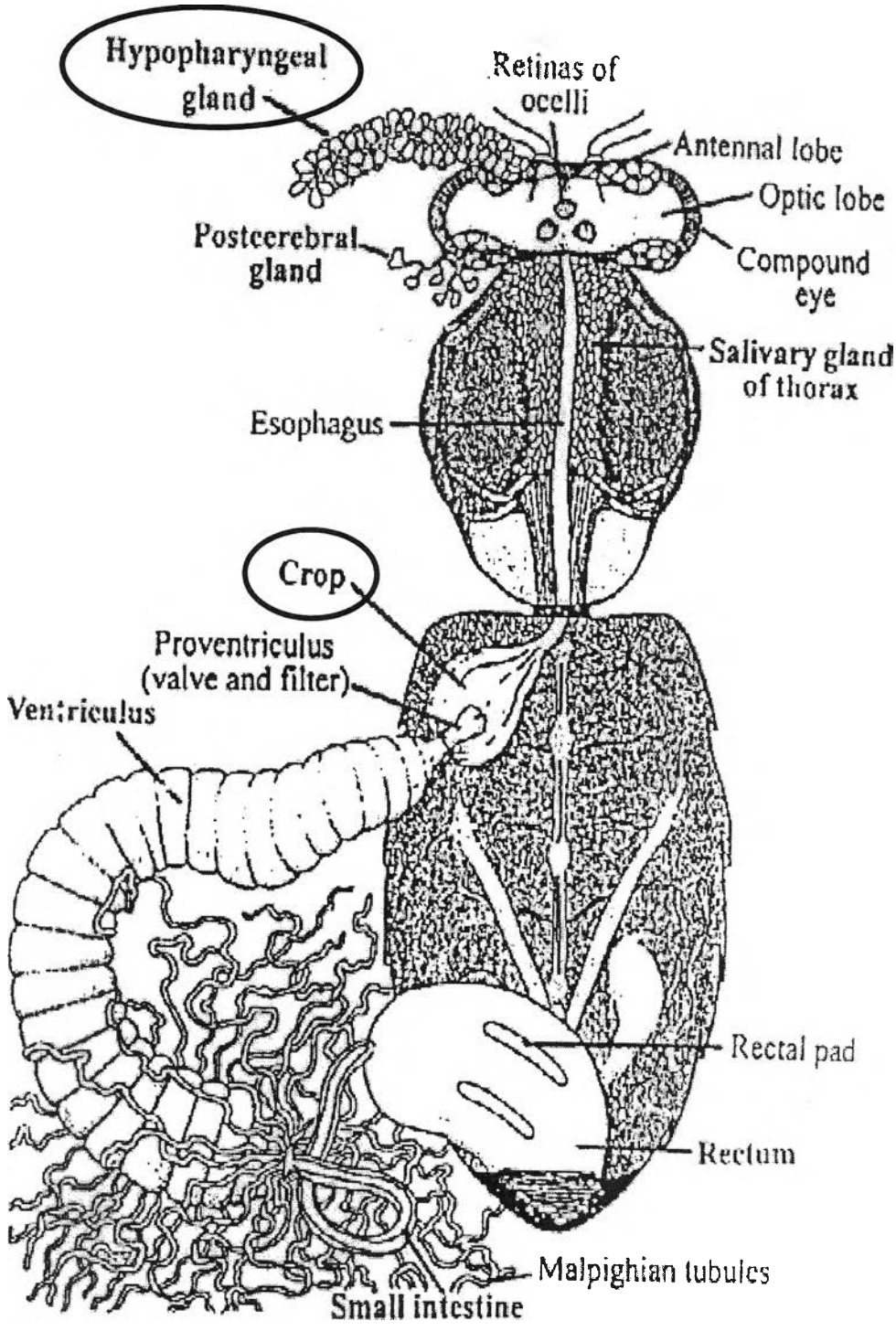

Figure 2.3 Location of hypopharyngeal glands and honey crop (Dade, 1994).

2.4 Royal jelly (Bee milk)

Royal jelly (RJ) is a creamy product secreted by nurse bee. It is fed to queen and other young larvae by nurse bee. It is totally synthesized in HPGs and mandibular glands. The nurse bee has best developed HPGs which produce large amounts of RJ (Crailsheim, 1991). RJ consists of proteins and nutrients from pollen. Also, it is composed of an emulsion of proteins, sugars, and lipids in a water base (Schmidt, 1996).

Table 2.1 Compositions of *A. mellifera* royal jelly (Srisuparbh *et al.*, 2003).

Content	Percent
Moisture content	65 - 70
Crude protein	12 - 17
Crude lipid	3 - 4
Total sugar	10 - 12

Tomoda *et al.* (1977) fractionated RJ protein of Italian hybrid strain of *A. mellifera*. Water - soluble protein, the major fraction, was separated on gel filtration (Sephadex G - 200). MW of two fractions containing water - soluble major protein are rather low about 14 and 33 kDa. The electrophoresis and ion exchange column (DEAE - cellulose) revealed that the water - soluble protein in RJ contains at least five different proteins. In nurse bee glands of *A. mellifera*, 3 major proteins (50, 56, and 64 kDa) were against immunoblotting by using affinity - purified antibodies. The 3 major protein are synthesized selectively and secreted as bee-milk protein (Kubo *et al.*, 1996). Major RJ protein from the HPGs of *A. mellifera* was isolated by ion - exchange chromatography and SDS - PAGE. It showed only one band at the MW of 57 kDa. In addition, protein from HPGs, abdomen, thorax, and head of honeybee were analysed by antiserum raised against major RJ protein. The result showed the major RJ protein was found in HPGs and head. MW of major proteins from HPGs and from RJ are 55 kDa and 57 kDa, respectively (Hanes *et al.*, 1992).

Srisuparbh *et al.* (2003) established expressed sequence tag (EST) library. It revealed that major RJ protein of *A. cerana* was identified as homologues of major RJ protein of *A. mellifera* families. Moreover, purification of RJ protein of *A. cerana*

on Q - Sepharose and Sephadex G - 200 chromatography showed protein at the MW of 115, 55, 50, and 300 kDa.

2.5 Honey

Honey is a sweet and viscous solution derived from nectar of flowers. It is a supersaturated solution of sugars, mainly fructose, glucose, and maltose. Flavor and color of honey are largely determined by flowers which nectar is gathered. Moisture content of natural raw honey varies from 14 - 18% so honey is preserved. Because of its high sugar concentration, it can kill bacteria by osmotic lysis Honey is used largely in baking, because it is hydroscopic. It is an excellent natural preservative. Honey has numerous medicinal properties which it is used as a folk remedy for burns, cataracts, ulcers, and wounds. Moreover, skin - care products, skin creams, and moisturizers also consist of honey which provides antioxidant activity.

2.6 Alpha glucosidase

Alpha glucosidase or AG (EC 3.2.1.20, α - D - glucoside glucohydrolase) is an enzyme that can hydrolyze 1, 2 - linked - alpha - glucosidase bond in sucrose to be fructose and glucose. AG catalyzes the splitting of an α - glycosyl residue from the non - reducing terminal side of a substrate having an α - glucosidic linkage to release α - glucose (Chiba, 1997). AG is often called maltase and sucrase or invertase because of the sucrose - hydrolyzing property (Terre *et al.*, 1994).

In 2000, Kimura *et al.* reported that AG in honeybee is classified into family I which attacks heterogeneous substrates such as sucrose and aryl α - glucosides faster than malto – oligosaccharides. It has no or less activity to α - glucan (so - called real "α - glucosidase" to recognize α - glucosyl structure). AG in other group, family II, has opposite characteristics to AG in family I (so - called "maltase" to recognize maltosyl structure).

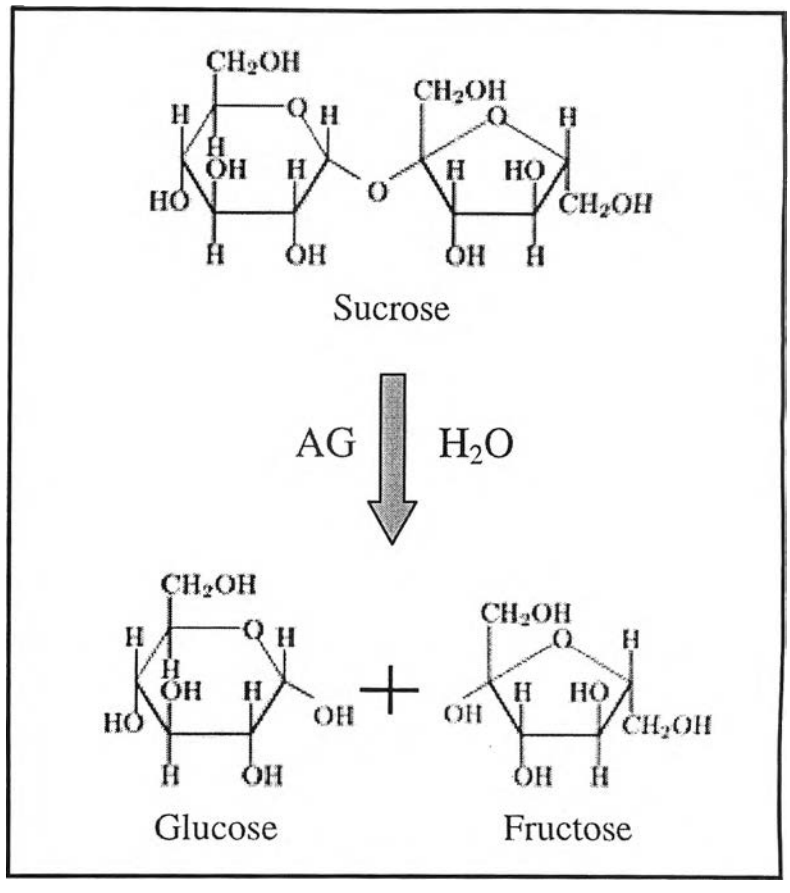

Figure 2.4 Hydration reaction of alpha glucosidase.

In honeybee, a major role of the enzymes seems to be the breakdown of sucrose in the gathered nectar. This enzyme is produced in HPGs, secreted into its mouth, and then passed through a digestive system.

AG has been isolated from various insects such as the fruit fly *Drosophila melanogaster* (Tanimura *et al.*, 1979), the house fly *Musca domesta*, the honeybee *Apis mellifera*, and the American cockroach *Periplaneta Americana* (Kimura *et al.*, 1990).

There are lots of reports about AG in *A. mellifera*. In HPGs of forager bee, there are at least 3 genes coding for carbohydrate - metabolizing enzymes, AG (sucrase), amylase, and glucose oxidase. These genes are not expressed in HPGs of nurse bee (Ohashi *et al.*, 1999).

There are more researches to support that AG is highly synthesized in foragers only. In nurse bee HPGs, there are 3 major proteins (50, 56, and 64 kDa) synthesized selectively and secreted as bee milk protein. After that, the major protein in forager bee is changed to be 70 kDa. It was purified by Mono Q FPLC column and

Superose 12 gel filtration FPLC (Kubo *et al.*, 1996). Immunoblotting analysis using affinity - purified antibodies against a major 70 kDa protein synthesized specifically in the forager bee HPGs indicated that it was AG.

Moreover, the *AG* cDNA was isolated and sequenced. The encoded protein consisting of 650 amino acids has high sequence identity to maltase and related enzymes in fruit fly and mosquito. By using RT – PCR, it shows that *AG* is expressed specifically in the HPGs of forager (Ohashi *et al.*, 1996).

AG of whole adult honeybee *A. mellifera* was purified by DEAE Sephadex, Sephadex G - 200, DEAE Sephadex A - 25, and Sepharose - 4B. The MW of the purified enzyme was 70 kDa or a little higher. There is no evidence in subunit structure of this enzyme. Then, optimum pH of purified sucrase was determined to be 5.5 (Huber, 1975).

There are 3 forms of AG in *A. mellifera.* AGI was purified by chromatography on CM - cellulose and gel filtration on Sephadex G - 100. The MW of AGI was estimated to be approximately 98 kDa. AGII was purified by chromatography on DEAE - cellulose, CM - cellulose, and by gel filtration on Bio - Gel P - 150. The MW of AGII was estimated to be approximately 76 kDa (Takewaki *et al.*, 1980). In addition, AGIII was purified by salting - out chromatography, DEAE - cellulose, DEAE Sepharose CL - 6B, Bio - Gel P - 150, and CM - Toyopearl 650M chromatographies. The MW of AGIII was estimated to be approximately 68 kDa (Nishimoto *et al.*, 2001).

Not only AG activity depends on age of honeybee but it also depends on tissue types. AGI, AGII, and AGIII are localized in different honeybee organs. Purified AGI, AGII, and AGIII from honeybee were checked by Ouchtelony double diffusion tests (Table 2.2). It shows that AGI is localized in ventriculus, and AGII, in ventriculus and haemolymph while AGIII is concluded to be honey AG, localized in the HPGs. Moreover AGIII was detected in ventriculus during the winter. It is possible that AGIII can be diffused out of the synthesized organ and be accumulated in other organs such as honey crop, small intestine, and rectum (Kubota *et. al.*, 2004).

Table 2.2 Ouchterlony double diffusion tests for AG in honeybee organs.

AG, organ extract and haemolymph	Response of antisera			
	Antiserum against			
	AGI	AGII	AGIII	Honey AG
AGI	+	-	-	-
AGII	-	+	-	-
AGIII	-	-	+	+
Honey AG	-	-	+	+
Ventriculus	+	+	-, (+)[a]	-, (+)[a]
HPGs	-	-	+	+
Haemolymph	-	+	-	N.E.
Postcerebral gland	-	-	-	N.E.
Salivary gland	-	-	-	N.E.
Crop	-	-	-	N.E.
Small intestine	-	-	-	N.E.
Rectum	-	-	-	N.E.

[a] During the winter (no flower season), AGIII is often detected also in ventriculus.

N.E., not examined.

To study AG in *A. cerana*, HPGs size at various ages was measured. The result shows that HPGs is in largest size on day - 15, and get decreasing after day - 18. Under renaturation condition, a positive band of AG is visible at the MW of 96 kDa. In addition, *AG* in *A. cerana* is highly expressed in foragers (Srimawong, 2003).