CHAPTER I

INTRODUCTION

Honeybees are one genus (*Apis*) of the most economically important insect not only in their ability to help crop pollination that increase their yield but also improve the seed and fruit qualities. The valuable hive products such as honey, beeswax, royal jelly and pollen used as supplement food, ingredient in foods, cosmetics and medicine products.

Every honeybee colony is a family consisting of 3 caste honeybees, that a single fertile female, **the queen**, which is the mother of the colony, a large number (approximately 10-30 thousand) of infertile females, **the workers**, and from zero to few thousand male **drones** (during mating times of the year). The females, the queen and workers, are heterozygote (diploid 2n = 32) grown from fertilized eggs whereas the drones are hemizygotes (haploid individuals n = 16) come from unfertilized eggs (Ruttner 1988, Wongsiri *et al.*, 1989).

1.1. Colony Members

1.1.1. Honeybee Queen

Being the sole member of the female reproductive caste, the queen is indispensable for the survival of the colony. A mated queen serves the colony in two essential functions: laying fertilized and unfertilized eggs, and secreting substances known as pheromones, required for the stability of the colony's social order.

Within a few days of having mated with several drones, the queen begins to lay eggs, and continues to do so until she is physiologically exhausted. The larvae of female bees (queens and workers) will hatch from the fertilized eggs, while the

unfertilized eggs yield drone larvae. During the mating process, the queen stores sperm from the drones in a storage organ, the spermatheca, within her abdomen (Wongsiri, 1989). By controlling the opening and closing of the spermatheca, the queen can allow sperm cells to fertilize her eggs or prevent them from doing so. The fertilized eggs are deposited in small worker cells, while the unfertilized eggs are laid in the larger drone cells.

Worker bees recognize their queen not by her physical structure but by her scent, given off by the pheromones she secretes. Queen pheromones, 9-oxodextrans-2-ecenoic acid and 9-hydrozydec-2-eonic acid substances, have both direct and indirect effects on the colony's social behavior (Robinson, 1991). During the nuptial flight, they serve as sex attractants, drawing the drones to the queen. Inside the hive, they assist in stabilizing the colony: the workers are aware of the queen's whereabouts by the presence or absence of pheromones. Under certain circumstances, the presence within the hive of pheromones will inhibit the untimely construction of queen cells: they also inhibit the development of the workers' ovaries (Liadlaw, 1992), and during swarming they exercise a direct influence on swarm stabilization.

Before an old queen dies, or departs to start another hive, she lays an egg in a large queen cell. The nurse bees feed the larva a diet of only royal jelly (RJ, rich mixture of food made from the hypophargeal and mandibular glands throughout development.

1.1.2. Honeybee Worker

The workers are an infertile caste of female bees, developed from fertilized eggs (diploid 2n: 32). They are suited by their physiological and anatomical features to perform virtually all kinds of chores except reproduction, to increase the chances of

the colony's survival. Factors determining the type of task to be executed by a worker include its physiological and anatomical state of readiness, and environmental stimuli, as well as the requirements of the colony to have a particular job done at a particular time.

Soon after emerging from its cell, a young worker receives food, in the form of either nectar or honey, from mature workers, and also helps herself to honey and pollen she finds in the colony's storage cells. In the first few days after she emerges, she is too weak to do anything except inspect and clean empty cells in preparation for food storage by the colony or egg-laying by the queen. During this period she consumes relatively large amounts of honey and pollen, and this directly affects the development of her hypopharyngeal and wax glands.

The secretion from nurse bees' hypopharyngeal glands and mandibular glands is fed to the larvae, those of all ages in queen cells receiving large quantities; for this reason it is referred to as "royal jelly". Larvae in worker and drone cells receive this special diet only during the first days after hatching; during their later larval life they are fed on a mixture of honey and pollen. At about the same time as the hypopharyngeal glands of the nurse bee develop, or shortly afterward, four pairs of wax glands, located below her abdominal segments also develop, under the stimulation of consumption of large amounts of honey. From these glands she secretes flakes of whitish wax, which are manipulated by worker bees, using their mandibles, in the process of comb construction and repair and in capping cells.

Usually, a worker bee when she reaches the age of about 5 - 15 days is physiologically exhausted from the tasks of secreting royal jelly (Lercker, 1981). After this period, they are spent packing pollen in storage cells, the mouth-to-mouth

retrieval of nectar from returning foragers. and occasionally guarding the hive entrance. When she is about three weeks old she stops to be a "house bee" and becomes a "field bee". At this stage her flight muscles are sufficiently developed, and after orientation flights which enable her to locate the hive in relation to surrounding landmarks, she collects nectar, pollen, water and propolis and carries them back to the hive until she dies (Robinson, 1991; Page and Peng, 2001).

1.1.3. Honeybee Drone

The drones are the male members of the honeybee society reared by the colony shortly before the swarming season begins. In queenless colonies, workers whose ovaries have developed as a result of the lack of inhibiting action by the queen's pheromones can also lay eggs which, being unfertilized because the worker is unmated, also yield drones.

Drones possess no food-gathering apparatus: their sole biological function is to mate with queens. During the mating season, they are well fed by the workers before taking flight. To ensure successful mating, several thousands of drones must be in the area, although the queen will mate with only about ten. The drone dies shortly after copulation.

When the mating season is nearing its end, the colony reduces its drone-rearing, and when the season is over, the rearing of drones ends completely. The drones remaining in the hive gradually die of old age, negligence by the worker bees or starvation, or they may simply be expelled from the hive.

1.2. Honeybees in Thailand

Bees are insects of the Order Hymenoptera which feed on pollen and nectar. They constitute a group of about 20,000 species throughout the world, known taxonomically as the superfamily Apoidea. Honeybees of the genus *Apis* belong to the family Apidae, a sub-group of this superfamily. Member of this genus could be allocated to three different lineages based primarily on morphology and behavior: 1) the dwarf, or midget, bee composing of *A. florae*, and *A. andrenifrmis*, 2) the giant, or rock, bee composing of *A. dorsata*, and *A. laboriosa*, 3) the cavity-nesting bees composing of *A. cerana*; the oriental, or eastern (Indian, Chinese, Japanese, etc.) honey bees, *A. mellifera*; the common, or western (European, African, etc.) honey bees. (Smith, 1991)

Four species including *A. andrenifermis*, *A. cerana*, *A. dorsata,* and *A. florae* are indigenously distributed in Thailand whereas *A. mellifera* was introduced from Europe and Africa for a bee keeping purpose (Wongsiri *et al.*, 2000). Among these species of *Apis*, only *A. mellifera* and *A. cerana* are kept for commercial beekeeping due to non-aggressive behavior and simple management. Behavioral limitations of the dwarf and giant honeybees, prevents their being kept in man-made hives for reasonably long periods, while hiving colonies in specially-constructed containers is essential in that it enables the colonies to be manipulated.

Generally, there are two possible approaches to the development of commercial beekeeping: the introduction of modern beekeeping with *A. mellifera* or the improvement of existing techniques for using *A. cerana*. Notwithstanding the difficulties involved in establishing new apiaries of the introduced colonies and in developing colony management techniques suitable to local conditions, *A. mellifera*

colonies are generally more productive than those of *A. cerana* where forage is abundant. On the other hand, where forage is available only marginally, colonies of *A. cerana* survive better due to high level of resistance to nosema disease and the parasitic Asian mites, *Varroa jacobsonii* and *Tropilaelaps clarae* which plague *A. mellifera* (Verma, 1992). In general, *A. cerana* is gentle, diligent, industrious, managed easily as well as an ability to detect and remove bee mites from the colonies result in *A. cerana* is a suitable species for beekeeping in Thailand (Wongsiri *et al.*, 1990).

1.3. Royal Jelly (RJ)

A characteristic feature of honeybee society is the phenomenon of cast determination named insect polyphenisms. Female honeybees are destined to become queens or workers during the first few days of larval development. Young female larvae fed on royal jelly (RJ) develop into queen bees while genetically identical female larvae fed on worker jelly (nutritionally less valuable than RJ) develop into worker bees. This is a case of insect polyphenisms regulated by differential nourishment (Evans and Wheeler, 2001).

RJ is a secretion product of the cephalic glands; hypopharyngeal and mandibular gland with located in the head of nurse bees (Figure 1.1), mainly between the sixth and twelfth days of their life (Haydak, 1970) and serves as a food for young bee larvae and the adult queen bees and through prophylactic behavior is distributed between individuals of colony (Crailsheim, 1992). RJ is always secreted and fed directly, not stored, to the queen over their life span and first three days of worker and drone larvae. Subsequently, a mixture of honey and pollen was supplied as worker and drone larvae diets for the remaining time (Iannuzzi, 1990; Johansson, 1995).

When the larvae destined to become queen bees are supplied with an over-abundance of royal jelly. The queen larvae cannot consume the food as fast as it is provided and royal jelly accumulates in the queen cells (see Figure 1.2).

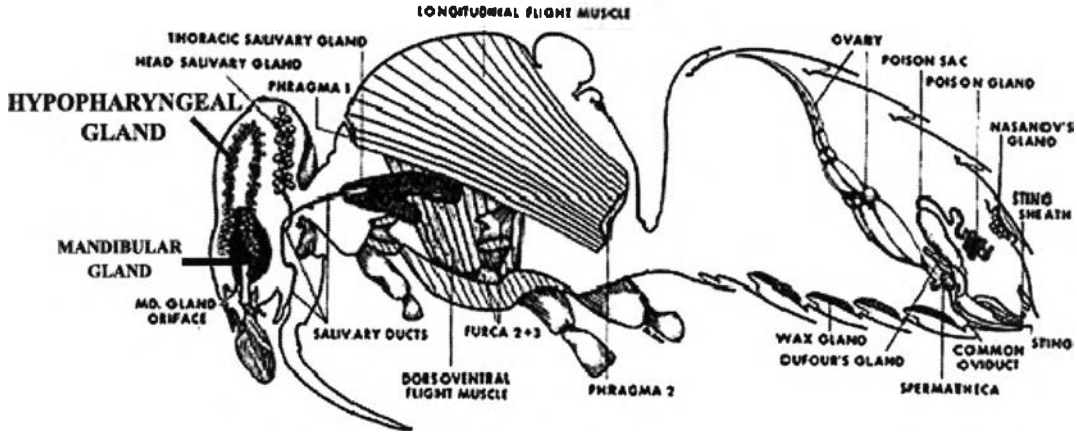

Figure 1.1 Diagram showing the ogan systems of an adult female honeybee.

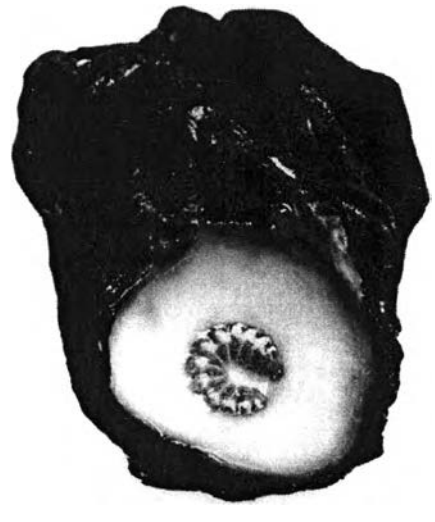

Figure 1.2 A 3-day-old queen larva floating in royal jelly.

Between queen and worker bees, the differentiation is related to feeding during the larval stages. Indeed, all female eggs can produce a queen bee during the whole development of the larvae and particularly the first four days, but this occurs only when they are cared for and fed "like a queen". Queen rearing, regulated by complex mechanisms within the hive, induces in a young larva a series of hormonal and biochemical actions and reactions that make it develop into a queen bee. A queen bee differs from a worker bee in various ways. Queen is superior size compared to the worker; with a longer abdomen; the reproductive organ is well developed to mature stage and lay up to several thousand eggs a day. The time required for queen development period is average in 15.5 days and lives for several years. On the contrary, workers are smaller and fully develop organ related to their tasks such as pollen baskets, stronger mandibles, hypopharyngeal glands and wax glands. The time for worker development period require in 21 days with only a few months of life span (Krell, 1996). It is mainly the spectacular fertility and long life-span of the queen, exclusively fed on RJ, which have suggestively led people to believe that royal jelly produces similar effects in humans.

Royal jelly (RJ, also called bee-milk); which a white-yellow colloid, a slightly pungent phenolic odor and a characteristic of sour flavour with a pH between 3.6-4.2; is one of essential and high valuable bee products widely produced in beekeeping. It can be sold in various forms including the fresh RJ, unprocessed except for being frozen or cooled, mixed with other product such as various juices, or freeze-dried RJ for subsequently used in other preparations. In its unprocessed form, RJ can also be included directly in many food and dietary supplement as well as medicine-like products or cosmetics industry. The largest producer and exporter of RJ in the world

are come from China for approximately 60% of world production. In Taiwan, RJ produced nearly 90% of the international market share (Lin, 2004). While the highest domestic consumption of RJ is Japan, a large part of which was imported from other Asian countries included Thailand. In Asia, the use of royal jelly in cosmetics has led to some very successful products, as in Thailand, a business originally based on cosmetics with RJ and other related bee products were successful and consistently grew into a multimillion dollar enterprise (Krell, 1996).

RJ is believed to be a potentially useful supplement because of the queen bee's superior size, strength, stamina and longevity compared to other bees. In several studies have been shown the various advantageous effects of RJ such as cancer-preventive properties; RJ might inhibit mild and slow growing tumors, but not rapid-growing tumor (Tamura *et al* 1985). Other research suggests RJ manifested the ability to decrease the mutagenic effects of some chemical and physical mutagens (Bariliak *et al.*, 1996). RJ may also lower cholesterol and general blood lipids (Cho, 1977 and Vittek, 1995); Vittek stated that RJ at approximately 50-100 mg per day can decrease total serum cholesterol levels by about 14%, and total serum lipids by about 10% in studied group. Due to these effects it may help prevent atherosclerosis. For immune-stimulating effect, Sver *et al.* (1996) informed RJ exhibited immunomodulatory properties by stimulating antibody production and immunocompetent cell proliferation in mice or depressing humoral immune function in rats. Whereas, Oka (2001) reported RJ could act prevents allergic reactions and histamine release.

For anti-inflammatory and wound-healing properties, RJ activated macrophages for inhibiting proinflammatory cytokine production (Kohno *et al.*, 2004). However, the allergic reaction was found to be the common side effect for

people who extremely allergic to bee products when using RJ. Allergic reactions from inter-muscular injection were the automatic imbalance symptoms such as malaise, caumesthesia and hypersensitive responsibility. To more severe reactions including mild gastrointestinal upset, asthma, anaphylaxis, intestinal bleeding, and even death when RJ was ingested (Thien *et al.*, 1996; Leung *et al.*, 1997 and Yonei *et al.*, 1997).

In addition. RJ exhibited antibiotic activity against a variety of microorganisms. The effective protein, royalisin, was the first RJ protein of *A. mellifera* which the complete amino acid sequence was characterized. The primary structure is composed of 51 amino acid residues containing three intramolecular disulfide bonds with 5.5 kDa of calculated molecular weight which found to have this activity against fungi and Gram-positive bacteria such as *Lactobacillus spp.*, *Bifidobacterium spp.* and *Leuconostoc spp.* at a low concentration but not Gram-negative bacteria (Fujiwara *et al.*, 1990, Bilikova *et al.*, 2001). In the freeze-dried form of crude RJ, it showed the growth inhibiting of *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* at the minimum inhibition concentration (MIC) of 20, 31 and 71 mg/ml, respectively (Sanguandeekul and Nimachaikool, 1993). Moreover, the jelleines are an antimicrobial family of peptides against Gram-positive, Gram-negative bacteria and yeasts (Fontana *et al.*, 2004). Recently, the protein fractions of royal jelly were confirmed to possess a high antioxidative activity and scavenging ability against reactive oxygen species (Nagai and Inoue, 2004).

1.3.1. Hypopharyngeal Gland Secretions

Hypopharyngeal, or food, glands are pains acinous glands (secretory glands) each of which are composed of about a dozen of secretary cells. Protein rich-

substances that are component of RJ were synthesized from these glands (Brouwers, 1982). Furthermore, hypopharyngeal glands of nurse bees were found to be the major synthesizing and secretory organ of RJ (Hanes and Simuth, 1992). The ultrastructural changes of hypopharyngeal gland in different development bees were analyzed. The number of rough endoplasmic reticulum (RER) in hypopharyngeal cells increased within a few days after bee emerged, reached to maximum number during the nursing phase and decreased in foragers (Kattz and Knecht, 1990). In other words, developments of hypopharyngeal glands in nurse bees are better than in forager bees (Kubo *et al.*, 1996). A nature of brood signal involved and activated protein synthesis in these glands of the nurse bees (Huang *et al.*, 1989). The secreting activity of HYPOPHARYNGEAL GLANDof newly emerged bees was studied under different social conditions such as singly isolated, caged bees with 6-day-old companion bees or living with their parents and the results showed that HYPOPHARYNGEAL GLANDof newly emerged bees lived with their parents were significantly better developed and had higher protein content than those in other conditions (Naiem *et al.*, 1999).

Carbohydrate-metabolizing enzymes including α-glucosidase, amylase and glucose oxidase, considerable in process nectar into honey are produced in hypopharyngeal glands and associated with an age-dependent role change of the worker result in mRNAs of these enzymes were only detected in the HYPOPHARYNGEAL GLANDof the forager bee, but not in the nurse-bee gland (Kubo *et al.*,1996 and Ohashi *et al.*, 1996). Previously, 70-kDa protein was purified and immunoblotted, this protein was positively detected in hypopharyngeal glands of foragers. Subsequently, the 70-kDa protein was further characterized and identified as

α -glucosidase (Kubo *et al.*, 1996).Molecular cloning and characterization of α -glucosidase cDNA in *A. mellifera* were reported. The deduced amino acid sequences of 650 residues revealed 41.9 % identity with maltase of the mosquito (*Aedes aegypti*) and 42.2 %, 46.3 % and 46.2 % of maltase 1, 2 and 3 of the fruit fly (*Drosophilla melonogaster*), respectively (Ohashi *et al*, 1996).

In addition, genes encoding α -amylase and glucose oxidase in *A. mellifera* were cloned and characterized. The deduced amino acid sequence showed 60.5 % identity with *D. melanogaster* α-amylase and 23.8 % identity with *Aspergillus niger* glucose oxidase. Recently, proteins having molecular masses of 57 and 85 kDa were purified from hypopharyngeal glands of *A. mellifera* foragers and determined by SDSPAGE These proteins showed amylase and glucose oxidase activity, respectively (Ohashi *et al*, 1999).

1.3.2. Composition of Royal Jelly

Chemical compositions of RJ from *A. mellifera* have been widely analyzed and reported based on different analytical procedures, sample collection and preservation methods. In commercial, fresh RJ is usually sampled from cells containing 72 hours old queen-designate larvae and is mainly composed of water (60-70%), proteins (12-15%), carbohydrates (10-12%), lipids (3-7%), and traces of minerals salts and vitamins (Howe *et al.*, 1985 ; Simuth *et al.*, 2004; Koya-Miyata *et al.*, 2004; Nagai *et al.*, 2004).

According to Association of Official Analytical Chemists (AOAC), organic nitrogen content of RJ was determined by the micro-Kjeldahl method while the moisture by automatic volatility or freeze drying or heating drying (Howe *et al.*, 1985,

Karaali *et al.*, 1988 and Plama, 1992; Table 1.1). The range of moisture and protein content was 65.32-69.4 % and 11.6 % to 16.7 %, respectively. For investigation of amino acids composition in RJ protein, the rich essential amino acids were found range 39.3 % - 51.4 % which aspartic acid and glutamic acid were the major amino acids of RJ and accounted for 16.1 % and 10.19 % of the RJ protein content, respectively (Howe *et al*, 1985, Table 1.4)

From either a modified Folch extraction or a Soxthlet method, the analyzed of lipid content in average was 4.76% which highest 8.2% in *A. mellifera* from Turkey (Karaali *et al.*, 1988; Table 1.1) followed from the United state (3.2%-5.6%; Howe *et al.*, 1985) and Brazil (2.9%-3.9%; Plama, 1992). The free fatty acids in lipids fraction with unusual and uncommon structures, whether saturated or unsaturated and linear or branched, which mostly short chain (8 to 10 cabon atoms) hydroxyl fatty acids or dicarboxylic acids, in contrast to the fatty acids with 14 to 20 carbon atoms which are commonly found in animal and plant material (Lercker *et al.*, 1981, 1982). These fatty acids are responsible for most of the recorded biological properties of royal jelly (Schmidt and Buchmann, 1992). The major fatty acids in RJ is 10-hydroxy-2-decanoic acid (10-HDA) at an average concentration of 50.3 % of the total fatty acids content (Howe *et al.*, 1985), followed by its saturated equivalent, l0-hydroxydecanoic acid. In criteria for RJ quality, 10-HAD content has been proposed as a freshness parameter if greater than 1.8%, this RJ is considered to be fresh and authentic. Under the controlled storage temperature at -18 °C and 4 °C, the rate of 10-HAD was loose 0.1% and 0.2% per year, respectively (Antinelli *et al.*, 2003)

As in Table 1.1, the total sugar content in *A. mellifera* RJ was 10.45%-11.5%. In many cases fructose and glucose together account for 90 % of the total sugars

which fructose was frequently in all RJ samples (Palma, 1992).Recently, compositions of fresh RJ from *A. cerana indica* and *A. cerana japonica* were also examined compared to that of *A. mellifera*. The chemical composition of RJ produced by the species is show in the Table 1.2. Crude protein, carbohydrate and acidity in Fresh RJ of *A. cerana indica* from Thailand were higher than that of *A. cerana japonica* from Japan. Specifically, carbohydrates content was approximately 2.5 times significant higher (Takenaka and Takenaka, 1996; Trongnipatt, 2002).

1.3.3. Major Royal Jelly Proteins (MRJPs) of *A. mellifera*

The crude proteins of RJ consist in water-insoluble protein fractions and water-soluble proteins (WSPs) which contained 46-89% of the total proteins in RJ (Takenaka and Echigo, 1983). The WSPs fraction of RJ contains several major proteins that belong to one protein family designated MRJPs (from major royal jell proteins). These MRJPs are closely related to each other and share sequence homologies with *yellow* proteins of *Drosophila*. All of the MRJPs, yellow proteins of *Drosophila* and other insects, together with several bacterial proteins compose with a protein family termed MRJP (Albertova *et al.*, 2005). MRJPs of *A. mellifera* RJ (AmMRJPs) account for 82-90% of total larval jelly protein and they contain a relatively high amount of essential amino acid as in Table 1.3. In addition, AmMRJPs have been extensively studied, which focuses on characterization of both proteins and cDNAs.

For AmMRJPs protein characterization, AmMRJPs were purified by a DEAE cellulose column chromatography. Three peaks of proteins were eluted out and further purified by rechromatography on DEAE cellulose column and then characterized by

Table 1.1 Compositions of fresh RJ of *A. mellifera*.

Sample : Location (Reference) / Composition	*A. mellifera* : U.S.A (Howe *et al.*,1985)		*A. mellifera* : Turkey (Karaali *et al.*, 1988)		*A. mellifera* : Brazil (Plama, 1992)	
	Method	Content	Method	Content	Method	Content
Moisture (%)	Automatic volatility computer, AVE™-MP	66.3 – 67.7	Freeze drying	65.32	Heating at 65 °C	67.8 – 69.4
Crude protein (%)	Total N , Micro-kjeldahl method	11.6 – 12.2	Total N , micro-Kjeldahl method	13.6	Total N , Micro-kjeldahl method	15.8 – 16.7
Lipid (%)	Modified Folch extraction	3.2 – 5.6	Soxthlet method	8.2	Soxthlet method	2.9 – 3.9
Total Sugar (%)	ND	ND	Norris and Ribbons, 1977	10.4	Phenol-sulfuric acid method	11.4 – 11.5
Acidity (pH value)	ND	ND	Titration with NaOH	3.65	Titration with NaOH	3.65

ND = not determined

Table 1.2 Compositions of fresh RJ of *A. cerana indica* and *A. cerana japonica*.

Sample : Location (Reference) / Composition	*A. cerana* : Northern (N) and southern (S) in Thailand (Trongnipatt, 2002)			*A. cerana japonica* : Japan (Takenaka and Takenaka 1996)	
	Method	Content		Method	Content
		N	S		
Moisture (%)	Heating at 70 °C	48.8	49.6	Heating at 105 °C	65.3
Crude protein (%)	Total N , Kjeldahl method	20.1	22.6	Total N , micro-Kjeldahl method	16.4
Lipid (%)	Soxthlet method	4.6	6.9	Soxthlet method	7.4
Ash (%)	Heating at 550 °C until weight constant	1.8	1.7	Heating at 550 °C for 3 hours	1.5
Acidity (ml/100 g RJ)	Titration with NaOH	47.1	45.0	Titration with NaOH	39.3
Carbohydrate (%)	ND	ND	ND	Subtracting the foregoing amounts from the total amount	9.4

SDS-PAGE. Two peak proteins showed a single band with the molecular mass of 49 and 55 kDa, whereas another showed two separate bands with the molecular mass of 55 and 60 kDa. This chromatographic purification did not recover all proteins compared to crude RJ protein. To classify families of AmMRJPs, crude RJ protein was separated by SDS-PAGE, electroblotted onto PVDF membrane and N-terminal amino acid sequenced. Four families (AmMRJP1, AmMRJP2, AmMRJP3 and AmMRJP5) were identified from the N-terminal amino acid sequences (Schmitzova *et al.*, 1998), whereas AmMRJP4 protein has been identified in royal jelly of *A. mellifera* by using two-dimensional gel electrophoresis and the N-terminal amino acid sequencing (Sano *et al.*, 2004).

To isolated cDNA clones coding for RJ protein, cDNA library was prepared by the Uni-ZAP XR expression from the head of 8-day-old nurse honeybees (*A. mellifera*) which was done in parallel with electrophoretic analysed and N-terminal sequencing of RJ proteins. AmMRJPs were immunologically screened with polyclonal anti-MRJPs raised in mice. Two selected clones, pRJP57-1 and pRJP57-2 were characterized by nucleotide sequencing and designated AmMRJP3 and AmMRJP4 respectively (Klaudiny *et al.*, 1994a). Two additional clones, pRJP120 and pRJP95, were subsequently identified as AmMRJP1 and AmMRJP2, respectively (Ohashi *et al.*, 1997; Schmitzova *et al.*, 1998). Subsequently, AmMRJP5 was found using the same procedure (Albert *et al.*, 1999a).

Recently, three new cDNA members of AmMRJPs were identified as AmMRJP6, AmMRJP7, and AmMRJP8. These nucleotide sequences were assembled by using the honeybee brain expressed sequence tags (EST) sequence database, honeybee genomic sequence data and nucleotide sequence from amplification product

of the nurse honeybee head cDNA library (Albert and Klaudiny, 2004). A summary for molecular characterization of cDNA and deduced amino acid sequences of the AmMRJPs is illustrated in Table 1.4

AmMRJP1

AmMRJP1, the protein of the dominant band that identical to the protein derived from RJP120 cDNA (Schmitzova *et al.*, 1998), possesses the N-terminal amino acid sequence of "NILRGESLNKS". This protein is the most abundant of RJ protein (31% of the total protein) which shows high amount of the 10 essential amino acids composition of 48%. Molecular weights were rather small about 55 kDa - 57 kDa on SDS-PAGE which may reflect some modification of the proteins during transport, or storage in, the honeybee mouth cavity. AmMRJP1 was classified to acidic protein according to Hanes and Simuth (1992); the studied informed that at least eight isoelectrophoretic variants ranging from 4.5-5.0 were found.

For indicating that native 56-kDa glycoprotein (AmMRJP1), purified 56 kDa protein is treated with N-glycosidase F. The molecular weight of the resulting digestion product is 47 kDa, which is closed to that of the putative protein lacking the signal sequence (46.8 kDa) (Ohashi *et al.*, 1997, and Schmitzova *et al.*, 1998).

AmMRJP1 was reported to have three different forms; 55 kDa of a monomer, and approximately 420 kDa oligomer, and water insoluble aggregates resulted from interaction with fatty acids (Simuth, 2001). In the royal jelly, AmMRJP1 is able to strongly bind with a small peptide named Apisimin (Bilikova *et al.*, 2002) and possibly with other compounds in a large complex of 420 kDa. The oligomeric form of AmMRJP1 is water-soluble (Kimura *et al.*, 1996; Simuth, 2001).

Table 1.3 Amino acid composition of *A. mellifera* MRJPs.

	MRJP1	MRJP2	MRJP3	MRJP4	MRJP5	MRJP6*	MRJP7*	MRJP8*
Ala	3.9	6.2	4.9	4.3	3.8	5.8	4.3	4.6
Arg	**3.4**	**3.8**	**4.9**	**4.1**	**9.0**	**3.1**	**3.8**	**3.6**
Asn	6.9	11.3	15.9	13.8	8.7	11.0	9.5	9.1
Asp	8.6	7.1	7.5	7.5	12.0	6.5	8.1	5.5
Cys	2.5	1.5	1.1	1.3	1.0	1.2	1.4	1.7
Gln	3.9	5.1	7.1	6.3	3.8	5.3	5.0	4.3
Glu	3.9	3.8	3.8	3.9	2.5	4.1	4.3	3.6
Gly	5.6	6.0	6.4	4.1	4.0	5.0	5.2	6.2
His	**2.3**	**2.4**	**2.2**	**3.9**	**1.8**	**2.6**	**1.4**	**1.2**
Ile	**6.0**	**5.1**	**4.0**	**3.2**	**4.8**	**7.4**	**7.5**	**7.7**
Leu	**9.5**	**8.2**	**6.8**	**9.7**	**5.2**	**7.9**	**8.6**	**10.8**
Lys	**5.1**	**6.9**	**5.8**	**5.0**	**4.3**	**6.0**	**5.2**	**4.8**
Met	**3.5**	**2.4**	**2.2**	**2.4**	**11.4**	**3.6**	**2.7**	**1.4**
Phe	**4.2**	**4.4**	**1.7**	**2.2**	**2.6**	**3.8**	**4.1**	**4.3**
Pro	3.7	3.1	2.5	2.2	2.6	2.9	2.7	2.6
Ser	8.1	5.8	5.9	8.4	6.2	8.2	6.8	7.7
Thr	**6.3**	**4.6**	**4.0**	**4.7**	**5.6**	**3.4**	**6.6**	**7.0**
Trp	**1.2**	**1.3**	**0.9**	**1.3**	**1.1**	**1.4**	**1.6**	**2.0**
Tyr	4.4	3.5	3.1	3.9	3.3	5.0	4.5	4.8
Val	**6.5**	**7.5**	**6.8**	**8.0**	**5.6**	**5.8**	**7.0**	**6.6**
Ess. aa.	48 %	47 %	39.3 %	44.5 %	51.4 %	45 %	48.5%	49.4%

Percent content of amino acid in native protein was obtained by computer analysis of its sequence (Schmitzova *et al.*, 1998).

Essential amino acids are marked in boldface.

* Amino acid composition of AmMRJP6 was obtained by computer analysis employing the program ProtParam (Albert and Klaudiny, 2004).

Table 1.4 Molecular characterization of cDNAs and deduced amino acid sequences of AmMRJPs.

Family	DNA insert size* (bp)	Deduced amino acid (residues)	No. of N-glycosylation site**	Amino acid residues without signal peptide**	Molecular weight (kDa)**	Reference
AmMRJP1	1444	432	3	416	46.8	Schmitzova *et al.* (1998)
AmMRJP2	1579	452	2	435	48.9	Schmitzova *et al.* (1998)
AmMRJP3	1719	467	1	528	59.5	Klaudiny *et al.* (1994)
AmMRJP4	1625	464	8	449	50.9	Klaudiny *et al.* (1994)
AmMRJP5	1966	598	4	581	68.0	Albert *et al.* (1999a)
AmMRJP6	1529	437	5	417	47.6	Albert *et al.* (2004)
AmMRJP7	1427	443	4	426	48.7	Albert *et al.* (2004)
AmMRJP8	1329	416	8	400	45.1	Albert *et al.* (2004)

* including polyA tail/ **Partial of data obtained from Schmitzova *et al.* (1998)

The cDNA encoding AmMRJP1 was cloned into pQE32 vector without signal peptide sequence for express in *E. coli* system. The recombinant protein was expressed and purified. Purified recombinant protein was characterized by SDS-PAGE and the molecular weight was 47 kDa as compared with 55 kDa (glycoprotein) in native AmMRJPs (Judova *et al.*, 1998). The sequence of AmMRJP1 cDNA was determined by three independent research teams and showed 100% identical with each other (Kucharski *et al.*, 1998; Ohashi *et al.*, 1997; Schmitzova *et al.*, 1998) and further proof that the AmMRJP1 sequence in highly conserved and without a single nucleotide change (Whitfield *et al.*, 2002).

AmMRJP2

The second most abundant of RJ proteins, AmMRJP2, possessed the N-terminal amino acid sequence of "AIVRENSPRNLEK". The relative content of this protein is 16% in total royal jelly protein with 47% of the 10 essential amino acid compositions (Schmitzova *et al*, 1998). The apparent molecular weight of native AmMRJP2 protein is 49 kDa that at least eight variants with different isoelectric points of pH 7.5-8.5.

The cDNA encoding AmMRJP2 without signal peptide sequence was cloned and expressed in *E. coli* expression system. The recombinant AmMRJP2 protein was purified and characterized by SDS-PAGE. The result showed the molecular weight of recombinant AmMRJP2 protein to be 49 kDa which was the same as the native AmMRJP2 protein (Schmitzova *et al.*, 1998; Bilikova *et al.*, 1999).

In addition, AmMRJP2 was found that contain repetitive regions (Albert *et al.*, 1999b) which appearing a similar pentapeptide repeat of in the C-terminal region.

AmMRJP3

The N-terminal amino acid sequence of AmMRJP3 protein is AAVNHQ (R/K) KSANNLAHS and exhibits a size polymorphism. The apparent molecular masses were between 60 and 70 kDa revealed by SDS. AmMRJP3 is approximately 26% of total royal jelly protein with lowest essential amino acid content is 39.3% (Schmitzova *et al.*, 1998) and deduced amino acid contains a repetitive region at the C-terminal part, repetitive motifs of XQNXX, typically with 20 repeated units (Klaudiny *et al.*, 1994a).

To detect the polymorphism of the AmMRJP3 repetitive region, the PCR analysis of genomic DNA result showed highly polymorphic of AmMRJP3 repetitive region with as many as five alleles found in 10 individuals from the same colony and similar to AmMRJP2 which appeared a pentapeptide repeat in the C-terminal region (Albert *et al*, 1999b). In other species, the study of repetitive sequence motifs in Gaint bee, *A. dorsata* found that repetitive sequence also existed in AmMRJP3 gene liked those in *A. mellifera* (Albert *et al.*, 2002).The AmMRJP3 protein was reported to have two different forms; a monomer (70 kDa) and trimer (210 kDa) (Okamoto *et al.*, 2003).

AmMRJP4

The N-terminal amino acid AmMRJP4 was characterized by using two-dimensional gel electrophoresis (Sano *et al.*, 2004) and possessed the "GVVRENSSRK" and "AVVRENSSRK" *N*-terminus for European and Africanized honeybee, respectively. Therefore, the N-terminal amino acid sequences of the 2 species were different just one amino acid residue.

Only a clone (RJP57-2) containing cDNA encoding AmMRJPs from the head cDNA library of nurse bees was characterized and designated AmMRJP4 which showed the low expression level (2% of total mRNA using Northern blot hybridization analysis). The deduced amino acid of AmMRJP4 contains 44.5% essential amino acid content that was lower in overall essential amino acid content, but possesses high amount of amino acid Leu (9.7%) and Val (8%). The calculated isoelectric focusing point of AmMRJP4 is 6.2 (Klaudiny *et al.*, 1994a, Schmitzova *et al*, 1998).

The molecular weight and isoelectric focusing point of AmMRJP4 of Africanized and European honeybee RJ were compared and showed in average was 60 kDa and 5-6, respectively (Sano *et al.*, 2004).

AmMRJP5

Although, AmMRJP5 exhibits two different molecular weights (77 kDa and 87 kDa) on SDS-PAGE, They possess an identical N-terminal amino acid sequence of "VTV (R/N) E (N/Q) SPR". The relative content of AmMRJP5 is 9% of total royal jelly protein with containing 51.4% essential amino acid, dominant in Arg (9%) and Met (11.4%) (Schmitzova *et al.*, 1998).

From AmMRJP5 cDNA, deduced amino acid shows the extensive repeat region located between 367 th and 540 th amino acid residues. The consensus sequence (GATAGAATG) which encodes for tripeptide as DRM: aspartic acid (D), arginine (R) and methionine (M) occurred 58 times and interrupted a conserved region at the C-terminal of this protein and invariant in repetitive unit size (Albert *et al.*, 1999a). In addition, the totally different tripeptide repetitive motif of AmMRJP5 appeared at a different position in AmMRJP3 (Albert *et al.*, 1999b).

The AmMRJP5 repetitive region was characterized in *A. dorsata*. The repetitive region was located at the same position as found in *A. mellifera* but smaller in size, and occurred 23 times compared with 58 times in *A. mellifera* (Albert *et al.*, 2002). From two-dimensional gel electrophoresis, the AmMRJP5 proteins were found in both the Africanized and the European honeybee RJ. The AmMRJP5 protein from this 2 species posses the identical N-terminal amino acid sequence (VTVRENSPRK), however, molecular weight and pI value were different (Sano *et al.*, 2004).

AmMRJP6-8

Three new cDNA members of the AmMRJPs family (AmMRJP6, AmMRJP7, and AmMRJP8) were identified (Albert and Klaudiny, 2004). They were assembled using the honeybee brain expressed sequence tags (EST) sequence database, honeybee genomic sequence database and nucleotide sequence from amplification product of the nurse honeybee head cDNA library (Klaudiny *et al.*, 1994a).

AmMRJP6 cDNA sequence is highly homologous to AmMRJP5, but does not have repeat sequence encoding the tripeptide motif (DRM). The 5′ non-coding region of AmMRJP6 cDNA sequence contains a 3′ part of intron0 with the conserved AG motif, intron0 is found in AmMRJP1 genomic sequence (Malecova *et al.*, 2003).

AmMRJP7 cDNA sequence was assembled from only the honeybee brain EST sequence database. The deduced amino acid sequence of AmMRJP7 shows high homology to AmMRJP2 protein which 73% identity in 433 amino acid overlap.

AmMRJP8 cDNA sequence was found only one clone in the honeybee brain expressed sequence tags (EST) sequence database. The complete AmMRJP8 cDNA sequence was identified by using genomic sequence database and nucleotide sequence from amplification product of the nurse honeybee head cDNA library.

1.4. Expression and Biological Activities of AmMRJPs Genes

Although AmMRJPs are structurally closely related to each other, the expression profiles of AmMRJPs genes are not identical. The mRNA of AmMRJP1 is present in higher amounts in the HYPOPHARYNGEAL GLANDof both nurses and foragers, while AmMRJP2 - 4 are synthesized predominantly in the hypopharyngeal gland of nurses (Klaudiny *et al.*, 1994b; Kubo *et al.*, 1996; Ohashi *et al.*, 1997).

The AmMRJP1 mRNA was found to be differentially expressed in the heads of early emerged honeybees (Kucharski *et al.*, 1998), nurse and also forager honeybees (Klaudiny *et al.*, 1994b). Its expression was localized to hypopharyngeal glands (Ohashi *et al.*, 1997), and also to a subset of Kenyon cells (intrinsic neurons) of mushroom bodies-presumed centers of learning and memory in the honeybee brain (Kucharski *et al.*, 1998). Therefore, it would seem that MRJP1 not only functions as a component of larval food but also plays a role in the honeybee brain

Immunoblotting analysis could only detect AmMRJP2 mRNA in hypopharyngeal gland of nurse bee but not in forager bee (Kubo *et al.*, 1996), whereas microarrays and northern blot hybridization analysis (Kucharski and Malezka, 2002) was found the expression of AmMRJP2 mRNA in heads of experienced foragers. MRJP3 mRNA and protein are expressed specifically in hypopharyngeal gland of nurse honeybees (Kubo *et al.*, 1996, Ohashi *et al.*, 1997). The expression level of AmMRJP4 mRNA in hypopharyngeal gland is very low compared to the expression of the other AmMRJPs (Klaudiny *et al.*, 1994b; Kubo *et al.*, 1996; Ohashi *et al.*, 1997).

Due to their high relative content of essential amino acid in the structure of some MRJPs ; such as MRJP1, MRJP4 and MRJP5; and the presence of the extensive

repetitive regions consisting of high amounts of nitrogen-rich amino acids in several MRJPs (MRJP2, MRJP3 and MRJP5) indicates that MRJPs are nutritive components of RJ serving as a supply of essential amino acid and a storage of biologically accessible nitrogen (Albert *et al.*, 1999b). Besides AmMRJP3, two other AmMRJPs (MRJP2 and MRJP5) contain repetitive regions. All repetitive regions in AmMRJPs contain high amounts of nitrogen-rich amino acids such as asparagines and glutamine. Their presence significantly increases the nitrogen content of the MRJP protein. Therefore, the repetitive regions may be domains storing nitrogen in biologically processable form. Albertova *et al.* (2005) reported additionally that the presence of an extensive repeat region of MRJP3 in four traditional honeybee species (*A. mellifera, A. cerana, A. dorsata, and A. florea*) showed size and sequence polymorphisms in all species and correlation between repeat length and nitrogen content; an essential component of biogenic polymers. In another word, the repeat occurred due to a selection for an increase in nitrogen storage for a more efficient nutrition of queens and larvae.

Moreover, biological activities of AmMRJPs have been reported in various systems. MRJP1 which showed diverse biological activities in several heterologous system was not only informed to enhanced cell proliferation of rat hepatocytes (Kamakura *et al.*, 2001a) but also stimulate the growth of human lymphocytes in a serum-free medium (Watanabe *et al.*, 1996), and showed an anti- exhaustion effect in mice (Kamakura *et al.*, 2001b). Recently, jelleines and antimicrobial peptides was indentified in RJ as tryptic digests of the C terminus of MRJP1 (Fontana *et al.*, 2004), suggest that one of the physiological functions of MRJP1 might be to serve as a precursor of jelleines, protecting the RJ against bacterial infections. Furthermore,

MRJP3 exhibited potent immunoregulatory effects *in vitro* and *in vivo* (Okamoto *et al.*, 2003).

1.5. Characterization and Regulatory Regions Analysis of AmMRJPs Genes

The recently published sequence of the AmMRJP1 genomic locus contains seven substitutions, which do not change the amino acid sequence of the encoded protein and show the genomic locus contains 6 exons (Malecove *et al.*, 2003). It has been reported that MRJPs gene families (AmMRJP1-5) were a single-copy gene per haploid honeybee genome when examined by Southern blot analysis(Malecove *et al.*, 2003). Furthermore, the genomic structure of the gene coding for AmMRJP1 and putative promoter regions of AmMRJP1-5 were studied. The AmMRJP1 gene sequence spans over 3038 bp and contains 6 exons separated by 5 introns. The nucleotide sequences flanking the 5′ ends of AmMRJP2-AmMRJP5 genes were obtained by using inverse polymerase chain reaction. From computer analysis, putative promoters were predicted upstream of all AmMRJPs genes and contained the TATA motif (TATATATT), highly conserved both in sequence and position across AmMRJPs gene families. Two ultraspiracle transcriptional factor (USP-TF) binding sites in AmMRJP1 gene was reported, but just only one in AmMRJP2-5 gene immediately downstream following predicted TATA box. The predicted CAAT regulatory box (CAAT) is located between 69-65 nt downstream from the transcription starting point in AmMRJP1, differ to AmMRJP2-5 gene with absent. USP-TF is a member of the ligand-modulated transcription factors that regulate cell homeostasis, reproduction, differentiation and development. In *Drosophila melanogaster,* the juvenile hormones were activated when USP-TF specifically bonded (Malecova *et al.*, 2003).

1.6. Characterization of MRJPs in *A. cerana*

In contrast to *A. mellifera*, the study of MRJPs in *A. cerana* (hereafter called AcMRJPs) is rather limited. Previously, Takenaka and Takenaka (1996) reported the comparing of chemical composition between *A. cerana* royal jelly and *A. mellifera* royal jelly. The different of proteins level, 10-hydroxydecenoic acid level and glucose/fructose ratio in RJ of both species were showed. Analysis of water soluble proteins in RJ by electrophoresis revealed 21 protein bands in each species where 14 protein bands were shared between the royal jelly of these bees. Four (bands 6, 7, 12 and 16) of six major bands (bands 4, 6, 7, 12, 16 and 21) in the royal jelly of *A. cerana* were more heavily stained than those of *A. mellifera*. In addition, two protein bands (no.10 and 11 with the range of 42.7-66.2 kDa in size) were major and specific to *A. mellifera* royal jelly.

Recently, MRJPs have been first characterized in *A. cerana* for both the expression level and the protein level. The expressed sequence tag (EST) library of *A. cerana* hypopharyngeal glands were constructed (Srisuparbh *et al.*, 2003). From Sixty-six recombinant clones that had insert sizes greater than 500 bp, forty two of these (63.6%) were identified as cDNA homologues of AcMRJP1 (50%), AcMRJP2 (6.06%), AcMRJP3 (6.06%) and AcMRJP4 (1.52%). The MRJPs 1, 2 and 3 of *A. cerana* showed high expression level, whereas AcMRJP4 was found only one clone that showed low expression level whereas the AcMRJP5 was not found in these 66 recombinant clones. Moreover, a cDNA library from 8-day-old worker heads of *A. cerana cerana* was constructed (Songkun *et al.*, 2005). One hundred and twenty positive clones were identified and characterized. Thirty one clones were homologous with AmMRJPs cDNA. The most abundant MRJPs cDNA homologue, similar as

above, was MRJP1 (11 clones).The next was MRJP3 (10 clones) and MRJP2 (7 clones), respectively. In differently, MRJP4 cDNA was not found, while MRJP5 cDNA was discovered 3 clones.

According to characterization of sequences encoding AcMRJPs, the open-reading frame (ORF) of the AcMRJP1 cDNA was 1299 nucleotides (Srisuparbh *et al.*, 2003) while Songkun *et al.* (2005) informed MRJP1 of *A. cerana cerana* (AccMRJP1) was 1445 nucleotides (poly [A] tail not included) which similar encoding a protein of 433 deduced amino acids with three predicted N-linked glycosylation site. The complete nucleotide sequence of AcMRJP2 cDNA is composed of 1392 bp that encodes for 463 amino acid residue with two predicted N-linked glycosylation site (Imjongjiruk *et al.*, 2005) whereas Songkun *et al.*, 2005 reported ORF of 1404 nucleotides encoding a protein of 468 amino acids with the same two predicted N-linked glycosylation site. The AcMRJP3 cDNA was identified by RT-PCR of hypopharyngeal gland (Srisuparbh, 2002). The AcMRJP3 ORF was composed of 1824 bp that encoded for 608 amino acid residues with five predicted N-linked glycosylation site. In another studies, AccMRJP3 cDNA was 1977 bp long contained an ORF (nucleotides 46-1824), which encoded 593 amino acid residues, and AccMRJP5 comprised 1970 nucleotides contained an ORF encoding a protein of 598 amino acid residues (Songkun *et al.*, 2005). AcMRJP4, AcMRJP5 and AcMRJP6 were isolated from hypopharyngeal gland of *A. cerana* nurse bee by RT-PCR (Cenphakdee, 2003). The RT-PCR products were cloned and nucleotide sequencing. The complete nucleotide sequence of AcMRJP4, AcMRJP5 and AcMRJP6 cDNA were identified with 1608, 1881 and 1450 bp and encoding for 485, 579 and 435 amino acids, respectively.

The AcMRJPs from the RJ were purified and characterized using Q-sepharose and Sephadex G-200 column chromatography (Srisuparbh *et al.*, 2003). The N-terminal and internal peptide sequencing were used to identify the purified proteins. The molecular weights of denatured proteins were determined by SDS-PAGE. Three types of AcMRJPs (AcMRJP1, AcMRJP2 and AcMRJP3) that homologue to AmMRJPs were found in *A. cerana* RJ. The AcMRJP1 was reported to have two different forms, 50kDa for a monomer and 300kDa for oligomer with isoelectric points of 5.2-5.7 and 5.7, respectively. The molecular weight of AcMRJP2 was 55 kDa with 7.0-8.0 of isoelectric points. The native form of AcMRJP3 had the molecular weight of 115 kDa, whereas denatured form was 80 kDa. The AcMRJP3 isoelectric point was 8.3. Additionally, the total protein quantities of AcMRJP1 (monomer) : AcMRJP1 (oligomer) : AcMRJP2 : AcMRJP3 (dimer) was 1 : 12.21 : 4.72 : 2.52.

Additionally, AcMRJP2, AcMRJP3, and AcMRJP5 were exhibited a repeat length polymorphism (Srisuparbh *et al.*, 2003, Songkun *et al.*, 2005). From tested polymorphism of AcMRJP2 and AcMRJP5 repeat regions by PCR with genomic DNAs of individual honeybees, the distribution of polymorphic alleles seems to be phenotypically neutral as there was no repeat length bias found in the population. Thus, the family of MRJPs gene alone provides three midisatellite polymorphic loci of VNTR (variable number of tandem repeat) type, which were suitable for genotyping *A. cerana* individuals (Songkun *et al.*, 2005)

Moreover, the complete gene sequences and genomic structure of both AcMRJP1 and AcMRJP2 were determined (Imjongjiruk *et al.*, 2005). Both of genes contained 6 exons and 5 introns, where all boundaries conformed to GT/AG rule. The

putative TATA boxes of AcMRJP1 gene and AcMRJP2 gene were found at -31 and -32 nt upstream from the transcription initiation sites, respectively. As identical position to AmMRJP1, the putative CAAT box was also found in AcMRJP1 gene whereas the consensus sequence was not found in AcMRJP2 gene. Both of them contained a single USP-TF binding site at the 5′ UTR immediately downstream from the predicted TATA box.

Furthermore from EST library as in Srisuparbh *et al.* (2003), AcApisimin, a serine-valine-rich peptide, was identified with ORF 234 bp in length, encoding 78 amino acid residues, as in *A. mellifera.* The deduced amino acid sequence possessed K-T-S-I-S-I-K, which was nearly identical to K-T-S-I-S-V-K that was found from the *N*-terminal sequencing of natural Apisimin that was purified from royal jelly of *A. mellifera*. Like *A. mellifera* Apisimin (AmApisimin), Cys, Met, Pro, Arg, His, Tyr, and Trp residues were not found in that of *A. cerana*. The sequence showed a 92.7% and 94.9% similarity with the nucleotide and amino acid sequences of AmApisimin, respectively. Recently from two-dimensional gel electrophoresis, Sontos *et al.* (2005) demonstrated the protein complement of the secretion from hypopharyngeal gland of nurse-bees (*A. mellifera* L.) was constituted of AmApisimin that identified with presenting a MW value higher than the expected value from the honeybee genome suggesting that it may be produced in a precursor form. The author informed this protein may be a regulator of AmMRJP1 oligomerization which referred to Biliková *et al.* (2002) who suggested this acidic peptide might play important physiological roles in honeybee colonies because of the relative high expression level throughout the whole life span.

1.7. Quantification of mRNA Level

1.7.1. Quantitative RT-PCR Using Internal Standard

For RNA quantitation, Northern blots are widely used. However, this technique is impractical because of their limited sensitivity which requires at least 10 mg of total RNA for semi-quantitation while RT-PCR requires is useful when only small amounts of tissue are available (Chung, 2001). Quantitation using RT-PCR is an greatly sensitive method of mRNA analysis, however, the method is not simple. Primarily reason, there are two sequential enzymatic steps involved: the synthesis of cDNA from the RNA template and PCR amplification of that cDNA. Generally, the exponential nature of PCR and the practical aspects of performing PCR pose the most serious obstacles for quantity information. Due to exponential PCR reaction, small variations in efficiency amplification (E) of each cDNA can yield large changes in the amount of PCR products. Furthermore, later cycles of PCR exhibit the plateau effect, in which the rate of amplification slows and eventually level off. However, RT-PCR can yield accurate quantitative results with some adaptations.

Reverse transcription (RT)-PCR has been shown to be several orders of magnitude more sensitive than traditional techniques (Wang *et al.*, 1989), and it is one of the most widely used approaches for the quantification of mRNA. Most commonly, the internal standards was used to control tube-to-tube variations in amplification efficiency. Two types of internal standards can be applied: an endogenous sequence or gene transcript that is normally present in the sample or an exogenous fragment

added to the amplification reaction. Each type of internal control has advantages and limitations.

1.7.1.1.Endogenous Internal Standard

An endogenous sequence was known to be present at constant levels throughout a series of samples to be compared that can be used as an internal standard in quantitative PCR reaction. Typically, house-keeping genes or genes that are structurally or functionally related to target mRNA have been used to determined relative levels of specific mRNAs (Horikoshi *et al.,* 1992 and Kinoshita *et al.*, 1992). Furthermore, ribosomal RNA was used as an endogenous internal standard for quantitation of mRNAs (Khan *et al.,* 1992). A disadvantage of using an endogenous internal standard is the need for a second pair of primers for co-amplification of the endogenous internal standard gene. Under conditions used for amplification of the target gene, another pair of primers may amplify with different efficiency. In addition, preliminary experiment must be tested to ensure that the endogenous control mRNA does not change during analysis due to expression of many housekeeping genes may change as a result of the experimental treatment. Unfortunately, some housekeeping genes are expressed in a strictly constitutive manner such as β-actin (Elder *et al.,* 1988). Moreover, the data must be collected before the amplification reaction reaches the plateau phase especially when the relative levels of expression of the standard and target sequences differ greatly. Murphy *et al.,* (1990) found that their internal standard mRNA, β_2- microglobulin, entered the plateau phase before the target, *mdr*-1 mRNA, was even detectable. Therefore, optimization of PCR condition such as primer and

MgCl2 concentration, though cycle number must be determined that lead to amplification products of equal intensity.

1.7.1.2. Exogenous Internal Standard

As an alternative approach to quantitative analysis of a target gene by PCR, the competitive synthetic template (competitor) can be added and co-amplified in the same tube with the same pair of primers as used for the target gene amplification. As differences in reaction conditions will equally affect amplification of competitive template as well as target gene, the relative ratio of both amplification products will remain independent of variations in conditions during amplification. Competitor is added in various and known amounts to several PCR mixtures with the constant amount of target gene. Quantification is performed after competitive amplification by distinguishing the two PCR products from each tube by differences in size, hybridization properties, or restriction enzyme sites.

This quantification methods described in the section employ mathematical models base on PCR equation:

$$N = N_0 (1+E)^n \qquad (1)$$

For convenience, Eq. (1) may also be written as:

$$A = A_0 (1+E)^n \qquad (2)$$

The relative initial amounts of a target sequence and the internal standard (the ratio N_0t / N_0s) can be determined from Eq.3

$$N_0t / N_0s = Nt (1 + Es)^n / Ns (1 + Et)^n \qquad (3)$$

When the amplification efficiencies (E) of the target and standard molecules are identical, the equation can be simplified to Eq. 4

$$N_0t / N_0s = Nt / Ns = At/As \quad (4)$$

$$\log N_0t / N_0s = \log Nt / Ns = \log At/As \quad (5)$$

From Eq. 5, if $N_0t = N_0s$, $Nt = Ns$, $At = As$, then:

$$\log N_0t / N_0s = \log Nt / Ns = \log At/As = 0 \quad (6)$$

Where
- N = the number of amplified molecules
- N_0 = the initial number of molecules
- E = the amplification efficiency
- n = the number of amplification cycles
- A = the amount of amplified product
- A_0 = the initial amount of cDNA
- N_0t = the initial number of target molecules
- N_0s = the initial number of standard molecules
- Nt = the number of amplified target molecules
- Ns = the number of amplified standard molecules
- Et = amplification efficiency of the target
- Es = amplification efficiency of the standard
- At = the amount of amplified target product
- As = the amount of amplified standard product

Following PCR, the amplification products are analyzed by gel electrophoresis. The logarithm of the ratio of At/As is graphed as a function of the logarithm of the initial

molar amount of the standard (N_0s). Assuming that N_0t is equal to N_0s added result in amount of amplified target (At) and standard product (As) was the same, therefore, the log of At/As = log of 1 = 0. In other words, X-intercept of this logarithm graph is determined that an identical amount of initial concentration of the target gene.

There is many ways to synthesis of competitor both homologous competitor fragment and heterologous competitor fragment. For homologous competitor fragment, Gilliand *et al.* (1990) used two types of internal standard: a genomic fragment corresponding to the target mRNA sequence, but containing a small intron and a cDNA which is modified to contain a unique restriction site. Unless there is a known small intron in the target gene, the construction of homologous competitors is time-consuming due to site-directed mutagenesis and multiple cloning steps. However, several methods that use simple PCR amplification with composite primers (Diviacco *et al.*, 1992, Celi *et al.*, 1993, Vanden *et al.*, 1993) or artificial linker sequence (Hoegh and Hviid, 2001) have been developed to generate DNA standard. Other than homologous competitor as above, DNA fragments that share the same primer template sequence but contain a completely different intervening sequence; heterologous competitor; can also be used for competitive PCR (Siebert *et al.*, 1993). Advantages and limitations of using homologous and heterologous competitor DNA fragment as internal standards for quantitative PCR was, in summary, homologous competitor fragments have the same amplification efficiency as their corresponding target but can form heteroduplexes that can complicate the measurement of PCR products. On the other hand, heterologous competitor fragments cannot form heteroduplexes, but their amplification efficiencies must be shown to be equal (or vary similar to) that of the target.

An important advantage of competitive PCR is that because the ratio of the target to standard remains constant during the amplification it is not necessary to obtain data before the reaction reaches the plateau phase. Nevertheless, two conditions must be met to use competitive PCR. First, the molar quantity of the competitor DNA must be known. Generally, competitor concentration can be measured by UV spectrophotometry, in case of DNA < 250 ng/ml, comparing with UV-induced fluorescence yield (which emitted by ethidium bromide) of a series of standards was another choice (Sambrook and Russell, 2001). Second was the amplification efficiency of the competitor and target must be identical. This is often true because the competitor and target possess the same primer binding sequences. The other possible problem with the use of competitor fragments that are homologous to the target is that during later stages of PCR, when the concentration of products is high, heteroduplexes can form between the standard and target sequences. However, Henco and Heibey (1990) resolved this problem by temperature-gradient gel electrophoresis.

The goal of quantitative PCR is to find the initial number or relative starting levels of target gene from the final amount of PCR product. There are several methods to measure these products such as incorporation of labeled nucleotides or primers into PCR products , the problem was trace amounts of unincorporated level often remain in the electrophoresis gel as the product bands migrate, resulting in a "trail" be complicated measuring the amount of incorporated label. Other strategies are base on hybridization. The most common of these methods is to probe a Southern blot hybridization of the PCR products using a radioactively labeled probe complementary to the specific amplified sequences. To quantify the amount of probe,

the bolt can either be exposed to X-ray film and the resulting autoradiogram densitometrically scanned, or the PCR product band can be excised from the blot and measured radioactivity using a scintillation counter. Fluorescent labels also can be used instead of radioactivity. Pannetier *et al.* (1993) described that a fluorescently labeled internal primer is annealed to one strand of the PCR product and extended using *Taq* DNA polymerase. Run-off extension products are electrophoresed in an automated DNA sequencer that quantitatively detects the incorporated fluorescent label. Moreover, the EtBr luminescence emitting from PCR products resolved by gel electrophoresis and quantified using CCD imaging system (Nakayama *et al.*, 1992). Recently, the computer integrated system was used for digital acquisition of gel images and for quantification of the band fluorescence intensities. (Alvarez *et al.*, 2000, Pagliarulo *et al.*, 2004, Goriya, *et al.*, 2005)

1.7.2. Real-Time PCR

Another in method for quantification was real-time PCR. In conventional PCR, the amplified product was detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time" (Wong and Medrano, 2005).

This reaction can be broken into four major phases as linear ground phase ;where baseline fluorescence was calculated, early exponential phase; where the value is representative of the starting copy number, log-linear (also known as exponential) phase; where PCR reach its optimal amplification period, and plateau phase.

There are two types of real-time quantification as absolute quantitation and relative quantitation. In absolute quantitation, the standard curve generated from the serial diluted standards of known concentration which produces a linear relationship between Ct and initial amounts of target. This method assumes all standards and target have approximately equal amplification efficiencies. While relative quantitation, changes in target gene expression are measured based on either an external standard or a reference sample. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays.

1.8. Objective of This Thesis

As above-mentioned, RJ that serves as food for young bee larvae and the adult queen bees contains MRJPs which synthesized from hypopharyngeal glands of nurse bees. If MRJPs plays a biological role only as nutrient, the hypopharyngeal glands of forager bees, which have not the task of RJ synthesis and feeding larvae, should not synthesize MRJPs. Therefore, in this study the mRNA levels of each MRJPs in hypopharyngeal gland of forager bees will be quantitated.

Only AcMRJP1, AcMRJP2 and AcMRJP3 were purified and characterized from RJ with different amount similar to those of AmMRJPs. It has been reported that AmMRJPs genes were a single-copy gene per haploid genome in the genomic DNA. Usually, the transcription step is the most of 2 controlling steps in regulation of gene expression. Therefore, the mRNA levels of AcMRJPs quantitated in this study could show whether MRJPs mRNA were the direct proportion to MRJPs content. If not, the

other factors should be involved such as controlling of translation and/or the protein degradation.

From EST library of nurse *A. cerana* hypopharyngeal gland, the short peptide, AcApisimin, was found as that in *A. mellifera* (AmApisimin). Because a relatively high level expression of AmApisimin mRNA was observed during the whole life span of the honeybee, this peptide might play some biological functions apart from the oligomerization of AmMRJP1. The quantification of AcApisimin mRNA level will show whether the transcription profile (which correlated to biological function of this protein) is constant or depend on each development stages.

In this study, the mRNA levels of 6 Major Royal Jelly Proteins (MRJPs) and Apisimin in hypopharyngeal gland each stage (newly emerged, 5-10 -day-old nurse, 11-15-day-old nurse and forager bees) of *A. cerana* worker bees will be quantitated using RT-PCR. The transcription profiles of each protein in different stage of honey bees will provide the basic knowledge to understand the biological function of each protein.