CHAPTER I

INTRODUCTION

Honeybee is an economically important insect. Beside pollination that helps increasing the crop yield and improving the seed and fruit qualities, honeybee produces many valuable products used for health foods, cosmetics and medicine-like products. There are two types of honeybee products, one collected from the hive such as honey, pollen and propolis and the other secreted by glandular cells such as royal jelly (RJ), wax and venom. These products can give marketable income to Thailand.

The honeybee is a highly eusocial insect that lives together as the colony. The social system of the honey bee colonies was clear between a queen, a large number of workers and zero to a few hundred of drones depending on different season of the year. The queen and workers are heterozygotes (diploid 2n = 32) grown from fertilized eggs whereas drones are hemizygotes (haploid individuals) arising from unfertilized eggs. The queen, a mother of all members in the colony, mates with several drones after developing into the adult and stores the sperm in her spermatheca for the remaining fertilization (Hamilton, 1984; Wongsiri, 1989).

The queen also releases 9-oxodextran-2-ecenoic acid and 9-hydroxydec-2-eonic acid substances (Queen pheromone) to control her offspring and suppress the development of worker's ovaries. Unlike the queen, workers are usually sterile and play all of the tasks associated with colonial living. A division of labor among workers is a distinctive feature of workers. Different tasks of workers are dependent on their ages. Newly emerged workers with 3-5 days old have no task performing owing to low secretary activity of the hypopharyngeal gland. Young workers are called nurse bees, generally ages less than 14 days posteclosion, involve in synthesizing, secreting and feeding the royal jelly to larvae. The older workers are called foragers, ages more than 14 days posteclosion, forage for nectars and pollens (Robinson, 1991; Pankiw and Peng, 2001; Page and Peng, 2001).

1.1 Honeybees in Thailand

Honeybees are distributed in all parts of the world and recognized as a single genus *Apis*. Member of this genus could be allocated to three different lineages based primarily on morphology and behavior; 1) the cavity-nesting bees composing of *A. mellifera*, *A. cerana* and *A. koschevnikovi*, 2) the dwarf bees composing of *A. florea* and *A. andreniformis* and 3) the giant bee composing of *A dorsata* and *A. laboriosa* (Smith, 1991).

Four species including *A. andreniformis*, *A. cerana*, *A. dorsata* and *A. florea* are indigenously distributed in Thailand whereas *A. mellifera* was introduced from Europe and Africa for a bee keeping purpose (Wongsiri *et al.*, 2000).

Commercial beekeeping can only be accounted to *A. mellifera* and *A. cerana.* Basically, beekeeping of the former is more successful due to non-aggressive behavior and simple management. *A. cerana* beekeeping over a million colonies has been mostly found in Chaina (Wongsiri *et al.*, 1990a). However, *A. cerana* is a promising species for beekeeping in Thailand because it shows more disease resistance to bee mite, does not require sugar feeding and exhibits better climatic adaptability than does *A. mellifera.* Moreover, *A. cerana* has an ability to detect and remove bee mites from the colonies (Wongsiri *et al.*, 1990b).

Taxonomic identification of *A. cerana* is as follow (Borroe *et al.*, 1976; Gojmerac, 1980)

Phylum Arthropoda

Classs Insecta

Order Hymenoptera

Super-family Apoidea

Family Apidae

Genus Apis

Species cerana

Scientific name: *Apis cerana*

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1.2 Population genetics of the Asian honey bees (*A. cerana*) in Thailand

The Asian honey bee, *Apis cerana*, has been classified into four subspecies: *A. cerana cerana*, *A. cerana himalaya*, *A. cerana indica* and *A. cerana japonica*, based on morphology and geographic distribution (Ruttner,1988). This species is found over a vast geographic area of Asia ranging from Iran to China and from Japan to the south of Indonesia (Smith & Hagen, 1997).

Sylvester *et al* (1998) examined 128 samples of *A. cerana* collected throughout its range in Thailand, and from two populations in Malaysia (Selangor and Johor) using morphometric analysis. Geographic areas showed distinct morphologies and the populations could be divided into four different groups: (1), the north to central region; (2), peninsular Thailand and two of the Malaysian populations; (3) Phuket Island; and (4), Samui Island.

Among honeybee species indigenous to Thailand (*Apis cerana*, *A. florea*, *A. andreniformis* and *A. dorsata*), only *A. cerana* is of interest for commercial beekeeping because it can be maintained in hives like *A. mellifera* (Wongsiri *et al.*, 1986). Nevertheless, knowledge of the levels of genetic diversity and population subdivision of *A. cerana* in Thailand is not well studied. This basic information is important for understanding distribution patterns and colonization of this species.

The use of mtDNA for population genetic studies in eusocial species like honey bees allows simpler sampling strategies than those of nuclear DNA (Garnery *et al.*, 1995). The mtDNA of individuals within a particular colony is identical; as a result, one individual can represent a genetic pattern of the colony obviating the effects of within-colony variation when nuclear markers are utilized.

Deowanish *et al.*, (1996) investigated genetic relationships of four subspecies of *A. cerana* (*A. c. indica, A. c. cerana*, *A. c. japonica* and *A, c. himalaya*) covering most of their geographic distributions using restriction analysis of total *A. cerana* DNA with ten restriction endonucleases (*Hae* III, *Hinf* I, *Bcl* II, *Bgl* II, *Eco* RI, *Eco* RV, *Hinc* II, *Hind* III, *Nde* I and *Spe* I) and probed with the amplified fragment between $tRNA^{leu}$ and cytochrome oxidase subunit II (COII). Population

differentiation patterns of *A. cerana* samples were consistent with those based on morphometric studies (Ruttner, 1988).

Biogeographic differentiation of *A. cerana* in most of its range was recently examined by sequencing of the non-coding COI-COII region (n = 110). The first major group (western) is composed of bees from India, Sri Lanka, and the Andaman Islands whereas the other group (eastern) of *A. cerana* could be further divided to the Asian mainland (India, Nepal, northern and southern Thailand, Hong Kong, Korea, Japan and Taiwan), the Sundaland (Malaysian Peninsula, Borneo, Java, Bali, Lombok, Timor and Flores) and the Philippines (Luzon, Mindanao and Sangihe) (Smith and Hagen, 1997).

1.3 Royal jelly (RJ)

RJ (also called bee-milk) is one of the essential bee products widely produced in beekeeping. It can be sold in various forms including the fresh RJ that mixed with other products and freeze-dried RJ for subsequently used. China is the world's largest producer and exporter of RJ. In Thailand, businesses 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 the secretion of nurse bees. It is a combined product of hypopharyngeal and mandibular glands, which are located in the head of nurse bees (Figure 1.1). It is whitish in color with yellow creamy, acidic material with slightly pungent phenolic odor and taste. It is always fed directly to the queen throughout her larval and adult stages. For non-selective queen larvae (worker and/or drone larvae), the RJ are generously supplied only the first three days after emerging. Subsequently, a mixture of honey and pollen was supplied as their diets for the remaining time (Johansson, 1955; Iannuzzi, 1990 and Cardiff, 1994).

Due to different dietary feeding which is particular rich in queen, mechanisms between queen and workers in the process of female cast determination and differentiation is observed. In addition, queen attains a larger size than workers and the reproductive organ is well developed to a mature stage and is able to lay several thousand eggs a day. In contrast, workers are smaller in size. The reproductive organ is not well developed but organs that related with their tasks such as pollen baskets,

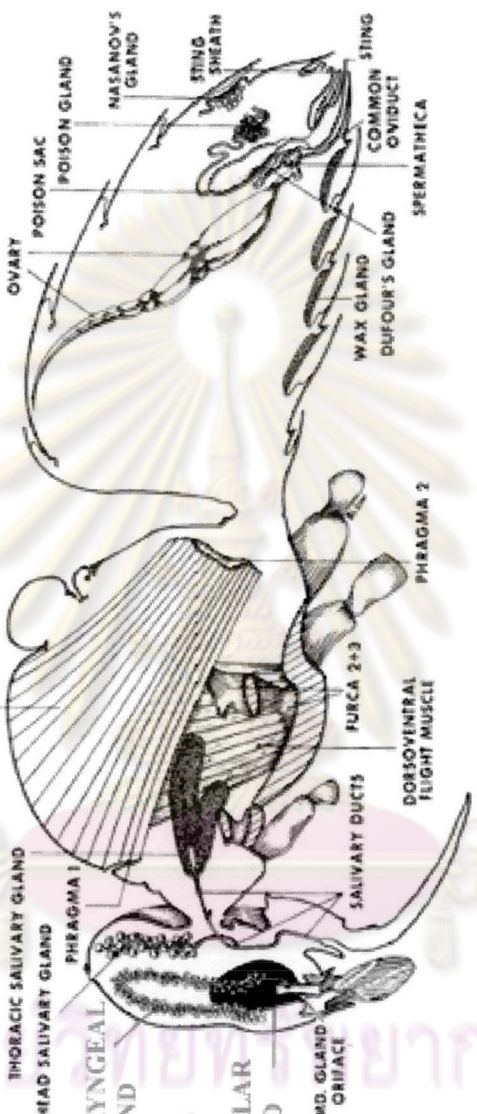

Figure 1.1 Diagram showing the organ systems of an adult female honeybee

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mandiblar, hypopharyngeal and wax glands are fully developed. Occasionally, when the queen is absent in the hive, workers can lay eggs instead. Basically, the time required for development of the queen larvae to the adult stage is about 15.5 days. The life span of the adult queen was several years, while workers require 21 days for growing up with only a few months of life span (Krell, 1996).

RNA-differential display of mitochondrial genes of *Apis mellifera*, including a gene homologous to the nuclear-encoded mitochondrail translation initiation factor 2 ($AmIF2_{mt}$), cytochrome oxidase subunit I (COX-I; mitochondrial-encoded), and cytochrome C (cyt c; nuclear-encoded) have been reported (Corona *et al.*, 1999). These genes revealed greater expression in queen larvae than did worker larvae.

Several studies have been carried out to examine advantageous effects of RJ. For example, RJ was successfully used to control cholesterol and triglyceride level in blood (Cho, 1977). Moreover, it was reported that RJ might inhibit mild- and slow-growing tumors, but not rapid-growing tumor (Tamura *et al.*, 1985). O'Conner and Baxter (1985) reported that RJ showed insulin-like activity, which stimulated glucose incorporation into rat adipocytes. RJ could also ameliorate the physical fatigue after exercise. Mice administered with RJ showed significantly decreased accumulation level of lactate and ammonia in the serum and depletion of glycogen after swimming (Kamakura *et al.*, 2001).

However, side effects resulted from inter-muscular injection of RJ were found. The automatic imbalance symptoms such as malaise, caumesthesia and hypersensitivity responsibly were exposed after RJ injection (Kushima, 1985). The symptoms of asthma and anaphylaxis were found in 39 (accounting for 52 %) of 75 subjects when they ingested RJ and an allergic cross-reactivity was significantly responded to RJ (Thien *et al.*, 1996).

For immunological effects, RJ could act as a potential immunomodulator for stimulating antibody production and immunocompetent cell proliferation in mice. However, RJ depressed humoral immune functions in rat (Sver *et al.*, 1996). The suppression of allergic reactions by RJ was investigated in DNP-KLH immunized mice by oral administration. This significantly decreased the serum levels of antigen-specific IgE and histamine release from mast cells in association with restoration of

macrophage function and improvement of Th1/Th2 cell responded in DNP-KLH mice (Oka *et al.*, 2001).

Additionally, an antibacterial activity of RJ was also found. The effective protein, royalisin, was proposed and purified from acid extraction, gel filtration and reverse-phase HPLC. The primary structure is composed of 51 amino acid residues containing three intramolecular disulfide bonds, with the calculated molecular weight of 5.5 kDa. It showed activity against Gram-positive bacteria such as *Lactobacillus spp.*, *Bifidobacterium spp.* and *Leuconostoc spp.* at a low concentration (< 10 μM) but not Gram-negative bacteria (Fujiwara *et al.*, 1990). Crude RJ in the freeze-dried form inhibited the growth of *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* at the minimum inhibition concentration (MIC) of 20, 31 and 71 mg/ml, respectively (Sanguandukul and Nimachaukool, 1993). Recently, RJ was purified by DEAE-Toyopearl 650M column chromatography. The DIII protein with the molecular mass of 58 kDa wes characterized by SDS-PAGE and reported that it had the growth stimulating activity to the U-937 human myeloid cell line in a serum-free condition (Watanabe *et al.*, 1998).

1.3.1 Compositions of RJ

RJ from *A. mellifera* has been extensively analyzed. Chemical compositions have been reported based on different analytical procedures, sample collection methods and preservation methods. The moisture content of 65.32-69.4 % in *A. mellifera* RJ was reported, when determined by different methods either automatic volatility or freeze drying or heating drying (Howe *et al.*, 1985; Karaali, Meydanoglu and Eke, 1988 and Plama, 1992; Table 1.1)

According to Association of Official Analytical Chemists (AOAC), the micro-kjeldahl method was used for the determination of organic nitrogen content of RJ. The protein content was then calculated from the percentage of nitrogen content multiplying by the conversion factor of 6.25 and protein content ranged from 11.6 % to 16.7 %. In addition, the amino acids composition of RJ protein was also investigated. RJ proteins are rich in essential amino acids (39.3 % - 51.4 %). Major amino acids of RJ were aspartic acid and glutamic acid accounted for 16.1 % and 10.19 % of the RJ protein content, respectively (Howe *et al*, 1985).

The average percentage of lipid content determined by either a modified Folch extraction or a Soxthlet method was 4.76 %. The highest lipid content (8.2 %) of previously analyzed *A. mellifera* RJ was found in that from Turkey (Karaali *et al.*, 1988; Table 1.1) followed by RJ from the United state (3.2 % – 5.6 %; Howe *et al.*, 1985) and Brazil (Plama, 1992). The most common form was found in a short chain organic acids containing 8 or 10 carbon atoms, whether saturated or unsaturated, linear or branched (Lercker *et al.*, 1981). The major fatty acid was present as 10-hydroxy-2-decenoic acid (10-HAD) at an average concentration of 50.3 % of the total fatty acid content (Howe, *et al.*, 1985). A liquid chromatography and HPLC was additionally used to determine 10-HDA (Genc and Aslan, 1999). Among the criteria for RJ quality, 10-HDA content has been proposed as a freshness parameter. RJ with 10-HDA content greater than 1.8 % is considered to be fresh and authentic. Under the controlled storage temperature, rates of 10-HAD loss were 0.1 % and 0.2 % per year at –18 °C and 4°C, respectively (Antinelli *et al.*, 2003).

As can be seen from Table 1.1, the total sugar content in *A. mellifera* RJ was 10.45-11.5 %. The sugar fraction was further dissolved in methanol and determined by HPLC. Various sugars; ribose, fructose, glucose, sucrose, mannose, trehalose, erythritol, adonitol and mannitol where fructose was commonly found in all RJ samples (Palma, 1992).

Recently, compositions of fresh RJ from *A. cerana indica* and *A. cerana japonica* were also examined (Table 1.2). *A. cerana* RJ contained 52.1 - 65.3 % moisture, 16.4 - 19.5 % crude proteins, 9.4 - 23.0 % carbohydrates, 3.9 - 7.4 % lipid and 1.5 % ash, respectively. Interestingly, carbohydrate content and acidity of *A. cerana indica* RJ from Thailand was significant higher than that of *A.cerana japonica* from Japan (Kavinseksan, 1994 and Takenaka and Takenaka, 1996).

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

Sample : Location	*A. mellifera* : U.S.A.		*A. mellifera* : Turkey		*A. mellifera* : Brazil	
Composition	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 , Microkjeldahl method	11.6 – 12.2	Total N , Microkjeldahl method	13.6	Total N , Microkjeldahl 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
Reference	Howe *et al.* (1985)		Karaali *et al.* (1988)		Plama (1992)	

ND = not determined

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

Sample : Location	*A. c. indica* : Thailand		*A. c. japonica* : Japan	
Composition	Method	Content	Method	Content
Moisture (%)	Heating at 70 °C	52.1	Heating at 105 °C	65.3
Crude protein (%)	Total N , Kjeldahl method	19.5	Total N , Microkjeldahl method	16.4
Carbohydrate (%)	Subtracting the foregoing amounts from the total amount	23.0	Subtracting the foregoing amounts from the total amount	9.4
Lipid (%)	Soxthlet method	3.9	Soxthlet method	7.4
Ash (%)	Heating at 550 °C until weight constant	1.5	Heating at 550 °C for 3 hours	1.5
Acidity (ml/100 g RJ)	Titration with NaOH	56.2	Titration with NaOH	39.3
Reference	Kavinseksam (1994)		Takenaka and Takenaka (1996)	

1.3.2 Hypopharyngeal gland secretions

Hypopharyngeal or food glands are paired acinous glands, which synthesize and secrete a protein-rich substance (Brouwers, 1982). The ultrastructural changes of hypopharyngeal glands in different developmental bees were analyzed ((Figure 1.2, Knecht and Kaatz, 1990). The number of RER in hypopharyngeal cells increased within a few days after bee emerged, reached the maximum number during the nursing phase and decreased in foragers. A nature of brood signal involved and activated protein synthesis in these glands of the nurse bees (Huang *et al.*, 1989). In addition, the secreting activity of hypopharyngeal glands of newly emerged bees was studied under different social conditions (e.g. singly isolated, caged bees with 6-day-old companion bees or living with their parents) and the results showed that hypopharyngeal glands of the 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).

Many enzymes constituents; α-glucosidase, amylase and glucose oxidase are produced in hypopharyngeal glands with an age-dependent role. The electrophoretic profile of proteins in hypopharyngeal glands from different ages of *A. mellifera* workers was analyzed. Three major proteins with the molecular mass of 50, 56 and 64 kDa were restrictively found in nurse bees, whereas a major 70-kDa protein was specifically found in foragers. Immunoblotting analysis against 50-, 56- and 64-kDa proteins confirmed that they were only detected in hypopharyngeal glands of nurse bees and also existent in RJ. These results suggested that these proteins were synthesized in hypopharyngeal glands of the nurse bee and secreted as constituents of RJ. A 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 an 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

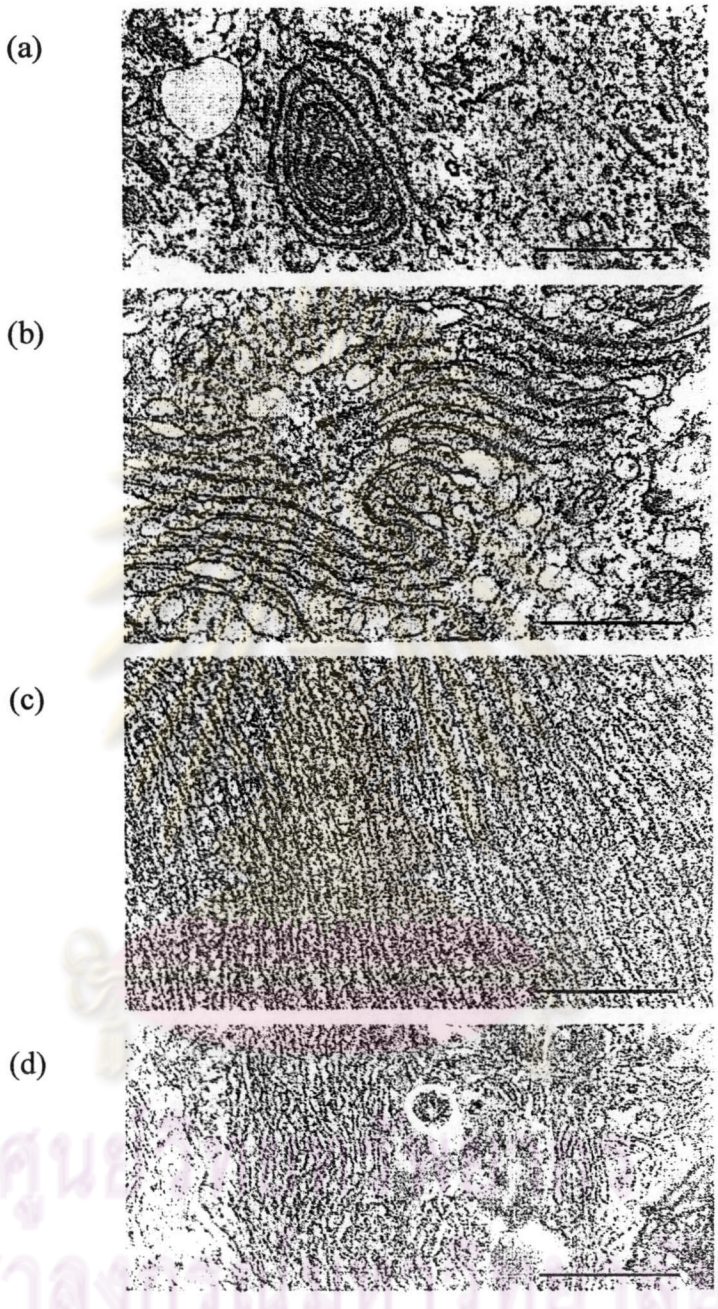

Figure 1.2 Transmission electron microscopy illustrating high numbers of Rough Endoplasmic Reticulum (RER) in hypopharyngeal cells of *A. mellifera* workers: (a) 12 hours before eclosion, (b) 2 days, (c) 8 days nurse bee and (d) 29 days forager. Bars = 1 μm.

et al, 1996). Moreover, genes encoding α-amylase and glucose oxidase in *A. mellifera* were cloned and characterized. These genes were specifically expressed in hypopharyngeal gland of foragers. 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 SDS-PAGE These proteins showed amylase and glucose oxidase activity, respectively (Ohashi *et al*, 1999).

1.3.3 Purification and characterization of major royal jelly proteins (MRJPs)

MRJPs of *A. mellifera* RJ (hereafter called **AmMRJPs**) have been extensively studied and usually focused on purification and characterization of these major proteins. DEAE cellulose column chromatography (anionic exchanger), has been initially used to purify AmMRJPs. The purification process was performed by eluting the proteins out with a linear gradient of NaCl concentration at pH 7.2. The elution profile showed three protein peaks eluted out at NaCl concentrations of 0.05 M, 0.10 M and 0.20 M, respectively. Nevertheless, molecular masses of purified AmMRJPs were not determined (Tomoda and Matsuka, 1977).

Hanes and Simuth (1992) purified AmMRJPs from RJ purchased from the Institute of Honeybee Research using a DEAE cellulose column chromatography. Two peaks of proteins eluted out at 0.05 M and 0.2 M NaCl concentrations at pH 7.5 were characterized. The former showed several bands whereas the later peak exhibited only one band with a molecular mass of 57 kDa when analyzed by SDS-PAGE. The 57-kDa protein was further analyzed by IEF and fractionated into at least eight proteins with isoelectric points between pH 4.5 to pH 5.0.

Recently, AmMRJPs were also isolated by a DEAE cellulose column chromatography. Three peaks of proteins were eluted out with a linear gradient 0.11-0.16 M (peak A), 0.16-0.22 M (peak B) and 0.22-0.30 M (Peak C) NaCl concentrations at pH 7.5. Each peaked protein was further purified by rechromatography on DEAE cellulose column and characterized by SDS-PAGE. Peak A and C proteins showed a single band with the molecular mass of 49 kDa and 55

kDa, respectively, whereas a peak B protein showed two separate band with the molecular mass of 55 kDa and 60 kDa. This chromatographic purification did not recover all proteins compared to crude RJ proteins. To classify families of AmMRJPs, crude RJ proteins were separated by SDS-PAGE, electroblotted onto PVDF membrane and *N*-terminal amino sequenced. Four families (MRJP1, MRJP2, MRJP3, and MRJP5) were identified from the *N*-terminal amino acid sequences as described below. The AmMRJP4 amino acid sequence was deduced from the nucleotide sequence of the cDNA clone RJP57-2. All components of MRJPs were glycoproteins (Schmitzova *et al.*, 1998).

AmMRJP1 possessed the *N*-terminal amino acid sequence of "NILRGESLNKS". This protein family was the most abundant protein (31 % of the total protein) in RJ of *A. mellifera* and exhibited the apparent molecular masses of 55 kDa or 57 kDa on SDS-PAGE. The essential amino acid content of AmMRJP1 was 48 % of total amino acid composition (Schmitzova *et al.*, 1998). Previously, Hane and Simuth (1992) purified and characterized AmMRJP1. The result showed that at least eight isoelectrophoretic variants ranging from 4.5 to 5.0 were found suggesting that AmMRJP1 was an acidic protein.

For localization of AmMRJP1 production sites, proteins from various organs of honeybees (head, thorax, abdomen and hypopharyngeal gland) were prepared and separated by SDS-PAGE and immunochemically detected with antiserum against the MRJP1. Results suggested that MRJP1 was localized in head and hypopharyngeal gland. However, sizes of cross-reacting proteins in head and hypopharyngeal gland were smaller than those in RJ reflecting modifications of AmMRJP1 during transportation to, or storage in, the honeybee mouth cavity.

Subsequently, cDNA encoding AmMRJP1 was cloned to a pQE-32 vector without the signal peptide sequence and expressed. The recombinant AmMRJP1 (rAmMRJP1) protein was purified and characterized. The different molecular mass was found between rAmMRJP1 (47.9 kDa) and native AmMRJP1 (55 kDa). The rAmMRJP1 will be used for testing allergic reactions with patients (Judova *et al.*, 1998).

More recently, AmMRJP1 was isolated by ultracentrifugation and was characterized by SDS-PAGE and size exclusion column chromatography. Three different forms of MRJP1; a monomer (55kDa), oligomer (approximately 420 kDa) and water insoluble aggregates resulted from interaction with fatty acids, were obtained. The oligomeric form of AmMRJP1 is water-soluble (Simuth, 2001).

To make an indicator for freshness of RJ, stability of AmMRJP1 was determined during storage under various conditions (at 4°C, room temperature, 30 °C, 40 °C and 50 °C for 0 to 7 days). The monomeric AmMRJP1 was gradually degraded with a direct proportion to both storage temperature and period (Kamakuma *et al.*, 2001).

AmMRJP2 which is the second most abundant of RJ proteins possesses the "AIVRENSPRNLEK" *N*-terminus. The apparent molecular weight Of AmMRJP2 was 49 kDa on the basis of SDS-PAGE analysis. AmMRJP2 represented 16 % of total RJ protein with 47 % of the essential amino acid composition (Schmitzova *et al.*, 1998).

Recently, cDNA encoding AmMRJP2 was cloned and expressed in a pQE-30 vector (without signal peptide sequence). The recombinant AmMRJP2 (rAmMRJP2) protein was isolated from crude bacterial lyzate, characterized and compared to native AmMRJP2. The molecular mass of rAmMRJP2 and AmMRJP2 was identical when electrphoretically estimated by SDS-PAGE. The post-translation modification of the native MRJP2 did not affect the molecular mass of this protein. The native MRJP2 was resolved into at least eight variants with different isoelectric points of pH 7.5-8.5. Therefore AmMRJP2 is a basic protein (Bilikova *et al.*, 1999).

AmMRJP3 possessed the *N*-terminal amino acid sequence of "AAVNHQ (R/K)KSANNLAHS" and exhibited size polymorphism. Therefore, an apparent molecular mass of MRJP3s was between 60 and 70 kDa revealed by SDS-PAGE analysis. The essential amino acid content of AmMRJP3 was 39.3 %. A relative content of AmMRJP3s was approximately 26 % of total protein (Schmitzova *et al.*, 1998). The deduced amino acid of MRJP3 contained a repetitive region at the end of the C-terminus, which contained motifs of XQNXX, typically with 20 repeated units. The amount of AmMRJP3 mRNA was 8 % of total mRNA (Klaudiny *et al.*, 1994).

AmMRJP4 was not obtained from direct purification of *A. mellifera* RJ (Schmitzova *et al.*, 1998). Only a clone containing cDNA encoding MRJP4 from the head cDNA library of nurse bees was previously characterized. The lowest expression level (2 % of total mRNA) of AmMRJP4 transcript was found compared with other AmMRJPs. The deduced amino acid of AmMRJP4 contained 44.5 % essential amino acid content. The calculated isoelectric point of AmMRJP4 was 6.2 (Klaudiny, *et al.*, 1994).

AmMRJP5 exhibited different molecular weight (77kDa and 88 kDa on SDS-PAGE) possessed an identical *N*-terminal amino acid sequence of "VTV(R/N)E(N/Q) SPR". The amount of AmMRJP5 accounted for 9% of total protein and contained 51.4 % essential amino acid (Schmitzova *et al.*, 1998). From the cDNA encoding AmMRJP5 nucleotide sequence, AmMRJP5 was composed of a 58 repeated unit of tri-amino acid motif where DRM was predominate at the C-terminus (Albert *et al.*, 1999a).

Gel filtration chromatography is another technique used to purify RJ. This chromatography does not require binding between proteins and the matrix. Proteins are separated due to size differences. The water-soluble RJ proteins were firstly isolated by Sephadex G-200. Two protein peaks, with molecular weight of 14 kDa (peak AI) and 33 kDa (Peak AII) were obtained. Each protein peak was further purified by re-chromatography on Sephadex G-75 column. No further separated protein was found. Protein peaks AI and AII were characterized by disc electrophoresis. The fraction AI contained 4 components while the fraction AII was composed of 3 components. The 5th main band was found in both fractions. Obviously, RJ proteins were not successfully purified and the molecular mass of these proteins was not determined in that study (Tomoda *et al.*, 1977).

1.3.4 Molecular cloning of cDNA encoding MRJPs

Previously, the Uni-ZAP XR expression cDNA library was prepared from the head of *A. mellifera* nurse bees. AmMRJPs were immunologically screened with polycolnal anti-MRJPs raised in mice. Two selected clones, pRJP57-1 and pRJP57-2, were characterized by nucleotide sequencing and assigned to be AmMRJP3 and AmMRJP4 respectively (Klaudiny *et al.*, 1994). Two additional clones, pRJP120 and

pRJP95, were subsequently identified as AmMRJP1 and AmMRJP2, respectively (Ohashi *et al.*, 1997; Schmitzova *et al.*, 1998). Recently, a new member of RJ proteins AmMRJP5, was also found using the same procedure (Albert *et al.*, 1999a). A summary for molecular characterization of cDNA encoding AmMRJP families is illusttrated in Table 1.3.

Putative promoter and genomic structure of AmMRJP1 were currently analyzed (Malecova *et al.*, 2003). The genomic structure and an upstream binding site map of AmMRJP1 are illustrated in Figure 1.3. The AmMRJP1 gene-sequence spans over 3038 bp containing six exons. The predicted promoter with the TATA motif of AmMRLP1 was found and highly conserved for both the sequence and the position across AmMRJP gene families.

Regulation of AmMRJP transcription was examined. Ultraspiracle (USP), a steroid hormone receptor superfamily, also acts as a transcription factor and binding hormone ligand. USP acts together with others receptors to activate transcriptton of hormone responsive genes that involve with cell differentiation and development as well as homeostasis and reproduction. Thus, juvenile hormone (JH) was proposed to be a physiological regulator for binding of USP and acting as a mediator on AmMRJP expression. The USP-TF binding sites were computationally predicted in the promoter region. Moreover, the dead ringer (Dri) is one of DNA-binding proteins that share an A/T rich interaction domain (ARID) responded either positive or negative transcriptional regulation. The clusters of Dri-TF binding site were also located in upstream of the promote region of AmMRJPs.

AmMRJP3 is highly polymorphic as suggested by size differences on SDS-PAGE. A pair of primers in the flanking regions of repetitive sequences was designed and amplified in the individual recombinant plasmid harboring AmMRJP3 cDNAs. Polymorphism of AmMRJP3 is supported because at least 10 PCR products with distinct sizes were obtained from random sampling clones. The repetitive region consists of two distinguishable segments (Albert *et al.* 1999b).

Table 1.3 Molecular characterization of AmMRJP cDNAs.

Family	DNA insert size* (bp)	Deduced amino acid (residues)	Signal peptide cleavage site	No. of *N*-glycosylation site	References
MRJP1	1444	432	Gly_{19}-Asn_{20}	3	Ohashi *et al.* (1997)
MRJP2	1579	452	Gly_{17}-Ala_{18}	2	Schmitzova *et al.* (1998)
MRJP3	1719	467	Gln_{16}-Asp_{17}	1	Klaudiny *et al.* (1994)
MRJP4	1625	464	Cys_{15}-Gln_{16}	8	Klaudiny *et al.* (1994)
MRJP5	1979	598	Gln_{16}-Gly_{17}	4	Albert *et al.* (1999a)

* including poly A tail

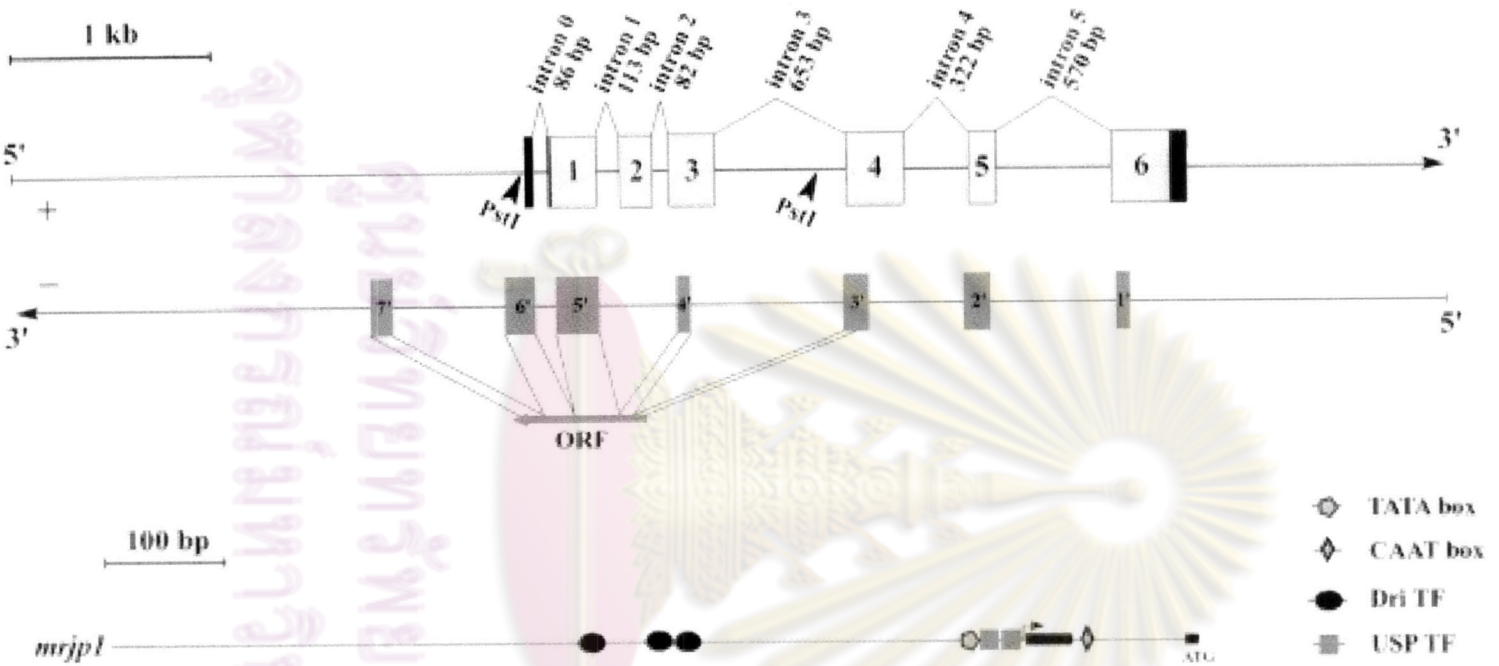

Figure 1.3 (a) Genomic structure of MRJP1 gene. For the sense (+) strand, light-gray numbered boxes (1-6) indicate exons transcribed into mRNA of MRJP1 while black lines indicate untranslated regions . Arrows showed *Pst* I restriction sites. For the antisense (-) strand, darked-gray numbered boxes (1'-7') indicated putative exons prediction. (b) Binding site mapped on upstream sequence of MRJP1 gene. An arrow indicates transcriptional starting point. Black boxed represented 5' UTR.

The first segment was located in a more proximate region of the N-terminus and more conserved. The repeat consisted of NQNA(D/N/G) encoded by AATCAGAATGCT(A/G)A(C/T), where 13 nucleotides of these were 100% conserved. This motif appeared in 6 – 8 copies in characterized AmMRJP3.

The second repeated segment was less conserved. It consisted of (K/R)QN (D/G)N encoded by A(A/G)(A/G)CA(A/G)AATG(A/G)TAA(C/T). The invariant glutamine-asparagine (QN) motif is conserved. The repeated unit ended with asparagines at the 5^{th} position. The unit in this segment started with lysine or arginine (positive charges) causing a basic character of this part of AmMRJP3 (Albert *et al.*, 1999b).

The extensive repeat region found in AmMRJP5 was recently characterized (Albert *et al.*, 1999a). This region was located between 367^{th} and 540^{th} amino acid residues. The consensus sequence "GATAGAATG" which encoded asparatic acid, arginine and methionine occurred 58 times and interrupted a conserved region at the C-terminus of this protein

Using the differential display-reverse transcription-polymerase chain reaction (DD-RT-PCR) technology, gene expression in brain of various developmental stages (newly emerged bees, young workers and adult foragers) of *A. mellifera* were examined. A cDNA encoding AmMRJP1 (56 kDa) was found in a subset of Kenyon cells in mushroom bodies of the brains of the workers. Levels of mRNA expression in this organ were examined by northern blot hybridization analysis. A high level of transcription was observed approximately 48 hours after emergence (Kucharski and Maleszka, 1998).

Recently, a new serine-valine rich peptide called "Apisimin" was isolated and characterized from a cDNA library prepared from the head of *A. mellifera* nurse bees. Full-length cDNA of apisimin was obtained after sequencing analysis. Apisimin had an open reading frame (ORF) of 234 nucleotides encoding a precursor peptide of 78 amino acid residues where the signal peptide was located at residues 1-24. Therefore, a cleavage signal peptide site was located between Ala_{24} and Lys_{25}. Apisimin of *A. mellifera* was valine- (18.5 %) and serine-rich (16.7 %), and contains only one aromatic amino acid, phenylalanine. Apisimin was synthesized for the entire life span

of *A. mellifera* and may be involved in activation of different cellular processes (Bilikova *et al.*, 2002).

1.3.5 Phylogeny and evolution of MRJPs

Sequences of all AmMRJP cDNAs were translated to amino acid sequences and used to search the EMBL/GenBank databases with the BLITZ program. Similarity was observed between AmMRJPs and the *yellow* proteins of *D. melanogaster* and *D. subobscura* (27 % identity and 51 % similarity) and four genomic sequences (loci) of *D. melanogaster*. Amino acid sequences of AmMRJPs and *Drosophilla yellow* proteins were then aligned and subjected to phylogenetic analysis based on parsimony (exhaustive maximum parsimony search and branch-and-bound bootstrapping analysis) and distance (bootstrapping of the original protein sequence data) approaches (Figure 1.4).

Phylogenetic analysis strongly supported a monophyletic relationship between AmMRJP families but the analysis did not resolve evolutionary relationships within the AmMRJPs family. The phylogenetic tree revealed that AmMRJP genes occurred as a consequence of multiple gene duplication at nearly simultaneous period of time. Nevertheless, MRJP4 may possibly exhibit the earliest divergence within these gene families (Albert *et al.*, 1999b).

1.4 Importance and objectives of this research

Genetic differentiation between different geographic regions of *A. cerana* in Thailand have been reported with limited sample sizes (n = 5 and 6 colonies in Deowanish *et al.*, 1996 and Smith and Hagen, 1997, respectively). Accordingly, identification of patterns of population differentiation and estimation of genetic variability of *A. cerana* in Thailand are necessary for construction of genetic based-management and conservation programs in this important bee species.

Molecular characterization of AmMRJPs at both nucleotide and DNA levels has been reported and well studies. However, this basic information is rather limited for *A. cerana*.

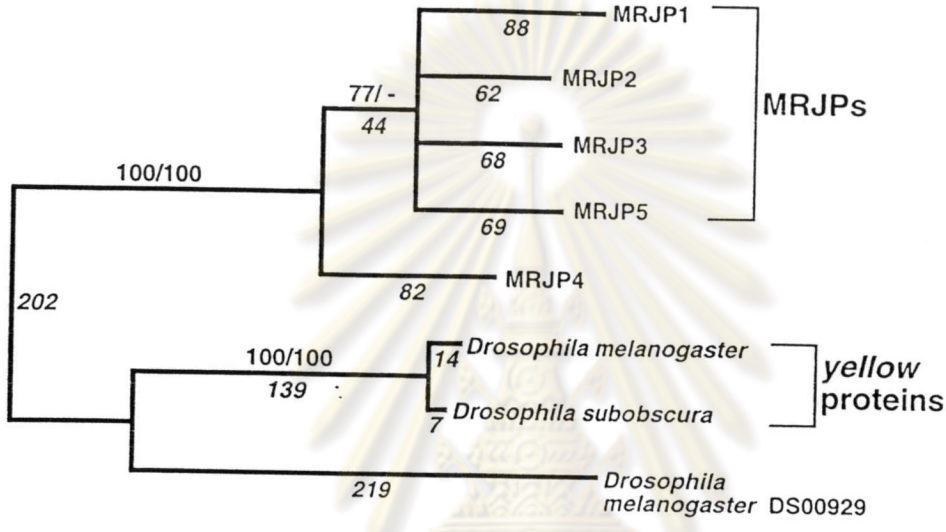

Figure 1.4 A phylogenic tree of AmMRJPs constructed using the maximum-parsimony method (PAUP 3.1.1) searched by an exhaustive procedure. Branch and bound bootstrapped analysis (2000 replications) are shown above lines, on the left side of the slash. Bootstrapped results (2000 replications) from PROTDIST (PHYLIP Version 3.5c) analysis are shown above lines, on the right of the slash. The most parsimonious step between branches is italicized below the line. The tree was rooted with *Drosophilla* protein sequences.

Recently, Takenaka and Takenaka (1996) reported that chemical compositions of *A. mellifera* and *A. cerana* major royal jelly protein components (levels of proteins, 10-hydroxydecanoic acid and glucose/fructose ratio) were different. Analysis of water soluble proteins in royal jelly by electrophoresis revealed 21 protein bands in each species where 14 protein bands were shared between the royal jelly of these bees. A highly aggregated protein was found in *A. cerana* but not in *A mellifera*. 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. mellifera* were more heavily stained than those of *A. cerana*. Additionally, 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.

Currently, *A. cerana* is widely used for commercial beekeeping in Thailand due mainly to its disease resistance against bee mites. Queen rearing experiments supported differences of *A. mellifera* and *A. cerana* royal jelly because *A. cerana* queens could not be successfully reared with *A. mellifera* royal jelly and *vice versa* (Pothichot and Wongsiri, 1993; Takenaka and Takenaka, 1996).

The objectives of this study were determination of genetic diversity and population genetic differentiation of *A. cerana* in Thailand and characterization of major royal jelly cDNAs and proteins of *A. cerana*.

As a result, relatively rapid and convenient method for genetic diversity analysis was established based on restriction of the large subunit of ribosomal (lr) RNA gene against a large sample size of *A. cerana* in Thailand. Representatives of *A. cerana* from different locations in Thailand were collected. Mitochondrial (mt) DNA polymorphism of these specimens was examined by sequencing of the amplified lrRNA gene region. Nucleotide sequences obtained were compared and searched for the existence of any informative restriction endonuclease site to simplify the analysis to a diagnostic PCR-RFLP.

For the characterization of major royal jelly cDNAs and proteins of *A. cerana*, an EST library from hypopharyngeal glands of *A. cerana* was established. The cDNAs encoding AmMRJP homologues and other important cDNAs (apisimin, α-glucosidase and glucose oxidase) were searched. Expression levels of three major royal jelly protein genes in *A. cerana* (AcMRJP1, AcMRJP2 and AcMRJP3) were

semi-quantified using a target/standard competitive PCR. In addition, major royal jelly proteins of *A. cerana* were chromatographically purified and further characterized by SDS-PAGE, isoelectric focusing and amino acid sequencing.

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