CHAPTER I

INTRODUCTION

Honeybees are social insects in Genus *Apis*. They are an economical insect. They help pollination that increase crop yield and improve the seed and fruit qualities. The honeybees produce many valuable products such as honey, bee wax, royal jelly and pollen. They are used as supplement food, ingredient in foods, cosmetics and medicine-like products.

Honeybees are social insects that live as colony. One colony is composed of 3 caste honeybees, that are queen, worker and drone bees. The colony has only one queen, a large number of workers and zero to a few hundred of drones depending on different annual period. The queen and worker bees are female, heterozygotes (diploid 2n = 32) grown from fertilized eggs whereas drones are male, hemizygotes (haploid individuals) arising from unfertilized eggs. (Wongsiri, 1989)

Queen bee has the greatest size in the colony when compared with the others and has fully developed ovary. She lays eggs and secretes queen pheromone to control her offspring and suppress development of worker's ovaries. Worker bees are sterile and have all tasks in the colony. Distribution of task among workers depends on their ages. Young workers, called nurse bees, generally age less than 14 days after eclosion, are involved in synthesizing, secreting and feeding the royal jelly to young larvae and the queen. The older workers, called foragers, age more than 10 day after eclosion, forage for nectar and process it into honey. Role of drone bees is mating with the queen bee (Robinson, 1991; Page and Peng, 2001).

Honey bees in Thailand

There are five species of honey bee in Thailand, 1) the giant or rock honey bees, *Apis dorsata*, 2) the dwarf honey bees, *Apis florea*, 3) the small dwarf honey bees, *Apis andreniformis*, 4) the eastern honey bees, *Apis cerana* and 5) the western honey bees, *Apis mellifera*. For *A. mellifera*, this species is not native to Thailand, they are imported from Europe and Africa for a beekeeping purpose. *A. mellifera* and *A. cerana* can be kept and managed in hive for commercial beekeeping due to non-aggressive behavior and simple management. Commercial beekeeping with *A. mellifera* is better studied than those for *A. cerana*, but *A. cerana* shows more disease resistance to bee mite especially *varroa jacobsoni* mite and exhibits better climatic adaptability than *A. mellifera*. From this reason *A. cerana* was suitable for beekeeping in Thailand (Wongsiri *et al.*, 1990).

Royal jelly (RJ)

RJ (also called bee-milk) is the high valuable bee product widely produced in beekeeping. It can be sold in various forms such as fresh RJ, freeze-dried RJ and mixed with other product. It is used as supplement food and used in cosmetics industry. China is the world's largest producer and exporter of RJ. Moreover, Japan has the highest domestic consumption of royal jelly, a large part of which is imported from other Asian countries. In Thailand, a business with royal jelly and other bee products is successful that it grows into a multimillion dollar enterprise (Krell, 1996).

Royal jelly is secreted from the hypopharyngeal and mandibular glands of the worker honeybees mainly between the sixth and twelfth days of their life (Haydak, 1970).

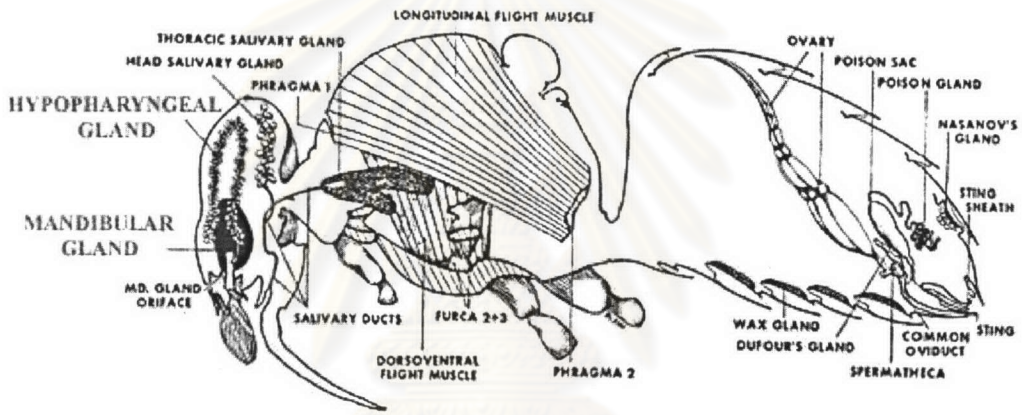

Figure 1.1 Diagram showing the organ systems of an adult female honeybee

These glands are located in the head of nurse bees (Figure 1.1). Royal jelly is a thick, whitish in color, has a slightly pungent phenolic odor and a characteristic sour flavour (Iannuzzi, 1990; Krell, 1996). 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 is supplied only the first three days after emerging. Subsequently, a mixture of honey and pollen is supplied as their diets for the remaining time (Johansson, 1995).

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 are related with their tasks such as pollen baskets, mandibular, hypopharyngeal and wax glands are fully developed. 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 is several years, while workers require 21 days for growing up with only a few months of life span (Krell, 1996).

Royal jelly has been demonstrated to possess several pharmacological activities in experimental animal. For example, RJ can lower the cholesterol in blood (Vittek, 1995), possesses the anti-inflammatory and wound healing properties (Fujii *et al.*, 1990) and can act as a potential immunomodulator for stimulating antibody production and immunocompetent cell proliferation in mice. However, RJ depresses humoral immune functions in rat (Sver *et al.*, 1996). In human, RJ consumption in the amounts of 50-100

mg per day can reduce total cholesterol levels by about 14% in people with moderately high cholesterol levels (Vittek, 1995).

However, allergic reactions are the most common side effect when consume royal jelly. Allergic reactions from oral intake of royal jelly can range from very mild (e.g., mild gastrointestinal upset) to more severe reactions, including asthma, anaphylaxis (shock), intestinal bleeding, and even death in people who are extremely allergic to bee products (Thien *et al.*, 1996; Leung *et al.*, 1997 and Yonei *et al.*, 1997).

Composition of RJ

Numerous chemical analyses of royal jelly have been published. Royal jelly is acidic substance with pH between 3.6 to 4.2. The chemical composition of fresh RJ of *A. mellifera* contains moisture (66-70%), protein (12-17%), sugars (11-12.5%), lipids (3-5.5%) and mineral salts. In addition, the amino acids composition of RJ protein is also investigated. RJ proteins are rich in essential amino acids (39.3 % - 51.4 %), the most important being aspartic acid (16.1 %) and glutamic acid (10.19 %). For lipids fraction, the major fatty acid is 10-hydroxy-2-decenoic acid (10-HDA) at an average concentration of 50.3 % of the total fatty acid content. The sugars in royal jelly are dominant in fructose and glucose; fructose is prevalent in all RJ samples. In many cases, fructose and glucose together account for 90% of the total sugars (Howe, *et al.*, 1985, Palma, 1992 and Krell, 1996).

Recently, compositions of fresh RJ from *A. cerana indica* and *A. cerana japonica* were also examined and compared with that of *A. mellifera* (Table 1.1). *A. cerana* RJ contains 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. Interestingly, moisture content of *A. cerana indica* RJ

Table 1.1 Composition of fresh RJ of *A. cerana indica*, *A. cerana japonica* and *A. mellifera*.

Composition	*A. cerana indica*	*A. cerana japonica*	*A. mellifera*
Moisture (%)	52.1	65.3	68.3
Crude protein (%)	19.5	16.4	12.7
Carbohydrate (%)	23.0	9.4	11.9
Lipid (%)	3.9	7.4	6.1
Ash (%)	1.5	1.5	1.0
Acidity *	56.2	39.3	42.2
Reference	Kavinseksan (1994)	Takenaka and Takenaka (1996)	Takenaka and Takenaka (1996)

* Acidity: Volume of 1N NaOH (ml)/100g of fresh RJ)

from Thailand is lower than that of *A.cerana japonica* from Japan and *A mellifera*, whereas crude proteins content, carbohydrate content and acidity of *A. cerana indica* RJ from Thailand are higher than those of *A. cerana japonica* from Japan and *A mellifera* (Kavinseksan, 1994 and Takenaka and Takenaka, 1996).

Hypopharyngeal glands secretions

Hypopharyngeal glands of the worker bee are a pair of long tuberous organ connecting to many acini (secretory glands), each of which is composed of about a dozen of secretory cells. This glands synthesizes and secretes royal jelly (Brouwers, 1982; Knecht and Kaatz, 1990). The ultrastructural changes of hypopharyngeal glands in different developmental bees were analyzed. The number of rough endoplasmic reticulum (RER) in hypopharyngeal cells increased within a few days after bee emerged, reached to the maximum number during the nursing phase and decreased in foragers (Knecht and Kaatz, 1990). Immunofluorescence study by Kubo, *et al.* (1996) showed that the protein condensed in the duct of the gland was secreted from acini as royal jelly proteins.

Royallizin was the first royal jelly protein which the complete amino acid sequence was characterized. Royalizin of *A. mellifera* bee is composed of 51 amino acid residues, with the calculated molecular weight of 5.5 kDa. It is found to have potent antibacterial activity against Gram-positive bacteria (Fujiwara, *et al.*, 1990). Further proteins of RJ were characterized by cloning and sequencing of their complementry DNAs (cDNAs) : RJP 57-1 (MRJP3) and RJP 57-2 (MRJP4) (Klaudiny *et al.*, 1994), α glucosidase (Ohashi, *et al.*, 1996) and the dominant 56 kDa protein (MRJP1) (Ohashi, *et al.*, 1997).

Subsequently, Schmitzova (1998) isolated cDNA clones coding for RJ proteins from uni-ZAP XR expression cDNA library, which prepared from the head of 8-day-old nurse honeybees (*A. mellifera*). It was done in parallel with electrophoretic analysed and N-terminal sequencing of RJ proteins. The result of N-terminal sequences of the proteins and by analysis of the newly obtained and known cDNA sequence data concerning this proteins. It was found that royal jelly contained major proteins and that all the proteins belong to one protein family designated MRJP (from major royal jelly protein). The family consists of five main members (MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5) which comprise 82-90% of total larval jelly protein. MRJPs contain high amount of essential amino acids (39.3-51.4%), presumably that MRJPs have nutritional function in honeybee larval food. Amino acid compositions of *A. mellifera* MRJPs are illustrated in Table 1.2. All members of MRJP are glycoprotein. In addition, The MRJP gene family encodes a group of closely related proteins that share a common evolutionary origin with the yellow protein of *Drosophila melanogaster*. Yellow protein has a functions in cuticle pigmentation in *D. melanogaster* (Albert *et al.*, 1999a).

Recenty, three new members of the MRJP family (MRJP6, MRJP7 and MRJP8 were identified in *A. mellifera*. Novel cDNA of MRJP family were identified 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. Excluding MRJPs, cDNA coding for orthologues of *Drosophila* yellow protein was reported. From its homology with the yellow-f gene product of *Drosophila*, the cDNA was designated am-yellow-f (Albert and Klaudiny, 2004).

Table 1.2 Amino acid composition of *Apis mellifera* MRJPs

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

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).

Characterization of major royal jelly proteins (MRJPs)

MRJPs of *A. mellifera* RJ (hereafter called AmMRJPs) have been extensively studied, which focuses on characterization of both cDNAs and proteins. The Uni-ZAP XR expression cDNA library was prepared from the head of 8-day-old nurse honeybees (*A. mellifera*). 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.*, 1994). Two additional clones, pRJP120 and pRJP95, were subsequently identified as AmMRJP1 and AmMRJP2, respectively (Ohashi *et al.*, 1997; Schmitzora *et al.*, 1998). Subsequently, AmMRJP5 was found using the same procedure (Albert *et al.*, 1999a).

Recently, three new cDNA members of MRJPs 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 1.3

For AmMRJPs characterization, AmMRJPs were purified by a DEAE cellulose column, further purified by rechromatography on DEAE cellulose column and then characterized by SDS-PAGE. The result showed that only 3 bands with molecular weight of 49, 55 and 60 kDa can be purified. The purification did not recover all proteins compared to crude RJ protein. To classify families of AmMRJPs, crude RJ protein was

Table 1.3 Molecular characterization of cDNAs and deduced amino acid sequences of AmMRJP.

Family	DNA insert size* (bp)	Deduced amino acid (residues)	No. of N-glycosylation site**	Amino acid residues without signal peptide**	Molecular weight (kDa)**	Reference
MRJP1	1444	432	3	416	46.8	Schmitzova *et al.* (1998)
MRJP2	1579	452	2	435	48.9	Schmitzova *et al.* (1998)
MRJP3	1719	467	1	528	59.5	Klaudiny *et al.* (1994)
MRJP4	1625	464	8	449	50.9	Klaudiny *et al.* (1994)
MRJP5	1966	598	4	581	68.0	Albert *et al.* (1999a)
MRJP6	1529	437	5	417	47.6	Albert *et al.* (2004)
MRJP7	1427	443	4	426	48.7	Albert *et al.* (2004)
MRJP8	1329	416	8	400	45.1	Albert *et al.* (2004)

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

separated by SDS-PAGE, eletroblotted onto PVDF membrane and N-terminal amino acid sequenced. Four families (MRJP1, MRJP2, MRJP3 and MRJP5) were identified from the N-terminal amino acid sequences, which correlated with cDNA sequences obtained (Schmitzova *et al.*, 1998). More recently, 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).

MRJP1

AmMRJP1 protein has the N-terminal amino acid sequence NILRGESLNKS. From deduced amino acid sequence, this protein shows high amount of the 10 essential amino acids (48%). This protein is the most abundant protein in RJ of *A. mellifera*, that comprises 31% of the relative content as determined by SDS-PAGE. This protein exhibits the apparent molecular weights of 55 kDa or 57 kDa, which consists of at least eight isoelectrophoretic variants in the range of 4.5-5.0. These rather small differences in molecular weights may reflect some modification of the proteins during transport, or storage in, the honeybee mouth cavity. (Hanes and Simuth, 1992; Schmitzova *et al.*, 1998).

For indicating that native 56-kDa protein (AmMRJP1) is a glycoprotein, 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).

MRJP1 was reported to have three different forms; a monomer (55 kDa), oligomer (approximately 420 kDa) and water insoluble aggregates resulted from interaction with fatty acids (Simuth, 2001). In the royal jelly, MRJP1 associates 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).

MRJP1 mRNA was found to be differentially expressed in the heads of early emerged honeybees (Kucharski *et al.*, 1998), nurse and also forager honeybees (Ohashi *et al.*, 1997). 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.

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 in native AmMRJPs (Judova *et al.*, 1998).

MRJP2

The N-terminal amino acid sequence of AmMRJP2 is AIVRENSPRNLEK. The relative content of this protein is 16% in total royal jelly protein with 47% of the essential amino acid composition (Schmitzova *et al*, 1998). The apparent molecular weight of native AmMRJP2 protein is 49 kDa that can be resolved into 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 MRJP2 protein to be 49 kDa which was the same as the native AmMRJP2 protein (Schmitzova *et al.*, 1998; Bilikova *et al.*, 1999).

The 50 kDa protein that had the same molecular weight with MRJP2 as detected in nurse bee hypopharyngeal gland, but not in the forager bee hypopharyngeal gland when using immunoblotting analysis (Kubo *et al.*, 1996). Interesting; MRJP2 mRNA was found to be expressed in heads of experienced foragers when using microarrays and northern blot analysis (Kucharski and Malezka, 2002).

MRJP3

AmMRJP3 protein exhibits a size polymorphism as detected by SDS-PAGE. The apparent molecular masses of MRJP3 are between 60 and 70 kDa. They have almost identical N-terminal amino acid sequence: AAVNHQ (R/K) KSANNLAHS, which is identical to the amino acid sequence inferred from RJP57-1 cDNA. A relative content of AmMRJP3 is approximately 26% of total royal jelly protein. The essential amino acid content is 39.3% (Schmitzova *et al.*, 1998). The deduced amino acid of MRJP3 contains a repetitive region at the C-terminal part, repetitive motifs of XQNXX, typically with 20 repeated units (Klaudiny *et al.*, 1994). From the PCR analysis of genomic DNA for the detection of the polymorphism of the MRJP3 repetitive region, the result showed that repeat region of the MRJP3 was highly polymorphic with as many as five alleles found in 10 individuals from the same colony (Albert *et al*, 1999b). In other species, the study of repetitive sequence motifs in Gaint bee, *Apis dorsata* found that repettitive sequence also existed in MRJP3 gene liked those in *A. mellifera* (Albert *et al.*, 2002).

The MRJP3 protein was reported to have two different forms; a monomer (70 kDa) and trimer (210 kDa) (Okamoto *et al.*, 2003).

MRJP3 mRNA and protein are expressed specifically in hypopharyngeal gland of nurse honeybees (Kubo *et al.*, 1996, Ohashi *et al.*, 1997). The amount of AmMRJP3 mRNA is 8% of total mRNA (Klaudiny *et al.*, 1994).

MRJP4

Only a clone (RJP57-2) containing cDNA encoding MRJP from the head cDNA library of nurse bees was characterized and designate MRJP4. It showed the lowest expression level (2% of total mRNA). 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.*, 1994, Schmitzova *et al*, 1998).

MRJP4 can not be obtained from purification experiment and SDS-PAGE analysis of RJ. It can be characterized by two-dimensional gel electrophoresis of RJ following by N-terminal amino acid analysis. From two-dimensional gel electrophoresis of European honeybee (*A. mellifera*) RJ, two spots; HBRJ E27 and E28, were identified as MRJP4. They possessed the N-terminal amino acid sequence (GVVRENSSRK) identical to those of MRJP4 (RJP57-2) cDNA previously reported by Klaudiny *et al.* (1994). For Africanized honeybee RJ, five spots; HBRJ A24 to A28, were identified as MRJP4, and possessed the N-terminal amino acid sequence of AVVRENSSRK. The N-terminal amino acid sequence of the 2 species were different by only one amino acid residue.

The molecular weight and isoelectric focusing point of MRJP4 of Africanized honeybee RJ and European honeybee RJ were compared. The results showed that the molecular weight average of MRJP4 was 60 kDa and isoelectric focusing point was 5-6 (Sano *et al.*, 2004).

MRJP5

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 and contains 51.4% essential amino acid, dominant in Arg (9%) and Met (11.4%) (Schmitzova *et al.*, 1998).

The deduced amino acid of AmMRJP5 inferred from MRJP5 cDNA shows the extensive repeat region located between 367 th and 540 th amino acid residues. This repeat unit is composed of a consensus sequence (GATAGAATG) which encodes for tripeptide (DRM): aspartic acid (D), arginine (R) and methionine (M) occurred 58 times and interrupts a conserved region of the MRJP consensus sequence. This repeat region is located at the C-terminal of this protein and invariant in repetitive unit size (Albert *et al.*, 1999a).

The MRJP5 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 MRJP5 proteins were found in both the Africanized and the European honey bee royal jelly. The MRJP5 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).

MRJP6-8

Three new cDNA members of the MRJP family (MRJP6, MRJP7, and MRJP8) 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.*, 1994).

MRJP6 cDNA sequence is highly homologous to MRJP5, but does not have repeat sequence encoding the tripeptide motif (DRM). The 5' non-coding region of MRJP6 cDNA sequence contains a 3' part of intron0 with the conserved AG motif, intron0 is found in MRJP 1 genomic sequence (Malecova *et al.*, 2003).

MRJP7 cDNA sequence was assembled from only the honeybee brain EST sequence database. The deduced amino acid sequence of MRJP7 shows high homology to MRJP2 protein (73% identity in 433 amino acid overlap with MRJP2).

MRJP8 cDNA sequence was found only one clone in the honeybee brain expressed sequence tags (EST) sequence database. The complete MRJP8 cDNA sequence was identified by using genomic sequence database and nucleotide sequence from amplification product of the nurse honeybee head cDNA library.

Genomic structure of MRJP

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 six exons separated by five 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 AmMRJP genes. The predicted promoters contained the TATA motif (TATATATT), highly conserved both in sequence and position across AmMRJP gene families. Ultraspiracle (USP) transcription factor (TF) binding sites and clusters of dead ringer TF binding sites were predicted in putative promoter regions. The juvenile hormone (JH) was proposed to be a physiological regulator for the binding of USP and acted as a regulator of MRJPs expression. Moreover, MRJPs gene family (MRJP1-5) were a single-copy gene per haploid honeybee genome when examined by Southern blot analysis (Malecova *et al.*, 2003).

Characterization of MRJPs in *Apis cerana*

Nowadays, the study of MRJPs in *A. cerana* is limited. Previously, Takenaka and Takenaka (1996) reported the chemical composition of *A. cerana* royal jelly compared with *A. mellifera* royal jelly. They showed that the levels of proteins, 10-HDA and glucose/fructose ratio in RJ of this 2 species were different. 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 jellys 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). Sixty-six recombinant clones that had insert sizes greater than 500 bp were sequenced in unidirection. The nucleotide sequences showed that forty two of these (63.6%) were identified as homologues of AmMRJPs, homologues of AmMRJP1 (50%), AmMRJP2 (6.06%), AmMRJP3 (6.06%) and AmMRJP4 (1.52%). The MRJPs 1, 2 and 3 of *A. cerana* (hereafter called AcMRJPs) showed high expression level, whereas AcMRJP4 was found only one clone that showed low expression level. Moreover, the AcMRJP5 was not found in these 66 recombinant clones.

The complete and partial sequences of AcMRJP1 and AcMRJPs 2, 3 and 4 were reported. The open-reading frame of the AcMRJP1 cDNA was 1299 nucleotides that encoded for 433 deduced amino acids with three predicted N-linked glycosylation site (Srisuparbh *et al.*, 2003). The AcMRJP3 cDNA was identified by RT-PCR of hypopharyngeal gland. The AcMRJP3 ORF was composed of 1824 bp that encoded for 608 amino acid residues with five predicted N-linked glycosylation site (Srisuparbh, D., 2002). Nowadays, the genomic structure and complete cDNA sequence of both AcMRJP1 and AcMRJP2 have been reported. The complete nucleotide sequence of AcMRJP2 cDNA is composed of 1565 bp that encodes for 463 amino acid residue with two predicted N-linked glycosylation site (Imjongjailuk, C., 2004 In publish).

To characterize MRJP of *A. cerana*, the royal jelly proteins were purified and characterized using Q-sepharose and Sephadex G-200 column chromatography. 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 MRJP (MRJP1, MRJP2 and MRJP3) that homologue to AmMRJP were found in

A. cerana royal jelly. AcMRJP1 was reported to have two different forms, a monomer (50kDa) and oligomer (300kDa) with isoelectric points of 5.2-5.7 and 5.7, respectively. The molecular weight of AcMRJP2 was 55 kDa which had isoelectric points of 7.0-8.0. The native form of AcMRJP3 had the molecular weight of 115kDa, whereas denatured form was 80 kDa. The AcMRJP3 isoelectric point was 8.3 (Srisuparbh *et al.*, 2003).

Advantages of MRJPs in other biological system

Several advantages of MRJPs in other biological system have been studies. The MRJP1 protein enhances the cell proliferation of rat hepatocytes (Kamakura *et al.*, 2001b), stimulates the growth of human lymphocytes in a serum-free medium (Watanabe *et al.*, 1996) and shows an antifatigue effect in mice (Kamakura *et al.*, 2001a). The MRJP3 protein has immunosuppressive functions in a mammalian immune system. This proteins inhibits the production of IL-2, IL-4 and IFN-γ by anti-CD3 mAb-stimulated T cell *in vitro*. Furthermore, MRJP3 inhibited IgE production *in vivo* when MRJP3 was administered intraperitoneally to OVA/Alum-immunized BALB/C mice (Okamoto *et al.*, 2003)

Recently, royal jelly which had MRJP3 in one component, showed anti-inflammatory actions through inhibiting proinflammatory cytokine production such as TNF-α, IL-6 and IL-1 by activated macrophages (Kohno *et al.*, 2004).

The protease N treated royal jelly (ProRJ) and peptides from ProRJ (ILe-Tyr (IY), Val-Tyr (VY), Ile-Val-Tyr (IVY)) inhibited angiotensinI-converting enzyme (ACE) activity and had an antihypertensive effect in repeated oral administration for 28 days on spontaneously hypertensive rats (SHR) (Tokunaga *et al.*, 2004).

Phylogeny and evolution of MRJPs

MRJP gene family encode a group of closely related proteins that share a common evolutionary origin with the yellow protein of *D. melanogaster*. The duplications and subsequent divergence within this gene family, which accompany the development of social behavior in the honeybee. This results in the novel nutritional function of the MRJP proteins (Albert *et al.*, 1999; Albert *et al.*, 2004).

Amino acid sequence of AmMRJPs, *A. mellifera* yellow protein and *Drosophila* yellow protein were aligned and subjected to phylogenetic analysis using exhaustive maximum parsimony search and Branch-and-bound bootstrap analysis. The analysis showed that both honeybee and *Drosophila* Yellow-f protein was a monophyletic group distant from MRJPs, with MRJP8 exhibited the earliest divergence within MRJPs gene families (Albert *et al.*, 2004) (Figure 1.2).

Objectives of this research

The objectives of this study is identification and characterization of MRJP4 and MRJP5 cDNA in hypopharyngeal gland of *A. cerana*. Then nucleotide and deduced amino acid sequences obtained will be used in phylogenetic study between *A. cerana* and *A. mellifera*. Furthermore, expression of AcMRJP4 in *E. coli* expression system will be performed.

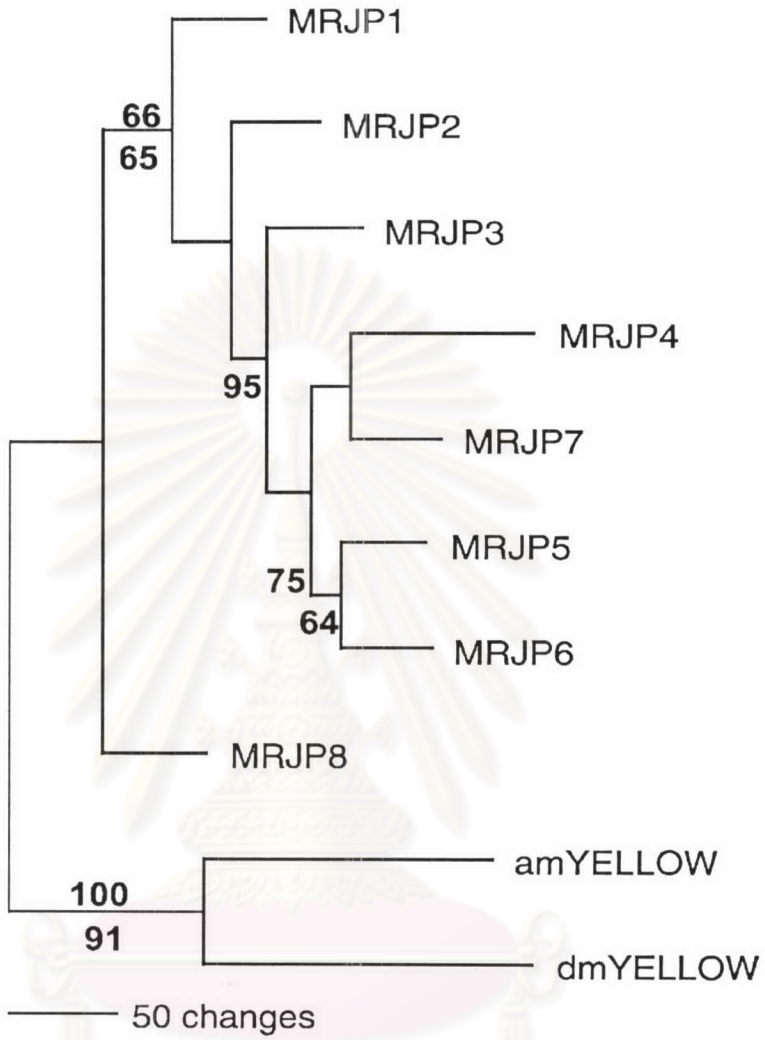

Figure 1.2 Phylogeny of MRJP/YELLOW proteins of *A. mellifera*. This is an unweighted maximum parsimony tree (938 steps) found with an exhaustive search. The results of bootstrap analyses using the branch-and-bound method (200 replications) are shown above the branches. The numbers below the branches are maximum likelihood quartet-puzzling values found using TREEPUZZLE V5.0 (WAG + C evolutionary model, 5000 puzzling steps). Only bootstrap or puzzle values >60% are shown (Albert and Klaudiny, 2004)