CHAPTER IV

DISCUSSION

Initially, semi-quantitative PCR for estimating transcription levels of AcMRJPs and AcApisimin mRNA in hypopharyngeal glands of *A. cerana* nurse bees (11-15 -day-old) was attempted by comparing with the expression level of the internal standard as an endogenous sequence of housekeeping genes (28S rRNA). Using this method, Plateau effect problem must be aware.

According to determining of PCR cycle number at the starting to plateau phase demonstrated that only the AcMRJP3-6 mRNA (20^{th}, 20^{th}, 24^{th} and 22^{nd} cycles respectively) could be compared with 28S rRNA (20^{th} cycles) but AcMRJP1 (14^{th} cycles), AcMRJP2 (16^{th} cycles) and AcApisimin mRNA (18^{th} cycles) could not. This result indicated that mRNA levels of AcMRJP1, AcMRJP2 and AcApisimin were much higher than those of AcMRJP3, AcMRJP4, AcMRJP5, AcMRJP6, and 28S rRNA. In addition to determining the PCR-cycle number, the result showed that the amplification efficiencies (E) of AcMRJPs and AcApisimin cDNA were different as shown by the different slopes of graphs of the log intensity of PCR products and the cycle numbers (Appendix B, I). Moreover, the important factor, which must be consider of comparing AcMRJPs and AcApisimin mRNA levels between different stages of worker bees was that the endogenous internal standard (28S rRNA) must be constant in all stage, (the newly emerged bees, the 5-10- day-old nurse bees, the 11-15- day- old nurse bees and the forager bees). In this study, total RNA content in hypopharyngeal gland of forager bees was about 5 times lower than those in nurse bees. Therefore it is unlikely that the level of 28S rRNA is the same in hypopharyngeal gland of all stages.

Quantitative determination of mRNA levels by competitive PCR method using an exogenous sequence (added genomic DNA or the cDNA competitor the competitive) as the internal standard did not affect by Plateau effect problem. Pannetier *et al.* (1993) demonstrated that the ratio of the competitor and the target was maintained in a parallel manner both in the exponential phase and the plateau phase. In addition the amplification efficiency of the target and the internal standard, added genomic DNA competitor or cDNA competitor, were identical because of using the same primers for amplification reaction.

Since the target and the competitor must be truly competitive in PCR, therefore the non-specific PCR product must not be found in the reaction. In this study, the non-specific PCR product was found when the genomic DNA was used as competitor, even though the condition had been optimized. Therefore, the cDNA competitors, which were 19-44 nt shorter sequence (Δn), were used instead. The cDNA competitor was synthesized by PCR amplification of 1st stranded cDNA template according to the method of Celi *et al.* (1993), using the specific forward primer of target and the reverse composite primer.

To achieve the accuracy performance of competitive PCR, two important conditions must be fulfilled. First, the cDNA competitor and cDNA target were amplified with the same efficiency or amplification kinetics. Preparation of cDNA competitor by the method of Celi *et al.* (1993), Loitsch *et al.* (1999) found that the amplification kinetics of the target and the corresponding competitor were the same. They had been demonstrated that the two types of genes with different amplification kinetics by using the different primer sets, these differences were eliminated by titrating against their corresponding cDNA competitors. The second critical point in

competitive PCR was the accuracy of quantitatively measuring the ethidium bromide-stained intensity of the amplification product. To overcome this problem, a suitable PCR template concentration (cDNA target and cDNA competitor) must be determined to make sure that the intensities of both amplified PCR products did not reach the saturated intensity and directly correlated with the amount of the amplified PCR products. Usually, the numerous ranges of serial dilution of the cDNA competitor concentration were simultaneously amplified with the cDNA target. Furthermore, in this study at least 2 concentrations of cDNA target (the non-dilute and the 2-fold dilution) were confirmed by repeating amplify with same suitable competitor range. The results showed the direct correlation of intensity and amount of PCR product.

The relative transcription level of AcMRJP1 : AcMRJP2 : AcMRJP3 mRNA obtained from this study was 19 : 11 : 1 and 22 : 11 : 1 in the 5-10 -day-old and 11-15 -day-old nurse bees, respectively, which was different from the report of Schmitzova *et al.* (1998) and Srisuparbh (2002). Schmitzova *et al.* (1998) reported that the ratio of MRJP1 : MRJP2 : MRJP3 mRNAs was 3 : 1 : 1 in nurse bees whereas Srisuparbh (2002) similarly reported the ratio of 3.3 : 1.6 : 1. The difference of the results might be the results of using the different analysis methods which have different sensitivity and/or the accuracy of the stage of bee samples.

According to the report of Schmitzova *et al.* (1998), they used the northern blot analysis of total RNA from whole head of nurse bees (*A. mellifera carnica*). This method is known to be less sensitive and less accurate because of the saturation effect of the hybridization signals. It had been reported by Kucharski *et al.* (1998) that AmMRJP1 was also expressed in Mushroom bodies in honey brain. Therefore, using of total RNA extracted from whole head might give the wrong ratio of mRNA levels

if the other organs could express MRJPs. Although, Srisuparbh (2002) quantified mRNA levels using the same quantitative analysis of competitive PCR but the difference of the sample collection method might cause the different result. In this study, bees were collected at accurate day by color marking on the thorax of newly emerged bees whereas Srisuparbh (2002) collected the bee samples base on the bee behavior.

Previously, Malecova *et al.* (2003) reported that AmMRJPs genes were a single-copy gene per haploid genome in the genomic DNA. From EST library (Srisuparbh *et al.*, 2003) which was prepared from the hypopharyngeal gland of *A. cerana* nurse bees, the most abundant clones were AcMRJP1 (50%) followed by AcMRJP2 (6.06%), AcMRJP3 (6.06%) and AcMRJP4 (1.52%). The transcription levels of AcMRJP1-6 mRNA quantitated in this study also showed the different transcription level. The results showed that AmMRJPs genes might differ in their transcriptional regulation and/or mRNA stability.

Moreover, Schmitzova *et al.* (1998) reported the levels of MRJP1, MRJP2, MRJP3 and MRJP5 in *A. mellifera* RJ were 31%, 16%, 26%, and 9%, respectively. Srisuparbh *et al.* (2003) reported the ratio of the total protein quantities of MRJP1 in monomeric forms : MRJP1 in oligomeric forms : MRJP2 : MRJP3 in *A. cerana* RJ was 1 : 12.21 : 4.72 : 2.52. According to these results, MRJP1, MRJP2 and MRJP3 in both *A. mellifera* and *A. cerana* were the main secreted MRJPs. However, the relative ratio of mRNA of AcMRJP1 : AcMRJP2 : AcMRJP3 as 18.89 : 11.32 : 1 and 23.97 : 12.10 : 1 in 5-10 -day-old and 11-15 -day-old nurse bees, respectively which as not corresponding to the amount of AcMRJPs in RJ. This result suggested that expression of each protein may differ in translational efficiency and/or might be occurred by the

protein degradation. Srisuparbh (2002) reported that degradation of AcMRJP3 was directly proportional to both storage temperature and period whereas Kamakura *et al.* (2001) demonstrated these same events were found in AmMRJP1.

The expression levels of each AcMRJPs mRNA at different developmental stages of *A. cerana* workers were examined in this study. The result revealed that all of AcMRJPs mRNA was expressed in hypopharyngeal glands of the nurse bees and the forager bees, but not in the newly emerged bees. Usually, AcMRJP1-6 produced in hypopharyngeal gland of nurse bees served as components of RJ. From this study, all AcMRJPs mRNA were found in hypopharyngeal gland of forager bees which were not synthesis RJ. This result suggested that AcMRJP1-6 should have the other additional roles than nutrient especially in forager bees.

The mRNA levels of AcMRJP1-5 were higher in nurse bees than those in forager bees. On the contrary, the mRNA levels of AcMRJP6 in the forager bees as greater than at in the nurse bees. The result implied that AcMRJP6 may have differed in biological function compares to other AcMRJP1-5 in honeybees. In addition from the transcription profile, the maximum level of AcMRJP1 mRNA was found at 5-10 -day-old nurse bees whereas the others AcMRJPs mRNA was found at 11-15 -day-old nurse bees. Thus, AcMRJP1 must be the first expressed gene in AcMRJPs family.

Bilikova *et al.* (2002) suggested that AmApisimin seem to be involved with the oligomerization of AmMRJP1. In this study, AcApisimin mRNA was found in all bee stages (the newly emerged bees, the 5-10- day-old nurse bees, the 11-15- day- old nurse bees and the forager bees). The maximal level was found in nurse bees, followed by forager bees and newly emerged bees, respectively. In contrast with AcApisimin mRNA, AcMRJP1 mRNA was absent in newly emerged bees, therefore

it suggested that Apisimin should involved in the other biological functions than the involvement in oligomerization of AmMRJP1.

The result from this study showed only the transcription levels of AcMRJPs and AcApisimin mRNA. The mRNA levels may not indicate the levels of proteins produced by the cell, because of many regulations occur at the post-transcriptional stage (Gygi *et al.*, 1999). Therefore, complete and biologically relevant analysis of gene expression requires additional information especially that derived from immunohistochemistry and biochemical assays.