CHAPTER III

RESULTS

3.1 Population genetic studies and identification of population origins of Thai honey bees, *A. cerana*.

The amplified lrRNA gene segment of *A. cerana* was approximately 750 bp in length. The number of readable nucleotides was either 653 or 654. Similarity between the sequences was 97.4% - 99.8%. The GC content of these sequences was approximately 15.5%.

A total of 57 point mutations (19 transitions and 38 transversions) were observed (Figure 3.1). The percentage sequence divergence between pairs of sequences was 0.15 % - 1.70 % (average sequence divergence = 1.13 %) indicating a close relatedness among the investigated samples.

Based on the 5 different sequences, *Dra* I (which recognizes TTT/AAA) could be used to differentiate *A. cerana* specimens according to their geographic origins. Restriction analysis of the lrRNA gene of 225 bees with *Dra* I resulted in 4 different haplotypes (Figure 3.2). The percent sequence divergence between pairs of haplotypes ranged from 1.59% (A and D) to 4.37% (C and D). These haplotypes can be parsimoniously related by a single loss or gain of restriction sites. The A haplotype was the most common, found in 98.08%, 95.12% and 86.67% of bees from the north, north-east and central (collectively called the north-to-central) samples. Haplotype D was also observed at low frequency in these samples (Table 3.1). The other common haplotype, B, was found in all individuals originating from peninsular Thailand and Phuket Island. This haplotype was also found (52.94 %) along with haplotype C (47.06 %) in the Samui sample.

The average nucleotide divergence between the north-to-central and southern latitude (peninsular Thailand, Phuket and Samui Island) *A. cerana* in Thailand (2.657%) was greater than that within each region (0.005% and 0.144%, respectively) (Table 3.2). Significant genetic differentiation was observed across overall samples and between north-to-central and southern regions ($P < 0.0001$ for a Monte Carlo

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                        13777 (A. mellifera)
North-to-Central1       ATAGAGACAGTTGTTATTTCATCAATTCATTCATTCAATTCTTCAATTAAAAGACAATTT  60
North-to-Central2       ATAGAGACAGTTGTTATTTCATCAATTCATTCATTCAATTCTTCAATTAAAAGACAATTT
Phuket Island           ATAGAGACAGTTGTTATTTCATCAATTCATTCATTCAATTCTTCAATTAAAAGACAATTT
Samui Island            ATAGAGACAGTTGTTATTTCATCAATTCATTCATTCAATTCTTCAATTAAAAGACAATTT
A. mellifera            TTAGAGACAGTTATTATTTCATTAATTCTTTCATACAATTCTTCAATTAAAAGACAATTT
                        ********** ********* ***** ***** ************************

North-to-Central1       ATTATGCTACCTTTGTACAGTCAATATACTGCAGCTATTTAAATTTATTTCATGGAGCAG  120
North-to-Central2       ATTATGCTACCTTTGTACAGTCAATATACTGCAGCTATTTAAATTTATTTCATGGAGCAG
Phuket Island           ATTATGCTACCTTTGTACAGTCAATATACTGCAGCTATTTAAATTTATTTCATGGAGCAG
Samui Island            ATTATGCTACCTTTGTACAGTCAATATACTGCAGCTATTTAAATTTATTTCATGGAGCAG
A. mellifera            ATTATGCTACCTTTGTACAGTCAACATACTGCAGCTATTTAAAAT-AATTCATTGAGCAG
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North-to-Central1       ATCGACCTAAAATTATACTCAATAGGCCATGTTTTTGTTAAACAGGTGAATAATCAATTT  180
North-to-Central2       ATCGACCTAAAATTATACTCAATAGGCCATGTTTTTGTTAAACAGGTGAATAATCAATTT
Phuket Island           ATCGACCTAAAATTATATTCAATAGGCCATGTTTTTGTTAAACAGGTGAATAATCAATTT
Samui I Bland           ATCGACCTAAAATTATATTCAATAGGCCATGTTTTTGTTAAACAGGTGAATAATCAATTT
A. mellifera            ATCGACCTAAAATTATAATCAACAGGACATGTTTTTGATAAACAGGTGAATAATATATTT
                        ***************** **** *** ********** ***************  ****

North-to-Central1       TGCCGAGTTCCTTTAAATTATATATATATAAATAATTTATATATTATTAAATATACTTTT  240
North-to-Central2       TGCCGAGTTCCTTTAAATTATATATATATAAATAATTTATATATTATTAAATATACTTTT
Phuket Island           TGCCGAGTTCCTTTAAATTATATATATATAAATAATTTATATATTATTAAATATACTTTT
Samui Island            TGCCGAGTTCCTTTAAATTATATATATATAAATAATTTATATATTATTAAATATACTTTT
A. mellifera            -GCCGAATTCCTTTAAATTATATA---ATACAAAATATC---ATTATAAAATATAACTTT
                         ***** *****************   *** * *** *    ***** *******  ***

North-to-Central1       ATTACTAATTTAATCATTATCACTATATCTTAAAAATTAAAATATATGTTTTTATAGAAT  300
North-to-Central2       ATTACTAATTTAATCATTATCACTATATCTTAAAAATTAAAATATATGTTTTTATAGAAT
Phuket Island           ATTACTAATTTAATCATTATCACTATATTTCAAAAATTAAAATATATATTTTTATAGAAT
Samui Island            ATTACTAATTTAATCATTATCACTATATTTTAAAAATTAAAATATATATTTTTATAGAAT
A. mellifera            ATTACTAATCTAATCACTATTTCTATATTTTATTAATTAAAATATAAATTTTTATAGAA-
                        ********* ****** ***  ****** * *  ************  ************

North-to-Central1       AAATAAAATTCAAAATTTAAATTTTTTAAAATTAATAACTA---AATTATTAAATTTTTT  360
North-to-Central2       AAATAAAATTCAAAATTTAAATTTTTTAAAATTAATAACTA---AATTATTAAATTTTTT
Phuket Island           AAATAAAATTTAAAATTTAAATTTTTTAAA-TTAATAACTA---AATTATTAAATTTTTT
Samui Island            AAATAAAATTTAAAATTTAAATTTTTTAAA-TTAATAACTA---AATTATTAAATTTTTT
A. mellifera            AAATAAAATATAAA-TTTAAATCATTATTAATTTATAAATATTAAAATTATTAAATTATTA
                        *********  *** *******  **   * ** **** **    ************ **

North-to-Central1       ATATTAATAAAAAATATTAACTTCATAATATTATAAATAAAATCAAAAATTTTTATAAAT  420
North-to-Central2       ATATTAATAAAAAATATTAACTTCATAATATTATAAATAAAATCAAAAATTTTTATAAAT
Phuket Island           ATATTAATAAAAAATATTAACTTCATAATATTATAAATAAAATCAAAAATTTTTATAAAT
Samui Island            ATATTAATAAAAAATATTAACTTCATAATATTATAAATAAAATCAAAAATTTTTATAAAT
A. mellifera            AAATTAAAGAAAAATATTATCTCTATAACATTAAAAATTAAAATTAAA-TTTTTATCT-T
                        * *****  ********** **  **** **** **** ***   *** *******   *

North-to-Central1       AAATTTATAGTTTATCCCATAAATTTTTAATATAAAAATTAATACTATAAA-TAAATTTT  480
North-to-Central2       AAATTTATAGTTTATCCCATAAATTTTTAATATAAAAATTAATACTATAAA-TAAATTTT
Phuket Island           AAATTTATAGTTTATCCCATAAATTTTTAATATAAAAATTAATACTATAAA-TAAATTTT
Samui Island            AAATTTATAGTTTATCCCATAAATTTTTAATATAAAAATTAATACTATAAA-TAAATTTT
A. mellifera            AAATTTATAGATTATCCCATAAATTTTTAATATAAAAATTAATAAATTAAAATAAAATTT
                        ********** ********************************   **** **** ***

North-to-Central1       AAGGTATTAAAAATTTTATATCTAAATTAAATTTATTTCTAAAAAAACTAGATATCAATA  540
North-to-Central2       AAAGTATTAAAAATTTTATATCTAAATTAAATTTATTTCTAAAAAAACTAGATATCAATA
Phuket Island           AATGTATTAAAAATTTTATATCTAAATTAAATTTATTTCTAAAAAAACTAGATATCAATA
Samui Island            AATGTATTAAAAATTTTATATCTAAATTAAATTTATTTCTAAAAAAACTAGATATCAATA
A. mellifera            AATTTACTAAAATAATTATATCTAAATTAAATTTATTTCTAAAAAAACTAGATATTAATA
                        **  ** *****   ****************************************** ****

North-to-Central1       ACTTCGAATGACATTTAATCTCTAAATTTATATTTATAATTTTATTGCAACAAAAAAAAT  600
North-to-Central2       ACTTCGAATGACATTTAATCTCTAAATTTATATTTATAATTTTATTGCAACAAAAAAAAT
Phuket Island           ACTTCGAATGACATTTAATCTCTAAATTTATATTTATAATTTTTATGCAACAAAAAAAAT
Samui Island            ACTTCGAATGACATTTAATCTCTAAATTTATATTTATAATTTTTATGCAACAAAAAAAAT
A. mellifera            AGTTCGTTTAACATTTAATTTCTAAATCTATATTTATAATTTTATTGCTACAAAAAAAAT
                        * ****  * ********* ******* ***************  *** ***********

                                                                (A. mellifera)14422
North-to-Central1       ATTACAAATTTAGCTCACTTATTTTCGAGATATTTAAATTTATTAAATAAATTTTAAT    658
North-to-Central2       ATTACAAATTTAGCTCACTTATTTTCGAGATATTTAAATTTATTAAATAAATTTTAAT
Phuket Island           ATTATAAACTTAGCTCACTTATTTTCGAGATATTTAAATTTATTAAATAAATTTTAAT
Samui Island            ATTATAAACTTAGCTCACTTATTTTCGAGATATTTAAATTTATTAAATAAATTTTAAT
A. mellifera            AATATAAATTTAGCTCCCTTATTTTCGAGATATTTAAAATCATTAAATAAATTTTAAT
                        * ** *** ******* ********************** * *****************
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Figure 3.1 Nucleotide sequences of the lrRNA gene of *A. cerana* (GenBank accession number 140508-140511). The homologous *A. mellifera* sequence positions 13777-14422 (Crozier and Crozier, 1993) was used as an outgroup reference. *Dra* I-recognition sites are illustrated in boldface. Asterisks indicate identical nucleotides among aligned sequences.

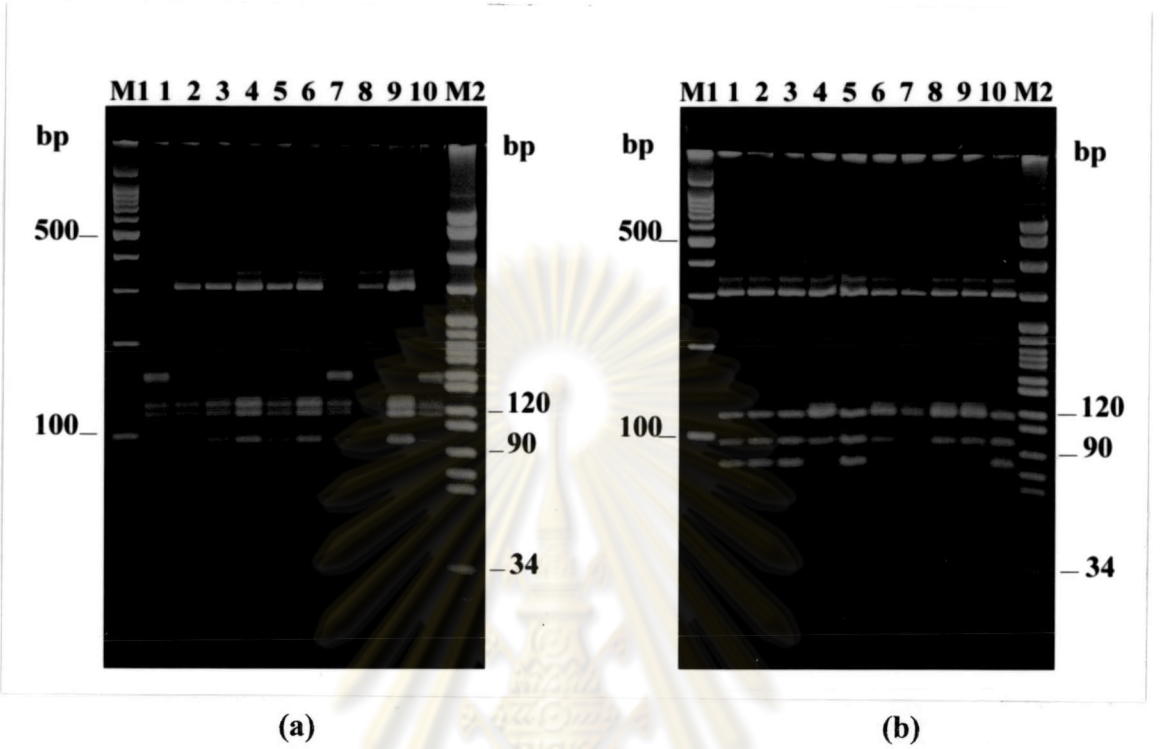


Figure 3.2 Restriction pattern of amplified lrRNA gene of *A. cerana* digested with *Dra*I. Four haplotypes were observed (Haplotype A = lane 2-6 & lane 8-9 in panel a, Haplotype B = lane 4 & lane 6-9 in panel b, Haplotype C = lane 1-3, 5 & 10 in panel b, Haplotype D = lane 1, 7 & 10 in panel a). Lane M1 and M2 are 100 bp DNA ladder and pBR322/*Msp I*, respectively.

Table 3.1 Restriction fragment size of *Dra* I digested lrRNA gene from *A. cerana* in Thailand

Haplotype	Number of fragments at indicated size in base pairs													Total length (bp)
	350	300	152	148	130	120	120	90	80	60	50	40	7*	
A	0	1	0	0	1	1	0	1	0	1	1	0	0	750
B	0	1	0	0	0	1	1	1	0	1	1	0	1	747
C	0	1	0	0	0	1	0	1	1	1	1	1	1	747
D	0	0	1	1	1	1	0	1	0	1	1	0	0	750
E	1	0	0	0	0	1	1	1	0	1	0	0	0	740

* this fragment was not observed on the electrophoretic gel but inferred from DNA sequenceing data of this gene region.

Table 3.2 Geographic distribution of *Dra* I digested lrRNA gene of *A. cerana* samples in Thailand

Haplotype	Geographic distribution						Total
	North	Central	North-East	Peninsular Thailand	Phuket Island	Samui Island	
A	51	26	39	0	0	0	116
B	0	0	0	61	7	18	86
C	0	0	0	0	0	16	16
D	1	4	2	0	0	0	7
Total	52	30	41	61	7	34	225

Table 3.3 Nucleotide divergence between six geographic samples of *A. cerana* in Thailand

	North	Central	North-East	Peninsular Thailand	Phuket Island	Samui Island	*A. mellifera*
North	-						
Central	0.012	0.005					
North-East	-0.001	0.003	-				
Peninsular Thailand	2.566	2.563	2.562	-			
Phuket Island	2.566	2.563	2.562	0.000	-		
Samui Island	2.847	2.840	2.842	0.216	0.216	-	
A. mellifera	5.310	5.341	3.315	4.372	4.373	4.695	-

Table 3.4 Geographic heterogeneity test based on a Monte Carlo simulation (below diagonal) and *F*st estimate (above diagonal) between six geographic samples *A. cerana* in Thailand

	North	Central	North-East	Peninsular Thailand	Phuket Island	Samui Island
North	-	0.083 ns	-0.008 ns	0.982**	0.966**	0.764**
Central	0.0602 ns	-	0.017 ns	0.919**	0.814**	0.618**
North-East	0.5872 ns	0.3876 ns	-	0.962**	0.920**	0.710**
Peninsular Thailand	<0.0001**	<0.0001**	<0.0001**	-	0.000 ns	0.544**
Phuket Island	<0.0001**	<0.0001**	<0.0001**	1.0000 ns	-	0.298 ns
Samui Island	<0.0001**	<0.0001**	<0.0001**	<0.0001**	0.0325 ns	-

ns = not significant.
** = P<0.0001.

Table 3.5 AMOVA test and hierarchical F-statistics of *A. cerana* samples based on 20000 permutations

Component	Df	Variance component	% of total variance	*F*-statistics	P-value
Among groups	2	0.3803	84.79	0.849	<0.0001
Among samples	3	0.0005	0.12	0.007	0.893 ns
Within groups					
Within geographic samples	219	0.0677	15.09	0.848	0.0162 ns
Total	224	0.4485			

ns = not significant.

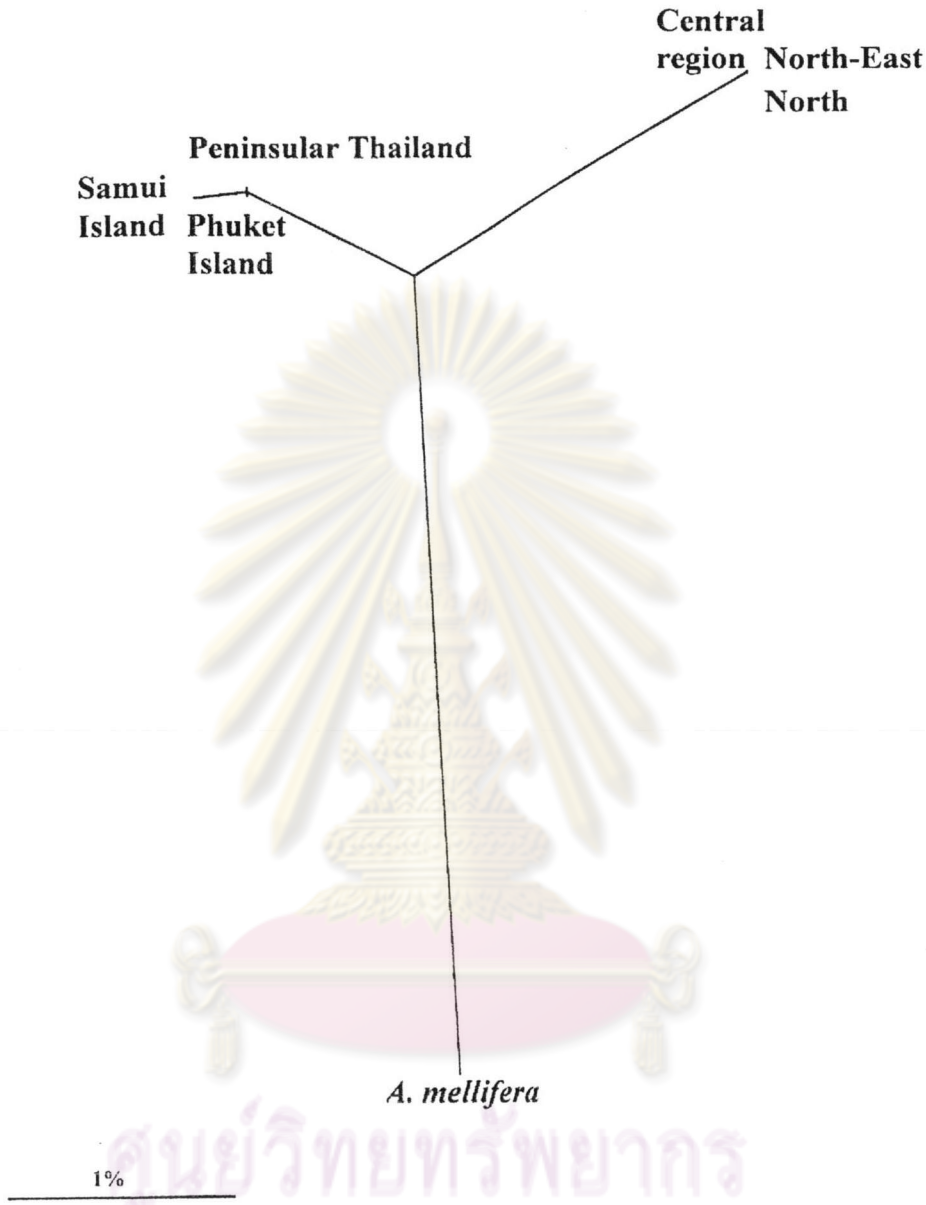


Figure 3.3 A Neighbor-joining tree indicating genetic relationships of *A. cerana* in Thailand based on the percentage of sequence divergence of *Dra* I digested lrRNA gene

3.2 Isolation, purification and characterization of AcMRJPs

3.2.1 Production of RJ

Bee colonies originating from northern ($N = 2$) and peninsular Thailand ($N = 3$) were queen-reared to produce RJ. Alternatively, RJ was directly collected from the colony ($N = 1$ from the Samui Island). A 100 % of queen cup-acceptance before larvae grafting was observed in colonies originating from both northern and peninsular Thailand whereas the average queen cup-acceptance of 65.48% and 67.54 % was observed after larvae grafting (Table 3.6). The average quantity of RJ production derived from the peninsular samples (29.12 mg/cup/day) was comparable to that of the northern samples (32.16 mg/cup/day) ($P > 0.05$). The RJ derived from peninsular population (southern latitude *A. cerana*) was used for purification and characterization of AcMRJPs.

Crude *A. cerana* RJ was electrophoretically examined on SDS-PAGE and compared to commercial *A. mellifera* RJ. Patterns of crude RJ proteins are shown in Figure 3.4. Different patterns of major proteins in RJ of *A. cerana* and *A. mellifera* were observed. Crude RJ of *A. cerana* was composed of three major discrete bands (50, 80, 82 kDa) where a 50 kDa band was the most intense stained protein band compared to a doublet of 80 and 82 kDa. On the other hand, a pattern of *A. mellifera* crude RJ proteins was composed of four major bands (55, 60, 65 and 80 kDa). The most intense stained band was 60 kDa while the faintest band was a 80 kDa band. Several additional faint bands exhibiting sizes less than 50 kDa, were also found in RJ of *A. cerana* and *A. mellifera*. Nevertheless, only major royal jelly proteins are further purified and characterized.

3.2.2 Ammonium sulfate precipitation

To determine suitable concentrations of ammonium sulfate for precipitation of AcMRJPs, a step-wise increment (a 10 % increment from 20 % to 80 % saturation) was performed. AcMRJPs (50 kDa and 80 kDa) were precipitated in all saturation fractions up to a 70 % saturation (Figure 3.5). Only a 50 kDa protein was still precipitated in 70-80 % saturation of ammonium sulfate.

Table 3.6 RJ production from *A. cerana* colonies originating from peninsular Thailand (colony no 1, 2 and 3) and the north to central Thailand (colony no 4 and 5)

Colony no.	Date of production	Percentage of cup acceptance before grafting	Percentage of cup acceptance after grafting	Quantity of RJ (mg / cup/day)
1	May-2001	100	67.44	28.62 [a]
2	Aug-2001	100	66.67	31.57 [b]
3	Aug-2001	100	68.52	36.31 [b]
Average		100	67.54	32.17
4	Apr-2000	100	65.85	30.62 [b]
5	Mar-2002	100	65.11	27.62 [b]
Average		100	65.48	29.12

a. Incubation time after grafting = 2 days
b. Incubation time after grafting = 3 days

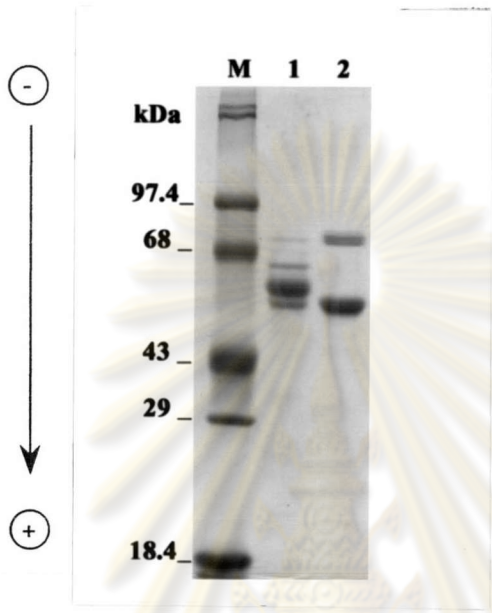


Figure 3.4 10 % SDS-PAGE analysis of crude RJ from *A. mellifera* and *A. cerana*.

Lane M = Standard protein markers
Lane 1 = *A. mellifera* RJ
Lane 2 = *A. cerana* RJ

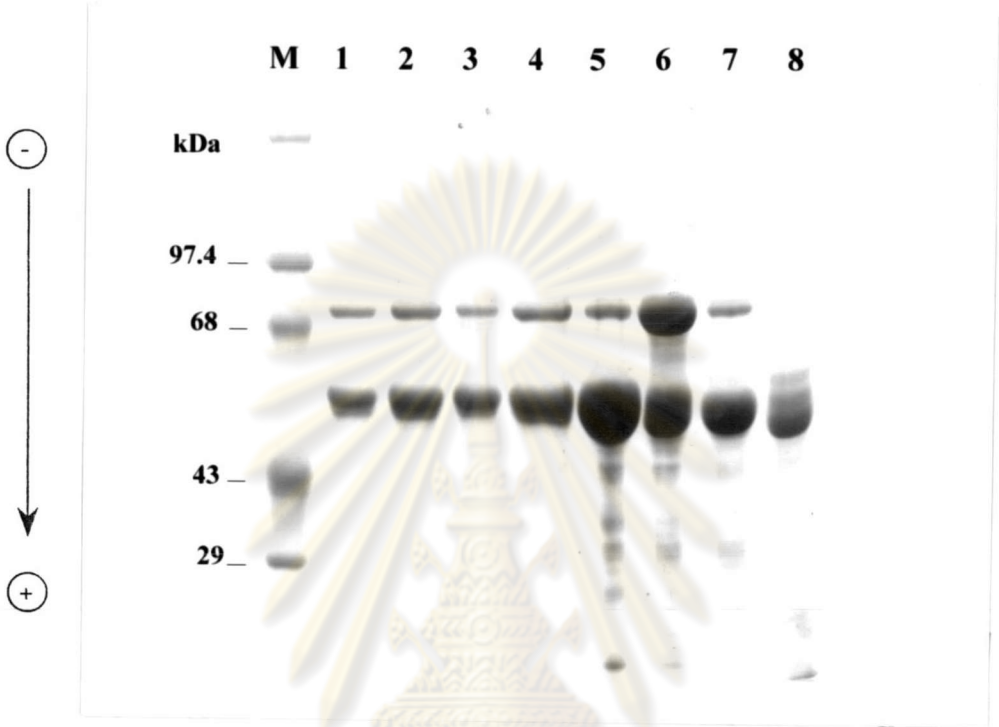


Figure 3.5 10% SDS-PAGE analysis of AcMRJPs precipitation under various percentages of saturated ammonium sulfate.

Lane M = Standard protein markers
Lane 1 = Crude RJ protein
Lane 2 –8 = RJ protein precipitated at 20%, 30%, 40%, 50%, 60%, 70%, and 80% saturated ammonium sulfate, respectively.

Different precipitation rate of each protein was observed (Figure 3.5). The 50 kDa protein showed the heaviest staining in the 40-70 % saturation fraction (lane 5-7). The availability of proteins with sizes smaller than 50 kDa and a larger protein (more than 97.4 kDa in size) were also found in the 40-50 % saturated fraction (lane 5). The 80 kDa protein exhibited the highest stained intensity in the 50-60% saturation fraction (lane 6). The ability to be precipitated in a broad range of ammonium sulfate saturation resulted in a possible loss of parts of MRJPs during salting out. Therefore, crude RJ was directly used as a starting material for purification of AcMRJPs by column chromatography.

3.2.3 DEAE cellulose column chromatography

Crude RJ dissolved in the phosphate buffer was dialyzed against 0.1 M EDTA followed by deionized water and adjusted to 20 mM Tris-HCl pH 7.5 and 1 mM EDTA. Protein recovery was approximately 54.24 % after dialysis. The dialyzed solution was loaded onto a DEAE cellulose column and chromatographically analyzed (Figure 3.6). Two peaks (DEAEP1 and DEAEP2) of bound protein were eluted at 0.05 M and 0.15 M NaCl, respectively. The amount of protein DEAEP1 and DEAEP2 was 14.20 % and 19.27 % of total protein eluted from the column, respectively. Protein from each peak was then analyzed by 10 % SDS-PAGE (Figure 3.7). Three bands (55, 80 and 82 kDa) were observed from the protein DEAEP1 where 80 and 82 kDa band was much fainter than that of a 55 kDa. The 55 kDa bands also comigrated band with another band exhibiting a slightly smaller molecular weight. In contrast, only a single band with the molecular weight of 50 kDA was observed in the protein DEAEP2.

N-terminal and internal amino acid sequences of these proteins indicated high similarity of proteins DEAEP1 and DEAEP2 to AmMRJP2 and AmMRJP1, respectively (Table 3.7).

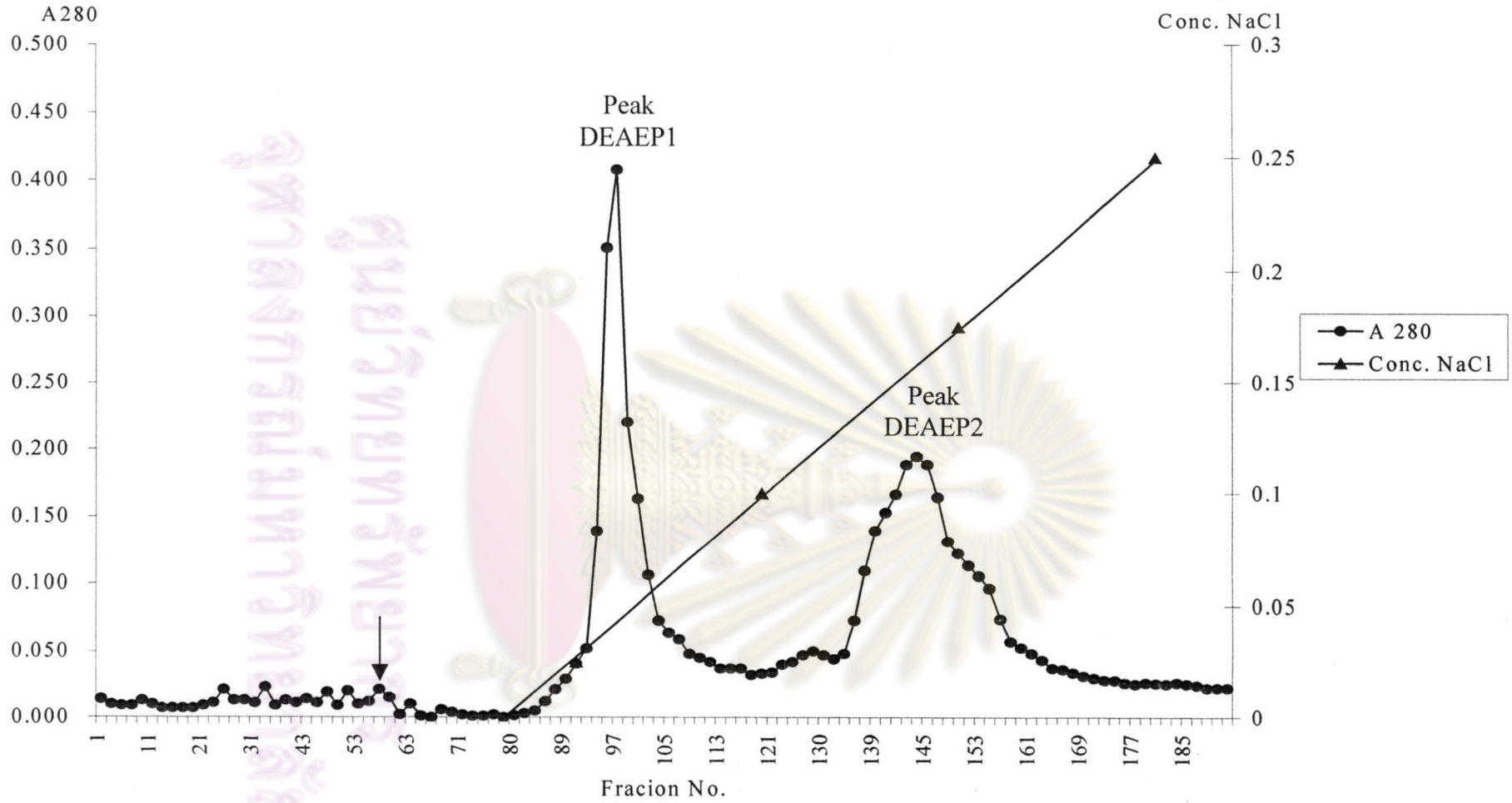


Figure 3.6 Chromatographic profile of *A.cerana* crude RJ on DEAE cellulose column (2.5 ∅x 12 cm.) at pH 7.5. Aliquots of 2 ml were collected. An arrow indicates the starting point of the NaCl gradient.

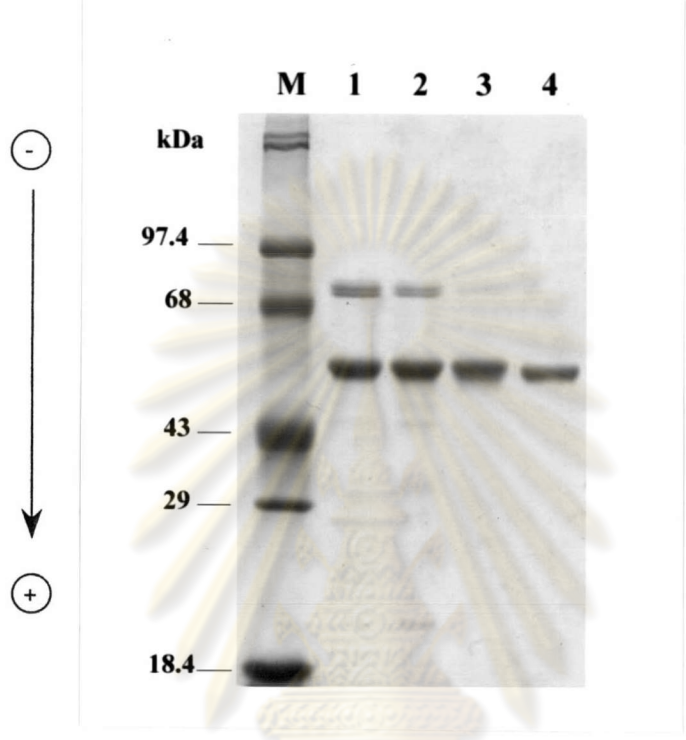


Figure 3.7 10% SDS-PAGE analysis of AcMRJPs after purified by DEAE cellulose column chromatography.

Lane M = Standard protein markers
Lane 1 = Crude RJ protein
Lane 2 = Dialyzed fraction
Lane 3, 4 = Protein peaks DEAEP1 and DEAEP2, respectively.

3.2.4 Protein purification using Q-Sepharose and Sephadex-G200 column Chromatography

Q-sepharose is a stronger anionic exchanger which was used instead of DEAE-cellulose. Crude RJ was dialyzed against 20 mM Tris-HCl pH 7.5 and 1 mM EDTA. The percentage of protein recovery after dialysis was 90.95 %. Any unbound proteins were not found following protein monitoring at A_{280}.

Three peaks (A, B and C) of AcMRJPs were eluted at 0.075 M, 0.175 M and 0.250 M NaCl, respectively (Figure 3.8). SDS-PAGE analysis revealed that peak A showed three bands of protein with the denatured molecular weight of 55, 80 and 82 kDa while peaks B and C showed an identical denatured molecular weight of 50 kDa (Figure 3.9). Notably, faint band at approximately 80 kDa was co-purified in the peak B. The peak C also contained very faint bands exhibiting the molecular weight of 80 kDa and 115 kDa. Fractions representing the same peak were pooled and concentrated for a Sephadex G-200 chromatographic separation. The total protein in peaks A, B and C was 22.79 %, 3.15 % and 38.44 % of the total protein of the starting crude RJ, respectively.

Concentrated proteins peak A, B and C was separately loaded onto a Sephadex G-200 column. Only the Q-sepharose-purified peak A protein could be further separated to sub-peak A1 and A2 while proteins B and C did not show any sub-peak (subsequently called peak B1 and C1 to indicate an additional purification step, respectively). The chromatographic profile is shown in Figure 3.10. Using SDS-PAGE analysis (Figure 3.11), denatured protein peaks B1 and C1 showed a single band with the equal molecular weight of 50 kDa whereas denatured protein peaks A1 and A2 showed doublet bands at approximately 80 kDa and 55 kDa, respectively.

During purification of the A1 protein through a Sephadex G-200 column chromatography, degradation of the A1 was found. Degradation of the A1 protein under different conditions was studied, results revelaed degradation of the protein A1 when stored in the purification buffer (20 mM Tris-HCl pH 7.5 and 1 mM EDTA) A similar effect was found when the protein A1 was stored in the same buffer containing 0.5 mM DDT or 5 mg of protein A2 (a co-purified protein from Q-Sepharose). Only the buffer supplemented with 0.5 mM PMSF eliminated degradation of this protein.

As a result, PMSF was added to a pooled Q-sepharose-purified protein A before further chromatographic separation by Sephadex G-200 (Figure 3.12).

Purification of a pooled protein peak A through a Sephadex G-200 column did not show complete separation of protein A1 and A2 (Figure 3.10a). For further purification of these proteins, ammonium sulfate added to a 50 % - 60 % saturated level showed high quantity of a 80 kDa protein (in this case representing protein A1) was used to purify through Sephadex G-200 column. The chromatographic profile is shown in Figure 3.13. Protein peak Y of this profile was nearly found in the same mobility as that of the protein A1 after Q-Sepharose and Sephadex G-200 column chromatography and the same molecular weight after analysis with 10 % SDS PAGE (Figure 3.14). Moreover, other proteins (peaks X and Z) were also isolated. Two protein peaks exhibiting the native molecular weight of 300 kDa and 55 kDa were observed. These proteins showed identical mobility on a Sephadex-G200 to that of proteins C1 and either A2 or B1, respectively.

To determine native molecular weight of proteins A1, A2, B1 and C1, Sephadex G-200 column was calibrated with standard molecular weight markers. A molecular weight calibration curve was constructed (Figure 3.15). The native molecular weight of protein peak A1, A2, B1 and C1 was examined and estimated to be 115 kDa, 55 kDa, 50 kDa and 300 kDa, respectively. When the molecular weight of denatured and native forms of each protein was compared, results indicated that A1 and C1 naturally were present in dimeric and oligomeric forms while A2 and B1 exhibited natural monomeric forms.

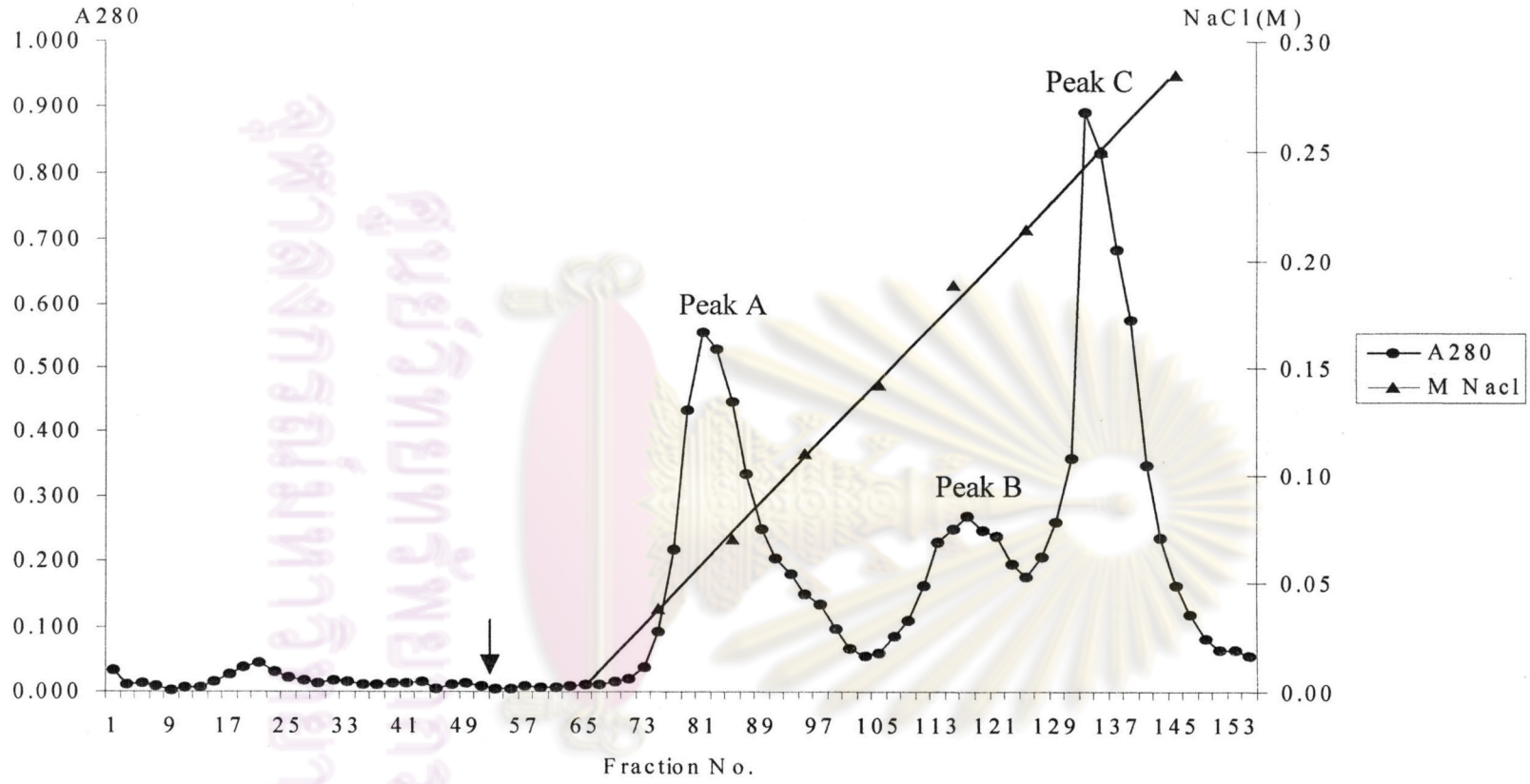


Figure 3.8 Chromatographic profile of *A.cerana* crude RJ on Q-Sepharose column (1.25∅ x 20 cm.) at pH 7.5. Aliquots of 2 ml were collected. An arrow indicates the starting point of NaCl gradient.

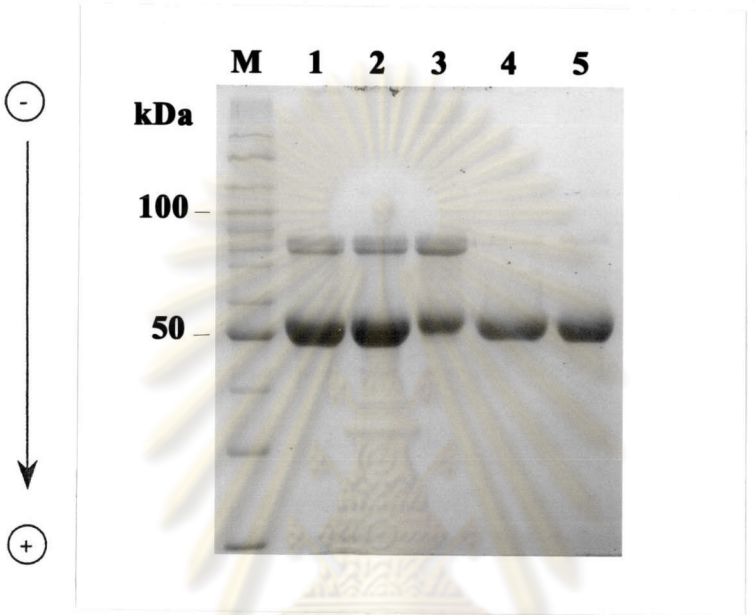


Figure 3.9 10% SDS-PAGE analysis of AcMRJPs after purified by Q-Sepharose column chromatography.

Lane M = A BanchMark™ protein Ladder
Lane 1 = Crude RJ protein
Lane 2 = Dialyzed fraction
Lane 3, 4, 5 = Protein peaks A, B and C, respectively.

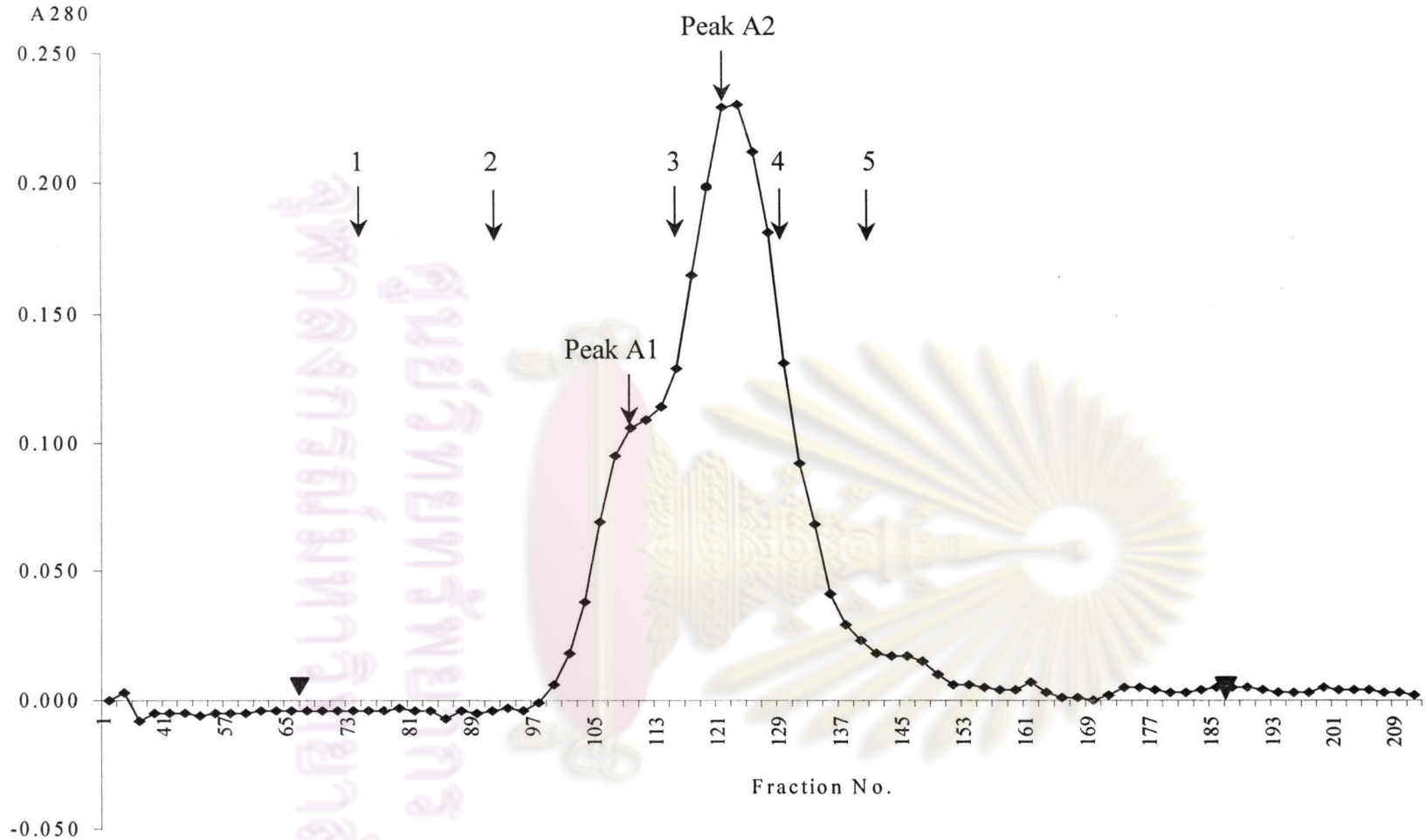


Figure 3.10(a) Chromatographic profile of Q-Sepharose purified protein peak A on Sephadex G-200 column (2.5 ∅ x 80 cm.) at pH 7.5. Aliquots of 2 ml were collected. Triangles indicate void and total volumes, repectively. Arrows (with numbers) indicate the number of collected fraction containing protein markers (1 = ferritin, 2 = catalase, 3 = BSA, 4 = ovalbumin and 5 = chymotrypsinogen).

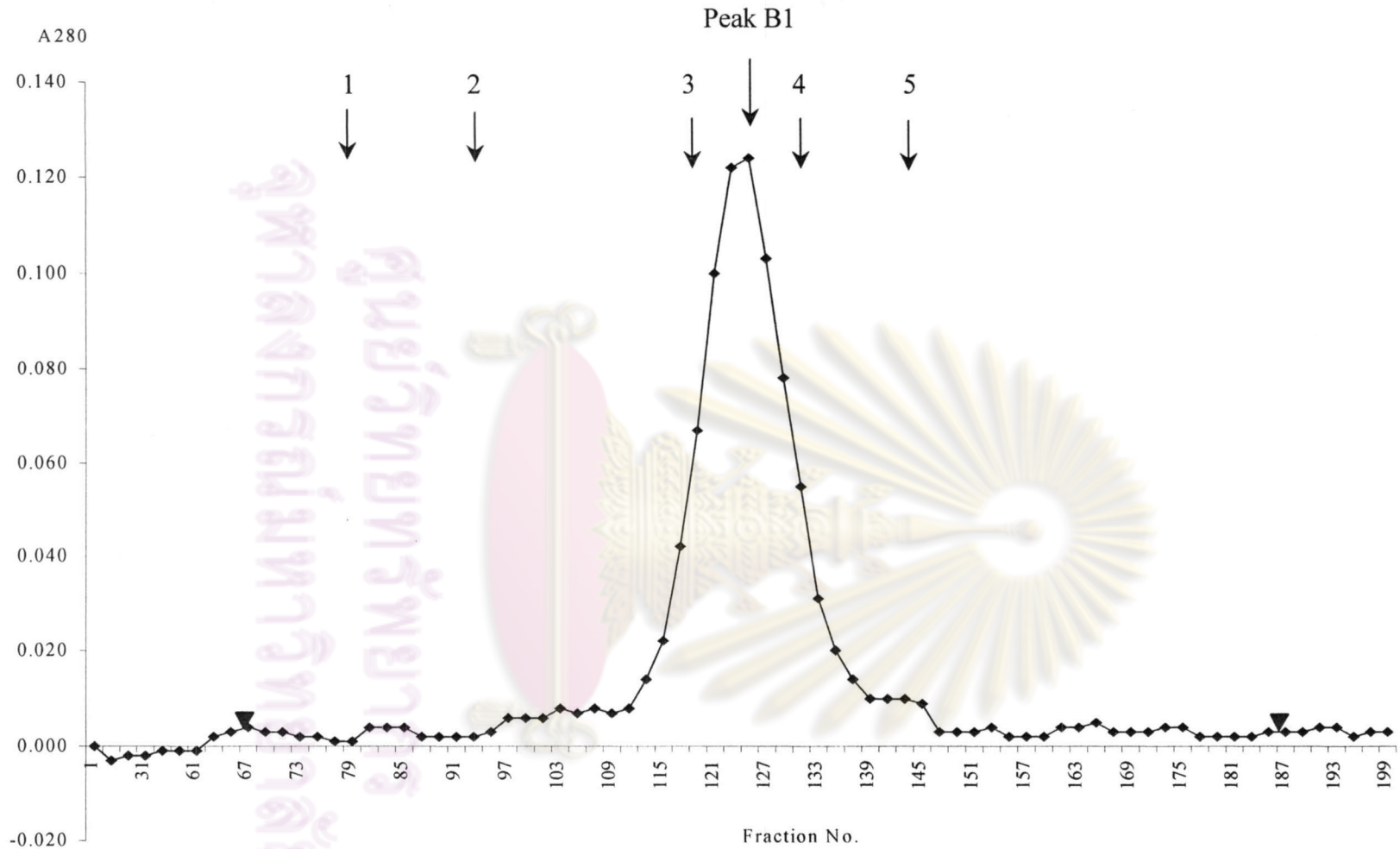


Figure 3.10(b) Chromatographic profile of Q-Sepharose purified protein peak B on Sephadex G-200 column (2.5 ∅ x 80 cm.) at pH 7.5. Aliquots of 2 ml were collected. The void and total volumes and standard molecular weight marker were indicated as in Figure 3.10(a.).

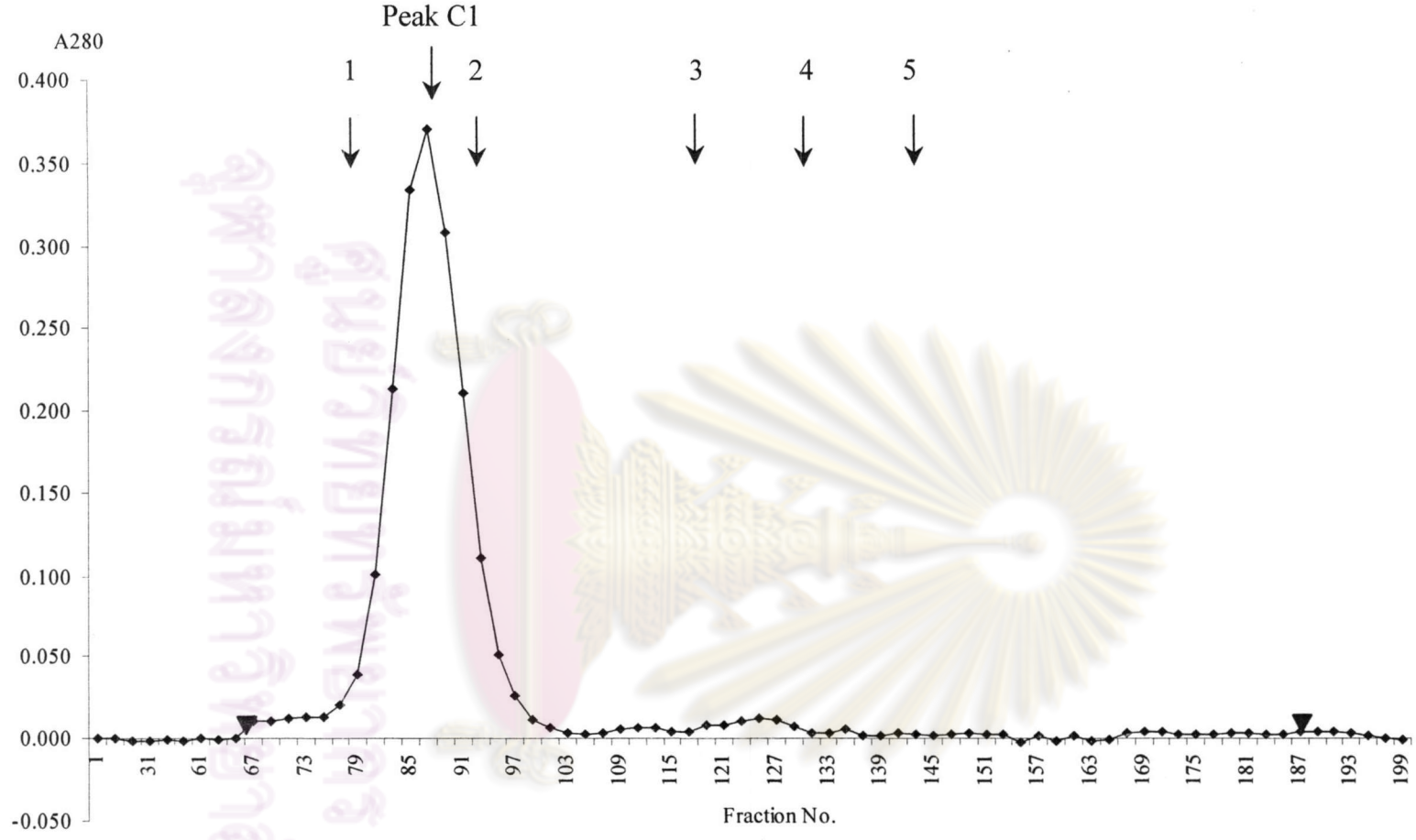
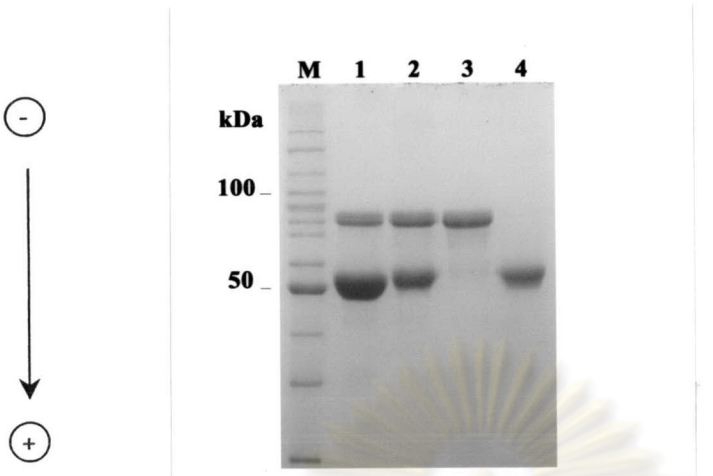


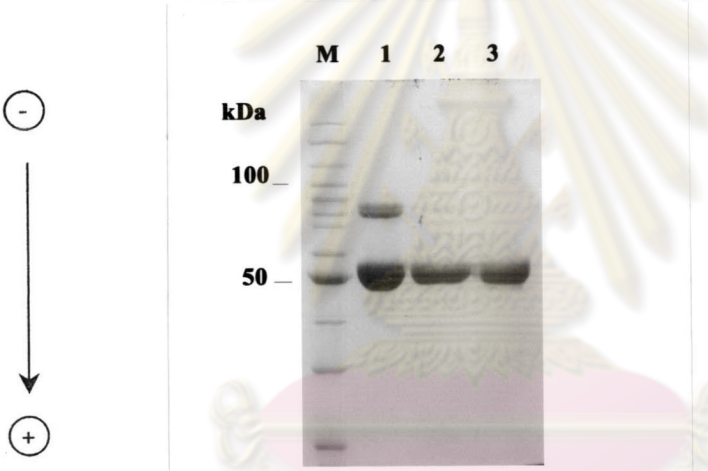
Figure 3.10(c) Chromatographic profile of Q-Sepharose purified protein peak C on Sephadex G-200 column (2.5 ∅ x 80 cm.) at pH 7.5. Aliquots of 2 ml were collected. The void and total volumes and standard molecular weight marker were indicated as in Figure 3.10(a.).

Figure 3.11 10% SDS-PAGE analysis of AcMRJPs after purified by Q-sepharose followed by Sephadex G-200 column chromatography.

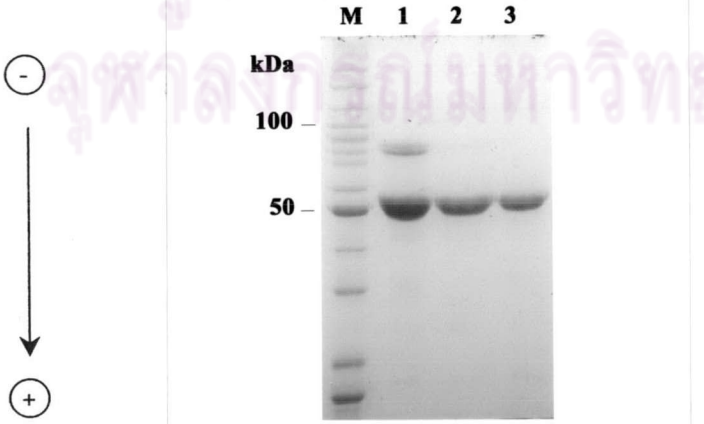
Lanes M panels a, b and c = A BanchMark™ protein Ladder
Lane 1 panels a, b and c = Crude RJ protein
Lane 2 panels a, b and c = Q-Sepharose purified protein A, B and C, respectively.
Lanes 3 and 4 panel a = Protein A1and A2, respectively
Lane 3 panel b = Protein B1
Lane 3 panel c = Protein C1



(a)



(b)



(c)

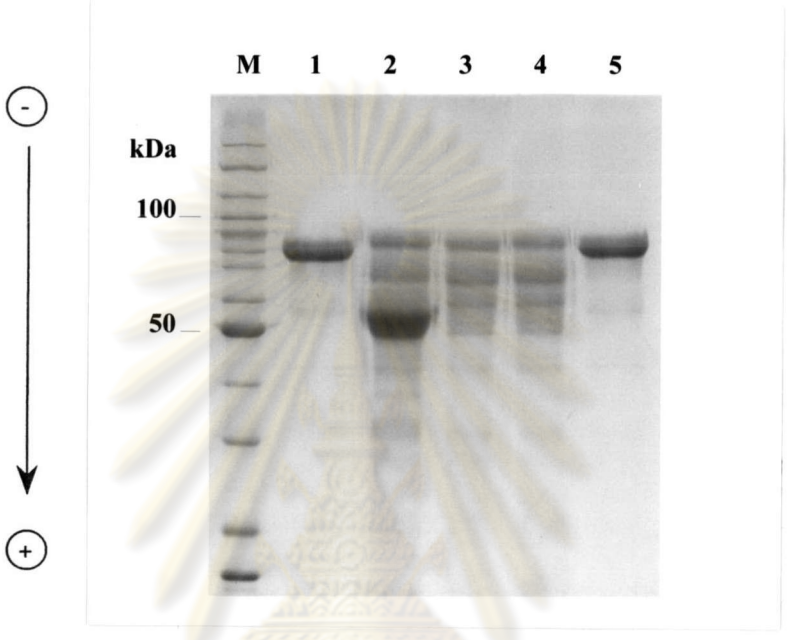


Figure 3.12 10% SDS-PAGE analysis illustrating degradation (lane 2-4) and stability (lane 1) of protein A1 when mixed with protein A2 (lane 2) or treated with EDTA (lane 3), DDT (lane 4) and PMSF (lane 5) in Tris-HCl pH 7.5.

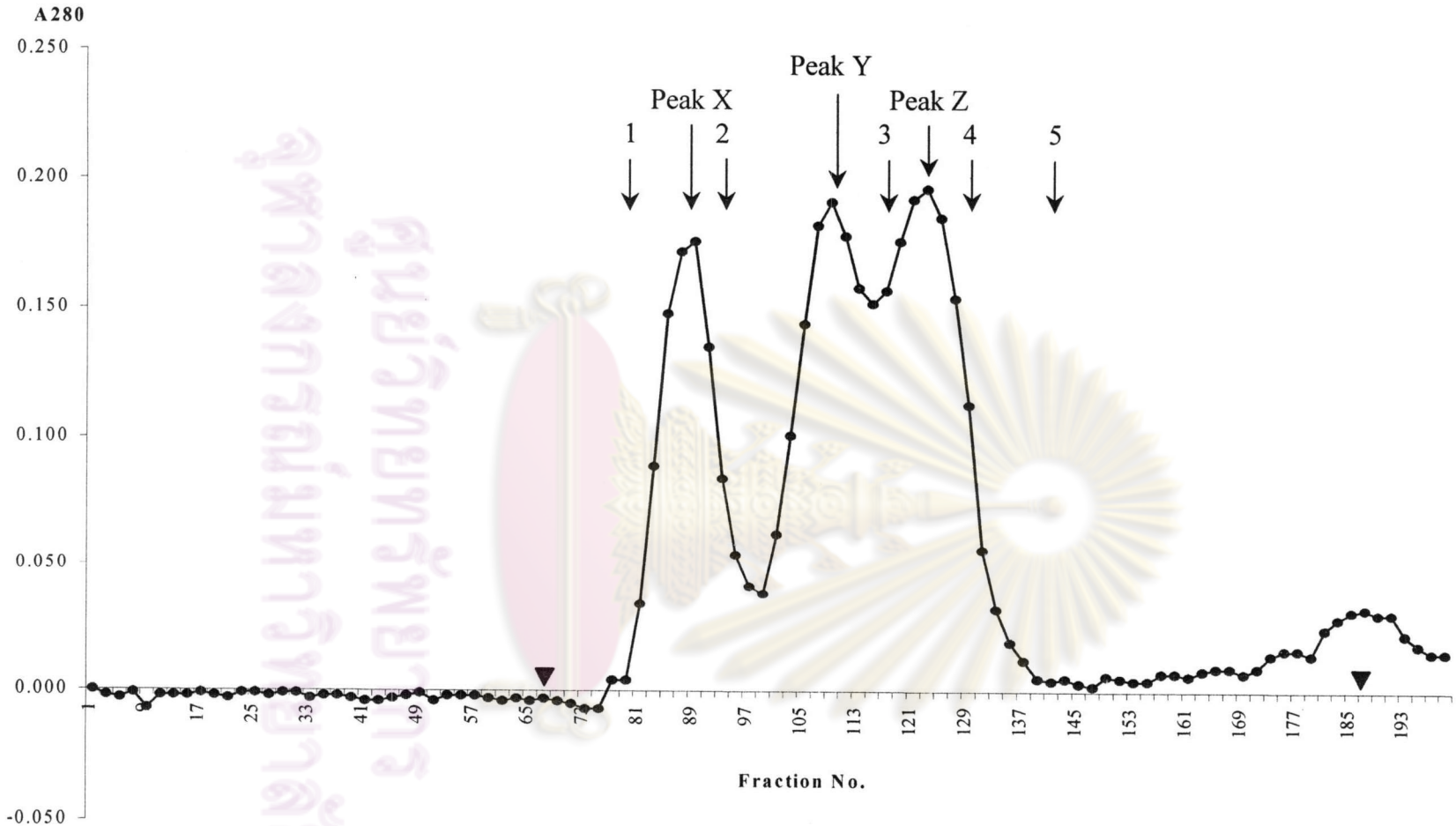


Figure 3.13 Chromatographic profile of 50–60 % saturated ammonium sulfate of crude RJ purified on Sephadex G-200 column (2.5 ∅ x 80 cm.) at pH 7.5. Aliquots of 2 ml were collected. The void and total volumes and standard molecular weight marker was indicated the same as Figure 3.10(a.).

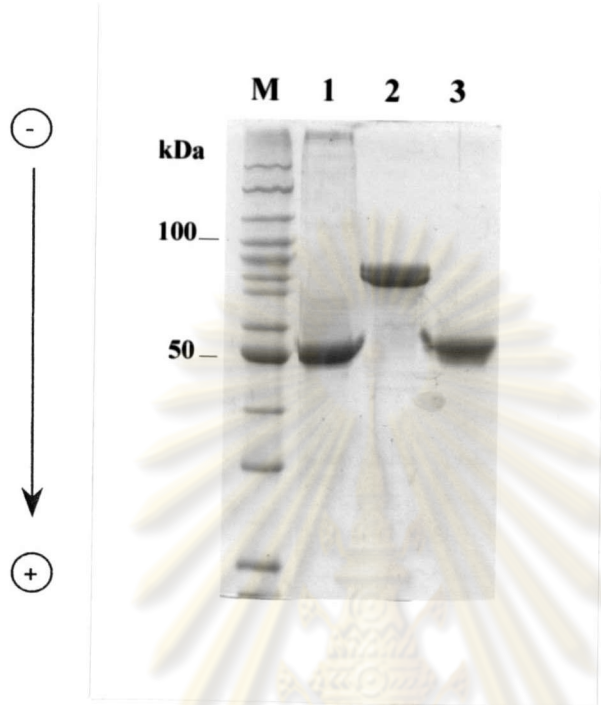


Figure 3.14 10 % SDS-PAGE analysis of AcMRJPs after purified by 50-60 % saturated ammonium sulfate followed by Sephadex G-200 column chromatography.

Lane M = A BanchMark™ protein Ladder
Lane 1 = Protein peak X identical the mobility to C1
Lane 2 = Protein peak Y identical the mobility to A1
Lane 3 = Protein peak Z identical the mobility to A2 or B1

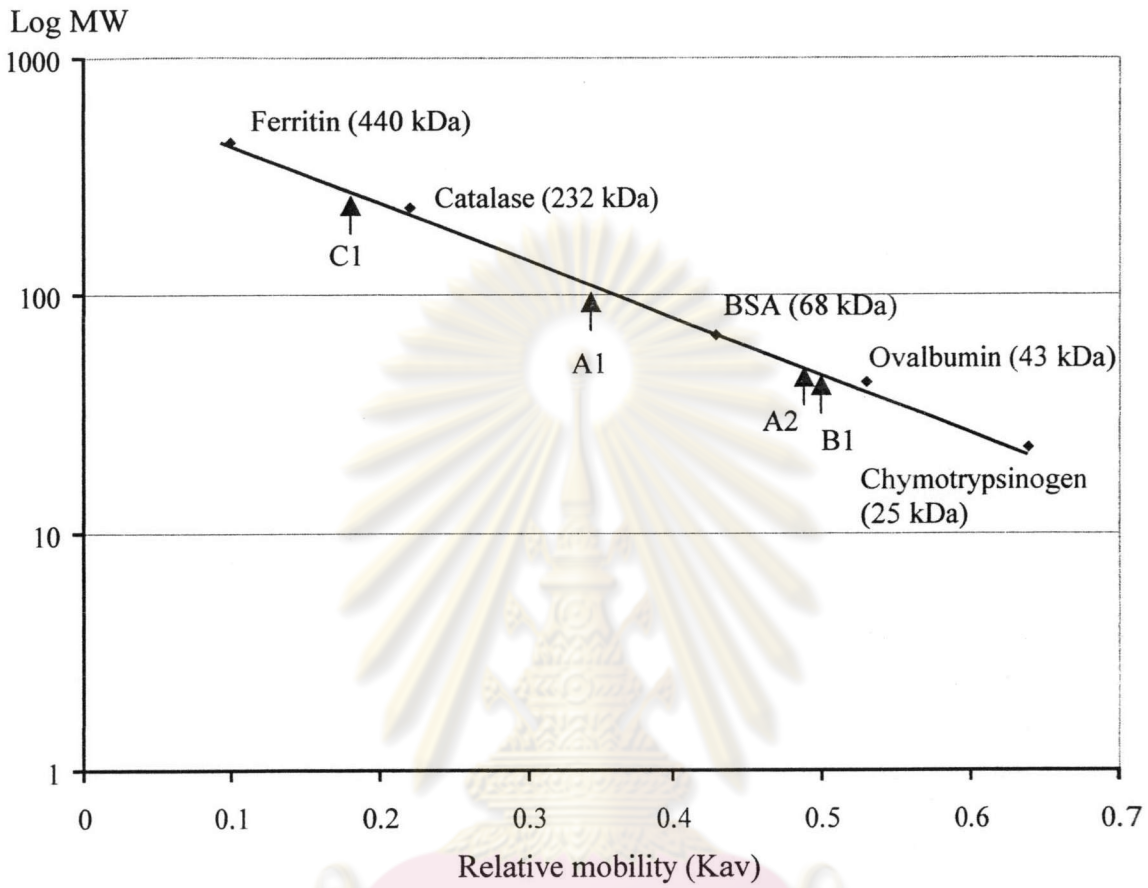


Figure 3.15 Calibration curve for estimation of native molecular weight of purified protein chromatographically analyzed on Sephadex G-200 column used in this study. Arrows indicate relative mobility of purified proteins A1, A2, B1 and C1.

3.2.5 Determination of carbohydrate side chain

An aliquot of purified proteins A1, A2, B1 and C1 from Sephadex G-200 column was fractionated through 10 % SDS-PAGE and stained with PAS reagents and Commassie blue (Figure 3.16). All purified proteins were positively stained with PAS reagents, as was transferrin (a positive control). The result suggested that these purified proteins were glycoproteins. Digestion of *N*-glycosidase F shifted the mobility of these proteins to smaller sizes (Figure 3.17). This suggested that *N*-linked oligosaccharides should have occurred in purified proteins of *A. cerana* RJ. Results were congruent to those predicted from deduced amino acid sequence inferred from cloned AcMRJP nucleotide sequences (3.3.1 and 3.3.2).

3.2.6 Determination of isoelectric point (pI) of AcMRJPs

An aliquot of purified proteins A1, A2, B1 and C1 from Sephadex G-200 column was analyzed for their pI values using IEF gel electrophoresis (Figure 3.18). Comparing with the relative mobility of standard pI markers (Figure 3.19), purified protein A1 and A2 were basic proteins having pI values of of 7.4-8.4 and 7.0-8.2, respectively, while purified proteins B1 and C1 were acidic proteins having pI values of 5.2-5.7 and 5.7, respectively. All purified protein except C1 showed several bands with different pI values.

3.2.7 Effects of temperature on stability of AcMRJPs

An aliquot of purified protein A1, A2, B1 and C1 from Sephadex G-200 column was incubated under various temperatures (–20 °C, 4 °C and 37 °C for 1 to 30 days) in 20 mM Tris-HCl pH 7.5 as described in 2.12.5. The result is shown in Figure 3.20, 3.21 and 3.22.

All purified proteins exhibited the highest stability when incubated at –20 °C. Initially, protein A1 was not stable after stored at 4 °C. The band intensity of protein A1 was decreased by 50 % compared to that of the control at 4 °C for 7 days. In addition, a more rapid degradation of the protein A1 was observed at 37 °C as degradation of this protein was nearly complete after incubated for 3 days.

Disregarding the protein A1, electrophoretic patterns of the remaining proteins were identical throughout the storage time at 4 °C. Proteins A2, B1 and C1 did not show any degradation when incubated at 4 °C for 15 days. At 37 °C, purified proteins A1 lost its stability after incubated at this temperature for only 1 days whereas protein A2 and C1 lost their stability after 3 days. Basically, the protein B1 was the most stable RJ protein followed by A2, C1 and A1 in order.

3.2.8 *N*-terminal amino acid sequences of AcMRJPs

N-terminal amino acid sequences of undigested and lysyl endopeptidase digested AcMRJPs were used to assign their protein families by compared with that of *A. mellifera*. Sequences of proteins DEAEP1 and DEAEP2 purified from a DEAE cellulose column chromatography were highly similar to AmMRJP2 and AmMRJP1, respectively. Similarity of amino acid sequences of AcMRJP1 and AcMRJP2 compared to their homologues were 70-89 % and 92-100 %, respectively (Table 3.7).

When AcMRJPs purified by Q-Sepharose were re-chromatographically loaded on DEAE Cellulose column. Purified proteins A2 and C1 were eluted from the column at the same concentration of NaCl as were DEAEP1 and DEAEP2, respectively. Therefore, only the remaining protein A1 and B1 were further sequenced. *N*-terminal amino acid sequences of proteins A1 and B1 showed 90% and 80% similarity to AmMRJP3 and AmMRJP1, respectively. The protein DEAEP2 (referred as C1) and B1 were recognized as AmMRJP1 with monomeric (B1) and oligomeric (C1). Some amino acid residues of B1 and C1, for example, asparagine (N) and glutamic acid (E) at the 1st and the 9th position C1 *N*-terminal sequences, were different from Serine (S) and aspartic acid (D) of B1. Polymorphism at the 1st residue (G and A) of the protein A1 was also found.

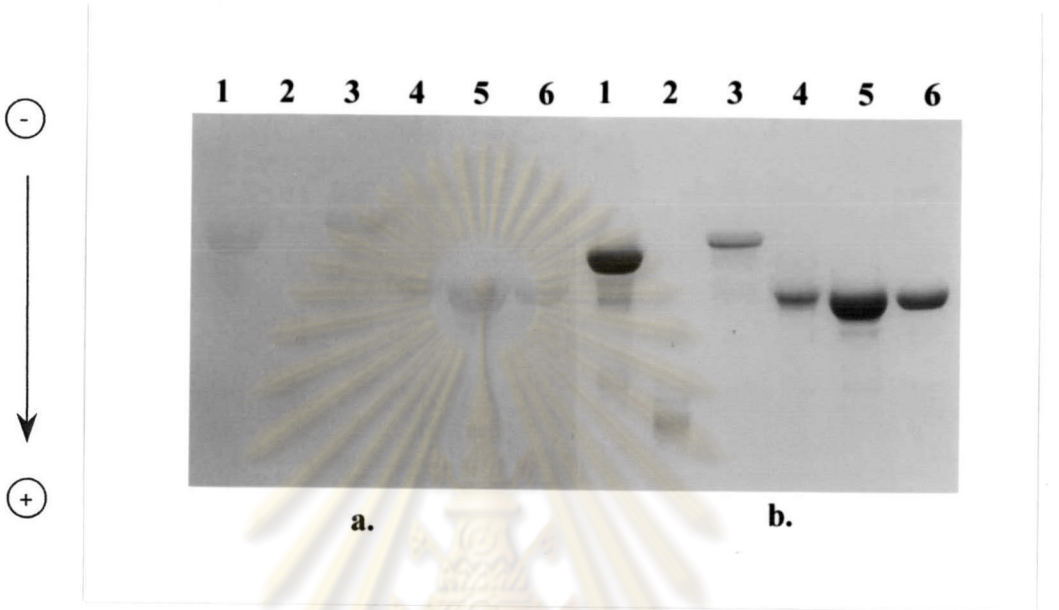


Figure 3.16 10% SDS-PAGE showing identification of purified AcMRJPs as glycoproteins. Purified proteins A1, A2, B1 and C1 loading in lanes 3 – 6, respectively were stained with PAS (panel a.) and Coomassie blue (panel b.). Transferrin (lane 1) and Haemoglobin (lane 2) were included as the positive and negative control, respectively.

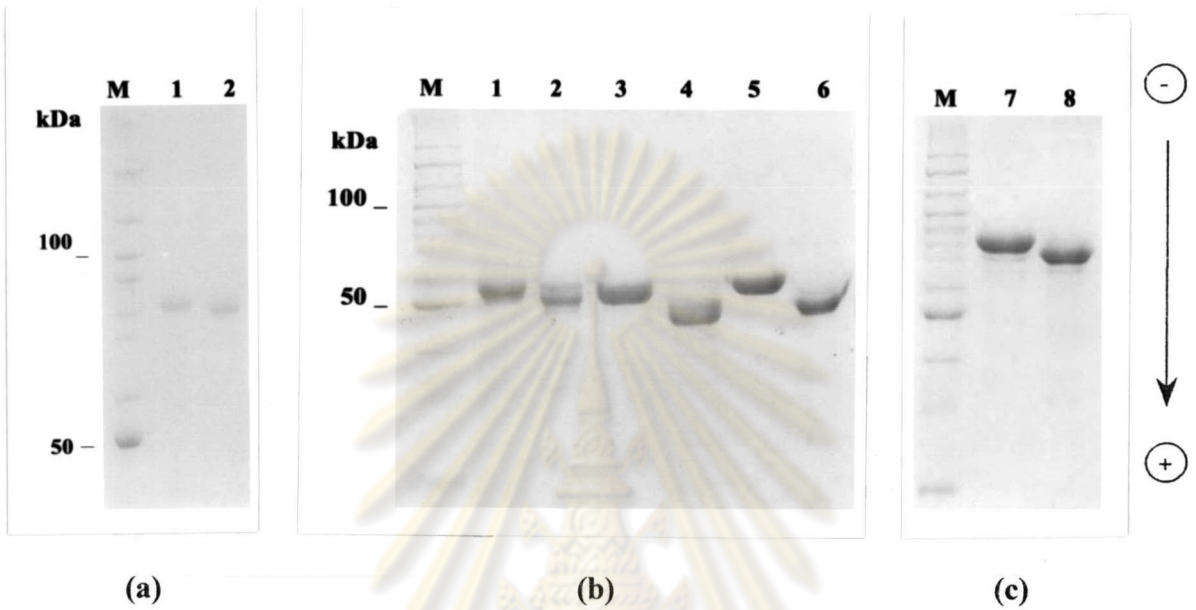


Figure 3.17 Undigested and PNGaseF digested purified AcMRJPs were analyzed on 7.5% (panel a) and 10% SDS-PAGE (panel b and c). Lanes M = A BanchMark™ protein Ladder.

Panel a	Lane 1	Undigested purified protein A1
	Lane 2	Digested purified protein A1
Panel b	Lanes 1,3,5	Undigested purified protein A2, B1, C1
	Lanes 2,4,6	Digested purified protein A2, B1, C1 and
Panel c	Lane 7,8	Undigested and digested Transferrin (positive controls)

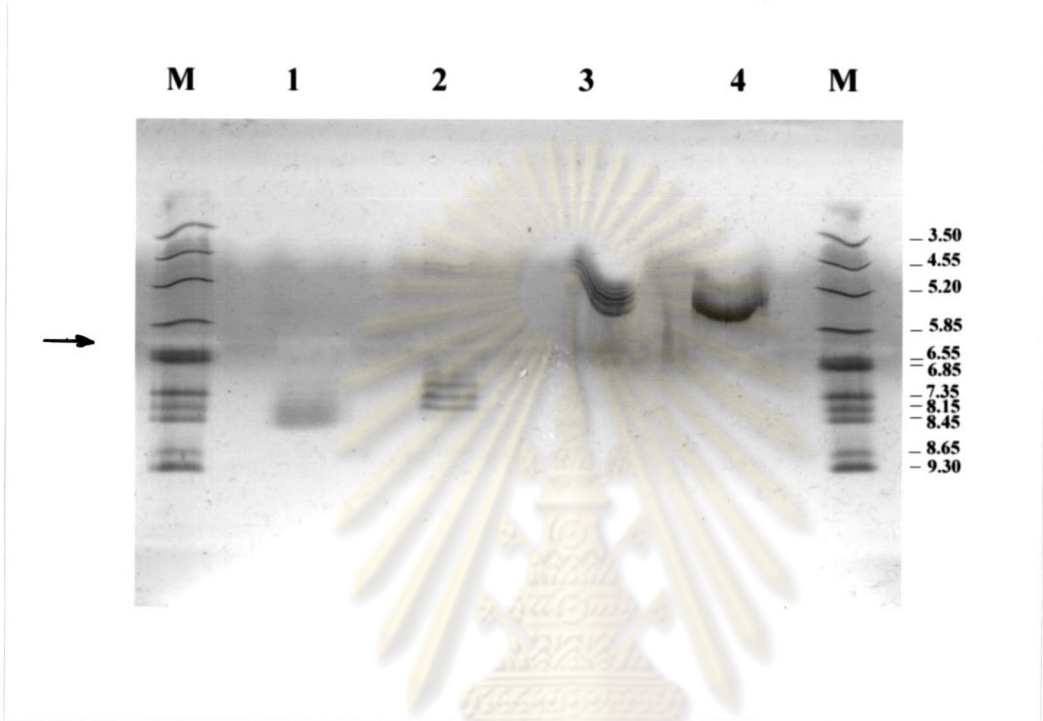


Figure 3.18 Isoelectrofocusing analysis of purified AcMRJPs using ampholyte pH 3.0-10.0. An arrow indicates the position of sample strips.

Lane M = A standard pI protein marker
Lanes 1,2,3,4 = Proteins A1, A2, B1 and C1, respectively.

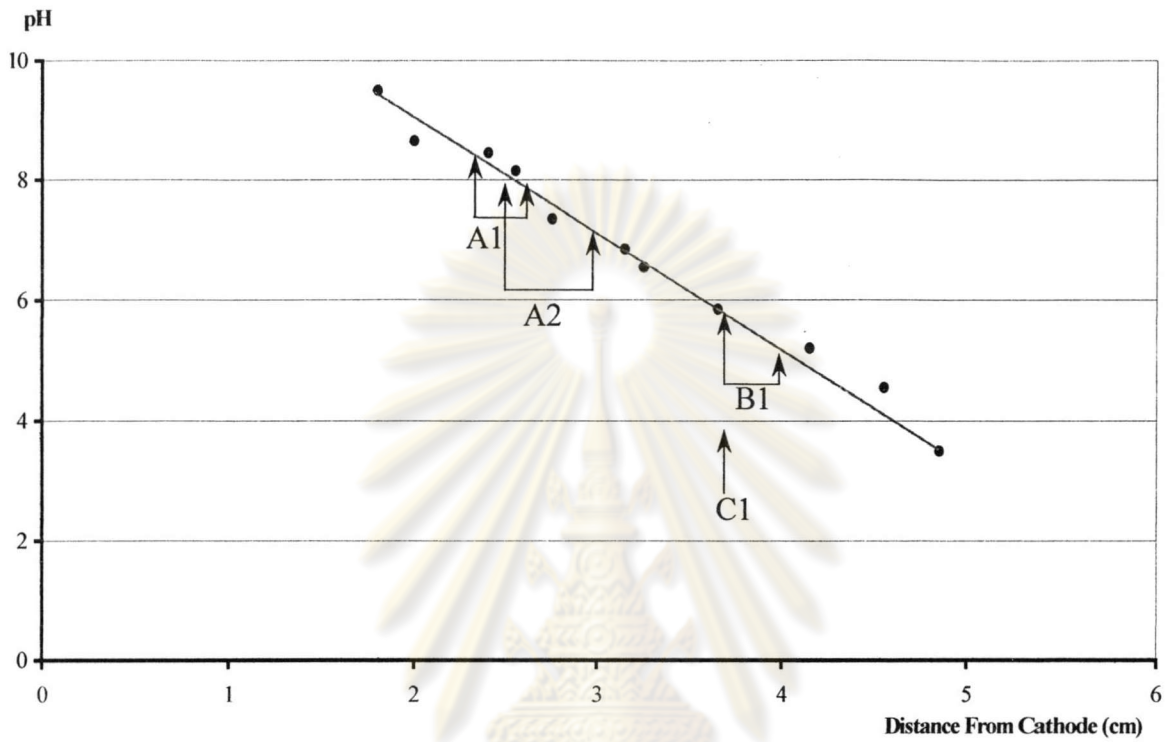


Figure 3.19 The calibration curve for pI estimation on IEF gel. Arrows indicate the mobility from the cathode of each protein A1, A2, B1 and C1.

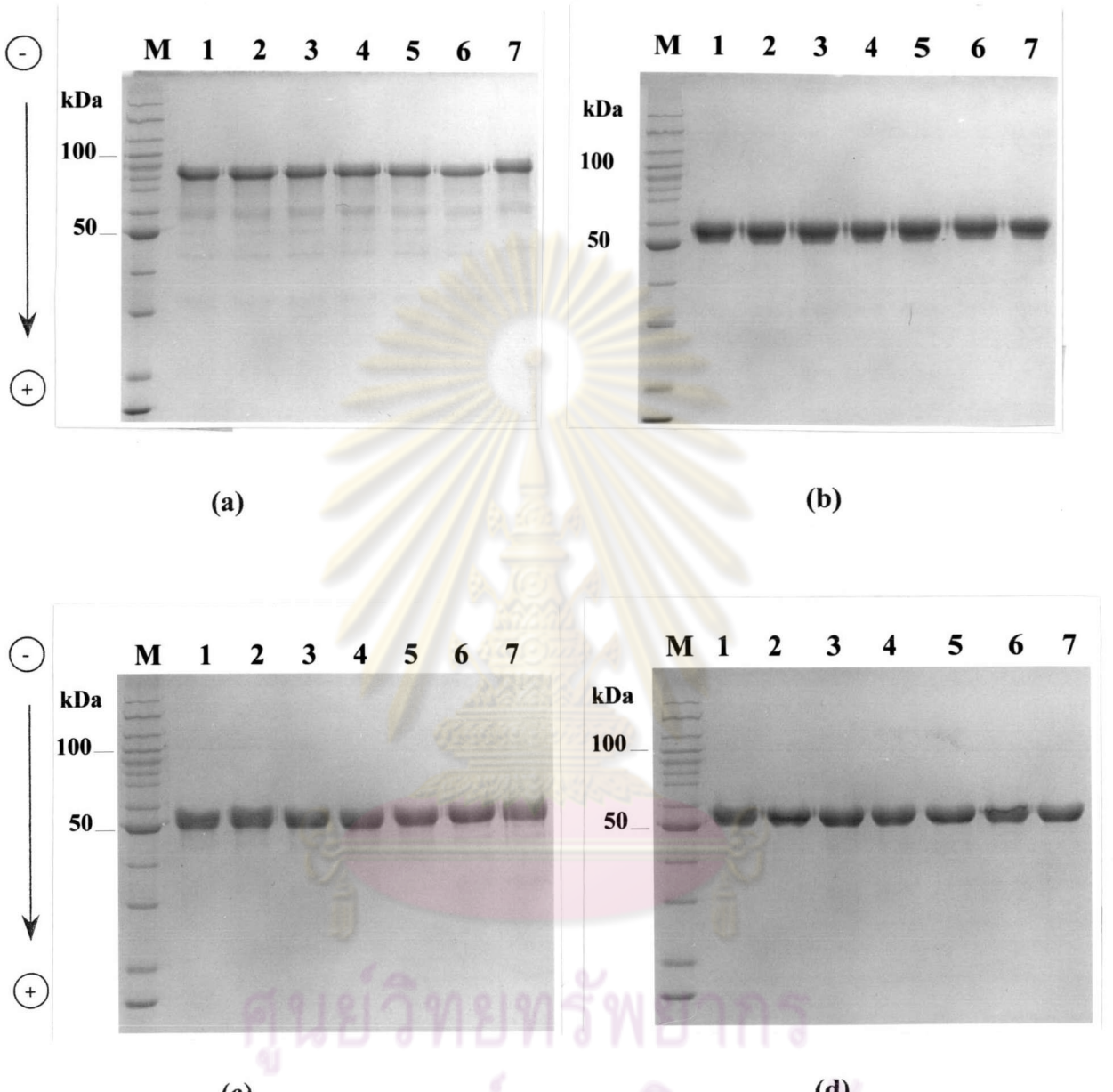


Figure 3.20 Effects of temperature at (-20 °C) on stability of purified AcMRJPs peak A1, A2, B1 and C1 (panel a, b, c and d, respectively)

Lanes M = A standard protein marker

Lanes 1 - 7 = purified proteins incubated for 0, 1, 3, 5, 7, 15, 30 days, respectively.

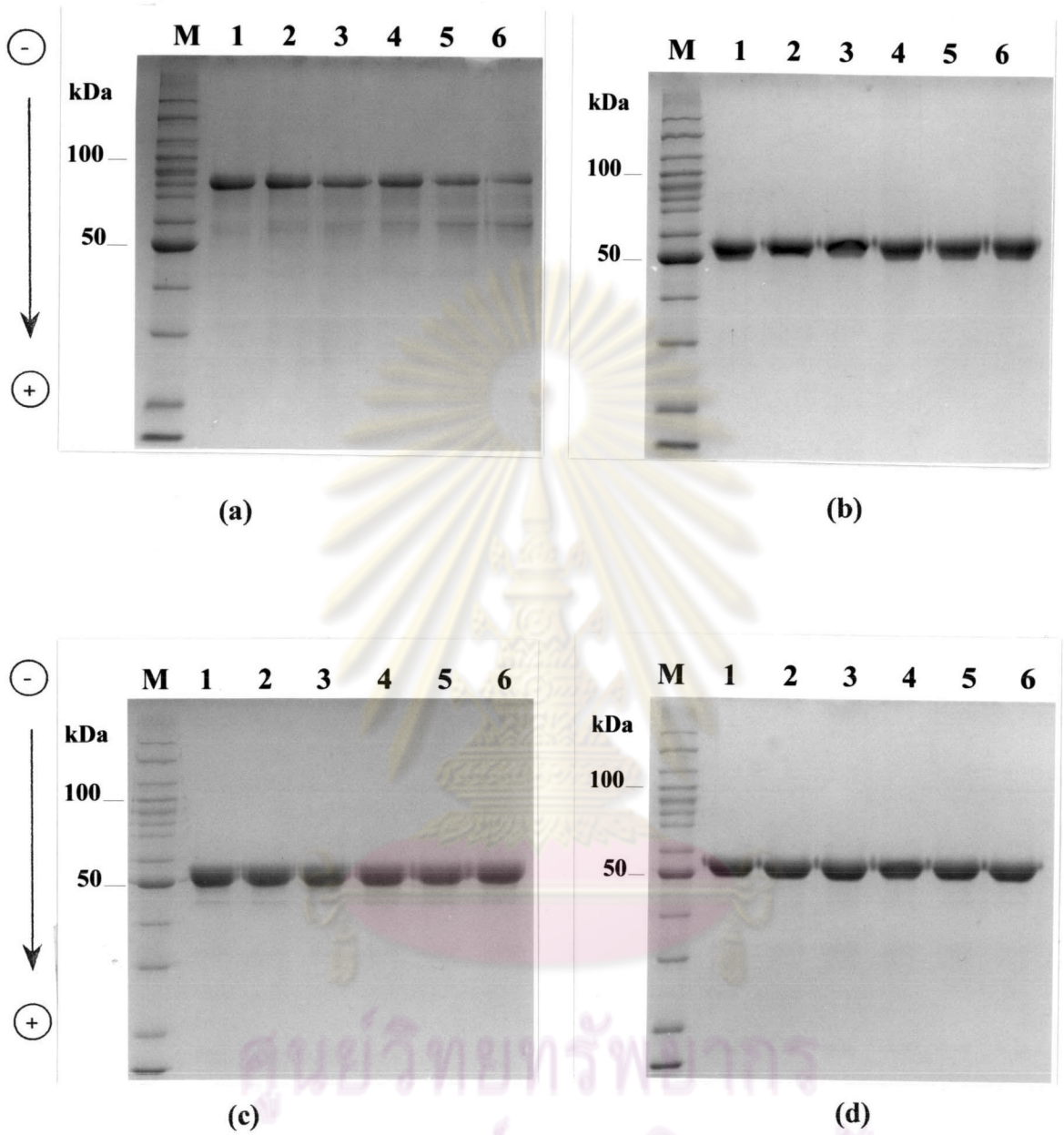


Figure 3.21 Effects of temperature (4 °C) on stability of purified AcMRJPs peak A1, A2, B1 and C1 (panels a, b, c and d, respectively)

Lanes M	Standard protein marker
Lanes 1 – 6	Purified proteins incubated for 0, 1, 3, 5, 7, 15 days, respectively.

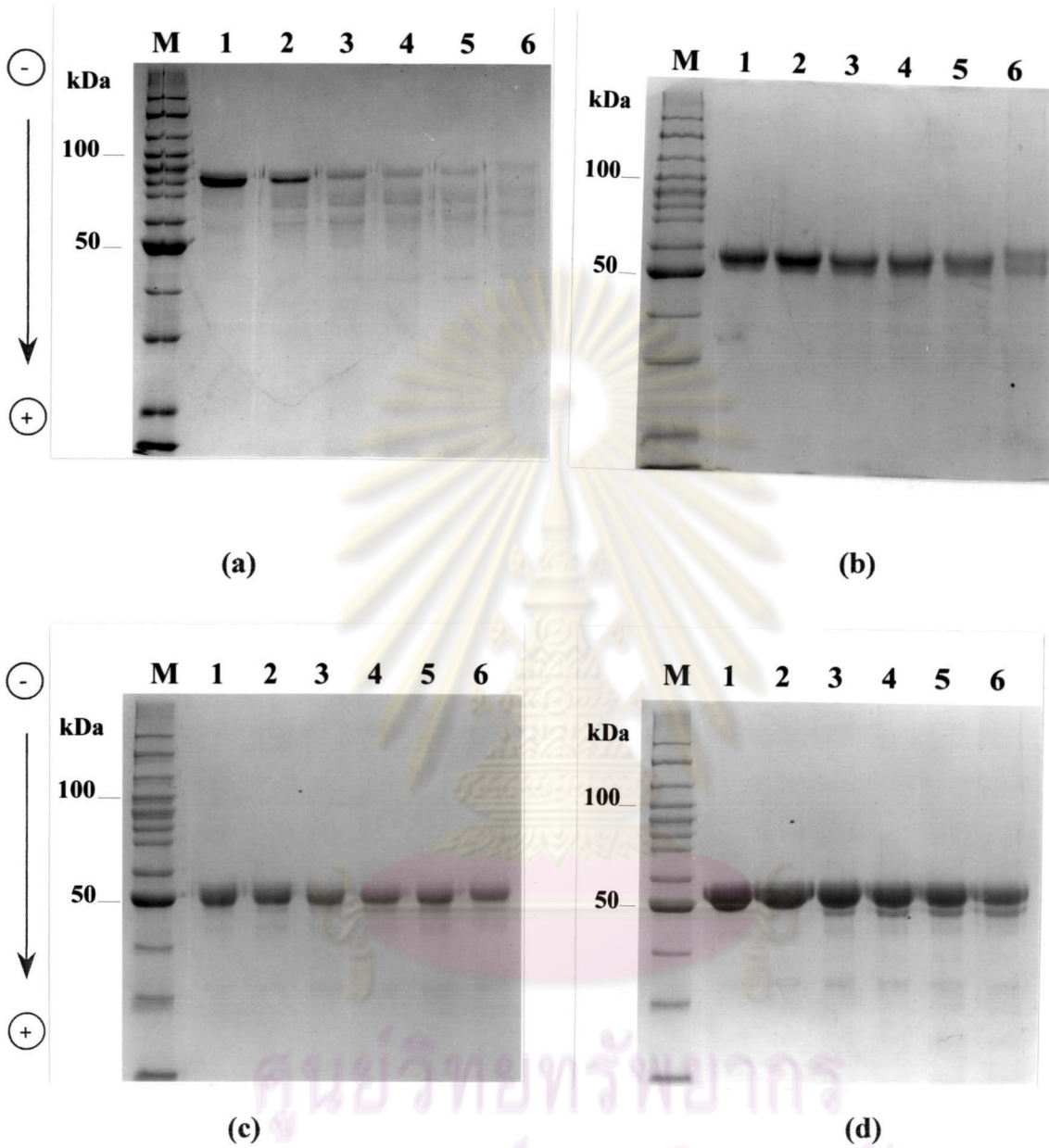


Figure 3.22 Effects of temperature (37 °C) on stability of purified AcMRJPs peak A1, A2, B1 and C1 (panels a, b, c and d, respectively)

Lanes M	A standard protein marker
Lanes 1 – 6	Purified proteins incubated for 0, 1, 3, 5, 7, 15 days, respectively.

Table 3.7 *N*-terminal amino acid sequences of undigested and lysyl endopeptidase digested AcMRJPs.

Table 3.7 *N*-terminal amino acid sequences of undigested and lysyl endopeptidase digested AcMRJPs.

Peak	Sequence	Homology compared to AmMRJP
A1	(G/A)AVNHQRKSA	AmMRJP3 : 90%
DEAEP1 (A2)	LHVFDLK	AmMRJP2 : 100%
	GDALIVYQNSDDFHR	AmMRJP2 : 100%
	NLENSLNVIHEWK	AmMRJP2 : 100%
B1	SILRGESLDK	AmMRJP1 : 80%
DEAEP2 (C1)	NILRGESLEKXLSVLHEIXF	AmMRJP1 : 70%
	IGDGGPLLQPYPDXSFAK	AmMRJP1 : 89%
	SAVLFFGLVGDSALGXXNEHNSLE	AmMRJP1 : 83%
	MENNDYNENDYNFRIMDANVRDLI	MRJP1 : 71%

3.2.9 Ratio of purified RJ protein.

The ratio of total purified protein quantity (A1(AcMRJP3) : A2(AcMRJP2) : B1(monomeric AcMRJP1) : C1(Oligomeric AcMRJP1)) in RJ of different bee populations (northern Thailand, Peninsular Thailand and Samui Island) was determined after semi-purified by a Q-Sepharose column chromatography. Proteins A, B and C were pooled. The protein content was measured. The protein A was separated to two distinct protein bands when analyzed by 10 % SDS-PAGE. The intensity ratio was further determined by a densitophotometer to quantify these proteins. Since results from purification of proteins B and C with a Sephadex G-200 column chromatography did not further differentiate these proteins, the protein content of B and C was used to represent that of B1 and C1, respectively.

The ratio between all proteins in three RJ samples derived from different populations of *A. cerana* was different (Table 3.8). The highest quantity of AcMRJPs was C1. The percentage of monomeric AcMRJP1compared to oligomeric AcMRJP1 varied greatly among RJ from different *A. cerana* populations. Considering the ratio of purified proteins according to families, the most abundant protein was AcMRJP1 followed by AcMRJP2 and AcMRJP3, respectively. Different ratios of AcMRJPs in the RJ from different population may reflect different quality of RJ *per se*. This should be tested in the future.

Table 3.8 Ratios of purified AcMRJPs representing from different geographic locations

Colony no.	Geopraphic origin	Ratio A1 (AcMRJP3) : A2 (AcMRJP2) : B1 (monomeric AcMRJP1) : C1 (oligomeric AcMRJP1)	Ratio AcMRJP3:AcMRJP2:AcMRJP1
2	Peninsular Thailand	2.52 : 4.72 : 1 : 12.21	1 : 1.89 : 5.24
5	Northern Thailand	0.84 : 4.57 : 1 : 9.73	1 : 5.45 : 12.77
6	Samui Island	0.88 : 2.04 : 1 : 4.97	1 : 2.32 : 6.78

3.3 Molecular cloning and characterization of AcMRJP cDNAs

3.3.1 Establishment of Expression Sequence Taq (EST) Markers from a Hypopharyngeal Gland cDNA Library

The mRNA with the molecular weight ranging of 0.6 up to greater than 9.4 kb was observed (Figure 3.23). High abundant transcripts were estimated at the molecular weight of 1.5 kb. The concentration of mRNA was then determined by spectrophometry at the wavelength of 260 nm and calculated following the formula: [mRNA] = A260 x 40 μg/ml. Approximately 8.5 μg mRNA was obtained from 50 hypoharyngeal glands of *A. cerana* nurse bees.

To establish a cDNA (an EST) library of hypopharyngeal glands, 3 μg of mRNA was used as the template for cDNA synthesis. Size-fractionated cDNAs (>500 bp) were obtained by column chromatography (Figure 3.24). The 9^{th} – 13^{th} fractions were pooled and used to construct a cDNA library. Sizes of insert were screened by colony PCR (Figure 3.25). One hundred and seventy-three (86.5 %) of 200 colonies were recombinant clones. Sixty-six recombinant clones having insert sizes greater than 500 bp were randomly picked up and unidirectionally sequenced.

Nucleotide sequences were analyzed by homology searches against the GenBank database using nucleotide (BlastN) and translated protein (BlastX). Results are shown in Table 3.9. A total of 53 clones (80.3 %) significantly matched to gene homologues, whereas the remaining (19.7 %) did not match to any sequences previously deposited in the GenBank. Forty-one clones (63.6%) were homologous of AmMRJPs. These included AmMRJP1 (50.0 %), AmMRJP2 (6.06 %), AmMRJP3 (6.06 %) and AmMRJP4 (1.52 %) homologue. The AcMRJP5 was not found in this library.

Others encoding protein homologues (16.7%) found in this library were apisimin (3.03%), glucose oxidase (3.03%), α-glucosidase (1.52%), heat shock protein (1.52%) and housekeeping genes such as elongation factor-1αF2, cytochrome oxidase II, 16S ribosomal DNA, NADH 4L, 60 ribosomal RNA gene (7.6%).

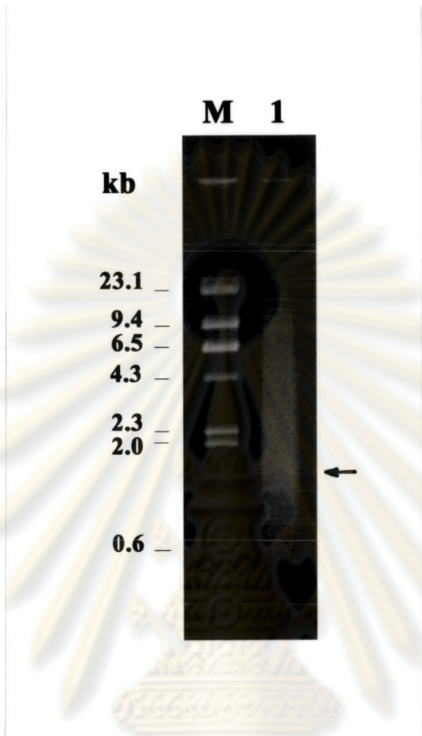


Figure 3.23 The mRNA extracted form hypopharyngeal glands of *A. cerana* nurse bees. Abundant transcript is illustrated by an arrow.

Lane M = A λ/*Hin*d III standard DNA marker

Lane 1 = two microliters of purified mRNA from 50 hypopharyngeal glands

Figure 3.24 Size-fractionation of *Not* I – digested cDNA using gel filtration

Lane M = A λ/*Hin*d III standard DNA marker

Lanes 1-22 = Eluted cDNA fractions no. 1-22

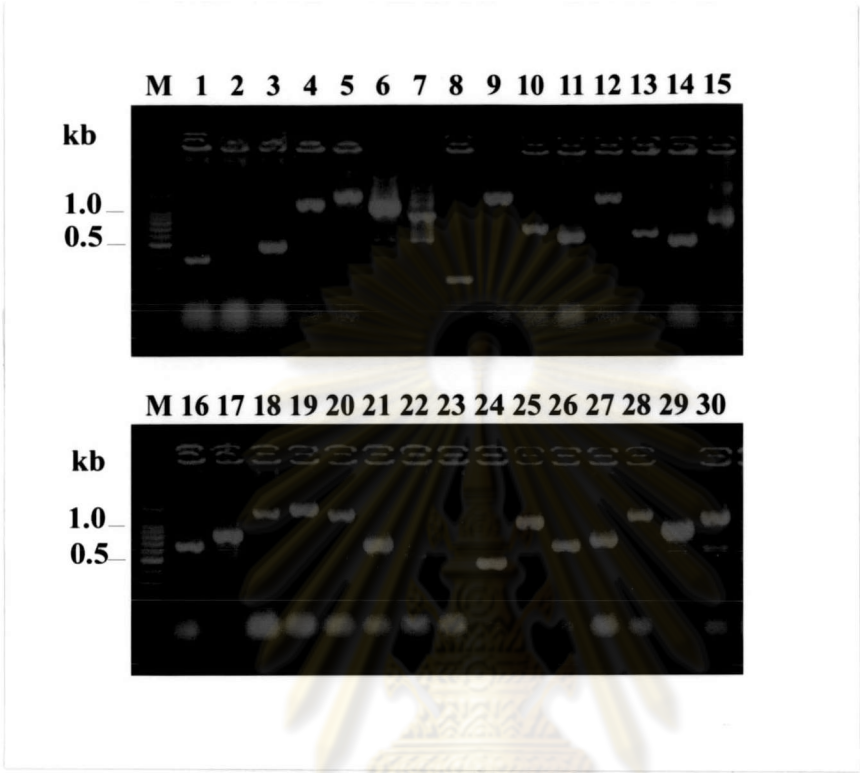


Figure 3.25 Colony PCR to determine sizes of insert.

Lane M = A 100 bp DNA ladder

Lane 1-30 = Products of colony PCR

Table 3.9 Gene homologues of ESTs from hypopharyngeal glands of *A. cerana* nurse bees

Gene homologues	Closest species	Redundancy	% EST	Probability
MRJP1	*A. mellifera*	33	50.0	1×10^{-111} - 0.0
MRJP2	*A. mellifera*	4	6.06	1×10^{-14}-0.0
MRJP RJP57-1 (MRJP3)	*A. mellifera*	4	6.06	6×10^{-79} -0.0
MRJP RJP57-2 (MRJP4)	*A. mellifera*	1	1.52	1×10^{-150}
Apisimin	*A. mellifera*	2	3.03	1×10^{-139}- 1×10^{-144}
Glucose oxidase	*A. mellifera*	2	3.03	1×10^{-170} -0.0
α-glucosidase	*A. mellifera*	1	1.52	0.0
Elongation factor-1αF2 gene	*A. mellifera*	1	1.52	0.0
Heat shock protein 86	*Mus musculus*	1	1.52	1×10^{-77}
Cytochrome oxidase II	*A. cerana*	1	1.52	1×10^{-151}
16S ribosomal DNA	*A. cerana*	1	1.52	4×10^{-46}
NADH 4L	*A. mellifera*	1	1.52	1×10^{-14}
60S ribosomal RNA gene	*Spodoptera frugiperda*	1	1.52	9×10^{-31}
Unknown	-	13	19.70	$> 1 \times 10^{-4}$

The full length of AcMRJP1 nucleotide sequence could be deduced from three recombinant clones: pCUAC322 (accession no. 17086885), pCUAC 147 (accession no. 17086860) and pCUAC171(accession no. 17086866). The open reading frame (ORF) of AcMRJP1 was 1299 nucleotide encoded for 433 amino acid residues (Figure 3.26). AcMRJP1 showed high similarity (93.1 % and 90.3 % at nucleotide and protein levels, respectively) to AmMRJP1. The deduced AcMRJP1 contained 44.6 % hydrophobic, 28.0 % neutral and 27.5 % hydrophilic amino acid residues. The essential amino acid content was 48.3 %. The estimated pI-value of AcMRJP1 was 5.5. The cleavage site of signal peptidase was located after Ser_{19}. Three putative *N*-like glycosylation sites were predicted at the nucleotide positions of 124-132, 472-480 and 571-579, respectively.

In addition, two full-length transcipts (pCUAC146: accession no. 17086895 and pCUAC165: accession no. 17086897) exhibiting 92.4 % and 94.9 % similarity at the nucleotide and protein levels to *A. mellifera* apisimin were also found. The ORF of both sequences was 234 bp encoding 78 amino acid residues (Figure 3.27). Silent substitutions between these clones were found in the position 110^{th} and 143^{rd} of nucleotide sequences. Deduced amino acids of *A. cerana* apisimin were composed of 61.2 % hydrophobic, 27.5 % neutral, 8.8 % hydrophilic and 2.5 % other groups of amino acid residues. It is highly enriched in Val (18.8 %), Ala (16.3 %) and Ser (13.8 %), where Pro, Trp, Arg, Tyr and His were not found. The estimated pI value and molecular weight was 4.7 and 7.9 kDa, respectively. The position of signal peptidase site was located between Ala_{24} and Lys_{25}. Transmembrane segments were predicted at the amino acid positions 5^{th} – 24^{th} and 39^{th} -61^{st}.

ESTs representing α-glucosidase (1.52 %) and glucose oxidase (3.03 %) homologues were found. To verify α-glucosidase expression, a pair of primer (5'-TCG ACT TCT AGT TGG TAG CAT GAA GG –3' and 5'-CCT TTC TCA TGT GCA GCA CTG ACT AG-3') (Ohashi *et al* 1996) was used to amplify the 1st strand cDNA template synthesized from different developmental stages of *A. cerana*. An expected 350 bp fragment were found in hypopharyngeal glands of both nurse bees and foragers (Figure 3.28). The 28S ribosomal RNA (a positive control amplified by a pair of primers: 5'-AAA GAT CGA ATG GGG AGA TTC-3' and 5'-CAC CGG GTC CGT ACC TCC-3') was successfully amplified.

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GTACAATATCCATTGCTTCGTTACTCGCAGCCTAGAAAAATGACAAGGTGGTTGTTTATG     60
                                          M  T  R  W  L  F  M

GTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAAGCAGCATTCTTCGAGGAGAATCT    120
 V  V  C  L  G  I  V  C  Q  G  T  T  S  S  I  L  R  G  E  S

TTAAACAAATCATTAAGCGTCCTTCACGAATGGAAATTCTTTGATTATGATTTCGATAGC    180
 L [N  K  S] L  S  V  L  H  E  W  K  F  F  D  Y  D  F  D  S

GATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATACGACTACAGGAAAAATTATCCA    240
 D  E  R  R  Q  D  A  I  L  S  G  E  Y  D  Y  R  K  N  Y  P

TCCGACGTTGATCAATGGCATGGTAAGATTTTTGTCACCATGCTAAGATACAATGGCGTA    300
 S  D  V  D  Q  W  H  G  K  I  F  V  T  M  L  R  Y  N  G  V

CCTTCCTCTTTGAACGTGATATCTAAAAAGATCGGTGATGGTGGACCTCTTCTTCCACCT    360
 P  S  S  L  N  V  I  S  K  K  I  G  D  G  G  P  L  L  P  P

TATCCCGATTGGGCGTTTGCTAAATATGACGATTGCTCTGGGATCGTGAGCGCCACAAAA    420
 Y  P  D  W  A  F  A  K  Y  D  D  C  S  G  I  V  S  A  T  K

CTTGCGATCGACAAATGCGACAGATTGTGGGGTCTGGACTCAGGTCTTGTCAATAATACT    480
 L  A  I  D  K  C  D  R  L  W  G  L  D  S  G  L  V [N  N  T]

CAACCCCTGTGTTCTCCCAAACTGCTCACCTTTGATCTGACTACCTCGCAATTGCTCAAG    540
 Q  P  L  C  S  P  K  L  L  T  F  D  L  T  T  S  Q  L  L  K

CAAGTCGAAATACCGCATGATGTTGCCGTAAATGCCACCACAGGGAAGGGGAGACTATCA    600
 Q  V  E  I  P  H  D  V  A  V [N  A  T] T  G  K  G  R  L  S

TCTCTAGCTGTTCAACCTTTAGATTGCAATATAAATGGTGATACTATGGTATACATAGCA    660
 S  L  A  V  Q  P  L  D  C  N  I  N  G  D  T  M  V  Y  I  A

GACGAGAAAGGTGAAGGTTTAATCGTGTATCATGATTCTGATAATTCTTTCCATCGATTG    720
 D  E  K  G  E  G  L  I  V  Y  H  D  S  D  N  S  F  H  R  L

ACTTCCAAAACTTTCGATTACGATCCTAAATTTACCAAAATGACGATCAATGGAGAAAGT    780
 T  S  K  T  F  D  Y  D  P  K  F  T  K  M  T  I  N  G  E  S

TTCACAACGCAAAGTGGAATTTCTGGAATGGCTCTTAGTCCTATGACTAACAATCTCTAT    840
 F  T  T  Q  S  G  I  S  G  M  A  L  S  P  M  T  N  N  L  Y

TACAGTCCTGTAGCTTCTACCAGTTTGTACTATGTTAACACGGAACAATTCAGAACATCC    900
 Y  S  P  V  A  S  T  S  L  Y  Y  V  N  T  E  Q  F  R  T  S

AATTATGAACAAAATGCCGTACATTATGAAGGAGTTCAAAATATTTTGGATACCCAATCG    960
 N  Y  E  Q  N  A  V  H  Y  E  G  V  Q  N  I  L  D  T  Q  S

TCTGCTAAAGTAGTATCGAAAAGTGGCGTCCTCTTCTTCGGACTGGTGGGCGATTCAGCT   1020
 S  A  K  V  V  S  K  S  G  V  L  F  F  G  L  V  G  D  S  A

CTTGGCTGCTGGAACGAACATCGATCACTTGAAAGACACAATATCCGTACCGTCGCTCAA   1080
 L  G  C  W  N  E  H  R  S  L  E  R  H  N  I  R  T  V  A  Q

AGTGATGAAACACTTCAAATGATCGTTGGCATGAAGATTAAGGAAGCCCTTCCACACGTG   1140
 S  D  E  T  L  Q  M  I  V  G  M  K  I  K  E  A  L  P  H  V

CCCATATTCGATAGATATATAAACCGTGAATACATATTGGTTTTAAGTAACAGAATGCAA   1200
 P  I  F  D  R  Y  I  N  R  E  Y  I  L  V  L  S  N  R  M  Q

AAAATGGCGAATAATGACTATAACTTCAACGATGTAAACTTCAGAATTATGGACGCTAAT   1260
 K  M  A  N  N  D  Y  N  F  N  D  V  N  F  R  I  M  D  A  N

GTAAATGACTTGATATTGAACACTCGTTGCGAAAATCCTAATAATGATAACACACCTTTC   1320
 V  N  D  L  I  L  N  T  R  C  E  N  P  N  N  D  N  T  P  F

AAAATTTCAATACATCTGTAAAATCTGTTTTTTTCGATATATATTAAATATTGTTCGAGA   1380
 K  I  S  I  H  L  *

TTTCTTATGAATGTATTATGAATGTATAAAATAAATATTGTTTTCGCATAAAAAAAAAAA   1440

AAAAAAAAAAAAAAAAAAAAA 1461
```

Figure 3.26 Nucleotide and deduced amino acid sequences of AcMRJP1. Initiation and termination of translational codons and putative polyadenylation signal are boldfaced. The signal peptide is underlined. *N*-liked glycosylation sites are boxed.

```
GAACCGAGCTTTCTAGAAGCAATTCCAAACAACACACAAATCAAAATGAGCAAAATCATT  60
                                                M  S  K  I  I

GCTGTCGTCGTCCTAGCTGCCTTCTGCGTAGCCATGTTGGTCAGCGATGTATCCGCCAAA 120
 A  V  V  V  L  A  A  F  C  V  A  M  L  V  S  D  V  S  A  K

ACATCGATCAGTGCCAAGGCCGAGTCGAATGTAGATGTCGTTTCCCAAATCAACAGTTTG 180
 T  S  I  S  A  K  A  E  S  N  V  D  V  V  S  Q  I  N  S  L

GTTTCATCTATCGTAGCTGGTGCCAACGTGTCGGCAGTTCTTCTAGCTCAAACTTTAGTT 240
 V  S  S  I  V  A  G  A  N  V  S  A  V  L  L  A  Q  T  L  V

AATATCCTCCAAATTCTCATCGACGCTAATGTTTTTGCTTAATTTATATATTCTTTAGCT 300
 N  I  L  Q  I  L  I  D  A  N  V  F  A  *

TTGTATTGCGCGCATACAACGCATTCGAATAAAATAAGTAATAAAATTCAAAAAAAAAAA 360

AAAAAAAAAAAAA 373
```

Figure 3.27 Nucleotide and deduced amino acid sequences of *A. cerana* apisimin from clone pCU AC165. Initiation and termination of translational codons and putative polyadenylation signal are boldfaced. The signal peptide is underlined. *N*-liked glycosylation sites are boxed. Silent mutations of pCU AC 146 are illustrated in blue boxes.

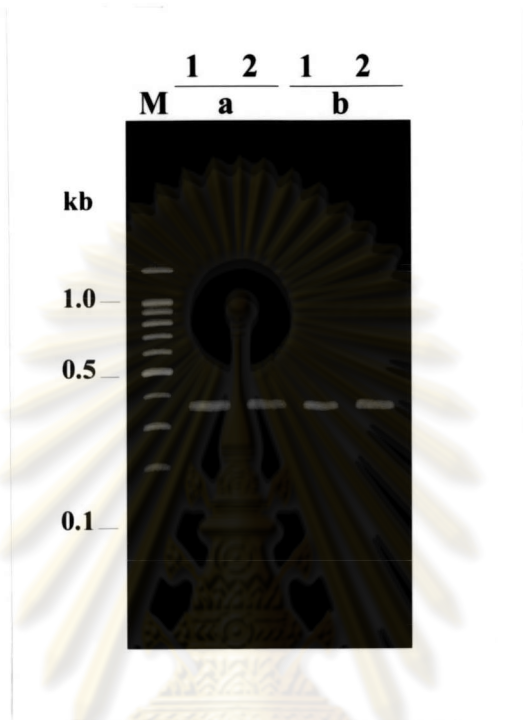


Figure 3.28 Expression of α-glucosidase was investigated by amplification of hypopharyngeal gland mRNA of *A. cerana* nurse bees and foragers.

Lane M = A 100 bp DNA ladder

Panel a lane 1 and 2 = 28S ribosomal RNA gene from nurse bees and foragers, respectively.

Panel b lane 1 and 2 = α-glucosidase gene from nurse bees and foragers, respectively

3.3.2 RT-PCR

To characterize the full length of other AcMRJPs (AcMRJP2 and AcMRJP3), family-specific primers were designed. The sequence of AmMRJP3 was retrieved from the GenBank and used for amplification of its homologue in *A. cerana* (5'-GTC AAT TGG AAA ATA TCT GTA TTA T-3' and 5'-TTT TAA TTG ATA ATT GAT TGA TTT AAT G-3'). The nucleotide sequence of AcMRJP2 was kindly provided by C. Imjongjailuk (personal communication).

The expected full length of AcMRJP3 and AcMRJP2 were about 1,900 bp and 1400 bp in length. The amplification products were cloned. Recombinant plasmids were mapped by digestion with restriction endonucleases to confirm the inserts. A recombinant plasmid containing an AcMRJP3 insert digested with *Ssp* I, *Sal* I and *Cla* I are shown in Figure 3.29. Sizes of each fragment were identical to those expected from the AmMRJP3 sequence. Four primers were used for sequencing of the entire AcMRJP3 cDNAs. Initially, AcMRJP3 was sequenced using M13 forward and reverse M13 primers. Internal sequencing primers for 5' and 3' directions of these genes were then designed from nucleotide sequences obtained. Nucleotide sequences of AcMRJP2 and AcMRJP3 are shown by Figure 3.30 and 3.31, respectively.

AcMRJP2 contained an ORF of 1389 nucleotides encoding 463 amino acid residues. Eight repeated units of "AATCAGAA(G/C/A)ATA(C/A)C" encoding "NQ (K/N)N(N/T)" were found at the C-termini. Disregarding this repeated sequences, AcMRJP2 showed 92.1 % of nucleotide and 87.7 % of deduced amino acid similarity to that of AmMRJP2. The deduced amino acid composition of AcMRJP2 comprised 42.3 % hydrophobic, 31.3 % neutral and 26.4 % hydrophilic amino acid residues. The essential amino acid content was 46.4 %. AcMRJP2 was a basic protein with an estimated pI value of 8.02. The cleavage site of the signal peptide was located between Gly_{17} and Ala_{18}. Two putative *N*-link glycosylation sites were found.

The nucleotide sequence of AcMRJP3 were composed of 1824 bp ORF enclosed 608 amino acid residues. Two sets of repeated unit, "NQN(A/D)(N/T)" and "(R/K/N) (Q/R) N (G/D/A/S) N" were found and occurred in five and twenty-nine times, respectively. Disregarding the repeated units, AcMRJP3 sequences showed 91.5 % and 87.4 % similarity to AmMRJP3 sequence at the nucleotide and protein

levels, respectively. The amino acid composition of AcMRJP3 was 33.1 % hydrophobic, 39.1 % neutral and 27.8 % hydrophilic amino acid residues. The essential amino acid content of AcMRJP3 was 39.5 %. The pI value was estimated to be 8.8. The cleavage site of signal peptide was located between Ser_{20} and Ala_{21}. Five putative *N*-link glycosylation sites were observed.

3.3.3 Phylogenetic relationships between AcMRJP and AmMRJP families

Nucleotide and deduced amino acid sequences of AcMRJP1, AcMRJP2 and AcMRJP3 found in this study were aligned (Figure 3.32, 3.33). The 68.22 % and 55.7% of nucleotide and deduced amino acid sequences were conserved across AcMRJP families. Sequences of AmMRJPs were retrieved from the GenBank and aligned with those of AcMRJP1, AcMRJP2 and AcMRJP3 (Appendix H-L). Genetic distances of each MRJP were calculated at both nucleotide and protein levels. The lowest and highest divergence at the nucleotide level was 0.0706 (AcMRJP1-AmMRJP1) and 0.4499 (AmMRJP4-AmMRJP5), respectively. At the protein level, the lowest divergence was 0.1017 (AcMRJP1-AmMRJP1) whereas the highest divergence was 0.8239 (AcMRJP3-AmMRJP5) (Table 3.10).

The original data was then bootstrapped 1000, and 200 times for nucleotide and protein data, respectively. Bootstrapped neighbor-joining trees were then constructed (Figure 3.34 and 3.35). Relationships at both nucleotide and protein levels of MRJPs indicated phylogenetically closer relationships between the same MRJPs families from different species rather than different families of MRJPs within the same species.

The same families from different species were grouped together. The different trees were obtained from two types data either nucleotide and amino acid sequences. Clustering of AmMRJP5 to a AmMRJP1-AcMRJP1 branch with the high bootstrapped value suggested the requirement for isolation and sequencing of MRJP5 from different bee species for unambiguously conclusion for phylogenetic relationships of MRJPs in bees

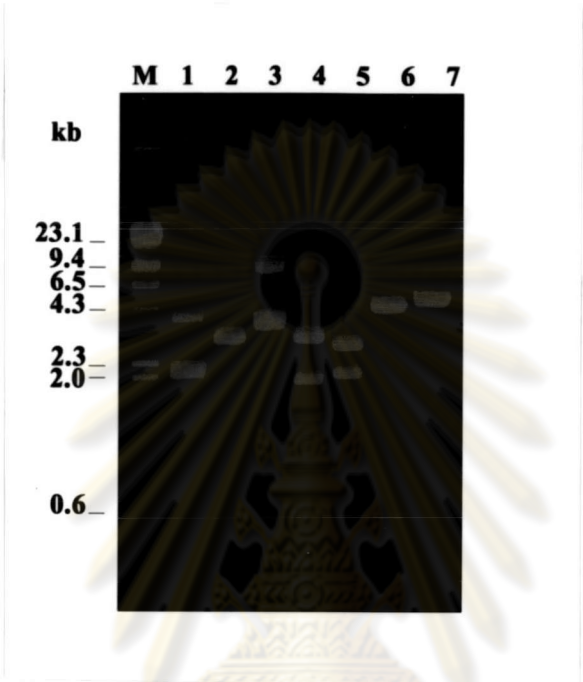


Figure 3.29 Restriction analysis of recombinant plasmid containing an AcMRJP3 insert.

Lane M = A λ/*Hin*d III standard DNA marker
Lane 1 = Undigested pGEM
Lane 2 = pGEM digested with *Eco*R I
Lane 3 = Undigested recombinant plasmid
Lane 4 = Recombinant plasmid digested with *Eco*R I
Lane 5 = Recombinant plasmid digested with *Ssp* I
Lane 6 = Recombinant plasmid digested with *Sal* I
Lane 7 = Recombinant plasmid digested with *Cla* I

Figure 3.30

```
ATGACAAGGTGGTTGTTCATGGTGGCATGCCTTGGCATAGCTTGTCAAGGCGCCATTATT   60
 M  T  R  W  L  F  M  V  A  C  L  G  I  A  C  Q  G  A  I  I

CGACAAAATTCTGCAAAAAACTTGGAAAATTCGTTGAACGTAATTCACGAATGGAAATAT  120
 R  Q  N  S  A  K  N  L  E  N  S  L  N  V  I  H  E  W  K  Y

ATCGATTATGATTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATAC  180
 I  D  Y  D  F  G  S  E  E  R  R  Q  A  A  I  Q  S  G  E  Y

GATCATACGAAAAATTATCCCTTCGATGTCGATCAATGGCATGATAAGACTTTTGTCACC  240
 D  H  T  K  N  Y  P  F  D  V  D  Q  W  H  D  K  T  F  V  T

ATACTAAAGTACGATGGTGTGCCTTCTACTTTGAACATGATATCTAACAAAATCGGTAAG  300
 I  L  K  Y  D  G  V  P  S  T  L  N  M  I  S  N  K  I  G  K

GGTGGACGCCTTCTACAACCATATCCTGATTGGTCGTGGGCAGAGAATAAAGATTGCTCT  360
 G  G  R  L  L  Q  P  Y  P  D  W  S  W  A  E  N  K  D  C  S

GGAATCGTGAGCGCTTTCAAAATTGCGATTGACAAATTCGACAGATTGTGGGTTTTGGAT  420
 G  I  V  S  A  F  K  I  A  I  D  K  F  D  R  L  W  V  L  D

TCAGGTCTTATCAATAGAACTGAACCTATATGTGCTCCAAAATTGCATGTCTTTGATCTG  480
 S  G  L  I  N  R  T  E  P  I  C  A  P  K  L  H  V  F  D  L

AAAAACACAAAGCACCTTAAGCAAATCGAAATACCGCATGATATTGCCGTAAATGCCACC  540
 K  N  T  K  H  L  K  Q  I  E  I  P  H  D  I  A  V  N  A  T

ACAGGAAAGGGAGGGCTAGTCTCTCTAGTTGTTCAAGCCATGGATCCTATGAATACTTTA  600
 T  G  K  G  G  L  V  S  L  V  V  Q  A  M  D  P  M  N  T  L

GTATACATAGCAGACCATAAGGGTGATGCTTTGATCGTCTATCAAAATTCCGATGATTCC  660
 V  Y  I  A  D  H  K  G  D  A  L  I  V  Y  Q  N  S  D  D  S

TTCCATCGAATGACTTCCAACACTTTCGATTACGATCCCAGATATGCCAAAATGACGATC  720
 F  H  R  M  T  S  N  T  F  D  Y  D  P  R  Y  A  K  M  T  I

AATGGAGAAAGTTTCACATTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTGACG  780
 N  G  E  S  F  T  L  K  N  G  I  C  G  M  A  L  S  P  V  T

AACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATGTCAACACGGAACCA  840
 N  N  L  Y  Y  S  P  L  A  S  H  G  L  Y  Y  V  N  T  E  P

TTTATGAAATCACAATTTGGAGACAATAATAACGTGCAATATGAAGGATCCCAAGATACT  900
 F  M  K  S  Q  F  G  D  N  N  N  V  Q  Y  E  G  S  Q  D  T

TTGAACACGCAATCATTGGCTAAAGCAGTATCGAAAGATGGCGTCCTCTTCGTCGGACTT  960
 L  N  T  Q  S  L  A  K  A  V  S  K  D  G  V  L  F  V  G  L

GTGGGTAATTCAGCTCTTGGATGCTTGAACGAGCATCAACCACTTCAGAGAGAAAATTTA 1020
 V  G  N  S  A  L  G  C  L  N  E  H  Q  P  L  Q  R  E  N  L

GAACTGGTCGCCCAAAATGAAAAAACACTTCAAATGATCGCAGGTATGAAAATTAAGGAA 1080
 E  L  V  A  Q  N  E  K  T  L  Q  M  I  A  G  M  K  I  K  E

GAGCTTCCACATTTCGTAGGAAGTAACAAACCTGTAAAGGACGAATATATGTTAGTTTTA 1140
 E  L  P  H  F  V  G  S  N  K  P  V  K  D  E  Y  M  L  V  L

AGTAACAAAATGCAGAAAATAGTAAATAATGATTTTAATTTCAACGACGTAAACTTCCGA 1200
 S  N  K  M  Q  K  I  V  N  N  D  F  N  F  N  D  V  N  F  R
```

Figure 3.30 (continued)

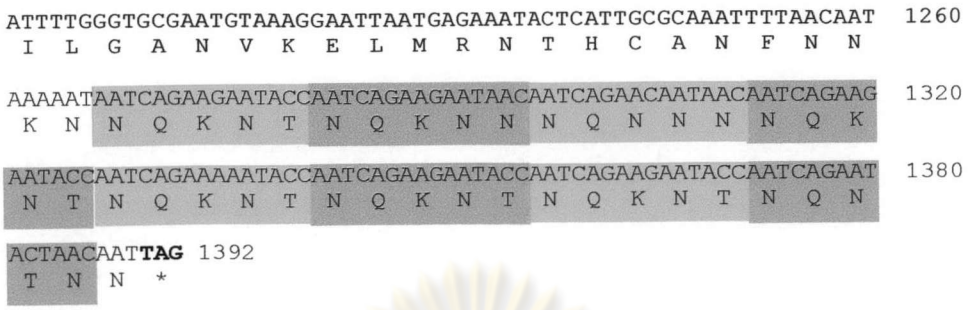


Figure 3.30 Nucleotide and deduced amino acid sequences of AcMRJP2. Initiation and termination of translational codons are boldfaced. The signal peptide is underlined. *N*-liked glycosylation sites are boxed. The repeated units are illustrated in the blue colored boxes.

Figure 3.31

```
ATGACAAAGTGGTTGTTGCTGGTGGTGTGTCTTGGTATAGCTTGTCAAGATGTGACAAGC      60
 M  T  K  W  L  L  L  V  V  C  L  G  I  A  C  Q  D  V  T  S

GCAGCTGTGAACCATCAAAGAAAATCTTCAAAAAATTTGGCACATTCGATGAAGGTGATC     120
 A  A  V  N  H  Q  R  K  S  S  K  N  L  A  H  S  M  K  V  I

TACGAATGGAAACATATTGATTATGATTTTGGTAGCGTTGAAAGAAGAGATGCTGCGATT     180
 Y  E  W  K  H  I  D  Y  D  F  G  S  V  E  R  R  D  A  A  I

AAATCTGGCGAATTTGATCACACAAAAAATTACCCTTTCGATGTGGATAGATGGCGTGAT     240
 K  S  G  E  F  D  H  T  K  N  Y  P  F  D  V  D  R  W  R  D

AAGACATTTGTCACCGTAGAAAGGTTCGATGGTGTACCTTCTTCTTTGAACGTGGTAACT     300
 K  T  F  V  T  V  E  R  F  D  G  V  P  S  S  L  N  V  V  T

AATAAAAAAGGCAAAGGTGGACCTCTTCTACATCCATATCCTGATTGGTCGTGGGCGAAC     360
 N  K  K  G  K  G  G  P  L  L  H  P  Y  P  D  W  S  W  A  N

TATAAAGATTGCTCTGGAATTGTGAGCGCTTTCAAAATTGCGGTCGACAAATTCGACAGA     420
 Y  K  D  C  S  G  I  V  S  A  F  K  I  A  V  D  K  F  D  R

TTATGGGTTCTGGACTCAAGTCTTGTCAATAATAATCAACCCATGTGCTCTCCAAAATTG     480
 L  W  V  L  D  S  S  L  V  N  N  N  Q  P  M  C  S  P  K  L

GTAACCTTCGATTTGAATACCTCAAAATTGCTTAAGCAAGTCGAGATACCACATAATATT     540
 V  T  F  D  L  N  T  S  K  L  L  K  Q  V  E  I  P  H  N  I

GCCGTAAATGCCACCACCGAATGGGGAGAATTAGTATCACTAGCTGTTCAAGCTGTAGAT     600
 A  V  N  A  T  T  E  W  G  E  L  V  S  L  A  V  Q  A  V  D

CCTACGAATACTATGGTGTACATAGCAGACGAAAGAGGTGAAGCTTCAATCATCTATCAA     660
 P  T  N  T  M  V  Y  I  A  D  E  R  G  E  A  S  I  I  Y  Q

AATTCCGACGATTCCTTCCATCGATTGACTTCCAATACTTTCGATTACGATCCCAGATAT     720
 N  S  D  D  S  F  H  R  L  T  S  N  T  F  D  Y  D  P  R  Y

ACCAAACTGACAGTCGCTGGAGAAAGTTTCACAGTGAAAAATGGAATTTGTGGAATTGCA     780
 T  K  L  T  V  A  G  E  S  F  T  V  K  N  G  I  C  G  I  A

CTTAGTCCCGTGACGAACAATCTTTATTACAGTCCTCTCGCTTCTCACAGTTTGTATTAT     840
 L  S  P  V  T  N  N  L  Y  Y  S  P  L  A  S  H  S  L  Y  Y

GTTAACACAGAACAATTCAGGAATCCACAATATGAAGAAAATAACGTCCAATATGAAGGA     900
 V  N  T  E  Q  F  R  N  P  Q  Y  E  E  N  N  V  Q  Y  E  G

TCCCAAGATATTTTGAACACTCAATCATTCGCTAAAGCAGTATCGAAAAATGGCGTCGTT     960
 S  Q  D  I  L  N  T  Q  S  F  A  K  A  V  S  K  N  G  V  V

TTCTTGGGACTCGTGAGTAATTCAACTGTTGGCTGTGTGAATGAACATCAAGTACTTCAG    1020
 F  L  G  L  V  S  N  S  T  V  G  C  V  N  E  H  Q  V  L  Q

AAAGAAAATTTTGATGTTGTCGCTCAGAATGAAGAGACACTTCAAATGATCGTTAGTATG    1080
 K  E  N  F  D  V  V  A  Q  N  E  E  T  L  Q  M  I  V  S  M
```

Figure 3.31 (continued)

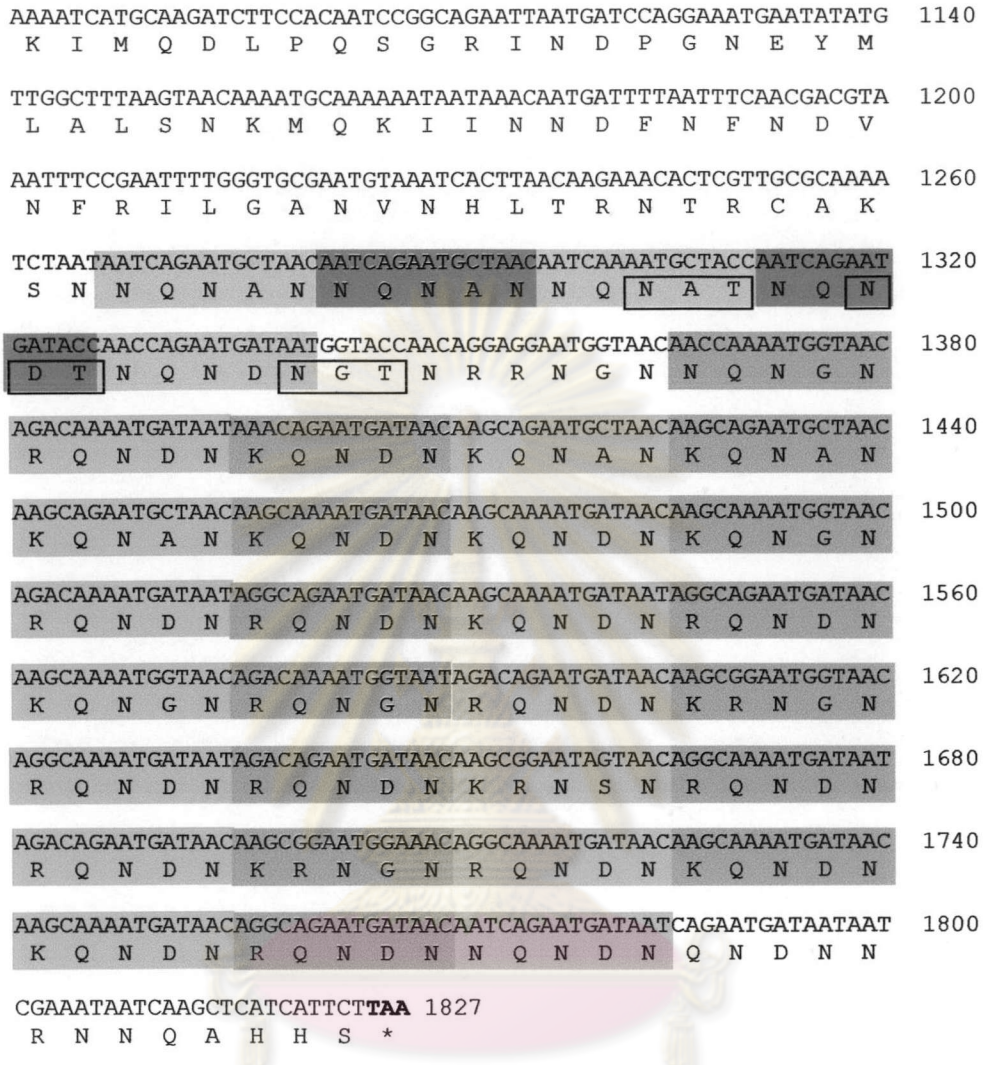


Figure 3.31 Nucleotide and deduced amino acid sequences of AcMRJP3. Initiation and termination of translational codons are boldfaced. The signal peptide is underlined. *N*-liked glycosylation sites are boxed. The repeated units are illustrated in the blue and green colored boxes. Internal primers used for sequencing are illustrated in the purple boxes.

Figure 3.32

```
AcMRJP2     ----------------------------------------------------ATGACAAGGT  60
AcMRJP3     ----------------------------------------------------ATGACAAAGT
AcMRJP1     GCAGTTCACGTACAATATCCATTGCTTCGTTACTCGC--AGCCTAGAAAAATGACAAGGT
                                                               ******* **

            GGTTGTTCATGGTGGCATGCCTTGGCATAGCTTGTCAAG---------GCGCCATTAT--  120
            GGTTGTTGCTGGTGGTGTGTCTTGGTATAGCTTGTCAAGATGTGACAAGCGCAGCTGTGA
            GGTTGTTTATGGTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAAGCAGCATTCT--
            *******  ******  ** ***** **** ********         **     * *

            ----TCGACAAAATTCTGCAAAAAACTTGGAAAATTCGTTGAACGTAATTCACGAATGGA  180
            ACCATCAAAGAAAATCTTCAAAAAATTTGGCACATTCGATGAAGGTGATCTACGAATGGA
            ----TCGAGGAGAATCTTTAAACAAAT---------CATTAAGCGTCCTTCACGAATGGA
                ** *  * * ***  *** ** *          *  * *  **  *  *********

            AATATATCGATTATGATTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCG  240
            AACATATTGATTATGATTTTGGTAGCGTTGAAAGAAGAGATGCTGCGATTAAATCTGGCG
            AATTCTTTGATTATGATTTCGATAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGCG
            **    * ********** * *****  ********* * * *** ***  ********

            AATACGATCATACGAAAAATTATCCCTTCGATGTCGATCAATGGCATGATAAGACTTTTG  300
            AATTTGATCACACAAAAAATTACCCTTTCGATGTGGATAGATGGCGTGATAAGACATTTG
            AATACGACTACAGGAAAAATTATCCATCCGACGTTGATCAATGGCATGGTAAGATTTTTG
            ***  **  * *  ******** ** * *** ** ***  ***** ** *****  ****

            TCACCATACTAAAGTACGATGGTGTGCCTTCTACTTTGAACATGATATCTAACAAAATCG  360
            TCACCGTAGAAAGGTTCGATGGTGTACCTTCTTCTTTGAACGTGGTAACTAATAAAAAAG
            TCACCATGCTAAGATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGATCG
            ***** *   **  * * **** ** *****  ******** ** ** **** ** *  *

            GTAAGGGTGGACGCCTTCTACAACCATATCCTGATTGGTCGTGGGCAGAGAATAAAGATT  420
            GCAAAGGTGGACCTCTTCTACATCCATATCCTGATTGGTCGTGGGCGAACTATAAAGATT
            GTGATGGTGGACCTCTTCTTCCACCTTATCCCGATTGGGCGTTTGCTAAATATGACGATT
            *  * *******  ***** *  ** ***** ****** ***  **  *  ** * ****

            GCTCTGGAATCGTGAGCGCTTTCAAAATTGCGATTGACAAATTCGACAGATTGTGGGTTT  480
            GCTCTGGAATTGTGAGCGCTTTCAAAATTGCGGTCGACAAATTCGACAGATTATGGGTTC
            GCTCTGGGATCGTGAGCGCCACAAAACTTGCGATCGACAAATGCGACAGATTGTGGGGTC
            ******* ** ********    *** ***** * ******* ********* **** *

            TGGATTCAGGTCTTATCAATAGAACTGAACCTATATGTGCTCCAAAATTGCATGTCTTTG  540
            TGGACTCAAGTCTTGTCAATAATAATCAACCCATGTGCTCTCCAAAATTGGTAACCTTCG
            TGGACTCAGGTCTTGTCAATAATACTCAACCCCTGTGTTCTCCCAAACTGCTCACCTTTG
            **** *** ***** ******  * * ****  * **  **** *** **     *** *

            ATCTGAAAAACACAAAGCACCTTAAGCAAATCGAAATACCGCATGATATTGCCGTAAATG  600
            ATTTGAATACCTCAAAATTGCTTAAGCAAGTCGAGATACCACATAATATTGCCGTAAATG
            ATCTGACTACCTCGCAATTGCTCAAGCAAGTCGAAATACCGCATGATGTTGCCGTAAATG
            ** ***  * * *  *    ** ****** **** ***** *** ** ***********
```

Figure 3.32 (continued)

```
AcMRJP2     CCACCACAGGAAAGGGAGGGCTAGTCTCTCTAGTTGTTCAAGCCATGGATCCTATGA---  660
AcMRJP3     CCACCACCGAATGGGGAGAATTAGTATCACTAGCTGTTCAAGCTGTAGATCCTACGA---
AcMRJP1     CCACCACAGGGAAGGGGAGACTATCATCTCTAGCTGTTCAACCTTTAGATTGCAATATAA
            ******* *    ***     **   ** **** ******* * * ***   * *

            ------ATACTTTAGTATACATAGCAGACCATAAGGGTGATGCTTTGATCGTCTATCAAA  720
            ------ATACTATGGTGTACATAGCAGACGAAAGAGGTGAAGCTTCAATCATCTATCAAA
            ATGGTGATACTATGGTATACATAGCAGACGAGAAAGGTGAAGGTTTAATCGTGTATCATG
                  ***** * ** ************ * *  ***** * **  *** * *****

            ATTCCGATGATTCCTTCCATCGAATGACTTCCAACACTTTCGATTACGATCCCAGATATG  780
            ATTCCGACGATTCCTTCCATCGATTGACTTCCAATACTTTCGATTACGATCCCAGATATA
            ATTCTGATAATTCTTTCCATCGATTGACTTCCAAAACTTTCGATTACGATCCTAAATTTA
            **** **  **** ********* ********** ***************** * ** *

            CCAAAATGACGATCAATGGAGAAAGTTTCACATTGAAAAATGGAATTTGTGGAATGGCTC  840
            CCAAACTGACAGTCGCTGGAGAAAGTTTCACAGTGAAAAATGGAATTTGTGGAATTGCAC
            CCAAAATGACGATCAATGGAGAAAGTTTCACAACGCAAAGTGGAATTTCTGGAATGGCTC
            ***** ****  **  ***************  * *** ******** ****** ** *

            TTAGTCCCGTGACGAACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATG  900
            TTAGTCCCGTGACGAACAATCTTTATTACAGTCCTCTCGCTTCTCACAGTTTGTATTATG
            TTAGTCCTATGACTAACAATCTCTATTACAGTCCTGTAGCTTCTACCAGTTTGTACTATG
            *******  **** ******** ************ * ******  * ******* ****

            TCAACACGGAACCATTTATGAAATCACAATTTGGAGACAATAATAACGTGCAATATGAAG  960
            TTAACACAGAACAATTCAGGAATCCACAATATGAAGAAAATAA---CGTCCAATATGAAG
            TTAACACGGAACAATTCAGAACATCCAATTATGAACAAAATGC---CGTACATTATGAAG
            * ***** **** *** *  *   *  * * ** * * ***     *** ** *******

            GATCCCAAGATACTTTGAACACGCAATCATTGGCTAAAGCAGTATCGAAAGATGGCGTCC 1020
            GATCCCAAGATATTTTGAACACTCAATCATTCGCTAAAGCAGTATCGAAAAATGGCGTCG
            GAGTTCAAAATATTTTGGATACCCAATCGTCTGCTAAAGTAGTATCGAAAAGTGGCGTCC
            **   *** *** **** * ** ***** *  ******* **********  *******

            TCTTCGTCGGACTTGTGGGTAATTCAGCTCTTGGATGCTTGAACGAGCATCAACCACTTC 1080
            TTTTCTTGGGACTCGTGAGTAATTCAACTGTTGGCTGTGTGAATGAACATCAAGTACTTC
            TCTTCTTCGGACTGGTGGGCGATTCAGCTCTTGGCTGCTGGAACGAACATCGATCACTTG
            * *** * ***** *** *  ***** ** **** **   *** **  **** *  ****

            AGAGAGAAAATTTAGAACTGGTCGCCCAAAATGAAAAAACACTTCAAATGATCGCAGGTA 1140
            AGAAAGAAAATTTTGATGTTGTCGCTCAGAATGAAGAGACACTTCAAATGATCGTTAGTA
            AAAGACACAATATCCGTACCGTCGCTCAAAGTGATGAAACACTTCAAATGATCGTTGGCA
            * * * * *** *       ***** ** * ***  * ***************   * *

            TGAAAATTAAGGAAGAGCTTCCACATTTCGTAGGAAGTAACAAACCTGTAAAGGACGAAT 1200
            TGAAAATCATGCAAGATCTTCCACAATCCGGCAGAATTAATGATCCAGGAAATGA---AT
            TGAAGATTAAGGAAGCCCTTCCACACGTGCCCATATTCGATAGATATATAAACCGTGAAT
            **** ** * * ***  ********         *   *         ***      **

            ATATGTTAGTTTTAAGTAACAAAATGCAGAAAATAGTAAATAATGATTTTAATTTCAACG 1260
            ATATGTTGGCTTTAAGTAACAAAATGCAAAAAATAATAAACAATGATTTTAATTTCAACG
            ACATATTGGTTTTAAGTAACAGAATGCAAAAAATGGCGAATAATGACTATAACTTCAACG
            * ** ** * ********** ****** *****    ** ***** * *** *******
```

Figure 3.32 (continued)

```
AcMRJP2     ACGTAAACTTCCGAATTTTGGGTGCGAATGTAAAGGAATTAATGAGAAATACTCATTGCG 1320
AcMRJP3     ACGTAAATTTCCGAATTTTGGGTGCGAATGTAAATCACTTAACAAGAAACACTCGTTGCG
AcMRJP1     ATGTAAACTTCAGAATTATGGACGCTAATGTAAATGACTTGATATTGAACACTCGTTGCG
            * ***** *** ***** ***  ** ********  * ** *     ** **** *****

            CAAATTTTAACAATAAAAAT----A-ATCAGAAGAATACCAATCAGAAGAATAACAATC 1380
            CAAAATCTAATAATCAGAATGCTAACAATCAGAATGCTAACAATCAAAATGCTACCAATC
            AAAATCCTAATAATGATAACAC-ACCTTTCAAAA---TTTCAAT-ACATCTGTAA-AATC
             ***   *** *** * **         *** **   *  **** * *    **  ****

            AGAACAATAACAATCAGAAGAATA------CCAATCAGAAAAAT---ACCAATCAGAAGA 1440
            AGAATGATACCAACCAGAATGATAATGGTACCAACAGGAGGAATGGTAACAACCAAAATG
            TGTTTTTTTCGATATATATTAAATATTGTT-----------------------------
             *     *   *   * *   *

            ATACCAATCAGAAGAATACCAATCAGAATACTAACAATTAG------------------- 1500
            GTAACAGACAAAATGATAATAAACAGAATGATAACAAGCAGAATGCTAACAAGCAGAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1560
            CTAACAAGCAGAATGCTAACAAGCAAAATGATAACAAGCAAAATGATAACAAGCAAAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1620
            GTAACAGACAAAATGATAATAGGCAGAATGATAACAAGCAAAATGATAATAGGCAGAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1680
            ATAACAAGCAAAATGGTAACAGACAAAATGGTAATAGACAGAAAGATAACAAGCGGAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1740
            GTAACAGGCAAAATGATAATAGACAGAATGATAACAAGCGGAATAGTAACAGGCAAAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1800
            ATAATAGACAGAATGATAACAAGCGGAATGGAAACAGGCAAAATGATAACAAGCAAAATG
            ------------------------------------------------------------

            ------------------------------------------------------------ 1860
            ATAACAAGCAAAATGATAACAGGCAGAATGATAACAATCAGAATGATAATCAGAATGATA
            ------------------------------------------------------------

            ------------------------------------------------------------ 1920
            ATAATCGAAATAATCAAGCTCATCATTCTTAAAAATCACATTAAATCAATCAATTATCAA
            ------------------------------------------------------------

            --------- 1929
            TTAAAAAAT
            ---------
```

Figure 3.32 Alignment of nucleotide sequence among AcMRJP families using Clustal X. Asterisks indicate the same bases. The sequences used to estimate the homology are underlined. The initial and terminal codons are bold-faced.

```
AcMRJP2    MTRWLFMVACLGIACQG---AIIRQN--SAKNLENSLNVIHEWKYIDYDFGSEERRQAAI 60
AcMRJP3    MTKWLLLVVCLGIACQDVTSAAVNHQRKSSKNLAHSMKVIYEWKHIDYDFGSVERRDAAI
AcMRJP1    MTRWLFMVVCLGIVCQGTTSSILRGE---S--LNKSLSVLHEWKFFDYDFDSDERRQDAI
           **:**::*.****.**.   : :. :   :  * :*:.*::***.:****.* ***: **

           QSGEYDHTKNYPFDVDQWHDKTFVTILKYDGVPSTLNMISNKIGKGGRLLQPYPDWSWAE 120
           KSGEFDHTKNYPFDVDRWRDKTFVTVERFDGVPSSLNVVTNKKGKGGPLLHPYPDWSWAN
           LSGEYDYRKNYPSDVDQWHGKIFVTMLRYNGVPSSLNVISKKIGDGGPLLPPYPDWAFAK
           ***:*: **** ***:*:.* ***: :::****:**::::* *.** ** *****::*:

           NKDCSGIVSAFKIAIDKFDRLWVLDSGLINRTEPICAPKLHVFDLKNTKHLKQIEIPHDI 180
           YKDCSGIVSAFKIAVDKFDRLWVLDSSLVNNNQPMCSPKLVTFDLNTSKLLKQVEIPHNI
           YDDCSGIVSATKLAIDKCDRLWGLDSGLVNNTQPLCSPKLLTFDLTTSQLLKQVEIPHDV
           .******** *:*:** **** ***.*:*..:*:*:*** .***..:: ***:****::

           AVNATTGKGGLVSLVVQAMDP-MN--TLVYIADHKGDALIVYQNSDDSFHRMTSNTFDYD 240
           AVNATTEWGELVSLAVQAVDP-TN--TMVYIADERGEASIIYQNSDDSFHRLTSNTFDYD
           AVNATTGKGRLSSLAVQPLDCNINGDTMVYIADEKGEGLIVYHDSDNSFHRLTSKTFDYD
           ******  * * **.**.:*   *  *:*****.:*:. *:*::**:****:**:*****

           PRYAKMTINGESFTLKNGICGMALSPVTNNLYYSPLASHGLYYVNTEPFMKSQFGDNNNV 300
           PRYTKLTVAGESFTVKNGICGIALSPVTNNLYYSPLASHSLYYVNTEQFRNPQYEENN-V
           PKFTKMTINGESFTTQSGISGMALSPMTNNLYYSPVASTSLYYVNTEQFRTSNYEQNA-V
           *:::*:*: ***** :.**.*:****:********:** .******* * ..:: :*  *

           QYEGSQDTLNTQSLAKAVSKDGVLFVGLVGNSALGCLNEHQPLQRENLELVAQNEKTLQM 360
           QYEGSQDILNTQSFAKAVSKNGVVFLGLVSNSTVGCVNEHQVLQKENFDVVAQNEETLQM
           HYEGVQNILDTQSSAKVVSKSGVLFFGLVGDSALGCWNEHRSLERHNIRTVAQSDETLQM
           :*** *: *:*** **.***.**:*.***.:*::** ***: *::.*:  ***.::****

           IAGMKIKEELPHFVGSNKPVKDEYMLVLSNKMQKIVNNDFNFNDVNFRILGANVKELMRN 420
           IVSMKIMQDLPQSGRINDPG-NEYMLALSNKMQKIINNDFNFNDVNFRILGANVNHLTRN
           IVGMKIKEALPHVPIFDRYINREYILVLSNRMQKMANNDYNFNDVNFRIMDANVNDLILN
           *..*** : **:    :     **:*.***:***: ***:**********:.***:.*  *

           THCANFNNK-------NNQKNTNQKNNNQNNN------------NQKNTNQKNTNQKNTN 480
           TRCAKSNNQNANNQNANNQNATNQNDTNQNDNGTNRRNGNNQNGNRQNDNKQNDNKQNAN
           TRCENPNND-------NTPFKISIHL---------------------------------
           *:* : **.       *.    . :

           QKNTN-QNTNN------------------------------------------------- 540
           KQNANKQNANKQNDNKQNDNKQNGNRQNDNRQNDNKQNDNRQNDNKQNGNRQNGNRQKDN
           ------------------------------------------------------------

           ------------------------------------------------------------ 600
           KRNGNRQNDNRQNDNKRNSNRQNDNRQNDNKRNGNRQNDNKQNDNKQNDNRQNDNNQNDN
           ------------------------------------------------------------

           ------------ 613
           QNDNNRNNQAHHS
           -------------
```

Figure 3.33 Alignment of deduced amino acid sequence among AcMRJP families using Clustal X. Asterisks indicate the same residues. The sequences used to estimate the homology are underlined.

Table 3.10 Estimated genetic distance among *A. cerana* and *A. mellifera* RJ families obtained from nucleotide (above diagonal) and deduced amino acid (below diagonal) sequences.

	AcMRJP1	AmMRJP1	AcMRJP2	AmMRJP2	AcMRJP3	AmMRJP3	AmMRJP4	AmMRJP5
AcMRJP1	-	0.0706	0.2777	0.2916	0.3385	0.3000	0.3555	0.3613
AmMRJP1	0.1017	-	0.3083	0.3170	0.3391	0.3296	0.3789	0.3936
AcMRJP2	0.4533	0.4463	-	0.1064	0.2138	0.2247	0.3175	0.3097
AmMRJP2	0.4380	0.4270	0.1411	-	0.2667	0.2622	0.3336	0.3438
AcMRJP3	0.4988	0.4960	0.3817	0.3889	-	0.1362	0.3655	0.4432
AmMRJP3	0.4799	0.4857	0.4106	0.3963	0.1374	-	0.3599	0.4356
AmMRJP4	0.6143	0.6012	0.6302	0.5984	0.6019	0.5919	-	0.4499
AmMRJP5	0.6016	0.6137	0.5639	0.5284	0.8238	0.7385	0.6995	-

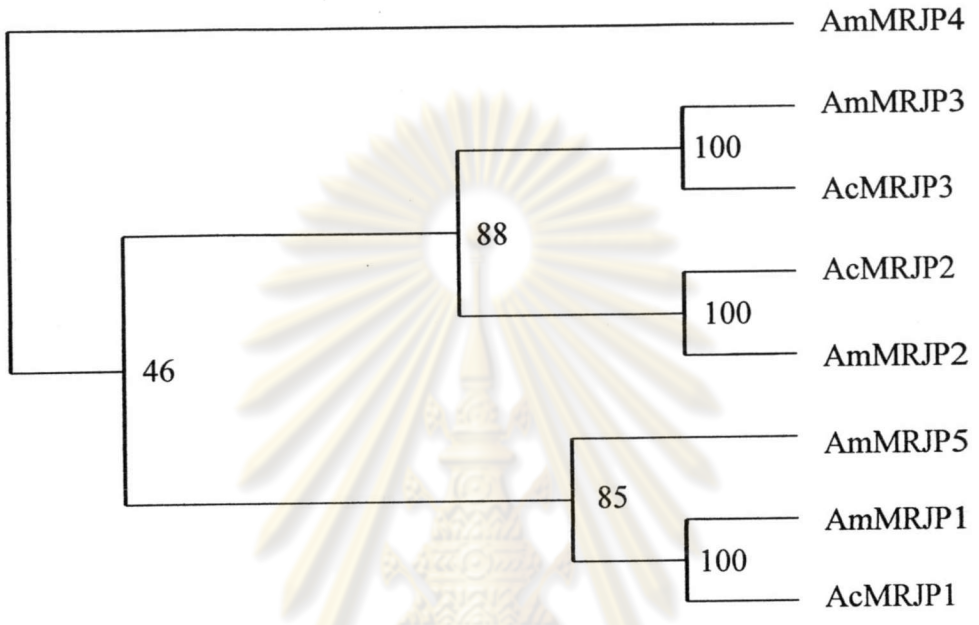


Figure 3.34 A bootstrapped tree illustrating relationship of MRJPs of *A. cerana* and *A. mellifera*. The original nucleotide sequence data was bootstrapped 1000 times. Values at the node indicate the percentage of times occurred out of 1000 trees.

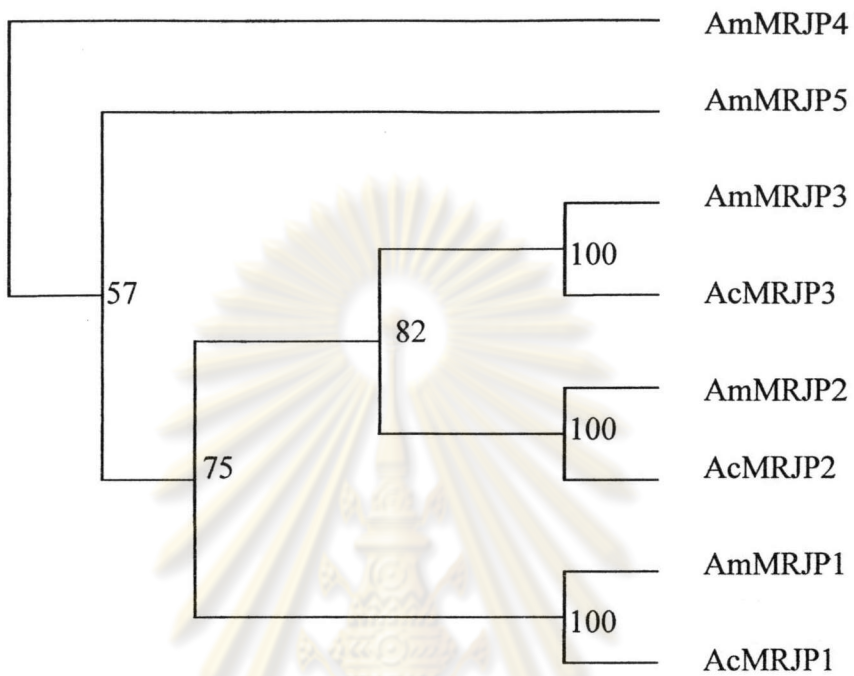


Figure 3.35 A bootstrapped tree illustrating relationship of MRJPs of *A. cerana* and *A. mellifera*. The original deduced amino acid sequence data was bootstrapped 200 times. Values at the node indicate the percentage of times occurred out of 200 trees.

3.4 Semi-quantification of AcMRJP mRNA levels using competitive PCR

3.4.1 Optimization of PCR conditions.

Family-specific primers for AcMRJP1, AcMRJP2 and AcMRJP3 were designed from *A. cerana* cDNA sequences. PCR reactions for each pair of primers were optimized for the most appropriate primer and $MgCl_2$ concentrations and cycle numbers. Optimal PCR conditions were previously described in Table 2.2. Specific primers of each family provided different sizes of products. Moreover, the amplification product was also mapped by digestion with restriction endonucleases to verify the target amplification fragment (Figure 3.36, Table 3.11).

The AcMRJP1 amplified product was individually digested with *Rsa* I, *Dra* I and *Sau*3A I and showed the same restriction fragment sizes as expected. The AcMRJP2 amplified product digested with *Hinf* I and *Taq* I and AcMRJP3 amplified product digested with *Sau*3A I and *Rsa* I yielded expected digestion patterns from nucleotide sequences. This suggested specificity of family-specific primers developed by this study

For amplification of an AcMRJP1 gene (350 bp), $MgCl_2$ concentration (0 - 4.0 mM) was optimized when a sufficient primer concentration was constantly used at 0.15 μM of each primer (Figure 3.37 panel a). PCR product firstly appeared at 0.5 mM $MgCl_2$ and gradually increased until 1.5 mM $MgCl_2$. Non-specific amplification product was not observed. An optimal $MgCl_2$ concentration for an AcMRJP1 gene was then chosen at 1.5 mM $MgCl_2$. Subsequently, primer concentration (0.05 - 0.30 μ M) was evaluated in the same manner at the constant concentration of 1.5 mM $MgCl_2$ (Figure 3.37 panel b). PCR product was firstly visualized at the primer concentration of 0.05 μM and increased with higher primer concentration until 0.20 μM. Although non-specific was slightly observed, it did not significantly interfere the PCR reaction. Thus, an optimal primer concentration for amplification of an AcMRJP1 gene was used at 0.20 μM.

Likewise, amplification of an AcMRJP2 gene (440bp) was optimized with $MgCl_2$ concentration ranging from 0 to 4.0 mM at the 0.15 μM primer concentration (Figure 3.38 panel a). The PCR product was initially observed at 0.5 mM $MgCl_2$ and

consistently increased with 0 - 1.5 mM $MgCl_2$ concentrations. Thus, the $MgCl_2$ concentration used for amplification of an AcMRJP2 gene was 1.5 mM. Together with this, an optimal primer concentration for this gene was also examined (0-0.30 μM at 1.5 mM $MgCl_2$ concentration; Figure 3.38 panel b). The PCR product was gradually increased from 0.05 to 0.20 μM. Thus, the optimal primer concentration for amplification of an AcMRJP2 gene was chosen at 0.20 μM.

Optimization of $MgCl_2$ and primer concentrations were also carried out for amplification of an AcMRJP3 (270 bp; Figure 3.39 panel a). Comparable amount of the PCR product was obtained in all concentrations of $MgCl_2$ but non-specific products were increased in higher concentrations of $MgCl_2$. The most suitable $MgCl_2$ concentration for an AcMRJP3 was then chosen at 1.0 mM. The optimal primer concentration was also examined (Figure 3.39 panel b). The amplification yield of an AcMRJP3 was not increased above the primer concentration of 0.15 μM. Therefore, a primer concentration of 0.15 μM was selected for amplification of an AcMRJP3.

The cycle number of amplification of each AcMRJP gene was also determined to ensure that the amplification reached a plateau phase of reaction. As a result, PCR was carried out for 10 – 26 cycles. The yield of the PCR product was gradually increased until the 16^{th}, 20^{th} and 20^{th} cycles for AcMRJP1, AcMRJP2 and AcMRJP3 genes, respectively. For simplification of the experiment, the appropriate cycle number for PCR was selected at 25 cycles for all primer sets (Figure 3.40).

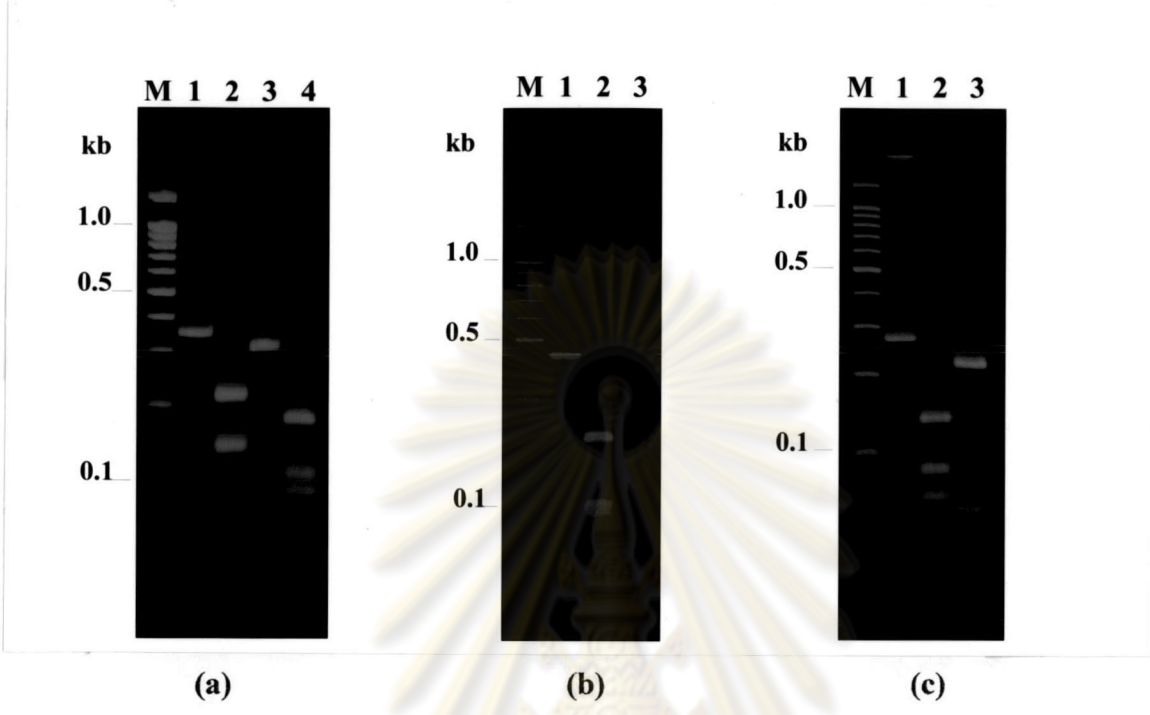


Figure 3.36 Restriction analysis of the amplification products from AcMRJP1 (a), AcMRJP2 (b) and AcMRJP3 (c).

Lane M = A 100 bp DNA ladder

Panel a lane 1 = Undigested AcMRJP1

lane 2 = AcMRJP1 digested with *Rsa* I

lane 3 = AcMRJP1 digested with *Dra* I

lane 4 = AcMRJP1 digested with *Sau*3A I

Panel b; lane 1 = Undigested AcMRJP2

lane 2 = AcMRJP2 digested with *Hinf* I

lane 3 = AcMRJP2 digested with *Taq* I

Panel c; lane 1 = Undigested AcMRJP3

lane 2 = AcMRJP3 digested with *Sau*3A I

lane 3 = AcMRJP3 digested with *Rsa* I

Table 3.11 A summary of digestion pattern of AcMRJPs amplification products on various restriction enzymes

PCR products	AcMRJP1			AcMRJP2		AcMRJP3	
Restriction enzymes	*Rsa* I	*Dra* I	*Sau3A* I	*Hinf* I	*Taq* I	*Sau3A* I	*Rsa* I
Digested size (bp)	210	310	165	200	200	130	215
	140	40	105	100	120	80	55
			80	80	120	60	
				60			
Undigested size (bp)	350	350	350	440	440	270	270

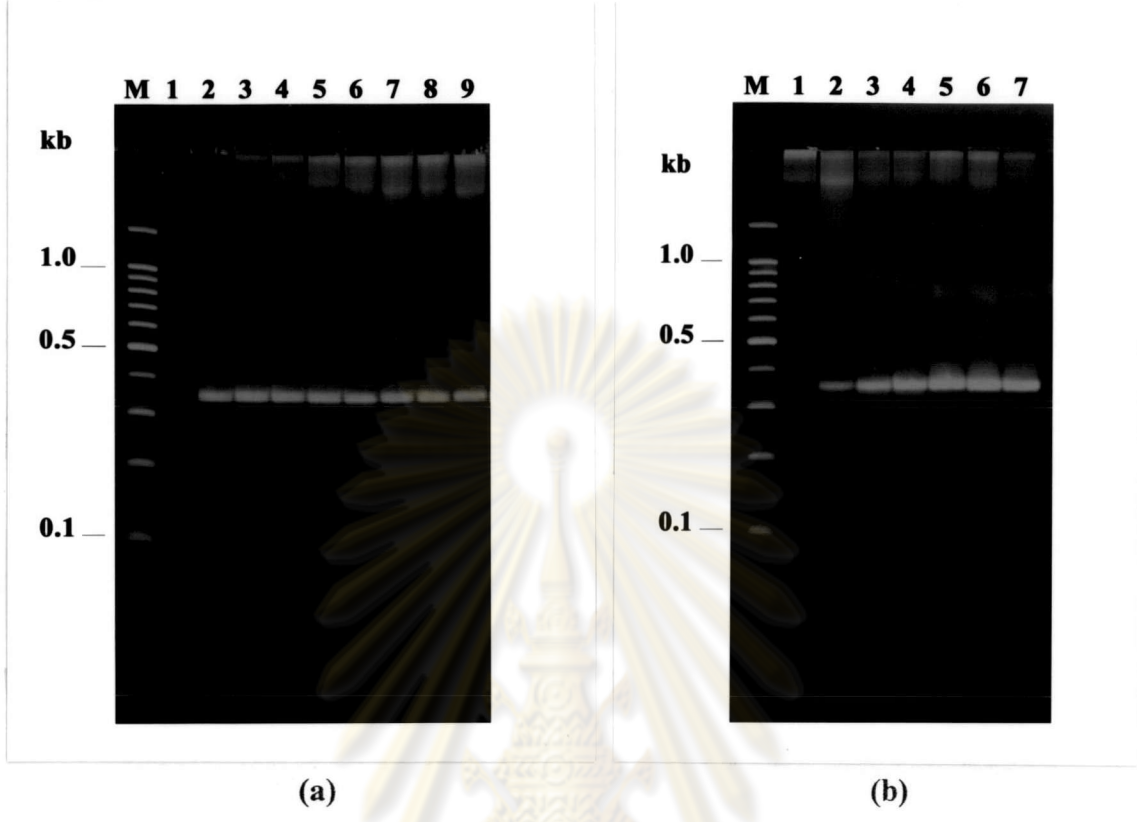


Figure 3.37 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM (a) and optimization of primer concentration at 1.5 mM $MgCl_2$ concentration (b) used for AcMRJP1 amplification.

Lanes M = A 100 bp DNA ladder

Panel a; Lane 1-9 = Amplification product using of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM of $MgCl_2$, respectively.

Panel b; Lane 1-7 = Amplification products using 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μM of each primer, respectively.

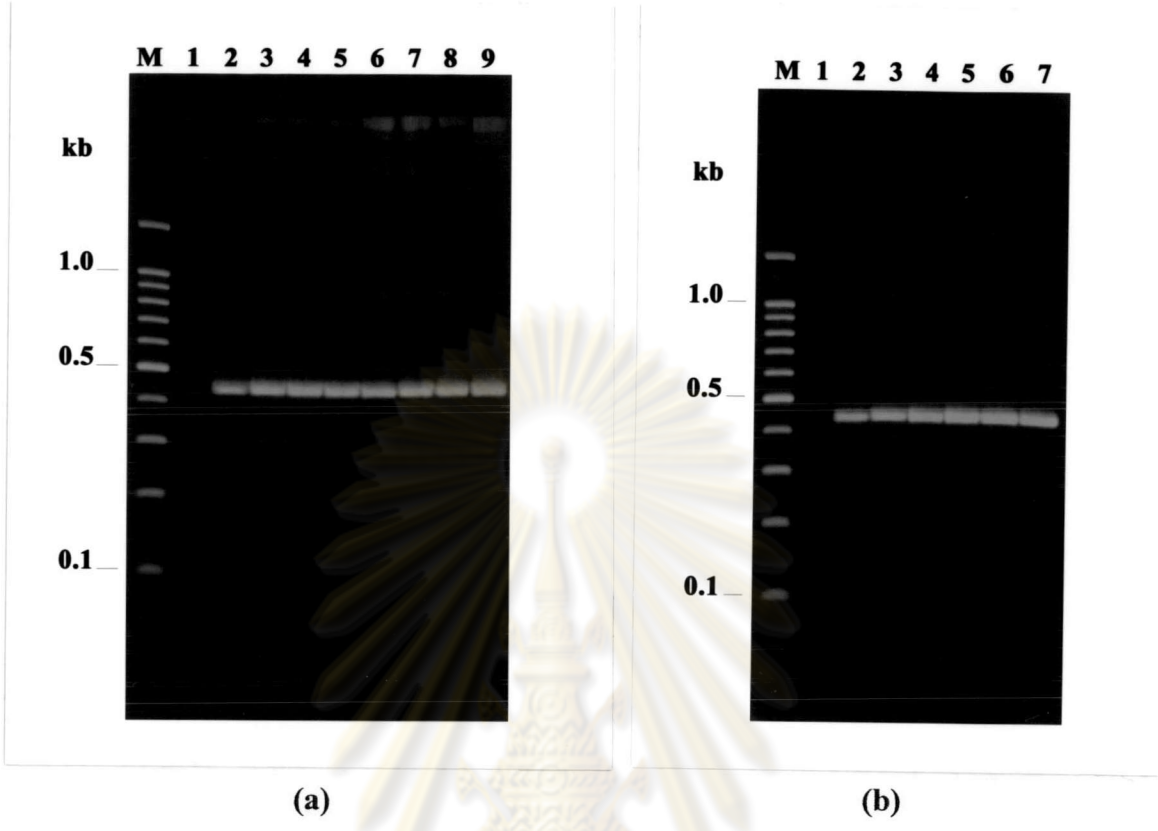


Figure 3.38 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM (a) and optimization of primer concentration at 1.5 mM $MgCl_2$ concentration (b) used for AcMRJP2 amplification.

Lanes M = A 100 bp DNA ladder

Panel a; Lane 1-9 = Amplification product using of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM of $MgCl_2$, respectively.

Panel b; Lane 1-7 = Amplification products using 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μM of each primer, respectively.

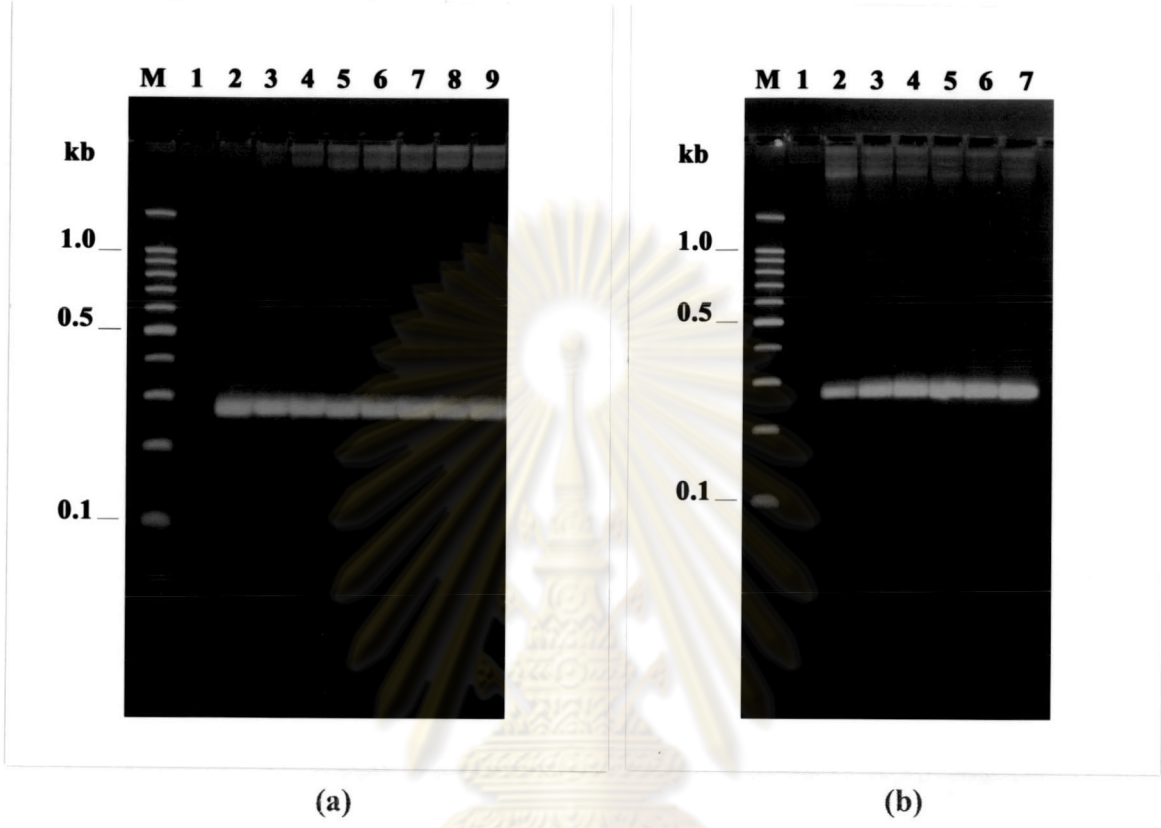
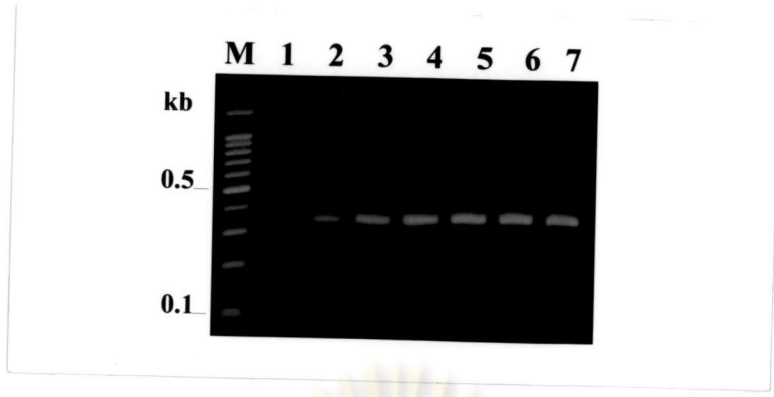


Figure 3.39 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM (a) and optimization of primer concentration at 1.0 mM $MgCl_2$ concentration (b) used for AcMRJP3 amplification.

Lanes M = A 100 bp DNA ladder
Panel a; Lane 1-9 = Amplification product using of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM of $MgCl_2$, respectively.
Panel b; Lane 1-7 = Amplification products using 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μM of each primer, respectively.



(a)

(b)

(c)

Figure 3.40 Determination of cycle number for amplification of AcMRJP1 (a), AcMRJP2 (b)and AcMRJP3 (c).

Lane M = A 100 bp DNA ladder

Panel a,b Lane 1- 7 = PCR product amplified at 10, 12, 14, 16, 18, 20 and 22 cycles, respectively.

Panel c Lane 1-7 = PCR product amplified at 12, 14, 16, 18, 20, 22 and 24 cycles, respectively.

3.4.2 Internal standard DNA preparation

To produce the internal standard DNA, total DNA was individually extracted from the thorax of each *A. cerana* bee and was amplified by a pair of family-specific primers separately. The PCR product of each AcMRJP gene family was then purified. Subsequently, its quantity was estimated by comparing the fluorescent intensity to known concentrations of λDNA (Figure 3.41).

A 10-fold serial dilution between 1 ng/μl to 1 fg/μl was generated and preliminary used in the PCR reactions. Due to the existence of intervening sequences in the genomic DNA, larger sizes of the amplification product than that from cDNA were found. Internal standard size for AcMRJP1, AcMRJP2 and AcMRJP3 was 450 bp, 660 bp and 450 bp in length, respectively.

3.4.3 Quantitative PCR assay

The expression level of each gene family (AcMRJP1, AcMRJP2 and AcMRJP3) in hypopharyngeal glands of nurse bees was determined by semi-quantitative PCR. The mRNA was used as the template to construct first and second stranded AcMRJP1, AcMRJP2, and AcMRJP3 cDNAs (sections 2.16.1, 2.23). This second stranded cDNA was used as target cDNA and co-amplified with known concentrations of internal standard DNA.

As can be seen in Figure 3.42, 3.43 and 3.44, the PCR products showed competitive relationships between the intensity of the target and the internal standard DNAs. Ratios of amount PCR fragments between these DNAs were densitometrically evaluated and plotted versus the known amounts of the standard DNAs. At the equivalent amount of target and internal standard DNAs (ratio = 1), AcMRJP1, AcMRJP2 and AcMRJP3 expression were estimated to be 1 pg/ng mRNA, 0.48 pg/ng mRNA and 0.3 pg/ng mRNA, respectively. Therefore, a ratio of mRNA quantity between different protein families (AcMRJP1: AcMRJP2 : AcMRJP3) was 3.3 : 1.6 : 1.

Expressions of the same AcMRJP family in hypopharyngeal glands of different developmental stages of worker bees (newly emerged bees, nurse bees and frager bees) were also determined by the same method. In this case, total RNA was

extracted from 10 hypopharyngeal glands of each stage of *A. cerana* workers and briefly electrophoretically analyzed through a 1.0 % agarose gel to adjust the concentration of total RNA from different samples (Figure 3.45).

In the newly emerged bees, the PCR product of each family in ACMRJPs was not found, while the PCR product of a positive control (28s ribosomal RNA) was observed in 350 bp (Figure 3.46). In contrast, AcMRJP1, AcMRJP2 and AcMRJP3 were expressed at different levels in both nurse bee and forager bees. The expression level of AcMRJP1 in nurse bees was approximately 1.8 times higher than that in forger bees (Figure 3.47 and 3.48). Likewise, the level of an AcMRJP2 transcript in nurse bees was about 2.5 times greater than that in forager bees (Figure 3.49 and 3.50). Determination of the AcMRJP3 expression was not successful when total was used. Therefore, mRNA purified from total RNA of hypopharyngeal glands was used instead. The corresponding result showed that the expression level of an AcMRJP3 transcript in nurse bees was approximately 2.0 times greater than that in forager bees, conforming as results from AcMRJP1 and AcMRJP2 (Figure 3.51, 3.52).

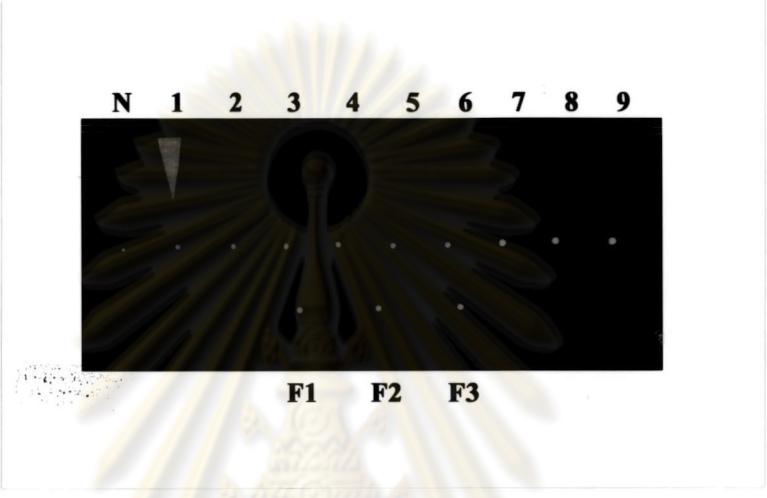


Figure 3.41 Determination of standard DNA concentration using ethidium bromide intensity comparison.

Lane N = A negative control

Lane 1-9 = 2, 4, 6, 8, 10, 12, 14, 16 and 18 ng of DNA, respectively.

Lane F1-F3 = Standard DNA for AcMRJP1, AcMRJP2 and AcMRJP3, respectively.

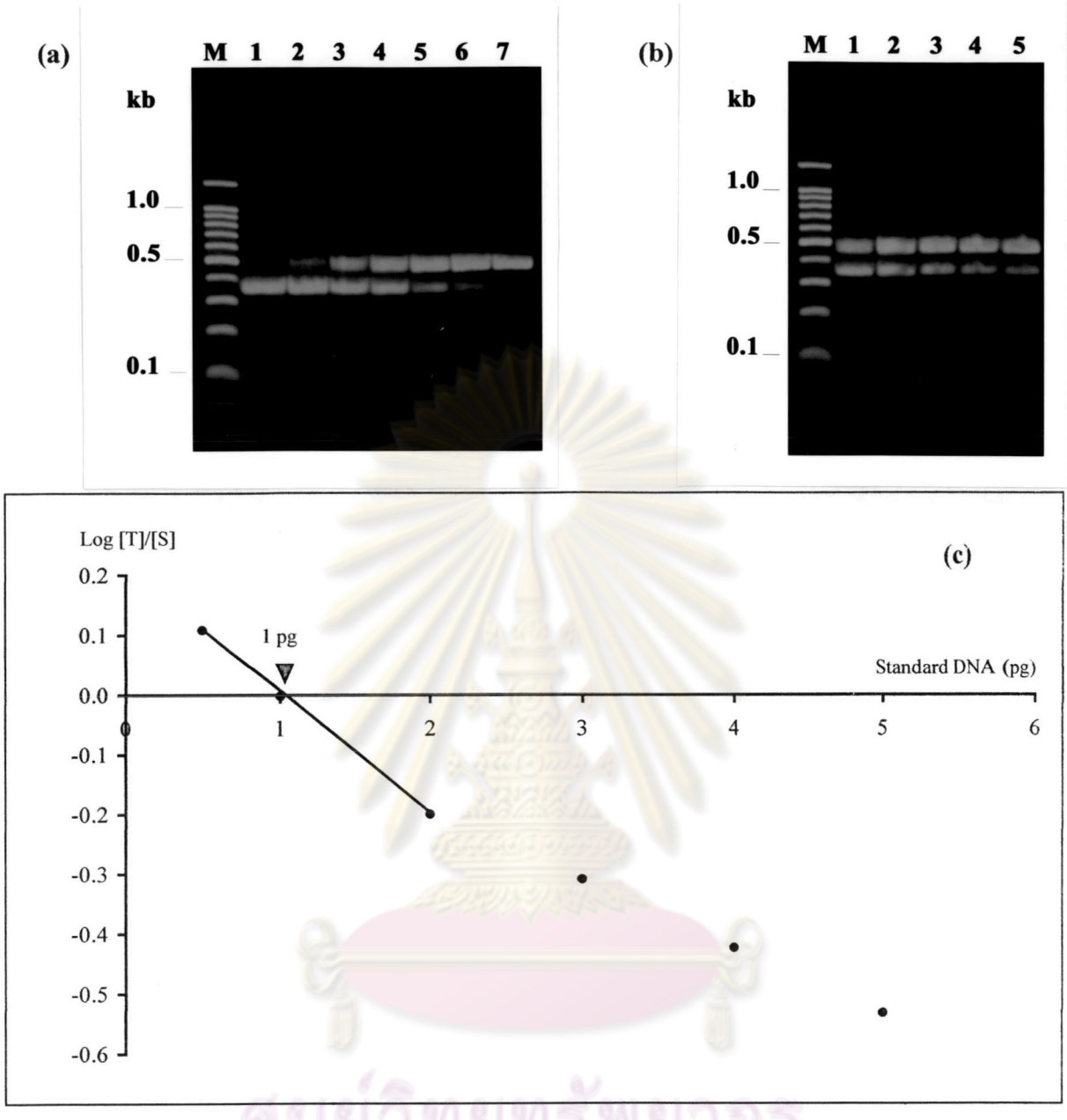


Figure 3.42 Agarose gel electrophoresis (a, b) and a semi Log graph (c) for quantification of AcMRJP1 mRNA in hypopharyngeal gland of *A. cerana* nurse bees using competitive PCR at the constant 1 ng target DNA.

Lanes M = A 100 bp DNA ladder

Panel a; Lane 1-7 = AcMRJP1 co-amplified in the presence of product in 0, 0.1, 0.5, 1, 5, 10 and 20 pg of internal standard DNA, respectively.

Panel b; Lane 1-5 = AcMRJP1 co-amplified in the presence of 1, 2, 3, 4 and 5 pg of the internal standard DNA, respectively.

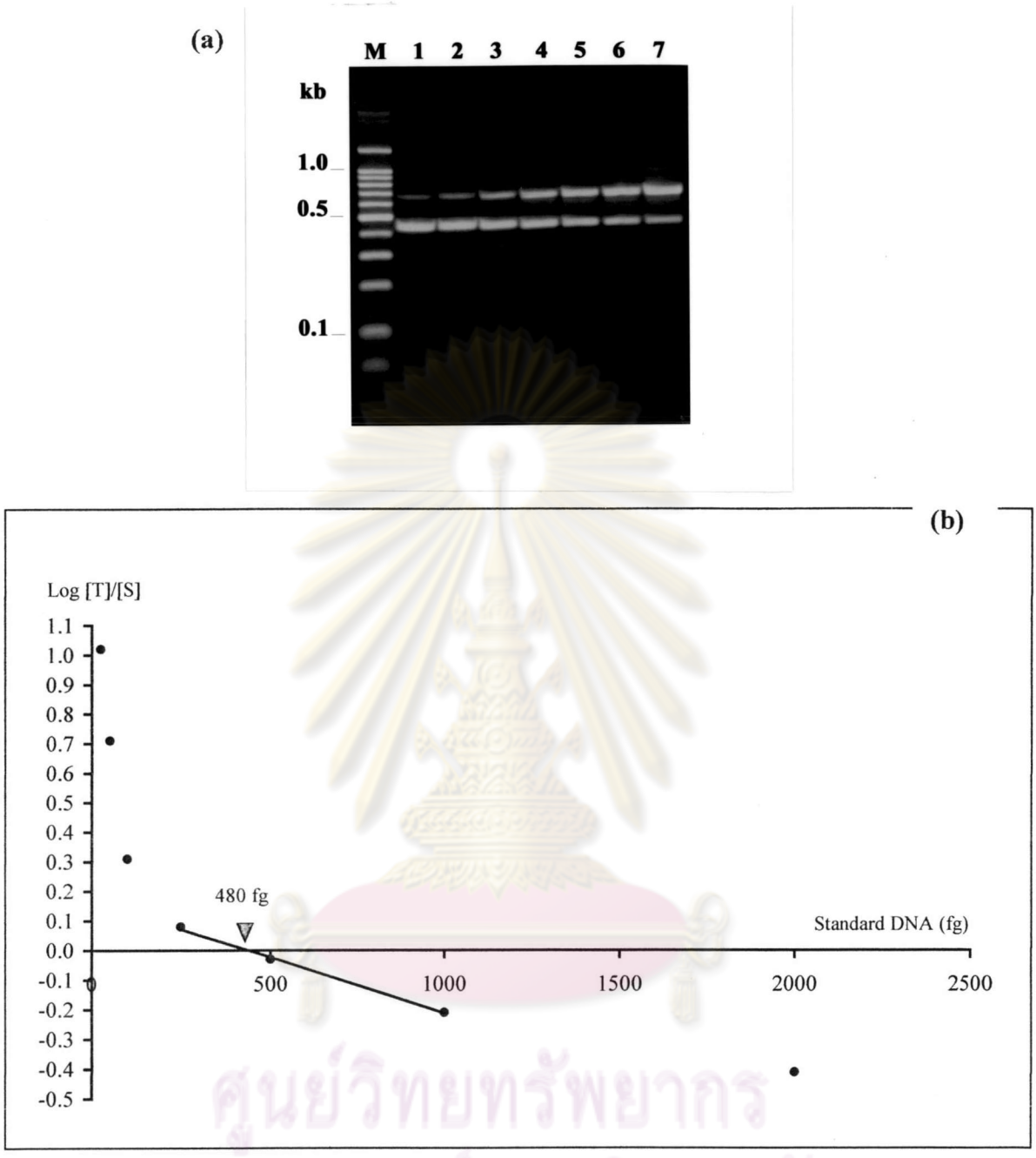


Figure 3.43 Agarose gel electrophoresis (a) and a semi Log graph (b) for quantification of AcMRJP2 mRNA in hypopharyngeal gland of *A. cerana* nurse bee using competitive PCR at the constant 1 ng target DNA.

Lanes M = A 100 bp DNA ladder

Panel a; Lane 1-7 = AcMRJP2 co-amplified in the presence of 25, 50, 100, 250, 500, 1,000 and 2,000 fg of the internal standard DNA, respectively.

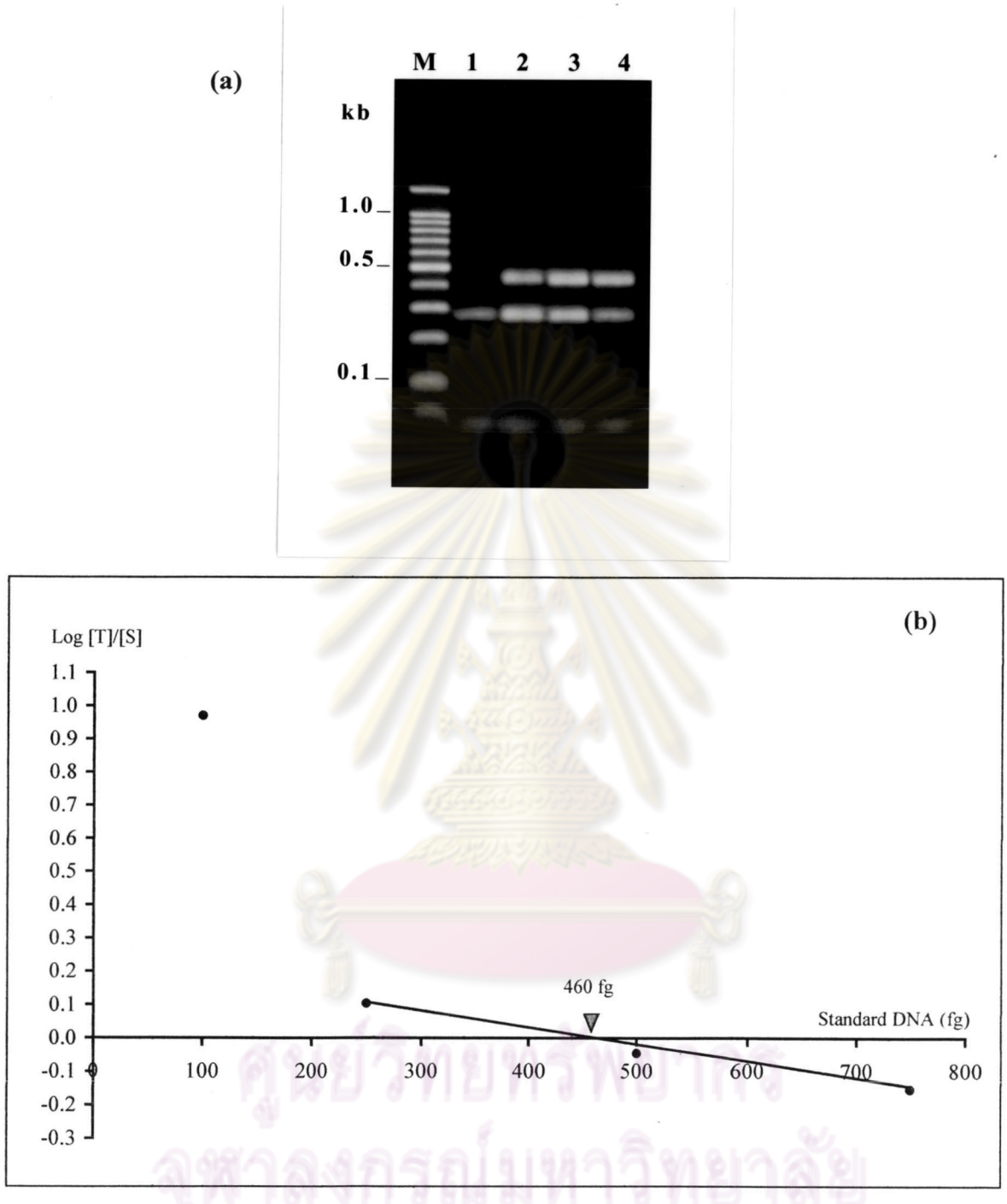


Figure 3.44 Agarose gel electrophoresis (a) and a semi Log graph (b) for quantification of AcMRJP3 mRNA in hypopharyngeal gland of *A. cerana* nurse bees using competitive PCR at the constant 1.5ng of target DNA.

Lanes M = A 100 bp DNA ladder

Panel a; Lane 1-4 = AcMRJP3 co-amplified in the presence of 100, 250, 500, and 750 fg of the internal standard DNA, respectively.

Figure 3.45 Total RNA extracted from 20 hypopharyngeal glands of newly emerged bees (lane 1), nurse bees (lane 2) and foragers (lane 3) was briefly electrophoresed through 1.0 % agarose gel.

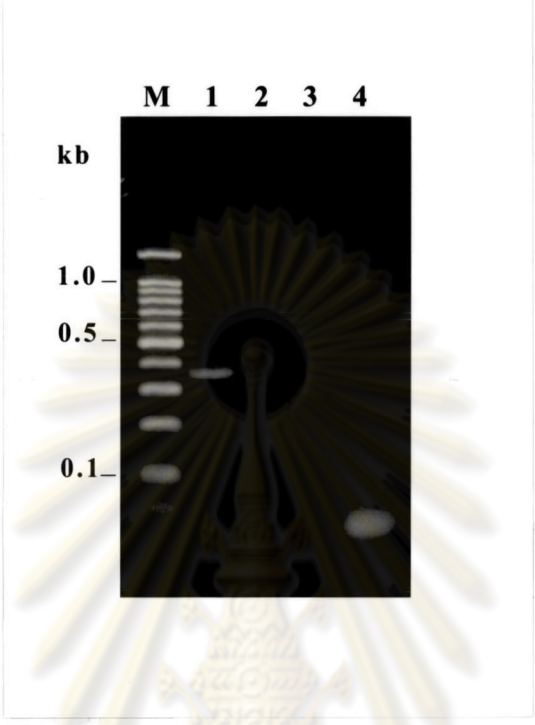


Figure 3.46 Amplification of AcMRJP1 (lane 2), AcMRJP2 (lane 3) and AcMRJP3 (lane 4) of newly emerged *A. cerana*, Lane M is a 100 bp DNA ladder. Lane 1 was 28S rDNA amplified product.

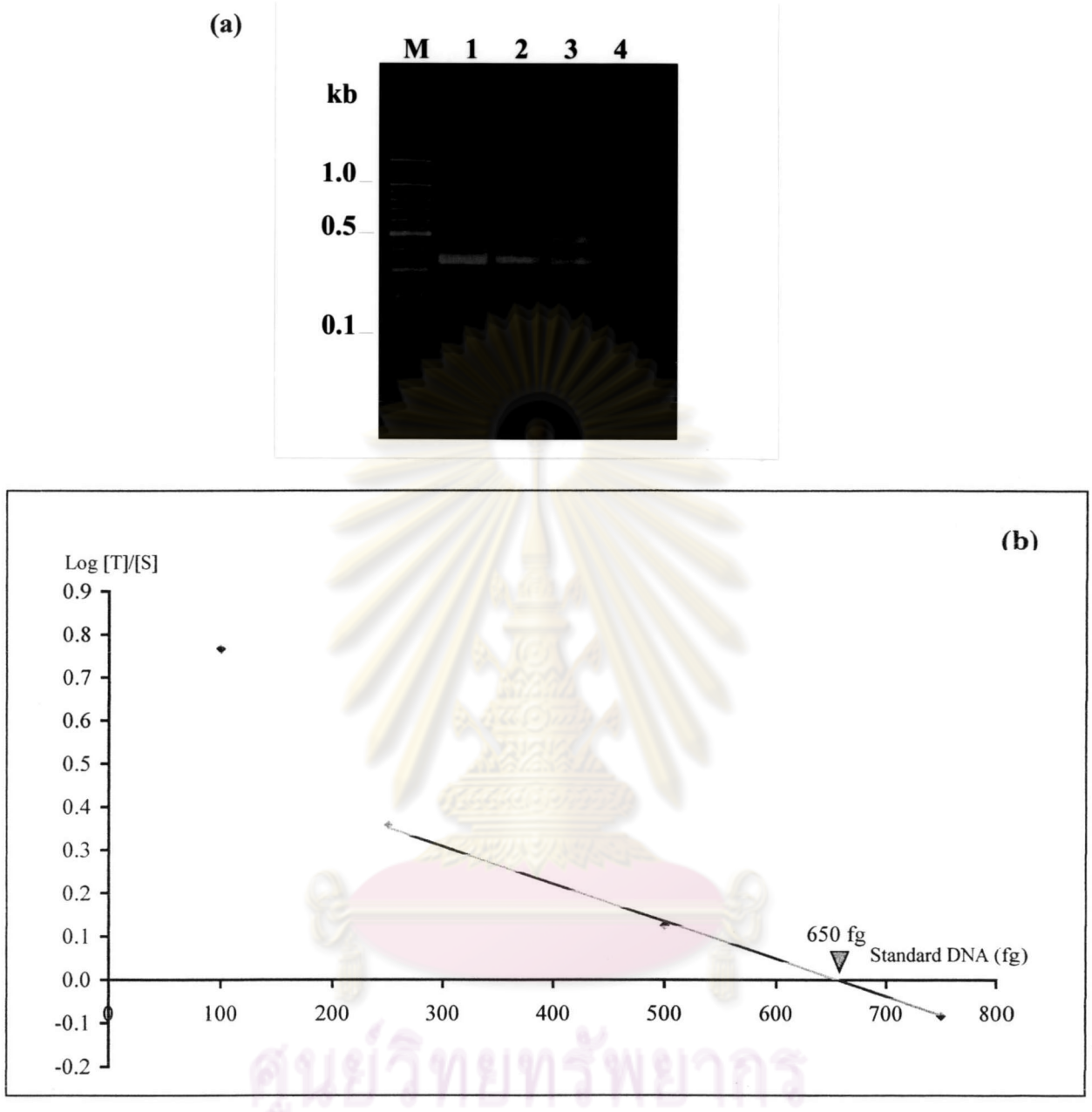


Figure 3.47 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP1 mRNA in hypopharyngeal gland of *A. cerana* nurse bees using competitive PCR at the constant 3.2 µl of the target DNA.

Lane M = A 100 bp DNA ladder

Panel a; lane 1-4 = AcMRJP1 co-amplified in the presence of 100, 250, 500 and 750 internal standard DNA, respectively.

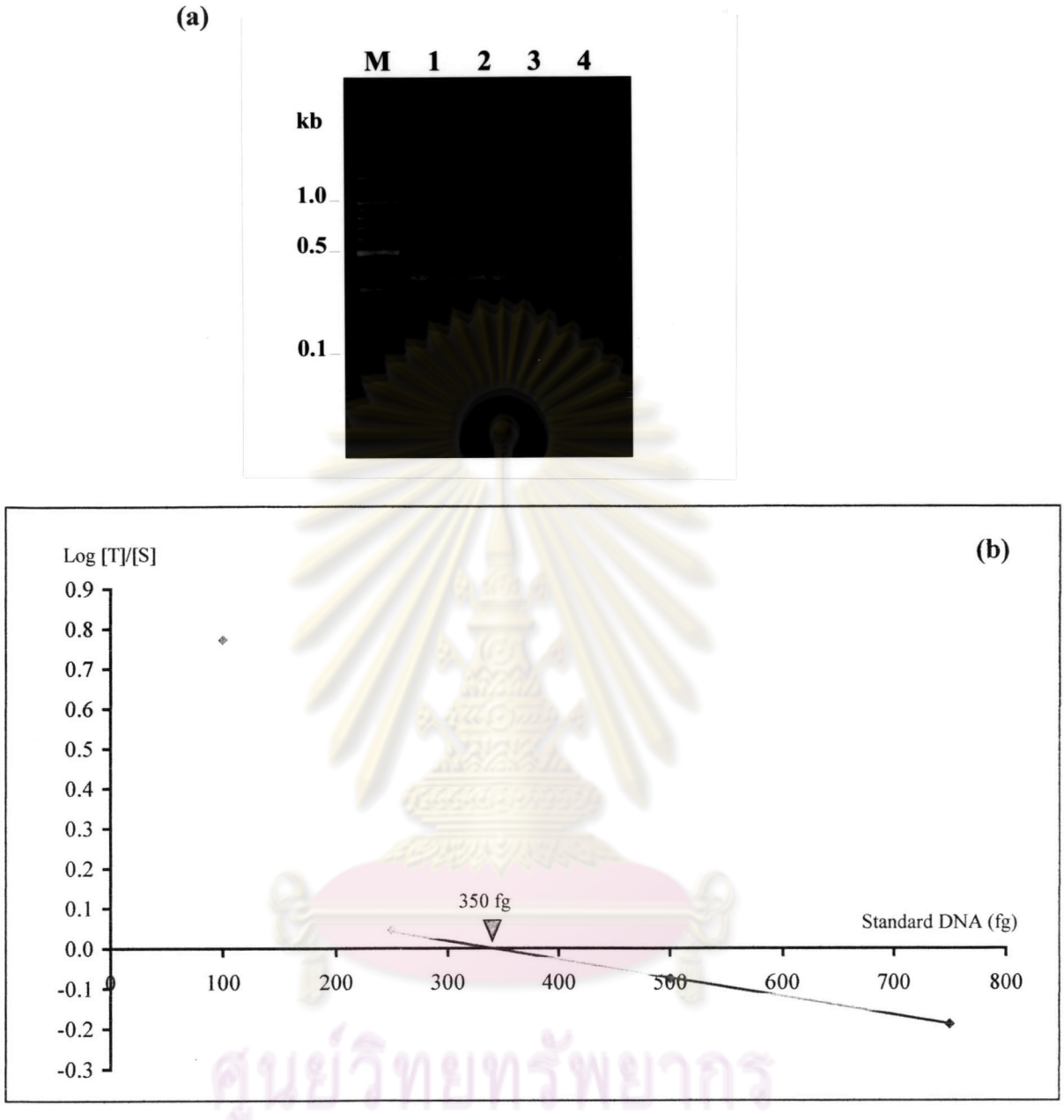


Figure 3.48 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP1 mRNA in hypopharyngeal gland of *A. cerana* foragers using competitive PCR at the constant 3.2 µl of the target DNA.

Lane M = A 100 bp DNA ladder

In panel a; Lane 1-4 = AcMRJP1 co-amplified in the presence of 100, 250, 500 and 750 internal standard DNA, respectively.

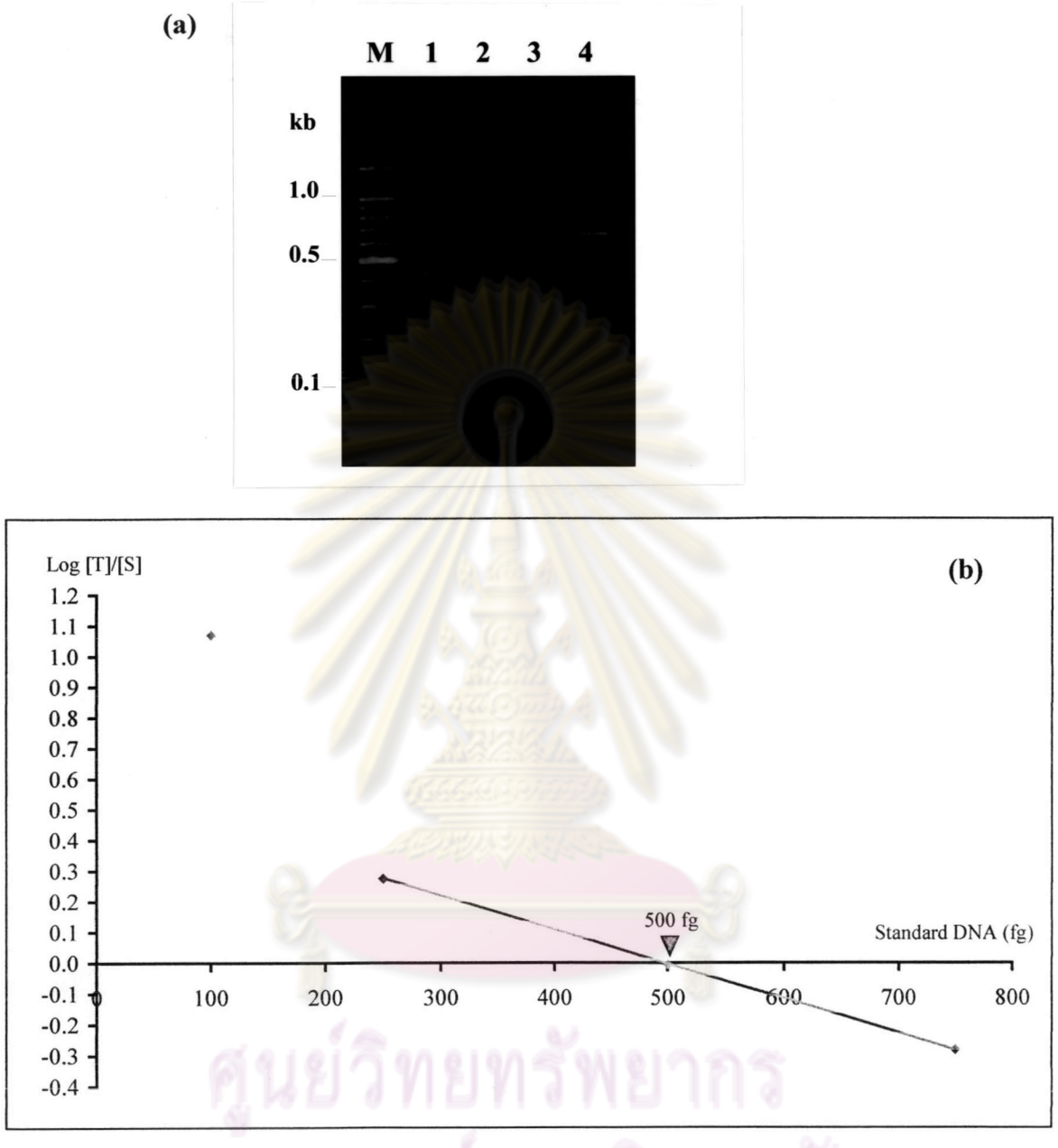


Figure 3.49 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP2 mRNA in hypopharyngeal gland of *A. cerana* nurse bees using competitive PCR at the constant 3.5 μl of the target DNA.

Lane M = A 100 bp DNA ladder

In panel a; lane 1-4 = AcMRJP2 co-amplified in the presence of 100, 250, 500 and 750 internal standard DNA, respectively.

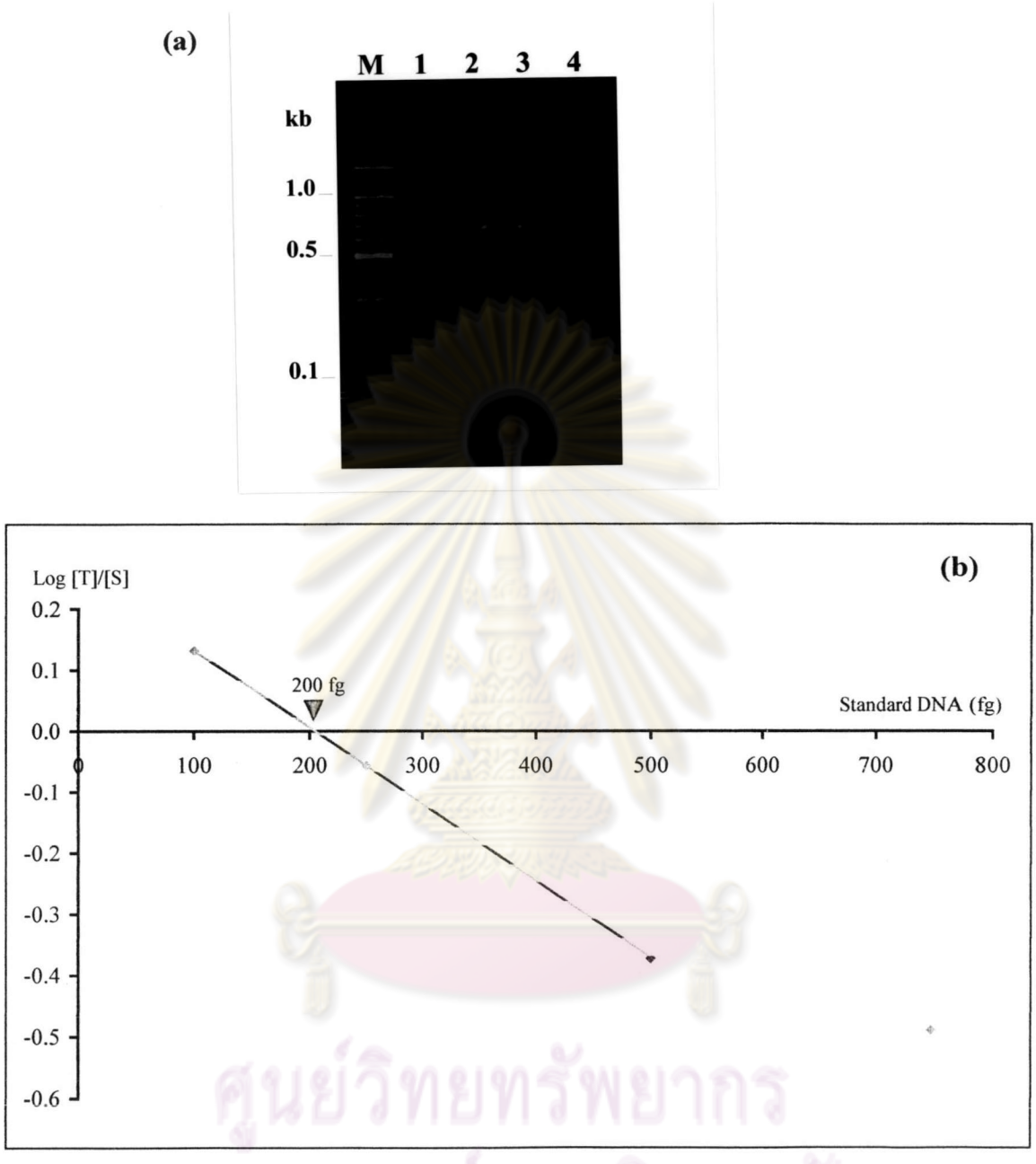


Figure 3.50 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP2 mRNA in hypopharyngeal gland of *A. cerana* foragers using competitive PCR at the constant 3.5 µl of the target DNA.

Lane M = A 100 bp DNA ladder

In panel a; lane 1-4 = AcMRJP2 co-amplified in the presence of 100, 250, 500 and 750 internal standard DNA, respectively.

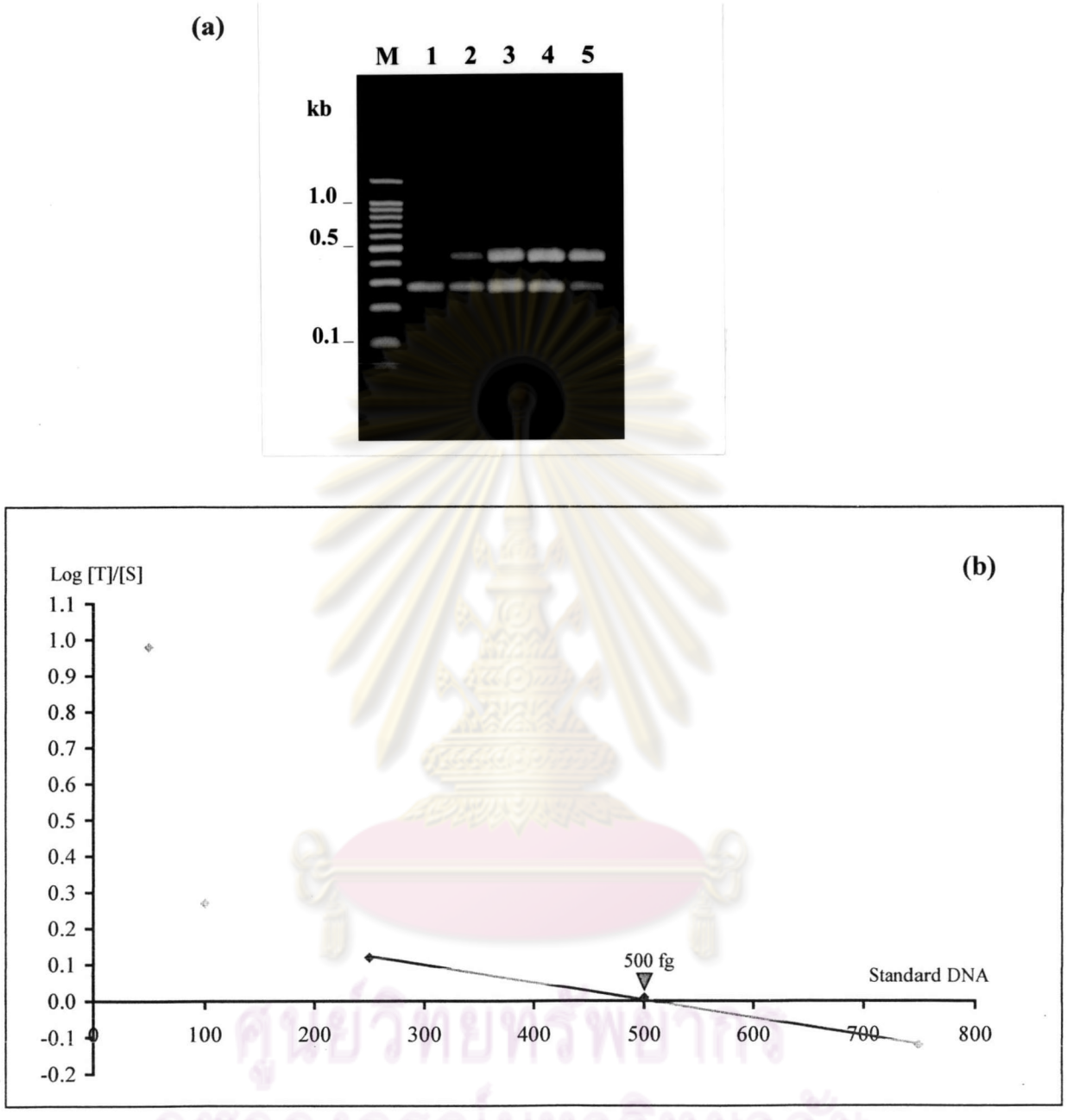


Figure 3.51 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP3 mRNA in hypopharyngeal gland of *A. cerana* nurse bees using competitive PCR at the constant 4.0 µl of the target DNA.

Lane M = A 100 bp DNA ladder

In panel a; lane 1-5 = AcMRJP3 co-amplified in the presence of 50, 100, 250, 500 and 750 internal standard DNA, respectively.

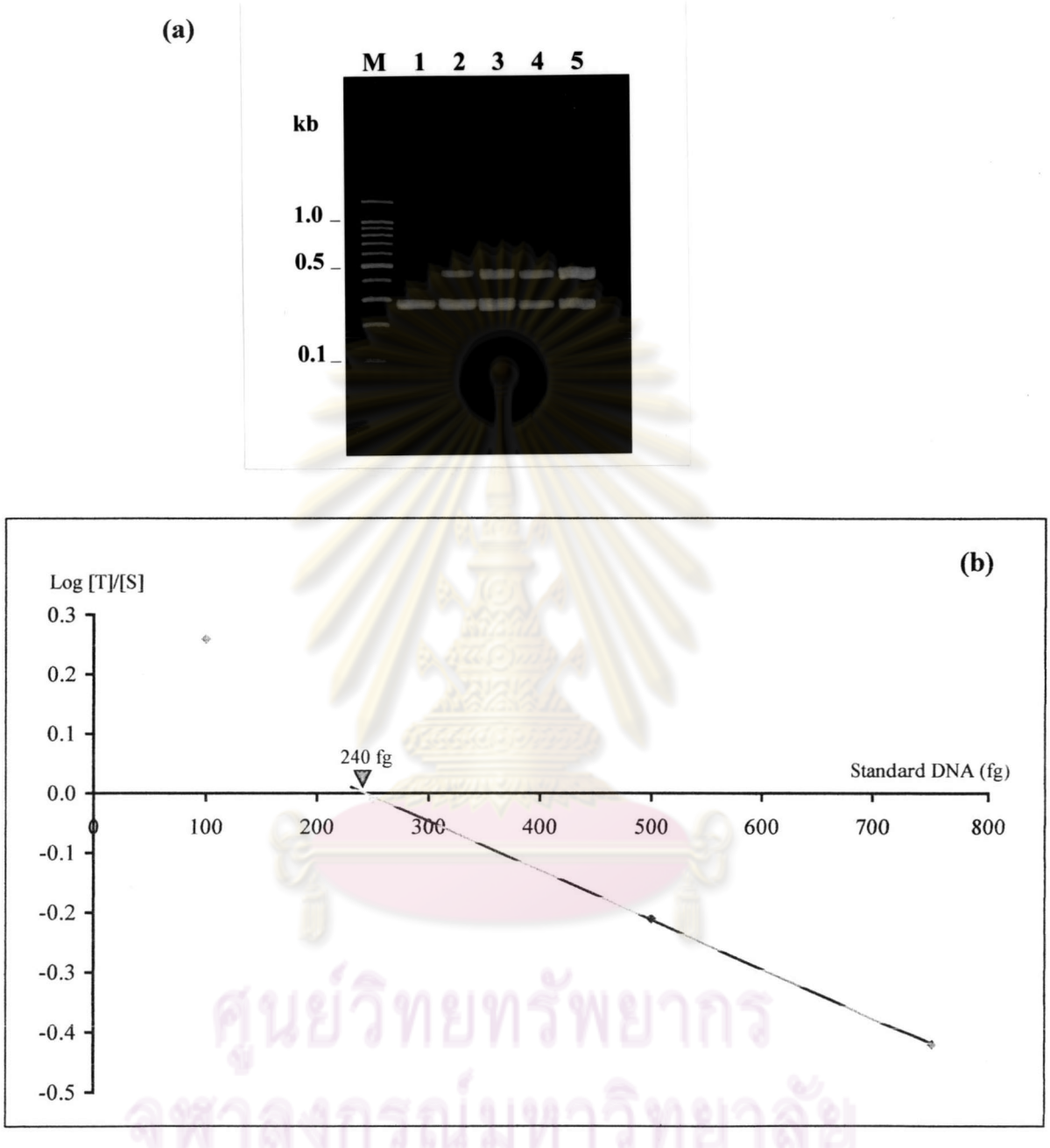


Figure 3.52 Agarose gel electrophoresis (a) and semi Log graph (b) for quantification of AcMRJP3 mRNA in hypopharyngeal gland of *A. cerana* foragers using competitive PCR at the constant 4.0 µl of the target DNA.

Lane M = 100 bp DNA ladder

In panel a; lane 1-5 = AcMRJP1 co-amplified in the presence of 50, 100, 250, 500 and 750 internal standard DNA, respectively.