CHAPTERII

MATERIAL AND METHODS

2.1 Chemicals

Absolute ethanol (Merck, Germany)

Agarose gel (FMC Bioproducts, USA) : Seakem LE Agarose

Ampicillin (Sigma, USA)

AMV First Strand cDNA Synthesis Kit (Biotechnology Department Bio Basic Inc., Canada)

Bacto-agar (DIFCO, USA)

100 Base pair DNA ladder (Promega Coperation, USA)

Boric acid (Merck, Germany)

5-bromo-4-chloro-3-indole-beta-D-galactopyranoside; X-gal (Sigma, USA)

Bromophenol blue (Merck, Germany)

Chloroform (Merck, Germany)

Deoxynucleotide triphosphate:dNTPs (Promega Coperation, USA)

Diethyl pyrocarbonate (Sigma, USA)

Ethylene diamine tetra-acetic acid di-sodium; Na_2EDTA(Fluka, Switzerland)

Ficoll type 400 (Sigma, USA)

Hydrochloric acid (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

NucleoSpin® Extract kit (Macherey-Nagel, Germany)

Phenol crystal (BDH, England)

Protein ladder, 10-200 kDa (Fermentas, Lithuania)

QIAquick Gel Extraction kit (QIAGEN, Germany)

QuickPrep Micro mRNA Purification kit (Amershem Pharmacia Biotech Inc., USA)

Sodium acetate (Merck, Germany)

Sodium chloride (BDH, England)

Sodium dodecyl sulfate: SDS (Sigma, USA)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

Tryptone (DIFCO, USA)

Xylene cyanol FF (Sigma, USA)

Yeast extract (DIFCO, USA)

2.2 Equipments

Autoclave: H-88LL (Kokusan Ensinki Co. Ltd., Japan)

Automatic micropipette:pipetman P2, P20, P100, P200, P1000 (Gilson Medical Electronics S.A., France)

Camera: Pentax K1000 (Asahi Opt. Co., Japan)

Centrifuge:J2-21 (Beckman Instrument Inc.,USA)

Electronic balance: Alsep EY220A (A&D Co. Ltd., Japan)

-20° C Freezer (Krungthai Ltd., Thailand)

-80° C Freezer (Bara laboratory Co. LTD., Thailand)

Ultrasonic: 28H (Ney Dental Inc., USA)

Incubator: BM-600(Memmert Gambh, Germany)

Incubator shaker: GALLENKAMP

Magnetic stirrer and heater (Fisher Scientific, USA)

Microwave Oven: TRX1500 (Turbora International Co. Ltd., Korea)

Power supply: POWERPAC 300 (BioRad Laboratories, USA)

Thermocycler:GeneAmp PCR system 2400 (Perkin Elmer Cetus, USA)

UV transilluminator: 2001microwave (San Gabriel California, USA)

Vortex: K-550-GE (Scientific Industries, USA)

2.3 Inventory supplies

Black and white print film Tmax-400 (Eastman Kodak Company, USA)

Microcentrifuge tubes 0.5, 1.5 ml (Axygen Hayward, USA)

Pipette tips 10, 20, 100 ul (Axygen Hayward, USA)

Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, USA)

2.4 Enzymes

DyNazyme^{TM}II DNA Polymerase (Finnzymes, Finland)

Pfu DNA Polymerase (Promega, USA)

Proteinase K (USB, USA)

Restriction endonucleases

: *Bam*HI (New England Biolab, England)

: *Eco*RI (Amershem Pharmacia Biotech Inc., USA)

: *Nde*I (New England Biolab, England)

: *Ssp*I (New England Biolab, England)

T4 DNA ligase (New England Biolab, England)

Taq DNA Polymerase (Amershem Pharmacia Biotech Inc., USA)

2.5 Primers

Oligonucleotides : BioBasic INC,Bio service unit, Gibco BRL.

2.6 Bacterial strains

Escherichia coli JM109, genotype: F' *tra*D36 *pro*AB^{+} *lac*I^{q} *lac* ZΔM15 *rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ (lac-proAB)

E. coli Rosetta (DE3) pLysS, genotype: F^{-} *omp*T *hsd*S$_{B}$ (r_{B}^{-} m_{B}^{-}) *gal dcm lac*Y1 (DE3) pLysSRARE(Cm^{R})

2.7 Plasmids

pGEM®-T Easy vector (Appendix A)

pET-19b vector (Appendix B)

2.8 Sample preparations

2.8.1 Honeybee samples

Honeybee samples, *Apis cerana*, were collected from Chumphon Province in manage beekeeping colonies. Nurse honey bees were collected in grafting colony when they were feeding the brood onto the queen cup. Honey bee samples were immediately preserved in liquid nitrogen and then stored at –75°C for later use.

2.8.2 Hypopharyngeal gland samples

Hypopharyngeal gland was dissected out from the head of each frozen nurse bee under a binocular microscope at 4°C. A knife was used to cut through the wall of the mask, across the vertex, round the margins of the compound eyes, and round the edges of the mask. The mask was then taken off. A hypopharyngeal gland located in front of the brain was removed and placed in a tube containing pre-chilled buffer constituting of guanidium thiocyanate and *N*-lauroyl sarcosine and stored in liquid nitrogen.

2.9 Molecular Cloning of AcMRJPs cDNA

For cloning of AcMRJPs cDNA, the full length cDNAs were isolated from hypopharyngeal gland of *A. cerana* by the Reverse transcription-polymerase chain reaction (RT-PCR) method. The mRNA was extracted from hypopharyngeal gland of nurse honeybee. In the RT reaction, the oligo(dT) primers were used to transcribe all the mRNA species into cDNAs. It anneal to the 3' poly(A) tails of mRNA molecules, and then Avian myeloblastosis virus (AMV) reverse transcriptase was synthesized the first-stranded cDNAs. The first - stranded cDNAs were used as template in PCR reaction that contain *Taq* DNA polymerase, MRJP specific forward and reverse primers. The amplified AcMRJPs cDNA product was electrophoresed analyzed through a 0.7% agarose gel and then purified from agarose gel by QIAquick spin column. The purified AcMRJPs cDNA were ligated with pGEM®-T easy vector. The ligation product was electro-transformed to *E. coli* JM109. The recombinant clones contained the cDNA inserts were screened by blue-white colony screening on selective plate. Positive clones were selected and cultured in LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl pH

7.5) containing 50 μg/ml of ampicillin. The recombinant plasmids were extracted using an alkaline lysis method . The extracted plasmid was digested with restriction enzyme *Eco*RI and *Ssp*1. The digested product was electrphoretically analyzed through 1% agarose gel. The clones that show restriction map correlated with restriction map of AmMRJP were selected for nucleotide sequencing. The universal M13 forward and reverse primers were initially used for sequencing. Internal sequencing primers were subsequently designed from nucleotide sequence obtained, and used for sequencing along the entire length of the cDNA insert. The nucleotide sequences were identified by blasted against the nucleotide sequence that deposited in the GenBank database. The AcMRJP nucleotide sequences were further characterized by various computer programs.

2.9.1 mRNA extraction

The mRNA was extracted from 50 hypopharyngeal glands of nurse bees using QuickPrep® *Micro* mRNA Purification kit (Amershem Pharmacia Biotech Inc., USA). Hypopharyngeal glands were homogenized in 0.4 ml of extraction buffer containing guanidinium thiocyanate and *N*-lauroyl sarcosine. The homogenate was diluted with 0.8 ml of the elution buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA), mixed and centrifuged at 10,000xg for 1 minute. This can be done in parallel with centrifugation of 1 ml Oligo(dT)-cellulose. Clear homogenate was obtained and transferred to the tube containing Oligo(dT)-cellulose pellet and gently mixed for 3 minutes and centrifuged at 10,000xg for 1 minute at room temperature.

The pellet was washed 5 times with 1 ml of the high-salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.5 M NaCl) and 2 times with 1 ml of the low-salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 M NaCl). In each step, Oligo (dT)-cellulose

was mixed and centrifuged at 10,000xg for 1 minute. After washing, Oligo(dT)-cellulose was resuspended in 0.3 ml of the low-salt buffer and transferred to MicroSpin column which was placed in a microcentrifuge tube. The column was then centrifuged at 10,000xg for 1 minute. Three additional times of of low-salt washing were performed. The column was placed in a new microcentrifuge tube and 0.2 ml of the pre-warmed at 65 °C elution buffer was added on the top of the resin bed. The mRNA was collected by centrifugation at 10,000xg for 1 minute. The second 0.2 ml of pre-warmed at 65 °C elution buffer was added to recover the residual mRNA. To concentrate mRNA, 10 µl of 10 mg/ml glycogen solution and 40 µl of 2.5 M potassium acetate solution pH 5.0 were added followed by two volumes of cold absolute ethanol. The resulting solution was kept in –20 °C for a minimum of 30 minutes. Precipitated mRNA was collected by centrifugation at 10,000xg for 10 minutes at room temperature, air dried and redissolved in DEPC-treated water.

Quantity of mRNA was then spectrophometrically determined. The concentration of mRNA was calculated following the formula: [mRNA] = A260 x 40 x dilution factor.

2.9.2 Synthesis of MRJP cDNA by Reverse transcription-polymerase chain reaction (RT-PCR)

2.9.2.1 First stranded cDNA synthesis

The first stranded of total cDNA was synthesized using an AMV First Strand cDNA Synthesis Kit (Biotechnology Department Bio Basic Inc., Canada). Approximately 0.6 µg of mRNA and 0.5 µg of Oligo(dT)18 primer in 12 µl reaction volume was gently mixed and incubated at 70 °C for 5 min. The mixture was quickly chilled on ice for at

least 5 minutes and spun down. Subsequently, 20 units of ribonuclease inhibitor, 2 µl of 10 mM each of dNTP mixture and 4 µl of 5X reaction buffer were added, mixed and incubated at 42 °C for 5 minutes. Finally, 20 units of AMV reversetranscriptase was added in the reaction, mixed and further incubated at 42 °C for 60 minutes. At the end of the incubation period, the reaction was terminated by heating at 70 °C for 10 minutes before chilled on ice for 2-3 minutes.

2.9.2.2 PCR amplification of the full length *A. cerana* MRJP4 and 5 cDNA

Primers for amplification of the full length *A. cerana* MRJP4 cDNA were designed from *A. mellifera* MRJP4 sequences (Klaudiny *et al.*, 1994). The forward primer was designed over start codon. The reverse primer was designed on poly A region. A pair of primers for amplify AcMRJP4 were 5'-ATA TCC TAG AAA AAA AAT GAC AAA ATG GTT GC- 3'(primer name MRJP4) and 5'- GGG GTA CCC TTT TTT TTT TTT TTT TTT TTT TTT TTT TA- 3'(primer name RMJ2). Amplification reaction was carried out in a 25 µl reaction volume containing 1 µl of the 1st stranded cDNA (2.9.2.1), 1X PCR buffer with $MgCl_2$ (10mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 100 µM of each dNTP, 0.1 µM of forward and reverse primers and 1 unit of *Taq* DNA polymerase. The reaction was predenatured at 94°C for 5 minutes following by 35 cycles of denaturing at 94 °C for 50 seconds, annealing at 50 °C for 1 minute and extension at 72 °C for 1.50 minutes. The final extension was performed at 72°C for 10 minutes.

Primers for amplification of the full length AcMRJP5 cDNA were designed from AmMRJP5 sequences (Albert *et al.*, 1999). The forward primer was designed before start codon. The reverse primer was designed on poly A region. A pair of primers for

amplify AcMRJP5 were 5'- CTG TCG TTT GCA AAA TAT TTG CAG C - 3'(primer name MRJP5_2) and 5'- GGG GTA CCC TTT TTT TTT TTT TTT TTT TTT TTT TTT TA- 3'(primer name RMJ2). Amplification reaction was carried out in a 25 μl reaction volume containing 1 μl of 20 fold dilution 1st stranded cDNA, 1X PCR buffer with $MgCl_2$ (10mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 100 μM of each dNTP, 0.1 μM of forward and reverse primers and 1 unit of *Taq* DNA polymerase. The reaction was predenatured at 94°C for 3 minutes following by 25 cycles of denaturing at 94 °C for 50 seconds, annealing at 51 °C for 1 minute and extension at 72 °C for 3 minutes. The final extension was performed at 72°C for 10 minutes.

After amplification process, 5 μl of reaction mixture was electrophoretically analyzed using a 0.7 % agarose gel.

2.9.2.3 Analysis of cDNA products by agarose gel electrophoresis

Agarose gel electrophoresis was standard method used to size-fractionate and purification of DNA fragments. Different concentrations of agarose gel were prepared depending on sizes of DNA fragments. Generally, 0.7-1.0 % agarose gels were used for general purposes. An appropriate amount of agarose was weighted out and dissolved in the appropriate volume of 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.3). The gel slurry was heated until completely solubilize by microwave oven. The agarose solution was incubated at 65°C and further left to 50°C before poured into the electrophoretic gel mould. The comb was inserted. After the gel was completely set , the comb was carefully removed. The gel was placed in the electrophoresis chamber. An enough volume of 1X TBE was poured to cover the gel for 2-3 cm. One-fifth volume

of loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 15 % Ficoll 400) was added into the sample and loaded into the gel. Electrophoresis was usually operated at 100 volts for 0.7-1.5% gel until bromophenol blue reached approximately 1 cm from the bottom of gel. The gel was stained with a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained in deionized water for 15 minutes. The DNA was visualized under a long wavelength UV light and photographed through a red filter using a Kodax Tri - Xpan 400 film. The concentration or molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA fragment.

2.9.3 Purification of cDNA product by recovery from agarose gel

An appropriate amount of amplified AcMRJP4 and AcMRJP5 cDNA product (2.9.2.2) was electrophoresed analyzed through a 0.7 % agarose gel. After electrophoresis was complete, the marker lane (λ/*Hin*d III) and partial of amplified product lane was cut off and stained with ethidium bromide solution (2.5 μg /ml) for 5 minutes. The position of desired cDNA band was located and excised from the gel and placed into the pre-weight microcentrifuge tube. The gel slices were weighted. Three volumes of QG buffer to one volume of gel (100mg estimated 100μl) were added. The gel mixture was incubated at 50 °C for 10 minutes or until the gel slices were completely dissolved. The color of the gel mixture was yellow.

The gel mixture was applied to a QIAquick spin column, which was placed into a provided 2-ml collection tube and centrifuged at 10,000xg for 1 minute at room temperature. The effluent was discarded. Optionally, 0.5 ml of QG buffer was added to remove all traces of agarose from the column and centrifuged at 10,000xg for 1 minute.

After that, 0.75 ml of the PE buffer was added, left for 2-5 min and centrifuged at 10,000xg for 1 minute. The column was placed into a new microcentrifuge tube. Finally, DNA was eluted by adding 50 µl of the EB buffer (10 mM Tris-HCl pH 8.5) and centrifuged at 10,000xg for 1 minute.

2.9.4 Ligation of MRJP cDNA to pGEM®-T easy vector

The pGEM®-T easy vector (Appendix A) was used for cloning of MRJP cDNA. The ligation reaction was performed in the total volume of 10 µl containing 250 ng of purified cDNAs from 2.9.3, 50 ng pGEM®-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2mM ATP and 10 % PEG 8000), 3 units of T4 DNA ligase. The ligation mixture was mixed and incubated at 4 °C overnight. The ligation product was electro-transformed to *E. coli* JM109.

2.9.5 Electro-transformation of recombinant DNA into *E. coli* JM109

2.9.5.1 Preparation of host cells for electro-transformation

A 5 ml overnight culture of *E. coli* JM109 was inoculated to 500 ml of LB broth (1% tryptone, 0.5% yeast extract and 1.0 % NaCl). The cell was incubated at 37°C with shaking at 250 rpm for 2-3 hours until the optical density at 600 nm of culture reached 0.5-0.7. The culture was chilled on ice for 20-30 minutes and harvested by centrifugation at 8,000xg for 15 minutes at 4 °C. The supernatant was carefully decanted. The cell pellet was washed two times with 500 ml and 250 ml of cold water, and then washed with 20 ml of ice-cold 10% glycerol. The cells were collected by centrifugation at 8,000xg for 15

minutes at 4 °C. Finally, the concentrated cells were resuspended in a total volume of 1.0 ml of ice-cold 10% glycerol, divided into 40 µl aliquots and stored at –80 °C until used.

2.9.5.2 Electro-transformation

An aliquot of 40 µl of concentrated cell in 2.9.5.1 was thawed on ice and mixed with 1 µl of the ligation product. The mixture was transferred into the narrow gap of cold electroporation cuvette (0.2 cm) and tapped to the bottom. The cuvette was then placed in the chamber slide, pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber and pulsed once. The condition of electrophoration was set as follows; 25µF, 200Ω. and 2.50 kV of the pulse controller unit.

After one pulse was applied, 1 ml of the LB broth (1.0 % tryptone, 0.5 % yeast extract and 1.0 % NaCl pH 7.5) was immediately added to the cuvette and the cells were immediately resuspended with a Pasteur pipette. The cell suspension was transferred to the tube and incubated at 37 °C for 1 hour. Aliquots of the cells were spread on the selective plates and incubated for 16 hours as describe in 2.9.6.

2.9.6 Blue-White colony screening for recombinant plasmid

The selective plate (1% tryptone, 0.5% yeast extract, 1.0 % NaCl and 1.5 % Bacto-agar) containing 50 µg /ml ampicillin and coating with 4 µl of 20 % ITPG, 40 µl of 20 mg/ml X-gal was prepared. Aliquots of the cultured cell from electro-transformation (2.9.5.2) (200, 100 and 50 µl) were spread to LB agar plates and incubated at 37 °C for 16-18 hours. White colonies were selected for further analysis.

2.9.7 Characterization of the insert DNA of recombinant plasmid

For characterization of the insert DNA of recombinant plasmid, the recombinant plasmids were extracted by alkaline lysis method. Subsequently, the recombinant plasmids were characterized by digested with selected restriction enzymes. The restriction pattern of digested products were compared with restriction map of AmMRJP to identified the recombinant plasmid. Finally, the recombinant plasmids that had restriction map correlated with desire MRJP were selected for nucleotide sequencing. The nucleotide sequences obtained were blasted against those deposited in the GenBank database to identified type of MRJP.

2.9.7.1 Plasmid extraction

A recombinant clone (white colony on selected plate, 2.9.6) was inoculated into 3 ml of LB broth containing 50 μg/ml of ampicillin and incubated at 37 °C with constant shaking at 250 rpm for 16-18 hours. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 10,000xg for 1 min. The cell pellet was collected and resuspended with 100 μl of solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA and 50 mM glucose). The mixture was completely dispersed by vortexing, and then placed on ice. The mixture was then added with 200 μl of freshly prepared solution II (0.2 N NaOH and 1% SDS), gently mixed and placed on ice. Additionally, 150 μl of solution III (3 M sodium acetate, pH 4.8) was added, gently mixed and placed on ice for 5 minutes. To separate the cell debris, the mixture was centrifuged at 12,000xg for 10 minutes. The supernatant was transferred into a new microcentrifuge tube and extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). The mixture was centrifuged at 8,000xg for 5 minutes. The supernatant were removed to a new microcentrifuge tube.

Plasmid DNA was precipitated with the addition of 2 volumes of ice-cold absolute ethanol for 15 minutes at -80°C and recovered by centrifugation at 10,000xg for 10 minutes at 4°C. The DNA pellet was washed with 70% ethanol, centrifuged at 10,000xg for 10 minutes, air died and dissolved in 30 µl TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA) containing 20 µg/ml RNase A. Then plasmid was incubated at 37 °C for 1 hours and stored at –20 °C until used.

2.9.7.2 Restriction mapping for MRJP cDNA in the recombinant plasmid

The extracted recombinant plasmid from positive clone was separately digested with *Eco*RI (Amershem Pharmacia Biotech Inc., USA) and *Ssp*I (New England Biolab) restriction endonuclease. The reaction was carried out in 15 µl containing approximately 500 ng of recombinant plasmid, 10 unit of each restriction enzyme and 1X reaction buffer (supply by the manufacturer). The reaction mixture was incubated at 37 °C overnight. The digested product was electrophoretically analyzed through 0.7 % agarose gel.

2.9.7.3 Nucleotide sequencing and data analysis

Recombinant plasmid containing cDNA insert was extracted from recombinant clone and sequenced at Bioservice unit (BSU), Thailand. The universal M13 forward and reverse primers were initially used for sequencing by an automated DNA sequencer,by the dideoxynucleotide chain termination method of Sanger *et al.*, using an Applied Biosystems 373 A DNA sequencer with a PRISM kit (Perkin Elmer, U.S.A.) Internal sequencing primers were subsequently designed by Oligo program (Table 2.1 and Figure 2.1), and used for sequencing along the entire length of the insert.

Nucleotide sequences obtained were blasted against those deposited in the GenBank database using the BlastN and BlastX programs (http://www.ncbi.nlm.nih.gov).

The consensus polyadenylation signal sequences were scanned by HcpolyA : Hamming Clustering poly-A prediction in Eukaryotic Genes (http://l25.itba.mi.cnr.it/~webgene/wwwHC_polya.html).

Nucleotide sequences were translated to amino acid sequence and were calculated hydrophobic / hydrophilic characteristic of amino acid residues using Genetyx program.

Deduced amino acid sequences were predicted for signal peptidase cleavage site using SignalP V2.0.b2 program (http://www.cbs.dtu.dk/services/SignalP-2.0/).

Deduced amino acid sequences after eliminate the signal peptide were used to predicted MW, Theoretical pI and amino acid composition using ProtParam program (http://tw.expasy.org/tools/protparam.html).

The putative *N*-glycosylation sites were predicted using ScanProsite program (http://tw.expasy.org/tools/scanprosite/).

Nucleotide and deduced amino acid sequence of AcMRJPs were compared by aligned with AmMRJPs using Clustal X program.

2.9.7.4 Phylogeny reconstruction

Nucleotide and deduced amino acid sequences of AcMRJPs, AmMRJPs and yellow-f protein of *A. mellifera* were aligned using Clustal X. Genetic distance (*d*) between sequences was calculated based on a Kimura's method using Prodist in PHYLIP. The original data of nucleotide and deduced amino acid sequences was bootstrapped

1000 times and 500 times, respectively using Seqboot. Maximum parsimony trees for multiple data were constructed using dnapars (DNA) and propars (proteins). A consensus tree for bootstrapped DNA and protein data was constructed using consense. All phylogenetic program were implemented in PHYLIP 3.56c (Felsenstein, 1993). The bootstrapped treefile was transferred and properly viewed using TREEVIEW.

Table 2.1 Nucleotide sequence of all primers used in AcMRJP gene amplification and sequencing

Primer	Sequence(5' ▶ 3')
M13 Forward	GTTTTCCCAGTCACGAC
M13 Reverse	GTTGTGTGGAATTGTG
MRJP4	ATATCCTAGAAAAAAAATGACAAAATGGTTGC
MRJP4_2	GGTGTGCCTTCCTCTTTGAAC
MRJP4_4	GCGCAACGACTATTCCGTATC
MRJP5_2	CTGTCGTTTGCAAAATATTTGCAGC
MRJP5_A	GTGTACCTTCCTCTTTAAACG
MRJP5_B	TACATTTGCAGCCAAAATTCG
MRJP5_C	ATATCAAAATGATGGCCGCAG
MRJP6_A	TGCATTCTGTTACTTAAAGCC
RMJ2	GGGGTACCCTTTTTTTTTTTTTTTTTTTTTTTTTTTA

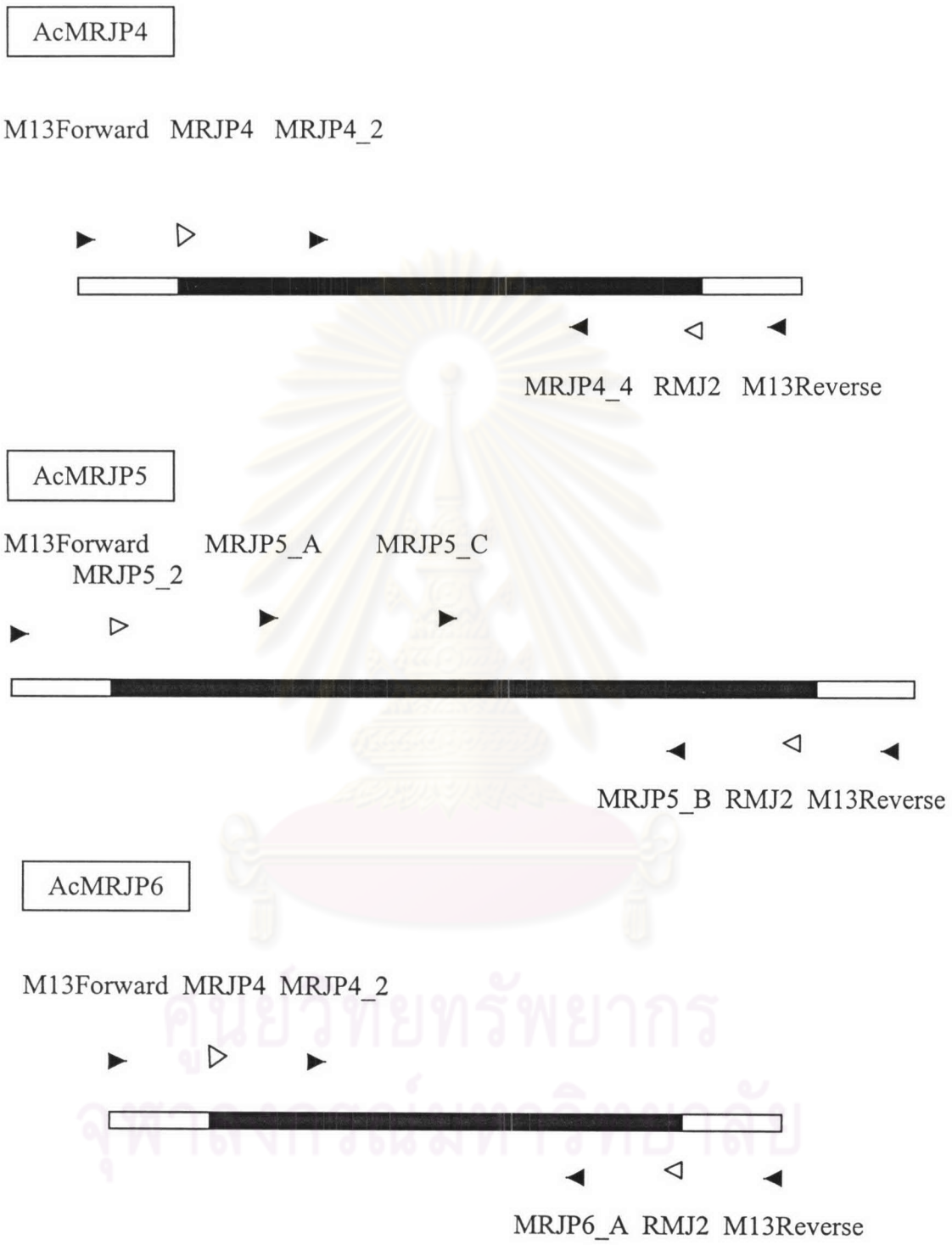

Figure 2.1 Position of primers for AcMRJP cDNA amplification (white arrowheads) and sequencing (black arrowheads).

2.10 cDNA cloning and expression of AcMRJP4 protein in *E.coli*

2.10.1 PCR Amplification

Amplification Primers of the full length AcMRJP4 cDNA for overexpression in *E. coli* were designed from AcMRJP4 sequence (Figure 3.1). The forward primer was designed over N-terminal amino acid sequence without signal peptide, *Nde*I restriction site (CA/TATG) was added to the 5'-end of the primer. The reverse primer was designed over stop condon of deduced amino acid sequence, *Bam*HI restriction site (G/GATCC) was added. A pair of primer were 5'-GGA ATT CCA TAT GGC CGT TGT TCG AGA AAA TTC C-3' (MRJP4_For) and 5'-CGG GAT CCG AAT GAT GAA CCT AAT TGT TAT ATT G-3' (MRJP4_Rev)

Amplification reaction was carried out in a 25 µl reaction mixture containing 4-5 ng of the plasmid template, 1x PCR buffer with $MgSO_4$ (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton®X-100 and 0.1 mg/ml nuclease-free BSA), 200 µM of each dNTP, 0.6 µM of forward and reverse primers and 0.5 unit of *Pfu* DNA polymerase. The reaction was predenatured at 94°C for 2 minutes following by 30 cycles of denaturing at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 3.30 minutes. The final extension was performed at 72°C for 10 minutes. The Amplification product was electrophoretically analyzed through 1% agarose gel.

2.10.2 Purification of PCR product

For purification of PCR product, PCR product from 5 reaction mixtures (125 µl) were pooled and adjusted to 150 µl volume with TE buffer (10 mM Tris-HCl pH 8.0 and

1 mM EDTA), then 15 µl of the 10X proteinase K buffer (100 mM Tris-HCl pH 7.8, 50mM EDTA and 5% SDS) and 10 µl of (10 mg/ml) proteinase K were added. The reaction mixture was incubated at 50°C for 30 minutes and then incubated at 37°C for 30 minutes. The reaction was further purified by using NucleoSpin® column (Macherey-Nagel, Germany). Four volumes of NT_2 buffer were added to one volume of reaction mixture and then mixed.

The PCR mixture was loaded in a NucleoSpin® column, which was placed into a 2 ml collecting tube and centrifuged at 10,000xg for 1 minute at room temperature. The effluent was discarded. Then 0.6 ml of NT_3 buffer was added and centrifuged at 10,000xg for 1 minute. The flow through was discarded. The column was put back to the collecting tube. Then 200 µl of NT_3 buffer was added and centrifuged at 10,000xg for 2 minutes. The column was placed into a new 1.5 ml microcentrifuge tube. Finally, PCR product was eluted by added 50 µl of the elution buffer NE (5 mM Tris-HCl pH 8.5), left at room temperature for 1 minute and centrifuged at 10,000xg for 1 minute.

2.10.3 Recombinant DNA preparation

2.10.3.1 Vector DNA preparation

The vector pET19b was linearized with *Nde*I and *Bam*HI restriction endonuclease. Firstly, *Nde*I was used for digestion in 20 µl reaction mixture containing approximately 600 ng of plasmid vector, 10 units of *Nde*I and 1X reaction buffer (50 mM potassium acetate pH 7.9, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT). The reaction mixture was incubated at 37°C for 16 hours. The digested product was electrophoretically analyzed and linearized vector was eluted from agarose gel by using

QIAquick gel extraction kit. The *Nde*I linearized vector was then digested with *Bam*HI. The reaction was carried out in 20 µl containing approximately 300 ng of *Nde*I digested plasmid, 10 unit of *Bam*HI, 100 µg/ml BSA and 1X reaction buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$ and 1 mM dithiothreitol). The reaction mixture was incubated at 37°C for 6 hours. The digested product was eletrophoretically analyzed and eluted from agarose gel as described above.

2.10.3.2 The AcMRJP4 cDNA fragment preparation

The purified AcMRJP4 cDNA fragment from 2.10.2 was digested with both *Nde*I and *Bam*HI. The reaction was carried out in 20 µl containing approximately 200 ng of purified PCR product, 5 unit of both *Nde*I and *Bam*HI, 100 µg/ml BSA and 1X *Bam*HI reaction buffer. The reaction mixture was incubated at 37°C for 6 hours. Finally, the digested product was eletrophoretically analyzed and eluted from agarose gel.

2.10.3.3 Ligation of cDNA fragment and vector DNA

The *Nde*I and *Bam*HI digested cDNA fragment was ligated to the *Nde*I and *Bam*HI digested pET 19b vector. The ligation reaction was performed in the total volume of 20 µl containing 500 ng of purified PCR product from 2.10.3.2, 150 ng digested pET 19b vector from 2.10.3.1, 1X ligation buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 25µg/ml BSA) and 200 units of T4 DNA ligase (New England Biolab). The ligation mixture was mixed and incubated at 16°C for 18 hours.

2.10.4 Tranformation to *E. coli* JM109 host

The ligation product was eletro-transformed to *E.coli* JM 109 prepared from 2.9.5.1 and eletro-transformed as describe in 2.9.5.2. The eletro-transformed cells that incubated at 37°C for 1 hour were spread on the LB agar plate containing 50μg /ml ampicillin. Then incubated at 37°C for 16-18 hours. Recombinant clones were cultured in LB broth containing 50μg/ml ampicillin. The recombinant plasmid was extracted using an alkaline lysis method as describe in 2.9.7.1. The extracted plasmid was double digested with *Nde*I and *Bam*HI, then eletrophoretically analyzed through 0.7% agarose gel.

2.10.5 Nucleotide sequencing

Recombinant plasmid contained AcMRJP4 cDNA was extracted and sequenced [Bioservice unit (BSU), Thailand]. The T7 forward primer was used for sequencing. Nucleotide sequence obtained was compared with pET19b vector and AcMRJP4 cDNA sequences. Clustal X program and Genetyx program were used for verifying the reading frame.

2.10.6 Transformation of recombinant plasmid to Expression host

E.coli Rosetta (DE3) pLysS was used as host for overexpression of AcMRJP4 protein. Fifty ng of both the recombinant plasmid and pET19b vector were eletro-transformed to a competent *E. coli* Rosetta (DE3) pLysS prepared by method described in 2.9.5.1. The eletro-transformation method was performed as described in 2.9.5.2.

Transformed cells were spreaded on the LB agar plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and then incubated at $37^{O}C$ for 16-18 hours.

2.10.7 Identification of recombinant clones by colony PCR

Recombinant clone was identified by colony PCR using T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and MRJP4_Rev (5'-CGG GAT CCG AAT GAT GAA CCT AAT TGT TAT ATT G-3') as forward and reverse primers, respectively. The colony were picked up and added to a 25 μl amplification reaction containing 1X PCR buffer (10mM Tris-HCl pH 8.8, 50mM KCl, 0.1% Triton X-100), 2 mM $MgCl_2$, 100 μM of each dNTP, 0.4 μM of forward and reverse primers and 1 unit of DyNazyme^{TM}II DNA polymerase. The reaction was predenatured at 94°C for 2 minutes following by 30 cycles of denaturing at 94°C for 1 minute, annealing at 52°C for 1 minutes and extension at 72° C for 1.30 minutes. The final extension was performed at 72°C for 10 minutes. After amplification process, 5 μl of reaction product was eletrophoretically analyzed using a 0.7% agarose gel.

2.10.8 Expression of AcMRJP4 protein in *E.coli*

E.coli Rosetta cells carried the recombinant plasmid or pET 19b plasmid were cultured in 2 ml LB broth containing 50 μg/ml ampicillin, 34 μg/ml chloramphenicol and 1% glucose. The cultures were incubated at 37°C with constant shaking at 250 rpm for 3-4 hours until the optical density at 600 nm reached 0.6. The cultures were stored at 4°C overnight. The cells were collected by centrifugation at 8,000xg for 30 seconds. The cell pellet was resuspended in 2 ml LB Broth containing 50 μg/ml ampicillin, 34 μg/ml

chloramphenicol and 1% glucose, gentle mixed and inoculated into 50 ml LB Broth containing 50 μg/ml ampicillin, 34 μg/ml chloramphenicol and 1% glucose. The cultures were incubated at 37^{O}C with constant shaking at 250 rpm for 2-3 hours until the optical density at 600 nm reached 0.6. Then 1 ml of cultured cell was collected and centrifuged at 8,000xg for 2 minutes. The cell pellet was stored at -20^{O}C. To induced protein production, IPTG was added to the culture to 1 mM final concentration. The cultivation was continued at 37°C for 5 hours, 1 ml of culture cell was collected every 1 hour. After centrifugation, the cell pellets were prepared for further analyzed by SDS-PAGE.

2.10.9 Protein detection by SDS-PAGE

2.10.9.1 Protein sample preparation

Sample buffer (Appendix F) of 5X was diluted with deionized water to 1X. The cell pellet collected at 0 hour of induction was mixed with 50 μl of 1X sample buffer, whereas cell pellet collected at 1-5 hours was mixed with 100 μl of 1X sample buffer. After the cells were completely suspended, the suspensions were boiled for 5 minutes. Collected the cell debris by centrifugation at 10,000xg for 5 minutes. 5 μl of clear homogenate was applied to the SDS-polyacrylamide gel.

2.10.9.2 SDS-polyacrylamide gel electrophoresis

The molecular weight of denatured protein was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE system was performed according to the method of Laemmli (1970). A discontinuous slap gel (10x10x0.75 cm) composed a 10% separating and 4% stacking gels in the presence of 0.1% SDS (w/v) was

prepared. Tris-glycine buffer (25 mM Tris, 192 mM glycine; pH 8.3 and 0.1% SDS (w/v)) was used as electrode buffer. The gel preparation was described in Appendix F. The protein sample preparation was described in 2.10.9.1 before loading to the gel. The electrophoresis was performed at 20 mA constant current per slab gel at room temperature. A 10-200 kDa Protein ladder was used as the standard molecular weight marker.

After electrophoresis, the gel was stained with staining solution (0.1% coomassie brilliant blue R-250 (w/v) in 10% acetic acid (v/v) and 45% methanol (v/v)) for at least 20 minutes. Destaining was performed by immersing the gel in destaining solution (10% acetic acid (v/v) and 10% methanol (v/v)) followed by several change of destaining solution until gel background was clear.