VI. SUMMARY

Dengue viruses are insect-borne viruses that can cause dengue fever and dengue hemorrhagic fever, which are public health problems in tropical and subtropical countries. The dengue viruses consist of a single stranded positive-sense RNA genome, three viral structural proteins and seven non-structural proteins. The immature virions of dengue and other flaviviruses contain three structural proteins; C, prM, and E, whereas, mature virions are much more infectious than immature ones that contain C, M and E structural proteins. The prM proteins have a cleavage site sequence of -Arg-Xaa-(Arg/Lys)-$Arg_{205}\downarrow$- that is processed the prM to M protein and pr-peptide by subtilisin-like proproteins convertase enzymes in acidic post-Golgi vesicles. This study analyzed amino acid in the P1 and P2 position of the proprotein convertase recognition site in the prM protein.

This study used three plasmids that were fundamental from pBluescript II KS. These three plasmids contained three overlapping-sequences and differential sized dengue genes under the control of an SP6 promoter. The mutations at prM-M cleavage site were introduced into the sequence of dengue serotype 2 strain 16681 by the PCR based site-directed mutagenesis. Ten of mutant cDNA clones were constructed by molecular cloning. Five of these cDNA clones, K204A, K204D, K204H, K204R and K204S, contained mutations at amino acid residue 204 that was the P1 position of the proprotein convertase recognition site. The other five mutations, R205A, R205D, R205H, R205K and R205S contained mutation at amino acid residue 205, P2 position of proprotein convertase recognition site. The mutated sequence of all these cDNA were proved to be mutated by the restriction enzyme digested pattern and/or nucleotide sequence analysis.

The full-length cDNA plasmids were linearized by *Xba* I at the last nucleotide of the dengue sequence, and purified for RNA transcription and transfection to the

C6/36 mosquito cell-line. After transfection, the infectious viruses were monitored for 8 weeks using dot blot immunoassay and viral titration in Ps clone D cells followed by immuno-peroxidase staining. Only K204R and R205K mutants were infectious viruses. The other mutants: K204A, K204D, K204H, K204S, R205A, R205D, R205H and R205S could not be detected as infectious viruses throughout 56 days after transfection. The first detection of the K204R virus occurred on day 11 after transfection, which started at a low titer. The K204R virus raised its titer on day 14 to a peak that was as high as non-mutant plasmid derived dengue virus, and maintained the higher titer throughout the 8 weeks of monitoring. The burst size of K204R viruses showed large sizes, similar to the parental plasmid-derived viruses.

Two transfections of the R205K mutant could produce infectious viruses that were detected for the first time on day 14 after transfection. In the first transfection, some R205K mutants days 28 post-transfection reversed the phenotype to wild type and multiplied to a high level on day 42 post-transfection. Mutant R205K virus collected on days 28 and 35 post-transfection displayed a mixture of intended sequence and a reversion of the nt 710 (A to G), which resulted in the amino acid arginine at the P1 position (position 205 of the polyprotein) instead of the intended lysine. Finally, almost all R205K mutant viruses on day 49 post-transfection were taken over by the revertant viruses. The second transfection of the R205K mutant was found to have the same reversion on day 35 post-transfection and it remained in the mixture throughout the monitoring period. This appearance related of the burst size, which showed adulteration of large- and small size after four weeks transfection and maintain throughout eight weeks. For eight non-multiply mutants were assumed that the prM-M cleavage did not occur. The uncleaved prM prevented conformational change of prM-E interaction and much decreased specific infectivity. The results indicated strong dibasic amino acids at P1 and P2 were necessary for cleavage by proprotein convertase-like enzyme. This result suggested that efficient dengue virus

replication required the arginine side chain at the P1 position of the prM-M cleavage junction; lysine side chain, although is also positively charged, is not optimal for virus multiplication. Beside the P1 position, the result suggested that the positively charged amino acid residue is required at the amino acid 204 of the polyprotein (P2 of the prM-M junction) for efficient dengue virus replication. Actually, most, viral glycoproteins and cellular proprotein precursor contain arginine at the P1 position of proprotein convertase target sequence. Moreover, the P2 position of the majority of flavivirus members, with an exception of the dengue virus serocomplex, contains arginine residue instead of lysine.