CHAPTER VII
CONCLUSION

The 5'-halves of PB1, PB2 and PA coding sequences, *i.e.*, PB1-5', PB2-5' and PA-5' from cDNA of HPAI H5N1 virus (influenza A/duck/144/Thailand/2005) were successfully amplified by using oligonucleotide primers designed from the PA, PB1 and PB2 gene sequences of the database. The sizes of the PCR products of 5'-ends of PB1, PB2 and PA coding sequences were 1,134, 1,212 bp and 720, respectively. These amplicons were cloned into the cloning vector (pTZ57R/T). Subsequently, PB2-5' and PA-5' coding sequences were subcloned into protein expression vectors pQE30 and pQE31, respectively, whereas PB1-5' coding sequences was subcloned into expression vector pET20b(+). DNA sequence analyses of the PB1-5', PB2-5' and PA-5' inserts in cloning and protein expression vectors showed 99-100% identity to nucleotide sequences of the respective genes in Genbank database.

The recombinant N-terminal PA and PB2 proteins could be successfully expressed by M15 *E. coli* transformants carrying the recombinant pQE expression vector. The recombinant N-terminal PB1 protein also could be expressed by BL21(DE3)pLysS *E. coli* transformant carrying the recombinant pET-20b(+). The recombinant N-terminal PB1, PB2 and PA had the relative molecular masses of approximately 43, 41 and 26 kDa, respectively. The recombinant N-terminal PA protein contained two nuclear localization signals, region I (amino acid residues 124 to 139) and partial region II (amino acids residues 186-240). Recombinant N-terminal PB1 protein (corresponding to amino acids residues 1-378) contained the PA binding site at amino acid residues 1-25, vRNA binding site at amino acid residues 1-83 and 249-256 as well as two nuclear localization signals at amino acid residues 180-195 and 202-252. Recombinant N-terminus of PB1 corresponding to amino acids residues 1-404 amino acids of the native counterpart contains the PB1 binding site at amino acid residues 206-259, cap binding site at amino acid residues 242-282 and NP binding site at amino acid residues 1-269. The recombinant N-terminal PA protein was found in both soluble cytoplasmic and insoluble cytoplasmic fractions. On the other hands, the recombinant N-terminal PB1 and PB2 were detected exclusively from

insoluble cytoplasmic fraction. Both N-terminal hexahistidine fusion proteins (recombinant N-terminal PA and PB2) and C-terminal hexahistidine fusion protein (recombinant N-terminal PB1) were successfully purified by using Ni-NTA affinity chromatography.

In order to obtain human ScFv antibody (HuScFv) specific to PA, PB1 and PB2 of HPAI H5N1 virus from the HuScFv phage display library. The purified recombinant N-terminal PA, PB1 and PB2 were used as antigen in the single round bio-panning process to select the phage clones displaying HuScFv specific to the polymerase protein epitopes. The soluble HuScFv proteins were successfully prepared and analyzed by western blotting using mouse anti E-Tag antibody. The specific binding of soluble HuScFv antibodies to recombinant N-terminal PA, PB1 and PB2 were successfully demonstrated by using indirect ELISA, dot and Western blot analyses.

The function analysis of recombinant N-terminal PA protein was determined by using nuclear import and RNA binding assays. Nuclear import assay was carried out using digitonin-permeablilized Vero cells. The recombinant N-terminal PA efficiently accumulated in the nucleus of digitonin-permeabilized Vero cells as visualized by confocal microscopy. However, the N-terminal portion of PA was unable to bind to biotinylated 5'-vRNA promoter as examined by RNA binding assay. In addition, the HuScFv specific to recombinant N-terminal PA was used to block the PA nuclear import. The significant reduction of PA nuclear import was found when HuScFv was added to the PA exposed permeablilized Vero cells.