CHAPTER 3

MATERIALS AND METHODS

3.1 Bacteria, yeast, plasmid, primers and gene

3.1.1 Bacteria

Escherichia coli strain XL1-blue (Stratagene, La Jolla, USA) was provided by Prof. Dr. Chatchai Tayapiwatana, Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.

3.1.2 Yeast

Pichia pastoris strain GS115 and X33 was kindly given by Dr. Christopher P. Marquis, (School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Australia).

3.1.3 Plasmid

Expression vectors pPICZA and pPICZαB (Invitrogen, CA, USA) were kindly given by Dr. Cristopher P. Marquis (School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Australia).

3.1.4 Gene

Haemagglutinin domain 1 gene (H5N1 HA1_OPT) was optimized, synthesized and cloned into pPICZA by GenScript, USA.

Haemagglutinin domain gene of avian influenza A (H5N1) virus (64.434 kDa) was kindly given by Dr. Chaisuree Suphawilai, Research Institute for Health Sciences, Chiang Mai University.

The sequence of H5N1 HA1_OPT gene is shown in appendix A.

3.1.5 Primers

Primers for H5N1 HA1_OPT and H5N1 HA2 were synthesized from 1st BASE Pte. Ltd. The forward primer for H5N1 HA2 was designed to add restriction enzyme sites *Xho*I and signal sequence to the 5' end. The reverse primer for H5N1 HA2 was designed with the restriction enzyme sites *Not*I added to the 3' end.

Primers for H5N1 HA1_OPT were designed to bind to H5N1 HA1_OPT at position 116-135 bp and 845-864 bp of PCR product, respectively.

The sequences of both sets of primers are shown in Table 3.1 and Table 3.2 respectively.

Table 3.1 Sequence of specific primers for H5N1 HA1_OPT gene

Primer	**Sequence**
Forward primer	5’-TCGTCTTGTTGTTCGCAATC-3’
Reverse primer	5’-ATCGTTTGGCTTCAAAATGG-3’

Table 3.2 Sequence of specific primers for H5N1 HA2 gene

Primer	**Sequence**
Forward primer	5’-AGTCCTCGAG**AAAAGAGAGGCTGAAGCT**ATGTTTAT AGAGGGAGGATGG-3’
Reverse primer	5’-ATATGCGGCCGCAAGAATAAATTGACAGTATTTG-3’

Note: Restriction enzyme site are underlined, signal sequence is bold.

3.1.6 Positive and negative human serum against avian influenza A virus CMU strain

The human serum samples which were used to detect the recombinant H5N1 HA protein were corrected at the same time of avian influenza transmission occurred in 2004. Serum sample from patient recovered from avian influenza infection who had their HI titer more than 1:40 was collected and renamed to PO3 (positive serum). The non-exposed individual serum was also coleected and renamed to CS (negative serum). All of the human serum samples were provided by Dr. Chaisuree Suphawilai, Research Institute for Health Sciences, Chiang Mai University.

3.2 General equipments and chemical

3.2.1 Equipments

The equipments used in this study are shown in Table 3.3

Table 3.3 Equipments

Equipment	**Manufacturer**
Analytical balance	Oerting Ltd., Kent, UK
Autoclave	Iwaki, Japan
Autopipette	PZ HTL S.A., Poland
Gel Electrophoresis apparatus	BioRad, USA
Gel documentation system	BioRad, USA
Hot air oven	Memmert, Germany
Incubator shaker	Daihan Labtech Co. Ltd., Korea
Larminar air flow	Labconco, Thailand

Table 3.3 Equipments (continued)

Equipment	Manufacturer
Microcentrifuge	Hettich, Germany
PCR thermal cycler	BioRad, USA
pH meter	Cyberscan, USA
Refrigerated centrifuge	Hettich, Germany
Ultrasonicator	Sonics & Materials Inc., USA
UV-Vis spectrophotometer	Shimadzu, Japan
Vortex mixer	Germany industrial, Germany
Water bath incubator	Memmert, Germany

Analytical balance (2 digits) (Oerting Ltd., Kent, UK) was used to prepare all medias and reagents. For more accuracy, 4 digits analytical balance (Oerting Ltd., Kent, UK) was employed.

3.2.2 Chemicals

The chemicals used in this study are shown in Table 3.4.

Table 3.4 Chemicals

Product	Manufacturer
Acetic acid	Lab-scan, Ireland
Agar	Difco®, Becton Dickinson ,USA
Agarose	Vivantis, Poland

Table 3.4 Chemicals (continued)

Product	**Manufacturer**
Ammonium sulphate	Lab-scan, Ireland
Ammonium hydroxide	J.T. Baker, USA
Biotin	Sigma, USA
Calcium chloride	Ajax Finechem, Australia
Calcium sulphate di-hydrate	QRëC™, New Zealand
Ethanol	Merck, Germany
Ethidium bromide	QRëC™, New Zealand
Ethylenediaminetetre acetic acid di-sodium salt (EDTA di-sodium salt)	Ajax Finechem, Australia
Glycerol	Fisher Chemical, UK
Methanol	Merck, Germany
Peptone	Criterion, USA
Potassium hydrogen phosphate	QRëC™, New Zealand
Potassium di-hydrogen phosphate	Lab-scan, Ireland
Sodium acetate trihydrate	Fisher Scientific, UK
Sodium chloride	Lab-scan, Ireland
Sodium dodecyl sulphate	Vivantis, Poland
Sulfuric acid	Lab-scan, Ireland
Tris base	Vivantis, Poland

Table 3.4 Chemicals (continued)

Product	Manufacturer
Trichloroacetic acid	Emsure, Germany
Tryptone	Criterion, USA
Yeast extract	Criterion, USA
Yeast Nitrogen base (YNB)	Himedia, India
Zeocin	Invitrogen, USA

3.3 Media

All media were prepared in reverse osmosis (RO) water and were autoclaved at 121^{o}C for 15 minute. Heat labile components were separate by prepared filtered sterile using 0.22 µm syringe filter (Whatman, England). Filter sterilzed supplement was aseptically added to the media when required. The details for preparing madia are shown in appendix B.

3.4 Buffers and reagents

The details for preparing buffers used in this study are shown in appendix C.

3.5 Construction of recombinant vectors

3.5.1 Vector preparation

Escherichia coli with pPICZA or pPICZαB expression vector was inoculated into 5 ml LB low salt broth containing 50 µg/ml zeocin. The culture was grown overnight at 37^{o}C, 250 rpm. Bacterial cells were harvested by centrifugation at 14,000 rpm for 5 minutes at 4^{o}C. The expression vectors were isolated using Favor Prep ^{TM} Plasmid DNA Extraction Mini Kit (FAVORGEN $^{®}$ BIOTECH CORP., Taiwan). The bacterial cells

were resuspended in 200 µl of resuspension buffer (FAD1 Buffer). Then, 200 µl of lysis solution (FAD2 Buffer) was added and gently mixed. Next, 300 µl of neutralization solution was added and gently mixed. The lysate was then cleared by centrifugation (14,000 rpm for 5 minutes) and was applied onto the FAPD column in a collection tube. The column was centrifuged for 30 seconds and the flow trough was discarded. After that, it was washed by 400 µl of W1 buffer and 600 µl of wash buffer. The column was centrifuged for 30 seconds and flow through was discarded. The column was dried by centrifuged again for an additional 3 minutes. The plasmid DNA was eluted with ddH_2O (Omega Bio-tek, Inc., USA). The plasmid DNA was used freshly or stored at -20°C.

3.5.2 Amplification of H5N1 HA2 gene

The H5N1 HA2 gene was amplified by High Fidelity PCR using hanging primers shown in table 3.2. The composition of PCR mixture was shown in Table 3.5. The reactions were performed in 50 µl containing approximately 25 ng of the DNA template, 1 µM of each primer, 0.2 mM dNTPs, and 1 unit of Phusion High-Fidelity DNA polymerase (Finnzymes, Finland). The PCR reaction were carried out in a thermal cycler (Mycycler®, BioRad, USA) and composed of heating step for 5 minutes at 94°C followed by 35 cycles of denaturating for 30 seconds at 94°C, annealing for 30 seconds at 50°C, extension for 90 seconds at 72°C, and final extension for 10 minutes at 72°C. The reaction was then kept at 4°C.

Table 3.5 The reaction mixture of high-fidelity PCR.

Reagent	Volume (µl)
5x Phusion HF Buffer	10.0
dNTPs (10 mM)	1.0
DMSO	1.5
Forward primer	1.0
Reverse primer	1.0
Template DNA	1.0
Phusion High-Fidelity DNA polymerase (2 U/µl)	0.5
ddH_2O	34.0
Total volume (µl)	50.0

Note:- Template DNA was haemagglutinin domain 2 gene of avian influenza A (H5N1) virus (H5N1 gene).

- For negative control, template DNA was replaced by ddH_2O.

3.5.3 Purification of DNA product

The DNA samples or PCR products were purified using GF-1 Ambiclean Kit (PCR & Gel) of GF-1 Nucleic Acid Extraction Kits (Vivantis, Poland).

3.5.3.1 Sample Preparation

The PCR products was adjusted to 100 µl using ddH_2O. One volume of Buffer DB was added to 100 µl of the PCR products and mixed vocationally to ensure complete solubilization.

The DNA samples were separated using 1% agarose gel electrophoresis. The agarose gel band containing desired DNA was excised and the gel was weighed in pre-weight microcentrifuge tube. One volume of Buffer DB was added to 1 volume of gel (100 µl of buffer to 0.1 g of gel). After that, the tube was centrifuged briefly to make sure the gel slice stayed at the bottom of the tube and was incubated at 50°C until the gel had melted completely and mixed vocationally to ensure complete solubilization.

3.5.3.2 DNA Purification

The sample solution from section 3.5.3.1 was transferred into a column assembled in a clean collection tube and centrifuged at 10,000×g for 1 minute. The flow through was discarded and the column was washed with 750 µl wash buffer and centrifuged at 10,000×g for 1 minute. The flow through was discarded and the column was dried by centrifugation at 10,000×g for 1 minute to remove residual ethanol. Finally, the column was placed into a clean microcentrifuge tube and 50 µl of ddH_2O was added onto the column membrane. The column was stood for 2 minutes and centrifuged at 10,000×g for 1 minute. The DNA was used freshly or stored at -20°C.

3.5.4 *Xho*I and *Not*I digestion

To ligate H5N1 HA gene to the expression vector, the H5N1 HA gene and expression vector were separately digested with *Xho*I and *Not*I (Fermentas, Lithuania) for 16 hours at 37°C. The composition of digestion is shown in Table 3.6.

Table 3.6 The digestion mixture of expression vector and H5N1 gene

Reagent	**Volume (µl)**	
	H5N1 HA1_OPT or H5N1 HA2 gene	**Expression vector**
DNA	10.0	5.0
10x Buffer O	3.0	5.0
*Xho*I (10 U/ µl)	1.0	1.0
*Not*I (10 U/ µl)	0.5	0.5
ddH_2O	15.5	28.5
Total volume (µl)	30.0	40.0

Then, the solution was incubated for 5 minutes at 65°C in water bath in order to inactivate restriction enzymes. After that, they were analysed by agarose gel electrophoresis. The gene and vector were purified using GF-1 Ambiclean Kit (PCR &

Gel) of GF-1 Nucleic Acid Extraction Kits (Vivantis, Poland) (section 3.5.3) and eluted with 50 µl of ddH_2O.

3.5.5 Ligation

After digestion with *Xho*I and *Not*I, the H5N1 HA gene was ligated into the digested expression vector using T4 DNA ligase (Fermentas, Lithuania). The molar ratio of expression vector and insert was 1:3. The reaction volumes was 20 µl and contained mixture as shown in Table 3.7. In this study the appropriate quantities of expression vector and H5N1 HA gene were calculated using the following equations:

(Insert gene weight / Insert gene size) x 3 = (Vector weight / Vector size)

The ligations was incubated for 16 hours at 4°C. The control reaction was also carried out that H5N1 HA2 gene was replaced by distilled water.

Table 3.7 The ligation mixture of expression vectors and H5N1 HA genes

Reagent	**Volume (µl)**
Insert gene	6.0
Vector (100 ng/µl)	2.0
10X T4 ligase buffer	2.0
Enzyme T4 ligase (5 U/µl)	1.0
ddH_2O	9.0
Total volume (µl)	20.0

Note : - The H5N1 HA1_OPT gene was ligated into the digested pPICZαB vector.
- The H5N1 HA2 gene was ligated into the digested pPICZA or pPICZαB vector.

3.5.6 Preparation of competent *Escherichia coli*

The competent *Escherichia coli* was prepared by $CaCl_2$ methods (Sambrook *et al.*, 1989). *E. coli* strain XL1-blue was inoculated into 10 ml LB broth and incubated overnight (8-10 hours) at 37°C with 250 rpm shaking. Then, the cells were collected by centrifugation at 4°C, 2500 rpm for 10 minutes and were kept on ice in all further steps. The cells were resuspended in 10 ml of 0.1 M ice-cold $CaCl_2$ and were collected as before. The cells were once more resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and further held on ice for 1 hour. The cells were collected and were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. Then, competent cells were stored by adding ice-cold steriled glycerol to a final concentration of 10% (v/v). After mixing, the mixture was aliquoted into 0.2 ml in a sterile microcentrifuge tube and was stored at -80°C.

3.5.7 Tranformation of recombinant plasmid into *Escherichia coli*

Two hundred microliters of competent *Escherichia coli* (XL1-blue) were retrieved and thawed on ice. Then, 20 µl of ligation mixture were added to competent *E. coli* in transformation tubes and incubated for 60 minutes on ice. Next, *E. coli* was heat-shocked at 42°C for 1 minutes 30 seconds and further incubated on ice for 1 minute. After addition of 2 ml LB broth, the bacteria were incubated at 130 rpm for 3 hours at 37°C. Then, transformed products were spread onto LB low salt agar containing 50 µg/ml zeocin. Plates were incubated overnight at 37°C.

3.5.8 Confirmation of recombinant clones using colony PCR technique

Integration of H5N1 HA gene into expression vector was evaluated by colony PCR (Sambrook *et al.*, 1989). Colonies from cultured plates were randomly selected, slightly picked and resuspended in 20 µl ddH_2O. The composition of PCR mixture was shown in Table 3.8. The reactions were performed in 50 µl containing DNA template, 1 mM of each primer, 0.1 mM dNTPs, and 1.25 unit of *Taq* DNA polymerase (Vivantis, Poland). The PCR protocol composed of heating step for 5 minutes at 94°C followed by 35 cycles of denaturating for 30 seconds at 94°C, annealing for 30 seconds at 50°C, extension for 90 seconds at 72°C, and final extension for 10 minutes at 72°C. The reaction was then

kept at 4°C. Negative control was similar to other reaction except that water was used instead of plasmid.

Next, the colonies positive with colony PCR technique were inoculated into 5 ml LB low salt and incubated overnight. The glycerol stock was made and stored at -20°C.

Table 3.8 The reaction mixture of colony PCR.

Reagent	**Volume (µl)**
10x Buffer A	5.0
dNTPs (10 mM)	0.5
$MgCl_2$ (50 mM)	5.0
Forward primer	1.0
Reverse primer	1.0
Template DNA	1.0
Taq DNA (5 U/µl)	0.5
ddH_2O	36.0
Total volume (µl)	50.0

Note: Negative control for reaction control, template DNA was replaced by ddH_2O

3.5.9 Isolation of plasmid DNA and restriction analysis

The *Escherichia coli* colonies contain the expression vector with H5N1 HA1_OPT or H5N1-HA2 gene (pPICZA-H5N1 HA1_OPT or pPICZαB-H5N1 HA1_OPT or pPIZA-H5N1 HA2 or pPIZαB-H5N1 HA2) were selected from cultured plates and grown in 5 ml LB low salt containing 50 µg/ml zeocin. Plasmid DNA (pPICZA-H5N1 HA1_OPT or pPICZαB-H5N1 HA1_OPT or pPIZA-H5N1 HA2 or pPIZαB-H5N1 HA2) was isolated using Favor Prep ™ Plasmid DNA Extraction Mini Kit (FAVORGEN ® BIOTECH CORP., Taiwan) (section 3.5.1). The DNA was eluted with 50 µl of ddH_2O into a new microcentrifuge tube.

For restriction analysis, the isolated plasmid was digested with *Xho*I and *Not*I for 16 hours at 37°C. The digestion mixture of recombinant vector was shown in Table 3.9.

Finally, H5N1 HA2 from clone confirmed by PCR and restriction analysis was subjected to nucleotide sequencing (1st BASE Pte. Ltd.).

Table 3.9 The digestion mixture of recombinant expression vector (*Xho*I and *Not*I)

Reagent	**Volume (µl)**
Vector	10.0
10x Buffer O	2.0
*Xho*I (10 U/µl)	1.0
NotI (10 U/µl)	0.5
ddH_2O	6.5
Total volume (µl)	20.0

3.6 Transformation of recombinant vector in *Pichia pastoris*

3.6.1 Vector preparation

Escherichia coli with recombinant plasmid (pPICZA-H5N1 HA1_OPT or pPICZαB-H5N1 HA1_OPT or pPIZA-H5N1 HA2 or pPIZαB-H5N1 HA2) was inoculated into 5 ml LB low salt broth containing 50 µg/ml of zeocin. The culture was grown overnight at 37°C, 250 rpm. Bacterial cells were harvested by centrifugation at 13,000 rpm for 5 minutes at room temperature. The expression vectors were isolated using Favor Prep ™ Plasmid DNA Extraction Mini Kit (FAVORGEN ® BIOTECH CORP., Taiwan) (section 3.5.1). Then the products were analysed by agarose gel electrophoresis.

3.6.2 Linearization of recombinant vector by *Sac*I

Prior to transformation, the recombinant vector (pPICZA-H5N1 HA1_OPT or pPICZαB-H5N1 HA1_OPT or pPIZA-H5N1 HA2 or pPIZαB-H5N1 HA2) was digested with *Sac*I (Fermentas, Lithuania) for 16 hours at 37°C. The composition of digestion mixture is shown in Table 3.10.

Table 3.10 The digestion mixture of expression vector.

Reagent	Volume (µl)
Vector	15.0
10x Buffer *Sac*I	2.0
*Sac*I (10 U/µl)	1.0
ddH_2O	2.0
Total volume (µl)	20.0

Then, the reaction mixture was incubated for 5 minutes at 65°C in order to inactivate restriction enzyme. Products were analysed on agarose gel. Next, the linearlized vector was concentrated by isopropanol precipitation (section 3.6.3).

3.6.3 Precipitation of DNA with isopropanol

In order to precipitate the DNA, the recombinant vector (section 3.6.2) was mixed with 0.1 volume of 3 M sodium acetate pH 7.0, and 0.7 volume of isopropanol to precipitate the DNA at room temperature. The precipitated DNA was recovered by centrifugation at 10,000 rpm for 15 minutes. After the isopropanol was removed, an extra wash with 1 ml of 70% ethanol was performed to remove excess salt from the pellet. Then, the DNA were collected by centrifugation at 10,000 rpm for 15 minutes. The ethanol was thoroughly drained from the microcentrifuge tube. The DNA pellet was air-dried before dissolved in 20 µl of TE buffer.

3.6.4 Preparation of competent yeast cell

The competent yeast cells were prepared as described in a manual of methods for expression of recombinant protein in *Pichia pasroris* (Invitrogen Corp., 1998). A single colony of *P. pastoris* stain GS115 or X33 was inoculated into 5 ml of YPD broth in 50 ml centrifuge tube. It was incubated at 30°C with 250 rpm agitation for 18 hours and 100 µl of overnight culture was inoculated into 100 ml of YPD medium in a baffled flask. The culture was then incubated until the cell density reaches 1.3-1.5 OD_{600} nm unit. The

culture was centrifuged at 1500 xg for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended with 100 ml of ice-cold sterile water. The process was repeated 3 times and the pellet was resuspended with 20 ml of ice-cold sterile water, 10 ml of ice-cold 1 M sorbitol and 1 ml of ice-cold 1 M sorbitol, respectively. The competent cells were kept on ice and used immediately for transformation.

3.6.5 Transformation of yeast cells by electroporation

Eighty microliter of the competent yeast cells was mixed with 5-20 µg of linearized plasmid DNA (in 5-10 µl TE Buffer) and incubated on ice for 5 minutes before transferred to an ice-cold electroporation cuvette with 2 mm gap (BioRad, USA). It was further incubated on ice for 5 minutes. After the cuvette was put into electroporator (BioRad, USA), the pulse was delivered at 25 µF, 2000 V and 200 Ω using Bio-Rad Gene Pulser (BioRad, USA). One ml of ice-cold 1 M sorbitol was immediately added to the cuvette. The content was transferred to a sterile 15 ml tube and incubated for three hours at 30°C. Then, 100 µl of transformant suspension was spread onto selective medium (YPD agar containing 100 µg/ml zeocin) before incubated at 30°C for 3 days.

3.7 PCR analysis of *Pichia* integrants

Twelve colonies from cultured plates were randomly selected to test for the present of H5N1 HA1_OPT or H5N1 HA2 gene in *Pichia* genome. The PCR-based method for detection of integration was described by Burdychova *et al.* (2002). The single colony on selective medium was slightly picked and resuspended into 30 µl ddH_2O. The suspension was heated at 80°C for 10 minutes. After centrifugation, supernatant was collected and used in PCR reaction. The composition of PCR mixture is shown in Table 3.11. The PCR reaction composed of heating step for 5 minutes at 94°C followed by 35 cycles of denaturating for 30 seconds at 94°C, annealing for 30 seconds at 50°C, extension for 90 seconds at 72°C, and final extension for 10 minutes at 72°C. The reaction was then kept at 4°C. After that, the products were analysed by agarose gel electrophoresis.

Table 3.11 The reaction mixture of colony PCR

Reagent	Volume (µl)
10x buffer A	5.0
dNTPs (10 mM)	0.5
$MgCl_2$ (50 mM)	5.0
Forward primer	1.0
Reverse primer	1.0
Template DNA	5.0
Taq DNA polymerase (5 U/µl)	0.5
ddH_2O	32.0
Total volume (µl)	50.0

Note: Negative control for reaction control, template DNA was replaced by ddH_2O.

3.8 Agarose gel electrophoresis

Unless indicated, all agarose gel electrophoresis were carried out in 1% agarose gel. Agarose solution was made to 1% (v/v) in 1x TAE buffer. The mixture was heated to dissolve agarose gel. After the solution was cooled down to about 60^{o}C, it was poured into the gel rack with the comb inserted at one side of gel. When the gel was cooled down and became solid, the comb was carefully removed. The gel was put together with the rack into a tank with 1x TAE buffer. Samples were mixed with 6x loading dye and loaded into agarose gel. Samples were then electrophoresed at 100 volts for 30-45 minutes until the tracking dye reached the bottom of the gel. DNA ladder, 1 kb (Fermentas, Lithuania), was also applied to estimate the size of the samples. After separation, the agarose gel was stained with 0.5 µg/ml of ethidium bromide for 10-15 minutes followed by destaining in water. The gel was observed under ultraviolet light and the image was documented with gel documentation (BioRad, USA). Size and concentration of DNA were estrimated by Quality One 4.6; a software supplied with gel documentation.

3.9 Cultivation and induction of *Pichia pastoris* containing H5N1 HA1_OPT or H5N1 HA2 gene

The expression of H5N1 HA gene in *Pichia pastoris* was evaluated in shake flask fermentation. The clone containing H5N1 HA1_OPT or H5N1 HA2 gene was inoculated into 5 ml of YPD broth containing 100 µg/ml zeocin and was incubated overnight at 30°C, 250 rpm. One hundred microliter of overnight culture was inoculated into 25 ml of BMGY and was incubated overnight at 30°C, 250 rpm. Methanol was added to final concentration of 1% (v/v) every 12 hours. The culture was centrifuged at 6,000 rpm for 10 minutes at room temperature. The samples of culture supernatant was collected from *P. pastoris* containing recombinant vector pPICZαB-H5N1 HA1_OPT or pPICZαB-H5N1 HA2 and the samples of the cell pellet was collected from *P. pastoris* containing recombinant vector pPICZA-H5N1 HA1_OPT or pPICZA-HA2 which will be used to extract endogenous protein by using ultrasonicator.

3.10 Preparation of yeast cell lysate using ultrasonicator

The extraction of endogenous protein from yeast cells was conducted as described in manual of methods for expression of recombinant proteins in *Pichia pastoris* (Invitrogen Corp., 1997). The cell pellets were resuspended with 2 ml of PBS buffer and transfer into a sterile 15 ml centrifuge tube and was kept on ice in all further steps. Next, the cells was sonicated for 20 minutes with a number of short pulse (2 seconds) with pause (3 seconds) by using ultrasonicator 3 mm diameter microtip (Vibra cell™, Sonic and Material Inc.). The soluble and insoluble protein fractions were separated by centrifugation for 20 minutes, 6,000 rpm, 4°C. After centrifugation the supernatant (containing the target protein) was purified by HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition and analysed by western blot.

3.11 Purification of recombinant H5N1 HA protein from *Pichia pastoris* using HisPur™ Ni-NTA Spin Column under native condition

HisPur Ni-NTA™ Spin Column (Thermo Fisher Scientific Inc., USA) was chosen to purify the recombinant H5N1 HA with Histidine-tagged proteins and all purifications steps was performed at 4°C.

3.11.1 Sample preparation

The recombinant H5N1 HA were extracted from a crude cell by ultrasonicator 3 mm diameter microtip (section 3.10). The total volume of sample recombinant H5N1 HA was adjusted to be ≥ 2 resin-bed volumes using an equal volume of equilibration buffer (20 mM sodium phosphate, 300 mM sodium chloride with 10 mM imidazole; pH 7.4).

3.11.2 Purification of H5N1 HA protein

The HisPur Ni-NTA™ Spin Column (Thermo Fisher Scientific Inc., USA) was placed into a 15 ml centrifuge tube and centrifuged at 700×g for 2 minutes to remove storage buffer. The column was equilibrated with two resin-bed volumes of equilibration buffer and centrifuged at 700 × g for 2 minutes to remove buffer. Next, the prepared sample recombinant H5N1 HA from section 3.11.1 was added to the column and incubated on rocking mixer for 30 minutes at 4°C. The column was centrifuged at 700×g for 2 minutes to collect the flow-through in a microcentrifuge tube. Then, the bound protein was washed with two resin-bed volumes of wash buffer (20 mM sodium phosphate, 300 mM sodium chloride with 25 mM imidazole; pH 7.4). The column was centrifuged at 700×g for 2 minutes and the fraction was collected in a microcentrifuge tube to measure the absorbance until an OD 280 nm returned to baseline. Once the baseline was stable, elution of the bound recombinant H5N1 HA protein was carried out using one resin-bed volume of elution buffer (20 mM sodium phosphate, 300 mM sodium chloride (PBS) with 250 mM imidazole; pH 7.4). The eluted recombinant H5N1 HA protein was collected from column by centrifugation at 700×g for 2 minutes and repeat elution 4 more times. Next, the eluted recombinant H5N1 HA protein was analyzed by SDS-PAGE and western blot.

3.12 Analysis of recombinant H5N1 HA1_OPT or H5N1 HA2 protein using SDS-PAGE and Western blot

3.12.1 SDS-PAGE gel electrophoresis

The SDS-PAGE gel electrophoresis was performed as described by Laemmli (1970) using 15% (v/v) polyacrylamide gels. Chromatein Prestained Protein Ladder (Vivantis, Poland), was also applied to estimate the size of the samples. Next, the protein was electrophoresed at 120 volts, until the tracking dye reached the bottom of the gel.

3.12.2 Coomassie Blue Staining of SDS-PAGE gels

After running SDS-PAGE gel (section 3.12.1), the gel was stained for 30 minutes. After that, the gel was destained with destaining solution until the background was clear. The gel was stored in water and photographed.

3.12.3 Western blot

After running SDS-PAGE gel (section 3.12.1), the proteins were then transferred to polyvinylidine difluoride (PVDF) membrane (Immobilon-P; Millipore, USA) 15 volts for 15 minutes.

3.12.3.1 Detection with HisDetector™ Western Blot Kits

The recombinant H5N1 HA protein on the membrane was detected by HisDetector™ Western Blot Kits (KPL, Inc., USA). Firstly, the membrane was blocked by completely immersing in 20 ml 1X Detector Block Solution for 1 hour at room temperature with gentle agitation. The recombinant H5N1 HA protein was probed with HisDetector™ Nickel-AP conjugate dilution 1:2,000. It was washed for 5 minutes by 1X TBST with gentle agitation for three times. Color was developed by adding BCIP/NBT 3 ml on the membrane and incubauted for 5-15 minutes.

3.12.3.2 Detection with Anti-myc antibody

The recombinant H5N1 HA protein on the membrane was detected by anti-myc antibody. Firstly, the membrane was blocked with 5% skim milk in PBS at 4ºC overnight. The recombinant H5N1 HA was probed with anti-myc antibody dilution 1:5,000 for 1 hour. on rocking mixer. It was washed for 5 minutes with gentle agitation for three times. After that, it was probed with goat anti-mouse IgG-HRP (GenScript Corp., USA) dilution 1:2,000 for 1 hour. The membrane was washed for 5 minutes with gentle agitation for four times. Color was developed by adding TMB substrates (contains3,3',5,5'–Tetramethyl benzidine) 3 ml on the membrane and incubauted for 5-15 minutes.

3.12.3.3 Detection with human serum

The recombinant H5N1 HA protein on the membrane was detected by PO3 (positive serum) or CS (negative serum). Firstly, the membrane was blocked with 5% skim milk in PBS at 4ºC overnight. The recombinant H5N1 HA was probed with PO3 (positive serum) or CS (negative serum) dilution 1:3,000 for 1 hour. It was washed for 5 minutes with gentle agitation for three times. After that, it was probed with anti-human IgG-HRP dilution 1:2,000 for 1 hour. The membrane was washed for 5 minutes with gentle agitation for four times. Color was developed by adding TMB substrates (contains 3,3', 5,5' – Tetramethyl benzidine) 3 ml on the membrane and incubauted for 5-15 minutes.

3.13 Estimation of protein concentration

The protein concentration of purified protein was estimated by absorbance at 280 nm. The ration of BSA (OD280 = 0.67 for 1 mg/ml) was used in estimation (Willott, 1999).