CHAPTER 5

DISCUSSIONS

Avian influenza is a disease of birds caused by influenza viruses. The highly pathogenic avian influenza virus (HPAI) such as H5 subtype is important pathogen for the poultry industry, and these subtype can be transmitted between avians and mammals which can result in hybrid viruses, and pandemics may occur due to antigenic shift generating novel strains (Wang *et al.*, 2007). Therefore, the antigenic proteins produced by recombinant protein was used for detection of avian influenza virus via clinical and laboratory, which first step to control the spread of infection and to prevent progression to more severe disease (Beck *et al.*, 2003).

Recombinant influenza HA proteins can be expressed in mammalian and insect cells (Wei *et al.*, 2008; Cornelissen *et al.*, 2010; De Vries *et al.*, 2012), plant cells (Shoji *et al.*, 2009; Kalthoff *et al.*, 2010), and *Escherichia coli* cells (Shen *et al.*, 2008; Biesova *et al.*, 2009; Chiu *et al.*, 2009; Khurana *et al.*, 2011). The advantage of expression in mammalian and insect cells are that the post-translational modifications such as disulfide bond protein production and complex type glycosylation are possible (Lin *et al.*, 2013). However, they are complicated, laborious and costly. Prokaryotic expression system such as *Escherichia coli* offers a rapid and economical means of recombinant protein production but it may lack biological activity due to the incorrect folding. HA1 and HA2 are usually expressed in the form of inclusion bodies in prokaryotic expression systems (Chiu *et al.*, 2009; Liu *et al.*, 2011), and procedures to solubilise and refold them are required to provide biological active protein which are time-consuming (Chiu *et al.*, 2009; Aguilar-Yáñez *et al.*, 2010; Khurana *et al.*, 2010) while the expression of HA in yeast (*P. pastoris*) provides many advantages over other systems as it can express the proteins that need post-translation modification processes such as glycosylation (Cregg *et al.*, 1987). Moreover, it can grow to very high cell densities in mineral based media, high protein

yield due to strongly inducible promoters, potential of secretion of recombinant proteins into the culture medium and is not involved with endotoxin contamination (Cregg *et al.*, 1993; Ebrahimi *et al.*, 2010).

5.1 Cloning of haemagglutinin domain (H5N1 HA gene) in *Pichia pastoris*

In this study, the sequences of haemagglutinin domain (HA1 and HA2 sequences) of avian influenza A virus stain CMU H5 isolate (A/Chicken/Chiang Mai/1/2004 (H5N1) were chosen for expression in *P. pastoris*. The H5N1 HA1 contains the majority of antigenic determinants that are responsible for generation of virus-neutralizing antibodies (Shehata *et al,* 2012), which contains 320 amino acids with five glycosylation sites. The H5N1 HA2 is the light chain of influenza HA, represents the conserved part of HA and is responsible for the fusion of the virus and the endosomal membrane during the entry of the virus into the cell (Gocnik *et al.*, 2007). First, the HA2 sequences was amplified by PCR technique and cloned into pPICZA and pPICZαB expression vector, and was renamed to pPICZA-H5N1 HA2 and pPICZαB-H5N1 HA2, respectively. On the other hand, the H5N1 HA1 gene could not be amplified by PCR technique when using the same PCR condition for amplification as the H5N1 HA2 gene. The melting temperature (T_m) of PCR condition was charged form 50^{o}C to 51-60^{o}C but it still not generated the band of PCR product (H5N1 HA1) (data not shown). Thus, the H5N1 HA1 gene was codon optimized, synthesized and cloned into pPICZA by GenScript. Then, the HA1_OPT gene was excised and cloned into pPICZαB and was renamed to pPICZαB-H5N1 HA1_OPT. The recombinant expression vectors (pPICZA-H5N1 HA1_OPT, pPICZαB-H5N1 HA1_OPT, pPICZA-H5N1 HA2 and pPICZαB-H5N1 HA2) were transformed into *Pichia pastoris.* The transformation efficiency obtained of recombinant vector pPICZA-H5N1 HA1_OPT and pPICZαB-H5N1 HA1_OPT into *P. pastoris* strain X33 was 2.02 x 10^3 cells/µg of DNA and 5.19 x 10^3 cells/µg of DNA, respectively. Moreover, the electroporation yield obtained from the transformation of recombinant vector pPICZA-H5N1 HA2 into *P. pastoris* strain GS115 was 1.11 x 10^3 cells/µg of DNA which was in the range of 10^3 to 10^4 transformants per µg of linearized DNA but the transformation efficiency for pPICZαB-H5N1 HA2 could not be calculated, because the colonies of *Pichia* grown on selective medium could not be counted precisely.

5.2 Expression of haemagglutinin domain (H5N1 HA gene) by *Pichia pastoris*

5.2.1 Secretory expression of haemagglutinin domain (H5N1 HA gene) by *Pichia pastoris*

In case of secretory expression (pPICZαB-H5N1 HA1_OPT or pPICZαB-H5N1 HA2), the selected PCR positive clones were used for expression in shake flask. The culture supernatants were analyzed by SDS-PAGE and Western blot for the present of recombinant protein. But there was no protein band observed on the SDS-PAGE gel and the membrane, which might be cause by *Pichia* produced protein at the lower level for detected. Therefore, the culture supernatants was purified by Ni-NTA affinity chromatography (Thermo Fisher Scientific Inc., USA) prior to detection.

After purification under native condition, the recombinant protein (H5N1 HA1_OPT or H5N HA2) could not be detected by SDS-PAGE and Western blot, which might be cause by the extracellular protease enzyme that *Pichia* produced while growing could affect the secreted protein during cultivation process. According to Clare *et al.* (1991), the pH-dependent extracellular proteases by *Pichia* which could be repressed by free amino acid in the growth medium. The effect of such proteases is further reduced by the presence of other substrates such as the peptide components of yeast extract, peptone and casamino acid. Thus, 1.5% of casein amino acid was added into BMGY to prevent protease enzyme from yeast cell. After the recombinant *Pichia* was cultivated and induced, the culture supernatant was collected and purified. Then, the purified protein was analyzed with SDS-PAGE and Western blot. The results showed that there was no H5N1 HA1_OPT and H5N1 HA2 present in the culture supernatant, which might cause by the recombinant proteins get stuck in the cell. It has been reported that recombinant proteins could not separate from secretory signal by the action of host specific endopeptidase resulting in the release of the mature protein that it was bound to the membrane of *Pichia* (Athmaram *et al.,* 2011). Moreover, the influence of extracellular pH might play a role in reaction as the trimerization and transport of HA from the mammalian host cells (Matlin *et al.,* 1988). Any trimerization of HA happening within the yeast cell, the aggregated HA trimers of high molecular weight could traverse the yeast cell wall (Athmaram *et al.,* 2011). Even though the pH of the induction medium

was initially adjusted to pH6, during the methanol induction, drop in pH to pH 4-5 was possible which probably would play a critical role in the stability and integrity of the HA protein expressed extra-cellulary. Since the medium was acidified, it was possible that a direct effect of pH on the HA might have led to the observed inhibition in transport. The influenza virus HA exhibits acid-dependent conformational changes leading to the exposure of latent epitopes and protease-sensitive sites, and unmasking a potent membrane fusogenic activity (White *et al.,* 1981; Doms *et al.,* 1985; Doms and Helenius, 1986). This activity plays an important role in uncoating the virus during infection (Matlin *et al.,* 1981). Therefore, the acidic pH was not suitable for the stability of the expressed protein. As it was shaker flask culture, it was practically difficult to continuously monitor the culture pH.

To prove if the protein was get stuck inside the cell or not, the *P. pastoris* cells was lysed by using ultrasonicator and the lysate was purified before analyzed with SDS-PAGE and Western blot. But there was no protein band observed on the SDS-PAGE gel and the membrane. Therefore, it could be concluded that the *P. pastoris* could not expressed and secreted H5N1-HA1_OPT and H5N1 HA2 protein.

5.2.2 Intracellular expression of haemagglutinin domain (H5N1 HA gene) by recombinant *Pichia pastoris*

In case of intracellular, the selected PCR positive clones were used for expression in shake flask. The endogenous protein was extracted by using ultrasonicator and was analyzed by SDS-PAGE and Western blot but the specific protein band could not be observed because of story background from non-specific protein (cellular protein from yest cells). For reduced the background from non-specific protein, the membrane was washed with washing buffer with 20-50 mM imidazole before developed the color but the protein band still could not be observed (data not shown). Therefore, the lysate was purified by Ni-NTA affinity chromatography. After purified under native condition, the sample was analyzed by SDS-PAGE and Western blot. The result showed that the recombinant proteins (H5N1-HA1_OPT) was not expressed as no protein band was observed on the SDS-PAGE gel and on the membrane. Murugan *et al.* (2013) suggested that under native condition, a non-specific protein was getting co-purified when the Ni-

NTA affinity chromatography was used for purification that might cause the recombinant antigenic proteins (H5N1-HA1_OPT) could not be absorbed with the Ni-NTA affinity chromatography and purified. Hence the recombinant proteins (H5N1-HA1_OPT) was purified under denaturing condition. However, no protein band was observed, imply that *P. pastoris* cloud not express H5N1-HA1_OPT protein. Furthermore, the H5N1 HA1 protein was more difficult to expression in *P. pastoris* than H5N1 HA2 because of the hydrophobicity of H5N1 HA1 was less than H5N1 HA2, this effected the expression of the recombinant antigenic proteins (H5N1-HA1_OPT) (Suphawilai *et al.*, 2015).

On the other hand the recombinant proteins (H5N1 HA2) was successfully expressed and purified by Ni-NTA affinity chromatography under native condition. The results showed the single band of protein with approximate molecular weight 35-40 kDa. However, the recombinant antigenic proteins (H5N1 HA2) (including Myc-tag, linker and His-tag) has a theoretical molecular mass of 24.724 kDa (by calculation) but the protein expressed in *P. pastoris* was larger than expected. Similar to this study, Shehata *et al.* (2012) has obtained a broad smear protein (70 kDa) above the expected size (theoretical molecular mass 39.67 kDa) when expressing HA1 protein in *P. pastoris*. Riley *et al*. (2002) found that recombinant FLAG tagged prion protein expressed in *P. pastoris* with pPICZB expression vector which is intracellular vector, is highly glycosylated. Kopera *et al.* (2014) found that recombinant hemagglutinin (HA0, HA1 and HA2) protein expressed in *P. pastoris* displayed three bands with molecular weight about 65, 47 and 22 kDa, respectively, which higher molecular weight than the expected (the theoretical molecular weight 59.5, 39.5 and 20 kDa, respectively) but the recombinant protein still gave a positive signal in Western blot analysis using anti-His and anti-HA1 antibodies and elicited a high immune response in mice. Moreover, glycosylation is one of the most common post-translational modifications present in *P. pastoris*. The degree of oligosaccharide chains added post-translationally might affect immunogenicity by masking or changing the conformation of important neutralizing epitopes (Martinet *et al.*, 1998). Although, the recombinant protein (H5N1 HA2) in this study was larger than the theoretical molecular weight possibly due to glycosilation, it could reacted with human serum from patient recovered from avian influenza infection (PO3, positive serum) and did not reacted with normal serum (CS, negative serum). These findings strongly

suggested that the recombinant proteins (H5N1 HA2) might be useful as potential antigen for diagnostics against natural/experimental HPAI infections.