Cloning and Expression of HA2 gene of Avian Influenza A (H5N1) Virus in *Pichia pastoris*

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Abstract

Avian influenza or bird flu is a highly infectious viral disease and is one of the most major concerns to both poultry industry and human health. The haemagglutinin (HA) protein is one of the markers for detection of influenza viral infection. There is no efficient protection and therapy for the disease, hence, rapid and accurate detection of avian influenza virus infection is an important tool to control outbreaks. In this study, the HA2 gene was amplified and cloned into pPICZA expression vector. The recombinant plasmid was verified by PCR, restriction analysis and nucleotide sequencing. After transformed into *Pichia pastoris*, the integration of HA2 gene into host genome was confirmed. The expression of HA2 was performed and the protein was internally expressed as tagged fusion protein. The recombinant HA2 protein was extracted and purified from cell lysate using nickel affinity chromatography under native condition. A single band of 35-40 kDa was observed by SDS-PAGE. Western blot analysis revealed that the protein could be reacted with His Detector™ and anti myc antibody, indicating that this was protein of interest. The HA2 recombinant protein could react with serum sample from patients recovered from avian influenza infection who had their hemaglutination inhibition (HI) titer over 1:40, while it did not react with the non-exposed individual serum. The recombinant HA2 expressed by *P. pastoris* could be useful as a potential antigen for epidemiological study of highly pathogenic avian influenza (HPAI) infection.

Keywords: Avian influenza, Haemagglutinin, H5N1, HA2, *Pichia pastoris*

1. Introduction

Avian influenza viruses are influenza A viruses belonging to the Orthomyxovirus family. The haemagglutinin (HA) is a transmembrane protein and a major surface antigen of the influenza virus. The HA (HA0) is synthesized and cleaved into two disulfide-linked chains, HA1 and HA2 which are responsible for binding to host cell receptors and for fusion between the virion envelope and the host cell (Mitnaul *et al*., 2000; Skehel and Wiley, 2000). The avian influenza viruses are classified according to their pathogenicity and the antigenicity of the surface proteins haemagglutinin (HA) and neuraminidase (NA) (Fouchier *et al*., 2005).

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It was demonstrated that a highly pathogenic avian influenza (HPAI) H5N1 subtype is considered a great threat to worldwide human and animal health. Several out brakes in human were reported with high mortality (Duan *et al*., 2008; Subbarao *et al*., 1998).

Due to the continuous outbreaks of avian influenza virus in the several countries, the development of laboratory techniques for efficient isolation and detection of avian influenza virus from surveillance samples continues to be a high priority. Production of viral antigen by traditional technique is not suitable. The antigenic protein produced by recombinant DNA technique was far more practical way for diagnosis and epidemiologic study of avian influenza virus. The serological test using recombinant protein was reported to be better sensitivity and also specificity because it is free of other proteins present with whole cell preparation (Errington *et al*., 1995; Mohan *et al*., 2006).

The methylotrophic yeast stain *Pichia pastoris* has raised an increasing interest for the production of viral antigens offering the capability of post-translational modifications such as glycosylation, disulfide bond formation and protein folding similarly to mammals, fast growth rates in low cost media, ease of genetic manipulation and no viral contamination. Recently, several research groups successfully cloned and expressed fully function recombinant HA in *P. pastoris*. HA1 and its truncated sequences of Influenza A virus subtype H5 could be expressed in *P. pastoris*. (Shehata *et al*., 2012). The aims of this study were to clone, express, and purify the haemagglutinin domain 2 (HA2) of avian influenza A virus in *P. pastoris* for diagnosis and epidemiological study.

2. Materials and Methods

2.1 Microorganisms, vector and serum samples

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. The expression vector pPICZA (Invitrogen, CA, USA) and *Pichia pastoris* GS115 strain were kindly given by Dr. Christopher P. Marquis, (School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Australia). Serum samples were corrected at the same time of avian influenza transmission occurred in 2004. Positive serum was collected from patients recovered from avian influenza infection who had their HI titer more than 1:40. The non-exposed individual serum was used as negative control.

2.2 Cloning of HA2 gene into pPICZA

The HA2 gene was amplified from HA gene of avian influenza A (H5N1) (accession AY577314.2) by high fidelity PCR (Finnzymes, Finland) as directed by manufacturer using forward primer (5'-AGTCCTCGAGAAAAGAGAGGCTGAAGCTATGTTTATAGAGGGAGGATGG-3') and reverse primer (5'-ATATGCGGCCGCAAGAATAAATTGACAGTATTTG-3'). The primers were designed to add the restriction enzyme sites *Xho I* and *Not I* to 5' and 3' end of the amplified products. The expression vector and PCR gene were separately digested with *Xho I* and *Not I*. The gene was then ligated into the vector and was transformed into competent *Escherichia coli* strain XL1-blue by heat-shock method. The transformants were screened on LB low salt agar (10 g/L tryptone, 5 g/L NaCl, and 5 g/L yeast extract), containing 50 µg/mL zeocin (Invitrogen, USA) overnight at 37°C. The clones of *E. coli* containing HA2 gene were verified by PCR technique, restriction analysis and nucleotide sequencing (1st BASE Pte. Ltd.). The recombinant vector was named pPICZA-HA2.

2.3 Transformation of pPICZA-HA2 into *Pichia pastoris*

The recombinant vector (pPICZA-HA2) was linearlized with *Sac*I (Fermentas, Lithuania) and transformed into competent yeast cells by electroporation technique (25 µF, 200 Ω, 1500 V). The transformants were selected on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), containing 100 µg/ml zeocin. The PCR-based method for detection of integration of expression vector to yeast genome was described by Burdychova et al. (2002).

2.4 Cultivation and induction of *Pichia pastoris* containing HA2 gene

The expression of HA2 gene in *P. pastoris* was conducted in shake flask fermentation. The clone containing HA2 gene was inoculated into 5 ml of YPD broth containing 100 µg/ml zeocin and was incubated overnight at 30°C with 250 rpm agitation. One hundred microliter of overnight culture was inoculated into 25 ml of BMGY (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L glucose, 20 g/L, 13.4 g/L YNB, 10 g/L glycerol, 400 µg/L biotin and 100 mM potassium phosphate pH 6.0) and was incubated overnight at 30°C with 250 rpm agitation. Methanol was added to final concentration of 1% (v/v) every 12 hours for 3 days. After that, the culture was centrifuged at 3000 x g for 10 minutes at 4°C, the supernatant was discarded and the pellet was collected for HA2 purification

2.5 Purification of HA2 protein using HisPur™ Ni-NTA Spin Column under native condition

The extraction of endogenous protein from yeast cells was performed by sonication technique. The HA2 protein (containing His-tagged) was purified using HisPur™ Ni-NTA Spin Column with 1 ml resin volume (Thermo Fisher Scientific Inc., USA) under native condition as suggested by manufacturer. The HA2 protein was eluted from the resin with one resin-bed volume of elution buffer (20mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole pH7.4) by

centrifugation at 700 × g for 2 minutes. The eluate was collected. The elution step was repeated 4 more times. The protein in eluate was monitored by measuring the absorbance at 280 nm. The eluted protein was analyzed by SDS-PAGE and western blotting.

2.6 Analysis of recombinant HA2 protein by SDS-PAGE and western blotting

The SDS-PAGE was performed as described by Laemmli (1970) using 12% (v/v) polyacrylamide gels. The protein was electrophoresed at 120 volts, until the tracking dye reached the bottom of the gel. The gel was stained with coomassie blue. For western blotting, the resolved protein in the SDS gel was electro-transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, USA). To probe with HisDetector™, membrane was blocked with His detector blocking solution. The HA2 gene was probed by HisDetector™ Western Blot Kits (KPL, Inc., USA) as directed by manufacture.

To probe with anti-myc antibody, membrane was blocked with 5% skim milk in PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4) pH 7.4 at 4°C overnight. The HA2 was probed with anti-myc antibody (GenScript Corp., USA) diluted 1:5000 in PBST (0.1% Tween 20 in PBS) and incubated 1 hour on rocking mixer. It was washed for 5 minutes using PBST with gentle agitation for three times. After that, it was probed with goat anti-mouse IgG-HRP (GenScript Corp., USA) diluted 1:2000 in PBST. The membrane was washed for 5 minutes with gentle agitation for four times. Color was developed using TMB substrates (KPL, Inc., USA).

To probe with positive and negative human sera, membrane was blocked with 5% skim milk in PBS at 4°C overnight. The HA2 was probed with positive serum or negative serum diluted 1:3000 in PBST. It was washed for 5 minutes using PBST with gentle agitation for three times. After that, it was probed with anti-human IgG-HRP (GenScript Corp., USA) diluted 1:2000 in PBST. The membrane was washed for 5 minutes with gentle agitation for four times. Color was developed using TMB substrates (KPL, Inc., USA)

3. Results

3.1 Construction of recombinant vectors

The HA2 gene was amplified by High Fidelity PCR with restriction enzymes sites *Xho*I and *Not*I added into the 5' end and 3' end, respectively. The concentration and size of HA2 gene were approximately 120 ng/µL and 600 bp, respectively (Figure 1).

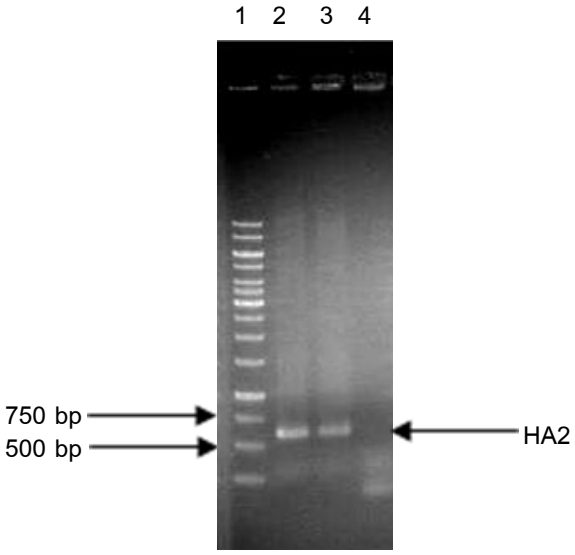

Figure 1 Agarose gel electrophoresis of PCR products (HA2 gene) amplified by using High Fidelity PCR. Lane 1 is 1 kb DNA ladder, lane 2 and 3 are HA2 genes, and lane 4 is negative control (ddH_2O)

After amplification, the HA2 genes was ligated into pPICZA vector, and were transformed to *E. coli* XL1-blue. The clones of *E. coli* containing HA2 gene were verified by colony PCR and restriction analysis with *Xho*I and *Not*I which indicated that the HA2 gene was successfully cloned into pPICZA vector (Figure 2). Moreover, the nucleotide sequence of the expression vector containing HA2 gene was sequenced and the result is show in Figure 3. There was one base shift from C to T at position 438 (boxed). However, the amino acid sequence was not affected.

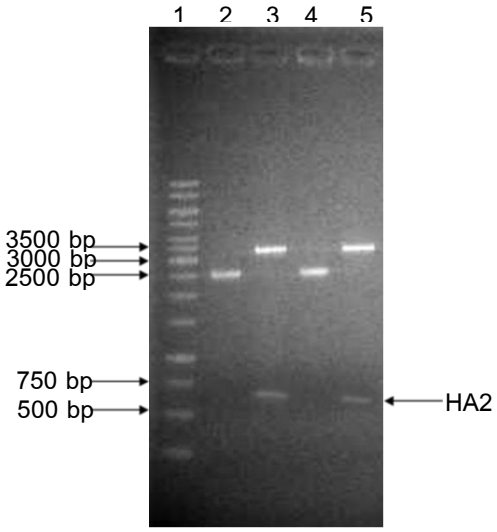

Figure 2 Agarose gel electrophoresis of recombinant plasmid (pPICZA-HA2) with *Xho* I and *Not* I restriction analysis. Lane 1 is 1 kb DNA ladder, lane 2 and 4 are un-digested recombinant plasmid, lane 3 and 5 are digested recombinant plasmid. Arrow indicates H5N1 HA2 gene, which is approximate 600 bp

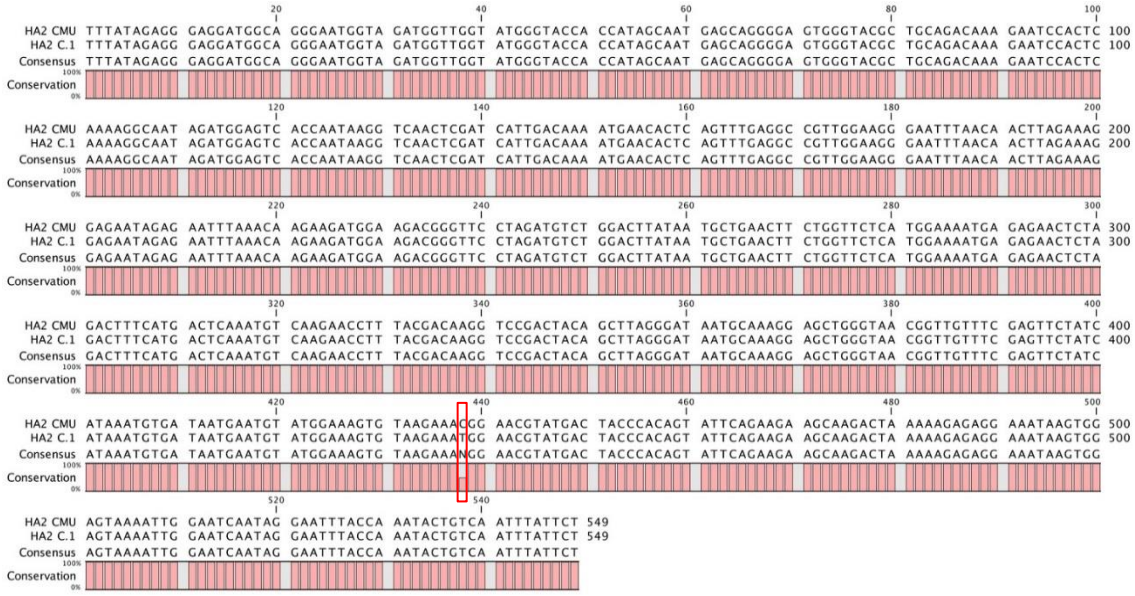

Figure 3 Nucleotide sequence of the HA2 gene from recombinant plasmid (pPICZA-HA2) compared with the haemagglutinin domain gene of avian influenza A (H5N1) virus (AY577314.2)

3.2 Transformation of recombinant vector containing HA2 gene in *to Pichia pastoris*

The recombinant vector (pPICZA-HA2) was transformed into the competent *P. pastoris* using electroporation technique. Two hundred and fifty one colonies were found on selective medium. The transformation efficiency was 1.11 x 10^3 cell/µg DNA. Twelve colonies were randomly selected for confirmation of integration. All of selected colonies were positive with PCR using primers specific to HA2 gene. This indicates that the HA2 gene had been integrated into the *Pichia* genome (Figure 4).

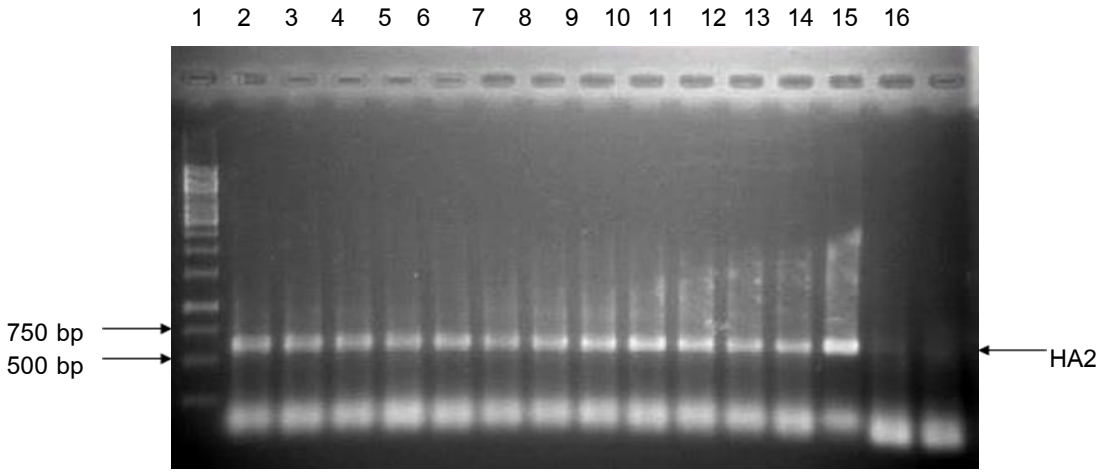

Figure 4 PCR products of twelve selected recombinant *Pichia* colonies containing pPICZA-HA2 vector. Lane 1 is 1 kb DNA ladder, lane 2–13 are PCR products of clone 1–12, lane 14 is HA2 gene (positive control), lane 15 and 16 are distilled water and *Pichia* with plain expression vector (pPICZA) (negative control), respectively.

3.3 Expression and purification of HA2 protein

The selected PCR positive clone was used for expression in shake flask. The endogenous protein was extracted by using ultrasonicator and was analyzed by Western blot. However, no specific protein band was observed. Therefore, the lysate was purified by Ni-NTA affinity chromatography under native condition. After that, the present of recombinant protein was analyzed by SDS-PAGE and Western blot using HisDetector™ and Anti-myc antibody. The recombinant HA2 protein was observed with molecular weight approximate 35-40 kDa when detecting by SDS-PAGE (Figure 5), HisDetector™ (Figure 6), Anti-myc antibody (Figure 7), respectively.

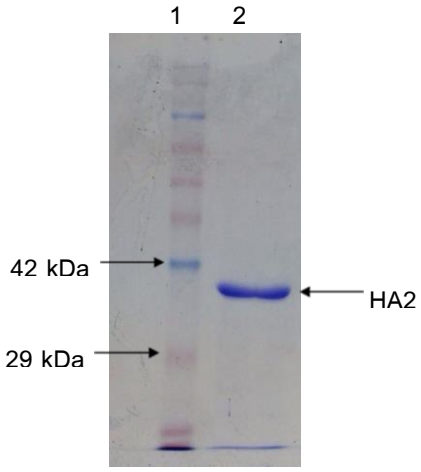

Figure 5 SDS-PAGE analysis of HA2 protein with Coomassie blue staining. Lane 1 is protein molecular weight marker, lane 2 is purified HA2 protein. Arrow indicates the band of the recombinant HA2 protein which is approximately 35–40 kDa.

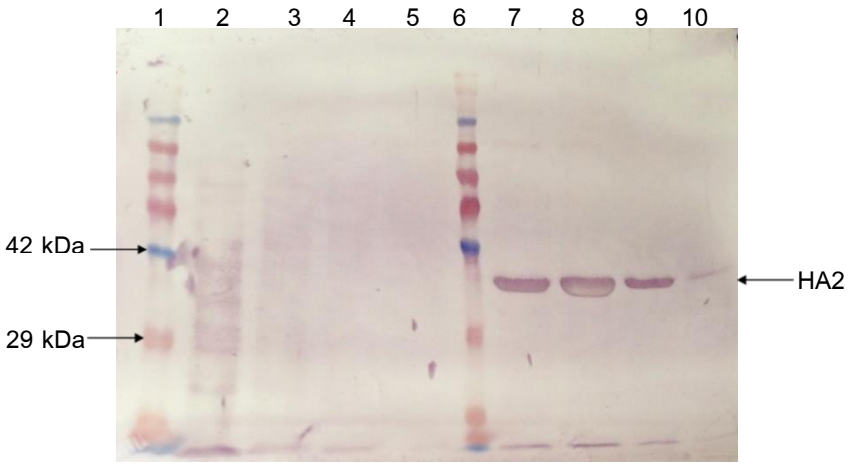

Figure 6 Western blot analysis of HA2 protein detected by HisDetector™ Western Blot Kits (KPL, Inc., USA). Lane 1 is molecular weight marker, lane 2 is supernatant from cell lysate, lane 3 is unabsorbed of cell lysates, lane 4–5 are first and second wash, respectively, lane 6 is molecular weight marker, lane 7–10 are elution1, 2, 3 and 4 respectively. Arrow indicates recombinant HA2 protein.

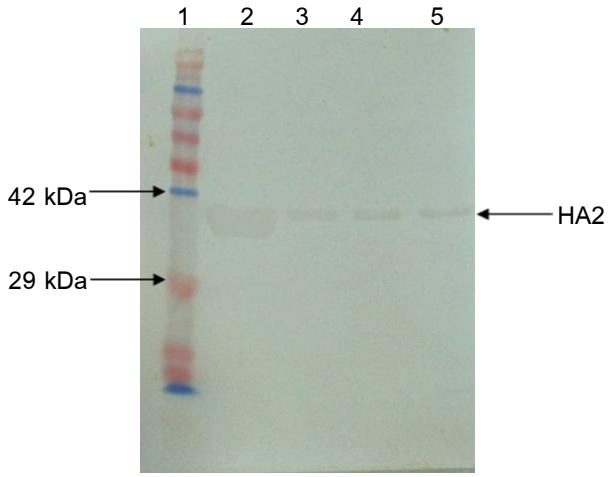

Figure 7 Western blot analysis of HA2 protein detected by Anti-myc antibody. Lane 1 was protein molecular weight marker, lane 2–5 were eluate 1, 2, 3 and 4 respectively. Arrow indicates recombinant HA2 protein.

Furthermore, the recombinant HA2 protein could reacted with human serum from patient recovered from avian influenza infection (positive serum) [Figure 8 (a)] and did not reacted with normal serum (negative serum) [Figure 8 (b)], confirming the expression of the target proteins. These findings strongly suggested that the recombinant HA2 proteins could be useful as potential antigen for diagnostics against natural/experimental HPAI infections.

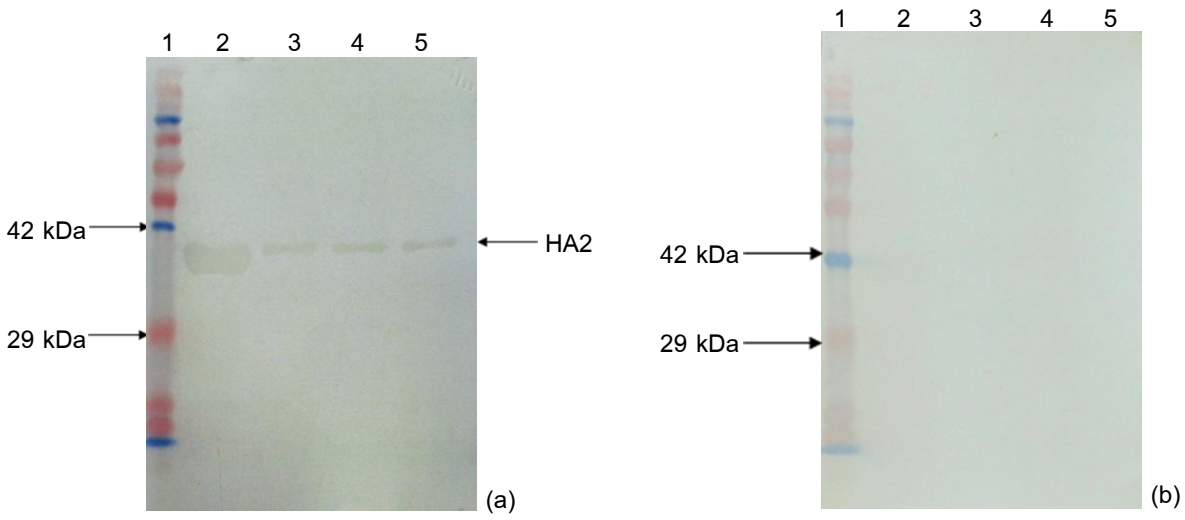

Figure 8 Western blot analysis of HA2 protein detected by positive serum and negative serum. Lane 1 is protein molecular weight marker, lane 2-5 are eluted fraction 1,2,3 and 4 respectively. (a) Western blot analysis of HA2 protein detected by positive serum. Arrow indicates the band of the recombinant HA2 protein which is approximately 35-40 kDa. (b) Western blot analysis of HA2 protein detected by negative serum.

4. Discussion and Conclusion

Avian influenza is a disease of birds caused by influenza viruses. The highly pathogenic avian influenza virus (HPAI) such as H5 subtype is an important pathogen for the poultry industry, and this 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 protein produced by recombinant protein was used for detection of avian influenza virus via clinical and laboratory, which is the first step to control the spread of infection and to prevent progression to more severe disease (Beck 2003 *et al*., 2003).

In this study, the sequences of haemagglutinin gene (HA2 sequences) of avian influenza A virus (AY577314.2) was chosen to internally expressed in *P. pastoris*. The yeast cell was lysed by using ultrasonicator and was analyzed by SDS-PAGE and Western blot but the specific protein band could not be observed. Therefore, the lysate was purified by Ni-NTA affinity chromatography under native condition. After that the sample was analyzed by SDS-PAGE and Western blot. 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 were 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 may affect immunogenicity by masking or changing the conformation of important neutralizing epitopes (Martinet *et al*., 1997). A study by Yang *et al*. (2012) demonstrated that low-glycosylated NA expressed in the och1-defective *P. pastoris* (defective α-1, 6-mannosyl-transferase gene (OCH1) has elicited the higher antibody titer with less antigens and less booster times than hyperglycosylated one. Although, the recombinant HA2 protein expressed in this study was larger than the theoretical molecular weight, it could

reacted with human serum from patient recovered from avian influenza infection (positive serum) and did not reacted with normal serum (negative serum). These findings strongly suggested that the HA2 recombinant proteins might be useful as potential antigen for diagnostic against natural/experimental HPAI infections

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