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Construction and heterologous expression of a truncated Haemagglutinin (HA) protein from the avian influenza virus H5N1 in *Escherichia coli*

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Abstract. Malaysia first reported H5N1 poultry case in 2004 and subsequently outbreak in poultry population in 2007. Here, a recombinant gene encoding of peptide epitopes, consisting fragments of HA1, HA2 and a polybasic cleavage site of H5N1 strain Malaysia, was amplified and cloned into pET-47b(+) bacterial expression vector. DNA sequencing and alignment analysis confirmed that the gene had no alteration and in-frame to the vector. Then, His-tagged truncated HA protein was expressed in *Escherichia coli* BL21 (DE3) under 1 mM IPTG induction. The protein expression was optimized under a time-course induction study and further purified using Ni-NTA agarose under reducing condition. Migration size of protein was detected at 15 kDa by Western blot using anti-His tag monoclonal antibody and demonstrated no discrepancy compared to its calculated molecular weight.

INTRODUCTION

Influenza type A virus is a member of the *Orthomyxoviridae* family which consists of single-stranded eight-segment negative-sense genomic RNAs that encoding for 10 proteins, including two major antigenic surface proteins: haemagglutinin (HA) and neuraminidase (NA). HA functions as the surface glycoprotein of virus particles that is capable to bind to sialic acid cell surface receptors (Wiley & Skehel, 1987), while the NA protein facilitates the mobility of virions by removing sialic acid residues from the viral HA during entry and release from cells (Palese *et al.*, 1974; Lamb & Choppin, 1983; Els *et al.*, 1989). Based on their antigenic characteristics of HA and NA envelope glycoprotein, influenza A viruses are further classified into 16 HA subtypes and 9 NA subtypes (Horimoto & Kawaoka, 2005).

Highly pathogenic avian influenza (HPAI) viruses are restricted to H5 and H7 subtypes because these subtypes are capable to mutate into HPAI and cause severe respiratory disease and high mortality in poultry populations (Alexander, 2000). HPAI H5N1 was the main virus caused outbreak in poultry and reported human fatality in Hong Kong in 1997. However, Malaysia (peninsular) first reported H5N1 poultry cases in August 2004. In February 2006, Malaysia reported another H5N1 case in a free-range poultry (Yee *et al.*, 2009) and first outbreak in poultry in 2007 (WHO, 2011).

Effective vaccination against H5N1 is generally considered an important first-line tool to reduce influenza virus morbidity and mortality. However, the development of H5N1 vaccines using the embryonated egg-based method is technically complicated and requires high biosafety facilities (Lin *et al.*, 2011). Therefore, several approaches have been developed as the alternatives (Lin *et al.*, 2008; Wei *et al.*, 2008; Shoji *et al.*, 2009). One of them is the recombinant protein expression in *Escherichia coli*, which is simple, fast, cost-effective, robust with the maximal amount

yielded, and has been used in several H5N1 studies (Shen *et al.*, 2008; Biesova *et al.*, 2009; Chiu *et al.*, 2009; Khurana *et al.*, 2011). In this study, we present the methods of construction, expression and purification of the truncated HA protein from H5N1 Malaysia strain in bacterial expression system, *E. coli*. HA, a major "antigenic change" envelope protein, is the primary target for neutralizing antibodies and therefore becomes the main focus of influenza vaccines development (Wei *et al.*, 2012; Kanekiyo *et al.*, 2013; Dilillo *et al.*, 2014). Recently, Herfst *et al.* (2012) shows that only four amino acid substitutions in the hemagglutinin, and one in the polymerase basic 2, are capable to transform H5N1 virus be airborne transmitted between ferrets. Remarkably, the influenza virus strain A/Indonesia/5/2005 from our neighbour was selected in their study as concerns this virus could initiate a pandemic (Herfst *et al.*, 2012).

MATERIALS AND METHODS

Amplification and construction of truncated HA gene

To construct the truncated HA gene, the forward primer: **GTCGAC**AGGCCTA GGAGCTACA and the reverse primer: **AAGCTT**AGGCCTTTCTTCTGAA (First base, Malaysia) were used to amplify the DNA from our lab collection plasmid pCR®-TOPO®2.1~HANA, which carries the fusion of truncated haemagglutinin (HA) (NCBI GenBank accession number: DQ320934.1) and neuraminidase (NA) gene, by a standard polymerase chain reaction (PCR) using recombinant *Taq* DNA polymerase (Fermentas, USA). *Sal*I and *Hind*III excision sites were indicated in boldface letters for forward primer and reverse primer, respectively. The amplified DNA fragments were cloned into pCR®2.1-TOPO® vector (Invitrogen, USA), and then chemically transformed into *E. coli* strain TOP10 cells on selected LB agar plate containing 50 µg/mL kanamycin and 40 mg/mL X-gal. The selected clones were screened by restriction analysis and verified by M13 forward and reverse DNA sequencing (First base, Malaysia). The DNA fragments were then excised at *Sal*I and *Hind*III sites and in-frame ligated to linearized pET-47b(+) expression vector (Novagen, USA) using T4 DNA ligase (Fermentas, USA), and subsequently transformed into *E. coli* strain BL21 (DE3).

Protein Expression and Optimization

Escherichia coli strain BL21 (DE3) cells harbouring plasmids were streaked on LB agar plate supplemented 150 µg/mL kanamycin and cultured overnight at 37°C. A single colony of bacteria was inoculated into 10 mL LB broth and incubated agitating overnight at 37°C. The LB medium (10 mL) was added with 100 µL overnight bacterial culture and incubated agitating at 37°C. At an OD600 of ≅ 0.5, 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration was added to induce the protein expression, and then continued incubated agitating and collected at 0 hour, 0.5 hour, 1 hour, 2 hours and 3 hours, respectively. The harvested cells were washed three times with ice-cold PBS (2.7 mM KCl, 137 mM NaCl, pH 7.4) and lysed in urea lysis buffer (7 M urea, 20 mM HEPES, pH 7). The cells were frozen at -80°C overnight and vortexed vigorously. Supernatant was collected after centrifugation at 9,000 x g for 30 minutes at 4°C.

Stepwise protein elution

An amount of 600 µL of lysis buffer (7 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, 300 mMNaCl, pH 8.0) was used to equilibrate Ni-NTA Spin Column (QIAGEN, Germany) and centrifuged for 2 min at 890 x g. Then, 600 µL of the cleared lysate supernatant was loaded onto a pre-equilibrated spin column and centrifuged for 5 min at 270 x g. Flow through was collected. The spin column was washed with 200 µL of wash buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, 300 mMNaCl, pH 8.0) containing 10 mM imidazole and centrifuged for 2 min at 890 x g. The eluted fraction was collected. The wash and centrifugation steps were repeated with wash

buffers containing 20, 30, 40 and 500 mM imidazole, subsequently. All the collects were analyzed by 12% SDS-PAGE.

Protein purification

A total amount of 6 mL bacterial cleared cell lysate was loaded on 1 mL bed volume of pre-equilibrated nickel-nitrilotriacetic acid agarose, Ni-NTA (QIAGEN, Germany) and flow through was collected. The column was then washed with 10 mL wash buffer (30 mMimidazaole, 8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, 300 mMNaCl, pH 8.0) and wash fraction was collected. Purified protein was eluted 4 times with 500 µL of elution buffer (500 mM imidazole, 8 M urea, 100 mM NaH2PO4, 100 mM Tris-Cl, 300mMNaCl, pH 8.0) and all fractions were collected.

SDS-PAGE and Western blotting

Sample proteins were resolved on reducing 12% SDS-PAGE and then visualized after Coomassie Brilliant Blue staining. For Western blotting, the gel was transferred to Immobilon P polyvinylidenedifluoride filter (Millipore, USA) by semi-dry electroblotting (Biorad, USA) for 1 h room temperature at 12 V. The membrane was then blocked with blocking buffer [1x TBS, 0.5% (w/v) skimmed milk, 0.1% (v/v) Tween 20] for 90 minutes at room temperature under agitating. The membrane was washed three times with washing buffer [0.5% (v/v) Tween 20, 1 x TBS], each for 10 minutes at room temperature. The membrane was probed with an anti-6x His tag® (ab18184) mouse monoclonal antibody [His.H8] (Abcam, USA) for overnight at 4ºC and washed again three times with washing buffer. Then, the membrane was probed with a goat anti-mouse antibody (sc-2005) (Santa Cruz Biotechnology, USA) under agitating for 1 hour at room temperature, followed by three times washing. Image was developed using ECL solution (Millipore, USA) and exposed to Hyperfilm (Amersham, UK) varied for 1-10 minutes, processed using Fuji medical film processor (Fuji, Japan).

Silver Staining

After electrophoresis, the gel was removed from the cassette and rinsed briefly with ultrapure water. The staining protocol was followed according to the manufacturer's instructions (SilverQuest™ Silver Staining Kit, Invitrogen, USA).

Antibody Epitope Prediction Tools

Bepipred Linear Epitope Prediction tool and Emini surface accessibility scale were used to design the truncated HA protein. Both tools can be accessed at http://tools.immuneepitope.org/tools/bcell/iedb_input.

Pairwise sequence alignment

EMBOSS Needle was used to identify regions of similarity of truncated HA gene in default settings (available at: http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

Protein molecular weight determination

Theoretical molecular weight of proteins was computed using Compute pI/Mw tool from ExPASy (available at: http://web.expasy.org/compute_pi/). The apparent migration size of proteins on 12% SDS-PAGE gel was estimated and referred to the Spectra multicolor broad range protein ladder or PageRuler prestained protein ladder (Fermentas, USA).

RESULTS

Amplification and construction of truncated HA gene

Based on the HA gene fragment (A/chicken/Malaysia/5858/2004(H5N1) (Figure 1 a), the truncated form of HA gene was designed and showed in Figure 1b. Amplification of truncated HA gene derived from plasmid pCR®2.1-TOPO®-HANA was performed using a standard PCR. Our unpublished work showed the amplification by a standard PCR at 55ºC produced a specific band at the size about 160 bp. Restriction analysis of cloned HA gene in the expression vector was shown in Figure 2. DNA sequencing using T7 promoter and pairwise sequence alignment analysis of this gene indicated that the constructed truncated HA gene contained no alteration.

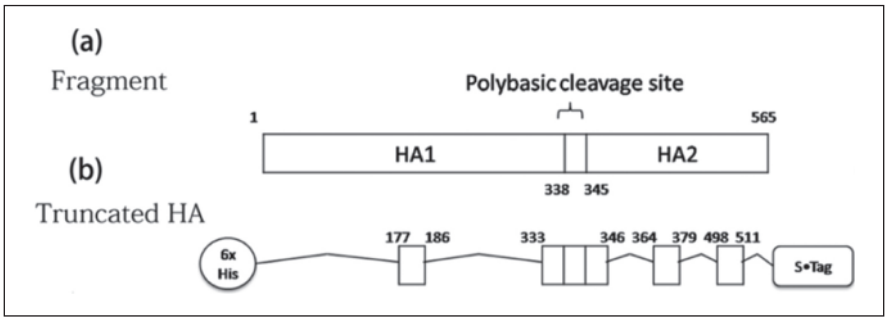

Figure 1. Schematic illustration of full-length HA protein and the truncated form. (a) Primary structure of HA polypeptide: HA1 subunit, a polybasic cleavage site (RERRRKKR) and HA2 subunit. (b) The 6x histidine-tagged truncated HA protein and tailed by S•Tag

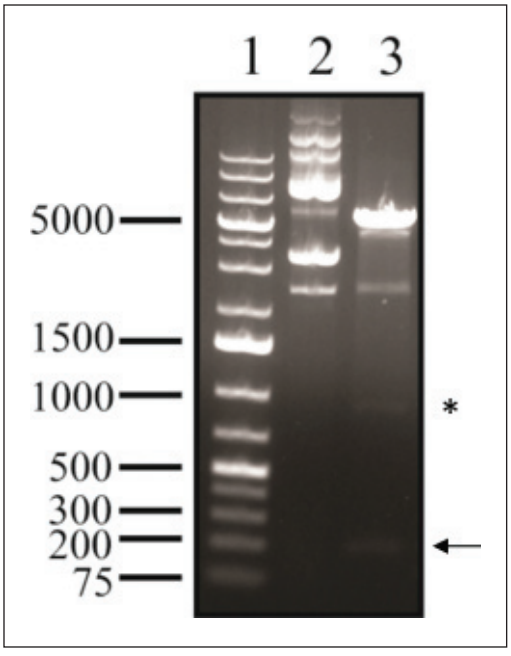

Figure 2. Digestion of pET-47b(+) expression vector containing truncated HA gene by enzymes *Sal*I and *Hind*III. Lane 1: GeneRuler^{TM} 1kb Plus DNA Ladder (Fermentas, USA); Lane 2: Undigested plasmid; Lane 3: Digested plasmid. An arrow indicates the excised truncated HA gene. Asterisk indicates the extra DNA band resulted from the enzyme star activity

Optimizing expression of truncated HA protein

Expression of truncated HA protein in *E. coli* strain BL21 (DE3) was optimized through small-scale time-course induction study under induction of 1 mM IPTG at 37°C. Visual inspection of the apparent thickest band on Western blot probed by anti-His tag antibody was justified as a maximal protein yield. Figure 3 showed a highest level expression of truncated protein was obtained at 3 hours of IPTG-induction at 37°C. Anti-His tag mouse monoclonal antibody was validated using a cleared lysate of non-harbouring plasmid *E. coli* strain BL21 (DE3) as a negative control and zinc fingers of CTCF protein as a positive control.

Protein Purification and Optimization

Purification of truncated HA proteins was initially performed through a small-scale stepwise protein elution study using Ni-NTA spin column (QIAGEN, Germany). Our data (Figure 4) demonstrated 6x histidine tagged recombinant protein was eluted at 500 mM of imidazole. Thus, the large-scale purification of truncated HA protein was washed with 30mM imidazole and all purified proteins were eluted at 500 mM imidazole buffer. The purification of truncated HA protein was resolved on 12% SDS-PAGE gel and then stained with Coomassie Briliant Blue (Figure 5) or more sensitive silver staining (Figure 6).

Molecular weight and migration size determination

Migration of denatured HA protein was analyzed on reducing 12% SDS-PAGE and probed by Western blot using anti-His-tag antibody (Figure 3). The migration size of HA protein was compared to its theoretical

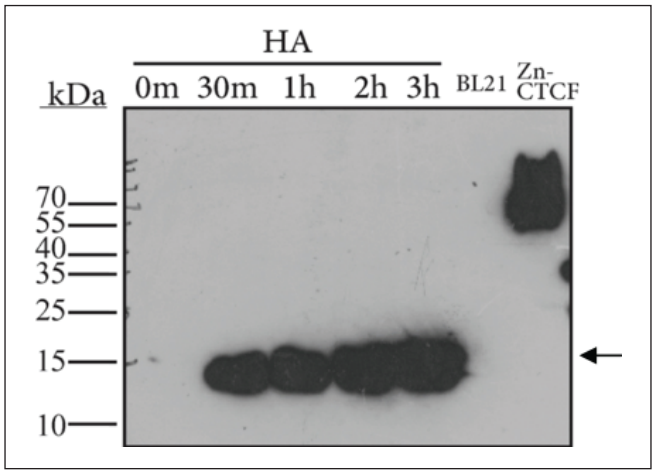

Figure 3. Time-course induction study in *Escherichia coli* strain BL21 (DE3). Expression of truncated HA protein was induced by 1 mM IPTG at final concentration and harvested at 0 min, 30 min, 1 hour, 2 hours and 3 hours. Cleared cell lysates containing the truncated protein were resolved on 12% gel and probed by Western blot using with anti-His tag monoclonal antibody in 1:1000 dilution. BL21: cleared lysate of non-harbouring plasmid *E. coli* strain BL21 (DE3); Zn-CTCF: 70-kDa zinc fingers of CTCF protein. An arrow indicates HA protein detected

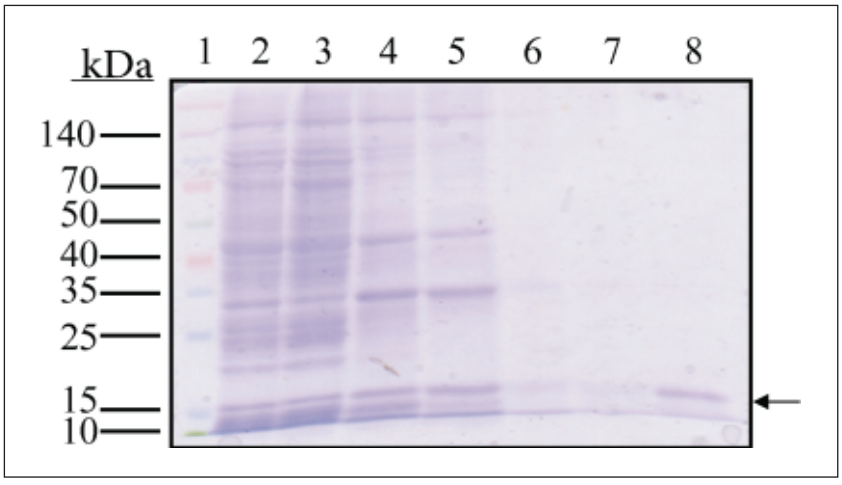

Figure 4. 12% SDS-PAGE gel analysis of stepwise elution of truncated HA protein. Protein was eluted with a stepwise gradient of 10-500 mM Imidazole. Lane 1: Pre-stained protein ladder; Lane 2: Cleared lysate; Lane 3: Flow-through fraction; Lane 4-8: Eluted fractions (10, 20, 30, 40, 500 mM imidazole, respectively). Arrow indicates truncated HA protein

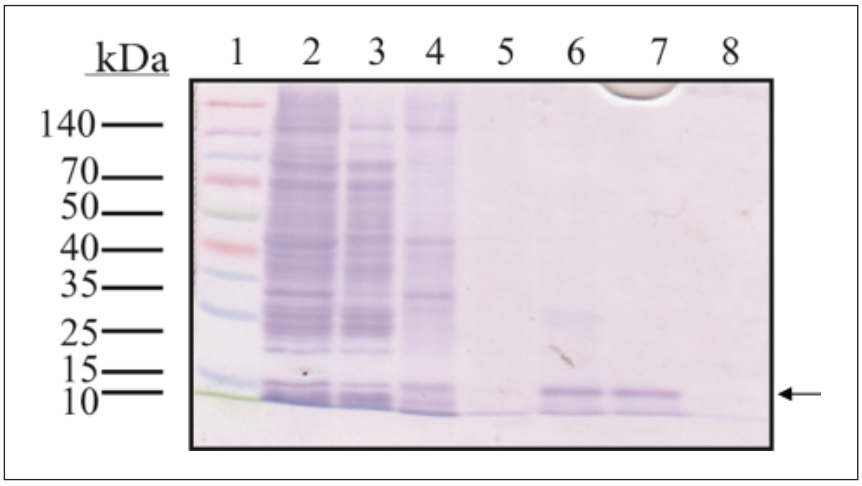

Figure 5. 12% SDS-PAGE gel analysis of the large-scale truncated HA protein purification. Lane 1: Pre-stained protein ladder; Lane 2: Cleared lysate; Lane 3: Flow-through fraction; Lane 4: Wash fraction; Lane 5-8: Eluted fractions I-IV (500 mM imidazole). Arrow indicates truncated HA protein

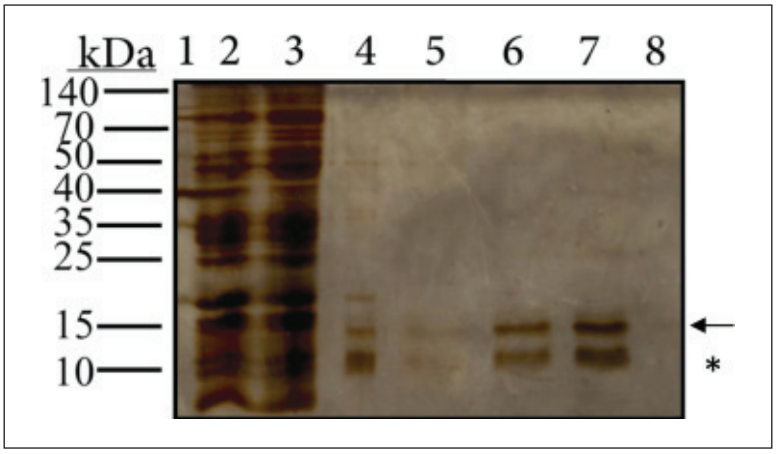

Figure 6. Silver staining of the truncated HA protein purification. Lane 1: Pre-stained protein ladder; Lane 2: Cleared lysate; Lane 3: Flow-through fraction; Lane 4: Wash fraction; Lane 5-8: Eluted fractions I-IV (500 mM imidazole). Arrow indicates truncated HA protein. Asterisk indicates a degradation product of truncated HA protein

molecular weight and percentage of aberrant migration calculated in Table 1. The percentage of aberrant migration of HA protein was calculated as following:

$$\%\ of\ Aberrant\ Migration = \frac{SDS\text{–}PAGE\ (kDa) - Theoretical\ (kDa)}{Theoretical\ (kDa)} \times 100\%$$

DISCUSSION

Influenza A viruses can be classified into two groups: low-pathogenicity (LPAI) and high-pathogenicity (LPAI) viruses on the basis of the virulence in chickens. The very virulent HPAI viruses can cause mortality as high as

Table 1. The molecular weight determination of truncated HA protein. Theoretical molecular weight of truncated HA protein was calculated from ExPASy compute pI/Mw tool and compared to the apparent migration on 12% SDS-PAGE. Its discrepancy in migration is shown in percentage

Protein	pI	Calculated molecular weight* (kDa)	Migration on 12% SDS-PAGE (kDa)	Aberrant migration (%)
HA	8.93	14.87	15	≅ 0.87%

* Calculation including endogenous pET-47b(+) expression vector containing 6 x His tag and S•Tag

100%, whereas LPAI viruses cause a mild or no symptoms (Alexander, 2000). HPAI viruses differ from all other strains by possessing the characteristics of polybasic amino acids motif (-RXR/KR-) in their glycoprotein haemagglutinin (Kaiyawet *et al.*, 2013). These polybasic amino acids are also termed as the polybasic cleavage site because they are recognized by the ubiquitous host protease furin and cleaved by the haemagglutinin precursor (HA0) to HA1 and HA2 subunits.

Based on H5N1 Guangdong sublineage (A/chicken/Malaysia/5858) (Chen *et al.*, 2006) partial cDNA fragment (GenBank: DQ320934.1) analysis, we had identified that the polybasic cleavage site (RERRRKKR) was located at 338-346 amino acid residues between HA1 and HA2 subunits (Figure 1a). HA1 subunit functions mediating initial contact with cell membrane, whereas HA2 subunit is responsible for membrane fusion (Xu & Wilson, 2011). The activation of cleaved HA undergoes a conformational change and mediates the fusion of the virion envelope with the endosomal membrane, which is associated to virus pathogenicity (Stieneke-Grober *et al.*, 1992; Klenk & Garten, 1994). However, the HA2 is more conserved and hydrophobic than the variable HA1. In addition, HA2 is poor immunogenic to elicit the neutralizing antibodies compared to HA1 and the molecular reason behind this poor immunogenicity is less clear (Rappuoli, 2011). On the other hand, the polybasic cleavage site on HA is also highly conserved among those highly pathogenic H5N1 viruses and this polybasic peptide is an interesting candidate as a universal vaccine for influenza A viruses (Tsai *et al.*, 2012). More importantly, the acquisition of this artificial polybasic HA cleavage site into LPAI non H5/H7 is one of the several alterations that are necessary for viruses to evolve into HPAI viruses (Stech *et al.*, 2009).

Since H5N1 virus is highly pathogenic and requires a high level biosafety laboratory, we opted to use one-step assembly PCR (Wu *et al.*, 2006) to generate a synthetic gene (unpublished work) composed of a hybrid of HA and NA based on their linear epitope using Bepipred Linear Epitope Prediction tool (Larsen *et al.*, 2006) and surface accessibility using Emini surface accessibility scale (Emini *et al.*, 1985). Assembly PCR is a simple, rapid, high-fidelity and cost-effective PCR-based DNA synthesis techniques (Xiong *et al.*, 2004). Bepipred Linear Epitope method is a combination of the hidden Markov model and the propensity scale methods to predict linear B-cell epitopes (Larsen *et al.*, 2006). Emini surface accessibility is a comparative surface features analysis between VPI sequences of hepatitis A virus (HAV) and poliovirus type I to identify their probable neutralizing antigenic sites (Emini *et al.*, 1985).

Thus, in this work, we isolated and cloned the truncated HA gene (Figure 1b), which are composed of peptide epitopes of HA1 and HA2 subunits, and a polybasic cleavage site, into pET-47b(+) downstream of powerful T7 promoter. DNA sequencing and pairwise alignment analysis confirmed that the cloned gene contained no alteration and in-frame to the expression vector. Subsequently, we expressed the truncated HA protein in a common workhorse *E. coli* strain BL21 (DE3) under IPTG induction. The small-scale time-course induction study justified that

the maximal yield of protein was obtained at 3 hours of induction.

Electrophoretic migration size of denatured truncated HA protein revealed no aberrant migration and almost identical to their theoretical molecular weight by Western blot analysis using anti-His tag monoclonal antibody. However, further protein N-terminal sequencing analysis is required to verify this result. In order to obtain the purified protein, we employed nickel chromatography to isolate polyhistidine-tagged protein from bacterial contaminant proteins due to the high specific affinity of the Ni-NTA resins for histidine residues (Janknecht *et al.*, 1991). Prior to large-scale protein purification production, a small-scale stepwise protein elution study was used to determine the stringency of wash condition using a gradient concentration of imidazole buffers. Gel analysis of protein purity (Figures 5 and 6) confirmed this method was feasible to obtain the high purified protein. The resultant protein obtained in this study requires further study to determine its capability to elicit the neutralizing antibodies raised in mouse by using micro-neutralizing assay (Rowe *et al.*, 1999). If reactive, the truncated HA protein expressed from bacterial system could be a potential candidate for the development of a safe and efficient vaccine for HPAI (H5N1).

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REFERENCES

Alexander, D.J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology* **74**(1-2): 3-13.

Biesova, Z., Miller, M.A., Schneerson, R., Shiloach, J., Green, K.Y., Robbins, J.B. & Keith, J.M. (2009). Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. *Vaccine* **27**(44): 6234-6238.

Chen, H., Smith, G.J., Li, K.S., Wang, J., Fan, X.H., Rayner, J.M., Vijaykrishna, D., Zhang, J.X., Zhang, L.J., Guo, C.T., Cheung, C.L., Xu, K.M., Duan, L., Huang, K., Qin, K., Leung, Y.H., Wu, W.L., Lu, H.R., Chen, Y., Xia, N.S., Naipospos, T.S., Yuen, K.Y., Hassan, S.S., Bahri, S., Nguyen, T.D., Webster, R.G., Peiris, J.S. & Guan, Y. (2006). Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proceedings of the National Academy of Science of the United States of America* **103**(8): 2845-2850.

Chiu, F.F., Venkatesan, N., Wu, C.R., Chou, A.H., Chen, H.W., Lian, S.P., Liu, S.J., Huang, C.C., Lian, W.C., Chong, P. & Leng, C.H. (2009). Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. *Biochemical and Biophysical Research Communications* **383**(1): 27-31.

Dilillo, D.J., Tan, G.S., Palese, P. & Ravetch, J.V. (2014). Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. *Nature Medicine* **20**(2): 143-151.

Els, M.C., Graeme Laver, W. & Air, G.M. (1989). Sialic acid is cleaved from glyco-conjugates at the cell surface when influenza virus neuraminidases are expressed from recombinant vaccinia viruses. *Virology* **170**(1): 346-351.

Emini, E.A., Hughes, J.V., Perlow, D.S. & Boger, J. (1985). Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *Journal of Virology* **55**(3): 836-839.

Herfst, S., Schrauwen, E.J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V.J., Sorrell, E.M., Bestebroer, T.M., Burke, D.F., Smith, D.J., Rimmelzwaan, G.F., Osterhaus, A.D. & Fouchier, R.A. (2012). Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336**(6088): 1534-1541.

Horimoto, T. & Kawaoka, Y. (2005). Influenza: lessons from past pandemics, warnings from current incidents. *Nature Reviews Microbiology* **3**(8): 591-600.

Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A. & Stunnenberg, H.G. (1991). Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proceedings of the National Academy of Science of the United States of America* **88**(20): 8972-8976.

Kaiyawet, N., Rungrotmongkol, T. & Hannongbua, S. (2013). Probable polybasic residues inserted into the cleavage site of the highly pathogenic avian influenza A/H5N1 hemagglutinin: Speculation of the next outbreak in humans. *International Journal of Quantum Chemistry* **113**(4): 569-573.

Kanekiyo, M., Wei, C.J., Yassine, H.M., McTamney, P.M., Boyington, J.C., Whittle, J.R., Rao, S.S., Kong, W.P., Wang, L. & Nabel, G.J. (2013). Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* **499**(7456): 102-106.

Khurana, S., Verma, S., Verma, N., Crevar, C.J., Carter, D.M., Manischewitz, J., King, L.R., Ross, T.M. & Golding, H. (2011). Bacterial HA1 vaccine against pandemic H5N1 influenza virus: evidence of oligomerization, hemagglutination, and cross-protective immunity in ferrets. *Journal of Virology* **85**(3): 1246-1256.

Klenk, H.D. & Garten, W. (1994). Host cell proteases controlling virus pathogenicity. *Trends in Microbiology* **2**(2): 39-43.

Lamb, R.A. & Choppin, P.W. (1983). The gene structure and replication of influenza virus. *Annual Review of Biochemistry* **52**: 467-506.

Larsen, J.E., Lund, O. & Nielsen, M. (2006). Improved method for predicting linear B-cell epitopes. *Immunome Research* **2**: 2.

Lin, S.C., Huang, M.H., Tsou, P.C., Huang, L.M., Chong, P. & Wu, S.C. (2011). Recombinant trimeric HA protein immunogenicity of H5N1 avian influenza viruses and their combined use with inactivated or adenovirus vaccines. *PLoS One* **6**(5): e20052.

Lin, Y.J., Deng, M.C., Wu, S.H., Chen, Y.L., Cheng, H.C., Chang, C.Y., Lee, M.S., Chien, M.S. & Huang, C.C. (2008). Baculovirus-derived hemagglutinin vaccine protects chickens from lethal homologous virus H5N1 challenge. *The Journal of Veterinary Medical Science* **70**(11): 1147-1152.

Palese, P., Tobita, K., Ueda, M. & Compans, R.W. (1974). Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* **61**(2): 397-410.

Rappuoli, R. (2011). The challenge of developing universal vaccines. *F1000 Medicine Reports* **3**: 16.

Rowe, T., Abernathy, R.A., Hu-Primmer, J., Thompson, W.W., Lu, X., Lim, W., Fukuda, K., Cox, N.J. & Katz, J.M. (1999). Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *Journal of Clinical Microbiology* **37**(4): 937-943.

Shen, S., Mahadevappa, G., Oh, H.L., Wee, B.Y., Choi, Y.W., Hwang, L.A., Lim, S.G., Hong, W., Lal, S.K. & Tan, Y.J. (2008). Comparing the antibody responses against recombinant hemagglutinin proteins of avian influenza A (H5N1) virus expressed in insect cells and bacteria. *Journal of Medical Virology* **80**(11): 1972-1983.

Shoji, Y., Bi, H., Musiychuk, K., Rhee, A., Horsey, A., Roy, G., Green, B., Shamloul, M., Farrance, C.E., Taggart, B., Mytle, N., Ugulava, N., Rabindran, S., Mett, V., Chichester, J.A. & Yusibov, V. (2009). Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine* **27**(7): 1087-1992.

Stech, O., Veits, J., Weber, S., Deckers, D., Schroer, D., Vahlenkamp, T.W., Breithaupt, A., Teifke, J., Mettenleiter, T.C. & Stech, J. (2009). Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. *Journal of Virology* **83**(11): 5864-5868.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.D. & Garten, W. (1992). Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO Journal* **11**(7): 2407-2414.

Tsai, H.J., Chi, L.A. & Yu, A.L. (2012). Monoclonal antibodies targeting the synthetic peptide corresponding to the polybasic cleavage site on H5N1 influenza hemagglutinin. *Journal of Biomedical Science* **19**: 37.

Wei, C.J., Xu, L., Kong, W.P., Shi, W., Canis, K., Stevens, J., Yang, Z.Y., Dell, A., Haslam, S.M., Wilson, I.A. & Nabel, G.J. (2008). Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. *Journal of Virology* **82**(13): 6200-6208.

Wei, C.J., Yassine, H.M., McTamney, P.M., Gall, J.G., Whittle, J.R., Boyington, J.C. & Nabel, G.J. (2012). Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure. *Science Translational Medicine* **4**(147): 147ra114.

WHO. (2011). H5N1 avian influenza: Timeline of major events. [Online] [Accessed 13 July, 2013], Available from World Wide Web: http://www.who.int/influenza/human_animal_interface/avian_influenza/H5N1_avian_influenza_update.pdf.

Wiley, D.C. & Skehel, J.J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual Review of Biochemistry* **56**: 365-394.

Wu, G., Wolf, J.B., Ibrahim, A.F., Vadasz, S., Gunasinghe, M. & Freeland, S.J. (2006). Simplified gene synthesis: a one-step approach to PCR-based gene construction. *J Biotechnol* **124**(3): 496-503.

Xiong, A.-S., Yao, Q.-H., Peng, R.-H., Li, X., Fan, H.-Q., Cheng, Z.-M. & Li, Y. (2004). A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequences. *Nucleic Acids Research* **32**(12): e98.

Xu, R. & Wilson, I.A. (2011). Structural characterization of an early fusion intermediate of influenza virus hemagglutinin. *Journal of Virology* **85**(10): 5172-5182.

Yee, K.S., Carpenter, T.E. & Cardona, C.J. (2009). Epidemiology of H5N1 avian influenza. *Comparative Immunology, Microbiology and Infectious Disease* **32**(4): 325-340.