CHAPTER 1

INTRODUCTION

Avian influenza virus (H5N1) is a negative strain RNA virus, which belong to the *Orthomyxoviridae* family. The subtype of the virus is classified based on the surface proteins haemagglutinin (HA) and neuraminidase (NA) (Aggarwal *et al.,* 2010). As an RNA virus, it is rapidly mutated during replication. Therefore, the mutation rate is very high and is the cause of no vaccine available for this disease. The H5 influenza virus subtype (HPAI) H5 is highly pathogenic in poultry. The first outbreak of this HPAI was reported in China in 1996 (World Health Organization, 2016) and the first human infection was detected in 1997 in Hong Kong (Claas *et al.*, 1998; Subbarao *et al.,* 1998). The current outbreak of HPAI H5N1 subtype began in 2003 and continued to spread in an unpredictable manner to almost all Asian countries as well as some countries in Europe and Africa (The World Organisation for Animal Health, 2009). Efficient virus binding to host cell receptors and the subsequent fusion of the viral and cellular membranes are mediated by the HA protein, which are important determinant of virulence and host restriction and critical to virus dissemination during epidemics and pandemics. The influenza HA (HA0) glycoprotein plays a crucial role in the virus infection by specifically binds to cellular receptors consisting of sialic acid on the surface of the host cell. Cellular proteases of the host cleave the HA precursor (HA0) into two smaller proteins linked by disulfide bond designated HA1 (36 kDa) and HA2 (27 kDa) (Skehel *et al.,* 1982; Boulay *et al.,* 1987; Singh *et al.,* 1990; Skehel and Wiley, 2000; Stevens *et al.,* 2006; DuBois *et al.,* 2011). The HA1 surface subunit mediates the binding to cellular receptors consisting of sialic acid on the surface of the host cell while the HA2 transmembrane subunit mediates membrane fusion between viral and endosomal membranes after endocytosis (White *et al.,* 2008).

Furthermore, HPAI H5N1 subtype was highly contagious among birds, and become entrenched in poultry in some countries, resulting in millions of poultry infections, several

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human cases, and human deaths (World Health Organization, 2016). This virus strain is highly susceptible to antigenic drift and shift and has already caused several outbreaks in human subjects with very high mortality rate (Duan *et al.,* 2008; Subbarao *et al.,* 1998). There is no effective and specific treatment for HPAI in poultry, therefore, the early detection of avian influenza virus via clinical case definition and rapid laboratory confirmation may identify patients who can be treated early with an antigenic proteins produced by recombinant protein, which is the first step to control the spread of infection and to prevent progression to more severe disease (Beck *et al.,* 2003). The haemagglutination inhibition (HI) assay, the standard method for serologic detection of influenza virus infection in humans, was less sensitive than the indirect enzyme-linked immunosorbent assay (ELISA) in detecting antibodies to avian influenza viruses (Rowe *et al.,* 1999). However, indirect ELISA require highly purify antigen in order to avoid cross reaction with other surface proteins.

Due to the continuous outbreaks of avian influenza virus in the fast two decades, the high priority in attempts fight AI are the laboratory techniques such as efficient isolation technique and sensitivity detection of AI/AI antibody from surveillance samples. The cell culture systems and the embryonated chicken egg (ECE) and are generally used as the basic method for isolation and cultivation which are able to support the growth of a large spectrum of avian influenza virus and their subtypes. The inoculation of specific pathogen free (SPF) embryonated chicken eggs or specific antibody negative (SAN) eggs are the preferred method for growing influenza A viruses, which inoculated into the allantoic sac of three to five embryonated SPF or SAN chicken eggs of 9–11 days incubation for 2–7 days at 37°C (range 35–39°C), and they can be subtyped with specific antisera in haemagglutination and neuraminidase inhibition tests, by RT-PCR, or by sequence analysis of the viral HA and NA genes (The World Organisation for Animal Health, 2007). Moreover, the influenza vaccines have traditionally been prepared in the ECE system as it is the possibility to acquire a large volume of the viral stock from a single egg (Palese and Shaw, 2007) and it also allowed high recovery of low-titre field samples, depending, however, on species, viral subtype and host specificity. However, the limiting factors which might be considered in the ECE system are the virulent strains avian influenza virus (H5N1 subtype) rapidly kill the embryos, yield low viral titres,

length of time necessary for the growth, microbial contamination and the unavailability of specific pathogen free (SPF) eggs. Production of viral antigen by traditional technique is not suitable. The recombinant DNA technology of protein expression and production of recombinant viral protein are widely used for expression the protein in various systems for diagnostics and vaccines. Therefore, the antigenic proteins produced by recombinant protein was the way for diagnosis and epidermiologic study of avian influenza. Moreover, using recombinant protein as an antigen in serological tests has an advantage for antigen prepared from whole cell preparations in terms of sensitivity and specificity as no only specific antigen is employed. Therefore exclude the nonspecific protein present in the whole cell preparations (Errington *et al.,* 1995; Mohan *et al.,* 2006).

Recombinant influenza HA proteins can be expressed in a variety of systems such as mammalian, insect cell lines, or in plants (Crawford *et al.,* 1999; Wei *et al.,* 2008; Wu *et al.,* 2010; Liu *et al.,* 2011; Kanagarajan *et al.,* 2012). The HA glycoprotein of influenza virus can be expressed constitutively either in continuous lines of cells stably transformed by recombinant vectors or during the course of lytic infections by recombinant viruses such as high levels of the HA protein of influenza virus were successfully expressed in simian CV-1 or COS-1 cells when infected with recombinant SV40-HA virus vectors (Gething and Sambrook, 1981), Roth *et al.* (1983) was successfully express HA from cloned cDNA accumulated preferentially on the apical surface of polarized cells, and the HA produced was displayed at the cell surface in a form which indistinguishable in its antigenic and immunogenic properties from that produced in cells infected with influenza virus (Gething *et al.,* 1984). Kuroda *et al.* (1986) expressed the fowl plague virus HA gene in insect cells using baculovirus expression vector and these observations demonstrate that HA was processed in insect cells similar fashion as in fowl-plague-virus-infected vertebrate cells. Shoji *et al.* (2009) expressed the haemagglutinin protein from the H5N1 influenza virus strain A/Indonesia/05/05 in plants using in transient gene expression method. The recombinant protein was successfully induce protective immunity against infection HPAI in mice and ferrets. Kalthoff *et al.* (2010) sucessfully expressed full-length hemagglutinin (rHA0) of HPAIV H5N1 in plant with the immunogenic capacity for several vaccine formulations within the highly relevant host species chicken. However the HA proteins which expressed in mammalian, insect cell lines, or in plants are costly, requires high-tech mass cell production that is under constant

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threat of foreign pathogen introduction. The methylotrophic yeast *P. pastoris* has raised an increasing interest for the expression of HA for the production of viral antigens as it can produce large amount of recombinant protein with proper folding and posttranslational modification, no viral contamination, ease of genetic manipulation, fast growth rates in low cost media, and it is relatively simpler than mammalian or insect cell systems (Wu *et al.,* 2009). Moreover, *P. pastoris* has been demonstrated to be a good expression vector for influenza proteins. For example, Wang *et al.* (2007) successfully expressed the HA0 protein from H5N2 virus in *P. pastoris*. However, some HA1 protein was also found, possibly due to digestion by proteases from the host. *P. pastoris* was also used to express biologically active neuraminidase (NA) of H1N1 which was considered as a potential protein-based vaccine or useful in anti-influenza drug screening (Yongkiettrakul *et al.,* 2009). Abubakar *et al.* (2011) were successfully clone and express highly pathogenic avian influenza virus full-length nonstructural (NS1) gene in *P. pastoris*. Shehata *et al.* (2012) reported the expression of two truncated polypeptide of full-length HA1 in *P. pastoris*. In this report, the HA protein was glycosylated. Moreover, *P. pastoris* was used to express a soluble secreted haemagglutinin protein (H1N1). The recombinant H1N1 could elicit neutralizing antibodies in rabbits and mice (Athmaram *et al.,* 2011). A recent study, Murugan *et al.* (2013) successfully expressed the haemagglutinin protein of H5N1 virus in the cytosol and in the membrane fractions of *P. pastoris* which was observed the antigen specific immune response when used in a mouse trial.

The aims of this study is to clone, express, and purify the haemagglutinin domain of avian influenza A virus CMU strain in *P. pastoris* for epidermiologic study.

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