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Expression and Purification of NS1 Protein of Highly Pathogenic Avian Influenza Virus H5N1 in *Escherichia coli*

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ABSTRACT

The highly pathogenic avian influenza (HPAI) is a viral disease of poultry and is a zoonosis. The eighth segment of the viral genomic RNA encodes a nonstructural protein, NS1. NS1 is a viral regulatory protein responsible for virus virulence. This study, produced the 6xHis-tag fusion protein. The full length NS1 cDNA of A/Chicken/TH/KU14/04 (H5N1) was cloned into an expression vector, pQE80L. The recombinant NS1 was expressed in the *E. coli* strain DH5α. The results showed that the polyhistidine-tagged NS1 fusion protein was successfully expressed in bacterial cells, but the fusion protein accumulated in the inclusion bodies. The fusion protein was isolated as either a native or denatured form and further purified by precipitation or immobilization on a nickel ion affinity column. Purification using affinity chromatography under denaturing conditions yielded greater amounts of purer NS1 protein. However, sufficient purity and concentration of the native NS1 protein was obtained after purification using a precipitation method. Both native and denatured, purified NS1 protein reacted specifically with the anti-NS1 chicken serum in a western blot assay. The results indicated that the NS1 protein may be useful for further applications.

Key words: highly pathogenic avian influenza, nonstructural protein, NS1 expression, NS1 purification, western blot

INTRODUCTION

Avian Influenza (AI) is an infectious disease caused by the avian influenza virus (AIV) belonging to type A of the family *Orthomyxoviridae*. Influenza A viruses are comprised of 16 HA and nine NA subtypes based on the antigenicity differences of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Palese and Young, 1982). AI is divided into two groups according to the virulence of the pathogenicity: low or mildly pathogenic avian influenza (LPAI or MPAI) and highly pathogenic avian influenza (HPAI). HPAI is a lethal disease in poultry.

Recently, HPAI virus subtype H5N1 caused disease outbreaks in poultry in Asia and spread to Europe and Africa resulting in concern about global health and the international poultry product trade (Wu *et al.*, 2007). Thus, serological surveillance of antibodies against AI and AI vaccines are indispensable for the prevention and control of AI. However, vaccination may interfere with serological surveillance and thus affect

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international trade in poultry and poultry products. Therefore, heterologous neuraminidase vaccines, live influenza virus vaccines with an altered nonstructural protein 1 (NS1) gene, virus vector vaccines and subunit vaccines have been developed to differentiate infected from vaccinated animals from vaccinated animals (DIVA) (Suarez, 2005). However, an inactivated (dead) whole virus vaccine is currently used in various countries. This type of vaccine consists of all viral proteins with the exception of the NS1 protein.

NS1 is the most important viral regulatory protein that interferes with host native immunity by inhibiting the interferon-mediated immune response and cellular protein synthesis (Bergmann *et al*., 2000). The NS1 protein is translated from the co-linear-unspliced transcript of segment 8 (García-Sastre *et al*., 1998). It contains two domains, the RNA-binding domain and the effector domain (Chien *et al*., 2004). The RNA binding domain is at the N-terminus of the NS1, comprises the first 73 amino acid residues and forms a symmetric homodimer in solution (Hatada *et al*., 1999). Arginine and lysine residues at positions 38 and 41, respectively, are required for RNA binding activity (Wang *et al*., 1999). Recently, it was found that the RNA-binding domain was responsible for binding with nucleolin (Murayama *et al*., 2007). The C-terminus of the NS1 protein or the effector domain, interacts with many viral and cellular factors involved in translation and post-transcriptional processing of RNA (Burgui *et al*., 2003). In addition, glutamic acid at position 92 in this domain may be involved in increased virulence and cytokine resistance in H5N1 strain (Seo *et al*., 2004).

The NS1 protein is abundantly synthesized in infected cells, but is not incorporated into the virions (Egorov *et al*., 1998). Thus, the NS1 protein presents only in the infected animals and not in animals vaccinated with the inactivated virus. This makes NS1 a better antigen for use in the development of a diagnostic tool that can differentiate between infected and vaccinated animals (DIVA). Recently, Tumpey *et al*. (2005) developed an enzyme-linked immunosorbent assay (ELISA) for DIVA on the basis of antibodies to NS1, which could be used as a serodiagnostic tool for the determination of LPAI virus infection of poultry. However, its use was not confirmed for the detection of the HPAI virus infection in poultry.

This study describes the production of the NS1 protein of the HPAI virus, A/Chicken / TH/ KU14/04 (H5N1), in *E. coli*. The protein expression vector, pQE80L, containing the NS1 gene was constructed. The recombinant NS1 was expressed in the *E. coli* strain DH5α and purified by precipitation and affinity chromatography. The protein was characterized by SDS-PAGE and Western blot analysis.

MATERIALS AND METHODS

Virus

A/Chicken/TH/KU14/04 (H5N1) is a HPAI virus. It was isolated from a field sample in Thailand. The subtype was identified as H5N1 using reverse transcription-polymerase chain reaction (RT-PCR).

RNA extraction and RT-PCR

The viral RNA was extracted from the allantoic fluid of an embryonated chicken egg (ECE) using Trizol® reagent (invitrogen) according to a protocol provided by the manufacturer. The cDNA was synthesized from total RNA using a specific primer (Hoffmann *et al*., 2001). The 20 µl reverse transcription mixture contained a 10 µl RNA template, 1 mM primer, 0.5 mM dNTPs, 0.01 M DTT, 200 U SuperScript™ III RT, 40 U RNase inhibitor and 4 µl of 5X First-Strand Buffer. The RT reaction mixture was incubated at 70°C for 10 minutes, followed by 42°C 50 minutes and 70°C for 15 minutes. The cDNA was used as a template for NS1 gene

amplification by using NS1-*Bam*HI forward primer: 5′-ACT TGG ATC CGA TTC CAA CAC TGT GTC-3′ and NS1-*Sal*I reverse primer: 5′-CAG TGT CGA CTC AAA CTT CTG ACT CAA TTG-3′. The positions of the forward and reverse primers correspond to nucleotide 4-20 and 684-704, respectively, on segment 8 of the viral genome. The 100 µl of PCR reaction contained 4 µl of cDNA, 10 µl of 10x PCR buffer (200 mM Tris-HCl and 500 mM KCL), 2 mM $MgCl_2$, 0.5 mM dNTP, 0.5 mM of each primer and 2.5 units Taq DNA Polymerase (Invitrogen). The PCR cycles consisted of 35 cycles of 30s at 94°C, 30s at 55°C and 45 min at 72°C. The PCR product is approximately 675 bp in length and carries the linker-restriction *Bam*HI and *Sal*l sites at 5′-end and 3′-end, respectively. The PCR products were purified using the QIAEXII Agarose Gel Extraction Kit (Qiagen).

Construction of expression vector containing NS1 gene

To construct the expression vector containing the NS1 gene, the purified NS1 DNA was first cloned into pGEM®-T Easy Vector (Promega) using T4 DNA ligase (Invitrogen). The integrity of the NS1 nucleotide sequence was verified by sequencing. The PCR fragment and pQE80L expression vector (Qiagen) were digested with the restriction enzymes *Bam*HI and *Sal*l. The linearized vector and NS1 gene were gel purified using the QIAEXII Agarose Gel Extraction Kit (Qiagen). The NS1 gene was inserted into pQE80L (Qiagen) using T4 DNA ligase (Invitrogen). This ligation product was transformed into Subcloning Efficiency™ DH5α™ Competent Cell (Invitrogen) by heating shock of the cells at 42°C for 20 seconds. The transformed *E. coli* was plated on LB agar containing 100 µg/ml ampicillin. The transformants were screened by PCR using specific primers. Plasmid containing the NS1 gene was isolated from positive clones prior to being sequenced at the Bioservice Unit, Thailand.

Induction and expression of 6x His-tag–NS1 fusion protein

The transformed *E.coli* with the NS1 insert were grown in LB broth containing 100 µg/ml ampicillin at 37°C in a shaking incubator. The shaking platform was set at 225 rpm. The cells were incubated until the optical density (OD) reached 0.5 to 0.7 at 600 nm. The cells were then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated for an additional four hours at 37°C, 225 rpm. The culture was harvested aseptically by centrifugation at 10,000xg for 30 minutes at 4°C. The supernatant was removed and the pellet was stored at -20°C until used.

Extraction and purification of 6x His-tag–NS1 fusion protein

The pellets were lysed and the fusion protein was extracted and purified using the following three methods.

1. Denaturing condition

The pellets were resuspended in native lysis buffer A (25 mM Tris-HCl; pH7.4, 5 mM $MgCl_2$, 0.1% Triton-X 100, 0.1% *N*-lauroyl-sarcosine, and 10 mM imidazole). Three protease inhibitors, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 10 µg/ml PMSF as well as 1 mg/ml lysozyme were also added into the resuspended pellet. The lysate was mixed and incubated on ice for an hour. The cells were sonicated on ice for 30s pulses with a one minute pause for at least six times. They were then centrifuged at 10,000xg at 4°C for 30 minutes. The supernatant was collected in fresh tubes and stored at -20°C until analyzed. The pellets were resuspended in a denatured lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8 M urea), and stored frozen at -20°C until used. Contaminating proteins were separated from the 6x His-tag-NS1 fusion protein by denaturing Ni^{2+}-chelate affinity chromatography. The immobilized metal ion affinity Protino® Ni-IDA resin column (Macherey-nagel) was equilibrated with

denaturing solubilization buffer (50 mM NaH_2PO_4, 300 mM NaCl and 8 M urea, pH 8.0). The crude extract was loaded onto the columns and the protein was allowed to pass through the resin under the force of gravity. The column was washed with denaturing solubilization buffer and the 6x His-tag–NS1 fusion protein was eluted with denaturing elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea and 250 mM imidazole, pH 8.0). Five fractions of elements were collected and the OD of the protein was determined using a DU 800 spectrophotometer (Beckman).

2. Native condition

The *E. coli* pellets were resuspended in native lysis buffer A containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml PMSF and 1 mg/ml lysozyme, and incubated on ice for an hour. The cells were sonicated on ice as previously described and centrifuged at 10,000xg at 4°C for 30 minutes. The supernatant was discarded. The pellets were resuspended in native lysis buffer B (25 mM Tris-HCl; pH7.4, 5 mM $MgCl_2$, 0.3% Triton-X 100, 0.3% *N*-lauroyl-sarcosine, and 10 mM imidazole), and stored frozen at -20°C until used. The suspension was dialyzed in Lysis-Equilibration-Wash buffer (LEW buffer; 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0) for four hours at 4°C. Contaminated proteins were separated from the 6x His-tag-NS1 fusion protein by using native Ni^{2+}- chelate affinity chromatography. The crude extract was loaded onto the Protino® Ni-IDA resin column (Macherey-nagel), which was pre-equilibrated and washed with LEW buffer. The 6x His-tag–NS1 fusion protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0). Five fractions of the native protein were collected and the concentration of the protein was determined using a spectrophotometer.

3. Precipitation method

The *E. coli* lysate was partially purified using a precipitation method modified from a previously published procedure (Young *et al.*, 1983). After lysing in the native lysis buffer A, the pellets were resuspended in lysis buffer C (50 mM Tris-HCl; pH8.0, 2 mM EDTA, 0.1 mM dithiothreitol and 5% (v/v) glycerol), containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml PMSF and 1 mg/ml lysozyme, and incubated on ice for 20 minutes. The cells were sonicated on ice as described previously and centrifuged at 12,000 xg at 4°C for 30 minutes. The pellets were resuspended in the lysis buffer C again and the 10% (w/v) of sodium deoxycholate was added into the suspension until a final concentration was 0.05%. The mixture was vortexed several times and incubated on ice for five minutes before being incubated at 15°C for 30 minutes. The mixture was centrifuged at 12,000xg at 4°C for 45 minutes. The supernatant was collected in a fresh tube and the 5 M NaCl/ 1 M $MgCl_2$ mixture was added into the supernatant until the final concentration was 1 M and 100 mM, respectively. The protein was allowed to precipitate for an hour at 4°C and the precipitin was collected by centrifugation at 12,000xg for an hour at 4°C. The pellets were dissolved in buffer D (40 mM Tris-HCl; pH 7.5 and 2 mM $MgCl_2$). The protein was precipitated again by adding 1 M $MgCl_2$ into the protein suspension until the final concentration was 100 mM. The precipitin was collected by centrifugation at 12,000xg for an hour at 4°C. The pellets were dissolved in buffer D and dialyzed overnight at 4° C in buffer D.

Production of antibodies specific to NS1

Antibodies specific to the NS1 protein of A/Chicken/TH/KU14/04 were produced in chickens. The *E.coli* lysate was electrophoresed in 12% SDS-PAGE. The protein band specific to the NS1 protein was excited and eluted from the gel. The eluted protein was injected into three chickens. Each chicken was boosted once and the sera were collected at three weeks after immunization. Blood was collected and coagulated at 4°C overnight. The blood was centrifuged at

2,000 rpm for 30 minutes to remove the clotted cells. The serum was collected and used for Western blot analysis.

SDS-PAGE and western blotting

The protein suspension was analyzed by being electrophoresed through 12% SDS-PAGE according to the Laemmli method. The 5 µl of protein suspension was denatured in 5 µl of Laemmli's sample buffer (Bio-Rad Laboratories) and boiled for five minutes before being separated in 12% SDS-PAGE at 100V for two hours. The proteins in the gel were stained using coomassie brilliant blue overnight. The gel was destained in destaining solution I (50% (v/v) methanol and 10% (v/v) acetic acid) for an hour and destaining solution II (7% (v/v) acetic acid, 5% (v/v) methanol) until the protein bands were clearly visible. For Western blot, the proteins were transferred from polyacrylamide gel onto a 0.2 µ nitrocellulose membrane using standard techniques at 150V for two hours. The membrane was blocked in washing buffer (500 mM Tris and 2 M NaCl, pH 7.5) containing 1% (w/v) BSA overnight at 4°C. The membrane was washed three times in the washing buffer containing 0.1% Tween 20. The blocked membrane was incubated in anti-His mouse IgG antibody (USbiological) or anti-NS1 chicken serum at a dilution of 1:5,000 and 1:100, respectively, at room temperature for two hours. After incubation, the excess antibodies were washed three times in the washing buffer containing 0.1% Tween 20. Subsequently, the bound antibodies were allowed to react with anti-mouse IgG or anti-chicken IgY peroxidase conjugate (Sigma) at the dilution of 1:3,000 and 1:8,000, respectively, for two hours at room temperature. The membrane was equilibrated in Tris buffer, pH 7.4 before being stained with TMB substrate solution (KPL) for 5-10 minutes. The reaction was ceased by rinsing in water.

Protein concentration assay

A bicinchoninic acid (BCA) protein assay kit (Pierce) was used to determine the total protein concentration. BSA was used as a standard protein. Two ml of the working reagent were added into 100 µl of each standard and unknown sample. The mixture was incubated at 60°C for 30 minutes before being incubated at room temperature and then allowed to until cool down. The protein concentrations were determined using a spectrophotometer at 562 nm.

RESULTS

RT-PCR and nucleotide sequence analysis of the NS1 gene

Amplification of NS1 cDNA synthesized from A/Chicken/TH/KU14/04 RNA resulted in a DNA fragment of 675 bp in length (Figure 1). The purified PCR product was cloned and sequenced. The sequence analysis revealed a full length NS1 open reading frame (ORF) fused in frame with 6x histidine tag in a correct orientation. The NS1 sequence of the KU14 isolate was compared to that of the HPAI virus H5N1 deposited in the GenBank database, A/Cat/Thailand/KU-02/04

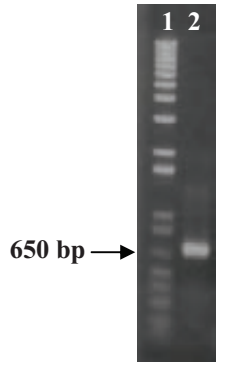

Figure 1 Agarose gel electrophoresis of NS1 RT-PCR product. Lane 1 is 1 kb DNA marker and lane 2 is the RT-PCR product of NS1 gene amplified from A/Chicken/TH /KU14/04.

(H5N1) (DQ236083.1) and A/Chicken/Vietnam/19/2003 (H5N1) (DQ493203.1). The result showed that the nucleotide sequence of the NS1 was 100% identical to that of A/Cat/Thailand/KU-02/04 and 99.6% identical to A/Chicken/Vietnam/19/2003.

Expression of the histidine-NS1 fusion protein

The recombinant plasmid pQE80L containing the NS1 gene fused in frame with a 6x histidine tag was transformed into *E. coli* DH5α. The positive clones with the NS1 gene were screened for the presence of the NS1 gene using PCR. When the selected clone was cultured and the protein expression was induced by IPTG, the protein of approximately 26 kDa was presented after induction for an hour, the first observed time point. The size of the protein corresponded to the NS1 protein. Yield of the protein increased with time, with the highest level of protein expression at four hours post IPTG induction. Therefore, the NS1 protein was collected at four hours for further purification.

The results of NS1 protein expression are shown in Figure 2. The band of 26 kDa was observed in the *E. coli* extracts containing recombinant NS1 gene after induced with IPTG (Figure 2 lane 6-7). This particular band was not found in the negative controls, *E.coli* containing only the expression vector, pQE80L, and non-induced *E.coli* containing plasmid pQE80L-NS1 (Figure 2 lane 1-4). The results showed that the NS1 fusion protein has been successfully expressed in *E. coli* strain DH5α.

Purification of the recombinant NS1 protein

The recombinant NS1 protein was purified using the three different methods. The results of the three different purifications are depicted in Figure 3. The crude extracts lysed in the denaturing lysis buffer and purified using a nickel affinity column, Ni-IDA purification system, under denaturing condition yielded the greatest amount of NS1 protein (142.06 μg/ml) and contained less amount of host proteins. However, the protein purified under native

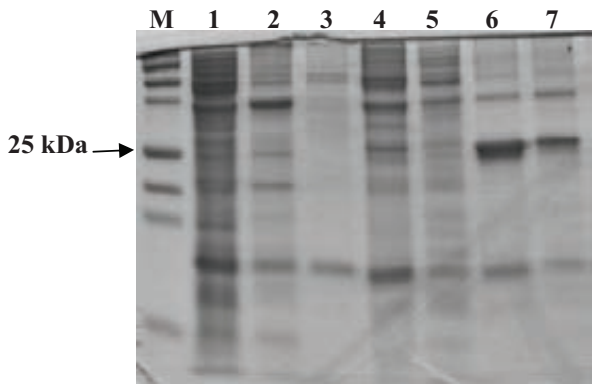

Figure 2 SDS-PAGE analyses of proteins produced in *E. coli* strain DH5α. M is the protein marker. Lanes 1 and 2 are crude extracts of pQE80L transformed *E. coli* after being induced by IPTG under native and denaturing conditions, respectively. Lanes 3 and 4 contain proteins extracted from pQE80L-NS1 transformed *E. coli* without IPTG induction under native and denaturing conditions, respectively. Lanes 5 and 6 shows the 26 kDa recombinant NS1 proteins from IPTG-induced pQE80L-NS1 transformed *E. coli* under native and denaturing conditions, respectively. Lane 7 contains the proteins similar to those in lane 5 and 6 but it was lysed twice under native conditions.

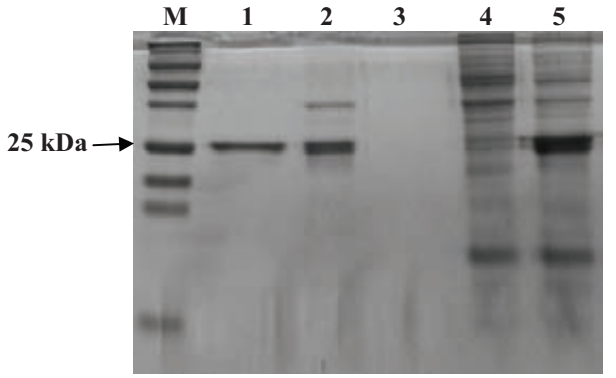

Figure 3 SDS-PAGE analysis of the NS1 protein purified using the three methods. M is the protein marker. Lane 1 is the NS1 protein purified by immobilization on a nickel affinity column under denaturing conditions. Lane 2 is the NS1 protein purified by the precipitation method. Lane 3 is the protein lysed twice using native lysis buffer and purified using histidine affinity chromatography. Lane 4 and 5 are crude extracts from pQE80L-NS1 transformed *E. coli* without IPTG induction and after induced by IPTG under denaturing conditions, respectively.

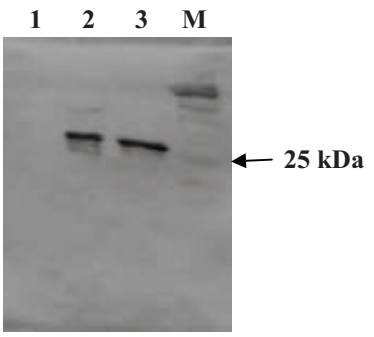

Figure 4 Western blot demonstrating the reactivity between the protein purified from *E. coli* containing pQE80L/NS1 and anti-histidine monoclonal antibody. M is the protein marker. Lane 1 is the protein extracted under native conditions, lane 2 is the protein purified by the precipitation method, and lane 3 is the protein extracted under denaturing conditions. The proteins of 26 kDa appears in lanes 1-3 are the NS1 fusion proteins.

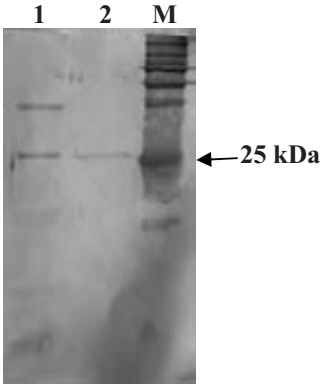

Figure 5 Western blot demonstrating the reactivity of the protein purified from *E. coli* containing pQE80L/NS1 and the NS1 specific chicken serum. M is the protein marker. Lane 1 is the protein purified using the precipitation method and lanes 2 is the denatured NS1 protein purified by immobilization on the nickel resin.

condition resulted in a very low amount of NS1 protein (96.33 μg/ml) (Figure 3 lane 5). The amount of NS1 protein purified by the precipitation method (163.92 μg/ml) was less than that purified under denatured conditions and containeds a small amount of *E. coli* protein. Note that the amount of the precipitated protein also included the contaminated host proteins.

Western blot

Western blots using a monoclonal antibody to the histidine tag were carried out to confirm the expression of the fusion protein. The expressed protein reacted strongly with anti-His monoclonal antibody (Figure 4) indicating that it was the histidine fused protein. In addition, the protein also reacted specifically with antibodies raised against the NS1 protein (Figure 5). This confirmed that the expressed protein was the NS1 protein.

DISCUSSION

This study successfully expressed and purified the 6x His-Tag-NS1 fusion proteins in *E. coli*. High level expression of the recombinant NS1 fusion protein was obtained mainly in the form of inclusion bodies. Proteins in the inclusion bodies were considered to have several advantages such as a high degree of purity of the protein in the aggregate fraction and protection from proteolytic degradation. The protein aggregates in the inclusion bodies could be easily purified from a large amount of contaminating soluble bacterial protein. To remove contaminating soluble cytoplasmic protein, the cell pellet was washed with a mild detergent buffer before the inclusion bodies were disrupted with high salts and detergent -strength buffer. The majority of the expressed protein was in the inclusion bodies and thus, the protein extracted using denaturing conditions yielded a larger amount. However, the denatured NS1 protein may lack biological activity unless it is re-natured and refolded.

Although the protein had undergone extraction several times using a high concentration of detergents under native conditions, the majority of proteins in the extracts were not NS1. These host-contaminated proteins interfered with the binding between the chelated nickel and the NS1 protein. In addition, the NS1 protein may not bind to the chelated nickel (Gaberc-Poreker and Menart, 2001), resulting in a great reduction in the NS1 yield. These reasons led this study to find another way of purification, to increase the amount of the protein while retaining its the native form. Therefore, the protein extracts were further purified using the protein precipitation method. Although the procedure was somewhat tedious, it gave rise to significant amount of native NS1 protein with a small amount of the contaminated host protein. This study also showed that the protein reacted specifically with the chicken sera containing antibodies specific to the NS1 protein. This implied that the NS1 proteins produced in this study still maintained some epitopes which were able to bind to the NS1 specific antibodies in the chicken serum. Thus, the NS1 protein may be used to develop monoclonal antibodies to NS1 and also diagnostic tools for the detection of the avian influenza virus.

In general, most recombinant proteins used in diagnostic ELISA are synthesized in either bacterial or insect cells. The recombinant proteins produced in *E.coli* are not post-transitionally modified. However, in some case, it may be a more effective antigen than the recombinant protein produced in insect cell systems (Spencer *et al*., 2007). Previous studies demonstrated that the NS1 protein produced in *E. coli* still maintained some function and immunogen, although the recombinant proteins were not post-transitionally modified. The modification of the protein may have been different from that which occured in the native infected cells and may have affected the quality of the antigen. For example, the mutant

of the porcine reproductive and respiratory syndrome virus, that caused a loss of glycan residues and N-linked glycosylation in the ectodomain of GP5 proteins, enhanced the sensitivity to neutralization and the immunogenicity of the nearby neutralizing epitope (Israrul *et al*., 2006). In this study, the NS1 protein expressed in *E. coli* still maintained its immunogenicity. However, it was not known if lack of modification in the NS1 protein would enhance its serological activities.

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