The HN protein is one of the two surface glycoproteins of NDV (Yusoff and Tan, 2001). It is a multifunctional protein and plays a key role in the infection. HN protein recognizes sialic acid on the receptor of host cells, what leads to the virus attachment. Also, it contains neuraminidase activity that is involved in hydrolysis of progeny's sialic acid to prevent self-agglutination. Finally, the HN protein together with the fusion protein supports a viral penetration process and determines tropism and virulence of the virus (Huang et al., 2004). The HN is highly antigenic and together with the fusion protein stimulates the production of protective antibodies (Yusoff and Tan, 2001). Studies using monoclonal antibodies revealed a presence of various epitopes within the HN protein (Long et al., 1986). The hemagglutination-inhibition test based on the HN protein is widely employed in the diagnosis of NDV infection (Alexander, 1989).

The HN protein has become a dominant target in many immunological studies. Advances in recombinant DNA technology led to the intensive studies of HN protein using various expression systems. The HN gene was cloned into Vaccinia virus (Nishino et al., 1991), Fowlpox virus (Boursnell et al., 1990; Edbauer et al., 1990; Iritani et al., 1991), retrovirus (Morrison et al., 1990; Cosset et al., 1991), herpesvirus of turkey (Heckert et al., 1996), baculovirus (Nagy et al., 1991; Niikura et al., 1991; Ong et al., 1999), Cytomegalovirus (Loke et al., 2005), and plant (Berinstein et al., 2005) recombinant vectors.

However, there is a relatively limited knowledge about the immunological properties of the recombinant HN protein.
tein produced in _Escherichia coli_. The rapid growth of this bacterium provided in simple and inexpensive culture media has made an advantage over eukaryotic systems. Numerous viral proteins used in immunological studies were produced in _E. coli_ as hemagglutinin of influenza virus (Davis _et al._, 1981), glycoprotein of Rabies virus (Yelverton _et al._, 1983), gC glycoprotein of Varicella-zoster virus (Ellis _et al._, 1985), gC-II glycoprotein of Human cytomegalovirus (Kari _et al._, 1994), Pseudorabies virus glycoprotein gE (Ro _et al._, 1995), and glycoproteins (gC, gE, and gp60) of Infectious laryngotracheitis virus (Chang _et al._, 2002).

To investigate the immunogenicity of the recombinant HN protein in chickens, we cloned and expressed the HN gene using the _E. coli_ expression system. We produced the insoluble and soluble forms of the recombinant HN proteins through the pRSET and pET-43.1a vectors and the purified recombinant proteins were used to immunize chickens. We examined the production of antibodies against the recombinant HN proteins using ELISA and immunoblot analysis. We found that the recombinant HN proteins triggered significant antibody titers with reactivity toward the authentic and recombinant HN proteins.

### Materials and Methods

**Bacterial strains and plasmids.** _E. coli_ TOP10 [F mcrA Δ(mrr-hsdSABC-mcrBC) ΔlacZΔM15 ΔproAΔproC ΔproE ΔproK ΔlacY1 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG; Invitrogen] was used as a general host for plasmid propagation. _E. coli_ BL21-SI [F- ompT hsdSB (rB-mB-) gal dcm endA1 proU::T7 RNAP::malQ-lacZ TTrap; Gibco] is a salt-inducible host for expression of the pRSETA/HN’ plasmid construct. _E. coli_ Origami B(DE3) [F ompT hsdSB (rB-mB-) gal dcm lacY1 ahpC gmr522::Tn10 (TcR) trxB::kan (DE3); Novagen] was employed to harbor the pET-43.1a/HN’ expression construct. The pPICZαA (Invitrogen) and the pCITE2a (Novagen) are plasmid yeast and _in vitro_ expression vector, respectively. The pRSETA vector (Invitrogen) harbors the T7 promoter and an N-terminal hexa-histidine (His) detection/affinity tag. The pET-43.1a vector (Novagen) carries the T7 promoter, N-terminal (His), tag, and NusA solubility carrier. _E. coli_ cells were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 µg/ml) with the exception that _E. coli_ BL21-SI was cultured in NaCl-free LB medium.

**Cloning of HN gene.** Oligonucleotides 5’-GAAGAAATTCAT GGAGGGTACACACACCGGAGCACAA-3’ (forward) and 5’-GATAGGTTAC CTGACTCAAACCCTCCAGATCT-3’ (reverse) were designed based on the nucleotide sequence of NDV strain AF2240 (Tan _et al._, 1995). The underlined nucleotides represent the cutting sites of
EcoRI and KpnI, respectively. The HN gene (nt 142–1739; Fig. 1) was amplified from the viral genome using RT-PCR. The reaction conditions were 48°C for 15 mins, 94°C for 2 mins, 30 cycles of 94°C for 30 secs, 55°C for 30 mins, and 68°C for 30 secs. The PCR products were cloned into pPICZαA and pCITE2a plasmids and inserted between the BamHI-KpnI restriction sites of pRSETA vector to produce the pRSETA/HN' plasmid. The plasmid was transformed into E. coli Origami B(DE3) harboring the pRSETA/HN. The HN protein was expressed in E. coli Origami B(DE3) harboring the pRSETA/HN' plasmid (Fig. 1).

Expression of recombinant HN protein. The pRSETA/HN' and pET43.1a/HN' plasmids were used to transform E. coli BL21-SI and Origami B(DE3), respectively, by heat shock method (Sambruk et al., 1989). Expression of the recombinant HN proteins was induced when cell density reached by A600 reached 0.6–0.8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mmol/l) and NaCl (0.3 mol/l) were added as inducers for E. coli Origami B(DE3) and BL21-SI, respectively. The expression cultures were incubated at 30°C for 3 hrs for BL21-SI, and at 37°C for 6 hrs for Origami B(DE3).

Purification of recombinant HN proteins. The expression cultures were harvested and resuspended in 1/25 volume of lysis buffer (20 mmol/l Na2PO4, 0.1 mol/l NaCl, pH 7.4). Cells were lysed by lysozyme (1 mg/ml of cells) at 4°C for 1 hr followed by sonication at power output of 40 W (Braun) for 10 cycles of 30 secs. The lystate was spun at 2,500 g for 5 mins to remove cell debris followed by centrifugation at 20,000 g at 4°C for 20 mins. The supernatant contained soluble proteins, while the insoluble proteins remained in the pellet.

The soluble (His)6-tagged NusA-HN protein was precipitated with ammonium sulfate at 30% saturation. The pellet was dialyzed twice against phosphate buffer (20 mmol/l Na2PO4, 0.1 mol/l NaCl, pH 7.6) at 4°C for 3 hrs prior to purification with immobilized metal affinity chromatography (IMAC) using ProBond + resin (Invitrogen). The bound recombinant protein was eluted from the column with PBS (4.3 mmol/l Na2HPO4, 1.4 mmol/l KH2PO4, 137 mmol/l NaCl, 2.7 mmol/l KCl, pH 7.4) containing imidazole (0.1 mol/l). The inclusion bodies containing the recombinant HN protein were extracted with increasing concentrations 0.1, 0.2, 0.5, and 1% of Triton X-100 made in PBS. The inclusion bodies were washed twice in PBS (5 ml) to remove excess of the detergent and recovered by centrifugation at 20,000 g at 4°C for 15 mins.

Total protein assay. The amount of NusA-HN protein was measured by the Bradford assay (Bradford, 1976). The insoluble recombinant HN protein was analyzed with SDS-PAGE. The protein bands were stained with Coomassie Brilliant Blue and analyzed in the BioRad imaging system using the Quantity One software (Version 4.2.2). Bovine serum albumin was used as a standard protein.

Immuno blot analysis. The recombinant HN, NusA-HN proteins and NDV (0.1–0.5 μg) were analyzed by SDS-PAGE and blotted onto nitrocellulose membranes. Skim milk (5% in PBS) was used as a blocking agent. The blot was probed with chicken serum samples (e.g. antibodies against NDV, recombinant HN and NusA-HN proteins) at 1:500–1:1,000 dilutions in PBS at room temperature for 1 hr. The blot was washed three times in PBS containing Tween 20 (0.01%) followed by incubation with goat anti-chicken IgG conjugated to alkaline phosphatase (1:5,000 dilution in PBS) for 1 hr at room temperature. Color development was achieved by adding nitroblue tetrazolium (66 μl) and bromochloroindolyl phosphate (33 μl) in the alkaline phosphatase buffer (100 mmol/l Tris, 100 mmol/l NaCl, 5 mmol/l MgCl2, pH 9.5).

Results and Discussion

Cloning, expression, and purification of recombinant HN proteins

The gene portion (nt 141–1739) encoding the ectodomain (aa 47–580) of the HN protein was amplified and cloned (Fig. 1). The recombinant HN protein was expressed in E. coli BL21-SI harboring the pRSETA/HN. The HN protein was observed as a band with Mw of 65 K in SDS-PAGE (Fig. 2).

SDS-PAGE of different fractions obtained during purification of recombinant HN protein

Protein size markers (M), total bacterial lysate (1), soluble protein fraction (2), insoluble protein fraction (3), proteins extracted with 0.1, 0.2, 0.5, and 1% Triton X-100 (4, 5, 6, 7, respectively). The arrows indicate the HN protein.
It was extensively produced at the expression level of 6.7% of the total host cell protein. The recombinant HN protein was found in the pellet of the bacterial cell lysate (Fig. 2) indicating that HN protein was expressed in insoluble aggregates or deposits known as inclusion bodies (Marston, 1986). Formation of inclusion bodies is a common obstacle in preserving the native structure and biological function of the recombinant protein. However, these insoluble protein aggregates have an advantage in the purification process, since they can be easily isolated and enriched with centrifugation and in addition, they are protected from a proteolysis (Rudolph and Lilie, 1996; Jonasson et al., 2002). However, the inclusion bodies containing recombinant protein are often contaminated with other bacterial membrane proteins such as OmpF, OmpC, and OmpA (Rinas and Bailey, 1992). Therefore, we extracted the preparation of insoluble HN protein with solutions of Triton X-100 in different concentrations, what successfully removed most of the bacterial proteins (Fig. 3).

*E. coli* Origami B(DE3) cells transformed with the pET-43.1a/HN plasmids expressed the NusA-HN fusion protein. The NusA-HN fusion protein (M_r = 125 K) was produced at the level of 4.2% of the total cell protein (Fig. 4). Unlike the recombinant HN protein, the NusA-HN fusion protein was expressed in bacterial host as a soluble protein. The pET-43.1a vector contains an NusA gene at the 5’-end of its multiple cloning sites. The product of the gene, NusA protein has been identified as the highly soluble protein with a potential to improve solubility of a protein fused to its C-terminus (Davis et al., 1999). Fusion of the HN polypeptide to the C-terminus of NusA protein led to the expression of NusA-HN protein that was present in the soluble fraction of the bacterial cell lysate. The soluble NusA-HN fusion protein contained a (His)_6 tag that interacted with a bivalent ions such as Ni^{2+}. Unlike the insoluble recombinant HN protein, which was contaminated with relatively low number of the host proteins, the crude preparation of NusA-HN protein contained a quantity of bacterial cytoplasmic proteins. Hence, a differential precipitation with ammonium sulfate was carried out to fractionate this mixture. The procedure was useful for removal of a portion of the soluble protein contaminants that might bind non-specifically to the Ni^{2+}.
resin and decrease the efficiency of IMAC purification process. The NusA-HN protein was efficiently eluted from the column by 0.1 mol/l of imidazole. However, multiple bands were detected below the band of full-length NusA-HN protein suggesting that the expressed protein was partially degraded (Fig. 4). A degradation of the expressed protein might be attributed to the absence of glycosylation due to the protein expression in a prokaryotic system. It is known that the oligosaccharide chains protect the native glycoproteins against proteolysis (Olden et al., 1982; Rudd et al., 1994).

**Immunogenicity of recombinant HN proteins**

The IDEXX Newcastle disease antibody test kit quoted that the antibody titers beyond 396 (or log >2.60) indicated an exposure to NDV or a vaccination of the tested birds. The ELISA results showed that all chickens immunized with the recombinant HN and NusA-HN proteins showed positive antibody titers (Fig. 5). The control birds injected with PBS or the NusA protein gave insignificant antibody titers (Fig. 5). None of the pre-immunization serum samples tested positive for the anti-NDV antibodies. It was observed that the insoluble recombinant HN protein elicited a more rapid and stronger immune response than the soluble NusA-

**Fig. 5**

Serum antibody titers in chickens immunized with recombinant HN and NusA-HN proteins

PBS and NusA were used for preparation of control antisera. x-axis: days of immunization; \-------- cut off line

HN protein. Chickens immunized with the recombinant HN protein showed significant antibody titers 2 weeks

**Fig. 6**

**Immunoblot analysis**

The blotted antigens represent recombinant HN protein (A), recombinant NusA-HN protein (B), and purified NDV (C, D). Sera of chickens immunized with NDV (A, B), recombinant HN protein (C), and recombinant NusA-HN protein (D).
after the primary injection. The antibody titers had doubled even after the first injection of the recombinant HN protein (log = 1.77–3.41). After a booster injection the titers went up steadily to log = 3.74. In contrast, chickens injected with the soluble NusA-HN protein did not yield positive titers until the booster injection was given. The antibody titers increased slightly from log = 1.23–1.70 after the first injection of the NusA-HN protein. After the second injection, the titers increased nearly 2-fold to a significant level of log = 3.27. However, the soluble antigen could have influenced the immune response of the chickens. The insoluble recombinant HN protein in inclusion bodies was in the state of protein aggregates that could increase the likelihood of effective T-cell epitope response and engage the antigen-processing cells (Hanly et al., 1995).

**Immunoblot analysis of recombinant HN proteins**

ELISA represents a quantitative analysis of antibodies against both continuous and discontinuous epitopes on the antigen. On the other hand, immunoblot analysis involves mainly reaction of continuous epitopes and corresponding antibodies. The NDV, HN and NusA-HN proteins were denatured by SDS and 2-mercaptoethanol, separated in SDS-PAGE, and electroblotted. The results demonstrated positive reactions between all examined chicken sera raised against NDV, HN and NusA-HN proteins and the corresponding blotted proteins. The anti sera against the recombinant HN and the NusA-HN proteins detected the authentic HN glycoprotein of the NDV (Fig. 6). Conversely, the anti-NDV serum reacted with both recombinant HN and the NusA-HN proteins detected the authentic HN glycoprotein of the NDV (Fig. 6). Several studies defined the nature of the epitopes present on the blotted HN protein. Most of them were continuous epitopes or epitopes able to regenerate by refolding after denaturation in SDS-PAGE (Russell et al., 1983; Long et al., 1986; Chambers et al., 1988; Yusoff et al., 1988). Furthermore, E. coli cells are devoid of any glycosylation machinery and are unable to add oligosaccharide chain to the recombinant HN and NusA-HN proteins. Therefore, binding of the antibodies to the recombinant proteins involved epitopes devoid of carbohydrates.

The detection of the authentic SDS-denatured NDV HN glycoprotein with the antibodies against the recombinant HN and NusA-HN proteins revealed the potential application of these antibodies as probes for the viral glycoprotein in immunoblot analysis. In addition, reactivity of the anti-NDV serum toward the denatured recombinant HN and NusA-HN proteins in immunoblotting opens the possible use of these recombinant proteins as antigens for diagnosis of Newcastle disease in poultry. The recombinant HN and NusA-HN proteins produced in E. coli may be used to substitute the conventional use of whole NDV as an antigen with advantages in terms of safety, cost, and technical convenience of production.

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