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Purification of the extra-cellular domain of Nipah virus glycoprotein produced in *Escherichia coli* and possible application in diagnosis

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Abstract

The glycoprotein (G) of Nipah virus (NiV) is important for virus infectivity and induction of the protective immunity. In this study, the extra-cellular domain of NiV G protein was fused with hexahistidine residues at its N-terminal end and expressed in *Escherichia coli*. The expression under transcriptional regulation of T7 promoter yielded insoluble protein aggregates in the form of inclusion bodies. The inclusion bodies were solubilized with 8 M urea and the protein was purified to homogeneity under denaturing conditions using nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography. The denatured protein was renatured by gradual removal of the urea. Light scattering analysis of the purified protein showed primarily monodispersity. The purified protein showed significant reactivity with the antibodies present in the sera of NiV-infected swine, as demonstrated in Western blot analysis and enzyme-linked immunosorbent assay (ELISA). Taken together, the data indicate the potential usefulness of the purified G protein for structural or functional studies and the development of immunoassay for detection of the NiV antibodies.

Keywords: NiV G protein; Protein purification; Inclusion body; ELISA

1. Introduction

Nipah virus (NiV) is a negative sense, nonsegmented RNA virus that was first isolated from cerebrospinal fluid of the human patients (Chua et al., 2000). It was found to be the etiologic agent for both human and swine diseases and classified to the family *Paramyxoviridae* under a new genus *Henipaviruses* (Chua et al., 2000; Mayo, 2002). Its genome encodes six structural proteins: nucleocapsid (N), phospho- (P), matrix- (M), fusion- (F), glyco- (G) and large- (L) proteins (Harcourt et al., 2000; Wang et al., 2001).

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The natural reservoir of the virus is fruit bat with no signs of infection but causes fatal encephalitis in humans and a respiratory syndrome in pigs (Chua et al., 2002). The infected pigs can transfer the virus to other animals such as dogs, cats, and horses (Chua et al., 1999, 2000; Paton et al., 1999). Most recently, Guillaume et al. (2004) found that destruction of the natural habitat of fruit bats caused the migration of these bats to live closer to humans and domesticated animals, and as a result the virus can be transferred to new species. Recent outbreaks of the NiV and Nipah-like viruses in Bangladesh (WHO, 2004a,b) demonstrate the existence of a time bomb that poses a major health problem worldwide, which could destroy the economies of many countries. Therefore, there is a demand for rapid detection as well as serological diagnosis of the virus for monitoring the presence of the virus and its antibodies in individuals and animals in high-risk areas. Currently, production of immunological reagents for these assays require biohazard level 4 (BL4) laboratories which are limited only to a few countries worldwide.

The G protein plays a central role in the viral replication process since it is responsible for the viral attachment to sialic acid-containing host cell receptors (Bossart et al., 2001). Thus, the G protein appears to be essential for paramyxovirus replication, and as such could represent the primary target for neutralizing antibodies as well as potential targets for antiviral agents.

In this study, the extra-cellular domain of the G protein of NiV was cloned and expressed in the *Escherichia coli* system. The purified product was used as the capturing antigen in an enzyme-linked immunosorbent assay (ELISA) to determine the presence of the anti-NiV antibodies in serum samples collected from naturally infected swine. It was found that the purified G protein reacted only with antibodies in NiV positive samples, suggesting a potential replacement for currently used whole virus antigen that requires containment facilities.

2. Materials and methods

Swine anti-NiV sera with known serum neutralizing test (SNT) and inactivated NiV from infected cell culture medium were generous gifts from the Veterinary Research Institute (VRI), Ipoh, Malaysia. A volume of 250 μl of NiV-infected cell culture medium was used to extract total vRNA using the TRIZOL LS reagent (Life Technologies, USA), as recommended by the manufacturer. Extracted vRNA was used as a template for cDNA synthesis using the Superscript II RNaseH (−) reverse transcriptase (Life Technologies, USA), which was subsequently used in a PCR amplification using the Platinum^{TM} high fidelity Taq DNA polymerase (Life Technologies, USA). Two synthetic oligonucleotides, TGNiV forward (5′-GGGGGATCC-ATGGACAATCAGGCCGTGATC-3′) and TGNiV reverse primers (5′-GGGGGGAAGCTTCTCAACCA-ATGATATGCACCA-3′) were used to amplify the coding sequence of the *NiV G* gene between nucleotides 231 and 1737. The underlined nucleotides represent *Bam*HI and *Hin*dIII cutting sites, respectively. These two primers were designed to eliminate the transmembrane and signal regions of the G protein of NiV. The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into the pRSET plasmid (Life Technologies, USA) that had been digested with the same restriction endonucleases. The resulting construction was transformed into *E. coli* strain BL21 SI (Life Technologies, USA). The expression was confirmed with SDS-PAGE and Western blot analysis before subjecting to purification and further analysis. Broad range protein markers (Gibco, BRL) were used in SDS-PAGE and Western blot analysis. Swine anti-NiV polyclonal antibodies (1/500 dilution) were used as the primary antibody. Appropriate species-specific immunoglobulin conjugated to alkaline phosphatase (1/5000 dilution) was used as the secondary antibody.

The recombinant protein was extracted from bacterial cells using the Bacterial Protein Extraction Reagent (B-PER) (Pierce, USA), as recommended by the manufacturer with some modifications. Briefly, bacterial cells from 250 ml culture were centrifuged at $3440 \times g$ for 10 min and the pellet was washed with PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.5) and centrifuged as above. The cells were resuspended in 20 ml of B-PER reagent and the mixture was gently shaken at RT for 2 h before centrifugation at $27{,}000 \times g$ for 20 min to separate inclusion bodies from soluble proteins. After repeating the above extraction for two times, the inclusion bodies were resuspended in 10 ml of B-PER reagent containing 200 μg/ml lysozyme (Sigma, USA), and the mixture was incubated at RT for 20 min before adding 100 ml

PBS. The inclusion bodies were collected by centrifugation at 27,000 × *g* for 20 min. The pellet was resuspended in 100 ml of B-PER reagent in PBS (1:10 dilution) and centrifuged as above. The extracted inclusion bodies were dissolved in 5 ml of denaturing buffer (6 M guanidine HCl; 20 mM $NaPO_4$, pH 7.8; 500 mM NaCl) and loaded onto a pre-equilibrated column packed with 5 ml of nickel–nitrilotriacetic acid (Ni–NTA) agarose (Life Technologies, USA). The protein-bound Ni–NTA resin was first washed with 10 ml of denaturing binding buffer (8 M urea; 20 mM $NaPO_4$, pH 7.8; 500 mM NaCl) followed by washing with 20 ml of denaturing wash buffer (8 M urea; 20 mM $NaPO_4$, pH 6.0; 500 mM NaCl). The column was washed with another 20 ml of the above washing buffer at pH 5. The bound recombinant protein was finally eluted with 10 ml denaturing elution buffer (8 M urea; 20 mM $NaPO_4$, pH 4.0; 500 mM NaCl) and the sample fractions were analyzed by SDS-PAGE and Western blotting. The fractions containing the purified recombinant protein were pooled and first dialyzed against 6 M urea for 6 h. The dialysis was continued by the addition of 250 ml of 25 mM Tris–HCl (pH 7.5) every 12 h for three times and finally the dialysis solution was replaced with 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% Triton X-100 and 5 mM β-mercaptoethanol, and dialyzed for another 12 h. The purity of the sample was studied with a dynamic light scattering (DLS) instrument (DynaPro-MS/X, Proterion Inc., UK) at 20 °C, and at least 20 measurements were taken at each experiment. All DLS measurements were performed at 824.8 nm wavelength laser at 90° angle.

The titers of the anti-G antibodies in swine sera were determined using ELISA. All washing steps were carried out three times with TTB [0.05% Tween-20 in TBS (50 mM Tris–HCl, 150 mM NaCl; pH 7.5)]. All antigen and antibody dilutions were done in TBS. Flat-bottom polystyrene microtiter plates were used as solid-phase adsorbents (TPP Immunomax high binding flat-bottomed ELISA plate, USA). The plates were first coated with anti-swine IgG (1/3000) (KPL, USA) for overnight at RT. After washing, the plates were blocked with 200 μl of SEA BLOCK blocking buffer (Pierce, USA) for 1 h at RT. Subsequently, the plates were washed and incubated for 2 h at RT with appropriate dilution (1/100) of the swine sera from infected and non-infected animals. Following another round of washing, 100 μl of optimum concentration (100 ng/well) of the recombinant G protein in TBS was added in the wells and the plates were incubated at RT for further 1 h. After washing with TTB, the appropriate dilution (1/3000) of mouse anti-His (Amersham Biosciences, UK) was added. Following 1 h of incubation at RT, the plates were washed and 100 μl of the anti-mouse IgG alkaline phosphatase (KPL, USA) was added and the microtiter plates were incubated further for 1 h at RT. Subsequently, the enzyme–substrate solution, containing 1 mg/ml PNPP (*p*-Nitrophenyl phosphate, Sigma, USA) in 0.1 M diethanolamine (Sigma, USA), pH 10.3 was added. The reaction was stopped after 30 min at RT by the addition of 50 μl of 1N NaOH to each well and the A_{405} values were measured with a microtiter plate reader (Bio Rad, USA).

3. Results and discussion

The extra-cellular domain of the *NiV G* gene was cloned downstream of a T7 promoter in the pRSET vector system, which allows the expression proteins via transcription by T7 polymerase. *NiV G* gene was expressed as a protein attached at its amino-terminus to a polyhistidine peptide. As shown in Fig. 1A, an extra protein band with the expected M_r of approximately 57 kDa appeared in the insoluble fraction of the extract of bacteria containing the recombinant plasmid and was absent in the corresponding fraction of bacteria containing the vector without an insert. The time course study showed that the highest level of expression could be achieved after 5 h post induction (data not shown). An immunoreactive band of approximately 57 kDa was detected by the pooled anti-NiV sera on a Western blot (Fig. 1B), confirming the expression of the recombinant G protein.

The inclusion bodies, which were extracted from bacterial cells using B-PER reagent, were washed several times with the same reagent to remove most of the contaminants. Two reasonably pure protein bands of approximately 57 and 47 kDa were observed in a polyacrylamide gel stained with Coomassie blue (Fig. 1A). The lower band was detected with swine anti-Nipah sera but not with anti-His (data not shown), indicating that it could be either a bacterial protein or His-tag deleted proteolytic product derived from the recombinant protein. The extracted inclusion bodies were

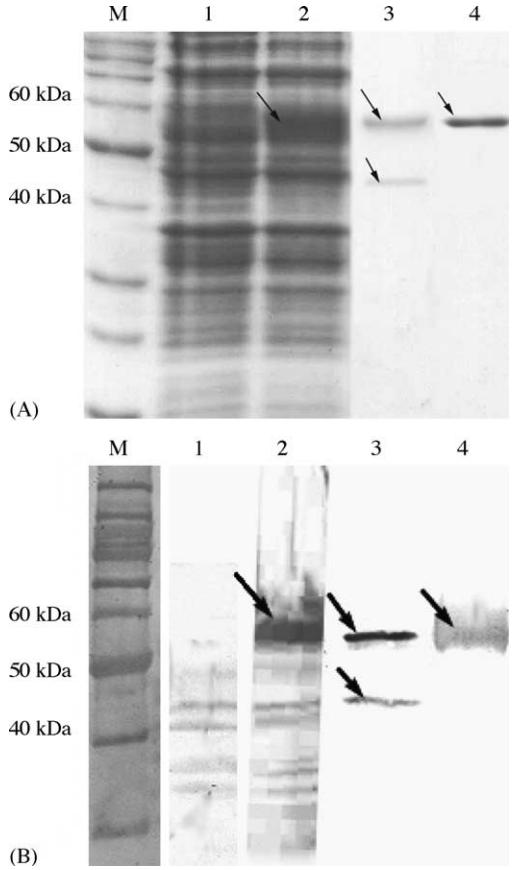

Fig. 1. (A) SDS-PAGE of bacterial extract after 5 h induction with 0.3 M NaCl after 5 h induction. (1) Cells containing the vector without an insert, (2) recombinant G before purification, (3) purified inclusion bodies, (4) purified by affinity chromatography. (B) Western blot of the above samples using swine anti-NiV sera. The same number of the bacterial cells was used for the first two lanes.

solubilized in 6 M guanidine HCl containing buffer and directly purified on Ni–NTA matrix under denaturing conditions. Subsequently, the urea was gradually eliminated from the purified protein by dialysis and the protein renatured again. The concentration of the purified recombinant G protein from the total bacterial cell proteins was estimated to be approximately 0.01–0.03 mg g^{-1} of the wet weight of bacterial cells.

Light scattering of the purified protein showed monodispersity with a hydrodynamic radius (R_h) of 14 nm, whereas the inclusion bodies were found to be polydispersed, and polymodal regression analysis indicated 98% of one species with R_h 200 nm and 2% of the another species with R_h 1700 nm.

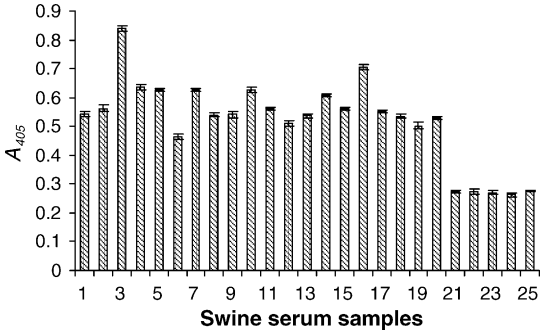

Fig. 2. Reactivity of a panel of 25 swine sera in sandwich ELISA test based on plates coated with purified recombinant G protein (1–20: positive serum samples; 21–25: negative serum samples).

In order to determine the specificity and sensitivity of the recombinant G protein as antigen for detecting antibodies in NiV positive swine sera a quantitive immunoassay was carried out. A total of 20 NiV positive sera and 5 negative sera from healthy swine were tested, and the results showed that the purified G protein reacted specifically with antibodies in NiV positive samples (Fig. 2). This reflects the reliability of the purification procedure in eliminating bacterial contaminants from the G protein.

Inactivated NiV is currently being used as a coating antigen in ELISA for detection of the anti-NiV antibodies in serum samples. The process of growing the virus in cell cultures, however, requires BL4 laboratories. In addition, the use of NiV is restricted in certain countries due to biosecurity considerations. Therefore, an ELISA based on a recombinant protein, as capturing antigen, is simpler to produce and perform than ELISA based on inactivated virus. The latter is hazardous and not suitable for large-scale surveys.

In order to assess the suitability of the G protein as an ELISA antigen for the detection of specific anti-NiV antibodies, a recombinant pRSET vector was constructed, which could be used to express the recombinant G protein in *E. coli* BL21 SI. Production of such recombinant proteins in *E. coli* as capturing antigens for diagnosis of diseases caused by viruses have been widely used including turkey coronavirus (Loa et al., 2004), Marburg virus (Saijo et al., 2001), Norwalk-like virus (Yoda et al., 2000), measles virus (Warnes

et al., 1995), African fever swine virus (Freije et al., 1993), Lassa virus (Barber et al., 1987), and hepatitis B virus (Stahl et al., 1982). Furthermore, the advantages of using a prokaryotic host to produce recombinant G protein would be considerable due to the ease of scale-up, and the low costs involved in growing bacteria.

A strong specific signal was observed in Western blots confirming the specific immunoreactivity of the recombinant G protein. The results of the Western blot analysis and ELISA test suggest that the recombinant G protein exhibits the antigenic epitopes and conformation necessary for specific antigen–antibody recognition. The inclusion bodies before purification showed negligibly low background on Western blot but when used as the coating antigen in ELISA, a relatively high background was observed. This was probably either due to the presence of bacterial protein (s) and/or existence of some antibodies recognizing conformational epitopes in ELISA. The results of the light scattering also confirmed the impurity of the inclusion body sample and monodispersity of the purified recombinant protein. Based on the molecular mass of the recombinant G protein (57 kDa) and the assumption that it is spherical form, the calculated radius of the protein is about 3.4 whereas the R_h of the purified protein detected by DLS was about 14 nm. This implies that the protein most probably exists in multimer forms (trimer or tetramer).

In order to ensure a more consistent binding pattern of the coated antigen to the ELISA plate and to avoid the high background, it was decided to use affinity-purified recombinant G protein as the coating antigen. The validation of the recombinant G protein ELISA was carried out using 25 samples of swine sera. The aim was to determine the variability of negative and positive sera using ELISA based on recombinant G protein. The newly established assay was able to distinguish clearly the two groups of samples based on their reactivity. However, the result of the ELISA should be interpreted cautiously, since proper standardization of the ELISA requires sera from experimentally infected animals followed by testing a more significant number of field serum samples. Unfortunately, it was not possible to obtain sera from experimentally infected animal sera due to lack of the BL4 laboratory throughout this study and also to the limited availability of the field sera.

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