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Short communication

Enhancement of the yield of long helical structure of recombinant nucleocapsid protein of Newcastle disease virus

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ABSTRACT

A deletion mutant of the nucleocapsid protein ($NP_{\Delta c375}$) of Newcastle disease virus self-assembles into a long helical structure when expressed in *Escherichia coli*. However, the $NP_{\Delta c375}$ subjects to proteolytic activity of host cell endogenous proteases during the protein recovery process. Image analysis of Western blots using the Quantity One software was performed to identify the size of the degraded bands and hence the potential proteases cleavage sites were predicted. The data obtained from this image analysis were compared to those identified with the PeptideCutter program; the potential proteases that degrade the $NP_{\Delta c375}$ were identified to be mainly the metallo and serine proteases. Combination of ethylenediaminetetraacetic acid and phenylmethylsulfonyl fluoride at their optimal concentration gave a synergistic effect and increased the $NP_{\Delta c375}$ yield by 2.1-fold. The antigenicity and self-assembled long helical structure long helical structure of $NP_{\Delta c375}$ were preserved after treatment with the protease inhibitors.

1. Introduction

Newcastle disease virus (NDV) is the prototype of paramyxoviruses that causes diseases in many avian species [1]. The viral RNA genome is about 15 kb in size which encodes six structural proteins: hemagglutinin-neuraminidase (HN), nucleocapsid (N), fusion (F), large (L), matrix (M) and phospho- (P) proteins [1,2]. The N protein of NDV involved in the viral replication [1]. It is constituted of 489 amino acids with a molecular mass of approximately 53 kDa. The recombinant N protein of NDV expressed in *Escherichia coli* (*E. coli*) formed virus-like particle (VLP) [3]. It has been exploited as a biocarrier for subunit vaccine development [4,5] and as a diagnostic reagent [6]. The full-length N protein assembles into ring-like and short helical structures, but its C-terminal end is prone to proteolytic digestion [3]. Interestingly, when the C-terminal region of 114 residues was deleted, the mutant, namely $NP_{\Delta c375}$, assembles into long helical structure [7]. Although the $NP_{\Delta c375}$ is produced easily by *E. coli*, the product was found to degrade easily during the purification process. It is postulated that the degradation of $NP_{\Delta c375}$ is due to the endogenous proteases that co-released during cell disruption process. Therefore the aim of this study was to identify the potential endogenous proteases that cause the degradation of $NP_{\Delta c375}$ with a bioinformatics tool and the application of suitable protease inhibitors to inhibit its proteolytic activities.

Proteases, a protein hydrolysis enzyme [8] can be categorized into six major groups based on their catalytic mechanisms: serine, aspartic, metallo, cysteine, threonine and glutamic proteases [9]. They play a prominent role in regulating cell homeostasis. Besides, proteases are elementary to the life cycles of pathogenic viruses and bacteria, particularly in their infection processes [8]. Proteases have therefore become important targets for therapeutic intervention to combat virus infections, for instance, HIV protease has been targeted to block the propagation of the virus [10].

Protease inhibitors are molecules that present naturally in many organisms [11]. They prevent proteins from proteolytic digestion triggered by particular proteases that are released during cellular structure disruption. Generally, a protease inhibitor forms a stable complex with its target protease by interacting at the catalytic region and consequently inactivates the protease [12]. Some of the protease inhibitors are chelating agents which act by lowering the concentration of metals until they are eliminated from the active sites of proteases and thus inactivate their activity. For instance, ethylenediaminetetraacetic acid (EDTA) is a type of metalloprotease inhibitor [13]. Other inhibitors may act by alter an amino acid residue at the active domain of a protease. For example, phenylmethylsulfonyl fluoride (PMSF) inactivates serine protease by reacting with the serine [14].

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Recombinant proteins produced in *E. coli* are often recognized by the cells as foreign proteins and therefore they are a burden to the cell metabolism. As a consequence, these recombinant proteins are susceptible to host cell's endogenous proteolytic activity [15]. Endogenous proteolytic activities of the host cells that degrade recombinant proteins reduce the yields of the synthesized protein significantly and the degraded products might complicate the subsequent protein purification process. The identification of the potential proteases and the inhibition of its proteolytic activities are essential to improve the yield of the target protein and simplify the downstream processing.

2. Materials and methods

2.1. Identification of potential proteases and their possible cleavage sites

The PeptideCutter program (http://www.expasy.ch/tools/peptidecutter) was employed to predict the possible proteases that degrade the recombinant $NP_{\Delta c375}$ and their possible cutting sites. $NP_{\Delta c375}$ is a deletion mutant of the full-length N protein of NDV. It was constructed by deleting 114 amino acid residues from the C-terminal end of the full-length N protein. The amino acid sequence of the full-length N protein of NDV (GenBank ID: AF284646) obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) was used as a reference to obtain the amino acid sequence of the $NP_{\Delta c375}$.

2.2. Protease inhibitor

Protease inhibitors, PMSF (0.5, 1.0, 1.5, and 2.0 mM; Calbiochem, Germany) and EDTA (25, 50, and 75 mM; Vivantis Technologies, Malaysia) were employed in this study to inhibit the *E. coli* endogenous protease activity.

2.3. Cultivation of E. coli

E. coli cells harboring the plasmid containing the $NP_{\Delta c375}$ gene was cultivated in Luria–Bertani broth [10% (w/v) tryptone, 5% (w/v) yeast extract and 5% (w/v) NaCl; pH 7.0] at 37 °C by agitating at 250 rpm as described by Kho et al. [3]. At the end of the fermentation, the *E. coli* biomass was collected by centrifugation at 3840 × *g* for 10 min (Kubota 6500, Tokyo, Japan).

2.4. Modulation of proteolytic degradation of the recombinant N protein of NDV

The cell pellet harvested from the culture was resuspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 4 mM $MgCl_2$, 0.1% (v/v) Triton X-100 and 0.2 mg/ml lysozyme] to yield a 10% (w/v) cell suspension [3]. The cell suspension was added immediately with different concentrations of protease inhibitor(s) stated in Section 2.2. The cells were lysed by ultrasonication (Vibra-Cell™ VCX 130, Sonics, USA) [16] for 2 min with 5 s intervals between pulses. The cell lysate was clarified at 12,100 × *g* (Kubota 6500, Tokyo, Japan) for 20 min [17]. The clarified supernatant was first subjected to ammonium sulfate precipitation from 0 to 20% saturation, followed by 20–30% saturation. Dialysis was performed in dialysis buffer (50 mM Tris and 100 mM NaCl; pH 8.0) to remove the salts. Dialyzed samples were then collected for protein analysis and quantification.

2.5. Sucrose gradient ultracentrifugation

After the modulation study, purification of $NP_{\Delta c375}$ was performed as described by Tan et al. [17]. The dialyzed sample supplemented with optimal concentration of protease inhibitor(s) was purified by 10–50% (w/v) sucrose gradient ultracentrifugation at 247,000 × *g* (SW 41 Ti rotor, Beckman, USA), 4 °C for 5 h. The collected purified fractions were analyzed by using SDS-PAGE, Western blot analysis, ELISA and electron microscopy.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins were separated by using 12% SDS–PAGE as described by Laemmli [18]. Western blotting was performed as described by Chong et al. [19]. The primary antibodies used were anti-*myc* antibody conjugated to alkaline phosphatase (1:2000 dilution; Invitrogen, USA) to detect the intact $NP_{\Delta c375}$ and rabbit anti-N protein antisera (1:5000 dilution; Genetic Improvement and Farm Technologies, Malaysia) to detect the $NP_{\Delta c375}$.

2.7. Protein quantitation

The adjusted volume of intact $NP_{\Delta c375}$ (45 kDa) was quantitated from Western blots (WB) with an imaging system (Gel Doc™ XR+ System, Bio-Rad, USA) supported by the Quantity One® Quantitation software (Bio-Rad, USA) as described by Tey et al. [20]. The relative concentration of intact $NP_{\Delta c375}$ (45 kDa) was calculated based on the following equation:

$$\text{Relative concentration of } NP_{\Delta c375} = \frac{\text{Adjusted volume of } NP_{\Delta c375} \text{ from WB (with protease inhibitor)(intensity} \times \text{mm}^2)}{\text{Adjusted volume of } NP_{\Delta c375} \text{ from WB(control)(intensity} \times \text{mm}^2)} \quad (1)$$

2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described by Tan et al. [21]. U-shaped high binding microtiter plate wells were first coated with purified $NP_{\Delta c375}$ (1–50,000 ng, in triplicate) and incubated at 4 °C for overnight. The wells were then blocked with BSA [0.5% (w/v)] for 2 h at room temperature followed by incubation with primary antibody, chicken anti-NDV antibody (1:1000 dilution) for 1 h. The wells were washed 3 times with TBS-T [50 mM Tris–HCl (pH 7.6), 150 mM NaCl and 0.1% (v/v) Tween 20] before incubating with goat anti-chicken antibody conjugated to alkaline phosphatase (1:2500 dilution; Kirkegaard and Perry Lab., Gaithersburg, USA) for 1 h. The wells were then washed with TBS-T buffer. Substrate solution containing p-nitrophenyl phosphate (1 mg/ml; Sigma, USA) in diethanolamine (0.1 M; pH 9.8; Sigma, USA) was added and the reaction was terminated after 15 min of incubation by adding NaOH (1 M; 50 μl) to the wells. The intensity of the developed color was recorded by reading its absorbance at a wavelength of 405 nm (A_{405}) in a microtiter plate reader (Bio Tek Instruments Inc., Mode ELX 800, USA).

2.9. Electron microscopy

Carbon-coated grids (200 meshes) were coated with the $NP_{\Delta c375}$ purified with sucrose density gradient ultracentrifugation and the protein was stained with uranyl acetate [2% (w/v)]. The grids were then examined under a transmission electron microscope (TEM; Hitachi-T-700, Tokyo, Japan).

3. Results and discussion

E. coli has been exploited extensively as an expression host for the synthesis of recombinant proteins [22]. Nevertheless, overexpression of the recombinant proteins was reported to have adverse effects to the host cells, particularly the stability of the recombinant proteins. These effects include inhibition of cell growth [23], cell death after protein induction [24], and homeostasis imbalance of the cells due to the change in regulatory and metabolism function [25]. Therefore, recombinant proteins are considered by the cells as foreign substances and could be degraded by endogenous proteases [26].

Fig. 1a illustrates the SDS-PAGE profile of the ammonium sulfate precipitated $NP_{\Delta c375}$. The anti-*myc* antibody was used in this study to detect the intact 45 kDa band of $NP_{\Delta c375}$, comprising His-tag and *myc* epitope at its C-terminus (Fig. 1b, lane 2). However, a few extra protein bands of approximately 41, 36, 35, 33, 32 and 31 kDa were observed in addition to the intact 45 kDa band of $NP_{\Delta c375}$ in Western blot analysis probed with the rabbit anti-N protein serum (Fig. 1b, lane 3). These extra protein bands are believed to be the degraded products of $NP_{\Delta c375}$ as they were detected by the rabbit anti-N protein serum. Similar observation was reported by Tan et al. [17] in a study using the full-length N protein of NDV. The potential protease cleavage sites that gave rise to various degraded protein bands on a Western blot are tabulated in the first 2 rows of Table 1. Since the degraded proteins possess molecular mass around 31, 32, 33, 35, 36 and 41 kDa, the possible cleavage sites of endogenous proteases are calculated to be at residues 281, 292, 300, 316, 326 and 370, respectively (Table 1). These degraded proteins with possible cleavage sites before amino acid residue 377 are assumed to lose their *myc* epitope located at the C-terminal end. Western blot analysis (Fig. 1b, lane 2) showed that the degraded proteins could not be detected by the anti-*myc* antibody. Chong et al. [27] also reported a similar observation in which the degraded recombinant N protein of Nipah virus has lost the His-tag located at its C-terminal end. Table 1 also summarizes the potential proteases predicted by the PeptideCutter program that cleave the $NP_{\Delta c375}$