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A preparative hydrophobic interaction chromatography for purification of recombinant nucleocapsid protein of Nipah virus from clarified *Escherichia coli* homogenate

Fui Chin Chong[a,b], Wen Siang Tan[c,d], Dayang R.A. Biak[b], Tau Chuan Ling[d,e], Beng Ti Tey[b,d,*]

[a] Department of Chemical and Natural Resources Engineering, Faculty of Engineering, Universiti Malaysia Pahang, 25000 Kuantan, Pahang, Malaysia
[b] Department of Chemical and Environmental Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[c] Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[d] Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[e] Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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ABSTRACT

The downstream processing of the recombinant nucleocapsid (N) protein of Nipah virus (NiV) from *Escherichia coli* homogenate using a preparative hydrophobic interaction chromatography (HIC) was investigated in the present study. Ammonium sulfate precipitation experiment was performed and it showed that 15% saturation of the salt was the most suitable salt concentration for the binding buffer. Batch binding of the N protein of NiV was performed using Sepharose™ 6 Fast Flow (FF) adsorbents coupling separately with four different types of ligand; phenyl low substitution, phenyl high substitution, butyl and octyl. The phenyl low substitution ligand was selected for subsequent optimization process due to its highest yield and purity of the N protein achieved from the batch binding experiment. The HIC for purification of the N protein of NiV was further scaled-up using a 10 cm column packed with phenyl low substitution Sepharose™ adsorbent. A recovering yield of 81% of the N protein of NiV with a purification factor of 9.3 was achieved from this scaled-up operation. The antigenicity of the purified N protein was still preserved as shown in ELISA analysis.

1. Introduction

Nipah virus (NiV) is a new member of the genus *Henipavirus* which is grouped under the family *Paramyxoviridae* [1]. Its RNA genome is about 18.2 kb nucleotides and encodes six main structural proteins; nucleocapsid (N), phospho- (P), matrix- (M), fusion- (F), glyco- (G) and large- (L) proteins [2]. The viral nucleocapsid (N) protein is the essential component of helical nucleocapsid [2] and the most abundant structural protein found in NiV. The gene encoding the N protein of NiV has been successfully cloned and expressed in *Escherichia coli* (*E. coli*) [3] and in baculovirus system [4]. The recombinant N protein of NiV has the potential to be used as an epidemiological diagnostic reagent [4]. Yu et al. [5] demonstrated that the recombinant N protein of NiV is a safe and cost-effective antigen for the detection of NiV infection. Due to its potential application in the epidemiological surveillance, hence, the purification of the N protein of NiV using a rapid and efficient method is needed urgently.

Hydrophobic interaction chromatography (HIC) was first developed and reported by Tiselius [6]. In HIC separation, protein adsorption is promoted by high salt concentrations and elution of the bound protein is achieved by a linear decreasing gradient of salt concentration in the binding buffer [7]. The performance of HIC is influenced by the characteristics of protein and the operating conditions of the chromatographic system (as reviewed in [8]). The property of proteins that determines the recovery of a purification process are the hydrophobicity of protein [7], the surface hydrophobicity distribution of protein [9] and the molecular size of protein [10]. On the other hand, the operating conditions that promote ligand–protein interactions in HIC are the characteristics of the mobile phase such as the type, concentration and pH of the salt [11], the properties of the stationary phase, mainly the chemical nature of the medium base, the type of hydrophobic ligand [12], and the ligand density [13].

The availability of different chromatography methods with different selectivity has simplified the purification process of most recombinant proteins. However, the selection of the right technique and operating condition is important to ensure a successful protein purification process. In this work, the development of a packed bed adsorption protocol using HIC matrix for the direct recovery of the N protein of NiV from clarified *E. coli* homogenate

* Corresponding author at: Department of Chemical and Environmental Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia. Tel.: +60 3 89466289; fax: +60 3 86567120.
E-mail address: btey@eng.upm.edu.my (B.T. Tey).

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was investigated. In order to study the effects of type of ligand on the recovery of the N protein, a set of experimental work using a commercially available Sepharose™ product with the same matrix base, but different types and densities of ligand is presented here. The effect of salt concentration in salting out the contaminant proteins was investigated. The optimization procedure was carried out using a 1 ml packed bed column. Subsequently, the optimized condition was applied in a scaled-up column to purify the N protein from the clarified *E. coli* homogenate.

2. Materials and methods

2.1. Adsorbents

All the HIC media: Sepharose™ 6 Fast Flow (FF) phenyl low substitution (low sub), phenyl high substitution (high sub), butyl and octyl were purchased as prepacked 1 ml HiTrap™ from GE Healthcare (Sweden). The matrix is the cross-linked agarose beads with a diameter of 90 μm. The HIC ligands are coupled to the monosaccharide units via glycidylethers. The HIC column was connected to the Äkta FPLC chromatography system (GE Healthcare, Sweden) throughout the protein purification process.

2.2. Feedstock preparation

The harvesting procedure of the *E. coli* strain BL21 (DE3) harbouring plasmid pTrcHis$_2$ expressing the N protein of NiV [3] was performed as that reported by Chong et al. [14]. The *E. coli* cells were cultured in Luria–Bertani (LB) medium containing ampicillin (50 μg/ml) at 25 °C in a shaking incubator at 200 rpm. The expression of N protein was induced once the culture reached an optical density at 600 nm (OD_{600}) of about 0.6–0.8, by adding isopropylthio-β-D-galactoside (IPTG) (final concentration of 1 mM) and was further incubated for another 5 h at 25 °C. The cells were centrifuged at 8000 × *g* (JLA 16.25 rotor, Beckman, USA) for 20 min at 4 °C.

The centrifuged cell pellet was resuspended in five types of salt buffer. The salt buffers were prepared by adding ammonium sulfate at saturation of 5%, 10%, 15%, 20% and 30% into 20 mM sodium phosphate buffer, pH 7.5. The cell suspension was then mixed with 0.2 μg/ml lysozyme and 4 mM $MgCl_2{\cdot}6H_2O$. The ultrasonic disruption of cell was performed as that described by Ho et al. [15]. The disrupted cell was then centrifuged at 18,000 × *g* (JA 20 rotor, Beckman, USA) for 20 min at 4 °C. The clarified homogenate was collected and analysed for total protein and N protein concentration. The specific amount of N protein in the clarified homogenate was calculated based on Eq. (1):

$$\text{specific amount of N protein} = \frac{\text{amount of N protein}}{\text{amount of total protein}} \quad (1)$$

2.3. Precipitation of N protein from disrupted cell homogenate

Ammonium sulfate was ground and added slowly into the clarified homogenate to obtain 10% saturation with continuous stirring for 2 h at 4 °C. The resulting solution was centrifuged at 18,000 × *g* for 20 min. The centrifuged supernatant was pooled and subjected to 20% ammonium sulfate saturation in a procedure similar to that of 10% saturation. Subsequently, the centrifuged supernatant was subjected to 30% saturation in the similar procedure. After centrifugation, the pellets of all the three steps precipitation were resuspended and dialysed with Tris–NaCl buffer (50 mM Tris, 100 mM NaCl; pH 8.0) for 24 h at 4 °C. The dialysis buffer was changed four times during the process. The N protein in the dialysed sample was quantitated based on the method described previously [14].

2.4. Selection of hydrophobic ligand

Selection of ligand was carried out in the HiTrap™ 1 ml column (0.7 cm diameter, 2.5 cm length) (GE Healthcare, Sweden) prepacked with the same Sepharose™ FF matrix, but with four different types of ligands: phenyl high sub, phenyl low sub, butyl and octyl. The dialysed protein solution from Section 2.3 was resuspended in Buffer A (20 mM sodium phosphate buffer, 15% ammonium sulfate, pH 7.5).

Columns were equilibrated with 10 column volumes (CV) of Buffer A. The feedstock was fed to the column at a linear flow rate of 150 cm/h. Unbound protein was washed out with 10 CV of Buffer A. The proteins were eluted by a linear decreasing salt gradient buffer (by mixing Buffer A and Buffer B (20 mM sodium phosphate buffer, pH 7.5)) over 10 CV, followed by a regeneration step with 5 CV of distilled water and Buffer A. The binding capacity for the N protein (mg of N protein adsorbed per ml of packed bed volume of adsorbent) was calculated using Eq. (2):

$$\text{binding capacity for N protein} = \frac{I - F - W}{V} \quad (2)$$

where I is the amount of N protein in the feedstock, F is the amount of N protein lost in flowthrough, W is the amount of N protein lost in washing, and V is the volume of packed bed adsorbent.

2.5. Purification of N protein of NiV using HIC

A scaled-up HIC process was performed using the HiScreen™ Phenyl FF low sub adsorbent prepacked in a polypropylene column (0.77 cm diameter, 10 cm length). The column was installed onto the Äkta FPLC. The feedstock was loaded onto the column at a linear flow rate of 300 cm/h, which had been equilibrated with 10 CV of Buffer A. One CV is equivalent to 5 ml. Then the column was washed with 5 CV of Buffer A to remove unbound or loosely bound proteins. The adsorbed proteins were eluted with 10 CV linear gradient of Buffer A from 15% to 0% ammonium sulfate saturation. The column was regenerated by washing with 5 CV of Buffer A. Cleaning in place was first conducted with 5 CV of distilled water, and followed by 5 CV of Buffer A. The entire chromatographic process was operated at 25 °C.

2.6. Protein analysis and quantitation

The recovery of N protein from the chromatographic process was investigated in this study. The SDS-PAGE of protein sample was performed under denaturing condition with 0.1% (w/v) SDS [3,16]. Electrophoresis separation was run using a Mini-Protean 3 apparatus (Bio-Rad, USA). For Western blotting, the polyacrylamide gel was transferred onto the nitrocellulose membrane using a Transblot apparatus (Bio-Rad, USA). The band of N protein on the transferred blot was detected by alkaline phosphatase-conjugated antihistidine antibody (1:5000 dilution; Invitrogen, USA).

The quantity of the N protein was determined by comparing the intensity of the protein band on the Western blot to the standard curve using the Quantity One® Quantitation software (Gel Doc; Bio-Rad, USA) [14,17]. The Bradford assay [18] was performed to estimate the protein content using bovine serum albumin as the standard and was measured at the optical density of 595 nm with a microplate reader (Model Elx 800; Bio Tek Instruments Inc., USA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The antigenicity of the N protein of NiV was determined with ELISA as described by Tan et al. [3]. After the coating of the N protein and the blocking with milk diluent on a microtiter plate,