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Comparative Evaluation of Three Purification Methods for the Nucleocapsid Protein of Newcastle Disease Virus from *Escherichia coli* Homogenates

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In the present study, the performances of conventional purification methods, packed bed adsorption (PBA), and expanded bed adsorption (EBA) for the purification of the nucleocapsid protein (NP) of Newcastle disease virus (NDV) from *Escherichia coli* homogenates were evaluated. The conventional methods for the recovery of NP proteins involved multiple steps, such as centrifugation, precipitation, dialysis, and sucrose gradient ultracentrifugation. For the PBA, clarified feedstock was used for column loading, while in EBA, unclarified feedstock was used. Streamline chelating immobilized with Ni^{2+} ion was used as an affinity ligand for both PBA and EBA. The final protein yield obtained in conventional and PBA methods was 1.26% and 5.56%, respectively. It was demonstrated that EBA achieved the highest final protein yield of 9.6% with a purification factor of 7. Additionally, the total processing time of the EBA process has been shortened by 8 times compared to that of the conventional method.

Key words: NP protein, NDV, *Escherichia coli,* expanded bed adsorption, packed bed adsorption

The effectiveness of the treatment of a disease is very much dependent on the delivery of the therapeutic molecules to the proper target. Various kinds of biological molecules have been proposed or used as carriers for the delivery of nucleic acids and other therapeutic compounds. The nucleocapsid protein (NP) of Newcastle disease virus (Yusoff and Tan, 2001), a poultry virus, can be produced in *Escherichia coli* as ring-like structures which have the potential to be used both as biocarriers for the development of subunit vaccines (Rabu *et al.,* 2002) and as a diagnostic reagent. Kho *et al.* (2001) reported that a 29 amino acid peptide which included the *myc* epitope and a hexa-His tag being inserted into the C-terminus of the NP did not impair the ring-like assembly of the viral nucleocapsid. These extra amino acid sequences were shown to be exposed on the surface of the ring-like particles, suggesting that individual particles can be used in the development of subunit vaccines as molecular carriers for presenting desired epitopes (Rabu *et al.,* 2002). This recombinant NP protein is abundantly expressed in *E. coli* and the His tag can aid in the purification of the recombinant protein (Kho *et al.*, 2001).

The recovery of proteins from various recombinant organisms is a crucial step in the production of proteins for therapeutic and diagnostic use. This is quite challenging because the products must be concentrated from a very dilute condition in the feedstock and purified from other protein impurities, which may have very similar properties. The feedstock, undisrupted fermentation broth or disrupted biomass homogenate, is very complex in nature and contains various sizes of dissolved solid, biomass, and cell debris. In the conventional method, the ability to obtain a final product of guaranteed purity and potency can only be achieved by a combination of several unit operations (Anspach *et al.,* 1999). Prior to the concentration and purification steps, the biomass or cell debris must be separated from the fermentation broth or cell homogenates through a process of centrifugation or filtration. Centrifugation is usually combined with a microfiltration step to obtain a particle-free solution prior to further purification by packed-bed chromatography or the combination of precipitation and ultracentrifugation (Lee, 1989). However, in the large scale process of protein recovery, these clarification methods show limitations in practice. Although microfiltration yields cell-free solutions, in many cases the flux of liquid per unit membrane area is dramatically decreased during the filtration process (Chase and Draeger, 1992). On the other hand, in terms of capital investment and operational cost, centrifugation is an expensive process. Moreover, a combination of centrifugation and filtration operations often results in long processing times. The additional unit operations involved

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would not only cause a longer processing time, but would also result in higher product loss and decrease in the throughput and final product yields. Therefore, a single-step protein purification process that combines feedstock clarification, concentration, and purification is needed to improve protein recovery yields and reduce processing time.

Expanded bed adsorption (EBA) is a single pass operation in which desired proteins are purified from crude, particulate-containing feedstock without the need for separate clarification, concentration, and initial purification. The expansion of the adsorbent bed creates a distance between the adsorbent particles, which allows for unhindered passage of cells, cell debris, and other particulates during the application of crude feed to the column (Ling, 2002). EBA has been proven to be a versatile tool which can be applied to all commonly used source materials such, as *E. coli* and yeast homogenates, mammalian cell culture fluid, and transgenic milk (Batt *et al.*, 1995; Thömmes *et al.*, 1995; Chang and Chase, 1996; Noppe *et al.*, 1996; Willoughby *et al.*, 1999; Ling *et al.*, 2004). Immobilized metal affinity chromatography (IMAC) has been widely used for the separation of His tagged recombinant proteins. The separation is based on the differential affinity of proteins, derived from the coordination bonds formed between metal ions and histidine side chains exposed on the surface of the protein molecules (Chaga, 2001; Hu *et al.*, 2003; Ueda *et al.*, 2003).

The aim of this study was to develop a rapid, simplified primary capture step for the recovery of NP protein from particulate-containing feedstock. The IMAC expanded bed adsorption was used to capture the NP protein. The purification of the NP protein from unclarified *E. coli* homogenates with EBA has not been reported elsewhere in the literature. In this work, we investigated the performance of three different modes of operations employed for the recovery of recombinant NP protein, namely conventional methods (combination of precipitation and ultracentrifugation), packed bed adsorption (PBA) and EBA. The final yield and purity of the product and processing times of these three methods are compared.

Materials and Methods

Materials

Streamline 25 Column (ID = 25 mm), XK 16/20 Column (ID = 16 mm) and Streamline chelating were purchased from Amersham Bioscience (Uppsala, Sweden). The adsorbent has a mean particle density of 1.2 g/ml and a size distribution of 100 - 300 μm. It has metal chelating ligand iminodiacetic acid (IDA) attached to it.

Preparation of feedstock

The *E. coli* cells harbouring the recombinant plasmid of the NP_{cfus} gene, pTrcHis2- NP_{cfus} (Kho *et al.*, 2001), were cultured in Luria Bertini (LB) broth supplemented with 50 μg/ml ampicillin. The cultures were incubated at 37°C with vigorous shaking at 250 rpm until the biomass density reached an optical density (OD_{600}) of 0.6 to 0.8. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, Promega, USA) at a final concentration of 1.0 mM. After a further 5 h of incubation, the cells were harvested by centrifugation at 3,840 × g (JA 14 Rotor, Beckman, USA) for 10 min at 4°C. The cell pellets were resuspended in lysis buffer [20 mM sodium phosphate, 0.5 M sodium chloride (pH 8.0), 0.1% Triton X-100, 0.2 mg/ml lysozyme, and 4 mM magnesium chloride]. The cells were lysed by sonication at 33 W (watt) with 5 × 15 sec pulse with 5 sec intervals. The samples were kept in a salt-ice bath (0°C) during the cell disruption to prevent overheating. The cell homogenate was then treated with DNase (5 μg/ml) for 1 h at room temperature (25°C) with gentle shaking.

Purification

Conventional Purification Methods: After cell disruption, the cell homogenate was clarified by centrifugation at 12,100 × g (JA 20 rotor, Beckman, USA) for 20 min at 4°C. The clarified supernatant was first subjected to ammonium sulphate precipitation from 0 to 10% saturation, followed by 10 to 25% saturation. The precipitate was isolated by centrifugation at 12,100 × g (JA 20 rotor, Beckmen) for 20 min at 4°C and dialysed overnight in dialysis buffer (50 mM Tris, 100 mM NaCl, pH 8.0). The dialyzed solution was then subjected to sucrose gradient ultracentrifugation. A decreasing sucrose solution from 10 to 50% was used in the present study. Centrifugation was performed at 247,000 × g (SW 41 Ti rotor, Beckman, USA), 4°C for 5 h. Protein fractions were collected and subjected to protein analysis and quantification.

Packed Bed Adsorption (PBA): PBA was carried out using the Streamline chelating adsorbent. The adsorbent was packed into an XK 16/20 column and charged with 0.1 M nickel sulphate solution. The column was then equilibrated with equilibration buffer (0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0) at 100 cm/h. Clarified feedstock was applied onto the column followed by washing with equilibration buffer. Protein was first eluted with elution buffer 1 (50 mM imidazole in 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0), followed by elution buffer 2 (350 mM imidazole in 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0) at 10 cm/h. Protein fractions were collected and subjected to protein analysis and quantification. All steps were performed in a downward flow.

Expanded Bed Adsorption (EBA): Streamline chelating was loaded onto a Streamline 25 Column and charged with 0.1 M nickel sulphate solution at 100 cm/h. Adsor-

bent was first equilibrated with equilibration buffer (0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0) at 200 cm/h. The adsorption was performed by loading the unclarified feedstock onto the equilibrated column at the same flow rate at room temperature. The column was then washed with equilibration buffer. After washing, the bed was allowed to settle and the adapter of the column was lowered to the top of the settled bed. The elution was performed in the downward flow packed-bed mode, first with elution buffer 1 (50 mM imidazole in 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0), followed by elution buffer 2 (350 mM imidazole in 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 8.0) at 10 cm/h. Protein fractions were collected and subjected to protein analysis and quantification. Clean-in-place (CIP) was done by pumping the washing buffer (0.5 M sodium hydroxide and 1 M sodium chloride) into the column with a flow rate of 40 cm/h for 4 h, followed by rinsing with distilled water. The adsorbent was preserved in 20% ethanol.

Protein Analysis

SDS-PAGE: Aliquots of the fractions were analysed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue to analyze their purity (Laemmli, 1970). Electrophoresis was performed at 32 mA for 90 min using the Mini Protean 3 apparatus (Bio-Rad, USA).

Western Blotting and Quantification of NP Protein: Following SDS-PAGE, proteins were transferred to nitrocellulose membranes using the Transblot SD semidry transfer cell (Bio-Rad, USA) for 2 h at 15 V. The membranes were blocked for 1 h with milk diluent and incubated overnight at room temperature with a solution of anti-*myc* antibody alkaline phosphatase conjugated (Invitrogen, USA) in TTBS (25 mM Tris, 137 mM NaCl, 2.68 mM KCl, 0.01% Tween-20, pH 7.5) at a dilution of 1:2000. To remove the unbound antibodies, the membranes were then washed 3 times with TTBS washing solution for 5 min each. The colour development was performed by immersing the membrane in BCIP/NBT (1-bromo-3-chloro-3-indolyl phosphate/ nitro blue tetrazolium) reagent. The NP protein concentrations for various samples were obtained by comparing the intensity of their bands from Western blots with a standard curve. The bands were quantified using the Sophisticated Volume Tools from the Bio-Rad imaging devices supported by Quantity One (Bio-Rad, USA). A volume is the intensity data inside a defined boundary drawn on the images. The intensity of the data inside the boundary and that of other objects can be compared using the Volume Analysis Report (Xiang *et al.*, 2002; Tey *et al.*, 2004). The entire assay was performed in duplicate.

Total Protein Quantification: The amount of total protein was estimated according to the Bradford Assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Calculation: The specific NP protein concentration, yield of NP protein, and purification factor were determined using the following equations:

$$\textit{Specific NP Protein Concentration} = \frac{\textit{Amount of NP Protein}}{\textit{Amount of Total Protein}} \quad (1)$$

$$\textit{Yield of NP Protein} = \frac{\textit{Amount of NP Protein}}{\textit{Amount of NP Protein in the feedstock}} \times 100 \quad (2)$$

$$\textit{Purification Factor} = \frac{\textit{Specific NP Prote in Concentration}}{\textit{Specific NP Protein Concentration in the feedstock}} \quad (3)$$

Results and Discussion

In the present study, the performance of conventional, PBA, and EBA purification methods on NP protein recovery was compared. Conventional purification methods consist of several unit operations, including centrifugation, precipitation, dialysis, and sucrose gradient ultracentrifugation. In the PBA, the cell homogenate was clarified using centrifugation prior to loading onto the XK 16/20 column, which had been packed with the Streamline chelating adsorbent. A Streamline 25 column was chosen as the contactor for the EBA using Streamline chelating adsorbent to recover the NP protein from crude, particulate-containing feedstock. The specific NP protein concentration, yields, and purification factor of NP protein, processing time, and number of unit operation involved in these three recovery processes were compared.

Table 1 summarises the specific NP protein concentration, yields, and the purification factors of NP protein in these three methods. The final protein yield obtained in the conventional method was 1.26%; only 1.3 mg of the NP protein was recovered after 5 stages of purification. In the PBA, the number of stages involved was reduced to only 2; hence, the final protein yield increased 4.3-fold compared to the conventional method. However, the clarification processes (see Fig. 2) employed in both the conventional and PBA methods faced some limitations, such as long process time, and high capital and operating costs; these were more pronounced for large scale processes (Ersson *et al.*, 1998; Anspach *et al.*, 1999). It was demonstrated that a single-step purification using EBA recorded a 7.6-fold higher final protein yield compared to the conventional method. Fig. 1 shows the SDS-PAGE gel of the NP protein purified from all three protein purification methods. The intensity of the 58 kDa band corresponding to the NP protein band can be clearly observed

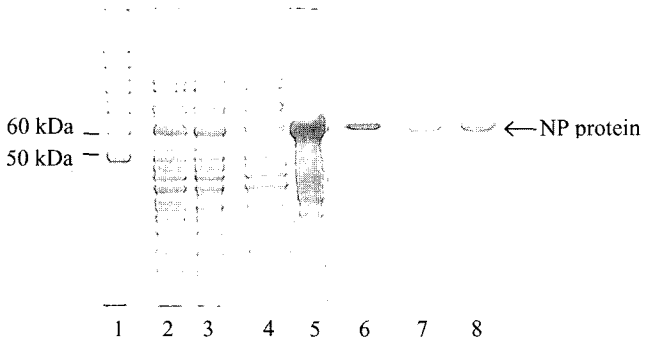

Fig. 1. SDS-PAGE of the NP Protein purified from the conventional methods, packed bed adsorption, and expanded bed adsorption. Lane 1: Molecular mass markers in kDa. Lane 2: Sample from crude feedstock. Lane 3: Sample after centrifugation, Lane 4: Sample of supernatant after ammonium sulphate precipitation. Lane 5: Sample after dialysis. Lane 6: Sample after sucrose gradient ultracentrifugation. Lane 7: Sample eluted from packed bed adsorption, and Lane 8: Sample eluted from expanded bed adsorption. The arrow shows the location of NP protein (58 kDa).

on the gel. The purity obtained for EBA is comparable to that from PBA (Table 1).

The process schemes for the recovery of recombinant NP protein from 400 ml of *E. coli* cell homogenate using all three purification methods are illustrated in Fig. 2. The conventional method employed several unit operations, while the EBA has only one. In the EBA, the number of purification steps was greatly reduced from 5 steps to 1 step. Table 2 summarises the operational conditions and total processing time for the operation of all three purification methods. The total processing time to handle a 400 ml cell homogenate is about 56 h in the conventional purification method. In the EBA, it has been reduced by 8 times to only 7.5 h. The reduction of unit operations and processing time could reduce the loss of targeted product and/or product activity, since product modification (e.g. chemical and biological processes) is time-dependent (Thömmes *et al.*, 1996, Kaufmann, 1997).

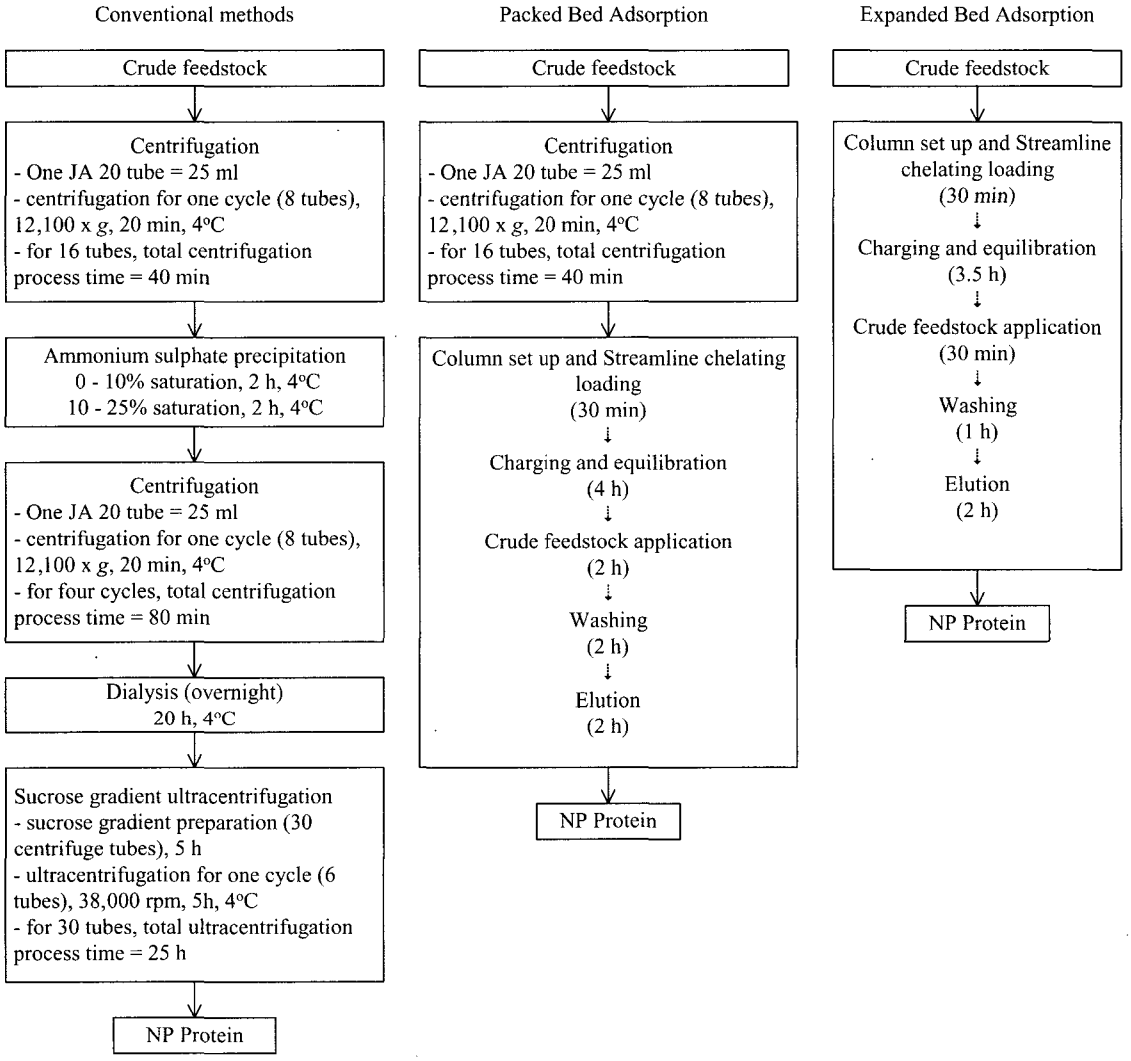

Fig. 2: Flowchart of the purification of NP Protein. Left hand panel shows the steps involved in the conventional methods. Center panel shows the packed bed adsorption purification steps and the right hand panel shows the steps in expanded bed adsorption. Parameters listed are for a feedstock volume of 400 ml. The total process time for conventional methods, packed bed adsorption, and expanded bed adsorption are 56 h, 11 h, and 7.5 h, respectively.

Table 1. Comparison of the purification of NP protein using conventional methods, packed bed adsorption, and expanded bed adsorption

Purification stages	Amount of NP protein (mg)	Specific Protein Concentration (mg of NP / mg of total protein)	Yield of NP protein (%)	Purification factor
Conventional methods				
Crude	104.04	0.106	100.00	1.00
Centrifugation	22.03	0.112	21.17	1.06
Ammonium sulphate precipitation	17.39	0.101	16.71	0.95
Dialysis	5.25	0.298	5.05	2.82
Sucrose gradient ultracentrifugation	1.31	0.930	1.26	8.79
Packed bed adsorption				
Crude	86.08	0.096	100.00	1.00
Centrifugation	11.81	0.120	13.72	1.26
Eluted protein	4.79	0.697	5.56	7.29
Expanded bed adsorption				
Crude	393.04	0.105	100.00	1.00
Eluted protein	37.67	0.690	9.59	6.56

Table 2. Comparison of the operational conditions and processing time for conventional methods, packed bed adsorption, and expanded bed adsorption

Operational conditions	Conventional method	Packed bed adsorption	Expanded bed adsorption
Operation temperature	4°C	Room temperature	Room temperature
Total unit operations	5	2	1
Total process times for 400 ml	56 h	11 h	7.5 h

Conclusion

The direct recovery of NP Protein from *E. coli* feedstock utilizing an expanded bed column is a viable operation. EBA using Streamline chelating adsorbent immobilized with Ni^{2+} ions was successfully employed to purify the recombinant NP protein from unclarified feedstock with a 7.6-fold higher final protein yield than that achieved by the conventional purification method. The EBA has greatly reduced the number of purification steps from 5 steps to 1 step and the processing time has been shortened by 8 times. Additionally, it has been demonstrated that EBA chromatography is a scalable operation (Ameskamp *et al.*, 1999; Sumi *et al.*, 1999) and can be operated at room temperature. In conclusion, EBA is an attractive alternative to the conventional purification method, with the advantages of less unit operation involved, shorter processing time, higher yield, lower capital investment, and lower operational costs.

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