Journal of Chromatography A, 1217 (2010) 1293–1297

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Direct recovery of recombinant nucleocapsid protein of Nipah virus from unclarified *Escherichia coli* homogenate using hydrophobic interaction expanded bed adsorption chromatography

Fui Chin Chong[a,b], Wen Siang Tan[c,d], Dayang Radiah Awang 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

ARTICLE INFO

Article history:
Received 23 September 2009
Received in revised form 9 December 2009
Accepted 14 December 2009
Available online 22 December 2009

Keywords:
Expanded bed adsorption
Hydrophobic interaction chromatography
Recombinant nucleocapsid protein
Nipah virus
Escherichia coli
Phenyl ligand

ABSTRACT

A direct recovery of recombinant nucleocapsid protein of Nipah virus (NCp-NiV) from crude *Escherichia coli* (*E. coli*) homogenate was developed successfully using a hydrophobic interaction expanded bed adsorption chromatography (HI-EBAC). The nucleic acids co-released with the recombinant protein have increased the viscosity of the *E. coli* homogenate, thus affected the axial mixing in the EBAC column. Hence, DNase was added to reduce the viscosity of feedstock prior to its loading into the EBAC column packed with the hydrophobic interaction chromatography (HIC) adsorbent. The addition of glycerol to the washing buffer has reduced the volume of washing buffer applied, and thus reduced the loss of the NCp-NiV during the washing stage. The influences of flow velocity, degree of bed expansion and viscosity of mobile phase on the adsorption efficiency of HI-EBAC were studied. The dynamic binding capacity at 10% breakthrough of 3.2 mg/g adsorbent was achieved at a linear flow velocity of 178 cm/h, bed expansion of two and feedstock viscosity of 3.4 mPa s. The adsorbed NCp-NiV was eluted with the buffer containing a step gradient of salt concentration. The purification of hydrophobic NCp-NiV using the HI-EBAC column has recovered 80% of NCp-NiV from unclarified *E. coli* homogenate with a purification factor of 12.5.

1. Introduction

In an expanded bed adsorption chromatography (EBAC) operation, the adsorbents are expanded in the column with an upward flow of liquid and the target protein is captured by the adsorbent from unclarified crude feedstock while allowing the passage of suspended solids of cell debris and contaminant proteins [1,2]. EBAC combines the process of clarification, concentration and initial purification into one unit operation; thus, a significant increase in product yield is expected due to the decrease in the number of process steps and the shortened overall process time [1,3]. EBAC has been demonstrated to be able to process a large volume of feedstock [4] from various sources such as *Escherichia coli* [5,6], and yeast [7] homogenates, mammalian cell culture [8], and chicken egg white [9].

The hydrodynamic behaviour of the EBAC and the adsorption efficiency in the column are affected significantly by the complex nature of feedstock (liquid viscosity and density) and the superficial velocity [10,11]. The presence of solids and other colloidal components such as DNA in the loading of crude feedstock leads to a non-specific interactions between cells and adsorbent, which cause aggregation of adsorbents that deteriorates the bed stability of adsorbent and protein adsorption efficiency [12–14]. Flow velocity affects the retention time of the EBAC column, hence when EBAC is operated at high flow velocity, early breakthrough of protein occurs and thus reduces the adsorption efficiency [15–17]. In order to improve the hydrodynamic performance of the bed during the washing stage, the use of enhanced viscosity of washing solution with glycerol was found to be efficient in removing weakly adsorbed proteins from the expanded bed [18,19].

The nucleocapsid protein of Nipah virus (NCp-NiV) has been successfully cloned and expressed in *E. coli* [20] and in baculovirus system [21]. The recombinant NCp-NiV self-assembles into herringbone-like structures, resembling the native nucleocapsid isolated from the live virus [20–22]. The recombinant NCp-NiV is a safe and cost-effective epidemiological diagnostic reagent which

* 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).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.12.039

can be used to detect the anti-NiV antibodies in the event of an outbreak [21,23]. Chong et al. [24] demonstrated the purification of NCp-NiV using a conventional packed bed adsorption (PBA) of HIC adsorbent, which requires clarification of crude feedstock prior to chromatography separation. The removal of biomass is one of the downstream operation steps that causing a longer processing time, and also resulting in higher product loss and decrease in the throughput and final product yield [3,25]. Therefore, a single step protein purification process combining feedstock clarification, concentration, and purification is needed to improve the yield of NCp-NiV and reduce overall processing time. Purification of recombinant NCp-NiV using a HI-EBAC column has not been described so far. In the present study, the adsorption of recombinant NCp-NiV from unclarified *E. coli* homogenate was performed using a HIC adsorbent, streamline phenyl, in an EBAC column.

2. Materials and methods

2.1. Materials

Streamline phenyl was purchased from GE Healthcare (Uppsala, Sweden). It has phenyl groups coupled to highly cross-linked 6% agarose quartz core based with a size distribution of 100–300 μm and average density of 1.2 g ml^{-1}.

All EBAC operations in the present study were conducted using a commercially available Fastline™ 10 contactor (UpFront Chromatography A/S, Copenhagen, Denmark). A peristaltic pump (Watson Marlow, UK) was used to circulate the liquid to the EBAC contactor throughout the EBAC operation in this study.

2.2. Preparation of unclarified feedstock

E. coli BL21(DE3) containing plasmid pTrcHis$_2$ carrying the NCp-NiV gene [20] was cultured as reported by Chong et al. [26]. The biomass harvested from the culture was first weighed and resuspended in buffer A (20 mM sodium phosphate buffer, 615 mM ammonium sulfate, pH 7.5) to yield a 5% (w/v) of cell biomass. Cell disintegration was performed with ultrasonication as described by Ho et al. [27]. The homogenate was then treated with DNAse (15 μg/ml) for 1 h with gentle shaking at 4 °C.

2.3. Measurements of viscosity

The viscosity of the various feedstocks (mobile phase) was measured using the Spindle 14–18 of the DV-II+ Viscometer (Brookfield Eng. Lab., USA). The sample was stirred at a constant speed of 60 rpm, for 10 s to achieve equilibrium before measuring viscosity.

2.4. Equilibrium of adsorption isotherm

A series of batch adsorption experiments of NCp-NiV onto streamline phenyl were obtained by challenging with a range of unclarified feedstock with various biomass concentration values. The unclarified feedstocks were prepared in buffer A (20 mM sodium phosphate buffer, 615 mM ammonium sulfate, pH 7.5) and mixed with pre-equilibrated 1 ml adsorbents. The mixtures were mixed well on a rotator for 2 h at 25 °C to allow equilibrium between the solid and liquid phase. The NCp-NiV concentration during the equilibration, C, was measured and the adsorbed NCp-NiV, Q, was calculated based on the mass balance of NCp-NiV in the feedstock before and after adsorption.

2.5. Operation of EBAC

The Fastline 10 EBAC column was filled with 8 ml of streamline phenyl adsorbent corresponding to a settled bed height of 10 cm. Bed expansion and equilibration was done by pumping buffer A through the mixing chamber and onto the column at a flow rate of 2.33 ml/min (linear flow velocity of 178 cm/h) until a constant bed height was obtained. The bed was allowed to expand to a bed height of 20 cm (corresponding to a two-fold expansion) and the adaptor was positioned at 22 cm prior to the loading phase. The unclarified feedstock was then loaded into the column followed by washing with buffer A with and without the addition of 10% (v/v) glycerol to remove weakly or unbound proteins from the voids of bed. Elution was carried out with buffer B (20 mM sodium phosphate buffer, 410 mM ammonium sulfate, pH 7.5) and followed by buffer C (20 mM sodium phosphate buffer, 205 mM ammonium sulfate, pH 7.5). The adsorbents were then regenerated based on the supplier's protocols.

2.5.1. Expansion characteristic of adsorbent

The expansion characteristics of streamline phenyl adsorbent were investigated in a Fastline 10 column at 25 °C. The adsorbent bed was expanded using equilibration buffer (buffer A) or otherwise stated with increasing superficial velocity. The degree of bed expansion corresponding to each velocity was recorded and expressed as a ratio of the height of the expanded, H, to the sedimented, $H_{0,}$ bed adsorbent. The data were then fitted with the Richardson–Zaki correlation:

$$u = u_t \varepsilon^n \quad (1)$$

where u is the superficial velocity, u_t is the terminal settling velocity, ε is the expanded voidage, and, n is the bed expansion index. The settled bed voidage, $\varepsilon_{0,}$ was assumed to have a value of 0.4 [28].

2.5.2. Dynamic binding capacity

Dynamic binding capacity was studied in an EBA column loaded with streamline phenyl to a settled bed height of 10 cm. The adsorbent bed was expanded to a degree of 2 with buffer A at a superficial velocity of 178 cm/h. Subsequently, the unclarified feedstock was loaded and protein fractions were collected and assayed for the NCp-NiV concentration. The dynamic binding capacity, Q_B (mg of protein adsorbed per ml of settled adsorbent), was calculated as

$$Q_B = \frac{C_0 V_b}{V_s} \quad (2)$$

where C_0 is the initial concentration of the feedstock (mg/ml), V_b is the volume at 10% breakthrough (ml) and V_s is the settled volume of the adsorbent (ml).

2.6. Analysis and quantitation of protein

The yield and purity of the NCp-NiV were determined by comparing the intensity of the protein bands on Western blots to the standard curve using the Quantity One® Quantitation software (Gel Doc; Bio-Rad, USA) [26,29]. The Bradford assay [30] was performed to estimate the total protein content at the optical density of 595 nm with a microtiter plate reader (Model Elx 800; Bio Tek Instruments Inc., USA).

The antigenicity of the NCp-NiV was determined with ELISA by measuring the optical density at 405 nm using the microtiter plate reader, as described by Tan et al. [20].

3. Results and discussion

3.1. Equilibrium adsorption isotherm

The equilibrium adsorption isotherm of NCp-NiV from unclarified feedstock onto the streamline phenyl was observed and fitted

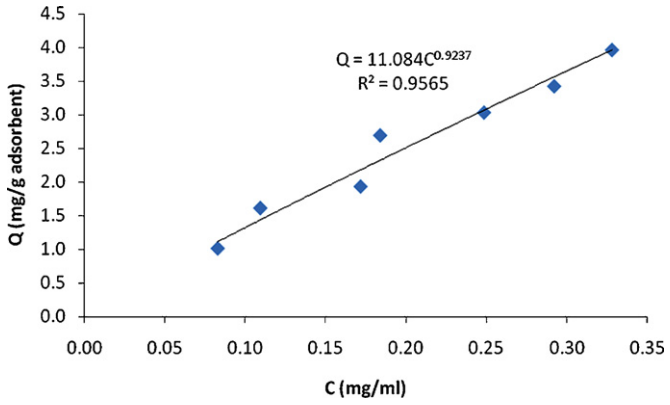

Fig. 1. Adsorption isotherm of NCp-NiV from unclarified feedstock on the streamline phenyl adsorbent.

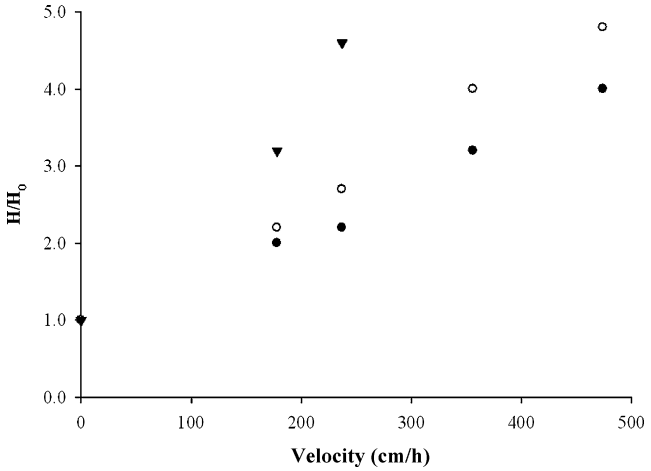

Fig. 2. Bed expansion characteristics of streamline phenyl in equilibration buffer (●), DNase-treated (○) and non-DNase-treated (▼) unclarified feedstocks.

to the Freundlich isotherm [31] as shown in Fig. 1 (R^2 = 0.9565).

$$Q = k(C)^n \quad (3)$$

where Q and C are the adsorbed and the unbound concentration of NCp-NiV, respectively, during equilibrium; k is the dissociation constant and; n is the Freundlich coefficient. The Freundlich isotherm has an n value of 0.924 and the k of the adsorption of NCp-NiV onto the streamline phenyl was 11.08 (mg/g)(ml/mg)^n. This result suggests that the binding of NCp-NiV by streamline phenyl in 615 mM ammonium sulfate is a favorable adsorption. Indeed, as reported by Tiselius [32], Porath et al. [33] and Hjertén et al. [34], the binding of hydrophobic proteins to HIC adsorbents was promoted at high concentration of salt, which stabilizes protein structures.

3.2. Expansion characteristic of adsorbent

The degree of bed expansion is influenced by liquid superficial velocity, density of adsorbent particles and viscosity of feedstock, as reviewed by Anspach et al. [1]. The bed expansion characteristics of streamline phenyl were evaluated in various mobile phases, taken as the 5% (w/v) biomass containing unclarified feedstock with and without treatment of DNase (15 μg/ml), and the equilibration buffer, as shown in Fig. 2. The results show that the bed expansion increased linearly with the superficial velocity, until 474 cm/h. At a constant superficial velocity of 178 cm/h, the adsorbent was expanded evenly at a degree of 2 by using the equilibration buffer. When the non-DNase-treated unclarified feedstock was loaded at 178 cm/h, the bed expanded was higher by a degree of 3. As a result, channeling within the bed, over-expansion and unstable bed were observed. These phenomena were also observed by increasing the superficial velocity. This observation contradicts our previous work [35], using the feedstock from a different *E. coli* strain which has a lower DNA content, in which the increase in liquid dispersion within the bed was minimum, with 5% (w/v) biomass of feedstock. The highly viscous feedstock as a result of high level content of DNA, can be overcome by adding DNase prior to purification [36,37]. Thus, the crude feedstock was further treated with DNase and its viscosity was measured using a viscometer (Table 1). The DNase-treated unclarified feedstock had a 24% lower viscosity compared to that of non-DNase-treated one. Other researchers also showed that adding DNase into the unclarified feedstock can improve the stability of the expanded bed significantly [36,37].

A linear regression of the Richardson–Zaki equation was used to assess the hydrodynamic stability of the fluidization of the adsorbent bed. The values of n and u_t for different mobile phases were obtained and are summarized in Table 1. An increase in the viscosity in the mobile phase had resulted in a decrease in the value of terminal settling velocity. This is in line with the findings as by other researchers [38]. The n value obtained for the DNase-treated feedstock is 5.07, which is close to the value in the laminar flow regime of 4.8 [17]. Therefore, the DNase-treated feedstock was applied for HI-EBAC operation throughout this study.

3.3. Dynamic binding capacity

Frontal analysis observed from the breakthrough curve is important to estimate the maximum loading amount of target protein passed through adsorbent bed with a target permissible loss of 10% of protein in the effluent. The breakthrough curve of the NCp-NiV adsorbed onto the streamline phenyl described above is shown in Fig. 3. At breakthrough of 10%, the dynamic binding capacity, Q_B calculated from Eq. (2) was 3.2 mg/g adsorbent.

3.4. Direct recovery of NCp-NiV from unclarified feedstock

The results of the comparative EBAC profile with two different washing strategies are shown in Fig. 4. The performance of the HI-EBAC for the recovery of NCp-NiV from unclarified feedstock is summarized in Table 2. When the bed expansion was stable at a degree of 2, the DNase-treated unclarified feedstock containing the NCp-NiV and other contaminant proteins was applied onto the column. The solid particles flowed through the bed voids and left the column through the outlet pipe.

Table 1
Viscosity of equilibration buffer, non-DNase-treated and DNase-treated unclarified feedstocks and the Richardson–Zaki coefficient, n and the terminal settling velocity, u_t obtained from the linearized plot of Richardson–Zaki equation.

Mobile phase	Viscosity (mPa s)	Richardson–Zaki coefficient (n)	Terminal settling velocity (u_t, cm h)
Equilibration buffer (20 mM phosphate, 615 mM ammonium sulfate, pH 7.5)	1.80 ± 0.2	4.70	993.3
Unclarified feedstock (5% (w/v) biomass, non-DNase-treated)	4.50 ± 0.2	5.49	541.5
Unclarified feedstock (5% (w/v) biomass, DNase-treated (15 μg/ml))	3.40 ± 0.2	5.07	871.3

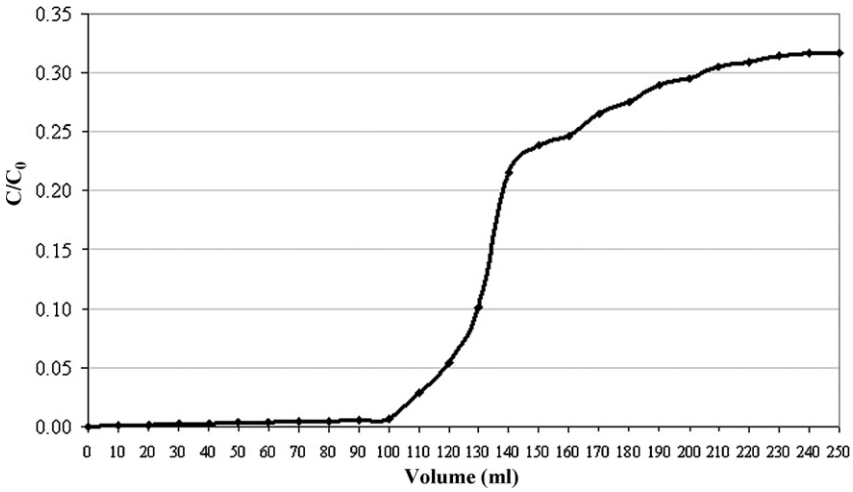

Fig. 3. Breakthrough curve of the NCp-NiV from unclarified feedstock onto streamline phenyl.

Subsequently, an intensive washing (approximately 10 CVs) was used to remove contaminant proteins and unbound proteins from the expanded bed (Fig. 4A). During the washing step, the sudden change in the viscosity in the washing buffer has led to a buoyancy-induced mixing. This resulted in an improper washing, thus more washing buffer was used to remove the contaminant proteins. This large volume of washing buffer applied has resulted in a 19.5% loss of the NCp-NiV. Fee [18] has addressed this issue by adding glycerol into the washing buffer to reduce the overall volume consumption of buffer and to shorten the operation time. In this study as shown in Fig. 4B, after applying 10% (v/v) glycerol in the washing buffer, the buffer consumption was reduced by half and the contaminant proteins were removed efficiently with minimal loss of the NCp-NiV (2%). Moreover, this study demonstrates that the washing buffer with enhanced viscosity was 1.5 times more efficient in removing the contaminant proteins.

The strongly adsorbed NCp-NiV was eluted from the streamline phenyl adsorbent in an expanded mode. As shown in Fig. 4B, the adsorbed NCp-NiV was eluted with a step gradient elution buffer at salt concentration of 410 mM and 205 mM. Fig. 5 shows the SDS-PAGE analysis (Fig. 5A) on a 12% polyacrylamide gel and Western blot analysis (Fig. 5B) of the protein fractions collected from the HI-EBAC operation. A yield of 80% and a purification factor of 12.5 were obtained in this single step direct recovery of the NCp-NiV from unclarified feedstock (Table 2). Moreover, the antigenicity of the purified NCp-NiV from the HI-EBAC operation was still preserved as demonstrated by ELISA (Fig. 6) using an anti-NiV rabbit serum.

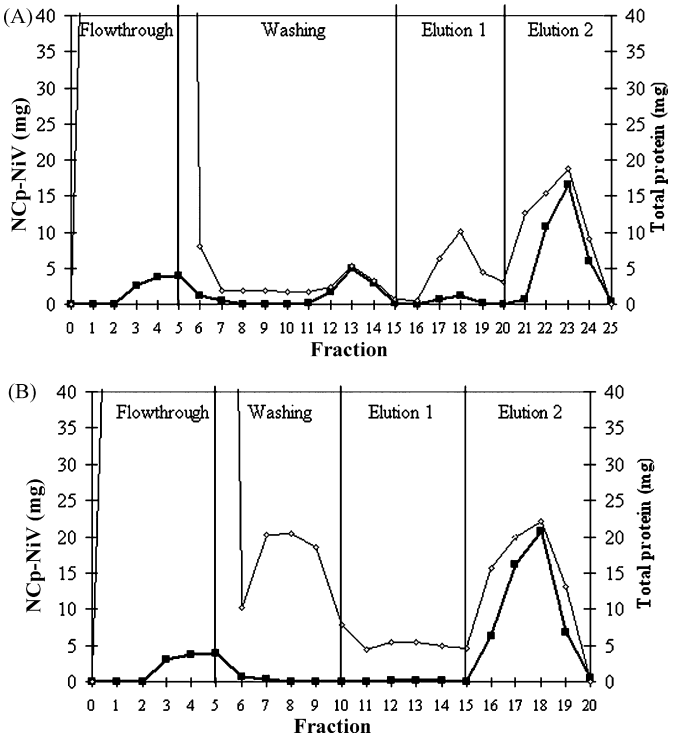

Fig. 4. Separation of 250 ml unclarified *E. coli* feedstock on streamline phenyl in EBA mode at a superficial velocity of 178 cm/h. (A) The washing buffer without glycerol. (B) The washing buffer with 10% (v/v) glycerol. (■) NCp-NiV, (◊) total protein.

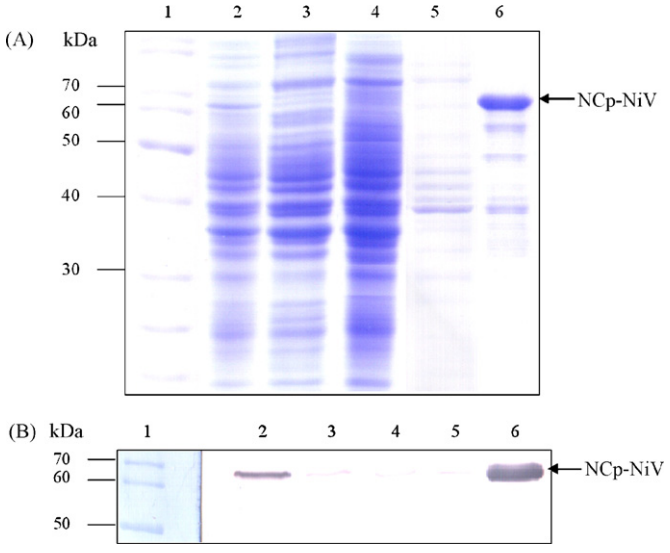

Fig. 5. Graphical illustration of the pooled protein fractions collected in HI-EBAC operation. SDS-PAGE analysis (A) and Western blot analysis (B) of the protein fractions. The NCp-NiV bands in (B) were detected by anti-histidine antibody. Lane 1: molecular mass markers; lane 2: unclarified *E. coli* homogenate; lanes 3: flowthrough; lane 4: washing; lane 5: elution with 410 mM salt concentration; lane 6: elution with 205 mM salt concentration.

Table 2
The purification performance of NCp-NiV from unclarified feedstock using HI-EBAC column.

Operation time: 3 h 15 min				Flow rate: 178 cm/h		
Purification stage	Total volume (ml)	Total protein (mg)	Amount of NCp-NiV (mg)	NCp-NiV purity (%)	Yield (%)	Purification factor
(A) Washing buffer without glycerol						
Unclarified feedstock (5% (w/v) biomass)	250	1045.6	58.6	5.6		
Flowthrough	250	936.0	10.4	1.1		
Washing	100	28.9	11.4	39.4		
Elution 1	50	24.6	2.1	8.4		
Elution 2	50	56.1	34.4	60.5	58.7	10.8
Operation time: 2 h 55 min				Flow rate: 178 cm/h		
Purification stage	Total volume (ml)	Total protein (mg)	Amount of NCp-NiV (mg)	NCp-NiV purity (%)	Yield (%)	Purification factor
(B) Washing buffer with 10% (v/v) glycerol						
Unclarified feedstock (5% (w/v) biomass)	250	1110.9	63.0	5.7		
Flowthrough	250	938.1	10.8	1.2		
Washing	50	77.1	1.3	1.7		
Elution 1	50	24.7	0.4	1.7		
Elution 2	50	71.0	50.5	71.1	80.1	12.5

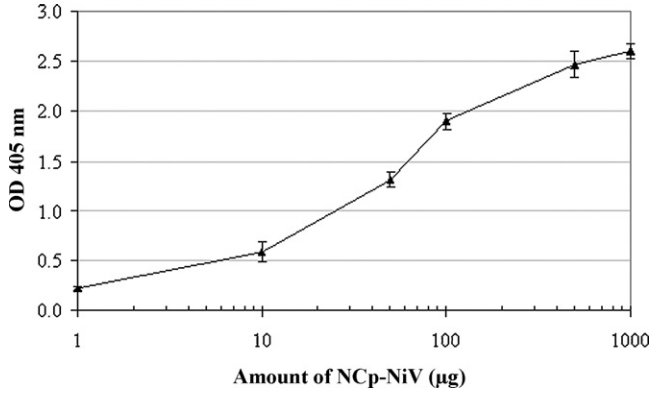

Fig. 6. ELISA analysis of the purified NCp-NiV samples from HI-EBAC operation with the rabbit anti-NiV antibody. The uninfected rabbit serum was used as the negative control in every assay. The mean ± standard deviation of triplicate assays is shown.

4. Conclusion

HI-EBAC has been applied successfully to purify the NCp-NiV from a DNase-treated unclarified *E. coli* homogenate. The EBAC mode combined clarification, capture and purification of the NCp-NiV in a single step process, thus gave rise to a high product recovery. The washing buffer containing 10% (v/v) glycerol has enhanced the removal of contaminant proteins efficiently and thus reduced the volume of washing buffer. The elution of the NCp-NiV using a step gradient elution buffer containing 410 mM and 205 mM salt concentration has eluted successfully all the adsorbed NCp-NiV which led to a yield of 80% and purification factor of 12.5.

Acknowledgements

This study was funded by the Research University Grant Scheme (Project No: 05/01/07/0225RU) from the Universiti Putra Malaysia. F.C. Chong was supported by Universiti Malaysia Pahang.

References

[1] F.B. Anspach, D.H. Curbelo, R. Garke, W.D. Deckwer, J. Chromatogr. A 865 (1999) 129.
[2] H.A. Chase, Trends Biotechnol. 12 (1994) 296.
[3] Y.P. Tan, T.C. Ling, K. Yusoff, W.S. Tan, B.T. Tey, J. Microbiol. 43 (2005) 295.
[4] R. Hjorth, Rev. TBTech. 15 (1997) 230.
[5] C.W. Ho, W.S. Tan, S. Kamaruddin, T.C. Ling, B.T. Tey, Biotechnol. Appl. Biochem. 50 (2008) 49.
[6] J. Abendroth, S. Chatterjee, D. Schomburg, J. Chromatogr. B 737 (2000) 187.
[7] M.P. Smith, M.A. Bulmer, R. Hjorth, N.J. Titchener-Hooker, J. Chromatogr. A 968 (2002) 121.
[8] F. Poulin, R. Jacquemart, G. de Crescenzo, M. Jolicoeur, R. Legros, Biotechnol. Prog. 24 (2008) 279.
[9] Y.K. Chang, I.P. Chang, Biochem. Eng. J. 30 (2006) 63.
[10] E. Güzeltunç, K.O. Ulgen, J. Chromatogr. A 914 (2001) 67.
[11] H.J. Johansson, C. Jagersten, J. Shiloach, J. Biotechnol. 48 (1996) 9.
[12] R. Bermejo, F.G. Acién, J. Ibáñez, J.M. Fernández, E. Molina, J.M. Alvarez-Pex, J. Chromatogr. B 790 (2003) 317.
[13] Z. Zhang, S. Burton, S. Williams, E. Thwaites, A. Lyddiatt, Bioseparation 10 (2001) 113.
[14] H.M. Fernández-Lahore, R. Kleef, M.R. Kula, J. Thömmes, Biotechnol. Bioeng. 64 (1999) 484.
[15] X.-D. Tong, T.Y. Sun, Biochem. Eng. J. 16 (2003) 265.
[16] F. Raymond, D. Rolland, M. Gauthier, M. Jolivet, J. Chromatogr. B 706 (1998) 113.
[17] Y.K. Chang, H.A. Chase, Biotechnol. Bioeng. 49 (1996) 512.
[18] C.J. Fee, Chem. Eng. Proc. 40 (2001) 329.
[19] H.A. Chase, N.M. Draeger, J. Chromatogr. 597 (1992) 129.
[20] W.S. Tan, S.T. Ong, M. Eshaghi, S.S. Foo, K. Yusoff, J. Med. Virol. 73 (2004) 105.
[21] M. Eshaghi, W.S. Tan, S.T. Ong, K. Yusoff, J. Clin. Microbiol. 43 (2005) 3172.
[22] S.T. Ong, K. Yusoff, C.L. Kho, J.O. Abdullah, W.S. Tan, J. Gen. Virol. 90 (2009) 392.
[23] F. Yu, N.S. Khairullah, S. Inoue, V. Balasubramaniam, S.J. Berendam, L.K. Teh, N.S.W. Ibrahim, S.A. Rahman, S.S. Hassan, F. Hasebe, M. Sinniah, K. Morita, J. Clin. Microbiol. 44 (2006) 3134.
[24] F.C. Chong, W.S. Tan, D.R.A. Biak, T.C. Ling, B.T. Tey, Sep. Purif. Technol. (2009), doi:10.1016/j.seppur.2009.11.007.
[25] I. Theodossiou, O.R.T. Thomas, J. Chromatogr. A 971 (2002) 73.
[26] F.C. Chong, W.S. Tan, D.R.A. Biak, T.C. Ling, B.T. Tey, J. Chromatogr. B 877 (2009) 1561.
[27] C.W. Ho, W.S. Tan, F.C. Chong, T.C. Ling, B.T. Tey, J. Microbiol. Biotechnol. 19 (2009) 416.
[28] X.-D. Tong, Y. Sun, J. Chromatogr. A 943 (2001) 63.
[29] Y.P. Tan, T.C. Ling, W.S. Tan, K. Yusoff, B.T. Tey, Proc. Biochem. 41 (2006) 874.
[30] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[31] G. Bayramoglu, H.A. Oktem, M.Y. Arica, Biochem. Eng. J. 34 (2007) 147.
[32] A. Tiselius, Ark. Kemi. Min. Geol. 26B (1948) 1.
[33] J. Porath, L. Sundberg, N. Fornstedt, I. Olson, Nature 245 (1973) 465.
[34] S. Hjertén, J. Rosengren, S. Pahlman, J. Chromatogr. 101 (1974) 281.
[35] Y.T.M. Ng, W.S. Tan, N. Abdullah, T.C. Ling, B.T. Tey, J. Biotechnol. 138 (2008) 74.
[36] W.-S. Choe, R.H. Clemmitt, H.A. Chase, A.P.J. Middleberg, J. Chromatogr. A 953 (2002) 111.
[37] R.H. Clemmit, H.A. Chase, J. Chromatogr. A 874 (2000) 27.
[38] Y.T.M. Ng, W.S. Tan, N. Abdullah, T.C. Ling, B.T. Tey, J. Chromatogr. A 1172 (2007) 47.