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Purification of His-tagged hepatitis B core antigen from unclarified bacterial homogenate using immobilized metal affinity-expanded bed adsorption chromatography

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ABSTRACT

Hepatitis B core antigen (HBcAg) is used as a diagnostic reagent for the detection of hepatitis B virus infection. In this study, immobilized metal affinity-expanded bed adsorption chromatography (IMA-EBAC) was employed to purify N-terminally His-tagged HBcAg from unclarified bacterial homogenate. Streamline Chelating was used as the adsorbent and the batch adsorption experiment showed that the optimal binding pH of His-tagged HBcAg was 8.0 with a binding capacity of 1.8 mg per ml of adsorbent. The optimal elution condition for the elution of His-tagged HBcAg from the adsorbent was at pH 7 in the presence of 500 mM imidazole and 1.5 M NaCl. The IMA-EBAC has successfully recovered 56% of His-tagged HBcAg from the unclarified *E. coli* homogenate with a purification factor of 3.64. Enzyme-linked immunosorbent assay (ELISA) showed that the antigenicity of the recovered His-tagged HBcAg was not affected throughout the IMA-EBAC purification process and electron microscopy revealed that the protein assembled into virus-like particles (VLP).

1. Introduction

Human hepatitis B virus (HBV) is the prototype member of family *Hepadnaviridae*. Its genome is about 3.2 kb and consists of four open reading frames (ORF), namely the polymerase (P), the X, the precore/core (preC/C) and the surface (preS/S) antigen [1]. HBV infection is responsible for about one million deaths worldwide annually [1,2]. Therefore, it is always a need to develop an effective diagnostic reagent to detect the viral infection.

HBV core antigen (HBcAg) is highly immunogenic both in humans and animal models [1]. It can be expressed in bacteria [3,4], either with an N-terminal extension [5–7] or a C-terminal fusion tag [8]. The recombinant HBcAg self-assembles into virus-like particles (VLP) with two distinctive triangulation numbers, $T=3$ and $T=4$ [9]. The immunodominant region of HBcAg is located at the tip of spikes that protrude from the surface of the viral capsid [10]. Due to its ability to form VLPs, HBcAg has been exploited extensively as a platform for the presentation of foreign epitopes [11]. Thus, it is of importance to establish an effective method to purify the VLPs.

Expanded bed adsorption chromatography (EBAC) has been employed widely in the purification of various bioproducts such as α-amylases [12], β-galactosidase [13] and human single-chain Fv antibody [14]. This method allows direct purification of the bioproducts from unclarified feedstocks [15–18] where the additional solid–liquid separation and concentration of bioproducts are not necessary prior to the purification processes [13,16,18,19]. Hence, biodegradation or proteolysis of the target products can be circumvented as a result of the fast operation of this method [13,15,20].

Various types of adsorption such as ion-exchange [15,21–23], immobilized metal affinity (IMA) [3,19] and dye-ligand [24] affinity have been exploited extensively in the EBA operation. In an EBAC operation, the crude feedstock is loaded upwards through a resin bed with a constant linear flow velocity. The resin bed is allowed to expand to a factor of 2–3 of the initial bed height [18]. The stable bed expansion increases the voids between adsorbent particles which allow high mass transfer rate and fast adsorption of the target bioproduct onto the adsorbent.

IMAC is by far the most widely used method in the purification of His-tagged proteins [25–27]. The adsorbent matrices conjugated to iminodiacetic acid (IDA) ligands, for instance Streamline Chelat-

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ing, are commonly utilized in IMA-EBAC [13,14,20,28]. Metal ions such as nickel(II) or copper(II) [27–29] are commonly immobilized on the ligands and act as a Lewis acid that accepts electron from the histidyl residues of the His-tagged proteins [26]. In addition, adsorptive refolding of protein inclusion bodies directly from cell homogenate using IMA-EBAC was also demonstrated by Hutchinson and Chase [29].

Purification of the His-tagged HBcAg directly from crude feedstock using EBA chromatography was developed in this study. The yield, purity, intactness and antigenicity of the purified His-tagged HBcAg were determined.

2. Materials and methods

2.1. Materials

Streamline Chelating was purchased from GE Healthcare (Uppsala, Sweden). It is made of highly cross-linked 6% agarose which entraps an inert quartz core to give a mean particle density of 1.2 g/ml (data from the manufacturer's manual). It is also linked to IDA groups via stable covalent bonds. The average particle size is approximately 200 μm. Fastline™ 20 EBA column with an inner diameter of 20 mm and height of 750 mm was purchased from Upfront Chromatography (Copenhagen, Denmark). The feedstock was loaded into the column by a peristaltic pump (Watson Marlow, UK).

2.2. Cultivation of cells expressing His-tagged HBcAg

E. coli BL21 (DE3) expressing His-tagged HBcAg (His-β-L-HBcAg) was cultured in LB (Luria-Bertani) broth supplemented with 100 μg/ml of ampicillin at 30 °C on a shaking incubator as described in Yap et al. [6]. The culture was induced with isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM when the OD_{600} reached about 0.6–0.8. The cells were pelleted by centrifugation at 4000 × *g* at 4 °C for 10 min and subsequently resuspended in binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 8) to a biomass concentration of 5 and 10% (w/v).

2.3. Cell disruption

2.3.1. Ultrasonication

The cell suspension at 10% (w/v) biomass was homogenized according to the conditions as described previously [6] with slight modifications. However, the sonication time was prolonged to 30 min due to higher biomass concentration compared to that of Yap et al. [6].

2.3.2. Bead milling

The cell suspension at 10% (w/v) biomass concentration was homogenized by a bead mill (Dynomill Type Multilab, Switzerland) at an impeller tip speed of 10 m/s for 10 min at 4 °C [23].

2.4. Measurement of viscosity

Prior to the measurement of viscosity, the cell lysate was added with DNase I (10 μg/ml). The viscosity of cell homogenate was determined with a viscometer (Brookfield DV-II+, USA) equipped with a spindle SC 18 that rotated at a speed of 60 rpm.

2.5. Optimization of adsorption and elution of His-tagged HBcAg

2.5.1. Binding pH

Nickel(II) (0.1 M)-charged adsorbent (1 ml) was pre-equilibrated with 5 ml of binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 5–9) and then mixed with 1 ml of 10% (w/v) biomass. The mixture was incubated at room temperature on a rotator (Stuart Scientific, UK) for 2 h. The supernatant was collected from the settled adsorbent. The amount of unbound His-tagged HBcAg in the supernatant was quantified.

2.5.2. Elution conditions

Optimization of elution conditions of bound His-tagged HBcAg was performed in batch mode. 0.1 M nickel(II) charged adsorbent was incubated with unclarified feedstock of His-tagged HBcAg for 2 h at room temperature. The supernatant was decanted and adsorbent was washed thoroughly with the binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 8). The adsorbed protein was eluted with sodium phosphate (20 mM) buffer in a linear gradient in which the pH ranged from 6 to 8 and contained 0.5–2.0 M sodium chloride and 50–500 mM imidazole.

2.6. Equilibrium adsorption isotherm

The unclarified feedstock containing different concentrations of His-tagged HBcAg was prepared in the binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 8). The biomass was mixed with 1 ml of pre-equilibrated nickel(II) charged adsorbent and incubated on a rotator at room temperature for 2 h. Then, the supernatant was collected and the amount of unbound His-tagged HBcAg was quantified.

2.7. Operation of EBA column

The 0.1 M nickel(II) treated adsorbent was loaded manually into the column. The loading of feedstock and the collection of fractions were performed in the down-up mode.

2.7.1. Bed expansion characteristics

The characteristic of bed expansion under various feedstock concentrations was studied at room temperature. About 12-cm settled bed height of nickel(II) charged adsorbent was loaded into the column. The feedstock containing various biomass concentrations [0–10% (w/v)] was pumped into the column. Bed expansion was allowed to stabilize for 10–15 min before a reading was taken. The linear flow velocity was increased gradually and the corresponding bed expansion was recorded. The degree of bed expansion was calculated as a ratio of height of the expanded bed (H) to the settled bed height (H_0).

2.7.2. Dynamic binding capacity

The EBA column was loaded with 40 ml of nickel(II)-charged Streamline Chelating adsorbent to a settled bed height of 12 cm. The adsorbent was equilibrated with 100 ml of binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 8) at a linear flow velocity of 150 cm/h. The adsorbent bed was allowed to expand to an expansion degree of 2. Subsequently, the unclarified feedstock (100 ml) was pumped into the expanded bed and protein samples were collected in fractions (10 ml per fraction) from the outlet of the column and the concentration of His-tagged HBcAg was quantified. The dynamic binding capacity (Q_B) (mg of protein adsorbed per ml of settled adsorbent) was calculated as follows:

$$Q_B = \frac{C_0 V_b}{V_s} \quad (1)$$

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

2.7.3. Direct purification of His-tagged HBcAg from unclarified feedstock

The purification of His-tagged HBcAg via IMA-EBAC was performed in down-up mode at room temperature. About 40 ml of nickel(II) charged Streamline Chelating adsorbent was loaded into the EBA column to a bed height of 12 cm. In order to avoid the leaching of nickel(II) ions throughout the purification of His-tagged HBcAg, the adsorbent was first washed with 100 ml of elute buffer (20 mM sodium phosphate, 500 mM imidazole, 1.5 M sodium chloride, pH 7) at 150 cm/h. Then, the adsorbent was equilibrated with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, pH 8). The adsorbent bed was allowed to expand and stabilize to a degree of 2. The unclarified feedstock (100 ml) was then loaded into the column at a linear flow velocity of 150 cm/h, followed by washing with 100 ml of wash buffer A (20 mM sodium phosphate, pH 6, 10 mM imidazole, 500 mM sodium chloride, 10% glycerol) and 100 ml of wash buffer B (20 mM sodium phosphate, pH 6, 50 mM imidazole, 500 mM sodium chloride). Elution of His-tagged HBcAg was also performed in down-up mode with 50 ml of elute buffer (20 mM sodium phosphate, 500 mM imidazole, 1.5 M sodium chloride, pH 7). Protein samples were collected in fractions (10 ml per fraction) and analyzed for the amount of total protein and His-tagged HBcAg.

2.8. Ultracentrifugation

The cell homogenate was spun down at 4000 × *g* for 10 min at 4 °C. The suspended His-tagged HBcAg was precipitated with ammonium sulfate at 35% saturation and dialyzed overnight at 4 °C against 2 L of dialysis buffer (50 mM Tris–HCl, 150 mM sodium chloride, pH 8). The dialyzed sample was then separated from contaminating proteins and debris by sucrose density gradient [8–40% (w/v)] ultracentrifugation as described by Tan et al. [4]. The protein samples were collected in fractions (500 μl per fraction) and analyzed on 15% (w/v) – sodium-dodecyl-sulfate polyacrylamide gels.

2.9. Protein analysis

The eluted His-tagged HBcAg was analyzed on a sodium-dodecyl sulfate polyacylamide (15%) gel [30] while the amount was quantified by the Bradford assay [31]. An imaging system (GelDoc Bio-Rad) linked to the Quantity One Quantitation software was used to quantify the amount of His-tagged HBcAg. An area of interest on the polyacrylamide gel was scanned and the purity of His-tagged HBcAg was calculated as a relative quantity of the His-tagged HBcAg band on the gel against the total amount of protein determined by the Bradford assay.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The antigenicity of purified His-tagged HBcAg was determined by ELISA as described previously by Yap et al. [6]. Briefly, 50–1000 ng of His-tagged HBcAg were coated onto the wells of an ELISA plate in triplicate and incubated overnight at 4 °C. The wells were blocked with milk diluent at room temperature (1:10 dilution, Kirkegard and Perry Laboratories, Gaithersburg, U.S.) for 2 h and then incubated with anti-HBc monoclonal antibody (1:2500 dilution, Chemicon, Temecula, U.S.) for 1 h. The wells were washed three times with TBS-T (50 mM Tris–HCl, pH 7.6; 150 mM NaCl; 0.1% Tween 20). Then, the wells were incubated with goat anti-mouse IgG conjugated to alkaline-phospatase (1:2500 dilution; Kirkegard and Perry Laboratories, Gaithersburg, U.S.) for 1 h and washed three times with TBS-T. The substrates, nitro-blue tetrazolium chloride (NBT; Fermentas, Glen Burnie, U.S.) and 5-bromo-4-chloro-3′-indolyl phosphate (BCIP; Fermentas, Glen Burnie, U.S.) were added to the wells for colour development.

2.11. Calculations

Purity is defined as

$$\text{Purity} = \frac{\text{Amount of recovered His-tagged HBcAg}}{\text{Amount of total protein}} \quad (2)$$

Yield is represented by

$$\text{Yield (\%)} = \frac{\text{Amount of recovered His-tagged HBcAg}}{\text{Amount of His-tagged HBcAg in the feedstock}} \times 100\% \quad (3)$$

Purification factor is defined as

$$\text{Purification factor (PF)} = \frac{\text{Purity of His-tagged HBcAg}}{\text{Purity of His-tagged HBcAg in the feedstock}} \quad (4)$$

3. Results and discussion

3.1. Homogenization

Bead milling and ultrasonication were employed to disrupt the bacterial host (*E. coli*). Feedstock containing 10% (w/v) of biomass was prepared in the binding buffer and then subjected to ultrasonication and bead milling separately. Bead mill disruption produced feedstock with a significantly low viscosity (2.4 cP) compared to that of ultrasonication (3.5 cP; Fig. 1). The viscosity of the feedstock was further reduced by the addition of DNase I in which the viscosity was reduced by 20 and 15% in ultrasonicated and bead mill disrupted feedstocks, respectively (Fig. 1). Genomic DNA was released along with the target protein after the disruption of bacterial host and this increased the viscosity of the solution [32]. Indeed, genomic DNA induced cross-linking of adsorbent and formed an aggregated expanded bed; hence it caused channelling in the fluidized bed due to an increase in the bed voidage [33,34]. Therefore, addition of DNase I can prevent the formation of the aggregation network that interferes the stability of the expanded bed [13,23]. As a result, the feedstock was disrupted using a bead mill and the viscosity of the unclarified cell lysate reduced tremendously by the addition of DNase I to a final concentration of 10 μg/ml before it was loaded into the column.

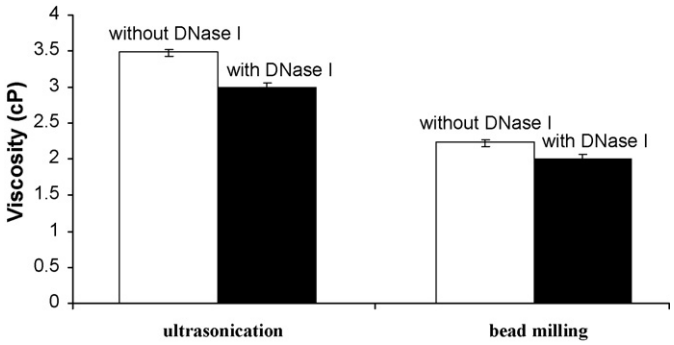

Fig. 1. Viscosity measurement of 10% (w/v) unclarified feedstock after ultrasonication and bead milling homogenization.

Table 1
Optimization of the binding pH of His-tagged HBcAg onto 0.1 M Ni^{2+}-charged adsorbent.

	pH				
	5.00	6.00	7.00	8.00	9.00
Binding capacity (mg/ml adsorbent)	0.60	0.51	1.20	1.80	0.90

3.2. Optimization of binding pH

pH plays an essential role in the binding of a target protein onto the adsorbent because it can easily influence the binding strength between functional groups on the resin and target protein [34]. Among the pH values examined in this study, pH 8 gave the highest protein binding efficiency (Table 1). This is in good agreement with the finding of Tan et al. [35] who showed that binding of His-tagged nucleocapsid protein of Newcastle disease virus onto immobilized metal ions was enhanced at a pH higher than the pK_a of histidine residues (pK_a ~6–7). Besides, the concentration of hydrogen ion (H^+) increases in an acidic pH. This creates a competitive adsorption between H^+ and Ni^{2+} [36]. This resulted in low binding of the His-tagged HBcAg onto immobilized Ni^{2+} at pH 5 and 6. As a result, sodium phosphate binding buffer was adjusted to pH 8 prior to any experimental applications in this study.

3.3. Elution conditions

Three parameters were investigated in order to determine the optimal conditions to elute the His-tagged HBcAg from the adsorbent after protein adsorption. Imidazole is commonly used as a competitive agent to displace proteins from metal ions as it is inexpensive and barely affects the biological properties of proteins [13]. The amount of eluted His-tagged HBcAg increased gradually with a rise in the imidazole concentration. About 30% of His-tagged HBcAg (0.6 mg) was successfully recovered with a purity of 0.75 (Table 2(a)). The result shows that the buffer containing 50 mM imidazole eliminated the majority of host cell proteins (HCPs) efficiently with minimum amount of target protein in the eluate (Table 2(a)). Therefore, 50 mM imidazole was incorporated in the wash buffer B to remove loosely bound contaminants from the adsorbent.

The yield was increased from 5 to 35% when the concentration of sodium chloride in the elution buffer was increased from 0.5 to 2.0 M, while the purity of the target protein was increased from 0.05 to 0.70 (Table 2(b)). Indeed, Gaberc-Porekar and Menart [26] reported that relatively high-ionic strength (containing 0.1–1.0 M sodium chloride) reduced the nonspecific electrostatic interactions between contaminating proteins and adsorbent particles. This is further supported by the finding of Clemmitt and Chase [13] in which the ionic and hydrophobic forces were interrupted by high-ionic strength.

Elution buffers with relatively low pH are frequently used for the elution of target protein [26]. However, a decrease in pH lower than 6 tends to cause protein degradation especially for pH sensitive proteins [13,26]. Therefore, pH 6, 7 and 8 were examined for the elution of His-tagged HBcAg in this study. The purity of the eluted His-tagged HBcAg increased drastically from 0.1 to 0.7 when the pH value was increased from 6 to 8 (Table 2(c)). This is in good agreement with that reported by Stein and Kiesewetter [37] whereby the majority of HCPs were removed at pH 6. In addition, the yield was increased to 45% at pH 7. Therefore, pH 6 and 7 were adopted in the wash and elute buffers while 1.5 M sodium chloride was incorporated in the elute buffer for the purification of His-tagged HBcAg in the IMA-EBAC operation.

3.4. Equilibrium adsorption isotherm

The adsorption isothermal data of His-tagged HBcAg onto Streamline Chelating were fitted with the Sigma Plot version 11 and the fitted curve showed that the data obeyed the Langmuir's adsorption isotherm as shown in Fig. 2 (R^2 = 0.9989),

$$q = \frac{q_{max}c}{k_e + c} \quad (5)$$

where q is the amount of target protein adsorbed onto the resin while c represents the equilibrium concentration of unbound target protein in the suspension. q_{max}, the maximum binding capacity and k_e, the equilibrium constant can be derived from the linear regression of the Langmuir's plot. The maximum binding capacity (q_{max}) of His-tagged HBcAg was 5.6 mg per ml of adsorbent whilst its equilibrium constant (k_e) was 3.3 mg/ml. Tsai et al. [38] reported that high molecular mass of a target protein is a limiting factor that causes low maximum binding capacity of the target protein onto the adsorbent. Therefore, the low maximum binding capac-

Table 2
(a) The effect of the concentration of imidazole on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent. (b) The effect of the concentration of NaCl on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent. (c) The effect of pH on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent.

Concentration of imidazole (mM)	Total protein in the elution (mg)	His-tagged HBcAg in the elution (mg)	Yield (%)	Purity
(a) The effect of the concentration of imidazole on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent				
50	1.10	0.10	5.00	0.09
100	0.83	0.25	12.50	0.30
200	0.83	0.25	12.50	0.30
300	0.83	0.25	12.50	0.30
400	0.89	0.40	20.00	0.45
500	0.80	0.60	30.00	0.75
Concentration of sodium chloride (M)	**Total protein in the elution (mg)**	**His-tagged HBcAg in the elution (mg)**	**Yield (%)**	**Purity**
(b) The effect of the concentration of NaCl on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent				
0.5	1.80	0.10	5.00	0.05
1.0	1.67	0.50	25.00	0.30
1.5	1.75	0.70	35.00	0.40
2.0	1.00	0.70	35.00	0.70
pH	**Total protein in the elution (mg)**	**His-tagged HBcAg in the elution (mg)**	**Yield (%)**	**Purity**
(c) The effect of pH on the elution of His-tagged HBcAg from the IMA-EBAC adsorbent				
6	3.00	0.30	15.00	0.10
7	1.29	0.90	45.00	0.70
8	1.14	0.80	40.00	0.70

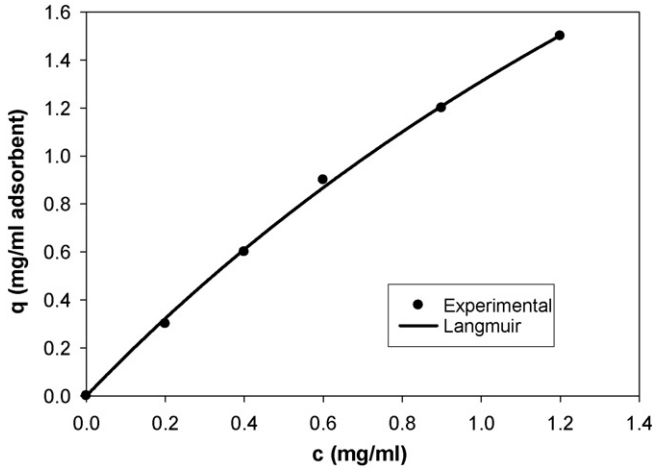

Fig. 2. The equilibrium adsorption isotherm of His-tagged HBcAg. The adsorption isotherm was fitted using the Sigma Plot version 11. A series of different concentrations of His-tagged HBcAg was prepared in unclarified feedstock and allowed to adsorb onto Streamline Chelating adsorbent until equilibrium was achieved.

ity of the His-tagged HBcAg onto the adsorbent could be due to the high molecular mass of the capsid containing 180 and 240 subunits of HBcAg. The bound HBcAg capsid exerts a steric hinderance to other target protein that binds concomitantly onto the adsorbent. Besides, cell debris also forms unspecific interactions with the adsorbent through the surface charge, thereby reducing the binding capacity of the target protein to the adsorbent [39,40].

3.5. Bed expansion characteristics

The mode of bed expansion affects significantly the protein adsorption and yield. The bed expansion was examined using the binding buffer containing 0, 5 and 10% (w/v) biomass. The bed height increased linearly with the linear flow velocity as shown in Fig. 3. The increased bed height resulted in an increase in the bed void and this is best characterized by the Richardson–Zaki equation:

$$U = U_t \varepsilon^n \quad (6)$$

where U represents the superficial velocity, ε and U_t are the bed void and terminal velocity of settling resin, respectively. The bed void, ε can be calculated from the following equation:

$$\frac{H}{H_0} = \frac{(1-\varepsilon_0)}{(1-\varepsilon)} \quad (7)$$

The settled bed void, ε_0 is always regarded as 0.4 [39,41].

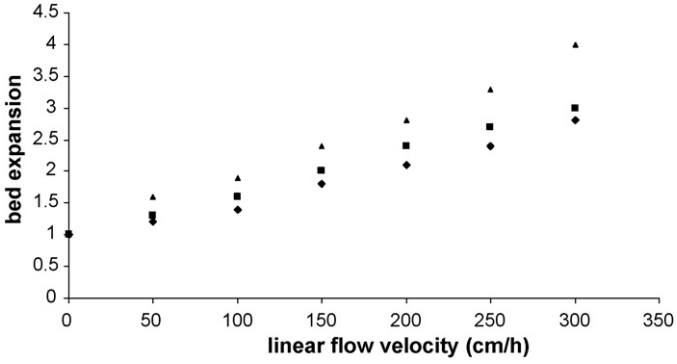

Fig. 3. Bed expansion characteristic of the Streamline Chelating adsorbent in the binding buffer containing 0, 5 and 10% biomass: (♦) 0%, (■) 5% and (▲) 10% biomass.

Table 3
The Richardson–Zaki coefficient, n obtained from a linear regression of Richardson–Zaki equation.

	Richardson–Zaki coefficient, n
Binding buffer (20 mM sodium phosphate, pH 8, 500 mM NaCl, 10 mM imidazole)	3.6
5% (w/v) unclarified feedstock	4.2
10% (w/v) unclarified feedstock	5.4

The values of n were derived from a linear regression of the Richardson–Zaki equation and are summarized in Table 3. The n value ranges from 3.6 to 5.4 which are reasonably close to the value of 4.8 in the laminar flow regime [39,41]. The n values obtained show that the expanded bed was stable in the presence of biomass. The bed expansion of 0 and 5% (w/v) biomass of feedstock was approximately 2.5 at a linear flow velocity of 300 cm/h (Fig. 3). On the other hand, the high bed expansion in 10% (w/v) biomass is most likely due to its high viscosity (Fig. 1). The high viscosity resulted by high DNA content in the feedstock can easily lead to channelling, enlargement of bed void and eventually collapse of the expanded bed [15,33,34]. As a result, 5% (w/v) biomass was applied at a linear flow velocity of 150 cm/h in the IMA-EBAC operation which the bed expansion was maintained at 2.

3.6. Dynamic binding capacity

Frontal analysis was performed to determine the dynamic binding capacity (Q_B) of Streamline Chelating adsorbent for His-tagged HBcAg in IMA-EBAC. Q_B helps to estimate the maximum volume of the unclarified feedstock that can be loaded into the column prior to the breakthrough. Fig. 4 presents the breakthrough curve of direct capture of His-tagged HBcAg from unclarified feedstock. The Q_B of the adsorbent at 10% breakthrough was derived from Eq. (1). About 5.6 mg of target protein per ml of adsorbent was recovered and this corresponds well to the q_{max} (5.6 mg/ml of adsorbent) obtained in this study. This phenomenon is probably due to the majority of contaminants were readily removed from the expanded bed during the application of the wash buffers in the IMA-EBAC operation. However, similar to the maximum binding capacity, the low Q_B was most likely due to the high molecular weight of the target protein that inhibits its binding onto the adsorbent.

3.7. Direct purification of His-tagged HBcAg from unclarified feedstock

The optimal conditions described above were employed in the direct purification of His-tagged HBcAg using IMA-EBAC. The

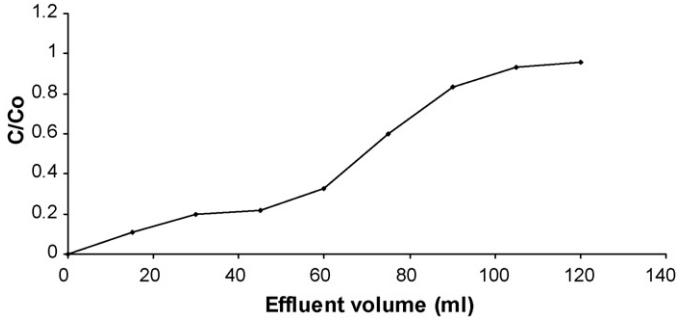

Fig. 4. The breakthrough curve of the His-tagged HBcAg from the expanded bed at a linear velocity of 150 cm/h. The volume of effluent at which 10% of breakthrough occurred was used to quantify the dynamic binding capacity of Streamline Chelating adsorbent. The settled bed height, V_s was about 12 cm and the initial concentration of feedstock was 15 mg/ml.

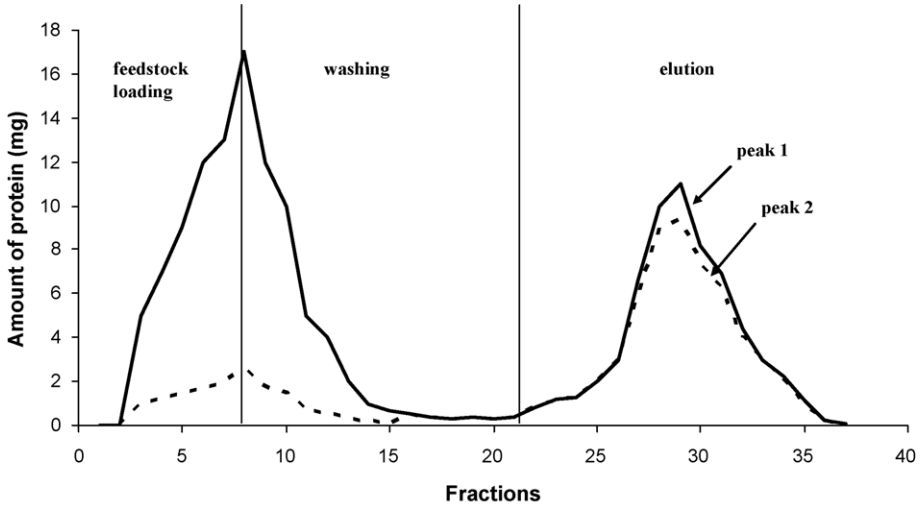

Fig. 5. The purification profile of His-tagged HBcAg from 5% unclarified feedstock via IMA-EBAC. 10 ml of sample was collected per fraction. Peak 1 indicates the washing profile for the removal of weakly bound host cell proteins while peak 2 represents the elution profile for the elution of His-tagged HBcAg from the IMA-EBAC adsorbent.

nickel(II)-charged Streamline Chelating adsorbent was loaded into an EBA column and pre-equilibrated with binding buffer (pH 8) at a linear flow velocity of 150 cm/h prior to the loading of unclarified feedstock. Several reports [13,14,27] suggest that use of low-concentration-imidazole containing binding buffers which can reduce the unspecific binding of contaminating proteins onto the adsorbent. Therefore, 10 mM imidazole was included in the binding buffer.

The loading of unclarified feedstock was performed after the expanded bed became stable at an expansion degree of 2. A linear flow velocity of 150 cm/h was applied throughout the purification process. Wash buffer A (20 mM sodium phosphate, pH 6, 500 mM NaCl, 10 mM imidazole and 10% glycerol) was entailed to remove the majority of cell debris and contaminating proteins. The adsorption of weakly bound HCPs onto the resin was reduced under acidic pH [37], thus, allowing HCPs to be removed from the adsorbent during washing step. Besides, 10% glycerol was also used to remove residual particulates and debris that entrapped in the fluidised adsorbent [39]. The removal of HCPs was further enhanced by feeding wash buffer B containing 50 mM imidazole. A rather low concentration of imidazole is commonly recommended in the subsequent washing in order to maximize the removal of contaminating proteins but minimize the desorption of the target protein [13].

Fig. 5 demonstrates the purification profile of His-tagged HBcAg and Fig. 6 shows the protein samples collected via IMA-EBAC and analyzed on a 15% polyacrylamide gel. The majority of weakly bound HCPs were removed during the washing steps. The bound His-tagged HBcAg was successfully eluted from the adsorbent with the elute buffer (pH 7) containing 500 mM imidazole and 1.5 mM NaCl. A yield of 56% and purity factor of 3.64 were obtained from the elution step (Table 4).

Table 4
Purification of His-tagged HBcAg from the IMA-EBAC operation.

	Amount of total protein (mg)	Amount of His-tagged HBcAg (mg)	Purity	Yield (%)
Feedstock	436.30	109.07	0.25	100.00
Flow-through	325.00	32.50	0.12	29.00
Washing	43.00	6.45	0.15	6.00
Elution	68.00	62.00	0.91	56.00

Hu et al. [42] reported that the leaching of nickel(II) ions from Ni-IDA was approximately 15–60 ppm, while Ni-NTA showed a nickel(II)-ion-leakage that ranged from 5 to 25 ppm during the purification of His-tagged VP3 protein of infectious bursal disease virus. This is likely due to a weaker coupling between Ni-IDA than that of Ni-NTA. Therefore, leakage of nickel(II) ions is expected in the purification of His-tagged HBcAg using IMAC in an expanded bed mode. However, the free nickel(II) ions can be removed by passing through a non-charged IMAC column [43].

3.8. Antigenicity of His-tagged HBcAg

The effects of pH on the antigenicity of His-tagged HBcAg were examined using ELISA. The His-tagged HBcAg remained antigenic in pH 6, 7 and 8. However, its antigenicity was affected drastically in pH 5 and 9. The absorbance readings obtained were only 0.5 and 0.8 when compared to that of pH 6, 7 and 8 (Fig. 7(a)). Fig. 7(b) shows that the EBA-eluted His-tagged HBcAg remained antigenic although the purification process was carried out at room temperature. The absorbance readings increased with the amount of protein

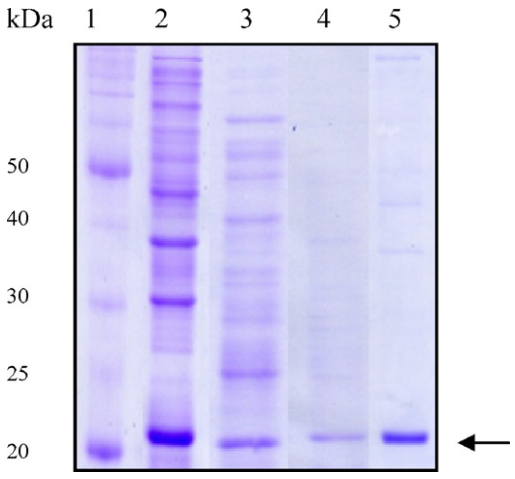

Fig. 6. SDS-PAGE of the purification of His-tagged HBcAg with IMA-EBAC. Samples collected at the binding, washing and elution steps were pooled separately and analyzed on a 15% polyacrylamide gel. Lane: 1, molecular weight markers in kDa; lane 2, 5% unclarified feedstock; lane 3, flow-through; lane 4, washing with wash buffers A and B; lane 5, eluted His-tagged HBcAg. The arrow indicates the protein band of His-tagged HBcAg (~21 kDa).

Table 5
Comparison between IMA-EBAC and sucrose density gradient ultracentrifugation for the purification of His-tagged HBcAg.

	Purification steps	Operation time (h)	Total protein (mg)	Total His-tagged HBcAg (mg)	Total eluted protein (mg)	Total eluted His-tagged HBcAg (mg)	Yield (%)	Purity	Purification factor
IMA-EBAC	Single	2–3	436.30	109.08	68.00	62.00	56.00	0.91	3.64
Ultracentrifugation	Multiple	48–72	4.23	1.06	0.64	0.60	57.00	0.93	3.72

coated onto the wells in an ELISA and the lowest concentration of the protein (50 ng) gave a significant reading of ~0.5, demonstrating that the antigenicity of the purified His-tagged HBcAg is in good agreement with that demonstrated by Yap et al. [6]. Besides, the eluted His-tagged HBcAg also formed particles similar with that reported by Yap et al. [6] when viewed under a transmission electron microscope (Fig. 8). In fact, a reduction of purification steps in the IMA-EBAC is advantageous in minimizing the possibility of proteolytic damage and other functional destructions [13].

3.9. Comparison of IMA-EBAC and sucrose density gradient centrifugation for the purification of His-tagged HBcAg

Table 5 summarizes the differences between IMA-EBAC and sucrose density gradient ultracentrifugation in the purification of His-tagged HBcAg from an unclarified feedstock. IMA-EBAC is more advantageous as it allows the purification of His-tagged HBcAg from an unclarified feedstock in a short duration. Sucrose density gradient centrifugation is only suitable for lab-scale purification of the target protein because it is a multi-step operation that involves feedstock clarification and ammonium sulfate precipitation [39,44] prior to the ultracentrifugation. This might be detrimental to the proteins especially those are sensitive to proteolysis and time-dependent modifications [13]. The purity of His-tagged HBcAg purified from IMA-EBAC is almost similar to that of sucrose density gradient centrifugation (Table 5).

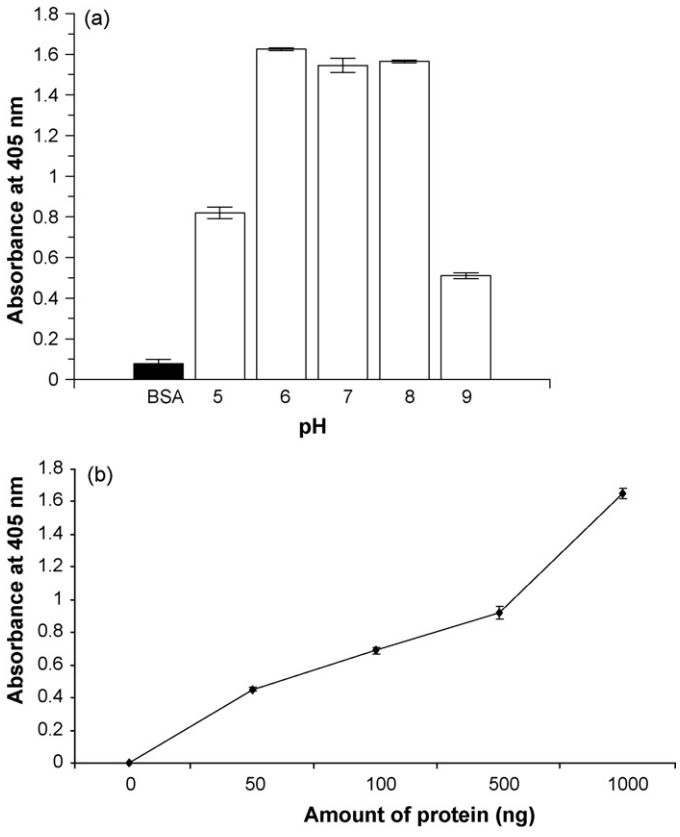

Fig. 7. Determination of the antigenicity of His-tagged HBcAg using ELISA. (a) The antigenicity of His-tagged HBcAg collected in different pH during the batch mode optimization. One microgram of protein was coated onto the ELISA-plate wells. Bovine serum albumin (BSA) was used as a negative control. (b) His-tagged HBcAg purified from the EBA column. Different concentrations of proteins were coated onto the ELISA-plate wells. The absorbance readings are mean ± standard deviation of triplicate determinations.

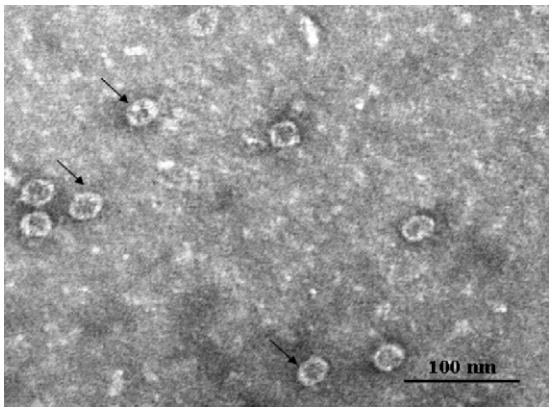

Fig. 8. Transmission electron micrograph of the purified His-tagged HBcAg. The arrows indicate the virus-like particles of His-tagged HBcAg. The diameter of the His-tagged HBcAg is approximately 30 nm.

4. Conclusion

The results obtained in the current study demonstrate that IMA-EBAC provides an ideal alternative for the purification of His-tagged HBcAg. It allows direct adsorption of the target protein onto the adsorbent from the unclarified bacterial homogenate without impairing the biological properties of His-tagged HBcAg which assembles into VLPs. Target protein with a purification factor of 3.64 was eluted from the column. The antigenicity of His-tagged HBcAg and its particulate form were preserved throughout the purification process. Therefore, this study demonstrated an easy, cost- and labour-effective method in the purification of VLPs using IMA-EBAC.

Nomenclature

q	amount of target protein adsorbed onto the resin
c	concentration of unbound target protein in the suspension
q_{max}	maximum binding capacity
k_e	equilibrium constant
U	superficial velocity
ε	bed void
U_t	terminal velocity of settling resin
H	height of the expanded bed
H_0	settled bed height
cP	centipoises

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