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Purification of recombinant hepatitis B core antigen from unclarified *Escherichia coli* feedstock using phage-immobilized expanded bed adsorption chromatography

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ABSTRACT

Fusion M13 phage with disulfide constrained heptapeptide, C-WSFFSNI-C, inserted into the minor coat protein (gpIII), has been selected in the current study as ligand in direct purification of hepatitis B core antigen (HBcAg) from unclarified *Escherichia coli* (*E. coli*) feedstock. The selected fusion phage showed strong association with the surface of the core particle. In the present study, this fusion M13 phage was immobilized onto Streamline base matrix via epoxy activation and used as adsorbent to capture HBcAg from crude *E. coli* homogenate. The maximum binding capacity for the adsorbent was 3.76 mg/mL with equilibrium coefficient of 1.83 mg/mL. Due to the slow uptake rate of HBcAg by M13 phage-immobilized adsorbents, a modified EBAC operation with recirculation of feedstock into the expanded bed has been investigated in this study. The introduction of feedstock recirculation has led to an 18% increase in yield; however, the purity of the eluted product was reduced by 15% compared with typical EBAC operation. The level of antigenicity exhibited by the core particles purified by both EBAC operations employed in the present study was comparable to that purified using sucrose ultracentrifugation.

1. Introduction

Hepatitis B core antigen (HBcAg) forms the empty and non-infectious recombinant core particles, which are immunologically and morphologically indistinguishable from the native capsids. HBcAg can be efficiently produced in great variety of expression host systems [1–4]. The particles produced in bacteria can be separated into two sizes; 32 nm particles with triangulation number $T=3$ (180 subunits) or 36 nm particles with $T=4$ (240 subunits) icosahedral symmetry [5]. Due to its unique ability to self assemble into particles and induce strong immune responses, it has been widely studied as a promising carrier for various foreign epitopes in the development of vaccines, diagnostic kit and also as a gene therapy tool [6]. Ultracentrifugation [7], size-exclusion [8,9] and packed bed chromatography [10] are the most common method employed in HBcAg purification. Although these methods are able to yield purity of more than 90%, they require multi-conditioning steps that dictate higher production cost. In view of these, EBAC has been employed as a primary capture step to purify HBcAg from particulate containing feedstock using anion exchangers [11]. However, due to the unspecific binding of the negatively charged host cell biomolecules at the operating condition to the anion exchangers, the purity obtained was lower compared to the conventional methods. Recently, Yap et al. [12] have demonstrated the usage of metal chelated adsorbents in purifying His-tagged HBcAg from unclarified feedstock. Although the yield and purity are comparable to that obtained using the conventional method, additional step will be required to cleave off the His tag that may compromise the product safety and efficacy, particularly when clinical use is concerned [13].

Therefore, this study has taken the advantage of phage display system to develop adsorbent loaded with a more target specific ligand against HBcAg that can be incorporated in EBAC operation. Bacteriophage is unlikely to impact negatively on human health and has long been used for human therapy [14]. In addition, M13, a single stranded DNA filamentous phage has been employed to purify various bio-products such as lipase [15] and lactoferrin from defatted milk [16]. Furthermore, the association between the core particle and large surface protein (L-HBsAg) during viral morphogenesis [17] was shown to be greatly inhibited by M13 phages that carry disulfide constrained heptapeptides, bearing the sequence C-WSFFSNI-C on their gpIII proteins [18]. These

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phages bearing the sequence C-WSFFSNI-C, have also exhibited great potential in detection of HBcAg in human serum as well as in precipitation of HBcAg from bacterial lysate [19]. Therefore, the aim of this study was to develop a method to purify HBcAg from unclarified *Escherichia coli* feedstock using M13 phage bearing the heptapeptides sequence, C-WSFFSNI-C, immobilized onto epoxidated Streamline base matrix via EBAC.

2. Materials and methods

2.1. Materials

Fastline™ 20 contactor (length: 70 cm, diameter: 2 cm) from UpFront Chromatography A/S (Copenhagen, Denmark) was used throughout this study. Streamline™ Quartz Base matrix was purchased from GE Healthcare (Uppsala, Sweden). It is a macroporous adsorbent with 6% cross-linked agarose containing crystalline quartz core to give an average density of 1.2 g/mL. The bead size range is between 100 and 300 μm, with mean particle diameter of 200 μm. All the chemicals used in this study were molecular biology grade and the M13 phage bearing disulfide constrained heptapeptide C-WSFFSNI-C was from a disulfide constrained 7-mer phage display peptide library (New England Biolabs, MA, USA).

2.2. Methods

2.2.1. Preparation of M13 phage

Propagation and purification of M13 phage bearing disulfide constrained heptapeptide C-WSFFSNI-C was based on the method described by Smith and Scott [20] with some modifications. Briefly, M13 phage was grown in Luria Bertani (LB) medium containing *E. coli* ER 2738 grown at log phase. The M13 phage was then recovered by centrifugation at 6300 × *g* for 15 min at 4 °C (JA 14 rotor, Beckman J2-MI, Beckman Coulter, California, USA) and subsequently precipitated with PEG/NaCl [20% (w/v) in 2.5 M NaCl]. The M13 phage was then further purified with cesium chloride density gradient centrifugation at 174,000 × *g* for 18 h at 4 °C (Sorvall, rotor T-865, Thermo Scientific, MA, USA).

2.2.2. Epoxy activation and immobilization of M13 phage onto Streamline base matrix

Streamline™ Quartz Base matrix in this study was activated with 60% (v/v) epichlorohydrin (ECH) and 4 mol/L NaOH solution containing sodium borohydride (1.2 mg/mL of adsorbent). The reaction mixture was then mixed well at 45 °C for 3 h. The epoxidated adsorbents were then washed with copious amount of distilled water before immobilization with M13 phages suspended in 0.3 M sodium bicarbonate buffer, pH 8.5 with final concentration of 2.5×10^{12} pfu/mL of adsorbents. Immobilization of M13 phage onto the epoxidated adsorbents was performed at 25 °C for 18–20 h. The non-immobilized phages were then removed and residual reactive sites were then blocked with 1.0 M ethanolamine for 2 h at 25 °C. Excess blocking reagent was then removed by washing the adsorbents with 0.3 M sodium bicarbonate buffer, pH 8.5 and followed by 50 mM Tris–HCl, pH 8.4 prior to batch adsorption and EBAC purification of HBcAg from unclarified *E. coli* feedstock.

2.2.3. Preparation of unclarified E. coli feedstock

Preparation of unclarified *E. coli* feedstock was carried out based on the method described by Ng et al. [11]. Briefly, *E. coli* strain W31101Q harboring the pR1-11E plasmid encoding the truncated HBcAg [21] was cultivated in Luria Bertani (LB) medium containing 100 μg/mL ampicillin and incubated at 37 °C with shaking at 250 rpm. HBcAg expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) with final concentration of 0.5 mM. The cells were then harvested with centrifugation at 3836 × *g* (JA 14 rotor, Beckman, Beckman Coulter, California, USA) for 15 min at 4 °C. Cell disintegration was carried out based on the method described by Ho et al. [22] using a horizontal bead-mill (Type Multilab, Willy A. Bachofen AG, Basel, Switzerland).

2.3. Method scouting and optimization

2.3.1. Optimization of binding pH

Optimal binding pH of HBcAg with M13-immobilized adsorbents was determined based on batch adsorption experiments with unclarified feedstock containing 5% (w/v) biomass. Prior to batch adsorption, 2 mL settled volume of M13-immobilized adsorbents was equilibrated separately with 0.1 M sodium phosphate buffer, pH 6 and 50 mM Tris–HCl, pH 7–9. Equal volumes of feedstock comprising 5% (w/v) of wet biomass at different pH ranges from 6 to 9 were then added to the adsorbents. The final pH of feedstock was achieved by suspending the biomass in 0.1 M sodium phosphate buffer for pH 6, and pH 7, 8 and 9 with 50 mM Tris–HCl buffer of respective pH. The mixtures were mixed well on a roller mixer for 5 h at 25 °C in order to ensure a good mass transfer of protein to the adsorbents. The adsorbents were then allowed to sediment by gravitational force and the protein solution was removed from each reaction in order to determine the amount of total protein and HBcAg bound.

2.3.2. Optimization of elution condition

Initial screening for suitable eluting agent was carried out using various salts comprising (a) 1 M NaCl, (b) 3 M KSCN, (c) 2.5 M NaI, (d) 2 M $MgCl_2 \cdot 6H_2O$, (e) 1 M urea. All eluting solutions were buffered with 20 mM Tris–HCl (pH 8). As urea was chosen as the most suitable eluting agent to use in this study, urea concentrations ranging from 1 M to 8 M in 20 mM Tris–HCl (pH 8) were tested to determine the optimum concentration to use for efficient HBcAg elution. Briefly, 2 mL settled volume of M13-immobilized adsorbents was challenged with equal volume of unclarified feedstock containing 5% biomass for 5 h at 25 °C. Unbound or loosely bound proteins or cell debris were removed by washing the HBcAg loaded adsorbents thoroughly with 50 mM Tris–HCl buffer, pH 8.4 for two times. Following washing step, the adsorbents were added with eluting solutions. The mixtures were mixed again for 30 min at 25 °C. The purity and yield of HBcAg in elution were determined based on the SDS polyacrylamide gel electrophoresis and the Bradford assay.

2.3.3. Ligand leakage evaluation

Phage titration assay was conducted to access ligand leakage at every purification step. The amount of phage was determined based on the number of blue plaques formed on the LB agar plates and calculated as pfu. All titrations were carried out in triplicates.

2.4. EBAC operation

2.4.1. Dynamic binding capacity

Frontal analysis was carried out to determine the dynamic binding capacity of M13-immobilized adsorbents against HBcAg in unclarified feedstock. Sedimented bed height of 19 cm was first expanded to 33 cm at linear flow velocity of 101 cm/h. This was followed by loading of unclarified feedstock containing 5% (w/v) biomass. Fractions collected throughout the process were assayed for HBcAg concentrations to determine the value C/C_o. The dynamic binding capacity (Q_B) (mg of protein adsorbed per mL of settled adsorbent) can be calculated as follows:

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

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

2.4.2. Purification of HBcAg from unclarified feedstock

Two different EBAC operations were investigated in the present study; the typical single pass EBAC operation and modified EBAC operation with recirculation of depleted feedstock into the expanded bed. In both EBAC operations, approximately 60 mL of M13-immobilized adsorbents corresponding to sedimented bed height of 19 cm was used in the current study. Prior to feed loading, the adsorbents were first expanded at linear flow velocity of 101 cm/h with 50 mM Tris–HCl, pH 8.4 to give an expanded bed height of 33 cm. When the bed was stable, approximately 100 mL of unclarified feedstock containing 5% of biomass was pumped into the column and fractions of protein between 10 and 15 mL exited the column were collected. However, in the modified EBAC operation, the entire depleted feedstock exited the column during adsorption stage was directed to the expanded again for up to 1 h. This was then followed by washing with similar equilibration buffer to remove weakly bound proteins and elution was carried out using 20 mM Tris–HCl (pH 8.0) containing 3 M urea for both EBAC operations. The adsorbent bed was then regenerated using 2 M NaCl, followed by distilled water and finally stored in 0.3 M sodium bicarbonate buffer (pH 8.5) containing 0.05% sodium azide at 4 °C until the following use. Fractions collected throughout the purification process were assayed for total protein and HBcAg concentrations to determine the purity and yield of HBcAg obtained.

2.5. Quantitation of total protein and HBcAg

2.5.1. Bradford assay

The amount of total protein was estimated as described by Bradford [23] using bovine serum albumin (BSA) as standard. All the samples were analyzed in duplicate.

2.5.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were fractionated with 15% (w/w) reducing SDS-PAGE [24] using a Mini-Protein 3 apparatus (Bio-RAD, CA, USA) with a discontinuous buffer system. After electrophoresis, the gels were stained with Coomassie® Brilliant Blue R-250 (CB) (Sigma, St. Louis, USA) and destaining of the gels was carried out with solution containing 10% (v/v) methanol and 10% (v/v) acetic acid.

2.5.3. Quantitation of HBcAg

Quantitation of HBcAg was based on the method previously described by Ng et al. [25]. Quantity One® Quantitation software (Gel Doc; Bio-Rad, CA, USA) was employed to determine the amount of HBcAg in the samples. The amount of HBcAg was determined based on the relative quantity of the HBcAg band on the SDS-polyacrylamide gel against the amount of total protein obtained from the Bradford assay.

2.6. Qualitative analysis of purified HBcAg

2.6.1. Particle size analysis

Fractions collected from elution were pooled and urea present in elution was removed by dialyzing it with buffer containing 50 mM Tris (pH 8.0) and 100 mM NaCl overnight at 4 °C. The dialyzed protein solution was then concentrated using 300 kDa cut-off microconcentrator (Vivaspin, Sartorius) prior to analysis with light scattering machine (DynaPro, Wyatt Technology, CA, USA). The size of HBcAg particles purified using EBAC was analyzed based on the method as previously described by Tan et al. [7]. Particles present in the concentrated fraction were determined based on the intensity of the laser light scattered or reflected by the particles at 25 °C. The acquisition time for the machine was set to 10 s for a total of 20 acquisitions for each sample tested. Triplicates readings were carried out for each eluted fraction.

2.6.2. Antigenicity analysis

Enzyme-linked immunoassay (ELISA) was carried out to determine the antigenicity of HBcAg purified using M13-immobilized adsorbents as previously described by Ho et al. [26]. Core particles purified with sucrose gradient ultracentrifugation as described by Ng et al. [25] were used as a positive control. Briefly, the clarified cell lysate after cell disruption was precipitated using ammonium sulfate with 35% saturation. The precipitate obtained was dialyzed and subjected to 8–40% continuous sucrose gradient and centrifuged at 210,053 × *g* (SW 41 Ti rotor, Beckman, Beckman Coulter, California, USA) for 5 h at 4 °C. In ELISA, different amount of purified HBcAg (10–1000 ng; 120 μL) was coated onto U-shaped high binding microtitre plate wells (TPP, Switzerland) for 16 h at 4 °C. This was followed by blocking with 10% (w/v) BSA diluent (KPL, USA) for 2 h at 4 °C. The wells were washed three times, 5 min each, with Tris Buffer Saline [TBS; Tris–HCl (50 mM, pH 7.6, NaCl (150 mM)] supplemented with 0.05% (v/v) of Tween 20 (TBST). Anti-HBcAg monoclonal antibody [mAb clone C1-5, original concentration (8 mg/mL)]; (1:2500; 100 μL, Chemicon, MA, USA) was then added and incubated for 1 h at 25 °C. This was followed by anti-mouse antibody conjugated with alkaline phosphatase (1:2500, 100 μL; Chemicon, MA, USA). After 1 h incubation at 25 °C, unbound antibodies was decanted and the wells were washed three times, 5 min each, with TBST. This was followed by developing the assay using ρ-nitro-phenyl phosphate substrate (1 mg/mL, 200 μL; Sigma, St. Louis, USA). The intensity of the color developed was determined with absorbance at 405 nm in a microtitre plate reader (KCjunior, Bio-Tek Instrument, VT, USA). The assays were performed in triplicates.

2.7. Calculations

Purity is defined as the amount of HBcAg in relation to the total protein present in the eluted fraction:

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

Yield (%) is defined as the amount of HBcAg obtained in elution divided by the initial amount of HBcAg present in the feedstock and this is usually expressed in percentage:

$$\text{Yield (\%)} = \frac{\text{Amount of HBcAg}}{\text{Initial amount of HBcAg}} \times 100 \quad (3)$$

Purification factor (PF) is determined based on the purity of HBcAg in elution divided by the purity of HBcAg in the feedstock:

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

Elution efficiency (%) is determined based on the amount of HBcAg in elution divided by the amount of HBcAg bound to the adsorbents:

$$\text{Elution efficiency (\%)} = \frac{\text{Amount of eluted HBcAg}}{\text{Amount of bound HBcAg}} \times 100. \quad (5)$$

Table 1
Effect of different pH on the binding of HBcAg onto M13 phage-immobilized adsorbents.

Binding pH	Initial amount of HBcAg (μg)	Percentage (%) of bound HBcAg
6	1870.43	24.4
7	1870.43	35.3
8	1870.43	37.2
9	1870.43	36.5

Table 2a
Elution efficiency of different chaotropic salts as eluting agents.

Eluting agent	Amount of HBcAg bound (μg)	Elution efficiency (%)
NaCl	556.14 ± 0.02	20
KSCN	717.3 ± 0.05	6
$MgCl_2 \cdot 6H_2O$	424.61 ± 0.01	6
NaI	755.89 ± 0.02	5.3
Urea	509.55 ± 0.02	57

3. Results and discussion

3.1. Method scouting and optimization

3.1.1. Optimal binding pH

The possible binding mechanism between the core particle and linear heptapeptide WSFFSNI has yet to be identified, however, it was demonstrated that the association of this peptide to the core particles was primarily due to hydrophobic bonding [8]. Unlike the linear hexapeptide sequence, LLGRMK that contains two basic residues separated by a hydrophobic residue with net charge of +2 [27], ionic interactions was shown to play a major role in binding to the acidic residues (Glu 77 and Asp 78) at the tip of the spike of the HBV capsid [28].

The inhibitory effect on the association of L-HBsAg and HBcAg exhibited by this particular linear heptapeptide was higher compared with that observed using cyclic heptapeptide, C-WSFFSNI-C, due to increased hydrophobicity contributed by the cysteine residues [18]. However, when this disulfide constrained heptapeptide was displayed at the gpIII protein coat of M13 filamentous phage, it was demonstrated that M13 phages bearing this particular disulfide constrained heptapeptide formed a tighter binding with truncated HBcAg compared with free cyclic peptide bearing the same sequence as well as phages bearing the sequence LLGRMK [18]. Spontaneous disulfide bonds would form and the displayed peptides would gain a defined conformation, which subsequently altered the properties of the displayed residues, different from how they exist as free peptide or in linear forms [18,29]. In addition, it has been described that most of the peptide ligand would only function when they are an integral part of the phage coat protein [30]. Therefore, higher affinity against HBcAg exhibited by the peptide expressing phages compared with free cyclic heptapeptides may be attributed to the presence of both phage coat protein and the peptide. This is in accordance with that reported by Gaskin et al. [15] in affinity purification of lipase using peptide expressing phages.

In this experiment, the effect of pH buffers on the adsorption of HBcAg onto the M13 phage-immobilized adsorbents was investigated. The binding of HBcAg to M13-immobilized adsorbents was higher at alkaline pH buffers (pH 7–9) compared with slightly acidic buffer at pH 6 (Table 1). In the present study, more than 30% of HBcAg from the crude feedstock was bound to the M13-immobilized adsorbents with buffers pH higher than the neutral pH and pH 8 bound the highest amount of HBcAg from unclarified feedstock compared with pH 7 and 9. Therefore, 50 mM Tris–HCl, pH 8.4 was employed throughout this study, similarly with that employed by Ng et al. [11] in direct capture of HBcAg using anion-exchanger EBAC.

It is also should be noted that the interactions between the peptides expressing phages and HBV capsid could be due to several possible interactions that occur between the peptide and certain regions on the capsid's surface [18]. The constrained heptapeptide sequence C-WSFFSNI-C, which consist of hydrophobic residues (W, F, I), two hydroxylic side chains (S) and one amide-bearing residue (N) may contribute to both hydrophobic and hydrogen bondings between the selected peptides and the surface of the core particles [18]. Although the sequences of the constrained peptide used in this study were different from that of linear peptide (LLGRMK), the crystal structure of HBV capsid showed that the region where the linear peptide bound to is flanked by hydrophobic residues [31], which may play a substantial role in hydrophobic interaction with the cyclic peptides employed in this study. Further experiments were carried out to determine the possible binding forces between the two components based on elution condition used as described in Section 3.1.2.

3.1.2. Optimal elution condition

Affinity chromatography that based on specific protein–protein interactions often involve a combination of binding forces such as Van der Waals, ionic and hydrophobic interactions [32], which contributed to more selective binding to the target protein compared with those only utilizing one binding force between the ligand and target proteins [33]. Similar observations have also been described on the association between the M13 phage with disulfide constrained heptapeptides and HBV capsids [18]. Therefore, chaotropic salts such as urea, guanidine hydrochloride and inorganic salts such as magnesium chloride and potassium thiocyanate are essential in order to disrupt these bindings. Various chaotropes have been used to disrupt affinity binding between peptide ligand [31,34] and peptide expressing M13 phage ligand [16] with their captured target biomolecules. In the present study, chaotropic salts such as chloride, thiocyanate and iodide were used in desorption of HBcAg from M13-immobilized adsorbents. Although these salts were able to abolish antigen–antibody interactions by changing the structure of the protein [35], the amount of HBcAg eluted with these salts was the lowest (Fig. 1a) (less than 10% of HBcAg eluted) compared with elution using NaCl (Fig. 1b) and urea (Fig. 1c). As NaCl has been shown to successfully used in desorption of lactoferrin from peptide expressing phage ligand [17], however in this study, elution buffer containing 1 M NaCl was only able to elute about 20% of bound HBcAg (Table 2a). This could be attributed to the presence of ionic interaction that exists between the cyclic peptide in gpIII coat protein and core particle, thus resulted in low amount of eluted HBcAg. As chloride ion is also considered one of the chaotropic ion but of the weaker type [36], therefore, the possible binding force that is present here could also be due to hydrophobic interaction and NaCl is not suitable to use as it may enhance the interaction. When elution was carried out with harsher chaotropic agent such as urea, which is able to disrupt both hydrophobic interaction and hydrogen bonding, more than 50% of bound HBcAg was eluted (Table 2a). Therefore, this indicated that most of the binding force that is involved in the interaction between the cyclic heptapeptide and core particle was attributed to either hydrogen or hydrophobic bindings.

When 1–4 M of urea were tested on the recovery of HBcAg, it was demonstrated that elution with 3 M urea recovered the highest amount of bound HBcAg with the highest purification factor of 2.3 as depicted in Table 2b. Although the yield of HBcAg obtained with 4 M urea was more than those obtained with low urea concentrations (1–2 M), the purity of the eluted HBcAg was not as high as that obtained using 3 M urea. Therefore, 3 M urea was selected as the optimal eluting agent to use in the present study.

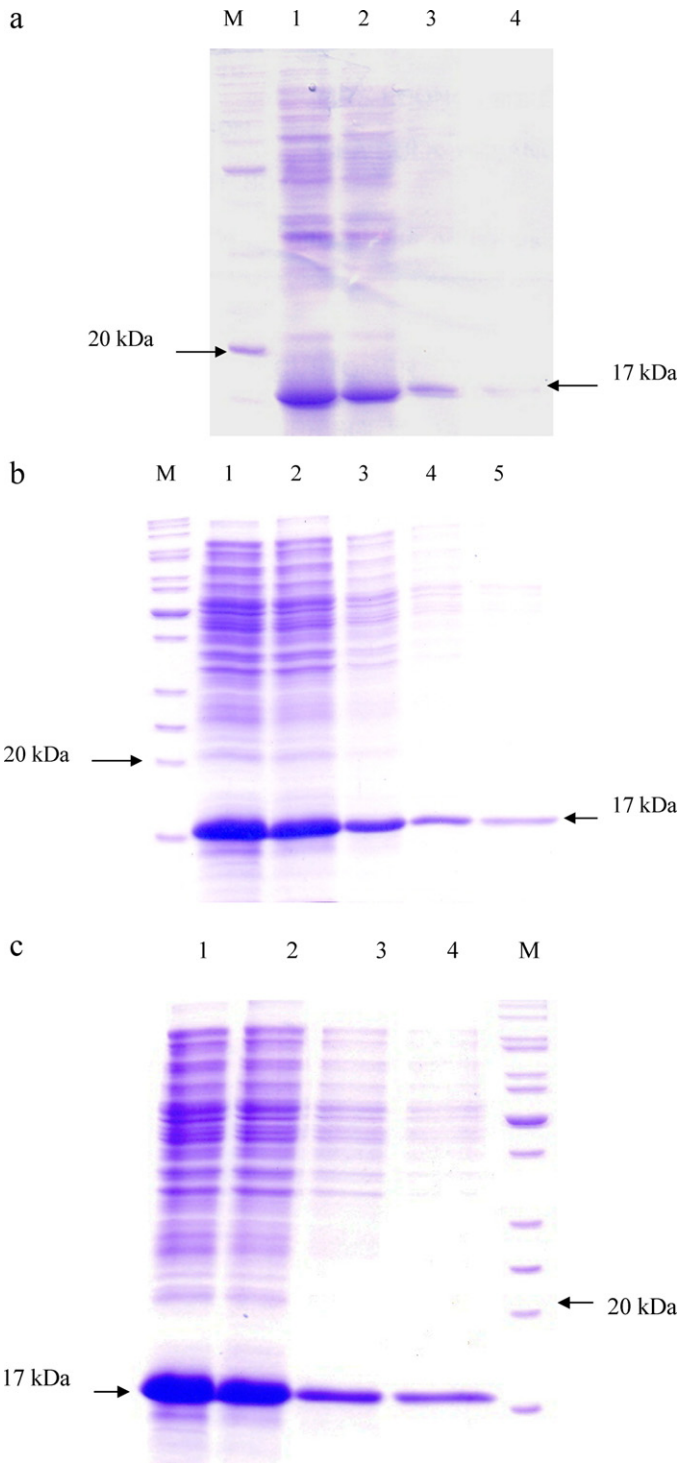

Fig. 1. (a) Elution of HBcAg with 3 M KSCN. The 17 kDa band on gels is the monomer of HBV core particle. Lane M: molecular mass marker in kDa (Benchmark, Invitrogen), Lane 1: initial unclarified feedstock, Lane 2: binding at 5 h, Lane 3: washing, Lane 4: elution. (b) Elution of HBcAg with 1 M NaCl. The 17 kDa band on gels is the monomer of HBV core particle. Lane M: molecular mass marker in kDa (Benchmark, Invitrogen), Lane 1: initial unclarified feedstock, Lane 2: binding at 5 h, Lanes 3 and 4: washing, Lane 5: elution. (c) Elution of HBcAg with 3 M urea. The 17 kDa band on gels is the monomer of HBV core particle. Lane M: molecular mass marker in kDa (Benchmark, Invitrogen), Lane 1: initial unclarified feedstock, Lane 2: binding at 5 h, Lane 3: washing, Lane 4: elution.

Table 2b
Effect of different urea concentrations on elution.

Urea concentrations (M)	Yield of HBcAg in elution (%)	Purification factor
1	10.99	1.72
2	10.51	1.59
3	18.58	2.28
4	18.35	2.19

3.2. Operation of EBAC

3.2.1. Dynamic binding capacity (DBC)

Frontal analysis was employed to estimate the volume of unclarified feedstock that can be loaded per volume of adsorbent prior to significant breakthrough of HBcAg occurs. Loading of feedstock is generally terminated at $C/C_o = 0.1$ in most of the industrial practice, in order to prevent loss of target product [37]. Fig. 2 shows the breakthrough curve of HBcAg on expanded bed of M13-immobilized adsorbents with unclarified *E. coli* feedstock containing 5% (w/v) of biomass at sedimented bed height of 19 cm with flow velocity of 105 cm/h. The dynamic binding capacity (DBC) at 10% breakthrough capacity calculated based on formula (1) was 1.98 mg/mL.

3.3. Purification of HBcAg from unclarified feedstock

Affinity chromatography, which exploits the principle of biospecific recognition and forms reversible complexes between ligand and target molecule, has become a powerful and effective tool to use in various bio-molecule purifications [38]. In the present study, M13 phage with fusion of disulfide constrained heptapeptide, C-WSFFSNI-C, at the gpIII protein has been exploited as affinity ligand to directly capture HBcAg from unclarified feedstock. In a typical EBAC operation, it is suggested that the settled bed height to use is about 1/5 of the length of the column [39]. However, considerations on both the settled bed height and flow velocity are of great importance to ensure efficient capture of target proteins [40,41]. This is particularly important when longer adsorption time between adsorbent and adsorbate is necessary to achieve maximum binding capacity of the adsorbents used [42,43]. Different modes of chromatographic operations for adsorption stage to increase the contact time and surface interactions between adsorbent and adsorbate for higher product recovery have been described in previous studies [43].

In the current study, two different modes of EBAC operations were investigated; a typical single pass operation and modified EBAC operation with recirculation of feedstock into the expanded

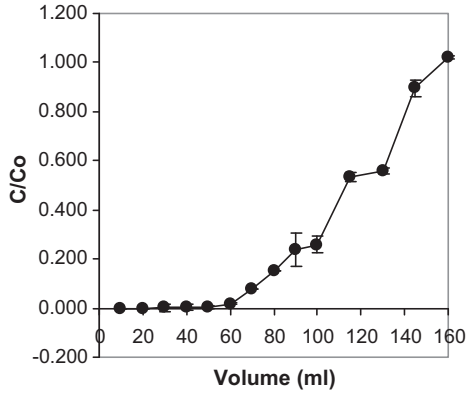

Fig. 2. Dynamic binding capacity of the expanded bed using M13 phage-immobilized adsorbents against HBcAg. Error bars: duplicate determinations.

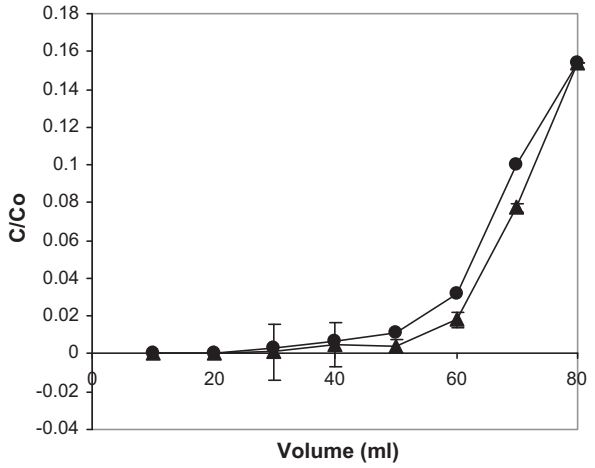

Fig. 3. This figure shows the breakthrough curves of total protein (●) and HBcAg (▲) in typical EBAC operation. Error bars: duplicate determinations.

bed. The settled bed height as well as the superficial velocity employed in the current study was different compared with EBAC operation with anion-exchanger as described in [11]. In this study, settled bed height of 19 cm with flow velocity of 101 cm/h was employed throughout this study.

In typical EBAC operation, the bed was expanded until a steady-state degree of expansion of approximately 1.74 was obtained. During this stage, there was very limited movement of the adsorbent particles and the bed behaved similar to packed bed chromatography. When feed application was carried out, low level of bed expansion to 34 cm was observed. The flow of the loaded feedstock through the expanded bed was close to plug flow and the inter-particle space was sufficient to provide unhindered passage for cell debris to pass through the column without disturbing the stability of the expanded bed. Due to higher sedimented bed height and low superficial velocity employed in the study, the feedstock took approximately 9 min to appear on the surface of the expanded bed and the breakthrough curves for both HBcAg and total protein were depicted in Fig. 3. It is observed that both breakthrough curves for total protein and HBcAg exhibited similar trend at the beginning of feedstock application. However, when the volume of the loaded feedstock was increasing, it shows that the total protein appeared first in the outlet stream followed by HBcAg at breakthrough capacity of $C/C_o = 0.1$. This was a desirable situation as it indicated that HBcAg was adsorbed more selectively than other non-HBcAg proteins from the applied feedstock by M13 phage-immobilized adsorbents. In addition, the gradually increasing incline of HBcAg breakthrough curve (within 20–70 mL of feed application) as shown in Fig. 3, also indicated that longer interaction time was needed for HBcAg to bind onto the adsorbents before achieving breakthrough at $C/C_o = 0.1$. The purity and yield of recovered HBcAg using this mode of operation was as summarized in Table 3. It is shown that the purity of the recovered HBcAg was increased by 4-fold whereas the yield was approximately 12% (Fig. 4). As feed application was halted at $C/C_o = 0.1$, approximately 20 min after feed application, the time allowed for binding may not be sufficient as that obtained in batch adsorption, hence resulted in low yield.

Therefore, in this study, an alternative mode of EBAC operation where feedstock from the outlet stream of the column was re-circulated back to the expanded bed to pro-long the exposure time between the adsorbents and adsorbate. This modified technique is a combination of three modes of operations, which involved fluidized, suspended and expanded bed chromatographies. In fluidized bed purification, recycling of feedstock has been employed

Table 3
Effect of different EBAC operations on direct capture of HBcAg from crude feedstock.

	Typical EBAC	Modified EBAC
Feedstock		
Total protein (mg)	576.39 ± 0.04	576.39 ± 0.04
Total amount of HBcAg (mg)	114.70 ± 0.04	114.70 ± 0.04
Elution		
Eluted total protein (mg)	16.99 ± 0.02	23.59 ± 0.02
Eluted total HBcAg (mg)	14.00 ± 0.02	16.54 ± 0.02
HBcAg recovery		
Yield (%)	12.21	14.42
Purity	0.82	0.70
Purification factor	4.14	3.52
Retention time (min)	~20	~60

in order to increase the target protein binding and it is only feasible to use for stable protein purification [44]. As this method is rather laborious and time-consuming [44], therefore, Levison et al. [45] reported a new approach, which combined the advantages in batch adsorption and packed bed chromatographies. This method does not only reduce the overall processing time compared with stirred tank and packed bed column but also increases the yield of target protein compared with that obtained using batch mode [42]. However, this technique is only applicable in processing clarified feedstock. Therefore, in this study, with combination of these techniques and EBAC operation, the need of clarification of feedstock was thus eliminated. Unlike continuous counter-current protein chromatography, whereby the entire stream exiting the wash stage was directed back to adsorption stage [46], in this study, the depleted feedstock exited the column during adsorption was continuously pumped into the expanded bed again for approximately 1 h to increase the interaction time for both adsorbent and adsorbate, to mimic the adsorptive stage in batch mode. The purity and yield of HBcAg recovered using this mode of operation is as summarized in Table 3. Based on Table 3, there was an increase of about 18% in the yield of HBcAg obtained from unclarified feedstocks containing 5% (w/v) of biomass compared with that obtained using single pass operation. However, the purity obtained was slightly reduced by 15% in the eluted fraction obtained from feedstock containing 5% (w/v) biomass compared with that obtained in single pass operation (Fig. 5). Although presence of cell debris in the feedstock in this mode of operation did not affect the yield of HBcAg similarly to that described by Chase and Draeger [47], continuous

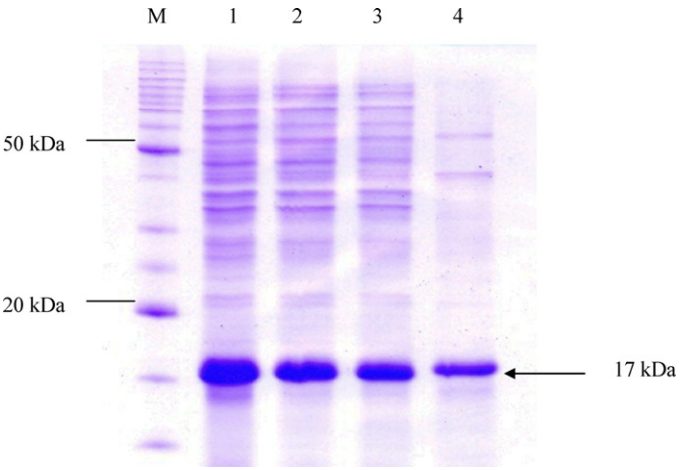

Fig. 4. SDS-PAGE gels of the pooled fraction collected throughout the EBAC purification of HBcAg from unclarified feedstock using typical single pass operation. The 17 kDa band on gels is the monomer of HBV core particle. Lane M: marker (Benchmark, Invitrogen), Lane 1: initial unclarified feedstock, Lane 2: flowthrough, Lane 3: wash, Lane 4: elution (desalted and concentrated fraction).

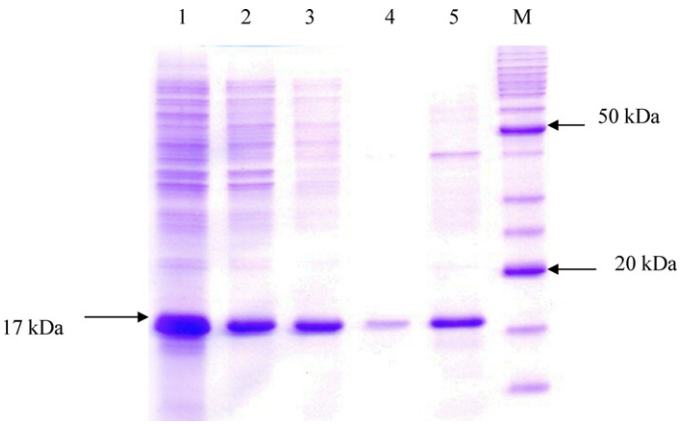

Fig. 5. SDS-PAGE gels of the pooled fraction collected throughout the EBAC purification of HBcAg from unclarified feedstock using modified operation with recirculation of depleted feedstock into the expanded bed. The 17 kDa band on gels is the monomer of HBV core particle. Lane M: marker (Benchmark, Invitrogen), Lane 1: initial unclarified feedstock, Lane 2: after binding (depleted feedstock exited the column), Lane 3: flowthrough (depleted feedstock that remained in the column when recirculation of feedstock stopped), Lane 4: wash, Lane 5: elution (desalted and concentrated fraction).

recirculation of protein exhausted feedstock into the expanded bed could simultaneously increase the probability for the interactions to occur between the adsorbents and HBcAg but also with other contaminants present in the crude feedstock, thus leading to lower purity level. In addition, increased time during adsorption stage could also lead to higher possibility for the contaminants to diffuse into the internal volume of the macroporous adsorbents. Furthermore, as previously demonstrated by [48] using macroporous EBAC adsorbents, contaminants that were trapped within the pores of the adsorbent could not be easily removed, subsequently leading to lower product purity when desorption was carried out. Indeed, the results depicted in Fig. 6 clearly demonstrated that there was not any host cell protein being bound non-specifically on to the developed affinity adsorbent (Fig. 6a). The contaminant host protein was most probably trapped inside the porous structure of the adsorbent, and it was only released out from the adsorbent after it was eluted using 3 M urea (Fig. 6b).

However, when comparison was made between both methods of EBAC operations employed in the present study with conventional methods, the purity of HBcAg obtained was lower compared with that purified using sucrose ultracentrifugation [27] and size-exclusion chromatography [8]. Although the purity of HBcAg obtained in the current study was not as high as the conventional methods, the yield of HBcAg with the current methods was significantly higher compared with that previously reported by Rolland et al. [9] using sucrose ultracentrifugation and size-exclusion chromatography. In addition, longer overall processing time is also necessary in the conventional purification compared with the current methods discussed in this study due to the need for additional pre-clarification and precipitation steps to yield a concentrated and particulate free protein solution. Similar to that observed in purification of core particles with histidine-tagged at the C-terminal, additional denaturation and renaturation steps are necessary when purified with metal-chelated adsorbents [49].

As affinity purification, particularly with biological ligand has a reputation of being less robust to use compared with other adsorbents and ligand leakage is the main concern in affinity purification [39]. In the present study, it was demonstrated that the rate of total M13 phage leakage detected in the liquid phase throughout both typical and modified EBAC operations were 6.6×10^{-3}% and 7.8×10^{-3}%, respectively (Table 4).

When desalted and concentrated HBcAg purified using both typical EBAC and modified EBAC operations in the current study was analyzed with light scattering machine (Table 5), the radius of more than 95% of the particles with highest mass percentage present in the solution were between 14 and 22 nm, which correspond to the size of HBcAg determined previously using light scattering [7] and X-ray crystallography [50]. It is worth noting that the percentage polydispersity of the capsids purified using single pass EBAC

Table 4
Amount of ligand leakage at every purification step in typical and modified EBAC operations.

Steps in purification	Typical EBAC operation	Modified EBAC operation
Initial bound	9.6×10^{14} pfu	9.68×10^{14} pfu
After binding	–	5.3×10^{10} pfu
Flowthrough	2.74×10^{10} pfu	1×10^{10} pfu
Washing	3.2×10^{10} pfu	1.08×10^{10} pfu
Elution	3.26×10^{9} pfu	2.06×10^{9} pfu
Total leakage	6.3×10^{10} pfu	7.59×10^{10} pfu
Total leakage rate	6.6×10^{-3}%	7.8×10^{-3}%

Table 5
Light scattering analysis on typical and modified EBAC purified core particles.

	Typical EBAC	Modified EBAC
Particle size radius (nm)	14–15	16–22
%Mass	95.45 ± 1.2	96.9 ± 0.42
%Polydispersity	0–4.1	16.3–26

Note: %Mass refers to concentration of a particular species present in the solution.

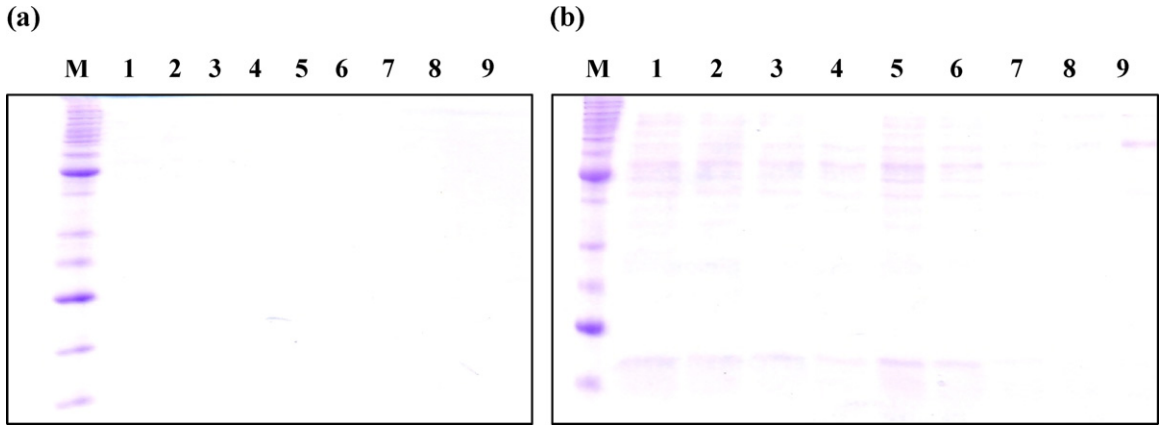

Fig. 6. (a) No binding of *E. coli* proteins was observed using non-M13-immobilized Streamline base matrix in EBAC operation. (b) Traces of *E. coli* host W31101Q proteins were observed using M13-immobilized Streamline base matrix when elution was carried out using 3 M urea. Lane M: marker (Benchmark, Invitrogen), Lanes 1–9: elution.

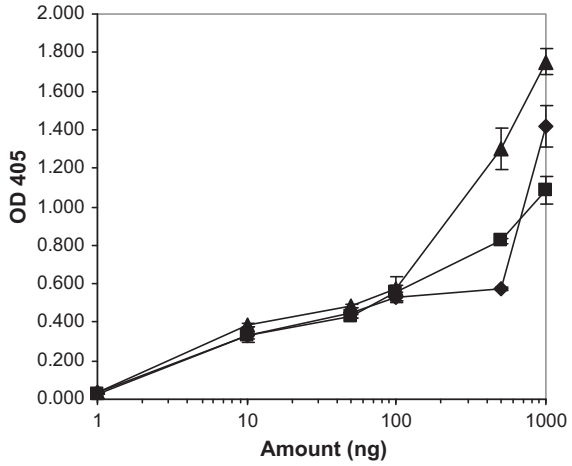

Fig. 7. This figure shows the antigenicity of HBcAg purified from unclarified feedstock by using single pass EBAC operation (■), modified EBAC operation (▲) and sucrose ultracentrifugation purified HBcAg (♦). Data points are mean ± standard deviation of triplicate determinations. Error bars: triplicate determinations.

operation was less than 15%, indicating that the particles present were more homogenous. On the other hand, the percentage polydispersity of the purified particles using modified EBAC was higher, between 15% and 30%, indicating different sizes of particles, which could be due to the presence of $T=3$ and $T=4$ capsids as well as HBcAg dimers in the solution. Only a small fraction (between 0 and 4.5%) of contaminants with larger particle size (78–240 nm) were detected in the protein solution. In addition, no traces of smaller particle size than the core particles (approximately 6 nm in diameter) were detected thus indicated absence of M13 phage (length: 6 μm and diameter: 1 nm) in the concentrated protein solution.

ELISA was also carried out in the current study to determine the antigenicity of capsids purified using both EBAC operations. Based on Fig. 7, it is shown that the immunoreactivity of the capsids purified using M13 phage-immobilized adsorbent in both EBAC operations is comparable to that exhibited by sucrose gradient ultracentrifugation purified core particles. Therefore, this study has demonstrated that M13 phage with fusion disulfide constrained heptapeptides, C-WSFFSNI-C, can be used in both EBAC operations in direct capture of HBcAg from unclarified feedstock.

4. Conclusion

The findings obtained from the present study have created a new platform in affinity purification of HBcAg using fusion M13 phage as affinity ligand. M13 phage with fusion of disulfide constrained heptapeptide, C-WSFFSNI-C at its gpIII protein exhibited high selectivity against HBcAg even in the presence of cell or cell debris. Direct recovery using either typical EBAC operation or modified EBAC operation by recirculating the feedstock into the expanded bed using M13 phage-immobilized adsorbent have shown great potential in purification of recombinant HBcAg from unclarified *E. coli* feedstock. When tested with ELISA, the antigenicity level of the purified core particles was comparable with that purified using sucrose ultracentrifugation. Therefore, this study has successfully demonstrated the usage of M13 phage-immobilized adsorbents in direct capture of HBcAg from unclarified feedstock via EBAC although improvement on ligand and adsorbent design is necessary to improve protein capture efficiency.

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