

## A Preparative Purification Process for Recombinant Hepatitis B Core Antigen Using Online Capture by Expanded Bed Adsorption Followed by Size-Exclusion Chromatography

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**Hepatitis B core antigen (HBcAg) is an important serological marker used in the diagnosis of hepatitis B virus (HBV) infections. In the current study, a fast and efficient preparative purification protocol for truncated HBcAg from *Escherichia coli* disruptate was developed. The recombinant HBcAg was first captured by anion exchange expanded bed adsorption chromatography integrated with a cell disruption process. This online capture process has shortened the process time and eliminated the “hold-up” period that may be detrimental to the quality of target protein. The eluted product from the expanded bed adsorption chromatography was subsequently purified using size-exclusion chromatography. The results showed that this novel purification protocol achieved a recovery yield of 45.1% with a product purity of 88.2%, which corresponds to a purification factor of 4.5. The recovered HBcAg is still biologically active as shown by ELISA test.**

**Keywords:** Hepatitis B core antigen, *Escherichia coli*, online capture process, expanded bed adsorption chromatography, size-exclusion chromatography

Hepatitis B virus (HBV) is the causative agent of hepatitis B, one of the widespread infectious diseases in the world. The inner nucleocapsid of HBV is composed of multiple copies of a single core protein, known as HBcAg. This virus-based nanoparticle (VBNP) has been successfully expressed in a variety of microbial systems including *Escherichia coli* (*E. coli*), and the protein was able to self-

assemble into two sizes of icosahedral particles (containing 180 or 240 subunits with triangulation number of T=3 and T=4, respectively), which resemble the morphologies of the authentic nucleocapsids isolated from HBV-infected livers [9, 11]. The electron cryomicroscopic analysis has revealed that the estimated diameters of the large and small particles are 30 nm and 34 nm, respectively [11].

A full-length hepatitis B core antigen (HBcAg) has a molecular mass of about 21 kDa and comprises 183 residues that are highly rich in arginine residues at the C-terminal regions. On the other hand the recombinant HBcAg used in this study is a 17 kDa truncated HBcAg, which lacks the arginine-rich region [36]. The yield of this truncated HBcAg derivative in *E. coli* is considerably higher than that of the full-length HBcAg expressed from equivalent plasmid constructions [35, 36]. Similar to the full-length HBcAg, the truncated mutant also self-assembles into particles, indicating that the arginine-rich region is not involved in capsid assembly [44]. In addition, no nucleic acids were detected in the T=4 capsid formed by the truncated HBcAg [34]. The deleted region can be replaced with foreign epitopes or polypeptides, and because of its ability to self-assemble into particles, the HBV capsid has been widely explored as a molecular and vaccine carrier [10, 26, 29]. The chimeric HBcAg is not only able to induce T-helper, B, and cytotoxic lymphocyte (CTL) responses, but also allows the inserted epitopes to display the appropriate immunological properties [29, 40]. Hence, the HBcAg-based chimeras may be incorporated into a pharmaceutical composition for prophylactic or therapeutic vaccination, for instance against human influenza A [13] and HBV [1]. The great potential of recombinant HBcAg may prompt high demand of VBNP. In view of this, we have developed

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a fast, simple, scaleable, and cost-effective protocol to purify this VBNP from *E. coli* disruptate.

The direct product sequestration protocol by integrating the cell disruption with expanded bed adsorption chromatography (EBAC) can be applied to achieve a higher product yield, reduce process steps, and shorten process time. For instance, it has significantly improved the recovery yield and molecular quality of an extracellular acidic protease from oleaginous yeast *Yarrowia lipolytica* [15, 25]. In addition, another integrated process for the primary recovery of glyceraldehydes 3-phosphate dehydrogenase (G3PDH) [3] has achieved a higher specific activity and purification factor of recovered product, compared with those obtained in extended time-scale of sequential batch operations of bead milling, centrifugation, and fixed-bed chromatography. The purity of the recovered product from this primary recovery process can be further improved by using size exclusion chromatography. Successful SEC applications have been demonstrated on the purification of various bioproducts such as enzymes [20], polysaccharides [21], peptides [41], proteins [30], viruses [24], and virus core particles [5, 8, 31].

In this study, we have demonstrated the feasibility of a novel HBcAg purification protocol utilizing an online primary capture process based on EBAC and a polishing step using SEC.

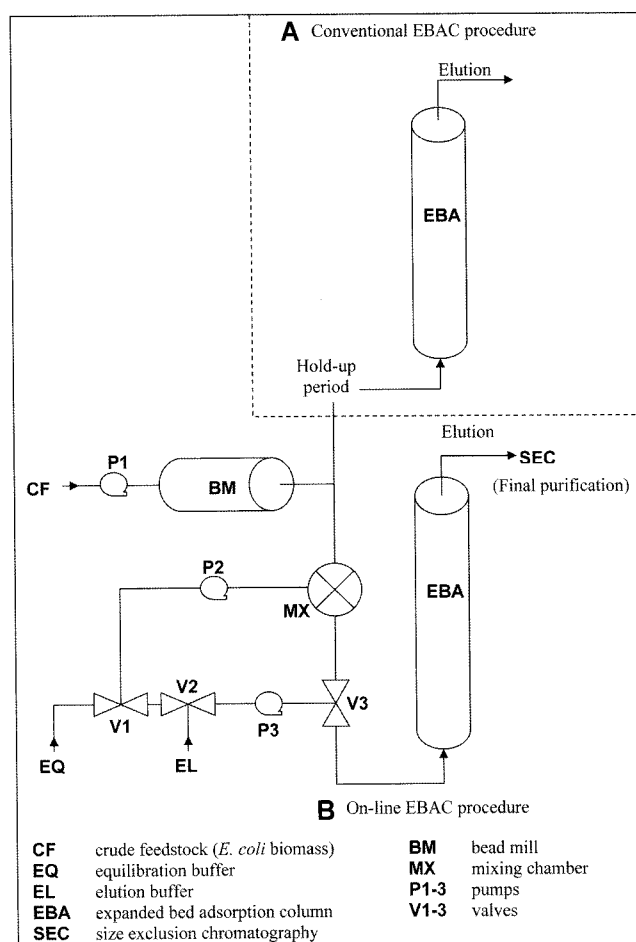
## MATERIALS AND METHODS

### Cultivation of *Escherichia coli*

The W3110IQ *E. coli* strain harboring plasmid PR1-11E, which encodes for truncated HBcAg [34, 42], was cultured in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin. The culture was incubated at 37°C under shaking at 250 rpm. The protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) (Promega, U.S.A.) at a final concentration of 0.5 mM when the biomass concentration had reached an optical density at 600 nm ( $OD_{600}$ ) of 0.8–1.0. The culture was further incubated overnight at 250 rpm, 37°C, for another 16–20 h. The *E. coli* cells were harvested by centrifugation at  $3,750 \times g$  (Avanti JLA-16.250; Beckman-Coulter, U.S.A.) for 15 min at 4°C. Prior to the cell disruption, the cell pellet was washed with distilled water and centrifuged again at  $3,750 \times g$  (Avanti JLA-16.250; Beckman-Coulter, U.S.A.) for 15 min at 4°C.

### Online Capture of HBcAg by Expanded Bed Adsorption Chromatography

The pelleted *E. coli* cells were suspended in lysis buffer [50 mM Tris-HCl (pH 8.0)] to a biomass concentration of 10% (w/v). Cell disruption was carried out using batch mode bead milling under an impeller tip speed of 10 m/s for a period of 17 min, as described in our previous work [17]. The disruptate (approximately 100 ml) was immediately mixed with an equal volume of equilibration buffer [50 mM Tris-HCl (pH 8.4)] to produce a diluted feedstock of 5% (w/v) biomass concentration before being fed directly to the EBAC contactor (Fastline 20 column; Upfront Chromatography, Copenhagen, Denmark) containing equilibrated Streamline DEAE (GE Healthcare, Uppsala, Sweden) adsorbents. Streamline DEAE, a weak anion exchanger, is based on highly cross-linked 6% agarose, modified by



**Fig. 1.** Experimental configuration of the purification processes of HBcAg from *E. coli* biomass.

The feedstock was initially disrupted by batch mode bead milling [10% (w/v) biomass concentration, 10 m/s impeller tip speed, 80% (v/v) bead loading], and the disruptate was then immediately diluted with equilibration buffer to achieve a 5% (w/v) biomass concentration and subsequently loaded into the equilibrated EBA contactor. The integration of bead milling and EBAC (B) successfully eliminated the unfavorable “hold-up” period (A). The partially purified HBcAg was further purified by SEC.

including an inert quartz core to give the desired density of 1.2 g/ml. The average bead diameter is 200 µm with a bead size distribution of 100–300 µm. The feedstock was fed at a linear flow rate of 100 cm/h (or 300 ml/h). A rotating magnetic bar created a narrow mixed zone at the bottom of the expanded bed, which ensured efficient distribution of feedstock. This “open” system effectively eliminated the risk of clogging while an efficient adsorption was maintained in the entire expanded bed. The speed of the magnetic bar at the bottom of the expanded bed was controlled by the regulator of a hotplate/magnetic stirrer. The collection of sample fractions was started when the feedstock reached the column outlet. The bed was then washed with equilibration buffer until no residue particulate was visible in the effluent. The adsorbed protein was eluted by pumping in elution buffer containing 50 mM Tris-HCl and 1.0 M NaCl (pH 8.4). The fractions of elution were pooled before being further purified by size-exclusion chromatography. The experimental configuration of the HBcAg purification processes is illustrated in Fig. 1.

### Final HBcAg Purification by Size-Exclusion Chromatography

Agarose BioGel A-0.5M (Bio-Rad, Hercules, CA, U.S.A.) with particle size in a range of 38–75  $\mu\text{m}$  was packed into a glass column with an internal diameter of 1.6 cm and a length of 20 cm (Column C 16/20) fitted with the AC 16 Adaptor (Amersham Biosciences, Uppsala, Sweden) to a bed height of approximately 17 cm (34 ml bed volume) using the gravity slurry-sedimentation method. The chromatography column was then equilibrated with equilibration buffer [50 mM Tris-HCl buffer (pH 8.4)]. Feedstock containing eluted product from EBAC was applied to the column at 0.1 ml/min with a peristaltic pump (Econo Pump; Bio-Rad, Hercules, CA, U.S.A.), followed by one bed-volume of elution buffer [50 mM Tris-HCl buffer (pH 8.4)].

### Protein Determination and Densitometric Analysis

The SDS-PAGE was performed under denaturing condition [22]. The reducing reagent used in the electrophoresis buffer was 0.1% (w/v) SDS, whereas the 2 $\times$  loading dye contained 4% (w/v) SDS and 200 mM  $\beta$ -mercaptoethanol. Electrophoresis was conducted using a Mini-Protean 3 apparatus (Bio-Rad, U.S.A.). The protein content was estimated by the Bradford assay [4] using bovine serum albumin as the standard. The sample was read with a microplate reader (Model 550; Bio-Rad, Japan) at 595 nm wavelength.

Quantitation of HBcAg was based on the method previously described [27, 38]. The relative quantity of HBcAg band on the SDS-PAGE gels, which represents the relative quantity of HBcAg in the sample, was determined using the Quantity One Quantitation software (Gel Doc; Bio-Rad, U.S.A.). The amount of HBcAg was determined by calculating the relative quantity of the HBcAg against the amount of total protein obtained from the Bradford assay. The ratio of the amount of HBcAg to the amount of total protein in the sample is defined as purity and was expressed in percentage.

### Enzyme-Linked Immunosorbent Assay (ELISA)

The antigenicity of the HBcAg was determined by ELISA as previously described [16, 17]. After the coating of HBcAg and the blocking with milk diluent, anti-HBcAg monoclonal antibody [mAb clone Cl-5, original concentration (8 mg/ml); (1:1,000 or 1:1,500; 100  $\mu\text{l}$ ); Chemicon] was added and incubated for 1 h at room temperature, followed by anti-mouse antibody conjugated to alkaline phosphatase (1:5,000, 100  $\mu\text{l}$ ; Chemicon). After 1 h incubation at room temperature, the HBcAg was detected by adding *p*-nitrophenyl phosphate (NPP) (Sigma, 200  $\mu\text{l}$ ) as substrate for the enzyme. The antigenicity of HBcAg was detected by measuring the optical density at 405 nm using a microplate reader (Model 550; Bio-Rad, Japan). The assays were carried out in triplicates.

### Calculations

The binding capacity of total protein or HBcAg for anion exchange adsorbents in EBAC was calculated based on Eq. (1), while the EBAC elution yield of HBcAg was calculated based on Eq. (2):

$$\text{Binding capacity for total protein or HBcAg} = \frac{I - F - W}{\text{Volume of settled adsorbent applied}} \quad (1)$$

where

I=Amount of total protein or HBcAg in the initial feedstock

F=Amount of total protein or HBcAg lost in flowthrough

W=Amount of total protein or HBcAg lost in washing

Elution efficiency of HBcAg

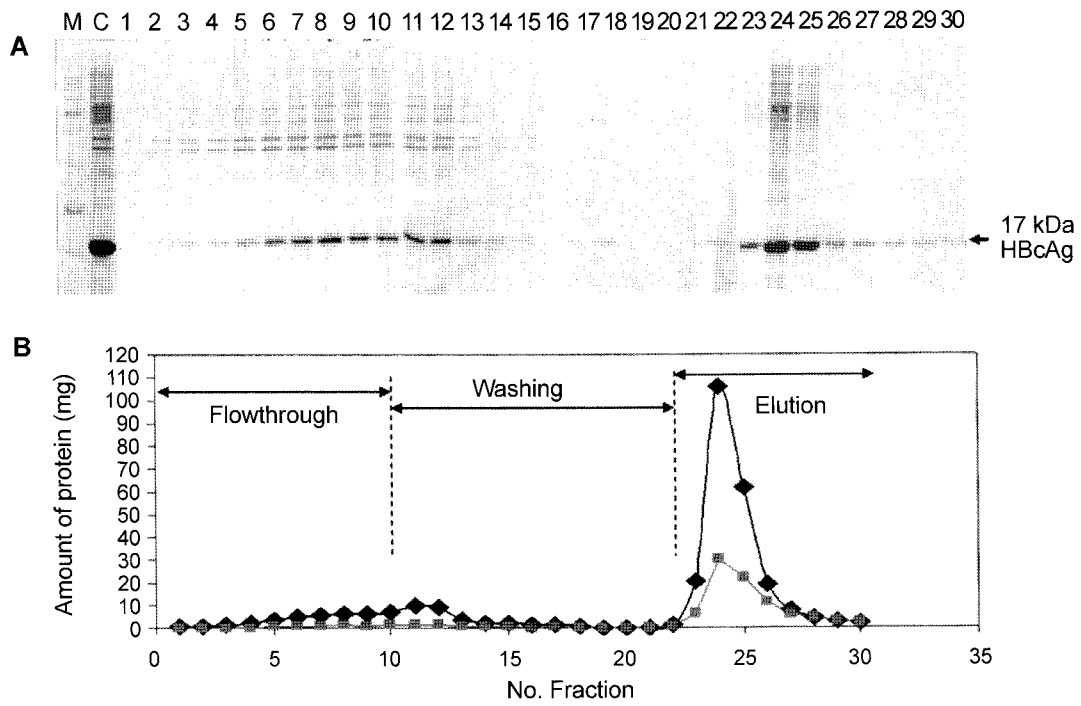
$$= \frac{\text{Total amount of HBcAg successfully eluted out}}{\text{Total amount of HBcAg bound to the adsorbent}} \times 100\% \quad (2)$$

## RESULTS

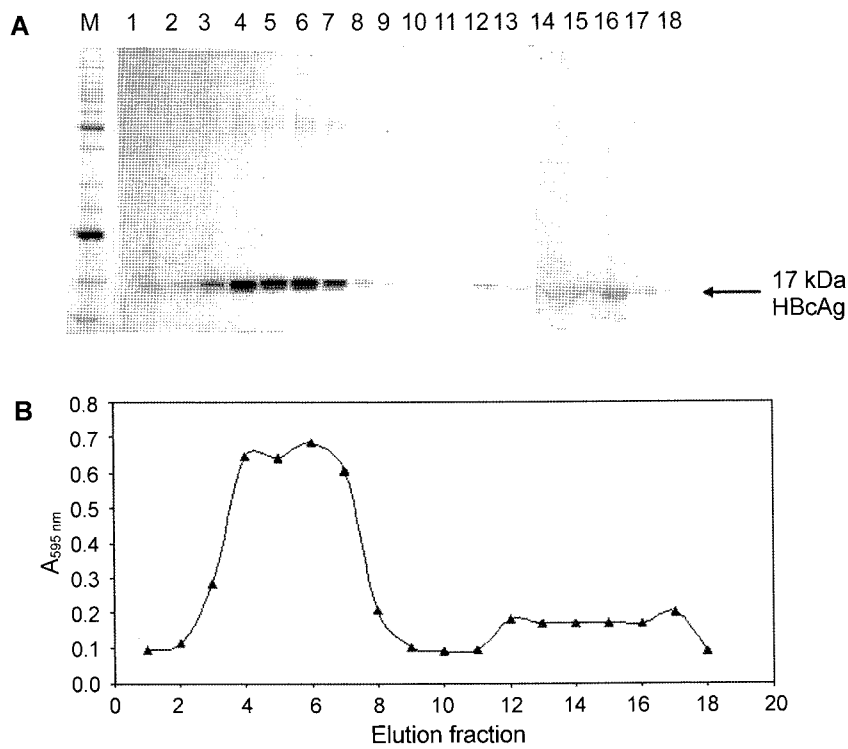
### Online Capture of HBcAg by Expanded Bed Adsorption Chromatography

In the current study, *E. coli* suspension corresponding to 10% (w/v) biomass concentration was initially disrupted in a bead mill, operated batchwise for a period of 17 min. The resulting disruptate was immediately mixed with an equal volume of equilibration buffer to produce a feedstock of 5% (w/v) biomass concentration, and it was loaded directly to the equilibrated EBAC contactor. The dilution step prior to irrigation of feedstock into the EBAC is necessary to decrease feedstock viscosity and to preserve a laminar flow regime in the EBAC. The feedstock was applied into the EBA contactor at a flow rate similar to that used during the adsorbent bed equilibration (100 cm/h) and this flow rate was kept constant throughout the entire EBAC process (equilibration, flowthrough, washing, and elution stages), which resulted in the inconsistency of the degree of bed expansion. Most of the EBAC literatures involved flow rate altering to maintain a constant bed expansion; nevertheless, there are some reports on successful EBAC procedures utilizing a constant flow velocity [7, 32]. In the current study, the constant flow rate approach was employed because of the simplicity in EBAC operation without the need to change the flow rate throughout the entire EBAC process.

The chromatographic profile of the online EBAC purification of HBcAg from unclarified *E. coli* disruptate is illustrated in Fig. 2. About 26.4% of the total protein was lost in the flowthrough and washing stages. These total protein losses comprised 39% of the total amount of HBcAg in the initial feedstock. The loss of HBcAg in the flowthrough was anticipated and was attributed to the competitive adsorption that had occurred between the target protein and other contaminant proteins at the binding sites of the adsorbent, as indicated by the low ratio of HBcAg to total protein bound to the adsorbents (approximately 1:5). The binding capacity of HBcAg and total protein calculated was 1.08 mg/ml and 5.78 mg/ml adsorbent, respectively. Streamline DEAE is a porous adsorbent designed for the fractionation of small macromolecular proteins rather than the VBNP such as HBcAg. Thus, the inner pore compartments of the adsorbent are restricted to large particles such as HBcAg but are easily accessible to small-size impurities such as cell debris and linear fragment of DNA [39, 43]. Indeed, the lower recovery yield of total protein than HBcAg, as shown in Table 1, may suggest that these impurities were strongly trapped in the inner pore compartments of the



**Fig. 2.** The chromatographic profile of online HBcAg purification by EBAC operation. The *E. coli* feedstock was disrupted by bead milling and was diluted immediately to 5% biomass (w/v) and loaded into the equilibrated EBA contactor. The initial purification was carried out under room temperature and constant linear flow rate of 100 cm/h. (A) SDS-PAGE analysis shows the distribution of HBcAg, and (B) graphical illustration indicates the amount of total protein (◆) and HBcAg (■) collected in the entire EBA process. In (A), lane M: molecular mass markers; lane C: unclarified *E. coli* disruptate; lanes 1–10: flowthrough; lanes 11–22: washing; lanes 23–30: elution.



**Fig. 3.** The eluted protein from the on-line EBAC process was subjected to final purification by SEC. The (A) SDS-PAGE analysis and (B) graphical illustration showing elution profile of SEC. In (A), lane M: molecular mass markers; lanes 1–18: SEC elution fractions.

**Table 1.** Results of the initial purification of HBcAg by integrated EBAC method.

Purification stages	Total volume (ml)	Total protein (mg)	HBcAg purity (%)	Amount of HBcAg (mg)	Specific HBcAg yield (mg/g protein)	Recovery yield (%)	Elution efficiency (%)	Purification factor
Initial	100	365.08	19.8	72.29	0.20			
Flowthrough	100	54.48	29.2	15.91	0.29			
Washing	150	41.83	29.9	12.51	0.30	58.5 <sup>a</sup>		
Overall elution	150	100.02	42.3	42.31	0.42	(27.4) <sup>b</sup>	96.4	2.1
Un-eluted		168.75		1.57				

<sup>a</sup>The figure indicates recovery yield of HBcAg.

<sup>b</sup>The figure in brackets indicates the recovery yield of total protein.

porous adsorbents. It has been reported that the application of solid, nonporous, and pellicular adsorbents is capable to address the problems associated with restrictive pore dimensions and to increase the EBAC recovery yield [39, 43].

A high elution efficiency of HBcAg (96.4%) was obtained in the current study. It is observed that a large portion of contaminant proteins were co-eluted with HBcAg in the first few elution fractions from EBAC (Fig. 3A). The elution process yielded 42.3 mg of HBcAg, which was collected in EBAC fractions 23–30. Because of the loss of HBcAg during the flowthrough and washing stages, the EBAC only managed to achieve a recovery yield of 58.5%. However, the eluted fractions contained a satisfactory 42.3% purity of HBcAg, which corresponds to a purification factor of 2.1. The overall performance of the EBAC purification process is summarized in Table 1. The partially purified HBcAg derived from online EBAC was further purified using SEC.

#### Final Purification by Size-Exclusion Chromatography

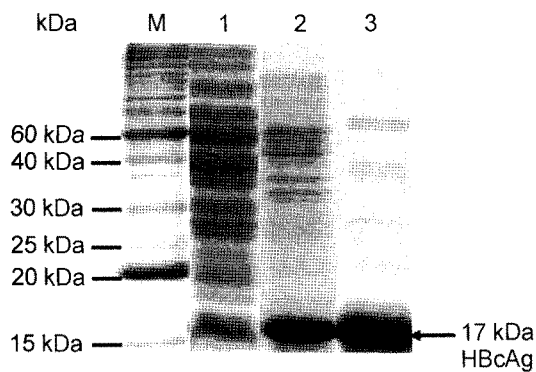
In the current study, the elution fractions from integrated EBA chromatography comprising partially purified HBcAg were subjected to SEC. Multiple components in the sample were separated according to molecular size as they passed through a 20-cm-long column packed with agarose gel (particle size in a range of 38–75  $\mu\text{m}$ ). Larger molecules such as HBcAg particles did not enter the gel matrix and directly pass through the interspaces between the particles in the column and elute from the column faster compared with the smaller molecules such as cell debris and DNA fragments. Molecules with partial access to the pores of the gel matrix move along the column at reduced velocity and elute from the column in a sequence of decreasing size.

The elution profile of SEC purification is illustrated in Fig. 3. A chromatographic peak at fractions 3–8 can be clearly observed. In Figs. 2A and 3A, the purified HBcAg protein bands appeared as a doublet at 17 and 16 kDa. The HBcAg doublet bands could be due to the presence of a different degree of oxidation/reduction of the intramonomeric disulfide bonds in the HBcAg monomer. The faint 16 kDa band probably represents the small amount of HBcAg monomer with an intradisulfide bridge, which migrates faster than the denatured 17 kDa monomer in SDS-PAGE gels. Similar observations for other proteins containing disulfide bonds have been reported by Hackstadt *et al.* [14] and Sun *et al.* [33].

Table 2 summarizes the results of purification of HBcAg by integrated EBAC and SEC. The purity of HBcAg in each purification step was determined using densitometric analysis of polyacrylamide gels (Fig. 4). The results show that the final purification step of SEC achieved a recovery yield of 45.1% and a product purity of 88.2% that led to a purification factor of 4.5. Indeed, a similar purity but at a much lower recovery yield (5.8%) was achieved in a recent purification protocol proposed by Broos *et al.* [5], which involved a multistep purification using DEAE Sepharose, heat treatment, ammonium sulfate precipitation, SEC, and anion-exchange chromatography. They introduced an additional step of heparin Sepharose chromatography to discriminate HBcAg with or without RNA. The step was shown to increase the purity of HBcAg to above 95% but at the expense of recovery yield (3.2%). However, this additional step is unnecessary for our purification work owing to the fact that the truncated HBcAg produced in this study is an empty capsid free of bacterial contaminant RNA. Nucleic acids were not observed in the crystal structure of the similar truncated HBcAg [34]. In another recent work on HBcAg, Tang *et al.* [37]

**Table 2.** Results of the HBcAg purification protocol developed in the current study.

Purification step	Total protein (mg)	Total HBcAg (mg)	HBcAg purity (%)	Recovery yield (%)	Purification factor
Crude disruptate (after batch bead milling)	356.08	72.29	19.8	(100)	(1.00)
Anion-exchange EBAC (Streamline DEAE)	100.02	42.31	42.3	58.5	2.1
Size exclusion (BioGel A-0.5M)	36.98	32.61	88.2	45.1	4.5



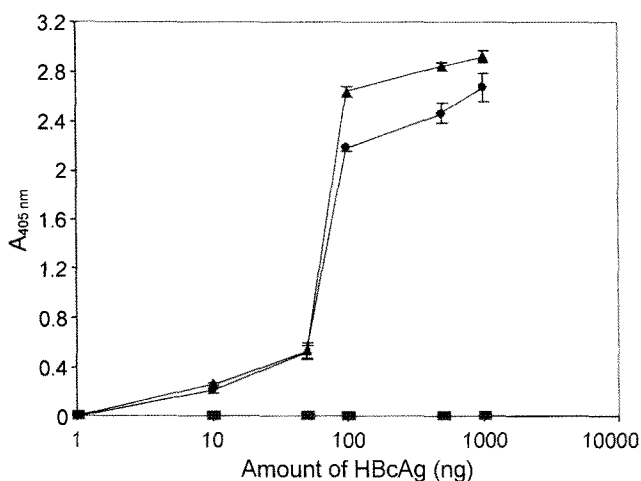
**Fig. 4.** SDS-PAGE analysis of the HBcAg recovered from unclarified feedstock.

Lane M: molecular mass markers; lane 1: unclarified *E. coli* disruptate; lane 2: eluted product from online EBAC; lane 3: eluted product from SEC. The arrow shows the position of 17-kDa truncated HBcAg.

established a small-scale purification method utilizing ammonium sulfate precipitation and the HPLC system (using a size-exclusion column containing zirconium-stabilized silica). This method gave rise to a purity of about 98.7%, but it is not suitable for large-scale purification of HBcAg.

#### Antigenicity of HBcAg

The antigenicity of HBcAg purified in the current purification protocol was determined by evaluating the reactivity of the HBcAg with the anti-HBcAg monoclonal antibody using ELISA (Fig. 5). The antigenicity test is of great importance because we have recently shown that HBcAg particles



**Fig. 5.** Antigenic study of the purified HBcAg with an anti-HBcAg monoclonal antibody *via* ELISA.

Sample tested contained purified HBcAg derived from integrated EBAC and SEC processes (▲), partially purified HBcAg derived from the online EBAC operation (●), and milk diluent as negative control (■). The absorbance at 405 nm was determined after a standard incubation time of 20 min. The data represent the mean±standard deviation of triplicate determinations.

were vulnerable to deformation due to extreme high shear induced during high-speed bead mill disruption [17]. The antigenicity exhibited by the partially purified HBcAg derived from online EBAC was slightly lower than the fully purified HBcAg derived from the combination of online EBAC and SEC, which might be a result of the presence of impurities in the partially purified sample. For both samples, the anti-HBcAg monoclonal antibody can detect as low as 10 ng (0.1 ng/ml) of HBcAg, and therefore confirmed the intactness of the particulate form and the antigenicity of the HBcAg recovered. Hence, the potential of the purified HBcAg to be applied as a reagent for detection of anti-HBcAg antibodies in a diagnostic kit was unaffected.

#### DISCUSSION

In the non-integrated protein purification protocol, the EBAC is a discrete unit operation located downstream of a fermentation operation or cell disruption process (Fig. 1A), and it is usually operated in a single or multi pass mode. The major drawback of this discrete operation is its detrimental “hold-up” period, in which batches of feedstock are stored before being subjected to the EBAC operation [23]. The “hold-up” period may lead to product degradation due to proteases activity and other harsh physical conditions [19]. In addition, the long “hold-up” time will also promote higher electrostatic and/or hydrophobic product-debris interactions, which may affect the recovery yield and molecular fidelity of the product [2]. Our online product capturing procedure, which is achieved by merging the bead mill disruption process with EBAC, is successful in sequestering the product immediately after the cell disruption processes, and hence eliminating the adverse effects of “hold-up” period (Fig. 1A).

The adoption of EBAC in our protocol has compressed the long sequence of conventional primary recovery steps such as clarification, concentration, and initial purification into a single process, and hence simplified the purification process and reduced the number of unit operations. The conventional purification protocols often resulted in low recovery yield due to massive amount of protein being lost in the multi-step procedure [5, 18, 31]. Apart from that, the simplification of the purification process has also contributed to significant reduction in process time and cost. The integrated EBAC procedure takes less than 2 h, whereas other more common initial purification steps such as ammonium precipitation and chemical treatment requires one full day [8, 16, 31, 42]. In addition, our integrated EBAC technique has excluded from our protocol the preliminary clarification of feedstock that is necessary prior to packed bed adsorption chromatography or sucrose gradient ultracentrifugation. The clarification step is often

achieved by means of high-speed centrifugation, which is laborious, expensive, and high power consuming.

Most of the studies on HBcAg have isolated the core particles based on their physical properties such as size and density. One of the dedicated techniques widely used for HBcAg purification is sucrose density gradient ultracentrifugation [35, 36]. An alternative method popularly used is SEC, which is adopted in this study. SEC separates HBcAg molecules on the basis of size differences [37]. A HBcAg isolation protocol using 30% ammonium sulfate precipitation, followed by final purification employing SEC, has been described by Crowther *et al.* [11], Tan *et al.* [34], and Böttcher *et al.* [6]. A comparison study on HBcAg purification by using ultracentrifugation and SEC was conducted by Rolland *et al.* [31]. The yield and productivity of HBcAg purified from *E. coli* E3CB using SEC was found to be 10-fold higher compared with those recovered from sucrose density gradient ultracentrifugation. It has been suggested that density gradient ultracentrifugation could induce deformation and aggregation of VBNP due to over concentration or dehydration of particles at high sucrose density, which could lead to protein degradation [31]. Furthermore, density gradient ultracentrifugation not only requires tedious and meticulous preparations, but is also time- and cost-consuming. In contrast, SEC is favorable because of its simple operation, mild operating conditions, low cost requirement, and, most importantly, scalability.

Recently, improvement on the SEC process has been done based on the implementation of high operating temperature. The elevated temperature increases the viscosity of the mobile phase and increases the diffusivity of molecules, thus shortening the SEC process time without serious deterioration in the resolution [28]. However, high-temperature SEC is not suitable for protein work owing to the risk of thermal denaturation, and therefore, the recent studies on high-temperature SEC centered on separation of non-protein products such as polymers. However, the target protein in this study is heat-stable, and has been demonstrated to withstand high temperature up to 80°C [12, 27]. Hence, the application of high-temperature SEC to reduce the process time might be a possible strategy for HBcAg purification.

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