UNIVERSITI PUTRA MALAYSIA

DIRECT RECOVERY OF RECOMBINANT HEPATITIS B CORE ANTIGEN FROM UNCLARIFIED *ESCHERICHIA COLI* FEEDSTOCK USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

MICHELLE NG YEEN TAN

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By

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August, 2008

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Institute: Bioscience

The capsid of hepatitis B virus (HBV), which consists of hepatitis B core antigen (HBcAg) has become one of the most frequently studied viral-like-particle (VLP) for the display of foreign epitopes. Many studies have been carried out to purify this capsid. However, the conventional method of purification requires multiple steps of operation, which could lead to excessive product loss and high production costs. Therefore, it is of importance to develop a fast and cost effective protein recovery method such as expanded bed adsorption chromatography (EBAC) to ensure a better and efficient recovery of protein, especially in large scale downstream process.

In this study, thermal treatment of the *Escherichia coli* cell feedstock at 60^{o}C for 30 min prior to solid removal in the conventional method has resulted in 1.4 times and 18% higher in purity and recovery yield respectively compared to non-heat-treated feedstock. In direct capture of HBcAg from unclarified feedstock using the STREAMLINE DEAE (weak anion-exchangers) in batch adsorption, heat treatment at 60^{o}C for 45 min has increased the recovery yield and purity by 2.3 and 3.8 times

respectively compared with non-heat-treated feedstock. When these conditions were applied in large scale purification of HBcAg via EBAC, the yield and purity have increased by 1.2 and 1.8 folds respectively, compared with that purified from non-heat-treated feedstock. Heating the crude feedstock has resulted in denaturation and precipitation of contaminants in the feedstock, hence reducing non-specific interactions between the cell debris and anion-exchanger. The present study has also demonstrated that purification of HBcAg from heat-treated unclarified feedstock was most efficient when EBAC operation using Fastline^{TM} 20 contactor was operated at constant velocity (127.9 cm/h) in feedstock containing 5% of biomass. Although the current study showed that heat-treatment of unclarified feedstock could increase the purity of HBcAg and reduced non-specific binding of contaminant onto Streamline DEAE, the purity obtained was lower compared with that purified using conventional methods. Therefore, development of an affinity adsorbent using M13 phage bearing a disulfide constrained heptapeptide at the gpIII protein coat with the sequence, C-WSFFSNI-C as the ligand has been carried out in this study. M13 phage immobilised onto Streamline Base Matrix via epoxy activation was used in direct capture of HBcAg from unclarified feedstock via two different modes of EBAC operations; typical single pass operation and modified EBAC operation with recirculation of feedstock.

Higher yield of HBcAg was obtained using modified EBAC operation due to increase in protein residence time in the column, however, the purity was reduced by 15% compared with typical EBAC operation, which could be due to diffusion of contaminants into the internal volume of the macroporous adsorbents. Although the purity of HBcAg recovered using M13 phage ligand adsorbents was higher (70-80%)

but the yield was lower compared with that purified using anion-exchanger. Therefore, this study showed that peptide displayed on M13 phage can be employed as an affinity ligand in direct capture of HBcAg from unclarified feedstock using EBAC. When analysed with ELISA, the antigenicity of HBcAg purified using both adsorbents in EBAC was still preserved.

Abstrak tesis yang dikemukakan kepada Senat Univerisiti Putra Malaysia sebagai memenuhi keperluan ijazah Doktor Falsafah

PEMULIHANAN SECARA LANGSUNG REKOMBINAN ANTIGEN TERAS HEPATITIS B DARIPADA SUAPAN *ESCHERICHIA COLI* TANPA PERJERNIHAN MENGGUNAKAN PENJERAPAN LAPISAN TERKEMBANG KROMATOGRAFI

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Kapsid virus hepatitis B (HBV) yang terdiri daripada antigen teras (HBcAg) telah menjadi salah satu daripada partikel bak virus (VLP) yang kerap digunakan untuk mempamerkan epitop asing. Pelbagai kajian telah dijalankan untuk penulenan HBcAg, akan tetapi, kaedah konvensional memerlukan beberapa langkah penjernihan yang boleh mengakibatkan kehilangan protein, kapital dan kos buruh yang tinggi serta masa pemprosesan yang panjang. Oleh itu, kaedah penulenan yang berkesan, murah dan pantas seperti penjerapan lapisan terkembang kromatografi (EBAC) harus digunakan, terutamanya dalam pelaksanaan skala besar.

Dalam kajian ini, rawatan haba ke atas suapan *Escherichia coli* dalam penulenan konvensional pada suhu 60^{o}C selama 30 min telah menyebabkan peningkatan ketulenan sebanyak 1.4 kali dan 18% dalam hasilan terakhir jika dibandingkan dengan suapan tanpa rawatan haba. Di dalam penulenan langsung HBcAg dari suapan tanpa penjernihan menggunakan Streamline DEAE (penjerap penukaran

anion lemah) dalam penjerapan kelompok, rawatan haba pada suhu 60^{o}C selama 45 min telah meningkatkan hasilan terakhir sebanyak 2.3 kali dan 3.8 kali dalam ketulenan HBcAg berbanding dengan suapan tanpa rawatan haba. Apabila ciri-ciri rawatan haba ini digunakan dalam penulenan HBcAg pada skala besar menggunakan EBAC, peningkatan sebanyak 1.2 dan 1.8 kali masing-masing dalam hasilan terakhir dan ketulenan HBcAg berbanding dengan suapan tanpa rawatan. Rawatan haba ke atas suapan telah menyebabkan penyahaslian dan pemendakan bendasing lalu mengurangkan interaksi di antara bendasing dengan penjerap penukaran anion. Kajian ini juga telah menunjukkan bahawa penulenan HBcAg daripada suapan tanpa penjernihan yang telah dirawat adalah lebih berkesan jika operasi EBAC menggunakan turus Fastline^{TM} 20 dilaksanakan pada halaju malar (127.9 cm/j) dengan suapan tanpa penjernihan yang mengandungi 5% berat basah biojisim (berat/isipadu). Walaupun ketulenan HBcAg diperolehi daripada suapan yang telah melalui rawatan haba telah meningkat jika dibandingkan dengan tanpa rawatan, ketulenannya masih tidak setaraf dengan apa yang diperolehi dengan kaedah konvensional. Oleh itu, penjerapan affiniti menggunakan faj M13 yang membawa peptida C-WSFFSNI-C telah digunakan dalam pemulihan secara langsung HBcAg melalui EBAC. Faj M13 telah dipegunkan pada Streamline matrik basal melalui pengaktifan epoksi dan telah digunakan dalam dua operasi EBAC yang berlainan iaitu operasi biasa EBAC dan operasi EBAC yang telah diubahsuaikan dengan pengitaran semula suapan secara berterusan melalui lapisan terkembang dalam turus.

Walaupun hasilan terakhir HBcAg melalui penulenan operasi EBAC yang telah diubahsuai adalah lebih tinggi berbanding dengan operasi biasa, tahap ketulenan yang diperolehi telah menurun sebanyak 15% akibat daripada resapan bendasing ke

isipadu dalaman penjerap bermakroliang. Ketulenan HBcAg yang diperolehi melalui penjerapan affiniti ini adalah lebih tinggi jika berbanding dengan penjerapan penukaran anion (melebihi 80% ketulenan) walaupun hasilan terakhirnya adalah lebih rendah. Dengan itu, kajian ini telah membuktikan bahawa pembawa peptida faj M13 boleh digunakan sebagai penjerap affiniti dalam pemulihan secara langsung HBcAg melalui EBAC. Apabila HBcAg yang diperolehi melalui kedua-dua kaedah penjerapan dianalisis dengan menggunakan kaedah ELISA, tahap keantigenannya masih dikekalkan.

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I certify that an Examination Committee has met on 8 August 2008 to conduct the final examination of Michelle Ng Yeen Tan on her degree thesis entitled "Direct Recovery of Recombinant Hepatitis B Core Antigen from Unclarified *Escherichia coli* Feedstock Using Expanded Bed Adsorption Chromatography" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

MICHELLE NG YEEN TAN

Date: 21 August 2008

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LIST OF ABBREVIATIONS

aa	amino acid
BSA	bovine serum albumin
CIP	clean-in-place
cm	centimetre
cm/h	centimetre per hour
cP	centipoise
C-terminus	carboxy-terminus
DNA	deoxy-ribonucleic acid
DNAse	deoxyribonuclease
EBAC	expanded bed adsorption chromatography
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
g	gram
g/ml	gram per milliliter
h	hour
HBcAg	hepatitis B core antigen
HBV	hepatitis B virus
HCl	hydrochloric acid
HPLC	high performace liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
KCl	potassium chloride
KH_2PO_4	potassium di-hydrogen phosphate
KSCN	potassium thiocyanate
mg	milligram

min	minute
mL	milliliter
mM	millimolar
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	sodium chloride
NaI	sodium iodide
NaOH	sodium hydroxide
Ni^{2+}	nickel ion
N-terminus	amino-terminus
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming unit
pH	*Puissance hydrogene*
p-npp	ρ-nitro-phenyl phosphate
RNA	ribonucleic acid
rpm	revolution per minute
s	second
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
T	triangulation number
TEMED	tetramethyl ethylenediamine
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Capsid of the hepatitis B virus (HBV), which is composed of multiple subunits of core antigens (HBcAg) has become one of the most studied viral-like-particle (VLP) that possess similar morphology and self-assemble ability to the native capsids without involvement of the viral genome (Pumpens and Grens, 2001). HBcAg is a strong immunogen (Milich *et al.*, 1997; Milich and McLachlan, 1986), and its capability in generating both humoral and cell-mediated immunity has made hepatitis B virus (HBV) core particle an excellent tool in delivering antigens for vaccination purpose (Nassal *et al.*, 2008; Chen *et al.*, 2004). This has also benefited in development of various immunoassays that are promising to use as diagnostic reagents (Li *et al.*, 2007; Touze *et al.*, 1999). Therefore, the quality of HBcAg should not be compromised as presence of impurities could lead to various problems such as inaccuracies in detection of specific antibodies in diagnostic kit (Li *et al.*, 2007). Therefore, due to the wide spread application of HBcAg, it is essential to produce this protein at low cost in both upstream and downstream processes (Mechtcheriakova *et al.*, 2006). However, apart from being too tedious to perform as it requires rounds of clarification steps, the available current method of HBcAg purification such as size-exclusion chromatography (Tang *et al.*, 2007; Broos *et al.*, 2007) and sucrose gradient ultracentrifugation (Huang *et al.*, 2006; Tan *et al.*, 2003), also involves higher production cost, longer processing time and low in product yield (Rolland *et al.*, 2001).

Therefore, alternative method such as expanded bed adsorption chromatography (EBAC) can be sought to improve the downstream process of HBcAg. EBAC has long been used in direct recovery of biomolecules from various sources such as *E. coli* and yeast cells homogenate (Chow *et al.,* 2007; Tan *et al.,* 2005; Chase and Draeger, 1992) as well as more complex feedstocks such as plant extracts (Valdès *et al.,* 2003) and chicken egg white (Chang and Chang, 2006) without the need of pre-clarification steps. Unlike the conventional packed-bed chromatography that requires particulate-free feed stream, this method of purification forms a stable expanded bed that allows unhindered passage for cell or cells debris to flow through the column without the risk of clogging up the bed (Chase, 1994). Furthermore, EBAC combines three important steps in purification; clarification, capture and concentration steps into one single operation, hence, resulted in shorter processing time (Anspach *et al.,* 1999; Chase and Draeger, 1992).

At present, the commonly-used EBAC matrices are ion-exchangers (Clemmitt and Chase, 2002) and metal-chelating (Tan *et al.,* 2005) adsorbents. However, EBAC operation using ion-exchangers is greatly influenced by electrostatic interactions between cell or cell debris in the feedstock and the adsorbent (Lin *et al.,* 2003) as well as the viscosity and ionic strength of the applied feedstock (Chang and Chase, 1996a; Anspach *et al.,* 1999). Therefore, affinity ligands are preferred as they are less likely to be affected by the aforementioned factors (Chase and Draeger, 1992). Unlike packed bed chromatography resin, affinity chromatographies in EBAC are usually carried out using commercially-available affinity ligands such as metal ions (Tan *et al.,* 2005) and dye-ligand (Reichert *et al.,* 2001). In view of these, many new solid phases, which can be chemically-linked with various synthetic and biological