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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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 \textit{Escherichia coli} cell feedstock at 60°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 \textsc{STREAMLINE DEAE} (weak anion-exchangers) in batch adsorption, heat treatment at 60°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™ 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

PEMULIHAN 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°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°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 penulenenaan 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 penulenenaan HBcAg daripada suapan tanpa penjernihan yang telah dirawat adalah lebih berkesan jika operasi EBAC menggunakan turus Fastline™ 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 penulenenaan operasi EBAC yang telah diubahsuaikan 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
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>viii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>ix</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxi</td>
</tr>
</tbody>
</table>

## CHAPTER

1 **INTRODUCTION**  

2 **LITERATURE REVIEW**  

2.1. Hepatitis B virus  

2.1.1. Virion structure  

2.2. Hepatitis B core antigen (HBcAg)  

2.2.1. HBcAg as carrier protein  

2.2.2. Production and purification of HBcAg  

2.3. Expanded bed adsorption chromatography (EBAC)  

2.3.1. Protein adsorption equilibrium  

2.3.2. Principles of operations in EBAC  

2.4. EBAC matrices  

2.5. Types of ligands  

2.5.1. Ion-exchanger  

2.5.2. Affinity ligand  

3 **HEAT-TREATMENT OF UNCLARIFIED FEEDSTOCK**  

IMPROVED THE RECOVERY EFFICIENCY OF RECOMBINANT HEPATITIS B CORE ANTIGEN.  

3.1. **INTRODUCTION**  

3.2. **MATERIALS AND METHODS**  

3.2.1. Cultivation of *E. coli* cells  

3.2.2. Conventional method of HBcAg purification and heat treatment  

3.2.3. Preparation of unclarified feedstock  

3.2.4. Optimisation of thermal treatment of unclarified feedstock direct capture of HBcAg  

3.2.5. Batch adsorption  

3.2.6. The Bradford assay  

3.2.7. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)  

3.2.8. Quantitation of HBcAg  

3.2.9. Enzyme-linked immunosorbent assay (ELISA)
3.3. RESULTS AND DISCUSSION 43
   3.3.1. Application of heat treatment in the 43
   conventional method of HBcAg purification
   3.3.2. Direct capture of HBcAg from heat-treated
   unclarified feedstock
   3.3.3. Effect of heat treatment on the antigenicity of
   HBcAg

3.4. CONCLUSION 50

4  DIRECT PURIFICATION OF RECOMBINANT HEPATITIS 51
   B CORE ANTIGEN FROM TWO DIFFERENT
   PRE-CONDITIONED UNCLARIFIED FEEDSTOCKS

   4.1. INTRODUCTION 51

   4.2. MATERIALS AND METHODS 53
       4.2.1. Materials 53
       4.2.2. Feedstocks preparation 54
       4.2.3. Method scouting and optimisation 54
       4.2.4. Equilibrium adsorption isotherm 55
       4.2.5. Operation of the EBAC column 56
       4.2.6. Quantitation of total protein and HBcAg 58
       4.2.7. Qualitative analysis of HBcAg 58
       4.2.8. Calculations 59

   4.3. RESULTS AND DISCUSSION 60
       4.3.1. Optimisation of binding pH 60
       4.3.2. Optimisation of elution condition 62
       4.3.3. Equilibrium adsorption isotherm 64
       4.3.4. Operation of the EBAC column 65
       4.3.5. Operation of EBAC 70

   4.4. CONCLUSION 78

5  EFFECT OF DIFFERENT OPERATING MODES 79
   AND BIOMASS CONCENTRATIONS ON THE
   RECOVERY OF RECOMBINANT HEPATITIS B CORE
   ANTIGEN

   5.1. INTRODUCTION 79

   5.2. MATERIALS AND METHODS 81
       5.2.1. Materials 81
       5.2.2. Preparation of heat-treated unclarified feedstock 81
       5.2.3. Viscosity measurement 81
       5.2.4. Operation of EBAC 82
       5.2.5. Total protein and HBcAg quantitation 84

   5.3. RESULTS AND DISCUSSION 85
       5.3.1. Liquid dispersion behaviour in the expanded bed 85
       5.3.2. Effect of different bed heights and biomass
               concentration on the performance EBAC operation

   5.4. CONCLUSION 97
IMMOBILISATION OF FUSION M13 BACTERIOPHAGE ON EBAC ADSORBENT IN AFFINITY CAPTURING OF HEPATITIS B CORE ANTIGEN

6.1. INTRODUCTION

6.2. MATERIALS AND METHODS
   6.2.1. Materials
   6.2.2. Large scale preparation and purification of M13 bacteriophage
   6.2.3. Preparation of unclarified E. coli feedstock
   6.2.4. Selection of a suitable activation method
   6.2.5. Immobilisation of M13 phage
   6.2.6. Batch adsorption
   6.2.7. Quantitation of total protein and HBcAg
   6.2.8. Qualitative evaluations on M13-immobilised adsorbents

6.3. RESULTS AND DISCUSSION
   6.3.1. Selection of a suitable activation method
   6.3.2. Effect of different ECH concentrations
   6.3.3. Effect of different NaOH concentrations
   6.3.4. Effect of reaction time and temperature
   6.3.5. Optimisation of M13 immobilisation
   6.3.6. Effect of ligand density
   6.3.7. Blocking of residual groups
   6.3.8. Stability and reusability of M13 immobilised adsorbents

6.4. CONCLUSION

DIRECT PURIFICATION OF RECOMBINANT HEPATITIS B CORE ANTIGEN USING M13 PHAGE-IMMobilised ADSORBENTS

7.1. INTRODUCTION

7.2. MATERIALS AND METHODS
   7.2.1. Materials
   7.2.2. Preparation of M13 phage
   7.2.3. Epoxy activation and immobilization of M13 phage onto Streamline Base Matrix
   7.2.4. Preparation of unclarified E. coli feedstock
   7.2.5. Method scouting and optimization
   7.2.6. EBAC operation
   7.2.7. Quantitation of total protein and HBcAg
   7.2.8. Qualitative analysis of purified HBcAg
   7.2.9. Calculations

7.3. RESULTS AND DISCUSSION
   7.3.1. Method scouting and optimization
   7.3.2. HBcAg uptake rate
   7.3.3. Equilibrium adsorption isotherm
   7.3.4. Operation of EBAC

7.4. CONCLUSION
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Summary of products purified from various sources using EBAC.</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparison of the purity, purification factor and yield of HBCAg of the thermal and non-thermal treated unclarified feedstock.</td>
</tr>
<tr>
<td>3.2</td>
<td>Comparison between the yield and the purity obtained after batch adsorption with unclarified feedstocks treated at different temperatures.</td>
</tr>
<tr>
<td>3.3</td>
<td>Comparison between the yield and the purity obtained after batch adsorption with crude feedstocks treated at 60°C of different treatment durations.</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of pH on the binding efficiency of HBCAg from unclarified feedstock.</td>
</tr>
<tr>
<td>4.2</td>
<td>Effect of NaCl concentrations on the elution of HBCAg.</td>
</tr>
<tr>
<td>4.3</td>
<td>Results of the linear regression of Richardson-Zaki plot.</td>
</tr>
<tr>
<td>4.4</td>
<td>Purification of HBCAg from heat-treated and non heat-treated unclarified feedstocks.</td>
</tr>
<tr>
<td>5.1</td>
<td>Velocities employed to maintain constant bed expansion degree of 2.</td>
</tr>
<tr>
<td>5.2</td>
<td>Liquid dispersion of the expanded bed operating at constant velocity of 127.9 cm/h with degree expansion of 2.</td>
</tr>
<tr>
<td>5.3</td>
<td>The adsorption performance of Streamline DEAE in different mode of EBAC operations.</td>
</tr>
<tr>
<td>5.4</td>
<td>Viscosity of equilibration buffer, non-heat treated and heat-treated biomass containing feedstock. The data represent the mean ± standard deviation of triplicate measurements.</td>
</tr>
<tr>
<td>6.1</td>
<td>Effect of different activation methods on the immobilization of M13 and binding of HBCAg from unclarified E. coli feedstock with Streamline base matrix.</td>
</tr>
<tr>
<td>6.2</td>
<td>Effect of pH on the immobilization of M13 onto epoxidated Streamline base matrix and efficiency of HBCAg binding from unclarified E. coli feedstock onto M13-immobilised adsorbents.</td>
</tr>
</tbody>
</table>
6.3 Effect of different ligand densities on HBcAg binding. 124
6.4 Effect of different ethanolamine concentrations on residual epoxy groups. 125
6.5 Stability of M13-immobilised adsorbents during storage. 127
6.6 Reusability of M13-immobilised adsorbents. 127
7.1 Effect of different pH on the binding of HBcAg onto M13 phage–immobilised adsorbents. 138
7.2 Elution efficiency of different chaotropic salts as eluting agents. 141
7.3 Effect of different urea concentrations on elution. 142
7.4 Effect of different EBAC operations on direct capture of HBcAg from crude feedstock. 150
7.5 Amount of ligand leakage at every purification step in typical and modified EBAC operations. 155
7.6 Light scattering analysis on typical and modified EBAC purified core particles. 156
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>A schematic representative of HBV virion structure.</td>
<td>6</td>
</tr>
<tr>
<td>2.2</td>
<td>A schematic diagram of an EBAC column.</td>
<td>21</td>
</tr>
<tr>
<td>3.1</td>
<td>SDS-PAGE gel of the heat-treated (60°C, 30 min) and the non-heat-treated feedstock in the conventional method of HBcAg purification.</td>
<td>45</td>
</tr>
<tr>
<td>3.2</td>
<td>SDS-PAGE gel of the batch binding assay by using anion-exchange adsorbents and crude feedstock without heat treatment.</td>
<td>48</td>
</tr>
<tr>
<td>3.3</td>
<td>SDS-PAGE gel of the batch binding assay by using anion-exchange adsorbents and heat-treated feedstock at 60°C for 45 min.</td>
<td>48</td>
</tr>
<tr>
<td>3.4</td>
<td>The antigenicity level exhibited by the core particles after thermal-treated at different temperatures.</td>
<td>49</td>
</tr>
<tr>
<td>4.1</td>
<td>Elution of HBcAg using different NaCl concentrations.</td>
<td>63</td>
</tr>
<tr>
<td>4.2</td>
<td>Equilibrium adsorption isotherm of HBcAg from non-heat-treated and heat-treated unclarified feedstocks on Streamline DEAE.</td>
<td>65</td>
</tr>
<tr>
<td>4.3</td>
<td>Bed expansion characteristics of Streamline DEAE with different liquid phases.</td>
<td>66</td>
</tr>
<tr>
<td>4.4</td>
<td>Breakthrough curve of the HBcAg from unclarified feedstock containing 5% of biomass onto Streamline DEAE in EBAC.</td>
<td>69</td>
</tr>
<tr>
<td>4.5</td>
<td>EBAC recovery of HBcAg.</td>
<td>71</td>
</tr>
<tr>
<td>4.6</td>
<td>SDS-PAGE gels of the pooled fractions collected throughout the EBAC purification of HBcAg from two different pre-conditioned unclarified feedstocks.</td>
<td>74</td>
</tr>
<tr>
<td>4.7</td>
<td>Breakthrough curves of HBcAg and total protein from two different pre-conditioned feedstocks in EBAC.</td>
<td>75</td>
</tr>
<tr>
<td>4.8</td>
<td>Light scattering analysis of HBcAg and other contaminating proteins present in two different pre-conditioned unclarified feedstocks.</td>
<td>77</td>
</tr>
<tr>
<td>4.9</td>
<td>ELISA analysis of purified HBcAg from heat-treated unclarified feedstock.</td>
<td>78</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>5.1</td>
<td>EBAC operation at constant velocity.</td>
<td>90</td>
</tr>
<tr>
<td>5.2</td>
<td>EBAC operation at constant bed expansion.</td>
<td>91</td>
</tr>
<tr>
<td>5.3</td>
<td>Breakthrough curves of the HBcAg and total protein onto Streamline DEAE in EBAC operated at constant bed expansion.</td>
<td>96</td>
</tr>
<tr>
<td>5.4</td>
<td>Breakthrough curves of the HBcAg and total protein onto Streamline DEAE in EBAC operated at constant superficial velocity.</td>
<td>96</td>
</tr>
<tr>
<td>6.1</td>
<td>Optimisation of epoxy activation on Streamline Base Matrix using different concentrations of epichlorohydrin.</td>
<td>115</td>
</tr>
<tr>
<td>6.2</td>
<td>Effect of different concentrations of NaOH on epoxy activation of Streamline Base Matrix.</td>
<td>116</td>
</tr>
<tr>
<td>6.3</td>
<td>Effect of different temperatures on epoxy activation.</td>
<td>118</td>
</tr>
<tr>
<td>6.4</td>
<td>Epoxy activation of Streamline Base Matrix at 25°C.</td>
<td>118</td>
</tr>
<tr>
<td>6.5</td>
<td>SEM micrograph of epoxidated Streamline Base Matrix.</td>
<td>119</td>
</tr>
<tr>
<td>6.6</td>
<td>Macroporous surface of Streamline Base Matrix.</td>
<td>121</td>
</tr>
<tr>
<td>6.7</td>
<td>Immunofluorescence staining with non-epoxidated adsorbents.</td>
<td>122</td>
</tr>
<tr>
<td>6.8</td>
<td>Immunofluorescence staining of M13 phage-immobilised adsorbents.</td>
<td>122</td>
</tr>
<tr>
<td>6.9</td>
<td>Immunofluorescence staining of epoxidated Streamline Base Matrix.</td>
<td>126</td>
</tr>
<tr>
<td>7.1</td>
<td>Optimisation of HBcAg elution using different eluting agents.</td>
<td>140</td>
</tr>
<tr>
<td>7.2</td>
<td>Uptake rate of HBcAg using M13 phage–immobilised adsorbents.</td>
<td>143</td>
</tr>
<tr>
<td>7.3</td>
<td>Equilibrium adsorption isotherm of HBcAg on M13 phage–immobilised adsorbents.</td>
<td>145</td>
</tr>
<tr>
<td>7.4</td>
<td>Dynamic binding capacity of the expanded bed using M13 phage-immobilised adsorbents against HBcAg from unclarified feedstock containing 5% biomass.</td>
<td>146</td>
</tr>
<tr>
<td>7.5</td>
<td>The breakthrough curves of total protein and HBcAg from unclarified feedstock containing 5% of biomass in typical EBAC operation.</td>
<td>148</td>
</tr>
<tr>
<td>7.6</td>
<td>Recovery of HBcAg using typical single pass EBAC.</td>
<td>150</td>
</tr>
<tr>
<td>Section</td>
<td>Content</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>7.7</td>
<td>Recovery of HBcAg from modified EBAC operation.</td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>Elution profile from typical single pass EBAC operation.</td>
<td></td>
</tr>
<tr>
<td>7.9</td>
<td>Elution profile from modified EBAC operation.</td>
<td></td>
</tr>
<tr>
<td>7.10</td>
<td>ELISA analysis of HBcAg purified from different modes of EBAC operations.</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>Process scheme for the recovery of HBcAg from <em>E. coli</em> unclarified feedstock via EBAC purification using Streamline DEAE.</td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>Process scheme for the recovery of HBcAg from <em>E. coli</em> unclarified feedstock via EBAC purification using M13 phage-immobilised adsorbents.</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
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<td></td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>clean-in-place</td>
<td></td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
<td></td>
</tr>
<tr>
<td>cm/h</td>
<td>centimetre per hour</td>
<td></td>
</tr>
<tr>
<td>cP</td>
<td>centipoise</td>
<td></td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy-terminus</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy-ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DNAse</td>
<td>deoxyribonuclease</td>
<td></td>
</tr>
<tr>
<td>EBAC</td>
<td>expanded bed adsorption chromatography</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
<td></td>
</tr>
<tr>
<td>g/ml</td>
<td>gram per milliliter</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
<td></td>
</tr>
<tr>
<td>HBeAg</td>
<td>hepatitis B core antigen</td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
<td></td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>potassium di-hydrogen phosphate</td>
<td></td>
</tr>
<tr>
<td>KSCN</td>
<td>potassium thiocyanate</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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</tr>
</tbody>
</table>
min minute
mL milliliter
mM millimolar
$\text{Na}_2\text{HPO}_4$ di-sodium hydrogen phosphate
NaCl sodium chloride
NaI sodium iodide
NaOH sodium hydroxide
$\text{Ni}^{2+}$ nickel ion
N-terminus amino-terminus
OD optical density
PAGE polyacrylamide gel electrophoresis
PEG polyethylene glycol
pfu plaque forming unit
pH $Puiissance\ hydrogene$
p-npp $\rho$-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-$\beta$-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