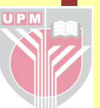
UNIVERSITI PUTRA MALAYSIA

# DEVELOPMENT OF AN INTEGRATED RECOVERY PROCESS FOR RECOMBINANT HEPATITIS B CORE ANTIGEN

HO CHIN WOI

FK 2007 27



# DEVELOPMENT OF AN INTEGRATED RECOVERY PROCESS FOR RECOMBINANT HEPATITIS B CORE ANTIGEN

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MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA
2007

**DEVELOPMENT OF AN INTEGRATED RECOVERY PROCESS FOR RECOMBINANT HEPATITIS B CORE ANTIGEN**

**By**

**HO CHIN WOI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science**

**May 2007**

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**DEVELOPMENT OF AN INTEGRATED RECOVERY PROCESS FOR RECOMBINANT HEPATITIS B CORE ANTIGEN (HBcAg)**

By

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**May 2007**

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Hepatitis B core antigen (HBcAg) expressed intracellularly in *Escherichia coli* (*E. coli*) has great potential and high demand in pharmaceutical market. The main objective of this study was to develop an efficient and cost effective protocol for the recovery of recombinant HBcAg. Cell disruption is the prerequisite step to release the intracellular HBcAg to the surrounding medium prior to subsequent purification processes. In the current study, *E. coli* was disrupted by mechanical method of bead milling. Bead milling can be operated either in batch or continuous recycling mode. The performance of bead milling is affected by parameters such as impeller tip speed, biomass concentration, bead loading and feed flow rate. Thus, in the first chapter of the study, the effects of these parameters on different modes of operation were investigated. The effect of these operation modes on the subsequent downstream processing was also examined. In the second chapter of the study, a

purification protocol for the recovery of HBcAg was developed. The HBcAg was initially purified by the integrated operation of batch mode bead milling and expanded bed adsorption (EBA), and subsequently purified by size exclusion chromatography (SEC).

The results of the cell disruption study show that the optimum disruption of *E. coli* and release of intracellular HBcAg in batch mode bead milling was achieved at impeller tip speed of 14 m/s, biomass concentration of 20% (w/v) and bead loading of 80% (v/v), whilst the performance of continuous recycling bead milling was peak at impeller tip speed of 14 m/s, biomass concentration of 10% (w/v) and feed flow rate of 15 L/h. Batch mode was ideal for the batch anion-exchange adsorption, in which a HBcAg yield of 34.3%, a HBcAg purity of 65% and a purification factor of 2.86 was achieved.

In the purification study, the product yield, product purity and purification factor achieved in the integrated EBA process was 55%, 42.3% and 1.96, respectively. The partially purified HBcAg was then further purified by SEC, in which a product yield of 42.4% was obtained. The SEC purification has also yielded a HBcAg purity of 88.2%, which corresponded to a purification factor of 4.08. The purified HBcAg was confirmed to be functionally active and hence, can be used in the development of hepatitis B virus (HBV) diagnostic kits.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PEMBANGUNAN SATU PROSES PEMULIHAN INTEGRASI UNTUK HEPATITIS B CORE ANTIGEN (HBcAg)**

Oleh

**HO CHIN WOI**

**Mei 2007**

**Pengerusi:** **Professor Madya Tey Beng Ti, PhD**

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Hepatitis B core antigen (HBcAg) yang diekspresi secara intrasel dalam *Escherichia coli* (*E. coli*) mempunyai potensi dan permintaan yang tinggi dalam pasaran farmaseutik. Objektif utama kajian ini ialah membangunkan satu protokol yang cekap dan kos-berkesan untuk pemulihan HBcAg rekombinan. Pemecahan sel ialah langkah pertama untuk membebaskan HBcAg intrasel ke medium sekeliling sebelum proses penulenan berikutnya. Dalam kajian ini, *E. coli* dipecahkan oleh kaedah mekanikal pengisaran manik. Pengisaran manik boleh dioperasi sama ada dengan mod kelompok atau mod berkitar berterusan. Prestasi pengisaran manik adalah dipengaruhi oleh parameter seperti kelajuan hujung pendesak, kepekatan biojisim, pengisian manik dan kadar aliran suapan. Oleh itu, dalam bab pertama kajian ini, kesan parameter-parameter ini terhadap mod operasi yang berlainan telah disiasat. Kesan mod pengisaran manik terhadap proses hiliran berikutan juga telah diperiksa.

Dalam bab kedua kajian ini, satu protokol penulenan bagi pemulihan HBcAg telah dibangunkan. HBcAg pada awalnya ditulenkan oleh operasi berintegrasi antara pengisaran manik mod kelompok dan penjerapan lapisan terkembang (EBA), dan kemudiannya ditulenkan oleh komatografi penyisihan saiz (SEC).

Keputusan kajian pemecahan sel menunjukkan bahawa pemecahan sel *E. coli* dan pembebasan HBcAg intrasel yang optimum dengan pengisaran manik mod kelompok telah dicapai pada kelajuan hujung pendesak selaju 14 m/s, kepekatan biojisim sebanyak 20% (w/v) dan pengisian manik sebanyak 80% (v/v), sementara persembahan pengisaran manik berkitar berterusan adalah kemuncak pada kelajuan hujung pendesak selaju 14 m/s, kepekatan biojisim sebanyak 10% (w/v) dan kadar aliran suapan bernilai 15 L/j. Kesan mod pengisaran manik terhadap proses hiliran berikutan juga telah diperiksa. Mod kelompok adalah ideal untuk penjerapan kelompok pertukaran anion, dalam mana hasil HBcAg setinggi 34.3%, ketulenan HBcAg setinggi 65% dan faktor penulenan setinggi 2.86 telah dicapai.

Dalam kajian penulenan, hasil produk, ketulenan poduk dan faktor penulenan yang dicapai dalam proses berintegrasi EBA adalah masing-masing setinggi 55%, 42.3% dan 1.96. HBcAg separa tulen kemudiannya ditulenkan lagi dengan SEC, dalam mana hasil produk setinggi 42.4% telan diperolehi. Proses penulenan SEC juga menghasilkan ketulenan produk sebanyak 88.2%, yang mana selaras dengan faktor penulenan sebanyak 4.08. HBcAg yang ditulenkan telah disahkan berfungsi secara aktif dan boleh digunakan dalam pembangunan kit diagnostik virus hepatitis B (HBV).

## ACKNOWLEDGEMENTS

I wish to express my utmost gratitude to my esteemed supervisor, **Assoc. Prof. Dr. Tey Beng Ti** for his patience, encouragement and inspiration throughout this fascinating project. Sincere acknowledgements are extended to my respected co-supervisors, **Assoc. Prof. Dr. Tan Wen Siang, Dr. Ling Tau Chuan and Pn. Suryani Kamarudin** for their invaluable advises and constructive criticisms. Their suggestions and comments during the lab meeting have helped me immensely in tackling many problems encountered in my study.

A special note of appreciation goes to my colleagues at laboratory of molecular virology, Faculty of Biotechnology and Biomolecular Science especially Swee Tin, Michelle Ng, Kie Hie and Fui Chin for their valuable cooperation and suggestions in so many ways.

I would also like to thank all the laboratory members in Faculty of Biotechnology and Biomolecular Science, for their friendship and sharing of scientific knowledge throughout the period of my study: Dr. Firoozeh, Dr. Raha Raus, Dr. Tan Geok Hun, Yan Peng, Kah Fai, Han Koh, Lin Kiat, Kok Song, Thong Chuan, Taznim, Siti Salwa, Max, Jaffri, Samira, Rajik, Hamzah, Hidayah.

Last but not least, to my parents and my siblings, I wish to dedicate my heartiest gratitude for their understanding and never-ending love.

I certify that an Examination Committee met on 31st May, 2007 to conduct the final examination of Ho Chin Woi on his Master of Science thesis entitled "Disruption of *Escherichia coli* by bead milling and development of an integrated recovery process for recombinant HBcAg" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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**HO CHIN WOI**

Date : 10 JUNE 2007

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| β | beta |
| °C | degree centrigrade |
| % | percent |
| µg | microgram ($10^{-6}$ g) |
| µg/mL | microgram per mililitre |
| µL | microlitre ($10^{-6}$ l) |
| µM | micromolar ($10^{-6}$ M) |
| Å | Ångstrom |
| 1/min | per minute |
| APS | ammonium per sulphate |
| ASP | aspartic acid |
| BSA | bovine serum albumin |
| CBB | Coomassie Brilliant Blue |
| CIP | clean-in-place |
| cm | centimeter ($10^{-2}$ m) |
| cm/h | centimeter per hour |
| C-O | carbon-oxygen |
| C-terminal | carboxyl terminal |
| dcw/v | dry cell weight/volume |
| DEAE | diethylaminoethyl |
| $dH_2O$ | distilled water |
| DNA | deoxy-ribonucleic acid |
| DPS | direct product sequestration |

| | |
|---|---|
| DSP | downstream processing |
| EBA | expanded bed adsorption |
| *E. coli* | *Escherichia coli* |
| ELISA | enzyme-linked immunosorbent assay |
| G3PDH | glyceraldehydes 3-phosphate dehydrogenase |
| Glu | glutamic acid |
| g/mL | gram per mililitre |
| h | hour |
| HBcAg | hepatitis B core protein |
| HBsAg | hepatitis B surface antigen |
| HBV | hepatitis B virus |
| HCC | heptocellular carcinoma |
| HCl | hydrochloric acid |
| IPTG | isopropyl-β-thiogalactopyranoside |
| IU | international unit |
| *k* | rate constant |
| kDa | kilodalton |
| L | litre |
| LB | Luria-Bertani |
| L/h | litre per hour |
| L-HBsAg | large surface antigen |
| M | molar |
| mA | miliAmpere ($10^{-3}$ A) |
| M-HBsAg | middle surface antigen |

| | |
|---|---|
| mL/h | mililitre per hour |
| mg | milligram ($10^{-3}$ g) |
| mg/mL | milligram per litre |
| $MgCl_2$ | magnesium chloride |
| mg/g | milligram per gram |
| min | minute |
| mL | mililitre ($10^{-3}$ l) |
| mm | milimetre ($10^{-3}$ m) |
| mM | milimolar ($10^{-3}$ M) |
| m/s | meter per second |
| $N$ | number of passage in a continuous mode bead milling |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| ng | nanogram ($10^{-9}$ g) |
| $(NH_4)_2SO_4$ | ammonium sulphate |
| nm | nanometer ($10^{-9}$ m) |
| NPP | p-nitro phenyl phosphate |
| OD | optical density |
| PHB | poly-β-hydroxybutyrate |
| $Q$ | volumetric feed flow rate |
| $R$ | the amount of protein/HBcAg released in a determined time |
| $R_m$ | maximum amount of protein/HBcAg released |
| S-HBsAg | small surface antigen |
| SP | sulphopropyl |

| | |
|---|---|
| pH | *Puissance hydrogene* |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEC | size exclusion chromatography |
| s | second |
| t | residence time |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline buffer supplemented with Tween-20 |
| TEMED | N,N,N'-N'-tetramethyenediamine |
| UK | United Kingdom |
| USA | United States of America |
| uv | ultraviolet |
| *V* | void volume of a grinding chamber |
| VLP | virus-like-particle |
| v/v | volume/volume |
| W | watt |
| WHO | World Health Organisation |
| w/v | weight/volume |
| x *g* | centrifugal force |

## CHAPTER 1

## INTRODUCTION

Biotechnology is a highly multidisciplinary field and has its foundations in biology, microbiology, biochemistry, molecular biology, genetics, chemistry, and chemical and process engineering (Smith, 2004). In the twentieth century, modern biotechnology has generated a wide range of new and novel products, such as nucleic acid-based products and therapeutic proteins. By mid-2003, some 118 nucleic acid-based products and therapeutic proteins (produced by genetic engineering and hybridoma technology) have gained marketing approval and one in four genuinely new drugs on the market are now produced by biotechnological means (Spada and Walsh, 2005). In the industrial manufacturing of these products, downstream processing (DSP) is economically vital. DSP is the general term used to describe the separation processes used to recover biological products (Liddel, 1994). Some of these products are intracellular and have to be released to the surrounding prior to subsequent DSP. This is achieved by cell disruption. Cell disruption is closely connected to the performance of subsequent DSP due to its effects on the quality (eg. purity, concentration, molarity and amount of cell debris) and physical conditions (eg. viscosity, conductivity, density) of the cell disruptate derived.

The common cell disruption methods include ultrasonication and enzymatic (Harison, 1991; Garcia, 1993; Middelberg, 1995). These two methods are usually utilized in laboratory scale operation and are not suitable for large-scale disruption (Geciova *et al.*, 2002). Another popular cell disruption technique is bead milling. In

bead milling, the cells are agitated in suspension with small abrasive particles such as glass beads, silica, or sands. Cell walls are ruptured by shear forces of the grinding action of rotating beads and from the impact resulting from collision with cascading beads. Bead milling can be operated either in batch or continuous mode. Bead milling is widely used for large-scale cell disruption due to its relatively low power consumption and capability to process large volume of biomass (Garcia, 1993; Middelberg, 1995).

A suitable DSP is required to purify and isolate the intracellular product released during cell disruption. Ion exchange adsorption chromatography is a widely used DSP unit operation for primary capture of proteins and other charged biomolecules (Bonnerjea *et al.*, 1986). Ion exchange chromatography can be subdivided into cation and anion exchange chromatography. In cation exchange chromatography, positively charged ions are bound to negatively charged adsorbents, whilst negatively charged ions are attracted to positively charged resins in anion exchange chromatography. Ion exchange chromatography can be operated either batchwise or in expanded bed adsorption (EBA) mode (Amersham 2004a; Amersham 2004b).

The target protein used in the current study is a recombinant hepatitis B core antigen (HBcAg) that was expressed in *Escherichia coli* (*E. coli*). HBcAg plays an important role in diagnosis and monitoring of hepatitis B infection. It is widely used as antigen for the detection of anti-HBcAg antibodies in serum samples (Hoofnagle *et al.*, 1974; Korec *et al.*, 1989; Tordjeman *et al.*, 1992). It assembles into virus like particle (VLP), which has been widely used to display immunodominant epitopes of various virus such as hepatitis B, C and E, Hanta, foot-and-mouth, influenza, human

immunodeficiency virus and other viral proteins (Pumpens and Grens, 2001). Due to the vast potential and high demand of HBcAg in the pharmaceutical market, there is a need to produce this intracellular protein in large scale. Hence, the primary aim of this study was to develop an efficient and cost effective protocol for the production of recombinant HBcAg. In the current study, the cell disruption of *E. coli* expressing recombinant HBcAg was achieved by bead milling. The expression level of this protein in *E. coli* was about 20-25% of the total host protein, which is considered high. Furthermore, this protein has a pI (iso-electric point) of 4 and therefore has a negative net charge at pH 8.4. Thus, it can be purified using anion exchange chromatography. The combination of bead milling and anion exchange would result in a fast, simple, efficient and relatively cheap HBcAg production process. Therefore, the objectives of the current study were:

1. To optimize the operating conditions of both batch and continuous recycling mode of bead milling and to investigate the effects of these operation modes on the performance of batch anion-exchange adsorption chromatography.
2. To develop a protocol for HBcAg purification by utilizing integrated EBA procedure and size-exclusion chromatography (SEC).

## CHAPTER 2

## LITERATURE REVIEW

### 2.1 Hepatitis B

Hepatitis B is a worldwide public health problem. The causative agent of this B-type hepatitis in human is hepatitis B virus (HBV). It belongs to the virus family *Hepadnaviridae* (Seeger and Mason, 2000). The number of HBV carriers worldwide is estimated to be 2 billion, of which about 360 million are chronically infected (WHO, 2004). Most individuals who contracted Hepatitis B infection have a mild or entirely symptom-free disease that disappears by itself and requires no medical treatment. It leaves the affected person with lifelong immunity against hepatitis B. However, the remaining infected individuals will develop chronic HBV infection that may lead to liver cirrhosis and heptocellular carcinoma (HCC), which resulted in about 500-700 thousand deaths per year worldwide (WHO, 2004).

#### 2.1.1 Hepatitis B Virus (HBV)

HBV has been divided into 6 genotypes: A to F, by the comparison of its complete nucleotide sequences (Norder *et al.*, 1992, Norder *et al.*, 1994, Kramvis *et al.*, 2005). The genotypes show a distinct geographical distribution between and even within regions, and are proving to be an invaluable tool in tracing the molecular evolution and patterns and modes of spread of hepatitis B virus (Kramvis *et al.*, 2005). Structural HBV serotypes and genotypes are not uniformly distributed around the