UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF OPTIMISE STRATEGIES IN PRODUCTION OF RECOMBINANT HEPATITIS B SURFACE ANTIGEN FOR CHIP-BASED ANTIBODY DETECTION APPROACH

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By

TAM YEW JOON

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2015

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DEDICATION

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

ESTABLISHMENT OF OPTIMISE STRATEGIES IN PRODUCTION OF RECOMBINANT HEPATITIS B SURFACE ANTIGEN FOR CHIP-BASED ANTIBODY DETECTION APPROACH

By

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May 2015

Chairman : **Zeenathul Nazariah Allaudin, Ph.D**.
Faculty : **Institute of Bioscience**

Hepatitis B surface antigen (HBsAg) antibody (anti-HBs) has shown to be an important serological marker in monitoring the success in hepatitis B vaccination. In this, the HBsAg protein plays a vital role as the basic component for anti-HBs detection assays due to its high reactivity and specificity. As such, the present thesis focuses on assessing and optimising key areas related to the downstream processing conditions specifically in the production of HBsAg from recombinant *Pichia pastoris* and in the establishment of chip-based anti-HBs detection prototype.

A study of the Mut$^+$ phenotype for the expression of recombinant hepatitis B surface antigen (HBsAg) in *P. pastoris* strain GS115 with a two-phase fed-batch protocol is described. Expression levels of HBsAg protein of 6.74 g/L Dry Cell Weight (DCW) and 26.07 mg/L of HBsAg concentration were achieved 48 h of expression. The use of the pPIC3.5K-HBsAg plasmid in the Mut$^+$ phenotype enhanced the expression of HBsAg by a nearly 13 times higher volumetric productivity in the first 24 h and 35 times higher at peak production concentration. Comparison of *AOX* expression promoters relative to the HBsAg gene in the role of mRNA secondary structure during translation initiation revealed that HBsAg possesses lower folding stability with *AOX1* Mut$^+$ phenotype and was found better suited for HBsAg expression, which correlates with the ease of translation initiation under shake flask conditions.

Subsequently, cell disruption strategies by high pressure homogenizer were optimized using response surface methodology (RSM) to correlate influencing factors affecting cell disruption capability and specific protein release of HBsAg

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from *P. pastoris* cells. From the results, an optimised cell disruption strategy with up-scale adaptability consisted of a number of passes set at 20 times, biomass concentration of 7.70 g/L of dry cell weight (DCW) and pulse pressure at 1,029 bar was demonstrated and was shown to increase cell disruption efficiency by 2-fold and 4-fold for specific protein release of HBsAg when compared to glass bead method yielding 75.68% cell disruption rate (CDR) and HBsAg concentration of 29.20 mg/L, respectively.

Following the release of HBsAg from cell disruption, univariate screening approaches on factors affecting the purification performance of HBsAg on ion exchange chromatography (IEC) and size exclusion chromatography (SEC) techniques and the establishment of a two-step purification strategy were performed. The main results drawn showed that the IEC Q Sepharose XL column and optimum conditions of 4 mL sample loading volume, an elution buffer with 1.5 M NaCl concentration at pH 8 with an elution gradient length of 20 column volume and subsequent purification with SEC at a sample volume of 5 mL and a flow rate of 1 mL/min was able to efficiently purify HBsAg. An up-scaled version the established purification strategy comprising of the two techniques further demonstrated adaptability for scale-up operations with a final total PF of 94.82 ± 16.20, HBsAg purity of 95.48% and recovery yield of 78.07%.

Finally, detection and quantification of anti-HBs using direct surface plasmon resonance (SPR) chip-based assay approach with recombinant HBsAg obtained as capturing agent was performed. Recombinant HBsAg derived from *P. pastoris* was immobilized under optimum binding conditions at a concentration of 150 mg/L, sodium acetate buffer at pH 4 and 0.6% Triton X-100 while effective regeneration of sensor surface was able to be done with 20 mM HCl. A dynamic range of detection from ~0.00098 to 0.25 mg/L was achieved and no cross-reactivity was found for the other known antibodies tested. Comparison of SPR chip-based assay with ELISA in terms of limit of detection generated an approximate 7-fold increase in sensitivity and a 2-fold increase in accuracy of the replicated results. The ability of the assay to detect anti-HBs in human sera samples was demonstrated with minor differences in comparison to that of the results obtained with ELISA.

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

PEMBENTUKAN STRATEGI YANG OPTIMA DALAM PENGHASILAN REKOMBINAN HEPATITIS B PERMUKAAN ANTIGEN UNTUK CIP BERASASKAN PENGESANAN ANTIBODI

Oleh

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Hepatitis B antigen permukaan (HBsAg) antibodi (anti-HBs) telah terbukti sebagai penanda rujukan yang penting untuk pemantauan keberkesanan hepatitis B vaksinasi. Dengan ini, protein HBsAg memainkan peranan penting sebagai komponen asas untuk ujian pengesanan anti-HBs berikutan kereaktifan dan specifikasi yang tinggi terhadap antibodi tersebut. Oleh itu, tesis ini memberi tumpuan kepada penilaian dan pengoptimuman bidang-bidang utama yang berkaitan dengan keadaan pemprosesan hiliran khususnya dalam penghasilan HBsAg dari rekombinan *Pichia pastoris* di samping penubuhan prototaip cip pengesanan terhadap anti-HBs.

Satu kajian terhadap fenotip Mut^+ untuk ekspresi rekombinan HBsAg daripada *P. pastoris* jenis GS115 dengan protokol suapan sesekelompok dua fasa telah dilakukan. Tahap ekspresi protein HBsAg sebanyak 6.74 g/L keberatan sel kering (DCW) dan HBsAg berkepekatan 26.07 mg/L telah dicapai dalam masa 48 jam dari titik permulaan induksi. Penggunaan plasmid pPIC3.5K-HBsAg dalam fenotip Mut^+ mampu meningkatkan ekspresi HBsAg hampir 13 kali lebih tinggi dari segi produktiviti isipadu dalam jangka masa 24 jam yang pertama dan 35 kali lebih tinggi daripada puncak kepekatan pengeluaran. Perbandingan ekspresi promotor *AOX* relatif kepada gen HBsAg dalam peranan mRNA struktur sekunder semasa permulaan terjemahan menunjukkan bahawa HBsAg mempunyai kestabilan lipatan lebih rendah dengan *AOX1* Mut^+ fenotip dan didapati lebih sesuai untuk ekspresi HBsAg, jika dikaitkan dengan kesenangan dalam permulaan terjemahan di bawah keadaan kelalang bergoncang.

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Seterusnya, strategi pemecahan sel oleh homogenizer bertekanan tinggi untuk membebaskan rekombinan HBsAg daripada sel *P. pastoris* telah dioptimasikan dengan menggunakan kaedah respon permukaan (RSM) dalam penentuan factor-faktor yang mempengaruhi kecekapan pemecahan sel dan di dalam pembebasan HBsAg. Daripada keputusan yang didapati, rumusan strategi gangguan sel dengan kebolehan penyesuaian pengskalaan naik yang optima terdiri daripada bilangan 20 kali laluan, kepekatan biojisim 7.70 g/L DCW dan tekanan pada 1,029 bar didapati meningkatkan kecekapan pemecahan sel dan pelepasan protein spesifik HBsAg secara ketara dengan masing-masing 2 kali ganda dan 4 kali ganda jika dibandingkan dengan kaedah manik kaca yang menghasilkan 75,68% kadar gangguan sel (CDR) dan kepekatan HBsAg daripada 29.20 mg/L, masing-masing.

Susulan pembebasan HBsAg secara pemecahan sel, pendekatan saringan secara univariat telah digunakan dalam mengkaji kesan daripada faktor-faktor yang mempengaruhi prestasi penulenan HBsAg pada teknik kromatografi pertukaran cas (IEC) dan teknik kromatografi penyisihan saiz (SEC) serta pembentukan strategi penulenan dua langkah telah dilaksanakan. Keputusan yang diperolehi membuktikan bahawa IEC penjerap Q Sepharose XL berserta dengan syarat optima 4 mL isipadu sampel pemuatan, kepekatan garam 1.5 M NaCl pada pH 8 dengan tempoh elusi kecerunan linear 20 isipadu penjerap dan penulenan selanjutnya dengan SEC pada syarat optima isipadu sampel 5 mL dan kadar aliran 1 mL / min berkesan untuk menulenkan HBsAg. Strategi penulenan versi penskalaan naik yang terdiri daripada gabungan teknik IEC dan SEC menunjukkan keupayaan adaptasi terhadap operasi penskalaan naik dalam mencapai jumlah akhir PF sebanyak 94.82 ± 16.20 dengan ketulenan HBsAg 95.48% dan hasil pengutipan 78.07%.

Akhir sekali, satu kaedah untuk pengesanan dan kuantifikasi anti-HBs berdasarkan cip plasmon permukaan resonans (SPR) yang langsung dengan menggunakan rekombinan HBsAg yang diperolehi telah dibentuk. Rekombinan HBsAg daripada *P. pastoris* telah ditetapkan di atas permukaan dengan keadaan optima pada kepekatan 150 mg/L, natrium asetat penampan pada pH 4 dan 0.6% Triton X-100 manakala penjanaan semula permukaan pengesan secara berkesan dapat dilakukan dengan 20 mM HCl . Rangkaian pengesanan yang linear dianggarkan pada 0.00098 hingga 0.25 mg/L telah dicapai dan tiada tindak balas silang dapat diperhatikan terhadap antibody-antibodi lain yang diuji. Perbandingan kaedah ujian pengesan berasaskan cip SPR dengan ujikaji ELISA dari segi had pengesanan dapat meningkatkan sensitiviti ujian lebih kurang 7 kali ganda dan peningkatan sebanyak 2 kali ganda dari segi ketepatan pengulangan keputusan. Kaedah ini telah diaplikasikan kepada analisis yang lebih meluas dengan menggunakan sampel sera manusia yang mengandungi anti-HBs, memberikan data bersamaan dengan ujikaji ELISA.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AE	adsorption efficiency
ALT	alanine aminotransferase
ANOVA	analysis of variance
AOX	alcohol oxidase
BSA	bovine serum albumin
CCD	central composite design
CDR	cell disruption rate
CV	column volume
DCW	dry cell weight
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EE	elution efficiency
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
ICD	intact cell density
IEC	ion exchange chromatography
IFN	interferon
LOD	limit of detection
LOQ	limit of quantification
mAU	milli absorbance unit
mfe	minimum free energy
MIT	molecular imprinted technology
Mut	methanol utilization pathway
NHS	N-hydroxysuccinimide
OD	optical density
PF	purification factor
PHEMAT	poly(hydroxyethyl methacrylate-N-methacryloyl-L-tyrosine methyl ester
PMSF	phenylmethanesulfonylfluoride
RSM	research surface methodology
RU	response unit
SEC	size exclusion chromatography
SPR	surface plasmon resonance
TMB	3, 3', 5, 5'-tetramethylbenzidine
WCW	wet cell weight
YH	recovery yield of HBsAg concentration
YP	total protein yield concentration

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CHAPTER 1

INTRODUCTION

1.1 Background

Hepatitis B disease poses a major public health problem in many parts of the world, particularly developing countries in Asia, which comprises populations that show high endemicity of chronic hepatitis B virus (HBV) infection (Yap, 1994; Liaw, 2009). Although HBV is regarded as non-pathogenic, on its own, the virus does not trigger innate immunity and the outcome of its infection in causing liver diseases, often leads to fatality (Lavanchy, 2005). In Malaysia, significant numbers of chronic HBV carriers have been detected amongst the different ethnic groups with an increasing trend on the incidence rate of Hepatitis B infection in recent years especially in the productive age group (Ministry of Health, 2012; Ministry of Health, 2014). Apart from vaccination, constant screening for anti-HBs is an essential measurement needed to be taken to help in lowering infection cases (Atkinson *et al.*, 2011). Notably, anti-HBs serves as an important serological marker in monitoring the success of hepatitis B vaccination and as an indicator for disease resolution as well as in determination of the recovery of hepatitis B infected individuals (Bauer and Jilg, 2006). Presently, a variety of anti-HBs detection assays have been developed such as immuno-chromatography assay (ICA) (Li *et al.*, 2009), chemiluminescence assay (CL) (Zhang *et al.*, 2007), protein microarray (Xu *et al.*, 2007) and more importantly, enzyme linked immunosorbent assay (ELISA), which has been the gold standard assay for anti-HBs detection in the last 3 decades (Tsitsilonis *et al.*, 2004; World Health Organization, 2014b).

In this, the basic component for the detection of anti-HBs, hepatitis B surface antigen (HBsAg) has shown to play a vital role in meeting the quality control requirements of these assays in terms of sensitivity, specificity and accuracy as it has highly antigenic properties as well as the highest density of epitomes against anti-HBs (Bruss and Ganem, 1991; Kouichi *et al.*, 1991; Zhang *et al.*, 2001). As a diagnostic antigen, particularly the recombinant HBsAg, has been reported to possess high reactivity and specificity towards anti-HBs, with additional benefit as a safe and stable reagent for the development of qualitative and quantitative immunoassays (Bo *et al.*, 2005; Tan *et al.*, 2005; Ottone *et al.*, 2007). However, although these assays performed well in providing accuracy and reliable results, current short comings including time factor, indirect format, labour intensity, cost and the need for multiple washing steps which could lead to increased risk of contamination remain. Thus, there is still a continuous demand for more specific and effective alternative assays for anti-HBs detection. In addition, the current commercially produced HBsAg is still considered relatively expensive with limitations to downstream processing. These limitations were identified to be caused by low and prolonged expression levels, inefficiency in HBsAg release

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from host cells and low recovery yield of the protein during purification (Hardy *et al.*, 2000; Kumar *et al.*, 2003). The impact of the limitations, affects the viability of detection assay development.

As such, with the realization on the economic importance of recombinant HBsAg as well as the need for alternative assay for the detection of anti-HBs, the general objective of this study was directed towards optimising the production route for *P. pastoris* derived HBsAg and in the establishment of chip-based assay specific for anti-HBs detection for the purpose of achieving self-sufficiency in sustaining production of the chip-based assay in the long run. It is hypothesized that by assessing process parameters and coming up with alternative optimised strategies in crucial areas of production comprising of HBsAg expression, cell disruption and purification, the overall production process efficiency can be improved while attaining the biological activity of the protein applicable for the use as a diagnostic agent. Using the HBsAg produce, it is also hypothesized that the protein can be integrated into an SPR chip-based assay as a recognition element for the detection of anti-HBs through similar means of assessment and optimisation. Ideally, the success criteria of established prototype would be able to detect and quantitate anti-HBs in human remnant sera samples without major interference. This is important to omit additional steps needed in sample preparation which could allow a higher risk of false interpretation due to increased risk of exposure to contamination.

1.2 Objectives

The main objectives of the present study were as follows:

1) to develop a HBsAg expression strategy using Mut^+ phenotype of *P. pastoris* strain and study its feasibility as an alternative to Mut^S phenotype expression in the production of recombinant HBsAg protein.

2) to assess the characteristics of cell disruption for the release of recombinant HBsAg performed with a high pressure homogenizer based on the effects influenced by important variables and their correlation to each other and optimisation of the process strategy with the possibility of process scale-up.

3) to determine the significant factors influencing the purification performance of recombinant HBsAg for assay development and develop an optimum purification strategy for a better recovery yield of the purified protein.

4) to develop and evaluate a chip-based SPR detection assay for the quantification of anti-HBs using purified *P. pastoris* derived HBsAg as the recognition element.

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1.3 Null hypotheses:

1) Optimizing process parameters in the area of HBsAg expression, cell disruption of *P. pastoris* host cells and purification of HBsAg could not improve overall process efficiency.

2) The established recovery strategies would not be feasible for up-scaled processes.

3) The integration of HBsAg produce onto the sensor surface of SPR chip-based assay would be unsuccessful.

4) The established SPR chip-based assay was unable to detect anti-HBs from human remnant sera samples.

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