# UNIVERSITI PUTRA MALAYSIA

# SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE DISPLAYED CYCLIC PEPTIDE LIBRARY

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# SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE DISPLAYED CYCLIC PEPTIDE LIBRARY

By

HO KOK LIAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

March 2002

*This thesis is dedicated to my family, beloved one and friends……*

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

**SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE-DISPLAYED CYCLIC PEPTIDE LIBRARY**

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**March 2002**

**Chairman: Dr. Tan Wen Siang, Ph.D.**

**Faculty: Science and Environmental Studies**

Hepatitis B virus is the prototype member of the family *Hepadnaviridae* which causes acute and chronic liver diseases worldwide. The viral nucleocapsid containing a partially double stranded DNA is surrounded by an envelope comprises three distinct but related surface proteins (HBsAg), termed as small (S), medium (M) and large (L)-HBsAg. The essential subunit of the nucleocapsid is a polypeptide comprising 183 amino acids known as core protein (HBcAg). HBcAg produced in *Escherichia coli* is capable of self-assembly into core-like particles and can be purified easily with ammonium sulphate precipitation and sucrose gradient centrifugation. Core particles make of full-length HBcAg were used as substrate in biopanning with a cysteine constrained phage-displayed heptapeptide library. The most frequently identified phage clones displayed the cyclic peptides C-WSFFSNI-C and C-WPFWGPW-C. The relative dissociation constant ($K_d^{rel}$) values for the interaction between the phages and HBcAg were determined by an equilibrium binding assay in solution. The $K_d^{rel}$ values for

phage bearing peptides C-WSFFSNI-C and C-WPFWGPW-C for full-length and truncated HBcAg are less than 10 and 30 nM, respectively, which are 17- and 7-fold stronger than that of phage bearing the linear peptide LLGRMK. The selected phages were able to compete with monoclonal antibody C1-5 for a binding site on the surface of core particles, suggesting that the docking site of these phages may partially overlap with the epitope of mAb C1-5, which was mapped at amino acid positions 78 to 83 at the tips of the core particles. The heavy chain of mAb C1-5 is hydrophobic and was proposed to be the contact region for HBcAg. Interestingly, the isolated peptides C-WSFFSNI-C and C-WPFWGPW-C are mainly composed of hydrophobic amino acids and may bind to the same region as mAb C1-5. A synthetic linear peptide bearing the sequence WSFFSNI inhibited the binding of L-HBsAg to core particles *in vitro* with an inhibition concentration ($IC_{50}$) approximately 9.8 μM. The additional of cysteine residues to both the N- and C-termini of the peptide greatly reduced the solubility of this cyclic peptide, and as a result the $IC_{50}$ is approximately 20-fold higher than that of WSFFSNI. A suitable recombinant carrier therefore is needed in order to reduce the hydrophobicity of the peptides and subsequently acts as a delivery system for targeting the peptide to virally infected cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan bagi mendapat Ijazah Master Sains.

**PEMILIHAN PEPTIDA-PEPTIDA BERAFFINITI TINGGI TERHADAP ANTIGEN TERAS HEPATITIS B DARIPADA PERPUSTAKAAN PEPTIDA PAMERAN FAJ YANG TERBATAS SECARA DISULFIDA**

**Oleh**

**HO KOK LIAN**

**Mac 2002**

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Virus Hepatitis B adalah ahli kepada keluarga *Hepadnaviridae* yang menyebabkan masalah kesihatan sedunia serta merupakan punca utama penyakit hati kronik dan akut. Nukleokapsid HBV mengandungi DNA bebenang dua separa yang diselaputi oleh sarung yang terdiri daripada tiga jenis protein permukaan (HBsAg) iaitu HBsAg kecil (S), sederhana (M) dan besar (L). Subunit nukleokapsid ini merupakan satu polipeptida yang terdiri daripada 183 asid amino yang dikenali sebagai protein teras (HBcAg). Unit-unit HBcAg yang dihasilkan dalam *Escherichia coli* bergabung untuk membentuk partikel teras yang boleh ditulenkan dengan pemendakan ammonium sulfat dan pengemparan kecerunan sukrosa. Partikel teras yang diperbuat daripada HBcAg berpanjangan asal telah digunakan sebagai substrat dalam "*biopanning*" dengan menggunakan perpustakaan peptida pameran faj yang terbatas secara disulfida. Faj-faj yang membawa peptida C-WSFFSNI-C dan C-WPFWGPW-C merupakan faj-faj yang paling banyak dipilih. Pemalar penceraian relatif ($K_d^{rel}$) antara faj-faj dan HBcAg

telah ditentukan dalam asai keseimbangan pengikatan dalam cecair. Nilai-nilai $K_d^{rel}$ bagi faj yang membawa peptida C-WSFFSNI-C dan C-WPFWGPW-C dengan HBcAg berpanjangan asal dan bundung adalah kurang daripada 10 dan 30 nM masing-masing, iaitu, 17 dan 7-kali lebih kuat daripada faj yang membawa peptida lurus LLGRMK. Faj-faj yang terpilih juga berupaya bersaing dengan mAb C1-5 untuk tapak pengikatan pada permukaan partikel teras. Penemuan ini mencadangkan bahawa tapak pengikatan faj-faj tersebut adalah bertindih secara separa dengan epitop mAb C1-5 yang telah dipetakan dalam kedudukan asid amino 78 hingga 83 pada penghujung duri partikel teras. Selain daripada itu, rantai berat C1-5 adalah kaya dengan asid amino yang hidrofobik dan sifat ini telah disarankan sebagai bahagian yang bergabung dengan HBcAg. Peptida-peptida yang terpilih juga terdiri daripada asid amino yang hidrofobik, maka, peptida-peptida ini mungkin ikat pada bahagian yang sama dengan rantai berat mAb C1-5. Peptida sintetik lurus WSFFSNI berupaya menyekat pengikatan di antara L-HBsAg dan partikel teras secara *in vitro* dengan kepekatan penyekatan ($IC_{50}$) lebih kurang 9.8 μM. Penambahan asid amino sisteina yang bersifat hidrofobik pada kedua-dua penghujung C dan N peptida WSFFSNI telah menurunkan keterlarutannya, dan seterusnya mengakibatkan $IC_{50}$ peptida gelang ini 20-kali lebih tinggi daripada peptida WSFFSNI. Sesuatu pembawa diperlukan untuk mengurangkan hidrofobik peptida-peptida tersebut dan seterusnya bertindak sebagai sistem penghantaran peptida-peptida ini ke sel-sel yang dijangkiti oleh virus.

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I certify that an Examination Committee met on 7th March 2002 to conduct the final examination of Ho Kok Lian on his Master of Science entitled "Selection of High Affinity Peptides against Hepatitis B Core Antigen from A Phage-Displayed Cyclic Peptide Library" in accordance with 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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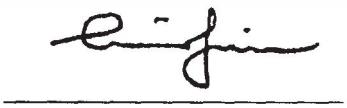

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## DECLARATION

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.

HO KOK LIAN

Date: 8th March 2002

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ε | encapsidation signal |
| α | alpha |
| β | beta |
| °C | degree centrigrade |
| µg | microgram ($10^{-6}$ g) |
| µl | microlitre ($10^{-6}$ l) |
| µM | micromolar ($10^{-6}$ M) |
| ρmole | picomole |
| A | adenine |
| Å | Ångstrom |
| Amp | ampicillin |
| ATP | adenosine triphosphate |
| bp | basepair |
| BSA | bovine serum albumin |
| C | cytosine/ core |
| ccc | covalently closed circular |
| Ci | curies |
| CITE | Cap-independent translation enhancer |
| cpm | count per minute |
| C-terminus | carboxy terminus |
| CTLs | CD8+ cytotoxic T lymphocytes |
| dATP | 2'-deoxy-adenosine-5'-triphosphate |
| dCTP | 2'-deoxy-cytidine-5'-triphosphate |
| ddATP | 2',3'-dideoxy-adenosine-5'-triphosphate |
| ddCTP | 2',3'-dideoxy-cytidine-5'-triphosphate |
| ddGTP | 2',3'-dideoxy-guanosine-5'-triphosphate |
| ddNTP | dideoxy-nucleoside triphosphate |
| ddTTP | 2',3'-dideoxy-thymidine-5'-triphosphate |

| | |
|---|---|
| dGTP | 2'-deoxy-guanosine-5'-triphosphate |
| DHBV | duck hepatitis B virus |
| DNA | deoxy-ribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| DR | direct repeat |
| dsDNA | double-stranded DNA |
| DTT | 1,4-dithiothreitol |
| dTTP | 2'-deoxy-thymidine-5'-triphosphate |
| ELISA | enzyme-linked immunoabsorbent assay |
| ER | endoplasmic reticulum |
| g | gram |
| GSHV | ground squirrel hepatitis virus |
| h | hour |
| HBcAg | hepatitis B core protein |
| HBsAg | hepatitis B surface protein |
| HBV | hepatitis B virus |
| HBxAg | hepatitis B x protein |
| HCC | hepatocellular carcinoma |
| HIV | human immunodeficiency virus |
| HSV | herpes simplex virus |
| IPTG | isopropyl-β-d-thiogalactopyranoside |
| kb | kilobase |
| $K_d$ | dissociation constant |
| kDa | kilodalton |
| $K_d^{rel}$ | relative dissociation constant |
| l | litre |
| LB | Luria broth |
| L-HBsAg | large surface antigen |
| LTR | long terminal repeat |
| M | molar |
| mAb | monoclonal antibody |

| | |
|---|---|
| mg | milligram ($10^{-3}$ g) |
| M-HBsAg | medium surface antigen |
| MHC | major histocompatibility complex |
| min | minute |
| ml | millilitre ($10^{-3}$ l) |
| mm | millimetre ($10^{-3}$ m) |
| mRNA | messenger ribonucleic acid |
| NDV | Newcastle disease virus |
| NET-gel | sodium-Tris-EDTA-gelatin buffer |
| nM | nanomolar ($10^{-9}$ M) |
| NP-40 | Nonidet p40 |
| N-terminus | amino terminus |
| OD | optical density |
| ORF | open reading frame |
| P | polymerase protein |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| pfu | plaque forming unit |
| pH | *Puissance hydrogene* |
| *pol* | polymerase protein |
| PreS1 | N-terminal region of L-HBsAg comprising 108 or 119 amino acids |
| PreS2 | region of M and L-HBsAg comprising 55 amino acids |
| PVDF | polyvinylidene difluoride |
| RF | replicative form |
| RNA | ribonucleic acid |
| RNAsin | RNA inhibitors |
| rpm | revolutions per minute |
| s | second |

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| S-HBsAg | small surface antigen |
| ssDNA | single stranded DNA |
| STE | sodium-tris-EDTA buffer |
| SV40 | simian virus 40 |
| T | thymine/ triangulation number |
| TBE | tris-buffered EDTA solution |
| TBS | tris-buffered saline |
| TE | tris-EDTA buffer |
| TEMED | tetramethyl ethylenediamine |
| TP | terminal protein |
| tRNA | transfer RNA |
| U | unit |
| UV | ultraviolet |
| v | volt |
| v/v | volume/volume |
| vol | volume |
| w/v | weight/volume |
| WHV | woodchuck hepatitis virus |
| x *g* | centrifugal force |
| X-gal | 5-bromo-4-chloro-3-indol-β-D-galactopyranoside |
| Y | fraction bound |

## AMINO ACID ABBREVIATIONS

| | **One letter code** | **Three letter code** |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |

| | | |
|---|---|---|
| Aspartic acid | D | Asp |
| Cysteine | C | Cys |
| Glutamic acid | E | Glu |
| Glutamine | Q | Gln |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

# CHAPTER I

## INTRODUCTION

Hepatitis B virus (HBV) is an enveloped DNA virus of the family *Hepadnaviridae*, which causes a variety of acute and chronic liver diseases such as cirrhosis and hepatocellular carcinoma in human and other higher primates such as chimpanzees (Ganem and Varmus, 1987). The virus is transmitted through direct contact with serum of an infected patient or body fluid, such as saliva, semen and vaginal fluid. In endemic areas, perinatally transmission from an HBV-infected mother to her offspring is more common (Mahoney, 1999). Generally, HBV infection becomes clinically apparent in less than 50% of all infected individuals after an incubation period of 35-150 days and a complete remission occurs in 90-95% of the cases within 3 to 4 months (Caselmann, 1996). The people infected with HBV either recover from the infection or they may remain chronically infected. According to World Health Organisation (WHO), there are about 400 million carriers worldwide and approximately 2 million carriers die annually (Caselmann, 1996), despite the existence of an effective HBV vaccine.

Currently, there is no safe and effective therapeutic agent treatment available for hepatitis B infection. Several inhibitors have been used in the therapy of chronic liver disease such as arabinoside A, acycloguanosine, β- and α-interferon

(Bassendine *et al.*, 1981; Weller *et al.*, 1983; Thomas and Scully, 1985). At present, α-interferon appears to hold the best hope to clear the virus. However, the overall respond rate is only approximately 50 % (Thomas and Scully, 1985). Lamivudine (also known as 3TC) is the currently licensed chemotherapeutic, which proved to be actively suppressed hepatitis B infection (Hilleman, 2001). Until recently, there is no safe and effective antiviral compound against the viral assembly and infectivity. To overcome these problems, small molecules such as peptide inhibitors, which bind to subunit interfaces that interfere the virus morphogenesis have been extensively studied.

Filamentous bacteriophage displaying millions of random peptide sequences on the minor coat proteins has been used to define ligand-binding sites that are difficult to identify by conventional methods (Scott and Smith, 1990). Peptide sequences that react with ligands such as monoclonal antibodies (D'Mello *et al.*, 1997), carbohydrate (Harris *et al.*, 1997), virus receptor (Ramanujam *et al.*, 2002) and animal organ (Pasqualini and Ruoslahti, 1996) were successfully isolated. Peptide sequences that bind to the core antigen of HBV were successfully isolated from a random linear hexapeptide library displayed on gpIII proteins of filamentous phage (Dyson and Murray, 1995). The relative dissociation constants for the linear hexapeptide and the core particles are in micromolar range. The peptides block the association between the core particles and the long surface antigen (L-HBsAg) *in vitro* and also inhibit the virus assembly in cell culture system (Dyson and Murray, 1995; Böttcher *et al.*, 1998). The linear peptide

sequences were not found as a continuous sequence within the L-HBsAg polypeptide, suggesting that some of the amino acids are brought from different positions of the polypeptide to form a discontinuous binding region or mimotope. Furthermore, Tan *et al.*, (1999) showed that the interaction of L-HBsAg and core particles is rather complex and involves at least two binding sites. It is therefore of interest to select for tighter ligands that bind to these sites with a disulfide constrained phage-displayed peptide library.

Isolation of cyclic peptides that associate with the core particles from a conformational phage display peptide library would be advantageous in providing high affinity binding clones to the core particles. As a result, synthetic peptides based upon the selected sequences would inhibit the association of HBcAg and L-HBsAg and thus block the assembly of HBV. Therefore, the objectives of this study were:

1. To select filamentous phage bearing cyclic peptide sequences that interact with HBcAg by biopanning;
2. To determine the relative dissociation constants ($K_d^{rel}$) between the selected phages and core particles;
3. To study the binding site of the phages on core particles and;
4. To evaluate the inhibitory effects of the synthetic peptides derived from the selected sequences upon the association of core particles and L-HBsAg.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Hepatitis B Virus (HBV)

### 2.1.1 Hepatitis B Virus Classification

HBV is an etiologic agent of human liver diseases, which poses the major public health problem, causing acute, chronic and fulminant hepatitis (Tiollais *et al.*, 1981). HBV is the prototype of the family *Hepadnaviridae* and subdivided into genus *Orthohepadnavirus*.

### 2.1.2 Epidemiology and Transmission

HBV is transmitted by exposure to blood or body fluid from HBV-infected individuals, and also sexually contact with HBV-infected patients (Mahoney, 1999). In the high endemicity areas such as Southeast Asia and China, perinatal transmission from infected mother to her offspring and intra-familial spread are most common. However, in the moderate and low endemicity areas such as United States, Canada, Western Europe, Middle East and Japan, most infections occur among the high-risk group for HBV infection such as intravenous drug users, sexual contact, haemodialysis patients and occupational exposure to HBV-