UNIVERSITI PUTRA MALAYSIA

EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERISATION OF THE HEPATITIS B VIRUS CORE ANTIGEN WITH AN N-TERMINAL EXTENSION

YOON KAM YEE

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By

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EXPRESSION, PURIFICATION AND STRUCTURAL CHARACTERISATION OF THE HEPATITIS B VIRUS CORE ANTIGEN WITH AN N-TERMINAL EXTENSION

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August 2013

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Hepatitis B virus (HBV) belongs to the family of *Hepadnaviridae* which contains an enveloped, nucleocapsid consisting of multiple copies of core antigen (HBcAg). HBcAg expressed in *Escherichia coli* self assembles into large and small spherical particles with 240 (triangulation number *T=4*) and 180 (triangulation number *T=3*) HBcAg subunits, respectively. Previous X-ray analysis of the wild type HBcAg showed that the N-terminal region of HBcAg is displayed on the surface of the capsid but the additional 11 residues of β-galactosidase and a linker could only be observed with weak electron density in the absence of atomic features due to the low atomic resolution (~8.9Å). Previous purification of HBcAg particles performed with sucrose density gradient ultracentrifugation and gel filtration methods could not separate the large and small particles homogenously. Therefore, an improved method to purify and isolate the large and small HBcAg particles homogenously was developed using the native agarose gel electrophoresis and electro-elution method (NAGE-EE). Dynamic light scattering (DLS) and transmission electron microscopic analyses further confirmed the homogeneity of the purified and separated *T=3* and *T=4* HBcAg particles. However, the isolated *T=3* and *T=4* HBcAg particles could not produce the desired X-ray diffractable crystals for structural elucidation of HBcAg with an N-terminal extension. To improve the resolution, Tyr132 of the HBcAg polypeptide was substituted with an Ala (N-Y132A) in order to create a mutant that forms a dimer. The morphology of the dimer is similar to that of the HBcAg dimeric structure of the capsid. The mutant was expressed, purified and crystallised with 18% PEG 2,000, 200 mM calcium acetate and 100 mM sodium cacodylate, pH 6.5 at 291 K. Crystals soaked in 20% glycerol, which functions as a cryo-protectant, was diffracted with X-ray to a maximum resolution of 1.8 Å using synchrotron radiation sources. The crystal belongs to a space group $P3_1$ with the unit cell parameters a=103.86, b=103.86 and c=88.11 Å. The electron density map revealed the molecular details of the N-terminal extension displayed consistently on the surface of the nucleocapsid. Hence, this finding provides an insight into the N-terminal extension which may offer a route for the development of multicomponent vaccines and molecular carriers.

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

KAJIAN PENGEKSPRESAN, PENULENAN DAN STRUKTUR ANTIGEN TERAS VIRUS HEPATITIS B DENGAN PEMANJANGAN N-TERMINAL

Oleh
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Virus hepatitis B (HBV) tergolong dalam ahli keluarga *Hepadnaviridae* yang terdiri dengan nukleokapsid bersampul yang terbentuk daripada banyak salinan antigen teras (HBcAg). Pengekspresan HBcAg dalam *Escherichia coli* menghasilkan zarah besar dan kecil dengan 240 (nombor triangulasi *T=4*) dan 180 (nombor triangulasi *T=3*) subunit, masing-masing. Kajian sinar X-ray analisis yang lepas dengan HBcAg virus tabii telah menunjukkan bahawa bahagian N-terminal HBcAg terpapar pada permukaan luar kapsid tetapi penambahan 11 asid amino kepunyaan β-galaktosidase dan seutas pemaut hanya mempamerkan sebahagian peta ketumpatan elektron yang lemah. Butir-butir molekul tidak dapat diperolehi kerana resolusi atomik yang rendah (8.9 Å). Penulenan zarah-zarah HBcAg yang lepas dengan menggunakan pengemparan ultra ketumpatan gradien sukrosa dan penurasan gel tidak dapat mengasingkan zarah besar dan kecil dengan homogen. Oleh sebab itu, kaedah penulenan dan pengasingan zarah besar dan kecil HBcAg telah diperbaiki dengan menggunakan kaedah elektroforesis gel agaros asli dan elektro-elusi (NAGE-EE). Kehomogenan zarah HBcAg *T=3* dan *T=4* dapat disahkan dengan analisis dinamik penyerakan cahaya (DLS) dan transmisi mikroskopi elektron. Walau bagaimanapun, zarah HBcAg *T=3* dan *T=4* yang diasingkan tidak dapat menghasilkan hablur yang dapat memberi penyerakan sinaran X-ray yang tinggi untuk kajian struktur HBcAg dengan perlanjutan N-terminal. Untuk memperbaiki resolusi, HBcAg kapsid telah dimutasikan dengan menggantikan asid amino Tyr132 dengan Ala (N-Y132A) untuk menghasilkan mutan yang boleh membentuk dimer. Morfologi dimer mutan ini adalah sama dengan HBcAg dimer dalam kapsid. Mutan ini telah diekspreskan, ditulenkan dan dihablurkan dengan 18% PEG 2,000, 200 mM kalsium asetat dan 100 mM natrium kakodilat, pH 6.5 pada 291 K. Hablur yang direndam dalam 20% gliserol yang berfungsi sebagai pembekuan pelindung telah membelau sinar X-ray kepada resolusi maksimum 1.8 Å dengan menggunakan sumber radiasi sinkrotron. Hablur ini tergolong dalam kumpulan $P3_1$ dengan parameter sel unit a=103.86, b=103.86 and c=88.11 Å. Peta ketumpatan elektron menonjolkan butir-butir molekul di N-terminal yang terdiri daripada β-galaktosidase yang terpamer pada permukaan kapsid. Kajian ini dapat meningkatkan pemahaman dalam interaksi molekul yang menglibatkan sambungan N-terminal dan dapat menawarkan satu cara untuk perkembangan vaksin multisubunit dan pembawa molekul.

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APPROVAL

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DECLARATION

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LIST OF ABBREVIATIONS

α	alpha
Å	Ångström
β	beta
µg	microgram (10^{-6} g)
µl	microliter (10^{-6} l)
µM	micromolar (10^{-6} M)
Amp	ampicillin
bp	basepair
BSA	bovine serum albumin
ccc	covalently closed circular
CCP4	Collaborative Computational Project Number 4
C-terminal	carboxy terminal
DHBV	duck hepatitis B
DNA	deoxy-ribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTT	1,4-dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunoabsorbent assay
ER	endoplasmic reticulum
FPLC	fast protein liquid chromatography
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl-β-d-thiogalactopyranoside
K	Kelvin
kb	kilobase
K_d	dissociation constant
kDa	kilodalton
LB	luria broth
L-HBsAg	large surface antigen
mAb	monoclonal antibody
MAD	multiwavelength anomalous dispersion
mg	milligram (10^{-3} g)
M-HBsAg	medium surface antigen
MHC	major histocompatibility complex
min	minute
mRNA	messenger ribonucleic acid
nM	nanomolar (10^{-9} M)
NAGE	native agarose gel electrophoresis
NAGE-EE	NAGE electro-elution
N-terminus	amino terminus
N-Y132A	N-terminal extension of HBcAg dimer

OD	optical density
ORF	open reading frame
P	polymerase protein
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
RMSD	root mean square deviation
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
S-HBsAg	small surface antigen
TBE	tris-buffered EDTA solution
TBS	tris-buffered saline
TEMED	tetramethyl ethylenediamine
tRNA	transfer RNA
U	unit
UV	ultraviolet
v	volt
v/v	volume/volume
w/v	weight/volume
WHV	woodchuck hepatitis virus
x *g*	centrifugal force
Y132A	truncated HBcAg mutation amino acid 132 from Tyr to Ala

AMINO ACIDS 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 1

INTRODUCTION

Hepatitis is due to inflammation of the liver tissues which is caused by a few different hepatitis viruses known as hepatitis A, B, C, D and E viruses. The main characteristic of hepatitis can be seen in the development of jaundice. The patients' sera could be tested for the existence of specific viral antigens or antibodies. Among all hepatitis viruses, hepatitis B virus (HBV) has become globally important because it has affected millions of people throughout the world with common infectious liver disease (Block *et al.*, 2013; Lok and McMahon, 2009). The severity of persistent HBV infection includes the development of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC). Infections occurred during early childhood are asymptomatic and about 90% of the cases may develop into a chronic stage (Franco *et al.*, 2012).

To date, more than 2 billion people are infected with HBV. Of these, about 300 million people remain infected chronically and become carriers of the virus (Franco *et al.*, 2012). HBV, which belongs to the family of *Hepadnaviridae*, is an enveloped virus containing a 3.2 kb partially double stranded circular DNA (Pasek *et al.*, 1979). HBV replicates in hepatocytes and may disturb normal liver functions. In the presence of the viral infectious particles, the host immune system is activated in order to neutralise the infectious agent. As a consequence, the liver is damaged and inflamed.

The route of HBV transmission is similar to human immunodeficiency virus (HIV) transmission which involves percutaneous or parental contact with virally infected blood and body fluids. HBV could survive outside the host for about a week without losing its infectivity (Hollinger FB, 2001). Vaccinated individuals or those have been exposed to the virus and developed anti-HBsAg are immunised naturally against the viral infection.

Based on the prevalence of chronic HBV infection, the world can be divided into 3 regions with high, intermediate and low endemicity (Franco *et al.*, 2012). In the high endemic regions such as South East Asia and sub Saharan Africa, the HBV carrier rate is higher than 8%. Whereas in low endemic regions such as the United States, Northern Europe, Australia and parts of South America, the prevalence is less than 2%. The Middle East and some Eastern Europe countries are considered as regions with intermediate endemicity with a chronic carrier rate between 2 to 8%.

The most effective way to reduce the infection is through vaccination during early childhood. In 1981 and 1982, the first vaccine was produced by purifying non-infectious particles from HBsAg carriers' sera. The particles were then inactivated by combination of urea, pepsin, formaldehyde and heat treatment. This plasma-derived vaccine was then replaced by vaccine produced via recombinant DNA technology in late 1980's. The recombinant vaccine has a huge global impact in decreasing the risk of infection in non-developing countries when a campaign of vaccination was made by WHO between 1991 and 1993 (Franco *et al*., 2012).

Since hepatitis B is a viral disease, the use of antibiotics is of no value for treatment of this infection. Early treatment of acute hepatitis B with steroid has worsen the scenario leading to persistent infection. Chronic HBV infection can be treated with α-interferon but its efficacy was limited and has been use until today (Hoofnagle *et al.*, 1988). Subsequently, lamivudine was developed and this compound has been proven to be able to inhibit the HBV replication by suppressing the HBV-polymerase (Dienstag *et al.*, 1995). However, long term usage of lamivudine could lead to viral resistance and result in high levels of alanine transferase besides incomplete suppression of viral replication (Lai *et al.*, 2003; Leung, 2002).

In recent years, the viral capsid formed by the core protein of hepadnavirus has been exploited extensively as vaccine platforms (Peters *et al.*, 2005). Thus, the development of the first synthetic peptide parenteral vaccine against a communicable disease was tested in human and is safe to be used (Herrington *et al.*, 1987). HBcAg was the pioneer recombinant virus like particle shown to deliver high level of immunogenicity to foreign sequences. The insertion of foreign sequences into the N- or C-terminal HBcAg or in the major immunodominant region (positions 78 to 83 amino acids) have been expressed and purified from *E. coli* and assembles into particles, revealing the potential of HBcAg as a molecular carrier in multicomponent vaccine development. An insertion at the N-terminal with the 8 residues of β-galactosidase and a linker sequence has shown the first example of such modified particles in *E. coli* system (Stahl *et al.*, 1982). X-ray crystallography has revealed that the N-terminal of HBcAg is located at the external surface of the capsid (Tan *et al.*, 2007; Wynne *et al.*, 1999). However, the available 3-D structure data for insertion of β-galactosidase residues at the N-terminus of HBcAg could not be traced. Therefore, in this study, the structure of the inserted β-galactosidase fragment at the N-terminus and its interactions with the endogenous HBcAg residues were studied.

The objectives of this study were:
i) to purify, isolate and characterise the HBcAg particles,
ii) to construct, express, purify and to characterise HBcAg dimers,
iii) to crystallise HBcAg dimers,
iv) to study the structure of HBcAg dimers with an N-terminal extension.

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