UNIVERSITI PUTRA MALAYSIA

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DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYA

2010

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By

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

February 2010

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

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February 2010

Chairman: **Associate Professor Tey Beng Ti, PhD**

Faculty: **Engineering**

The nucleocapsid protein (NCp) of Nipah virus (NiV) expressed in *Escherichia coli* (*E. coli)* is antigenic and immunogenic. NCp-NiV is a potential serological antigen that can be used in the diagnosis of NiV infections. The yield of NCp expressed in *E. coli* is low due to the proteolytic degradation by host endogenous proteases. Therefore, it is important to inhibit the endogenous proteolytic degradation activity and shorten the protein recovery process to avoid or reduce the action of protease on the recombinant NCp.

A method to predict the type of potential protease that attacks the NCp-NiV and its potential cleavage sites in *E. coli* to enhance the recovery of NCp was developed. A bioinformatics tool, PeptideCutter was used to identify potential protease and its cleavage sites from the amino acid sequences deduced from the published DNA

sequence of the NCp-NiV. The predicted proteases were serine proteases, hence, a range of serine protease inhibitors were tested to improve the yield of NCp. The yield of NCp was increased by 2-fold after the phenylmethylsulphonyl fluoride (PMSF) supplementation.

The downstream processing of the NCp-NiV from clarified *E. coli* homogenate was investigated. Two types of preparative chromatographic purification in a packed bed column; immobilised metal affinity chromatography (IMAC) and hydrophobic interaction chromatography (HIC) were studied and compared. A direct recovery of recombinant NCp-NiV from unclarified *E. coli* homogenate based on EBA chromatography was then developed by using the type of chromatography that can obtain high yield of the NCp with high antigenicity. In the IMAC system, HisTrap™ 6 Fast Flow was applied to purify the recombinant histidine-tagged NCp. A histidine hexamer tag was placed at the C-terminus of the NCp and this enabled the purification of NCp by IMAC system. The optimal binding was achieved at pH 7.5 and superficial velocity of 75 cm/h. The bound NCp was successfully recovered by a stepwise elution with a range of imidazole concentration (50, 150, 300 and 500 mM). The NCp was captured and eluted from an inlet NCp concentration of 0.4 mg/ml in a scale-up IMAC packed bed column of Nickel Sepharose™ 6 Fast Flow with the optimized conditions obtained from the scouting method. The purification of histidine-tagged NCp using IMAC packed bed column has resulted a 68.3% yield and a purification factor of 7.94.

In the HIC system, ammonium sulfate precipitation experiment was performed and it showed that 15% saturation of the salt was the most suitable concentration for the

binding buffer. Batch binding of the NCp was performed using Sepharose™ 6 Fast Flow adsorbents coupling separately with four different types of ligand; phenyl low substitution, phenyl high substitution, butyl and octyl. The phenyl low substitution ligand was selected for subsequent optimization process due to its highest yield and purity of the NCp achieved from the batch binding experiment. The HIC for purification of the NCp was further scaled up using a 10 cm column packed with phenyl low substitution Sepharose™ adsorbent. A recovering yield of 81% of the NCp with a purification factor of 9.3 was achieved from this scaled-up HIC operation.

Hence, the HIC adsorbent was used to capture the NCp in an EBA column due to its higher yield and purity obtained in the third chapter than the IMAC purification in the second chapter of this study. DNase was added to reduce the viscosity of feedstock and improve the axial mixing prior to the loading of the feedstock to the EBA column packed with the Streamline^{TM} HIC adsorbent charged with phenyl. The addition of glycerol to the washing buffer has reduced the volume of washing buffer applied, and thus reduced the loss of the NCp during washing stage. The dynamic binding capacity at 10% breakthrough of 3.2 mg/g adsorbent was achieved at a linear flow velocity of 178 cm/h, bed expansion of two and viscosity of 3.4 mPas. The adsorbed NCp was eluted with the buffer containing a step gradient of salt concentration. The purification of hydrophobic NCp using HIC-EBA column has resulted an 80% yield and a purification factor of 12.5.

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

PEMULIHAN KROMATOGRAFIK PROTEIN REKOMBINAN NUKLEOKAPSID VIRUS NIPAH DARIPADA HOMOGENAT *ESCHERICHIA COLI*

Oleh

CHONG FUI CHIN

February 2010

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

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Protein nukleokapsid (NCp) virus Nipah (NiV) terekspres dalam *Escherichia coli* (*E. coli*) adalah antigenik dan imunogenik. NCp-NiV adalah satu antigen serologis yang berpotensi untuk diagnosis jangkitan NiV. Hasil NCp terekspres dalam *E. coli* adalah rendah disebabkan oleh degradasi proteolitik endogenus protease *E. coli*. mengurangkan hasil NCp. Maka adalah penting untuk merencatkan aktiviti degradasi proteolitik dan memendekkan proses pemulihan protein untuk mengurangkan tindakan protease ke atas rekombinan NCp.

Satu cara untuk meramalkan jenis protease protensi yang bertindak ke atas rekombinan NCp-NiV dan tapak pemotongan potensi dalam *E. coli* untuk peningkatan hasil pemulihan NCp telah dibangunkan. Satu alat bioinformatik, PeptideCutter telah digunakan untuk mengenalpasti jenis protease dan tapak pemotongannya daripada jujukan asid amino yand dideduksikan dari jujukan DNA

NCp-NiV yang terterbit. Protease yang diramalkan adalah protease serine, oleh yang demikian, satu julat perencat protease serine telah diuji bagi mempertingkatkan hasil NCp. Hasil NCp telah dipertingkatkan sebanyak dua kali ganda selepas penambahan fenilmetilsulfonil fluorida (PMSF) ke dalam buffer.

Pemprosesan hilir rekombinan NCp-NiV daripada homogenat *E. coli* yang dijernihkan disiasat. Dua jenis kromatografi sediaan dalam satu turus lapisan terpadat; afiniti logam pegun (IMAC) dan kromatografi sedian interaksi hidrofobik (HIC) telah dikaji dan dibanding. Satu pemulihan secara langsung rekombinan NCp-NiV dari homogenat *E. coli* tanpa dijernihkan dalam penjerapan lapisan terkembang (EBA) telah dibangunkan dengan menggunakan jenis kromatografi di mana NCp yang didapati dengan antigenisiti yang tinggi. Dalam sistem IMAC, HisTrap™ 6 Fast Flow telah digunakan untuk menulen NCp rekombinan bertag histidina. Satu tag heksamer histidina telah ditempatkan pada hujung-C NCp untuk membolehkan penulenan NCp dengan menggunakan sistem IMAC. Keadaan penjerapan optimum yang diperolehi adalah pada pH 7.5 dan halaju permukaan 75 cm/j. NCp yang dijerap telah berjaya dipulihkan dengan elusi berperingkat dengan satu julat kepekatan imidazole (50, 150, 300 and 500 mM). Satu naik-skala turus lapisan terpadat dengan menggunakan suapan Nickel Sepharose™ 6 Fast Flow dan keadaan teroptimum yang diperolehi daripada aktiviti peninjauan telah dijalankan untuk memulih NCp. Penulenan NCp bertag histidina dengan IMAC telah membawakan hasil setinggi 68.3% dan faktor penulenan sebanyak 7.94.

Dalam sistem HIC, ujikaji pemendakan ammonium sulfate telah dijalankan dan ia menunjukkan 15% ketepuan garam adalah kepekatan yang paling sesuai digunakan untuk penimbal penjerapan. Penjerapan kelompok NCp telah dijalankan dengan

menggunakan penjerap Sepharose™ 6 Fast Flow yang diganding berasingan dengan empat jenis ligan berlainan; fenil berpekali penggantian rendah, fenil berpekali penggantian tinggi, butil dan oktil. Fenil berpekali penggantian rendah telah dipilih untuk proses pengoptimuman seterusnya disebabkan oleh hasil yang tertinggi dan ketulenan NCp yang dicapai daripada eksperimen penjerapan kelompok. Penulenan NCp dari HIC telah dinaik-skala selanjutnya dengan penggunaan turus lapisan terpadat 10 cm dengan penjerap fenil berpekali penggantian rendah Sepharose™. Satu hasil pemulihan NCp sebanyak 81% dengan satu faktor penulenan 9.3 telah dicapai daripada operasi naik-skala ini.

Dengan demikian, penjerap HIC telah digunakan untuk menyerap NCp dalam turus EBA kerana hasil dan ketulenan yang didapati dalam Bab Ketiga lebih tinggi daripada hasil penulenan IMAC dalam Bab Kedua dalam kajian ini. DNase telah dicampurkan untuk merendahkan kelikatan suapan sebelum muatan suapan dan mempertingkatkan pencampuran paksi dalam turus EBA yang dipadatkan dengan penjerap Streamline™ HIC terganding dengan fenil. Penambahan gliserol dalam penimbal pembersih telah mengurangkan penggunaan isipadu penimbal pembersih dan seterusnya mengurangkan kehilangan NCp semasa peringkat pembersihan. Kapasiti penjerapan dinamik pada 10% terobosan bernilai 3.2 mg/g penjerap telah dicapai pada halaju permulaan 178 cm/h, tahap dua lapisan terkembang dan kelikatan 3.4 mPas. NCp yang terjerap telah dielusikan dengan penimbal yang mengandungi langkah kecerunan kepekatan garam berkurangan. Penulenan hidrofobik NCp dari HIC dengan turus EBA telah membawa hasil sebanyak 80% dan faktor penulenan 12.5.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and respect to my main supervisor, **Assoc. Prof. Dr. Tey Beng Ti** for his professional competence and guidance throughout the research period. My sincere appreciations are also extended to my supervisory committee members, **Prof. Dr. Tan Wen Siang, Assoc. Prof. Dr. Ling Tau Chuan** and **Dr. Dayang Radiah Awang Biak,** for their excellent advice and constructive suggestions in helping to shape my project. I want to extend my sincere thanks to all laboratory assistants of Department of Microbiology, Department of Chemical and Environmental Engineering, and Department of Process and Food Engineering, UPM, for their consistent help and advice. And also thanks to Universiti Malaysia Pahang for funding my study. This study was supported by the Research University Grant Scheme (Project No: 05/01/07/0225RU) from the Universiti Putra Malaysia.

Deepest affection is also due to my friends especially Swee Tin, Ho, Rattana, Senthil, Kah Fai, Few Ne, Jason Lee and others labmates in Lab 134, who have helped and supported me spiritually. I have gained a lot of invaluable lab skills and advice from them to work my very best throughout this project.

Finally, my earnest love is to my wonderful husband, Ceg Sen and my caring parents and brother, Yew Hong for their encouragements, love and supports. Special thanks to my younger sister, Fui Yin and her boyfriend, Kok Seong. I could not have done this without all of them.

I certify that an Examination Committee has met on 4th February 2010 to conduct the final examination of Chong Fui Chin on her Doctor of Philosophy thesis entitled "Chromatographic Purification of Recombinant Nucleocapsid Protein of Nipah Virus from *Escherichia coli* Homogenate" 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 degree of Doctor of Philosophy. Members of the Examination Committee are as follows:

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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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Date: 8 April 2010

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.

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CHONG FUI CHIN

Date: 1 April 2010

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A	alanine
AEBSF	4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride
APS	ammonium persulfate
BSA	bovine-serum albumin
C	cysteine
CBB	coomassie Brilliant Blue
CIP	clean-in-place
ClpP	caseinolytic peptidase
cm	centimetre
cm/h	centimetre per hour
C-terminus	carboxy-terminus
D	aspartic acid
DegP	trypsin-like serine protease
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E	glutamic acid
E. coli	*Escherichia coli*
EBA	expanded bed adsorption
ELISA	enzyme-linked immunosorbent assay
F	phenylalanine
FF	fast flow
FPLC	fast protein liquid chromatography
G	glycine

g	gram
H	histidine
H_2O	dihydrogen monoxide
h	hour
HCl	hydrochloric acid
HIC	hydrophobic interaction chromatography
I	isoleucine
IDA	iminodiacetic acid
IMAC	immobilised metal affinity chromatography
IPTG	isopropylthio-β-d-galactoside
K	lysine
L	leucine
Lon	adenosine-5'-triphosphate dependent protease
M	methionine
mg	milligram
mg/ml	milligram per millilitre
$MgCl_2.6\ H_2O$	magnesium chloride hexahydrate
min	minute
ml	millilitre
mM	millimolar
MW	molecular weight
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide
NCp	nucleocapsid protein

NCp-NiV	nucleocapsid of Nipah virus
Ni^{2+}	nickel ion
NiV	Nipah virus
NTA	nitrilotriacetic acid
N-terminus	amino-terminus
OD	optical density
OmpT	outer membrane protease
P	proline
PAGE	polyacrylamide gel
PBA	packed bed adsorption
pH	*Puissance hyrogene*
pI	isoelectric point
PMSF	phenylmethylsulphonyl fluoride
p-npp	ρ-nitrophenylphosphate
Q	glutamine
R	arginine
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature
S	serine
s	second
SDS	sodium dodecyl sulfate
T	threonine
TEMED	tetramethyenediamine
V	valine

W	tryptophan
Y	tyrosine
µg	microgram
µg/ml	microgram pre millilitre

CHAPTER 1

INTRODUCTION

Target proteins can be separated and are purified using purification methods that separate them from other contaminant proteins based on its specific properties such as the charge, hydrophobicity, size and combinations of several properties. For instance, affinity chromatography separates proteins based on the specific chemical interaction between the target protein and the specific ligand coupled to a chromatography matrix. Meanwhile, hydrophobic interaction chromatography (HIC) separates proteins according to the surface hydrophobicity. The selection of suitable purification methods for a target protein is challenging due to the complexity of the feedstock contains dissolved solids, biomass and cell debris. Protein purification is a series of processes to produce an active and stable target protein, which is isolated from those potential contaminant proteins and proteases in the complex feedstock. Protein purification plays important role in obtaining the target protein at the desired concentration while maintaining their biological characteristic. Therefore, a well-developed purification protocols are needed to provide the target protein with acceptable purity and well preserved.

Nipah virus (NiV), a paramyxovirus was isolated from an outbreak of encephalitis and respiratory illness among humans through close contact with infected swine in Malaysia. A mass culling of infected and non-infected swine was carried out, causing large economic loss to the swine industry of the affected countries. Therefore, there is a need for surveillance programs in preventing future NiV epidemics by