UNIVERSITI PUTRA MALAYSIA

STRUCTURAL ANALYSIS OF APEPTIDE (CTLTTKLYC) THAT INTERACTS WITH NEWCASTLE DISEASE VIRUS

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FBSB 2005 22

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

June 2005

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

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Chairperson: Professor Datin Khatijah Yusoff, PhD

Faculty: Biotechnology and Biomolecular Sciences

A peptide with the sequence Cys-Thr-Leu-Thr-Thr-Lys-Leu-Tyr-Cys (CTLTTKLYC) has previously been identified to inhibit the propagation of Newcastle disease virus (NDV) in embryonated chicken eggs and tissue culture. It has two different dissociation constants (K_d^{rel}), in which the first constant can be used as a determinant to classify NDV strains into two groups: the velogenic strains in the first group, whereas the mesogenic and lentogenic strains are in the second group. The peptide, $C^1T^2L^3T^4T^5K^6L^7Y^8C^9$, displayed on the pIII protein of a filamentous M13 phage was mutated by oligonucleotide-directed mutagenesis in order to identify the amino acid residues involved in the interactions with NDV. Mutations of Cys at first position (C^1) and Lys at the sixth position of the peptide (K^6) to Ala (A), which produced mutants C^1A and K^6A, did not affect the binding between the peptide and the virus significantly, but substitution of Tyr at eighth position (Y^8) alone with Ala (A) dramatically reduced the interaction. This suggests that Y^8 could play an important role in the peptide-virus interaction. Double mutations were carried out on K^6 and Y^8 to produce mutants K^6A-Y^8A, K^6R-Y^8A, K^6A-Y^8F, and K^6R-Y^8F, to determine

whether the mutated amino acids could improve the binding capability. However, the mutations did not improve the binding capability significantly.

Fmoc-solid phase peptide synthesis was employed to synthesize the peptide, CTLTTKLYC. Crude peptide was purified with HPLC and analysed with a mass spectrometer. The secondary structure of the peptide was analysed with circular dichroism (CD) and the three dimensional conformation of the peptide was determined by nuclear magnetic resonance (NMR) and molecular modelling. A mixture conformation of β-turn and β-sheet (intermolecular interaction) was observed for the linear peptide by using CD. However, the three-dimensional structure of the linear peptide could not be arrived due to the mixture of conformation which made the sequence assignment of NMR extremely difficult. On the other hand, the disulfide-constrained cyclic peptide, which has a more rigid structure, exhibited only a β-turn structure. Two models were obtained: one of it consists of a β-turn and a distorted β-turn, while the other structure is an extended structure.

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

PENGANALISAAN STRUKTUR PEPTIDA (CTLTTKLYC) YANG BERINTERAKSI DENGAN VIRUS PENYAKIT SAMPAR AYAM

Oleh

CHIA SUET LIN

Jun 2005

Pengerusi: **Profesor Datin Khatijah Yusoff, PhD**

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Peptida dengan jujukan Cys-Thr-Leu-Thr-Thr-Lys-Leu-Tyr-Cys (CTLTTKLYC) telah dikenalpasti sebagai perencat untuk pembiakan virus penyakit sampar ayam (NDV) dalam telur ayam yang berembrio dan juga kultur tisu. Ia mempunyai dua pemalar pengasingan (K_d^{rel}) di mana pemalar yang pertama boleh digunakan sebagai penentu untuk mengasingkan strain NDV kepada dua kumpulan: kumpulan pertama adalah strain velogenik manakala kumpulan kedua merupakan strain mesogenik dan juga lentogenik. Peptida berjujukan $C^1T^2L^3T^4T^5K^6L^7Y^8C^9$ yang dipaparkan pada protein pIII faj M13 telah dimutasikan dengan menggunakan teknik mutagenesis oligonukleotida. Ini adalah untuk mengenalpastikan residu asid amino yang memainkan peranan yang penting dalam interaksi antara peptida dan NDV. Mutasi pada Cys pada posisi pertama (C^1) dan Lys pada posisi keenam (K^6) kepada Ala (A) tidak mempengaruhi interaksi di antara peptida dan NDV manakala penggantian Tyr pada posisi kelapan (Y^8) kepada Ala (A) pula mengurangkan interaksi tersebut secara mendadak. Keadaan ini mencadangkan bahawa Y^8 mungkin memainkan peranan yang penting dalam interaksi antara peptida dan NDV. Mutasi berganda telah dijalankan pada K^6 danY^8 untuk menghasilkan mutasi K^6A-Y^8A, K^6R-Y^8A, K^6A-

Y^8F, dan K^6R-Y^8F bagi mengenalpastikan sama ada mutasi asid amino ini dapat meningkatkan interaksi tersebut. Keputusan yang didapati menunjukkan bahawa mutasi berganda tidak berkesan dalam meningkatkan interaksi antara peptida and NDV.

Sintesis peptida secara fasa pejal Fmoc telah digunakan untuk mensintesiskan peptida. Peptida kasar telah ditulenkan dengan menggunakan HPLC dan dianalisiskan dengan menggunakan spektrometer jisim. Struktur dua dimensi peptida ditentukan dengan menggunakan "circular dichroism" (CD) terlebih dahulu dan struktur tiga dimensi peptida pula dikenalpastikan dengan menggunakan resonan magnetik nuclear (NMR), dan pemodelan molekul. Berdasarkan data yang diperolehi daripada CD, peptida yang linear menunjukkan campuran struktur β-pusingan dan juga kepingan-β (interaksi antara molekul). Walau bagaimanapun, struktur tiga dimensi peptida yang linear ini tidak dapat dikenalpastikan kerana campuran kedua-dua struktur tersebut telah menyebabkan analisis jujukan NMR sangat sukar. Peptida siklik yang dikekangkan oleh ikatan dwisulfida mempunyai struktur yang lebih tegap dan ia hanya menunjukkan struktur pusingan-β. Terdapat dua model yang diperolehi daripada analisis pemodelan molekul: satu daripadanya mempunyai satu pusingan-β dan satu pusingan-β yang tidak sempurna manakala struktur yang lain pula mempunyai struktur yang longgar.

ACKNOWLEDGEMENT

There are endless numbers of people that I would like to express my deepest appreciation. They have not only given me the physical support in completing the experiments but also their moral support and sincere caring.

My first thanks will go to none other than my most wonderful supervisors: **Prof. Datin Dr. Khatijah Yusoff**, for believing in me in whatever that I am doing, giving me all the support, either physically or mentally, and most importantly giving me a chance to be a student under her supervision; **Assoc. Prof. Dr. Tan Wen Siang,** a passionate scientist, for his valuable comments, thoughtful discussions, and useful suggestions throughout the research and thesis writing; **Assoc. Prof. Dr. Khozirah Shaari**, for her valuable knowledge and time in helping me to appreciate the beauty of Nuclear Magnetic Resonance (NMR), as well as endless support in interpretation of the spectra.

I would also like to convey my deepest gratitude to **Asst. Prof. Dr. Seetharama D. S. Jois**, in the Department of Pharmacy, National University of Singapore, for helping me derive the peptide structure by using NMR and also molecular modelling. This project will not be successful without his unconditional guidance and help. Not forgetting the most generous staff and students in the department, Jining, Siew Eng, Lau, Wai See etc. for helping me in handling machine, spectrum analysis, and thoughtful suggestions.

Special thanks to **Prof. Dr. Noorsaadah Abdul Rahman**, in the Department of Chemistry, University Malaya, for teaching and guiding me during the process of peptide synthesis.

Of course, I would also like to convey my deepest appreciation to Swee Tin and Chiew Ling, who have been sisters to me, for guiding me, supporting me, and also helping me throughout the project. Not forgetting, all the members in the Virology Laboratories of the Faculty of Biotechnology and Biomolecular Sciences, Dr. Majid, Geok Hun, Thong Chuan, Suhana, Lalita, Zul, Onie, Rafidah, Nazreen, Kah Fai, Andrew, Wawa, Taznim, Budy, and Mukrish, who have been giving me a lot of supports and happy memory in the laboratories. These thanks would also go to all the staff members in the Department of Microbiology and Biochemistry, Encik Hussein, Ibrahim, Shamsudin, Burhannudin, Khalid, Puan Rosema, Long, Yati, Su, Helen, and Kamariah.

I wish to express my deepest thanks to my parents, brothers and sisters for their unconditional love and support. I would also like to thank my dearest roommate, Douglas, who always there to listen to my complaints, happiness and sadness.

Finally, I would like to thank the Ministry of Science, Technology and Innovation of Malaysia for providing me the National Science Fellowship.

I certify that an Examination Committee met on 7th of June, 2005 to conduct the final examination of Chia Suet Lin on his Master of Science thesis entitled "Structural analysis of a peptide (CTLTTKLYC) that interacts with Newcastle disease virus" 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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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 11 AUG 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for equations 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.

CHIA SUET LIN

Date: 20/7/05

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LIST OF ABBREVATIONS

α	alpha
A	adenine/ alanine
Å	Ångstrom unit (10^{-8} cm)
ABTS	$[C_{18}H_{18}N_4O_6S_4(NH_2)_2]$– 2',2'-Azinobis (3-ethylbenzothiazoline-6-sulforic acid) diammonium
Amp	ampicillin
ATP	adenosine triphosphate
β	beta
BOC	tert-butyloxycarbonyl
bp	base pair
C	cytosine/ cystein
°C	degrees centigrade
CD	circular dichroism
Clt	2-chlorotrityl
CLTR	2-chlorotrityl resin
COSY	2D correlated spectroscopy
C-terminus	carboxy terminus
Cvff	consistent valence force field
δ	delta
DCM	dichloromethane
DIPEA	N,N-diisopropylethylamine
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide

DNA	deoxy-ribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DQF	double quantum filter
DTT	1, 4-dithiothreitol
ε	epsilon
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
F	fusion protein
Fmoc	9-fluorenylmethyloxy carbonyl
g	gram
h	hour
HA	haemagglutination activity
HBTU	N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium
HCl	hidrochloride acid
HN	haemagglutinin-neuraminidase protein
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HR	heptad repeat
Hz	Hertz
IPTG	isopropyl-β-D-thiogalactopyranoside
K	Kelvin/ lysine

kb	kilobase
kcal	kilacalories
KCl	potassium chloride
kDa	kilodalton
K_d^{rel}	relative dissociation constant
λ	lambda
L	large protein/ leucine
l	litre
LB	Luria Bertani
Ltd.	limited
μg	microgram
μl	microlitre
μM	micromolar
M	molar/ Matrix protein
MD	molecular dynamic
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MS	mass spectrometry
ms	millisecond
Mtt	4-methyltrityl
NDV	Newcastle disease virus
ng	nanogram

nm	nanometre
NMR	nuclear magnetic resonance
NOE	nuclear overhauser enhancement
NOESY	2D nuclear overhauser spectroscopy
NP	nucleocapsid protein
nt	nucleotide
N-terminus	amino terminus
OD	optical density
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pH	*Puissance hydrogene*
ps	picosecond
RNA	ribonucleic acid
PNK	polynucleotide kinase
RMSD	root mean square distance
rNTP	ribonucleoside triphosphate
ROESY	rotating frame overhauser enhancement speactroscopy
r	distance
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SPPS	solid-phase peptide synthesis
ssDNA	single-stranded DNA

T	thymine/ threonine
TBS	tris-buffered saline
tBu	tert-butyl
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TIS	triisopropylsilane
TMS	tetramethyl silane
TOESY	total correlation spectroscopy
Trt	trityl
U	unit
UV	ultraviolet
vol	volume
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside
Y	tyrosine
YT	yeast-tryptone

CHAPTER 1

INTRODUCTION

Random peptide library displayed on the pIII protein of bacteriophage M13 has been utilized extensively to select peptide ligands that bind to target molecules such as cell receptors, enzymes, and viral surface proteins. Nucleotide sequences encoding these peptides were cloned into the *gIII* gene of the phage, which is then translated and displayed on the pIII protein as a fusion molecule. Ramanujam *et al.* (2002) employed a disulfide-constrained phage display library to select ligands that interact with Newcastle disease virus (NDV) that had been immobilized on microtitre plate wells. After three rounds of affinity selection, peptides with the sequence CTLTTKLYC and other related sequences were obtained.

Synthetic peptides with the sequence TLTTKLY, either in linear or cyclic forms, were shown to inhibit the propagation of NDV in embryonated chicken eggs (Ramanujam *et al.*, 2002). This inhibition could be due to the ability of the peptide to bind tightly to the surface proteins of the virus which then interferes with the fusion activity between NDV and the host cell. The binding site of the peptides on the viral surface proteins, however, remained unknown. The two surface proteins on the virus, the haemagglutinin-neuraminidase (HN) and fusion (F) proteins have been known to be involved in attachment and entry into the host cell. They are, however, rather difficult to be isolated while retaining their structural integrity. Several reviews have shown that the co-expression of these homologous proteins is crucial for the

infection activity making the determination of the peptide-NDV binding site very difficult (Stone-Hulslander and Morrison, 1997; McGinnes *et al.*, 2002).

The relative dissociation constants (K_d^{rel}) of the phage-NDV have been determined by using an equilibrium-binding assay in solution (Ramanujam *et al.*, 2002; 2004). The phage displayed two widely different binding affinities towards NDV with the first binding affinity almost 1000-fold higher than the second one. It was suggested that the system has two or more classes of binding sites with different affinities. The first K_d^{rel} value has been shown to be able to differentiate the pathotypes of NDV into two groups (Ramanujam *et al.*, 2004): one of the groups consists of lentogenic and mesogenic strains whilst velogenic strains form the other group. This finding is particularly important because there are no detection tools capable of differentiating between the mesogenic and velogenic strains (Li *et al.*, 2002).

The functional activity of any protein is always associated with its structure, which in turn is influenced by its sequence. Proteins with different structures and sequences account for the diverse functions. Not all amino acid residues in a protein are involved in functional activities. Some amino acid residues are the key residues or regions whereas the others serve as a 'holder'. Nevertheless, these 'holder' amino acids may play an important role in ensuring proper folding of the protein. In order to determine which of the amino acids in the above novel peptide are the key residues involved in the peptide-virus interaction, a detailed analysis on each of the amino acid residues in the sequence CTLTTKLYC by mutagenesis should be performed. In addition, information on the tertiary structure(s) of this peptide would be useful in developing a model for synthesizing a secondary drug as in peptidomimetics.

The main objective of this study was to determine the three-dimensional structure(s) of the inhibitory peptide, CTLTTKLYC, and the key residue(s) in the phage-NDV interactions. In order to achieve these objectives, the study has been divided into three major sections:

1. Phage-NDV binding study:

   The amino acid residues in peptide CTLTTKLYC displayed on the pIII protein of the M13 phage were substituted by site-directed mutagenesis and used in phage-NDV binding study to determine the key residues involved in the interaction.

2. Fmoc-solid phase peptide synthesis:

   The CTLTTKLYC peptide was synthesized by using the Fmoc-solid phase peptide synthesis and purified by using RP-HPLC to obtain sufficient peptide powder for structural analysis.

3. Conformational study of the peptide:

   The two- and three-dimensional structures of the peptide were studied with circular dichroism (CD) and nuclear magnetic resonance (NMR), and the structures of the peptide were modelled using molecular modelling software.

The conformation study of the peptides will provide information on the functional activities of the peptide, in particular the two K_d^{rel} values.