UNIVERSITI PUTRA MALAYSIA

PURIFICATION ON A RECOMBINANT NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

TAN YAN PENG.

FK 2005 28

PURIFICATION ON A RECOMBINANT NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

By

TAN YAN PENG

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

PURIFICATION ON A RECOMBINANT NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

By

TAN YAN PENG

June 2005

Chairman: Tey Beng Ti, PhD

Faculty: Engineering

The purification of histidine-tagged nucleocapsid protein (NP) of Newcastle disease virus (NDV) in expanded bed adsorption (EBA) directly from unclarified *Escherichia coli* feedstock was investigated. Streamline 25 Column (ID = 25 mm) was used as a contactor and Streamline chelating immobilised with Ni^{2+} ion was used as affinity adsorbent. Unclarified *E. coli* feedstock of 8% biomass wet mass (w/v) was loaded onto the stable expanded bed.

The adsorption properties of the Streamline chelating were investigated by batch adsorption. The adsorption of NP protein onto the Streamline chelating was observed to follow the Langmuir isotherm. Optimal binding and elution conditions for the application of EBA were developed. Batch binding experiments results showed that the optimal pH for adsorption and elution buffer is 8.0. Elution buffer which contained 50 mM imidazole was used to remove the unbound proteins and elution buffer with 350 mM imidazole was used to elute NP protein from the adsorbent. Elution in packed bed column showed that the highest NP protein yield was achieved at a flow velocity of 10 cm/h. The bed expansion characteristics of the

Streamline chelating with buffer and unclarified feedstock were determined by visually monitoring the bed height as a function of increasing superficial velocity. The dynamic binding capacity of the adsorbent for NP protein was determined in the expanded bed column to be 2.9 mg/mL adsorbent. These results were used to develop a large scale purification of NP protein in Streamline 25 Column.

The performance of a conventional purification method, packed bed adsorption (PBA) and EBA for the purification of the NP protein from *E. coli* feedstock was assessed and compared. The conventional way for the recovery of NP proteins involved multiple steps such as centrifugation, precipitation, dialysis and sucrose gradient ultracentrifugation. For the PBA, feedstock clarified by centrifuge was used for column loading, while in EBA; unclarified feedstock was used. The final protein yield obtained in conventional and PBA methods was 1.3% and 5.6% respectively. It was demonstrated that the EBA achieved the highest final protein yield of 9.6% with a purification factor of 6.6. In addition, the total processing time was reduced from 56 h to 7.5 h for EBA compared to that of the conventional method. Within the range of the experimental works, result from this study suggested that EBA technique allowed clarification, concentration and initial purification to be combined into a single step operation. The expanded bed purification for NP protein was efficient and scalable, promising its implementation in the large scale production of proteins.

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

PENULENAN REKOMBINAN PROTEIN NUKLEOKAPSID PENYAKIT SAMPAR AYAM MENGGUNAKAN KROMATOGRAFI PENJERAPAN LAPISAN TERKEMBANG

Oleh

TAN YAN PENG

Jun 2005

Pengerusi: **Tey Beng Ti, PhD**

Fakulti: **Kejuruteraan**

Penulenan secara langsung protein nukleokapsid (NP) virus penyakit Sampar Ayam (NDV) yang mengandungi tag enam histidin dari suapan tanpa penjernihan *Escherichia coli* dengan penjerapan lapisan terkembang (EBA) telah diselidiki. Kolum Streamline 25 yang berdiameter 25 mm telah digunakan sebagai kontaktor dan Streamline chelating diselat dengan ion nikel telah digunakan sebagai bahan jerap afiniti. Suapan tanpa penjernihan *E. coli* dengan 8% berat basah biojisim (berat/isipadu) dimuatkan ke dalam lapisan terkembang yang stabil.

Ciri penjerapan Streamline chelating telah dikaji dalam penjerapan kelompok. Penjerapan protein NP ke atas Streamline chelating yang diperhati adalah mematuhi isoterm Langmuir. Keadaan penjerapan dan elusi yang optimum untuk aplikasi penjerapan lapisan terkembang telah dibangunkan. Hasil dari ujikaji penjerapan kelompok menunjukkan pH 8.0 adalah pH optimum untuk penimbal penjerapan dan elusi. Penimbal elusi yang mengandungi 50 mM imidazol telah digunakan untuk mengeluarkan protein yang tidak terjerap manakala penimbal elusi yang

PERPUSTAKAAN SULTAN ABDUL SAMAD
UNIVERSITI PUTRA MALAYSIA

mengandungi 350 mM imidazol digunakan untuk memulihkan protein NP dari bahan jerap. Elusi dalam lapisan terpadat menunjukkan hasilan protein NP yang tertinggi boleh diperolehi pada halaju 10 cm/j. Ciri lapisan pengembangan Streamline chelating dengan penimbal and suapan tanpa penjernihan telah diperiksa dengan memerhatikan secara visual ketinggian lapisan sebagai fungsi kepada penambahan halaju superfisial. Kapasiti dynamik pengikatan bahan jerap untuk protein NP telah diperiksa dalam lapisan terkembang dan bernilai 2.9 mg/mL. Penemuan ini akan digunakan untuk membangunkan proses penulenan protein NP di kolum Streamline 25 dalam skala besar.

Prestasi kaedah penulenan konvensional, penjerapan lapisan terpadat (PBA) dan EBA untuk penulenan protein NP dari suapan tanpa penjernihan *E. coli* telah diselidiki dan diperbandingkan. Cara penulenan secara konvensional untuk memulihkan protein NP meliputi pelbagai langkah seperti pengemparan, pemendakan, dialisis dan pengemparan kecerunan sukrosa. Dalam PBA, suapan *E. coli* yang dijernihkan dengan pengempar telah digunakan untuk dimuatkan ke dalam kolum, sementara dalam EBA, suapan *E. coli* tanpa penjernihan telah digunakan. Hasilan akhir protein yang diperolehi dalam cara penulenan konvensional dan kaedah PBA adalah 1.3% dan 5.6% masing-masing. Penulenan secara EBA membawa hasilan akhir protein yang tertinggi dengan nilai 9.6% dan factor penulenan sebanyak 6.6. Di samping itu jumlah masa pemprosesan telah disingkatkan dari 56 j ke 7.5 j dalam EBA dibandingkan dengan kaedah penulenan konvensional. Dalam lingkungan eksperimen, kajian ini mencadangkan teknik EBA membolehkan penggabungan proses penjernihan, pemekatan dan penulenan awal kepada satu unit operasi. Penulenan protein NP secara EBA adalah efisien dan boleh

dinaik-skalakan dan peluang untuk melaksanakan dalam skala besar adalah cemerlang.

UPM

ACKNOWLEDGEMENTS

I profoundly express my gratitude to my esteemed advisor, **Dr. Tey Beng Ti** for his momentous guidance and advice with encouragement throughout the research period. Sincere acknowledgments are extended to **Prof Datin Dr. Khatijah Yusoff**, **Dr. Ling Tau Chuan** and **Assoc. Prof. Dr. Tan Wen Siang**, the members of the thesis advisory committee, for their valuable suggestion and constructive comments.

I would like to extend my heartfelt gratitude to my colleagues at Fermentation Technology Unit, Institute of Bioscience especially Siew Ling, Chin Ming, Alan, Teck Seong, Shiau Tsuey, Norita, Yan Mei, lecturers and all staffs for their critical suggestions and valuable technical information on fermentations.

I wish to thank my colleagues at laboratory of virology of the Faculty of Biotechnology and Biomolecular Sciences including Chiew Ling, Sing King, Andrew, Kah Fai, Han Koh, Firozeh, Siti, Taznim, Geok Hun, Lalita, Raha, Suhana, Thong Chuan, Dr. Majid, Swee Tin and Eddie for their valuable cooperation and suggestion in so many ways.

Finally and most importantly, I would like to express my gratitude to my family for their love and guidance. With love and affection, my achievement is dedicated to my beloved sister, Yan Yan.

I certify that an Examination Committee met on 8th June 2005 to conduct the final examination of Tan Yan Peng on her Master of Science thesis entitled "Purification on a Recombinant Nucleocapsid Protein of Newcastle Disease Virus Using Expanded Bed Adsorption Chromatography" 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:

SURAINI ABDUL AZIZ, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

ARBAKARIYA ARIFF, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

RAJA NOOR ZALIHA RAJA ABDUL RAHMAN, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

SHALIZA IBRAHIM, PhD
Associate Professor
Faculty of Engineering
Universiti Malaya
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 2 1 JUL 2005

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:

TEY BENG TI, PhD
Lecturer
Faculty of Engineering
Universiti Putra Malaysia
(Chairperson)

KHATIJAH YUSOFF, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

TAN WEN SIANG, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

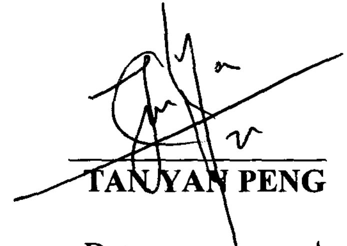
LING TAU CHUAN, PhD
Lecturer
Faculty of Engineering
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

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 dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



TAN YAN PENG

Date: 9 JULY 2005

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	iv
ACKNOWLEDGEMENTS	vii
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvi
CHAPTER	
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1 Nucleocapsid Protein (NP) of Newcastle Disease Virus	4
2.1.1 Recombinant Protein	4
2.1.2 Studies on the Recombinant NP Protein	5
2.1.3 Purification Processes	6
2.2 Product Recovery in Biotechnology	8
2.2.1 Bioseparation Processes	8
2.2.2 Problems associated with the Conventional Purification Processes	11
2.3 Immobilised Metal Affinity Chromatography (IMAC)	12
2.3.1 Principles of IMAC	12
2.3.2 Chelate Structure and Metal Ions	15
2.4 Expanded Bed Adsorption (EBA)	17
2.4.1 Principles of Operation	17
2.4.2 EBA and Packed Bed Adsorption (PBA)	20
2.4.3 Streamline Chelating	22
2.5 Method Scouting and Optimisation	22
2.5.1 Protein Adsorption Equilibrium	22
2.5.2 Bed Expansion	23
2.5.3 Adsorption	24
2.5.4 Washing	24
2.5.5 Elution	25
2.6 Application of EBA in Biotechnology	26
3. MATERIALS AND METHODS	27
3.1 Materials	27
3.2 Production and Cell Disruption of *E. coli*	27
3.2.1 Protein Expression	27
3.2.2 Cell Disruption of *E. coli*	28
3.3 NP Protein Analysis	28
3.3.1 SDS-PAGE	28
3.3.2 Western Blotting	30
3.3.3 Densitometric Analysis	31

3.3.4 The Bradford Assay 32
3.4 Calculations 32
3.5 Conventional Purification Method 33
3.5.1 Centrifugation 33
3.5.2 Ammonium Sulphate Precipitation 33
3.5.3 Dialysis 34
3.5.4 Sucrose Gradient Ultracentrifugation 34
3.6 Method Scouting and Optimisation 34
3.6.1 Equilibrium Adsorption Isotherm 34
3.6.2 Optimisation of Binding pH 35
3.6.3 Optimisation of Elution pH 36
3.6.4 Optimisation of Imidazole Concentrations 36
3.6.5 Optimisation of Elution Flow Velocities 37
3.7 Packed Bed Adsorption 38
3.7.1 Column Specifications and Set-up 38
3.7.2 Purification of NP Protein 38
3.8 Expanded Bed Adsorption 39
3.8.1 Column Specifications and Set-up 39
3.8.2 Bed Expansion 41
3.8.3 Dynamic Binding Capacity 41
3.8.4 Direct Purification of NP Protein from Unclarified Feedstock 42
3.9 Comparative Evaluation of Conventional Purification Method, Packed Bed Adsorption and Expanded Bed Adsorption 43

4. RESULTS AND DISCUSSION 44
4.1 Conventional Purification Processes 44
4.2 Method Scouting and Optimisation 52
4.2.1 Adsorption Isotherm 52
4.2.2 Optimisation of Binding pH 54
4.2.3 Optimisation of Elution pH 58
4.2.4 Optimisation of Imidazole Concentration 60
4.2.5 Optimisation of Elution Flow Velocities 64
4.3 Purification of the NP Protein by Packed Bed Adsorption 68
4.4 Expanded Bed Adsorption 74
4.4.1 Bed Expansion 74
4.4.2 Dynamic Binding Capacity 80
4.4.3 Direct Purification of the NP Protein from Unclarified Feedstock 83
4.5 Comparative Evaluation of Conventional Purification Method, Packed Bed Adsorption and Expanded Bed Adsorption 92

5. CONCLUSION AND FUTURE PERSPECTIVES 100

REFERENCES 103
BIODATA OF THE AUTHOR 114

LIST OF TABLES

Table		Page
4.1	Purification of NP protein using the conventional method	49
4.2	Influence of pH on the binding of NP protein from unclarified feedstock [10% biomass wet mass (w/v)] and clarified feedstock to Ni^{2+}-Streamline chelating	55
4.3	Effects of pH on eluting the NP protein of unclarified feedstock from Ni^{2+} -Streamline chelating	59
4.4	Influence of the imidazole concentration in elution buffer on the elution of NP protein of unclarified feedstock from Ni^{2+}-Streamline chelating	63
4.5	Effects of elution flow velocities on eluting NP protein of unclarified feedstock from Ni^{2+}-Streamline chelating	67
4.6	The PBA of NP protein from clarified feedstock	71
4.7	Results of the linear regression of the Richardson-Zaki plots	78
4.8	The EBA of NP protein from unclarified feedstock	86
4.9	Comparison of the purification of NP protein using conventional method, PBA and EBA	94
4.9a	Comparison of the purification of NP protein using conventional method, PBA and EBA [Normalised based on biomass concentration of 8% (w/v) and feedstock volume of 400 ml]	95
4.10	Comparison of the operational conditions for the conventional method, PBA and EBA	97

LIST OF FIGURES

Figure		Page
2.1	Implementation of EBA into a conventional procedure of protein purification	9
2.2	Schematic illustration of the protein adsorption and desorption to a metal-chelated affinity support	14
2.3	Structure of IDA chelating ligand	15
2.4	Schematic illustration of an expanded bed adsorption cycle	18
2.5	A packed bed and a stable expanded bed	21
3.1	Schematic diagram of Streamline 25 Column in a basic configuration set-up with one pump	40
4.1	SDS-PAGE and Western blot analysis of the NP protein purified from the conventional method	45
4.2	SDS-PAGE of the NP protein fractions collected from the 10-50% sucrose gradient ultracentrifugation	48
4.3	Adsorption isotherm of NP protein from unclarified *E. coli* feedstock onto Ni^{2+}-Streamline chelating	53
4.4	Linear plot of Langmuir isotherm for determination of maximum adsorption capacity of NP protein onto Streamline chelating and the dissociation constant	53
4.5	SDS-PAGE analysis of the NP protein after IMAC purification	62
4.6	SDS-PAGE of the NP protein fractions eluted from different flow velocities	66
4.7	SDS-PAGE of the purification of NP protein from a clarified *E. coli* feedstock using a packed bed of Ni^{2+}- Streamline chelating	69
4.8	SDS-PAGE of the NP protein fractions collected from PBA	72
4.9	Elution profile of the purification of NP protein from clarified *E. coli* feedstock using immobilized metal affinity PBA	73
4.10	Bed expansion characteristic of the Streamline chelating, which used binding buffer and unclarified feedstock [8% biomass wet mass (w/v)]	75

4.11 Ln/ln plot of superficial velocity against bed voidage for estimation of Richardson-Zaki parameters for Streamline chelating fluidised by binding buffer 77

4.12 Ln/ln plot of superficial velocity against bed voidage for estimation of Richardson-Zaki parameters for Streamline chelating fluidised by unclarified feedstock [8% biomass wet mass (w/v)] 77

4.13 Breakthrough curve for the NP protein on expanded bed of Ni^{2+} loaded Streamline chelating with unclarified *E. coli* feedstock 82

4.14 SDS-PAGE analysis of the purification of NP protein from an unclarified *E. coli* feedstock using an expanded bed of Ni^{2+} Streamline chelating 87

4.15 SDS-PAGE of the NP protein fractions collected from the EBA 88

4.16 SDS-PAGE and western blot analysis of the purification of NP protein from an unclarified *E. coli* feedstock using an expanded bed of Ni^{2+}-Streamline chelating 90

4.17 Elution profile of the purification of NP protein from unclarified *E. coli* feedstock using immobilised metal affinity EBA 91

4.18 Flowchart of the purification of NP protein 96

4.19 Process effectivity of three different purification methods for NP protein 99

LIST OF ABBREVATIONS

BCIP	1-bromo-3-chloro-3-indolyl phosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CIP	clean-in-place
cm	centimeter
Co^{2+}	cobalt ion
C-terminus	carboxyl terminus
Cu^{2+}	copper ion
DNA	deoxy-ribonucleic acid
DNase	deoxyribonuclease
EBA	expanded bed adsorption
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
h	hour
HCl	hydrochloric acid
ID	internal diameter
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
L	litre
LB	Luria Bertani
μg	microgram

μL	microlitre
M	molar
mA	milliampere
$MgCl_2$	magnesium chloride
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
NBT	nitro blue tetrazolium
nm	nanometre
NaCl	sodium chloride
NaOH	sodium hydroxide
NDV	Newcastle disease virus
Ni^{2+}	nickel ion
NP	nucleocapsid protein
N-terminus	amino terminus
OD	optical density
pH	*Puissance hydrogene*
PAGE	polyacrylamide gel electrophoresis
PBA	packed bed adsorption
pKa	dissociation constant
RNA	ribonucleic acid
rpm	revolution per minute
s	second

SDS	sodium dodecyl sulphate
TEMED	tetramethyl ethylenediamine
V	volt
v/v	volume/volume
W	watt
w/v	weight/volume
Zn^{2+}	zinc ion

CHAPTER 1

INTRODUCTION

The effectiveness of the treatment of a disease is very much depending on the delivery of the therapeutic molecules to its target. Various kinds of biological molecules have been proposed for use as carrier for the delivery of nucleic acids and other therapeutic compounds. The nucleocapsid protein (NP) of a poultry virus, Newcastle disease virus (NDV) (Yusoff and Tan, 2001) can be produced in *Escherichia coli* as ring-like structures which has the potential to be used as a biocarrier for subunit vaccines development and also as a diagnostic reagent (Kho *et al.*, 2001; Rabu *et al.*, 2002). The recombinant NP protein consists of the insertion of a hexa-histidine residues on its C-terminus can be expressed abundantly in *E. coli* (Kho, 2003). The engineered histidine tag structures can aid the purification of the recombinant protein. Immobilised metal affinity chromatography (IMAC) has been widely used for the separation of recombinant proteins harbouring histidine-tags (Ueda *et al.*, 2003). The separation is based on the differential affinity of proteins, derived from the coordination bonds formed between metal ions and histidine side chains exposed on the surface of the protein molecules (Chaga, 2001).

In the production of therapeutic proteins, the purification process is often characterised by a large number of subsequent unit operations, which is not only contributed to the fixed and variable costings but also reduce the overall yield of the target protein. Up to 80% of the overall cost of a protein production are contributed from product recovery processes (Thommes *et al.*, 1996). Downstream processing is

challenging because the products must be concentrated from a very dilute condition in the feedstock and purified from other protein impurities having very similar properties. The feedstock is generally very complex in nature and contains various sizes of dissolved solid, biomass and cell debris. In the conventional way, a final product of guaranteed purity and potency can only be achieved with a combination of several unit operations (Anspach *et al.*, 1999). Prior to the concentration and purification steps, the biomass or cell debris must be separated from the fermentation broth or cell homogenate by centrifugation or filtration. In the large-scale process of protein recovery, these clarification methods often show limitations in practice (Lee, 1989). The more the number of unit operations involved would not only cause a longer processing time but also results in higher product loss. Hence, the throughput and the final product yield will decrease. Therefore, a single step protein purification process that combines feedstock clarification, concentration and purification is needed to improve the yields of protein recovery and reduce the processing time.

Expanded bed adsorption (EBA) is a single pass operation in which desired proteins are purified from crude, particulate containing feed-stock without the need for separate clarification, concentration and initial purification (Chase, 1998; Miyauchi *et al.*, 1998; Sandgathe *et al.*, 2003). The expansion of the adsorbent bed creates a distance between the adsorbent particles, which allows for unhindered passage of cells, cell debris and other particulates during application of crude feedstock to the column (Hjorth, 1997). The EBA has been proven to be a versatile tool that can be applied on all commonly used source materials such as *E. coli* (Clemmitt and Chase, 2000a; Cho *et al.*, 2002), yeast homogenates (Chang and Chase, 1996b; Willoughby

et al., 1999), hybridoma cell (Thommes *et al.*, 1995a), mammalian cell culture fluid (Batt *et al.*, 1995) and transgenic milk (Degener *et al.*, 1998).

Currently, the NP protein was purified in the laboratory scale by a four step conventional method which involving centrifugation, precipitation, dialysis and sucrose gradient ultracentrifugation (Kho, 2003). However, there was no literature report on the purification of the NP protein using EBA chromatography. Due to the wide application of NP protein, there is a need to produce and purify this protein in large scale. Hence, the prime aim of this study was to develop a simplified method for fast and cost effective purification process of the recombinant NP protein. The combination of EBA and IMAC would result in a simple and efficient protein purification process. Therefore, the objectives of the present study were:

1) Optimisation of the capture of NP protein onto IMAC adsorbent from clarified and unclarified feedstock.
2) Optimisation of the elution of NP protein from IMAC adsorbent.
3) Development and optimisation of a packed bed chromatography for the purification of NP protein from clarified feedstock.
4) Development and optimisation of an EBA chromatography for the purification of NP protein from unclarified feedstock.

The final protein yield, purity and purification factor from EBA chromatography would be compared with that of packed bed adsorption (PBA) and conventional purification method.

CHAPTER 2

LITERATURE REVIEW

2.1 Nucleocapsid Protein (NP) of Newcastle Disease Virus (NDV)

2.1.1 Recombinant Protein

The development of recombinant DNA techniques presents a great opportunity for the integration of the 'upstream' gene cloning steps and 'downstream' protein purification operations. Production of recombinant protein has been successfully performed in several host systems, such as *E. coli* (Gibert *et al.*, 2000), yeast (Raymond *et al.*, 1998), insect (Michel *et al.*, 2001) and mammalian cells (Batt *et al.*, 1995). *E. coli* has been the popular choice of host because a wide choice of cloning vectors is available, large yields of recombinant protein, easy to grow and recombinant proteins can be expressed as fusion proteins (Pharmacia Biotech, 1993).

Expression vectors have been developed where the expressed protein is fused to a protein of known composition so that the product can be easily captured by a highly specific affinity chromatography step prior to further purification (Jonasson *et al.*, 2002). For example, the histidine-tagged fusion protein can be recovered by using IMAC, which will be discussed in detail in section 2.3. The application of IMAC together with EBA chromatography can simplify the recovery process by integrating several unit operations such as clarification, concentration and initial purification,

PERPUSTAKAAN SULTAN ABDUL SAMAD
UNIVERSITI PUTRA MALAYSIA

thereby increasing the overall efficiency in the downstream purification process (Hu *et al.*, 2003; Ueda *et al.*, 2003).

2.1.2 Studies on the Recombinant NP Protein

Newcastle disease virus (NDV) is the prototype of the *Paramyxoviridae* family and the causal agent of the major disease outbreak in avian species worldwide (Seal *et al.*, 2000). NDV contains a linear single-stranded RNA of negative polarity, which is associated with the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemaglutinin-neuraminidase (HN) and large proteins (L) (Seal *et al.*, 2000; Yusoff and Tan, 2001).

The NP protein is the most abundant of the viral proteins and plays a prominent role in viral replication (Kho *et al.*, 2003). The NP proteins of many viruses are highly conserved, immunogenic and abundantly expressed during infection. These features enabled their potential uses as diagnostic reagents, such as enzyme-linked immunosorbent assays (ELISA) (Errington *et al.*, 1995; Rabu *et al.*, 2002). Kho *et al.* (2001) reported that the NP protein on NDV produced in *E. coli* assembled into ring-like particles and the insertion of a 29 amino acid peptide including the *myc* epitope and a hexa-histidine tag into the C-terminus of the NP protein did not impair ring-like assembly of the viral nucleocapsid. These extra amino acid sequences were shown to be exposed on the surface of the ring-like particles, suggesting that the particle can be used as a molecular carrier for presenting desired epitopes in the development of subunit vaccines (Kho *et al.*, 2001; Kho, 2003).

Errington *et al.* (1995) have discovered earlier that the NP protein of NDV was antigenic and could be used in ELISA to detect the presence of anti-NDV antibodies. These results indicated the potential of the NP protein as a diagnostic reagent in ELISA. For example, the recent outbreak of the novel coronavirus, Severe Acute Respiratory Syndrome (SARS-CoV) has utilised this potential in its diagnosis. Timani *et al.* (2004) noted that the NP protein of SARS-CoV is highly immunogenic and has successfully been used in ELISA for detecting the antibodies for the sero-diagnosis of SARS. Other examples of the nucleocapsid proteins of viruses, such as mumps (Slibinskas *et al.*, 2003), rabies (Reid-Sanden *et al.*, 1990), measles (Samuel *et al.*, 2003) and infectious bronchitis virus (Seah *et al.*, 2000) were shown to have the potential to be developed as a diagnostic reagent.

2.1.3 Purification Processes

NP protein of NDV has been reported to be successfully expressed abundantly in *E. coli* (Rabu *et al.*, 2002; Kho *et al.*, 2003) and baculovirus (Errington *et al.*, 1995) and many researches have reported on the study of the functionality of the possibility to use NP protein as a diagnostic reagent. However, there are not many published data on the purification of the NP protein expressed in recombinant hosts.

In general, the NP protein was purified conventionally with cesium chloride gradient ultracentrifugation (Slibinskas *et al.*, 2003; Juozapaitis *et al.*, 2005). Slibinskas *et al* (2004) reported that the NP protein expressed in yeast, was first purified with sucrose gradient ultracentrifugation for 3 h, followed by cesium chloride gradient