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BAHAGIAN B – Untuk Kegunaan Pejabat Sekolah Pengajian Siswazah

Tesis ini telah diperiksa dan diakui oleh:

Nama dan Alamat Pemeriksa Luar : Prof. Dr. Arbakariya Ariff
Department of Bioprocess Technology,
Faculty of Biotechnology & Biomolecular
Sciences, UPM 43400 Serdang, Selangor.

Nama dan Alamat Pemeriksa Dalam : Prof. Madya Dr. Firdausi Razali
Department of Bioprocess Engineering,
Faculty of Chemical & Natural Resources
Engineering (FKKKSA), UTM, 81310
UTM, Skudai, Johor.

Nama Penyelia Lain (jika ada) : .....
.....
.....
.....

Disahkan oleh Timbalan Pendaftar di SPS:

Tandatangan : ..... Tarikh : .....

Nama : .....

CHROMATOGRAPHIC PURIFICATION STRATEGIES FOR RECOMBINANT HUMAN TRANSFERRIN FROM SPODOPTERA FRUGIPERDA

WEE CHEN CHEN

A thesis submitted in fulfilment of the
requirements for the award of the degree of
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To my beloved grandparents, parents and brothers

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ABSTRACT

Insect cell-baculovirus system is an excellent artificial system for the production of recombinant glycoprotein despite its glycosylation deficiencies. In this study, laboratory scale production of recombinant human transferrin (rhTf) from insect cell-BEVS was conducted and chromatographic purification strategies were employed to obtain rhTf in high yield and high recovery. Research was started with the amplification of recombinant baculovirus, using low multiplicity of infection (MOI). Virus stock in a 1.2 x 10^9 pfu/ml infected suspension culture of *Spodoptera frugiperda* (*Sf9*) at 15 MOI had produced 31µg/ml of rhTf. To purify the rhTf, hydrophobic interaction chromatography, dialysis and ion exchange chromatography were performed. For hydrophobic interaction chromatography, elution strategy, flowrate and rhTf loading capacity of phenyl sepharose were optimized. By loading 38µg rhTf/ml of gel, employing step elution with 50% 1.2M $(NH_4)_2SO_4$/0.4M $Na_3C_6H_5O_7$, pH6 (buffer A) and 25% buffer A and flowrate at 1ml/min, 74.6% of rhTf had been recovered from phenyl sepharose. For ion exchange chromatography, batch purification in reduced size was used to select suitable anion exchange matrix, suitable pH of equilibration buffer and concentration of equilibration buffer. 20mM Tris/HCl buffer, pH8.5 and gradient elution with the increase of of 5mM NaCl/CV succeeded in giving pure rhTf with 52.5% recovery from Q-sepharose. The overall recovery of pure rhTf was 34% with 200 purification fold. A brief glycan characterization of the recovered pure rhTf was performed for a better understanding of the glycosylation feature of this protein expressed using optimized medium from BEVS. The carbohydrate component of the purified rhTf was determined. The purified rhTf was hydrolyzed and the release sugar was labeled with 1-Phenyl-3-Methyl-5-Pyrazolone (PMP) before analysis with High performance Liquid Chromatography (HPLC). The molar fractions of Man, GlcNAc and Gal of rhTf were 3.78, 1.69 and 0.93, respectively.

ABSTRAK

Sistem pengekspresan sel serangga-bakulovirus merupakan sistem pilihan yang baik untuk menghasilkan rekombinan glikoprotein meskipun kekurangan glikosilasi. Penghasilan produksi skala makmal mendapat rekombinan human transferrin (rhTf) dari sistem sel serangga-bakulovirus dan strategi purifikasi jenis kromatografi telah dijalankan untuk mendapatkan rhTf yang tulen dan perolehan yang tinggi. Kajian bermula dengan peningkatan kuantiti rekombinan bakulovirus dari gandaan jangkitan (MOI) yang rendah. Stok virus dalam 1.2 x 10^9 pfu/ml menjangkiti kultur ampaian sel *Spodoptera frugiperda* (*Sf9*) dengan 15 MOI telah menghasilkan 31µg/ml rhTf. Dalam proses purifikasi, kromatografi saling tindak hidrofobik, dialisis dan kromatografi penukaran ion telah dijalankan. Bagi kromatografi saling tindak hidrofobik, strategi elusi, kelajuan dan kapasiti muatan rhTf ke atas phenyl sepharose telah dioptimumkan. Penggunaan muatan 38µg rhTf/ml gel dengan elusi berperingkat menggunakan 50% 1.2M $(NH_4)_2SO_4$/0.4M $Na_3C_6H_5O_7$, pH6 (larutan penimbal A) and 25% larutan penimbal A dan kelajuan pada 1ml/min berjaya memperoleh 74.6% rhTf daripada phenyl sepharose. Bagi kromatografi penukaran ion, purifikasi dalam saiz kecil telah digunakan untuk memilih matrik penukar ion, pH larutan penimbal pada fasa keseimbangan dan kepekatan larutan penimbal pada fasa keseimbangan. 20mM Tris/HCl larutan penimbal, pH8.5 and elusi cerun dengan peningkatan 5mM NaCl/CV berjaya menghasilkan rhTf tulen dengan 52.5% perolehan daripada Q-sepharose. Perolehan rhTf tulen secara keseluruhan ialah 34% dengan 200 lipat purifikasi. Pencirian glikan secara kasar telah dijalankan ke atas rhTf tulen untuk mendapat pemahaman tentang ciri-ciri glikosilasi bagi protein ini yang diekspresikan dengan sistem pengekspresan sel serangga-bakulovirus dan media optimum. Komposisi karbohidrat untuk rhTf tulen telah dikenalpasti. rhTf yang tulen telah dihidrolisis. Gula telah dilepaskan, dan dilabelkan dengan 1-Phenyl-3-Methyl-5-Pyrazolone (PMP) sebelum dianalisis dengan menggunakan kromatografi cecair prestasi tinggi (HPLC). Nilai fraksi molar Man, GlcNAc and Gal daripada rhTf ialah 3.78, 1.69 and 0.93.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ ABBREVIATIONS

%	Percentage
α	Alpha
β	Beta
µm	Micro meter
°C	Degree Celsius
µg	Micro gram
µg/ml	Micro gram per milliliter
µl	Microliter.
µm	Micrometer
µmol/ml	Micro mol per milliliter
AAGR	Average annual growth rate
A_{blank}	Absorbance for blank
*Ac*MNPV	*Autographa californica* multiple nuclear polyhedrosis virus
ACN	Acetonitrile
*Ac*NPV	*Autographa californica* nuclear polyhedrolysis
A_{sample}	Absorbance for sample
Asn-X-Ser	Asparagine-X-Serine
Asn-X-Thr	Asparagine-X-Threonine
$A_{standard}$	Absorbance for standard
ATCC	American Tissue Culture Collection
BEVS	Baculovirus expression vector system
BHK	Baby hamster kidney cells
Bm	*Bombyx mori*
*Bm*NPV	*Bombyx mori* nuclear polyhedrosis virus.

BV	Budded virus
*Bm*NPV	*Bombyx mori* nuclear polyhedrosis virus.
BV	Budded virus
BVs	Budded viruses
cDNA	Complementary deoxyribonucleic acid
cells/ml	Cells per milliliter
CHO	Chinese Hamster Ovary
CM	Carboxymethyl
cm/hr	Centimeter per hour
cm^2	Centimeter square
CMP-NeuAc	Cytidine-5'-monophospho *N*-acetylneuraminic acid
Cu^{1+}	Cuprous ion
$CuSO_4 \bullet 5H_2O$	Copper (II) sulfate pentahydrate
CV	Column Volume
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DO	Dissolved oxygen
DPA	Dipicolylamine
e^-	Electron
E.coli	*Escherichia coli*
ELISA	Enzyme linked immunorsorbent assay
ER	Endoplasmic recticulum
FBS	Fetal bovine serum
FDA	Food and Drugs Administration
Fe^{3+}	Ferric ion
Fuc	Fucose
g	Gravitational
g/l	Gram per liter
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GDP-mannose	Guanosine diphoshate mannose
GlcN	Glucosamine
GlcNAc	N-Acetylglucosamine

GLDH	Glutamate dehydrogenase
GLP-1	Glucagons-like peptide 1
GLP-1-R	Glucagons-like peptide 1-receptor
GMP	Good manufacturing practice
gp	Glycoprotein
GV	Granuloviruses (GV)
H^+	Hydrogen cation
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HIC	Hydrophobic interaction chromatography
His6	Hexahistidine
HPLC	High performance Liquid Chromatografi
HRP	Horseradish peroxidase
Hrs	Hours
hTf	Human transferrin
IEX	Ion exchange chromatography
IgG	Immunoglobulin G
IMAC	Metal affinity chromatography
k	constant
Kb/kbp	Kilo base pair
kDa	Kilo Dalton
M	Molar
Man	Mannose
Man3–1GlcNAc2	3(Mannose)-2(N-Acetyl Glucosamine)
$Man_3GlcNAc_2$	3(Mannose)-2(N-Acetylglucosamine)
Man8–GlcNAc2	8(Mannose)-2(N-Acetylglucosamine)
$Man_9GlcNAc_2$	9(Mannose)-2(N-Acetylglucosamine)
MeOH	Methanol
mg	Milligram
mg/ml	Milligram per milliliter
min	Minutes
ml/min	Milliliter per minutes
mmol/L	milli mol per liter
MOI	Low multiplicity of infection

MPa	Mega Pascal
MW	Molecular weight
MWCO	Molecular Weight Cut Off
N	Normal
N.D	Not defined
NaCl	Sodium Chloride
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
$Na_3C_6H_5O_7$	Sodium citrate
NaOH	Sodium hydroxide
ng/ml	Nanogram per milliliter
NH_3	Ammonia
$(NH_4)_2SO_4$	Ammonium Sulphate
Ni^{2+}	Nickel ion
nm	Nano meter
NPV	Nucleopolyhedoviruses
O_2	Oxygen
OB	Occlusion bodies
ODS	Octadecyl silica
ODV	Occlusion derived virus
OV	Occluded virus
p10	Phage-encoded protein-10
PBS	Phosphate buffered saline
pfu/ml	Plug performing unit per milliliter
pH	Potential hydrogen
pI	Isoelectric point
PIBs	Polyhedral inclusion bodies
pmol	Pico mol
PMP	1-Phenyl-3-Methyl-5-Pyrazolone
QAE	Quaternary Aminoethyl
Q-sepharose	Quaternary ammonium
rhTf	Recombinant human transferrin
RP-HPLC	Reversed phase HPLC
rpm	Rotation per minutes
RT	Retention time

S	Methyl sulphonate
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFM	Serum Free Medium
SP	Sulphopropyl
T.ni	*Trichoplusia ni*
TBS	Tris buffered saline
$TCID_{50}$	50 % Tissue Culture Infectious Dose
$TCID_{50}$/ml	50 % Tissue Culture Infectious Dose per milliliter
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TFA	Trifluoroacetic acid
™	Trademark
TMB	3,3',5,5'-tetramethylbenzidene
TN5B1-4	High 5
TOI	Time of Infection
Tris-HCl	Tromethamine and Hydrochloric Acid
UDP	Uridine-5'-diphophate
UDP-Gal	Uridine-diphosphate galactose
UDP-Glc	Uridine-diphosphate glucose
UDP-GlcNAc	Uridine-diphophate N-acetylglucosamine
V	Volts
W.R	Working reagent

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Preface

The biopharmaceutical industry has experienced a significant transformation based on the development of recombinant DNA and hybridoma technologies in the 1970s. The industry has moved beyond simple replication of human proteins (such as insulin or growth hormones) and played a key role in the development of large-molecule drugs such as any protein, virus, therapeutic serum, vaccine, and blood component. These genetically engineered therapeutic drugs are targeting some of the major illnesses such as cancer, cardiovascular, and infectious diseases and they have the full potential to tackle a whole array of new diseases effectively and safely.

By mid 2003, 148 biopharmaceuticals proteins were approved in the United States and Europe compared to 84 in 2000 (Birch and Onakunle, 2005). The total global market for protein drugs was $47.4 billion in 2006 and the market is presumed to reach $55.7 billion by the end of 2011 with an average annual growth rate (AAGR) of 3.3% (Figure 1.1). It is expected that current cell culture facilities are unlikely to meet expected demand. The imbalance of supply-demand is

expected to get worse in the future, as more biotech therapeutics proteins are approved. 20–50% of potential therapeutics could be delayed due to the lack of manufacturing capacity (Fernandez *et al*., 2002). Hence, the ability in expanding the existing capacity and producing a larger variety of products are crucial in order to meet future demand. Drug companies and biotech firms are considering alternative manufacturing platforms, besides increasing fermentation capacity (Table 1.1) (Elbehri, 2005).

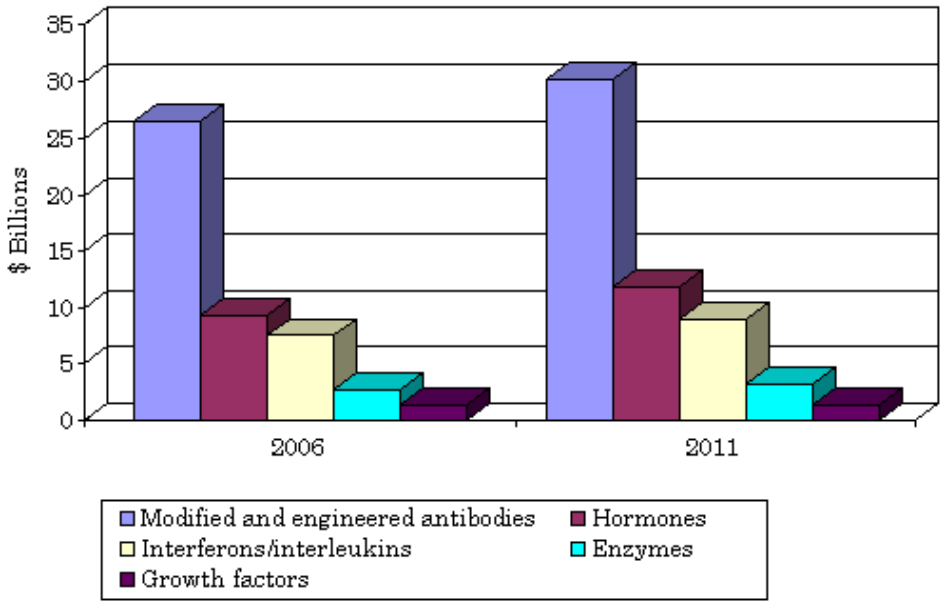

Figure 1.1: Worldwide sales forecast for protein drugs, 2006 and 2011 (Talukder, 2007).

Generally, recombinant therapeutic protein can be generated and produced in various prokaryotic and eukaryotic expression systems. Until the early 1990s, the majority of recombinant proteins were expressed in either microbial or mammalian cell culture systems. The first approved recombinant therapeutic glycoproteins, insulin is produced from *Escherichia coli*. Today, the manufacturing of biotechnology products relies heavily on the use of mammalian cells, chiefly on Chinese Hamster Ovary (CHO) cells. The well-known drugs Avonex (interferon beta 1-a, Biogen, Inc) and EPOGEN/EPREX (epoetin alfa, Amgen Inc/ Ortho Biotech) are produced in CHO. Insect, transgenic plant, transgenic animal and yeast

cells are also attractive as hosts for the production of recombinant proteins, as they represent potentially inexpensive and versatile expression systems. Optimal expression system can be varied, based on different critical parameters of the protein of interest. Selecting an appropriate expression system for the protein of interest will affect factors such as time to market, cost of goods, product characteristics, regulatory hurdles, and intellectual property (Figure 1.2).

Table 1.1: Comparison of pharmaceutical expression system (Elbehri, 2005).

Expression System	Advantages	Disadvantages	Applications	Cost per gram
Bacteria	Established regulatory track; well-understood genetics; cheap and easy to grow	Proteins not usually secreted; contain endotoxins; no posttranslational modifications	Insulin (E. coli; Eli Lilly); growth hormone (Genentech); growth factor; interferon	N.R
Yeast	Recognized as "safe;" long history of use; fast; inexpensive; posttranslational modifications	Overglycosylation can ruin bioactivity; safety; potency; clearance; contains immunogens/antigens	Beer fermentation; recombinant vaccines; hepatitis B viral vaccine; human insulin	$50-100
Insect cells	Posttranslational modifications; properly folded proteins; fairly high expression levels	Minimal regulatory track; slow growth; expensive media; baculovirus infection (extra step); mammalian virus can infect cells	Relatively new medium; Novavax produces virus-like particles	N.R
Mammalian cells	Usually fold proteins properly; correct posttranslation modifications; good regulatory track record; only choice for largest proteins	Expensive media; slow growth; may contain allergens/ contaminants; complicated purification	Tissue plasminogen activator; factor VIII (glycoprotein); monoclonal antibodies (Hercepin)	$500–5,000
Transgenic animals	Complex protein processing; very high expression levels; easy scale up; low-cost production	Little regulatory experience; potential for viral contamination; long time scales; isolation/GMPs on the farm	Lipase (sheep, rabbits; PPL Therapeutics); growth hormone (goats; Genzyme); factor VIII (cattle)	$20–50
Transgenic plants	Shorter development cycles; easy seed storage/scaling; good expression levels; no plant viruses known to infect humans	Potential for new contaminants (soil fungi, bacteria, pesticides); posttranslational modifications; contains possible allergens	Cholera vaccine (tobacco; Chlorogen, Inc.); gastric lipase (corn; Meristem); hepatitis B (potatoes; Boyce Thompson)	$10–20

N.R- Not Reported

The baculovirus expression vector system (BEVS) has a number of significant advantages over other methods of recombinant protein production. It is best known as providing quick access to biologically active proteins and used as a research tool (Cox, 2004). The major advantages of BEVS over bacterial and mammalian expression system is the very high expression of recombinant proteins which in many cases are antigenically, immunogenically and functionally similar to their native counterparts (Goosen, 1993). Lack of adventitious viral agents that could replicate in mammalian cells (John Morrow, 2007), make BEVS a powerful manufacturing platform for health care solutions to pandemic, biodefense, and emergency scenarios (Cox, 2004). However, BEVS also has its limitation in producing authentic mammalian proteins and glycoproteins. An absence of complex sugars in BEVS-produced proteins may result in poor pharmacological activity in vivo due to the rapid clearance from the circulatory system of glycoproteins with non-human glycans (Betenbaugh *et al*., 2004)

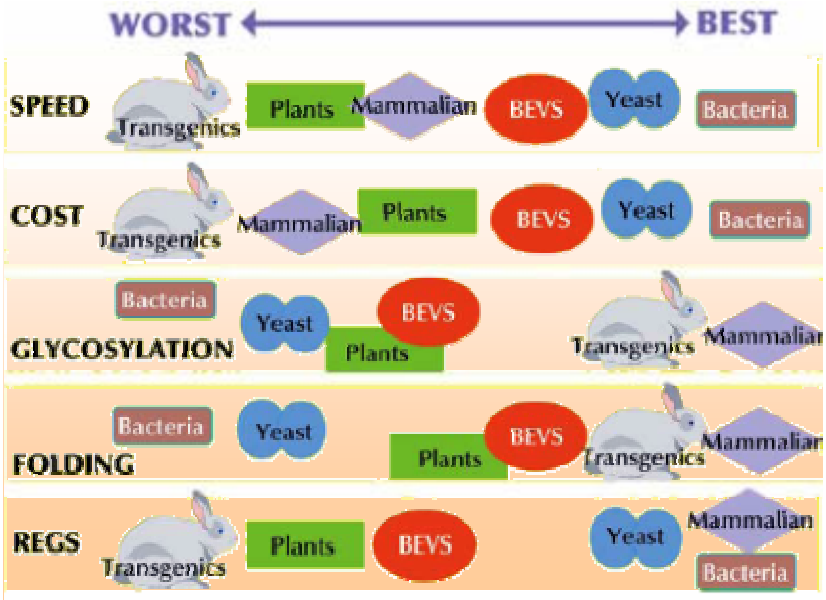

Figure 1.2: Strength and weaknesses of various expression systems (Cox, 2004).

The deficiency of BEVS in producing mammalian like-glycoproteins of potential therapeutic is a hot topic among researchers in this field. BEVS had been reported to produce sialylated complex type N-glycan through the modification of its metabolic engineering pathway (Betenbaugh *et al*., 2004; Viswanathan *et al*., 2005;

Yun *et al*., 2005). Protein Sciences Corporation (PSC) had developed technology for large-scale (600 L) production of proteins in insect cells using the BEVS (Cox, 2004). Although currently there are no FDA-approved therapeutic proteins expressed using BEVS, a number of products are in advanced clinical trials and several are about to get acceptance. Among these, three vaccines that are close to market are Provenge™, a prostate cancer immunotherapy from Dendreon (www.dendreon.com); Ceravix™, a papilloma virus vaccine from GlaxoSmithKline (www.gsk.com); and FluBIOk™ from Protein Sciences, a non-egg based flu vaccine (John Marrow, 2007).

BEVS have tremendous potential to become the next therapeutic manufacturing system. In this study, recombinant human transferrin was used as a model protein. Transferrin was chosen because of the simplicity of its structure and its recent important role in protein engineering. Non-glycosylated transferrin had been used as a scaffold to extend the half life of peptide and proteins. Various chromatographic methods for purification of transferrin have been reported. Among these reports, Ali *et al*. (1996) and Ailor *et al*. (2000) had purified rhTf from *sf9* and *Tn* cells using phenyl sepharose and Q-Sepharose. In this study, hydrophobic interaction chromatography utilizing phenyl sepharose was used as the capture step and IEX chromatography utilizing Q-sepharose was used for further purification of rhTf. To obtain pure rtTf, optimization of both chromatographic techniques had been carried out. Basic characterization of the carbohydrate content of the pure rhTf had also been carried out to get a better understanding of the glycan.

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