OPTIMIZATION OF RECOMBINANT HUMAN TRANSFERRIN EXPRESSION IN INSECT CELLS BACULOVIRUS SYSTEM

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Specially dedicated to:

my father Marcellus Ongkudon, mother Juanah Ungit,

Sisters Sibylla; Clarice; Stella; Mellisa, Brother McMarshall,

Uncle Bacon, Auntie Jane,

and my beloved Jessica @ Jess

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ABSTRACT

Insect cells-baculovirus expression system is a promising new artificial system for the production of many therapeutic glycoproteins. This system owns many of the protein processing and folding mechanisms of mammalian cells and is capable of expressing a large amount of recombinant proteins. This work aimed at expressing, optimizing, and characterizing recombinant human Transferrin (rhTf), a model glycoprotein, at a laboratory scale. In this research, time course expression profiles of rhTf at various multiplicities of infection (MOI), seeding densities (SD), times of infection (TOI), and harvest times (HT) were studied. Screening experiments were conducted to identify the medium components in Sf900-II SFM and the recombinant baculovirus stock that resulted in improved production of rhTf. Finally, Response Surface Methodology (RSM) was employed to hunt for optimum medium composition. The results showed that the optimum HT for rhTf was between 24 to 72 hours post infection, at SD of 1.6 x 10^6 viable cells/ml, TOI of day 2 post seeding, and MOI of 5 pfu/cell. Glucose and glutamine were found to have the most positive effect on rhTf production with more than 95% significance. In addition to that, the best recombinant baculovirus stock was identified at 98.7% purity. With the optimized parameters, rhTf production had increased three-fold from 19.89μg/ml to 65.12μg/ml.

ABSTRAK

Sistem ekspresi sel serangga-bakulovirus adalah satu sistem alternatif dalam penghasilan pelbagai jenis glikoprotein teraputik. Sistem ini memiliki banyak mekanisma pemprosesan dan penglipatan protein sel mamalia serta mampu untuk menghasilkan protein rekombinan dalam kuantiti yang besar. Penyelidikan ini bertujuan untuk mengekspresi, mengoptimum dan mencirikan model glikoprotein iaitu Transferin manusia rekombinan (rhTf) pada skala makmal. Di dalam penyelidikan ini, kajian dilakukan ke atas profil ekspresi lawan masa bagi rhTf pada pelbagai gandaan jangkitan (MOI), kepekatan pembenihan (SD), masa jangkitan (TOI) dan masa penuaian (HT). Eksperimen penyaringan dilakukan untuk mengenalpasti komponen dalam medium Sf900-II SFM dan juga stok bakulovirus rekombinan yang dapat meningkatkan lagi penghasilan rhTf. Akhirnya, Metodologi Permukaan Tindakbalas (RSM) dijalankan untuk mencari komposisi medium yang optimum. Hasil kajian mendapati bahawa, nilai optimum untuk HT ialah pada 24 hingga 72 jam selepas jangkitan pertama, SD sebanyak 1.6 x 10^6 sel produktif/ml, TOI pada hari ke-2 selepas pembenihan pertama dan MOI sebanyak 5 pfu/ml. Glukosa dan glutamina didapati mempunyai kesan yang paling positif terhadap penghasilan rhTf dengan nilai signifikan melebihi 95%. Stok bakulovirus rekombinan yang terbaik dikenalpasti pada 98.7% ketulinan. Melalui parameter-parameter yang telah dioptimumkan, penghasilan rhTf telah meningkat sebanyak 3-kali ganda iaitu daripada 19.89ug/ml kepada 65.12ug/ml.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	BLANK PAGE	**-**
	THESIS DECLARATION	**-**
	SUPERVISOR'S DECLARATION	**-**
	TITLE PAGE	**i**
	DECLARATION OF ORIGINALITY	**ii**
	DEDICATION	**iii**
	ACKNOWLEDGEMENT	**iv**
	ABSRACT	**v**
	ABSTRAK	**vi**
	TABLE OF CONTENTS	**vii**
	LIST OF TABLES	**xiii**
	LIST OF FIGURES	**xv**
	LIST OF SYMBOLS/ABBREVIATIONS/TERMINOLOGY	**xviii**
	LIST OF APPENDICES	**xxii**
1	**INTRODUCTION**	**1**
	1.1 Preface	**1**
	1.2 Research Problem Background	**3**
	1.3 Research Objective	**4**
	1.4 Research Scopes	**4**

	1.5	Research Contributions	**5**
2	**LITERATURE REVIEW**		**6**
	2.1	Insect Cells - Baculovirus Expression System	**6**
		2.1.1 Baculovirus Characteristics	**6**
		2.1.2 Insect Cell Lines	**8**
		2.1.3 The Pros and Cons of the Insect Cells – Baculovirus Expression System	**9**
		2.1.4 Generation of Recombinant Baculovirus	**10**
	2.2	Model Glycoprotein	**11**
		2.2.1 Native Human Transferrin (nhTf)	**11**
		2.2.2 Recombinant Human Transferrin (rhTf)	**14**
		2.2.3 Biosynthesis of N-Glycans	**15**
	2.3	Insect Cell Culture Medium	**16**
		2.3.1 Protein Hydrolysates (Peptones)	**16**
		2.3.2 Carbohydrates	**17**
		2.3.3 Amino Acids	**17**
		2.3.4 Lipids	**18**
		2.3.5 Albumin	**19**
		2.3.6 Serum Free Medium (SFM)	**19**
	2.4	Optimization of Protein Expression in BEVS	**20**
		2.4.1 Physical Factors that Ensure Success of Expression	**20**
		2.4.2 Optimization of Recombinant Baculovirus Stock	**21**
		2.4.3 Medium Optimization	**23**
	2.5	Design of Experiments	**25**
		2.5.1 Factorial Experiments in Completely Randomized Designs	**25**
		2.5.2 Interactions	**26**
		2.5.3 Coded Variables	**26**

	2.5.4	Factor Levels Combinations	**27**
	2.5.5	Fractional Factorial Experiments	**28**
	2.5.6	Screening Experiments	**29**
	2.6	Analysis of Experiments	**30**
	2.6.1	Correlation	**30**
	2.6.2	Regression Analysis	**31**
	2.6.3	Nonlinear and Higher-Order Regression Analysis	**32**
	2.7	Optimization of Experiments	**33**
	2.7.1	Improvements of RSM	**34**
	2.8	Specialized Protein Analysis: Theories and Principles	**34**
	2.8.1	Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)	**34**
	2.8.2	Enzyme Linked Immunosorbent Assay (ELISA)	**38**
	2.8.2.1	Basic Immunology	**38**
	2.8.2.2	Principles of ELISA	**40**
3		**RESEARCH METHODOLOGY**	**43**
	3.1	Materials	**43**
	3.1.1	Cell line and Recombinant Baculovirus	**43**
	3.1.2	Equipments	**43**
	3.1.3	Chemicals	**44**
	3.2	Insect Cells Techniques	**45**
	3.2.1	The Preparation of TC100 Medium From Powdered Formulation	**45**
	3.2.2	Maintenance and Regeneration of Sf9 Cells Monolayer Culture	**45**
	3.2.3	Cells Freezing	**46**
	3.2.4	Cells Thawing	**46**
	3.2.5	Cells Counting	**47**

3.2.6 Adaptation of Sf9 Cell Culture in Serum Free Medium **48**

3.2.7 Adaptation of Sf9 Cells in Suspension Culture **48**

3.2.8 Sf9 Cells Maintenance in Suspension Culture **49**

3.3 Baculovirus Techniques **50**

3.3.1 Viral Amplification **50**

3.3.2 Viral Titration by End Point Dilution Method **51**

3.3.3 Generation of Pure Recombinant Virus Stocks by End Point Dilution Method **52**

3.4 Protein Analysis Techniques **53**

3.4.1 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis, SDS-PAGE under Denaturing Condition **53**

3.4.2 Bicinchoninic Acid (BCA) Assay **54**

3.4.3 Enzyme Linked Immunosorbent Assay, ELISA **55**

3.4.4 ELISA-Conversion of Calibrated Data to Actual Product Concentration **56**

3.5 Recombinant Human Transferrin (rhTf) Expression and Optimization **57**

3.5.1 Optimization of rhTf Expression in Monolayer Culture **57**

3.5.2 Medium Screening **57**

3.5.3 Medium Optimization in Suspension Culture **58**

3.6 Response Surface Methodology, RSM (Method of Steepest Ascent) **59**

4 RESULTS AND DISCUSSIONS **61**

4.1 The Study of Sf9 Insect Cells Culture Growth Profiles **61**

4.1.1 Sf9 Cell Growth in T-flask (Monolayer) and Shake flask (Suspension) **61**

4.1.2 Development of Sf9 Suspension Culture System in 24-well Plate **63**
4.1.3 Growth Profiles of Infected Cells **65**
4.1.4 Growth Analysis **67**
4.2 Study on the Expression Profiles of rhTf in Infected Sf9 Insect Cells Culture **69**
4.2.1 rhTf Expression at Different MOIs **69**
4.2.2 rhTf Expression at Different Seeding Densities **71**
4.2.3 rhTf Expression at Different Times of Infection **73**
4.3 Optimization of the Recombinant Human Transferrin Expression **76**
4.3.1 Recombinant Baculovirus Screening **76**
4.3.2 Medium Screening **81**
4.3.3 Medium Optimization using Response Surface Methodology **90**
4.3.3.1 Regression Model **90**
4.3.3.2 Nutrients Interactions **94**
4.4 Characterization of the Optimized Recombinant Human Transferrin Expression **98**

5 CONCLUSIONS 106
5.1 Summaries **106**
5.1.1 rhTf Expression in Sf9 Insect Cells Monolayer Culture **106**
5.1.2 Utilization of 24-well Plates for Insect Cells Suspension Culture **107**
5.1.3 Medium and Baculovirus Screening **107**
5.1.4 Response Surface Methodology **108**
5.2 Recommendations **108**

5.2.1	Large Scale Study in Bioreactor	**109**
5.2.2	Expression and Purification of Biologically Active Glycoprotein	**110**

REFERENCES **111**

APPENDICES **130**

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Seeding densities for typical vessel sizes	**21**
2.2	Example of a 4-Factor, 2-level Full Factorial Experiment	**28**
2.3	Example of 12-Run, 11-Factor, 2-Level, Screening Design	**29**
4.1	Comparison of Sf9 growth in T-flask, Shaker, and 24-well plate	**65**
4.2	rhTf yield coefficients at various seeding density, MOIs, and time of infection	**75**
4.3	Concentration of rhTf in each well of a 96-well plate	**77**
4.4	Poisson distribution data sheet	**79**
4.5	Factors affecting the end point dilution method	**81**
4.6	Real values for the screening of 13 selected nutrients using *Plackett-Burman* design	**82**
4.7	13-factor (nutrients), 33-run, 2-level *Plackett-Burman*	**83**

	screening design	
4.8	Estimated effects on rhTf yield based on the results of Plackett-Burman screening experiments	**87**
4.9	Central composite design for the optimization of glutamine, glucose and lipid mixtures 1000x	**91**
4.10	Analysis of Variance (ANOVA) of the CCD	**93**
4.11	Summary of the characteristics of optimized rhTf expression	**98**

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	*Spodoptera frugiperda* Sf9 Cells	**8**
2.2	Insect Cells Baculovirus Expression System	**10**
2.3	3D structure of the first domain of Human Transferrin	**12**
2.4	Samples being electrophorase in SDS-PAGE	**35**
2.5	Chemical structure of a polyacrylamide	**36**
2.6	Movement of molecules in the porous gel	**36**
2.7	Basic principles of Direct Sandwich ELISA	**41**
4.1	Growth curves of Sf9 monolayer culture in 25cm^2 T-flask at different seeding densities, SD	**62**
4.2	Growth curves of Sf9 suspension culture in 250ml shake flask at different seeding densities, SD	**62**
4.3	Growth curves of Sf9 suspension culture in 24-well plate at different seeding densities, SD	**64**

4.4	Growth curves of Sf9 suspension culture in 24-well plate at different seeding densities, SD	**64**
4.5	Growth curves of Sf9 infected with AcMNPV	**66**
4.6	Comparison between uninfected (U), wild-type (WI), and recombinant (R) virus-infected Sf9 cells	**66**
4.7	Growth rate constants of Sf9 in various cultivators and at different seeding densities	**68**
4.8	Doubling time of Sf9 in various cultivators and at different seeding densities	**68**
4.9	SDS PAGE analysis of rhTF expression	**70**
4.10	rhTf expression profiles at different MOIs	**70**
4.11	rhTf expression profiles at different seeding densities	**72**
4.12	Surface plot of figure 4.11	**72**
4.13	rhTf expression profiles at different times of infection	**74**
4.14	Surface plot of figure 4.13	**74**
4.15	3D plot of Table 4.3	**77**
4.16	Infected cells appearance in medium A (lipid mixtures added) and medium B (no lipid mixtures added)	**84**
4.17	rhTf concentration at different medium compositions based on *Plackett-Burman* screening experiments	**84**

4.18	SDS-PAGE analysis of medium screening	**85**
4.19	Effect of nutrients on rhTf yield	**87**
4.20	Amino Acids in Human Transferrin (679 residues)	**89**
4.21	Observed and predicted experimental data for the optimization of glutamine, glucose and lipid mixtures	**92**
4.22	Glutamine (Gln) vs Glucose (Gluc) vs rhTf	**95**
4.23	Glutamine(Gln) vs Lipid Mixtures 1000x (Lip) vs rhTf	**95**
4.24	Glucose (Gluc) vs Lipid Mixtures 1000x (Lip) vs rhTf	**96**
4.25	Sf9 growth in controlled and optimized expression.	**99**
4.26	Total protein and rhTf contents in controlled and optimized expression	**99**
4.27	Total protein and rhTf production rates in controlled and optimized expression	**101**
4.28	Glucose and lactate concentrations in controlled and optimized expression	**101**
4.29	Lactate production and glucose uptake rate in controlled and optimized expression	**103**
4.30	SDS PAGE gel for non optimized medium	**104**
4.31	SDS PAGE gel for optimized medium	**105**

LIST OF SYMBOLS/ABBREVIATIONS/TERMINOLOGY

a	-	Constant
ABTS	-	2, 2'-azino-bid (3-ethylbenzthiazoline-6-sulfonic acid)
AcMNPV	-	Autographa Californica Multiple Nuclear Polyhidrosis Virus
apo-hTf	-	Low iron binding human trasferrin
Arg	-	Arginine
Asn-X-Thr/Ser	-	Asparagine-X-Threonine/Serine
ATCC	-	American Tissue Culture Collection
b	-	Constant
BEVS	-	Baculovirus Expression Vector System
b-Gal	-	Beta Galactosidase
BL1	-	Biosafety Level 1
BSA	-	Bovine Serum Albumin
BTI-Tn-5B1-4	-	Insect cell line
CDG	-	Carbohydrate-Deficient Glycoprotein
cm^2	-	Surface area in centimeter square
CO_2	-	Carbon dioxide
Cys	-	Cystine
DMSO	-	Dimethylsulfoxide
DNA	-	Diribonucleic Acid
doub	-	Doubling
dpi	-	Days post infection
E	-	Global error
E. coli	-	Escherichia Coli

ELISA	-	Enzyme Linkage Immunosorbent Assay
ER	-	Endoplasmic Reticulum
exp	-	Exponential
FBS	-	Fetal Bovine Serum
FP	-	Few Polyhedra
Fruc	-	Fructose
$g\ l^{-1}$	-	Gram per liter
g/cell	-	Gram per cell
g/ml	-	Gram per milliliter
GC	-	Gas Chromatography
Glc	-	Glucose
Glcp3-Manp9-GlcNAcp2	-	3(Glucose)-9(Manose)-2(N-Acetylglucosamine)
Gln	-	Glutamine
Gluc	-	Glucose
H_2O	-	Water
H_2SO_4	-	Sulphuric acid
HRP	-	Horseradish Peroxidase
Htf	-	Human Transferrin
Interc.	-	Intercept
k	-	Number of factors in experimental design
kbp	-	Kilo base pair
kDa	-	Kilo Dalton
KOH	-	Kalium Hidroxide
Lys	-	Lysine
M	-	Molar
Malt	-	Maltose
Man	-	Mannose
Man(alpha-1,6)	-	Mannose (alpha-1,6)
Manp8-GlcNAcp2	-	8(Mannose)-2(N-Acetylglucosamine)
max	-	Maximum
Met	-	Methionine
mg/ml	-	Milligram per milliliter
mM	-	Millimolar

MOI	-	Multiplicity of Infection
MS	-	Mass Spectrometry
n	-	Number of possible combinations in experimental design
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hidroxide
ng	-	Nanogram
nm	-	Nanometer
NPV	-	Nuclear Polyhidrosis Virus
OD	-	Optical Density
OPD	-	O-phenylene diamine
OV	-	Occluded virus
p	-	Proportion of cultures receiving particular number of infectious units.
p	-	Probability in analysis of variance
PCR	-	Polymerase Chain Reactor
PD	-	Proportionate Distance
PFU	-	Plug Performing Unit
pfu/cell	-	Plug performing unit per cell
pH	-	Potential hydrogen
r	-	Number of infectious units
R	-	Recombinant
RER	-	Rough Endoplasmic Reticulum
rhTf	-	Recombinant human trasferrin
SD	-	Seeding Density
SDS	-	Sodium Dodecyl Sulphate
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electophoresis
SEAP	-	Human Secreted Alkaline Phosphatase
Ser	-	Serine
Sf21	-	Insect cell line
Sf9	-	Insect cell line
SFM	-	Serum Free Medium
Std. Err.	-	Standard Error

t	-	Student's test
TBS	-	Tris Buffered Saline
TCA	-	Tricarboxylic Acid
TCI	-	Time Course of Infection
TCID50	-	50 % Tissue Culture Infectious Dose
TEMED	-	Tetramethylethylenediamine
Thr	-	Threonine
TMB	-	Tetramethyl benzidine
TOI	-	Time of Infection
Tris	-	Tromethamine
Tris-HCL	-	Tromethamine and Hydrochloric Acid
Tyr	-	Tyrosine
U	-	Uninfected
u_{net}	-	Net Growth Constant
USA	-	United State of America
UTM	-	Universiti Teknologi Malaysia
V	-	Voltage
Val	-	Valine
w/v	-	Weight per Volume
w/w	-	Volume per Volume
WI	-	Wild-Type
X	-	Cell concentrations at time t
X_0	-	Cell concentrations at time 0
x_i, y_i	-	Data pairs
μ	-	Mean concentration of the infectious units
μl	-	Microliter
%	-	Percentage
^{0}C	-	Temperature level in degree Celcius

LIST OF APPENDICES

APPENDIX NO.	TITLE	PAGE
A1	Kinetics Analysis of Sf9 Insect Cells Growth at Various Conditions	**130**
A2	Growth kinetic coefficients of Sf9 cells monolayer culture	**133**
B1	TCID50 Calculation	**134**
B2	Calculation of End Point Dilution based on Reed and Muench method	**135**
C1	RSM spreadsheet	**136**
D1	Flowchart of the major steps involved in this research	**138**

CHAPTER 1

INTRODUCTION

1.1 Preface

The Internet created a sky rocketed investment that made history in this modern era. It is a matter of time in some circles that biotechnology is the next computer revolution that will change the world, spawn new industries and create multi-millionaires.

Global manufacturing of biopharmaceuticals has increased significantly over the last decade due to a number of reasons. Biopharmaceuticals offer several advantages such as highly effective and potent action, fewer side effects and the potential to actually cure diseases rather than merely treating the symptoms. These advantages, combined with the increasing number of new diseases that can be treated with biopharmaceuticals, are driving enhanced production of these drugs worldwide.

According to a report by PRNewswire, London dated November 30th 2004; the global manufacturing capacity of biopharmaceuticals was around 2.27 million liters in 2004. This included the capacity held by both captive use and contract

manufacturers. It is expected to increase to 3.69 million liters in 2011 at a compound annual growth rate (CAGR) of 7.2 per cent.

A variety of systems can be employed to produce biopharmaceuticals. The most important ones are derived from bacteria and yeasts, but eukaryotic systems become more and more important because the proteins produced are almost similar to native proteins. In the recent past, the baculovirus insect cell system has attracted wide attention as vectors for high level and faithful expression of a variety of heterologous proteins. In many cases the products are chemically, antigenically, immunologically and functionally similar, if not identical to their authentic counterparts (Vlak, 1997).

The baculovirus expression vector system (BEVS) is frequently a method of choice for the expression of recombinant mammalian proteins (O'Reilly *et al.*, 1994). Apart from the simplicity and cost-effectiveness of this method, the insect host cells possess many of the protein-processing and -folding mechanisms of mammalian cells (O'Reilly *et al.*, 1992) therefore functional and antigenic differences are rarely seen. The technology called the BEVS for the safe, abundant and rapid production of recombinant proteins in insect cells and insects was pioneered in the laboratory of Dr. Max D. Summers of Texas A&M University USA in 1982.

The BEVS has become a core technology for the cloning and expression of genes for study of protein structure, processing and function. It is also important for the production of biochemical reagents and study of regulation of gene expression. It has a wide application in the commercial exploration, development and production of vaccines, therapeutics and diagnostics; drug discovery research; as well as exploration and development of safer, more selective and environmentally compatible biopesticides consistent with sustainable agriculture.

Studies of proteins for the development of drug therapies, vaccines, and insights into biological function depend upon the ability to produce large amounts of structurally complex proteins. It is important that these proteins are biologically active, processed correctly, assume a native shape, and locate to the proper place in the cell. The inability to generate large quantities of structurally complex eukaryotic proteins with these characteristics has been a major limitation for many years. Thus, this thesis hoped to give necessary foundations on how to develop a process that will produce greater amount of recombinant proteins for therapeutic purpose.

1.2 Research Problem Background

The development of new recombinant therapeutic proteins requires extensive studies on the expressional host and product. In this research, human transferrin, a model protein was chosen as the expressional product and insect cell as the expressional host. The selection was based on many reports from other researchers which indicate that insect cell baculovirus system is a promising new artificial system for the production of large amount of recombinant proteins. Insect cells S*podoptera Frugiperda* (Sf9) and recombinant Autographa Californica Multiple Nuclear Polyhydrosis Virus (rAcMNPV) were utilized in this research. Human transferrin was chosen because it is a simple form of glycoprotein which is easier to study than the complex and hybrid forms.

To complement metabolic engineering works involving the humanization of recombinant glycoprotein, it is important that the recombinant protein can be generated in large quantities. The understanding of the insect cells and baculovirus behaviour is as critical as the expressional behaviour of the recombinant protein at various settings. Various yields of recombinant human transferrin (rhTf) using the baculovirus system have been reported. Among those were Tomiya *et al.*, (2003) who reported rhTf yield of 7μg/ml and Ali *et al.*, (1996) with 20 μg/ml of rhTf.

1.3 Research Objective

The ultimate objective of this research was to optimize the expression level of recombinant human transferrin in insect cells baculovirus expression system in terms of its concentration (μg/ml) and protein percentage.

1.4 Research Scopes

This research focused on the optimization of expression of recombinant human transferrin gene which had already been cloned into the baculovirus DNA. The scopes of this research were as follows:

a) Expression and optimization of rhTf in Sf9 insect cells monolayer culture using conventional method. Variables studied were seeding density (SD), multiplicity of infection (MOI), time of infection (TOI), and harvest time (HT).
b) Screening of the Sf900-II SFM insect cell culture medium and recombinant baculovirus stock that resulted in improved production of rhTf.

c) Expression and optimization of rhTf in Sf9 insect cells suspension culture using experimental design. Variables studied were dominant medium components that were screened earlier.

1.5 Research Contributions

Some major contributions of this research are listed below.

1) Establishment of methods for optimizing recombinant protein expression in insect cells culture.
2) Trained (hands-on-experienced) personnel in Baculovirus Insect Cells Expression System.
3) Two research papers (proceedings) were published (Ongkudon *et al.*, 2004; Ongkudon *et al.*, 2005) and two other papers are in preparation.

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