DEVELOPMENT OF NOVEL GALACTOSYLATION METHOD FOR THE EXPRESSION OF RECOMBINANT HUMAN TRANSFERRIN IN INSECT CELL CULTURE

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical and Natural Resources Engineering
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MARCH 2006

ABSTRACT

The objective of this research is to develop a novel galactosylation method for the expression of recombinant human transferrin (hTf) with better *N*-glycan quality. The baculovirus-insect cell system, consisting of hTf as the model protein, β1,4-galactosyltransferase (β1,4-GalT) as the enzyme, and uridine-diphospho-galactose (UDP-Gal) as the sugar nucleotide, has been successfully established. In the early part of the study, fundamental works were carried out to optimize *Spodoptera frugiperda* (*Sf*-9) cells growth and mock infection. Serum concentration, different type of media, cell subculturing condition, initial cell density and spent medium carry over had been found to significantly influence the growth kinetics of *Sf*-9 cells. Multiplicity of infection (MOI) and spent medium carry over were found to have direct impact on viral infectivity. The optimized parameters were then used to evaluate the expression of recombinant hTf and β1,4-GalT in *Sf*-9 cells. Subsequently, native UDP-Gal levels at normal and upon baculovirus infection produced in *Sf*-9 cells were monitored using Reverse Phase High Performance Liquid Chromatography. UDP-Gal concentration was discovered to decrease gradually once infected with the recombinant baculovirus. Finally, baculovirus coinfection study was carried out to evaluate the recombinant glycoprotein quality. However, lectin binding analysis using *Ricinus communis* agglutinin-I, revealed that co-expression between rhTf and β-1,4GalT (*in vivo*) did not show encouraging result due to the reduction of UDP-Gal upon baculovirus infection. This finding suggested that the introduction of β-1,4GalT alone was not sufficient for successful galactosylation. However, another strategy was used to overcome the problem. Commercial GalT and UDP-Gal were introduced artificially to the rhTf after it was secreted from cell culture. It was found that the *in vitro* strategy promoted better *N*-glycan quality in insect cells.

ABSTRAK

Kajian ini bermatlamat untuk mengkaji proses galaktosilasi yang baru bagi penghasilan rekombinasi human transferrin (hTf) dengan kualiti *N*-glikan yang lebih baik. Sistem bakulovirus-sel serangga yang terdiri daripada hTf sebagai protein model, β1,4-galaktositransferasa (β1,4-GalT) sebagai enzim, dan uridina-diphosphogalaktosa (UDP-Gal) sebagai gula nukleotida telah dibentuk dengan berjaya. Dalam kajian awal, kerja asas mengenai pengoptimuman telah dilakukan bagi pertumbuhan sel dan jangkitan bakulovirus tanpa membawa gen tertentu dalam sel *Spodoptera frugiperda* (*Sf*-9). Kepekatan serum, medium yang berbeza, keadaan sel subkultur, ketumpatan sel awal dan medium telah-guna telah memberi kesan yang ketara terhadap kinetik pertumbuhan sel *Sf*-9. Gandaan Jangkitan dan medium telah-guna menunjukkan kesan terus terhadap infektiviti virus. Semua parameter yang telah dioptimumkan telah digunakan untuk menilai ekpresi bagi rekombinasi hTf and β1,4-GalT dalam sel *Sf*-9. Seterusnya, tahap UDP-Gal semulajadi pada normal dan atas jangkitan bakulovirus yang dihasilkan dianalisis dengan menggunakan Fasa Terbalik Kromatografi Cecair Pertunjukkan Tinggi. Didapati bahawa kepekatan UDP-Gal menurun secara perlahan sebaik sahaja dijangkiti dengan rekombinasi bakulovirus. Akhirnya, jangkitan serentak bakulovirus telah dilakukan bagi menilai kualiti glikoprotein rekombinasi. Tetapi, analisis lektin perlekatan dengan menggunakan *Ricinus communis* agglutinin-I, menunjukkan *in vivo* galaktosilasi tidak cukup berkesan disebabkan kekurangan UDP-Gal semasa jangkitan bakulovirus. Keputusan yang menarik ini mencadangkan bahawa penambahan β1,4-GalT sahaja tidak cukup untuk menjayakan galaktosilasi. Oleh itu, strategi lain telah digunakan untuk mengatasi kelemahan ini. GalT mamalia dan UDP-Gal yang diperolehi secara komersil diperkenalkan kepada supernatan hTf yang dikumpul. Didapati bahawa kaedah ini berjaya meningkatkan kualiti *N*-glikan dengan baik.

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LIST OF SYMBOLS/ ABBREVIATIONS

2-ADN	-	2-acetamide-1,2-dideoxynojirimycin
AcMNPV	-	*Autographa californica* multicapsid nucleopolyhedrovirus
Asp	-	Asparagine
$Ba(OH)_2$	-	barium hydroxide
BEVS	-	baculovirus expression vectors system
bIFN- γ	-	bovine interferon-γ
Bm	-	*Bombyx mori*
BSA	-	bovine serum albumin
BVs	-	budded viruses
$CaCl_2$	-	calcium chloride
CHO	-	chinese hamster ovary
CMP	-	cytidine-5'-monophosphate
CMP-NeuNAc-		cytidine-5'-monophospho *N*-acetylneuraminic acid
CMP-SAS	-	CMP-NeuNAc synthase
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
E.Coli	-	*Escherichia coli*
Ea	-	*Estigmene acrea*

EDTA	-	ethylenediamine tetraacetic acid disodium salt dehydrate
ELISA	-	Enzyme Linked Immunosorbent Assay
ER	-	endoplasmic reticulum
FBS	-	fetal bovine serum
Fuc	-	fucose
FucT	-	Fucosyltransferases
Gal	-	galactose
GalNAc	-	*N*-Acetylgalactosamine
GDP-Fuc	-	guanosine 5'-diphoshate-β-L-fucose
GDP-Man	-	guanosine 5'-diphoshate-D-mannose
Glc	-	glucose
GlcNAc	-	*N*-Acetylglucosamine
GlcNAcT II	-	*N*-Acetylglucosaminyltransferase II
GlcNAcT-I	-	*N*-Acetylglucosaminyltransferase I
H_2O_2	-	peroxidase
H_3PO_4	-	phosphoric acid
HCl	-	hydrochloric acid
HRP	-	horseradish peroxidase
hTf	-	human serum transferrin
IgG	-	immunoglobulin G
kbp	-	kilobasepairs
kDa	-	kilodalton
LacNAc	-	*N*-Acetyllactosamine
M	-	molar
Man	-	mannose
ManNAc	-	*N*-Acetylmannosamine
ManNAc	-	*N*-acetylmannosamine
ManNAc-6-P	-	*N*-acetylmannosamine-6-phosphate
MB	-	*Mamestra brassicae*
Mg	-	magnesium
min	-	minute
mm	-	mililiter
$MnCl_2$	-	manganese chloride
MOI	-	Multiplicities of Infection

MOPS	-	4-Morpholinepropanesulfonic acid
MWCO	-	molecular weight cut off
NaCl	-	sodium chloride
NAG	-	*N*-acetylglucosamine
NAL	-	*N*-Acetyllactosamine
NeuNAc	-	*N*-acetylneuraminic acid
NeuNAc-9-P	-	*N*-Acetylneuraminic acid-9-phosphate
nm	-	nanometer
NOV	-	non-occluded virus particles
NPV	-	nucleocapsid nuclear polyhedrovirus
OBV	-	occlusion body-derived virus particles
PBS	-	Phosphate Buffer Saline
PBST	-	PBS containing 0.05% Tween 20
PI	-	Post infection
RCA I	-	*Ricinus communis* agglutinin 1
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
rpm	-	rotation per minutes
SAS	-	*N*-Acetylneuraminate-9-phosphate synthase
SDS	-	sodium dodecyl sulfate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sf	-	*Spodoptera frugiperda*
SiaT	-	sialyltransferase
TBAS	-	tetrabutylammonium hydrogen sulfate
TBS	-	Tris-buffered Saline
$TCID_{50}$	-	Tissue Culture Infectious Dose 50
TEMED	-	*N,N,N′,N′*-tetramethylethylenediamine
TLC	-	Thin Layer Chromatography
TMB	-	3,3’,5,5’-tetramethylbenzidene
Tn	-	*Trichoplusia ni*
TOI	-	Time of infection
UDP-Gal	-	uridine-diphosphogalactose
UDP-GlcNAc	-	uridine-5’-diphopho-*N*-acetylglucosamine
UDP-hexose	-	uridine-5’-diphopho-D-hexose
UF	-	ultrafiltration

UTP	-	uridine 5'-triphosphate sodium
UV	-	ultraviolet
$ZnSO_4.7H_2O$	-	zinc sulfate 7-hydrate
α2,6-ST	-	α2,6-sialytransferase
β1,4-GalT	-	β1,4-galactosyltransferase
µl	-	microliter
µm	-	micrometer
^0C	-	degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Many glycoproteins have been produced by a variety of expression systems including cell cultures of mammalian or insect cell lines. Of particular interest has been the baculovirus expression system that generates high levels of recombinant proteins from insect cells such as *Spodoptera frugiperda* (*Sf*-9). The potential production of therapeutic glycoproteins in these systems has stimulated the desire to monitor the glycosylation pattern of specific insect-cell-produced glycoproteins and the glycosylation potential of insect cells in general. The glycan moieties can significantly affect a protein's stability, biological activity, antigenicity, immunogenicity, solubility, cellular processing, secretion and pharmacokinetic behaviour such as *in vivo* metabolic clearance rate (Takeuchi *et al.*, 1990, Takeuchi and Kobata, 1991, Munk *et al*., 1992).

It is well documented that the *N*-glycans found in recombinant glycoproteins expressed by lepidopteran cells using the baculovirus vector are predominantly high mannose type glycans and short truncated glycans (paucimannose) with α1,3/ α1,6-linked fucose residue on its asparagines-bound *N*-acetylglucosamine (GlcNAc)

(Jarvis and Summers, 1989; Wathen *et al.*, 1991; Grabenhorst *et al.*,1993; Yeh *et al.*, 1993; Manneberg *et al.*, 1994; Ogonah *et al.*,1995; Hsu *et al.*,1997; Opez *et al.*, 1997). In contrast, mammalian cells usually produce sialylated complex-type *N*-glycans. Generation of complete forms of sialylated complex-type *N*-glycans in insect cells may increase the value of insect cell derived products as vaccines, therapeutic and diagnostics.

The glycosylation process in the cultured cells can be controlled by various factors, which are sugar acceptor as model protein, substrate donor also known as sugar nucleotide and glycosyltransferase as enzyme. Activity measurements of several glycosyltransferases involved in the elongation of *N*-glycans have demonstrated that insect cells contain α1,6-fucosyltransferase (Staudacher *et al.*,1992) and a significant level of β1,2-*N*-acetylglucosaminyltransferase I activities (Velardo *et al.*,1993, Altmann *et al.*,1993) but they lack significant β1,4-galactosyltransferase (Butters *et al.*,1981; van Die *et al.*, 1996) and sialytransferase activities (Hooker *et al*., 1999). In addition to glycosyltransferases, another important factor in protein glycosylation is the sugar nucleotides essential in the biosynthesis of glycoconjugates. Since these are the substrate donor of glycosyltransferases that construct the glycan chains, the intracellular levels of sugar nucleotides can affect the glycosylation potential of the cultured cells as well.

In this study, we will focus on the galactosylation processing pathway rather than the whole glycosylation process. In the galactosylation process, recombinant human transferrin (hTf) is the substrate acceptor, β1,4-galactosyltransferase (β-1,4GalT) is the glycosyltransferase, and uridine-5'-diphosphogalactose (UDP-Gal) is the substrate donor. Recombinant hTf will be used as a model protein simply due to its simple biantennary *N*-glycan structure. Ailor *et al.* (2000) revealed that the *N*-glycan structures of hTf produced in insect cells included high mannose, paucimannosidic, and hybrid structures with over 50% these structures containing one or two fucoses linked to the Asn-linked *N*-acetylglucosamine. Furthermore, neither sialic acid nor galactose was detected on any of the *N*-glycan.

In this study, establishment of the native UDP-Gal level at normal and upon baculovirus infection was performed. It is proposed that analysis of the intracellular concentration of sugar nucleotides could provide important information on the potential of galactosylation in *Sf*-9 insect cells as little is known about the level of native UDP-Gal level especially upon baculovirus infection. To evaluate the quality of the recombinant hTf, different levels of galactosylation were conducted to obtain the galactosylated hTf, including *in vivo* and *in vitro* study. *In vivo* refers to the baculovirus coinfection by coexpressing β-1,4GalT and hTf simultaneously in cultured cell, meanwhile the introduction of commercial GalT and UDP-Gal to the hTf after it was secreted from insect cell cultures called as *in vitro*.

1.2 Objectives

(1) To develop a method for the expression of galactosylated recombinant hTf in insect cells
(2) To optimize the expression of the galactosylated recombinant hTf

1.3 Scopes of Research

(1) Recloning of recombinant virus stock, virus propagation and virus titration
(2) Establishment of an assay system for the detection of β1,4-GalT and UDP-Gal
(3) Monitoring of native UDP-Gal level at normal and upon baculovirus infection
(4) Evaluation of the quality of the glycoprotein obtained through baculovirus coinfection study to coexpress β1,4-GalT and hTf (*in vivo* study) and the

artificial introduction of commercial GalT and UDP-Gal to secreted hTf (*in vitro* study)

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