Production of a camelid antibody in CHO cells and its potential for the *in vitro* modification of its N-glycan using N-acetylglucosaminyltransferase III produced in insect cells

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Monoclonal antibodies have been extensively used for clinical diagnosis over the years. Beyond their ability to bind antigens, their ability to invoke or evade immune responses has also led to their use in therapeutic applications. These additional characteristics have been linked to the sugar molecules naturally attached to the proteins through glycosylation – a post-translational modification performed in higher order organisms. Certain N-glycans, glycans attached to protein at asparagine residues, have been shown to influence antibody Fc-receptor mediated effector functions. Of these, glycans with a bisecting N-acetylglucosamine show promising characteristics. The goal of this project is to create a process to remodel a novel therapeutic mAb (EG2 camelid antibody against Epidermal Growth Factor Receptor (EGFR)) produced from Chinese Hamster Ovary (CHO) cell culture to have a larger fraction of proteins having bisecting N-acetylglucosamine. The driving hypothesis is that a robust *in vitro* process will be better than what can be achieved in *vivo*. To achieve this goal, expression and purification of truncated N-acetylglucosaminyltransferase III (GnT3) using the baculovirus expression vector system (BEVS) in the *Sf9* insect cell line was used to gain an adequate supply of glycosyltransferase. The truncated form of GnT3 contained a C-terminal 6xHistidine-Tag and was cloned downstream of a honeybee mellitin (HBM) secretion signal. Activity of the GnT3 produced in insect cells was compared to a commercial GnT3 acting on our product of interest and on an IgG glycosylated protein. Active GnT3 was utilized to develop a scalable remodeling process to produce bisecting glycan enriched antibody pools.

Acknowledgements

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Dedication

I would like to dedicate my work to my family: Jack and Cynthia Ang, Mike and Joanna Nguyen, Clarissa Roberto and Victoria Ang

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List of Abbreviations

2-AB	2-Aminobenzamide
AcMNPV	Autographa californica nuclear polyhedrosis virus
ADCC	*Antibody-dependent cellular cytotoxicity*
Asn	Asparagine
BCA	Bicinchoninic acid
BEVS	Baculovirus expression vector system
BmNPV	Bombyx mori nuclear polyhedrosis virus
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CR	Constant region
CDC	Complement-dependent cytotoxicity CDR
Complementarity-determining regions CHO	
Chinese Hamster Ovary	
CMP	Common myeloid progenitors
DHFR	Dihydrofolate reductase
DO	Dissolved Oxygen
E.coli	*Escherichia coli*
EG2	Eosinophil Granules 2
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
Fc	Fragment Crystallizable Region
FR	Framework Regions
GalNAc	N-acetylgalactosamine
GalT	Galactosyltransferase
GlcNAc	N-Acetylglycosamine
GnT2	GlcNAc transferase II
GnT3	N-Acetylglucosaminyltransferase III GU
Glucose units	
GVs	Granulosis viruses
H	Heavy chain
HBM	*Honeybee* melittin
hCSAT	Human CMP sialic acid transporter
HPLC	High-performance liquid chromatography IMAC
Immobilized metal ion affinity chromatography Ig	
Immunoglobulin	
L	Light chain
mAbs	Monoclonal Antibodies MOI
Multiplicity of Infection MSDS	
Material safety data sheet NK	
Natural killer	
NPVs	Nuclear polyhedrosis viruses
NP-HPLC	Normal Phase High-performance liquid chromatography
OSTase	Oligosaccharide Transferase
PBS	Phosphate Buffer Saline
PNGase F	Peptide-N-Glycosidase F

PPE	Personal protective equipment
Polh	Polyhedron
PTM	Post-translational modification
RB	Russell bodies
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9	*Spodoptera frugiperda*
SRP	Signal Recognition Particle
TEV	Tobacco Etch Virus
UPR	Unfolded Protein Response
VHH	Variable domain of the heavy immunoglobulin chain
VR	Variable region
WHMIS	Workplace hazardous materials information system

Chapter 1: Introduction

The use of monoclonal antibodies (mAbs) for therapeutic applications against cancer and autoimmune disorders has widely developed in the past 50 years. Whereas the antigen binding functionality of antibodies is not affected by post-translational modifications (PTMs), the overall effectiveness of a mAb as a therapeutic does indeed hinge on key PTMs. Of these PTMs, glycosylation plays a major role. Glycosylation is traditionally an *in vivo* process that results in the addition and modification of sugar moieties on a protein molecule. A specific type of glycosylation, N-glycosylation has shown to be vital for the Fc-receptor mediated effector function of antibodies[1].

Although all mAbs produced in culture will have the same specificity for a certain epitope to which they can bind, these mAbs are not truly identical to each other. This is because the process of glycosylation is somewhat stochastic in nature and results in glycoform variants – mAbs with different glycan structures attached to them. This means that in a collection of antibodies produced in culture, a number of different populations can exist, some having greater potency than others in terms of therapeutic effects. To make a more effective therapeutic, a number of initiatives have been undertaken to create processes that can produce single-type glycoforms.

Of all the means possible to alter glycoforms, it is thought that *in vitro* remodeling may be the easiest way to achieve specific glycoforms of interest. And of the glycoform variants, glycoforms having a bisecting N-acetyl glucosamine, are thought to hold significant promise. Hodonickzky *et al.* have previously shown that commercial antibodies such as Rituxan and Herceptin can indeed be remodeled from a G0 state using rat N-acetylglucosaminyltransferase III (GnT3)[2]. A number of questions still remain regarding the feasibility of such a process. Can the process work with human GnT3 instead of rat GnT3? How accessible is GnT3 (rat or human)? Can

it be produced? Can it be produced in insect cells? What modifications to the GnT3 gene need to be made for it to be made for it to be made in insect cells? Can GnT3 work on a novel camelid heavy chain antibody?

Although the primary goal of this project was to produce an active GnT3 using the baculovirus expression vector system (BEVS) in Sf9 cell culture to remodel a camelid antibody (EG2), it became clear that understanding the substrate, the EG2 antibody, and its initial glycopattern was of importance. As such, this thesis explores the production of EG2 in CHO cells and the glycopattern of soluble secreted EG2, as well as cell-associated/non-secreted EG2.

In the second chapter of this thesis, a detailed literature review is given on the protein production in Chinese Hamster Ovary cells, and why these cells remain the primary workhorse for therapeutic production in industry. This is followed by a detailed look at antibody production in CHO cells and notable problems with secretion and aggregation of antibodies. The second half of the literature review is dedicated to the baculovirus expression vector system, which serves as the platform for the production of the in-house GnT3 used in this work. The baculovirus expression vector system (BEVS) consists of a baculovirus vector and an insect cell host; however, for the creation of the baculovirus there is a need for genetic manipulation of plasmid DNA, which requires the use of a lab strain of *Escherichia coli.* For this reason, there is a section of the literature review dedicated to lab strains of *E.coli.* A portion of the literature review on BEVS also focuses on the limitations of insect cells in terms of their glycosylation ability and some of the current genetic improvements that have been made to improve the system.

Following the literature review, Chapter 3 describes the materials and methods used in this work. Specifically, this chapter details the production and purification of EG2 from CHO cells

cultured in shake flasks; the production and purification of GnT3 in a 3L bioreactor; and finally the enzymatic transformation of EG2 using GnT3 and UDP-GlcNAc.

Chapter 4 describes the results of the production of EG2. Eosinophil granule monoclonal antibody or also known as EG2 recognizes the epidermal growth factor receptor (EGFR) which is strongly expressed on specific cancer cells[48]. EG2 is a camelid antibody. Usually, camelid antibodies originates from the serum of camels, dromedaries and llamas. It is a unique type of antibody devoid of light chains. The structure is made of only heavy chains and the antibody only has one single domain: the variable domain of the heavy immunoglobulin chain (VHH). Camelid antibodies are known to have high thermostability, relatively high proteolytic resistance and high expression yields in various expression systems[49].

The EG2 clone is fused with a human constant region structure to make it less immunogenic and transfected into Chinese hamster ovary (CHO cells)[50]. There are various mammalian cell lines available but CHO cells are often used due to its advantages. CHO cells are the traditional host expression system for the pharmaceutical industry since it has a low risk of transmission of human viruses since viral entry genes are not expressed in the cells. Also, it has the ability to withstand broad changes in pH, temperature, oxygen level and pressure[50, 51]. Most importantly, CHO cells are able to modify therapeutic glycoproteins with human-like glycans through post-translational modifications, thus enabling these therapeutic antibodies to be more stable and compatible for treatment[50, 51]. The degree of glycosylation of EG2 that is found in the CHO cells are mostly the sialic acid containing glycans and glycans with glucose moieties attached to mannose-1,3 and -1,6 alpha branch.

This chapter also describes the discovery of so-called Russell Bodies in the CHO cells studied in this work. The monoclonal antibody, EG2, is produced and secreted.

Most proteins in eukaryotes undergo post-translational modifications (PTM) that influence the structure and function of proteins. Glycosylation takes place within the endoplasmic reticulum and Golgi apparatus of cells and is known to be cell specific[54]. Monoclonal antibodies contain an N-glycosylation site on the Fc fragment on both heavy chains. The glycosylation profile of an antibody can impact its bioactivity, clinical efficacy, pharmacokinetics, safety and antigenicity[55]. All secreted proteins must undergo a complex pathway before secretion from the cells. Poor protein secretion can occur due to improper processing and assembling of the protein[56].

Aggregation of misfolded proteins is likely to occur when the expression of protein is much greater than the breakdown of misfolded proteins. Protein aggregation is a problem for therapeutic antibodies because it reduces their effectiveness, and may affect the quality and safety of the product. Aggregation of antibodies can occur at any of the steps throughout production including: cell culture, purification, formulation and storage.[57] Antibody aggregation during cell culture likely affects secretion of antibody and impacts the quantity of antibody produced[58]. Molecular chaperones and folding catalysts are involved in ensuring proteins are properly folded[59]. Stress in the endoplasmic reticulum (ER) may impact the performance of these catalysts or chaperones and result in misfolded proteins leading to aggregation. Misfolding may also be due to over accumulation of polypeptides in the ER, called unfolded protein response (UPR) [60].

Visual inspection of the cells showed signs of aggregation of proteins that are suspected to be EG2. Further analysis is discussed in detail in this chapter.

Chapter 5 describes the production and purification of GnT3. GnT3 catalyzes the addition of N-acetylglucosamine (GlcNAc) to the core mannose that will produce a bisecting GlcNAc. This enzyme is produced in insect cells, specifically *Sf9* cells, which are more cost effective. Once the enzyme was produced, it was purified using a His-tag high affinity column. Once the activity of

the enzyme was verified, the active GnT3 was used to catalyze the *in vitro* modification of EG2 antibody.

In order for the GnT3 to be properly secreted, the construct was designed to contain a HBM tag which is a secretion tag. The activity of the GnT3 is measured using a non-radioactive assay. In looking at the activity of this enzyme, two factors involving the donor and acceptor substrates were investigated. The donor substrate that is used is UDP-GlcNAc while the acceptor substrate is the EG2 monoclonal antibody. The assay involves the use of a coupling phosphatase which liberates a free phosphate from nucleotides generated from the glycosyltransferase reaction. The free phosphate levels are assessed using a malachite green phosphate detection reagent. The concentration of released phosphate is directly proportional to the number of sugar molecules transferred. Other factors examined include reaction times, concentration of the acceptor substrate, and a comparison of different sources of GnT3.

Lastly, Chapter 6 discussed the problems and opportunities with the use of insect cell derived human GnT3 for the remodeling of antibodies, and presents what can be concluded from this work and identifies new questions that have arisen as a result of this work.

Chapter 2: Literature Review

2.0 Expression Systems

2.1 Mammalian Cells

2.1.1 Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells are the most widely used cell line for commercial protein production. Some of the most prominent qualities of this cell line include: the inability to propagate human pathogenic viruses, the ease in which foreign DNA is transferred into its genome and their rapid and robust growth[1]. CHO cells encompass a large number of very different cell lines. Industrial CHO lines include those having a dihydrofolate reductase (DHFR) gene mutation – a defect that facilitates the introduction of transgenes with a functional copy of DHFR, which then acts as a selectable marker[21].

The specific cell line used in this thesis is the CHO DUKX cell line, which contains a deletion of one DHFR allele and an inactivating mutation in the second allele[22]. A stable cell line is generated through the integration of exogenous DNA containing the gene of interest into a transcriptionally active site of host genome[22]. Due to cost, another way to develop a stable cell line is through the delivery of a plasmid DNA containing the gene of interest into host cells capable of exponential growth. The common biophysical methods or chemical agents that are used include: electroporation, cationic lipids, polycations, and calcium phosphate[22].

2.2 Antibodies

Immunoglobulins are glycoproteins that are produced by plasma cells in response to the presence of antigens. The two main functions of immunoglobulins (Ig) are antigen binding and effector function stimulation. The basic structure of an IgG monomer consists of one or more units, each containing four polypeptide chains: two identical heavy chains (H) and two identical light chains (L). Beyond consisting of light and heavy chains, antibodies can be described as having

antigen binding fragment and a crystallizable fragment. Antibodies can also be described by their variable and constant regions. Constant region is the part of the light or heavy chain of antibody with a free carboxyl group end. The constant region has similar amino acid sequence from one antibody to another. Whereas, variable region differs in all antibodies produced by a single B cell or B cell clone. The variable (V) regions are found at the terminal ends of the polypeptide chains and shows variation in amino acid composition compared to the constant (C) regions[1] **(Figure 2.1)**. Each L chain consists of one variable domain and one constant domain, whereas, the heavy chain consists of a variable domain and three constant domains. A bilaterally symmetric structure consisting of a combination of noncovalent interactions and covalent disulfide bonds hold the light and heavy chains together[7]. Another feature of an antibody is a paratope, it is a small region on the Fv region (variable domain) that recognizes an epitope on a specific antigen during the antigen binding mechanism.

Immunoglobulins can be digested with a proteolytic enzyme called papain, which cleaves the Ig at the hinge region on the H-H inter-chain disulfide bonds. The treatment results in three fragments of approximately identical sizes. Two of the fragments are called fragment antigen binding (Fab) since they contain the antigen binding sites of the antibody. The V_H and V_L regions create the combining site, portion of the antibody that makes a physical contact with corresponding antigen, of the antibody that allows the antibody to bind to a specific epitope. Different combinations of V_H and V_L result in a variation of antibodies that can bind to a specific epitope. The third fragment that is produced is the fragment crystallizable region (Fc region). The Fc region contains the remainder of the two heavy chains, where each heavy chain fragment is comprised of C_{H2} and C_{H3}. The effector function of IgGs is mediated by this Fc region by means of binding to complement proteins such as C1q and cell surface receptors (Fc receptors, Fc gamma RI, II and II)

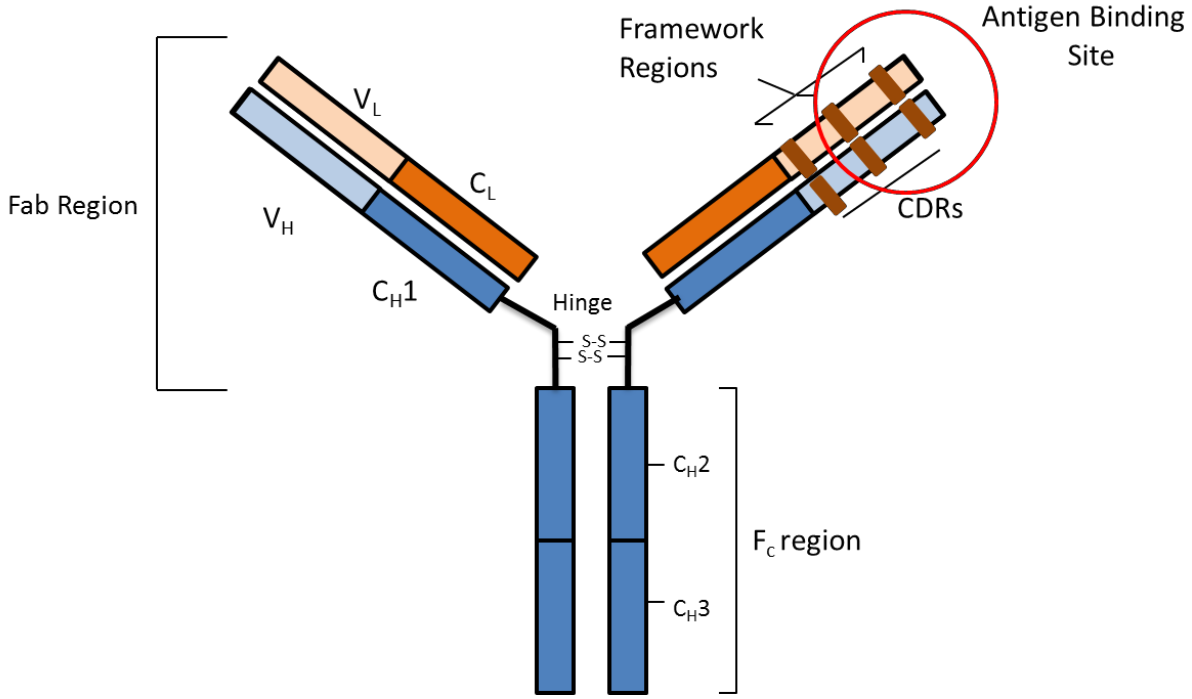


Figure 2.1: Schematic picture of an IgG antibody. Immunoglobulins consist of four polypeptide chains: two heavy chains, represented in blue, and two light chains, represented in orange. Each light and heavy chain consists of variable and constant regions that are represented by a lighter shade and a darker shade, respectively. The Complementarity determining regions (CDRs), which determine the protein's affinity and specificity for specific antigens, are represented by brown strips. Lastly, the antibody-binding site is circled in red.

There are five primary classes of immunoglobulins: IgG, IgM, IgA, IgD and IgE. These classes are differentiated by the molecular differences in their respective heavy chains. IgG is the most common Ig antibody present in humans and consists of gamma-chains that are used for secondary immune responses to an antigen[23]. IgG is the main antibody used in therapeutics and immunological research.

2.3 Polyclonal vs. Monoclonal Antibodies

To use antibodies for immunoassays, antibodies can be produced as either polyclonal or monoclonal antibodies. Polyclonal antibodies are produced by different B-lymphocytes in response to an antigen injected into a live animal. The heterogeneous mixture containing the polyclonal antibodies recognizes different epitopes on the same antigen **(Figure 2.2)** [23]. Polyclonal antibodies are purified from the serum of immunized animals such as mice, rabbits, goats or horses. The serum contains a diverse pool of antigen-binding immunoglobulins of several different classes and subclasses[23]. The main disadvantage of polyclonal antibodies production is that some antibodies generated are non-specific. In immunoassays, this lack of specificity causes a decrease in accuracy and an increase in background noise[24]. Furthermore, every batch of polyclonal antibodies produced can vary, resulting in problems of consistency[24].

In contrast, monoclonal antibodies (mAbs) bind to one specific epitope on an antigen. Monoclonal antibodies are purified from an individual B-lymphocyte providing a source of homogeneous antibody binding to a specific epitope on an antigen [25]. The major disadvantage of monoclonal antibodies is that the B-lymphocytes must be isolated from spleen or lymph node cells. B-lymphocytes, however, have short life spans and are not able to be cultured directly to produce useful amounts of antibodies[25.] Fortunately, the development of mouse hybridoma cells by Kohler and Milstein overcame these drawbacks[24, 25]. Ever since, the field of protein therapeutics has

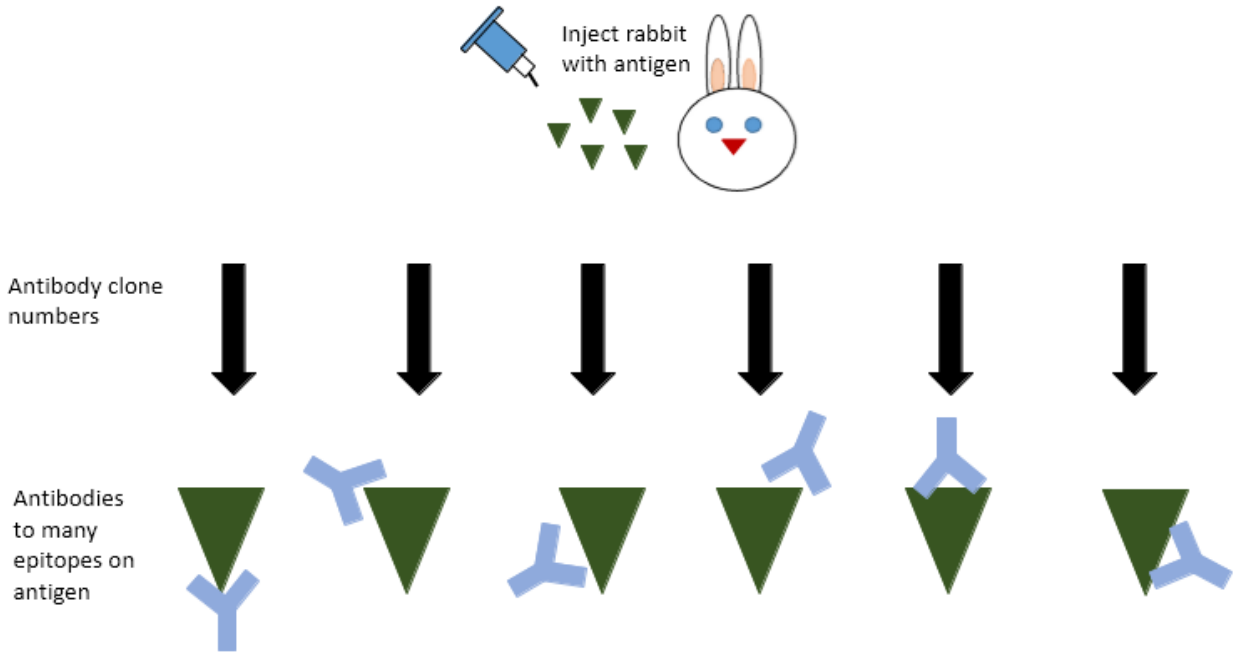


Figure 2.2: Production of Polyclonal Antibodies. The animal, a rabbit in this case, is injected with an antigen that will generate B-cell clones that produces antibodies with multiple epitopes[41]

expanded significantly, with the ability to produce antibodies in a variety of cell lines, including the most industrially used Chinese Hamster Ovary (CHO) cells.

2.3.2 Camelid Antibodies

Camelid antibodies are derived from Bactrian camels, dromedaries and llamas. They are different from a conventional antibody consisting only of heavy chains. The single N-terminal domain of camelid antibodies bind antigen without domain pairing, combination of heavy and light chains of antibodies[26]. Furthermore, these heavy-chain antibodies lack the C_H1 domain that interacts with the light chains. Camelid are unique due to their single N-terminal domain also known as VHH domain which are also referred to as a Nanobody. As shown on **Figure 2.3,** these heavy-chain antibodies do have VHH domains. The VHH domains contain four framework regions (FR) that form the core structure of the immunoglobulin domain and three complementarity-determining regions (CDR), which are important in antigen binding. An interesting feature of the VHH region is the presence of amino acid substitutions at four FR2 (positions 37, 44, 45 and 47) positions that are also conserved in conventional VH domains[26]. The advantages of a camelid single-domain antibody include their high solubility, high physiochemical stability, rapid tissue penetration and fast clearance making them appealing for therapeutic applications[26].

2.4 Beyond Antigen Binding

Beyond antigen binding, antibodies are recognized by the immune system. Binding of antibody to a specific target cell can induce apoptosis, agglutination and precipitation of the antigen-antibody complex. The precipitation is eliminated by phagocytosis or by immunogenic responses such as antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [23]. ADCC involves natural killer (NK) cells that have receptors on the surface to bind to the Fc portion of certain subclasses of IgG. Once the NK cells are bound to the Fc region, it releases perforins, proteolytic enzymes (granzymes) and chemokines that will lead to apoptosis of the targeted cell[8].

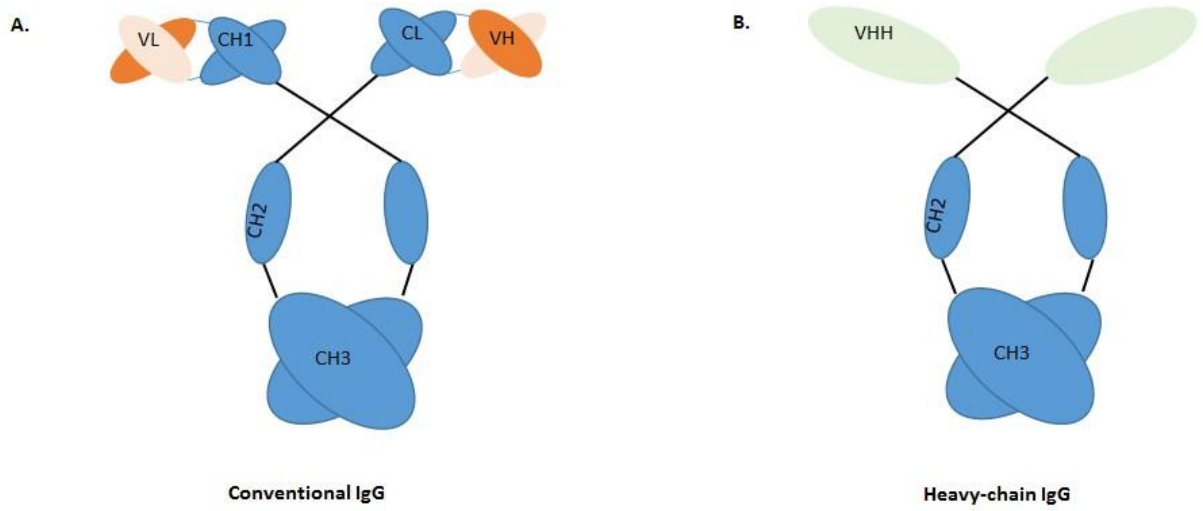


Figure 2.3: Schematic diagram of conventional (a) and heavychain (b) antibodies and fragments thereof. Variable domains derived from the antibody heavy (VH) and light (VL) chains are shaded dark gray and light gray, respectively, whereas constant domains (CH and CL) are not shaded. Note the absence of the light chain and CH1 domain in heavy-chain antibodies. Antibody domains that pair by noncovalent interactions are indicated by overlaying them. The VHH domains contain four framework regions (FR) that form the core structure of the immunoglobulin domain and three complementarity-determining regions (CDR), which are important in antigen binding.

On the other hand, CDC uses the complement cascade to mediate the lysis of cells by utilization of the C1q component of the complement cascade[2, 23].

Due to the importance of antibodies in the immune system, they are greatly used as alternative therapies to treat diseases. Monoclonal antibodies from murine hybridomas were once used as an initial step in treatment regiments to cure various diseases. For example when organ transplantation is needed, early hybridoma antibodies elicited severe immune responses that caused patients to reject their transplants. Therefore, surface molecules on cells crucial for allograft rejection was identified and mAb-based therapy was used in preventing the rejection. In 1986, Ortho Biotech introduced a chimeric antibody, called Muromonab-CD3 (Orthoclone OKT3), to inhibit transplant organ rejection. Muromonab-CD3 combined a human Fc region with a murine Fab fragments, retaining the antibody's specificity without triggering an adverse immune response. The development of chimeric antibodies significantly advanced success of antibodies as therapeutic agents. Antibody products such as Avastin, Herceptin, Humira, Rituxan and Xolar are all humanized antibodies and are currently amongst the leading therapeutics on the market against certain cancers and autoimmune diseases[25].

2.5 Baculovirus Expression System

Baculoviruses are a diverse group of occluded viruses found mostly in insects. This large family of occluded viruses are composed of two genera which are differentiated by the size of their occlusion bodies. Nuclear polyhedrosis viruses (NPVs) produce large polyhedron-shaped structures called polyhedral which contain many virions. In contrast, granulosis viruses (GVs) have smaller occlusion bodies called granules, which normally contain a single virion[3]. Common to both is the structure of the virion. They are usually 40-50 nm in diameter and 200 – 400 nm in length[3]. The virion is composed of a viral nucleocapsid: a 130 Kbp double-stranded circular DNA condensed into a nucleoprotein structure surrounded by the capsid.

As part of the Baculovirus Expression System these viruses are genetically modified to carry foreign genes that are introduced into a region of the viral genome that is nonessential for virus replication through homologous recombination using a transfer vector.

The Baculovirus Expression Vector (BEV) system is commonly used for production of proteins because of its various advantages. This system is capable of producing very high levels of recombinant proteins. When a virus infects insect cells, up to 50% of the total cellular protein that is produced can be the protein of interest[4]. In addition, the proteins that are produced using the BEV system are biologically active since they end up having post-translational modifications similar to those present in the native form of the protein[5].

The foreign genes are usually controlled by polyhedron and p10 promoters. The baculovirus that are commonly used for the expression of recombinant proteins are the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and the *Bombyx mori* nuclear polyhedrosis virus (BmNPV). Both viruses have genomes of approximately 130 kbp[6].

2.6 Insect Cells

The very first insect cell line was established from ovarian tissue of *Antheraea eucalypti*, which was grown in medium that contained silkworm (*Bombyx mori)* plasma [7]. The two most common insect cell lines are derived from the immature ovaries of *Spodoptera frugiperda* pupae (Sf9 and Sf21)[8].

2.6.1 Spodoptera frugiperda cells

2.6.1.1 IPLB-*SF*-21

The Sf21 cell line was cloned from pupal ovarian tissue of the Fall Army Worm *Spodoptera frugiperda*. This cell line was developed by Vaughn et al. with hemolymph-supplemented medium. The population doubling time of this cell line is approximately 26 to 30 hours[8]. The Sf21 cell line

supports replication of the *Autographa californica* nuclear polyhedrosis virus and the *S. frugiperda* nuclear polyhedrosis virus[8].

2.6.1.2 Sf9 Cells

As described by Smith and Summers in 1987[6], *Sf9* is a subclone of IPLB-SF-21. The IPLB-SF-21 cell line was observed to support the replication of the *Autographa californica* nuclear polyhedrosis virus[8]. *Sf9* consists of different key features like *Sf*21 such as the cells can grow either adherent or in suspension, and can be adapted to serum free medium. Compared to mammalian cells, a CO_2 incubator is not needed to grow these cultures since the media is buffered with phosphate rather than carbonate. Notably this cell line can also be grown in a carbonate buffer. Also, these cells have an optimal temperature of 27-28°C rather than 37°C[9].

2.6.1.3 *Trichoplusia ni* (High Five)

This cell line is derived from the eggs of the cabbage looper and was established in serum free medium. High five cells are susceptible to two distinct baculoviruses and are highly productive in serum-containing and serum-free medium compared to the other insect cell lines[10]. Compared to *Sf9* and *Sf21* cell lines, *Trichoplusia ni* which infected with vectors expressing the same genes (LacZ and SEAP gene) showed higher levels of both ß-galactosidase and secreted alkaline phosphatase per cell[11].

2.6.1.4 *SfSWT6* Cells

One of the major limitations for production of proteins in insect cells is the production of therapeutic proteins lacking complex type N-glycans, which in turn reduces their efficacy[12]. Specifically, the N-glycans found on insect cell produced proteins are mainly of a high mannose type or non-fucosylated and core-fucosylated tri-mannose structures[13] **(Figure 2.4)**. To overcome these limitations, the *N*-glycan processing pathway in insect cells has been extended through the co-infection of *Sf9* cells with recombinant baculoviruses that encode mammalian

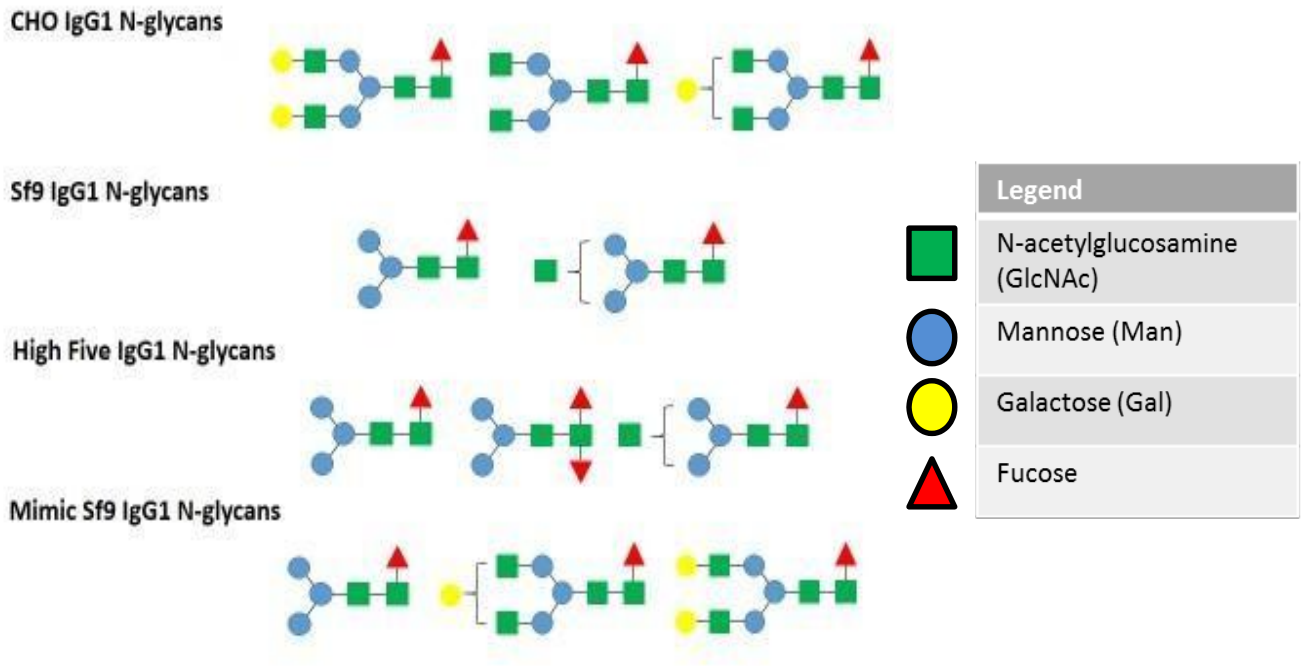


Figure 2.4: Comparison of IgG1 N-glycan structures expressed in CHO, Sf9, "High Five" and Mimic Sf9 cell lines. There are different N-glycan structures that are present in the different cell lines. It was observed that more complex N-glycan structures are found in CHO cells compared to the other cell lines. (a) The CHO population majorly consists of fucosylated biantennary N-glycan structures with two terminal N-acetylglucosamine residues. (b) Whereas, the IgG1 purified from Sf9 cells primarily consists of smaller biantennary structures which has no terminal galactose residues. (c) Comparable to the structures found in Sf9 IgG1, "High Five" cells carries two fucose residues. (d) The structures identified in analysis of N-glycans from IgG1 expressed in Mimic Sf9 cells, closely resemble the ones found in IgG1 expressed in CHO cells. Nevertheless, the amount of galactosylated IgG1 in Mimic Sf9 cells is considerably lower than in CHO cells.

glycosyltransferase genes, enabling the production of proteins carrying glycans that are structurally identical that can complex *N*-glycans produced by mammalian cells[14].

SweetBac has the capability for N-linked glycosylation modifications. It was constructed using a glycol-module that consists of open reading frames coding for *C. elegans* GnTII and a bovine GalT controlled by p10 and polyhedron (polh) promoter, respectively, which was integrated into the loxP site of a MultiBac genome[12] **(Figure 2.5)**. The functionality of this system was tested through the introduction of heavy and light chain open reading frames of the human HIV anti-gp41 antibody 3D6[12].

Sf9 and High Five cells lack cytosine-5'-monophospho (CMP)-sialic acid which is known to be required as a donor substrate for glycoprotein sialylation. It was discovered that these insect cells has an endogenous sialic acid salvaging pathway which produces and imports CMP-sialic acid that is utilized by mammalian sialytransferases which supports recombinant glycoprotein sialylation[15]. Therefore, in developing genetically modified insect cell lines to express mammalian proteins, *Spodoptera frugiperda (Sf9)* was transformed with six mammalian genes, which resulted in the generation of the SfSWT-4 cell line that is able to produce sialylated glycoproteins when cultured with the sialic acid precursor. SfSWT-6 was obtained by SfSWT-4 transforms with a human CMP- sialic acid transporter (hCSAT) gene. The new daughter cell line showed higher levels of cell surface sialylation and recombinant glycoprotein sialylation[15].

2.7 Bacteria

Escherichia coli (E .coli) cells are gram-negative, anaerobic, rod-shaped bacteria that are commonly found in the lower intestine of warm-blooded organisms. This bacterium is the most widely studied prokaryotic model organism and serves as the host organism for the majority of the work performed using recombinant DNA. This is due to *E. coli*'s ability to be grown easily under

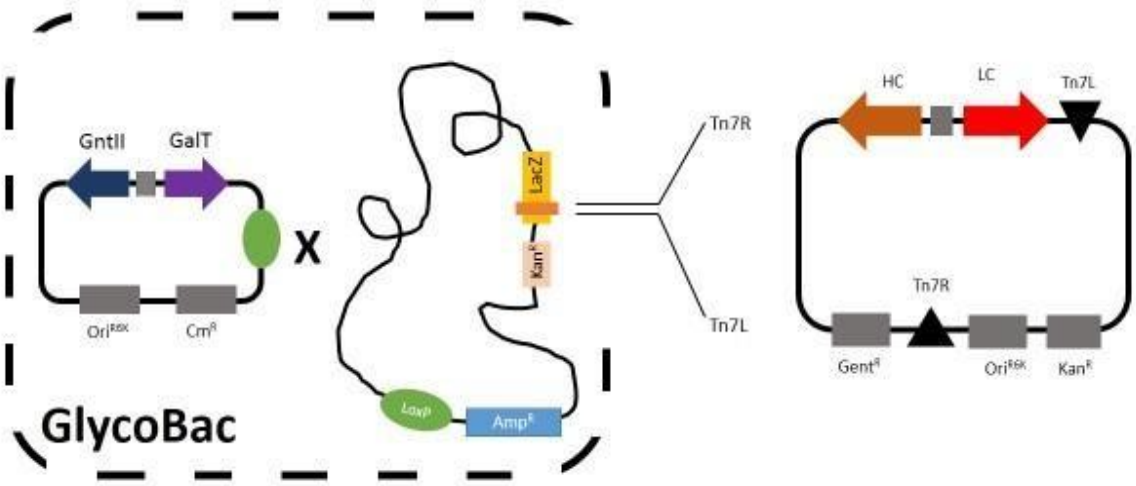


Figure 2.5: Schematic representation of SweetBac. A glycomodule that consists of the open reading frames of *C. elegans N*-acetylglucosaminyltransferase II (GnTII) and bovine B4- galactosyl-transferase I (GalT) was integrated in the loxP site of the MultiBac genome. The generated viral backbone was used for the expression of 3D6 antibody heavy (HC) and light (LC) chain genes by integration in the standard Tn7 site. The vector which consists the HC and LC genes, consists of different features such as kanamycin resistant gene and ori site. Whereas, the MultiBac genome has double antibody selection gene which are Ampicillin and Kanamycin. This will ensure of the expression of only the protein of interest. Adapted from: Palmberger, D. *et.al.* (2012). SweetBac: A New Approach for the production of mammalian glycoproteins in insect cells. *PLoS ONE,* **7(8).**

conditions where it takes approximately 20 minutes to reproduce. *E .coli* has various strains and the specific strain that was used in this work was DH10Bac.

2.7.1 DH10Bac

The DH10Bac is an *E .coli* strain which was engineered to generate baculovirus vectors in insect cells. This cell line contains a parent bacmid, bMON14272, and a helper plasmid, pMON7124. The parent bacmid contains the mini-F replicon, the kanamycin resistance gene, the attTn7 site and the lacZα complementation factor. The helper plasmid contains the *tns*ABCD regions that supplies the transposition proteins required for insertion of the mini-Tn7 from the donor plasmid, which contains the gene of interest into its target site on the parent bacmid. The shuttle vectors are constructed by the transposition of the mini-Tn7 from the donor plasmid to the attachment site on the parent shuttle vector in *trans* by the helper plasmid. The mini-Tn7 consists of the expression cassette that has a gentamicin resistance gene, a baculovirus promoter, a foreign gene and an SV40 poly (A) signal inserted between the left and right arms of Tn7. This cell line is resistant to the effects of ligase and ligase buffer allowing it to tolerate the addition of small amounts of undiluted ligation reactions. Furthermore, its genotype consists of φ80dlacZDM15, which provides an alpha- complementation of the B-galactosidase gene from the bacmid vector.

2.8 Glycosylation

The majority of proteins produced in eukaryotes undergo some type of post-translational modification (PTM) that influences the structure and function of the specific protein[27]. One of the most common PTM is glycosylation. Glycosylation takes place in the endoplasmic reticulum (ER) and the Golgi apparatus. It is known to be species- and cell-specific therefore the glycosylation of each protein is dependent on the cell or tissue in which it is produced[27]. Glycosylation is differentiated into two groups: N-linked and O-linked, depending on the glycan-peptide linkage regions[28] **(Figure 2.6).**

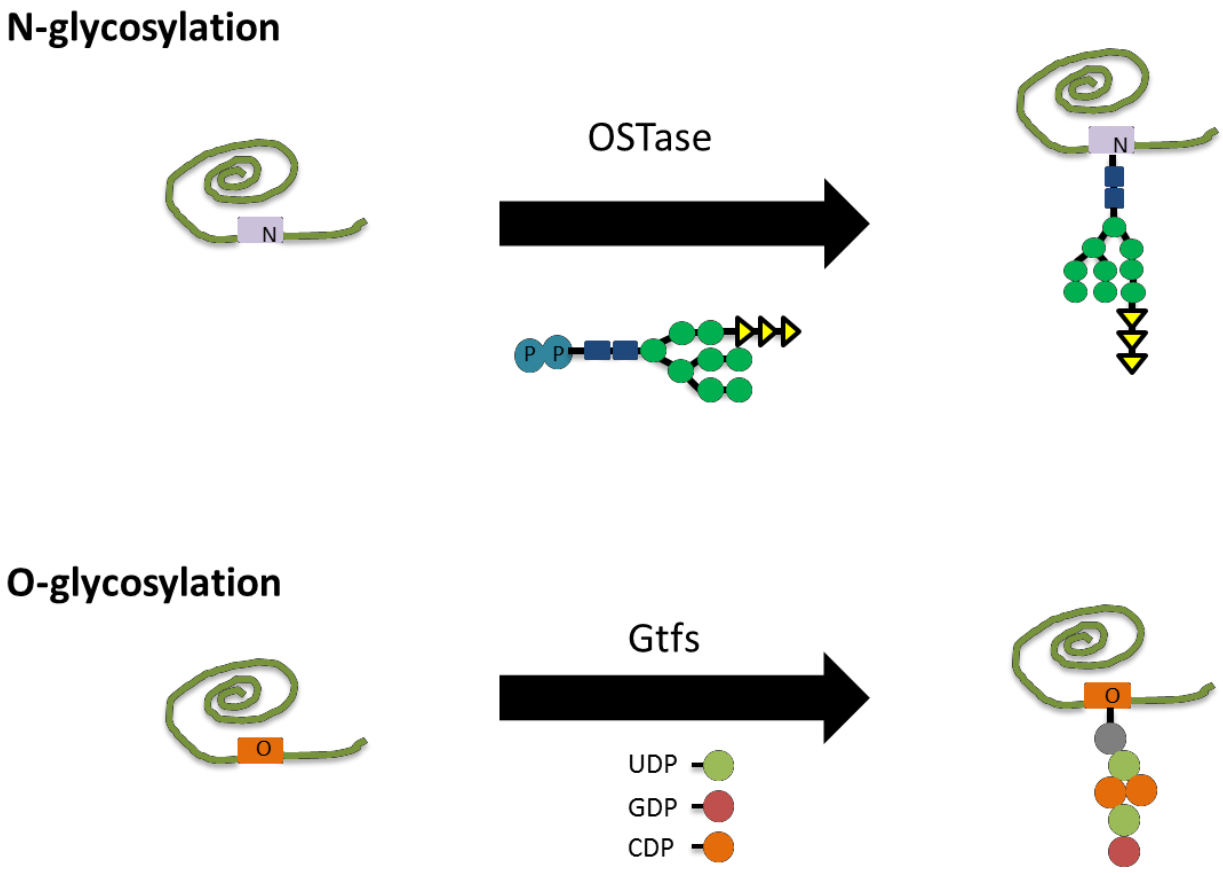


Figure 2.6: Various Glycosidic Linkages. N-linked glycosylation is a glycan that binds to the amino group of asparagine in the ER. While O-linked glycosylation are monosaccharides which bind to the hydroxyl group of serine or threonine in the ER, Golgi, Cytosol and nucleus. Reference: ThermoScientific (2013). Protein Glycosylation. Adapted from: Rudd P. M. and Dwek R. A. (1997) Glycosylation: Heterogeneity and the 3D structure of proteins. Crit Rev Biochem Mol Biol. 32, 1-100.

N-glycans are linked to asparagine (Asn) whereas O-glycans are linked to serine (Ser) or threonine (Thr) residues. More specifically, N-glycans have a reducing terminal N-acetylglucosamine attached to the amide group of Asn through an aspartylglycosylamine linkage[29]. All N-glycans are produced through the same common pathway, which starts in the ER with the transfer of a preformed oligosaccharide to an Asn residue. The Asn residue is typically found as part of an Asn-X-Ser/Thr sequence, where X is any amino acid except for proline[30]. There are three classes of N-glycans: High-mannose, Hybrid and Complex. High-mannose N-glycans contain several highly branched mannose residues on both branches of the glycans forming a simple structure **(Figure 2.7)**. Hybrid glycans consist of a single complex high-mannose branch. Complex glycans are the most frequent glycans found in mammalians and can be bi-, tri- or tetra-antennary. The structure of the complex type glycan can have a bisecting N-acetylglucosamine (GlcNAc), a core-fucose or terminal sialyation[28]. In contrast, O-glycans are extremely diverse in nature. For O-glycans, N-acetylgalactosamine (GalNAc) is attached to the hydroxyl groups of Ser or Thr residues within the polypeptide[31]

2.9 N-glycosylation

N-glycosylation occurs co-translationally where the glycan is attached to the forming protein as it goes through the ER pathway[29]. This type of glycosylation starts by the assembly of a precursor glycan, then the attachment of the precursor glycan to the protein, followed by trimming and maturation of the glycan as it passes through the various organelles containing the necessary enzymes for the process[32]. An enzyme called oligosaccharide transferase (OSTase) then scans the nascent polypeptide for the conserved sequence Asn-X-Ser/Thr and attaches the precursor glycan at that site[30.]

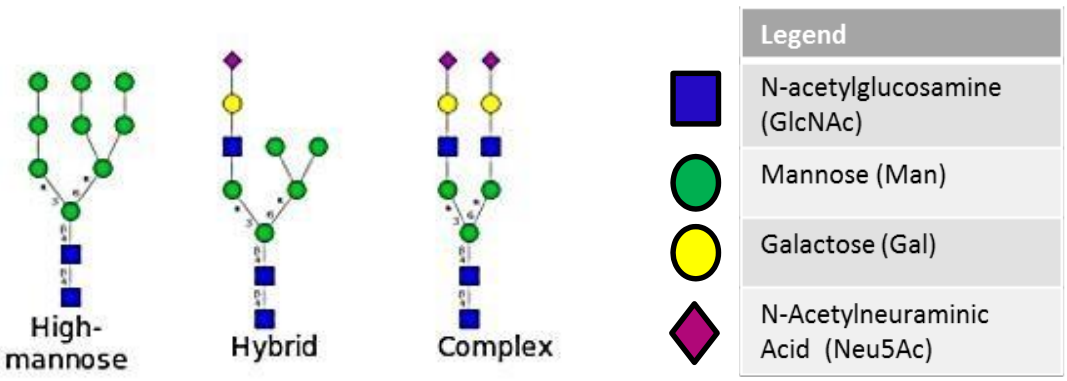


Figure 2.7: Three major type of N-glycans. High mannose glycans contain unsubstituted terminal mannose sugars which typically contain between five and nine mannose residues attached to the GlcNAc core. Whereas, Hybrid glycans contains both unsubstituted terminal mannose residues and substituted mannose residues with an N-acetylglucosamine linkage. Lastly, Complex has GlcNAc residues at both the alpha-3 and alpha-6 mannose sites. Made using Glycowork Bench 2.

2.10 Glycosyltransferases

Glycosyltransferases are enzymes involved in glycan biosynthesis; they typically add one monosaccharide at a time to specific positions on specific glycan precursors[31]. These enzymes found within the ER-Golgi pathway catalyze reactions involving the transfer of monosaccharide components from high-energy nucleotide sugar donors to the glycan[3]. A specific glycosyltranferase that modifies glycans includes the B-1,4-galactosyltransferase 1, an enzyme found in the golgi that is responsible for the galactosylation of both glycoproteins and glycolipids. Another enzyme is *N*-acetylglycosaminyltransferase I (GnT I), also found in the Golgi that transfers GlcNAc residue to oligomannose core structures and results in hybrid and complex *N*- linked oligosaccharides[33]. Gnt- V catalyzes the attachment of a B1-6 GlcNAc residue which results in branched N-glycans[34]. Subsequently, *N*-linked oligosaccharides can be further modified by *N*-acetylglucosaminyltransferase III.

2.10.1 N-acetylglucosaminyltransferase III (GnT3) and bisecting GlcNAc

β-1,4 *N*-acetylglucosaminyltransferase III (GnT3) catalyzes the addition of *N*-acetylglucosamine in β-1-4 linkage to the *B*-linked mannose in the core regions of *N*-glycans to form a bisecting GlcNAc **(Figure 2.8)**[35]. GnT3 is a type II membrane glycoprotein composed of 536 amino acids and its size is about 60 kDa[35]. The domain structure is composed of a short amino terminal cytoplasmic tail, transmembrane and neck regions and a long carboxyl terminal catalytic domain projecting into the Golgi lumen **(Figure 2.9)**[36]. The catalytic domain has three potential N-glycosylation sites at Asn243, Asn261 and Asn399[36]. Previous research has suggested that the activity of GnT3 is regulated by post-translational modification such as phosphorylation and glycosylation[36]. The importance of these post-translational modifications have been investigated by inhibiting glycosylation and phosphorylation modification of GnT3.

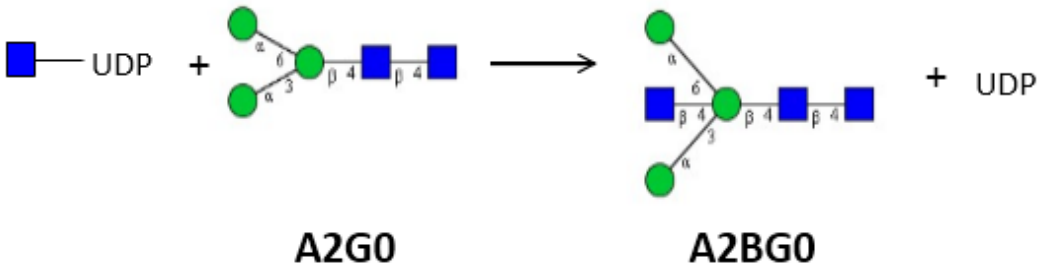


Figure 2.8: Addition of bisecting GlcNAc catalyzed by GnT3. The glycan structures are shown in CFG nomenclature. Each symbol represents a certain moiety. The blue square represents Glucose, green circle represents Mannose, yellow circle represents Galactose and red triangle represents Fructose. The structures were generated using GlycoWorkBench.

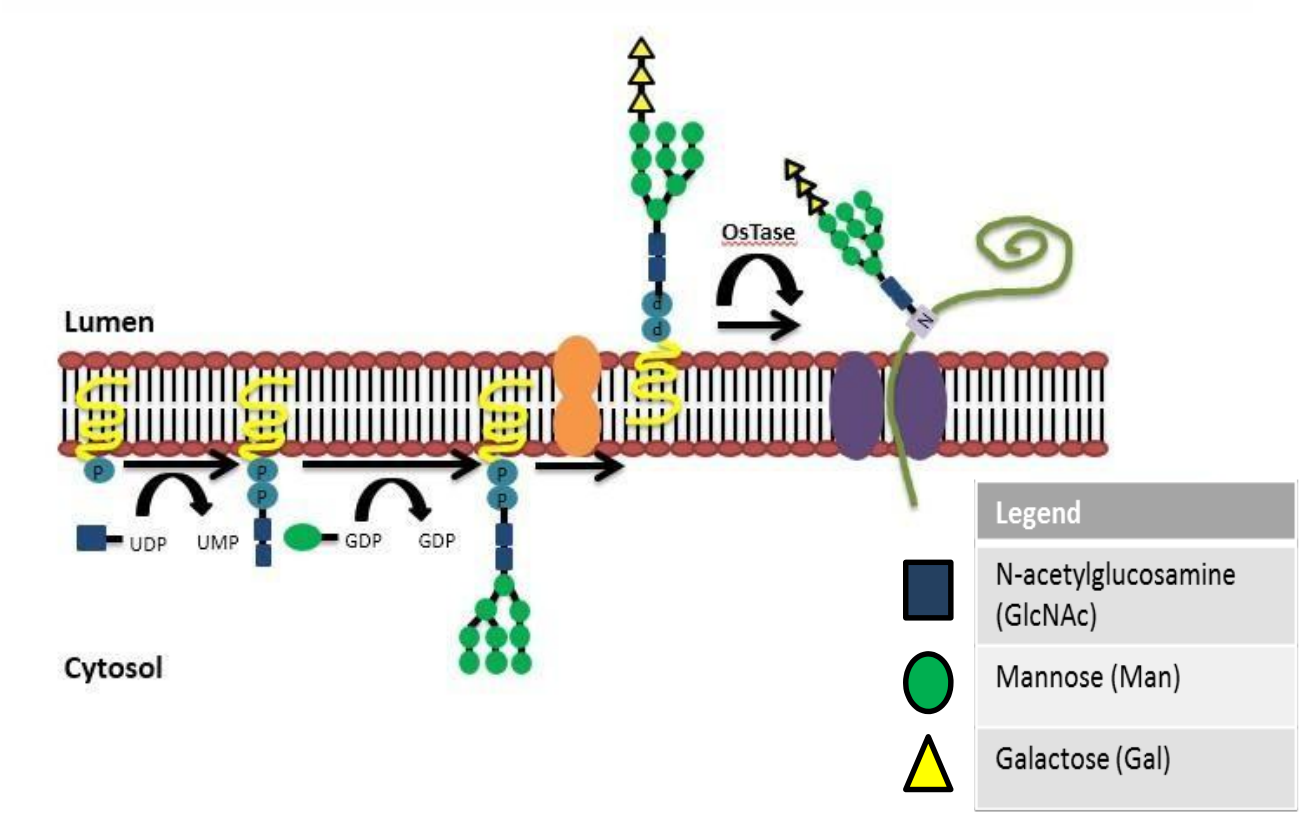


Figure 2.9: Glycan Assembly and Attachment. The precursor glycan synthesis begins on the cytosol of the Endoplasmic Reticulum and is flipped into the ER lumen. OSTase transfers the precursor glycan to the Asn residue on the nascent protein.

Tunicamycin which blocks the core glycosylation and prevents for the initial step of N-glycan processing has been used[36]. GnT3 catalyzes the transfer of a GlcNAc from UDP-GlcNAc to the core β-mannosyl moiety of an N-glycan acceptor[37]. The addition of the GlcNAc creates a β1-4 linkage between the core-mannosyl, which results in a bisecting GlcNac[31] **(Figure 2.8).** The reaction catalyzed by GnT3 is inhibited by β1-4-galactosylation of the Man-α1-3 branch. α1-6 core-fucose has no effect on GnT3. Interestingly, the presence of a bisecting GlcNAc was seen to increase the activity of adenylyl cyclase III, which plays a role in Alzheimer's disease[38]. Specifically, it also has an effect on integrin mediated cell adhesion and cell signaling.

2.10.2 Background on GnT3 and its activity

As formerly studied, post-translational modification is an important factor for proteins to properly function or secreted to the cytosol. In mammalian cells, there are a wide variety structures of the oligosaccharides of glycoproteins and glycoproteins. Due to the findings that these structures change during the embryogenesis and cell differentiation shows that the surface oligosaccharides play a specific role in cell-cell interaction[36,41.] Therefore, studies on glycosyltransferases are vital since it controls the synthesis of different types of oligosaccharides. The specific glycosyltransferase that is studied is the *N*-acetylglucosaminyltransferase III (GnT3) which acts on the production of a bisecting GlcNAc residue in the *B*-linked mannose of the trimannosyl core of the oligosaccharides. The first GnT3 activity that was observed was in hen oviduct and further studies was done and extracted from rat kidney and human B lymphocytes[36, 41]. Therefore, previous studies on GnT3 was done where it was sequenced and each domain was identified.

In this specific study, the human GnT3 will be looked at. Taniguchi *et. al* have compared the predicted amino acid and nucleotide sequences for both rat and human Gnt3. When it was compared, it was shown that there is 86% homology at the nucleotide coding sequence level, 50% at the nucleotide 3' noncoding sequence level, and 91% at the amino acid sequence level. Both

amino-terminal transmembrane domain and the catalytic domain are well conserved in the two species. The only difference that was identified is that the human GnT3 is a few amino acid shorter compared to rat GnT3 since it has four amino acid deletion and difference in the COOH terminal[34].

2.11 Protein Secretion and Aggregation

Protein trafficking in eukaryotes involves the compartmentalization that includes elaborate mechanisms in targeting nascent proteins to the correct compartment[61]. Typically each protein has a code within their sequences that are scanned in each stage of their trip to the specific target. Depending on the destination of each protein, it is synthesized in a different compartment. Such as, proteins that are targeted to the cytosol, mitochondria, peroxisomes or chloroplasts are synthesized on free ribosomes whereas proteins that are targeted for secretion, ER, Golgi or lysosomes are synthesized on the membrane bound ribosomes of the rough ER.

Secretory proteins are directed to the ER by a signal sequence that is characteristically a stretch of 9 or more hydrophobic residues. This signal sequence is recognized by the signal recognition particle (SRP) which initiates the assembly of Sec61 complex. The Sec61 complex acts as the translocator to allow passage of the polypeptide chain across the bilayer[62]. The ER signal sequence is cleaved off by a signal peptidase except for the signal sequences that are found within the polypeptide instead of the N-terminus which are not cleaved. The orientation of the polypeptide depends on the charge distribution on either side of the signal sequence[62]. The folding of the protein is aided by chaperones and protein disulphide isomerase within the ER lumen[63].

Most proteins in eukaryotes undergo post-translational modifications (PTM) that influences the structure and function of the specific protein (25T). One of the common PTM is glycosylation which take place within the endoplasmic reticulum and Golgi apparatus of cells which is species and cell specific[27]. The more common type of glycosylation is N-linked glycosylation which

involves the attachment of precursor oligosaccharide to asparagine residues in the ER. The precursor is transferred from a dolichol lipid moiety in the ER membrane by oligosaccharyl transferase. Immediately after the transfer, all three glucose residues and one mannose residue are removed. The precursor is further modified in the Golgi to produce the mature glycoprotein by trimming certain sugars and addition of others[63].

Improper processing and assembling of the protein leads to poor protein secretion[56]. Thus, misfolded proteins can be a cause of protein aggregation. Typically, protein aggregation involves the formation of insoluble precipitates due to the protein concentrations exceeding the solubility limit. There are different types of protein aggregation that are identified as either ordered or disordered[64]. For example, amyloid fibrils which are found both in *in vivo* and *in vitro* are considered as ordered aggregates whereas inclusion bodies are considered to be *in vivo* disordered aggregates. In particular, protein aggregation was always assumed to form due to misfolding of protein. The formation of inclusion bodies are believed to arise from hydrophobic aggregation of unfolded or denatured states. Inclusion bodies are common when proteins are overexpressed[65].

The process of aggregation starts off with the intermolecular interaction of the hydrophobic surfaces on the structural subunit of one molecular to a matching hydrophobic surface area of a neighboring molecule. Initially, when dimer and tetramer aggregates are formed, it remains soluble until it reaches a size which exceed the solubility limit. Aggregation often appears to be irreversible due to the slow rates of disaggregation[65].

Specific intercellular protein aggregates are called the Russell bodies (RB), condensed immunoglobulins are contained in the endoplasmic reticulum cisternae. They are one example of protein aggregation inside cells. Aggregation of misfolded proteins is likely to occur when the expression of protein is much greater than the breakdown of misfolded protein[66]. Protein aggregation is a problem for therapeutic antibodies because it reduces their effectiveness, and may

affect the quality and safety of the product. Aggregation of antibodies can occur at any of the steps throughout production including: cell culture, purification, formulation and storage. Antibody aggregation during cell culture likely affects secretion of antibody and impacts the quantity of antibody produced. Newly formed polypeptide chains must be properly folded[59, 60]. Stress in the endoplasmic reticulum (ER) may impact the performance of these catalysts or chaperones and result in misfolded proteins leading to aggregation[59] or misfolding due to over accumulation of polypeptides in the ER, called unfolded protein response (UPR) [60].

2.12 Previous Work

Post-translational modifications can confer various properties to proteins. There's a variety of modifications that can be done to the glycans such as sialylation, fucosylation, sulfation, galactosylation and addition or removal of GalNAc. Most work that was done in modifying glycans has been *in vivo*. Weikert *et al.* engineered CHO cells by the overexpression of human B-1,4-galactosyltransferase and alpha-2,3-sialyltransferase[39]. *In vitro* processing has greater advantage due to its ability of having controlled conditions for glycan modification. Therefore, *in vitro* modification has been used to improve glycans for better delivery or enhancement of its therapeutic function. In 2001 Raju *et al.,* performed *in vitro* galactosylation and sialyation of glycoproteins. Hence as described in this paper, modification using GnT3 causes for the increase of ADCC[2].

Taniguchi *et al.* have done a great deal of research on the enzymatic properties and biological functions of GnT3[41]. Human GnT3 has been isolated and characterized using a human fetal cDNA library and has been shown that its mRNA had a size of approximately 4.7 kb. Human GnT3 contains 531 amino acid. Its typical domain structures include a short amino terminal cytoplasmic tail, a transmembrane region, and a COOH-terminal catalytic domain[41].

Studies have shown that high mannose type oligosaccharides are not an active substrate and that B-1,2-GlcNAc linked to alpha-1,3-mannose is required for GnT3 activity[36]. Based on the kcat/Km values, GnT3 specificity for UDP-GlcNAc is 2000 times higher than other sugar moieties[36].

Hodoniczky *et. al*[2] have remodeled Fc *N*-glycans of Rituxan and Herceptin using a rat GnT3 produced in insect cells *in vitro*. *In vitro* remodeling of the Fc *N*-glycans of therapeutic mAb with the addition of a bisecting GlcNAc results in modulation of ADCC and not CDC. However, the use of galactosidase for the removal of β-1, 4-linked galactose residues can modulate CDC. In remodeling the *N*-glycan *in vitro*, they were able to control the selective transfer of a bisecting β-1,4-linked GlcNAc to the core β-linked mannosyl residue on the *N*-glycan. Furthermore this remodeled antibody had increased efficacy *in vivo*. A 10-fold increase in ADCC was achieved by the introduction of a bisecting GlcNAc to a pure G0 antibody pool[2].

Chapter 3: Materials and Methods

3.1 mAb Production

3.1.1 Flask Culture

Chinese Hamster Ovary[1A7] (CHO) cells were cultivated in BIOGRO-CHO serum-free culture medium (Biogro Technologies, Manitoba, Canada) at 37°C with a 5% CO_2 atmosphere, rotation speed of 120 rpm in a polycarbonate flask (Sigma-Aldrich, St. Louis, USA). At about 3.0 x 10^6 cells/mL at passage 8, the cells were harvested and centrifuged at 500 rcf for 10 minutes. The supernatant containing the EG2 mAb was collected.

3.1.2 Concentrating EG2 mAb

A 400 mL Amicon Stirred Cell (Millipore) having a 10 kDa MW cut-off membrane was used to concentrate the EG2 mAb from culture supernatant. The supernatant was concentrated 10x from its original concentration.

3.1.3 mAb Purification

All mAb samples were purified using a 1 mL HiTrap Protein A column (GE Healthcare, Mississauga, Canada) prior to being remodeled. Briefly, the resin in the column was activated by the addition of the binding buffer (20 mM sodium phosphate, pH 7.0) and the samples were added onto the column and incubated for 4 minutes. Following a wash with binding buffer, the mAb was eluted with 0.6 mL of 50 mM glycine-HCl, pH 2.7. Eluted mAb was neutralized with 30 uL of 1M Tris-base pH 8.0 (10%v/v).

3.1.4 mAb Quantification

Monoclonal antibody was quantified by high performance liquid chromatography (HPLC). The particular that was used in the quantification of EG2 is POROS Protein A Affinity Columns (1 ml). The protein A column has a specific epitope on the Fc region of the antibody. The resins exhibit high speed and efficiency. The samples were run on the HPLC and different fractions will

be eluted. The detector that was used in the HPLC is UV light at absorbance 280 nm. The peak which is found at approximately 4 for the EG2 antibody at a specific retention time will be identified and the area under the curve will then be used to calculate the concentration of EG2.

3.1.5 Protein Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to characterize proteins produced in culture. The proteins were prepared by dilution 1:2 into the sample buffer (Bis-Tris 0.24 pH 6.5; SDS 2%; glycerol 20%, DTT 50 mM) and boiled for 10 minutes at 95°C. SDS-PAGE was performed in a discontinuous gel system with the following buffer composition: resolving gel: Tris-HCl 1.5 M pH 8.8 whereas stacking gel: Tris-HCl 0.5 M pH 8.8. It was run with running buffer (100 mM Tris, 0.01% SDS, and Glycine) at 150 V for an hour. The gels were stained and the bands were visualized using silver nitrate.

Immunoblots or commonly known as Western Blots were used as analytical techniques to detect specific proteins in a sample. Western blots were performed on the CHO supernatant, cell lysate and purified EG2 that were separated on 15% SDS-PAGE gels and transferred to Immun-blot PVDF Membrane (Bio-rad, Canada). Anti-Human IgG (Fc specific) goat HRP antisera (Sigma- Aldrich, USA) were diluted 1:100,000 with 5% wt/vol skim milk (Bio-Rad, Canada). The Immunoblots were developed by chemiluminescence using ECL (Amersham Biosciences).

3.1.6 Cell Lysis Procedure

CHO cell pellet were lysed using a detergent, Triton X-100 (Sigma-Aldrich, St. Louis, USA). Approximately 15 x 10^6 cells were collected were centrifuged at 1200 rpm for 5 minutes at 4°C to obtain a pellet. Subsequently, the pellet was washed with 1x PBS three times. The lysis buffer which consists of 1M Hepes pH 7.4, 5M NaCl, glycerol, Triton X-100, 0.5 M EGTA pH

8.0 were added to the sample and incubated on ice for 30 minutes. The cells were spun at 13,000 rcf for 15 minutes at 4°C. The supernatant which contains the proteins are collected and used for further analysis.

3.1.7 In vitro remodeling of EG2 N-glycans to produce G0 antibodies

Purified EG2 mAb underwent a buffer exchange into 50 mM sodium phosphate, pH 6.0 using centrifugal ultrafiltration (10 kDa cutoff, Millipore, Massachusetts, USA). The samples were reacted with *Streptococcus pneumonia ß*-1,4 galactosidase (Prozyme, California, USA) and sialidase (Prozyme, California, USA) at 37 °C for 24 h with 1:500 ratio for enzyme:EG2 sample.

3.2 GnT3 Production and Purification

Gene Synthesis

The GnT3 sequence **(Appendix)** was designed for gene synthesis that was ordered from Life Technologies (Ontario, Canada). The GnT3 gene sequence consists of BamHI and EcoRI restriction sites to allow for insertion of the gene into the pFastbac1 vector. It also contains TEV Cleavage Site to allow for the removal of the 6x His-tag **(Figure 3.1)**.

3.2.1 Culturing Sf9 cells

Spodoptera frugiperda Sf9 cells were grown in SF900 SFM III (Gibco, Life Technologies, Ontario, Canada) at a shaker incubator at 27 °C and with rotation speed of 120 rpm using glass flasks.

3.2.2 Generation of Recombinant Bacmid

The baculovirus stock was amplified twice where the multiplicity of infection (MOI) that was used was 0.5 for both P1 and P2. For the large-scale expression of the recombinant protein of interest, MOI of 3 was used.

3.2.3 Quantification of the Virus Titer

The plaquing medium also known as the Agarose overlay consists of 4% melted Agarose gel and Sf-900 SFM III Medium with 1:3 ratio. The plaque assay was performed in a 6-well plate with a total of 1 x 10^6 cells in each well. An 8-log serial dilution (10^{-1} to 10^{-8}) of clarified baculoviral stock in Sf-900 II SFM media was prepared and infected the adhered cells on each well. After an hour incubation, the viral stock was removed and replaced with the Agarose overlay. The plates are incubated at 27 °C in a humidified incubated for approximately 7 to 10 days.

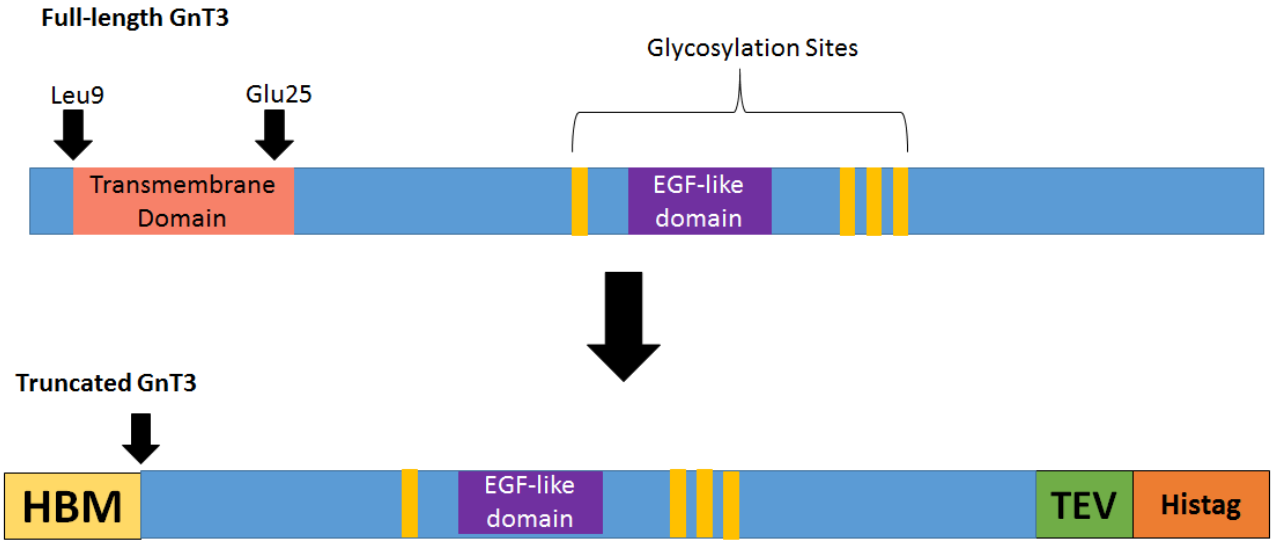


Figure 3.1: Schematic representation of the GnT3 construct. This shows the schematic version of the full-length GnT3 and its truncated version. The truncated version was added into a construct with various features such as the HBM tag, TEV cleavage site and His tag.

3.2.4 Production of Truncated GnT3

3.2.4.1 Flask Culture

The cells were seeded at 500,000 cells/mL and was grown to 2.0 x 10^6 cells/mL and infected at this point at MOI of 3. Samples were taken post infection at 24, 48 and 72 hours.

3.2.4.2 Bioreactor

The Applikon Bioreactor is a 3 L autoclavable stirred tank bioreactor was used to grow *Sf9* cells in SF900 Serum-free culture medium III. The bioreactor was operated at temperature of 27°C, DO of 55, and Stirring speed of 100 rpm. To prevent the clumping of the cells, 0.01% methylcellulose was added into the culture. When the *Sf9* cells have adapted to the bioreactor and seeded at density of 400,000 cells/mL. When the cell density reached 1.2 x 10^6 cells/mL, it was infected with the GnT3 generated baculovirus with MOI of 0.01. The Bioreactor ran for 4 days post infection and the cells were at 24 hours and 96 hours. The cells were harvested and centrifuged at 500 rcf for 10 minutes. The supernatant was collected since it contains the GnT3 protein.

3.2.5 Concentration of the GnT3 Protein

The supernatant that was collected from the bioreactor run was subjected through ultrafiltration using a 400 mL Ultrafiltration Stirred Cell with a 10 kDa cutoff filter (Millipore, Massachusetts, USA). Most of the supernatant samples were 10x concentrated after the ultrafiltration. Concentrated GnT3 samples were purified using a Histrap column (GE Healthcare, Ontario, Canada).

3.2.6 Quantification of GnT3 protein

The purity of the GnT3 was checked using SDS-PAGE stained with Silver Staining. Prior to use in assays, the concentration of the purified GnT3 was determined using the bicinchoninic

acid protein assay kit (Pierce, Rockford, USA) measuring at the absorbance of 280 nm calibrated with bovine serum albumin (BSA).

3.3 Protein Analysis

The Immunoblots in this experiment were performed on the GnT3 supernatant, GnT3 lysate and purified GnT3 that were separated on 12% SDS-PAGE gels and transferred to Immun-blot PVDF Membrane (Bio-Rad, Canada). The primary antibody that was used was a 6x-His Epitope Tag Antibody (Thermo-Scientific, Massachusetts, USA) that was diluted 1:10,000 with 5% wt/vol skim milk (Bio-Rad, Canada). The secondary antibody was used a F(ab') 2-Goat anti-Mouse IgG (H+L) Cross Adsorbed Secondary Antibody (Thermo-Scientific, Massachusetts, USA) that was diluted 1:100,000 with 5% wt/vol skim milk (Bio-rad, Ontario, Canada). The immunoblots were developed by chemiluminescence using ECL (Amersham Biosciences, Canada).

3.4 Glycosyltransferase Activity Assay

Activity of the N-acetylglucosaminyltransferase III (GnT3) was measured using a non-radioactive high throughput assay that utilizes nucleotidase ENTPD3/CD39L3 as a coupling phosphatase to remove inorganic phosphate from the leaving nucleotide disphosphate, UDP, generated during the glycosyltransferase reaction. The buffer that the purified EG2 samples are in were exchanged with 50 mM sodium phosphate, pH 6.0 using centrifugal ultrafiltration (10 kDa cutoff, Millipore,Billerica, USA) and the other half of the samples were exchanged with 25 mM MES Buffer, pH 6.7.

For each reaction, a phosphate standard curve was generated. The assay from R&D systems (Minneapolis, USA) was performed in a 96 well plate and each sample, control and standards were done in duplicates. Both commercialized and in-house GnT3 was used to measure the activity of each enzymes. The commercialized GnT3 was acquired from R&D

systems (Minneapolis, USA) which were derived from CHO cells. The controls that were used in this assay include both negative and positive controls. The first negative control consist of no GnT3, 1 mM UDP-GlcNac, 3 µg/mL EG2. The second negative control consists of 5 µg/mL GnT3, 1 mM UDP-GlcNac, and no EG2. Lastly, the third negative control consists of 5 µg/mL GnT3, 3 µg/mL IgG, and no UDP-GlcNac. Whereas, the positive control consists of 5 µg/mL GnT3, 1 mM UDP-GlcNac, 3 µg/mL IgG. A coupling phosphatase control was performed where it only contains buffer and UDP-GlcNac. The IgG that was used in the positive control was produced from CHO DP-12 and obtained from University of Manitoba. The reactions were incubated for various time points: 30 mins, 40 mins, 50 mins and 60 mins at 37°C and then malachite green reagent A, deionized water and malachite green reagent B were added. The 96-well plate is read at 620 nm at 25°C.

3.5 mAb Glycan Analysis

The EG2 antibody glycan structures were analyzed at the University of Manitoba. Briefly the process combine SDS-PAGE and high performance liquid chromatography (HPLC) analysis with the use of Exoglycosidase Array Digestions. The glycoproteins were initially visualized using SDS-PAGE stained with Coomassie blue. The glycoproteins were treated with Peptide-N- Glycosidase F (PNGase F), an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. Once released, the glycans were labelled with the fluorophore 2-aminobenzamide (2-AB) and run on normal phase HPLC (NP-HPLC). The elution times of glycans are expressed in glucose units (GU) by reference to a dextran ladder.

Chapter 4: Production of EG2 camelid antibody from DUKX CHO cells

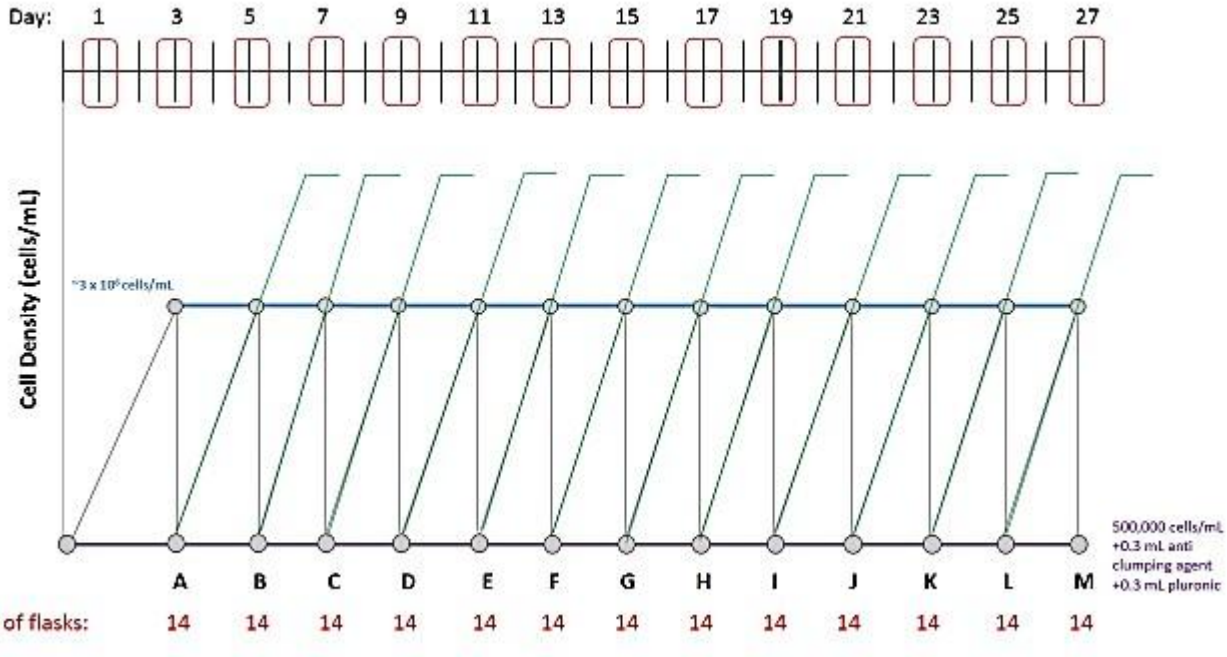
4.1 EG2 Antibody Production

The CHO DUKX cell line used in this experiment produces EG2 mAb. The CHO cells were grown into two different media: BIOGRO (University of Manitoba) and CD-CHO Media (Gibco, Life Technologies). The cells were seeded at 500,000 cells/mL and were passaged every two days **(Figure 4.1)**. Based on observations and calculations of the cell density, the cells have an average of doubling time of 19 hours.

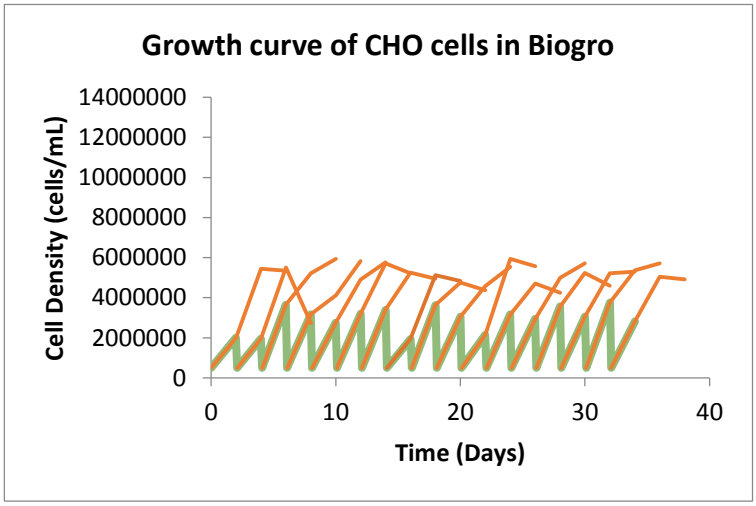
In parallel, the cells were left to grow for four days where they reached a cell density of up to 5.0 x 10^6 cells/mL. The EG2 mAb that are produced over the growth of the CHO cells are believed to increase at it reaches the later phase, therefore the cells were harvested at this point. The titre of EG2 was quantified using HPLC. The flask supernatant was quantified at 255.82 ug/mL and were purified using the protein A spin trap which is an antibody affinity chromatography which is specific to the Fc region of the antibody. After purification, the sample was quantified with HPLC where the purified flask culture is 152.81 ug/mL.

The purified samples for EG2 including the flowthrough, two washes and the elution samples were ran on a SDS-PAGE gel to show the presence of the EG2 in the elution samples. The cell supernatant was diluted 1:10 to be loaded on the gel whereas all the other samples were diluted 1:2. As shown on **Figure 4.2**, there is a band shown at approximately 45 kDa which corresponds to the size of the EG2 protein. Based on the results, the culture have grown exponentially over the number of days. The shaker flask had a constant cell viability of over 95%. Also, it was observed that the EG2

A



B



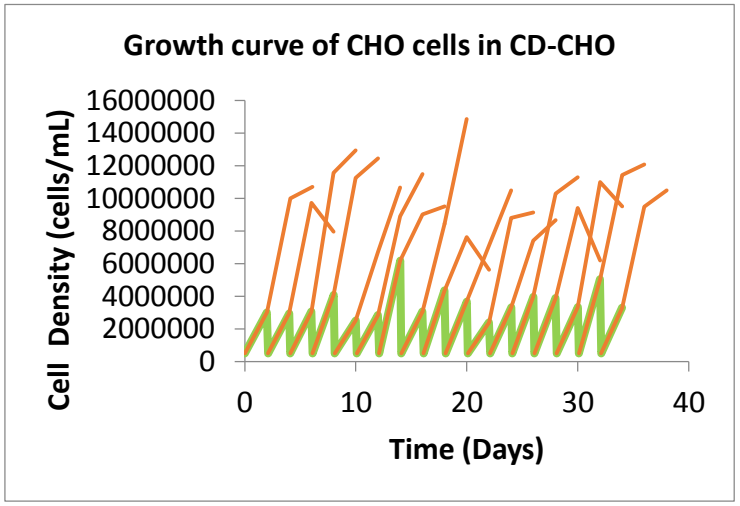


Figure 4.1: Growth curve for CHO cells in flask culture. A. Schematic graph of the design of the experiment where the passage number and extent of growth are studied. **B.** CHO DUKX cells were seeded at approximately 5.0 x 10^5 cells/mL at day 0 with a viability of 98.6%. The doubling time of CHO cells is approximately 19 hours and the maximum cell density before its passage is 3.0 x 10^6 cells/mL. Haemocytometer was used in counting the live and dead cells in the culture to calculate the cell density and viability of the culture over time up to 96 hours. The cell density was shown to roughly double within 24 hours, it may have lagged after day 0 since the cells were transferred into a new flask with fresh media. For each flask culture, one culture was left to grow for an additional two days to test the extent of growth.

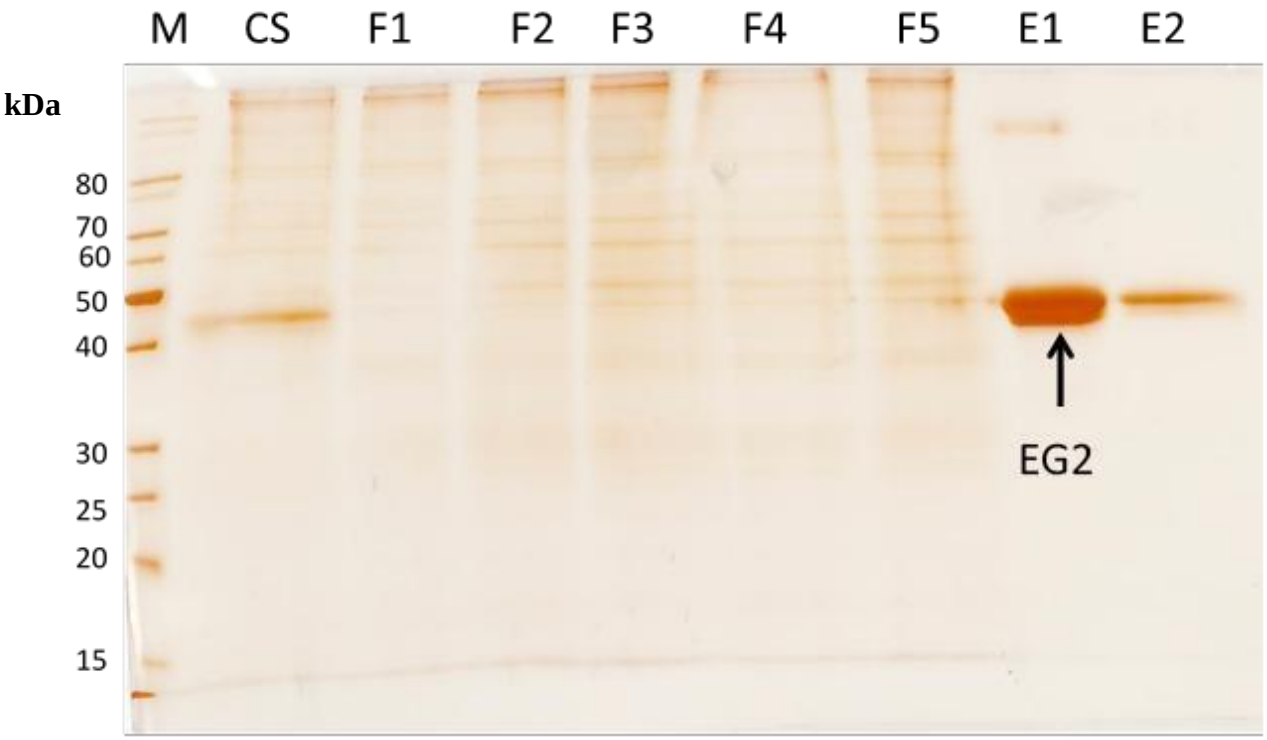


Figure 4.2: SDS-PAGE of purified EG2 samples. The CHO supernatant producing EG2 was purified using Protein A spin trap column. The samples were run on a 12% SDS-PAGE gel at 150 V for an hour and 15 minutes. The gel was stained using silver staining. CS is the culture supernatant, F1 to F5 are the flowthrough, E1 and E2 are elutions.

concentration for the purified samples are lower than the supernatant due to the loss of product during the handling of the sample for the purification.

In order to verify the presence of EG2 in the purified samples, the samples for each fraction of purification: flowthrough, two washes and the two elutions were visualized on the SDS-PAGE. The molecular weight of EG2 is approximately 90 kDa. Although when the samples subjected to SDS and heat, the protein is denatured which causes for the dimer to become a monomer which leads for the molecular size to be approximately 45 kDa. As shown on the SDS gel **(Figure 4.2)**, there was no bands at approximately 45 kDa on the flowthrough and washes which means that the EG2 protein is properly bound to the column. Due to the change of pH on the elution fractions, the EG2 protein is present and a band found at approximately 45 kDa.

4.2 Aggregation of intracellular EG2

Visual inspection of the morphology of CHO cells in the late exponential phase was observed under a Bright field with 20x magnification. Black dots were detected around the cell membrane, as shown in **Figure 4.3**. It was hypothesized that these were the result of protein aggregation. Further analysis was needed to identify the nature of these dots. In order to do a controlled and extensive study on this, different factors were studied. The first factor that was looked at was the type of media. Two types of media were used, Biogro and CD-CHO, in culturing these CHO DUKX cells. The second factor is the passage number when the black dots will start to show up. Lastly, the extent of growth where the CHO cells are left to grow until they reached their maximum cell density and if black dots will show up.

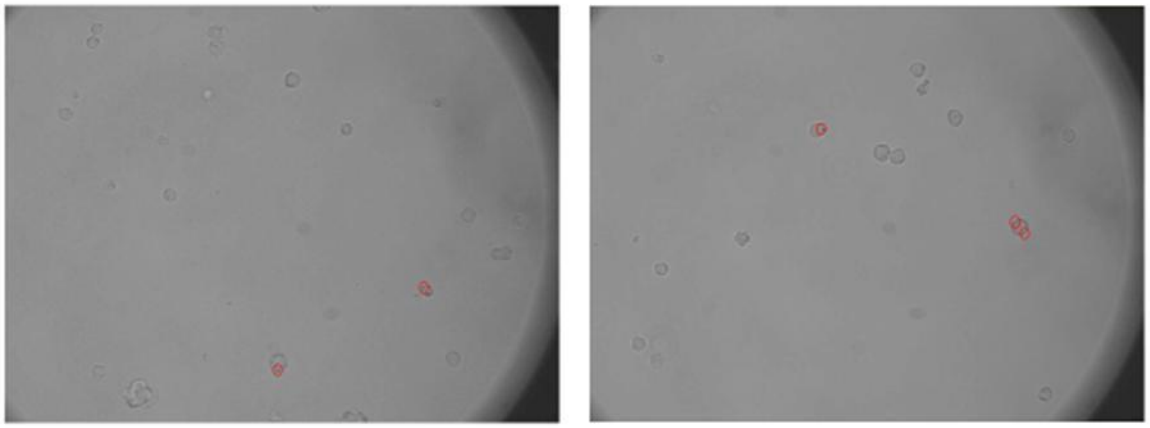


Figure 4.3: Bright field microscopy of CHO DUKX in Biogro media. The cells were harvested at 1.6 x 10^6 cells/mL at passage 2 with viability of 67%. Black dots emphasized with red circles were observed along the cell membrane. These black dots were suspected to be protein aggregation intercellularly.

The experiment was controlled by growing CHO cells in parallel with the two different types of media. Also, the flasks are done in triplicate. In order to observe the passage number in which the black dots are visible, the cell density for each flask were seeded at is 500,000 cells/mL and it was constantly passaged at approximately 3.0 x 10^6 cells/mL. Subsequently, one flask is left to grow until it reaches its maximum cell density to test for the extent of growth.

The CHO cells were collected when the black dots has appeared. Its currently at passage number is 11 for both Biogro and CDCHO cells and no black dots were observed in cells that were grown to 3.0 x 10^6 cells/mL. Interestingly, the black dots were observed at the extended growth when the cells reached 5.0 x 10^6 cells/mL **(Table 4.1 and Table 4.2)**. These cells were collected; the supernatant and pellet were separated through centrifugation at 500 rpm for 10 minutes. The cell pellet was prepared for cell lysis using Triton X-100 detergent with protease (Aprotinin and Pepstatin), fucosidase (Deoxyfuconojinimycin) and glucosidase (acarbose) inhibitors added into the lysis solution. Following the cell lysis procedure, the cell lysate supernatant was collected containing all solubilized proteins. The remaining cell debris after Triton lysis was considered as non-soluble protein, and was resuspended in 1x PBS at pH 7.2. The proteins found in the resuspended lysed pellet, lysate, culture supernatant and unlysed cell pellet (resuspended in 1x PBS) were compared on an SDS-PAGE reducing gel as shown in **Figure 4.4 and Figure 4.6**, and by western blot analysis as seen in **Figure 4.5**. The western blot analysis used an antibody binding to the Fc region of IgG. EG2 appears at the 45 kDa mark on a reducing gel and bands of proteins can be seen at 45 kDa for the cell lysate, supernatant, and unlysed cell pellet from the western blot analysis. A band found at approximately 30 kDa in

purified EG2 sample was thought to be caused by long term (1 month) storage at 4°C. Although, further experiments should be performed such as mass spectrometry to verify this hypothesis.

The concentration of EG2 found in each sample (supernatant, lysate, and insoluble proteins) were 181.93 ug/mL, 747.14 ug/mL, 123.25 ug/mL respectively. Once the samples were purified and the amounts of EG2 were quantified, the glycan forms that were found in antibody pool was investigated. All three samples: supernatant, lysate and insoluble proteins were sent to University of Manitoba for glycan analysis. Based on the glycan analysis shown in **Figure 4.7**, glycans in each sample were unique. Fucosylated glycoforms were found in the supernatant fraction while more complex glycoforms with more sugar moieties were found in the lysate and insoluble proteins.

Based on the experimental results, the visual inspection of CHO cells in the late exponential phase using an inverted light microscope revealed that there were signs of protein aggregation within the cells **(Figure 4.3)** which were identified as black dots. The stained SDS gel **(Figure 4.6)** showed a band around 45 kDa in samples of the solubilized pellet. Western blot **(Figure 4.5)** verified that this was EG2 protein. The EG2 present in the sample was quantified using HPLC and it was shown that most of the EG2 was found in the cell lysate. This showed that the overproduction of the protein caused the antibodies to aggregate and stay within the membrane of the cell. The aggregated proteins may be what others referred to as Russell Bodies.

One of the major questions that was asked is what caused these proteins to be stuck inside the membrane. A glycan analysis on the samples was performed to show whether or not PTMs affected the ability of each protein to be secreted. Based on the analysis **(Figure 4.7),** most of the glycoforms which contained fucose were found in the supernatant; whereas, the less complex glycoforms were found in the cell lysate or to be part of the insoluble proteins.

Therefore, it was identified that the black dots are the aggregated EG2 that were overproduced within the cell. The exact cause of the aggregates is still under investigation. To further investigate the identity of these black dots, immunofluorescent staining can be performed in these cells and CHO cells that do not produce EG2 mAb. Thus, in looking at the three factors that may have been causing these black dots, it can be postulated that it can be due to the sufficiency of nutrients present in the media. Also, the passage number and extent of growth where the cells are reaching their maximum density and the viability of the cells are decreasing which may lead to a mechanism which causes the antibody to aggregate.

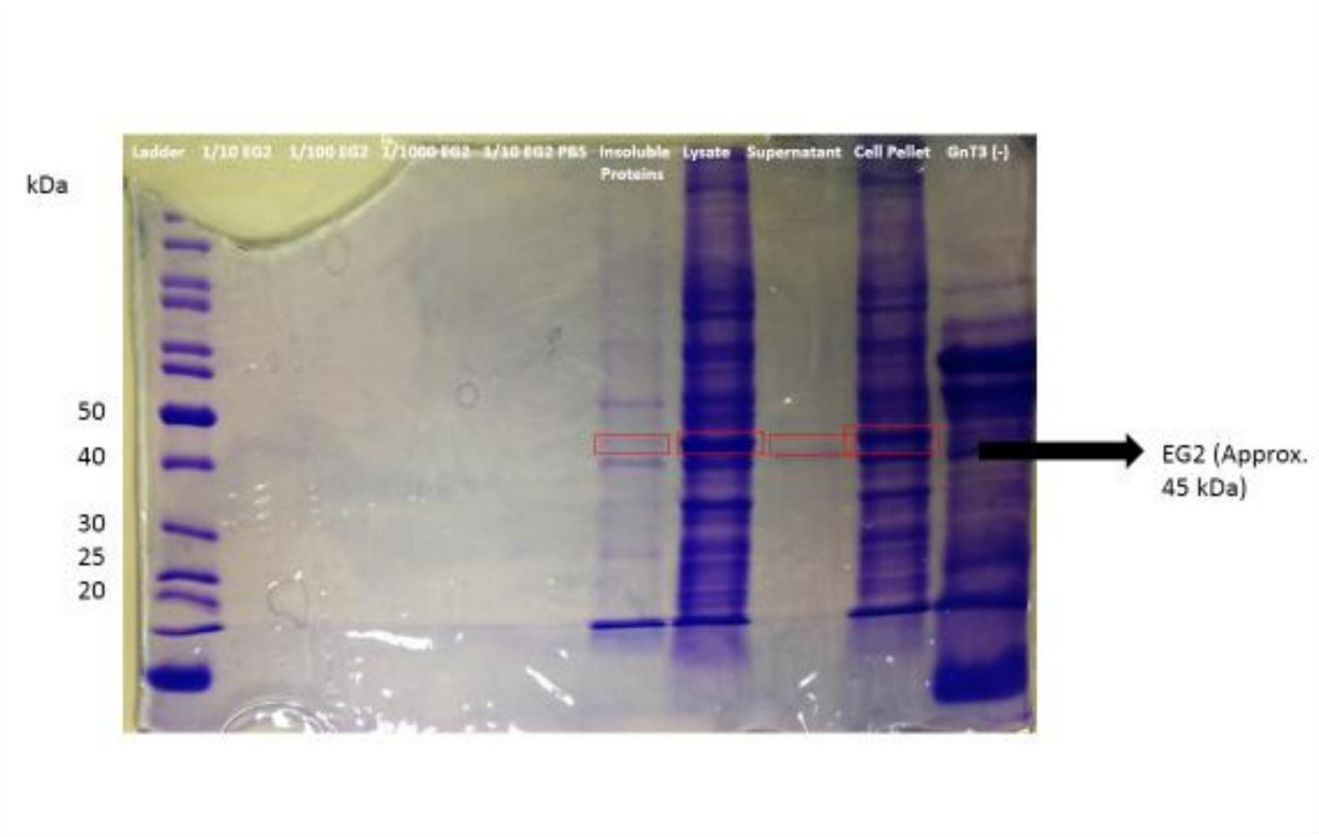


Figure 4.4: Coomassie Blue staining of SDS-Gel with different samples from cell lysis. The cells that was harvested were separated using a centrifuge at 500 rpm for 10 minutes. The cell pellet was subjected to lysis using a lysis solution with Triton X-100. The different samples were run on the gel including the purified EG2 that were dilution into 1/10, 1/100, and 1/1000 but Coomassie stain is not sensitive enough to detect them. The other samples that were run on the gel were insoluble proteins, lysate, cell supernatant, cell pellet and GnT3 cell lysate as a negative control. In all of the samples, there is a band observed at approximately 45 kDa that can be identified as EG2.

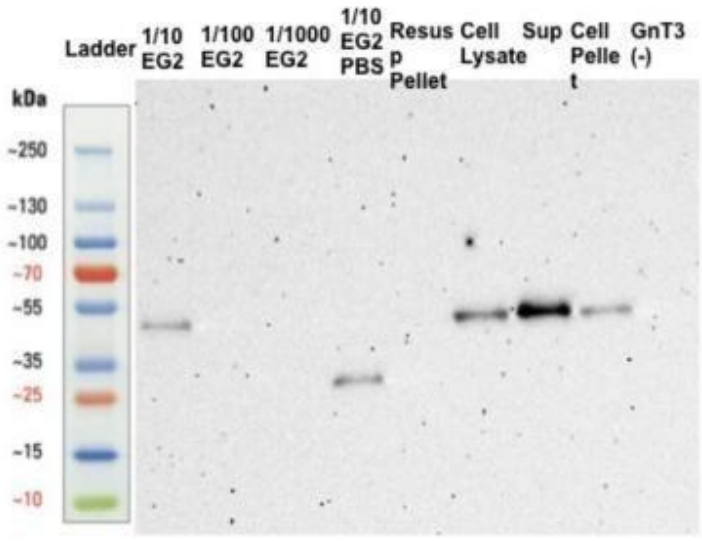


Figure 4.5: Western Blot analysis on samples from cell lysis. The samples from the cell pellet that were subjected to cell lysis were separated using 15% SDS-gels. The order that the sample was loaded on the gel is exactly the same for the gel that was stained with Coomassie blue. After the SDS-PAGE, the samples were transferred into a PVDF membrane and probed with Anti-Human IgG (Fc specific) goat HRP antisera. Bands showed up at around 45 kDa for the 1/10 EG2, Cell lysate, cell supernatant and cell pellet samples which is identified to be EG2.

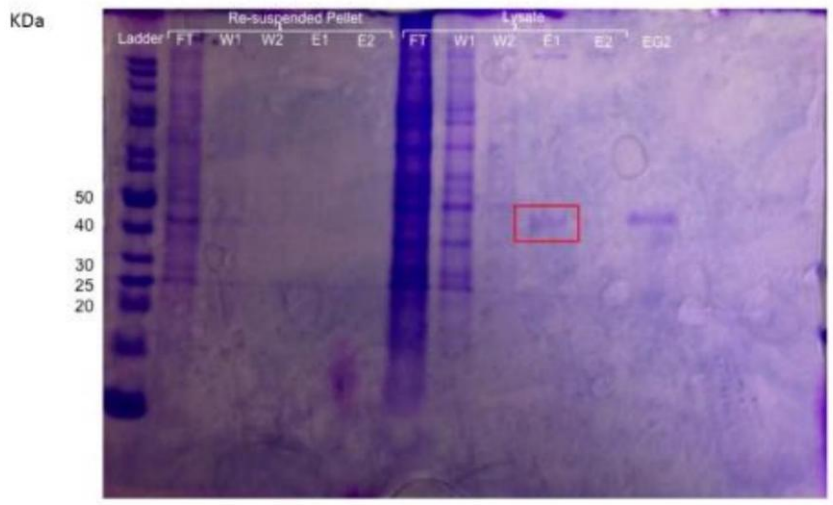


Figure 4.6: Coomassie Blue staining on purified samples. The insoluble proteins and cell lysate were purified using protein-A spin trap. The flowthrough, washes and elutions for both samples were run on a 15% SDS gel and stained with Coomassie blue. Purified EG2 was used as a positive control. There was a band at approximately 45 kDa that was identified for the first elution on the cell lysate that shows the presence of EG2 in the cell lysate.

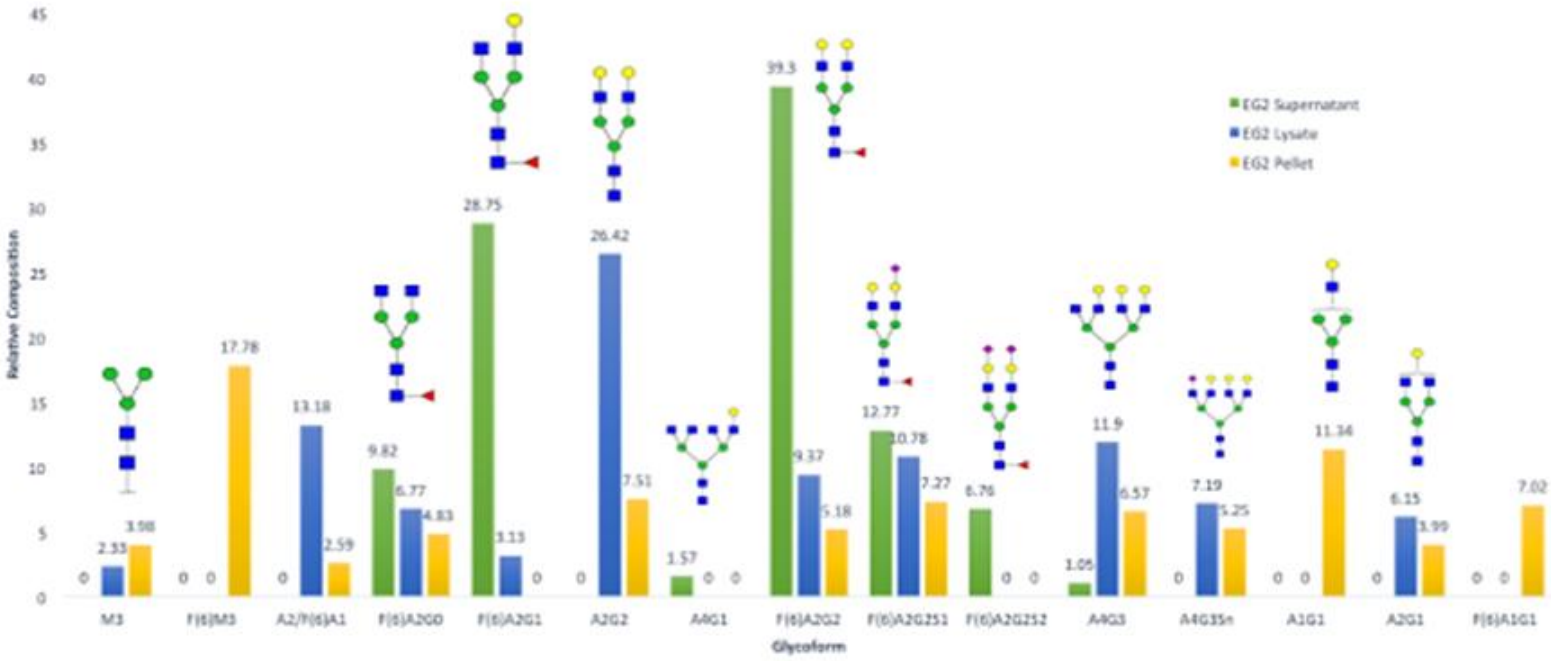


Figure 4.7: Glycan Analysis of the EG2 supernatant, lysate and pellet. Prior to the glycan analysis, each EG2 lysate sample was subjected to cell lysis using Triton x-100. A buffer exchange was performed on those samples since Triton X-100 is a detergent so it may interfere with the column. The Glycan analysis was performed using a protein A column and HPLC. The process was previously described in the thesis

Type	Passage	# of Black Dots/# of Total Cells	Cell Density	Cell Viability
Biogro	1	23/108	3650000	68%
CDCHO	1	0/104	9950000	93%
Biogro	2	32/140	1180000	43%
CDCHO	2	2/168	11575000	98%
Biogro	3	19/107	4121250	69%
CDCHO	3	3/292	4720000	87%
Biogro	4	8/154	4252500	73%
CDCHO	4	0/145	9450000	99%
Biogro	5	9/40	1370000	95%
CDCHO	5	23/137	4640000	87%
Biogro	6	26/89	3412500	65%
CDCHO	6	9/116	3176250	27%
Biogro	7	16/61	2825000	57%
CDCHO	7	8/60	5325000	57%

Table 4.1: Number of Black Dots per cell recorded

Type	Passage	Cell Density	Cell Viability
Biogro	1	5220000	95%
CDCHO	2	7840000	98%

Table 4.2: Cell Density when the Black Dots first appeared for both media

Chapter 5: Production, Quantification and Purification of *N*-Acetylglucosaminylferase III (GnT3) and Testing its Activity

5.1 Production and Purification of GnT3

A feature that was added is the honeybee melittin (HBM) which allows for the enzyme to be secreted and properly glycosylated that will allow for the GnT3 to be active. Once the protein was successfully expressed, an IMAC column was used to purify the protein with 6x His tag. Lastly, a TEV cleavage site is added which is used for controlled cleavage of the fusion of the 6x His tag protein to the GnT3 protein.

Comparably the bioreactor was seeded at 500,000 cells/mL and since the cells were originally growing in a flask, the cells had to adapt when it was grown in a bioreactor. The viability of the cells after the inoculation of the cells on the bioreactor dropped. Other than the adaptation of the cells to the bioreactor, this may have been caused by the fact that there was no heating jacket to maintain the temperature to 27°C. Therefore as an alternative, the bioreactor vessel was placed into a 27°C water bath. As previously stated, the conditions that are regulated for the growth of the *Sf9* insect cells are the temperature, stirring speed, and DO. Due to the lack of heating pad, the temperature only reached up to 25°C that may have slowed down the growth of the insect cells. Also, the DO level was set to 55% but it was fluctuating throughout from 30% to 75%. The cells ran for four days prior to infection until cell density reached 1.2 x 10^6 cells/mL, the *Sf9* insect cells were infected with the Gnt3 baculovirus. Once the cells were infected, the cells stopped growing and the cell viability started to decreased. The cells were left for four days post infection until it reached the cell viability of 73% when it was collected **(Figure 5.1)**. After the samples were collected, they were concentrated 10x and followed by purification of the GnT3 protein that was produced using Histrap column. Although before the purification, a

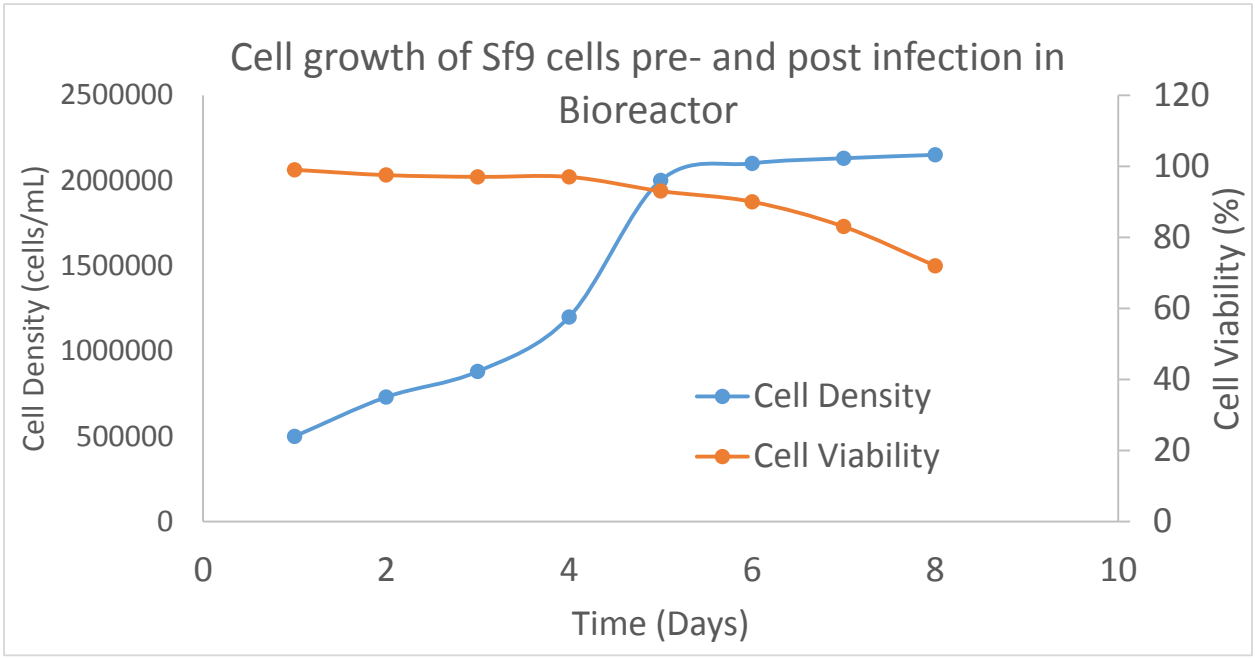


Figure 5.1: Growth Curve of Sf9 cells in an autoclavable Bioreactor. The *Sf9* cells were seeded at approximately 5.0 x 10^5 cells/mL at day 0 with a viability of 99%. The doubling time of *Sf9* cells is approximately 20 hours and the maximum cell density it can reach is about 8.0 x 10^6 cells/mL. Haemocytometer was used in counting the live and dead cells in the culture to calculate the cell density and viability of the culture over time up to 96 hours. The cell density was shown to roughly double within 24 hours, it may have lagged after day 0 since the cells were transferred into a new flask with fresh media. Once the cell viability reached 1.2 x 10^6 cells/mL, the cells were infected with the GnT3 baculovirus with MOI of 0.01 for protein production.

buffer exchange was performed on the supernatant to change the media Sf900 SFM III into the binding buffer for the Histrap purification is consists of 20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 5 mM imidazole, pH 8.0. It was determined that there are contents from the Sf900 SFM III media that will interfere with the resin of the Histrap column. The supernatant, cell lysate, all the fractions of purification were visualized using SDS-PAGE and stained with silver staining **(Figure 5.2)**. The purified samples were quantified using BCA Assay and it was determined to be 1.8 ug/mL for the flask culture and 67.9 ug/mL for the bioreactor run. For further analysis, the proteins were transferred onto a PVDF membrane and were probed with antibodies specific to the His-tag. Unfortunately, no bands showed up not even for the positive control that was used: a calmodulin protein with 6x-Histag.

The GnT3 protein that was synthesized was truncated at glutamine 25 since the transmembrane domain was needed to be removed which is located prior to the amino acid residue 25. This allowed for the protein not to get stuck in the membrane when it was produced within the cell. It was previously stated that GnT3 protein needs to be secreted in order to get properly glycosylated. Therefore, a honey bee melittin (HBM) tag was added to the construct. Honey Bee Melittin secretion signal allows for efficient translocation of proteins into the ER in *Spodoptera frugiperda (Sf9)* cells[69]. Another feature that was added was the 6x His-tag at the C-terminus. This allows for the purification of the protein using affinity chromatography. In order to get a properly folded protein, a TEV cleavage site was added preceding to the 6x His-tag so it can be easily removed after purification.

Since the production of proteins in insect cells in a flask does not give a huge amount of proteins, the infection of *Sf9* cells were up scaled into a bioreactor. After doing the plaque assay, the titre of the virus was identified and a low multiplicity of infection was performed. Low MOI infection was performed since it is feasible due to the amount of virus needed.

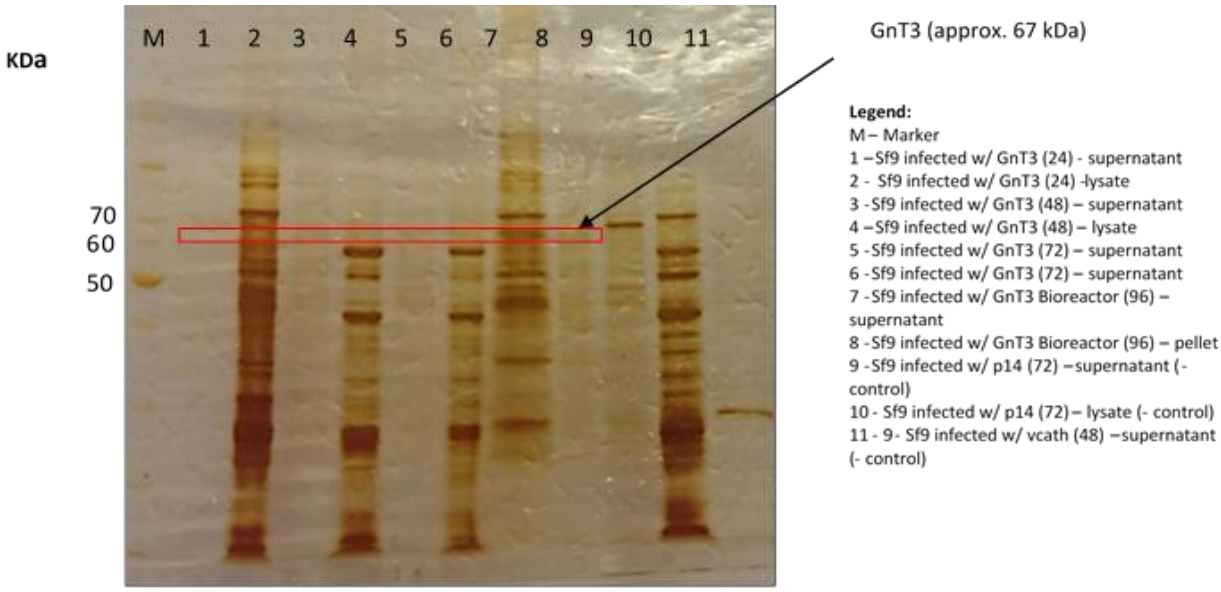


Figure 5.2: Visualization of GnT3 protein using SDS-PAGE. The protein samples were run on 12% SDS gel at 150 V for an hour and fifteen minutes. The gel was stained using silver nitrate. The legend is described on the side of the image. There were three negative controls used which are lanes 9 to 11. It was a different baculovirus infecting the *Sf9* cells. .Also, having a low MOI lets the cells grow for a longer periods of time since it will take longer to infect all of the cells in the culture.

After the production of GnT3, the proteins were harvested and purified using Histrap columns. According from previous studies, the SF900 SFM III media might interfere with the resins on the Histrap columns therefore a buffer exchange is needed. The harvested supernatant was exchanged into the binding buffer in order to prevent interference during purification. In order to verify that there is a pure sample, all the elutions were ran on a 12% SDS-PAGE gel and stained with silver staining. There were no protein bands present on the elution lanes **(Figure 5.3).** Therefore, the concentration of the purified samples were measured using the BCA Assay and it was shown that the amount of proteins that are loaded into each well is approximately 16 ng. Even though the gel was stained using silver nitrate which is highly sensitive (can detect as low as 25 ng), no band has appeared. Therefore, instead of just doing a 40x concentrate, it was increased to 200x that resulted for the appearance of the GnT3 band at approximately 68 kDa.

For the western blot analysis, no band showed up even for the positive control which showed that there might have been a problem with the antibodies that were used specific to the 6x Histag. One of the concern is the secondary polyclonal antibodies. As previously described, polyclonal antibodies are produced from different B cell lineages which allow for recognition of multiple epitopes. Therefore, there is a possibility that the secondary antibody that was used was produced in various lineages except for the one that is compatible with the insect cells since GnT3 is produced in insect cells.

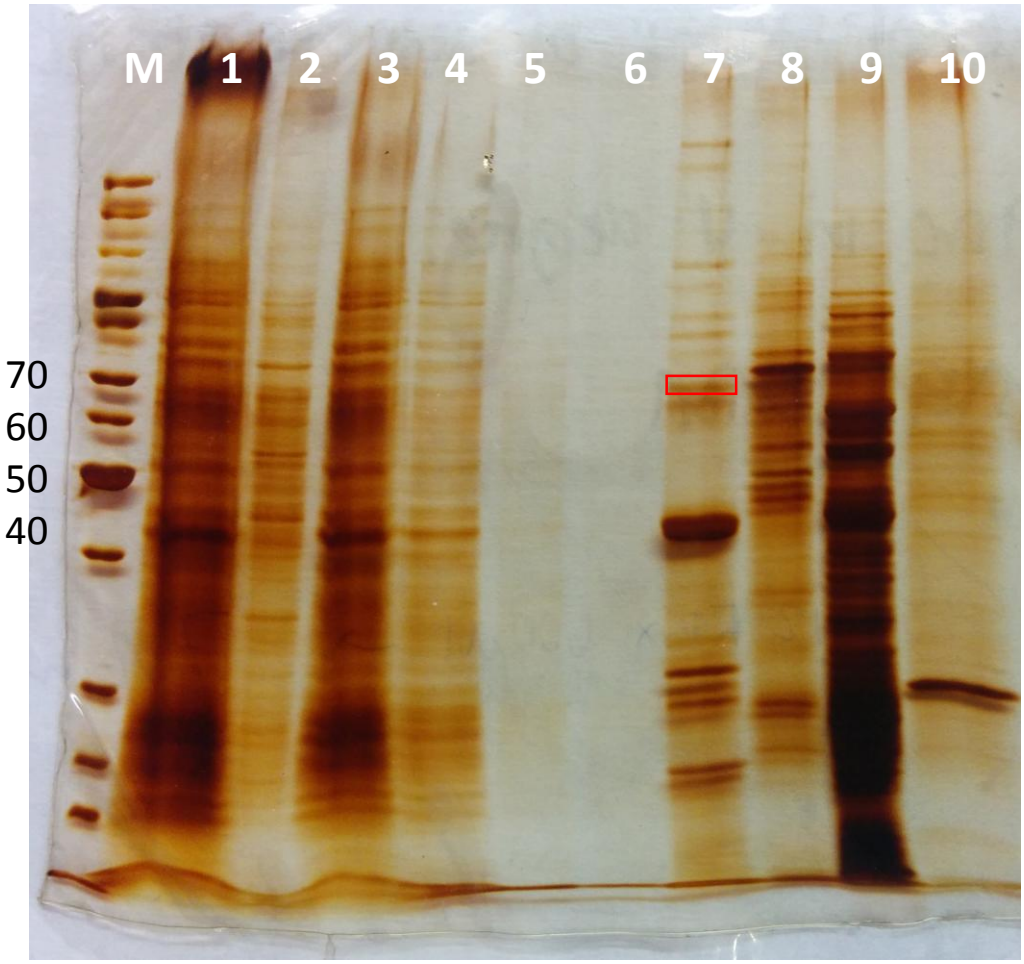


Figure 5.3: SDS-PAGE gel of purified GnT3. The protein samples were ran on 12% SDS gel at 150 V for an hour and fifteen minutes. The gel was stained using silver nitrate. M is marker, 1 is Sf9 supernatant, 2 is pellet resuspended in 1x PBS, 3 is Flowthrough, 4 to 6 are washes, 7 is the elution, 8 to 11 are negative controls which are another Sf9 infected cultures.

5.2 Activity of GnT3

Even though there was no band that showed up for the western blot, the activity of GnT3 was tested using the glycosyltransferase assay. Due to the contents that the SF900 SFM III media might have which will interfere with the assay, a buffer exchange to 1X Assay Buffer was performed on the in-house GnT3 that was produced from *Sf*9. The calibration curve that was used in this assay consisted of a phosphate standard curve dissolved in 1X Assay Buffer **(Appendix).**

In order to make sure that the activity that is being measured is accurately from the GnT3 enzyme, controls were executed while doing this assay. The positive control consisted of using another acceptor substrate which is the IgG DP-12 clone 1934 from Maureen Spearmann at University of Manitoba instead of the EG2 mAb. Various negative controls were performed in this assay. The first major ones was No GnT3 enzyme, no donor substrate and no acceptor substrate. Additionally, a negative control which consists of another baculovirus infected culture other than the GnT3 virus to make sure that there are no enzyme in the culture which gives a positive reading. The activity of GnT3 was calculated using a phosphate calibration curve **(Figure 5.4 and Figure 5.5)**.

As shown in the graphs for **Figure 5.4** and **Figure 5.5**, there's a visible decrease of enzyme activity as the incubation time gets longer. Also, the negative controls gave small absorbance readings that the activity observed in these wells were considered negligible. The positive control shows a great enzymatic activity for the GnT3 towards a different acceptor substrate. Since there was a decrease in activity, another factor that should be studied is the concentration of the acceptor substrate that is present in the reaction **(Figure 5.6)**. Lastly, the in-house GnT3 that was used in the previous reactions is the crude supernatant. Therefore, the GnT3 supernatant was purified using a Histrap column since the GnT3 protein has a 6x His tag

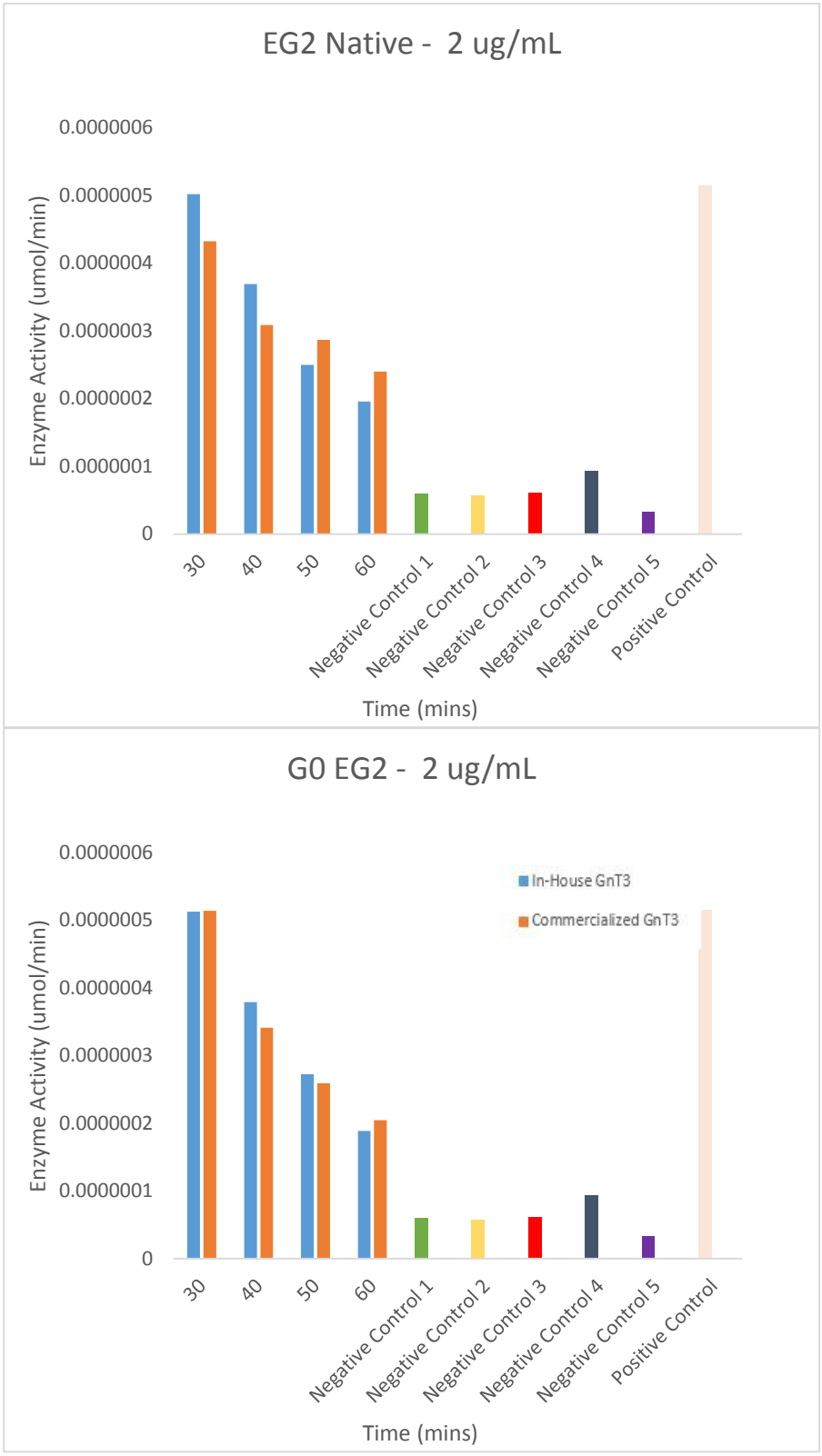


Figure 5.4: GnT3 enzyme activity with various times. The GnT3 reaction was incubated at different times to acquire the ideal time for the highest activity. Two types of EG2 that were used: EG2 G0 antibody which means that the terminal sugars were removed and native EG2. The concentration of the acceptor substrate that was used is 2 ug/mL and 1 mM donor substrate. The in-house GnT3 that was used is a crude sample.

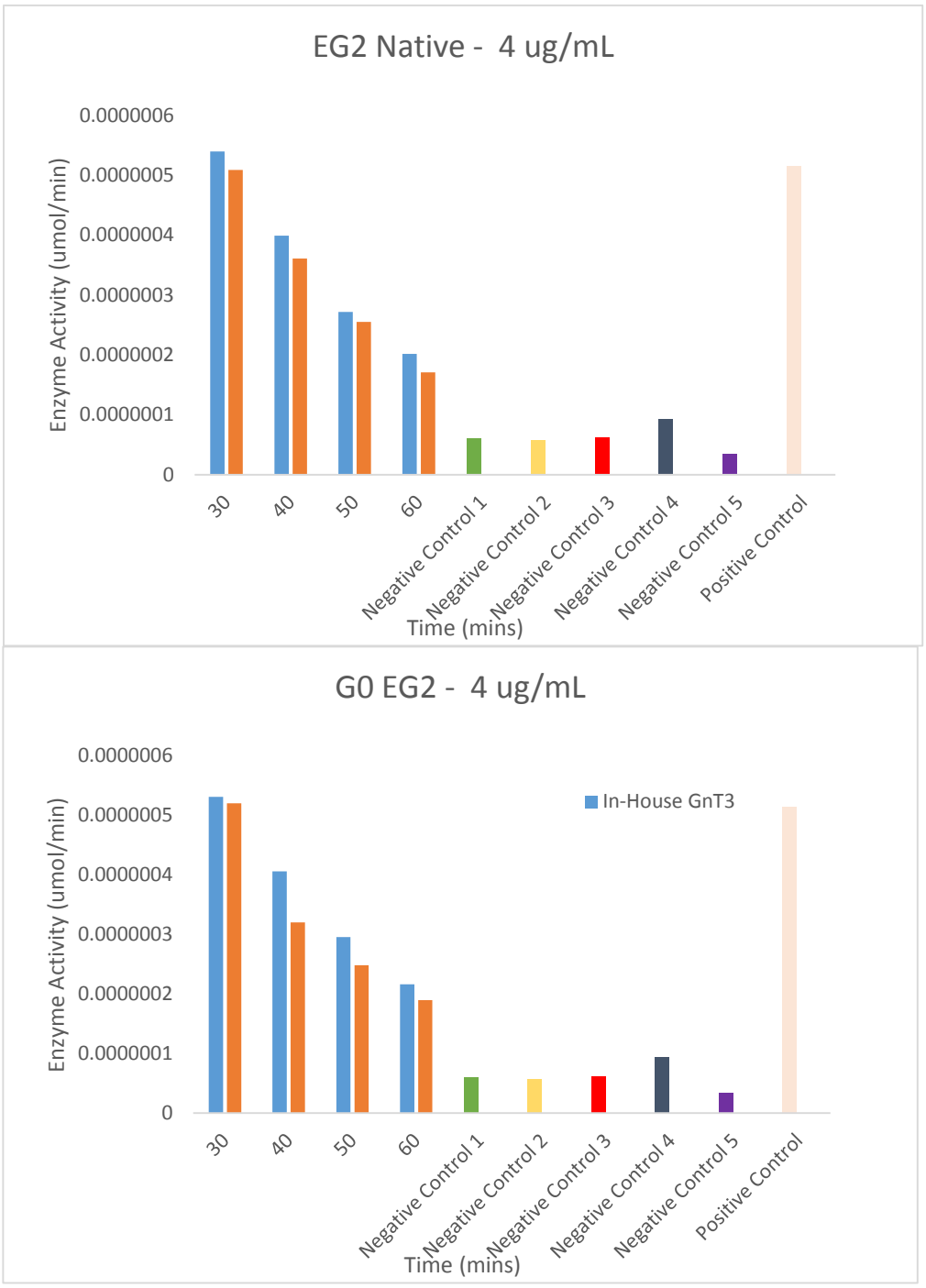
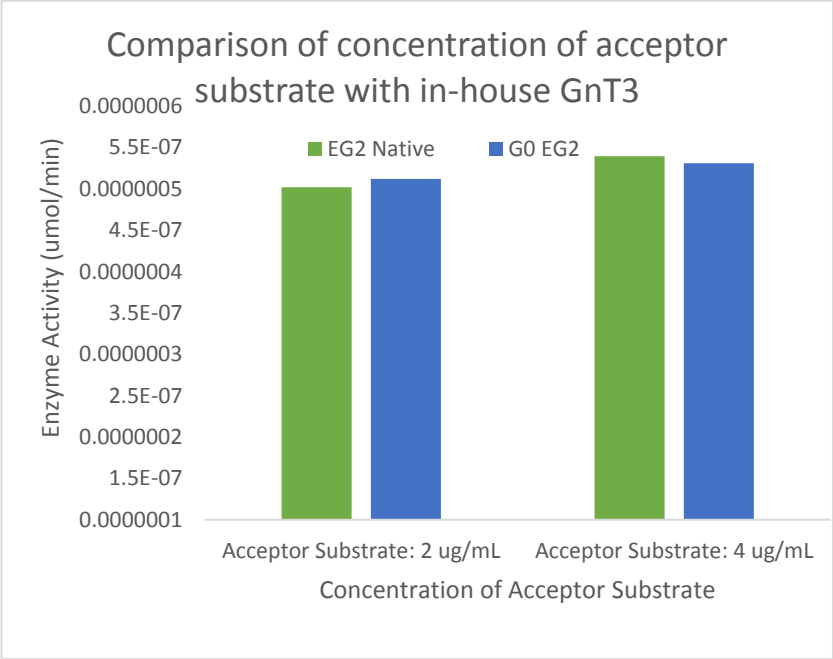


Figure 5.5: GnT3 enzyme activity with various times. The GnT3 reaction was incubated at different times to acquire the ideal time for the highest activity. Two types of EG2 that were used: EG2 G0 antibody which means that the terminal sugars were removed and native EG2. The concentration of the acceptor substrate that was used is 4 ug/mL and 1 mM donor substrate. The in-house GnT3 that was used is a crude samples.



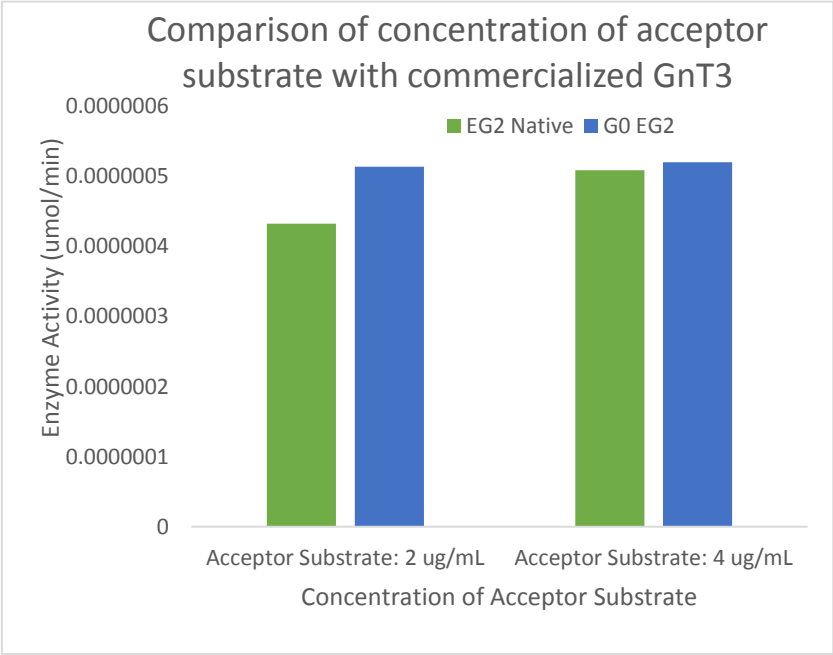


Figure 5.6: GnT3 enzyme activity with different concentrations of acceptor substrate. The GnT3 reaction was incubated at two different concentration of EG2: (a) 2 ug/mL and (b) 4 ug/mL to identify whether the enzymatic activity will increase with increase of acceptor substrate concentration. Two types of EG2 that were used: EG2 G0 antibody which means that the terminal sugars were removed and native EG2. The concentration of the acceptor substrate that was used is 4 ug/mL and 1 mM donor substrate. The in-house GnT3 that was used is a crude sample.

As previously described, the folding of the protein is vital in the enzymatic activity of an enzyme. For this experiment, the GnT3 had an HBM tag to ensure that it is secreted properly for the enzyme to be properly glycosylated in the three potential N-glycosylation sites. Thus, the activity of the GnT3 enzyme was measured using an assay that utilize the by-product of the reaction which is the nucleotide. A phosphatase is used to release a free phosphate which is directly proportional to the activity of the enzyme.

For every assay, there should always be controls that are executed. The positive control which involves the use of another acceptor substrate instead of EG2 mAb. This control was chosen to ensure that if there was no activity detected, that it wasn't because of the acceptor substrate. Thus, it makes sure that the active site is not buried in the EG2 protein since it has a mutation in the Fc region which may affect the protein folding. The first three negative controls are common since it is assumed that no activity should be detected if any of the factors that are involved in the reaction is not present. Whereas, the last two controls where a crude supernatant and purified baculovirus infected culture was used in the reaction instead of the GnT3. This allows for the verification that there is nothing in the *Sf9* culture or media that contributes to the activity that was being detected by the assay.

Two factors that were studied in this assay are: time and concentration of acceptor substrate. For the study involving time shown in **Figure 5.4** and **Figure 5.5,** there was a noticeable decrease in the enzyme activity as the time increases. This trend might have occurred due to the fact that there is not enough acceptor substrate in the reaction. Therefore this has led to increasing the acceptor substrate to double. Although, as seen in **Figure 5.6**, the enzymatic activity was not observed to have doubled even if the acceptor substrate increased by double. Therefore, there's another factor that should be studied which is the donor substrate. The GnT3 enzyme does not fall under the Michaelis Menten type mechanism but it can be hypothesized

to fall under the ping pong mechanism. The ping pong mechanism is a double-displacement reaction where the enzyme change into an intermediate form prior to the formation of the product[71]. This reaction may fall into this category since it involves three factors: enzyme, donor and acceptor substrate. Another key feature to this mechanism is that one product is formed and released before the second product substrate binds. In perspective, the GnT3 enzyme forms a temporary complex (intermediate) with GlcNac from UDP-GlcNac (donor substrate). The first product will then be released which is the UDP prior to the transfer of GlcNac to the monoclonal antibody (acceptor substrate)

The activity of the GnT3 enzyme can be measured using an assay which relates the release of free phosphate from the nucleotide to the enzymatic activity. The positive control gave a huge amount of activity whereas the values that were acquired for negative controls were negligible. The trends that were observed was a decrease in activity as the post incubation time increases which led to a subsequent experiment in doubling the concentration of the acceptor substrate. Although, this change did not give the expected results. Therefore, there might have been a shortage of UDP-GlcNac as well which hinders the activity of the enzyme. Lastly, a pure GnT3 enzyme must be used to avoid interferences in the absorbance readings of the assay.

Chapter 6: Conclusion and Future Work

The purpose of this project was to modify this antibody population *in vitro* to have a uniform glycoform. The specific glycan that was modified is the EG2 antibody that was produced from CHO DUKX cells. It was shown that the overproduction of the antibodies may have led to the aggregation of the protein. This was observed through the presence of black dots looking at the CHO cell under an inverted light microscope. The identity of the different morphology of these cells were tested through SDS-PAGE, column purification using protein-A column and western blot. Further analysis must be performed in order to verify that the black dots that were observed are in fact EG2. Immunofluorescent staining can be completed through the use of antibodies that are specific to the Fc region of EG2 which are conjugated onto a fluorescent protein which can be observed under the confocal microscope. If the black dots fluoresce, it can be concluded that the aggregated proteins are EG2.

Based on this report, the glycan patterns have been hypothesized to have an effect on the proper secretion of EG2 antibodies based on the glycan analysis that was performed. Therefore, an additional factor that can be studied is the production of EG2 in a specific cell line. An alternative cell line is *Sf9* insect cells, since the glycan patterns that are produced in insect cells are less complex. The glycans produced by insect cells are less complex since they do not have a glycan pattern with fucose, sialic acid and galactose. Therefore, if black dots are also observed in insect cells, this can verify the hypothesis that the absence of fucose or galactose affects the aggregation and secretion of the antibodies.

The enzyme that was used for the *in vitro* modification was the N-acetylglucosaminyltransferase III (GnT3). GnT3 catalyzes the transfer of bisecting GlcNAc into the mannose core region of glycan. Throughout this project, the GnT3 enzyme was synthetically synthesized in *Sf9* insect cells. The presence of GnT3 was verified using different

techniques such as SDS-PAGE and western blot. Lastly, the activity of the GnT3 enzyme was measured using glycosyltransferase activity assay. The activity assay showed activity for the in-house GnT3 although it was observed that higher concentration of the enzyme is needed to properly test and measure its activity.

As previously stated in this report, the GnT3 that was produced from *Sf9* insect cells after purification has a low concentration. Therefore, a different method in changing the buffer is needed to obtain higher recovery. Another bioreactor run should be performed in order to produce more GnT3 protein, then the enzyme can be purified and then be used to modify the antibody population. Glycan analysis is needed in order to verify that the EG2 antibody population was uniformly transformed into having a glycan pattern with a bisecting GlcNAc.

The main struggle for this part of the project is the production of high amounts of GnT3 protein which can be the bottleneck of successful *in vitro* modification of the EG2 antibody. Another limitation that was faced is that, the acceptor substrate, EG2 was not expressed as much as it was expected due to the aggregation that was observed. Therefore, a lot of questions arose throughout my master's thesis;

- Is the active site for the EG2 buried due to the mutation at the Fc region, that GnT3 is not able to catalyze the addition of bisecting GlcNAc?
- Would it be better to use a commercial antibody such as Rituxan and Herceptin?
- What additives can be added to the media in order to prevent for nutrition starvation which might have caused the aggregation?
- Would high MOI or low MOI be better for protein production?
- What buffer can be used that is general for all reactions and purification that are performed prior to the testing of the GnT3's activity in order to prevent for the loss of the protein?

The work demonstrated in this thesis can be used as a tool in expanding the *in vitro* modification of the glycans. The first step is understanding how the EG2 is folded and how this can affect any inhibition for the addition of bisecting GlcNAc. Also, try another type of IgG antibody such as Rituxan and Herceptin to test whether the uniform population of glycan patterns in the antibody population will be achieved. At the same time, the production of GnT3 should be optimized. This can be done through doing an infection with various high MOIs and identifying which will result to a highest protein production. Once the ideal MOI was identified, more GnT3 baculovirus will be generated in order to do a bioreactor run. A bioreactor run producing GnT3 is required in order to acquire higher concentration of proteins. Also, doing optimization of the concentration of imidazole is needed to acquire the purest GnT3 protein. In having a pure GnT3 enzyme stock, it would be easier to detect the concentration of enzyme and its activity.

After the verification of the activity of GnT3 and its ability to modify the glycoform to achieve a uniform antibody population, a process can be outlined an efficient process of producing human GnT3. Thus, this can be up-scaled to industry level by attaining the perfect conditions that will allow remodeling of antibodies directly from culture supernatant using immobilized glycosyltransferases.

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Appendix

Molecular Cloning of GnT3 with HBM secretion tag

Human N-acetylglucosaminyltransferase III (GnT3) has a molecular weight of approximately 60 kDa and is highly conserved between human, rat, chimpanzee, cow, mouse, chicken and zebra fish. The main function of this enzyme is it catalyzes the transfer of GlcNAc to the core β-mannosyl residue on an acceptor N-glycan (**Figure 1)**. The reaction can be inhibited by the presence of β-1,4-gal residue on the Manα-1,3 branch. It has a transmembrane domain shown in blue below, and a glycosyltransferase catalytic domain shown in green in the diagram below **(Figure 2).**

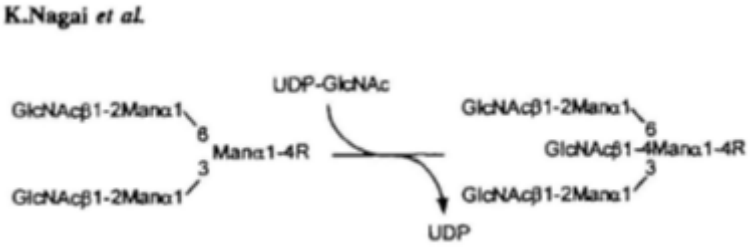


Figure 1: *Reaction catalyzed by GnT3.*

```
  1 MKMRRYKLFL MFCMAGLCLI SFLHFFKTLS YVTFPRELAS LSPNLVSSFF WNNAPVTPQA
 61 SPEPGGPDLL RTPLYSHSPL LQPLPPSKAA EELHRVDLVL PEDTTEYFVR TKAGGVCFKP
121 GTKMLERPPP GRPEEKPEGA NGSSARRPPR YLLSARERTG GRGARRKWVE CVCLPGWHGP
181 SCGVPTVVQY SNLPTKERLV PREVPRRVIN AINVNHEFDL LDVRFHELGD VVDAFVVCES
241 NFTAYGEPRP LKFREMLTNG TFEYIRHKVL YVFLDHFPPG GRQDGWIADD YLRTFLTQDG
301 VSRLRNLRPD DVFIIDDADE IPARDGVLFL KLYDGWTEPF AFHMRKSLYG FFWKQPGTLE
361 VVSGCTVDML QAVYGLDGIR LRRRQYYTMP NFRQYENRTG HILVQWSLGS PLHFAGWHCS
421 WCFTPEGIYF KLVSAQNGDF PRWGDYEDKR DLNYIRGLIR TGGWFDGTQQ EYPPADPSEH
481 MYAPKYLLKN YDRFHYLLDN PYQEPRSTAA GGWRHRGPEG RPPARGKLDE AEV
```

Figure 2: *Human GnT3 Protein Sequence.*

In order for the enzyme to be fully active, it has to be prevented from binding to the membrane. Therefore, the GnT3 enzyme that would be expressed is truncated at position 61 where the transmembrane was removed from the sequence.

Since the Baculovirus system will be used to express this enzyme, the vector that will be used is the pFastBac1 vector **(Figure 3)**. The Bac-to-Bac TOPO Expression System consists of the pFastBac plasmids which allow for the rapid generation of an expression construct containing the gene of interest which under the control of a Baculovirus-specific strong polyhedron (P_H) promoter. The *E.coli* host strain that was used is DH10Bac which contains a Baculovirus shuttle vector (bacmid) and a helper plasmid, which allows for the generation of a recombinant bacmid following transposition of the pFastBac expression construct.

Prior to retransforming of the plasmid with GnT3 into the DH10Bac system, it is transformed into an amplifying vector which is the Top10 cells. Top10 cells are ideal for high-efficiency cloning and plasmid propagation. It also allows for stable replication of high-copy number plasmids. After the plasmid with GnT3 has been successfully amplified with the Top10 cells, the

purified plasmid will be retransformed into the DH10Bac where it would be recombined into the Baculovirus.

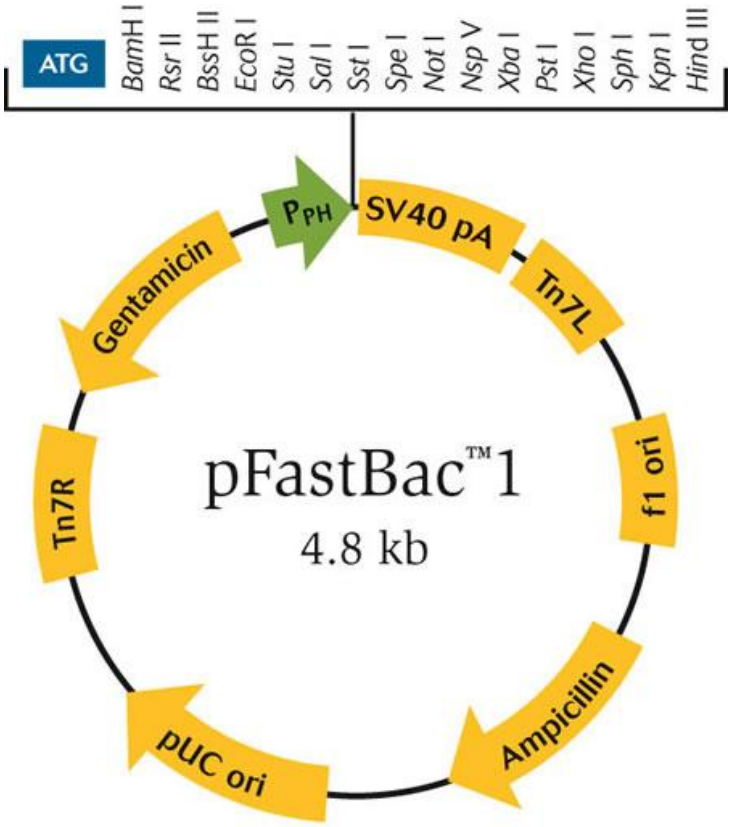


Figure 3: *Schematic map of pFastBac1 vector*

A couple of different ways were tried in order to generate an active GnT3 enzyme.

First: Conventional Molecular Cloning

Prior to the generation of the Baculovirus, the gene of interest, the truncated GnT3 was amplified using polymerase chain reaction using pAcSG2_GnT3 template and the following primers and conditions:

Forward primer:

EcoRI Glu$_{61}$ Truncated CDS

5’ GCACTGTCTAACGTACTCATAGCGAATTC**ATG**GAGCCAGGAGGCCCTGACCTGCT 3’

Reverse primer:

XhoI 6 x His Tag

5’ CAATCTCGAGCTAGCCGTGATGGTGATGGTGATGGCCgacttccgcctcgtccag 3’

Table 1: PCR Conditions

Cycles	Temperature	Time
Di	98 Celsius	30 seconds
D	98 Celsius	15 seconds
A	69 Celsius	15 seconds
E	72 Celsius	1:45 seconds
Ef	72 Celsius	5 minutes

Table 2: Composition of the PCR reaction

Composition	Volume
H_2O	37.3 uL
5x HF Buffer	10 uL
10 mM dNTPs	1 uL
100 uM Forward Primer	0.25 uL
100 uM Reverse Primer	0.25 uL
Phusion Polymerase	0.2 uL
Template from pAcsG2	1 uL

The gene of interest, GnT3, was properly amplified shown in the following figure:

Legend:
Lane M: 1 kbp Marker [kbp]
Lane 1: tGnT3-His (Start)
Lane 2: tGnT3-His (Start)
Lane 3: tGnT3-His (Fusion)
Lane 4: tGnT3-His (Fusion)
Lane 5: tGnT3-His (Start)
Lane 6: tGnT3-His (Start)

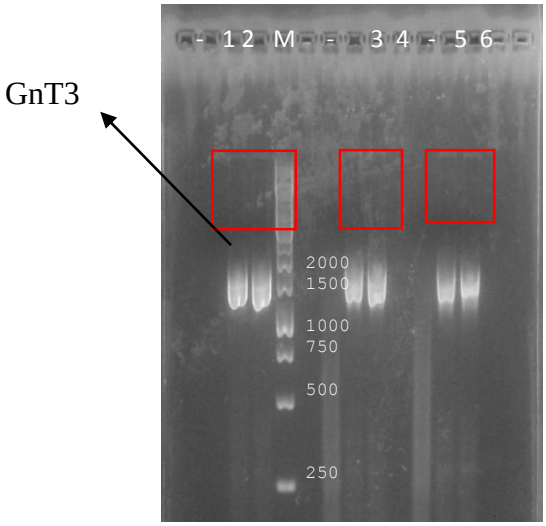


Figure 4: *1% Agarose Gel of the amplification of GnT3 gene*

In order for the GnT3 to be secreted and properly glycosylated, *honeybee* melittin (HBM) tag is needed. This was amplified using overlapping PCR. The primers that were used are overlapping 28 bp and no template is needed in generating the product. The primers and conditions that were are used for this PCR are the following:

Primers:

FWD_HBM-Overlap

BamHI

5’

ATG**GATCCG**CTAGCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTAT ACA

REV_HBM (O2)

EcoRI

5’

ATG**AATTCT**CGATCCGCATAGATGTAAGAAATGTATACGACCATAAAAACAAGGGC AACG

Table 3: Two step protocol (30 cycles) for HBM PCR Conditions

Cycles	Temperature	Time
Di	98 Celsius	30 seconds
D	98 Celsius	15 seconds
A/E	72 Celsius	20 seconds
Ef	72 Celsius	5 minutes

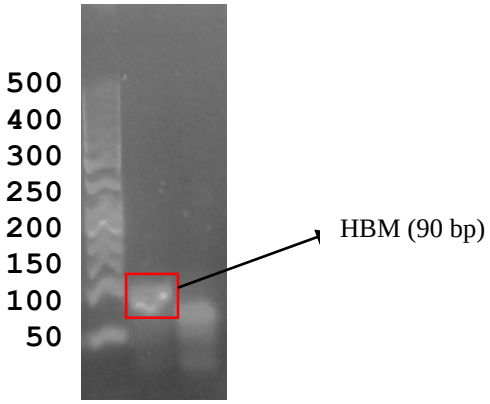
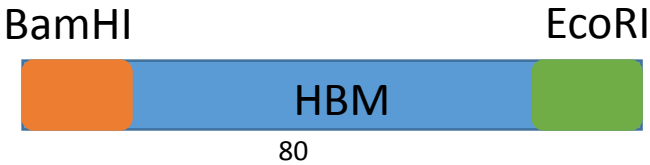


Figure 5: *3% Agarose gel of HBM Tag*

For both PCR reactions, Phusion polymerase was used. After acquiring the amplified products, gel extraction purification was performed to isolate the desired fragment of the intact DNA. After the DNA purification, the purified PCR products were digested with specific restriction enzymes shown in the following figure.

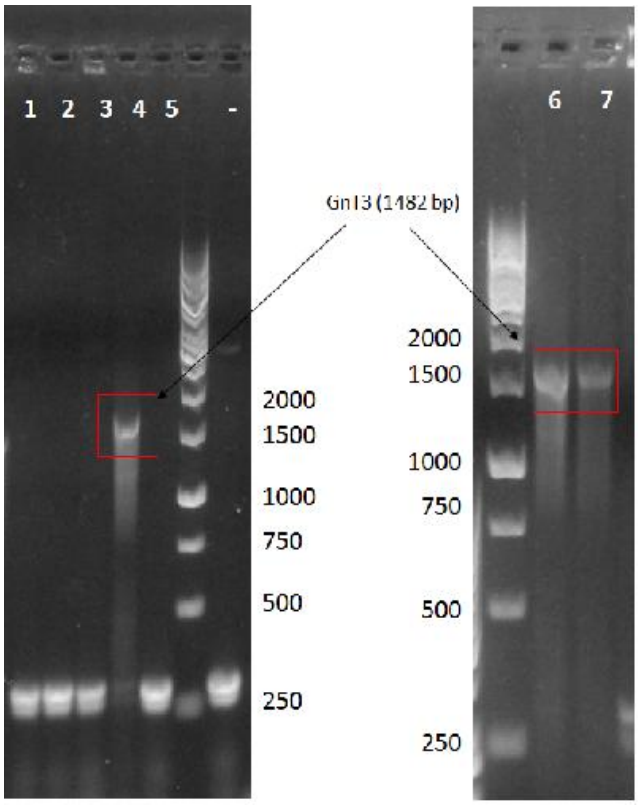
Digested the HBM fragment with BamHI and EcoRI at 37 degrees Celsius for an hour (Slow Digest)



Digested the GnT3 fragment with EcoRI and XhoI at 37 degrees Celsius for an hour (Slow Digest)

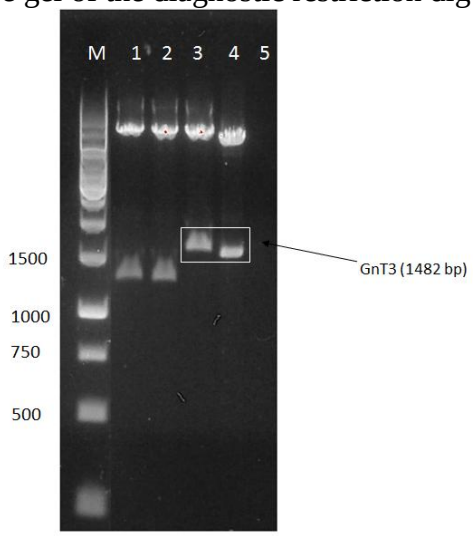
After the digestion of the DNA fragments, ligation was performed. The initial approach to the ligation is having a 3:1 ratio of the DNA products to the pFastbac1 vector. In this approach, the GnT3 was successfully ligated in the pFastbac1 vector. After digestion diagnostics and sequencing this was confirmed. Therefore, the ligation of HBM tag and pFastbac1_GnT3 vector was performed in having a 3:1 ratio as well of the HBM DNA product to the pFastbac1 vector. Although this approach has not been successful therefore another approach was done. The second approach is the ligation of the two DNA products: GnT3 and HBM PCR products in having a 1:1 ratio and ligating the GnT3 with HBM tag into pFastBac1 vector with a 3:1 ratio of the DNA to the vector. The ligated products were transformed into Top10 *E.coli* cells using the heat shock method. The negative plate consists of just the Top10 *E.coli* cells without the vector, therefore no colonies showed up. The positive plate consists of the Top10 *E.coli* cells with the pFastbac1 vector without the insert and a lawn of colonies showed up. Lastly, the plate with pFastbac1 with the insert have shown a couple of colonies and colony PCR was performed on these colonies. There were possible positive colonies that may have the vector with the insert.

The following figure shows the colony PCR of the pfastbac1_Gnt3+HBM



The primers that were used for this diagnosis are specific to the GnT3 gene therefore the product was found approximately at 1482 bp. The negative control had pFastbac1 without the insert and

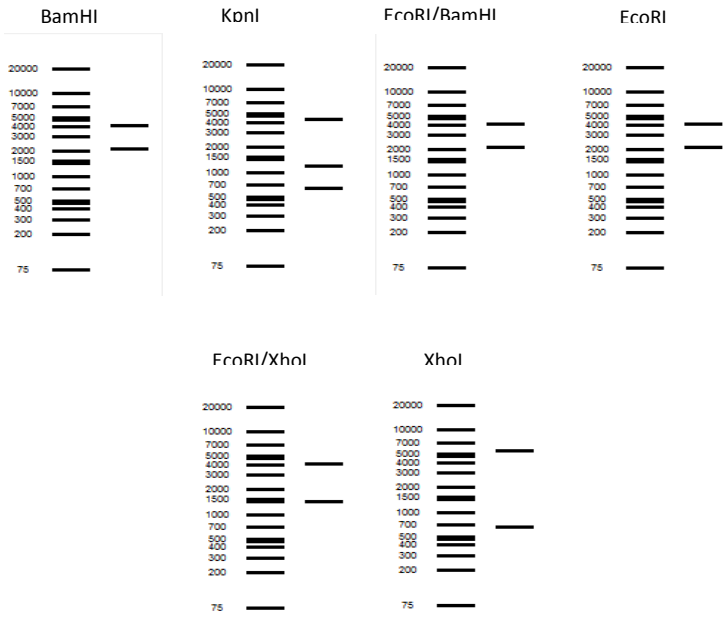
no band showed up. On the other hand, colonies 4, 6 and 7 shows insertion of the tGnT3His with HBM.
The vector DNA from the positive colonies were isolated using mini-prep and were digested with EcoRI and XhoI restriction enzymes for a diagnostic restriction digest test. The following figure shows the 1.5% Agarose gel of the diagnostic restriction digest.

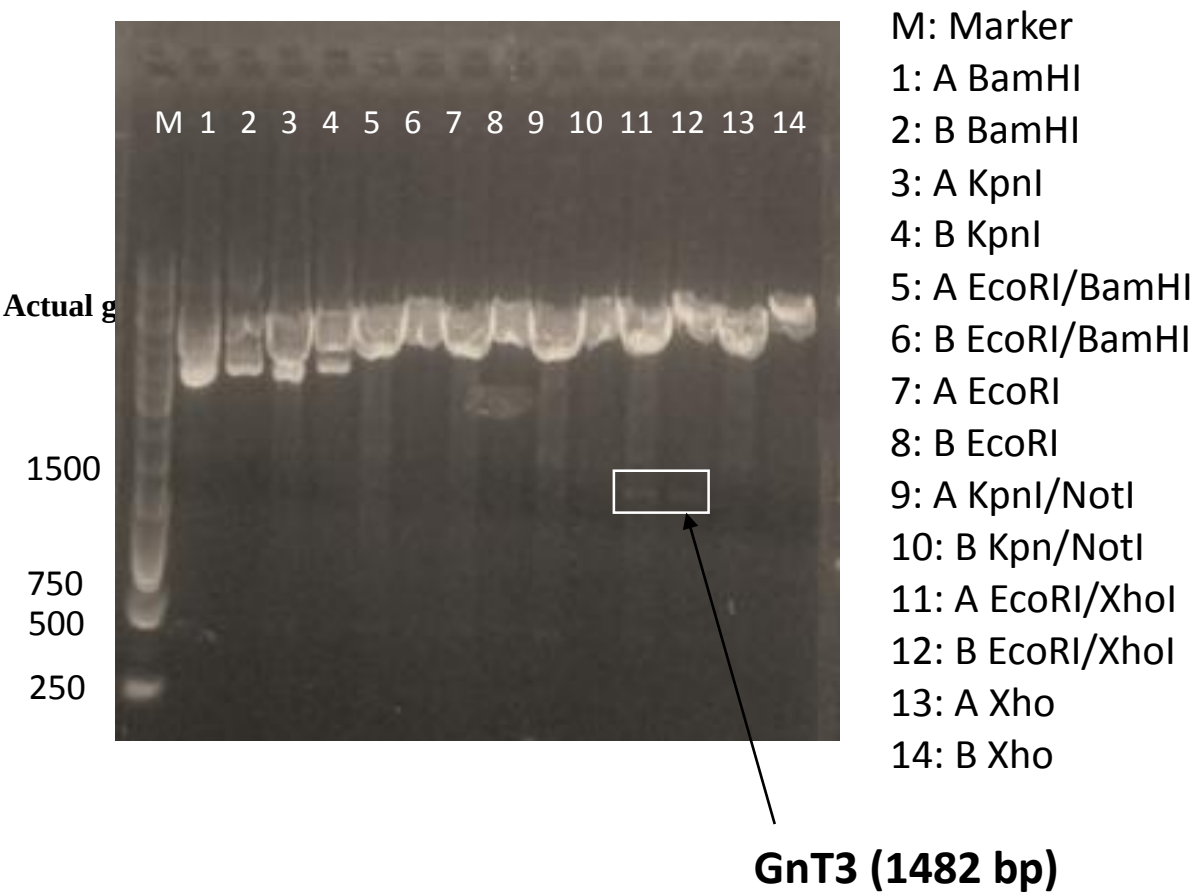


Legend:
M: Marker
1 – 4: Colonies digested with EcoRI/XhoI
5: Negative control: pFastbac1 without insert

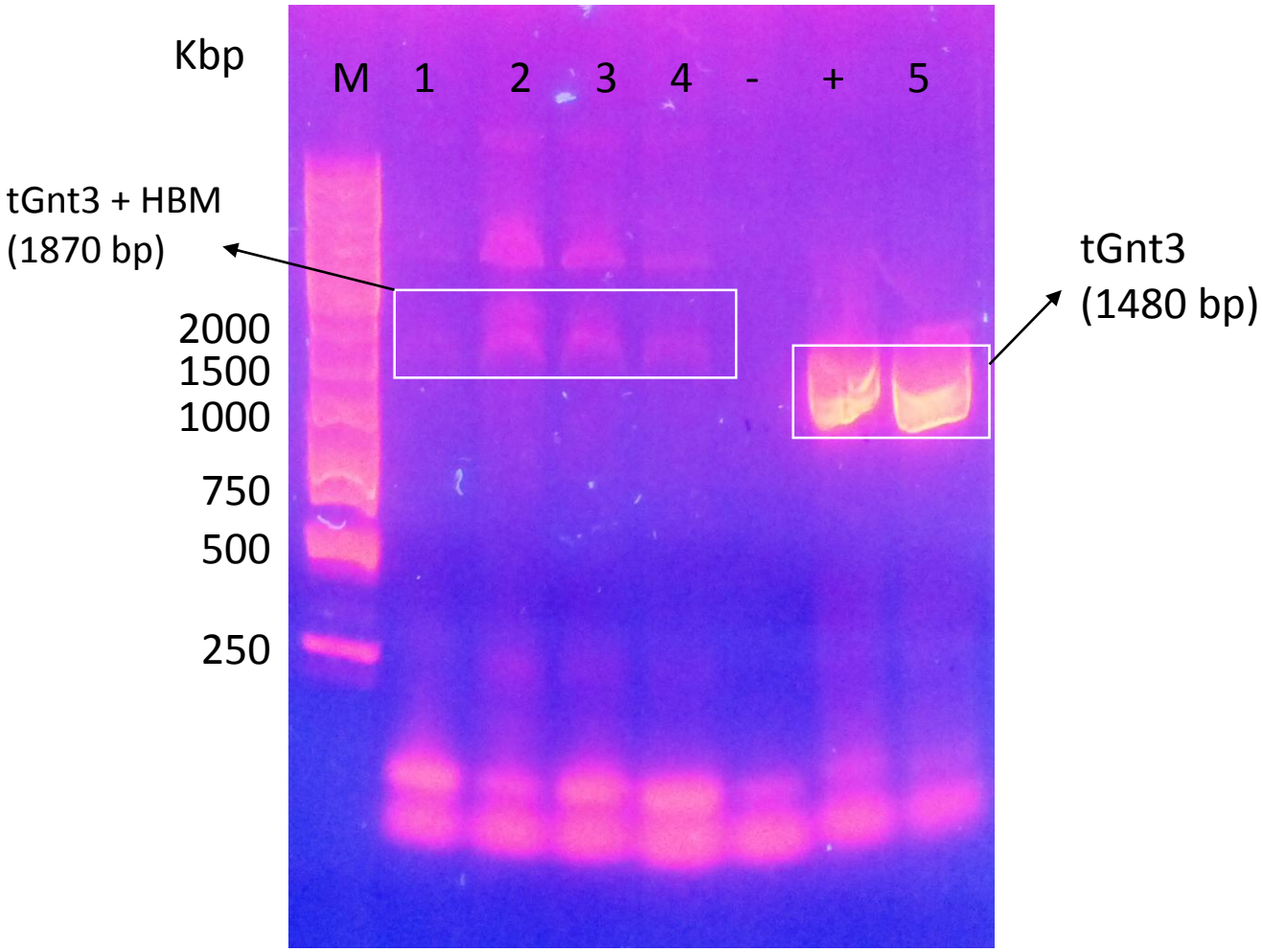
Colonies 3 and 4 were sent for sequencing although the results showed that the gene of interest was not properly ligated into the vector. Another restriction digest diagnostic was performed with different restriction enzymes in order to confirm the validity of the presence of the pfastbac1 vector with GnT3_HBM gene. The following figure shows the restriction digest diagnostic:

Expected gel results:





Also, another PCR amplification with the mini-prep was performed using different primers where the forward primer is specific to sequence 300 bp before the HBM tag and the reverse primer is specific to the GnT3 gene. The following figure shows the amplified gene product at approximately 1870 bp for the tGnT3 with HBM enzyme.

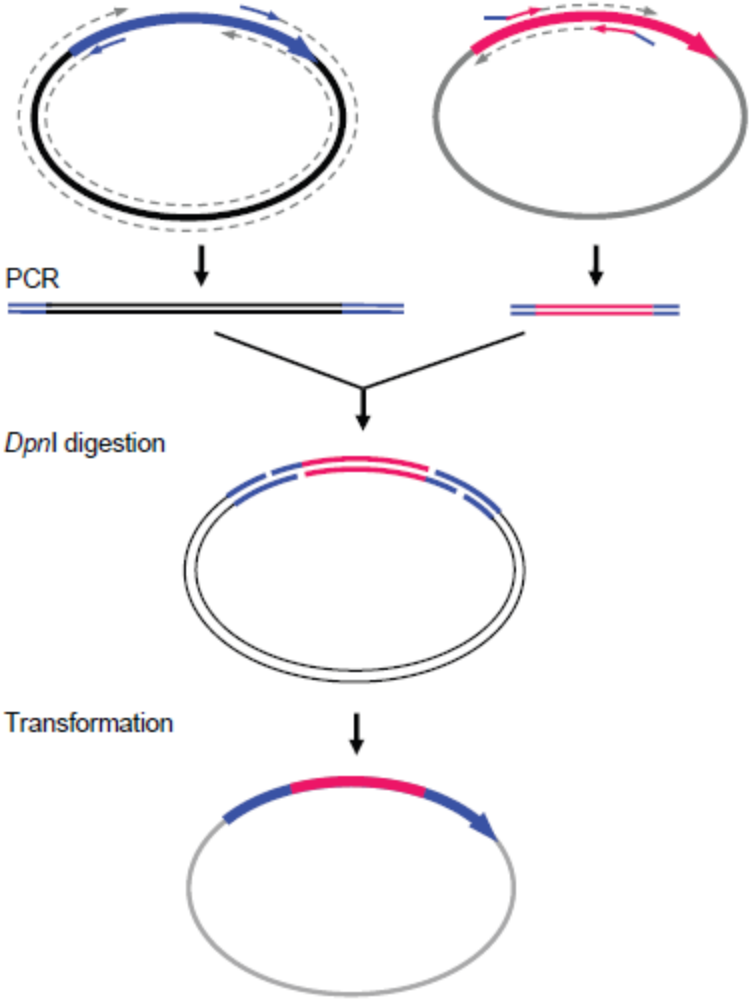


Legend:

Negative Control: no template; has all buffers, dNTPs, Taq polymerase
Positive Control: pAcSG2-Gnt3 template, all buffers, dNTPs, Taq polymerase
Lanes 1 – 4: Four different samples of mini-prep from various positive colonies (pFastbac1 with tGnt3 + HBM) with Phusion
Lane 5: Mini-prep sample of the positive colony for pfastbac1 + tGnT3 with Taq polymerase

Second: Fast Cloning

Fast cloning is where the vector is initially linearized and then the primers that were used had a bit of the vector and the gene of interest. The following figure briefly explains the process of Fast Cloning.



This idea was approached due to the restriction digests and ligation problems that were encountered since this approach does need restriction digestion and ligation. The primers that were used are the following:

Forward Primer:

5'CTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGAATTCatggagccaggagg

Reverse Primer:

5'GACCATAAAAACAAGGGCAACGTTGACTAAGAATTTCATTTCGGACCGGGATCCGCGCC

Although, this protocol did not work as well.

Third: Megaprimer PCR

Lastly, the last approach that was performed is megaprimer PCR. Megaprimer PCR is a method that involves two rounds of PCR which utilize "flanking" primers and one internal mutagenic primer containing the desired base substitution. Unlike other two step protocols, which uses Agarose gel electrophoresis whereas in megaprimer PCR this can be eliminated. It has been eliminated through the use of primers with significantly different melting temperatures to initiate the PCR steps which will ensure the selective synthesis of the only two primers present in the reaction. This approach consists of multiple PCR reactions and DpnI digest. The primers that were used are the following:

Forward Primer:

ACCATCGGGCGCGGATCTCGGTCCGAAACCATGAAATTCTTAGTCAACGTTGCCCTTGTT

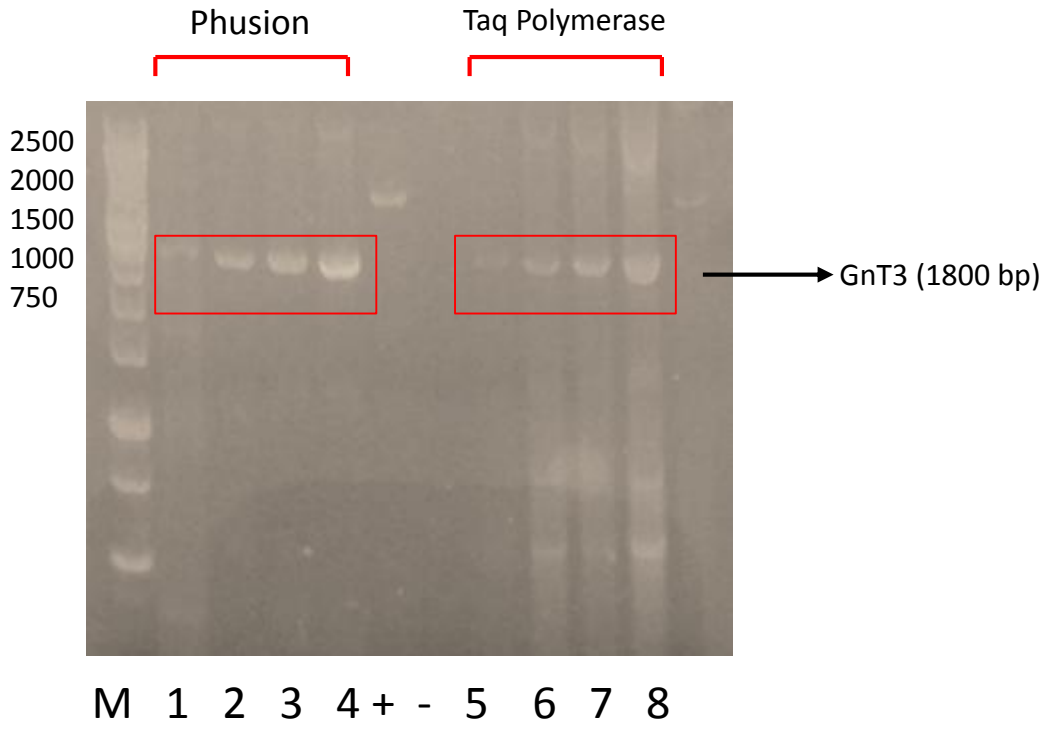
Reverse Primer:

AAGCGGCCGCACTAGTTGAGCTC**GTCGAC**GCGCATAGATGTAAGAAATGTATACGACCAT

Unfortunately, this approach didn't work as well. There were no positive colonies present when transformation using heat shock was performed.

Additional Update

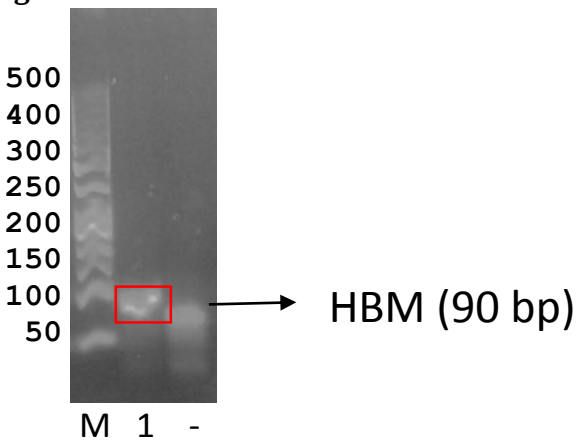
The previous colonies that were shown to be positive, the plasmid were isolate using the miniprep kit. It was amplified using Phusion and Taq polymerase to compare whether the band is more intense due to the polymerase or due to the amount of DNA in the miniprep



Legend:
M - Marker
1 and 5 – Colony 1
2 and 6 – Colony 4
3 and 7 – Colony 5
4 and 8 – Colony 6
+ - positive control (pFastbac1)
- - negative control (no template)

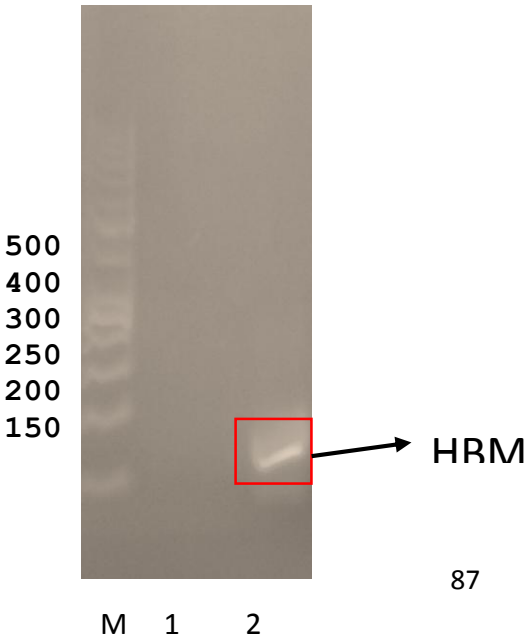
As shown on the gel, the bands from the Phusion enzyme is more intense than the Taq polymerase. Although, there not that much difference between the intensities.

Reamplification of HBM Tag



Legend: M – Marker, 1 – HBM PCR Product, 2 – No Template
The HBM tag was generated using overlapping PCR with the same conditions stated in **Table 3.** The PCR product was purified using the PCR kit and another Agarose gel electrophoresis was performed in order to make sure that there is enough DNA found in the solution after purification.

Product



Legend:
M – Marker
1 and 2 – Purified HBM

+ - Unpurified PCR Product

According to the PCR protocol, 100% isopropanol must be added if the DNA size is less than 500 bp, due to this the sample would not settle at the bottom the wells therefore as seen on the gel, there were no bands at around 90 bp.
Therefore, it was assumed that there are still DNA present in the sample and the digestion of the enzymes is performed.

Table 4: Components of Digestion Reaction

Fast Digest (BamHI/EcoRI)

	Double Digest
Water	16 uL
10x Buffer	2 uL
Enzymes (BamHI/EcoRI)	1 uL of each
DNA	10 uL
Total	30 uL

Slow Digest (EcoRI/XhoI)

	Double Digest
Water	11 uL
5x Buffer	5 uL
Enzymes (BamHI/EcoRI)	1 uL of each
DNA	10 uL
BSA	3 uL
Total	30 uL

The samples were incubated at 37 degrees Celsius for 5 hours and overnight. Then the enzymes are deactivated at 80 degrees Celsius.
After the enzymes were deactivated, the ligation reaction on the samples were performed

Table 5: Components of Ligation Reaction (using T4 Ligase)

Linear Vector (pFastBac with GnT3)	50 ng (1 ul)
Insert DNA (HBM)	5:1 molar ratio
10x T4 DNA Ligase Buffer	2 uL
T4 DNA Ligase	1 uL
H2O	11 uL
Total	20 uL

The ligation reaction was incubated at room temperature for 1 hour. After the ligation reaction, heat shock transformation was performed using Top10 *E.coli* cells.
Although after incubation of the transformed *E.coli* cells plated on LB-agar with 125mg/mL, there were no colonies found on the plate unlike the positive control with a lot of colonies with just the pFastBac vector with GnT3.
Therefore, there must be a possibility that the HBM DNA was lost during the purification therefore the digestion, ligation and transformation must be performed again.

Sequence of synthesized GnT3

BamHI — HBM Tag

ata **GGA TCC** **ATG** aaa ttc tta gtc aac gtt gcc ctt gtt ttt atg gtc

EcoRI

gta tac att tct tac atc tat gcg gat cga **GAA TTC** cat ttt ttt aaa

acc ctg agc tat gtg acc ttt ccg cgc gaa ctg gcg agc ctg agc ccg
aac ctg gtg agc agc ttt ttt tgg aac aac gcg ccg gtg acc ccg cag
gcg agc ccg gaa ccg ggc ggc ccg gat ctg ctg cgc acc ccg ctg tat
agc cat agc ccg ctg ctg cag ccg ctg ccg ccg agc aaa gcg gcg gaa
gaa ctg cat cgc gtg gat ctg gtg ctg ccg gaa gat acc acc gaa tat
ttt gtg cgc acc aaa gcg ggc ggc gtg tgc ttt aaa ccg ggc acc aaa
atg ctg gaa cgc ccg ccg ccg ggc cgc ccg gaa gaa aaa ccg gaa ggc
gcg aac ggc agc agc gcg cgc cgc ccg ccg cgc tat ctg ctg agc gcg
cgc gaa cgc acc ggc ggc cgc ggc gcg cgc cgc aaa tgg gtg gaa tgc
gtg tgc ctg ccg ggc tgg cat ggc ccg agc tgc ggc gtg ccg acc gtg
gtg cag tat agc aac ctg ccg acc aaa gaa cgc ctg gtg ccg cgc gaa
gtg ccg cgc cgc gtg att aac gcg att aac gtg aac cat gaa ttt gat
ctg ctg gat gtg cgc ttt cat gaa ctg ggc gat gtg gtg gat gcg
tttgtg gtg tgc gaa agc aac ttt acc gcg tat ggc gaa ccg cgc ccg
ctg aaa ttt cgc gaa atg ctg acc aac ggc acc ttt gaa tat att cgc
cat aaa gtg ctg tat gtg ttt ctg gat cat ttt ccg ccg ggc ggc cgc
cag gat ggc tgg att gcg gat gat tat ctg cgc acc ttt ctg acc cag
gat ggc gtg agc cgc ctg cgc aac ctg cgc ccg gat gat gtg ttt att
att gat gat gcg gat gaa att ccg gcg cgc gat ggc gtg ctg ttt ctg
aaa ctg tat gat ggc tgg acc gaa ccg ttt gcg ttt cat atg cgc aaa
agc ctg tat ggc ttt ttt tgg aaa cag ccg ggc acc ctg gaa gtg gtg
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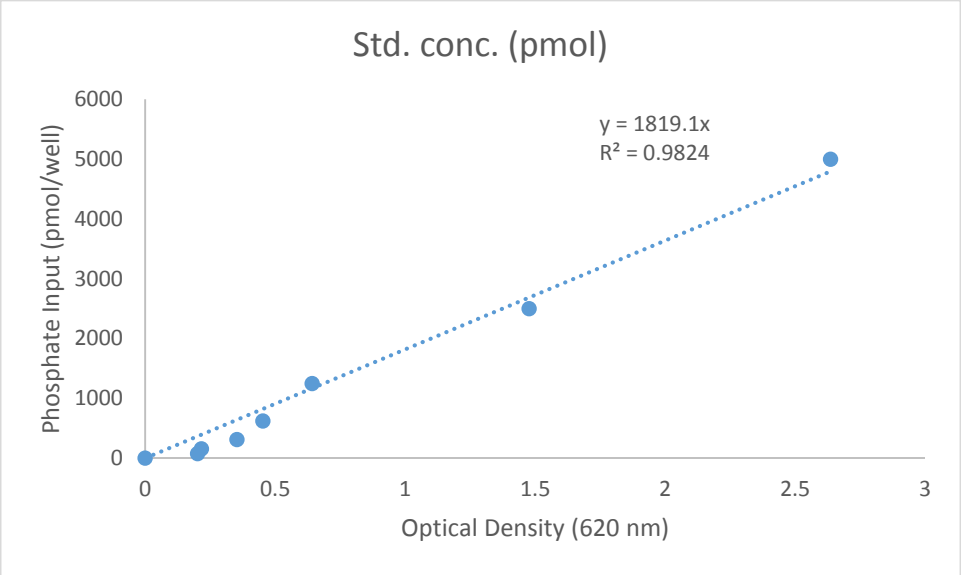
tat gaa aac cgc acc ggc cat att ctg gtg cag tgg agc ctg ggc agc
ccg ctg cat ttt gcg ggc tgg cat tgc agc tgg tgc ttt acc ccg gaa
ggc att tat ttt aaa ctg gtg agc gcg cag aac ggc gat ttt ccg cgc
tgg ggc gat tat gaa gat aaa cgc gat ctg aac tat att cgc ggc ctg
att cgc acc ggc ggc tgg ttt gat ggc acc cag cag gaa tat ccg ccg
gcg gat ccg agc gaa cat atg tat gcg ccg aaa tat ctg ctg aaa aac
tat gat cgc ttt cat tat ctg ctg gat aac ccg tat cag gaa ccg cgc
agc acc gcg gcg ggc ggc tgg cgc cat cgc ggc ccg gaa ggc cgc ccg
ccg TEV Cleavage Site
gcg cgc ggc aaa ctg gat gaa gcg gaa gtg aag ggc gaa aac ttg
tac

6x His-Tag

ttt caa ggc cat cac cat cac cat cac **TAG**



Appendix 2: ***Phosphate Standard curve determined using 1x Assay Buffer.*** The slope of the linear regression line, 1819 pmol/OD, which represents the amount of phosphate corresponding to a unit of absorbance at 620 nm. This is referred as the phosphate conversion factor (CF) which will be used for subsequent calculations.