Doctoral Dissertation

Engineering of Chinese hamster host cell line highly and stably producing sialylation using gene amplification

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DECLARATION

"I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which has been used in the thesis. This thesis has also not been submitted for any degree in any university/institution previously"

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Nguyen Thi Sam

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Contents

List of abbreviations

β-galactoside α2,6-sialyltransferase I	ST6GAL-I
β-galactoside α2,3-sialyltransferase I	ST3GAL-I
Antibody-dependent cellular cytotoxicity	ADCC
Asparagine	Asn
Bovine serum albumin	BSA
Chinese hamster ovary	CHO
Dihydrofolate reductase	DHFR/Dhfr
Fragment crystallizable	Fc
Immunoglobulin G	IgG
Internal ribosome entry site	IRES
Methotrexate	MTX
Minimum Essential Medium Eagle – Alpha Modification medium	α MEM
Liquid chromatography–mass spectrometry	LC-MS
N-Acetylglucosamine	GlcNAc
N-Acetylneuraminic acid	Neu5Ac
N-Glycolylneuraminic acid	NeuGc
Galactose	Gal
Mannose	Man
Phosphate buffered saline	PBS
Pyridylamine	PA
Reverse-phase high performance liquid chromatography	RP-HPLC
UDP-GlcNAc 2-epimerase/ManNAc kinase	GNE/Gne

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Chapter 1 General Introduction

1.1 Chinese hamster ovary cells for biopharmaceutical production

The first therapeutic protein approved for use, a human tissue plasminogen activator, was derived from recombinant mammalian cells in 1986. Since then, there has been emerging interest in mammalian cells as host cell lines for biopharmaceutical production. Even though there are various choices of platforms for protein expression, such as microorganisms, plants, and insects, mammalian cell lines have been prominently used as host cell lines for commercial therapeutic protein production. From 2014 to 2018, 155 biopharmaceuticals were approved and put on the US and EU market. Among them, about 79 % has been produced in mammalian cells (Walsh et al. 2018). Moreover, there are many kinds of available mammalian cells, such as those of mouse myeloma-derived NS0, baby hamster kidney, human retina-derived PerC6, Chinese hamster ovary (CHO), and human embryonic kidney HEK-293. Among these, CHO cells have been used as a main workhorse for biopharmaceutical production, accounting for 70% of the approved biopharmaceutical proteins on the market (Walsh et al. 2018).

CHO cells have gained popularity as a mammalian cell line for protein production due to its superior characteristics. Since CHO cells have proved to be safe expression systems for biopharmaceuticals for more than two decades, regulatory approval of CHO-based biopharmaceuticals is easier to obtain (Jayapal et al. 2007). Also, this cell line is well known in the field, so many researchers can easily access its characteristic for gene manipulation. Due to CHO cells' structural similarity to human glycan cells, proteins produced in CHO cells have been demonstrated as safe to human and less immunogenic than other cell types (Walsh et al. 2010). This cell type has efficient post-translational modification and thus can produce suitable bioactive proteins for human use. Furthermore, CHO cells can easily be adapted to serum-free medium in suspension conditions, a superior characteristic for industrial settings. However, one of the disadvantages of CHO cells compared to other mammalian cell lines is their low

specific productivity and their differences with human glycan structures. To solve this problem, gene amplification systems have been developed, such as dihydrofolate reductase/methotrexate (DHFR/MTX) or glutamine synthetase/methionine sulfoximine (GS/MSX).

The use of CHO as a host cell line has increased yearly by an average of 35% since 2001 (Aggarwal 2014). Thus, CHO cells may remain a favorite cell line for pharmaceutical production. Nevertheless, to improve this cell line type for a bio-better product there is demand to engineer characteristics of this cell type.

1.2 Glyco-engineering Chinese hamster ovary cells for pharmaceuticals productions

Sales of therapeutic glycoproteins have risen to tens of billions of dollars per year (Aggarwal 2014). These glycoproteins include many protein types, such as growth factors, hormones, cytokines, enzymes, clotting factors, Ig-Fc fusion proteins, and monoclonal antibodies (Ghaderi et al. 2012, Hossler et al. 2009, Lepenies and Seeberger 2014). The main force behind the development of these glycoprotein classes is the high demand for biopharmaceuticals in the treatment of infectious diseases, immune disorder, genetic disorders, cancers, and other diseases.

Glycan binding to proteins can be heterogeneous. It varies depending on the site of glycan occupancy and glycan structures (Varki et al. 2009). Various enzymes can participate in the formation of the final glycan structures. A number of glycans are generated by interaction between glycol-enzymes and oligosaccharide substrates for most proteins through endoplasmic reticulum (ER) and the Golgi compartments (An et al. 2003, Stavenhagen et al. 2013).

Specifically, in mammalian cells *N*-linked glycosylation occurs by a complex network reaction of glycol-enzymes such as glycosidases and glycosyltransferases in the ER and the Golgi compartments. Being initiated in the ER membrane, *N*-glycans start with the transfer of GlcNAc-P to dolichol phosphate (Dol-P) to form dolichol pyrophosphate *N*-acetylglucosamine (Dol-P-P-GlcNAc). Next, oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) has been formed by

adding 14 sugars to Dol-P-P-GlcNAc (Varki et al. 2009). Then, oligosaccharyltransferase (OST) transfers $Glc_3Man_9GlcNAc_2$ to the Asn of the polypeptide and releases Dol-P-P during the process (Aebi 2013). The oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ is trimmed by ER glucosidase enzymes such as α-glucosidases I and II, which are involved in the control of protein folding. Next, the precursor is cut by ER α-mannosidase I to form $Man_8GlcNAc$ glycoproteins once the glycoprotein is folded correctly. The $Man_8GlcNAc_2$ glycoproteins then exist from the ER compartment before translocating into cis-Golgi (Aebi 2010). Here, the $Man_8GlcNAc_2$ glycoproteins are further cut by other glycosidases in the Golgi, such as α-mannosidase I, to form $Man_5GlcNAc_2$ glycoproteins. These glycoproteins are key structures in the formation of other complex and hybrid *N*-glycoprotein structures (Aebi 2010).

The biosynthesis of complex *N*-glycoprotein in the medial Golgi is initiated by adding GlcNAc to the $Man_5GlcNAc_2$ glycoproteins by enzyme *N*-acetylglucosaminyltransferase (GnT-I) (Varki et al. 2009). Next, $GlcNAcMan_5GlcNAc_2$ is trimmed to form $GlcNAcMan_3GlcNAc_2$ by Golgi α-mannosidase II. The resultant *N*-glycan linked to proteins is then either does not extend further or is cut to release mannose residues to form a glycan with one or two Man residues. GlcNAc also can be added to the Man residues in the medial Golgi catalyzed by enzyme ß1,4-*N*-acetylglucosaminyltransferase III (GnT-III), which results in the bisection of GlcNAc structures. Next, the $GlcNAcMan_3GlcNAc_2$ linked to proteins is added to GlcNAc to form $GlcNAc_2Man_3GlcNAc_2$, a precursor for all multi-antennary structures of *N*-glycans, the reaction of which is catalyzed by ß1,2-*N*-acetylglucosaminyltransferase II (GnT-II). A more complex antennary can be generated by adding GlcNAc residues catalyzed by *N*-acetylglucosaminyltransferase IV (GnT-IV) and *N*-acetylglucosaminyltransferase V (GnT-V). In addition, another type of hybrid *N*-glycans can be formed in the trans-Golgi by adding core α1,6-fucose to the GlcNAc linked to Asn using α1,6-fucosyltransferase. Moreover, the branch can be elongated to form Gal ß1,4GlcNAc *N*-glycoproteins by adding ß-linked galactose to

GlcNAc using enzyme galactosyltransferase. Here, sialylated structures can be achieved by adding sialic acid to the terminal Gal residues by sialyltransferase enzymes (Varki et al. 2009). As mentioned above CHO cells have been predominantly used as a host cell line for biopharmaceutical production because its proteins are easily accepted by the human immune system (Butler and Meneses-Acosta 2012, Ghaderi et al. 2012). However, CHO also contains two types of nonhuman glycan structure: *N*-glycolylneuraminic acid (NeuGc) and Gal α1,3-Gal linkages. These differences in glycan structures can be immunogenic to human (Ghaderi et al. 2012, Padler-Karavani 2008). Glycosylation is an important factor for the measurement of biopharmaceutical qualities because of its effects on aggregation, half-life, thermal stability, efficacy, protease resistance, stability, immunogenicity, and solubility (Sareneva et al. 1995, Sola and Griebenow 2010, Spearman and Butler 2015, Wright and Morrison 1997). Thus, a number of studies have been performed to alter the glycan profiles of CHO for biopharmaceutical production.

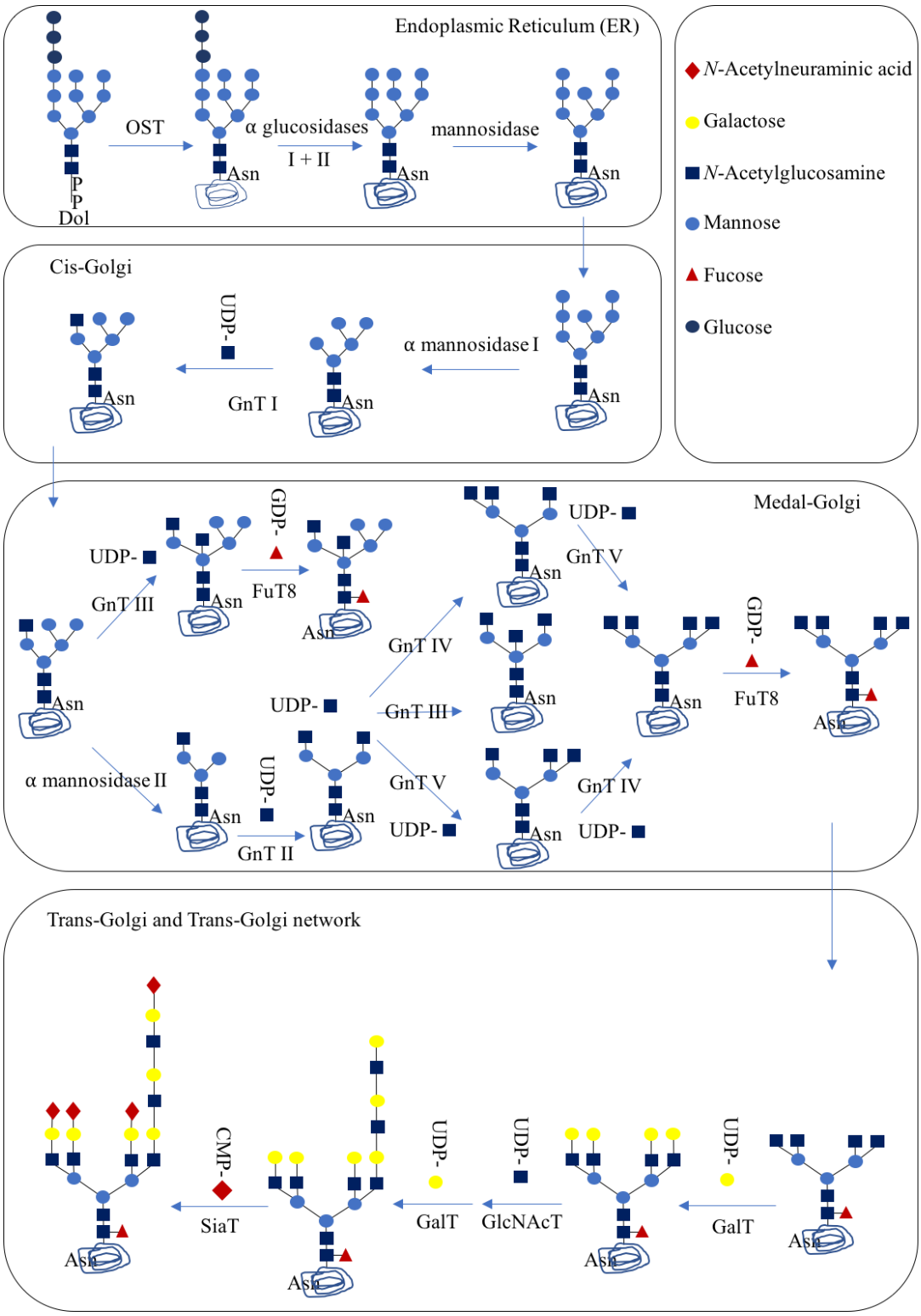


Fig. 1 Glycosylation pathway in CHO cells
OST: Oligosaccharyltransferase; GnT I-V:β-*N*-acetylglucosaminyltransferase I-V; FuT8: α1,6-fucosyltransferase; GlcNAcT: *N*-Acetylglucosaminyltransferase; GalT: β1,4-galactosyltransferase; SiaT: sialyltransferase

1.3 Sialylation pathway in mammalians cells and glyco-engineering Chinese hamster ovary cells for improving sialylation

The sialic acid terminal of glycosylation, *N*-acetylneuraminic acid (Neu5Ac), plays a more important role in biopharmaceutical half-life than other sugar residues in glycans. Sialic acid is a negatively charged acidic 9-carbon moiety. It is linked to the C8-, C6-, or C3-hydroxyl group of the galactose terminal in glycan structures via sialyltransferase enzyme β-galactoside α2,3-sialyltransferases (ST3GAL-I to ST3GAL-VI) or β-galactoside α2,6-sialyltransferases (ST6GAL-I and II) in human (Li et al. 2012). Sialylation can occur through α2,3; α2,6; or α2,8-linkage to galactose residues (Angata and Varki 2002, Angata and Varki 2002, Wang et al. 2015). In CHO, Neu5Ac terminals are linked to glycoproteins by only the α2,3-linkage due to the lack of expression of ST6GAL enzymes. Sialic acid on glycoprotein can improve its bioactivity and circulatory half-life. Terminal sialic acid can be used as a mask for glycoproteins. Thus, it protects galactose residues from removal in the circulation of blood because of the interaction between hepatocytes and asialoglycoprotein receptors in the endocytosis-mediated pathway (Wang et al. 2015, Ashwell and Harford 1982, Cole et al. 1993). Thus, there is strong demand to maximize the sialic acid content of glycoprotein in CHO cell lines.

Many studies have attempted to optimize the conditions for bio-parameter processing in glycoprotein production. Bioprocess parameters include pH, dissolved oxygen (DO), temperature, and time on glycan profiles. However, the results obtained have not been definitive, because the effects rely on the cell types and targeted glycoproteins. Also, number of studies have altered sialylation. Trummer et al. (2006) demonstrated that reducing culture temperature reduced the number of sialylated patterns. The reduction of temperature after the exponential growth phase causes a big decrease in NeuGc (Borys and Abu-Absi 2010). DO is also considered a parameter proved to increase the sialic acid content of human follicle-

stimulating hormone expression (Chotigeat and Gray 1994). According to Yang et al. (2000a), ammonium supplements resulted in a decrease in the number of sialylated glycan structures. Thus, the results obtained vary and are not so effective at altering glycosylation by the modulation of process parameters, even though potential effectiveness is expected due to its easy manipulation.

Although many efforts have been made to modulate the glycosylation pathway in CHO cell lines, the resultant glycoforms did not show big improvements or cost effectiveness. Thus, there is strong demand for a more direct, stable, and cost-effective method by gene modification, such as inserting or deleting targeted genes involved in glycosylation pathways.

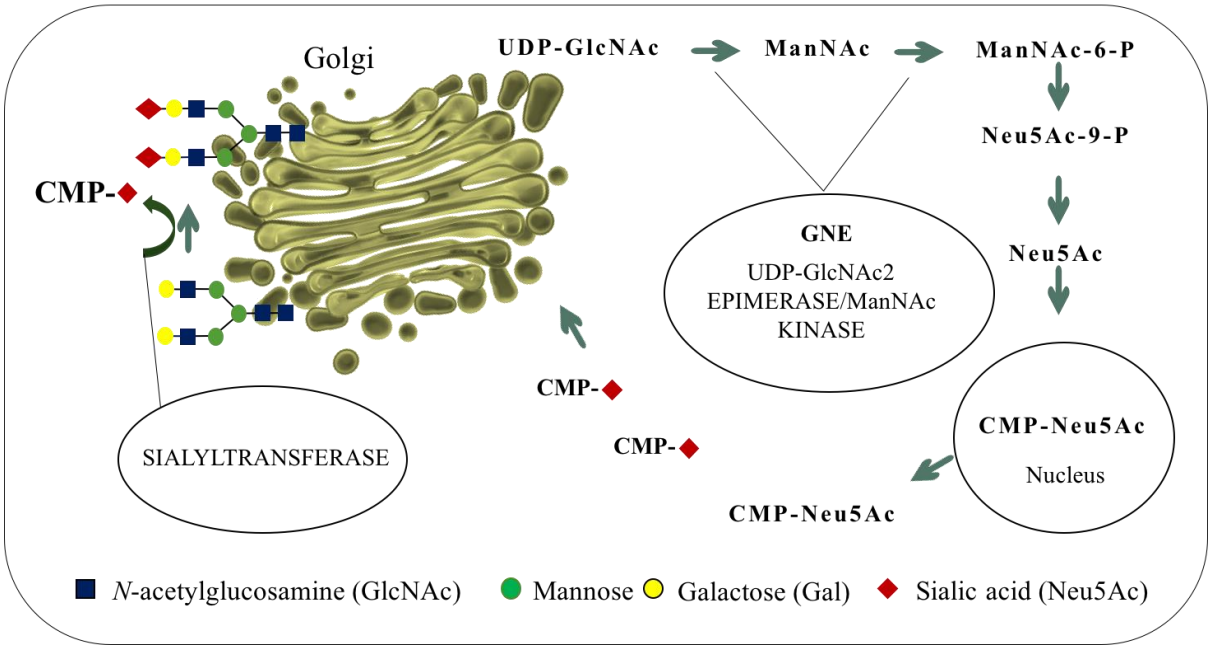


Fig. 2 Sialylation pathway in mammalian cells

1.4 Dihydrofolate reductase / methotrexate (DHFR/MTX) for genes amplification in Chinese hamster ovary cells

Low yield is one of the disadvantages of CHO cells as a host cell line for the production of therapeutics. Targeted genes have been amplified to solve this problem. Thus far, however, studies using this amplification method have focused mostly on amplifying therapeutics for a

better product. Since the spontaneous amplification frequency of genes in CHO cells is very low, few studies have used gene amplification to improve targeted homogeneous genes in CHO cells. Several cytotoxic drugs, such as methotrexate, methionine sulfoximine, PALA, cadmium, and histidinol, help to induce the amplification of genes of interest. The first study was performed in 1957 (Hakala 1957). DHFR/MTX is a common gene amplification method used in CHO cells. MTX is known to inhibit DHFR, which is involved in nucleotide synthesis. Thus, DNA replication stops when DHFR is absolutely inhibited (Pallavicini et al. 1990, Gandor et al. 1995). The majority of cells die after 2 to 3 weeks, but some cells can overproduce DHFR. The cells obtained through the single-cell selection can have hundreds to thousands of gene copies of plasmids integrated in chromosomes (Pallavicini et al. 1990, Wurm et al. 1986). The enrichment of specific productivity obtained through gene amplification varies and depends on individual clones, but the increase in productivity can be 10- to 20-fold (Zettlmeissl et al. 1988). Gene amplification has been widely used for the production of therapeutics. However, the utility of this method for enriching sialylation in therapeutics is still unconfirmed, since not many such studies have been conducted.

1.5 Autoimmune disease: causes, symptoms, and treatments

About 70 different kinds of disorders are defined as autoimmune disease (AD), afflicting about 5% of the population of Western countries. They can occur under various conditions such as in certain age ranges, as a result of some treatments, and some targeted tissues. It was reported that AD appears because of the complexity of interactions between environmental factors, defective regulation, and genetic susceptibility such as autoantibodies, loss of immune homeostasis, or upregulation of inflammatory cytokine (Selmi et al. 2004, Burek et al. 2009, Dedeoglu 2009, Selmi and Gershwin 2009). All AD mechanisms are believed to advance in phases from initiation and propagation to resolution. In human AD, many antibodies in serum work directly against functional components of cells such as receptors, nucleic acids, or nuclear

molecules. Thus, these antibodies can be used as important factors to classify and diagnose AD. Many studies have pointed out that AD patients can carry autoantibodies (AAbs) for a long time before the first symptom emerges. Thus far, however, the reason for the presence of AAbs in AD patients is still unclear. It is believed that AAbs can occur naturally or result from the progress of cancer, tissue damage, and paraneoplastic syndromes. These abnormal reactions of the autoimmune system can lead to some common type of diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis, and Type 1 diabetes mellitus. The common symptoms are fatigue, joint pain and swelling, skin problems, fever, and abdominal pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) and immune-suppressing drugs have been developed to treat AD; the latter provide better and longer effects. The immunosuppressive drugs most commonly used are cytokines, checkpoint inhibitors, B-cell targeting agents, and antibodies. Meanwhile, plasma human immunoglobulin G (IgG) has been long used to modulate immunity for diseases such as chronic inflammatory demyelinating polyneuropathy, systemic lupus erythematosus, and immune thrombocytopenia. There is great interest in improving the quality of this IgG to improve treatment for AD patients.

1.6 Antibodies IgG and sialylation as treatment for autoimmune disease

IgG antibodies are well known for their ability to protect the body against pathogens. These antibodies also play important roles in the inflammation and destruction of tissue in autoimmune diseases such as systemic lupus erythematosus and RA. However, intravenous immunoglobulins, IgG antibodies prepared from thousands of human donors, are known as effective treatments for chronic inflammation and autoimmune diseases. Several studies have proved the functions of oligosaccharides attached to the Fc fragment of IgG. Glycans can block or enrich the pro- and anti-inflammatory activities of antibodies. It was reported that an IgG with low levels of sialic acid terminal and galactose terminal in *N*-glycan is related to active autoimmune disease including Crohn's disease, SLE, and RA (Lauc et al. 2013, Parekh et al.

1985, Scherer et al. 2010, Shinzaki et al. 2008). In addition, the infusion of IVIg in Kawaski disease patients was shown to induce sialylated IgG glycovariants and was associated with enhanced treatment response in these patients (Ogata et al. 2013). An IVIg with tetra-antennary sialylation of Fc showed up to a 10-fold increase in inflammatory activity compared to random IVIg (Washburn et al. 2015). Sialic acid residues can help IgG antibodies bind to receptors on the surfaces of immune cells such as neutrophils, macrophages, mast cells, and complement component C1q by modulation of carbohydrate chains. Thus, these residues can avoid the induction of autoimmune reactions and inflammation (Nimmerjahn and Ravetch 2007). Recently, bone loss in RA patients was found to be associated with desialylated IgG. *N*-Acetylmannosamine (ManNAc) supplementation results in an acceleration of IgG sialylation to suppress inflammation, thus inhibiting bone loss. Sialic acid moieties in IgG glycans can have effects on anti- and pro-inflammatory as well as cytotoxic activities (Anthony et al. 2008, Aschermann et al. 2010). IgG antibodies with low sialylation can activate cytotoxic activity by binding to the FcγR receptor. The highly sialylated IgG in the Fc regions can reduce the binding activity of receptor FcγR to antibodies, resulting in the loss of cytotoxic activity. We can infer that IgG carrying a high level of sialylation is an attractive anti-inflammatory therapeutic for AD to against auto-antibodies. IgG was naturally found with a low percentage of sialylation around 5% (Kaneko et al. 2006). Taking all of these results together, IgG was chosen as a good model protein for our study to prove of the concept that the engineered cell line can produce highly sialylated glycoproteins.

1.7 Objectives of this study

Because of their superior quality and long history of production as a therapeutic, it is possible that CHO cells might persist as the main host cell line among mammalian cells. Thus, many efforts have been made to improve this cell line. One strategy to improve the quality of CHO cells is to modify their glycan patterns. Glycosylation is known as an important factor in the

quality of therapeutics. Meanwhile, sialylation of the therapeutics produced in CHO cells can modulate the functions of these proteins. Collectively, in this research I aimed to glyco-engineer this CHO cell line by improving its sialylation. Multiple experiments were performed toward this goal. I have established a CHO host cell line carrying Neu5Ac-based key enzymes to improve sialylation using the DHFR/MTX method.

To demonstrate the ability of the engineered CHO cell line to produce highly sialylated therapeutics, I produced IgG subclass antibodies on it. The IgG produced in this targeted cell line is expected to carry a high level of sialylation on its *N*-glycan structures at Asn 297. Since Neu5Ac terminal IgG is known as an effective therapeutic for AD, the resultant IgG can be a potential candidate for AD treatment with high sialic acid content in glycosylation binding to antibodies.

This approach aims to contribute to a better method of engineering CHO host cell lines to produce better therapeutics.

Chapter 2: Generation of glyco-engineered Chinese hamster ovary cell lines

2.1 Introduction

Because the glycan structures derived from some mammalian cells are close to human glycoforms (Xu et al. 2011), the mammalian has been prominently used as host cell lines for biopharmaceutical production. There are many available types such as CHO, 3T3, HEK293, BHK, HeLa and HepG2, rodent- or human-derived cells, which are often used in biopharmaceutical research for numerous protein expressions (Jayapal et al. 2007). Currently, nearly 70% of recombinant protein therapeutics produced in CHO cells are commercial in the market (Jayapal et al. 2007). Since the use of biopharmaceuticals generated from CHO cells has escalated in the last twenty years, these therapeutics are now among the ten best-selling biopharmaceutical products (Huggett and Lähteenmaki 2011).

Numbers of biopharmaceutical products produced in CHO cells have been recognized as safe for their potential clinical use, CHO cells have become a biopharmaceutical workhorse for production of therapeutics (Jayapal et al. 2007). Moreover, CHO cells show its resistance to many human pathogenic viruses such as HIV, influenza, polio, and herpes (Jayapal et al. 2007, Shukla et al. 1999, Tyagi et al. 2001). Also, CHO cells can be readily scaled-up in industrial settings because of their adaptability and their ease of genetic modification such as its ease of adapting in serum-free suspension culture (Jayapal et al. 2007). Therefore, CHO cells can be used on an industrial scale with a high accretion in cell suspension cultures. Taken together, at least into the near future CHO cells may persist as a premier host cell line for biopharmaceutical production.

The terminal sialylation of *N*-glycosylation in therapeutics is one of the most important considerations for the quality attribution. Thus, this aspect should be carefully elucidated in recombinant protein production process (Lipscomb et al. 2005). As mentioned previously, the Neu5Ac, known as sialic acid, is well-known as an important moiety because of its biological

functions. Neu5Ac can modulate the stabilization and the interaction between molecules and membranes in various organisms because of its strong electronegativity. Moreover, Neu5Ac terminals can enhance the stability of glycoprotein formation and to protect it from digestion by proteases and glycosidases. This moiety is also believed to alter signaling in the transmembrane, as well as the differentiation and growth of cells. Also, the Neu5Ac terminal glycans is reported to have antioxidant effects and help itself to against apoptosis (Varki et al. 2009). With so many superior effects, maximizing Neu5Ac terminals in glycoproteins are highly desired in biopharmaceutical glycoprotein production field.

The α2,6-linkages which linked terminal Neu5Ac to galactose residues are predominant in human proteins using enzyme β-galactoside α2,6-sialyltransferase (ST6GAL-I and II). Nevertheless, CHO glycans have primarily only α2,3-linkages (Xu et al. 2011, Santell et al. 1999, Takeuchi et al. 1989). Therefore, contain α2,6-linked Neu5Ac terminal therapeutics would be more favourable in human. Thereupon, maximizing the α2,6-linked Neu5Ac terminal in recombinant proteins are believed to be desirable. The α2,6-linked sialic acid residues are known as important factor for the anti-inflammation activity of human intravenous immunoglobulin (Anthony et al. 2008, 2010). This aspect might also be important for contributing to the quality of the therapeutics. Accordingly, CHO host cell line containing superior α2,6 linked Neu5Ac contents might be beneficial for host cell line engineering field.

NeuGc exists in CHO-derived glycoproteins but it does not occur in humans. This is because the gene encoding cytidine monophosphate (CMP)-*N*-Neu5Ac hydroxylase in humans does not have an *N*-terminal domain required for its activity (Irie and Suzuki 1998). Also, another key enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), is known for Neu5Ac synthesis. Glycoproteins with NeuGc residues are not desirable products in the biopharmaceutical field since humans can sometimes produce high level of circulating anti-NeuGc antibodies (Padler-Karavani et al. 2008).

In consideration of the above, by introducing ST6GAL-I and Gne genes, we attempted the glyco-engineering of CHO *N*-glycans. We elucidated the effects of the stable expressions of these two Neu5Ac-based genes on the glycosylation profiles of CHO cell lines. In specific, by using reverse phase-high-performance liquid chromatography (RP-HPLC), the free sialic acid content and enzyme activity were measured. Additionally, the *N*-linked glycosylation profiling was also revealed by using liquid chromatography-mass spectrometry (LC-MS). The results obtained reveals an increase of free sialic acid content and an increase of sialylation in the total *N*-glycans of total proteins. We therefore conducted the present study to obtain proof of concept that the resulting glycosylation in engineered CHO cells would consistently exhibit superior sialylation.

2.2 Materials and Methods

2.2.1 Cells and culture media

The host cell lines CHO-DG44 were cultured in a Minimum Essential Medium Eagle – Alpha Modification medium (α MEM) (Invitrogen, Massachusetts, USA) with nucleosides and L-glutamine supplemented with 10% fetal bovine serum (FBS). The transfected CHO cell lines were cultured in α MEM supplemented with L-glutamine, 10% FBS and 100 µg/mL neomycin. Next, the selected transfectants were cultured in an (-) α MEM medium (Invitrogen) without nucleosides, supplemented with L-glutamine, 10% dialyzed FBS and 100 µg/mL neomycin. All of cell lines were cultured in an incubator maintained at 37^{o}C and 5% CO_2.

2.2.2 Expression construct

An attenuated internal ribosome entry site (IRESmt) was established by modifying the IRES wild-type (IRESwt) with point mutation from the Encephalomyocarditis virus (Invitrogen) at the ATG 10th, 12th. IRESwt was point mutated from A to G by using primers, as described previously (Koh et al. 2013). The plasmid named as pcDNA-SG2 was next obtained by the insertion of open reading frame sequences for glycosylation genes ST6GAL-I, Gne and Dhfr

linked by IRESwt and IRESmt, respectively, into a pcDNA3.1 (+) expression plasmid (Invitrogen). This plasmid contained a neomycin resistance coding sequence. These DNA fragments were obtained by using PCR in a 96-well thermal cycler (Applied Biosystems, Massachusetts, USA) with the following primers:

5’ GTTGGTGTGAATCATCAAGCTTAAGTTTAAACGCTAGCCAG 3’ (sense for pcDNA 3.1 (+), 5’ GTTGGTGTGAATCATCAAGCTTAAGTTTAAACGCTAGCCAG 3’ (anti-sense for pcDNA 3.1(+),

5’ TTAAACTTAAGCTTGATGATTCACACCAACCTGAAGAAAAAGTTCAGCTG 3 ‘(sense for ST6GAL-I), 5’ GGGGAGGGAGAGGGGTTAGCAGTGAATGGTCCGGAAGCC 3’ (anti-sense for ST6GAL-I),

5’ ACCATTCACTGCTAACCCCTCTCCCTCCCCCCC 3’ (sense for IRESwt), 5’ CCCATTCTTCTCCATAACATGGTTGTGGCAACATATTATAACATCGTGTTTTTCAAAG 3’ (anti-sense for IRESwt),

5’ AGCCACAACCATGTAATGGAGAAGAATGGGAATAACCGAAAGCTTC 3’ (sense for Gne), 5’ GGGGAGGGAGAGGGGCTAGTGGATCCTGCGGGTTGTG 3’ (anti-sense for Gne),

5’ CGCAGGATCCACTAGCCCCTCTCCCTCCCCCCC 3’ (sense for IRESmt), 5’ CAATGGTCGAACCATCACGGTTGTGGCCATATTATCACCGTG 3’ (anti-sense for IRESmt),

5’ ATGGCCACAACCGTGATGGTTCGACCATTGAACTGCATCG 3’(sense for Dhfr) and 5’TCAGCGGGTTTAAACTTAGTCTTTCTTCTCGTAGACTTCAAACTTATACTTGATGCC 3’ (anti-sense for Dhfr).

The prepared DNA fragments were then ligated by using an In-fusion cloning kit (Takara, Shiga, Japan). This plasmid pcDNA-SG2 was next transfected into CHO-DG44 cells using

Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The plasmid of pcDNA-SG2 was not cut to linear. Then, the plasmid was transfected into 80 – 90% frequency of CHO wild-type cells using Lipofectamine 2000 supplemented with the final concentration of G418 (Wako, Tokyo, Japan) 800 μg/mL. Then, the resultant transfectants were maintained in the concentration of 100 μg/mL G418 in (-) alpha MEM medium without nucleosides.

2.2.3 Quantitative real-time PCR (qRT-PCR)

Cultured cells (1x 10^7) were harvested after washing well with phosphate buffered saline (PBS). To extract total RNA from collected cells, an RNeasy Mini Kit (Qiagen, Hilden, Germany) was employed following the manufacturer's instructions. The RNA was next used to synthesize complementary DNA (cDNA) using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) as described by the manufacturer's instructions. A qRT-PCR using FastStart Universal SYBR Green Master Mix (Rox) (Roche, Basel, Switzerland) was performed using a StepOnePlus™ real-time PCR system (Applied Biosystems) with the following primers: 5' TGTGTGGAAGGAAAAGAAGAAAGG 3' (sense primer for ST6GAL-I), 5' GGGTGCTGCTTGAGGATACA 3' (antisense primer for ST6GAL-I), 5' GTGGAAGGGATGTCAGTACCAAA 3' (sense primer for Gne);, and 5' CCAAGTCCCAAAGCAGTTCC 3' (antisense primer for Gne) to examine the expression of the investigated genes in transfected cells.

2.2.4 Preparation of cell extracts in selected cell lines

The investigated CHO cell lines were homogenized by using ultra-sonication with 25 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 50 mM KCl, and protease inhibitor (Roche). Next, this mixture was ultra-centrifuged for 1 hr at 4°C and 112,000×g. The resultant supernatant was used for sialic acid quantitation assays and the pellet was used to perform ST6GAL-I enzymatic assay.

2.2.5 ST6GAL-I enzymatic assays in CHOmt17-0

The microsomes were re-suspended in 20 mM cacodylic acid (pH 6.0) with 0.1% Triton X-100. The mixture was then incubated centrifuged at 10,000×g for 10 min at 4°C for 1 hr to collect the supernatant. The harvested supernatant was then added to an assay mixture consisting of 20 mM cacodylic acid (pH 6.0), 20 mM $MgCl_2$, 5 mM CMP-Neu5Ac, 10 pmol Gal2-Gn2-M3-PA (Gal: galactose, Gn: *N*-acetylglucosamine, M3: 3 mannose and 2 Gn, PA: pyridylamine) and protease inhibitor. Next, the mixture was incubated at 37°C for a defined time. The mixture was heated at 100°C for 3 min and centrifuged at 10,000×g for 5 min after incubation. The resultant supernatant was next examined using a RP-HPLC. The glycan standards used are Neu5Ac(B)α2,6-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,6Gal2-Gn2-M3-PA, Neu5Ac2α2,6-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA, M8C (Takara) and Neu5Ac(B)α2,3-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,3-Gal2-Gn2-M3-PA, and Neu5Ac2α2,3-Gal2-Gn2-M3-PA (Masuda Chemical Industries, Kagawa, Japan). PA-sugar chains were detected on an RP-HPLC apparatus (Hitachi 7000 HPLC system, (Hitachi, Tokyo, Japan)) at the excitation and emission wavelengths of 310 nm and 380 nm, respectively using a Cosmosil C18 column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan). The fraction is eluted by linearly increasing the acetonitrile concentration from 0% to 35% in 0.02% trifluoroacetic acid (TFA) at 30^{o}C for 50 min at a flow rate of 1.2 mL/min.

2.2.6 Free sialic acid quantitation in CHOmt17-0

According to the manufacturer's instructions, the free Neu5Ac and NeuGc from the supernatant of cell extracts was labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) using a sialic acid fluorescence labeling kit (Takara). The DMB-sialic acids level were next monitored on an RP-HPLC apparatus (Hitachi 7000 HPLC system) using a Cosmosil C18 column (4.6 × 250 mm; Nacalai Tesque) and were eluted with an isocratic solution containing 4% acetonitrile and 7% methanol at 1.2 mL/min flow rate at the excitation and emission wavelengths of 373

nm and 448 nm, respectively. The amounts of free sialic acids were estimated by measuring the peak area on the RP-HPLC profiles.

2.2.7 Glycan structure analysis of total proteins in CHOmt17-0

Investigated host cell culture (1 x 10^8) were well washed with PBS before adding lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% sodium dodecyl sulfate (SDS). By using peptide-*N*-glycosidase F (Takara), *N*-glycans were released from lyophilized lysate according to the manufacturer's instructions. The resultant sugar chains were then labeled with PA and subjected to phenol/chloroform extraction for removal of unreacted PA as previously described (Kondo et al. 1990). Next, the PA-sugar chains were fractionated by using RP-HPLC under the same assay conditions mentioned above. By using an LC-MS system (Agilent Technololies, CA, USA), the fractions eluted from RP-HPLC were then analyzed with HTC plus software (Bruker Daltonics, MA, USA) as previously described (Limkul et al. 2016). In brief, the mobile phase of the LC was consisted of acetonitrile/acetic acid used as solvent A (98/2, v/v). The solvent B was composed of water/acetic acid/trimethylamine (92/5/3, v/v/v). The column Shodex Asahipak NH2P-50 2D (2.0 mm ID x 150 mm, Showa Denko, Tokyo, Japan) was used for separation of PA-sugar chain by linearly increasing concentration of solvent B from 20% to 55 % at 0.2 mL/min flow rate over 35 min. The MS/MS parameters conditions were following as: nebulizer flow at 5.0 psi; scan range *m/z* at 350-2750. Hence, the peak area of the LC was used for evaluation the *N*-glycan relative amount.

2.3 Results

2.3.1 Establishment of cell lines highly producing ST6GAL-I and Gne expressions

The tri-cistronic expression of ST6GAL-I, Gne and Dhfr was established and driven by a single CMV promoter. In Figure 3, these three DNA fragments were then linked to each other by IRESwt and attenuated IRESmt. This expression vector was named as pcDNA-SG2.

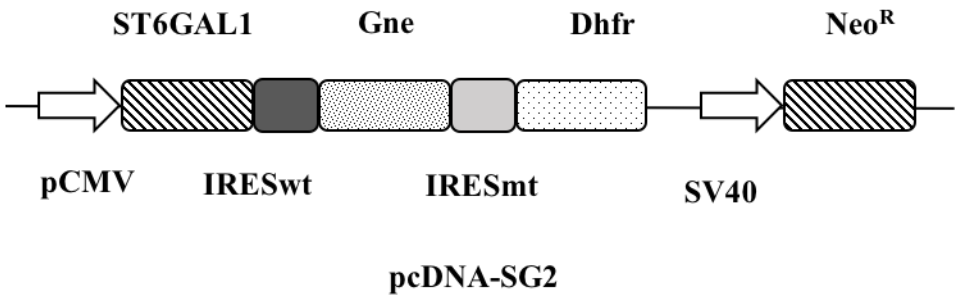


Fig. 3 Schematic representation of expression vector pcDNA-SG2. Plasmid pcDNA-SG2 carrying ST6GAL-I and Gne. pCMV: the human cytomegalovirus promoter; ST6GAL-I: coding region of the ST6GAL-I gene; IRESwt: IRESwt coding region, Gne: coding region of the Gne gene; Dhfr: Dhfr coding region used as selection marker; NeoR: neomycin resistance gene; SV40: simian virus 40 promoter

Six clonally derived cell lines stably expressing ST6GAL-I and Gne was established after transfecting of the expression vector pcDNA-SG2 into CHO-DG44. The PCR products amplified by specific primer sets to further confirm that these two genes were successfully expressed in the stable transfectants were electrophoresed (Fig. 4A). β-actin, a housekeeping gene used as an internal control, was expressed at the same level in all transfectants. Additionally, no PCR product band was obtained in the wild-type (WT) as shown in the Fig. 4A. A qRT-PCR was employed to evaluate the expression level of the ST6GAL-I and Gne genes, hence we can select the cell lines highly expressing these targeted genes. Among the six positive investigated cell lines expressing these genes, the cell line number 17 exhibited the highest expression level for both genes of interest as shown in Figure 4B.

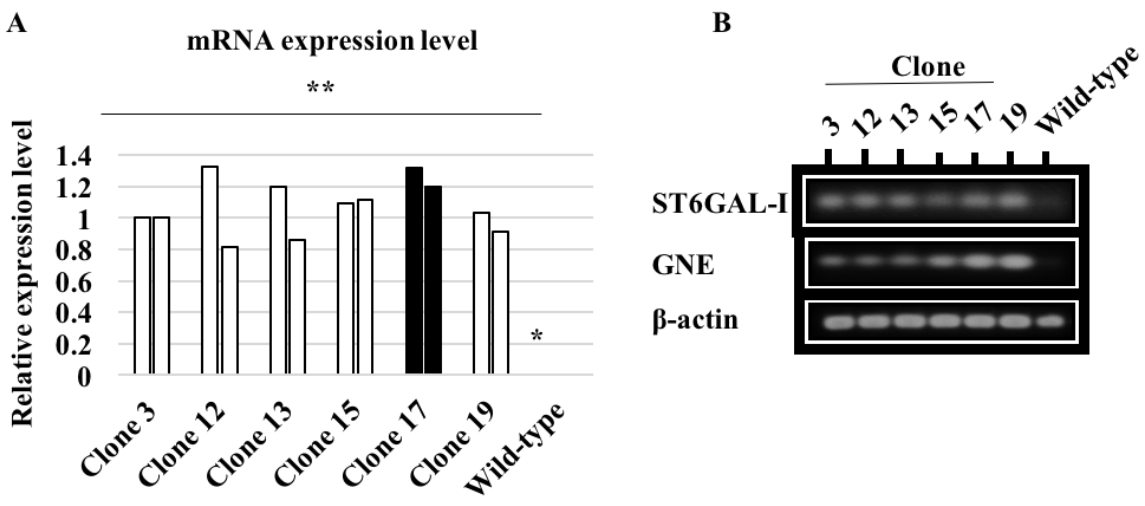


Fig. 4 The relative gene expression analysis data using qRT-PCR in stable expressing CHO cell lines (A) The expression of the two Neu5Ac-based key enzymes genes were measured by using quantitative RT-PCR in both wild-type and stable transfected cell lines. Left column: ST6GAL-I; Right column: GNE; *Under detectable amount; **P<0.05; (B) Gel electrophoresis of the Neu5Ac-based key enzymes genes PCR products using specific primers in the WT and transfected CHO cells

With these current results, I thus could confirm and select stable cell lines which the successful expressed of both ST6GAL-I and GNE. By using both gel electrophoresis and qRT-PCR, cell line 17 (CHOmt17-0) among six selected cell lines revealed the highest expression. Thus, CHOmt17-0 is potential as a candidate for further elucidated profile characterization.

2.3.2 Enzymatic activity assays of ST6GAL-I in CHOmt17-0

To examine the *in vitro* ST6GAL-I activity in both CHOmt17-0 and wild-type using a galactosylated structure as an accepter substrate and CMP-Neu5Ac as a donor substrate, an RP-HPLC was employed. The enzymatic products were analyzed using the RP-HPLC. I used M8C as an internal control and other standard glycans as following: PA-sugar chains of Neu5Ac(B)α2,6-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,6-Gal2-Gn2-M3-PA, Neu5Ac2-α2,6-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA, Neu5Ac(B)α2,3-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,3-Gal2-Gn2-M3-PA, Neu5Ac2-α2,3-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA (Fig.5A, Fig.5B). The RP-HPLC results revealed that there were no peaks corresponding to the sialylated

structures in the wild-type as showed in Figure 5C or the inactivated state of CHOmt17-0 as shown in Figure 5D. In contrast, in the microsome fraction obtained from CHOmt17-0 was incubated for 16 hrs. After reaction with donor and acceptor substrates, I observed a new peak corresponding to Neu5Ac(A)α2,6-Gal2-Gn2-M3-PA as revealed in Figure. 5E. Thus, these results may suggest that the active ST6GAL-I was successfully produced in CHOmt17-0. Thus, this enzyme produced in CHOmt17-0 was potentially able to transfer the donor substrate Neu5Ac to the acceptor substrate galactosylated bi-antennae *N*-glycan to form the sialylated structures. Here, I confirmed the activity of the ST6GAL-I produced in CHOmt17-0, which is an agreement with highly expression detected using qRT-PCR previously.

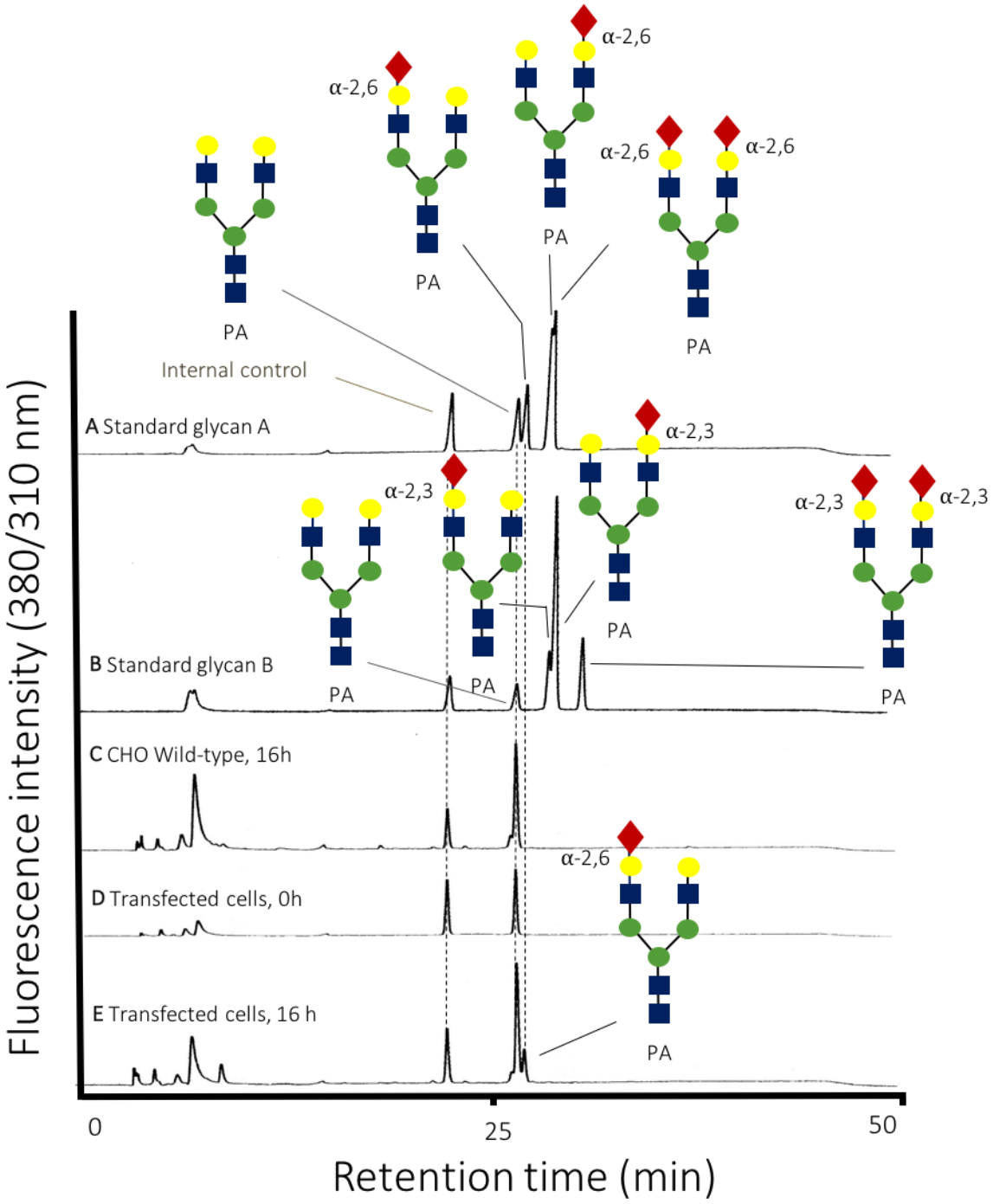
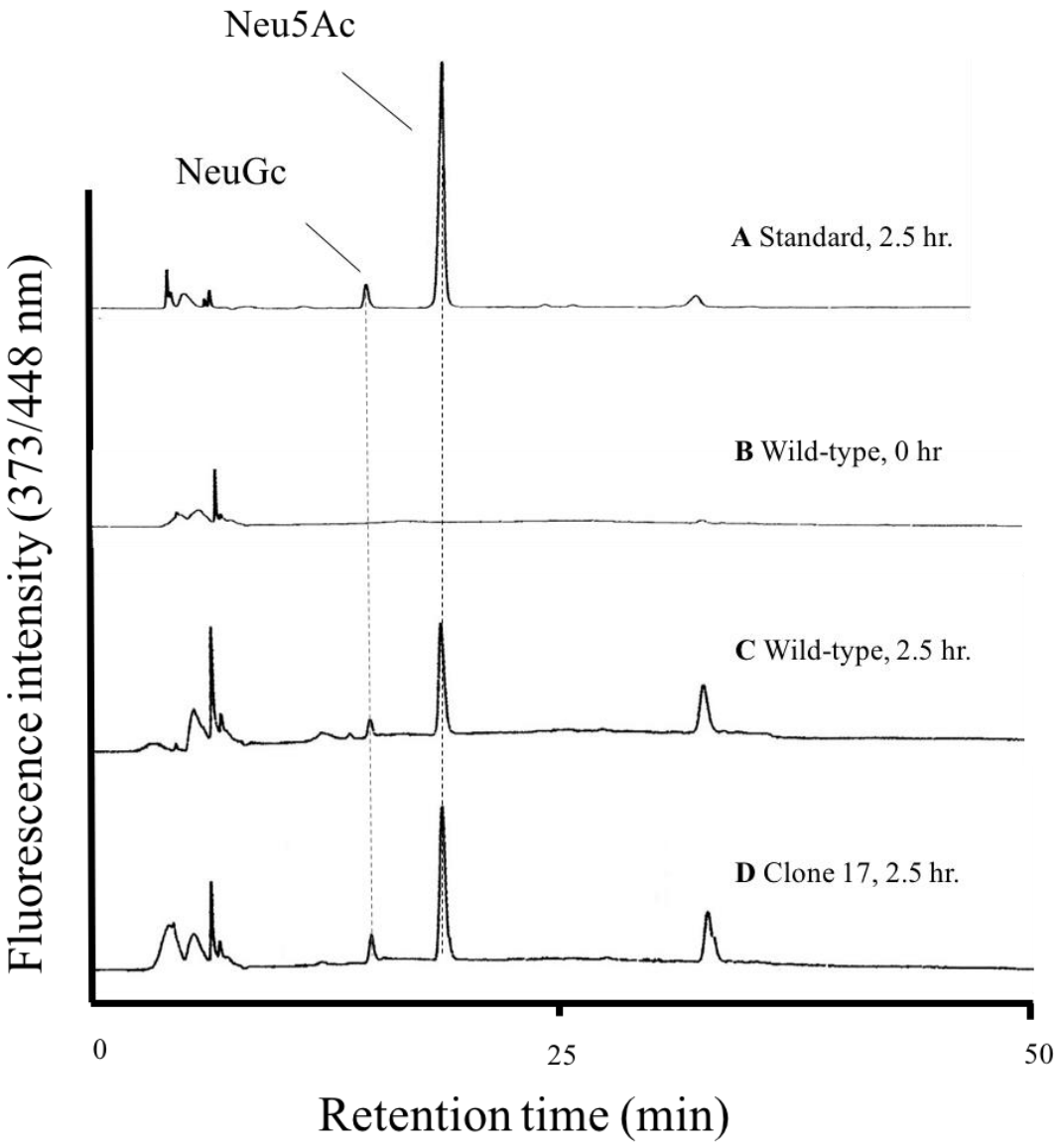


Fig. 5 Reverse phase HPLC profile for the identification of ST6GAL-I activity in the WT and transfected cells (A) & (B) RP-HPLC data of PA-sugar chains of Neu5Ac(B) α2,6-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,6-Gal2-Gn2-M3-PA, Neu5Ac2-α2,6-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA and M8C; Neu5Ac(B)α2,3-Gal2-Gn2-M3-PA, Neu5Ac(A)α2,3-Gal2-Gn2-M3-PA, Neu5Ac2-α2,3-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA, and M8C respectively (C) RP-HPLC profiles for ST6GAL-I activity in the WT for 16 hrs reaction (D) & (E) RP-HPLC analysis for sialyltransferase activity in CHOmt17-0 for 0 hr and 16 hrs enzyme reaction.

2.3.3 Significant enhancement of free sialic acid in cytosol in the CHOmt17-0

The free DMB-sialic acid obtained in CHOmt17-0 could be detected using RP-HPLC (Fig. 6). The standards of sialic acid were set as defined amounts of Neu5Ac and NeuGc. An equivalent amount of cytosol extracts collected from the wild-type cells was mixed with labeling solution without incubation. This mixture was used to ensure the background conditions of the assay.



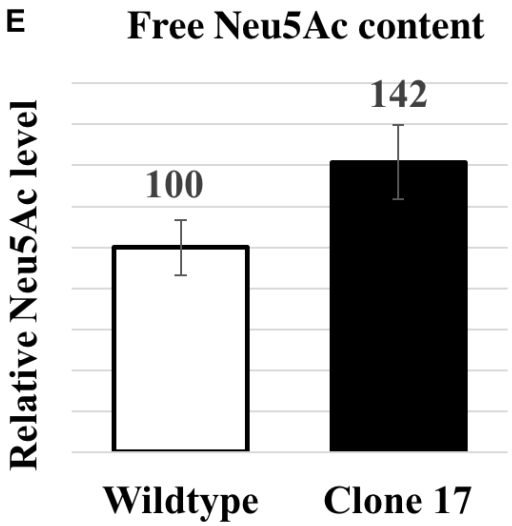


Fig. 6 Reverse phase HPLC profile in cell extracts of WT and CHOmt17-0 for the identification of free sialic acid; (A) RP-HPLC profile of standard DMB labeled Neu5Ac and NeuGc respectively; (B) & (C) RP-HPLC result obtained from WT after 0 hr and 2.5 hrs reaction (D) Identification of DMB-sialic acid in CHOmt17-0 using RP-HPLC for 2.5 hrs reaction (E) Relative quantitation of the amounts of free Neu5Ac in both cell lines. This experiment was carried out on using three independent experiments.

Here, RP-HPLC profile of the wild-type exhibited no peak corresponding to sialic acid after 0 hr of incubation. To examine the increase in the amount of sialic acid in the CHOmt17-0, I performed the same amount of total cytosolic proteins in both CHOmt17-0 and wild-type. Indeed, the free Neu5Ac amount in CHOmt17-0 was 41.6% higher than that in the wild-type (Fig. 6E). Interestingly, an insignificant increase of NeuGc has been found in the transfected cell line compared to that of the wild-type. Here, the overexpression of GNE possibly led to an increase in sialic acid and mostly found in Neu5Ac moiety.

2.3.4 Highly sialylated antennary structures generated in stable transfected cell lines

Due to the superior characteristics of CHOmt17-0, the detailed glycan profiles of this cell line were further examined. Next, the CHOmt17-0 cells were then incubated at 37ºC for 7 days in a culture medium supplemented with 100 µg/mL G418 and 10% dialyzed FBS. The viability,

was not different between the wild-type and CHOmt17-0 cells, was more than approximately 90% at the time of harvesting and cell growth.

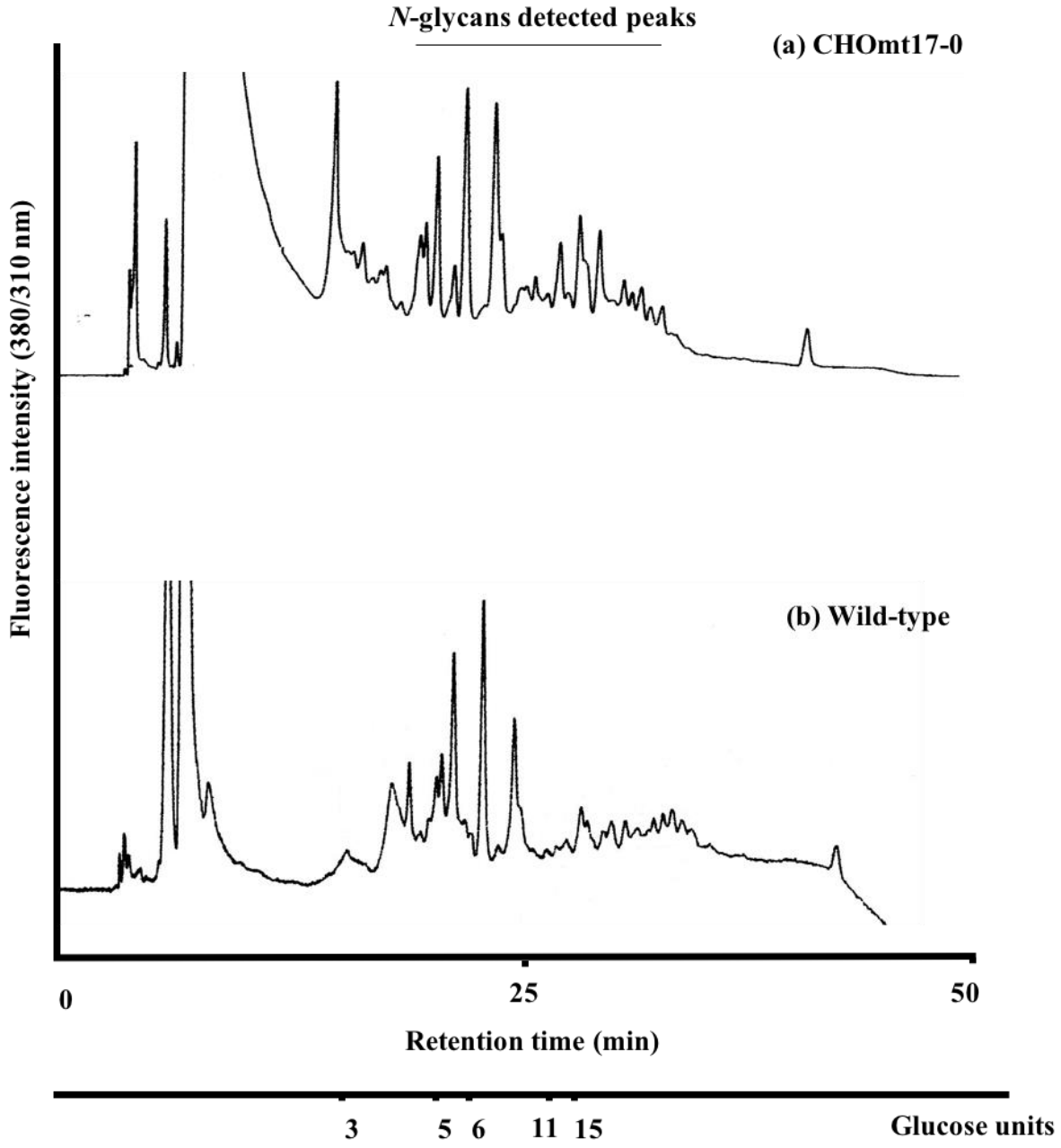


Fig. 7 Glycan profiles of (A) WT and (B) CHOmt17-0 cells. The total *N*-glycans extracted from glycoproteins were labeled with PA. The PA- glycans were fractionated using RP-HPLC. Then, the collected peaks were used to perform LC-MS; Glucose units: RP-HPLC profile for identification of glucose oligomer 3-15.

Table 1 Composition of predicted terminal sialic acid residue and predicted *N*-glycan structures

Structures	**Relative amount (%) in total *N*-glycan**	
	CHOmt17-0	**Wild-type**
Sialylated glycans	11.3	1.3
Neu5Ac terminal	11.3	1.0
NeuGc terminal	-	0.3
α2,3 linked sialic acid residues	3.3	1.3
High-mannose type	46.1	68.1
Galactosylated glycans	8.2	11.7
Fucosylated glycans	30.4	13.4

Before the PA-labeled *N*-glycans were analyzed by LC-MS, they were fractionated by RP-HPLC. The RP-HPLC profiles were indeed significantly different between CHOmt17-0 and the wild-type as shown in Fig. 7.

Specifically, the late-eluted peak exhibited a dramatic increase in the CHOmt17-0 as compared to the wild type. Hence the results revealed a big jump in the relative amounts of highly galactosylated and sialylated structures (Table 1). This result suggests that considerably enriched the antennary sialylated glycan structures in this stable transfected cells line was caused by the overexpression of ST6GAL-I and GNE. NeuGc was detected in both the wild-type and CHOmt17-0, Neu5Ac terminal mostly found in this stable cell line. Additionally, there was a great increase in di- and tri-antennary structures in CHOmt17-0 as compared to the wild type. Interestingly, among 11.3% of the sialylated structures in total *N*-glycans found in CHOmt17-0, α2,3 linked sialic acid accounted for 3.3% (Fig. 8). Table 2 exhibits the composition of predicted abbreviation in glycan structures in CHOmt17-0 and wild type cell

line. Indeed, the transfected cell line CHOmt17-0 showed a big jump in the amount of sialylated structures, to approximately 7-fold the amount in the wild-type. Collectively, these results indicated that the remarkable accretion of sialylated terminal is due to glycans overexpression of both GNE and ST6GAL-I.

Table 2 Predicted structures found in total proteins derived from highly sialylated cell lines using LC-MS

Predicted structures	**Relative amount (%) in total *N*-glycan**	
	Wild type	**CHOmt17-100**
M2	1.1	0.3
M2F	4.3	0.9
M3	1.0	12.0
M3F	3.5	4.9
M4	5.1	6.5
M4F	0.2	-
M5	14.5	21.5
M6	26.7	9.7
M6F	-	0.03
M7	5.3	3.3
M8	7.1	2.9
M9	14.4	8.7
M9Glc	1.4	-
GnM3	0.2	-
GnM3F	1.1	6.3
Gn2M3F	0.9	1.6

Gn3M3F	0.1	1.8
GalGnM4	7.2	0.2
GalGnM5	0.05	0.07
GalGnM3	0.3	-
GalGnM3F	0.3	0.7
GalGn2M2F	-	4.0
GalGn2M3	0.2	0.03
GalGn2M3F	0.4	0.01
GalGn3M3	0.7	-
GalGn3M3F	-	0.6
Gal2Gn2M3	0.2	-
Gal2Gn2M3F	1.6	1.6
Gal2Gn3M3F	0.2	0.3
Gal3Gn3M3F	0.3	0.6
Gal3Gn3M3	0.1	-
Gal4Gn4M3F	-	0.05
Neu5AcGalGnM3	-	1.2
Neu5AcGalGnM3F	-	4.0
Neu5AcGalGnM4	-	0.02
Neu5AcGalGn2M3F	-	0.8
Neu5AcGal2Gn2M3	0.4	0.6
Neu5AcGal2Gn2M3F	0.4	2.1
Neu5AcGal3Gn3M3F	-	0.1
Neu5Ac2Gal2Gn2M2	-	0.3

Neu5Ac2Gal2Gn2M3	0.2	2.3
NeuGcGal2Gn2M3	0.3	-

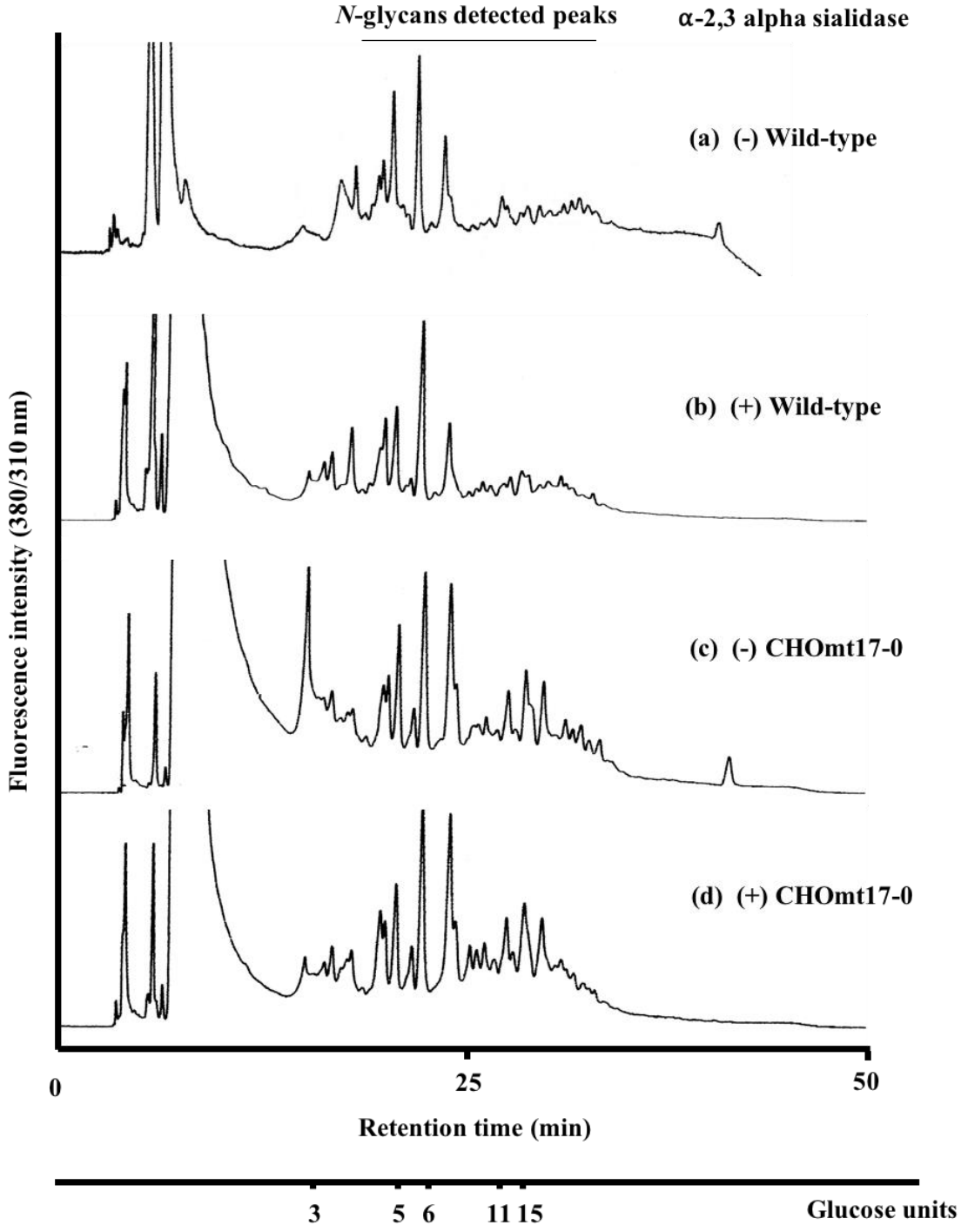


Fig. 8 Glycan profiles of WT and CHOmt17-0 cells digested with α2,3 sialidase. The total *N*-glycans extracted from glycoproteins were labeled with PA. The PA- glycans were

fractionated using RP-HPLC. Then, the collected peaks were used to perform LC-MS; Glucose units: RP-HPLC profile for identification of glucose oligomer 3-15; F: Fucose

2.4 Discussion

As mentioned above, CHO cells are the most predominant host cell line in the biopharmaceutical field due to their advantageous genetic properties.

Sialylation is one of the most important factors to determine the efficacy of therapeutics. Significant studies aim to alter host cell lines producing desired glycan structures in targeted proteins. It includes the addition of various supplements to the culture media or the optimization of processing methods. The decrease of culture temperature indicated to reduce sialylation and level of NeuGc (Trummer et al. 2006, Borys et al. 2010). Addition of DO concentrations could enhance sialyltransferase activity. This thus increases the sialic acid content of glycoproteins (Chotigeat et al. 1994). Sugars, nucleotide sugars or metals are used widely to alter CHO host cell line as media supplements methods. Additions of glucose and galactose were reported to increase the sialylation of glyproteins (Chee and Gek 2005, Liu and Fan 2015). Glucosamine was reported to reduce sialylation of therapeutics (Yang and Butler 2000, Baker and James 2001). However, ManNAc can slightly enhance the sialylation (Baker and James 2001, Wong et al. 2010). Nucleotide sugars which are known as building blocks for biosynthesis of glycoproteins are also utilized to modulate glycan patterns of therapeutics. Even though altering process parameters to modulate glycosylation is considered as an attractive approach, the results in return is highly variable. Hence, the host cell engineering methods by deletion/insertion of glycosylation-related genes or up/down regulation of is considered as more direct, stable and efficient.

To our knowledge, this is the first study approached to modify CHO host cells through the stable co-expression of two Neu5Ac-based key genes in the same construction for highly sialylated expression. Our results exhibit a profile of the host cell line obtained by the overexpression of glycosylated enzymes. The overexpression of ST6GAL-I and GNE in CHOmt17-0 enrich greater sialylation in total proteins. In this research, CHOmt17-0 exhibited

more than 7-fold higher in sialylation and 41.6% higher in free Neu5Ac content as compared to the wild-type (Table 1, Fig. 6). Previous study reported that the overexpression of GNE alone is sufficient to increase the free Neu5Ac in CHO cells (Bork and Horstkorte 2007). Thus, in our current results, the great improvement of sialylation in CHOmt17-0 may have been due to the expression of both ST6GAL-I and GNE. Additionally, because we have overexpressed solely ST6GAL-I without ST3GAL-(I =>VI) in this research, the sialylation glycans in total proteins was possibly formed mostly through ST6GAL-I transferring activity. The level of α2,6-linked sialic acids resulted in CHOmt17-0 cell line thus revealed more predominated over α2,3-linked sialic acid possibly due to the overexpression of ST6GAL-I as an agreement with the results of Lin et al. 2015. Also, the total amount of α2,3-linked Neu5Ac obtained in the CHOmt17-0 was slightly higher than that in the wild-type. The overexpression of GNE may result in an increase of donor substrate CMP-Neu5Ac linked to galactose terminal glycan as an acceptor substrate to form a sialylated structures. Hence, this slight increase in the amount of α2,3 linked sialic acid glycan profiling in CHOmt17-0 may have resulted from the overexpression of GNE.

In conclusion, the current results indicate that co-expression of these two genes successfully yielded a host-engineered CHO-cell line which can produce a noticeably large amount of sialylation, CHOmt17-0. Due to the importance of the sialylation for their functions, the CHOmt17-0 may thus be a potential candidate for industrial applications for therapeutics, such as antibodies, the production of vaccines or certain hormones productions

2.5 Summary

Here, I can confirm that ST6GAL-I and GNE were very important enzymes which playing a role in the biosynthesis pathway of sialic acid. In this study, the tremendous increase of sialylation in total proteins of CHOmt17-0 as compared to the wild type is resulted from the introduction of two investigated genes ST6GAL-I and Gne. For this reason, I thus strongly

believe that this research possibly assists in the pursuit of new development in the glycosylation field. Hence, it opens a new aspect for us to enhance sialylation using this CHOmt17-0 using gene amplification method.

Chapter 3: Enhancement of sialylation in engineered Chinese hamster ovary host cell line by using gene amplification

3.1 Introduction

Gene amplification is the method for the selection and repeated genes replication without an increase of other genes in the whole genome. This method has been long utilized as an important strategy for development of pharmaceuticals for the enrichment of protein production. The DHFR/MTX was described as the first gene amplification method applied in mammalian cells (Omasa 2002). This method widely utilized in the cell line development for enhancement of the productivity of biopharmaceutical proteins (Takagi et al. 2016). The system mechanism is to utilize the MTX for inhibition of selection enzyme marker DHFR which is crucial for the metabolism of cellular in the DHFR deficient CHO cells. The DHFR deficient CHO cells named as CHO-DG44 has been used for the first time in 1980 (Wigler et al. 1980, Urlaub and Chasin 1980). The expression vector carrying genes of interest and selection marker is transfected into CHO cells line. The cells were then selected and repeated amplified targeted genes using MTX. Thus, the genes of interest copies are increased in numbers resulting in an upregulation of productivity of targeted recombinant proteins (Schimke 1984).

Although this method has been widely used in the mammalian cells for recombinant protein production, the utility of the DHFR/MTX genes amplification to modulate the glycosylation in the host cell line remains very limited. Here, this study approached to obtain better sialylation CHO host cell line using two Neu5Ac-based key located same expression cassette using gene DHFR/MTX amplification system. The selected CHO cell line has been named as CHOmt17-0. I used MTX as an acceleration drug for upregulation of DHFR enzyme leading to the enrichment of Neu5Ac based key enzymes expression in the CHO cells. Thus, I attempted to

achieve to CHO cell line with better sialylation pattern in their glycan structures as compared to the CHOmt17-0. This study was carried out to prove that gene amplification method DHFR/MTX may be good tool to enhance the sialylated glycoforms because of great acceleration of Neu5Ac-bases key enzymes.

3.2 Materials and Methods

3.2.1 Cells and culture media

I established the cell line CHOmt17-0 by co-overexpressing two related human enzymes as described in chapter 2. These cells were cultured in an (-) α MEM. This medium was added with 100 µg/mL G418 without nucleosides or L-glutamine and 10% dialyzed FBS. The antibodies IgG-producing transfectants were cultured in an (-) α MEM without nucleosides, supplemented with 100 µg/mL G418, 10% dialyzed FBS, 1 µg/mL puromycin and 100 µg/mL hygromycin. High-resistance MTX cell lines, CHOmt17-100, CHOmt17-200, CHOmt17-300 were also cultured in an (-) α MEM (Invitrogen) without nucleosides, added with 100 µg/mL G418, 10% dialyzed FBS and an MTX concentration of 100 nM, 200 nM and 300 nM. All of investigated cells were then cultured in a cell culture incubator monitored at 5% CO_2 and 37^{o}C.

3.2.2 Quantitative real-time PCR (qRT-PCR)

The protocol to prepare a sample for the qRT-PCR was described in chapter 2. In brief, harvested cultured cells (1x 10^7) was well washed using PBS. Next, according to the manufacturer's instructions, the total RNA from the collected cells was extracted using an RNeasy Mini Kit (Qiagen). cDNA was then synthesized using a SuperScript® VILO™ cDNA Synthesis Kit using the RNA. The qRT-PCR was used in the StepOnePlus™ real-time PCR system (Applied Biosystems) to elucidate the expression of the investigated genes in the cell lines. The following primers were employed for the PCR reaction: 5' TGTGTGGAAGGAAAAGAAGAAAGG 3' (sense primer for ST6GAL-I), 5' GGGTGCTGCTTGAGGATACA 3' (antisense primer for ST6GAL-I), 5'

GTGGAAGGGATGTCAGTACCAAA 3’ (sense primer for Gne), and 5’ CCAAGTCCCAAAGCAGTTCC 3’ (antisense primer for Gne) as described in chapter 2.

3.2.3 Generation of highly methotrexate-resistant cell lines

The glyco-engineered cell lines (1 x 10^7) were added with various concentrations of MTX into culture medium containing 100, 200, or 300 nM of MTX to establish highly MTX-resistant cell lines to upregulate genes of interest in MTX- free CHOmt17-0 cell line. After 3 weeks of culture, stable highly MTX-resistant cell lines were generated using an incubator at 5% CO_2 and 37^{o}C. After selection, the stable cell lines CHOmt17-100, CHOmt17-200, and CHOmt17-300 were generated, exhibiting high resistance to 100, 200, and 300 nM of MTX, respectively,

3.2.4 Cell viability of highly MTX-resistant cell lines

The cultured cells lines (1 x 10^7) were washed twice with PBS to perform cell viability. Before adding 9 mL of fresh α MEM into each dish, the harvested cells were then detached by using 1 mL 0.25% trypsin (Life technologies, California, USA). Then, 50 µL of the sample containing cells was gently added with 50 µL of 0.4% trypan blue (Sigma, Tokyo, Japan). Next, 15 µL of the mixture was moved into each chamber in a hemocytometer. Counts were carried on in three experiments on days 1, 3, 5, 7, 9, and 11, followed by analysis under a 40x microscope objective as described previously (Louis et al. 2011).

3.2.5 Glycan structure analysis total proteins in highly MTX-resistant cells

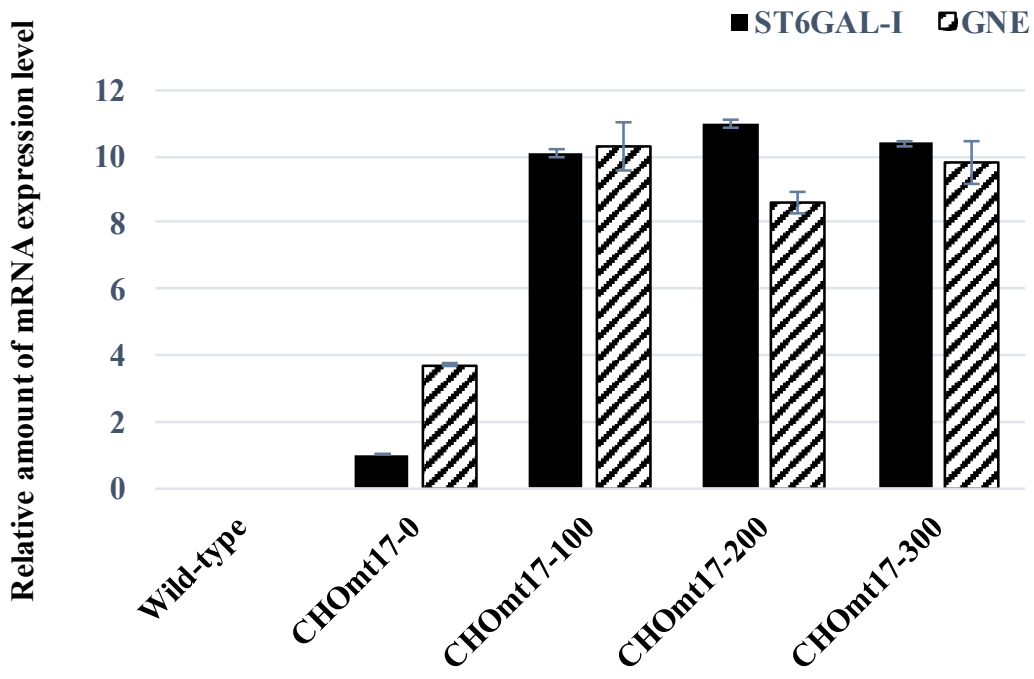
Investigated host cell culture (1 x 10^8) were well washed with PBS before adding lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS. The protocol was described in chapter 2.

3.3 Results

3.3.1 Establishment of cell lines highly expressing both ST6GAL-I and Gne with increased MTX concentrations from CHOmt17-0

I established a tri-cistronic expression plasmid in our previous studies. The plasmid pcDNA-SG2 carries three genes ST6GAL-I, Gne, and a Dhfr. These genes are linked to each other by IRESwt and IRESmt for gene amplification purpose. And these genes of interest were driven using a single CMV promoter as described in detail in chapter 2. The transfection of the expression vector pcDNA-SG2 into CHO-DG44 cells resulted in the establishment of clone CHOmt17-0 derived cell lines stably expressing ST6GAL-I and Gne. From here, due to its superior quality to produce high sialylation in total proteins, CHOmt17-0 was chosen to be host cell lines for further modification. The qRT-PCR was used to examine the expression levels of the ST6GAL-I and Gne genes in the highly MTX-resistant transfectants CHOmt17-100, CHOmt17-200, and CHOmt17-300 which supplied with 100 nM, 200 nM, 300 nM of MTX as described in the materials and methods.

(A) mRNA expression level of CHOmt17 in various concentration of MTX



(B) Gel electrophoresis

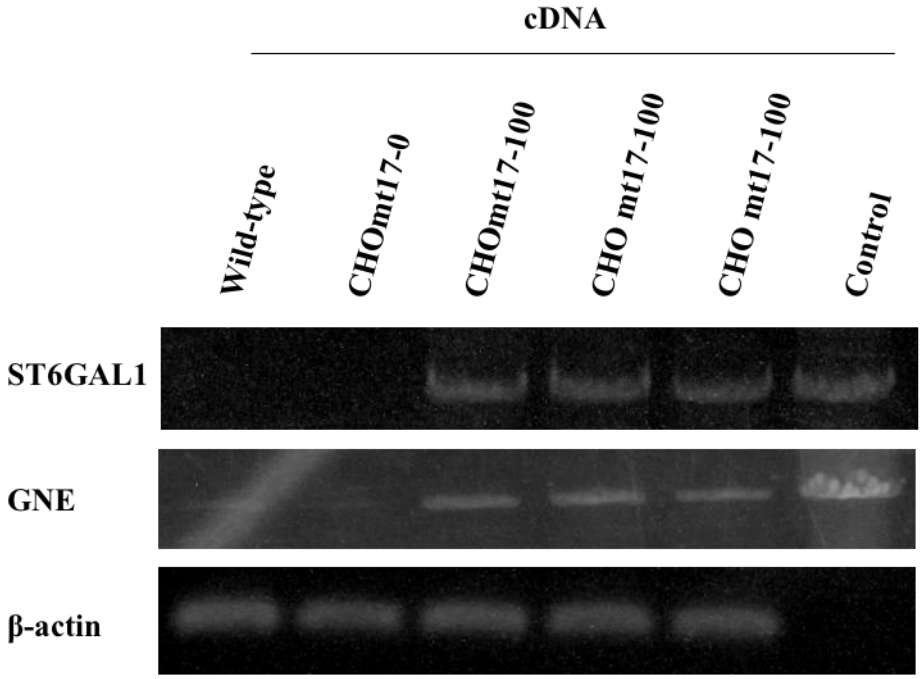


Fig. 9 mRNA expression level analysis data of investigated genese in CHO host cell line (A) The expression levels of the investigated genes were evaluated using quantitative RT-PCR in both wild-type and stable transfected cell lines. Left column: ST6GAL-I; right column: GNE; $P<0.05$. (B) Gel electrophoresis of the investigated genes' PCR products using specific primers in the WT, transfected CHO cells. Control: plasmid pcDNA-SG2 (Thi Sam et al. 2018)

Thus, when the MTX concentration added, the genes of interest indeed exhibited higher expression levels than that of the wild type and CHOmt17-0 (Fig. 9A). Interestingly, the expression levels of ST6GAL-I and Gne in CHOmt17-100, CHOmt17-200, and CHOmt17-300 obtained from qRT-PCR results revealed approximately three-fold upregulation as compared to those without MTX treated CHOmt17-0. Additionally, the host cell line CHOmt17-200 and CHOmt17-300 with the higher MTX concentrations of 200 nM and 300 nM did not show a noticeably increase in the mRNA expression level as compared to that of the lower concentration of 100 nM (Fig. 9A). Next, I amplified the full-length sequences of ST6GAL-I and Gne using cDNA as a template in each host cell lines. The results obtained showed high expression levels of the genes of interest in the MTX-treated clones to confirm

the qRT-PCR results, as shown in Fig. 9B. This gel electrophoresis result is an agreement with the relative mRNA expressions of these investigated then. With the current results, I was thus able to confirm success in upregulating the expression levels of both ST6GAL-I and Gne in the cell lines in the presence of MTX.

3.3.2 Highly sialylated antennary structures generated in highly MTX-resistant cell lines

The glycan profiles of the cell lines with the addition of various MTX concentrations CHOmt 17-100, CHOmt17-200, CHOmt17-300 and MTX-free CHOmt17-0 were further analyzed to evaluate the effects of MTX on the amplification of sialylation.

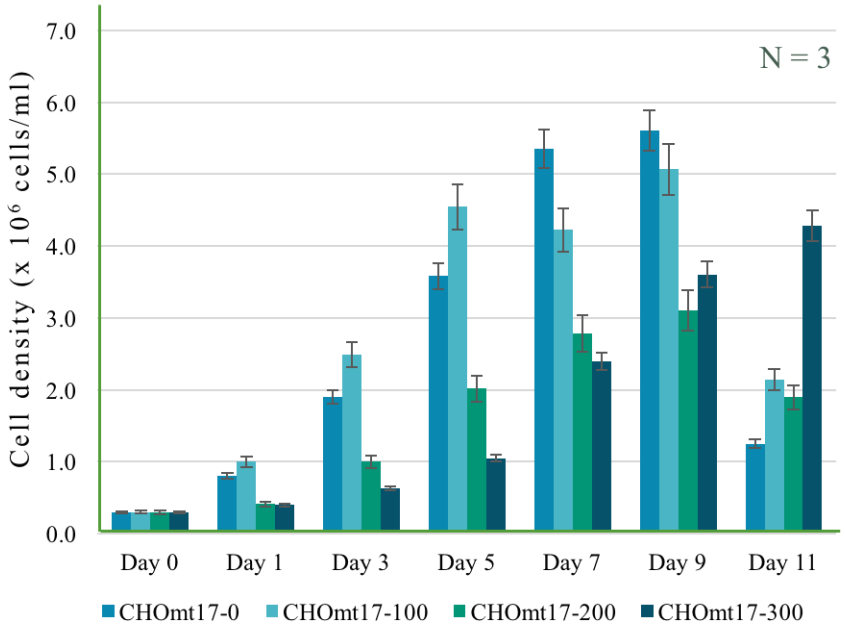


Fig. 10 Growth profiles of the unamplified and amplified CHO cells cultured in α MEM. Error bars represent the standard deviation calculated from data obtained in three independent experiments (n = 3).

The CHOmt17-0 cells were incubated in culture medium added with 100 µg/mL G418 and 10% dialyzed FBS in MTX concentrations of 100, 200, and 300 nM at 37°C for 7 to 9 days. The cell viability of these cell lines was more than approximately 90% at the time of harvesting

for examination (Fig. 10). As described in the materials and methods, the PA-labeled *N*-glycans obtained from these investigated cell lines were fractionated using RP-HPLC. Next, their structures were detected by LC-MS. Collectively, the RP-HPLC profiles of these host cell lines were easily distinguishable among the wild type, CHOmt17-0, CHOmt17-100, CHOmt17-200, and CHOmt17-300, as shown in Fig. 11. Specifically, the late-eluted peaks shown in the RP-HPLC exhibited a dramatic increase in the intensity of the transfected cell lines as compared to the wild type and CHOmt17-0. Hence, this change showed in a big jump in the relative amounts of highly galactosylated and sialylated structures in the highly MTX resistance cell lines CHOmt17-100, CHOmt17-200 and CHOmt17-300 (Table 3). Interestingly, CHOmt17-100 showed a great jump of 12-fold in sialylation of total proteins as compared to the wild type. Also, the cell line CHOmt17-100 illustrated about 1.4-fold higher sialylation compared to the MTX-free CHOmt17-0. Additionally, there is no big increase in the level of sialylation in the cell lines CHOmt17-200 and CHOmt17-300 as compared to CHOmt17-100 (Table 3). These results were indeed an agreement with the mRNA expression level obtained previously shown in Fig. 9. Collectively, the results obtained initiate that the overexpression of ST6GAL-I and GNE with MTX supplementation considerably improve the antennary sialylated glycan structures in the investigated stable transfected cell lines (Table 3). Although the terminal NeuGc of glycoproteins was detected in all investigated cell lines, the amount of terminal NeuGc structure obtained was similar to that of the wild type. The terminal sialic acid was mostly indeed Neu5Ac. These results indicated that due to gene amplification using MTX, the overexpression of both GNE and ST6GAL-I resulted in a remarkable accretion of sialic acid terminals in total protein glycans in MTX-resistance stable cell lines. Also, the amount of sialylation of total proteins produced in CHOmt17-100 was similar level to that of the cell lines with higher amounts of MTX such as CHOmt17-200 and CHOmt17-300 (Table 3). Thereupon,

CHOmt17-100 was chosen as the host cell line to produce recombinant antibodies IgG as a proof of concept using the gene amplification method due to its better viability.

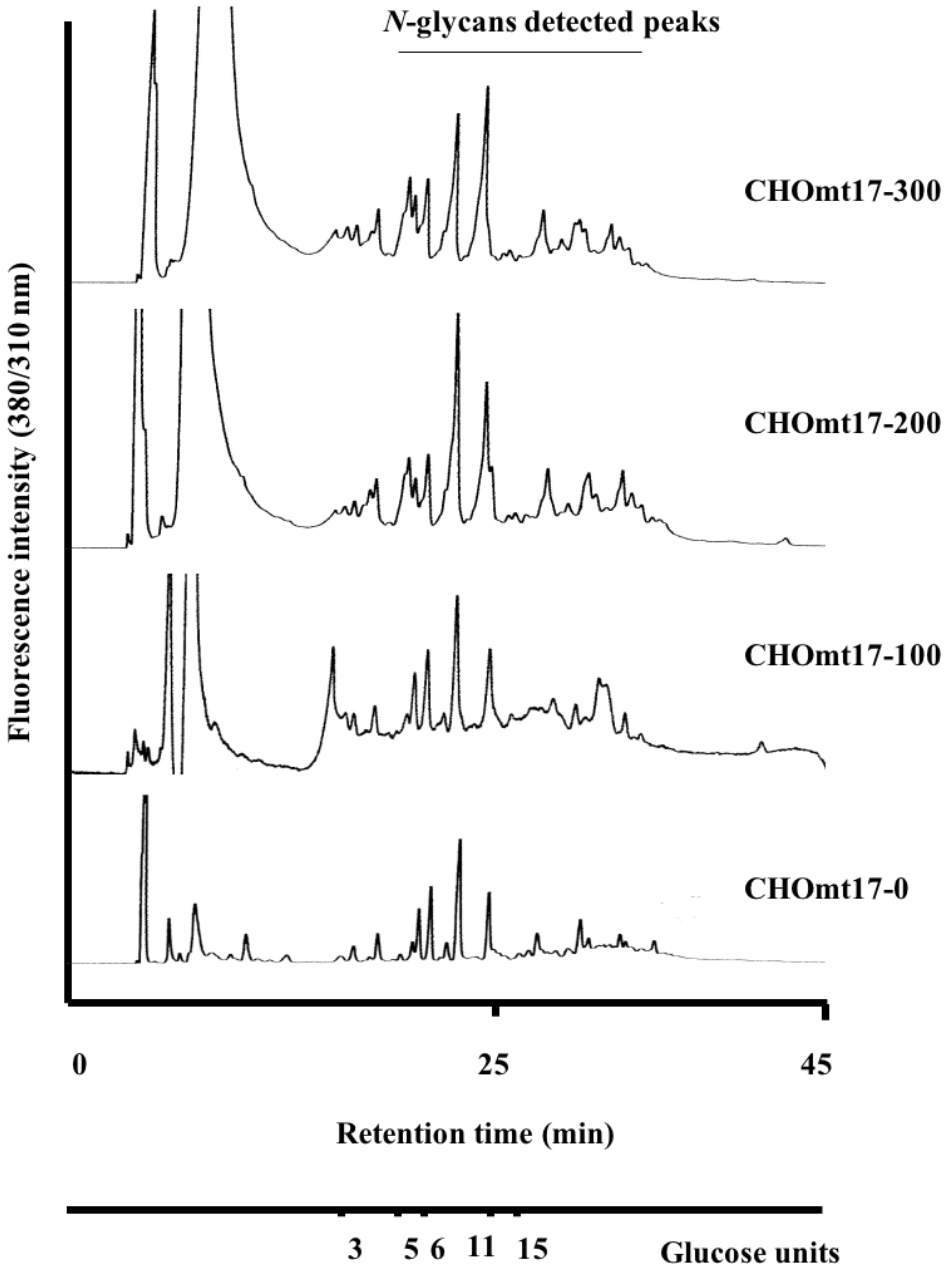


Fig. 11 Glycan profiles of CHOmt17-0, CHOmt17-100, CHOmt17-200, and CHOmt17-300 cells using RP-HPLC. The total *N*-glycans extracted from glycoproteins in investigated cells were labeled with PA. The PA-glycans were fractionated using RP-HPLC. Then, the eluted peaks were utilized to perform LC-MS. Glucose units: RP-HPLC profile for identification of glucose oligomer 3-15.

Table 3 Composition of predicted terminal sialic acid residue and predicted *N*-glycan structures

Structures	Relative amount (%) in total *N*-glycan			
	CHOmt17-0	**CHOmt17-100**	**CHOmt17-200**	**CHOmt17-300**
Sialylated glycans	11.3	15.5	15.8	15.9
Neu5Ac terminal	1.0	11.3	14.4	14.6
NeuGc terminal	0.3	-	1.1	1.2
High-mannose type	68.1	46.1	52.6	62.7
Galactosylated glycans	11.7	8.3	9.9	1.4
Fucosylated glycans	13.4	30.4	40.5	38.1

Table 4 Predicted structures found in total proteins derived from highly sialylated cell lines using LC-MS

Predicted structures	Relative amount (%) in total *N*-glycan			
	CHOmt17-0	**CHOmt17-100**	**CHOmt17-200**	**CHOmt17-300**
M2	0.3	0.5	-	-
M2F	0.9	2.6	1.4	1.6
M3	12.0	0.9	1.3	0.4
M3F	4.9		7.3	3.4
M4	6.5	3.1	6.1	6.0
M4F	-	1.8	-	-
M5	21.5	8.4	24.5	33.7

M5F	-	0.4	0.2	0.3
M6	9.7	17.9	27.4	17.0
M6F	0.03	1.0	-	-
M7	3.3	3.4	2.8	3.9
M7F	-	8.6	-	-
M8	2.9	2.0	5.7	8.3
M9	8.7	1.5	2.2	3.6
M9Glc	-	0.5	-	0.3
GnM3	-	0.4	0.7	0.7
GnM3F	6.3	7.2	1.9	1.3
GnM5	-	0.1	-	0.1
GnM5F	-	6.4	-	-
Gn2M2F	-	0.9	-	-
Gn2M3	-	0.2	0.1	-
Gn2M3F	1.6	-	1.3	0.1
Gn3M3	-	0.3	-	-
Gn3M3F	1.8	1.8	-	0.3
GalGnM4	0.2	0.2	-	-
GalGnM5	0.07	0.1	-	-
GalGnM3	-	-	-	0.2
GalGnM3F	0.7	-	1.1	1.7
GalGnM4F	-	6.0	-	-
GalGn2M2F	4.0	-	-	-
GalGn2M3	0.03	0.1	-	1.3

GalGn2M3F	0.01	-	-	-
GalGn3M3	-	-	0.3	-
GalGn3M3F	0.6	-	-	-
Gal2Gn2M3	-	7.0	-	-
Gal2Gn2M3F	1.6	-	-	-
Gal2Gn3M3F	0.3	-	-	-
Gal3Gn3M3F	0.6	-	-	-
Gal3Gn3M3	-	0.8	-	-
Gal4Gn4M3F	0.05	-	-	-
Neu5AcGalGnM2	-	3.3	-	-
Neu5AcGalGnM2F	-	-	0.6	-
Neu5AcGalGnM3	1.2	-	-	-
Neu5AcGalGnM3F	4.0	-	0.2	1.2
Neu5AcGalGnM4	0.02	-	0.1	
Neu5AcGalGnM4F	-	-	0.2	0.2
Neu5AcGalGnM5	-	-	0.1	0.4
Neu5AcGalGnM5F	-	-	0.1	
Neu5AcGalGn2M3	-	-	-	0.5
Neu5AcGalGn2M3F	0.8	-	0.6	1.3
Neu5AcGal2Gn2M3	0.6	-	0.4	0.3
Neu5AcGal2Gn2M3F	2.1	4.8	1.8	1.1
Neu5AcGal2Gn3M3	-	-	-	2.7
Neu5AcGal3Gn3M3	-	4.7		
Neu5AcGal3Gn3M3F	0.1	-		0.6

Neu5Ac2GalGn2M3	0.3	-		-
Neu5Ac2Gal2Gn2M3	2.3	-	0.2	0.3
Neu5Ac2Gal2Gn2M3F	-	1.7	10.1	5.7
NeuGcGalGnM3	-	0.9		-
NeuGcGal2Gn2M3	-	0.2		-
Neu5AcNeuGcGal2Gn2M3	-	-	0.7	0.7
Neu5AcNeuGcGal2Gn2M3F	-	-	0.4	0.8

3.4 Discussion

Post-translational modification is one of the most important factor to determine the glycoprotein quality such as efficiency and stability. Thereupon, mammalian cell lines are prominently used as host cell line for recombinant protein production. Meanwhile, among the mammalian cell line, CHO cells are known as a star in the biopharmaceutical production due to its superior characteristics. The CHO cell line used in this study can provide the stable expression recombinant protein with high amount Neu5Ac terminals attached in their glycan structures.

I achieved to establish stable cell line expressing Neu5Ac –based key enzyme ST6GAL-I and GNE resulting in a great increase of sialylation in total proteins using DHFR/MTX gene amplification method. I have used IRES-driven for *dhfr* promoter in the expression plasmid. The IRES fragments has been attenuated to reduce its efficiency, thus able to induce higher expression of DHFR enzymes for selection clones. In this present study, I used the effect of MTX on accelerating the genes of interest expression level. With supplementation of MTX to co-overexpress ST6GAL-I and Gne genes, the highly MTX-resistant cell lines CHOmt17-100, CHOmt17-100, and CHOmt17-300 revealed considerably better sialylation glycoforms in the total proteins about 1.4-fold as compared to that of CHOmt17-0 with MTX-free. With the supplement of MTX as an inhibitor of DHFR selection enzyme, the co-expression of ST6GAL-I and GNE showed an significant upregulation of sialylation of total protein in the amplified clones CHOmt17-100, CHOmt17-200 and CHOmt17-300 up to 12-fold as compared to unamplified clone. Nevertheless, the mRNA expression level of two genes of interest in the amplified clone treated with 100 nM of MTX, CHOmt17-100, did not reveal a great jump in CHOmt17-200 and CHOmt17-300 with an increase of concentration of MTX. This result remained unchanged that the composition of the sialylated glycoforms in total proteins of CHOmt17-200, CHOmt17-300 did not show great acceleration as compared to CHOmt17-100

cell line. The increase of genes expression in highly methotrexate concentration in CHOmt17-100 is about 7-fold higher as compared to the CHOmt17-0. However, in glycan analysis results, the sialylation from CHOmt17-100 showed only 1.4-fold higher as compared to that from the CHOmt17-0. Since there are multiple of glycosylated enzymes have been evolved in the sialylation pathway as well as the involvement of multiple substrates. However, in this research we only overexpress two sialylated genes ST6GAL-I and Gne. Thus, it does not ensure a same ratio of expression in glycan structures increase as compared to that of the mRNA expression. Furthermore, the cell viability of CHOmt17-200 and CHOmt17-300 were lower as compared to the CHOmt17-100. This result might be caused of toxicity derived from MTX drug. Hence, CHOmt17-100 host cell line can be utilized as the best candidate for further elucidation due to its superior characteristics.

3.5 Summary

Our results indicate that the stable established cell lines generating high expression of sialylation can be obtained by engineer CHO cells using gene amplification DHFR/MTX. Here, this gene amplification method is proved as effective method to upregulate the sialylation of the total protein in amplified cells to 12-fold as compared to unamplified cells. These results indicated that CHOmt17-100 host cell line might possibly be a great choice for therapeutic production.

Chapter 4: Production of antibodies IgG in glyco-engineered Chinese hamster ovary cells

4.1 Introduction

Sialic acid, Neu5Ac, is believed to be able to modulate signaling in cell transmembrane, differentiation, and growth. Moreover, Neu5Ac is reported to have antioxidant and anti-apoptotic effects (Varki et al. 2009). Thus, Neu5Ac-terminal glycoproteins are highly desirable for therapeutic production in CHO cells. I described previously the establishment of transfected CHO cell lines called CHOmt17-0, carrying two Neu5Ac-based key enzymes, ST6GAL-I and GNE, using the DHFR/MTX gene amplification method. I could select CHOmt17-100 as a host cell line due to its superior sialylation in total proteins. In this chapter, I suggested another approach to proof concept of highly sialylation in therapeutics production of CHOmt17-100 using antibodies immunoglobulin as model protein.

IgG, a glycoprotein in serum, can protect vertebrates from unknown substances and pathogens (Beale and Feinstein 1976; Raju 2003). IgG is the most common component in serum, composited of more than about 75% of it. The “Fragment antigen binding” (Fab) regions of IgG provide the body to detect antigenic substance and contribute to the diversity of the antibodies. Additionally, the antibodies have “Fragment crystallizable” (Fc) regions that help the antibodies interacting with Fc gamma receptors on phagocytes. While Fab regions can resist proteases, the Fc fragment and the hinge are more sensitive toward proteolysis (Ryan et al. 2008; Raju et al. 2006; Raju et al. 2007). IgG relies itself on their Fc regions to be functionally binding antigens. The domain of Fc fragment CH2 attached to asparagine 297 (Asn297) composed of glycan structures in the heavy chain (Lund et al. 1996; Krapp et al. 2003). These glycan structures can help IgG to activate the binding function between complement and FcγR receptors (Krapp et al. 2003). While the Fc region interacts with Fcγ receptors of immune cells, IgG can bind to the surface of antigen-presenting cells. These immune cells then release

cytotoxic substances to cause cell death (Strome et al. 2007). About more than 30 glyco-patterns linked to the Fc regions of IgG were found (Burton and Dwek 2006). The glycan structures in the antibodies include variable monosaccharides. And the effects of these glycans on the biological functions of IgG are not fully elucidated. Thus, examination the effects of glycan structures on the functions of antibodies by altering glycosylation is highly interested. It was reported that the Fc regions of antibodies without core fucose were able to improve the antibody cellular cytotoxicity (ADCC) (Beale and Feinstein 1976; Shields et al. 2002; Shinkawa et al. 2003). Recently, the enrichment of α2,6 sialylation of IgG was demonstrated to result in superior ADCC (Kurogochi et al. 2015). Additionally, the Fc region carrying the Neu5Ac-terminal glycans was proved to show strong anti-inflammatory ability. Interestingly, when it contains a large amount of α2,6 sialylation the study reported that intravenous immunoglobulin is the most effective for anti-inflammation (Kaneko et al. 2006, Anthony et al. 2008).

Collectively, I approached to engineer a CHO cell line by overexpressing two Neu5Ac-based key enzymes, so that the host cell line can exhibit greater sialylation profiles in IgG antibodies to prove the concept of using gene amplification to enhance sialylation using CHOmt17-100 host cell line.

4.2 Materials and Methods

4.2.1 Cells and culture media

I established the cell line CHOmt17-0 by co-overexpressing two related human enzymes as described in chapter 2 and chapter 3. These cells were cultured in a (-) α MEM. This medium was added with 100 μg/mL G418 without nucleosides or L-glutamine and 10% dialyzed FBS. The antibodies IgG producing transfectants were cultured in an (-) α MEM without nucleosides, supplemented with 100 μg/mL G418, 10% dialyzed FBS, 1 μg/mL puromycin and 100 μg/mL hygromycin. High-resistance MTX cell lines, CHOmt17-100, were also cultured in an (-) α

MEM without nucleosides, added with 100 µg/mL G418, 10% dialyzed FBS and an MTX concentration of 100 nM. All cells were cultured in a cell culture incubator monitored at 5% CO_2 and 37^oC.

4.2.2. Purification of antibodies using Protein G Sepharose

Protein G Sepharose™ (GE, Illinios, USA) was used to purify the antibodies from the crude sample due to its specific binding to the antibodies. The IgG1-producing cultured cells (2 x 10^8) were cultured in an α MEM without FBS supplementation. Next, the antibodies were then purified from the medium using Protein G Sepharose™ after 5 days in culture. The collected sample was first subjected to centrifugation for 5 min at 4^oC and at 5000 x g. Four milliliters of gel column were next loaded by 10 mL binding buffer composed of 0.02 M sodium phosphate pH 7.0. The gel column was next washed with binding buffer and eluted with 0.1 M glycine – HCl pH 2.7 after running the medium sample. The resultant elution was neutralized using 1 M Tris – HCl pH 9.0. The purification process was performed at 4^oC condition.

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

The ninety-six-well ELISA plates coated with 2.5 µg/mL of goat anti-human IgG (H+L) (MBL, Aichi, Japan) was then blocked by 50 µL of 5% bovine serum albumin (BSA) diluted in PBS. The plates were next each incubated with 50 µL of crude/purified sample. Then, the plates were washed three times before incubation with 50 µL of goat anti-human IgG (H+L) Fab_horseradish peroxidase (HRP)-conjugate by using 150 µL of PBS containing 0.05% Tween 20. The solution was finally mixed with SIGMAFASTTM *o*-phenylenediamine dihydrochloride (Sigma, MO, USA) before the addition of 3M HCl. The amount of bound IgG1 antibody was examined by using absorbance at 450 nm.

4.2.4 Silver staining

The recovered antibodies IgG in the elution from purification using Protein G Sepharose™ were subjected to perform SDS-PAGE and silver staining. Twenty µL of purified sample was

mixed with 2x sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, 0.01% bromophenol blue) before heating at 100^{o}C for 5 min. The collected mixture was then loaded into 10% polyacrylamide gels to run electrophoresis. The bands were detected by using a silver staining kit, Sil-Best Stain One (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions.

4.2.5 Glycan structure analysis of recombinant antibodies

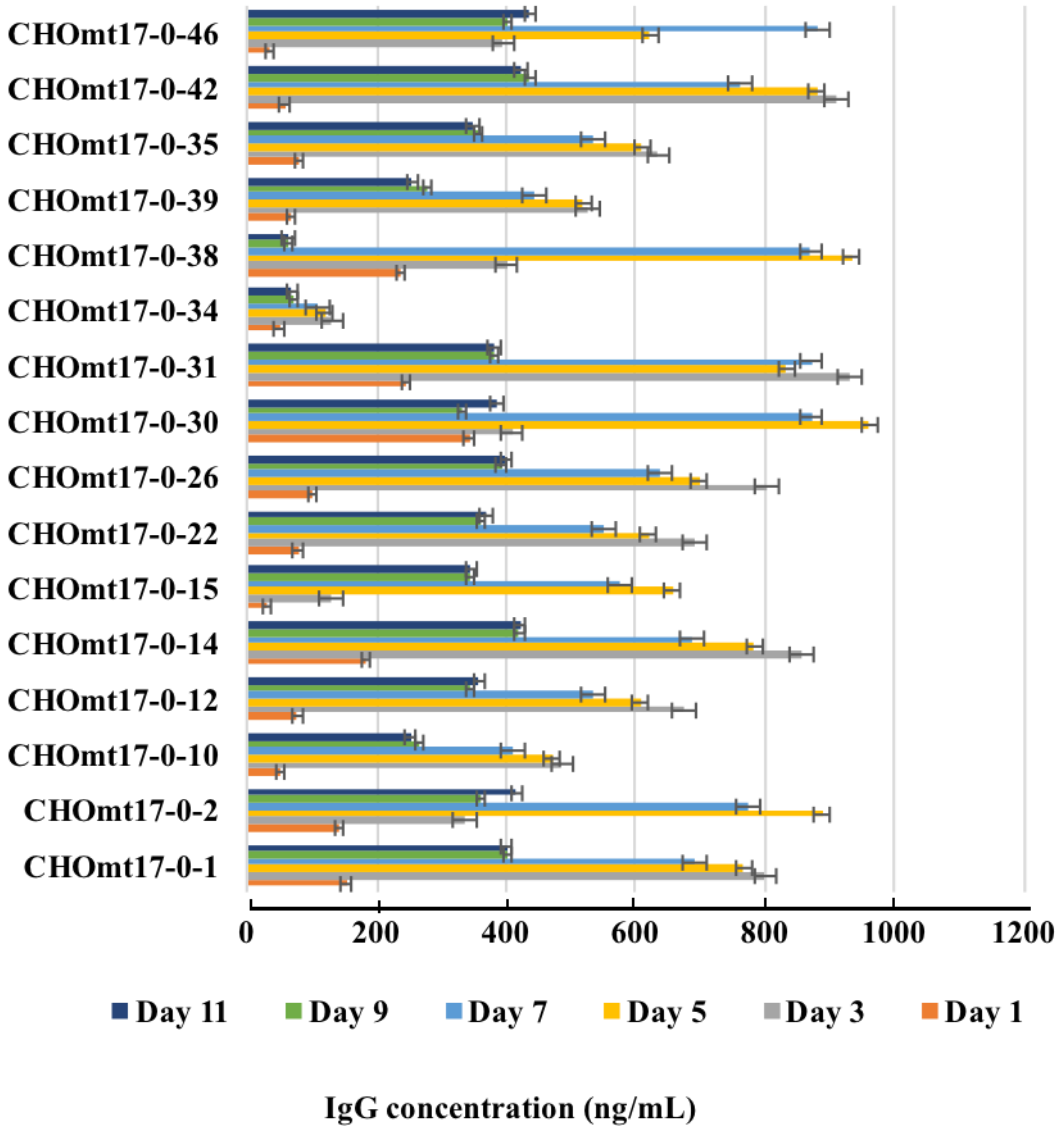
The antibodies resulted from purification using Protein G Sepharose™ were then subjected to glycan pattern analysis as described in details in chapter 2.

4.3 Results

4.3.1 Examination of recombinant IgG production in cell pools produced in investigated host cell lines

I transfected plasmid carrying heavy- and light-chain IgG1 into CHOmt17-0 and CHOmt17-100 to prove the concept of cell lines highly expressing sialylation. Then, I established stable cell lines expressing higher levels of antibodies. The crude samples collected were then analyzed using ELISA to check their production.

(A) IgG production in CHOmt17-0



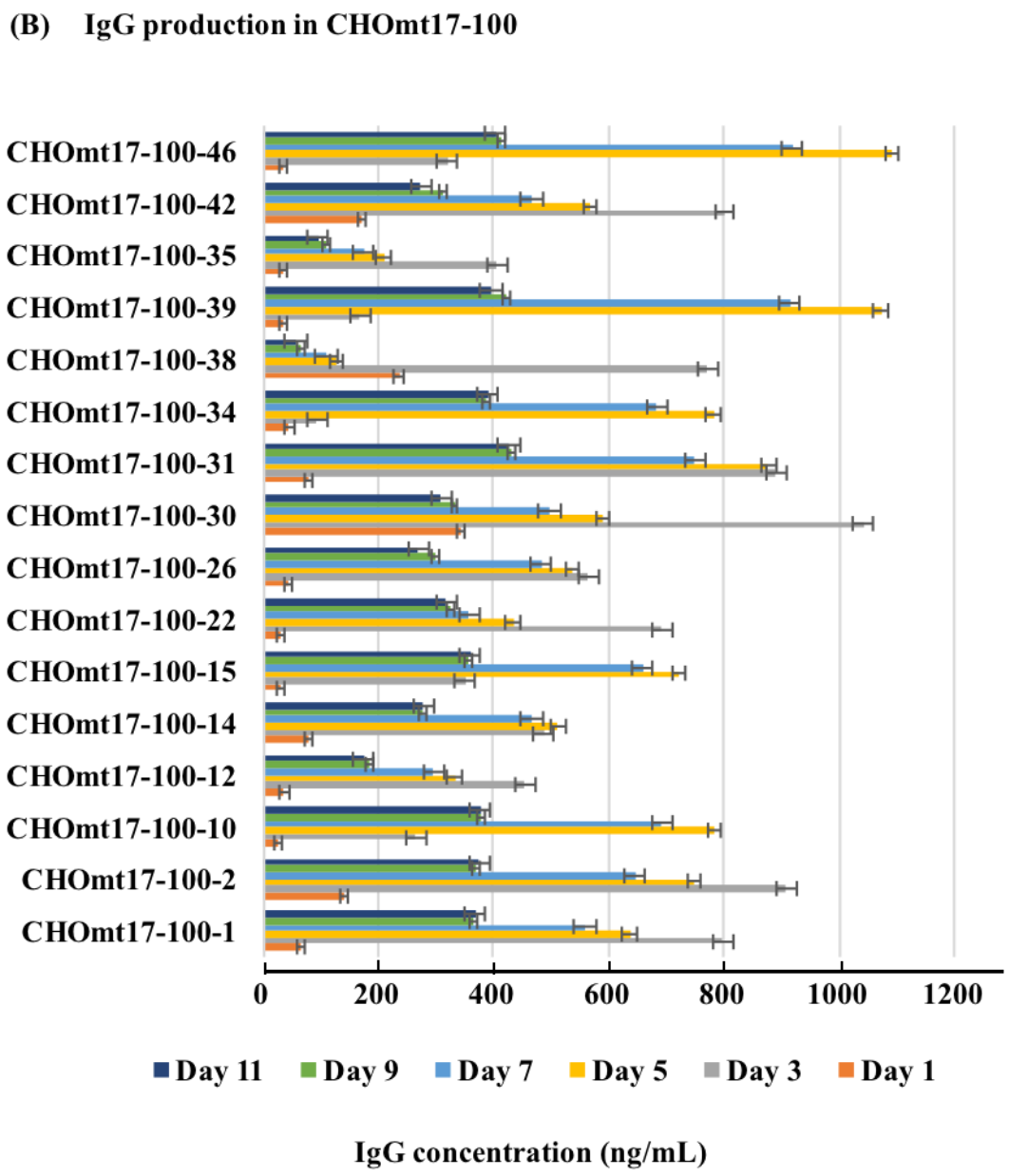


Fig. 12 (**A)** Production of IgG derived from CHOmt17-0 (**B)** Productivity of antibodies IgG derived from CHOmt17-100 cell pools, in α MEM on days 1, 3, 5, 7, 9, and 11 using ELISA. Error bars represent the standard deviation calculated from data obtained in three independent experiments (n=3)

By using equivalent sample amounts for each cell pool in both CHOmt17-0 and CHOmt17-100, ELISA was performed to elucidate their productivity as shown in Fig. 12A and Fig. 12B. The productivity of each antibody was examined on days 1, 3, 5, 7, 9, and 11 in three independent experiments. Interestingly, the cell pools derived from both CHOmt17-0 and

CHOmt17-100 illustrate comparable capabilities in producing antibodies: about 1 µg/mL on day 5. The clone exhibited the highest levels of antibodies were then chosen for purification. The antibodies harvested from CHOmt17-0 and CHOmt17-100 were next purified using Protein G Sepharose.

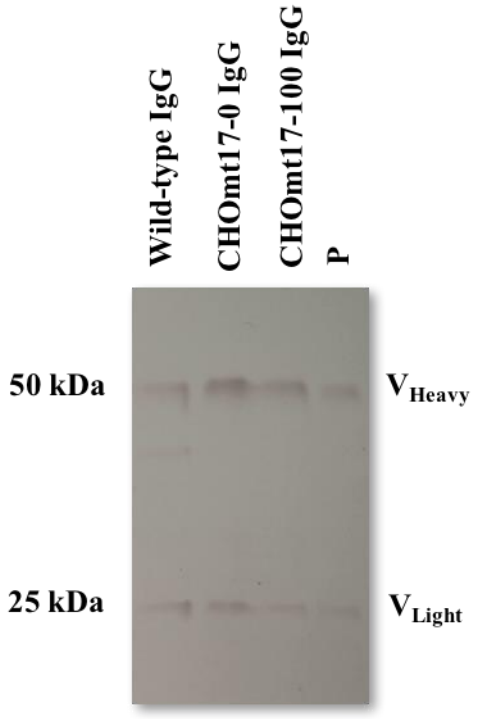


Fig. 13 Analysis data of 100 ng IgG-purified proteins using silver staining of SDS-PAGE gels in investigated wild-type, CHOmt17-0, CHOmt17-100 cell lines. P: human plasma IgG (50 ng)

Then, the resultant antibodies from purification were detected by ELISA and silver staining. In reduced-condition SDS-PAGE, Figure 13 shows the variant heavy chain and variant light chain at 50 kDa and 25 kDa, respectively. Collectively, stable cell lines highly producing IgG1 have been established using host cell lines CHO17mt-0 and CHO17mt-100. The resultant antibodies IgG1 were next subjected to glycan profiling using HPLC and LC-MS to exhibit glycoforms in these antibodies.

4.3.2 Highly sialylated antennary structures generated in the recombinant antibodies IgG using host cell line CHOmt17-100

To examine the *N*-glycan structures in the recombinant antibodies, an RP-HPLC was employed, followed by LC-MS in the wild type, CHOmt17-0, and CHOmt17-100. By using peptide-*N*-glycosidase F, the resultant antibodies from purification using Protein G Sepharose were then released *N*-glycans. The glycan structures were then analyzed by LC-MS after the PA-labeled *N*-glycans were fractionated by RP-HPLC. Interestingly, the resultant glycan profiles of antibodies derived from the wild type, CHOmt17-0, and CHOmt17-100 were significantly distinguishable (Fig. 14). In specific, the peaks occurring in the late elution in the RP-HPLC profile revealed a noticeable upregulation of intensity in the IgG glycoforms produced in CHOmt17-100 compared to that of the CHOmt17-0 and wild type (Fig. 14). These results obtained in glycan profiling of IgG were seen as an agreement with that of glycan patterns in total protein glycan patterns (Fig. 11). The eluted peaks in each clone were then collected and transferred to perform glycan analysis by using LC-MS. As a result, Table 5 indicates the composition of glycan pattern in antibodies IgG derived from wild type, CHOmt17-0, and CHOmt17-100 cell lines. Thus, the Neu5Ac terminal glycans binding to IgG produced in CHOmt17-0 exponentially jumped nine-fold, with about 37% of Neu5Ac terminal structure as compared to 3.7% of the wild type. Interestingly, by supplementation of MTX, the antibodies derived from CHOmt17-100 showed more than 53% sialylated glycan structures with about 14.5-fold higher as compared to the wild type. The results are a strong agreement with the glycan profiling in cell host lines CHOmt17-0 and CHOmt17-100 in total proteins. Also, Neu5Ac-terminal structures occur for most of the upregulation in sialylated structures as shown in Table 5. Despite the exponentially increase in sialylation, the proportion of the NeuGc terminal in IgG derived from CHOmt17-100 and CHOmt17-0 persisted unchanged at less than 5% compared to that of the wild type. These results proved that the two Neu5Ac-based key

enzymes expression using DHFR/MTX sufficiently improve the Neu5Ac-terminal glycoforms in IgG produced in investigated antibodies.

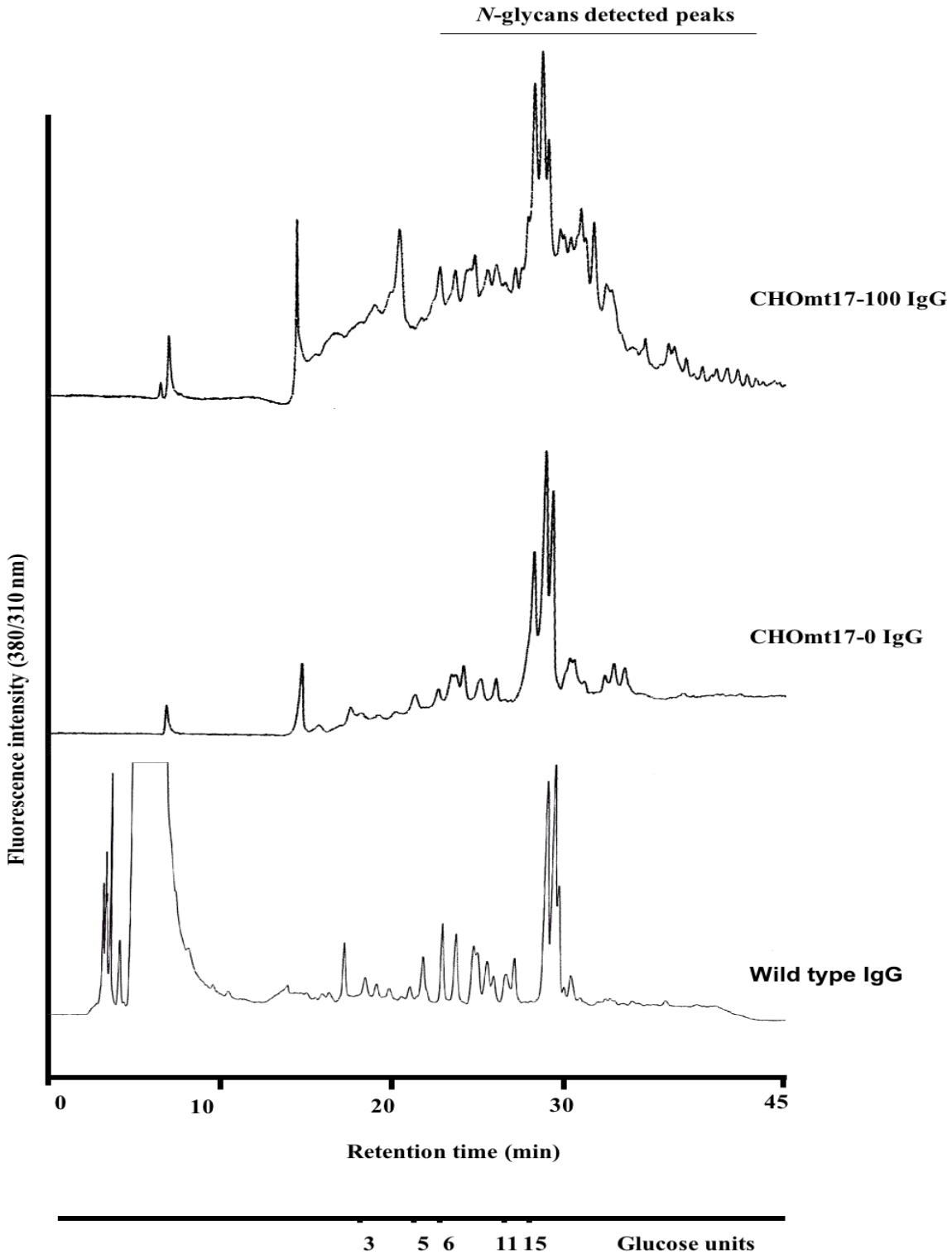


Fig. 14 The RP-HPLC profiles of glycan data of IgG antibodies produced in wild-type, CHOmt17-0, and CHOmt17-100 cells. The *N*-glycans derived from the IgG antibodies were labeled with PA. The PA-glycans were fractionated using RP-HPLC. Then, the eluted peaks were collected to perform LC-MS. Glucose units: RP-HPLC profile for identification of glucose oligomer 3-15.

Table 5 Composition of predicted terminal sialic acid residue and predicted *N*-glycan structures in antibodies produced in engineered cells

Structures	Relative amount (%) in total *N*-glycan		
	Wild-type	CHOmt17-0	CHOmt17-100
Sialylated glycans	3.7	37.4	53.5
Neu5Ac terminal	-	35	53.5
NeuGc terminal	3.7	2.4	-
High-mannose type	12.4	-	-
Galactosylated glycans	51.8	13.7	13.6
Fucosylated glycans	64.6	60.6	80.2

Table 6 Predicted structures found in IgG derived from highly sialylated cell lines using LC-MS

Predicted structures	Relative amount (%) in total *N*-glycan	
	CHOmt17-0	CHOmt17-100
M2	2.6	-
M3F	-	6.1
M5	2.2	-
GnM3	0.0	-
GnM3F	31	4.2
Gn2M3	1.4	-
Gn2M3F	10.1	-
GalGnM3F	6.4	-
GalGnM4F	-	-
GalGn2M2F	0.3	22.6

GalGn2M3	0.0	2.6
GalGn2M3F	3.0	3.0
Gal2Gn2M3	0.8	-
Gal2Gn2M3F	2.3	7.9
Gal2Gn2M4	2.2	-
Gal2Gn2M4F	0.1	-
Gal2Gn2M5	0.1	-
Gal3Gn3M3	0.0	-
Neu5AcGalGnM2	2.9	-
Neu5AcGalGnM3F	2.0	-
Neu5AcGalGnM4	0.6	-
Neu5AcGalGnM5	-	0.6
Neu5AcGalGn2M3	0.5	-
Neu5AcGal2Gn2M3	1.2	20
Neu5AcGal2Gn2M3F	2.4	0.9
Neu5AcGal3Gn3M3	3.5	2.5
Neu5Ac2Gal2Gn2M3	1.5	9.0
Neu5Ac2Gal2Gn2M3F	2.9	8.0
Neu5Ac2Gal3Gn3M3	17.4	6.0
NeuGcGal2Gn2M4F	0.1	-
NeuGcGal2Gn2M3F	1.4	-
NeuGc2Gal2Gn2M3	0.2	-
NeuGc2Gal2Gn2M4	0.3	-

		-
Neu5AcNeuGcGal2Gn2M3	0.4	

4.4 Discussion

CHO cells have been well known as the main host cell lines for therapeutic production for about 20 years. A large number of studies achieved to modify the glycoforms of CHO cell lines because of the contribution of glycosylation to the efficiency of therapeutics. These studies included modulation of glycosylation by culture media supplement, process optimization, or genetic modification. Additionally, modulation of glycoforms by altering glycosylation-related genes is the most common used.

1. The DHFR/MTX gene amplification method is well known as the first amplification system used in mammalian cells (Omasa 2002, Kaufman and Sharp 1982). Up to date, the system is the most common utilized in CHO cells to enhance therapeutics production (Takagi et al. 2017). Thus, the DHFR/MTX gene amplification system applied in altering glycan patterns stands uncommon. We believe that this tool has great potential to enrich sialylation patterns by greatly upregulating Neu5Ac-based key enzymes. To our knowledge, the current research is the first study obtained stable host cell lines using a gene amplification method that can overexpress two Neu5Ac-based key genes driven in the same expression vector. Goh et al. (2014) utilized the DHFR/MTX genes amplification to restore glycan enzyme GnT-I in the CHO cells with GnT-I-deficient. In laboratory conditions, the sialylation obtained in erythropoietin (EPO) glycans derived from the engineered CHO cell line were 25% more abundant than that in wild-type cells. EPO sialylation with industrial-scale conditions was about 2.5-fold greater than that in laboratory conditions. In another study, the IgG derived from CHO cells produced double times of the total sialic acid content as compared to the control with a similar effort of ST6GAL-I overexpression (Lin et al. 2015). In our study, the resultant sialylation patterns obtained in the antibodies noticeably escalated in engineered CHOmt17-100 with about 14.5-fold increase, and 53% of glycoforms carrying Neu5Ac residues, as compared to that the wild type. Additionally, the Neu5Ac residues mostly occurred in the glycoforms of the engineering

cell line, meanwhile the NeuGc residues persists unchanged compared to the wild type. Interestingly, these results are a strong agreement with the glycan analysis using total proteins of CHOmt17-100 cell line. The result obtained may possibly confirm the important role of ST6GAL-I and GNE in production of Neu5Ac. Since NeuGc is considered an immunogenic factor in human (Higashi et al. 1977; Merrick et al. 1978), with these qualifications CHOmt17-100 cells might provide itself greater favorably host cell line. Also, Fig. 10 showed that cell viability of CHOmt17-100 was not greatly changed as compared to that of CHOmt17-0 cell line, suggesting that concentration of 100 nM MTX might not dampen the viability of the CHOmt17-100. The antibodies IgG concentrations derived from CHOmt17-0 and CHOmt17-100 both highest detected at approximately 1 g/L on day 5 (Fig. 12). Thereupon, the supplementation of MTX might not change the antibodies productivity in CHOmt17-100 cell line. The investigated antibodies IgG have one *N*-glycosylation at the Asn297 posited in the Fc region. Here, the resultant glycan showed improvement in sialylation in CHOmt17-100 as compared to that of CHOmt17-0. As mentioned in our previous studies, the wild type CHO cells have α2,3-sialyltransferase enzymes but not α2,6-sialyltransferase enzymes. The upregulation of sialic acid in engineered cell lines mainly caused by ST6GAL-I and GNE. Thus, the linkage derived from the increased sialylation of the engineered cells is α2,6 linkage sialic acid. Due to its dynamic quality of the glycan in the Fc region, it can be accessible to approach to glycosylation enzymes (Barb et al. 2012, Ramasamy et al. 2005). These researches might possibly explain that the increase of sialylation is due to the upregulation of Neu5Ac-based key enzymes.

4.5 Summary

In conclusion, due to its superior characteristics, the cell line CHOmt17-100 can possibly be an attractive host cell for therapeutic production. Additionally, the DHFR/MTX gene

amplification was demonstrated as an effective method for CHO cell lines in stably amplifying the glycosylation genes.

Chapter 5: General Conclusions and Perspectives

A number of therapeutics derived from Chinese hamster ovary cells have been approved for clinical use. CHO cells have long been demonstrated as a safe workhorse for biopharmaceutical products (Jayapal et al. 2007). Meanwhile glycosylation, specifically sialylation, is an important factor in therapeutic qualities such as stability and half-life. Thus, CHO cell lines that can efficiently produce highly sialylated therapeutics are desirable.

Many researchers are keen to elucidate methods to modify sialylation. Some common methods are alteration of the process, supplementation of media, and engineering of glycosylation genes. However, genetic modification is considered the most attractive method due to its stability, permanence, and cost-effectiveness. The approaches often used to manipulate sialylation are genetic engineering of sialylation pathways, inhibition of sialidase activity, or overexpression of *N*-acetylglucosaminyltransferase genes. In two studies to modify the sialylation of CHO and HEK cells, Chung et al. (2015) and Krzewinski-Recchi (2003) elucidated the functions of a total of six genes involved in the sialyation pathways of mammalians cells ST3GAL-I to ST3GAL-IV and ST6GAL-I to ST6GAL-II, respectively. By combining siRNA knockdown and overexpression, those authors reported that ST3GAL-IV is the key enzyme for the synthesis of Neu5Ac residues in CHO cells. They also found out that ST6GAL-I preferred the ß-1,4 linkage of galactose terminal substrates that linked to the proteins; meanwhile, ST6GAL-II preferred the free ß-1,4 linkage of galactose terminal substrates. In another study, Bragonzi et al. (2000) aimed to overexpress ST6GAL-I without ST3GAL genes and found that interferon -γ exhibited about 40% of the sialic acid content of the wild type (Bragonzi et al. 2000). Apart from the sialyltransferase level and the step prior to that is galactosyl transferase or the nucleotide sugar substrates, the overexpression of genes for branching *N*-glycan structure, and the transportation of them into the Golgi can also have effects on the sialylation of targeted proteins. Some highly advanced techniques can be used to upregulate sialylation, such as

CRISPR/CAS9, RNA interference, and TALEN nucleases. Moreover, some combination with other techniques, such as next-generation sequencing and the editing of other genes, can be used to modify glycosylation. These tools could enhance the efficiency with which targeted glycosylation modifications can be made.

In this study, I used two Neu5Ac-based key enzymes to modify the genetic of CHO cells in an attempt to obtain high-sialylation cell lines. To achieve this, I employed the DHFR/MTX gene amplification method to amplify those two genes. The results obtained were very attractive. With the presence of MTX, I established a clone, CHOmt17-100, with 12-fold increase in sialylation compared to the wild type. To my current knowledge, this is a significant increase in sialic acid content in *N*-glycan of CHO host cell lines. This might prove that gene amplification systems are efficient for improving sialylation by overexpressing genes of interest. Next, I aimed to express a therapeutic based on this cell line to prove the concept of a high-sialylation cell line. The Fc region of IgG carrying sialylation has great potential for autoimmune treatment. IgG was thus determined as a model protein for CHOmt17-100. Interestingly, the resultant glycans obtained in this IgG transfected in CHOmt17-100 revealed a tremendous, 14.5-fold increase in sialylation profiles, with about 53% of the total glycans being sialylated. In the future, I believe that to accrete sialylation in pharmaceutical proteins, we can combine genome editing methods such as CRISPR/CAS9 to knockdown/knockout sialidase enzymes and gene amplifications of other sialyltransferase enzymes in CHO cells line. To improve the sialylation, the supplements of substrate and altering cell-culturing parameters of engineered cell lines can also be utilized.

I believe that this gene amplification system has high potential to serve as a great tool with which to boost superior glycosylation patterns by greatly increasing levels of Neu5Ac-based key enzymes in antibodies derived from amplified cell lines. I thus believe that the present results could strengthen the position of the bio-better products field.

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