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ORIGINAL PAPER

Galactose supplementation enhance sialylation of recombinant Fc-fusion protein in CHO cell: an insight into the role of galactosylation in sialylation

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Abstract Sialic acid levels of therapeutic glycoprotein play an important role in plasma half-life. An undesirable decrease of sialic acid content was observed when we increased Fc-fusion protein productivity fourfold in a GS-CHO cell line by bioprocess optimization. We investigated the potential mechanism for the sialic acid content reduction. We found that limited nucleotide sugar precursor and the extracellular sialidase were not responsible for the reduction of the sialic acid content after titer improvement. Oligosaccharide analysis revealed that the lack of protein galactosylation was the potential cause for the reduction of sialic acid content. Thus we validated this notion by evaluated galactose supplementation in 2 L bioreactors. Cell culture performance was not impacted by addition of up to 40 mM galactose except for the glucose consumption rate. Addition of 20 mM galactose to the bioreactor resulted in the increase of 44 % for total sialic acid content and 20.3 % for sialylated glycans. These data were further validated when the process was run on 200 L scaled bioreactor. These data together show that the galactosylation plays an apparent role in sialylation in our current system.

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Keywords Fc-fusion protein · CHO cell · Glycosylation · Sialylation · Galactose · Galactosylation

Introduction

In the last two decades, the application of genetic engineering to the bio-production of recombinant human therapeutic proteins has been made a great strike. Mammalian cells, especially Chinese hamster ovary (CHO) cells, are commonly employed as the host for production of various therapeutically recombinant proteins because of their robust growth and ability to perform complex post-translational modifications including glycosylation (Rasmussen et al. 1998; Wurm 2004). Approximately 70 % of the therapeutic proteins are glycoprotein (Sethuraman and Stadheim 2006). The glycosylation of protein showed to have impact on protein stability, susceptibility to proteases, bioactivity, pharmacokinetics, half-life of commercial biologics, as well as immunogenicity (Dalziel et al. 2014; Hossler et al. 2009; Sethuraman and Stadheim 2006). The terminal sialic acid content of glycoprotein has been shown to be intricately linked to plasma half-life (Liu et al. 2013), since it prevents recognition of the glycoprotein by asialoglycoprotein receptor (Weiss and Ashwell 1989). Thus, it is commonly considered as a critical quality attribute (CQA) in protein therapeutics due to its pivotal role in plasma half-life of protein therapeutics (Jedrzejewski et al. 2013; Rathore 2009). The raising of the biosimilar industry in recent years has drawn much attention to optimization of sialylation since the biosimilar products are required to replicate this CQA of the originator's products.

One of the main challenges during process development is to balance the high productivity and desirable sialylation of the recombinant protein. One way to circumvent this

problem is to utilize a cell line has the better sialylation capacity to manage the over-expressed protein (Ghaderi et al. 2012; Malykh et al. 1998). Moreover, researchers enhanced the cellular sialylation capacity by overexpression of glycosyltransferases or knockdown the glycosidases (e.g. sialidase) in cells. Both of these genetic modifications in cells were shown to have significant impact on the glycan synthesis pathways and/or degradation of glycan. Therefore, it affected the sialylation of the recombinant protein (Maszczak-Seneczko et al. 2011; Zhang et al. 2010). Recently, some studies showed that optimization of the physical and chemical environment of the cells can also improve its sialylation capacity of the cells. For example, alteration of the temperature, pH and DO was showed to improve the protein glycosylation in cells (Ahn et al. 2008; Restelli et al. 2006; Sajan et al. 2010; Yoon et al. 2003). Optimization of cell culture media by feeding precursors, nucleotide sugar substrates, hormones was also showed to enhance the glycosylation pathway for more desirable sialylation in cell (Clark et al. 2005; Crowell et al. 2007; Gramer et al. 2011; Gu and Wang 1998; Gu et al. 1997b; Jenkins et al. 1994; Jing et al. 2010; Rouiller et al. 2012; Wong et al. 2010). Cell metabolism byproducts such as ammonia were also found to play a significant role in the activity of enzymes of glycosylation pathways (Gawlitzek et al. 1998, 2000). While these studies provided methodologies to enhance sialylation on cellular level, it did not provide the insight of how the enhancement occurs in the cellular level. Therefore, a study to investigate the pathway for sialylation enhancement is necessary to provide insight of desirable sialylation in a high titer mammalian cell.

In our current study, a dramatic decrease in protein sialic acid content was observed after a high yield (>1 g/L) fed-batch process of an Fc-fusion protein had been developed in CHO cell. We found that the lack of galactosylation was the potential cause of sialic acid content reduction. Based on these findings, we were able to improve the sialylation of the Fc-fusion protein by correlating the galactose concentration in the media and sialic acid synthesis pathway in the cell.

Materials and methods

Cell line and cell culture media

A stable GS-CHO cell line expressing an Fc-fusion protein (the human tumor necrosis factor receptor linked to the Fc portion of human IgG1) was used in this study. The cell line was a gift from Hisun Biopharmaceutical (Hangzhou) Co., Ltd. The Fc-fusion protein consists of 934 amino acids with an apparent molecular weight of 140 kDa. The protein is highly glycosylated containing 6 N-glycans and several O-glycans. The basal medium for cell culture was EX-CELL^{TM} 302 serum-free medium (SAFC, Bioscience, USA). The feed medium for the fed-batch culture was chemical defined and composed of concentrated amino acids, vitamins and glucose. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were maintained as suspension cultures in shake flasks under conditions of 37 °C, 5 % CO_2 and 150 rpm rotation in a humidified incubator (Adolf Kühner AG, Birsfelden, Switzerland) before production.

Cell cultures in bioreactor

For fed-batch culture, exponentially growing cells were inoculated at a concentration of 0.6–0.8 × 10^6 cells/mL. All production cultures were performed in 2 L bench scale bioreactors with a 1 L initial working volume (Applikon, Foster City, CA, USA). Each bioreactor has an angled three-blade impeller operating with an agitation rate of 150 rpm. Dissolved oxygen was controlled at 50 % air saturation by sparging with air and pure oxygen gas via a micro sparger. Temperature was controlled at 37 °C and shifted to 31 °C at day 5. Culture pH was controlled at 7.0 with a dead band of 0.03 by addition 1 N Sodium hydroxide or sparging with CO_2. Starting from day 2, the feeding medium was added into the bioreactors at 3 % of the initial culture volume until the end of the process.

For large-scale production, 200 L pilot scale bioreactor (Sartorius BBI System, Melsungen, Germany) was used with a 150 L initial working volume. The bioreactor was equipped with two segment impellers and operated with an agitation rate of 70 rpm. Other operating procedure was the same as the 2 L bioreactor.

Cell count and cell metabolite measurement

Cell suspension samples were taken daily from the bioreactor. Viable cell density (VCD) and viability were measured daily using TC20^{TM} Automated Cell Counter (Bio-Rad, Hercules, CA, USA). pCO2, glucose, lactate and ammonia were measured by Bioprofile 400 (Nova Biomedical, Walthan, MA, USA). Galactose concentration was analyzed by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method (Harazono et al. 2011). Cell culture supernatants, after centrifugation, were aliquoted and kept frozen at −80 °C for later analysis.

Fc-fusion protein titer analysis

Titer was determined by high performance liquid chromatography (HPLC) using Applied Biosystems (Foster City, CA, USA) POROS protein A column (2.1 mm ×

30 mm). Running at the conditions recommended by the manufacturer. The eluted protein was detected by absorbance at 280 nm and quantified using reference standards.

Fc-fusion protein purification

Cell culture samples were purified by Protein A chromatography using MabSelect SuRe resin (GE Healthcare, Amersham, UK). The column was equilibrated in a tris/sodium chloride buffer at pH 7.4. Bioreactor supernatant samples were loaded directly onto the column, washed with tris/sodium chloride buffer at pH 7.4, eluted in citrate buffer at pH 3.6, and neutralized using a tris buffer (pH 8.0).

Protein glycosylation analysis

The N-linked glycans of Fc-fusion protein were analyzed by NP-HPLC method (Royle et al. 2008). In short, N-linked glycans were released from purified samples by overnight incubation with Peptide-N-Glycosidase F (Prozyme, San Leandro, CA, USA). The released glycans were recovered with 75 % ethanol precipitation and then the oligosaccharides were labeled with the fluorophore 2-aminobenzamide (2-AB; Prozyme). A cleaning Cartridge (Prozyme) was used to remove excess 2-AB. Glycans were analyzed on a TSKgel Amide-80 (150 mm × 4.6 mm) column (Tosoh Bioscience, Montgomeryville, PA, USA) equilibrated in acetonitrile and eluted with a gradient of 125 mM ammonium formate, pH 4.4 on a Waters HPLC with a Multil-fluorescence detector 2475 (excitation at 330 nm, emission at 420 nm) (Royle et al. 2008). Each peak was collected and analyzed by MALDI-TOF mass spectrometer (AB Sciex, Foster City, CA, USA). The amount of each structure such as A1, A1F, A2, et al. was expressed as the percentage of total peak area.

Total sialic acid content assay

Total sialic acid content was measured using Resorcine method (Svennerholm 1957). In brief, purified samples was mixed with resorcinol reagent (2 % Resorcine, 0.1 mol/L $CuSO_4$, 25 % HCl). The mixture was then heated in a water bath at 100 °C for 30 min. An extraction solution (N-butyl acetate and 1-butanol, 4:1 v/v) was then added to this mixture after it was cooled to room temperature. The mixture was shaken vigorously and kept still for 10 min to allow the separation of the organic layer from the aqueous layer. The organic layer was transferred to a cuvette and read at 580 nm by a spectrometer (DU730, Beckman Coulter, CA, USA). A blank organic solution was used as the control.

Extracellular sialidase activity

The commercial Amplex Red Neuraminidase (sialidase) assay kit (Life Technology, Carlsbad, CA, USA) was used to test the sialidase activity in cell culture supernatants. The assay was performed in 96-well plates according to the manufacturer's protocol.

Determination of biological activity of antibody

The biological activity of Fc-fusion protein was tested using a standard method according to Pharmacopoeia of the People's Republic of China. Briefly, a TNF-sensitive L929 cell was used to assess the bioactivity of the Fc-fusion protein when bound to TNF. 4×10^4 cells/well of L929 cells were incubated in 100 μL RPMI 1640 medium supplemented with 10 % FBS in a 96-well tissue culture plate (Corning, NY, USA) and cultured overnight (18–24 h) at 37 °C, 5 % CO_2. 100 μL of prepared serially diluted samples were added to each well of the plate. After 18–22 h of incubation at 37 °C, 10 μL chromogenic agent (Cell Counting Kit-8, Dojindo Molecular Technologies, Japan) was added before another 1–2 h of incubation. The absorbance was read and recorded at 490 and 630 nm. The ED_{50} value was obtained by four parameter logistics curve fitting.

Statistical analysis

Statistical analysis was carried out using SPSS 19 software (IBM Corp., NY, USA). One-way ANOVA followed by least significant difference (LSD) tests were used to determine the statistical significance of differences between the means. All the experiments were carried out in triplicate. p values < 0.05 were considered as statistically significant.

Results

Reduction of sialylation by titer increase

Using our platform fed-batch culture process, the final titer of the Fc-fusion protein was 0.31 g/L in a 12 day fed-batch culture process. Amino acid analysis showed that arginine, leucine, isoleucine, lysine and threonine were nearly depleted at the end of the culture (data not shown). After several rounds of feeding medium optimization, the specific protein production rate (Q_p) was improved to 3.6 fold, resulting in a final titer fourfold titer improvement (Fig. 1). However, the sialylation level decreased 31.1 % (Fig. 2). Other quality attributes (SDS-PAGE, HIC and bioactivity) were similar between the original process and

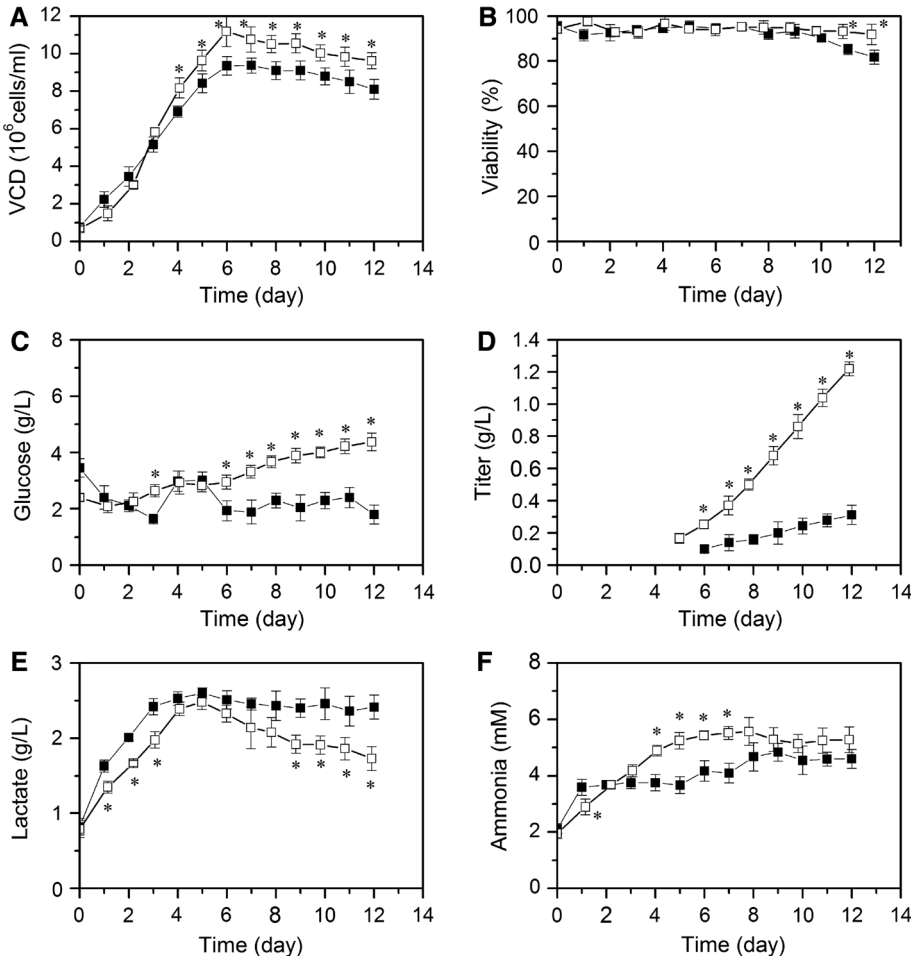


Fig. 1 Cell culture performances of the original process (*solid square*) and the optimized process (*open square*). Time profiles of **a** viable cell density (VCD), **b** viability, **c** glucose, **d** titer, **e** lactate, **f** ammonia. The data are represented as mean (±standard deviation, SD) of three independent experiments. $^*p < 0.05$

optimized process (data not shown). The major parameters of the two process were shown in Table 1.

Effect of sialylation pathway on Fc-fusion protein sialylation

In order to find out the reason for the reduction of protein sialylation in our current system, we investigated the potential mechanism for the sialic acid content reduction. It is possible that the activity of extracellular sialidase, which removes sialic acid from glycoproteins and glycolipids, was also increased by the optimized process (Chee Furng Wong et al. 2005; Chuan et al. 2006; Gramer and Goochee 1993; Gu et al. 1997a; Jing et al. 2010). To evaluate this possibility, we measured the activity of extracellular sialidase. Figure 3 showed that there was no significant difference of the sialidase activity between the original and optimized process. Therefore, the decreased level of sialic acid moieties in optimized process was not due to the extracellular sialidase activity. It should note that sialidase activity increased by approximately 55 % between day 7 and day 12 in both processes.

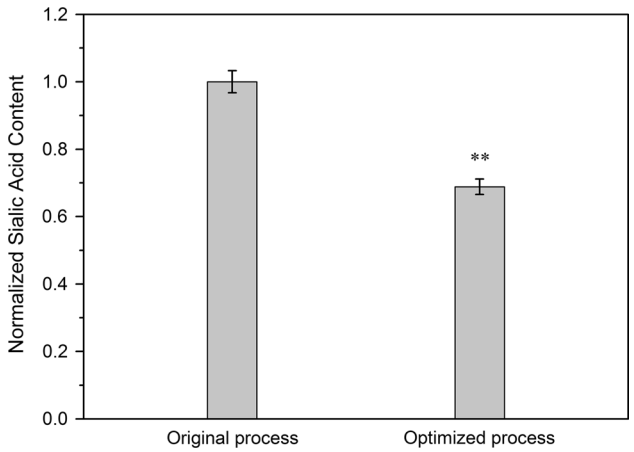


Fig. 2 Total sialic acid content of original and optimized processes. The *data* are represented as mean (±standard deviation, SD) of three independent experiments. $^{**}p < 0.01$

Another possible reason for the overall loss in protein sialylation might be the lack of the sialic acid precursor in the culture environment (Clark et al. 2005; Gu and Wang 1998; Wong et al. 2010). To increase the sialic acid pool,

Table 1 Comparison of the major parameters between the original and optimized process

Parameters	Original process	Optimized process
pH	7.0 ± 0.03	7.0 ± 0.03
DO (%)	50	50
Temperature (°C)	37 (day 0–5)	37 (day 0–5)
	31 (day 5–12)	31 (day 5–12)
Basal medium	EX-CELL™ 302	EX-CELL™ 302
Feeding medium	A chemical defined medium composed of concentrated amino acids, vitamins and glucose	A chemical defined medium with amino acids (arginine, leucine, isoleucine, lysine and threonine) optimization

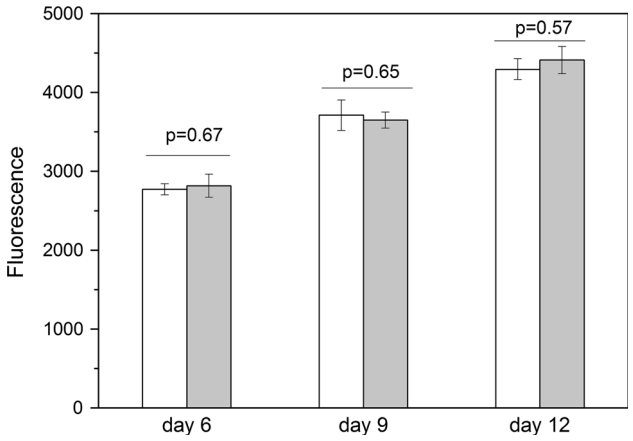


Fig. 3 Extracellular sialidase activity in the original process (*square blocks*) and the optimized process (*shaded blocks*). Values of each parameter are reported as average ± standard deviation (n = 3). Fluorescence units measured after 40 min incubation with the assay kit. Measurements performed after 6, 9 and 12 days of culture

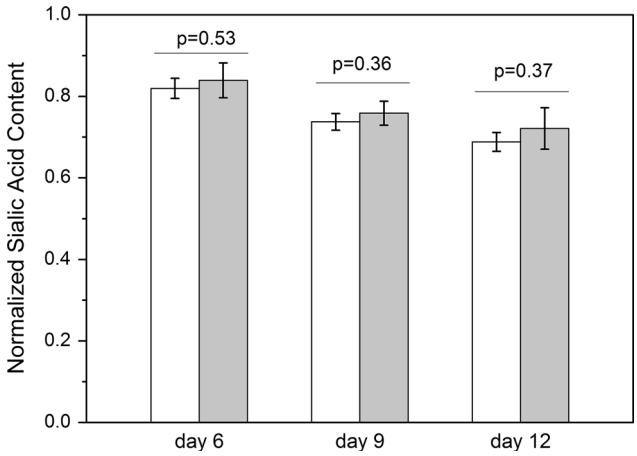


Fig. 4 Impacts of N-Acetylmannosamine and cytidine feeding on total sialic acid content of Fc-fusion protein. Optimized process (*square blocks*), optimized process with ManNAc and cytidine addition (*shaded blocks*). Values of each parameter are reported as average ± standard deviation (n = 3)

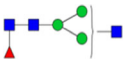
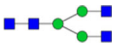
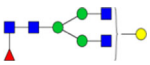
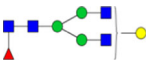
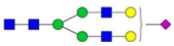
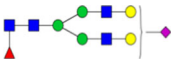
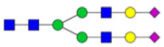
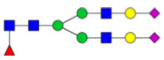
both *N*-Acetylmannosamine (ManNAc) and cytidine were fed to the culture. The concentrated ManNAc and cytidine was fed to the optimized process at the day 5 as the final concentration of each was 20 and 10 mmol/L, respectively. Figure 4 showed that there was no statistical difference of the sialic acid content on day 6, day 9 and day 12 when ManNAc and cytidine were added. The fact that feeding ManNAc and cytidine did not significantly improve the protein sialic acid content, implying the sialic acid pool in cell culture was sufficient. In addition, the decrease of total sialic acid content was observed during the culture time course, this might be caused by the increased extracellular sialidase activity we previously observed in Fig. 3.

In order to gain insight into the reduction of sialylation in our current system, we profiled the glycans of the protein (Table 2). Oligosaccharide analysis showed that the sialylated glycans dropped from 41.4 to 19.0 % after process optimization (Table 3). Interestingly, the N-glycans with no galactose was remarkably increased from 23.1 % of the original process to 47.4 % of the optimized process (Table 3), indicating that there is not enough galactosylation sites for sialic acid addition. Since sialyltransferase transfers sialic acid to the glycan substrates (i.e. galactosylated glycans) during the sialylation process, it seems that the reduced galactosylation might be the limited step in sialylation due to the lack of galactose sites for sialic acid addition (Fig. 5). Therefore, one can potentially improve the sialylation by increasing the galactosylation level of Fc-fusion protein.

Effect of galactose on bioprocess

To investigate our hypothesis, our first attempt is to increase the galactosylation, by adding the key precursor of the galactosylation process (e.g. galactose) (Baker et al. 2001; Crowell et al. 2007). Several experiments were conducted to test this hypothesis. The culture was grown in 2 L bioreactor and concentrated D-galactose was fed to the optimized process as the final concentration was 0, 10, 20 and 40 mmol/L at the day 5. Cell growth, viability, metabolism and protein production were tested. As shown in Fig. 6, galactose feeding had little impact on viable cell density, viability, cell metabolism and Fc-fusion protein

Table 2 Impact of galactose feeding on Fc-fusion protein glycoform characteristics

Structure[1]	Proposed structure[2]	Percentage of glycans from each culture conditions (%)					
		2 L scale					200 L scale
		Original	Optimized	10 mM	20 mM	40 mM	20 mM
	A1	0.4 ± 0.1[a]	1.2 ± 0.2[b]	1.0 ± 0.1[b]	1.1 ± 0.2[b]	0.9 ± 0.3[b]	1.2 ± 0.1[b]
	A1F	0.3 ± 0.1[a]	0.8 ± 0.3[b]	0.7 ± 0.2[b]	0.5 ± 0.2[b]	0.6 ± 0.1[b]	0.6 ± 0.2[b]
	A2	3.2 ± 0.3[a]	11.6 ± 0.7[b]	7.0 ± 0.1[c]	3.1 ± 0.1[a]	3.3 ± 0.4[a]	3.1 ± 0.3[a]
	A2F	16.7 ± 1.3[a]	30 ± 1.0[b]	24.1 ± 2.1[c]	15.7 ± 0.4[a]	15.4 ± 1.2[a]	15.3 ± 1.8[a]
	Man5	2.5 ± 0.2[a]	3.8 ± 0.4[b]	3.3 ± 0.4[b]	3.6 ± 1.1[b]	3.2 ± 0.3[b]	3.3 ± 0.6[b]
	A2G1	2.5 ± 0.3[a]	4.9 ± 0.1[b]	4.2 ± 0.1[c]	4.2 ± 0.3[c]	4.6 ± 0.3[c]	4.4 ± 0.5[c]
	A2G1F	12.8 ± 0.2[a]	12.2 ± 0.6[a]	12.2 ± 1.3[a]	12.1 ± 1.1[a]	12.4 ± 0.9[a]	12.2 ± 2.2[a]
	A2G2	6.7 ± 0.2[a]	5.7 ± 0.3[b]	6.6 ± 0.3[a]	6.9 ± 0.2[a]	6.5 ± 0.4[a]	6.3 ± 1.1[a]
	A2G2F	12.5 ± 0.6[a]	9.5 ± 0.3[b]	11.0 ± 1.1[a]	12.3 ± 0.4[a]	12.1 ± 0.3[a]	13.0 ± 0.2[c]
	A2G2S1	13 ± 0.3[a]	5 ± 0.4[b]	8.1 ± 0.7[c]	11.8 ± 2.2[a]	12.6 ± 0.6[a]	13.4 ± 0.4[a]
	A2G2S1F	19 ± 2[a]	9.1 ± 1.2[b]	14.7 ± 0.6[c]	19.2 ± 1.1[a]	18.7 ± 0.9[a]	18.5 ± 2.2[a]
	A2G2S2	3.2 ± 0.2[a]	1.4 ± 0[b]	1.7 ± 0.2[c]	2.7 ± 0.3[d]	2.6 ± 0.1[d]	2.8 ± 0.3[d]
	A2G2S2F	6.2 ± 0.2[a]	3.5 ± 0.2[b]	4.3 ± 0.3[c]	5.6 ± 0.5[d]	5.5 ± 0.1[d]	5.4 ± 0.2[d]

Significant differences ($p < 0.05$) between values within a row are indicated by different letters

[1] Assignment of symbols: *blue square* N-acetylglucosamine, *green circle* mannose, *yellow circle* galactose, *red triangle* fucose, *purple diamond* sialic acid. Glycan structures are drawn according to the symbol nomenclature defined by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml)

[2] *A* antennary, *G* galactose, *S* sialic acid, *F* fucose, *Man* mannose

Table 3 The relative percentage of N-linked glycans under different culture conditions

Galactose (mM)	Number of galactose in glycans (%)			Total galactosylated glycans (%)	Number of sialic acid in glycans (%)		Total sialylated glycans (%)	Fucosylated glycans (%)
	0	1	2		1	2		
Original	23.1 ± 1.2[a]	15.3 ± 0.6[a]	60.6 ± 0.9[a]	75.9 ± 1.2[a]	32.0 ± 1.6[a]	9.4 ± 0.3[a]	41.4 ± 1.7[a]	67.5 ± 0.9[a]
Optimized	47.4 ± 1.4[b]	17.1 ± 0.6[b]	34.2 ± 0.8[b]	51.3 ± 0.8[b]	14.1 ± 0.4[b]	4.9 ± 0.2[b]	19.0 ± 0.3[b]	65.1 ± 1.0[b]
10	36.1 ± 1.8[c]	16.4 ± 0.5[c]	46.4 ± 0.6[c]	62.8 ± 0.5[c]	22.8 ± 1.2[c]	6.0 ± 0.2[c]	28.8 ± 0.5[c]	67.0 ± 0.6[a]
20	24.0 ± 2.1[a]	16.3 ± 0.6[c]	58.5 ± 0.6[d]	74.8 ± 1.0[a]	31.0 ± 1.1[a]	8.3 ± 0.3[d]	39.3 ± 1.1[d]	65.4 ± 1.1[b]
40	23.4 ± 1.2[a]	17.0 ± 0.7[c]	58.0 ± 0.7[d]	75.0 ± 1.4[a]	31.3 ± 1.4[a]	8.1 ± 0.2[d]	39.4 ± 1.4[d]	64.7 ± 0.8[b]

The proportion of glycans was estimated using the relative peak area from Table 2

Significant differences ($p < 0.05$) between values within a column are indicated by different letters

production. Only 40 mM galactose suppressed glucose consumption rate. The final glucose concentration was increased from 4.4 g/L at 0 mM galactose to 4.7 g/L at 20 mM galactose; and to 5.4 g/L at 40 mM galactose. The galactose concentration of different processes was also analyzed (Fig. 7). The galactose of 10 mM feeding condition was completely consumed at day 10. Slightly increasing levels of residual galactose were found in bioreactors up to the 20 mM galactose feeding condition; however, >86 % of the galactose was still consumed. More

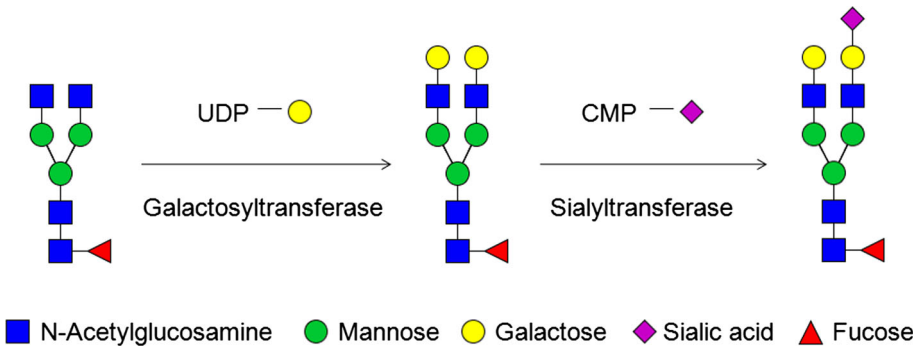


Fig. 5 Simplified oligosaccharide processing pathway: sialic acid was transferred to galactosylated glycan by sialyltransferase

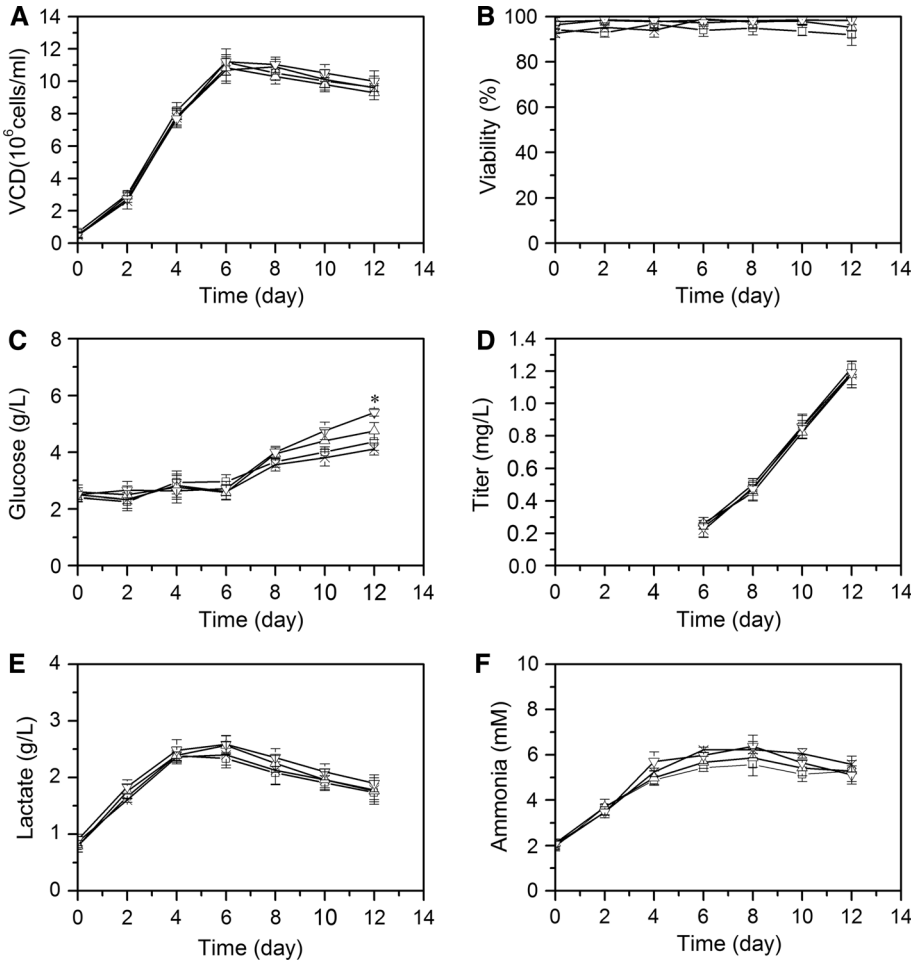


Fig. 6 Effects of galactose feeding on process performance: time profiles of **a** viable cell density (VCD), **b** viability, **c** glucose, **d** titer, **e** lactate, **f** ammonia in culture. *Open square*, 0 mM; *cross*, 10 mM; *up triangle*, 20 mM; *down triangle*, 40 mM. The *data* are represented as mean (±standard deviation, SD) of three independent experiments. $^*p < 0.05$

substantial galactose accumulation was observed in the 40 mM galactose feeding condition.

Effects of galactosylation pathway on Fc-fusion protein sialylation

The effect of feeding galactose on the glycosylated isoform distribution of the Fc-fusion protein was tested by NP-HPLC method. Table 3 summarized the percentage of glycans containing galactose and sialic acid. The increase of the 2-galactose-glycans with 20 and 10 mM galactose addition was 1.71 and 1.36 fold, respectively, when compared to 0 mM galactose addition. However, no significant changes were observed at the percentage of the 1-galactose-glycans. Therefore, we concluded that the overall increase in galactosylation by galactose supplementation was primarily due to the increase of 2-galactose-glycans.

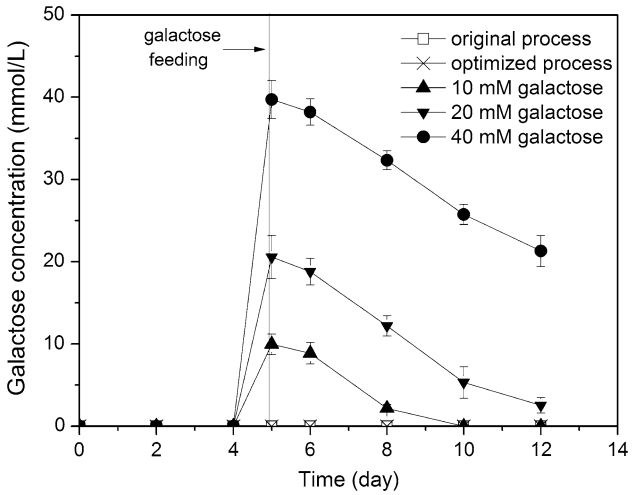


Fig. 7 Residual galactose concentration in bioreactors. The *data* are represented as mean (±standard deviation, SD) of three independent experiments

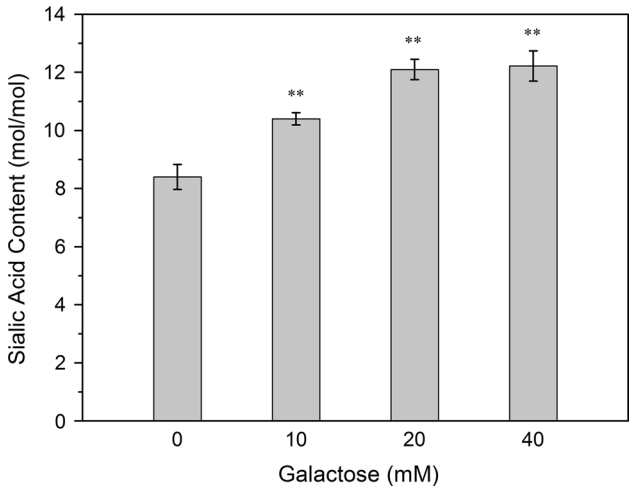


Fig. 8 Impacts of galactose supplementation on total sialic acid content of Fc-fusion protein. The *data* are represented as mean (±standard deviation, SD) of three independent experiments. A significant difference from 0 mM data is indicated by asterisks (**$p < 0.01$)

As shown in Table 3, the sialylated glycans of the protein increased from 19.0 to 28.8 % when 10 mM galactose was supplemented; and further improved to 39.3 % where 20 mM galactose was supplemented. However, there was no significant difference between the 20 mM galactose and 40 mM galactose supplement. The improved galactose in glycans when 20 mM galactose was supplemented during cell culture also translated into improvement of sialylated glycans. The mono-sialylated glycans for the 20 mM galactose condition increased from 14.1 to 31.0 %, and the di-sialylated glycans increased from 4.9 to 8.3 %. Similar phenomenon was also found when 40 mM galactose was supplemented. Meanwhile, the reduction of agalactosylated glycans from 47.4 % (control) to 24.0 % (20 mM galactose) was observed. To validate whether such conversion improves total sialic acid content, we performed total sialic acid content analysis. It was found that the sialic acid content was improved to 1.24, 1.44 and 1.45 fold when 10, 20 and 40 galactose were supplemented respectively (Fig. 8). Therefore, the sialic acid content was improved by supplementation of 20 or 40 mM galactose to the optimized process. It should note that supplementation of 20 mM galactose showed a slight effect on the Man5 (<3 %) (Table 2) as well as fucosylation (<5 %) (Table 3) compared to the original process.

Validation in the pilot scale

Several 200 L pilot scale runs were evaluated with 20 mM galactose feeding. The run performances were similar to that at 2 L scale (data not shown). Furthermore, The glycosylation profiles (Table 2) and the bioactivity of the protein (Fig. 9) at larger scale reflected that found at 2 L scale, suggesting that this fed-batch process was stable to scale up.

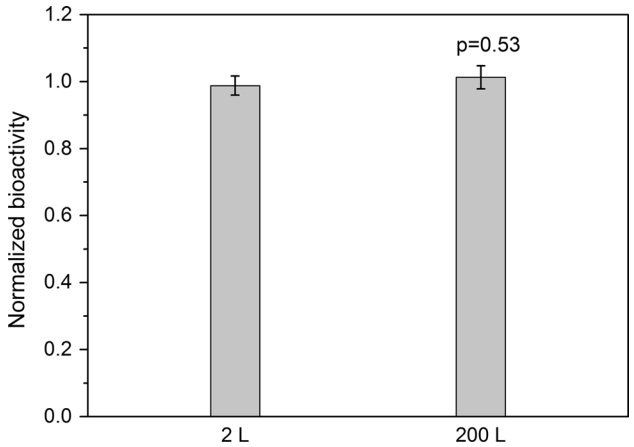


Fig. 9 The bioactivity of Fc-fusion protein at different culture scales. The *data* are represented as mean (±standard deviation, SD) of three independent experiments

Effect of galactose on the original process

The effect of feeding galactose (20 mM) on the original process was also studied. However, total sialic acid content analysis showed that there was no statistical difference of the sialic acid content between the original process and the original process with galactose feeding (Fig. 10). This result indicated that galactose feeding was not always useful, except that galactosylation was insufficient in sialylation. Therefore, it is necessary to identify the limited steps in sialylation during process optimization.

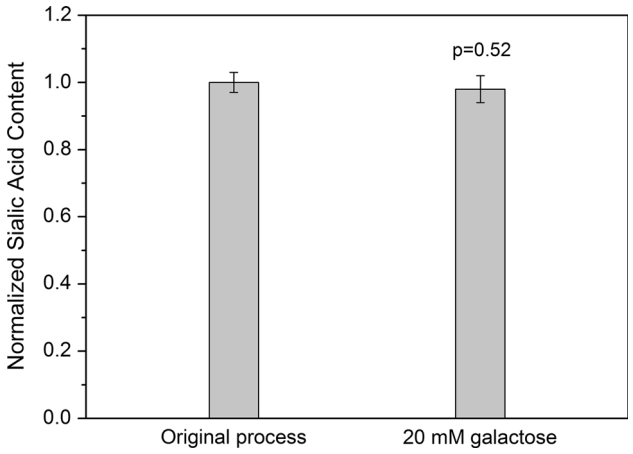


Fig. 10 Impacts of galactose supplementation on the original process. The *data* are represented as mean (±standard deviation, SD) of three independent experiments

Discussion

In our current study, we showed that a bioprocess reduced sialylation of Fc-fusion protein after the titer improvement. Interestingly, protein glycosylation analysis revealed that the galactosylation of the Fc-fusion protein was also decreased along with the decreasing of the sialylation. Upon these findings, we investigated to increase galactosylation as the potential method to improve sialylation since sialic acid was transferred to the galactose of glycan by sialytransferase (Kornfeld and Kornfeld 1985). Therefore, we found that the galactosylation pathway, plays an apparent role in sialylation this system.

In order to gain insight into the reduction of sialylation in our current system. We first compared the extracellular sialidase activity of the original process and optimized process, and determined that there was no significant difference between the two processes (Fig. 3). These data suggested that the sialidase activity was unaffected by the process optimization. Limited nucleotide sugar precursor of sialic acid synthesis can also be the potential cause of reduced sialylation after the process optimization. Therefore, ManNAc and cytidine was added to test this hypothesis. However, the sialic acid content was not changed, implying that the lack of sialic acid precursor in cell culture was sufficient. However, glycosylation analysis showed that the galactosylation was remarkably decreased after process optimization. Since, sialic acid was added to the galactose site of the glycan, it was reasonable to hypothesize that there was not enough galactosylation sites for sialic acid addition. Thus, we validated this notion by evaluated galactose supplementation in the culture. Both the glycosylation and sialylation level of the glycans were improved by supplementation of 20 or 40 mM galactose to the optimized process.

Similar observations were made by increased sialylation of interferon-γ (Wong et al. 2010), or monoclonal antibody (Mab) (Grainger and James 2013; Gramer et al. 2011) due to galactose addition. However, our result revealed that galactose feeding will not always lead to the increase of sialylation. The result of feeding galactose to the original process showed that there was no significant difference of the sialic acid content between the original process and original process with galactose feeding. These data together showed that galactosylation pathway, instead of sialylation pathway, plays an apparent role in sialylation in our current system.

Previous studies have demonstrated that addition of cell culture media component including galactose (Hills et al. 2001; Wong et al. 2010), uridine (Gramer et al. 2011), or Mn^{++} (Crowell et al. 2007), can improve the galactosylation. The synergistic effect of these components was also reported (Grainger and James 2013; Gramer et al. 2011). We chose galactose as the primary component of test because galactose, unlike uridine and Mn^{++}, did not significantly reduce IVCC or titer (Altamirano et al. 2000, 2001, 2006). In addition to galactose feeding effects on galactosylation and sialylation, we observed that supplementation of galactose at or below 20 mM show no significant effect on the bioprocess performance.

Although our studies showed that the galactosylation, instead of sialic acid metabolism, plays an apparent role in sialylation, there are still some limitations about this work. First, the reasons of sialic acid decline after process optimization still need to be investigated. Second, more studies will be needed to investigate precursors and enzymes in galactosylation and sialylation pathway. However, this study provides a methodology to improve the sialylation of protein during process development.

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