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Baculovirus Coinfection Strategy for Improved Galactosylation of Recombinant Glycoprotein Produced By Insect Cell Culture

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Abstract

Baculovirus Expression Vector System (BEVS) is widely used for the production of recombinant glycoproteins, but it is not ideal for pharmaceutical glycoprotein production due to incomplete glycosylation. The factors that ensure successful glycosylation are the presence of sufficient amount of glycosyltransferases, sugar nucleotides as the substrate donor and the recombinant protein as the substrate acceptor. In this study, we analyze the galactosylation process by the introduction of β -1,4galactosyltransferase (β -1,4GalT) as the glycosyltransferase of interest, and uridine-5'-diphosphogalactose (UDP-Gal) as the substrate donor. Recombinant human transferrin (rhTf) as a model protein was used as the substrate acceptor. Insect cell lines have been reported to produce a small amount of β -1,4GalT and thus insufficient for effective galactosylation. In this study, we developed a method to produce galactosylated rhTf and optimized the expression of rhTf with better N-glycan quality. Recombinant β -1,4GalT was introduced during protein expression by the coinfection of the BEVS with baculovirus carrying bovine β -1,4GalT. To evaluate the extent of galactosylation by the coinfection strategy, a binding assay was established. In this binding assay, glycoprotein acceptor was absorbed onto ELISA plate surface. A lectin known as Ricinus communis agglutinin-I (RCA-I) labeled with peroxidase, was added and allowed to recognize Gal β 1 \rightarrow 4GlcNAc group on the N-glycan of the glycoprotein, followed by appropriate color reaction measurements. Coexpression between rhTf and β -1,4GalT did not show encouraging results due to the reduction of UDP-Gal upon baculovirus infection. This interesting finding suggested that the introduction of β -1,4GalT alone was not sufficient for successful galactosylation. Alternatively, post harvest glycosylation method strategy seems to be a promising technique in the improvement of glycoprotein quality.

Keywords: Coinfection, Galactosylation, β -1,4Galactosyltransferase, Human transferrin, Uridine-5'-diphosphogalactose

Introduction

The Baculovirus Expression Vector System (BEVS) is widely used for the production of a large number of glycoproteins due to its ability to express high levels of heterologous proteins. This protein expression system primarily uses cell lines established from lepidopteran insects and the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) mediated expression vector [1]. However, incomplete glycosylation processing pathway due to the inability of lepidopteran insect cells to synthesize sialylated complex-type N-glycans have limited the insect cells as host cells for production of pharmaceutical glycoproteins.

The factors that ensure successful glycosylation are the presence of sufficient amount of glycosyltransferases, sugar nucleotides as the substrate donor and the recombinant protein as the substrate acceptor. In the galactosylation process, β 1,4-galactosyltransferase (β 1,4-GalT) is the glycosyltransferase, uridine-5'-diphosphogalactose (UDP-Gal) is the substrate donor and in our study, recombinant human transferrin (rhTf) is chosen to be the substrate acceptor due to its simple biantennary N-glycan structure. Many experimental evidences suggested that insect cells contain only a small amount of β -1,4GalT [2,3] and thus insufficient for the galactosylation processing. One of the strategies to overcome the limitation of the enzyme is to introduce β -1,4GalT into the system artificially.

In this work, baculovirus coinfection study was performed for the coexpression of hTf and β -1,4GalT. Recombinant glycoprotein quality with a terminal Galactose residue biosynthesis was evaluated. Ailor E. *et al.* [4] reported that the N-glycan of human serum transferrin produced in insect cells were at high mannose, paucimannosidic [2, 5, 6, 7, 8] and some hybrid structures [9, 10, 11, 12] with

over 50% of these structures containing one fucose, $\alpha(1,6)$ - or two fucoses, $\alpha(1,6)$ - and $\alpha(1,3)$ -, linked to the Asn-linked *N*-acetylglucosamine. Neither sialic acid nor galactose was detected on any of the *N*-glycans. In order to determine if altering the intracellular level of enzyme $\beta 1,4$ -GalT in the oligosaccharide processing pathway can promote elongation of the hTf *N*-glycan, a recombinant $\beta 1,4$ -GalT was overexpressed in *Sf*-9 insect cells in conjunction with hTf.

Approach and Methods

Materials and Equipments

Spodoptera frugiperda (*Sf*-9) insect cells was purchased from ATCC cat. No. 1711 (Rockville, MD). The recombinant baculovirus containing the gene coding for hTf and $\beta 1,4$ -GalT were provided by Prof Dr Michael J. Betenbaugh of Johns Hopkins University, USA. *Sf*-900 II Serum Free Media (SFM) was from GIBCO BRL (Gaithersburg, MD). TMB (3,3',5,5'-tetramethylbenzidine) Peroxidase Substrate and Peroxidase Solution B (water soluble) were obtained from Bethyl Laboratories Inc (Texas). Peroxidase-labeled RCA I (*Ricinus communis* agglutinin-I), bovine serum albumin, were purchased from Sigma (Missouri, USA). Trypan blue, ethanol, Tween 20, potassium phosphate dibasic, potassium dihydrogen phosphate were from Fluka (Missouri, USA). All chemicals were used as received.

Shimadzu UV-160 spectrophotometer (Minnesota, USA) was used to measure absorbance at 450nm. Laminar flow hood was from Telstar Bio-II-A (Germany). Inverted phase contrast microscope and Compound microscope (optional) were from Zeiss Instruments (Germany). Incubator was from Memmert (Germany).

Insect Cell Culture

Sf-9 insect cells were maintained in 25 cm³ tissue culture flasks (Techno Plastic Products, Switzerland) containing 5 ml of SF900 II SFM and kept in a humidified 27°C incubator. Cell viability was determined using the trypan blue exclusion test and cell counts were performed using an inverted microscope.

Recloning of Recombinant Virus Stock, Virus Propagation, Virus Titration and Cell Infection

The generation of pure recombinant hTf and $\beta 1,4$ -GalT involves the preparation of a stock starting from a single infectious unit. The method was described by David R. O'Reilly *et al.* [13]. *Sf*-9 cells were inoculated with purified virus stock by adding 20 μ l inoculum to the cells. The infected culture was incubated at 27°C for 7 days, and was visually examined daily to ensure the cells were well infected. To collect extracellular virus, the infected cell culture were transferred to a centrifuge tube and spinned at 1000 x g for 15min. The concentration of the infectious virus particles in recombinant virus stock was calculated by end point dilution technique, which was developed by Reed and Muench [14] and modified by Nielsen *et al.* [15]. Infection of insect cells involved inoculation of

exponential stage cell cultures with a small volume of broth supernatant of the known viral titer from the previously infected monolayer culture. All experiments involved viral inoculation at multiplicities of infection (MOI) of 5 pfu/cell.

Coexpression of Recombinant Human Transferrin and $\beta 1,4$ -Galactosyltransferase

Two sets of *Sf*-9 insect cell cultures were infected with AcMNPV-hTf baculovirus. One of the cultures was coinfecting with recombinant baculovirus carrying the gene for $\beta 1,4$ -GalT (*in vivo*). For the post harvest *in vitro* analysis, 2.0 mU/ml commercial mammalian GalT and 0.32 mM commercial UDP-Gal were introduced to the harvested AcMNPV-hTf supernatant. There were three negative controls in this experiment which were *Sf*-9 cell culture infected with AcMNPV- $\beta 1,4$ -GalT, uninfected *Sf*-9 insect cell culture and harvested uninfected *Sf*-9 cell culture mixed with 2.0 mU/ml commercial mammalian GalT and 0.32 mM commercial UDP-Gal. All samples were harvested at time 24 hours post infection (PI). For the time course upon coinfection, the cell culture supernatants from recombinant baculovirus hTf and $\beta 1,4$ -GalT coexpression were collected at every 24 hours PI time intervals until 120 hours PI.

Enzyme Linked Immunosorbent Assay

HTf production rate was determined using Direct Sandwich Enzyme Linked Immunosorbent Assay (ELISA). It was performed in 96 wells microtiter plate (TPP, Switzerland) which were coated with primary antibody and incubated at 4°C overnight. The following day, the plate was washed with washing solution (Tris-buffered Saline (TBS), pH 8.0 containing 0.05% Tween 20) 3 times. The plate was blocked with blocking solution (TBS, pH 8.0 containing 1% BSA) for 30 min and washed with washing solution 3 times. The plate was subsequently incubated with serial dilutions of standards - Human Reference Serum (Bethyl Laboratories Inc, Texas) and samples in sample/conjugate diluent (TBS, pH 8.0 containing 1% bovine serum albumin (BSA) and 0.05% Tween 20) for 60 min and washed with washing solution 5 times. Next, the plate was incubated with HRP-conjugated secondary antibody in sample/conjugate diluent for 60 min at 37 °C and washed with washing solution 5 times. Color development by the enzyme substrate reaction was performed by adding to each well 100 μ l of equal volumes of Trimethyl Benzene (TMB) Peroxidase Substrate and Peroxidase Solution B (H₂O₂). After 5-30 min, the reaction was stopped with 100 μ l of 1 M Phosphoric Acid (H₃PO₄). The absorbance at 450 nm was determined.

Lectin Binding Assay for Recombinant $\beta 1,4$ -Galactosyltransferase Detection

Asialoagalactofetuin was used as the substrate acceptor. It was prepared by dissolving 25 mg of asialofetuin in 1 ml of 0.2 M sodium phosphate buffer (pH 4.5) containing 0.1 M citric acid. 0.4 units (2.2 mg) of bovine β -galactosidase was added to the mixture. The mixture was incubated for 72 h at 37 °C to remove galactose residues. The sample

was diluted at least 20 times with 0.1 M sodium phosphate-buffered saline (PBS), pH 7.2 containing 1mM CaCl₂, 0.15 M NaCl and 1mM MnCl₂ and concentrated using Amicon Model 8010 UF with MWCO of 10 000. This procedure was repeated three times to remove any remaining sugars which had been released from protein by the enzyme treatments. The protein produced was asialoagalactofetuin.

Each well of the 96 wells plate was coated with 100 µl of the asialoagalactofetuin (1µg/ml in 0.05 M Sodium Carbonate, pH 9.6 containing 2% glutaldehyde) at room temperature for 60 min, washed 3 times with washing solution (PBS containing 0.05% Tween 20, PBST) and then blocked with 1% BSA in PBST at room temperature for 60 min. The plate was washed and the enzyme reaction was started by adding to each well 100 µl of enzyme-donor substrate mixture (10 mM MnCl₂, 0.1% BSA, 0.32 mM UDP-Gal and sample β1,4-GalT) in 30 mM Mops (pH 7.4). The plate was incubated at 37°C for 60 min, the reaction was stopped by discarding the reaction mixture. The plates were washed and incubated with peroxidase-labeled RCA 1 (0.16 mg/ml in PBST containing 1% BSA) at 37 °C for 90 min. Color development by the enzyme substrate reaction was performed by adding to each well 100 µl of equal volumes of TMB Peroxidase Substrate and Peroxidase Solution B (H₂O₂). After 5-30 min, the reaction was stopped with 100µl of 1 M Phosphoric Acid. The absorbance at 450 nm was determined.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Analysis

Sf-9 cells or infected *Sf*-9 cells (about 1 x 10⁶ cells) was collected by centrifugation (1000 x g, 15min at 4°C). Pellets were washed with PBS buffer, pH 7.4. Cells were lysed in ice-cold 75% ethanol (300µl) by freeze-thawing and homogenizing. Soluble fractions were obtained by centrifugation (16 000 rpm x 10min at 4 °C). Supernatant was filtered through 10 000 MWCO membranes.

RP-HPLC was operated at 1ml/min and the column was kept at 30°C. UDP-Gal was detected at the absorbance of 260nm. The ion pair RP-HPLC was carried out using a LC-10Dvp HPLC system and a NovaPac C18 column (3.9 x 150mm, 4µm) with a NovaPac C18 guard cartridge. The following two solvents were used as eluents: 5mM tetrabutylammonium sulfate (TBAS) – 50mM ammonium phosphate, pH 5.0 (E1) and 5mM TBAS-methanol (E2). A portion of the cell extract samples prepared as above was injected into a NovaPac C18 column equilibrated with a mixture (98:2, v/v) of E1 and E2.

Lectin Binding Assay for coexpression of human transferrin and β1,4-Galactosyltransferase

Each well of the ELISA plate was coated with 100 µl of the glycoprotein (1µg/ml in 0.05M Sodium Carbonate, pH 9.6 containing 2% glutaldehyde) at room temperature for 60 min, washed 3 times with washing solution (PBST) and

then blocked with 1% BSA in PBST at room temperature for 60min. The plate was washed and the enzyme reaction was started by adding to each well 100 µl of enzyme-donor substrate mixture (10mM MnCl₂, 0.1% BSA, 0.32mM UDP-Gal and 0.2 units of β1,4-GalT) in 30mM Mops (pH 7.4) for the post harvest *in vitro* galactosylation samples. The plate was incubated at 37 °C for 60 min, and the reaction was stopped by discarding the reaction mixture. The plates were washed and incubated with peroxidase-labeled RCA 1 (0.16 mg/ml in PBST containing 1% BSA) at 37 °C for 90min. Color development by the enzyme substrate reaction was performed by adding to each well 100 µl of equal volumes of TMB Peroxidase Substrate and Peroxidase Solution B (H₂O₂). After 5-30 min, the reaction was stopped with 100 µl of 1 M Phosphoric Acid. The absorbance at 450 nm was determined.

Results and Discussion

To evaluate the extent of galactosylation that occurred in insect cells by the coinfection strategy, an assay was established. In this binding assay, glycoprotein was adsorbed onto the ELISA plate surface. A lectin known as *Ricinus communis* agglutinin-I (RCA-1) labeled with peroxidase, is a carbohydrate-binding proteins with the high affinity to recognize the linkage Galβ1→4GlcNAc on the *N*-glycan of glycoprotein. Unbound lectin was removed by washing and the bound lectin was determined by adding equal volumes of TMB and Peroxidase Solution B, which can be measured at 450 nm wavelength.

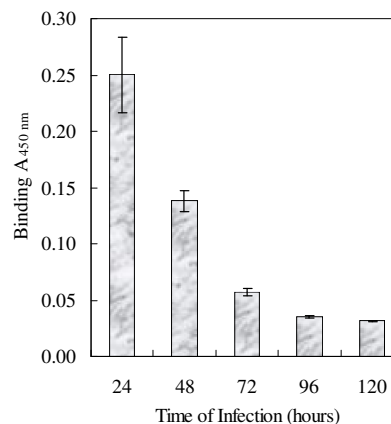


Figure 1: Galβ1→4GlcNAc linkage binding values at 450nm for the time course upon coinfection between recombinant baculovirus hTf and β1,4-GalT. Supernatants from each of the coexpressed cell culture were collected at hour 24, 48, 72, 96 and 120 respectively.

Time course assay for coinfection between recombinant hTf and β1,4-GalT was investigated. The medium from each of the coexpressed cell cultures was collected every

24 hour PI until 120 hours PI. As shown in Figure 1, the coinfection strategy was a success. However, *in vivo* galactosylation efficiency decreased gradually upon time of infection. This observation revealed that sufficient amount of UDP-Gal is also essential to ensure successful galactosylation apart from the present of sufficient amount of glycosyltransferase. This can be proved by monitoring the native UDP-Gal level at normal and upon time of infection using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) (to be reported separately). The data showed that UDP-Gal concentration dropped once infected with recombinant baculovirus. A simple explanation for this is that the existing UDP-Gal was utilized to construct the *N*-glycans terminating in galactose.

Clearly, the *in vivo* galactosylation as performed did not show encouraging result due to the limitation of the sugar nucleotide. Thus, another strategy, post harvest *in vitro* galactosylation was developed to improve the glycoprotein quality. In this study, commercially available GalT and UDP-Gal were introduced to the hTf after it was secreted from *Sf*-9 cell culture. Harvested hTf concentration was determined using ELISA. To determine the optimal conditions for the reaction, different concentrations of commercial GalT were added to the harvested hTf in the presence of excess amount of sugar nucleotide, 0.32 mM UDP-Gal. Lectin RCA-1 was later added to interact with the β 1,4-linkage on the *N*-glycan of hTf, followed by TMB color reaction measurement. As shown in Figure 2, the reaction increased with the increase of commercial GalT concentration up until approximately 2.0 mU/ml of commercial GalT was introduced into the system. Thus, for the subsequent experiments, 2.0 mU/ml of mammalian GalT was selected to ensure sufficient amount of enzyme in the reaction. Also, the substrate donor will always be in excess for the *in vivo* galactosylation process.

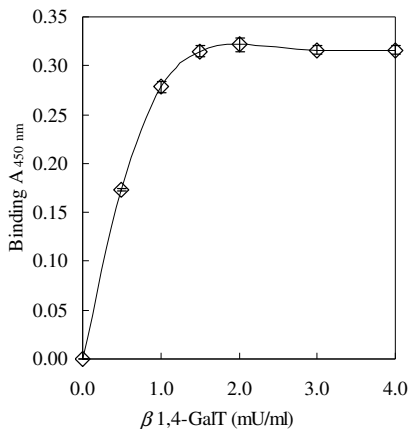


Figure 2: Effect of the mammalian GalT on the rate of *in vitro* galactosylation process. Commercial mammalian GalT and UDP-Gal were introduced to the hTf after it was secreted from *Sf*-9 cell culture. Different concentrations of enzyme were added to the hTf in the presence of excess amount of sugar nucleotide, 0.32 mM.

Lectin RCA-1 was later added to recognize the β 1,4-linkage on the *N*-glycan of hTf, followed by TMB color reaction measurement.

Post harvest technique for *in vitro* glycosylation was developed. Different levels of galactosylation were investigated which include positive controls, negative controls, *in vivo* and *in vitro* galactosylation as shown in Figure 3. The positive control in this experiment was the commercial apo hTf with two *N*-glycosylation sites which include galactose residues at each branch. As for the negative controls, there were the *Sf*-9 cell culture infected with AcMNPV- β 1,4-GalT, uninfected *Sf*-9 insect cell culture as well as 2.0 mU/ml commercial mammalian GalT and 0.32 mM commercial UDP-Gal added into harvested uninfected *Sf*-9 cell culture. For the samples, two *Sf*-9 insect cell cultures were infected with AcMNPV-hTf. One of the cultures was coinfecting with AcMNPV- β 1,4-GalT (*in vivo*). For the post harvest *in vitro* analysis, 2.0 mU/ml commercial mammalian GalT and 0.32 mM commercial UDP-Gal were added to the harvested AcMNPV-hTf supernatant. In this study, all the infected cell cultures and uninfected cell cultures were harvested at time 24 hours PI. The concentration of glycoprotein absorbed onto the ELISA plate was constant, which was 1 μ g/ml. A lectin, peroxidase labeled-RCA 1, was used to interact with the Gal β 1 \rightarrow 4GlcNAc-linkage on the *N*-glycan of glycoprotein, which can be measured by TMB color reaction.

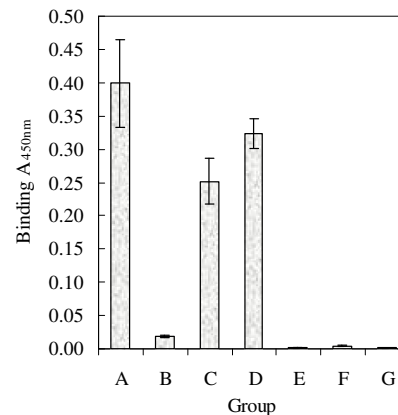


Figure 3: Gal β 1 \rightarrow 4GlcNAc linkage binding values at 450nm for the different level of galactosylation process. A: Apo human transferrin (Standard); B: AcMNPV-rhTf supernatant harvested at 24 h PI; C: Coexpression of rhTf and β 1,4-GalT supernatant harvested at 24 h PI (*in vivo*); D: Introduction of commercial GalT and UDP-Gal to harvested AcMNPV-rhTf supernatant at 24 h PI (post harvest *in vitro*); E: Uninfected *Sf*-9 cell culture; F: Introduction of commercial GalT and UDP-Gal to uninfected *Sf*-9 cell culture; G: AcMNPV- β 1,4-GalT supernatant harvested at 24 h PI.

As illustrated in Figure 3, the various data for the conditions described above represent different level of galactosylation. Apo hTf was used as a guide for the comparison with other groups due to its *N*-glycan oligosaccharide chains containing Galactose residues. AcMNPV-hTf produced in insect cell culture did not achieve satisfactory galactosylatin, as expected because it was missing the enzymes necessary to construct the *N*-glycan chain. However, this phenomenon took a positive turn once the AcMNPV-hTf was coinfecting with the AcMNPV- β 1,4-GalT. The absorbance value for the *in vivo* coexpression was 12.5 times higher compared to the AcMNPV-hTf culture. This result confirmed that the success of galactosylation did not depend on the presence of substrate acceptor and substrate donor only, but also on the enzyme needed to elongate the chains. Hence, post harvest *in vitro* galactosylation potential was studied, where harvested AcMNPV-hTf supernatant at time 24 hours PI was introduced with GalT and UDP-Gal artificially. Surprisingly, the post harvest *in vitro* galactosylation was more successful compared to the coinfection method (Figure 3). This observation indicated that in addition to GalT, sufficient amount of sugar nucleotide was another key factor in guaranteeing the success of the galactosylation process. As for the negative controls, they were showed to be insignificantly galactosylated.

To ensure successful galactosylation, cooperation of the substrate acceptor, substrate donor and enzyme is needed. The relationships among the three main elements of the *in vivo* galactosylation process, which were hTf as the substrate acceptor, β 1,4-GalT as the enzyme and UDP-Gal as the substrate donor is illustrated in Figure 4. *Sf*-9 cell culture infected with AcMNPV-hTf and coinfecting with the AcMNPV- β 1,4-GalT was used to demonstrate the relationship. The supernatants and lysates were collected every 24 h. HTf and β 1,4-GalT expression was determined using ELISA and lectin binding assay. Meanwhile, UDP-Gal monitoring was performed using RP-HPLC analysis. UDP-Gal concentration decreased gradually once the *Sf*-9 cells were coinfecting with hTf and β 1,4-GalT. Figure 4 reveals that β 1,4-GalT and hTf accumulation rates increased proportional to the time of infection. Thus, it can be concluded that even though hTf and β 1,4-GalT accumulation increased upon the time of coinfection, the gradual decrease of sugar nucleotide (UDP-Gal) still affect the effectiveness of the galactosylation process.

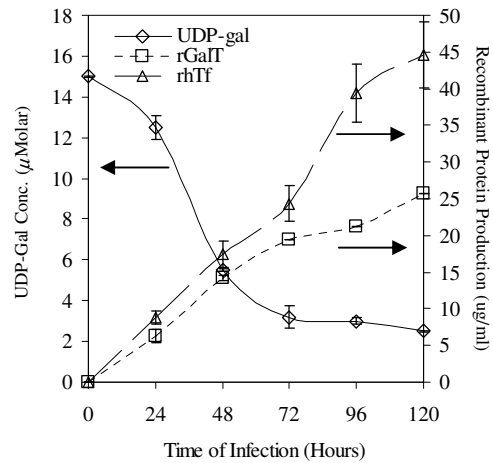


Figure 4: Relationship among the three main elements in *in vivo* galactosylation process. hTf as sugar acceptor, β 1,4-GalT as enzyme and UDP-Gal as substrate donor in the process.

Conclusion

Three main elements for successful protein galactosylation, are the presence of sufficient amount of hTf as the substrate acceptor, β 1,4-GalT as the enzyme and UDP-Gal as the substrate donor. Unfortunately, the limitation in the elongation of the *N*-glycan processing of hTf in insect cells occurs due to the lack of β 1,4-GalT needed to produce galactosylated hTf. In this report we proposed a strategy for galactosylated hTf by the coexpression of the hTf and β 1,4-GalT. However, the reduction of the native UDP-Gal level upon baculovirus infection decreased the galactosylation efficiency. Alternatively, post harvest *in vitro* technique was developed to overcome the limitation. Interestingly, post harvest *in vitro* galactosylation shows great potential as a technique that can be developed to improve glycoprotein quality.

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