Preliminary Study on the Optimization of Recombinant Human Transferrin Expression in Insect Cells Baculovirus System

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ABSTRACT

Insect cells Baculovirus system is a promising artificial system for producing many biopharmaceuticals products. In this study, the expression profiles of recombinant human transferrin (rhTf), a model protein expressed in Sf9 insect cells monolayer and baculovirus culture were investigated. Time course expression profile of rhTf at various multiplicity of infection (MOI), seeding density (SD), time of infection (TOI), and harvest time (HT) were conducted as well as growth control (GC) for non infected Sf9 cells. Growth kinetics of non infected and infected Sf9 cells were also performed to monitor the progress of infection. SDS-PAGE, Western Blot, and ELISA analyses were utilized in this study to confirm the progress of the rhTf production. The data shows that maximum production of rhTf was between 24 to 72 hours post infection, at the seeding density of 1.6 x 10^6 viable cells/ml, infection at day 2 post culture, and MOI of 5. The maximum rhTf yield was 11.2 g/ml. The results suggested that rhTf yield could be increased further provided that the nutrient and cells density are not limiting.

Key words*:* Insect Cells Baculovirus System; Human Transferrin; Time Course; SDS-PAGE; Western Blot; ELISA

1.0 INTRODUCTION

Wild type baculoviruses exibit both lytic and occluded life cycles that develop independently throughout the three phases of virus replication. The early phase occurs 0.5 to 6 hours after infection. Late phase which is the release of extracellular virus occurs 18 to 36 hours after infection. In the very late phase the polyhedrin and p10 genes are expressed. Between 24 and 96 hours after infection, the cells start to produce occluded virus (OV). A polyhedrin protein with a molecular weight of 29 kDa is the major structural component of the viral occlusions. In infected *Spodoptera frugiperda* cell cultures, polyhedrin accumulates to very high levels, routinely 1mg/ml per 1.0-2.0 x 10^6 infected cells accounting for 50-75% of total "stainable" protein of the cell detected on SDS-PAGE. (Summers and Smith, 1988).

Success with the baculovirus expression system is dependent on the ability to infect cells efficiently with AcMNPV, thus obtaining maximum virus replication and hence optimum production of the desired protein (King and Possee, 1992).Recombinant proteins have been produced as fusion or nonfusion proteins at levels ranging from 1-500 mg/L (Luckow and Summers, 1988). The polyhedrin protein expression depends on the use of log phase Sf9 cells that are at least 97% viable, a multiplicity of infection (MOI) of at least 5-10, high quality medium and fetal bovine serum. Some cells are infected later than the others and as a result, reach maximum expression at a later time. Therefore, it is of some importance that sufficient virus is used to ensure synchronous infection of all of the insect cells in a culture. Some proteins may not be stable in virus-infected cells. If these proteins are harvested too late, considerable amounts may be lost (King and Possee, 1992).

2.0 MATERIALS AND METHODS

2.1 *CELL LINES, RECOMBINANT BACULOVIRUS AND MEDIUM*

The Sf9 cells were obtained from American Tissue Culture Collection (ATCC). Sf-900 II SFM culture medium was obtained from GIBCO^{TM}. Autographa Californica Multiple Nuclear Polyhydrosis Virus encoding for Human Transferrin gene (rhTf-AcMNPV) was provided by Professor Michael Betenbaugh of Johns Hopkins University USA.

2.2 *ROUTINE SUBCULTURE OF SF9 CELLS MONOLAYER*

Confluent monolayer of Sf9 cells was detached from the flask surface by gentle shaking. 4 x 10^6 viable cells were inoculated into a 25 cm^2 T-flask and distributed evenly over the surface. The cells were left for 10-15 minutes to attach to the surface before the spent medium and floating cells were removed. 5 ml of SF-900 II medium was added into the flask and incubated at 27 $\pm$ 0.5 ^oC. When the monolayer reached >80% confluency, the cells were subcultured again. Cell number and viability were determined by staining with Trypan Blue and counted using a haemocytometer.

2.3 *AMPLIFICATION AND TITRATION OF BACULOVIRUS*

Virus stock was diluted by serial dilution in the range of 10^{-1} – 10^{-8}. 10 µl of each virus dilution was mixed with 90 µl of 0.5 x10^6 cells/ml in each well (1 row per dilution) of the 96-well plate. The plate was incubated in a humidified environment for 4-7 days. The aim was to dilute the virus such that, when exposed to the Sf9 cells culture, 50% or less of the culture was infected. This represented the end point dilution or Tissue Culture Infectious Dose ($TCID_{50}$).

For amplification of baculovirus stock, a 75 cm^2 flask was seeded with 5 x 10^6 Sf9 cells and infected with seed virus stock at low MOI (less than 1 pfu/cell if seed virus stock had been titrated or use 0.25 – 0.5 ml if it had not). It was incubated at 27^oC for 4-6 days until the cells were well infected. Viruses were harvested by centrifuging the infected medium at 250 x g (1800rpm) 5 minutes and stored at 4^oC.

2.4 *RECOMBINANT HUMAN TRANSFERRIN EXPRESSION AND ANALYSIS*

All experimental works were conducted at Sf9 cells viability of at least 90%. Each 25 cm^2 T-flask was seeded with 4 x 10^6 Sf9 cells in 5ml fresh SF-900 II medium. Virus innoculums at different MOI ranging from 1-100 MOI were tested. 100 µl of each flask sample was collected every 2 days and undergone an ELISA analysis for rhTf expression Expression at different seeding densities ranging between 0.8-5.6 x10^6 cells/ml was studied using 5 MOI virus innoculum. Expression at different time of infection was done by introducing virus innoculum at day 0 to day 6 post culture.

Protein quality was assessed using SDS PAGE analysis. The gels consisted of 10% separating gel and 5% stacking gel. Loading volume was 10 µl. The loaded gel was electrophorase at 200V for 45 minutes. The gels were removed, washed, and immersed in 25-30 ml of Commassie Blue Solution. After 1 hour, the gels were washed and immersed in 50 ml of destaining solution for 24 hours. Photographs of the gels were taken for record and further analysis.

Protein quantity was analyzed using ELISA analysis. The materials were obtained from Bethyl Lab. Each well was coated with 100 µl of goat-anti-hTf antibody for 60 minutes; washed 3 times; blocked with 200 µl of blocking solution for 30 minutes; washed 3 times; added with 100 µl of samples and standards for 60 minutes; washed 5 times; detected with 100 µl goat-anti-hTf-HRP conjugate; washed 5 times and reacted with 100 µl of enzyme substrate for 5-30 minutes. Reaction was stopped using 200 µl 2 M H2SO4. Finally, absorbance at 450 nm was read using microtiter plate reader.

3.0 RESULTS AND DISCUSSION

3.1 *GROWTH KINETICS OF NON INFECTED CELLS*

At lower initial densities, the cells were able to multiply efficiently thus achieved maximum density. As the seeding density was increased, the exponential phase became shorter thus unabling the cells to reach optimum density. For lower seeding densities, the cells viability could also be maintained for a longer period. For seeding at 5.6 x 10^6 viable

cells/ml, the Sf9 cells could not propagate anymore and viability dropped as soon as the cells were inoculated (Figure 1). At high densities, the cells monolayer are over confluent and resulted in high competition among the cells to utilize the medium as well as mass transfer problems. Therefore, the optimum seeding density for Sf9 monolayer was in the region of 0.8 x 10^6 cells/ml (Figure 2).

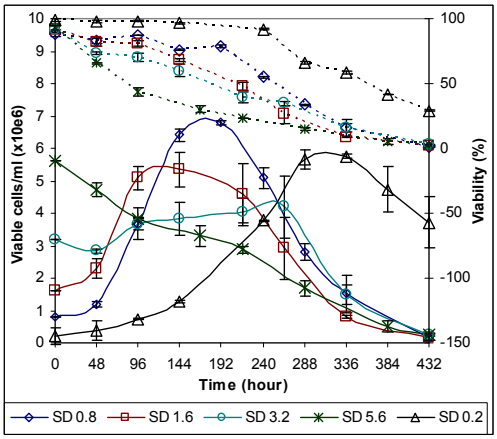

Figure 1: Growth curves of Sf9 at different seeding densities

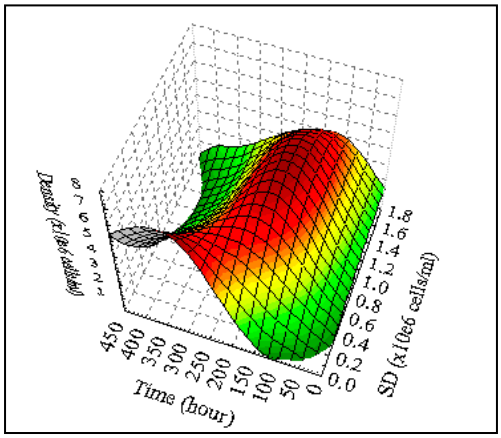

Figure 2: Surface plot of figure 1

3.2 *GROWTH KINETICS OF INFECTED CELLS*

During the early phase of Sf9 infection with recombinant AcMNPV, adsorptive endocytosis takes place, followed by DNA replication (Volkman and Goldsmith, 1985). Late phase occurs within 6 to 24 hours after infection where budded viruses are produced logarithmically (Knudson and Harrap, 1976). At the end of this phase, infected cultures stop growing and budded viruses cease its production. In this study, the infected Sf9 cells stopped growing from 24 - 48 hours post infection (Figure 3) which suggested that the budded viruses were still being produced at a later time. In the very late phase, cells ceased production of budded virus and begin the expression of recombinant gene product. The cells viability remained high for about 2 days and dropped to 50% at day 3.

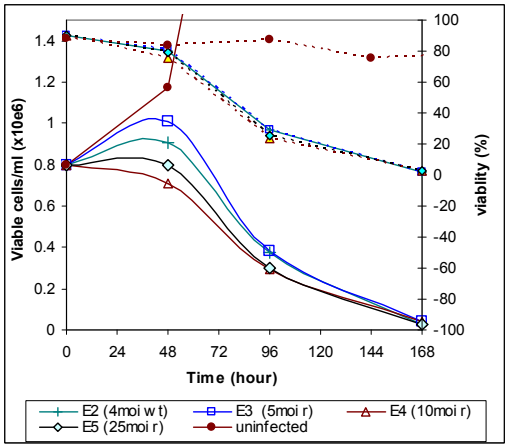

Figure 3**:** Growth curves of Sf9 infected with AcMNPV

3.4 *SDS PAGE ANALYSIS*

Production of heterologous proteins ranging from 25-225 kDa was observed. The SDS PAGE analysis showed that Sf9 cells infected with AcMNPV resulted in the decline or shut off of host gene expression (Figure 4). In lane 4 of figure 5, almost all of the major proteins in the healthy Sf9 cells were not expressed. Many of the proteins in lane 4 consisted of polyhedrin and p10 viral protein. For recombinant baculovirus infection, the analysis clearly showed that the recombinant hTf was expressed as the major protein. However the rhTf molecular weight was slightly lower than its native counterpart (apo-hTf) (Figure 4). This might be due to incomplete glycosylation and lack of iron bonded to

the transferrin molecule. The absence of particular glycosylation-related enzymes could be the reasons of incomplete glycosylation.

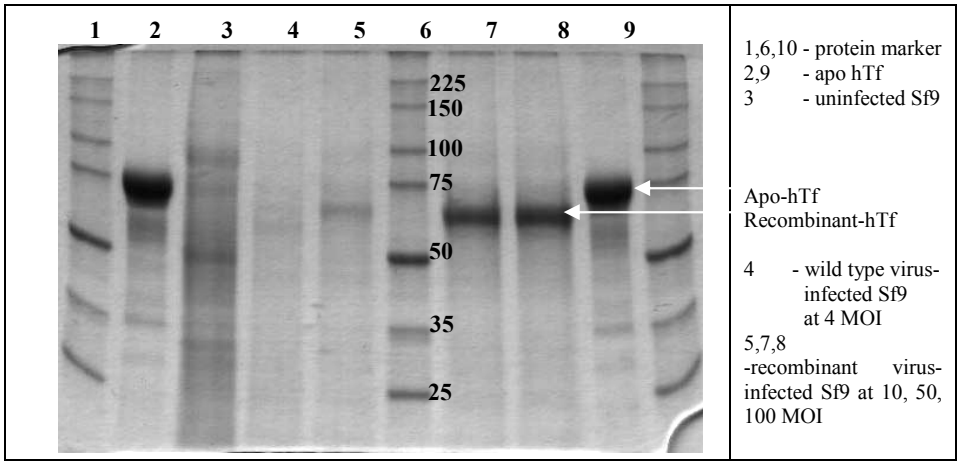

Figure 4: Commassie Blue Stained -SDS PAGE analysis of the recombinant hTf expression. Samples were collected at day 4 post infection. Loading volume is 20 µl.

3.5 *RECOMBINANT HUMAN TRANSFERRIN EXPRESSION PROFILES*

RhTf was produced extensively 48 to 96 hours post infection after which the production rate decelerated (Figure 5). The deceleration phase might be due to the sudden drop in Sf9 cells viability after day 2 post infection. After the deceleration phase, rhTf concentration was still increasing in a relatively small quantity. It was also shown that infection at low MOI produced higher rhTf yield, which in this case was 5 MOI. At lower MOIs, the Sf9 cells were allowed to propagate further thus increasing the concentration of rhTf. The maximum rhTf yield when seeding at a lower density (0.8 x 10^6 cells/ml) was approximately 4.0 µg/ml (Figure 6). This was relatively equivalent to the yield when seeding at 3.2 x 10^6cells/ml and 5.6 x 10^6cells/ml. The optimum yield was found at the seeding density of 1.6 x 10^6cells/ml (Figure 7) with approximately 10.0 µg/ml rhTf. At this density, the Sf9 cells monolayer was 100% confluent. This suggested that the infection with recombinant AcMNPV was optimum as well as the mass transfer of medium through the cells membrane. At seeding densities higher than 1.6 x 10^6cells/ml,

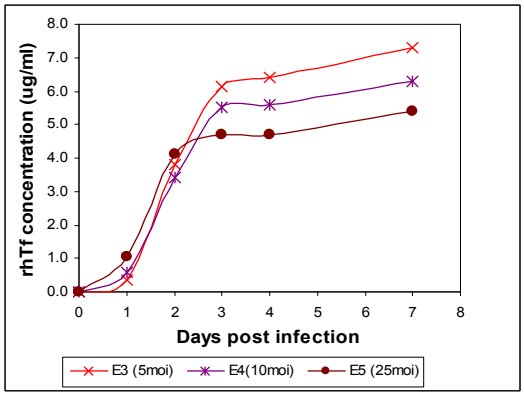

Figure 5: rhTf expression profiles at different MOI

the cells monolayers were already over confluent when virus innoculum was introduced into the culture. Although the cells were at higher concentrations, only a portion was able to express rhTf. Thus, it can be inferred that the relationship between rhTf yields and seeding densities higher than 1.6 x 10^6cells/ml did not depend on MOI and nutrient consumption.

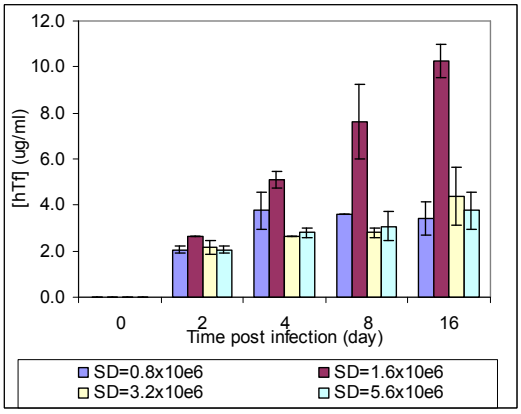

Figure 6: rhTf expression profiles at different seeding density

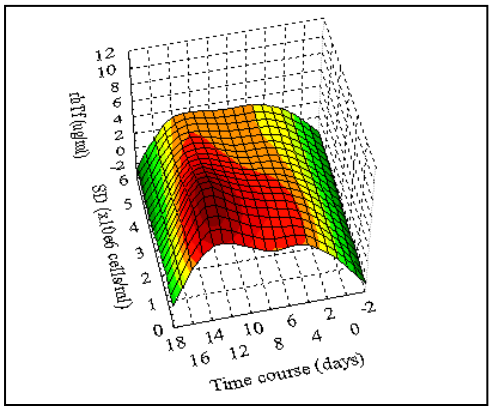

Figure 7: Surface plot of figure 6

The initiation of infection at day 1 and 2 post culture gave significant rhTf yield. The initial cells density was 0.8 x 10^6 viable cells/ml. At day 2 post culture, the cells have reached approximately 1.6 x 10^6 viable cells/ml (Figure 1). This was two times the initial density. When recombinant AcMNPV was introduced into the culture during this time, rhTf yield was maximum compared to time of infection at day 0, 1, 4, and 6 (Figure 9). For time of infection at day 2(cells density at time of infection=1.6 x 10^6 cells/ml), rhTf yield was also found to be higher than time of infection at day 0 (with same cells density at time of infection=1.6 x 10^6 cells/ml). This suggested that for the same cells density at time of infection, cells which were infected at a later time post culture would produced higher rhTf yield (Table 1).

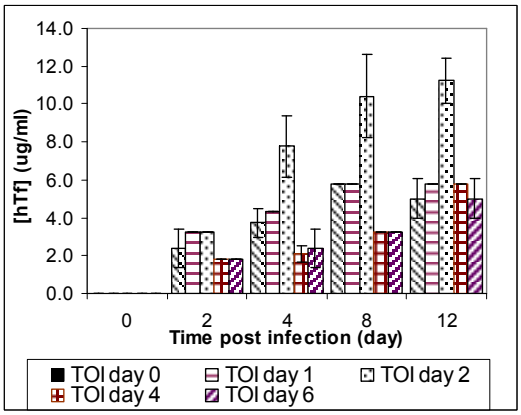

Figure 8: rhTf expression profiles at different time of infection

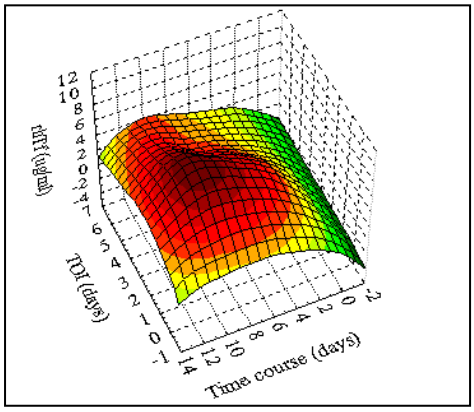

Figure 9: Surface plot of figure 8

Spent medium may contain secreted growth promoting factors with a positive effect on protein production (Jesionowski and Ataai 1997). When the Sf9 cells were first cultured in the T-flask, synthesis of some growth or expression promoting factors might still be at a low level. When infection was initiated at this time, the rhTf yield was not really good. When the cells were infected at a later time when there were enough growth promoting factors, the rhTf yield was higher than before.

When the cells were infected at a later time, they were actually allowed to propagate further thus achieving higher density. Higher density allowed these cells to express more rhTf and therefore increased the rhTf yield even further. If the cells were infected too late, the cells would become over confluent. This would reduce the mass transfer efficiency. Furthermore, some of the nutrients might have been fully consumed. Eventually rhTf expression could not reach higher concentration (Figure 8) and the yield was minimal too (Table 1).

Table 1: rhTf yield at various SD, MOI, and TOI obtained on day 4 post infection.

MOI	Yield at day 4 post infection ($ng/10^3$cell)	SD (10^6viable cells/ml)	Yield at day 4 post infection ($ng/10^3$cell))	TOI (day post culture)	Yield at day 4 post infection ($ng/10^3$cell)
1.0	1.76	0.8	4.71	0	4.66
2.5	2.82	1.6	3.18	1	3.82
5.0	1.70	3.2	0.83	2	4.87
7.5	1.58	5.6	0.50	4	0.65
10.0	1.76			6	0.38
12.5	1.70				

After day 4, there were still some viable cells in the culture (Figure 3). Supplementation of methionine and tyrosine was found to retard cell death in Sf9 culture (Mendonca et al., 1999). The SF-900 II medium used in this study might have these supplements or other death retarding nutrients that supported the cells to remain viable for a longer period thus enabling the cells to produce more proteins.

Since that the ELISA analysis was only a quantitative analysis, it accounted for whatever form of rhTf in the culture. Therefore the rhTf produced very late in the culture might be the biologically inactive ones or could be the degraded ones. For this study, reactivity analysis of rhTf was beyond the scope.

4.0 CONCLUSIONS

This study helped to understand the rhTf expression in insect cells monolayer and baculovirus culture. MOI, TOI, SD, and HT were found to significantly affect the production of rhTf. However the rhTf yield was still low compared to the average yield of other recombinant proteins expressed in this system. Further studies on the expression and optimization of rhTf expression would be carried out based on these findings. This work would help to monitor any changes that might occur when scaling up into suspension culture and decide on what and how to optimize the rhTf expression.

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