Observation and modeling of induction effect on human transferrin production from stably transfected Drosophila S2 cell culture

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Received 1 November 2004; received in revised form 23 September 2005; accepted 21 October 2005

Abstract

Human transferrin (hTf) is a serum glycoprotein involved in iron transport. We performed, for the first time, mathematical modeling of stably transfected insect Drosophila melanogaster S2 cell culture, a nonlytic plasmid-based system that secretes recombinant hTf under control of the copper sulfate-inducible Drosophila metallothionein promoter. Cell growth patterns at various inducer concentrations revealed that the specific growth rate of S2 cells was substantially reduced as the specific rate of recombinant hTf production increased, and recovered to some extent when recombinant hTf production was nearly stopped. Additionally, the time profiles of specific production rates exhibited a maximum in the early culture period. Longer times and lower values of the maximum specific production rate were observed at lower inducer concentrations, and shorter times and higher values at higher inducer levels. Although the proposed S2 cell culture model was slightly limited with regard to prediction of cell growth profile at the late stage, we ensured that it gave reasonable predictions of the dynamics of glucose consumption and recombinant hTf production and confirmed its validity through simulating other culture experiments under different conditions. The S2 model proposed in this study can contribute to the elucidation of cell culture dynamics, and optimization of each culture variable to enhance heterologous protein production.

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Keywords: Insect cell culture; Drosophila S2 cells; Modeling; Simulation; Optimization; Human transferrin

1. Introduction

Among the insect cells introduced as high eukaryotic expression systems to overcome some deficiencies in prokaryotic and mammalian cell systems, Schneider line 2 (S2) cells derived from Drosophila melanogaster have been developed as a plasmid-based, nonlytic integration system [1,2]. High copy numbers of recombinant plasmid vector are inserted into the S2 cell genome, with the advantage that foreign proteins are expressed stably without destroying cells [3,4]. The nonlytic process is more efficient for secreted proteins. Therefore, the S2 cell system is suitable for the efficient expression and secretion of functional heterologous eukaryotic gene products [5–8].

Human transferrin (hTf), a β1 globulin synthesized in the liver, is a serum glycoprotein whose main role is transporting Fe³⁺ in the blood and delivering it to all cells in the body [9,10]. hTf is composed of two globular lobes, each containing an affinity binding site for iron. The transferrin binds Fe³⁺, enters cells by interacting with the receptor on the plasma membrane, releases Fe³⁺, then exits and repeats this activity [11]. hTf contains 38 cysteine residues that are all engaged in disulfide linkages (11 in the C-lobe and 8 in the N-lobe) [12]. This extensive system of disulfide bonds makes it difficult to produce functional recombinant hTf in the Escherichia coli system [11]. The insect cell system is thus an ideal alternative for the expression of functional hTf. The recombinant protein has been expressed in baculovirus-based Spodoptera frugiperda or Trichoplusia ni cells [13–15], and plasmid-based Drosophila S2 cells [16].

Recombinant protein expression is generally an important factor that impedes cell growth. High foreign protein formation competes with the synthesis of cellular components, and draws major resources to product generation, defined as ‘metabolic burden’, consequently leading to a reduction in cell growth rate and respiratory capacity [17–19]. Over-expression of the recombinant protein causes ‘dilution’ of the native cellular protein and it is also proposed as a major process that leads to decrease in glycolytic flux and growth rate [18]. Recent studies show that,
in some bacterial systems, induction of the recombinant protein inhibits the maximum specific uptake capacity for glucose [19]. However, recombinant protein production is also affected by cell growth, although it competes with the process of cell proliferation [20]. The Drosophila metallothionein (MT) promoter employed in this work is strong and highly inducible, and thus useful for the expression of foreign genes in S2 cells [21,22]. However, we also observed hindrance of cell growth upon strong induction of recombinant protein in S2 cell culture [16]. Following the addition of copper sulfate inducer, the rate of cell growth was significantly reduced. Consequently, the total cell number remained at a similar level to that observed at the induction time.

By setting up a culture model, we can clarify the relationships between different culture variables, and generalize the system in each related variable. In addition, using the model, we can predict production efficiency of the system as culture conditions are altered, facilitating optimization of the cell culture process. Culture models have been proposed that describe the production of foreign proteins in microorganisms [20,23,24], insect cells [25–27], and stably transfected mammalian cells [28–30]. To date, however, no culture models have been reported for insect Drosophila S2 cells.

In the present work, we have developed a S2 cell culture model to describe recombinant hTf production, based on the observation of cell growth and foreign protein expression patterns under various culture induction conditions.

2. Materials and methods

2.1. Strains and plasmids

D. melanogaster S2 cells (in vitro) and recombinant plasmid pMT/BpPVS-His/hTf [16] that contains copper sulfate-inducible Drosophila metallothionein (MT) promoter and signal sequence of immunoglobulin binding chaperone protein (BiP) were used to facilitate the secretion used for expressing the protein.

2.2. Cell culture

Stably hTf gene-transfected S2 cells [16] grown to 2 x 10⁶ cells ml⁻¹ with over 90% viability were transferred into a 500 ml spinner flask (Wheaton, USA) containing 150 ml of serum-free M3 medium (Shirels and Sang M3 insect medium, Sigma, USA). Since transformin is a serum protein, serum-free medium was used to avoid contamination. In addition, using serum-free medium can be advantageous in subsequent protein purification. Cells were cultured at 27 °C with constant stirring (80 rpm). Copper sulfate was added to induce production and secretion of recombinant hTf.

2.3. Analytical methods

Cell number was counted using a hemacytometer (Fisher Scientific, USA) and cell viability was determined by trypan blue (Sigma) exclusion using ≥0.4% (v/v) solution. Total protein amount was measured at 595 nm by a UV–vis spectrophotometer (Shimadzu Corp., Japan) using BraddFord method. Biovine serum albumin (BSA, Bio-Rad, USA) was used as a standard protein. The quantity of recombinant hTf was determined using purified human apo-hTf (Sigma) as a calibration standard, and goat anti-human transferrin (Sigma) for Western blot. Glucose concentrations of the culture medium were determined by an enzymatic method using a glucose assay kit (YD Diagnostics, Korea).

2.4. Western blot analysis

Samples were mixed with sample buffer (10% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 0.1 M Tris–HCl (pH 6.8), 0.05% bromophenol blue, and 50% glycerol), boiled for 5 min, and resolved by 12% or 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to Hybond™-PVDF membranes (Amersham Pharmacia Biotech). After blocking for 1 h in Tris buffered-saline (TBS; 20 mM Tris–HCl (pH 7.5), and 500 mM NaCl) containing 5% non-fat dry milk, the membrane was incubated in antibody solution (1% non-fat dry milk in TBS (TBS with 0.05% Tween-20) washing solution containing goat anti-hTf polyclonal antibody (1:2000, v/v) (Sigma) and probed with anti-goat monoclonal antibody conjugated with alkaline phosphatase (1:3000, v/v; Sigma). After successive washing with TBS and TBS, FAST Red TR/Naphthol AS-MX (4-chloro-2-methylbenzene diazonium/3-hydroxy-2-naphthol acid 2,4-dimethylindole phosphate; Sigma) was added for detection and the reaction was quenched with distilled water. The detected membrane was scanned and the digitized image was analyzed by Gel-pro Analyzer software (Media Cybernetics, USA).

2.5. Modeling and simulation

To set up a culture model equations for growth behavior, glucose consumption, and expression of recombinant hTf in S2 cells, each time-point was analyzed, and parameters were identified using the non-linear least square regression method (SigmaPlot version 7.0, Systat Software, Inc., USA). The Polymath program (Version 5.1, CACHE Corp., USA) was employed for simulation of the proposed S2 cell culture model.

3. Results and discussion

3.1. Observation and modeling of S2 cell culture

To determine the effects of recombinant protein production on the growth of S2 cells, four sets of cells, each cultured with a different initial copper sulfate concentration (0, 200, 500, and 1000 μM), were grown in 500 ml spinner flasks at a working volume of 150 ml. Parameters such as cell growth, glucose consumption, and recombinant hTf formation in each culture were investigated (Fig. 1). In the case of non-induction, cell growth increased steadily for 3 days from commencement of the culture, while growth in induction cases was significantly inhibited (Fig. 1A). Our results confirm a close correlation between the presence of inducers and cell growth. Total cell numbers were gradually reduced after 4 days in all cases (Fig. 1A), as glucose uptake was reduced (Fig. 1B).

The total cell number of S2 was defined as:

\[ X = X^i + X^d \]  

where \( X^i \) represents the total number of cells (cells ml⁻¹), \( X^v \) the number of viable cells (cells ml⁻¹), and \( X^d \) is the number of dead cells (cells ml⁻¹).

The growth rate (\( R_g \)) and death rate (\( R_d \)) of S2 cells were defined as:

\[ R_g = \frac{dX^i}{dt} = \mu - K_d X^i \]  

(2)

\[ R_d = \frac{dX^d}{dt} = K_d X^i \]  

(3)

where \( t \) is the culture time (h), \( \mu \) the specific growth rate (h⁻¹), and \( K_d \) is the specific death rate (h⁻¹).
Fig. 1. Time-course of (A) total cell growth, (B) glucose consumption, and (C) recombinant hTf production at different inducer amounts. Symbols: (●), no induction; (▼), 200 μM inducer; (■), 500 μM inducer; (♦), 1000 μM inducer. S2 cell culture was performed in 150 ml serum-free M3 medium using a 500-ml spinner flask. Each value represents the mean of two independent experiments.

S2 cell culture was performed in 150 ml serum-free M3 medium using a 500-ml spinner flask. Initial total cell number for each culture was $2 \times 10^6$ cells ml$^{-1}$ with over 90% cell viability. Copper sulfate inducer was added to the initial medium. Copper sulfate was added to the initial medium from plots of specific death rate against copper sulfate concentration (data not shown).

Glucose, a primary nutrient in most cells, is involved in the production of ATP, NADPH, and precursors for cellular synthesis of macromolecules. Therefore, glucose metabolism is an essential process required by most cells for survival and proliferation. Alterations in the glucose metabolic conditions due from plots of specific death rate against copper sulfate concentration (data not shown).

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The specific death rate of S2 cells was defined as:

$$K_d = A e^{bt}$$  \hspace{1cm} (4)

The specific death rate, $K_d$, increased with culture time (Fig. 2A). Inhibitor or toxic components in growth medium might also affect the $K_d$ value. In this study, the coefficients $A$ and $b$ that are functions of the inducer concentration, $I$ (μM), were determined (Table 1) via non-linear least square regression.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Value</th>
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<tbody>
<tr>
<td>$a$</td>
<td>$\left(1.8 + \frac{3.15I}{\left(110 + I^2\right)}\right) \times 10^{-3}$</td>
</tr>
<tr>
<td>$b$</td>
<td>$\left(1.3 + \frac{1.69I}{\left(110 + I^2\right)}\right) \times 10^{-2}$</td>
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to environmental changes can exert a negative influence on cell growth [31]. We assume that glucose is a unique growth-limiting carbon source for S2 cell cultures, and therefore, the specific growth rate in the case of non-induction may be described by the Monod Equation:

\[
\mu = \frac{\mu_{\text{max}} S}{K_s + S}
\]  

(5)

whereby \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), \( K_s \) the saturation constant (g l\(^{-1}\)), and \( S \) is the glucose concentration (g l\(^{-1}\)) in the medium. By plotting \( \ln X \) against culture time (data not shown), \( \mu \) was determined, while \( \mu_{\text{max}} \) and \( K_s \) were calculated (Table 2) from the plots of \( 1/\mu \) versus \( 1/S \) (data not shown). However, once production of recombinant hTf commenced, the growth of S2 cells was inhibited (Figs. 1A and 2B). Therefore, inclusion of a product inhibition term in the equation of specific growth rate should be considered.

The production rate (\( R_p \)) of recombinant hTf was defined as:

\[
R_p = \frac{dP}{dt} = fx^\alpha
\]  

(6)

where \( P \) is the recombinant hTf concentration (mg ml\(^{-1}\)), and \( f \) is specific production rate (mg cells\(^{-1}\) h\(^{-1}\)). Analysis of cell growth patterns at various inducer concentrations disclosed that the specific growth rate of S2 cells was substantially reduced with increasing specific production rate of recombinant hTf, and recovered to some degree (Fig. 2B) when recombinant hTf production was nearly stopped (Fig. 2C). Based on these observations, a modified model equation for growth behavior of S2 cells induced with foreign protein production may be obtained from the above Monod Equation (5):

\[
\mu = \frac{\mu_{\text{max}} S}{K_s + S} \exp\left(-\frac{f}{K_f}\right)
\]  

(7)

where \( K_f \) represents the product inhibition constant (mg cells\(^{-1}\) h\(^{-1}\)), determined (Table 2) by plotting the reduction rate of \( \mu \) versus \( f \) (Fig. 3A). This type of product inhibition as clearly shown in Fig. 3A may arise from a metabolic burden on growing cells caused by foreign protein formation [17–19].

Upon fusing hTf cDNA to the BiP signal sequence, recombinant hTf was successfully secreted into the culture medium. Interestingly, most recombinant hTf proteins were secreted into the medium by stably transfected S2 cells in a previous study [16]. Therefore, in this modeling analysis of stable S2 cell culture, we assumed that secretion efficiency of recombinant hTf is 100%, and all hTf protein produced is present in culture medium.

We performed time course analyses of recombinant hTf release into the medium to investigate the effects of inducer concentration on production. Addition of copper sulfate to the initial culture medium ensured that recombinant hTf proteins were produced from the initial period in all cultures. The order of production levels was 1000 \( /H_9262 \) M > 500 \( /H_9262 \) M > 200 \( /H_9262 \) M, as expected (Fig. 1C). Upon the addition of copper and cadmium ions into the medium, it is known that the inducible MT promoter begins to work, and the level of induction depends on the concentration of ions added [21]. However, after 3 days culture time, the recombinant hTf concentration was higher in the system with 500 \( /H_9262 \) M copper sulfate, compared to that with 1000 \( /H_9262 \) M. This may be due to 1000 \( /H_9262 \) M copper sulfate having the greatest inhibition effect on S2 cell growth (Figs. 1A and 2A). The data reveal an optimal inducer amount for maximum production yield. Each time profile of specific production rates for recombinant hTf displayed a maximum in the early culture period (Fig. 2C). Interestingly, the times (\( t_{\text{max}} \)) and values (\( f_{\text{max}} \)) for maximum specific production rate depended on the inducer concentration.

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**Table 2**

Parameters of the model equation for the specific growth rate of stably transfected S2 cells

<table>
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<th>Coefficient</th>
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<tr>
<td>( \mu_{\text{max}} )</td>
<td>0.0315 h(^{-1})</td>
</tr>
<tr>
<td>( K_s )</td>
<td>3.95 g l(^{-1})</td>
</tr>
<tr>
<td>( K_f )</td>
<td>( 1.9 \times 10^{-10} ) mg cells(^{-1}) h(^{-1})</td>
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**Fig. 3.** (A) Reciprocal relationship between specific S2 cell growth rate and specific recombinant hTf production rate. (B) Time at maximum specific production rate (\( t_{\text{max}} \)) and velocity coefficient \( \alpha (\%) \), and (C) maximum specific production rate of recombinant hTf as a function of inducer concentration.
centrations. The longest time and lowest value were observed with 200 μM copper sulfate, and the shortest time and highest value with 1000 μM copper sulfate. From a plot of $t_{\text{max}}$ and inducer concentration, a reciprocal curved line was obtained (data not shown). Therefore, $t_{\text{max}}$ was inversely proportional to the square root of copper sulfate concentration (Fig. 3B and Table 3). Generally, the maximum specific production rate, $f_{\text{max}}$, increased with copper sulfate concentration but displayed a saturation profile at high concentrations (Fig. 3C). Based on these observations, the model equation for the specific production rate was proposed as:

$$ f = f_{\text{max}} e^{-\alpha t} \left( \frac{I}{K_I + I} \right)^2 $$

where $f_{\text{max}}$ is the maximum specific production rate (mg cells $^{-1}$ h $^{-1}$) of recombinant hTf, $t_{\text{max}}$ represents the time at maximum specific production rate (h), $K_I$ the saturation constant (μM) of inducer, $I$ the inducer concentration (μM), and $\alpha$ is the velocity constant related to $f_{\text{max}}$. Generally, after time $t_{\text{max}}$, the specific production rate decreased significantly, and this tendency was more evident at higher inducer concentrations (Fig. 2C). The decrease in production rate was reflected in the velocity constant, $\alpha$, and decreased with copper sulfate concentration (Fig. 3B). $\alpha$ was determined (Table 3) as a function of inducer concentration by plotting the change in the specific production rate of hTf against inducer concentration (data not shown). From these results, we concluded that the production of recombinant hTf in stably transfected S2 cells was definitely affected by the inducer concentration and induction point.

The model equation for glucose consumption was proposed as:

$$ -\frac{dX}{dt} = \frac{1}{Y_{ps}} \frac{dP}{dt} + mX^2 + \frac{1}{Y_{ps}} \frac{dP}{dt} $$

where $Y_{ps}$ is the cell growth yield on glucose (cells mg $^{-1}$), $Y_{psr}$ represents the yield of recombinant hTf on glucose (mg h $^{-1}$), and $m$ is the maintenance coefficient (mg cells $^{-1}$ h $^{-1}$). Cell growth yield and the maintenance coefficient on glucose were estimated (Table 4) from the profiles of cell growth and glucose consumption in non-induced cultures (data not shown). Glucose consumption was considerable in recombinant hTf-expressing cultures, similar to non-induced cultures (Fig. 1B), while cell growth was much lower than expected (Fig. 1A). Based on these observations, the yield of recombinant hTf on glucose was determined (Table 4) from changes in the glucose concentrations after excluding cell growth and maintenance parts.

### Table 3

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<th>Coefficient</th>
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<tr>
<td>$t_{\text{max}}$</td>
<td>$2.5 + 2.1$ h</td>
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<tr>
<td>$\alpha$</td>
<td>$4.57 + 0.51$</td>
</tr>
<tr>
<td>$f_{\text{max}}$</td>
<td>$2.66 + 10^{-5}$ mg cells $^{-1}$ h $^{-1}$</td>
</tr>
<tr>
<td>$K_I$</td>
<td>$428$ μM</td>
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### Table 4

<table>
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<tr>
<td>$Y_{ps}$</td>
<td>$2.35 \times 10^6$ cells (mg glucose)$^{-1}$</td>
</tr>
<tr>
<td>$m$</td>
<td>$2.5 \times 10^{-3}$ mg cells$^{-1}$ h$^{-1}$</td>
</tr>
<tr>
<td>$Y_{psr}$</td>
<td>$9.8 \times 10^{-3}$ mg hTf (mg glucose)$^{-1}$</td>
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### 3.2. Simulation based on the proposed S2 cell culture model

We confirmed the validity of the proposed mathematical model by comparison with other culture experiments under varying conditions of induction time, inducer amount, and initial glucose concentration (Fig. 4). These proposed culture model equations were reliable for predicting the growth of S2 cells at

\[\text{Fig. 4. Validation of the proposed model under culture conditions of (A) non-induction} \]

\[\text{and 13 g l}^{-1} \text{initial glucose concentration, (B) induction with 1000 μM copper sulfate at 36 h and 9.5 g l}^{-1} \text{initial glucose concentration, and (C) induction with 500 μM copper sulfate at 48 h and 13 g l}^{-1} \text{initial glucose concentration. Symbols represent experimental data; (○), total cell number; (●), glucose concentration; (●) hTf concentration. The lines signify simulation results. Initial total cell number for each culture was 2} \times 10^6 \text{cells ml}^{-1}, \text{with over 90% cell viability. Each value represents the mean of two independent experiments.}\]
the early stage. However, the experimental data and simulation results showed poorer agreement at the late stage (open circle in Fig. 4). This discrepancy may be due to the fact that the model was developed based on assumption that glucose is the single growth rate-limiting factor, but there may be other important factors, such as glutamine [31] and by-products accumulation [32].

Although the proposed culture model was slightly limited with regard to prediction of cell growth profile at the late stage, it gave reasonable predictions of the dynamics of glucose consumption (closed circle in Fig. 4) and recombinant hTf production (triangle in Fig. 4). The final concentration of recombinant hTf generated increased to 45 μg ml⁻¹ when induction was initiated after 36 h culture for the system with an initial glucose concentration of 9.5 g l⁻¹ (Fig. 4B). Note, it was about 32 μg ml⁻¹ when the inducer was added to the initial culture (recall Fig. 4C).

Upon increase of the initial glucose concentration to 13 g l⁻¹, the recombinant hTf level increased to 62 μg ml⁻¹ owing to the promotion of cell growth induced by the higher initial glucose concentration (Fig. 4C).

Cell density was an important factor for the production of recombinant hTf, although protein generation also interfered with S2 cell growth. Specifically, higher cell density at the time of induction correlated with the greater production of foreign proteins. Therefore, induction is generally performed during the exponential growth phase when cells multiply rapidly, and increasing the cell density before induction is an important factor in protein generation. We performed a model simulation to obtain optimal induction times for maximum production yield and productivity of recombinant hTf (Fig. 5A). The initial total cell number and glucose concentration for each simulation were set to 2 × 10⁶ cells ml⁻¹ with over 90% cell viability and 9.5 g l⁻¹, respectively. The inducer concentration was set to 500 μM. Maximum production yield was obtained when induction commenced at 80 h after the culture start time, while maximum productivity was obtained at an induction time of 70 h (about 3 days). Our data confirm that both optimal induction points were at the later stage or middle of the exponential growth phase. Based on the optimal induction point determined for productivity, we performed simulations to determine the inducer concentrations that gave the maximum yield and productivity of recombinant hTf (Fig. 5B). Following the induction of expression at 72 h (determined as the optimal induction point under the same initial culture conditions), the final recombinant hTf concentration reached a maximum value at around 700 μM copper sulfate, while maximum productivity was achieved at over 1000 μM (Fig. 5B). This finding indicates that the production rate is promoted at higher inducer concentrations, possibly due to changes in the transportation rate as a result of different inducer concentrations in the culture medium and cellular cytoplasm [33,34]. On the other hand, the optimal inducer concentration tended to increase at higher S2 cell densities (data not shown). This shift may result from alterations in the net sensory concentration at different cell densities.

4. Conclusions

We have proposed model equations for stable recombinant hTf-expressing S2 cell culture, and confirmed their validity by comparing data from other culture experiments with predictions using the proposed model. Although this proposed S2 cell culture model was slightly limited in the prediction of cell growth at the late stage, it successfully described glucose consumption and productivity changes under various experimental conditions. Based on this model, we were able to suggest the optimum value of each culture variable. These optimal conditions may be practically applied to stably transfected Drosophila S2 cell culture to improve production of target foreign proteins.

Acknowledgments

The authors would like to acknowledge support of this work by the Korea Research Foundation (KRF-2004-041-D00181) and the Brain Korea 21 program issued from the Ministry of Education, Korea.

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