Study of kinetic parameters for the production of recombinant rabies virus glycoprotein

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Abstract Gene expression in insect cells is an advantageous system for recombinant protein production, mainly because of its capacity to produce complex proteins with correct post-translational modifications. Recently, we identified and purified a protein from *Lonomia obliqua* hemolymph able to increase the production of rabies virus glycoprotein, expressed in *Drosophila melanogaster* cells, by about 60%. In this work, the kinetic parameters for cell growth and recombinant rabies virus glycoprotein production were determined in cultures of transfected *Drosophila melanogaster* Schneider 2 (S2) cells expressing recombinant rabies virus glycoprotein (rRVGP), enriched and non-enriched with the hemolymph of *Lonomia obliqua* (Hb). The highest concentration of rRVGP was achieved at the beginning of the culture enriched with Hb, indicating that the cells produce greater amounts of rRVGP per cell

(specific rRVGP concentration) at the early exponential growth phase. After day 8, a decrease in the concentration of rRVGP (ng/mL) was observed, probably due to protein decomposition. The average specific rRVGP production rate (μ_{rRVGP}) was 30 ng rRVGP/ 10^7 cell.day, higher than that observed in the non-enriched culture.

Keywords Insect cells recombinant · Rabies glycoprotein · Hemolymph · *Drosophila melanogaster* cells · *Lonomia obliqua*

Introduction

Animal and plant gene expression systems allow the large scale production of heterologous proteins. Contextually, insect cell cultures are advantageous systems for the recombinant proteins production, mainly because of its capacity to produce complex proteins with correct post-translational modifications, such as folding and glycosylation. Nevertheless, maximizing the production of recombinant proteins is necessary to optimize cellular growth and protein expression. Several optimization strategies have been developed: metabolic or genetic manipulation, the replacement of some culture medium components and the addition of stimulating agents which promote cell growth and protection. These procedures aim to allow high cellular densities at low cost and with reduced operation complexity. Several studies have reported

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the presence of substances with pharmacological activity in the insect hemolymph (Shiotsuki et al. 2000; Yamamoto et al. 1999; Guerrero et al. 1999; Jiang et al. 1999; Rosenfeld and Vanderberg 1998; Hamdaoui et al. 1998; Maranga et al. 2003; Huberman et al. 1979; Lin et al. 1998; Kurata et al. 1994; Jones et al. 1993; Zhu et al. 2000; Lowenberger et al. 1999; Lamberty et al. 1999; Gross et al. 1998; Johns et al. 1998; Lanz-Mendoza et al. 1996; Peters et al. 1993; Souza et al. 2005; Raffoul et al. 2005; Kanaya and Kobayashi 2000; Mendonça et al. 2008). Kanaya and Kobayashi (2000) isolated and characterized a protein from silkworm hemolymph able to increase the activity of a recombinant protein (luciferase) by approximately 6,000 times. However, few studies have succeeded in isolating and characterizing the factors involved in these effects (Nussbaumer et al. 2000; Shishikura et al. 1996, 1997; Ochanda et al. 1992). These factors, once identified and isolated, can be of great importance in the optimization of cell growth and recombinant protein production.

Recently, we identified, isolated and characterized a protein from *Lonomia obliqua* hemolymph which enhances recombinant rabies virus glycoprotein (rRVGP) production by transfected S2 cells (Mendonça et al. 2008). This work aims to extend the previous work by determining the kinetic parameters for cell growth and recombinant protein (i.e., maximum specific rates— $\mu_{X_{\max}}$ and $\mu_{rRVGP_{\max}}$) and the relationship between the production of the recombinant protein and the phase of the cell cycle on the addition of the hemolymph.

Materials and methods

Cell line and culture conditions

Drosophila melanogaster Schneider (S2) cells expressing recombinant rabies virus glycoprotein (S2AcRVGP) were grown in 100 mL Schott flasks containing TC-100 medium (Gibco), supplemented with 10% fetal bovine serum (FBS). The flasks were inoculated with S2 cells to provide an initial concentration of 1×10^6 cells/mL. The cultures were then incubated at 29 °C and at 100 rpm in a incubator shaker. S2 cells expressing rRVGP were kindly provided by Yokomizo et al. 2007.

Hemolymph collection

The hemolymph of *Lonomia obliqua* was collected from sixth-instar larvae after setae had been cut off. The collected hemolymph was clarified by centrifugation at 1,000g for 10 min. Afterwards, the supernatant was heat-treated at 60 °C for 30 min. The heat-treated hemolymph was then filtered through a 0.2 µm membrane and stored at 4 °C.

Hemolymph fractionation by chromatography

After centrifugation and filtration, 6 mL of hemolymph was fractionated by gel filtration chromatography using an AKTA Purifier chromatography system equipped with a Hi-prep 26/60 Sephacryl 200 column (Amersham Pharmacia Biotech) at a flow rate of 1 mL/min. The elution was monitored at 280 nm and 120 fractions (4 mL each) were collected. The purified protein fractions obtained were then analyzed by SDS-PAGE and added to transfected S2 cell cultures for studies on cell growth and recombinant protein production.

Enhancing effect of hemolymph on recombinant protein production

In order to investigate the enhancing effect of hemolymph on recombinant protein production, whole hemolymph and hemolymph protein fractions were added to the culture medium to a final concentration of 1% (v/v) or 2% (v/v), respectively, at the time of inoculation. Experiments were carried out in quadruplicate.

Analytical procedures

rRVGP expression analysis by ELISA

The recombinant rabies virus glycoprotein (rRVGP) levels produced by S2AcRVGP cells were estimated by ELISA as described by Perrin et al. (1996). Cell culture samples were centrifuged at 900g for 5 min. Pellets were recovered and cells lysed with a lysing buffer. The lysate was then centrifuged at 10,000g for 10 min and the supernatant analyzed for rRVGP concentration determination.

Measurement of cell viability

Culture samples were taken daily and cell concentration was measured using a haemocytometer. Cell viability was determined by the trypan blue exclusion test under light microscopy.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Statistical analysis was performed using the Student's *t*-test and the level of significance was set at $p \leq 0.01$.

Results and discussion

Effect of the hemolymph and its fractions on cell growth and recombinant protein production

The effect of whole hemolymph (Hb) and its fractions on cell growth and recombinant protein production was assessed. The results obtained are presented and discussed below.

As shown in Fig. 1, the addition of whole hemolymph or its fractions at the beginning of the culture resulted in higher maximum cell concentrations, in comparison with that in the control culture. The highest value was found to be 6.10×10^6 cells/mL for the culture supplemented with whole hemolymph, which corresponds to an increase of 20% compared to the value observed in the control culture (5.16×10^6 cells/mL). The variation between replicates was minimum (level of significance— $p < 0.01$).

Table 1 presents rRVGP production in cultures supplemented with whole hemolymph (Hb) and its fractions. When the cell cultures were enriched with whole Hb and pool 1, specific rRVGP yields increased to maximum values of 51.4 and 53.1 ng rRVGP/ 10^7 cells, respectively. On the other hand, a lower maximum specific rRVGP yield (40.6 ng RVGP/ 10^7 cells) was observed in the non-enriched control culture (level of significance— $p < 0.01$). It can be also observed that pool 1 yielded the best results among the tested fractions.

Unlike the control run, where the maximum yield was achieved on day 5, rRVGP reached its maximum yield in enriched cultures earlier (day 3). rRVGP levels were 26.6 and 31.5% higher in the cultures

Fig. 1 Effect of hemolymph on transfected S2 cell growth. Whole hemolymph and hemolymph protein fractions (pools 1, 2 and 3) were added to the culture medium to a final concentration of 1% (v/v) or 2% (v/v), respectively, at the time of inoculation. Samples were taken daily and the cell number was determined using a haemocytometer. The error bars represent the standard deviation in a single experiment performed in quadruplicate

treated with whole hemolymph and with pool 1, respectively, than that in the control run (Table 1).

The average specific rRVGP concentrations after 6 days of culture are shown in Table 2. The whole hemolymph-enriched culture attained a 30.5% higher value than that observed in the control culture, indicating the positive effect of the hemolymph on the production of the recombinant protein.

Figure 2 shows a comparative study between the specific concentrations of rRVGP (ng/cell) and the concentrations of rRVGP in the supernatant (ng/mL) from hemolymph-treated (1% (v/v)) and non-treated cultures. Almost throughout the treated experiment, the rRVGP concentrations, per cell and per mL, were higher. The specific concentration fluctuated slightly from day 2 to day 5, while recombinant protein concentration profile paralleled cell growth.

Figure 3 exhibits the time profiles for cell growth and rRVGP production in whole hemolymph-enriched (1% (v/v)) and non-enriched cell cultures. The maximum specific concentration of rRVGP was reached at the early exponential phase, but not at the late exponential phase, when cell concentration was higher, suggesting that the production of rRVGP

Table 1 Specific rRVGP yields (ng/10⁷ cells) in transfected S2 cell cultures

Day	Mean control	(1)	Mean Hb	(1)	Mean pool 1	(1)	Mean pool 2	(1)	Mean pool 3	(1)
1	17.7	3.89	20.1	1.94	19.1	1.06	16.9	0.97	18.2	2.56
2	30.4	2.79	48.4	1.25	36.9	0.82	28.7	1.51	39.66	6.29
3	39.6	0.80	51.4	1.18	53.4	2.61	49.2	0.06	50.0	1.08
4	38.7	0.25	46.0	0.17	47.2	0.04	36.5	3.55	36.4	3.45
5	40.6	4.46	48.6	8.09	33.8	2.01	37.1	6.04	38.4	1.18
6	22.8	0.37	24.6	8.87	24.4	0.50	22.6	1.28	23.8	0.30
7	16.4	1.47	30.0	1.41	26.7	1.35	23	1.14	22.8	0.76

Whole hemolymph and hemolymph protein fractions (pools 1, 2 and 3) were added to the culture medium to a final concentration of 1% (v/v) or 2% (v/v), respectively, at the time of inoculation

(1) Standard deviation

Bold = Maximum value

Table 2 Average specific concentrations of rRVGP (ng/10⁷ cell) after 6 days in transfected S2 cell cultures non supplemented (control) and supplemented with whole hemolymph at 1% (v/v) or its fractions (pools 1, 2 and 3) at 2% (v/v)

	Control	Hb	Pool 1	Pool 2	Pool 3
Mean	29.5	38.5	34.4	30.6	32.7
Standard deviation	9.0	11.5	7.01	8.9	9.6

takes place under the physiological conditions characterized by cells at early exponential growth phase.

Table 3 presents the kinetic parameters for cell growth and rRVGP production, in term of maximum specific cell growth rate ($\mu_{X_{max}}$), doubling time (td) and productivity (maximum specific rRGVP production rate). Specific growth rate (μ) is defined as the rate of the instantaneous growth rate by biomass concentration at this moment and it is measured as h⁻¹ (day 1). In the exponential growth phase (when the specific growth rate is constant and his value is maximum) there is a relationship between this maximum specific growth rate value, μ_{max} , and the doubling time, which is the time necessary to cell population double in number. In our work we have observed no difference in the ratio of maximum cell growth (μ_{max}) in both, control and treated culture as well in the doubling time of cells (td) (46.2 h in control culture to 53.3 h in treated culture). Moreover, the productivity of the cultures in the production of recombinant protein was very different. While the maximum specific rRGVP production rate

Fig. 2 Comparative study between the specific concentrations of rRVGP (ng/cell) and the concentrations of rRVGP in the supernatant (ng/ml) from hemolymph-treated (1% (v/v)) or non-treated cultures. The cultures were performed in Schott flasks containing 20 mL of TC-100 medium. The flasks were inoculated with S2 cells to provide an initial concentration of 1 × 10⁶ cells/mL. Samples were taken daily and quantified by ELISA. The results are expressed as ng of rRVGP/10⁷ cells. The error bars represent the standard deviation in a single experiment performed in quadruplicate

Fig. 3 Time profiles for cell growth and rRVGP production in whole hemolymph-enriched (1% (v/v)) and non-enriched cell cultures. The cultures were performed in Schott flasks containing 20 mL of TC-100 medium. The flasks were inoculated with S2 cells to provide an initial concentration of 1×10^6 cells/mL. Samples were taken daily and quantified by ELISA. The results are expressed as ng of rRVGP/ 10^7 cells. The error bars represent the standard deviation in a single experiment performed in quadruplicate

(μ_{rRVGPmax}) was 60 ng rRVGP/ 10^7 cell.day in the treated culture, a 2.5 times lower maximum rate (24 ng rRVGP/ 10^7 cell.day) was observed in the control culture (level of significance— $p < 0.01$). In this case, the productivity of the cultures treated with hemolymph was clear. Productivity studies analyze technical processes and engineering relationships such as how much of an output can be produced in a specified period of time. It is related to the concept of

efficiency. Productivity improves when the quantity of output increases relative to the quantity of input, or, as occurs in our case, when there is an increase in the amount of product per unit of cell. So, the productivity was measured as, increase in recombinant production by the time required for this increase and with a constant cell number.

Cell growth and rRVGP production in the non-supplemented culture

The time profiles for cell growth, rRVGP production, specific growth and rRVGP production rates in the non-treated (control) cultures are shown in Fig. 4. According to Fig. 4a and Table 3, a maximum cell concentration (X_{max}) of 5.18×10^6 cell/mL and a maximum rRVGP concentration (rRVGP max) of 14.4 ng/mL were achieved on days 7 and 5, respectively. The concentration of rRVGP increased in the cell culture until the end of the exponential growth phase (day 5), as shown in Fig. 4b. Figures 4b and c depict the plots of cell and rRVGP concentrations (X and rRVGP) and corresponding specific rates (μ_X and μ_{rRVGP}) against time. As shown in Fig. 4b, the exponential growth phase took place between days 1 and 5 of the experiment. Cell concentrations started to decrease on the seventh day. Cell death was observed at a constant rate after the eighth day of cultivation (estimated at 0.11 day^{-1}). Similarly, rRVGP concentrations grew exponentially at 19 ng rRVGP/ 10^7 cell.day from days 1 to 4 (Fig. 4c) but, after day 5, dropped sharply to the end of the culture (degradation rate estimated at $0.41 \text{ ng rRVGP}/10^7 \text{ cell.day}$).

Figure 4d compares the time profiles for specific growth and RGPV productions rates. The production of rRVGP paralleled growth. As it can be observed in Fig. 4c and d, the concentration of rRVGP (ng/mL or ng/ 10^6 cell) and its specific rate (μ_{rRVGP}) started to decrease after the fifth day of culture, when the specific growth rate was still at its maximum value, indicating that the dissociation between cell growth

Table 3 Kinetic parameters for cell growth and rRVGP recombinant protein production

	X_{max} cell/mL	rRVGP max (ng/mL)	$\mu_{X_{\text{max}}}$ (h^{-1})	Td (h)	μ_{rRVGPmax}
Control	5.18×10^6	14.4	0.015	46.2	24 ng rRVGP/ 10^7 cell.day
Hemolymph	6.10×10^6	17.0	0.013	53.3	60 ng rRVGP/ 10^7 cell.day

X_{max} , maximum cell concentration (cell/mL); rRVGP max, maximum rRVGP concentration (ng/mL); $\mu_{X_{\text{max}}}$, maximum specific cell growth rate (h^{-1}); μ_{rRVGPmax} , maximum specific rRVGP production rate (ng rRVGP/ 10^7 cell.day); td, doubling time (hour)

Fig. 4 Time profiles for cell growth, rRVGP production, specific growth and rRVGP production rates in the non-treated (control) culture. **a** Cell (X) and rRVGP (rRVGP) concentrations versus time; **b** Cell concentrations (X) and specific

growth rates (μ_X) versus time; **c** rRVGP (rRVGP) concentrations and specific production rates (μ_{rRVGP}) versus time; **d** specific growth rates (μ_X) and specific production rates (μ_{rRVGP}) versus time

and protein production may be due to either the decomposition of the protein or higher fragility of the membrane as growth occurs. Degradation of the protein produced took place at $0.41 \text{ ng rRVGP} / 10^7 \text{ cell.day}$. After calculating the specific rates by $\mu_X = \frac{1}{X} \frac{dX}{dt}$ and $\mu_{\text{RGPV}} = \frac{1}{X} \frac{d\text{RGPV}}{dt}$, the maximum specific rates were determined. The $\mu_{X_{\text{max}}}$ and $\mu_{\text{rRVGP}_{\text{max}}}$ values obtained were 0.356 day^{-1} (0.015 h^{-1}) and $19 \text{ ng rRVGP} / 10^7 \text{ cell.day}$ in the non-enriched control culture. In the culture treated with hemolymph these

parameters were 0.314 day^{-1} (0.0137 h^{-1}) and $60 \text{ ngRVGP} / 10^7 \text{ cell.day}$, respectively. These data are summarized in Table 3.

Cell growth and rRVGP production in hemolymph-supplemented culture

The time profiles for cell growth, rRVGP production, specific growth and rRVGP production rates in the hemolymph-treated cultures are shown in Fig. 5.

Fig. 5 The time profiles for cell growth, rRVGP production, specific growth and rRVGP production rates in the hemolymph-treated (1% v/v) cultures. **a** Cell (X), rRVGP (rRVGP) and specific rRVGP concentrations versus time. **b** Cell

concentrations (X) and specific growth rates (μ_X) versus time; **c** rRVGP (rRVGP) concentrations and specific production rates (μ_{rRVGP}) versus time; **d** specific growth rates (μ_X) and specific production rates (μ_{rRVGP}) versus time

Overall, the addition of the hemolymph had a positive effect on cell growth and rRVGP production.

According to Fig. 5a, the addition of the hemolymph to the culture resulted in higher maximum cell (6.1×10^6 cells/mL) and rRVGP (17.0 ng/mL) concentrations compared to those observed in the control culture. Unlike the control culture, the treated culture attained its highest cell concentration later (day 8). Cells grew exponentially over a longer period of time (between days 1 and 7), in comparison with the control culture (Fig. 5b). Cell number started to decrease after day 8 (at a estimated death rate of 0.16 days^{-1}). Unlike in the control culture, the specific rates of cell growth (μ_X) and rRVGP production (μ_{rRVGP}) behaved differently (Fig. 5b, c). Linear increase in the concentration of rRVGP occurred; consequently, μ_{rRVGP} decreased over the course of the experiment over eight days, ranging from a maximum value of $4.1\text{--}0.0 \text{ ngRVGP}/10^7 \text{ cell.day}$. After the eight day, the concentration of rRVGP dropped sharply to the end of the culture (degradation rate at the end of the culture = $4.3 \text{ ng rRVGP/cell } 10^7 \text{ day}$).

The comparison between the time profiles for specific growth and rRVGP production rates are shown in Fig. 5d. The average value of the specific production rates during the exponential growth phase was higher than that in the control experiment.

Different time profiles for rRVGP (ng/mL) and specific rRVGP (ng/cell) concentrations were obtained (Fig. 5a, c). The highest value of specific rRVGP concentration was achieved at the beginning of the culture (day 2), indicating that cells initially produce more rRVGP per cell. It then decreased linearly until the tenth day of culture. The drop in the rRVGP concentration (ng/mL) after the eight day of the culture suggests protein decomposition.

As for the specific rates, the maximum specific growth rate (μ_{max}) observed in the treated culture (0.33 d^{-1} or 0.013 h^{-1}); between days 2 and 7, was slightly slower than that in the control culture. However, a longer exponential phase took place, indicating that the hemolymph may minimize the effects of growth-limiting factors. The specific rates of rRVGP production varied from 10 to $60 \text{ ng rRVGP}/10^7 \text{ cells.day}$ with an average of $30 \text{ ng rRVGP}/10^7 \text{ cells.day}$, which was higher than that observed in the non-treated experiment. Its values throughout the treated culture were higher

than those observed in the control. As a consequence, a higher concentration of rRVGP was reached (rRVGP max = 17 ng/mL). These results suggest that the *Lonomia obliqua* hemolymph has a protein which acts as a potent enhancer of the recombinant protein production by S2 cells.

The differences between the average of the variables for the two groups, hemolymph-enriched and non-enriched (control) cultures, were statistically significant at $p < 0.01$, meaning that there was a statistically significant increase in rRVGP production due to hemolymph addition. Recently, we identified and purified a protein from *Lonomia obliqua* hemolymph able to increase the production of rabies virus glycoprotein, expressed in *Drosophila melanogaster* cells, by about 60% (Mendonça et al. 2008). This work aimed to extend the previous work by determining the kinetic parameters for cell growth and recombinant protein production (i.e., maximum specific rates— $\mu_{X_{\text{max}}}$ and μ_{rRVGPmax}) and the relationship between the production of the recombinant protein and the phase of the cell cycle on the addition of the hemolymph. The mechanism by which the protein in our study promotes rRVGP production remains to be solved. Kanaya and Kobayashi (2000) reported that the supplementation of the culture medium with *Bombyx mori* hemolymph led to an up to 6,000-fold increase in recombinant luciferase production in a polyhedron baculovirus system, suggesting that the increase in recombinant protein production could be related to the increase in the baculovirus replication (10,000 times). However, we speculate that this increase may be due to some other mechanism. Souza et al. (2005) have isolated an anti-apoptotic protein from *Lonomia obliqua* hemolymph. Thus, it is possible that this protein also plays an essential role in the enhancement of rRVGP production. Another relevant observation was the relationship between cell growth and the production of the protein in the cultures studied. Specific rRVGP production (ng/cell) increased as cells grew and decreased as cells entered the stationary phase, indicating that the production of rRVGP may be directly related to cell activity.

The importance of this study was not only to obtain a protein that can enhance the production of recombinant proteins but also to understand the kinetics of this process in order to optimize productivity.

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