Using cell size kinetics to determine optimal harvest time for *Spodoptera frugiperda* and *Trichoplusia ni* BTI-TN-5B1-4 cells infected with a baculovirus expression vector system expressing enhanced green fluorescent protein

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Received: 15 November 2006 / Accepted: 27 February 2007 / Published online: 14 April 2007
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Abstract Infecting insect cells with a baculovirus expression vector system (BEVS) is an increasingly popular method for the production of recombinant proteins. Due to the lytic nature of the system, however, determining the optimal harvest time is critical for maximizing protein yield. We found that measuring the change in average diameter during the progress of infection with an automated cell analysis system (Cedex HiRes, Innovatis AG) could be used to determine the time of maximum protein production and, thus, optimal harvest time. As a model system, we use insect cells infected with a baculovirus expressing enhanced green fluorescent protein (EGFP). We infected two commonly used insect cell lines, *Spodoptera frugiperda* (Sf-9) and *Trichoplusia ni* BTI-TN-5B1-4 (Hi5) with an *Autographa californica* nuclear polyhedrosis virus (AcNPV) encoding EGFP at various multiplicities of infection (MOI). We monitored the progress of infection with regard to viability, viable cell density and change in average cell diameter with a Cedex HiRes analyzer and compared the results to the EGFP produced. Peak protein production was reached one to two days after the point of maximum average diameter in all

conditions. Thus, optimal harvest time could be determined by monitoring the change in average cell diameter during the course of an infection of a cell culture.

Keywords Baculovirus · BEVS · Sf-9 · Hi5 · Cell size kinetics · Average cell diameter · EGFP · Cedex HiRes

Abbreviations

BEVS	baculovirus expression vector system
Sf-9	<i>Spodoptera frugiperda</i>
Hi5	<i>Trichoplusia ni</i> BTI-TN-5B1-4
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
EGFP	enhanced green fluorescent protein
MOI	multiplicity of infection
Pfu	plaque forming units
RFI	relative fluorescence intensity
PI	post-infection

Introduction

The infection of insect cells with the baculovirus expression vector system (BEVS) has become an increasingly popular platform for the production of recombinant proteins. Compared to mammalian cells, insect cells offer a number of advantages including ease of cultivation and higher expression levels when

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infected with a recombinant baculovirus. Insect cells can also perform many of the necessary post-translational modifications and correct protein folding (see Ikonomou et al. 2003 for a recent review of the use of insect cells for industrial production of recombinant proteins).

However, a critical issue limiting the usefulness of BEVS is proteolysis since the system is inherently lytic in nature. Besides the addition of protease inhibitors to baculovirus infected cultures (Ikonomou et al. 2003), one approach to dealing with the problem of proteolysis has involved the creation of BEVS that are less lytic in nature. Some researchers have focused on eliminating specific protease activity from a particular vector (e.g. Suzuki et al. 1997; Monsma and Scott 1997), while others have looked at the possibility of using different promoters to replace the commonly used polyhedrin promoter that is activated at the very late phase of a baculovirus infection (e.g. Bonning et al. 1994; Hill-Perkins and Possee 1990; Kost et al. 1997; Lawrie et al. 1995).

Another important approach has involved looking for more effective ways to monitor the entire process in order to predict the time of maximum protein yield and hence optimum harvest time. Proposed methods for monitoring infected cell cultures have included, among others, measurement of oxygen uptake rate (Hensler and Agathos 1994; Kamen et al. 1996; Palomares and Ramirez 1996), intracellular ATP measurement (Olejnik et al. 2004), viability (Jain et al. 1991), change in modal cell diameter (Jain et al. 1991; Palomares et al. 2001) and peak cell diameter (Palomares et al. 2001).

Many of the proposed monitoring methods are labor and time intensive. Viability, cell density and cell size kinetics, on the other hand, are particularly easy parameters to monitor due to the availability of rapid, automated systems for monitoring cell density, viability and diameter. These parameters, therefore, have been considered as potentially useful methods for monitoring infected cell cultures to determine the point of peak protein production and to predict the maximum protein yield. Proposed monitoring methods have included using a specific drop in viability as an indication of peak protein production (Jain et al. 1991), finding a direct relationship between modal cell diameter and

peak protein production (Jain et al. 1991; Palomares et al. 2001) and using peak cell size as a predictor of maximum protein yield (Palomares et al. 2001). In these reports, one cell line and one multiplicity of infection (MOI) were used.

To our knowledge, researchers looking into possible correlations between viability and cell size kinetics to peak production and maximum yield have generally only used a cell line derived from the fall armyworm, *Spodoptera frugiperda* (Sf-9), as the model insect cell culture. However, a cell line derived from a cabbage looper, *Trichoplusia ni* BTI-TN-5B1-4 (Hi5), is becoming an increasingly popular choice for infection with BEVS due to the much higher product yield often obtainable with Hi5 compared to Sf-9 (Ikonomou et al. 2003; Taticek et al. 2001). Sf-9 and Hi5 cell lines, derived from two different organisms, differ markedly from each other in their general behavior and metabolism. Hi5 cells produce alanine, ammonium and lactate in high concentrations during cell growth, and require both glutamine and asparagine for growth (Yang et al. 1996a; Rhiel et al. 1997). Sf-9 cells, in contrast, produce ammonium and lactate only at very low levels (Bedard et al. 1993) and can grow in glutamine-, glutamate- and aspartate-free medium if ammonium ions are present (Öhman et al. 1996).

Since the metabolism is so different between these two cell lines, we opted to use both Hi5 and Sf-9 in combination with an *Autographa californica* nucleopolyhedrosis virus (AcNPV) expressing enhanced green fluorescent protein (EGFP) to test whether viability, viable cell density and cell size kinetics determined with the automated Cedex HiRes system could be used to predict the time of peak protein production and the maximum protein production in both cell lines. In addition, we tested each cell line at different MOIs to see if varying the amount of baculovirus used to infect cell cultures would result in different patterns of viable cell density, viability and cell size kinetics, as well as a different pattern of correlation of these parameters with peak EGFP production and maximum yield. We found that peak EGFP production invariably occurred one to two days after peak average diameter for both cell lines and all MOIs of baculovirus used to infect the cultures.

Materials and methods

Cell culture

Trichoplusia ni BTI-TN-5B1-4 (Hi5), a continuous cell line, was obtained from Invitrogen (cat. # B85502). The cells were cultured in Express Five SFM (Invitrogen, cat. # 10486-025). *Spodoptera frugiperda* (Sf-9), a continuous cell line, was obtained from Invitrogen (cat. # 11496-015). The cells were cultured in Sf900 II (Invitrogen, cat. # 10902-088). Both cell lines were cultured in suspension in 250 ml flasks (Corning, cat. #431144) with a 60 ml working volume. All cultures were incubated in an Infors Multitron shaking incubator at 27 °C and agitated at 97 rpm.

Virus stock and infection

The recombinant *Autographa californica* nuclear polyhedrosis virus was used. The recombinant EGFP-AcNPV baculovirus, which produces the enhanced green fluorescent protein (EGFP), was provided by Catherine Heddle, Astra Biotech Lab, Astrazeneca, Södertälje, Sweden. The EGFP was expressed under the polyhedrin promoter. The infection was performed at the mid-exponential growth phase of the cell culture for both Hi5 and Sf-9 cells when the cells had reached a density of 1×10^6 cells ml⁻¹. Both cell lines were infected at 3 different multiplicities of infection (MOI): 0.5, 1, and 5 plaque forming units (pfu) cell⁻¹, as well as a negative control with no virus added (MOI = 0). The titer of the viral stock used for infecting the insect cells was 1.3×10^8 pfu ml⁻¹. Four measurements were taken from two independent experiments for all combinations of insect cell lines and different MOIs.

Analytical methods

Viral titer was determined using the BD BackPAK Baculovirus Rapid Titer Kit (BD Biosciences, cat. # K1599-1). Cell concentration, viability and average diameter were determined using a Cedex HiRes automated cell counter (Innovatis AG, Bielefeld, Germany), which uses image analysis software to determine the diameter of each cell in a sample and to determine the cell density and viability via the Trypan blue exclusion method (0.2% Trypan blue).

Recombinant EGFP synthesis was identified using a fluorescence reader, Spectra MAX GEMINI XS (Molecular Devices) and read at the following setup: Excitation: 485, Emission: 520 and cut-off: 515. Both secreted and intracellular EGFP were analyzed. Cell samples were removed from culture flasks and centrifuged for 6 min at $5,300 \times$ rcf to separate cells from medium. The supernatant was removed from the pellet and both cell pellets and supernatants were stored at -80 °C until analyzed. Secreted EGFP was determined by rapidly thawing samples and analyzing the supernatant on the fluorescence reader directly to determine the fluorescence intensity. Intracellular EGFP was extracted from the pellet fraction using BugBuster (Novagen, cat. #70584). Pellets were incubated with BugBuster for 45 min on ice, then centrifuged for 5 min at 10,000 rpm. The resultant supernatant was then analyzed using the fluorescence reader. All samples were placed in 96 well black microtiter plates (Nunc, cat. #137103) along with fresh medium (Sf900 II and Express Five SFM for Sf-9 and Hi5 cells, respectively) as blanks. The relative fluorescence intensity (RFI) for each sample was calculated by subtracting the fluorescence intensity of the appropriate blank from the fluorescence intensity of the sample. The RFI was used to determine the relative protein yields of the various samples analyzed.

The average of all measurements taken for each combination of insect cell line/MOI was used for the construction of the graphs, and standard deviations were calculated using Microsoft Excel 2003.

Results

Infected Hi5 and Sf-9 cell lines gave very different results in terms of EGFP production, change in average cell diameter over time, growth rate and viability over time. In addition, at each MOI Hi5 cells gave very different results with regard to the above parameters. The results from infection of Sf-9 cells at different MOIs, on the other hand, more closely resembled each other.

For each condition of cell line and MOI, duplicate cultures were set up in order to determine the reproducibility of the results. In addition, each culture was sampled twice. The results from the separate cultures very closely matched each other with low standard deviations (Figs. 2, 4). The average of all results was taken for each culture condition, and the

averages were used to calculate and compare EGFP production, viability, viable cell density and change in average cell diameter over time.

Recombinant EGFP production

Green fluorescence protein (GFP), from the jellyfish *Aequorea Victoria*, is a highly stable protein that is easily detected using a fluorescence reader and is not easily degraded by proteases (Chalfie et al. 1994; Li et al. 1998; see March et al. (2003) for a recent review of biotechnology applications of GFP). Enhanced green fluorescence protein (EGFP) is a variant of GFP that gives a stronger signal, but otherwise behaves in a similar fashion (Yang et al. 1996b; Cormack et al. 1997). EGFP is produced as an intracellular protein in the insect cell/baculovirus system but is later released into the medium as post-infection time increases due to increasing lysis of the cells (Ho et al. 2004). Our results showed this same pattern of EGFP production in both infected Hi5 and Sf-9 cells. EGFP first appeared intracellularly (pellet fractions of the centrifuged samples removed from the cultures), then appeared in increasing amounts in the medium (supernatant fractions of the centrifuged samples removed from the cultures) after day 3 post-infection (PI) in both cell types (Figs. 1A, 3A).

The stability of EGFP was demonstrated by the fact that the relative fluorescence intensity hit a plateau once the peak intensity was reached, despite all cell cultures being allowed to run for several days after peak EGFP RFI and until all cultures were nearly 0% viable. As the maximum amount of EGFP found in the pellet fraction was significantly lower than the maximum amount found in the supernatant fraction, we added the results from the pellet and supernatant fractions to determine the total EGFP produced based on the RFI (Figs. 1B, 3B).

Using the RFI as an indication of level of EGFP production, Hi5 cultures yielded a much higher total amount of EGFP volumetrically than Sf-9 cells. Infected Hi5 cell cultures attained a maximum of 33,000 RFI compared to the maximum of 8,500 RFI obtained with the same volume of Sf-9 cells. This was a four-fold difference in intensity despite the fact that peak Hi5 viable cell density was less than twice that of peak Sf-9 viable cell density for MOI = 0.5 and MOI = 1 and nearly identical in the case of MOI = 5 (Figs. 2B, 4B). In Hi5 cultures, the point of

peak production of EGFP and the total yield varied depending on the MOI of the virus used to infect the cultures. Cultures infected at MOI = 5 and MOI = 1 reached the point of peak EGFP production much earlier than cultures infected at MOI = 0.5 (3 days PI, 3 days PI and 6 days PI, respectively). However, the Hi5 cultures infected at MOI = 0.5 produced the most EGFP, with an RFI of approximately 60% and 20% greater than cultures infected at MOI = 1 and MOI = 5, respectively (Fig. 1B).

Sf-9 cultures infected at MOI = 0.5 and MOI = 1 reached the point of peak EGFP production on day 5 PI, while cultures infected at MOI = 5 reached the point of peak EGFP production on day 4. Total EGFP yield from the infected Sf-9 cultures varied somewhat, though not as much as the yield from infected Hi5 cultures. Sf-9 cultures infected at MOI = 5 had the highest yield, while cultures infected at MOI = 1 and MOI = 0.5 had lower yields (Fig. 3B).

Viable cell density

Hi5 cultures showed very distinct differences in the viable cell density depending on the MOI of the virus used to infect the cells. Cultures infected at MOI = 0.5 and MOI = 1 continued to grow for the first 3 days PI, although cultures infected at MOI = 1 grew more slowly and reached a lower peak viable cell density than those infected at MOI = 0.5. Cultures infected at MOI = 5, on the other hand, barely grew for the first day post-infection before rapidly decreasing in viable cell density. The peak viable cell density reached by the cultures ranged from 2.9×10^6 cells ml⁻¹ (MOI = 0.5) to 2.3×10^6 cells ml⁻¹ (MOI = 1) and 1.2×10^6 cells ml⁻¹ (MOI = 5), compared to the peak density of 4.6×10^6 cells ml⁻¹ reached by uninfected cells (MOI = 0) (Fig. 2A, B).

Sf-9 cultures also showed different growth rates depending on the MOI used to infect the cultures, but the differences between peak viable cell densities of the cultures infected at different MOIs were less pronounced than in the Hi5 cells. Cultures infected at a MOI of 0.5 reached a peak viable cell density of 1.6×10^6 cells ml⁻¹ compared to 1.4×10^6 cells ml⁻¹ (MOI = 1) and 1.2×10^6 cells ml⁻¹ (MOI = 5) (Fig. 4A, B). Uninfected cultures (MOI = 0) reached a much higher peak density of 13.8×10^6 cells ml⁻¹ (Fig. 4A).

Fig. 1 Hi5 uninfected cell cultures (MOI = 0 (◆)) and cell cultures infected with MOI = 0.5 (▲), MOI = 1 (■), MOI = 5 (●) tracked for up to 10 days post-infection: (A) intracellular (---) and released (—) enhanced green fluorescence protein levels produced over time, based on relative fluorescence intensity;

(B) Total protein levels (secreted + intracellular). Standard deviation calculations, represented by (⊥), are shown for all points on all graphs. In some cases, the standard deviation was so small that the standard deviation bar is hidden by the symbol used for that point

Uninfected Hi5 cell cultures have been grown in batch culture up to $8\text{--}10 \times 10^6$ cells ml^{-1} (Rhiel et al. 1997). In our uninfected Hi5 cultures, however, both viable cell density and viability sharply plummeted after the cultures reached a cell density of 4.6×10^6 cells ml^{-1} at 2 days PI. The uninfected Sf-9 cultures grew to a much higher peak cell density. The differences in peak viable cell density in uninfected cultures reached by the two different cell lines may possibly be due in part to the differences in the metabolism of the two cell lines. The uninfected Hi5 cultures may have been limited in growth due to the rapid increase in cell density in the first 2 days

leading to a rapid depletion of nutrients in the medium and the production of potentially inhibitory byproducts such as ammonia and lactate (Yang et al. 1996a; Rhiel et al. 1997). In contrast, Hi5 cells infected at the rates of MOI = 0.5 and MOI = 1 remained viable for longer, but grew more slowly and reached much lower peak cell densities (Fig. 2A, C).

Viability

Infected Hi5 cells showed marked differences in viability during the progress of infection. Cultures infected at MOI = 5 were 50% viable at 3 days PI,

Fig. 2 Hi5 uninfected cell cultures (MOI = 0 (◆)) and cell cultures infected with MOI = 0.5 (▲), MOI = 1 (■), MOI = 5 (●) tracked for up to 10 days post-infection with regard to: (A) change in viable cell density over time, with uninfected cell cultures included; (B) change in viable cell density over time, without uninfected cell cultures included;

whereas cultures infected at MOI = 1 and MOI = 0.5 were 50% viable at 4.5 and 5 days PI, respectively. Cultures infected at MOI = 5 were under 10% viable by approximately 7 days PI, while cultures at MOI = 1 and MOI = 0.5 were under 10% viable by 9 and 8 days PI, respectively (Fig. 2C). Uninfected Hi5 cultures were below 10% viable considerably earlier (day 5 PI, data not shown) for the reasons outlined in the previous section.

Again, the Sf-9 cultures infected at the various MOIs more closely matched each other in their change in viability over time. MOI = 5 cultures were 50% viable by day 3 PI, whereas MOI = 1 and MOI = 0.5 were 50% viable at 3.6 and 3.8 days, respectively. MOI = 5 cultures were below 10% viable by approximately day 5 PI. Cultures infected at MOI = 1 and MOI = 0.5 were below 10% viable by

(C) change in viability over time; and (D) change in average cell diameter over time. Standard deviation calculations, represented by (□), are shown for all points on all graphs. In some cases, the standard deviation was so small that the standard deviation bar is hidden by the symbol used for that point.

day 6 PI. Uninfected cultures (MOI = 0) were still 70% viable after 7 days (Fig. 4C).

Average cell diameter

As was the case with viable cell density and viability, infection of Hi5 cells at different MOIs had markedly different effects on cell size kinetics. Cells infected at MOI = 5 most rapidly swelled and reached a higher peak average diameter more quickly (22 μm at 2 days PI) than cultures infected at MOI = 1 (peak diameter of 20.2 μm at 2 days PI) and MOI = 0.5 (peak diameter of 20.8 μm at 4 days PI) (Fig. 2D). The average cell diameter at the start of infection for all Hi5 cultures was approximately 16.3 μm , so the diameter increased by 35% in the case of MOI = 5, 24% for MOI = 1 and 28% for MOI = 0.5 (Table 1).

Fig. 3 Sf-9 uninfected cell cultures (MOI = 0 (◆)) and cell cultures infected with MOI = 0.5 (▲), MOI = 1 (■), MOI = 5 (●) tracked for up to 7 days post-infection: (A) intracellular (---) and released (—) enhanced green fluorescence protein levels produced over time, based on relative fluorescence

intensity; (B) Total protein levels (secreted + intracellular). Standard deviation calculations, represented by (⊥), are shown for all points on all graphs. In some cases, the standard deviation was so small that the standard deviation bar is hidden by the symbol used for that point

Although cells infected at MOI = 1 and MOI = 5 reached their peak average diameter at the same time (day 2 PI), the pattern of change in average cell diameter over time was very different for the two different conditions. Cells infected at MOI = 5 rapidly increased in size until 2 days PI, after which the average cell diameter clearly decreased. Cells infected at MOI = 1 increased in size rapidly until day 2 PI, after which the average diameter hit a plateau before starting to decrease after day 5 PI. Cells infected at MOI = 0.5 increased in size more slowly than either

cells infected at MOI = 1 or MOI = 5. Peak average diameter was also reached later: 4 days PI (Fig. 2D). Uninfected cells also showed some increase in average diameter (peak of 19.3 μ m at 3 days PI, an increase of 18%). The pattern of increase in the uninfected cultures was, however, different from the patterns found in the infected cultures. The average diameter of the cells in the uninfected cultures increased very slowly, with the first noticeable increase at 2 days PI, whereas all the infected cultures showed a dramatic increase in average diameter by 10 h PI.

Fig. 4 Sf-9 uninfected cell cultures (MOI = 0 (◆)) and cell cultures infected with MOI = 0.5 (▲), MOI = 1 (■), MOI = 5 (●) tracked for up to 7 days post-infection with regard to: (A) change in viable cell density over time, with uninfected cell cultures included; (B) change in viable cell density over time, without uninfected cell cultures included;

(C) change in viability over time; and (D) change in average cell diameter over time. Standard deviation calculations, represented by (□), are shown for all points on all graphs. In some cases, the standard deviation was so small that the standard deviation bar is hidden by the symbol used for that point

Table 1 Relative fluorescence intensity, viable cell density, viability and average cell diameter for Sf-9 and Hi5 cell cultures before dilution of all cultures to 1×10^6 cells ml⁻¹ and before the addition of the recombinant EGFP-AcNPV baculovirus

	Relative fluorescence intensity	Viable cell density ($\times 10^6$ cells ml ⁻¹)	Viability	Average cell diameter, (μm)
Hi5	-96	3.05	98%	16.3
Sf-9	-34	5.09	97%	14.0

In the case of the Sf-9 cells, the change in average cell diameter followed a similar pattern for all infected cell cultures regardless of the MOI used for infection. The peak average diameters of 16.9 μm (MOI = 5) and 17.1 μm (MOI = 1 and MOI = 0.5)

were reached at 3 days PI by all infected Sf-9 cultures (Fig. 4D). The average cell diameter at the start of infection for all Sf-9 cultures was approximately 14 μm, so the average diameter increased by 21% for MOI = 5 and 22% for both MOI = 1 and MOI = 0.5. Uninfected Sf-9 cultures increased slightly in average diameter to 14.4 μm by day 4 PI, an increase of 3% (Table 1).

In all cases of both cell lines and all MOIs used to infect the cultures, peak average diameter was reached 1–2 days before peak protein production. Hi5 cultures infected at MOI = 0.5 reached the peak average diameter on day 4 PI and the peak EGFP production on day 6. For MOI = 1 and MOI = 5, the peak average diameter was reached on day 2 PI and peak EGFP production on day 3 PI (Figs. 1B, 2D).

All Sf-9 cultures reached peak average diameter on day 3 PI. Sf-9 cultures infected at MOI = 5 reached peak EGFP production on day 4, whereas cultures infected at MOI = 0.5 and MOI = 1 reached peak EGFP production on day 5 (Figs. 3B, 4D).

Discussion

Here we report that the peak in average cell diameter was the sole reliable predictor of the point of peak protein production in the case of two different insect cell lines, Hi5 and Sf-9, infected with a recombinant EGFP-AcNPV baculovirus. Various other factors, including viable and maximum cell density, drop in viability, maximum average cell size, and time of peak modal cell diameter proved to be less useful predictors of either point of peak protein production or total protein produced. These results differed somewhat from published results of studies involving infections of insect cells with recombinant baculoviruses expressing other proteins.

Viability, viable cell density and maximum cell density

Jain et al. (1991) found that a 50% drop in viability coincided with the peak production of antistatin when Sf-9 cells were infected with a recombinant AcNPV encoding antistatin at a MOI = 0.1 (Jain et al. 1991). However, we found no correlation between a particular percent drop in viability and the time of peak EGFP production for both Sf-9 and Hi5 cell cultures when the cultures were infected at different MOIs. In the case of Hi5 cell cultures, the viability of cells at the time of peak EGFP production varied from 20% to 70% depending on the MOI used (Fig. 2C). In Sf-9 cell cultures, the viability was very low at the point of peak EGFP production and was more consistent: the viability ranged 17%–19% depending on the MOI used (Fig. 4C).

There was no correlation between peak EGFP levels and the point of peak viable cell density. Peak EGFP levels in the case of Hi5 cells, for example, were reached at the same time as the point of peak viable cell density in the case of cultures infected at MOI = 1 but were reached 3 days and 2 days after peak viable cell densities for cultures infected at MOI = 0.5 and MOI = 5, respectively (Fig. 2B).

We expected to find some correlation for each cell line between maximum cell density and maximum EGFP production but found no strong connection between the two, particularly in the case of Hi5 cells. Hi5 cultures infected at MOI = 1 reached a maximum viable cell density of 2.34×10^6 cells ml⁻¹, but produced less EGFP than cultures infected at MOI = 5, which only reached a cell density of 1.16×10^6 cells ml⁻¹ (Figs. 1B, 2B).

Maximum EGFP production in Sf-9 cells was also unrelated to the maximum viable cell density. Cell cultures infected at MOI = 5 produced the most EGFP but had the lowest peak viable cell density. Cultures infected at MOI = 0.5 and MOI = 1 produced nearly identical amounts of EGFP, but cells infected at MOI = 1 reached a lower maximum viable density than cells infected at MOI = 0.5 (Figs. 3B, 4B).

Maximum mean cell size

Palomares et al. (2001) infected Sf-9 cells with a baculovirus encoding the VP8 protein of a rotavirus and found a correlation between the maximum mean cell diameter and the total amount of protein produced, even when media conditions were varied. The cultures that achieved the largest maximum mean cell size produced the most protein (Palomares et al. 2001). We did not find the same correlation when the MOI used to infect cell cultures was varied. Hi5 cell cultures infected at a MOI = 5 produced less total EGFP but reached a larger average cell size than cultures infected at MOI = 0.5, which produced the most total protein (Figs. 1B, 2D). Infected Sf-9 cells all reached roughly the same maximum average cell size but produced somewhat different amounts of EGFP (Figs. 3B, 4D).

Modal cell diameter versus average cell diameter

We calculated the modal cell diameter (i.e. the most frequently occurring cell diameter in each set of data) for each individual sample taken as Palomares et al. had found the peak modal cell diameter to be a more useful predictor of the point of peak VP8 production than peak average cell diameter (Palomares et al. 2001). In our experiments, however, peak modal cell diameter was not always reached before the point of peak EGFP production. In the case of Hi5 cultures infected at MOI = 1 and MOI = 5, for example, peak modal cell diameter was reached after peak protein production had already been reached for all samples

drawn from the cultures. In Hi5 cell cultures infected with $\text{MOI} = 5$ the peak modal cell diameter was reached 4 to 5 days PI, whereas peak EGFP production was reached at 3 days PI. This was also the case for Hi5 cell cultures infected at $\text{MOI} = 1$. In addition, the day PI of the peak modal cell diameter was highly inconsistent from sample to sample drawn from identical cultures and conditions. Peak modal diameter for individual samples drawn from identical cultures and conditions ranged from 4 to 5 days PI in the case of Hi5 cultures for all MOIs.

The modal cell diameter was a somewhat better indicator of peak protein production in the case of Sf-9 cells, since maximum modal cell diameter was reached before peak EGFP production in most of the samples drawn from the various culture conditions. However, the variation with regard to modal cell diameter from sample to sample in cultures with identical conditions was also much greater than the variation in the case of average cell diameter. For individual sample series from Sf-9 cultures, peak modal diameter ranged from 2 to 5 days PI ($\text{MOI} = 0.5$), 3–5 days PI ($\text{MOI} = 1$) and 2–5 days PI ($\text{MOI} = 5$). In some cases for sample series taken from Sf-9 cultures, the peak modal cell diameter was concurrent with, or later than, the peak production of EGFP (data not shown).

In contrast, nearly all individual sample series taken from each culture condition indicated a peak average cell diameter on the same day PI. For example, all of the samples taken from Hi5 cells infected at $\text{MOI} = 0.5$ indicated that the peak average cell diameter was reached on day 4 PI, while all of the samples taken from cultures infected at $\text{MOI} = 5$ indicated that a peak average cell diameter was reached on day 2 PI. However, in the case of cultures infected at $\text{MOI} = 1$, the peak average cell diameter indicated by each individual sample ranged from day 2 to day 5 PI. $\text{MOI} = 1$ infected Hi5 cells had a fairly steady plateau in average diameter from day 2 to day 5 PI, with only a slight increase in average cell diameter over that time. Nearly all samples from Sf-9 cells infected at $\text{MOI} = 0.5$, $\text{MOI} = 1$ and $\text{MOI} = 5$ indicated that the peak average cell diameter was reached on day 3 (data not shown).

Using peak average cell diameter to predict peak EGFP production

In the case of both Sf-9 and Hi5 cells infected with recombinant EGFP-AcNPV baculovirus, we found

that the only factor that consistently correlated with the time of peak EGFP production in both Sf-9 and Hi5 cells regardless of which MOI was used to infect the cells, was the time at which peak average cell diameter was reached. For Hi5 cells, peak EGFP production was reached 1–2 days after the peak average diameter was reached for all MOIs, despite vastly different times of peak EGFP production and different amounts of EGFP produced by the cell cultures infected at various MOIs. For Sf-9 cells, peak EGFP production also occurred 1–2 days after the point of peak mean cell diameter. The results were remarkably consistent for all samples from every culture condition tested.

Osmolarity can have an effect on cell size, particularly in the case of insect cells. Rosinski et al. (2000) for example, found that the osmolarity of the medium changes during culturing of cells, falling from approximately 360 mOsm/kg during the early-to mid-exponential growth phase to approximately 290 mOsm/kg during the stationary phase. There is, generally, a much higher increase in viable cell density in the uninfected cultures and, therefore, a possibly larger overall osmolarity decrease in the culture medium of uninfected cultures compared to infected cultures in which the cell division immediately ceases (e.g. both Sf-9 and Hi5 cultures infected at $\text{MOI} = 5$) or ceases 2–3 days post-infection (e.g. both Sf-9 and Hi5 cultures infected at $\text{MOI} = 0.5$ and $\text{MOI} = 1$). However, the authors found that during the first 5 days of a cell culture, the decline in osmolarity was similar for infected and uninfected cells (Rosinski et al. 2000). Since the change in osmolarity is similar for both infected and uninfected cell cultures, the measured differences in diameter found in infected cultures compared to uninfected cultures are not likely to be due to differences in osmolarity.

In addition, Rosinski et al. (2000) found that differences in osmolarity between fresh media (approximately 360 mOsm/kg) or PBS (approximately 290 mOsm/kg) versus the cell culture media in which cells were grown had an effect on the measured cell volume. The volume of cells suspended in fresh medium, which had a higher osmolarity than the culture medium in which the cells had been grown, was slightly underestimated when compared to the measured volume of cells suspended in the culture medium (Rosinski et al. 2000), whereas the volume of cells suspended in PBS, which had a lower osmolarity than culture medium, was overestimated. In

our experiments, cells in culture medium were diluted 1:1 with a 0.2% Trypan blue in PBS solution to determine viability. This may have led to an increase in measured cell size compared to actual cell size in the culture. However, all cells were handled identically and mixed in the same volume ratio with trypan blue/PBS solution. While there may have been a general overestimation of the cell volume due to exposure to the trypan blue/PBS dye mixture, the degree of overestimation would be the same for all samples. The relative differences in measured cell diameter between the samples, therefore, would still be valid.

Variability of results

As indicated above, other studies of the effect on insect cells infected with recombinant baculovirus with regard to cell size kinetics, viability and viable cell density gave different results than we report here. Jain et al. (1991) found a drop in viability and the change in modal cell diameter to be the most useful parameters for predicting the point of peak production of antistasin. Palomares et al. (2001) tested various medium components and found that the peak modal cell diameter more closely correlated to the point of peak production of the VP8 protein of rotavirus than the peak average cell diameter for all medium conditions tested. Both VP8 and antistasin are secreted proteins, and peak production was reached approximately 3–5 days after infection in both studies, which was similar to our results with regard to the time of peak EGFP production.

Cell size kinetics, either average cell diameter or modal cell diameter, did correlate to some extent with the time of peak protein production for VP8, antistasin and EGFP, which would indicate that changes in cell size during infection may be a useful parameter for predicting the time of peak production in the case of other secreted proteins. However, differences in results with regard to exactly which parameters were most useful for predicting the point of peak production, as well as how those parameters correlated to the time of peak protein production, would suggest that each unique combination of insect cell line and BEVS expressing a particular protein may give unique results with regard to cell size kinetics, change in viability over time, and change in viable cell density over time. It may be possible to find parameters that can be used to predict optimal time of

harvest and approximate yield for a particular BEVS/insect cell combination. However, it may not be possible to apply the results from one combination of BEVS/insect cell line more generally to other combinations of BEVS/insect cell lines.

Differences in results may be particularly large when comparing results from BEVS expressing labile proteins that are produced intracellularly to BEVS expressing secreted recombinant proteins that may be less sensitive to proteolysis. In this study, we used EGFP as a model protein since it has the advantage of being a very stable and easily quantifiable protein. This allowed for the comparison of EGFP production to changes in average cell diameter, viable cell density and viability until cultures were less than 10% viable without having to take into account the possible effects of proteolysis. The main limitation of using EGFP, however, is that it may not be a good model for BEVS expressing proteins that are produced intracellularly or sensitive to proteolysis.

Reproducibility of results and monitoring of repeat infections

Although patterns of change in cell size, viability and viable cell density over the course of an infection will most likely vary for different combinations of insect cell lines and BEVS, the pattern of change in those parameters may be consistent for all cell cultures infected with a particular BEVS as long as the same insect cell line is used and all other conditions are identical.

For example, we found that the results from cell size kinetics, viability and viable cell density were highly consistent between the different cultures with identical conditions in the case of both Sf-9 and Hi5 cells infected with EGFP-AcNPV baculovirus. In the case of separate Hi5 cell cultures with identical conditions the results from the different cultures were remarkably consistent given that each culture condition gave very different results with regard to the different parameters. The change in diameter over time, for example, followed nearly exactly the same pattern in different cultures with identical conditions (i.e. the same MOI used when infecting the cells), despite wide differences in cell size kinetics, time of peak EGFP production and total EGFP produced at each condition.

This suggests that it may be interesting to monitor a particular insect cell line/BEVS combination with

regard to cell size kinetics, as well as changes in viability and viable cell density, during the course of an infection. It may be possible to determine expected patterns in the change in these parameters over the course of the infection and apply those expectations to future infections run under identical culture conditions. A number of factors may have an effect on the course of infection for a particular combination, and thus on the change in average cell diameter, viability and viable cell density during the course of infection. Factors may include, for example, changes in the behavior of the insect cell line due to e.g. higher passage numbers, changes in the medium components or changes in the titer of the baculovirus stock.

Stability of baculovirus stock

Baculovirus stocks are commonly produced for the purpose of multiple rounds of protein production, whereby insect cells are infected repeatedly with baculovirus from a single batch of viral stock, or multiple batches of viral stock expressing the same protein. A baculovirus stock is, thus, often prepared in large batches and stored for future use. However, it is well-known that such stored stocks can be unstable over time with regard to infectivity. Jorio et al. (2006) for example, tested the stability of a baculovirus supernatant and found that the supernatant was only stable at 4 °C for about 100 days, after which titers decreased, most likely due to particles aggregating. The baculovirus stock was completely unstable at –20 °C and showed some initial loss of infectivity at –80 °C (Jorio et al. 2006). The relative instability of baculovirus supernatant at 4 °C is of some concern as this is a common method for baculovirus storage.

The determination of the actual pfu of a baculovirus stock each time it is used is time-consuming and laborious. A difference in actual pfu, and thus MOI used if protocols assume a stable pfu for a particular viral stock, could have a large effect on the time that the peak protein production is reached. Many laboratories commonly follow an established protocol based on the assumption that the baculovirus stock is stable with regard to infectivity. This could lead to a loss in amount of protein harvested if the assumption is incorrect. We found that the actual MOI used to infect cells did have an effect on cell size kinetics in the case of insect cells infected with baculovirus

expressing EGFP. In Hi5 cells, for example, peak EGFP production was reached at 3 days PI for cells infected at MOI = 1 and MOI = 5, but was not reached until 6 days PI when the MOI was decreased to 0.5 (Fig. 1B). If a standard protocol had specified harvesting the cells on day 3 PI based on an assumed MOI = 1, nearly half of the potential protein yield would have been lost (Fig. 1B).

Peak average cell diameter, however, could be used to pinpoint the time of peak EGFP production in all cases regardless of actual MOI used to infect the cells. Therefore, even if the MOI used to infect cells were essentially unknown due to instability of the baculovirus stock, it could still be possible to predict the point of peak EGFP production using the peak average cell diameter as an indicator and harvest the cells at the optimal time. Thus, determining the pfu of a baculovirus stock each time would not have been necessary for determining optimal harvest time.

Measuring the change in average cell diameter could, therefore, be conveniently used to control for changing quality of baculovirus stock. The cell size kinetics were very different between all of the MOIs used to infect Hi5 cells. MOI = 1 and MOI = 5, for example, gave very different results with regard to the pattern of change in average cell diameter over time even though the point of peak protein production was reached at the same time in both cases. Comparing a pattern of change in the average cell diameter over time to the expected pattern for a particular combination of insect cell line/MOI of a baculovirus stock may give an indication of whether or not the stock has lost some of its infectivity without the extra work involved in determining the new pfu.

Conclusion

After infecting Hi5 and Sf-9 cells at various MOIs with a baculovirus encoding EGFP and following the progress of the infection with regard to viability, cell density, average cell diameter and EGFP produced, we found a direct relationship between the time that the peak average cell diameter was reached and the time of peak EGFP production. Peak EGFP was reached 1–2 days after the peak average diameter had been reached. This relationship was found in both infected Hi5 and Sf-9 cell cultures, regardless of the MOI used to infect the cultures.

We infected different Sf-9 and Hi5 cell cultures at three different MOIs and found that the results were highly consistent between independent cell cultures with identical conditions despite the fact that the change over time in all three parameters differed depending on the MOI used to infect the cells. In the case of Hi5 insect cells infected with a BEVS expressing EGFP, the differences in all three parameters were large enough that it could be possible to track even small changes in MOI via the pattern of change in cell size throughout the course of an infection.

However, EGFP is very stable while many other recombinant proteins produced using an insect cell/baculovirus system are much more sensitive to proteolysis. For such proteins it is critical to harvest the cells and extract the protein at the point of peak production before proteolysis starts having an effect on the protein yield. The exact correlation between any of the three parameters and optimal harvest time is likely to be different from our results with an EGFP expressing baculovirus and unique for each specific combination of BEVS and insect cell line. For repeated infections, however, tracking changes in average cell diameter, viability and viable cell density over time may be an effective way to monitor the course of infection and predict optimal harvest time once the expected changes in those parameters have been established and the infection optimized.

The advantage of using cell size kinetics, as well as changes in viability and viable cell density over time, to monitor infection and determine the optimal harvest time is that determining these parameters during the progress of infection can be done quickly and easily.

Acknowledgements The authors would like to thank Per-Erik Strömstedt and Philippe Cronet for their unfailing support of this project; Catherine Heddle for very kindly providing the recombinant EGFP-AcNPV baculovirus; and Thomas Becker for critically reading the manuscript.

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