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The indirect effects of multiplicity of infection on baculovirus expressed proteins in insect cells: secreted and non-secreted products

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Abstract

The baculovirus expression vector system was employed to produce human apolipoprotein E and β-galactosidase in order to study the effect of multiplicity of infection on secreted and non-secreted recombinant protein production. Prior knowledge of the influence of other cell culture and infection parameters, such as the cell density at time of infection and the time of harvest, allowed determination of the direct and indirect influences of multiplicity of infection on recombinant protein synthesis and degradation in insect cells. Under non-limited, controlled conditions, the direct effect of multiplicity of infection (10^{-1}–10 pfu/cell) on specific recombinant product yields of non-secreted β-galactosidase was found to be insignificant. Instead, the observed increased in accumulated product was directly correlated to the total number of infected cells during the production period and therefore ultimately dependent on an adequate supply of nutrients. Only the timing of recombinant virus and protein production was influenced by, and dependent on the multiplicity of infection. Evidence is presented in this study that indicates the extremely limited predictability of post-infection cell growth at very low multiplicities of infection of less than 0.1 pfu/cell. Due to the inaccuracy of the current virus quantification techniques, combined with the sensitivity of post-infection cell growth at low MOI, the possibility of excessive post-infection cell growth and subsequent nutrient limitation was found to be significantly increased. Finally, as an example, the degree of product stability and cellular and viral protein contamination at low multiplicity of infection is investigated for a secreted recombinant form of human apolipoprotein E. Comparison of human apolipoprotein E production and secretion at multiplicities of infection of 10^{-4}–10 pfu/cell revealed increased product degradation and contamination with intracellular proteins at low multiplicities of infection.

Abbreviations: AcNPV – *Autographa californica* nuclear polyhedrosis virus; APOE4 – human apolipoprotein E4; HPI – hours post-infection; MOI – multiplicity of infection; TOI – cell density at time of infection; TOH – time of harvest

Introduction

The wide variation of recombinant product yields reported for the baculovirus expression vector system has been attributed to variables at the molecular genetics, cloning, cell culture or infection level. In particular infection variables are most readily manipulated to maximise production yields. These include: MOI, TOI and TOH. The apparent effect of MOI has previously been documented (Brown and Faulkner, 1975; Dougherty *et al*., 1981; Maiorella *et al*., 1988; Murhammer and Goochee, 1988; Schopf *et al*., 1990;

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Licari and Bailey, 1991, 1992; Lazarte *et al.*, 1992). Other sources of variation, however, during both cell growth and viral infection phases may be responsible for the range of reported yields. The inherent inability to estimate the true MOI, protein instability, or more frequently variation in other cell culture or infection parameters, such as oxygenation or the TOI may be underlying. It still remains unclear what the effect of varying this infection parameter alone has on potential product yields.

Recently protocols at very low MOI's have been suggested for efficient production of both recombinant virus and protein (Peter *et al.*, 1995). The study was based on an intracellular product, β-galactosidase which appeared relatively stable at the various MOI's tested. It is of interest to determine the effect of low MOI protocols on secreted products or potentially unstable proteins. The effect of MOI on process times and down-stream processing also needs to be established for optimisation of this infection parameter for efficient production of recombinant products. The following study determines the effect of MOI in *Sf9* suspension cultures infected in SF900II expressing intracellular β-galactosidase (0.1–10 pfu/cell) and secreted APOE4 (0.0001–10 pfu/cell) under strictly non-limited conditions. Additionally the use of low multiplicity of infection, which is often necessary for large scale production where limited virus stock is available, is assessed in terms of expression, degradation and contamination by intracellular proteins.

Materials and methods

Cell line, culture conditions and virus inoculum

A one litre bioreactor (Setric, France) was inoculated with exponentially growing Sf9 insect cells (*Spodoptera frugiperda*, ATCC no. CRL-1711) at approximately 5×10^5 cells/ml and allowed to grow to approximately 4×10^6 cells/ml in SF900II (Gibco, USA). At this density these cells have previously been shown not to be limited for recombinant product expression (Radford *et al.*, 1992). Culture aliquots of 150 ml volumes were removed from the parent bioreactor directly into 250 ml capacity glass Erlenmeyer shaker flasks (Schott). The cultures were centrifuged at 1000x g for 5 min and resuspended in fresh medium, pooled and re-aliquoted as 150 ml cultures into the original shaker flasks. Maximum shaker volumes of 150 ml were chosen and rotated at 170 rpm in the 250 ml capacity shaker flasks to ensure adequate oxygen supply in the non-sparged environment (under these conditions we have not observed significant differences between post-infection growth and recombinant product parameters obtained in shaker flasks and bioreactor cultures infected at the same MOI). For determination of the effect of MOI on the post-infection growth and intracellular protein production, each culture was infected with recombinant baculovirus (β-galAcNPV: Amrad, Australia) at MOI's ranging from 0.1 to 10 pfu/cell. All cultures were sampled throughout the infection period for determination of cell density, viability and recombinant virus and protein production.

For determination of the effect of MOI on a secreted, unstable product we chose to employ recombinant virus encoding APOE4. The AcNPV recombinant virus, constructed in our Institute (Bernard Allet, Glaxo Imb., Geneva, Switzerland) contained genes encoding for the entire APOE4 protein under control of the very late polyhedrin promoter. APOE4 has been implicated in cardiovascular disorders and is a suggested indicator of Alzheimer's disease. To determine the effect of MOI on the production, degradation and contamination of secreted protein, 150 ml shaker flasks of the *Sf9* cells grown in SF900II were infected with recombinant APOE4-AcNPV at MOI's from 0.0001–10 pfu/cell. Again all cultures were sampled throughout the infection period for determination of cell density, viability and recombinant protein production.

Cell density and viability estimations

For all cultures cell densities were determined via triplicate haemocytometer counts employing a statistically valid protocol (Nielsen *et al.*, 1991) and culture viabilities were calculated via the 0.1% trypan blue exclusion method.

Virus titration

An adaptation of the endpoint dilution titration assay was used for recombinant virus quantification, where each virus titre represents an estimate from 60 to 100 repeats (Reed and Meunch, 1938; Nielsen *et al.*, 1992). In these assays virus endpoints were determined by detection of β-galactosidase activity upon the addition of the substrate, Xgal.

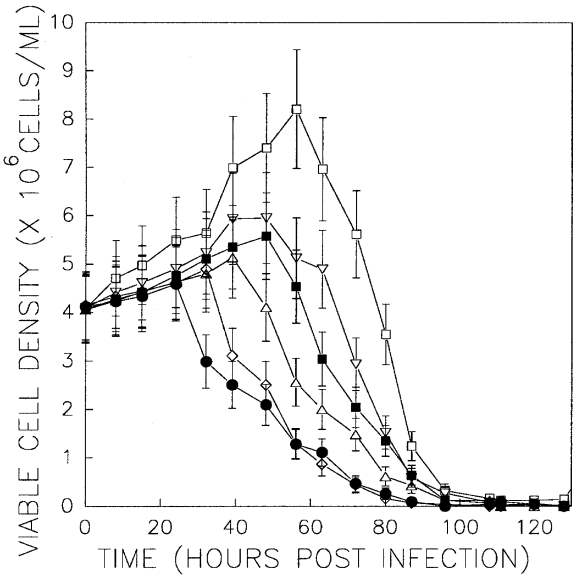

Figure 1. The effect of MOI on post-infection viable cell densities of Sf9 cultures in SF900II medium. (□) 0.1, (▽) 0.5, (■) 1, (△) 2, (◊) 5, (●) 10 pfu/cell.

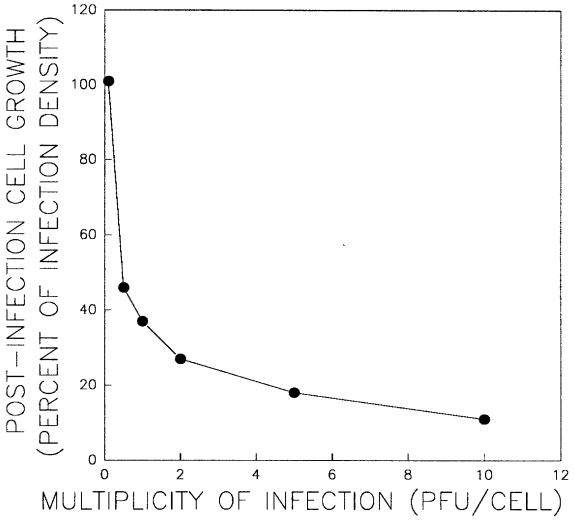

Figure 2. Relationship between MOI and proportion of maximum post-infection cell growth in recombinant β-gal-AcNPV infected SF9 cultures in SF900II. Maximum viable cell density achieved is given as a percentage of the original cell density at infection. That is for example, 100% cell growth indicates that the cell density doubled following infection.

β-galactosidase activity

Recombinant β-galactosidase activity of each culture broth was monitored throughout the infection period via the protocol of Miller (1972). These β-galactosidase estimations were based on the total amount of enzyme activity including that released from cells by hypo-osmotic lysis.

Gel electrophoresis and western blots

The secreted APOE and its degradation products in culture supernatants were visualised by enhanced chemiluminescence western blotting (Amersham, United Kingdom) using a murine monoclonal anti-human apolipoprotein antibody (Synbio, The Netherlands) coupled to horse radish peroxidase conjugated anti-murine immunoglobulin (Kirkegard & Perry Laboratories Inc., USA). Coomassie staining was used to visualise contaminating proteins released during the infection process.

Results and discussion

Direct effect of MOI on cell growth

MOI was shown to directly effect cell growth in an inverse manner. Figure 1 shows the post-infection growth trajectories of baculovirus infected Sf9 shaker suspension cultures at 0.1, 0.5, 1, 2, 5 and 10 pfu/cell. At low MOI a high maximum viable cell density was achieved and conversely at high MOI growth was arrested quickly resulting in lower maximum viable cell density. Post-infection cell growth was the result of asynchronous infection. Cessation of cell growth was brought about by eventual complete infection of the entire cell population. The onset of the cell lysis phase was delayed and the rate of cell lysis was decreased in cultures infected at the lowest MOI's.

At the lowest MOI tested, 0.1 pfu/cell, the culture continued to grow for 60 hours after infection. During this period the apparent post-infection growth rate was only slightly lower than the apparent uninfected growth rate (results not shown). This period of largely unaffected cell growth corresponded to the time required for inoculum virus adsorption, infection and initiation of the first round of progeny virus release. Uninfected cells during this time period continued to grow presumably at a normal rate, and the slight decrease in

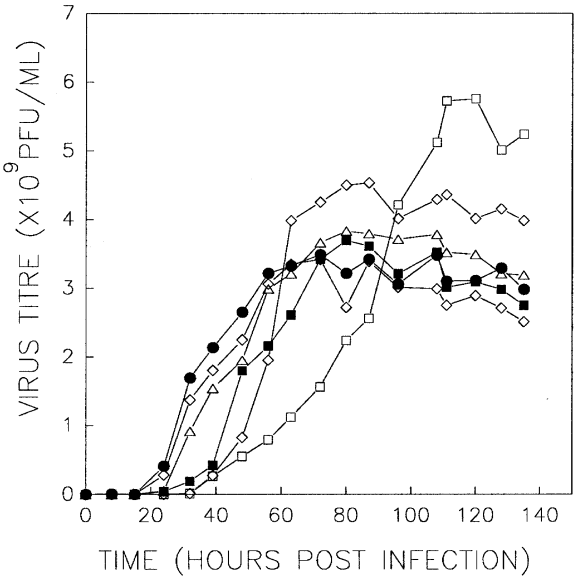

Figure 3. Effect of MOI on virus secretion in recombinant β-gal-AcNPV infected SF9 cultures in SF900II medium. (□) 0.1, (▽) 0.5, (■) 1, (△) 2, (◊) 5, (●) 10 pfu/cell.

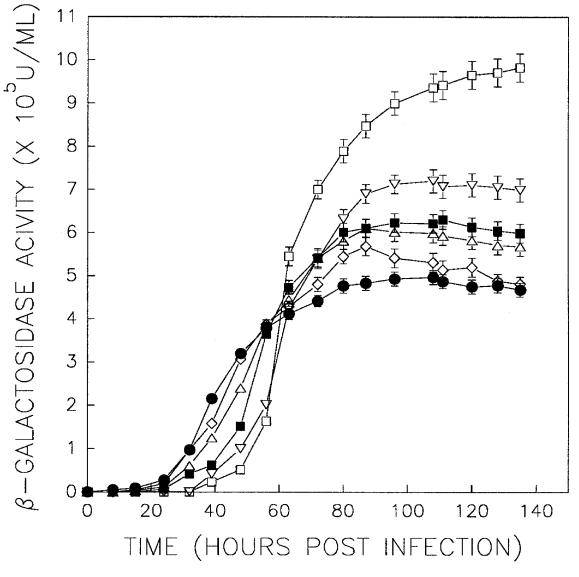

Figure 4. Effect of MOI on β-galactosidase expression in recombinant β-gal-AcNPV infected SF9 cultures in SF900II medium. (□) 0.1, (▽) 0.5, (■) 1, (△) 2, (◊) 5, (●) 10 pfu/cell.

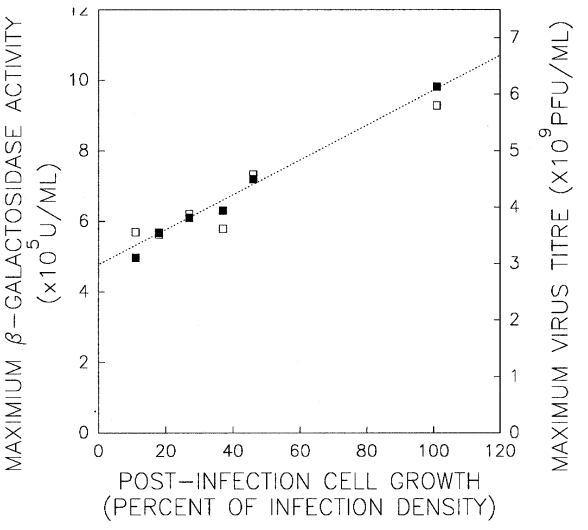

Figure 5. The relationship between the amount of post-infection cell growth and the maximum volumetric extracellular virus (□) and β-gal (■) yields achieved at decreasing MOI in recombinant β-gal-AcNPV infected SF9 cultures in SF900II medium.

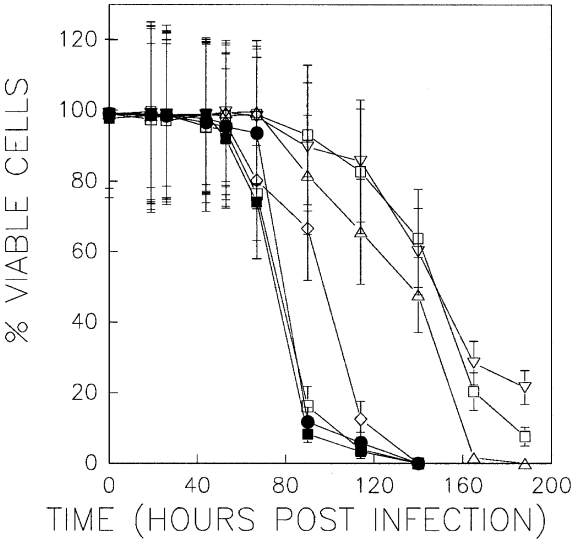

Figure 6. Degree of cell lysis in SF9 cultures infected at 10 (□), 2 (■), 1 (●), 0.1 (◊), 0.01 (△), 0.001 (○) and 0.0001 (▽) pfu/cell with recombinant baculovirus expressing APOE4 protein.

the overall population growth rate was only attributed to those cells already infected that were incapable of replication. Subsequent cell growth within the culture population was arrested after secondary adsorption and infection of the previously non-infected cell population. This occurred due to the release of infective progeny virus released from the cells successfully infected in the initial round of virus replication.

At the highest MOI, 10 pfu/cell, a smaller proportion of the cell population remained uninfected after initial adsorption and infection of the culture with inoculum virus. Consequently, the cell lysis rate was

higher because a greater proportion of the cells were lysing over the same time period.

Predictability of post-infection cell growth at low MOI

Although prediction of post-infection cell growth and subsequent product yields is possible theoretically by a carefully chosen combination of TOI and MOI (Power *et al.*, 1994, Peter *et al.*, 1995), translation to practical application remains problematic. The practical importance of utilising MOI's approaching unity or above is expressed by the plot of the percentage of post-infection growth against the MOI (Figure 2). Due to the exponential nature of growing cells combined with the inherent inaccuracy of current virus quantification techniques, cultures infected at low MOI are sensitive to unwanted deviation from predicted final cell yields.

Slight underestimation of the true low MOI leads to excessive cell growth, and increases the risk of substrate limitation. For example, due to the exponential nature of growing cells, a culture infected at a MOI of 0.05 pfu/cell instead of 0.1 pfu/cell would result in more than 100% post-infection cell growth (Figure 2). If the culture was originally infected at 4×10^6 cell/ml it would attain a post-infection cell density of beyond 8×10^6cell/ml (Figure 1). Since a finite amount of nutrients are available in the batch culture environment, increasing the proportion available to cell growth will decrease the proportion available for recombinant virus and protein production (Radford, 1994; Radford *et al.*, 1995).

Conversely, slight overestimation of the true low MOI leads to sub-optimal post-infection cell growth, and therefore decreased predicted volumetric yield. It remains far more predictable, and therefore reproducible to arrest post-infection cell growth as soon as possible so that the final cell yield does not exceed 8×10^6 cell/ml. This approximates the final post-infection density that allows maximum potential recombinant virus and protein production in SF900II medium before substrate limitation occurs. Therefore it would be necessary to infect before 5×10^6 cell/ml, if a true MOI of at least 1 pfu/cell was employed for Sf9 cells cultured in SF900II. Similar values can be determined experimentally for other media.

Indirect effect of MOI on recombinant product

Both recombinant virus titres and protein yields were apparently increased at lower MOI. Figures 3 and 4 show the volumetric production of extracellular virus and β-galactosidase respectively in Sf9 cultures infected at various MOI's. The specific productive capacity of both virus and protein, however, were not found to be dependent on the MOI (Figure 5). When each culture was monitored for a sufficient period of time the same specific productive capacity could be attributed to each culture (Table 1). This may explain why some early investigators have reported significant effects associated with MOI. Recombinant product expression was found to be dependent only on the amount of post-infection growth experienced by the cultures infected at various MOI's (Figure 5). Both recombinant virus and β-galactosidase accumulated yields correlate directly to the percentage of post-infection growth. This was due to the fact that lower MOI cultures eventually consisted of higher numbers of infected cells.

Delayed production kinetics

In the culture infected at a MOI of 0.1 pfu/cell significant β-galactosidase production could not be detected until after 36 hours post-infection, possibly after secondary infection of the culture had occurred (Figure 4). The production continued for about 99 hours. The virus release period was also extended, from about 20 to 110 hours post-infection (Figure 3). In contrast, the culture infected at 10 pfu/cell indicated more discrete infection and production trajectories. Significant β-galactosidase expression began earlier at 20 hours and accumulated for a shorter time, 64 hours. Similarly the virus production period was shorter beginning at 20 and ending at 68 hours post-infection. Thus the apparent volumetric production improvements observed at low MOI are at the expense of production time. The improved production yields obtained are simply the result of infection of a higher number of cells. Thus, by increasing the TOI to the critical maximal post-infection cell density attained at low MOI and infecting at an MOI above unity, similar production yields are achieved after a shorter production period.

Secreted product degradation and contamination by intracellular protein

Consideration of the effect of MOI on a secreted product such as APOE4 was used to determine the significance of this infection parameter for production of secreted, membrane associated and/ or non-stable intracellular recombinant proteins. Figure 6 indicated that the lysis kinetics of Sf9 cultures infected with APOE4-AcNPV at various MOI's was not significant-

Table 1. Effect of multiplicity of infection on recombinant product yields in SF9 cultures in SF900II

Multiplicity of infection (pfu/cell)	Volumetric virus titre (10^9 pfu/ml)[a]	Specific virus titre (pfu/cell)	Volumetric β-galactosidase (10^5 U/ml)[a]	Specific β-galactosidase (10^5 U/10^6cells)[a]
0.1	5.8 (4.6–7.3)	707 ± 150	9.8 ± 0.3	1.2 ± 0.2
0.5	4.6 (3.6–5.8)	770 ± 168	7.2 ± 0.3	1.2 ± 0.2
1	3.6 (2.9–4.5)	648 ± 139	6.3 ± 0.2	1.1 ± 0.2
2	3.9 (3.1–4.8)	754 ± 162	6.1 ± 0.2	1.2 ± 0.2
5	3.5 (2.6–4.7)	719 ± 160	5.7 ± 0.2	1.2 ± 0.2
10	3.6 (2.8–4.7)	776 ± 174	5.0 ± 0.2	1.1 ± 0.2

[a] Skewed errors associated with virus titre estimation are calculated as reported by Nielsen *et al.* (1992). Errors associated with β-galactosidase activity represent 95% confidence intervals based on three separate estimations.

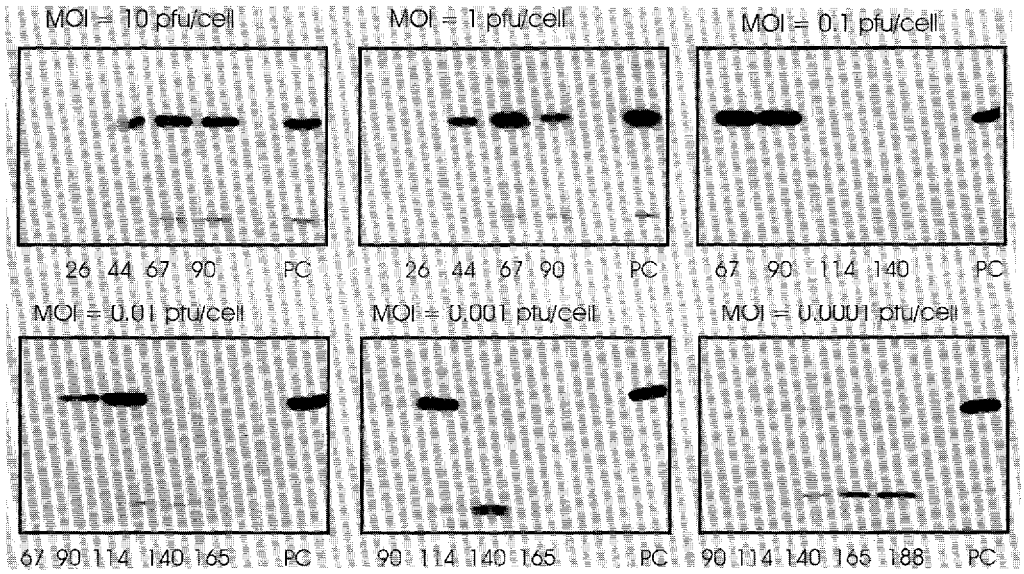

Figure 7. Production of APOE4 protein and antibody positive degradation products in SF9 insect cells grown and infected at multiplicities of infection of 0.0001 to 10 pfu/cell in SF900II medium visualised by western blot analysis. Gels were loaded with samples taken at increasing times post-infection.

ly different to that observed for the virus encoding β-galactosidase. Post-infection cell growth was increased as MOI decreased, as was the dispersion of virally induced cell lysis. The increased dispersion of cell lysis at low MOI increased the time of product exposure to cell debris and intracellular proteins, including proteases. Western blot analysis of samples taken throughout the infection period at various MOI's indicated at higher MOI's (1–10 pfu/cell) the product is expressed sooner and the maximal yield is maintained for a longer period of time without significant degradation (Figure 7). At lower MOI it was observed that the optimisation of time of harvest becomes very difficult, as the trade off of maximal protein expression for minimal product degradation becomes a significant issue. For the very low MOI of 0.0001 pfu/cell it appears that the protein is degraded almost instantaneously with no evidence of the full length APOE4. Infection regimes employing higher MOI of infection appear to prevent significant overlap of the time at which maximum product expression occurs and the onset of significant degradation, allowing a wider window for prediction of optimal TOH. Observations of reduced protein stability associated with low MOI have been reported previously in the literature. Lazarte *et al.* (1992) concluded that a very high MOI of 580 pfu/cell led to an optimised protocol for the production of CD4, membrane associated human glycoprotein, in a 6-l air-

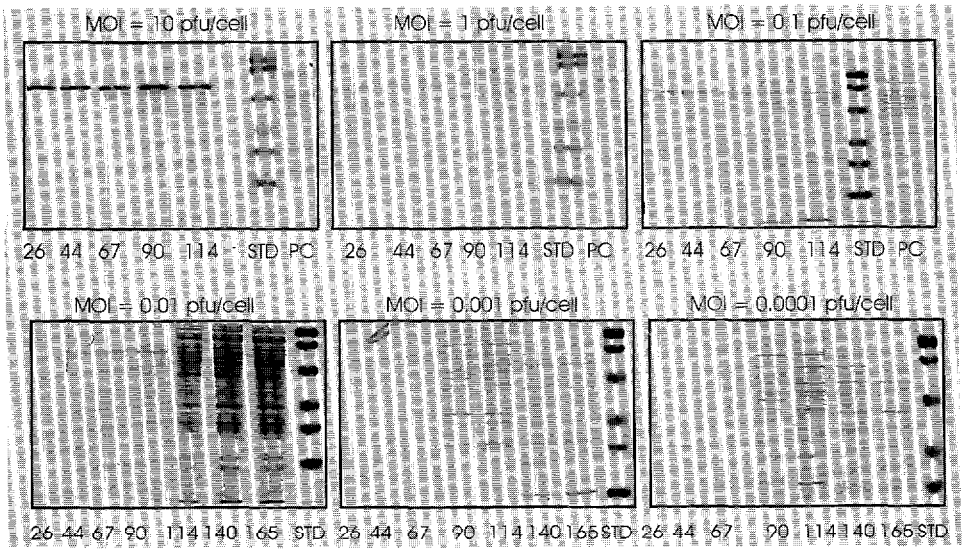

Figure 8. Coomassie staining of contaminating proteins released after infection of SF9 insect cells with recombinant APOE4-AcNPV baculovirus at multiplicities of infection of 0.0001 to 10 pfu/cell in SF900II medium. Gels were loaded with samples taken at increasing times post-infection.

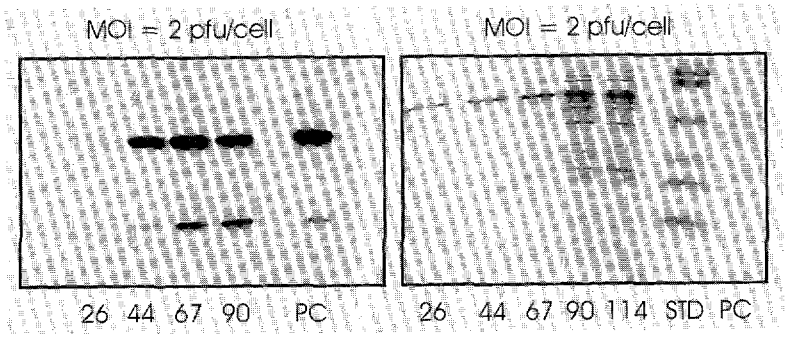

Figure 9. Western blot analysis of APOE4 protein and Coomassie staining of contaminating proteins in an *Sf9* culture infected at 1.5×10^6cell/ml at a MOI of 2 pfu/cell.

lift fermenter culture. In this case the normal renewal of cell membrane may have led to suboptimal yields at low MOI, a problem that was easily reversed by decreasing the production time by using a very high MOI. Such high MOI values however are not normally recommended due to the addition of exhausted medium in the virus inoculum.

Apart from protease degradation or loss of protein via membrane renewal, the release of unwanted host cell or virus induced intracellular proteins are a separate issue and can only serve to further complicate downstream processing. Secreted human proteins such as APOE4 may only be produced as a few percent of the total baculovirus expressed proteins. Coomassie staining of APOE4 expressed over the infection period at various MOI's clearly demonstrates the increased release of intracellular proteins occurred when cultures were infected at decreased MOI (Figure 8). Therefore the difficulties of purification of a product produced at very low MOI are exacerbated not only due to immunoreactive degradation products but also due to increased proportion of other non related contaminating proteins. Furthermore even in the case of non-secreted stable products, contaminating proteins remain an issue for low MOI infection protocols.

Investigation of degradation and contamination by intracellular proteins at increased density

Results reported thus far in this work, indicate that low density (5×10^5cell/ml) combined with MOI of 1 or 10 pfu/cell increase the duration of expressed product stability concurrent with decreased release of contaminant intracellular proteins. Increased infection

cell density, however, is necessary to permit maximal product yield in this range of MOI. To ensure that increased cell density at time of infection did not simply increase the amount of contaminating proteins by virtue of cell number alone, expression and protein production was investigated in a culture infected at MOI of 2 pfu/cell at a density of 1.5×10^6cell/ml with recombinant AcNPV-APOE4 virus. The final cell density achieved by the culture was 3.7×10^6cell/ml and therefore could be compared to the culture infected at MOI of 0.001 pfu/cell at 5×10^5cell/ml, that grew to a final cell density around 4×10^6cell/ml (Figure 9). Western blot analysis revealed that protein stability was maintained for a longer period of time when the high infection density, high multiplicity protocol was employed compared to the low infection density, low MOI protocol. Possible harvest time was extended from 44 to 90 hpi at high MOI and the expressed APOE4 did not significantly degrade during this time, compared to the low MOI equivalent where APOE4 was mainly produced at 114 HPI and degraded within 26 hours.

Conclusions

Our results indicated that MOI's greater than unity should always be used to maximise final process yield. Low MOI strategies can only be employed in situations where sufficient virus inoculum volume is unavailable, and strictly in cases where product stability has been established. Regardless of the product stability it is impossible to produce larger amounts of protein and virus per cell by utilising low MOI. We have demonstrated that increased volumetric yield associated with decreased MOI is directly correlated to the degree of post-infection cell growth. The same volumetric yields can be obtained utilising high MOI more predictably, reproducibly and rapidly by simply adjusting the TOI.

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