When Less is More: Enhanced Baculovirus Production of Recombi- nant Proteins at Very Low Multiplicities of Infection

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Baculovirus expression systems for recombinant proteins have found widespread use because they produce biologically active proteins more reliably than do bacterial or yeast expression systems, and the yields generally exceed those from mammalian expression systems. However, optimization of baculovirus yields remains an important issue, especially when biophysical studies are contemplated. Recently we sought to produce the recombinant cysteine proteases, cathepsins B and S (1,4,16), from baculovirus for structural studies. Here, we report that production of these recombinant cathepsins reached the high levels we needed for this objective when the cultures were inoculated at multiplicities of infection (MOIs) several orders of magnitude lower than those conventionally recommended.

For baculoviral expression of preprocathepsins B and S, the respective cDNAs were cloned from human liver cDNA by reverse transcription polymerase chain reaction (RT-PCR) using primers designed according to published nucleotide sequences (4,16). The cDNA encoding the 30-kDa fragment of fibronectin (FN-30) (5,8) was obtained by RT-PCR of total RNA from human dermal fibroblasts, again using primers corresponding to the published cDNA sequence. These cDNA inserts, which each contained endogeneous signal sequences for secretion, were separately subcloned into the pFASTBAC™ 1 donor plasmid (Life Technologies, Gaithersburg, MD, USA). A bacterial strain (DH10B) containing a vendor-supplied baculovirus shuttle vector (bacmid; Life Technologies) was transformed with recombinant pFASTBAC 1 plasmid, and colonies containing recombinant bacmids were grown for isolation of the resulting viral DNA. Transfections of Sf9 Insect Cells (PharMingen, San Diego, CA, USA) in

Table 1. Time Course for Cell Viability

MOI	Time (h)				
	48	72	96	120	144
No. of Viable Cells					
No virus	3.9	6.0	9.0	7.2	6.0
0.00003	4.4	5.4	5.5	4.2	1.0
0.0003	3.3	4.6	3.9	2.2	0.7
0.003	2.9	2.8	2.3	0.9	<0.1
0.03	1.8	1.8	1.0	0.5	<0.1
0.3	1.6	0.8	0.4	<0.1	<0.1
Irrelev BV	1.1	0.8	0.6	0.3	<0.1

Cell density readings (expressed as 1×10^6 viable cells/mL culture) were taken at defined time intervals after inoculation of Sf9 cells with baculovirus expressing preprocathepsin S (Figure 2), cells were counted using a hemacytometer.

Table 2. Cathepsin S Activity in Media Expressed as a Ratio to Cell Number

MOI	Time (h)				
	48	72	96	120	144
mU Activity/ 1×10^6 cells					
No virus	0	0	0	1	2
0.00003	0	0	5	27	238
0.0003	0	2	19	48	202
0.003	0	12	28	89	*
0.03	2	16	37	101	*
0.3	8	21	54	*	*
Irrelev BV	0	0	0	1	*

Values were divided by the number of viable cells (1×10^6) in corresponding media samples (see Table 1 and Figure 2). Asterisks indicate cases where cell density was too low to be counted reliably by hemacytometer (i.e., $<0.1 \times 10^6$ cells/mL).

log-phase growth were performed using CELLFECTIN™ Reagent (Life Technologies). Supernatants were used to infect Sf9 cells through two or three stages of amplification, initially in monolayer cultures, then at the final stage in shaker flasks. High viral titers (ca. $1-3 \times 10^8$) were obtained after final amplification, as indicated by plaque assays and end-point dilution assays. At all times during amplification and subsequent culture, insect cells were maintained at 27°–28°C.

Following inoculation of 1×10^6 Sf9

cells/mL in Grace's insect cell media containing 10% fetal bovine serum (FBS) (the presence of which facilitated initial infection), 0.1% pluronic acid, antibiotics and amphotericin (Life Technologies), cells were grown in shaker culture at 125 or 250 mL/flask. At 24 h after inoculation, cells were centrifuged, resuspended in Sf-900II Serum-Free Media (Life Technologies) supplemented with 1 mL of 10% pluronic acid per 100 mL media (which we had found to increase yields approximately twofold) and cultured as before. This transfer to

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serum-free media was essential because the presence of serum was found to suppress the subsequent production of active recombinant cathepsins B and S, and to interfere with immunodetection of recombinant FN-30. Aliquots (12–15 mL) were taken from the cultures at approximately 48, 72, 96, 120 and, in some cases, 144 h postinoculation, viable cell counts were determined by trypan blue exclusion, and the remainder of the aliquots were centrifuged at approximately 1000× *g* to separate cell pellets from media before storage at -70°C.

Recommended and commonly used MOI values for optimizing recombinant protein production, including secreted proteins, are usually in the range of 1–10 infectious viral units per cell (6,10,12,13), although MOI values as low as 0.005 have been reported to be effective in producing β -galactosidase (β -gal) or chloramphenicol acetyltransferase (CAT) as model proteins (9,14,15,17). As previously reported for β -gal (9) and cathepsin D (7), longer incubation times up to 120 h yielded greater amounts of cathepsin B in the culture medium. We noted that at this time interval, the amount of cathepsin B activity was greater at a MOI of 0.03 than at 0.3 or 3.0. In a subsequent series of experiments, optimal yields of cathepsin B in media were found to result from an MOI of 0.003 when cultures were harvested 120 h postinoculation (Figure 1).

Figure 1. Relative activity of cathepsin B in samples of media from cultures inoculated with baculovirus at different MOI. Samples were taken at 48, 72, 96 and 120 h after inoculation, centrifuged to remove cells and debris and stored at -70°C. Before the assay, samples from preprocathepsin B culture media were passed through PD-10 columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA), then stored overnight at 4°C in sodium acetate, pH 5.0, to activate cathepsin B. Assays were performed by combining enzyme with the substrate, Z-Arg-Arg-AMC (Peptides International, Louisville, KY, USA) in buffer [88 mM KH₂PO₄, 12 mM HPO₄, 1.3 mM EDTA, 2.7 mM dithiothreitol (DTT), 0.03% Brij®-35 (Sigma Chemical, St. Louis, MO, USA), pH 5.8] and measuring the resulting emission spectrofluorometrically (1). Samples from uninoculated Sf9 cell cultures and from cultures infected by baculovirus expressing a different recombinant protein were subjected to identical assay conditions. Activities were expressed as mU per mL of clarified culture media, where 1 U of activity was defined as one μ mol of product per min under standard assay conditions.

Figure 2. Relative activity of cathepsin S in samples of media and cell pellets. The cultures sampled were inoculated with baculovirus at different MOI. Samples were taken at 48, 72, 96, 120 and 144 h after inoculation. Procedures were similar to those for cathepsin B (Figure 1) except that (i) activation of cathepsin S was achieved by buffer-exchanging the culture media to 100 mM sodium acetate, pH 4.5 (containing 5 mM EDTA and 5 mM DTT), and (ii) samples were incubated at 37°C for 2.5 h. For cathepsin S activity assays (2), the buffer was the same as for cathepsin B, but the substrate used was Z-Val-Val-Arg-AMC (Bachem, Bubendorf, Switzerland).

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Cathepsin S activity was determined under similar conditions in both pellets and media, and the optimal MOI for producing active recombinant cathepsin S in both pellets and media was found to be ≤ 0.0003 (Figure 2). This MOI value was lower than that for cathepsin B by approximately two orders of magnitude. Media and pellet yields were comparable through 120 h of culture, but at 144 h, activity in media greatly exceeded that in pellets, coinciding with observed, near-complete cell lysis at this time point (Table 1). In a previous report documenting baculoviral production of cathepsin S (2), most of the enzyme activity was found to be in the pellet following inoculation at a conventional MOI of 1.5 and harvest at approximately 84 h of culture. The present results (Figure 2) do not necessarily contradict this observation, but they show that both the MOI and the harvest time point greatly influence the ratio of yields between pellets and media. The expression of β -gal has also been reported to peak earlier in cell pellets than in culture medium (9,14,17).

It has been shown elsewhere that cell lysates from both uninfected and baculovirus-infected Sf9 cells contain endogenous cathepsin-like activities (3). The use of culture media in the present experiments as the source of recombinant enzyme would be expected to minimize the presence of these endogenous cellular proteins in the experimental samples. Additionally, to control for this possibility, cultures containing either uninoculated Sf9 cells or Sf9 cells inoculated with baculovirus expressing a non-protease protein were incubated side by side with test cultures and assayed for cathepsin B or cathepsin S in an identical manner. For both cathepsin B (data not shown) and cathepsin S (Figure 2), enzyme activity in these control cultures was negligible at all harvest time points. Furthermore, the identity of the recombinant cathepsin B produced by this method was verified by enzyme kinetic and dynamic light-scattering studies (11), and confirmatory N-amino terminal sequencing was performed on both recombinant cathepsin samples (data not shown).

Because these lysosomal thiol proteases are highly proteolytic enzymes, the possibility was considered that the

atypical MOI relationships observed might result from either some complex interaction between enzyme-induced proteolysis and baculoviral titer or viability of infected Sf9 cells. If this were the case, a conventional MOI optimum might be expected from the expression of a different class of protein. Accordingly, the optimal MOIs at different time points were determined for the baculoviral expression of secreted FN-30, a fragment of the extracellular matrix constituent, fibronectin. Optimal yields were again observed at 96–120 h of incubation and at a very low MOI (<0.001 ; Figure 3). No detectable immunostaining was produced by uninoculated Sf9 cultures or cultures inoculated with baculovirus expressing a different recombinant protein (data not shown). These results, together with several previous reports of low MOIs used for selected model proteins (9,15,17), suggest that the observed low MOI optima are not merely an idiosyncratic as-

pect of thiol protease expression.

Cell viability declined more slowly at low rather than at high MOI values, as previously reported (9), but infection (swelling, reduced cell count, absence of cell division) was still detectable at MOI values as low as 0.0003. The time course for cell viability shown in Table 1 after infection with preprocathepsin S baculoviral stock was representative of all three secreted baculoviral expression systems studied. The amount of detectable active enzyme in culture media, when expressed as a ratio to the number of viable cells in the same culture sample (Table 2), increased continuously during culture even after cell density had begun to decline.

Wickham et al. (15) have described conditions under which the production of β -gal declines at higher MOI conditions. They have concluded that this decline occurs because defective interfering viral particles accumulate in high-passages cultures and inhibit the

Figure 3. Relative immunostaining of FN-30 in Western blots as quantitated by densitometry. Samples were taken as described in the Figure 1 legend. Media from cultures expressing FN-30 were concentrated, protein content was assayed, and samples that had been adjusted to contain equivalent total protein were electrophoresed in a 14% acrylamide Tris-Glycine gel, then transferred to nitrocellulose membranes (Novex, San Diego, CA, USA). Membranes were incubated with Anti-fibronectin Monoclonal Antibody, O5-138 (Upstate Biotechnology, Lake Placid, NY, USA), followed by horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (Calbiochem-Novabiochem, La Jolla, CA, USA) and were visualized using TMB Stabilized Substrate for HRP (Promega, Madison, WI, USA). Using Sigma-Gel™ Gel Analysis Software (SPSS, Chicago, IL, USA), immunostaining was converted to arbitrary, linearly scaled units by reference to a positive-control sample of FN-30, 25 μ g per lane (Sigma Chemical) as a standard, and a negative-control sample was derived from the lane containing the corresponding lysate from uninoculated Sf9 cells, which showed undetectable immunostaining.

replication of standard virus particles. The present viral stocks were produced according to standard protocols and were not considered to be high-passage stocks. Nonetheless, in additional experiments, a single-passage viral stock was used that had been obtained by inoculating cells with transfection media. Sf9 cells were inoculated at MOI values of approximately 0.2, 0.002 and 0.00002. Cultures inoculated with this single-passage stock showed more production of cathepsin S at an MOI of 0.002 than at 0.2 plaque-forming units (pfu) per cell, and production of cathepsin S peaked at later time intervals as previously observed (data not shown). Thus, the MOI relationship described could also occur in cultures that are inoculated with conventionally derived, low-passage baculoviral stocks.

Although these findings do not preclude the possible presence of defective interfering particles in the viral stocks tested, alternative interpretations of the inverted MOI curve are possible. At low MOI values, when fewer viral particles are present per cell, only a small fraction of the cells in culture are infected immediately. Under these conditions, infection appears to propagate primarily by transmission from infected to uninfected cell (9). Low MOI values permit infected cells to remain intact for a longer period of time and to proliferate to higher densities (cf. Table 1). In the present experiments, maximal recombinant cathepsin S activity was detected at time points when cell counts were declining from initially high values. Several factors could contribute to the observed high levels of recombinant protein production at low MOIs and later time intervals: (i) low MOIs permit more infected cells to accumulate in culture before lysis takes place (14); (ii) rates of intracellular degradation of recombinant protein could decrease later in infection, as shown for β -gal (9); (iii) the increased cell survival time in culture after inoculation could permit intracellular accumulation of more recombinant protein and release of greater amounts into the media before lysis takes place.

In conclusion, the present results demonstrate optimal production of three different recombinant proteins at baculoviral MOI values of 0.003 or

lower. To our knowledge, the MOI optimum observed for one of these, namely cathepsin S, was lower by at least two orders of magnitude than any other MOI optima previously reported. From a practical standpoint, the identification of low MOI optima greatly reduces the volume of baculoviral stock required to produce recombinant proteins (9,14). For example, high yields of cathepsin S could be obtained by inoculating 125 mL of media with 20 μ L of a 1:10 or 1:100 dilution of preprocathepsin S baculoviral stock having a titer of approximately 2×10^8 pfu per mL. Under these conditions, baculoviral density in culture immediately after inoculation can be calculated to be in the range of 10^2 – 10^3 pfu/mL of culture volume.

Regardless of the possible underlying mechanism(s), this complex relationship among MOI, harvest time and recombinant protein yield might characterize the baculoviral production of other recombinant proteins. Although low MOI values would not necessarily be expected to improve the yields of all proteins, the search for baculoviral MOI optima should be aided by the aphorism "less is more".

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