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# Production of recombinant herpes simplex virus protease in 10-L stirred vessels using a baculovirus–insect cell expression system

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**A gene expression system using recombinant *Autographa californica* nuclear polyhedrosis virus (baculovirus) and Sf-9 cells has been scaled up to the 10-L tank level and shown to be capable of producing herpes simplex virus (HSV) protease in serum-free media. High densities of *Spodoptera frugiperda* (Sf-9) cells were achieved by modifying two 10-L Biolafitte fermenters specifically for insect cell growth. The existing Rushton impellers were replaced by marine impellers to reduce shear and the aeration system was modified to allow external addition of air/$O_2$ mixtures at low flow rates through either the sparge line or into the head space of the fermenter. To inoculate the tanks, Sf-9 cells were adapted to grow to high cell densities (6–10 × $10^6$ cells $ml^{-1}$) in shake flasks in serum-free media. With these procedures, cell densities of 5 × $10^6$ cells $ml^{-1}$ were routinely achieved in the 10-L tanks. These cells were readily infected with recombinant baculovirus expressing the 247-amino acid catalytic domain of the HSV-1 strain 17 protease UL26 gene as a glutathione-*S*-transferase (GST) fusion protein (GST-247). Three days after infection at a multiplicity of infection (MOI) of 3 pfu $cell^{-1}$, the GST-247 fusion protein was purified from a cytoplasmic lysate by Glutathione Sepharose 4-B affinity chromatography with reproducible yields of 11–38 mg $L^{-1}$ of recombinant protein and ≥ 90% purity. Maximum production of this protein was observed at a cell density of 5.0 × $10^6$ cells $ml^{-1}$.**

**Keywords:** baculovirus–insect cell expression vector system (BEVS); Sf-9; HSV protease; glutathione-*S*-transferase

## Introduction

The baculovirus–insect cell expression vector system (BEVS) has been shown to be capable of producing a wide variety of heterologous proteins [6]. This system was developed in the early 1980s in the laboratories of Max Summers [9,10] and Lois Miller [8] and takes advantage of the highly expressed and regulated *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedron promoter modified for the insertion of foreign genes. This allows expression of prokaryotic or eukaryotic genes to produce recombinant proteins in *Spodoptera frugiperda* (Sf-9) cells infected with specifically engineered virus. The abundant expression of recombinant proteins, which in many cases are antigenically, immunogenically, and functionally similar to the mammalian protein, is the major advantage of this system. Another important advantage is that baculoviruses are not pathogenic to vertebrates or plants and do not employ transformed cells or transforming elements as do other expression systems.

Scale-up of the BEVS into sparged bioreactors has been difficult because of the insect cell's sensitivity to agitation and sparging and by their relatively high oxygen demand [7]. Literature reports at the time we began our development centered around spinner flask fermentations or expensive fermentors of atypical design such as air-lift, helical ribbon or perfusion vessels [2,3,13]. Until recently, media had to be supplemented with serum to achieve high cell density, making protein purification more difficult. New serum-free medium formulations have allowed cost reductions and easier purification of recombinant proteins.

The objectives of the research described in this paper was to scale up the BEVS into the 10-L tanks we had available in our laboratory. To achieve this goal it was necessary to develop techniques to provide high density inocula for our bioreactors and to optimize recombinant protein production at as high a cell density of insect cells as possible. The viral clone used in this BEVS is a herpes simplex virus type 1 (HSV-1) strain 17 UL26 gene product that has glutathione *S*-transferase (GST) fused to its amino terminus. GST can be purified from baculovirus/Sf-9 cells and bacterial lysates by one-step affinity chromatography using the Glutathione Sepharose 4B matrix. The HSV-1 strain 17 UL26 gene encodes a 635-amino acid protease that cleaves itself and the HSV-1 assembly protein ICP35 (infected cell protein 35) [5]. Two proteolytic processing sites within the protease have been identified between $Alanine^{247}$ and $Serine^{247}$ and between $Alanine^{610}$ and $Serine^{611}$ [1]. Cleavage at the 247–248 site releases the catalytic domain of the protease which resides within the first 247 amino acids of the protease [12]. It is this 247-amino acid sequence which has been fused to GST and expressed in the BEVS.

## Materials and methods

### *Cells, viral DNA and antibodies*

SF-9 cells were obtained from Gibco-BRL (Grand Island, NY, USA). BaculoGold viral DNA and the pVL1393 bacu-

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lovirus transfer vector were obtained from Pharmingen (San Diego, CA, USA). A GST expression vector (pGEX-1λT) was obtained from Pharmacia (Piscataway, NJ, USA). Monoclonal antibody raised against GST was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and a 20-amino acid peptide rabbit polyclonal antibody was raised against the amino terminus of the 247-amino acid catalytic domain.

### *Tank modifications*

To minimize cell disruption, two Biolafitte 10-L tanks were modified to accommodate insect cell fermentation. The existing three Rushton (paddle-type) impellers (90 mm diameter) were removed and replaced by a single marine impeller (100 mm diameter) placed near the shaft base. The single marine impeller created less shear than the multiple Rushton impellers. The aeration system was modified to provide for metered, external oxygen enrichment, both through the head-space of the fermenter as well as through the sparge line. The oxygen supplementation increased the oxygen transfer rate thereby reducing the volumetric flow rate requirement. This system minimized the cell damage caused by bubbling high volumes of air through the vessel [4,11].

### *Growth optimization of Sf-9 cells*

Initially, Sf-9 cells for inoculation into fermentation tanks were grown in spinner flasks (Bellco 500-ml jar with 500 ml of culture) and stirred at 100–120 rpm (two 1-inch Teflon paddles with a 3-inch diamter) at 27°C. The cells were grown in Grace's medium (Gibco) supplemented with lactalbumin hydrolysate (Difco), yeastolate (Difco), and 10% fetal calf serum (Hyclone). No external air supplementation was supplied to the spinner flasks. Cells were grown for 4–6 days until the cell density was sufficient (1.5–$3.7 \times 10^6$ cells ml$^{-1}$) and viabilities were above 95% to pool several spinner flasks and inoculate the 10-L Biolafitte tank to a cell density between 3 and $5 \times 10^5$ cells ml$^{-1}$. Typically, 1.5–2 L of inocula were added to 8–8.5 L of Sf-900 medium, a proprietary medium developed by Gibco for the serum-free growth of insect cells. The growth of Sf-9 cells in spinner flasks was severely impaired due to dissolved oxygen (DO) limitations at cell densities above $10^6$ cells ml$^{-1}$. Cell viability, measured by Trypan Blue staining, decreased with a dramatic fall in pH and concomitant rise in lactate accumulation at cell densities above $3 \times 10^6$ cells ml$^{-1}$. When used for tank inocula, cells provided from spinner flasks were quite variable in their growth characteristics. Lag-times prior to cell growth varied from 24–72 h and in some cases failed to grow after 5 days.

In the Spring of 1992, we began our efforts to optimize Sf-9 inoculum growth by culturing cells in shake flasks. Although there were no literature reports of successful shake flask culturing of insect cells at this time, we were encouraged by our ability to grow Sf-9 cells in the Biolafitte 10-L stirred vessel. Using 250-ml and 1-L Erlenmeyer flasks containing various volumes of Sf-900 II medium (a new formulation from Gibco), Sf-9 cells were adapted to shake flask conditions. Initially, cells were grown in media containing 5% fetal bovine serum (JRH Biosciences). The Sf-9 cells were gradually weaned off the serum requirement by subculturing in reduced serum and serum-free media. Cell densities of $1 \times 10^7$ cells ml$^{-1}$ with viabilities above 98% were routinely achieved in shake flasks. Achieving cell densities previously described, we were able to reduce the volume of inocula and achieve higher initial cell densities in our 10-L tanks. In all fermentors described in this report, gentamicin was added to Sf-900 II media to a concentration of 10 μg ml$^{-1}$.

### *Cell storage and revival*

High density cell stocks for storage were prepared by centrifuging Sf-9 cells at cell densities above $6 \times 10^6$ cells ml$^{-1}$ at $1000 \times g$ for 30 min. The cells were resuspended at 0.1 volume in Sf-900 II medium containing 10% serum and 10% DMSO. The cell concentrate was stored as 3-ml aliquots at −78°C. For initial adaptation of the cells from frozen stocks, one thawed vial of cells was added to 50 ml of Sf-900 II medium containing 5% serum and shaken at 28°C as previously described for 24–48 h. Cell viability was monitored daily and the cells diluted to 100 ml in Sf-900 II medium containing 5% serum when viability reached 80%. Subsequent cell dilution did not require additional serum. Cells were diluted in fresh serum-free media (to a density of $5–10 \times 10^5$ cells ml$^{-1}$) when cell densities reached $6–8 \times 10^6$ cells ml$^{-1}$ with a viability above 98%. Doubling times of approximately 24 h were achieved.

### *BEVS recombinant protein expression in 10-L tanks*

For 10-L fermentations, 5 L of SF-900 II medium in the 10-L Biolafitte tank were inoculated with 1 L of inoculum at a cell density of $6–10 \times 10^6$ cells ml$^{-1}$ (maximum cell viability). The tank was agitated at 135–140 rpm at a temperature of 28°C. A 1:1 mixture of air/$O_2$ was introduced to the head space at a metered rate between 50–100 ml min$^{-1}$ (Cole-Palmer Flowmeter). Cell count, viability, pH, and DO were monitored daily. As cell density increased above $2 \times 10^6$ cells ml$^{-1}$, the air/$O_2$ addition was altered to flow through the sparge line in order to maintain DO above 50%. When the cell density reached $3–5 \times 10^6$ cells ml$^{-1}$, the tank was diluted to 10 L with Sf-900 II medium (to a cell concentration of $2–3 \times 10^6$ cells ml$^{-1}$). Incubation was continued for an additional 24 h, allowing for a doubling of cell numbers to $4–6 \times 10^6$ cells ml$^{-1}$. The tank was then infected with the recombinant virus. Successful recombinant protein expression was achieved at Sf-9 cell densities between 2.0 and $5.5 \times 10^6$ cells ml$^{-1}$. Cells were infected with recombinant virus at an MOI of 1–5 for 72 h and were monitored daily for viability. When cell viability decreased to 70–80% (~72 h), infected cells were harvested by centrifugation at $1000 \times g$ for 25 min in a Beckman J-6 large head centrifuge.

### *Cloning of HSV protease*

The 247-amino acid sequence of the catalytic domain of the HSV-1 UL26 gene (strain 17, obtained from Valerie Preston at the MRC Virology Unit, Glasgow, UK) was cloned into the *Eco*RI site of a GST expression vector, pGEX-1λT, and was designated pJWC50. The GST-247 fusion protein gene was subcloned as a 1.9-kb *Ssp*I fragment from pJWC50 into the *Sma*I site of a baculovirus transfer vector, pVL1393, such that expression of the fusion

gene would be under the control of the baculovirus polyhedron promoter. This construct was designated pJWC57. The GST-247 construct also contains a thrombin cleavage site which releases the GST from the 247-amino acid sequence.

### *Expression and purification of HSV protease*

The pJWC57 construct was co-transfected with linearized BaculoGold virus DNA into Sf-9 cells. The recombinant baculovirus mix was obtained 4 days post-transfection. The recombinant baculovirus/GST-247 virus was identified by plaque assay and expression of the recombinant gene products was identified by immunoblotting analysis. High titer stocks of the baculovirus/GST-247 were purified and amplified by Invitrogen and used to infect, at an MOI of 3 pfu cell$^{-1}$, 10-L fermentors containing Sf-9 cells prepared as described above. After an initial experiment to determine the optimum infection time, cells were harvested routinely after 72 h post-infection. Infected Sf-9 cell pellets were then resuspended in buffer B (50 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM DTT, 20 ng ml$^{-1}$ antipain, 10 ng ml$^{-1}$ leupeptin, and 100 μM PMSF) containing 1% Triton X-100 and 150 mM NaCl. The lysate was incubated on ice for 30 min and then Dounce homogenized 10 times using a hand-held Wheaton homogenizer. The lysate was then clarified at 17 000 rpm for 30 min at 4°C using an SS-34 rotor in a Sorvall centrifuge. The clarified supernatant was batch absorbed with Glutathione Sepharose 4B beads (2 ml per liter lysate) for at least 30 min at 4°C and then the beads were packed into a column. The column was washed with buffer B plus 1 M NaCl and the GST-247 fusion protein was eluted from the beads with 10 mM glutathione (Boehringer Mannheim) in buffer B and analysed by SDS-PAGE, silver and Coomassie stains, and Western blot analysis. Also, the GST-247 purified protein from the BEVS system was compared to a GST-247 protease expressed and purified from *E. coli*.

### *Protease activity assay*

The protease activity was monitored by an *in vitro trans* cleavage assay. In this assay, the GST-247 was incubated with its substrate which was synthesized and $^{35}$S-labeled in an *in vitro* transcription and translation system (Promega). Cleavage of the 40-kD labeled substrate by GST-247 results in the release of two products: one 37-kD product and one 3-kD product. The 40-kD substrate and the 37-kD cleavage product were visualized by autoradiography.

## Results

### *Baculovirus/GST-247 protein expression*

An initial time course experiment (designated Batch 1) was conducted to determine the optimum harvest time of the infected Sf-9 cells. Infected cells were harvested and lysed at 48, 72, and 96 h post-infection. Cell lysates were purified and analyzed by SDS-PAGE, Coomassie, and Western blot analysis. Cell densities were monitored and ranged from $3.5 \times 10^6$ to $5.5 \times 10^6$ cells ml$^{-1}$ while the percentage of viable cells dropped from 96% to less than 50%. The largest amount of purified protein, 37.3 mg L$^{-1}$, was obtained at 72 h post-infection at an MOI of 3 pfu cell$^{-1}$. The results from Batch I are shown in Table 1. Six more production fermentations were carried out using the 72-h harvest time and the results of the cell densities, the % viability, and the protein yields from these batches are listed in Table 1. Protein yields varied from 11–38 mg of purified protein per liter of cells. Highest GST-247 recombinant protein yields were observed at cell densities between 5–5.5 $\times 10^6$ cells ml$^{-1}$.

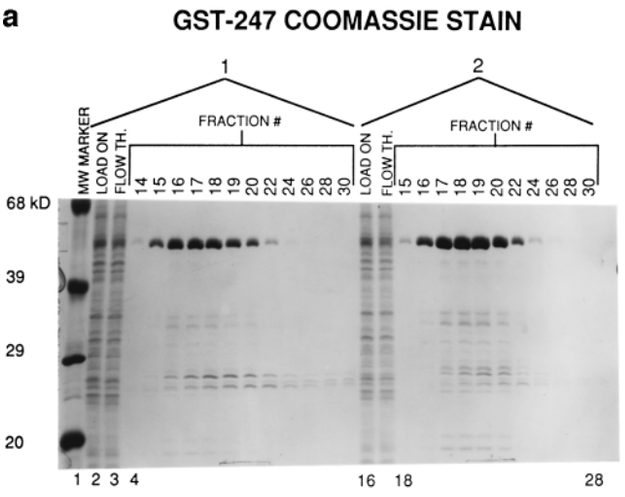
To assess the reproducibility of the BEVS as well as the purification scheme, the GST-247 recombinant protein expression of two separate fermentations (Batch #1 and Batch #2 from Table 1) were analyzed by SDS-PAGE followed by Coomassie stain. As shown in Figure 1a, Coomassie stain analysis of the elution patterns and protein profiles of Batch #1 (lanes 4–12) and Batch #2 (lanes 18–25) were nearly identical. The purity of the fusion protein was also similar for both fermentations as evidenced by the high molecular weight band (54 kD) corresponding to the GST-247 fusion protein. The identity of the purified recombinant GST-247 fusion protein was confirmed in fermentation 2, lanes 2 and 4–11, by Western blot analysis as indicated in Figure 1b. No substantial loss of the GST-247 protein was detected in the flow through (lane 3) resulting in a high purification recovery of the fusion protein.

### *Baculovirus/GST-247 protease activity*

The ability of recombinant GST-247 to cleave its substrate is shown in Figure 2. The same two fermentations that appear in Figure 1 were compared in respect to protease activity in the protease cleavage assay (*in vitro* transcription-translation system). In this system, the substrate alone

**Table 1** Results of seven Sf-9 cell fermentations infected with baculovirus/HSV GST-247 showing cell count, viability, and protein yield

| Time post-infection | Cell count ml$^{-1} \times 10^6$ | % Viable | Yield (mg L$^{-1}$) |
|---|---|---|---|
| Batch I (5/23/94 clone 4-57-10) | | | |
| 0 | 3.5 | 96 | |
| 24 | 5.0 | 97 | |
| 48 | 5.5 | 85 | 27.3 |
| 72 | 5.5 | 74 | 37.3 |
| 96 | | < 50 | 7.4 |
| Batch II 7/22/94 clone 3-57-4B) | | | |
| 72 | 3.4 | 82 | 15 |
| Batch III (7/22/94 clone 5-57-11) | | | |
| 72 | 2.6 | 77 | 11 |
| Batch IV (8/29/94 clone 5-57-11) | | | |
| 72 | 4.7 | 76 | 22.3 |
| Batch V (9/2/94 clone 5-57-11) | | | |
| 72 | 4.2 | 82 | 12.3 |
| Batch VI (9/6/94 clone 5-57-11) | | | |
| 72 | 3.2 | 76 | 19.2 |
| Batch VII (9/19/94 clone 5-57-11) | | | |
| 72 | 5.0 | 67 | 38.0 |



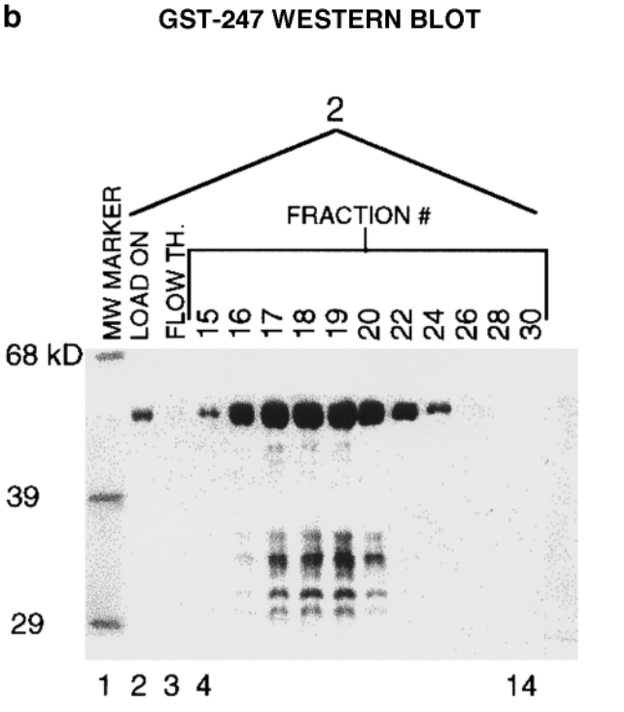




**Figure 1** (a) An SDS-PAGE Coomassie stained protein gel of two different fermentations designated 1 and 2. Lanes 4–12 of fermentation 1 and lanes 18–25 of fermentation 2 show the protein profile for the purified recombinant GST-247 fusion protein (54 kD). (b) A Western blot analysis of fermentation 2 confirming the identity of the GST-247 fusion protein (lanes 2, 4–11) as well as the lack of detectable GST-247 protein in the flow through (lane 3).

(lane 1) ran as a doublet and in the presence of a positive control for protease activity, was cleaved to a lower molecular weight doublet product (lane 2). Both batches had similar activities in that the substrate was virtually 100% cleaved (lanes 5–10 and 19–24). Based on these results, it was concluded that the BEVS reproducibly produces active protease.

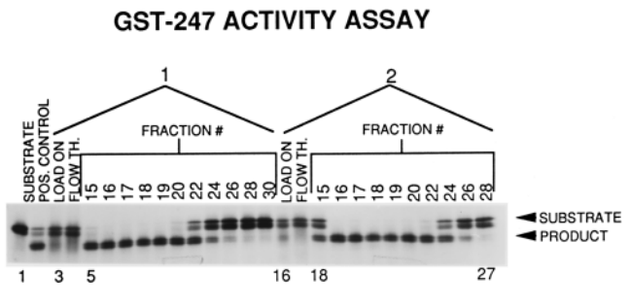



**Figure 2** Results of the GST-247 protease activity assay revealing similar cleavage activity of the purified recombinant GST-247 fusion protein from two different fermentations (#1 and #2). Both recombinant fusion proteins cleave the labeled substrate to a lower molecular weight product equally well (lanes 5–11 and 19–25).

### *Baculovirus/GST-247 and E. coli/GST-247 comparison*

The purified baculovirus GST-247 fusion protein was compared to a purified GST-247 produced in *Escherichia coli* by SDS-PAGE followed by silver and Coomassie staining. Although the total protein yield was similar in the *E. coli* and baculovirus systems, the GST-247 produced by baculovirus was more pure than the protein purified from *E. coli* using this particular purification scheme. As shown in Figure 3 (lanes 4, 5, 9, and 10), the baculovirus-expressed GST-247 electrophoresed as a predominant single band whereas the *E. coli*-expressed GST-247 displayed multiple lower molecular weight species which were not proteolytically active (data not shown).

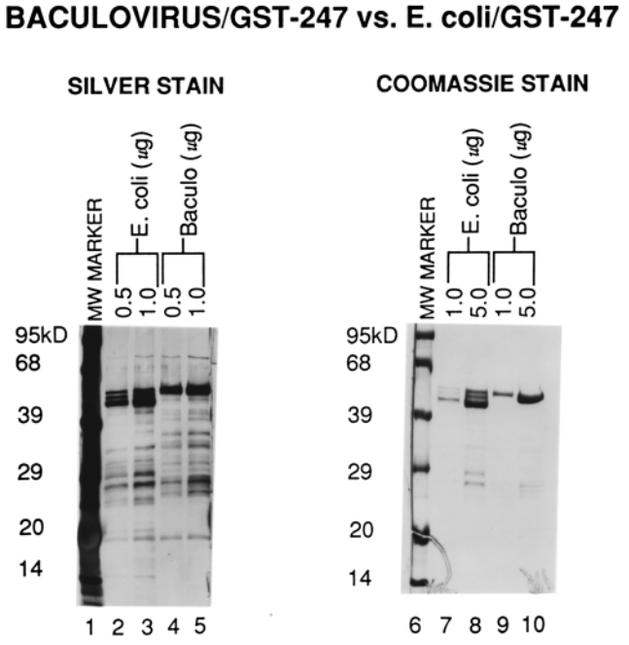



**Figure 3** Results of a comparison between a recombinant GST-247 fusion protein purified from baculovirus (lanes 4, 5, 9 and 10) and a recombinant GST-247 fusion purified from *E. coli* (lanes 2, 3, 7, and 8). Shown on the left is an SDS-PAGE silver-stained gel and shown on the right is a SDS-PAGE Coomassie-stained gel.

## Discussion

The baculovirus–insect cell expression vector system has been developed and utilized to reproducibly express the HSV protease recombinant fusion protein. Our ability to culture inocula in shake flasks to cell densities greater than $1 \times 10^7$ cells ml$^{-1}$ with a viability above 95% enabled us to inoculate our 10-L bioreactor to an initial cell density of $>1 \times 10^6$ cells ml$^{-1}$. The specific modifications to our 10-L tank minimized cell stress associated with agitation and air bubbling while providing adequate mixing and dissolved oxygen concentrations. The lag phase and variability previously observed with Sf-9 growth was considerably reduced. As a consequence, we were able to successfully culture Sf-9 cells in our bioreactor to cell densities equivalent to those grown in shake flasks ($8–10 \times 10^6$ cells ml$^{-1}$ with a viability greater than 95%). Initial experiments with viral infection and recombinant protein expression were successful and encouraged further development with this expression system. Sf-9 cells were successfully infected with a baculovirus encoding for HSV protease at cell densities up to $5.5 \times 10^6$ cells ml$^{-1}$ without loss of productivity. With respect to HSV-247, expression levels were equal to or greater than those produced by an *E. coli* construct. These results suggest that infections at higher cell densities and further optimization of the BEVS are quite possible.

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