Production of a self-activating CBM-factor X fusion protein in a stable transformed Sf9 insect cell line using high cell density perfusion culture

Volker M. Gorenflo^{1,2,*}, Tom A. Pfeifer³, Gary Lesnicki¹, Emily M. Kwan⁴, Thomas A. Grigliatti³, Douglas G. Kilburn^{1,4} and James M. Piret^{1,2}

¹Biotechnology Laboratory, 6174 University Boulevard, ²Department of Chemical and Biological Engineering, ³Department of Zoology, ⁴Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada; (*Author for correspondence; current address: Aventis Pasteur, 1755 Steels Ave. West, Toronto, ON, M2R 3T4, Canada, e-mail: volkergorenflo@web.de)

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Abstract

Factor Xa is a serine protease, whose high selectivity can be used to cleave protein tags from recombinant proteins. A fusion protein comprised of a self-activating form of factor X linked to a cellulose-binding module, saCBMFX, was produced in a stable transformed Sf9 insect cell line. The activity of the insect cell produced saCBMFX was higher than the equivalent mammalian cell produced material. A 1.5 l batch fermentation reached a maximum cell concentration of 1.6×10^7 cells ml⁻¹ and a final saCBMFX concentration of 4 mg l⁻¹. The production of saCBMFX by this cell line was also analyzed in a 1.5 l perfusion system using an ultrasonic filter as a cell-retention device for flow rates up to 3.5 l day⁻¹. The cell-retention efficiency of an air backflush mode of acoustic filter operation was greater than 95% and eliminated the need to pump the relatively shear sensitive insect cells. In the perfusion system over 4×10^7 Sf9 cells ml⁻¹ were obtained with a viability greater than 80%. With a doubling of viable cell concentration from 1.5 to 3×10^7 cells ml⁻¹ the saCBMFX production rate was doubled to 6 mg l⁻¹ day⁻¹. The saCBMFX volumetric productivity of the perfusion system was higher than the batch fermentations (0.6 mg l⁻¹ day⁻¹) by an order of magnitude.

Introduction

Stable transformed insect cell line systems have recently been used to produce a number of important mammalian proteins (Pfeifer et al. 1997; Hegedus et al. 1998, 1999). A beneficial aspect of using insect cells is their ability to perform most mammalian cell post-translational modifications (Wallin and Martin 1988; Stanton and Wallin 1992; Chu and Robinson 2001). They also have high growth rates, the culture readily scales up and protein-free/serum-free medium is available.

With these advantages in mind, several investigators have reported the use of stable, transformed insect cell lines for the batch production of secreted proteins (Leytus et al. 1984; Wallin and Martin 1988; Messier et al. 1991; Rezaie et al. 1993; Pfeifer et al. 1997; Rudolph et al. 1997).

Human factor X is a vitamin K-dependent glycoprotein zymogen that is synthesized as a single-chain precursor (Wallin and Martin 1988; Stanton and Wallin 1992). It is a complex glycoprotein with >50% of the carbohydrates on the activation peptide, and is also glycosylated at the C-terminus of

the heavy chain (Assouline et al. 1993; Inoue and Morita 1993; Di Scipio et al. 1977; Cook et al. 2000). The mature plasma form of factor X is a disulfide-linked two-chain molecule consisting of a light chain and a heavy chain, formed after intracellular proteolytic cleavage of the precursor (Leytus et al. 1984). Factor X is activated to factor Xa (FXa) by removal of the activation peptide using Russell viper venom (RVV) protease. FXa is a highly specific serine protease that can be used to cleave fusion proteins. The levels of expression and production of recombinant human factor X in mammalian cells are usually low, $\sim 1 \text{ mg l}^{-1}$ (Messier et al. 1991; Rezaie et al. 1993; Rudolph et al. 1997). In cultures of Chinese hamster ovary cells concentrations of up to 120 mg l^{-1} of a different recombinant human FX can be obtained (Himmelsbach et al. 2000). However, yield of the biologically active protein have been between 22% and 43% from CHO cells and 30% from Cos-1 cells because of impaired translational modification, propeptide removal and/or protein processing (Stanton et al. 1996; Himmelsbach et al. 2000).

We have previously shown that a stably transformed Sf9 insect cell line produced up to 18 mg l^{-1} of a non-self-activating CBMFX in batch mode (Pfeifer et al. 2001). This protein requires activation by RVV prior to use. Recently, a self-activating factor X derivative with a N-terminus cellulose-binding module (CBM) and a C-terminus H6 (saCBMFX) was engineered and expressed in baby hamster kidney (BHK) cells (Kwan et al. 2002). The protein was stable when bound to Perloza and could be used at a 1 : 200 ratio for protein substrate hydrolysis. Production of this form of factor X would eliminate the complication and expense of adding RVV for protein activation; however, the expression was low, $\leq 2 \text{ mg l}^{-1}$.

This very low expression level led us to the decision to study the use of stably transfected Sf9 insect cells for the expression of saCBMFX. In addition, converting from batch to perfusion should provide an order of magnitude increase in productivity (e.g. Trampl et al. 1994), which is particularly valuable when an organization is coping with increasing demands for production capacity (Garber 2001). Semi-continuous or continuous processes are frequently selected for larger-scale clinical production (Chu and Robinson 2001). Here we investigate the production of a self-activating form of CBMFX in

Sf9 insect cells and determine the productivity of a continuous high cell density culture system under a range of operating conditions.

Materials and methods

Molecular biology

For protein production in BHK cells, a previously described construct using the expression vector pcDNA (Invitrogen) was used (Kwan et al. 2002). For insect cell expression, a 1.5-kb XhoI/EcoRI fragment containing the mammalian transferrin secretion signal peptide, CBM2a and the E2 domain of human factor X containing mutations in the activation peptide (Kwan et al. 2002) were cloned into the XhoI/EcoRI sites of the insect expression vector p2ZOp2F (Hegedus et al. 1998). The resulting recombinant vector was designated p2ZOp2F-saCBMFX and the DNA prepared using Endo-free columns (Qiagen, Germany).

Isolation of BHK clones

Baby hamster kidney clones were isolated as previously described (Kwan et al. 2002). Briefly, plasmid DNA was introduced into BHK cells by transfection in the presence of lipofectamine reagent. The transfected cells were cultured for 3 days in DMEM-F12 supplemented with 5% FCS (Life Technologies) and screened for resistance to $0.5 \text{ mg G418 ml}^{-1}$. Stable clones were isolated and cultured in DMEM-F12 supplemented with $0.5 \text{ mg G418 ml}^{-1}$ and 0.5% knockout serum replacement (Life Technologies). After 48–72 h, $10\text{-}\mu\text{l}$ samples of the culture supernatants from all clones were screened for the presence of the fusion protein by non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) through 12% gels, followed by Western blotting with rabbit anti-human FX serum (Assouline et al. 1993) and detection using anti-rabbit Ig HRP (Amersham).

Insect cell line transformations

ESF921 medium and Sf9 insect cells adapted to it were obtained from Expression Systems (Woodland, CA). p2ZOp2F-saCBMFX DNA was introduced into Sf9 cells using Cellfectin (Invitrogen) as

previously stated (Pfeifer et al. 1997) and after 48 h, 1 mg ml^{-1} Zeocin (Invitrogen) was applied. After confluence of cells was achieved in a 30 mm dish, the cells were scaled up to T25 flasks and passed three times on medium containing 1 mg ml^{-1} Zeocin. A relatively high producing cell line designated SGE6, was selected and used in further studies.

Production in BHK cells

The saCBMFX from BHK cells was produced and purified as described previously (Kwan et al. 2002). Briefly: stable, high saCBMFX producing clones, were expanded from T25 flasks and cultured in 200 ml of DMEM-F12, 5% FCS, 0.5 mg of G418 ml^{-1} in expanded-surface roller bottles (Corning) at 1 rpm, 37°C until semi-confluent. The medium was removed and replaced with DMEM-F12, containing 0.5% knockout serum replacement and $0.1 \text{ mg G418 ml}^{-1}$. After 72 h, the saCBMFX containing culture supernatant was recovered and subjected to protein analysis and purification.

Insect cell batch and perfusion culture maintenance

The transformed Sf9 cell line, SGE6, expressing saCBMFX was inoculated at $5 \times 10^5 \text{ cells ml}^{-1}$ into a 125 ml screw cap Erlenmeyer containing 20 ml of ESF921 medium and placed in a rotary shaker at 80 rpm and 27°C . When cells reached a density of $1 \times 10^7 \text{ cells ml}^{-1}$, the entire 20 ml was used to inoculate a 1 l screw cap Erlenmeyer flask containing 200 ml of ESF921, and once grown, these cells were used to inoculate the bioreactors. A 3 l bioreactor (Applikon, Schiedam, Netherlands) containing 1.5 l of ESF921 medium with 0.05% PluronicF68 (Sigma) added was inoculated at $0.5 \times 10^6 \text{ cells ml}^{-1}$. Culture temperature and dissolved oxygen (DO) were automatically maintained using an ADI 1030 Bio Controller (Applikon), and monitored by a personal computer. DO was maintained by controlling pure oxygen addition using a GFC mass flow controller (Aalborg, Orangeburg, NY). Cell number and viability were measured by trypan blue dye exclusion using a hemocytometer. The culture supernatant was analyzed for glucose, lactate and ammonium content with a Stat 10 Blood/Gas Analyzer (NOVA Biomedical, Waltham, MA).

Starting from a batch culture inoculated at $\sim 1.5 \times 10^6 \text{ Sf9 cells ml}^{-1}$, perfusion was typically initiated on day 4 and adjusted to maintain a glucose concentration of $27.5 \pm 0.4 \text{ mmol l}^{-1}$ for the duration of the first 7 days. The bioreactor temperature (T) was controlled at 26.8°C , DO at 60% air saturation and the pH varied, without control, between 5.8 and 6.2. Cells were retained in the reactor using a BioSep 10 l acoustic separator (AppliSens, division of Applikon). Cole-Parmer (Vernon Hills, IL) Masterflex peristaltic pumps (7521-40 and 7550-20, L/S pump heads) added fresh medium, harvested cells, pumped the culture recirculation and the backflush air. Between experiments the culture volume was maintained using a level sensor that triggered the harvest pump. The cell concentration was maintained at the set-point between 15 and $30 \times 10^6 \text{ viable cells ml}^{-1}$ by bleeding the contents of the bioreactor once per hour at a rate matching the culture growth rate.

Acoustic separator operation

A 10 l acoustic separator (AppliSens) was operated in the air-backflush mode as recently described by Gorenflo et al. (2003). Briefly: injection of bioreactor air downstream of the separator periodically returned the captured cells to the reactor by completely emptying the separator while the acoustic power and harvest pump were turned off. The separator was refilled from the bioreactor by reversing the direction of the backflush air pump, followed by turning on the acoustic power and harvest pump. This mode of operation eliminates the need to pump shear sensitive insect cells through the recirculation line and pump. It also allows the selection of short cell residence times in the separator. The backflush flow rate was 100 ml min^{-1} and perfusion typically resumed within $\sim 35 \text{ s}$. The separator was operated with a duty cycle run and stop time of 45 and 4.5 s, respectively and the backflush frequency was set to 6 h^{-1} . In the conventional recirculation mode of operation, the contents of the bioreactor were continuously pumped to the base of the acoustic filter via the "recirculation line" using L/S 16 PharMed tubing (Cole-Parmer). A portion of this stream was clarified and harvested, while the remainder flowed back to the bioreactor through the "settling tube" (Gorenflo et al. 2003).

Analytical methods

Sf9 and BHK samples were clarified at $3000 \times g$ in a micro-centrifuge, 2 mM benzamidine added to be frozen for subsequent Western analysis, substrate hydrolysis and peptide sequencing.

Estimates of factor X concentration were made using a Western dot-blot dilution technique as described previously (Pfeifer et al. 2001).

N-terminal sequencing of proteins was performed on an Applied Biosystems 476A automated sequencer using standard gas phase Edman chemistry by the Nucleic Acids Protein Service Unit at the University of British Columbia.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described previously (Laemmli 1970).

Purified saCBMFXa (10 μg) was bound to 1 mg of Avicel PH101 (FMC) or 2 mg of Perloza MT500 beads (Sigma) overnight at 4 °C in 20 mM Tris/HCl, pH 8.0, 0.15 M NaCl (TBS), 2 mM benzamidine (Sigma). The cellulose beads were washed with $2 \times$ TBS. The bound samples were analyzed by 12% SDS-PAGE using the non-reducing loading buffer.

Maltose-binding protein (MBP)–calbindin fusion protein was produced as described previously (Guarna et al. 2000) and purified by ion-exchange chromatography on Macro-PrepQ resin (Bio-Rad). For substrate hydrolysis 500 μg of MBP–calbindin was added to $\sim 2.5 \mu\text{g}$ of the Avicel or Perloza bound activated saCBMFXa in 100 μl of TBS. The samples were incubated at room temperature in a micro-centrifuge tube on a tube roller. Five microliter samples were removed at time points 0, 1, 2, 4, 5, 6 h and added to 45 μl of non-reducing loading buffer. Two microliters of each sample was analyzed on a 12% SDS-PAGE. The coomassie blue stained gels were analyzed by densitometry.

Results and discussion

Properties/hydrolytical activity of saCBMFX produced by BHK and Sf9 cells

The saCBMFX fusion protein (Figure 1) was produced in BHK and Sf9 cells to study the impact of the host cell on its productivity and activity. Stable, high producing clones were isolated (data not

shown), cultivated in shake flasks and the secreted saCBMFX purified and concentrated. N-terminal sequencing of both Sf9 (this study) and BHK (Kwan et al. 2002) produced Avicel-bound saCBMFXa samples, revealed that 100% of the protein had the 2 N-termini of VPDKT and IVGGQ (the light chain and the heavy chain), showing that the correct protein was produced, processed and self-activated to saCBMFXa upon binding to cellulose.

To compare the enzyme kinetics of saCBMFX produced by Sf9 and BHK cells, identical quantities were bound to the cellulose materials Perloza MT500 and Avicel PH101, respectively, and incubated with 500 μg of MBP–calbindin at a substrate : enzyme ratio of 200 : 1. The influence of the two different binding materials as well as the host organism on the activity of the enzyme was evaluated. The Perloza bound protein produced by Sf9 cells hydrolyzed 90% of the substrate within the first hour and 100% after 6 h and showed the highest activity (Figure 2). In comparison, the Perloza bound BHK produced protein hydrolyzed only 65% of substrate within the first hour and 95% after 6 h revealing significantly lower activity than the cognate Sf9 produced protein. The same host cell line dependant trend can be seen for the Avicel bound protein with significantly lower overall activities. Only $\sim 50\%$ of the substrate could be hydrolyzed after 1 h for both host cell lines and after 4 h, 75% of the substrate was hydrolyzed by the Sf9 produced protein but only 65% by the BHK produced material. At 4 °C, the immobilized protein was stable and retained $\sim 70\%$ of original activity after 1 year (data not shown). In addition the Sf9 cells produced more protein than the BHK cells (4 mg l^{-1} vs. $\leq 2 \text{ mg l}^{-1}$). Therefore, the Sf9 cell clone, SGE6, was subsequently chosen for the further investigation of saCBMFX production in batch and perfusion systems. Although the overall productivity was lower than seen when expressing other complex proteins in insect cell systems, the specific nature of this protein likely limits the productivity (Fann et al. 1999).

Production of saCBMFX in Sf9 batch

A 1.5 l batch culture of Sf9 cells (clone SGE6), producing saCBMFX, was inoculated at $\sim 5 \times 10^5 \text{ cells ml}^{-1}$ at a culture viability of 90%

Figure 1. The processing of saCBMFX. The full-length protein (A) is processed to form two protein chains linked by a disulphide bridge (B). Removal of the activation peptide (AP) at the factor X cleavage site (IEGR) creates the activated form of saCBMFX, saCBMFXa (C). TF – transferrin secretion signal, CBM – cellulose binding module, E2 – factor X E2 domain, H6 – Histidine 6 tag.

Figure 2. Kinetics of hydrolysis of MBP-calbindin by immobilized saCBMFXa (200:1). saCBMFXa produced by BHK (batch) and Sf9 (perfusion) host cells was bound to Avicel and Perloza, respectively and bound activities were compared. The error bars indicate the standard error of the mean of 2–4 replicates.

(Figure 3). DO was controlled at 50% air saturation and pH varied without control between 6.0 and 6.2 until the last 10 h of the cultivation when it increased to 6.7. The culture grew exponentially to a maximum total cell concentration of 1.1×10^7 cells ml^{-1} at 120 h, with viability at $\sim 95\%$. During the following stationary phase of the culture (120–225 h) the viability decreased to 40% with a total cell concentration of 1.0×10^7 cells ml^{-1} at the end of the culture. During the batch cultivation the glucose concentration decreased from its starting point of 58 to 1 mmol l^{-1} . The saCBMFX concentration augmented in a growth associated manner to 3.9 mg l^{-1} within the first 120 h of cultivation, then declined to 3.0 mg l^{-1} by the end of the culture. The maximum saCBMFX concentration coincided with the maximum total cell concentration and the onset of the drop in the viability at a glucose concentration of $\sim 30 \text{ mmol l}^{-1}$ (see arrow, Figure 3).

Figure 3. Total Sf9 cell concentration (—■—), viability (—●—), glucose concentration (—◇—) and saCBMFX concentration (—▽—) for a 9-day batch culture. The arrow indicates maximum saCBMFX concentration.

Figure 4. Viability of Sf9 insect cell culture for indicated rates and durations of recirculation pumping. The initial cell concentration was 3.2×10^7 cells mL^{-1} in a 1.5 l bioreactor.

Production of saCBMFX in perfusion

In the conventional recirculation mode of the acoustic filter operation for perfusion, the contents of the bioreactor are continuously pumped to the base of the acoustic filter via the “recirculation line”. Although commonly used for mammalian cells,

recirculation pumping at a flow rate of 25 l day^{-1} rapidly decreased the viability of the Sf9 cells from 65% to 35%, within 3.5 h (Figure 4). This result is consistent with a previous report of Hi-5 insect cell sensitivity to recirculation pumping (Merten 2000). The pump was stopped for the following 3 h, allowing the viability to stabilize above 30%. When the recirculation pump was restarted with a flow rate of 15 l day^{-1} , the viability decreased again but with a reduced slope in comparison to 25 l day^{-1} , leading to a culture viability of 4% at 35 h (Figure 4). The higher shear sensitivity of insect cells may be the cause of cell death upon recirculation pumping. Sf9 insect cells have ~2- and ~8-fold higher shear sensitivity than CHO and hybridoma cells, respectively (Ma et al. 2002). However, Sf9 perfusion cultures up to 30×10^6 cells mL^{-1} with a viability maintained at ~90% have been reported despite using the ultrasonic separator in the recirculation mode (Zhang et al. 1998). The cultivation medium used in that study contained 1% serum (FBS), which can be an effective protecting agent against hydrodynamic stress (McQueen and Bailey 1989; Kunas and Papoutsakis 1990; Chisti 2001; Ma et al. 2002; Keane et al. 2003). In Figure 4, serum free medium was used, which could explain the greater sensitivity of the insect cell

Figure 5. (A) Viable Sf9 cell concentration (—■—), viability (—●—), glucose concentration (—◇—) and cell specific saCBMFX production (—▽—); (B) Total Sf9 cell concentration (—■—), separation efficiency (—▲—), saCBMFX production (—●—) and perfusion rate (—◇—) for a 35-day perfusion culture. Horizontal lines represent means of corresponding results.

culture to shear stress created by recirculation pumping.

To eliminate the need to pump cells through the recirculation line we chose to use the recently

developed air-backflush mode of operating the 10 l acoustic cell separator (Gorenflo et al. 2003). No detrimental effects of the acoustic separator were observed during the 800 h run (Figure 5A).

Table 1. Comparison of saCBMFX productivity.

	Batch	Perfusion
Maximum saCBMFX concentration (mg l^{-1})	4.0	5.0
Volumetric saCBMFX productivity ($\text{mg l}^{-1} \text{ day}^{-1}$)	0.6*	6.0

*Only first 6 days of batch cultivation considered and turn around (down time) not considered.

Figure 6. Cross-plot of cell specific saCBMFX productivity (■) and volumetric saCBMFX production (▲) vs. viable cell concentration. Glucose concentration during the run was adjusted between 26 and 40 mmol l^{-1} (see Figure 5).

Throughout the runs the culture viability typically remained above 80% and a separation efficiency of >95% could be maintained at perfusion rates of up to 3.5 l day^{-1} (Figure 5B).

The air-backflush Sf9 insect cell perfusion system yielded a 10-fold increase in volumetric saCBMFX productivity in comparison to a batch culture system (Table 1). The cross-plot in Figure 6 shows no significant impact of the viable cell concentration on the cell specific saCBMFX productivity ($\sim 0.2 \text{ pg cell}^{-1} \text{ day}^{-1}$). As a result, the volumetric saCBMFX production of the system doubles from ~ 3 to $\sim 6 \text{ mg l}^{-1} \text{ day}^{-1}$ with doubling cell concentration from 15 to 30 $\times 10^6 \text{ cells mL}^{-1}$, independent of the glucose concentration between 28 and 40 mmol l^{-1} (Figure 5). The volumetric productivity of saCBMFX can likely be further

Figure 7. Cross-plot of cell specific (■) and volumetric (○) saCBMFX production vs. glucose concentration. Viable cell concentration varied between 21 and 23 $\times 10^6 \text{ cells mL}^{-1}$. Error bars represent the standard error of the mean of last 3 days at pseudo steady state.

increased by greater cell concentrations. In addition, the productivity of the system should be further improved by adaptation of the medium composition to perfusion cultivation. Figure 7 shows that reducing the glucose concentration to an average of 17.5 mmol l^{-1} resulted in a significant, ~ 2 -fold, reduction in the cell specific, and volumetric production rates of the perfusion culture compared to a glucose concentration above 28 mmol l^{-1} . This reduction in the cell specific production rate indicates an unknown nutrient is limiting at glucose levels below 28 mmol l^{-1} . The ESF921 culture medium used in this study was not optimized for the perfusion cultivation of insect cells. It may be that the reduction in the cell specific protein production rate was due to an amino acid limitation.

Conclusions

The expressed fusion protein saCBMFX was readily purified and fully self-activated by binding to cellulose. The saCBMFXa produced in Sf9 insect cells had a higher specific activity in comparison to saCBMFXa produced in BHK cells and with the availability of a protein-free/serum-free medium this insect system presents an attractive alternative

to mammalian cell systems. The implementation of the air-backflush mode of operation facilitated the production of saCBMFX in an Sf9 insect cell perfusion system by avoiding detrimental levels of recirculation pumping shear stress. The perfusion system increased volumetric saCBMFX productivity 10-fold in comparison to the conventional batch mode of operation while maintaining the saCBMFX concentration. The productivity of the perfusion system can be enhanced even further by operating the system at higher cell concentrations or optimizing the medium for perfusion.

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