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PROTEIN PRODUCTION (β–GALACTOSIDASE) FROM A BACULOVIRUS VECTOR IN *SPODOPTERA FRUGIPERDA* AND *TRICHPOLUSIA NI* CELLS IN SUSPENSION CULTURE

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Abstract: Protein production capabilities of *Trichpolusia ni* (TN 368) cells and *Spodoptera frugiperda* (Sf9) cells were compared in GTC100 medium in suspension culture using as a vector a genetically engineered *Autographa californica* nuclear polyhedrosis virus. TN 368 produces more ß-galactosidase than Sf9, on a per cell basis ($2.2x10^5$ and $1.7x10^5$ units/ 10^6 cells[1] respectively). In growth experiments serum-free medium supported a higher maximum Sf9 cell density ($4\pm1x10^6$ cells/ml) than the serum- based media ($1.5\pm.5x10^6$ cells/ml in GTC100 and $2\pm1x10^6$ cells/ml in TNM-FH). However, using a cell density of $5x0^5$ cells/ml, the productivity per cell varied, from a low of $4.5x10^4$ units in EX-CELL-400 medium to a high of $7.6x10^4$ units in TNM-FH. The TN 368 cells were twice a large as Sf9 cells and appeared to be more shear sensitive than Sf9 cells.

Introduction

Baculoviruses have become an important part of the biotechnology repertoire. More than 100 laboratories are currently using this viral system for the production of recombinant proteins, chiefly because it is capable of producing very high levels of expression of recombinant proteins (1). Also because invertebrates possess the protein modification systems present in higher eucaryotes, in most cases the recombinant proteins produced are functionally similar to the original protein (1). In the past there has been no systematic examination of the recombinant protein producing capabilities of the insect cell lines available for *in vitro* protein production. Different cell lines have been used to produce the same recombinant protein, but it is difficult to compare the results because in most cases a different constructed vector was used, or the experimental conditions were dissimilar. For baculovirus vectors the levels of protein expression depends greatly on the construction, ie. the exact place in the viral genome that the recombinant protein gene is inserted. Even among similar constructs , differences in the point of insertion by a few base pairs can have a profound effect on the levels of expression which can differ by as much as 20 fold(2). To compare protein production with various hosts it is necessary to use precisely the same vector. Previous results(3) indicate that in the case of wild type viruses, TN 368 cells are much more productive than *Spodoperta frugiperda*

[1] One unit of ß- galactosidase hydrolyzes $1x10^{-9}$ moles of O.N.P.G.

cells. In this paper we compare the recombinant protein production capability of TN 368 cells and Sf9 cells in suspension culture.

Materials and methods

Cell Lines: The *Trichopolusia ni* 368 (TN 368) was obtained from Dr F.Hink, Ohio State University , while the S.*frugiperda* 9 (Sf9) cells were purchased from American Type Culture Collection, 12301 Park-lawn Drive, Rockville, MD, 0852-1776, Accession Number CRL1711.

Virus: The virus is a recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV 246) that produces a fusion r-protein, *E coli* ß-galactosidase, which is fused to the start of the polyhedrin gene. The vector was obtained from MicroGeneSys Inc., West Haven, Ct. All virus inocula used in the experiments described in this report were in the third or earlier passage . In all cases the Multiplicity of Infection was 5. Multiplicity of infection, or M.O.I., is the ratio of the number of infectious viral particles to viable cell. Our experience has shown that for passage number less than or equal to 3 recombinant protein production is unaffected by passage number if experimental conditions remain constant (ie; M.O.I., cell density at infection).

Media Formulations : GTC100 consist of Grace's insect cell culture medium (4) supplemented with 2.6 g/liter bacto-tryptose broth, 4.2mM $NaHCO_3$ and 10% (v/v)fetal bovine serum(Hyclone Labatories Inc., Logan Utah). TNM-FH media is also based on Grace's insect cell culture medium. It is prepared by the addition of 3.3g / liter yeastolate and 3.3g/liter lactalbumin hydrosolate(VWR Scientific) to GTC100. The serum free medium EX-CELL 400, is a defined medium designed specifically for Sf9 cells and is available commercially from J .R. Scientific company, (Woodlands Ca).

Culture conditions: All experiments were done in 50 ml. spinner flasks, in suspension at 28°C and 60 rpm. The screw caps of the spinner flasks were removed and silicone closures (Belco Glass, Vineland,NJ.) were used instead. The use of silicone closure allowed for increased gas exchange between the reactor and the environment while allowing the reactor to remain sterile.

Virus Inoculation: Cells in exponential phase of growth were centrifuged @1000*g* for 10 minutes and then resuspended in fresh media. The viral inoculum was added so that the final cell density was approximately $1X10^7$ cells /ml. The cells were allowed to incubate @28°C for 1 hour, after which fresh medium was added so that the final cell density was $5X10^5$ viable cells / ml. The suspensions were then transferred to spinner flasks and the infection allowed to proceed..

Analytical methods: Glucose was assayed using the Sigma Diagnostic Glucose HK kit (procedure no. 16-uv). β-galactosidase activity was assayed by measuring the hydrolysis of the O-nitrophenyl ß-galactopyranoside (5). Samples were diluted appropriately in Z buffer and ONPG added @ 28°C. After ten minutes the reaction was terminated by the addition of 1.0 M Na_2CO_3. The change in absorbance was measured using a spectrophotometer set at a wavelength of 420 nm. The nmoles of (O-nitrophenyl ß-galactopyranoside) ONPG hydrolyzed per minute were calculated using the Lambert-Beer Law. One unit of enzyme hydrolyzes 1.0 nm ONPG per minute at 28°C.

Cell viability was measured using the trypan-blue dye exclusion viability test. An aliquot of 0.1 ml of 4.0% dye solution was added to 1.0 ml cell suspension, incubated for 4 min. and then examined under a microscope(120X). The fraction of cells that had absorbed the dye were assumed to be proportional to the percent of the total cells that were non-viable. Cell concentration was determined using a Coulter Electronics cell counter, model Zb (Coulter Electronics, Hialea, Florida), with a 140 micron aperture.

Results and Discussions

Comparison of cell lines in GTC100: TN 368 cells are much larger than Sf9 cells and the difference in average cell diameter, 13 *vs* 18 μm (Table 1) results in a cell volume for TN 368 cells twice that of Sf9 cells. Unlike Sf9 cells, TN 368 cells require a period of adaptation (~15 days) before becoming fully acclimatized to the suspension culture environment. This adaptation is not only illustrated in the growth curves, but also in the glucose metabolism curves as well as the changes in morphology that occur (Fig. 1 &2). Unadapted cells rapidly utilize glucose during exponential growth and are very fibroblastic in appearance. For adapted cells the glucose uptake during growth is minimal, but at the onset of stationary phase, glucose metabolism increases markedly . Accompanying the changes in metabolism are changes in morphology. By the end of the period of adaptation the cells are no longer fibroblastic in appearance. The cells have become spheroid, although the average cell volume remains unchanged. The glucose metabolism of Sf9 cells is very similar to that of unadapted TN 368 cells and these cells rapidly deplete extracellular glucose during the exponential phase of growth.

After infection with virus, for both cell lines mitosis ceases, but protein production continues. The total cell number remains constant until late in the infection cycle when cell viability decreases and the cells begin to lyse. Along with an increase in cellular protein content there is an increase in average cell volume. Cell volume increases steadily and by 50 hours post infection (p.i.) the average cell volume has doubled (fig 3). Significant ß-galactosidase production begins by about 36 hours post infection. The protein accumulates until a maximum is reached after which there is a reduction in total activity (fig.4). Because the β-galactosidase assay used detects only enzymatically active enzyme, the reductions in activity may be due to proteolytic degradation. An examination of SDS gels of extracellular media samples showed that accompanying this reduction in activity was a decrease in the intensity of the 114 kd band that represents ß-galactosidase. Simultaneously, lower molecular weight bands representing smaller size fragments appeared. These bands did not appear to represent proteins normally produced during the course of the infection and may represent degradation products.

Although TN 368 cells grow and produce recombinant protein in suspension culture, we have had difficulty reproducing results. TN 368 cells grow well after a period of adaptation, but the culture canot be maintained after 30-50 days in suspension. Consequently suspension cultures have to be reestablished. It thus appears that the cells that grow in suspension are a variant that is not very stable.

One of the reasons for the difficulty we experienced with the TN 368 cells may be an adverse response to high shear stress in our spinner flask. Insect cells are known to be shear sensitive and Tramper *et al* (6) calculated the shear stress above which cell growth and division are suppressed. This shear stress was designated the 'critical shear

stress'. They demonstrated that this value varies according to the cell line, and that Sf21 cells (the parent cell line for Sf9) have a higher tolerance for shear stress than TN 368 cells. Tramper used correlation charts to estimate the shear stresses present in his reactor. However for spinner flask there are no correlation charts available to determine the shear stresses present. Thus an alternative method is needed. Because the flow in our flask is turbulent we were able to use theories available for the description of turbulent flows to analyze our spinner flask flow patterns.

In a turbulent flow field there exist a wide spectrum of eddy sizes, the largest being as big as the width of the flow and the range extends down to the viscous dissipation regime. Short term hydrodynamic forces arise through the motion of these turbulent eddies and the maximum shear stresses are produced by the smallest eddies. If the cell sizes(~10-20 μm) are on the same order of magnitude as the flow patterns on the microscopic scales, the shear stresses seen by the cells will be produced on these small scales.

Using Kolmogorv's universal equilibrium theory (7) it was possible to calculate the microscopic eddy size(~7.5 μm), and the shear stress produced by these microscopic eddies. The maximum shear stress present in our reactor was calculated to be $1.7x10^{1}Nm^{-2}$. This theory was also applied to the data presented by Tramper *et al* and the critical shear stress was recalculated($3.6x10^{2}Nm^{-2}$). These results indicated that the shear stresses present in our reactor were more than an order of magnitude(20X) lower than the critical shear stress for TN 368 cells. Thus cell lysis due to shear is not the likely cause of deterioration of TN 368 suspensions, but a rather a more subtle effect may be responsible.

Effect of Media composition on Sf9 cells: Media composition has a profound effect on growth characteristics. The doubling time, maximum cell density and cell size varies with media composition (Table 1). In order to insure maximum protein production, cells were infected while still in exponential phase of growth. Different media formulations support cell growth to different extents, and to make a comparison of the effect of media composition on protein production the conditions were maintained as uniform as possible. Thus all infection experiments were conducted at a cell density of $5x10^{5}$/ml. At this cell density in all instances the cells were in the exponential phase of growth, and thus producing protein at the maximum possible rate. The yields of recombinant protein, as well as the start of recombinant protein production, vary greatly. Sf9 cells grown in GTC100 produced more than twice as much protein per cell as cells grown in EX-CELL 400 or TNM-FH (Table 2). Since different media formulations are able to support different levels of cell growth it is possible to infect some of these cultures at higher cell densities and the cells will produce recombinant protein at its maximum levels. Although the recombinant protein yield per cell varies on a per cell basis, the yield per unit reactor volume is quite similar (Table 3).

Summary

TN 368 cells produce twice as much recombinant protein as Sf9 cells per flask volume, but because of their ease of cultivation, and resistance to shear, Sf9 cells may be more suitable for large scale production of recombinant proteins. Media composition effects the amount of protein produced per cell by Sf9 cells, but protein production in serum containing and serum free media are quite similar on a per unit volume basis.

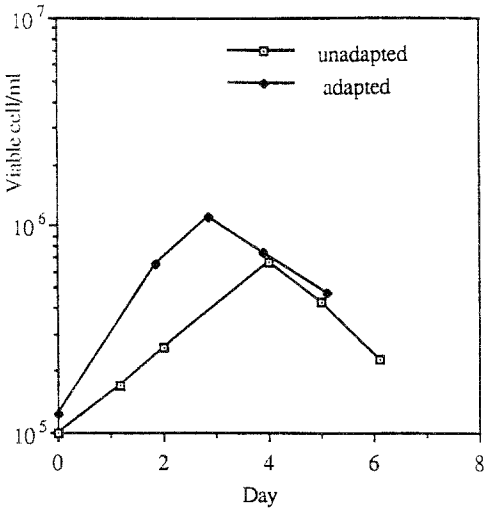

Fig. 1 Growth curve for T.ni. 368 cells.

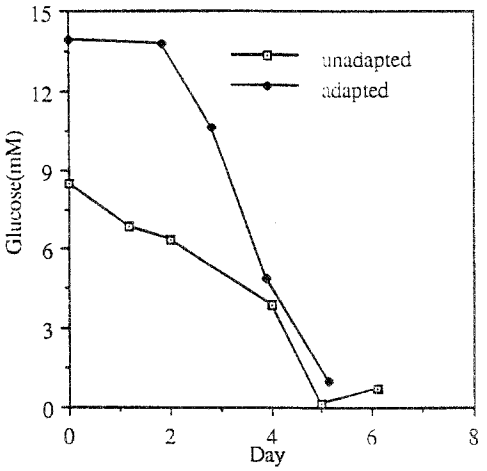

Fig.2 Glucose uptake curves for adapted and unadapted T.ni. 368 cells

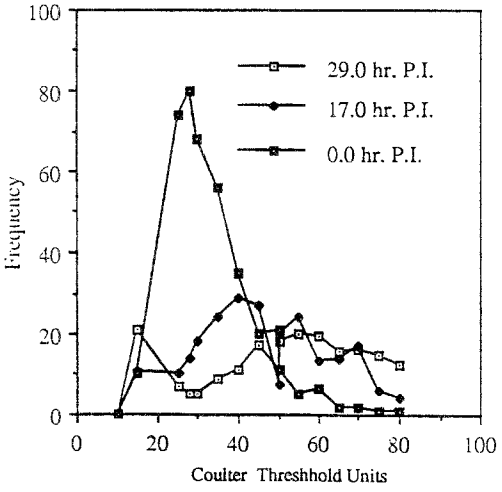

Fig.3 Change in cell volume with time P.I for TN 368 cells. 100 Threshold units = 9.63E3 cubic microns.

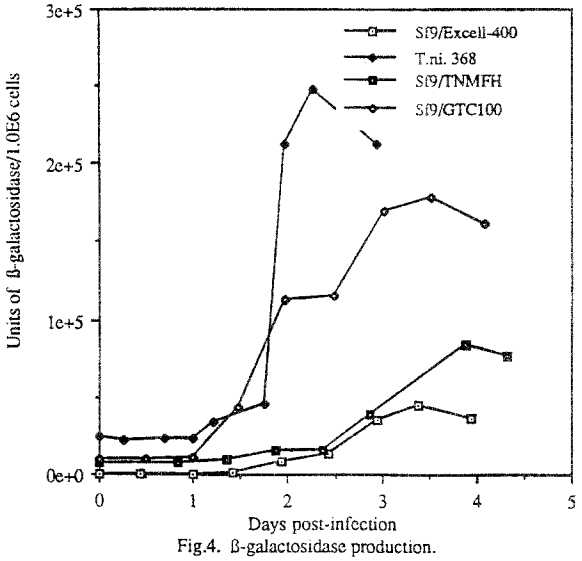

Fig.4. ß-galactosidase production.

Table 1. Growth Characteristics of Sf9 and TN 368 cells

	TN 368 GTC100	Sf9 GTC100	Sf9 TNM-FH	Sf9 EX-CELL 400
Max cell density/10^6	1.5±5	1.5±1	2±1	4±1
Doubling time(hr)	20±1	24±2	18±2	24±2
Cell diameter (μm)	18±2	13±2	12±2	14±2

Table 2. ß-galactosidase production in Sf9 and TN 368 cells. Cell density at inoculum is $5X10^5$ cells / ml. M.O.I.~5

	TN 368 GTC100	Sf9 GTC100	Sf9 TNM-FH	Sf9 EX-CELL 400
Time to peak conc.(day)	~2.5	~3.5	~3	~3.5
Yield ; Units/10^6 cells	$2.2X10^5$	$1.7X10^5$	$7.6X10^4$	$4.5X10^4$

Table 3: Yield of recombinant protein per unit spinner flask volume.

	TN 368 GTC100	Sf9 GTC100	Sf9 TNM-FH	Sf9 EX-CELL 400
cells/ml	$1X10^6$	$6X10^5$	$1X10^6$	$2X10^6$
Yield ; Units/ml	$2.2X10^5$	$1.0X10^5$	$7.6X10^4$	$9.0X10^4$

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