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Factors Affecting Recombinant Protein Yields in Insect Cell/Baculovirus Expression System

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The optimum conditions for the infection of *Spodoptera frugiperda* (Sf) 21 cells with recombinant virus were revealed. The maximum activity of *Escherichia coli* β-galactosidase was obtained by using infected cells with 5th-passage recombinant virus. The repetition of passage for recombinant virus purification resulted in a sharp decrease in protein production, and the expressed enzyme activity with 11 th-passage virus dropped one fortieth of that with 5th-passage. The β-galactosidase productivity by Sf21 cells infected in the exponential and stationary stages of growth was almost in the same level in final product yield, indicating the absence of a "culture age" effect. An increase in cell density on initial infection, moreover, did not result in a proportional increase in the final product levels. The increase in final product was observed for multiplicity of infection (MOI) values greater than 4 in cultures infected with recombinant virus. The maximum yield of recombinant protein occurred in Sf21 cells infected with an MOI of 10.

INTRODUCTION

The baculovirus expression system in insect cells constitutes a promising alternative to classical bacterial fermentation systems for the production of recombinant proteins, and is well suited to the large-scale production of valuable recombinant proteins. While some experiments have been done on the effects of initial cell density on baculovirus titers and polyhedrin levels (Stockdale and Gardiner, 1977; Murhammer and Goochee, 1988; Maiorella *et al.*, 1988; Hink *et al.*, 1990), very little is known pertaining to infection of Sf21 cells with recombinant baculovirus.

In previous papers (Hara et *al.*, 1992 and 1993), we reported that a cell line from *Spodoptera frugiperda* (IPLB-Sf21-AEII) is a good producer of *Escherichia coli* β-galactosidase, that its productivity was greatly stimulated at 30°C and that the final product yield reached 7.1 mg/ml, or more than 36% of the total cell proteins.

Previous work by other investigators suggests that some of the factors such as medium condition (Stockdale and Gardiner, 1977), cell density (Wood *et al.*, 1982), and aeration (Wiss *et al.*, 1986), may be important for optimizing recombinant protein synthesis in insect cell/baculovirous expression system. Lazarte *et al.* (1992) reported that the efficient recombinant protein production was achieved with extremely high MOI at 580. However, Schopf *et al.* (1990) reported that the MOI did not have a dramatic effect in the cells infected with the recombinant baculovirus. Licari and Bailey (1992) reported that maximum heterologous protein synthesis was dependent on the growth phase of the insect cells prior to infection.

*Corresponding author. Abbreviation: MOI, multiplicity of infection; p.i., postinfection.

Maximization of the desired product is important in any biological process. The present paper describes the factors affecting the recombinant polyhedrin/β-galactosidase yield in insect cell/baculovirus expression system.

MATERIALS AND METHODS

Cell culture

A cell line from *Spodoptera frugiperda* (IPLB-Sf 21-AEII) (Vaughu *et al., 1977)* was cultured in IPL-41 medium (Stockdale and Gardiner, 1977) supplemented with 10% fetal bovine serum (Gibco Labs., New York, USA). Cells were maintained as monolayers in stationary 25-ml TC flask (Falcon", Becton Dickinson Labware, New Jersey, U.S.A.) and infected with a recombinant virus at 27°C. Cell counts were determined with the use of a hemacytometer on an optical microscope (Olympus Ltd., Tokyo; 10x eye piece and 40x objective). Viable cells were determined via trypan blue exclusion (Kuchler, 1977).

Virus stock and infection

A recombinant baculovirus, Ac360-*501β-gal*, described previously (Hara *et al.,* 1992), was used to infect Sf21 cells. It contained the *Escherichia coli lacZ* gene of β-galactosidase. The fusion protein (Mr=120 kDa) contsisted a segment of the polyhedrin protein fused with β-galactosidase. A virus stock solution was added to the monolayer of cell cultures at time zero after the medium was aspirated off from them. The cultures were placed on a rocking platform for 1 h. After one additional hour, the medium was replaced with fresh one. In mock infected cell cultures, fresh medium was added in place of the viral inoculum.

Virus titer was determined using the end-point dilution method (Summers and Smith, 1987) by the addition of 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside to the medium.

β-Galactosidase assay

The activity of β-galactosidase was determined by the o-nitrophenyl-β-D-galactopyranoside (ONPG) assay (Miller, 1992). The ONPG (ZOO µl) was added to 1,000 µl of Z-buffer (16.1 g/L $Na_2HPO_4 H_2O$, 5.5 g/L $NaH_2PO_4 \cdot H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4 \cdot 7H_2O$ and 2.7 ml/L β-mercaptoethanol) with 1 µl of sample at 28°C. The intracellular enzyme farction in the culture fluid was prepared by harvesting, suspending into water and disrupting cells with TOMY UD-201 (TOMY Co., Ltd., Tokyo, Japan). The conversion to 0-nitrophenyl was measured the absorption at 420 nm so that a faint yellow color developed for 1 min after the ONPG addition. The reaction was stopped with 500 µl of 1 M Na_2CO_3. β-Galactosidase activity defined as follows: Units of β-galactosidase = 1000 X OD_{420} / time (min) / sample volume (ml)

RESULTS

Effect of serial passage of recombinant virus on β-galactosidase expression

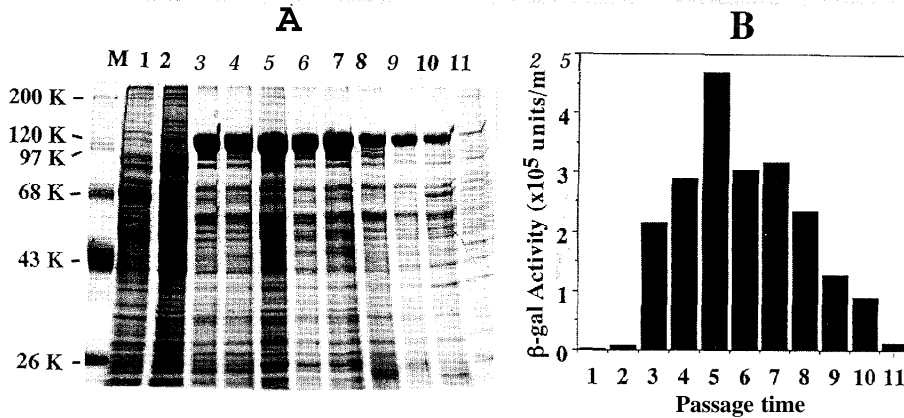

Fig. 1. Effect of Passage Number-of Recombinant Virus on β-Galactosidase Expression Level.
(A) SDS-polyacrylamide gel electrophoresgram profiles of proteins produced by infected Sf21 cells. Sf21 cells (5.0×10^5 cells/ml) were infected with th addition of 0.5-ml of a recombinant virus inoculum and incubated at 30°C for 4 days. Lysates of 2.3×10^4 cells were placed in each well. Uninfected cells (lane 1, mock) were similarly treated. Lanes 2-1 1 indicate passage numbers for recombinant virus purification from 1 to 11, respectively. The molecular mass and positions of standard proteins (lane M) are indicated to the left (myosin, 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa). (B) Intracellular β-galactosidase activity expressed by Sf21 cells which have been infected with virus at different passage numbers of recombinant virus. The enzyme activity was assayed according to the method of Miller (1972).

To investigate the effects of serial passage of recombinant virus on β-galactosidase expression, the total protein contents of infected Sf21 cells were determined by SDS-polyacrylamide gel electrophoresis (Fig. 1A). The amount of 120-kDa proteins corresponding to β-galactosidase expressed by infected Sf21 cells with the 5th-passage time was the highest (lane 5, Fig. 1A). As shown in figure 1B, the intracellular activity of β-galactosidase expressed by Sf21 cells was the greatest when they were infected with the 5th-passage recombinant virus. However, when the virus was further passaged the protein production decreased. The enzyme activity level obtained with the 1 lth-passage virus was one fortieth of that obtained with the 5th-passage virus.

Effect of cell growth stages on β-galactosidase expression

To determine the effects of cell growth stage on the production of β-galactosidase, replicate monolayer cultures of Sf21 cells (1.5×10^7 cells/ml) were set up at 2×10^5 cells/ml (Fig. 2A). Figure 2B shows the effects of cell growth stage on β-galactosidase expression. Though the β-galactosidase productivity was strongly dependent on initial cell viability, the cell viabilities declined within days of the time of infection (data not shown). Sf21 cells which had been infected at the exponential (I and II, Fig. 2A) and stationary (III, IV and V, Fig. 2A) stages expressed the β-galactosidase almost in same

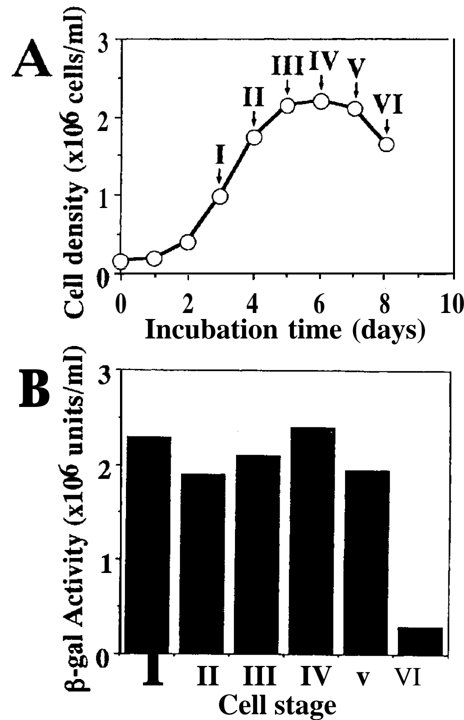

Fig. 2. Effect of Cell Stage on ,3-Galactosidase Expression Level.

(A) Sf21 cells (5.0×10^5 cells/ml) were cultured at 30°C for 3, 4, 5, and 6 days, respectively. The arrow indicates the cell stage used for viral infection. (B) The infected cells with recombinant virus at an MOI of 20 were incubated at 30°C for 4 days. The intracellular activity of β-galactosidase was measured by the method described of Miller (1972).

level, indicating the absence of a "culture age" effect. However, β-galactosidase activity declined rapidly in final product yield after 8 days p.i. (VI, Fig. 2B).

Effect of cell density on β-galactosidase expression

Determination of optimal cell density for infection is an essential factor in the efficient use of the baculovirus system for recombinant protein production. Figure 3 shows the effects of cell density ranging from 2.0×10^5 to 2.0×10^7 cells/flask at the time of infection on β-galactosidase expression. Cell viabilities declined within several days after infection (data not shown). The expressed β-galactosidase activity on a culture volume basis was the highest when the insect cells were infected at 2.0×10^6 cells/flask, and

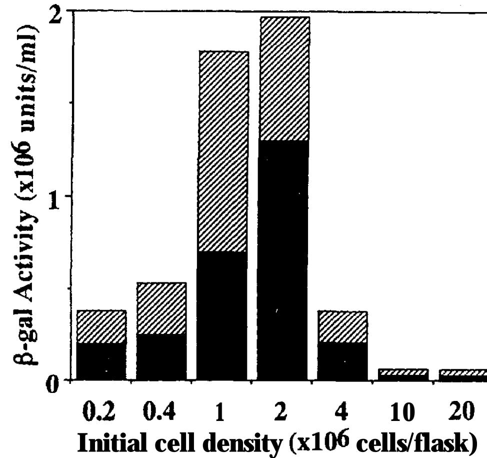

Fig. 3. Effect of Cell Density on ,3-Galactosidase Expression.

Sf21 cells in the late exponential phase of growth were harvested, suspended in fresh medium at various cell densities, infected with recombinant virus at an MOI of 20, and incubated at 30°C for 4 days. Intra- (■) and extracellular (▨) enzyme activities were assayed according to the method described in the text.

declined sharply when the cell density was further increased.

Effect of MOI on β-galactosidase expression

The effects of MOI, ranging from 0 to 600, on the yields of β-galactosidase were determined. Final β-galactosidase activity as a function of MOI is presented in Fig. 4. The increase in final product was observed for MOI values greater than 4 in cultures infected with recombinant virus. The maximum yield of recombinant protein occurred in the infected cells with a MOI of 10. If MOI was higher than 100, cell viability decreased in proportion to the MOI (data not shown), leading to a slight decrease in final products.

DISCUSSION

In order to develop an efficient process for recombinant protein production, various factors which affect the productivity of Sf21 insect cells when using the baculovirus expression system were investigated. The final yield of cloned β-galactosidase by the infected Sf21 cells was affected by the serial passage of recombinant baculovirus and MOI. Especially, the repetition of passage of recombinant baculovirus resulted in a sharp decrease in β-galactosidase productivity, and the expressed enzyme activity obtained

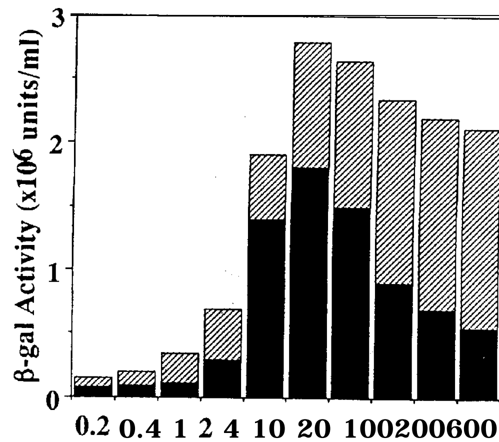

Fig. 4. β-Galactosidase Expression Level by Sf21 Cells Infected with Various MOIs.

Sf21 cells (2.0×10^6 cells/ml) were infected with various MOIs, and incubated at 30°C for 4 days. The intra- (■) and extracellular (❑) enzyme fractions were prepared and assayed.

with 11th-passage virus dropped one forith of that obtained with 5th-passage (Fig. 1B). The decrease of virion formation was observed by the infected insect cells with AcNPV. Therefore, the decrease in recombinant protein productivity might due to the propagation of baculovirus itself.

A significant difference in the enzyme activity of β-galactosidase expressed by the infected Sf21 cells was not detected at any cell stage of the growth. The β-galactosidase activity on a culture volume basis showed a sharp increase in was extremely influenced with the initial cell density. However, the enzyme activity on a per cell basis was found to be almost same beyond 2×10^6 cells/ml. Lindsay and Betenbaugh (1992) stated that insect cells generated higher product yields when infected with recombinant baculovirus at low ceil densities (3 X 10" cells/ml) than at high cell densities (9×10^5 cells/ml). These results indicate that MOI might be quite important as a factor affecting recombinant protein expression in baculovirus insect cell system.

Lazarte et al. (1992) reported that the infection at high MOI of 580 allowed an efficient recombinant protein production by infected Sf9 cells with AcCD4 encoding the full length human CD4. However, as shown in Fig. 4 in this work, the β-galactosidase productivity by infected Sf21 cells at higher MOI than 100 resulted in decrease remarkably. Licari and Bailey (1992) reported that it is more feasible to infect the culture earlier in the exponential phase with a lower MOI (e.g., 0.1) and obtain the final product concentrations of the same order of magnitude as that by the infection with an MOI of 100. It seems that, by using a lower MOI and relying on secondary infection of the culture (Schopf et al., 1990), the amount of stock virus needed will be substantially reduced. However, the higher enzyme productivity at a lower MOI than 1 has not been observed

with recombinant virus in this study (Fig. 4). At an MOI of 10, the β-galactosidase activity expressed by the infected Sf21 cells amounted to the highest. Therefore, it is quite interesting that the suitable range of MOI for recombinant protein production by Ac360-*501 β-gal*-infected Sf21 cells was found to be very narrow as between 4 and 20.

To improve the product yields from a biochemical engineering perspective, it will try to achieve the mass production of *E. coli* β-galactosidase in the stirred vessels.

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