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Heterologous Protein Production in Baculovirw-Insect Cell System

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The baculovirus (AcNPV)-insect cell expression system was employed for the production of *Escherichia coli* β-galactosidase as a model heterologous protein. Preliminary experiments made clear that most of all *Spodoptera* cells were adapted well to IPL-41 medium supplemented with 10% fetal bovine serum. Sf9 cells provided a maximum cell density of 4.9 X 10^6 cells/ml, while the *E. coli* β-galactosidase expression level of Sf9 cells infected with recombinant baculovirus (Ac360-*501 β-gal*) was found to be less than that of Sf21 cells. The Ac360-*501 β-gal*-infected Sf21 cells expressed *E. coli* β-galactosidase at a high level, and the amount of 120 kDa-proteins corresponding to β-galactosidase was densitometrically estimated to be 4.1 mg per 5.0 x 10^5 cells in a milliliter of culture broth, representing more than 36% of total cellular protein. The β-galactosidase activity expressed by infected Sf21 cells grown in IPL-41 medium was 1.4 x 10^5 units per 5.0 X 10^5 cells, and the enzyme expression level was equivalent to 7 times of that expressed by Sf9 cells under the same conditions.

INTRODUCTION

Baculoviruses, causative agents of fatal diseases in insects, are not only of interest because of their application as control agents of insect pests in agriculture (Martignoni, 1984), but have increased importance since they are used as expression vectors for the production of heterologous proteins of pro- and eukaryotic origin (Luckow and Summers, 1988a). Therefore, baculovirus-insect cell systems have shown great promise recently for the production of biologically active proteins such as medical, pharmaceutical, and veterinary importance (Wu et al., 1989). The cells used to support *Autographa californica* nuclear polyhedrosis virus (AcNPV) replication are either from the cell line IPLB-Sf21-AE II (Vaughn *et al.*, 1977) derived from cultured ovaries of *Spodoptera furgiperda* or a clonal isolate (Sf9) (Summers and Smith, 1987) derived from this line. Nowadays, the Sf9 cells has mainly been used as a host. Development of the AcNPV transfer vectors has been described (Matsuura *et al.*, 1987; Miller *et al.*, 1986) and reviewed (Martignoni, 1984; Miller, 1988). Much of the recent efforts to maximize gene expression in this system has focused on optimizing the placement of foreign gene with respect to transcriptional and translational signals of the polyhedrin gene (Luckow and Summers, 1988b). Quantifying the significance of cell culture factors is essential to the development of an optimized culture process. In generaly, culture medium composition

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has considerable influence on insect cell growth and product formation, but there are only a few publications except Mitushashi (1982) and Broussard and Summers (1989). TNM-FH and Grace's media are routinely used in many laboratories for the propagation and manipulation of S. *frugiperda* cells. However, TNM-FH medium was developed to establish primary cultures of *Trichoplusia ni* cells (Hinh, 1970).

In this study, we performed to achieve a suitable and efficient insect cell culture system to produce large quantities of *Escherichia coli* β-galactosidase by baculovirus encoding a fusion β-galactosidase.

MATERIALS AND METHODS

Insect cell lines and growth medium

Four insect cell lines were assessed as shown in Table 1; a fall armyworm, S. *frugiperda* (Sf) designated IPLB-Sf21-AE II (Vaighn *et al.*, 1977) and a clonal isolate (Sf9) (Summers and Smith, 1987), S. *littoralis* designated CLS79, and S. *litura* designated S1224. Analysis of elemental composition of the *Spodoptera* cells was entrusted to the Kyushu University analysis center. Cells were cultured in IPL-41 medium (Dougherty *et al.*, 1981), modified TNM-FH medium (Hinh, 1970) (Grace's medium (Grace, 1963) supplemented with 3.3 g/L Difco yeastolate, 3.3 g/L lactalbumin hydrolysate and 0.35 g/L $NaHCO_3$), modified M3 medium (Shields *et al.*, 1975), and TC-100 medium (Gardiner and Stockdale, 1975), with 10% fetal bovine serum (Gibco Labs., New York, U.S.A.). Cultures grew as monolayers in stationary 25-cm^2 TC flasks (Falcon ®, Becton Dickinson Labware, New Jersey, U.S.A.) maintained at 27°C. Cells were passaged by flushing the monolayer off the surface of the TC flask with a Pasteur pipette.

Virus stocks and infection

Wild-type *A. californica* nuclear polyhedrosis virus (AcNPV) and recombinant virus encoding a fusion β-galactosidase under the control of the polyhedrin promoter (Ac360-*501 β-gal*) (Luckow and Summers, 1988b) were used in this study. The fusion protein contains 11 amino acids of the polyhedrin protein and lacks 7 amino acids of the native *E. coli* β-galactosidase. Infection began when medium was aspirated off the monolayer culture, and virus stock solution was added. The virus added at a multiplicity of infection (MOI) of 20 per cell was allowed to absorb for 1 hr at room temperature with gentle rocking. The inoculum was removed and replaced with appropriate medium, and incubation was carried out at 27°C for the indicated periods.

Cell counting

Cells were counted with a hemacytometer on an optical microscope (Olympus Ltd., Tokyo, Japan) (15 Xeye piece and 10 X objective). Viable cells were determined via trypan blue exclusion (Kuchler, 1977).

β-Galactosidase assay

The activity of β-galactosidase were determined by the *O*-nitrophenyl-β-D-galactopyranoside (ONPG) assay (Miller, 1972). The ONPG (200 μl) was added to 1,000 μl of Z-buffer (16.1 g/L $Na_2HPO_4 \cdot H_2O$, 5.5g/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 fraction in culture fluid was preparated 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 1M Na_2CO_3. β-Galactosidase activity defined as follow:
Units of β-gal = 1,000 $\times OD_{420}$ / time (min) / sample volume (ml)

Gel *electrophoresis*

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of total cellular proteins were done in 10% gel by the method of Laemmli (1970). After electrophoresis, protein bands were stained with coomassie brilliant blue R-250 and then destained by 20% methanol plus 7% acetic acid solution.

RESULTS AND DISCUSSION

Adaptation of insect cells in various media

Figure 1 shows the adaptation profiles of *Spodoptera* cells in various media. After successive passage of each cell lines in 25-cm^2 TC flask, the initial cell density was adjusted to 3.0 x 10^5 cells/ml. As shown in Fig. 1, the Sf9 and Sf21 cells established from S. *frugiperda* adaptated well to IPL-41 medium, and the maximum cell density of both cells reached 5.0 x 10^6 and 3.5 x 10^6 cells/ml, respectively. As shown in Table 1, the size of both S. *frugiperda* cells in diameter differed overall: Sf9 cells, 15.7$\pm$2.5 μm; Sf21

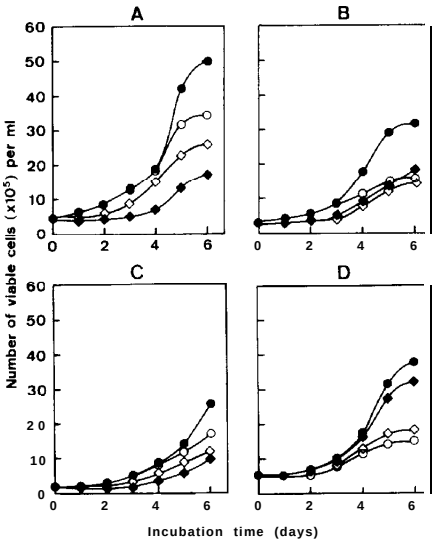

Fig. 1. Adaptation profiles of *Spodoptera cells* lines such as Sf9 (A), Sf21 (B), CLS79 (C), and S1224 (D) in various insect media.

The cells inoculumed at 5.0×10^5 cells/ml were adaptated to the used media supplemented with 10% fetal bovine serum and cultured at 27°C. Symbols: ● , IPL-41 medium; 0, modified TNM-FH medium; ◆, modified M3 medium; *0,* TC-100 medium.

Table 1. Insect cell lines used in this study

Species	Cell line	Size (μm)
Spodoptera frugiperda	Sf9	*15.7f2.5*
Spodoptera frugiperda	Sf21	21.1 f2.3
Spodoptera littoralis	CLS79	16.6±1.4
Spodoptera litura	*S1224*	*14.3* ± 1.9

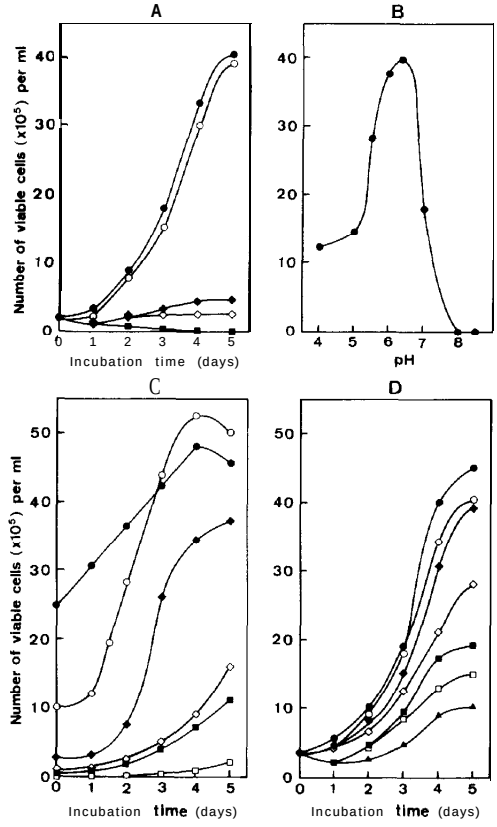

Fig. 2. Growth profiles of *Spodoptera* insect cells.

Sf9 cells inoculated at 5.0 X 10^5 cells/ml as a initial cell density were cultured with IPL-41 medium supplemented with 10% fetal bovine serum at 27°C except indicated. (A) Effect of growth temperature. Symbols: ■ , 37°C; ● ,3O"C; **0, 27°C; ● , 20°C; 0, 15°C.** (B) Effect of initial pH. (C) Effect of initial cell density. Symbols: ● , 2.5 ×10^6 cells/ml; ○, 1.0 X 10^6 cells/ml; ◆, 3.0 ×10^5 cells/ml; 0, 1.0 X 10^5 cells/ml; ■, 6.0 ×10^4 cells/ml; □, 1.0 X 10^4 cells/ml. (D) Effect of fetal bovine serum concentration. Symbols: ●, 20%; 0, 15%; ◆, 10%; 0, 5%; ■ , 2.5%; □, 1%; A, no addition.

cells, $21.1 \pm 2.3\ \mu m$. The elementary composition of *Spodoptera* cells at exponential growth phase was quite similar (date not shown). Therefore, the net cellular mass of Sf21 cells per a milliliter of culture broth might be equivalent to 1.5-folds as that of Sf9 cells. These results suggest that Sf21 cells are superior to Sf9 cells as a host in this system, although Sf9 cells has mainly been used as a host.

Growth profile of Spodoptera cells in IPL-41 medium

The growth profiles of *Spodoptera* cells used in this study were investigated (Fig. 2). Figure 2A shows the effects of temperature on cell growth of *S. frugiperda* Sf9 cells, representing a maximum cell density. Sf9 cells grew well at 30°C, while the increase of cell density was not observed below 20°C and at 37°C. Eppler (1980) reported that the optimum growth temperature for insect cells is found to be at between 25°C and 28°C, which correspond with optimum growth temperature for the insect individuals, and that the growth rate of established insect cell lines is sharply decreased above 30°C. In a baculovirus-insect cell system, therefore, all experiments for insect cell cultures including expression of foreign gene products were carried out at 27°C. It is, therefore, quite interesting that the maximum cell density at 30°C was higher than that at 27°C. As shown in Fig. 2B, the optimum range of initial pH for cell growth was quite narrow and limitted only among pH 6.0 to 6.6. Kurtti and Brooks (1972) has suggested that insect cells are quite sensitive against pH change of culture medium, and that even at pH 7.0 cell growth was sharply decreased. Figure 2C indicates the effects of initial cell density on cell growth. The lag time in growth phases was found to become shorter with the increase of initial cell density, and a higher inoculum such as 2.5×10^6 cells/ml accomplished directly logarithmic phase in growth. However, the maximum cell density was not increased above 6.0×10^6 even though the initial cell density had been increased to 5.0×10^6 cells/ml (data not shown). Therefore, we concluded that the 3.0×10^5 cells/ml as a initial cell density was desirable for the monolayer formation in a 25 $-cm^2$ TC flask. As shown in Fig. 2D, the effects of serum on cell growth were investigated. Although the increase of fetal bovine serum concentration on upto 20% had a beneficial effect on cell growth, 10% fetal bovine serum added to the medium is found to be enough for Sf9 cells growth.

From these results, we decided the media conditions for insect cell culture as follows: media, IPL-41 medium (pH 6.4); a initial cell density, 3.0×10^5 cells/ml; fetal bovine serum concentration, 10%.

Expression of E. coli β-galactosidase in Spodoptera cells

To clarify the expression level of *E. coli* β-galactosidase gene in various *Spodoptera* cells, the total protein content of infected cells with Ac360-*501β-gal* virus at a MOI of 20 was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). The cellular lysates prepared by sonication at 3 days postinfection (p.i.) were put on the gels. Coomassie blue staining of total infected-cell proteins showed that Sf9 and Sf21 cells expressed a considerable amount of β-galactosidase, but the expression level of CLS79 and S1224 cells were quite low. The β-galactosidase expression level of Sf21 cells appears to be the highest among *Spodoptera* cells, and the amount of 120 kDa-proteins corresponding to β-galactosidase expressed by Sf21 cells was densitometrically estimated to be 4.1 mg per 5.0×10^5 cells in a milliliter of culture broth, representing

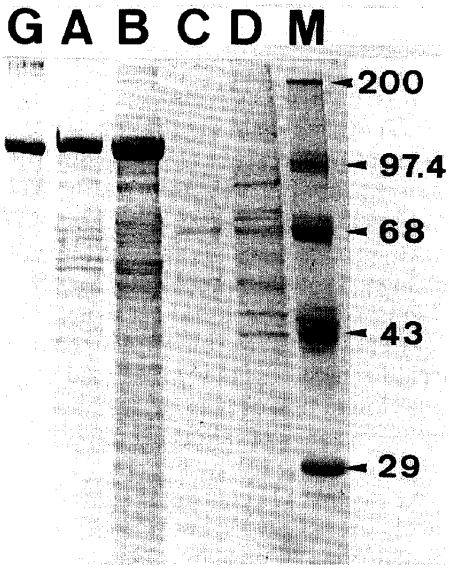

Fig. 3. SDS-polyacrylamide gel electrophoresis profiles of proteins from various *Spodoptera* cells infected with the recombinant baculovirus, Ac360-*501 p-gal*, expressing *E. coli* β-galactosidase.

Spodoptera cells were infected with Ac360-*501* p-gal at a MOI of 20 and incubated at 27°C. Cellular lysates of 2.3 x 10^4 dells of Sf9 (lane A, 3 days p.i.), Sf21 (lane B, 4 days p.i.), CLS79 (lane C, 3 days p.i.), and S1224 (lane D, 4 days p.i.) are put in each well. Molecular weight standards (lane M) and the authentic *E. coli* β-galactosidase (lane G) were put on as a marker. The molecular masses and positions of the standard proteins are indicated to the right (myosin, 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa).

Table 2. Comparison of intracellular β-galactosidase activity expressed by infected *Spodoptera* cells

Cell line	β-Galactosidase activity (units/ml)
Sf9	20,167
Sf21	142,042
CLS79	1,293
51224	1,925
Mock"	1,000

The experiments were carried out under the conditions described in MATERIALS AND METHODS.
"A level of spontaneous conversion occured in the mock-infected cells.

more than 36% of total cellular protein (see lane B in Fig. 3).

Comparison of E. coli β-galactosidase activity by infected Spodoptera cells

To clarify the net productivity of p-galactosidase by insect cells, the β-galactosidase activity of intracellular fractions expressed by infected *Spodoptera* cells per 5.0 X 10^5 cells in a milliliter of culture broth was compared (Table 2). The highest activity of expressed β-galactosidase was observed by infected Sf21 cells, and the enzyme activity amounted to 1.4 X 10^5 units per 5.0×10^5 cells. Hink et al. (1990) compared the expression level of *E. coli* p-galactosidase using baculovirus vector at a MOI of 20 in 23 insect cell lines, and they concluded that the p-galactosidase activity by *S. frugiperda* Sf9 cells (8.5 X 10^4 units per 10^6 cells) was the highest among the others, including Sf21 cells. Ogonah et al. (1991) reported that *E. coli* p-galactosidase activity by *S. frugiperda* Sf9 cells infected with different baculuvirus (pAcNPV246) at a MOI of 5 was 1.7~ 10^5 units per 10^6 cells. Therefore, the *E. coli* β-galactosidase expression level by Sf21 cells was 3.3 times higher than that of Sf9 cells reported by Hink et *al.* (1990), and was found to be 1.6 times higher than that reported by Ogonah (1991).

Baculovirus-insect cell systems, as a high-level protein expression system, have been developed but only a few published results of recombinant proteins approach the 1 g/liter potential associated with the polyhedrin promoter (Miller, 1988), which is expressed at levels approaching 1.2 g/liters (Luckow and Summers, 1988a). The β-galactosidase fusion protein was highly expressed by Sf21 cells infected with Ac360-501 *β-gal* (lane B in Fig.3). The long-term objective of our work is to develop the biological conversion technology for large-scale production of foreign proteins by a baculovirus-insect cell system. Now, it progress to achieve the mass production of *E. coli* β-galactosidase in this baculovirus (Ac360-*501 β-gal*)-insect cell (Sf21) system.

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