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A Simple Overexpression Technique and Purification of CSK, A Mammalian Tyrosine Kinase Using Baculovirus Expression Vector System and its Activity

Satoru Takeuchi
Department of Protein Research, Hibergenome, 85 NE, Takamatsu,
Kahoku, Ishikawa 929-1215, Japan

Abstract: The *Escherichia coli* expression system is the most popular and frequently available system because its various recombinant proteins are economically and quickly obtainable. However, production of cell cycle proteins and toxic genes, in particular, expression of mammalian proteins would be difficult in that system. Baculovirus Expression Vector System (BEVS), which uses insect cells, offers more advantages than the *E. coli* expression system because most of the produced mammalian proteins retain their correct folding and activity. However, cell maintenance costs much time and expense. Our simple technique in BEVS has demonstrated its expression efficiency with such a high level that expression of C-SRC tyrosine kinase (CSK), an oncogenesis controllable protein, increased 2-3 times over that of cultured *Spodoptera frugiperda* Sf9 insect cells in 2 days than by the ordinary technique.

Key words: *E. coli* expression system, Sf9 insect cells, Baculovirus, CSK

INTRODUCTION

The SRC family tyrosine kinases (SFKs) play important roles in cancer tumor progression and metastasis, along with cellular contact, migration, invasiveness, proliferation, disruption and facilitating anti-apoptosis and angiogenesis, as well as signaling process regulation in normal cells[1-4]. Interestingly, the activities of all SFKs are controlled by phosphorylation of conserved tyrosine residues (tyrosine 527 in the case of c-SRC) in C-termini of the SFKs by C-SRC tyrosine kinase (CSK) and CSK homologous kinase (CHK). Clarifying the transformation mechanism is important because cell transformation is caused by uncontrolled phosphorylation. *Spodoptera frugiperda* Sf9 cell suspension culture was performed to produce CSK, a key protein in cancer tumors. However, the ordinary technique costs much time and effort to produce CSK in sufficient quantities. Using a simple technique that adds a small amount of Fetal Bovine Serum (FBS) was immediately applied to Sf9 cell suspension culture before infecting the cells using recombinant Baculovirus, CSK expression increased 2-3 times over that of the ordinary technique. The success will provide instructive information for experimental CSK-based protein therapy of colon cancers. And also, the simple technique might be applicable to production of other tyrosine kinases such as CSK from other sources and CHK.

MATERIALS AND METHODS

Growth measurement of Sf9 cells and SDS-PAGE analysis of CSK expression: A Sf9 cell suspension culture was performed using Grace's medium (Invitrogen) including 10% FBS, based on the manufacturer's instruction manual (Invitrogen). Harvested viable cells were counted using trypan blue dye exclusion every day. The Sf9 cells were infected using recombinant Baculovirus expressing CSK (RZPD, RZPDo834E0634-pDEST10, N-His6) when the cell density reached 1.5-2×10^6 cells mL^{-1} of log-phase. Normally, after 4 days, the Sf9 cells were harvested and lysed in 50mM HEPES (pH 7.4) with 0.15M NaCl, 2% Nonidet P40 (NP40), 5mM dithiothreitol (DTT), 10 μg mL^{-1} leupeptin, 10 μg mL^{-1} aprotinin and 1mM phenyl methane sulfonyl fluoride (PMSF). After centrifugation to remove insoluble fractions, supernatants in Laemmli sample buffer were subjected to SDS-PAGE (10% acrylamide) and visualized by Coomasie Brilliant Blue R-250 staining and CSK expression was confirmed.

Simple technique's effect on CSK expression: The effect on CSK expression by adding FBS prior to recombinant Baculovirus infection was examined at amounts of 10, 25 and 50 mL of FBS addition. Change of CSK expression was analyzed using SDS-PAGE, as described.

Purification and activity assay of CSK: Sf9 cells expressing CSK were freeze-thawed 3 times in 50 mM HEPES (pH 7.4) with 0.5M NaCl, 10 mM imidazole, 1 mM DTT and 1mM PMSF. After homogenization and centrifugation, CSK was purified by passing supernatants including the CSK through TALON metal resin (Clontech) column. Finally, the active fractions were processed on a Sephacryl S-400 High-Resolution column (resin; Amersham Biosciences, column; BIO-RAD, 1.5×50 cm empty column) pre-equilibrated using 50 mM HEPES (pH 7.4) with 0.15 M NaCl, 1mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM PMSF. CSK purity was evaluated by SDS-PAGE analysis as described.

CSK activity is evaluated by measuring its molar amount when phosphate is incorporated into the synthetic polyamino acid poly(Glu-Tyr) 4:1 substrate (Sigma) per minute per mg protein at 30°C using 50 μM[^{32}P] of ATP (a final concentration)[5].

RESULTS AND DISCUSSION

The produced recombinant proteins using Baculovirus Expression Vector System (BEVS) retain proper folding including S-S bond formation, tertiary structure formation with chemical functionalization and oligomerization[6,7]. Furthermore, recombinant protein is producible at levels of 0.1-50% of the total insect cell protein; the produced protein size is not restricted in BEVS. In contrast, overexpression of mammalian protein in the *E. coli* expression system often causes incorrect folding and/or inclusion body occurrence of the protein[8]. Consequently, BEVS offers some advantages in comparison to the *E. coli* expression system.

Generally, insect Sf9 cell culture should be started at an initial density of approximately 5×10^5 cells mL^{-1} (Fig. 1A) under constant stirring at 90rpm in a spinner flask at 27°C. The Sf9 cell maintenance of suspension cultures requires subculturing when the cell density reaches ca. 2×10^6 cells mL^{-1} (2-3 times a week). Routine processes then remove 65-75% of the cell suspension and replace it with fresh medium. Recombinant protein is produced moderately when the cells are infected using recombinant Baculovirus of optimal amounts and titers at the log-phase of the cell growth (after 70 h culture in the case of Fig. 1A). First, recombinant CSK showed little or no expression in Sf9 cells. Through experience, the CSK expression was observed through recombinant Baculovirus-infection times 2 days (Fig. 1B). Finally, the ordinary technique demonstrated CSK expression reaching a maximal level of 20 mg per 1 litter of Sf9 cell suspension culture at the recombinant Baculovirus-infection time of 4 days based on SDS-PAGE analysis.

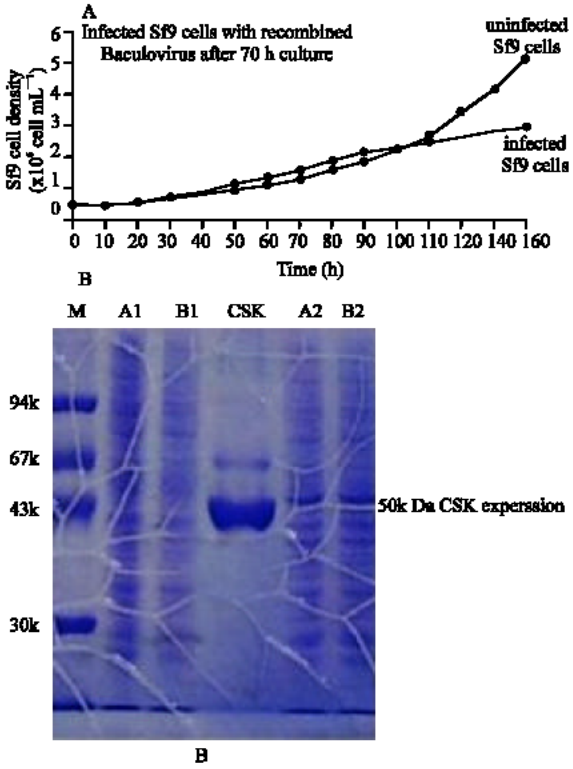

Fig. 1: Growth curves of Sf9 cells in normal suspension cultures and SDS-PAGE analysis of CSK expression in recombinant Baculovirus-infected Sf9 cells. Sf9 viable cell count was performed on harvested Sf9 cells with trypan blue dye exclusion. (A) Growth curves of the Sf9 cells cultured using an ordinary technique were indicated. (B) 15 μL of protein samples (extracted from an equal number of non-infected and infected Sf9 cells) in Laemmli sample buffer were subjected to SDS-PAGE (10% acrylamide) analysis. M indicates Low molecular standard (BIO-RAD). CSK (MW: 50kDa) indicates the purchased sample as a marker (Sigma). Each of A1 and B1 lanes indicates CSK expression in recombinant Baculovirus-infected Sf9 cells (1 day later). Each of A2 and B2 lanes indicates CSK expression in recombinant Baculovirus-infected Sf9 cells (2 days later).

However, a problem exists: it is generally difficult to obtain constant expression of recombinant protein because insect cells are transformed with cellular passage in BEVS. For that reason, we must prepare some monolayer cultures of Sf9 cells with a low passage at least every two months and a recombinant Baculovirus-infected suspension or monolayer culture of the cells at a multiplicity of infections (MOI) of 0.01-0.1 to amplify viral stocks.

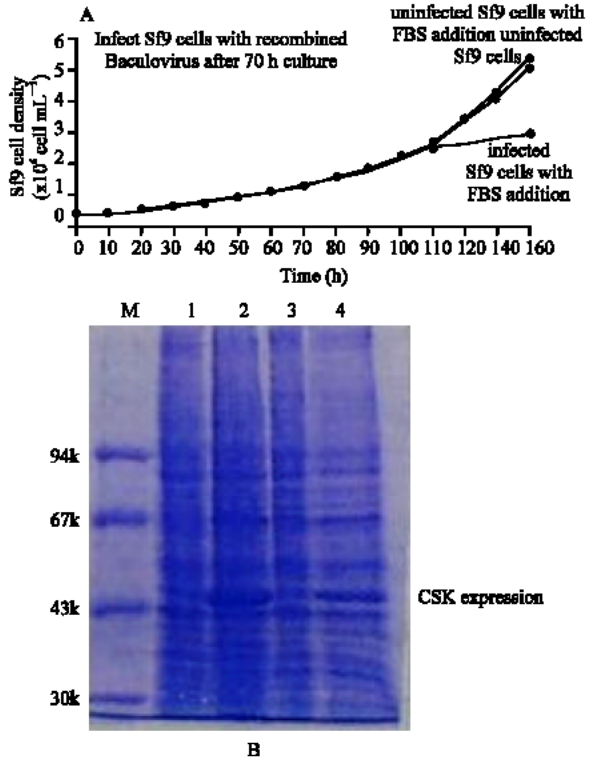

Fig. 2: Growth curves of Sf9 cells in suspension cultures using a simple technique and CSK expression difference in the presence and absence of FBS addition before infecting the cells using recombinant Baculovirus. (A) Growth curves of Sf9 cells cultured using a simple technique are indicated. (B) Protein samples were prepared and subjected to SDS-PAGE analysis as described. M indicates Low molecular standard. Lanes 1 and 2, respectively indicate the CSK expression caused by recombinant Baculovirus infection after 25 mL of FBS addition, 1 and 2 days later. Lanes 3 and 4, respectively indicate the CSK expression caused by an ordinary recombinant Baculovirus infection, 1 and 2 days later.

Different studies concerning protein require large quantities of protein. Furthermore, production on a commercial basis requires economical and quick methods for protein mass production. Therefore, we examined CSK expression in Sf9 cell suspension culture in the following conditions:

- Aeration in suspension culture
- Infection timing
- Feature of serum and its amount in medium (except for a difference of LOT (i.e., product number))
- Addition amount of Baculovirus (with recommended titers)

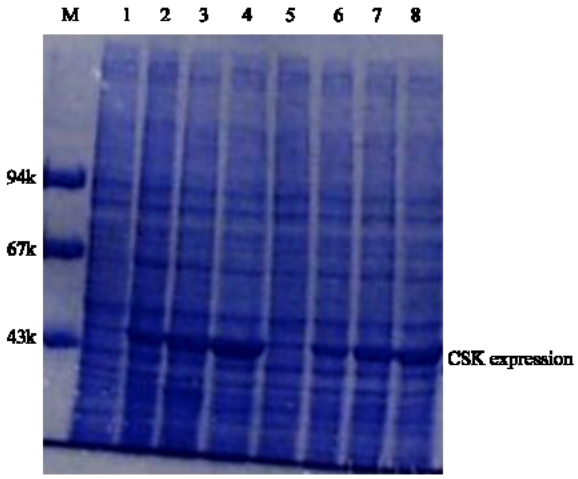

Fig. 3: Correlation with added amounts of FBS and CSK expression. Protein samples were prepared and subjected to SDS-PAGE analysis as described. M indicates Low molecular standard. Lanes 1, 2, 3 and 4, respectively indicate CSK expression in the case of 10 mL of FBS addition, 1, 2, 3 and 4 days later. Lanes 5, 6, 7 and 8, respectively indicate CSK expression in the case of 50 mL of FBS addition, 1, 2, 3 and 4 days later.

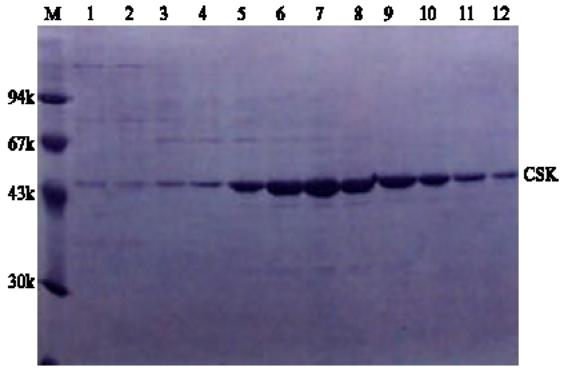

Fig. 4: Analysis of Gel filtration step using SDS-PAGE. Recombinant CSK was purified as described. 15 μL of CSK samples were prepared and subjected to SDS-PAGE analysis as described. M indicates Low molecular standard. Other lanes indicate the final elution step of CSK.

First, we reduced the speed of rotation to limit Sf9 cell damage in the suspension culture. Although the speed of rotation was reduced to 50rpm, CSK expression was unchanged. The expressed Sf9 cells were slight in the monolayer culture, whereas CSK tended to express at a high level in the monolayer culture rather than in the suspension culture. Second, we examined infection timing

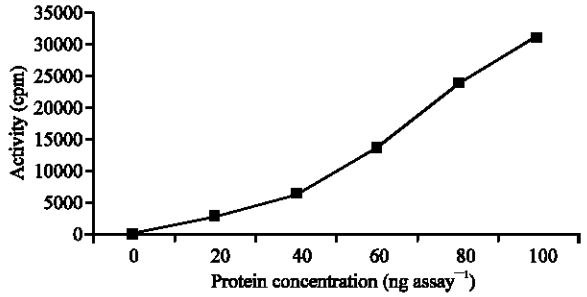

Fig. 5: Kinase assay Kinase activity of CSK was measured in 50mM HEPES (pH 7.4) reaction buffer with 5mM $MgCl_2$ in the presence of ATP and poly (Glu-Tyr) as described.

by which Sf9 cells infect using recombinant Baculovirus at the latter half of cell growth (after 120 h culture in the case of Fig. 1A). However, the total amount of CSK showed no change. Third, Sf9 cells were cultured using various media, each including 10% newborn calf serum (NCS), 10% calf bovine serum (CBS), or 10% donor horse serum (DHS). In this experiment, CSK expression was down-regulated by these serums. In addition, they were cultured in media including amounts of FBS of 3, 5, 8 and 10%, but CSK showed no expression in percentages of < 8% (data not shown). Finally, we examined difference of CSK expression by varying the added amounts of the recombinant Baculovirus retained the recommended titers. Reduction of CSK production resulted from the diluted Sf9 cell suspension culture by increasing the Baculovirus amounts. For consideration, we noted and examined the effective usage of FBS as a growth enhancer. We carried out one technique by which FBS is added to Sf9 cell suspension culture before infecting the cells using recombinant Baculovirus. First, we preliminarily examined the effect on Sf9 cell growth by adding 25 mL of FBS to the cell suspension culture. The technique shows a significant difference in CSK expression, although the effect appears to be inactive in the cell growth as shown in Fig. 2A. Surprisingly, the expression efficiency of CSK with the technique was much higher than that of CSK in the case of no FBS addition (Fig. 2B). Respective correlations with additional amounts of FBS and CSK expression were confirmed by adding 10 and 50 mL of FBS to 500 mL of Sf9 cell suspension culture. Consequently, the expression seems to be high in the case of 10 mL of FBS addition, as shown in Fig. 3. Our simple technique shows that the total amount of the produced CSK at recombinant Baculovirus-infection time for 4 days using an ordinary technique can be obtained at infection times of at least 2 days. As shown in Fig. 4, we could be purified the CSK quickly and greatly due to the reduction effect of Sf9 cellular endogenous proteins was caused by the simple technique (i.e., by CSK overexpression), compared with our preliminary experiment (Fig. 1B) and previous study[9]. Unlike *E. coli* expression system, although CSK was overexpressed, most of the CSK was soluble in Sf9 cells using BEVS. Finally, the gel-purified CSK retains kinase activity (Fig. 5), perhaps because of its correct structure shown in previous study[10]. Fortunately, we were able to produce ca. 60mg of active recombinant CSK per liter of Sf9 cell suspension culture at the Baculovirus-infection time of 4 days.

The SRC family comprises nine proto-oncogenic proteins in vertebrates: BLK, FGR, FYN, HCK, LCK, LYN, SRC, YES and YRK. These proteins play important roles in normal cells and cancer tumors as described above. Establishment of the mass-production and purification methods of these proteins will contribute markedly to various bio-industries as well as cancer therapeutic study.

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