Enhanced Production of Recombinant Protein in *Escherichia coli* Using Silkworm Hemolymph

Ji Eun Kim, Eun Jeong Kim, Won Jong Rhee, and Tai Hyun Park*

School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Korea

Abstract The effect of silkworm hemolymph on the expression of recombinant protein in *Escherichia coli* was investigated. The addition of silkworm hemolymph to the culture medium increased the production of recombinant β-galactosidase in *E. coli*. The production was dependent on the concentration of the added silkworm hemolymph, which increased 2-, 5-, and 8-fold in media supplemented with 1, 3, and 5% silkworm hemolymph, respectively. To identify the effective component, the silkworm hemolymph was fractionated by gel filtration column chromatography. A fraction, with a molecular weight of about 30 K was identified as the effective component.

Keywords: *E. coli*, recombinant protein, silkworm hemolymph

INTRODUCTION

Escherichia coli is one of the most popular host organisms for the high-level production of recombinant proteins. It has many advantages, including its rapid cell growth, economic aspects and ease of handling. The efficient production of recombinant proteins depends on many factors, such as the expression level, expression system, cell growth characteristics, biological activity of the protein and economic considerations [1,2]. Many strategies have been developed to increase the productivity of recombinant proteins by optimizing culture conditions [3-7] or using molecular genetic tools [8-10]. However, if productivity could be increased using a medium supplement, this would be the easiest way.

The silkworm, *Bombyx mori*, has been effectively used in various biological researches [11]. Silkworm hemolymph is the most studied insect hemolymph, which can be easily and economically collected as the silkworm is a large and cheaply domesticated insect. Silkworm hemolymph has previously been used as a medium supplement in insect cell cultures [12], and has been successfully used to increase the production of recombinant β-galactosidase in an insect cell-baculovirus system [13,14]. It was also found to inhibit apoptosis in various animal cell systems, including insect and human cells [15-20]. Moreover, the anti-apoptotic component of silkworm hemolymph has been isolated and characterized [21]. In this study, we investigated the effect of silkworm hemolymph on the expression of recombinant protein in *E. coli*.

*Corresponding author
Tel: +82-2-880-8020   Fax: +82-2-875-9348
e-mail: thpark@plaza.snu.ac.kr

MATERIALS AND METHODS

Bacterial Strain and Culture Condition

The *lacZ* gene coding for β-galactosidase was inserted into the pET22b(+)vector, and the β-galactosidase produced under the control of the T7 promoter. *E. coli* BL21(DE3)/pET22b(+) was cultivated in LB broth, and whole or fractionated silkworm hemolymph were used as medium supplements. The components fractionated from silkworm hemolymph (FI and FII) were added to the medium at the amounts contained in 5% silkworm hemolymph. Cells were grown at 37°C and 250 rpm in shake flasks. The *lacZ* gene expression was induced by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactoside).

Fractionation of Silkworm Hemolymph

The silkworm hemolymph was collected from 5th-instar larvae by clipping the side of an abdominal leg. The hemolymph was collected from hundreds of silkworms to make a stock, and the same stock was used throughout the experiments. The collected hemolymph was heat-treated at 60°C for 30 min, and then centrifuged (12,000 rpm, 1 h and 4°C). The supernatant was filtered through a 0.2-μm millipore filter and used for supplementing the medium. Hemolymph prepared using the above method was loaded onto a Superdex 200 HR column (1 × 30 cm, Amersham-Pharmacia Biotech) and eluted with 50 mM sodium phosphate buffer (pH 7.0), containing 0.15 M NaCl, at a flow rate of 0.5 mL/min. The eluent was monitored at 280 nm, and 1 mL fractions collected.

Analytical Procedure

The β-galactosidase activity was assayed by measuring

Table 1. Effect of silkworm hemolymph (SH) on the extracellular, intracellular, total, and specific β-galactosidase activities. The activity was measured at its maximum point (8 h)

Concentration of SH added to medium (%)	Extracellular β-galactosidase activity (10^6 unit/L)	Intracellular β-galactosidase activity (10^6 unit/L)	Total β-galactosidase activity (10^6 unit/L)	Specific activity of β-galactosidase (10^6 unit/g cell)
0	0.113	1.078	1.191	0.655
1	0.893	1.979	2.872	1.642
3	0.992	5.549	6.541	3.479
5	1.385	9.018	10.403	5.361
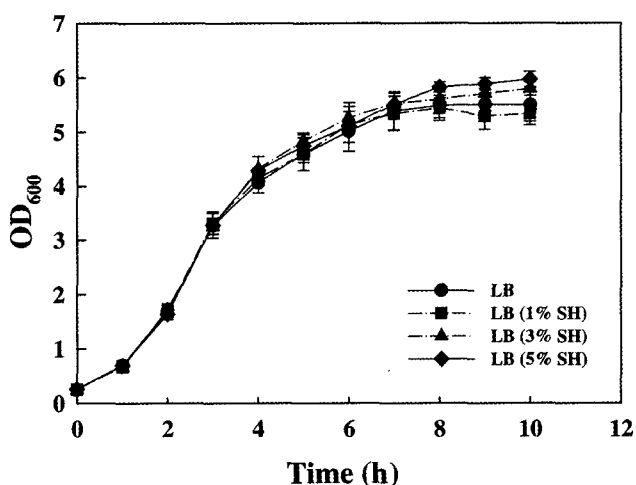
Fig. 1. Effect of silkworm hemolymph on cell growth.

Fig. 2. Effect of silkworm hemolymph on the total activity of β-galactosidase.

the hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG), using the method described by Miller [22]. The cell concentration was measured by a spectrophotometer (SPECTRONIC® GENESYS™). The extracellular β-galactosidase activity was determined from the sample supernatant after centrifugation (13,000 rpm, 5 min, 4°C). To disrupt the cells, the pellet was resuspended in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 $MgSO_4$, 0.05 M 2-mercaptoethanol, pH 7.0) and treated with toluene, and after the toluene had been evaporated, the cell debris was separated by centrifugation. (13,000 rpm, 20 min, 4°C). The supernatant obtained was used to assay the intracellular β-galactosidase activity. To compare of the amount of protein with protein activity, SDS-PAGE was carried out using a 7.5% polyacrylamide separating gel and 5% polyacrylamide stacking gel, employing Laemmli's method [23].

RESULTS AND DISCUSSION

To investigate the effect of silkworm hemolymph on recombinant protein expression, silkworm hemolymph was added at various concentrations to the culture medium. Silkworm hemolymph contains nutrients that may affect cell growth. The cell growth was slightly activated by the addition of silkworm hemolymph to the M9 minimal me-
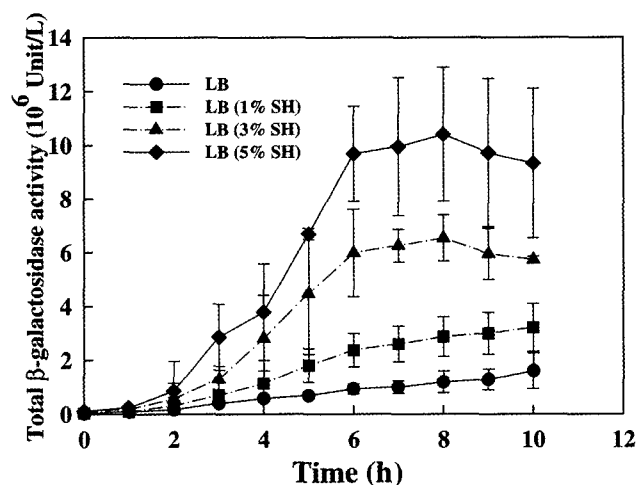
dium (data not shown). However, when cells were cultured in rich medium (LB), the cell growth was not significantly influenced by the addition of silkworm hemolymph, as shown in Fig. 1.

Fig. 2 shows the effect of silkworm hemolymph on the production of recombinant β-galactosidase. The y-axis represents the total β-galactosidase activity, which is the sum of the intracellular and extracellular β-galactosidase activities. This activity increased with increasing additions of silkworm hemolymph. The addition of 1, 3, and 5% silkworm hemolymph increased the production of recombinant β-galactosidase by 2-, 5-, and 8-fold, respectively; maximum activity was reached at 6 h in most cases.

More detailed data are summarized in Table 1, which shows the extracelluar, intracellular, total, and specific β-galactosidase activities. These activities were measured after they had reached their maxima. Although the extracellular activity was significantly lower than the intracellular activity in every case, it also increased on the addition of silkworm hemolymph. Since the addition of silkworm hemolymph does not significantly influence the cell concentration, as shown in Fig.1, its effect on the specific activity showed a similar trend to that exerted on the total activity; the specific activity represents the activity per gram of cells.
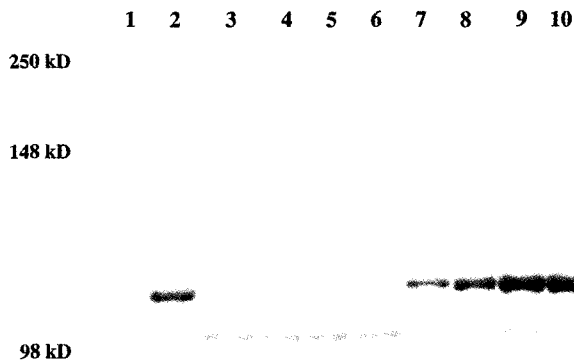
The results shown in Fig. 2 and Table 1 may indicate

Fig. 3. Effect of silkworm hemolymph on the production of β-galactosidase. Lane 1: Marker, Lane 2: Standard β-galactosidase (116 kD), Lanes 3~6: 0, 1, 3, and 5% SH (before induction), Lanes 7~10: 0, 1, 3, and 5% SH (4 h after induction).

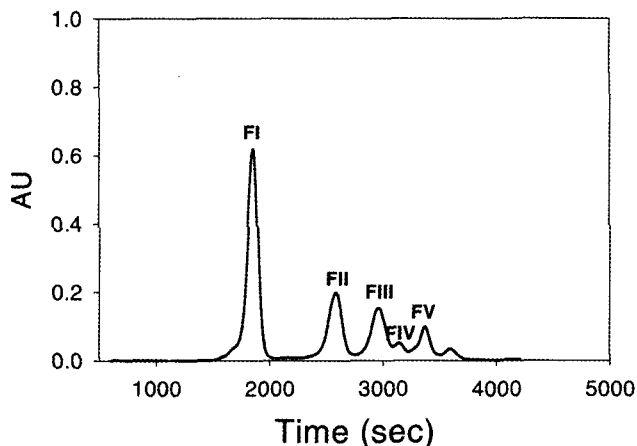Fig. 5. Effect of fractions FI and FII on the β-galactosidase activity.

Fig. 4. Fractionation of silkworm hemolymph by gel filtration chromatography.

that silkworm hemolymph increased the production of recombinant protein. However, we can also think another possibility that silkworm hemolymph may improve the enzyme activity of β-galactosidase. In this case, for example, β-galactosidase produced in the presence of silkworm hemolymph may have different protein structure. To examine these two possibilities, the effect of silkworm hemolymph on the production of β-galactosidase was confirmed by SDS-PAGE, as shown in Fig. 3. Lanes 3~6 show the results for 0, 1, 3 and 5% concentrations of silkworm hemolymph prior to the induction of β-galactosidase by IPTG; lanes 7~10 show the results at 6 h (*i.e.* 4 h after induction). The results show that silkworm hemolymph increased the production of β-galactosidase. At this point (*i.e.* 4 h after induction), the intracellular activities per gram of cells were 0.509×10^6, 1.068×10^6, 3.079×10^6 and 5.171×10^6 unit/g cell for 0, 1, 3 and 5% concentrations of silkworm hemolymph, respectively. This result indicates that silkworm hemolymph increased
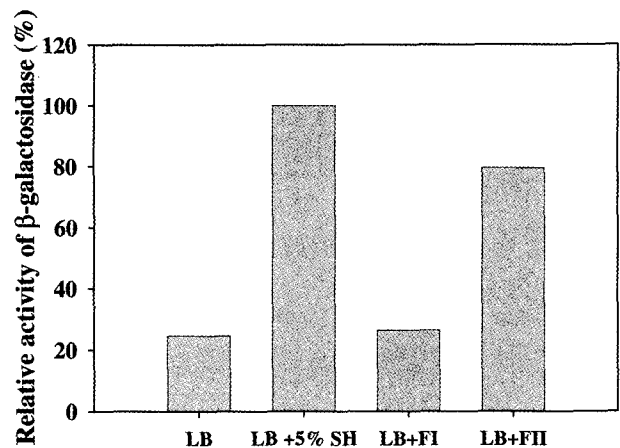
the amount of β-galactosidase produced, and the second possibility mentioned above is not the case.

To identify the effective component, the silkworm hemolymph was fractionated by gel-filtration chromatography, and produced five fractions, as shown in Fig. 4. Among these fractions, FI and FII, which were the major fractions, were used, as the amounts of protein in the other fractions were insignificant, so these were not used. Although fraction FIII showed a significant peak in the chromatogram, no significant amount of protein was detected by SDS-PAGE. The molecular mass of the major protein contained in FI was ca. 70,000 Da, which accounted for about 60% of the silkworm hemolymph proteins. The major protein contained in FII was found to have a molecular mass of ca. 30,000 Da, which accounted for 30% of the protein present. These two proteins accounted for almost all of the hemolymph protein [21].

Fig. 5 shows the effects of FI and FII on the activity of β-galactosidase. The amounts of FI and FII added to the medium were equivalent to that contained in 5% silkworm hemolymph. FI showed the same activity as the negative control (LB without silkworm hemolymph), while FII resulted in a positive effect, which was similar to that of the positive control (LB + 5% whole silkworm hemolymph). The percentage relative activity shown on the y-axis represents the β-galactosidase activity relative to that of the positive control (100%).

In this research, it was found that silkworm hemolymph can be effectively used to enhance the efficient production of recombinant protein in *E. coli*. Moreover, except for the addition of the silkworm hemolymph, the other culture conditions were unchanged, implying the components in the silkworm hemolymph had influenced the gene transcription and/or translation. We suggest that silkworm hemolymph may act as a transcription activator or stabilizes mRNA. However, further study will be required to fully understand the mechanism of increased recombinant protein production due to silkworm hemolymph.

CONCLUSION

The addition of a medium supplement is a convenient way of increasing productivity during fermentation. In this article, we investigated the effect of silkworm hemolymph on the expression of recombinant protein in *E. coli*, and obtained an increased level of recombinant β-galactosidase expression by the addition of silkworm hemolymph to the culture medium. The production was dependent on the concentration of the silkworm hemolymph added, and increased 2-, 5- and 8-fold in medium supplemented with 1, 3 and 5% silkworm hemolymph, respectively. Although there are many components in silkworm hemolymph that could possibly affect the expression level, FII was found to be the main component responsible for this effect. Therefore, this silkworm hemolymph fraction can be used to supplement the medium for enhanced recombinant protein production.

REFERENCES

[1] Hockney, J. (1993) Expression systems: A user's guide. *Bio/Technology* 11: 887-893.

[2] Marino, M. H. (1989) Expression systems for heterologous protein production. *BioPharm.* 2: 18-33.

[3] Yoon, S. K., W. K. Kang, and T. H. Park (1994) Fed-batch operation of recombinant *E. coli* containing *trp* promoter with controlled specific growth rate. *Biotechnol. Bioeng.* 43: 995-999.

[4] Yoon, S. K., S. H. Kwon, M. G. Park, W. K. Kang, and T. H. Park (1994) Optimization of recombinant *Escherichia coli* fed-batch fermentation for bovine somatotropin. *Biotechnol. Lett.* 16: 1119-1124.

[5] Yoon, S. K, W. K. Kang, and T. H. Park (1996) Regulation of *trp* promoter for production of bovine somatotropin in recombinant *Escherichia coli* fed-batch fermentation. *J. Ferment. Bioeng.* 81: 153-157.

[6] Park, S. and T. H. Park (2000) Analysis of two-stage continuous operation of *Escherichia coli* containing bacteriophage λ vector. *Bioprocess Eng.* 23: 557-563.

[7] Ramisetti, S., H. A. Kang, S. K. Rhee, and C. H. Kim (2003) Production of recombinant hirudin in galactokinase-deficient saccharomyces cerevisiae by fed-batch fermentation with continuous glucose feeding. *Biotechnol. Bioprocess Eng.* 8: 183-186.

[8] Choi, J. I. and S. Y. Lee (2004) High level production of supra molecular weight poly(3-hydroxybutyrate) by metabolically engineered *Escherichia coli*. *Biotechnol. Biopro-*

cess Eng. 9: 196-200.

[9] Lin, C. S., B. Y. Chen, T. H. Park, and H. C. Lim (1998) Characterization of bacteriophage λ Q' mutant for stable and efficient production of recombinant protein in *Escherichia coli* system. *Biotechnol. Bioeng.* 57: 529-535.

[10] Kim, T. S. and T. H. Park (2000) Optimization of bacteriophage λ Q'-containing recombinant *Escherichia coli* fermentation process. *Bioprocess Eng.* 23: 187-190.

[11] Yun, E. S. and T. H. Park (2000) Quantitative measurement of general odorant using electoantennogram of male silkworm moth, *Bombyx mori. Biotechnol. Bioprocess Eng.* 5: 150-152.

[12] Ha, S. H., T. H. Park, and S. E. Kim (1996) Silkworm hemolymph as a substitute for fetal bovine serum in insect cell culture. *Biotech. Tech.* 10: 401-406.

[13] Ha, S. H. and T. H. Park (1997) Efficient production of recombinant protein in *Spodoptera frugiperda*/AcNPV system utilizing silkworm hemolymph. *Biotechnol. Lett.* 19: 1087-1091.

[14] Woo, S. D., W. J. Kim, H. S. Kim, J. Y. Choi, B. R. Jin, and S. K. Kang (1997) Effect of silkworm hemolymph on the expression of *E. coli* β-galactosidase in insect cell lines infected with recombinant baculovirus. *Mol. Cell* 7: 572-574.

[15] Rhee, W. J., E. J. Kim, and T. H. Park (1999) Kinetic effect of silkworm hemolymph on the delayed host cell death in an insect cell-baculovirus system. *Biotechnol. Prog.* 15: 1028-1032.

[16] Rhee, W. J. and T. H. Park (2000) Silkworm hemolymph inhibits baculovirus-induced insect cell apoptosis. *Biochem. Biophys. Res. Commun.* 271: 186-190.

[17] Rhee, W. J. and T. H. Park (2001) Flow cytometric analysis of the effect of silkworm hemolymph on the baculovirus-induced insect cell apoptosis. *J. Microbiol. Biotechnol.* 11: 853-857.

[18] Rhee, W. J., E. J. Kim, and T. H. Park (2002) Silkworm hemolymph as a potent inhibitor of apoptosis in Sf9 cells. *Biochem. Biophys. Res. Commun.* 295: 779-783.

[19] Choi, S. S., W. J. Rhee, and T. H. Park (2002) Inhibition of human cell apoptosis by silkworm hemolymph. *Biotechnol. Prog.* 18: 874-878.

[20] Kim, E. J. and T. H. Park (2003) Anti-apoptosis engineering. *Biotechnol. Bioprocess Eng.* 8: 76-82.

[21] Kim, E. J., W. J. Rhee, and T. H. Park (2001) Isolation and characterization of an apoptosis-inhibiting component from the hemolymph of *Bombyx mori. Biochem. Biophys. Res. Commun.* 285: 224-228.

[22] J. H. Miller (1972) *Purification of β-Galactosidase. In: Experiments in Molecular Genetics.* pp. 398-404. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

[23] U. K. Laemmli (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.