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Expression of Recombinant Human Growth Hormone in a Soluble Form in *Escherichia coli* by Slowing Down the Protein Synthesis Rate

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Abstract Formation of inclusion bodies is usually observed when foreign proteins are overexpressed in *E. coli*. The formation of inclusion bodies might be prevented by lowering the rate of protein synthesis, and appropriate regulation of the protein expression rate may lead to the soluble expression. In this study, human growth hormone (rhGH) was expressed in a soluble form by slowing down the protein synthesis rate, which was controlled in the transcriptional and translational levels. The transcriptional level was controlled by the regulation of the amount of RNA polymerase specific to the promoter in front of the rhGH gene. For lowering the rate of translation, the T7 transcription terminator-deleted vector was used to synthesize the longer mRNA of the target gene because the longer mRNA is expected to reduce the availability of free ribosomes. In both methods, the percentage of soluble expression increased when the expression rate slowed down, and more than 93% of rhGH expressed was a soluble form in the T7 transcription terminator-deleted expression system.

Keywords: Soluble form, inclusion body, T7 transcription terminator, human growth hormone, *E. coli*

E. coli has been widely investigated for various purposes such as the production of industrial chemicals [25], regulatory analysis of pathways [11, 14], and effective gene manipulation [2, 20]. Overexpression of recombinant proteins is important in every case; however, the overexpression in *E. coli* often results in the formation of inclusion bodies. Inclusion bodies are insoluble aggregates of the recombinant protein and do not have biological activity. Various reasons have been suggested to account for the formation of inclusion bodies when eukaryotic proteins are expressed in *E. coli*. One of the reasons is that the cytoplasm of *E. coli* is a very reducing environment that may prevent the formation of disulfide bonds. It was also suggested that the inability of *E. coli* to glycosylate the protein may reduce the solubility of the intermediate, resulting in inclusion bodies. Although the inclusion body has some advantages such as high level of expression, protection from protease attack, and production of protein nanoparticles [23], it causes a complex process, low yield, and mass buffer requirement for recovery [7, 21]. To produce foreign proteins as a soluble form in *E. coli*, there have been many efforts made such as culture at lower temperature [24], use of enriched media [16], culture with some additives [17], secretion of the expressed protein [5, 15, 19], fusion of the target protein with soluble fusion partners [3, 4, 6, 13, 18], and coexpression with molecular chaperone and foldase [1, 12, 22].

There have been many successful results to express the foreign protein as a soluble form in *E. coli*, but no universal method exists. The feature of inclusion body formation is that it only appears when a foreign protein is overexpressed in *E. coli*. Thus, overexpression of protein is a common reason for the formation of inclusion bodies, and the rate of protein expression and folding may determine the solubility of the expressed protein [8]. Whereas glycosylation is important for the biological activity of glycosylated proteins [10], human growth hormone is a non-glycosylated protein consisting of 191 amino acids and also often results in the formation of inclusion bodies in *E. coli*. In this study, we tried to express rhGH in a soluble form in *E. coli* by lowering the target protein expression rate. The expression rate was controlled in the transcriptional and translational levels. The amount of RNA polymerase and the length of mRNA transcript were regulated for this purpose.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

The host strain used in this study was *E. coli* BL21(DE3) [F^- *ompT* *hsd*$S_B(r_B^- m_B^-)$ *gal dcm* (DE3)]. This strain

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carries the T7 RNA polymerase gene under the control of the *lac* promoter in its chromosome. The plasmid pET-3a (Novagen, Madison, WI, U.S.A.) harboring rhGH was provided by the Daewoong Biotechnology Research Center (Yongin, Korea), and named pET-hGH. This plasmid contains the T7 promoter and termination sites. It carries 10 histidine residues and enterokinase cleavage site (EK) at the N-terminus of rhGH for the efficient purification of rhGH. Another plasmid, which does not contain a T7 termination site, was constructed. The His10-EK-hGH DNA fragment was amplified by PCR with a forward primer (5'-ATA CAT ATG GGC CAT CAT CAT CAT CAT CAT-3') and reverse primer (5'-CTA AAG CTT TTA CTA GAA GCC ACA GCT GCC-3'). The primers contain NdeI and HindIII sites, respectively. The PCR product was inserted into the NdeI-HindIII sites of the plasmid pET-3a to construct the T7 terminator-deleted plasmid, which was named pET-hGH$_{\Delta T7T}$.

Cell Culture and Protein Expression

The medium consisted of 20 g of yeast extract, 10 g of casamino acid, 0.24 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 3 g of KH_2PO_4, 2.5 g of $(NH_4)_2HPO_4$, 5 g of glucose, and 100 mg of ampicillin per liter. Seed culture was grown in 50 ml of medium in a shaking incubator at 37°C, 250 rpm, for 16 h. Main culture was carried out in a 2.5-l jar fermentor (Ko-Biotech, Incheon, Korea) containing 1 l of medium. The pH was controlled at 6.8 by adding 5 N HCl and 50% (v/v) NH_4OH, and the dissolved oxygen concentration was maintained above 30% air saturation. The rhGH expression was induced by adding isopropyl thio-β-D-galactopyranoside (IPTG) to the cultures at various concentrations.

Analysis of Protein Expression

Cultured cells were collected by centrifugation at 10,000 ×*g* for 2 min at room temperature. The cell pellets were resuspended with sonication buffer (20 mM Tris, 0.5% Triton X-100, 1 mM EDTA, pH 8.0) and disrupted by sonication for 1.5 min on ice. The sonicated samples were centrifuged at 22,000 ×*g* for 20 min at 4°C. The supernatants were used as intracellular soluble protein fractions, and the pellets were washed twice and resuspended in 10 mM PBS (pH 7.2) and used as inclusion body fractions. The protein expression was analyzed by SDS-PAGE, and the total protein concentration was measured by the Bradford assay. The amount of the rhGH in each sample was determined by scanning the SDS-PAGE gel using the Total Lab v1.10 program (Nonlinear Dynamics, Durham, NC, U.S.A.) and comparing the bands of the rhGH with the band of a known amount of protein.

Western Blot Analysis of T7 RNA Polymerase

Six hours after IPTG addition, the same amounts of cells were collected and the intracellular soluble fractions were prepared by the method mentioned above. The samples were loaded on SDS-polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The membrane was probed with T7 RNA polymerase monoclonal antibody (Novagen, Madison, WI, U.S.A.), followed by incubation with HRP-conjugated goat anti-mouse IgG (Novagen, Madison, WI, U.S.A.). The immunoreactive bands were visualized using the ECL detecting system (Amersham Bioscience, Uppsala, Sweden).

Northern Blot Analysis

To analyze the rhGH mRNA transcripts, 1×10^9/cells were collected and total RNA was isolated using a High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Ten μg of total RNAs was subjected to electrophoresis on a 1.2% formaldehyde-agarose gel and transferred onto a Nylon$^+$ membrane (Invitrogen, Carlsbad, CA, U.S.A.). Preparation of DIG-labeled probe DNA and hybridization were performed using a DIG High Prime DNA Labeling and Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. After hybridization of the target mRNA transcript with DIG-labeled probe DNA, the membrane was washed twice with 2×SSC containing 0.1% SDS for 5 min at room temperature and 0.5×SSC containing 0.1% SDS for 15 min at 68°C. The membrane was treated with anti-DIG-AP conjugate (Roche Diagnostics GmbH, Mannheim, Germany) and visualized with NBT/BCIP solution (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DIG-labeled RNA molecular weight marker I (Roche Diagnostics GmbH, Mannheim, Germany) was used as a molecular size marker.

RESULTS AND DISCUSSION

Regulation of the Rate of rhGH Synthesis

The tendency to form aggregates depends on the rate of expression and the rate of refolding. If the rate of expression exceeds the rate of refolding, the proteins expressed come into contact with one another and aggregate prior to reaching their native state [8]. Appropriate regulation of the protein expression rate may lead to the expression of rhGH in a soluble form. The rate of rhGH synthesis was regulated using two different methods in this study. The first method was regulation of the amount of RNA polymerase specific to the promoter in front of the rhGH gene. This is the method to regulate the rate of rhGH synthesis on the transcriptional level. The *E. coli* BL21(DE3)/pET expression system used in this study has the T7 RNA polymerase gene in the chromosome, and the expression of T7 RNA polymerase gene is under the control of the *lac*

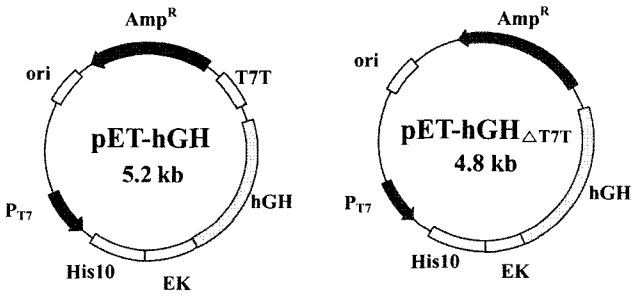

Fig. 1. Schematic diagram of the vectors, pET-hGH and pET-$hGH_{\Delta T7T}$. P_{T7} is a T7 promoter.
Ten histidine residues (His10) and the enterokinase cleavage site (EK) at the N-terminus of the human growth hormone are for the metal affinity chromatography purification and the cleavage of the target protein, respectively. T7T represents the T7 transcription terminator. Amp^R is an ampicillin-resistant gene that produces β-lactamase.

UV promoter. The target rhGH gene, which is under control of the T7 promoter, is located in the pET vector. When IPTG is added, it induces the expression of T7 RNA polymerase, the expressed T7 RNA polymerase binds to the T7 promoter, and the target protein is expressed [26, 27]. Therefore, it is expected that the rate of target protein expression depends on the amount of T7 RNA polymerase synthesized, which can be regulated by the concentration of IPTG.

In the second method, it was assumed that the protein synthesis rate would slow down if the longer mRNA of the target gene is synthesized. The longer mRNA is expected to reduce the availability of free ribosomes because it retains the ribosomes on it for a longer period. Although the longer mRNA also retains the RNA polymerase for a longer period, the availability of free ribosomes rather than RNA polymerase is the limiting factor for the target gene expression. RNA polymerase is expressed sufficiently with high concentration of IPTG. Therefore, this method is for the regulation of the rate of rhGH expression on the translational level. To synthesize the longer mRNA of the target gene, the T7 transcript terminator was deleted from the pET vector, and the T7 transcript terminator-deleted vector was named pET-$hGH_{\Delta T7T}$ as mentioned before. The expression vectors used in this study are illustrated in Fig. 1. The only difference between pET-hGH and pET-$hGH_{\Delta T7T}$ is the T7 transcription terminator. Both plasmids have 10 histidine residues and an enterokinase cleavage site at the N-terminal of the target protein for the efficient purification using a Ni-chelating column and to obtain the natural form of the target protein by the site-specific protease reaction.

Effect of the Amount of RNA Polymerase on the Soluble Expression

To investigate the effect of the amount of RNA polymerase, cells containing pET-hGH were cultured in different IPTG concentrations. When cells were cultured without IPTG, the maximum specific growth rate was 1.38 h^{-1}. The specific growth rate decreased to 0.827 and 0.519 h^{-1} with the addition of 0.02 mM and 1.0 mM of IPTG, respectively. However, the final cell concentration was not much different, even when the IPTG was added.

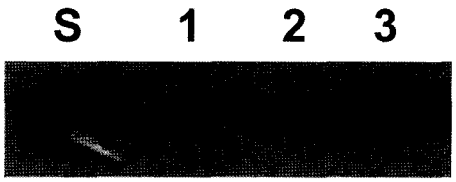

Fig. 2. Western blot analysis of T7 RNA polymerase.
Samples were collected 6 h after the addition of various concentrations of IPTG. The intracellular fractions obtained from the same amount of cell were loaded on SDS-PAGE gel, and Western blot analysis was performed. S, T7 RNA polymerase standard (5 units); 1, 0 mM IPTG; 2, 0.02 mM IPTG; 3, 1 mM IPTG.

Western blot analysis was performed to confirm that the expression of T7 RNA polymerase varies with IPTG concentration. Fig. 2 shows that the amount of T7 RNA polymerase increased with the IPTG concentration. When a small amount of T7 RNA polymerase is synthesized with a low concentration of IPTG, a small amount of rhGH mRNA will be transcribed. This may result in slowing down the rhGH synthesis rate, and consequently, it is expected to provide adequate time for the correct folding of the target protein.

Fig. 3 shows the expression pattern of rhGH at different IPTG concentrations. Even when IPTG was not added, basal-level expression was observed and approximately 90% of rhGH was expressed in a soluble form over the culture time. With the addition of IPTG, the total amount of rhGH increased; however, two-thirds of the rhGH was expressed as an inclusion body. When 0.02 or 1 mM of IPTG was added, the concentration of a soluble form was higher than that of inclusion bodies until approximately 6 h; however, the inclusion body exceeded the soluble form thereafter, as the rhGH accumulated in the cytoplasm. The expression in the soluble form was somewhat higher without IPTG. These results indicate that the regulation for the lower amount of RNA polymerase, which results in a lower transcription rate, is favorable to the soluble expression.

Effect of the mRNA Transcript Length on the Soluble Expression

As mentioned above, a longer mRNA is expected to reduce the availability of free ribosomes and consequently decrease the rate of rhGH synthesis. It was reported that the synthesis of protein in *E. coli* is limited by the concentration of free ribosomes [28], and *E. coli* growing in glycerol minimal medium has approximately 15,000 ribosomes per cell

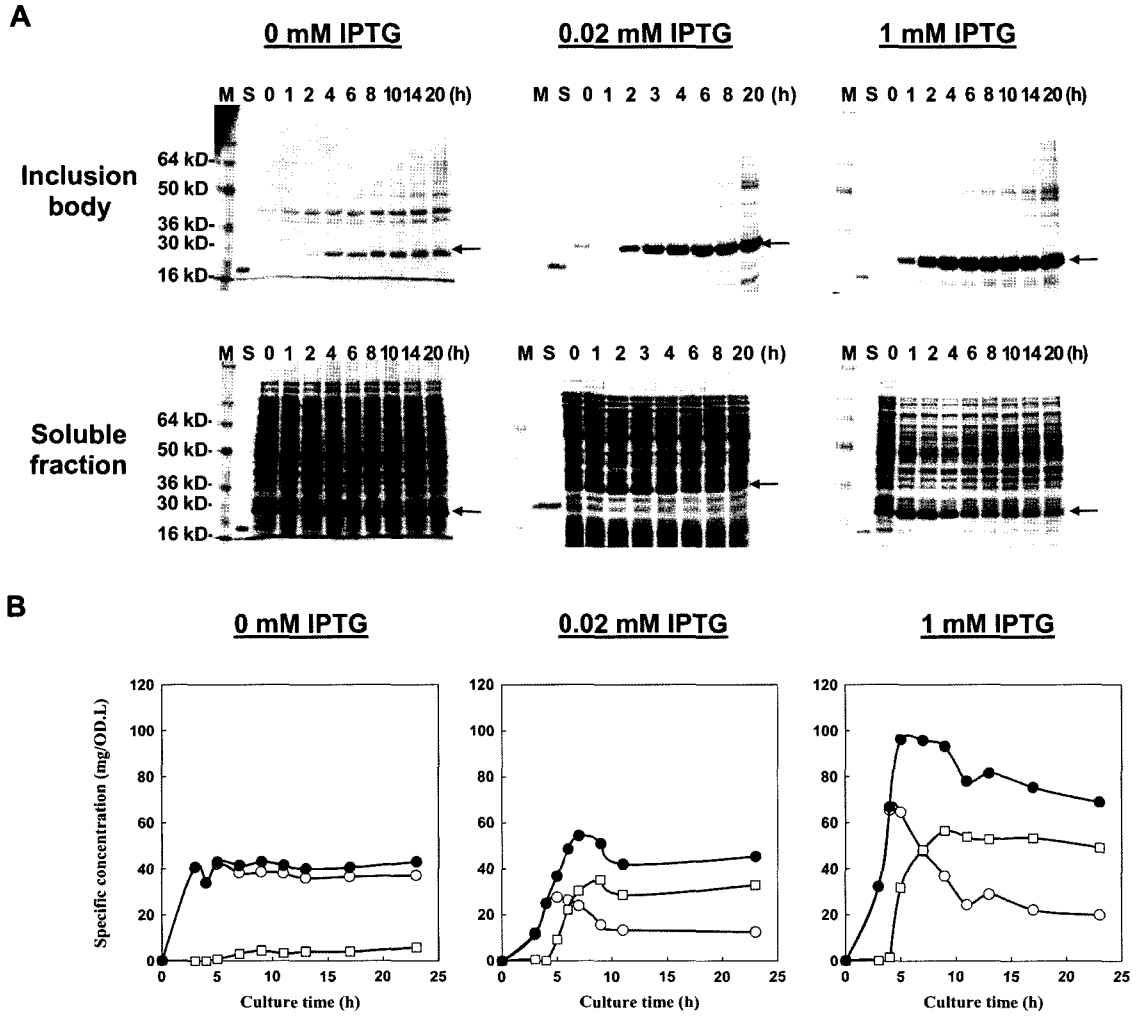

Fig. 3. Expression patterns and levels of rhGH in *E. coli* BL21(DE3)/pET-hGH with different amounts of IPTG addition.
A. SDS-PAGE. IPTG was added to each culture 3 h after inoculation at different concentrations. The expressed rhGH shows a larger size than the standard hGH because of the additional 10 histidine residues and the enterokinase cleavage site. M, molecular size marker; S, hGH standard (2 μg); Numbers represent the time post-induction (h). The arrows indicate rhGH. **B.** The amount of rhGH expressed per unit cell (mg/OD.L). ●, total rhGH; ○, soluble form; □, inclusion body.

[9]. The T7 transcription terminator-deleted vector, pET-hGH$_{\Delta T7T}$, was used to synthesize the longer mRNA of the target gene.

The specific growth rate of the *E. coli* containing pET-hGH$_{\Delta T7T}$ was 1.29 h^{-1} and decreased to 0.787 h^{-1} after the induction with 1 mM of IPTG. Fig. 4A shows the expression pattern of rhGH in *E. coli* BL21(DE3)/pET-hGH$_{\Delta T7T}$. Most rhGH was expressed in a soluble form during the overall cultivation. The β-lactamase gene is located right behind the rhGH gene in this vector. Because the T7 terminator, which is supposed to be between the two genes, was deleted, the β-lactamase gene was also translated from the same transcript. High expression of β-lactamase was also observed as shown in Fig. 4A, whereas the β-lactamase expression was not so high in Fig. 3A. Deletion of the terminator site enabled more than 90% of the total rhGH and β-lactamase to be expressed in a soluble form as shown in Fig. 4B.

Northern blot analysis was performed to confirm that the deletion of the T7 transcription terminator results in the longer mRNA transcript. As shown in Fig. 5, the mRNA band with approximately 700 bases was detected in the presence of the T7 transcription terminator. This corresponds to the size of the rhGH gene. In contrast, the mRNA band with approximately 2,000 bases was detected for the cells containing the pET-hGH$_{\Delta T7T}$. This corresponds to the size of the region from the rhGH gene to the β-lactamase gene in the pET-hGH$_{\Delta T7T}$. Compared with the mRNA from pET-hGH (lanes 1 and 2), the amount of mRNA produced from pET-hGH$_{\Delta T7T}$ was much less. This is believed to be caused by the lower availability of free T7 RNA polymerase because the longer mRNA retains the RNA polymerase

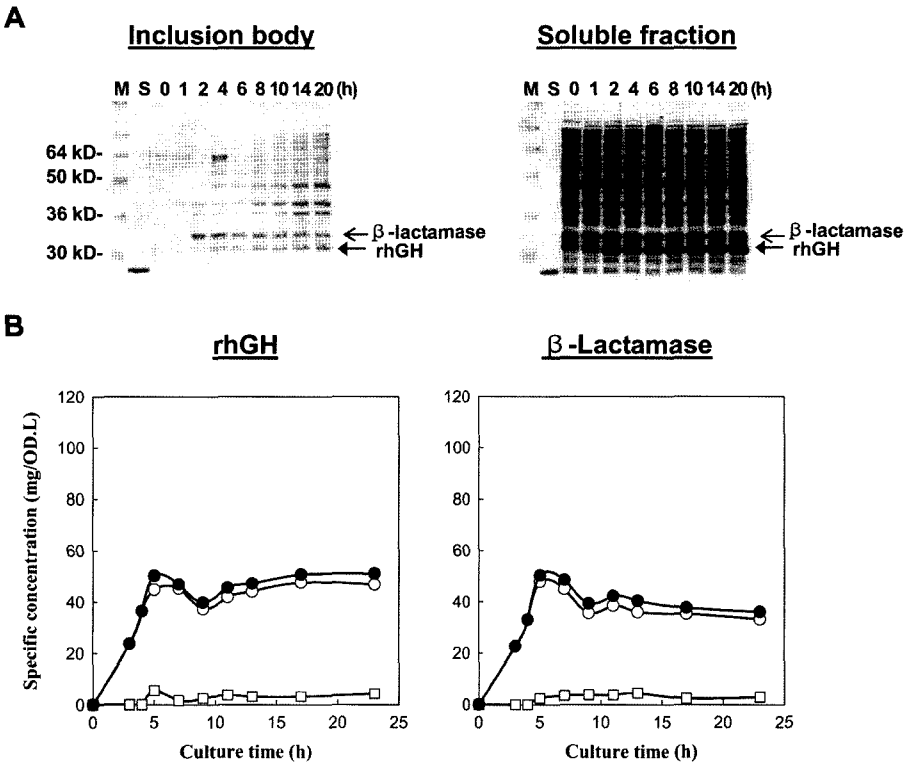

Fig. 4. Expression patterns and levels of rhGH and β-lactamase in *E. coli* BL21(DE3)/pET-hGH$_{\Delta T7T}$.
A. SDS-PAGE. 1 mM IPTG was added to the culture 3 h after inoculation. M, molecular size marker; S, rhGH standard (2 μg). Numbers represent the time post-induction (h). **B.** The amount of rhGH and β-lactamase expressed per unit cell (mg/OD.L). ●, total; ○, soluble form; □, inclusion body.

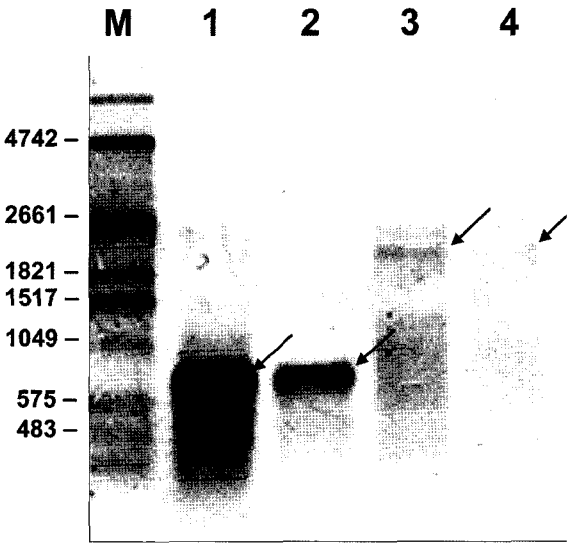

Fig. 5. Northern blot analysis of the mRNAs synthesized from pET-hGH and pET-hGH$_{\Delta T7T}$.
Samples were collected 3 and 4 h after IPTG addition. Ten μg of total RNAs was loaded on a 1.2% formaldehyde-agarose gel and Northern blot analysis was performed. M, RNA molecular weight marker; 1, total RNAs from pET-hGH 3 h after IPTG addition; 2, total RNAs from pET-hGH 4 h after IPTG addition; 3, total RNAs from pET-hGH$_{\Delta T7T}$ 3 h after IPTG addition; 4, total RNAs from pET-hGH$_{\Delta T7T}$ 4 h after IPTG addition. The arrows indicate the mRNAs detected.

longer on it. This implies that the rate of rhGH synthesis was restricted by not only the translational level but also the transcriptional level in this method. The lower amount and longer length of the mRNA contributed to a slowdown of the protein synthesis rate.

The expression pattern and amount of rhGH per unit cell are summarized in Table 1 for the cases with and without the T7 transcription terminator, when the IPTG concentration was 1 mM. Approximately 69% of rhGH was aggregated to inclusion bodies in the presence of the T7 transcription terminator (pET-hGH). On the contrary, in the T7 transcription terminator-deleted expression system (pET-hGH$_{\Delta T7T}$), more than 93% of rhGH was expressed in a soluble form and β-lactamase was co-overexpressed also in a soluble form. Although the total rhGH was much less in the pET-hGH$_{\Delta T7T}$ system, soluble rhGH was approximately twice as much as that in the pET-hGH system.

This system was also used for the soluble expression of other mammalian proteins such as G-CSF and interferon-α, and more than 80% of the target proteins were expressed in soluble forms (data not shown). The T7 transcription terminator-deleted expression system, which generates the long mRNA transcript, has another advantage in another sense: two different foreign genes can be expressed in a soluble form at the same time from one long mRNA

Table 1. Comparison of recombinant protein expression with and without a T7 transcription terminator. One mM IPTG was added. Two vector systems, pET-hGH and pET-hGH$_{\Delta T7T}$, were used for the cases with and without the T7 transcription terminator, respectively.

	pET-hGH	pET-hGH$_{\Delta T7T}$		
	rhGH	rhGH	β-lac	rhGH+β-lac
Soluble fraction (mg/OD.L)	23.7	43.9	35.7	79.6
Inclusion body (mg/OD.L)	53.0	3.05	3.30	6.35
Total (mg/OD.L)	76.7	47.0	39.0	86.0
Soluble expression ratio (%)	30.9	93.4	91.5	92.6

transcript. In conclusion, the T7 transcription terminator-deleted expression system would be a valuable tool for the production of foreign proteins in a soluble form in *E. coli*.

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REFERENCES

1. Bukau, B. and A. L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* **92:** 351–366.
2. Chen, J.-Y., Y.-L. Liao, T.-H. Wang, and W.-C. Lee. 2006. Transformation of *Escherichia coli* mediated by magnetic nanoparticles in pulsed magnetic field. *Enzyme Microbial Technol.* **39:** 366–370.
3. Collins-Racie, L. A., J. M. McColgan, K. L. Grant, E. A. Di-Blasio, S. J. M. McCoy, and E. R. LaVallie. 1995. Production of recombinant bovine enterokinase catalytic subunit in *E. coli* using the novel secretory fusion partner DsbA. *Bio/Technology* **13:** 982–987.
4. Davis, G. D., C. Elisee, D. M. Newham, and R. G. Harrison. 1999. New fusion protein systems designed to give soluble expression in *E. coli*. *Biotech. Bioeng.* **65:** 382–388.
5. Eom, G. T., J. S. Rhee, and J. K. Song. 2006. An efficient secretion of type I secretion pathway-dependent lipase, TliA, in *Escherichia coli*: Effect of relative expression levels and timing of passenger protein and ABC transporter. *J. Microbiol. Biotechnol.* **16:** 1422–1428.
6. Goh, L. L., P. Loke, M. Singh, and T. S. Sim. 2003. Soluble expression of a functionally active *Plasmodium falciparum* falcipain-2 fused to maltose-binding protein in *E. coli*. *Protein Expr. Purif.* **32:** 194–201.
7. Guan, Y.-X., H.-X. Pan, Y.-G. Gao, S.-J. Yao, and M. G. Cho. 2005. Refolding and purification of recombinant human interferon-c expressed as inclusion bodies in *Escherichia coli* using size exclusion chromatography. *Biotechnol. Bioprocess Eng.* **10:** 122–127.
8. Guise, A. D., S. M. West, and J. B. Chaudhuri. 1996. Protein folding *in vivo* and renaturation of recombinant proteins form inclusion bodies. *Molec. Biotechnol.* **6:** 53–64.
9. Ingraham, J. L., O. Maaloe, and F. C. Neidhardt. 1983. *Growth of the Bacterial Cell.* Sinauer Associates, Inc., Sunderland, MA, U.S.A.
10. Jeong, H. Y., J. Y. Lee, and T. H. Park. 2004. Specificity of enzymatic *in vitro* glycosylation by PNGase F: A comparison of enzymatic and non-enzymatic glycosylation. *Enzyme Microbial Technol.* **35:** 587–591.
11. Jin, J. H., K. K. Choi, U. S. Jung, Y. H. In, S. Y. Lee, and J. Lee. 2004. Regulatory analysis of amino acid synthesis pathway in *Escherichia coli*: Aspartate family. *Enzyme Microbial Technol.* **35:** 694–706.
12. Kim, S.-G., J.-A. Kim, H.-A. Yu, D.-H. Lee, D.-H. Kweon, and J.-H. Seo. 2006. Application of poly-arginine fused minichaperone to renaturation of cyclodextrin glycosyltransferase expressed in recombinant *Escherichia coli*. *Enzyme Microbial Technol.* **39:** 459–465.
13. Kim, Y. S. and H. J. Cha. 2006. Solubility dependency of co-expression effects of stress-induced protein Dps on foreign protein expression in *Escherichia coli*. *Enzyme Microbial Technol.* **39:** 399–406.
14. Lee, S. G., K. S. Hwang, and C. M. Kim. 2005. Dynamic behavior of regulatory elements in the hierarchical regulatory network of various carbon sources-grown *Escherichia coli*. *J. Microbiol. Biotechnol.* **15:** 551–559.
15. Loo, T., M. L. Patchett, G. E. Norris, and J. S. Lott. 2002. Using secretion to solve a solubility problem: High yield expression in *E. coli* and purification of the bacterial glycoamidase PNGase F. *Protein Expr. Purif.* **24:** 90–98.
16. Moore, J. T., A. Uppal, F. Maley, and G. F. Maley. 1993. Overcoming inclusion body formation in a high-level expression system. *Protein Expr. Purif.* **4:** 160–163.
17. Nigro, M., V. Martin, F. Kaufer, L. Carral, S. O. Angel, and V. Pszenny. 2001. High level of expression of the *Toxoplasma gondii* recombinant Rop2 protein in *E. coli* as a soluble form for optimal use in diagnosis. *Mol. Biotechnol.* **18:** 269–273.
18. Nygren, P.-A., S. Stahl, and M. Uhlen. 1994. Engineering proteins to facilitate bioprocessing. *Trends Biotechnol.* **12:** 184–188.
19. Oh, J. S. and T. H. Park. 2006. Late gene mutants of bacteriophage λ as an efficient expression vector. *Enzyme Microbial Technol.* **39:** 420–425.

20. Ow, D. S.-W., P. M. Nissom, R. Philp, S. K.-W. Oh, and M. G.-S. Yap. 2006. Global transcriptional analysis of metabolic burden due to plasmid maintenance in *Escherichia coli* DH5α during batch fermentation. *Enzyme Microbial Technol.* **39:** 391–398.
21. Park, H. J., E. J. Kim, T. Y. Koo, and T. H. Park. 2003. Purification of anti-apoptotic recombinant 30 K protein produced in *Escherichia coli* and its anti-apoptotic effect in mammalian and insect cell systems. *Enzyme Microbial Technol.* **33:** 466–471.
22. Park, S.-L., E.-J. Shin, S.-P. Hong, S.-J. Jeon, and S.-W. Nam. 2005. Production of soluble human granulocyte colony stimulating factor in *E. coli* by molecular chaperones. *J. Microbiol. Biotechnol.* **15:** 1267–1272.
23. Ro, H. S., H.-K. Park, M.-G. Kim, and B. H. Chung. 2005. *In vitro* formation of protein nanoparticle using recombinant human ferritin H and L chains produced from *E. coli*. *J. Microbiol. Biotechnol.* **15:** 254–258.
24. Scein, C. H. 1989. Production of soluble recombinant proteins in bacteria. *Bio/Technology* **7:** 1141–1148.
25. Song, H. and S. Y. Lee. 2006. Production of succinic acid by bacterial fermentation. *Enzyme Microbial Technol.* **39:** 352–361.
26. Studier, F. W. 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **219:** 37–44.
27. Studier, F. W. and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189:** 113–130.
28. Vind, J., M. A. Sorensen, M. D. Rasmussen, and S. Pedersen. 1993. Synthesis of proteins in *E. coli* is limited by the concentration of free ribosome. *J. Mol. Biol.* **231:** 678–688.