Research article

Solubility enhancement of aggregation-prone heterologous proteins by fusion expression using stress-responsive *Escherichia coli* protein, RpoS

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Abstract

**Background:** The most efficient method for enhancing solubility of recombinant proteins appears to use the fusion expression partners. Although commercial fusion partners including maltose binding protein and glutathione-S-transferase have shown good performance in enhancing the solubility, they cannot be used for the proprietary production of commercially value-added proteins and likely cannot serve as universal helpers to solve all protein solubility and folding issues. Thus, novel fusion partners will continue to be developed through systematic investigations including proteome mining presented in this study.

**Results:** We analyzed the *Escherichia coli* proteome response to the exogenous stress of guanidine hydrochloride using 2-dimensional gel electrophoresis and found that RpoS (RNA polymerase sigma factor) was significantly stress responsive. While under the stress condition the total number of soluble proteins decreased by about 7%, but a 6-fold increase in the level of RpoS was observed, indicating that RpoS is a stress-induced protein. As an N-terminus fusion expression partner, RpoS increased significantly the solubility of many aggregation-prone heterologous proteins in *E. coli* cytoplasm, indicating that RpoS is a very effective solubility enhancer for the synthesis of many recombinant proteins. RpoS was also well suited for the production of a biologically active fusion mutant of *Pseudomonas putida* cutinase.

**Conclusion:** RpoS is highly effective as a strong solubility enhancer for aggregation-prone heterologous proteins when it is used as a fusion expression partner in an *E. coli* expression system. The results of these findings may, therefore, be useful in the production of other biologically active industrial enzymes, as successfully demonstrated by cutinase.
Background

_Escherichia coli_ have been widely used as a host to produce valuable commercial, industrial, and therapeutic proteins. There are several disadvantages with this system, however, especially for the expression of eukaryotic proteins. For example, when randomly selected 2,078 full-length genes of _Caenorhabditis elegans_ were expressed in _E. coli_ cytoplasm, only 11% of genes yielded significant amounts of soluble material [1]. Several different approaches have been taken to resolve the solubility problem in the past and include (1) truncation of long multi-domain proteins into short and separate domains [2]; (2) co-expression of molecular chaperones or foldases [3]; (3) enabled secretion to the periplasm where disulfide bonds can be properly formed with the help of an oxidative environment and Dsb protein families [4]; (4) co-expression of aminoacyl tRNA cognates to amino acids encoded by rare codons [5]; and more recently (5) use of a fusion expression partner [6,7]. The most efficient method for enhancing solubility and folding efficiencies of recombinant proteins appears to be the latter (fusion expression partners) which includes maltose binding protein (MBP) [8], thioredoxin (Trx) [9], human ferritin heavy chains (hFTN-H) [10], and glutathione-S-transferase (GST) [11]. Although these fusion partners have shown good performance in enhancing the solubility and folding of some recombinant proteins [12], they likely cannot serve as universal helpers to solve all protein solubility and folding issues. Thus, novel fusion partners will continue to be developed through systematic investigations including proteome mining.

In the present study, we found that the level of RpoS significantly increased during the stress caused by guanidine hydrochloride through proteome-wide mining involving the stress response of _E. coli_ BL21(DE3). As an N-terminus fusion expression partner, RpoS dramatically increased the solubility of the following heterologous proteins (human minipro-insulin (mp-INS), human epidermal growth factor (EGF), human ferritin heavy chains (hFTN-H), human ferritin light chain (hFTN-L), human interleukin-2 (hIL-2), human transcription elongation protein NusA (55 kD), DNA gyrase subunit A (97 kD), formate acetyltransferase 1 (85 kD), succinate dehydrogenase flavoprotein subunit (64 kD), etc. Since the synthesis yield of target protein can be significantly reduced, the molecular mass of proteins to be used as fusion expression partner should not be too high. That is, although the large amount of fusion protein is synthesized, the actual amount of the target fusion-free protein could be very low if the fusion partner is too big. RpoS (38 kD) is a relatively small protein among the stress-responsive proteins we found and was highly effective in enhancing the solubility of target proteins.

_RpoS_, stress responsive RNA polymerase sigma factor is a well-known universal stress regulator controlling many proteins under various stress conditions, e.g. the onset of the stationary phase [19], and carbon starvation [20]. RpoS was previously reported to be induced by osmotic stress [16] and heat shock [21]. We also observed that the RpoS expression level increased 3- to 5-fold in response to heat shock (data not shown). Muffler _et al._ [21] reported that the duration of stability of RpoS in response to heat shock is maintained by the direct- or indirect-binding of DnaK. Bound DnaK appears to assist the effective folding of RpoS and also protect RpoS against the action of ClpP, a protease that degrades RpoS [21]. From this point of view, RpoS may serve as a solubility enhancer in...
**E. coli** cytoplasm, when used as a fusion expression partner upon the expression of aggregation-prone heterologous proteins.

**Expression of aggregation-prone heterologous proteins using RpoS as fusion partner**

We used RpoS as an N-terminus fusion expression partner for the synthesis of numerous heterologous proteins [mpINS, EGF, ppGRN, hIL-2, AID, GAD_{448-585}, CUT, hFTN-L, G-CSF, and NACHT (Fig. 2 and Table 2 for expression system construction)]. Stenström et al. [22] reported that the codon following an AUG start triplet (+2 codon) significantly affects gene expression in **E. coli**, and the second codon starting with A is most advantageous to achieve an enhanced expression level. The second codon of RpoS is AGT (encoding Ser) that seems to be favorable second codon based on the report of Stenström et al. [22]. As shown in Figure 3B and Table 3, all heterologous proteins expressed directly without RpoS fusion formed insoluble inclusion bodies resulting in nearly negligible solubility. Compared to these results, it seems surprising that when the same heterologous proteins were expressed with the fusion of RpoS, the solubility of these foreign proteins dramatically increased (Fig. 3A and Table 3), thereby indicating that **E. coli** RpoS is a highly effective solubility enhancer for aggregation-prone heterologous proteins. Table 3 compares the effect of RpoS- and GST fusion on the solubility enhancement for heterologous proteins. In the fusion expression of CUT, GAD_{448-585}, and mpINS, the effect of RpoS fusion was much higher, whereas GST fusion was significantly more effective in the expression of AID, NACHT, and hFTN-L. This result indicates that there

**Figure 1**

**E. coli** proteome profiles under non-stress and GdnHCl-stress condition. (A) 2-DE gel image of **E. coli** proteome under the non-stress condition. (Arrow indicates RpoS spot under non-stress condition.) (B) 2-DE gel image of **E. coli** proteome under GdnHCl-stress condition (Arrow indicates RpoS spot under GdnHCl-stress condition). (Figure in a box presents relative spot intensities of RpoS, analyzed under non-stress and GdnHCl-stress conditions.)
are no universal helpers to solve all protein solubility issues. Table 3 also shows that the results of direct- and fusion expression of heterologous proteins are highly reproducible.

Moreover, the plasmid vector for the expression of polyhistidine-tagged fusion mutant of G-CSF [(His)6::RpoS::(D4K)::G-CSF] was constructed (Fig. 2) to purify fusion-free G-CSF. After (His)6::RpoS::(D4K)::G-CSF was bound onto the ProBond resin (Ni+2) column, the enterokinase proteolysis was carried out in a batch mode, and subsequently the digested product was collected and centrifuged. SDS-PAGE and Western blot analyses of the supernatant show that the recombinant G-CSF was easily released from the Rpos-fusion protein and was

Table 1: Result of proteome-wide finding of some aggregation-resistant *E. coli* proteins

| Gene name | Access No. | Protein name | pI/MW (kDa) | Sequence coverage | Scorec | Fold change |
|-----------|------------|--------------|-------------|------------------|--------|-------------|
| rpoS      | P13445     | RNA polymerase sigma factor rpoS | 4.89/37.97 | 5.08/38.95 | 19     | 36          | 6.21         |
| groL      | P0A6F5     | HSP60 chaperonin | 4.85/57.20 | 4.90/58.63 | 16     | 34          | 2.13         |
| htpG      | P0A6Z3     | Chaperone protein htpG | 5.09/71.38 | 5.14/66.37 | 9      | 27          | 3.10         |
| nusA      | P0AFF6     | Transcription elongation protein nusA | 4.53/54.87 | 4.69/59.09 | 16     | 44          | 3.46         |
| gyrA      | P0AE54     | DNA gyrase subunit A | 5.09/96.96 | 5.20/92.23 | 21     | 29          | 1.95         |
| pflB      | P09373     | Formate acetyltransferase | 5.69/85.23 | 5.68/83.51 | 19     | 39          | 3.26         |
| sdhA      | P0AC41     | Succinate dehydrogenase flavoprotein subunit | 5.85/64.42 | 5.70/65.73 | 25     | 43          | 2.16         |

a Gene name, accession number, and protein name were obtained from ExPASy Proteomics Server [35].
b Theoretical values of pI and Mw were calculated using "Compute pI/Mw tool" [36].
c Experimental values of pI and Mw were estimated through 2-DE gel image analysis in the present study.
d Sequence coverage and score values were calculated using "ALDENTE: PEPTIDE MASS FINGERPRINTING TOOL" [37].

Table 2: Primers used for the cloning of genes encoding various heterologous proteins

| Heterologous proteins | Direct expression | Primer sequences Fusion expression |
|-----------------------|-------------------|-----------------------------------|
| mp-INS                | cat atg ttt gtc aac cca cat | ctc gag ttt gtc aac cca cat |
|                       | aag cct tta gtc aca gta gtc c | aag ctt tta gtc aca gta gtc c |
|                       | aag ctt tta gtc aca gta gtc c | aag ctt tta gtc aca gta gtc c |
| EGF                   | cat atg aac tct gac tcc gaa tgc | ctc gac aac tct gac tcc gaa tgc |
|                       | aag ctt tta aac gag ttc cca cca | aag ctt tta aac gag ttc cca cca |
|                       | aag ctt tta aac gag ttc cca cca | aag ctt tta aac gag ttc cca cca |
| ppGRN                | cat atg ggc tcc agc ttc ctg t | ctc gac ggc tcc agc ttc ctg t |
|                       | aag ctt tca ctt gtc ggc t | aag ctt tca ctt gtc ggc t |
|                       | aag ctt tca ctt gtc ggc t | aag ctt tca ctt gtc ggc t |
| hIL-2                | cat atg gca cct act tca agt | ctc gag gca cct act tca agt |
|                       | aag ctt tca aag ctc att cag t | aag ctt tca aag ctc att cag t |
|                       | aag ctt tca aag ctc att cag t | aag ctt tca aag ctc att cag t |
| AID                   | cat atg gac agc ttc tgg aag aac | ctc gag gac agc ttc tgg aag aac |
|                       | aag ctt tca taa caa aac tgg ca | aag ctt tca taa caa aac tgg ca |
|                       | aag ctt tca taa caa aac tgg ca | aag ctt tca taa caa aac tgg ca |
| GAD46–585             | cat atg cgc cac gtt gat gt | ctc gag cgc cac gtt gat gt |
|                       | aag ctt tca taa atc tgg tcc | atc gag tta taa atc tgg tcc |
|                       | aag ctt tca taa atc tgg tcc | atc gag tta taa atc tgg tcc |
| CUT                   | cat atg gct ccc ctg ccc gat ac | ctc gag gct ccc ctg ccc gat ac |
|                       | aag ctt tta aac ccc ggc ggc ct | aag ctt tta aac ccc ggc ggc ct |
|                       | aag ctt tta aac ccc ggc ggc ct | aag ctt tta aac ccc ggc ggc ct |
|                       | atc gat tta taa atc tgg tcc | atc gat tta taa atc tgg tcc |
|                       | atc gat tta taa atc tgg tcc | atc gat tta taa atc tgg tcc |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
|                       | ctc gag gac ggc ggc ggc ccc ccc gcg cgg ct (for metal affinity purification) | |
present in the form of soluble protein in the supernatant (Fig. 4).

Bioactivity of the recombinant fusion mutant of cutinase, \textit{RpoS::CUT}

Cutinase has been used as a lipolytic enzyme in the composition of laundry and dishwashing detergents to more efficiently remove immobilized fats [23,24]. In addition, the oleochemistry industries [25], and pollutant degradation [26,27] represent other potential uses of cutinase. In recent years, the esterification and transesterification properties of cutinase have been intensively exploited and could be applied usefully in other chemical synthesis processes [28]. Because of these extensive potential applications, we were particularly interested in the production of a bioactive recombinant cutinase. Thus, we cloned the cutinase gene from the genome of \textit{Pseudomonas putida} and expressed it in \textit{E. coli} using the fusion of \textit{RpoS}. Cutinase is known for its hydrolytic activity for a variety of esters ranging from soluble \textit{p}-nitrophenyl esters to insoluble long-chain triglycerides. The hydrolytic activity of cutinase, especially on \textit{p}-nitrophenyl esters of fatty acids, is extremely sensitive to fatty acid chain length. Previously, Lin \textit{et al}. [29] reported that microbial cutinase lacked a large hydrophobic surface around the active site, in contrast to other lipases and esterases. This structural characteristic of cutinase may be strongly related to high substrate-specificity, i.e. the extremely low activity on \textit{p}-nitrophenyl palmitate (PNP).

\textbf{Figure 2}

The plasmid vector constructions for direct and fusion expression of heterologous proteins in \textit{E. coli}. (A) Direct expression vector, (B) \textit{RpoS-} and GST-fusion expression vector, (C) Hybrid vector for metal (Ni\textsuperscript{2+}) affinity purification of \textit{RpoS-CUT-(His)}\textsubscript{6}, (D) Hybrid vector for metal (Ni\textsuperscript{2+}) affinity purification of (His)\textsubscript{6}-\textit{RpoS-D\textsubscript{4K}-G-CSF}, followed by enterokinase cleavage.
Table 3: Solubility of the expressed recombinant proteins

| Heterologous proteins | Solubility* of the expressed recombinant proteins (%) | RpoS-fusion expression | GST-fusion expression | Direct expression |
|-----------------------|------------------------------------------------------|------------------------|----------------------|------------------|
| G-CSF                 |                                                      | 87.4 ± 2.1             | 91.5 ± 1.5           | 4.6 ± 0.5        |
| CUT                   |                                                      | 44.9 ± 2.7             | 26.2 ± 1.1           | 3.1 ± 0.2        |
| AID                   |                                                      | 18.1 ± 3.5             | 41.7 ± 1.5           | 8.7 ± 1.6        |
| hFTN-L                |                                                      | 47.8 ± 2.1             | 87.6 ± 2.8           | 1.7 ± 0.4        |
| NACHT                 |                                                      | 36.4 ± 4.3             | 74.8 ± 2.2           | 8.4 ± 1.3        |
| GAD_{448-585}         |                                                      | 59.0 ± 1.8             | 7.8 ± 2.7            | 1.9 ± 0.5        |
| hiL-2                 |                                                      | 60.1 ± 2.4             | 85.7 ± 1.7           | 1.3 ± 0.3        |
| EGF                   |                                                      | 80.4 ± 0.9             | 92.9 ± 1.2           | 3.0 ± 0.9        |
| ppGRN                 |                                                      | 89.2 ± 3.5             | 90.1 ± 2.3           | 7.6 ± 0.6        |
| mp-INS                |                                                      | 75.9 ± 2.8             | 13.3 ± 1.0           | 1.3 ± 0.4        |

* The solubility was defined as the fraction of the soluble recombinant protein compared to the synthesized total (soluble + insoluble) recombinant protein. Average and standard deviation values were calculated based on the results of repeated triplicate experiments.

Figure 3

Results of direct and fusion expression of heterologous proteins. SDS-PAGE analyses of the RpoS-fusion expressed proteins (A), directly (non-fusion) expressed proteins (B), and GST-fusion expressed proteins (C).
We assayed the enzymatic activity of our fusion mutant of cutinase (RpoS::CUT) and demonstrated the same selective bioactivity as native cutinase to degrade p-nitrophenyl butyrate (PNB) but not to degrade PNP (Fig. 5A and 5B). We also purified RpoS::CUT-His6 (Fig. 6A) through Ni\(^{2+}\) affinity chromatography and analyzed the purified RpoS::CUT-His6 using reversed phase HPLC (Fig. 6B). As shown in Figure 6B, RpoS::CUT-His6 was analyzed as a single peak, which seems to indicate that the crafted mutant molecules of cutinase have uniform and correctly folded conformation due probably to the help of fusion partner, \textit{E. coli} RpoS.

**Conclusion**

Using 2-dimensional gel electrophoresis, we found that \textit{E. coli} RpoS, RNA polymerase sigma factor was GdnHCl stress-responsive and highly effective as a strong solubility enhancer when used as fusion partner for the expression of aggregation-prone heterologous proteins in \textit{E. coli} BL21(DE3). The results of these findings may, therefore, be useful in the production of other biologically active industrial enzymes, as successfully demonstrated by cutinase.

**Methods**

**Bacterial strain and plasmids**

\textit{E. coli} strain BL21(DE3) (\textit{FompT\ hsdS\_(rB\ mB)}) was selected under both non-stress and GdnHCl-stress conditions for 2-dimensional gel electrophoresis analysis. Through PCR amplification using appropriate primers (Table 2), the genes encoding mp-INS, EGF, ppGRN, hIL-2 [30], AID, GAD\textsubscript{448–585} [31], CUT, hFTN-L [32], G-CSF, and NACHT were cloned using previously cloned heterologous genes, except for CUT gene that was cloned from chromosomal DNA of \textit{Pseudomonas putida} (ATCC 53552). The gene clones of mp-INS, ppGRN, AID, G-CSF, and NACHT were kindly donated by other researchers, as acknowledged (see Acknowledgements). The EGF gene was cloned using pCMV6-XL vector ( OriGene Technologies, USA). Each of the recombinant genes above and various fusion/hybrid genes [rpo\textsubscript{S}(or GST gene)::(each heterologous gene)] were inserted into the \textit{Ndel-HindIII} site of the same plasmid pT7-7 to construct the expression vectors. All the heterologous genes above were fused directly to the RpoS gene (cloned from the chromosomal DNA of \textit{E. coli} BL21(DE3)) or GST gene [cloned from the GST-fusion vector pET42a(+) (Novagen, USA)] without any linker sequence. Therefore, each expression vector has no enzymatic cleavage site between rpo\textsubscript{S} (or GST gene) and heterologous gene, except for the case of G-CSF. For the purification of fusion-free recombinant G-CSF, the \textit{D}_{4}K sequence for enterokinase digestion was inserted between RpoS and G-CSF genes to synthesize the polyhistidine-tagged fusion mutant of G-CSF, i.e. (His)\textsubscript{c}::RpoS::(D\textsubscript{4}K)::G-CSF. For the purification of RpoS::CUT, hexahistidine (His\textsubscript{c}) was added to the C-terminus of CLT by PCR (Fig. 2). For this, the sequence coding for N-RpoS::CUT-His\textsubscript{c}-C was inserted into the \textit{Ndel-HindIII} site of plasmid pT7-7.

After complete DNA sequencing of all gel-purified hybrid plasmids, the \textit{E. coli} strain BL21(DE3) (\textit{FompT\ hsdS\_(rB\ mB)}) was transformed with the hybrid plasmids, and ampicillin-resistant transformants were subsequently selected using LB-agar plates supplemented with ampicillin (100 mg/l).

**Recombinant \textit{E. coli} culture, gene expression, and recombinant protein purification**

For shake flask experiments, 250 ml Erlenmeyer flasks containing 50 ml LB media and ampicillin at 100 mg/l of culture (37°C and 150 rpm) was used. When the culture turbidity (OD\textsubscript{600}) reached 0.5, gene expression was induced with the addition of IPTG (1 mM), and after a further 4 h of cultivation the all recombinant cells (80 mg wet cell mass) were harvested by centrifugation (13,000 rpm (MICRO17TR, Hanil Science Industrial, Korea) \times 5 min) and cell pellets resuspended in 5 ml lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA). Cell disruption was accomplished by sonication, and the supernatant was loaded onto a ProBond resin (Ni\(^{3+}\)) column for metal affinity purification; lane 2, soluble fraction of enterokinase(EK)-digested product of (His)\textsubscript{c}-RpoS-\textit{D}_{4}K-G-CSF, containing RpoS and fusion-free G-CSF (indicated by an arrow); lane 3, purified soluble fusion-free G-SCF. -Western blot analysis (lane 4): result of immunoblotting analysis of purified soluble fusion-free G-SCF (loaded onto lane 3).

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**Figure 4**

Results of SDS-PAGE and Western blot analysis of RpoS-fusion and fusion-free G-CSF. - SDS-PAGE analysis (lane M-3): lane M, molecular markers; lane 1, supernatant of recombinant \textit{E. coli} cell lysates containing recombinant (His)\textsubscript{c}-RpoS-\textit{D}_{4}K-G-CSF, which was loaded onto ProBond resin (Ni\(^{3+}\)) column for metal affinity purification; lane 2, soluble fraction of enterokinase(EK)-digested product of (His)\textsubscript{c}-RpoS-\textit{D}_{4}K-G-CSF, containing RpoS and fusion-free G-CSF (indicated by an arrow); lane 3, purified soluble fusion-free G-CSF. - Western blot analysis (lane 4): result of immunoblotting analysis of purified soluble fusion-free G-SCF (loaded onto lane 3).
achieved using a Branson Sonifier (Branson Ultrasonics Corp., Danbury, CT). The cell-free supernatant and insoluble protein aggregates were separated at 13,000 rpm (MICRO17TR, Hanil Science Industrial, Korea) for 10 min. The isolated inclusion bodies, if any, were washed twice with 1 % Triton X-100. Cell-free supernatants and the washed inclusion bodies were subjected to polyacrylamide (14%) gel electrophoresis (PAGE) analysis. Coomassie-stained protein bands were ultimately scanned, and the intensity of each recombinant protein band was estimated using densitometry (Duoscan T1200, Bio-Rad, Hercules, CA). The solubility of recombinant proteins was determined by analyzing the fraction of the soluble recombinant protein compared to the synthesized total (soluble + insoluble) recombinant protein. Average and standard deviation values of the solubility were calculated based on the results of repeated triplicate experiments.

The purification of recombinant G-SCF was accomplished using metal affinity chromatography. That is, polyhistidine-tagged fusion mutant of G-CSF [(His)₆::RpoS::(D₄K)::G-CSF] (Fig. 4) were loaded onto ProBond resin (Ni²⁺) column. Prior to sample loading, the resin was washed twice with 10 column volumes of binding buffer (50 mM potassium phosphate, 300 mM KCl, 20 mM imidazole, pH 7.0). Binding buffer contains 20 mM imidazole to minimize non-specific binding of untagged protein contaminants, and binding was carried out in a batch mode at 4 °C. Afterwards the resin was

![Figure 5](http://www.biomedcentral.com/1472-6750/8/15)

**Figure 5**

**Bioactivity of recombinant fusion mutant, RpoS::CUT.** Assay results using cell-free supernatants from (A) *E. coli* BL21 (DE3) host and (B) recombinant *E. coli* BL21 (DE3) [pT7-RpoS-CUT] producing RpoS::CUT. Both PNB (●) and PNP (○) were used as substrates for the cutinase activity assay. (Concentrations: PNB = 6.6 mM; PNP = 6.6 mM).
washed twice with 5–8 ml Tris-HCl (10 mM Tris, pH 8.0) prior to enterokinase digestion step. The enterokinase digestion was carried out in a batch mode at 4°C for 10 h using 5-unit enterokinase (Invitrogen, CA, USA). Then, the proteolytic product was collected and centrifuged [3,000 rpm (MICRO17TR, Hanil Science Industrial, Korea) for 10 min], and the supernatant fraction was subjected to being analyzed by SDS-PAGE and western blotting.

Purification and HPLC analysis of cutinase fusion mutant
For the purification of RpoS::CUT-His6, the soluble fraction was separated after cell disruption by centrifugation (13,000 rpm (MICRO17TR, Hanil Science Industrial, Korea)) for 30 min. The cell-free supernatant containing RpoS::CUT-His6 was loaded onto the ProBond resin (Ni2+) column (Invitrogen) for affinity purification. Before the sample loading, the resin was washed twice with ten column volumes of binding buffer (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole). Binding was carried out in a batch mode at 4°C. Afterwards, the resin was washed twice with 8 ml washing buffer (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl, 50 mM imidazole) and eluted with elution buffer (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole). The elution buffer in the eluted solution containing the purified RpoS::CUT-His6 was changed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) using Amicon Ultra-4 centrifugal filter (Millipore, Ireland). Analysis of the purified RpoS::CUT-His6 was performed with high-performance liquid chromatography (HPLC) (LC-20A Prominence, Shimadzu Co. Ltd., Japan). A Shim-pack CLC-NH2 column (60 × 150 mm, Shimadzu Co. Ltd., Japan) was equilibrated with 10 % acetonitrile containing 0.1 % trifluoroacetic acid at a flow rate of 1 ml/min. The sample was loaded onto the column and eluted with 75 % acetonitrile. The elution profile was monitored at 215 nm.

Sample preparation for proteome analysis and 2-dimensionsal gel electrophoresis
Flask culture conditions were identical to those for recombinant gene expressions. Cells were grown at 37°C and then 100 mM GdnHCl was added for the GdnHCl-induced proteome response when culture turbidity (OD600) reached 0.5 in the LB media. After further 3 h cul-
tivation, the cells were harvested by centrifugation at
6,000 rpm (MEGA17R, Hanil Science Industrial,
Korea) for 15 min (4 °C) and then washed twice with 40
mM Tris buffer (pH 8.0). Cell pellets were resuspended in
500 µl of lysis buffer (8 M urea, 4% (w/v) CHAPS, 40 mM
Tris, protease inhibitor cocktail; Roche Diagnostics
GmbH, Mannheim, Germany) and disrupted by sonication.
After sonication (Branson Sonifier 450, USA), the
cell debris and the aggregated proteins were removed by
centrifugation at 12,000 rpm (MICRO17TR, Hanil Sci-
ence Industrial, Korea) for 60 min (4°C), and only soluble
proteins were obtained. 2-dimensional gel electrophoresis
was performed as described previously Kim et al. [33]. Stained gels were scanned using a UMAX
powerlook 1100 scanner and Image Master software v
4.01 (Amersham Biosciences, Uppsala, Sweden) was used
for gel image analysis, including quantification of spot
intensities performed on volume bases (i.e. values calcu-
lated from the integration of spot optical intensity over
spot area).

MALDI-TOF-MS analysis and protein identification

Samples for MALDI-TOF MS analysis were prepared
through the extraction from silver-stained protein spots
according to the previous protocol [33]. Enzymatic diges-
tion was performed with 10 mg/ml of sequencing grade
modified trypsin (Promega, WI, USA) in 25 nM ammo-
nium bicarbonate (pH 8.0) for overnight at 37°C in a sta-
tionary incubator. The trypsin-digested protein spots was
analyzed using a MALDI-TOF-MS system (Voyager DE-
STR, PE Biosystem, Framingham, MA, U.S.A.) by the
Korea Basic Science Institute (Seoul, Republic of Korea),
and peptide mass fingerprinting for protein identification
was performed using MS-Fit [34]. Spectra were calibrated
using a matrix and tryptic autodigestion ion peaks as
internal standards. Peptide mass fingerprints were ana-
yzed using the MS-Fit [34]. The identification of a protein
with respect to theoretical parameters (pl, molecular
mass, etc.) was accepted if the peptide mass matched
within a mass tolerance of 10 ppm.

Bioactivity assay

A hydrolytic enzyme activity of the recombinant cutinase
fusion mutant was assessed as described below. The hydrolysis reactions occurred in 96-well microplates at
37°C for 15 min where each well contained 200 µl
enzyme/substrate solution comprised of 106.7 µl phos-
phate buffer (0.1 M, pH 8.0), 13.3 µl Triton X-100 solu-
tion (4 g/l), cutinase solution. The reaction was initiated
by adding 66.7 µl of substrate reagent solution to each
well in the 96-well microplate. Absorbance changes
(ΔA1150nm Per min) were measured using a Bio-Rad micro-
plate reader (Tecan, Austria), and solution A with no
enzyme solution was used as blank. From the absorbance
changes measured at each column, an average absorbance
for a specific reaction condition could then be calculated.

Abbreviations

2-DE, 2-dimensional gel electrophoresis; AID, human
activation induced cytidine deaminase; CUT, Pseudomonas
putida cutinase; EGF, human epidermal growth factor;
GAD148-585, deletion mutant of human glutamate decar-
boxylase; G-CSF, human granulocyte colony-stimulating
factor; GdnHCl, Guanidine hydrochloride; GST, gluthath-
one-S-transferase; hFTN-H, human ferritin heavy chain;
hFTN-L, human ferritin light chain; hIL-2, human inter-
leukin-2; MBP, maltose binding protein; mp-INS, mini-
pro-insulin; NACHT, human cold autoinflammatory
syndrome 1 protein (NALP3) NACHT domain; PNB,
p-nitrophenyl butyrate; PNP, p-nitrophenyl phamitate;
ppGRN, human prepro-ghrelin; Trx, thioredoxin.

Authors’ contributions

JSP, KYH, and JAS carried out all experiments. SHS and
KYA carried out 2-dimensional gel electrophoresis and its
analysis. JHL and SWK performed HPLC analysis. JSP and
SJS drafted and revised the manuscript in collaboration
with JL. JL also participated in the planning, design, and
coordination of the research. All authors read and
approved the final manuscript.

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