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Expression of Recombinant Human Stem Cell Factor (hSCF) Protein using *Bombyx mori* Protein Disulfide Isomerase (bPDI)

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Protein disulfide isomerase (PDI) catalyzes the oxidation of disulfides and the isomerizatiob of incorrect disulfides in new polypeptides during folding in the oxidizing environment of the endoplasmic reticulum (ER). To increase recombinant protein hSCF (human stem cell factor) production, we have developed expression system using the *Bombyx mori* PDI (bPDI) as a fusion partner. bPDI gene fusion was found to improve the production of recombinant hSCFs. Thus, we conclude that bPDI gene fusion will be very useful for the large-scale production of biologically active recombinant proteins.

Key words: *Bombyx mori*, Protein Disulfide Isomerase (PDI), Stem Cell Factor (SCF)

Introduction

The human stem cell factor (hSCF) is a hematopoietic cytokine that triggers its biologic effects by binding to its receptor, c-kit. hSCF can act on hematopoiesis by promoting the survival, proliferation, and differentiation of hematopoietic stem cells and progenitor cells by itself or synergizing with other cytokines such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3) (Galli *et al*., 1994; Matous *et al*., 1996). In addition, the hSCF has been used as potential therapeutic applications including the treatment of anemia, increasing the effectiveness of gene therapy, and boosting the mobilization of hematopoietic stem/progenitor cells to the peripheral blood for harvest and transplantation (Galli *et al*., 1994; Hsu *et al*., 1997; Langley *et al*., 1992; Lu *et al*., 1991; McNiece *et al*., 1995).

Insect cell systems including the baculovirus expression vector system (BEVS) and the stably transformed cell system are now popular and used widely to produce proteins from higher eukaryotes because insect cells have a similar pattern and capacity of co-translational and posttranslational modifications as mammalian cells, including glycosylation, phosphorylation, and protein processing (Kato *et al*., 2010). However, recombinant proteins in these systems often have low bioactivity and poor secretion because of protein aggregation and degradation in the endoplasmic reticulum (ER) lumen. Endogenous protein biosysthesis in the insect cell is essentially shutdown because of the high levels of exogenous gene production. This shutdown leads to insufficient expression of molecular chaperones and foldases in the ER, resulting in protein aggregation (Fath-Goodin *et al*., 2006). Protein disulfide isomerase (PDI) catalyzes the oxidation of disulfides and the isomerization of incorrect disulfides in new polypeptides during folding in the oxidizing environment of the ER. We previously isolated *Bombyx mori* protein disulfide isomerase (bPDI), which has two thiol oxidoreductase sites and enzymatic activity for reduced and scrambled RNase, like other PDI family members (Goo *et al*., 2002). In addition, our most recent studies have demonstrated that a co-expressed PDI increased the folding and secretion of recombinant proteins in the baculovirus expression system (Goo *et al*., 2008a, Goo *et al*., 2008b).

For more efficient expression as compared to independent expression of hSCF gene in insect cells, we con-

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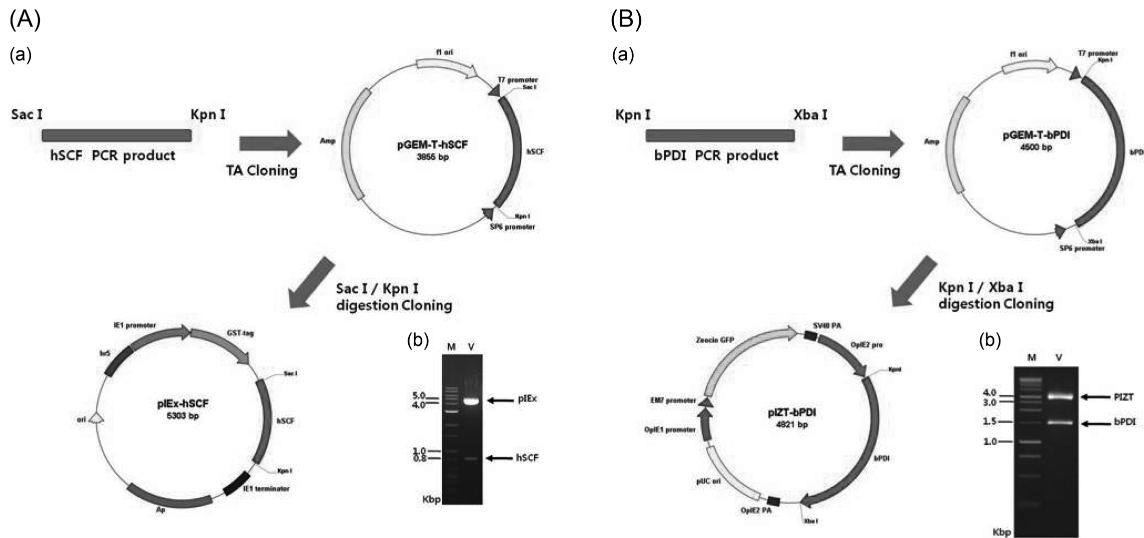

Fig. 1. Construction of the transfer vectors, pIEx-hSCF and pIZT-bPDI. A) Construction of the pIEx-hSCF vector. (a) The PCR product for ORF flanked by *Sac* I/*Kpn* I stie was inserted into pGEM-T vector. The pGEM-T-hSCF was digested with *Sac* I/*Kpn* I, and then DNA fragment was subcloned into the *Sac* I/*Kpn* I site on pIEx vector. (b) M, 1kb DNA marker; V, the pIEx- hSCF were digested with *Sac* I/*Kpn* I. B) Construction of the pIZT-bPDI vector. (a) The PCR product for ORF flanked by *Kpn* -I/*Xba* I stie was inserted into pGEM-T vector. The pGEM-T-bPDI was digested with Kpn I/Xba I and then DNA fragment was subcloned into the *Kpn* I/*Xba* I site on pIZT. (b) M, 1kb DNA marker; V, the pIZT-bPDI were digested with *Kpn* I/*Xba* I.

structed the stably transformed Sf9 insect cell line co-expressing the hSCF and bPDI gene. In addition, transformed Sf9 insect cell line was successfully co-expressed recombinant hSCF and bPDI proteins, its expression efficiency was investigated and compared to independent expression of hSCF in this study

Materials and Methods

Cell culture

The culture cell lines, Sf9, are derived from the pupal ovarian tissue of *Spodoptera frugiferda*. The transformed insect cell lines generated in this study, Sf-hSCF and Sf-hSCF-bPDI, were routinely maintained as adherent cultures in TC-100 medium (Sigma, USA) with 10% (v/v) heat-inactivated fetal bovine serum and 1 mg/ml G418 (Calbiochem, Germany) and 400 ug/ml zeocin (Invitrogen, USA), respectively (Summers & Smith, 1987; Yun *et al.*, 2005).

Construct of tranfer vectors

The human stem cell factor (hSCF) (Clone ID: 30915176) was prepared at the Open Biosystems (US). The linear DNA fragment encoding hSCF (1-280 aa) was obtained by PCR performed with a sense primer (5′-GAGCTCGTTATGAA-GAAGACACAAACT-3′) and an anti-sense primer (5′-GGTACCGTGGTGGTGGTGGTGGTGCCGCACTTCT-TGAAACTCTCT-3′), in which the *Sac* I and *Kpn* I sites were introduced. The hSCF fragment was subcloned into pGEM-T-easy vector (Promega, USA), double digested with *Sac* I and *Kpn* I, purified by agarose gel electrophoresis, and subcloned into insect expression vector pIEX-2 (Novagen, Germany). The resulting experimental vector was named pIEX-hSCF (Fig. 1A). The The bPDI cDNA was obtained using the reverse transcription-polymerase chain reaction (RT-PCR) method with specific primers and poly(A)$^+$ mRNA from the Bm5 cells (Goo et al., 2002). The linear DNA fragment encoding bPDI (1-485 aa) was obtained by PCR performed with a sense primer (5′-GGTACCAT-GCGTGTTTTAATTTTCACG-3′) and an anti-sense primer (5′-TCTAGATCTAGATTATAACTCGTCTTTG-GCAGG-3′), in which the *Kpn* I and *Xba* I sites were introduced. The bPDI fragment was subcloned into pGEM-T-easy (Promega, USA), double digested with *Kpn* I and *Xba* I, purified by agarose gel electrophoresis, and subcloned into insect expression vector pIZT-V5/His. The resulting experimental vector was named pIZT-bPDI (Fig. 1B). FuGENE HD Transfection Reagent (Roche, Germany) was used to transfect the pIEX-hSCF plasmids into Sf9 cells and the pIZT-bPDI plasmids into the transformed insect cell line, Sf-hSCF. In conclusion, we constructed stably transformed insect cell line, Sf-hSCF-bPDI co-expressing hSCF and bPDI gene.

Expression assay

The normal Sf9 cells (6×10^5 cells) and stably transformed Sf9 cells (6×10^5 cells) in 6 well plates were washed twice with cold 1X phosphate buffered saline (PBS) and scraped. The cells lysed by the addition of I-PER Insect Cell Protein Extraction Reagent (Pierce, USA) as described under material and methods. The protein concentration was determined using Bradford's method (Bio-Rad Laboratories, USA) and recrystallized BSA was used as a standard. The cell lysates were mixed with SDS sample buffer (62.5 mM Tris-HCl pH6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03 (w/v) bromphenol blue) and separated by 12% (v/v) SDS-PAGE. The separated gel was fixed and stained with 0.1% Coomassie Brilliant Blue R-250. For Western blot analysis, SDS-PAGE was performed as described above, and the proteins were blotted onto a nitrocellulose membrane in transfer buffer (25 mM Tris/HCl, pH 7.6 and 192 mM glycine in 20% methanol) at 30 V overnight at 4°C (Towbin *et al*., 1979). The membrane was then blocked by incubation in a 1% BSA solution for 2 h at room temperature (RT). The blocked membrane was incubated with the rabbit anti-SCF monoclonal antibody (Stesssgen, USA) and the mouse anti-PDI monoclonal antibody (abcam, UK), respectively. The membrane was washed in buffer containing 10 mM Tris/HCl (pH 8.0), 100 mM NaCl, and 0.05% Tween 20 (TBST) and incubated with HRP-conjugated goat anti-Rabbit IgG (abcam, UK) and HRP-conjugated rabbit anti-Mouse IgG (abcam, UK) for 1 h at RT, respectively. After washing with TBST, the membrane was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). Densitometry of Western blot signal was obtained using the Image J program (NIH Image, USA).

Results and Discussion

Co-expression of hSCF and bPDI in Sf9 cells

A recombinant human stem cell facto (hSCF)r was produced using *B. mori* protein disulfide isomerase (bPDI) in the insect cell. The ER contains molecular chaperones and foldase that optimize the folding and assembly of newly synthesized proteins. Recombinant protein was produced using the insect cell expression system often had poorly secretion, because of protein aggregation and degradation in the ER lumen. The bPDI was isolated from *B. mori* and known to enhance protein secretion (Goo *et al*., 2002). In this study, we attempted to increase expression of the recombinant hSCF proteins through co-expression of bPDI gene

The hSCF was obtained at the Open Biosystems contained 822 bp encoding 273 amino acid residues. The DNA fragment encoding hSCF (822 bp) was inserted into the insect cell expression vector pIEx-2 described in Material and Methods (Fig 1A). The constructed pIEx-hSCF was co-transfected with pIE1-neo vector into Sf9 cells. After treatment with G418 (1 mg/ml), G418-resistant colonies (Sf-hSCF) were isolated by the limiting dilution method. To analyze whether the hSCF gene was correctly introduced in Sf9 cell genome, genomic DNA was extracted from Sf9-hSCF cells. The extracted genomic DNA was used as template for PCR with sense and antisense primer used in construction of the pIEx-hSCF. The PCR product of 822 bp size was correctly amplified in band (Data not shown). Also, Western blot analysis was used for confirmation of hSCF recombinant protein expression (Fig. 2A). As a result, we confirmed that the hSCF recombinant proteins were expressed in Sf9 cells.

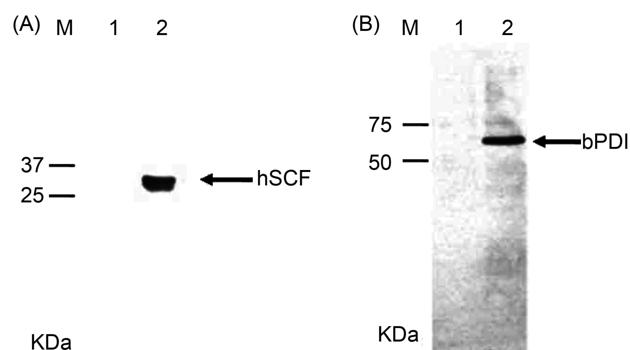

Fig. 2. Western blot analysis of hSCF and bPDI recombinant proteins in Sf-hSCF/bPDI cells. Samples were electrophoresed on 12% denaturing polyacryamide gel and transferred to a PVDF membrane. A) The proteins were detected with the rabbit monoclonal antibody against SCF. M, protein molecular mass markers (kDa); lane1, Sf9 cells; lane2, Sf-hSCF cells. B) The proteins were detected with the mouse monoclonal antibody against PDI. M, protein molecular mass markers (kDa); lane1, Sf9 cells; lane2, Sf-hSCF/bPDI cells.

The DNA fragment encoding bPDI (1592 bp) was inserted into the insect cell expression vector, pIZT described in Material and Methods (Fig. 1B). To construct Sf-hSCF-bPDI cell line, the pIZT-bPDI vector was transfected into Sf-hSCF cells. The Sf-hSCF/bPDI cells were subsequently selected by treatment with zeocin (400 ug/ml). To analyze whether the bPDI gene was correctly introduced in Sf-hSCF cell genome, genomic DNA was extracted from Sf-hSCF/bPDI cells. The extracted genomic DNA was used as template for PCR with sense and antisense primer used in construction of the pIZT-bPDI. The PCR product of 1,592 bp size is correctly

amplified in band (Data not shown). Also, SDS-PAGE and Western blot analysis were used for confirmation of bPDI and hSCF recombinant protein expression. In SDS-PAGE, extra bands as expected were not found. But, Western blot analysis verified that the selected Sf-hSCF and Sf-hSCF/bPDI cells expressed hSCF (30.9 kDa) and bPDI (55.6 kDa), respectively (Fig. 2). As these results, we confirmed that the bPDI recombinant protein was expressed in hSCF-transfected Sf9 cells.

Comparison of hSCF recombinant protein expression in Sf-hSCF and Sf-hSCF/bPDI

We investigated whether bPDI improved the production of hSCF or not. In the Western blot analysis, hSCF recombinant proteins in Sf-hSCF/bPDI cells were indentified higher expression than that of Sf-hSCF cells (Fig. 3B). Also, we confirmed approximately 2.3-fold increase of hSCF protein level in the Sf-hSCF/bPDI cells as compared to Sf-hSCF cells by relative quantification analysis of Western blot signal using the image J program. These results suggest that bPDI assists in the folding of newly synthesized polypeptides for oxidation and/or disulfide isomerization, which improves hSCF production. Thus, we conclude that bPDI gene fusion will be very useful for the large-scale production of biologically active recombinant proteins including hSCF.

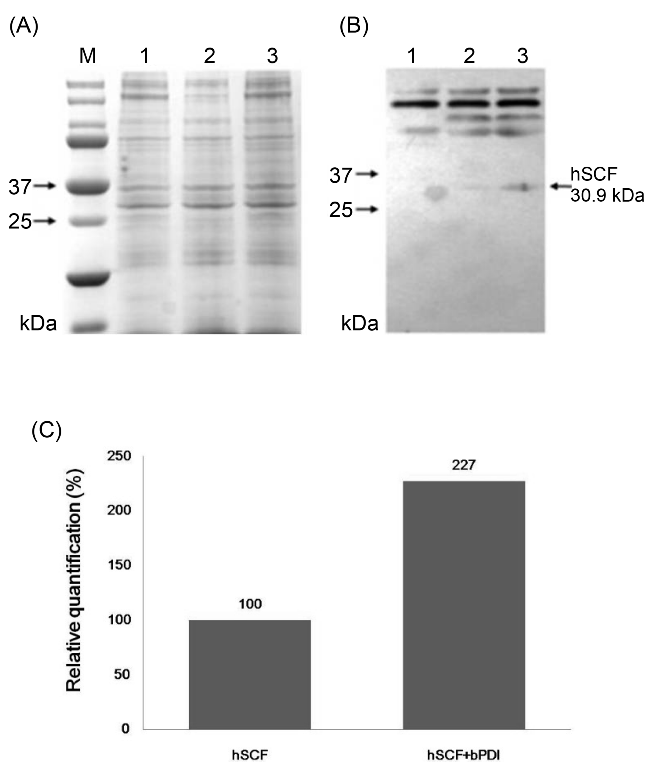

Fig. 3. Comparison of hSCF recombinant protein expression in Sf-hSCF cells and Sf-hSCF/bPDI cells. A) Samples were electrophoresed on 12% denaturing polyacryamide gel and stained with coomassie bryant blue R250. B) The celsl of overexpressing hSCF were analyzed by Western blot using the rabbit monoclonal antibody against SCF for each lysate (20 ug). Protein concentrations were determined by Bradford's method; lane1, Sf9 cells; lane2, Sf9 cells were transfected with pIEx- hSCF; lane3, Sf9 cells were co-transfected with pIEx- hSCF and pIZT-bPDI. C) The band intensities of hSCF were measured using the Image J program; hSCF, hSCF overexpressing Sf9 cell; hSCF+bPDI, hSCF and bPDI co-expressing Sf9 cell. Mean values from three independent experiments done in duplicates at $p < 0.05$ are presented.

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