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Expression of Human Nerve Growth Factor β and Bacterial Protein Disulfide Isomerase (DsbA) as a Fusion Protein (DsbA::hNGF) Significantly Enhances Periplasmic Production of hNGF β in *Escherichia coli*

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Abstract

Production of human nerve growth factor β, a neurotrophin required for the development and survival of the nerve cells, in Escherichia coli forms insoluble aggregates because it exhibits complex three disulfide bonds in its structure. Also periplasmic expression of it using a usual bacterial signal peptide often has multiple difficulties. In this study a useful method was used to improve periplasmic expression of hNGF- β. To promoting NGF production and translocation through the inner membrane, the pET39b plasmid that permits DsbA expression as a fusion protein with hNGF in comparison to pET22b plasmid with a routine signal peptide (pelB) were used. Studies carried out in the present work indicated high expression and periplasmic translocation of rhNGF when pET39b-DsbA::hNGF plasmid (over expression of DsbA) was used. In contrast, when pelB signal sequence was used for periplasmic expression, no rhNGF production was obtained. These results indicate the efficiency of DsbA over expression to increase the total yield and periplasmic expression of the recombinant proteins with multiple disulfide bonds.

Keywords: DsbA protein, nerve growth factor, pelB signal sequence, periplasmic production.

1. Introduction

Human nerve growth factor (hNGF), discovered for the first time by Rita-Levi Montalcini and her co-workers is a member of the neurotrophin family (LEVI-MONTALCINI & al. [1]; LEVI-MONTALCINI [2]; DECHANT and NEUMANN [3]). This high molecular weight (130-140 kDa) protein consists of three subunits; α, β and γ subunits. The β subunit responsible for NGF biological activity is a dimmer composed of two similar 118- amino acid subunits associated non-covalently. Each monomer of β-NGF has a cysteine knot motif which is formed by three disulfide bonds (WIESMANN & al. [4]; WIESMANN and DE VOS [5]). β-NGF like other neutrophins plays a fundamental role in development, maintenance and survival of the central and peripheral nervous systems; so it can be used as a therapeutic agent for the treatment of neurodegenerative diseases such as Alzheimer's disease (AD) (CATTANEO & al. [6]; SNIDER [7]; HEESE & al. [8]). Besides, it has also other important functions; for examples accelerating wound healing in diabetic mice and working as an effective factor in treating several ocular diseases (MUANGMAN & al. [9]; WANG & al. [10]). NGF extracted from natural source, the male mice submaxillar glands, is

unsuitable for therapeutic goals due to the fact that these extracts are heterogeneous mixtures of partially degraded dimmers (BOCCHINI and ANGELETTI [11]; MOBLEY & al. [12]). Therefore, many studies have attempted to produce recombinant hβ-NGF using prokaryotes. Expression of β-NGF containing disulfide bonds in its native structure in the reducing environment of cytoplasm often produces aggregates called inclusion bodies (IBs) (DE BERNARDEZ-CLARK & al. [13]). However, IBs can be isolated and subsequently renatured but in the case of β-NGF these renaturation and refolding processes not only are time consuming and costly but also yield the protein at very low concentrations (RATTENHOLL & al. [14]). Therefore, in this study, two different vectors (pET22b(+) and pET39b(+)) were utilized in order to secret the recombinant NGF to the oxidative environment of *E. coli* periplasm. Also the periplasm of *E. coli* contains at least four enzymes called Dsb (disulfide bond formation) proteins, DsbA, DsbB, DsbC, and DsbD, which are involved in disulfide bond formation (CHOI and LEE [15]; BERKMEN [16]). As recent studies have revealed that overexpression of Dsb proteins in the periplasm of *E. coli* increases the periplasmic expression of a number of heterologous proteins with multiple disulfide bonds, we used pET39b(+) vector containing DsbA gene; DsbA is a periplasmic enzyme that can assist disulfide bond formation in nascent polypeptide chains, in order to produce correctly folded β-NGF with high efficiency (CHOI and LEE [15]; BERKMEN [16]; KUROKAWA & al. [17]).

2. Materials and Methods

2.1. Bacterial strains, culture conditions and recombinant DNA technology

The BL21(DE3) strain (Novagene-USA), carrying T7 RNA polymerase gene was used as the expression host throughout the experiments. pET22b(+) (Novagene-USA) plasmid, carrying pelB signal peptide coding sequence was used for periplasmic expression. Also pET39b(+) (Novagene-USA) plasmid contained the signal peptide and entire coding region of *dsbA* gene was used to ensure disulfide bond formation in periplasmic space. The above vectors also carry histidine-tag sequence and T7 promoter. The cDNA of mature human β-NGF (obtained from NCBI gene bank) was synthesized and cloned using *BamHI* and *XhoI* (Fermentas-USA) restriction enzymes according to standard procedures into each vector (SAMBROOK and RUSSEL [18]). Some codons rarely used in *E. coli* were replaced by those used more frequently and also the GC content was optimized in order to improve translational efficiency of the mature form of the NGF gene. The resulting vectors, pET22b::hNGF and pET39b::hNGF were transformed individually in *E. coli* (DE3) host strain. The cells were cultured in Luria-Bertani medium (Sigma-USA). 100 μg ml^{-1} ampicillin (Sigma-USA) or 50 μg ml^{-1} kanamycin (Sigma-USA) were used for selection of recombinant bacteria. In order to detect the colonies with hNGF coding sequence, after isolating recombinant clones on selective media, the plasmid DNA from each clone was extracted using alkaline lysate method as well as commercially prepared columns (Bioneer-Korea) and then analyzed by restriction mapping and sequence analysis (SAMBROOK and RUSSEL [18]); MASSA & al. [19]). Double digestion of purified plasmid using appropriate restriction enzymes was used; in addition recombinant clones were sequenced to confirm the insertion of the hNGF gene into the vectors without any base deletion or substitution.

2.2. Protein expression

For gene expression, 1% dilution of an overnight culture of BL21(DE3) transformants was grown at 30°C in LB medium with the appropriate antibiotics to a OD_{600} of 0.5-0.8. Gene expression was induced by addition of isopropyl thio-β-D-galactoside (IPTG) (Sigma-USA)

at a final concentration of 1mM. The cells were grown for additional 4 hours at 30°C and harvested by centrifugation at 5000 g for 15 min and subjected for protein analysis.

2.3. Preparation of cytoplasmic and periplasmic proteins

To obtain periplasmic proteins of bacterial cells, osmotic shock procedure with some modifications was used (LIBBY & al. [20]). Briefly, cell pellets were re suspended in 40 ml g^{-1} cells of ice cold TES buffer (0.5 M sucrose, 0.03 M Tris-HCl and 1mM EDTA) pH 8.0 and mixed for 15 min. The mixture was then centrifuged at 10000 g for 10 min at 4°C. Then ice cold MgSo4 was added rapidly to the pellet and incubation was done for 10 min. Resultant mixture was centrifuged as described previously and the pellet was saved as cytoplasmic fraction. Tri-chloro acetic acid (TCA) was added to the supernatant up to 12% of the final volume. After centrifugation the pellet was collected as periplasmic fraction for further protein analysis. To obtain cytoplasmic proteins of bacterial cells, urea 8M (Merck-Germany) was added to the cellular pellet and the resultant suspension was then sonicated at least for six cycles of 30s on/1min off on ice in order to fragment the cytoplasmic membrane. After overnight incubation at 37°C and centrifugation of mixture at 10000 g, the supernatant was collected as cytoplasmic fraction.

2.4. SDS-PAGE and immunoblot analysis of the recombinant hNGF

SDS-PAGE was carried out according to the modified method explained by Laemmli (LAEMMLI [21]). The prepared protein samples (under denaturing and reducing conditions) were subjected to electrophoresis on a 15% polyacrylamide gel and were stained by Coomassie Brilliant blue (Merck- Germany). For western blotting, the proteins from gel were transferred onto a nitrocellulose membrane (Millipore-USA) in a transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol) at 200 mA (DEMAIO [22]). The membrane subsequently was treated with anti-his tag monoclonal antibody (Sigma-USA) conjugated with horseradish peroxidase with 1:1000 dilution in blocking buffer (PBS; 3% W/V skimmed milk). Dot blotting was carried out using rabbit anti-hNGF polyclonal antibody (S.Cruz-USA) with 1:500 dilution and anti-his tag monoclonal antibody (Sigma-USA) conjugated with horseradish peroxidase with 1:1000 dilution individually. For the first reaction, peroxidase conjugate anti-rabbit IgG (Sigma-USA) was added as a secondary antibody with 1:2000 dilution. Proteins were detected using a solution of DAB (Biobasic-Canada) with hydrogen peroxide as enzyme substrate.

3. Results and Discussion

3.1. Construction of the pET22b-hNGF and pET39b-hNGF expression vectors for the periplasmic production of rh-NGF

With the aim of addressing the recombinant mature hNGF in *E. coli* periplasmic space, two recombinant plasmids, pET22b-pelB::hNGF and pET39b-DsbA::hNGF, after codon and GC content optimization were constructed. Figure1 shows the coding sequence of hNGF before and after optimization. The above vectors differ in the signal peptide that allows the periplasmic translocation of the recombinant protein. In particular, pET39b::hNGF is characterized by the presence of the DNA region encoding the *DsbA*. The above recombinant plasmids were moved into the BL21(DE3) strain of *E. coli* and a number of colonies in each case were isolated from selected media containing antibiotics. Verification of the isolated bacteria was performed by restriction enzyme (RE) analysis of the isolated plasmid DNA with *BamHI* and *XhoI* to release the hNGF insert with the size of 360 bp (Figure 2). Also the recombinant plasmids were sequenced using T7 terminator primer (Data not shown). The confirmed recombinant bacteria were subjected for subsequent expression analysis.

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Optimized     7   TCTTCCAGCCATCCGATTTTCCATCGCGGTGAATTTTCTGTCTGTGATAGCGTGTCTGTG
Original      7   TCATCATCCCATCCCATCTTCCACAGGGGCGAATTCTCGGTGTGTGACAGTGTCAGCGTG

Optimized    67   TGGGTGGGTGATAAAACGACGGCAACGGATATTAAAGGCAAAGAAGTGATGGTTCTGGGT
Original     67   TGGGTTGGGGATAAGACCACCGCCACAGACATCAAGGGCAAGGAGGTGATGGTGTTGGGA

Optimized   127   GAAGTCAACATCAACAACAGTGTGTTCAAACAGTATTTCTTTGAAACGAAATGCCGTGAT
Original    127   GAGGTGAACATTAACAACAGTGTATTCAAACAGTACTTTTTTGAGACCAAGTGCCGGGAC

Optimized   187   CCGAACCCGGTTGACAGCGGCTGTCGCGGTATTGATAGCAAACATTGGAACAGCTACTGC
Original    187   CCAAATCCCGTTGACAGCGGGTGCCGGGGCATTGACTCAAAGCACTGGAACTCATATTGT

Optimized   247   ACCACGACCCACACGTTCGTCAAAGCGCTGACGATGGATGGCAAACAAGCGGCCTGGCGT
Original    247   ACCACGACTCACACCTTTGTCAAGGCGCTGACCATGGATGGCAAGCAGGCTGCCTGGCGG

Optimized   307   TTCATCCGCATTGATACCGCCTGTGTCTGTGTCCTGTCCCGTAAAGCCGTCCGTCGTGCC
Original    307   TTTATCCGGATAGATACGGCCTGTGTGTGTGCTCAGCAGGAAGGCTGTGAGAAGAGCC
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Figure 1. cDNA of mature human β-NGF before and after optimization. Changed bases are shown grey.

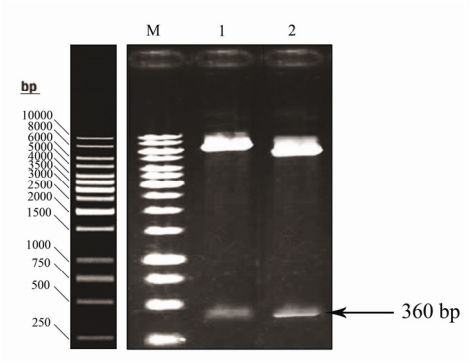

Figure 2. Restriction enzyme analysis of hNGF recombinant by agarose gel (1% W/V) electrophoresis. M: DNA Ladder (Fermentas-USA). Lane 1: digestion of recombinant pET22b::hNGF DNA with *BamH*I and *Xho*I. Lane 2: digestion of recombinant pET39b::hNGF DNA with *BamH*I and *Xho*I.

3.2. Analysis of the periplasmic rhNGF production

Total protein patterns from the bacteria containing two different plasmids, 4 hours after induction with IPTG were analyzed by SDS-PAGE and dot blotting using specific anti-histag.HRP monoclonal antibody to evaluate the production of rhNGF. As shown in Figure 3 A, there were no differences between the total protein patterns of the negative control or uninduced bacterial extracts (without IPTG) and the total protein patterns of the bacteria after induction with 1mM of IPTG. This is because of basal transcription (promoter leakage) (TEGEL & al. [23]). It has to be noted that in the case of pET22b-pelB::hNGF plasmid the expressed protein is thought to be the pelB::hNGF or hNGF, so the recombinant protein band must be in the range of 15 and 13 kD, respectively. But, when pET39b-DsbA::hNGF plasmid is used to express the NGF, the molecular weight of the recombinant protein is approximately 42-44 kD which is fusion protein of hNGF and DsbA enzyme. The results obtained from dot blot analysis revealed that proteins obtained from bacteria containing pET39b-DsbA::hNGF

plasmid were reactive to anti-histag.HRP antibody; a strong dark color dot indicates reactivity with antibody (Figure 3, panel B). On the contrary, no rhNGF production obtained when pET22b-pelB::hNGF plasmid was used; no stain was observed. To confirm the above results, proteins extracted from bacteria harboring pET39b-DsbA::hNGF plasmid were analyzed by another dot blot assay using specific anti-h-NGF polyclonal antibody. As shown in Figure 3, panel C, the proteins were reactive to anti-NGF polyclonal antibody confirming that the expressed protein is hNGF; a strong dark color dot indicated the presence of hNGF protein compared to the negative control (uninduced bacterial extracts) that has very light color dot stain when using the anti-hNGF polyclonal antibody.

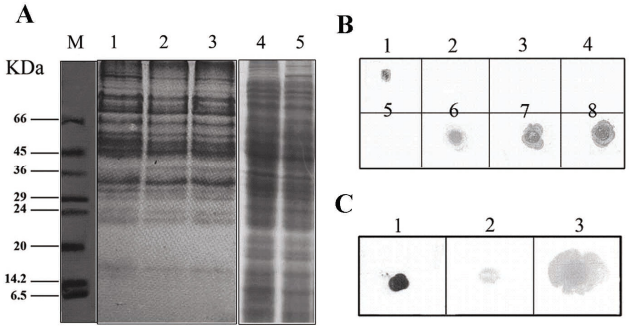

Figure 3. Analysis of recombinant hNGF production. (A) 12% SDS gel electrophoresis of the recombinant bacterial total proteins extracted by urea 8M. Lane 1 is the total protein pattern of recombinant bacteria carrying pET39b plasmid without induction with IPTG; Lanes 2-3 are the total protein pattern of two different colonies of recombinant bacteria carrying pET39b plasmid after induction with 1mM of IPTG; Lanes 4-5 are the total protein pattern of recombinant bacteria carrying pET22b plasmid without and with induction with IPTG respectively. M shows the protein size marker (Sigma-USA). (B and C) Dot blot assay of total proteins extracted from recombinant bacteria using anti-histag monoclonal antibody (B) and anti-h-NGF polyclonal antibody (C). Dots 1-8 in B are standard hNGF (Sigma-USA), total proteins of bacteria lacking recombinant pET22b plasmid, total proteins from an *E. coli* host carrying pET22b plasmid but without induction with IPTG, total proteins from an *E. coli* host carrying pET22b plasmid after 4 h induction with 1 mM of IPTG, total proteins of bacteria lacking recombinant pET39b plasmid, total proteins from an *E. coli* host carrying pET39b plasmid but without induction with IPTG, 7 and 8 are total proteins from two different colonies of *E. coli* host carrying pET39b plasmid after 4 h induction with 1 mM of IPTG, respectively. Dotes 1-3 in C are commercial standard hNGF (Sigma-USA), total proteins from an uninduced *E. coli* host carrying pET39b and total proteins from an *E. coli* host carrying pET39b plasmid after 4 h induction with 1 mM of IPTG, respectively.

To confirm that the DsbA sequence directed the correct and total translocation of rhNGF in the host periplasmic space, a cellular fractionation (cytoplasm and periplasm) was carried out. SDS-PAGE and western blot experiments using anti-his tag monoclonal antibody were done to analyze the protein patterns. As shown in Figure 4, the western blot analysis revealed three protein bands among the total periplasmic cell proteins. As indicated in the SDS-PAGE (Figure 4 panel A), the lightest band is due to protein breakage with his.tag at the N-terminal and the heavier bands were considered as processed and unprocessed form of NGF-DsbA fusion protein (42 and 44 KDa). Comparison of the periplasmic and cytoplasmic protein pattern showed that the recombinant BL21(DE3) bacteria harboring pET39b-DsbA::hNGF

plasmid secreted detectable amount of mature NGF in the periplasm in the presence of 1 mM of IPTG and the recombinant hNGF was accumulated in the periplasmic space of *E. coli* only when DsbA signal sequence was used. The NGF found in the periplasmic fraction of *E. coli* might probably represent soluble form of correctly folded protein. These results are in agreement with previous data suggesting that the co-expression of Dsb proteins is effective for obtaining soluble NGF in the periplasm (KUROKAWA & al. [17]). But in contrast to the previous works we produced DsbA in the form of fusion protein associated with expressed NGF (DsbA-NGF).

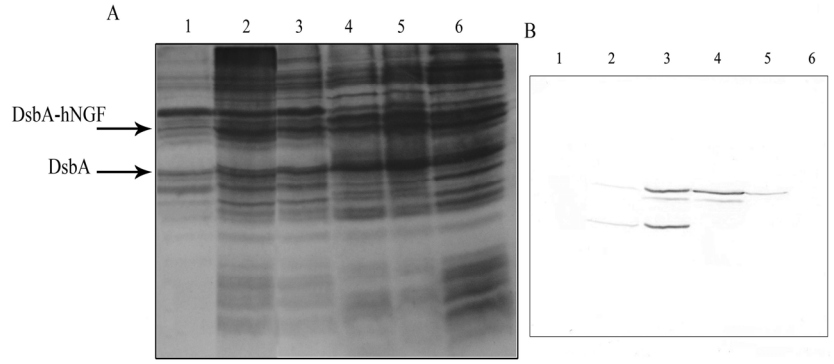

Figure 4. SDS-PAGE (A) and anti-histag.HRP western blot (B) analysis of the cytoplasmic and periplasmic protein pattern of a recombinant bacteria carrying the pET39::hNGF plasmid. Lanes 1 and 6 are the periplasmic and cytoplasmic proteins of bacteria lacking recombinant plasmid; Lanes 2 and 5 are the periplasmic and cytoplasmic proteins of recombinant bacteria without induction with IPTG and lanes 3 and 4 are the periplasmic and cytoplasmic proteins of recombinant bacteria after induction with 1 mM of IPTG respectively.

4. Conclusion

Studies carried out in the present work; indicated high expression and periplasmic translocation of rhNGF when pET39b-DsbA::hNGF plasmid was used. In contrast, when pelB signal sequence was used for periplasmic expression, no rhNGF production was obtained. Taken together, the data presented here clearly indicate that the co-expression of DsbA in the form of fusion protein with recombinant proteins containing a number of disulfide bonds (eg., NGF) not only aids to oxidative folding but also makes high expression and periplasmic translocation of the heterologous proteins possible. Also our results show that for expression of some heterologous proteins selection of signal peptide and fusion partner is very important as some of them are not effective at all.

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