

بیان هم‌زمان فاکتور رشد عصبی نو ترکیب انسانی با چاپرون تریگر فاکتور در *E. coli*

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چکیده. فاکتور رشد عصبی (NGF) یک فاکتور نوروتروفیک است که در حفظ، بقا و تمایز سلول‌های سیستم عصبی نقش دارد. پروتئین NGF دارای سه زیر واحد است که زیر واحد β آن فعالیت اصلی را بر عهده دارد. این پروتئین از موتیف گره سیستئینی که در آن رشته‌های β با پیوندهای دی سولفیدی به یکدیگر متصل شده‌اند، تشکیل شده است. NGF برای درمان بسیاری از بیماری‌ها استفاده می‌شود. به دلیل اینکه NGF استخراج شده از منابع طبیعی برای درمان نامناسب است، بسیاری از محققین برای تولید β -NGF نو ترکیب تلاش کرده‌اند. در این مطالعه، به منظور افزایش بیان NGF، این پروتئین به طور هم‌زمان با چاپرون Trigger Factor در *E. coli* بیان شد. بدین منظور pET39b(+): β -NGF و پلاسمید چاپرونی pTF16 به *E. coli* BL21(DE3) انتقال یافتند. پس از القای هر پروموتور، کل محتوی پروتئینی و پروتئین‌های پری پلاسمی بطور جداگانه استخراج شدند. به منظور تأیید تأثیر TF بر میزان بیان کلی و تولید β -NGF محلول، تکنیک‌های برادفورد و دات بلات و نرم‌افزار ImageJ استفاده شدند. سپس β -NGF با استفاده از ستون کروماتوگرافی تمایلی (Ni^{2+} -NTA) تخلیص شد. همچنین به منظور مطالعه عملکرد β -NGF تخلیص شده، رده سلول‌های PC12 با پروتئین به مدت یک هفته تیمار شدند. داده‌ها نشان دادند که بیان هم‌زمان با چاپرون TF می‌تواند سبب افزایش تولید پری پلاسمی و محلول β -NGF و نه کل محتوی پروتئینی شود. همچنین تیمار سلول‌های PC12 با β -NGF تخلیص شده (بیان‌شده با چاپرون TF)، منجر به تمایز سلول‌های PC12 به سلول‌های عصبی شد، که نشان‌دهنده عملکردی بودن پروتئین تولید شده است. داده‌های این مطالعه نشان‌دهنده این است که بیان هم‌زمان چاپرون سیتوپلاسمی TF با پروتئین NGF ممکن است یک روش مؤثر در تولید مناسب فاکتور رشد عصبی نو ترکیب (rhNGF) محلول و فعال باشد.

واژه‌های کلیدی. بیان هم‌زمان، تولید پری پلاسمی، فاکتور راه انداز، فاکتور رشد عصبی، کروماتوگرافی تمایلی

Co-expression of recombinant human nerve growth factor with trigger factor chaperone in *E. coli*

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Abstract. Nerve growth factor (NGF) is a neurotrophic factor that is functional in the survival, maintenance and differentiation of nervous system cells. This protein has three subunits, of which the beta subunit has the main activity. Its structure consists of a cysteine knot motif made up of beta strands linked by disulfide bonds. It can be used as a therapeutic agent in the treatment of many diseases. As NGF extracted from natural sources is unsuitable for therapeutic goals, many studies have attempted to produce recombinant β -NGF. In this study, Trigger Factor (TF) chaperone was expressed simultaneously with β -NGF in *E. coli* in order to obtain increased yield of soluble recombinant human β -NGF. For this purpose, pET39b(+): β -NGF and chaperone plasmid pTF16 were transferred to *E. coli* (DE3 strain). After the induction of each promoter, the total proteins and periplasmic proteins were extracted. To confirm the effects of TF on total protein and soluble β -NGF expression level, Bradford and Dot blot techniques and ImageJ software were used. Then, β -NGF was purified using affinity chromatography column (Ni^{2+} -NTA). Also, the PC12 cells were treated with the protein for one week in order to study the function of purified NGF. Our data indicated that co-expression of TF could increase the soluble and periplasmic production of β -NGF but not total proteins. Also, the treatment of PC12 cell line with purified β -NGF, co-expressed with TF chaperone, showed differentiation of these cells to nerve cells. This indicated that the purified NGF is fully functional. Our data suggest that the co-expression of cytoplasmic chaperone (TF) with recombinant nerve growth factor might be an efficient approach to produce a proper quantity of soluble and active rhNGF.

Keywords. affinity chromatography, co-expression, nerve growth factor, periplasmic production, trigger factor

INTRODUCTION

The Nerve Growth Factor (NGF) is the prototype of the neurotrophins protein family (Wang *et al.*, 2014). It is involved in the survival and growth of sensory neurons as well as the maintenance of neurons in the central nervous system (Wang *et al.*, 2014; Dreyfus, 1980; McAllister, 2001; Lindsay & Harmar, 1989). In addition to its neuronal targets, NGF has been shown to act on a number of non-neuronal targets, including other cells of the brain such as astrocytes and microglia, cells of the immune system such as mast cells and basophils, keratinocytes, blood vessel endothelial cells and many others (Wei *et al.*, 2015; Wang *et al.*, 2014; Aloe & Levi-Montalcini, 1977; Bischoff & Dahinden, 1992; Di Marco *et al.*, 1992; Raychaudhuri *et al.*, 2001). NGF was isolated for the first time from snake venom and mouse submaxillary glands (Levi-Montalcini & Cohen, 1956). As NGF extracted from natural sources is unsuitable for therapeutic goals, many studies have attempted to produce recombinant β -NGF using different hosts (Barnett *et al.*, 1990; Fan & Lou, 2010; Manni *et al.*, 2013).

E. coli is the best-studied organism for heterologous protein production because it is easy to transform, grows quickly in simple media, requires low-cost equipments for growth and storage and has well-known genetics. However, high level expression of recombinant proteins in this host often results aggregation in the form of inclusion body (Lilie *et al.*, 1998; Schein & Noteborn, 1988; King & Betts, 1999; Schrödel & de Marco, 2005; Choi & Lee, 2004). One of the approaches used most extensively to improve the yield of soluble protein in *E. coli* involves the co-expression of molecular chaperones.

Molecular chaperones contribute to the folding of newly synthesized proteins to the native state and provide a quality control system which is helpful in refolding misfolded and aggregated proteins (De Marco *et al.*, 2007). A variety of heterologous proteins co-expressed with cytoplasmic or periplasmic chaperones increasing the solubility has already been reported (Sonoda *et al.*, 2011; Pei *et al.*, 2015; Tong *et al.*, 2016; Mahamad *et al.*, 2016). In this study, the co-expression of TF chaperone was carried out in order to obtain high level production of active and soluble recombinant β -NGF.

MATERIALS AND METHODS

Material

pET39b (+) (carried signal peptide and entire coding region of *dsbA* gene to ensure disulfide bond formation in the periplasmic space) and pTf16 chaperone plasmid (carried trigger factor chaperone) were purchased from Novagene and TaKaRa, respectively.

Unless otherwise specified, all reagents were purchased from Merck (Germany).

Gene synthesis

Gene synthesis and cloning of the β -NGF cDNA to the pET39b expression vector was done by ShineGene (China).

Transformation

Transformation of pET39b (+):: β NGF into *E. coli* BL21(DE3) was done using heat shock method. Transformants were selected on LB agar with kanamycin (70 μ g/mL for pET39b (+):: β NGF) and chloramphenicol (20 μ g/mL for pTf16 chaperone plasmid) (Sambrook *et al.*, 2006).

Expression of recombinant β -NGF

In brief, overnight cultures were inoculated into LB medium containing 70 μ g/mL of kanamycin. After the culture was grown in an OD_{600nm} of 0.6 with vigorous shaking (180 rpm) at 37°C, Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for the expression of β -NGF in *E. coli*. The incubation period continued for another four hours at 37°C with shaking at 180 rpm. Then, the cells were harvested by centrifugation and total proteins were extracted using 8M Urea (Sambrook *et al.*, 2006).

Co-expression of β -NGF with trigger factor chaperone

The single colony of transformants containing pET39b:: β -NGF and pTf16 chaperone plasmids was inoculated into LB medium containing 20 μ g/ml chloramphenicol and 70 μ g/ml kanamycin for plasmid selection and 0.5 μ g/ml L-arabinose for induction and expression pTf16 chaperone. IPTG was added to the medium to final concentration of 1mM, when OD_{600 nm} of the culture reached 0.5-0.7. Then, the cells were grown for an additional 4h at 37°C and were harvested by centrifugation.

Extraction of periplasmic proteins

To obtain periplasmic proteins of bacterial cells, osmotic shock procedure with some modifications was used (Libby *et al.*, 1987).

In Brief, 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. The resultant mixture was centrifuged as described previously. 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.

Dot blot analysis

The same quantities of samples (5 µg) were loaded on the nitrocellulose membrane. Non-specific interaction sites were blocked by incubating the membrane for 1h in TBS-T buffer (Tris-HCl, NaCl and Tween 20) containing 5% skimmed milk. The membrane was then incubated with anti-his-tag monoclonal antibody conjugated with horseradish peroxidase (Sigma-USA) at 1:1000 dilution in TBS-T buffer containing 1% skimmed milk. Proteins were then detected using a solution of DAB (Biobasic-Canada) and hydrogen peroxide as enzyme substrates.

Purification of β-NGF by Ni²⁺-NTA affinity chromatography

The resin (ABT-Spain) was packed into a column. The column was then washed with binding buffer (50 mM NaH₂PO₄ and 300 mM NaCl at pH 8). The sample proteins were loaded and then the column was washed with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole at pH 8) in order to wash out the unbound proteins. Finally, the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 500 mM imidazole at pH 7) was added to elute the bound proteins (β-NGF with His-tag tail). The purified protein was dialyzed against PBS at 4°C overnight in order to eliminate the imidazole.

Gel electrophoresis

SDS-PAGE was carried out according to the modified method explained by Laemmli (Laemmli, 1970). The prepared protein samples (under denaturing and reducing conditions) were subjected to electrophoresis on a 12% polyacrylamide gel and were stained by Coomassie Brilliant blue.

Bradford assay

The concentration of protein in the samples was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Cell culture

PC12 cells were cultured in plastic flasks in a DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% horse serum in a humidified atmosphere with 5% CO₂ at 37°C. The cells were allowed to reach log phase growth (2 day incubation). At this time, fresh medium was added together with 50 ng/ml of purified β-NGF co-expressed with TF chaperone. The medium was changed twice (days 4 and 6) during the experiment (1 week period).

RESULTS AND DISCUSSION

1- NGF production

Total protein patterns from the bacteria containing pET39b::β-NGF and pTf16 chaperone plasmids, 4 hours after induction with IPTG were analyzed by SDS-PAGE and dot blotting using specific anti-his-tag monoclonal antibody to evaluate the production of β-NGF. It has to be noted that when pET39b::β-NGF plasmid is used to express the β-NGF, the molecular weight of the recombinant protein (DsbA-β-NGF fusion protein) is approximately 42-44 kDa, which is the sum of β-NGF and DsbA enzyme molecular weights (Fig. 1A). The results obtained from dot blot analysis revealed that proteins obtained from bacteria in both cases (with and without TF chaperone co-expression) were reactive to anti-his-tag antibody; a strong dark color dot indicates reactivity with the antibody (Fig. 1B).

The concentration of the total protein extracted from *E. coli* was determined in each case by Bradford assay. The amounts of 2.417 ± 0.096 and 1.620 ± 0.081 mg/ml were obtained for expression without and with co-expression of TF chaperone, respectively. This indicated that TF chaperone reduced the total protein expression level. However, these values are the sum of bacterial proteins, cytoplasmic and periplasmic recombinant β-NGF. As the main goal of this study is definitely the periplasmic production of β-NGF and not bacterial proteins or cytoplasmic fraction of β-NGF, periplasmic proteins in each case were extracted and the produced β-NGF was detected using dot blotting. Then, quantitative comparison of soluble β-NGF production in both experiments (with and without TF expression) was done using Image J software (Schneider *et al.*, 2012).

As it can be seen in Fig. 2, the co-expression of pTf16 (encoded TF) increased the amount of soluble β-NGF protein in the periplasmic space compared with its expression without chaperone.

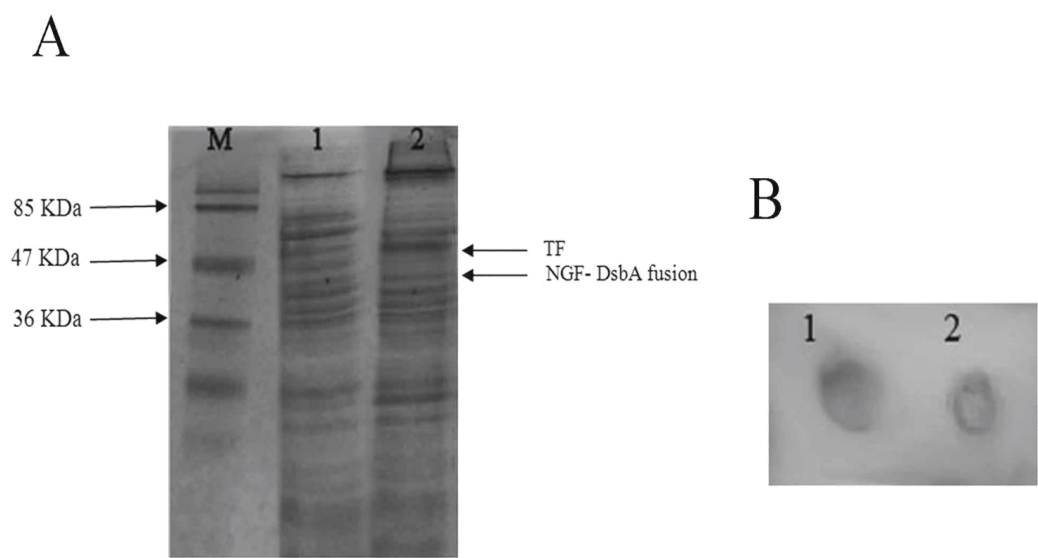


Fig. 1. A: 12% SDS gel electrophoresis of the recombinant bacterial total proteins extracted by urea 8M. **B:** Dot blot assay of total proteins extracted from recombinant bacteria using anti-his-tag monoclonal antibody. Lane 1 is the total proteins of recombinant bacteria carrying pET39b:: β NGF without chaperone plasmid; Lane 2 is the total proteins of bacteria carrying pET39b:: β -NGF and pTf16 chaperone plasmid. M shows the protein molecular weight marker.

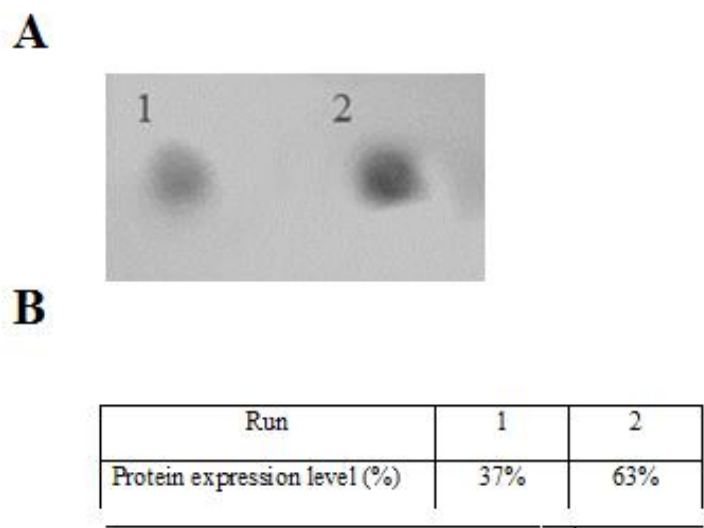


Fig. 2. Periplasmic expression level analysis of β -NGF. A: Dot blot analysis of periplasmic proteins from recombinant bacteria carrying only pET39:: β -NGF (1) and pET39:: β -NGF and pTf16 chaperone plasmids (2) using anti-histag monoclonal antibody. **B:** % Recombinant β -NGF periplasmic expression level measured by ImageJ software.

2- Purification

Ni²⁺-NTA column was used for purification. After protein purification, its accuracy was studied by SDS-PAGE and dot blot techniques. As shown in Fig. 3, two bands were seen in the range of 42 and 21 KDa; the latter is probably DsbA protein with his-tag tail at the N-terminal due to protein breakage or incomplete translation.

2- Cell culture

After protein concentration determination 50 ng/ml of purified β -NGF was added to PC12 cell line. Commercial β -NGF (Sigma-USA) was used as control too. As shown in Fig. 4, after 7 days PC12 cells were differentiated to the nerve cells. This indicated that the purified β -NGF could be fully functional.

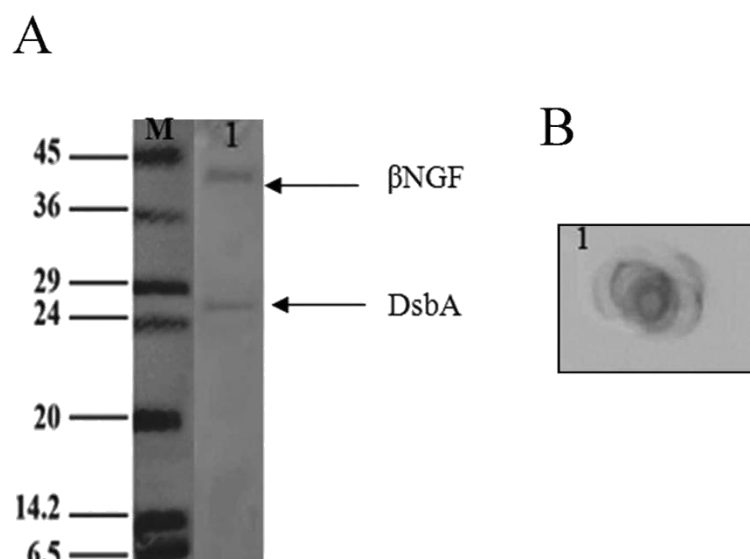


Fig. 3. A: 12% SDS gel electrophoresis of purified β-NGF. **B:** Dot blot assay of purified β-NGF using anti-histag monoclonal antibody. Lane 1 is the purified β-NGF co-expressed with pTf16 chaperone plasmid. M is protein molecular weight marker.

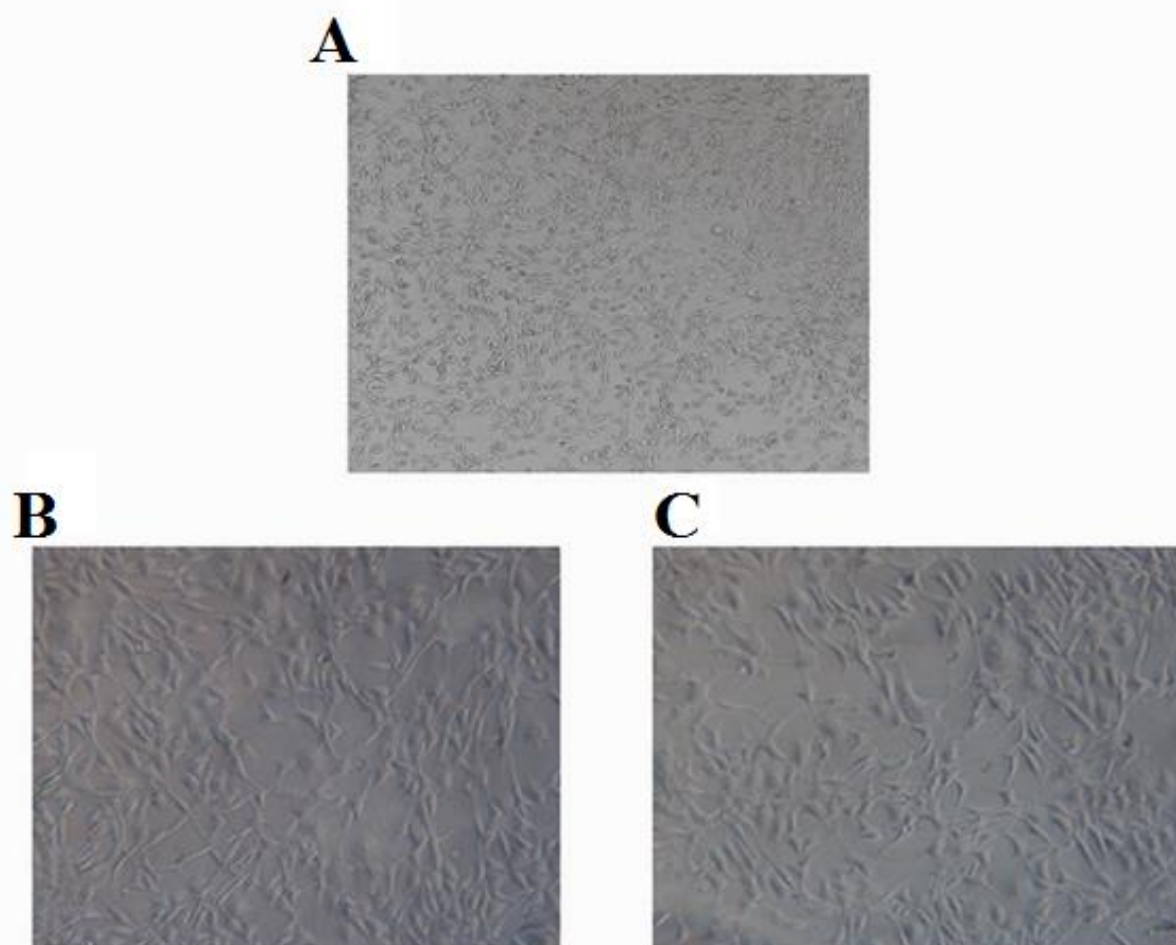


Fig. 4. A: The PC12 cells before treatment with β-NGF protein. **B, C:** PC12 cells after treatment with commercial β-NGF protein and purified β-NGF. Differentiation of PC12 cells to nerve cells is obvious in 2 and 3 (inverted microscope, ×40).

High level expression and production of recombinant proteins is the goal of many researches. However, this usually leads to the formation of aggregated and misfolded proteins called inclusion body. One of the best strategies to overcome this problem is to use fusion proteins, e. g. DsbA, as used in this research. DsbA is an enzyme that can assist disulfide bond formation in nascent polypeptide chains (Ke & Berkmen, 2014).

Another strategy for the prevention of inclusion body formation is the co-expression of molecular chaperones. As molecular chaperones are involved in the protein-folding process, their over-expression was usually carried out in order to increase the stability and solubility of the produced target recombinant protein (De Marco *et al.*, 2007; Tegel *et al.*, 2011; Makhoba *et al.*, 2015; Gounel *et al.*, 2016). *E. coli* cytoplasm involves three chaperone systems: trigger factor, DnaK-DnaJ-GrpE and GroEL-GroES (Voziyan *et al.*, 1998; Baneyx & Mujacic, 2004). Newly-synthesized proteins in bacteria are associated with the chaperone trigger factor as soon as they leave the exit tunnel of the ribosome (Deuerling *et al.*, 1999; Valent *et al.*, 1997; Schaffitzel *et al.*, 2001). The effects of different chaperone teams on protein solubility and folding have been studied in recent researches. For example, in one experiment done by Sia and colleagues, GroEL team and TF increased the production of soluble human-like collagen (HLC) by assisting its correct folding (Jia *et al.*, 2014). In another study, the co-expression of different chaperones was performed to produce a soluble and active human cyclinA (Grigoroudis *et al.*, 2015). Our goal in this study was the production of soluble and active β -NGF, a useful treatment for neurodegenerative diseases like Alzheimer's and MS (Althaus, 2004; Heese *et al.*, 2006).

In our investigation we hypothesized that the attachment of a DsbA whole sequence including the signal sequence to the N-terminal of β -NGF and co-expression of TF, a cytoplasmic chaperone, could increase the secretion of β -NGF to the oxidative environment of periplasmic space and enhance protein solubility. Comparing the total expression level of proteins with and without TF co-expression (using Bradford assay) indicated that the total expression level in the absence of TF chaperone is higher than the total expression level when the TF chaperone is expressed simultaneously in the cell. On the contrary, the highest soluble periplasmic β -NGF was obtained when TF chaperone was expressed in the cell as dot blot analysis and quantitative comparison of the periplasmic expression of β -NGF showed. Therefore, the co-expression of TF chaperone can increase the

periplasmic expression of β -NGF in *E. coli* because TF chaperone probably binds to newly synthesized β -NGF and helps its translocation to the periplasmic space with the aid of secretory machinery. Our results also were in agreement with the results obtained by Wang and colleagues in 2013 as they showed that the co-expression of only TF could enhance the HSP soluble expression level (Wang *et al.*, 2013). According to previous studies, soluble proteins are not necessarily active and functional with native conformation. For example, in the case of the luciferase, the co-expression of chaperones increased the solubility of the protein, but enzyme activity was reduced (Agashe *et al.*, 2004).

Therefore, in this investigation, the bioactivity of β -NGF was studied after its purification by Ni^{+2} -NTA chromatography. The differentiation of PC12 cell line in the presence of purified β -NGF to the nerve cells demonstrated that the obtained β -NGF was fully functional.

CONCLUSION

In conclusion, our data suggest that the co-expression of cytoplasmic chaperone TF with recombinant β -NGF might be an efficient approach to produce a proper quantity of soluble and active β -NGF for further clinical studies.

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