Brain-Derived Neurotrophic Factor is Produced in Escherichia Coli Using Transforming Plasmids

Reza Taherian[1], Mahdi Taherian[2]

[1] *Students' Research Office, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[2] *Department of Biology, Semnan University of Medical Sciences, Semnan, Iran*

ABSTRACT

Background and purpose: Brain-derived neurotrophic factor (BDNF) is present in the hippocampus and cerebral cortex in high concentrations and has an important role in development, maintenance and regeneration of the nervous system. The aim of this study was to produce active BDNF in E.coli using transforming plasmid.
Methods: To construct an expression plasmid of BDNF, a gene coding for it was obtained from human BDNF DNA. The plasmid was then transformed in the E.coli. After the extraction of plasmids, they were treated by BamHI and SacI restrictions endonuclease and the produced fragments were inserted in pTRSBDNF vector.
Results: After transformation of the recombinant vector into E.coli HB101 cells, the recombinant plasmid was transformed into E.coli C600 cells to express BDNF gene. SDS-PAGE confirmed the expression of recombinant protein of BDNF in the E.coli.
Conclusion: BDNF, as an important protein of nervous system, can be produced in the E.coli by use of transforming plasmids.

Keywords: Brain-derived neurotrophic factor; Plasmid; Escherichia coli

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Correspondence to: Mahdi Taherian, MSc; Department of biology; Semnan University of Medical Sciences, Semnan, Iran; Tel: +98 912 215 4574; Email: Mahdi_Taherian@hotmail.com

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INTRODUCTION

Brain-derived neurotrophic factor, also known as BDNF, is a member of the neuro-trophic family of growth factors [1]. This protein gene is on 11 p human chromosome [2]. The precursor protein contains 247 amino acids and in the later process the protein yields a 119 amino acid residue [3]. These amino acids are the functional division of this protein in which there are three disulfide bonds [4]. Also, this protein is active in both monomer and dimer forms [5] and stimulates tyrosine kinase receptors which leads to a series of cascades within the cell [6]. These cascades activate proteins within cells. These proteins increase expression of genes involved in the development of nervous system especially those involved in neurogenesis and differentiation[7]. Moreover, they increase hippocampal neurons [8,9] and development of the visual cortex and formation of dendrites [10]. Furthermore, this protein increase ganglion cells survival and prevents neuronal degeneration [11]. Besides, this protein Stimulates microglia and synaptic Glutamate pathway [12]. Microglia are non-neuronal cells in the central nervous system (CNS). Previous studies have shown the effect of physiological and environmental factors on the expression of the BDNF. Stress and fear reduce its expression[13] but physical activity, light and learning increase its expression [14,15]. Moreover, level of this protein changes in different diseases. Level of this protein is markedly reduced in multiple sclerosis [16], Alzheimer's disease [17], Huntington's disease [18,19], Parkinson's disease [20] and Wilson disease [21]. Hence, production and injection

of BDNF may be used as a treatment option in these patients. This study aimed to produce active BDNF in E.coli using transforming plasmid. As this protein has no glycosylation, we used E.coli for cloning of this protein. Besides, we used pTRSBDNF vector in order to make disulfide bonds. This vector secrets the protein into the periplasmic space. This space is a suitable environment for this type of linkage [22].

MATERIALS AND METHODS

Construction of cDNA of BDNF protein

BDNF gene sequences was taken from the NCBI database access number NG_011794.1. Then, using the database UniProt, the coding sequences of the functional part of this protein was determined which had 357 bp. In the next step, we used "codon adaptation tools" database in order to optimize codons and to remove rare codons of this sequence. Then, the nucleotide sequences of Bam HI enzyme and SacI enzyme identifier were placed at 5′and 3′ ends respectively. Moreover, the nucleotide sequence of cutting of the restriction enzyme invertase was placed. The final sequence was ordered to Pishgam Company, Tehran. The sequence, which was 375 bp after addition of aforementioned sequences, was placed in the PUC57 vector in its synthetic form (Figure 1)

Amplification of the coding DNA of BDNF

In order to increase the coding DNA of BDNF, PUC57 vector was transformed into E.coli strain DH5. Given that this plasmid has ampicillin antibiotic resistance genes, medium with this antibiotics was used to ensure the transformation of it. After the bacteria was propagated sufficiently, vector extraction was performed using the Biobasic kit.

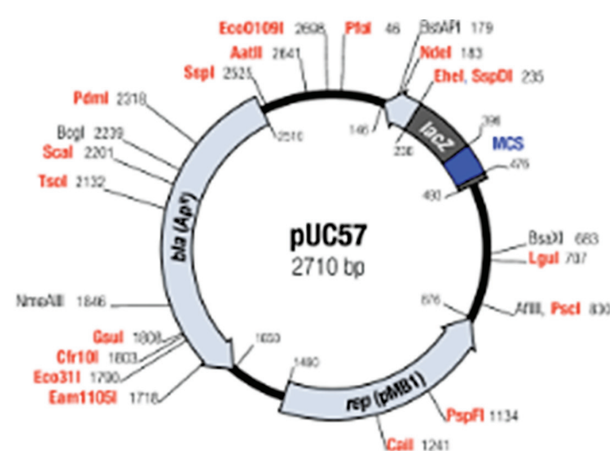

Figure 1. PUC57 vector

Cloning of BDNF protein coding sequence

The propagated vector contains the BDNF protein coding sequence. To separate the pieces and create sticky ends, the vector was treated with the enzymes Bam H1 (Fermentase, Germany) and SacI (Fermentase, Germany) according to the manufacturer's instructions. In each phase, samples were analyzed by electrophoresis. Finally, the piece with sticky ends of these two enzymes was introduced into the BamH1 and SacI places of vectors. The ligation technique was performed by Vivantis kit (Malaysia). To replicate recombinant vector, it was transmitted into the E. coli strain HB101 cells. In the next day, the colonies were analyzed using colony PCR and specific primers carriers. Forward Primer sequences was 5′CACTCCGACCCCGCCCGCCG 3′ and reverse primer sequence was 5′GATCCTATTATCTTCCCCTTTTAATGG 3′. The reaction was performed under the following conditions: A primary cycle which included a 95°C incubation for 4 minutes, 30 next cycles involved the denaturation in 96°C for 30 seconds, annealing in 54°C for 30 seconds and extension phase in 72°C for 1 min in a PCR equipment. The amounts used in PCR reactions are as follows: 3 ml of a mixture of 40 ml with a single colony

PCR Buffer (10X): 2.5µl, MgCl2 (50mM): 1µl, dNTP (2mM): 1µl, Primer Forward (10mM): 1µl, Primer Reverse (10mM): 1µl, Taq Polymerase: 0.3µl, DDW: .15 .2µl. Besides, a PCR reaction was performed on purified plasmid colonies as a positive control. One of the recombinant colonies was removed and was cultured overnight. Then, the vector extraction was performed using the Biobasic kit (Canada). Finally, to sequence and ensure of its existence, the piece was sent to Tehran Gene Technologists Company.

Analysis of BDNF protein expression

The recombinant vector was transformed in the E.coli HB101 strain. After one night, a colony was cultured in 5 ml LB medium. Then, 1 ml of that was transferred to 100 ml. When the culture OD reached to 0.6, BDNF expression was stimulated by adding IPTG (Fermentas, Germany) and the cells were maintained for 6 hours in growth conditions (37°C). Then the medium was centrifuged at 400rpm. To better separate proteins of periplasmic space, the sediment bacteria was dissolved in 20% sucrose and then was centrifuged in 11000g acceleration. The remaining sediment was dissolved in phosphate buffer with pH = 7/4 and the bacterias were destroyed in five cycles of 30 seconds and 90 seconds of rest according to sonication method. After centrifugation

at 14000 rpm, the bacteria extract was separated form waste and it was loaded and stained on 15 ml of SDS-PAGE gel.

RESULTS

Cloning of the gene expression of BDNF protein

PUC57 vector which contained the piece was extracted after transformation and replication in the bacteria. To separate the piece, the vector was treated with restriction enzymes. Pure plasmid has 2710 bp and plasmid with intended fragment has 3085 bp (Figure 2). Intended fragment was connected to the sticky ends of pTRSBDNF vector of these two enzymes (Figure 3). The movement of the vector was different before and after the incorporation reaction (Figure 4). Colony PCR was done to investigate the presence of recombinant vector in the produced colonies. A 634 bp fragment of recombinant colony and a 270 bp fragment of the colonies with pure plasmid (positive control) was replicated. Colony PCR analysis showed that the recombinant vector is one of the three investigated colonies (Figure 5).

Expression analyzes BDNF protein

To study the expression of BDNF protein, extracts of cells transformed with pTRSBDNF and were electrophoresed. BDNF expression with molecular weight of 14 kDa was clearly identified on the gel SDS-PAGE (Figure 6).

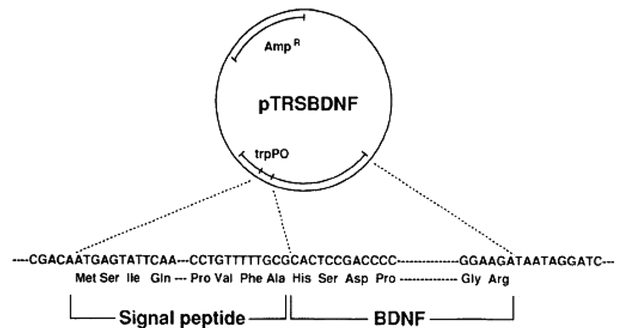

Figure 3. pTRSBDNF Vector.

Purification of BDNF

The mature BDNF protein produced by E.coli cells harboring pTRSBDNF was purified. After cells were intermittently sonicated, a cell lysate was obtained by removing cell debris from the disrupted cell suspension through centrifugation. The cell lysate was centrifuged at 26,000rpm for 16 h to obtain the supernatant and the precipitate. The supernatant and the precipitate were called the soluble protein fraction and the insoluble protein fraction, respectively. The soluble protein fraction included 20% of total BDNF protein and the insoluble protein fraction included 80% of total BDNF protein. The soluble protein fraction was purified using ion exchange chromatography, gel filtration, and reverse-phase chromatography. After lyophilization, the BDNF was dissolved in a PBS buffer (PH7.0) for bioassay.

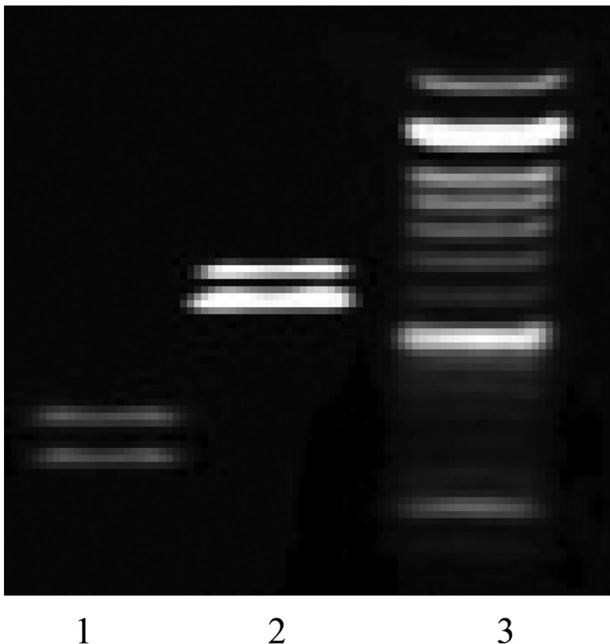

Figure 2. PUC57 vector. Lane 1: DNA Ladder 1 kb, lane 2: Extracted PUC57 Vector, lane 3: Treated PUC57 with two enzymes (Pure plasmid has 2710 bp).

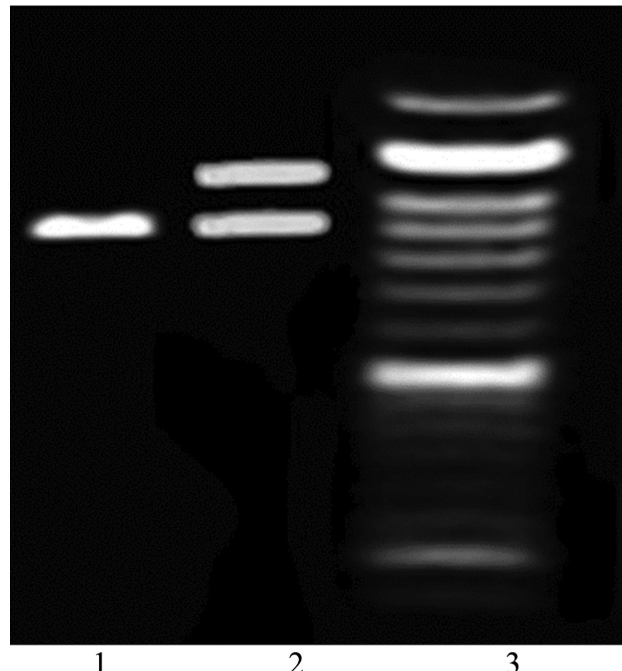

Figure 4. pTRSBDNF vector. Lane 1: DNA Ladder 1 kb, lane 2: Natural pTRSBDNF 26b Vector, lane 3: Recombinant pTRSBDNF Vector.

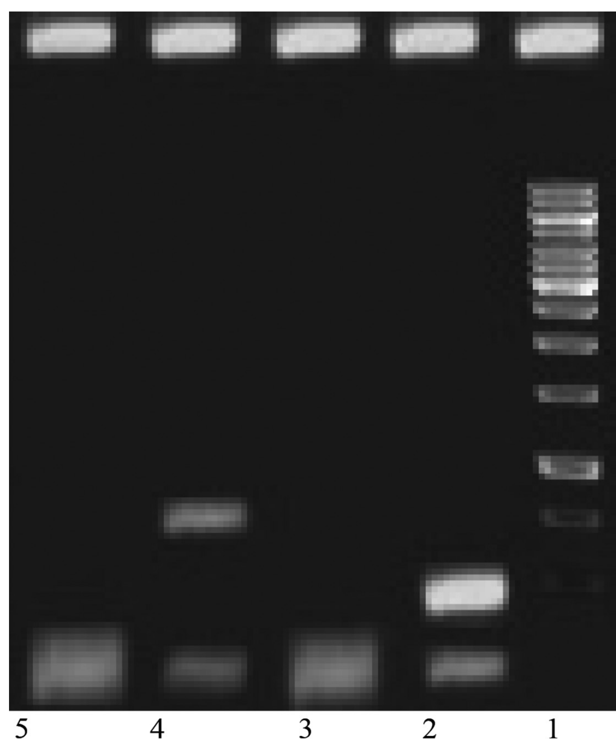

Figure 5. Colony PCR. Lane 1: DNA Ladder 1 kb, lane 2: Positive control (Colony has purified plasmid and the amplified fragment of 270 bp), lane 3,5: No piece, lane 4: Recombinant colony(amplified fragment of 634 bp).

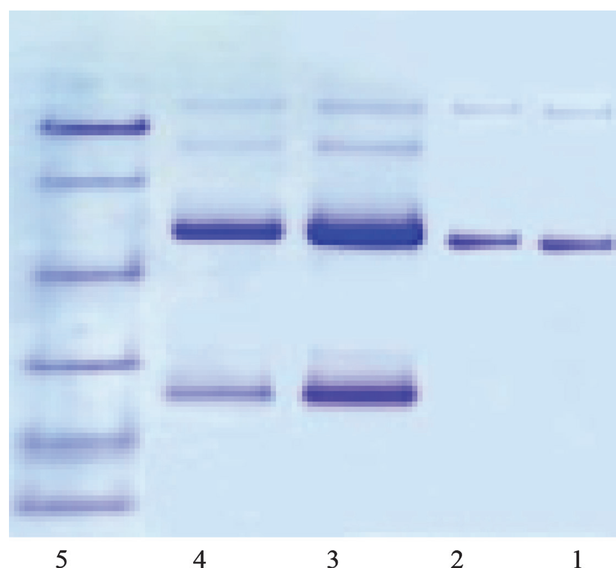

Figure 6. SDS-PAGE gel. Lane 1: Cytosol sample with Recombinant protein, lane 2: Periplasmic Space sample with Recombinant protein, lane 3: The final sediment bacteria sample with Recombinant protein, lane 4: Destruction of bacteria sample with vector, No piece, lan5: The size of protein view.

Its purity was measured at 98% from the intensity of staining a SDS-PAGE gel. On the other hand, the insoluble protein fraction was dissolved in 6M guanidine hydrochloride solution. After the solution was dialyzed against 10mM acetate buffer (PH4.5), the aggregates formed were dissolved in an 8 M urea solution. The BDNF in the 8 M urea solution was purified by ion exchange chromatography and gel filtration in an 8 M urea solution, and reverse-phase chromatography.

DISCUSSION

Due to diagnostic and therapeutic benefits of BDNF protein, production of this protein is in a great attention. In 1990, Quinn et.al extracted the RNA of this protein from frozen brain and cloned it after replication. Also, the produced Protein included the pre-peptide piece [23]. Moreover, in 2007, this protein with its pre-peptide piece was cloned in PUC19 vector and expressed in pET23c The produced protein needed cutting in addition to denatured protein solution to take its own structure [24].

In the current study, only the coding sequence of the functional part of this protein was selected for cloning purposes to avoid other steps, such as cutting enzyme. Avoiding these steps causes a lower cost in addition to reducing the needed time. Also, pTRSBDNF vector contains pelb sequence that transfers the recombinant protein to periplasmic space. Since this protein has three disulfide bonds, periplasmic space is a suitable environment to create these bonds. Furthermore, pTRSBDNF vector is designed in such a way that the cloned gene in it can be connected to six CAC sequences. This causes the produced recombinant protein to be connected to six histidine which enables the protein to be purified by the nickel column chromatography. Given the importance of BDNF protein in the pharmaceutical industry and health features as well as diagnostic applications, the production of this protein could be an important step towards the development of the pharmaceutical industry. Our recombinant protein production system enabled us to produce biologicaly-active, mature BDNF, although it was difficult for E.coli transformants to produce biologicaly active BDNF [25]. Moreover, it can be produced in large scale by prokaryotic expression vectors using cloning technique. pTR expression vectors are one of the most widely used expression systems used in the prokaryotes. These carriers have a great speed and efficacy in the producing recombinant proteins due to their viral T7 promoter [26]. Also, designing new structures of it using bioinformatics methods can be an effective step towards novel researches in the field of production and functional adjustment of this protein and opens the way of further researches. Furthermore, since this protein is not produced in our country yet, further research could create

conditions for mass production and commercialization of this therapeutic protein.

Disclosure statement

The authors have nothing to disclose.

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