Research Article

Production and Purification of Recombinant B Subunit of *Vibrio cholerae* Toxin in *Escherichia coli*

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Abstract

Cholera toxin B subunit (CTB) is a non-toxic and immunostimulatory component of *Vibrio cholera* toxin. CTB is one of the most studied protein compounds for adjuvant design. This study aimed to produce and purify recombinant CTB (rCTB) by pET-22a plasmid in *Escherichia coli* BL21(DE) strain, focusing on cost-effectiveness and ease of use. The target gene was identified in the genome of *Vibrio cholerae* biotype *El Tor* through the NCBI database, and its specific primers were designed. The gene fragment was amplified by PCR and cloned into pET-22a plasmid by *Nco*I and *Sac*I restriction enzymes and transformed into *E. coli*. Transformed bacteria were inoculated into a 2×YT medium. Stimulation of recombinant protein production was performed by adding IPTG with a final concentration of 0.4 mM. Finally, recombinant protein was purified by a Ni-IDA column. The concentration of recombinant protein was determined by GM1-ELISA and Bradford tests. The Western blotting technique verified recombinant CTB, So results showed the expected bands at a molecular weight of about 12.76 kDa (denatured) and 63 kDa (non-denatured). GM1-ELISA and Bradford tests showed the final protein concentrations of 11 and 9.57 mg/L, respectively. GM1-ELISA confirmed the biological activity of rCTB in the presence of GM1 ganglioside receptor. Recombinant CTB produced by the method proposed in this research has high purity and appropriate concentration and can be used in immunological studies, especially adjuvant design.

***Keywords*:** Cholera toxin B subunit, *Escherichia coli*, Molecular cloning, *Vibrio cholerae*

Introduction

Cholera toxin is one of the most studied bacterial toxins of the AB5 family, which is widely used as a potent mucosal adjuvant in various studies due to its ability to increase the immune response to the antigen injected with it. This toxin is an important virulence factor of *Vibrio cholerae* that causes severe diarrhea in infected people and consists of two proteins, subunit A, which is a monomer in the complex and has a molecular weight of 28 kDa, subunit B (CTB) is a Pentamer with a molecular weight of 11.6 kDa (Lebens and Holmgren, 1994). CTB is a circular structure consisting of five CTB monomers; Each monomer interacts with two adjacent molecules through hydrogen bonding and salt bridges, without covalent bonding. Centre of this pentameric structure accepts a tunnel-like structure whose inner wall is composed of 5 alpha-helical structures, each belonging to a monomer (Sixma et al., 1993).

Each CTB monomer consists of 124 amino acids, including 21 residues of leader peptide and mature form contains 103 amino acids. After producing CTB in the cytoplasm, it is directed to the periplasmic space; Here, CTB monomers come together to form a pentameric structure that may be secreted separately or in association with CTA as holotoxin AB5(Lebens and Holmgren, 1994).

The B subunit of the cholera toxin is the non-toxic part of this toxin. Its tendency to monosialotetrahexosylganglioside(GM1) receptor, which is widely distributed in cell types, including intestinal epithelial cells, antigen-presenting cells (APCs), macrophages, dendritic cells, and B cells, allows optimal access to the immune system. The identification of its cellular receptor regulates the cellular uptake of cholera toxin; CTB interacts with this receptor via the pentasaccharide fragment of GM1 (Baldauf et al., 2015).

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Because CTB is a non-toxic part of the cholera holotoxin, interest in it has grown over the past few decades. This subunit can form stable pentamers and exhibit multifaceted biological activity (Holmgren et al., 2005). For this reason, CTB has been considered as a potential vaccine and adjuvant scaffold (Boustanshenas et al., 2013a). In addition, CTB has been shown to have potent anti-inflammatory and immunomodulatory effects (Baptista et al., 2014). Since many non-recombinant CTB on the market contains small amounts of cholera toxin and its A subunit, high purity recombinant CTB are essential for immunological research.

Non-toxicity combined with stability and relative ease of expression of CTB makes it easy to use as a flexible adjuvant. The possibility of expressing this protein in many types of organisms expands its application potential(Lebens et al., 2016). Due to its effective binding to APCs and epithelial surfaces, its ability to reduce antigen amounts up to 100 times for immunization creates a valuable and cost-effective adjuvant(Stratmann, 2015).

One of the essential applications of CTB is its use in biotechnology and immunology studies, especially the development of effective adjuvants for commercial vaccines. Past studies(Alam et al., 2013; Baptista et al., 2014; Boustanshenas et al., 2013a; Charles et al., 2014; Khastar et al., 2021) have shown the widespread use of CTB as a vaccine adjuvant (co-administration or conjugation), cholera vaccine immunogen and immunomodulator. These studies have led to various rCTB expression systems for more efficient access to this protein. Given that CTB appears to be more effective for killed bacterial-based vaccines(Charles et al., 2014), the development of alternative methods of producing and transmitting recombinant CTB could play a significant role in preventing and controlling cholera. Due to its ability to stimulate mucosal humoral immune responses, the antigen-CTB combination provides a promising strategy for vaccination against enteric pathogens and mucosal diseases(Baldauf et al., 2015).

Unfortunately, many researchers cannot access this protein due to international sanctions related to bioterrorism and the very high cost of commercially recombinant CTB. So this study aimed to produce rCTB by focusing on cost-effectiveness and ease of production by a pET-based expression system in *Escherichia coli*.

Materials and Methods

Culture of Vibrio cholerae and DNA Extraction

Vibrio cholerae serotype *Inaba* and biotype *El Tor* were purchased from the Iranian research organization for science and technology (IROST) with PTCC.No 1611. Bacteria were inoculated in 100 cc of Craig's medium (Atlas, 2010) containing 3 g/L of casein hydrolyzate (Merck®, Germany), 0.4 g/L of yeast extract (Merck®, Germany), 0.05 g/L of K_2HPO_4 (SAFC®, USA), and 2mL of 20% glucose(Merck®, Germany) solution (w/v) based on Khastar and colleagues (Khastar et al., 2020) described. The inoculated culture medium was incubated at 48 °C without shaking. After culture, bacterial DNA was extracted by a genomic DNA extraction kit (Denazist®, Iran) according to the manufacturer's instructions and stored for further studies.

Gene Amplification by PCR

The nucleotide sequence of the Cholera toxin B subunit gene was extracted from the Gene Bank of the United States National Center for Biotechnology Information (NCBI, GenBank accession no. AY307389.1), with a size of 321 base pairs. Forward(5′-CATGACACACCTCAAAATATTACTGA-3′)and Reverse (5′CGAGCTCGGTACCATTTGCCATACT-3′) primers were designed based on the above sequence and synthesized by BioNEER Company of South Korea.

The target gene fragment was amplified by *pfu* DNA polymerase (G-biosciences, USA). DNA amplification was performed at a final volume of 25µL, which included 12.5µL of Mastermix solution, 0.5µL of each primer (10 picomols), 2µL DNA template, and 9.5 µL of molecular-grade nuclease-free water. The heat cycle of the reaction mixture was set for 35 cycles, beginning with a period of initial denaturation at 95°C for 10 min. Temperature and time profiles of each cycle were as follows: 95 ºC for 30 seconds (denaturation), 48ºC for 20 seconds (annealing), 72ºC for 20 seconds (extension).

According to the manufacturer's instructions, the target gene was removed from the agar gel by PCR extraction kit (Pioneer Gene Transfer Co, Iran) and stored for further studies.

Gene Cloning in pET-22a

The target gene was cloned into pET-22a by the *Sac* I (Thermofisher®, USA) and *Nco* I (Thermofisher®, USA) restriction enzymes and T4 DNA ligase (Thermofisher®, USA). Finally, the cloned plasmid was transformed into *Escherichia coli* BL21 (DE3) strain, cultured on 2xYT plates (containing 100µg/mL ampicillin), and incubated overnight at

37 °C. Plasmid-free bacteria will not be able to grow in this medium.

In order to investigate the entry of the gene fragment into the plasmid, PCR was performed randomly from several colonies using universal specific primers of the T7 promoter and terminator (Sigma-Aldrich®, Germany). Typically, the distance between these two regions is about 310 bp, which is expected to reach 631 bp if the gene fragment enters.

Expression of Recombinant Proteins

In this study, a 2xYT bacterial culture medium containing 16g/L of tryptone (Merck®, Germany), 10g/L of yeast extract (Merck®, Germany), and 5g/L of NaCl (Fakhr Razi®, Iran) was used to culture the transformed bacteria., Initially, 10cc of the culture medium (containing 100 µg/mL ampicillin) was inoculated with a colony of bacteria and incubated for 16h at 37°C with a 150×g shaker (starter culture). Finally; the starter culture was added to 1 liter of culture medium (containing 100µg /mL ampicillin) and incubated under the same conditions. When the OD_{600} reached 0.7 (range between 0.6 to 1), Isopropyl β- d-1-thiogalactopyranoside (IPTG) with a final concentration of 0.4mM was added to the culture medium and incubated for 3 hours under the conditions described above. Then the culture medium was placed on ice for 5min and centrifuged at 13000×g at 4°C (SIGMA®, Germany), and the precipitate was stored in a -20°C freezer for further investigation.

Extraction of Recombinant Protein from Periplasmic Space

In order, to lysis the cells and extract the recombinant protein, a lysis buffer containing 50 mM potassium phosphate, 400mM NaCl, 100mM KCl, 10% (v/v) glycerol, 0.5% (v/v) Triton ×100, and 10mM imidazole was used at a final pH of 7.8. First, the Frozen cell precipitate of the previous step was slowly melted and dissolved well in a lysis buffer, then transferred to a liquid nitrogen tank to freeze again. After freezing, the sample was melted at 42°C. This process was repeated three times. Finally, the samples were centrifuged at maximum speed for 1 minute at 4°C. The supernatant containing soluble proteins was stored for further evaluation.

Purification of rCTB by Ni-IDA Column

Due to six histidine residues in the C-terminal of the rCTB and the affinity of histidine for nickel metal, it can be easily purified by nickel-containing chromatographic columns. The above column was purchased from Pars Tous Biotechnology Company. The purification process was performed according to the manufacturer's instructions with some changes. After passing the previous stage solution from the nickel column, the column was washed with a washing solution containing 500mM NaCl, 50mM sodium phosphate, and 10mM imidazole at a final pH of 7.8. In order to purify the rCTB from nickel seed, the column was washed three times with an elution solution containing 500mM NaCl, 50mM sodium phosphate, and 150mM imidazole at a final pH of 7.8, and the column output was stored each time.

Confirmation of rCTB by SDS-PAGE and Western Blotting

SDS-PAGE method was used to evaluate the quality of purified protein. 15µL of the purified protein was combined with a suitable ratio of loading buffer (Cytomatingene® co, Iran) and, after boiling, was loaded on 15% acrylamide gel. After electrophoresis, the gel was stained with silver nitrate(panreac®, Spain) as the silvering agent.

Also, to ensure the proper production of recombinant protein by *Escherichia coli*, the Western blotting technique was used. After washing the nitrocellulose paper with TBS (Novagen®, USA) and TBS/TT (Novagen®, USA) solutions, the paper was blocked with bovine serum albumin (BSA, Sigma-Aldrich®, Germany) at room temperature for one hour. In this study, a conjugated anti-polyhistidine antibody with HRP (Sigma-Aldrich®, Germany) in a ratio of 1: 1000 was used. For staining the paper, 3,3 ', 5,5'-Tetramethylbenzidine (TMB) solution (Suitable for Western blot, Cytomatingene® co, Iran) was used according to the manufacturer's instructions.

Determination of Purified rCTB Concentration by GM1-ELISA and Bradford Methods

The GM1-ELISA technique, previously described by Khastar and colleagues (Khastar et al., 2020), was used to determine the amount of the rCTB with slight modifications. The microtiter plates were coated with GM1gangloside receptor in a ratio of 1:500. 100 µL of purified rCTB, which was well combined with a potent protease inhibitor (Thermofisher®, USA), was added to the plate wells. HRP-conjugated anti-polyhistidine antibody (Sigma-Aldrich®, Germany- diluted in PBS solution containing 0.1% BSA) was used in a ratio of 1:1000. The estimated time for blocking the wells with BSA (Sigma-Aldrich®, Germany) and using anti-his-tag antibody was 30-60 min, respectively. TMB solution was used to stain the substrate. Finally, the microtiter plate was read at 450 nm.

The study also used the standard Bradford protocol (Kruger, 2009) to calculate the concentration of

purified protein. BSA was used as a protein standard in this technique.

Results

PCR and Cloning of Gene Fragments

The results of the PCR technique based on the specific primers showed a band with the expected size (321bp) on the agarose gel (Figure 1). The presence of about 631 bp bands from PCR of the T7 promoter and terminator region from some of the colonies grown in 2xYT plates on the agarose gel indicates that the target gene has entered the plasmid quite correctly (Figure 1).

Qualitative and Quantitative Evaluation of rCTB

The results of SDS-PAGE showed that the rCTB was produced by *Escherichia coli* BL21(DE3) strain (Figure 2). In this study, the culture medium before adding IPTG was considered as negative control. If the bacterium produced the protein, a protein band of about 12.76kDa (due to the addition of six histidine residues to the C-terminal of the rCTB) in denature form and 63kDa in non-denature form was expected to be observed in the acrylamide gel (Figure 2). After staining the nitrocellulose paper, a protein blot of about 12.76kDa was observed, indicating the correct expression of the recombinant B subunit of the cholera toxin (Figure 3). Table 1 shows the results of measuring the concentration of purified rCTB.

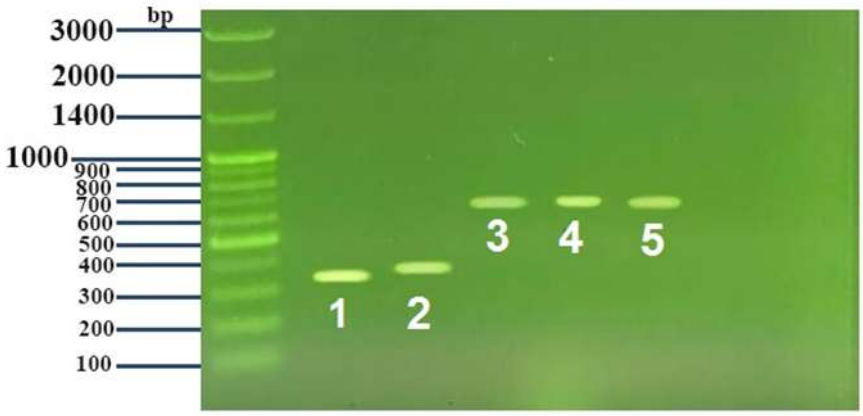

Figure 1. Results of amplification of gene fragments. Columns 3 to 5 result from amplification of the region between the T7 promoter and terminator from three random colonies. Column 1 shows the distance between the T7 promoter and terminator without the insertion of the target gene, and column 2 shows the result of amplification of the desired gene by the primers.

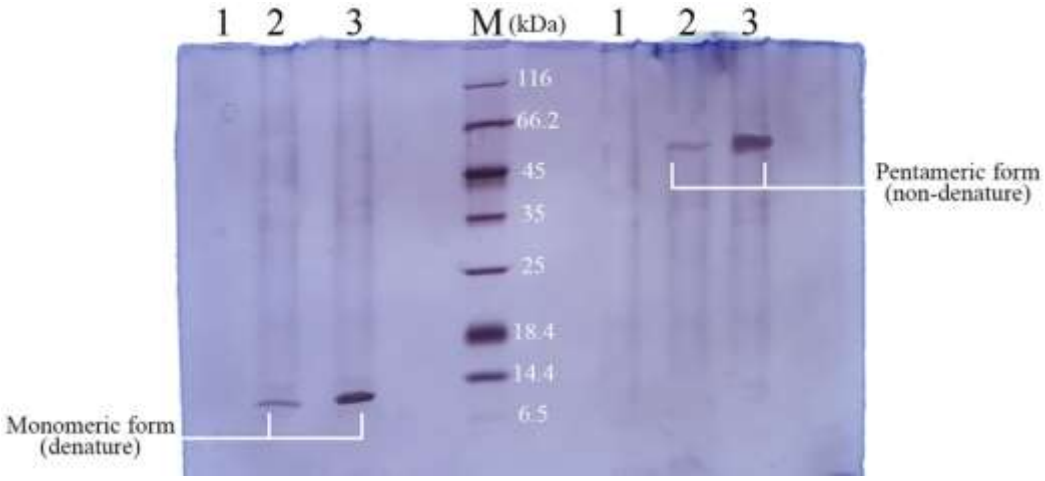

Figure 2. Results of quality assay of recombinant protein by SDS-PAGE. Column 1: Culture medium before induction by IPTG. Column 2: Culture medium after induction with IPTG. Column 3: Recombinant protein purified

using Ni-IDA column. The molecular weight in denatured and non-denatured forms is about 12.76 and 63 kDa, respectively.

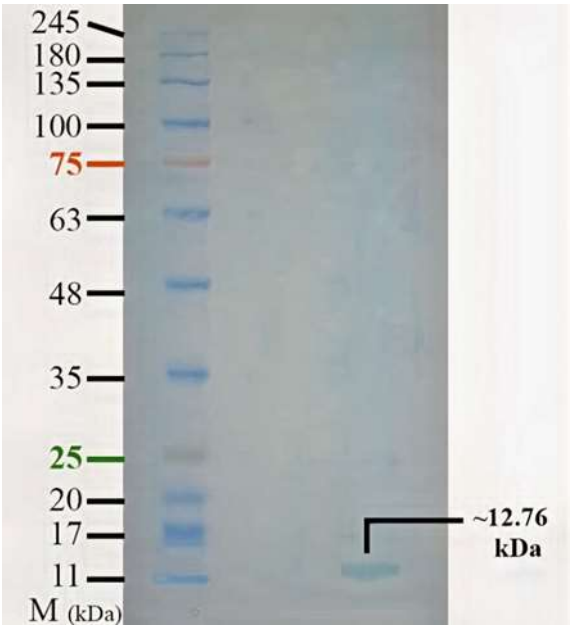

Figure 3. The result of the Western blot technique confirms the correct production of rCTB.

Table 1. Results from quantitative rCTB assays

Assessment method	First wash	Second wash	Third wash
GM1-ELISA	11 µg/mL	0.33 µg/mL	-
Bradford	9.57 µg/mL	0.1 µg/mL	-

Discussion

The main virulence factor of Vibrio cholerae is cholera toxin (CT), which consists of two subunits: subunit A (CTA) and subunit B (CTB) (Kim et al., 2009). CTB is a non-toxic protein with a high affinity for the GM1 ganglioside receptor on the surface of mammalian cells (Chinnapen et al., 2007). Today, CTB is recombinantly produced and is part of an internationally licensed cholera vaccine. This protein can build potent humoral immunity, which neutralizes the cholera toxin in the body (Alam et al., 2013; Charles et al., 2014; Clemens et al., 1986; Schaetti et al., 2012). In addition, many studies show that CTB administration can play an anti-inflammatory role in the body (Baldauf et al., 2015).

In this study, first; a gene fragment related to the synthesis of the B subunit of cholera toxin was identified, and its specific primers were designed. After amplification, the target gene was cloned into plasmid pET22a by *Sac* I and *Nco* I restriction enzymes. Recombinant CTB can be produced in various expression systems, including plants, yeast and fungi (Arakawa et al., 1998; Hamorsky et al., 2013; Hiramatsu et al., 2014; Li et al., 2014; Meng et al., 2011; Okuno et al., 2013). Nevertheless, the most efficient and cost-effective way is to use a pET-based expression system. In addition, this expression plasmid contains the T7 promoter, which is known for its high expression of recombinant proteins and reduced costs and increased efficiency of protein

synthesis. This plasmid has a His-tag coding sequence at the C-terminal, allowing the recombinant protein to be efficiently purified by affinity chromatography with a nickel ion column and using the selective AmpR (ampicillin resistance) marker. Also, The pelB leader sequence in pET-based vectors prevents the inclusion bodies formation and allows the extraction of recombinant proteins from the periplasmic space of *E. coli*, which reduces the cost of protein synthesis. In this study, we tried to provide the most optimal method for rCTB production in *Escherichia coli*. Recombinant CTB can be used as a potent Immunostimulant and non-toxic adjuvant in various commercial vaccines. As Figures 2 and 3 and the quantification results (Table 1) show, the recombinant CTB produced by our method is of high purity.

Due to the strong mucosal immunogenicity of CTB, its recombinant production by various techniques has been considered by many researchers. This recombinant protein is currently used in the WHO-approved cholera vaccine. CTB can be produced in many hosts, including prokaryotes such as *Lactobacillus*, *Escherichia coli*, and even genetically modified *Vibrio cholerae* strains. Other hosts for the production of the CTB are simple eukaryotic cells such as yeast or more complex organisms such as silkworms and plants (Bakhshi et al., 2014; Boustanshenas et al., 2013b; Gong et al., 2005; Hiramatsu et al., 2014; Jani et al., 2002; Kim et al., 2006; Kim et al., 2009; Li et al., 2014; Meng et al., 2011; Nochi et al., 2007; Oszvald et al., 2008; Yasuda et al., 1998; Yuki et al., 2013). However, due to the use of bioreactors or controlled culture chambers and unique greenhouses, the production of CTB in eukaryotic cells is not cost-effective. Okuno and colleagues (Okuno et al., 2013) produced recombinant CTB protein in *Lactobacillus casei* and *Lactobacillus reuteri* at a concentration of 0.05 to 1 mg /L, respectively. Their study used the pHIL253 shuttle vector, which can produce maximum recombinant protein in lactic acid bacteria. The immunization of rCTB was also confirmed in female mice with a lifespan of 4 weeks and binding of the recombinant protein to the GM1 receptor. In another study performed to produce recombinant CTB in *Lactobacillus casei* by Hiramatsu and colleagues(Hiramatsu et al., 2014), recombinant CTB protein was purified at a 1 mg/L concentration. In this study, rCTB was fused with the anti-inflammatory YVAD tetrapeptide (tyrosine, valine, alanine, aspartic acid), which inhibits explicitly caspase-1, which catalyzes the production of interleukin (IL) -1 from its inactive precursor. Their results showed that this fused rCTB can inhibit interleukin-1 in intestinal epithelial cells with high efficiency and can be used as a carrier to deliver fused compounds such as vaccine antigen. Other research has been done to produce this protein in different expression systems such as plants and silkworms (*Bombyx mori*), which can be found in the research of Li and colleagues(Li et al., 2014), As well as Meng and colleagues (Meng et al., 2011). They produced 0.5 mg/g of silkworm pupae and 0.23 mg/g of silkworm larvae, respectively. In order to produce CTB in plant expression systems, Hamorsky and colleagues(Hamorsky et al., 2013) were able to produce this protein at a concentration of more than 1 g/kg of fresh leaves of tobacco (*Nicotiana benthamiana*) leaves. Miyata and colleagues were able to produce a recombinant CTB fused to a protein matrix in the yeast *Pichia pastoris*, which dramatically increased the efficiency of the immune response to the antigen in mice. Although they did not specify the final production rate of this recombinant CTB, it is possible to obtain up to 50 mg / L in this expression system(Stratmann, 2015). One of the advantages of the yeast-based expression system is its similarity to bacterial culture without the production of endotoxins. Due to the need for better folding of proteins fused with CTB, yeast-based systems have a tremendous advantage over other systems(Miyata et al., 2012; Song et al., 2004). However, using eukaryotic expression systems is expensive and complicated.

The present study could produce CTB at an 11 mg/L concentration in a very inexpensive 2xYT medium. As Table 1 shows, the accuracy of the GM1-ELISA technique for quantitative CTB assays is higher due to the higher sensitivity of CTB to the GM1 ganglioside receptor. Small molecular size and low weight, along with the high similarity of this protein to the *Escherichia coli* heat-sensitive enterotoxin B subunit, facilitate the expression of this protein in *Escherichia coli*.

Conclusion

In recent years, extensive studies have been conducted on the immunological effects of the cholera toxin B subunit on the effective stimulation of the immune system. Non-toxicity combined with stability and relative ease of expression of CTB makes it easy to use as a flexible adjuvant. The possibility of expressing this protein in many types of organisms expands its application potential. CTB is a combination with diverse applications and promises a practical approach to evolving vaccine development. Therefore, in this study, we aimed a

convenient method for the recombinant production of this protein for use in vaccine design.

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Conflict of Interest
The authors declare no conflict of interest.

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