DOI: 10.56511/JIPBS.2017.v4.i01.pp66-69

e-ISSN: 2349-2759 p-ISSN: 2395- 1095

Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)

www.jipbs.com



Research article

Expression and purification of cholera toxin fragment B (CTB) in *E. coli*

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Key words: cholera toxin fragment B (CTB), *E. coli*, IPTG, temperature.

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Abstract

Cholera toxin fragment B (CTB), a non-toxic fraction of cholera toxin produced by Vibrio cholera, is able to bind to epithelial or M cells in the intestine through the GM1-ganglioside receptor. Because of this property, CTB is a powerful mucosal immunogen and adjuvant for mucosal vaccines. In this study, *E. coli* BL21 (DE3) was used a host to produce the CTB. The induction conditions, including time, temperature, and IPTG concentration, were optimized in order to increase the expression of soluble CTB. The biological activity of the purified CTB was measured in a series of GM1-ELISA experiments. Our results indicated that the optimal IPTG concentration and induction time for CTB expression are 1 mM and 2-3 hr, respectively. Protein expression was found to decrease upon the induction time and the purified CTB protein was found to be stable at temperatures below 4°C. This study provides insight into the optimized conditions for CTB expression in *E.coli* as well as the suitable temperatures for CTB storage, and information that will be useful for future studies.

Introduction

In many developing countries, cholera is a major cause of disease and mortality in children. The symptoms of cholera are mainly caused by the secretion of Cholera Toxin (CT). CT is a protein complex produced from *Vibrio cholera* and is composed of one subunit of fragment A (CTA) and five subunits of fragment B (CTB) [1]. CTA is responsible for the toxic activity and is constituted of two polypeptides linked by a disulfide bridge. CTB is composed of five identical peptides arranged in a ring-like pentameric configuration [2].

CTB is a subunit vaccine candidate antigen for cholera. It has been shown to be effective in inducing the mucosal immune response [2] because it can bind to the GM1-ganglioside receptors on the epithelial cells of the intestine [3]. Therefore, CTB is a possible candidate as a mucosal vaccine adjuvant [3]. Previously, CTB has been expressed in different systems such as *Escherichia coli* (*E. coli*) [4,5], yeast [6], plants [7,8], and insects [7]. *E. coli* is widely used in recombinant protein expression, though it can produce insoluble or nonfunctional target proteins [9]. These undesirable outcomes can occur because of the absence of cofactors or post-translational modifications required for function or folding. Moreover, previous studies have found that cholera toxin is difficult to express in E. coli because of the former's toxicity [10, 11] and high homology to the labile toxin of *E. coli* [12].

However, using *E. coli* has many advantages such as its low cost, fast growth, and ability to express labeled protein [7]. Thus, it is important to find the best conditions for CTB expression in *E. coli*. In this study, our goal was to determine the optimal conditions for inducting CTB expression in *E. coli* and then storing the CTB produced. To test this, we cloned the recombinant CTB gene with 6XHis, expressed it in E. coli BL21 (DE3), and characterized by SDS-PAGE and Western blot analyses. Different IPTG concentrations and induction times were tested for their effects on protein expression. In addition, the temperature for the optimal storage of purified CTB was determined.

Experimental

Construction of Bacterial Plasmids

The pET21a vector was purchased from EMD Biosciences, Inc. (Madison, WI, USA) [13]. The pET21a-containing signal sequence of synthetic CTB was constructed according to the following parameters. Synthetic CTB was cloned into

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the N-terminus using *Nde*I site and the C-terminus using the *Xho*I (pET21a-CTB) site. To clone synthetic CTB into pET21a, the polymerase chain reaction (PCR) product regenerated by the pMY053 plasmid digested with *Nde*I and *Xho*I was directly cloned into the expression vector pET21a, which was digested with *Nde*I and *Xho*I. The sequence of the forward primer for PCR is 5′-ggaattccat ATGGTG AAGCTCAAGTTCGGAG-3′ and that of the reverse primer is 5′-ccgctcgag TTTCTCAGAGTAGTTAGCCATGC-3′ (restriction sites are underlined). This process produced a pET21a-CTB vector with a synthetic CTB gene, a N-terminal signal sequence of wild-type CTB, and a C-terminal 6XHis.

Expression and Purification of CTB in *E. coli*

The *E. coli* BL21 (DE3) (EMD Biosciences, Inc.) was used for expressing all the vectors. The BL21 (DE3) bacterial cells containing the plasmid were grown at 37°C in Luria-Bertani (LB) media with ampicillin (100 µg/ml) on a ratory shaker. Isopropyl-ß-D-thiogalactopyranoside (IPTG) at concentrations of 0.2, 0.5, or 1 mM was added to the culture at an optical density of 0.6 at 600 nm. The cells were then cultured at 22, 28, or 37°C and harvested after 2, 3, or 5 hours. For the purification of CTB proteins, transformed cells were suspended in 20 mMTris-HCl, pH 7.9 containing 5 mM imidazole and 0.5 M NaCl, and then the sonication and centrifugation at 12,000 rpm for 20 min at 4°C were performed. The supernatant was added into a Ni-NTA column. The column was washed with 50 ml of 20 mMTris-HCl (pH 7.9) containing 60 mM imidazole and 0.5 M NaCl. The recombinant CTB proteins were then eluted with 20 mMTris-HCl containing 1 M imidazole and 0.5 M NaCl, pH 7.9.

SDS PAGE and Western blot

The harvested cells were centrifuged at 4000 rpm for 10 min. The pellets were resuspended in 200 µl of 1xSDS buffer (125mM This-HCl pH 6.8, 12% SDS, 10% Glycerol, 22% 2-mercaptoetanol, 0.001% Bromophenol blue) and boiled. The eluted proteins were separated on 15% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Whatman, NJ, USA). To detect CTB protein, the membrane was probed with mouse anti-His diluted 1:5000 in 1% skim milk in PBST for 1 hr at room temperature. After three 10 min washes with PBST, the membrane was incubated with goat anti-mouse IgG-horse a dish peroxidase (HRP) conjugate diluted 1:10,000 in 1% skim milk in PBST for 1 hr at room temperature. The membrane was washed for 10 min four times and developed by chemiluminescence using ECL plus detection reagent (Amersham, NJ).

GM1-Ganglioside Binding Assay

The binding of *E. coli*-expressed CTB for GM1-ganglioside receptor was determined by GM1-ELISA [7]. Ninety six-well microtiter plates were coated with monosialoganglilside-GM1 (Sigma G-7641, MO, USA) by incubating the plate with 100 µl of 3 µg/ml GM1 in 15 mM Na_2CO_3, 25 mM $NaHCO_3$ in coating buffer at 4 ºC overnight. The plates were blocked with 5% skim milk in PBS for 2 hr at 37ºC. After washing three times with PBS containing 0.05% Tween 20 (PBST), bacterial-expressed CTB diluted in PBS (50 µl/well) was added and incubated for 2 hr at 37ºC. The plates were washed three times with PBST and incubated with rabbit anti-CTB (diluted 1:1000 in 1% skim milk) in PBST for 2 hr at 37ºC. The plates were then washed three times with PBST and incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (diluted 1:5000 in 1% skim milk) in PBST for another 2 hr at 37ºC. Next, the plates were washed four times with PBST and developed with 100 µl of TMB substrate (Pierce, IL, USA) for 2 min at room temperature. The reaction was ended by the addition of 100 µl of 1M H_2SO_4 and the absorbance was read at 450 nm.

Results and Discussion

The demand for the production of biopharmaceuticals has increased dramatically because of their use in a variety of product such as vaccines, antibodies, and recombinant proteins. The goal of research in recombinant protein production is to maximize its productivity. Among the different protein expression systems, bacteria are particularly popular because of the product's enhanced biological activity, high stability and solubility, and simplified downstream processing [14, 15].

In this study, our goal was to optimize the expression conditions of functional Cholera toxin fragment B (CTB) in *E. coli* BL2 (DE3). CTB has been used as a cholera vaccine, mucosal adjuvant, and oral tolerance inductor, depending on whether the antigen is conjugated or co-administered[16].To express CTB protein in *E. coli*, the presence of the CTB gene in pET21a-CTB was confirmed using *Nde*I-*Xho*I-digestion and sequencing. The pET21a-CTB vector was then transformed into *E. coli* BL21 (DE3). To determine the optimal conditions for CTB expression in E. coli, the IPTG concentrations, temperatures, and induction times were varied. The results showed that a high level of CTB expression in *E.coli* was induced at 1 mM IPTG for all temperatures (22°C, 28°C, and 37°C) after 2-3 hours of induction (Figure 1). This result confirmed the methods utilized by previous reports [17-19], which used 1 mM IPTG to induce CTB expression. However, 5 hours of induction decreased CTB expression presumably because of toxic effect to the host cells. Thus, the optimal conditions for CTB expression in *E. coli* are induction with 1 mM IPTG for a few hours.

The purification of CTB proteins tagged with 6XHis at their C-terminus was performed on a Ni-NTA column. The purified protein was re-suspended in 5x SDS buffer, boiled, and separated on 15% SDS-PAGE gel. SDS-PAGE analyses showed a single band of purified CTB in monomeric form

with an expected molecular weight approximately 13 kDa (Figure 2). To confirm the specific affinity of bacterial CTB, the GM1 ELISA method was performed. The result demonstrated that the purified bacterial CTB was able to bind to GM1-ganglioside suggesting structurally function (Figure 3). The binding to GM1 ganglioside on intestine cells confirmed the function of CTB as an immunogen and adjuvant [7, 20].

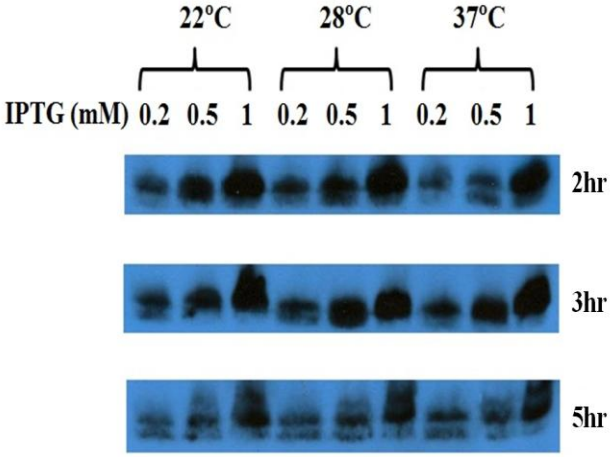


Figure 1. Western blot analysis of CTB protein expressed in E. coli. Various IPTG concentrations, temperatures, and induction time were applied to find an optimal expression condition.

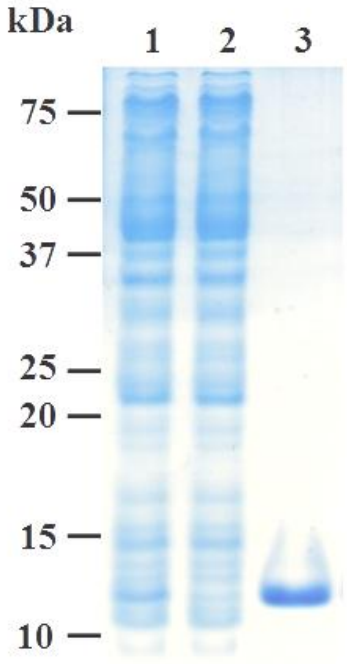


Figure 2. Purification of CTB protein from E. coli using a Ni-NTA column. Collected protein samples during purification were separated by SDS-PAGE and Coomassie stained. Lane 1: Crude extract, lane 2: Ni-NTA flow-through fraction, 3: Elution fraction.

In addition to investigating the optimal conditions for CTB expression, we studied the effects of storage temperature on the CTB protein. The stability of purified bacterial CTB was detected by incubation at -80, -20, 4, 25, and 37°C for seven days. The purified bacterial CTB protein was stable at -80°C, -20°C, and 4°C (Figure 4). However, the aggregation of CTB protein occurred when it was incubated at 25°C and 37°C for seven days, which gives important effect to CTB function for immune response.

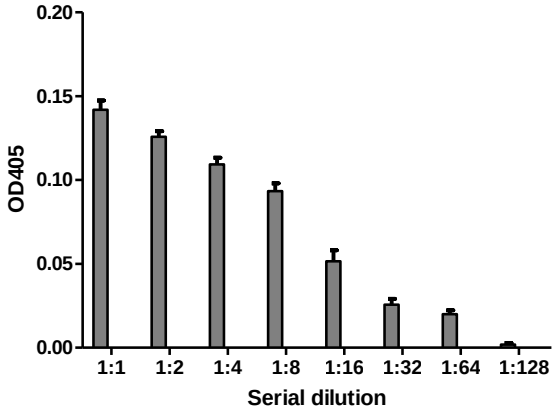


Figure 3. GM1 binding of purified bacterial CTB. The purified CTB was serial diluted starting from 100 ng/ml. The detection with rabbit anti-CTB and HRP-labeled goat anti-rabbit IgG yielded the OD405 measurements. Data is shown as means ± SD of samples from three independent infiltration experiments.

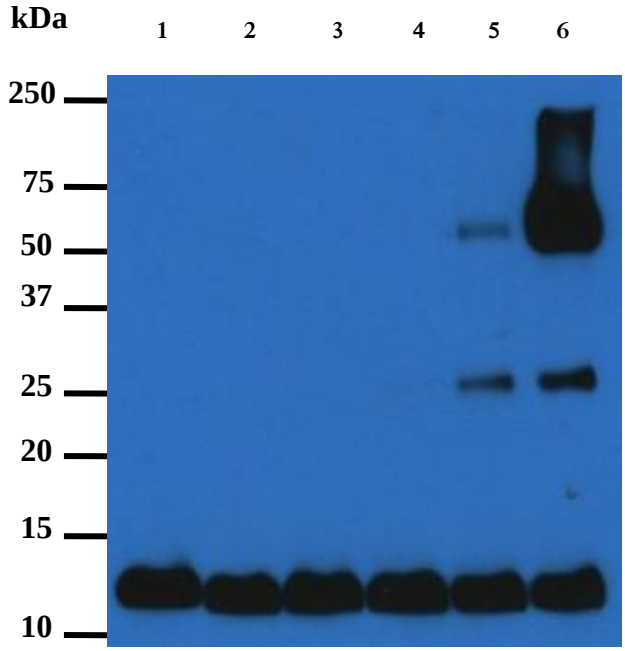


Figure 4. Optimization of purified CTB storage temperature. The purified CTB was stored in -80°C (lane 2), -20°C (lane 3), 4°C (lane 4), 25°C (lane 5), and 37°C (lane 6). After seven days, Western blot analysis was used to compare the stored CTB with freshly expressed and purified CTB (lane 1).

Conclusion

Cholera toxin fragment B (CTB) is an effective subunit vaccine candidate antigen for cholera and a good candidate for using as a mucosal vaccine adjuvant due to its ability to the GM1-ganglioside receptors on the epithelial cells of the intestine. The optimal IPTG concentration and induction time for CTB expression in *E. coli* are 1 mM and 2-3 hr, respectively. This study provides insight into the optimized conditions for CTB expression in *E.coli* as well as the suitable temperatures for CTB storage, information that will be useful for future studies.

Acknowledgements

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ009007), Rural Development Administration, Republic of Korea.

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