Archives of Razi Institute, Vol. 73, No. 1 (2018) 27-38

Copyright © 2018 by
Razi Vaccine & Serum Research Institute

Original Article

High-level expression of tetanus toxin fragment C in *Escherichia coli*

Aghayipour*, K., Teymourpour, R.

Department of Genomics and Genetic Engineering, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Karaj, Iran

Received 15 June 2016; accepted 18 February 2017

Corresponding Author: Khosrow@rvsri.ac.ir

ABSTRACT

Fragment C is the C-terminal domain of the heavy chain of tetanus toxin that can promote the immune response against the lethal dose of this toxin. Therefore, this portion can be considered as a candidate vaccine against tetanus infection, which occurs by *Clostridium tetani*. The present study aimed to compare the expression of tetanus toxin fragment C in Escherichia coli BL21 (DE3) pLysS cells having a high tolerance to toxins between two different expression vectors, namely pET22b and pET28a, using the sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analyses. After DNA extraction from Harvard CN49205 strain of *C. tetani*, the gene of interest was amplified using polymerase chain reaction, and then sequenced and cloned into the expression vectors of pET22b and pET28a, transformed into competent BL21 (DE3) pLysS cells, and finally expressed using an optimized protocol. The cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at four different incubation temperatures (i.e., 37, 33, 30, and 25 °C) and three different incubation times (i.e., 1, 2, and 3 h). Although the SDS-PAGE and western blot analyses confirmed the expression of the recombinant fragment C (r-fragment C) ligated into both of the expression vectors, pET28a showed a higher r-fragment C expression level than the other vector (38.66 mg/L versus 32.33 mg/L, $P<0.05$). An optimal expression condition was acquired 3 h after 1 mM IPTG induction at 25 °C. The results demonstrated that E. coli BL21 (DE3) pLysS as an expression host in combination with pET-28a as an expression vector was a more compatible expression system to express the fragment C of tetanus toxin, compared to E. coli BL21 (DE3) pLysS/pET-22b expression system. Overall, these results may represent an opportunity to improve the expression system for the production of tetanus toxin vaccine using recombinant protein strategy.

Keywords: *Clostridium tetani*, Fragment C, pET22b expression vector, pET28a expression vector, *E. coli BL21 (DE3) pLysS*

La forte expression du fragment C de la toxine tétanique dans *Escherichia coli*

Résumé: Le fragment C'est le domaine C-terminal de la chaîne lourde de la toxine tétanique. Ce dernier favorisé la réponse immunitaire contre la dose létale de cette toxine et peut donc être considéré comme un candidat potentiel pour le vaccin contre l'infection tétanique causée par Clostridium tetani. Dans cette étude, l'expression du fragment C de la toxine tétanique dans des cellules BL21 (DE3) pLysS *d'E. coli* ayant une tolérance élevée aux toxines a été comparée en utilisant deux vecteurs d'expression différents, pET22b et pET28a, par SDS-PAGE et western blot. Après l'extraction de l'ADN de la souche Harvard CN49205 de C. tetani, le gène d'intérêt a été amplifié par PCR, séquencé, cloné dans les vecteurs d'expression, pET22b et pET28a, transformé dans des cellules compétentes BL21 (DE3) pLysS et enfin exprimé selon un protocole optimisé. Les cellules ont été induites avec IPTG à quatre températures d'incubation différentes (37, 33, 30 et 25° C) et trois temps d'incubation différents (1 à 3 h). Bien que l'expression du fragment recombinant C (fragment C) ligaturé dans les

deux vecteurs d'expression ait été confirmée par les analyses SDS-PAGE et western blot, pET28a a montré une expression plus élevée du fragment r C comparé à pET22b (38,66 mg / l contre 32,33 mg / l, p <0,5). La condition optimale d'expression a été obtenueà 5° C, IPTG 1 mM, et 3 h après l'induction de l'IPTG. Ces résultats ont démontré que l'hôte d'expression E. coli BL21 (DE3) pLysS en combinaison avec le vecteur deux vecteurs d'expression ait été confirmée par les analyses SDS-PAGE et western blot, pET28a a montré une expression plus élevée du fragment r C comparé à pET22b (38,66 mg / l contre 32,33 mg / l, p <0,5). La condition optimale d'expression a été obtenueà 5° C, IPTG 1 mM, et 3 h après l'induction de l'IPTG. Ces résultats ont démontré que l'hôte d'expression *E. coli BL21(DE3) pLysS* en combinaison avec le vecteur d'expression pET-28a représentait le système d'expression le plus compatible pour exprimer le fragment C de la toxine tétanique. Dans l'ensemble, ces résultats montrent qu'il est possible d'améliorer la production de vaccin contre la toxine tétanique à base de protéines recombinantes en optimisant leur système d'expression.

Mots-clés: *Clostridium tetani*, Fragment C, vecteur d'expression pET22b, vecteur d'expression de pET28a, *E. coli BL21 (DE3) pLysS*

INTRODUCTION

Tetanus is an infection caused by Clostridium tetani, which is an obligate anaerobic spore-forming bacterium (Gil et al., 2001). Tetanus toxin is a potent neurotoxin that is intracellularly synthesized by C. tetani as a single 150 kDa polypeptide chain. Following cell lysis, the toxin is released into the medium and cleaved by endogenous proteases generating a 50 kDa N-terminal light chain (fragment A) disulfide bonded to a 100 kDa C-terminal heavy chain (fragments B and C) (Bruggemann and Gottschalk, 2004). Fragment C, the 50 kDa C-terminal portion of the heavy chain, has ganglioside (Helting and Zwisler, 1977) and protein (Schiavo et al., 1991) binding activities in the tissues of neural origin. These gangliosides are considered to be potential relevant eukaryotic cell receptors (Herreros et al., 2000). Fragment C is completely nontoxic in animals, whereas fragment B retains some residual toxicity at high doses (Helting et al., 1977). Fragment C has been demonstrated to retain the ganglioside binding activity of intact tetanus toxin (Morris et al., 1980). Therefore, purified fragment C is used to successfully immunize the animals against tetanus. This indicates that the entire molecule is not essential for protection (Helting and Nau, 1984). It seems that this fragment can be used as a novel efficient vaccine without any neurotoxicity. In some studies, the recombinant fragment C (r-fragment C) was expressed alone or in fusion with other antigens on different strains of Escherichia coli (Fairweather and Lyness, 1986; Makoff et al., 1989; Halpern et al., 1990; Ribas et al., 2000; Yousefi et al., 2013), Salmonella (Chatfield et al., 1992), Lactobacillus (Maassen et al., 1999), Bordetella (Stevenson and Roberts, 2004), yeast cells (Romanos et al., 1991), and cultured insect cells (Charles et al., 1991), which in some cases resulted in the induction of protective antibodies against tetanus toxin. However, each of these studies encountered some deficiencies, such as the presence of several fortuitous polyadenylation sites within r-fragment C in S. cerevisiae (Romanos et al., 1991), presence of glycosylation sites in S. cerevisiae and Pichia pastoris, which would result in inactive antigen (Romanos et al., 1991), phenotypic alterations, including chlorotic phenotype and male sterility in tobacco leaves (Tregoning et al., 2003), low ganglioside binding activity of r-fragment C of baculovirus expression system (Charles et al., 1991), and ethical issues regarding live vector vaccines. *E. coli* expression system also has some problems, such as low expression level and low solubility rates of r-fragment C. Despite the many attempts made to improve the expression level and solubility rate of r-fragment C (Fairweather et al., 1986; Makoff et al., 1989; Halpern et al., 1990; Ribas et al., 2000; Wang et al., 2008; Yousefi et al., 2013), the suggested solutions in this area have not been analyzed yet. It seems that some limitations in the

expression level of r-fragment C are related to the probable toxicity of this antigen to *E. coli* as the expression host. On the other hand, the low solubility rate of this protein is probably due to its high tendency to form inclusion bodies in overproduction condition (Miyake et al., 2007). The two major factors that reduce the production rate of the recombinant proteins, such as r-fragment C, are the formation of inclusion bodies resulting in a low solubility rate (Miyake et al., 2007) and degradation by lon and ompT outer membrane proteases (Goff and Goldberg; Grodberg and Dunn, 1988). Another problem in this area is the limitations related to the traditional expression method (Sambrook and Russell, 2001). Therefore, it could be highly desirable to investigate the expression of r-fragment C in the BL21 (DE3) pLysS strain of *E. coli* containing a pLysS plasmid with a high tolerance to toxins and lacking lon and ompT outer membrane proteases and an expression vector with a strong promoter using a modified protocol. With this background in mind, the present study, aimed to investigate the expression of the gene encoding fragment C of tetanus toxin in *E. coli* BL21 (DE3) pLysS using pET22b and pET28a vectors. In addition, a comparative study was performed between pET22b and pET28a expression vectors in order to determine the plasmid that is more compatible with *E.coli* BL21 (DE3) pLysS cells for expressing r-fragment C. We also used a recently-optimized expression method in our laboratory (Bahreini et al., 2014) for the overexpression of r-fragment C. Moreover, different temperature and time levels were analyzed in order to determine an optimal expression condition, which is an important factor in the overexpression and overproduction of a recombinant protein.

MATERIALS AND METHODS

Polymerase chain reaction, cloning, and DNA sequencing. Total DNA was extracted from Harvard CN49205 strain of C. tetani using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Germany). The gene encoding fragment C of tetanus toxin was amplified using specific primers, including 5'GGAATTC<u>CATATG</u>AAAAATCTGGATTGTTGG GT3' as forward primer containing NdeI restriction site (shown as underlined letters) as well as 5'CCG<u>CTCGAG</u>***TTA***ATCATTTGTCCATCCTT3' and 5'CCC<u>AAGCTT</u>***TTA***ATCATTTGTCCATCCTT3' as reverse primers, including XhoI and HindIII restriction sites, respectively (shown as underlined letters), and stop codon (shown as italic and bold letters). Based on the kind of the reverse primer (XhoI or HindIII), two different PCRs with similar conditions were performed. The PCRs were carried out with 300 ng of total DNA in a final volume of 50 µl containing 10X PCR buffer, 0.2 mM dNTP (Roche Diagnostics, Germany), 1 unit/reaction of Pfu DNA polymerase (Fermentas, Russia) combined with 0.5 unit/reaction of Taq DNA polymerase (Fermentas, Russia), 1.5 mM MgCl2, 10 pmol/reaction of each forward and reverse primers, and double-distilled H2O up to the total volume of 50 µl. Both of the PCR reactions were performed by a Gene Cycler (Bio-Rad, USA) with the following thermo-cycling profile: initial denaturation at 95 °C for 5 min, followed by 5 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and then 30 cycles of 95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. The final extension was 10 min at 72 °C. The PCR products were visualized by electrophoresis on 1% agarose gel. Subsequently, they were purified from agarose gel by the High Pure PCR Template Preparation Kit and ligated into pTZ57R/T cloning vector (Fermentas, Germany). The ligated mixtures were transformed into *E. coli* DH5α (Invitrogen, USA) competent cells using the heat shock method (Sambrook and Russell, 2001). Then, the recombinant transformed colonies were screened on lysogeny broth (LB) agar media containing 100 µg/mL ampicillin, 20 mg/mL5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 0.1 M IPTG. Two different recombinant pTZtetc plasmids were extracted using the High Pure Plasmid Isolation Kit (Roche Diagnostic, Germany). Then, the sequencing was

performed in both directions using T7 promoter and M13 (-20) primers, a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Canada), and an ABI 3730 XL DNA Analyzer (BioNeer, South Korea). The obtained nucleotide sequence of the gene encoding fragment C was analyzed using BioEdit software (Hall, 1999).

Subcloning into expression vectors and gene expression. One of the two distinctive recombinant pTZtetc plasmids harboring NdeI and XhoI restriction sites was ligated into pET22b expression vector (Novagen, USA) at NdeI and XhoI cloning sites. The other recombinant pTZtetc plasmid containing NdeI and HindIII restriction sites was ligated into pET28a expression vector (Novagen, USA) at NdeI and HindIII cloning sites. In order to express r-fragment C, initially, the recombinant pET22b and pET28a expression vectors were separately transformed into *E. coli* BL21 (DE3) pLysS competent cells using the heat shock method. Subsequently, the mixtures were cultured on (LB) agar plates containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol obtained from recombinant *E. coli* BL21 (DE3) pLysS transformed with pET22b, as well as 30 µg/mL kanamycin and 34 µg/mL chloramphenicol obtained from recombinant *E. coli* BL21 (DE3) pLysS transformed with pET28a. In order to overexpress the gene of interest, we used an optimized expression method reported in our previous study (Bahreini et al., 2014) rather than the traditional method described by Sambrook and Russell (2001). To this end, three steps were considered for the bacterial growth at 37oC in a shaking incubator at 200 rpm (Bahreini et al., 2014). At the first step, a single colony from each of the two recombinant transformed *E. coli* BL21 (DE3) pLysS cells, cultured on the LB plates, was inoculated in 5 mL of LB broth supplemented with 1% w/v glucose (50 µg/mL ampicillin and 30 µg/mL kanamycin obtained from pET22b and pET28a, respectively). The resulting mixtures were incubated for 7 h. At the second step, the first-step media were transferred into 100 mL of LB broth (1% w/v glucose), containing 300 µg/mL ampicillin and 200 µg/mL kanamycin obtained from pET22b and pET28a, respectively, and incubated overnight. At the third step, the overnight media were exchanged by centrifuging (1200 g, 4 °C for 5 min). Subsequently, the fresh LB media (1% w/v glucose), containing 50 µg/mL ampicillin and 30 µg/mL kanamycin obtained from pET22b and pET28a, respectively, were added to the bacterial pellets and diluted until obtaining an optical density(OD) of 10 at 600 nm. The last media were incubated for 75 min in order to adapt bacteria to the fresh media until adding inducer and beginning protein expression. Afterwards, each medium was incubated with 1 mM IPTG at four different incubation temperatures (i.e., 37, 33, 30, and 25 °C) and three different incubation times (i.e., 1 to 3 h after IPTG induction) in order to determine the optimal condition for the expression of r-fragment C.

Immunoblotting. Prior to IPTG induction and 1, 2, and 3 h after that, sampling from each medium was performed, and the cells from each sample were immediately harvested by centrifuging at 10,000 g and 4 °C for 5 min. Each of the harvested bacterial cell pellets was diluted and lysed in 5X sample buffer (Laemmli, 1970). The total protein concentration in cell lysate was determined by the Bradford Protein Assay Reagent Kit (Bio-Rad, USA). For the SDS-PAGE analysis, the protein samples were loaded on a 10×10 cm and 12.5% SDS-PAGE gel following the method described by Laemmli (1970). The mixtures were electrophoresed using Protean II xi cells (Bio-Rad, USA) in electrophoresis buffer (Laemmli, 1970) for 24 h at 30 V. The gel was stained with coomassie brilliant blue R-250. Finally, the specific band of r-fragment C protein was cut out from the gel, and its concentration was evaluated using Bradford assay for both of the expression vectors. For western blot, fractioned proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Roche Diagnostic, Germany) using an Electro Blot System (Bio-Rad, USA) in a transferring buffer (pH=8.3), containing 25 mM Tris, 192 mM glycine, and 15% methanol. The solution was kept overnight at 20 V and 4°C, blocked

with 5% bovine serum albumin (BSA) with gentle rocking at room temperature for 1.5 h, and washed for three times in TBST (pH=7.2), containing 20 mM Tris-HCl, 150 mM CaCl2, and 0.05% W/V Tween 20. Subsequently, the membrane was probed with horse anti-tetanus toxin polyclonal antibody (Razi Vaccine and Serum Research Institute, Karaj, Iran) (1:1000 dilution in TBST) using gentle rocking at ambient temperature for 1.5 h. The membrane was then washed four times with TBST and incubated with horse radish peroxidase-conjugated Anti-Goat IgG (Abcam, UK) (1:1000 dilution in TBST) using gentle rocking at room temperature for 1.5 h. After washing the membrane again, the color was developed using 3'-Diaminobenzidine.

Statistical analysis. The concentrations of total cell protein and r-fragment C were determined through the estimation of the mean and standard deviation. Additionally, the percent yield was calculated. The t-test was used to analyze the differences between the two expression systems in terms of protein concentration. All data analysis was performed in SPSS software. P-value less than 0.05 was considered statistically significant.

RESULTS

Polymerase chain reaction, gene cloning, and DNA sequence analysis. Two different PCR products, both of which encoded the fragment C of tetanus toxin, were obtained using total DNA from *C. tetani* as a template and designed primers. The agarose gel electrophoresis of the two amplicons confirmed them to be ~ 1.4 kb in size (Figure 1). Both of the PCR products were then cloned into pTZ57R/T cloning vector. The recombinant constructs were verified by means of DNA sequencing and digestion using NdeI and XhoI restriction endonucleases producing one of the two amplicons (Figure 2a) and NdeI and HindIII restriction endonucleases generating the other one (Figure 2b). DNA sequence analysis confirmed both amplicons to be exactly 1356 bp in size as reported by the sequences of *C. tetani* strain CN3911, a derivative of Harvard strain (Fairweather and Lyness, 1986) and Massachusetts strain of *C. tetani* (Eisell et al., 1986).

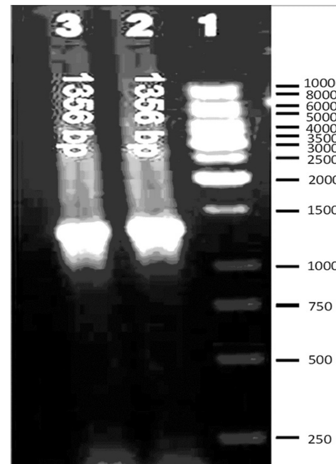

Figure 1. Electrophoresis on 1% (w/v) agarose gel for two different polymerase chain reaction products, both encoding fragment C of tetanus toxin, verifying the amplification of the gene of interest. Lane 1: DNA size marker 1 kb (Fermentase, Germany). Lanes 2 and 3: polymerase chain reaction products of fragment C gene obtained by specific primers, including NdeI/XhoI and NdeI/HindIII restriction sites, respectively.

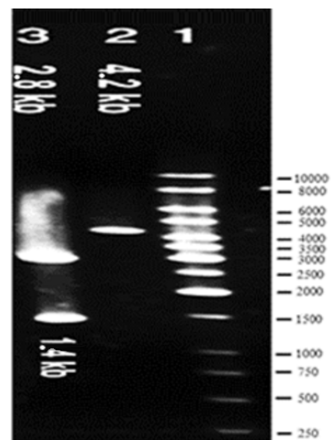

Figure 2a. Electrophoresis on 1% (w/v) agarose gel for two different polymerase chain reaction products, both encoding fragment C of tetanus toxin, verifying the amplification of the gene of interest. Lane 1: DNA size marker 1 kb (Fermentase, Germany). Lanes 2 and 3: polymerase chain reaction products of fragment C gene obtained by specific primers, including NdeI/XhoI and NdeI/HindIII restriction sites, respectively.

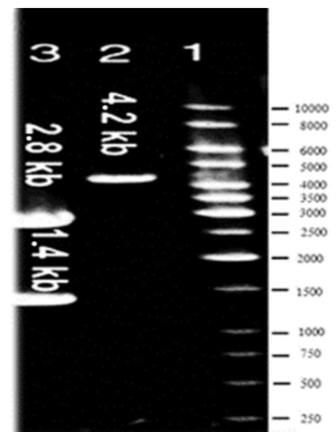

Figure 2b. Electrophoresis on 1% (w/v) agarose gel for the restriction enzyme digestion of the recombinant cloning vector (pTZ57R) using NdeI/XhoI, confirming the insertion of the gene encoding tetanus toxin fragment C into this vector. Lane 1: DNA size marker 1kb (Fermentase, Germany); lane 2: single digestion product of recombinant pTZ57R using NdeI endonuclease; lane 3: double digestion products of recombinant pTZ57R using NdeI and XhoI.

Based on the sequence analysis, in the two samples sequenced in the present study, no mutations or polymorphisms were found within the gene encoding fragment C obtained from Harvard CN49205 strain of *C. tetani* (DTP department, Razi Vaccine and Serum Research Institute, Karaj, Iran). The nucleotide sequence of tetanus toxin fragment C analyzed in the present study was deposited into the NCBI GenBank (GenBank ID: FJ917402).

Construction of recombinant expression plasmids. Figure 3 displays a schematic representation of two recombinant pET22b and pET28a expression vectors designed by SnapGene software, version 2.8 (www.snapgene.com). These two vectors both containing fragment C gene of *C. tetani* (Figure 3) were constructed in order to express the r-fragment C protein in *E. coli* BL21 (DE3) pLysS. The electrophoresis of the two recombinant expression vectors on agarose gel confirmed them to have a higher length than the non-recombinant ones (~ 6.7 kb versus ~ 5.4 kb For the recombinant and non-recombinant pET22b, respectively, and ~ 6.6 kb versus ~ 5.3 kb in the recombinant and non-recombinant pET28a, respectively) (Figures 2c and 2d).

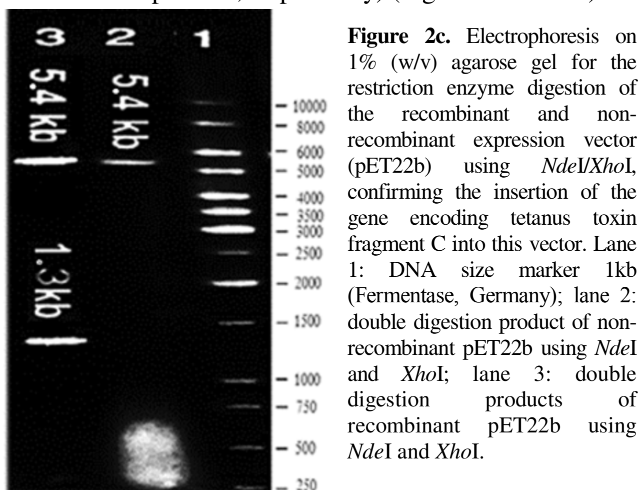

Figure 2c. Electrophoresis on 1% (w/v) agarose gel for the restriction enzyme digestion of the recombinant and non-recombinant expression vector (pET22b) using *Nde*I/*Xho*I, confirming the insertion of the gene encoding tetanus toxin fragment C into this vector. Lane 1: DNA size marker 1kb (Fermentase, Germany); lane 2: double digestion product of non-recombinant pET22b using *Nde*I and *Xho*I; lane 3: double digestion products of recombinant pET22b using *Nde*I and *Xho*I.

Expression of r-fragment C. As shown in Figure 4a, the r-fragment C was successfully expressed in E. coli BL21 (DE3) pLysS as the fusion and native proteins. The production of the recombinant protein was detectable after 1 h of IPTG induction and reached a maximal level after 3 h in both expression vectors. Furthermore, the high expression level of r-fragment C was achieved at the incubation temperature of 25 °C (Figure 4a). The expressed r-fragment C was either approximately 51.6 kDa as a native protein in pET22b vector or almost 54 kDa as a fusion protein in pET28a (Figure 4a). As shown in Table 1, no significant differences were observed between the two expression systems in terms of total soluble protein in cell lysate. However, Bradford analysis showed that the concentration of r-fragment C obtained by pET28a was higher than that obtain from pET22b (38.66 mg/L versus 32.33 mg/L, P<0.5) (Table 1).

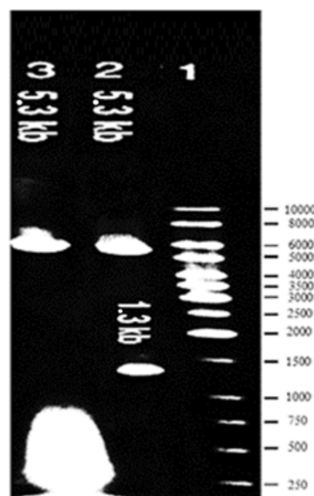

Figure 2d. Electrophoresis on 1% (w/v) agarose gel for the restriction enzyme digestion of the recombinant and non-recombinant expression vector (pET28a) using *Nde*I/*Hin*dIII, confirming the insertion of the gene encoding tetanus toxin fragment C into this vector. Lane 1: DNA size marker 1kb (Fermentase, Germany); lane 2: double digestion products of recombinant pET28a using *Nde*I and *Hin*dIII; lane 3: double digestion product of non-recombinant pET28a using *Nde*I and *Hin*dIII.

Table 1. Mean of total soluble protein and recombinant fragment C concentrations in cell lysates and percentage of the yield obtained from the two expression vectors of pET28a and pET22b

Protein source	Expression vector		
	pET28a	pET22b	P-value
Total soluble protein (mg/L)	381.67±14.0	395.0±9.85	NS
Recombinant-fragment C (mg/L)	32.33±3.05	38.66±2.08	P<0.5
Yield (%)	8.5±0.5	9.8±0.4	P<0.5

NS: not-significant, Note: The data presented in this table is related to the *Escherichia coli* cultures with log phase of “10” and expression condition of 25 °C, 1 mM IPTG, and 3 h IPTG induction.

Western blot analysis. To detect the r-fragment C, a western blot assay was performed using anti-tetanus

toxin-specific polyclonal antibody under reducing conditions.

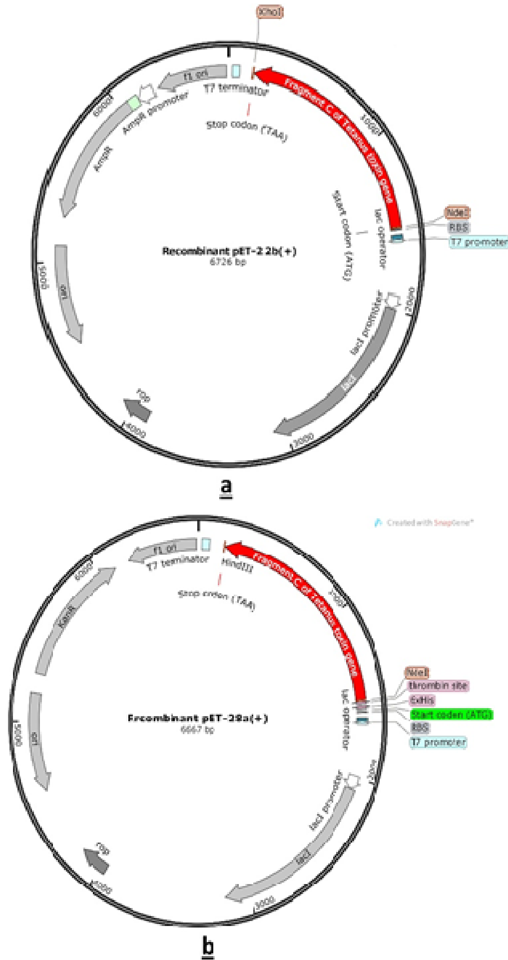

Figure 3a. Schematic representation of the recombinant pET22b expression vector designed by SnapGene software version 2.8. The gene encoding fragment C of tetanus toxin was cloned into the *Nde*I/*Xho*I restriction sites of pET22b vector.

Figure 3b. Schematic representation of the recombinant pET28a expression vector designed by SnapGene software version 2.8. The gene encoding fragment C of tetanus toxin was cloned into the *Nde*I/*Hin*dIII restriction sites of pET28a vector, in frame with the 6-histidin tag and thrombin cleavage site in N-terminal proportion of pET28a.

As shown in Figure 4b, the western blot by polyclonal horse antisera against r-fragment C of tetanus toxin resulted in the emergence of only one band on the membrane in both recombinant native (51.6 kDa) and fusion (54 kDa) proteins, representing that horse antibodies specifically bound to the r-fragment C produced by both expression vectors, namely pET22b and pET28a.

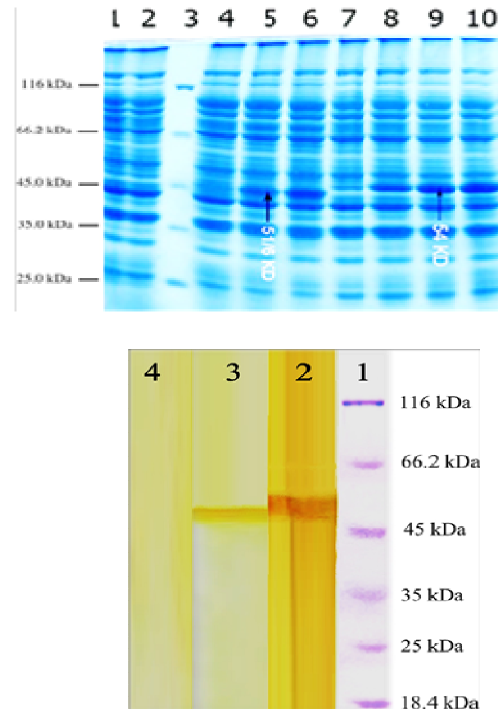

Figure 4a. Analysis of total cell lysates of transformed *E. coli* BL21 (DE3) pLysS, containing recombinant pET22b or pET28a expression vectors by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, confirming high expression level of recombinant fragment C of tetanus toxin. Samples of cell lysates including total protein content were collected at 24 h intervals after methanol induction, separated by 12.5% SDS-PAGE gel, and visualized by staining with coomassie blue R-250. Lane 1: negative control; lanes 2 and 7: total cell protein obtained from *E. coli* BL21 (DE3) pLysS cells bearing the recombinant expression constructs in pET22b and pET28a, respectively, grown in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG); lane 3: protein molecular weight standards (Fermentas, Lithuania); lanes 4, 5, and 6: the same as lane 2, but the samples were taken 1, 2, and 3 h after IPTG induction, respectively; lanes 8, 9, and 10: the same as lane 7, but the samples were taken 1, 2, and 3 h after IPTG induction, respectively.

Figure 4b. Analysis of total cell lysates of transformed *E. coli* BL21 (DE3) pLysS, containing recombinant pET22b or pET28a expression vectors by western blot, confirming high purification of recombinant fragment C of tetanus toxin. Fractioned proteins were transferred from the SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane, and a single band representing recombinant fragment C was detected in both recombinant native

(51.6 kDa) and fusion (54 kDa) forms; lane 1: schematic representation of a broad-range protein size marker (Fermentas, Lithuania); lanes 2 and 3: PVDF membranes probed with total cell protein from *E. coli* BL21 (DE3) pLysS cells containing the recombinant pET28a and pET22b, respectively; lane 4: negative control.

DISCUSSION

There are many reports confirming the antigenicity of fragment C of tetanus toxin, since it has been identified as an immunodominant (Francis et al., 2004; Carlton et al., 2008) and nontoxic (Helting and Zwisler, 1977; Helting and Nau, 1984) antigen. Therefore, some research teams have used this fragment to produce a recombinant vaccine against tetanus (Maassen et al., 1999; Ribas et al., 2000; Stevenson and Roberts, 2004).

In the present study, we successfully expressed the r-fragment C of tetanus toxin in E. coli regarding the production of recombinant subunit vaccine against tetanus toxin. Although several studies have hitherto succeeded to clone and express the fragment C of tetanus toxin, there are still some problems in the industrial production of this antigen. A problem occurring in the overproduction process of many proteins by a recombinant protein expression system is the limitations of the traditional, widely used standard system for protein expression in the shaking flask cultures described by Sambrook and Russell (2001). In this regard, some solutions to this problem are available, which were used in the present study. Based on the traditional expression methods, the recombinant protein synthesis is induced, when the (OD_{600}) reaches to 0.6, at a point about the middle of the exponential growth. Such low cell densities produce a low yield value. As a result, a large culture volume is required for high protein production. On the other hand, the expression of recombinant plasmid gene is a biochemical pathway, decreasing the specific rate of population growth and cell density owing to two factors. Firstly, this pathway in bacteria is a long process; therefore, nutrient depletion, accumulation of waste and toxic metabolites, and inhibitory compounds in the medium will provide antigrowth conditions, such as plasmid loss (Hannig and Makrides, 1998), limited availability of dissolved oxygen (DO), and increased CO2 levels, which cause significant reduction of medium pH (Khushoo et al., 2004). Therefore, under such circumstances, high cell densities cannot be reached. Secondly, for the high processivity of T7 RNA polymerase and IPTG induction, most of the transcription and translation machinery of the cell might be employed for recombinant protein expression, resulting in the reduction of bacterial growth rate and cell density (Sambrook and Russell, 2001). The low cell density often causes a low protein production. To overcome these problems, in the present study, we used a recently-optimized protocol (Bahreini et al., 2014), which was set in our laboratory using ASNase II as a recombinant protein model rather than the traditional protocol (Sambrook and Russell, 2001). This modified method resulted in a higher number of plasmid copies in each cell, and subsequently a higher copy number of the fragment C gene per cell using a high antibiotic concentration (i.e., 200 and 300 μg/mL) before the induction time, which would lead to a significant increase in protein expression per cell (Bahreini et al., 2014). Furthermore, a fresh medium with a very higher initial cell density (OD_{600}=10 versus OD_{600}=0.6) at induction time was provided using a three step-culture, the third step of which included exchanging the overnight medium by centrifuging, adding fresh LB medium to the bacterial pellet, and diluting until reaching a high cell density. As fragment C is part of a toxic protein, we supposed that its basal levels may prevent the full establishment of the plasmid carrying its codding gene in *E. coli* strains, which will result in the incomplete expression of this gene in this bacterium. On the other hand, one of the major factors reducing the overproduction of recombinant proteins, such as r-fragment C, is protein degradation by lon and ompT outer membrane proteases (Goff and Goldberg; Grodberg and Dunn, 1988). One way of providing additional stability to the target genes is to express them in the host strains containing a compatible chloramphenicol-resistant plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA

polymerase (Studier, 1991). In the present study, BL21 (DE3) pLysS strain of E. coli, a high-stringency expression host, which has the pLysS plasmid, was used. This plasmid confers resistance to chloramphenicol. The lysozyme produced by this plasmid does not interfere with the transformation of the cells containing it and has no negative effect on the growth rate. The presence of pLysS increases the tolerance of expression host in the plasmids with toxic inserts, and also has the further advantage of facilitating the preparation of the cell extracts. This strain is also deficient in the lon protease and lacks the ompT outer membrane protease. Therefore, fragment C should be more stable in BL21 (DE3) pLysS than in the host strains containing the mentioned proteases. An alternative approach to increase the stability of the target gene is to use a vector that contains a T7lac promoter ((Dubendorf and Studier, 1991; Studier, 1991). In this regard, in the present study, two different expression vectors, namely pET-28a and pET-22b, both containing T7lac promoter were used. These plasmids contain a lac operator sequence just downstream of T7 promoter. They also carry the natural promoter and coding sequence for the lacI so that the T7lac and lacI promoters diverge. When this type of vector is used in DE3 lysogens to express the r-fragment C, the lac repressor acts both at lacUV5 promoter in the host chromosome to repress the transcription of the T7 RNA polymerase gene by the host polymerase and at the T7lac promoter in the vector to block the transcription of the target gene by T7 RNA polymerase (Dubendorf and Studier, 1991; Studier, 1991). The results of the present study revealed that the employment of BL21 (DE3) pLysS strain of E. coli as an expression host and pET-28a and pET-22b as expression vectors could overcome the probable toxic effects of fragment C on the establishment of the plasmid and the subsequent expression level of its encoding gene. These results showed that the recently optimized protocol set in our laboratory for high expression level of ASNase II (Bahreini et al., 2014) could be also applied for the expression of tetanus toxin fragment C. Another important factor reducing the overproduction of the recombinant proteins is the low solubility rate (Gerday et al., 1990). It should be mentioned that in some cases, the overexpression of a special protein does not result in its overproduction. The high-level expression of both heterologous and host proteins in E. coli is often accompanied by the formation of inclusion bodies, which are irreversible aggregates mainly consisting of the overexpressed polypeptide (Ho et al., 1990). Proteins in form of inclusion bodies are insoluble agents resulting in low production rate. In this regard, the protein expression at low temperatures would be favorable to increase the overproduction of the proteins, which tend to form inclusion bodies. This is due to the fact that the formation of inclusion bodies is sometimes avoided by lowering the cultivation temperature (Vasina and Baneyx, 1996). When E. coli is used as the host, the cultivation temperature is sometimes decreased to suppress the formation of inclusion bodies (Miyake et al., 2007). A low-temperature expression system is also expected to alleviate the heat denaturation of proteins and would be suitable for the production of thermolabile proteins (Miyake et al., 2007). Protein expression at low temperatures is also useful for the production of the enzymes whose activities are harmful to the host cells, such as proteases that degrade the essential components of the host, because the enzyme activities can be suppressed by lowering the temperature (Miyake et al., 2007). In the present study, the transformants were induced with IPTG at four different incubation temperatures (i.e., 37, 33, 30, and 25 °C) and three different incubation times (i.e., 1-3 h). The optimal expression condition, in this study, was acquired at 25 °C, 1 mM IPTG, and 3 h after IPTG induction (Figure 4a), which was desirable to suppress the formation of the inclusion bodies, resulting from the overproduction of r-fragment C in E. coli expression system. Ribas et al. (2000) similarly reported the optimal expression condition at 1 mM IPTG and 3 h

after IPTG induction. In contrast with our results, (Yousefi et al., 2013) reported the optimal expression time of 8 h after IPTG induction. The transformed E. coli BL21 (DE3) pLysS with pET-28a produced a fusion protein (~54 kDa) consisting of a 6-histidin tag and a thrombin cleavage site in N-terminal proportion, and the cells transformed with pET-22b produced a protein (~ 51.6 kDa) in a native form. The presence of a histidin tag in the expressed protein facilitates its purification via one-step metal-affinity chromatography. On the other hand, as any additional amino acid sequence may affect the structure, spatial conformation, and the subsequent function of the native protein, they should be removed from the native protein sequence in order to be used in the clinical trial testing and final product manufacturing. In this regard, the presence of thrombin cleavage site (N-Leu-Val-Pro-Arg-Gly-Ser-C) within the expressed protein produced in the present study enabled us to cleave the 6-histidin tag from protein sequence in the future studies. Although the western blot analysis confirmed the expression of both recombinant native and fusion fragments C, the yield obtained by pET28a was higher than that of pET22b (9.8% versus 8.5%, P<0.5). Therefore, it seems that BL21 (DE3) pLysS, as an expression host, was more compatible with pET28a expression vector than with pET22b to express the r-fragment C. The results of the present study revealed the occurrence of no mutations or polymorphisms during gene amplification or cloning procedure, which is a desirable characteristic for a bacterial strain to be applied as a vaccinal strain.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was financially supported by Razi Vaccine and Serum Research Institute, Karaj, Alborz, Iran.

Acknowledgement

We appreciate the staff of the Departments of Genomics and Genetic Engineering, DTP, and Anaerobic Vaccines, Razi Vaccine and Serum Research Institute, Karaj, Alborz, Iran

References

Bahreini, E., Aghaiypour, K., Abbasalipourkabir, R., Goodarzi, M.T., Saidijam, M., Safavieh, S.S., 2014. An optimized protocol for overproduction of recombinant protein expression in Escherichia coli. Prep Biochem Biotechnol 44, 510-528.

Bruggemann, H., Gottschalk, G., 2004. Insights in metabolism and toxin production from the complete genome sequence of Clostridium tetani. Anaerobe 10, 53-68.

Carlton, E., Teng, Q., Federici, T., Yang, J., Riley, J., Boulis, N.M., 2008. Fusion of the tetanus toxin C fragment binding domain and Bcl-xL for protection of peripheral nerve neurons. Neurosurgery 63, 1175-1182; discussion 1182-1174.

Charles, I.G., Rodgers, B.C., Makoff, A.J., Chatfield, S.N., Slater, D.E., Fairweather, N.F., 1991. Synthesis of tetanus toxin fragment C in insect cells by use of a baculovirus expression system. Infect Immun 59, 1627-1632.

Chatfield, S.N., Fairweather, N., Charles, I., Pickard, D., Levine, M., Hone, D., et al., 1992. Construction of a genetically defined Salmonella typhi Ty2 aroA, aroC mutant for the engineering of a candidate oral typhoid-tetanus vaccine. Vaccine 10, 53-60.

Dubendorf, J.W., Studier, F.W., 1991. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. Journal of Molecular Biology 219, 45-59.

Fairweather, N.F., Lyness, V.A., 1986. The complete nucleotide sequence of tetanus toxin. Nucleic Acids Res 14, 7809-7812.

Fairweather, N.F., Lyness, V.A., Pickard, D.J., Allen, G., Thomson, R.O., 1986. Cloning, nucleotide sequencing, and expression of tetanus toxin fragment C in Escherichia coli. J Bacteriol 165, 21-27.

Francis, J.W., Figueiredo, D., vanderSpek, J.C., Ayala, L.M., Kim, Y.S., Remington, M.P., et al., 2004. A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. Brain Res 995, 84-96.

Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.-P., Claverie, P., Collins, T., et al., 1990. Cold-adapted enzymes: from fundamentals to biotechnology. Trends in Biotechnology 18, 103-107.

Gil, C., Chaib-Oukadour, I., Blasi, J., Aguilera, J., 2001. HC fragment (C-terminal portion of the heavy chain) of tetanus toxin activates protein kinase C isoforms and phosphoproteins involved in signal transduction. Biochem J 356, 97-103.

Goff, S.A., Goldberg, A.L., Production of abnormal proteins in E. coli stimulates transcription of ion and other heat shock genes. Cell 41, 587-595.

Grodberg, J., Dunn, J.J., 1988. ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification. Journal of Bacteriology 170, 1245-1253.

Halpern, J.L., Habig, W.H., Neale, E.A., Stibitz, S., 1990. Cloning and expression of functional fragment C of tetanus toxin. Infect Immun 58, 1004-1009.

Hannig, G., Makrides, S.C., 1998. Strategies for optimizing heterologous protein expression in Escherichia coli. Trends in Biotechnology 16, 54-60.

Helting, T.B., Nau, H.H., 1984. analysis of the immune response to papain digestion products of tetanus toxin. Acta Pathologica Microbiologica Scandinavica Series C: Immunology 92C, 59-63.

Helting, T.B., Zwisler, O., 1977. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. J Biol Chem 252, 187-193.

Helting, T.B., Zwisler, O., Wiegandt, H., 1977. Structure of tetanus toxin. II. Toxin binding to ganglioside. J Biol Chem 252, 194-198.

Herreros, J., Lalli, G., Schiavo, G., 2000. C-terminal half of tetanus toxin fragment C is sufficient for neuronal binding and interaction with a putative protein receptor. Biochem J 347 Pt 1, 199-204.

Khushoo, A., Pal, Y., Singh, B.N., Mukherjee, K.J., 2004. Extracellular expression and single step purification of recombinant Escherichia coli L-asparaginase II. Protein Expr Purif 38, 29-36.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Maassen, C.B.M., Laman, J.D., Heijne den Bak-Glashouwer, M.J., Tielen, F.J., van Holten-Neelen, J.C.P.A., Hoogteijling, L., et al., 1999. Instruments for oral disease-intervention strategies: recombinant Lactobacillus casei expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine 17, 2117-2128.

Makoff, A.J., Oxer, M.D., Romanos, M.A., Fairweather, N.F., Ballantine, S., 1989. Expression of tetanus toxin fragment C in E. coli: high level expression by removing rare codons. Nucleic Acids Res 17, 10191-10202.

Miyake, R., Kawamoto, J., Wei, Y.L., Kitagawa, M., Kato, I., Kurihara, T., et al., 2007. Construction of a low-temperature protein expression system using a cold-adapted bacterium, Shewanella sp. strain Ac10, as the host. Appl Environ Microbiol 73, 4849-4856.

Morris, N.P., Consiglio, E., Kohn, L.D., Habig, W.H., Hardegree, M.C., Helting, T.B., 1980. Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. J Biol Chem 255, 6071-6076.

Ribas, A.V., Ho, P.L., Tanizaki, M.M., Raw, I., Nascimento, A.L., 2000. High-level expression of tetanus toxin fragment C-thioredoxin fusion protein in Escherichia coli. Biotechnol Appl Biochem 31 (Pt 2), 91-94.

Romanos, M.A., Makoff, A.J., Fairweather, N.F., Beesley, K.M., Slater, D.E., Rayment, F.B., et al., 1991. Expression of tetanus toxin fragment C in yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. Nucleic Acids Res 19, 1461-1467.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press.

Schiavo, G., Ferrari, G., Rossetto, O., Montecucco, C., 1991. Tetanus toxin receptor specific cross-linking of tetanus toxin to a protein of NGF-differentiated PC 12 cells. FEBS Letters 290, 227-230.

Stevenson, A., Roberts, M., 2004. Intranasal immunisation against tetanus with an attenuated Bordetella bronchiseptica vector expressing FrgC: improved immunogenicity using a Bvg-regulated promoter to express FrgC. Vaccine 22, 4300-4305.

Studier, F.W., 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. Journal of Molecular Biology 219, 37-44.

Tregoning, J.S., Nixon, P., Kuroda, H., Svab, Z., Clare, S., Bowe, F., et al., 2003. Expression of tetanus toxin Fragment C in tobacco chloroplasts. Nucleic Acids Res 31, 1174-1179.

Vasina, J.A., Baneyx, F., 1996. Recombinant protein expression at low temperatures under the transcriptional control of the major Escherichia coli cold shock promoter cspA. Appl Environ Microbiol 62, 1444-1447.

Wang, M.Y., Zhang, Y.N., Lei, M., Zuo, D.M., Zhang, L.Y., Chen, Z.L., 2008. [Gene cloning, optimized expression and immunogenicity evaluation of tetanus toxin fragment C]. Nan Fang Yi Ke Da Xue Xue Bao 28, 731-735.

Yousefi, M., Khosravi-Eghbal, R., Hemmati, A., Shokri, F., 2013. Production and characterization of recombinant light chain and carboxyterminal heavy chain fragments of tetanus toxin. Avicenna J Med Biotechnol 5, 220-226.