

Full Length Research Paper

Expression and purification of recombinant Shiga toxin 2B from *Escherichia coli* O157:H7

Marwa E. A. Aly¹, Amro S. Hanora^{2*}, Tamer M. Essam¹ and Magdy A. Amin¹

¹Microbiology and Immunology Department and Biotechnology Centre, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo11562, Egypt.

²Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, Ismailia, 41522, Egypt.

Received 11 December, 2015; Accepted 2 March, 2016

Enterohemorrhagic *Escherichia coli* are important human food-borne pathogens. Recently, Shiga toxin-producing *E. coli* (STEC) causes life-threatening hemolytic-uremic syndrome (HUS). In this study, Stx2B gene, a subunit of Shiga toxin, was amplified via polymerase chain reaction (PCR) from the chromosomal DNA of clinical fecal sample using appropriate primers. The PCR product was cloned to commercially available plasmid pH6HTN His6HaloTag® T7 containing two purification tags, namely, six histadine tag and Halo tag. The integrity of the constructed plasmid was confirmed using restriction enzyme mapping and sequencing. Then, Stx2B protein expressed after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) in *E. coli* JM109 (DE3) under the control of the T7 promotor. The two step purification trains were used to purify native Stx2B. First step purification was Ni-immobilized metal ion affinity chromatography (IMAC) column, followed by second step using HaloLink resin. The native Stx2B was obtained after column cleavage of halo-tag using HaloTEV protease. Maximum protein expression of Stx2B economically was obtained using 1 mM IPTG for 4 h at 37°C. Protein identity was confirmed by a band at ~11.4 kDa using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and StxB2 yield was 450 µg ml⁻¹ confirmed by Bradford assay. Recombinant Stx2B protein was produced in highly pure yield using HaloTag technology.

Key words: *Escherichia coli* O157:H7, StxB gene, expression, HaloTag technology, purification.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) strains are important human food-borne pathogens (Kaper et al., 2004). The clinical manifestations of EHEC infections range from watery diarrhea or hemorrhagic colitis (HC), to the most severe outcome, the life-threatening hemolytic-uremic syndrome (HUS) (Nataro and Kaper,

1998). Currently, there are no specific protective measures or therapy against EHEC infection other than supportive therapy; as the utility of antibiotic or anti-diarrhetics treatment may be a risk factor for HUS and there is insufficient evidence to recommend antibiotic treatment for EHEC infection as reported by Nguyen and Sperandio

*Corresponding author. E-mail: ahanora@yahoo.com. Tel: +2064-3230741 or +201000323406. Fax: +2064-3230741.

(2012).

Although, the magnitude of the social and economic impacts caused by EHEC infections is high, no licensed vaccine or effective therapy is presently available for human use. So far, a number of experimental approaches are being investigated in animals. For example, Donohue-Rolfe et al. (1989) reported that a monoclonal antibody against the Stx2B subunit recognizes and neutralizes both Stx1 and Stx2 in the HeLa cell cytotoxicity assay. The immune-prophylactic potential of the Stx1B subunit has been proven in the publication from different laboratory such as Boyd et al. (1991) and Acheson et al. (1996). In recent study, the immune-modulatory potential of recombinant Shiga toxin B subunit (rStxB) protein in BALB/c mice was evaluated. Animal protection with recombinant StxB was conferred through both humoral and cellular immune responses (Gu et al., 2011). Recently, the large scale production of stx2 in *E. coli* for toxoid vaccine antigen was achieved by Hideyuki et al. (2013).

Several studies showed evidence that a closer association between Stx2 expression by Shiga toxin-producing *E. coli* (STEC) and a more severe course of illness than Stx1 as were reported in both Scotland et al. (1987) and Hashimoto et al. (1999) studies. For example, in a recent outbreak involving 131 Japanese patients infected with Stx1-producing O118:H2 STEC serotype, no case of HUS was reported, and the gastrointestinal symptoms were relatively mild (Marcato et al., 2001). In addition, it has been proposed that a cellular vaccine consisting of a nontoxic Stx2B or combined with other proteins (Dubendorff and Studier, 1991) might provide safe and effective protection against the most severe complications of EHEC infections (Hanahan, 1983). This is better than the use of holotoxin Stxs as the expression of holotoxin may lead to instability of the expression strain or mutations accumulation due to the enzymatic activity of A subunits (Hopwood, 2003). Therefore, these epidemiological findings make Stx2B a compelling candidate for vaccine development in present study.

In this research, an easy way was developed to build recombinant plasmid pH6HTN His₆HaloTag® T7-Stx2B to get high yield of recombinant Stx2B (rStx2B) that could be applied in large scale production within vaccine candidate.

MATERIALS AND METHODS

Bacterial strains, plasmid and media

One clinical isolate of *E. coli* containing *stx*_{1AB} and *stx*_{2AB} genes was collected from Abu El-Reesh Hospital, Cairo University's fecal samples. A clinical EHEC was tested for the inability to utilize sorbitol by plating on sorbitol MacConkey (SMA) agar (Oxoid, USA) at 37°C. Also, biochemical profile was performed by API 20E (BioMérieux, France) according to the manufacturer's instructions. *E. coli* DH5α (Promega, USA) (Hanahan, 1983) and *E. coli* JM109 (DE3) (Promega, USA) were used as hosts for cloning and protein expression, respectively.

The prokaryotic plasmid pH6HTN His₆HaloTag® T7 (4,014 kb, Promega, USA) was used for gene cloning and expression in *E. coli*. All *E. coli* strains were grown on Luria Bertani (LB) (Oxoid Ltd., Basingstok, Hampshire, England) liquid or agar medium at 37°C and with 100 µg.ml⁻¹ of ampicillin as a selective medium whenever necessary.

Construction of Stx2B expression plasmid

DNA cloning and further manipulations were carried out according to methods described by Hopwood (2003) and Sambrook and Russell (2001). The DNA of clinical EHEC isolate was used as template for polymerase chain reaction (PCR) amplification of Shiga toxin 2B gene after purified by wizard genomic DNA purification kit (Promega, USA) according to manufacturer's structures. PCR was performed in a programmable thermo-cycler PCR Machine (Touchgene Gradient, USA) using the specific primers (IDT DNA, USA): P1:5-cgTCTAGAATGAAGAAGATGTTTATGGCGGTT-3 and P2:5-ttGCGCCGCTCAGTCATTATTAATACTG-3. The forward primer (P1) contained an engineered *Xba*I site (underlined) and the reverse primer (P2) incorporated an engineered *Not*I site (underlined). The amplification of stx2B by PCR was performed using Flexi GoTaq DNA polymerase (Promega, USA) and primer P1 and P2 under the following conditions: one cycle of denaturation (95°C for 10 min); 30 cycles of denaturation (95°C for 30 s), annealing (54°C for 30 s), extension (72°C for 45 s); followed by a final extension (72°C for 10 min).

Both the purified amplicon of *stx*_{2B} gene and his6halotag t7 plasmid were double digested with FastDigest *Xba*I/*Not*I (Thermo-Scientific, USA) according to the manufacturer's specifications. Ligation took place under optimal reaction conditions using T4-DNA ligase according to New England BioLabs (NEB) manufacturer's protocol, with an insert: vector ratio of 3:1, respectively. The reaction was performed at 16°C for 18 min in thermo-cycler PCR machine at a final reaction volume of 20 µl containing 0.2 µg.µl⁻¹ for digested PCR fragment, 1 µg.µl⁻¹ for linearized vector, 2 µl for 10x ligation buffer and 1 µl for (1 unit) T4 ligase. The mixing was done gently by pipetting the solution up and down.

The cloned vector was used to transform chemically prepared competent *E. coli* DH5α and was cultured on Luria Broth (LB) medium containing 100 µg.ml⁻¹ of ampicillin. The resulted colonies were tested for further analysis for the presence of an insert into the multiple cloning sites (MCS) by using colony PCR primer for PCR amplification reaction that proceed part from MCS of pH6HTN His₆HaloTag T7 vector using the specific primers (IDT DNA, USA); P1 (forward primer): 5GGTCTGAATCTGCTGCAAGAA-3 and P2 (reverse primer): 5-ACATGGCGATAGCTAGACTG-3. The thermal cycler protocol was performed according to the previous conditions except annealing temperature was 52.7°C for 30 s. The *E. coli* cells which carry empty vector were used as a negative control.

The positive colonies were subjected to another colony PCR analysis for identifying the correct orientation (in frame) of the gene insert (*stx*_{2B}) in the pH6HTN His6HaloTag vector. Moreover, the positive *E. coli* DHα5 colonies harboring correct recombinant vector of *stx*_{2B} gene were subjected to *Xba*I/*Not*I double digestion restriction analysis to ensure insertion of the desired insert.

The clone harbouring plasmid DNA from positive clone was isolated with QIAGEN® Plasmid Purification kit (Qiagen, Germany). The resulting purified plasmid pH6HTN His6HaloTag®T7-Stx2B was transformed into final host of chemically competent *E. coli* JM109 (DE3) and was cultured on LB medium containing 100 µg. ml⁻¹ of ampicillin.

Stx2B expression and purification

Expression of Stx2B in *E. coli* was carried out as described by

Studier et al. (1990). Expression was performed under the control of the T7 promoter using *E. coli* JM109 (DE3) strains. Single colony harbouring the plasmid His6HaloTag T7-Stx2B and His6HaloTag T7 (empty vector as negative control) were grown overnight in 3 ml LB medium containing $100 \mu\text{g}\cdot\text{ml}^{-1}$ ampicillin at 37°C on a shaker incubator 250 rpm. 200 μl of these pre-cultures were used to inoculate 20 ml fresh LB medium in 250 ml normal flasks and let them grow at 37°C on shaker incubator 120 rpm to an $\text{OD}_{600 \text{ nm}}$ of 0.5 to 0.6.

Optimization of the Stx2B expression

Five flasks of previous cultures were induced by the addition of different concentration of isopropyl β -D-1-thiogalactopyranoside (IPTG; 0.1, 0.25, 0.5, and 1 mM) and one culture was un-induced. Induction was continued for different time periods (0 h, 4 h and overnight) at incubation temperature (37°C) with shaking at 120 rpm. The above experiment was repeated at 30°C incubation temperature instead of 37°C. Then aliquots were removed at different time points for analysis. Induced cultures were transferred to falcon tube to harvest by centrifugation for 30 min at 13,000 rpm at 4°C and the pellets were washed twice with lysis buffer (ice cold 50 mM $\text{Na}_2\text{HPO}_4/\text{MOPS}$, 300 mM NaCl, 5 mM Imidazole, and 0.25 $\mu\text{g}/\mu\text{l}$ (p-amidinophenyl)methanesulfonyl fluoride (APMSF), pH at 7.0) then were kept in -20°C until used.

Batch purification Stx2B protein from *E. coli* under native condition

Purification of His₆HaloTagged Stx2B proteins using IMAC by gravity-flow

The pellets were re-suspended in lysis buffer in ratio 1:10. The extent of sonication was tightly controlled: sonication in 4 cycles (each at 20 s on at 35% power, 60 s off, at 0°C) (Elma, USA). The resulting cell lysate was centrifuge at $12,000 \times g$ for 20 min at 4°C to remove the unlysed cells and insoluble proteins. The supernatant was collected and loaded onto a profanity nickel metal charged resin (Bio-Rad, Hercules CA, USA) Econo-Pac® column that had been pre-equilibrated with 5 column volumes (CVs) buffer A (50 mM $\text{Na}_2\text{HPO}_4/\text{MOPS}$, 300 mM NaCl and 5 mM imidazole, pH at 7.0).

Then, the column was washed with 5 CVs of buffer A to remove unbound sample and the resulted fractures (0.5 ml/fraction) were pooled for further analysis. After that, the His₆HaloTagged Stx2B protein was eluted with buffer A containing 500 mM imidazole and then protein containing fractions (0.5 ml/fraction) were pooled and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Further purification of His₆HaloTagged Stx2B proteins using halo-link resin

The His₆HaloTagged Stx2B protein resulted from previous purification was subjected to further purification by HaloLink resin (Promega, USA) by batch purification. About 2 ml (0.5 ml settled) HaloLink resin was equilibrated with 10 CVs of Halotag purification buffer (50 mM MOPS and 120 mM NaCl, pH at 7.0) by inverting the tube until thoroughly mixed. Then, resin was centrifugation at $1,000 \times g$ for 10 min at 4°C and washed twice with the same buffer followed by centrifugation. 1 ml of His₆HaloTagged Stx2B IMAC purified protein previously purified was incubated with the HaloLink resin at 4°C overnight with constant mixing, and collected by centrifugation at $1,000 \times g$ for 10 min at 4°C. The resin was washed with 10 CVs of Halotag purification buffer and mixed by inverting

the tube, the resin centrifugation at $1,000 \times g$ for 10 min at 4°C and the supernatant was discarded, and a total of two washed was performed.

Afterwards, the resin was incubated with 0.55 ml cleavage solution (50 mM MOPS and 120 mM NaCl, pH at 7.0) containing 33 μl of HaloTEV protease (Promega, USA) for 90 min at room temperature. The supernatant was separated by centrifugation at $10,000 \times g$ for 5 min and the resin was washed with Halotag purification buffer and both supernatants were combined. The eluted purified protein was analyzed by 15% SDS-PAGE.

Protein analysis

Electrophoresis of proteins was performed using SDS-PAGE according to Laemmli (1970) where the final concentration of acrylamide in the stacking and separating gels was 4 and 15%, respectively.

The gels were scanned with a Digital Camera (Cannon, Japan) and analyzed using the software package GelCompar II, 6.5 (Applied Maths, Belgium). The pertained protein ladder" Broad Range (6.5-200 kDa) (Serva, USA) was used as internal reference of molecular weight for normalization. The concentration of resulted Stx2B protein was determined using the Bradford assay (Thermo Scientific, USA), using BSA as a protein standard (Bradford, 1976).

RESULTS

Construction of Stx2B expression plasmid

Genomic DNA from EHEC clinical isolate was used to isolate the gene coding for Stx2B. Thus, the 270 bp PCR amplicon size carrying *Xba*I/*Not*I restriction sites on the 5' and 3' flanking regions, respectively (Figure 1B; Lane 1) was cloned into the pH6HTN His6HaloTag® T7 vector resulting in intermediate plasmid pH6HTN His6HaloTag® T7-Stx2B.

The digestion of double restriction the pH6HTN His6HaloTag® T7 vector and Stx2 insert with Fast Digest *Xba*I/*Not*I restriction enzymes resulting in the linearization of vector producing a band size ~3974 bp (Figure 1A; Lane2), while the PCR product of *stx2B* gene was observed at ~270 bp, (Figure 1B; Lane 1).

Screening for recombinant *stx2B* gene for confirmation of insert

The recombinant construct containing *stx2B* gene obtained after ligation was used for *E. coli* DH5 α cells transformation. Among the colonies appeared on the LB Agar/ampicillin ($100 \mu\text{g}\cdot\text{mL}^{-1}$), the positive clone was screened initially by colony PCR using the primers designed from MCS of vector. The 350 bp of PCR product size corresponded to negative clone, while the 595 bp amplicon size corresponded to positive clone (Figure 1B, Lanes 3 and 4, respectively).

The positive clone was subjected to colony PCR screening for correct insert orientation resulting in 435 bp amplicon size (Figure 1B; Lane 5). The 4.244 kb plasmid DNA of positive clone was double digested after

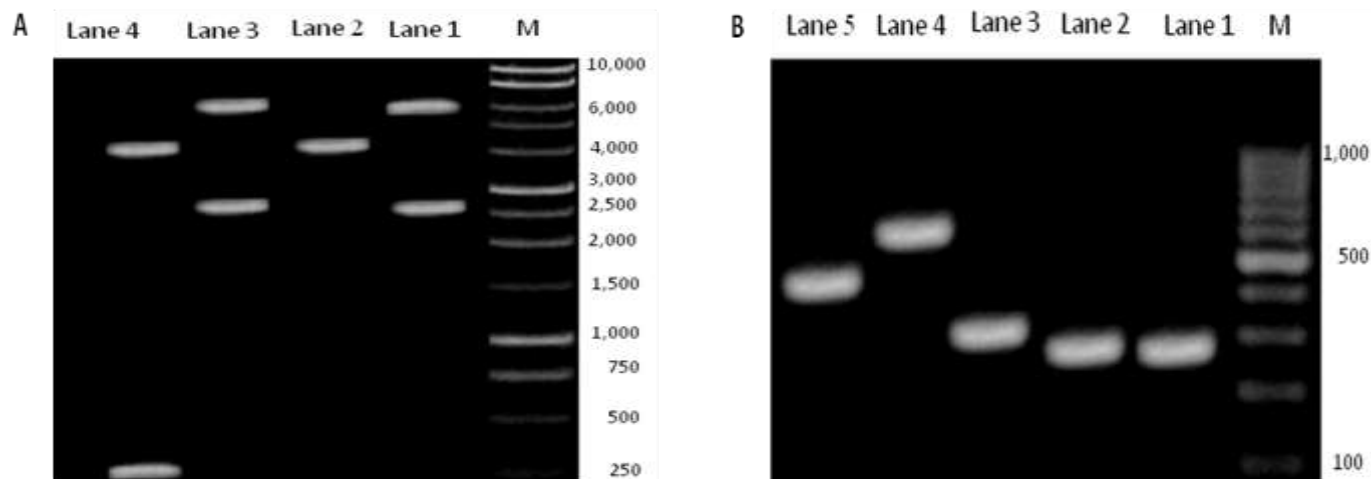


Figure 1. Gel-electrophoresis of the PCR amplification products for cloning, restriction digests, and screening for correct clone. M: 1 kb DNA Marker, Lane 1: plain super-coiled vector at 4.014 kb, Lane 2: linearized digested plasmid product at 3.974 kb by double digestion with *XbaI/NotI* for cloning, Lane 3: super-coiled positive plasmid at 4.244 kb, Lane 4: two DNA bands of *stx2B* at 0.27 kb and linearized digested plasmid at 3.974 kb after restriction analysis (A). M: 100 bp DNA Marker, Lane 1: *stx2B* gene with *XbaI/NotI* restriction sites at 270 bp, Lane 2: digested *stx2B* gene at ~270 bp, Lane 3: Negative clone at 350 bp, Lane 4: positive clone at 594 bp, Lane 5: correct *stx2B* insert orientation at 453 bp (B).

purification (Figure 1A; Lane 3) resulting in two fragments; one of larger size which correspond to linearized vector (3.974 kb) and another of smaller size, which corresponded *stx2B* gene (270 bp) (Figure 1A; Lane 4).

Stx2B protein expression and purification

The construct containing the target gene (*stx2B*) was used to transform JM109 (DE3) cell. The cell has been confirmed to be transformed in the gene (*stx2B*) in the correct orientation insertion. The transformed cells were grown to test the ability of the host cell containing construct to produce recombinant protein.

As evidenced by on GelComparII6.5 software after normalization; the expression of His₆ Halo tagged recombinant Stx2B protein increased gradually by the gradual increase in IPTG concentration (0.1, 0.2, 0.5 and 1 mM), respectively (Figure 2). Moreover, 37°C induction temperatures showed higher His₆ Halo tagged recombinant Stx2B protein expression (Figure 2B) than at 30°C induction temperature (Figure 2A). Overnight induction showed higher expression of the His₆ Halo tagged recombinant Stx2B protein when compared with the observed expression when induction was conducted for 4 h (Figure 2A and B).

Batch purification Stx2B protein from *E. coli* JM109 (DE3) under native condition

Purification of Stx2B protein using IMAC

His₆ Halo tagged recombinant Stx2B protein was

obtained in a soluble form in a relatively high purity level with a band corresponding ~47.5 kDa, Lanes 5 to 9, when phosphate buffer was replaced by MOPS buffer through Profinity IMAC Ni-charged resin (Figure 3).

Further purification of His₆HaloTagged Stx2B proteins using halo-link resin

The resulted ~47 kDa His₆Halo Tagged recombinant Stx2B protein from previous purification was subjected to undergo further purification by HaloLink resin with HaloTEV protease treatment. The resulted protein band was observed at ~11.4 kDa, Lane 10 corresponded to highly pure Stx2B protein (Figure 3). The final concentration of resulted protein was 450 µg.ml⁻¹ using Bradford assay.

DISCUSSION

Several vaccine strategies have been used with variable success in a number of animal models. The strategies have involved the use of recombinant virulence proteins such as Stx, intimin and *E. coli* secreted protein A (EspA) (Gu et al., 2009) or peptides (Wan et al., 2011) or fusion proteins of A and B subunits of Stx2 and Stx1 such as Stx2Am-Stx1B (Cai et al., 2011) or avirulent host cells of EHEC O157:H7 (Cai et al., 2010). The application of live attenuated bacteria such as *Salmonella* as a carrier for vaccine proteins against mucosal pathogens including EHEC has obvious advantages (Hideyuki et al., 2013).

The choice of Stx2B in the present study as compelling candidate for vaccine development in present study was

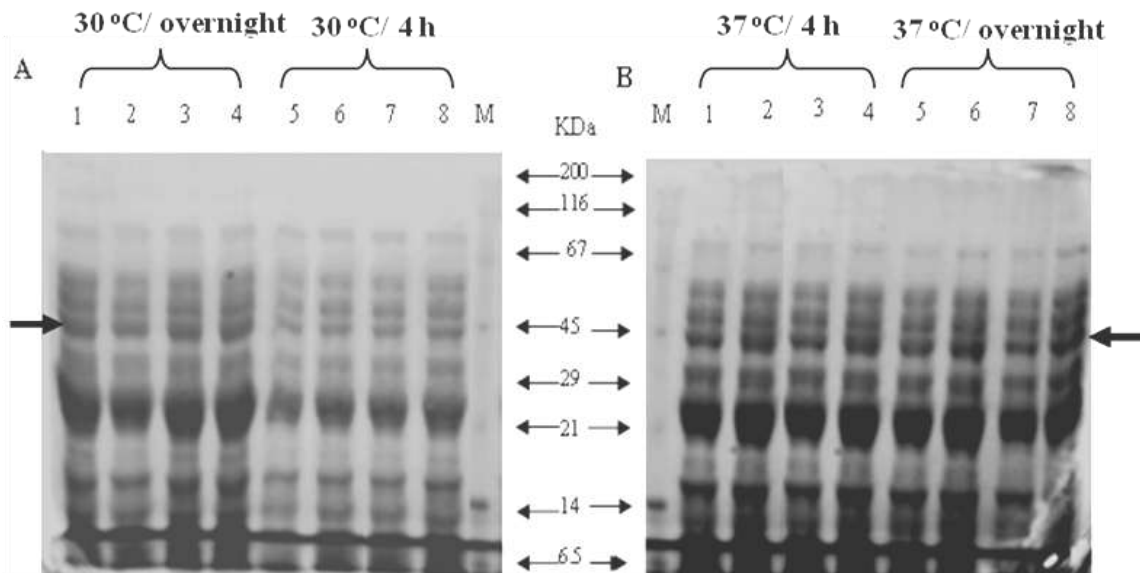


Figure 2. SDS-PAGE analysis of optimization His₆Halo tagged recombinant Stx2B protein expression in *Escherichia coli* JM 109(DE3) cells transformed with pH6HTN His6HaloTag® T7. M: protein molecular weight marker (6.5-200 kDa), Lane 1-4: culture growth under 0.1, 0.2, 0.5, 1 mM of IPTG in 30°C at overnight, Lane 5-8: culture growth under 0.1, 0.2, 0.5, 1 mM of IPTG in 30°C at 4 h (A). M: protein molecular weight marker (6.5-200 kDa), Lane 1-4: culture growth under (0.1, 0.2, 0.5 and 1 mM of IPTG in 37°C at 4 h, Lane 5-8: culture growth under (0.1, 0.2, 0.5 and 1mM of IPTG in 37°C overnight) (B). Arrows indicated predicted protein size (~47 kDa) in supernatant fractions.

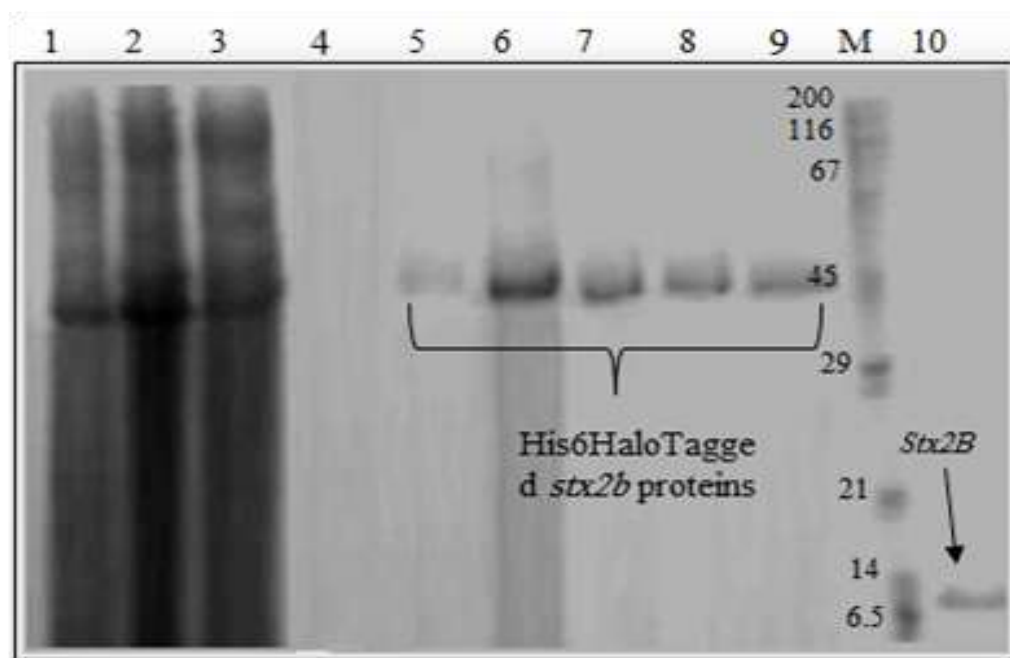


Figure 3. An image of a Coomassie blue-stained SDS-PAGE gel of cell lysates of *Escherichia coli* JM109(DE3) strain carrying the recombinant Stx2B plasmid that were either not induced with IPTG (-) in Lane 1 or induced with 1 mM IPTG for overnight (+) in Lane 2. Lane 3 contained a sample of the column flow through FT, Lane 4 contained a sample of the column wash (W), while Lanes 5-9 contain samples of the fractions eluted from the Profinity IMAC Ni-charged resin which indicates the position of the main component eluted from the column with a band size of approximately 47.5 kDa of 6xHis-Halo tagged Stx2B protein. (M) Contained protein molecular weight marker (Serva, USA) and the sizes of the respective bands were indicated. Lane 10 contained pure Stx2B ~11.4 kDa elution after removal of HaloTEV protease by Profinity IMAC Ni-charged resin. The Mops Buffer was used in all of steps of purification.

consistent with what have been reported in recent studies such as Fujii et al. (2012) and Mejias et al. (1996). However, some other studies showed contrasts, such as Liu et al. (2009) and Tu et al. (2009), where they used truncated Stx2A1 and the holotoxin Stx2 in their candidate vaccine, respectively.

In this study, different conditions for the induction of Shiga toxin expression were examined including temperature, IPTG concentration and the induction period in order to obtain the maximum level of protein production. Thus, Stx2B expression showed higher level of expression when Stx2B was induced at 37°C compared with 30°C. Similar results were obtained by Shan et al. (2010) and Madanchi et al. (2011) who reported that StxB-pQE40-DHFR and CtxB-linker-StxB-pET-28a expression systems showed a higher expression rate at 37°C. In contrast, Liu et al. (2009) and Tu et al. (2009) have reported that 30 and 16°C was considered optimal for Stx2 holotoxin pET32b and for truncated Stx2A1 pET-22b expression systems, respectively.

In the present study, the expression of Stx2B protein was gradually increased when the concentration of IPTG was gradually increased. The expression was optimal at 1 mM IPTG concentration. In contrast, Shan et al. (2010) study has employed 0.3 mM final IPTG concentration for mature StxB induction.

The overnight induction of Stx2B protein at 30°C expression, have resulted in higher yield compared to induction for 4 h also the overnight induction period at 37°C expression have little increase in yield compared to 4 h induction. The induction for 4 h at 37°C appeared more economically preferred compared to overnight induction, as it has more productivity as well as saved the energy that was consumed for shaking. These results were consistent with that of Shan et al. (2010), as they used 37°C for 3 h for induction of mature Stx protein. However, Liu et al. (2009) reported that 30°C for 14 h time of induction of truncated Stx2A1 was the optimal. Hence, the optimum condition to induce the expression of Stx2B in pH6HTN His6HaloTag vector is 4 h incubation period with 1 mM IPTG at 37°C.

The two tag system is one example of tandem affinity purification (TAP) methods which offers an effective and highly specific means to purify target protein, protein capture, quantification, and reduces contaminants significantly (Esposito and Chatterjee, 2006; Puig et al., 2011). To the best of our knowledge, this is the first report of the expression of a Halo-His6 fusion of the Stx2B protein. Despite, there were reports of purification of Stx2B as a fusion protein with glutathione S-transferase (GST) (Mukhopadhyay et al., 2013) or hexahistidine (Madanchi et al., 2011). Block et al. (2009) has reported that protein purified using His6Tag have significantly lower purity, which might be attributed to that fact that endogenous proteins have high affinity to metal ions, which inevitably bind to the resin and co-purify with the fusion protein.

Moreover, Ohana et al. (2009) have compared the Halo tag backbone protein for expression of 23 human proteins in *E. coli* relative to GST and His6Tag revealed that 74% of the proteins were produced in soluble and pure form when fused to HaloTag compared to 39 and 22%, respectively, for the other tags.

One of the obstacles of studying protein-protein interaction is the contamination of the target protein especially with no any prior knowledge. In the present study, the His6HaloTag protein purification was affinity purified using IMAC by use of phosphate buffer. The use of phosphate buffer resulted in unstable pure protein and/or contaminated protein. In this work therefore, the pellets were incubated on ice with APMSF protease inhibitor before sonication to limit proteolytic clipping of the protein of interest. Unfortunately such treatment did not result on successful protein purification and separation. The phosphate buffer produced unstable pure protein and/or contaminated protein or non-purified protein, as all protein appeared in the flow through during purification.

In this work, an experimental purification technique was designed using MOPS buffer instead of phosphate buffer. Such procedures well separated Shiga toxin and higher protein yield was obtained. The superiority of MOPS buffer in purification and separation of Shiga toxin protein, could be explained by the fact that MOPS is a weaker metal ion "stripping" buffer than phosphate when used in the imidazole gradient elution (Hutchens et al., 2009). In contrast Hideyuki et al. (2013) used phosphate buffer in TALON resin for purification Stx2 holotoxin. The discrepancy between the results might be attributed to the use of different experimental design than that used in this study.

To improve the purity and yield of the target protein, further purification step by Halo tag technology coupled with proteolytic tag removal (Ohana et al., 2009) was performed. The final concentration of free tagged Stx2B reached to 450 µg/ml by using Bradford assay. This result was approximately two times higher compared to Halo tag-based purification of PKC γ Kinase free tag protein (Ohana et al., 2011) which produced 244 µg/ml. In the present study, the final concentration of free tagged Stx2B protein suggested the large scale production of Shiga toxin by the method used in this work could be more economic and industry more feasible.

Conclusion

To the best of our knowledge, this study represents the first example of cloning, expression and purification of a Stx2B protein using HaloTag technology in highly pure form. Cloning of the Stx2B was successful in a new technology vector "His₆ Halo dual tags" and led to expression of soluble protein. Interestingly, due to problems that arose in all the steps of purification stage

using phosphate buffer for production contaminated unstable Stx2B protein resulted. This has been overcome and improved by using the MOPS buffer rather than phosphate buffer in all steps of purification stage.

According to the results, Stx2B protein yields obtained in the small scale experiments described in the present study were encouraging. Determination of the immunogenicity or the sensitivity, stability, specificity, accuracy and linearity of the purified Stx2B is still required to prove the usefulness of the application of this product in both developing and developed countries against Shiga toxin type 2B enterohemorrhagic *E. coli* in future prospective.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

The authors gratefully thank 'Abou El-Reesh' Children's Hospital, Cairo University, Egypt staffs' laboratory for their assistance to collect clinical specimens for our study.

REFERENCES

- Acheson DW, Levine MM, Kaper JB, Keusch GT (1996). Protective immunity to Shiga-like Toxin I following oral immunization with Shiga-like Toxin I B subunit-producing *Vibrio cholerae* CVD 103-HgR. *Infect. Immun.* 64:355-657.
- Block H, Maertens B, Spriestersbach A, Brinker N, Kubicek J, Fabis R, Labahn J, Schafer F (2009). Immobilized-metal affinity chromatography (IMAC). A review. *Methods Enzymol.* 463:439-473.
- Boyd B, Richardson S, Garipey J (1991). Serological responses to the B subunit of Shiga-like Toxin 1 and its peptide fragments indicate that the B subunit is a vaccine candidate to counter action of the Toxin. *Infect. Immun.* 59:750-757.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72:248-254.
- Cai K, Gao X, Li T, Hou X, Wang Q, Liu H, Xiao L, Tu W, Liu Y, Shi J, Wang H (2010). Intra-gastric immunization of mice with enterohemorrhagic *Escherichia coli* O157:H7 bacterial ghosts reduces mortality and shedding and induces a Th2-type dominated mixed immune response. *Can. J. Microbiol.* 56:389-398.
- Cai K, Gao X, Li T, Wang Q, Hou X, Tu W, Xiao L, Tian M, Liu Y, Wang H (2011). Enhanced immunogenicity of a novel Stx2Am-Stx1B fusion protein in amice model of enterohemorrhagic *E. coli* O157:H7 infection. *Vaccine* 29:946-952.
- Donohue-Rolfe A, Acheson DW, Kane AV, Keusch GT (1989). Purification of Shiga Toxin and Shiga-like Toxins I and II by receptor analogue affinity chromatography with immobilized P1 glycoprotein and production of cross-reactive monoclonal antibodies. *Infect. Immun.* 57:3888-3893.
- Dubendorff JW, Studier FW (1991). Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *J. Mol. Biol.* 219:45-59.
- Esposito D, Chatterjee DK (2006). Enhancement of soluble protein expression through the use of fusion tags. *Curr. Opin. Biotechnol.* 17:353-358.
- Fujii J, Naito Mo, Yutsudo T, Matsumoto S, Heatherly D, Yamada T, Kobayashi H, Yoshida S, Obrig T (2012). Protection by a Recombinant Mycobacterium bovis Bacillus Calmette-Guerin Vaccine Expressing Shiga Toxin2B Subunit against Shiga Toxin-Producing *E. coli* in Mice. *Clin Vaccine Immunol.* 19(12):1932-1937.
- Gu J, Ning Y, Wang H, Xiao D, Tang B, Luo P, Cheng Y, Jiang M, Li N, Zou Q, Mao X (2011). Vaccination of attenuated EIS-producing *Salmonella* induces protective immunity against enterohemorrhagic *E. coli* in mice. *Vaccine* 29:7395-7403.
- Gu J, Liu Y, Yu S, Wang H, Wang Q, Yi Y, Zhu F, Yu XJ, Zou Q, Mao X (2009). Enterohemorrhagic *E. coli* trivalent recombinant vaccine containing EspA, intimin and Stx2 induces strong humoral immune response and confers protection in mice. *Microbes Infect.* 11:835-841.
- Hanahan D (1983). Studies on transformation of *E. coli* with plasmids. *J. Mol.* 166:557-580.
- Hashimoto H, Mizukoshi K, Nishi M, Kawakita T, Hasui S, Kato Y, Ueno Y (1999). Epidemic of Gastrointestinal Tract Infection Including Hemorrhagic Colitis Attributable to Shiga Toxin 1-producing *Escherichia coli* O118: H2 at a Junior High School in Japan. *Pediatrics* 103(1):e2-e2.
- Hideyuki A, Keiko S, Takeshi S, Kentaro T, Toshiyasu S, Takao T (2013). Large scale production of Shiga Toxin 2 in *E. coli* for toxoid vaccine antigen production. *J. Microb. Immunol.* 57:38-45.
- Hopwood DA (2003). The *Streptomyces* genome-be prepared *Nat. Biotechnol.* 21:505-506.
- Kaper JB, Nataro JP, Mobley HL (2004). Pathogenic *E. coli*. *Nat. Rev. Microbiol.* 2:123-140.
- Hutchens TW, Yip TT (1991). Metal ligand-induced alterations in the surfaces of lactoferrin and transferring probed by interaction with immobilized Cu (II) ions. *J. Chromatogr.* 536:1-15.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Liu L, Zeng H, Luo P, Wu J, Chen H, Shi Y, Zhang W, Mao X, Xiao B, Zou Q (2009). Cloning a Truncated Fragment (stx2a1) of the Shiga-Like Toxin 2A1 Subunit of EHEC O157:H7: Candidate Immunogen for a Subunit Vaccine. *Mol. Biotechnol.* 43(1):8-14.
- Madanchi H, Honari H, Sadraei M, Hesaraki M (2011). Fusion of CtxB with StxB, Cloning and Expression of in *Escherichia coli*: A challenge for Improvement of Immune Response Against StxB. *Iran. J. Pharm. Sci.* 7(3):185-190.
- Marcato P, George M, Randy J, Kathleen V, Patrick N, Glen D (2001). Immunoprophylactic Potential of Cloned Shiga Toxin 2 B Subunit. *J. Infect. Dis.* 183:435-43.
- Mukhopadhyay S, Redler B, Linstedt AD (2013). Adam Shiga Toxin-binding site for host cell receptor GPP130 reveals unexpected divergence in Toxin-trafficking mechanisms. *Mol. Biol. Cell* 24(15):2311-2318.
- Nataro JP, Kaper JB (1998). Diarrheagenic *E. coli*. *Clin. Microbiol. Rev.* 11:142-201.
- Nguyen Y, Sperandio V (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Front. Cell Infect. Microbiol.* 12:2-90.
- Ohana RF, Hurst R, Vidugiriene J, Slater MR, Uhr M, Wood KV (2011). HaloTag-based purification of functional human kinases from mammalian cells. *Protein Express Purif.* 76:154-164.
- Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Uhr M, Wood KV (2009). HaloTag7: a genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. *Protein Express Purif.* 68:110-120.
- Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Séraphin B (2011). The Tandem Affinity Purification (TAP) Method: A General Procedure of Protein Complex Purification. *Methods* 24(3):218-229.
- Sambrook J, Russell DW (2001). *Molecular cloning: a laboratory manual*, 3rd Eds. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Scotland SM, Smith HR, Rowe B (1987). Two distinct Toxins active on Vero cells from *E. coli* O157. *Lancet* 2:885-886.
- Shan G, Lin K, Jinglin W (2010). Expression of overlapping PCR-generated Shiga Toxin B Gene Fragment in *E. coli* and Its Ascitic Polyclonal Antibody. *J Life Sci.* 4(1):1934-7391.
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60-89.

Tu W, Cai K, Gao X, Xiao L, Chen R, Shi J (2009). Improved production of holoToxin Stx2 with biological activities by using a single-promoter vector and an auto-induction expression system. *Protein Express Purif.* 67:169-174.

Wan CS, Zhou Y, Yu Y, Peng LJ, Zhao W, Zheng XL (2011). B-cell epitope KT-12 of enterohemorrhagic *E. coli* O157:H7: a novel peptide vaccine candidate. *Microbiol. Immunol.* 55:247-253.