Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Purification of Shiga-like Toxin from *Escherichia coli* O157: H7 by a Simple Method

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Abstract

Human infection by *Escherichia coli* enterohemorhagic (EHEC) can lead to watery diarrhea, blood flow, or hemolytic uremic syndrome (HUS). This syndrome occurs in 5 to 10 % of patients with *E. coli* O157: H7 infection. Children under the age of 5 years old and the elderly and people with immune deficiency are the most prone to severe complications caused by this pathogen. The entry of this bacteria that has the ability to produce Stx-like toxin causes gastrointestinal symptoms including diarrhea and intestinal mucus. This toxin is a hexamer protein with a molecular weight of 70.5 kDa and is composed of A and B units. The purpose of this study is to purify the Shiga-like toxin, which can be used to provide a diagnostic kit, antibody production and vaccine studies. First, *E. coli* O157: H7 was confirmed by PCR technique and cultured in LB medium. After centrifugation, the cell wall of the bacteria was destroyed by a sonication. Since the toxin is secreted both in the medium and intra-cellular, to increase the concentration of toxin, the precipitate and supernatant were mixed together then the mixture was precipitated with ammonium sulfate salt, it was dialyzed against the salt in a PBS buffer. The presence of toxin was confirmed by SDS-PAGE and Western Blot techniques. In order to confirm the toxicity of protein, supernatant, lysed sediment and a mixture of both were injected into mice groups. In this experiment, the yield of toxin production was 650 µg/ml and the final purity was 90%. Our results demonstrate that Shiga-like toxin (Stx) can be purified without chromatographic methods whit an acceptable purity and yield.

Keywords: *Escherichia coli* O157: H7 ، *Escherichia coli* enterohemorhagic, Atypical Hemolytic Uremic Syndrome, Shiga-like Toxin, Watery Diarrhea

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Submission Date: 8/11/2017
Accepted Date: 10/23/2017

Introduction

Escherichia coli is a Gram-negative bacterial strain of the Enterobacteriaceae family commonly found in the intestine of most warm-blooded animals. Most of the *E. coli* strains are non-pathogenic and intestinal flora. But some serotypes such as *E. coli* O157: H7 cause food poisoning and diarrhea [1]. *Escherichia coli* strains are divided into two groups of extra-intestinal pathogens (EXPEC) and pathogenic diarrhea (DEC). Species that cause gastrointestinal tract infections in humans are classified into 6 groups based on their invasive properties. Enteroaggregation *Escherichia coli* (EAEC), Enterohemorrhagic *Escherichia coli* (EHEC), Enterinvasive *Escherichia coli* (EIEC), Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli* (ETEC) and Diffusely Adherence *Escherichia coli* (DAEC) [2]. EHEC O157: H7 is one of the major pathogens in humans which found in contaminated food and water. Infection of this bacterium occurs in humans as diarrhea, often without bleeding, and in severe cases, in the form of severe inflammation of the intestine accompanied by hemorrhage (hemorrhagic colitis).In some cases, urinary hemorrhagic syndrome (hemolytic uremia) is also observed [3]. Livestock is the main source of this bacterium and is the main source of transmission to humans [4]. Severe degenerative damage caused by enterohaemorrhagic bacterial infection in the host cells is responsible for bloody diarrhea in humans [5]. The infection has been reported worldwide, including Iran. In Iran, contamination of this bacterium is often transmitted through livestock and contaminated meat and milk [6]. Shiga-like toxin (Stx) is one of the most important virulence factors of *Escherichia coli* O157: H7. This toxin is a hexamer protein with a molecular weight of 70.5 kDa and consists of a toxic subunit of StxA and a non-toxic StxB receptor-binding homopentamer. StxB is essential for the entry of the StxA toxic subunit. The StxA has a molecular weight of about 32 kDa, consisting of two subdomains which connected together with a covalent bundle. The first subdomain is called A1, weight about 28 kDa, and the second subdomain called A2, weight about 4 kDa. Two subunits of the stxA are connected through disulfide bonds that the proteolytic cut is necessary for the separation of the two subunits.Stx1 causes depurination (deletion of adenosine) in the 28S RNA subunit of the 60S ribosome. This change makes the tRNA unable to attach to the ribosome leads to inhibition of the protein expression in the target cells [7].

Several purification methods have been used for Shiga-like toxin. Yoshifumi Takeda et al purified Shiga-like toxin by using the ammonium sulfate treatment method, molecular sieve and high performance liquid chromatography [8]. Skinner *et al*., purified the Stx2 using molecular sieve and ion exchange chromatography methods. They used precipitation with ammonium sulfate, ion exchange chromatography, cation exchange and molecular sieve chromatography respectively [9]. The above methods require a long cultivation of poisonous bacteria due to the need to use advanced laboratory equipment, use of multiple and

prolonged steps to purify and yet not reaching the proper concentration of Shiga-like toxin, makes the purification process long and costly. In the method that we used in this study, we tried to achieve a good concentration of Shiga like-toxin with the lowest culture and use of simple laboratory equipment. The purpose of this study is to purify Shiga-like toxin that can be used in some cases, including antibody production, the production of a diagnostic kit, the targeting of cancer cells, the delivery of drugs and vaccines by subunit B of this toxin [10].

Materials and Methods

E. coli O157: H7 bacteria was taken from Imam Hossein biology research center. The medium of Loria Bertoni (LB) and ammonium sulfate salt were prepared from Sigma (Missouri, United States) company, respectively. Dialysis bag, KCl, NaCl, KH_2HPO_4 Na_2HPO_4, Chicken conjugate and DAB color producing substrates were prepared from Sigma Company. Protein markers was prepared from Sinaclon, Tehran, Iran. Female Syrian mice were prepared from Baqiyatallah University of Medical Sciences, Tehran, Iran.

Confirmation of E. coli O157: H7 bacteria

E. coli O157: H7 was cultured in 10 ml of LB broth without antibiotics and incubated at 37°C overnight. To confirm the *E. coli* O157: H7 bacteria, PCR technique was employed using the specific primers *rfbE* and *stx2* genes. To this purpose, the sequences of these two genes were first extracted from the NCBI bioinformatics database. Specific primers for this gene were developed using oligo software (version 7) (Table 1) [11]. *E. coli* O157: H7 strain was cultured in LB broth, and bacterial genome was extracted via the CTAB-NaCl method. The PCR was carried out in a reaction composed of 50 ng/ml of DNA, 4 pM of forward and reverse primers, 0.4 mM of dNTP mix, 3 mM of $MgCl_2$, 1X PCR buffer and 4U Taq DNA polymerase (Cinnagen Tehran, Iran) in a volume of 25 µl. Thermocycler stages set for 94°C for 5 min as initial denaturing and 30 cycles of 94°C (30 sec), 58°C (30 sec) and 72°C (60 sec) respectively for denaturing, annealing and a final 5 min at 72°C. [12]. It should be noted that the number of cycles for replication from the secondary phantom phase to the phase of the expansion of the synthesis sequence was 30 repetitions. 1% Agarose gel was prepared and the sample was electrophoresed [11].

Table 1. The sequence of primers used for PCR detection.

Gene	Primer	Sequences	Size
rfbE	Forward	5′ GTGCTTTTGATATTTTTCCGAGTAC 3′	239 bp
	Reverse	5′ TTTATATCACGAAAACGTGAAATTG 3′	
stx2	Forward	5′ GGCACTGTCTGAAACTGCTCC 3′	255 bp
	Reverse	5′ TCGCCAGTTATCTGACATTCTG 3′	

Toxin extraction

The culture medium containing bacteria was centrifuged at 4°C and 10,000 rpm for 10 minutes. As regards to the fact that the bacteria present in the sediment possess toxin, the sediment was dissolved in 5 ml phosphate buffer and was disrupted with sonication, with 70 Hz in cycle of 0.7 for seven periods and for 30 sec per round. Then the sample was incubate in 37°C for 2 hours to separate the sediment from the supernatant then it was centrifuged at 4°C and 10,000 rpm for 10 minutes and the supernatant was isolated and stored.

Bio assay

SDS-PAGE and western blotting were used to confirm the presence of the toxin. In SDS-PAGE, the samples were examined on 12% polyacrylamide gel and protein purity was evaluated using Bio Doc Analyze software. The western blot method was used to confirm the toxin. Blotting of samples was done on nitrocellulose paper. At first, 30 micrograms of the net sample protein was loaded in 12% polyacrylamide electrophoresis and the BSA protein was used as negative control. The gel was then isolated from the electrophoresis cassette and washed in a blotting buffer containing 25 mM Tris, 192 mM glycine and 20% ethanol. After washing, the gel was packed in western blot sandwich and placed in a western tank. Blotting process was carried out at a voltage of 100 mV for 90 minutes. In order to fill the vacancies (blocking), the paper was kept in a 5% dry milk solution in PBST at 4°C for one night. Then it was washed three times with PBST. The paper was coated with anti-stxB antibody 1:1000 dilution. The washing process was performed with PBST three times. The Anti-Chicken/Bird Anti-IgG Horseradish Peroxidase Conjugated with 1:2000 dilution was used as a diagnostic antibody. As in the previous step, the heating was done. The washing process was carried out as in the previous steps. Finally, nitrocellulose paper was placed in a dye substrate solution of DAB (0.6 mg/ml, 50 mM buffer with pH 8) until a protein band emerged. To stop the reaction, the paper was placed in distilled water. Then paper was checked that the bands are in the desired location [13].

Final purification of toxin

After confirming the presence of the toxin in the supernatant of centrifuged and lysed bacteria by SDS-PAGE and western blotting, the bacteria were lysed and the supernatant mixed together and placed in three 1-liter drops for lyophilization. The lid of the dishes was closed with aluminum foil and kept at -80°C for 24 hours. After 24 hours, the aluminum foil was opened and the specimens were transferred to a lyophilization machine to reduce the sample volume from 900 ml to 80 ml. The sample was digested for 24 hours against the PBS buffer; the dialysis sample was centrifuged at about 4°C for 15 minutes at 10,000 rpm. The precipitate was discarded and the supernatant from centrifugation at 4°C was deposited with 65% saturated ammonium sulfate salt. The sample was kept at 4°C for 24 hours to allow the toxin to precipitate. The sample was centrifuged at 20°C for 20 min at 10,000 rpm, the supernatant was discarded and the precipitate was obtained. The precipitate was dissolved in 3 ml of PBS buffer and dialyzed against PBS buffer for 24 hours then; 0.3 ml of

the sample was boiled and injected into two mice in two steps. Two mice were injected by 0.3 ml of non-boiled sample in two steps [14]. All injections were performed intraperitoneally.

Results

Confirming the bacteria and presence of rfbE and stx2 genes

To confirm the *E. coli* O157: H7 bacteria, PCR technique was used using the specific primers *rfbE* and *stx2* genes. As shown in Fig. 1, two target genes were successfully amplified. They include a 239 bp region of the *rfbE* gene and a 255 bp region of the *stx2* gene.

Protein estimation

At different stages of purification of the Shiga-like toxin, the concentration was measured by the Bradford method. The results are listed in Table 2, separately. Total Concentration of protein after precipitation with 65% saturated ammonium sulfate was 6.5 mg/L.

Toxicity assay

In different stages of the experiment, the toxin was injected into four mice groups to confirm the presence of the protein in the sample and confirm its toxicity. Survival rate of boiled and non-treatment sample of each fraction was 0 and 100%, respectively (Table 3).

Table 2. Estimation of protein content in each step of purification.

Concentration in each step	Volume (ml)	Concentration (µg/ml)	Total Concentration (mg/L)
The supernatant after the first stage of centrifugation	900	140	126
The sediment contains a bacteria lysed after the first stage of centrifugation	20	230	4.6
The mixture of bacterial lysate and the supernatant after lyophilize	80	300	2.4
Lyophilized mixture of lysed bacteria after precipitation with 65% saturated ammonium sulfate	10	650	6.5

Table 3. Confirming protein toxicity and injection into laboratory mice.

Protein injection stage	Sex	Race	Weight (G)	Diluted injected (µg/ml)	Survival rate (Boiled sample) (%)	Survival rate (Non-boiled sample) (%)
Supernatant after the first stage of centrifugation	female	Syrian	18-20	42	100	0
The sediment contains a bacteria lysed after the first stage of centrifugation	female	Syrian	18-20	69	100	0
The mixture of bacteria is leached and Super Nutan after liveliness	female	Syrian	18-20	90	100	0
The supernatant and bacteria lyophyllised mixtures were precipitated with 65% saturated ammonium sulfate	female	Syrian	18-20	195	100	0

Confirming the presence of Shiga-like toxin by SDS-PAGE

After lyophilization and precipitating with 65% saturated ammonium sulfate, SDS-PAGE indicated the presence of a Shiga-like toxin at the desired location on the gel (Fig. 2). Protein purity analyzing using Bio Doc software determined to be more than 90% (585 µg/ml).

Western blotting analysis with anti-Stx chiken antibody

The Stx toxin was confirmed by western blot analysis (Fig. 3). Western blot analysis by anti-Stx antibody, confirmed a single band of native Stx protein.

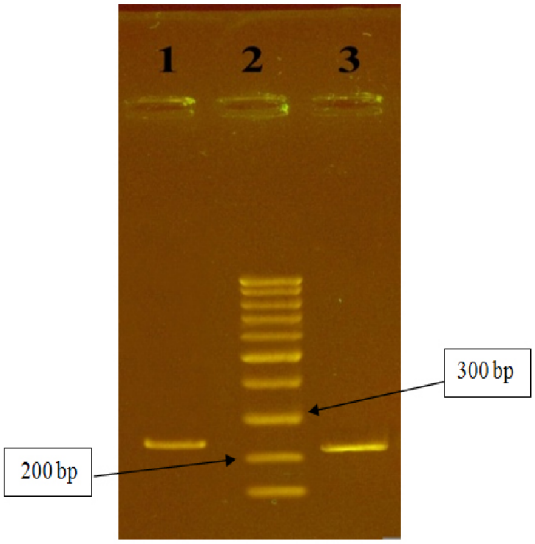

Figure 1. Electrophoretic pattern of PCR amplification of target genes on 1% agarose gel. Lane 1: *stx2* gene PCR product (255 bp) Lane 2: 100 bp DNA Ladder marker, Lane 3: *rfbE* gene PCR product (239 bp).

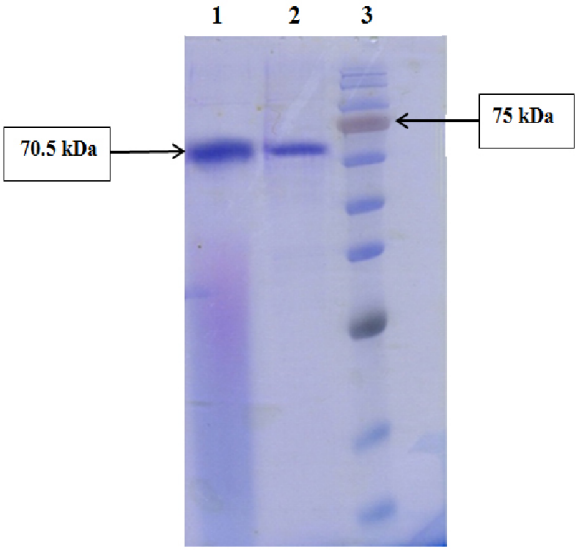

Figure 2. SDS-PAGE analysis of purified Stx protein. Lane 1: Sample after saturation with 65% saturated ammonium sulfate, Lane 2: Sample after lyophilize, Lane 3: Protein marker.

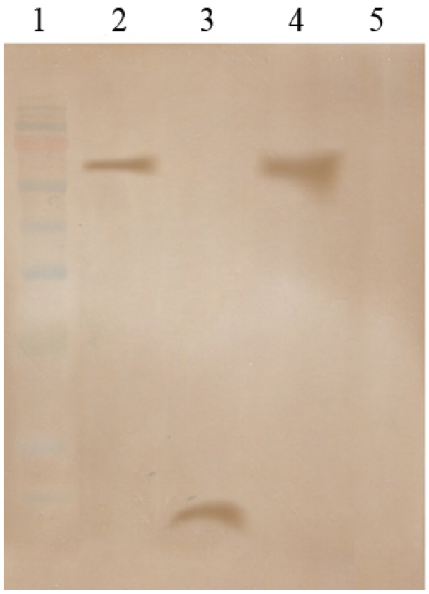

Figure 3.Western blatting analysis of purified Stx protein using anti-StxB antibody. Lane 1: Protein marker. Lane 2: Sample without mercapto ethanol. Lane 3: Sample with mercapto ethanol. Lane 4: Positive sample without mercapto ethanol. Lane 5: Negative sample (BSA).

Discussion

Shigellosis is always known as acute bacilli bloody diarrhea, which is accompanied by watery stools with blood, mucus associated with clinical symptoms such as fever and abdominal cramps that may be accompanied by hemolytic uremic syndrome. Most of the symptoms are due to the production of shiga-like toxins, which can cause neurotoxin and cytotoxic problems [15].

The extraction of Shiga-like toxin can be very useful for use in diagnostic kits, produce antiserum to combat primary poisoning and vaccine candidates. The StxB subunit acts as a StxA subunit translocator. Subunit B can be used as a deliverer medicine, vaccine and chemicals for many reasons, including stability against pH changes, the presence of proteases, the ability to cross the tissue barriers, enter the cells, and resistance to cellular inactivitors [10]. Globotriaosylceramide which is a StxB receptor is affected to be more express by cancer cells such as colon cancer [12, 16], breast and ovarian cancer [17, 18]. These findings suggest that the proprietary StxB binding to Gb3 can be used to target cancer cells. In studies carried out by Urirat Kongmuang *et al*., for 1 liter of *E. coli* O157: H7 culture by use of ammonium sulfate precipitation, 3.2 mg of Shiga-like toxin was obtained [19]. In a work by Hui Wang et al., expression and purification was done using nickel affinity gel column chromatography, 3 mg of Shiga-like toxin by 85% purity was obtained out of 1 liter cultured media [20]. In other research Skinner *et al*., employed anion exchange chromatography, cation exchange chromatography and hydrophobic interaction in which got 5.2 µg/ml of stx2 in 450 ml of *E. coli* O157: H7 culture [9]. In our method the amount of toxin obtained was 6.5 mg for 1 liter of culture, which is almost two times more than the amount that was obtained in the method mentioned. In-

crease of high yield due to bacterial lysis; so extraction of Stx toxin was performed from lysed bacteria, besides secreted form.

Conclusion

Our results demonstrate that Shiga-like toxin (Stx) can be purified without chromatographic methods whit a respected purity and acceptable yield.

Acknowledgments

The authors wish to thank Imam Hossein University for supporting this work.

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