A New Purification Method for Beta-Toxin of *Clostridium perfringens* Type C Vaccinal Strain

Ehsan Zayerzadeh [1*], *Azadeh Fardipour* [2], *Ahmad Reza Jabbari* [3]

[1]*Department of Biology, Faculty of Food Industry and Agriculture, Standard Research Institute, Karaj, IR Iran*

[2]*Department of Vaccine Quality Control, Razi Vaccine and Serum Research Institute, Karaj, IR Iran*

[3]*Department of Anaerobic Bacterial Vaccine Research & Production, Razi Vaccine and Serum Research Institute, Karaj, IR Iran*

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ABSTRACT

***Background*:** *Clostridium perfringens* type C strains cause severe necrotizing enteritis in sheep, calves, goats, pigs and humans. Beta-toxin is introduced to be the essential virulence factor of this microorganism. In the present study, a new method was established for the purification of beta toxin from culture supernatant fluid of *C. perfringens* type C vaccinal strain.

***Methods*:** The four steps of the purification scheme involved ammonium sulfate precipitation, cation exchange chromatography (CM- Sepharose), anoion exchange chromatography (DEAE-Cellulose) and gel filtration (Sephadex G-100).

Results: Beta toxin was purified about 78-fold from the Sephadex G-100 column with a yield of about 16.7% in terms of lethality of the toxin. The molecular weight of the beta toxin as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was approximately 37 KD. The LD50 for adult mice was 2.21 µg/kg. Human umbilical vein endothelial cells (HUVEC) exposed to CPB showed a cell border retraction, cytoplasmic blebbing, cell shrinkage and cell rounding. The toxin was heat labile and was inactivated by trypsin.

Conclusions: In conclusion, the results of this study showed the new protocol is suitable for purification of beta toxin of *C. perfringens* type C regarding good purity, good yields and high activity of beta toxin.

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[*]Corresponding Author: Ehsan Zayerzadeh, Standard Research Institute, Karaj, IR Iran. *Tel*: +98 26 32806031-8, *Email*: zayerzadeh@standard.ac.ir

Introduction

Clostridium perfringens type C is a gram positive, anaerobic, spore-forming pathogen of most mammalian species (1, 2). *C. perfringens* type C strains causes necrotic enteritis in sheep, cattle, pigs, goats and chickens. This disease most frequently occurs in the young animals of these species (2, 3). Beta toxin is secreted by *C. perfringens* type C strains and plays a key role in the lethal outcome of type C strains infections. Beta toxin plays an important role in the early stages of *C. perfringens* type C enteritis in pigs (4). However, this microorganism can produce other toxins, such as alpha toxin, beta2-toxin, enterotoxin, perfringolysin and TpeL (2, 5). Beta toxin is pore-forming toxin which generates potential-dependent, cation-selective channels in membranes of susceptible cells (6). The importance of beta toxin in the pathogenesis of *C. perfringens* type C disease has been documented and several investigators have established the purification method of this toxin (7, 8, 9). Purified beta toxin also is necessary to control quality of commercial enterotoxemia vaccine for doing potency test. This toxin also is urgent for diagnosis of enterotoxemia disease in the field. ELISA test is one of the most important diagnostic tests that need purified beta toxin. Up to our knowledge, this study is the first study about purification and characterization of beta-toxin from *C. perfringens* type C vaccinal strain in Iran. In this study, we introduced a new simplified and economical method for purification of beta toxin that represents a significant improvement over the other procedures used in other laboratories. In the present study some characteristics of purified beta toxin were determined.

Material and method

Bacterial cultivation

Clostridium perfringens type C vaccinal strain (CN 301) was used to purify beta-toxin. The bacterial culture media used throughout the present study included casein hydrolysate (3%), chopped meat (0.8%), yeast extract (0.4%), Nacl (0.25%), glucose (1%), cysteine hydrochloride (0.02%) (Sigma).

Purification of beta-toxin

Following culture of microorganism, 1 liter of culture supernatant was centrifuged at 10,000 *g* for 30 min. Proteins in the culture supernatant were precipitated using 50% ammonium sulfate, with constant stirring, at 4°C for 1 h. The precipitate was then collected by centrifugation at 8,000 *g* for 45 min. The pellet resulting from the 50% saturation ammonium sulfate cut was re-suspended in 30 ml of 20 mM Tris-HCl buffer (pH 6) and dialyzed overnight against the same buffer with several changes at 4°C. After

The dialyzed solution was again centrifuged at 8,000 *g* for 50 min, the supernatant was filtered through a 0.45-µm-pore size filter (EMD Millipore, USA) and loaded onto a CM-Sepharose column (Sigma-Aldrich, USA). This column was pre-equilibrated with 20 mM Tris-HCl buffer (pH 6) After loading of the sample, the CM- sepharose column was washed with 200 ml of 20 mM Tris-HCl buffer (pH 6) and unbound CPB and different anionic proteins were then eluted from the column. Fractions containing the CPB were pooled and dialyzed with ice-cold 20 mM Tris-HCl buffer (pH 7.5) at 4°C overnight. Pooled fractions were then loaded on to a DEAE-Cellulose column (Sigma-Aldrich). The DEAE-Cellulose column was washed with 300 ml of 20 mM Tris-HCl buffer (pH 7.5), and bound CPB was then eluted from the column using a gradient of NaCl (0 to500 mM) in 20mM Tris-HCl buffer (pH 7.5). Fractions containing the CPB from DEAE-cellulose column were pooled and dialyzed with 10 mM Tris-HCl buffer (pH 7.5) at 4°C overnight. Pooled fractions were then loaded on a Sephadex G-100 column (2 by 100 cm), previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Elution of the column was done with the same buffer and five-milliliter fractions were collected.

Lethality assay

The minimum lethal dose (MLD) was determined by intravenously injecting mice (18-20 g) with 0.5 mL of serial dilutions of test samples and monitoring the mice over 72 h for lethality. Doses of beta-toxin were between 0.5 to 5 µg per mouse. Two mice were used for each dose of toxin.

Purity and molecular mass determination of the CPB toxin preparation and western blot

The purity and molecular mass of the beta toxin was analyzed using SDS-PAGE. An SDS-12% polyacrylamide gel was prepared. Following SDS-PAGE, the proteins were electroblotted and transferred from a gel to nitrocellulose membrane. The membrane was then probed using a monoclonal anti-CPB antibody.

Determination of protein concentration

The protein concentration of concentrated crude culture supernatant and target fractions containing beta toxin following purification steps was determined by the method of Lowry *et al* using bovine serum albumin (BSA) as the standard (10).

LD50 determination

Adult male mice (approximately 20 g) were used to determine the 50% lethal dose (LD50) For the beta toxin. The mice were injected intraperitoneally with 0.1 ml of various toxin dilutions and then monitored for 24 h. The time of death was recorded for each animal. Eight mice were used in each group. The LD50 was calculated based on the method of Reed and Muench (11).

Cell culture

HUVEC (human umbilical vein endothelial cells) cell lines were obtained from the National Cell Bank of Iran (NCBI). The cells were cultured in DMEM containing 10% FBS, 2 mM glutamine, antibiotics (penicillin G, 60 mg/L; streptomycin, 100 mg/L; amphotericin B, 50 µL/L) and were maintained at 37C in a humidified atmosphere containing 5% CO.

Microscopy Examination

For light microscopic study, HUVEC cell lines were grown on 24-well plates and incubated with CPB for different time intervals at 37ºC in 5% CO2 incubator. Treated cells and untreated control cells were examined in a phase-contrast microscope and photographed.

Indirect enzyme-linked immunosorbent assay (ELISA)

To determine immunogenicity of beta toxin, the presence of CPB was determined by indirect ELISA. A commercial kit (Cypress diagnostics, Belgium) was used according to the instructions of the manufacturer. The purified CPB sample was diluted into dilution buffer and used to coat the wells of polystyrene plates (100 µl/well). The plate was incubated overnight at room temperature (18-24°C) for 1 hr and then washed two times with washing solution. Then 100 µl of the conjugate solution was added to each well and incubated at room temperature for 1 hr and washed two times with washing solution after which 100 µl of the chromogen solution was added to each well on the plate and incubated 10 minutes at room temperature away from light. Then, 50 µl of stop solution was added to each microwell. Finally, the optical densities in the microwell were read using a plate reader and a 450 nm filter. Comparing the OD of the samples with the OD of positive control.

Effects of temperature and pH

Purified beta toxin was stored at -20, 4, 20, 30, 37, 40, 50, 60, 70, 80 and 100 °C at different time interval (5, 30, 60, 120 min). Samples were examined for loss of cytotoxicity and lethality. Purified beta toxin was diluted in the following different pH buffers and incubated for 3 hr at room temperature and then examined for loss of cytotoxic and lethal activities: 0.02 M glycine hydrochloride (pH 2.0), 0.02 M acetate (pH 4.0), 0.02 M Tris hydrochloride (pH 6), 0.02 M Tris base (pH 8) and 0.02 M Tris base (pH 10.0).

Result

Purification of beta toxin

The purification of beta toxin produced by *C. perfringens* type C was followed by a mouse lethality assay. Only the unbound fraction from the first cation exchange column (CM-Sepharose) showed detectable lethal activity. This showed that the beta toxin was an acidic protein since it did not bind to this column at pH 6. The isoelectric point of beta toxin was later confirmed to approximately 5.5. Analysis of target fraction by SDS-PAGE showed more than 60% contaminating proteins was bound to the column, whereas beta toxin passed through without binding due to its acidic isoelectric point. Thus, a considerable amount of the contaminating proteins were eliminated, which may have removed a potential endogenous inhibitor of the beta-toxin. This step resulted in a 3.7-fold purification with a specific activity 400 MLD/mg. At the second step of purification, fractions from the DEAE-cellulose column was eluted 100 mM NaCl and exhibited toxic activity. Beta toxin was detected throughout the purification steps by SDS-PAGE. This step resulted in a 41.5-fold purification with a specific activity 4444.5 MLD/mg. At the third step, proteins were separated in two peaks. Pure beta toxin was detected in 55 to 72 fractions in the second peak. This step resulted in a 77.8-fold purification with a specific activity 8333.3 MLD/mg (Table 1). LD 50 of beta toxin was also determined approximately 2.21 µg/kg in mice.

Table 1. Summary of purification of beta toxin from *Clostridium perfringens type C*

Step	Activity (MLD)	Protein (mg)	Purification (fold)	Specific activity (MLD/mg)	Yield (%)
Ammonium Sulfate precipitation	6 x 10^3	56	-	107.1	-
CM-Sepharose	4 x 10^3	10	3.7	400	66.6%
DEAE-Cellulose	2 x 10^3	0.45	41.5	4444.5	33.3%
Sephadex-G100	1 x 10^3	0.12	77.8	8333.3	16.7%

Characterization of beta toxin

The preparation of beta toxin which eluted from sephadex-G100 column displayed one major band in SDS-PAGE. The major band had an estimated native molecular weight of 37000 D (Fig. 1). Immunoblot was also done for identification of beta toxin in the crude culture filtrate and purified beta toxin fraction. Monoclonal antibody reacted with beta toxin in concentrated crude culture filtrate and target fraction from sephadex-G100 step (Fig. 2). An indirect ELISA procedure was performed to study the reaction of beta toxin antibody and beta toxin. The response observed with monoclonal antibody as the detecting antibody showed target sample is positive when compared with positive control.

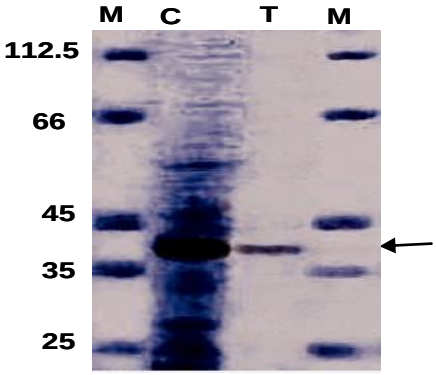


Figure 1. SDS-PAGE of beta toxin purified from C. perfringens type C. Lane M, molecular weight marker; lane C, concentrated crude culture supernatant; lane T, purified beta toxin fraction after purification step 3. The arrow indicates the purified beta toxin.

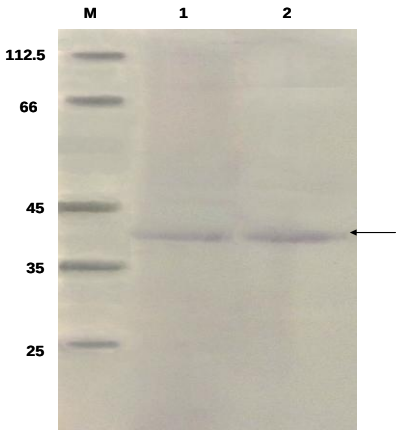
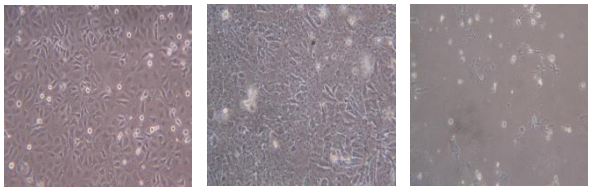


Figure 2. Western blotting of beta toxin. Lane M, molecular weight marker; lane 1, concentrated crude culture supernatant. Lane 2, purified beta toxin fraction after third step purification. The arrow indicates the purified beta toxin.

Morphological effects CPB on HUVEC

HUVEC showed morphological changes when they were exposed to CPB (100 ng/ml). After 1 h of exposure to CPB (100 ng/ml) the cells showed marked cell border retraction, cytoplasmic blebbing, cell shrinkage and cell rounding. Following these morphological changes, there was a decrease in the number of attached cells and cell remnants in suspension. Higher concentrations of CPB (200 to 900 ng/ml) also induced the same effects at shorter times (10 to 50 min; data not shown). These alterations were not observed in non-treated control cells (Figure 3).



A B C

Figure 3. Representative images of human umbilical vascular endothelial cells (HUVEC). A. Normal HUVEC. Images B and C show the induced morphological alterations following treatment with the beta toxin (100 ng/kg) for 30 and 60 min at 37ºC, respectively.

Thermal and PH stability of beta toxin

Purified beta toxin was thermolabile. Biological activity of beta toxin was retained up to 30 ° C. Activity was decreased between 30 ° C to 60 ° C. Finally, lethal activity of beta toxin completely inactivated at 60° C for 1 hour incubation. Beta toxin activity was also decreased at PH 2 and 3. Loss of biological activity of beta toxin was completely observed following treatment with trypsin and pepsin.

Discussion

Clostridium perfringens type C causes enterotoxemias in almost all livestock species and enteritis necroticans in humans which is clinically characterized by severe bloody diarrhea and abdominal pain (5). It is evident that *C. perfringens type C* beta toxin (CPB) is most important and sufficient to induce type C pathologic effects in ileal loop of rabbit (12) Beta toxin and enterotoxin have potential synergistic toxin interactions during *C. perfringens* intestinal infections in some human enteritis necroticans cases (13). Several studies also have proven lethal activity of CPB in mice (14, 15). In the present study here, we purified beta toxin by cation exchange chromatography (CM-Sepharose), anion exchange chromatography (DEAE-Cellulose) and gel filtration (Sephadex-G100). Following this purification experiment, the CPB purification factor, approximately 77.8 was achieved, and this was an increase of approximately 5-fold compared with previous purification strategy (8). This protocol was simple, economic and fast in comparison with previous strategies for purification of CPB. Shatursky *et al* purified beta toxin of *C. perfringens* by column of glutathione Sepharose following complex sample preparation (15). However, this protocol is more expensive than our protocol. In another study beta toxin of *C. perfringens* type C was purified by ammonium sulfate precipitation and DEAE-CL6B Sepharose column (16). This method was also more complex and expensive than our protocol. Previously investigations have reported that molecular weight of beta toxin produced by *C. perfringens* type C was between 30 and 40 KD (7, 8, 15, 16). Our observed molecular weight for purified beta toxin was approximately 37 KD and is similar to previous findings. In the present study, LD50 of purified beta toxin was determined 2.21 µg/kg for mice. LD50 of CPB was previously reported 1.87 µg/kg for mice 7) and lethal amount of CPB was <400 ng/kg for mice (8, Thermal stability studies of CPB demonstrated that CPB is heat labile. In our study, biological activity of CPB was retained up to 30°C. The activity was decreased between 30°C to 60°C. Finally, lethal activity of beta toxin was completely inactivated at 60°C for 1 hr incubation. Previous investigations demonstrated that 90% of lethal activity of CPB was decreased following incubation for one hr at 50°C or 10 min at 100°C. However, our results are compatible with previous studies results (7). The pH stability investigations showed that this toxin has no sensitivity to pH changes. In contrast, in our study, lethal activity of CPB was dramatically decreased following incubation with low pH buffers for 1 h. Biological activity of CPB was also inactivated following pepsin and trypsin treatment. Previous investigators have shown sensitivity of CPB to trypsin and pepsin (9, 15). In the studies on the beta toxin of *C. perfringens* type C, investigators have reported cytotoxic effects of this toxin for HUVEC, HL60, Intestinal 407 and porcine endothelial cells. In the present study, we observed cytotoxic effects of beta toxin including cell shrinkage, retraction of the cell borders and vacuolization on HUVEC cells (6, 17, 18).

Conclusion

Our findings showed good yields and good purity of beta toxin can be prepared relatively using this simplified and economic protocol.

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Conflict of interest

None declared conflicts of interest.

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