Comparison of Two Purification Methods for Beta-Toxin of *Clostridium perfringens* Type B

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ABSTRACT: *Clostridium perfringens is an anaerobic bacterium that among its strains, only types B and C can produce beta-toxin. Beta-toxin plays an important role in many human and animal diseases and has detrimental effects on the intestine. The present study aimed to compare two purification methods for the extraction of beta-toxin from C. perfringens type B. As the first method, beta-toxin was purified using ammonium sulfate precipitation, column chromatography (Sephadex G-25), and ion-exchange chromatography (DEAE Sephadex), while the second method employed affinity chromatography. Hemolysis activity and protein content were measured in each step of purification. The purity of beta-toxin was monitored in each step using SDS-PAGE. According to the results, a comparison of these two methods indicated that the yield of the first method was 82.8%, and the yield of the second method was 90.1%. The specific activity values for the first and second methods were calculated to be 2368.1 U/mg and 164.5 U/mg, respectively. Our results show that affinity chromatography could be used to purify beta-toxin from clostridium perfringens type B with high purity and specific activity (4370 HU/mg).*

KEYWORD: *Clostridium perfringens type B; Beta toxin; Ion exchange chromatography; Affinity chromatography.*

INTRODUCTION

Clostridium perfringens is a rod-shaped, immobile, gram-positive bacterium capable of forming resistant spores to harsh environmental conditions [1, 2]. *C. perfringens* strains produce four major toxins called alpha, beta, epsilon, and iota toxins with lethal and necrotic activity and are divided into five isotypes, namely A, B, C, D, and E [3]. The current study focused on the comparative yield of two purification methods for the extraction of Beta-toxin, which is produced only by types B and C strains [4]. The cellular function of beta-toxin includes initial binding to a receptor located on the plasma membrane of mammalian cells. It has been indicated that the binding of beta-toxin to its cognate receptors activates some intracellular pathways and causes cytopathic effects that eventually lead to cell death [5]. It is known as a major agent for the induction of necrosis and enterotoxemia

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in humans and animals; therefore, proper purification of beta-toxin is of great importance for improving the quality of vaccines against enterotoxemia [6].

Proteins can be purified using various modern and classical methods, such as precipitation with ammonium sulfate or organic solvents, electrophoresis, gel filtration, and various types of chromatography[7]. Chromatographic processes are mostly preferred, and they can be used specifically for binding and removing high-purity proteins [8]. According to different reports, various chromatographic methods have been developed including column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion-exchange chromatography, gel filtration chromatography, high-pressure liquid chromatography, and affinity chromatography[9] Chromatographic methods with columns at high flow rates are preferred in the industry because they can hold samples in place during equilibrium and washing procedures [10]. Specific binding reactions between an analyte of interest in the mobile phase and a binding portion or ligand in the stationary phase is the basis of affinity chromatography[11]. Ion exchange chromatography is a historical technique widely used for the purification of therapeutic proteins [12] by which proteins are separated based on differences in the surface charge of molecules [13].

In 2016, the recombinant beta protein was extracted from *C. perfringens* type B strain using a Ni-NTA column [14]. In 2013, Sayadmanesh *et al.* purified botulinum neurotoxin from *C. botulinum* by affinity chromatography (nickel column) [15]. In 2014, Zaerzade *et al.* combined four methods of ammonium persulfate precipitation, cation chromatography (CM), anion chromatography (DEAE Sephadex), and gel filtration (G-100) for the purification of beta-toxin from *C. perfringens* [16]. In 2020, Najafi et al. purified alpha-toxin from *C. Perfringens* by ammonium sulfate precipitation followed by column chromatography and ion-exchange chromatography [17]. In 2016, Johnson *et al.* purified BoNT/A3 neurotoxin from *C. Botulinum* [18]. Using ion-exchange chromatography. In this study, affinity chromatography was compared with ammonium sulfate precipitation, column chromatography, and ion-exchange chromatography for beta-toxin purification.

EXPERIMENTAL SECTION

Microorganism and growth

C. perfringens type B was taken from Razi Vaccine and Serum Research Institute, Mashhad, Iran. The bacterium was grown in the liver extract medium for 16 h; then, the culture was sub-cultured into peptone proteose medium for toxin production. After incubation for 5-6 hours in anaerobic conditions, the culture was centrifuged, and the supernatant was subjected to purification steps. The hemolysis activity and protein concentration were determined during all steps. The culture supernatant was then aliquoted into two parts to be used in the two purification methods [17]

Hemolysis assay

Hemolysis assay was performed according to the method established by Mollby and Wadstrom [19] with some modifications. The reaction was performed by the addition of 0.2 ml of the culture supernatant obtained in the previous step to 1 ml of red blood cells (RBCs). Then, the mixture was washed by the addition of 20 mM phosphate-buffered saline PBS (pH 6) and then incubated at 37°C for proper time incubation and centrifuged at 3000 rpm for 5 minutes. The optical absorbance of the released hemoglobin was read at a wavelength of 570 nm using a spectrophotometer (Shimadzu).

The positive control was prepared by the addition of 0.2 ml of PBS to 1 m of RBC, and the mixture was incubated at 37°C for 5 minutes. After centrifugation (3000 rpm for 5 minutes), the supernatant was discarded, and 1.2 m of water was added to the pellet. Finally, the optical absorbance of the sample was recorded at a wavelength of 570 nm. The negative control was prepared by the addition of 0.2 ml of PBS to 1 ml RBC, and the resulting mixture was incubated at 37 °C for 5 minutes. After centrifugation (at 3000 rpm for 5 min), the supernatant was used as a baseline correction. Hemolysis activity was defined by the amount of one unit of beta-toxin that can induce RBC lysis and increase one percent of RBC lysis according to positive control per minute under an optimized condition[19].

Protein estimation

Protein concentration was measured with Bradford method [20], in which bovine serum albumin was utilized as standard. In this method, 100 mg of Coomassie Brilliant

Blue G-250 was dissolved in 50 ml of 95% ethanol. Then, 100 ml of 85% (w / v) phosphoric acid was added to the mixture. When the dye was completely dissolved, the volume of the mixture was adjusted to 1 L. Afterward, the standard diagram was plotted against different concentrations of BSA (10, 20, 40, 80, 100, 200, 250 mg/ml). Finally, 25 µl of the sample was added to 125 µl of the Bradford solution, and its optical absorption was read at a wavelength of 595 nm. The protein content of each sample was calculated using the slope of the standard diagram line.

BETA-TOXIN PURIFICATION

First method

The first method for beta-toxin purification was carried out in three steps, namely ammonium sulfate precipitation, column chromatography, and ion-exchange chromatography (the steps are described below). After each step, the protein content and its activity were determined, according to the methods described in previous sections. In addition, the presence of beta-toxin was monitored in each step using SDS-PAGE**.**

Ammonium sulfate precipitation

Solid ammonium sulfate (up to 70% of saturation) was added to the culture supernatant in a cold-water bath and then stirred continuously using a magnetic stirrer at 4 °C. The mixture was kept at 4°C for 24h for better precipitation of the desired protein. The precipitated protein was collected by centrifugation (8000 rpm, 4 °C, 20 min) by dissolving the resulting mixture in Tris buffer (20 mM, pH 7.0).

Gel chromatography

Beta-toxin obtained from the previous step was loaded onto a Sephadex G-25 column (1.2 x 135 cm), pre-equilibrated with 20 mM Tris-HCl (pH 7), at a flow rate of 30 ml/h. Fractions with a volume of 3 ml were collected, and those having high hemolysis activity were pooled and used for ion exchange chromatography [21].

Ion exchange chromatography (DEAE Sephadex)

The partially purified enzyme obtained from Sephadex G-25 chromatography was adsorbed into a DEAE-Sephadex column (2 x 10 cm), pre-equilibrated at pH 7 with 100 mM Tris-buffer. The column was washed with the same buffer until the eluent showed no toxin activity. The bound toxin was eluted with stepwise application in 100 mM Tris-buffer, pH 7 and NaCl gradient ((400, 500, 600, 800, 1000mM). The positive fractions were stored at –20ºC for further use. The purity of toxin was evaluated by SDS-PAGE[22].

Second method

In this method, the culture supernatant was purified using affinity chromatography after determining the protein content and hemolysis activity.

Affinity chromatography

In order to wash lecithin, at first, 1g of lecithin was dissolved in 100 ml of Tris buffer (20 mM, pH=7) and centrifuged at 10000 rpm for 10 minutes. The culture supernatant and washed lecithin (at a ratio of 50:50) in Tris buffer (20 mM, pH7) were kept at 4°C to cool down. After temperature equilibration, the supernatant and lecithin were mixed and incubated at 4ºC for 15 minutes. The mixture was centrifuged at 10000 rpm at 4ºC for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet was washed with the same buffer. The washed pellet was mixed with the same volume of Tris buffer and incubated at 37°C to release beta-toxin from lecithin. After 10 minutes of incubation, the mixture was centrifuged, and the supernatant was collected as purified beta-toxin. The final purified toxin was subjected to SDS-PAGE, protein content calculation, and hemolysis assay.

SDS-PAGE analysis of beta-toxin

The purified toxin was analyzed by SDS-polyacrylamide gel electrophoresis. SDS-PAGE was carried out in 12.5% (w/v) polyacrylamide gel at a pH value of 8.3, according to a study conducted by Laemmli [23]. After electrophoresis (SDS-PAGE), the bands were visualized by the silver staining method. Protein molecular weight markers were used to determine the molecular weight of the desired protein.

RESULTS

Microorganism and Growth

According to SDS-PAGE and cytotoxicity assay, beta-toxin was detected at the early stages of bacterial growth. The cytotoxicity assay indicated that beta-toxin has low

Table 1: Beta-toxin purification conditions.

No	Method	T(C^0)	t (h)	PH	V(ml)
1	Sephadex G-25	25	3.5	6	1
2	DEAE–Sephadex	25	2	6	3
3	Adsorption chromatography	4	1	7	20

Table 2: Purification procedures of beta-toxin by column chromatography (method 1).

Steps	Total activity(U)	Total protein(mg)	Specific activity(U/mg)	Yield(%)	folds
Culture supernatant	92100	560	164.5	100	1
Ammonium sulfate, 70%	91470	488	187.4	99.3	1.1
Sephadex G-25	89340	109	819.7	97.7	4.9
DEAE–Sephadex	75780	32	2368.1	82.8	14.3

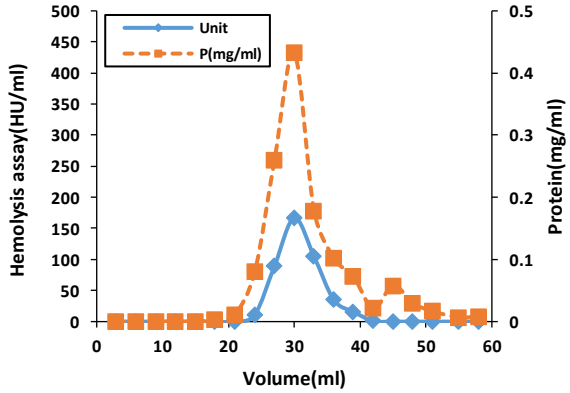

Fig 1: Chromatogram of beta-toxin in sephadex G-25 column.

shelf-life and long-term storage plays an important role in the inactivation of beta-toxin.

Purification of beta-toxin

Comparison of different physicochemical properties between the two applied methods

Purification of beta-toxin was performed by two different methods, and purification conditions were summarized and compared in Table 1.

The first method of purification (combined chromatography)

Beta-toxin was precipitated by ammonium sulfate followed by gel chromatography (Sephadex G-25) to remove low molecular weight proteins and desalting (Fig. 1).

Ion exchange chromatography (DEAE Sephadex) was used to remove some other proteins and determine the specific activity. Finally, a homogeneous beta-toxin was purified with a specific activity of 2370 U/mg, 14.3 folds of purification, and an 82.8 % yield. The results of purification are summarized in Table 2. Proteins purified in each step were analyzed using SDS-PAGE (Fig. 2).

The second purification method (affinity chromatography)

Beta-toxin was purified using affinity chromatography at 4°C by binding of beta-toxin to lecithin as a substrate. In this method, impurities were removed, and beta-toxin was separated from the substrate at 37°C. Finally, homogeneous beta-toxin was purified with a specific activity of 4372 U/mg, 26.5 folds of purification, and a 90.1% yield. Beta-toxin appeared as a single band in SDS-PAGE (Fig. 3). As shown in SDS-PAGE, beta-toxin extracted from *clostridium perfringens* type B was highly purified in one-step affinity chromatography.

DISCUSSIONS

Clostridium perfringens is abundantly found in the environment and is a part of the normal intestinal flora of humans and warm-blooded animals. It can act as a pathogen under specific conditions. Beta-toxin produced by *C. perfringens* type B strain is one of the major causes of intestinal diseases in developed countries [24, 25]. Vaccination seems to be the most effective way to control intestinal clostridiosis[26]. The production and availability of pure toxin are of great importance for measuring the immunogenicity of vaccines as well as conducting highly accurate experiments, such as ELISA and other diagnostic methods. Therefore, the selection of an appropriate

Table 3: Purification steps of homogeneous beta-toxin by one-step affinity chromatography (method 2)

Steps	Total activity(U)	Total protein(mg)	Specific activity(U/mg)	Yield (%)	folds
Culture supernatant	92100	560	164.5	100	1
Affinity chromatography	83050	19	4371.5	90.1	26.5

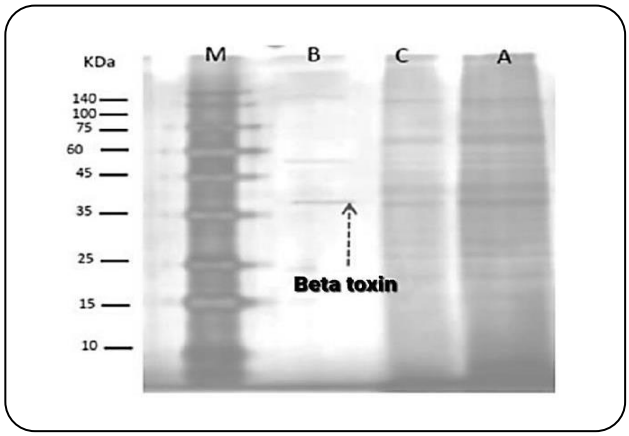

Fig. 2: Gel electrophoresis of purified beta-toxin; A: ammonium sulfate precipitation of the sample, C: G-25 column chromatography sample outflow, B: ion exchange chromatography sample outflow, M: Marker

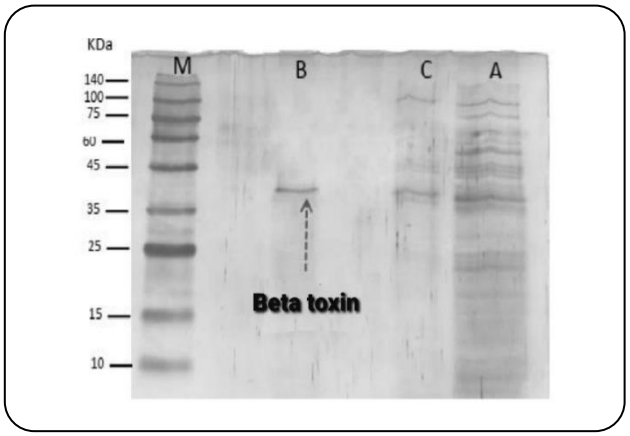

Fig. 3: Analysis of the two purification methods; A: beta-toxin supernatant, B: adsorption chromatography sample outflow C: ion exchange chromatography sample outflow, M: Marker.

purification method plays a significant role in the biological and medicinal fields. Although beta-toxin has been successfully purified in previous studied [27], some purification efforts have not yielded high levels of purity[28]. In this study, a combination of column chromatography and ion-exchange chromatography was used for the first time to purify beta-toxin, and the results were compared with the method that used affinity chromatography in terms of purification steps and time, cost of purification, simplicity and, most importantly, toxin activity. Beta-toxin is known as a major cause of necrosis and enterotoxemia in humans and animals; therefore, proper purification of beta-toxin is of great importance for improving the quality of vaccines against enterotoxemia.

In the first method (ammonium sulfate precipitation, gel chromatography, and ion-exchange chromatography), beta-toxin was purified with a specific activity of 2370 U/mg, whereas in the second method (affinity chromatography), it was purified with a specific activity of 4372 U/mg. These results are consistent with the results of Zaerzade *et al*., who purified beta-toxin from *clostridium perfringens* Type C [16]. Beta-toxin produced by *C. perfringens* type C strain was purified by Sakurai *et al.* using four consecutive purification methods consisting of the addition of ammonium sulfate, gel filtration with Sephadex G-100, isoelectrofocusing at a pH range of 3-6, and immunoaffinity chromatography, leading to 80% purity. In the present study, using gel chromatography (Sephadex G-25), the purity of C. *perfringens* beta-toxin was low, but its purity reached a moderate level when ion-exchange chromatography was applied (Fig 2). Of note, beta-toxin was obtained with high purity when single-step affinity chromatography was used (Fig. 3), which indicated the high efficiency of this method. In a study performed in the Keizo-Asam immunopathology lab in 2004, the yield of immunoaffinity chromatography was reported to be about 24%; thus, it was introduced as a superior method for beta-toxin purification [29]. The percent yield obtained using affinity chromatography in this study was 90.1% (Table 3). Bharti *et al.* (2014) purified beta-toxin by ammonium sulfate precipitation and then used pure cellulose phosphate column chromatography [27]. However, the affinity chromatography method performed in the present study has two main advantages over the method used by Bharti,

such as lower costs and fewer steps as well as the ability to produce toxins at an industrial scale. Sample volume is of crucial importance in the purification process. The sample volume applied in column chromatography is low in comparison with ion-exchange chromatography (Table 1). In the one-step affinity chromatography, the sample volume was 20 times higher than with other types of chromatography, indicating the efficiency of this method in large volume and industrial quantity. In addition, affinity chromatography is cost-effective in terms of material availability and cost. The required purification time is another significant parameter in the purification procedures. According to Table 1, the purification time in affinity chromatography was about one-third of the time required for column chromatography, and one-half of the time was needed for ion-exchange chromatography.

CONCLUSIONS

Affinity chromatography is a cost-effective and efficient method for the purification of beta-toxin produced by *C. perfringens* type B strain in terms of high purity, less time required for purification, and large production volume compared with others methods.

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