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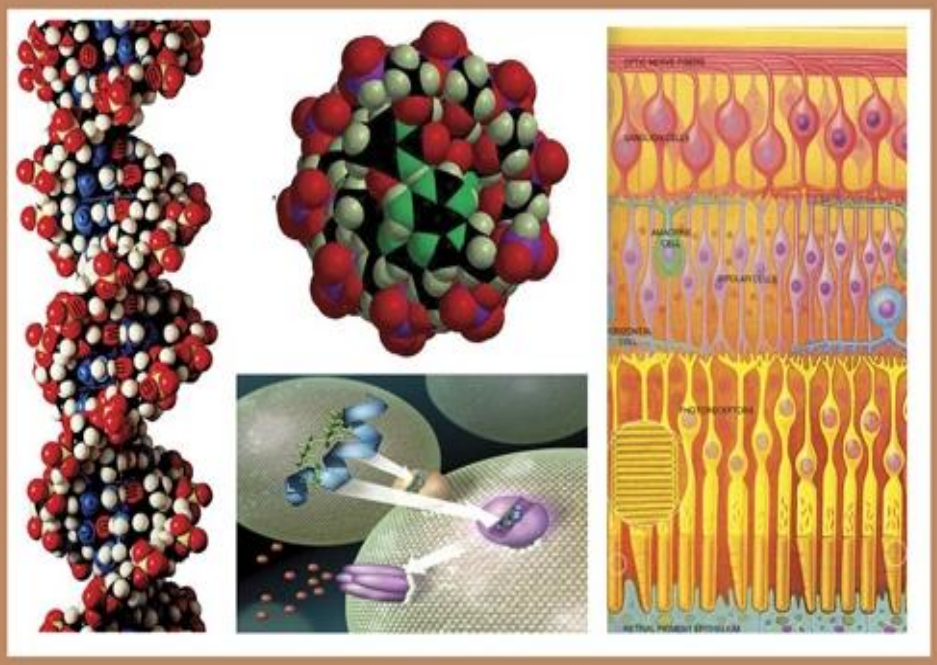

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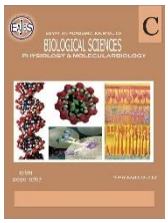

Purification and Characterization of Beta Toxin *of Clostridium perfringens* Type B

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ABSTRACT

Clostridium perfringens is a gram-positive and anaerobic bacterium that is categorized into five types of A-E. Regarding the type of toxins produced, *Clostridium perfringens* is classified into four types; among them, types B and C strains can produce beta-toxin. Beta-toxin plays a critical role in the development of various diseases in humans and animals and can intoxicate epithelial cells in the intestine. Purification of the beta toxin was performed using affinity chromatography and the obtained product was used for injection into rabbits and production of specific antibodies. Finally, the characteristics of the produced pure toxin were measured. Beta-toxin was purified by one-step affinity purification. Beta-toxin was purified to homogeneity with an overall recovery of 90.1% and specific activity of 4371.5 CU/mg. The purified toxin exhibited a molecular weight of ~37KDa, as shown by SDS-PAGE. The Purified beta-toxin was evaluated using standard antibodies by Western blot and ELISA tests. The physicochemical properties of beta-toxin were also determined and optimum pH and temperature values, as well as working buffer for the purification of beta-toxin, were 6, 37°C, and phosphate buffer, respectively. The K_m and V_{max} values of beta-toxin were reported to be 9.907 mg/ml and 0.406, respectively. The purified beta-toxin could be applied for different purposes, such as antibody production and determination of the serum rate of anti-toxin antibodies in animals.

INTRODUCTION

The clostridium genus includes gram-positive and anaerobic bacteria that are capable of producing a diverse range of toxins. Toxins produced by the clostridium family affect the host by different mechanisms. *Clostridium perfringens* is a gram-positive, rod-shaped, immobile bacterium that is able to form spores resistant to environmental factors *et al.,* 2010; Milach *et al.,* 2012). *C. perfringens* strains produce four major toxins called beta, epsilon, alpha, and iota toxins, possessing necrotic and lethal activity, and they are categorized into five types, namely A, B, C, D, and E, according to the type of toxins produced (Mehrvarz *et al.,* 2020; J. Sakurai *et al.,* 2006). Beta-toxin is produced only by types C and B (Bakhshi *et al.,* 2016). The monomeric form of beta-toxin is readily transformed into the oligomeric form in buffer solution. The monomeric form is toxic, whereas the oligomeric form is not, implying that beta-toxin is simply converted from the active into the inactive form *et al.,* 2006).

Moreover, beta-toxin is extremely sensitive to heat and proteinase enzymes. For these reasons, the activity of beta-toxin sharply vanishes from the culture supernatant of the type C strain following the growth period; thus, it would be difficult to purify the toxin from the culture supernatant (Fekete *et al.,* 2015). Sayadmanesh et al. reported that beta toxin could be extracted from *C. perfringens* type B using the Ni-NTA column *et al.,* 2013). The molecular weight of beta-toxin is about 35 kDa, and its gene is located on the virulence plasmid of *C. perfringens* (Gurjar *et al.,* 2010). Studies demonstrated that beta-toxin is necessary for lethal enterotoxaemia caused by type C isolates as well as necrotizing enteritis (Sayeed *et al.,* 2008; Uzal et al., 2009). Steinthorsdottir et al. found that beta-toxin forms an oligomeric complex in the human umbilical vein endothelial cells (Steinthorsdottir *et al.,* 2000). This toxin is able to switch from monomeric to a multimeric form in-vitro (Tweten, 2001). Tweten and colleagues reported that beta toxin could form potential-dependent and cation-selective channels in planar lipid bilayers containing cholesterol and phosphatidylcholine (1:1) (Tweten, 2001). They found that the size of the pore was approximately 12 Å in diameter, denoting that the toxin is a pore-forming protein. Beta-toxin causes disruption and swelling in the human leukemic HL-60 cell line(Nagahama *et al.,* 2003). Beta-toxin extracted from *Clostridium perfringens* Type B, in the form of the vaccine (after conversion of toxin into toxoid), can control infectious diseases; therefore; gaining insight into the properties of the purified beta-toxin could be useful for the production and modification of vaccines as well as other purposes. The current study aimed to purify and evaluate beta-toxin extracted from *Clostridium perfringens* type B for vaccine production and the evaluation of its toxicity.

MATERIALS AND METHODS

Bacterial Culture and Maintenance:

The seeds of *Clostridium perfringens* type B, with the ability to produce high levels of beta-toxin, were gifted by Dr. Fathi-Najafi from the Department of Anaerobic Vaccines-Razi vaccine and serum research institute (Iran). The bacterial cell culture was performed by growing the active bacteria in medium culture supplemented with the liver extracts of sheep at 37°C for 18 h in anaerobic and sterilized conditions. The resulting colonies were stored at 4°C until use. Microbiological features of the strain were determined according to the methods described in Bergey's Manual of Systematic Bacteriology (Rainey, Hollen, & Small, 2009).

Preparation of Toxin:

The seeds of Clostridium perfringens type B were grown according to a method established by Ballard and colleagues (Ballard et al., 1993) with some modifications. The production and purification of beta-toxin were monitored by cytotoxicity and ELISA assays in all steps (Borrmann et al., 1999). Then, the grown cells were harvested by centrifugation at 5000 × g for 10 min at 10°C, and the resulting supernatant was used as a starting point for the purification.

Hemolysis Assay:

The hemolysis assay was carried out in accordance with a method developed by Mollby and Wadstrom(Möllby et al., 1976) with slight modifications. The reaction was initiated by adding 0.2 ml of the culture supernatant to 1 mL of red blood cells. The mixture was washed with the addition of 20 mM phosphate-buffered solution (PBS). The mixture was poured into tubes and then incubated at 37°Cfor 10 min. afterward; the tubes were centrifuged at 3000 × g for 5 min. Finally, the optical absorbance of the released hemoglobin was read at a wavelength of 570 nm using a spectrophotometer (Shimadzu).

Positive Control of The Reaction: 0.2 ml of PBS buffer was centrifuged with 1 ml of RBC at 2000 rpm for 10 minutes, and the resulting precipitate was mixed with 1.2 ml of water. The optical absorbance of the sample was measured at a wavelength of 570 nm.

Negative Control of The Reaction: 0.2 ml

PBS was incubated with 1 ml RBC and then centrifuged at 2000 rpm for 20 minutes at 37 ° C for 5 minutes. Afterward, the optical absorbance of the supernatant was recorded at a wavelength of 570 nm. Then enzymatic activity was calculated using the following formula:

$$\%\text{Hemolysis} = \frac{optical\ absorbance\ of\ the\ sample}{absorbance\ of\ positive\ control} \times 100$$

Hemolysin Activity: The amount of enzyme capable of producing 1% RBC cell lysis per unit time and volume.

$$\text{Hemolysin} = \frac{\text{hemolysis percentage} \times \text{Dilution coefficient}}{Time}$$

Protein Estimation:

The protein concentration was measured based on the Bradford method (Bradford, 1976), in which bovine serum albumin was utilized as a standard.

Toxin purification:

Affinity chromatography:

The supernatant of the bacterial cell culture and washed lecithin (dissolved in 20mM Tris buffer; pH=7) were kept at 4°C to cool down. Following the temperature equilibration, lecithin and the supernatant were mixed and then incubated at 4°C for 15 min. Next, the resulting mixture was centrifuged at 10000 × g at 4°C for 10 min. When centrifugation was terminated, the supernatant was removed, and the resulting precipitate was washed with the same buffer solution. The precipitate was then mixed with the same volume of Tris buffer and incubated at 37°C for 10 min to allow releasing the bacterial toxin from lecithin. Finally, the mixture was centrifuged, and the resulting supernatant was used as a purified toxin. The obtained purified toxin was further analyzed by hemolysis assay, determination of the protein content, and SDS-PAGE.

SDS-PAGE:

The purified beta-toxin was analyzed by the SDS-PAGE technique. SDS-PAGE was conducted according to a method introduced by Laemmli and colleagues(Laemmli, 1970) in 12.5% (w/v) polyacrylamide gel. After electrophoresis, the obtained bands were visualized by the silver staining method (Schirmer *et al.*, 2004).

Determination of Anti-Serum Beta-Toxin:

The ELISA method was used to analyze the presence and concentration of Purified beta-toxin produced against standard antibodies. Beta-toxin purified was assayed in a 96-well flat-bottomed A polyvinyl chlorideplate (Nunc, Denmark). The assay was performed as described by McGregor et al.(1994)(McGregor *et al.*, 1994)

Western Blot:

The western blot technique was applied for the evaluation of beta-toxin purification. At first, SDS-PAGE electrophoresis was performed by running the samples containing beta-toxin on the gel along with a standard molecular weight marker. The nitrocellulose paper was then cut to the size of acrylamide gel and placed in the western blot buffer (Table 1) for 10 minutes with filter paper and sponge layers. In the next step, the grille of the plastic basket specified for transfer was opened, and the western blot sandwich system was prepared, then bubbles between the layers were removed, and the clamps were connected, and finally, the sandwich set was placed inside the western blot tank. The membrane was placed to the positive pole, and the gel was positioned to the negative pole and electrophoresed at 100 volts for 90 minutes. It was placed in an ice tank to keep the electrophoresis system cool.

Table 1: Materials and solutions required to prepare western blot buffer

Materials	Amounts
Glycine	14.4 g
Tris-buffer	2.2 g
SDS	1 g
Methanol*	200 ml

Volume was increased to 1000 ml with distilled water.

After removing the nitrocellulose membrane from the electrophoresis system, the membrane was first transferred to a container on a shaker, and 5 ml of 3% BSA solution was poured on it and incubated for 30 minutes (blocking step). After washing three times with Tris buffer, it was added to the conjugated anti-His-Taq nitrocellulose membrane and incubated again for 30 minutes. Then it was washed three times with Tris buffer, and finally, DAB (Diaminobenzidine) was added to the medium to visualize the protein band.

Characterization of Beta-Toxin:

In order to analyze the physicochemical characteristics of beta-toxin, purified samples of beta-toxin were used, and the effects of pH, buffer solution, temperature, metal ions, and EDTA were assessed. Also, the Km value, as well as the activity and stability of the enzyme, was investigated.

Effect of pH and Buffer Solution on Beta-Toxin Activity:

Sodium acetate buffer at pH values of (4.5 and 5), phosphate buffer at pH values of 5, 6, 7, and Tris buffer at pH values of 6, 7, 8, and 9 at a concentration of 1M were prepared. Then, 920 µl of the substrate (red blood cells) and 200 µl of each of the prepared buffer solutions at a final concentration of 150 mM were added to microtubes. Next, 80 µl of beta-toxin was added to each microtube and incubated at 37°C for 60 min. The optical absorbance of each sample at 570 nm was then recorded using a spectrophotometric device. According to the "hemolysis assay" section mentioned earlier, the enzyme activity in each of the buffer solutions with certain pH values was determined.

Effect of Temperature on Beta-Toxin Activity:

In order to evaluate the stability of the toxin at different temperatures, 200 µl of 150 mM phosphate buffer (pH = 6) and 920 µl of the RBC solution were poured into 11 microtubules, and each microtube was heated to reach 6, 10, 15, 20, 25, 30, 37, 40, 50, 55 and 60 °C, respectively. When the contents of all microtubes reached the temperature equilibrium, 80 µl of the enzyme was added to each microtube. After 1 hour, the reaction stopped, and the amount of enzyme activity at each temperature was calculated and evaluated using the hemolysin test (described earlier).

Effect of Metal Ions on Beta-Toxin Activity:

After considering the three optimal factors (buffer, pH, and temperature) determined in the previous section, the impact of metal ions on the activity of beta-toxin was investigated. First, a 5 mM solution was prepared from each metal ion (Ca, Na, Mn, Ag, Co, Mg, K, Ba, Zn, and Cu) and the sample separately. Under optimal conditions, the hemolysin reaction of the prepared solutions was performed over 60 minutes. After stopping the reaction of hemolysin and centrifugation at 2000 rpm for 3 minutes, the optical absorbance of each sample was measured at a wavelength of 570 nm. The enzyme activity of each solution was determined using calculations related to the hemolysin test.

Effect of EDTA on Toxin:

In order to assess the stability of beta-toxin against a chelating reagent, EDTA, the enzyme was incubated with various concentrations of EDTA (from 1 to 15 mM)

at 37°C for 1 h at a pH value of 8. Finally, the residual activity of the treated beta-toxin was determined by assaying the enzyme under standard assay conditions and compared to control (EDTA-free experiments).

Steady-State Enzyme Kinetics:

Initial rates of the enzyme were measured as a function of the substrate concentration. The initial rate of the reaction was measured at different substrate concentrations, and the kinetic parameters V_{max}, K_{cat}, and Km, were calculated. A concentration of the substrate that results in a rate of $½V_{max}$ is called K_m. the k_{cat} value is defined by dividing V_{max} by [ET], which is a given enzyme concentration.

RESULTS

Bacterial Culture and Maintenance:

According to the cytotoxicity and SDS-PAGE results, the production of beta-toxin was confirmed at the early stages of bacterial growth. Aging is an important factor for the inactivation of beta-toxin. The cytotoxicity assay indicated that beta-toxin has a low shelf life.

Hemolysis:

The hemolysin test was used to quantitatively measure the amount of beta toxin activity. As shown in Figure (1), the amounts of hemolysin isolated from the supernatant of the bacterial culture and purified sample were calculated and evaluated for the positive control over 5 minutes.

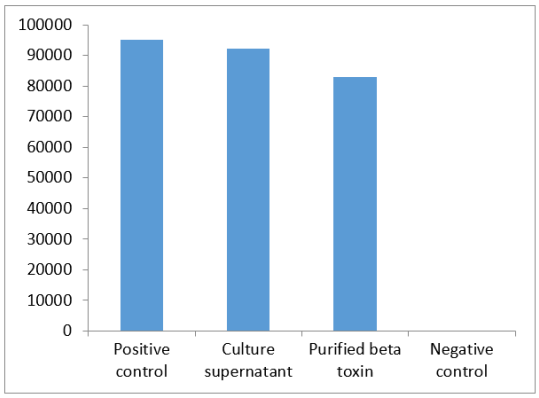

Fig. 1- Based on the formulas presented in the "Materials and Methods" section, the amount of activity obtained from the hemolysin test was measured and compared with positive and negative controls.

Purification of Beta-Toxin:

Beta-toxin was purified by one-step affinity chromatography. In this method, beta-toxin binds to lecithin as a substrate at 4°C, impurities are removed, and beta-toxin is separated from lecithin at 37°C. Finally, a homogeneous toxin was purified with a specific activity of 4372 U/mg at a yield of 90.1% and 26.5 folds of purification. Purification results are summarized in Table 2. Beta-toxin was visualized as a single band in SDS-PAGE (Fig. 2).

The results of SDS-PAGE and Western blot analyses verified the purification steps of beta-toxin extracted from *Clostridium perfringens* type B, as well as the proper production of pure beta toxin as depicted in Figure 2.

Table 2: Purification procedures of beta-toxin.

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

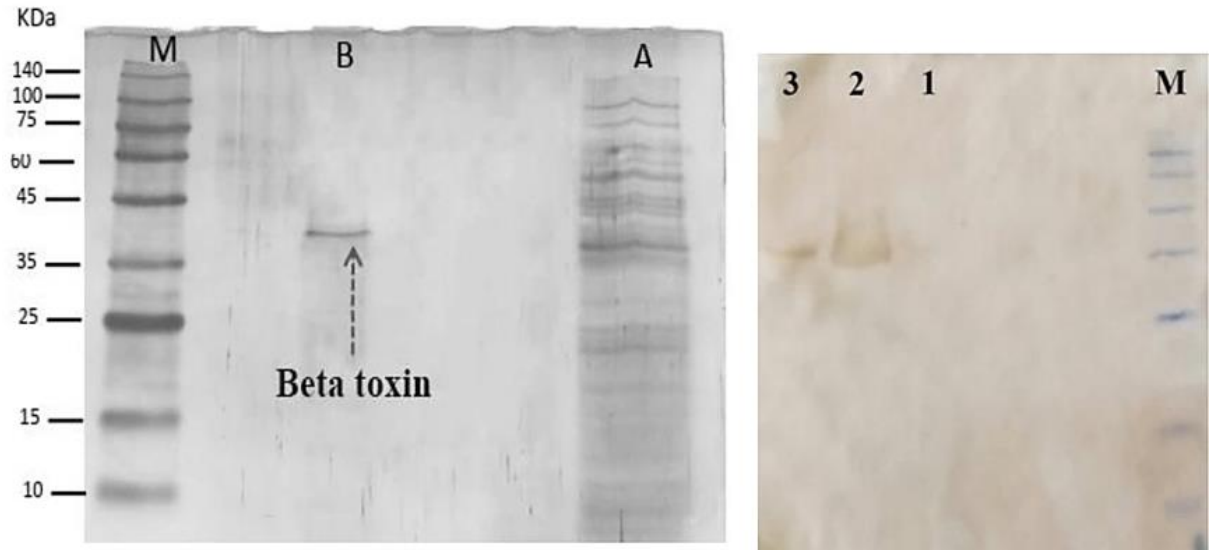

Fig. 2- Left: Analysis of the purification step. A: beta-toxin supernatant, B: Sample outflow from affinity chromatography, M: Marker, Right: Western blot, 1without beta-toxin, 2: positive control of beta-toxin, 3: purified sample of beta-toxin, M: Marker.

Antibody Responses:

According to the results obtained from the ELISA technique, the standard antibodies against beta-toxin showed cross-reaction with the purified form of the protein and are able to detect beta-toxin. The calculated international unit in comparison with standard antibody issued by the European Directorate for the Quality of Medicines & HealthCare (EDQM) was 9.45 IU.

Effect of pH and Buffer Solution on Beta-Toxin Activity:

Three types of buffers solutions at a concentration of 150 mM with different pH values were applied in this study. The comparison of the three buffer solutions determined the most enzyme activity at the most optimal pH value as well as the most appropriate buffer. According to Figure 3, the activity of beta-toxin was higher in phosphate buffer than Tris and sodium acetate buffers. The maximum activity of the enzyme was detected in a pH range of 5-7, and the highest activity was found at a pH value of 6 in 150 mM phosphate buffer.

Effect of Temperature on the Beta-Toxin Activity:

The effect of temperature on the beta-toxin activity at a range of 4-60ºC was also evaluated. As displayed in Figure 4, the maximum enzyme activity was detected at 37°C (80%), while the minimum activity was found at 50° (10%).

Effect of Metal Ions:

The impact of metal ions on the activity of the purified beta-toxin revealed that the barium ion has a 90% inhibitory effect, whereas zinc and copper ions increase the activity of beta-toxin (Fig. 5).

Effect of EDTA on Beta-Toxin Activity:

The results demonstrated that the activity of beta-toxin was almost retained intact in response to different concentrations of EDTA (1-15 mM). Besides, 75% of the initial activity of beta-toxin was maintained even after 1 h incubation with 15 mM EDTA (Fig. 6).

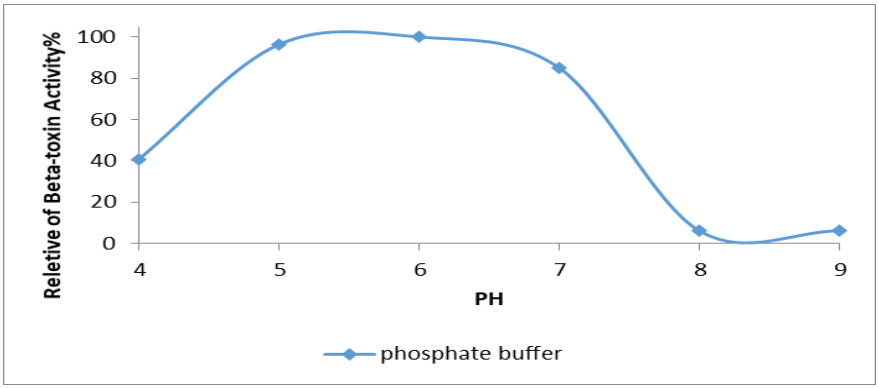

Fig. 3- The effect of the nature of buffer solutions and pH values on beta toxin activity; 1- Sodium acetate buffer at pH values of 4.5-5; 2- Phosphate buffer at pH values of 5, 6, 7; 3- Tris buffer at pH values of 6,7,8,9.

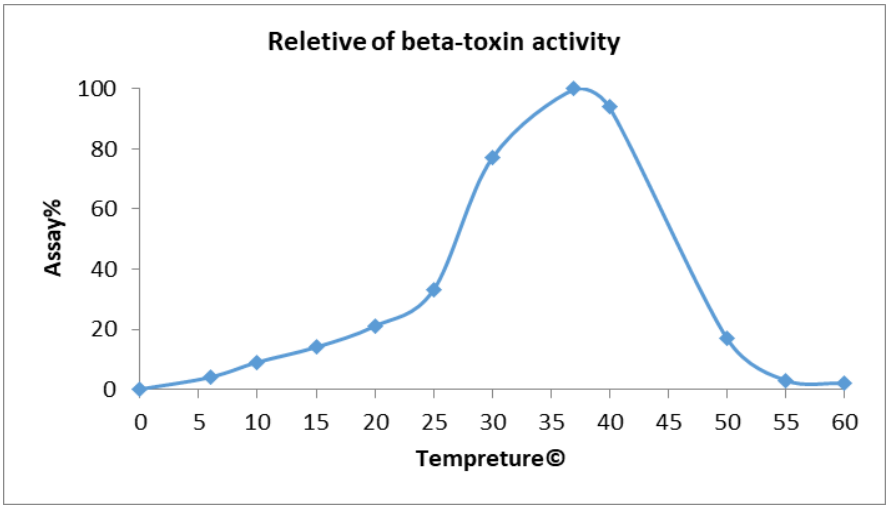

Fig. 4- The impact of temperature on beta-toxin activity.

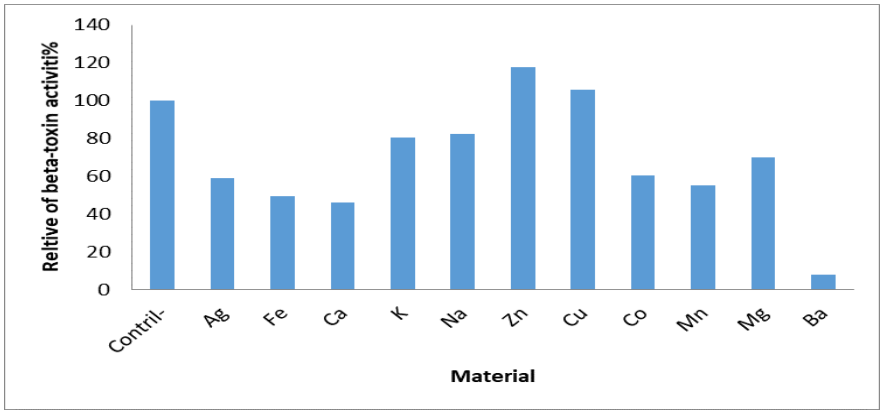

Fig. 5- The effect of metal ions on beta-toxin activity.

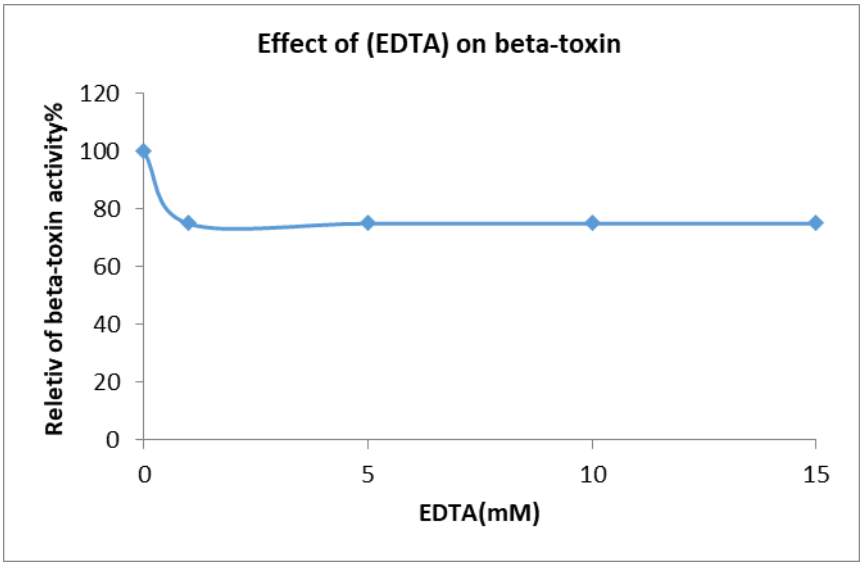

Fig. 6- Effect of different concentrations of EDTA on enzyme activity during 1 h

Steady-State Enzyme Kinetics:

The results indicated that the activity of beta-toxin is increased with an increase in the concentration of the substrate, implying that the activity and kinetics of the enzyme are directly related to the substrate concentration. Upon reaching the concentration of 0.5%, the enzyme exhibits the maximum activity. Of note, a further increase in the concentration of the activity does not result in further enzyme activity at which the diagram becomes a steady-state. The values of V_{max} and Km were reported to be 0.406 and 9.907, respectively.

The conclusion of this research can be seen in Table 3.

Table 3: Properties of beta-toxin *Clostridium perfringens* type B

Properties	Value
SDS-PAGE	37 kDa
Optimum pH	6
Optimum temperature	35-37 °C
Temperature stability	4°C for 1 month, -20 °C for 5 months, in the form of lyophilized for two years
Metal ion requirement	Cu^{2+}, Zn^{2+}
Metal ion Inhibitor	Ba^{2+}, Ca^{2+} ,Fe^{2+} , Mn^{2+}, Co^{2+}, Ag^{+} , Na^{+}, K^{+}
Suitable buffer	phosphate buffer
State kinetic by K_m and V_{max}	9.907 and 0.406

DISCUSSION

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 some conditions. Beta-toxin produced by C. *perfringens* type B strain is one of the major causes of intestinal diseases in developed countries (Gkogka *et al.*, 2020). The most effective way to control intestinal clostridiosis is vaccination (Nijlandr *et al.*, 2007). The availability and production of pure toxin would be necessary for the analysis of immunogenicity and conducting other highly accurate techniques, such as ELISA and diagnostic experiments.

Therefore, the selection of an appropriate purification method would be

requisite. Although beta-toxin has been successfully purified in previous studies (Bhatia *et al.,* 2014), some purification efforts are yet to be made to achieve high levels of purity (Kouguchi, Watanabe, Sagane, & Ohyama, 2001). In this study, the beta toxin was purified using one-step affinity chromatography. The presence of a 37 KDa band in SDS-PAGE denotes the existence of the N-terminal amino acid sequence of the enzyme. The first 27 amino acids of the protein could encode a signal sequence that is responsible for the export of the toxin across the cell membrane of C. *perfringens*. Previous studies have found that the beta-toxin gene actually encodes an approximately 34 kDa protein that is subsequently converted into an approximately 28 kDa protein. In fact, the signal sequence is removed from the N-terminal region of the enzyme, as determined by gel-filtration chromatography or SDS-PAGE (Hunter *et al.,* 1993). The beta toxin was purified with a specific activity of 4372 U/mg. These results are consistent with the results of Zayerzadeh et al., who purified beta-toxin from *clostridium perfringens* type C(Zayerzadeh *et al.,* 2014). However, the method used in our study was more cost-effective and faster than theirs. One of the fastest and most efficient purification procedures is affinity chromatography. The immunization protocol and adjuvant type were the same as those applied in a study performed by Hang and colleagues. Inactivation of alpha-toxin was carried out by formalin in the study of Hang *et al.,* while Ballard and colleagues did not apply formalin for the inactivation of the enzyme (Ballard *et al.,* 1992; Hang'ombe *et al.,* 2005). Indirect ELISA and western blot analyses were performed to analyze the immunogenicity of beta-toxin. For the indirect ELISA, purified antigens were diluted at ratios of 1:10, 1:100, and 1:200 and then used to react with the standard antibody.

Beta-toxin is very unstable when diluted in phosphate buffer or saline or being bubbled, and its activity is rapidly decreased. The inactivation seems to be due to the denaturation of the protein caused by oxidation or being exposed to the gas-liquid interface. In the present study, we have found that the nature of the buffer solution, pH, and temperature have remarkable effects on the toxin activity. The maximum activity was obtained in phosphate buffer (150 mM) at a pH value of 6 at 37ºC and 50ºC. Previous reports have also shown that alpha-toxin Clostridium preifrigense type A has a similar optimum temperature of 45-50°C(Fathi Najafi *et al.,* 2020). These results were in line with the findings of Nagahama et al.(Nagahama *et al.,* 2015). The impact of metal ions on the activity of the purified beta-toxin revealed that the enzyme does not rely on metal ions to be activated, and the presence of some metal ions had only stimulatory effects. In fact, the activity of beta-toxin was decreased in response to 5mM of Ba^{2+} and Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ag^{+}, Na^{+}, K^{+} by 90%, 50%, 40% and 20%, respectively. Contrarily, the presence of zinc and copper ions in the buffer solution increased toxin activity. These findings were consistent with the findings of Najafi et al., who studied alpha-toxin (Fathi Najafi & Biology, 2020).

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