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A New Practical Purification Method for Type D *Clostridium Perfringens* Epsilon Toxin by Size-Exclusion Chromatography (SEC) and Ultrafiltration (UF)

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Running title: Epsilon toxin purification

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Abstract

BACKGROUND: The high potential toxicity of epsilon toxin (Etx) produced by *Clostridium perfringens (C. perfringens)* type D, has made it the third most lethal clostridial toxin behind botulinum and tetanus, therefore, having a pure and concentrated Etx is very important.

OBJECTIVES: The aim of this study was to purify Etx as pure as possible with an applicable, cost-effective, multistep purification protocol with the lowest and shortest time.

METHODS: The purification of the Etx was carried out in multiple consecutive steps; ammonium sulfate precipitation, dialysis, size exclusion chromatography by G_{50}, two concentration steps, and ultrafiltration. The Etx activity after different steps was evaluated by the minimum lethal dose (MLD) calculation, according to the standard operating procedure. Toxin quantification was determined using Lowry technique, and its presence and specificity was tracked to identify pure Etx by SDS- PAGE and western blotting. Finally, the purity of Etx was estimated by capillary electrophoresis.

RESULTS: The purified Etx formed a single band of about 32.9 kDa in SDS-PAGE and blotting. The pure Etx concentration was calculated to be 3.9 mg/ml and its MLD value was the dilution of 1/24000 after the ultrafiltration step. The presented purification processes to purify Etx resulted in ~ 87-fold concentration and 88.6% purity.

CONCLUSIONS: Due to this high Etx purity, the processes used in this study can provide the technical knowledge of toxin production in a larger industrial scale that can be used in development of clostridial toxoid vaccines, as well as quality control and/or diagnostic tests.

KEYWORDS: *Clostridium perfringens*, Concentration, Epsilon toxin, High purity, Purification

Introduction

Clostridium perfringens (*C. perfringens*) is a Gram-positive anaerobic bacterium that is not motile. It is a normal flora (i.e., microbiota) of human and animal digestive tracts, which is dominant in a range of environments, including foods, soils, dusts, feces, sewages, and waste waters (Garcia *et al.*, 2013).

C. perfringens is known to be a life-threatening factor because it usually does not attack to the intestinal tissue directly, but rather, multiplies rapidly in the gut and produces large amounts of potent toxins, which can cause toxemia (Najafi, 2012). It is divided into seven toxinotypes (A, B, C, D, E, F and G) based on its producing toxins (Alpha, Beta, Epsilon, Iota, Enterotoxin, and NetB).

This spore-forming bacterium is able to produce an assortment of other toxins/enzymes. Different studies have shown that *C. perfringens* toxins are involved in the pathogenesis of numerous types of intestinal and/or non-intestinal diseases in most animals (Boarer *et al*., 1988).

C. perfringens type D is one of the most common causes of enterotoxemia in ruminants. Type D enterotoxemia is a major economic and hygienic concern for small ruminant farmers across the world. This is a disease caused by excessive levels of toxins being absorbed from the intestines into the circulatory system (Garcia *et al*., 2013).

Epsilon toxin (Etx) is the most lethal toxin produced by *C. perfringens* type D. It is produced to a lesser extent by toxinotype B, but not other toxinotypes (Nagahama *et al*., 2020). Etx has a greater pathogenic potential than the other *C. perfringens* toxins (Garcia *et al*., 2013) and, it ranks the third most lethal clostridial toxin behind botulinum and tetanus, with an LD_{50} of ~110 ng/kg in mice. It is also considered as a potential biological warfare and bioterrorism agent (Alves *et al*., 2014).

Etx is secreted as a very low-active polypeptide, the epsilon prototoxin (~33 kDa), and becomes fully active by the digestive protease enzymes like trypsin and chymotrypsin, creating the mature Etx (~29-32 kDa) and its toxicity increases up to 100 times than the prototoxin (Najafi, 2012; Zayerzadeh *et al*., 2015).

Since Etx is the main toxin of *C. perfringens* that used to make enterotoxemia vaccine, having pure and concentrated Etx is very important. High purity Etx was also used for different diagnostic as serum neutralization test, immunological assays, and the antibody detection in the immunized animals and/or vaccine quality control tests as potency test (Alves *et al*., 2021).

To date, many attempts have been made to purify Etx, based on single or a combination of several protocols. Precipitation and subsequently purification with different chromatographic techniques is the most common used purification procedure for Etx (Zayerzadeh *et al*., 2015). Ammonium sulfate precipitation is used in many studies to protein precipitation, because of its simplicity and cost-effectiveness (Andler and Steinbüchel, 2017; Habeeb, 1969; Kaushik *et al*., 2019), albeit some disadvantages as the effect of high salt concentrations on the structure of proteins (Alves *et al*., 2021). Methanol precipitation was also used by Verwoerd (1960).

Chromatography is an important techniques for Etx purification, and is usually used as a purification step in almost all studies. The most used chromatographic methods to purify Etx are hydrophobic interaction, ion-exchange, gel filtration, and affinity chromatography. These can be used in single-step (Mathur *et al*., 2010) or a combination of several consecutive steps (Boarer *et al*., 1988; Payne *et al*., 1994; Zayerzadeh *et al*., 2015). The most approach for Etx purification is ion exchange chromatography (Habeeb, 1969). Although the use of ion exchange chromatography

provides acceptable results (Alves *et al*., 2021; Zayerzadeh *et al*., 2015), the effect of buffer type on peak resolution can't be ignored. As well as, unlike gel filtration chromatography, excessive and frequent use of columns in this method may affect the analysis results (Alves *et al*., 2021).

Another way for protein purification is ultrafiltration that can be performed in lab or industrial scales. Ultrafiltration is a pressurized process using semi-permeable membranes that separates sample components based on size and response to a driving force. This separation involves the removal of large amounts of solvent from the sample and the concentration of the particles (Diaz *et al*., 2018).

Until today, the best purified Etx (77-fold purification) was obtained via precipitation and ion exchange chromatography (DEAE-C) (Worthington *et al*., 1973). There are still defects in chromatography purification process to obtain high pure Etx, including the use of two chromatographic steps as ion exchange followed by gel filtration chromatography in the study of Alves et al. (2021), which, although increasing purity, reduces performance.

Furthermore, applying two chromatographic techniques is also not cost-effective. For these reasons, researchers prefer to utilize a good applicable one-step or multistep purification method based on chromatography, filtration, and/or a combination of both (Alves *et al*., 2021; Worthington *et al*., 1973). The most important bottlenecks face by researchers in choosing the best purification procedure, are low mobile phase volume, cost saving, sample structure stability, simplicity, good sensitivity and etc. (Ó'Fágáin *et al*., 2017). The aim of this study was to purify Etx as pure as possible with an applicable, cost-effective, multistep purification protocol with the lowest and shortest time.

Materials and Methods

Ethical statement

All experimental manipulations were performed according to the local institutional guidelines and standard operating producers (SOPs) for the care and use of laboratory animals, Institute of Razi, Iran. This study was carried out under the 3Rs (replacement, reduction and refinement) principles and efforts were made to minimize animal suffering.

C. perfringens culture and toxin preparation

C. perfringens type D vaccinal strain (CN409) was received from Razi Vaccine and Serum Research Institute (RVSRI) and cultivated under anaerobic condition in a specific liquid culture medium to produce Etx, consisted of meat peptone (3%), yeast extract (0.5%), L-cysteine (0.02%), sodium chloride (0.24%), Na_2HPO_4 (1%), Dextrin (1%) and a surreptitiously trace vitamin solution.

Under controlled circumstances and at a constant temperature (37 ˚C), the incubation time was six hours at a pH of 6.5–6.9 (Zayerzadeh *et al*., 2015) in a fermenter system. Quality control of the used strain was performed based on British Pharmacopoeia (Veterinary) 2019 and the Etx production was confirmed by calculation of the Minimum Lethal Dose (MLD), according to a standard SOP (ANB.0024.SOP, RVSRI, Iran). Protein quantification of the crude toxin was determined using the technique of Lowry *et al*. (1951).

Purification

The toxic filtrates used were obtained from 1 liter of culture *C. perfringens* type D. The purification of the Etx was carried out in multiple stages; Precipitation, Dialysis, Size exclusion chromatography (gel filtration by G_{50}), Concentration, and Ultrafiltration.

Protein Precipitation

Following culture of bacterium, the cell debris were separated from supernatant using centrifugation (6000 rpm for 40 minutes at 4 ˚C). Proteins in supernatant were precipitated by adding 500g of 50% ammonium sulfate (AM04011000, Scharlau, Spain) in two steps, stir at room temperature for 2h, and stored at 4 ˚C for 24 hours (Payne *et al*., 1994). Then, the pellet was collected after centrifugation (6000 rpm for 40 minutes at 4 ˚C), resuspended in Tris-Hcl (0.02 M, pH 6.3), and dialyzed by dialysis tubing MWCO 10kDa (Cat Number 71748-3, Millipore) to remove salt. Dialysis was performed against the same buffer for 4 h at 4 ˚C and the sample was collected at the end and kept at -20 ˚C for further purification.

Size exclusion chromatography

Following dialysis, the Etx was purified by size exclusion chromatography (gel filtration with G50). Sephadex G-50 Fine powder (9048-71-9, Sigma) was dissolved in Tris-Hcl buffer (0.02 M, PH 6.3) and packed into the chromatography column (2 x 55 cm). The concentrated sample was loaded on the prepared column and the flow rate was set by the peristaltic pump to 30 ml/hour. Then the column was eluted by the same buffer, and the output fractions (3 ml per tube) were collected. The fractions' optical absorption at 280 nm was measured, and peaks with high Etx concentration were selected and pooled to obtain more amount of pure toxin.

Etx Concentration

The pooled sample passed through a 0.45 µm syringe filter (CHROMAFIL CA-45/25 S, 729025, Macherey-Nagel) to remove particulate impurities and sample filtration. At the next step, Etx was concentrated in two step by a dialysis tubing cellulose membrane (MWCO 10 KDa, D9402, Sigma) with sucrose crystals around it to allow water to flow out for 1 hour. This procedure should be

continued until the sample volume is halved (Noman *et al*., 2006). Then, the concentrated Etx were collected and stored at -20 ˚C.

Etx Ultrafiltration

This procedure is performed to eliminate the smaller proteins and to improve the Etx purity. Amicon® Ultra-4 Centrifugal Filter Unit (NMWL: 30kDa, UFC803008, Millipore) was used since the molecular weight of Etx is around 32 to 34 kDa. The sample was loaded into the tube and centrifuged for 45 minutes at 5000 rpm at 4 ˚C (Boarer *et al*., 1988). The ultra-pure Etx was stored at -70 ˚C for further analysis.

Evaluation of Etx purification

The presence of Etx at different purification steps was confirmed by calculation of the MLD at BALB/c mice (weight 20±2 g, 6-8 weeks). Samples obtained by different purification steps including precipitation, chromatography and ultrafiltration, were first activated by trypsin enzyme (500 µl toxin + 5 mg trypsin), then different dilutions were prepared and injected in tail vein (2 mice per dilution). Finally, mice were followed for 72 hours later to estimate toxin lethality. Toxin quantification was also evaluated using the technique of Lowry *et al*. (1951).

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) method was used to track Etx in unpurified samples and/or to identify pure Etx. SDS-PAGE was performed using the Laemmli method (1970). The coomassie brilliant blue R-250 was used to stain the bands of the chromatographic fractions, and silver nitrate was used to stain the sample bands concentrated by ultrafiltration and the pooled fractions following chromatography (Hochstrasser *et al*., 1988). The specificity of pure Etx was evaluated with 5 IU/ml of the specific sheep Anti-epsilon HP serum (RVSRI, Iran) as the primary antibody and 0.2 µg/mL of the rabbit poly anti-sheep IgG (H&L), HRP (KO211798, Koma Biotech Inc.) as the secondary conjugate antibody by western blotting, according to the standard protocol (Sayeed *et al*., 2005). Bands were detected using DAB substrate and specific bands of the purified Etx were observed.

The purity of Etx was evaluated by capillary electrophoresis using the Agilent 7100 CE (Agilent Technologies, USA), with an ultraviolent- visible (UV–vis) detector at 200 nm. For this work, uncoated fused silica capillaries (50 m ID x 360 m OD x 70 cm length) were utilized. Prior to loading the sample, 100 µl of pure Etx was mixed with 1900 µl of 10 mM acetic acid, and this prepared sample was then analyzed using Agilent ChemStation software.

Results

Toxin Production

Primary culture of *C. perfringens* type D was performed in a specific medium. The Etx expression was improved by consecutive cultures of bacterium with increasing the medium volume, and subsequently in a toxin-producing medium in the fermenter under controlled pH and temperature. In all stages, there was good growth. The purity of cultures were controlled and confirmed by Gram staining smears. The presence of Etx in the supernatant of one liter of culture medium in fermenter was confirmed by IV injection of 0.5 ml of supernatant solution to two mice. Death of animals in this group compared to the control group (normal saline recipient) showed the activity of Etx. Toxin activity was estimated by MLD assay, which it was 1/8000.

Precipitation and gel filtration chromatography

The final volume of solution after precipitation and concentrated dialysis was 30 mL and 20 mL, respectively. After these primary precipitation and purification steps, the total protein concentration and MLD value was estimated to be 50 mg /mL and 1/14000, respectively.

After precipitation and dialysis, column chromatography (Sephadex G-50 gel filtration) was used to purify Etx. The output fractions were collected and evaluated for 280 nm optical absorption of each by spectrophotometer and a chromatogram was plotted (Figure 1).

The protein concentration of chromatographic fractions was measured by Lowry method at a wavelength of 750 nm and theirs proteins banding pattern was examined by vertical electrophoresis of polyacrylamide gel (SDS-PAGE) (Figure 2). According to the electrophoresis results, the fractions containing pure Etx were mixed to obtain a higher concentration of toxin and were used for the next purification step. The protein content of the pooled fractions was estimated at about 2.1 mg/ml and its MLD was determined the dilution of 1/19000.

The purification of culture supernatant by ammonium sulfate precipitation and dialysis, followed by Sephadex G-50 filtration chromatography, resulted in ~ six-fold and ~13-fold concentration of the Etx, respectively.

Concentration and ultrafiltration

The purified Etx was concentrated by two dialysis steps and its purity were improved by ultrafiltration. The volume of the toxin-containing solution was 8 ml and 3.5 ml after the first and second concentration steps, respectively. The final volume of purified Etx after ultrafiltration step was measured 1.5 ml. The protein concentrations were calculated to be 2.4 mg / ml and 3.9 mg / ml after the concentration and the ultrafiltration steps, respectively. The activity of the purified Etx after ultrafiltration was also determined by measuring the MLD in different dilutions and the MLD value was the dilution of 1/24000.

The evaluation of the purified Etx showed an excellent purification after ultrafiltration than G-50 chromatography by SDS-PAGE (Figure 3) and western blotting (Figure 4). Purified Etx appeared as an approximately 33 kDa protein on SDS-PAGE and western blot using sheep anti-epsilon HP serum as primary antibody and anti-sheep IgG (H&L), HRP conjugated secondary antibody. The electrophoretic pattern indicates excellent purification of Etx and its high purity after ultrafiltration. The purity of the purified Etx after ultrafiltration was also measured by capillary electrophoresis (Figure 5), which the highest peak related to the purified Etx with 88.6% purity. The final purification of Etx by ultrafiltration resulted in ~ 87-fold concentration. The purification process of *C. perfringens* Etx was summarized in table.

Discussion

The high potential toxicity of Etx produced by *C. perfringens* type D, has made it as a very important toxin among other clostridial toxins (Alves *et al*., 2014; Garcia *et al*., 2013). It is responsible for the pathogenesis of pulpy kidney disease or enterotoxemia. The production of purified Etx has many application as quality control tests, serum neutralization, and antibody detection in vaccinated animals (Alves *et al*., 2021; Mathur *et al*., 2010), and its industrial purification by ultrafiltration can be used for toxoid vaccine production. With the aim of increasing Etx purification, the current study focused on the procedures of precipitation, chromatography, concentration, and ultrafiltration, eventually reaching a purity of 88.6% of the Etx.

The best strategy used to purify toxins is to use a cost-effective, simple, relatively fast method that yields the maximum purity (Garcia *et al*., 2013; Morcrette *et al*., 2019). Ammonium sulfate precipitation and chromatography techniques are the most common used purification procedure for Etx (Kaushik *et al*., 2019; Najafi, 2012; Zayerzadeh *et al*., 2015). The optimum concentrations of ammonium sulfate for the Etx precipitation are reported to be 50-70% (Kaushik *et al*., 2019; Zayerzadeh *et al*., 2015). In this study, a concentration of 50% ammonium sulfate was used, because due to the sensitivity and high degradability of Etx, high concentrations of ammonium sulfate can be led to its degradation (Alves *et al*., 2021). The amount of total protein after precipitation (50 mg) indicates that the concentration of ammonium sulfate is appropriate.

Various chromatographic techniques are commonly used for the Etx purification in almost all studies (Singh *et al*., 2020; Zayerzadeh *et al*., 2015). In some studies, two chromatographic techniques was utilized to improve the purity of Etx, which, in addition to decreasing yield, increases the danger of protein breakdown and reduces its effectiveness. For example, it was found that the use of ion exchange and gel filtration chromatography, as well as anion exchange repeat chromatography, did not provide any obvious advantage compared to single-step chromatography

(Alves *et al*., 2021). This result was observed in the study of Zayerzadeh *et al*. (2015), which also used cation exchange chromatography followed by gel filtration, and in Habib's research (1969), which applied two ion exchange chromatography steps. Therefore, it seems that using a chromatographic step not only saves time and money, but also provides superior outcomes in lab scale purification. In the present study, single-step gel filtration chromatography was used. Following that, the fractions with the highest concentration of Etx were pooled together based on the SDS-PAGE result to obtain a higher concentration of Etx.

An important advantage of gel filtration is that the parameters may be adjusted without affecting the separation depending on the kind of sample or the requirement for additional purification, analysis, or storage conditions (Ó'Fágáin *et al*., 2017). Because molecules do not interact to the chromatographic medium in the same way that they do in ion exchange or affinity chromatography, the buffer content has no direct influence on the degree of separation between the peaks. This is probably why the purification factor in the study of Worthington *et al*. (1973), who used an ion exchange chromatography step after precipitation with ammonium sulfate, was 77-fold, whereas in this study and following gel filtration and ultrafiltration, the Etx was purified 87-fold. Capillary electrophoresis results indicate this level of purity.

In the present study, by performing a single-step chromatography and an additional ultrafiltration step, the final yield of Etx was 20.31%. In the study of Zayerzadeh *et al*. (2015), despite one more ion exchange chromatography step, the final yield was 16.7%. On the other hand, because the chromatography was performed in one step, the toxin degradation was largely prevented following a reduction in the purification time. The type of usage and the final objective of purification determine how each purification procedure is implemented (Alves *et al*., 2021). The final yield in the Boarer *et al*. (1988) study after anionic chromatography and ultrafiltration was reported to be 0.331 mg/ml (Boarer *et al*., 1988), while in the present study it was 3.9 mg/ml, which indicates the high efficiency of the method used. As a result, it seems that in similar conditions, performing gel filtration chromatography compared to ion exchange chromatography greatly increases the yield of the Etx.

According to previous studies, the inactive form (protoxin) of Etx weighs around 33 kDa, whereas the fully active form weighs around 29 kDa (Bokori *et al*., 2011; Payne and Oyston, 1997). Based on the current gel electrophoresis results, the molecular weight of the purified Etx in this study was about 32.9 kDa, which corresponded to the molecular weight of the studies using different chromatographic methods (Nagahama *et al*., 2020; Najafi, 2012; Payne *et al*., 1994), so the type of purification method has no significant effect on the molecular weight of the resulting pure Etx.

Ultrafiltration method was used to concentrate and to purify Etx. This method, which is based on the separation of compounds based on particle size, allows smaller particles to pass while particles larger than the membrane pores are preserved (Diaz *et al*., 2018). Since the molecular weight of Etx is between 32 and 34 kDa (Najafi, 2012; Zayerzadeh *et al*., 2015), in this study 30 kDa ultrafiltration membrane was used. In the most of the previous studies (Alves *et al*., 2021; Payne *et al*., 1994; Zayerzadeh *et al*., 2015), preparation of pure toxin were considered, while in the current study, as pure as possible, in addition to the high purity of the toxin (which is the main goal), the attainment of a concentrated toxin is also considered. Therefore, it is necessary to concentrate toxin after the purification steps to obtain a higher amount of pure Etx.

Conclusions

This study will provide technical knowledge on the production and purification of Etx with high concentration from type D *C. perfringens*. This pure Etx in industrial production scale by ultrafiltration systems can be used in development of clostridial toxoid vaccines, as well as quality control and/or diagnostic tests.

Conflict of Interests: The author declares that he has no conflict of interest.

Article Responsibilities: The scientific and ethical responsibility of the article belongs to its authors.

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یک روش کاربردی نوین برای تخلیص توکسین اپسیلون باکتری کلستریدیوم پرفرنژنس تیپ D، توسط کروماتوگرافی اندازه طردی (SEC) و اولترافیلتراسیون (UF)

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نویسنده مسئول

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چکیده

زمینه مطالعه: سمیت بالقوه بالای توکسین اپسیلون تولید شده توسط باکتری کلستریدیوم پر فرنژنس (Clostridium perfringens) تیپ D، آن را به سومین سم کشنده کلستریدیال پس از بوتولینوم و کزاز تبدیل کرده است، بنابراین داشتن توکسین خالص و غلیظ اهمیت زیادی دارد.

هدف: هدف از این مطالعه خالص سازی توکسین اپسیلون تا حد ممکن با اجرای پروتکل های کاربردی، مقرون به صرفه، چند مرحله ای و در کمترین زمان بود.

روش کار: تصفیه توکسین اپسیلون در چندین مرحله متوالی انجام شد; رسوب سولفات آمونیوم، دیالیز، کروماتوگرافی اندازه طردی با استفاده از ستون Sephadex-G50 ، دو مرحله تغلیظ و اولترافیلتراسیون. فعالیت توکسین اپسیلون پس از مراحل مختلف با توجه به روش عملیاتی استاندارد با محاسبه حداقل دوز کشنده (MLD)

تعیین شد. میزان توکسین با استفاده از روش Lowry و حضور و اختصاصیت آن نیز توسط SDS-PAGE و وسترن بلات پیگیری شد. در نهایت، ارزیابی خلوص توکسین اپسیلون با اجرای الکتروفورز مویرگی صورت گرفت.

نتایج: توکسین اپسیلون خالص یک باند واحد، در حدود 32.9 کیلو دالتون در SDS-PAGE و وسترن بلات تشکیل داد. غلظت خالص توکسین اپسیلون، 3.9 mg/ml محاسبه شد و میزان MLD آن، رقت 1/24000 پس از مرحله اولترافیلتراسیون بود. فرآیندهای تصفیه ارائه شده جهت تخلیص توکسین، منجر به تغلیظ آن تا 87 fold و خلوص 88.6 درصد شد.

نتیجه گیری نهایی: با توجه به خلوص بالای توکسین اپسیلون، فرایند های مورد استفاده در این مطالعه می تواند دانش فنی تولید توکسین در مقیاس صنعتی را فراهم کند که می تواند در تولید واکسن توکسوئید کلستریدیدیال، همچنین کنترل کیفیت و یا آزمایشات تشخیصی استفاده شود.

کلمات کلیدی: کلستریدیوم پرفرنژنس، توکسین اپسیلون، تخلیص، تغلیظ، خلوص بالا

Table: Summary of the purification process of the Etx from *C.perfringens* type D strain CN409

Step	Volume (mL)	Protein (mg/ml)	Total protein (mg)	Activity (MLD)	Specific activity (MLDs/mg)[a]	Purification (fold)[b]	Yield (%)[c]	Efficiency[d]
Culture medium supernatant	100	1.7	170	8000	47	1	100	1
Precipitation	20	2.5	50	14000	280	5.96	88.24	5.26
G50 Chromatography	15	2.1	31.5	19000	603	12.83	92.67	11.89
Ultrafiltration	1.5	3.9	5.85	24000	4102	87.28	20.31	17.73

[a] Specific activity (MLD/mg) = Activity (MLDs)/ mg of total protein

[b] Purification (fold) = final specific activity / initial specific activity

[c] Yield (%) = (final Total Toxin / initial Total Toxin) × 100

[d] Efficiency = (Purification (fold) × Yield (%))/100

Figure Captions:

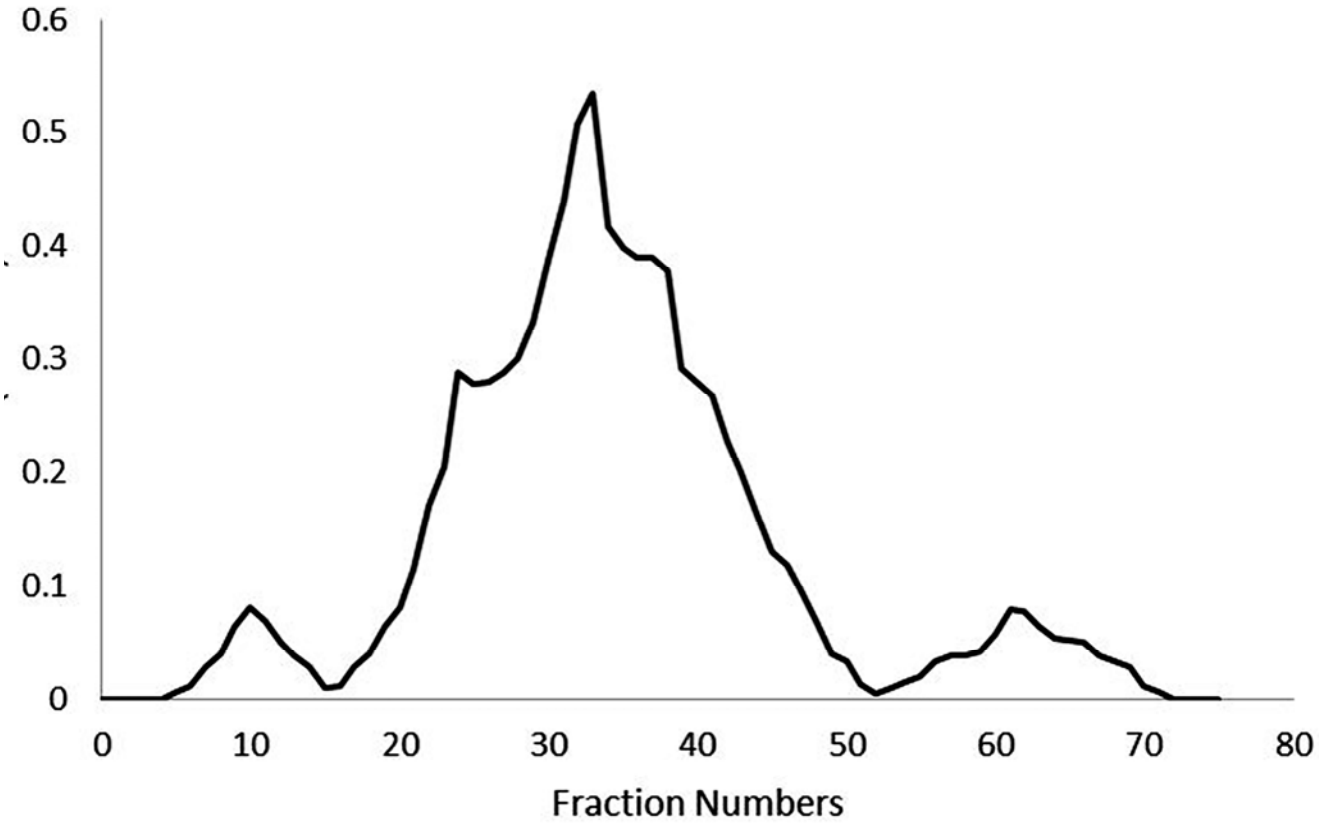
Figure 1: Chromatogram of gel filtration chromatography with Sephadex G-50

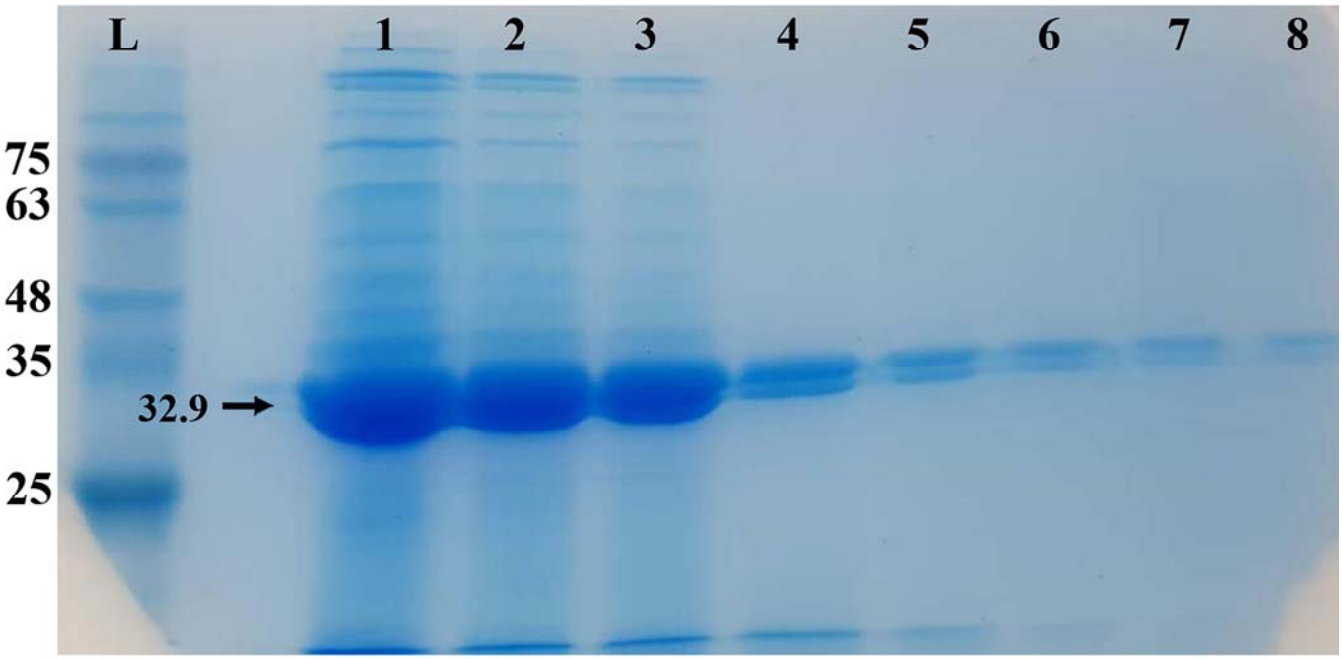
Figure 2: Vertical polyacrylamide gel electrophoresis (SDS-PAGE) on some of fractions after column chromatography with Sephadex G-50 gel and coomassie blue staining. Lane L: The protein size marker; Lane 1: The crude culture supernatant containing Etx after precipitation, prior to chromatography; Lanes 1-8: Some of the fractions after chromatography that show the relative purified Etx. Note the purified Etx in lanes 4-8, which weighing about 32.9 kDa and indicated by the arrow.

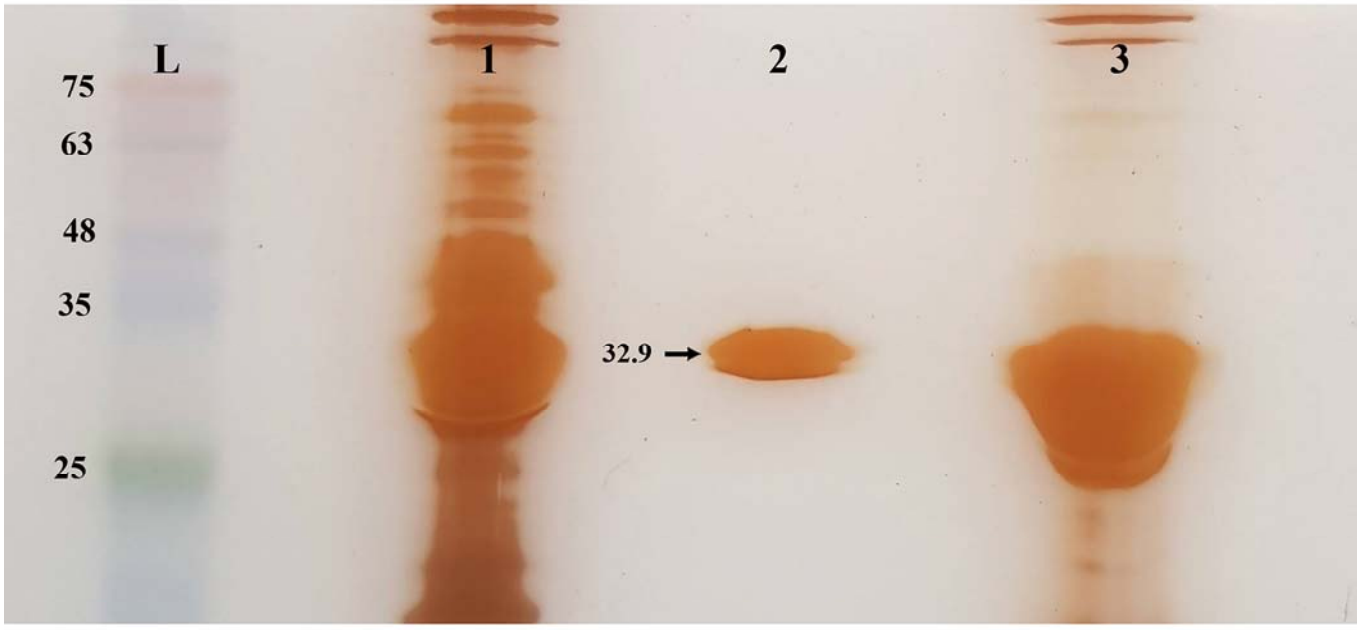
Figure 3: The vertical polyacrylamide gel electrophoresis (SDS-PAGE) of pooled fractions containing purified Etx after chromatography with Sephadex G-50 and ultrafiltration with Amicon® Ultra-4 Centrifugal Filter staining with the silver nitrate. Lane L: The protein size marker; Lane 1: The crude culture supernatant containing Etx after precipitation, prior to chromatography; Lane 2: High pure Etx after ultrafiltration; Lane 3: The purified Etx after G50 column chromatography. The pure Etx weighing about 32.9 kDa is indicated by arrow.

Figure 4: Western blot analysis of the purified Etx after chromatography and ultrafiltration. Lane L: The protein size marker; Lane 1: The purified Etx after G50 column chromatography; Lane 2: High pure Etx after ultrafiltration. The pure Etx weighing is about 32.9 kDa.

Figure 5: Graph of the purity of the purified Etx by capillary electrophoresis technique. The highest peak based on the relative passage time of soluble components from the detector is related to the purified Etx with 88.6% of purity.







495