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Alpha Toxin Purification and Antibody Production Against Local Strain of *Clostridium septicum* NH2

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Abstract

BACKGROUND: *Clostridium septicum* has played a significant role as a causative agent of many acute fetal diseases in man and animals. Alpha- toxin is the main factor in the pathogenesis of *C. septicum* with hemolytic, necrotic and lethal activities.

OBJECTIVES: The study was designed to evaluate alpha-toxin purification and antibody production rate against a local strain of *C. septicum* NH2 which could be applied in diagnosing kits, potency test of the vaccines, and other related applications.

METHODS: Local strain of *C. septicum* NH2 was cultured in liver broth. Alpha-toxin in supernatant purified by three steps: the first step was done by 25% and 60% of ammoniums sulfate precipitation and continued by DEAE-Sephadex ion exchange chromatography, and finally finished in gel filtration on Sephadex G-50. Alpha-toxin was assayed in all steps and purification procedures were analyzed by SDS-PAGE. After immunization of rabbits with alpha- toxin and serum collection, immunoglobulin was separated by three purifying steps: ammoniums sulfate, ion exchange chromatography, and gel filtration. Serum purification process was evaluated by electrophoresis, double immunodiffusion (DID), single radial immunodiffusion (SRID), western blot, and SDS-PAGE.

RESUTLS: SDS-PAGE results showed the alpha-toxin and anti-alpha-toxin were purified partially. Double immunodiffusion and single radial immunodiffusion methods detected the specific antibody. Heavy and light chains of anti-alpha-toxin separated by 2ME in electrophoresis reacted with 48 kDa alpha-toxin during the western blot without any reaction to other proteins in nitrocellulose paper.

CONCLUSIONS: The present study showed a modified protocol for *C. septicum* alpha-toxin and anti-alpha-toxin production. The purification method is more economical and faster than previously reported procedures, and anti-alpha-toxin production is an advantage in detection of *C. septicum* infection.

KEYWORDS: alpha-toxin, anti-alpha-toxin, *Clostridium septicum*, polyclonal antibody, purification

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Introduction

Clostridium septicum, an anaerobic gram-positive rod-shaped bacteria has played a significant role as a causative agent of many acute diseases in man and animals. One of the acute fetal infections of the bacterium is malignant edema or gas gangrene in all kinds of animals and humans (Chakravorty et al., 2015, de Assis et al., 2012, Pilehchian Langroudi, 2015, Thachil et al., 2012).

C. septicum produces several extracellular toxins that include alpha-toxin, beta-toxin (DNase), gamma-toxin (hyaluronidase), and delta-toxin (thiol-activated toxin), protease and neuraminidase (Ballard et al.,1992). Alpha- toxin, the main tool in the pathogenesis of *C. septicum*, is a hemolysin, necrotizing and lethal factor (Ballard et al.,1991, Bozorgkhoo et al., 2014, Lancto et al., 2014).

Alpha-toxin is secreted as a pro-toxin that needs to be activated by a proteolytic cut in the carboxyl-terminal, and connects the cell membrane by glycosylphosphatidylinositol receptors (GPI) (Gordon et al.,1999). A protease can release a 4.1 kDa peptide. Then the active form of the alpha-toxin with seven monomers is formed which makes the pore in the membrane, disturbs the ion balance of the cell, and causes cell lysis. Some proteases like trypsin, chymotrypsin, *C. septicum* secreted proteases, and furin (on the surface of eukaryotic cells) can activate alpha-toxin (Hang'ombe et al.,2007). Some commercial Elisa kits have been developed for identification of *C. septicum* (Thachil et al.,2013) but none of them are specific enough for alpha-toxin detection. It is important to develop a short procedure to produce alpha-toxin as the unique known lethal factor of *C. septicum* and its specific antibody (Vazquez-Iglesias et al.,2017). In our previous work (2006), ten strains of *C. septicum* were identified in Razi Vaccine and Serum Research Institute of Mashhad-Iran with alpha-toxin production capability. This study was designed to purify alpha-toxin of *C. septicum* and the antibody which could be used in diagnosis kits, potency tests of the vaccines, and other related applications.

Material and Methods:

Preparation of Bacteria: *C. septicum* strains NH2 (NCBI: Eu482189) were isolated and characterized previously from sheep dung (Hemmaty et al., 2005, Hemmaty et al., 2006). This strain shows high alpha-toxin production in comparison to other strains in our previous work.

Alpha-toxin production: Five ml of freshly prepared *C. septicum* strains NH2 was inoculated in 500 ml of liver broth (liver powder 1 g, peptone 15 g, glucose10 g, NaCl 1.25 g, pH =7.3) and incubated for 24 h at 37 °C in an anaerobic jar with gas pack type A (Merck). Biomass of the culture was removed by centrifugation at 5000 rpm for 15 min at 4 °C. The supernatant containing toxins was stored at -20 °C for further use.

Purification and characterization of alpha-toxin: All purification steps were conducted at 4°C (Jonson and Ryden1989). This method was chosen because it is one of the most temperature sensitive toxins. The first purification step was done by adding 25 percent ammonium sulfate and spinning at 5000 rpm for 15 min. Ammonium sulfate up to 60% was added to the supernatant followed by centrifugation at 5000 rpm for 15 min. The pellet was dissolved in 20 ml of 50 mM Tris buffer (pH= 7.4) (using Tris, dH2O, HCl). The solution was dialyzed in 50 mM

Tris buffer with pH= 9.5 overnight (dialysis bag cut off 25 KDa) for removing ammonium sulfate residues and to change the buffer.

In the next step, DEAE-Sephadex (Diethylaminoethyl Sephadex, Sigma) was used for ion-exchange chromatography (Adiguzel et al., 2015, Sato et al., 2012). Three ml of the last step's prepared protein solution was passed from the column after equilibration with 50 mM Tris buffer (pH=9.5) for 15-20 min. Then the proteins were eluted with different concentrations of 5 ml NaCl from 0.1-0.6 M with 0.1 M intervals. Hemolysin and protein assays were performed on each fraction by hemolysis method and Bradford method respectively. The pool fractions of alpha-toxin were used for the next step. One percent of the previous fraction with higher hemolysis activity was applied to gel filtration chromatography Sephadex G-50 (100 cm ×1 cm) with a flow rate of 0.2 ml/ min. The samples were collected by a fraction collector and the optical density of the fractions was measured at 280 nm. Each sample was tested for hemolysin activity with rabbit RBC.

The purification process was analyzed by SDS-PAGE electrophoresis. Two gel concentrations, stacking, and separating were used in discontinuous electrophoresis method. Separating gel concentration was 12.5 percent. 10 µl of sample was heated at 100 °C for 3 min pre-loaded with 3 µl sample buffer (4x) accompanied with SDS and 2-mercaptoethanol. The sample was centrifuged for removing any insoluble part of the sample. The Voltage of electrophoresis was 50 and 110 V in thickening and separating gel respectively. Bromophenol blue of buffer showed the finished sample at the end of the gel. The gel was fixed and finally stained with silver staining method.

Alpha-toxin assay

Five types of red blood cell (man, mice, rat, sheep, rabbit) were evaluated for sensitivity to alpha-toxin and one substrate was chosen. The hemolytic activity of alpha-toxin was determined with some modifications as described by different researchers (Ballard et al.,1995, Fatmawati et al., 2013, Uppalapati et al., 2013). Briefly, 200 µl of alpha-toxin sample was added to 1% washed rabbit red blood cell (RBC) in a total volume of 1ml of PBS (phosphate buffer saline) and incubated for 1 h at 37 °C. The reaction mixture was centrifuged for 5 min at 1000 rpm. The supernatant absorbency was measured by the spectrophotometer (Mitsubishi) at 565 nm for hemoglobin content. All of the tests were performed in duplicate tubes. Deionized water for the positive control (100% hemolysis) and phosphate buffer for negative control were added on RBC suspension. One hemolysin unit(HU) is equal to reduction of one percent hemolysis of RBC against control positive at one minute and one ml.

Antibody production

Alpha-toxin rabbit antibody production : As a recommended method for immunization of animal(Leenaars and Hendriksen,2005), two New Zealand healthy 6 month-old female rabbits with 350-450 gr body weight were chosen. Alpha-toxin (250 µl) with complete Freund's adjuvant (500 µl) was prepared and a total 750 µl emulsion was injected laterally over the thorax of animals subcutaneously. Two weeks later, alpha-toxin (250 µl) with incomplete Freund's adjuvant (500 µl) was injected subcutaneously (SC). The third injection was performed just 15 days after the second injection in the same manner.

Bleeding and serum collection: To evaluate the antibody levels, rabbits were bled

from their marginal vein three days after the last injection. Serum was separated with centrifugation and stored at 4 °C after sodium azide (0.1%) addition.

Purification of Antibodies

The following steps were performed for antibody purification:

Precipitation of total immunoglobulin: Ammonium sulfate (2.95 gr) was gently added to the rabbit serum (10 ml) with continuous stirring in an ice bath, after solving of ammonium sulfate the mixture was kept at 4 ºC for overnight. The precipitated proteins were isolated by centrifugation at 3500 rpm for 20 min at 4 oC, and the supernatant was discarded. The sediment was dissolved in PBS(10 ml, pH 7.4), and dialyzed overnight against PBS pH 7.4 overnight (dialysis bag cut off 25KDa) for removing ammonium sulfate and buffer changing.

Ion exchange chromatography: DEAE-Sephadex column was equilibrated with 100 mM Tris-base buffer. One ml of dialysis sample was applied to DEAE-Sephadex for 20 min and washed out with 0.2 ml/min flow rate. The outed fraction was collected and used for next step(Fathi Najafi et al., 2005) .

Gel filtration chromatography: Sample was poured into gel, Sephadex G-50 column (100 cm × 1 cm) equilibrated with PBS buffer (pH =7.4). Fractions of the chromatography with 10 ml/h flow rate were collected in 4-ml vials and absorbency was read at 280 nm (Curling 1980).

Alpha-toxin antiserum evaluation

Double immunodiffusion (DID): In this method, a 2% agarose in PBS buffer (pH =7.3) was prepared and the gel was poured on slide (about 2 mm height) and allowed to solidify. The agarose gel was drilled to form small holes for the antigen or antibody. The purified alpha-toxin (20 µl) in the central cavity and the serums (20 µl) in the round cavities were loaded. For sediment formation, the plates were incubated for 24 h at 37 °C in humid condition and then for 2 days at 4 °C.

Single radial immunodiffusion (SRID): According to the Standard Operating Procedure (SOP), agarose (2%) was melted using PBS buffer (pH =7.3) plus 0.05% (w/v) sodium azide and the purified alpha-toxin antibody was added. Twenty microliters of a series of the diluted samples (1:2, 1:4, 1:8, 1:16) added into each rounded well were allowed to diffuse into the matrix and react with the antiserum for 24 h at room temperature. The precipitin zones were stained with Coomassie brilliant solution (Choi et al.,2017).

Electrophoresis: Purity of antiserum was analyzed from different stages of purification by SDS-PAGE according to the Laemmli method (Costa et al., 2013). Separating gel concentration was 12.5% and staking was prepared with 5%. Four volumes of purified samples of each fraction were added in one volume of sample buffer (5x) and the mixture was boiled for 5 min at 100 °C. To each well 15 µl of mixtures and to last well standard molecular weight marker were loaded. Gel electrophoresis was performed at 120 voltage in concentrating gel and 100 volts for separating gel. Finally, the gel was stained with silver nitrate (Noshahri et al.,2016).

Western blot

The Western blotting technique was used for alpha-toxin purification evaluation. At first SDS-PAGE electrophoresis was done with alpha-toxin samples and standard molecular weight marker. Bands were transferred to nitrocellulose membranes by wet transmission at 120 volts for 45 min. After saturation of nitrocellulose paper with a solu-

tion of bovine serum albumin 1% (BSA), the IgG conjugated with horseradish peroxidase (HRP) was added before substrate hydrogen peroxide (H2O2) and was finally stained by di-amino benzidine (Dabaghian et al.) to investigate non-specific bands (Noshahri, Fathi Najafi, Kakhki, Majidi, Mehrvarz, 2016).

Results

Purification and characterization of Alpha-toxin

According to the study, the maximum alpha-toxin production of *C. septicum* was acquired at the end of the logarithmic phase before the stationary phase. Results on the alpha-toxin effect on different RBCs showed that alpha-toxin is able to lyse all five types of RBC (man, mice, rat, sheep, rabbit). However, the hemolysis rate (percentage of RBC lysis) in rats, sheep, humans, mice, and rabbits is 100, 97, 81, 62, and 55, respectively (Fig. 1).

Ammonium sulfate concentration results showed that the maximum amount of alpha-toxin is deposited in 60% concentration. Ammonium sulfate in 25% concentration is able to precipitate the non-specific proteins that are almost 40% of the total protein present in the supernatant of cell culture. This step helps to remove large amounts of unwanted proteins and purification process will be facilitated (Table 1). Trace amounts of ammonium sulfate were removed by Tris buffer dialyzing.

The results of ion exchange chromatography with NaCl gradient concentrations showed that 0.4M NaCl could dissociate most of the alpha-toxin that binds to DEAE. The results of gel filtration chromatography, hemolysin test results, and absorbency at 280 nm are shown in Fig. 1. Alpha-toxin purification steps are summarized in Table 1.

Alpha-toxin with the special activity of 394 U/mg in the first culture medium reached to the special activity of 13666 U/mg at the last stage of purification. The purification methods gave 35 times purity with 47% toxin recovery. The alpha-toxin purification analysis by SDS-PAGE showed that the toxin has a molecular weight of about 48 KDa (Fig. 2).

Antibody Preparation

By the method of antibody preparation

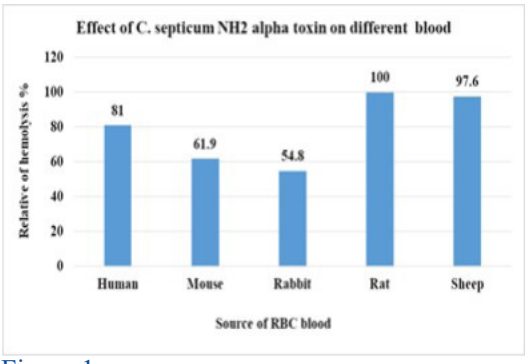

Figure 1.

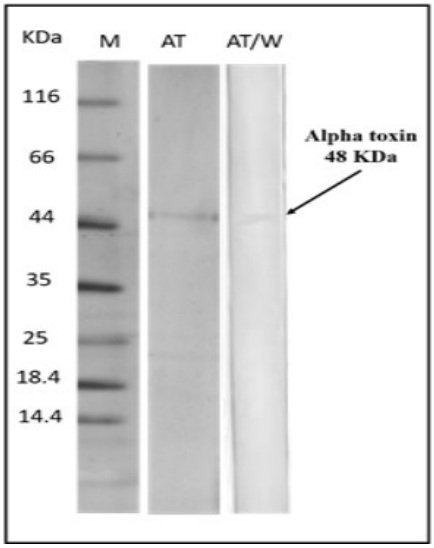

Figure 2.

Table1. Purification table of Clostridium septicum NH2 alpha toxin

step	Volume(ml)	total HU (Hemolysin Unit)	total protein(mg)	Specific activity (HU/mg protein)	Rate	yield
cell supernatant	250	35000	88.75	394.4	1	100
ammonium sulfate	50	32500	28	1160.7	2.9	92.9
dialysis	55	32450	26.4	1229.2	3.1	92.7
DEAE- Sephadex	20	24900	3.06	8137.3	20.6	71.1
Sephadex G50	100	16400	1.2	13666.7	34.7	46.9

based on standard immunization pharmacopeia, the anti-toxin that was prepared and used as an antibody detected the *C. septicum* alpha-toxin. The partially purified serum by ammonium sulfate precipitation, ion exchange, and gel filtration showed that purified immunoglobulins had a good percentage of purity and results were confirmed by SDS-PAGE, and Western Blot (Fig. 3).

Antitoxin evaluation

In DID test, alpha-toxin was poured into the central hole and the primary serum with dilutions of 1:2, 1:4, and 1:8 in the holes. The high-density sediment line visibly appeared between the antigen and the initial serum after Coomassie blue staining. Also, precipitation line formed between the holes of serum dilution of 1/2 and 1/4 were weaker. Increasing the antibody dilution in well 4 and the lack of observation of precipitation line with the dilution of 1/8 indicates un-equilibration between Ab and Ag concentrations (Fig.3-A).

Figure 3-B show results of SRID test. The white halo is clearly displayed, indicating an antibody production against the alpha-toxin

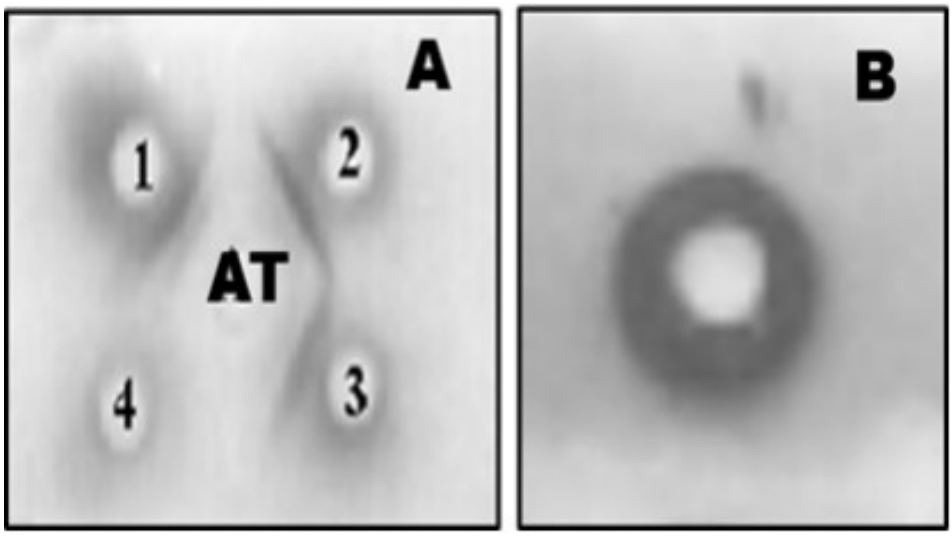

Figure 3.

in the rabbit.

The results showed that the 48 KDa purified alpha-toxin has high specificity to anti-toxin on Western Blot. The purified antibody was of the best quality and the heavy and light chains of Ig displayed on SDS-PAGE (Fig. 2).

Discussion

In previous studies 9 local *C. septicum* strains were isolated and confirmed by biochemical tests, indirect immunofluorescence and PCR (Hemmati et al. 2006). The bacterial growth process and the level of alpha-toxin secretion were investigated and then the hemolysin gene sequence was determined in all strains and recorded in the Gene Bank (Fathi Najafi and Hemmaty 2007).

Based on present and previous studies (Ballard et al. 1991) the highest amount of toxins release is in the logarithmic phase which increases during growth progression before the stationary phase.

The effect of the alpha-toxin on the different RBCs showed that the toxin activity can be related to the type of animal; therefore, the bacterium pathogenicity could be dependent on the host. The same results were reported by other researchers (Gordon et al. 1999, Hang'ombe et al. 2005, Hang'ombe et al. 2004). According to reports, GPI receptor and its level, as well as the amount of toxin tendency to the cellular receptor, can be effective in alpha-toxin activity (Gordon et al. 1999, Mukamoto et al. 2013). In this study, the results showed that the most hemolytic activity of alpha-toxin was on RBCs of rat, sheep, man, mouse, and rabbits respectively. Hang'ombe et al showed that the alpha-toxin adheres to a protein larger than 100 kDa in the rat's mucus membrane while on the mice, horses, cows, pigs, and poultry it adheres to proteins weighing 45-30 kDa. In Hang'ombe's study, alpha- toxin hemolysis on rat's blood was 86 times higher than sheep's blood (Hang'ombe et al. 2005).

Alpha-toxin is produced as a pro-toxin weighting 46.5 KDa and needs to be subsequently cut to yield the active form (41.3 KDa)(Ballard et al. 1991). Research by Hang'ombe showed that if the alpha- toxin is treated by proteinase K, it will decrease activity against RBC hemolysis and other proteases treatment such as trypsin and chymotrypsin will not significantly alter the hemolytic activity of alpha- toxin (Hang'ombe et al.,2005). The best activity of the toxin will be observed when the pro-toxin converts to the toxin near the cell surface. If alpha-toxin is activated earlier, its activity will be lost. It is oligomerized and cytolytically inactivated. In our study, no proteases' treatment on alpha-toxin was used prior to hemolysin test, similar to Ballard (Ballard et al. 1991).

Although alpha-toxin has the main role in the pathogenesis of the *C. septicum*, some other factors effect on the lethality by the bacterium (Kennedy et al. 2005). Ballard suggested that alpha-toxin has the role up to 70% of the *C. septicum* lethality. Therefore, the use of LD50 or MLD tests cannot be a standard assay for detection of the direct alpha-toxin effect. In this study, the quantitative hemolysin test was used to assay alpha-toxin during the purification steps according to Hang et al (Hang'ombe et al. 2005).

The immunization course and the type of the adjuvant were equal in the present and Hang's study. Inactivation of alpha toxin was done by formalin in Hang's study but in Ballard's and our study, it wasn't (Ballard et al. 1992, Hang'ombe et al. 2005).

Anion exchange and cation exchange chromatography, and gel filtration were per-

formed for alpha-toxin purification by Ballard et al (1992) in the first complete report procedure. Ballard found that during the alpha-toxin purification some other factors influence the hemolysin test such as a protein with 44 KDa weight and a larger 230 kDa protein. The 44-KDa protein is formed by removal of a part of the toxin due to the proteolytic effect of some secreted proteases by the bacterium. The 230 KDa protein is less when the alpha-toxin concentration is near to 2 mg/L and increases when the concentration reaches to 8 mg/ml. Therefore, it is a concentration-dependent protein that has much less hemolytic activity than in monomeric form. A phenomenon that probably consists of the aggregation of 5-6 monomer units (Ballard et al. 1992). In our study, these proteins were not observed, which could be the result of faster purification process and differences in purification steps and conditions, or the higher stability of the alpha-toxin secreted by the local *C. septicum* NH2 strain.

Hang and Ballard's purification steps were the same, with the difference that Hang used 60% saturated ammonium sulfate in the first step. However, our purification method started with 25% ammonium sulfate, which removed many of the proteins and continued with 60% saturated ammonium sulfate followed by three stages of chromatography instead of five Ballard steps. Fathi Najafi et al started the protein purification with ammonium sulfate precipitation simultaneous with Sephadex G-50 and DEAE- cellulose chromatography (Fathi Najafi et al. 2005).

The purification of alpha-toxin was begun with a specific hemolysin activity (U/mg) of 394, it was estimated to increase to 13666 after three purification steps. This method, with 47% efficiency, also made up about 34 times the purity. In a similar study conducted by Ballard on another strain and with five purification steps, it resulted in a purity of 84, with a 56% productivity.

Our method is more economical and faster than Hang and Ballard's purification procedure. The electroelution method used by Vázquez-Iglesias is faster but can just extract a small amount of the toxin (Vazquez-Iglesias et al. 2017). One of the faster and the most efficient purification procedures is immunoaffinity chromatography. Polyol-responsive antibody mimetics for single-step protein purification named nanoCLAMPs is based on a carbohydrate binding module domain of C. perfringens hyaluronidase by single-step affinity chromatography and polyol elution (Suderman et al. 2017). The procedure has not yet been used for purification of *C. septicum* alpha toxin.

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Conflicts of Interest

The author declared no conflict of interest.

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خالص سازی آلفا توکسین سویه بومی NH2 *کلستریدیوم سپتیکوم* و تولید آنتی بادی آن

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چکیده

زمینهٔ مطالعه: *کلستریدیوم سپتیکوم* عامل ایجاد بیماری های حاد متعددی در انسان و حیوانات است. آلفاتوکسین مهمترین فاکتور بیماریزایی *کلستریدیوم سپتیکوم* است و فعالیتهای همولیتیکی، نکروتیکی و کشندگی دارد.

هدف: درمطالعه حاضر، خالص سازی آلفاتوکسین سویه بومی *کلستریدیوم سپتیکوم* NH2 و تهیه آنتی بادی علیه آن انجام شده که می تواند در تهیه کیت های تشخیصی، آزمون توانایی واکسن و موارد مرتبط مورد استفاده قرار گیرد.

روش کار: سویه بومی *کلستریدیوم سپتیکوم* NH2 در محیط جگر کشت داده شد. آلفاتوکسین مترشحه در محیط کشت، طی سه مرحله خالص سازی شد: رسوب دادن با آمونیوم سولفات ۲۵ و ۶۰ % اشباع، کروماتوگرافی تغییر یونی با ستون DEAE-Sephadex و ژل فیلتراسیون بر روی SephadexG-۵۰. در تمامی مراحل میزان آلفا توکسین مورد اندازه گیری قرار گرفت و روش خالص سازی با SDS-PAGE ارزیابی شد. متعاقب ایمن سازی خرگوش با آلفاتوکسین و تهیه سرم از حیوان، ایمونوگلوبین با یک فرایند سه مرحله ای خالص شد: آمونیوم سولفات، کروماتوگرافی تغییر یونی و ژل فیلتراسیون. عملیات مسیر خالص سازی آنتی بادی به وسیله آزمون های الکتروفورز، ایمونودیفیوژن دوطرفه و شعاعی یکطرفه(SRID)، وسترن بلات و SDS-PAGE مورد ارزیابی قرار گرفت.

نتایج: مراحل خالص سازی که با آمونیوم سولفات ۲۵٪ آغاز و با ۶۰٪ ادامه یافت موجب حذف بسیاری از پروتئین ها گردید. نتایج کروماتوگرافی تعویض یونی با غلظت های مختلف نمک نشان داد که غلظت ۰,۴ مولار نمک می تواند از همه بیشتر آلفاتوکسین را از ستون DEAE جدا نماید. آلفاتوکسین در مرحله اول، در محیط کشت باکتری فعالیت مخصوص ۳۹۴ U/mg را داشت و در نهایت، پس از انجام مراحل خالص سازی فعالیت مخصوصش به ۱۳۶۶۶ U/mg رسید. روند خالص سازی توانست توکسین را ۳۵ برابر خالص کرده و ۴۷ ٪ توکسین اولیه را تخلیص و بازیابی نماید. نتایج الکتروفورز روی ژل SDS-PAGE حاکی از خلوص حدودی آلفاتوکسین بود. نتایج ایمونودیفیوژن دوطرفه و شعاعی یکطرفه وجود آنتی بادی مربوطه را تایید نمود. در فرایند وسترن بلات، دو زنجیره سبک و سنگین ایمونوگلوبین توسط مرکاپتواتانول از هم جدا شده و در الکتروفورز فقط با پروتئین ۴۸ کیلودالتونی بر روی غشاء نیتروسلولزی واکنش داد.

نتیجه گیری نهایی: نتایج این تحقیق منجر به دستیابی به روش خالص سازی آلفاتوکسین *کلستریدیوم سپتیکوم* و آنتی بادی علیه آن سریعتر و اقتصادی تر از روش‌های سایر محققین گردید.

واژه‌های کلیدی:

کلستریدیوم سپتیکوم، آلفاتوکسین، خالص سازی، آنتی آلفاتوکسین، پلی کلونال آنتی بادی

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