ANTIGENICITY DETERMINATION OF PURIFIED ALPHA-TOXIN OF CLOSTRIDIUM SEPTICUM

Jasem Estakhr, Nasim Javdan

Science and Research Branch, Islamic Azad University, Fars, Iran.

Corresponding Address: Jasem Estakhr, j.estakhr@yahoo.com. Tel: +989179283966

Summary

Polyclonal antibodies and their components from animal's immunized serum have many applications in designing variety of immunity measurements as well as treatment of poisoning and diseases. The aim of this study is to design an efficient and economic method to produce and purify antibodies against clostridium septicum alpha- toxin to diagnose suspicious patients. Purified alpha- toxin was used to produce polyclonal antibody in 2 three-month male rabbits. After immunization, single radial immunodiffusion (SRID) and double immunodiffusion (DID) were used to detect produced antibodies. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse antibodies purity. IgG molecule was separated with three methods: ammonium sulphate, ion exchange chromatography and gel filtration. Western blot was used to confirm inclusive bands absent. SDS-PAGE showed high purity IgG can be achieved by mentioned three purifying methods. Primary and elementary purifying of serum containing anti alpha- toxin by precipitating with 50% ammonium sulphate and salt fraction dialyze against phosphate buffer saline (PBS) helped to remove impurities before main purifications on ion exchange and gel filtration column. White halo observed clearly in SRID indicated antibody production against alpha- toxin in rabbits. Coloured 47 kD band and no coloured bands in other parts of nitrocellulose membrane in Western Blot showed produced antibody reacted with alpha toxin only. Saturated ammonium sulphate, ion exchange chromatography by DEAE-Sephadex, Sephadex G-50 gel filtration and Western Blot were successfully used to purify IgG antibody against clostridium septicum alpha- toxin.

Keywords: clostridium septicum, Alpha- toxin, Antigenecity.

Introduction

Closteridium septicum is a gram forming anaerobe that has been implicated as a cause of malignant edema in animals (1). Alpha- toxin is a pore forming toxin which is secreted from Clostridium septicum (2). The organism produces several extracellular factors that include deoxyribonuclease, hyaluronidase, neuraminidase and alpha-toxin (3). Alpha toxin is the main factor of pathogenesis with hemolytic, necrosis, and lethality activities (4). Alpha- toxin is categorized in the group of pore forming toxins and has been formed of three domains. Domain one is receptor binding site of the toxin, domain two and domain three are involved in oligomer formation and assembly, respectively. Alpha- toxin and aerolysin have many structural and functional similarities. Aerolysin is a toxin which is secreted by Aeromonas Hydrophila. Previously crystalline structure of Aerolysin was used for determining molecular model of Alpha- toxin. Aerolysin is a two-lobar protein that its smaller lobe is formed of domain one (D1) and larger lobe is formed of domains two and four (D2 – D4). Primary structure of Alpha- toxin is similar to larger lobe of aerolysin but without smaller lobe of 38 amino acids. These two proteins have a similar mechanism of pore formation (5-7) Alpha- toxin is secreted in the form of a water soluble protoxin and is activated by a protolytic cut at the carboxylic end. A few proteases mediate in activation of this toxin, the most important of which is eukaryotic protease furin (8).

Furin detects the RGKR sequence in Alpha- toxin and causes 5.1kDa to be released from carboxylic end (9). Polyclonal antibodies and their components, which are produced from serum of immunized animals, are applicable in designing immunity measurements as well as curing of diseases and poisonings. Producing polyclonal immunoglobulin from animal serums (rabbit, sheep, goat, and horse) requires repeated cupping, serum preparing procedures, separation and purification of antibodies, as well as suitable and proper storage. Moreover, these antibodies cause positive error with rheumatoid factors in immunochemical tests (10). Various immunochemical methods such as single radial immunodiffusion (SRID), double immunodiffusion (DID), imunoelectrophoresis, western blot as well as various electrophoresis methods such as cellulose acetate electrophoresis and SDS-PAGE are used to confirm immunoglobulin's purity (11). The purpose of this study is designing and establishing an efficient method to produce and purify polyclonal antibody against alpha- toxin of clostridium septicum which is used to diagnose suspicious diseases with clostridium septicum.

Materials and Methods

Rabbits Immunization with Alpha- toxin

Purified alpha- toxin which has been detected and separated before (12,13) was used to produce polyclonal antibody in rabbits. Two three-month male New Zealand white rabbits were subcutaneously injected with 250 µl immunogen and 500µl Freund's complete adjuvant (FCA) (BioGene, U.K.). Then, 250 µl immunogen and 500µl Freund's incomplete adjuvant (FIA) (BioGene, U.K.) was subcutaneously injected after 15 days. In third injection (15 days later), rabbits were injected again with 250 µl immunogen and 500µl (FIA) subcutaneously. Three days after last injection, blood was bled from marginal vessels of rabbit's ear. Blood was left at 4˚C for 12 hours in order to separate serum. Then clotted blood was removed from tube and centrifuged (Eppendorf 5415R, Germany) at 3000 rpm for 15 minutes. Supernatant (serum) was separated and stored at 4˚C by adding 0.1% sodium azide (Merck, Germany)

Antibody Detection

To detect produced antibody in rabbit's serum, two methods were used:

1. Double Immunodiffusion (DID) Test

2% agarose in PBS (Sigma Aldrich,USA) buffer (pH=7.3) was used. Wells in gel were encompassed with 20 µl antigen or antibody aliquots. Purified alpha- toxin was added into central well and serum was added into other wells with different distances from central well. Then, lams were incubated for 24 hours at 37˚C followed by 2 days at 4˚C.

2. Single Radial Immunodiffusion (SRID) Test

2% agarose in PBS buffer (pH=7.3) was mixed with 300 µl antibody. The mixture was poured into several lam up to 2 mm in height. Diffusion wells in gel were filled with 20 µl of antigen. Lam was left for 24 hours at 37˚C followed 2 days at 4˚C in order to loops sedimentation appears.

Antibody Purification

Depositing with Ammonium Per sulphate

2.95 g Ammonium per sulphate (Merck, Germany) was added to 10 ml of serum (50% saturated). Serum container temperature reached to 0˚C in ice bath. While stirring, ammonium per sulphate was added to serum as much as not to foam and proteins to settle gradually. Produced precipitation was separated by centrifugation (3500rpm, 20 minutes) and

solved in PBS buffer (0.1 M, pH=7.4) and dialyzed overnight in order to remove ammonium per sulphate.

Ion Exchange Chromatography

Sample purified with above method was purified with ion exchange chromatography after being dialyzed with starter buffer. DEAE Sephadex (Sigma Aldrich, USA) bed was applied which have been equilibrated with BPS of 20 mM (pH=7.4). 1 ml of dialyzed sample was added in column. Stream velocity was 0.2 ml per minute. Optical absorption of purified samples was read with a spectrophotometer (Shimadzu UV-160A, Japan) in 280 nm and absorption diagram was drawn against fractions volume. Fractions related to diagram's peak were mixed together and after precipitation with ammonium sulphate were stored at 4˚C.

Gel Filteration Chromatography with Sephadex G-50

G-50 Sephadex (Biogene, U.K.) gel was poured in a column with height of 1 m and 1cm diameter and its final length was 80 cm. Gel in column was balanced by PBS buffer (pH=7.4). After being dissolved in PBS buffer, precipitated result from previous stage was placed into column. Liquid leaving chromatography column at stream velocity of 10 ml per hour and fractions of 4 ml were gathered. Optical absorption was read at 280 nm and absorption diagram was drawn against fractions volume.

SDS-PAGE

To examine sample purity from different stages of purification SDS-PAGE technique based on Laemmeli was used. 12.5% polyacrylamid (Fluka, U.K.) was used as separator gel. Four volumes of purified sample from each fraction were added to one volume of sample buffer (5x), and mixture was boiled at 100˚C for 5 minutes. 15µl of mixture was added into each well. Standard marker was added to last well. Electrophoresis was performed under constant voltage of 120 V in condenser gel (Bio Rad, USA) and of 100 V in separator gel. Then, gel was coloured by argentums nitrate.

Examination for existence or absence of unspecified bands

Western blot technique was performed for existence or absence of unspecified bands. Sample containing alpha- toxin was considered as standard of molecular weight under SDS-PAGE. Resulted bands were transferred to nitrocellulose membrane (Merck, Germany) by damp transfer method at 120 V for 45 minutes. After saturation of nitrocellulose papers with 1% solution of bovine serum albumin (BSA) (Merck, Germany), IgG sample conjugated with peroxidase (HRPO) was added and finally examined due to existence of unspecified band by adding hydrogen peroxide (H2O2) (BDH Labratory, UK) substrate and chromogenic reactor of Diaminobenzidine (DAB) (Merck, Germany).

Results

Purified alpha- toxin was used to produce polyclonal antibody in male rabbit. SRID and DID were applied to detect antibodies. SDS-PAGE was done to analyse antibodies purity. IgG molecule was separated with ammonium sulphate, ion exchange chromatography and gel filtration. Western blot was done to confirm inclusive bands absent. Figure 1 showed a precipitated line with high density was appeared between antigen and undiluted serum. A weak precipitated line was also appeared between diluted serums (1/2 and 1/4) and antigen

well. No precipitated line was appeared among 1/8 diluted serum and antigen well. A white halo was also observed clearly in SRID test indicating antibody production against alpha-toxin in rabbits. Figure 3 showed IgG was not connected to the column and removed from the column by rinsing buffer. The absorption of collected fractions was measured at 280 nm (ion exchange chromatography of DEAE-Sephadex).

Fig 1: Double Immunodiffusion – central well has been filled with alpha- toxin and surrounding wells have been filled with rabbit serum of different concentrations.

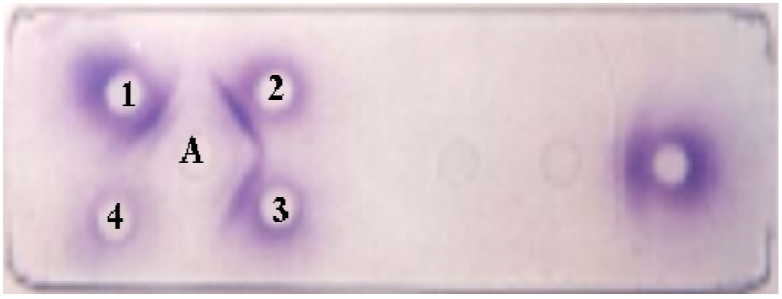


Fig 2: Single Radial Immunodiffusion – wells have been filled with alpha-toxin.

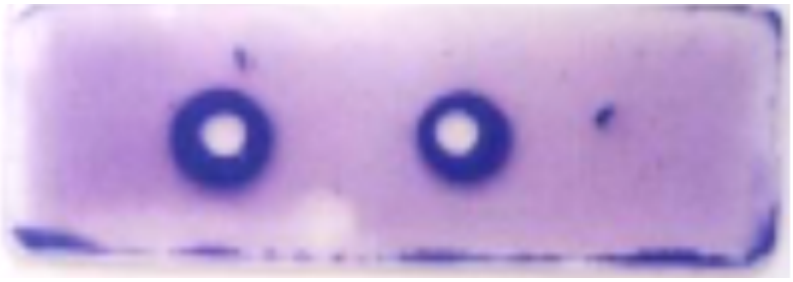
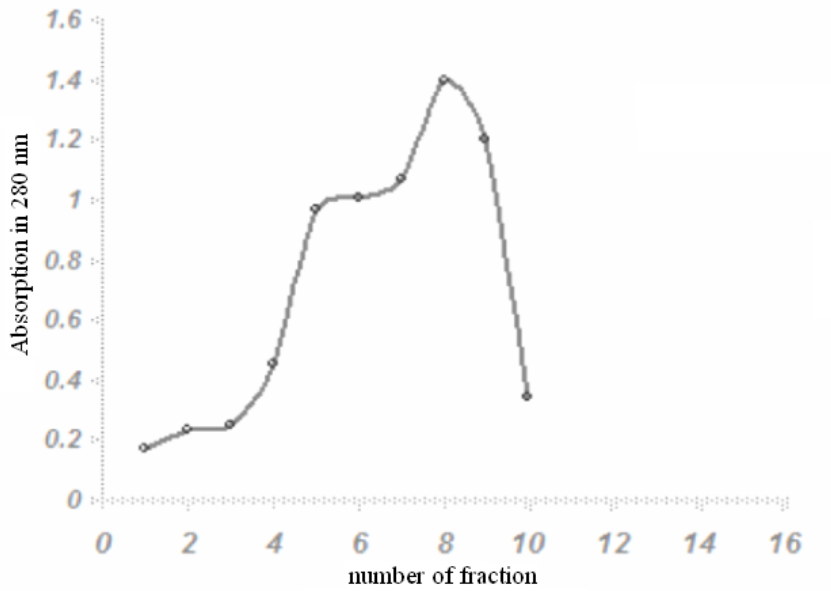


Fig 3: Absorption chromatogram of fractions of ion exchange chromatography 280 nm.



Gel filtration with Sephadex G-50 has been shown in figure 4. Fractions were collected and their absorption was measured at 280 nm. IgG was collected in fraction No 7 due to its heavy weight. SDS-PAGE was used to detect purification level. Light and heavy chains of IgG were separated from each other due to reduction method. The gel was stained with argentums nitrate after electrophoresis. Figure 5 illustrated that purification level has been increased after performing different processes and samples have higher purity followed by gel filtration. Western blot showed produced antibody is peculiar to alpha-toxin (Figure 6).

Fig 4: Absorption chromatogram of fractions from gel filtration chromatography at 280 nm.

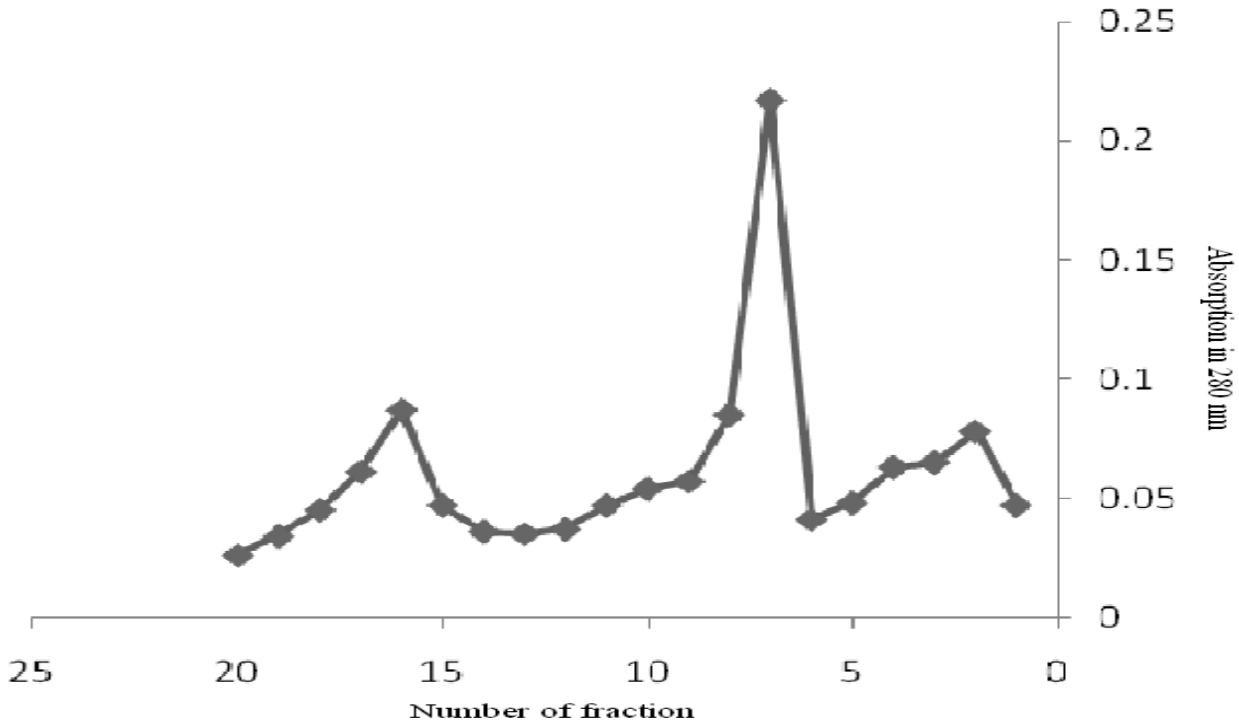


Fig 5: Electophoresis under denatured conditions. Molecular weight marker (M), original serum (Lane 1), precipitated sample with ammonium sulphate (Lane 2), after ion exchange chromatography (Lane 3), after gel filtration (Lane 4).

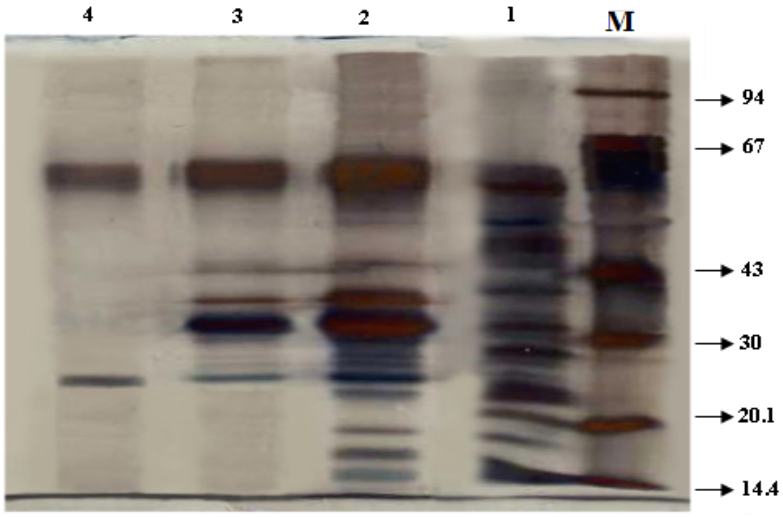
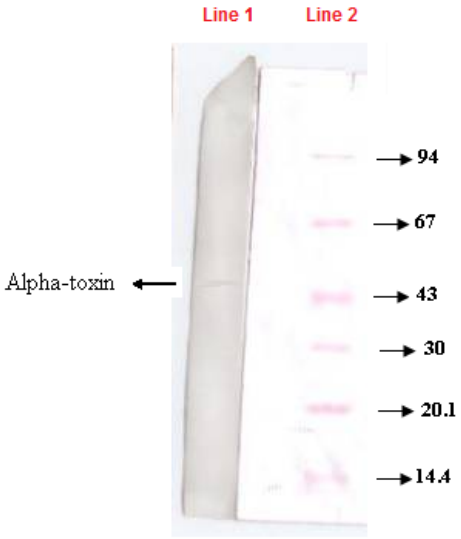


Fig 6: Purified polyclonal antibody binds 47 KD alpha-toxin band but not other sample proteins in Western blot analyses. Alpha-toxin (Lane 1) and sample proteins (Lane 2) are shown following staining with purified polyclonal antibody in a Western blot.



Discussion

In this present study, we provided an efficient and economic method to produce and purify antibodies against clostridium septicum alpha- toxin to diagnose suspicious patients. Three purification methods were used in this study. The first stage of purification is using saturated ammonium sulphate which is a starting point for other purification stages (13,14). Product of this method has some impurities. Other purification stages are needed for more purity. Key point is all stages have to be done at cold conditions and concentration of ammonium sulphate has to reach to 50 percent of saturation after being mixed with serum. It is necessary to say primary purification of serum containing anti alpha- toxin through precipitating with 50 percent ammonium sulphate and dialyzing salt fraction against PBS helps considerably in removing main impurities before performing main purifications on the columns of ion exchange and gel filtration (15). Due to high differences among isoelectric pH of immunoglobulin and other proteins existing in serum, ion exchange chromatography is a suitable technique to separate immunoglobulin of rabbit's serum (Smith, 1975). DEAE-Sephadex bed is suitable method for separating IgG and Sephadex G-50 gel filtration was used to continue purification process. The product showed high purity. SDS-PAGE was used to measure purity level of final product in this study. This method indicated we can achieve a highly purified IgG antibody by three purification stages as mentioned above. De sulphide bands were broken and light and heavy chains were separated due to existence of 2-mercapto ethanol in buffer. So, two bands were generated with molecular weight of 27 and 57 kD. 27 kD band could be attributed to light chain and 57 kD band could be attributed to heavy chain of IgG. Western Blot technique was used to examine unspecific reaction probability between produced anti alpha-toxin with other sample proteins and specific reaction with lpha-toxin. Due to colour in 47 kD band and bands with no colour in other parts of nitrocellulose

membrane it can be concluded that produced antibody reacted only with alpha- toxin and didn't cause unspecific reactions with other proteins. It can be concluded that Anti clostridium septicum alpha toxin is a polyclonal antibody which is applied for detecting diseases suspicious of infection with clostridium septicum. Saturated ammonium sulphate, ion exchange chromatography using DEAE-Sephadex bed and Sephadex G-50 gel filtration were successfully used to purify IgG anti clostridium septicum alpha toxin. Affinity chromatography is recommended for more purification.

Acknowledgment

We express our special thanks to anaerobic division of Razi Vaccine and Serum Research Institute (Mashhad, Iran) for providing necessary facilities for this study.

References

1- Mudena BHO, Tomoko K, Masafumi M, Shunji K, (2004). Relationship between Closteridium septicum alpha toxin activity and binding to erythrocyte membrane. Bacteriology, 69-74.
2- Geny B, Popoff M R. (2006). Bacterial protein toxins and lipids: pore formation or toxin entry into cell. Biol Cell. 98: 667-678.
3- Ballard J, Bryant A, Stevens D, Tweten RK. (1991). Purification and Characterization of the Lethal Toxin (Alpha-Toxin) of Clostridium septicum. Infection and Immunity. 60: 784-790.
4- Melton JA, Bentsen LM, Tweten RK. (2006). Identification of Functional Domains Clostridium septicum Alpha- toxin . Biochem. 45:14347-14354.
5- Melton JA, Parker MW, Rossjohn JT, Tweten RK. (2004). The identification and structure of the membrane-spanning domain of the Clostridium septicum alpha- toxin . J Biol. Chem. 279:14315-14322.
6- Ballard J, Crabtree J, Roe B.A., Tweten RK. (1994). The primary structure of Clostridium septicum alpha-toxin exhibits similarity with that of Aeromonas hydrophila aerolysin. Infection and Immunity. 63: 340-344.
7- Ballard J, Sokolv Y, Yuan WL, Kagan BL, Tweten RK. (1993). Activation and mechanism of clostridium septicum Alpha- toxin . Mol Microbiol. 10:627-634.
8- Gordon VM, Benz R, Fujii K, Leppla ST, Tweten RK. (1997). Clostridium septicum alpha-toxin is proteoliticaly activated by furin. Infection and Immunity. 65:4130-4134.
9- Larsson A, Mellestedt H. (1992). Chicken antibodies : a tool to avmid interferense by human anti mouse antibodies in ELISA after invivvo treatment with murine monoclonal antibodies. Hybridoma. 11(1):33-39.
10- Hansen P, Scole J A, Hanson B, Hoogenraad N J. (1998) . Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatology . J Immunol Methods. 215: 1-7.
11- Hemmaty M, Morshedi A, Yousofbeigi GH, Fathi M. (2006). Isolation and identification of Clostridium septicum from sheep dung. Archives of Razi Institute. 61(3): 167-172.
12- Jonston A, Thorpe R. (2004). purification of antibody, Immunochemistry in practice, Blackwell scin pub. 102-120.
13- Valker JM. (2006). protein precipitation, the protein protocols handbook .Human press INC. 65-80.
14- Laemmli UK. (1977). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.
15- Stec J, Bicka L, Kuzmak J (2004). Isolation and purification of polyclonal IgG antibodies from bovine serum by high performance liquid chromatography. Bull Vet Inst Pulawy. 48: 321-327.