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Purification of Exotoxin A from Locally Isolate of *Pseudomonas aeruginosa*

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Abstract: Twenty-five isolates of *Pseudomonas aeruginosa* were isolated from wounds, burns, inflammation of the urinary tract(UTI), sputum and ear infections. These isolates were found producing exotoxin A as indicated by ELISA test. Isolates from different infections (burn, wounds and UTI) were selected according to their productivity. An amount of 0.045, 0.064 and 0.040 mg/ml protein which represent and 23.31, 29.73 and 19.01 ng/ml exotoxin A were produced respectively. Extoxoxin A was partially purified using precipitation by 90% ammonium sulphate followed by ion exchange chromatography with DEAE-cellulose then gel filtration chromatography by sepharose 4B was using to detection the molecular weight for the purified toxin was estimated as 65 KD.

Key words: Exotoxin A; *Pseudomonas* Aeruginosa; ELISA test.

تنقية السم الخارجي من نوع A المنتج من قبل العزلة المحلية لبكتريا *Pseudomonas aeruginosa*

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الخلاصة: تم إختبار 25 عزلة من بكتريا *Pseudomonas aeruginosa* المعزولة محلياً من الجروح، الحروق، التهابات المسالك البولية، القشع والتهابات الأذن. تم الكشف عن قابلية هذه العزلات على إنتاج السم الخارجي من نوع A بأستعمال نظام ELISA، وقد تم إختيار هذه العزلات من الجروح، الحروق والتهابات المسالك البولية وفقا لأنتاجيتها إذ بلغت 0.045، 0.064 و 0.040 ملغم/مل للبروتين و 29.73، 23.31 و19.01 نانوغرام/مل للسم الخارجي نوع A على التوالي. تمت التنقية للسم الخارجي بواسطة الترسيب بكبريتات الامونيوم بنسبة إشباع90% تليها التنقية بالمبادل ألايوني DEAE-cellulose، والتنقية بالهلام من نوع Sepharose 4B وتعيين الوزن الجزيئي للسم الخارجي الذي قدر بـ 65 كيلودالتون.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen and is a leading cause of infection in compromised hosts (1,3). Exotoxin A is a potentially important virulence factor found in *Pseudomonas aeruginosa*. Functionally exotoxin A mimicthat of diphtheria toxin in its potent inhibitory action to mammalian protein synthesis (2, 4), it is Synthesized as a proenzyme that

catalyzes the transfer of ADP ribose moiety from NDA^+ into covalent linkage with elongation factor 2. Detectable quantities of exotoxin A were found in some but not all supernatants of media in which toxigenic strains of *P. aeruginosa* were grown (7,8). Furthermore, individual strains of *Pseudomonas aeruginosa* reportedly differ in their requirements for optimal toxin production (9). Since exotoxin A is not formed constitutively by toxigenic strains of *P. aeruginosa*, it is likely that specific regulatory systems or factors control its synthesis and secretion of exotoxin A in a manner that is distinct from the regulation of bulk protein synthesis. This detection could be done by the use of enzyme-linked immune sorbent assay (ELISA) which is sensitive, specific and quantifiable.(10). The aim of this study is isolation, and purification of exotoxin A from local toxigenic isolates of *Pseudomonas aeruginosa*.

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Materials and Methods

Microorganism

Twenty- five *P. aeruginosa* isolates were obtained from AL-Yarmok hospital and child center hospital Baghdad city/Iraq. For identification of the isolates, a number of biochemical and cultural tests were done including growth in brain heart infusion broth at 42°C, oxidation of maltose and mannitol, nitrate production, motility, ornithine and arginine dearboxylation, indolphenol oxidation reaction and liquefaction of gelatin (11), *Pseudomonas aeruginosa* differ from other species of *Pseudomonas* by growth in selective media (cetramid agar) and characterized by PBH media, TSA media and arginine dehydrolyse media (14). Api-20E system (BioMerieux-France) was also included as a confirmatory test system for identification of these isolates.

Detection of Exotoxin A

The ability of the twenty-five isolates to produce Exotoxin A was done by using ELISA (Enzyme- Linked immune sorbent Assay),(12,13). The principle of this assay is the microtiter plate provided in this kit has been pre-coated with an antibody specific to PEA(*Pseudomonas aeruginosa* Exotoxin A). standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated at 37°C for 1hour. Then a TMB (3,3‘,5,5‘tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain PEA, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of PEA in the samples is then determined by comparing the O.D. of the samples to the standard curve (15).

Exotoxin A production

Stock cultures were streaking on Trypticase soy agar(TSA) plates, and incubated overnight at 30°C**,** and then isolated colonies were picked, streaked on TSA slants, and incubated overnight. The growth was suspended in Trypticase soy broth dialysate (TSBD)

which contain 15% (vol/vol) glycerol and was stored in aliquots at -70°C. For each experiment, an aliquot was thawed and used to inoculate a TSA slant. After overnight incubation at 30°C, loopfull growth from this slant was suspended in TSBD and used to inoculate 40 ml of TSBD in a 300-ml flask. To inhibit protease activity (16), nitrilotriacetic acid (NTA) was added to the culture mixture in a final concentration of 5 µm. Cultures were centrifuged for 20 min at 10,000 rpm, and supernatants were taken for purification.

Protein concentration

Cultures were grown in flasks containing 100 ml of TSBD media containing NTA and NTA free media. The protein concentration was assayed by adding 0.1 ml of cultural liquid and 0.4 ml of the Tris-HCl buffer in the test tube and 2.5 ml of thecommasie solution was added, mixed well and left for 5 minutes, the absorbance was measured at 595 nm. Standard curve was used to calculate protein concentration (17).

Purification of exotoxin A

Precipitation by ammonium sulphate

The supernatant (crude extract) was fractionated with ammonium sulphate at (0,10,20,30,40,50,60,70,80,90)% saturation was used to obtain complete precipitation of the toxin, then the precipitant was separated by centrifugation at 10,000 rpm for 30 min. The precipitant was resuspended in 10 ml normal saline.

Ion exchange chromatography

DEAE-cellulose ion exchange column was prepared according to (18). The sample obtained with 90% ammonium sulphate saturation, was dialyzed in distilled water for 24 hr., the concentrated protein solution then applied to DEAE-cellulose column (2x18 cm) previously equilibrated with Tris-HCL buffer pH 8.1, the isoelectric point of exotoxin A is 5.5, the column was washed with the same buffer and eluted with a linear salt gradient with the same buffer containing (0.1-1)MNaCl. The fractions were collected in test tubes at flow rate of 30 ml/hr. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions of the protein peaks were assayed for exotoxin A. fractions containing exotoxin A were collected and concentrated for measurements.

Gel filtration chromatography

1-Determination of the void volume (Vo) of the column

Sepharose-4B column (56×1.5cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden). The column was equilibrated overnight with 0.02 M Tris-HCl buffer pH 8.0 with a flow rate of 50 ml/hr. A 2 ml blue dextran 2000 solution was passed through the column, and 225 ml of Tris-HCl buffer pH 8.0 was added to the column. Fractions of 5 ml were collected. The absorbency at 600 nm for each fraction was measured.

2-Determination of exotoxin elution volume (Ve)

Sepharose-4B column (56 × 1.5 cm) was prepared, packed and equilibrated

for a second time. A 3 ml of purified exotoxin A sample was passed through the column carefully, and equilibrated with 0.02 M Tris-HCl buffer pH 8.0, with a flow rate of 50 ml/hr. Fractions of 5 ml were collected. The elution volume (Ve) was estimated for the separated fractions of purified exotoxin, by following the absorbency at 280 nm.
rate of 50 ml/hr, as shown in table (1).

3-Measurement of standard proteins elution volume (Ve)

Different standard proteins were applied through sepharose-4B column, and then eluted with 0.02 M Tris-HCl buffer pH 8.0, with a flow

Table (1): Molecular weight of standard proteins

Standard proteins	Molecular weight Dalton
Chymotrypsin	23000
Ovalbumin	43000
Bovine serum albumin	67000
Aldolase	158000

The elution volume was estimated for each standard protein by following the absorbency for the separated fractions at wavelength 280 nm. The (Ve/Vo) ratio was calculated for each standard protein and for the dissolved fractions and separated fractions of purified exotoxin A, then standardization was done, by plotting the elution volume (Ve) of each standard proteins to the void volume (Vo) of the blue dextran 2000 (Ve/Vo) versus the log value of molecular weight (19,20). The exotoxin molecular weight was accordingly calculated**.**

Results and Discussion

Detection of Exotoxin A

ELISA kit was used for detection of exotoxin A produced by isolates of *Pseudomonas aeruginosa*. The kit was also used for concentration measurements as results indicated in Table (2). Screening of these isolates indicates that, most of the isolates show positive results. However, three isolates were selected as the highest producer. Accordingly, these isolates were designated as, PA 10, PA 11 and PA 15 produce (23.31) (19.01) (29.73) ng\ml respectively. Productivity was further specified for purified product.

Table (2):The exotoxin A con. Produced by different isolates

Isolate no.	Location	Concentration (ng/ml)
PA 1	Wound	12.06
PA 2	Wound	16.17
PA 3	Burn	10.22
PA 4	Ear	8.67
PA 5	Wound	10.54
PA 6	Burn	13.87
PA 7	UTI	6.90
PA 8	sputum	4.43
PA 9	Ear	4.16
PA 10	Burn	23.31
PA 11	UTI	19.01
PA 12	Wound	17.07
PA 13	UTI	5.12
PA 14	sputum	3.87
PA 15	Wound	29.73
PA 16	UTI	3.12
PA 17	Wound	18.05
PA 18	Ear	6.51
PA 19	UTI	3.40
PA 20	Ear	4.60
PA 21	Wound	9.22
PA 22	Burn	11.54
PA 23	Burn	15.23
PA 24	Wound	11.30
PA 25	UTI	16.40

Effect of nitrilotriaetic Acid (NTA) on exotoxin A production (monitored as protein concentration) was studied. Results shown in table(3) indicate that protein concentration was found to be higher in cultures contain NTA than that of free cultures, the productivity of proteins for the three isolates in media containing NTA more than that of free NTA media.

This result encourageس us to fallow concentration of exotoxin A for the three isolates after purification to found if any other proteins participate in the results as long as NTA inhibit protease activity and stop protein turn over.

Precipitation of proteins by ammonium sulphate

In order to concentrate the crude extract of toxin and remove as much as possible water and some protein molecules, ammonium sulphate were used at (10,20,30,40,50,60,70,80,90)% saturation, the saturation ratio of 90% was chosen to precipitate exotoxin A. This step allows the salting out of molecules from water.

Since ammonium sulphate has the ability to neutralize charges at the surface of the protein and to disrupt water layer surrounding the protein, it will eventually cause a decrease in the solubility of protein which, in turn leads

to the precipitation of the protein by the effect of salt(15,16). Ammonium sulphate is widely used because of availability of ammonium sulphate, high solubility, low cost and it stabilizes the protein (18).

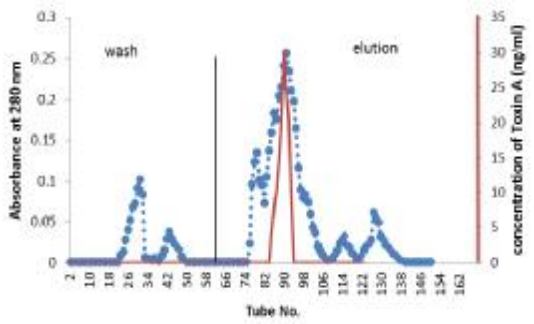
Ion Exchange chromatography

Purification of exotoxin A was done by ion-exchange chromatography using (DEAE-cellulose). Figure (1) showed the wash and elution of DEAE-cellulose column for three isolates. No exotoxin A was detected in the wash steps, while the eluted fractions revealed. The presence of three peaks. However, only one peak for each elution of three isolates shows activity as detected by ELISA kit. The amounts of purified proteins indicate in table (3).

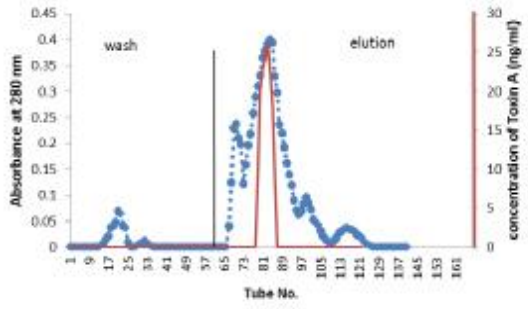
Table (3):Exotoxin A production by the three of *P.aeruginosa* isolates.

No. of isolate	Protein concentration (mg/ml)		
	Media NTA free (mg/ml)	NTA containing media(mg/ml)	Purified protein from NTA containing media
PA 15	0.064	0.069	0.063
PA 11	0.040	0.049	0.044
PA 10	0.045	0.058	0.051

A.



B.



C.

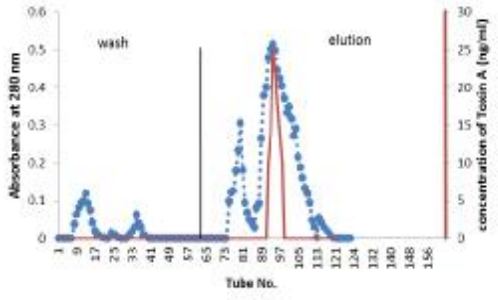


Figure (1):Purification of exotoxin A produced from local isolates, A. isolate PA 15, B. isolate PA11 and C. isolate PA 10 respectively, by DEAE-cellulose ion exchange chromatography column (2x18)cm equilibrated with 0.01 M Tris-HCL buffer PH 8.1, exotoxin eluted with linear salt gradient 0.1-1 M NaCl, flow rate 45ml/hr.

Detection of Exotoxin

Detection of exotoxin in fractions eluted from ion-exchange chromatography was done using ELISA kit, the results shown in table (4) indicate the presence of only one peak in the elution steps of the three isolates giving positive result, as measured spectrometrically at 450 nm wavelength.

Table (4):Detection of exotoxin A using ELISA kit

No. of isolate	Toxin concentration (ng/ml)		
	Media NTA free (ng/ml)	NTA containing media(ng/ml)	Purified protein from NTA containing media (ng/ml)
PA.15	29.73	56.48	32.08
PA.10	19.01	38.22	24.79
PA.11	23.31	41.05	26.11

The results indicate that, isolate PA.15 is the highest producing of exotoxin A.

Purification by gel chromatography

The sample passed through Sepharose-4B then fractionated on the gel fractions were collected up to 30 fractions. Exotoxin A was present in fractions 21-23 depended on using acetic acid assay and increase absorbency of fractions. The result of purification showed one-peak as indicated in figure(2).

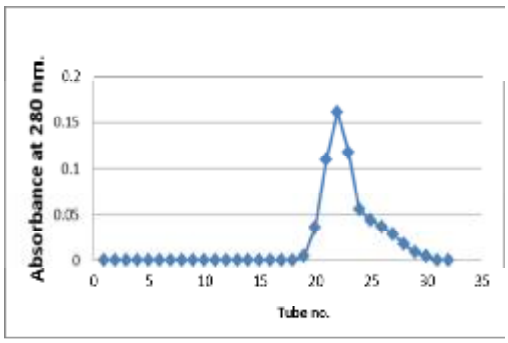


Figure (2):Purification of exotoxin A by using Sepharose-4G column (63×1.5 cm) with flow rate of 50 ml/hr.

Determination of molecular weight of exotoxin A

Molecular weight of exotoxin A was determined using Sepharose-4B column (56×1.5 cm). The void volume (Vo) of the column was calculated by estimating the void volume of blue dextran 2000 to the elution volume (Ve) for each one of standard proteins and for the separated fractions of purified exotoxin. The ratio of the elution volume of each standard protein as well as the separated fractions of the purified exotoxin, to that of void volume of the blue dextran 2000 was calculated.

Results in figure(3), show that the (Ve/Vo) ratio of purified exotoxin was about (65KD), the ratio of (Ve/Vo) of each standard protein to the log molecular weight of each standard protein was plotted.

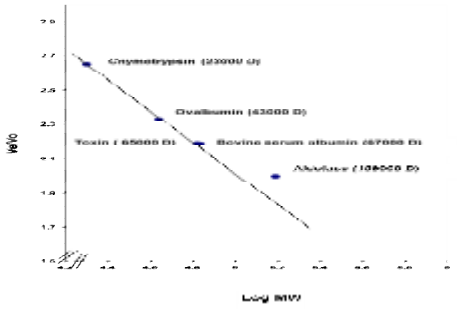


Figure (3):The (Ve/Vo) ratio of standard proteins and purified exotoxin A

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