Extraction and Purification of LasA Enzyme from pseudomonas aeruginosa

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Abstract

Staphylolycin is one of the important enzymes produced by Pseudomonas aeruginosa as a competitive way against other bacterial species such Staphylococcus aureus. Pseudomonas aeruginosa was isolated from 100 clinical specimens, including urine, wound, and burn swabs. On Tryptic soya agar with heat killed Staphylococcus aureus at temperature 100°C, the capacity of local isolates to produce staphylolysin enzyme was investigated. Staphylolysin enzymes A and D activity was determined by measuring the capacity of the boiled Staphylococcus aureus cells of P. aeruginosa culture supernatants for lysis.

The results showed only two isolates from 23 P.aeruginosa isolates that were collected from patients suffering from burns and UTIs were shows positive production for Staphylolysin A (LasA), depending on the appearance of the transparent aura around its colonies the isolate no.1 was the most productive of this enzyme, as it formed a transparent halo of about 14.16 mm, and Staphylolytic activity was extracted by cooling centrifugation and partially purified by ammonium sulphate precipitation at a saturation percentage of 80%, followed by Ion exchange chromatography using a Dowex-column with purification folds and recovery of 4.3 fold, while SEC gave 1.5 fold.

Keywords: *staphylolysin, Pseudomonas aeruginosa, Purification.*

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gammaproteobacterium found in different environmental niches such as soil and water. As an opportunistic pathogen, it also causes severe infections in mammals and other animals and in plants (Wallner et al., 2019).

During these infections, P. aeruginosa secretes many virulence factors, including pro-teases, toxins, phenazines, and pyocyanin, that neutralize the host immune defense and cause damage to organs (Bai et al., 2020).

Elastase is the most abundant endopeptidase, additionally, it exhibits an exceptionally strong substrate specificity and is capable of degrading a large number of host proteins (Al Maeni etal.,2021).

This involves elastin, a significant constituent of connective tissues that is resistant to hydrolysis by the majority of proteases, Elastase is thus considered a major virulence factor of P. aeruginosa, alkaline proteinase has a broad specificity for cleavage but is less

potent than elastase and lacks elastolytic activity (Veetilvalappil etal.,2022).

Elastase A, D (LasA , LasD) are staphylolytic enzymes that are extracellular proteolytic enzymes that are believed to be involved in the pathogenesis of this organism (Al Maeni etal.,2021).

This study aimed to isolate and purify of staphylolysin enzyme from P. aueroginosa.

Materials and Methods:

Patients and Specimens collection

One hundred clinical samples (50 mid-stream urine samples) were collected in sterile screw-cap containers from patients suffering from urinary tract infections (UTIs), (25 burns swabs) from burns patients and (25 wounds swabs) from injured patients whom attending to Baghdad Teaching Hospital, Ghazi Hariri Hospital, Central Laboratories in Medical City and Al-Yarmok hospital in Baghdad /Iraq, from March 2022 to June 2022. Each specimen was immediately transferred under cooling conditions to the laboratory for analysis.

Isolation and Identification of Pseudomonas aeruginosa

All burns and wounds swabs samples were cultured in a B.H.I. broth medium and incubated at 37 ºC for 24 hours to promote bacterial growth , likewise , urine samples but they firstly were centrifuged at 1,500 rpm for 5 minutes , then removing the supernatant, the pellet was cultured in a B.H.I. broth. After that they were streaked on the general and differential culture media, also incubated for 24 hours at 37 ºC. Lactose non- fermenting colonies that were streaked on MacConkey agar picked and re-cultured on fresh MacConkey agar plates to obtain pure well isolated colonies. The obtained colonies were streaked on cetrimide agar to investigate the produce of fluorescein and pyocyanin dyes and the plates were incubated for another 24hrs at 37ºC (Wanger et al., 2017).

The positive growth was diagnosed depending on morphological (Shape, size, margin, consistency and color of colonies), microscopical features and biochemical tests; also, we used vitek II system in the identification of bacterial growth.

Estimation of Staphylolytic enzyme assay.

Staphylolysin enzymes A and D activity was determined by measuring the capacity of the boiled Staphylococcus aureus cells of P. aeruginosa culture supernatants for lysis. A 30 ml volume of an overnight S. aureus culture was boiled for 10 minutes and then centrifuged at 5000 rpm for 10 minutes, the precipitate was taken to prepare the bacterial suspension. The activity of staphylolysin enzyme D, A determinate (Gustin et al., 1996), and protein concentration were estimated by lowery method (1951).

Determination of the activity of the enzyme staphylolysin A

Fifty µl of enzyme to 1 ml of thermally killed substrate (suspended cells S. aureus) in the Eppendorf tube of 1.5 ml. The reaction solution was mixed using a mixture and the absorbance was measured using a spectrophotometer at wavelength 595nm (Gustin et al., 1996).

Determination of the activity of staphylolysin enzyme D

Added 1 ml of enzyme to 1 ml of the substance thermally killed (suspended in cells of S .aureus) 1:1 percentage . The reaction mixture was incubated at 37°C for a period of more than3 hours. Absorbance was measured at a

wavelength of 595 nm each 30 minutes (Diggle et al.,2002).

The unit of activity of an enzyme (unit) is defined as the amount of enzyme that It causes a decrease in absorbance at wavelength 595 .A nanometer is one absorption unit per minute (Folders etal.,2000).

The effectiveness was estimated based on the following equation:

$$\text{Activity (units/ml)} = \frac{\text{Absorbance 595 nm} \times \text{total volume in the device cell (mL)}}{\text{(Decrease in the spectrometer reading per minute) } 0.001x \text{ Reaction time (min) x volume of enzyme (ml)}}$$

Purification

1. Ammonium Sulphate precipitation :

Crude enzyme was precipitated with 80% saturation of ammonium sulfate by adding 51.6 g from salt to 100 ml crude enzyme under cooled conditions with constant stirring, then leave the solution at a temperature of 4 degrees with constant stirring. The solution was centrifuged at 12000 rpm for 30 minutes at 4 ° C (Gustin et al., 1996).

Precipitates were dissolved in small amount of Tris buffer -HCl (0.02 M and pH = (7.5) ,then activity of enzyme; protein concentration and specific activity were calculated.

2-Dialysis

The dialysis process of the enzymes was carried out after precipitation with ammonium sulfate using dialysis bag with cut off 10-KDa and Tris buffer -HCl (0.02 M and pH = (7.5), at 4 C for 24 hours, with constant stirring.

3-Ion exchange chromatography

This step was done with Dowex -400 ion-exchanger column that was previously washed and equilibrated with a Tris -Hcl (0.02 mL) and pH = 7.5 at a flow rate of 2 mL/Minute . Enzyme was eluted with 1 M of NaCl gradient buffer Tris -Hcl (0.02 mL) and pH = 7.5 .All enzyme purification steps were performed in in cooled conditions. The absorbance for each fraction was measured at 280 nm., the protein concentration and the enzyme's activity were estimated(Gustin et al., 1996).

4- Size exclusion chromotography using Sephacryl-300

Purification of the enzyme using gel filtration chromatography with Sephacryl S-300 employed an elution buffer of Tris-HCl 0.02 M, pH 7.5. The protein pattern (A280) and activity of staphylolysin as calculated by Sephacryl S-300 column chromatography .

Gel filtration chromatography (Sephacryl S-300). 1- An active concentrated fraction sample was loaded over the Sephacryl S-300.

2- An equilibration buffer was used to collect the fractions

3- The flow rate was organized to be 30 ml/hr., and a fraction volume of 5 ml was collected.

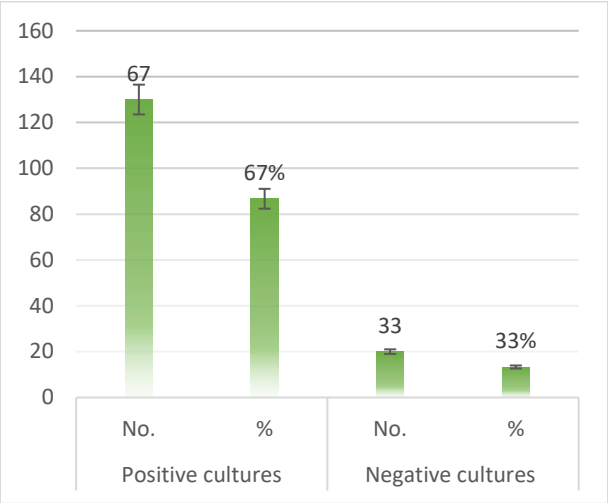
4-Absorbance at 280 nm was measured for each fraction peak (Al Maeni et al., 2021).

Results and Discussion:

Bacterial Isolates

In this study, 100 clinical samples (50 urine samples), (25 burns swabs) and (25 wounds swabs) were cultured on the nutrient agar, MacConkey agar plates, blood agar plates, CCA agar and cetrimide agar plates for isolation and identification of Pseudomonas aeruginosa, and 67% (67/100) samples showed positive bacterial cultures, whereas no growth was seen in the other 33% (33/100) samples.

Figure (1): Distribution of Samples according positive and negative bacterial cultures



Among 67 positive culture samples, only 34.3% (23/67) isolates belonged to P. aeruginosa, and 65.7% (44/67) isolates belonged to other genera of bacteria as show in table (1).

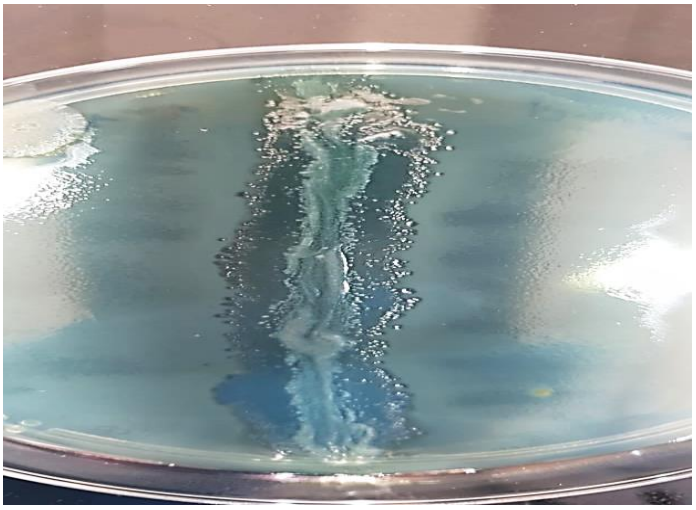
Table (2.1): Bacterial Isolates recovered from the Samples.

Bacterial Isolates	No.	%
Pseudomonas aeruginosa	**23**	**34.3%**
Others	**44**	**65.7%**
Total	**67**	**100%**

Screening of bacterial isolates for Staphylolysin A (LasA) production

Only two isolates from 23 P.aeruginosa isolates that were collected from patients suffering from burns and UTIs were shows positive production for Staphylolysin A (LasA). Tryptic soya agar + 0.2% (wt./vol.) of heat killed Staphylococcus aureus at temperature 100oC was the medium used for screening the activity of all isolated bacteria for Staphylolysin A (LasA) production. Depending on the appearance of the transparent aura around its colonies the isolate no.1 was the most productive of this enzyme, as it formed a transparent halo of about 14.16 mm when streaking on Tryptic soya agar and incubated for 24h (figure 2), while the isolate no.23 formed a transparent halo of about 9.48 mm.

Figure (2): The transparent halo)zone by P.aeruginosa no. 1 that had positive production for Staphylolysin A (LasA) grown on Tryptic soya agar + 0.2% (wt./vol.) of heat killed S. aureus at temperature 100oC at 37°C for 24 hrs.



The enzyme activity in different concentrations of ammonium sulfate is depicted in table 2, with staphylolysin exhibiting the highest specific activity at a fraction of 80% with a specific activity of 1.508 u/mg.The sedimentation step followed with ammonium sulphate, Tris-Hcl dialysis process 0.02 M, pH (7.5). Purification of the enzyme using the Dowex-400 column the activity of staphylolysin was 10.76, the purification folds were 4.3, and the enzymatic yield was 3.19 percent, as seen in figure (3). These findings corroborate the researchers' findings(Petersen etal.,2002, andAl Maeni etal.,2021).According to 4 , Purification of the enzyme using gel filtration chromatography with Sephacryl S-300 employed an elution buffer of Tris-HCl

0.02 M, pH 7.5.This figure(4) demonstrated that Specific activity to 29th with a ratio of 6.34 u/mg.

Table (2): The purification steps

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield %
Crude enzyme	250	10.8	8.449	1.278	2700	1	100
Precipitation with 80% saturation of (NH4)SO4	30	12.01	7.963	1.508	360.3	1.18	13.34
desalting by dialysis	15	12.51	6.802	1.839	187.65	1.43	6.95
Ion exchange chromatography (DEAE-Cellulose) and enzyme concentration by sucrose.	8	10.76	1.959	5.49	86.08	4.3	3.19
Gel filtrationSephacryl	5	66.5	10.2	6.34	333.5	1.5	9.81

Figure (3): Ion exchange chromatography

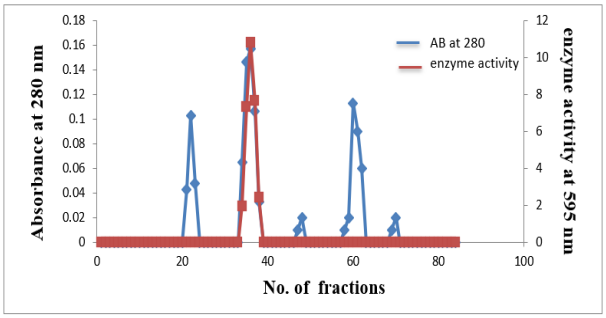
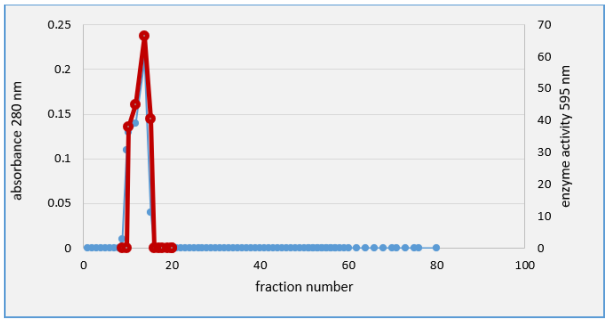


Figure (4): Chromatogram of staphylolysin from P. aeruginosa at molecular sieves column chromatography using Sephacryl S-300.



Staphylolysin is a secreted metalloendopeptidase that can lyse

Staphylococcus aureus cells by cleaving the pentaglycine bridges in their peptidoglycans, Moreover, it has the ability to break down elastin and cause cell-surface proteoglycans to shed, both of which have a role in the pathogenesis of P. aeruginosa infections(Biswas etal.,2022(

Many S. aureus strains have been shown to be lysed by the LasA protease, and S. aureus cell proliferation has also been demonstrated to be inhibited in vitro, this suggests that LasA protease may be yet another effective enzyme-based therapy for S. aureus infections, LasA protease is a minor virulence factor in experimental P. aeruginosa keratitis (Hotterbeekx etal.,2017).

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