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Separation and Purification of Hemolysin from Local Isolate of *Serratia marcescens*

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ABSTRACT

Twenty isolates of *Serratia marcescens* were isolated from inflammation of the urinary tract (UTI)., These isolates were found to produce hemolysin as indicated by blood agar plates in which the hemolysis of red blood cell indicate a positive result. Isolates were selected according to their hemolysis activity by measuring absorbance of hemoglobin at 405 nm that released from red blood cell. Hemolysin was completely purified using 50-75% saturation of ammonium sulphate followed by ion exchange chromatography with DEAE-cellulose then gel filtration chromatography by sepharose 4B. Accordingly molecular weight for the purified toxin was estimated as 45 KD.

Keywords: *Serratia marcescens,* Hemolysin, purification.

INTRODUCTION

Serratia marcescens is a gram-negative bacterium ubiquitous in nature that occurs in hospital environments and it is a nosocomial and opportunistic pathogen. Infections with this bacterium often cause septicemia, meningitis, endocarditis, and wound infections. The high and broad intrinsic resistance of this organism to various antibiotics makes *S. marcescens* infections difficult to treat [1]. Resistance of bacteria to antibiotics is generally attributed to alterations of the drug target, enzymatic modifications to reduced drug accumulation as a result of efflux pump-mediated antibiotics, or drug exclusion or a membrane barrier [2].

Patients with significant *Serratia* infections may have a variety of debilitating diseases such as diabetes mellitus, heart disease, renal insufficiency or malignant diseases. Relatively little is known about the virulence determinants of this bacterial species [3]. Clinical isolates of *S. marcescens* were shown to produce various exoenzymes [4]. A metalloprotease was described that induces pneumonia in laboratory animals, enhances vascular permeability through the activation of a Hageman factor-dependent kallikrein pathway *in vitro*, and causes fibrinolysis [5].

Recently, Braun [6] noticed that all *S. marcescens* strains rapidly lyses human erythrocytes in solution. These observations stood in contrast to the narrow lysis zone around *Serratia* colonies on blood agar. The cell-bound hemolysin activity required metabolizing Serratia cells. No hemolysis activity was found in the culture supernatants. Hemolysis might initiate human invasive nosocomial infections with subsequent spread and blood stream invasion [7].

Serratia marcescens synthesizes a hemolysin [8] encoded by the shlA gene. It also acts as a cytotoxin on epithelial cells and fibroblasts, where it causes ATP depletion and potassium efflux [9]. The ShlA toxin is secreted across the outer membrane by the ShlB protein, encoded by the ShlB gene [10], there by distinguishing this type of secretion from all other known secretion systems [11]. The aim of this research is isolation, and purification of hemolysin from local toxigenic isolates of *Serratia marcescens*.

MATERIALS AND METHODS

Microorganisms

Twenty isolates suspected to be *Serratia marcescens* were obtained from AL-Yarmok hospital, Baghdad

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 40°C, neuclease production, citrate utilization, motility, indol phenol oxidation reaction and liquefaction of gelatin, according to systematic key proposed by purges. Api-20E system (Bio Merieux-France) was also included as a confirmatory test system for identification of these isolates.

Hemolysis assay

For the determination of hemolysis in an exponentially growing bacterial culture, 20 ml of cultures of *S. marcescens* were incubated at 37°C to a time until an optical density at 578 nm of 0.5 was reached. From the sediment of washed erythrocytes, 0.66 ml was added to the culture, and hemolysis was determined after 15min incubation at 37°C. The bacterial cells and unlysed erythrocytes were removed by centrifugation, the supernatant was diluted to 1:100 in distilled water, and the absorbance at 405 nm of the released hemoglobin was measured [12].

Hemolysin production

Stock cultures were streaking on brain heart infusion agar plates, and incubated overnight at 37°C**,** and then isolated colonies were picked, streaked on brain heart infusion slants, and incubated overnight. The growth was scraped and suspended in brain heart infusion broth 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 slant. After overnight incubation at 37°C, loopfull growth from this slant was suspended in brain heart infusion broth and used to inoculate 100 ml of brain heart infusion broth. Cultures were centrifuged for 20 min at 10,000 rpm, and supernatant was taken for purification.

Protein concentration

Cultures were grown in flasks containing 100 ml of brain heart infusion broth medium. 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 the coomassie solution was added, mixed well and left for 5 minutes, the absorbance was measured at 595 nm. Standard curve of BSA (bovine serum albumin) was used to calculate protein concentration [13].

Purification of Hemolysin

Precipitation by ammonium sulphate

The supernatant (crude extract) was fractionated with ammonium sulphate at (0-25, 25-50, 50-75 and 75-100) % saturation, maximum precipitation of the toxin was obtained at 50-75 % saturation and then the precipitant was separated by centrifugation at 10,000 rpm for 20 min. The precipitant was resuspended in 10 ml Tris-HCL buffer.

Ion exchange chromatography

DEAE-cellulose ion exchange column was prepared according to (14). The sample obtained with 50-75 % ammonium sulphate saturation, was dialyzed in distilled water for 24 hr., the concentrated protein solution then applied to DEAE-cellulose column (3.5x7 cm) previously equilibrated with Tris-HCL buffer pH 8, the column was washed with the same buffer and eluted with a linear salt gradient with the same buffer containing (0-1) M NaCl. The fractions were collected in test tubes at flow rate of 100 ml/ hr. Protein concentration in each fraction was monitored by spectrophotometer at 280 nm. Fractions of the protein peaks were assayed for hemolysin. Fractions containing hemolysin was collected and concentrated for measurements.

Gel filtration chromatography:

Determination of the void volume (Vo) of the column

Sepharose-4B column (77.5×1.5 cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden). The column was equilibrated overnight with 0.01 M Tris-HCl buffer pH 8.0 with a flow rate of 50 ml/hour. 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.

Determination of hemolysin elution volume (Ve)

Sepharose-4B column (77.5×1.5 cm) was prepared, packed and equilibrated for a second time .A 5 ml of purified hemolysin sample was passed through the column carefully, and equilibrated with 0.01 M Tris-HCl buffer pH 8.0, with a flow rate of 50 ml/hour. Fractions of 5 ml were collected. The elution volume (Ve) was estimated for the separated fractions of purified Hemolysin, by following the absorbency at 280 nm.

Measurement of standard proteins elution volume (Ve)

Different standard proteins were applied through sepharose-4B column and then eluted with 0.01 M Tris-HCl buffer pH 8.0, with a flow rate of 50 ml/hour, as shown in table (1).

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 Hemolysin , 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. The Hemolysin-molecular weight was accordingly calculated.

Table 1. Molecular weight of standard proteins

Standard proteins	Molecular weight (Dalton)
Inuline	5000
Casein	23600
Bovine serum albumin	67000
Catalase	232000

RESULTS AND DISCUSSION

Screening of isolates for hemolysin:

Twenty isolates were screened for their productivity of hemolysin as detected by determining of hemolytic activity spectrophotometrically at 405 nm. Results shown in table (2) indicate that isolate Sm 11 gives the highest absorbance; accordingly it was selected for further experiments.

Table 2. Production of *S. marcescens* for hemolytic activity

No. of isolate	O.D at 405 nm
Sm 1	0.4
Sm 2	0.5
Sm 3	0.4
Sm 4	0.6
Sm 5	0.8
Sm 6	0.7
Sm 7	0.6
Sm 8	0.2
Sm 9	0.4
Sm 10	0.7
Sm 11	0.9
Sm 12	0.7
Sm 13	0.5
Sm 14	0.8
Sm 15	0.3
Sm 16	0.7
Sm 17	0.6
Sm 18	0.4
Sm 19	0.4
Sm 20	0.6

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 (0-25, 25-50, 50-75, 75-100) % saturation, the saturation ratio of 50-75% was chosen which give highest precipitate. 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 lead to the precipitation of the protein by the effect of salt [15 , 16]. Ammonium sulphate is widely used because of its availability, high solubility, low cost and it stabilizes the protein [14].

Ion exchange chromatography

Purification of hemolysin was done by ion-exchange chromatography using (DEAE-cellulose). Fig (1) showed the wash and elution peaks for precipitated products of isolate Sm 11. No hemolysin was detected in the wash steps, while the eluted fractions revealed. The presence of two peak indicate that we have two purified proteins but Only one peak for elution shows activity as detected spectrophotometrically by measuring absorbance of hemoglobin at 405 nm, which in turn indicate hemolytic activity. Results show that as much as 29 µg/ml hemolysin was quantified as estimated by comparison of absorbance (0.82) with that of standard protein.

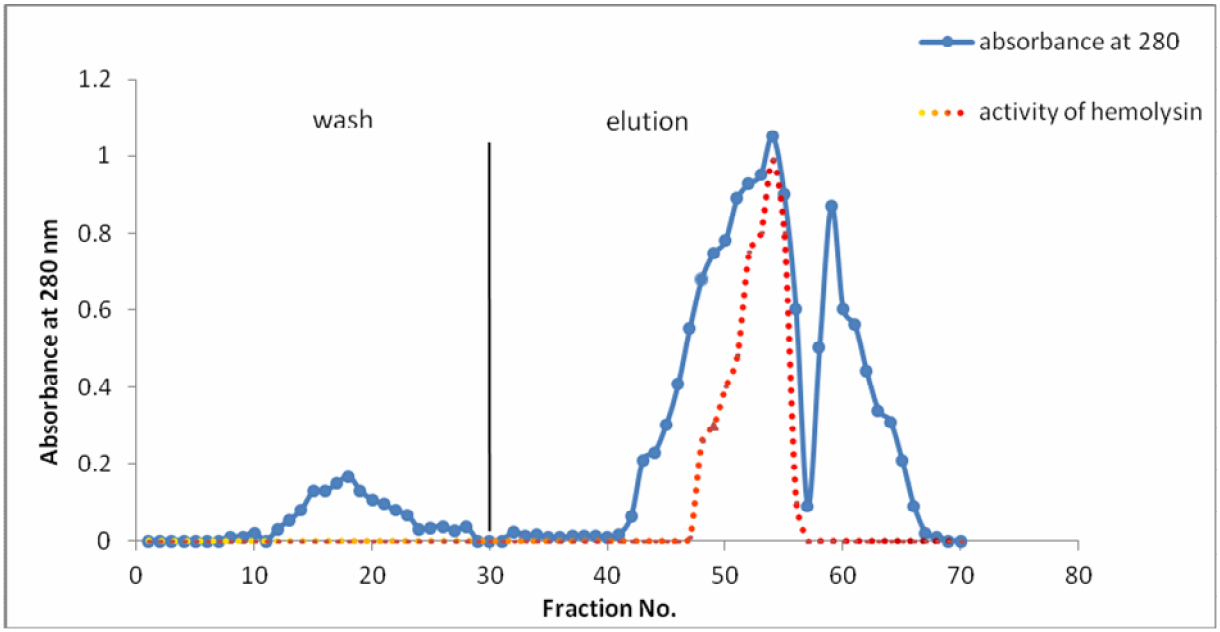

Figure 1. Purification of Hemolysin separated from culture liquid of isolate Sm 11, by DEAE-cellulose ion exchange chromatography column (3.5x7)cm equilibrated with 0.01 M Tris-HCL buffer PH 8, hemolysin eluted with linear salt gradient 0 - 1 M NaCl, flow rate 50ml/hr.

Detection of hemolysin

Detection of hemolysin in fractions eluted from ion-exchange chromatography was done by measuring absorbance at 405 nm of released hemoglobin from red blood cell incubated with the toxin as reported by Ralf [12] who used this procedure for detecting hemolytic activity, it was shown that as much as 22 µg/ml of hemolysin was quantified as estimated by comparison of absorbance (0.71) with that of standard protein.

Purification by gel Filtration chromatography

The two eluted proteins passed through Sepharose-4B then fractionated on the gel fractions were collected up to 40 fractions. Hemolysin was present in fractions 24-31 depended on measuring of activity and increase absorbency of fractions. The result of purification showed one-peak as indicated in figure 2.

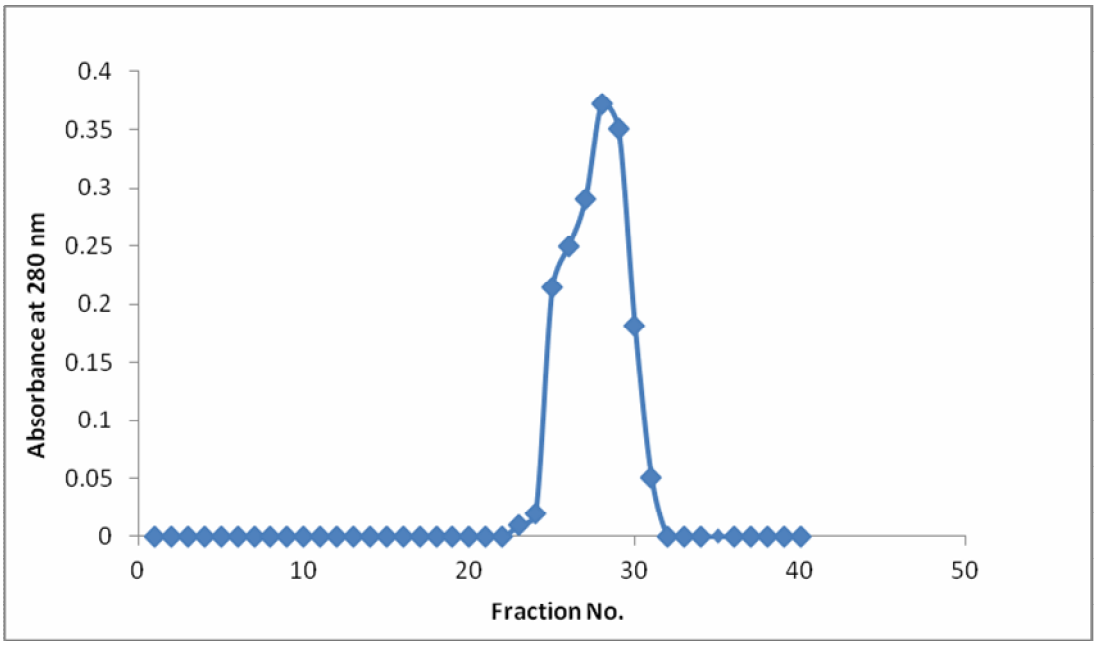

Figure 2. Purification of hemolysin by using Sepharose-4B column (77.5×1.5 cm) with flow rate of 50 ml/hour.

Determination of molecular weight of hemolysin

Molecular weight of hemolysin was determined using Sepharose-4B column (77.5×1.5 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 hemolysin. The ratio of the elution volume of each standard protein as well as the separated fractions of the purified hemolysin, to that of void volume of the blue dextran 2000 was calculated. Results shown in figure (3), indicate that the (Ve/Vo) ratio of purified Hemolysin was about (45 KDa), the ratio of (Ve/Vo) of each standard protein to the log molecular weight of each standard protein was plotted. Tiwari [17] reported that molecular weight of hemolysin also estimated as (45 KDa) by using ammonium sulphate fractional precipitation and gel filtration through Sephadex G-75 column. Homogeneity was determined by gel electrophoresis and purified hemolysin was tested for its stability and other characteristics. Other published paper mentioned that molecular weight of hemolysin estimated as (165 KDa) [18]. These differences are possible to be due to the purification method used in the purification of the toxin.

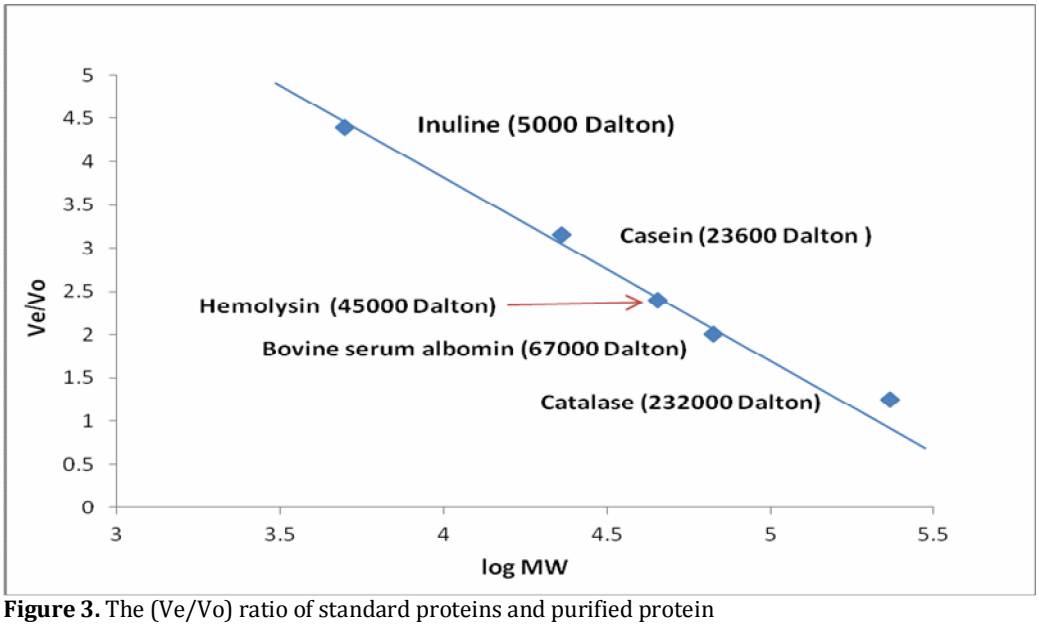

Figure 3. The (Ve/Vo) ratio of standard proteins and purified protein

S. marcescens is known for its production of exoproteins, yet a hemolytic activity identified in this organism was demonstrated to be cell associated. This stands in contrast to the majority of bacterial hemolysin reported to date, which are secreted extracellularly [18]. In the present study, the molecular weight of the *S. marcescens* hemolysin was estimated as 45 KDa which is mimicking that reported by Braun [10].

CONCLUSION

S. marcescens was shown to have the ability to produced different exotoxin and hemolysin is shown to be one of them. Isolation and complete purification was done to hemolysin to determine the molecular weight of it and it was 45 KDa. Purified hemolysin can be used in further studies.

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REFERENCES

1. Hideaki M. ,Yumiko H., Rumi K., Akihiro S. et al (2009). Mutational Upregulation of a Resistance-Nodulation-Cell Division-Type Multidrug Efflux Pump, SdeAB, upon Exposure to a Biocide, Cetylpyridinium Chloride, and Antibiotic Resistance in Serratia marcescens . American Society Microbiology, 53: 5230-5235.
2. Poole, K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. J. Appl. Microbiol., 92:55S-64S.
3. Clegg, S., S. Hull, R. Hull, and J. Pruckler. (1985). Construction and comparison of recombinant plasmids encoding type 1 fimbriae of members of the family Enterobacteriaceae. Infect. Immun. 48:275-279.
4. Matsumoto, K., H. Maeda, K. Takata, R. Kamata, et al (1984). Purification and characterization of four proteases from a clinical isolate of Serratia marcescens. J. Bacteriol., 157:225-232.
5. Traub, W. H., and D. Bauer. (1985). Fibrinolytic activity of purified Serratia marcescens metalloproteases. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 260:35-40.
6. Braun, V. H.; Gunther, B. NeuBl, et al (1985). Hemolytic activity of Serratia mnarcescens. Arch. Microbiol., 141:371-376.
7. Traub, W. H. (1982). Virulence of nosocomial isolates of Serratia mnarcescens for NMRI mice: correlation with 0-antigens 06 and 014. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 252:360-369.
8. Bernheimer, A.W. (1988). Assay of hemolytic toxins. Methods Enzymol., 165: 213-217.
9. Hertle R, Hilger M, Weingardt-Kocher S. et al (1999). Cytotoxic action of Serratia marcescens hemolysin on human epithelial cells. Infection and Immunity, 67: 817-825.
10. Braun, V., B. Neuss, Y. Ruan, E. et al (1987). Identification of the Serratia marcescens hemolysin determinant by cloning into Escherichia coli. J. Bacteriol. 169:2113–2120.
11. Braun, V., R. Schönherr, and S. Hobbie. (1993). Enterobacterial hemolysins: activation, secretion and pore formation. Trends Microbiol., 1:211-216.
12. Ralf. H, Martin. H, Sandra. W. (1999). Cytotoxic Action of Serratia marcescens Hemolysin on Human Epithelial Cells. Infection and immunity, 67: 817–825.
13. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
14. Whitaker, J. R. and Bernard, R. A.(1972). Experiment for introduction to enzymology. The Wiber Press Davis. Inc. New York, USA. Segel, I. H. (1976). Biochemical Calculations, 2nd edition, John and Sons. Inc. New York.
15. Matthew P., Nancy S. Taylor, et al (1977). Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immu., 3(17):776-780.
16. Micheal, J. Bjorn, Barbara h. Iglewiski, and Sylvia K. Ives. et al (1987). Effect of Iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immu., 3(19):785-791.
17. Tiwari RP ,Singh J, Grewal JS, et al. (2000). Purification and characterisation of haemolysin from Serratia marcescens B-91. Indian J Med Res. 112:186-90.
18. Keith, P., Elmar, S.and Volkmar, B.(1988). Molecular Characterization of the Hemolysin Determinant of Serratia marcescens. J. Bacteriol., 170:3177-3188.

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