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PRODUCTION AND PURIFICATION OF APHLA- HEMOLYSIN FROM *PROTEUS MIRABILIS* PMBS41 FROM URINARY TRACT INFECTION

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ABSTRACT

The *Proteus mirabilis* pathogenic isolates (53 isolates), which isolated for urine samples from patients suffering from urinary tract infection (UTI) were found to produce hemolysin as indicated by greenish zone around colonies grown on blood agar plates. Ten isolates were screened for their production and the highest producing isolate PMBS41 which gave (42.734 Unit/ml) was selected for further experiments. Hemolysin was completely precipitated using 25-50% saturation of ammonium sulphate which gave enzyme activity (128.20unit/ml). Then followed by ion exchange chromatography using DEAE-cellulose (3.5 x 15 cm) gave only one of the many peaks showed hemolytic activity gave (48.42 unit/ml) and a yield (45.42 %), then gel filtration using Sepharose 6B column (1.5 x85 cm) gave (42.24 unit/ml) and a yield (14.90 %).

KEYWORDS: Purification of hemolysin, *Proteus mirabilis* PMBS41, DEAE-cellulose, Sepharose 6B.

INTRODUCTION

Proteus mirabilis is a Gram-negative bacterium which is well-known for its ability to robustly swarm across surfaces in a striking bulls'-eye pattern. Clinically, this organism is most frequently a pathogen of the urinary tract, particularly in patients undergoing long-term catheterization.

The urinary tract infection (UTI) is defined as infection or colonization of the urinary tract (urethra, bladder, ureter and kidney) by microorganisms. The most common bacterial uropathogens in UTI are*: Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis* and *Enterobacter cloacae.*[1]

The routine use of antibiotics leads to the prevalence of antibiotic resistance and development of antibiotic resistance genes particularly, within gram negative organisms and this consider as the most serious problems in the field of medicine.[2, 3] In *P. mirabilis* the antimicrobial resistance is increasing, such as the resistance to extended-spectrum cephalosporin's due to the production of extended-spectrum β-lactamases (ESBLs), which causes epidemiologic effect of *P. mirabilis* bacteremia.[4]

P. mirabilis produces hemolysin HpmA, that used to damage the kidney tissues. This hemolysin is associated to the cell, calcium-independent, former of pores, Encodes by two genes, *hpmA* and *hpmB*, that codify the HpmA (166 kDa) and HpmB (63 kDa) proteins, respectively.[5]

HpmA hemolysin is responsible for tissue damage and is activated when its N-terminal peptide is cleaved, resulting in active HpmA (140 kDa), and HpmB is responsible for HpmA activation and transport.[6, 5] The HpmA haemolysin toxin of *Proteus mirabilis* production is upregulated co-ordinately with the synthesis and assembly of flagella during differentiation into hyperflagellated swarm cells.[7] The levels of hemolysin in *P. mirabilis* correlate with its ability to invade cultured kidney cells, and an isogenic *P. mirabilis hpmA* mutant is minimally invasive in cultured cells.[8, 9] The aim of the study is to production and purification of Alpha Hemolysin form *P. mirabils* PMBS41 isolate from patients with urinary tract infections.

MATERIALS AND METHODS

Alpha-hemolysin was extracted from the selected local producer isolate of *P. mirabils* by centrifugation, applied on an 24 hrs cultured at 37°C of brain heart infusion broth, at 10000 xg for 20 min at 4°C for the removal of cells from the bacterial culture medium. The cell-free supernatant fluid was decanted and used as crude extract.

The hemolytic activity assay was determined using Senior and Hughes[9] methods with some modification as follow. 0.1 ml of an overnight nutrient-broth culture of each strain was added to 10 ml of BHI broth and incubated with shaking at 37°C, small amount of samples (150µl) were removed into micro-centrifuge tubes containing 1 ml of a washed suspension of red blood cells 2% v/v in saline with and without 150 mM $CaCl_2$. The tubes were incubated with gentle agitation in a water bath at 37°C for 1hr., after that the tubes were then centrifuged at 13000 rpm for 1 min. to pellet the red blood cells and bacteria. The amount of free haemoglobin in the supernate (measured by its absorbance at 540 nm) indicated the degree of hemolytic activity. Protein concentration was carried out using the Bradford.[11]

The optimal calcium chloride concentration that effected on hemolytic activity

To the 0.1 ml of an overnight nutrient-broth culture of each strain was added to 10 ml of BHI broth and incubated with shaking at 37°C, small amount of samples (150µl) were removed into micro-centrifuge tubes containing 1ml of a washed suspension of red blood cells 2% v/v in saline with different concentration of $CaCl_2$ (20,50,100,150,200,250 and 300mM), all tubes were incubated at 37^{o}C for 1 hr. after that centrifuged at 13000 rpm for 1 min, The absorbance of Supernatant was monitored at 540nm and that indicated the degree of hemolytic activity.

The optimal incubation time of *P.mirabilis* isolates that effected on hemolytic activity

To the 0.1 ml of an overnight nutrient-broth culture of each strain was added to 10 ml of BHI broth and incubated with shaking at 37°C, small amount of samples (150µl) were removed into micro-centrifuge tubes containing 1ml of a washed suspension of red blood cells 2% v/v in saline with 150m $CaCl_2$. After that the tubes were incubated with different time (15, 60, 90, 120 minute) a water bath at 37 ^{o}C. The tubes were then centrifuged at 13000 rpm for 1 min. The absorbance of Supernatant was monitored at 540nm and that indicated the degree of hemolytic activity.

Precipitation of Protein by Ammonium Sulphate.[13]

The supernatant (crude extract) was fractionated with ammonium sulphate at (0-25, 25-50, 50-75, 75-100) % saturation. This was done by adding the salt slowly to the crude extract with continuous agitation. The precipitated product of 25-50% saturation was used to obtain complete precipitation of the hemolysin, and then the precipitant was separated by centrifugation at 10000 xg at 4ºC for 30 min then the precipitate was taken and dissolved in a

minimal amount of 10mM Tris-base buffer (pH 8.0) and dialyzed overnight against The same buffer.

Purification of Bacteriocin by Ion Exchange Chromatography

The exchanger DEAE-Cellulose was prepared and packed into a column according to Whitaker and Bernhard [14], as follows: 10 mM Tris-base buffer pH 8.0 after that pour the slurry in to the column 3.5x7.5 cm, then the column equilibrated with the same buffer overnight. Partially purified concentration hemolysin 10 ml were separately passed after loaded onto the column carefully. Then 100 ml of 10 mM Tris-base buffer pH 8 was added. Flow rate estimated as 50 ml / hr. proteins were eluted by using 200 ml of a gradient from 0.5was 0-1 M sodium chloride. Fractions of 5ml were collected and absorbency was monitored at 280 nm. Protein concentration and Hemolytic activity were determined for the collected active fraction was carried [11, 9] respectively. The presence of the Hemolysin was estimated from each fraction of the major peaks. Hemolytic activity was then determined for the collected active fractions.

Purification of Bacteriocin by Gel Filtration Chromatography

Sepharose 6B column 80 x 2.5cm was prepared and packed according to Stellwagen [12], the column was equilibrated with 10mM Tris-base buffer pH 8.0 at a flow rate of 50 ml / hr. A 5 ml sample of each concentrated partially purified hemolysin was added to the column. Elution's of proteins were done with the application of 200ml of 10 mM Tris-base buffer PH 8.0. A 5 ml fraction was collected for each hemolysin then protein concentration was estimated by measuring the absorbency at 280 nm. The beaks for each hemolysin were determined by plotting the absorbency of protein fraction versus the elution volumes. Hemolysin activity was determined for each fraction of the major peaks.

RESULTS AND DISCUSSION

The production of hemolysin from *P.mirabilis* of the isolates has been done. From fifty three isolates were screened for their production of hemolysin as detected by determining the hemolytic activity was monitored at 540nm after examined by culturing on blood agar plates and appear greenish area as a results of alpha hemolysin production, but these results were not ascribed to hemolytic activity these agreement with Senior *et al.*[9] The results shown in table (1) indicate that the hemolytic activity of isolates (PMBS3, PMBS10, PMBS14, PMBS26 and PMBS29) are very close to each other, but isolate PMBS41 gave the highest

hemolytic activity (42.734 U\ml), which means that this isolate has the highest ability to lyse red blood cells due to highest production of hemolysin, and it was selected for further tests.

Table (1): The most efficient bacterial isolation in hemolysin production.

Bacterial isolate	Hemolytic activity (U\ml)
PMBS 3	36.123
PMBS 10	31.718
PMBS 14	37.885
PMBS 19	25.991
PMBS 24	20.704
PMBS 26	31.720
PMBS 29	32.158
PMBS 34	22.466
PMBS 37	27.753
PMBS 41	42.734

SURVEY OF THE OPTIMAL CALCIUM CHLORIDE CONCENTRATION USED Detection Of Hemolytic Activity Of *P.mirabilis*

P.mirabilis hemolysin like *Serratia*-type calcium-independent hemolysin, but unlike *E.coli* calcium dependent hemolysin type as mentioned by Al-Shammai.[15] In this study investigate the optimal $CaCl_2$ concentration that used in the detection of hemolysis activity by *P.mirabilis* and the results of survy after used seven $CaCl_2$ concentration show that the most efficient concentration was 150mM see figure (1), these results was not agree with Senior and Hughes,[9] that used 20mM of $CaCl_2$ in haemolytic activity detection but these concentration is not the optimal for the hemolysin produced by Local isolates of *P.mirabilis*.

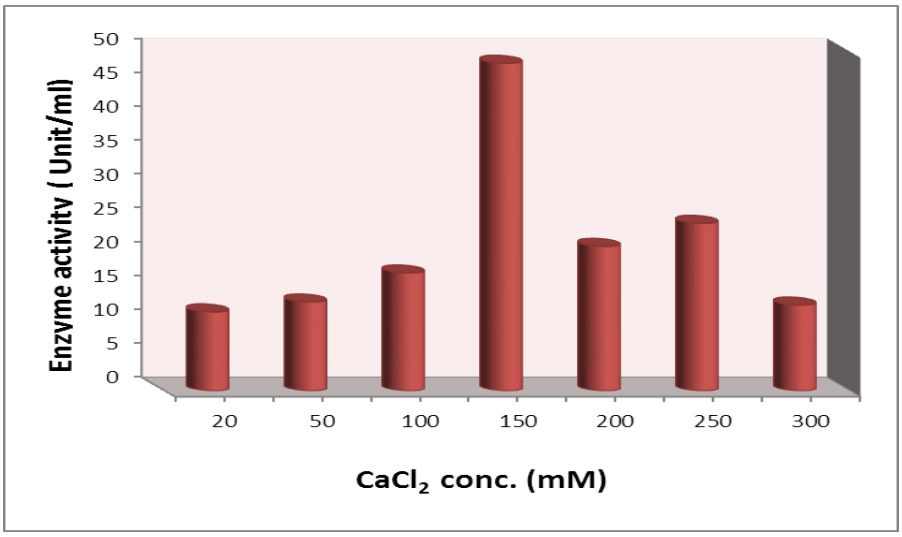


Figure (1): The optimal concentration of $CaCl_2$ used in detection of hemolysin production by *P.mirabilis* PMBS41.

The hemolysin of *P.mirabilis* produced at mid-to-late logarithmic phase growth Senior and Hughes.[9] In this study investigate the most efficient incubation time for production and activity of hemolysin and the results of used of 15, 60 and 90 minutes. and 120 min. show that the optimal incubation time in 60 min. see figure (2), these results is not agree with the incubation time demonstrate by [9] was 15 min.. This the difference in the incubation time for the production and activity of hemolysin because the hemolysin production by local isolates of *P.mirabilis* isolation from the UTI patient.

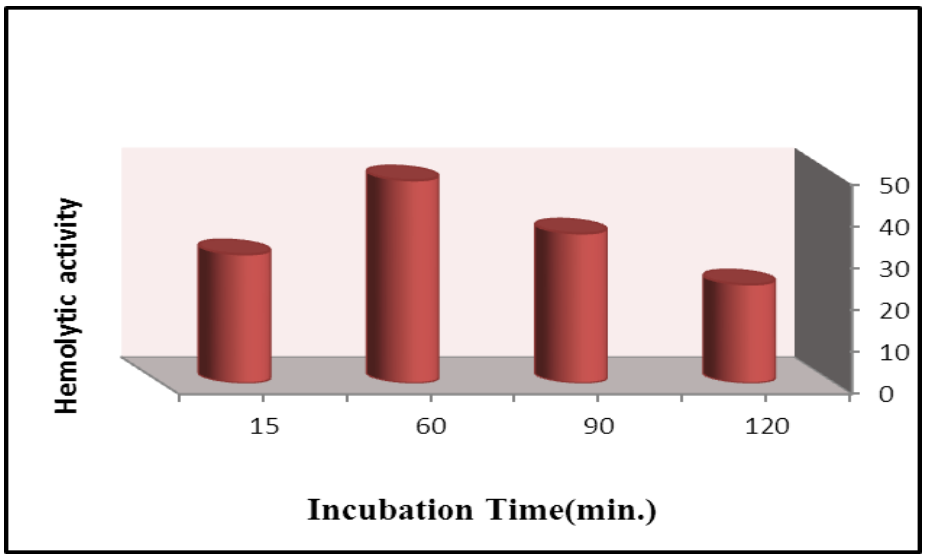


Figure (2): The optimal incubatiom time for the production and activity of hemolysin of *p.mirabilis* PMBS41.

Alpha- Hemolysin Purification

Several procedures were developed for the purification of bacterial exotoxin depending on (the bacterial type, chemical structure and the purpose of purification).To purify α-hemolysin produce from *P.mirabilis* strain PMBS4, three purification steps were set up to obtain purified α–hemolysin for further study of its characterization. These steps were reduced to get as much as possible minimum loss of the amount the hemolysin produced. These purification steps include the following:

Ammonium Sulfate precipitation

Precipitation of α-hemolysin by ammonium sulfate is a useful method of concentration and is ideal as an initial step in purification.[16] In order to concentrate the crude extract of toxin and remove as much as possible water, ammonium sulfate was used at (0-25 , 25-50 , 50-75 , 75-100) % saturations, the saturation ratio of 25-50% was chosen which gave the highest precipitates of α-hemolysin as well which agrees with Rennie And arbuthnott[17] who

reported that the ratio of 50% ammonium sulfate saturation was sufficient for complete precipitation of α-hemolysin and no loss of activity was observed. This step allows the salting out of molecules from water. Since ammonium sulfate 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.[18] The advantages of ammonium sulfate are at saturation; it is of sufficiently high molarity that it causes the precipitation of most proteins.[19] Ammonium sulfate is widely used because of availability of ammonium sulfate, high solubility, cheapness, lack of toxicity to most enzymes and proteins, and its stabilizing effect on some enzymes. Its large-scale use, however, is limited as it is corrosive except with stainless steel.[16, 20]

Purification of α–Hemolysin by Ion-Exchange Chromatography

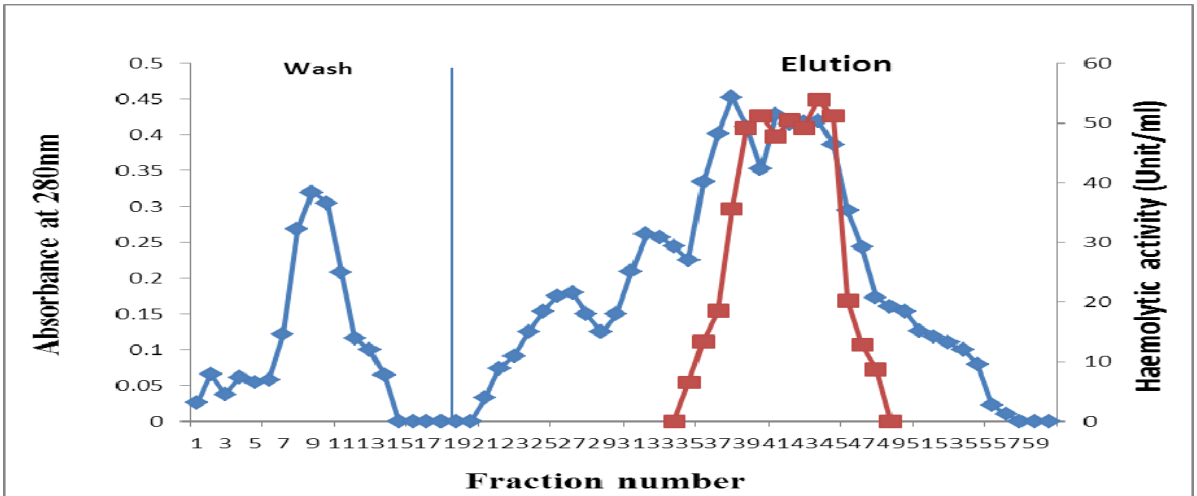
Two types of proteins can be distinguished the difference between them being a single amino acid. The concentrated protein from an ammonium sulfate precipitation step passed to the ion exchanger column (DEAE-Cellulose) a negative exchange. An ideal buffer should be in the physiological pH range of 6.0 to 8.0. At this pH range, most of the proteins have been observed to be negatively charged. Hence, proteins would bind to positively charged molecules of the resin. Change in the buffer pH condition could make the protein relatively positive, thereby allowing it to bind to a negatively charged resin material. Among the most commonly used charged molecules are DEAE-Cellulose.[21] This is used to separate acidic and neutral proteins.[16] The protein is loaded onto this packed column and is allowed to bind.

The column was washed and the bound proteins are eluted depending on their tightness of binding, by subjecting them to either increasing concentrations of salt or changes in pH as well as proteins with low charge will elute at the first.[21]

Pure α–hemolysin was obtained by using buffer solution at concentration of 0.01M Tris – base pH 8.0. Absorbance for washing portions was measured at 280 nm upon the arrival of absorbance to the line of zero (line base), after that recovery of protein operation associated with the ionic exchanger was conducted (proteins that carry a negative charge). The recovery was by using the same buffer mentioned above with the gradient NaCl (0 -1M).

Ionic exchange chromatography results showed that four peaks appeared when reading absorbance at a wavelength of 280nm figure (3), but only one peak for elution shows activity

as detected by was monitored at 540nm, which in turn indicates hemolytic activity. The fractions were collected, protein concentration, the hemolytic activity and specific activity are estimated. The result shown in table (2) indicated that the amount of 48.34 U/ml, 5.31 with purification fold of 5.60 and the yield of the α–hemolysin is 45.24%.



Frgure (3): Ion Exhange chromatgraphy for α–hemolysin for *Proteus marb.* PMBS 41 through DEAE-Cellulose (3.5 x 15 cm) . The column was calibrated with Tris -base pH 8.0 . flow rate 60ml/hrs and 5 ml fraction.

Purification by Gel Filtration Chromatography

The eluted protein was passed through Sepharose-4B column then fractionated on the gel. Figure (4) demonstrates that the fractionation yielded three protein peaks as absorbance at 280nm and determined for α–hemyltic activity at 540nm, the most activity came between (24-26), the specific activity reached (927.5 Unit/mg protein), purification fold is 9.77 and a yield (19.53%) as mentioned in table (2) and figure (4).

Table (1): Purification steps of Alpha-Hemolysine Produced from *Proteus mirabilis.* PMBS 41.

Steps	Volume (mls)	Hemolytic activity (unit/ml)	Protein (mg/ml)	Specific activity (Unit/mg)	Total unit	Yield (%)	Purification fold
Crude	100	42.73	0.45	94.9	4273.4	100	1
Ammonium Sulphate (25-50%)	25	128.20	0.35	366.29	3205.05	75	3.86
Ion-exchange DEAE-Cellulose	40	48.34	0.10	531	1933.6	45.24	5.60
Gel-filtration Sepharose-6B	15	55.65	0.06	927.5	834.75	19.53	9.77

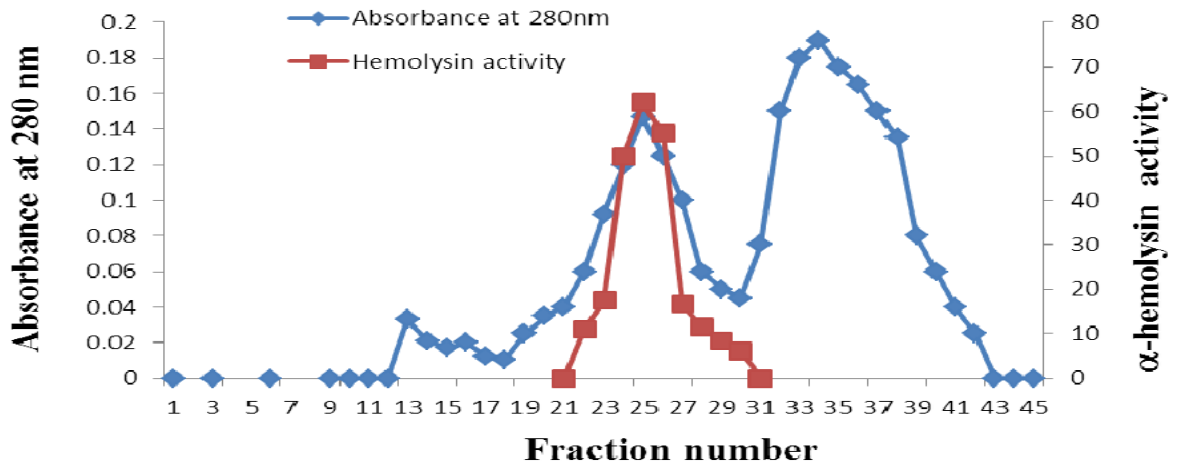


Figure (4) : Gel filtration chromatography for purified aphal-haemolysine from *Proteus mirabilis* . PMBS 41 by using Sepharose 6B column (2.5x 80)cm. The column was calbrated with Tris-base pH 8.0 ; flowrate 50ml/hr. and 5 ml/fraction.

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