Purification and Characterization of Hemolysin Produced By a Local Isolates of *Staphylococcus Aureus*

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Summary

Background: *Staphylococcus aureus* is a ubiquitous bacterium that is generating increasingly bad press coverage due to its propensity to adopt a pathogenic lifestyle in hospital and community settings. *S. aureus* colonies are found in approximately 30% of the general population. It colonizes the skin readily and can lead to a wide range of pathological conditions from skin lesions to osteomyelitis, endocarditis, and septicemia. Hemolysins are extracellular toxic proteins which are produced by many gram negative (e.g. *Escherichia coli*, *Serratia* spp., *Proteus* spp., *Vibrio* spp., *Pasteurella* spp., *Pseudomonas aeruginosa*) and gram positive bacteria (e.g. Streptococcus spp., *Staphylococcus aureus*, Listeria spp., *Bacillus cerius*, *Clostridium tetani*), all of which possess a certain pathogenic potential. Hemolysins have been therefore always considered as virulence factors. Most hemolysins cause lysis of erythrocytes by forming pores of varying diameters in the membrane and are designated as such because they have the ability to lyse red blood cells (RBCs).

Objectives

1-Purification of hemolysin from a local isolate of *S. aureus*.
2-Characterization of hemolysin produced by a local isolate of *S. aureus*.

Methods: Bacterial samples were identified by subjecting them to the standard laboratory procedures while semi quantitative screening on blood agar (containing 5% human blood) revealed that all isolates were hemolysin producer but in different efficiencies. Hemolysin was extracted by cooling centrifugation and purified by many steps including: precipitation by ammonium sulphate, dialysis, ionic exchange chromatography by using DEAE-Cellulose, and gel filtration chromatography by using Sephadex G-100. The molecular weight of hemolysin was determined by gel filtration chromatography on Sephadex G-100 while the optimum pH and temperature for hemolysin stability were also determined.

Results: The results showed that forty isolates out of 100 were identified as *Staphylococcus aureus*. Hemolysin was extracted by cooling centrifugation and purified by many steps including: precipitation by ammonium sulphate with 50-75% saturation percentage, dialysis, ionic exchange chromatography by using DEAE-Cellulose, and gel filtration chromatography by using Sephadex G-100. The results showed that hemolysin was purified 135 fold with a yield of 1.16%.The molecular weight of hemolysin determined by gel filtration chromatography on Sephadex G-100 column was about 35000 daltons, while the optimum pH for enzyme stability was 7 and the optimum temperature for enzyme stability was between 25-35^{o}C.

Conclusions

1.Conventional methods can be performed to extract hemolysins.
2.Hemolysin was maximally produced when the pH was near neutrality and incubation temperature was 37^{o}C and this conclusion indicates that hemolysin was produced when the conditions were similar to that of the host.

Key words: Hemolysin, ammonium sulphate precipitation,ion exchange chromatography, gel filtration chromatography

Introduction

Staphylococcus aureus is a ubiquitous bacterium that is generating increasingly bad press coverage due to its propensity to adopt a pathogenic lifestyle in hospital and community settings [(1)]. *S. aureus* colonies are found in approximately 30% of the general population [(2)].Hemolysins are extracellular toxic proteins which are produced by many gram negative (e.g. *Escherichia coli, Serratia* spp., *Proteus* spp., *Vibrio* spp., *Pasteurella* spp.,*Pseudomonas aeruginosa*) and gram positive bacteria (e.g. Streptococcus spp., *Staphylococcus aureus,* Listeria spp., *Bacillus cerius, Clostridium tetani*), all of which possess a certain pathogenic potential. Hemolysins have been therefore always considered as virulence factors. Most hemolysins cause lysis of erythrocytes by forming pores of varying diameters in the membrane and are designated as such because they have the ability to lyse red blood cells (RBCs). Many hemolysins can also attack – probably by a similar mechanism – other mammalian cells. Due to this cytolytic effect, they are also termed cytolysins [(3)].

Materials and methods

Staphylococcus aureus were identified according to the laboratory standard techniques relying on biochemical tests and identification was verified using API test. Afterwards, bacterial cells were harvested by cooling centrifugation at 7000 rpm at 4^{o}C for 15 minutes, and then the supernatant was collected in sterile test tubes. Crude extract was treated with ammonium sulphate to precipitate the intended protein. The column of ion exchange chromatography (DEAE-Cellulose) was prepared according to Schutte *et al.*,[(4)] and was equilibrated by adjusting its pH to approximately 7 through suspending overnight in 0.2M PBS. Fractions were collected and absorbance was measured at 280 nm; the tubes resembling the protein peak were collected and concentrated against sucrose and the resultant sample was applied onto sephadex G-100 (gel filtration column), absorbance at 280 nm was also measured and dialysis against sucrose was also done for the collected fractions that constituted the prominent protein peak. For the characterization of hemolysin several experiments were done regarding the optimum temperature and pH for hemolysin stability and also the determination of its molecular weight. Several incubation temperatures and multiple degrees of pH were tested for their effect on the purified hemolysin; while the method of Hu & Mobley,[(5)] was applied for the determination of the molecular weight of hemolysin. Protein concentration and enzyme activity was determined throughout the study by the methods of Burtis & Ashwood, [(6)]; Namadari & Botton,[(7)] respectively

Results

The results showed that only 40 specimens were identified as *S. aureus*, reflecting a total percentage of 40%. Table 1 shows the ranking of *S. aureus* being identified to be the causative agents of each type of the infections mentioned in the same table.

Hemolysins assays:

Hemolysin activity was measured by semi-quantitative screening method and the results demonstrated the appearance of clear zones of hemolysis after the end of incubation period around the growing colonies with different diameters. Bacterial isolates were also examined for their ability to produce hemolysin and the results showed that the highest titer of extract that caused hemolysis in HRBCs within the microtiteration plate was 1/32, and the hemolytic unit for each ml of the extract was 320 U/ ml (figure 1). Among the fourty identified pathogenic local isolates of *S. aureus* isolate SW-14, was selected as the highest hemolysin

producing isolate as it gave a zone of hemolysis of 15 mm and an enzyme activity of 320 U/ml relying on semi-quantitative screening method and micro-titeration plate assay, respectively. The results indicated that hemolysin production was maximal at pH near neutrality (pH 7) as indicated by an enzyme activity of 3905 U/ml and it was also clear that hemolysin production was significantly reduced as the pH of the medium shifted gradually towards acidity or alkalinity (figure 2).

Table 1. Shows the ranking (frequency) of *Staphylococcus aureus* and the percentage of each infection from a total of 40 cases.

Specimens	Frequency	Percentage
wounds(Swabs)	15	37.5%
Ear swabs	5	12.5%
Sputum	8	20%
Urine samples	4	10%
Urethral discharge	2	5%
Vaginal swabs	1	2.5%
Blood samples	5	12.5%
Total (40)	40	100%

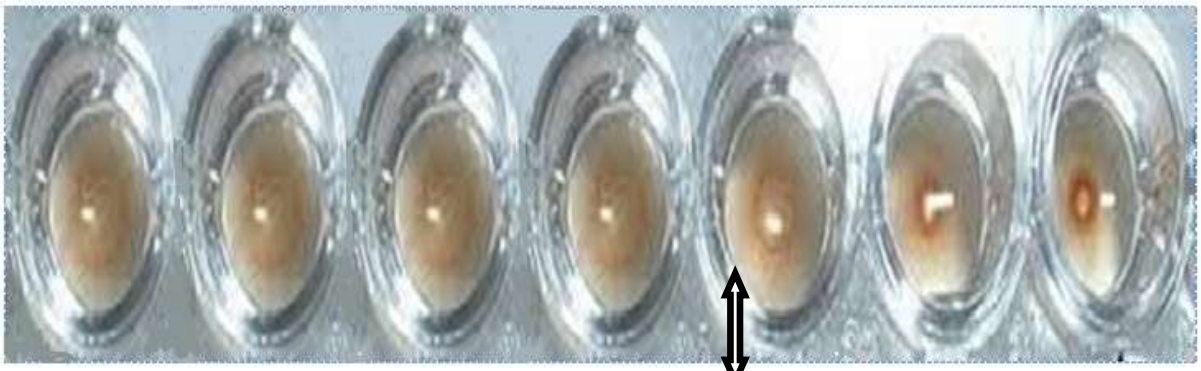

Figure 1. The titer of hemolysin on the microtiteration plate demonstrates the highest titer of the extract that cause hemolysis in HRBCs.

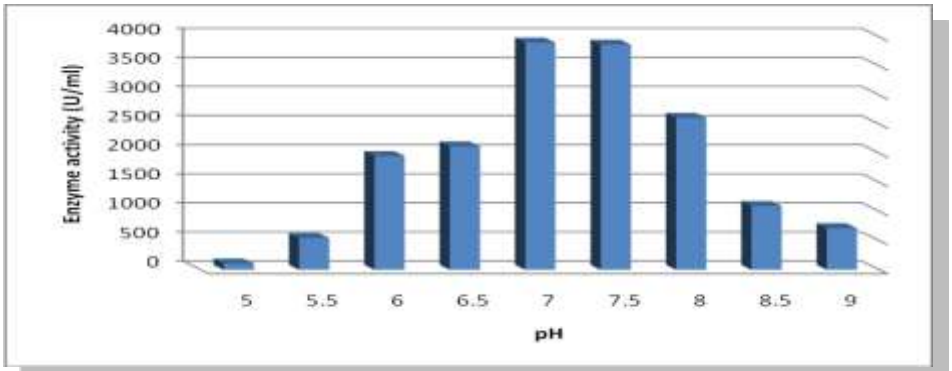

Figure 2. Effect of the pH on production of the hemolysin from the local isolate of *Staphylococcus aureus* (SW-14).

The results in figure 3 showed increasing hemolysin production when temperature of incubation ranged between 35°C - 40°C (3920 and 3732, respectively) which is the ordinary temperature of incubation suitable for almost all types of microorganisms while hemolysin specific activity decreased as far as the incubation temperature scheduled to change from normal degrees towards increased or decreased temperatures ofincubation.

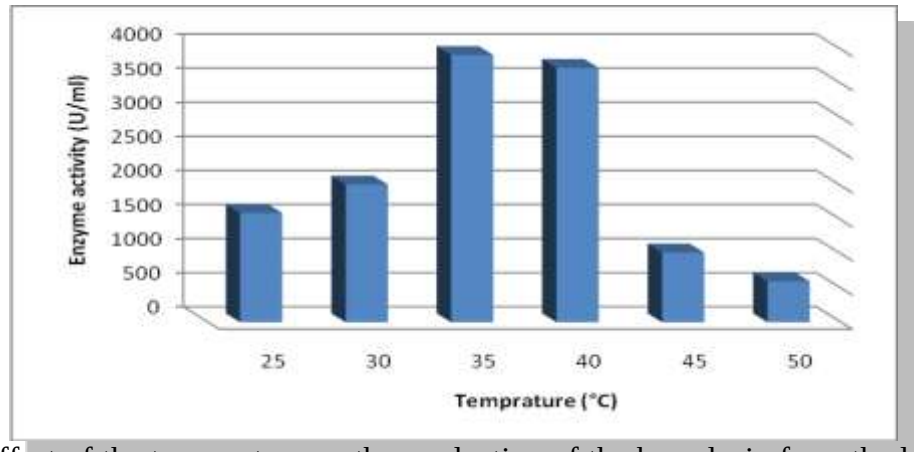

Figure 3. Effect of the temperature on the production of the hemolysin from the local isolate of *Staphylococcus aureus* (SW-14).

The results of the current study revealed that the crude enzyme solution had a total protein concentration of 17.4 mg/ml and an enzyme specific activity of 235 U/mg. The hemolysin was precipitated by ammonium sulphate with 50-75% saturation percentage as a first step of purification.The results of the ion exchange experiment showed that a prominent peak of protein resulted and it was around fraction number 36-41 and was characterized by a maximal enzyme specific activity as shown in figure 4Thefractions were collected, protein concentration was 0.46 mg/ml and the enzyme specific activity was 6345 U/mg. The collected fractions with higher enzyme activity were dialyzed against sucrose to reach a final volume of 5 ml.

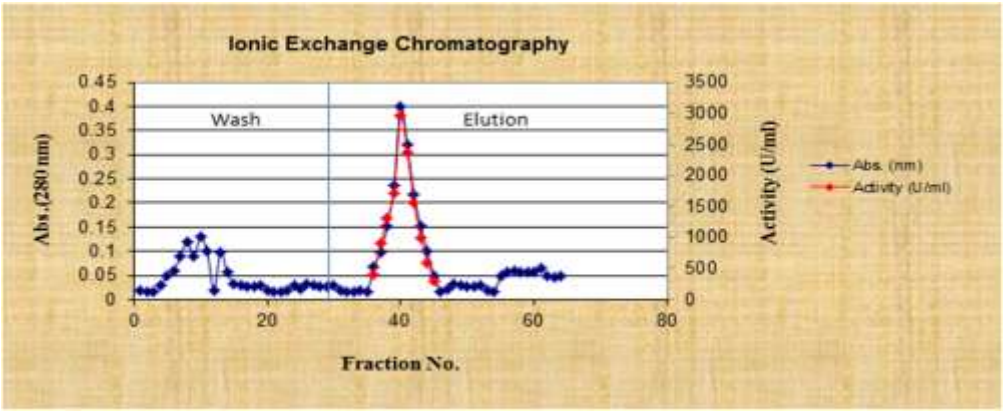

Figure 4. Ion exchange chromatography for hemolysin through DEAE-Cellulosecolumn (3×8) cm. The column was calibrated with phosphate buffer saline PBS 0.2 M and pH 7.0, flow rate 30 ml/hour and 5ml / fraction.

The enzyme was further purified using gel filtration and the product of dialysis (5 ml solution) was applied to Sephadex G-100 column. Figure 5 demonstrated that single

peak of protein was observed with a concentration of 0.03 mg/ml and the fractions number 45 through 51 correlated with that peak were collected and showed an enzyme specific activity of 31725 U/mg.

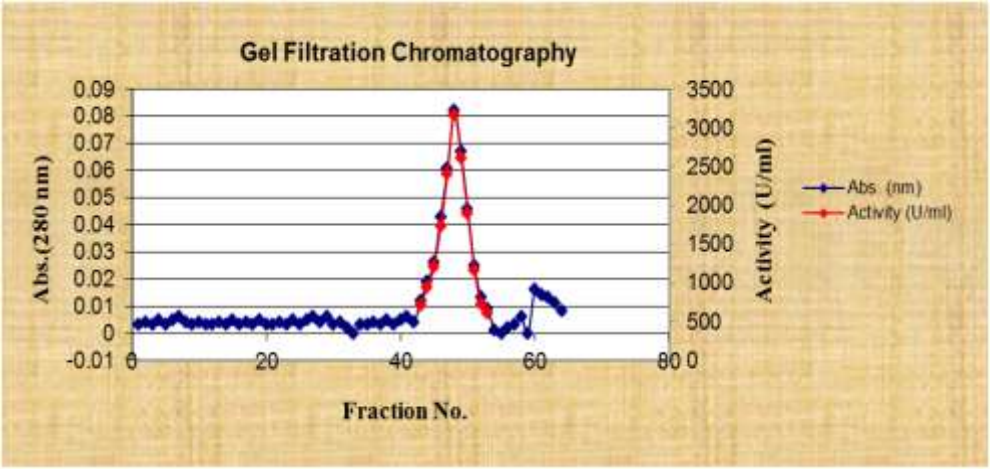

Figure 5: Gel filtration chromatography for hemolysin purification by using Sephadex G-100 column (2x61) cm. The column calibrated with phosphate buffer saline PBS 0.2 M and pH 7.0, flow rate 30 ml /hour and 5ml / fraction

Table 2. Purification steps of hemolysin from local isolate of *Staphylococcus aureus.*

Purification step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
Crude extract	100	4089	17.4	235	408900	100	1
Ammonium sulphate precipitation (50-75%)	15	4018.5	1.9	2115	60277.5	14.74	9
Ion exchange using DEAE-cellulose	10	2918.7	0.46	6345	29187	7.137	27
Gel filtration using Sephadex-G100	5	951.75	0.030	31725	4758.75	1.16	135

For the characterization of hemolysin enzyme, pH dependence experiment was carried out by using several buffers with pH range from 5-10, enzyme activity was assayed. The result revealed that the enzyme had a gradual increase in its activity with increasing pH and retained its activity at pH 7 and then the activity declines as the pH reaches 8 (Figure 6).

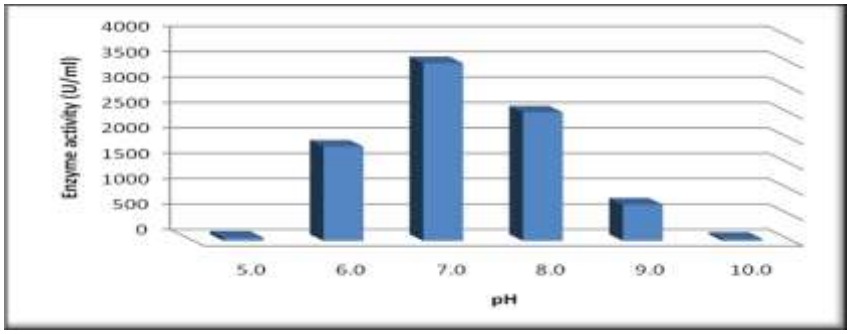

Figure 6: Effect of different pH on hemolysin activity (incubation with buffers).

For determining the optimum temperature required for attaining full activity of the enzyme, temperature experiment had been performed. The purified hemolysin was incubated under different degrees of temperature from 5-85^{o}C for 30 minutes, and enzyme activity was then measured after the end of the incubation period, the result showed that the optimum temperature for sustained activity of hemolysin ranged between 25^{o}C-35^{o}C; while hemolysin activity declined as the temperature of incubation shifted toward both higher and lower degrees of temperature (Figure 7).

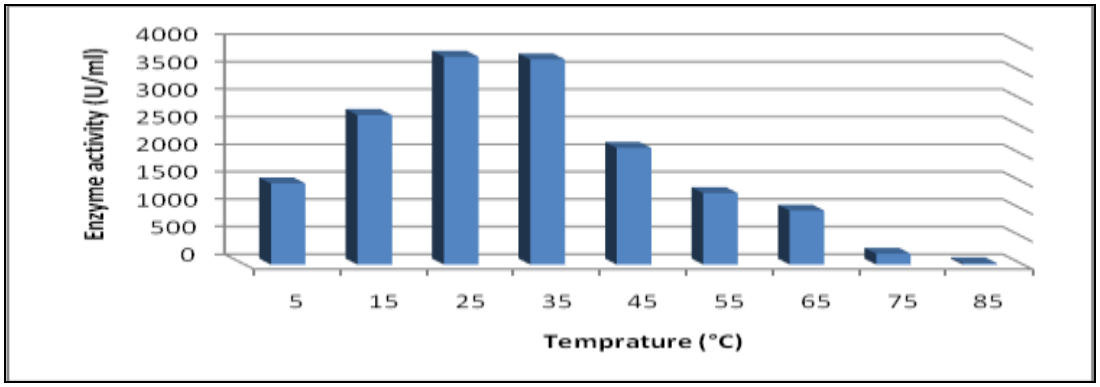

Figure 7. Effect of different temperatures on hemolysin activity (incubation for 30 minutes).

The results of the current study revealed that when the three standards proteins of known molecular weight were eluted through the Sephadex-G100 gel filtration column, they appeared within the elution buffer in a manner resembling their molecular weight starting from the highest to the lowest as shown in figure 8.

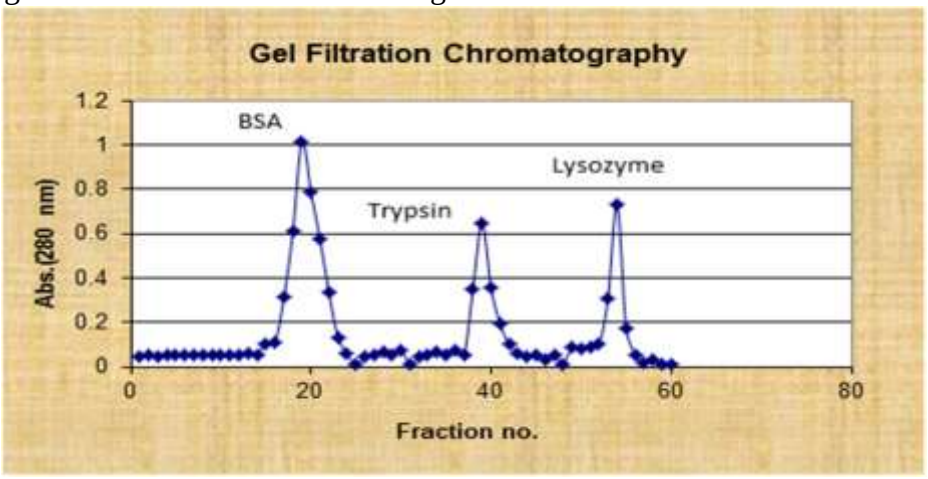

Figure 8. Gel filtration chromatography for the three standard proteins using Sephadex G-100 column (2x61) cm. The column calibrated with phosphate buffer saline PBS 0.2 M and pH

7.0, flow rate 30 ml /hour and 5ml / fraction The construction of standard curve resembling the v_e/v_o values for the standard proteins against their relevant log molecular weight directed the way to determine the molecular weight of the intended protein (hemolysin) which was about 35000 Dalton as shown in figure 9.

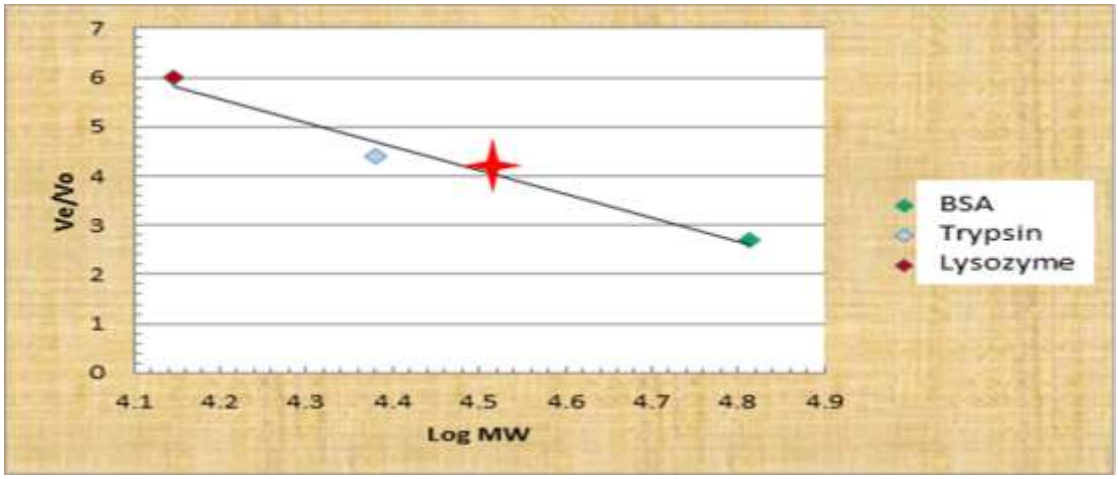

Figure 9: Determination of the molecular weight of *Staphylococcus aureus*hemolysin using standard proteins of different molecular weights.

Discussion

In a previous study done to purify and characterize Hemolysin of *Vibrio parahaemolyticus* (Kanagawa hemolysin) using Sephadex G-100 gel and ion-exchange columnchromatography, after the culture supernatant had been adsorbed on and eluted from diethylaminoethyl-Sepharose CL-6B, and acid precipitated. K-hemolysin was a heat-stable and an apparent molecular weight of 44,000 D[8], While Chung *et al*.,[9], who attempted to purify and characterize hemolysin of jelly fish (*Carbdyaalata*), reported that two chromatographic purification methods, size fractionation on Sephadex G-200 and anion exchange with quaternary ammonium, provided fractions in which hemolytic activity corresponded to the presence of a protein band with an apparent molecular weight of 42 kDa as determined by SDS-PAGE. As for the current study, fractionation using high pressure liquid chromatography, in the study mentioned above, was discouraged because the resultant loss of hemolytic activity. The current study utilized nearly the same methods for purification and characterization of hemolysin, applied in the above mentioned studies, and this might help to explain to some extent the similarity of the results of the three studies.The results of the current study showed a decrease protein yield after four steps of purification indicating to marked discrepancy to the results of others who revealed that the yield was 8.2%[10]. A low yield of hemolytic proteins after Sephadex chromatography has been reported by others and has been attributed to an interaction between the hemolytic protein and Sephadex[11].Cytolytic toxin produced by *Aeromonas hydrophila* and *Aeromonas. veronni* biotype sorbia were purified from culture supernatant in tow steps including ammonium sulphate precipitation and hydrophobic column chromatography, the elution pattern of this column showed that the two cytotoxins eluted as single peak or double peak produced by the first and second microorganism, respectively. Specific hemolytic activity of the cytotoxins increased gradually with the decline in the amount of protein suggesting true purification. Ammonium sulphate precipitation followed by dialysis (1.2 fold 27.5 fold) resulted in an increase of specific activity, following

chromatography, enzyme specific activity also increased by 32 folds [12]. On the one hand, the above mentioned results were in partial agreement with the results of the current study in terms of increased enzyme specific activity and decrease protein concentration as purification proceeded and also for the elution of the toxins as a single peak despite the dissimilar column used in both studies, on the other hands, some disagreement also observed in relation to the folds of increase of specific activity pointing that the current study had more folds of purification suggesting better results. Karzyminska *et al*.,[12] mentioned that the low grade of the purification suggested that culture supernatant and column chromatography fractions should be concentrated and this in turn might help clarify the difference of the results It was stated that the average activity of hemolysin of two separate subspecies of *Staphylococcus cohnii*, namely *Staphylococcus cohnii* spp. and *Staphylococcus cohnii* spp. *Urealyticus* were 74 and 43 HU/mg of protein, respectively. These results clearly contradicted the results of the current study which might be attributed to the fact that the strongest effects of lytic activities observed when the strains were grown on BHI medium [13]. The results of the present study were nearly close to the results of others who demonstrated that the purification steps involved ammonium sulfate precipitation of proteins which showed considerable activity when precipitated by 60 to 80% ammonium sulfate, then it followed by dialysis for desalting, followed by anion- and cation-exchange chromatographies which resulted in six peaks obtained by DEAE-cellulose column chromatography that showed considerable enzyme activity [14]. The above mentioned study showed a manner or sequence of purification steps that seemed similar to the methods applied by the current study despite the change in the results which might be explained by the different organism used as a source of isolating, purifying, and characterizing hemolysin.The results of a local study by Al-Makhzomi,[15], directed to purify and characterize the hemolysin from *V. cholerae* showed identical pattern of saturation to that obtained in the current study as far as ammonium sulphate precipitation was considered, while percentages of saturations of 50% and 60% were recorded by Honda & Finklestien,[16] and Yamamoto *et al*.,[10], respectively. Whereas when specific activity is considered, there is a marked inconsistency between the results reported by Al-Makhzomi,[15], reporting a specific activity of 41.77 U/mg, and the results of the present study while the results of Honda &Finklestien,[16] recording a final specific activity of 1173.3 U/mg came very close to the results of the current study.

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