Purification and Characterization of Thermo Stable DNase of Staphylococcus Aureus Isolated from Different Clinical Source

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Abstract

Hundred samples were collected from different clinical source. Sixty isolates were identified as *Staphylococcus aureus*. The ability of *S. aureus* to produce DNase was examined phenotypically on DNase agar medium and also by quantitative assay that revealed only 37(66%) of *S. aureus* were able to produce the enzyme. DNase was extracted, the crude activity and specific activity was 38 (U/ml) and 253.3(U/mg) respectively. The enzyme purified by precipitating with ammonium sulphate at (65-85 %) saturation then by using ion exchange chromatography in CM cellulose and gel filtration by using Sephadex G150. Purified DNase activity and specific activity was 42 (U/ml) and 4200(U/mg) respectively. The optimum PH for DNase was found to be 8 while the enzyme was stable at wide range of pH (8, 9 and 10) with remaining activity 100%, 90%, 86% respectively. The optimum temperature for DNase was 37 ºC while the stability was also at 37 ºC. Results indicate that DNase activity increased when the enzyme was incubated with 10 mM of each $MnCl_2$, KCl, NaCl, $MgCl_2$, and $CaCl_2$. The molecular weight of DNase was done by gel filtration and found to be approximately 19KDa.

Keywords: *Purification, thermo stable DNase, Staphylococcus aureus.*

Introduction

Staphylococcus aureus is a Gram-positive bacterium living as a commensal on the skin, mouth and upper respiratory system, making it a risk factor for opportunistic and nosocomial infections [(1)]. This group of microorganisms has various virulence factors subscribe to the ability of *S. aureus* to cause infection enzymes, cell-surface proteins, toxins, factors that assistance in avoiding the innate immune defense [(2)]. One of the distinguishing characteristics of *S. aureus* is its ability to produce a wide variety of exoenzymes [(3)].

Among these exoenzymes, nuclease (EC 3.1.31.1) which was originally identified in 1956 by Cunningham and was named such as micrococcal nuclease, thermonuclease, deoxyribonuclease and DNase, and hereafter we will refer to the enzyme as DNase due to its ease of purification [(4, 5)].

Nucleases are very important enzymes belonging to the group of hydrolases that degrade nucleic acids (6). Staphylococcal nuclease catalyzes the hydrolysis of both DNA and RNA at the 5' position of the phosphodiester bond yielding a free 5'-hydroxyl group and a 3'-phosphate monoester. The pH optimum is between 8.6 and 10.3 and varies inversely with Ca2+ concentration, but at any pH rather high levels of Ca, typically

1.1 M, are required for optimal activity [(7)].

A remarkable tolerance to prolonged heating and storage is exhibited by staphylococcal nuclease in foods and broth, and its presence is closely related with the occurrence of enterotoxins in food poisoning outbreaks[(8)]. The gene encoding for staphylococcal nuclease (nuc) has also been widely used as a specific marker for the detection of S. aureus in various types of food and clinical samples [(9, 10)].

Materials and Method

Isolation of bacteria: From 100 samples of different clinical sources (60) isolates primary diagnosed as *S. aureus* depending on cultural morphological and biochemical test. These characteristics include; colonial morphology, size of colony, ability to ferment mannitol. Bacterial isolates were examined and identified by

microscopic, biochemical test and Vitek2 system characteristics [11].

Phenotypic detection of thermo stable DNase: Sixty isolates of *S. aureus* and were cultured by streaking on DNase agar and incubate at 35ºC for 18-24 hr.[12, 13]

The quantitative assay thermo stable DNase: All *S. aureus* isolates were cultured in nutrient broth overnight in a concentration comparable to McFarland standard no. 0.5. Afterward, then centrifuged at 8000 ×g for 15 min. The supernatant was taken, boiled for 15 min and cooled down at 4 °C. Afterward, 50µl of supernatant was poured in wells punched in DNase agar and incubated overnight. A zone of pink or rose color around the well indicated a positive result; which were measured by an aid of metric ruler [14].

Extraction thermo stable DNase: The method described by Ohsaka *et al.* [15] was followed with some modifications for the purification of DNase. The isolate; which developed the largest zone of clearance on DNase agar, was cultured in 100 ml of nutrient broth at 32 °C for 24 hr. on a rotary shaker at 100 rpm. Subsequently, supernatant was obtained, heated in boiling water for 15 min then cooled.

Purification of thermo stable DNase: The crude DNase was subjected to different steps of purification including ammonium sulphate $(NH_4)_2SO_4$ precipitation, dialysis, CM- cellulose ion-exchange chromatography and gel filtration by using gradient elution buffer.

Thermo stable DNase Assay: *Pseudomonas aeruginosa DNA* was extracted by Presto™ Mini gDNA Bacteria Kit (Geneaid Corporation\ Korea). A volume of 2.5 µl of the sample was incubated with 7.5 µl *E. coli* DNA (1 mg/ml) and 40 µl DNase buffer (0.01 M CaCl2, 0.1 M Tris HCl; pH 8) for 60 min at 37°C. The nuclease reaction was stopped with 12.5 µl of 0.33 M EDTA (pH 8.0). One unit of activity was expressed as change in absorbance at 260nm [15, 16].

Characterization of thermo stable DNase

Determination of optimum pH for DNase activity and stability: The effect of pH on the purified DNase activity was done by adding the enzyme solution (0.1 ml) to one ml of (0.1M) Tris-HCl buffer at different pH values (5 to 11) and the activity was determined by performing the standard assay procedure. While the pH effect on DNase stability was done using Equal volumes of purified enzyme solution was reacted with different pH buffers range from (5 to 11) was incubated at a room temperature for 30 min. The enzymatic activity for each one was measured [17].

Determination of optimum temperature for DNase activity and stability: The DNase activity was measured at different temperatures (20, 37, 40, 60, 80 and 100 ºC). The remaining activity was plotted against the temperature. While for thermal stability, equal volumes of purified alkaline phosphatase was incubated in water bath at (30, 40, 50, 60, 70, 80, 90, and 100 ºC) for 30min., and immediately transferred into an ice bath. Enzymatic activity was measured and the remaining activity (%) was plotted against the temperature [17].

Determination of various metal ions effects on DNase activity: The enzyme was incubated with an equal volume of different metal ions $MnCl_2$, KCl, NaCl $MgCl_2$ and $CaCl_2$at a concentration of 10mM/mL at 37 ºC for 30 minutes. The enzyme activity was assayed for each treatment. The control was the enzyme solution without any of these compounds. The remaining activity was assayed for each treatment.

Molecular weight determination for DNase: Molecular weight was determined by gel filtration technique using the column Sephadex G-150 with dimensions 1.5 × 80 cm. The column was calibrated with Lysozyme (14KDa), carbonic anhydrase (29,000), albumin (66 KDa) and alcohol dehydrogenase (150,000). Dextran blue (2,000,000) was used to determine the void volume [18].

Results and Discussion

Isolation and Identification of taphylococcus aureus: From 100 samples from different clinical sources 60 isolates were primary diagnosing as *S. aureus* depending on biochemical, morphological and cultural characteristics. These samples were collected from different sources such as wound, urine, burn, wound, blood, stool, and sputum and nose table 1. These samples were collected from many hospitals in Baghdad. .All colonies from primary culture were purified by subculture on blood agar and then re inoculated on Mannitol Salt Agar at 37 °C for 24 hr [19].

Table 1: Percentage of *Staph aureus* with difference Source infection

Sources of isolation	Number of samples	*S.aureus* Percentages %
Urine	20	33
Burns	10	17
Wounds	9	15
Blood	8	13
Stool	7	12
Sputum	3	5
Nose	3	5

Phenotypic detection of thermo stable DNase: Sixty isolates of *S. aureus* isolates were cultured by streaking on DNase agar. Results showed that 56(93%) of *S. aureus* isolates gave positive result on DNase agar by changing the color from blue to pink or rose color while only 4 (7%) of *S. aureus* were non producer figure 1.

The quantitative assay thermo stable nuclease: The quantitative assay was done for fifty six isolates of S. aureus. Result revealed that only 37(66%) of *S. aureus* were able to produce thermo stable nuclease while 19 (34%) were non producer. Staphylococcus aureus 43 was chosen for DNase extraction since that it accomplished the highest zone of clearance on DNase agar figure 1.

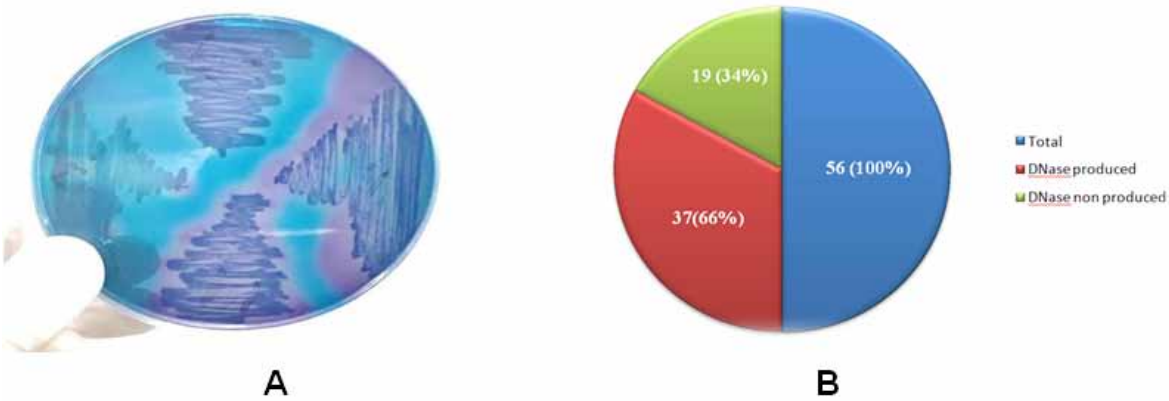

Figure 1: A-Screening of *S. aureus* DNase production; B- Percent of thermo stable DNase production by *Staphylococcus aureus*

Extraction of the enzyme: Supernatant of this S. aureus 43 c was boiled to achieve the inhibition of other enzymes activities and remaining bacterial cell. Tang *et al*.,[20] indicated that the nucleases secreted by strains still showed functional activity after 30 min at 121°C. The crude DNase activity and specific activity was 38 U/ml and 253.3 U/mg respectively.

Purification of the enzyme: After extraction, the supernatant was taken for (65-85%) ammonium sulphate precipitation. The DNase activity and specific activity was (31U/ml) and (606 U/mg) protein respectively. The sample was subjected to CM cellulose column by linear gradient of NaCl (0.1–1 M). The results showed four peaks in wash step, only the first peak of them showed DNase activity (29 U/ml) in fraction numbered 22 to 27 while the last three peaks had no DNase activity thus it was neglected. The fifth protein peak with 0.3M of NaCl at fraction numbered 71 to 78 showed the highest DNase activity reached to 49 U/ml figure 2. Ibraheem and Al-Mathkhury., [16] reported that the specific activity of DNase extracted from *Staphylococcus aureus* was 241.920 U/mg.

Further purification carried out by a gel filtration using Sephadex G150. Enzymic fractions from CM-cellulose were pooled and passed through gel filtration column. The fractionation yielded one protein peaks as absorbance reading at 280nm. DNase activity was (42U/ml), protein concentration (0.01mg/ml) with specific activity (4200U/mg) and the purification fold was (16.5) with yield of enzyme (46.4%) as mentioned in figure 2 and table 2.

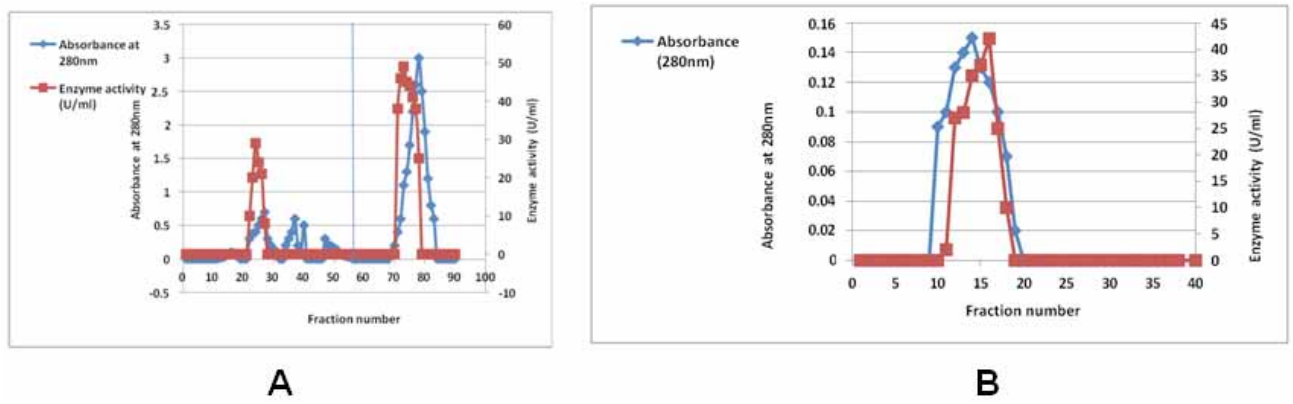

Figure 2: A- Ion exchange chromatography of DNase produced by using CM-cellulose column (2x20cm). The flow rate was a drop per 5 seconds, 3 ml per tube; temperature, 10 °C; B- Gel filtration chromatography for purified DNase from *St. aureus by* using Sepharose-6B column (1.5x80) cm. The column was calibrated with 0.1M Tris-HCl pH 8.

Table 2: Purification steps of DNase from staphylococcus aureus

Purification step	Volume (ml)	Enzyme activity (U/ ml)	Protein Concentration (mg/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	50	38	0.15	253.3	1900	1	100
Ammonium sulfate precipitation (65-85 %)	50	31	0.05	606	1550	2.3	80.4
Dialysis	40	32	0.02	1600	1280	6.2	66.3
Ion exchange chromatography on DEAE-cellulose Wash	18	29	0.02	1450	522	5.6	27
Gel filtration chromatography Sephadex G-150	21	42	0.01	4200	882	16.5	46.4

Characterization of thermo stable DNase:

Determination of optimum pH for DNase activity and stability: The optimal pH for *S.* aureus DNase activity was found to be at pH= 8. However, DNase of *S. aureus was* still active over a wide range (6 to 9) of pH values. DNase activity decreased at pH (5, 11, and 12) figure 3 A. Abdel-Gany, [21] reported that the pH optima of DNase from human, pig, bovine, rabbit, rat and mouse were ranged from 6.5 to 7.0.

PH stability for DNase activity: The purified enzyme was incubated at different pH values for 30 min at 37 ºC. DNase of S. *aureus was* stable in a wide range of pH (8, 9 and 10) with remaining activity 100%, 90%, 86% respectively figure 3 B.

Effect of temperature on DNase activity: The effect of temperature on DNase activity was examined by performing the enzyme assay at various temperature ranging from 20ºC to 100, Purified DNase from S. aureus showed the highest activity at 37 ºC with enzyme activity (42 U/ml figure 3 C. Each enzyme has an optimum pH at which the rate of the reaction that it catalyzes is at its maximum. Small deviations in pH from the optimum value lead to decreasing the activity due to changes in the ionization of groups at the active site of the enzyme, while larger deviations in pH lead to the denaturation of the enzyme protein itself due to interference with many weak non covalent bonds, maintaining its three dimensional structure [22].

Effect of temperature on DNase stability: Thermo stability of DNase was examined by pre incubation of pure enzyme in different temperatures (30–100°C) for 30mins and then the remaining activity was determined after assaying enzyme activity.

The maximum stability of DNase was observed to be at 37 with remaining activity 100% figure 3 D.

It has been reported that salt bridges play an important role in the high temperature tolerance of the protein. There are more salt bridges in thermophilic proteins [23].

Effect of metal ions on DNase activity: Results in table 3 illustrate that DNase activity increased when the enzyme was incubated with 10 mM of each $MnCl_2$, KCl, NaCl, $MgCl_2$, and $CaCl_2$, which gave a higher enzyme activity. DNase I activity is 100- fold lower in buffers that contain only one type of divalent cation compared to a Ca^{2+}/Mg^{2+} reaction mixture, without any divalent cations, DNase I activity is almost negligible[24].

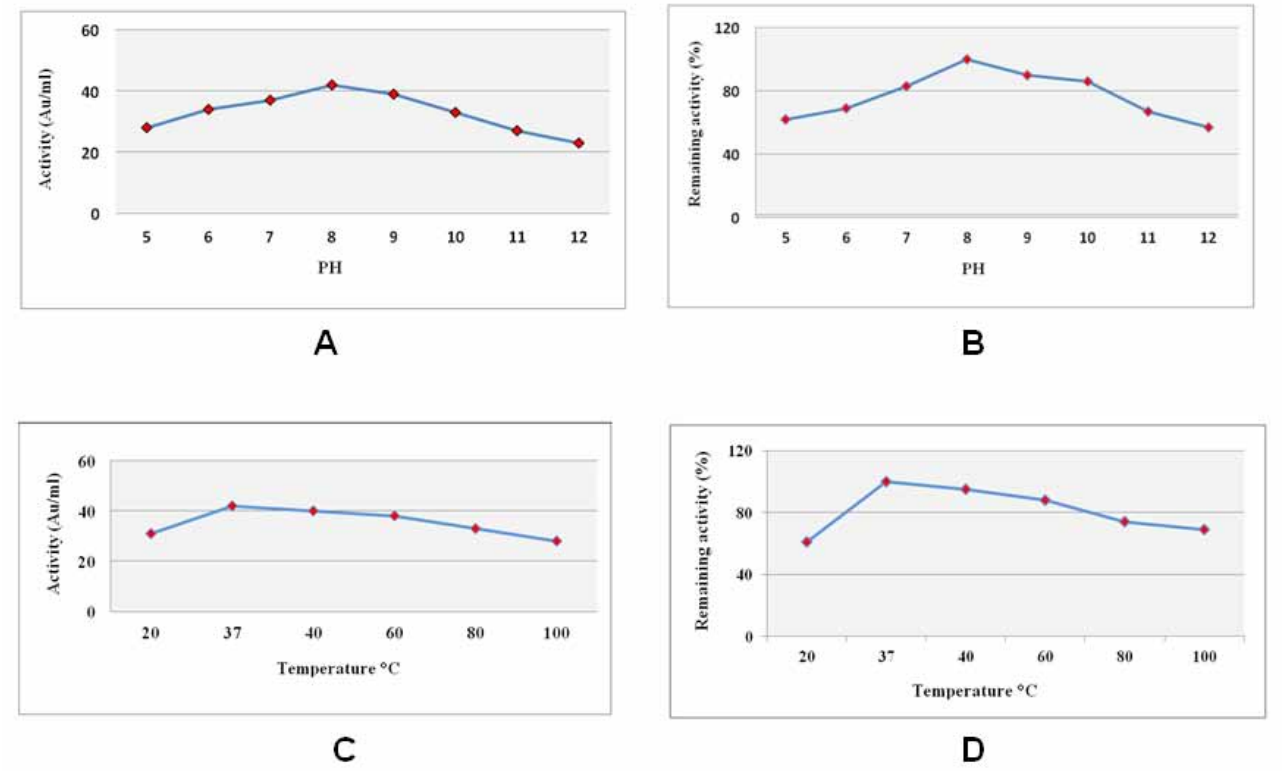

Figure 3: A- Effect of PH on Enzyme Activity; B- Effect of PH on Enzyme stability; C- Effect of Temperature on Enzyme Activity; D- Effect of Temperature on Enzyme stability

Table 3: Metal ions effect on DNase activity

Metal ion	Remaining activity (%)
$MnCl_2$	107
KCl	112
NaCl	119
$MgCl_2$	126
$CaCl_2$	129

Determination of molecular weight for DNase: The molecular weight was estimated by gel filtration according to the logarithm molecular weight and elution volume/void volume (Ve/Vo), the molecular weight for S. aureus DNase found to be 19 KDa. Ibraheem and Al-Mathkhury[16] stated that the molecular weight of purified DNase extracted from *Staphylococcus aureus* was 16.8 kDa by using SDS gel electrophoresis, while DNase purified from streptomyces by gel filtration was 19.9 kDa [25].

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Conflict of Interest: No conflict of interest

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