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Characterization of Protease isolated from Staphylococcus aureus local isolates at Hilla City, Iraq. Analytical chemical study

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Abstract

Background: Bacterial proteases play a vital role in clinical and industrial services. It regards virulence factor when produced from bacteria and can destroy the antibiotics (β-lactamases) and causes damage that may make the disease worsens or delay healing or may interfere with some portions of the immune system. Industrially proteases introduce for many applications like detergent production. The current study aims to isolate and characterize the proteases isolated from clinical isolates of staphylococci. Methodology: Sixty samples were collected from different clinical sources including swabs from wounds, burns, ulcers, urine, and blood, and diagnosed with mannitol salt agar medium using a biochemical test. Protease production, characterization, and purification were performed. Results: Thirty isolates were diagnosed as belonging to the genus *Staphylococcus*, which included 17 isolates of *S. epidermises* and 13 isolates of *S. aureus*. Only 10 isolates of *S. aureus* can produce protease enzyme using Colombia papain medium containing sorting milk. The optimum conditions for the production were determined by the method of submerged farms using fructose as a source of Carbon at a concentration of 0.5% and peptone and yeast extract as nitrogen sources at a concentration of 1% and 0.5% 6 at an initial pH of 8 after 24 X 710 CFU/ ml, respectively. The medium was incubated for 1 hour at 35°C in a vibrating incubator at 150 rpm. The number of purification times was 8.83 with an enzymatic yield. It was observed that the partially purified enzyme showed two protein bands when electrophoresis was performed on Polyacrylamide gel in the absence of protein teratogens. The molecular weight of the protease enzyme is 10000 Daltons by gel filtration method. The optimum conditions were: pH for enzyme activity is 8, and the optimum pH range for enzyme stability was between 7 and 9; temperature for enzyme activity was 40 °C. The value of the activation energy for converting the base material into a product was determined at 6626.7 calories/mol. The enzyme retained its full activity when incubated for 30 minutes at 25°C Up to 80% of its effectiveness when incubated at 55 °C. For the enzyme, it was 7.5 mg/ml and 1.5 Vmax, and the maximum speed was -6 Km. Conclusion: It was found that the enzyme under study belongs to the group of serine proteases with a molecular weight of approximately 10,000

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Keywords
Protease, *S. aureus*, Ion Exchange Chromatography, Kinetic Enzyme, Isolation Enzyme.

S. aureus is a major type of staphylococci that is pathogenic to humans. The occurrence of different types of injuries in different locations of the body, and the severity of this type of injury ranges from minor skin injuries such as infections and abscesses to life-threatening injuries [1, 2]. The virulent *S. aureus* is equipped with a determinant of antibiotic resistance and virulence suit. The most important resistance determinant was β-lactamases which were classified as serine and Metallo - β-lactamases [3]. Bacteria protease's vital roles include: acting as an immune evasion molecule via the destruction of some immune system components [4-6] and compromising host tissue stability via disruption of extracellular matrix and intercellular connections contributing to the dissemination of the infection [7]. *S. aureus* secretes many proteases, including two cysteine proteases (staphopain A, ScpA, and staphopain B, SspB), a serine protease (V8 or SspA), serine protease–like proteins (Spls) and a metalloproteinase (aureolysin, Aur) [8-10]. Both ScpA and Aur inhibit the classical and alternative pathways of complement activation [11]. Additionally S. aureus protease can trouble the regulation of host neutrophil serine proteases via the inactivation of α1-protease inhibitor and α1-antichymotrypsin [12], while cysteine proteases of *S. aureus* degrade elastin, fibrinogen, and collagen, potentially leading to tissue destruction and ulceration [13]. The present study aimed to characterize bacterial proteases as beneficial knowledge used to combat antibiotic hydrolyzing enzymes and virulence of *Staphylococcus aureus* infections, especially superficial skin infections.

Materials and Method

Sample Collection

As many as sixty clinical samples were collected from different clinical samples (wounds, burns, ulcers, urine, and blood) from Al-Hilla Teaching Hospital. The sample included different ages and both genders. They were planted after being collected and transported to the laboratory on medium blood agar and incubated at 37 °C for 24 hours. Then, the colonies were transferred to mannitol salt agar to differentiate between *S. aureus* and *S. epidermides* [14,15].

Bacterial Diagnosis

Gram-positive staphylococci on blood agar transferred to mannitol salt agar, coagulase test, and novobiocin susceptibility to differentiate between mannitol fermenter, coagulase-positive, novobiocin sensitive *S. aureus* from other staphylococci [16,17].

Media for Proteases Production

Fifty milliliters of each of the above mediums into 250 volumetric flasks 108 and incubate for 24 hours at 37 °C in the CFU/ml incubator with a volume of 1% inoculum and many Vibrating at 150 rpm. Bacterial cells were separated after the incubation period by centrifugation at 6000 rpm for 15 minutes. The enzymatic activity of the culture filter was estimated (Crude enzymatic extract), using casein as a reaction material with a pH of 7.5 as well, Bradford Method [18]

Estimation of Protease Activity

Enzymatic activity was estimated according to the method described above (Murachi, 1970.) [19], briefly, 1.8 ml of the base material solution was placed in 10 ml test tubes and placed in a water bath at 37 °C and left for 5 minutes, and adding 0.2 ml of the enzyme solution and re-incubated in a water bath at 37°C for 20 minutes. The reaction was stopped by adding 3 mL of the end solution. Centrifugal atomize at 6000 rpm for 15 minutes. To the base material solution, then %TCA. The control sample uses 3 ml of water. Then, the

enzyme was added and the same steps were followed as in the earlier steps. The optical absorbance of the supernatant was measured at a wavelength of 280 nm with a spectrophotometer.

Protein Concentration Estimation

Protein was measured according to the method described by Bradford [18].

Determination of Protein Concentration in Enzymatic Solutions

The protein concentration of the enzyme solutions was estimated by adding 0.1 ml of the enzyme solution and 0.4 mL of buffer hydrochloric acid gear solution in two duplicates to test tubes. Then, the mixture was mixed well. It was left for 5 minutes and the absorbance was read at a wavelength of 595 nm, and the device was zeroed with the aforementioned solution. Following that, the protein concentration in the enzymatic solutions was calculated regarding a standard curve for bovine serum albumin [20].

Determining the Optimal Carbon Source for Protease Production

Several carbon sources, including glucose, fructose, glycerol, starch, and mannose, were tested at a concentration of 0.5 percent to create (Refractometer and date juice) [21].

Determining the Optimal Nitrogen Source for Protease Production

To choose the appropriate nitrogen source for the production of the enzyme in the liquid peptone medium, various organic nitrogen sources were used with a concentration of 0.5% as the following: peptone, yeast extract, and trypton Casein and bovine serum albumin, as well as inorganic nitrogen sources, including chloride at a concentration of NaNO, sodium nitrate 3 (NH4)2 SO, ammonium sulfate 4 NHCl, % 0.5 [22].

Enzyme Extraction and Purification

Cells were harvested in optimal conditions for enzyme production, at a speed of 6000 rpm for 15 minutes. A clear solution was obtained and the next steps were taken to purify the enzyme: precipitation with ammonium sulfate, 60, 40, ammonium sulfate was added to the crude extract with varying saturation rates (20, 80, and 90%), and these different percentages were evaluated individually until saturation was achieved Best. The optimum saturation rate of 90% was chosen for the concentration of the enzyme by adding 60.3 g per 100. Atomize the solution at 6000 rpm at 4°C for 30 stirrer minutes, remove the floater, and dissolve the precipitate in a tiny amount of 0.05 M numbered gear buffer. At the pH of 7.5, the activity and protein concentration were estimated, and dialysis was conducted against several substitutions of the same buffer solution for 24 hours [23].

Ion Exchange Chromatography

Solutions and substances * buffer hydrochloric acid gear solution (5 mM), pH 7.5 The pH of the balancing and washing buffer solution was adjusted to 7.5 using a Tris-HCl hydroxide solution after dissolving 0.75 g of hydrochloric acid gear in distilled water. Sodium and distilled water, to a volume of one liter. A volume of 0.25 M hydrochloric acid solution was prepared by diluting 10.4 milliliters of concentrated hydrochloric acid in a given volume of distilled water. A volume of 0.25 M NaOH solution was prepared by dissolving 5 g of sodium hydroxide and 7.2 g of sodium chloride in a volume of distilled water and completed

Preparation of the ion exchanger Diethylaminoethyl Cellulose (DEAE-C) (Whitaker & Bernard, 1972) [24]

One liter of distilled water was added to 20 g of a heat exchanger in a graduated cylinder and left to stagnate. Then, the top liquid was added and the exchanger was washed with distilled water several times until the top liquid became clear. The precipitate was then filtered in a funnel under a vacuum, then the precipitate was suspended in a 0.25 M NaOH solution. 0.25 M NaCl, filtered and

rinsed several times with distilled water, followed by 0.25 M hydrochloric acid, washed several times with distilled water, and suspended in the gear buffer The air was evacuated 1.5) cm using a balanced degassing pump (with a pH of 7.5) and then filled the exchanger in the column to give an exchanger with dimensions (13) Vacuum pump. A gear buffer was used to align the Tris-HCl shafts.

Determination of Optimum pH for Enzyme Activity

A solution of the reactant (casein) was prepared at a concentration of 1% with different pH values ranging from 7- 11. It was distributed into tubes and the tubes were incubated at 37°C [25].

Determination of Optimum pH for Enzyme Stability

A total of 0.2 mL of the partially purified enzyme solution was added to tubes. The prepared buffer solutions were incubated with different pH values at 37 °C for 30 minutes, then placed in an ice bath and 0.2ml of the solution was drawn. The enzyme was added to a 1% casein solution with pH (8) as a reaction material, then incubated at a temperature of Heat 37 °C for 20 minutes. Then, the reaction was stopped, and the remaining enzyme was processed [26].

Determination of Optimum Temperature for Enzyme Activity

As much as 0.2 ml of the enzyme solution was added to a 1% casein solution with a pH (of 7.5). for 20 minutes, then - as a reaction material and then incubated at different temperatures ranging from (25-30) The reaction was stopped and the enzymatic activity was estimated. (Ea) [27].

Determination of Activation Energy

The activation energy to transform a reactant into a product Reaction Rate Constant Observed (Kobs) between the logarithm of the observed reaction rate constant (4-10-60) ° C - over a temperature range of between 4-10-60 ° C - was computed using the relationship Transformation Ea (25-30). (Ko) 1/T and the reciprocal of absolute temperature (Segal, according to Irhenius equation (1976) [28].

Determination of Thermal Stability for Enzyme

0.5 ml of the partially purified enzyme solution was incubated at different temperatures ranging from 25-60 °C for (30) minutes, immediately after which the tubes were placed in an ice bath and withdrawn. Then, 0.2 ml of the enzyme solution was added to the solution of the reaction material 1% casein with pH 8. It was incubated at 37 °C for 20 minutes, then the reaction was stopped and the remaining enzyme activity was estimated as % [29].

Determination of the Kinetic Constants for Enzyme

Several concentrations of the base material were created, ranging from (0.25 mg/ml) to (0.25 mg/ml). The values of Michaelis' constant were calculated using the graph of the relationship (Vmax) and the maximum speed (Km). According to the Line Weaver-Bruk reciprocal plot, the Lineweaver-Brk method (S) and the matrix concentrations (Vo) between the initial velocity 1976) and Segal's computation were used. Enzymatic activity based on the amount of tyrosine was released per minute under the measurement conditions based on the standard tyrosine curve. Preparation of the tyrosine 100) g/ml standard curve by dilution - tyrosine concentrations varied from (0) buffer tyrosine solution (1 mg/ml) to (0) buffer tyrosine solution (1 mg/ml) using 0.2 M HCl. At a wavelength of 275 nm, the absorbance was measured, and the standard s curve for tyrosine was obtained. The visual association between absorption values and tyrosine levels is presented [30].

Results and Discussion

Determining the optimal carbon source for the production of proteases

Six different carbon sources were used with a control treatment (without carbon source) and its effect on the production of proteases (glucose,

fructose, mannose, glycerol, date juice, and starch) at a concentration of 0.5% for each source. The results showed that fructose sugar is the best carbon source in the production of proteases (specific activity 40 units/mg protein), followed by glucose (33 units/mg protein), and starch with a specific activity (Figure 1). The enzyme could not be produced in a large amount, and the specific effects of this treatment did not exceed 10 units/mg of protein. Fructose is a simple sugar that acts as a catalyst for the growth of microorganisms and as an energy source.

In another study of proteases produced from other bacterial species, it was noted that 1% glucose is the level of effectiveness, and Streptococcus pyogenes is the best carbon source for enzyme production from bacteria. The specificity is 3.23 units/mg of protein Specificity is 188.35 units/mg of protein [31]

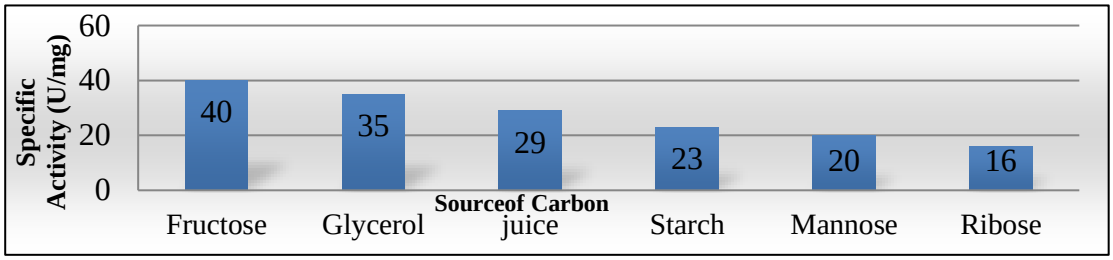

Figure 1. The effect of different carbon sources on the production of proteases from isolated S. aureus

Determining the optimal nitrogen source for protease production

Peptone and yeast extract together are the most efficient in the production of protease compared to the rest of the organic nitrogen sources (Figure 2), While the filtrate was cleaned from the food media to create proteases containing 44 units/mg protein, the specific efficacy reached 44 units/mg protein. Enzymatically, inorganic nitrogen sources are entirely efficient. S. aureus is a kind of bacteria. The expression of secreted proteases from the local isolate 10 was found to be high. It is triggered by the presence of small molecular weight protein molecules as basic materials, such as peptone and trypton. Studies have shown that adding an extract to liquid culture conditions used to manufacture protease enhances the productivity of this enzyme. [32] .For the culture medium, yeast is necessary to stimulate the production of this enzyme, so the researchers used the medium of Tod Huet 0.5% yeast extract to produce protease from the Todd Hewitt medium A previous study indicated that the nitrogen sources used in the production medium of proteases are the yeast extract at 0.5% and casein at 1.5%, as *S. aureus*.

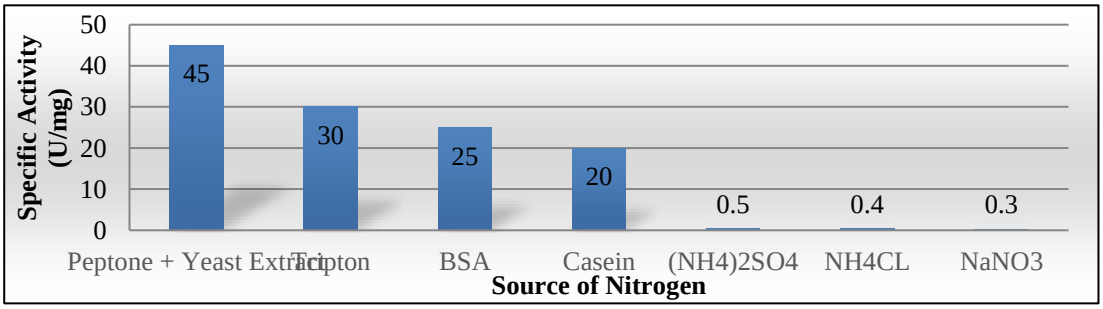

Figure 2. Effect of different nitrogen sources on the production of proteases from isolate S. aureus

Ion exchange chromatography

The precipitated protein was passed from the acetone concentration step into the ion exchanger column which was previously equilibrated with the buffer gear solution with the positive groups possessed by the ion exchanger, and the fractions

that have enzymatic activity were collected. The fractions were concentrated, and the specific efficacy reached 350 units/mg of protein (figure 3). This study is in line with another study that focused on purifying proteases from bacteria. The recovered fractions showed two peaks of DEAE-Cellulose by the S. aureus V8 ion exchange method. In another study on bacteria, [33] as DEAE-Cellulose showed itself treated with one of the mutagens, purified proteases were exchanged.

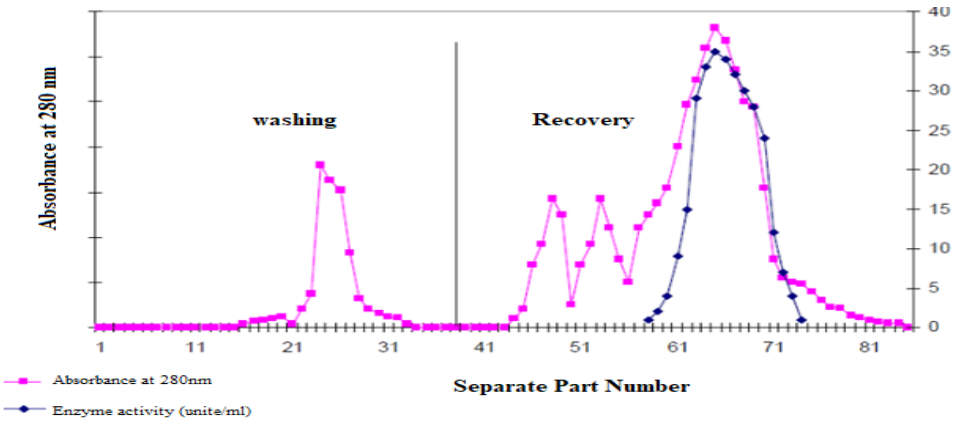

Figure 3. Ion exchange chromatography for purification of protease enzyme generated from a local S. aureus isolate employing column ion exchanger DEAE- Cellulose was recovered using a buffer solution with a saline gradient = pH, which was then equilibrated with another buffer solution (5 mM). 1), with a flow rate of 30 milliliters per hour and a 3 milliliters per part rate. - from linear (0.1-1 Muller)

Determination of the optimum pH for enzyme activity

The effect of pH on the activity of partially purified protease enzyme was studied. It was found that the optimum pH for the enzyme's activity is 8, as the pH ranged between 7 and 11. A decrease in efficacy was also observed at 30 units/ml (Figure 4). The pH range of *S. aureus* V is optimal for the activity of the protease produced from strain 8. From these findings, it may be deduced that the protease was created. The protease under investigation belongs to the group of basal proteases with the highest activity of isolate AG 10. It shares this feature with other proteases produced by different genera and species in the basal ocean. In contrast to microorganisms, the optimal hydrogen number for the activity of a protease synthesized from one of them is as follows. In the 8 B. stearothermophilus bacteria strains (Chopra & Mathur, 1985), Proteus mirabilis (miracle fish), the optimal pH for one of the strains' protease activity was measured. The same pH was recorded for Jilawi (1995). The optimum pH for protease activity is 7. The pH affects the ionic state of the enzyme through its effect on the acid chains Side aminos are necessary to maintain the triple structure of the enzyme and may lead to changing the ionic state of the reaction material, which is reflected in the effectiveness of the protease. The higher or extremely low pH values can lead to denaturation of the enzyme and loss of its effectiveness [34].

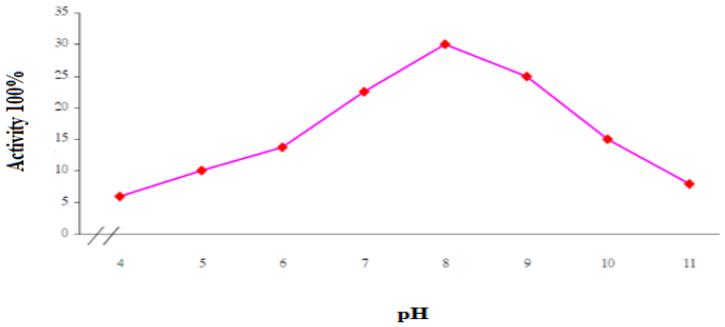

Figure 4. Effect of different values of pH (4 -10) on Enzyme purified from local isolate S. aureus incubated for half an hour, with different pH values at the temperature of 37 °C

Effect of temperature on the activity of protease

The results of the effect of different temperatures (25, 60) on the efficacy of proteases from isolation o (60-60) (Figure 5). Up to 40°C, the protease's activity increases as the temperature rises. 10 local S. aureus. This is since the rate of the enzymatic reaction increases - m form (3 o zero at 60), which increased until it reached a maximum of 32 units/ml, then decreased with increasing temperature until it reached 18), and this is because of the rate of the enzymatic reaction increases. An increase in temperature within a given range is caused by an increase in particle kinetic energy and increased collisions. As a result of increasing the kinetic energy of the molecules due to a temperature rise, there is more interaction between the enzyme molecules and the base material but high temperatures above certain limits lead to enzyme deformation and damage to the structure. Regarding other studies that dealt with proteases produced from other species of bacteria [35]

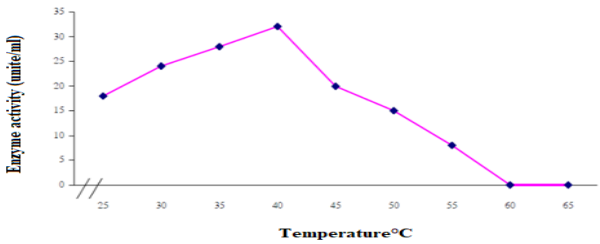

Figure 5. The effect of temperature on the effectiveness of proteases produced from the isolate of S. aureus. The reaction was run at different temperatures for 20 minutes using casein as a solution the reaction

Estimation of the kinetic constants of the enzyme for the purified protease enzyme

The values of the reaction speed in this experiment represent the concentration of amino acids released as a result of the hydrolysis which represents (Km) the base material casein by the action of the enzyme (figure 6). It is important to determine the value of the Michaels constant (Vmax is equal to half of the maximum velocity Vo concentration of the substrate Because it provides an approximation of the level of the substrate in the cell. It also takes the Michal constant as a criterion for recognition. it

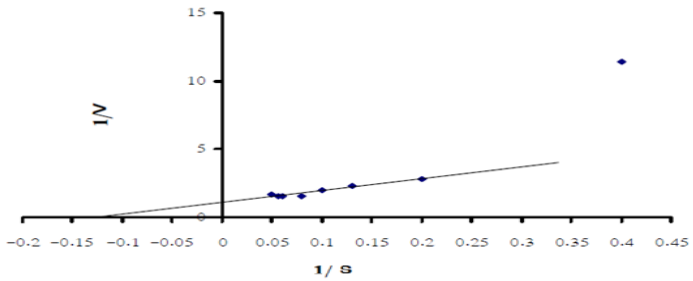

Figure 6. The curve of the relationship between the reciprocal of the reactant and the reciprocal of the initial velocity using the method, to estimate the kinetic constants of the protease enzyme produced by the Line Weaver-Burk Plot of S. aureus Local isolate.

Conclusions

It was found that the enzyme under study belongs to the group of serine proteases with a molecular weight of approximately 10,000 Dalton.

Conflict of interest

All authors stated that there is no conflict of interest.

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