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PRODUCTION, PURIFICATION AND DETERMINATION MOLECULAR WEIGHT OF COAGULASE ENZYME FROM STAPHYLOCOCCUS AUREUS ISOLATED FROM CLINICAL SAMPLES

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Abstract: Background: Coagulase is a critical enzyme produced by pathogenic strains of *Staphylococcus aureus*, which plays a significant role in the classification of staphylococci into coagulase-positive (CoP) and coagulase-negative staphylococci (CoNS). This enzyme interacts with prothrombin to induce blood clotting, which is essential for understanding its pathogenic mechanisms. **Knowledge Gap:** Despite its importance, detailed methods for extracting, purifying, and characterizing coagulase from clinical *S. aureus* isolates remain underexplored, particularly in relation to optimizing purification conditions and accurately determining its molecular weight. **Aims:** This study aimed to extract and purify coagulase from *Staphylococcus aureus* isolated from clinical samples and to determine the enzyme's molecular weight using SDS-PAGE. **Methods:** A total of 2,000 clinical samples were collected from hospitals in Baghdad, yielding 130 isolates of *S. aureus*. The optimum conditions for coagulase production were identified as pH 7.5 and 37°C. Coagulase was extracted and purified through ammonium sulfate precipitation (50-80% saturation), SDS-PAGE, ion exchange chromatography with DEAE cellulose, and gel filtration using Sephadex G150. **Results:** The crude coagulase extract exhibited an activity of 1.7 U/ml. Following purification, the enzyme's specific activity was measured, and the molecular weight of the coagulase was determined to be 36 kilodaltons (kDa). **Novelty and Implications:** This study provides a detailed protocol for the extraction and purification of coagulase from clinical isolates of *S. aureus*, along with the molecular weight determination of the enzyme. The findings enhance the understanding of coagulase's biochemical properties and its role in staphylococcal pathogenicity, potentially contributing to improved diagnostic and therapeutic strategies.

Keywords: Staphylococcus, Purification, Coagulase, Extraction, SDS-PAGE

Introduction

Staphylococcus aureus is a type of bacteria that is positive for the enzyme catalase and has a spherical shape. It forms colonies that resemble grapes in their structure. They lack spores and are incapable of movement **(Chalise, 2021)**. During the 1880s, there was a widespread outbreak of

"puerperal fever" (later known as "childbed fever") in Great Britain. The emergence of "puerperal fever" was initially linked to Staphylococcus aureus. In the 1890s, a post-surgical osteomyelitis and quinsy (appendicitis) involving Staphylococcus aureus was reported. In the mid-20th century, skin and soft tissue infections (SSiTs) were found in injection drug users due to Staphylococcus aureus **(Osama et al., 2024)**. Outbreaks of osteomyelitis were reported in children from a bacteremic interior osteosarcoma **(Asidah Mohamed, Mohamed, & Teow Chong, 2012)**. The ability of S. aureus to cause infections is often linked to various virulence factors. Bacterial coagulase is a virulence determinant of S. aureus. This enzyme converts the soluble plasma protein fibrinogen to insoluble fibrin clots, which have been suggested to protect S. aureus from opsonization and phagocytosis by host immune cells, thereby preventing clearance by the immune system (**Cheung, Bae, & Otto, 2021)**. S. aureus coagulase, the best known virulence factor among clumping factor family proteins also secreted with the same proprotein form, is the only coagulase of bacteria that has been characterized biochemically and genetically. S. aureus produces at least 50 different virulence factors and can cause diseases ranging from localized skin infections to life-threatening systemic infections. Pathogenicity is mediated by a number of virulence factors, including toxins, enzymes, and factors that interfere with host defence mechanisms such as the immune system or blood clotting **(Zengena, Al-Taai, & Al-Dulaimi, 2020)**. The earliest references to the coagulase activity in staphylococcal culture media appeared in 1942 (Ingram and Heller) and 1944 (Zern and Harris). The former isolated a culture medium specifically concerned with coagulase activity as opposed to toxin or hemolysin production. Later references included Farber, Moller, and Oritsh et al. The latter demonstrated, using standard rabbit plasma preparations, that three mNig/th Coagulase activity rendered plasma incapable of clotting within a 24 hour period. Gradation of animal sera in proteolytic power demonstrated that stability of Coagulase activity was as high as 10 per cent of the proteolytic activity active. When 50 mNig Coagulase was added, plasma was rendered only partially clotting (gentle). Subsequent filtration through seasoned coarse grass fibers, porous glass discs, and cellophane bags obtained partially purified Coagulase preparations **(Stewart, 2022).**

Methods

2.1. Collectiion of Sample

A total of two hundred samples from blood, biopsies, wounds, vaginal swabs, and urine were collected. The hospitals in Baghdad are Al-Shahed Alsadder Teaching Hospital and Ibn Al-Baladi Hospital. All samples were cultivated on selective media for isolation of *S.aureus* The media (from Himedia and others) were autoclave sterilized at 121°C for 15 minutes. All solutions and other materials have been prepared in advance **(MacFaddin & Wilkins, 2000).**

2.2. Isolation of Bacteria

In the laboratory and under aseptic conditions, the collected samples were cultured directly on a suitable selective media; the selective media used mannitol salt agar for 24 hours at 37ºC. Then sub-culture on the nutrient agar plate by streaking and incubation at 37°C for 24 hours to obtain pure well-isolated colonies, for identification **(Gupta, 1996)**.

2.3.Identification of Bacteria

In identifying S. aureus, Bergey's textbook on systematic bacteriology proved to be crucial **(Mirhosseini et al., 2010)**.

2.4 Phenotypic detection of Coagulase enzyme

The various methods used to detect coagulase rely on the principle of the coagulase enzyme reaction. Coagulase production (coagulase-free coagulase) binds to prothrombin. Several bacterial

species frequently produce coagulase and blood clotting properties by converting prothrombin (Factor II) to staphylocoagulase, thrombin (Factor IIa). This induces the formation of a fibrin clot **(Kearney, Ariëns, & Macrae, 2022)**. It was generated utilizing **(Ali & Abdallah, 2022)**.

The slide test was conducted to isolate bound coagulase. This involved suspending a single, uncontaminated bacterial colony in a mixture of normal saline and human plasma, while gently stirring. The test is classified as coagulase-positive, and the formation of clumps within a few seconds is observed. The control group consisted of regular saline and bacteria without the addition of plasma. This was done to verify that the bacteria did not form clusters spontaneously in normal saline.

Tube test; for this tube experiment, a solitary colony of the bacteria was mixed with milliliters of human plasma (diluted in normal saline at a ratio of 1:6) and incubated for 24 hours at a temperature of 37 °C. The process of testing the tubes required a duration of one to four hours. The result was affirmative when a clot formed, and negative when the tube was incubated for a further 24 hours and examined again.

2.5 Extraction of Coagulase from *S.aureus*

The extraction of coagulase was performed following the methods outlined in reference **(Mirhosseini et al., 2010)**, and the specified agencies. At first, a 250 ml volume of BHIB medium was produced and then contaminated with Coagulase-producing *S. aureus*. The medium was inoculated with four specifically chosen isolates of *S. aureus* bacteria, with the isolate that demonstrated the highest level of anti-growth activity being selected, and incubated at a temperature of 37 °C. The combination was left to incubate for a period of 24 to 48 hours. Next, the bacterial culture underwent centrifugation at a speed of 10,000 revolutions per minute for a duration of 30 minutes, and the solid particles were removed. Subsequently, the filtrate was subjected to heating at a temperature of 70 °C for a duration of 3 minutes. The liquid portion, which included undiluted aureus, was gathered after cooling.

2.6 Determination of Coagulase Protein Concentration

The Bradford **(Bradford, 1976)**, method was used to estimate the protein concentration of each Coagulase extract produced from the isolate of *S.aureus* bacteria. This was done by using the standard curve of bovine serum albumin (BSA) at concentrations of (10, 20, 30, 40, 50…100) µg/ml. The following test was carried out; 0.5 ml of the sample and 0.5 ml of each bovine serum albumin concentrate were separately added to the test tubes. Then, 4.5 millimeters of Commasie Brilliant Blue dye solution was added to each one. After shaking, the tubes were left for two minutes, and the absorbance was read at a wavelength of 590 nm. A phosphate buffer was used instead of the model in a blank efficiency solution to miniaturize the spectrophotometer. The protein concentration was calculated in the model, by referring to the standard curve of bovine serum albumin. This was drawn based on the relationship between albumin concentration and absorbance values.

2.7 Coagulase Purification of Ammonium sulphate precipitation

The previously acquired supernatant, obtained by subjecting the mixture to heating and subsequent cooling to a temperature of 4 °C, was gradually combined with the crude enzyme, along with a precise amount of ammonium sulfate. The mixture was agitated incessantly for 60 minutes to attain a saturation level ranging from 50% to 80%. The precipitate was diluted in a liter of Tris-HCl buffer (0.1 M, pH 7.5) and then tested for coagulase activity using a thermally stable assay to determine the ideal saturation percentage **(Duong-Ly & Gabelli, 2014; Ibraheem, 2012).**

Dialysis

Ammonium sulfate precipitate was filtered via an active dialysis membrane which the molecular cut-off weight of the dialysis membrane (8-14 KDa) and then added to a buffer solution in

a test tube (0.01M phosphatebuffer) **(Ponnumallayan, 2014)**.

Ion-exchange chromatography

In order to create the DEAE-cellulose column, Whitaker and Bernhard **(Whitaker & Bernhard, 1972)**, combined 20 grammes of resin with 1 litre of distilled water. The liquid underwent repeated rinses with distilled water until it achieved clarity after being allowed to settle. After being discharged, the suspension was filtered using a Buchner funnel with a Whattman No. 1 filter. The resin was dissolved in a 0.25 M solution of sodium hydroxide and sodium chloride. The suspension underwent many filtrations and rinses using a 0.25 M hydrochloric acid solution, as previously stated. Subsequently, it was balanced with a 0.05 M phosphate buffer solution at a pH of 7. After washing the column with an equal volume of the same buffer, the bound proteins were eluted progressively by adding increasing quantities of sodium chloride (0.1–1 M). The absorbance of each fraction was determined at 280 nm using a UV-VIS spectrophotometer, with the column operating at a flow rate of 30 ml/h. We quantified the coagulase activity of each component

Gel filtration

Sephadex G-150 was formulated based on the guidelines provided by Pharmacia Fine Chemicals Company. A glass column measuring 2×40 cm was filled with Sephadex G-150. The Sephadex G-150 was then treated to remove any trapped gases and placed in a suspension in a Tris-HCl buffer with a pH of 7.5, at a concentration of 0.1 M. Subsequently, the column was permitted to reach equilibrium. Following pre-equilibration with a 0.1 M Tris-HCl buffer at pH 7.5, a concentrated amount of Serratiopeptidase was added to the matrix. The elution process was carried out using the identical equilibration buffer at a flow rate of 3 ml each fraction. The measurement of the absorbance of each fraction was conducted at a wavelength of 280 nm.

2.8 Characterization of Coagulase

2.8.1. Molecular weight determination

The molecular weight was determined using gel-filtration chromatography. The column used was a Sephadex G-150 (2x40 cm) that was prepared and washed with a 0.05M phosphate buffer solution at pH 7. Alcohol dehydrogenase, with a molecular weight of 150,000, albumin with a molecular weight of 66,000, carbonic anhydrase with a molecular weight of 29,000, and lysozyme with a molecular weight of 14,300, were used as molecular weight markers. The void volume at 600 nm was measured using Blue Dextran. At a wavelength of 280 nm, the UV-Vis Bio-Rad spectrophotometer was used to find out how much of each reference protein was eluted. The molecular weight of Coagulase was measured by comparing it to the molecular weight of established reference proteins and the elution volume **(Kummari & Bose, 2022)**.

Results and Discussion

3.1. Collection of Samples

Twenty sample including blood, urine, wounds, blood, sputum and biopsy were collected from Ibn Al-Baladi Hospital in Baghdad. All samples were cultivated on selective media for isolation of S. *aureus*. The media (from Himedia and others) were autoclave sterilized at 121°C for 15 minutes. All solutions and other materials have been prepared in advance **(MacFaddin & Wilkins, 2000).**

3.2. Isolation & Identification of *Staphylococcus aureus*

In the laboratory and under aseptic conditions, the collected samples were cultured directly on a suitable selective media; the selective media used mannitol salt agar for 24 hours at 37ºC. Then sub-culture on the nutrient agar plate by streaking and incubation at 37°C for 24 hours to obtain pure well-isolated colonies, for identification also by using the microscopic examination. As well as

biochemical tests were done by VITEK 2 compact system **(Gupta, 1996)**. *Staphylococcus aureus* colonies appear in creamy yellow colonies on mannitol salt agar.

Figure1. Colonies of *Staphylococcus aureus* on Mannitol Salt Agar (Selective media)

The fermentation of mannitol by pathogenic Staphylococcus aureus on MSA causes the medium's color to change from red to yellow.A total of 130 isolates were obtained from a sample size of 200, using the technique outlined below: 75 isolates were obtained from urine samples, 11 from biopsy samples, 39 from wound and ear swabs, 22 from fluid samples, and 33 from sputum samples. A total of 45 isolates were collected from urine samples, 5 isolates were obtained from biopsy samples, 28 isolates were obtained from lesion samples, and 10 isolates were obtained from ear samples. In addition, 15 isolates were collected from fluids, and 27 isolates were collected from sputum. Table 1 displays the distribution of isolates within the total samples categorized by their type and origin.

Table (1): *S.aureus* isolates isolated from different clinical sources

Samples	No. of Samples	No. of isolates	Percentage of isolates according total samples	Percentage of isolates according samples source
Urine	75	45	22.5%	60%
Biopsy	11	5	2.5%	45.4%
Ear Swabs	20	10	5%	50%
Wound Swabs	39	28	14%	71.7%
Fluids	22	15	7.5%	68.1%
Sputum	33	27	13.5%	81.8%

3.3 Phenotypic detection of Coagulase enzyme

The coagulase test can be performed using two different procedures:

Slide Coagulase Mehtod

This technique entails creating a mixture of bacterial cells suspended in a solution, which is then blended with a small amount of rabbit plasma treated with EDTA on a slide for microscopic observation. If the bacterial cells have bound coagulase, the presence of plasma will cause them to come together and form aggregates. The cells will adhere to fibrinogen in the plasma due to the presence of the adhesin, a type of adhesion molecule belonging to the MSCRAMM family, which is responsible for the formation of clusters **(Blair, 1939)**. This phenomenon will result in the observable clustering of bacterial cells on the microscope slide. Figure 1 illustrates the identifiable clustering of cells on the microscope specimen. **Figure2**

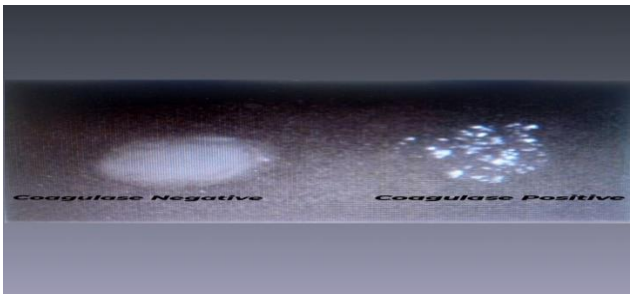

Figure2. Slide test. The coagulase-negative staphylococci are located on the left side of the image slide. The coagulase-positive staphylococci are located on the right side of the slide.

The tube coagulase test

The technique involves pooling bacterial cells with higher plasma concentration in a tiny test tube. The staphylocoagulase secreted by the bacteria into the plasma stimulates their proliferation. The activation of prothrombin by staphylocoagulase initiates the cycle of blood coagulation. staphylocoagulase catalyzes the rapid breakdown of fibrinogen into fibrin by creating a complex with it, therefore bypassing the blood clotting process and facilitating the immediate formation of a fibrin clot. Upon the completion of twenty-four hours, the formation of a clot indicates a successful reaction. Figure3

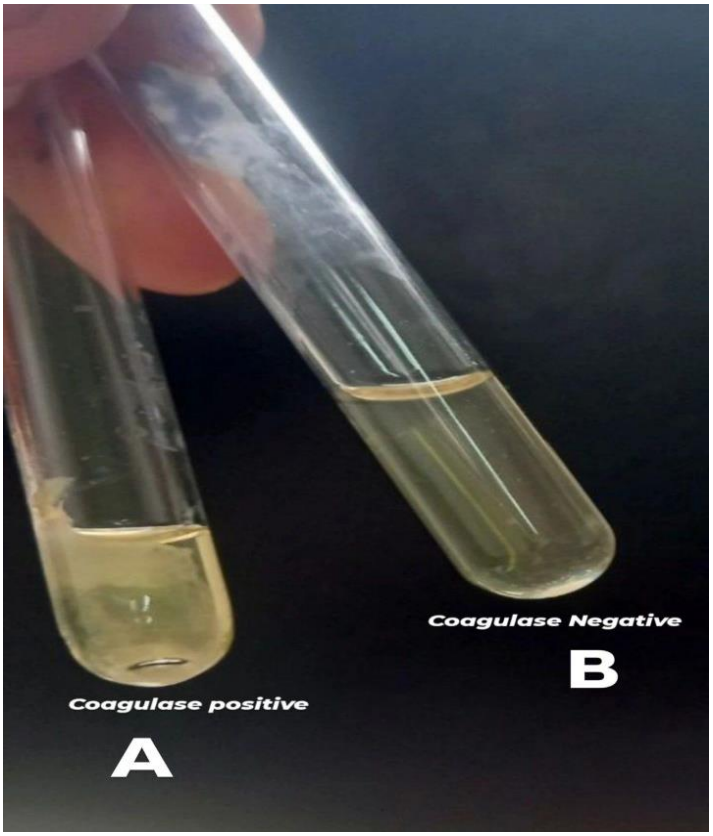

Figure. 3. (A) A Positive tube coagulase test reaction indicating coagulase-positive cells. (B) A negative tube coagulase test reaction indicating coagulase-negative cells.

Despite the potential for false negative outcomes, the slide test is user-friendly and provides data within a reasonable 10-second timeframe. Although the tube test is the exclusively reliable method, it may require a duration of up to 24 hours to provide results. The presence of any degree of

aggregation or clotting in either test indicates a positive outcome **(Hemker, Bas, & Muller, 1975; Turner & Schwartz, 1958))**. Furthermore, the level of bacterial infection is unaffected by the coagulation rate of the blood.

3.4 Detection Enzyme Activity

Using a dilution ratio of 1:10, the plasma was prepared by combining 0.2 ml of plasma with 1.8 ml of physiological saline. Three small test tubes labelled T (Test), P (Positive Control), and N (Negative Control) were obtained. Test duration ranges from 18 to 24 hours. An 18 to 24 hour incubated broth culture of S. aureus constitutes the positive control. An aseptic broth constitutes the negative control. Each tube was pipetted with 0.5 ml of diluted plasma. Five 0.1 ml drops of the Test organisms were introduced to tube "T", five droplets of S. aureus culture were placed in tube "P", and five droplets of sterile broth were added to tube "N". The three tubes were combined and then positioned in an incubator operated within the temperature range of 35 to 37 degrees Celsius. Analysis of coagulation after one hour of physical exertion. In order to investigate the agglutination events induced by tube coagulase, we conducted an experiment that mirrored the methodology outlined by Katz in 2010, but with certain modifications **(Cheng et al., 2010)**.

Calculate Enzyme Activity The enzyme activity was calculated using the following equation:

Enzyme activity =rate × reaction volume.

The specific activity is obtained by dividing the enzyme units (U) by the protein content.

The following equation was used to calculate the overall activity: The overall activity is determined by multiplying the enzyme activity by the whole volume produced during each stage. The purity (fold) was determined using the following equation: Purification (fold) is calculated by dividing the specific activity (for phases) by the specific activity (crude enzyme).

3.5 Extraction of Cured enzyme

Compound coagulase was synthesised from cardiac infusion broth.The staphylococci were cultivated in cardiac infusion broth over a period of three days on a rotating shaker set at 37°C. The orgamism was cleared by centrifugation in a cold centrifuge at 20,000 revolutions per minute. Table 2 displays a protein yield concentration of 10.5mg/ml, along with Coagulase activity and specific activity measurement of 1.7 units per millilititer and 0.16 units per millilitre, respectively.

3.6 Purification of Coagulase

3.6.1 Ion exchange chromatography

After ammonium sulfate precipitation, negatively charged Coagulase was purified using ion exchange chromatography. As shown in Fig. (4). Due to the gradient concentrations of sodium chloride, the washing stage produced one protein peak, while the subsequent elution step produced one protein peak. These protein peaks were examined to see if they had Coagulase activity. According to the findings, the proteins eluted (Fractions 25 to 30) accounted for the bulk of Coagulase activity. Analysis of pooled fractions was conducted to determine Coagulase activity at 2.3 U/ml, specific activity at 1.4 U/mg, and protein production at 1.6 mg/ml. The charge difference principle is the foundation of ion exchange chromatography. Therefore, the detection of a negatively charged Coagulase during the elution stage confirmed that it was produced by S. aureus.

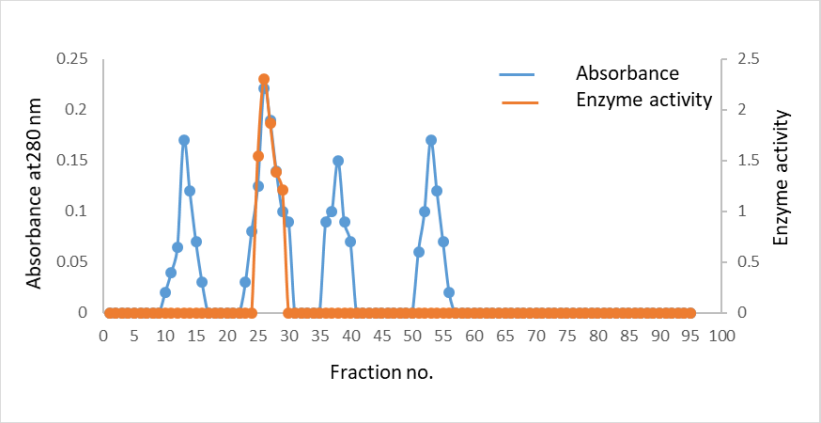

Figure4. Ion exchange chromatography on DEAE cellulose

3.6.2. Gel filtration chromatography

Coagulase-reflecting fractions were pooled and put into a Sephadex G-150 column following ion exchange purification. This column's separation limits range from (5,000) to (600,000) Dalton to produce superior separation efficiency and purification level. It may be utilized repeatedly for protein separations due to its minimal maintenance, ease of preparation, rapidity, and excellent recovery **(Stellwagen, 1990)**. The results in Fig. (5) show that Coagulase activity showed up as one peak after the Tris-HCl buffer was used to wash the sample. These separate parts were put together to make one protein. Coagulase was also measured for its concentration, activity, and specific activity. The fractionation yielded one protein peak with an absorbance reading of 280 nm. This peak (fractionation tubes 6-8) contained Coagulase activity (1.3U/ml), and specific activity (0.65 U/mg) with a yield of protein(2mg/ml)

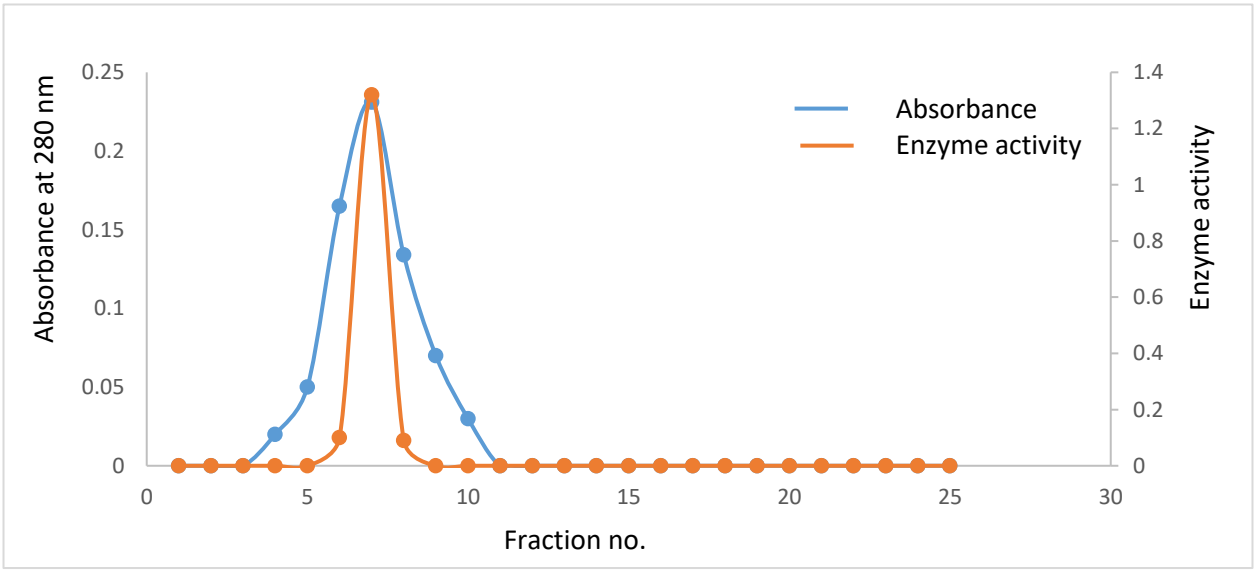

Figure5. Gel filtration chromatography on Sephadex G-150

3.6.3 Determination of Protein Concentration

Fig. 6 demonstrates the application of the Bradford approach with Coomassie brilliant blue-250 and BSA in purification procedures to determine the crude protein level obtained by supernatant filtering.

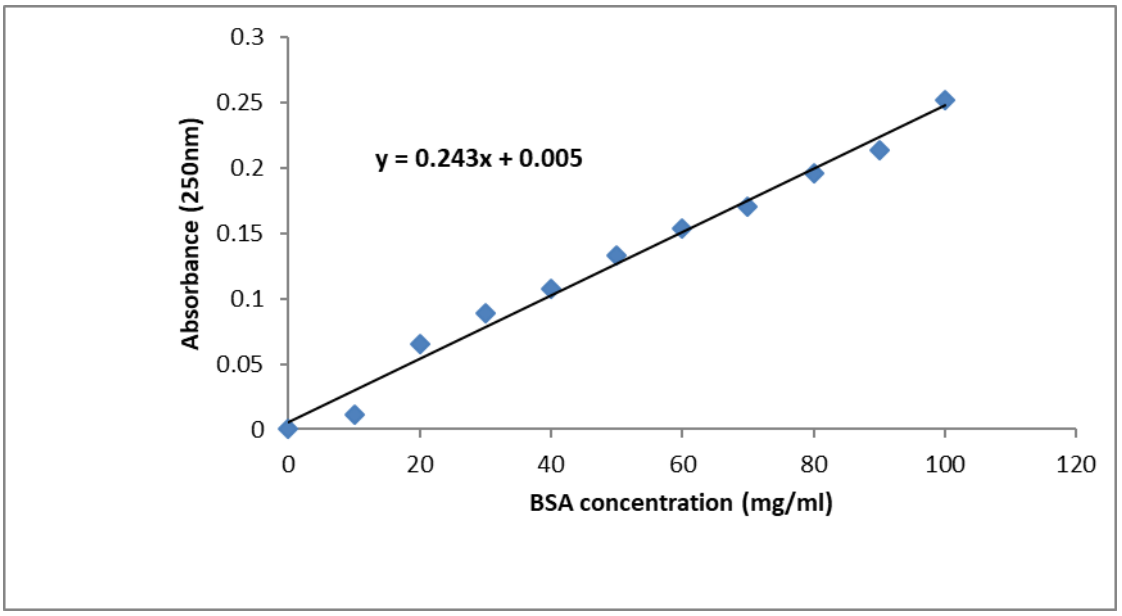

Figure6:Stander curve of protein (BSA) concentration determinatrion

The initial concentration of Coagulase was 9.6 Mg/ml, which decreased to 1.6 Mg/ml after ion exchange purification and further stabilised to 2 Mg/ml after gel filtration purification **(Stellwagen, 1990)**.

Table 2. Coagulase enzyme extracted by four different methods (Crude extract , Dialyzes,DEAD-cellulose,Sepharose6B)

Purification Step	Volume (ml)	Enzyme Activity (U/ml)	Protein concentration (mg/ml)	Specific Activity (U/mg)	Total Activity (U)
Crude enzyme	**450**	**1.7**	**10.5**	**0.16**	765
Ammonium Sulfate	**30**	**2.6**	**10.8**	**0.24**	78
Enzyme dialyzes	**27**	**2.8**	**9.6**	**0.29**	75.6
DEAD-cellulose	**25**	**2.3**	**1.6**	**1.4**	57.5
Sepharose 6B	**15**	**1.3**	**2**	**0.65**	19.5

3.6.4 Molecular weight determination by SDS-PAGE

By using SDS-PAGE, it was discovered that Coagulase had an apparent molecular weight of 36 kDa.as shown in figure 7.

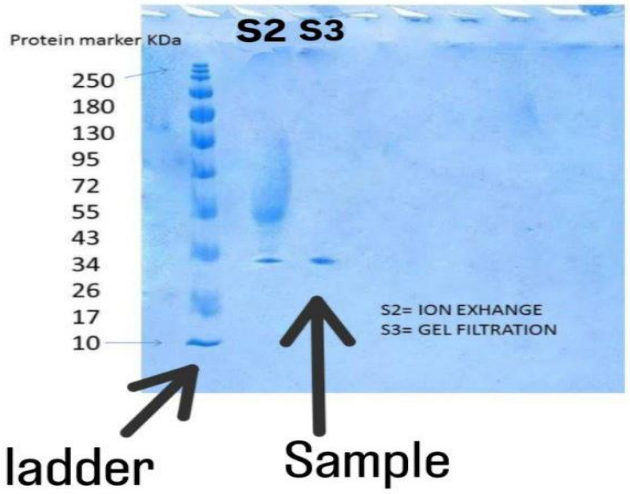

Figure7. SDS-PAGE after purification

5. Acknowledgements

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Conclusion

This study collected 200 samples from patients aged 18 to 45 located in Ibn Al-Baladi Teaching Hospital in Baghdad, including both males and females. Following diagnosis using culture media, biochemical assays, and the VITEK system, 130 isolates of Staphylococcus species producing the enzyme Coagulase were produced under optimal conditions: temperature of 37°C, pH of 7.5, and incubation period of 48 hours in a shaking incubator. The highest enzyme production was observed in Brain Heart Infusion Broth medium. The enzyme purification involved several steps, starting with ammonium sulfate precipitation at 70% saturation, followed by desalting using membrane dialysis, and subsequent application of an ion exchange column (DEAE-Cellulose) and a gel filtration column (Sephadex G-100). The highest specific activity of the enzyme was achieved at the dialysis step, measuring 2.8 units/mg of protein. Enzymatic activity was assessed at each purification step. The molecular weight of the enzyme was determined using SDS-PAGE, revealing a molecular weight of 36 kDa.

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