Isolation, Extraction, Purification and Characterization of Fibrinolytic Enzyme from *Pseudomonas aeruginosa* and Estimate the Molecular Weight of the Enzyme

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Abstract:

*Pseudomonas aeruginosa* was isolated from injuries of wounds and burned patient to ensure that the isolate was belonging to *Pseudomonas aeruginosa*. Several tests are performed such as staining techniques, biochemical test, morphological, Vitek 2 system and sensitivity test. The gram stain test results showed rod pink gram negative bacteria, these findings served as a proof that the isolate was belong to *Pseudomonas aeruginosa*. Growth optimization of bacterial were done by assessing different combination of pH and temperatures. It is revealed that the best conditions for increasing the number of bacteria achieved at 37°C with bacterial number of 5.53×10⁸ and pH 6 with bacterial number of 5.87×10⁸. Fibrinolytic enzyme is agent that lysis fibrin clots. This fibrinolytic factor has prospective use to treat cardiovascular diseases, such as stroke and heart attack. Cardiovascular diseases attracted worldwide attention for its elevation morbidity and mortality. Fibrinolytic enzyme was extracted by centrifugation at 10000 × g for 10 min in 4°C, supernatant was taking and the pellet having bacterial cells was discarding. Purification of the fibrinolytic enzyme was achieved by using salt precipitation, ion exchange and gel filtrations chromatographic techniques. The results showed that, the gel filtration chromatography has optimal specific activity and purification fold at 562.6 U/ml, the final specific activity of the purified enzyme increased 4.1 times. The molecular weight of the fibrinolytic enzyme was determined as 26 kDa by gel filtration chromatography. The purified fibrinolytic enzyme has optimum activity in pH 7 and 40°C. The pH stability for the enzyme activity found in pH 6–7 and range of 10°C from 30° to 40°C.

**Keyword:** Characterization, Isolation, Identification, *Pseudomonas Aeruginosa*, Fibrinolytic enzyme
1. Introduction

The fibrinolytic enzymes are a sub-family of the proteases EC 3.4. The importance of these enzymes is due to their applications in the field of medicine and the benefit of their effect in many ways. The enzymatic action on the fibrin protein (insoluble protein) that contributes to blood clotting is one of the most important medical applications of these enzymes. Fibrin deposits inside the blood vessels and you become vulnerable to disease or injury (1). The molecular weight ranges from 14 kDa to 97 kDa, as it contributes to dissolving blood clots (thrombi), thus maintaining a regular flow in the blood vessels. Thrombosis is a major risk factor for myocardial infarction, deep venous thrombosis and a group of cardiovascular diseases (2). These enzymes have the ability to degrade fibrin, as fibrin is usually composed of fibrinogen with the action of thrombin (EC 3.4.21.5). Thrombin, which is hydrolyzed by plasmin (EC 3.4.21.7), which is activated from plasminogen by the tissue plasminogen activator. Fibrin clots is hydrolyzed by plasmin to avoid blood clots in the blood vessels. In the pathological cases that result from physiological disorders, the fibrin sheets formed inside the blood vessels are not dissolved, which leads to their deposition, the occurrence of blood clots and other cardiovascular diseases (3). Thrombotic disturbances are a main cause of dying worldwide. This is show in the form of stroke, myocardial infarction, and embolism. The formation of intravascular thrombus leads to causes the various cardiovascular disorders (CVDs) and leads to death. In 2011 statistics of American Heart Association referring that 31.3% death was caused by thrombosis and on the report of the WHO, every year about 17 million persons are succumbing to thrombotic disorders (4). The Aim of this study was to investigate:

1. Isolation and diagnosis of \textit{P.aeruginosa} and confirm the production of Fibrinolytic enzyme.
2. Extraction and Purification of the molecular enzyme by salting out by ammonium sulphate deposition, ion exchange chromatography and gel filtration chromatography.
3. Estimate the molecular weight of the enzyme.
4. Characterization of enzyme during the determination of the optimal pH and temperature of fibrinolytic activity and stability.
2. Materials and Methods

2.1. Patients, specimens, collection

Through the period extending from September 2020 till December 2020, 107 specimens were collected from injuries of wounds and burns, where samples were collected by transport swabs with the transporting medium from the patients injured in the hospital of Al-Rusafa (Medical City Hospital), they were diagnosed externally, microscopically and diagnosed by Vitek 2 system (5).

2.2. Samples identification

All the isolates of bacteria were examined for gram stain ability (6). Structure and color of the cells were observed by light microscope using oil emersion, the collected samples were streak plate technique is used for the isolation into pure culture of the organisms (mostly bacteria), from a mixed population. *P. aeruginosa* was streaked over the agar surface. Some singular bacterial cells are separated and well-spaced from each other. As the original specimen is diluted by streaking it over successive quadrants and then incubate at 37°C for 24h, the number of organisms lowering and will show the bacterial morphology (7).

2.3. Optimal temperature and pH for the production of bacteria

The bacterial suspension was cultured once at constant pH but different temperature (32, 35 and 37°C) and once at constant temperature but different pH (5.5, 7 and 9) and measures the absorbance at 600 nm (8).

2.4. Extraction of enzyme

Taken 75 ml of production broth that contain the bacterial cells in which the extracellular fibrinolytic enzyme was found and carry it into centrifuge tubes, at 10000 × g the bacterial cells were cooling centrifuged for 15 minutes at 4°C. Supernatant containing extracellular protein was taking and the pellet having bacterial cells was getting rid of it (9).

2.5. Determination of Protein Concentration

Protein concentration was determined according to the technique of Bradford (1976). The protein concentration determined on the following method: a 20µl of crude enzyme was mixed with 50µl of 1 M NaOH with shaking for 2-3 minutes then 1 ml of Bradford solution was added with shaking, and by spectrophotometer the absorbance was measured at 595 nm (10).
2.6. Measurement of crude enzyme activity

The fibrinolysis activity was estimated according to the Chang method (11). 1.4 mL of 50 m MTris-HCl (pH 8.0) and 0.4 mL of a 0.72% fibrinogen solution (w / v) were taken in a sterile tube and incubated in Water bath at 37 ° C for 5 minutes. Then 0.1 ml of thrombin was added and the tubes were incubated in a water bath at 37 ° C for 10 minutes. Then (0.1 ml) of crude extract was added and the tube was incubated for 60 minutes. 0.2 M of trichloro acetic acid was added to it. Then the mixture was placed in a centrifuge at 10,000 rpm for 10 minutes, where the precipitate was discarded and the filtrate was taken and the efficacy was estimated based on measuring the absorbance at a wavelength of 275 nm. The same previous steps were followed in preparing the blank sample by adding the suspension solution before adding the raw enzymatic extract. One unit of fibrinolytic activity (FU) is describe as the amount of enzyme required to produce an increase in absorbance equal to 0.01 in 1 min at 275 nm .

2.7. Ammonium sulfate precipitation

Ammonium sulfate was added at more than one saturation ratio (20% to 80%), to reach the best ratio of ammonium sulfate by adding progressively the amount of salt to each 10ml enzyme solution in ice bath and magnetic stirrer for 1 hour, centrifuge the solution 10000 rpm/min for 10 min. Get rid of the supernatant and take the precipitate and dissolved it in 25ml Phosphate buffer Saline pH 7.2 and calculated the activity of enzyme and protein concentration (12).

2.8. Purification by dialysis tube

The dialysis process of the enzyme was carried out from the ammonium sulfate sedimentation step, where by 4 mL of the enzyme was placed in a dialysis tube with a diameter of 2.5 cm, which allows the passage of materials with a molecular weight of 8000 to 14000 Dalton, and the dialysis tube was placed in a container containing potassium phosphate, which leads to the separation of the brine solution and it was placed at a temperature of 4 ° C for a day and the solution was changed twice, and after the completion of the dialysis process, the absorbance was measured with a spectrophotometer with a wavelength of 275 nm (13).

2.9. Separation of enzyme through ione xchange resin (DEAE Cellulose)

DEAE cellulose was prepared according to a method conducted by Whitaker and Bernard (1972). The enzyme was separated by adding 35 mL of the crude enzyme purified by dialysis
tube slowly and diagonally to the walls of the ion exchanger column containing the exchanger material using the Dropper, then the separated fraction was collected in appropriate and sterilized tubes at a flow rate of 36 mL/hour at a volume of 3 mL for each part, then a step wash was performed with Phosphate buffer saline with a pH of 7.2. While the Elution retrieval step was performed using different concentrations of NaCl (0.1, 0.3, 0.5, 0.7, 0.9, and 1) molar, after which the absorbance of each retrieval led fraction was measured at a wavelength of 280 nm for each of the washing and retrieval steps, then the enzyme activity in the fractions was calculated. The enzyme activity was calculated from collect the parts of the ion exchanger to determine the fractions containing the enzyme activity by calculating the enzyme activity, concentration and protein volume (14).

2.10. Enzyme separation through Sephadex G-150 column

Sephadex G-150 gel filtration was prepared according to the manufacturer Pharmacia Fine. 27 mL of the enzyme purified by an ion-exchanger step was added slowly into the column on the column walls and the enzyme was retrieval led with the same solution used for calibration at a flow rate of 30 mL/hour per fraction, after which the absorbance of the protein fraction was measured at a wavelength of 280 nm. The enzymatic activity of the high absorbance peaks was measured, the enzyme activity was measured for all protein peaks and the protein concentration was measured (15).

2.11. Estimate the molecular weight of the enzyme

The molecular weight of the enzyme was estimated by gel filtration chromatography. A Sephadex G-150 (2x35 cm) column was used and titrated with 50 mM of phosphate buffer solution, and the recovery step was performed using the same phosphate buffer solution. The following standard crystal proteins act as molecular weight markers, where the partial weight of each alcohol dehydrogenase (150,000 Dalton), Albumin (66,000 Dalton), Carbonic anhydrase (29,000 Dalton), and lysozyme (14,300 Dalton). The void volume was estimated by Blue Dextran at a wavelength of 600 nm, and the recovery volume of each standard protein was measured at 280 nm using a spectrophotometer (UV Vis BioRad system). From the recovery volume of the enzyme, the molecular weight was determined depending on the molecular weight of the known standard protein (16).

2.12. Characterization of fibrinolytic enzyme

2.12.1 Optimum pH for Fibrinolytic enzyme activity
The optimum pH for the fibrinolytic enzyme activity was determined by used sodium acetate C2H3NaO2 was prepared with a concentration of 0.1 M and pH ranged between (5-6), Phosphate buffer saline with 0.1 M concentration and pH ranged from (7-8) and Tris-HCl 0.1 M concentration and pH ranged between (9-10). Then equal volumes of these buffers were mixed with the Substrate fibrinogen at 0.1 M concentrations was 1:1. After that, 0.1 ml of the purified enzyme was added to 0.9 ml substrate. Solution of substrate with pH values, and then plotted the relationship between enzymatic activity and pH to determine the optimal pH of enzyme activity (17).

2.12.2. Optimum temperature for fibrinolytic enzyme activity

The optimum temperature for fibrinolytic enzyme activity was determined by used 0.9 mL of the substrate Fibrinogen with 0.1M concentration was added to 0.1 mL of purified enzyme solution and then incubated for 10 minute in a water bath at different temperatures (30, 35, 40, 45°C) and then determined the enzyme activity for each temperature. Then plotted the relationship between enzymatic activity and temperature to determine the optimum temperature for enzyme activity (18).

2.12.3. Optimum pH for fibrinolytic enzyme stability

The optimal pH for fibrinolytic enzyme stability was determined by used equal volumes of purified enzyme (0.4) ml were mixed with each buffer with pH rang between (5-10) at 0.1M concentration and substrate. The solutions were incubated in a water bath at 37°C for 30 minute and then transferred to ice bath. The absorption was then measured with the optical spectrometer at a wavelength of 275 nanometers, calculated and then the relationship was plotted percentage of residual activity and optimal pH for enzyme stability (19).

2.12.4. Optimum temperature for Fibrinolytic enzyme stability

Determine the optimum temperature for fibrinolytic enzyme stability was determined by used 0.5 ml of purified fibrinolytic enzyme with 0.1 was incubated in a water bath at different temperatures (30, 35, 40, and 45) °C for 30 minutes. The enzyme-containing tubes were then moved directly to an ice bath. Estimation of residual activity, the relationship between temperature and percentage of residual activity was determined to determine the optimal temperature for enzyme stability (20).

3. Results and Discussion
3.1. Identification of bacteria

Identification of bacteria initiated primarily by culturing the specimens on nutrient agar and incubates at 48°C, finally Vitek 2 system were done to ensure that the isolate is belong to *P. aeruginosa* (21).

3.2. Optimal pH and temperature for bacterial Growth

The bacterial suspension was tested after growing it on the nutrient medium to find the best temperature and pH for the growth of the bacteria, where it was incubated at different temperatures (32, 35, and 37) and the best growth temperature was 37 °C, where the numbers of bacteria reached $5.53 \times 10^8$ cells / ml, compared to 35 °C, the number of bacteria was $3.46 \times 10^8$ cells / ml, and at a temperature of 32 °C, the number of bacteria was $2.63 \times 10^8$ cells / ml (Figure 1), after which the bacteria were grown in different culture media with pH (6, 7, and 9) with fixing the optimum incubation temperature, where the best growth was at pH 6, where the number of bacteria was $5.87 \times 10^8$ cells / ml compared with pH 7, where the number of bacteria was $4.33 \times 10^8$ cells / ml and at the pH of 9 the number of bacteria was $2.91 \times 10^8$ cells / ml (Figure 2). Where the current study agreed with (22) where it was proved in his study that the best temperature and pH respectively are 37 °C and 5.5 .

![Figure 1. Show optimum temperature of *P. aeruginosa*](image-url)
3.3. Extraction and purification of Fibrinolytic enzyme

In this work the fibrinolytic enzyme produced by *P. aeruginosa* in the culture broth was subjected to a purification protocol. After that the crude enzyme activity was estimated from supernatant. The enzyme activity of crude enzyme was 27.3 unit/ml and specific activity 136.5 unit/mg. The purification involved ammonium sulphate precipitation and dialysis tube followed by ion exchange and Gel filtration.

3.4. Ammonium sulfate

The greatest ratio for precipitate the crude extract of enzyme was 80 %, when the specific activity got to 151.5 U/mg, with purification fold 1.1 times and the yield 73.9 %. The result of this study was in accordance with (23) where the results were close, as the best saturation percentage was at a concentration of 75%.

3.5. Dialysis

In this step, ammonium sulfate salts were eliminated and the purification results showed an increase in the specific activity of the enzyme, reaching 275.3U/mg, compared to the specific efficacy after the sedimentation step with ammonium sulfate, which reached 150 U/mg, and purification fold was 2 and the enzyme yield was 70.6 %.

3.6. Ionic Exchange Chromatography

This is one of the most useful methods for protein purification. This process depending on the surface molecule charge, the protein and the buffer conditions, the protein will have net a
positive or negative charge. Fibrinolytic enzyme was obtained by using phosphate buffer solution (pH=7.2). Absorbance of eluted fractions were measured at 280 nm upon the arrival of absorbance to the line of zero, then same buffer with the NaCl gradient (0.1-1M) used to elute the bounded protein. Ionic exchange patterns showed two protein peak one in elution and one in wash and one peak of enzyme in gradient elution, represent enzyme activity (tubes \(\sim\)-\(\sim\)). Those fractions pooled and tested for specific activity (499 U/mg) a fold purification of (\(\times\)6 time) and enzyme yield of (65.8%) in parts (Figure 3).

![Diagram of fractionation](image)

**Figure 3.** Ion exchanger chromatography of Fibrinolytic produced by the *P. aeruginosa*

### 3.7. Gel filtration chromatography

Purification carried out by a gel filtration using Sephadex G-150. Enzymes fraction from Ionic exchange were pooled and passed through gel filtration column. The fractionation yielded two protein peaks, absorbance reading at wavelength (280 nm), only one peak where appeared when reading absorbance at wavelength of 280 nm and when determined for enzyme activity in resulting parts enzyme activity recorded in (20-29), the specific activity reached to (563.7U/mg), fold of (4.1) and a yield (52.8%) as mentioned in (table 1) and (Figure 4).
Figure 4. Gel filtration for the purification of fibrinolytic enzyme produced from local isolation \textit{P. aeruginosa}

Table 1. The purification steps of the fibrinolytic enzyme

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Purification (folds)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>75</td>
<td>27.3</td>
<td>0.2</td>
<td>136.5</td>
<td>2047.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation 80%</td>
<td>50</td>
<td>30.3</td>
<td>0.2</td>
<td>151.5</td>
<td>1515</td>
<td>1.1</td>
<td>73.9</td>
</tr>
<tr>
<td>Dialysis</td>
<td>35</td>
<td>41.3</td>
<td>0.15</td>
<td>275.3</td>
<td>1445.5</td>
<td>2</td>
<td>70.6</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>27</td>
<td>49.9</td>
<td>0.1</td>
<td>499</td>
<td>1347.3</td>
<td>3.6</td>
<td>65.8</td>
</tr>
<tr>
<td>Sephadex G150</td>
<td>24</td>
<td>45.1</td>
<td>0.08</td>
<td>563.7</td>
<td>1082.4</td>
<td>4.1</td>
<td>52.8</td>
</tr>
</tbody>
</table>
3.8. Molecular weight of Fibrinolytic enzyme

The molecular weight of the enzyme was determined by gel filtration chromatography. A Sephadex G-150(1.5*55 cm) column was used as for the enzyme purified from *P. aeruginosa* depending on the size and charge of the separated particles. As in (Figure 5) according to the algorithm of molecular weight Ve / Vo, where it was found that the molecular weight of an enzyme is (26,000) Dalton. This study is closely related to Devi, Mohanasrinivasan (24) where the molecular weight produced by *P. aeruginosa* was estimated (27,000) Dalton using the gel filtration method.

![Molecular weight of purified fibrinolytic enzyme from *P. aeruginosa*](image)

3.9. Characterization of fibrinolytic enzyme

3.9.1. pH and temperature optimum activity

The results of this study show that maximum Fibrinolytic activity was detected at 40°C and show decrease activity in the 45°C (Figure 6). The result agreed with Taneja, Bajaj (23) that maximum fibrinolytic activity was detected at 40°C. The pH activity curve showed that the maximum fibrinolytic enzyme was detected at the pH 7 and decrease in the pH 8 (Figure 7). These results agreed with Lee, Kim (25) about low activity curve of the fibrinolytic enzyme at that pH 8.
3.9.2. pH and Temperature stability

The pH stability curve showed that the fibrinolytic enzyme was stable at pH 6 and 7. The stability data showed a decline in fibrinolytic activity below 6 and above 7. However, 93 and 95% relative activity was retained at this pH (Figure 8 and 9). The result agreed with the study Taneja, Bajaj (23) that explained the purified enzyme showed higher stability between pH (6–8) retaining more than 90% of its activity while it retained 87.22 and 74.23% of its activity at
pH 5 and 9, respectively. Maximal temperature stability of the fibrinolytic enzyme was observed in the temperature range of (30–40) °C. At 80°C, the enzyme maintained 20% of enzyme activity.

Figure 8. Show Temperature stability of Fibrinolytic enzyme

Figure 9. Show pH stability of Fibrinolytic enzyme

In conclusion, we can extraction the fibrinolytic enzyme from *P. aeruginosa* bacteria, and it is possible to obtain pure fibrinolytic enzyme from bacteria using purification steps with high purity and good quality efficacy. The optimum conditions for the Fibrinolytic enzyme were the
optimum temperature 40 °C and the degree of its stability enzyme ranged between (30-40 °C), and the optimum pH for the performance of the fibrinolytic enzyme was 7 and its stability was (6-7). Molecular weight of purified fibrinolytic enzyme from *P. aeruginosa* (26,000) Dalton.

**Acknowledgement**

The authors express their sincere appreciation to the Department of Applied Science Laboratories, University of Technology, Baghdad, Iraq for its help in some matters of the current study. No funding support by our college was provided. The authors are responsible for their funding support.

**Reference**


