Extraction and Purification of Extracellular L-Glutamate Oxidase from *Streptomyces*

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Abstract

The bacterial isolates *Streptomyces* were obtained from the soil and cultivated in a wheat bran medium, which is used to produce the L-glutamate oxidase enzyme. The extracellular enzyme was then extracted using a cooling centrifugation process to obtain the filtrate that represents the crude enzyme, after that, the enzyme purification processes were carried out, which included precipitation with ammonium sulfate as a preliminary purification step, followed by dialysis to remove the salts. After that, ion-exchange chromatography and gel filtration were used to finish the purification process, and the enzyme activity was determined for each purification step. The results of purification of L-glutamate oxidase enzyme from *streptomyces* using ammonium sulfate showed that the specific activity was 8.25 units per milligram protein with a saturation ratio of 60% and the results of purification using dialysis tube showed that the specific activity was 9.5 units per milligram protein and the result of purification using DEAE cellulose ion column showed that the specific activity was 25 unit per milligram protein and the results of purification using gel filtration showed that the specific activity was 56 units per milligram protein which was the best step in purification process due to high enzyme’s specific activity. The optimum temperature and pH for the enzyme’s activity and stability were tested. The results showed that the optimum temperature for the enzyme’s activity was 37 °C and the optimum temperatures for the stability of the enzyme were (30 - 50) °C, while the optimum pH for the activity was 7.0 and the optimum pH for the enzyme stability was (5.0-7.0).

Keywords: L-glutamate oxidase, L-glutamate, *Streptomyces*, Specific activity
1. Introduction

Being the most important entity in multiple biological and non-biological processes, enzymes play a crucial role in maintaining and sustaining industrial, commercial and economical products. They include all biochemical response and accelerate the rate of response without being expressing themselves in the last product. L-Glutaminase is amidohydrolase enzyme belong to hydrolytic class which catalyzes the conversion of amino acid L-glutamine into L-glutamic acid, in the presence of water and releases ammonia. This enzyme plays an important role in nitrogen metabolism at cellular level. This enzyme is present in both microorganisms such as bacteria fungi and yeast as well as in macro-organisms, so this is ubiquitous in nature (1). The probable sources may include animals, plants, bacteria, actinomycetes, yeast and fungi (1-3). Numerous bacteria are involved in synthesis of extracellular and intracellular glutaminases such as Bacillus sp., Pseudomonas, Actinobacterium sp, and E. coli.

L-glutamate is an essential amino acid that is commonly utilized as a food additive due to its ability to enhance flavor. In neurochemistry, it is a main excitatory neurotransmitter of the CNS (central nervous system) and the ENS (enteric nervous system) (1). Based on this data, it is critical to determine appropriate analytical procedures for detecting this amino acid generally in simple and dependable methods (2). An enzymatic approach can be used to evaluate L-glutamate. Both Glutamate dehydrogenase (GDH), and Glutamate decarboxylase (GDC) have been used to determine levels of L-glutamate (3). The GDC and GDH have some problems because of their poor substrate specificity and the need for a costly coenzyme like NAD+. Instead, L-glutamate oxidase (GLOD) is utilized because it has a higher substrate specificity than GDH and GDC and requires no further coenzyme (4).

Extracellular L - glutamate oxidase (E.C 1.4.3.11), in the presence of water and oxygen, this enzyme stimulates the oxidative deamination of L-glutamate, which leads to the formation of α-ketoglutarate, ammonia, and hydrogen peroxide.

\[
\text{GLOD} \\
\text{L glutamate + O}_2 +\text{H}_2\text{O} \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_3 +\text{H}_2\text{O}_2
\]
Biosensors for detecting L-glutamate, L-glutamine, ammonia, and creatinine can be made using the analytic reagent of L-glutamate oxidase. These biosensors can be used in analytical chemistry for qualitative and quantitative enzymatic process tests, and food products to determine the quality of the food, and in clinical biochemistry for detecting heart and liver disorders early by evaluating glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase in biological fluids (5). The aim of the current study was to isolate, purify, and characterization of L-glutamate oxidase from *Streptomyces* culture filtrate.

2. Materials and Methods

2.1. Preparation of production medium

*Streptomyces* were grown in a medium of 2 g of wheat bran, 0.5 g of Monosodium Glutamate (MSG) as a substrate for the enzyme, and 0.5 g of NaCl in 100 mL of distilled water as a catalytic medium to the bacteria for enzyme production (6).

2.2. Extraction of enzyme

Bacterial cultures were placed in suitable Falcon tubes. Then the cells were precipitated in a cooling centrifuge at 8000 rpm for 20 minutes to extract the enzyme. Because the enzyme is extracellular, the filtrate is separated from the sediment and taken for the purpose of the purification process.

2.3. Estimating the Activity of L-glutamate oxidase

The peroxidase-catalyzed chromogenic technique was used to determine the enzyme activity. The reaction mixture was made up of 1 ml of 4-aminoantipyrine (2 mM), 2 ml of phenol (3 mM), 0.1 ml of horseradish peroxidase (60 U/ml), and 0.1 ml from the enzyme. The reaction was started by adding 0.1 ml of (0.01 mM) from monosodium glutamate to the reaction mixture after a 2-minute pre-incubation at 37 °C to the reaction mixture. After 30 minutes of incubation at 37 °C with gentle shaking, the absorbance at 500 nm was measured. Under the assay conditions, one unit of enzyme activity is described as the amount of enzyme required to generate 1 mol of H2O2 per minute (7, 8).

Enzyme Activity (U/ml) = (A×V) / (t× E ×v)
2.4. Estimation of Protein Concentration in the Sample

The protein concentration has been calculated according to Classics Lowry, Rosebrough (9) method. The total protein content of the L-glutamate oxidase enzyme was determined by a spectrophotometer at 600 nm. Bovine serum albumin (BSA) was used as standard protein (9).

2.5. Determination of Optimum Temperature for L- glutamate Oxidase Production

To calculate the optimum temperature for enzyme production, the prepared culture medium (wheat bran), which contains 1 ml of Streptomyces culture, and the age of culture was 48 hours were incubated at different temperatures (20, 25, 30, 37, 40) °C in the shaking incubator for 60 hours, after that the activity of the enzyme produced by bacteria was evaluated after extraction to find the optimum temperature for production (10).

2.6. Determination of Optimum pH for L- glutamate oxidase production

To determine the optimum pH for enzyme production, the pH of the prepared culture medium (wheat bran) was changed to (3,5,7,8,9), which contains 1 ml of Streptomyces culture, and the age of culture was 48 hours in the shaking incubator for 60 hours. After that, the activity of the enzyme produced by bacteria was measured after extraction to determine the optimum pH for production (11).

2.7. L-glutamate oxidase Purification process

2.7.1. Ammonium sulfate precipitation

The crude enzyme was precipitated with a saturation ratio of 60% of ammonium sulfate (NH₄)₂ SO₄ by gradually adding salt to the crude enzyme with continuous stirring for an hour.
on a magnetic stirrer, then the components were separated by a cooling centrifuge at 10000 cycle/min for 15 minutes, the filtrate was neglected, and the precipitate was dissolved in 15 ml of phosphate buffer (pH 7.4, 0.2 M), then the absorbance was measured at a wavelength of 500 nm to measure the enzyme activity and specific activity (12).

2.7.2. Purification by Dialysis Tube

The dialysis process was carried out for the enzyme resulting from the precipitation step with ammonium sulfate. 15 milliliters of the enzyme were placed in the dialysis tube, which allows the passage of materials less than 120 kDa. The tube was placed in a container containing a phosphate buffer (pH 7.4, 0.2 M) for 24 hours, which leads to the separation of the saline solution from the enzyme. So that the enzyme remains inside the tube. After the dialysis process is completed, the enzymatic activity and specific activity of the resulting enzyme are measured (13).

2.7.3. Enzyme separation from the ion-exchange column by DEAE cellulose

The method for preparing the ionexchange (DEAE-Cellulose) used by Whitaker and Bernhard (14) was applied. 20 g of resin dissolved in 1 L distilled water and left to settle, the supernatant was disposed of, and this process was repeated multiple times until supernatant became pure. DEAE-Cellulose was activated for 30 minutes with 0.25 M HCl, then filtered through a Buchner funnel with Whatman No.1 filter paper and washed twice with distilled water. The DEAE-Cellulose was then activated with 0.25 M NaOH, followed by two rounds of filtration and washing. The activated DEAE-Cellulose was equilibrated with phosphate buffer (pH 7.4, 0.2 M) and packed in column with dimension (3x13) cm, then 10 milliliters of the enzyme were slowly placed on the walls of the ion exchanger using a dropper, and then the separated portion was collected in appropriate tubes of 5 milliliters for each portion, then a washing step was performed using a phosphate buffer solution (pH 7.4, 0.2 M), and the elution step was conducted applying various sodium chloride concentrations (0.15-1 M NaCl), then the absorbance of each eluted fraction was measured at 280 nm wavelength for each of the washing and elution steps, and the enzymatic activity was calculated in the collected fractions of the exchanger to determine the fractions containing enzymatic activity (15).
2.7.4. Purification by gel filtration column using Sephadex G200

The gel filtration material was prepared according to the instructions of the supplied company by placing 6 grams in 200 milliliters of distilled water to remove the preservatives, then it was washed with a phosphate buffer solution at (pH 7.4, 0.2 M) and then heated at a temperature of 90 degrees Celsius for 3 hours and the air and bubbles were removed by a vacuum pump. Then the material was placed in a column with dimensions (21 x 1) cm, and the material was left to precipitate. Then the column was titrated using a phosphate buffer solution, then 3 ml of the purified enzyme was taken in the ion exchange step and slowly placed on the walls of the gel filtration column, and the enzyme was recovered using the solution used to wash the material. Then the absorbance of the protein part has been determined at a wavelength of 280 nm. Then the enzymatic activity of the protein peaks with high absorbency was measured. The enzymatic activity of all the protein peaks was measured (16).

2.8. Characterization of L–Glutamate Oxidase

2.8.1. Determination of Optimal pH for the Effectiveness of Enzyme

To determine the optimum pH for enzyme activity, sodium acetate buffer CH₃NaO₂ was prepared at a concentration of (0.1 M - pH 3, 4, 6), and phosphate buffer was prepared at a concentration of (0.1 M - pH 7, 8). Equal volumes of these solutions were mixed with the substrate solution in a ratio of 1:1, then 0.1 ml of this mixture was added to 0.1 ml of the enzyme. The enzymatic activity was estimated by measuring the absorbance at 500 nm. Then the relationship between the enzyme activity and the pH was plotted to calculate the optimum pH for the enzyme activity (17).

2.8.2. Determination of Maximum pH for Enzyme Stability

To find out what pH is best for enzyme stability, equal volumes of the pure enzyme were mixed with buffer solutions, where 0.1 ml of the enzyme was mixed with 0.1 ml of sodium acetate buffer solution at a concentration of (0.1 M, and pH 3, 4, 6), and 0.1 ml of the enzyme was mixed with 0.1 ml of phosphate buffer solution. (0.1 M - pH 7, 8) the solutions were incubated in the incubator at 37 Celsius degrees for 30 minutes. The activity of the enzyme was calculated by a spectrophotometer at 500 nm, and the enzymatic activity was calculated. The relationship between the percentage of residual activity and the optimum pH for the stability of the enzyme was drawn (18).
2.8.3. Determination of Optimal Temperature for the Effectiveness of Enzyme

To find out the optimum temperature for enzyme activity, 0.9 ml of the substrate solution was added with 0.1 ml of the pure enzyme, then the mixture was incubated for 10 minutes at different temperatures (20, 30, 37, 40, 50) °C, then the enzyme activity was determined for each temperature, and the relationship was drawn between temperature and enzyme activity to calculate the optimum degree of enzyme activity (19).

2.8.4. Estimation of Optimal Temperature for Enzyme Stability

To find out what temperature is better for enzyme stability, 0.1 ml of the pure enzyme was incubated at a range of different temperatures 20, 30, 40, 50, and 60 °C for 30 minutes, after which the enzyme activity was calculated, and the residual enzyme activity was plotted against temperature to find the optimum temperature for its stability (20).

3. Results and Discussion

3.1. The optimum temperature for L-glutamate oxidase production

The results revealed that the optimum temperature for the production of the enzyme is 30 °C, as shown in figure 1, with the highest specific activity reaching 4.5 units per milligram at the temperature 30 °C, and the lowest specific activity of the enzyme was 2.7 units per milligram at the temperature 20 °C.
3.2. Optimum pH for L-glutamate oxidase production

The results showed that the highest specific activity was 4.6 units per milligram at pH 7.0, while the lowest specific activity was 2.1 units per milligram at pH 3.0, indicating that 7 is the ideal pH for enzyme production. As illustrated in figure 2.
3.3. Enzyme purification

Ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and gel filtration were used in this study to purify L-glutamate oxidase produced by *Streptomyces* in the culture broth.

3.4. Ammonium sulfate

Ammonium sulfate does not affect pH and precipitates the largest proportion of proteins present with the enzyme. It also does not affect the enzymes since it does not induce denaturation of many proteins during deposition. The ammonium sulfate at a saturation ratio of 60% was chosen as the optimal ratio for precipitating the crude enzyme extract, with a specific activity of 8.25 units per milligram, purification fold 1.8, and yield of 20.8 percent.

3.5. Purification by dialysis

To get rid of ammonium sulfate salts and concentrated the enzyme, purification by dialysis was used. A volume of 15 milliliters of the enzyme obtained from the precipitation step with ammonium sulfate was taken and placed in dialysis tubes with a diameter of 2.5 cm. The purification results are shown increasing the specific activity of the enzyme reaching 9.5 units/mg
of protein compared to the specific activity after the precipitation step with ammonium sulfate was 8.25 units/mg, the number of purification times in this step was 2, and the yield was 18.6%.

### 3.6. Ion exchange chromatography

The enzyme solution after the concentration step by dialysis was passed through the DEAE-Cellulose ion-exchange column that was already equilibrated with the phosphate buffer (pH 7.4, 0.2 M). Absorbance is calculated for washing parts (positively charged proteins) at the wavelength of 280 nm. When the arrival of the absorbance of the line of zero, binding protein (negative proteins) was eluted with phosphate buffer (pH=7.4) supplement with NaCl (0.15-1) M, figure 3 illustrate the results two peaks, one in washing and other in elution and one of them show an enzyme activity in elution step. The specific activity in this step was 25units per milligram protein with purification fold 5.2 and yield 12.7%.

![Figure 3. Ion exchange chromatography for L-glutamate oxidase purification from *Streptomyces* using DEAE-Cellulose column (3x13) cm equilibrated with phosphate buffer (pH 7.4, 0.2 M), eluted with a phosphate buffer with NaCl gradient (0.15-1) M in phosphate buffer.](image-url)

### 3.7. Gel filtration chromatography
Gel filtration with Sephadex G200 has been used for purification, DEAE cellulose enzyme fractions were pooled and processed through a gel filtration column, the results in figure 4 shown the purity of enzyme increased when using a gel filtration column and protein peaks appeared, and the enzyme activity was concentrated in one peak recorded from (11-13), and the specific activity reached to 56 U/mg with purification fold 11.7 and yield 4%.

Figure 4. Gel filtration chromatography for L-glutamate oxidase purification from *Streptomyces* using Sephadex G200 (21x1) equilibrated with phosphate buffer (PH 7.4, 0.2 M)
Table 1. Summary of the purification steps of L-glutamate oxidase from *Streptomyces*

<table>
<thead>
<tr>
<th>Steps</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 (units)</th>
<th>Fold of purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>80</td>
<td>26.7</td>
<td>5.6</td>
<td>4.8</td>
<td>2,136</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation 60%</td>
<td>15</td>
<td>29.7</td>
<td>3.6</td>
<td>8.25</td>
<td>445.5</td>
<td>1.8</td>
<td>20.8</td>
</tr>
<tr>
<td>Purification by dialysis</td>
<td>15</td>
<td>26.5</td>
<td>2.8</td>
<td>9.5</td>
<td>397.5</td>
<td>2</td>
<td>18.6</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>10</td>
<td>27.3</td>
<td>1.1</td>
<td>25</td>
<td>273</td>
<td>5.2</td>
<td>12.7</td>
</tr>
<tr>
<td>By DEAE cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration</td>
<td>3</td>
<td>28.6</td>
<td>0.51</td>
<td>56</td>
<td>85.8</td>
<td>11.7</td>
<td>4</td>
</tr>
<tr>
<td>By Sephadex G200</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

3.8. Characterization of L-glutamate oxidase

3.8.1. The optimal temperature of enzyme activity and stability

To find out the ideal temperature for purified enzyme activity, the enzyme reaction was carried out at a varied temperature range (20-50 °C). Results in figure 5 showed an increase in enzyme activity
by increasing the temperature and reached a maximum value of 26.7 U/ml at 37 °C, therefore it began to decline with decreasing the temperature until reaching 16 U/ml at 20°C. Furthermore, the remaining activity was calculated at various temperature ranges (20–60) °C, with the results shown in figure 6. The enzyme maintained 100% of its activity when incubated at (30-50) °C. Then the activity began to decrease above and below this range.

Figure 5. Effect of different range of temperatures (20-50) °C on locally purified L-glutamate oxidase activity from *Streptomyces*
Figure 6. Effect of different range of temperatures (20-60 °C) on locally purified L-glutamate oxidase stability from *Streptomyces*

### 3.8.2. Optimal pH for enzyme activity and stability

The activity was measured at different pH. It was inferred that the L-glutamate oxidase enzyme has the maximum activity in (pH 7.0) which enzyme activity was 27 U/ml as shown in figure 7. It can be concluded that the activity of L-glutamate oxidase was greater at neutral or almost basic pH values than at nearly acidic pH values. For determination of the pH stability, the remaining activity was determined, it was observed that pH ranged from (5.0-7.0) was the optimum pH for L-glutamate oxidase stability where the remaining activity was 100% as shown in figure 8, this stability decreased in extreme acidic pH, the enzyme remained 40% of its activity at pH 3, and 75% of the activity has remained at pH 8.
Figure 7. Effect of different pH values (3.0-8.0) on locally purified L-glutamate oxidase activity from *Streptomyces*

Figure 8. Effect of different pH values (3.0-8.0) on locally purified L-glutamate oxidase stability from *Streptomyces*
4. Discussion

The experimental data of this research showed that the extracellular L-glutamate oxidase can be easily purified by the protocol used to purify proteins. One of the successful methods used to purify enzymes was the precipitation with ammonium sulfate, in this study 60% saturation ratio was used, and this percentage was favorable and close with Kusakabe, Midorikawa (21), who used a 50% saturation ratio in their study. After that ion exchange chromatography and gel filtration were used, and the results of these two steps reflect the difference between the previous steps in purification as the highest values of specific activity and high purity of enzyme were obtained in these two steps. Because enzymes can be used in different applications, it is necessary to identify the thermal stability and activity in a wide range of pH, and the results showed that the optimum temperature for L-glutamate oxidase activity was 37 °C, and thermal stability was ranged from (30-50) °C. This result is almost in agreement with the findings of BÖHMER, MÜLLER (22) they find that the enzyme was stable from (30 to 50 - 55) °C. This difference in the effect of temperature is due to the thermal effect on the enzyme structure followed by denaturation, as temperature affects the protein structure by breaking the bonds that stabilize the secondary and tertiary structures of the protein, leading to denaturation and thus loss of enzymatic activity (23). While the optimal pH for enzyme activity and stability was determined and according to the findings of this study the optimum pH for L-glutamate oxidase activity was 7.0, this is consistent with the results of Wachiratianchai, Bhumiratana (6), they report that the optimum pH was ranged from 7.0 to 7.4, while stability ranged from (5.0- 7.0). The reason for the decrease or increase in the activity of the enzyme at the different pH values is due to a change in the protein nature of the enzyme due to a change in the ionic state of the amino acid side chains, which is necessary to maintain the three-dimensional structure of the enzyme and pH affects the ionic groups of the active site in the enzyme. The optimum pH for enzyme stability is necessary to provide the appropriate environment for enzyme storage (24).

The results of this work showed that after 60 hours of incubation of *streptomyces* in a wheat bran medium, the L-glutamate oxidase enzyme can be obtained. The optimal temperature and pH for enzyme production were also investigated, with the results revealing that the best pH and temperature for enzyme production were 7.0 and 30 °C, respectively. Furthermore, ammonium sulfate precipitation, an ion-exchange column, and a gel filtration column can all be used to purify
the L-glutamate oxidase enzyme. Gel filtration chromatography was the best approach to purify the enzyme isolated from *Streptomyces*. The appropriate temperature and pH for the enzyme's activity and stability were evaluated to store the enzyme and preserve its activity and stability. And according to the findings of the study the maximum temperature for the L-glutamate oxidase enzyme activity was 37 °C, and its stability ranged from (30-50°C), whereas the enzyme's efficiency pH value was 7.0 and its stability ranged from (5.0-7.0).

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References