RESEARCH ARTICLE

Extraction, Purification, and Characterization of Chloramphenicol Acetyltransferase from *Pseudomonas aeruginos*a

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ABSTRACT

Chloramphenicol acetyltransferase (CATs) is a bacterial enzyme that detoxifies the antibiotic chloramphenicol and is implicated in bacterial chloramphenicol resistance. The acetyl group from acetyl-CoA is covalently bound to chloramphenicol by this enzyme, preventing chloramphenicol from binding to ribosomes. In this study, *Pseudomonas aeruginosa* was harvested to study its chloramphenicol acetyltransferase. *P. aeruginosa* was isolated from urinary tract infections and diagnosed morphologically, microscopically, and with VITEK 2 system. Then we examined the ability of bacteria to produce chloramphenicol acetyltransferase; initially was tested for resistance to the antibiotic chloramphenicol, and then cultured in the production media difco nutrient broth (supplemented with 1% of glycerol and addition of the substrate chloramphenicol) which was suitable for growing bacterial cells and extraction of the enzyme. Chloramphenicol acetyltransferase was extracted by ultra-sonication and then purified through precipitation with ammonium sulfate as a first purification step, ion-exchange chromatography, and gel filtration. The activity of the enzyme was measured for each purification step. Purification of chloramphenicol acetyltransferase from *P. aeruginosa* using ammonium sulfate revealed that the specific activity was 20 units/mg protein, and the best saturation ratio was 40%, the purification times were 1.5, and the enzymatic yield was 88.8%. Furthermore, the results of enzyme purification by using ionic exchange DEAE-Cellulose were: the specific activity was 47.5 unit/mg protein, the enzymatic yield was 88.6%, and the number of purification times was 3.7. The purification results using gel filtration Sephadex G-100 showed that the specific activity was 66.6 unit/mg protein, purification times, and enzymatic yield were 5.2 and 66%, respectively.

Keywords: Chloramphenicol acetyltransferase, Chloramphenicol acetylase, *Pseudomonas aeruginosa*, Purification.

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INTRODUCTION

Enzymes are the complex protein molecules that consist of a chain of amino acids bound together by peptide bonds. They can catalyze a single biochemical reaction or a set of closely related reactions in living organisms; they act to convert the substances involved in the reaction into products.[1]

Enzymes possess highly specific both in the reactions that they catalyze and in their selection of substrates. temperature and pH are essential variables that influence enzyme activity and alter amino acid structure leading to alteration in the primary, secondary, and tertiary structure of enzyme.[2]

CATs (EC 2.3.1.28) is a bacterial enzyme that detoxifies the antibiotic chloramphenicol and is responsible for high levels of antibiotic resistance in bacteria. It catalyzes the conversion of an acetyl group from acetyl CoA to chloramphenicol's primary hydroxyl. The general function of this enzyme is chloramphenicol O-acetyltransferase activity, and the specific function is an effector of chloramphenicol resistance in bacteria. The cat gene encodes CATs that inactivate the drug chloramphenicol by acetylation.[3]

P. aeruginosa is a gram-negative bacterium widely distributed in the environment and interacts with eukaryotic host organisms in various ways. It is an opportunistic pathogen distributed widely in humans, causing many infections in both the community and healthcare facilities. *P. aeruginosa* isolates show high-level resistance to chloramphenicol that is usually associated with the enzyme CATs.[4]

Enzymes are extracting from microorganisms according to their presence inside or outside of the cell. Microorganisms produce either extracellular enzymes that excrete outside of the cell into the medium and extract by centrifugation or intracellular enzymes which remain inside the cell and need to use a mechanism for cell lysis to release enzyme and extract it.[5]

Enzymes purification involves several steps, beginning with salt precipitation which involves precipitation of proteins, thus eliminating residual nutrients of the medium and other released byproducts. It is usually carried out using ammonium

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sulfate. Ammonium sulfate precipitation is followed by dialysis to remove the salt which is bound to the protein. After that enzyme will be purified with ion-exchange chromatography then gel filtration chromatography.[6]

This study aimed to extraction, purification, and characterization chloramphenicol acetyltransferase from *P. aeruginosa* to detect the biological properties of the purified enzyme through the following steps:

- Bacterial isolation, examination, and evaluation of their ability to produce the enzyme
- Extraction of the enzyme using ultrasonication
- Purification of the enzyme using these steps (salting out with ammonium sulfate, ion-exchange chromatography, and gel filtration chromatography)
- Characterization of the enzyme

MATERIALS AND METHODS

Bacterial Isolation and Identification

The bacteria were isolated from women with urinary tract infections from Fatima Al-Zahraa maternity hospital and Al-Elwiya Teaching Hospital in Baghdad, Iraq. These isolates were verified as *P. aeruginosa* morphologically, microscopically and with Vitek2 system diagnosis. *P. aeruginosa* identified by:

Gram Stain

A tiny bacterial smear was organized on an uncontaminated glass slide, air dehydrated and heat stabilized. The isolate was submerged with crystal violet for (0.5 to 2 minutes). The additional stain was detached by rinsing it under tap water. Later, the smear was stabilized with Gram's iodine for (1-minute), decolorized with alcohol, and then washed gently with running tap water. Finally, As a counterstain, safranin was used for (0.5 to 2 minutes) and detached the excess stain by washing it under tap water. The slide was dried and examined under 10x, 40x, and 100x objective lenses.[7]

Biochemical Test

Bacterial isolates were identified by biochemical tests such as oxidase test, catalase test, indole production, urease, and H_2S production.[8]

- *Oxidase test:* A commercially available oxidase disc was used which containing the reagent. The isolated colony was picked and rubbed in the disc to be tested, and then observed the inoculated area for a color change to deep blue or purple within 10 seconds.[9]
- *Catalase test:* A small bacterial isolate inoculum was placed on a microscope slide and mixed with a drop of hydrogen peroxide solution, then observed of oxygen bubbles elaboration.[10]
- *Indole production:* A sterilized test tube containing tryptophan broth was taken. A small amount of pure culture was inoculated into the tube, then incubated at 37°C for 24–48 hours. The broth culture was then provided 0.5 mL of Kovac's reagent and then observed for the presence or absence of a ring.[11]

Morphology

The bacteria from a mixed population were isolated into a pure culture using the streak plate technique. *P. aeruginosa* was streaked across the agar surface, and some individual bacterial cells were isolated and well-spaced. The original sample was diluted by streaking it over successive quadrants and then incubated at 37°C for 24 hours; the number of organisms decreased and will show the bacterial morphology.[12]

Microorganisms Media and Growth Conditions

The Medium difco nutrient broth (supplemented with 1% glycerol and addition of the substrate chloramphenicol) was prepared in 1000 mL of distilled water; sterilized in the autoclave at 121°C and pressure 15 pounds/inch^2 for 15 minutes. Bacterial cells were cultured and incubated for 24 hours at 37°C, then incubated in shaking incubator 150 rpm at 35°C which the optimum temperature for producing the enzyme that approved after studying the optimum conditions.[13]

The Optimum Conditions for the Enzyme Production

- *The Optimum Temperature:* Bacterial cells cultured in the production medium and incubated in different degrees of temperature ranged between (25, 30, 35, and 40°C) for 24 hours, and then the activity of the enzyme was measured to find out the optimum temperature.[14]
- *The Optimum pH:* The production medium pH was changed within (6, 7, 8 and 9), then the medium was inoculated with bacterial cells and incubated at 35°C for 24 hours. The activity of the enzyme was measured to find out the optimum pH.[15]

Extraction of Chloramphenicol Acetyltransferase from Bacteria

The intracellular enzyme was extracted by ultra-sonication. The broth medium, which contained bacterial culture, was centrifuged for 15 minutes at 4000 rpm, and the cells were harvested, packed cells were re-suspended in Tris-HCl buffer. Cells disruption was achieved by ultrasonication for 6 minutes in the ice bath, followed by centrifugation at 15000 rpm for 10 minutes; the supernatant was separated as a crude intracellular enzyme.[16]

Measurement of the Enzyme Activity

The activity of enzyme CATs was carried out according to the method of Shaw.[6] From the individual reagents, the reaction mixture is freshly prepared (DTNB, Tris-HCl buffer 1.0 M; pH 7.8 and acetyl CoA 5 mM), then 50 microliters of the crude enzyme were added to 1-mL of the reaction mixture and allowed to equilibrate with the water bath. After that, chloramphenicol (5 mM) was added to start the reaction. The activity of the enzyme was measured at 412 nm. 1 unit of CAT equal to (1-µmole) of chloramphenicol acetylated per minute under experimental conditions.

Purification of Chloramphenicol Acetyltransferase

Precipitation with Ammonium Sulfate

Different saturation ratios of ammonium sulfate were added to the crude enzyme (20, 30, 40, 50, 60, 70, and 80%). The

mixture was mixed gently on a magnetic stirrer for 30 minutes in the ice bath after that, centrifugation at 6000 rpm at 4°C for 20 minutes. The precipitate obtained was dissolved in a suitable volume of phosphate buffer saline; pH 7.3. The protein concentration and enzyme activity were both measured.[17] Under cooling conditions, the enzyme solution was dialyzed after precipitation with ammonium sulfate by distilled water for 24 hours, the distilled water was changed four times, then the enzyme activity and protein concentration was measured.[18]

Ion-exchange Chromatography

According to Whitaker and Bernard (1972), a column of DEAE-Cellulose (2×35 cm) was prepared by 20 g of DEAE-Cellulose resin suspended in 1 liter of distilled water. After allowing the mixture to settle, it was washed several times with distilled water until it was clear. Under discharging, the suspension was filtered using Buchner's funnel, and then the resin was re-suspended in 0.25 M solution of sodium chloride and sodium hydroxide. The suspension was filtered once more and washed several times, as previously reported with 0.25 M solution of hydrochloric acid and then by distilled water before it was equilibrated with 0.05 M Tris-HCl buffer pH 8.[19] The DEAE-Cellulose column was previously equilibrated with 0.05 M Tris-HCl buffer pH 8 and the enzyme solution obtained in the previous step was added to it. Then an equivalent amount of the same buffer was used to wash the column, while the attached proteins were eluted in steps with increasing sodium chloride concentrations (0.1–0.9M). Each fraction's absorbance was measured at 280 nm using a UV-vis spectrophotometer at the column's flow rate of 36 mL/hour. In each fraction, chloramphenicol acetyltransferase activity was measured. CATs activity was found in fractions that were pooled and held for further purification steps.[20]

Gel Filtration Chromatography

Sephadex G-100 was made according to the instructions given by Pharmacia Fine Chemicals Company. It was packed in a glass column (1.5×55 cm) after being suspended in 0.1 M Tris-HCl buffer pH 8, degassed. The gel was kept for 72 hours at room temperature for swelling then equilibrated with Tris-HCl buffer. The ion-exchange step yielded concentrated chloramphenicol acetyltransferase, which was added to the column. Elution was carried out at a flow rate of 30 mL/hour, and equilibration was carried out with the same buffer. Using a UV-vis spectrophotometer, the absorbance of each fraction was estimated at 280 nm. The activity of chloramphenicol acetyltransferase was measured in each fraction.[21]

Characterization of Chloramphenicol Acetyltransferase (CAT)

The Optimum pH and Temperature of CAT Activity:

The optimum pH was determined for chloramphenicol acetyltransferase activity at 37ºC by incubating the enzyme in buffers at different pH (6.0–8.0) for 10 minutes. For pH (6.0, 6.5) Sodium acetate buffer (0.05 M) was used. For pH 7.0 potassium phosphate buffer (0.05 M) was used and Tris-HCl buffer (0.05 M) , for pH (7.5, 8.0). Then the enzyme activity was measured.[22]

Also, the optimum temperature was determined, the reaction mixture was incubated for 10 minutes at different temperatures from 25 to 45ºC, and then the enzyme activity was determined.[23]

The Optimum pH and Temperature of CAT Stability

Chloramphenicol acetyltransferase stabilitywas examined at different pH values, and the enzyme was incubated in buffers ranging from 6.0 to 8.0 for 30 minutesat 37ºC. For pH (6.0, 6.5), sodium acetate buffer (0.05 M) was used,for pH 7.0 potassium phosphate buffer (0.05 M) was used,and Tris-HCl buffer (0.05 M) for pH (7.5, 8.0). Then, it was placed in ice,and residual enzyme activity was calculated and represented as a percentage of the relative enzyme activity.[24] The thermal stability of the test sample was determined by incubating it for 30 minutes at temperatures ranging from 25 to 45ºC for 30 minutes. The amount of residual enzyme activity was calculated and expressed as a percentage of the relative enzyme activity.[25]

RESULTS AND DISCUSSION

Bacterial Identification had been identified previously in the hospital, and even further identification was made by using Gram staining, morphological, and biochemical tests to ensure that the isolates were pure and belonged to *P. aeruginosa*. The result agreed with the previous identification of *P. aeruginosa*,[26] as shown in Table 1 and Figure 1.

The Optimum Temperature for CAT's Production

Temperature affected enzyme production that was determined by maintaining the flasks at a range of temperatures (25, 30, 35, and 40°C) for 24 hours (Figure 2).The maximum enzyme production was detected at 35°C when the specific activity was (23 U/mgprotein). The enzyme's optimum temperature corresponds to maximum activity. The enzyme's activity decreases as the temperature rises or falls.[27]

The Optimum pH for CATs Production

For the evaluation of chloramphenicol acetyltransferase production, the pH of the medium was adjusted to 6, 7, 8, and 9 (Figure 3). After 24 hours of incubation, the maximum enzyme production was observed at pH 7 when the specific activity was (22.4 U/mg protein).Each enzyme has an optimum pH; if the pH is too high or too low, the enzyme's activity is reduced.[27]

Table 1: Identification of *P. aeruginosa*

Test	*Test result*
Oxidase	Positive (+ve)
Catalase	Positive (+ve)
Indole production	Negative (-ve)
Urease	Negative (-ve)
H2S production	Negative (-ve)
lactose fermenter	Negative (-ve)
Colonies (morphological)	Forms 2–3 mm round colonies, smooth with irregular surface
Color and odor	Greenish color and fruity odor
Gram stain	Pink rod gram negative (-ve) bacteria

Precipitation with Ammonium Sulfate

The first step in the purification process is ammonium sulfate used to purified chloramphenicol acetyltransferase from *P. aeruginosa*, which involves precipitation of proteins, thus eliminating residual nutrients of the medium and other released byproducts. Different saturation ratios (20, 30, 40, 50, 60, 70, and 80%) were used, mixed gently with enzyme for 30 minutes in the ice bath, and centrifuge at 6000 rpm for 20 minutes at 4°C. The precipitate was dissolved in a sufficient amount of buffer. Enzyme activity and protein concentration were measured. The results showed that the best saturation ratio was 40%, which contradicts Shaw, 1972,[6] specific enzyme's activity was 20 unit/mg, the purification times 1.5, and the enzymatic yield 88.8% as shown in Table 2.

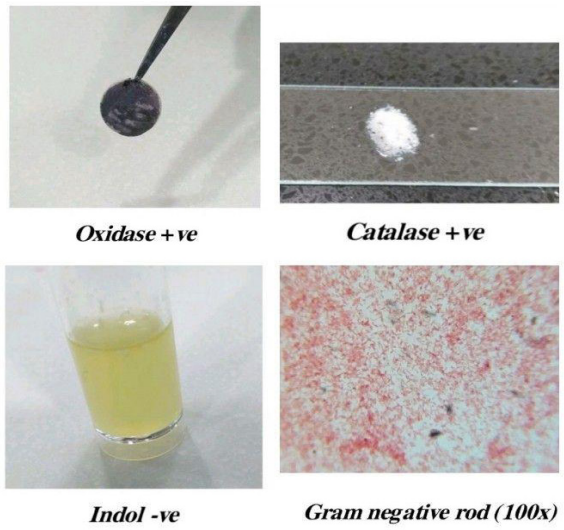

Figure 1: Identification of *P. aeruginosa* by biochemical test and gram staining

Purification by Ion-exchange Chromatography

The column of DEAE-Cellulose was used as an anion exchange; the enzyme solution was applied to the DEAE-Cellulose column equilibrated previously with 0.05 M Tris-HCl buffer pH 8. The results showed that only one distinctive protein band in the elution showed CAT activity (Figure 4). In the elution step, when the enzyme passes, the negative charge of the enzyme will bind to the positive charge of substance in the column while the proteins with opposite charge pass through the column, then the enzyme eluted by added elution buffer. As well as the results showed the specific activity was 47.5 unit/mg protein, the total activity was 39.9 unit, the purification times was 3.7, and the yield of enzyme was 88.6%.[28]

Purification by Gel Filtration Chromatography

The results of purification by gel filtration chromatography showed that only one distinctive protein band which showed the activity of CAT (Figure 5); Where the specific activity was 66.6 unit/mg protein, the total activity was 30 unit, the purification times was 5.2, and the yield of enzyme was 66%.[28]

Characterization of Chloramphenicol Acetyltransferase[29]

The Optimum pH and Temperature of CAT Activity

The optimum pH for CAT activity was determined by incubating the enzyme in various pH-adjusted buffers (6.0–8.0) for 10 minutes. The results showed that the optimum pH for enzyme activity was 7.0 where the maximum enzyme

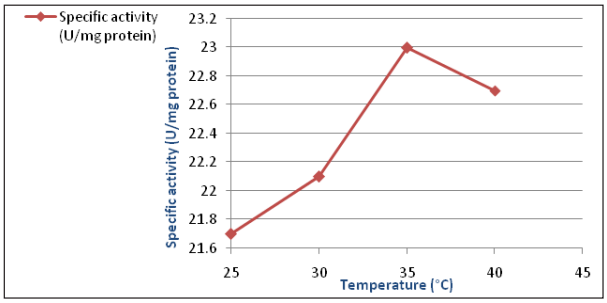

Figure 2: The effect of temperature on the CATs production

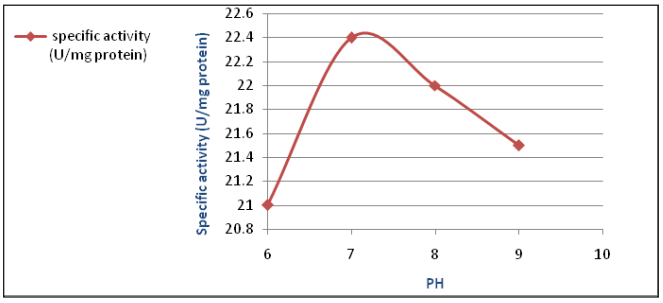

Figure 3: The effect of pH on the CAT production

Table 2: Results of purification of CAT from *P. aeruginosa*

Purification step	*Volume (mL)*	*Enzyme activity (U/mL)*	*Protein concentration (mg/mL)*	*Specific activity (U/mg protein)*	*Total activity (U)*	*Purification (folds)*	*Yield (%)*
Crude enzyme	50	0.9	0.07	12.8	45	1	100
Ammonium sulfate precipitation (40%)	40	1.0	0.05	20	40	1.5	88.8
Dialysis	40	1.0	0.045	22.2	40	1.7	88.8
Ion-exchange chromatography on DEAE-cellulose	21	1.9	0.04	47.5	39.9	3.7	88.6
Gel filtration chromatography on Sephadex G-100	15	2.0	0.03	66.6	30	5.2	66

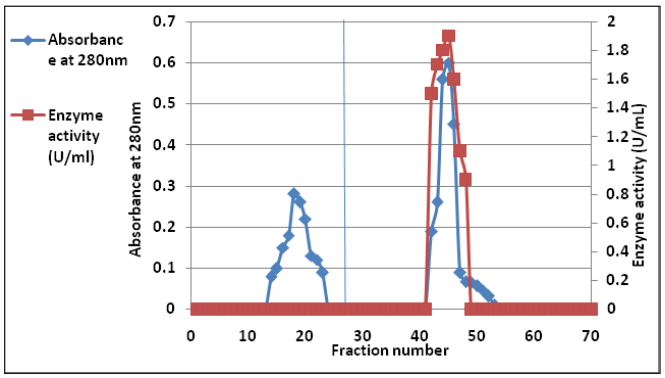

Figure 4: Ion exchange-chromatography for purification of CAT from *P. aeruginosa* using DEAE-Cellulose column (2×35 cm)

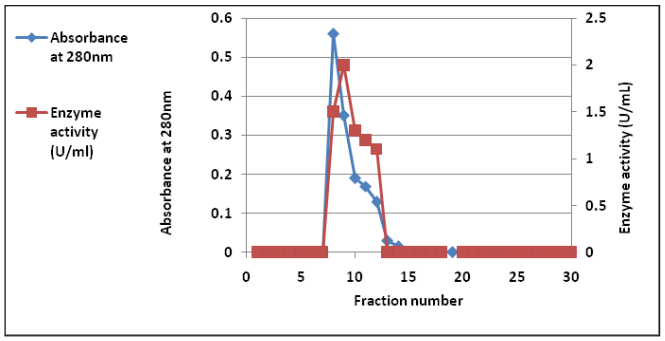

Figure 5: Gel filtration chromatography for purification of CAT from *P. aeruginosa* using Sephadex G-100 column (1.5×55 cm)

activity was 1.8 unit/mL while the minimum enzyme activity was 0.5 unit/mL at pH 6.0.

By incubating the reaction mixture for 10 minutes at various temperatures from 25 to 45ºC, the optimum temperature was determined. The results showed that the velocity of the reaction was increased when the temperature reached 30ºC where the enzyme activity was 1.88 unit/mL, then lowered when the temperature increased.

The Optimum pH and Temperature of CAT Stability

The optimum pH for chloramphenicol acetyltransferase stability was determined by incubating the enzyme in various pH buffers ranging from 6.0 to 8.0 for 30 minutes. The results showed that the chloramphenicol acetyltransferase had high stability at pH range (6.5–7.5).

The thermal stability of the test sample was examined by incubating it at various temperatures ranging from 25 to 45ºC for 30 minutes. The enzyme was found to be stable at temperatures ranging from 30 to 40ºC.

CONCLUSION

The enzyme will be increased in the neutral pH. Higher or lower than neutral, the activity of the enzyme will be lowered. Higher or lower temperatures will also affect enzyme activity.

The gel filtration shows the optimal purification, and the high specific activity was obtained through gel filtration chromatography because the protein concentration drops at each step.

The optimum temperature of chloramphenicol acetyltransferase activity was 30ºC, and the enzyme was stable at the temperature range 30 to 40ºC. The optimum pH for chloramphenicol acetyltransferase activity was 7.0, and this enzyme had high stability at pH range (6.5–7.5).

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