Open Access
Baghdad Science Journal **Vol.16(4) 2019**

DOI: http://dx.doi.org/10.21123/bsj.2019.16.4.0824

Extraction, Purification and Characterization of Peroxidase from *Pseudomonas aeruginosa* and Utility as Antioxidant and Anticancer

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Received 10/8/2018, Accepted 7/1/2019, Published 1/12/2019

Abstract

Peroxidase is a class of oxidation-reduction reaction enzyme that is useful for accelerating many oxidative reactions that protect cells from the harmful effects of free radicals. Peroxidase is found in many common sources like plants, animals and microbes and have extensive uses in numerous industries such as industrial, medical and food processing. In this study, *P. aeruginosa* was harvested to utilize and study its peroxidases. *P. aeruginosa* was isolated from a burn patient, and the isolate was verified as *P. aeruginosa* using staining techniques, biochemical assay, morphological, and a sensitivity test. The gram stain and biochemical test result show rod pink gram-negative bacteria, and ensure that the isolate was that of *P. aeruginosa*. Optimization for bacterial growth were done by used more than pH (5,7,9) and temperatures (32,35,37°C), and it was found that the best growth conditions were at pH 5.5, producing ($4.5x10^8$cells), and a temperature of 37°C, with ($5.25x10^8$cells) being produced. Intracellular enzymes were extracted by ultra-sonication that used frequencies of ultrasound 30 kHz for 20 min in 4 °C, and was centrifuged at 13000×g for 5min. The supernatant was then re-used as a crude enzymatic extract and the cell pellet was discarded. Purification of peroxidase was accomplished by using salt precipitation, dialysis, gel filtrations and ion exchange chromatographic techniques. The result shows that gel filtration has optimal specific activity and purification fold at (61 U/ml), purification fold 6 times and then the improvement enzyme was applied as H_2O_2 scavenging activity antioxidant by used three concentration of enzyme (10,40,60 µg/ml), and show higher scavenging activity at 60 µg/ml, which reached to 45% scavenging activity. The enzyme was also used as anticancer agent, which was verified by using three concentration of enzyme (10,15,20 µg/ml) which show a significant kill for Mcf-7cells at (15µg/ml), with cytotoxicity activity reaching (45%).

Key words: Anticancer, Antioxidant, Mcf-7, Peroxidase, *Pseudomonas aeruginosa.*

Introduction:

Enzymes have become more important products for the food and drug industry. Peroxidase (EC.1.11.17) is characterized as an oxidation-reduction enzymes, which and accelerates reaction rates among H_2O_2 as an electron acceptor and various kinds of substrates by O_2 release from H_2O_2.Harmful electrons such as super oxide and hydroxide radicals, can be found in cells due to the presence of oxygen. Peroxidases use H_2O_2 as an electron acceptor which catalyzes many oxidative reactions (1). Peroxidase is an enzyme which has iron that catalyzes the transport of oxygen from hydrogen peroxide to an appropriate substrate and thus takes about oxidation of the substrate (2). There are many ways to extract enzymes depending on location.

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Extracellular enzymes are extracted by centrifugation and intracellular enzymes are extracted by either chemical or mechanical method such as ultra-sonication(3).

The purification of a specific enzyme involves the elimination of other substances (proteins as well as non-proteins) existing in the preparation./ Purification of the enzyme is usually more than a stepwise procedure using a range of biophysical and biochemical features, such as its relative concentration in the source, solubility, charge, size (molecular weight) and hydrophobicity/hydrophobicity of the target protein. In general, the enzyme purification is done either by (ammonium sulfate, dialysis, gel filtration or ion exchange) (4). The problem with an antioxidant drug is their interference with anticancer drugs which reduce the effect of drug, causing ulcers and nausea (5). The side-effects of cancer drugs are loss of weight, loss of hair and cost (6). The aim of this study was to improve the peroxidase yield and

specific activity through purification, of local peroxidase enzyme that is lower cost than synthetically produced enzyme, which is used as an antioxidant and anticancer with no side effects, and to study the optimal temperature and buffer for peroxidase production from bacterial growth.

Materials and Methods:

Isolation and Identification of Bacteria:

Twenty bacterial isolate were obtained from Al-Yarmouk hospital from burns, UTI, and otitis media patients in 2017/11/3, and then test the best isolates for growth by spectrophotometry. It was found that the optimal isolate for enzyme production was burns isolate and 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 (30 sec to 2 min). The additional stain was detached by rinsing it under tap water. Later, the smear was stabilized with Gram's iodine for (1 minute) and was decolorized with alcohol, then it was washed under running tap water gently. Finally, the smear was counterstained with Safranin for (30 sec to 2 min.), with excess stain detached by washing it under tap water. The slide was spot dried with bibulous paper and experiential under 10x, 40x and 100x objective lenses (7).

Biochemical Test

Bacterial isolates were identified using (France Biomerieux API kits). First, 5 ml of sterilized distilled water was added into the tray to offer a moist atmosphere which avoid drying of the strip and a suspension of the bacteria was used to inoculate the wells then incubate at 37°C overnight at (8).

Morphology

Streak plate technique was used for the isolation into pure culture of the organisms (mostly bacteria), from a mixed population. The *P. aeruginosa* was streaked over the agar surface, and some individual bacterial cells are separated and well-spaced from each other. As the original sample is diluted by streaking it over successive quadrants and then incubate at 37°C for 24h, the number of organisms' decreased and will show the bacterial morphology (9).

Optimal Temperature and pH for Peroxidase Production from *P. aeruginosa*

The bacterial suspension was cultured once at constant temperature but different pH (5.5, 9, and 7) and once at constant pH but different temperature (32, 35, and 37°C), the absorbance was measured at 556 nm (10).

Extraction of Enzyme and Proteins

We used 60 ml of production broth (the broth that contain the bacterial cells in which the intracellular peroxidase was found) and transported it into centrifuge tubes at 4000 rpm for 10 minutes. Supernatant consuming extracellular protein was discarded and the pellet containing bacterial cells was collected. The mixture was sonicated by using frequencies of ultrasound 30 kHz for 20 min at 4 °C, then centrifuged in 4000 rpm for 5 min. The supernatant was used as a crude enzymatic extract (11).

Measurement of Crude Enzyme Activity

The activity was determined according to the methods in (12). The substrate solution was accomplished by put all the following volumes according to each ratio:

Guaicol: Hydrogen peroxide solution of 0.2mM: Sodium acetate solution 0.2mM: Distilled water
1: 1: 1: 7 (v: v: v: v).

Three ml of substrate solution was added to the cell of spectrophotometer (cuvette) when it was considered as a blank solution. 0.1 ml of enzyme solution was added and mixed well, and we measured the absorbance at 1 minute at 470nm and drew the relationship between the absorbance and time. Peroxidase activity (unit activity) the amount of enzyme that oxidize (1μM) of guaicol in one minute under experiment condition.

Ammonium Sulfate Precipitation

The ratio of ammonium sulfate was determined by adding gradually the amount of salt to each 10 ml enzyme solution in ice bath and magnetic stirrer for 1 hr. The solution was centrifuged at 4000rpm/min for 10 minute, and then the precipitate was isolated and dissolved in 10ml sodium acetate (0.2mM buffer), and we calculated the activity and protein concentration (13).

Dialysis Tube

15ml of isolated enzyme was put in the tube with 2.5 cm in diameter that allow molecules less than 12KD to pass in the sucrose solution, then the dialysis tube was put in the beaker contain sucrose for 3 hour to discard salt and retain the enzyme (14).

Separation of Enzyme Through Ion Exchange Resin (DEAE Cellulose)

The DEAE-Cellulose was accomplished along with the method suggested by Whitaker and Bernard (1972). Twenty grams from ion-exchange resin were put off in 1 liter distilled water, retained in the graduated cylinder to decay. Once the supernatant became pure, the ion exchange resin was washed away all contaminate manner by using Buchner's funnel under vacuum (without drying the ion exchange resin), then the resin was stimulated in 250 ml from buffer (0.25M sodium hydroxide) for 30 minutes. The resin was re-filtered and washed under vacuum using distilled water, then the resin

was solubilized in 250ml of -0.25M hydrochloride acid with agitation for 30 minutes. After that, the resin was cleaned with distilled water under vacuum, and the resin was suspended in potassium phosphate buffer (5mM, pH=7), and the ion exchange resin was degassed using a vacuum pump. The resin was boxed softly in glass column (2.5×16cm), and the equilibration was achieved by the same potassium phosphate buffer (15) .

Enzyme Separation Through Sephadex G-200 column

The preparation of the gel was achieved as recommended by a supplied company. 20 gram of gel Sephadex G-200 powder was put off in 500 ml distilled water and placed in a water bath at 90 ˚C for 3 hour and then washed twice with potassium phosphate buffer (0.2M, pH=7). After that, the gel was suspended, then placed in a vacuum pump, and the gel was boxed softly in glass column with dimensions (1.5×10) cm (16).

Peroxidase H_2O_2 Scavenging Activity

The unbound electron scavenging activity of the extracts was determined by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) as said by the earlier reported method by Shin. A 0.1mM solution of DPPH in methanol was accomplished and 1 mL of this solution was added to 3 ml of the solution in methanol at different concentration (20,40,60, µg/mL). The mixtures were shaken and allowed to settle at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer, with ascorbic acid as the control. Lower absorbance values of the reaction mixture show higher free radical scavenging activity and the ability of scavenging of the DPPH radical was calculated by using the following formula DPPH scavenging effect (% inhibition) = {(A0 –A1)/A0) x100 (17).

Cytotoxicity of Peroxidase on MCF-7 Cells Using MTT Assay

This assay was done in Al-Nahrain Biotechnology Research Center. MCF-7 cells were provided by Al-Nahrain Biotechnology Research and was incubated 24 hours for attachment. Peroxidase with concentrations (20, 10, 5 µg/mL) were added in triplicates and incubated for 48 hours at 5% CO_2 and 37°C. Afterwards, the cells were treated with MTT and incubated for 4 hours. After incubation, all of the medium including MTT solution were aspirated from the wells. The absorbance was measured at 492 nm using ELISA Micro Plate Reader. The cytotoxicity catalogue was firm, using the untreated cells as negative control. The percentage of cytotoxicity was calculated using the background corrected absorbance follows: Where A is the optical density of control, B is the optical density of treated sample. Inhibition rate % = (A - B/ A) X 100 (18).

Statistical Analysis

The obtained data were statically analyzed using unpaired t-test with Graph Pad Prism 6. The values were presented as the Mean ± S.E of the three replicates of each experiment.

Results and Discussion:

Identification of Bacteria

The bacterial isolates were previously identified in Al-Yarmouk hospital and farther identification was done by using Gram staining, morphological, and biochemical tests to ensure that the isolates were pure and belonged to *P. aeruginosa*. The result agreed with previously identification of *P. aeruginosa* (19), as shown in Table 1.

Table1. Identification of *P. aeruginosa*

Test	Test result
Oxidase	(+)
Catalase	(+)
H_2S production	(-)
Urease	(-)
Indole production	(-)
lactose fermenter	(-)
methyl-red	(-)
Glucose	(+)
Colonies(morphological)	Forms 2-3mm round colonies, smooth with irregular surface
Color and odder	Greenish color by procaine stain and fruity odder
Gram stain	Pink rod gram (-ve) bacteria

Optimal pH and Temperature for Peroxidase Production from *P. aeruginosa*

The bacterial suspension was incubated once at constant temperature but different pH (5.5, 9, and 7) and once at constant pH but different temperature (32, 35, 37°C), and the absorbance was measured at 556nm and compared with the McFarland number. It was establish that the best conditions for the creation of bacteria at pH 5.5 with ($4.5x10^8$cells) as compare with pH (9, 7) with ($3x10^8$)($0.5x10^8$cells) respectively and a temperature of 37°C with bacterial number ($5.25x10^8$cells). It was less at (32,35°C) with bacterial number ($2.25x10^8$, $3.5x10^8$cells) respectively, and this agreed with the result of (20), as in Fig.1 and 2.

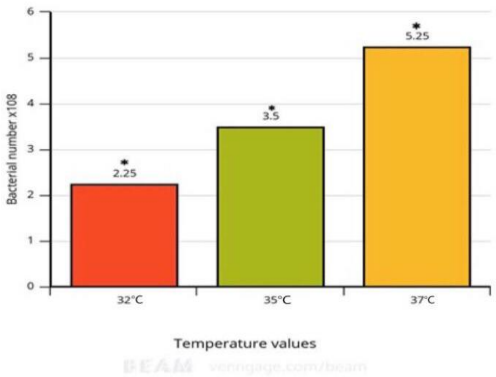

Figure 1. The optimal Temperature for Peroxidase production extracted from *P. aeruginosa* The values represents the Mean ± S.E *P <0.05.

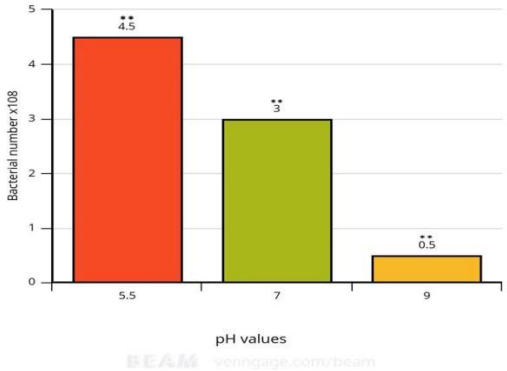

Figure 2. The optimal pH for peroxidase production extracted from *P.aerugionsa* The values represents the Mean ± S.E **P <0.01.

Ammonium Sulfate

The 80% ratio was chosen as the greatest ratio for precipitation of crude enzyme, in which the specific activity got to 12.64 U/mg, purification 2.17 fold and yield 53%, as shown in table (2). The results disagreed with (21), when they purified peroxidase from various vegetables sources, they used ammonium sulfate to precipitate peroxidase with 80% saturation ratio. The specific activity was 3.9 U/mg protein with purification 1.93 fold.

Dialysis

The results showed higher specific activity and purification than the ammonium sulfate step. The specific activity was 32.77 U/mg with purification 5.63 fold and the result was disagreed from (22), for peroxidase extract from *Aspergillus niger* with specific activity reached to 23 U/mg.

Purification Through Ion Exchange Chromatography

As shown in the Fig.3, four protein peaks appeared in the elution and wash steps, but only one peak showed enzyme activity in the fraction number (60-66) in the elution step. This step show increases in the specific activity and purification fold (45 U/mg) and (7.73 times). The result disagreed with (23), extracted peroxidase from strawberry with specific activity reached to 34 U/mg and purification was 1.17 fold.

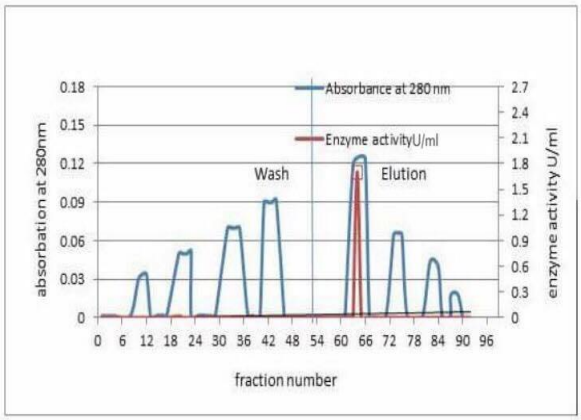

Figure 3. Ion exchange chromatography using DEAE-Cellulose column (1.5×10) cm with gradient salt (0.1-1) M NaCl, for purification peroxidase from *P. aeruginosa.* The column was eluted with 5mM phosphate buffer saline with flow rate (6 ml/h) and size of fractions are (0.1 ml).

Purification Through Gel Filtration

As shown in Fig.4, four peaks of protein were detected, but only one peak showed activity (21-25). In this step, the specific activity increased to (61.36 U/mg), purification was10.60 fold and the result was disagreed with (24), where peroxidase extracts from apple leaves was scored with specific activity at 9.2U/mg, as shown in Table 2.

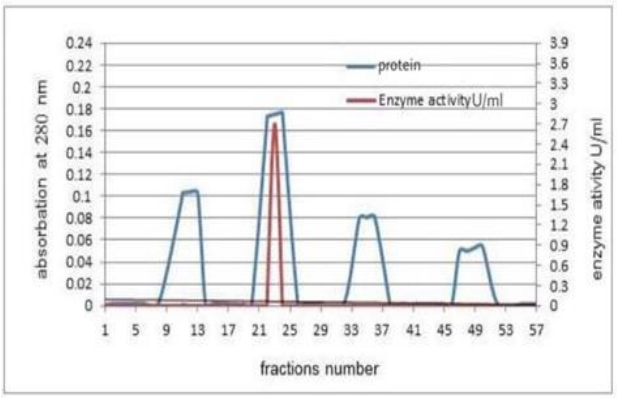

Figure 4. Gel filtration chromatography using sephadex G-200 column with dimensions (10×1.5) cm for purification the peroxidase from *P. aeruginosa* equilibrated with phosphate buffer 0.2M pH=7, the column was washed with 5mM phosphate buffer saline with flow rate (6ml/h) and fraction volume (0.5ml).

Table 2. The purification steps of peroxidase enzyme

Purification steps	volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	yield%
Crude enzyme	60	1.34	0.23	5.82	80.4	1	100
Ammonium sulfate precipitation in 80% ratio	20	2.15	0.17	12.64	43	2.17	53.34
Dialysis tube	10	2.95	0.09	32.77	29.5	5.63	36.70
Ion exchange chromatograph	9	1.8	0.04	45	16.2	7.73	20.14
Gel filtration	10	2.7	0.044	61.36	27	10.60	35.83

Peroxidase H_2O_2 scavenging activity by DPPH:-

The peroxidase has the ability to break down H_2O_2 and other free radicals to simple compounds, so it was used as scavenging activity by used DPPH reagent, which had extra free radicals shown as red color and then converted to yellow stain by the enzyme, with the stain breaking down the free radicals and measured at 517nm, as shown in the Fig.5. The tube with red color is the control, while the yellow is peroxidase enzyme and the white color is ascorbic acid (control positive) against free radicals. Three enzyme concentrations were used with triplicates (10, 40, 60, mg/mL), The results show scavenging activity of (20, 35, 43%) respectively and the result agreed with (24).

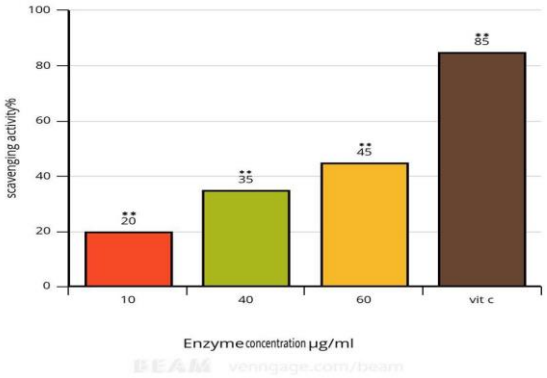

Figure 5. The relationship between enzyme concentration and scavenging activity. The values represent the Mean ± S.E **P <0.01.

Cytotoxicity determination using MTT assay:-

The cytotoxicity was done on MCF-7 cells by using MTT stain, which is a colorimetric method dependent on the conversion of yellow tetrazolium salt to purple formazan crystals according to cellular activity. By using three enzyme concentration with triplicate (10, 15, 20, µg/ml) and incubated for 48 hours In the CO_2 incubator, the results show that the concentration of control cells decreased with each concentration, and the cytotoxicity activity achieved was (20, 40, 45%). The peroxidase killed cancer cells by uptaking electrons from the lipid bilayer of cancer cells and rupturing the cancer cells in process known as lip-peroxidation, and the result was agreed with (25), as shown in Fig.6.

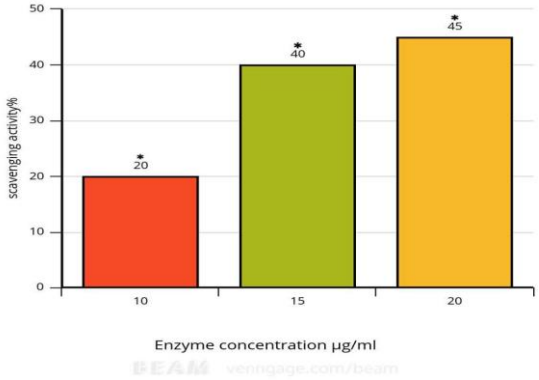

Figure 6. The effect of peroxidase enzyme produced by *P. aeruginosa* viability of cell line (MCF-7). The values represents the Mean ± S.E *P <0.05.

Conclusion:

The number of bacteria will be increased in the pH at 5.5 and decreased in the pH at 9, so the enzyme will be increased in the acidic pH and decreased in the basic pH. The ion exchange shows the optimal purification, the high specific activity was obtained through gel filtration chromatography because the protein concentration dropping in each step. The peroxidase shows antioxidant scavenging activity because of their effect on H_2O_2 by breaking down peroxide hydrogen. This extends to anticancer activity because of the peroxidase take the electrons in the cell wall and makes disturbances in the structure and electrons of cell wall, which ruptured the cancer cells.

Conflicts of Interest: None.

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استخلاص ، تنقية، توصيف انزيم البيروكسيديز من بكتريا الزوائف الزنجاريه واستخدمها كمضاد للاكسده ومضاد للسرطان

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الخلاصة:

يصنف انزيم البيروكسيديز من انزيمات التي تحفز تفاعل الاكسدة والاختزال والذي يحمي الخلايا الحيه من التاثير الضار من الجذور الحرة. يوجد البيروكسيديز في مصادر مختلفه مثل النباتات،الحيونات والمايكروبات.يمتلك البيروكسيديز تطبيقات واسعه في مجال الصناعه والطب وصناعة الاغذيه. في دراستنا الحالية ، تم استخدام بكتريا الزوائف الزنجاريه لانتاج انزيم البيروكسيديز.تم عزل البكتريا من مرضى الحروق وتشخيصها باستخدام طرق التصبيغ والفحوصات البايوكميائيه وفحص الحساسية وعن طريق شكل البكتريا حيث اظهرت النتائج بكتريا عصوية سالبة لصبغة كرام اما الفحوصات البايوكميائية فاكدت ان العزلات تعود لبكتريا الزائفة الزنجارية .تم دراسة الظروف المثلى لانتاج الانزيم ووجد ان افضل دالة حامضية هي بحدود 5,5 ودرجة حراره 37 درجه مئوية. تم تنقية الانزيم باستخدام الترسيب بالاملاح، الديلزة، المبادل الايوني، الترشيح الهلامي وكانت اعلى فعالية نوعيه قد اظهرت في خطوة المبادل الايوني بحدود (61وحدة/ملغم). تم استخدام الانزيم المحسن لكبت الجذور الحرة (مضاد للاكسدة) حيث حصلت اعلى نسبة كبت عد فعالية 60 بالمئة وكذلك استخدمه كمثبط للخلايا سرطان الثدي حيث سجلت اعلى نسبة كبح للخلايا السرطانية عند تركيز (15ملغم/مل).

الكلمات المفتاحيه: مثبط للخلايا السرطان، مضاد للاكسده، خلايا سرطان الثدي، بيروكسيديز،الزائفة الزنجاريه.