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Isolation and partial purification of heme oxygenase-1enzyme from the serum of children Iraqi patients with chronic myeloid leukemia

Assistant teacher Mustafa yassin khalf [1] , **Prof. Dr.** Wasan Nazhan Hussein[2]
Department of Chemistry , College of Education for Pure Sciences, Tikrit University, Iraq
1-my230038pep@st.tu.edu.iq , 2-Wasannnzhan@tu.edu.iq

Abstract :

Background: The present study was conducted to purify heme oxygenase-1 enzyme from the serum of patients with chronic myeloid leukemia (CML). The malignant genetic condition of hematopoietic stem cells leads to an increase in myeloid cells, so the cancerous cells are the myeloid cells in the blood. The study was conducted on the heme oxygenase-1 enzyme, which is an important indicator of this disease. It is a microsomal enzyme that has an antioxidant and anti-inflammatory role essential in the degradation of heme, **Objective:** To isolate, purify and study the kinetics of heme oxygenase 1 enzyme from the serum of patients with leukemia. **Materials and methods:** The enzyme was isolated and purified using chromatography (gel filtration, ion exchange) and precipitation using ammonium sulfate. The precipitated fraction, which has high enzyme activity, was dialyzed and finally, high-performance liquid chromatography (HPLC-SEC) was used to estimate the molecular weight, **Results:** The purification degree was 18.43, with an enzyme yield of 226.86 and a specific activity of 75.040 U/mg. Kinetic studies were conducted for the partially purified enzyme, where the optimum concentration of the substrate was 0.20 mmole/mL, the value of the Michaelis-Menten constant Km was 0.098, the maximum speed was 61.350 Vmax. **Conclusions:** Based on the results shown above, the isolation and purification process was carried out based on the basic principle of equalizing the charges on the surface of the protein, decomposing the water layer surrounding the protein, reducing the degree of hydration, and then dissolving the protein and precipitating.

Keywords: heme oxygenase-1, chronic myeloid leukemia, enzyme kinetics, enzyme molecular weight.

عزل وتنقية جزئية لأنزيم الهيم أوكسيجيناز-1 من مصل الأطفال العراقيين المصابين بسرطان الدم النقوي المزمن

م.م مصطفى ياسين خلف ، أ.د وسن نزهان حسين
my230038pep@st.tu.edu.iq Wasannnzhan@tu.edu.iq
قسم الكيمياء، كلية التربية للعلوم الصرفة، جامعة تكريت، العراق

مستخلص:

الخلفية: اجريت الدراسة الحالية لتنقية انزيم الهيم اوكسجنيز-1 من مصل مرضى المصابين بابيضاض الدم سرطان الدم النخاعي المزمن (CML) تؤدي الحالة الوراثية الخبيثة للخلايا الجذعية المكونة للدم إلى زيادة خلايا النخاع الشوكي فتكون الخلايا المتسرطنة هي الخلايا النخاعية في الدم، حيث اجريت الدراسة على انزيم الهيم اوكسجنيز-1 الذي يعد من المؤشرات المهمة لهذا المرض وهو انزيم ميكروسومي له دور مضاد للأكسدة ومضاد للالتهابات أساسي في تحلل الهيم، الهدف من الدراسة: عزل وتنقية ودراسة حركية انزيم الهيم اوكسجنيز-1 من مصل المرضى المصابين بأبيضاض الدم، المواد والطرق: تم عزل وتنقية الانزيم باستخدام تقنية كروماتوكرافيا (الترشيح الهلامي، التبادل الايوني) والترسيب باستخدام كبريتات الأمونيوم أجريت الديلزة للجزء الراسب الذي فيه فعالية الأنزيم عالية واخيراً استخدمت تقنية كروماتوغرافيا السائل عالي الاداء- كروموتوغرافيا استبعاد الحجم HPLC-SEC لتقدير الوزن الجزيئي النتائج: بلغت درجة التنقية18.43 وبحصيلة انزيمية226.86 والفعالية النوعية 75.040U/mg وتمت اجراء الدراسات الحركية للأنزيم المنقى جزئياً حيث كان التركيز الامثل للمادة الاساس 0.20mmole/mL، وقيمة ثابت ميكالس - منتن 0.098 Km وكذلك السرعة القصوى 61.350 Vmax ودرجة الحرارة المثلى لعمل الانزيم40 درجة مئوية والاس الهيدروجيني الامثل 6.8 وكانت أعلى فعالية للأنزيم في الحزمة الاولى وتم تعيين الوزن الجزيئي التقريبي للأنزيم المنقى جزئياً وكان حوالي 207.8554 كيلو دالتون.المناقشة: استناداً الى النتائج الموضحة اعلاه حيث تمت عملية العزل و التنقية اعتماداً على المبدأ الاساسي هو معادلة الشحنات على سطح البروتين وتحلل طبقة الماء المحيطة بالبروتين وتقليل درجة الترطيب ومن ثم ذوبان البروتين والترسيب.

1-1 Introduction

A group of cells that loses the normal principles of cell division and develops uncontrollably is referred to as cancer. The normal cell cycle is activated by signals that cancer cells respond to, resulting in aberrant cell proliferation and the promotion of altered cells.(1) Cancer is a collection of diseases that are characterized by the aberrant proliferation of cells that have the potential to invade other regions of the body or disseminate. In contrast, benign tumors do not have the ability to disseminate.(2) Leukemia is a blood malignancy that affects the bone marrow, which is the sponge-like tissue located in the interior of most bones and contains the majority of blood cells. The blood-forming stem cells in the bone marrow (an embryonic blood cell) are the origin of leukemia. The cell transforms into leukemia-type cells as a result of one or more malignant mutations.(3) They produce an increasing quantity of leukemia cells through the process of division and multiplication. Leukemia cells in the bone marrow may either obstruct or crowd out the production of healthy blood cells over time, and they may also seep into the circulation.(3)

1-2 Types of blood cancer

The classification of leukemia as lymphoma or myeloid is contingent upon the type of compromised stem cells. Blast leukemia or stem cell carcinoma typically indicate lymphoid or myeloid disease. Blood malignancies are referred to by a variety of names, which can be perplexing, depending on the cell in question. Four (4).

1-Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is a malignancy of lymphocytes (cells of origin) that develops in the bone marrow, peripheral circulation, and extra medullary sites. It is hypothesized that genes in cells induce an abnormal response to infection, which is the cause. As lymphocytic leukemia is a malignancy that impacts the cells of origin that will subsequently differentiate into B and T lymphocytes. (5)

2 -Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) has an irregular proliferation of myeloid cells. The number of fissions is more than 20% to diagnose chronic myeloid leukemia in the bone marrow. It is also

known as cancer that affects the stem cells in the bone marrow, which will later form granulocytes, erythrocytes, and megakaryocytic cells. (6)

3-Chronic myeloid leukemia (CML)

The malignant genetic condition of hematopoietic stem cells known as chronic myeloid leukemia leads to an increase in myeloid cells, red blood cells, and platelets in the peripheral blood, so the cancerous cells are myeloid cells in the blood, which include all types of cells found in the blood except lymphocytes.(7)

4-Chronic lymphoblastic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is a form of leukemia that primarily affects the elderly and exhibits a diverse array of symptoms. Blood type B and T lymphocytes are the cells that are implicated in carcinogenesis. Leukemia is transmitted as a result of specific genetic modifications that obstruct clonal apoptosis. (8)

1-3 Heme Oxygenase-1 Enzyme (HO-1)

Heme oxygenase 1, a stress protein, metabolic enzyme, stimulator of immune stress in the host, and alleviator of inflammation. HO activity is represented by two main forms, namely HO-1) (HO-2) (HO-3).

Which is encoded by two distinct genes (HMOX1) and HMOX2, respectively. Heme oxygenase-1 is a microsomal enzyme that has an essential antioxidant and anti-inflammatory role in the degradation of heme.

The synthesis of biliverdin and carbon monoxide, which is subsequently reduced by biliverdin reductase, leads to the production of bilirubin and unbound ferritin, Fe+2 (9). Bilirubin, which is derived from biliverdin, is an antioxidant that possesses anti-inflammatory properties. Recent studies have indicated that it may also contribute to overall health. By means of hepatic pathways (10).

A variant of heat-resistant and shock-resistant enzymes is the HO-1 enzyme. The class of these proteins, specifically the HO-1 enzyme, is referred to as Heat Shock Proteins (HSPs) by scientists.

The HMOX1 gene expresses 288 amino acid residues, which contribute to the molecular weight of 32 kDa. NADPH-Cytochrome P450 reductase is essential for the activity of HO-1. Cer-

tain investigations have demonstrated that agents that inhibit the HO-1 enzyme impede platelet aggregation and enhance fibrinolysis and phagocytosis, thereby preventing tissue injury and coagulation. Furthermore, hemin functions as an activator of neuroglobin, a protein that is involved in the transport and storage of oxygen in nerve cells, thereby increasing pressure. Neurons are protected from hypoxia by the intracellular oxygen percentage(11)(12) .

1-4 Materials and working methods

Sample collection

Research samples were collected for patients with leukemia from the Children's Central Hospital in Baghdad Governorate, and the control group for the period from November to May 2024.

The patient group included blood samples of patients with leukemia, and their number was (60) samples, and the number of males was (30) samples and females (30) samples, and their ages ranged between (1-14) years. Their infection with leukemia was confirmed through diagnostic procedures via blood tests, through their clinical symptoms diagnosed by the doctor and found in the patients' files and records. The special questionnaire form was relied upon and the required information for each patient was recorded in it. The control group included (30) healthy samples and no diseases, including (15) male samples and (15) female samples, and their ages also ranged from (1-14) years.

Diagnostic tests: Serum urea and creatinine concentrations were measured using the Spanish analysis kit, as well as the level of heme oxygenase-1 enzyme in the serum of patients and compared with the serum of the control group using the ELISA technique.

1-4-1 Separation and purification of heme oxygenase-1 enzyme from serum of leukemia patients (chronic myeloid leukemia)

Heme oxygenase-1 enzyme was separated and purified using the following steps:

1-Protein precipitation using ammonium sulfate at a rate of 75%.

(15) gm of solid ammonium sulphate was added gradually and gently at a saturation rate of (75%) to (20mL) of blood serum of leukemia patients prepared for purification, with continuous stirring for (60 minutes) us-

ing a magnetic stirrer to dissolve all ammonium sulphate, at a temperature of (4°C). After the completion of the hour period and to complete the protein precipitation process completely, the solution was left for (24 hours) at a temperature of (4°C). A centrifuge was then used at a speed of (2775 Xg) for (30 minutes) in order to separate the filtrate from the precipitate (13). The volume of each of the filtrate and precipitate solutions was calculated (after adding an amount of distilled water to it). The total protein content of the two solutions (filtrate and precipitate) was estimated using the diagnostic kit provided by Sun long Biotech (China) at a wavelength of (550 nm) (14). The activity of the HO-1 enzyme of the two solutions was also estimated at a wavelength of (340 nm) (15)

2- Dialysis (dialysis bag)

The protein-containing portion obtained from paragraph (1.5.2) above, which had high HO-1 enzyme activity, was placed in a semi-permeable dialysis bag and tightly tied from both sides. Then, the sample was placed in a beaker with a capacity of (1000 mL) containing a phosphate buffer solution with a concentration of (0.1 M) and a pH of (pH = 6.25). The dialysis process was carried out at (°C4), with continuous stirring by a magnetic stirrer for two days (48 hours) with the buffer solution in the beaker being replaced every (3 hours). After the dialysis process was completed, the final remaining volume of the sample solution was calculated (16). The total protein amount was estimated, and the HO-1 enzyme activity of the sample solution resulting from membrane sorting was estimated. Finally, the solution was kept frozen at a temperature of (°C20-) until later use.

3- Ion exchange chromatography

Diethyl amino ethyl cellulose (DEAE-Cellulose) powder weighing 20g was suspended in 1000 ml of distilled water and allowed to settle to create this technique. Until the supernatant was clear, the supernatant was discarded after each round of this process. After being activated with 0.25N HCl for 30 minutes, DEAE-Cellulose was filtered using a Buchner funnel and Whatman No. 1 filter paper, and washed twice with distilled water.

DEAE-Cellulose was then given a 0.25N NaOH activation. There were two iterations of the washing and filtering procedures. The activated DEAE-Cellulose was packed in a column with a diameter of 3× 18 cm after being equilibrated with (0.1M Tris buffer pH (7.2).

The ionexchanger column received the concentrated enzyme from earlier processes. For washed and eluted samples, which were collected in 3 ml for each fraction, flow rate was structured to be 1 ml per minute. HO-1 was washed in Tris buffer (0.1M, pH (7.2), and the enzyme was then extracted using the same buffer and NaCl (50–200 M). At 280 nm, the absorbance of each portion was measured. To measure enzyme activity, protein content, and specific activity, active fractions were collected.(17).

4-Gel filtration chromatography (using Sephadex (G100).

The fractions from the gel filtration process are collected using a fraction collector, and the volume of distilled water resulting from the displacement of each protein from the separation column is calculated, then the protein materials are isolated by reading the absorbance of the separated fractions by ultraviolet light at a wavelength of (280 nm) (18).

5- HbLc technique to estimate molecular weight. Explained in a paragraph(**1-5-2**)

1-4-2 Kinetics of heme oxygenase-1

The kinetics of heme oxygenase-1 enzyme were studied after its isolation and partial purification from leukemia patients' serum by gel filtration. The kinetic study included the following:

1- Effect of substrate concentration: Using different concentrations of substrate concentration (0.01250, 0.006250.05, 0.02500, 0.20, 0.10)mmol/ml.

2- Effect of temperature: Used to measure the activity of heme oxygenase-1 enzyme, the reaction was carried out at different temperatures (5, 20, 30, 40, 50, 60).

3-Effect of pH: Effect of pH on the reaction of heme oxygenase-1 enzyme where different solutions with pH (2, 4, 6, 8, 10, 12) were used

4- HbLc technique was used to estimate the molecular weight of the partially purified enzyme.

1-5 Results

1-5-1 Partial purification of heme oxygenase-1 from serum of patients with leukemia

The essential tenet

The objective of this procedure is to equilibrate the charges on the enzyme's surface, eliminate the water layer around the protein, and reduce the level of hydration. Thereafter, the protein will solubilize and precipitate(19).

The partial purification process was extracted and carried out for patients with chronic myeloid leukemia (CML) through the following steps: The first stage of purification is the protein precipitation process using ammonium sulfate, which is called salting out. Therefore, the enzyme was purified to a high concentration and the resulting salts were extracted using membrane sorting (dialysis) using the buffer solution PH 7.4 Tric-Hcl. When gel filtration was performed, the degree of purification of the enzyme reached (18.43), the enzyme production rate (226.86), and the specific activity (75.040), as shown in Table (1). The figure below shows the separation and purification process using gel filtration.

Table (1) Stages of purification of heme oxygenase enzyme in the blood serum of patients with chronic myeloid leukemia

Purification Steps	Total Volume mL	Activity U/L	Total Activity	Protein mg/mL	Total Protein	Specific Activity U/mg	Recovery Yielld %	Fold of Purification Total Protein
Crude	20	26.462	529.240	6.50	130	4.071	100.00	1
Precepitation	17	27.315	464.355	5.90	100.3	4.630	103.22	1.14
Dialysis	15	29.573	443.595	3.80	57	7.782	111.76	1.91
Ione-Exchange	5	48.582	242.910	0.7	3.5	69.403	183.59	17.05
Gel Filtration	3.5	60.032	210.112	0.8	2.8	75.040	226.86	18.43

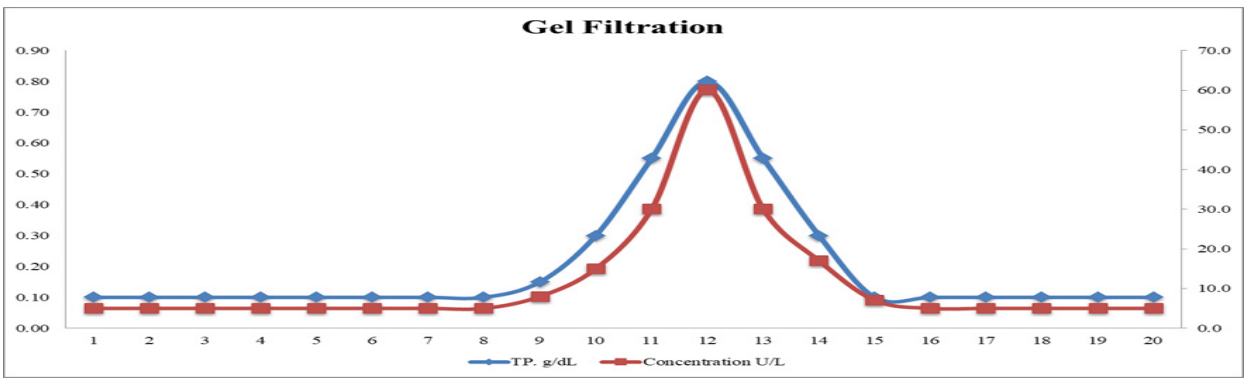


Figure (1) shows the package for purification of heme oxygenase-1 enzyme using gel filtration chromatography

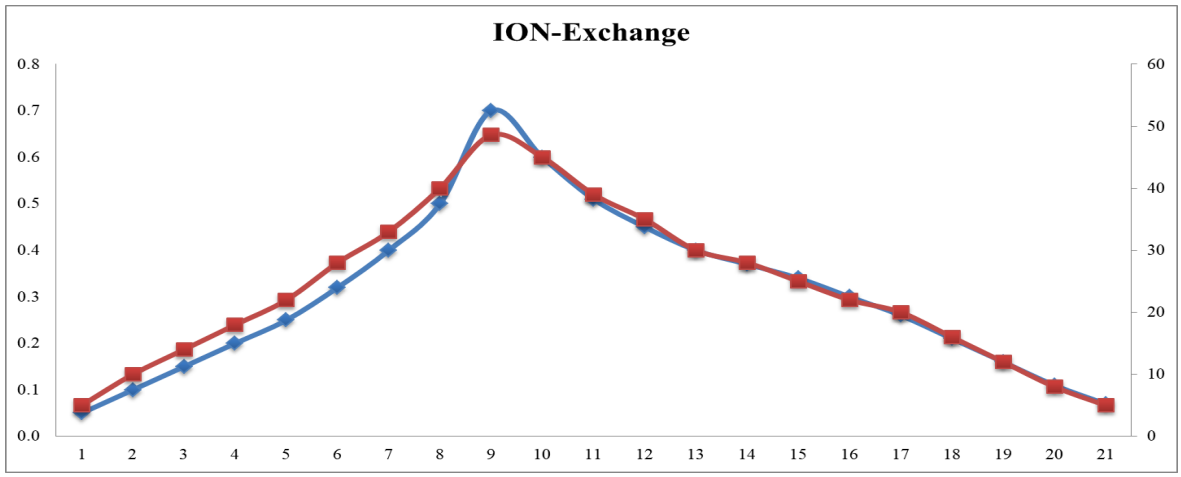


Figure (2) shows the package for purification of heme oxygenase-1 enzyme using ion exchange.

1-5-2 Determination of the molecular weight of heme oxygenase-1 enzyme using high-performance liquid chromatography (HpLC-SEC) technology

The method is summarized by using special columns used to separate compounds based on their molecular size. The column contains a gelatinous material consisting of small particles with holes (pores) of specific sizes. The basis of separation in it depends on the fact that small molecules are able to enter the pores and move through the column in a longer path (high retention time), while large molecules do not enter the pores and therefore move faster (low retention time). Through the difference in the path taken by the molecules inside the column, they are separated according to their molecular size (molecular weight). One of its important applications is separating proteins and determining their molecular weights by using standard proteins with different molecular weights. Proteins with a molecular weight rate of KDA (290.3-5.8) were used, where the retention time was determined for each protein.

Through the analysis, the calibration curve for the standard proteins was drawn, where the (X) axis represents the retention time and the (Y) axis represents the molecular weight. The straight line equation was found

as in Figure (3) where the straight line equation was extracted in the general formula.

$$Y = ax \pm b$$

where x represents the retention time

Y represents the molecular weight

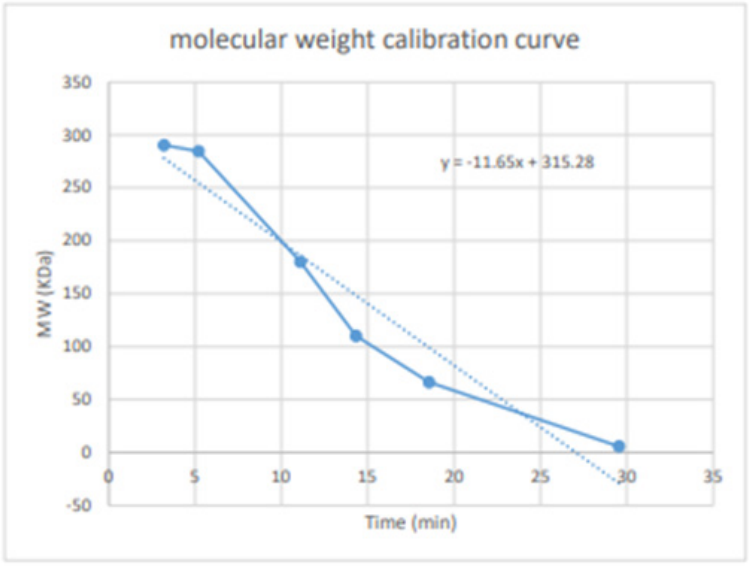


Figure (3) showing molecular weight calibration curve

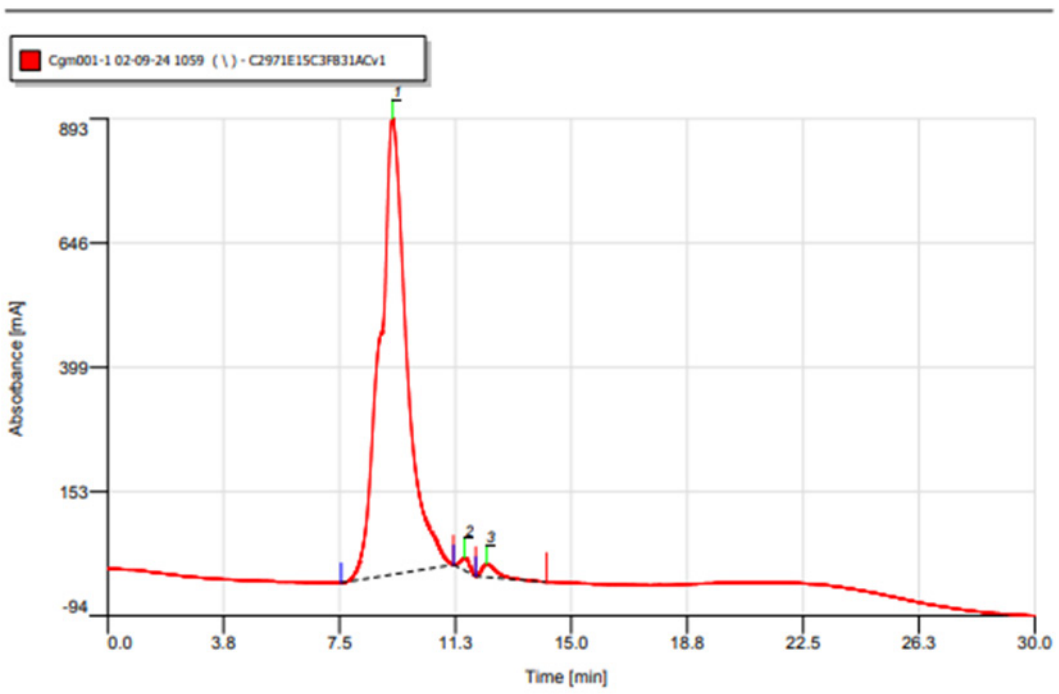
The molecular weight of the model is calculated after performing the analysis and extracting the retention time, where the value of x is replaced by the retention time in the equation and the value of Y (molecular weight) is calculated for each component in the model.

When the sample is analyzed by HpLC-SEC, the separation chromatogram appears as in the figure, where the main enzyme appears with a retention time of (9.221) minutes, and the corresponding enzymes (Co enzyme) appear at low rates. By applying the straight line equation in the calibration curve, Figure No. (4), the molecular weight of the main enzyme is KDA (207.8554). The table below shows the retention times of the enzyme and the corresponding enzymes, their percentages, and their molecular weights.

Table (2) shows the retention times of the enzyme and the corresponding enzymes, their percentages and molecular weights.

Sequence	Detention time	Percentage	Detention time
1	9,221	97.568%	9,221
2	11,546	0.911%	11,546
3	12,260	1.521%	12,260

That is, the main enzyme has a purity of 97.568% and a molecular weight of KDA (207.8554).



The Figure (4) of the separation chromatogram is placed when the sample is analyzed by HpLC-SEC.

1-5-3 Study of the kinetic properties of partially purified heme oxygenase-1 from patients with chronic leukemia

1-The effect of the substrate concentration

The effect of the substrate on the enzyme activity was studied, where the

optimum concentration of the substrate at which the enzyme is at its highest activity was (0.2) m mole/ml, and Figure (5) shows this. The Michaelis-Menten constant (Km) was also determined, which means the convergence between the enzyme and the substrate using the Lineover-Burke equation, where the constant value of the Michaelis-Menten coefficient for the substrate was (0.098) and the maximum speed value was (61.350).

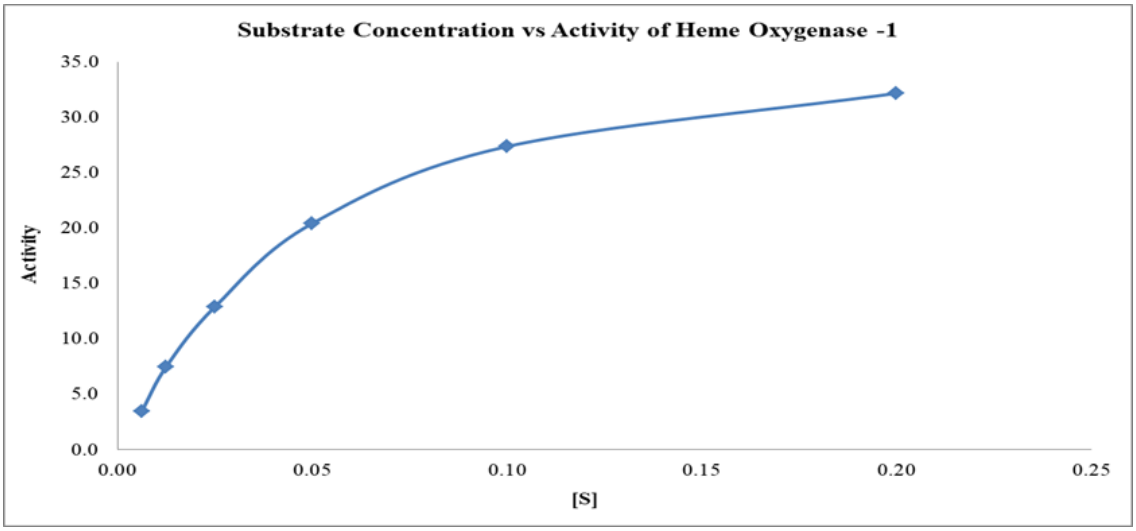


Figure (5) shows the effect of the concentration of the substrate on the activity of the heme oxygenase-1 enzyme.

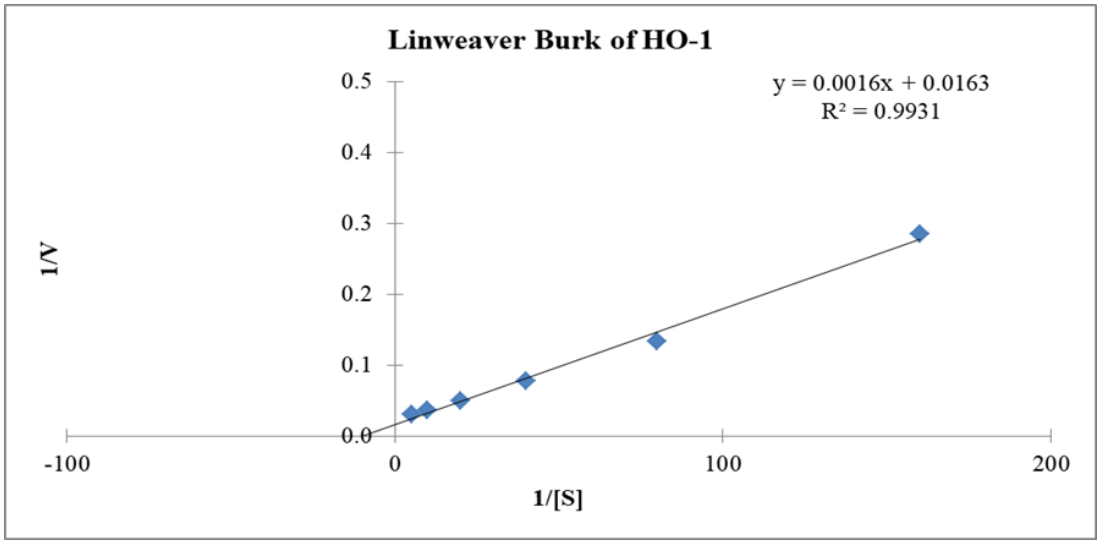


Figure (6) shows the Linover-Burke equation, the value of the Michaelis-Menten constant, and the maximum speed of the heme oxygenase-1 enzyme.

2-The effect of Ph

on the activity of heme oxygenase-1 enzyme was studied, as the optimal pH for the work of heme oxygenase-1 enzyme is (6.8). The pH is considered one of the important factors that have a great impact on the ideal work of enzymes, and the pH is considered one of the important features for controlling and stabilizing the work of enzymes, and the figure shows the optimal pH for the work of heme oxygenase-1 enzyme.

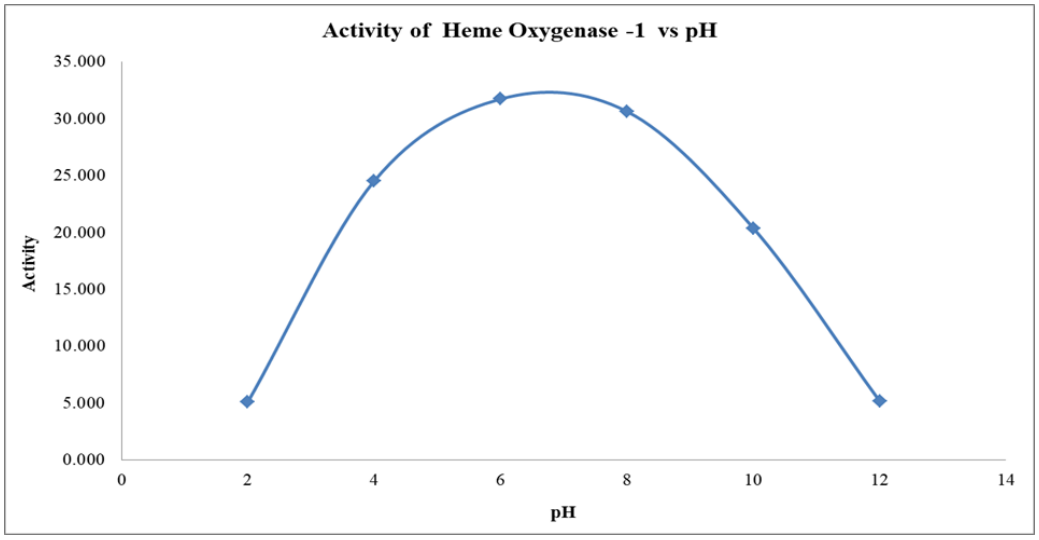


Figure (7) shows the optimal pH for the action of heme oxygenase-1 enzyme.

3-The effect of temperature

The optimum temperature for the activity of the heme oxygenase-1 enzyme was studied, which is the temperature at which the rate of the enzyme reaction is at its maximum activity, and the enzyme is very effective and the enzyme is affected by the acidity and other factors. Through the study that was conducted, it was shown that the optimum temperature for the enzyme to work when ensuring the concentration of the basic material and the pH is around (40) °C, and the figure shows the optimum temperature for the work of the heme oxygenase-1 enzyme.

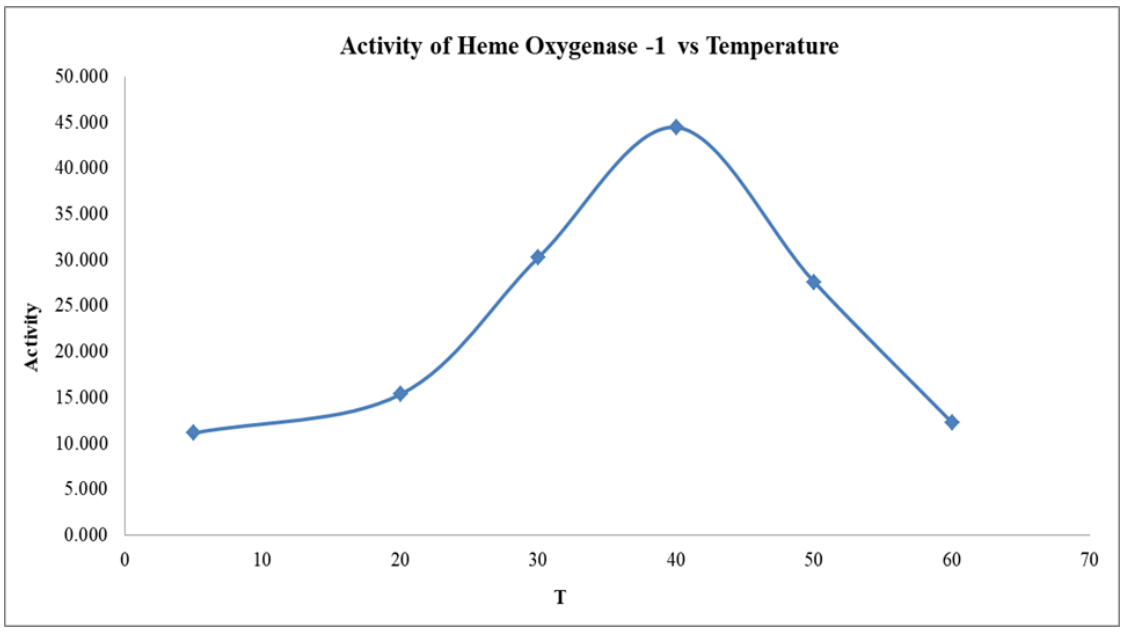


Figure (8) Effect of temperature on the activity of heme oxygenase-1 enzyme

1-6 Discussion

The current study was conducted to understand and know the optimal conditions for the work of the heme oxygenase-1 enzyme, as all enzymes work within specific conditions and factors and are affected by these factors, where the most important factors are temperature, pH, concentration of the substrate, etc. (20). The heme oxygenase-1 enzyme was isolated and purified using different biological techniques, where the enzyme was isolated using ammonium sulfate, followed by gel filtration chromatography, dialysis, ion exchange, and molecular weight estimation using HPLC-SEC.

Through the results we reached in our study, we found that the optimum temperature for the enzyme to work is approximately 40°C, and the optimum pH is 6.8, where the enzyme is at its highest activity under these conditions and the concentration of the substrate is 0.20 m mole/mL. In a previous study on the heme oxygenase-1 enzyme. The specific heme oxygenase-1 activity was shown to increase from 0.82±0.05 to 24.8±1.8 units/mg during the entire purification steps, thus the purification process can reduce the demand for raw material preparation and simplify the

purification process (21).

In a study conducted to purify the partial and reconstitute the heme oxygenase system from pig spleen microsomes at the Department of Biochemistry, Faculty of Medicine, Tohoku University, Japan, magnesium chloride salt was used instead of ammonium sulfate, which is almost identical to the salt used in the study, sucrose and Tris-HCI solution (pH 7.5) were used, and a centrifuge was used for sedimentation. After this process, the washing process was carried out using a 1 ml solution of potassium chloride containing 20 mmol of potassium phosphate solution (pH 7.5) and 10 mmol of EDTA, and also using a centrifuge. The resulting precipitate was placed in 180 ml of potassium phosphate solution, after which the other steps were completed until it was obtained by dissolving heme oxygenase from microsomes treated with DOC by adding one-tenth of the volume of Trition X-100 solution at a rate of 5.5%. After shaking for 20 min, the suspension was centrifuged at 77000 g for 90 min. Although the addition of DOC to the Triton fraction caused some decrease in heme oxygenase activity, DOC was necessary to obtain a sharp heme oxygenase escape peak on hydroxyl apatite column chromatography or DEAE-cellulose column chromatography. The obtained preparation, purified 110-fold in 0.1 M potassium phosphate buffer, did not contain any indication of heme oxygenase protein, as the preparation was as low as 0.07 nmol per mg protein when analyzed according to the Omura and Takesu method. The absence of protein is due to the low heme oxygenase content in pig spleen, less than 10% of its level in pig liver. Finally, the researcher did not address the enzyme kinetics and the optimum conditions for enzyme action, and the fact that the research is the only one on the subject of purification of heme oxygenase.(22).

The maximum velocity value Vmax was calculated using the Lenover-Burke chart as shown in Figure (6) where the Vmax value of the purified heme oxygenase-1 enzyme from chronic myeloid leukemia patients was 61.350 units per liter and using the same equation to calculate the value of the Michaelis-Menten constant Km which represents the inverse of the enzyme activity 1/v versus the inverse of the substrate concentration 1/s as

shown in Figure (6) where the value of km was (0.098) mol/liter.

Conclusions

The molecular weight of the partially purified enzyme was determined and its molecular weight value was (207.8554) kDa, the percentage was (97.568%), the optimum pH was 6.8, the optimum temperature was approximately 40°C, and the concentration of the substrate was (0.20 m mole/mL), in addition to the other conditions and factors mentioned in Table No. (1).

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