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Separation and purification of paraoxonase1 enzyme in women with hypothyroidism and assessment kinetic studies

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ABSTRACT

Background: Paraoxonases1 (PON1), are a type of Hydrolase enzyme that have a wide range of substrates.Paraoxonase-1 is produced in the liver and then released into the bloodstream, where it binds to high-density lipoprotein (HDL) particles. **Objectives :**the present study aims to Purifying PON1 enzyme from fresh human blood and study kinetics represented by the study of substance concentration , pH, temperature, and time of reaction.

Material and methods: the PON 1 enzyme were isolated from serum separated from blood sample withdrawal from fresh human hypothyroidism by many chromatographic methods. **Result**: The separation methods were carried out using Various biological technologies. The first step in the precipitation with ammonium Sulfate(at a level of saturation rate 60%) , the specific activity of protein increased from 0.207u/mg protein in crude extract to 0.212 u/mg protein with purification and the enzyme yield was 96.79% table(1) .The fold of PON-1 increases from (1 to 1.024) after precipitation. The second step in ion exchange chromatography : The fraction produced following this treatment had a specific activity of (0.653 IU/mg) and demonstrated a total purification of 3.15-fold. During the second step, the extract was subjected to chromatography on DEAE-Cellulose, a technique that effectively isolates the majority of the PON1 activity from the Apo lipoproteins. The yield of this phase was low, with just 34.9% of the original activity applied. This was due to a significant loss of active PON1 activity, but a relatively big amount of protein dissociated from the PON1 activity. In the last step it was with gel filtration chromatography using Sephadex G100 , fig.(2) showed the elution of the enzyme results in a solitary and symmetrical peak, which is observed in the PON1 enzyme fraction. According to table (1), the purified PON1 enzyme has an activity of (7.8 IU/mL), and its specific activity is increased by (4.1) folds to (0.866 IU/mg). The enzyme's yield is (32.09 %).

Keyword : Paraoxonase1, Purification, kinetic Studies, Hypothyroidism

1.Introduction

The Paraoxonase (PONs) family consists of three genes: paraoxonase 1 (PON1), paraoxonase 2 (PON2), and paraoxonase 3 (PON3). These genes have around 70% genetic sequence similarity and 60% same sequence of amino acid[1]. The genes that encode PONs in humankinds are situated throughout the long arm of chromosome 7, and are located in region 7q21.3–22.1. Paraoxonases, specifically PON1, are a type of Hydrolase enzyme that have a wide range of substrates.

Paraoxonase-1 is produced in the liver and then released into the bloodstream, where it binds to high-density lipoprotein (HDL) particles. Hight density lipoprotein is the most effective

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physiological acceptor complex for increasing the secretion of PON1 and stabilizing the released peptide[1]. Based on the structural study: Paraoxonase 1 is a type of glycoprotein consisting of 355 amino acid and has approximately molecular weight that is 43 kD. The structure of the object was determined using X-ray crystallography. It was found to have a six-bladed β-propeller shape, with each blade consisting of four β-strands joined by a disulphide bridge. Additionally, there were three helices located on the top portion of the propeller [2]. It appears that hydrophobic N-terminal sequence of the enzyme plays a vital function in anchoring PON1 to the HDL molecule. This sequence acts as signal peptide that retained through maturation, except for the methionine residue initiator. Paraoxonase-1 is shown to be extensively expressed in various tissues and organs, including those of the kidneys, liver, brain, heart, and gut [3]. The absence of PON1 mRNA expression in these organs provides evidence that HDL molecules are probable carriers that transport PON1 to different tissues where its function is needed . Epithelial cells, particularly those in the lungs,exhibit significant levels of PON1 production as a result of their frequent interactions with a wide variety of xenobiotics [4]. In the beginning, PON1 was classified particularly A-esterases because it has the potential to catalytically break down organophosphate (OP) substrates, in accordance through Aldridge's classification [5], Furthermore the Enzyme Commission of the International Union of Biochemistry and Molecular Biology has officially identified it into the category aryldialkylphosphatase (EC 3.1.8.1) [6].

2.Materials and methods

2.1. Purification of Paraxonase 1

Paraoxonase 1 is purified from serum of women with hypothyroidism in many steps:

a. Ammonium sulfate precipitation

The crude enzyme was precipitated by saturating it with ammonium sulphate (NH4)2SO4 using 4.74g, 4.1g, and 3.16g of salt, respectively, in a 10 ml volume. The mixture was chilled and continuously stirred for 60 minutes, followed by centrifugation at 6000 xg at 4°C for 30 min. Following the dissolution of the precipitates in a small amount of 10m phosphate buffer solution, then it was determined the activity of each enzyme, along with the total proteins and specific activity. The precipitate was dialyzed against a 10mM phosphate buffer solution at 4°C in pH 7.4 for 24 hours with stirring in order to remove salts and impurities. The particular activity was determined subsequent to the estimation of each enzyme activity and protein concentration.

b. Dialysis

This method involves placing the protein in a dialysis bag then soaking it in a 10mM phosphate pH 7.4 buffer solution. The objective of this technique is to eliminate any residual ammonium sulphate that was introduced during protein precipitation. The buffer solution underwent a change for a duration of 24 hours. The completion of this stage occurred at a temperature of 4 degrees Celsius. The enzymatic activities were assessed and the protein content was quantified at the end of the period.

c. Gel filtration chromatography

Preparation of Sephadex G-100 column

The Sephadix G100 gel has been prepared following the directions provided by the Pharmacia Fine Chemical Company. The gel was immersed in a 10mM phosphate solution with a pH of 7.4 and left at a temperature of 4°C for a duration of 24 to 20 hours. Throughout this time, the buffer solution was repeatedly replaced in order to eliminate small particles from the solution. The presence of these particles slows down the flow rate of the osmotic solution through the column.

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Sample loading

Subsequently, transferred the gel and poured into a glass Chromatography column inner diameter 2×15 cm .The column was equilibrated by adding a sufficient quantity of (10mM) phosphate buffer pH (7.4) when the pH of the leaching fractions reached 7.4. This was done at a flow rate of 5ml every 3 mint. After preparing the column, the concentrated enzyme solution generated by dialysis was applied to the surface of the column. The purification process was carried out using a 10mM phosphate buffer with a pH of 7.4, that contained 500 mM NaCl. The flow rate during elution was set at 5ml per 3 minutes. Every fraction requires a volume of 5ml.

d.Ion exchange chromatography

DEAE-Cellulose Preparation:

The pooled fractions obtained from the DEAE-cellulose column were subjected to dialysis overnight using 2 liters of buffer solution. The dialyzed fractions were then put onto a column (33 cm^2) filled with pre-equilibrated DEAE-cellulose at a flow rate of (0.5 ml/min). The enzyme that was attached to the column was released by washing it with a buffer solution. This was done by utilizing a linear salt concentration of NaCl (0 ±0.35 M) in a buffer solution, with a flow rate of (0.5 ml/min). The elution process used a volume of 300 ml. Fractions with a volume of (5 ml each) were obtained and the ones that had the highest PON1 activity were combined for additional purification [7]. The fraction produced following this treatment had a specific activity of (0.653 IU/mg) and demonstrated a total purification of 3.15-fold. During the second step, the extract was subjected to chromatography on DEAE-Cellulose, a technique that effectively isolates the majority of the PON1 activity from the Apo lipoproteins. The yield of this phase was low, with just 34.9% of the original activity applied. This was due to a significant loss of active PON1 activity, but a relatively big amount of protein dissociated from the PON1 activity. This study employed the previously mentioned technique to separate and purify the PON1 enzyme. The process involved the precipitation of ammonium sulfate and subsequent purification using an ion-exchange column.

2.2.Kinetic Study and Characterization of partially purified Paraoxonase 1 enzyme.

An analysis was conducted on the kinetic characteristics of the enzymes following their isolation and purification through the serum from people.

2.2.1.Factor effect

a. Effect of substrate concentration: This effect has been studied using different concentrations of substrate: Paraoxon on PON1 (1,0.5,0.25,0.125,0.0625,0.03125mM), enzymatic activity if the concentration utilized to find the optimal substrate concentration for enzyme action. The effect then plots the relationship between interaction velocity and substrates concentration to determine the ideal substrate concentration at which the interaction velocity is at its greatest (Vmax). To define Michaelis-Menten constant value (Km) by using diagram as in Line Weaver-Burk plot belonging with reverse values for the both velocity and concentration (1/v vs 1/[s]).

b. Effect of optimum pH: The influence of a buffer solution (10mM Tris–HCl pH 7.2) and (Sodium Carbonate–Bicarbonate) on PON1 velocity was studied. Solutions of various pH (2.4, 4.4, 6.4, 8.4, 10.4, 12.4) at 37°C. The measurement of enzyme activity was performed to establish the optimal pH through plotting the association across the velocity of interaction and pH.

c. Effect of Optimum Temperature: The assess enzymes activities by performing interactions at various temperatures (7, 17, 27, 37, 47, and 57 °C) with a buffer solution (sodium carbonate–

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Bicarbonate) at PH 7 and concentration of Paraoxon (1mM) as substrate. Then, in order to ascertain the optimal temperature over interaction, a correlation involving interaction both temperature and velocity was established across every enzyme.

d. Effect of optimum Incubation time: The effect of an incubation time interaction mixture on, PON 1 enzyme activity was at 37°C using the solution of a buffer (sodium carbonate – bicarbonate). The precise time periods used were (1, 5, 10, 15, 20, and 25) minutes. Then, to determine the impact of incubation time on enzyme interaction velocity, the relationship between enzyme activity and time was drawn.

3.Results and Discussion

3.1.Separation and Purification of Paraoxonase 1 Enzyme

a. Separation of enzyme

The PON 1 enzyme was isolated from serum separated from blood sample withdrawal from fresh human hypothyroidism by many chromatographic methods. The separation methods were carried out using Various biological technologies, including salting out, dialysis, and gel filtering on a Sephadex G-100 column the gel filtration approach was used to separate the enzyme and maintain it under ideal circumstances, with the highest peak being observed.

b. Purification

Paraoxonase 1was purified in three steps: in precipitation with ammonium Sulfate(at a level of saturation rate 60%) , the specific activity of protein increased from 0.207u/mg protein in crude extract to 0.212 u/mg protein with purification and the enzyme yield was 96.79% table(1) .The fold of PON-1 increases from (1 to 1.024) after precipitation. The obtained results are attributed to the absence of any negative effects of ammonium sulphate on enzyme function. Ammonium sulphate is successfully used to reduce complexity protein/enzyme solutions, eliminate interference from small molecules, and precipitate immune components [8]. The solubility of proteins is dependent on the ionic strength of the solution. During the initial stages of enzyme purification, the concentration of protein occurs by removing a substantial amount of water and attaining a high degree of purity. Due to their significant solubility in water, salts such as ammonium sulphate $(NH4)2SO4$ are commonly employed for this objective. Ammonium sulphate is an inexpensive compound that has minimal impact on enzyme composition[9]. It is highly suitable for the intended purpose. Salting out refers to the process when salt molecules equalize the charges on the protein's surface, leading to a decrease in protein solubility and subsequent deposition[10]. It enhances the efficiency of purification by removing the primary contaminants from the enzyme extracted. Due to its interference with enzyme activity, it increases the efficiency of purification by removing proteins and water from the enzyme extract. The enzyme extract is added incrementally to eliminate certain protein molecules that have precipitated[11]. ion exchange chromatography and gel filtration chromatography .the result shown in the table (1). In ion exchange chromatography step : The fraction produced following this treatment had a specific activity of (0.653 IU/mg) and demonstrated a total purification of 3.15-fold. During the second step, Ion exchange chromatography method by using the risen Cellulose DEAE Di Ethyl Amino Ethyl cellulose as this matrix used for purification because it has high capacity for bio separation, easy to prepare, multiple use, in addition to simplicity to separate different biomolecules[12]. a technique that effectively

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isolates the majority of the PON1 activity from the Apo lipoproteins. The yield of this phase was low, with just 34.9% of the original activity applied. This was due to a significant loss of active PON1 activity, but a relatively big amount of protein dissociated from the PON1 activity. This study employed the previously mentioned technique to separate and purify the PON1 enzyme. The process involved the precipitation of ammonium sulfate and subsequent purification using an ion-exchange column. The results are displayed in Figure (1).

Table 1: Steps of PON-1 purification from human hypothyroidism

Purification Steps	Volume Ml	Activity IU/ml	Total Activity IU	Protein Concentration Mg/ml	Specific Activity IU/mg U/mg	Recovery Yield %	Fold of Purification	Total protein mg
Crude	9	13.5	121.5	65	0.207	100	1	585
Precipitation	8	10.6	84.8	50	0.212	69.79	1.024	400
Dialysis	8	9.1	72.8	18	0.505	59.9	2.43	144
Ion exchange (DAEA-Cellulose)	5	8.5	42.5	13	0.653	34.9	3.15	65
Gel Filtration	5	7.8	39	9	0.866	32.09	4.1	45

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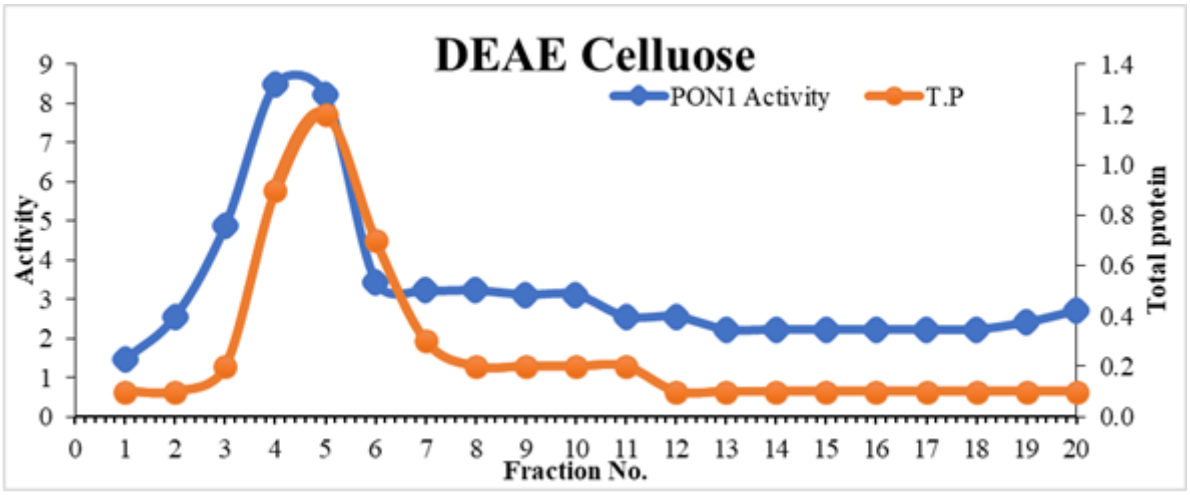

Figure1: Ion Exchange Chromatography of PON 1 in DEAE Cellulose

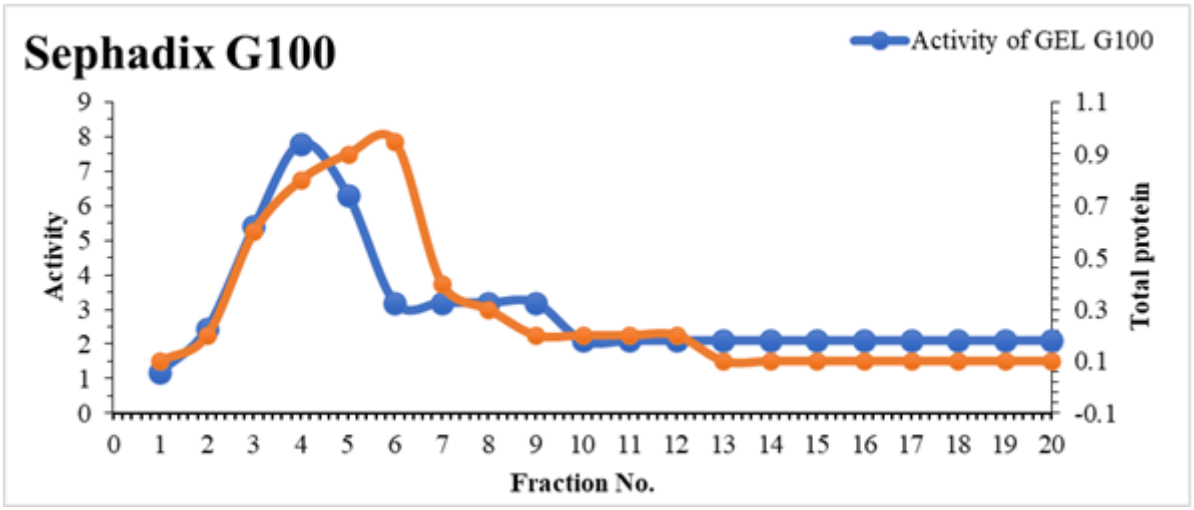

Figure 2: Gel Filtration Chromatography of PON1 by Sephadex G100

In the last step it was with gel filtration chromatography using Sephadex G100 , fig.(2) showed The elution of the enzyme results in a solitary and symmetrical peak, which is observed in the PON1 enzyme fraction. According to table (1), the purified PON1 enzyme has an activity of (7.8 IU/mL), and its specific activity is increased by (4.1) folds to (0.866 IU/mg).The enzyme's yield is (32.09 %). This chromatographic technique is employed for the purpose of separating, extracting, and refining proteins, enzymes, hormones, antibiotics, and nucleic acids that are dissolved in a solution. Gel filtration chromatography is the term used to describe the procedure of using an aqueous solution to transport the sample through the column[13]. It is mostly employed for the purpose of isolating proteins along with other polymers that dissolve in water. This method offers the benefit of effectively separating larger particles from smaller particles with minimal contamination filtering, while also enabling use of various solutions without disrupting the filtration procedure[14].

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3.2.Study of Optimal Parameters for Paraoxonase 1 Obtained after Partial Purification from Blood Serum

a.Effect of Varying Substrate Concentrations on Enzyme Activity and Maximum Velocity

The activity of the PON1 was measured in the presence of different concentrations of Paraoxon on PON1 (1,0.5,0.25,0.125,0.0625,0.03125mM) as substrate. It is found the maximum activity of PON1 in the concentration (1 mmol/L) of paraoxon. The table (2), figure (3) showed increasing enzyme interaction velocity with increase conc. of substrate.

Table2: The effect of substance concentration on the activity of PON1

Conc.[S] mmol/L	Activity IU/L	1/s	1/v	KM	Vmax
1	7.500	1	0.1333	1.176	19.607
0.5	6.200	2	0.1613		
0.25	4.500	4	0.2222		
0.125	3.210	8	0.3115		
0.0625	2.455	16	0.4090		
0.03125	0.689	32.000	1.4514		

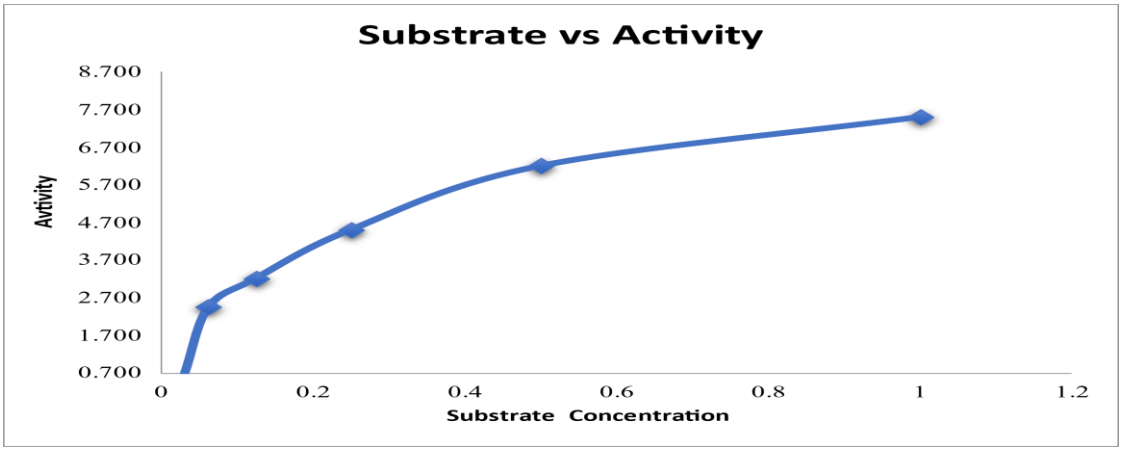

Figure Error! No text of specified style in document.**: Effect of substrate Concentration on the activity of enzyme**

There are multiple techniques available to determine the value of the constant Michaelis-Menten (Km), for enzyme by using equation of the Michaelis-Menten. The Line weaver-Berke method considers the most precise and effective due to its user-friendly nature, absence of complex computations, and efficiency in demonstrating experimental accuracy. The enzyme's Km and Vmax values (1.176mM and 19.607 IU/L) determine using Line weaver–Burk plots, resulting in a linear relationship. These values were found for the enzyme PON1, as shown in figure (3-17). The invers of substrate concentration (1/S) was graphed against the invers of reaction rate (1/V) using the given equation[15]:

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$$V_i = V_{max}[S] / K_m + [S]$$

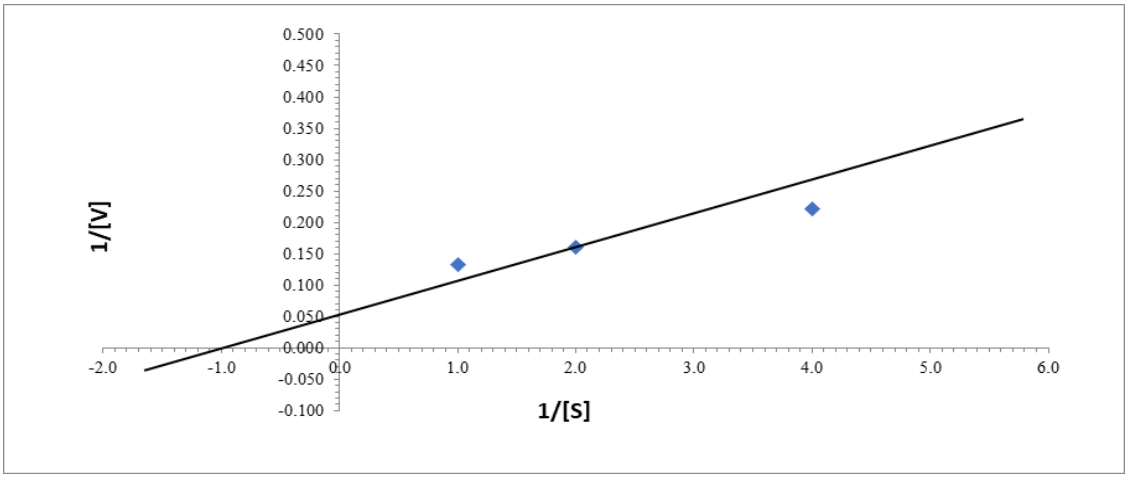

Figure 4: Line Weaver–Burk plot for partially purified of PON1

b.Effect of PH on Enzyme Activity

A study was conducted to investigate the varied effects of pH on the activity of the PON1 enzyme. Different pH levels have been studied to determine the most suitable pH for optimal PON1 enzyme activity (2.4,4.4,6.4,8.4,10.4 and 12.4 PH), showed in table (3) and figure (5). The highest level of enzyme activity was seen at (pH of 8.2) in the purified PON1 sample.

The unique attributes of the enzyme and its molecular composition, combine with the presence of many ionic groups transport by the enzyme, jointly determine how pH affects enzyme function. Enzymes exhibit optimal activity at various pH values due to their heightened sensitivity to change in concentration of hydrogen ion[16].

The pH affects the rate of enzyme-catalyzed reactions[17], pH is also crucial for maintaining the stability of enzymes, as enzymes have an optimal pH level and often exhibit a bell-shaped velocity curve. Graphs depicting the relationship between Ph and enzyme's activity is completely lost when its pH deviates from the optimal range, either being very high or very low enzyme activity can be reduced at low pH levels as a result of changes in the ionic state of substrate .Difference of pH conditions in the site of activity or intricate interactions between the enzyme and substrate when the substrate concentration exceeds Km. The activity of enzyme will be crucial when the concentration of the substrate is low. The pH also affects the tertiary structure of the enzyme. Consequently, the activity of enzyme can undergo irreversible denaturation at elevated pH levels.

Table Error! No text of specified style in document.**: Effect of pH on the enzyme activity**

pH	Activity IU/L
2.4	4.20
4.4	4.50
6.4	6.80
8.4	6.89
10.4	2.80
12.4	2.50

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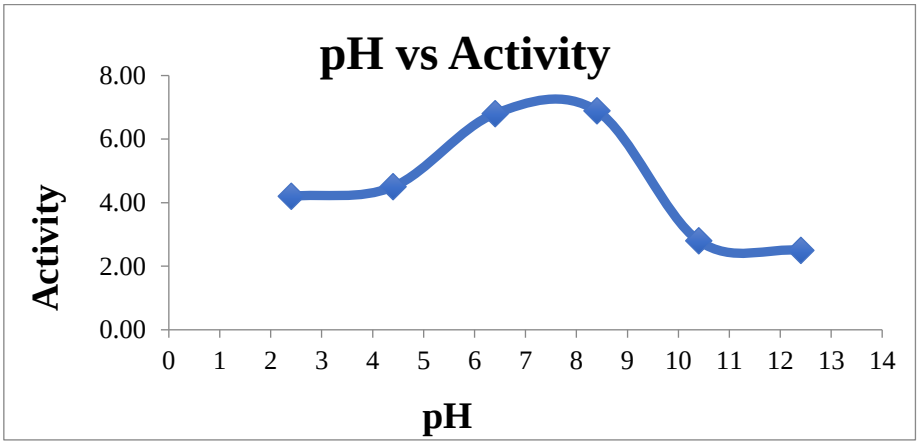

Figure 5: Effect of pH on the enzyme activity

C. Effect of Temperature on Enzyme Activity

The optimal temperature of PON 1 enzyme activity has been determined through testing at various temperatures (7,17, 27, 37, 47, and 57) °C. The optimal temperature was (37°C), The data indicate that raising the temperature resulted in an initial rise in PON1 activity, followed by a subsequent decrease in PON1 activity, as shown in the table (4) and Figure (6). It is widely recognized that the rate of a certain reaction increases as the temperature increases until it reaches the optimal temperature for the reaction, beyond which it gradually decreases. The enzyme molecule has undergone denaturation or damage, leading to this outcome. At elevated temperatures, the interaction between active amino acids weakens because of the heightened kinetic energy of molecules. As a result, the enzyme's functionality decreases. The decline may be ascribed to the variation in structural configuration of enzymes. Increased temperatures affect the ionization of groups found on the surface of both the enzyme and its substrate. Enzymes, being complex protein molecules, have a shape that directly affects their ability to promote chemical reactions[18].

The tertiary structure of an enzyme is mostly upheld by non-covalent interactions. However, excessive absorption of energy can lead to the destruction of the tertiary structure, resulting in denaturation of the enzyme and loss of its activity. At a temperature lower than (37 °C), the decline in PON1 activity is caused by insufficient energy to facilitate the formation of enzyme-substrate complexes.

Table 4: Effect of Temperature on enzyme activity

Temperature °C	Activity IU/L
7	3.800
17	4.500
27	6.900
37	7.000
47	6.800

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57	5.200

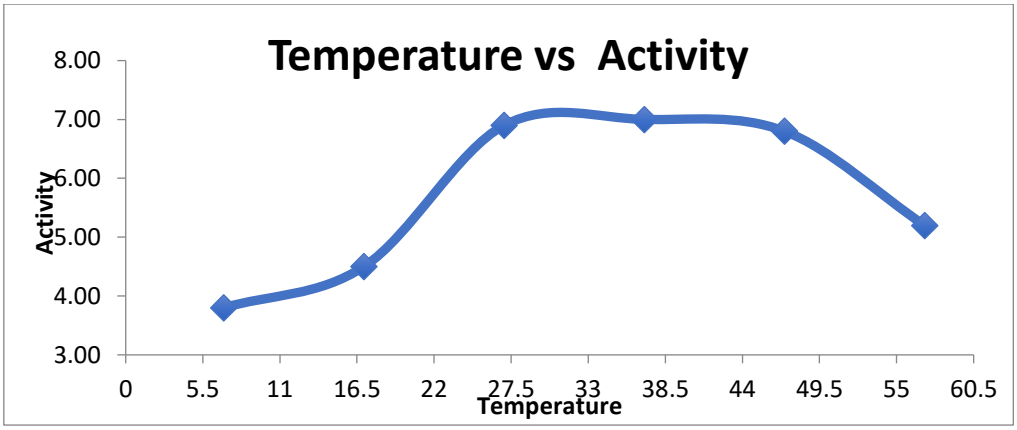

Figure 6: Effect of temperature on enzyme activity

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