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ORIGINAL ARTICLE

Molecular purification, and kinetic study of the enzyme glutathione peroxidase in patients with cardiovascular disease

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Abstract

This study was conducted to evaluate serum glutathione peroxidase activity and partial purification of the enzyme from the serum of heart attack patients using a precipitant or what is called salting out, ion exchange dialysis using DEAE-Cellulose, and gel filtration using Sephadex G100 gel. This study was conducted on 90 samples, (60) patients whose ages ranged between (35-75) years and control (30) individuals whose ages ranged between (23-55). It showed that the number of purification times was (4.64), and the enzyme yield was (40%). The specific activity was (1.3 U/mg). Likewise, kinetic studies were carried out in which values of Km and Vmax of glutathione peroxidase were (16.66 mM) of Vmax, and (0.909 IU/L) of Km, and the optimal concentration of the base material was (100 mmol/L). The optimal temperature for enzyme action was (37°C), while the optimal pH was (7.2), and the molecular weight of glutathione peroxidase was (23 kDa). The study also studied the effect of carvedilol as an inhibitor of the enzyme glutathione peroxidase, and the results showed a decrease in enzyme activity, and the type of inhibition was uncompetitive.

1. Introduction

1.1 Glutathione Peroxidase

Glutathione peroxidase (GPx) is a vital antioxidant enzyme responsible for mitigating oxidative stress by catalyzing the reduction of hydrogen peroxide and lipid hydroperoxides using glutathione as a substrate. As a selenium-dependent enzyme, GPx is crucial in maintaining redox homeostasis and protecting cells from oxidative damage [1]. The biochemical mechanisms underlying GPx's antioxidant functions have been extensively explored. Batten et al. provide foundational insights into the enzymatic processes involved. Beyond ROS neutralization, GPx's functions extend to immune regulation and inflammation. Ursini and Maiorino delve into the enzyme's role in immune responses, emphasizing its impact on overall cellular health [2, 3]. GPx's relevance in disease prevention, especially in cardiovascular disorders and neurodegenerative diseases, has been investigated. Godeas et al. highlights the protective effects of GPx in cardiovascular health, emphasizing its role in mitigating oxidative damage to blood vessels and tissues. Lubos et al. further explores potential therapeutic opportunities arising from understanding GPx's mechanisms, providing insights for interventions to enhance cellular resilience and prevent oxidative stress-related disorders [4, 5]. The multifunctional nature of glutathione peroxidase positions is critical in cellular defense against oxidative stress. The potential therapeutic opportunities arising from understanding GPx's mechanisms open avenues for interventions to enhance cellular resilience and prevent

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oxidative stress-related disorders [6].

1.2 Purification of enzyme

The isolation and purification of glutathione peroxidase (GPx) are critical steps in elucidating the enzyme's biochemical characteristics and exploring its potential therapeutic applications. Common methodologies involve the extraction of GPx from biological samples through techniques like differential centrifugation to isolate cellular components. Subsequent steps often include chromatographic techniques, such as ion exchange and affinity chromatography, to achieve high purity and preserve enzymatic [7,8]. Advances in protein purification technologies, examples include high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC), have further enhanced the precision and efficiency of the isolation process. However, protocol variations exist due to the diverse sources of GPx and the specific objectives of each study [9].

2. Materials and Methods

2.1 Study Design

This study was conducted on (60) patients whose ages ranged between (35-75) years, and controlled (30) individuals whose ages ranged between (23-55); these samples were collected from August 12th, 2022, to January 2nd, 2023. Samples were taken from Ibn Al-Beetar Hospital and SalahAlDin Labs. Five milliliters of venous blood have been collected from patients and control individuals and allowed to coagulate for 10 minutes at room temperature. The serum was then separated by centrifugation at 4000 rpm for 10 minutes and divided into three aliquots, each stored at -20 °C in 1.5mL capacity small Eppendorf tubes until analysis.

3. Results and Discussion

3.1 Measurement of Glutathione Peroxidase Activity

Estimating the effectiveness of the enzyme glutathione peroxidase (GPX) according to the method used by the researcher [10]. The GPX enzyme oxidizes reductive glutathione (GSH) using hydrogen peroxide and converting it to the oxidized form of glutathione (GSSG) (as in the equation below and the residue from reduced (unreacted) glutathione is measured later:

Table 1: Average standard deviation of GPX enzyme activity

Group	Mean ± SE
	Glutathione Peroxidase Activity (M moles/ml)
Patients	16.94 ±0.28
Control	13.89 ±0.76
T-test	1.335 *
* (P≤0.01)	

Regarding Glutathione Peroxidase Activity levels, the Patients group (16.94 ± 0.28 M moles/ml) has significantly higher levels compared to the Control group (13.89 ± 0.76 M moles/ml) at probability level (P≤0.05).

The results shown in the table above (Table 1) indicate that Glutathione Peroxidase Activity is significantly higher in patients with comparison to the control group.

A study by *Vassalle et al*. found that the activity levels of GPx were significantly higher in patients with coronary artery disease (CAD) compared to controls [11]. Similarly, research by *Zuzak et al.* discovered that GPx activity levels were significantly increased in patients with heart failure. These studies indicate that higher GPx activity may be associated with heart disease. Additionally, while higher GPx activity levels may be associated with heart disease, the exact mechanism and clinical significance of this association require further exploration. Overall, while some studies have found a significant difference in GPx activity levels between patients with heart disease and control groups [12].

3.2 Separation and Purification of Glutathione Peroxidase

The glutathione peroxidase was partial purified from the blood serum of patients, a number of biotechnological techniques, including salting out, dialysis, and Sephadex G-100 gel filtration, were used in the enzyme separation process. The highest peak found during the gel filtering process is then used to calculate the isolated enzyme's molecular weight and make sure its ideal parameters are kept.

3.3 Salting Out

When ammonium sulfate is added, the protein precipitates; this process is referred to as "salting out" [13]. A temperature of 4 °C and a saturation ratio of 65% are used in this precipitation process. The precipitate that results after precipitation is collected by centrifugation for 30 minutes at 4°C and 8000xg. Compared to the filtrate, the precipitate has a purification factor of 0.92 due to its concentrated high specific activity, which measures 0.260 enzyme units/mg of protein. As a result, the filtrate is ignored, and the precipitate is used to finish the purification process by dialysis.

3.4 Dialysis

The dialysis of the precipitate resulting from the salting out process demonstrates an increase in the specific activity of the isolated enzyme and its quantity relative to the total protein. This is evidenced by the specific activity of the enzyme measuring 0.566 enzyme units/mg of protein and a purification factor of 2 compared to the initial enzyme separation. This is attributed to the removal of protein compounds with small molecular weights (less than 10,000 Dalton) due to the properties of the cellophane utilized in this study. Additionally, the removal of the ammonium sulfate salt used in the protein precipitation in the previous step, along with other small particles, contributes to the purification process. Dialysis

effectively separates small molecules from large ones by allowing only the diffusion of small molecules through selectively permeable membranes created with cellulose membranes featuring pore sizes designed to exclude molecules below a selected molecular weight. These actions collectively work to increase the specific activity of the isolated enzyme in the solution obtained from dialysis [14].

Table 2: Steps of Glutathione Peroxidase purification from human blood serum.

Purification steps	Volume ml	Activity IU/ml	Total Activity	Protein Concentration mg/ml	Specific Activity U/mg	Recovery Yield %	Fold of purification	Total protein
Crude	10	19.2	192	68	0.28	100	1	680
Precipitation	8	14.6	116.8	56	0.260	60	0.92	448
Dialysis	9	11.9	99	21	0.566	51	2	180
Ion exchange (DAEA-Cellulose)	5	12.8	64	10	1.28	33	4.5	50
Gel Filtration	5	10.4	52	8	1.3	27	4.64	40

3.5 DEAE-Cellulose Ion Exchange Chromatography

Anion exchange diethylaminoethyl-cellulose, or DEAE-cellulose, is a polydextran derivative that has shown exceptional effectiveness in the selective adsorption and eluting of proteins, allowing for thorough and quick purification [15]. The results presented in Table 2 and Fig. 1 show that the ion exchange process was successful, and they also show that high-activity glutathione peroxidase emerged as a single peak. As shown in Table 2, the enzyme's specific activity is 1.28 enzyme U/mg, indicating an amazing 4.5-fold purification factor.

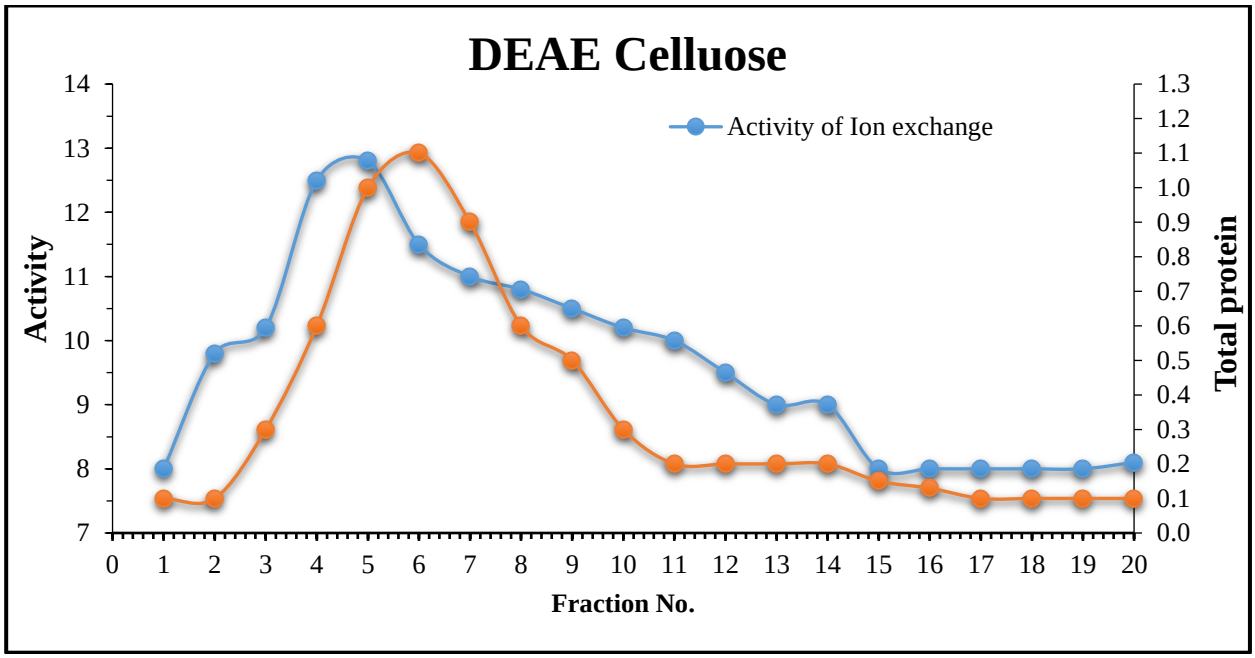

Figure 1: Ion exchange chromatography of GPX enzyme by DEAE-Cellulose.

3.6 Gel Filtration Chromatography

When proteinous compounds from the ion exchange process are to be separated using gel filtration, a 2.5 x 30 cm separation column filled with G-100 is utilized. Once the ion exchange-produced protein peak is passed by the gel filtration process, the results show the appearance of a single protein peak, which is shown in Fig. 2. It is clear from assessing the glutathione peroxidase enzyme activity that the peak, which reached 1.3 enzyme U/mg, shows a high specific activity of the enzyme. Furthermore, 4.64 times as many purification cycles are reached, demonstrating how well the gel filtration method purifies the enzyme.

3.7 Determination of the approximate molecular weight of Glutathione Peroxidase using SDS-Page Electrophoresis

The molecular weight of the partially purified glutathione peroxidase extracted from human blood serum has been estimated using the SDS-PAGE analysis and the standard curve of logarithm of molecular weight versus relative migration of ten standard ladders. After the protein was broken

down by the enzyme and treated with SDS, chains of varying lengths were encircled by negatively charged SDS molecules. Through this process, the protein's initial charge is eliminated, enabling the chains to migrate electrically. The ratio of charge to mass determines how far the proteins travel to reach the anode, or positive electrode. For glutathione peroxidase, the estimated molecular weight was found to be roughly 23 kDa, as demonstrated by the standard curve in Fig. 4.

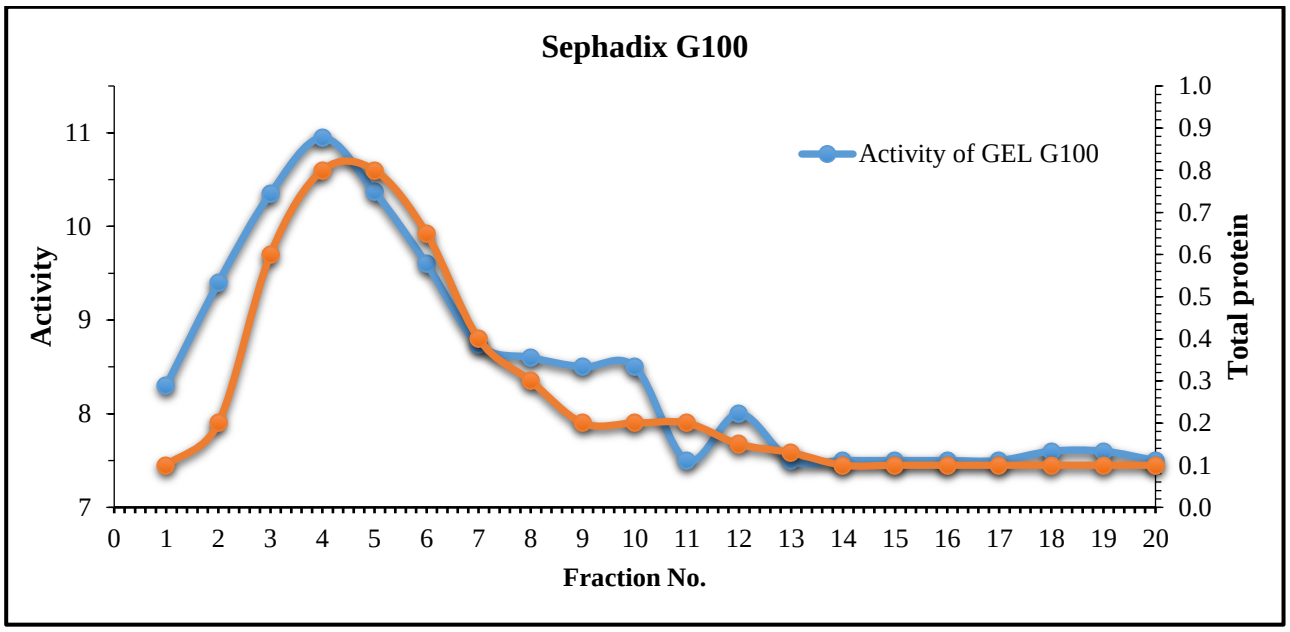

Figure 2: Purification of the GPX enzyme by gel filtration chromatography.

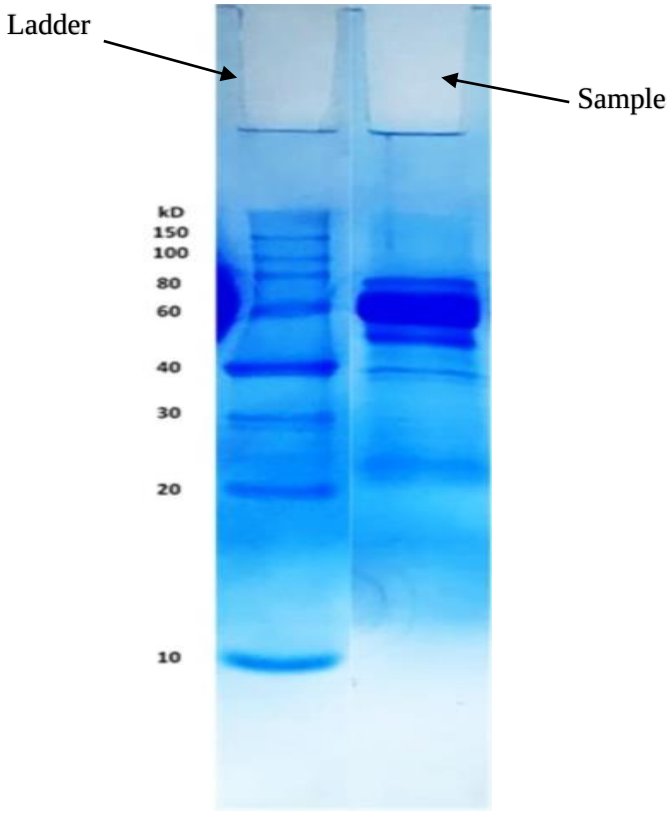

Figure 3: SDS-PAGE analysis bands of the purified glutathione peroxidase and the standard ladder proteins.

The crude extract, which includes a range of proteins with various molecular weights, is what causes the numerous protein bands seen on the gel. But only one band was visible in the refined enzyme sample, proving that other proteins had not contaminated it [17]. The molecular weight determination process using the standard curve is shown in Figure 4.

Table 3: Standard protein used in determination of molecular weight of glutathione peroxidase by SDS-Page

MW	Log	Cm
150	2.17	0.7
100	2	1.2
80	1.90	1.4
60	1.77	2
40	1.60	2.7
30	1.47	4
20	1.30	4.8
10	1	5.8

Purified glutathione peroxidase was analyzed using polyacrylamide gel electrophoresis with SDS, and the results showed that there was no contamination at all. Figure 5 shows that the molecular weight of the subunit was around 23 kDa, and there was only one distinct band. In earlier research, the use of polyacrylamide gel electrophoresis with SDS for the study of pure glutathione peroxidase was examined [18].

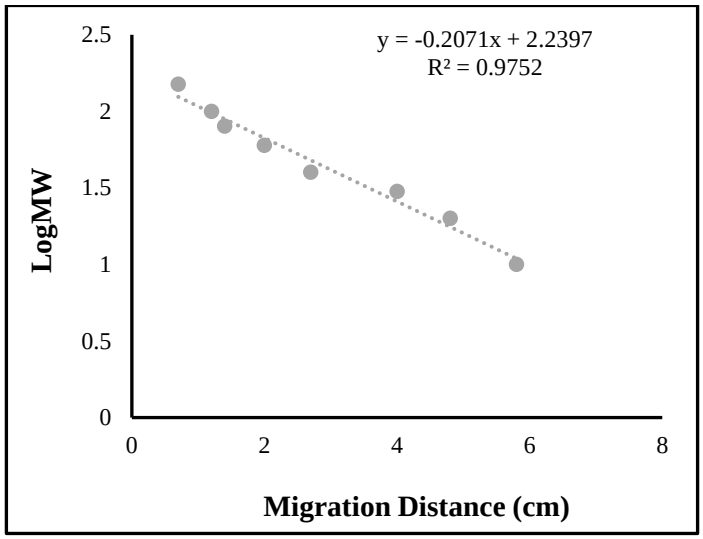

Figure 4: Standard curve of the migration distance versus ladder fragment size of the standard proteins.

3.8 Studying Kinetic and characterization of partially purified glutathione peroxidase

Kinetic enzymes, exemplified by glutathione peroxidase, represent a pivotal class of proteins essential for cellular homeostasis. As a key player in antioxidant defense, glutathione peroxidase efficiently scavenges reactive oxygen species, safeguarding cells from oxidative damage. Understanding the kinetic properties of this enzyme is paramount for unraveling its intricate regulatory mechanisms and potential therapeutic implications in oxidative stress-related pathologies. Many researches provides foundational insights into the kinetic behavior of glutathione peroxidase, guiding our comprehension of its role in cellular redox balance [19,20].

3.9 Effect of Different Substrate Concentration on GPx Enzyme Activity with Determination of Michaelis-Menten Constant (Km) and Maximum Velocity (Vmax)

The glutathione peroxidase enzyme's activity was assessed using various hydrogen peroxide concentrations (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mM) as a base material. It was discovered that the highest activity of GPX was achieved with a concentration of 1 mM hydrogen peroxide, or 15.800 IU/L. Figure 5 illustrates how an increase in substrate concentration causes the rate of the enzyme reaction to rise.

The linear Line weaver Burk equation was used in this investigation to plot the reciprocal of the starting velocity against the reciprocal of the matrix concentration. Using Line weaver Burk plots, a linear connection was produced with the value of km and the maximum velocity (Vmax) of (15.800 mM) and (Km) of (0.3 IU/L) for the enzyme GPX. The reaction rate was plotted against the reciprocal of the matrix concentration (1/S). Figure 6 shows the inverse (1/V).

With a Km (Michaelis-Menten constant) of 16.66 and a Vmax (maximum reaction velocity) of 0.909, the plot allows us to analyze the enzyme's affinity for its substrate and its catalytic efficiency. In the Line Weaver-Burk plot, the reciprocal of the initial reaction velocity (1/V) is plotted against the reciprocal of substrate concentration (1/S). The slope of the resulting line corresponds to Km/Vmax, and the y-intercept represents 1/Vmax. The Km reflects the substrate concentration at which the enzyme achieves half of its maximum velocity, indicating its affinity for the substrate.

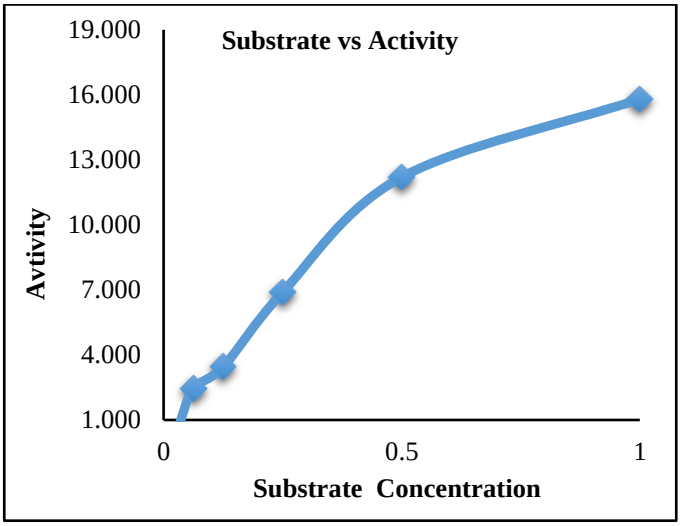

Figure 5: Michaelis-Menten plot show effect of substrate concentration on GPX activity.

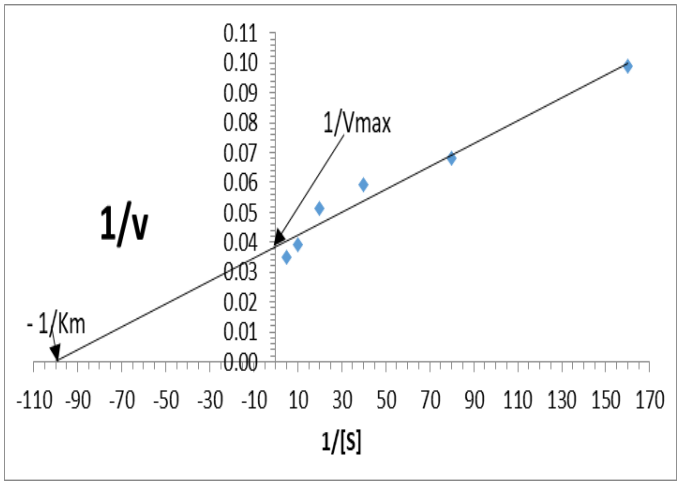

Figure 6: Line Weaver-Burk plot for partially purified GPX

A lower Km suggests a higher affinity. For partially purified GPX, a Km of 16.66 implies that the enzyme requires a relatively moderate substrate concentration for optimal activity. The Vmax of 0.909 signifies the maximum rate at which the enzyme can catalyze the reaction under saturating substrate conditions. These findings differed from prior studies that were undertaken to estimate the kinetic constants of the enzyme, and the discrepancies could be attributed to the diverse sources from which the enzyme was purified as well as the different purification methods used. According to the

findings of Y. Yamashita and his colleagues, who isolated and characterized GPX from the red muscle of the Pacific bluefin tuna Thunus orientalis, a linear connection was discovered in a non-Innover-Burke plot, with Km and Vmax values of 6.8mm and 1.25, respectively [21]. A study by *AU Lodziensis and his group* indicated that GPX purified from human placenta was determined to be 78.5Mm Km at optimal conditions for the enzyme [22]. The *Al-helaly* study also indicated that a linear relationship was obtained for the GPX enzyme purified from the pea plant, which uses GSH as a base material, giving a Km value of (1.25 mmol/liter) and a Vmax value of (2.1 mmol/min) [23].

3.10 Effect of pH on GPx Enzyme Activity

The influence of pH on GPx enzyme activity was studied, as illustrated in Fig.7. The isolated GPX enzyme showed the greatest activity at pH 7.2. Understanding glutathione peroxidase (GPX) function as an antioxidant enzyme is critical for protecting cells from oxidative damage. The pH of the enzyme's environment has a substantial impact on its activity, which is linked to the protein's three-dimensional structure and charge distribution.

Deviations from this optimum pH may result in reduced enzyme activity due to changes in the ionization state of crucial amino acid residues involved in catalysis. Notable studies have investigated the pH dependency of GPX activity. *Ursini et al.* explored the pH profile of GPX in various tissues and identified distinct optima for different forms of the enzyme. *Joshi et al.* examined the impact of pH on GPX activity in the context of cardiovascular diseases. The dynamic relationship between pH and GPX activity contributes to the regulation of cellular redox balance and antioxidant defense mechanisms. [24, 25]**.**

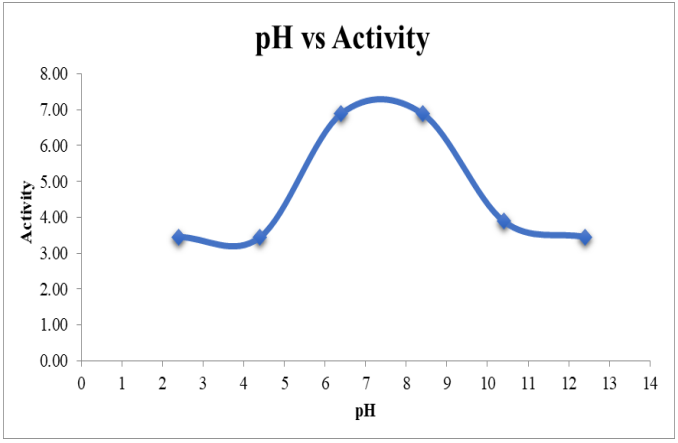

Figure 7: Effect of pH on GPx Enzyme Activity

3.11 Effect of Temperature on GPx Enzyme Activity

Different temperatures were assessed to determine the optimal temperature for GPx enzyme activity. The temperatures considered were (7, 17, 27, 37, 47, and 57°C). The results showed that the optimal temperature for GPx activity was found to be 37°C. As the temperature increased beyond the optimal temperature, there was a subsequent increase and then a decline in GPx enzyme activity. This trend can be observed in Fig.8. In addition to temperature optima, studies such as the one conducted by Li et al. [26] further enhance our understanding of GPx behavior under different thermal conditions. This research offers insights into the thermal stability of GPx, examining how the enzyme responds to varying temperature regimes and whether prolonged exposure to higher temperatures affects its catalytic efficiency. Furthermore, the work by Kim et al. [27] provides a contemporary perspective on the temperature sensitivity of GPx, addressing how fluctuations in temperature impact the enzyme's kinetic parameters. Taken together, these studies contribute to a dynamic understanding of the influence of temperature on GPx activity.

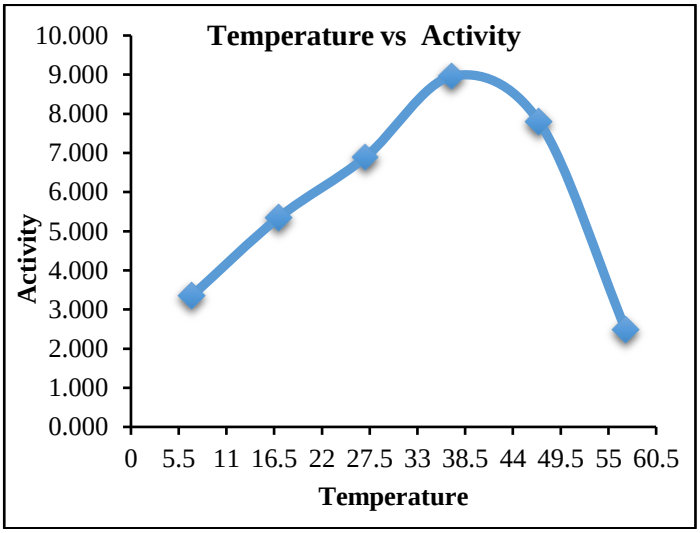

Figure 8: Effect of Temperature on GPx enzyme activity.

3.12 Effect of Reaction Time on Enzyme Activity

Enzyme activity dependence on incubation time was illustrated in Fig.9 , showing measurements at different time intervals (1, 5, 10, 15, 20, and 25 minutes) where the optimal incubation time was (5) minutes.. With increasing reaction time, the rate of substrate conversion by GPX initially rises, eventually reaching a peak where the enzyme achieves its maximum catalytic potential. The kinetics of GPX, influenced by reaction time, involve a delicate balance between substrate availability and enzyme saturation. However, as reaction time extends, the potential for substrate depletion or product inhibition may arise, leading to a plateau or decline in enzyme activity. Determining the optimal reaction time is crucial for accurately assessing GPX activity, ensuring sufficient substrate conversion while mitigating limitations associated with prolonged reaction durations [28]. Although *Ursini et al.'s* study primarily focused on the dual function of GPX during sperm maturation, it highlights the importance of understanding GPX dynamics in different cellular contexts [29].

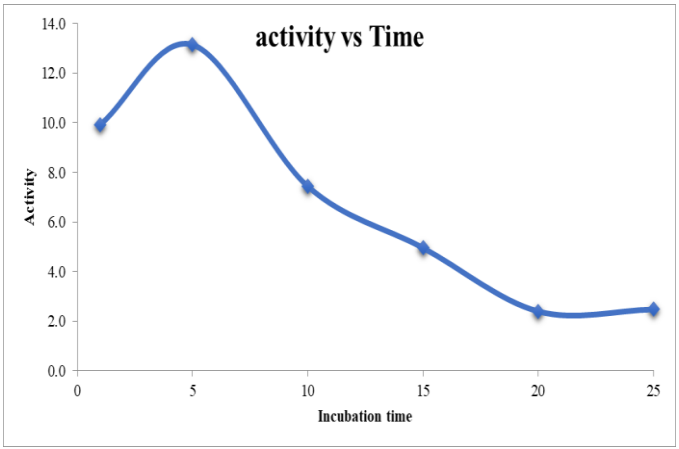

Figure 9: Effect of Reaction Time on Enzyme Activity.

3.13 Study of the effect of carvedilol on the activity of the enzyme glutathione peroxidase

The effect of manufactured particles on enzyme activity was assessed through in vitro assays. The results of these assays indicated a decrease in enzyme activity in the presence of carvedilol, as shown in table 4 and fig.10.

Table 4: The effect of carvedilol on glutathione peroxidase activity

Conc.	Activity IU/L
nil (control)	25 IU/L
100 mg/10 ml	6.1
10 mg/10 ml	8.38
1 mg/10 ml	10.85
0.1 mg/10 ml	13.32
0.01 mg/10 ml	17.44
0.001 mg/10 ml	20.11
0.0001mg/10 ml	24.7

Table 4 provides valuable data on the effect of carvedilol, acting as an uncompetitive inhibitor, on glutathione peroxidase activity at various concentrations. The control group without carvedilol exhibits an initial glutathione peroxidase activity of 25 IU/L, providing a baseline for comparison. Carvedilol Concentrations as the concentration of carvedilol increases, the glutathione peroxidase activity decreases. The observed relationship between increasing carvedilol concentration and decreasing glutathione peroxidase activity strongly suggests that carvedilol functions as an uncompetitive inhibitor of this enzyme. As the concentration of carvedilol increases, it seems to interfere with the enzyme's ability to carry out its catalytic function, resulting in a progressive decline in enzyme activity levels. Understanding the inhibitory effects of carvedilol on glutathione peroxidase activity is of clinical significance, as it may provide insights into the potential therapeutic applications of carvedilol in managing conditions and diseases associated with oxidative stress. Uncompetitive inhibitors are a unique category of enzyme inhibitors which are known for their distinct binding to the substrate-enzyme complex, establishing an enzyme-substrate-inhibitor (ESI) complex. This class of inhibitors differs from competitive and noncompetitive inhibitors, offering an innovative mechanism to control enzyme activity by *Segel* [30]. Notably, these inhibitors do not influence the maximum velocity (Vmax) whose value is (15.800 mM) of the reaction catalyzed by an enzyme. Study by *Cornish-Bowden* concluded that continued product formation is reduced, but substrate binding is unaffected [31]. Researchers *Johnson and Goody et al.* stated that the presence of an uncompetitive inhibitor lowers the perceived Michaelis-Menten constant (Km) whose value is (0.3 IU/L) for the enzyme, indicating higher enzyme-substrate affinity.

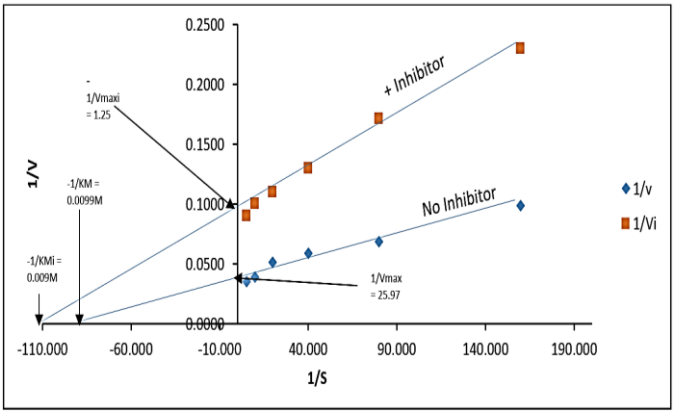

Figure 10: Line weaver-Burk illustrates the Michealis constant and the maximum velocity when using Carvedilol inhibitor

This alteration to the enzyme's efficacy can have implications on enzymatic reactions [32] With respect to a Lineweaver-Burk plot, study by *Dixon and Webb* concluded that the parallel lines are indicative of uncompetitive inhibition, demonstrating how both Km and Vmax are affected while the x-intercept (1/[S]) remains unchanged. This unique plot is often employed to identify this type of inhibition [33]. *Wlodkowic and Skommer* proved that uncompetitive inhibitors play a significant role in pharmaceutical research and treatment approaches, including potential cancer treatments and antiviral measures. Examples include HIV protease inhibitors and certain chemotherapy drugs [34]. Given their unique binding to substrate-enzyme complexes and their exclusive effects on Km and Vmax, uncompetitive inhibitors are a fascinating area of study in enzymology and drug development.

4. Conclusions

The study focused on investigating the serum glutathione peroxidase activity and partially purifying of the enzyme from the blood serum. Findings revealed that the purification of enzyme was achieved 4.64 times, and the enzyme yield was 40%. Specific activity was estimated as 1.3 U/mg. The study also carried out kinetic examinations, with Km and Vmax values at 15.800 mM and 0.3 IU/L, respectively. Optimal conditions for enzyme action were identified as a concentration of 100 mmol/L, a temperature of 37°C, and a

pH level of 7.2. The molecular weight of the glutathione peroxidase enzyme derived from heart attack serum was 23 kDa. The research also explored the effect of carvedilol on the enzyme glutathione peroxidase, revealing a decrease in enzyme activity and identifying it as an uncompetitive inhibitor.

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