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Partial Purification And Molecular Weight Determination Of Glutathione S-Transferase Enzyme By Gel filtration Method In Patient With Beta Thalassemia Major

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Summary

The glutathione S-transferase (GST) enzyme was partially purified by using gel filtration chromatography technique, from the blood serum of a patient with beta thalassemia major, using a separation column with dimensions (2 cm in diameter and 100 cm in height), and Sephadex G-75 gel was used. Precipitation was done using ammonium sulfate, and dialysis was performed for the precipitated part in which the enzyme activity was high. Three packages for the activity of GST enzyme were obtained, and the activity of GST in the second package (B) was higher than the other packages, and from it the approximate molecular weight of the enzyme was determined, Also, standard calibration curves were used for a number of standard proteins with known molecular weights to obtain the linear equation from which the approximate molecular weight of GST was determined to be about (25882 Daltons).

The kinetics of partially purified GST from patient serum was studied with the determination of its optimal conditions. The results of the optimal conditions for GST using the buffer solution at a concentration of (0.12 mol/L), when the concentration of purified protein was (25 mg/mL), pH (pH = 6.5), incubation time (12 minutes), temperature (25°C). And the concentration of the base substance 1-chlorodinitrobenzene (CDNB) was (22.463 mmol/L). The values of the maximum velocity (Vmax) and the Michaelis constant (Km) were found using the Lineweaver-Burk plot, where the value of (Vmax = 0.556 µmol/min), and the value of (Km = 12.7 mmol/L). The effect of one of the inhibitors on GST activity was studied by using Benzyl Chloride, and the type of inhibition was non-competitive.

Keywords:

Glutathione-S-transferase (GST), beta thalassemia major (BTM), substrate: 1-chlorodinitrobenzene (CDNB), Gel Filtration Chromatography (GFC).

Glutathione-S-transferase (GST) (EC 2.5.1.18) is a vital defense enzyme that detoxifies reactive oxygen species (ROS), GST genes associated with elimination of oxidative stress [1]. GST is secreted by the liver and has a major role as an antioxidant in cellular defense processes, used to remove toxins and heavy metals from

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the body.[2] It regulates cellular signaling, post-translational modification, and resistance to chemotherapeutic agents.[3]

The isoenzymes of GST, It is multifunctional because it is involved in many catalytic and non-catalytic processes, and works to catalyze the nucleophilic addition of glutathione (GSH) to a variety of non-polar substrates that come from outside the body. Such as chemical carcinogens, environmental pollutants, even antitumor agents, drugs, and internal substrates materials (consisting of various catabolic products inside the body), thus facilitating their removal and disposal [4]. It also catalyzes electrophilic substrates with GSH, which plays a major role in the detoxification of these toxic substances. [5]

In beta thalassemia major (BTM), glutathione-S-transferase forms affect the iron overload associated with the disease. Thalassemia is a congenital genetic disorder of the blood, because the body produces abnormal forms of hemoglobin.[6] t results from a partial or total deficiency in the synthesis of the alpha (α) or beta (β) chain that leads to an unbalanced production of the globin chain, so that the two main categories of thalassemia are alpha or beta. And the formation of inactive red blood cells, and a varying degree of anemia, and then hemolysis.[7] BTM is the most severe form of hereditary hemolytic anemia, resulting from the absence of the beta chain for the production of hemoglobin, which is necessary for the formation of normal hemoglobin. BTM patients suffer from ineffective erythropoiesis, which leads to hemolysis and severe anemia.[8] As excess iron leads to organ damage, and GST has an important role as an antioxidant in detoxification processes from toxic substances as a result of repeated blood transfusions that cause toxicity of inactive erythropoiesis. Iron has a catalytic role in the production of free radicals and ROS, and antioxidants have an essential role in protecting cells from oxidative stress.[9] The antioxidant role of GST isoenzymes comes from the fact that these enzymes possess tissue-specific gene expressions because they are from a family of important multigenes that catalyze the glutathione coupling reaction with a variety of harmful ionic molecules that are disposed of. [10] In fact, GST genes are upregulated in response to oxidative stress, genetically determined differences lead to changes in the levels of activity and expression of some GST genes that cause a decrease in the defense capacity against oxidative stress.[11]

2- The Aim Of The Research

Purification of glutathione-S-transferase enzyme using gel filtration column chromatography technique from blood serum of a patient with beta thalassemia major, estimation of its approximate molecular weight, study of enzyme kinetics, extraction of values of maximum velocity (Vmax) and Michaelis constant (Km), and determination of optimal conditions for enzyme activity.

3- Materials and methods

3.1- Estimation of glutathione-S-transferase levels in serum of patients with beta thalassemia major.

3.1.1- Basic Principle

The activity of the GST enzyme was estimated when stimulating the electrophilic aggregates in the aromatic ring of the enzyme dependent substrates. The substrate (1-Chloro-2,4-dinitrobenzene) CDNB is conjugated by a chlorine-hydrogen substitution of the (SH-)thiol group of the reduced glutathione (GSH). The product of the reaction is Dinitrophenyl thioether and chlorine ion, Cl-, as shown in Figure (1). The absorbance was measured at (λ=340 nm).[12]

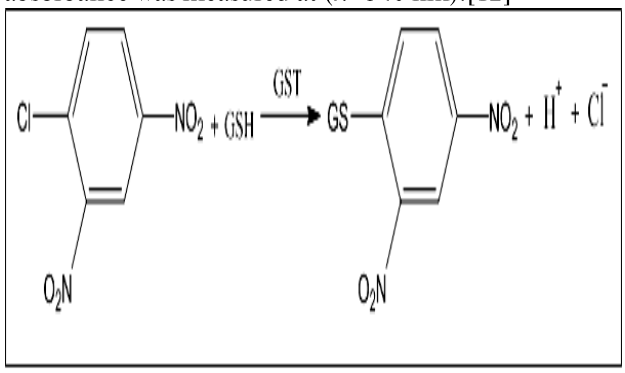

Figure (1) Clarification of the working principle of GST and the association of glutathione with CDNB

3.1.2- Working reagent

1 Prepare the buffer solution, Phosphate buffer (pH = 6.25).

2 Prepare (29.93 mM) from the standard solution of glutathione GSH.

3 Prepare (22.463 mM) of CDNB substrate.

3.1.3- Working Method

PROCEDURE		
Materials	Blank	Sample
Phosphate buffer (pH 6.25)	2700µL	2700µL
Sample	-	100µL
Distilled water	100µL	-
CDNB solution	100µL	100µL
Incubate for (3 minutes)		
Standard solution (GSH)	100µL	100µL
Mix the ingredients well, then read the absorbance difference after the first minute and the tenth minute at (λ=340nm)		

3.1.4- Calculations

GST activity (U/L) = (ΔA × Vt × 1000) / (Eo × Vs × d)

Where:- **[ΔA:** the difference in absorbance (absorbance at the tenth minute – absorbance at the first minute), **Vt:** total volume (3mL), **Vs:** sample volume (100µL), **Eo:** molar absorption coefficient estimated to be (9.6 mmol-1), **d:** cell length (1cm)].

3.2- Purification of glutathione-S-transferase in blood serum.

The GST enzyme was purified from a patient suffering from BTM, while he was attending the Thalassemia Center in Azadi Teaching Hospital in Kirkuk Governorate. The patient's gender was male, age (13 years), blood type (O+), Height (159 cm), weight (47 kg), body mass index (BMI) (18.6 kg / m2), normal weight, and the relationship between his parents is from cousins. Purification of GST was carried out at low temperatures (4 °C) and in several sequential steps, which can be explained below:

3.2.1- sedimentation using ammonium sulfate

Use the salt displacement method, which is a common method for precipitating proteins. The use of neutral ammonium sulfate salt, as it is one of the most common salts due to its high solubility. As ammonium sulfate works to stabilize proteins by preferential solubility, so that the protein is finally suitable for gel filtration. The buffer solution is replaced several times to get rid of the remaining ammonium sulfate.[13]

Solid ammonium sulfate salt (15gm) was added gradually and gently with a saturation rate of (75%) to (20mL) of the blood serum of a patient with BTM. With continuous stirring for a period of (60 minutes) by means of a magnetic stirrer, to dissolve all ammonium sulfate at a temperature of (4°C). After completing the hour period and to complete the protein sedimentation process completely, the solution is left for (24 hours) at a temperature of (4°C). Then, a centrifuge is used at a speed of (5000 rpm/min) for a period of (30 minutes) in order to separate the leachate from the precipitate. [14] The volume of the filtered and precipitated solution is calculated. The amount of total protein for both solutions (filter and precipitate) was estimated using the diagnostic kit prepared by the French company Biolabo at (λ =550 nm). [15] The GST activity of both solutions is also estimated at (=340 nmλ).[12] The protein fraction with high GST activity is kept frozen at a temperature (-20 °C) until it is used in the subsequent steps of purification.

3.2.2- Dialysis

The principle of dialysis depends on the osmosis process, where the liquid moves from areas of high water concentration to low ones through a semi-permeable membrane until it reaches equilibrium [16].

The part of the sediment containing the proteins obtained after the salting process was placed in a semi-permeable dialysis bag and tightened on both sides, then placed in a beaker (1000 mL) containing a buffered phosphate solution with a concentration of (0.1M), and (pH = 6.25). The dialysis was

performed at (4°C), with continuous stirring by means of the magnetic stirrer, for a period of two days (48 hours), with the buffer solution changed every (6 hours). After completion, the final remaining volume of the sample solution is calculated. [17] Both the amount of total protein and the GST activity of the sample solution are estimated. Then the solution is kept frozen at a temperature (-20°C) until it is used later.

3.2.3 Gel Filtration Chromatography (GFC).

GFC is a liquid phase chromatography technique for separating proteins and nanoparticles (less than 100 nm) according to their size in solution [18]. GFC is used in protein purification to obtain high purity proteins because protein molecules do not stick to the column during separation unlike ion exchange chromatography and affinity chromatography. GFC allows the extraction of proteins and their isolation or storage. [19] The delay of the protein molecule in the column is proportional to the time it spends inside the pores of the gel. That is, particles with a radius larger than the pore diameter of the gel do not enter the gel and are said to be excluded.[20]

3.2.3.1- Filling The Filter Gel Column

Separation column in GFC with dimensions (2 cm in diameter x 100 cm in height) to obtain high accuracy in the separation process [21]. The column was filled with a gel of the type (Sephadex G-75), which is available in the form of a powder, and it was prepared by soaking it in a sufficient amount of distilled water and stirring it gently to get rid of the air bubbles generated, then it was left for (72 hours) to fully swell at (20 °C).[22] Sephadex G-75 gel is filled to a height (95 cm) of the separation column and the remainder is left for sample and solvent additions. Depending on the dimensions of the column, the volume of the gel was (500 ml). The gel is poured gently and accurately from the top of the separation column with its lower outlet closed. The top of the column is moved all the time to ensure even filling and to avoid air bubbles. The bottom port is opened after the column has settled and then washed with buffer solution (mobile phase). A layer of solvent is always kept on top of the surface of the column to prevent it from drying out.[20]

3.2.3.2- Solutions And Materials Used In The Gel Filter Column.

1 The concentrated protein sample (2mL) obtained from dialysis
2 Deionized waters.
3 Add (2mL) of the following protein substances with known molecular weights: Urease, Pepsin, Bovine serum albumin, α-Amylase, Papain, Insulin, Tryptophan.
4 The aforementioned solutions are added accurately to the walls of the column in a circular manner. Then the packed gel was washed in a separation column with deionized water, which used an Elution solution.
5 The elution of protein materials was carried out at a flow rate of (55 ml/hour) at a specified time (5 minutes), using deionized water as an elution solution, and the fragments were collected for the specified time using a minute-based digital clock [20].
6 The amount of total protein added to the separation column was estimated by absorbance (UV / Visible) at (λ = 280 nm) [23], then GST activity by spectrophotometer at (λ = 340 nm). [12]
7 The volumes of functionalization were determined for the standard protein materials with known molecular weights that were injected into the separation column.[12]
8 A standard calibration curve is drawn between the volumes of the functionalities with the logarithm of the molecular weights of the proteins, to obtain the linear equation to extract the approximate molecular weight of GST [24].
9 The purified GST is kept in a tightly closed test tube at (-20°C) for the purpose of determining the optimal conditions affecting the enzyme activity, studying its kinetics, and extracting the values of Michaelis' constant (Km) and the maximum speed (Vmax).

4- Results and Discussion

The purification of the GST enzyme was carried out by gel filtration chromatography, according to the steps shown in Table (1).

Table (1) Partial purification steps of GST enzyme in blood serum.

1	2	3	4	5	6	7	8
Purification steps	Volume (mL)	Total protein concentration (mg/mL)	Activity (*U/mL)	Total Activity (U*)	Specific activity (U/mg)	Folds of purification	Recovery rate (100%)
Serum	20	10.32	2.38	47.6	0.231	1.0	100
Precipitation by ammonium sulfate, saturation 75%.	16.64	8.54	2.51	41.77	0.294	1.273	87.75
Leachate (supernatant)	6.55	3.37	0.96	6.29	0.285	1.234	13.21
Dialysis	17.90	6.22	2.18	39.02	0.350	1.515	81.98
Filtration by Cephadex-G75 gel to obtain the highest activity of the GST enzyme from the highest peak present							
Peak A	39.44	0.351	0.942	37.152	2.684	11.619	78.05
Peak B	23.08	0.207	1.853	42.767	8.951	38.749	89.85
Peak C	28.77	0.511	1.054	30.324	2.063	8.931	63.71

4.1- Gel filtration chromatography (GFC) technique

GFC is a versatile method that allows efficient separation of biological molecules with high throughput. [21] And using the appropriate Sephadex G-75 gel, the chromatography column gave three protein peaks of GST activity, which are shown in Figure (2). The elution volumes for the three peaks were as follows: the first peak A (129.1 mL), the second peak B (175.9 mL), and the third peak C (287.9 mL). By following up the GST activity, it was observed that it is more concentrated at the second band (peak B), as in Table (2).

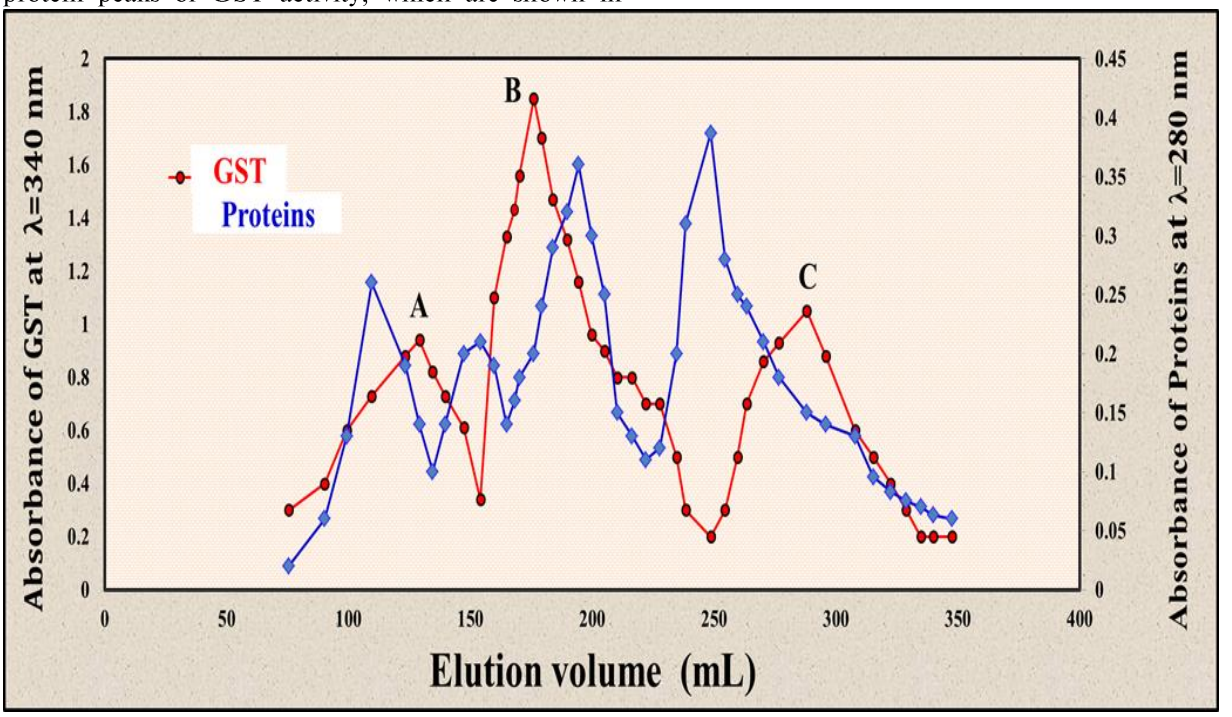

Figure (2) The Elution volumes of the protein bands of GST enzyme in blood serum obtained from the gel filtration separation column.

4.2- Estimation of the approximate molecular weight of glutathione-S-transferase by gel chromatography.

The approximate molecular weight of the second bundle (peak B) was estimated. Protein materials with known molecular weight between (204-480000 Dalton) were passed. Then the elution volumes are calculated on the separation column containing Sephadex G-75 gel, as shown in Table (2).

Table (2): The Elution volumes of standard protein materials with known molecular weights.

Materials	Molecular Weight	Elution Volume
Urease	480000	89.3
Bovine serum albumin	67000	125.6
α-Amylase	45000	143.2
Pepsin	36000	155.4
Papain	24717	200.3
Insulin	5700	225.1
Tryptophan	204	350.3

After completing table (3). The standard linear curve of the elution volumes is plotted against the logarithm of their molecular weights. To obtain the straight line equation (y = -0.012x + 6.524) and from it the approximate molecular weight of GST is extracted, as shown in Figure (3). This result was in agreement with the previous literature, where the approximate values for the effectiveness of GST were between (22000-29000 Dalton) from different tissues. [24,25,26,27,28,29,30,31,32

Table (3) The Elution volumes of standard protein substances and the logarithm of their molecular weights.

Materials	Molecular Weight	Elution Volume	Log M.wt
Urease	480000	89.3	5.681
Bovine serum albumin	67000	125.6	4.826
α-Amylase	45000	143.2	4.653
Pepsin	36000	155.4	4.556
Papain	24717	200.3	4.362
Insulin	5700	225.1	3.756
Tryptophan	204	350.3	2.310
Unknown (Peak B)	25882*	175.9*	4.413*

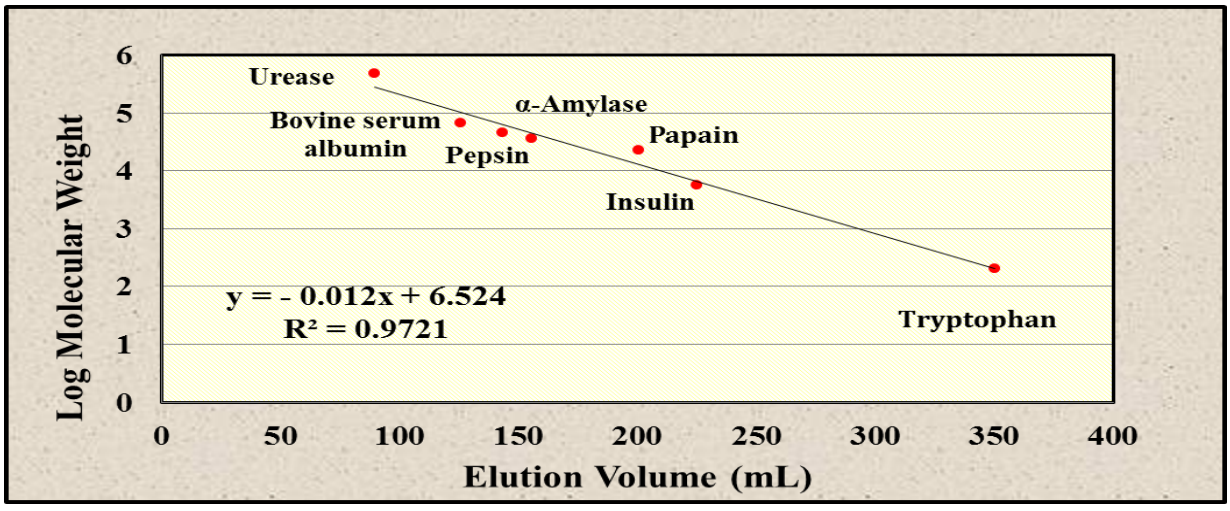

Figure (3) standard calibration curve to determine the approximate molecular weight of the GST enzyme when it was purified by gel filtration chromatography.

5.1- Effect of partially purified protein concentration on the activity of glutathione-S-transferase.

Different concentrations of partially purified GST enzyme were prepared, ranging between (5-40 mg/mL), with all other factors kept constant. It was observed in Figure (4) that the enzymatic reaction speed increases with increasing enzyme concentration. A constant concentration (20 mg/mL) of purified GST enzyme was used when studying other factors affecting the activity of the enzyme.

5- Study of factors affecting the activity of glutathione-S-transferase purified from blood serum

The second protein band (peak B) was used as the highest activity of GST purified from a gel filtration separation column. There are several important and influencing factors on the speed of enzymatic reactions, including: enzyme concentration, pH, temperature, and substrate concentration (CDNB). [24]

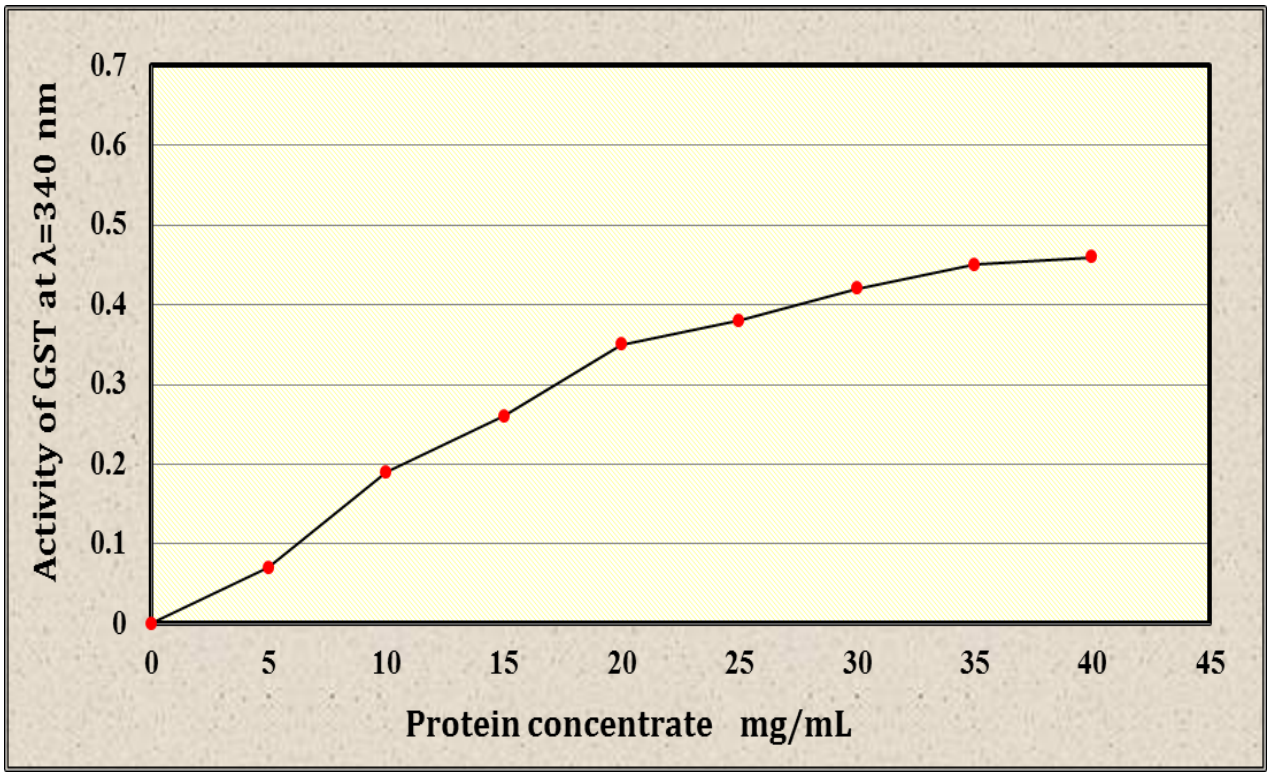

Figure (4) Effect of purified protein concentration on GST activity.

5.2- The effect of the buffer solution on the activity of the glutathione-S-transferase enzyme.

5.2.1- The effect of the concentration of the buffer solution on the activity of the glutathione-S-transferase enzyme.

Several different concentrations of the buffer solution were used at (pH = 6.25) ranged between (0.04-0.16 mol/L), the highest activity of the GST was obtained at the concentration (0.12 mol/L) of the phosphate buffer solution. As shown in Figure (5).

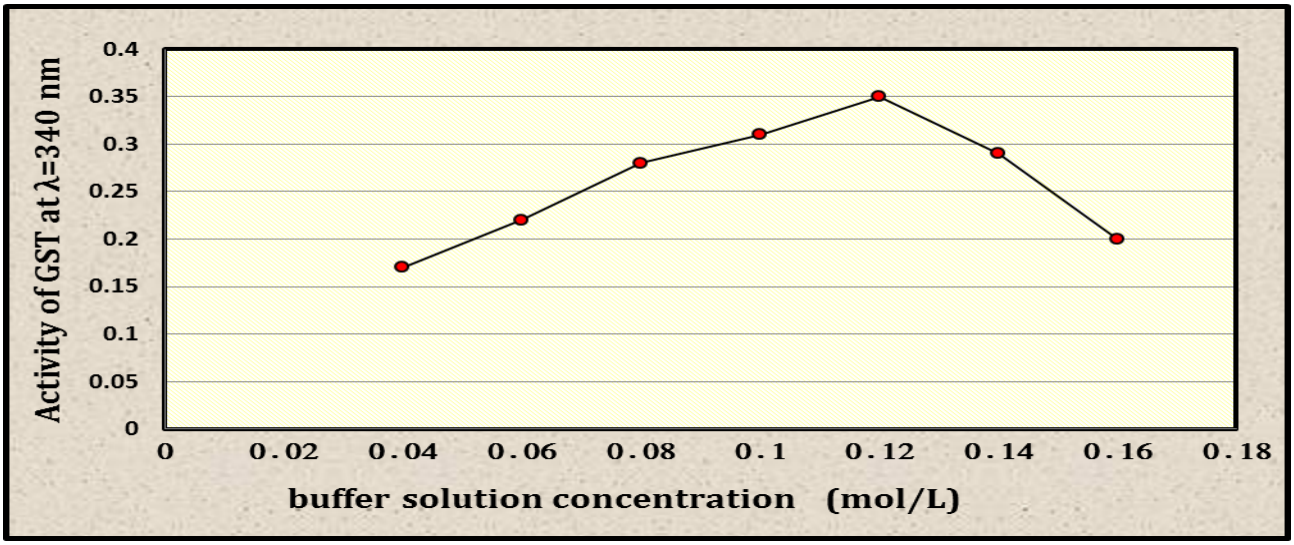

Figure (5) Effect of buffer solution concentration on GST activity.

5.2.2- The effect of pH on the activity of glutathione-S-transferase.

Several different pH values were used, which ranged between (4-8), with the concentration of the buffered phosphate solution fixed at (0.12 mol/L), and the highest activity of the GST enzyme was obtained at (pH = 6.5), as shown in Figure (6).

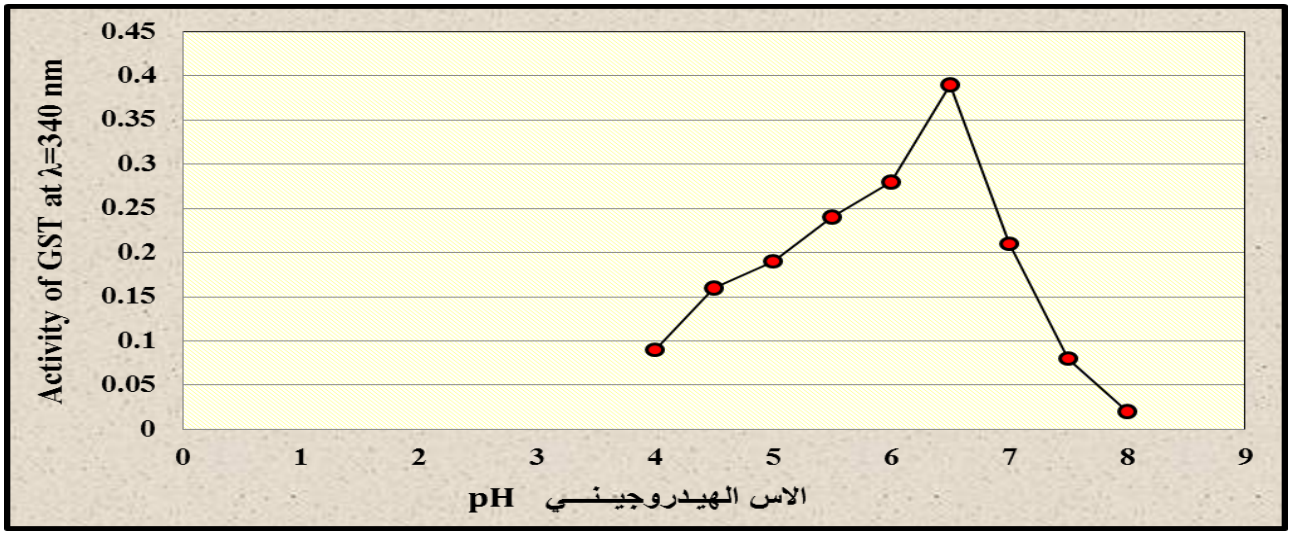

Figure (6) The effect of pH on the activity of GST.

5.3- The effect of reaction time (incubation period) on the activity of the glutathione-S-transferase enzyme.

The activity of the GST was measured in different periods of time ranging between (2-20 minutes) at room temperature (25 C), to reach a specific time in which the activity of the GST was as high as possible. The highest activity of the GST was at the time (12 minutes), as shown in Figure (7).

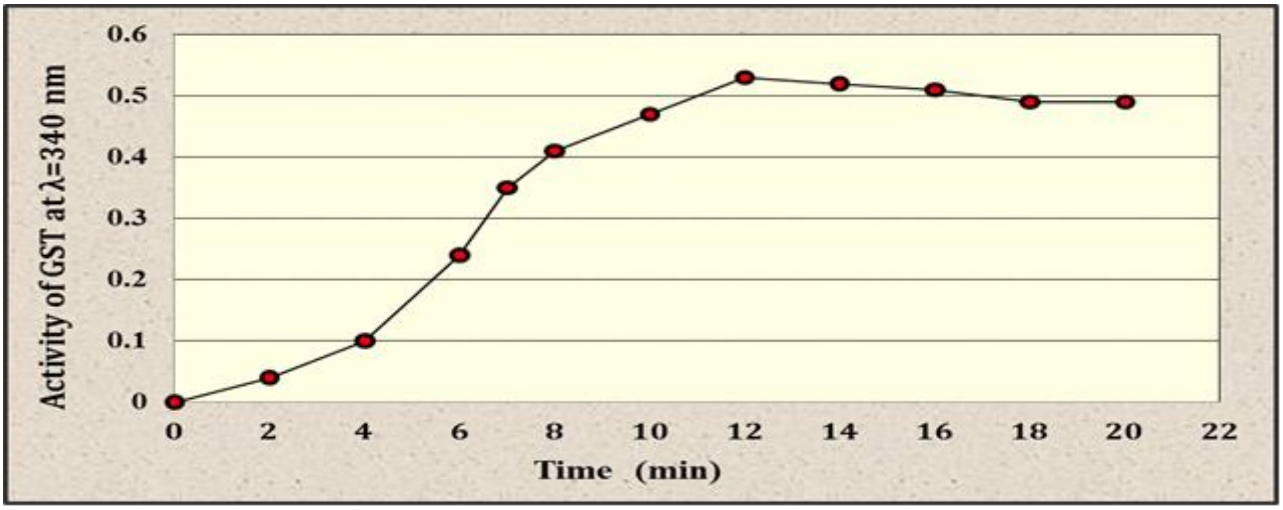

Figure (7) Effect of reaction time (incubation period) on GST enzyme activity.

5.4- The effect of temperature on the activity of the glutathione-S-transferase.

The speed of enzymatic reactions increases with increasing temperature until it reaches the optimal temperature for enzyme activity, in which the enzyme is at its highest activity. However, when the temperature rises and exceeds the optimum temperature, a deformation of the proteins (enzymes) occurs as a result of the dissolution of the hydrogen bonds and other forces responsible for the triple structure of the protein, and thus leads to a decrease in the enzymatic reaction rate.

The activity of the GST was measured at different temperatures, ranging from (10-45 °C) to reach the optimum temperature. The highest activity of the GST was at (25 °C), as shown in Figure (8).

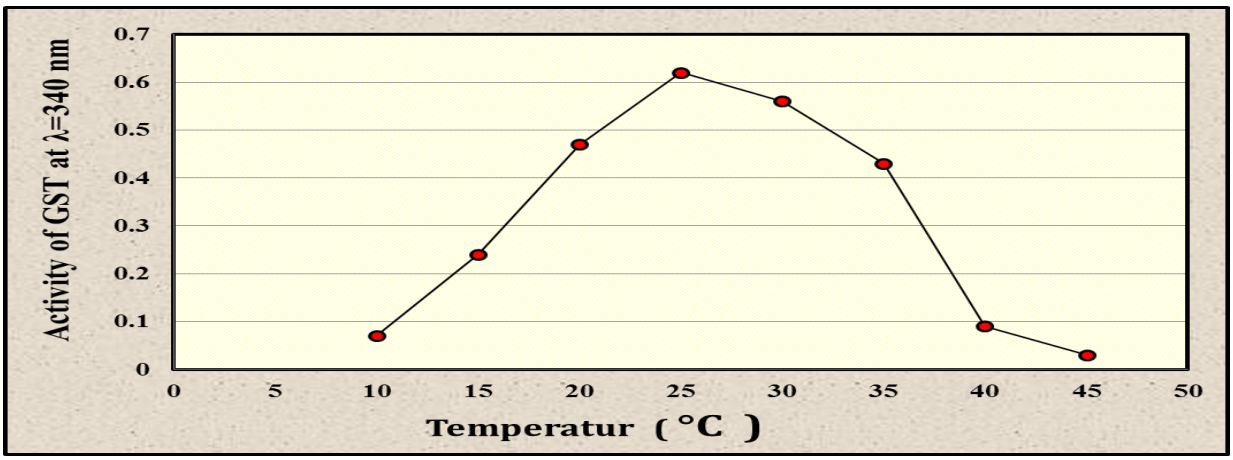

Figure (8) The effect of temperature on the activity of GST .

5.5- Effect of CDNB concentration on glutathione-S-transferase activity.

The rate of an enzymatic reaction usually increases with an increase in the concentration of the substrate (when the concentration of the enzyme is fixed). However, continuing to increase the concentration of the substrate leads to a decrease in the speed of the enzymatic reaction until the speed becomes constant no matter how much the concentration of the substrate is increased. The speed then at the highest concentration of the substrate is called the maximum enzyme speed (Vmax).[33]

The activity of the GST enzyme was measured in different concentrations of the substrate (CDNB) ranging between (10-40 mmol/L) as shown in Figure (9), which shows an increase in the speed of the activity of the GST by increasing the concentration of the substrate (CDNB). The activity of the GST enzyme was measured in different concentrations of the substrate (CDNB) ranging between (10-40 mmol/L) as shown in Figure (9), which shows an increase in the speed of the activity of the enzyme GST by increasing the concentration of the substrate (CDNB). Where the value of (Vmax = 0.556 µmol/min) and (Km = 12.7 mmol/L). Where Km is the concentration of the CDNB substrate when the enzyme activity (speed) is half of the maximum speed.

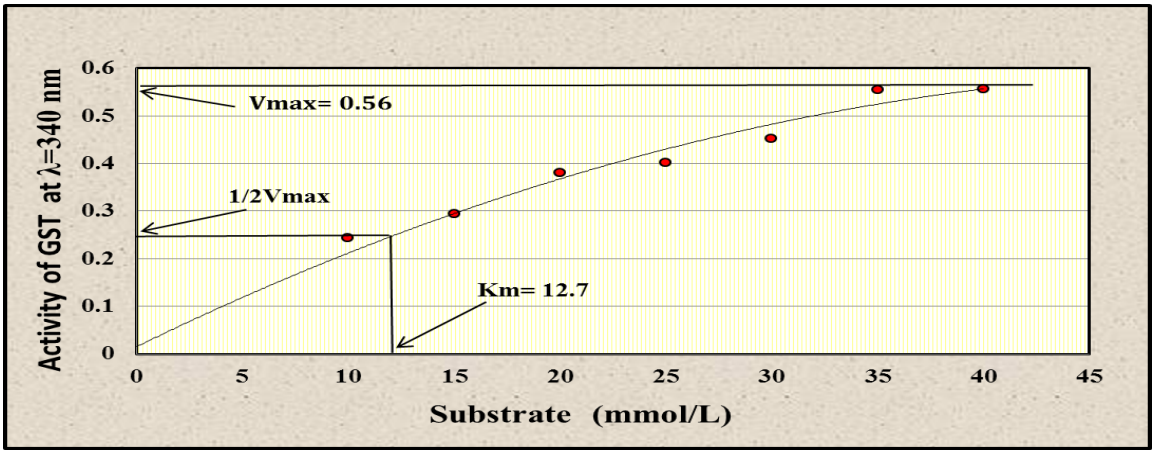

Figure (9) Effect of CDNB substrate concentration on GST activity.

Lineweaver-Burk was plotted between the inverse of the CDNB substrate concentration (1/[S]) against the inverse of the enzyme activity GST (1/V) to find the value of the maximum velocity Vmax and the Michaelis constant Km as shown in Figure (10).

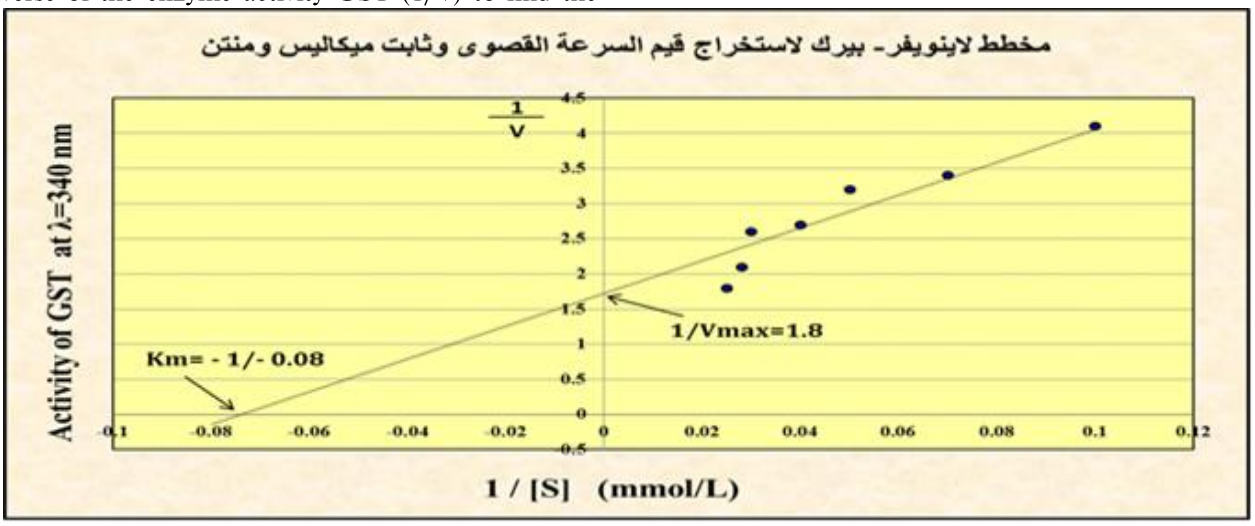

Figure (10) Lineweaver-Burk drawing to extract the values of Vmax and Km.

5.6- The effect of inhibitors (benzyl chloride) on the activity of the glutathione-S-transferase.

There are a number of compounds that inhibit the reaction rate of the GST enzyme and thus lead to a decrease in the activity of GST in its reactions. Benzyl chloride acts as an inhibitor when it interacts with the enzyme GST to form S-Bezyl glutathione, which reduces the activity of GST as its concentration increases in the reaction mixture. GST activity was measured in different concentrations of the inhibitor

Benzyl Chloride, which ranged between (10-40 mmol/L), as shown in Figure (11).

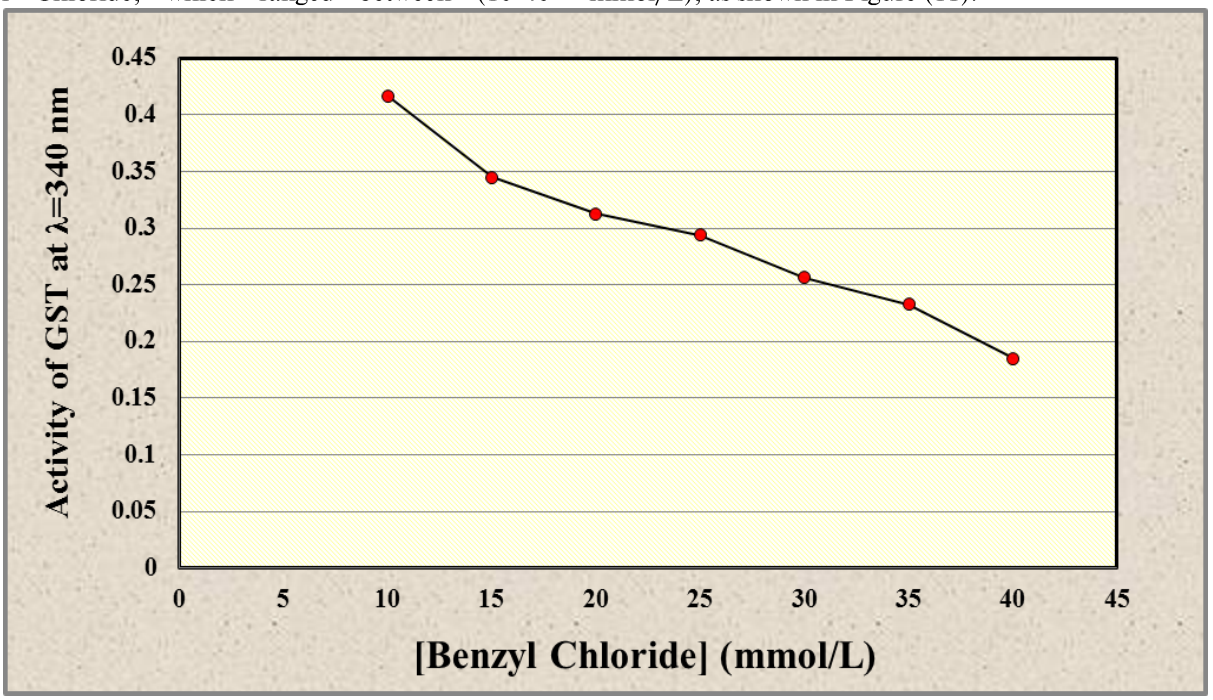

Figure (11) The effect of benzyl chloride concentration on the activity of GST.

To find the type of inhibition of benzyl chloride, the Lineweaver-Burk plot was used. Where the results showed in Figure (12) that the type of inhibition is non-competitive, and this was compatible with the result of the researcher [24].

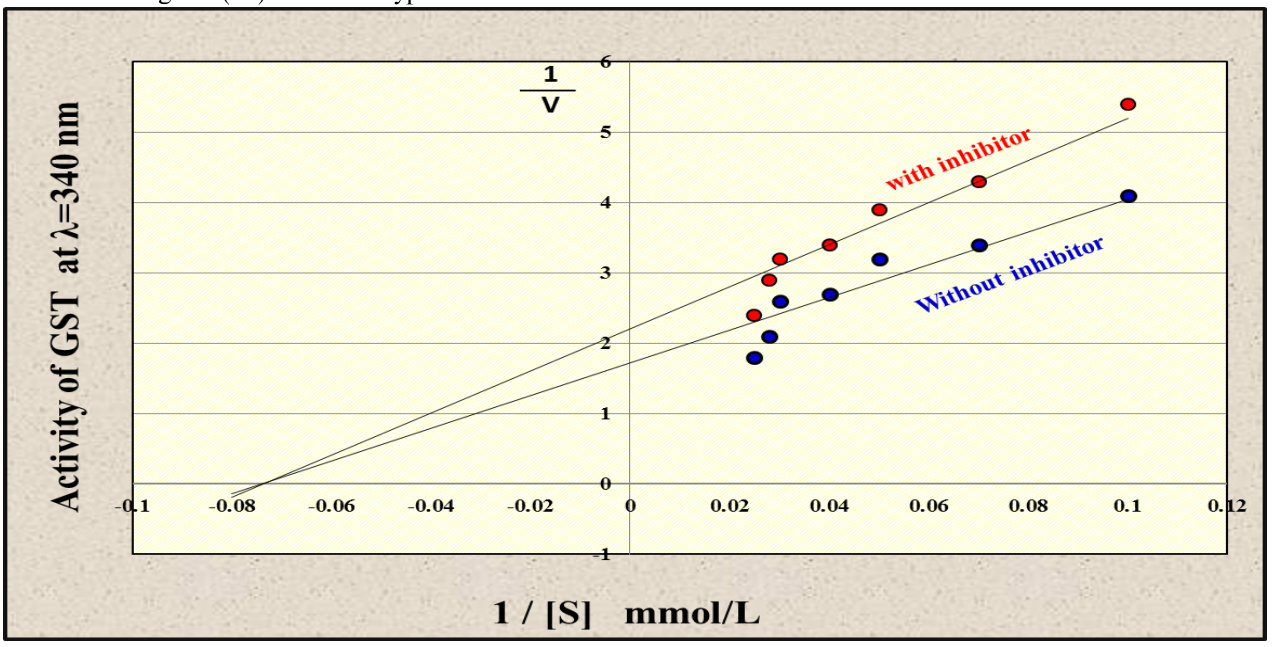

Figure (12) Drawing of Lineweaver-Burk with and without benzyl chloride inhibitor.

5.7- Optimal conditions for measuring the activity of the enzyme glutathione-S-transferase.

Upon completion of the study of the effect of factors affecting the activity of GST. The optimal conditions for the results of our study when purifying GST from blood serum were as shown in Table (13).

Table (13) Optimal conditions in the study to measure the activity of purified GST.

purified enzyme concentration	Phosphate buffer concentration	pH	Reaction Time	Temperature	Substrate concentration
20 mg/mL	0.12 mol/L	pH= 6.5	12 minutes	25°C	[S]=Km= 12.7

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