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PARTIAL PURIFICATION OF GLUTATHIONE S-TRANSFERASE FROM SERUM OF INFERTILE MEN

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Abstract
This study was carried out by partial purification of Glutathione S-transferase from patient's serum with infertile men by ion exchange and gel filtration chromatography sephadex G-100. The degree of enzyme purification was (24.06) fold, enzyme yield (46.18) and specific activity is (0.698) IU/mg. The kinetic studies of partially purified the enzyme technology demonstrated the maximum activity for GST was obtained using was (0.1) M of 1-chloro-2,4- dinitrobenzene (CDNB) as substrate, using lineweaver–burk plot, the Michaelis constant (K_m) and maximum velocity (V_{max}) were (0.010) mmol/L and (3.70) µmol/ min respectively. The optimum temperature was (27°C), pH (7.5) and optimization incubation time (25). The molecular weight of the partially purified enzyme was determined by a gel Electrophoresis method, in the presence of polyacrylamide gel and sodium Dodecyl Sulfate (SDS_PAGE) which showed that the approximate. The molecular weight was (24 KD).

Keywords: Glutathione S-transferase (GST), Purification, Optimum temperature, Ion exchange, Gel filtration chromatography.

Introduction

Glutathione (GSH), a tripeptide that is composed of glutamate, cysteine, and glycine it is found in low concentrations in most cells. GSH and several enzymes combine to form the glutathione system, which plays a crucial role in the regulation and prevents damage caused by ROS and RNS [1]. Its antioxidant activity is promoted by the cysteine sulphydryl group, enabling GSH participate in redox cycling [2]. Conjugation of GSH by the enzyme glutathione S-transferase (GST) to xenobiotic compounds yields nontoxic products, thereby effecting their detoxification [3,4].
Glutathione-S-transferases are usually composed of two identical subunits, each consisting of 199–244 amino acids [5]. Glutathione S-transferase has at least (7) isoenzymes: Alpha (α), Mu (µ), Omega (ω), Pi (π), Sigma (σ), Theta (θ), and Zeta (ζ), among which the correlation of GSTT1 and GSTM1 with events related to male infertility has been emphasised [6]. One of the defense systems against the damaging effects of oxidative stress in human semen are the GST, by catalyzing S-conjugation between the thiol group of GSH and electrophilic moiety in the hydrophobic and toxic substrate, and play a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH. It is known that GST activity is widely distributed in hepatic and extrahepatic tissues including the ovaries, testes and serum.

Although such enzymes operate as antioxidants in most mammalian cell types, sperm GST play a triple role:
(i) Cell detoxification: electrophilic compounds (EC) react with the reduced nucleophilic thiol group of GSH, in a reaction catalysed by GST, forming the reaction product (EC-SG) (preventing lipid membrane peroxidation and subsequent OS).
(ii) Cell signalling regulation: GST are involved in the regulation of signalling pathways, such as the JNK-C-jun pathway that regulates the apoptosis process. (involved in spermatogenesis and sperm capacitation).
(iii) Fertilisation: (since specific GST members are involved in sperm–oocyte binding and acceleration of sperm nuclear decondensation) [7].

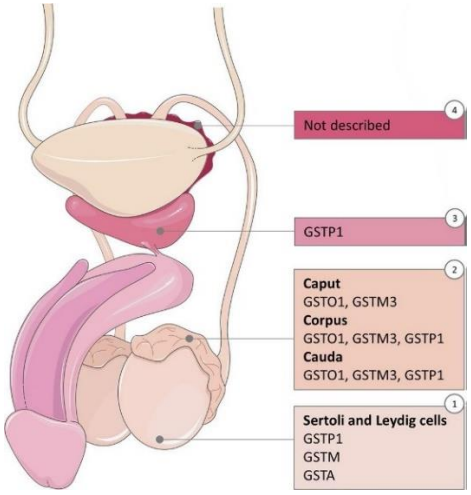

Figure (2): Proteomic annotation of GST in male reproductive tract [7].

Material and Methods

Determination of Glutathione S-transferase Activity Assay

A. principle

The activity of the Glutathione S-transferase GST(CDNB) were determined spectrophotometrically as described by Habig et al. with modifications. The enzyme Glutathione S-transferase catalyzes the binding of compounds that contain electrophilic groups, especially aromatic rings, such as the compound (1-Chloro-2,4-dinitrobenzene (CDNB)) with the thiol group (-SH) of reduced Glutathione (GSH). As in **Figure (3)** [8,9].

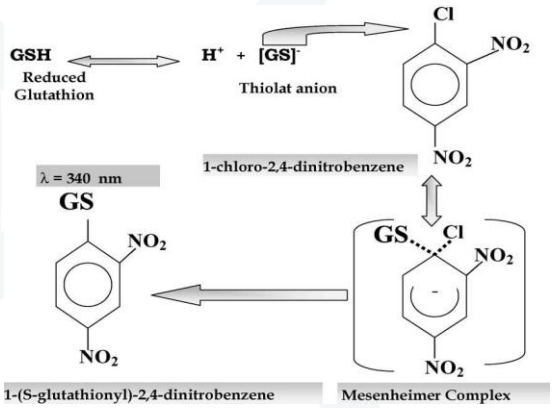

Figure (3): Mechanism of the glutathione conjugation to the CDNB [9].

B. Preparation of Reagents

- Preparation of **pH (6.5)**
- Preparation of Sodium Azide **GSH (9.2 mM)**
- Preparation of **CDNB (0.1M)**

C. Procedure

The working method for the determination of glutathione S-transferase activity is shown in the following table:

Reagent	Sample	Blank
pH	750 µl	750 µl
GSH solution	100 µl	100 µl
CDNB solution	10 µl	10 µl
De-lonized Water	-	50 µl
Sample	50 µl	-

The tubes were mixed, then the absorbance R1 was read at a wavelength of 340 nm for the sample and the plank, then the R2 was read after the fifth minute.

D. Calculation of Results

- Calculate **Δ R** for **Blank** and **Δ R** for **Samples**
- The Glutathione S-transferase activity (U/mL) was calculated according to the following equation:

Activity of GST = (Δ Abs Samples **-** Δ Abs of blank) × Dilution factor / (9.6 ×0.05)

Dilution factor = 5.41

Separation and Purification of Glutathione-S-transferase from Blood Serum of Male Infertiled Patients

The Glutathione-S-transferase (GST) was purified from blood serum samples of male infertiled patients who showed the highest percentage when measuring the concentration of the enzyme for them, taking into account that they were non-smokers and did not take medications three months before the sample collection period. The purification steps were carried out as follows**:**

1. Precipitation of Enzyme by Ammonium Sulfate

Ammonium sulfate $(NH_4)_2SO_4$ was added in its solid state to the blood serum and with full saturation (65%). by adding 9 g salt to 15 ml crude enzyme under cooled conditions, the addition of ammonium sulfate was gradual with continuous stirring with an electric stirrer for 60 minutes at a temperature of 4°C **Figure (4)**, and then the solution was left to settle for a period of (24 hours) at a temperature of 4°C (in the refrigerator). in order for the proteins to be completely precipitated. After that, the precipitate was separated from the clear liquid by centrifugation at a speed of (9000g) and for 40 minutes. After this process, one precipitate was obtained, which was dissolved in the least amount of distilled water. Then, according to the volume of the protein precipitate and the resulting clear liquid, the amount of protein was estimated according to the method provided by the company (BIOLABO), and the activity of GST was measured in The protein precipitate and the clear liquid

according to the method mentioned before the subsequent purification steps, then the precipitate and the clear liquid were kept at a temperature of -20°C until further use [10].

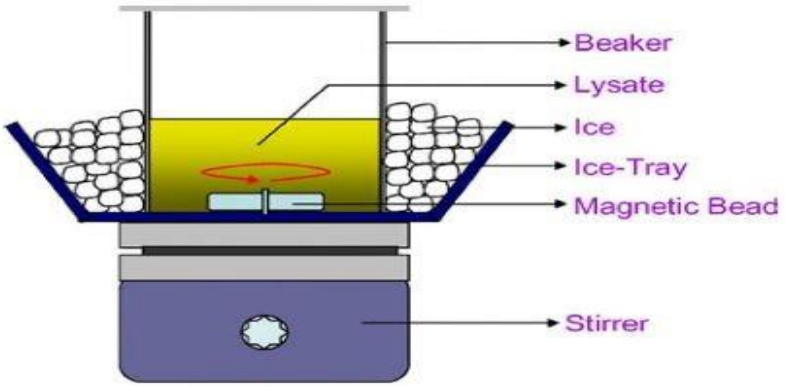

Figure (4): Protein precipitation using ammonium sulfate [11].

2. Dialysis

Dialysis is one of the oldest protein purification techniques. This step is done by placing the protein solution (clear liquid) produced from step (1) in a dialysis bag. we put the protein solution in the bag that was previously soaked and tied tightly from the bottom. After placing the sample in the bag, the top of the bag was tied tightly with a thread, then washed with distilled water and immersed in a volumetric container containing 1 liter of distilled water containing 10 mM Tris-HCl pH 7.2 buffer solution. The membrane sorting process was carried out with magnetic stirring, and the membrane sorting process continued for 24 hours at 4°C, taking into account the replacement of the membrane sorting solution every 4 hours. As shown in the **Figure (5)**.

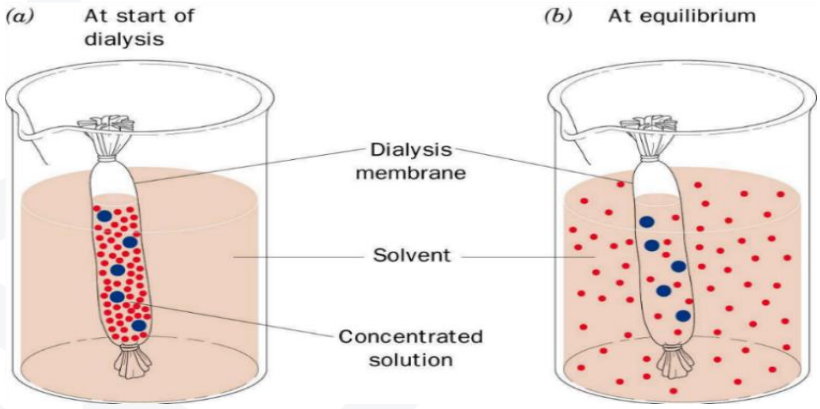

Figure (5): Membrane screening process.

After completing the membrane sorting process, the final volume of the resulting solution was calculated, the amount of protein was estimated, as well as the enzyme activity (GST) was measured in the solution produced by the membrane sorting.

3. Gel Filtration Chromatography

The gel filtration technique is one of the important techniques in the field of biochemistry, and it is used to separate compounds based on the size of their molecules or their molecular weights. Proteins with large molecular weights do not permeate through the gel, but rather move out of the gel layer with the sequentially displaced solvent, which is often distilled water or a dilute buffer solution.

Therefore, large molecules are filtered out first during the separation, while small molecules can enter into the gel granules. So, you finally apply. The fractions are collected from the gel filtrate using a fraction collector and the volume of distilled water (buffer) is calculated to displace each protein from the separation column. The protein substances were isolated by reading the absorbance of the separated parts using the readymade kit to measure the level of total protein. The gel used for purification is Sephadex G-100 [12]. as well as the enzyme activity (GST) was measured in the solution produced.

Electrophoresis

The movement of charged molecules in an electric field is known as electrophoresis. It is a fast and widely used technique for determining protein molecular weight and separating biological molecules such as nucleic acid, nucleotides, amino acids, and protein [13].

Determine the molecular weight of Glutathione S-transferase using SDS-PAGE

Laemmli's method was used to prepare the separation gel in determining the molecular weight of GST with some modulations [14].

Result and discussion

Purification of Glutathione S-transferase from Serum of person's male infertility and studying their kinetic properties and estimating their molecular weights

The GST were partial purified from the blood serum taken from sterile men (after taking all the questionnaire information from them). These samples were chosen over the others because these samples suffer from high amounts of oxidation. Several purification steps were used from including salting out, dialysis to prepare the sample for injection in the Sephadix G-100 gel filtration technique in order to later estimate the molecular weight of the enzyme using the top peak obtained during the gel filtering technique and to maintain the optimal conditions for it. The enzyme was obtained with a good purity ratio of 24 **Table (1)**. The following describes the stages of purification**.**

1. Salting out

Salting-out is a highly beneficial method for assisting in protein purification, Ammonium sulfate has already been widely used in salting out for protein purification as it is very water soluble. As ammonium sulfate dissolves, a substantial amount of water is bound to each ammonium sulfate molecule, which has no adverse influence on enzyme activity. When a result, as the amount of ammonium sulfate molecules in solution rises, there is less water available to interact with any proteins present [15].

Because of these characteristics that the ammonium sulfate salt contains, it was used to precipitate blood serum proteins, as the precipitation was done using a saturation ratio equal to 65%. The high specific activity is concentrated in the precipitate part, the specific activity of which is (0.023) enzyme units / mg of protein, and the number of purification times is by (0.793) as compared to the filtrate, so the filtrate is neglected and the purification process has been completed using the precipitate in the dialysis **(Table 1)**.

3.4.2. Dialysis

Dialysis is a method of separating molecules based on their size using semipermeable membranes with pores smaller than macromolecular diameters. Tiny molecules, such as solvents, salts, and small metabolites, can diffuse through the membrane through these pores, whereas larger molecules cannot [16].

Our results presented in **Table (1)** for the GST enzyme prove increase in the activity of GST due to the removal of protein compounds with small partial weights (less than 14000 Daltons) [17], which leads to an increase in the specific activity that many of proteins in Dialysis this result caused elevated in specific activity for partial purification GST enzyme (0.099 unit / mg) and the fold of purification were increase to (3.41) as up to the start of the enzyme separation.

Table (1): Steps of Glutathione S-transferase Purification from Serum of Male Fertility

Purification steps	Volume (mL)	Activity *(IU/mL)	Total Activity (U)	Protein Conc. (mg/mL)	Specific Activity (U/mg of protein)	Recovery Yield %	Folds	Total protein mg/mL
Crude serum GST	15	2.017	30.25	68	0.029	100	1	1020
Precipitation with ammonium sulfate 65%	13	1.341	17.47	57	0.023	57.61	0.793	741
Dialysis	12	1.995	23.8	20	0.099	78.6	3.41	240
Ion exchange (DAEA-Cellulose)	5	3.088	15.44	13	0.237	51.04	8.17	65
Gel Filtration	5	2.795	13.97	4	0.698	46.18	24.06	20

*Enzyme unit: It is the amount of glutathione S-transferase that converts one micromole of 1-chloro-2,4-dinitrobenzene into product per minute under the specified measurement conditions.

DEAE-Cellulose Ion Exchange Chromatography

Selective adsorption and elution of proteins from the polydextran derivatives anion exchange diethylaminoethyl-cellulose (DEAE-cellulose) have also been extremely successful for extensive and rapid purification [16]. The results indicated in **Figure (6)** and **Table (1)**, where the results of ion exchange show the emergence of one high-activity protein peak of GST and the specific activity of the enzyme is (0.237) enzyme unit/mg and the fold of purification were increase to (8.17) times with **Table (1)**.

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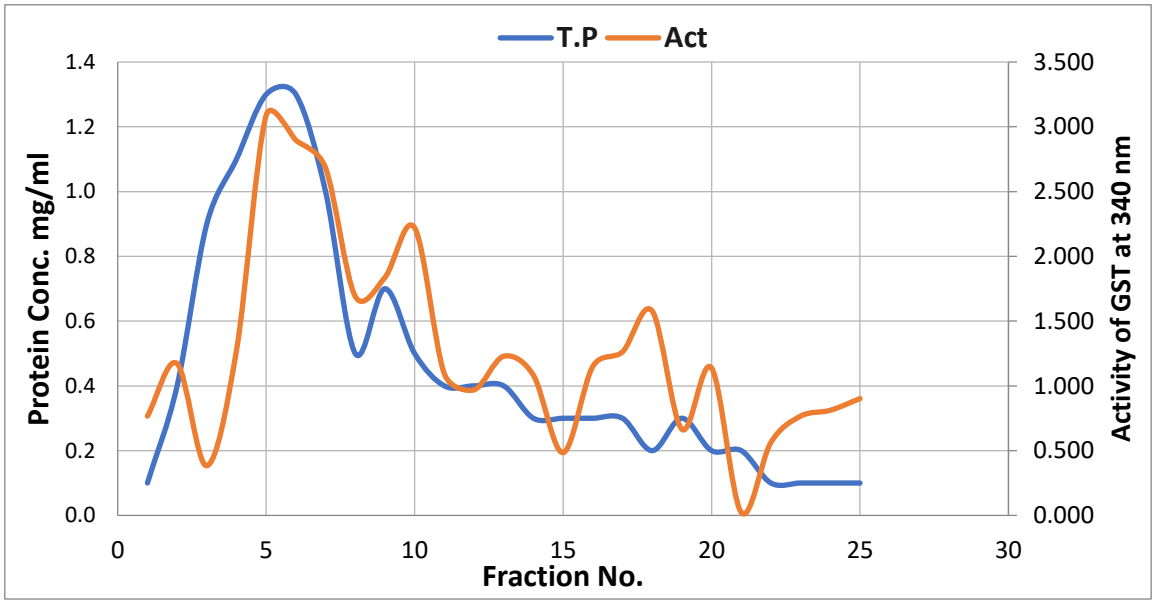

Figure (6): Ion exchange chromatography of GST by DEAE-cellulose

Gel Filtration Chromatography

With this chromatographic method, enzymes, proteins, hormones, and nucleic acids are fractionated, separated, and purified in solution. In this research, this technique was used to isolate and purify GST enzyme. This was achieved through ammonium sulfate precipitation and purification of it using a Sephadex G100 column and **Figure (7)** below show our results. It is used for separating the proteinous compounds obtained from the ion exchange process using a separation column containing Sephadex G-100. The results of gel filtration when passing the protein peak resulting from ion exchange indicate the appearance of a single protein peak, and its elution volume is (5 mL) (**Figure 7**). As indicated in **Table (1)**, the activity of purified GST enzyme reached (2.795 unit/mL), Upon tracking the activity of GST enzyme, it is found that the peak gives specific activity of the enzyme, reaching (0.698 unit/mg) by (24.06) folds and enzyme yields(46.18%), **Table (1)**.

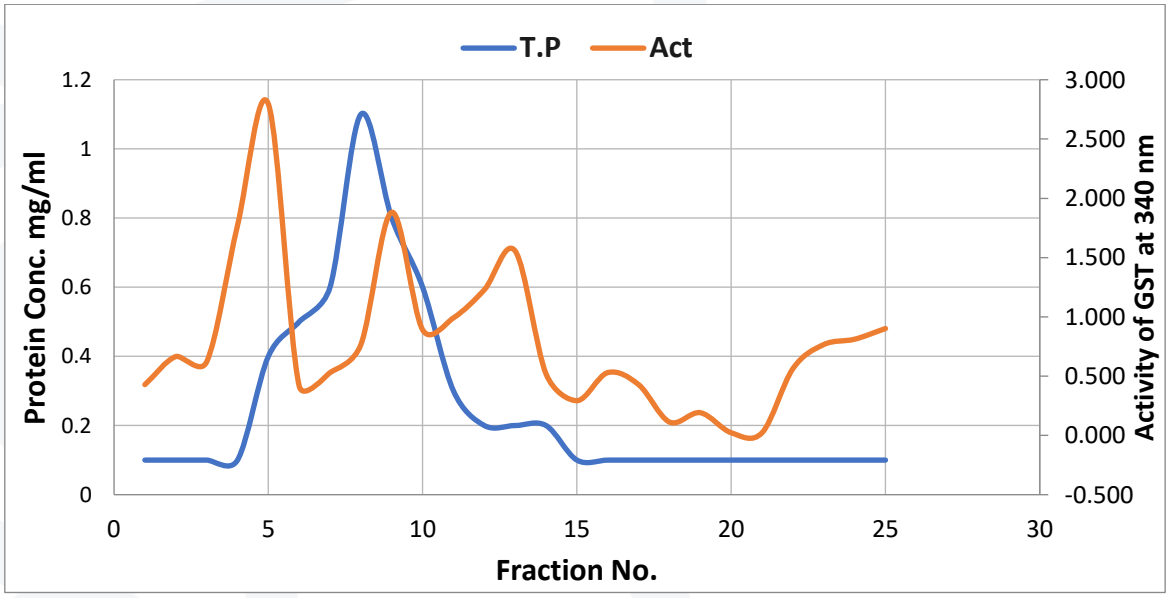

Figure (7): Gel filtration chromatography of GST by Sephadex G-100

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Determination of the approximate molecular weight of Partial Purified Glutathione S-transferase Using SDS-PAGE Electrophoresis

The molecular weight of GST which it partially purified from human blood serum of persons who infertiled men has been approximated by electrophoresis on SDS-PAGE using standard curve of logarithm of molecular weight versus relative migration of ten standard ladders between (17-250 kDa). Where the enzyme is treated with SDS to disassemble the protein, giving chains of variable sizes surrounded by negatively charged of SDS molecules, as it removes the original charge of the protein and these chains move by electrical migration, The distance traveled by proteins to the and (positive electrode) depending on the ratio of the charge to the mass, The movement of enzymes or proteins during electrical migration depends on several factors, including the molecular weight when other factors such as charge and electric current are fixed. The proteins will therefore move at various speeds based on their molecular weight [18]. as shown in **Figure (8)** below, molecular weight was calculated by estimating approximately about (24.356) Dalton for GST, resulting from comparison between enzyme bundle and standards bundle using the standard curve shown in **Table (2)** and **Figure (9**)

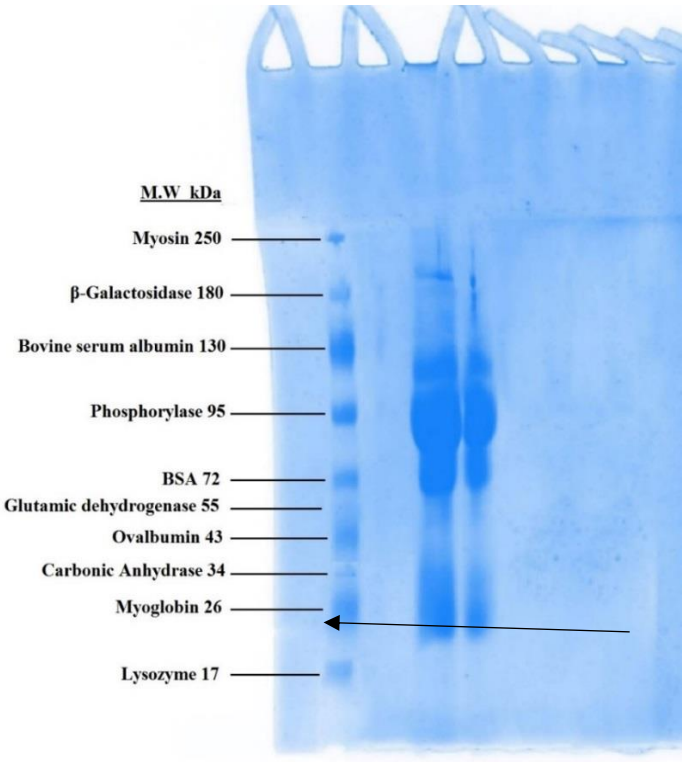

Figure (8): SDS-PAGE analysis bands of purified GST and the standard ladder proteins

Table (2): Standard Proteins used in Determination of Molecular Weight of Glutathione S-transferase by SDS-PAGE

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MW	Log	Cm	RF
250	2.4	1.3	0.12
180	2.26	2.2	0.2
130	2.11	3.1	0.28
95	1.98	4.2	0.38
72	1.86	5.3	0.48
55	1.74	5.7	0.52
43	1.63	6.2	0.56
34	1.53	6.8	0.62
26	1.41	7.4	0.67
17	1.23	8.5	0.77

Figure blow shown standard curve determination of molecular weight

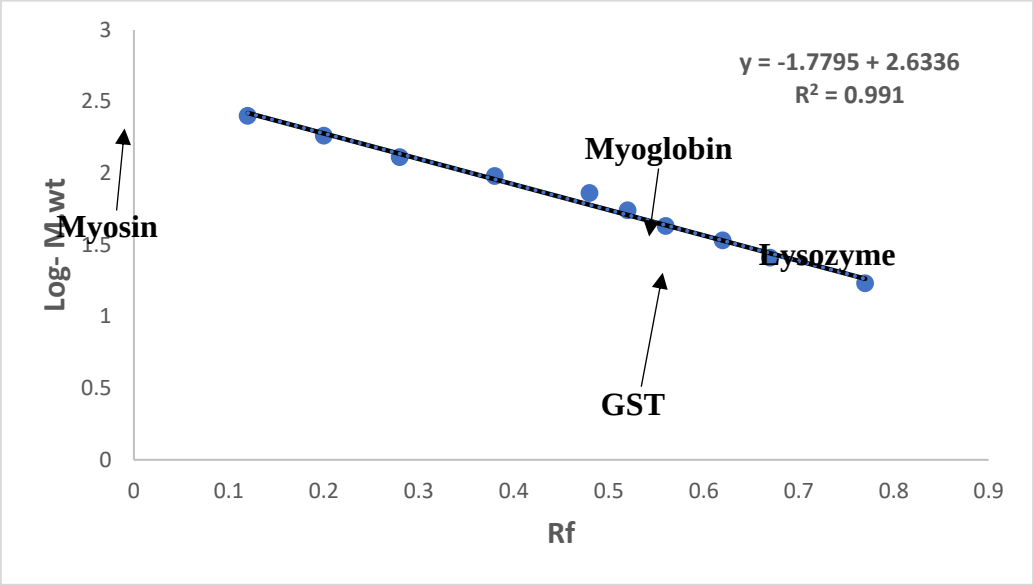

Figure (9): Determination of molecular weight of partial purified GST by SDS-PAGE

The analysis of purified GST using polyacrylamide gel electrophoresis with SDS revealed that there was no detectable contamination, as it represented just one distinct band and the molecular weight of the subunit about (24 kDa) shown in **Figure (8)**. Previous studies showed agreement with our result and detected that purified GST gives at (22 – 29 kDa)

Study of optimum conditions for Glutathione S-transferase Partially Purified from infertiled men

1. Effect of Different Substrate Concentrations on Enzyme Activity with Determination of Michaelis-Menten Constant K_m and Maximum Velocity V_{max}

In order to find out the relationship between the GST and the concentration of the substrate 1-Chloro-2,4-dinitrobenzene (CDNB), the enzyme activity was measured in the presence of different concentrations of the substrate ranging between (0.1-0.0031). **Figure (10)** shows that increasing the concentration of the substrate increases the speed of the enzymatic reaction until it reaches a

value after which no increase in the speed of the enzymatic reaction and thus can determine the concentration of the substrate required to obtain the velocity maximum V_{max}

This is identical to what was proven by the two scientists, Michaels and Menten, as they explained that the use of low concentrations of the substrate makes the active sites of the enzyme not saturated with the substrate, and therefore the speed of the enzymatic reaction depends on the concentration of the substrate, but when the concentration of the substrate used is greatly increased so that the active sites of the enzyme become saturated With the substrate, the rate of the enzymatic reaction is not dependent on the concentration of the substrate [19]. **Figure (10)** shows that the saturation of the enzyme GST with the substrate was at a concentration of (0.1M) from CDNB, This concentration was adopted in subsequent experiments because it gives the V_{max}, and using the Lineweaver-Burk plot, it was found that the value of the velocity maximum V_{max} and constant Michaelis K_m for GST is (3.70 µmol/min) and (0.010 mmol/L). respectively and as shown in the **Figure (11)**.

The K_m value is an important constant in enzyme studies, it is the substrate concentration which gives half of the maximum velocity, so it is an indicator of the enzyme affinity toward its substrate. An enzyme with high affinity for its substrate has low K_m value, the value of K_m establishes an approximate value for the intercellular level of the substrate, While V_{max} refers to the amount of active enzyme present [20].

The **Figure (11)** show a K_m and V_{max} value from Line weaver–Burk plot.

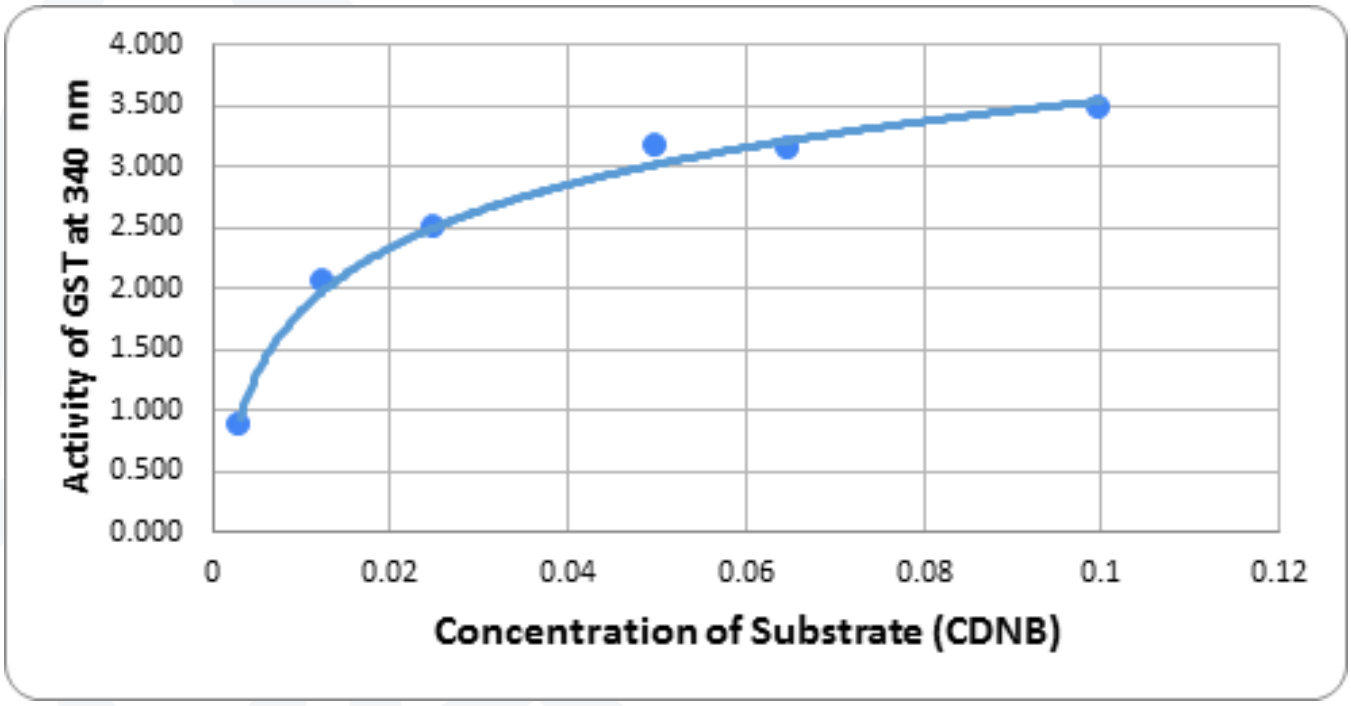

Figure (10): Michaelis–Menten plot show effect of substrate concentration on GST activity

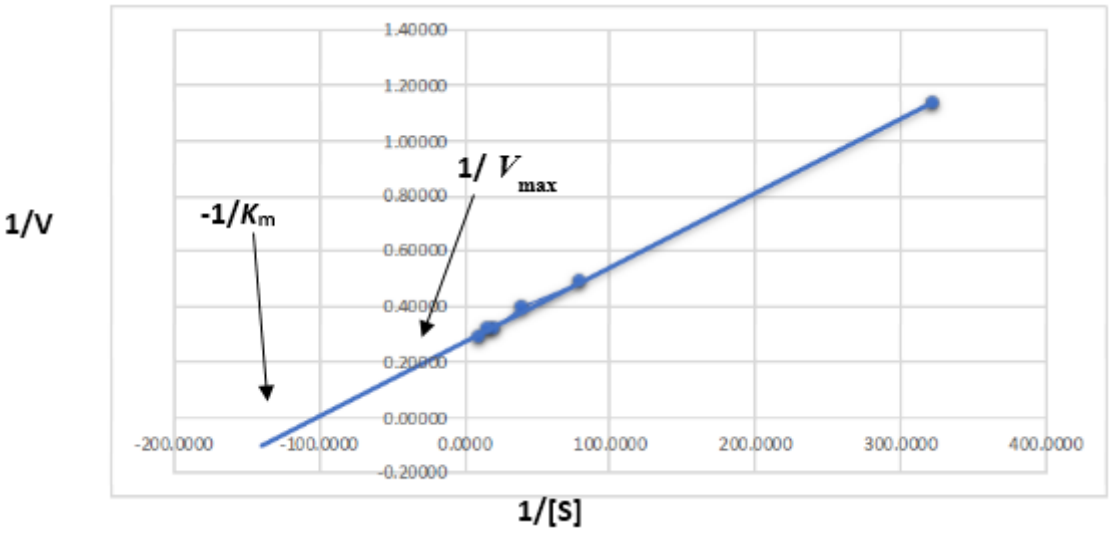

Figure (11): Line Weaver–Burk plot for partially purified GST

2. Effect of pH on Enzyme Activity

The various effect of pH on the GST enzyme activity was studied as shown in **Figure (12).** For purified GST, the maximum enzyme activity was found at (pH 7.5). The different nature of the enzyme, its chemical makeup, and the presence of multiple ionic groups carried by the enzyme all have an impact on how the pH affects the activity of the enzyme. Enzymes function best at an ideal pH because they are extremely sensitive to changes in hydrogen ion concentration (H^+) [21].

pH has an impact on how quickly enzyme-catalyzed reactions proceed. Also, it helps keep enzymes stable. Although other shapes have been seen, enzymes have an optimum pH and are frequently produced by enzymes give bell-shaped velocity versus pH graphs [22]. Each enzyme has a specific pH at which the enzyme shows its highest activity called the optimum pH. When their pH is too high or too low, most enzymes entirely lose their action. Enzyme activity may be decreased at low pH due to the pH environment of the reaction in the ionic groups of the active site, modifications in the ionic state of the substrate, or complex enzyme-substrate interactions at substrate concentrations larger than Km. If the substrate concentration is low, the enzyme will be the deciding factor since pH has an impact on the tertiary structure of the enzyme. As a result, at high pH levels, the enzyme's activity may be irreversibly denaturized [23].

There are many studies in the effect of pH on GST activity. The studies showed that the optimum pH for GST was range from (6.4 - 8).

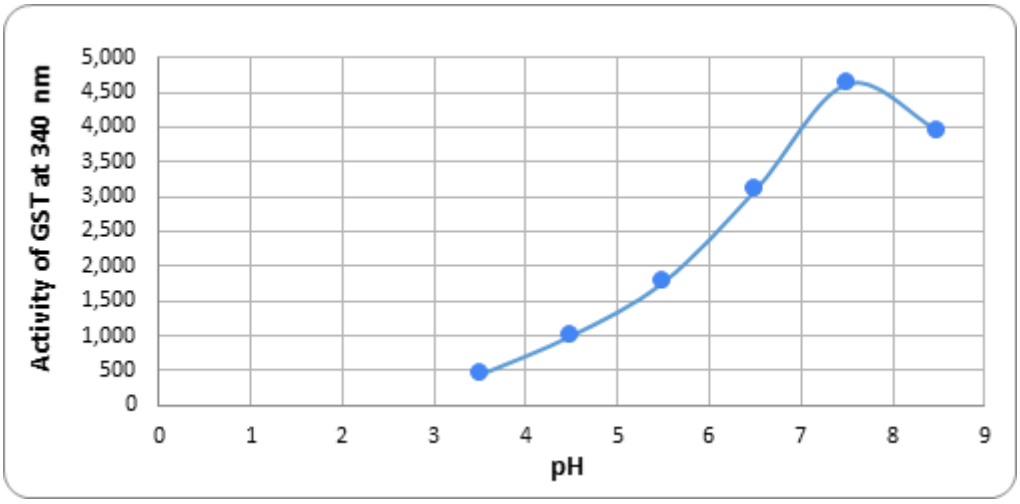

Figure (12): Effect of pH on GST activity

3. Effect of Temperature on Enzyme Activity

Different temperatures were tested to determine optimal temperature for the GST enzyme activity (7, 17, 27, 37, 47, and 57 °C). The optimal temperature was (27°C), and the results showed that increasing the temperature caused an increase in GST activity, followed by a decrease in GST activity, as shown in **Figures (13)**.

It is well knowledge that as the temperature rises, an enzyme reaction's speed climbs until it reaches its ideal temperature, at which point it begins to slow down. This is due to the denaturation or damage to the enzyme molecule. High temperatures increase the kinetic energy of molecules, which leads to a breakdown in the binding between active amino acids and the loss of enzyme action. The drop is explained by a change in the engineering arrangement of the enzyme. High temperatures have an impact on the ionization of groups on the surface of the enzyme and its substrate. Enzymes are complex protein molecules whose structure affects their catalytic activity. Most of an enzyme's non-covalent connections maintain the enzyme's tertiary structure, but when a molecule absorbs too much energy, the tertiary structure is broken and the enzyme is denatured, losing its function [24]. At (17°C) glutathione S-transferase activity decreases because to insufficient energy that required to perform enzyme substrate complex.

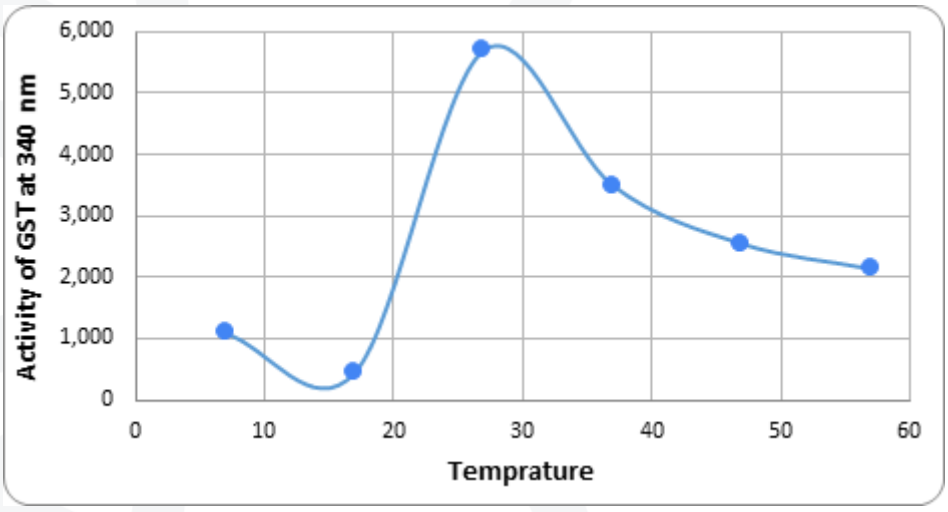

Figure (13): Effect of temperature on GST activity

4. Effect of Reaction Time on Enzyme Activity

The reaction time is studied to choose the optimal time period to give the highest possible activity of the enzyme. Where the activity of the enzyme was measured at (0, 5, 10, 15, 20 and 25) minutes. The results shown in **Figure (14)** indicate that the activity of the enzyme increases with the increase in the reaction time, so the 5th minute was adopted when making all subsequent measurements.

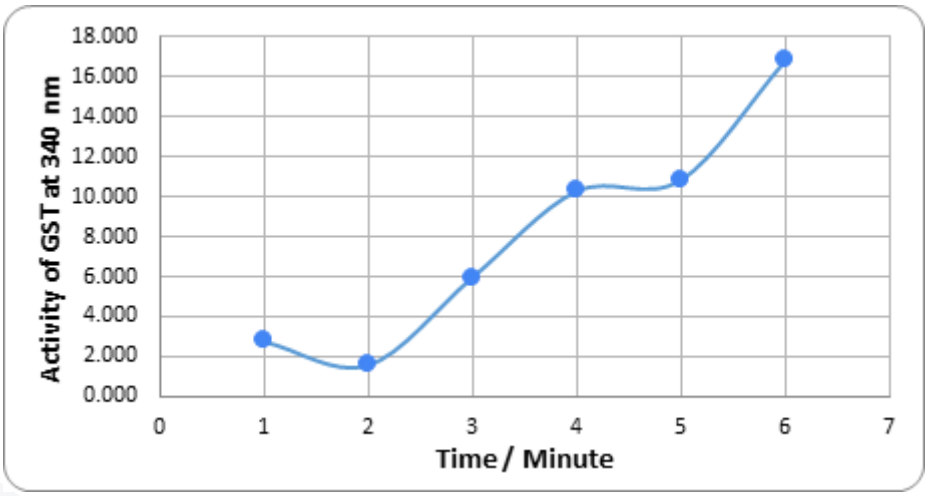

Figure (14): Optimization incubation time of GST

Summary of Optimal Conditions of Partially Purified Glutathione S-transferase from Serum the Infertiled Men

The optimum conditions for measuring the activity of GST enzyme purified from serum the infertiled men are shown in the following **Table (3)**.

Table (3): Optimum conditions for the partially purified Glutathione S-transferase

Substrate Conc. (M)	pH	Temp. (°C)	Time (min)	K_m (mmol/L)	V_{max} (µmol/min)	M.wt
0.1	7.5	27	25	0.010	3.70	24.356

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تنقية جزئية للكلوتاثيون S-ترانسفيراز من مصل الرجال المصابين بالعقم

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الملخص

أجريت هذه الدراسة عن طريق التنقية الجزئية لإنزيم الكلوتاثيون S-ترانسفيراز من مصل المرضى المصابين بالعقم باستخدام تقنيتي التبادل الايوني وكروماتوغرافيا الترشيح الهلامي سيفاديكس G-100. وكانت درجة تنقية الإنزيم (24.06) ضعفاً، وإنتاجية الإنزيم (46.18) والفعالية النوعية (0.698) وحدة دولية/ملغ. أظهرت الدراسات الحركية لتقنية الإنزيم المنقى جزئيًا أن الحد الأقصى لنشاط (GST) تم الحصول عليه باستخدام (0.1) مولاري من (1-chloro-2,4-dinitrobenzene (CDNB)) كركيزة، باستخدام مخطط لينوفر –بيرك، ثابت ميكاليس –منتن (K_m) والسرعة القصوى (V_{max}) كانت (0.010) ملي مول/لتر و(3.70) ميكرومول/دقيقة على التوالي. وكانت درجة الحرارة المثلى لعمل الانزيم هي (27 درجة مئوية)، والاس الهيدروجيني الامثل (7.5) ومدة الحضانة الأمثل (25). تم تقدير الوزن الجزيئي للإنزيم المنقى من المرضى بطريقة الترحيل الكهربائي على هلام متعدد الأكريل أمايد بوجود كبريتات دوديكايل الصوديوم (SDS_PAGE) والتي أظهرت أن الوزن الجزيئي تقريبي للأنزيم (24 كيلو دالتون).

الكلمات المفتاحية: الكلوتاثيون S-ترانسفيراز (GST)، تنقية، درجة الحرارة المثلى، التبادل الايوني، كروماتوغرافيا الترشيح الهلامي