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# Partial Separation and Some Kinetic studies of Glutathione S-Transferrase (GST) in Human Serum

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**ABSTRACT**

This study attempts to isolate the GST from serum of human in city center of Mosul. Three proteinous components had been isolated by gel filtration chromatography from the precipitate produced by ammonium sulfate. It was found that only the second peak had a high activity for GST. The apparent molecular weight of the isolated GST using gel filtration chromatography and SDS-PAGE was(24,717) and (24,807) dalton respectively.

Maximum activity for GST was obtained using (22.463) mmol/l of 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, phosphate buffer (0.11 M/l) as a buffer at pH (6.4) for (12) minutes in incubation at (25)°C. Using lineweaver–burk plot, the maximum velocity ($V_{max}$) and Michaelis constant ($K_m$) were (0.625) µmol/ min and (10.526) mmol/l respectively. Benzyl chloride inhibition on the GST activity were investigated and showed noncompetitive inhibition at a concentration of (30) mmol/l.

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# فصل جزئي ودراسة بعض الصفات الحركية لأنزيم كلوتاثايون S- ترانسفيريز من مصل الإنسان

**الملخص**

الدراسة حاولت عزل أنزيم كلوتاثايون S-ترانسفيريز من المصل لشخص يعيش في مركز مدينة الموصل.إذ تم فصل ثلاث حزم رئيسة من البروتينات بتقنية الترشيح الهلامي للراسب ألبروتيني الناتج من الترسيب بـ كبريتـات الامونيـوم، وأظهـرت الحزمـة الثانيـة فعاليـة عاليـة لأنـزيم كلوتاثـايون S-ترانسفيريز. قدر الوزن الجزيئي للأنزيم باستخدام تقنيتي الترشيح الهلامي والهجرة الكهربائية، ووجد أنه بحدود (24,717) و(24,807) دالتون على التوالي.

تم أيجاد الظروف المثالية لفعالية أنزيم كلوتاثايون S-ترانـسفيريز وكانـت كـالاتي: اسـتخدام (22.463) ملي مول/لتر من مادة ((1-chloro-2,4-dinitrobenzene (CDNB) كمادة أسـاس و(0.11) مول/لتر من المحلول المـنظم (Phosphate buffer) عنـد أس هيـدروجيني (6.4) ودرجـة حـرارة

تحضين(25) م بفترة زمنية مقدارها (12) دقيقة. وباستخدام رسم لاين ويفر– برك تم أيجاد قيمة السرعة القصوى ($V_{max}$) وثابت مكيلس ($K_m$) وكانتا مساوية لـ (0.625 مايكرومول/دقيقة) و (10.526) ملـي مول/لتر على التوالي. كذلك تمت دراسة تأثير المثبط بنزايل كلورايد على فعالية أنزيم كلوتاثايون S- ترانسفيريز، وبينت النتائج أن التثبيط غير تنافسي عند التركيز (30) ملي مول/لتر.

## INTRODUCTION

The glutathione S-transferases (GSTs) (EC 2.5.1.18) are dimeric enzyme found in mammals, insects, plants, and microbes that catalyze nucleophilic attack by the thiolate anion of GSH at electrophilic centers of hydrophobic molecules (Mannervik and Danielson,1988). GST contribute in conjugating drugs, poisons, and other compounds with reduced glutathione and neutralize the electrophilic side and dissolution in the aqueous cellular and extracellular media, to be excreted, out of the body (Mannervik, 1996; Bello et al.,2001;Raza *et al*.,2002; Baraczyk-Kuoma et al.,2004).

Role of GST in detoxification of xenobiotics has been classified into three distinct phases. Phases I and II involve the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble less toxic metabolite, which is then eliminated more easily from the cell (Cedeberg, 2001; Chauhan,2003).

The mammalian GST are localized in both the cytoplasm and the endoplasmic reticulum however, the cytosolic glutathione activities are usually 5-40 times greater than the microsomal activity (Chauhan,2003).

GST catalyze the general reaction:

$$GSH + R\text{-}X \longrightarrow GSR + HX$$

The main function of this enzyme is to bring the substrate into close proximity with GSH by binding both GSH and electrophilic substrate to the active site of the protein , and activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH in the electrophilic substrate (R-X). GST catalyze various reactions like nucleophilic aromatic substitution reactions, reversible Michael additions to α, β-unsaturated aldehydes and ketones, isomerizations, epoxide ring openings, and for few GST, peroxidase reactions (Chauhan,2003). The electrophilic functional center of the substrates can be carbon, nitrogen or sulfur(Stipanuk,2000)..

Although endogenous substrates for mammalian GST have not been clearly defined, there is evidence that α-GST protect against oxidative stress by detoxifying reactive products generated by lipid peroxidation (Singhal et al., 1992). This class, which is very heterogeneous in primary structure, also includes GST from microbes, insects, and mammals (Buetler and Eaton, 1992; Pemble and Taylor, 1992).

The aim of the research is to provide a detailed study of glutathione S-transferase (GST) involving isolation, characterization and purification from human serum in normal using different biochemical techniques.

## MATERIALS AND METHODS

### Assay the activity of serum Glutathione S-Transferase (GST)

**Principle**

The activity was determined by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al.,1974), Figure(1).

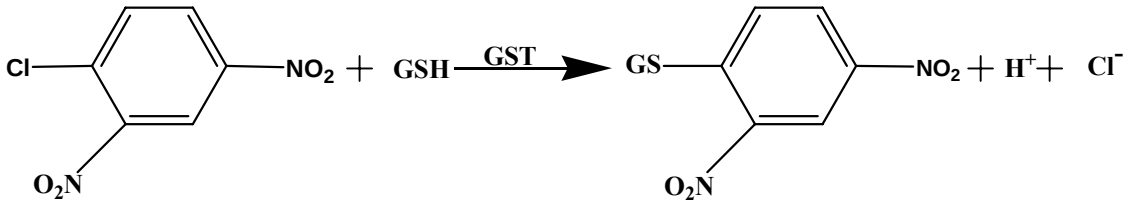


Figure1: Reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione in the presence of glutathione S-transferase enzyme,(Yildiz and Kuman,2004).

The conjugation of (CDNB) with GSH proceeds by nucleophilic aromatic substitution of chlorine by thiol group of GSH, producing a dinitrophenyl thioether and chloride ion, measured absorbance at 340 nm.

**Reagents:**

1.Glutathione solution(29.93 mM).
2. 1-chloro-2,4-dinitrobenzene (CDNB)(22.463 mM).
3.Phosphate buffer solution,pH 6.25.

**Procedure:**

1.Twe sets of tubes were prepared as follow:

| Reagents | Sample | Blank |
|---|---|---|
| Phosphate buffer pH 6.25 | 2.7 ml | 2.7 ml |
| Serum | 100µl | - |
| Distilled water | - | 100µl |
| CDNB solution | 100µl | 100µl |
| After 3 minutes add GSH solution | 100µl | 100µl |

2.Then, all tubes were mixed at room temperature and the absorbance after each 1 minutes for 10 minutes was read absorbance at 340 nm by using spectrophotometer.

**Calculation:**

Enzyme activity was expressed as (U/L) is calculated according to the following equation:

$$\text{Activity of GST (U/L)} = \frac{\Delta A \times V_t \times 1000}{\varepsilon. \times V_s \times d}$$

Where:

ΔA: the difference between the first absorbance at 1 st. minute and absorbance at tenth minute.

$V_t$: the total volume.

$V_s$: the sample volume.

ε.: extinction coefficient (9.6 $mM^{-1}$).
d: light path (1 cm).

$$\text{Activity of GST (U/L)} = \frac{\Delta A \times 3 \times 1000}{9.6 \times 0.1 \times 1}$$

**Purification of Glutathione S-Transferase (GST) from Human Serum.**

The method given here has yielded an enzyme preparation acceptable for human serum. All steps were performed at 4 $^{o}$C unless stated otherwise.

**Step I: Ammonium Sulfate Fractionation**

A human fresh serum was obtained from one normal male person (age: 32 year) and protein was precipitated using ammonium sulfate $(NH_4)_2SO_4$ saturation or protein salting out (Dixon and Webb, 1961). Solid ammonium sulfate (19.5)gm per (26 ml) human serum was added with stirring to the enzyme extract and the mixture was stirred until all of the ammonium sulfate had dissolved; the suspension was centrifuged at 10,000 Xg for (30) min. The protein in precipitate and supernatant are determined using the modified Lowry method (Schacterle and Pollack, 1973), and the GST is determined in each fraction (Habig et al.,1974).

**Step II: Dialysis**

Dialysis was made using a semipermeable cellophane dialysis membrane with M.wt. cut off (<10000) dalton.

The dialysis sac containing the suspension in (Step I) was dialyzed against 0.015M phosphate buffer, pH 6.25.The solution was stirred with a magnetic stirrer overnight at (4) $^{o}$C. The buffer was changed twice during dialysis (Robyt and White, 1987). The protein of the dialyzed enzyme was estimated by modified Lowry method (Schacterle and Pollack, 1973).

**Step III: Gel Filtration Chromatography**

The Sephadex gel G-75 supplied as a powder was suspended in adequate distilled water so that when it was stirred incorporated air bubbles that escape rapidly to the surface. It was then allowed to swell for 72 hours at 20 $^{o}$C in a complete swelling. This procedure was used for column packing and sample application (Robyt and White, 1987).

In the present study, the column of dimension 2×100 cm which contained a gel Sephadex G-75 to height of (95) cm. The exclusion limit for this type of the gel is (70,000) dalton (Robyt and White, 1987). Depending on the volume of this column which was 200 ml, it was packed with a slurry of the gel in water (Plummer, 1978).

A concentrated sample (2) ml of the proteinaceous material, which was obtained in (Step II), was applied on the top of a bed Sephadex G-75, followed by deionized water.

Elution of the proteinaceous materials was carried out at a flow rate (52.3)ml/ hour with a definite time (4) min, using deionized water, as eluant, The fractions were collected using a fraction collector apparatus which is worked on minute system. The proteinaceous compounds in each fraction collected were detected by following the absorbance at wave length (280) nm using UV/Visible Spectrophotometer. Peak was combined separately from the plot of an absorbance versus elution volumes and GST was determined in each fraction (Habig et al.,1974).

**Step IV: Freeze-Dryer (Lyophilization) Technique**

The enzyme fraction which was obtained from gel filtration was dried using a freeze-dryer (lyophilization) technique to obtain a powder or a concentrated protein. The enzyme was kept in a deep freeze at (-20)$^{o}$C in a tight sample tube to be used in further investigations.

**Step V: Electrophoresis**

Only one sample can be run per gel each tube, (Step IV) which was applied on Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using disc electrophoresis unit quick fit instrumentation (Laemmli, 1970).

## RESULTS AND DISCUSSION

**Enzyme Isolation**

The results predicted that the enzyme activity was found in the (75)% of a saturation ammonium sulfate precipitates (Habig et al.,1974).

As shown in Table (1), the specific activity was increased after dialysis. This might be due to the removal of the small molecules and increasing the purification of GST.

Table 1: Partial purification steps of glutathione S-transferase (GST)from human serum.

| Purification stage | Volume taken (ml) | Total protein mg/ml | Activity U*/ml | Total activity (U) | Sp.activity U/mg protein | Folds of Purification | Recovery % |
|---|---|---|---|---|---|---|---|
| **Serum** | 26 | 76.431 | 4.7 | 122.2 | 1.598 | 1.0 | 100 |
| **Precipitate by $(NH_4)_2SO_4$** | 21.5 | 61.154 | 5.17 | 111.155 | 1.82 | 1.1 | 91 |
| **Supernatant** | 8.0 | 15.192 | 1.27 | 10.16 | 0.67 | 0.4 | 8 |
| **Dialysis** | 24.4 | 47.056 | 4.45 | 108.58 | 2.31 | 1.4 | 89 |
| **Sephadex G-75 (Fractions)** | | | | | | | |
| **Peak A** | 52.45 | 1.172 | 0.38 | 19.931 | 17.0 | 10.6 | 16 |
| **Peak B** | 34.77 | 0.737 | 1.74 | 60.5 | 82.1 | 53.2 | 50 |
| **Peak C** | 32.75 | 1.254 | 0.82 | 26.855 | 21.4 | 13.4 | 22 |

U*: a mount of glutathione S-transferase (GST) catalyzing the formation of one micromole of product per min under of optimum conditions.

**Gel Filtration Separations**

This technique was applied to separate the protein as a source of enzyme, which was obtained after dialysis using a column containing sephadex G-75 gel as shown in((step III (A)). The result (Figure 2) indicated that there were mainly three peaks, similar results were published by others( Goughlin and Hall,2002). The elution volume of peak (A) was (107.95) ml, while the elution volume of peak (B) was (188.8) ml and the elution volume of peak (C) was (218.45 ) ml. The specific activity of the enzyme peak (B) was (53.2) folds of that in initial extract Table (1). Peak (A) and (C) were neglected for the time, because of their low activity.

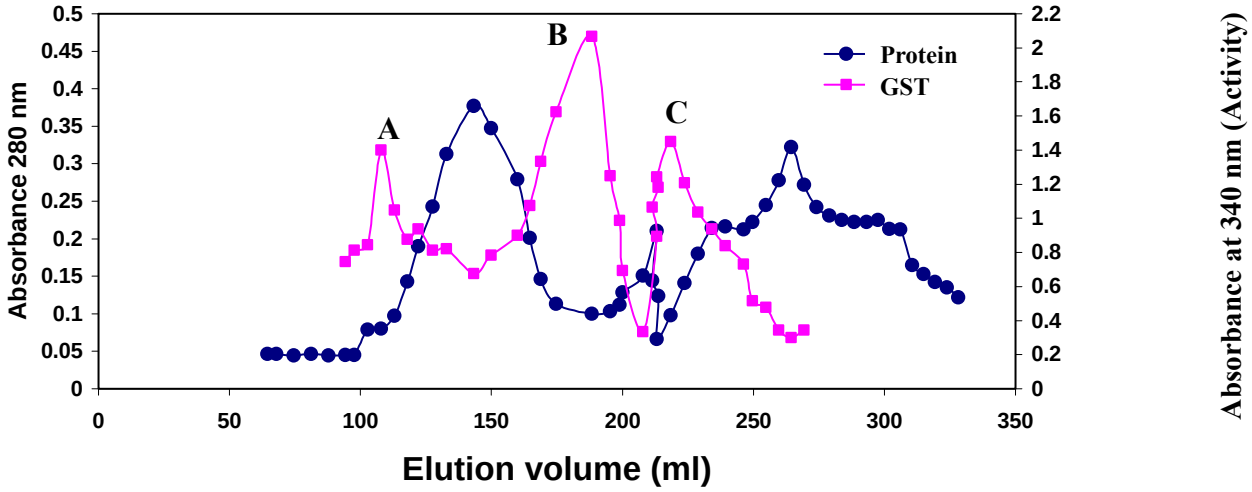


Fig. 2: Elution profile glutathione S-transferase (GST) for serum human on sephadex G-75.

**Molecular Weight Determination of GST by Gel Filtration**

The molecular weight of second peak (B) as a source of GST was determined by gel filtration chromatography using sephadex G-75 column (2 x 100) cm calibrated with known molecular weight proteins that were listed in Table (2).

Table 2: Elution volumes of known molecular weight materials on sephadex G-75.

| Materials | Molecular weight (Dalton) | Elution volume (ml) |
|---|---|---|
| **Urease** | 480,000 | 86.3 |
| **Bovine serum albumin (BSA)** | 67,000 | 118.5 |
| **α-Amylase** | 45,000 | 153.4 |
| **Egg albumin** | 45,000 | 151.5 |
| **Insulin** | 5,700 | 270.3 |
| **Pepsin** | 36,000 | 170.8 |
| **Unknown (peak B )** | 24,717* | 188.8* |

*This value was obtained from Figure (2).

A plot of logarithmic molecular weight of each material versus the elution volumes indicated in Table (2) gives a straight line as illustrated in Figure (3).

The molecular weight of unknown proteinous compound separated by the same column chromatography as shown in (step III) was determined from the standard curve, which was represented by Figure (3). The comparative molecular weight of peak (B) as a source of GST is approximately equal to (24,717) dalton. This finding was in a agreement with the previous results where it was reported that the molecular weight of GST was (22,000-29,000) dalton from different tissues (Gronwald and Plaisance ,1998; Hoarau et al.,2001; Goughlin and Hall,2002; Baraczyk-Kuoma et al.,2004).

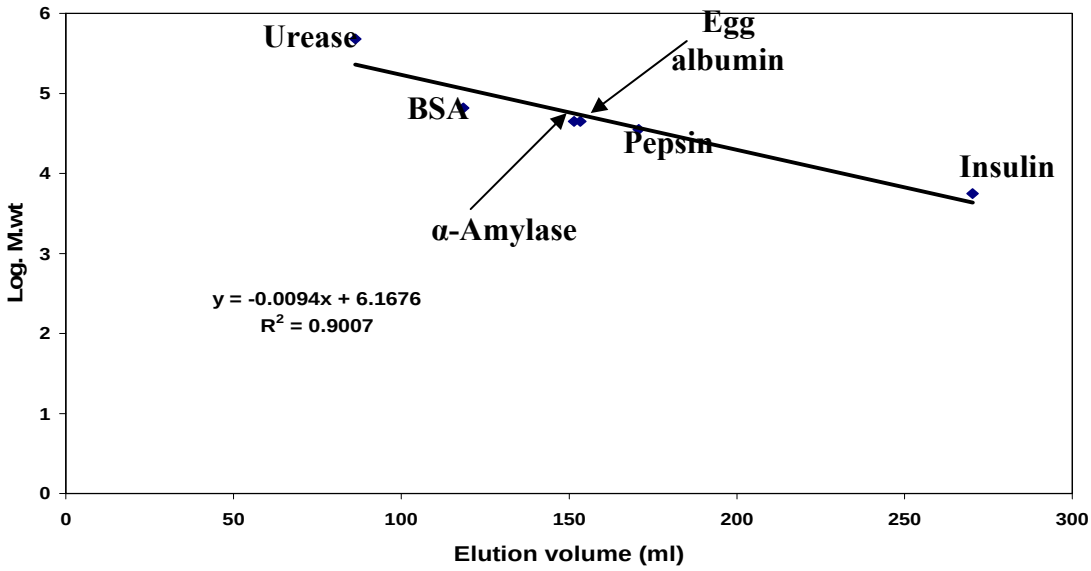


Fig. 3: A plot of the logarithm molecular weights of known proteins versus elution volumes on a Sephadex G-75.

**Molecular Weight Determination by SDS-PAGE**

The electrophoretic mobility of GST in SDS gels, the enzyme migrated as a single band in control only as shown in Figure (4) with an apparent molecular weight of (24,807) dalton which was determined by using known molecular weight compounds as shown in Figure (5).

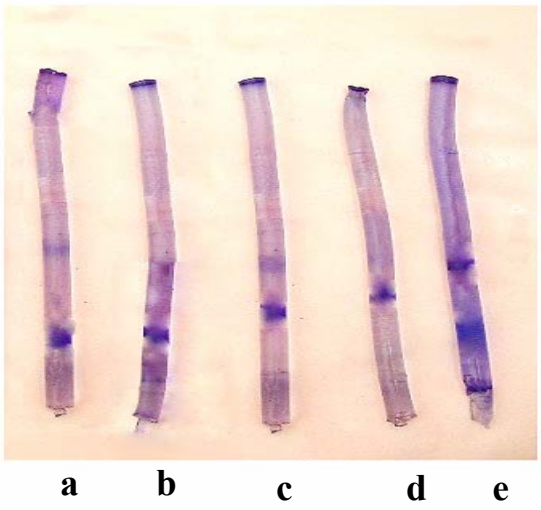


Fig. 4: Protein patterns obtained by SDS gel electrophoresis. The tubes from left to right contained (50)µg of standard protein employed to calibrate the columns were:

**a.** G.S.T separated. **b.** Egg albumin (M.wt. 45,000). **c.** Pepsin(M.wt. 36,000). **d.** Bovin serum albumin (BSA)(M. wt. 67,000). **e.** Urease (M.wt. 480,000).

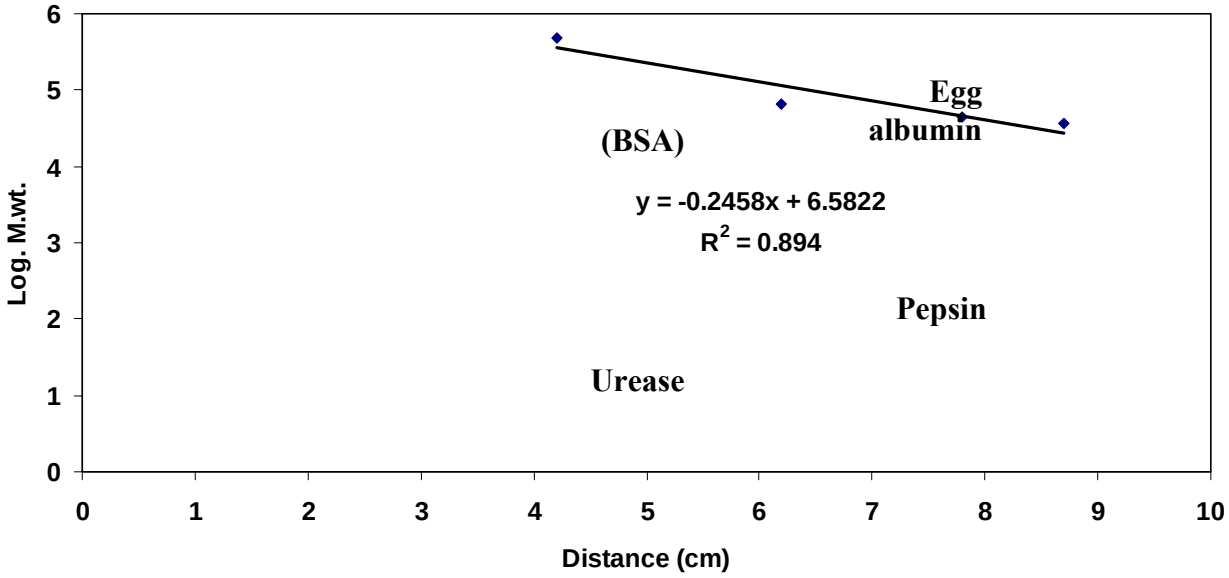


Fig. 5: Calibration plot for molecular weight estimation of GST by (SDS) gel electrophoresis using known molecular weight proteins.

**Optimum Conditions for GST Activity**

To develop assay conditions where GST from control individual shows a maximum activity, a series of experiments were performed. These included the enzyme concentration, the pH of the assay conditions, the incubation time, the incubation temperature and the substrate concentration.

**1.Effect of Enzyme Concentration on GST Activity:**

It is important to establish that the activity varies linearly with enzyme concentration. The activity of enzyme was measured in the presence of different concentrations of partially purified enzyme from serum between (10-80) µg/ml as shown in Figure (6).

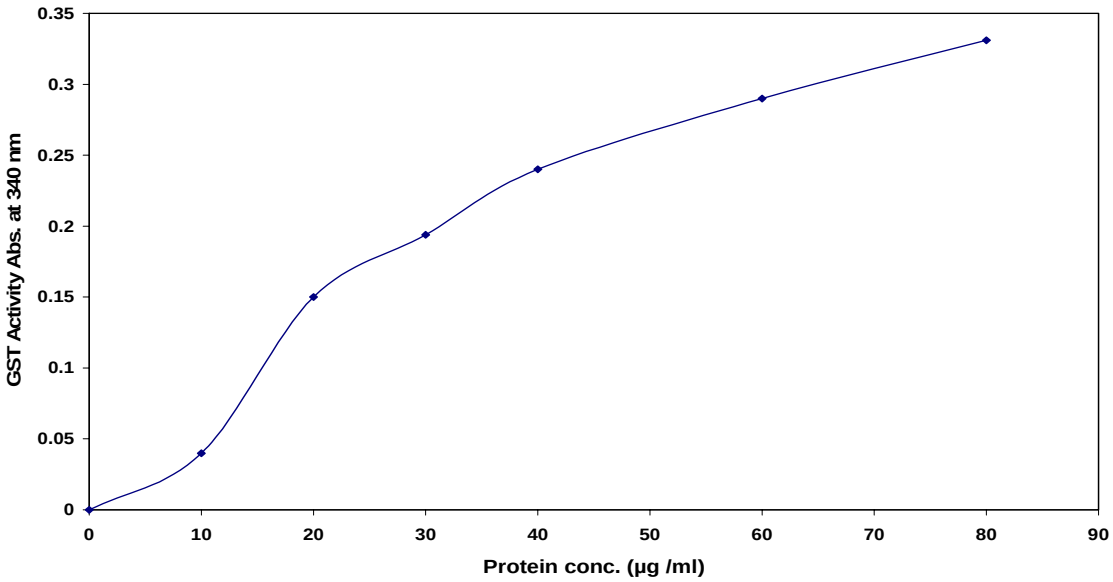


Fig. 6: Effect of different protein concentrations on GST activity

The result indicated that the enzyme activity increased with increasing the concentration of protein as a source of the enzyme. For the next experiment (40)µg/ml, as a source of the enzyme was selected for determination other optimum conditions.

**2.Effect of Buffer Solution:**

**2.1.Effect of Buffer Concentration on GST Activity:**

The activity of enzyme was measured in the presence of different concentrations of buffer solution within the range (0.05-0.17) mol/liter of phosphate buffer at pH 6.4. Maximum activity was obtained using (0.11)mol/liter of phosphate buffer (Table 3).

Table 3: Effect of buffer concentrations on GST activity.

| Phosphate buffer pH 6.25 (mol/liter) | GST activity (ΔA at 340 nm) |
|---|---|
| 0.05 | 0.22 |
| 0.07 | 0.27 |
| 0.9 | 0.30 |
| 0.11 | 0.33 |
| 0.14 | 0.24 |
| 0.17 | 0.21 |

**2.2.Effect of pH on the GST Activity:**

The influence of pH upon the activity of GST was investigated by using (40µg/ml) as a source for enzyme in (0.11)mol/liter phosphate buffer. The assay conditions were conducted in the same manner as described earlier at pH range of (4.2-6.76). Maximum GST activity was obtained at pH (6.4) as indicated in Figure (7).

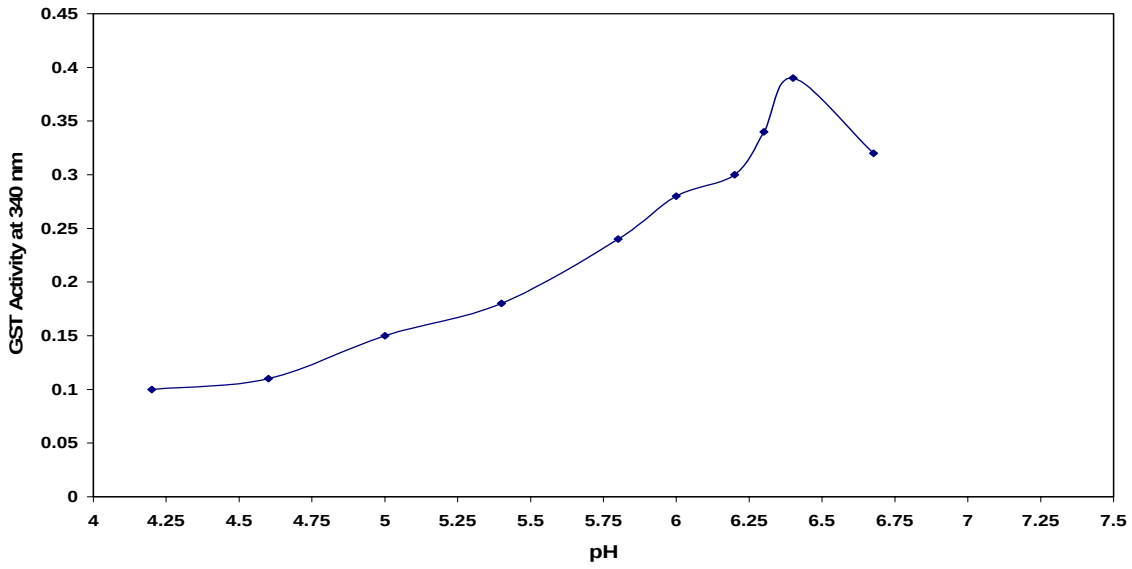


Fig. 7: Effect of pH on GST activity using (0.11)mol/liter phosphate buffer and (40µg/ml) as a source for the enzyme.

**3. Incubation Time as a Function of Enzyme Activity**

To determine the stability of GST activity under assay conditions, a series of experiments were performed at different time intervals. The results indicated that maximum enzyme activity was obtained after (12) mint. in (25 °C) incubation(Figure 8).

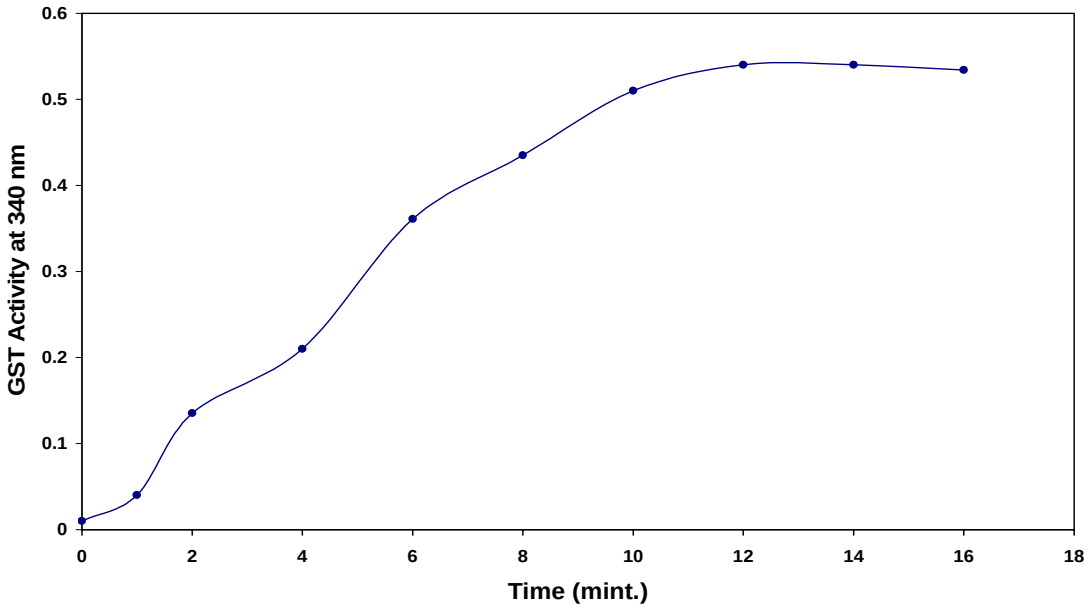


Fig. 8: Effect of incubation time on GST activity.

**4.Effect of Temperature on GST Activity:**

It has been found that as the temperature increased, there was a concave up increase in the enzyme activity until it reached a maximum value at a temperature of (25 °C) then dropped gradually after that Figure (9).

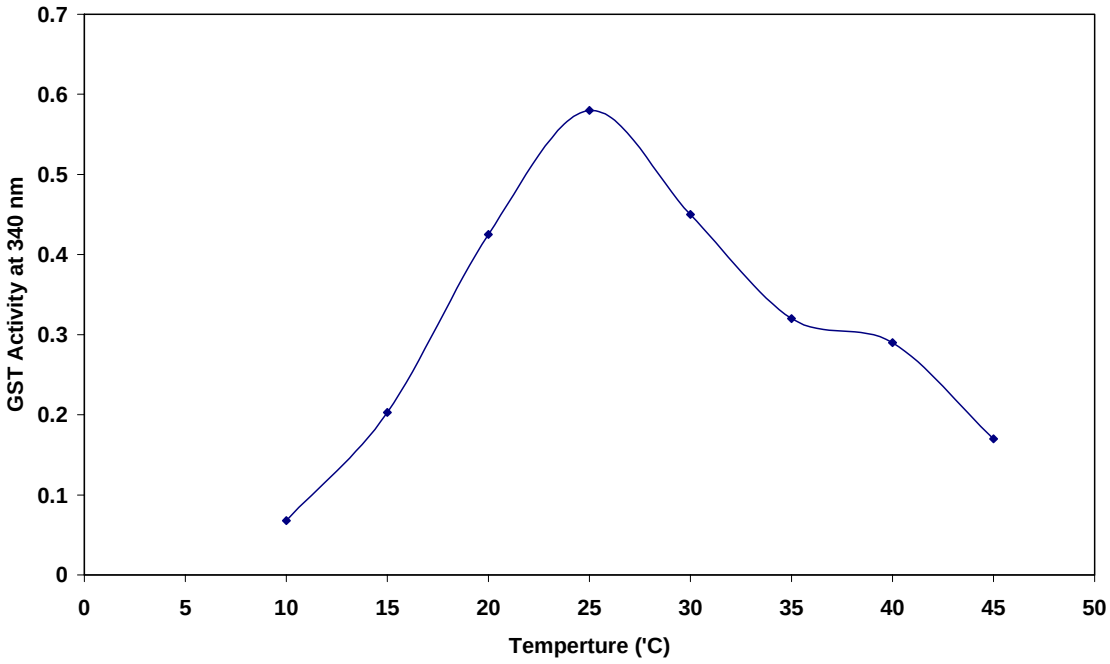


Fig. 9: Effect of temperature (°C) on GST activity.

**5.Effect of Substrate Concentration on the Enzyme Activity:**

To determine the effect of substrate concentration on the enzyme activity, a series of experiments were performed where the concentration of the substrate was varied Figure (10). Crystalline 1-chloro-2,4-dinitrobenzene (CDNB) was preferred for routine

use as the substrate, because of its greater stability and commercial availability in satisfactory purity.

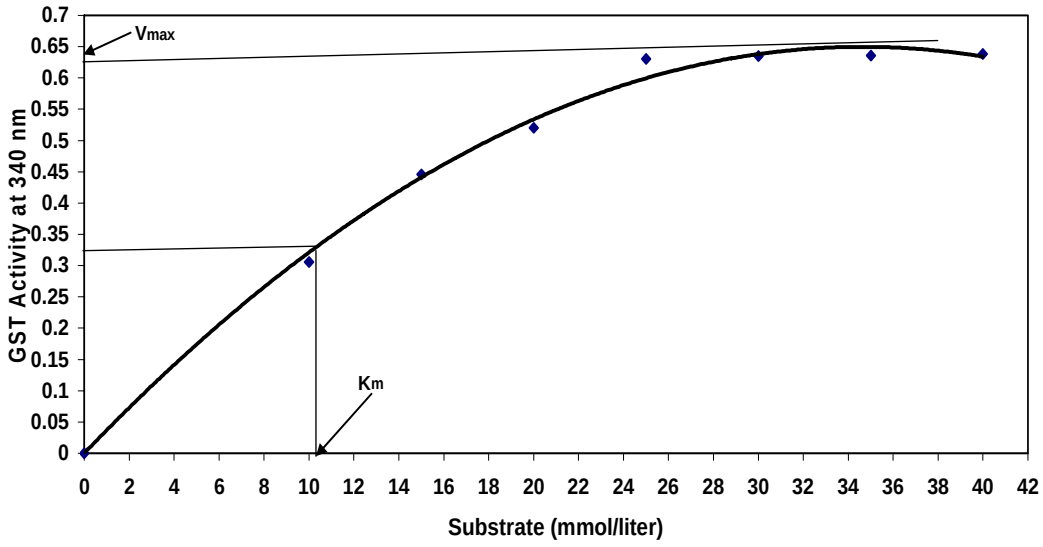


Fig.10: Effect of substrate concentration [CDNB] on the activity of partially purified GST.

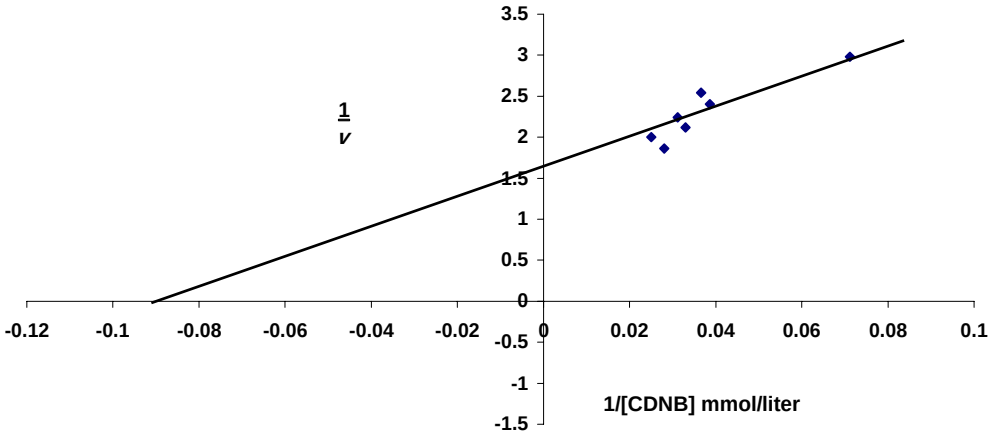


Fig. 11: Lineweaver-Burk plot of partially purified GST from serum human.

The Michaelis-Menten constant ($K_m$) of the enzyme was determined from Figure (9) and found to be (10.53) mmol/liter. A similar result was obtained using a Lineweaver-Burk plot by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained Figure (11) giving a $K_m$ value of (10.32mmol/liter) and $V_{max}$ (0.625 µmol/min.).

**6. Inhibition of GST:**

Many investigators observed that some chemical compounds have an inhibitory effect on GST activity. The results of adding benzyl chloride on the activity of partially purified GST were shown in Figure (12) . Benzyl chloride is a substrate for the enzyme, the production of S-benzylglutathione decreased activity of enzyme with increased concentration. Because benzyl chloride did not interfere with the spectrophotometric assays, its behavior as an inhibitor of the standard(Pabst et al.,1974).

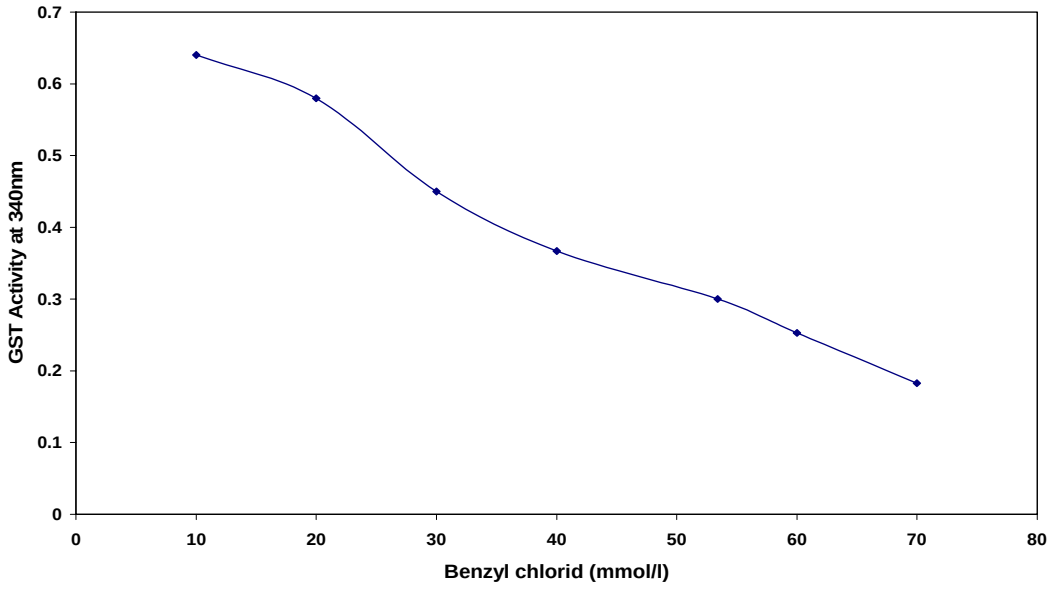


Fig. 12: Effect of Benzyl chloride on the activity of partially purified GST .

In this study, A lineweaver-Burk plot was performed Figure (18) where (30 mmol/liter) of benzyl chlorid as an inhibitor was used. The results showed that benzyl chlorid acted as a noncompetitive inhibitor. Noncompetitive-type inhibition was $K_m$ appears unaltered and $V_{max}$ was decreased proportionately to inhibitor concentration (Voet and Voet, 2004).

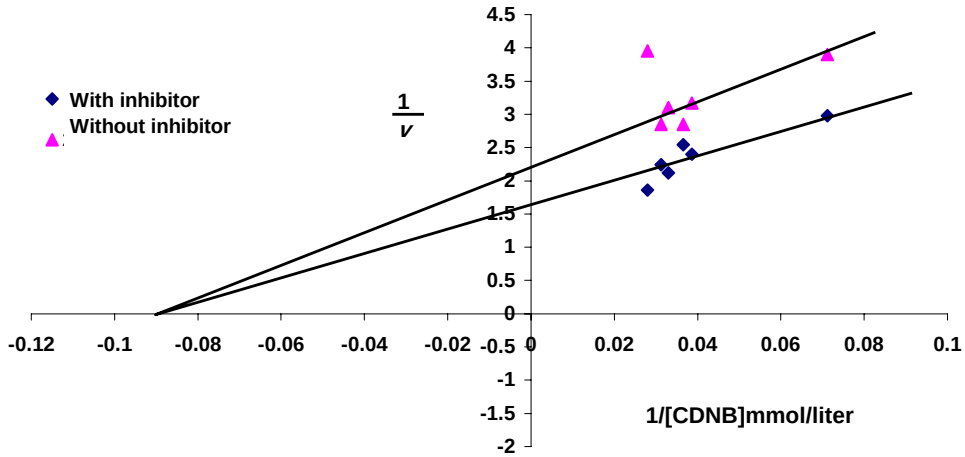


Fig.13: Lineweaver-Burk plot of GST with and without benzyl chloride as an inhibitor.

The optimum conditions of the partially purified glutathione S-transferase from serum were obtained in the following Table (4):

Table 4: Optimum conditions of the partially purified glutathione S-transferase.

| Substrate concentration (mmol/liter) | Temp.(°C ) | Time (mint.) | pH | Buffer concentration (mol/liter) | Enzyme concentration (µg/ml) |
|---|---|---|---|---|---|
| 22.463 | 25 | 12 | 6.4 | 0.11 | 40 |

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