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Effect Diabetic drugs on a novel Kinetic properties of Glutathione-S-Transferase Enzyme(GSTs) from human serum.

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ABSTRACT

Glutathione –S-transferase (GSTs)(EC 2.5.1.18) are dimeric enzyme present in many tissues including lungs ,liver and found in mammals ,insects ,plants ,microbes and divided into four classes including alpha,mu,pi,theta. GSTs are important phase II drug-metabolizing enzymes that a major role in protecting cells from toxic . This study attempt isolate GST enzyme from serum human .The partial purification of glutathione –S-Transferase were done using DEAE-Cellulose ,Then purification steps include precipitation by ammonium sulfate 70%, then using dialyzed by dialyze tube cut off number (10-14KD)overnight against the phosphate buffer pH=7 and further purified with DEAE-Cellulose column chromatography with a(55.14) purification folds then the glutathione –s-transferase enzyme purified also from serum human by using pre-packed column affinity chromatography by containing glutathione Sepharose 4% and compare activity between two separation ways the activity of GSTenzyme result from affinity column were more by using ion exchange column and found that enzymatic efficiency of GST by using affinity column (100%) while at using ion-exchange chromatography (55.14%) . The molecular weight of enzyme was determined by using SDS-PAGE analysis by using enzyme GST result from ion-exchange chromatography and affinity chromatography found (28KD). This study involved also some kinetic studies of GST , maximum activity for GST enzyme was obtained using 22.463mmol/l of 1-chloro-2,4-dinitrobenzene (CDNB)as substrate ,the enzyme showed maximum activity at 35℃ and optimum pH at 6.8 and time at 12 minutes in incubation at 35℃ . Using Lineweaver – Burk plot the maximum velocity (Vmax) and Michaelis constant Km were (11.12mmol/liter) and Vmax (1.254µmol/min) respectively .The thermodynamic constants of activation ,$(\Delta H, Ea, \Delta s, and \Delta G)$ were determined by using Arrhenius plot and found to be(35.866KJ/ mol,31.73KJ/mol,12.06 KJ/mol.K ,-3.559 J /mole.K) respectively .Metformin and Dionial inhibition on the GST activity were found that , this lead to drugs effect on enzyme GST.Where showed result that non comparative inhibitor at metformin and showed that comparative inhibitor at dionial .

Keywords, Glutathione-S-transferase, GST, human serum, Kinetic properties

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INTRODUCTION

Glutathione-S-Transferases (GSTs) (GSTs: EC 2.5.1.18) are dimeric enzyme from family of Phase II detoxification enzymes that promote catalyze the conjugation of glutathione (GSH) to large group of endogenous and exogenous electrophilic compounds. (Hayes et al.,2005).Some of these xenobiotic are produced free radicals .(Dibyajyoti S ,&Tamrakar A.,2011).The first GST enzyme structure was determined in 1991(Oakley.,2011).In 1961 extracts from rate liver were reported to catalyse the conjugation of GSH with either 1,2-dichloro-4-nitrobenzene (DCNB) or bromosulphophthalein.(Booth et al.,1961)(Coombes and stakelum.,1961). 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 was excreted out of the body.(Mannervik,1996; Bello et al.,2001;Raza et al.,2002; Baraczyk-Kuoma et al.,2004). GSTs represent a complex groping of proteins .

In human GST enzymes include cytosolic classes of GST such as (alpha, mu , pi, kappa, ,theta and zeta) GSTA(α), GSTM(μ),GSTP (π), GSTK(K), GSTT(θ) and GSTZ(Z) and one microsomal from (mGST ,microsomal).(Bolt et al.,2006).Among these classes of GST(GSTT1 ,GSTM1, GSTM3 GSTP1, and GSTZ1 have been shown to be polymorphically distributed.(Sheehan et al.,2001).(Wu B et al.,2012). The different GST was classified into families depend on amino acid sequences , Among cytosolic GSTs, members of the same class was possess greater than 40% amino acid sequence identity. Between classes was proteins have less than 25% sequence identity.(Oakley.,2011). The most of GSTs was present as soluble enzymes and was active as dimeric proteins with each subunit was having an active location composed of two distinct functional regions, involving a G-site for binding of the co-substrate GSH and a hydrophobic H-site binding structurally diverse electrophilic substrates.(Frova.,2006)(Dixon et al .,2002). (GSTs) are detoxifying enzymes found in birds, plants,microbes ,mammals,vertebrates,Yeasts , insects, and aerobic bacteria .(Hayes.,1995). GSTs play an important role in drug metabolism containing many cancer therapeutic agents(Wang et al,2012). It was recognized that GSTs can reduce lipid hydroperoxides through that was Se-independed glutathione peroxidase activity and these enzymes can too detoxify lipid peroxidation end products for example 4-hydroxynonenal .(Sharma et al.,2004).The key role of GST was catalyze formation of glutathione-S-conjugates with electrophiles that is decisive for inactivation and consequent excretion of other molecules. (Narasimhan et al.,2011)(Nosheen et al.,2011) .GSTs was catalyzed nucleophilic aromatic substitutions and, Michael type additions to anon saturated ketones and epoxide ring-opening reactions of which all result in the formation of GSH conjugates and the reduction of hydroperoxides was resulting in the formation of oxidized glutathione (GSSG). (J. A. Redick.,1982)(D. Sheehan et al.,2001)

Instruments

The instruments used throughout this study are as listen in table(1-1)

Table(1-1): Instruments and equipments used in this study.

Instrument	Supplied company
pH- meter	WTW inolab 740 Series
Sensitive balance	Stanton 461
Water bath	GFL
UV.visible spectrophotometer	Spectroscan 60 DV
Electrophoresis	Page Run-RWSE-1100
Oven	Hearson (England)
Nano drop	**UVS-99\ UVISDrop**
Lyphilization (Freeze-Dryer)	KV400
Ultracentrifuge	Universal 320 R Hettich
Vortex mixer	**Karl Kolb VF2**
Magnetic stirrer	**Baind&Tatlock., England**
Centrifuge	Hermle Z 200A

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Shaker water bath	**Tecam (England)**
Column affinity chromatograph	**GE Healthcare GSTPrep FF 16\10**

Glutathione –S-Transferase enzyme assay :

Principle

Glutathione –S- Transferase activity was using 1-Chloro- 2,4 dinitrobenzene (CDNB) as substrate, spectrophotometrically essentially was described by Habig (Habig et al.,1974).

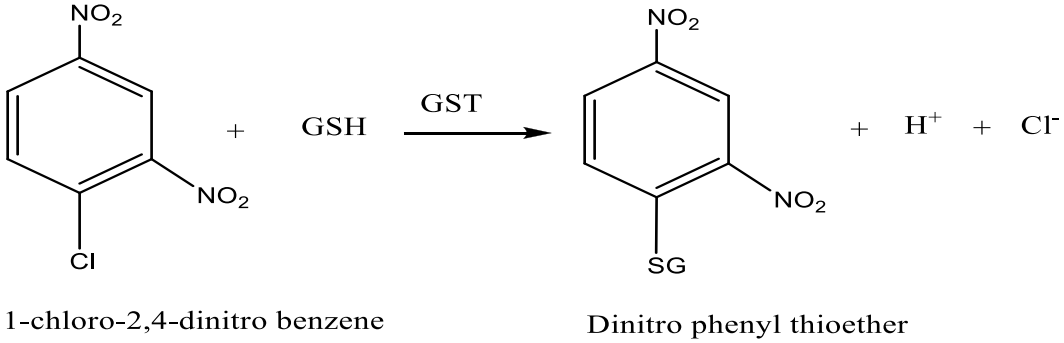

Figure1:- Reaction (1-Chloro- 2,4-di nitrobenzene) with reduced glutathione (GSH) in the present of glutathione –S-Transferase enzyme. (Yildiz and Kuman,2004).

Reagents

1-Reduced glutathione (GSH)(29.93mM) prepare from soluble 0.092g in 10 ml D.W .
2- (1-chloro-2,4-dinitrobenzene) (CDNB)(22.463m M) prepare from soluble (0.455g in 100ml from ethanol .
3- Phosphate buffer solution pH =6.25 at concentration 0.11 mol/L .

Procedure

1-Two sets of tubes were prepared as follow

Reagents	Sample	Blank
Phosphate buffer pH=6.25	2.7ml	2.7ml
Serum	100μl	———
Distilled water	———	100µl
CDNB	100μl	100μl
After 3minutes was added GSH	100μl	100μl

2- Then the tubes were mixed at room temperature, then the absorbance was measured after each 1 minutes for 10 minutes at (340nm) by using spectrophotometer.

Calculation :

Enzyme activity was expressed as (U\L) is Calculated by following equation :

$$\textbf{Activity of GST(U\L)} = \frac{\Delta A \times Vt \times 1000}{\varepsilon. \times Vs \times d}$$

Where

ΔA:the different between the first absorbance at (1) minute and absorbance at(10) minute.
Vt: the total volume.
Vs:the sample volume.

ε:extinction coffficient of complex CDNB with GSH which equal 9.6 mM^{-1} .
d:light path (1cm)

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

Partialy Purification of Glutathione -S-Transferase (GST) from Human Serum by using ion-exchange chromatography:

The method was given her as yielded of enzyme preparation acceptable for human serum. Every steps were performed at 4℃ unless stated otherwise.

Ammonium Sulfate Fractionation

The first step in purification of enzyme (GST) was isolated of enzyme from human fresh serum was obtained from one normal male person (age:25year).the extract crude was concentration by addition of solid ammonium sulfate (70%).The mixture were stirred until all of ammonium sulfate were dissolved the suspension was centrifuged at (1400xg at 4℃)for (20) min . The precipitate protein was for using determined .(Segel, I. P., 1976). at 280nm and the GST was estimated in each fraction have protein activity, and then the GST activity was checked at 340nm using CDNB as substrate. (Habig et al.,1974).

Dialysis

Dialysis was made using a semipermeable cellophane dialysis membrane with M.wt.cut off number (10-14)KD by overnight against potassium phosphate buffer (pH=7) at concentration (0.1M).

DEAE-Cellulose chromatography.(Aydemer.,2009).

The solution resulted from the dialysis was applied into DEAE-Cellulose Column (30 x 1.5 cm) previously equilibrated with 10 mM of Tris-HCl buffer pH 7.5 containing 0.1mM EDTA and 0.1 mM of reduced glutathione .The eluent solution was contained 10 mM of Tris- HCl buffer pH 7.5 , 0.1 mM EDTA, and 1mM of GSH and eluted with gradient sodium chloride solution NaCl (1M-0.5M-0.1M),was carried out at aflow rate of(0.5ml\min and(0.5ml) fractions were collected and measure the total proteins at 280 nm with spectrophotometer UV(Nano drop) , GST activity at 340nm was determined for using CDNB as substrate.(Habig et al.,1974). The fractions and GST activityies were pooled then using it to kinetic and electrophoretic studies.

Purification of GST using glutathione sepharose 4% by anffinity chromatography.(Simons and Vander.,1977).

Human serum was taking from normal male persons (age :20-25year) and the volume 10ml from serum were taken . Serum was filtered by using mile pore filter unit (0.45μm)and The solution of serum was applied to affinity column (GSTrap FF column according to GE healthcare the column is(16$\times$ 20mm).packed with matrix glutathione sepharose 4% fast flow and 10 carbon linker arm as ligand at concentration 120-320µmole GSH\ml medium.The structure of bead is highly crossed linked 4% agrose with average particle size of 90µm.the column was equilibrated with 100ml from binding buffer . The sample was applied to column by using aflow rate 1ml\min .Then washing with 200ml from binding buffer until no materials appeared.Eultion solution was appied by using 100ml at aflow rate 1ml\min the bound GST was eluted with 50mM from Tris-HCl buffer at (pH=7.3) containing 10mM GSH at aflow rate of 0.5ml\min . All fractions were collected,and the protein eluted from column was followed up by measuring absorbance at(280nm) by using with spectrophotometer UV(Nano drop) ,for GST activity at 340nm using CDNB as substrate. (Habig et al.,1974). The fractions and GST activityies were pooled then using it to kinetic and electrophoretic studies.

Freeze –Dryer (Lyophilization)Technique

The enzyme fraction were collected which was obtained from a affinity chromatography and ion -exchange chromatography were dried using a freeze – Dryer (Lyophilization) technique separately to obtain

concentered protein .The enzyme was kept in a freeze at (-4℃) in a tight sample tube to be used in further investigations.

Electrophoresis

Electrophoresis was carried out in presence of sodium dodecyl sulphate SDS at 10% concentration separation gel the sample was treated according to the method of Laemmli. (Laemmli, 1970). ((Page Run – RWSE-1100)and heated at (100℃ for 5 min)bromo phenol blue was working as front run marker.The gels were runed for (80min at 21mA) and 150 volt in a electrophoresis unit . After complted run , they were marked the protein by Comassie brilliant blue R-250 .All The fractions with (GST) activityies were pooled and electrophoresis was used the enzyme fractions which were obtained from affinity chromatography and ion - exchange chromatography .

Estimation of some enzyme properties(GSTs)

Effect of temperature

The optimum temperature of glutathione –S-transferase was determined by using the enzyme assay at different incubation temperatures of (20℃,25℃,30℃, 35℃, 40℃, 45℃, 50℃, 55℃, 60℃, 65℃, 70℃).

Effect of PH

The influence of the pH on the glutathione –S-transferase activity was studied using enzyme assay system at different potassium phosphate buffer solution (5.8 - 8.0).

Kinetic parameter

Kinetic experiments were performed at 35℃ and pH= 6.8 . The Michaelis –Menten and Lineweaver – Burk plots. (Al-Helaly, 2007). Kinetic parameters of enzyme can be determined using Michaelis-Menten Kinetics according to. (Lineweaver.,1934) .

Estimation(Km,and Vmax)

In order to determine Michaelis –Menten equation values a series of solutions of the (1-chloro -2,4-dinitrobenzene) at different concentration substrate were utilized.Solutions series from(1-chloro-2,4-dinitrobenzen) (2.5-35 mmol/L) . metformin and Daonil was prepared series of solution (2.5-35 mmol/L) from than measured the activity according to .(Al-Helaly, 2007).

Thermodynamic studies

The relationship between the rate an enzymatic of reaction and activation energy was given by empirical formula of Arrhenius equation(1):

$$K = Ae^{-Ea/RT} \text{ --------(1)}$$ (Segel,1976).

Where A is the pre-exponential factor,and Ea: is the activation energy(**kJ mol^{-1}**) , and **R**:is universal gas constant **(R=8.314Jmol^{-1}K^{-1})** which can be determined. (EL-Hefnawy et al.,2014). Thus when a reaction has a rate constant **K** that obetys Arrhenius equation a plot of (ln **K)** versus **(1/T**) gives astraght line whose gradient and intercept was used to determined **Ea** and **A**.

Taking the natural logarithm of Arrhenius equation yields:

$$\ln K = \ln A - \frac{Ea}{RT} \text{----------}(1)$$

Depended on(Eyring Polanyi) equation can find enthalpy change and entropy change.
(EL-Hefnawy et al.,2014).

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$$\ln\left(\frac{Vmax}{T}\right) = \ln\frac{KB}{h} + \frac{\Delta S}{R} - \frac{\Delta H}{R}.\frac{1}{T} \text{ ----------(2)}$$

The slope of the resulting line corresponds to $-\Delta H/R$

determine the ΔS of the reaction from the *y*-intercept which corresponds to ΔS.

$$\text{Intercept} = ln\frac{KB}{h} + \frac{\Delta s}{R}$$

And determined ΔG can using depended on equation(Gibbs-Helmholtz Equation)

$$\Delta G = \Delta H - T\Delta s \text{ ----------(3)}$$

Where Temperature in kelvin **(K)** at **25**℃ **.**

RESULTS AND DISCUSSION

Ammonium sulphate salt is a common salt using in protein concentration in view of cost cheap and higher solubility and stabilizes protein structure. The results predicted that the enzyme activity was found in the (70%) of saturation ammonium sulfate precipitates .As show in Table(1-1) that the specific activity was increased after each stage purification . This might be dut to the removal of the small molecules and increasing the purification of GST. Other studies by the same research have shown that GST can be precipitated highest activity obtained at 75% (Al-Helaly, 2007) of ammonium sulphate depending on the source of enzyme.Other studies suggested highest activity obtained at 85% saturation . (Zhang et al.,2013).

Table(1-1):Partial purification steps for human serum GST.

Purification Stage	Volume Taken (ml)	Total protein mg\ml	Activity U\ml	Sp.activity U\mg	Folds of purification	Total Activity Units	Recovery %
Serum	5	1.66	0.346	0.208	1	1.73	100
Precipitate by $(NH_4)_2SO_4$	4	1.43	0.321	0.224	1.07	1.28	73.98
Dialysis after lyphilization	3	1.34	0.353	0.263	1.26	1.05	60.69
EAE- Cellulose	3	1.01	0.318	0.314	1.50	0.954	55.14

$$Total\ activity = enzyme\ activity(U/ml) * Volume(ml) = U$$

$$\text{Specific activity} = \frac{enzyme\ activity\ (U/ml)}{Protein\ concentration(\ mg/ml)}$$

$$= \frac{Specific\ activity\ of\ purified\ enzyme}{Specific\ activity\ of\ crude\ enzyme}$$ (Lebda et al.,2012)Purification fold

$$\text{Yield\%} = 100 * \frac{Total\ activity\ of purified\ enzyme}{Total\ activity\ of crude\ \ enzyme}$$

International unit(IU): amount of glutathione –S-transferase (GST) catalyzing the formation of one micromole of produced per min under of optimum conditions. (Habig et al.,1974).

(A specific activity was defined that enzyme activity (units) per milligram of protein. Colowick et al .,1985).
This enzyme preparation was concentrated and further purified by DEAE-Cellulose chromatography (Fig.1)(1.50 fold) through the above purification steps with increased in specific activity(0.314U\mg) with yield (55.14%). The DEAE- Cellulose chromatography has several from advantage including high resolution power, good separation , easy handling ,high capacity ability of reactivation for using several times besides of

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separation principle which depending on charge differences.(AL-Kubaissy .,2011).

Table(1-2): Compare between enzymatic efficiency affinity chromatography and Ion-exchange chromatography for human serum GST .

Purification stage	Activity (U/ml)	Volume (ml)	Total protein (mg\ml)	Total Activity (Units)	Yield (%)
DEAE-Cellulose	0.318	3	1.01	0.945	55.14
(Serum) By using Glutathione Sepharose 4%	0.178	10	1.136	1.78	100

Where notes from table (1-2) that enzymatic efficiency affinity chromatography equal 100%while enzymatic efficiency of ion-exchange chromatography equal to 55.14% because of Column length cellulose and column smaller (Glutathione Sepharose 4%) and this lead to increase number of tubes result and which contain enzyme activity in state using DEAE-Cellulose column .Prepared enzyme was concentrated and further rand purified by Glutathione Sepharose 4% with (100%Yield).

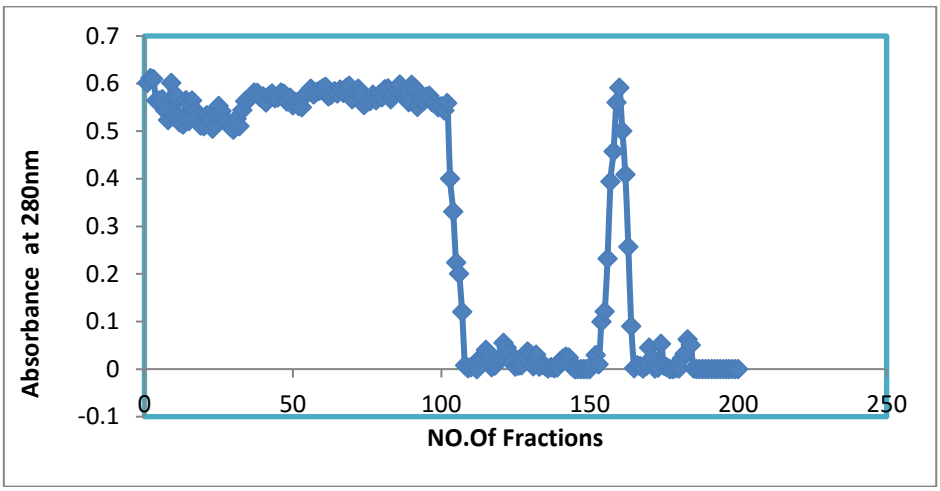

Fig.(1):Absorbance protein at 280 nm of GSTs by using affinity column (Glutathione Sepharose 4%).

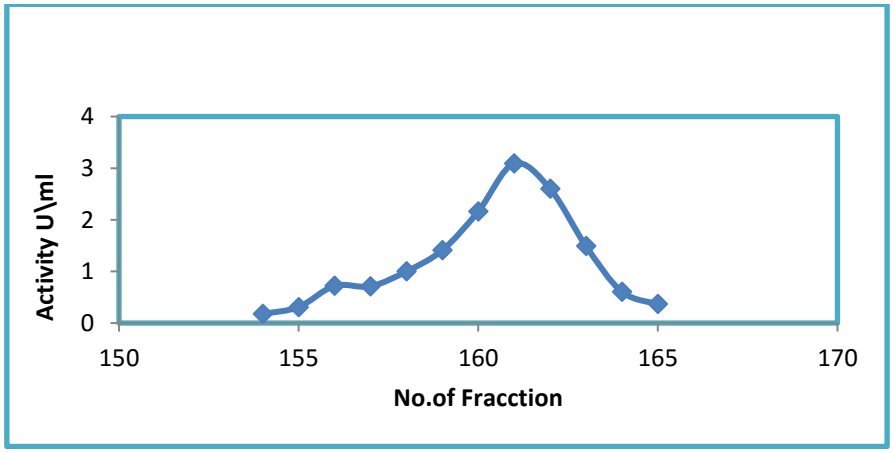

Fig.(2):Activity of GSTs enzyme by using affinity column (Glutathione Sepharose 4%)where appear the activity between (154 -165) tubes .

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Affinity chromatography elution the bound protein was eluted as indicated by single peak which contain on enzyme activity was showed in figure (2)the pooled active fraction were assayed for GST enzyme activity by using CDNB as substrate.

While ion-exchange chromatography it is observed the separation of two peaks summit of protein with one summit appearance of enzymatic activity as (fig.5) which contain on enzymatic activity. the pooled active fraction were assayed for GST enzyme activity by using CDNB as substrate.

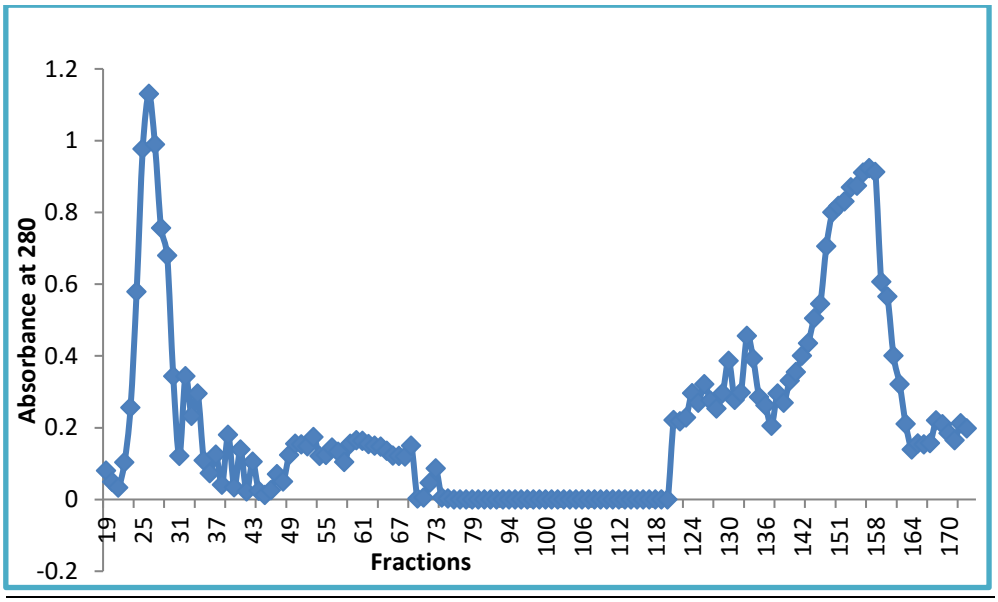

Fig.(3):Purification of GSTs on DEAE-Cellulose column (30×1.5)cm.

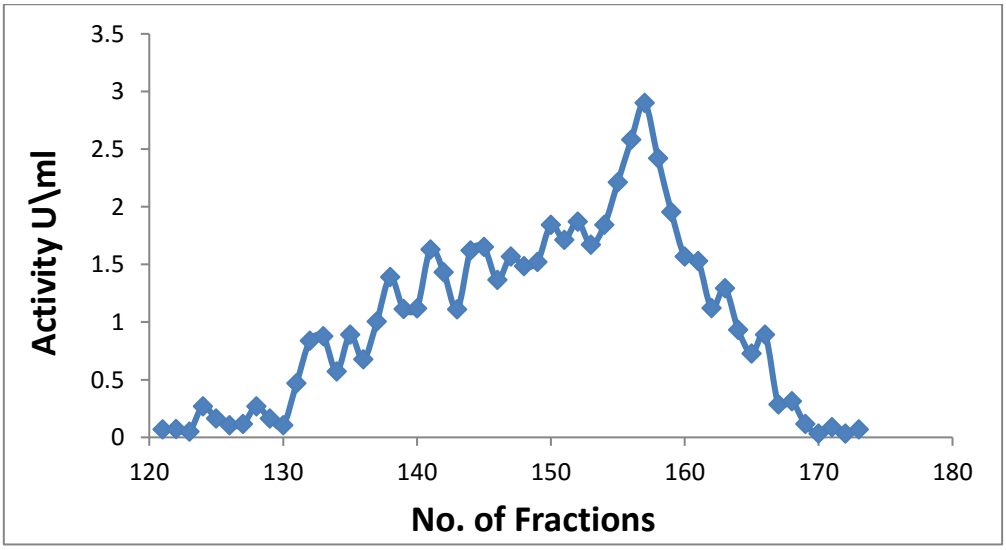

Fig.(4):Purification partial of GSTs by using DEAE-Cellulose column (30×1.5)cm where tubes activity between (121-173).

Molecular weight for determination of GST by using affinity chromatography and Ion –exchange chromatography (SDS-PAGE)

The electrophoretic mobility of GST in SDS gels that the enzyme migrated as single band in control only as show in (fig. 5) with apparent molecular weight of (28KD). The GST enzyme was purified from serum

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human .The crude extracts of serum human and filtered were loaded onto affinity chromatography column and the elution of the bound protein was eluted ,the enzyme was appeared single sharp peak which was contained on enzyme activity as show in(Fig. 2) .The pooled active fractions were assay for GST activity using (CDNB) as substrate. This result agreement with(Al-Helaly., 2007). ,showed only single protein at 24KD .Other studies by the same research have shown that the molecular weight of GST enzyme obtained was 50000 Dalton , showed only single protein band on the gel after staining by commassie brilliant blue (R-250)(Al-Jumaily.,2009). The crude extracts of serum human were loaded onto ion – exchange chromatography column and the elution the bound protein was eluted and the enzyme was appeared single peak which was contained on enzyme activity as show in (fig.2) The pooled active fractions were assay for GST activity using (CDNB) as substrate, with apparent molecular weight of (28KD) this lead increase in enzyme pure in each purification . Other studies different suggest that the apparent molecular weight contain on two bands of 24KD and 32KDwere observed for M.florea, one more band of 26KD was present in S.ribesii .(Nicolas.,2004).

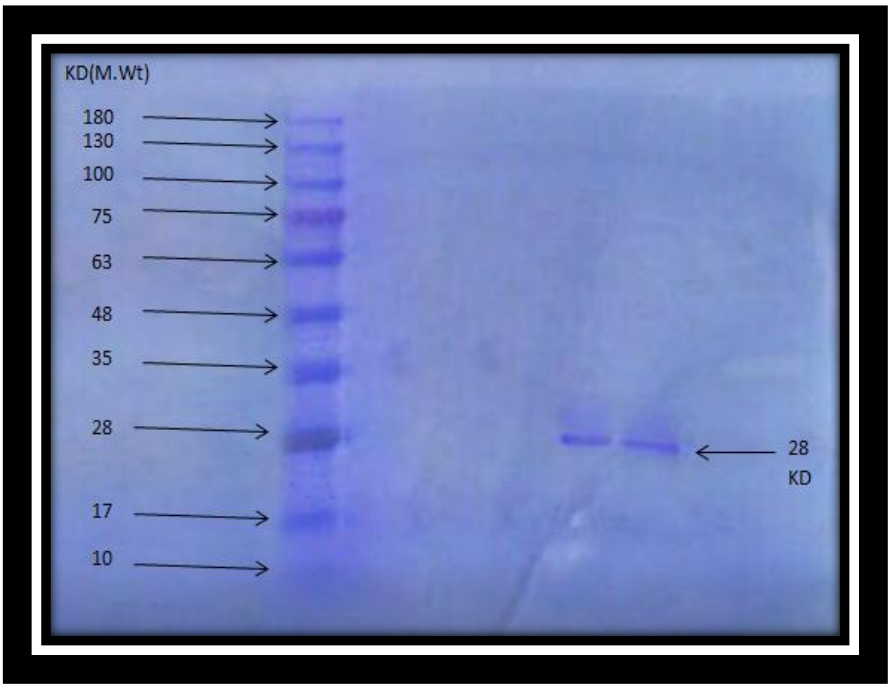

Fig.(5): The electrophoresis of patter GST in present SDS and mercaptoethanol and determining the molecular weight of purified enzyme by by using ion-exchange chromatography and anffinity chromatography.

Optimum Conditions for GST Activity

Effect of Enzyme Concentration on GSTs enzyme Activity

The activity of enzyme(GST) was measured in the presence of different concentrations from purified enzyme from serum human between (10-80) µg/ml as shown in Figure (6). With constant substrate (CDNB) concentration which work on it enzyme GST.Notes that enzymatic reaction rate increase with increase enzyme concentration.

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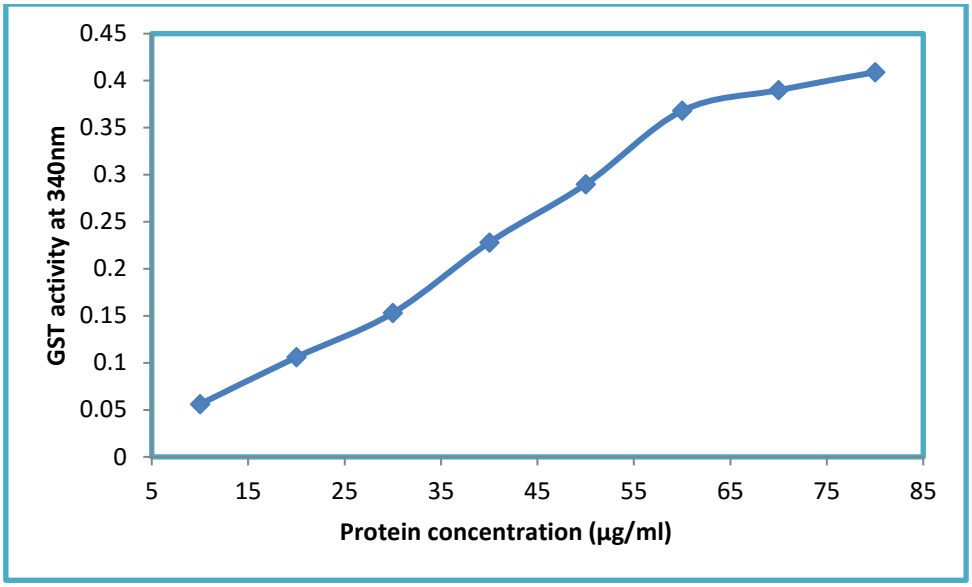
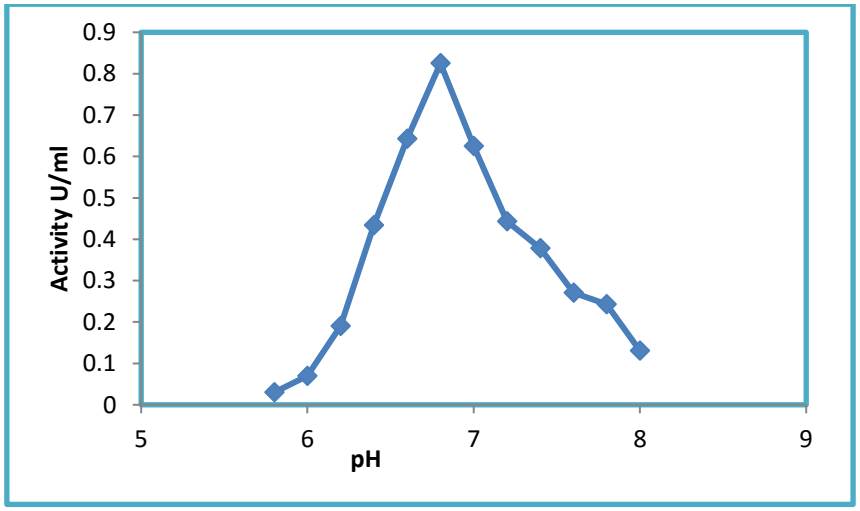


Fig. 6: Effect of different protein concentrations on GST activity.

Effect of pH on the GSTs enzyme Activity

That each enzyme optimum pH certain appear enzyme top activity was called optimum pH , where notes that at using pH too hight or too lower leaed to happing distortion in natural protein of the enzyme because losses of activity . The assay conditions were as described at pH range of (5.8-6.8). Optimum GST activity was obtained at pH (6.4) as indicated as showed in Figure (7).Other studies by the same research have shown that GST activity was obtained at pH=6.4.(Al-Helaly., 2007).Other studies different suggest that pH= 7 those of purification of enzyme GST from rates liver (Lebda et al.,2012).Also studies appear suggest that pH=7-7.5 the maximum activity of Purification and partial characterization of glutathione transferase from the teleost Monopterus albus. (Q. Huang.,2008).



(Fig.7). Effect of pH on activity enzyme GST.

Effect of Temperature on GST Activity

The reaction which catalystic enzymes effect temperature, The results indicated that , The maximum enzymatic activity occurred at at the optimum temperature (35℃) as shown in Fig(8). These results agree with those of purification of enzyme GST from rates liver. (Lebda et al.,2012). It was found that as the temperature increased,to reach to 35℃ after decrease rate of reaction obtained denaturation .

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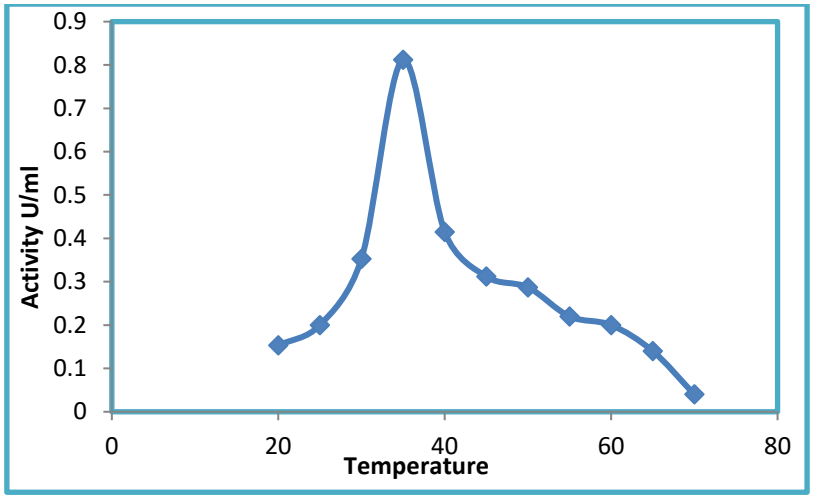
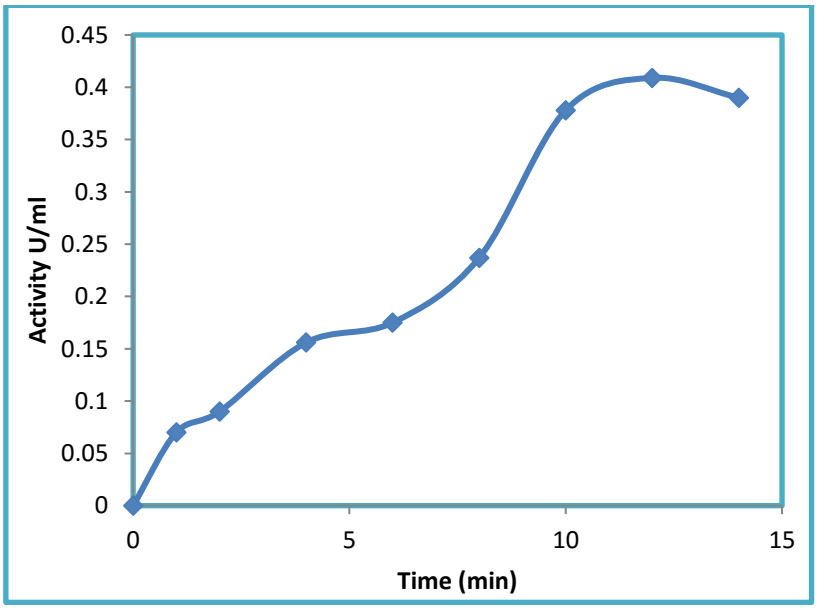


(Fig.8).Effect of temperature on activity of enzyme GST.

Reaction time

To determine the stability of GST activity under assay conditions, It has been studies reaction time to choose duration optimum, as showed in Figure (9) , The results indicated that optimum enzyme activity was obtained activity height of enzyme GSTs at after (12) minutes. in (35℃) incubation(Figure 9).



(Fig. 9): Effect of reaction time on GST activity.

Effect of Substrate Concentration on the Enzyme Activity

When the concentration of enzyme in mixture reaction constant ,the increase in substrate concentration it was causes at first a significant rise at the speed of enzymatic reaction rate , but continue to increase the concentration of substrate leads to get a decrease in the amount of increase in the speed of enzymatic reaction rate to become fixed speed of whatever increase concentration of substrate and was called the activity at the top the concentration of substrate is called maximum activity (Vmax). (Berg.,2007).

To determined the relation between GST enzyme and concentration of substrate (CDNB), It was measured of enzyme activity at different concentrations between (2.5-35mmol/L).As showed in figure (10)

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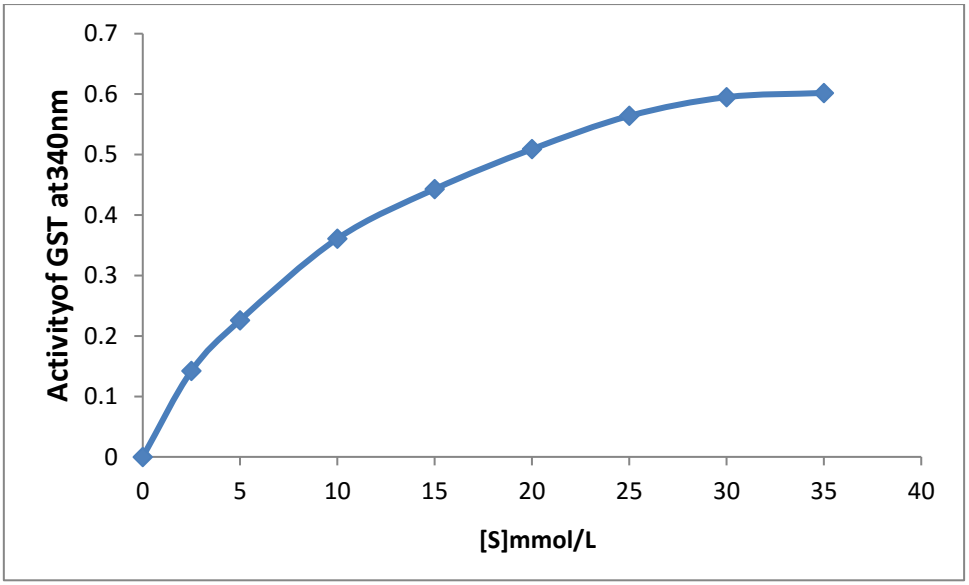

Fig.10: Effect of substrate concentration [CDNB] on the activity of purified GST.

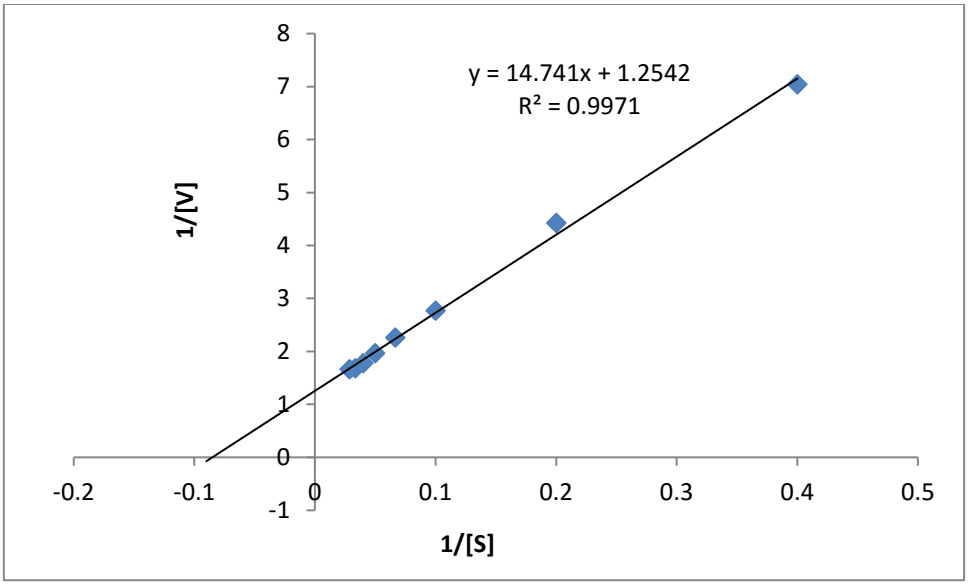

Fig. 11: Lineweaver-Burk plot of purified GST from serum human.

The Michaelis-Menten constant (Km) of the enzyme was determined from Figure (10) and found to be (10) mmol/liter.The 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 Km value of (11.12mmol/liter) and Vmax (1.254µmol/min).This inudicated that GST enzyme has a high affinity toward substrate(CDNB).

Effect of Inhibition on GST activity

Effect Metformin

The use of metformin inhibitory of GST enzyme .It was noted that reacts with GST enzyme and decrease from of enzymatic reaction rate.When using LineweaverBurk plot to find the type of work inhibitor metformin The results showed that metformin acted as a noncompetitive inhibitor. Noncompetitive-type inhibition was Km appears without change and Vmax was decreased proportionately to inhibitor concentration. (Voet and Voet., 2004). . This result agreement with.(Al-Helaly., 2007). lineweaver-Burk plot

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was performed Figure (12), Where Km value of (11.76mmol/liter) and Vmax (1.927µmol/min) in present inhibition. Other studies suggest that inhibition a noncompetitive inhibition of GST enzyme by α –tocopherols and α –tocopherols derivatives.(Van Haaften l.,2001).

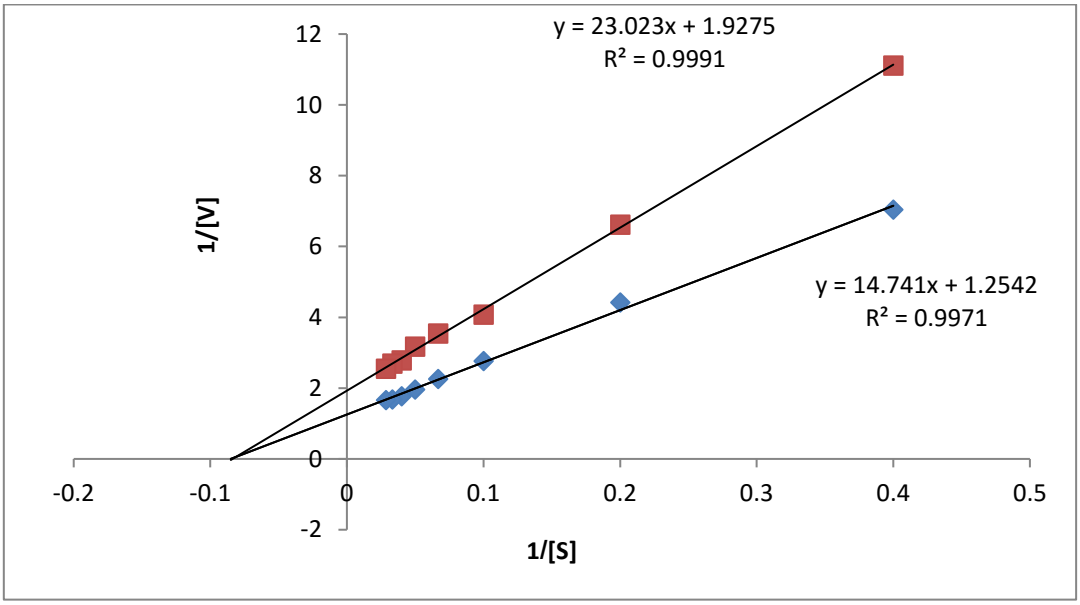
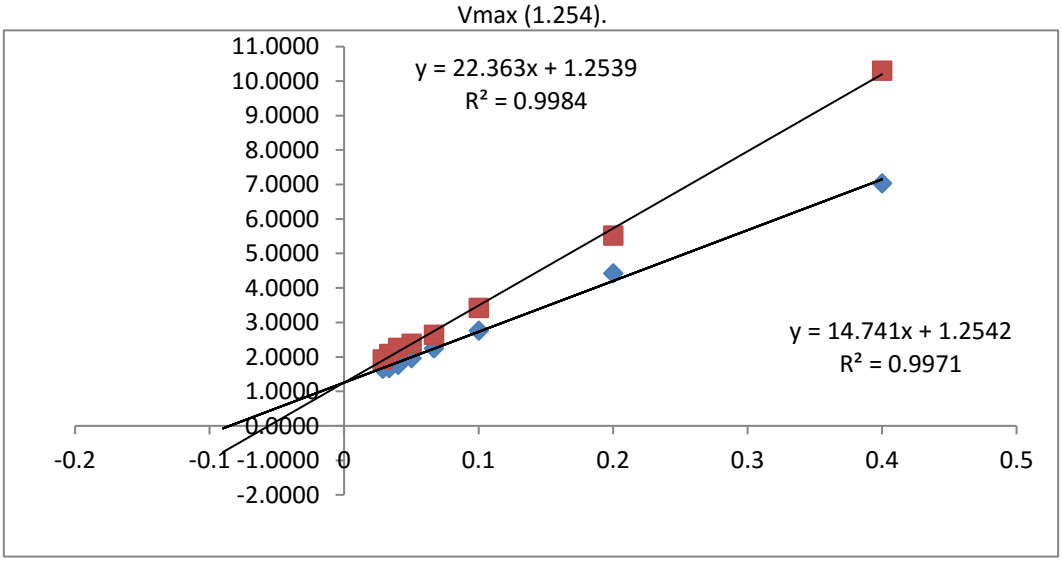


Fig.12: Lineweaver-Burk plot of GST present metformin as Non competitive inhibitor.

Effect Dionial

The use of dionial inhibitory of GST enzyme .It was noted that reacts with GST enzyme and then completes with substrate (CDNB) and decrease from of enzymatic reaction rate.When using LineweaverBurk plot to find the type of work inhibitor metformin The results showed that metformin acted as a competitive inhibitor. Competitive-type inhibition was Km appears change and Vmax was remained constant .This result agreement with(Sarhan.,2010).(Hamedet al.,2014) .Lineweaver-Burk plot was performed Figure (13), Where Vmax (1.254).



(Fig.13) Lineweaver-Burk plot of GST present dionial as competitive inhibitor.

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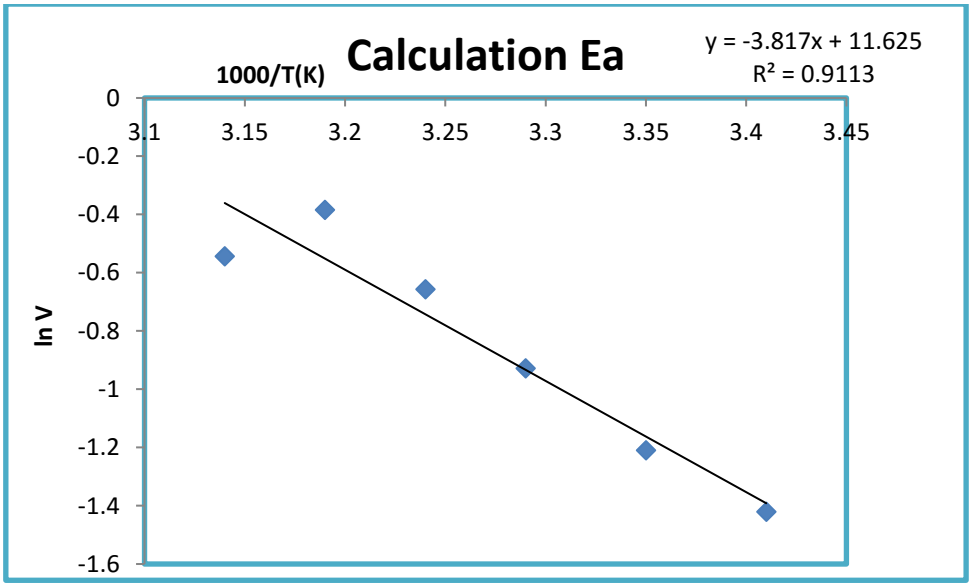
Table (1- 3): Optimum conditions of the purified glutathione S-transferase.

Enzyme concentration (µg/ml)	pH	Time (min)	Temperature (℃)	Substrate concentration (mmol/l)
60	6.8	12	35	10

Thermodynamic constants of GST enzyme

1. **Activation energy (Ea)**
   Activation energy was energy required occurred the reaction between the enzyme and substrate . Activation energy of enzyme was determined logarithm plot rate constant (ln K) versus 1/T(K) by application Arrhenius equation .The results predicted that the activation energy was found (31.734KJ/mol) , .As show in Table(1-4) . As figure (14).
2. **Enthalpy change (ΔH)and entropy change (ΔS)**
   Enthalpy change and entropy change was determined by using eyring equation the slope was resulting line correspond to (- $\Delta H/R$) . As figure (15). But intercept was correspound to ΔS. The results were showed in table(1-4).
3. **Gibbs free energy (ΔG)**
   For the chemical reaction , the change in Gibbs free energy (ΔG) was the energy which was available to do work. To determined **(ΔG)** was using Gibbs equation.



(Fig. 14): Calculation of activation energy for GST enzyme.

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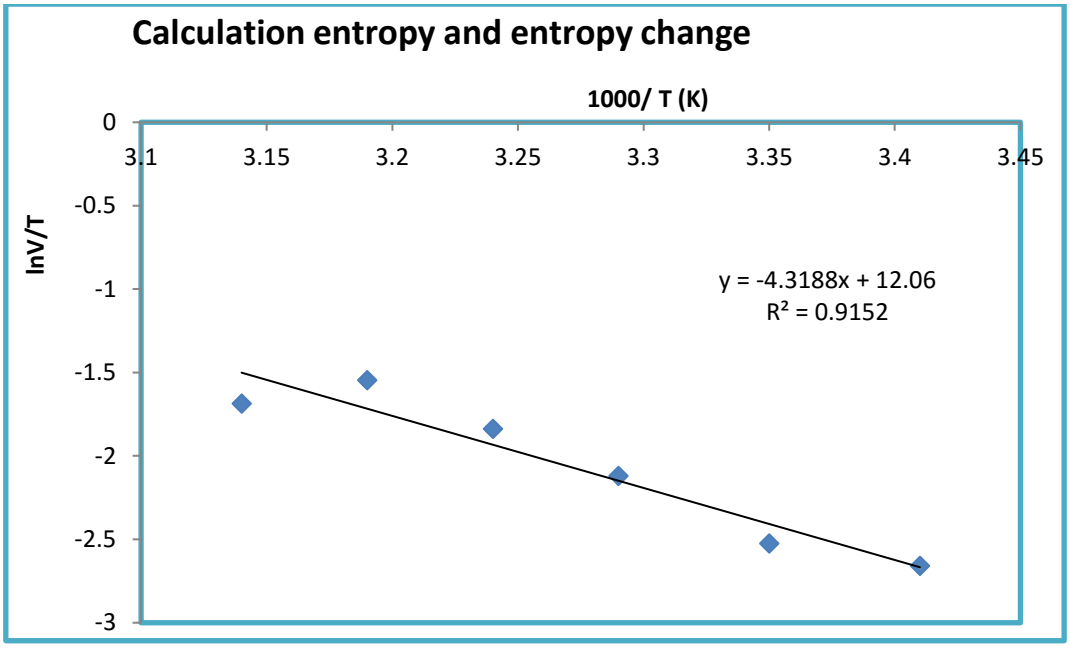

(Fig. 15): Calculation of Entropy change and enthalpy change for GST enzyme .

Table(1-4):Function was found Activation energy and enthalpy change and entropy change .

T(℃)	1000/T (K)	lnK	V/T	lnV/T	T(K)
20	**3.41**	**-1.42**	**0.07**	**-2.659**	**293.15**
25	**3.35**	**-1.21**	**0.088**	**-2.525**	**298.15**
30	**3.29**	**-0.928**	**0.12**	**-2.120**	**303.15**
35	**3.24**	**-0.657**	**0.159**	**-1.838**	**308.15**
40	**3.19**	**-0.384**	**0.213**	**-1.546**	**313.15**
45	**3.14**	**-0.543**	**0.185**	**-1.687**	**318.15**

The positive value $\Delta \mathbf{H}$ it was mean the reaction endothermic in this case the hight temperature as expected leads to a constant velocity increase and thus to increase enzymatic activity.The positive value $\Delta \boldsymbol{S}$ thereason for increase random because of the increase number of molecules in solution . This is because the correlation process many links to different ionic strength.The negative value $\Delta \boldsymbol{G}$ this mean the spontaneous process.To importance of determined the activation energy **Ea** to knowing the enzyme catalytic ability .The term given to activation energy which is called threshold energy.

Table(1-5):A s showed thermodynamic constants of GST enzyme.

Ea (KJ/mol)	ΔH $)(KJ/mol$	Δs $)(KJ/mol.K$	ΔG .K)$(J/mol$
31.73	**35.866**	**12.06**	**-3.559**

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