Kinetic and Inhibition Studies for Glutathione Peroxidase(GPx) Isolated from Pea (*Pisum sativum*) Locality

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Abstract

This research is included the isolation of glutathione peroxidase(GPx) from the pea (*Pisum sativum*), studied the factors effecting the activity of the enzyme and determination of its molecular weight. One proteinous band had been isolated by gel filtration sephadex (G-50) from the proteinous supernatant produced by ammonium sulfate saturation(65%) after dialysis and the product from (G-50) give two bands by sephadex (G-100). It was found that the first peak (Peak A) had a high activity for (GPx). The apparent molecular weight of the isolated enzymes using gel filtration chromatography was (89388± 850) Dalton for GPx .

The results also showed that the optimum conditions of GPx was obtained at (100µg/ml) of enzyme concentration using (25 mmol/l) of glutathione(GSH) as a substrate, phosphate buffer (0.5 mol/l) as a buffer at pH (7.5) for (12) minutes at (40°C). Using Lineweaver–Burk plot, the values of maximum velocity (V_{max}) and Michaelis constant (K_m) were (2.1 µmol/ min) and (1.25 mmol/l) respectively. Beside of, the study showed inhibition for antibiotics, analgesic and other types drugs on the enzyme activity, especially of psedeuphidrine and flagyl.

Keywords: Isolation, Glutathione peroxidase (GPx), *Pisum sativum, Drugs.*

الخلاصة

تم عزل إنزيم كلوتاثايون بيروكسيديز(GPx) من نبات البازاليا (*Pisum sativum*) ثم درس بعض العوامل المؤثرة على فعالية الانزيم فضلا عن تحديد وزنه الجزيئي. حيث تم فصل حزمة بروتينية واحدة بتقنية الترشيح الهلامي سيفادكس نوع G-50 للراسب البروتيني الناتج من عملية الترسيب بكبريتات الامونيوم(65%) بعد عملية الديلزة، اعطى الناتج من G-50 حزمتين بعد تمريره على الجل سيفادكس G-100. اذ أظهرت فعالية عالية للإنزيم GPx في الحزمة الاولى (قمةA)، بعدها قدر الوزن الجزيئي للإنزيم باستخدام تقنية الترشيح الهلامي التي كانت بحدود 89388 ± 850 دالتون.

تم درسة الظروف المثلى لقياس فعالية الإنزيم المنقاة من نبات البازاليا وأظهرت النتائج ان الظروف المثلى لعمل الإنزيم عند تركيز 100 مايكروغرام/مل باستخدام المحلول المنظم الفوسفات بتركيز 0.5 مول/لتر عند أس هيدروجيني 7.5 وزمن التفاعل 12 دقيقة ودرجة حرارة 40°م و25 ملي مول/لتر من مادة الأساس الكلوتاثايون واستخدمت رسومات لاين ويفر– برك لحساب قيمة السرعة القصوى (V_{max}) وثابت مكيلس (K_m) وكانت مساوية لـ 2.1 مايكرومول/دقيقة و1.25 ملي مول/لتر على التوالي. فضلا عن ذلك، أوضحت الدراسة ان

للمركبات الدوائية من المضادات الحيوية والمسكنات وأنواع أخرى تأثير تثبيطي على فعالية الإنزيم وبالأخص بسيدوفيدرين والفلاجيل.

الكلمات الدالة: عزل، كلوتاثايون بيروكسيديز، نبات البازاليا، الادوية.

Introduction

The glutathione peroxidases include the "classic" enzyme selenoglutathione peroxidase-I (GPx; GSH:H_2O_2 oxidoreductase, EC 1.11.19) [(1)]. GPx is present both in the cytosol and in mitochondria that major intracellular source of free radicals[(2)]. The formation of hydrogen peroxide and related oxygen radicals is suspected to be involved in the mechanism of nerve cell death and in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease and other diseases [(3)].

The important roles of glutathione peroxidase play in the organization of the level of peroxides different by accelerating transformation reduced glutathione (GSH) to oxidized glutathione (GSSG) after removal of peroxides such as hydrogen peroxide H_2O_2 or peroxides fat (LOOH) or organic peroxides (ROOH) as indicated in equations below, as a part of the GPx system protecting cells against oxidation and products also reduces the cell damage caused by the increase of free radicals (oxidative stress)[(4)].

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + LOOH \xrightarrow{GPx} GSSG + LOH + H_2O$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

The enzyme glutathione peroxidases plays a role in protecting cells and tissues against lipid peroxidation by removing hydrogen peroxide, as well as reduce the configuration of the hydroxyl radical and thus prevent one way to start lipid peroxidation that can occur in unsaturated fatty acids in the reproductive system[(5)].

The pea (*Pisum sativum*) is an important food-source because it provides high-quality portein, particularly for vegetarians. It contains 22–25% protein. Pea has been described as the world's second most important pulse crop after dry beans [(6)]. Peas produce isoflavonoid phytoalexins, and contain several antioxidant enzymes activities such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase (GPx) [(7)].

The aim of this research is to provide a detailed study of (GPx) involving isolation and purification from pea (*Pisum sativum*) using different biochemical techniques then characterization GPx and show the effects of some drugs on GPx .

Materials and Methods

Assay the activity of plasma glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was measured by the method of Rotruck *et al.*, 1984. Briefly, the reaction mixture contained 0.2 ml 0.4 M sodium phosphate buffer, pH 7.0, 0.1 ml 10 mM sodium azide, 0.2 ml plasma, 0.2 ml of 2 mM GSH, and 0.1 ml 0.2 mM hydrogen peroxide . The contents were incubate at 37°C for 10 min, the reaction was stopped with 0.4 ml 10% TCA and centrifuge. The supernatant was assayed for GSH content using Ellman reagent (19.5 mg DTNB [5,5^{-}- dithio bis (2-nitrobenzoic acid)] in 100 ml 0.1% sodium citrate) [(8)].

Purification of glutathione peroxidase (GPx) from pea (*Pisum sativum*)

The method given here has yielded an enzyme preparation acceptable for pea (*Pisum sativum*). All steps were performed at 4 ^{o}C unless stated otherwise.

Step I: Collection of pea (*Pisum sativum*)

Collected of the pea (*Pisum sativum*) from locality market in Mosul city and classification in Mosul university/College of science/ Biology department. Pea weighting 500 g were washed three times with distilled water and ground in a cold mortar with 1500 ml of distilled water. The plant material was frozen in liquid nitrogen three times and stored at -20° C or directly used in determination. The samples were centrifuged at 13,000 X g for 15 min at 2–5°C. The supernatants were used in protein and enzyme assays[8].

Step II: Ammonium sulfate fractionation

A supernatant was obtained from the pea (*Pisum sativum*) and protein was precipitated using 65% ammonium sulfate $(NH_4)_2SO_4$ saturation (Protein salting out)[9,10].

Step III: Cooling ultracentrifuge separation

The suspension was centrifuged at 9,000 Xg for (45) min at -4°C. The protein in precipitate and supernatant are determined using the modified Lowry method[11], GPx activity determined in each fraction[8].

Step IV: Dialysis

Dialysis was made using a semi permeable cellophane dialysis membrane with M.wt. cut off (<10000) dalton. The dialysis sac containing the suspension in (Step III) was dialyzed against 0.1M ammonium bicarbonate, was stirred with a magnetic stirrer overnight at 4 ^{o}C. The solution of dialysis was changed three times only per 3 hours during dialysis for 24 hour[10]. The protein of the dialyzed enzyme was estimated by modified Lowry method[11].

Step V: Gel filtration chromatography using Sephadex G-50 and Sephadex G-100

The sephadex gel G-50 and G-100 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 3 hours at 90 ^{o}C in a complete swelling. This procedure was used for column packing and sample application[10].

In the present study, the column of dimension 2.2×110 cm which contained a gel sephadex height of (102) cm. The exclusion limit for sephadex G-50 (30000) Dalton, while sephadex G-100 is (150000) [10]. Depending on the volume of this column which was 390 ml, it was packed with a slurry of the gel in water .

A concentrated sample (4) ml of the protein material (by freeze-dryer technique), which was obtained in (Step IV), was applied on the top of a bed sephadex G-50 and its results used to sephadex G-100, followed by distilled water.

Elution of the protein materials was carried out at a flow rate for G-50 (90)ml/ hour with a definite time (3) min, but for G-100 a flow rate (67)ml/ hour with a definite time (7) min. was used distilled water as eluant. The fractions were collected by using a fraction collector apparatus which was worked on minute system. The protein compounds in each fraction collected were detected by following the absorbance at wave length (280) nm by using UV/Visible Spectrophotometer. Peak was combined separately from the plot of an absorbance versus elution volumes with determined of GPx in each fraction[12].

Step VI: 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 kepted in a deep freeze at -20^{o}C in a tight sample tube to be used in further investigations.

Results and Discussion

Enzyme purification

The results predicted that the enzyme activity was found in the (50)% of a saturation ammonium sulfate precipitates . As shown in Table (1), the specific activity was increased after dialysis. This might be due to the removal of the small molecules (Below 14000 Dalton) and increasing the purification of GPx.

Table 1: Partial purification steps of glutathione peroxidase (GPx) from the pea (*Pisum sativum*).

Purification stage	Volume taken (ml)	Protein conc. (mg/ml)	Activity (U*/ml)	Total activity (U)	Sp.activity (U/mg protein)	Folds of Purification	Recovery %
Extraction crude	1211	6.25	0.373	451.7	0.05968	1	100
Precipitate by $(NH_4)_2SO_4$(65%)	645	7.26	0.655	422.47	0.09022	1.5	93.5
Supernatant	596	3.15	0.101	60.196	0.0321	0.5	13.3
Dialysis	661	1.89	0.263	173.84	0.1391	2.3	38.5
Sephadex G-50 (Fractions)Peak	77.2	1.01	0.89	68.71	0.881	15	15.2
Sephadex G-100 (Fractions) Peak A	27.5	0.23	1.44	39.6	6.26	105	8.8
Sephadex G-100 (Fractions) Peak B	53.5	0.31	0.51	27.285	1.645	28	6.0

U*: a mount of glutathione peroxidase (GPx) catalyzing the formation of one micromole of product per min under optimum conditions.

Gel filtration separations

This technique was applied to separate the protein as a source of enzyme, which was obtained after dialysis and by using a column containing sephadex G-50 and G-100 gel as shown in(step V). The result (Figure 1) indicated that there was mainly one peak in sephadex G-50 and after lypholization injected to G-100, were mainly two peaks(A and B) (Figure 2). The elution volume of peak (A) was (169.7) ml, while the elution volume of peak (B) was (288.3) ml. The specific activity of the enzyme peak (A) was (6.26 U/mg protein) and 105 folds of purification compared to initial extract Table (1). Peak (B) was neglected for the time, because of their low activity.

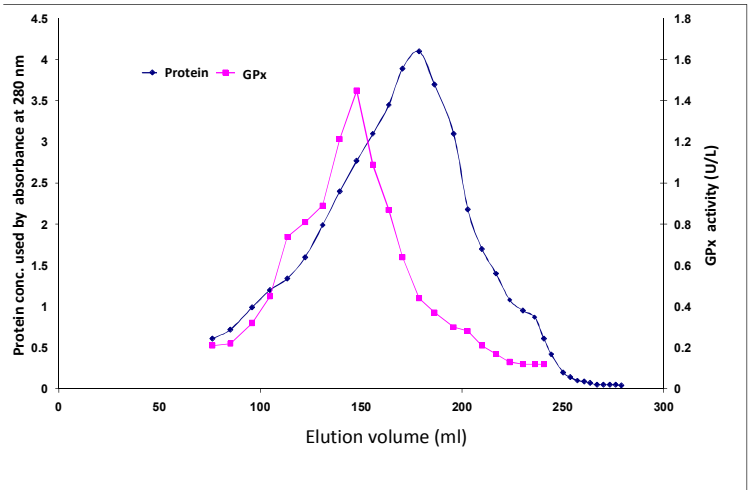

Figure 1: Elution profile Glutathione peroxidase (GPx) for pea (*Pisum sativum*) on sephadex G-50.

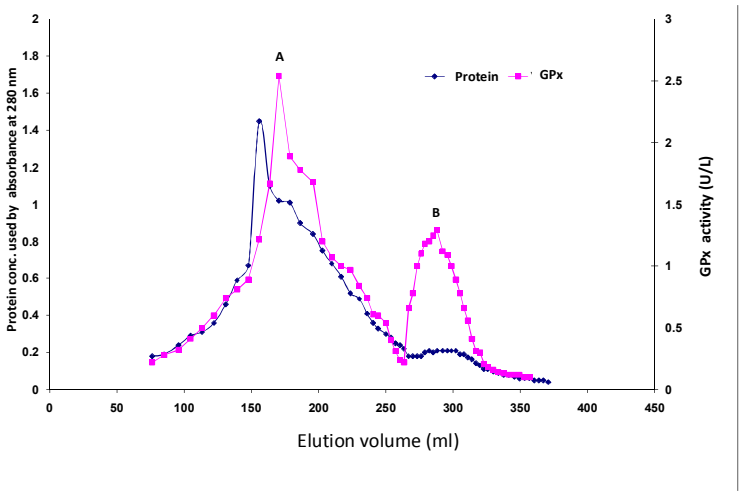

Figure 2: Elution profile Glutathione peroxidase (GPx) for pea (*Pisum sativum*) on sephadex G-100.

Molecular weight determination of GPx by gel filtration

The molecular weight of first peak (A) as a source of GPx was determined by gel filtration chromatography using sephadex G-100 column (2.2×110)cm calibrated with known molecular weight proteins that were listed in Table (2).

Table 2:

Materials	Molecular weight (Dalton)	Elution volume (ml)
Blue dextran (Void volume(V_o))	2000000	137.9
Hexokinase	100000	159.8
Bovine serum albumin	67000	187.5
α- amylase	58000	201.1
Egg albumin	45000	219.1
Pepsin	36000	239.4
Papain	23000	297.2
Tryptophan (Internal volume(V_i))	204	438.4
Unknown (peak A)	89388	*169.7

Elution volumes of known molecular weight materials on sephadex G-100.

***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).

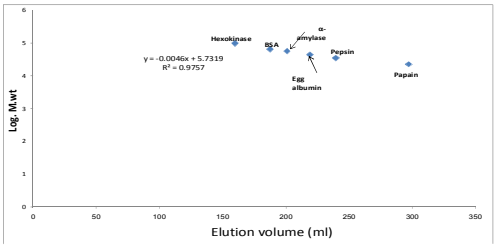

Figure 3: A plot of the logarithm molecular weights of known proteins versus elution volume on a sephadex G-100.

The molecular weight of unknown protein compound separated by the column chromatography as shown in (step V) was determined from the standard curve, which was represented by Figure (3). The comparative molecular weight of peak (A) as a source of GPx is approximately equal to (89388 ± 850) Dalton. This finding was in a agreement with the previous results where it was reported that the molecular weight of GPx was (88000) Dalton from different tissues[13] and the molecular weight of the enzyme of some mammals and fish species have been reported as follows: rat liver, 75000-76000 Dalton [14], 80000 Dalton [15] and the M.wt. from carp hepatopancreas 100000 Dalton [16]

Optimum Conditions for GPx Activity

To develop assay conditions where GPx from pea (*Pisum sativum*) shows a maximum activity, a series of experiments were performed. These included enzyme concentration, pH of the assay conditions, incubation time, incubation temperature and substrate concentration[17].

1.Effect of enzyme concentration on GPx 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 plasma between (0-100) µg/ml as shown in Figure (4).

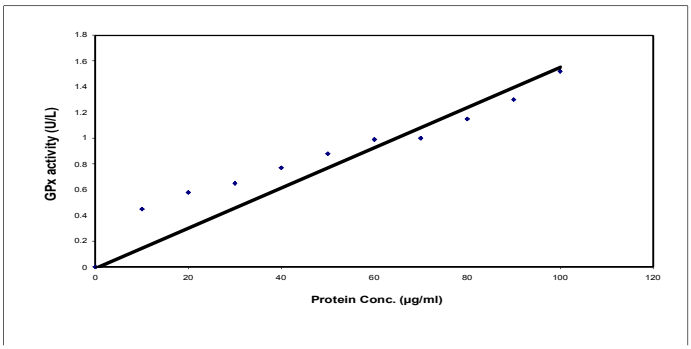

Figure 4: Effect of different protein concentrations on GPx activity

The result indicated that the enzyme activity increased with increasing the concentration of protein as a source of the enzyme. For the next experiment (100)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 GPx activity:

The activity of enzyme was measured in the presence of different concentrations of buffer solution within the range (0.1-0.7) mol/liter of phosphate buffer at pH 7.0. Maximum activity was obtained using (0.5) mol/liter of phosphate buffer (Figure 5).

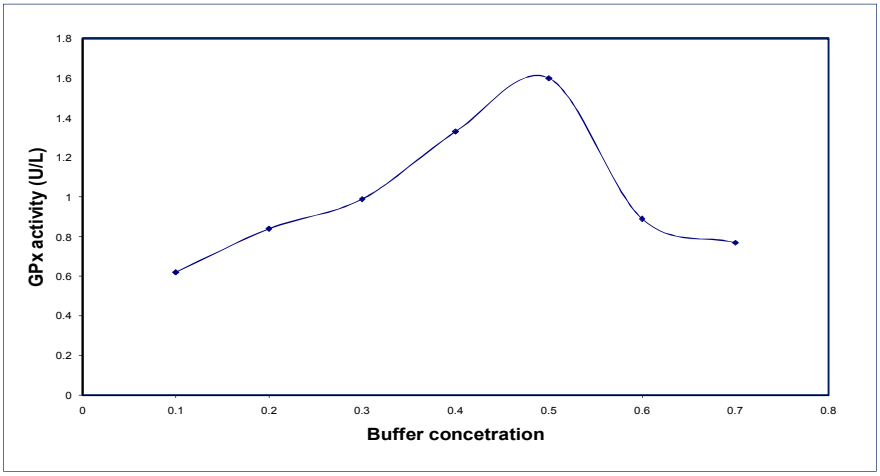

Figure 5: Effect of buffer concentrations on GPx activity.

2.2.Effect of pH on the GPx activity:

The influence of pH upon the activity of GPx was investigated by using (50 µg/ml) as a source for enzyme in (0.5) mol/liter phosphate buffer. The assay conditions were conducted in the same manner as described earlier at pH range of (2.0 - 9.0). Maximum GPx activity was obtained at pH (7.5) as indicated in Figure (6). Extremes of pH above of 7.5 can lead to denaturation of the enzyme and decreased activity, because the structure of the catalytically active site for enzyme depends on the ionic character of the amino acid side chains[9].

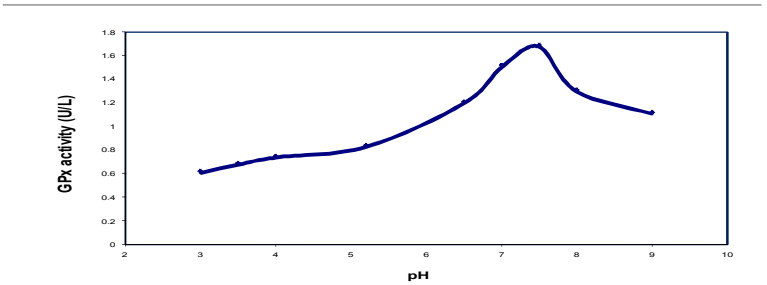

Figure 6: Effect of pH on GPx activity using (0.5) mol/liter phosphate buffer and (100µg/ml) as a source for the enzyme.

In other studies, the optimum pH of the enzyme was about (7.0) from the liver of Japanese sea bass [18], human erythrocyte (pH 8.5)[19] and carp hepatopancreas (pH 8.0) [16].

3. Incubation time as a function of enzyme activity

To determine the stability of GPx 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) min. in (25 °C) incubation(Figure 7).

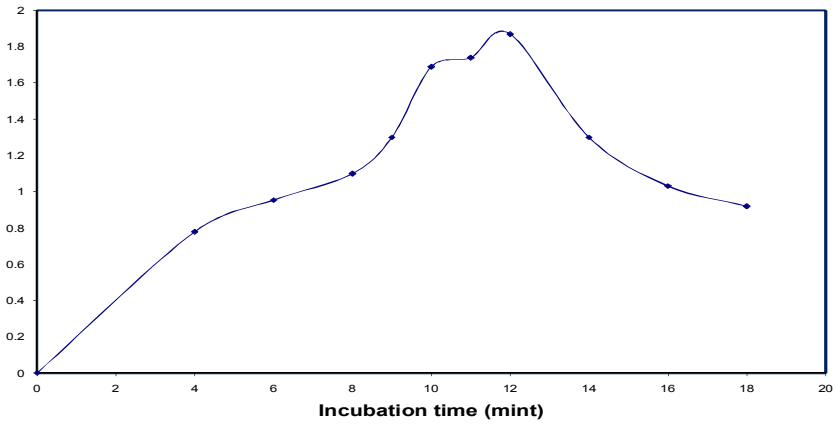

Figure 7: Effect of incubation time on GPx activity.

4.Effect of temperature on GPx 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 (40 °C) then dropped gradually after that (Figure 8). This increase is the result of the increased number of molecules having sufficient energy to pass over the energy barrier and form the products of the reaction. Further elevation of the temperature results in a decrease in reaction velocity as a result of temperature-induced denaturation of the enzyme [9].

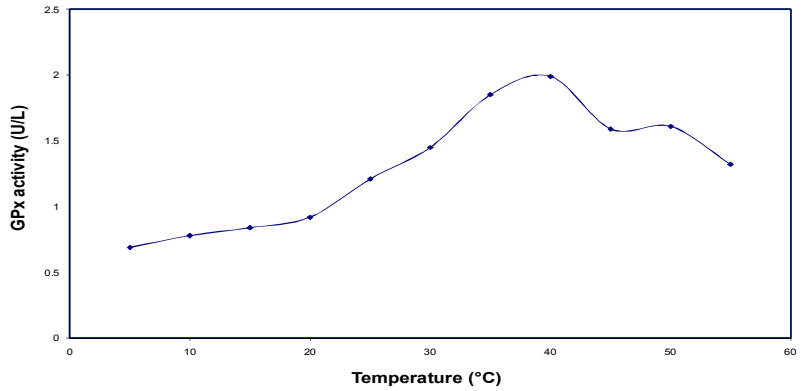

Figure 8: Effect of temperature (°C) on GPx activity.
Nagai *et al.* observed the optimum temperature for the enzyme activity was around 40°C [18] and Nakano *et al.* reported that enzyme were stable at 40°C for 10 min[16] .

5.Effect of substrate concentration on the enzyme activity:

To determine the effect of substrate concentration [GSH] on the enzyme activity, a series of experiments were performed where the concentration of the substrate was varied Figure (9).

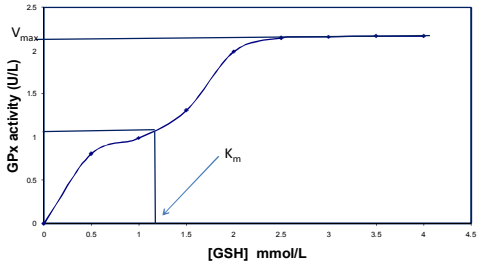

Figure 9: Effect of substrate concentration [GSH] on the activity of purified GPx.

The line Weaver-Burk plot by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained Figure (10) giving a K_m value of (1.25 mmol/liter) and V_{max} (2.1 µmol/min.).

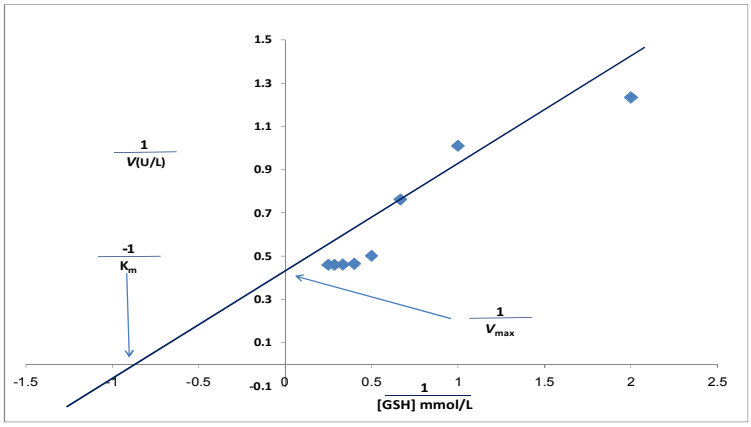

Figure 10: Line Weaver-Burk plot of partially purified GPx from pea (*Pisum sativum*).

6. Optimum conditions of the purified glutathione peroxidase:

The optimum conditions of the purified Glutathione peroxidase from pea (*Pisum sativum*) were obtained in the following Table (3):

Table 3: Optimum conditions of the purified Glutathione peroxidase.

Enzyme Conc. (µg/ml)	Buffer Conc. (mol/liter)	pH	Time (min)	Temp. (°C)	Time (min)	pH	K_m (mmol/liter)	V_{max} (µmol/min)
100	0.5	7.5	12	40	12	7.5	1.2	2.1

7. The effect of some drugs on the enzyme activity:

The results showed decreased activity of enzyme with increased concentration (20, 25, 30 mM) of different types of drugs especially of psedeuphidrine and flagyl (Table 4), which were similar in to others reported for example, Asha *et al*. for tetracycline[20] and Galal *et al*. for paracetamol [21].

The increased of pro-oxidants produced from many types of drugs for example, paracetamol (Acetaminophen)[22] that increased hydrogen peroxide and decreased activity of enzyme (GPx) by used of H_2O_2 (as indicated in equation below), and that might be induce oxidative stress, either through creating reactive oxygen species or inhibiting antioxidant systems[22].

$$2\,GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

The oxidative stress produced by these chemicals can damage cells and tissues, for example increased paracetamol can cause fatal damage to the liver, partly through its production of reactive oxygen species[23].

Table 4 : Effect of drugs compounds on GPx activity.

Chemical compounds	Concentration of Compounds (mM)	GPx activity (U/L)	*% Inhibition
Metoclopramide	20	1.2	42.85
	25	1.02	51.42
	30	0.91	56.66
Chloramphnicol	20	1.8	14.28
	25	1.3	38.1
	30	1.09	48.1
Paracetamol	20	2.0	4.76
	25	1.9	9.52
	30	1.6	23.8
Anhydrous theophylline	20	1.78	15.23
	25	1.65	21.42
	30	1.22	41.9
Anhydrous caffeine	20	1.74	17.14
	25	1.32	37.14
	30	1.11	47.14
Phenylphrine	20	1.96	6.66
	25	1.62	22.85
	30	0.98	53.33
Flagyl	20	1.154	45.04
	25	0.853	61.9
	30	0.29	86.19
Ceramide	20	2.04	2.85
	25	1.89	10.0
	30	1.38	34.28
Psedeuphidrine	20	1.15	45.23
	25	0.79	62.38
	30	0.35	83.33
Neomycin	20	2.04	2.85
	25	1.79	14.76
	30	1.56	25.71
Allopurinol	20	1.33	36.66
	25	1.09	48.1
	30	0.85	59.52
Tetracycline	20	1.89	10.0
	25	1.25	40.47
	30	1.03	50.95
Calamine	20	1.96	6.66
	25	1.66	20.95
	30	1.14	45.71

***The value of GPx standard activity (2.1 U/L).**

Beside of, GSH present in the reaction's solution can nonenzymatically forming many complexes with by bonding with a variety of heavy metals, therefore the calamine might be decreased the GPx activity with increased because of contain zinc[24].

It is generally thought that reactive oxygen species (ROS) are involved in a wide variety of diseases, including ischemia-

reperfusion, cancer and various types of inflammation, but when the therapeutic used produce oxidants compounds that may be decreased of antioxidant enzyme (for example GPx) and increased the oxidative stress. In this commentary, the author takes a look back at important oxidative drugs [25].

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