Purification of Glutathion Peroxidase and Determination of it's Relation with some biochemical parameters in type II Diabetic patients

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Abstract:

The research includes the determination of Glutathione peroxidase activity and its relationship with some biochemical parameters in serum of patients of diabetes type II. The activity Glutathione peroxidase, Glutathione-s-transferase and the level of Glutathione are significantly decreased ($p<0.05$). whereas the activity of Superoxide dismutase, Polyamine oxidase and the levels of glucose, Glycocylated hemoglobin, Malondialdehyde, insulin hormone and insulin hormone resistance are increased significantly in patients compared to that of healthy subjects.

A positive correlation is found between Glutathione peroxidase with Glutathione-s-transferase ($r=0.417$), Superoxide dismutase ($r=0.644$) and Glutathione ($r=0.521$), whereas a significant negative correlation is found between Glutathione peroxidase with Malondialdehyde ($r= -0.452$).

The present study also deals with partial purification of Glutathione peroxidase from serum of Type II diabetic patients by dialysis, and ion exchange DEAE-cellulose techniques. One proteinous peak of Glutathione peroxidase activity is obtained with specific activity of 0.128 U\mg protein and purification fold 10.27 compared to crude enzyme.

The kinetic characteristics of partially purified Glutathione peroxidase are studied. The maximum activity is obtained at 12.8μg of enzyme, Na-K phosphate buffer solution at pH 7.3, temperature 37°C, incubation time 8 min., with 10 mM Na^{+1} . Vmax and Km values of partially purified Glutathione peroxidase with the substrate, Glutathione are found 0.31unit/ml and 1.538 mM respectively.

Abbreviation: Diabetes mellitus: DM, Glutathione: GSH, Glutathione peroxidase: Gpx, Glutathione-S-transferase: GST, Glycosylated haemoglobin: HbA1c, Malondialdehyde: MDA, Polyamine oxidase: PAO, Red Blood Cells: RBC, Reactive Oxygen Species: ROS, Superoxide dismutase: SOD.

تنقية انزيم كلوتاثايون بيروكسيديز ودراسة علاقته مع بعض المتغيرات الكيموحيوية في مرضى النوع الثاني من داء السكر

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قسم الكيمياء   قسم الكيمياء   قسم التحليلات المرضية

كلية التربية   كلية التربية   المعهد التقني

جامعة الموصل   جامعة الموصل   الموصل

ملخص البحث:

تضمن البحث تحديد مستوى فعالية انزيم كلوتاثيون بيروكسيديز وعلاقته ببعض المتغيرات الكيموحيوية في مصل دم المصابين بالنوع الثاني من داء السكر. فقد وجد ان فعالية انزيمي كلوتاثايون بيروكسيديز وكلوتاثايون–S–ترانسفيريز فضلا عن مستوى الكلوتاثايون انخفضت معنويا ($p<0.05$)، في حين ان فعالية انزيمي سوبر اوكسايد ديسميوتيز وبولي امين اوكسيديز، ومستويات الكلوكوز والهيموكلوبين المتسكر والمالوندايالديهايد وهرمون الانسولين ومقاومة هرمون الانسولين ارتفعت معنويا في المصابين مقارنة بالسليمين.

كما وجد ان العلاقة طردية بين انزيم كلوتاثايون بيروكسيديز وكلا من انزيمي كلوتاثايون–S–ترانسفيريز($r=0.417$) وسوبر اوكسايد دسميوتيز($r=0.644$) والكلوتاثايون ($r=0.521$). بينما كانت عكسية بين انزيم كلوتاثايون بيروكسيديز والمالوندايالديهايد ($r= 0.542 -$)

كذلك تناولت الدراسة التنقية الجزئية لأنزيم كلوتاثايون بيروكسيديز من مصل دم المصابين بالنوع الثاني من داء السكر، باستخدام تقنيات الفرز الغشائي والتمرير عبر المبادل الأيوني DEAE-cellulose. وقد تم الحصول على قمة بروتينية واحدة تمتلك فعالية أنزيم كلوتاثايون بيروكسيديز بفعالية نوعية 0.128 وحدة انزيمية/ ملغم بروتين بعدد مرات تنقية 10.27 مرة مقارنة بالانزيم الخام.

ودرست الخصائص الحركية لأنزيم كلوتاثيون بيروكسيديز المنقى جزئيا وتبين ان الفعالية القصوى للأنزيم كانت عند 12.8 مايكروغرام من الانزيم، ومحلول الصوديوم– بوتاسيوم فوسفات المنظم عند دالة حامضية 7.3 ودرجة حرارة 37°م وزمن حضن 8 دقائق. وباستخدام ايون الصوديوم بتركيز 10 مليمولار، كما وجد ان قيمتي السرعة القصوى وثابت ميكايليس– مينتون كانتا 0.31 وحدة انزيمية / مل و1.538 ملي مولار على التوالي.

Introduction:

Diabetes mellitus (DM) is a metabolic disorder of the endocrine system. The disease is found in all parts of the world and it is rapidly increasing worldwide. People suffering from diabetes cannot produce or properly use insulin, so they have high blood glucose (Dallatu *et al*., 2009). Type II DM, non–insulin-dependent diabetes mellitus, in which the body does not produce enough insulin or properly use it. Type II DM is the most common form of the disease, accounting for 90%–95% of cases, Hyperglycaemia in diabetes generates reactive oxygen species (ROS) and disturbs the endogenous antioxidant defence system that causes oxidative stress.

Oxidative stress in turn generates several secondary complications in diabetic patients. (Rajeshwari *et al*., 2011). Free radicals are generated as by-products of normal cellular metabolism, however, several conditions are known to disturb the balance between ROS production and cellular defense mechanisms. This imbalance can result in cell dysfunction and lead to damage of cellular organelles and enzymes (Robertson, 2004). The role of oxidative stress has been reported in various diabetic complications. An increased reactive oxygen species and insufficient antioxidant activity are associated with diabetes mellitus, which is mainly responsible for diabetic pathogenesis (Shanmugam *et al*., 2011)

The level of antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes. Also, this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses (Moussa , 2008).

Glutathione peroxidase (GPx) (EC1.11.1.19), the selenium–containing peroxidases is enzyme found in the cytoplasm, mitochondria, and nucleus. Glutathione peroxidase metabolizes hydrogen peroxide to water by using reduced glutathione as a hydrogen donor (Costa *et al*., 2012). Glutathione disulfide is recycled back to glutathione by glutathione reductase, using the cofactor NADPH generated by glucose 6-phosphate, dehydrogenase (Shakya *et al*., 2012).

In erythrocytes and other tissues, GPx catalyzes the destruction of H_2O_2 and lipid hydroperoxides by reduced glutathione, protecting membrane lipids and hemoglobin against oxidation by peroxides (Murray *et al*., 2012). This reaction is important, since accumulation of H_2O_2 may decrease the life span of the erythrocyte by causing oxidative damage to the cell membrane, leading to hemolysis. Additionally, GPx also functions to remove harmful $ONOO^-$ (Murray *et al*., 2012).

Materials and Methods
Laboratory analyses

Sample collection : A total of 40 diabetic male patients and 40 healthy subjects aged between 45 to 50 years were included in the study. 10 ml of fasting venous blood samples were drawn from the cubital vein into blood tubes, and the serum was immediately separated from the cells by centrifugation at 13000 xg for 10 min, stored at −20°C, and then they were analysed.

Serum glucose was estimated by enzymatic method GOD – POD by using analysis kit syrbio-france (Tinder,1969).

Two milliltre of whole blood was collected in a tube containing ethylenediamine tetraacetate as an anticoagulant for determination of HbA1c for assessment of glyceamic state. Hb A1c was measured with a cation exchange chromatography method assessed glycaemic control. The procedure is a microchromatographic methodology for the quantitation of glycosylated haemoglobin (Maquart *et al*., 1984).

Fasting blood GSH was estimated by the method of (Sedlak and Lindsay 1968) and MDA was estimated by Akande and Akinyinka (2005) method.

Insulin was assayed by ELISA using monobind, USA Kit which depends on the high affinity and specificity, TREA Co. China. (Gerbitz,1980), and the Insulin Resistance (IR) was calculated by the following equation:

IR = [fasting insulin (μIU/mL) × fasting glucose (mmol/L)] / 22.5 (Maheria *et al*., 2011).

Polyamine Oxidase assay: PAO activity was monitored spectrophotome- trically. The oxidation of spermine by the enzyme is carried out with potassium ferricyanide as electron acceptor. The decrease in absorbance at 410 due to the reduction of potassium ferricyanide (Dahel *et al*., 2001).

Glutathione –S- transferase activity: GST activity was determined using the method of Habig *et al* (1974). This assay is based on the principle that GST catalyzes the conjugation of glutathione with 1-Cl-2,4-dinitrobenzene. The formation of 2,4 dinitrophenyl-S- glutathione, which has maximum absorbance at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

Superoxide dismutase assay: SOD was assayed by the modified method of Brown and Goldstein (1983), based on the ability of SOD to inhibit the formation of formazan at 560 nm by reducetion of NBT by superoxide.

Protein Determination: Total protein concentrations of serum samples were assayed by modified Lowry method (Scharcterle and Pollack

1973), using a calibration curve at 650 nm, established with bovine serum albumin as a standard.

Purification of Gpx:

Gpx assay: Gpx activity was measured by the method of Rotruck *et al.* (1984). Briefly , the reaction mixture contained 0.2ml 0.4M sodium phosphate buffer, pH 7.0, 0.1 ml 10 mM sodium azide, 0.2 ml of serum sample, 0.2 ml GSH, and 0.1ml 0.2mM Hydrogen peroxide. The content were incubated at 37°C for 10min, the reaction was stopped with 0.4 ml 10% TCA and centrifuged. The supernatant then was used to assayed unreactant GSH using Ellman reagent (19.8 mg DTNB in 100 ml 0.1% sodium citrate) spectrophotometrically at 412 nm.

Purification of Gpx from serum of diabetes mellitus type II patients
Step I : Dialysis

Ten milliliter of serum was dialyzed against 0.4 mM phosphate buffer, pH 7. The solution was stirred overnight with a magnetic stirrer at 4°C. The buffer was changed every 6 hrs. during dialysis (Robyt and White,1987).

Step II: Ion Exchange Chromatography

A ten milliliter of dialyzed enzyme solution was applied on DEAE-cellulose anion exchanger column (2.5x40)cm, followed by phosphate buffer, pH 7. Elution of the protein was carried out at a flow rate (50) ml/hour with a definite time (6) min, using phosphate buffer as eluant. The fractions were collected and the protein in each fraction was detected by following the absorbance at wavelengh 280 nm. Peak was combined separately from the plot of an absorbance versus elution volumes and Gpx was determined in each fraction (Rotruck *et al.*, 1984).

Lyophilization: The fractions which contain Gpx activity were collected and concentrated by lyophilizer at -20 °C .

Properties of the purified Gpx:

Enzyme concentration: The partially purified Gpx was added to the reaction mixture with different concentrations to study the best concentration of enzyme. The partially concentrated-elution volume used were 50,100,150, 200, 250,300 and 350 µl. of concentrated enzyme after Lyophilization (2.56, 5.12, 7.68, 10.24, 12.8, 15.36 and 17.92 µg), respectively.

Buffers type: The partially purified enzyme reaction mixture were incubated with the following buffers: Tris-HCl, Na-Na-phosphate, Na-K-phosphate, Citric acid, Sodium acetate.

pH range: The purified enzyme reaction mixture were incubated at different pH values 6.4, 6.7, 7, 7.3, 7.6, 7.9 and 8.2 using more effective buffer above on enzyme reaction.

Temperature: The partially purified Gpx activity was measured at 10, 20, 30,40, 50, 60, 70 and 80 °C.

Incubation time: The partially purified enzyme is incubated at different times (5,6,7,8,9,10,11,12,13,14 and 16 min) to study the best time for reaction.

Metal ions: All these metal ions Na^{+1}, K^+, Mg^{+2} and Ca^{2+} were applied on the reaction mixture with a concentration of 10mM.

Substrate concentration: The substrate, (GSH) was applied on the reaction mixture at different concentrations (0.5,1,1.5,2,2.5,3,3.5,4,4.5 and 5 mM).

Statistical analysis: All values are reported as mean ± SEM. Statistical significance was assessed using Student's t-test . P value less than 0.05 was accepted as the significance level.

Results and Discussion:

As shown in table 1, serum glucose was significantly increased in patients with type II DM compared to healthy subjects . A similar result was obtained by Lasker *et al*.(2010), who showed a significantly elevated in glucose levels in matching patients. This increase may be due to insulin resistance action , which lead to stop glycolysis or induce Gluconeogenesis and Glycogenolysis (Harvey and Ferrier, 2011).

A significantly higher in HbA1c levels were observed in patients with type II DM compare with healthy subjects (Table 1). HbA1c in RBC was determined, and it was shown that it was significantly higher in female diabetic patients type II DM than in normal persons (AL-Lehebe, 2013). HbA1c level was significantly higher in the diabetic subjects compared with control subjects(Chandrasekharan *et al.,*2011).

Baynes (1991) concluded that diabetes with complications is associated with increased chemical modification of protein and lipids. Hyperglycemia leads to protein glycation, glucose auto-oxidation and fatty acid oxidation, which may contribute to increased ROS generation.

The results showed a significant lowering in GSH levels in patients compared to controls (Table 1). Serum GSH showed significant decrease in patients with type II DM compared to controls (Kharb *et al*., 2000). Al-Bajari's study (2013) showed a significant decrease of antioxidants, GSH in patients of both sexes suffering from Type II DM.

GSH is an essential antioxidant for recycling of vitamin E and C and is very powerful in helping the body fight against the free radicals (El-Missiry, 2012). GSH also participates in the cellular defense system against oxidative stress by scavenging free radicals and reactive oxygen intermediates. Thus, a decrease in GSH level in diabetic patients increases the sensitivity of cells to oxidative stresses (Yoshida *et al*., 1995).

MDA was greatly increased in diabetic compared to non-diabetic subjects (Table 1). Aljarah (2005) found a significantly increasing of MDA in serum in diabetic compared to healthy control. MDA and acrolein (CH_2=CHCHO), potentially toxic agents which spontaneously formed from aminoaldehydes, and induce oxidative stress in mammalian cells (Bjelakovic *et al.*,2010). Elevated glucose levels induce oxidative stress that is ultimately reflected by the increased MDA levels in the erythrocytes and liver (Senel *et al.*, 1997)

Our results demonstrated a significantly increasing in insulin hormone and insuline resistance levels in patients compared to controls (Table 1). The results of Al-Bajari (2013) showed that there is an increase in the insulin hormone level and insulin resistance for patients a long time suffering from type II DM.

Obesity is considered major contributors to Type II diabetes, roughly 90% of individuals with type II are obese. These conditions can predispose one to the condition of insulin resistance, which is characterized by the hypersecretion of insulin (hyperinsulinemia) (Kaczmar, 1999).

Glucocorticoids play an important role in the development of peripheral insulin resistance by altering cellular glucose metabolism. Hence, both excess endogenous and exogenous glucocorticoids acutely impair insulin sensitivity and chronically promote development of the metabolic syndrome including obesity, diabetes and hypertension (Asensio *et al.*, 2004).

Table 1 Biochemical parameters in the serum of healthy controls and diabetic patients

Biochemical parameters	Control	Diabetic	
Glucose(mmol/L)	4.55±0.832	12.11±2.93	S
HbA$_{1c}$(%)	5.5±0.56	9.68±1.38	S
MDA(µmo/L)	5.10±0.56	7.65 ±1.84	S
GSH(µmo/L)	14.18±1.79	8.71±1.53	S
Insulin(µ lU/ml)	14.55±1.59	23.2±2.1	S
Insulin resistance	1.82±0.13	2.47±0.239	S

Data are represented as mean ± SD. *P<0.05 compared to control (Student t-test).
S: significant, NS: Not significant.

It has been found that the Gpx activity was significantly decreased in patients with type II DM diabetes compared to the controls (Table 2). These results agreed with the results of Ramakrishna and Jailkhani (2008) which observed that erythrocyte GPx activity was lower in type II DM patients compared to normal subjects. Also, the diabetic rats exhibited lower activity of GPx, in hepatic and renal tissues as compared to normal rats (Shanmugama *et al*., 2011). The reduction in GPx activity associated with enhanced oxidative stress in diabetic may be related to increased H_2O_2 levels (Arnaiz *et al*.,1997).

The present study indicates a significant decreasing of GST activity observed in patients than control group (Table 2). The activity levels of GST were significantly decreased in Type II DM males and females patients than in healthy subjects in a North Indian population (Verma *et al*., 2013), but Sarkar *et al.*, (2010) found that there was no significant change in GST between type 2 DM patients and healthy controls.

The antioxidant enzyme, GST in RBC, have a likley role in the glycation of hemoglobin in diabetic patients. Therefore, a significant decrease in the levels of GST indicated protection against oxidative stress (Nalini, 2011).

Table 2 explains a significant increase in SOD activity in patients compared with healthy subjects. Ahmed *et al*. (2006) also reported that serum SOD activity was significantly higher in type 2 diabetic patients as compared to the control. The activity of SOD increases in erythrocytes depend on the increase in electrolyte concentrations like copper and zinc which are parts of SOD enzyme (Trlvier *et al*., 1992).

PAO activity was significantly increased in patients with type II DM compared to healthy subjects. Recently, the determination of polyamine oxidase activity in female patients with type II DM was studied. It was found that the activity of PAO in red blood cells and plasma were significantly higher than that of normal subjects (AL-Lehebe, 2013). As well as, it was reported that the PAO activity in children with type I DM was very high (Bjelakovic *et al*., 2010). In fact, the pancreas is the organ containing the highest concentration of intracellular Spermine and Spermidine which they have a role in inhancing insulin secretion (Rosenthal and Tabor, 1956). Spermine and Spermidine are degraded by PAO, which modulates membrane fusion processes in the beta-cells (Bungay *et al*., 1984).

Table 2: Activity of enzymes in the serum of healthy controls and diabetic patients

Enzymatic parameters	Control	Diabetic	
GPx(μmol/min)	0.724±0.029	0.495±0.022	S
Gst(U/L)	12.26±1.01	6.3±1.19	S
SOD	0.015±0.007	0.025±0.015	S
PAO(U/ml)	0.045±0.011	0.092±0.014	S

Data are represented as mean ± SD. *P<0.05 compared to control (Student t-test). S: significant, NS: Not significant.

Correlation between Gpx and biochemical parameters

A significant positive correlation was found between Gpx and Gst (r=0.417), SOD(r=0.644), GSH(r=0.521), and non significant correlation with insulin hormone. The results also indicated a significant negative correlation of ARE with MDA(r= -0,452) whereas non significant negative correlation with PAO, glucose, HbA1c and IR.

Purification Gpx: A representative purification profile of the Gpx active fractions is summarised in Table 1. Diabetic patients Gpx was purified to about 10.27 fold compared to crude enzyme with 177% activity recovery. From the elution profile of proteins, which is shown in Fig.1, Gpx active fractions were represented in the fractions between 17 to 33.

Table 3: Glutathione peroxidase   purification steps from patients with type 2 diabetic individuals serum

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Specific activity (U/mg protein)	Yield %	Purification fold
Crude	10	12	5.15	0.429	100	1
Dialysis	10	8.75	5.22	0.596	101	1.38
Ion exchange	83	2.07	9.13	4.41	177	10.27

*U: A unit is defined as that amount of enzyme which reduce 1 micromole of GSH per min.
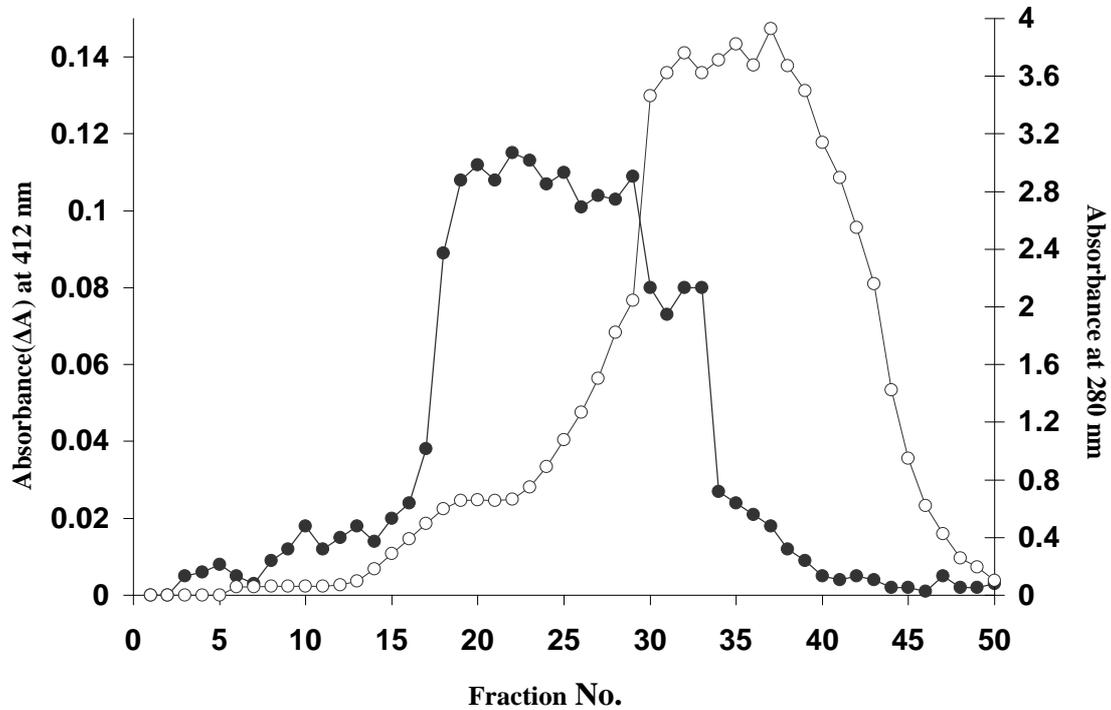
Figure 2: Elution profile of Gpx purification enzyme activity in Type II diabetic patients by DEAE- cellulose chromatography culomn (40×2.5 cm). ● **Gpx activity at 270nm ,** ○ **Protein at 280 nm**

By *et al* (1979) found one peak of GPx activity with 14.2 and 8.39 U/ml and the specific activity respectively which purified from human placenta. Purification of Gpx on DEAE-cellulose Column from erythrocyte appeared in the eluent fractions (110 to 290 ml.) with Sp. Ac. 274 units/mg protein (Mills, 1959).

Effect of enzyme conc. on reaction velocity : Fig.(2) showed that Gpx activity was linearly proportional to the amount of protein up to 12.8 µg .
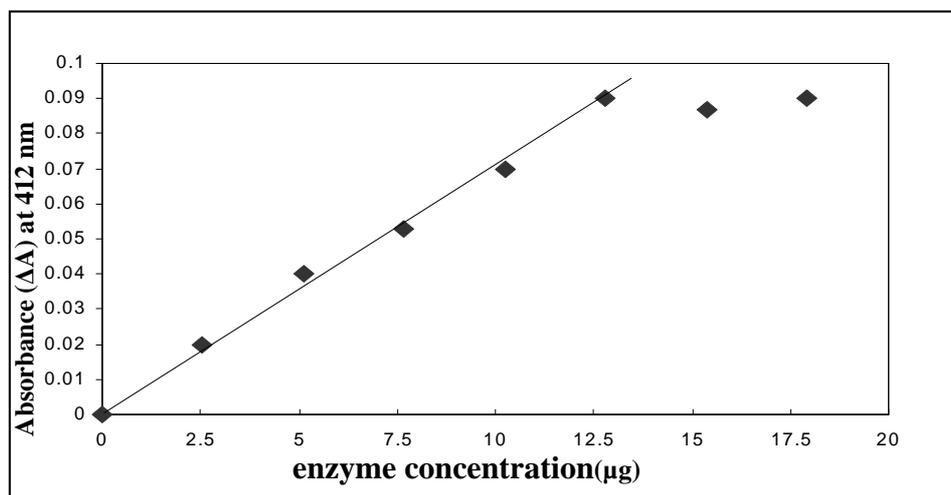

(Fig.2) Effect of enzyme concentration on the activity of the partially

Effect of buffers and pH on the reaction velocity : Gpx

activity assayed with different buffers. It was found that Gpx activity exhibited maximum activity with Na-K phosphate buffer Fig. (3)
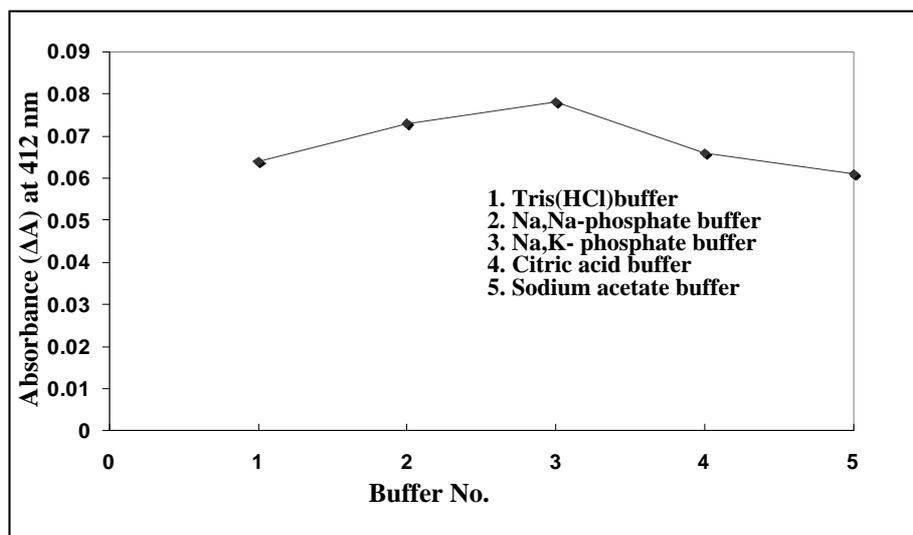

Fig. 3: Effect of buffer type on the activity of the partially purified Gpx

When assayed the Gpx activity at different pH (6.4 to 8) of Na,K-phosphate buffer, it exhibited optimum activity at pH=7.3(Fig.4).This value was different from those of human erythrocyte pH 8.5 (Awasthi *et al*., 1975) and rat lung pH 8.8 (Chiu *et al*., 1976). The optimum pH for different enzymes varies depending on the nature of catalytic groups. The stability of the tertiary and / or quaternary structures of the enzyme may also be pH dependent and may affect the velocity of the enzyme reaction , especially at extreme alkaline or acidic pH values (Robyt and White 1987).
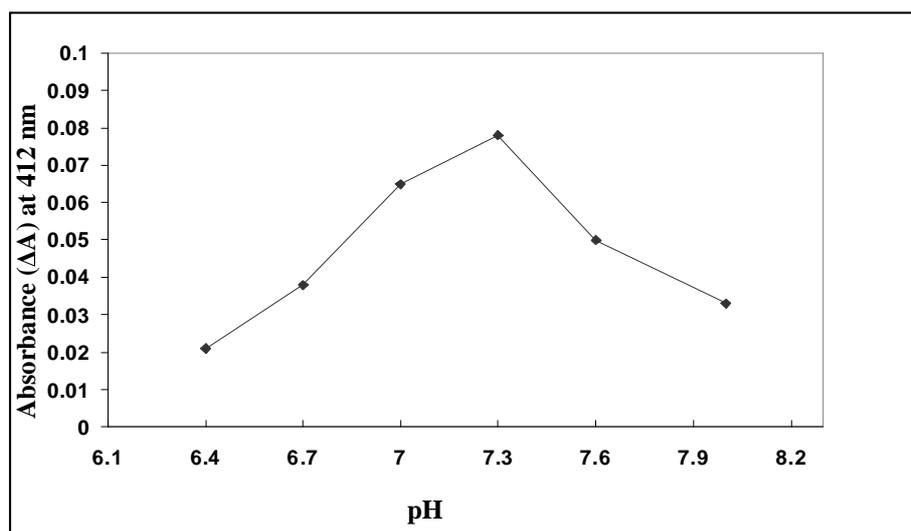
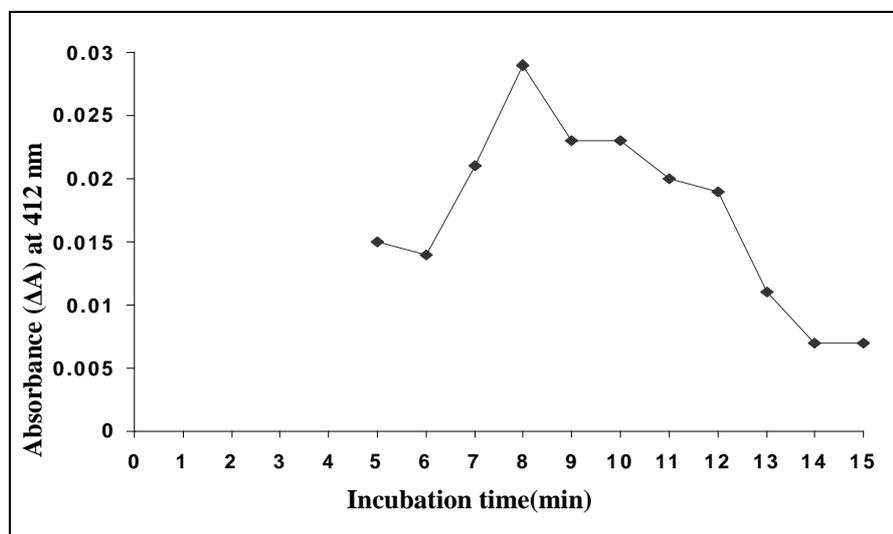**Fig.4: Effect of pH values on the activity of the partially purified Gpx**

Effect of temperature on the reaction velocity: The

activity of Gpx was determined at different temperature (10-80) °C. It is clear from the results presented in Fig.(5) that Gpx exhibited a maximum tempreture at 37°C .
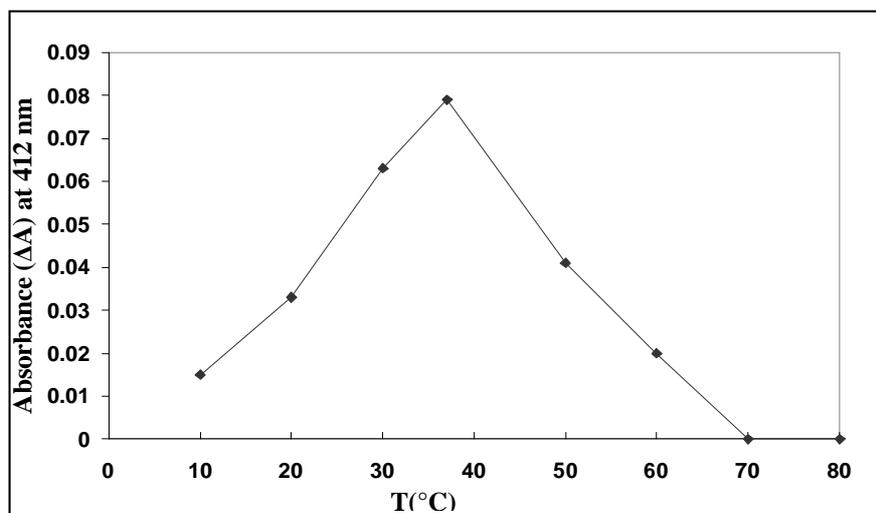
**Fig. 5: Temperature effect on the activity of the partially purified Gpx**

The optimum temperature for the enzyme activity from the Liver of Japanese Sea Bass *Lateolabrax japonicus* was around 40°C (Nagai *et al*.,2002). The enzyme reaction has optimum tempretures and then rapidly decrease with further tempreture increase. The loss of activity at the higher tempretures   is due to thermal conformational (denaturation) changes of the enzyme. Most enzymes are inactivated at tempretures above 55-60 °C (Robyt and White 1987).

Effect of enzyme incubation time on the reaction

velocity : The activity of Gpx was determined every 1 min. The maximum

activity was at 8 min (Fig.6)

(Fig.6) Effect of incubation time on the activity of the partially purified Gpx

Effect of ions on the activity of Gpx

Effect of ions on the activity of Gpx : Gpx activity was determined with different ions (Na^{+1}, K^{+1}, Mg^{+2}, Ca^{2+}) conc. at 10mM. The results in Fig (7) idicated that each of tested ions had an activation effect on Gpx activity. However Na^{+1} ion had the most activation effect on Gpx activity.
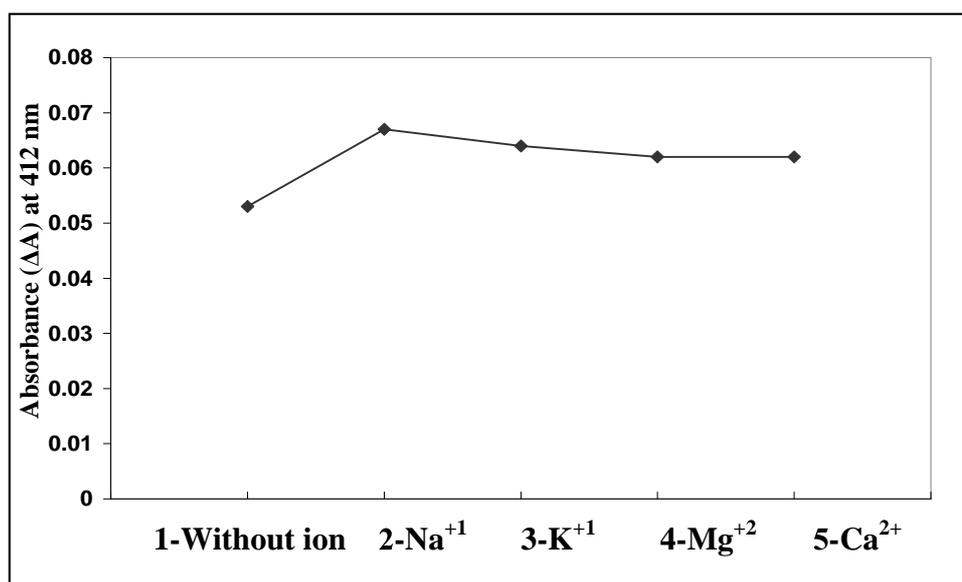
Fig.7: Effect of ions (10mM) on the activity of the partially purified Gpx

Effect of substrate concentration on enzymatic activity

Effect of substrate concentration on enzymatic activity: The effect of different substrate concentration was tested by incubating different substrate concentration (0.5 to 5 mM) with the same amount of the enzyme. The enzyme activity was plotted against substrate concentration. The results in Fig.(8) demonstrated that 3.5mM of substrate was the best of the optimal Gpx activity. Our study illustrated the values of Vmax and Km were and 0.31µmol/min and 1.538 mM respectively .
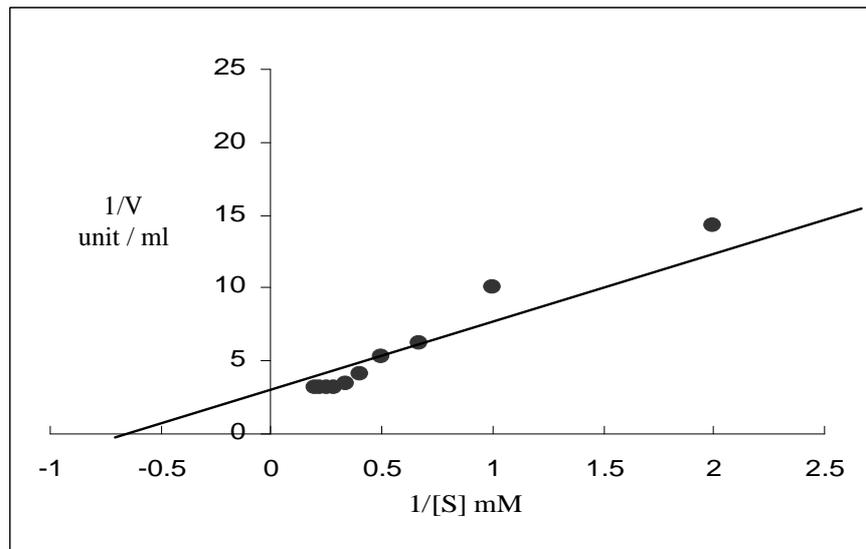
Fig. 8: Linewaver-Burk plot of Gpx

The Km value of human Erythrocyte Gpx was 4.1 mM by usin GSH, as substrate (Awasthi *et al.*, 1975), The velocity increases with the increase in substrate concentration up to a certain point and then becomes constant and reaches a maximum velocity. The high value of Km indicates that there is a low enzyme affinity toward substrate. The Km value is affected by substrate, pH and tempreture (Ahmed and ALhilali, 2010)

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