Purification And Characterization Of Two Forms Of Glutathione S-Transferase From *Bimphlaria Alexandrina* Snails

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ABSTRACT: Glutathione S-transferase (GST) have been purified and characterized from the tissue of *B. alexandrina* snails. The purification was carried out by chromatography on DEAE-cellulose. Five GST multiple forms (one unadsorbed and four adsorbed forms) were obtained. The major adsorbed forms GSTP2 and GSTP3 with the highest activity levels were purified to homogeneity by affinity chromatography Sepharose column. The pH optima for the purified GSTP2 and GSTP3 activity with CDNB as co substrate were at pH 6.5. The K_m values for GSH were 0.51 and 0.29 mM for GSTP2 and GSTP3 forms, respectively, while for CDNB were 0.41 mM for GSTP2 and 0.4 mM for GSTP3. The p*Ka* values were 6.0 and 8.0 for GSTP2 and GSTP3, respectively. The bi-substrate kinetics of the purified *B. alexanderina* GSTP2 and GSTP3 with CDNB and GSH as substrates did not obey Michaelis-Menten equation at most of the pHs tested. Hill plots of GSTP2 and GSTP3 for CDNB were non linear at pH 6.5 with values less than 1.0 at low substrate concentrations and higher than 1.0 at high substrate concentrations. Results may indicate that GSTP2 and GSTP3 display a mixture of positive & negative and non cooperativety. [Nature and Science. 2009;7(5):1-10]. (ISSN: 1545-0740).

Keywords: B. alexanderina, Km, GST, affinity chromatography.

INTRODUCTION

Schistosomiasis is one of the major communicable diseases of public health and socio-economic importance in the developing world. The success of these parasites is a result of their ability to switch rapidly between several different environments including snail tissue, fresh water and mammalian blood **(McKerrow & Salter, 2002)**. Snails are important horticultural and agricultural group of pests in that they act as intermediary hosts of animal as well as human parasites. In Egypt, nine species of snail's intermediate host for parasite were discovered **(Abo-Madyan *et al.*, 2005)**. Some snail species that have medical or veterinary importance in Egypt are *Biomphalaria alexanderina (B. alexanderina*), the intermediate host of *S. mansoni***,** *Bulinus truncatus* (Planorbidae, Bulininae), the intermediate host of *S. haematobium, Lymnea trancutula* (Lymnaeidae) the intermediate host of *Fasiola* and *Physa acuta* (Physidae) which is consider as a biological competitor for other snails **(Lardans & Dissous, 1998).**

It has been recognized that the successful control of the disease should include the control of the intermediate host snails. Several control techniques are now available, these include chemical **(Lowe *et al.*, 2005 and Ansaldo *et al.*, 2006)** cultural **(Teo, 2003)**, physical **(Schûder *et al.*, 2003 and Regoli *et al.*, 2005)** and biological control **(Teo, 2006)**.

Living organisms, from the simple bacteria to higher eukaryotes have developed a system for detoxication to protect important macromolecules from damage caused by reactive compounds that may be intracellular; produced during the metabolic processes or extracellular (xenobiotics), which enter into cells through polluted air, water or even food stuff, where they are continuously exposed to non-nutritional foreign chemical species. There are a variety of protection systems in the cell, including enzymes that can catalyze reactions that convert toxic molecules into harmless products. The modified molecules can be excreted from the organism or reused in the cell by some metabolic pathways. The metabolic detoxication of small molecules is carried out by a family of enzymes; specifically designed for that purpose. These enzymes are called detoxication enzymes **(Jakoby & Habig, 1980 and Armstrong, 1997)**.

The enzymatic detoxification of xenobiotics has been classified into three distinct phases which act in a tightly integrated manner. Phases I and II involve the conversion of a lipophilic, non-polar xenobiotics into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily

from the cell (phase III) **(Sheehan *et al.*, 2001).** Phase II enzymes catalyze the conjugation of activated xenobiotics to this endogenous water-soluble substrate, such as reduced glutathione (GSH), UDP-glucuronic acid, sulfate, certain amino acids e.g. glycine **(Mannervik *et al.*, 1989 and Meister, 1989).** The conjugation reaction serves the purpose of decreasing the biological activity and to increase the solubility of the original compound **(Falany, 1991)**. The phase II reactions are catalyzed by a number of different enzymes known as transferases. Quantitatively, conjugation to GSH, which is catalyzed by the glutathione transferases (GSTs; also known as glutathione S-transferases), is the major phase II reaction in many species **(Salinas & Wong, 1999).**

Glutathione S–transferases are mainly cytosolic, multifunctional detoxification enzymes, found in most aerobic eukaryotes and prokaryotes exist as dimeric proteins (homodimers or heterodimers). It was proposed that the soluble enzymes have two active sites per dimer each of which functions independently of the other **(Mannervik, 1985b)**.

The aim of the present work was to purify and characterize GST from the tissue of *B. alexandrina* snails and study the mechanisms of enzyme reaction.

MATERIALS AND METHODS

1. Snails

The snails *Biomphalaria alexandrina* used in the present study were maintained in the laboratory under standard conditions of aeration and temperature (25-30°C). They were fed fresh lettuce leaves and placed in dechlorinated water (aerated in a container for several days prior to being used in the experiments).

2. Chemicals

The reduced glutathione (GSH), Epoxy-activated Sepharose 6B, β-mercaptoethanol (β-ME), diethylaminoethyl (DEAE)-cellulose (DE-53) for chromatography and all resins and reagents for electrophoresis were obtained from Sigma Chemical Co. (St Louis, Mo). 1-chloro-2, 4-dinitrobenzene (CDNB), glutathione disulfide (GSSG) and H_2O_2 were purchased from Fluka Company. Other general chemicals were of the highest purity commercially available.

Protein determination

Protein was determined in the eluted fractions by the method of **Bradford (1976)** using bovine serum albumin as a standard.

Assay of GST

Enzyme activity was assayed by monitoring the change in absorbance, due to thioether formation from the substrate 1-chloro-2, 4-dinitrobenzene (CDNB), at 340 nm and 25°C as described by **Ajele & Afolayan (1992).** The assay reaction mixture contained in a total volume of 1.0 ml; 0.1 M potassium phosphate buffer, pH 6.5, 1.0 mM CDNB in ethanol (final concentration of ethanol less than 4 %), 1.0 mM GSH, and the enzyme solution. The extinction coefficient of product was taken to be 9.6 $mM^{-1}cm^{-1}$.

Preparation of Snail Extract

Snails (separated whole animals) were homogenized using Omni mixer in 20 % (w/v) of 0.1 M potassium phosphate buffer, pH 6.5 for the determination of glutathione transferase activity. The homogenate was then centrifuged at 16,000 g for 15 min. The supernatant was filtered through a plug of glass wool to remove floating lipids. The filtrate was designated crude homogenate and saved at -20°C for further analyses.

Preparation of DEAE -Cellulose Column

Diethylaminoethyl cellulose was treated as recommended by Pharmacia information book.

Preparation of GSH - Sepharose Affinity Matrix

Glutathione was coupled to epoxy - activated Sepharose 6B according to **Simons and Vander Jaget, (1977)**.

Purification of GST from *B. alexanderina* Snails

Unless otherwise stated all steps were performed at 4^0C. Crude extract which prepared as mentioned above was applied directly on DEAE-cellulose column previously equilibrated with 25 mM Tris-HCl buffer, pH 8.0. The adsorbed proteins were eluted using stepwise NaCl gradient in 0.02 M Tris-HCl buffer, pH 8.0. All the equilibration and elution buffers contained 2.0 mM mercaptoethanol. Fractions in 5.0 ml volume were collected at a flow rate of 60 ml/h. Fractions containing enzyme activity were pooled (unadsorbed, P1, P2, P3 and P4) according to their elution order. GSTP2 and GSTP3 DEAE-cellulose fractions were applied separately to a GSH-Sepharose column equilibrated with the same equilibration buffer of the DEAE-cellulose column and developed overnight at a flow rate of 10 ml/h. The enzyme was eluted with 50 mM Tris-HCl buffer, pH 9.6 containing 10 mM GSH. Two milliliter fractions were collected.

Characterization of GSTP2 and GSTP3:

The characterization of the purified two GST isoforms with respect to pH optimum and kinetic properties (effect of pH on Km and V_{max}) was thoroughly investigated and shown in result section.

RESULTS

Purification of GST from *B. alexanderina* Snails

The purification of GST is summarized in Table (1). Chromatography on DEAE-cellulose (Fig 1) produced five peaks of GST; named unadsorbed and adsorbed P1, P2, P3 and P4. Their specific activities were ranged from 0.047 to 0.22 units mg^{-1} protein. GSTP2 and GSTP3 were chosen for further purification using GSH-Sepharose affinity chromatography, Fig. (2).

Characterization of the Purified GSTP2 and GSTP3

The effect of pH on *B. alexanderina* GSTP2 and GSTP3 activities were examined between pH 4.5 and 9.0. GSTP2 and GSTP3 exhibited maximum activity at pH 6.5, Fig. (3).

The effect of substrate concentration on the enzyme reaction rates of GSTP2 and GSTP3 were investigated at 25°C and pH values between 5.5 and 9.0. The results were presented in Tables (2-5). The p*Ka* values were in the range from 6.0 -> 9.0 at low and high GSH and CDNB concentration for GSTP2 and GSTP3, (Table 6).

Hill plots were constructed by plotting v/Vmax-v versus [S] (GSH or CDNB concentrations in mM) on log-log scale at pHs ranging from pH 5.5 to pH 9.0 (graphs not shown). Hill coefficients (n; the slope of the plot of v/Vmax-v versus [S]) for GSTP2 and GSTP3 for GSH and CDNB were calculated and the results are summarized in Table (7).

Table (1): Purification scheme of GST from the tissue of *B.alexanderina* snails

Step		Total protein (mg)	Total activity (Units)*	Specific Activity (U/mg Protein)	Fold Purification	Recovery %
Crude Extract:		312.5	43.00	0.138	1.00	100.00
Chromatography on DEAE – cellulose:						
Unadsorbed	**- ve**	31.2	1.48	0.047	0.34	3.44
0.05 M NaCl	**P (1)**	89.8	6.80	0.076	0.55	15.80
0.075 M NaCl	**P (2)**	39.1	4.37	0.112	0.81	10.16
0.125 M NaCl	**P (3)**	48.4	10.63	0.220	1.59	24.73
0.2 M NaCl	**P (4)**	14.8	2.62	0.177	1.28	6.10
Gel filtration on GSH Sepharose:						
GSTP2		0.26	2.65	10.19	91.00	60.73
GSTP3		0.06	5.28	85.16	387.1	54.49

**One unit of glutathione transferase activity was defined as the amount of enzyme that catalyzes the formation of 1.0 µmole of thioether per min under standard assay conditions.*

Table (2): Kinetic parameters of the purified *B. alexanderina* GSTP2 when GSH was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
5.5	2.34	0.3	0.43	3.33	0.28	0.05	3.57	20.0
6.0	1.66	0.43	0.60	2.33	0.18	0.08	5.56	12.5
6.5	1.98		0.51		0.16		6.25	
7.0	0.45		2.22		0.09		11.1	
7.5	3.34		0.30		0.32		3.13	
8.0	2.90		0.34		0.40		2.50	
8.5	8.43	0.56	0.12	1.79	0.68	0.22	1.47	4.55
9.0	4.25		0.24		0.53		1.89	

Table (3): Kinetic parameters of the purified *B. alexanderina* GSTP2 when CDNB was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
5.5	2.52	0.95	0.40	1.05	0.27	0.18	3.70	5.56
6.0	7.6		0.13		0.28		3.57	
6.5	18.3	2.45	0.05	0.41	0.29	0.19	3.45	5.26
7.0	9.35	5.55	0.11	0.18	0.25	0.22	4.00	4.55
7.5	4.35		0.23		0.37		2.70	
8.0	3.06	0.44	0.33	2.27	0.58	0.19	1.72	5.26
8.5	2.78		0.36		0.49		2.04	
9.0	3.1		0.32		0.51		1.96	

Table (4): Kinetic parameters of the purified *B. alexanderina* GSTP3 when GSH was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	High	Low	High	Low	High	Low	High
5.5	2.15	0.60	0.47	1.67	0.33	0.15	3.03	6.67
6.0	1.55		0.65		0.26		3.85	
6.5	3.45		0.29		0.28		3.57	
7.0	1.75		0.57		0.30		3.33	
7.5	4.52		0.22		0.58		1.72	
8.0	3.94		0.25		0.35		2.86	
8.5	8.14	1.30	0.12	0.77	0.56	0.30	1.79	3.33
9.0	7.36	0.66	0.14	1.52	0.53	0.21	1.89	4.76

Table (5): Kinetic parameters of the purified *B. alexanderina* GSTP3 when CDNB was the varied substrate at pH 5.5 – 9.

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	High	Low	High	Low	High	Low	High
5.5	3.15		0.32		0.24		4.17	
6.0	8.22		0.12		0.23		4.35	
6.5	27.6	2.48	0.04	0.40	0.23	0.15	4.35	6.67
7.0	27.3	4.08	0.04	0.25	0.24	0.16	4.17	6.25
7.5	2.53		0.40		0.24		4.17	
8.0	3.35	0.42	0.30	2.38	0.50	0.15	2.00	6.67
8.5	2.05		0.49		0.35		2.86	
9.0	2.26		0.44		0.39		2.56	

Table (6): Effect of pH on log V_{max}/ K_m of the purified *B. alexanderina* GSTP2 and GSTP3 at different concentrations of GSH and CDNB

p*Ka*		
	Low	**High**
GSH		
GSTP2	**6.5, 7.5, 8.5**	**6.5, 7.5, >9.0**
GSTP3	**6.5, 8.5**	**6.5, 8.0**
CDNB		
GSTP2	**6.5**	**6.0, 7.0, 8.5**
GSTP3	**6.5, 7.0**	**6.0, 7.0, 8.5**

Table (7): Hill coefficients of the purified *B. alexanderina* GSTP2 and GSTP3 for GSH and CDNB at different pHs

pH	GSTP2				GSTP3			
	GSH		CDNB		GSH		CDNB	
	Low	High	Low	High	Low	High	Low	High
5.5	1.07	1.0	0.97	1.03	0.92	1.03	1.05	
6.0	0.91	1.06	1.03		1.01		1.22	
6.5	1.02		1.13	1.01	1.02		0.86	1.24
7.0	1.0		0.92	1.0	1.04		1.01	1.03
7.5	0.82		1.07		0.96		0.99	
8.0	1.0		1.0	1.05	1.01		1.01	1.02
8.5	1.0	0.64	1.06		1.05	0.83	1.05	
9.0	0.97		1.04		0.94	0.67	1.04	

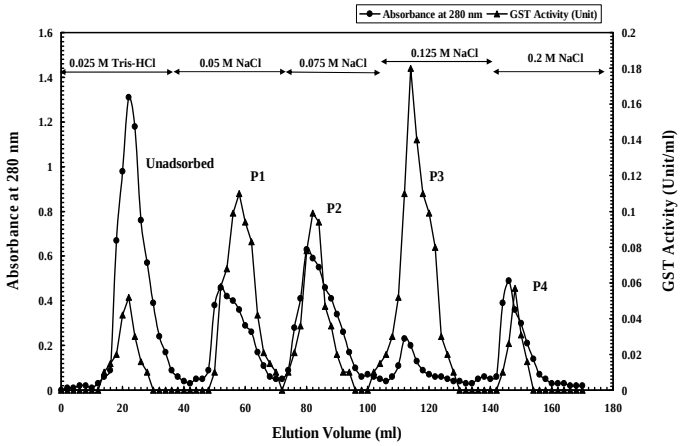

Fig. (1): Typical elution profile of *B. alexanderina* GST chromatography on DEAE-cellulose column (30 x 1.6 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 2 mM β-mercaptoethanol. Protein was eluted by stepwise NaCl gradient in the equilibration buffer.

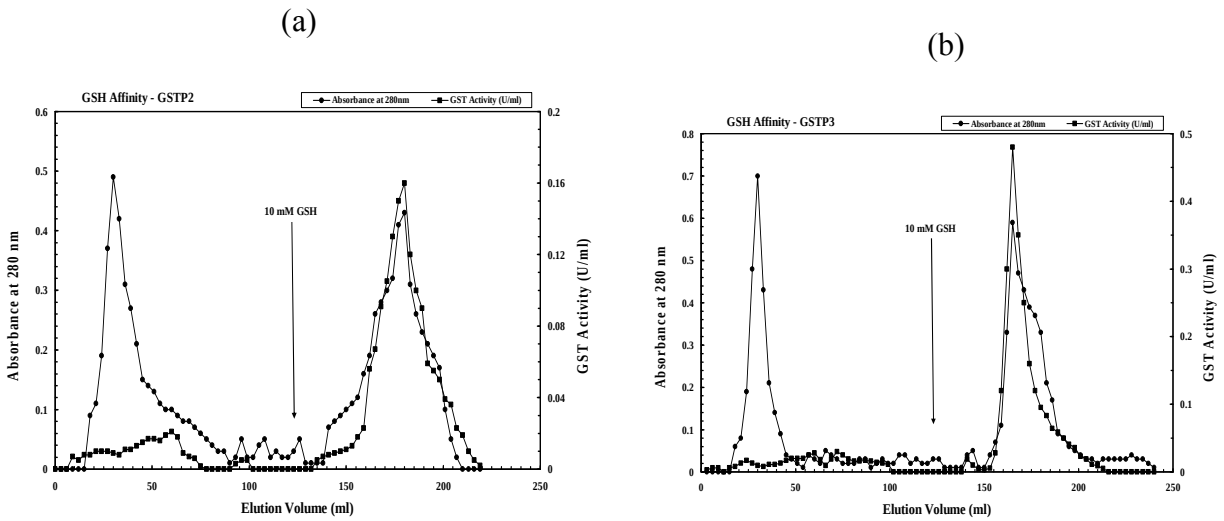

Fig. (2): Typical elution profile for the chromatography of (a) GSTP2 and (b) GST3 on GSH-Sepharose affinity column. The arrow indicates initiation of elution using 10 mM GSH

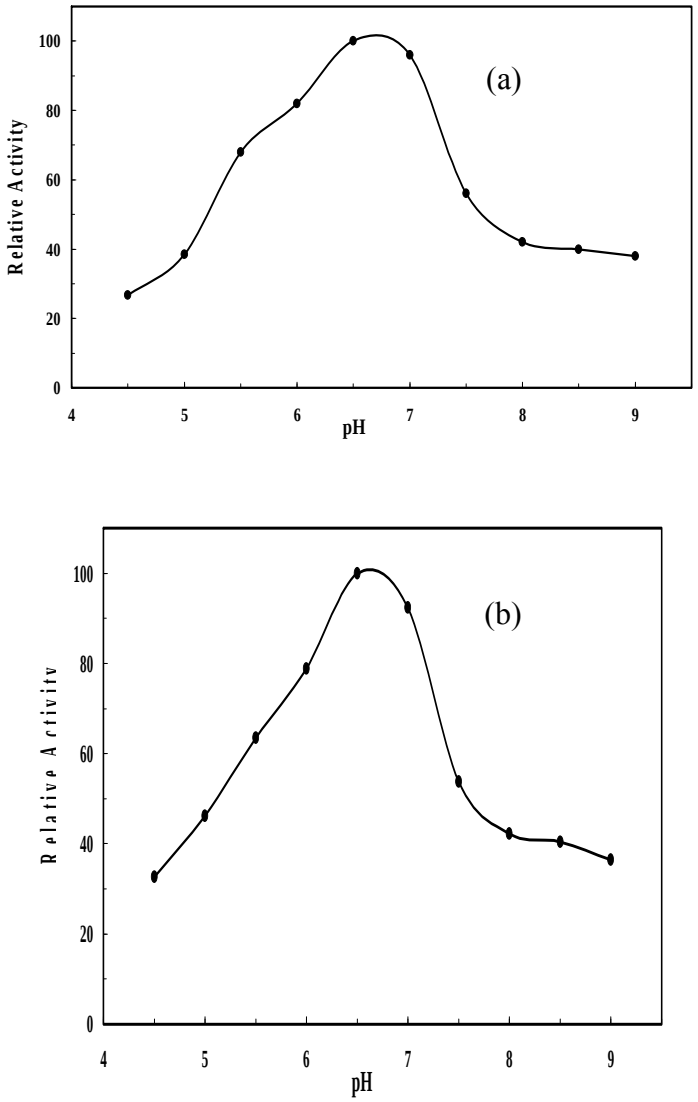

Fig. (3): Effect of pH on the activity of the purified (a) GSTP2 and (b) GSTP3. The buffers used were 0.1 M sodium acetate buffer for pH 4.5 to 5.5, 0.1 M potassium phosphate buffer for pH 6.0 to 8.0 and 0.1 M Tris-HCl buffer for pH 8.5 to 9.0.

DISCUSSION

In the present investigation, chromatographic separation of *B. alexandrina* tissue homogenate on DEAE cellulose indicated the presence of five multiple forms of GST (one unadsorbed and four adsorbed P2, P3, P4 and P5) eluted by NaCl stepwise gradient, **Fig. (1)**. The two major forms, GSTP2 and GSTP3 were further purified on GSH Sepharose according to the method described by **Abdullah (2000)**, The specific activities increased to 10.19 (91-fold purification) and 85.16 unit/ mg protein (387.1-fold purification) with 60.73 and 54.49 % recovery, for GSTP2 and GSTP3 respectively **Fig. (2a &b)**.

Kinetic studies of GSTP2 and GSTP3 of *B. alexanderina* snails, specially the changes in enzyme affinity to its substrates was examined at different pHs. pH optima for GSTP2 and GSTP3 activity with CDNB as co substrate were at pH 6.5, **Fig. (3a &b)**. Similar results were observed for the two GSTs purified from *Bulinus trancatus* **(Abdullah *et al.*, 2004)**.

The bi-substrate kinetics of the purified *B. alexanderina* GSTP2 and GSTP3 with CDNB and GSH as substrates did not obey Michaelis-Menten equation at most of the PHS tested (not shown).

The *Km* values for GSH were 0.51 and 0.29 for GSTP2 and GSTP3 at pH 6.5, respectively, **Table (2, 4)**. These values are comparable to the majority of values reported for invertebrate GSTs. The *Km* values for GSH were 0.23 mM and 0.15 mM for GST2 and GST3 isoenzymes of *B. trancatus*, respectively **(Abdullah *et al.*, 2006)** and 0.19 mM for the mussels *Atactodea striata* **(Yang *et al.*, 2003)**.

The *Km* values for CDNB were calculated with respect to GSTP2 at pH 6.5 to be 0.05 mM at low concentration and 0.41 mM at high concentration, **Table (3)**, while for GSTP3 *Km* values were 0.04 mM and 0.40 mM at low and high CDNB concentration respectively, **Table (5)**.

At the acidic side of the pH, *B. alexanderina* GSTP2 did not obey Michaelis-Menten equation at pH 5.5 and 6.0, for GSH and 5.5 for CDNB. Also GSTP3 exhibited the same behavior at pH 5.5 for GSH **(Fig. not shown)**. At the acid side of pH, one may predict a role for the unfolding of the GST on going to the more acidic side. The presence of the unfolded dimmer, the partially active dimmers may have different *Km* values resulting in the concave behavior for the substrate concentrations.

Results in **Tables (2 – 5)** showed that *Km* values for GSH and CDNB were affected by increasing the pH. The *Km* values for CDNB were decreased from 0.40 mM to 0.05 mM and from 1.05 to 0.41 at low and high CDNB for GSTP2 and from 0.32 mM to 0.04 mM for GSTP3 at low concentration and increased from 0.32 to 0.4 at high concentration by increasing the pH values from 5.5 to 6.5. The same behavior was observed for both GST2 and GST3 of *B. trancatus* isoenzymes, where the *Km* values for CDNB were decreased from 110 μM to 14 μM for GST2 and from 61 μM to 4 μM for GST3 by the increase in the pH from 6.0 to 8.5 **(Abdullah *et al.*, 2006)**. In contrast to the present results **Clark, (1989)**, observed that Michaelis constant for CDNB and DCNB from the insects *Costelytra zealandica* and *Galleria mellonella* were unaffected by increasing the pH from 6.0 to 8.0.

On the other hand, the *Km* values for GSH were increased from 0.43 mM to 0.51 mM at low GSH concentration and decreased from 3.33 mM to 0.51 mM at high GSH concentration, **Table (2)**, with respect to GSTP2. For GSTP3, *Km* values for GSH were decreased from 0.47 and 1.67 at low and high GSH concentration to 0.29 mM, **Table (4)** by increasing pH value from 5.5 to 6.5.

By increasing pH from 6.5 to 9.0, Km for GSH with respect to GSTP2 was decreased from 0.51 to 0.24 at low and high GSH concentrations. With respect to GSTP3 the Km values were decreased from 0.29 mM to 0.14 mM and increased from 0.29 to 1.52 at low and high GSH concentrations. While the *Km* values for CDNB was increased from 0.05 to 0.32 mM and decreased from 0.41 to 0.32 for GSTP2 (Table 3) at low and high CDNB concentrations. With respect to GSTP3, the Km values were increased from 0.04 and 0.4 to 0.44 at low and high CDNB concentrations by changing the pH value from 6.5 to 9.0, (Table 5).

A well documented property of GSTs is the ability to lower the p*Ka* of the sulfhydryl group of the bound GSH. The acidity constant of GSH in the active site ranges between 6.0 – 6.5 for α **(Bjornestedt *et al.*, 1995)**, μ **(Liu *et* al., 1992)** and θ **(Caccuri *et al.*, 1998)** class GSTs.

In the present investigation, the pH dependence of log *Vmax*/*Km*, which reflect the kinetically significant ionizations that occur in the GST-GSH complex suggested apparent p*Ka* values from 6.0 to >9, Table (6). These results suggest that during the catalysis, an amino acid residue with a p*Ka* value of 6.0 must be deprotonated and another residue with a p*Ka* value higher than 9 must be protonated to obtain maximum activity of the enzyme. The p*Ka* value of 6.0 most likely represent that of the thiol group of the bound GSH in the active site of GST. Similar behavior was observed from the kinetic studies of purified *M. mucedo* GST. It indicated that, the fungal GST isoenzymes lowers the p*Ka* of the sulfhydryl group of the GSH as occurs in other GST classes **(Hamed *et al.*, 2005)**.

If the binding of one substrate molecule induces structural or electronic changes that result in altered affinities for vacant sites, the velocity curve will no longer follow Michaelis-Menten kinetics. The phenomenon has been called cooperative binding. Generally, allosteric enzymes yield sigmoidal velocity curves. The binding of one substrate molecule facilitates the binding of the next substrate molecule by increasing the affinities of the vacant binding sites (positive cooperativity)**.** Negative cooperativity occurs when binding of each substrate molecule decreases the intrinsic affinities of the vacant sites. The velocity curve raises rapidly at low substrate concentrations, but then slopes off markedly as the affinities of the

vacant sites decreases. The curve is not sigmoidal. The reciprocal plot is non linear. The linear region at high 1/S values corresponds to the normal hyperbolic saturation of the first (normal affinity) site at low substrate concentrations. As the substrate concentration increases, the second, third and fourth sites fill (with low affinities). The reciprocal plot and scatchard plot are indistinguishes from the plots obtained for a mixture of enzymes with different substrate affinities, or one enzyme with multiple sites of different affinities. The Hill plot has a slope less than 1.0, but approaches a slope of 1.0 at very high and very low substrate concentrations **(Segel, 1993)**.

It was observed that GSTP2 exhibited a non cooperativity at most pHs under investigation at low and high concentrations of GSH and CDNB, except positive cooperativity (n = 1.13), at low CDNB concentration at pH 6.5, and negative cooperativity (n = 0.92) at pH 7.0. Regarding GSH, GSTP2 exhibited negative cooperativity at pH 6.0 at low GSH concentration (n = 0.91) also, at pH 7.5 (n = 0.82) at low and high GSH concentrations as well as at pH 8.5 at high GSH concentration (n = 0.64), **Table (7)**. Nearly the same behaviors were observed for GSTP3, it exhibited positive cooperativity at pH 6.0 (n = 1.22) and at pH 6.5 (n = 1.24) at high CDNB concentration, while at low concentration it exhibited negative cooperativity (n = 0.86). With respect to GSH, GSTP3 showed negative cooperativity (n = 0.92 and 0.94) at pH 5.5 and 9.0 at low GSH concentration and at pH 8.5 (n = 0.83) and pH 9.0 (n = 0.67) and high GSH concentrations, **Table (7)**.

This behavior may suggest that GSTP2 and GSTP3 display a mixture of none, positive and negative cooperativety in the binding of a ligand as though the binding of one substrate molecules induces structural or electronic changes that result in altered affinities for the vacant sites **(Segel, 1993)**. Such behavior has been observed in the sorghum GST B1/B2 **(Gronwald & Plaisance, 1998)**, GST2-2 **(Caccuri *et al.*, 2001)** and the fungus *M. mucedo* GST **(Hamed *et al.*, 2005)**.

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