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Research Article

Kinetic features and characterization of liver glutathione transferase in rats exposed to glyphosate

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Abstract. Glutathione-S-transferases (GSTs) are phase-II metabolizing enzymes which detoxify various compounds through their conjugation reaction. This study characterized and investigated the kinetic properties of liver glutathione-S-transferase in rats exposed to glyphosate. Rats of average weight 200 g were randomly divided into groups (n=4); A, B, C and D. Group A served as control and received distilled water alone while groups B, C and D were exposed to 200, 300 and 400 mg/kg body weight of glyphosate respectively. Three GST model substrates [1, 2-dichloro-4-nitrobenzene (DCNB), paranitrobenzylchloride (pNBCl), and 1-chloro-2, 4-dinitrobenzene (CDNB)] were used to determine the substrate utilization pattern of the GST isozymes in the liver crude homogenate. The liver homogenate was purified by combination of 80 % ammonium sulfate precipitation, gel filtration chromatography on Sephacryl S-200 column and ion exchange chromatography on CM-Sepharose column. Spectrophotometric methods were used to determine the activity of the GSTs and protein concentration. GST isozymes in the liver homogenate were unable to conjugate pNBCl to GSH, DCNB showed slight conjugation while CDNB shows a 2-fold conjugation. The optimum temperature for the induced isozymes of GST A and GST B were 400C and 500C respectively while the optimum pH were 8.0 and 8.5 respectively. In this study, pNBCl proved unsuitable due to its inability to conjugate GSH. Future study may provide additional information about the class of GST isozyme induced.

Keywords: Glutathione transferase (GST), Glyphosate, Metal ions, Thiol reagents,

1. Introduction

Glutathione-S-transferases (GSTs) are essential enzymes responsible for detoxifying xenobiotics in living organisms (Sheehan *et al*., 2001; Sau *et al*., 2010). They play a crucial role in biotransforming xenobiotics into less toxic glutathione conjugates, serving as a major phase II reaction in most organisms (Sheehan *et al*., 2001; Sau *et al*., 2010). Elevated activities of GSTs have been associated with cancer resistance to chemotherapy, bacterial resistance to antibiotics, plant resistance to herbicides, and insect resistance to insecticides (Ranson *et al*., 2001).

GSTs are broadly classified into three enzyme families: mitochondrial, microsomal, and cytosolic GSTs (Hayes *et al*., 2005; Glisic *et al*., 2015). While mitochondrial and cytosolic GSTs share a similar three-dimensional structure, they differ from microsomal enzymes (Ladner *et al*., 2003). Cytosolic GSTs exhibit a broad substrate specificity for xenobiotics, and are divided into multiple classes, including alpha (GSTA), mu (GSTM), pi (GSTP), omega (GSTO), sigma (GSTS), theta (GSTT), and zeta (GSTZ) (Sheehan *et al*., 2001; Hayes *et al*., 2005; Glisic *et al*., 2015). Microsomal GSTs, also known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), participate in the synthesis of leukotrienes and prostanoids (Glisic *et al*., 2015). The mitochondrial GSTs belong to the kappa-class (Thomson *et al*., 2004).

Various drugs and toxicants, including barbiturates, chemoprotectants, insecticides, microbial antibodies, heavy metals, and herbicides, have been linked to increased GST activity (Hayes & Pulford, 1995; Dagget *et al*., 1998; MxLellan & Wolf, 1999; Ranson *et al*., 2001). GSTs are known for their role in protecting cells by inactivating toxic metabolites (Glisic *et al*., 2015; Singhal *et al*., 2015).

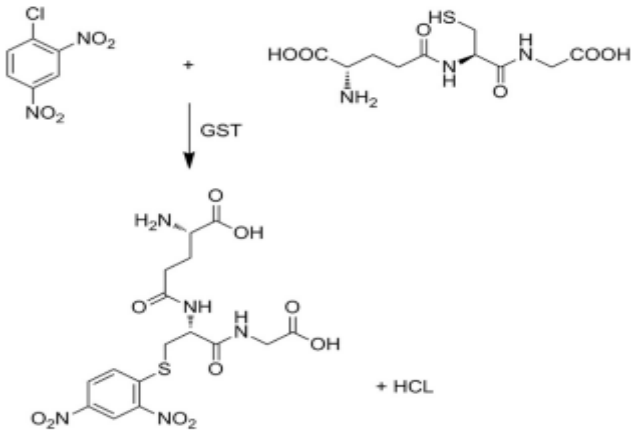

Figure 1: GST facilitates conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH) to form S-2,4-dinitrophenylglutathione (Ojopagogo *et al*., 2015)

Efficient detoxification mechanisms, such as GST activation, are crucial adaptations for living organisms to cope with environmental xenobiotics (Sau *et al*., 2010; Glisic *et al*., 2015; Singhal *et al*., 2015). While some studies have focused on the induction and purification of GSTs in various animal species (Ojopagogo *et al*., 2015; Adewale & Afolayan, 2005; Adewale & Afolayan, 2006), there is a significant gap in our understanding of the induction and purification of GSTs in animals exposed to environmental herbicides like glyphosate and the kinetics of GSTs in such exposed animals.

Previous studies on glyphosate's impact on glutathione-S-transferases (GSTs) have often lacked specificity in substrate selection, and this limits insight into how particular GST isozymes respond to such xenobiotic. Many researchers have used the generic substrate CDNB, which can be metabolized by multiple GST isozymes, but this approach may not reveal how specific GST classes respond to xenobiotics like glyphosate. This gap in knowledge has led to an incomplete understanding of the mechanisms through which glyphosate influences GST activity. Additionally, past research has typically focused on broad toxicological markers, without systematically evaluating how glyphosate alters the enzyme kinetics of different GST isozymes.

To add to our understanding of how toxic substances like glyphosate affect GST activity, this study looked at the kinetic parameters (such as Km and Vmax) of GST and characterized its isoforms especially when in response to glyphosate exposure, which has been less studied.

2 Materials and Methods

2.1 Materials

CM-sepharose (fastflow) was a product of GE Healthcare, Sweden while Sephacryl S-200, 1, 2-dichloro-4-nitrobenzene (DCNB), paranitrobenzylchloride (pNBCl), 1-chloro-2, 4-dinitrobenzene (CDNB) are products of Sigma Chemical Company, St. Loius, Mo, U.S.A. All other reagents and chemicals used were of analytical grade and obtained from trusted and reputable suppliers.

2.2 Animals

Sixteen male albino rats of average weight of 150 g were purchased from the Animal House of the College of Health Sciences, Obafemi Awolowo University (O.A.U.), Ile-Ife, Osun State, Nigeria. The rats were divided into four groups of four animals each (n=4) and housed in plastic cages with access to standard feed and water *ad libitum*. Dry wood shavings were used as bedding while they were maintained under ambient conditions of temperature, humidity, and a normal dark/light cycle. All experimental procedures used in this study were done according to the guidelines provided by the Institutional Animal Care Committee. We also strictly adhered to the European Commission Directive 86/609/EEC for animal experiments (Louhimies, 2002).

2.3 Experimental Design

The animals were acclimatized for one week before the administration of different doses of glyphosate, taking into consideration the LD_{50} of glyphosate for rats which is greater than 5000 mg/kg body weight (Duke *et al*., 2003; Cerdeira & Duke, 2006; Duke & Powles, 2006). Group A served as the control and received distilled water alone. Groups B, C

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and D received 200, 300 and 400 mg/kg body weight of glyphosate respectively. Glyphosate was administered once. After 24 hours, the animals were sacrificed and organ (liver) was harvested.

2.4 Preparation of Crude Enzyme Sample

Preparation of the crude enzyme sample was carried out according to the method of Agunbiade *et al*. (2014) with a slight modification. Briefly, freshly-excised liver was homogenized in 10 mM Tris-HCl buffer pH 7.8, containing 10 mM EDTA, 1 mM EGTA, 5 mM 2-Mercaptoethanol and 10 % (v/v) glycerol to obtain 10 % (w/v) homogenate. The homogenate was centrifuged at 4°C and 10,000 rpm for 30 minutes in a refrigerated centrifuge. The supernatant obtained was filtered to remove the floating lipid. The filtrate obtained was used as the crude extract of glutathione-S-transferase.

2.5 Protein Determination

Protein concentration was measured routinely during the period of the experiment according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

2.6 Substrate Utilization Features

GST activity, using 1-chloro-2,4-dinitrobenzene (CDNB), 1, 2-dichloro-4-nitrobenzene (DCNB) and paranitrobenzylchloride (pNBCl) as model substrates, was determined according to the method of Habig *et al*. (1974).

2.7 Ammonium Sulphate Precipitation

The crude enzyme sample was concentrated with ammonium sulfate to 80 % saturation. The mixture was kept at 4°C for 24 hours and then centrifuged at 5,000 rpm for 10 minutes. The supernatant was discarded and the pellets were dissolved in 10 mM Tris-HCl buffer pH 7.8, containing 10 mM EDTA, 1 mM EGTA, 5 mM 2-Mercaptoethanol and 10 % (v/v) glycerol. This was dialyzed against the same buffer for 24 hours with four (4) changes in buffer. The remaining sample after dialysis was used for further purification.

2.8 Gel Filtration Chromatography

The dialyzed enzyme sample was applied to a gel filtration column (Sephacryl S-100; 1.5 cm × 70 cm). The column was washed and equilibrated with buffer A (20 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA and 1 mM 2-mercaptoethanol). Enzyme was eluted with the same buffer at a flow rate of 25 ml/h and 5 ml fractions were collected. Protein profile of each fraction was determined at 280 nm. Fractions with GST activity was pooled.

2.9 Ion Exchange Chromatography

Fractions pooled from gel filtration column was applied to ion exchange column (Fastflow CM-Sepharose; 2 cm × 26 cm) equilibrated in buffer B (20 mM potassium phosphate buffer, pH 8.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol). The column was washed with the same buffer at a flow rate of 180 ml/h and 2 ml fractions were collected until unbound GST proteins were collected. Sodium chloride (NaCl) gradient of 0.1 to 1.0 M in buffer B was used to elute bound GST. The protein profile of both bound and unbound fractions was determined at 280 nm. Fractions with GST activity was pooled and used for further characterization.

2.10 Determination of Native Molecular Weight

The native molecular weight of the purified glutathione-S-transferase was determined by gel filtration (Sephacryl S-200) column chromatography using bovine serum albumin (66 KDa), ovalbumin (44 kDa), thermolysin (36 kDa), α-chymotrypsinogen A (25 kDa) and lysozyme (14 kDa).

2.11 Determination of Kinetic Parameters

The kinetic parameters (K_m and V_{max}) of the purified GST were determined by measuring GST activity at a fixed GSH concentration (1 mM) and varying concentration of CDNB (0.2, 0.4, 0.6, 0.8 and 1.0 mM) and at fixed CDNB concentration (1 mM) and varying concentration of GSH (0.2, 0.4, 0.6, 0.8 and 1.0 mM GSH). K_m and V_{max} were determined from Lineweaver-Burk's plot.

2.12 Determination of Activation Energy

The enzyme activation energy was determined by measuring the reaction velocity at different temperatures (25°C-65°C). At each temperature, the maximum velocity (V_{max}) was assayed and then the Arrhenius formula was used to determine the activation energy by making a plot of log Vmax versus T^{-1}.

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2.13 Effect of Temperature

The effect of temperature on the activity of the purified GST was monitored by incubating the enzyme for 5 minutes at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C. Enzyme sample was taken after the incubation period and GST activity was determined.

2.14 Effect of pH

The effect of pH on the activity of the purified GST was monitored by assaying for the activity of GST at different pH ranges (7.0, 7.5, 8.0, 8.5 and 9.0) of the assay buffer.

2.15 Effect of Thiol Reagents

Different concentrations (2, 4, 6, 8 and 10 mM) of thiol reagents [2-mercaptoethanol, 5′5-dithio-*bis*-(2-nitrobenzoic) acid (DTNB) and reduced glutathione (GSH)] were prepared in assay buffer (0.1 M phosphate buffer, pH 6.5). Aliquots containing 80 µl of the prepared thiol reagents and 20 µl of the enzyme sample was prepared and incubated for 20 minutes. After the incubation period, samples were taken and residual activity of the enzyme was measured. Parallel controls were maintained.

2.16 Effects of Metal Ions

Cobalt (Co^{2+}), iron II (Fe^{2+}), mercury (Hg^{2+}), magnesium (Mg^{2+}) and iron III (Fe^{3+}) ions (as their chloride salts) at concentrations of 0–100 mM (0, 20, 40, 60, 80 and 100 mM) were tested for their possible inhibitory effects on the activity of the purified enzyme. IC_{50} values for the inhibitors were measured in assays containing 1 mM GSH and 1 mM CDNB. IC_{50} values were determined by plotting a graph of fractional velocity (% residual activity) against the logarithm of the inhibitor concentrations.

2.17 Data Analyses

Statistical analysis of data was carried out using the combination of GraphPad Prism (version 6) and Microsoft excel (2010). Values were expressed as mean ± SEM of triplicates with $P < 0.05$ considered significant.

3. Results and Discussion

Glutathione-S-transferase (GST) isozymes, integral to phase II detoxification, are ubiquitously distributed across various organisms including microbes, insects, fungi, fish, birds, mammals, and plants (Dixon & Edwards, 2009), serving a pivotal role in the biotransformation processes (Basantani & Srivastava, 2007). These enzymes facilitate the conjugation of reduced glutathione (GSH) to a variety of electrophilic compounds, enhancing their solubility and excretion (Basantani & Srivastava, 2007; Dixon & Edwards, 2009). The cellular glutathione pool can be depleted by xenobiotics and potentially diminish antioxidative defense mechanisms (Rubino, 2015), because of this, enzyme inhibition studies are important especially for toxicology purposes.

In this study, male albino rats were subjected to varying concentrations of glyphosate, and subsequent GST activity was quantified in liver homogenates using three model GST substrates [1, 2-dichloro-4-nitrobenzene (DCNB), para-nitrobenzylchloride (pNBCl), and 1-chloro-2, 4-dinitrobenzene (CDNB)]. Since no significant difference was observed in GST activity across all groups, homogenate from the most responsive group was selected for purification based on their pronounced activity with CDNB substrate. The preferential utilization of CDNB by GST isozymes was evident and corroborated its status as a universal substrate for diverse GST isozymes.

In contrast, the conjugation efficiency of GST with DCNB was approximately halved relative to CDNB, aligning with other findings (Agunbiade *et al*., 2014; Wan *et al*., 2016). Conversely, pNBCl was unable to conjugate GSH, suggesting its unsuitability as a substrate for the induced rat liver GST isozymes. This observation is seen in the distinct substrate specificity of theta class GSTs, which exhibit little activity towards CDNB but heightened responsiveness to pNBCl (Fjellstedt *et al*., 1973; Spearman *et al*., 1985; Meyer *et al*., 1991). Hence, this study finds that there is no theta class GSTs in the rat liver crude homogenate. For theta class GSTs, CDNB is a relatively poor substrate, while pNBCl is often preferentially utilized. Theta class GSTs are not universally expressed across all tissues, and their activity can vary depending on factors such as the species, the type of xenobiotic exposure, and the specific tissue under investigation (Hayes & Pulford, 1995).

The absence of theta class GSTs in the rat liver homogenate suggests that the GST isozymes induced by glyphosate exposure are more likely to belong to other classes, such as alpha, mu, or pi. These classes are known to be more prevalent in liver tissue and are more likely to utilize CDNB as a substrate. Additionally, the lack of theta class GSTs could

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also be indicative of the nature of the xenobiotic stress induced by glyphosate. Glyphosate's impact on non-target organisms, including mammals, has been a subject of growing concern and the absence of theta class GST raises questions about the specific isoforms that are responsive to it. Glyphosate exposure could modulate the activity of GST isozymes (Diken *et al*., 2017; Borges *et al*., 2023), but the effects were highly dependent on the dose, duration of exposure, and the specific tissue examined.

Purification via gel filtration chromatography using Sephacryl S-200 column yielded enzymes with native molecular weight of 44 KDa. This molecular weight is similar with the range reported in other species, such as 62.13 KDa in Van Lake fish (Ozaslan *et al*., 2018), 40 KDa in Synechocystis PCC 6803 (Pandey *et al*., 2015), and 47 KDa in rainbow trout erythrocytes (Gadagbui & James, 2000).

On further subjection of the purified GST from Sephacryl S-200 gel filtration chromatography column (Figure 3) to CM-Sepharose ion exchange column, two main isozymes were detected (GST A and GST B). However, in contrast to other studies which showed that GST utilize GSH as substrate more than CDNB (Gadagbui & James, 2000; Huang & Han, 2007), this study indicated that CDNB is the more preferred substrate (Table 1) for the isozymes induced in the presence of glyphosate since low K_m mean more affinity of the isozymes for the substrates and vice versa. Studies (Ketterer *et al*., 1971; Ricci *et al*., 2003; Shimoji *et al*, 2007) have also noted that modification of cysteine residues in GSTs can alter enzyme activity and protection, and result in enzyme activation which suggests a dynamic equilibrium between activated and unactivated forms (Shimoji *et al*, 2007). However, these studies questioned whether this activation translates to increased protective capacity under physiological conditions, as the activation might not enhance the overall detoxification capacity under normal circumstances due to enzyme saturation with GSH thiolate.

Our study observed that, in the presence of glyphosate, isozymes of GST are able to adjust their kinetics in order to allow for a tighter binding in some cases and loose binding in others (Agunbiade *et al*., 2014). Substrates are not easily released for another round of catalysis when they are tightly bound to enzymes, and this may be disadvantageous by leading to compromise in turnover (Agunbiade *et al*., 2014). While earlier studies suggest that enzyme activation can shift the equilibrium toward a more activated state and potentially increase the protective capacity during high rates of reactive electrophile formation (Morgenstern, 2024), our findings suggest a nuanced role of substrate binding affinity. The observed tight binding in the presence of glyphosate appears to enhance the reaction velocity despite potentially compromising turnover rates due to reduced substrate release.

The complexity of GST kinetics under stress conditions is revealed since its tight binding enhances reaction velocity and limit enzyme turnover. Moreover, activation of GSTs in response to toxic conditions could be physiologically significant and potentially lead to rapid GSH depletion (Morgenstern, 2024). Hence the effect of glyphosate on GST substrate binding and reaction velocity highlights how environmental stressors can modulate GST function and alter detoxification dynamics.

The fact that the isozymes of GST A and GST B do not lose activity at 40^0C and 50^0C respectively indicated that the enzymes are relatively stable. Previous works have shown an optimum temperature of 65^0C in human blood serum, 50^0C in turkey liver and 50^0C in cyanobacterium-*Synechocystis* PCC 6803 (Turkanoglu, 2007; Akkemik *et al*., 2012; Pandey *et al*., 2015) and a pH range of 6.6-6.8 in velvet leaf, 8.0-8.5 in maize, 8.5 in turkey liver, 7.0 in *Laurocerasus officinalis Roem*, 8.0 in cyanobacterium- *Synechocystis* PCC 6803, 7.5 in gill and liver of fish and 7.5 in somatic tissue of *Gastrothylax crumenifer* (Guddewar & Dauterman, 1979; Gronwald & Plaisance, 1998; Akkemik *et al*., 2012; Turkan *et al*., 2014; Comakli et al., 2015; Pandey *et al*., 2015; Ahmed *et al*., 2017).

Table 1. Kinetic parameters of GST isozymes

Isozymes	Km (CDNB) (mM)	Km (GSH) (mM)	Vmax (U/mg protein)
A	0.065 ± 0.003	0.154 ± 0.010	2.632 ± 0.065
B	0.143 ± 0.007	0.179 ± 0.009	3.333 ± 0.167

Values were expressed as mean ± SEM of triplicates

Table 2. IC_{50} of some metal ions used as possible inhibitors

GST Isozymes	IC_{50} (mM)				
	Fe^{2+}	Co^{2+}	Hg^{2+}	Fe^{3+}	Mg^{2+}
GST A	77.631 ± 3.882	52.482 ± 2.624	26.923 ± 1.346	23.994 ± 1.110	45.711 ± 2.286
GST B	54.942 ± 2.747	38.023 ± 1.901	18.624 ± 0.931	28.841 ± 1.442	58.882 ± 2.944

Values were expressed as mean ± SEM of triplicates

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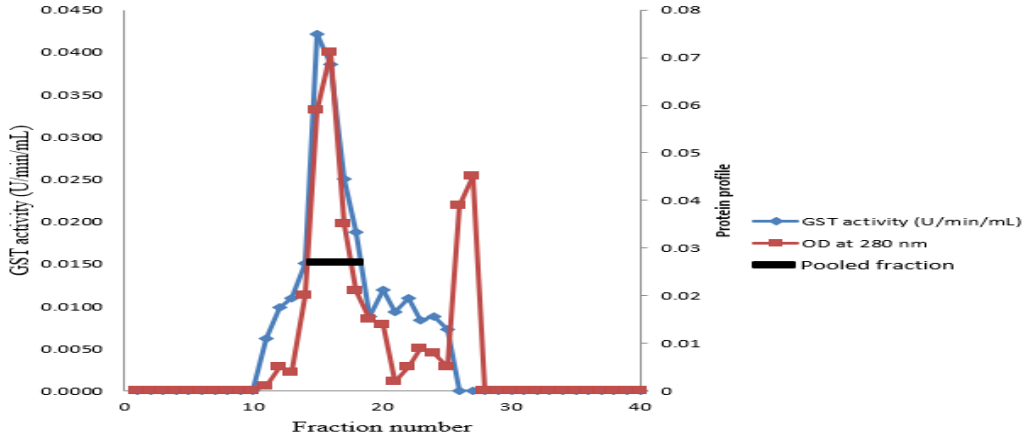

Figure 2. Elution profile of GST on Sephacryl S-200 column.

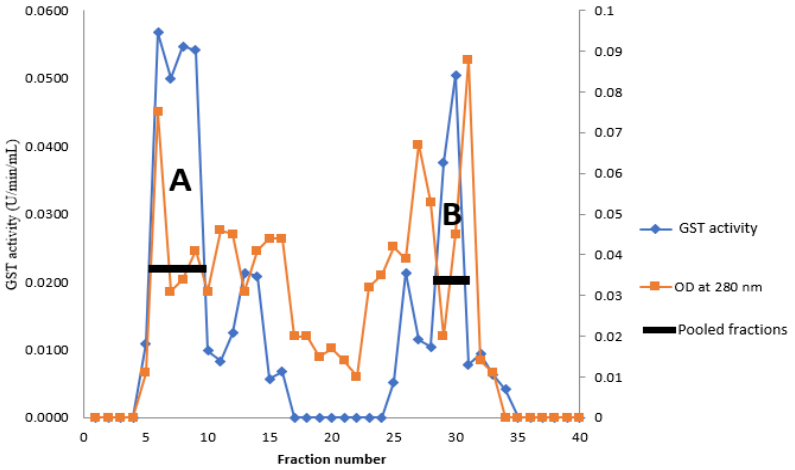

Figure 3. Separation of isozymes on CM-Sepharose ion exchange column.

Activation energy refers to the energy barrier that must be surmounted by reacting particles or compounds before any given reaction can take place (Pauling, 1946). The activation energy for an enzymatic reaction is crucial as it determines the rate at which the reaction occurs. Koshland's concept of enzyme specificity and transition state theory emphasizes that enzymes with lower activation energies are generally more effective in facilitating chemical transformations (Koshland, 1958). Enzymes function by stabilizing the transition state of the reaction, effectively lowering the activation energy required for the reaction to proceed. This stabilization facilitates the formation of the product and enhances the overall reaction rate (Garcia-Viloca *et al.*, 2004). In our study, we determined the activation energies for GST A and GST B to be 49.889 ± 2.495 and 18.283 ± 0.914 kJ/mol respectively. These values are reflective of the energy barriers these enzymes must overcome to catalyze their respective reactions. Thus, the lower activation energy of GST B suggests it may catalyze reactions most efficiently. Hence, the enhanced catalytic efficiency of GST B might contribute to more effective detoxification pathways and better protection against environmental toxicity.

Although there is paucity of information on the effect of thiol reagents on the activity of GST, this study revealed that thiol reagents generally activated the activities of all the isozymes of GST that were induced (Figure 4 & 5). The activation of GST by thiol reagents observed in our study suggests a potential mechanism by which these reagents enhance the enzymatic activity. This activation effect could be explained through several mechanistic pathways (Peter, 2013). One possible mechanism is the reduction of oxidized cysteine residues within the enzyme's active site (Biswas *et al*., 2006). GSTs, particularly those exposed to oxidative stress or toxic agents like glyphosate, may undergo oxidative modifications that impair their function. Thiol reagents, acting as reducing agents, could reverse these oxidative changes and restore enzyme activity.

Another mechanism could involve thiol reagents promoting the formation of enzyme-thiol reagent adducts that stabilize the enzyme's active conformation (Biswas *et al*., 2006). Such adduct formation could enhance the enzyme's affinity for glutathione (Vaskova *et al*., 2023). The precise nature of these adducts and their impact on enzyme kinetics

warrant further investigation. Thiol reagents that activate GSTs might be explored as potential therapeutic agents or adjuvants to boost the detoxification capacity in cases of exposure to toxic substances.

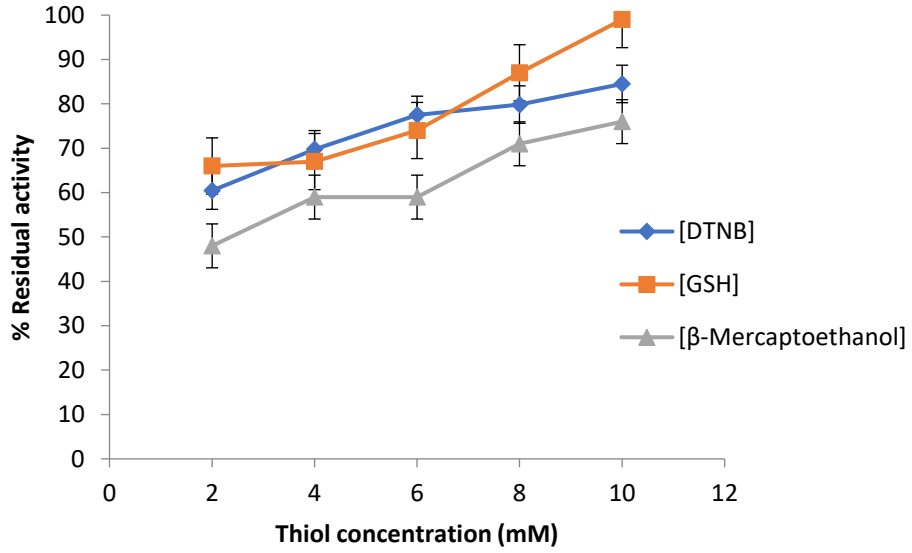

Figure 4. Effect of thiol reagent on GST A isozyme

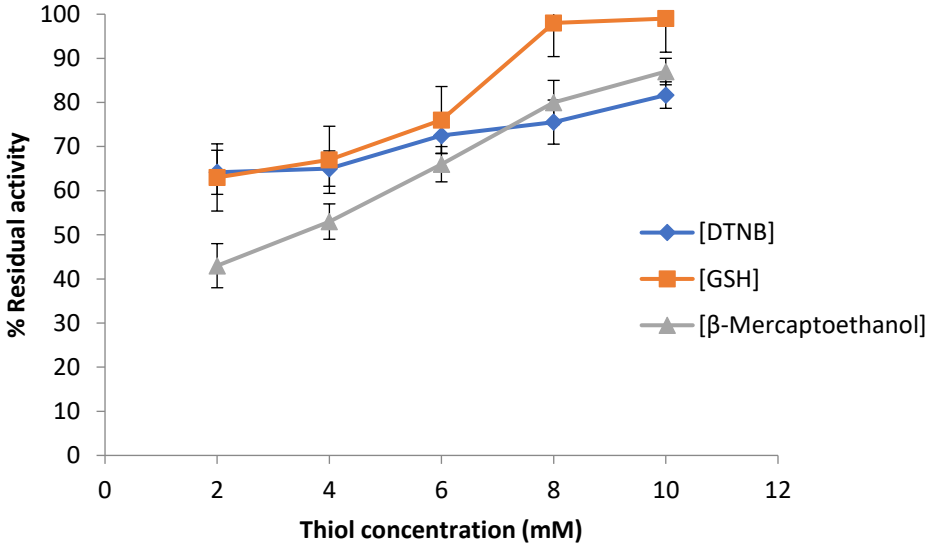

Figure 5. Effect of thiol reagent on GST B isozyme

Xenobiotic accumulation is a known contributor to environmental pollution (Basha & Rani, 2003). Prior research has addressed the *in vivo* and *in vitro* impact of metals on GST activity (Anushia *et al*., 2012; Agunbiade *et al*., 2014); however, data on GST responses in organisms exposed to herbicides, particularly glyphosate, remain scarce. Metal ions have been shown to inhibit the activities of the different isozymes of GST (Akkemik *et al*., 2012; Comakli *et al*., 2012; Demirdag *et al*., 2012; Ozaslan *et al*., 2018; Dobritzsch *et al*., 2020; Ozaslan and Cifti, 2022) and studies have suggested that this inhibition effect could be a result of binding of the metals to a site other than the active site of the enzyme (Aksoy *et al*., 2016). Corroborating this, our current investigation demonstrates that metal ions impede the enzymatic functions of GST isozymes. GST catalytic function and mRNA levels are established biomarkers for environmental xenobiotic exposure (Çomakli *et al*., 2015). Earlier findings have indicated that heavy metals markedly disrupt GST enzymatic processes (Comakli *et al*., 2012) with the half-maximal inhibitory concentration (IC_{50}) serving as a measure of metal ion inhibition.

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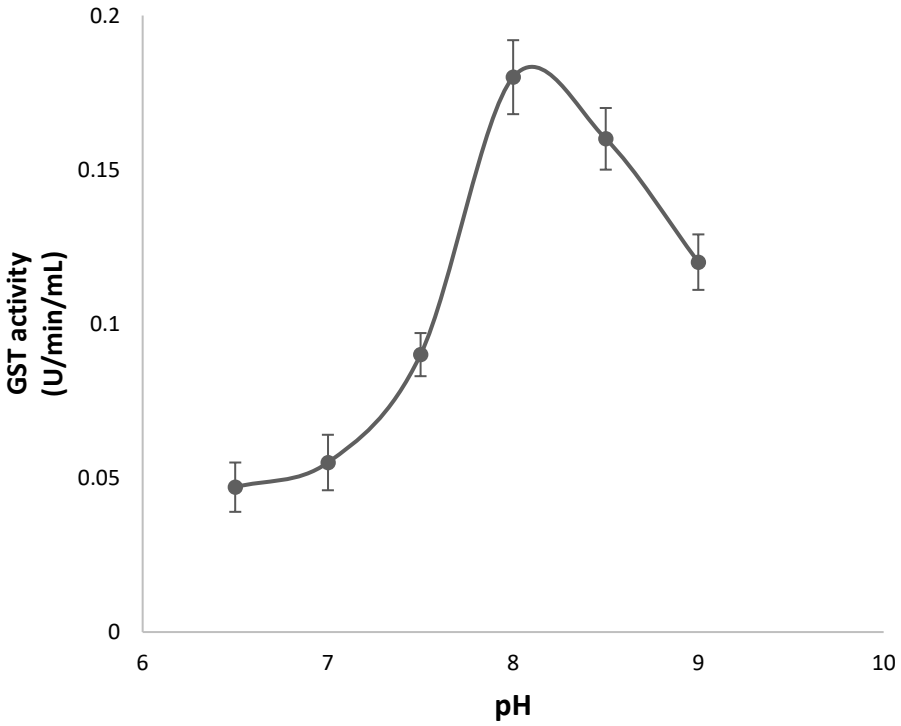

Figure 6. Effect of pH on GST A isozyme

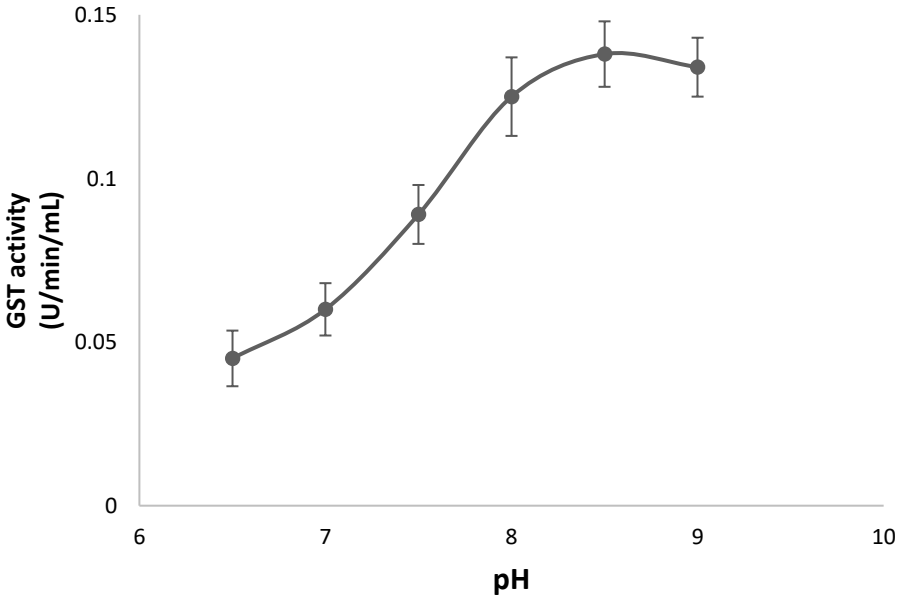

Figure 7. Effect of pH on GST B isozyme

Previous studies either focused on effects of metals or other environmental contaminants on GST, which leaves this crucial area of synergistic effect underexplored. While metal ions exert substantial inhibitory effects on GST in this present study, the presence of glyphosate could potentially alter these interactions. This suggests a more complex interplay between metal ions and environmental pollutants, with implications for understanding the broader impacts of pollution on enzymatic functions.

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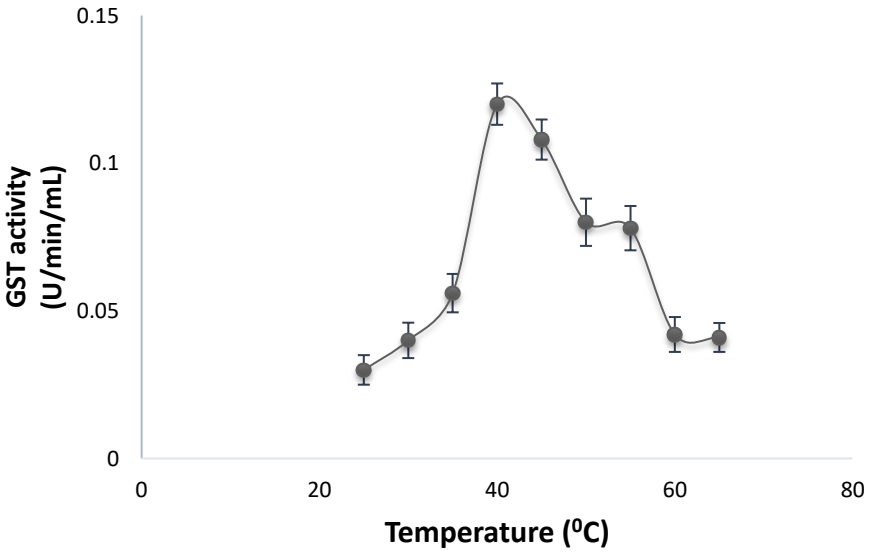

Figure 8. Effect of temperature on GST A isozyme

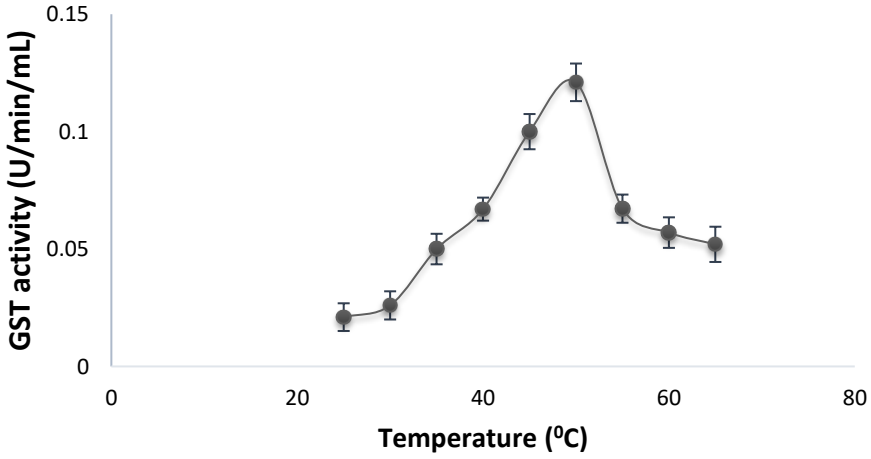

Figure 9. Effect of temperature on GST A isozyme

4. Conclusion

This research identified two cytosolic glutathione-S-transferase (GST) isozymes, GST A and GST B, in rat liver exposed to glyphosate; each of these isozymes exhibited unique kinetic properties. The optimal conditions for their activity were established, revealing that they remain stable under certain temperature and pH. GST B, in particular, demonstrated a lower activation energy, indicating a more efficient catalytic process.

The induced GST isozymes also exhibited a significant decrease in activity upon exposure to minimal concentrations of metal ions, indicating that such ions can impair the enzymes' protective roles against various xenobiotics. The activation of GST by thiol reagents and its inhibition by metal ions highlight the enzymes' sensitivity to environmental factors. This sensitivity positions GST as a crucial biomarker for monitoring exposure to xenobiotics shedding light on the broader implications for environmental pollution studies. This is especially relevant in the context of assessing the impact of herbicides such as glyphosate on the ecosystems.

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