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In-vitro inhibition of camel hepatic glutathione transferase by Quercetin

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Abstract: Glutathione S-transferases (GST) are a group of multifunctional ubiquitous enzymes widely present in animals and plants, which catalysis the conjugation of glutathione to different exogenous and endogenous electrophilic compounds. This study was carried out to characterize the purified GST enzyme from camel liver tissues and to investigate the *in-vitro* inhibitory effect of the flavonoid quercetin by measuring S-2,4-dinitrophenyl glutathione (DNP-GSH) formation from 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione(GSH) as substrates. The Km values for reduced GSH and CDNB were found to be 0.08438 and 0.6827 mM while Vmax values were 6.935 and 15.599 mM/min respectively. The IC_{50} was determined to be 1.8 mM. The inhibition constant (Ki) was estimated to be 1.91 mM at 0.5 mM and 1.76 mM at 2 mM. The mean inhibition constant (Ki) was estimated to be 1.835±0.075mM which revealed an uncompetitive profile and indicated quercetin as a weak inhibitor with the varied concentration of CDNB and fixed concentration of reduced GSH as a substrate.

Keywords: GST, Glutathione S-Transferase, CDNB, 1-Chloro-2,4-Dinitrobenzene, ($Vmax^{app}$), Apparent Vmax, (IC_{50}), The Inhibitor Concentration Causing 50% Inhibition, Km, Michaelis Constant, (Ki), Inhibitor Constant, (Km^{app}), Apparent Km

1. Introduction

Glutathione transferases also known as Glutathione S-transferases (EC 2.5.1.18) are a family of ubiquitous enzymes, present in the soluble fraction of many tissues, which are encoded by a large gene family (1-3). They are major phase II detoxification multifunctional cytosolic and membrane-associated microsomal proteins, that catalyzes a broad range of reactions that involve the addition of endogenous tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) to substrate compounds, but their archetypal functional role is in enzymatic detoxification of xenobiotic by facilitating their conjugation to glutathione, and the product is more water soluble, less toxic and more readily excreted (4, 5). These enzymes have broad substrate acceptance, although each GST possesses its own specific catalytic profile. Most GSTs are dimeric proteins. Each monomer is composed of a conserved thioredoxin domain containing the GSH binding pocket (G site) and a more variable α-helical domain (H site) containing the binding site for the GSH acceptor substrate (6).

GSTs are mainly classified into several classes (alpha, mu, pi, theta, sigma, kappa, zeta and omega) based on their primary structure, immunological and kinetic properties (7). In addition to its major role of sequestering carcinogen GST play an important role in cell signaling and other cellular processes. For example, GST pi has been shown to regulate c-Jun N-terminal kinase (JNK) signaling (8), and GST mu from mice forms inhibitory complexes with apoptosis signal-regulation Kinase 1 (ASK1), another member of the mitogen-activated protein (MAP) kinase pathway (9). Like other mammals, the camel exhibits GST activity in the liver and extra hepatic tissues (10) Camels are of great interest: although characterization of classical detoxification systems in such animals is not well known, they have unusual biochemical characteristics, differing from other mammals in a number of biochemical and physiological properties which led us to study at its detoxification enzyme GST kinetics and also explored the effect of common flavonoids quercetin on its enzyme activity.

Common flavonoids like Quercetin are present in most fruit and vegetables and it has been extensively studied because of its presumed antioxidant activity as well as its ability to modulate the activity of numerous enzymes involved in signal transduction, cell growth and

biotransformation (11, 12). *In vivo* studies indicated that dietary flavonoids like quercetin could significantly reduce the enzyme activity of the phase II enzymes GST and quinone oxireductase 1(NQO1) in rat liver, thus undermining the ability of the organism to detoxify endogenous and exogenous xenobiotics (13, 14).

The present study aimed to characterize camel hepatic GST and to investigate the inhibitory potential of quercetin on its activity *in vitro.*

2. Materials and Methods

Reduced GSH and CDNB and Quercetin were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). All starting materials and solvents used in this work were reagent grade in generally 98% pure.

2.1. Purification of the GST Enzyme from Camel Liver

To prepare the enzymes, twenty grams of camel liver were cut into small pieces and separately homogenized with 30 ml of 0.1 M potassium phosphate buffer pH 6.5, using a Wring blender for 1 min. All further steps were carried out at 4oC. The homogenates were centrifuged at 37,000 g for 30 min and the resulting supernatants filtered through glass wool to remove floating lipids. The supernatants were applied to a small GSH-Agarose affinity column and the glutathione S-transferases purified essentially as described by Hunaiti & Sarhan (1987). The activity of Glutathione S-transferase was assayed in a reaction mixture containing 100 mM phosphate buffer, pH 6.5, 1 mM 1- Chloro-2,4- dinitrobenzene (CDNB) and purified enzyme equivalent to 40 µg of purified enzyme. The reaction was initiated by addding 1 mM reduced glutathione (GSH) and formation of S-(2, 4-dinitrophenyl) glutathione (DNP-GSH) was monitored as an increase in absorbance at 340 nm as described by Hapig et al 1974 . Protein levels were determined spectrophotometrically at 600 nm using bovine serum albumin (BSA) as a protein standard (15).

2.2. Determination of Optimum Temperature

Purified GST enzyme (40 µg) in 1mM phosphate buffer(pH 6.5) was incubated at various temperatures 30, 35, 40, 45, 50, 55 and 60 ºC for 15 min before initiating the reaction by first adding 1mM CDNB followed by the addition of 1mM reduced GSH as described in materials and method section and monitoring the change in absorbance at 340nm for up to 2min.

2.3. Determination of Optimum pH

Glutathione S-transferase was incubated at 37 ºC for 5 minutes in varying pH concentration of 1mM potassium phosphate buffer (varying from pH 5 to 8).The GST activity was measured as described before.

2.4. Determination of Enzyme Kinetics

Two sets of experiments were conducted to determine the Km and Vmax of the enzyme. In the first set, the activity of glutathione S-transferase was measured at constant CDNB concentration of 1 mM with varying concentrations of reduced GSH ranging from 0.2 to 1.0mM, this set was used to determine the Km and Vmax values for GSH.

In the second set of the experiments, reduced GSH was held constant at 1.0 mM, but CDNB concentration was varied in five different concentration ranging from 0.2 to 1.0mM in order to determine the Km and Vmax values for CDNB as a substrate.

The Km and V-max values were calculated automatically by using the Michaelis-Menten equation using non-linear regression in GraphPad Prism 6 software (Graph Pad Prism Software Inc., USA).

2.5. Determination of IC_{50} and Ki

In order to determine IC_{50} value, enzyme activities were calculated in a reaction mixture which contained 100 mM reduced GSH, 100 mM CDNB with different quercetin concentration. The enzyme assay was carried out at 25^{o}C at a pH of 7.2 by adding 10µl of purified GST enzyme to the above reaction mixture. The enzyme activity was measured by the increase in absorbance for 2 min at 340nm. The reaction rate/min was determined by the slope Δ_{340} /min. The change in absorbance observed at 340nm for 2 min in the cuvette containing enzyme reaction mixture as described above without the inhibitor was taken as 100% enzyme activity.

The inhibitor concentration causing 50% inhibition (IC_{50}) was determined from the plot of GST enzyme activity verses different concentration of Quercetin ranged from 0.2 to 1mM. In order to determine Ki constant of the inhibitor (quercetin) in the reaction mixture, the substrate CDNB concentrations were varied from 0.2 to 0.8 mM with the fixed GSH substrate concentration to 100mM. Inhibitor solutions were added to the reaction medium, resulting in two different fixed concentrations (0.5 and 2 mM) of inhibitor in 1 ml of total reaction volume.

Lineweaver-Burk double reciprocal plots of the initial velocity against the concentration of CDNB in the presence and absence of inhibitor (quercetin) were used to calculate the ki and type of inhibition.

2.6. Statistical Analysis

Graph Pad Prism program (version 5 Graph Pad Software, Inc., San Diego, USA) was used to calculate Km and Vmax values and drawing the kinetic and inhibition graphs.

3. Results

3.1. Determination of the Optimum Temperature

Purified GST enzyme was incubated at different temperatures and enzyme activity was measured as described in materials and method section. The optimum temperature was determined from the plot of enzyme activity versus different temperature, which indicated that optimum temperature of the enzyme is 53^{o}C as shown in Fig.1.

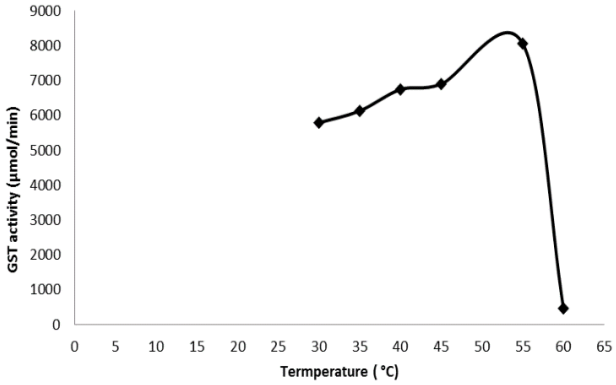


Fig 1. *The effect of different temperature on GST enzyme activity.*

3.2. Determination of the Optimum pH

Purified GST enzyme was incubated at different pH and its enzyme activity was measured as shown in Fig. 2. The bell shaped curve was obtained when enzyme activity was plotted against various pH. The optimum pH was determined using this graph. The optimum pH of GST enzyme was found to be 7.2.

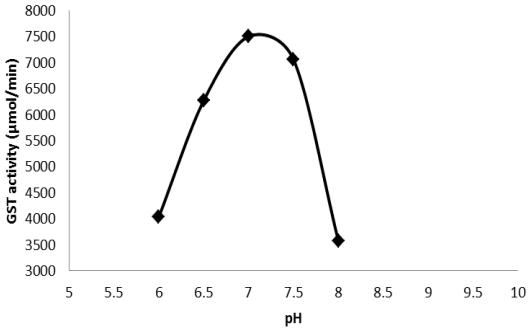


Fig 2. *Effect of different pH on GST enzyme activity.*

3.3. Kinetic Studies

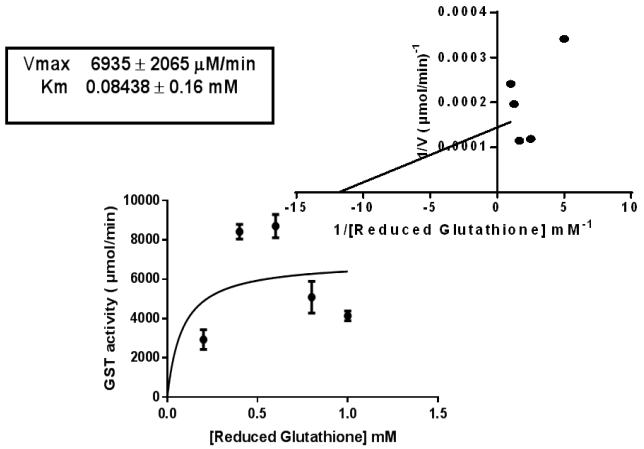


Fig 3. *A: Effect of reduced GSH substrate concentration on the enzyme activity. The initial velocity (amount of reduced GSH conjugated to CDNB per min.) was plotted against different reduced GSH concentration. Vmax and Km values were calculated by using nonlinear regression data analysis by Graph pad prism. B: Lineweaver burk plot of the reciprocal of the initial velocity versus reciprocal of substrate (reduced GSH) concentration.*

Km and Vmax values for CDNB and reduced GSH were obtained from the Michaelis menten plot of the GST enzyme activity with various concentration substrate concentration of reduced GSH and CDNB (Fig 3A and Fig 4A).

Lineweaver-Burk plot was obtained by plotting the reciprocal of the GST enzyme activity versus the reciprocal of reduced GSH and CDNB substrate concentration. A linear relationship was obtained (Fig 3B, Fig. 4b.) giving a Km and Vmax values.

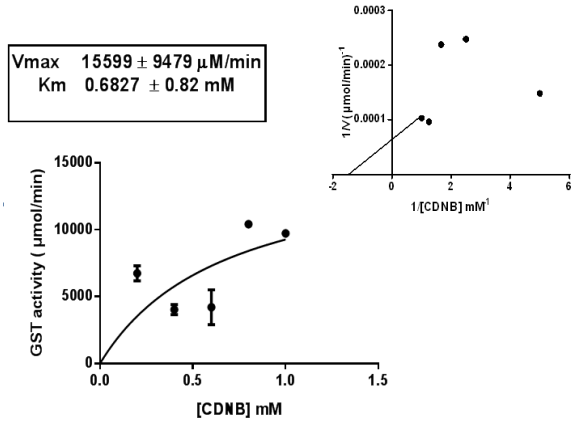


Fig 4. *A: Effect of CDNB substrate concentration on the GST enzyme activity. The initial enzyme reaction velocity (micromole of CDNB conjugated to reduced GSH per min)was plotted against different concentrations of CDNB. Vmax and Km were calculated as before. B: line weaver plot of the reciprocal of the initial velocity versus reciprocal of substrate (CDNB) concentration.*

3.4. Inhibition Studies

3.4.1. Determination of IC_{50}Value

Inhibitory kinetics of quercetin was investigated by initially calculating the IC_{50} value for quercetin. The GST enzyme activity was measured at different quercetin concentrations ranging from 0.2mM to 6mM. The IC_{50} was found to be 1.8mM of quercetin which exhibited about 50 % inhibition of enzyme activity. At 6mM concentration of quercetin no enzyme activity was observed and GST enzyme seemed to be inhibited completely (Fig. 5).

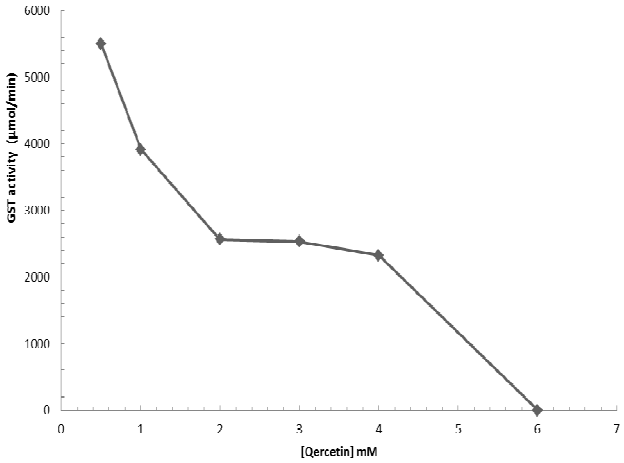
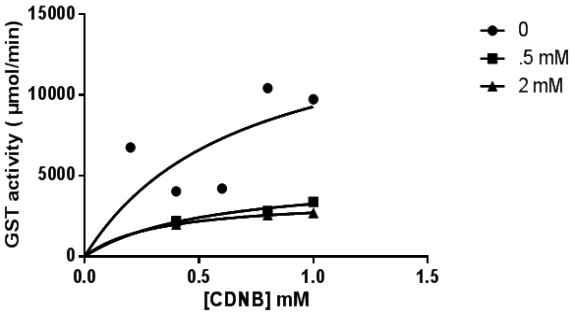


Fig 5. *Effect of different concentration of quercetin on the rate of enzyme activity.*

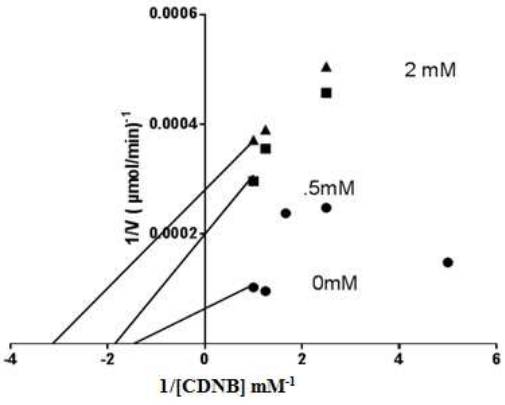
3.4.2. Determination of Ki

The inhibition kinetics of GST enzyme in the presence of quercetin was studied. The enzyme activity was measured in the absence and in the presence of 0.5mM and 1.8mM quercetin concentration, with varying concentration of CDNB (ranging from 0mM to 1mM) and fixed concentration of GSH. Michaelis menten curve was drawn using the average of each sample taken in triplicate. Vmax and Km values were calculated automatically using non-linear regression data analysis for enzyme kinetics by the latest edition of graph-pad prism programme. Lineweaver plot was used in order to find out the type of inhibition.

The Lineweaver-Burk plot (Fig. 6b) showed that inhibition by quercetin is in an uncompetitive manner. Ki values for both concentrations were calculated as shown in Table 2 from Fig. 6b.



A



B

Fig 6. *A: Effect of inhibitor (quercetin) on the rate of GST enzyme reaction velocity. The initial velocity of the enzyme reaction in mM/min was plotted against different concentrations of CDNB substrate with fixed amount of reduced GSH in the absence (■) and presence of 0.5mM (▲) and 2mM (●) of quercetin. B: line weaver burk plot of the reciprocal of initial velocity of GST enzyme versus reciprocal of CDNB substrate concentration .*

Table 1. *The values of Vmax, Km, Vmaxapp and Kmapp from the Micharlis-Menten plot using GraphPad Prism program for CDNB at different quercetin concentration.*

Quercetin concentration	Vmax (µM/min)	Km (mM)	$Vmax^{app}$ (mM/min)	Km^{app} (mM)
0 mM	15599 ±9479	0.6827± 0.82		
0.5 mM			5009±1174	0.5408±0.30
2 mM			3566±59.84	0.3193±0.017

Table 2. *The value of Ki from uncompetitive inhibition equation calculated from Lineweaver burk plot.*

Quercetin concentration	Ki from uncompetitive inhibition equation $(1/Km^{app} = \frac{1+(\frac{[I]}{Ki})}{Km})$
0.5 mM	1.90556730 mM ≈ 1.91 mM
2 mM	1.7572922 mM ≈1.76 mM

4. Discussion

Glutathione s transferase has been found in a wide variety of species. The difference in GST with respect to its expression, substrate specificity and activity of these enzymes can have a significant role in toxicity of xenobiotics**.** Free reactive electrophilic intermediate of Xenobiotics can produce damage to important cellular constituent. Reduced glutathione and glutathione transferase protects cells from this damage by capturing the reactive electrophilic before they can react at nucleophilic sites critical to cell viability (6)**.** The aim of this study was to characterize the purified camel hepatic GST enzyme and to investigate the role of quercetin a common flavonoids present in some food on its enzyme activity. The kinetic and inhibition studies were carried out with reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB), which are two substrates of GST. GST catalyzes the nucleophilic addition or substitution of glutathione at the electrophilic center of CDNB and formation of S-2,4-dinitrophenyl glutathione (DNP-GSH), which shows an increase in absorbance at 340 nm. Kinetic properties of camel liver GST was also studied with varying concentration for both of its substrate that is CDNB and reduced GSH, Vmax and Km values were calculate for each of its substrate. The Vmax and Km for CDNB was found to be 15599 ±9479 mM/min and 0.6827± 0.82 mM respectively. While in the case of GSH as a substrate we found the Vmax as 6.935±2.065mM/min and Km as 0.08438 ± 0.16 mM. The lower Km value for GSH indicated the higher affinity of GST enzyme towards GSH as compared to CDNB which has a higher Km. However, the maximum velocity Vmax of GST for CDNB was almost twice as that for GSH.

Flavonoids are known to modulate catalytic activity and expression of various enzymes. Glutathione *S*-transferases are the important biotransformation enzymes defending cells against potentially toxic xenobiotic. Naturally occurring flavonoids such as quercetin were indicated to be the inhibitor of GST enzyme in a number of studies (16-20)

In the present study the inhibitory kinetics of quercetin was investigated on CDNB concentration to find out the Ki values and the type of the inhibitor. From initial experiments IC_{50} was calculated to be 1.8mM when CDNB and GSH were used at fixed substrate concentration of 100mM. The Ki was estimated to be 1.91 mM at 0.5mM and 1.76 mM at 2mM quercetin concentration respectively with the varying concentration of CDNB and fixed concentration of GSH as a substrate.

In our study the Ki was found to be higher compared to

Km (Table 2) which indicated that quercetin was not a strong enzyme inhibitor for GST. The inhibition of GST enzyme with quercetin showed uncompetitive inhibition profile.

5. Conclusion

In conclusion, this study demonstrated that camel liver GST showed unique kinetics properties, showing optimum pH of 7.2 and optimum temperature of 53^{o}C. The Vmax and Km showed that GST enzyme has low Vmax and low Km for GSH as a substrate when compared to CDNB as a substrate, which has a much higher Vmax and Km. Camel hepatic GST was inhibited by quercetin reversibly in un-competitive manner with varied concentration of CDNB and 100mM of reduced GSH as substrates. The IC_{50} was calculated to be 1.8mM and the inhibitory potency of quercetin was investigated and found to be 1.91 mM at 0.5 mM and 1.76 mM at 2 mM concentration of quercetin.

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Conflict of Interest

The author declares that there is no conflict of interest with anybody or with any institution in preparation and submission of this work.

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