Inhibitory interactions of glutathione derivatives, coumarins and quercetins with dominant onion bulb glutathione *S*-transferases: a structural analysis

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Abstract: The dominant onion bulb glutathione *S*-transferases (GSTs) (named as GSTc, GSTd and GSTe) were separated by DEAE cellulose column chromatography, and the inhibitory interactions of glutathione (GSH) derivatives (*S*-methyl GSH, *S*-propyl GSH, *S*-butyl GSH, and *S*-hexyl GSH), four coumarins (coumarin, 7-hydroxy coumarin, esculetin and scopoletin) were tested with theses GSTs. The activities of GSTs were found to be inhibited differently by different compounds. The longer alkyl chained GSH derivatives viz. *S*-hexyl GSH (IC_{50} 25 μM) followed by *S*-butyl GSH (IC_{50} 28 μM) showed stronger inhibitory effect on the 1-chloro-2,4-dinitrobenzene (CDNB) conjugated activity of GSTe than shorter ones (*S*-propyl and *S*-methyl GSH). Among the coumarins tested, only esculetin had very strong inhibitory effects on GSTc and GSTd with IC_{50} values of 25 and 28 μM, respectively, but the activity of GSTe was poorly inhibited by this compound. Among the quercetins, quercetin-4′-clucoside showed strongest inhibitory effect on the activities of GSTc and GSTd with IC_{50}s of 8.6 and 7.1 μm, respectively. The IC_{50}s of quercetin, quercetin-3βD-glucoside and quercetin-3,4′-diglucoside were 21.1, 76.3, 76 μm on GSTc, respectively and 20.4, 69.3, 67.7 μm on GSTd, respectively. The structure-activity relationships suggested that hydroxyl groups at C6 and C7 positions of the coumarin skeleton played an important role in the expression of GST inhibitory activity. On the other hand, addition of glucoside at C4′ position of quercetin increased inhibitory activities on GSTc and GSTd.

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1. Introduction

Glutathione *S*-transferases (GSTs; E.C. 2.5.1.18) are a multigene family of enzymes that catalyze the conjugation of tripeptide glutathione (GSH) to a variety of hydrophobic, electrophilic, and usually cytotoxic substances (Mannervic and Danielson, 1988). In plant, GSTs have been proposed also to act as nonenzymatic carrier proteins/ligands for a wide variety of secondary metabolites (Edwards et al., 2000). GSTs also play an important role in protection cells against oxidative damage (Gronwald and Plaisance, 1998), intracellular transport of auxin (Watahiki et al., 1995), auxin-like substances (Droog et al., 1995) and cytokinin (Gonneau et al., 1998). However, information on interaction of plant GSTs with secondary metabolites is limited.

Plant GSTs are induced by different environmental and chemical stresses (Marrs, 1996; Hossain et al., 2006). On the other hand, the activities of plant GSTs have been reported to be inhibited by various natural and synthetic compounds. The activities of GSTs have been reported to be inhibited by *S*-hexyl glutathione (GSH) and its conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) (Cho and Kong, 2005), α,β-unsaturated carbonyl compounds and related aldehydes (Fujita and Hossain, 2003), auxin and related compounds (Droog et al., 1995; Watahiki et al., 1995), flavonoids (Mueller et al., 2000; Cummins et al., 2003) and a variety of other phenolic substances (Dean et al., 1995; Kitts and Wijewickreme, 1994). Recently, inhibitory approach has been reported as valuable tools to search physiological substrates of GSTs (Hossain et al., 2007a), though the information of physiological inhibitors of GSTs have been limited.

Onion bulb contains high level of GST activity

than other vegetable crops (Hossain et al., 2007b). On the other hand, alliums are rich source of a variety of sulfur compounds such as GSH derivatives (Lancaster and Shaw, 1989; Jones et al., 2004) and quercetins (Rohman et al., 2009a). Recently, in TLC analysis of onion bulb extracts, we observed some bands of florescent compounds and some of them might be some coumarin compounds. Therefore, the existence of GSH derivatives, quercetins and coumarins might have significant interactions with cellular GSTs of onion bulb. Considering all, interactions of some GSH derivatives, coumarins and quercetins were studied with dominant onion bulb GSTs. The GSTs were separated by DEAE column chromatography. In this paper, we report the structural relationship of the compounds in inhibiting the activities of the dominant onion bulb GSTs.

2. Materials and Methods

2.1 Preparation of crude enzyme:

Crude enzyme was extracted by homogenizing 150 g of fresh onion bulb tissue in an equal volume of 25 mM Tris-HCL buffer (pH 8.5), that contained 1 mM EDTA and 1% (w/v) ascorbate in a Waring blender. Homogenate was squeezed through two layers of nylon cloth and centrifuged at 11,500×*g* for 10 minutes and the supernatant was used as a crude enzyme solution. All procedures were performed at 4˚C.

2.2 Separation of GSTs:

Proteins were precipitated by ammonium sulphate at 65% saturation and centrifuged at 11,500×*g* for 10 minutes. The proteins were dialyzed against 10 mM Tris-HCl buffer (pH 8) containing 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA (Buffer A) overnight. The dialyzate was applied to a column (1.77 cm i.d. × 20 cm) of DEAE cellulose (DE-52; Whatman U.K.) that had been equilibrated with buffer A and eluted with a linear gradient of 0 to 0.2 M KCl in 600 ml of buffer A. Peaks eluted with high activity were collected and used to assay the inhibitory potencies of different chemicals.

2.3 Chemicals:

Five GSH derivatives (*S*-methyl GSH, *S*-propyl GSH, *S*-butyl GSH and *S*-hexyl GSH) from Sigma Company and four coumarins (coumarin, esculetin, scopoletin and 7-Hydroxycoumarin), and cinamic acid, caffeic acid, pyrocatechol, chlorogenic acid, dihydroascorbic acid and four quercetins (quercetin, quercetin-4'-glucoside, quercetin-3βD-glucoside and quercetin-3,4'-diglucoside) from Wako Pure Chemicals Ltd. were incorporated in this study.

2.4 Assay of Enzyme Activity:

GST activity was determined spectrophotometrically by the method of Rohman et al. (2009a) with some modifications. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, 1 mM CDNB, chemicals and enzyme solutions in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB, and A_{340} was monitored at 25˚C for 1 minute.

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3. Result and Discussion

3.1 Separation of onion bulb GSTs

To separate the component onion bulb GSTs from crude enzyme solution the proteins extract was precipitated by ammonium sulfate at 65% saturation and fractionated on a DEAE cellulose column (Fig. 1). Five peaks of GST activity eluted at approximately 28, 62, 107, 120 and 164 mM KCl. For convenience, GSTs responsible for these peaks were designated as GSTa, GSTb, GSTc, GSTd and GSTe, respectively. Among the five peaks, GSTa and GSTb had small activity with 2.6% and 1.4% of total activity and three dominant GSTs, GSTc, GSTd and GSTe were accounted for 27%, 36% and 33% of the total activity. Peak fractions of dominant GSTs were collected and tested their interaction with different chemicals used in this study.

3.2 Inhibition of dominant onion bulb GSTs by GSH derivatives

The inhibitory effects of four GSH derivatives namely, *S*-methyl, *S*-propyl, *S*-propyl, *S*-hexyl GSH were tested on the activities of GSTc, GSTd and GSTe towards CDNB (Fig. 2). Different GSTs were found to be interacted differently with different chemicals (Fig. 3). Among the GSH derivatives, *S*-hexyl GSH followed by *S*-butyl GSH showed strongest inhibition on the activity of GSTe, while other GSH derivatives showed poor inhibitory effects. However, activities of GSTc and GSTd were not sensitive to such inhibition. IC_{50}s of *S*-hexyl GSH and *S*-butyl GSH were found as 22, 33 μm, respectively on GSTe. *S*-propyl GSH showed poor inhibition and no inhibitory effect was observed by *S*-methyl. On the other hand, though the inhibitory potencies of the GSH derivatives were low on GSTc and GSTd, the longer chained GSH derivatives had

higher inhibitory effect than a shorter one.

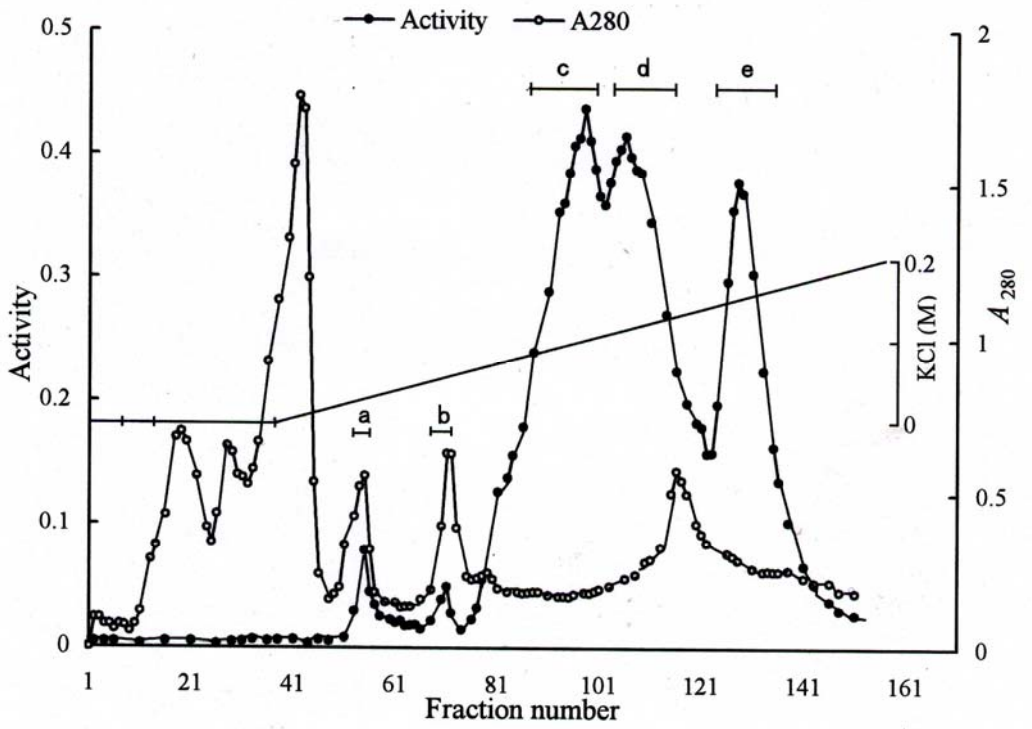
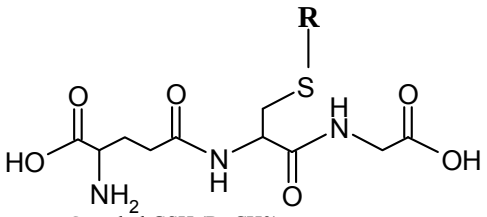


Figure 1. Column chromatography of DEAE-cellulose of 65% ammonium sulfate fraction from a crude enzyme solution extracted from 150 g onion bulb tissues. For each fraction, absorbance at 280 nm (○) and GST activity with 1-chloro-2,4-dinitrobenzene (●) were determined. Activity was expressed as μmol min^{-1} ml^{-1}. The fractions under the bar were pooled

3.3 Inhibition of dominant onion bulb GSTs by coumarins:

Four coumarins namely, coumarin, scopoletin, esculetin and 7-hydroxycoumarin were tested on the activities of different onion GSTs stated above (Fig. 4). Here also the sensitivities of the GSTs varied with different coumarins. Among the coumarins, only esculetin showed very strong inhibitory effects on the activities GSTc and GSTd with IC_{50} values of 25 μM and 28 μM, respectively, while other compounds showed negligible inhibitory effects (<10%) on other GSTs activities (Fig. 5). The other domonant GST, GSTe had no sensitivity to the inhibition of all the coumarins.

The two adjacent hydroxyl (OH) groups of esculetin in the coumarin skeleton have been reported to be responsible for inhibition of certain plant GSTs such as tyrosinase and polyphenol oxidase (Masamoto et al., 2003; Munoj-Munoj et al., 2007). Therefore, these two OH groups at the C6 and C7 positions of coumarin skeleton might play an important role in the expression of inhibitory activities on GSTc and GSTd. To check whether OH group(s) of other compound is also responsible for the inhibition of the GSTs activities, we investigated the effects of some chemicals possessing two adjacent OH groups namely, caffeic acid, chlorogenic acid, pyrocatechol and dihydroascorbic acid along with cinnamic acid on the activities of dominant onion GSTs (Fig. 5). None of the compounds showed any inhibitory effects on the GSTs (data not shown) suggesting that the inhibition caused by esculetin did not depend only on the general properties of adjacent OH groups.



S-methyl GSH (R=CH3)
S-propyl GSH (R=C3H7)
S-butyl GSH (R=C4H9)
S-hexyl GSH (R=C6H13)

Figure 2. Structures of derivatives of glutathione

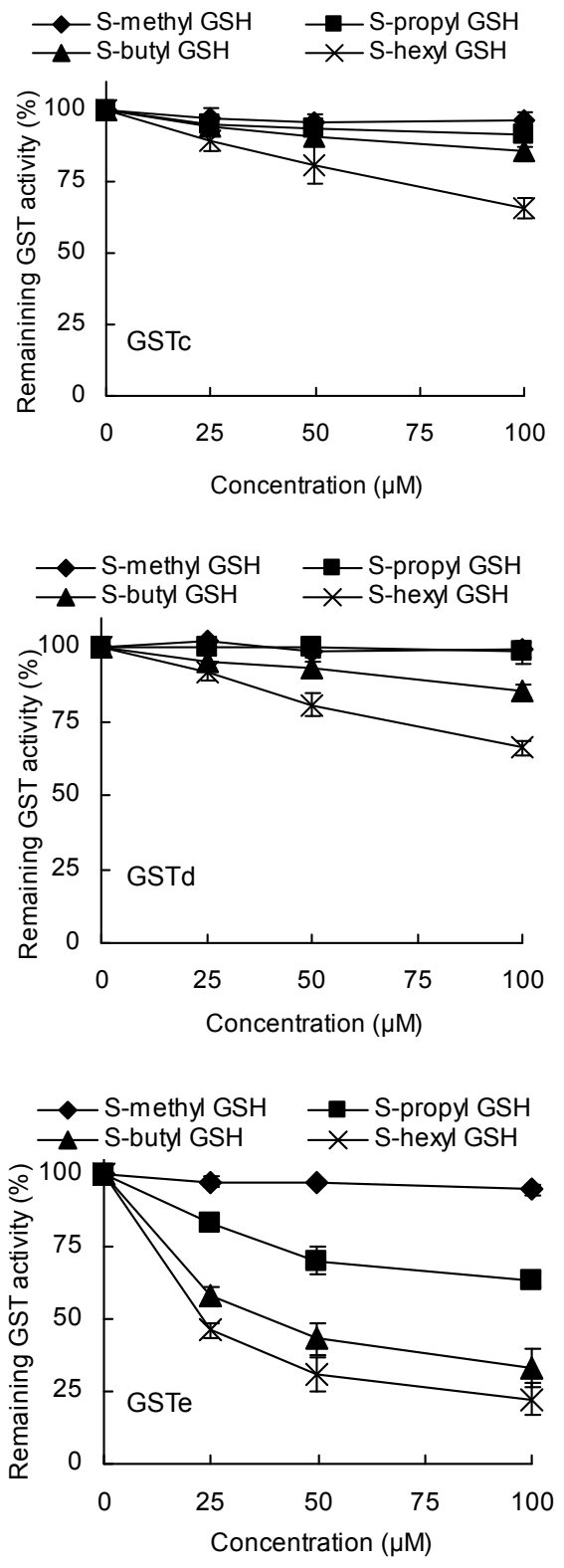


Figure 3. Inhibition of dominant onion bulb GSTs by different derivatives of glutathione. Results were obtained from 3 independent experiments and bars indicate standard error (SE)

3.4 Inhibition of dominant onion bulb GSTs by quercetins

Quercetins are important bioactive compound and their interactions have been extensively studying with animal GSTs. However, information on interaction of quercetins with plant GSTs have been limited. In this study, we tested the interaction of major quercetins of onion bulb with dominant onion bulb GSTs. The structures of major quercetins and the inhibitory potency of these compounds on dominant GSTs are showed in Fig. 6 and Table 1, respectively. It was observed that quercetin-4'-glucoside showed the strongest inhibitory effect on the activities of GSTc and GSTd with IC_{50} values of 8.6 and 7.1 μM, respectively. The IC_{50} values of quercetin, quercetin-3βD-glucoside and quercetin-3,4'-diglucoside were 21.1, 76.3 and 76.0 μM, respectively, for GSTc and 20.4, 69.3 and 67.7 μM, respectively, for GSTd. From this study, it was revealed that addition of one mole glucoside at C4′ position had tremendous inhibition on the GSTs. On the other hand, like esculetin, quercetins also showed poor inhibitory effects on GSTe (Table 1).

4. Discussion

Earlier reports also showed the high inhibitory potency of longer *S*-alkyl chained GSH derivatives on certain animal GSTs (Koehler et al., 1997; Ortiz-Salmeron et al., 2001). *S*-hexyl GSH has also been reported as a potent *in vitro* inhibitor of many animal GSTs occupying both G and H sites (Ong and Clark, 1986). Like previous study, we also found that the longer alkyl chained GSH derivatives had higher degrees of inhibitory effects on the activities of all GSTs than the shorter alkyl chained derivatives. *S*-hexyl GSH followed by *S*-butyl GSH which showed very strong inhibition on GSTe might also occupy both G and H sites of the enzyme. Although, several secondary sulfur compounds such as GSH derivatives are abundantly distributed in *Allium* spp. (Lancaster and Shaw, 1989; Jones et al., 2004), *S*-hexyl and *S*-butyl GSH which strongly inhibited the activity of GSTe, they are not present naturally in plants. Other physiological compounds, *S*-methyl and *S*-propyl GSH, showed low or no inhibitory activities towards the GSTs.

Coumarins are important physiological substances in plant cells (Costova, 2005) which control the action of plant growth hormones and growth regulators (Letham, 1978), decrease respiration and photosynthesis (Kupidlowska et al., 1994; Macias et al., 1999), inhibit root growth (Podbielkowska et al., 1995) and modify root morphology and histology (Kupidlowska et al.,

1994). Certain members of coumarins have been reported to act as strong inhibitors of various plant enzymes (Masamoto et al., 2003; Chang and Chiang, 1995). However, information on the effects of coumarin on plant GSTs is scarce. Previously, esculetin has been reported to be a strong inhibitor of lipoxygenase in soybean (Lee and Lillard, 1997) and tyrosinase in mushroom (Masamoto et al., 2003). It has also been reported that esculetin restricts the activities of antioxidant enzymes (GST and catalase) in Galleria mellonella larvae (Buyukguzel et al., 2006), but its inhibitory reports on plant GSTs is scare. In structural analysis, two hydroxyl groups at C6 and C7 position of esculetin are reported to have important role in the inhibitory activity of tyrosinase and polyphenol oxidase (Masamoto et al., 2003 and Munoj-Munoj et al., 2007). Therefore, two adjacent OH groups at those positions might also show inhibitory effect on the GSTs. This study also suggested that inhibitory effects not only depend on two adjacent OH groups, their poisons and compound skeleton are important.

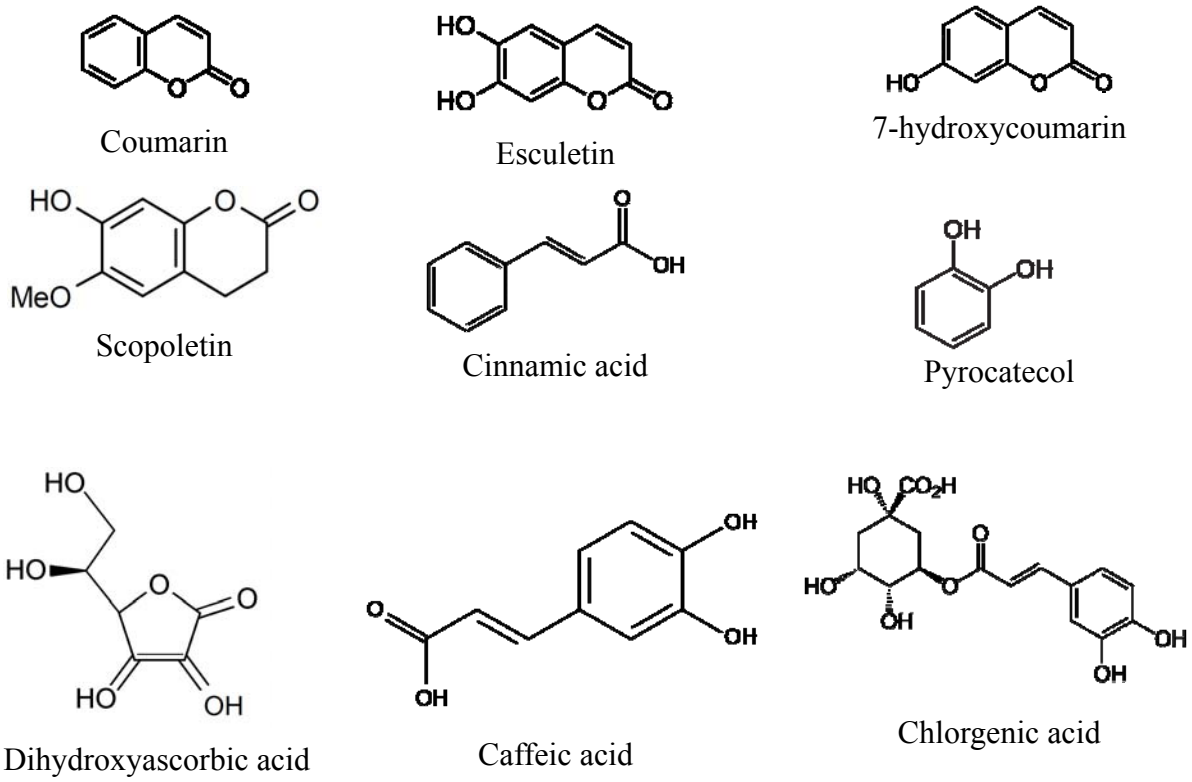


Figure 4. Structures of coumarins and other compounds with OH groups tested on the activities of dominant onion bulb GSTs

Table 1. Inhibition of dominant onion GSTs by major quercetins of onion (IC_{50} values in μM). IC_{50} values were graphically determined by plotting GST activities towards CDNB as a function of inhibitor concentration

Major flavonoids of onion	GSTc	GSTd	GSTe
Quercetin	21.1	20.4	Negligible inhibition (<10%)
Quercetin-4′-glucoside	8.6	7.1	Negligible inhibition (<10%)
Quercetin-3βD-glucoside	76.3	69.3	Negligible inhibition (<10%)
Quercetin-3,4′-diglucoside	76.0	67.7	Negligible inhibition (<10%)

Certain GSTs have been reported to be inhibited *in vitro* by quectentins, and GSTs are proposed to carry flavonoids without formation of GSH conjugation (Cummins et al., 2003). In our experiment, like esculetin, all of the quercetins inhibited the activities of GSTc and GSTd. Therefore, these two onion GSTs

might have metabolic relation with esculetin and quescetins. However, esculetin was not traced in onion bulb, and the predominant inhibitory substances are quercetin-4'-glucoside and quercetin-3,4'-diglucoside (Rohman et al., 2009a,b). Therefore, strong inhibitory interactions as well as simultaneous existence of high level GST and quercetin-4'-glucoside and quercetin-3,4'-diglucoside indicated that onion GSTS might have important physiological role in quercetin metabolism.

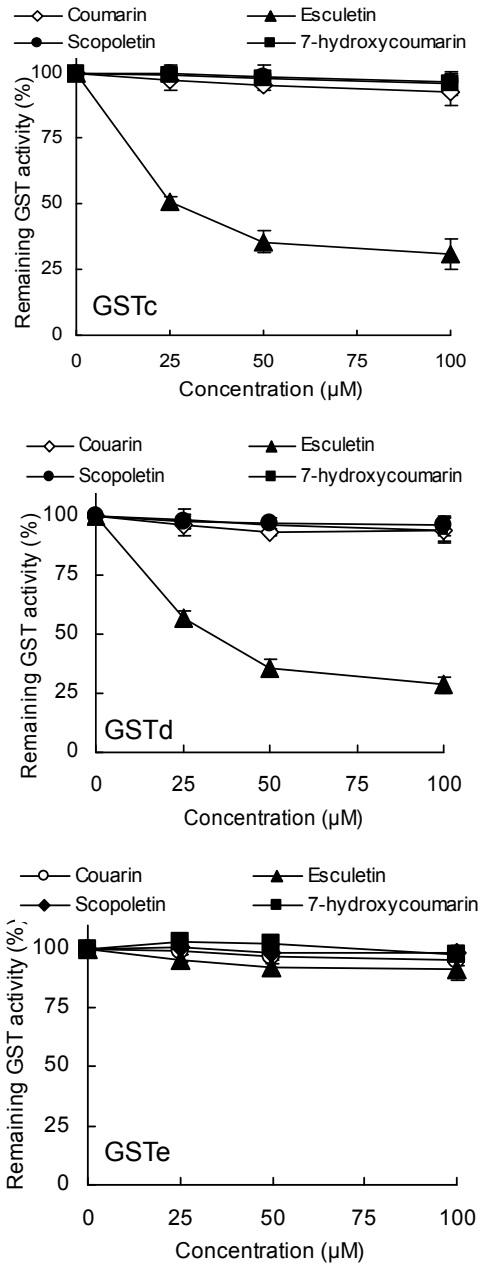
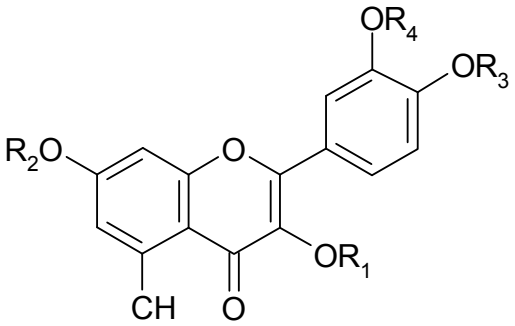


Figure 5. Inhibition of dominant onion bulb GSTs by different coumarin compounds. Results were obtained from 3 independent experiments and bars indicate SE



R1,R2,R3,R4=H: Quercetin
R1,R2,R4=H; R3=glu.: Quercetin-4′-glucoside
R2,R4=H; R1,R3=glu.: Quercetin-3,4′-diglucoside

Figure 6. Major quercetins in onion

5. Conclusions

In conclusion, this study demonstrated that inhibition depends on both types of GSTs and types of chemicals. It depends on alkyne chain of GSH, number and position of OH group of their skeleton and addition of glucoside at C4′ position of quercetin skeleton. However, this study thrust further research on the role of onion GSTs in quercetin metabolism in onion bulb.

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