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Identification of a glutathione *S*-transferase inhibitor in onion bulb (*Allium cepa* L.)

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Abstract

A glutathione *S*-transferase (GST, EC 2.5.1.18) inhibitor was isolated from the onion bulb by fractionation and purification using Sep-pak Vac C_{18} column, TLC and HPLC. Five partially purified onion bulb GSTs (GSTa and GSTb as minor GSTs, and GSTc, GSTd and GSTe as dominant GSTs) separated by DEAE column were used to detect the inhibitory substance in onion bulb extract. Among the three fractions of onion bulb extract, the water fraction showed the strongest inhibition on the CDNB conjugating activities of onion bulb GSTs followed by EtOAc faction. The *n*-hexane fraction had very low or all most no inhibition. Among the GSTs, GSTc and GSTd were found to be highly sensitive to the inhibition of the fractions; where GSTe was poorly sensitive, and GSTa and GSTb were almost insensitive to such inhibition. Substance(s) present in 45% methanol-elution of Sep-pak-Vac C_{18} column of water fraction was found to strongly inhibit the activity of GSTc. A yellow fraction (R_f = 0.48) on TLC representing 445 mg fresh onion bulb tissue showed 50% inhibition on the activity of GSTc. After purifying by HPLC, the inhibitory substance was identified as quercetin-3,4´-diglucoside by NMR and FAB-MS spectroscopes. The IC_{50}s of quercetin-3,4´-diglucoside were 76.3 μM and 69.3 μM on GSTc and GSTd, respectively. Quercetin-3,4´-diglucoside showed poor inhibitory expression on GSTe, and almost no inhibition on GSTa and GSTb.

Key words: Onion glutathione *S*-transferase; inhibitor; quercetin- 3,4´-diglucoside; onion bulb

Abbreviations: CDNB_1-chloro-2,4-dinitrobenzene; DEAE_Diethylaminoethyl; DW_ Distilled water; EDTA_ Ethylenediaminetetraacetic acid; EtOAc_ Ethyl acetate; FAB-MS_ Fast atom bombardment mass spectrometer; GST_ Glutathione *S*-transferase; HPLC_ High-performance liquid chromatography; NMR_ Nuclear magnetic resonance; SDS-PAGE_ Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC_ Thin layer chromatography; kDa_ kilo Dalton

Introduction

Plants produce numerous secondary metabolites that have versatile physiological and protective roles (Coleman et al., 1997; Steyn et al., 2002). At present, more than 200,000 secondary metabolites have been identified (Sirikantaramas et al., 2007). A large number of studies have demonstrated the importance of these metabolites as plant defense compounds (Tailor and Grotewold, 2005). On the other hand, many of these metabolites exert toxic effects on other organisms by interfering with cell protein function unless they are subcellular compartmentation (Marrs, 1996; Alfenito et al., 1998). Some of them interact with the molecules responsible for fundamental cellular function, such as DNA and the protein

involved in cell division. Therefore, to avoid self toxicity, plants employ different self-defense system to eliminate or modify the toxicity of the compounds.

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a multigene family of enzymes that constitute about 1% of the soluble protein in photosynthetic plant cells (Hayes and Pulford, 1995; Marrs, 1996). GSTs catalyze the conjugation of reduced glutathione (GSH) with a wide range of substrates (Droog, 1997). But GSTs are mostly studied for detoxification of diverse exogenous substrates including many drug administered to animals and herbicides applied to plants (Sandermann, 1992; Coleman et al., 1997). Due to economic importance of herbicides and safeners, they have been main focus of interest as substrates of plant GSTs. On the other hand, numerous toxic secondary metabolites are potential GST substrates, and in plant, GSTs are key enzyme in detoxification of endogenous toxins to lead vacuolar sequestration (Mueller et al., 2000). But little is known about the function of GSTs with endogenous compounds. Recently some physiological compounds have been studied as substrates of some plant GSTs (Hossain et al., 2007a; Cummins et al., 2003), and most of them have been found to inhibit the activities of GSTs. Therefore, inhibitory substrate might contribute basic information about physiological function of GSTs on plant secondary metabolites.

In our previous study, we found high GST activity in onion bulb compare to some other vegetable crops (Hossain et al., 2007b). In another experiment, we found inhibitory effect of onion bulb extract on crude onion bulb GST. Therefore, an attempt has been taken to identify the inhibitory substance(s) of onion GSTs in onion bulb. To search the inhibitory substance(s), the high active onion bulb GSTs were separated into its component GSTs by an anion exchange column chromatography, and used to detect the inhibitory substances in onion bulb. In this paper, we report the purification and identification of an inhibitor of highly expressed GSTs in onion bulb.

Materials and methods

Plant material

Mature onion (*Allium cepa* L., yellow variety) bulbs were obtained from a local market, and inhibitory substances and GSTs were extracted from onion bulb tissue.

Preparation of crude enzyme from onion bulb

Crude enzyme was extracted by homogenizing 150 g of fresh onion bulb tissues in an equal volume of 25 mM Tris-HCl buffer (pH 8.5), which contained 1 mM EDTA and 1% (w/v) ascorbate, with a Waring blender. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 11500×*g* for 10 minutes, and the supernatant was used as a crude enzyme solution.

Partial purification of GSTs from crude enzyme solution

Proteins were precipitated by ammonium sulfate at 65% saturation of the crude enzyme solution and centrifuged at 11500×*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 to completely remove low molecular inhibitors. 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. Each high active eluted peak was pooled separately and used to detect their inhibitory substances present in onion bulb extract.

Purification of GSTs

Each peak fraction (Fig. 1) of five partially purified GSTs (5 ml each) was separately applied on affinity column (0.76 cm i.d. × 4.0 cm) of *S*-hexylglutathione-agarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 1.5 mM *S*-hexylglutathione.

SDS-PAGE and silver staining

The purified five GSTs were subjected to SDS-PAGE (12.5% gel) according to Laemmli (1970). Protein was stained using a silver-staining protein detection kit (Stratagene, La Jolla, Calif., USA).

Enzyme assay

GST activity was determined spectrophotometrically by the method of Booth et al. (1961) with some

modifications as described by Fujita and Hossain (2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and inhibitor 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.

Protein estimation

Proteins of high active DEAE fractions and *S*-agarose hexyl glutathione fractions were estimated according to Bradford (1976) and Lowry et al., (1951), respectively.

Preparation of inhibitory substances from onion bulb

Low molecular compounds were extracted from the mature onion bulb as described by Masamoto et al. (2003) with little modification. One hundred g of fresh material was extracted three times with 50% methanol. The combined extracts were evaporated to dryness and suspended in 45 ml distilled water (DW). The substances were extracted three times with an equal volume of *n*-hexane followed by ethyl acetate (EtOAc), and remaining fraction was termed as water fraction. The *n*-hexane, EtOAc and water fractions were separately evaporated to dryness. The *n*-hexane and EtOAc-soluble fractions were each dissolved in 2.5 ml of 30% methanol, and the water fraction was dissolved in 40 ml DW.

Sep-pak Vac C_{18} column chromatography

Eight ml of water fraction (equivalent to 20 g fresh tissue) of mature onion bulb extract was put into a solid phase extraction kit (Sep-pak Vac C_{18} column, Waters, Ireland) and eluted with 20 ml of 0, 20, 45, 65 and 100% methanol in stages consecutively. The collected liquids were evaporated to dryness, dissolved in 0.50 ml of 50% ethanol and stored at -8˚C.

Thin layer chromatography (TLC)

TLC analysis was carried out on silica gel (Si 70, F_{254}, Wako, Japan) with solvent butanol-acetic acid-water (80:10:10, v/v/v). The silica gel was separated into 16 fractions in each 1-cm division from the origin to top and eluted with 10 ml of 50% methanol. Each eluate was evaporated to dryness and inhibitory activities were examined.

High-performance liquid chromatography (HPLC)

HPLC analysis was carried out on an LC-6AD Liquid Chromatograph (Shimadzu, Japan) fitted with a UV-VIS detector (SPD-6AV) and C-R6A Chromatopac. Separation was performed onto an Inertsil ODS-3 (4.6 mm i.d. × 100 mm, GL Sciences Inc., Japan) column with a methanol gradient from 30 to 80% for 40 min and at 100% for 20 min. The flow rate was 0.6 ml min^{-1} and detection was carried out at 220 nm.

NMR and FAB-MS spectroscopies

Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM Alpha-400 FT-NMR spectrometer (400 MHz) using magnetic stimulation (TMS) as an internal standard. Fast atom bombardment mass spectrometric (FAB-MS) spectra were acquired on a JEOL JMS-SX102A mass spectrometer in the positive ion mode with a magic bullet.

Results and discussion

Partial purification onion bulb GSTs

Five onion bulb GSTs were partially purified from crude onion bulb GSTs by DEAE-cellulose column chromatography, and were used to detect their inhibitory substances in onion bulb. The GST activities of all DEAE fractions were estimated towards model substrate CDNB. Five high active GST peaks were eluted at approximately 43, 65, 106, 117 and 157 mM KCl (Fig. 1). For convenience, the five GST of high eluted peaks were designated as GSTa, GSTb, GSTc, GSTd and GSTe, respectively. Among them, GSTa and GSTb, accounting for only 2.0% and 1.1% of the total activity, were termed minor GSTs, and GSTc, GSTd and GSTe, accounting for 27.7%, 35.2% and 33.6% of the total activity, were termed dominant GSTs.

Since GSTa and GSTb shoed very low activities towards CDNB, we assumed that these two GSTs might be quantitatively very low compared to dominant GSTs. Therefore, we directly applied the peak fraction of five GSTs (5 ml each) separately on to affinity column of *S*-hexylglutathione-agarose

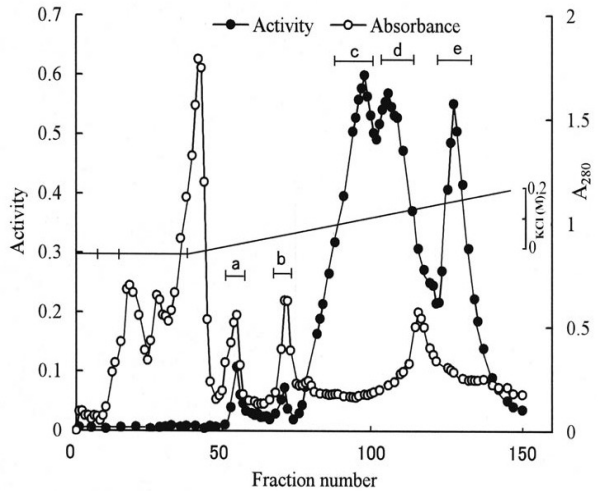

Fig 1. A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 150g onion bulb tissues. For each fraction, absorbance at 280 nm (o) and GST activity toward CDNB (●) were determined. Activity is expressed as μmol $min^{-1}ml^{-1}$. The fraction under the bar were pooled.

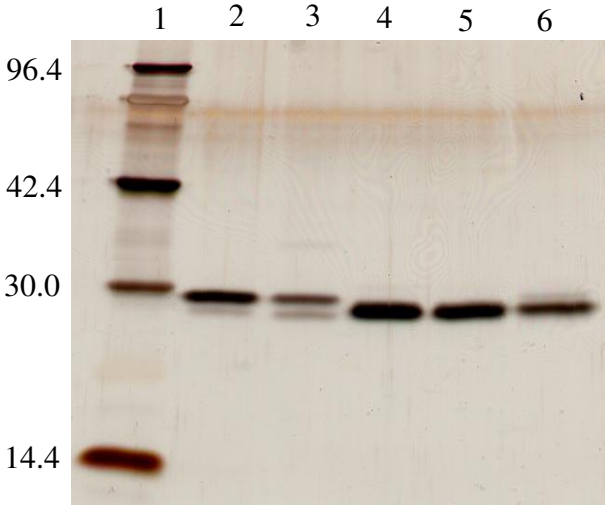

Fig 2. Silver staining of GSTs purified by affinity column to which each peak fraction of five partially purified GSTs was applied. Lane1, molecular weight marker; Lane 2, GSTa; Lane 3, GSTb; Lane 4, GSTc; Lane 5, GSTd and Lane 6; GSTe. Each lane contains 5.1 μg protein.

(Sigma, St. Louis, MO). Fractions of 2 ml of each GST were collected and applied on SDS-PAGE electrophoresis for silver staining. The comparative quantitative determination by silver-staining showed that the amount GSTa and GSTb are not as low as their activity showed (Fig 2). The model substrate CDNB used in this experiment is not a physiological substrate therefore, GSTa and GSTb might fully express to other physiological substrate. At the same time, this experiment also focused the molecular weight of onion bulb GSTs. In general GSTs are of 25-30 kDa protein. We also found that three dominant GSTs are of 27 kDa proteins, and GSTa and GSTb have higher molecule weight than those of the dominant GSTs, which also suggested that GSTa and GSTb might be different from other three dominant GSTs. However, all these five GSTs were used to detect their inhibitor(s).

Detection of inhibitory substance(s) of GSTs in onion bulb extracts

The methanol-water extract of onion bulb was divided into three fractions: i) *n*-hexane fraction, ii) EtOAc fraction and iii) water fraction. Present of inhibitory substance(s) in these fractions was tested by GSTa, GSTb, GSTc, GSTd and GSTe. Water fraction showed the strongest inhibition on the activities of GSTs followed by EtOAc fraction (Fig. 3). Very low inhibitory effect was apparent with the *n*-hexane fraction on all the GSTs. Among the GSTs, GSTc and GSTd were strongly inhibited by water fraction followed by EtOAc fraction. GSTe showed comparatively low sensitivity to the inhibition of all the three fractions, and GSTa and GSTb had very low or almost no inhibition. This experiment indicated that water fraction contained strong inhibitory substance(s) of onion bulb GSTs. Therefore, we investigated inhibitory substance(s) of the GSTs in water fraction.

Purification of inhibitory substance

Purification by Sep-pak Vac C_{18} column fraction

The water fraction was further fractionated by a Sep-pak Vac C_{18} column with 0, 20, 45, 65 and 100% methanol, and inhibitory substance eluted with 45% methanol - elution showed strongest inhibition on

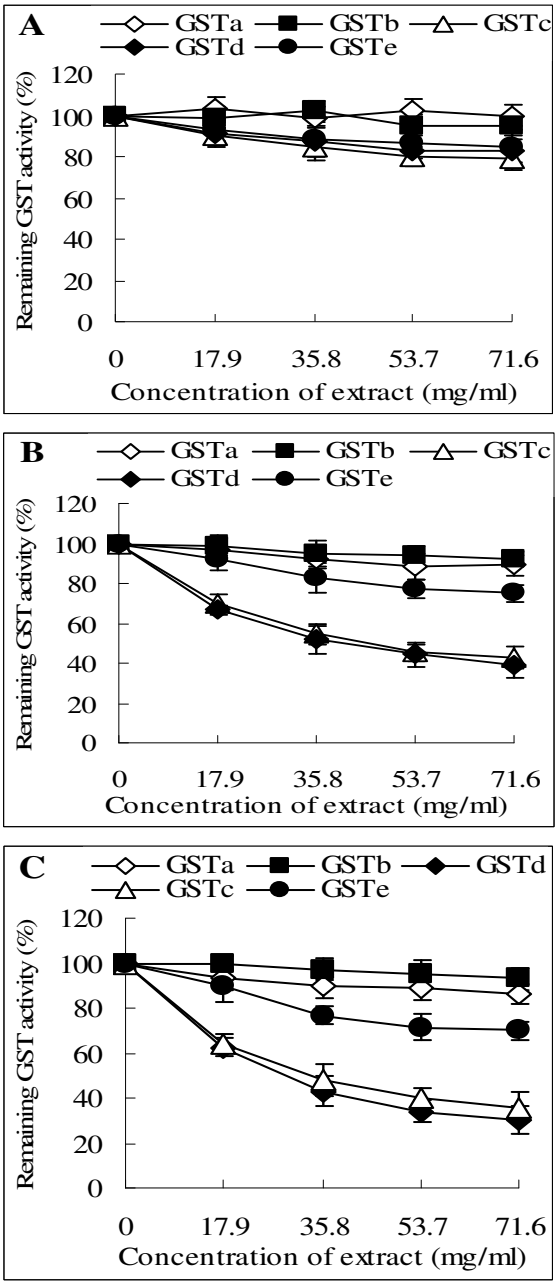

Fig 3. Inhibition of CDNB conjugating activities of onion bulb GSTs by **(A)** *n*-hexane fraction, **(B)** EtOAc fraction and **(C)** Water fraction. Concentration of extract is expressed as corresponding fresh weight of onion bulb tissues, from which fractions were extracted. Results were obtained from 3 independent experiments and bars indicate SE.

GSTc and GSTd. Since the activities of GSTc and GSTd were strongly inhibited by this fraction with a similar pattern, and other three GSTs were lessly sensitive to the inhibition of the extracts, inhibitory data are presented for GSTc (Fig. 4). IC_{50} was found from 142 mg/ml fresh tissue present in 45% methanol-elution. Since the inhibitory substance(s) eluted with 45% methanol-elution, it might be hydrophobic.

A considerable number of research findings indicated that plant natural products with different degrees of hydrophobicity exhibit significant interaction with GSTs which include phytoalexin medicarpin (Li et al., 1997), flavonoids (Marrs et al., 1995 ; Mueller et al., 2000), cinnamic acid, coumaric acid and some of their derivatives (Dean et al., 1995). In addition, some secondary sulfur compounds like glutathione derivatives and its structurally related substances are also reported as amphipathic inhibitors in Alliums (Neuefeind et al., 1997; Lucenta et al., 1998; Lancaster and Shaw, 1989).

Since onion is reported as rich source of flavonoids and sulfur compounds (Lachman et al., 2003; Lancaster and Shaw, 1989), there have been possibilities that onion bulb contain some of these phytochemicals which showed inhibitory effects on onion bulb GSTs. However, for more specific information about the inhibitory substance(s), 45% methanol-elution was further purified by TLC and HPLC.

Purification by TLC and HPLC

Fifteen µl (equivalent to 600 mg fresh tissue) of the 45% methanol-eluted Sep-pak Vac C_{18} fraction was applied on TLC plates. A yellow substance was visualized under UV absorption (R_f = 0.48) on the silica gel. Substance corresponding to 445 mg fresh tissue caused 50% inhibition on the activity of GSTc. The inhibitory substance(s) of the fraction was repeatedly applied on TCL, and thereafter it was applied on HPLC for further purification. The substance present in a high peak eluted at 26 min in fraction 5 showed strong inhibitory effect on CDNB conjugating activity of GSTc (Fig. 5). The HPLC was repeated many times to accumulate sufficient amount of the inhibitory substance for structural determination by NMR and MS spectrum.

Identification of inhibitor

The structural determination of the inhibitory substance was performed by ^{1}H and ^{13}C NMR spectra, including two dimensional correlation spectroscopic (COSY) and nuclear overhauser effect spectroscopic (NOESY) spectra. The data analyzed confirmed the structure of the inhibitory substance as quercetin-3,4´-diglucoside (a flavonoid) comparing the data of Fossen et al. (1998).

Inhibitory expression of quercetin-3,4´-diglucoside on onion bulb GSTs

The inhibitory expression of quercetin-3,4´-diglucoside on the activities of different onion bulb GSTs were investigated (Fig. 6). Quercetin-3,4´-diglucoside showed the strongest inhibitory effect on GSTd (IC_{50} with 69.3 μM) followed by GSTc (IC_{50} with 76.3 μM). The activity of GSTe was found to be inhibited to a lesser extent compared with these two GSTs. Quercetin-3,4´-diglucoside had very low or almost no inhibition on the minor GSTs, GSTa and GSTb.

Many research groups reported that onion in rich source of quercetin, both free and glycoside form (Lachman et al., 2003; Rhodes and Price, 1996). In his study, we found quercetin-3,4´-diglucoside as inhibitor of highly expressed onion bulb GSTs in onion bulb. According to the proposal of Walbot et al. (2000), plant GSTs act as nonenzymatic carrier proteins for plant natural compounds before transportation into the vacuole. Therefore, coexistence of high GST activity and quercetin-3,4´-diglucoside in onion bulb with inhibitory interaction suggested that the high levels of onion GSTs might function as carriers/ligands for quercetin-3,4´-diglucoside in the cytosol. However, though many hypothesizes have been proposed on flavonoids metabolism, it is still unclear to well understand.

Flavonoids are cytotoxic and xenotoxic compounds that can oxidize protein and intercalate into DNA (Ahmed et al., 1994). Therefore, these toxic flavonoids must be excluded from the cytoplasm to GSTs are thought to involve in preventing cellular synthesis pathway intermediates (Ahmed et al., 1994; limit both the mutagenic and oxidative effects of Rueff et al., 1995). damage by stabilizing flavonoids in cytosol or facilitating delivery of flavonoids to specific cellular compartments (Mueller et al., 2000). Therefore, Like other GSTs onion GSTs might have important role in quercetin-3,4´-diglucoside transportation. In addition to the role of transporters in vacuolar sequestration, GSTs have been known for their ability to detoxify various compounds, including toxic secondary metabolites and xenobiotic compounds.

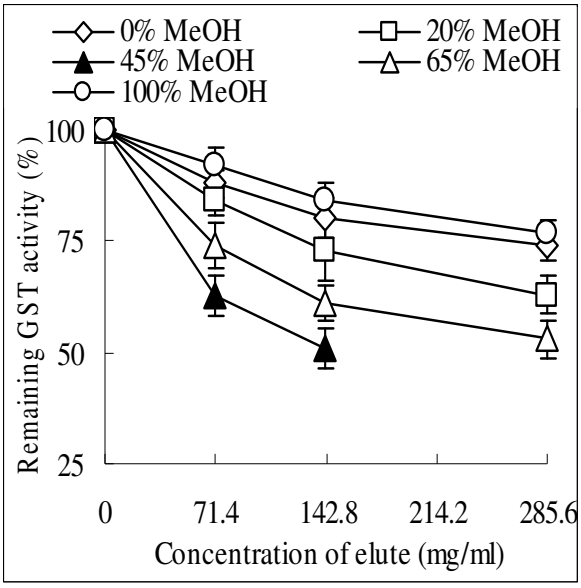

Fig 4. Inhibition of CDNB conjugating activity of GSTc by 0, 20, 45, 65 and 100% methanol fractions eluted from a Sep-pak Vac C_{18} column, to which water fraction containing tissue of mature onion bulb was applied. Concentration of eluate is expressed as corresponding fresh weight of onion bulb tissues, from which eluates were prepared. Results were obtained from 3 independent experiments and bars indicate SE.

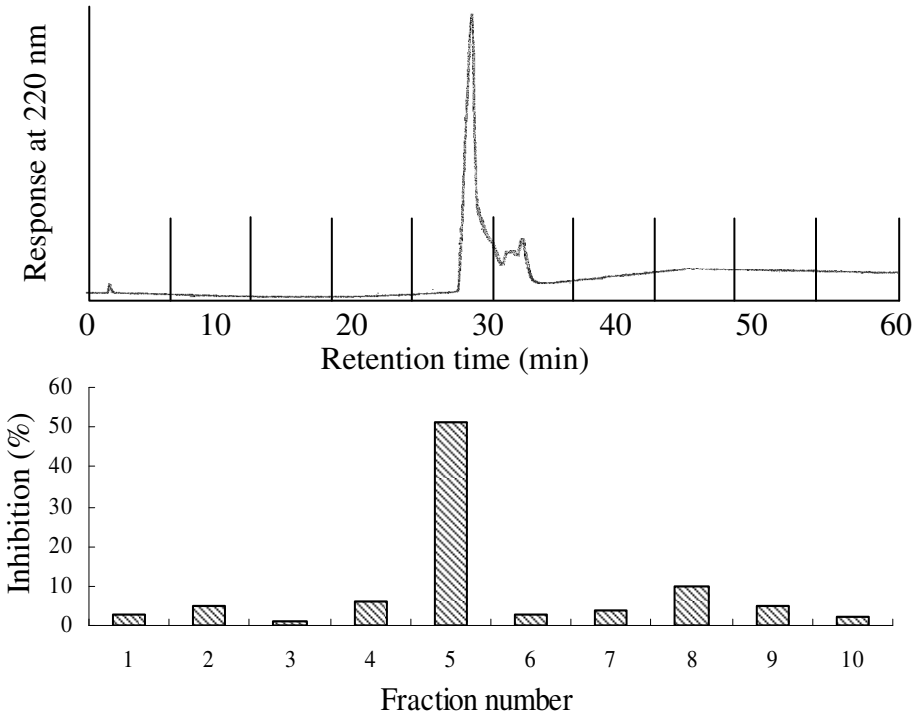

Fig 5. HPLC profile of inhibitory TLC fraction of the 45% methanol-eluted Sep-pak Vac C_{18} fraction of water fraction of onion bulb extract, and its inhibitory activity towards GSTc. A 50 microliter sample (equivalent to 600 mg fresh tissue) was injected into the column.

These enzymes conjugate the glutathione tripeptide (GSH) to a broad variety of substrates. As a result, the GSH-conjugated compound is transported to the vacuole via a tonoplast-located transporter (Lu et al., 1998). Three GSTs, namely, maize Bz2 (Marrs et al., 1995), petunia AN9 (Alfenito et al., 1998), and Arabidopsis TT19 (Kitamura et al., 2004), have been identified and characterized for their involvement in flavonoid (e.g. anthocyanin) accumulation in vacuoles.

However, Mueller et al., (2000) later showed that AN9 is a flavonoid-binding protein and is not involved in the covalent conjugation of GSH to flavonoids like anthocyanins. They also suggested that AN9 might serve as a cytoplasmic flavonoid carrier protein, and the formation of the GSH conjugate might not be required for transport into the vacuole. Therefore, the IC_{50}s of quercetin-3,4´-diglucoside on GSTc and GSTd (76.3 and 69.3 μM, respectively) and cell concentration (7.5mg quercetin-3,4´-diglucoside per 100 g fresh tissue equivalent

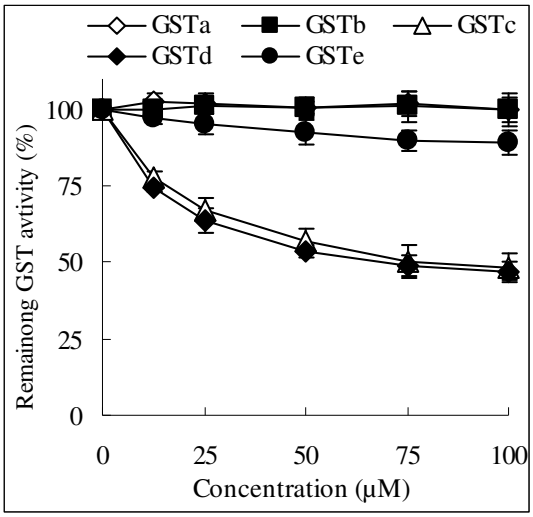

Fig 6. Inhibitory expression of quercetin-3,4´-diglucoside on CDNB conjugating activities of onion bulb GSTs. Results were obtained from 3 independent experiments and bars indicate SE.

to119 µM estimated in our experiment) suggested that as a carrier protein GSTs can deliver most of the cellular quercetin-3,4´-diglucoside into the vacuole like other flavonoids.

References

Ahmed MS, Ainley K, Parish JH, Hadi SM (1994) Free radical induced fragmentation of proteins by quercetin. Carcinogenesis 15: 1627-1630

Alfenito M, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione *S*-transferases. Plant cell 10: 1135-1149

Booth J, Boyland E, Sims P (1961) An enzyme from rat liver catalyzing conjugation. Biochem J 79: 516-524

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72: 248-254

Coleman JOD, Davies TGE, Blake-Kalff MMA (1997) Detoxification of xenobiotics in plant cells by glutathione conjugation and vacuolar compartmentalization: a fluorescent assay using monochlorobimane. Plant Cell Environ 20: 449-460

Cummins I, O`Hagen D, Jablonkai I, Cole DJ, Hehn A, Werck-reichhart D, Edwards R, (2003) Cloning, characterization and regulation of a family of phi class glutathione transferases from wheat. Pl Mol Biol 52: 591-603

Dean JV, Devarenne TP, Lee I-S, Orlofsky LE (1995) Properties of a maize glutathione *S*-transferase that conjugates coumaric acid and other phenyl propanoids. Plant Physiol. 108: 985-994

Debeaujon I, Peters AJ, Leon-Kloosterziel KM, Koornneef M (2001) The TRANSPARENT TESTA 12 gene of Arabidopsis encode a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13: 853-871

Droog F (1997) Plant glutathione *S*-transferases, a tale of theta and tau. J Plant Growth Regul 16: 95-107

Fossen T, Pedersen AT, Andersen ØM (1998) Flavonoids from red onion (*Allium cepa*). Phytochemistry 47: 281-285

Fujita M, Hossain MZ (2003) Molecular cloning of cDNAs for three tau-type glutathione *S*-transferases in pumpkin (*Cucurbita maxima*) and their expression properties. Physiol Plant 117: 85-92

Goodman CD, Casati P, Walbot V (2004) A multidrug resistance-associated protein involved in anthocyanin transport I Zea mays. Plant Cell 16: 1812-1826

Hayes JD, Pulford DJ (1995) The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol BIol 30: 445-600

Hossain MD, Suzuki T, Fujita MA (2007a) Preliminary Approach to identify the physiological substrates of pumpkin glutathione *S*-Transferase through inhibition studies. In: McConchie R Rogers G (eds) 3rd International Symposium on Cucurbits, vol 731. Acta Hort ISHS, Australia, p 217

Hossain MD, Rohman MM, Fujita M (2007b) Comparative investigation of glutathione *S*-transferases, glyoxalase-I and alliinase activities in different vegetable crops. J Crop Sci Biotech 10: 21-28

Kitamura S, Shikazono N, Tanaka A (2004) TRANSPARENT TESTA 19 is involved in the accumulation of both anthocyanins in Arabidopsis. Plant J 37: 104-114.

Lachman J, Proněk D, Hejtmánková A, Dudjak J, Pivec V, Faitová K (2003) Total polyphenol and main flavonoid antioxidants in different onion (*Allium cepa* L.) varieties. Hort Sci (Prauge) 30: 142-147

Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227: 680-685

Lancaster JE, Shaw ML (1989) Guma-glutamyl peptides in the biosynthesis of *S*-alkyl-L-cysteine sulphoxides (flavour precursors) in *Allium*. Phytochemistry 28: 455-460

Li Z-S, Alfenito M, Rea PA, Walbot V, Dixon RA (1997) Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. Phytochemistry 45: 689-693

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Lu Y-P, Li Z-S, Drozdowicz YM, Hörtensteiner S, Martinia E, Rea PA (1998) AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathione *S*-conjugates and chlorophyll catabolites: functional comparison with AtMRP1. Plant Cell 10: 267-282

Lucente G, Luisi G, Pinnen F (1998) Design and synthesis of glutathione analogues. II Farmaco 53:721-735.

Marrs KA (1996) The functions and regulation of glutathione *S*-transferases in plants. Ann Rev Plan Physiol Plant Mol Biol 47: 127-158

Marrs KA, Alfenito MR., Lloyd AM, Walbot VA (1995) Glutathione *S*-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature 375: 397-400

Masamoto Y, Ando H, Murata Y, Shimiishi Y, Tada M, Takahat K, Baba K (2003) Mushroom tyrosinase inhibitory activity of esculetin isolated from seeds of *Euforbia lathyris* L. Biosci Biotechnol Biochem 67: 631-634

Mueller LA, Godman CD, Silady RA, Walbot V (2000) AN9, a petunia glutathione *S*-transferases required for anthocyanin sequestration, is a flavonoid binding protein. Plant Physiol 123: 1561-1570

Neuefeind T, Huber R, Dasenbrock H, Prade L, Bieseler, B (1997) Crystal-structure of herbicide-detoxifying maize glutathione *S*-transferase-I in complex with lactoylglutathione: evidence for an induced-fit mechanism. J Mol Biol 274: 446-453.

Rhodes M, Price K (1996) Analytical problems in the study of flavonoid compounds in onions. Food Chemistry 57: 113-117

Rueff J, Gaspar J, Laires A (1995) Structural requirements for mutagenicity of flavonoids upon nitrosation: a structure-activity study. Mutagenesis 10: 325-328

Sandermann H (1992) Plant metabolism of xenobiotics. Ttrends Biol Sci 17: 82-84

Sirikantaramas S, Yamazaki M, Saito K (2007) Mechanisms of resistance to self-produced toxic secondary metabolites in plants. Phytochem Review 7:467-477

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G (2002) Anthocyanins in vegetable tissues: A proposed unified function in photo protection. New Phytol 155: 349-361

Tailor LP, Grotewold E (2005) Flavonoids as developmental regulators. Curr Opin Plant Biol 8: 317-323

Walbot V, Mueller LA, Silady RA, Goodman CD (2000) Do glutathione S-transferases act as enzymes or carrier proteins for their natural substrates? In: Brunold C et al. (eds) Sulfur nutrition and sulfur assimilation in higher plants. Paul Haupt, Bern, Switzerland, p 155