Inhibitory Substances of a Tau-Type Pumpkin Glutathione S-Transferase: Their Existence and Chemical Properties

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Abstract

Distributions of physiological inhibitors of a tau-type pumpkin glutathione S-transferase (CmGSTU3) have been investigated in different organs of pumpkin plants, as well as onion bulb and water hyacinth root. Inhibitory effects were observed in alcoholic extracts of all plant parts, but the extracts prepared from the roots of either water hyacinth or pumpkin plant showed the highest effect on CmGSTU3 toward 1-chloro-2,4- dinitrobenzene (CDNB). Results of various chromatographies indicated that a number of inhibitory substances were present in the alcoholic extract of each plant organ. Some macromolecules in the plant extracts exhibited inhibitory effects; however, the extracts might contain a large number of unknown, low-molecular-weight inhibitory substances. Some of the low-molecular-weight inhibitors in water hyacinth root extract showed characteristic fluorescence under UV light.

Key words: physiological inhibitors, alcoholic extracts, column chromatography, glutathione S-transferase activity.

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of enzymes that are present in almost all living organisms, including plants. GSTs have been extensively studied in plants and their several functions, including catalysis of glutathione (GSH) conjugation reactions with endo- and exogenous products, binding and transport of phytochemicals between cellular compartments, and catalysis of GSH-dependent biotransformation reactions have been proposed (Marrs 1996; Dixon et al. 2002).

Plant GSTs have been divided mainly into phi, tau, zeta, and theta groups, based on sequence homology. The two largest GST groups, phi and tau, are found exclusively in plants and catalyze GSH-dependent detoxification and peroxidation reactions or function as ligandins (Edwards et al. 2000). In our laboratory, three tau-type GST species (CmGSTU1, CmGSTU2, and CmGSTU3) have been isolated from pumpkin callus developed from sarcocarp tissues of mature fruit, and the cDNAs of the GSTs have been successfully cloned. Among the GSTs, CmGSTU3 has been reported to exhibit 48% identity and 66% similarity to tobacco Nt103, and 44% identity and 63% similarity to soybean Gmhsp 26-A (Fujita and Hossain 2003a). Tobacco

Nt103 is an auxin-induced GST-like protein that is expressed in tobacco root tips. Its enzymatic activity toward CDNB has been reported to be competitively inhibited by 2,4-D and its structurally-related compounds (van der Zaal et al. 1991). The soybean GST Gmhsp 26-A is a heat shock protein and has been reported to be induced by a wide variety of chemicals, including 2,4-D, abscicic acid, cadmium, and other heavy metals (Hagen et al. 1988).

In a bacterial expression system, CmGSTU3 exhibits the highest level of specific activity toward CDNB among all of the pumpkin GSTs studied (Hossain and Fujita 2006). Again, expression of the GST in pumpkin organs has been reported to be induced by different environmental and chemical stresses (Fujita and Hossain 2003b; Hossain et al. 2006b). Recently, we have also found that the CDNB-conjugating activity of the GST is inhibited by a few commercial glutathione derivatives and also by some unknown physiological substances present in pumpkin seedlings, leaf, and callus (Hossain and Fujita 2006; Hossain et al. 2006a, 2007b). Therefore, we selected CmGSTU3 to investigate the existence of its physiological inhibitors in different plant parts and to reveal their probable characteristics. In this study, we used different organs of the pumpkin plant (Cucurbita maxima), including the callus cultured from sarcocarp tissues of mature fruit. We also used mature onion (Alliums cepa)

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bulb, mainly because it was found to show the highest level of GST activity among different vegetable crops (Hossain et al. 2007a). In addition, water hyacinth (*Eichhornia crassipes*) roots were used in this study because it is very easy to grow healthy roots of the plant.

Materials and Methods

Preparation of plant extract

Different organs of one-month-old pumpkin plants (root, hypocotyl, leaf blade, leaf petiole, and cotyledon), six-day-old pumpkin seedling (excluding cotyledons), pumpkin callus, onion bulb, and water hyacinth roots were used for extraction. Each of the plant materials was extracted twice in methanol:chloroform:water (12:5:3, v/v/v) and once in 70% ethanol by a method used in our previous study (Hossain et al. 2006a).

Enzyme preparation and activity assay

The enzyme was extracted from *E. coli* cells, transformed with pBluescript [SK(-)] containing *CmGSTU3* cDNA in frame by a standard method (Hossain et al. 2007b). GST activity was determined spectrophotometrically by the method of Booth et al. (1961) with some modifications as described by Fujita and Hossain (2003b). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, 1 mM CDNB, 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 1min.

Column chromatography

To fractionate the alcoholic extracts from different plant parts, two types of gel filtration columns prepared with Sephadex G-25 (P10 column, Amersham Biosciences) and Sephadex G-15 (0.69 × 74 cm) were used. In the case of Sephadex G-25 column chromatography, 1.0 ml of the extract was applied onto the column and fractions of 0.855 ml were collected. In the case of Sephadex G-15 column chromatography, 2.0 ml of the extract was applied and fractions of 2.0 ml were collected. In all cases, the columns were equilibrated and eluted with distilled water. The inhibitory effect of the substances in each fraction was assayed toward *Cm*GSTU3.

Thin layer chromatography

The fractions with high levels of inhibitory activity obtained from Sephadex G-15 column chromatography of water hyacinth root extract were subjected to thin layer chromatography (TLC). The volume of the active fractions was reduced to 0.5 ml by evaporation, and 80 μl of the concentrated sample (corresponding to 0.8 g fresh tissue components) was applied to TLC plates (Si 70, F_{254}, Wako 2.5×20 cm) and developed with butanol-acetic acid-water (80:10:10, v/v/v). The fluorescent spots were marked under UV light at a wavelength of 254 nm. The solvent moving zone of the plate was fractionated into 18 equal divisions (2.5 ×

1 cm each) including two controls (lower and upper 2.5×1 cm areas). The silica gel in each fraction was scratched out and the substances were washed with 5.0 ml of 50% methanol. The solvent of each fraction was removed by evaporation, and dried substances were dissolved in 0.5 ml distilled water. From each of the concentrated fractions, 30 μl was used to assay the inhibitory activities toward *Cm*GSTU3.

Results and Discussion

Inhibition of *Cm*GSTU3 activity by plant extracts

In our previous studies, we found that alcoholic extracts of pumpkin seedling, leaf, and callus have different inhibitory effects on pumpkin GSTs (Hossain and Fujita 2006b; Hossain et al. 2006a) indicating a wide distribution of physiological inhibitors of the GSTs. We therefore prepared alcoholic extracts from different organs of the pumpkin plant along with onion bulb and water hyacinth root and examined their effects on *Cm*GSTU3 activity. As seen in Figure 1, the highest level of inhibition of the GST activity was revealed by the extracts of water hyacinth root (I_{50}= extract from 13.33 mg fresh tissue) followed by onion bulb (I_{50}= 28.33 mg fresh tissue) and pumpkin root (I_{50}= 54.17 mg fresh tissue). Other organs of the pumpkin plant showed small to moderate inhibitory effects.
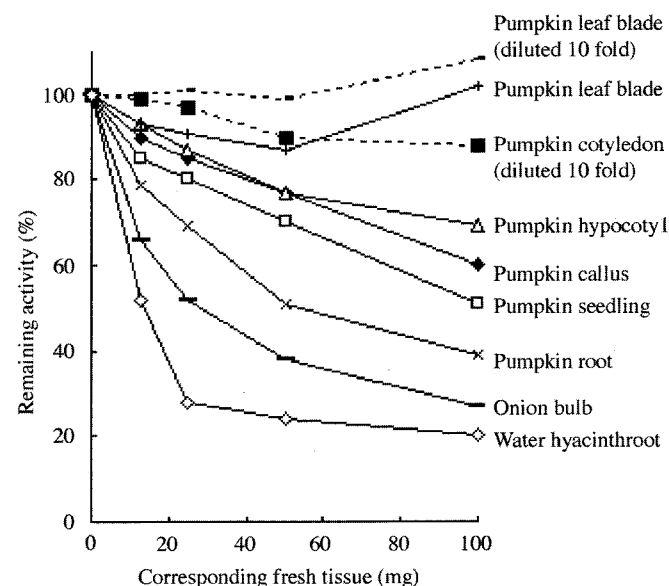

Fig. 1. Inhibition of *Cm*GSTU3 activity by alcoholic extracts from different organs of pumpkin plant, onion bulb and water hyacinth root. In the case of leaf blade and cotyledon, 10-fold diluted extracts were used for the study. Results were obtained from two independent experiments.

*Cm*GSTU3 has a relatively high similarity to auxin-induced tobacco GST Nt103, which is expressed in tobacco root tips. The expression of *Cm*GSTU3 in different organs of the pumpkin plant was reported to be under the detectable level (Fujita and Hossain 2003a). However, the GST could be expressed in the root of five-day-old pumpkin seedling, which was induced by different stresses (Hossain and Fujita 2002). But the microscopic

expression of the GST is still unknown. However, like tobacco Nt103, CmGSTU3 might be expressed in root tips of higher plants. In the present study, it was found that alcoholic extracts prepared from the roots of pumpkin or water hyacinth showed the strongest inhibitory effects on CmGSTU3 activity, indicating the existence of potent inhibitors in root tissues. These results, therefore, hypothesized that few physiological components in a particular plant cell have significant interaction with the existing GSTs.

Inhibitory substances in pumpkin seedling

To investigate the characteristics, particularly the molecular sizes of the inhibitory substances in the alcoholic extract of pumpkin seedling (PS), we conducted gel filtration column chromatography. The alcoholic extract was first fractionated by Sephadex G-25 column chromatography, and the inhibitory effects of different fractions (f) on CmGSTU3 activity were examined. The inhibitory substances were found to be eluted broadly (Fig. 2). Although the highest inhibitory potencies were observed in f_8 and f_9, the substances in f_{10-12} demonstrated the highest absorbance at 220 nm, indicating the elution of major substances of the extracts. The elution profile of different molecular weight markers (horse Cyt c, 12700; aprotinin, 6500; β-NADP$^+$, 743.41 and riboflavin, 376) indicated the approximate sizes of the inhibitors. The molecular weights of the potent inhibitors seemed to be very similar to that of β-NADP$^+$, suggesting the presence of some low-molecular-weight components in the extract.
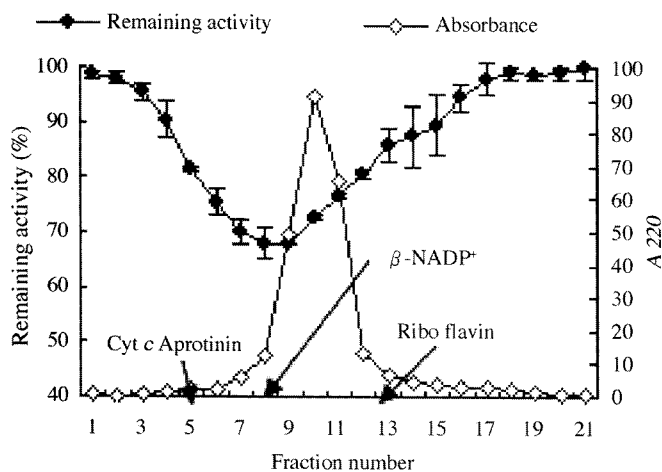

Fig. 2. Sephadex G-25 (P10) column chromatography with the alcoholic extract of pumpkin seedling. One-milliliter sample (corresponding to 2.5g fresh tissue) was applied to the column and eluted with water. Each fraction contains 0.855ml of eluates, and 100µl from each was used in the inhibition study. Results were obtained from two independent experiments.

To check whether the inhibitory substances represented NAD$^+$ or structurally-related compounds, we examined the spectra of the highly active fractions. The absorption spectra of f_7, f_8, and f_9 showed a shoulder at around 260 nm, which might indicate the existence of NAD$^+$ (Fig. 3A). However, we did not find any inhibitory effects of commercial NAD$^+$, NADP$^+$, or NADPH on CmGSTU3 activity (data not shown). For more confirmation, we separated the total nucleotides from PS extract through EtOH

precipitation which gave an absorption spectrum (Fig. 3B) similar to that of NAD$^+$, but did not show any inhibitory effects on CmGSTU3 activity (data not shown).
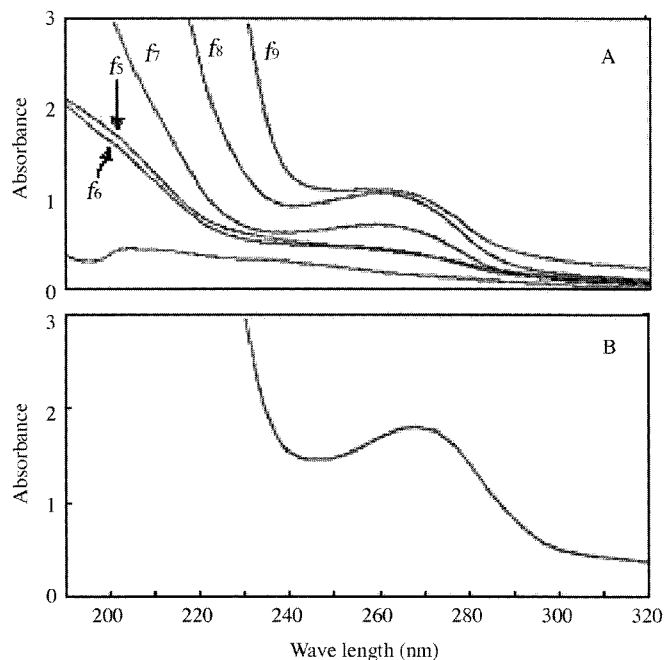

Fig. 3. Absorption spectra of the active fractions from Sephadex G-25 column chromatography of the PS extract (A) and total nucleotides separated from the alcoholic extract (B).

Although NAD(P)H has been reported to function as a cofactor in many enzymatic reactions (Colrat et al. 1999; Ryan et al. 2001), there are few reports on any of the dinucleotides used as a cofactor or substrate by plant GSTs. However, a number of low-molecular-mass products of DNA degradation and α,β-unsaturated aldehyde derivatives of nucleic acids and fatty acids have been shown to be endogenous substrates of human GSTs (Berhane et al. 1994, Eaton and Bammler 1999).

We also conducted a similar gel filtration column chromatography with PS extract using various solvent systems (mercaptoethanol-3mM, glycerol-10%, glucose-0.5M, CuSO$_4$/ MgSO$_4$/ EDTA-1mM, and KCl-100mM). For each of the solvents, the elution profile of PS extract was found to be very similar to that eluted with water (data not shown), indicating that the inhibitory substances in PS extract might have no or negligible interaction with the studied chemicals. On the other hand, such a small column (P10) might not be suitable for adequate separation of the substances in PS extract.

Since the inhibitory substances might not represent NAD$^+$ or structurally-related dinucleotides, in our next experiments we eliminated the total nucleotides from PS extract through EtOH precipitation. The inhibitory substances in the nucleotide eliminated extract of PS were found to be separated into two main groups, A (f_{25-32}) and B (f_{47-53}), by Sephadex G-15 column chromatography (Fig. 4). In NMR and mass spectroscopy analyses, the substances in groups A and B exhibited the signal of sugar molecules. However, the estimated concentration of total reducing sugar (in terms of glucose) was found to be higher in f_{37-45}, though

no apparent relation was found between the amount of total sugar and the pattern of inhibition among different fractions. This result suggests that the inhibitory substances might not address any simple sugar molecules.
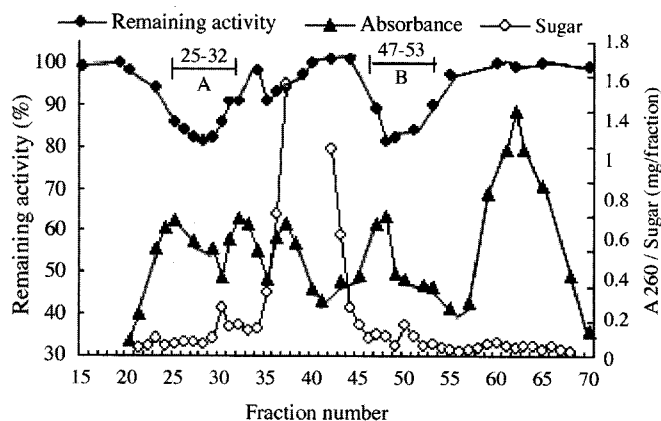

Fig. 4. Sephadex G-15 column chromatography of pumpkin seedling extract where the total nucleotides were eliminated. Total nucleotides from 4.0ml of extract (corresponding to 10g fresh tissue) were removed by precipitation with 10% (v/v) EtOH. The supernatant was evaporated, and dried substances were dissolved in 2.5 ml distilled water, 2.0ml (representing 8.0g fresh tissues) of which was applied onto the column. Fractionation was done by elution with water, and fractions of 2.0ml were collected. Eluates of 200µl from each fraction were used to assay their inhibitory potencies toward CmGSTU3.

To determine whether the inhibitors address any polysaccharides, we examined the effects of some polysaccharides on CmGSTU3 and found that polygalacturonic acid and acidic oligosaccharide showed inhibitory effects on the GST activity (Fig. 5). We applied acidic oligosaccharide to a Sephadex G-15 column which eluted in f_{25}. In another experiment, we fractionated the alcoholic extract from onion bulb with the same column chromatography and measured the concentrations of the substances in different fractions. The concentrations of total substances in the active fractions were found to be below 6 mg/ml, much lower than that of acidic oligosaccharide or polygalacturonic acid which showed a similar level of inhibition of CmGSTU3 activity. Therefore, the extract of onion bulb did not represent any poly-
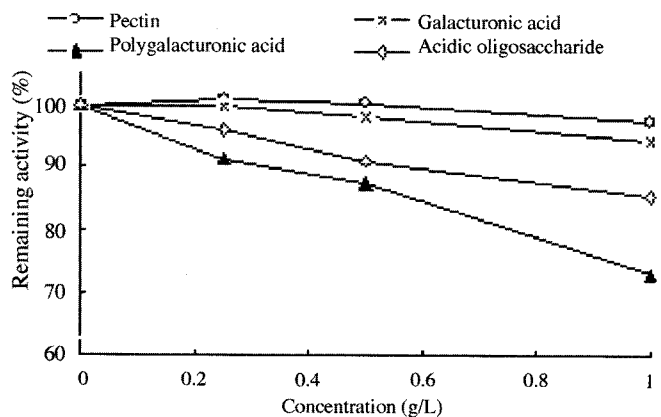

Fig. 5. Inhibition of CmGSTU3 activity by polysaccharides. Results were obtained from two independent experiments. Concentration was expressed as the final concentration in the enzyme reaction mixture.

saccharides as inhibitors of the GST. Plant polysaccharides have been shown to exert chemo-preventive effects such as inhibition of carcinogen activation systems and induction of detoxification enzymes (Devidson et al. 1990). However, to our knowledge, inhibition of GST activity by plant polysaccharides has not been reported. Although few polysaccharides showed inhibitory effects on the activity of CmGSTU3 in this experiment, the extracts of onion bulb or pumpkin seedling did not represent any polysaccharides or related macromolecules.

Inhibitory substances in pumpkin root

We removed the total nucleotides from pumpkin root extract by EtOH precipitation and fractionated by G-15 column chromatography. Figure 6 clearly indicates that the extract contains a number of inhibitory substances that were fractionated into at least five groups; however, the higher levels of GST inhibitory activity were observed in f_{28-33} and f_{40-45}. On the other hand, the levels of inhibitory activity were found to be much higher than those in PS extracts.
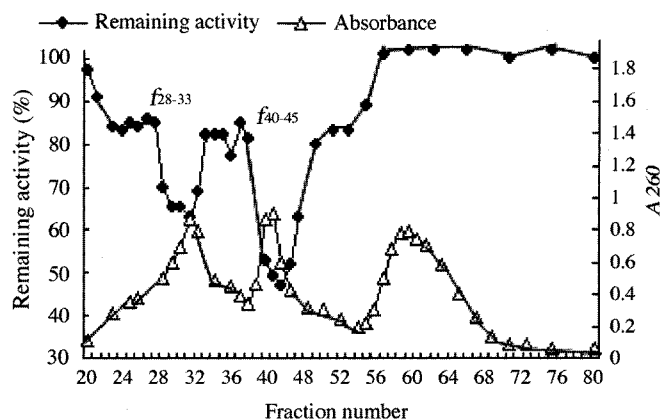

Fig. 6. Sephadex G-15 column chromatography of pumpkin root extract in which the total nucleotides were eliminated. Total nucleotides from 4.0ml of extract were removed by precipitation with 10% (v/v) EtOH. The supernatant was evaporated and dried substances were dissolved in 2.5ml distilled water, 2.0ml (representing 8.0g fresh tissue) of which was applied onto the column. Fractionation was done by elution with water and fractions of 2.0 ml were collected. Eluates of 200µl from each fraction were used to assay their inhibitory potencies toward CmGSTU3.

Inhibitory substances in water hyacinth root

To investigate the inhibitory substances in water hyacinth root extract, we conducted Sephadex G-15 column chromatography followed by thin layer chromatography. In Sephadex G-15 column chromatography, the inhibitory activities were found in many fractions and the substances were separated mainly into two major groups, f_{34-40} and f_{45-54} (Fig. 7). The inhibitory potencies of the active fractions were several-fold higher than those of PS extracts.

The inhibitors in f_{45-54} have great significance as they are low-molecular-weight compounds and showed high levels of inhibitory activity at lower concentrations. Therefore, we combined and evaporated the eluates of the fractions (f_{45-54}), and the concentrated solution was subjected to TLC on silica gel and developed with butanol:acetic acid:water (80:10:10 v/v/v). The
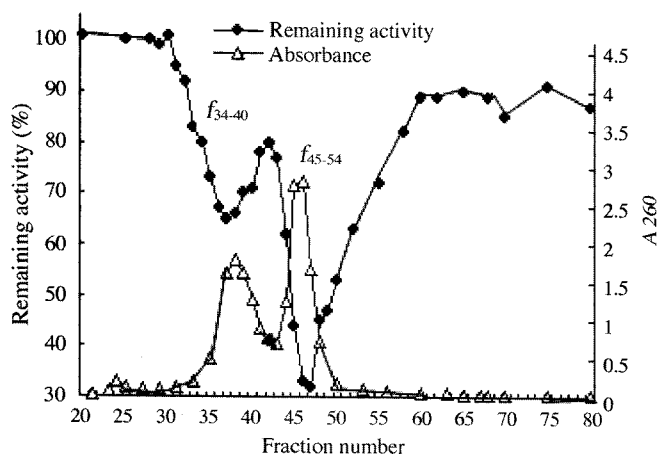
Fig. 7. Sephadex G-15 column chromatography of water hyacinth root extract. Two ml of extract (representing 5.0g fresh tissue) was applied to the column. Fractionation was done by elution with water and fractions of 2.0 ml were collected. Eluates of 200μl from each fraction were used to assay their inhibitory potencies toward CmGSTU3.

active substances in water hyacinth root extract were found to be separated further, indicating some characteristic spots/bands on the TLC plate (Fig. 8).
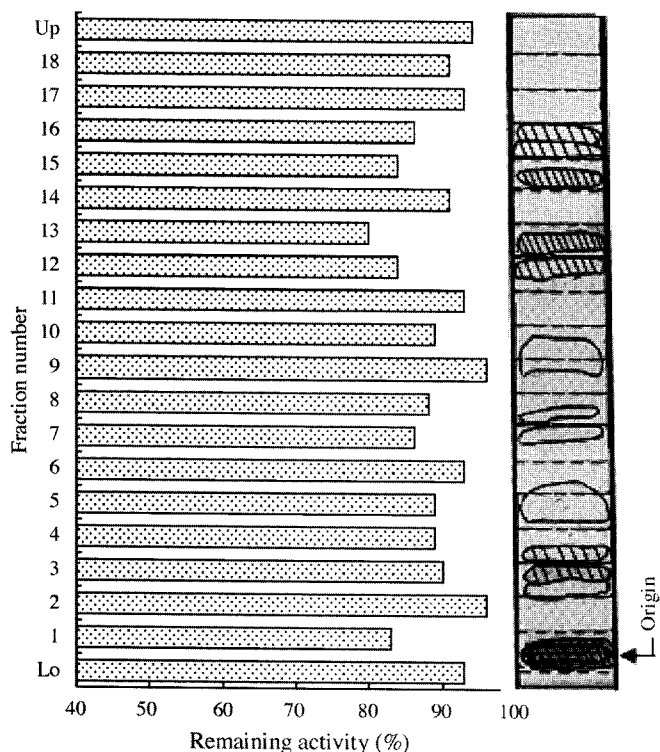

Fig. 8. Sephadex G-15 column chromatography of water hyacinth root extract. Two ml of extract (representing 5.0g fresh tissue) was applied to the column. Fractionation was done by elution with water and fractions of 2.0 ml were collected. Eluates of 200μl from each fraction were used to assay their inhibitory potencies toward CmGSTU3.

The substances in f_{12}, f_{13}, f_{15}, and f_{16} exhibited fluorescence under UV light at 254 nm and showed significant inhibitory effects on CmGSTU3 activity. The concentrations of the fluorescent substances in f_2 and f_3 seemed to be very low, but they showed

some inhibitory effects. On the other hand, f_1 might contain a number of non-polar quiescent as well as fluorescent substances, resulting in the highest inhibitory effects on CmGSTU3 activity. The results of this experiment indicated the existence of many active substances, including potent fluorescent inhibitors in water hyacinth root.

Conclusion

The results of the present investigation indicated that physiological inhibitors of a tau-type pumpkin GST are distributed in almost all plant parts but that the root contains potent inhibitors. Alcoholic extracts of six-day-old pumpkin seedlings and roots of one-month-old pumpkin plants and water hyacinth contain several types of inhibitors. The potent inhibitors are mainly low-molecular-weight compounds and might not represent any nucleotides, simple sugars or polysaccharides. In addition, most of the potent inhibitors in water hyacinth root have characteristic fluorescence under UV light.

References

Berhane K, Widersten M, Engström A, Kozarich JH, Mannervik B. 1994. Detoxification of base propenals and other $\alpha\beta$-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. Proc. Natl. Acad. Sci. USA 91: 1480-1484

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

Colrat S, Latché A, Guis M, Pech J-C, Bouzayen M, Fallot J, Roustan J-P. 1999. Purification and characterization of a NADPH-dependent aldehyde reductase from mung bean that detoxifies eutypine, a toxin from *Eutypa lata*. Plant Physiol. 119: 621-626

Davidson NF, Egner PA, Kensler TW. 1990. Transcriptional control of glutathione S-transferase gene expression by the chemopreventive agent 5-(2-pyrazinyl)-4- methyl-1,2-dithiole-3-thione (oltipraz) in rat liver. Cancer Res. 50: 2251-2255

Dixon DP, Lapthorn A, Edwards R. 2002. Protein family review, plant glutathione transferases. Genome Biol. 3(3): reviews 3004.1-3004.10

Eaton DL, Bammler TK. 1999. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. 49: 156-164

Edwards R, Dixon DP, Walbot V. 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. 5: 193-198

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

Fujita M, Hossain MZ. 2003b. Modulation of pumpkin glutathione S-transferases by aldehydes and related compounds. Plant Cell Physiol. 44: 481-490

Hagen G, Uhrhammer N, Guilfoyle TJ. 1988. Regulation of expression of an auxin-induced soybean sequence by cadmium. J. Biol. Chem. 263: 6442-6446

Hossain MD, Fujita M. 2006. Expression properties of three tau-type pumpkin glutathione *S*-transferases in bacteria and a search for their intrinsic inhibitors. in: Proceedings of Cucurbitaceae 2006. G.J. Holmes, ed. Universal Press, Raleigh, NC, U.S.A. pp 14-22

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

Hossain MD, Suzuki T, Fujita M. 2006a. Inhibitory substances to glutathione *S*-transferases in pumpkin seedlings. Asian J. Plant Sci. 5: 346-352

Hossain MD, Suzuki T, Fujita M. 2007b. A preliminary approach to identify the physiological substrates of pumpkin glutathione *S*-transferase through inhibition studies. Acta Horticulture 731: 217-222

Hossain MZ, Fujita M. 2002. Classification of pumpkin GSTs and introduction of a GST gene into rice. in: Proceedings of Cucurbitaceae 2002. D.N. Maynard, ed. ASHS Press, Alexandria, VA, U.S.A. pp 169-174

Hossain MZ, Hossain MD, Fujita M. 2006b. Induction of pumpkin glutathione *S*-transferases by different stresses and its possible mechanisms. Biologia Plant. 50 : 210-218

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

Ryan AD, Kwak M-K, Thomas RS, Thomas W. 2001. Antioxidative function and substrate specificity of NAD(P)H dependent alkenal/one oxidoreductase: A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. J. biol. Chem. 276: 40803-40810

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Chilperoort RA, Libbenga KR. 1991. Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol. Biol. 16: 983-998