

# Sweet potato trypsin inhibitor with thioltransferase-like and glutathione S-transferase-like activities

Shyh-Shyun HUANG<sup>1,2</sup>, Hsin-Jung HUNG<sup>1</sup>, Chuan-Sung CHIU<sup>1,3</sup>, Hsien-Jung CHEN<sup>4</sup>,  
Shiang-Shiou LIN<sup>1</sup>, Ying-Chih LIN<sup>5</sup>, Heng-Yuan CHANG<sup>1</sup>, Guan-Jhong HUANG<sup>1,\*</sup>, and  
Yaw-Huei LIN<sup>6,\*</sup>

<sup>1</sup>*Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung 404, Taiwan*

<sup>2</sup>*Department of Nursing, Hung Kuang University, Shalu, Taichung 433, Taiwan*

<sup>3</sup>*Nursing Department, Hsin Sheng College of Medical Care and Management, Taoyuan 325, Taiwan*

<sup>4</sup>*Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung 804, Taiwan*

<sup>5</sup>*Department of Optometry, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 356, Taiwan*

<sup>6</sup>*Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115, Taiwan*

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**ABSTRACT.** Our previous reports showed that sweet potato trypsin inhibitor (SPTI) exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities. SPTI also exhibited antioxidant activities against different radicals, inhibited growth of NB<sub>4</sub> cells, and induced apoptosis of NB<sub>4</sub> cells. In this work, we found novel enzyme activities of SPTI. The purified SPTI had thioltransferase (TTase)-like activity with a specific activity of  $1.6 \pm 0.3$  and  $0.58 \pm 0.02$  nmol/min/mg at pH 7.5 and 8.5, respectively. Soybean TI (STI) was used as a positive control. The purified SPTI also had glutathione S-transferase (GST)-like activity with a specific activity of  $0.094 \pm 0.005$  and  $0.43 \pm 0.03$   $\mu$ mol/min/mg at pH 6.0 and 7.0, respectively. The amino acids around disulfide bond of SPTI (Cys153~Cys160) were compared with those of published TTase and GST. The results provide a chemically and physiologically reasonable molecular basis on which SPTI exhibits both TTase-like and GSTs-like activities.

**Keywords:** Glutathione S-transferase-like activity; Sweet potato; Thioltransferase-like activity; Trypsin inhibitor.

## INTRODUCTION

Reactive oxygen species (ROS) play both good and bad roles. Whether it is good or bad depends on the cellular concentrations, the redox status of tissues and organs. For the bad side, ROS are generated in all aerobic organisms and cause serious damage to biological macromolecules, which eventually leads to pathological problems (Halliwell and Gutteridge, 1990). To counter the damage caused by ROS, cells are equipped with various antioxidant enzymes and maintain a reduced intracellular state (Zheng et al., 1998) with the help of redox-controlling proteins, such as protein disulfide isomerase (PDI), thioredoxin (Trx) (Vlami-Gardikas and Holmgren, 2002), and thioltransferase (TTase; or glutaredoxin, Grx) (Sasada, et al., 2002), together with their respective electron transferring systems. TTase contains two conserved cysteine residues with

sequences of (CP[Y/F]C) at its active site. With glutathione (GSH) as an electron donor, TTase has been shown to reduce dehydroascorbate (DHA) to ascorbate (Wells et al., 1990). Despite their ubiquitous distribution in all organisms, and unlike other redox proteins, the physiological functions of TTase proteins are not well characterized (Gan and Wells, 1986).

Glutathione S-transferases (GSTs, EC 2.5.1.18) constitute a family of multifunctional enzymes that mainly catalyze the nucleophilic attack of reduced glutathione (GSH) to a wide variety of electrophilic endogenous and exogenous compounds. GSTs are found in all living organisms tested to date, as unique enzymes in lower organisms and as a large number of tissue-specific isoforms in more complex species like mammals (Vuilleumier, 1997). The expression level of GSTs is regulated by treatment of many compounds including carcinogens, drugs and oxidative stress metabolites. Several additional functions were attributed to GSTs including the transport of hydrophobic ligands, binding to bilirubin and carcinogens (Litwack et al., 1971), the isomerization of maleylacetoacetate, and the regulation of stress kinases and apoptosis (Adler et al., 1999).

\*Corresponding authors: E-mail: boyhlin@gate.sinica.edu.tw, Fax: +886-2-2782-7954, Tel: +886-2-2787-1172 (Yaw-Huei LIN); E-mail: gjhuang@mail.cmu.edu.tw, Tel: +886-4-2205-3366 ext. 5508; Fax: +886-4-2208-3362 (Guan-Jhong Huang).

Protease inhibitors in plants may be important in regulating and controlling endogenous proteases and in acting as protective agents against insect and/or microbial proteases (Ryan, 1989). Sohonie and Bhandarker (1954) reported for the first time the presence of trypsin inhibitor (TI) in sweet potato. Later, it was indicated that TI activities in SP are positively correlated with concentrations of water-soluble protein (Sohonie and Bhandarker, 1954), and that SPTI activities increase in response to drought (Lin and Chen, 1980). Polyamines, including cadaverine, spermidine and spermine, bound covalently to SPTI, which might participate in regulating the growth and developmental processes of SP (Hou and Lin, 1997a). TI in SP storage roots account for about 60% of total water-soluble proteins and could be recognized as storage proteins. Maeshima et al. (1990) identified sporamin as the major storage protein in SP root, accounting for 80% of the total proteins there; a dramatic decrease of the amount of sporamin to 2% of the original value was found during sprouting. Lin (1993) considered sporamin and TI are various biochemical forms of the same polypeptide chain, a finding confirmed later by Yeh et al. (1997).

Our previous reports showed that SPTI exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou and Lin, 1997b). 33 kDa SPTI exhibited antioxidant activities against different radicals (Hou et al., 2001; Huang et al., 2007a). SPTI also exhibited growth inhibition activity and induction of apoptosis in NB4 cells (Huang et al., 2007b). In this work we report for the first time that SPTI also showed significant GSH dependent TTase-like and GSTs-like activities, which provide a chance to compare the amino acid sequences around conserved cysteine residues of the four proteins (TTase, GSTs, sporamin A and B) and give additional sequence-based mechanisms to explain why a single SPTI polypeptide chain can have so many biological activities.

## MATERIALS AND METHODS

### Chemicals

Tris and electrophoretic reagents were purchased from E. Merck Inc. (Darmstadt, Germany); Seebblue prestained markers for SDS-PAGE including myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamate dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa) were from Invitrogen (Groningen, The Netherlands); Coomassie brilliant blue G-250, Soybean trypsin inhibitor, other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### Plant materials

Fresh storage roots of sweet potato (*Ipomoea batatas* Lam. 'Tainong 57') were purchased from a local market. Samples were washed and peeled, and then cut into strips that were extracted immediately.

### Purification of sweet potato trypsin inhibitor

Extraction and purification of SPTI from sweet potato storage roots were carried out at 4°C according to the method of Huang et al. (2008a). The storage roots were cut into strips that were extracted immediately with four volumes (w/v) of 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl, 1% (w/v) ascorbate and 1% (w/v) polyvinylpyrrolidone (PVPP) in a homogenizer for 30s (four times). The homogenates were filtered through four layers of cheesecloth and centrifuged twice at 12,000 ×g for 30 min. The crude extracts were loaded directly onto a trypsin-Sepharose 4B affinity column (1.0 × 10 cm), and the adsorbed SPTI was eluted by changing pH value with 0.2 M KCl buffer (pH 2.0). The extracts were desalted and concentrated with Centricon 10 and then lyophilized for further use.

### Protein staining and activity staining of SPTI on 15% denaturing polyacrylamide gels

SPTI was detected with both protein and activity stainings on 15% SDS-PAGE gels. Samples were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, 0.1% bromophenol blue, with 5% 2-mercaptoethanol (for protein staining) or without 2-mercaptoethanol (for activity staining). Coomassie brilliant blue G-250 was used for protein staining. For SPTI activity staining, the gel was stained according to the method of Huang et al. (2004). When SDS-PAGE was finished, gel was immersed and shaken twice in 25% (v/v) isopropanol in 10 mM Tris buffer (pH 7.9) for 10 min each. The gel was then dipped into 10 mM hydrogen peroxide in the same buffer for at least 30 min with gentle shaking, and finally washed in 10 mM Tris buffer (pH 7.9) for 10 min. Then the gel was incubated in trypsin solution (50 µg/mL bovine trypsin, 10 mM Tris buffer pH 7.9) at 37°C for 20 min. After rinsing with the same buffer to remove excess trypsin, the gel was incubated in the dark at 37°C for at least 30 min with 160 mL of substrate-dye solution immediately prepared before use. The substrate-dye solution consisted of 40 mg *N*-acetyl-DL-phenylalanine β-naphthyl ester (APNE) in 16 mL of *N,N*-dimethylformamide that was brought to 160 mL with 144 mL of 10 mM Tris buffer (pH 7.9), in which 80 mg tetrazotized *o*-dianisidine were dissolved. After activity staining, the gel was destained with 10% acetic acid for 30 min.

### Thioltransferase activity assay

The TTase activity was measured spectrophotometrically using a modification of the standard assay (Morell et al., 2005). The reaction mixture contained 50 mM Tris-HCl (pH 7.5 or 8.5), 0.2 mM NADPH, 0.6 mM GSH, 0.5 units glutathione reductase and an aliquot of the SPTI sample solution. The reaction was started by adding 450 µL reaction mixture to a cuvette containing 50 µL of 15 mM β-hydroxyethyl disulfide (HED) dissolved in 20 mM Tris-HCl. Absorbance changes at 340 nm resulting from the

oxidation of NADPH were recorded. The TTase assay of HED reduction with GSH catalyzed by the SPTI sample solution was coupled to glutathione reductase and the consumption of NADPH was monitored at 340 nm. STI was used as a positive control. The disappearance of NADPH indicated by decrease at 340 nm was a measure of enzyme activity and extinction coefficient  $E^{mM}$  (340 nm) = 6.22 were used for calculations.

### Glutathione S-transferase assay

GSH conjugating activity was determined spectrophotometrically as described by Singh and Shaw (1988) with a slight modification. Enzyme activity was assayed by combining 50  $\mu$ l of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB), 50  $\mu$ l of 20 mM reduced glutathione (GSH), 850  $\mu$ l of 50 mM sodium phosphate buffer (pH 6.0 or 7.0), and 50  $\mu$ l of an aliquot of the SPTI sample solution, then monitoring the change of absorbance at 340 nm ( $\Delta$ 340 nm), which is the UV absorption maximum of conjugating product between GSH and CDBN, at 25°C for 10 min. All initial rates were corrected for the background nonenzymatic reaction. STI was used as a positive control.

### Statistical Analysis

Means of triplicates were measured. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when  $p < 0.05$ .

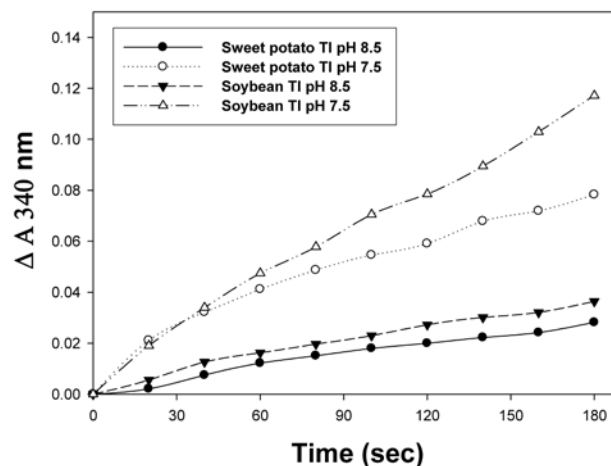
## RESULTS

### Effect of pH (7.5 and 8.5) on thioltransferase-like activity of SPTI

The assay using GSH and HED as substrates has been used to identify TTase in plants (Morell et al., 1995). SPTI catalyzed the reduction of HED by glutathione; and the oxidized glutathione was then reduced back to its original form by the glutathione reductase using NADPH as a reducing equivalent in the coupled reaction (Figure 1). The specific TTase activity of SPTI was  $1.6 \pm 0.3$  and  $0.58 \pm 0.02$  nmol/min/mg protein at pH 7.5 and 8.5, respectively. STI was used as a positive control with specific activity of  $2.4 \pm 0.5$  and  $0.75 \pm 0.03$  nmol/min/mg protein at pH 7.5 and 8.5, respectively. Morell et al. reported a specific TTase activity of STI as 1.3 nmol/min/mg protein at pH 8.7 (Morell et al., 1995). Since they used crude extract of soybean, the real value might be higher than what they reported. Very low TTase-like activity of SPTI was found at pH 8.5. SPTI exhibits a GSH-dependent TTase-like activity (Figure 2A) at both pH 7.5 and 8.5. With coupled reaction, the rate of NADPH consumption is essentially proportional to SPTI concentration (Figure 2B). Thus, SPTI exhibits a GSH-dependent TTase-like activity at pH 7.5 and 8.5.

### Effect of pH (6.0 and 7.0) on glutathione S-transferase-like activity

The GST activity of SPTI was determined with both



**Figure 1.** Effect of pH (7.5 and 8.5) on thioltransferase-like activity of SPTI. The reaction mixture contained 50 mM Tris-HCl (pH 7.5 or 8.5), 2 mM EDTA, 300  $\mu$ M NADPH, 200  $\mu$ M GSH, 0.5 units glutathione reductase, and 100  $\mu$ g SPTI. The reaction was started by adding 450  $\mu$ l reaction mixture to a cuvette containing 50  $\mu$ l of 15 mM HED. Decreases of absorbance at 340 nm due to the oxidation of NADPH were recorded for 3 min. STI was used as a positive control. Each data show the mean  $\pm$  SD of one experiment performed in triplicate.

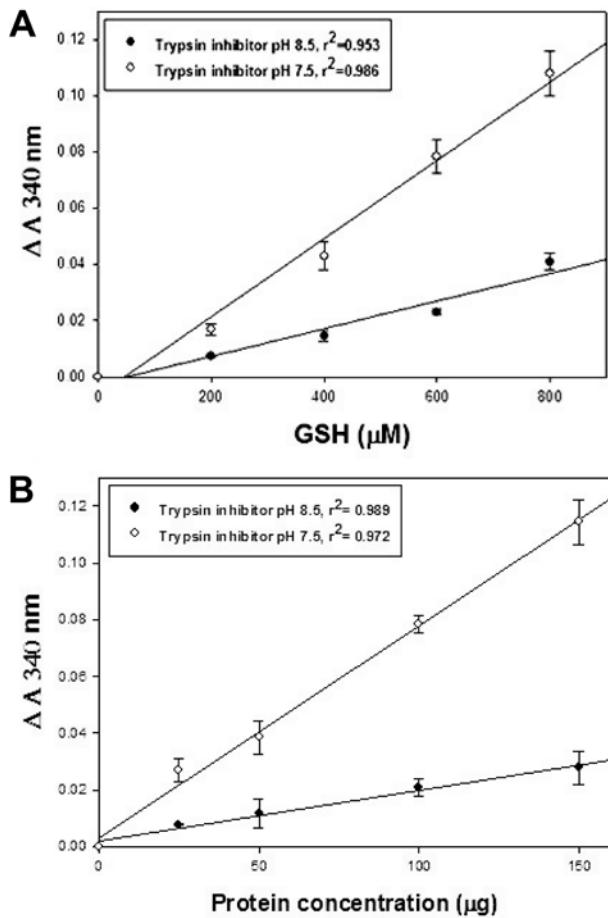
glutathione and CDNB as substrates. Figure 3 shows that SPTI exhibited GST-like activity at both pH 6.0 and 7.0. The specific GSTs activity of SPTI was  $0.094 \pm 0.005$  and  $0.43 \pm 0.03$   $\mu$ mol/min/mg protein at 6.0 and 7.0, respectively. STI was used as a positive control with a specific GSTs activity of  $0.37 \pm 0.08$  and  $0.94 \pm 0.06$   $\mu$ mol/min/mg protein at pH 6.0 and 7.0, respectively. However, very low GST-like activity of SPTI was found at pH 6.0. The rate of GSH-dependent GST-like activities at both pH 6.0 and 7.0, was dependant on concentrations of both GSH (Figure 4A) and SPTI (Figure 4B).

### Comparison of amino acid sequences around disulfide bond of SPTI (sporamin A and B) and glutathione S-transferase, and the active site of thioltransferase

Amino acids around disulfide bond (Cys153~Cys160) of SPTI (sporamin A and sporamin B) were compared with those of sweet potato GST (CB330391) and the active site of TTase by CLUSTALX 1.81 software (Table 1). The comparisons provide some molecular clues of the origin of both glutathione S-transferase and thioltransferase activities of SPTI polypeptides here.

## DISCUSSION

Although SPTI exhibit neither very high thioltransferase-like or glutathione S-transferase-like specific activities, these activities may still be of physiological significance because the large amounts of SPTI in sweet potato storage roots or leaves (Lin and Chen, 1980) may compensate for the low specific activities.



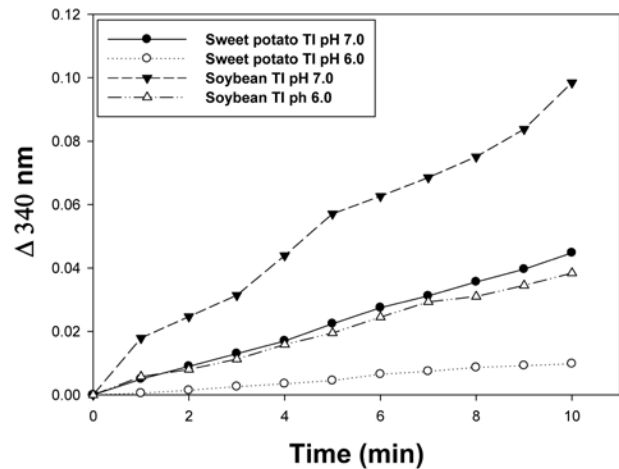
**Figure 2.** Dependence of thioltransferase-like activity of SPTI on GSH (A) and SPTI (B) concentration. (A) The reaction mixture contained 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 300 μM NADPH, 0.5 units glutathione reductase, and different concentrations of GSH (0, 200, 400, 600 and 800 μM) and 100 μg SPTI. (B) The reaction mixture contained 50 mM Tris-HCl pH 7.5 or 8.5, 2 mM EDTA, 300 μM NADPH, 0.5 units glutathione reductase, and 200 μM GSH and different concentrations of SPTI (0, 25, 50, 100 and 150 μg). The reaction was started by adding 450 μL reaction mixture to a cuvette containing 50 μL of 15 mM HED. Decreases of absorbance at 340 nm due to the oxidation of NADPH were recorded for 10 min. Each data show the mean ± SD of one experiment performed in triplicate.

**Table 1.** Comparison of amino acid sequences around thioltransferase active site and disulfide bond of SPTI (sporamin A and B), and of glutathione S-transferase (CB330391).

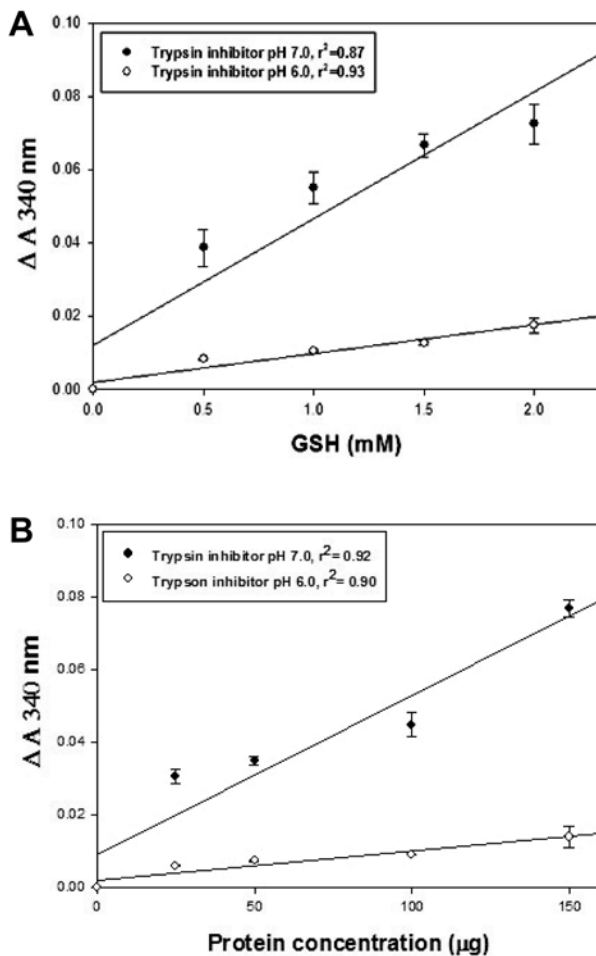
| Protein                                  | Partial sequence |
|--|------------------|
| Thioltransferase active site             | --CGPS--         |
| Sporamin A disulfide bond                | TYCQFGSD--KC     |
| Sporamin B disulfide bond                | SYCQFGTE--KC     |
| Glutathione-S-transferase disulfide bond | KFCRWKQDFLNC     |

TTase are small proteins, usually around 9-15 kDa, existing as large number of isoforms in basically all glutathione-containing life forms. Based on their active site motifs, TTase can be divided in two major categories: the dithiol TTase (active site Cys-Pro-Tyr-Cys) and the monothiol TTase (active site: Cys-Gly-Phe-Ser). These TTase are kept reduced by GSH. The resulting GSSG is converted by glutathione peroxidase (GPxs) to disulfide form (GSSG), which is recycled back to GSH by glutathione reductase (GR) with electrons from NADPH (Holmgren, 2005). The classical dithiol TTase efficiently reduce some protein disulfides like those in *E. coli* ribonucleotide reductase.

Numerous functions have been described for TTase, either catalyzing conversion between -SH and -S-S- or acting as regulators of cellular function in response to oxidative stresses such as sulfur assimilation (Besse and Buchanan, 1997; Berndt et al., 2004; Sun et al., 2007), dehydroascorbate reduction (Wells et al., 1990; Huang et al., 2008b); and the regulation of cellular differentiation (Takashima et al., 1999; Fan and Wu, 2005), transcription (Hirota et al., 2000; Hung et al., 2005), and apoptosis (Daily et al., 2001). Human TTases have been implied in several diseases (Berndt et al., 2007). TTase can be involved in protein folding in many different ways. The reduction of protein disulfides often results in subtle structural changes of the involved protein. But TTases do not necessarily act only as disulfide reductases, it could catalyze the reverse reaction; namely under certain conditions TTases promote disulfide bond formation and synergistically work with PDI and/or chaperones (Carsten et al., 2008). In higher plants, there are about 30 different TTase isoforms, which can be divided into three distinct subgroups depending on their active site sequences. The first class, which contains



**Figure 3.** Effect of pH (6.0 or 7.0) on glutathione S-transferase-like activity of SPTI. Enzyme activity was assayed by combining 10 μL of 20 mM CDNB, 10 μL of 1 mM reduced GSH, 170 μL of 50 mM sodium phosphate buffer (pH 6.0 or 7.0), and 10 μL of SPTI (100 μg), then monitoring the change in absorbance at 340 nm ( $\Delta A_{340}$ ) at 25°C for 10 min. STI was used as a positive control. Each data show the mean ± SD of one experiment performed in triplicate.



**Figure 4.** Dependence of glutathione S-transferase-like activity of SPTI on GSH (A) and SPTI (B) concentration. (A) Enzyme activity was assayed by combining 10  $\mu$ l of 20 mM CDNB, 170  $\mu$ l of 50 mM sodium phosphate buffer (pH 7.0 or 6.0), and 10  $\mu$ l of different concentrations of GSH (0, 0.5, 1, 1.5 and 2 mM) and 100  $\mu$ g SPTI, (B) Enzyme activity was assayed by combining 10  $\mu$ l of 20 mM CDNB, 170  $\mu$ l of 50 mM sodium phosphate buffer (pH 6.0 or 7.0), and 10  $\mu$ l of different concentrations of SPTI (0, 25, 50, 100 and 150  $\mu$ g), then monitoring the change in absorbance at 340 nm at 25°C for 10 min. Each data show the mean  $\pm$  SD of one experiment performed in triplicate.

TTases with C[P/G/S][Y/F][C/S] motifs other than CGFS, is homologous to the classical dithiol TTases such as *E. coli* glutaredoxins (Grx1 and 3). The second class has a strictly conserved CGFS active site sequence and includes Grxs homologous to *E. coli* Grx4. Plants have generally four members in this group (GrxS14 to S17). The properties of the third class TTases, which are specific to higher plants and involve a CC [M/L][C/S] active site, are largely unknown (Hung et al., 1994).

TTases plays an important role in the thiol/disulfide exchange reaction, as do thioredoxin, protein disulfide isomerase, and glutaredoxin. Thioredoxin has been reported to be reductants in cells, reacting similarly to GSH in the presence of thioredoxin reductase, which is different from TTases (Tomoyuki et al., 1992). TTases also can function

as a regulator of the restoration of SH-groups in oxidative damaged proteins. In our previous paper, the NADPH/thioredoxin system reduced SPTI lost their TI activity and decreased their protein stability. Disulfide bonds were essential for the SPTI activity. Disulfide bond is one of the main stabilizing forces in plant storage proteins, which results in increased structural stability and decreased solubility thus providing protection against proteolysis (Huang et al., 2004).

In one of our previous papers, SPTI exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou and Lin, 1997b); 33 kDa SPTI exhibited antioxidant activities against different radicals (Hou et al., 2001; Huang et al., 2007a). SPTI also exhibited growth inhibition activity against and induction of apoptosis of NB<sub>4</sub> cells. Thus, SPTI might prevent oxidative damage to sweet potato roots via the above-described biological activities. We have used synthetic peptides according to sequences of both sporamin A and B to measure both antioxidative and the ACE (angiotensin converting enzyme) inhibition activities. The IC<sub>50</sub> value of TYCQ for antioxidative activity was 206  $\mu$ M, when scavenging activity of DPPH radicals (%) was measured. The IC<sub>50</sub> value of TYCQ was 2.3  $\mu$ M for ACE inhibitory activity, suggesting that TYCQ might represent the main active site for the ACE inhibition (Huang et al., 2007a; 2008b). Cysteine residues with free -SH in whey proteins (Tong et al., 2000) were reported to have antioxidant activities. Our work suggests that TYCQ might represent the main active site for the antioxidative activity and ACE inhibition; there are marked structural similarities for peptides with antihypertensive, immunomodulatory and antioxidant activities and may be used as criteria for selecting or designing multifunctional ingredients of functional foods to control cardiovascular diseases.

In plants, a defense mechanism against chemical toxicants such as herbicides (Shimabukuro et al., 1970) is provided by GSTs. Plant GSTs also respond to pathogen attack, heavy metal toxicity, and oxidative stress resulting from normal metabolic processes. Yao et al. (2001) have shown that both local and systemic induction of sporamin gene expression occur when leaves are wounded. In mice, SPTI also exerts effects on plasma antioxidant activity and lipid levels (Huang et al., 2008c).

## CONCLUSION

In plants, TTase and GSTs activities have been the subject of intensive study. The present results provide a chemically and physiologically reasonable molecular basis on which SPTI exhibit both TTase- and GSTs-like activities. Our present results together with those already published provide both the molecular basis endowed to SPTI as a multi-functional versatile storage protein whether concerning the growth of sweet potato in field or as an important health food and interesting clues to the evolution of related enzyme activities.

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## 甘藷胰蛋白酶抑制因子具有類似硫基轉移酶和麩胱甘肽轉移酶活性

黃世勳<sup>1,2</sup> 洪心容<sup>1</sup> 邱傳淞<sup>1,3</sup> 陳顯榮<sup>4</sup> 林香秀<sup>1</sup>  
林穎志<sup>5</sup> 張恆源<sup>1</sup> 黃冠中<sup>1</sup> 林耀輝<sup>6</sup>

<sup>1</sup> 中國醫藥大學 中國藥學研究所

<sup>2</sup> 弘光科技大學 護理系

<sup>3</sup> 新生醫護管理專科學校 護理科

<sup>4</sup> 中山大學 生命科學系

<sup>5</sup> 仁德醫護管理專科學校 視光學科

<sup>6</sup> 中央研究院 植物暨微生物研究所

就我們先前報告表示，甘藷胰蛋白酶抑制因子 (SPTI) 具有去氫抗壞血酸還原酶和單去氫抗壞血酸還原酶活性，且 SPTI 也具有了抗氧化活性可對抗不同自由基、抑制細胞生長和誘導細胞凋亡。因此，我們本篇文章研究 SPTI 是否具有抗氧化相關酵素的活性。以純化的 SPTI 發現其具有類似硫基轉移酶 (thioltransferase, TTase) 的活性，其比活性在 pH 7.5 和 8.5 時分別為  $1.6 \pm 0.3$  and  $0.58 \pm 0.02$  nmol/min/mg，並以大豆胰蛋白酶抑制因子 (STI) 作為控制組。且純化的 SPTI 也發現其具有類似穀胱甘肽 S- 轉移酶 (glutathione S- transferase, GSTs) 的活性，其比活性在 pH 6.0 和 7.0 時分別為  $0.094 \pm 0.005$  和  $0.43 \pm 0.03$   $\mu$ mol/min/mg。利用 SPTI 雙硫鍵附近的胺基酸 (Cys153~Cys160) 與已發表之 TTase 和 GST 序列比較，結果顯示 SPTI 具有類似硫基轉移酶和穀胱甘肽 S- 轉移酶的活性主要的理由是其分子序列相似。

**關鍵詞：**胰蛋白酶抑制因子；甘藷；硫基轉移酶；穀胱甘肽 S- 轉移酶。