Antioxidant Activities of Trypsin Inhibitor, a 33 KDa Root Storage Protein of Sweet Potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57)

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Trypsin inhibitors (TIs), root storage proteins, were purified from sweet potato (*Ipomoea batatas* [L.] Lam cv. Tainong 57) roots by trypsin affinity column according to the methods of Hou and Lin (*Plant Sci.* 1997, *126*, 11−19 and *Plant Sci.* 1997, *128*, 151−158). A single band of 33 kDa TI was obtained by preparative sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels. This purified 33 kDa TI had scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. There was positive correlation between scavenging effects against DPPH (2 to 22%) and amounts of 33 kDa TI (1.92 to 46 pmol). The scavenging activities of 33 kDa TI against DPPH were calculated from linear regression to be about one-third of those of glutathione between 5 and 80 pmol. Using electron paramagnetic resonance (EPR) spectrometry for hydroxyl radical detection, it was found that 33 kDa TI could capture hydroxyl radical, and the intensities of EPR signal were significantly decreased from 1.5 to 6 pmol of 33 kDa TI compared to those of the controls. It is suggested that 33 kDa TI, one of the sweet potato root storage proteins, may play a role as an antioxidant in roots and may be beneficial to health when it is consumed.

Keywords: *Antioxidant; electron paramagnetic resonance; hydroxyl radical; sweet potato; trypsin inhibitor*

INTRODUCTION

Active oxygen species and free-radical-mediated reactions have been reported in degenerative or pathological processes such as aging (*1, 2*), cancer, coronary heart disease, and Alzheimer's disease (*3*−*6*). Meanwhile, there are many epidemiological data revealing an association between people who have a diet rich in fresh fruit and vegetable and a decrease in the risk of cardiovascular diseases and certain forms of cancer (*7*). Several natural compounds in fruits and vegetables have been proved to have antioxidant activities, such as echinacoside in *Echinaceae* root (*8*), anthocyanin (*9*), phenolic compounds (*10*), water extracts of roasted *Cassia tora* (*11*), and whey proteins (*12*−*14*).

Proteinaceous 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 (*15, 16*). Sohonie and Bhandarker (*17*) reported for the first time the presence of trypsin inhibitors (TIs) in sweet potato. We found that TIs in sweet potato roots accounted for about 60% of total water-soluble proteins and could be recognized as storage proteins (*18*). Maeshima et al. (*19*) identified the sporamin as the major storage protein in sweet potato root, which accounted for 80% of the total proteins in the root. Lin (*20*) proposed that sporamin should be one form of TIs in sweet potato, which was confirmed later by Yeh et al. (*21*). We found that TI activities in sweet potato are positively correlated with concentrations of water-soluble protein (*18*), and that a large negative correlation exists between the natural logarithm of TI activities and cumulative rainfall, which suggests that sweet potato TI activities may vary in response to drought (*22*). Sweet potato TIs were also proved to have both dehydroascorbate reductase and monodehydroascorbate reductase activities and might respond to environmental stresses (*23*).

Until now, most reports of plant proteinaceous protease inhibitors focus on their potential insecticidal activities (*16, 24, 25*). In this work we report for the first time that 33 kDa TI, one of the major sweet potato root storage proteins, had scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and hydroxyl radical.

MATERIALS AND METHODS

Sweet Potato TI Purification. Sweet potato (*Ipomoea batatas* [L.] Lam cv. Tainong 57) storage roots were purchased from a wholesaler. After the roots were washed and peeled, they were cut into strips for TI extraction and purification. After the root strips were extracted and centrifuged, the crude extracts were loaded directly onto a trypsin Sepharose 4B affinity column. The adsorbed TIs were eluted by pH changes with 0.2 M KCl (pH 2.0) according to the methods of Hou and Lin (*23, 26*). The purified TIs by trypsin affinity column were further purified by 10% preparative sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels to isolate the 33 kDa TI. After electrophoresis, sodium dodecyl sulfate (SDS) was removed (*27*) and the 33 kDa TI band on the gel was cut and extracted with 100 mM Tris-HCl buffer (pH 7.9) overnight. The extracts were desalted and concentrated with centricon 10 and then lyophilized for further use.

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10.1021/jf0100705 CCC: $20.00
Published on Web 05/12/2001

Protein and TI Activity Stainings on SDS−PAGE Gels. Four parts of samples were mixed with one part of sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue without 2-mercaptoethanol for TI activity stainings at 4 °C overnight. Coomassie brilliant blue G-250 was used for protein staining (*28*). After electrophoresis, gels were washed with 25% 2-propanol in 10 mM Tris-HCl buffer (pH 7.9) for 10 min twice to remove SDS (*27*) and then for TI activity staining. The gel was stained according to the method of Hou and Lin (*27*).

Scavenging Activity of 33 kDa TI or Glutathione against DPPH Radical. The scavenging activity of sweet potato TI or glutathione against DPPH radical was measured according to the method of Yamaguchi et al. (*29*) with some modifications. The 1.2 mL of different amounts of 33 kDa TI (1.92, 3.84, 5.76, 7.68, 15.36, 30.72, 38.4, or 46.08 pmol) or glutathione (1, 3, 5, 10, or 20 pmol) were added to 0.1 mL of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 1.2 mL of 500 μM DPPH in methanol for 20 min under light protection. The absorbance at 517 nm was determined. Deionized water was used instead of sample solution for control experiments. The decrease of absorbance at 517 nm was calculated and expressed as ΔA 517 nm for scavenging activity.

Scavenging Activity of 33 kDa TI against Hydroxyl Radical by Electron Paramagnetic Resonance (EPR) Spectrometry. The hydroxyl radical was generated by the Fenton reaction according to the method of Kohno et al. (*30*). The reaction solution included different amounts of 33 kDa TI (50, 100, and 200 μg corresponding to 1.52, 3.03, and 6.06 pmol, respectively), 5 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and 0.05 mM ferrous sulfate. After the solution was mixed, it was transferred to an EPR quartz cell and placed at the cavity of the EPR spectrometer, then hydrogen peroxide was added to a final concentration of 0.25 mM. After forty seconds, the relative intensity of the signal of DMPO−OH spin adduct was measured. Deionized water was used instead of sample solution for control experiments. All EPR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 EPR spectrometer equipped with WIN-EPR SimFonia software version 1.2. Following are the conditions of EPR spectrometry that were used: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constan,t 0.6 s; san time, 1.5 min.

Material. Trypsin (TPCK-treated, 40 U/mg), Tris, N-benzoyl-L-arginine-4-nitroanilide, and electrophoretic reagents were purchased from E. Merck Inc. (Darmstadt, Germany). Seeblue prestained markers for SDS−PAGE were from Novex (San Diego, CA); CNBr-activated Sepharose 4B were from Pharmacia Biotech AB (Uppsala, Sweden); DPPH, coomassie brilliant blue G-250, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and ferrous sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

Partial Purification of Sweet Potato Total TIs and Isolation of 33 kDa TI. Figure 1 (A) showed the protein staining (lane 1) and TI activity staining (lane 2) of partial purified TIs from the affinity column. There were two protein bands of 33 kDa and 22 kDa (lane 1), respectively, corresponding to trypsin inhibitory bands (lane 2). The 33 kDa protein was found to be the major TI band. Therefore, the 10% preparative SDS−PAGE gels were used to isolate the 33 kDa TI of partial purified TIs from affinity column. Figure 1 (B) showed the protein staining (lane 1) and TI activity staining (lane 2) of isolated 33 kDa TI. A single protein band of 33 kDa (lane 1) corresponding to trypsin inhibitory activity (lane 2) was found. This isolated 33 kDa TI was used for further investigations.

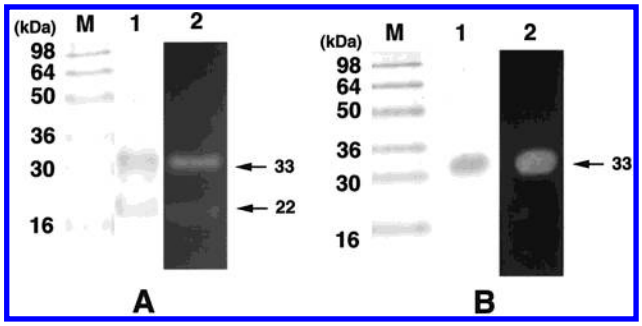

Figure 1. Protein staining (lane 1) and TI activity staining (lane 2) of purified TIs from trypsin affinity column (A) or isolated 33 kDa TI (B) from affinity column samples by preparative SDS−PAGE gels.

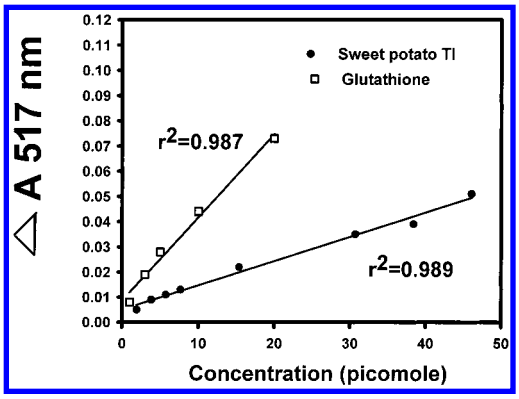

Figure 2. Scavenging activity of 33 kDa sweet potato TI (1.92, 3.84, 5.76, 7.68, 15.36, 30.72, 38.4, and 46.08 pmol) or glutathione (1, 3, 5, 10, and 20 pmol) against DPPH radical. Linear regression was plotted between scavenging activity (ΔA 517 nm) and concentration of 33 kDa TI or glutathione.

Scavenging Activity of 33 kDa TI versus Glutathione against DPPH Radical. DPPH radical has been widely used in model systems to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanin, or crude mixtures such as methanol extracts of plants (*8*, *9*, *31*, *32*). However, few reports concerned proteins except those addressing antioxidative enzymes on the direct antiradical effects. Therefore, we used 33 kDa TI to test the scavenging activities against DPPH radical (Figure 2). Figure 2 shows that the scavenging activity of 33 kDa TI against DPPH radical is concentration-dependent. This is the first report that 33 kDa TI, the major storage protein of sweet potato roots, could capture DPPH radical. There is a linear relationship between scavenging effects against DPPH radical (2 to 22%) and amounts of 33 kDa TI (1.92 to 46 pmol). The equation of linear regression is $\boldsymbol{Y} = 5.12 \times 10^{-3} + 9.62 \times 10^{-4}\,\boldsymbol{X}$ ($r^2 = 0.989$). Allen and Wrieden (*12*, *13*) found that whey proteins of α-lactalbumin and β-lactoglobulin exhibited antioxidative activities against copper-catalyzed lipid oxidation. Tong et al. (*14*) also found that high-molecular- weight fractions of whey proteins (molecular weight higher than 3.5 kDa) exhibited antioxidative activities against lipid peroxidation and peroxyl radical. They pointed out that free sulfhydryl groups in whey proteins might mainly contribute the antioxidative activities. Hou and Lin (*23*) found that sweet potato TIs exhibited dehydroascorbate reductase activities which were independent of glutathione, and intermolecular thiol-disulfide interchanges of TIs were found during dehydroascorbate reduction. It was postulated that the free sulfhydryl groups in sweet potato TIs could reduce

Table 1. Scavenging Effects of 33 KDa Sweet Potato Trypsin Inhibitor and Glutathione Against DPPH Radical

concentration (pmol, mg, mg)[b]	decrease of absorbance at 517 nm[a]		ratio (%)[c]
	33 kDa TI	glutathione	
5 (0.17, 0.0015)	0.0099	0.0252	39.28
10 (0.33, 0.0031)	0.0147	0.0417	35.25
20 (0.66, 0.0061)	0.0244	0.0748	32.62
40 (1.32, 0.0123)	0.0436	0.1410	30.92
80 (2.64, 0.0246)	0.0821	0.2734	30.03

[a] Different amounts of 33 kDa sweet potato TI or glutathione (1.2 mL) were added to 0.1 mL of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 1.2 mL of 500 μM DPPH in methanol for 20 min under light protection. The decrease of absorbance at 517 nm was calculated from each linear regression equation of Figure 2. [b] The amount of sweet potato TI (33 kDa) and glutathione (307.3 Da) in minigram, respectively, with the same pmol concentration. [c] The ratio of decreased absorbance at 517 nm of sweet potato TI to that of glutathione at the same pmol concentration.

dehydroascorbate to regenerate ascorbate to prevent oxidative damage to sweet potato roots. The scavenging activity of 33 kDa TI against DPPH radical must be due to its free sulfhydryl groups. At the same time, we also used glutathione for comparison of the scavenging activity against DPPH radical. A linear relationship between scavenging effects against DPPH radical and glutathione concentrations was also found (Figure 2). The equation of linear regression is $\boldsymbol{Y} = 8.6 \times 10^{-3} + 3.31 \times 10^{-3}\,\boldsymbol{X}$ ($r^2 = 0.987$). Table 1 shows the comparison of scavenging effects of 33 kDa and glutathione against DPPH radical under the same concentrations from each linear regression equation (Figure 2). The scavenging effects of 33 kDa sweet potato TI were about 30% to 39% of those of glutathione while the concentration was increased. On average, 33 kDa sweet potato TI concentrations between 5 and 80 pmol were about one-third as effective as glutathione at scavenging DPPH.

Scavenging Activity of 33 kDa TI against Hydroxyl Radical Determined by EPR Spectrometry. The hydroxyl radical was generated by the Fenton reaction according to the method of Kohno et al. (*30*) and was trapped by DMPO to form DMPO−OH adduct. The intensities of DMPO−OH spin signal in EPR spectrometry were used to evaluate the scavenging activity of 33 kDa sweet potato TI against hydroxyl radical. Figure 3 shows the scavenging activity using EPR spectrometry against the hydroxyl radical with different amounts of 33 kDa TI: (A) controls, (B) 50 μg (1.52 pmol), (C) 100 μg (3.03 pmol), and (D) 200 μg (6.06 pmol). It was found that the effect of 33 kDa TI as a scavenger of hydroxyl radical decreased intensities of DMPO−OH signals and was concentration-dependent. About one-half intensity was found when 50 μg (1.52 pmol) 33 kDa TI was added; about one-fourth and less than one-eighth intensities were found when 100 μg (3.03 pmol) and 200 μg (6.06 pmol), respectively, 33 kDa TI was added. Figure 3 provides the first piece of evidence that sweet potato TI exhibited scavenging activity against hydroxyl radical as shown by EPR spectrometry.

In conclusion, purified 33 kDa sweet potato TI exhibited antioxidant activity against both DPPH and hydroxyl radicals. It is suggested that 33 kDa TI, one of the sweet potato root storage proteins, may play a role as antioxidant in roots. The purified sweet potato TIs still retain partial inhibitory activities against trypsin after heating in boiling water for 10 min (data not shown). We propose that the cooked sweet potato as food might be beneficial to health because (1) the inhibitory activities of sweet potato TIs against trypsin were reduced, and (2) the remaining activities of TIs after cooking may have antioxidant effects as polypeptide forms before being ingested or as peptide forms after being ingested.

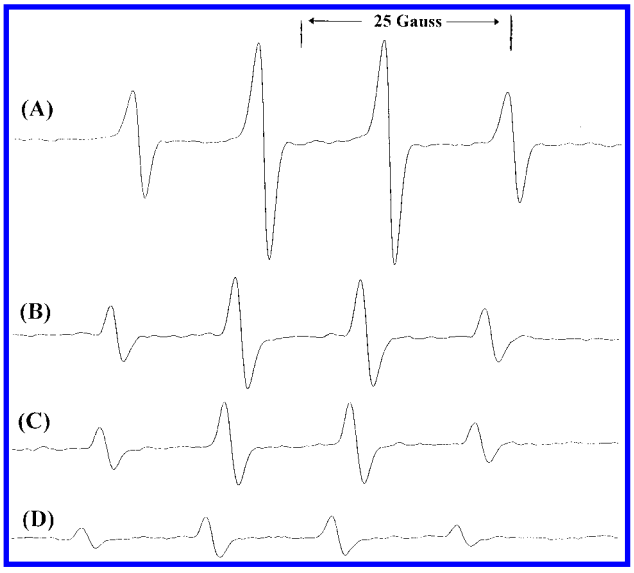

Figure 3. Scavenging activity against the hydroxyl radical by 33 kDa sweet potato TI measured by EPR spectrometry. (A) controls, (B) 50 μg (1.52 pmol), (C) 100 μg (3.03 pmol), (D) 200 μg (6.06 pmol) 33 kDa TI were added.

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Received for review January 17, 2001. Revised manuscript received March 22, 2001. Accepted March 22, 2001. The authors thank the National Science Council, Republic of China, for financial support (NSC 89-2313-B-038-002).

JF0100705