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Inhibition of Reactive Nitrogen Species in Vitro and ex Vivo by Trypsin Inhibitor from Sweet Potato 'Tainong 57' Storage Roots

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Peroxynitrite ($ONOO^-$), formed from a reaction of superoxide and nitric oxide, is one of the most potent cytotoxic species known to oxidize cellular constituents including essential proteins, lipids, and DNA. $ONOO^-$ induces cellular and tissue injury, resulting in several human diseases such as Alzheimer's disease, atherosclerosis, and stroke. Due to the lack of endogenous enzymes responsible for $ONOO^-$ scavenging activity, finding a specific $ONOO^-$ scavenger is of considerable importance. In this study, the ability of trypsin inhibitor (TI), isolated from sweet potato storage roots (SPTI), to scavenge $^{\bullet}ON$ and $ONOO^-$ was investigated. The data obtained show that TI generated a dose-dependent inhibition on production of nitrite and superoxide radicals. The IC_{50} value of TI on superoxide radical was 143.2 ± 4.29 μg/mL. SOD activity staining was used to confirm SOD activity of SPTI. SPTI also caused a dose-dependent inhibition of the oxidation of dihydrorhodamine 123 (DHR) by peroxynitrite. A calculated IC_{50} value of 809.1 ± 32.36 μg/mL was obtained on the inhibition of peroxynitrite radical. Spectrophotometric analyses revealed that TI suppressed the formation of $ONOO^-$-mediated tyrosine nitration through an electron donation mechanism. In further studies, TI also showed a significant ability to inhibit nitration of bovine serum albumin (BSA) in a dose-dependent manner. In vivo TI inhibited lipopolysaccharide-induced nitrite production in macrophages in a concentration-dependent manner with an IC_{50} value of 932.8 ± 29.85 μg/mL. The present study suggested that TI had an efficient reactive nitrogen species scavenging ability. TI might be a potential effective NO and $ONOO^-$ scavenger useful for the prevention of NO- and $ONOO^-$-involved diseases.

KEYWORDS: Peroxynitrite; nitric oxide; superoxide; trypsin inhibitor; sweet potato

INTRODUCTION

Nitric oxide (NO) is an important bioregulatory molecule, which has a number of physiological effects including control of blood pressure, neural signal transduction, platelet function, and antimicrobial and antitumor activities under aerobic conditions. Low concentrations of NO are sufficient to effect these beneficial functions. However, in the aerobic conditions, the NO molecule is very unstable and reacts with oxygen to produce intermediates such as NO_2, N_2O_4, N_3O_4, the stable products nitrate and nitrite (*1*, *2*), and peroxynitrite ($ONOO^-$). Recent studies indicate that NO may act by affecting the enzymatic activities of several thiol-rich DNA repair proteins, for example, DNA alkyl transferase, formamidopyrimidine−DNA glycosylase, and the DNA ligase that play critical roles in the maintenance of genome integrity (*3*).

Peroxynitrite ($ONOO^-$) is formed by the reaction of NO and superoxide ($O_2^{\bullet-}$). $ONOO^-$ is a cytotoxic reactive species that can be generated by endothelial cells, neutrophils, and macrophages (*4*, *5*). $ONOO^-$ is a relatively long-lived cytotoxicant with strong oxidizing properties toward various cellular constituents including sulfhydryls, lipids, amino acids, and nucleotides (*6*). $ONOO^-$ can induce oxidation of thiol (−SH) groups on proteins, nitration of tyrosine, and lipid peroxidation that affect cell metabolism and signal transduction (*6*−*8*). Excessive formation of $ONOO^-$ may also be involved in several human diseases such as Alzheimer's disease, atherosclerosis, and cancer (*7*). Naturally occurring and synthetic $ONOO^-$ scavengers such as glutathione (*9*), melatonin (*10*), and sinapic acid (*11*) have been recently reported. Ascorbic acid, α-tocopherol, flavonoids, and polyhydroxyphenols, which are constituents of fruits, wines, teas, and green vegetables, were demonstrated to be effective antioxidants against $ONOO^-$ (*4*). To prevent the formation of $ONOO^-$, the $O_2^{\bullet-}$ level can be regulated by a number of

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enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase (*4*), whereas the NO level can be controlled by oxyhemoglobin and NO synthase (*6*).

Sohonie and Bhandarker (*12*) reported for the first time the presence of trypsin inhibitor (TI) in sweet potato. TI in sweet potato roots accounted for about 60% of total water-soluble proteins and could be recognized as storage proteins (*13*). Maeshima et al. (*14*) identified sporamin as the major storage protein in sweet potato root, which accounted for 80% of total proteins in root. Lin (*15*) proposed that the sporamin should be one form of TI in sweet potato, which was confirmed later (*16*, *17*). In our papert, sweet potato TI (SPTI) exhibited dehydroascorbate reductase, monodehydroascorbate reductase, and antioxidant activities (*18*). In this work we report for the first time that SPTI has NO and $ONOO^-$ scavenging activities. The protective effect of trypsin inhibitor on nitration of tyrosine and bovine serum albumin (BSA) by $ONOO^-$ is very good. Our results suggest that TI may be a potential selective regulator of $ONOO^-$-mediated diseases via its direct scavenging activities.

MATERIALS AND METHODS

Materials. BSA was obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, OR) and Cayman Chemical Co. (Ann Arbor, MI). Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA). Anti-nitrotyrosine antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep were obtained from Upstate Biotechnology (Lake Placid, NY) and Amersham (Piscataway, NJ), respectively. All other chemicals were of the highest purity available from Sigma Chemical Co.

Plant Materials and Purification of SPTI. Fresh storage roots of sweet potato [*Ipomoea batatas* (L.) Lam. 'Tainong 57'] were purchased from a local market. Sweet potato storage roots were washed and peeled, cut into strips, and extracted. The crude extracts were loaded directly onto a trypsin Sepharose-4B affinity column. The adsorbed TI was eluted by pH changes with 0.2 M KCl (pH 2.0) according to the method of Huang et al. (*19*).

Scavenging Effect on NO. The scavenging effect of TI on NO was measured according to the method of Marcocci et al. (*1*). Purified TI at different concentrations was added to the test tubes containing 1 mL of sodium nitroprusside (SNP) solution (25 mM), and the tubes were incubated at 37 °C for 2 h. An aliquot (0.5 mL) of the incubation solution was removed and diluted with 0.3 mL of Griess reagent [1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride]. The absorbance of the chromophore that formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and compared to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Measurement of Superoxide Radical Scavenging Activity. Effects of TI and glutathione on superoxide radical were determined by the PMS-NADH superoxide-generating system (*19*, *20*). TI or glutathione (0−150 μg/mL) was added to a solution mixture that contained 200 μM NBT (nitro blue tetrazolium), 624 μM β-nicotinamide adenine dinucleotide (NADH), and 80 μM phenazin methosulfate (PMS) in 0.1 M phosphate buffer, pH 7.4. After 2 min of incubation at room temperature, the absorbance at 560 nm was measured. The capability to scavenge the superoxide radical was calculated using the following equation: scavenging effect (%) = (1 − absorbance of sample at 560 nm/absorbance of control at 560 nm) × 100.

Electrophoresis and SOD Activity Staining. Electrophoresis was carried out at 4 °C according to a procedure of Huang et al. (*21*, *22*) with 0.75 mM 12.5% polyacrylamide mini-slab gel in standard Tris−glycine buffer (pH 8.3). Samples were mixed with sample buffer, namely, 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue without 2-mercaptoethanol. Samples were loaded into each well and then electrophoresed at 80 V through the stacking gel for 15 min and at 120 V through the separating gel for 60 min. After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (*23*) was used to locate SOD activities on gels. The gel was first soaked in 25 mL of 1.23 mM NBT for 15 min, briefly washed, and then soaked in the dark in 30 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 28 μM riboflavin for another 15 min. The gel was briefly washed again and then illuminated on a light box with a light intensity of 30 mEinstein m^{-2} s^{-1} (measured by LI-COR LI 1000) for 15 min to initiate the photochemical reaction. All of the procedures were carried out at room temperature, and the two soaking steps were shaken at 75 rpm.

Protecting DHR 123 from $ONOO^-$-Mediated Oxidation by TI. The protection of DHR 123 against $ONOO^-$-mediated oxidation was detected according to the method of Kooy et al. (*24*). The total 180 μL reaction mixture included different amounts of TI (1−4 mg/mL), 0.9 mM DHR, and 5 μL of $ONOO^-$ in 50 mM phosphate buffer (pH 7.4) containing 90 mM NaCl. After 5 min of reaction, the fluorescence intensity was measured at the excitation and emission wavelengths of 500 and 536 nm, respectively, with excitation and emission slit widths of 2.5 and 3.0 nm, respectively. The control contained all reaction components except TI.

Inhibition of $ONOO^-$-Mediated Tyrosine Nitration by TI. The ability of TI to inhibit the formation of 3-nitrotyrosine was measured as the index of TI inhibition of tyrosine nitration utilizing spectrophotometric analysis and western blot analysis.

Spectrophotometric Analysis of Nitrated Proteins. Tyrosine (200 μM) was reacted with $ONOO^-$ (200 μM) in the presence of various concentrations of TI (0−100 μM). The formation of 3-nitrotyrosine was determined with a UV−visible spectrophotometer. Tyrosine was monitored at 275 nm, whereas 3-nitrotyrosine formation was monitored at 430 nm. The disappearance of the 3-nitrotyrosine peak at 430 nm in the presence of TI was taken as an indication of the inhibition by TI.

Western Blot Analysis. A 2.5 μL aliquot of TI was added to 95 μL of BSA (0.5 mg of protein/mL). The mixed samples were incubated with shaking at 20 °C for 1 h. After 1 h of incubation, 2.5 μL of $ONOO^-$ (100 μM) was added. The samples were then incubated for 30 min at 20 °C with shaking. After reaction, the samples prepared in gel loading buffer [pH 6.8; 0.125 M tris(hydroxymethyl)aminomethane (Tris), 4% mass per volume (m/v) of sodium dodecyl sulfate (SDS), 20% m/v glycerol, 10% m/v 2-mercaptoethanol, and 0.2% m/v bromophenol blue] in a ratio of 1:1 were boiled for 5 min. Twenty microliters of each sample was separated on an SDS−polyacrylamide mini-gel (10% for BSA) at 100 V and transferred to a poly(vinylidene fluoride) membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed into a blocking solution (10% m/v skim milk powder in TBS−Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM Tween-20, pH 7.5) at 4 °C overnight. The membrane was washed in TBS−Tween buffer for 30 min and then incubated with a mouse monoclonal anti-nitrotyrosine antibody (0.5% m/v skim milk, diluted 1:2000 in TBS−Tween buffer) at room temperature for 2 h. After four 10-min washings in TBS−Tween buffer, the membrane was reacted with rabbit alkaline phosphatase-conjugated secondary antibody against mouse antibody (0.1% m/v skim milk, diluted 1:1000 in TBS−Tween buffer) at room temperature for 2 h. After four 10- min washings in TBS−Tween buffer, blue color was developed using NBT/BCIP (5-bromo-4-chloro-3-indolylphosphate) (Sigma). A set of prestained blue protein markers was used for molecular weight determination.

Cell Culture. Murine macrophage cell line RAW 264.7 (BCRC 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin−0.02% EDTA in Ca^{2+},Mg^{2+}-free phosphate-buffered saline (DPBS).

Cell Viability. The cells (2×10^5) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with TI samples in the presence of 1 μg/mL lipopolysaccharide (LPS) for 24 h. The cells were

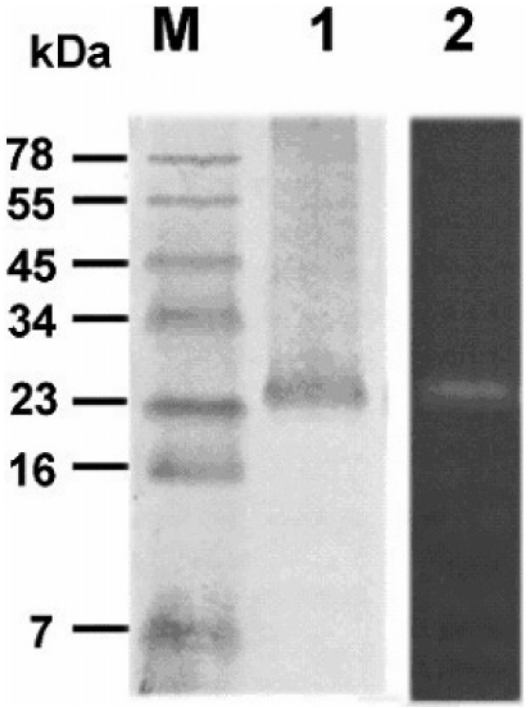

Figure 1. Protein (lane 1) and activity (lane 2) stainings of the trypsin inhibitor from sweet potato storage root on SDS-PAGE gels with 2-mercaptoethanol. The gel system contained 2.5 cm, 4% stacking gel and 4.5 cm, 15% separating gel. M indicates the Seeblue prestained markers of SDS-PAGE. Ten micrograms of trypsin inhibitor was loaded in each well.

then washed twice with DPBS and incubated with 110 μL of 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 2 h at 37 °C, testing for cell viability (*25*). The medium was then discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added. After 30 min of incubation, absorbance at 570 nm was read by using a microplate reader.

Effect of TI on Preventing NO Production in RAW 264.7 Cells. Cells were plated at a density of 2×10^5 cells/mL in a 96-well plate. After overnight incubation, TI samples and 1 μg/mL LPS were added and the culture was incubated for another 24 h. Aliquots of media (100 μL) were transferred to another 96-well plate, where 100 μL of Greiss reagent (50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% naphthylethylenediamine dihydrocholide in water) was added. Absorbance at 570 nm was determined using a microplate reader (Multiskan Spectrum, ThermoLabsystem). The concentration of nitrite was calculated from a standard curve obtained from the same procedure with sodium nitrite. The inhibition of nitric oxide production was calculated according to the following equation: inhibition (%) = (absorbance$_{positive}$ − absorbance$_{sample}$/absorbance$_{positive}$ − absorbance$_{negative}$) × 100. Absorbance$_{positive}$, absorbance$_{negative}$, and absorbance$_{sample}$ represent the absorbance of cultural media containing LPS, without LPS, and TI sample with LPS, respectively.

Statistical Analysis. Means of triplicate were calculated. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$, $p < 0.01$, or $p < 0.001$.

RESULTS AND DISCUSSION

Extraction and Purification of TI from Sweet Potato Storage Root. TI was purified (**Figure 1**) from sweet potato [*I. batatas* (L.) Lam. 'Tainong 57'] tubers according to the method of Huang et al. (*19*). The separation of TI was achieved by trypsin−Sepharose 4B affinity column (1.0 × 10 cm), and the purified enzyme was collected as TI for enzyme studies. SDS-PAGE analysis showed a monomer with a molecular mass of ca. 25 kDa. The yield was 8.3% (150 mg of protein of purified TI/1800 mg of total protein of crude extract = 8.3%).

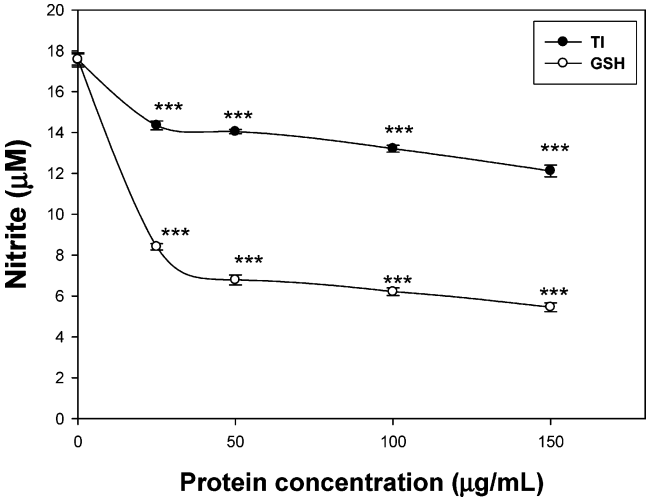

Figure 2. Scavenging effects of trypsin inhibitor on nitric oxide, generated with 5 mM SNP during incubation. Data are presented as mean ± SE for three different experiments performed in triplicate. ***, $p < 0.001$ (unpaired *t* test) compared with control group.

Scavenging Effect on NO. NO is a defense molecule with cytotoxic, microbiocidal, and microbiostatic activities. In the present study, the scavenging effect of TI on NO was investigated. Sodium nitroprusside (SNP) is known to decompose in PBS solution to produce NO. NO, under aerobic conditions, reacts with oxygen to form nitrate and nitrite, which can be determined using Griess reagent (*1*). As shown in **Figure 2**, TI in the range of 25−150 μg/mL generated a dose-dependent inhibition on nitrite production. Glutathione was used as a positive control. The nitrite levels of TI and glutathione at a concentration of 25 μg/mL were 14.4 ± 0.32 and 8.4 ± 0.23 μM, respectively, indicating that TI had a scavenging activity on NO. There was a significant decrease in the nitrite production of groups treated with 25, 25, 100, and 150 μg/mL TI and glutathione ($p < 0.001$).

Scavenging of Superoxide Radical ($^{\bullet}O_2^-$) Scavenging Activity. $^{\bullet}O_2^-$, the one-electron-reduced form of molecular oxygen, is a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage (*26*). According to the data of **Figure 3A**, the marked inhibitory effect of TI on $^{\bullet}O_2^-$ was in a dose-dependent manner. The IC_{50} value of TI on $^{\bullet}O_2^-$ was 143.2 ± 4.29 μg/mL. Glutathione was used as a positive control. These results reveal that TI is a potent scavenger of $^{\bullet}O_2^-$ and has SOD-like ability. There was a significant scavenging of $^{\bullet}O_2^-$ of groups treated with 0, 25, 25, 100, and 150 μg/mL TI and glutathione ($p < 0.05$, $p < 0.01$, or $p < 0.001$). Because TI is a potent scavenger of $^{\bullet}O_2^-$ and has SOD-like ability, we try to do the SOD activity staining in **Figure 3B**. The activity staining showed that TI has SOD-like activity. The most important findings from the present study are that the active component, TI, not only directly scavenged $ONOO^-$ but also was involved in the inhibition of $^{\bullet}O_2^-$ and $^{\bullet}NO$ radical formation.

Protecting DHR 123 from $ONOO^-$-Mediated Oxidation by TI. TI caused a dose-dependent inhibition of the oxidation of DHR 123 by $ONOO^-$ (**Figure 4**). The resulting IC_{50} value was 809.1 ± 32.36 μg/mL, and the positive control, glutathione, provided an IC_{50} of 946.9 ± 35.98 μg/mL. These results reveal that TI is a potent scavenger of $ONOO^-$ radical. $ONOO^-$ is formed from nearly diffusion-limited reaction between NO and

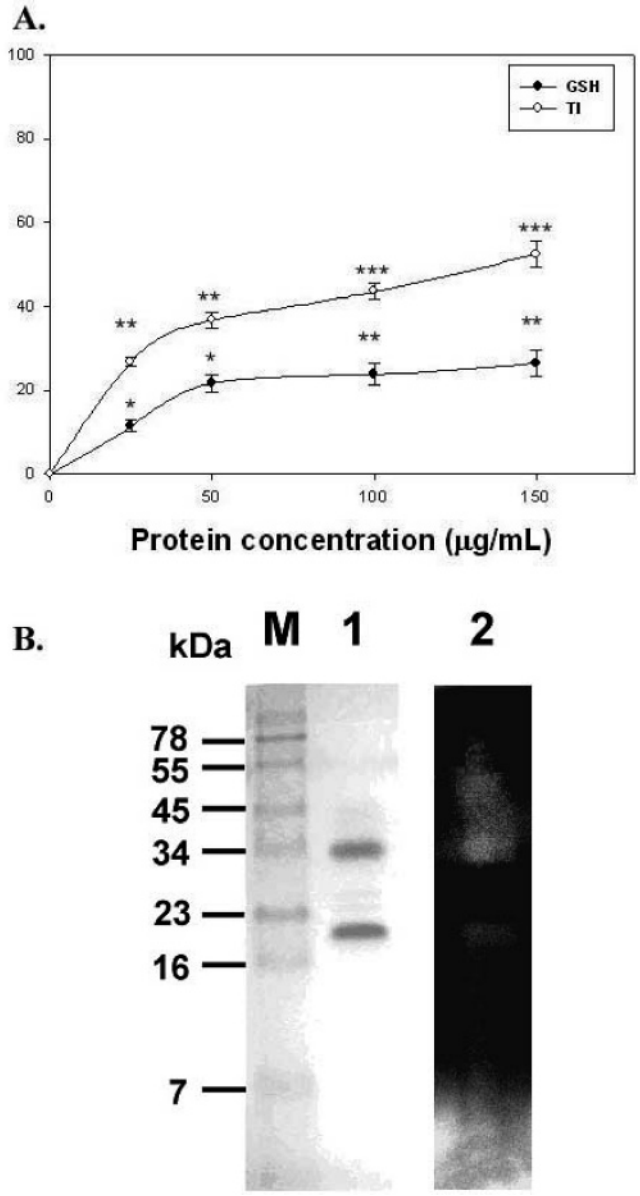

Figure 3. (**A**) Scavenging effect of trypsin inhibitor on superoxide radical; (**B**) SOD activity staining. Protein staining (lane 1) and SOD activity staining (lane 2) of purified trypsin inhibitor were from trypsin affinity column by SDS-PAGE gels. The gel system contained 2.5 cm, 4% stacking gel and 4.5 cm, 12.5% separating gel. Samples were mixed with sample buffer, namely, 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue without 2-mercaptoethanol. M indicates the Seeblue prestained markers of SDS-PAGE. Ten micrograms of trypsin inhibitor was loaded in each well. Data are presented as mean ± SE for three different experiments performed in triplicate. ∗, $p < 0.05$; ∗∗, $p < 0.01$; or ∗∗∗, $p < 0.001$ (unpaired t test), compared with control group.

$^{\bullet}O_2^-$ and acts as an initiator of potentially harmful oxidation reaction. There was a significant scavenging of $ONOO^-$ radical of groups treated with 25, 25, 100, and 150 μg/mL TI and glutathione ($p < 0.01$ or $p < 0.001$).

Effect of TI on $ONOO^-$-Mediated 3-Nitrotyrosine Formation. To explore the scavenging mechanisms of TI by which it undergoes either nitration reaction or electron donation after the addition of $ONOO^-$, spectrophotometric analysis at 400−450 nm was employed. Tyrosine was incubated without (**Figure 5A**) or with $ONOO^-$ (**Figure 5B**), followed by a spectrophotometric scan from 200 to 600 nm. Tyrosine undergoes nitration by $ONOO^-$ because there is a peak around 430 nm. TI at 50 μg/mL (**Figure 5C**) or 100 μg/mL (**Figure 5D**) was incubated with $ONOO^-$. Incubation of TI with tyrosine prior to the addition of $ONOO^-$ resulted in the disappearance of the nitrotyrosine peak at 430 nm (**Figure 5C**,**D**) in a dose-dependent manner, implying that TI inhibited the formation of 3-nitrotyrosine.

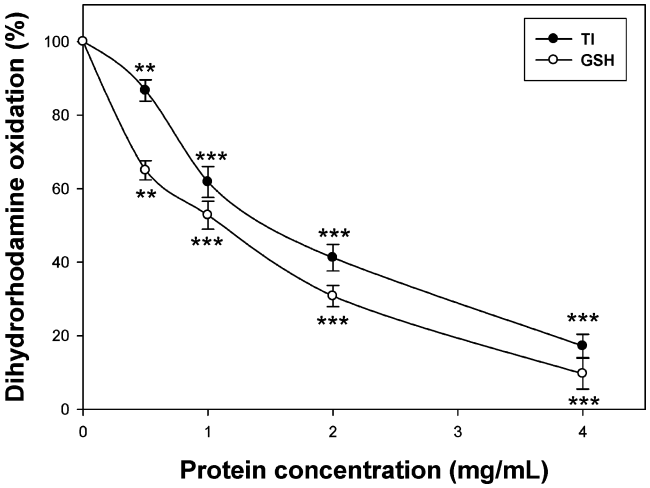

Figure 4. Inhibitory effect of trypsin inhibitor (0.5–4 mg/mL) on the oxidation of dihydrorhodamine 123 by peroxynitrite. Data are presented as mean ± SE for three different experiments performed in triplicate. ∗∗, $p < 0.01$; or ∗∗∗, $p < 0.001$ (unpaired t test), compared with control group.

In the present study we exposed TI to $ONOO^-$ to determine whether the TI scavenging mechanism involves the nitration reaction. The addition of $ONOO^-$ revealed no spectral change in the visible region, indicating that nitration of the aromatic ring did not occur. Furthermore, incubation of tyrosine and TI with $ONOO^-$ caused a decreased peak at 430 nm, which gave further evidence supporting the possibility of an electron donation reaction between TI and $ONOO^-$. The toxicity of $ONOO^-$ can be attributed to nitration of tyrosine and tryptophan residues and subsequent alterations of their functionalities (*27*). Plant food derived antioxidants and active principles such as flavonoids, β-carotene and other carotenoids, vitamins E and C, and tea are important dietary antioxidant substances (*28*, *29*) that may provide efficient $ONOO^-$ scavenging. This result suggested that TI directly blocked the formation of 3-nitrotyrosine by the action of $ONOO^-$ and possibly reduced the availability of $ONOO^-$.

Effect of TI on Nitration of BSA. Using mouse anti-3-nitrotyrosine antibody in western blot, we analyzed the ability of TI to suppress $ONOO^-$-mediated tyrosine nitration in common biological materials, such as BSA. For this purpose, TI was preincubated with BSA prior to the addition of 100 μM $ONOO^-$ and checked for the formation of 3-nitrotyrosine. The results revealed that preincubation of TI at concentrations of 0, 25, 50, and 100 μg/mL attenuated the nitration of BSA in a dose-dependent manner, as shown in **Figure 6**.

$ONOO^-$ could induce functional damage in some biological molecules, such as BSA and low-density lipoprotein (LDL), via nitrotyrosine (*29*). Protein tyrosine nitration by $ONOO^-$ may interfere with phosphorylation/dephosphorylation signaling pathways and alter enzyme functions (*30*−*32*). Nitrotyrosine has been reported in various hypertensive disorders, neurological disorders, and chronic renal disease (*33*−*35*). In this study evidence from western blot analysis showed that TI, even at a concentration of 50 μg/mL, could markedly reduce the nitrotyrosine present in BSA. With higher TI concentrations, BSA

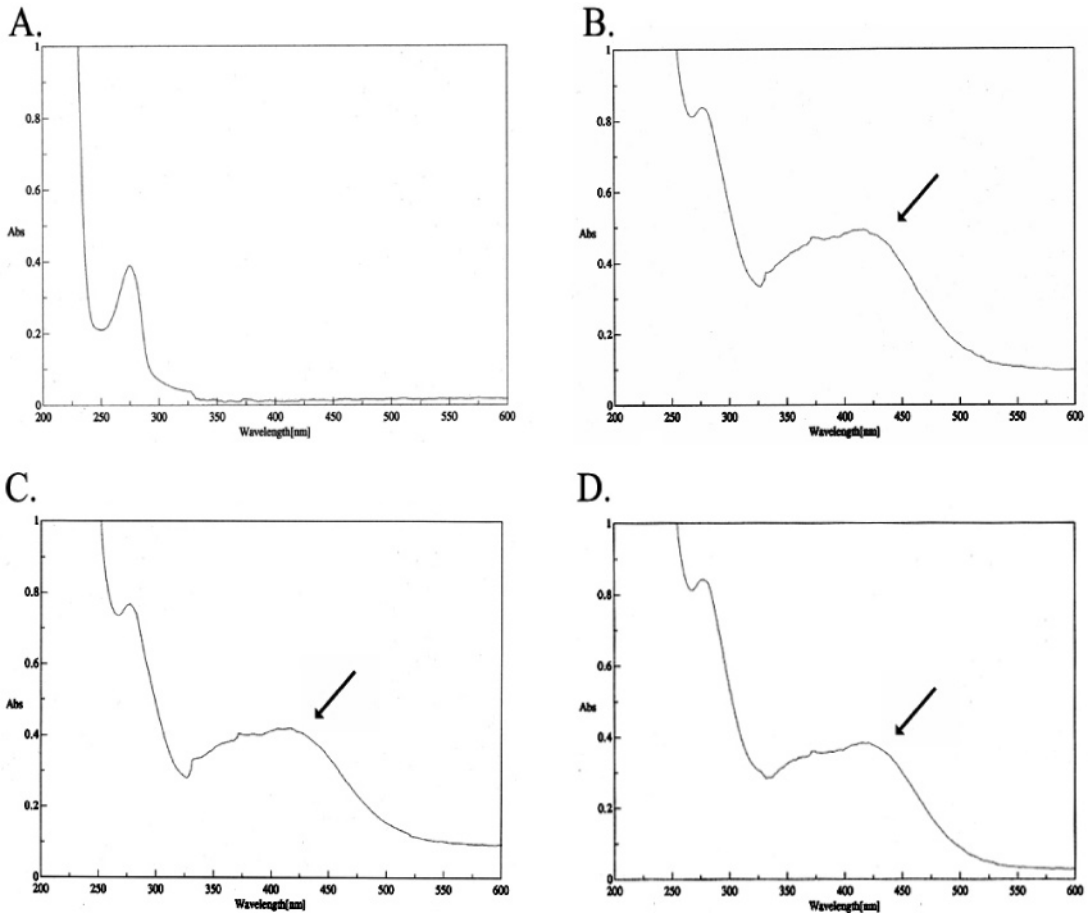

Figure 5. Interaction of trypsin inhibitor with peroxynitrite ($ONOO^-$). Tyrosine (200 μM) was incubated without (**A**) or with $ONOO^-$ (200 μM) (**B**) in 50 mM phosphate buffer at pH 7.0 at room temperature for 10 min, followed by a spectrophotometric scan from 200 to 600 nm. Trypsin inhibitor at 50 μg/mL (**C**) or 100 μg/mL (**D**) was incubated with $ONOO^-$ (200 μM) at 37 °C for 10 min. The arrow indicates a nitration peak at 400–450 nm.

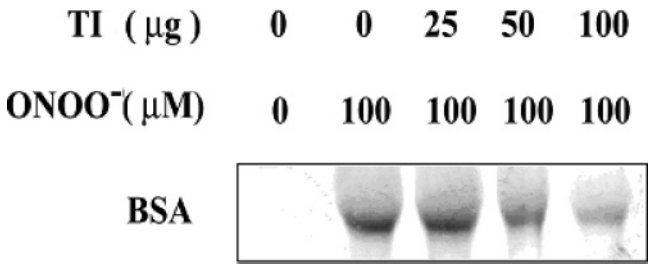

Figure 6. Effect of trypsin inhibitor on the nitration of BSA by $ONOO^-$. Trypsin inhibitor was added to BSA. The reaction samples were incubated with shaking at 25 °C for 1 h. After $ONOO^-$ was added, all samples were further incubated with shaking at 25 °C for 30 min.

nitration decreased, further suggesting its putative anti-$ONOO^-$ action in vivo.

MTT Assay for Cell Viability. The effects of TI on RAW 264.7 cell viability were determined by a MTT assay. TI alone did not exhibit cell cytotoxicity at the concentrations used for NO inhibitor compared to an LPS-treated control (data not shown).

Effect of TI on Preventing NO Production in RAW 264.7 Cells. The effect of TI on LPS-induced NO production in macrophage was examined. The cell culture medium was harvested, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method (**Figure 7**). Macrophages incubated with different concentrations of TI (0, 125, 250, 500, and 1000 μg/mL) together with LPS (1 μg/mL) for 24 h resulted in 0, 18, 35.6, 44.2, and 53.6% inhibition, respectively. TI inhibited LPS-induced nitrite production in macrophages in a concentration-dependent manner with an IC_{50} value of 932.8 ± 29.85 μg/mL. There was a significant

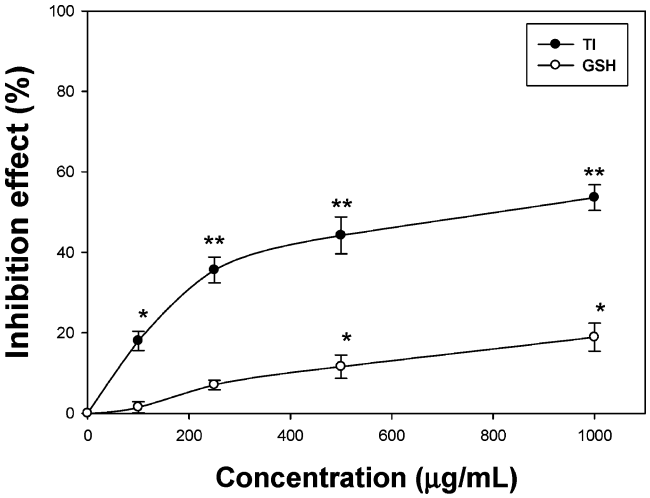

Figure 7. Inhibitory activities of trypsin inhibitor toward LPS-induced NO generation in macrophage RAW 264.7 cells. The cells were incubated for 24 h with 1 μg/mL LPS in the absence or presence of trypsin inhibitor (0, 125, 500, 100 μg/mL). Trypsin inhibitor was added 1 h before the incubation with LPS. Nitrite concentration in the medium was determined using Griess reagent. GSH was used as a positive control. Data are presented as mean ± SE for three different experiments performed in triplicate. $*$, $p < 0.05$; or $**$, $p < 0.01$ (unpaired t test), compared with control group.

inhibition on NO production in RAW 264.7 cells of groups treated with 25, 25, 100, and 150 μg/mL TI and glutathione ($p < 0.05$ or $p < 0.01$).

In conclusion, the tuber storage protein of sweet potato, TI, inhibited reactive nitrogen species both in vitro and ex vivo. TI caused a decrease of $ONOO^-$-mediated nitration of tyrosine through electron donation, and it also showed significant inhibition ability on nitration of BSA by $ONOO^-$ in a dose-dependent manner. TI might be a potentially effective NO and $ONOO^-$ scavenger useful for the prevention of the NO- and $ONOO^-$-involved diseases.

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