Running title: Inhibition of Reactive Nitrogen Species by Thioredoxin *h2*

Inhibition of Reactive Nitrogen Species *in Vitro* and *ex Vivo* by thioredoxin *h*2 from sweet potato 'Tainong 57' storage roots

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ABSTRACT

In this study, the ability of thioredoxin *h*2 (TRX *h*2) expressed in *E. coli* to scavenge •ON and $ONOO^-$ were investigated. The data obtained show that TRX *h*2 generated a dose-dependent inhibition on production of nitrite and superoxide radicals. TRX *h*2 also caused a dose-dependent inhibition of the oxidation of dihydrorhodamine 123 (DHR) by peroxynitrite. Spectrophotometric analyses revealed that TRX *h*2 suppressed the formation of $ONOO^-$-mediated tyrosine nitration through an electron donation mechanism. In further studies, TRX *h*2 also showed a significant ability of inhibiting nitration of bovine serum albumin (BSA) in a dose-dependent manner. *In vivo* TRX *h*2 inhibited LPS-induced nitrite production in macrophage in a concentration-dependent manner. The present study suggested that TRX *h*2 had an efficient reactive nitrogen species scavenging ability. TRX *h*2 might be a potential effective NO and $ONOO^-$ scavenger useful for the prevention of the NO and $ONOO^-$ involved diseases.

Keywords: Peroxynitrite; nitric oxide; superoxide; thioredoxin *h*2; sweet potato

1. 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, antimicrobial and antitumor activity. Recent studies indicate that NO may affect the enzymatic activities of several thiol rich DNA repair proteins e.g. DNA alkyl transferase, formamidopyrimidine-DNA glycosylase and the DNA ligase that play critical roles in the maintenance of the genome integrity (Wink, et al., 1991).

Peroxynitrite ($ONOO^-$) is formed by the reaction of NO with superoxide ($O_2\bullet^-$). $ONOO^-$ is a cytotoxic reactive species that can be generated by endothelial cells, neutrophils, and macrophages (Huang, Chu, Juang, & Wang, 2010). $ONOO^-$ is a relatively long-lived cytotoxicant with strong oxidizing properties toward various cellular constituents including sulfhydryls, lipids, amino acids, and nucleotides (Briviba, Klotz, & Sies, 1999). $ONOO^-$ can induce oxidation of thiol (-SH) groups on proteins, nitration of tyrosine, and lipid peroxidation that affect cell metabolism and signal transduction. Excessive formation of $ONOO^-$ may also be involved in several human diseases such as Alzheimer's disease, atherosclerosis and cancer (Bai, et al., 2010). Ascorbic acid, α-tocopherol, flavonoids, and polyhydroxyphenols, which are constituents of fruits, wines, teas, and green vegetables, were demonstrated to be effective antioxidants against $ONOO^-$ (Zou, Kim, Kim, Ceol, & Chung, 2002). To prevent the formation of $ONOO^-$, the $O_2\bullet^-$ level can be regulated by a number of enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase (Liu, Xu, & Cheng, 2008), whereas the NO level can be controlled by oxyhemoglobin and NO synthase (Briviba, Klotz, & Sies, 1999).

Thioredoxins, the ubiquitous small proteins with a redox active disulfide bridge,

are important regulatory elements in a number of cellular processes (Vianey-Liaud, Kobrehel, Sauvaire, Wong, & Buchanan, 1994). They all contain a distinct active site, WCGPC, which is able to reduce disulfide bridges of target proteins. Initially described as hydrogen carriers in ribonucleotide reduction in *E. coli*, they were found to serve as electron donors in a variety of cellular redox reaction (Bacon, Plumb, Howie, Beckett, Wang, & Bao, 2007). From genome sequencing data, a significant diversity of thioredoxin genes containing five different multigenic families (f, m, x, o and h) was observed (Mestres-Ortega & Meyer, 1999). Thioredoxin *h* is generally assumed to be cytosolic, which was supported by the absence of a transit peptide in the genes cloned for the isoforms from poplar (Gautier, Lullien-Pellerin, Lamotte-Guery, de Guirao, & Joudrier, 1998), germinating wheat seeds (Balmer & Buchanan, 2002) and barley seed proteome (Serrato, Crespo, Florencio, & Francisco, 2001).

Sweet potato is the fifth most important food crop in Taiwan. Sweet potato can attenuate oxidative damage, inflammation, aging and hypertension with its many antioxidant compounds, including polyphenolic compounds, flavonoids, and proteins (Huang, et al 2011). In our previously paper, we found that expressed sweet potato storage root recombinant thioredoxin *h2* (TRX *h*2) protein in *E. coli* with antioxidant activities (Huang**,** Chen, Hou, Lin, & Lin, 2004). However, no data relative to the NO and $ONOO^-$ scavenging activities of TRX *h*2 were found in the literature. Thus, the aim of this study was to evaluate the scavenging capacity of TRX *h*2 for the NO and $ONOO^-$ in order to understand their inhibition of reactive nitrogen species *in vitro* and *ex vivo*.

2. Materials and Methods

2.1. Materials.

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

2.2. Expression of Thioredoxin h2 in E. coli.

Thioredoxin *h*2 (TRX *h*2) was expressed in *E. coli*. The coding sequence was amplified from TRX *h*2 cDNA using an oligonucleotide (5´-GAG AGG ATC CAA TGG GAG GGG CT-3´), with a *BamH* I site (underlined) at the putative initial Met residue, and an oligonucleotide (5´- ATT TGA AGC TTG ATT GAT GCT -3´), with a *Hind* III site at the 3´ end (Huang, Chen, Hou, & Lin, 2004). The PCR fragment was subcloned in pGEM T-easy vector. The plasmid was then digested with *BamH* I and *Hind* III and subcloned in pQE32 expression vector (QIAexpress expression system, Qiagen, USA). The resulting plasmid, termed pQE-TRX *h*2, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-NTA columns (Qiagen), according

to the manufacturer´s instructions.

2.3. Scavenging Effect on Nitric Oxide.

The scavenging effect of TRX *h*2 on nitric oxide was measured according to the method of Marcocci et al. (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994). Purified TRX *h*2 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℃ 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 diazotization 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.

2.3.1. Measurement of superoxide radical-scavenging activity.

Effects of TRX *h*2 and GSH on superoxide radical were determined by the PMS-NADH superoxide generating system (Ardestani & Yazdanparast, 2007). TRX *h*2 or GSH (0-150 μg/mL) was added to a solution mixture that contained 200 μM NBT (nitro blue tetrazolium), 624 μM NADH (β-nicotinamide adenine dinucleotide) and 80 μM PMS (phenazin methosulfate) 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) x 100.

2.3.2. Protecting dihydrorhodamine 123 from peroxynitrite-mediated oxidation by thioredoxin h2.

The protection of dihydrorhodamine 123 (DHR) against peroxynitrite-mediated oxidation was detected according to the method of Kooy & Royall (1994). The total 180 μL reaction mixture included different amounts of TRX *h*2 (1–4 mg/mL), 0.9 mM DHR and 5 μL of peroxynitrite in 50 mM phosphate buffer (pH 7.4) containing 90 mM NaCl. After 5 min 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 TRX *h*2.

2.3.3. Inhibition of $ONOO^-$-Mediated Tyrosine Nitration by thioredoxin h2.

The ability of TRX *h*2 to inhibit the formation of 3-nitrotyrosine was measured as the index of TRX *h*2 inhibition of tyrosine nitration utilizing spectrophotometric analysis and western blot analysis.

(1) Spectrophotometric Analysis of Nitrated Proteins. Tyrosine (200 μM) was reacted with $ONOO^-$ (200 μM) in the presence of varying concentrations of TRX *h*2 (50 or 100 μg/mL). The formation of 3-nitrotyrosine was determined with a UV/visible spectrophotometer. Tyrosine was monitored at 275 nm while 3-nitrotyrosine formation was monitored at 430 nm. The disappearance of 3-nitrotyrosine peak at 430 nm in the

159 presence of TRX *h*2 was taken as an indication of the inhibition by TRX *h*2.

160 (2) Western blot analysis. A 2.5 μL aliquot of TRX *h*2 was added to 95 μL of BSA (0.5
161 mg of protein/mL). The mixed samples were incubated with shaking at 20℃ for 1 h.
162 After 1 h incubation, 2.5 μL of $ONOO^-$ (100 μM) was added. The samples were then
163 incubated for 30 min at 20℃ with shaking. After reaction, the samples prepared in gel
164 loading buffer [pH 6.8; 0.125 M tris-(hydroxymethyl)aminomethane (Tris), 4% mass per
165 volume (m/v) of sodium dodecyl sulfate (SDS), 20% m/v glycerol, 10% m/v
166 2-mercaptoethanol, and 0.2% m/v bromophenol blue] in a ratio of 1:1 were boiled for 5
167 min. Twenty microliters of each sample were separated on an SDS-polyacrylamide
168 mini-gel (10% for BSA) at 100 V and transferred to a poly(vinylidene fluoride)
169 membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA, USA).
170 The membrane was immediately placed into a blocking solution (10% m/v skim milk
171 powder in TBS-Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM
172 Tween-20, pH 7.5) at 4℃ overnight. The membrane was washed in TBS-Tween buffer
173 for 30 min and then incubated with a mouse monoclonal anti-nitrotyrosine antibody
174 (0.5% m/v skim milk, diluted 1:2000 in TBS-Tween buffer) at room temperature for 2 h.
175 After four 10-min washings in TBS-Tween buffer, the membrane was reacted with rabbit
176 alkaline phosphatase-conjugated secondary antibody against mouse antibody (0.1% m/v
177 skim milk, diluted 1:1000 in TBS-Tween buffer) at room temperature for 2 h. After four
178 10- min washings in TBS-Tween buffer, blue color was developed using NBT (nitro blue
179 tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Sigma). A set of prestained
180 blue protein markers was used for molecular weight determination.

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2.4. Cell culture.

A murine macrophage cell line RAW264.7 (BCRC No. 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℃ 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).

2.4.1. Cell viability.

RAW264.7 cells (2 x 10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with TRX *h*2 samples in the presence of 1 μg/mL LPS (lipopolysaccharide) for 24 h. Then, the cells were washed twice with DPBS and incubated with 110 μL of 0.5 mg/mL MTT (3-[4, 5-dimethylthiazol- 2-yl]-2, 5- diphenyltetrazolium bromide) for 2 h at 37℃ testing for cell viability. The medium was then discarded and 100 μL dimethylsulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

2.4.2. Effect of thioredoxin h2 on preventing nitric oxide production in RAW264.7 cells.

Cells were plated at a density of 2 x 10^5 cells/mL in a 96-well plate. After overnight incubation, TRX *h*2 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 Griess reagent (50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% naphthylethylenediamine dihydrochloride in water) were added. Absorbance at 570 nm was determined using a microplate reader (Multiskan Spectrum, Thermo Labsystem, USA). 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 (%) = [($\text{Absorbance}_{\text{positive}}$ - $\text{Absorbance}_{\text{sample}}$)/($\text{Absorbance}_{\text{positive}}$ – $\text{Absorbance}_{\text{negative}}$)] x 100. $\text{Absorbance}_{\text{positive}}$, $\text{Absorbance}_{\text{negative}}$ and $\text{Absorbance}_{\text{sample}}$ represented absorbance of cultural media containing LPS, without LPS and TRX *h*2 sample with LPS.

2.5. 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 $p < 0.05$.

3. Results and Discussion

3.1. Expression of thioredoxin h2 in E. coli.

To express sweet potato TRX *h*2 in *E. coli*, the coding sequence of TRX *h*2 was subcloned in a pQE-32 expression vector so that sweet potato TRX *h*2 was produced with a 6x His-tag at the N-terminus. SDS-PAGE analysis of crude extracts from transformed *E. coli* (M15) showed a high level of a polypeptide with the expected molecular mass (ca. 14 kDa). This polypeptide was found as a soluble protein in the supernatant and was

absent in protein extracts obtained from *E. coli* transformed with pQE-32 vector. The expressed protein was purified from crude extracts by Ni^{2+}-chelate affinity chromatography, which yielded highly purified His-tagged thioredoxin (Fig. 1).

3.2. Scavenging Effect on Nitric Oxide.

NO is a defense molecule with cytotoxic, microbiocidal, and microbiostatic activities. In the present study, the scavenging effect of TRX *h*2 on NO was investigated. Sodium nitroprusside (SNP) is known to decompose in PBS solution to produce NO. Nitric oxide, under aerobic conditions, reacts with oxygen to form nitrate and nitrite, which can be determined using Griess reagent (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994). As shown in Fig. 2A, TRX *h*2 in the range of 25 ~ 150 μg/mL generated a dose-dependent inhibition on nitrite production. GSH was used as a positive control. The nitrite levels each at TRX *h*2 and GSH in a concentration of 150 μg/mL were 13.47±0.33 and 5.45 ± 0.22 μM, respectively, indicating that TRX *h*2 had a scavenging activity on NO. The nitrite levels of control (SNP-induced oxidative stress) was about 17.57 ± 0.36 μM.

3.3. Superoxide radical-scavenging activity

Superoxide ($\bullet O_2^-$), 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 (Halliwell & Gutteridge, 1999). According to the data of Fig. 2B, the marked scavenging effect of TRX *h*2 on superoxide radical was in a dose-dependent manner. The superoxide radical-scavenging activity of TRX *h*2 and GSH

each at a concentration of 150 μg/mL were 23.1 ± 2.10 and 26.4 ± 3.20%. These results reveal that TRX *h*2 is a potent scavenger of superoxide radical and has SOD-like ability. The most important findings from the present study are that the active component, TRX *h*2, not only directly scavenged $ONOO^-$, but also was involved in the inhibition of $\bullet O_2^-$ and •NO radical formation.

3.4. Protecting dihydrorhodamine 123 from peroxynitrite-mediated oxidation by thioredoxin h2

TRX *h*2 caused a dose-dependent inhibition of the oxidation of dihydrorhodamine 123 (DHR) by peroxynitrite (Fig. 3). The inhibition of DHR oxidation by TRX *h*2 and GSH at a concentration of 4,000 μg/mL were 79.2 ± 5.21 and 90.3 ± 4.20%. The resulting IC_{50} value was 919.6 ± 38.62 μg/mL, and the positive control GSH, provided an IC_{50} of 946.9 ± 35.98 μg/mL.

3.5. Effect of thioredoxin h2 on $ONOO^-$-Mediated 3-Nitrotyrosine Formation.

Tyrosine was incubated without (Fig. 4A) or with $ONOO^-$ (Fig. 4B), followed by a spectrophotometric scan from 200 to 600 nm. Tyrosine undergoes nitration by $ONOO^-$ because there is a peak around 430 nm. TRX *h*2 50 μg/mL (Fig. 4C) or 100 μg/mL (Fig. 4D) was incubated with tyrosine before the addition of $ONOO^-$. TRX *h*2 prevented tyrosine from nitration as shown by the disappearance of the nitrotyrosine peak at 430 nm (Fig. 4C and 4D) in a dose dependent manner, implying that TRX *h*2 inhibited the

formation of 3-nitrotyrosine.

In the present study we exposed TRX *h*2 to $ONOO^-$ to determine whether the TRX *h*2 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 TRX *h*2 with $ONOO^-$ caused a decreased peak at 430 nm, which gave further evidence supporting the possibility of an electron-donation reaction between TRX *h*2 and $ONOO^-$. The toxicity of $ONOO^-$ can be attributed to nitration of tyrosine and tryptophan residues and subsequent alterations of their functionalities (Sies, Klotz, Sharov, Assmann, & Briviba, 1998). Plant food-derived antioxidants and active principles such as flavonoids, β-carotene and other carotenoids, vitamin C, vitamin E, and tea polyphenols are important dietary antioxidant substances (Afzal, Al-Hadidi, Menon, Pesek, & Dhami, 2001) that may provide efficient $ONOO^-$ scavenging. This result suggested that TRX *h*2 directly blocked the formation of 3-nitrotyrosine by the action of $ONOO^-$ and possibly reduced the availability of $ONOO^-$.

3.6. Effect of thioredoxin h2 on Nitration of BSA.

Using mouse anti-3-nitrotyrosine antibody in western blot, we analyzed the ability of TRX *h*2 to suppress $ONOO^-$-mediated tyrosine nitration in common biological materials, such as BSA. For this purpose, TRX *h*2 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 TRX *h*2 at concentrations of 0, 25, 50, and 100 μg/mL attenuated the nitration of BSA in a dose-dependent manner, as shown in Fig. 5A and 5B.

$ONOO^-$ could induce functional damage in some biological molecules, such as BSA

293 and LDL, via nitration of tyrosine residues (Liao, Guo, & Lin, 2011). Nitration of Protein
294 tyrosines by $ONOO^-$ may interfere with phosphorylation/dephosphorylation signaling
295 pathways and alter enzyme functions (Yen & Lai, 2003). Nitrotyrosine has been reported
296 in various hypertensive disorders, neurological disorders, and chronic renal disease
297 (Vaziri, Liang, & Ding, 1999). In this study evidence from western blot analysis showed
298 that TRX *h*2**,** even at a concentration of 50 µg/mL, could markedly reduce the
299 nitrotyrosine content present in BSA. With higher TRX *h*2 concentrations, nitration of
300 BSA decreased, further suggesting its putative anti-$ONOO^-$ action *in vivo*.
301
302 *3.7. MTT assay for Cell viability*
303 The effects of TRX *h*2 on RAW 264.7 cell viability were determined by a MTT assay.
304 TRX *h*2 alone did not exhibit cell cytotoxicity at the concentrations used for NO inhibitor
305 compared to an LPS-treated control (Fig. 6A).
306
307 *3.7.1. Effect of thioredoxin h2 on preventing nitric oxide production in RAW 264.7 cells*
308 The effect of TRX *h*2 on LPS-induced NO production in macrophage RAW 264.7
309 cells was examined. The cell culture medium was harvested and the concentration of
310 accumulated nitrite, the oxidative product of NO, was determined by Griess method (Fig.
311 6B). Macrophages incubated with different concentrations of TRX *h*2 (0, 125, 250, 500,
312 and 1,000 µg/mL) (0, 12.5, 25, 50, and 100 µg/well) together with LPS (1 µg/mL) for 24
313 h resulted in 0, 17, 25, 39.1 and 55.6% inhibition of nitrite production, respectively. An
314 IC_{50} value of 899.3 ± 2.52 µg/mL was found for TRX *h*2.
315 In conclusion, in this work we reported for the first time that TRX *h*2 has NO and

316 $ONOO^-$ scavenging activities. The protective effect of TRX *h*2 on nitration of tyrosine
317 and bovine serum albumin (BSA) by $ONOO^-$ is very good. Our results suggest that TRX
318 h2 may be a potential selective regulator of $ONOO^-$-mediated diseases via its direct
319 scavenging activity.

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