JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Active Recombinant Thioredoxin *h* Protein with Antioxidant Activities from Sweet Potato (*Ipomoea batatas* [L.] Lam Tainong 57) Storage Roots

DONG-JIANN HUANG,† HSIEN-JUNG CHEN,§ WEN-CHI HOU,# CHUN-DER LIN,† AND YAW-HUEI LIN*,†

Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan; Department of Horticulture, Chinese Culture University, Taipei 111, Taiwan; and Graduate Institute of Pharmacognosy Science, Taipei Medical University, Taipei 110, Taiwan

Recombinant thioredoxin *h* (Trx2) overproduced in *Escherichia coli* (M15) was purified by Ni^{2+}-chelated affinity chromatography. The molecular mass of Trx2 is ~1.4 kDa as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Total antioxidant status, 1,1-diphenyl-2-picrylhydrazyl (DPPH) staining, reducing power method, Fe^{2+}-chelating ability, ferric thiocyanate (FTC) method, and protection of calf thymus DNA against hydroxyl radical-induced damage were studied. The thioredoxin *h* protein with a concentration of 12.5 mg/mL exhibited the highest activity (expressed as 0.37 ± 0.012 mM ABTS• radical cation being cleared) in a total antioxidant status test. In the DPPH staining thioredoxin *h* appeared as white spots when it was diluted to 50 mg/mL (a final amount of 15 μg). Like the total antioxidant status, the reducing power, Fe^{2+}-chelating ability, FTC activity, and protection against hydroxyl radical-induced calf thymus DNA damage were found with the thioredoxin *h* protein. It was suggested that thioredoxin *h* might contribute to its antioxidant activities against hydroxyl and peroxyl radicals.

KEYWORDS: Sweet potato; thioredoxin *h*; cDNA sequence; gene expression; recombinant protein; antioxidant

INTRODUCTION

It is commonly accepted that in a situation of oxidative stress, reactive oxygen molecules such as superoxide ($O_2^{\bullet-}$, $HOO^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radicals are generated. The reactive oxygen molecules play an important role in the degenerative or pathological processes of various serious diseases, such as aging (*1*), cancer, coronary heart disease, Alzheimer's disease (*2*−*5*), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (*6*). The use of traditional medicine is widespread, and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several antiinflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity (*7*−*9*). In the search for sources of natural antioxidants, substances such as echinacoside in *Echinaceae* root (*10*), anthocyanin (*11*), phenolic compounds (*12*), water extracts of roasted *Cassia tora* (*13*), and whey proteins (*14*−*16*) have been extensively studied for their antioxidant activity and radical-scavenging activity.

Thioredoxins, the ubiquitous small proteins with a redox active disulfide bridge, are important regulatory elements in a number of cellular processes (*17*, *18*). 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 *Escherichia coli*, they were found to serve as electron donors in a variety of cellular redox reaction (*19*).

From genome sequencing data, a significant diversity of thioredoxin genes containing five different multigenic families (*f*, *m*, *x*, *o*, and *h*) was observed (*20*−*22*). The ferredoxin−thioredoxin system (thioredoxins *f* and *m*) has been proved to regulate several enzymatic activities associated with photosynthetic CO_2 assimilation in chloroplasts. Thioredoxin *x* contains a transit peptide similar to those required for chloroplast and mitochondria targeting; however, its function is not clearly defined (*20*). A new type of plant mitochondrial thioredoxin *o* was also shown to regulate the activities of several mitochondrial proteins by disulfide bond reduction (*23*).

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 tobacco (*24*, *25*), *Arabidopsis* (*26*, *27*), *Triticum aestivum* (*28*), poplar (*29*), germinating wheat seeds (*30*), and barley seed proteome (*31*). Moreover, the existence of several forms of thioredoxin *h* detected in spinach

* Corresponding author [fax 886 (2) 2782-7954; telephone 886 (2) 2789-9590, ext. 320; e-mail boyhlin@ccvax.sinica.edu.tw].
† Academia Sinica.
§ Chinese Culture University.
# Taipei Medical University.

10.1021/jf0498618 CCC: $27.50
Published on Web 06/26/2004

leaves (*32*), wheat flour (*33*), and rice phloem sap (*34*) supports the view that most higher plants possess multiple and divergent thioredoxin genes (*27*).

In humans the direct antioxidant properties of thioredoxin 1 include removal of hydrogen peroxide (*35*). Thioredoxin 1 has also been reported to be an efficient electron donor to human plasma glutathione peroxidase (*36*). Thioredoxin 1 has been suggested to have an antioxidant role in plasma (*37*).

The objectives of this work were to investigate the antioxidant property of thioredoxin *h* from sweet potato in comparison with chemical compounds such as butylated hydroxytoluene (BHT), reduced glutathione, or ascorbate in a series of in vitro tests.

MATERIALS AND METHODS

Expression of Thioredoxin *h* in *E. coli*. Thioredoxin 2 (Trx 2) was expressed in *E. coli*. The coding sequence was amplified from Trx2 cDNA using an oligonucleotide (5'-GAG AGG ATC CAA TGG GAG GGG CT-3'), with a *Bam*HI site (underlined) at the putative initial Met residue, and an oligonucleotide (5'-ATT TGA AGC TTG ATT GAT GCT-3'), with a *Hin*dIII site at the 3' end (*38*). The PCR fragment was subcloned in pGEM T-easy vector, and the plasmid was then digested with *Bam*HI and *Hin*dIII and subcloned in pQE32 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-Trx2, 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.

Protein Staining on 15% Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels. Thioredoxin was detected on 15% SDS-PAGE gels (*39*). Samples treated with sample buffer and β-mercaptoethanol (2-ME) with a final concentration of 14.4 mM were heated at 100 °C for 5 min before 15% SDS-PAGE.

Measurement of Total Antioxidant Status. The total antioxidant status of the thioredoxin *h* protein was measured using the total antioxidant status assay kit (Calbiochem Corp.) according to the manufacturer's instructions. The assay relies on the antioxidant ability of the protein to inhibit the oxidation of 2,2′-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) to $ABTS^+$ by metmyoglobin. The amount of $ABTS^+$ produced is monitored by reading the absorbance at 600 nm. Under these reaction conditions, the antioxidant ability of thioredoxin *h* protein suppresses the absorbance at 600 nm in proportion to its concentration. The final antioxidant capacity of thioredoxin *h* protein was calculated by the following formula: concentration of $ABTS^+$ being cleared (mmol/L) = [factor × (absorbance of blank − absorbance of sample)]; factor = [concentration of standard/(absorbance of blank − absorbance of standard)].

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining. An aliquot (3 μL) of each diluted sample of the thioredoxin *h* was carefully loaded on a 20 cm × 20 cm thin-layer chromatography (TLC) layer (silica gel 60 F254; Merck) and allowed to dry (3 min). Drops of each sample were loaded in order of decreasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (*40*). The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. Then the excess of solution was removed with a tissue paper, and the layer was dried with a hair dryer blowing cold air. The stained silica layer revealed a purple background with white spots at the location where radical scavenger capacity presented. The intensity of the white color depends on the amount and nature of radical scavenger present in the sample.

Determination of Antioxidant Activity by Reducing Power Measurement. The reducing powers of the thioredoxin *h* and glutathione were determined according to the method of Yen and Chen (*41*). Thioredoxin *h* (0, 18.8, 37.5, 75, 150, and 300 μg) and glutathione were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, during which time ferricyanide was reduced to ferrocyanide. Then an equal volume of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 6000 rpm for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2, and the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (Prussian Blue) formed. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

Determination of Antioxidant Activity by Fe^{2+}-Chelating Ability. The Fe^{2+}-chelating ability was determined according to the method of Decker and Welch (*42*). The Fe^{2+} was monitored by measuring the formation of ferrous iron−ferrozine complex at 562 nm. Thioredoxin *h* (0, 6.25, 12.5, 25, 50, and 100 μg) was mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10:1:2. The mixture was shaken and left to stand at room temperature for 10 min. The absorbance of the resulting solution at 562 nm was measured. The lower the absorbance of the reaction mixture, the higher the Fe^{2+}-chelating ability. The capability of the sample to chelate the ferrous iron was calculated using the following equation:

$$\text{scavenging effect (\%)} = [1 - \text{ABS}_{\text{sample}}/\text{ABS}_{\text{control}}] \times 100$$

Determination of Antioxidant Activity by the Ferric Thiocyanate (FTC) Method. The FTC method was adapted from the method of Osawa and Namiki (*43*). Samples (20 mg/mL) dissolved in 4 mL of 99.5% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer, pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in a screw-cap container at 40 °C in the dark. Then, to 0.1 mL of this solution was added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red color [$Fe(SCN)^{2+}$, Fe^{3+} was formed after lipid peroxide was produced and Fenton reaction occurred] was measured every 24 h until the day when the absorbance of the control reached the maximum value. The inhibition of linoleic acid peroxidation was calculated as % inhibition = 100 − [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

Protection of Thioredoxin *h* against Hydroxyl Radical-Induced Calf Thymus DNA Damage. The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*44*). The 15 μL reaction mixture containing thioredoxin *h* (2.5, 5, 10, or 20 mg/mL), 5 μL of calf thymus DNA (1 mg/mL), 18 mM $FeSO_4$, and 60 mM hydroxygen peroxidewere incubated at room temperature for 15 min. Then 2 μL of 1 mM EDTA was added to stop the reaction. Blank test contained only calf thymus DNA, and the control test contained all components except thioredoxin *h*. After agarose electrophoresis, the treated DNA solutions were stained with ethidium bromide and examined under UV light.

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

RESULTS AND DISCUSSION

Measurement of Total Antioxidant Status. To express sweet potato thioredoxin *h* in *E. coli*, the coding sequence of Trx2 (**Figure 1**) was subcloned in a pQE-32 expression vector so that sweet potato thioredoxin *h* was produced with a 6× 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 (~14 kDa). This polypeptide was found as a soluble protein in the supernatant (**Figure 1**, lane 2) and was absent in protein extracts obtained from *E. coli* transformed with the pQE-32 vector (**Figure 1**, lane 1). The expressed protein was purified from crude extracts by Ni^{2+}-chelate affinity chromatography, which yielded highly purified His-tagged thiredoxin *h* (**Figure 1**, lane 3).

The total antioxidant status of the thioredoxin *h* protein was measured using the total antioxidant status assay kit (**Figure 2**). Different amounts of thioredoxin *h* protein did not show a

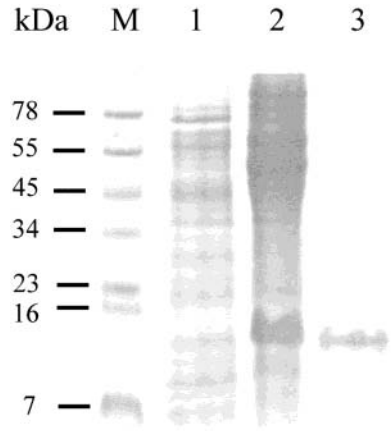

Figure 1. SDS-PAGE analysis of purified recombinant sweet potato thioredoxin *h*. Crude extracts from *E. coli* (M15) transformed with pQE32 (lane 1) or with pQE32-TRX2 (lane 2) were analyzed by 15% (w/v) SDS-PAGE with 5 μg of protein, and then the gel was stained with Coomassie blue G-250. Molecular masses of standard proteins are indicated at the left. His-tagged Trx2 was purified by Ni^{2+}-chelated affinity chromatography (lane 3). The experiments were done twice, and a representative one is shown.

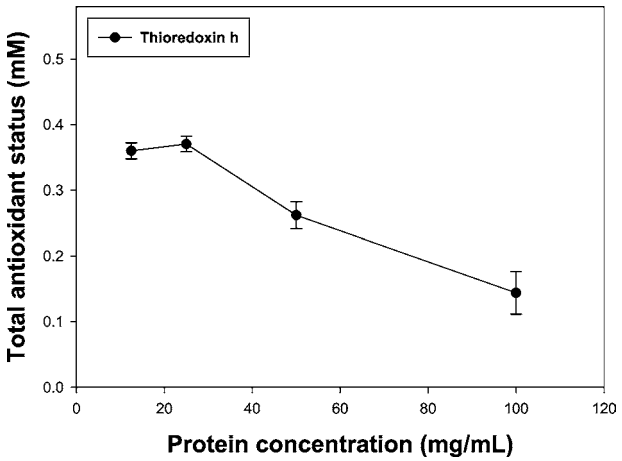

Figure 2. Total antioxidant activity of recombinant thioredoxin *h* from sweet potato, as measured by the total antioxidant status assay. Absorbance values represent triplicates of different samples analyzed.

dose-dependent total antioxidant activity within the applied concentrations (0, 12.5, 25, 50, and 100 mg/mL). At 12.5 mg/mL, thioredoxin *h* displayed the highest total antioxidant status (expressed as 0.37 ± 0.012 mM $ABTS^{\bullet}$ radical cation being cleared). At 100 mg/mL, thioredoxin *h* displayed the lowest total antioxidant status (0.144 ± 0.0326 mM $ABTS^{\bullet}$ radical cation being cleared).

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining. Antioxidant capacity of expressed sweet potato thioredoxin *h* was eye-detected semiquantitatively by a rapid DPPH staining method in TLC. Each diluted sample was applied as a dot on a TLC layer that was then stained with DPPH solution (**Figure 3**). This method is typically based on the inhibition of the accumulation of oxidized products, because the generation of free radicals is inhibited by the addition of antioxidants and scavenging the free radicals shifts the end point. The appearance of a white color spot versus a purple background has a potential value for the indirect evaluation of antioxidant capability of the expressed thioredoxin *h* in the dot blots (*40*, *45*). Fast-reacted and strong intensites of white spots appeared up to the dilution of 50 mg of thioredoxin *h*/mL (with

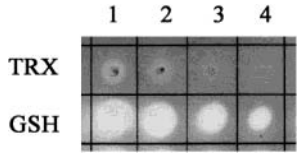

Figure 3. Dot blot assay of recombinant thioredoxin *h* from sweet potato on a silica sheet stained with a DPPH solution in methanol. For recombinant thioredoxin *h* extract, dots from left to right, respectively, each contained 3 μL of sample of 100, 50, 25, and 12.5 mg/mL. For GSH, dots from left to right, respectively, each contained 3 μL of sample of 10, 5, 2.5, 12.5, and 6.25 mg/mL.

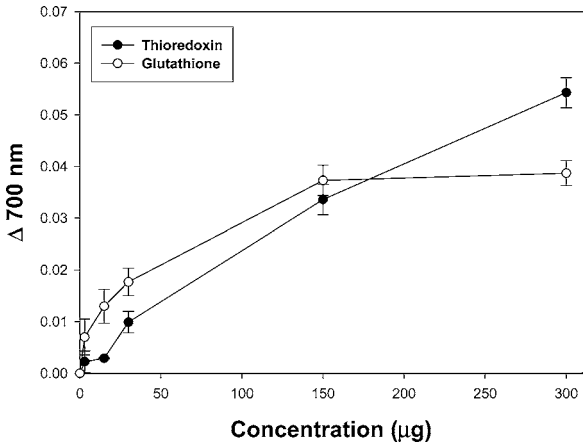

Figure 4. Antioxidative activities of recombinant thioredoxin *h* from sweet potato, as measured by the reducing power method. Absorbance values represent triplicates of different samples analyzed. Bars with different letters are significantly different ($p < 0.05$).

an absolute amount of 15 μg). The reduced glutathione was used as a positive control. Initial faint spots appeared, and 1 h later weak spots cound be observed in sample row. This explains why we got the negative results of DPPH-scavenging capacity of thioredoxin *h* in the spectrophotometric assay. Free cysteine residues in whey proteins were reported to have antioxidant activities (*46*, *47*). These findings suggest that cysteine residues in sweet potato thioredoxin *h* might also participate in antiradical activity.

Measurement of Reducing Power. We investigated the $Fe^{3+}-Fe^{2+}$ transformation in the presence of thioredoxin *h* to measure its reducing capacity. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*48*). The antioxidant activity of putative antioxidants has been attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (*49*). The reducing power of thioredoxin *h* is shown in **Figure 4** with reduced glutathione serving as a positive control. The reducing activity of thioredoxin *h* exhibited a dose dependence (significant at $p < 0.05$) within applied concentrations (0, 18.8, 37.5, 75, 150, and 300 mg/mL).

Measurements of Fe^{2+}-Chelating Ability. The metal-chelating capacities of thioredoxin *h* and standard antioxidants were determined by assessing their ability to compete with ferrozine for the ferrous ions. The Fe^{2+}-chelating ability of the thioredoxin *h* with a concentration-dependent mode is shown in **Figure 5**. EDTA was used as a positive control. The Fe^{2+}-chelating ability of thioredoxin *h* was lower than that of EDTA, and this difference was statistically significant ($p < 0.05$). The doses of 25, 50, and 100 μg of thioredoxin h exhibited 9.6,

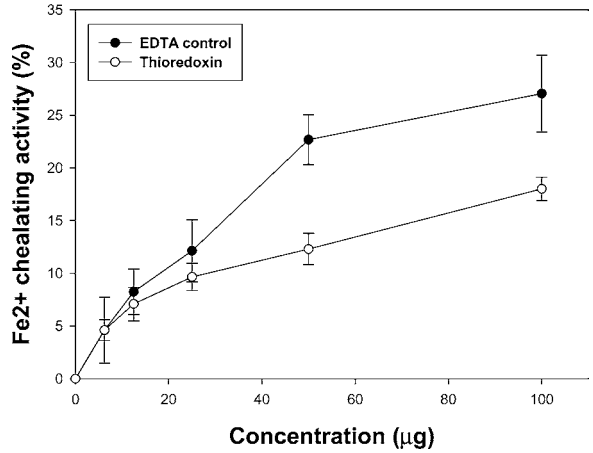

Figure 5. Antioxidative activities of recombinant thioredoxin *h* from sweet potato, as measured by the Fe^{2+}-chelating ability method. Absorbance values represent triplicates of different samples analyzed. Bars with different letters are significantly different ($p < 0.05$).

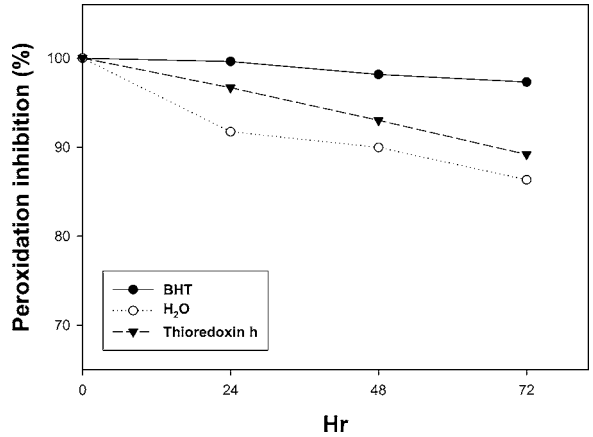

Figure 6. Inhibition of linoleic peroxidation by recombinant thioredoxin *h* from sweet potato, as measured by the FTC method. Absorbance values represent triplicates of different samples analyzed.

12.3, and 18.0% iron-binding capacities, respectively. On the other hand, the doses of 25, 50, and 100 μg of EDTA exhibited 12.1, 22.7, and 27.0% iron-binding capacities, respectively. Values obtained from **Figure 5** demonstrate that the action of thioredoxin *h*, as a peroxidation protector, may be mainly due to its iron-binding capacity.

Ferric Thiocyanate Method. Low-density lipoprotein (LDL) peroxidation has been reported to contribute to atherosclerosis development (*50*). Therefore, delay or prevention of LDL peroxidation is an important function of antioxidants. **Figure 6** shows the time course plots for the antioxidative activity of the thioredoxin *h* from sweet potato, butylated hydroxytoluene (BHT), and H_2O by the FTC method. BHT was used as a positive control and H_2O as a negative control. The results indicate that thioredoxin *h* has antioxidative activity. Thioredoxin *h* may act as a LDL peroxidation inhibitor (significant at $p < 0.05$).

Protection of Thioredoxin *h* against Hydroxyl Radical-Induced Calf Thymus DNA Damage. Free radicals could damage macromolecules in cells, such as DNA, proteins, and lipids in membranes (*51*). **Figure 7** shows that thioredoxin *h* protected calf thymus DNA against hydroxyl radical-induced damages. Compared to the blank test and control test, it was found that the thioredoxin *h* added above 5 mg/mL (the final absolute amount of 25 μg) could protect calf thymus DNA against hydroxyl radical-induced damages during 15-min reactions.

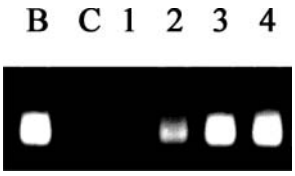

Figure 7. Protection against hydroxyl radical-induced calf thymus DNA damage by thioredoxin *h*. Thioredoxin *h* was added at a final concentration of 0, 2.5, 5, 10, or 20 mg/mL, respectively, from lane 1 to 4. Only calf thymus DNA was used for blank test (B), and the control test was without thioredoxin *h* (C).

In conclusion, the results from in vitro experiments, including total antioxidant status assay (**Figure 2**), DPPH staining (**Figure 3**), reducing power method (**Figure 4**), Fe^{2+}-chelating ability (**Figure 5**), FTC method (**Figure 6**), and hydroxyl radical-induced calf thymus DNA damage (**Figure 7**), demonstrated that the expressed thioredoxin *h* of sweet potato has various antioxidant activities. The measurement of total antioxidant activity is not just detection of free radicals. Thioredoxin *h* can serve as an electron donor in a variety of cellular redox reactions or during removal of hydrogen peroxide. Hence, thioredoxin *h* may contribute significantly to change the redox states and as a potent antioxidant against hydroxyl and peroxyl radicals when people consume sweet potato. The ex vivo or in vivo antioxidant activity of thioredoxin *h* should be examined soon.

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Received for review January 27, 2004. Revised manuscript received May 18, 2004. Accepted May 24, 2004.

JF0498618