

# Monothiol Glutaredoxin cDNA from *Taiwanofungus camphorata*: A Novel CGFS-type Glutaredoxin Possessing Glutathione Reductase Activity

Chuian-Fu Ken,<sup>S,||</sup> I-Jing Chen,<sup>†,||</sup> Chao-Ting Lin,<sup>S,||</sup> Shiu-Mei Liu,<sup>#</sup> Lisa Wen,<sup>†,||</sup> and Chi-Tsai Lin<sup>\*,†</sup>

<sup>†</sup>Institute of Bioscience and Biotechnology and Center for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

<sup>S</sup>Institute of Biotechnology, National Changhua University of Education, Changhua, Taiwan

<sup>#</sup>Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan

<sup>†</sup>Department of Chemistry, Western Illinois University, 1 University Circle, Macomb, Illinois 61455-1390, United States

**ABSTRACT:** Glutaredoxins (Grxs) play important roles in the redox system via reduced glutathione as a reductant. A TcmonoGrx cDNA (1039 bp, EU158772) encoding a putative monothiol Grx was cloned from *Taiwanofungus camphorata* (formerly named *Antrodia camphorata*). The deduced amino acid sequence is conserved among the reported monothiol Grxs. Two 3-D homology structures of the TcmonoGrx based on known structures of human Grx3 (pdb: 2DIY\_A) and *Mus musculus* Grx3 (pdb: 1WIK\_A) have been created. To characterize the TcmonoGrx protein, the coding region was subcloned into an expression vector pET-20b(+) and transformed into *E. coli* C41(DE3). The recombinant His6-tagged TcmonoGrx was overexpressed and purified by Ni<sup>2+</sup>-nitrilotriacetic acid Sepharose. The purified enzyme showed a predominant band on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The enzyme exhibited glutathione reductase (GR) activity via dithionitrobenzoate (DTNB) assay. The Michaelis constant ( $K_M$ ) values for GSSG and NADPH were 0.064 and 0.041 mM, respectively. The enzyme's half-life of deactivation at 60 °C was 10.5 min, and its thermal inactivation rate constant ( $k_d$ ) was  $5.37 \times 10^{-2} \text{ min}^{-1}$ . The enzyme was active under a broad pH range from 6 to 8. The enzyme retained 50% activity after trypsin digestion at 37 °C for 40 min. Both mutants C<sup>40</sup>→S<sup>40</sup> and C<sup>165</sup>→S<sup>165</sup> lost 40–50% GR activity, whereas the mutant S<sup>168</sup>→C<sup>168</sup> showed a 20% increase in its GR activity.

**KEYWORDS:** *Taiwanofungus camphorata*, monothiol glutaredoxin, three-dimensional (3-D) homology structure, glutathione reductase

## INTRODUCTION

*Taiwanofungus camphorata* is a medicinal mushroom found only in the forests of Taiwan and has traditionally been used for treating liver cancer and drug intoxication, among others. *T. camphorata* was first named *Antrodia camphorata* in 1997.<sup>1</sup> A phylogenetic analysis based on sequence data of rRNA genes of a large ribosomal subunit indicated that *A. camphorata* is distantly related to other species in *Antrodia*. The fungus was subsequently transferred to the new genus *Taiwanofungus*.<sup>2</sup> *T. camphorata* has been shown to exhibit anticancer properties,<sup>3,4</sup> and many studies are aimed at finding the exact bioactive compounds of the mushroom.<sup>5</sup> Recently, we established an expressed sequence tag (EST) from the fruiting bodies of *T. camphorata* to search for physiologically active components including antioxidant enzymes. We have cloned and characterized a 1-Cys peroxiredoxin,<sup>6</sup> a 2-Cys peroxiredoxin,<sup>7</sup> a superoxide dismutase,<sup>8</sup> a catalase,<sup>9</sup> a glutathione formaldehyde dehydrogenase,<sup>10</sup> a phospholipid hydroperoxide glutathione peroxidase,<sup>11</sup> a dithiol glutaredoxin,<sup>12</sup> and a 2-Cys peroxiredoxin isozyme<sup>13</sup> on the basis of the established EST from *T. camphorata*. This encourages us to further search for active components from *T. camphorata* for potential medical applications.

Glutaredoxins (Grxs) play crucial roles not only in redox reactions but also in iron–sulfur cluster assembly and heme biosynthesis.<sup>14</sup> Grxs can be classified into four categories on the

basis of their structures and catalytic properties.<sup>15</sup> The first category is exemplified by Grx1 and Grx3 from *Escherichia coli*, both of which contain the CPYC motif in their active site.<sup>16</sup> This group belongs to dithiol Grx, with molecular mass of 9–12 kDa. The second category is related to the glutathione S-transferase and is exemplified by *E. coli* Grx2 (with a larger molecular mass of 24.3 kDa).<sup>17</sup> Grx2 can catalyze disulfide formation between glutathione and low  $\beta$ -hydroxyethyl disulfide (HED) or high molecular mass substrates (such as arsenate reductase) with high efficiency.<sup>18</sup> The third category is defined by having a monothiol active site (CGFS).<sup>15</sup> Monothiol Grxs can be further categorized into single-domain monothiol Grxs that consist of only one Grx domain and multidomain monothiol Grxs that contain an N-terminal thioredoxin (Trx)-like domain and one to three C-terminal Grx domains.<sup>19</sup> The fourth category is called CC type due to the active site motif consisting of either CCXC or CCXS. This group appears to be specific for higher plants and might be involved in long-distance signaling.<sup>20</sup>

Here, we report the cloning of a novel CGFS-type monothiol Grx from *T. camphorata*, namely, TcmonoGrx. The coding

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region of the TcmonoGrx cDNA was introduced into an *E. coli* expression system and the active enzyme purified and characterized. Understanding the properties of this TcmonoGrx will be beneficial for its potential applications in the health food industry.

## MATERIALS AND METHODS

**Total RNA Preparation from *T. camphorata* and cDNA Synthesis.** Fruiting bodies of *T. camphorata*, which grew in hay of *C. kanehirai*, were obtained from Asian Co., Taiwan (<http://www.asian-bio.com/>). Fresh fruiting bodies (wet weight = 8 g) were frozen in liquid nitrogen and ground to powder in a ceramic mortar. PolyA mRNA (30  $\mu$ g) was prepared using Straight A's mRNA Isolation System (Novagen, Gibbstown, NJ). Four micrograms of the mRNA was used in the 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA synthesis using Clontech's SMART RACE cDNA Amplification Kit.

**Isolation of TcmonoGrx cDNA.** Using the *T. camphorata* 5'-RACE-Ready cDNA as a template and a UPM (universal primer A mix, purchased from BD Biosciences) primer and a degenerate primer (5' CAK CCS CCR AAK GCA AAA TA 3'), a 675 bp fragment was amplified by PCR. The degenerate primer was designed on the basis of the conserved sequences of sulfiredoxin (Srx) from HsSrx (*Homo sapiens*, Q9BYN0), MmSrx (*Mus musculus*, Q9D975), RnSrx (*Rattus norvegicus*, NP\_001041323), DmSrx (*Drosophila melanogaster*, Q9VX10), SpSrx (*Schizosaccharomyces pombe*, Q9URV9), LeSrx (*Lodderomyces elongisporus*, XP\_001528434), ScSrx (*Saccharomyces cerevisiae*, P36077), OsSrx (*Oryza sativa*, Q45GC6), and AtSrx (*Arabidopsis thaliana*, NP\_001077638). The 675 bp fragment was subcloned and sequenced. On the basis of this DNA sequence, a Trx Grx-1F primer (5' CGT CCG TTA AGC CCC TGT CC 3') and a Trx Grx-1R primer (5' GGA CAG GGG CTT AAC GGA CG 3') were synthesized. The primers allowed sequence extension from both ends of the 675 bp fragment. Two PCRs were carried out; each used 0.2  $\mu$ g of the 3'-RACE-Ready cDNA or 5'-RACE-Ready cDNA as a template. The primer pairs in each reaction were Trx Grx-1F primer and UPM and UPM and Trx Grx-1R primer. A 505 bp fragment (3'-RACE; 3'-DNA end) and a 553 bp DNA (5'-RACE; 5'-DNA end) were amplified. Both DNA fragments were subcloned into pCR4 vector and transformed into *E. coli* TOPO10. The nucleotide sequences of these inserts were determined in both strands. Sequence analysis revealed that the combined sequences covered an open reading frame of a putative monothiol Grx cDNA (TcmonoGrx, 1039 bp, EMBL no. EU158772). The identity of this TcmonoGrx clone was assigned by comparing the DNA sequence and the inferred amino acid sequence in various data banks using the basic local alignment search tool (BLAST).

**Bioinformatics Analysis of TcmonoGrx.** The BLASTP program was used to search homologous protein sequences in the non-redundant database (NRDB) at the National Center for Biotechnology Information, National Institutes of Health (<http://www.ncbi.nlm.nih.gov/>). Multiple alignments were constructed using the ClustalW2 program. Protein secondary structure was predicted by SWISS-MODEL program and represented as  $\alpha$  helices and  $\beta$  strands. There is no complete 3-D structure available for a monothiol Grx possessing both Trx-like domain in the N-terminus and monothiol Grx domain in the C-terminus; therefore, created two 3-D homology structures were created as follows: A 3-D homology structure of Trx-like domain pertaining to the N-terminal part of TcmonoGrx was created by SWISS-MODEL<sup>21</sup> (<http://swissmodel.expasy.org/SWISS-MODEL.html>) based on the known Trx-like structure (pdb: 2DIY\_A) of HsGrx3 (*H. sapiens*, O76003). The model superimposed with ScTrx1 (pink, pdb: 2I9H) via the SPDBV\_4 program was shown by using a protein solid ribbon. A 3-D homology structure of monothiol Grx domain pertaining to the C-terminal part of TcmonoGrx was created by SWISS-MODEL on the basis of the known monothiol Grx domain structure (pdb: 1WIK\_A) of MmGrx3 (*M. musculus*, Q9CQM9). The model

superimposed with EcGrx4 (pink, pdb: 1YKA) via the SPDBV\_4 program was shown by using a protein solid ribbon.

**Subcloning of TcmonoGrx cDNA into an Expression Vector.** The coding region of the TcmonoGrx cDNA was amplified using gene-specific flanking primers. The 5' upstream primer contains the *EcoRI* recognition site (5' GAATTCG ATG GCA ACA ACC ACC AAC 3') and the 3' downstream primer contains the *XhoI* recognition site (5' CTCGAG CTG AGT CAC AAG CTC CTG 3'). Using 0.2  $\mu$ g of TcmonoGrx cDNA as a template and 10 pmol of each 5' upstream and 3' downstream primer, a 0.7 kb fragment was amplified by PCR. The fragment was ligated into pCR4 and transformed into *E. coli*. The recombinant plasmid was isolated and digested with *EcoRI* and *XhoI*. The digestion products were separated on a 1% agarose gel. The 0.7 kb insert DNA was gel purified and subcloned into the *EcoRI* and *XhoI* sites of pET-20b(+) expression vector (Novagen, Darmstadt, Germany). The recombinant DNA was then transformed into *E. coli* C41(DE3). The recombinant protein was overexpressed in *E. coli* and its function identified by activity assay as described below.

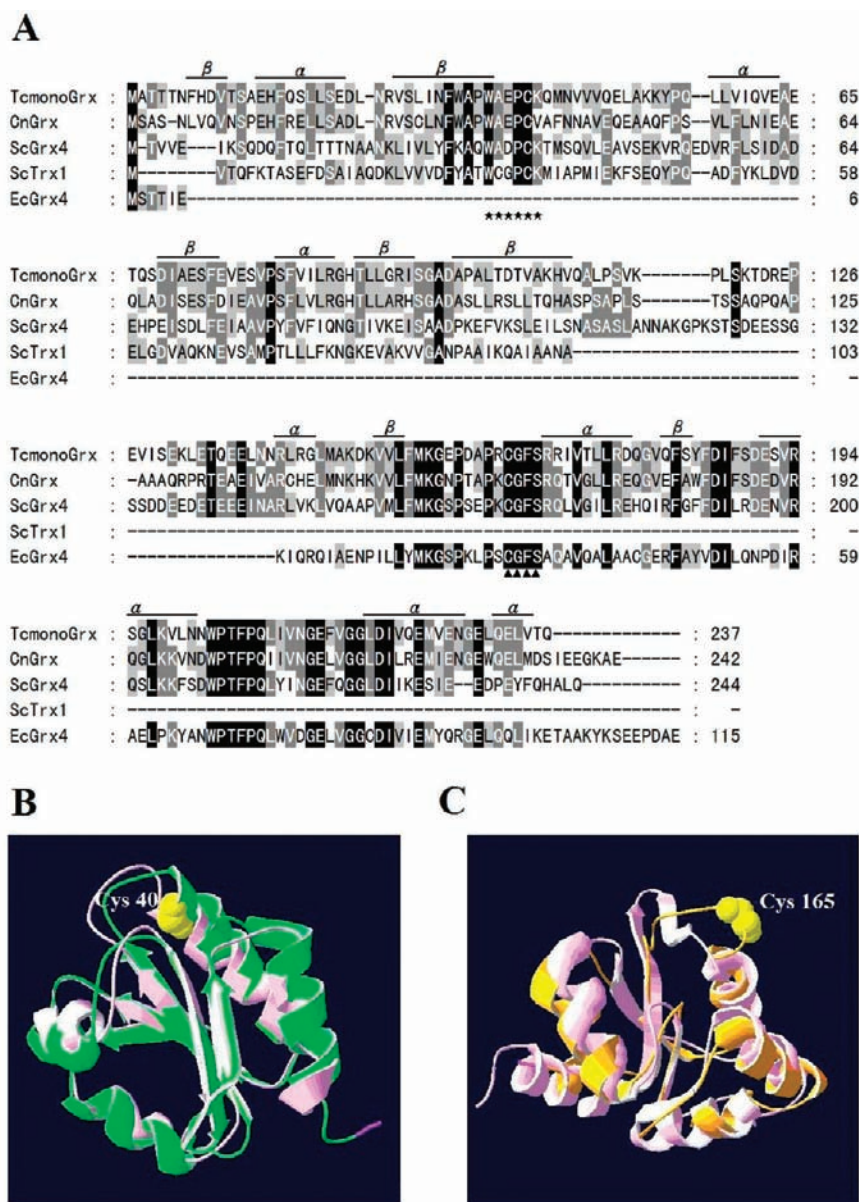
**Expression and Purification of the Recombinant TcmonoGrx.** The transformed *E. coli* containing the TcmonoGrx was grown at 37 °C in 200 mL of Luria-Bertani containing 50  $\mu$ g/mL ampicillin until  $A_{600}$  reached 0.7. Protein expression was induced by the addition of isopropyl  $\beta$ -D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated at 100 rpm for an additional 6 h at 32 °C. The cells were harvested and soluble proteins extracted in PBS with glass beads as described before.<sup>22</sup> The recombinant TcmonoGrx was purified by Ni-NTA affinity chromatography (elution buffer: 1/3  $\times$  PBS containing 100 mM imidazole) as per the manufacturer's instruction (Qiagen). The purified protein was checked by a 10% SDS-PAGE. Proteins on gel were detected by staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

**Molecular Mass Analysis via Electrospray Ionization Quadrupole-Time-of-Flight (ESI Q-TOF).** The purified recombinant TcmonoGrx (0.5 mg/mL) was prepared in 0.03  $\times$  PBS containing 0.05 mM imidazole and 0.05% glycerol. The sample (5  $\mu$ L) was used for molecular mass determination using an ESI Q-TOF mass spectrometer (Micromass, Manchester, U.K.).

**Glutaredoxin Activity Assay.** Grx activity was determined by measuring GSH-dependent reduction of  $\beta$ -hydroxyethylene disulfide (HED), (HOCH<sub>2</sub>CH<sub>2</sub>)<sub>2</sub>S<sub>2</sub>.<sup>23</sup> The reaction mixture contained 100 mM Tris-Cl (pH 7.4), 0.6 g of GR (glutathione reductase, Sigma), 0.3 mM HED, 0.2 mM NADPH, and 0.8 mM GSH. The reaction was started by the addition of Grx, and a mixed disulfide between HED and GSH was generally formed within 2 min. The reaction was followed by a decrease in  $A_{340}$  due to the oxidation of NADPH. This assay method is generally used to determine the dithiol Grxs' activity. Most of the monothiol Grxs are inactive using this HED assay.<sup>24</sup>

**Glutathione Reductase (GR) Activity Assay.** The GR activity of recombinant TcmonoGrx was tested at 25 °C by monitoring the oxidation of NADPH at  $A_{340}$  for GSSG reduction.<sup>25–27</sup> The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 0.2 mM NADPH, and 5 mM GSSG in a total volume of 100  $\mu$ L. The reaction was started by the addition of 10  $\mu$ g of TcmonoGrx or 4  $\mu$ M *E. coli* TR (thioredoxin reductase, Sigma) as a control. The reaction's progress was followed by the decrease in  $A_{340}$  due to the oxidation of NADPH between 10 and 40 min. To measure the amount of reduced GSH produced by TcmonoGrx, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added into the former reaction to 1 mM at the 40 min time point. One mole of 5-thio-2-nitrobenzoic acid (TNB) is formed per mole of GSH oxidized. The amount of TNB formed was measured at 412 nm for 1.0 min. The molar absorption coefficient of TNB at 412 nm is 13.6 mM<sup>-1</sup> cm<sup>-1</sup>.

**Enzyme Characterization.** The TcmonoGrx enzyme was tested for stability in terms of its GR activity under various conditions. Aliquots of the TcmonoGrx sample were treated as follows: (1) *Thermal effect.*



**Figure 1.** Alignment of the amino acid sequences of TcmonoGrx with other organisms' CGFS-type glutoredoxins or thioredoxin and 3-D homology structures. (A) Sequence alignment: TcmonoGrx (this study), CnGrx (*Cryptococcus neoformans*, Q5KJR8), ScGrx4 (*Saccharomyces cerevisiae*, P32642), ScTrx1 (P22217), and EcGrx4 (*Escherichia coli*, P0AC69). Identical amino acids in all sequences are shaded black; conservative replacements are shaded gray. Protein secondary structure was predicted by SWISS-MODEL program and represented as  $\alpha$  helices and  $\beta$  strands. (B) A 3-D homology structure of a Trx-like domain from N-terminal (Thr4–Ser115) TmonocGrx. The Trx-like structural model of the TcmonoGrx was created on the basis of the known Trx-like structure (pdb: 2DIY\_A) of HsGrx3 (*Homo sapiens*) via SWISS-MODEL program and was superimposed to obtain structure alignment via SPDBV\_4 program. Superimposition of the Trx-like domain of TcmonoGrx (green) and ScTrx1 (pink) is shown using protein solid ribbons. (C) A 3-D homology structure of monothiol Grx domain from the C-terminal end (Arg142–Thr236) of the TmonocGrx. The monothiol Grx domain structural model of the C-terminal TmonocGrx was created on the basis of the known monothiol Grx domain structure (pdb: 1WIK\_A) of MmGrx3 (*Mus musculus*) via SWISS-MODEL program and was superimposed to obtain structure alignment via SPDBV\_4 program. Superimposition of monothiol Grx domain of TcmonoGrx (orange) and EcGrx4 (pink) is shown using protein solid ribbons. The residues coordinating active sites Cys<sup>40</sup> and Cys<sup>165</sup> are conserved as they are present in all reported Trx-like domains and monothiol Grx domains of monothiol Grxs. The upper yellow ball represents the active site.

Enzyme sample was heated to 60 °C for 2, 4, 8, or 16 min. (2) *pH effect*. Enzyme sample was adjusted to desired pH by adding a half-volume of buffer with different pH values: 0.2 M citrate buffer (pH 2.5 or 4.0), 0.2 M Tris-HCl buffer (pH 6.0 or 8.0), or 0.2 M glycine–NaOH buffer (pH 10.0 or 11.0). Each sample was incubated at 37 °C for 1 h. (3) *Imidazole effect*. During protein purification, the TcmonoGrx enzyme was eluted with imidazole. Therefore, the effect of imidazole

on protein activity was examined. Imidazole was added to the enzyme sample to a level of 0.2, 0.4, or 0.8 M and incubated at 37 °C for 1 h. (4) *Proteolytic susceptibility*. The enzyme was incubated with  $1/10$  of trypsin or chymotrypsin (w/w) at pH 8.0 and 37 °C for a period of 5, 10, 20, or 40 min. Aliquots were removed at various time intervals for analysis. After each treatment, the residue GR activity was tested using the DTNB assay as described above.

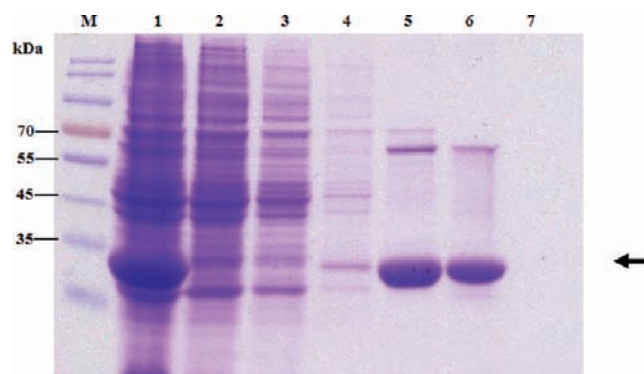
**Kinetic Studies.** The kinetics properties of the TcmonoGrx (3.7  $\mu$ M) were determined by varying the concentration of GSSG (from 0.06 to 3 mM) with a fixed amount of 0.2 mM NADPH or by varying the concentration of NADPH (from 0.04 to 0.2 mM) with a fixed amount of 5 mM GSSG. The change in absorbance at 340 nm was recorded between 20 and 40 s. The molar absorption coefficient of NADPH at 340 nm is  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ . The  $K_M$ ,  $V_{max}$ , and  $k_{cat}$  were calculated from Lineweaver–Burk plots.

**Site-Directed Mutagenesis ( $C^{40} \rightarrow S^{40}$ ,  $C^{165} \rightarrow S^{165}$ ,  $S^{168} \rightarrow C^{168}$ ).** The TcmonoGrx cDNA in pET-20b(+) prepared above was used as the wild-type template for site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Three mutants, C40S, C165S, and S168C, were created, and their sequences were verified by nucleotide sequencing. The expressed proteins were purified by affinity chromatography as described before. The purified mutant proteins were tested for Grx activity via HED method or GR activity.

## RESULTS

**Cloning and Characterization of a cDNA Encoding TcmonoGrx.** A putative TcmonoGrx cDNA clone was identified on the basis of the consensus pattern and sequence homology to the published Grxs in the NCBI data bank. The coding region of TcmonoGrx cDNA was 711 bp, which encodes a protein of 237 amino acid residues with a calculated molecular mass of 29 kDa (EMBL accession no. EU158772). Figure 1 shows the optimal alignment of the amino acid sequences of TcmonoGrx with four selected sequences. This TcmonoGrx shared 54% identity with CnGrx (*Cryptococcus neoformans*, Q5KJR8), 34% identity with ScGrx4 (*S. cerevisiae*, P32642), 17% identity with ScTrx1 (*S. cerevisiae*, P22217), and 38% identity with EcGrx4 (*E. coli*, P0AC69) using the Blast 2 sequence program. The TcmonoGrx enzyme belongs to the monothiol Grx subfamily because of the presence of a highly conserved monothiol catalytic motif  $C^{165}GFS$  (Figure 1). The secondary structure was predicted by SWISS-MODEL program and represented as  $\alpha$  helices and  $\beta$  strands (Figure 1A). The asterisks denote the conserved residues (WAEPC<sup>40</sup>K) possessing a Trx-like domain. The residue coordinating active site Cys<sup>40</sup> is conserved as it is present in all reported Trx-like domains. The triangles denote the conserved residues ( $C^{165}GFS$ ) belonging to the monothiol Grx domain. The residue coordinating active site Cys<sup>165</sup> is conserved as it is present in all reported monothiol Grx domains. A 3-D homology structure of the Trx-like domain pertaining to the N-terminal part of TcmonoGrx was created on the basis of the known Trx-like domain structure of HsGrx3. The model superimposed with ScTrx1 (pink) via the SPDBV\_4 program was shown using a protein solid ribbon (Figure 1B). A 3-D homology structure of the monothiol Grx domain pertaining to the C-terminal part of TcmonoGrx was created on the basis of known monothiol Grx domain structure of MmGrx3. The model superimposed with EcGrx4 (pink) via the SPDBV\_4 program was shown using a protein solid ribbon (Figure 1C).

**Expression and Purification of the Recombinant TcmonoGrx.** The coding region of TcmonoGrx (711 bp) was amplified by PCR and subcloned into an expression vector, pET-20b(+), as described under Materials and Methods. Positive clones were verified by DNA sequence analysis. The recombinant TcmonoGrx was expressed, and the proteins were analyzed by a 10% SDS-PAGE in the absence a reducing agent without boiling (Figure 2). The recombinant TcmonoGrx was expressed as a His6-tagged fusion protein and was purified by affinity



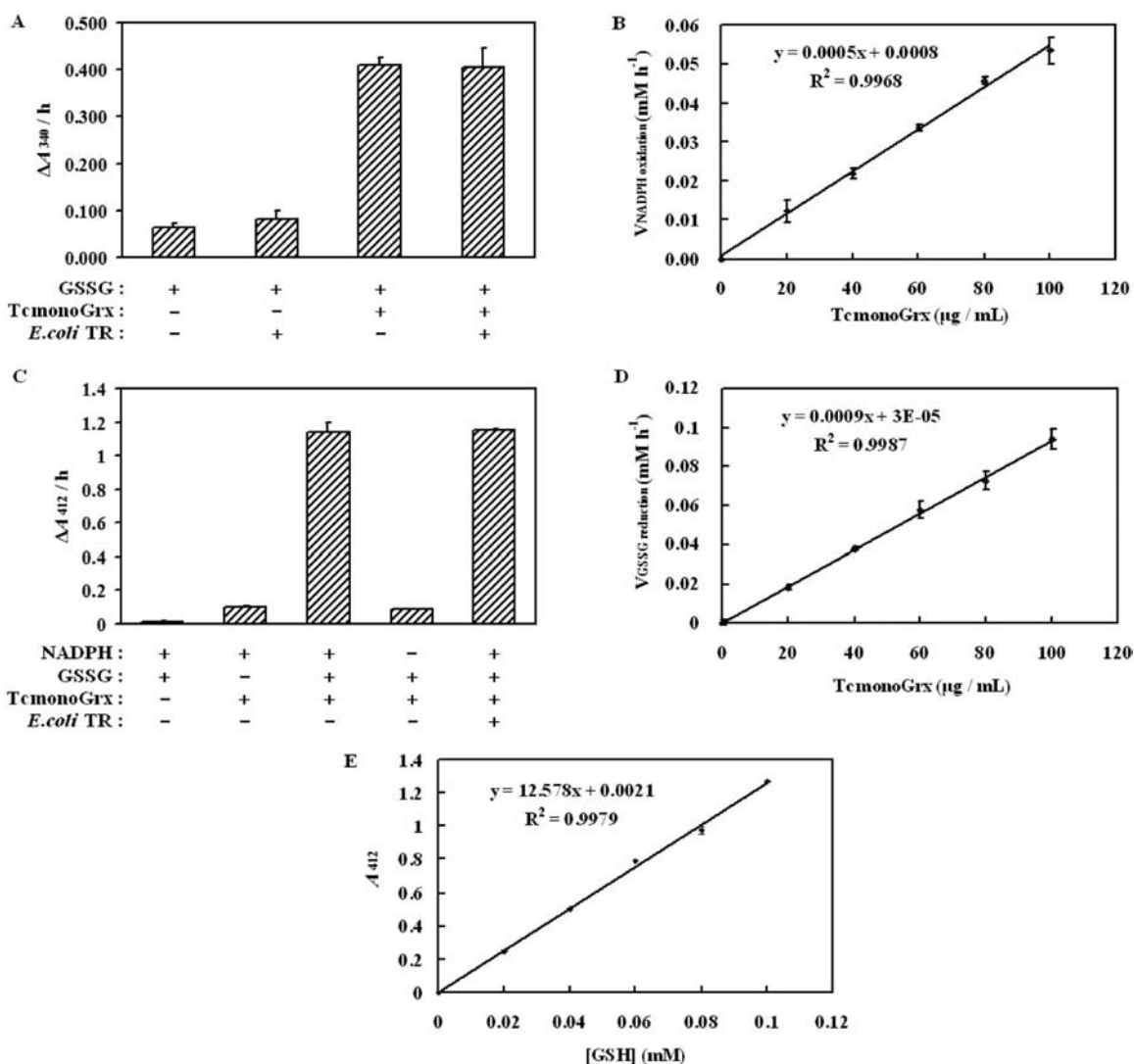
**Figure 2.** Expression and purification of recombinant TcmonoGrx in *E. coli*. Fifteen microliters (loading buffer without mercaptoethanol and without boiling) of each fraction was loaded into each lane of the 10% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Lanes: 1, crude extract from *E. coli* expressing TcmonoGrx; 2, flow-through proteins from the Ni-NTA column; 3, washed from Ni-NTA column; 4–7, TcmonoGrx eluted from Ni-NTA column. Molecular masses (in kDa) of standards are shown at left. The arrow indicates the target protein.

chromatography with nickel chelating Sepharose. A major band with a molecular mass of  $\sim 29$  kDa (the expected size of the TcmonoGrx monomer) was detected in Ni-NTA eluted fractions by SDS-PAGE (Figure 2, lanes 5 and 6). A minor band above 55 kDa, presumably the dimeric form of Grx, was observed. The Ni-NTA eluted fractions containing pure protein were pooled and characterized further. Analysis of the TcmonoGrx by ESI Q-TOF confirms the presence of one major protein band with a molecular mass of 28.6 kDa. This indicates that the enzyme is predominantly monomeric in nature. The yield of the purified His6-tagged TcmonoGrx was 300  $\mu$ g from 20 mL of culture. Functional TcmonoGrx was detected by activity assay as described below.

**Characterization of the Purified TcmonoGrx.** The TcmonoGrx enzyme was shown to possess novel GR activity by its ability to reduce the GSSG to GSH via NADPH. As shown in Figure 3A, the NADPH consumption in the reaction depended on the presence of GSSG (5 mM) and TcmonoGrx (10  $\mu$ g/0.1 mL) and was not caused by *E. coli* TR. The GSH produced in these reactions was used to reduce DTNB to TNB (Figure 3C). The GR activity of TcmonoGrx exhibits linearity between the enzyme levels and the reaction rate. As the TcmonoGrx levels increased from 20 to 100  $\mu$ g/mL, the reaction rate increased (Figure 3B,D).

Heat stability and imidazole effects of the TcmonoGrx were tested because the information is useful for developing enzyme purification protocols. To examine the effect of heat on GR activity, the purified TcmonoGrx was heat-treated as described under Materials and Methods and then analyzed for the residue GR activity. The enzyme appeared to be heat stable. TcmonoGrx's half-life of deactivation at 60  $^{\circ}$ C was 10.5 min, and its thermal inactivation rate constant ( $k_d$ ) was  $5.37 \times 10^{-2} \text{ min}^{-1}$  (Figure 4).

TcmonoGrx has an optimal GR activity at pH 6.0, and the enzyme retained significant activity at pH 4–10.0 (Figure 5A). The enzyme retained about 60% activity in the presence of 0.2–0.8 M imidazole (Figure 5B). The enzyme lost 20 or 40% activity after 40 min of incubation at 37  $^{\circ}$ C with  $1/_{10}$  of trypsin or chymotrypsin (w/w) (Figure 5C). The protease tests were useful in understanding the effect of the digestive enzymes on TcmonoGrx and its suitability as a health food.



**Figure 3.** TcmmonoGrx possesses GR activity. The oxidation of NADPH at 25 °C was followed spectrophotometrically at 340 nm in a coupled enzymatic assay (see Materials and Methods for further details). (A) Consumption of NADPH (0.2 mM) in the assay depended on GSSG (5 mM) and TcmmonoGrx (10 μg/0.1 mL) and was not caused by *E. coli* TR (thioredoxin reductase, 4 μM). Components that were varied in the sample cuvette are indicated. The sample without TcmmonoGrx contained elution buffer as a control. (B) Reaction velocities (NADPH oxidation) with 0.2 mM NADPH and 5 mM GSSG were proportional to the concentration of TcmmonoGrx at an enzyme concentration of ≤ 100 μg/mL. (C) Production of TNB (1 mM DTNB was reduced by GSH that was produced due to GSSG reduction) in the coupled assay depended on NADPH (0.2 mM), GSSG (5 mM), TcmmonoGrx (10 μg/0.1 mL) and was not caused by *E. coli* TR (4 μM). Components that were varied in the sample cuvette are indicated. (D) Reaction velocities (GSSG reduction) with 0.2 mM NADPH and 5 mM GSSG were proportional to the concentration of TcmmonoGrx at an enzyme concentration of ≤ 100 μg/mL. (E) Production of TNB was proportional to the amount of reduced GSH. The data shown in panels A and C were analyzed by ANOVA and Scheffe's test.

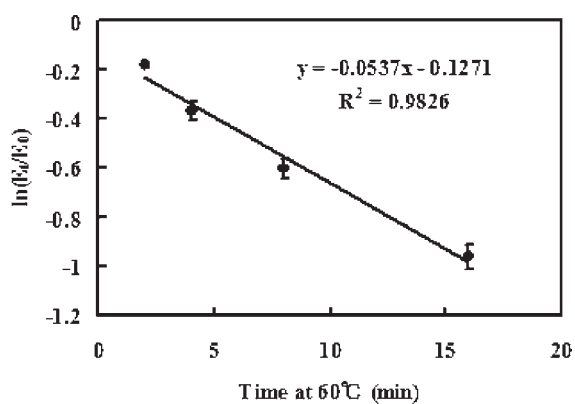
**Kinetics Studies of the Purified TcmmonoGrx.** As shown in Figure 6, the Lineweaver–Burk plot of the velocity ( $1/V$ ) against  $1/\text{GSSG}$  gave  $K_M = 0.06$  mM,  $V_{\max} = 0.03$  mM/h, and  $k_{\text{cat}} = 8.10$  h<sup>-1</sup>. The plot of the velocity ( $1/V$ ) against  $1/\text{NADPH}$  gave  $K_M = 0.04$  mM,  $V_{\max} = 0.06$  mM/h, and  $k_{\text{cat}} = 17.01$  h<sup>-1</sup> for TcmmonoGrx, respectively.

**Site-Directed Mutagenesis (C<sup>40</sup>→S<sup>40</sup>, C<sup>165</sup>→S<sup>165</sup>, S<sup>168</sup>→C<sup>168</sup>).** The effect that mutation of either the cysteine or the serine located in the active site (CGFS) had on GR activity was examined by creating amino acid substitution mutants C165S and S168C. When the enzyme was mutated to the dithiol (CGFC), the GR activity of the mutant was increased by 20% as compared to the wild-type protein (Figure 7). The dithiol mutant still lacks Grx activity by HED assay (results not shown). When the active site cysteine at position 165 was

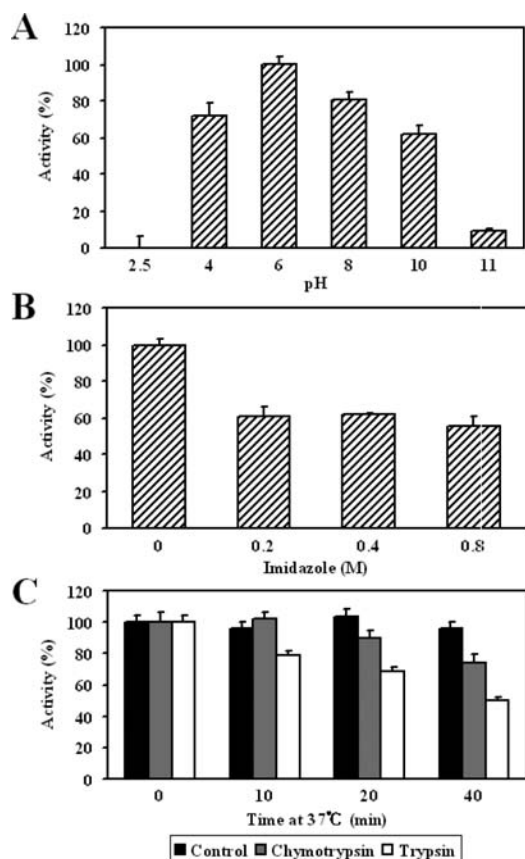
replaced with serine (SGFS), the GR activity of the mutant was decreased by approximately 60% as compared to the wild-type protein (Figure 7). Another conserved cysteine residue at position 40 (active site residue of Trx) was replaced with serine. This mutant showed decreased GR activity by approximately 50% as compared to the wild-type protein. The results demonstrated that these thiols play important roles in GR activity.

## DISCUSSION

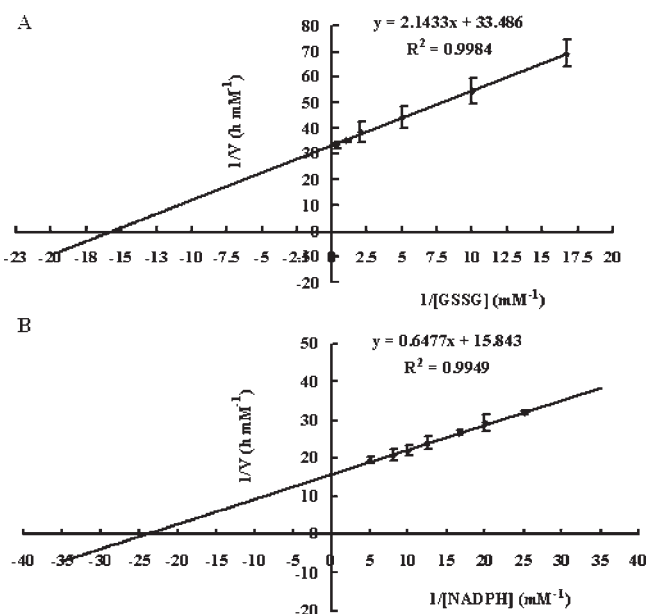
This study reported the first cloning and expression of an important reduction enzyme, TcmmonoGrx, from *T. camphorata*. The biologically active form of TcmmonoGrx has been successfully expressed in *E. coli*. The enzyme possesses GR activity and is



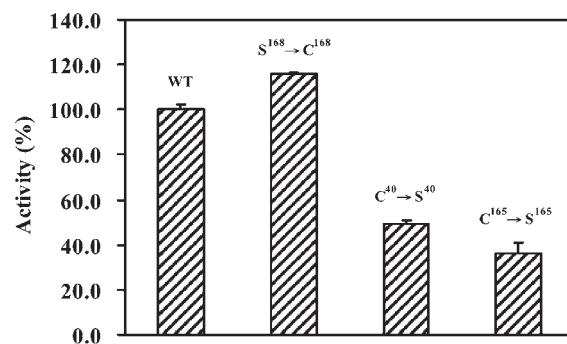
**Figure 4.** Effect of temperature on the purified TcmonoGrx. The enzyme sample was heated at 60 °C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8, or 16 min and assayed for GR activity. The figure shows a plot of the thermal inactivation kinetics of TcmonoGrx activity.  $E_0$  and  $E_t$  are original activity and residual activity after heating for different time intervals, respectively. Data are means of three experiments.



**Figure 5.** Effect of pH, imidazole, and chymotrypsin or trypsin on the purified TcmonoGrx. (A) The enzyme samples were incubated with buffers of different pH values at 37 °C for 1 h and then assayed for GR activity. (B) The enzyme samples were incubated with various concentrations of imidazole at 37 °C for 1 h and then checked for GR activity. (C) The enzyme samples were incubated with chymotrypsin or trypsin at 37 °C for various times and then checked for GR activity. The data shown in panels A–C were analyzed by ANOVA and Scheffe's test. Data are the mean  $\pm$  SD of three similar experiments.



**Figure 6.** Double-reciprocal plot of varying GSSG and NADPH on TcmonoGrx activity. The initial rate of the enzymatic reaction was measured at 0.2 mM NADPH with the GSSG concentration varied from 0.06 to 3 mM (A) and at 5 mM GSSG with the NADPH concentration varied from 0.04 to 0.2 mM (B).  $K_M$ ,  $V_{max}$  and  $k_{cat}$  were calculated from Lineweaver–Burk plots.



**Figure 7.** Mutation-altering enzymatic activity. Ten micrograms of TcmonoGrx (wild-type, WT) or mutants ( $S^{168} \rightarrow C^{168}$ ,  $C^{40} \rightarrow S^{40}$ ,  $C^{165} \rightarrow S^{165}$ ) was used to obtain GR activity in the presence of 0.2 mM NADPH or 2 mM GSSG. The data are the mean of three independent experiments.

capable of reducing GSSG to GSH. However, the enzyme lacks Grx activity when using the HED assay method. This is consistent with Mesecke et al.'s results<sup>24</sup> due to having an insertion of ( $G^{158}EPDA^{162}$ ) between  $K^{157}$  and  $C^{165}$  (Figure 8) to hinder its reduction by GSH. Using site-directed mutation to change a mutant ( $S^{168} \rightarrow C^{168}$ ), it looked like a dithiol Grx (having  $C^{165}GFC^{168}$  motif instead of original  $C^{165}GFS^{168}$ ); this mutant was still inactive using the standard Grx assay via HED assay method, although a 20% increase in GR activity was found. This further indicated that insertion  $G^{158}EPDA^{162}$  is crucial to TcmonoGrx. There are several examples as shown in Figure 8, which is consistent with Mesecke et al.'s paper; five monothiol Grxs, each containing an insertion sequence such as ScGrx3, ScGrx4, and ScGrx5 from *S. cerevisiae*,<sup>24,28</sup> EcGrx4 from *E. coli*,<sup>29</sup> and CrGrx3 from *Chlamydomonas reinhardtii*,<sup>30</sup> were inactive

TcmonoGrx	-----MATTTNFHDVTSAEHFQSLLEDLN-RVSLINFWAP	35
ScGrx3	MCSFQVPSAFSFNYSYCYKRHQARYYAAKLFQEMPVIEINDQEQFTYLTSTAAGDKLIVLYFHTS	67
ScGrx4	-----MTVVEIKSQDQFTQLTTTNAANKLIVLYFKAQ	32
ScGrx5	-----	-
EcGrx4	-----	-
CrGrx3	-----	-
TcGrx	-----	-
ScGrx6	-----	-
ScGrx7	-----	-
TomonoGrx	WAEPCKQMNVVQELAKKYP--QLLVIQVEAETQSDIAESFEVESVPSFVILRGHTLLGRISGADAP	100
ScGrx3	WAEPCKALKQVFEAISNEPSNSVNSFLSIDADENSEISELFEISAVPYFIIHKGTILKELSGADPK	134
ScGrx4	WADPCKTMSQVLEAVSEKVRQEDVRFSLIDADEHPEISDLFEIAAVPYFVFIQNGTIVKEISAADPK	99
ScGrx5	-----MFLPKFNPISRFSPI LRAKTLRLRYQN-----	26
EcGrx4	-----	-
CrGrx3	-----MASLQCNMRPATTILTRRPAQVARKSIGVARSQTVRVVTSAG-----	41
ToGrx	-----	-
ScGrx6	-----MIPSNKRNARISITLTLVVFFVAQANANFLTVEIKEE	39
ScGrx7	-----MAIVINKRNVRLVITNLLLIVVFFVLRNSNASVNESITT	40
TomonoGrx	ALTDTVAKHVQALPSVKPLSKTDREPEVIS-----	130
ScGrx3	EYVSLLEDCKNSVNSGSSQTHMENANVNEGSHNDEDDDEE-----	176
ScGrx4	EFVKSLEILSNASASLANNAGPKSTSDDESSGSSDD-----	136
ScGrx5	-----	-
EcGrx4	-----	-
CrGrx3	-----	-
ToGrx	-----	-
ScGrx6	TSKAFSTNMDNMAGGSSREYAAMPTSTTNKGSSEVDEEINEIKQKVLQQPISVDDSLSAIKNDKG	106
ScGrx7	HHPDSLVTDFDNGNAPGTHQSVHDTVNTQ-----DKEAEVVKNSG-----	81
TomonoGrx	EKLETQEELNNRLRGLMAKDKVVLFMKGEPDAPRCGFSRRIVTLRDOGVQ--FSYFDIFSD-----	191
ScGrx3	EEEEEEQINARLTKLVNAAPVLMKGGSPSEPKCGFSRQLVGLREHQVR--FGFFDILRDE-----	237
ScGrx4	EEDTEEEINARLVKLVAAPVLMKGGSPSEPKCGFSRQLVGLREHQIR--FGFFDILRDE-----	197
ScGrx5	-RMYLSTEIRKAIEDAIESAPVLFMKGTPPEFKCGFSRATIGLLGNQGVDPKFAAYNVLEDP--	89
EcGrx4	-----MSTTIEKIQRQIAENPILLYMKGSPKLPSCGFSQAQVQALAACGER--FAYVDILQNP--	56
CrGrx3	-----MAPDLKKSIDELIASNKVVFMKGTROFPKCGFSNTVVQILNVMDVP--YQTVNILEDD--	98
ToGrx	-----MSAAKIKAAKIIDENGVAV--SK--SYCPYCRATKQLLNDLGAK--YYTIELDEVDDGS	54
ScGrx6	SRI TKAFNVQKEYSLILDLSPIII--SK--STCSYSKGMKELLENEYQFIPNYIYIELDKHGHGE	168
ScGrx7	-----DAEFDAAAEYNKIMEQSPMIV--SK--TCPPYSKCLKALLTNSYTFSPSYVVELDRHEHTK	140
TomonoGrx	SVRSGLKVLNNWPTFPQLIVNCEFGGLDIVQEMVENGE--LOELVTO-----	237
ScGrx3	SVRQNLKKFSEWPTFPQLYINCEFGGLDIIKESLEEDPDFLQHALQS-----	285
ScGrx4	NVRQSLKKFSDWPTFPQLYINCEFGGLDIIKESI EEDPEYFQHALQ-----	244
ScGrx5	ELREGIKFSEWPTFPQLYVNKEFIGGCDVITSMARSGE--LADLLEEA--QALVPEEEETKDR--	150
EcGrx4	DIRAELPKYANWPTFPQLVWDGELVGGCDIVIMYQRGE--LQQLIKETAAYKSEEPDAE-----	115
CrGrx3	AIRSGMKEYSQWPTFPQVYINCEFGGCDIMMEAYQSGE--KEQLEIALNS-----	148
ToGrx	DIQAALKEINGQTSVPNIYIKQOHIGGNS--DLQARRSE--PELLKDAGAV-----	102
ScGrx6	ELQEYIKLVTGRGIVPPLLNVNVSRRGNEEIKKLHTGGK--LESQVWSDGKFSVEQREKPSNN--	231
ScGrx7	ELQDQIEKVTGRRIVPNIYIGTSRGGYTEIAELHKNDE--LDSFKKWSGGAFTVKANSQSESA--	203

**Figure 8.** Comparison of insertion sequence between K and CGFS of TcmonoGrx with other organisms on Grx activity. Five monothiol Grxs, each contained an insertion sequence such as ScGrx3, ScGrx4, or ScGrx5 from *S. cerevisiae*, EcGrx4 from *E. coli*, or CrGrx3 from *C. reinhardtii*, were inactive using the HED assay method. One TcGrx (a dithiol Grx from *T. camphorata*) and two monothiol Grxs (ScGrx6 and ScGrx7), without insertion sequence, were active using the HED assay method. The red box denotes insertion sequences between K and CGFS.

using the HED assay method. TcGrx (a dithiol Grx from *T. camphorata*)<sup>12</sup> and two monothiol Grxs (ScGrx6, ScGrx7)<sup>24</sup> without insertion sequences were active using the HED assay method. Furthermore, the active site domain (GGTC<sup>70</sup>-VIRGC<sup>75</sup>VPKK) and 2 Arg residues (R<sup>229</sup>-R<sup>235</sup>, a six distal-site for Arg residues) providing a NADPH binding site exist in sweet potato GR,<sup>25-27</sup> whereas this TcmonoGrx contains putative active sites (WAEPCK<sup>40</sup>K, RC<sup>165</sup>GFSRR) and 10 Arg residues

(R<sup>26</sup>, 87, 94, 124, 142, 164, 169-170, 176, 194); especially the ones located at the RC<sup>165</sup>GFSRR motif provide the six distal sites for Arg residues (R<sup>164-169</sup>, 170), and it is likely to be a NADPH binding site responsible for catalyzing GSSG reduction.

This TcmonoGrx from *T. camphorata* shows glutathione reductase activity and can regulate/reduce GSSG/GSH ratio, which can in turn reduce the oxidative stress in organisms. This property may be beneficial for its potential use as a health food.

## AUTHOR INFORMATION

## Corresponding Author

\*Postal address: Institute of Bioscience and Biotechnology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan. E-mail: B0220@mail.ntou.edu.tw. Phone: 886-2-24622192, ext. 5513. Fax: 886-2-24622320.

## Author Contributions

<sup>†</sup>Contributed equally to this paper.

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