

Cloning, Expression, and Characterization of an Enzyme Possessing both Glutaredoxin and Dehydroascorbate Reductase Activity from *Taiwanofungus camphorata*

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Glutaredoxins (Grxs) play important roles in the reduction of disulfides via reduced glutathione as a reductant. A cDNA (503 bp, EU193660) encoding a putative Grx was cloned from *Taiwanofungus camphorata* (Tc). The deduced amino acid sequence is conserved among the reported dithiol Grxs. A 3D homology structure was created for this TcGrx. To characterize the TcGrx enzyme, the coding region was subcloned into an expression vector pET-20b(+) and transformed into *Escherichia coli*. Functional TcGrx was expressed and purified by Ni²⁺-nitrilotriacetic acid Sepharose. The purified enzyme showed bands of ~15 kDa on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The TcGrx encodes a protein possessing both Grx and dehydroascorbate reductase (DHAR) activity. The Michaelis constant (K_m) values for β -hydroxyethyl disulfide (HED) and dehydroascorbate (DHA) were 0.57 and 1.85 mM, respectively. The half-life of deactivation of the protein at 100 °C was 8.5 min, and its thermal inactivation rate constant K_d was $6.52 \times 10^{-2} \text{ min}^{-1}$. The enzyme was active under a broad pH range from 6.0 to 10.0 and in the presence of imidazole up to 0.4 M. The enzyme was susceptible to SDS denaturation and protease degradation/inactivation.

KEYWORDS: *Taiwanofungus camphorata*; glutaredoxin; three-dimension homology structure (3D homology structure); expression; β -hydroxyethyl disulfide [HED, (HOCH₂CH₂)₂S₂]; dehydroascorbate (DHA)

INTRODUCTION

Taiwanofungus camphorata (*T. camphorata*, formerly named *Antrodia camphorata*, commonly known as Niuchangchih in Taiwan) is a medicinal mushroom species found only in the forests of Taiwan. *T. camphorata* has been traditionally used as a remedy for cancer and drug intoxication, among others, and has been shown to exhibit vasorelaxative (1), and anti-inflammatory (2) effects. Although *T. camphorata* shows physiological activities with great potential in medical applications, little is known about the exact bioactive compounds of the mushroom (3). Recently, we established an expressed sequence tag (EST) from fruiting bodies of *T. camphorata* to search physiologically active components for use. We have cloned and characterized a 1-Cys peroxiredoxin (4), 2-Cys peroxiredoxin (5), superoxide dismutase (6), catalase (7), glutathione formaldehyde dehydrogenase (8), and phospholipid hydroperoxide glutathione peroxidase (9) based on the established EST from *T. camphorata*.

Glutaredoxins (Grxs) are important antioxidative enzymes that can catalyze the reduction of disulfides via reduced

glutathione (GSH) in a coupled system with glutathione reductase and NADPH. The enzymes have been found in most aerobic organisms, including prokaryotes and eukaryotes. On the basis of their catalytic structure properties, Grxs can be classified into three categories (10). The first is exemplified by Grx1 and Grx3 from *Escherichia coli*. They share 33% sequence identity, and both have the CPYC (Cys-Pro-Tyr-Cys) dithiol motif at their active site (11). The second category is related to the glutathione *S*-transferase. The larger *E. coli* Grx2 (24.3 kDa) belongs to this category (12). Grx2 cannot reduce the intracellular disulfides of ribonucleotide reductase Ia but has high catalytic activity in reducing mixed disulfides formed between glutathione and low- or high-molecular-weight substrates (13), such as β -hydroxyethyl disulfide (HED) and arsenate reductase, respectively. The third category is defined by having a monothiol active site (CGFS) (10). The dithiol Grxs are believed to play an important role in protecting cells against oxidative stresses (14–17).

Here, we report the cloning of an antioxidant enzyme, TcGrx cDNA, from *T. camphorata* on the basis of EST. The coding region of the TcGrx cDNA was introduced into an *E. coli* expression system; the active enzyme was purified; and its properties were studied. Understanding the properties of this TcGrx might be beneficial for its potential applications in medicine or as health food.

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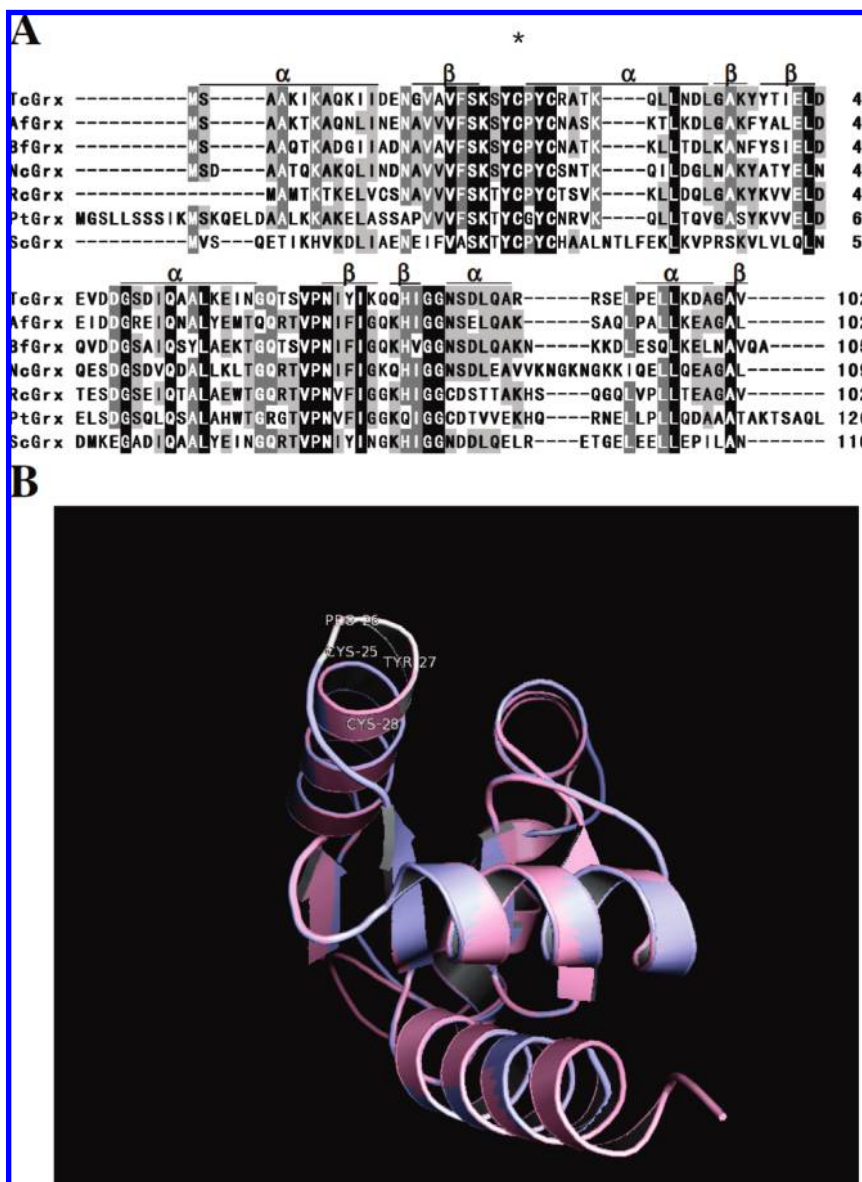


Figure 1. Alignment of the amino acid sequences of TcGrx with that of other organisms and 3D homology structure. (A) Sequence alignment: TcGrx (this study), AfGrx (*A. fumigatus*), BfGrx (*B. fuckeliana*), RcGrx (*R. communis*), NcGrx (*N. crassa*), PtGrx (*P. tremula* x *P. tremuloides*), and ScGrx (*S. cerevisiae*). Identical amino acids in all sequences are shaded black, and conservative replacements are shaded gray. Protein secondary structure was predicted by SWISS-MODEL and represented as α helices and β strands. (B) Three-dimensional homology structure of TcGrx. The structural model of TcGrx was created on the basis of the known structure of Grx C1 from *P. tremula* via SWISS-MODEL and was superimposed to obtain better structure alignment via SYBYL 7.3. Superimposition of TcGrx (light blue) and Grx C1 (pink) was shown using protein solid ribbons.

MATERIALS AND METHODS

RNA Extraction from *T. camphorata* and cDNA Synthesis.

Fruiting bodies of *T. camphorata*, grown in the hay of *C. kanehirai*, were obtained from Asian Nova Biotechnology, Inc. (<http://www.asian-bio.com/>). Total RNA was prepared from fresh fruiting bodies (wet weight of 5 g) using Straight A's mRNA isolation system (Novagen, Madison, WI). The total RNA (22 μ g) was obtained, and 3 μ g of the mRNA was used for cDNA synthesis using a ZAP-cDNA kit purchased from Stratagene (La Jolla, CA).

Isolation of TcGrx cDNA. We have previously established an EST database from fruiting bodies of *T. camphorata* and sequenced all clones with an insert size greater than 0.2 kb (data not shown). The identity of a Grx cDNA clone was assigned by comparing the inferred amino acid sequence in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST).

Bioinformatic Analysis of TcGrx. The BLASTP (protein–protein BLAST) program was used to search homologous protein sequences in the

nonredundant database at the National Institutes of Health (NIH, <http://www.ncbi.nlm.nih.gov/>). Multiple alignments were constructed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) and Blast 2 sequences (18). Structural modeling was carried out using SWISS-MODEL (19) (<http://swissmodel.expasy.org/SWISS-MODEL.html>) to create a 3D homology structure based on the known X-ray structure of Grx C1 of *Populus tremula*. The modeling data was then superimposed by SYBYL 7.3 (TRIPOS Associates, Inc., kindly provided by Dr. Nady Shaw, National Changhua University of Education, Taiwan).

Subcloning of the TcGrx cDNA Coding Region into an Expression Vector. The coding region of the TcGrx cDNA was amplified using two gene-specific primers. The 5' upstream primer contains the *Eco*RI recognition site (5'-GAA TTC GAT GTC CGC AAA GAT C-3'), and the 3' downstream primer contains the *Xho*I recognition site (5'-CTC GAG AAC AGC ACC GGC ATC CTT-3'). Using 0.1 μ g of *T. camphorata* cDNA as a template and 10 pmol of each 5' upstream and 3' downstream primer, a 0.3 kb fragment was amplified by the polymerase

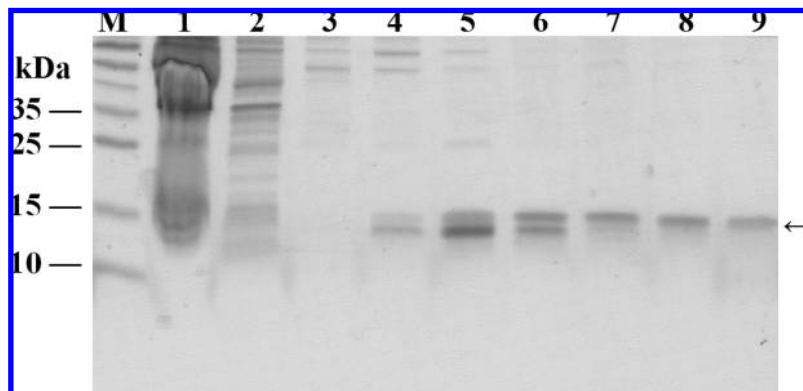


Figure 2. Expression and purification of recombinant TcGrx in *E. coli*. A total of 15 μL of each fraction eluted from the Ni-NTA column was loaded onto a 15% SDS-PAGE followed by Coomassie Blue staining. Lane 1, crude extract from *E. coli* expressing TcGrx; lane 2, flow-through proteins from the Ni-NTA column; lane 3, washed from the Ni-NTA column; lanes 4–9, TcGrx eluted from the Ni-NTA column. Molecular masses (in kilodaltons) of standards are shown at the left. The arrow indicates the target protein.

chain reaction (PCR). The fragment was ligated into pCR4.0 and transformed into *E. coli* Ecos101. Plasmid DNA was isolated from the clone and digested with *EcoRI* and *XhoI*. The digestion products were separated on a 1.5% agarose gel. The 0.3 kb insert DNA was gel-purified and subcloned into *EcoRI* and *XhoI* sites of pET-20b(+) vector (Novagen, Madison, WI). The recombinant DNA was then transformed into *E. coli* BL21(DE3)pLysS.

Expression and Purification of the Recombinant TcGrx. The transformed *E. coli* containing the TcGrx was grown at 37 °C in 80 mL of Luria–Bertani medium containing 50 $\mu\text{g}/\text{mL}$ ampicillin until A_{600} reached 0.75. Protein expression was induced by the addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated at 37 °C for an additional 4 h at 120 rpm shaking, and then the cells were harvested by centrifugation. Cells were suspended in 2 mL of phosphate-buffered saline (PBS) containing 1% glycerol and 1 g glass beads as described before (20). The content was vortexed for 5 min and centrifuged at 10000g for 5 min. The extraction procedure was repeated 3 times, and the supernatants were combined. The TcGrx was purified by nickel–nitrilotriacetic acid (Ni-NTA) affinity chromatography as per the instructions of the manufacturer (Qiagen) and then dialyzed as described before (20). The dialyzed sample was either used directly for analysis or stored at –20 °C until use. The protein concentration was determined by a Bio-Rad protein assay kit (Richmond, CA) using bovine serum albumin as a reference standard.

Molecular-Mass Determination via Electrospray Ionization Quadrupole-Time-of-Flight (ESI Q-TOF). The purified recombinant TcGrx (0.4 mg/mL) was prepared in 0.003 \times PBS containing 0.05 mM imidazole and 0.05% glycerol. The sample (5 μL) was used for molecular-mass determination using ESI Q-TOF mass spectrometry (Micromass, Manchester, U.K.).

Grx Activity Assay. Grx activity was determined by the HED [(HOCH₂CH₂)₂S₂] assay (21). The reaction mixture contained 100 mM Tris-Cl (pH 7.4), 0.6 μg of GR (glutathione reductase, Sigma), 0.3 mM HED, 0.2 mM NADPH, and 0.8 mM GSH in a total volume of 100 μL . A mixed disulfide between HED and GSH was formed within 2 min, and the reaction was started by the addition of 0.014–0.216 μg of TcGrx. The reaction rate was monitored by the decrease in A_{340} because of the oxidation of NADPH.

Dehydroascorbate Reductase (DHAR) Activity Assay (22). The DHAR assay method is same as the HED assay, except that 0.2 mM dehydroascorbate (DHA, Sigma) was used instead of HED as the substrate.

Peroxidase Activity Assay. This method is same as the HED assay, except that 0.3 mM hydrogen peroxide was used instead of HED as the substrate.

Kinetic Studies. The recombinant TcGrx protein was tested for Grx or DHAR activities at 25 °C by monitoring the consumption of NADPH at A_{340} for HED or DHA reduction as described above. The kinetic was studied using the recombinant TcGrx (0.18 μg) in a total volume of 100 μL .

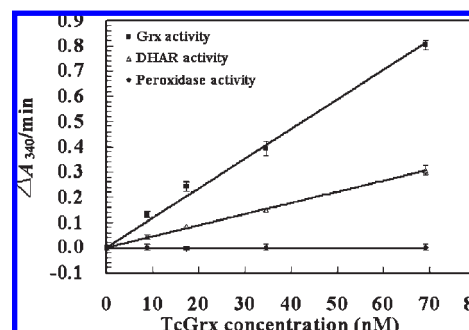


Figure 3. TcGrx possessing both Grx and DHAR activities. Linearity between TcGrx levels and the Grx and DHAR reaction rates was exhibited. Each activity was assayed and monitored the decrease in absorption at 340 nm as described in the text. Data are means of three experiments. TcGrx does not possess peroxidase activity.

at different concentrations of HED (0.3–2.4 mM) or different concentrations of DHA (0.2–3.0 mM). For each reaction, the NADPH was maintained at 0.2 mM and the initial rate was followed by measuring the decrease in A_{340} for 10–40 s. The extinction coefficient of NADPH at 340 nm is $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. K_m , V_{max} , and k_{cat} were calculated from Lineweaver–Burk plots.

TcGrx Activity Assay under Various Conditions. The enzyme sample was tested for Grx activity by the HED assay after various treatments. Aliquots of the TcGrx sample were treated as follows: (1) Thermal effect. The enzyme sample was heated to 100 °C for 2, 4, 8, or 16 min. (2) pH effect. The enzyme sample was adjusted to the 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 TcGrx enzyme was eluted with imidazole. Therefore, the effect of imidazole on protein activity was examined. Imidazole was added to the enzyme sample to the levels of 0.2, 0.4, or 0.8 M and incubated at 37 °C for 1 h. (4) Sodium dodecyl sulfate (SDS) effect. SDS, a denaturing reagent, was added to the enzyme sample to the levels of 0.5, 1, or 2% and incubated at 37 °C for 1 h. (5) Susceptibility to digestive proteases. The enzyme was incubated with one-tenth its weight of trypsin or chymotrypsin at pH 8.0 and 37 °C for a period of 5, 10, or 20 min. After each treatment, the Grx activity of the sample was tested by the HED assay.

RESULTS AND DISCUSSION

Cloning and 3D Homologous Modeling of TcGrx. Approximately 20 000 *T. camphorata* cDNA clones were sequenced. The nucleotide sequences and the inferred amino acid sequences were

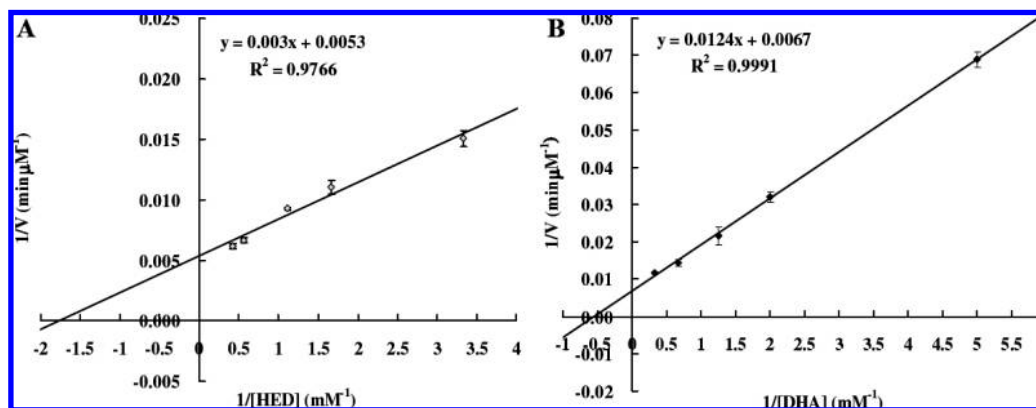


Figure 4. Double-reciprocal plots of varying HED and DHA on Grx and DHAR dual activities. The initial rate of the enzymatic reaction was measured at (A) 0.2 mM NADPH with the HED concentration varied from 0.3 to 2.4 mM and (B) 0.2 mM NADPH with the DHA concentration varied from 0.2 to 3.0 mM. K_m , V_{max} , and k_{cat} were calculated from the Lineweaver–Burk plots.

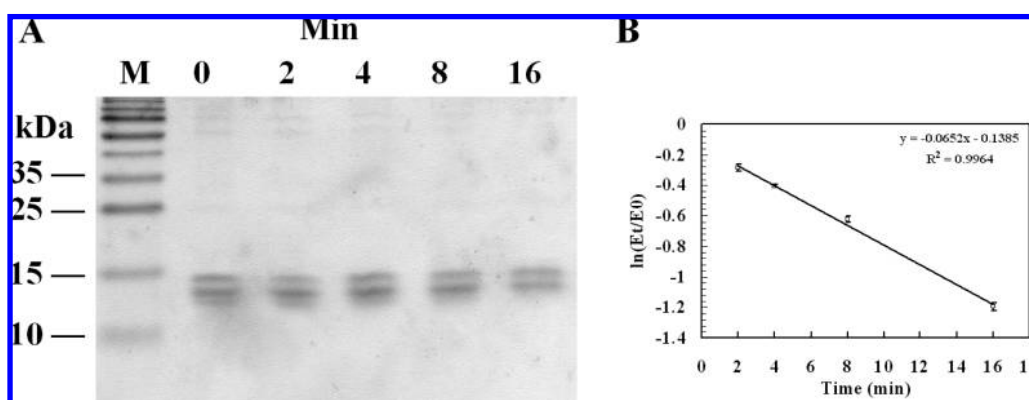


Figure 5. Effect of the temperature on the purified TcGrx. The enzyme sample was heated at 100 °C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8, or 16 min and analyzed by a 15% SDS–PAGE or assayed for enzyme activity by the HED assay. (A) Staining for protein (2.0 μ g of protein/lane) after separation on a 15% SDS–PAGE. (B) Plot of thermal inactivation kinetics of TcGrx activity (0.18 μ g of protein/time interval). E_0 and E_t are original activity and residual activity after being heated for different time intervals. Data are means of three experiments.

compared to the NCBI (www.ncbi.nlm.nih.gov) database using the BLAST programs. A putative TcGrx cDNA clone was identified by sequence homology to the published Grxs. The coding region of TcGrx cDNA was 306 bp that encodes a protein of 102 amino acid residues with a calculated molecular mass of 11 kDa (EMBL accession number EU193660). **Figure 1** shows the optimal alignment of the amino acid sequences of TcGrx with six other selected Grx sequences. This TcGrx shared 65% identity with AfGrx (*Aspergillus fumigatus*, XM_745266), 60% with BfGrx (*Botryotinia fuckeliana*, XM_001555685), 50% with RcGrx (*Ricinus communis*, Z49699), 56% with NcGrx (*Neurospora crassa*, EAA32349), 45% with PtGrx (*Populus tremula x Populus tremuloides*, AY833406.1, PDB ID 1z7p), and 41% with ScGrx (*Saccharomyces cerevisiae*, NP009895). The TcGrx enzyme appears to belong to the dithiol Grx subfamily because the characteristic catalytic motif C²⁵ PYC²⁸ (**Figure 1**) is conserved. The predicted secondary structure of TcGrx was represented as α helices and β strands (**Figure 1A**). The 3D structural model of TcGrx was created on the basis of the known structure of Grx C1 of *Populus tremula* (23), exhibiting a typical thioredoxin/glutaredoxin fold.

Expression and Purification of the Recombinant TcGrx. The coding region of TcGrx (306 bp) was amplified by PCR and subcloned into an expression vector, pET-20b(+), as described in the Materials and Methods. Positive clones were verified by DNA sequence analysis. The recombinant TcGrx was expressed as a 6His-tagged fusion protein and was purified by affinity

chromatography with nickel chelating Sepharose, and the proteins were analyzed by 15% SDS–polyacrylamide gel electrophoresis (PAGE). A band with a molecular mass of \sim 15 kDa (expected size of the His₆-TcGrx monomer) was detected in Ni–NTA eluted fractions as analyzed by SDS–PAGE (lanes 4–9 in **Figure 2**). Two TcGrx bands were seen in the affinity-purified protein (lanes 4–6 in **Figure 2** and **Figure 5A**). It is believed that Cys²⁵ and Cys²⁸ are far apart enough to form an intramolecular disulfide bond (**Figure 1B**). Therefore, it is possible that the lower band represented a more compact TcGrx with an intramolecular disulfide bond formed between Cys²⁵ and Cys²⁸. The fractions that contained pure protein were pooled and characterized further. An ESI Q-TOF of TcGrx reveals the presence of only the monomeric form of 15 kDa protein under the conditions of 0.003 \times PBS containing 0.005 mM imidazole and 0.05% glycerol. This indicates that the enzyme is monomeric in nature. The yield of the purified His₆-tagged TcGrx was 1.62 mg from 80 mL of culture. Functional TcGrx was detected by activity assays as described below.

TcGrx Displays Both Grx and DHAR Activities. The purified TcGrx protein was tested for Grx, DHAR, and peroxidase activities using HED, DHA, or hydrogen peroxide as the substrate, respectively. As shown in **Figure 3**, TcGrx possessed Grx activity because it can reduce disulfide bonds of HED. The TcGrx enzyme also possessed DHAR activity because it is able to catalyze the conversion of DHA to ascorbic acid (AsA). Both activities exhibit linearity between TcGrx levels and the reaction

Table 1. Kinetic Characterization of TcGrx and Comparison to Other Published Grxs^a

	<i>T. camphorata</i>	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>C. reinhardtii</i>	human
K_m (mM)	0.57	3.0	0.12	2.65	1.9
HED k_{cat} (min^{-1})	1633.0	6900	132	9696	19200
k_{cat}/K_m	2864.9	2300	1100	3658.9	10105
DHA K_m (mM)	1.85			0.39	
k_{cat} (min^{-1})	1291.8			100.2	
k_{cat}/K_m	698.3			256.9	

^a Values for Grx are from this work (*T. camphorata*) or from the literature [*E. coli* (24), *S. cerevisiae* (25), *C. reinhardtii* (22), and human (24)].

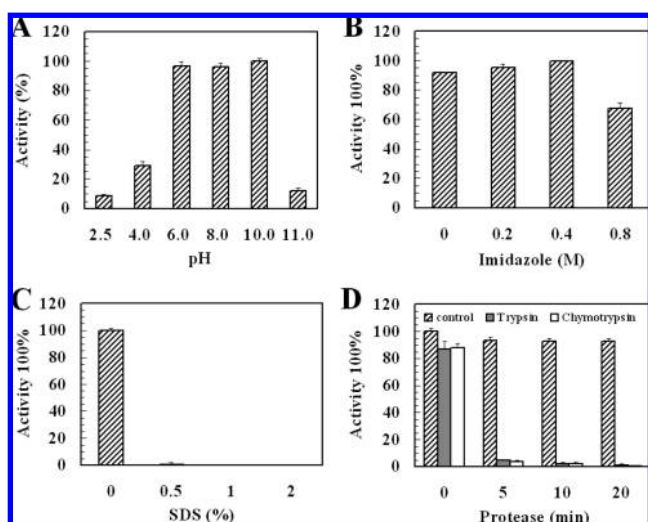


Figure 6. Effect of pH, imidazole, SDS, and trypsin or chymotrypsin on the purified TcGrx. (A) Enzyme samples were incubated with different pH buffer at 37 °C for 1 h and then assayed for TcGrx activity (0.18 μg of protein/pH point). (B) Enzyme samples were incubated with various concentrations of imidazole at 37 °C for 1 h and then checked for TcGrx activity (0.18 μg of protein/imidazole level). (C) Enzyme samples were incubated with various concentrations of SDS at 37 °C for 1 h and then checked for activity (0.18 μg of protein/SDS level). (D) Enzyme samples were incubated with trypsin or chymotrypsin at 37 °C for various times and then checked for TcGrx activity (0.18 μg of protein/time interval). Data are means of three experiments.

rate. As the TcGrx levels increased from 10 to 70 nM, both the Grx and DHAR reaction rates increased (Figure 3). DHAR activity has been reported in several Grxs, such as Grx from *Chlamydomonas reinhardtii* (22). However, the TcGrx did not possess peroxidase activity.

Kinetic Studies of the Purified TcGrx. As shown in panels A and B of Figure 4, the Lineweaver–Burk plot of the velocity ($1/V$) versus $1/[\text{HED}]$ gave $K_m = 0.57$ mM, $V_{\text{max}} = 188.68$ $\mu\text{M}/\text{min}$, and $k_{\text{cat}} = 1633.0$ min^{-1} for the Grx. The plot of the velocity ($1/V$) versus $1/[\text{DHA}]$ gave $K_m = 1.85$ mM, $V_{\text{max}} = 149.25$ $\mu\text{M}/\text{min}$, and $k_{\text{cat}} = 1291.8$ min^{-1} for the DHAR. Table 1 shows the comparison of the K_m , k_{cat} , and k_{cat}/K_m values from various organisms. As shown in Table 1, TcGrx has a relatively low K_m value for HED (0.57 mM), suggesting that TcGrx can reduce the disulfide bond efficiently. The TcGrx has a higher K_m value (1.85 mM) for DHA as compared to the enzyme from *C. reinhardtii* (0.39 mM), suggesting that the DHAR activity of TcGrx is less efficient.

Effects of the Purified TcGrx Activities under Various Conditions. The TcGrx activity was tested for its thermal and imidazole effects because the information is useful for developing enzyme purification protocols. To examine the thermo effect, the purified TcGrx was heat-treated as described in the Materials and

Methods and then analyzed by 15% SDS–PAGE or the HED assay. The enzyme activity appeared to be heat-stable. Approximately 50% of the TcGrx activity was retained at 100 °C for 8.5 min (Figure 5). The TcGrx activity was in a broad range of pH from 6.0 to 10.0, as shown in Figure 6A. The enzyme retained about 70% activity in 0.8 M imidazole (Figure 6B). The enzyme lost activity in 0.5% SDS (Figure 6C).

The TcGrx enzyme was tested for its susceptibility toward trypsin or chymotrypsin degradation. The results showed that only 10% of its Grx activity retained after 5 min of incubation at 37 °C with one-tenth its weight of trypsin or chymotrypsin (Figure 6D), suggesting that the TcGrx was quite susceptible to trypsin and chymotrypsin. The amino acid sequence of TcGrx as shown in Figure 1 contains 44 potential trypsin cleavage sites and 53 potential chymotrypsin high-specificity (C term to [FYW] and not before P) cleavage sites. Our results indicated that several of the cleavage sites are readily susceptible to trypsin and chymotrypsin. The protease tests were useful in understanding the effect of the digestive enzymes to the TcGrx and its suitability as a health food.

In conclusion, we have expressed, purified, and characterized TcGrx. The enzyme displays both Grx and DHAR activities, both of which are important antioxidants. The fruiting body of *T. camphorata* is well-known in Taiwan for treating cancer and inflammation. Little is known about the actions of its biological effects. This TcGrx may be one of the important physiological components in *T. camphorata* responsible for its medicinal efficacy. The availability of the TcGrx clone will facilitate further investigation in its medical applications.

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