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# A New Member of Human TSA/AhpC as Thioredoxin-dependent Thiol Peroxidase

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**A new type of the human TSA homologous gene was cloned from a HeLa cell cDNA and characterized. The gene product consists of 161 amino acids with a molecular mass of 16,900. The TSA homologous protein, as a new 6th member of the human TSA (hTSA VI), exerted a thiol-dependent peroxidase activity with the use of thioredoxin system as a physiological electron donor. The values of $V_{max}/K_m$ of hTSA VI for $H_2O_2$ and t-butyl hydroperoxide (t-BOOH) were calculated as $5.53 \times 10^{-2}$ and $3.70 \times 10^{-2}$, respectively. This implies that hTSA VI is a peroxidase, which reduces $H_2O_2$ and t-BOOH. The mutation of $Cys^{47}$ to serine resulted in a complete loss of the peroxidase activity. This suggests that $Cys^{47}$ acts as a primary site of catalysis. The analysis of the tryptic digest derived from hTSA VI revealed that the $Cys^{47}$ exists as a free thiol form. Taken together, these results suggest that the TSA homologous protein is a new type of the human family, which exerts thioredoxin-linked peroxidase activity toward $H_2O_2$ and alkyl hydroperoxide.**

**Keywords:** Antioxidant, Functional cysteine, New human thiol peroxidase, Reactive oxygen species, Thioredoxin.

## Introduction

Living organisms produce a reactive oxygen species (ROS) during physiological processes, such as respiration. This is in response to external stimuli such as UV radiation. Cells have evolved antioxidant defenses to protect them from damage of cellular constituents by ROS (Scandalios. 1997). Among these, cytosolic and mitochondrial superoxide dismutases eliminate the superoxide radical; cytosolic and peroxisomal catalases remove $H_2O_2$; and glutathione peroxidases reduce both $H_2O_2$ and alkyl hydroperoxides (Scandalios. 1997; Fridovich, 1978; Sies, 1993). A new type of peroxidase was first identified in *Saccharomyces cerevisiae* (Kim *et al.*, 1988; Chae *et al.*, 1993; Kim *et al.*, 1989). Instead of an active site of selenocysteine within the glutathione peroxidase, they have a cysteine within the TSA as a primary site of catalysis. In order to discriminate the difference between the peroxidase activities of TSA and GSH peroxidase, we called the peroxidase activity exerted by TSA "thiol peroxidase activity". The TSA homologues protein, referred to as a TSA/AhpC family, has been discovered from prokaryotes to eukaryotes (Kim *et al.*, 1989; Jacobson *et al.*, 1989; Lim *et al.*, 1994; Chae *et al.*, 1994; Cha and Kim, 1995; Sauri *et al.*, 1995; Cha *et al.*, 1995, 1996; Lim *et al.*, 1994; Kang *et al.*, 1998; Jeong *et al.*, 1999; Kwon *et al.*, 1994; Chae and Rhee, 1994; Chae *et al.*, 1999). At least, five types of TSA isoenzyme have been identified in human tissue (Lim *et al.*, 1994; Cha and Kim, 1995; Sauri *et al.*, 1995; Lim *et al.*, 1994). These enzymes reduced hydroperoxides with electrons that were donated by NADPH via thioredoxin (Trx), or other thiol-containing intermediate.

Yeast TSA exists as a homodimer and contains two essential cysteine residues, $Cys^{47}$ and $Cys^{170}$. The $Cys^{47}$-SH group is the primary site of oxidation by $H_2O_2$. The oxidized $Cys^{47}$ (probably Cys-SOH) rapidly reacts with $Cys^{170}$-SH of the other subunit to form an intermolecular disulfide (Chae *et al.*, 1994). The disulfide is reduced by the Trx system. Most members of the TSA/AhpC family contain two conserved cysteines that correspond to $Cys^{47}$ and $Cys^{170}$ of yeast TSA. However, a limited number of the TSA/AhpC family members, including *Escherichia coli* p20 and one type of human TSA, (named HORF6), contain one of the conserved cysteine residues that corresponds to the $Cys^{47}$ of yeast TSA. These exist as a monomer (Cha *et al.*, 1995; Kang *et al.*, 1998). Thus, members of the TSA/AhpC family can be divided into two subgroups, such as 1-Cys (HORF6) and 2-Cys (yeast TSA).

Recently, one additional human TSA protein was reported (Kang *et al.*, 1990). However, its kinetic properties and *in vivo* electron donor to the human TSA remain unsolved. In this paper, we characterized a new human TSA-homologous gene

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product. The TSA-homologous protein, designated as hTSA VI, was shown to be a new member of the human TSA as a 6th human, which reduces hydroperoxides with Trx as an electron donor. Also, the new type of human TSA contains one free cysteine residue that acts as a primary site of catalysis. The catalytic mechanism is discussed on the basis of a new type of TSA having a free cysteine as a functional thiol.

## Materials and Methods

**Cloning and mutagenesis of the gene coding for human TSA VI** The DNA sequence that corresponds to hTSA VI was obtained by the polymerase chain reaction (PCR) from a HeLa cell cDNA library using the forward primer (5-GGAA TTC CAT ATG GCC CCA ATC AAG GTG GGA G-3) It contained an *Nde*I (underlined) site and the initiation codon (boldface type). The reverse primer (5-CGC GGA TCC **TCA** GAG CTG TGA GAT GAT ATT G-3) contained the *Bam*HI site (underlined) and the stop codon (boldface type). The amplified products were purified and digested with *NdeI*/*Bam*HI. The digested fragments were subcloned into the T7 expression vector (pT7-7) and digested with *NdeI*/*Bam*HI. The resulting plasmid was used to transform the *E. coli* strain BL21 (DE3).

The C47S mutant protein, in which $Cys^{47}$ was replaced by serine, was generated by a standard PCR-mediated site-directed mutagenesis with complementary primers. It contained a 1-base pair mismatch that converts the codon for cysteine to one for serine.

**Expression and Purification of human hTSA VI and its C47S mutant** Transformed cells were cultured at 37°C overnight in a LB medium that was supplemented with ampicillin (100 μg/ml). It was then transferred to a fresh medium to the ratio of 1 to 200. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to the final concentration of 0.5 mM. After induction for 3 hours, cells were harvested by centrifugation and stored at -70°C until use.

Frozen cells were suspended in 50 mM Tris-HCl (pH 7.6) containing 2 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 mM EDTA, then disrupted by sonication. The supernatants clarified by centrifugation were loaded to a Q-Sepharose column that had been equilibrated with 50 mM Tris-HCl (pH 7.6). The unbound fractions were pooled and dialyzed against 50 mM Tris-HCl (pH 9.2). After dialysis, samples were applied to the Q-Sepharose column that had been equilibrated with 50 mM Tris-HCl (pH 9.2). The samples that were eluted with a linear gradient of NaCl, dialyzed against 10 mM Tris-HCl (pH 7.4) and stored at -70°C.

**Determination of thiol-dependent antioxidant activity** The thiol-dependent antioxidant activity was determined by measuring the activity needed to protect the inactivation of *E. coli* glutamine synthetase (GS) by a thiol metal-catalyzed oxidation system ($DTT/Fe^{3+}/O_2$) (thiol MCO system), as described previously [4,21]. Instead of DTT, an ascorbate was included as a non-thiol-reducing equivalent (non-thiol MCO system). The 30-μl reaction mixture (containing 100 mM Hepes-NaOH (pH 7.0), 1.0 μg of GS, 3 μM $FeCl_3$, of various concentrations of hTSA VI and either 10 mM DTT or 10 mM ascorbate) was incubated at 37°C and then 0.5 ml of a γ-glutamyltransferase assay mixture was added. After incubation at 37°C for 10 min, the remaining activity of GS was determined by measuring the absorbance at 540 nm. To determine the thioredoxin (Trx)-linked antioxidant activity of hTSA VI, the GS activity was measured in the reaction mixture containing 100 mM Hepes-NaOH (pH 7.0), 1.0 μg of GS, 3 μM $FeCl_3$, various concentrations of FC-TSA, 10 mM ascorbate, 3.6 μM Trx, 1.3 μM thioredoxin reductase (TR), and 0.5 mM NADPH.

**Determination of Trx-linked Peroxidase Activity of human hTSA VI** The peroxidase reaction was performed in a 400-μl reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.8 μM Trx, 0.3 μM TR, 0.26 mM NADPH, various concentrations of hTSA VI, and 0.1 mM $H_2O_2$ or 0.1 mM t-butyl hydroperoxide (t-BOOH) at 30°C. At the appropriate time, 20 μl of the reaction mixture was added to the 980 μl FOX1 reagent and then incubated at room temperature for a minimum of 30 min. At this time the color development is virtually complete. The remaining peroxides were monitored at 560 nm (Wolff, 1994). The peroxidase activity that is linked to the NADPH oxidation was monitored as the $A_{340}$ in a 400-μl of reaction mixture (containing 50 mM Hepes-NaOH (pH 7.0), 0.8 μM Trx, 0.3 μM TR, 1.2 μM hTSA VI, 0.2 mM NADPH and various concentrations of $H_2O_2$ or t-BOOH) decreased.

**Chemical modification of hTSA VI with iodoacetate** Protein was preincubated in the absence, or presence of 5 mM DTT at 30°C for 30 min. Then the chemical modification was carried out in a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.4 mg/ml hTSA VI and 10 mM iodoacetate at 30°C for 2 hours as previously described (Lundblad and Noyes, 1994). The remaining reagents were removed by an overnight-dialysis against 10 mM Tris-HCl (pH 7.4) at 4°C.

**HPLC separation of tryptic peptides** The hTSA VI protein was reductively or oxidatively denaturated by 200 mM Tris buffer (pH 8.0) containing 3 M guanidine-HCl in the presence or absence of 1 mM DTT. The cysteinyl thiol was labeled in the presence of 3 M guanidine chloride by an excess amount of DTNB (5.5-dithiobis-(2-nitrobenzoic acid)). The DTNB was used to identify the tryptic peptides containing the SH group. The resulting protein was twice washed with acetone, and the precipitate was suspended in a 100 mM Tris-HCl buffer, pH 8.0. Then the protein was digested with trypsin for 3 hr at 30°C. The additional digestion with fresh trypsin was carried out overnight. The tryptic peptides were separated by a reversed phase $C_{18}$ column (Kromasil, 4.6 mm × 250 mm) with a linear gradient of 10-45% acetonitrile in 0.1% trifluoroacetic acid for over 45 min at a 1 ml/min of flow rate. The peptides were detected using a photodiode array detector (Shimadzu SPD-M10$A_{vp}$) and identified by N-terminal sequencing.

## Results

**Cloning and Sequencing of hTSA VI gene** The gene of

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ATG GCC CCA ATC AAG GTG GGA GAT GCC ATC CCA GCA GTG GAG GTG   45
Met Ala Pro Ile Lys Val Gly Asp Ala Ile Pro Ala Val Glu Val   15

TTT GAA GGG GAG CCA GGG AAC AAG GTG AAC CTG GCA GAG CTG TTC   90
Phe Glu Gly Glu Pro Gly Asn Lys Val Asn Leu Ala Glu Leu Phe   30

AAG GGC AAG AAG GGT GTG CTG TTT GGA GTT CCT GGG GCC TTC ACC  135
Lys Gly Lys Lys Gly Val Leu Phe Gly Val Pro Gly Ala Phe Thr   45

CCT GGA TGT TCC AAG ACA CAC CTG CCA GGG TTT GTG GAG CAG GCT  180
Pro Gly Cys Ser Lys Thr His Leu Pro Gly Phe Val Glu Gln Ala   60

GAG GCT CTG AAG GCC AAG GGA GTC CAG GTG GTG GCC TGT CTG AGT  225
Glu Ala Leu Lys Ala Lys Gly Val Gln Val Val Ala Cys Leu Ser   75

GTT AAT GAT GCC TTT GTG ACT GGC GAG TGG GGC CGA GCC CAC AAG  270
Val Asn Asp Ala Phe Val Thr Gly Glu Trp Gly Arg Ala His Lys   90

GCG GAA GGC AAG GTT CGG CTC CTG GCT GAT CCC ACT GGG GCC TTT  315
Ala Glu Gly Lys Val Arg Leu Leu Ala Asp Pro Thr Gly Ala Phe  105

GGG AAG GAG ACA GAC TTA TTA CTA GAT GAT TCG CTG GTG TCC ATC  360
Gly Lys Glu Thr Asp Leu Leu Leu Asp Asp Ser Leu Val Ser Ile  120

TTT GGG AAT CGA CGT CTC AAG AGG TTC TCC ATG GTG GTA CAG GAT  405
Phe Gly Asn Arg Arg Leu Lys Arg Phe Ser Met Val Val Gln Asp  135

GGC ATA GTG AAG GCC CTG AAT GTG GAA CCA GAT GGC ACA GGC CTC  450
Gly Ile Val Lys Ala Leu Asn Val Glu Pro Asp Gly Thr Gly Leu  150

ACC TGC AGC CTG GCA CCC AAT ATC ATC TCA CAG CTC TGA  489
Thr Cys Ser Leu Ala Pro Asn Ile Ile Ser Gln Leu ***  162
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**Fig. 1.** Nucleotide sequence of the hTSA VI gene and its deduced amino acid sequence. Three cysteine residues are shaded and a putative peroxisomal sorting sequence is underlined. The gene has been assigned EBBL nucleotide sequence data base accession number, AJ249483.

the hTSA VI was cloned from a HeLa cell cDNA library by PCR using a partially identified sequence. The complete nucleotide sequence of a gene encoding hTSA VI, and a deduced amino acid sequence, are shown in Fig. 1. A 486-bp open reading frame encodes a polypeptide of 162 amino acids with a molecular mass of 17,030 Daltons. The hTSA VI contains 3 cysteine residues at the positions 47, 72 and 151 from the N-terminal. It also carries the C-terminal peroxisomal-like sorting sequence (SQL) (Gould and Keller. 1988) that is similar to the putative peroxisomal signal sequence S(A)K(H/R)L(I). A sequence comparison with the previously identified five types of human isoenzyme showed that $Cys^{47}$ is only conserved and a substantial homology is confined in a very short sequence surrounding the $Cys^{47}$ (Fig. 2).

**Thioredoxin-linked antioxidant activity of hTSA VI** In the presence of an electron donor, such as DTT or an ascorbate, $Fe^{3+}$ catalyzes the reduction of $O_2$ to $H_2O_2$. This is further converted to a hydroxyl radical by the Fenton reaction. Both the thiol MCO system (DTT, $Fe^{3+}$ and $O_2$) and the non-thiol MCO system (ascorbate, $Fe^{3+}$ and $O_2$) therefore inflict damage on the various enzymes including GS. This damage can be prevented by peroxidase that eliminates $H_2O_2$. All TSAs have protected GS from damage by the DTT oxidation system, but not by the ascorbate system. Human TSA VI was expressed in *E. coli* and homogeneously purified. Whether or not this protein protects GS from damage induced by the thiol or non-thiol MCO systems is being investigated. Fig. 3 revealed that the protein protected GS from the inactivation by the DTT oxidation system, but not from the ascorbate system. This suggests a thiol-dependent antioxidant activity of hTSA VI. It was suggested that Trx is one of the physiological

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hTPx VI  MAPI----------                                          4
hPAG     MSS-----------                                          3
hTSA     MAS-----------                                          3
hMER5    MAAAVGRLLRASVA------RHVSAIP-------------WGISATAALRP   32
hAOE372  MEAL--PLLAATTPDHGRHRRLLLLPLLLFLLPAGAVQGWETEERPRTRE   48
hORF6    MPGG-------------------------LLL-----------------      7
         *

hTPx VI  ------------------------------KVGDAIP---AVEVFEGEP   20
hPAG     ------------------------------GNAKICQPAPNFKATAVMPDGQ   25
hTSA     ------------------------------GNARIGKPAPDFKATAVV-DGA   24
hMER5    AACGRTSLTNLLCSGSSQAKLFSTSSSCHAPAVTQHAPYFKGTAVV-NGE   81
hAOE372  EECHFYAGGQVYPGEASRVSVADHSLHLSKAKISKPAPYWEGTAVI-DGE   97
hORF6    ------------------------------GDVAPNFEANTTVGRIR   24
                                          .. *  . ..

hTPx VI  GNKVNLAELFKGKKGVLFGVPGAFTPGCSKTHLPGFVEQAEALKAKGVQV   70
hPAG     FKHISLSD-YKGKYVVFFFYPLDFTFVCP-SEIIAFSDRAEEFKKLNCQV   73
hTSA     FKEVKLSD-YKGKYVVLFFYPLDFTFVCP-TEIIAFTTVKRTSAKLGCEV   72
hMER5    FKDLSLDD-FKGKYLVLFFYPLDFTFVCP-TEIVAFSDKANEFHDVNCEV  129
hAOE372  FKELKLTD-YRGKYLVFFFYPLDFTFVCP-TEIIAFGDRLEEFRSINTEV  145
hORF6    FHDF-LGD-SWG---ILFSHPRDFTPVCT-TELGRAAKLAPEFAKRNVKL   68
           ..  *..   *   ..*  * .**  *. ...   .  .   . ..

hTPx VI  VACLSVNDAFVTGEWGR---AHKAEG-----KVRLLADPTGAFGKETDLL  112
hPAG     IG-ASVDSHFCHLAW---VNTPKKQGGLGPMNIPLVSDPKRTIAQDYPVL  119
hTSA     LG-VSVDSQFTHLAW---INTPRKEGGLGPLNIPLLADVTRRLSEDYGVL  118
hMER5    VA-VSVDSHFSHLAW---INTPRKNGGLGHMNIALLSDLTKQISRDYGVL  175
hAOE372  VA-CSVDSQFTHLAW---INTPRRQGGLGPIRIPLLSDLTHQISKDYGVY  191
hORF6    IA-LSIDSVEDHLAWSKDINAYNCEEPTEKLPFPIIDDRNRELAILLGML  117
           .. *...    .*    . . ..      ...* . ..   .

hTPx VI  ----------LDDSLVSIFGNRRLKRFSMVVQDGIVKALNVE-------  144
hPAG     ----KADEGISF--RGLFIIDDKGILRQITVNDLPVGRSVDETLRLVQAF  163
hTSA     ----KNDEGIAY--RGLFIIDGKGVLRQITVNDLPVGRSVDEALRLVQAF  162
hMER5    ----LEGSGLAL--RGLFIIDPNGVIKHLSVNDLPVGRSVEETLRLVKAF  219
hAOE372  ----LEDSGHTL--RGLFIIDDKGILRQITLNDLPVGRSVDETLRLVQAF  235
hORF6    DPAEKDEKGMPVTARVVFVFGPDKKLKLSILYPATTGRNFDEILRVVISL  167
                      . . ...   .  .   . .. .

hTPx VI  --PDGTGLTCSLA----------PNI----------------------  158
hPAG     QFTDKHGEVCPAGWKPGSDTIK----PDVQKSKEY----FSK--------  196
hTSA     QYTDEHGEVCPAAWKPGRDTIK----PNVDDSKEY----FSK--------  196
hMER5    QYVETHGEVCPANWTPDSPTIK----PSPAASKEY----FQKV-------  254
hAOE372  QYTDKHGEVCPAGWKPGSETII----PDPAGKLKY----FDKL-------  270
hORF6    QLTAEKRVATPVDWKDGDSVMVLPTIPEEEAKKLFPKGVFTKELPSGKKY  217
                .  . . .

hTPx VI  ---ISQL  162
hPAG     -----QK  198
hTSA     -----HN  198
hMER5    -----NQ  256
hAOE372  -----N-  271
hORF6    LRYTPQP  224
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**Fig. 2.** Amino acid sequence alignment of 6 types of human TSA. Asterisk (*) and dot (●) indicate perfectly conserved and well-conserved position, respectively. The highly conserved N-terminal cysteine residues in VCP 1 region are shaded. The putative peroxisomal signal sequence (SQL) is underlined. The N-terminal conserved cysteine in VCP 2 region for 2-Cys TSA was underlined. Abbreviations: hTSA VI, a human TSA VI (accession no AJ249483.); PAG, proliferation-associated gene, named NKEF A (accession no. X67951); TSA, thiol-specific antioxidant protein from human brain. named hTSA II, HPRP, or NKEF B (accession no. Z22548 and L14286); MER5, MER5-like protein (accession no. D49396); AOE372, antioxidant enzyme AOE37-2 (accession no. U25182); ORF6, KIAA0106 gene (accession no. D14662).

electron donors for the catalytic function of TSA (Cha and Kim, 1995; Kwon *et al.*, 1994). Therefore, we examined the capability of hTSA VI to protect GS activity from inactivation by a non-thiol MCO system in the presence of the Trx system as a thiol electron donor. Taking advantage of the fact that the ascorbate oxidation system can inactivate GS, but not provide electrons required for the peroxidase function of TSA, we measured the remaining GS activity after incubation with a mixture of the ascorbate oxidation system with the Trx system. The GS protection activity of hTSA VI (in the

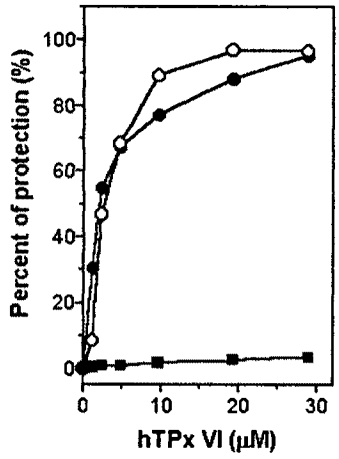


**Fig. 3.** Protection of glutamine synthetase by hTSA VI against the DTT or ascorbate oxidation system. Glutamine synthetase (1 μg) was inactivated in a 30-μl reaction mixture containing 100 mM Hepes-NaOH (pH 7.0), 3 μM $FeCl_3$, the indicated concentration of hTSA VI, 10 mM DTT (the DTT oxidation system: ○) or 10 mM ascorbate (the ascorbate oxidation system: ■). In the Trx system (●), 3.6 μM Trx, 1.3 μM TR, 0.5 mM NADPH and 10 mM ascorbate instead of 10 mM DTT were added to the reaction mixture. After inactivation for 14 min at 37°C, the residual glutamine synthetase activity was measured as described in "Experimental Procedure".

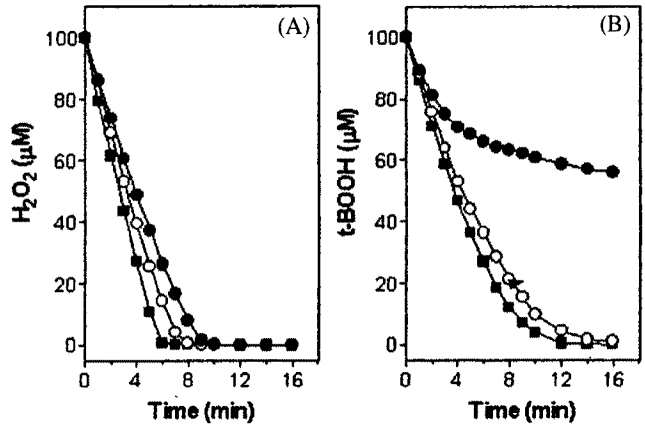


**Fig. 4.** Removal of $H_2O_2$ (A) and t-BOOH (B) by hTSA VI in the presence of Trx, TR and NADPH at 30°C. Peroxidase reaction was carried out in a 400-μl of reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.8 μM Trx, 0.3 μM TR, 0.26 mM NADPH, various concentrations of hTSA VI (●, 0.6 μM; ○, 1.2 μM; ■, 2.4 μM) and either 0.1 mM $H_2O_2$ (A) or 0.1 mM t-BOOH (B) at 30°C. At the indicated time, the remaining peroxide was measured by using FOX1 reagent as described.

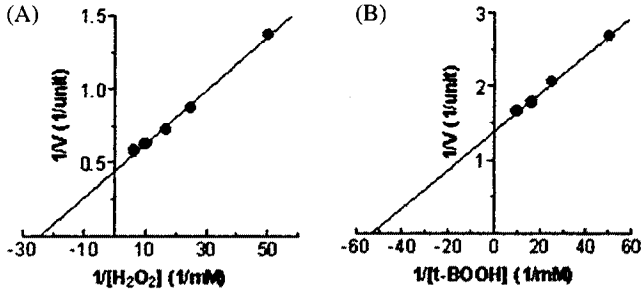


**Fig. 5.** Lineweaver-Burk plots of the initial rate of removal of $H_2O_2$ (A), t-BOOH (B) by hTSA VI. The initial rates of the peroxide removal were determined directly by measuring the amounts of peroxides reduced by hTSA VI in the presence of Trx system for 3 min at room temperature. One unit of peroxidase activity represents that 1 mg of hTSA VI can reduce 1 μmol of peroxide for 1 min.

presence of the Trx system) was similar to that in the presence of DTT (Fig. 3). These results suggest that hTSA VI is a new type of human TSA exerting a thiol-dependent antioxidant activity supported by Trx.

To investigate the kinetic property of hTSA VI, we monitored indirectly the peroxidase activity toward $H_2O_2$ and t-BOOH in the presence of the Trx system by following the decrease in $A_{340}$ that resulted from the oxidation of NADPH (data not shown). The rate of the peroxidase-dependent NADPH oxidation increased with the concentration of hTSA VI. To confirm the Trx-linked thiol peroxidase activity of hTSA VI, we measured the peroxidase activity of hTSA VI toward $H_2O_2$ and t-BOOH by directly following the removal of peroxides in the presence of the Trx system. The removal rate increased as a function of the hTSA VI concentration (Fig. 4). At the equivalent concentration of enzyme, hTSA VI was removed quicker with $H_2O_2$ than t-BOOH. The initial rate of the reduction of peroxide by hTSA VI was measured at varying concentrations of $H_2O_2$ and t-BOOH. The Lineweaver-Burk plots showed that the $K_m$ values for $H_2O_2$ and t-BOOH are 41.6 and 18.9 μM, respectively. The $V_{max}$ values for $H_2O_2$ and for t-BOOH are 2.3 and 0.7 μmol/min/mg, respectively (Fig. 5). The values of $V_{max}/K_m$ of hTSA VI for $H_2O_2$ and t-BOOH were calculated to be $5.53 \times 10^{-2}$ and $3.70 \times 10^{-2}$, respectively. This implies that hTSA VI has the capability to equally reduce $H_2O_2$ and t-BOOH. Taken together, these results suggest that hTSA VI is a new type of TSA that shows a thioredoxin-supported thiol peroxidase.

**Functional cysteine residues of Human hTSA VI** Most TSA proteins contain two conserved cysteines that correspond to $Cys^{47}$ and $Cys^{170}$ of yeast TSA [5]. However, several TSA, such as *E. coli* p20 (Cha and Kim, 1995; Cha *et al.*, 1996) and human ORF6 (Cho *et al.*, 1998), contain only one conserved cysteine residue that corresponds to the $Cys^{47}$ of yeast TSA. Yeast TSA exists as a dimer in which the conserved $Cys^{47}$ is linked to the $Cys^{170}$ of the second subunit via intermolecular disulfide bonds (Chae *et al.*, 1994). The *E. coli* p20 (Cha *et al.*, 1995) and human 1-Cys TSA (hORF6); however, exists as a monomer (Kang *et al.*, 1998). Human TSA VI contains three cysteine residues at the positions of 47, 72 and 151 from the N-terminal (see Fig. 1). Of the three cysteines, the conserved cysteine residue ($Cys^{47}$) was replaced with serine in

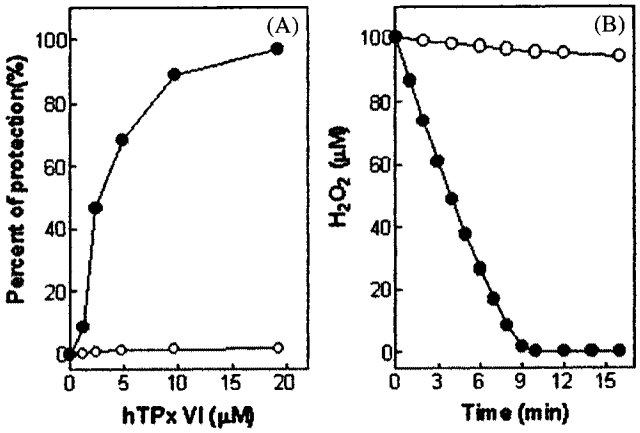


**Fig. 6.** Antioxidant and peroxidase activities of wild-type hTSA VI (●) and C47S (○). (A) Protection of glutamine synthetase by wild type and C47S against the DTT oxidation system. (B) Thioredoxin-linked peroxidase activity of wild-type and C47S. Removed $H_2O_2$ by wild-type hTSA VI (0.6 μM) and its C47S mutant (2.4 μM) in the presence of 0.8 μM Trx, 0.3 μM TR, and 0.26 mM NADPH was measured with FOX1 reagent.

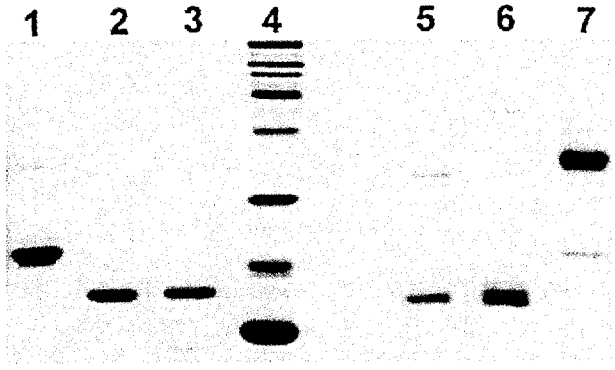


**Fig. 7.** Analysis of wild type hTSA VI and C47S on SDS-PAGE. For the non-reducing SDS-PAGE analysis (lanes 5, 6, 7), protein samples were mixed with a sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol), heated at 95°C for 5 min. For the reducing SDS-PAGE analysis (lanes 1-4), samples were mixed with the sample buffer containing mercaptoethanol. The resulting samples were analyzed on 14% SDS-PAGE. Lane 1 and 7 represent human TSA, a 2-Cys TSA (see Fig. 2). Lanes 2 and 6, hTSA VI mutant (C47S); lanes 3 and 5, hTSA VI. Lane 4 shows size markers (14.5-, 21.5-, 31.5-, 45-, 66-, 97.4-, 116-, and 200-kDa from the bottom).

order to identify the essential cysteine residue for peroxidase activity. The recombinant mutant (C47S) and wild-type proteins were expressed in *E. coli* and homogeneously purified from the soluble fraction of cell lysates. To identify whether or not $Cys^{47}$ is an active cysteine residue, we evaluated the ability of C47S to protect glutamine synthetase against damage by the thiol MCO system (Fig. 6A). The C47S protein revealed no GS protection activity. The peroxidase activity was determined directly by measuring the removal of $H_2O_2$ in the presence of the Trx system. It also

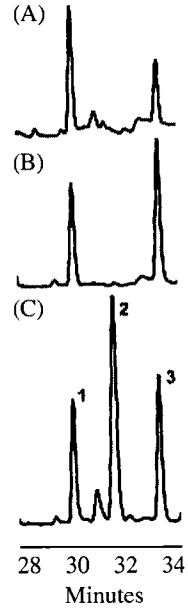


**Fig. 8.** HPLC separation of the tryptic peptides of hTSA VI and its C47S mutant proteins. Panels A and B show the peptide profiles for the tryptic digests of hTSA VI and C47S proteins detected at 328 nm. The profiles, B and C, represent the HPLC profiles from tryptic digests of the modified C47S and its wild proteins with DTNB, respectively, after preincubation with DTT in the presence of 3 M guanidine chloride. The profile A indicates the profile of tryptic digest derived from the wild-type protein, which is modified with DTNB in the absence of DTT.

showed that the C47S protein is completely inactive (Fig. 6B). These results suggested that the highly conserved $Cys^{47}$ acts as a primary catalysis in the reduction reaction of peroxide. The purified proteins were analyzed on SDS-PAGE under reducing (with DTT) and non-reducing (without DTT) conditions (Fig. 7). The C47S protein and its wild protein were detected at the same molecular sizes, corresponding to its monomer regardless of the presence or absence of DTT. This suggests that, in contrast to the 2-Cys TSA subfamily such as human TSA (see Fig. 2 and Fig. 7), hTSA 6 exists as a monomer. The minor formation of a dimer form of hTSA VI detected in non-reducing SDS-PAGE (Fig. 7) could be interpreted as the result of the self-condensation of cysteine residues during the SDS-PAGE sample preparation. To investigate the possibility of the existence of a disulfide bond in hTSA VI, we determined the disulfide in both proteins. Both proteins were modified with DTNB, a thiol-specific modification reagent in the presence of guanidine, and the resulting proteins were completely digested with trypsin. The tryptic digests were separated on a reversed phase HPLC column (Fig. 8). As expected, on the basis of the deduced amino acid sequence, three TNB-conjugated tryptic peptides were detected in the tryptic digestion that was derived from the hTSA VI protein, which reacted with the modification reagent in the presence of DTT and 3 M guanidine chloride

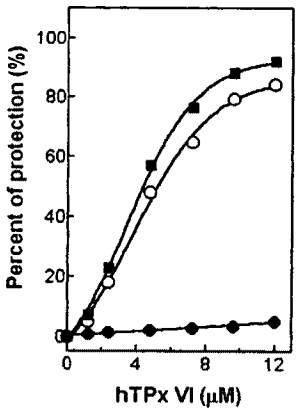


**Fig. 9.** The Chemical modification of hTSA VI with iodoacetate. After protein was incubated without (○) and with (●) 5 mM DTT at 30°C for 30 min, chemical modification was carried out in 100 μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.4 mg/ml hTSA VI and 10 mM iodoacetate at 30°C for 2 hours. The reaction mixture was dialyzed against 10 mM Tris-HCl (pH 7.4) and then glutamine synthetase protection activities of modified hTSA VI against the DTT oxidation system were determined. Symbol (■) represents the control experiment.

(profile C in Fig. 8). Two TNB-conjugated peptides (peaks 1 and 3) were detected in the profile of the tryptic digestion of the modified C47S protein that was in the same reaction condition (profile B in Fig. 8). The absence of the peptide peak 2 in the profile from the C47S protein suggests that peptide 2 in the C profile is the peptide containing $Cys^{47}$. Peptide 2 was also identified as the peptide containing $Cys^{47}$ by the N-terminal sequencing (i.e., $^{34}$GVLFGVPGAFTPGXS K[49]). Two TNB-conjugated peptides corresponding to peaks 1 and 3 were also detected in the profile of the tryptic digestion of the modified C47S protein in the absence of DTT (data not shown). This would suggest that $Cys^{71}$ and $Cys^{151}$ exist as free SH forms. We, therefore, would expect that $Cys^{47}$ should exist in a free SH form, which can be modified with DTNB in the absence of DTT. However, the corresponding tryptic peptide profile (profiles A and C in Fig. 8) shows that $Cys^{47}$ was modified in the reduced reaction condition, not in the oxidative reaction. It was previously reported that the functional Cys of 1-Cys TSA, such as ORF6, exists in an oxidized form (i.e., cysteine sulfenic acid, Cys-SOH), which can easily be reduced to Cys-SH by DTT (Kang *et al.*, 1995; Cho *et al.*, 1998) 25-26). The functional Cys of NADH peroxidase also exists as the Cys-SOH (Parsonage *et al.*, 1993). To gain insight into the nature of the functional cysteine residue ($Cys^{47}$), we reacted hTSA VI with a thiol-specific modification reagent, iodoacetate, in the absence or presence of DTT (Fig. 9). The modification of hTSA VI in the presence of DTT resulted in complete loss of antioxidant activity; whereas hTSA VI was not inactivated by iodoacetate in the absence of DTT. This indicates that Cys47 is modified, but readily converted to free SH by DTT. Therefore, our

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hTPx VI      MAPIKVGDAIPA----------VEVFE-GEPGNKVNLAELFKGKKGVLFGVPGAFTP 46
AOPP_MOUSE   MAPIKVGDAIPS----------VEVFE-GEPGKKVNLAELFKGKKGVLFGVPGAFTP 46
AAD28242.1.  MAPIAVGDVVPDGT---------ISFFDENDQLQTASVHSLAAGKKVILFGVPGAFTP 49
PM20_YEAST   MSDLVNKKFPAGDYKPQYIAISQSDADSESCKMPQTVEWSKLISENKKVIITGAPAAFSP 60
PM20_ASPFU   MSGLKAGDSFPSDVVFSYIPWSEDKGEITACGIPINYNASKEWAD-KKVILFALPGAFTP 59
             *: :  . .          .                    ** :: . *.**:*

hTPx VI      GCSKTHLPGFVEQAEALK-AKGVQVVACLSVNDAFVTGEWGRATRRKA--RFGSLADPTG 103
AOPP_MOUSE   GCSKTHLPGFVEQAGALK-AKGAQVVACLSVNDVFVIEEWGRAHQAEG--KVRLLADPTG 103
AAD28242.1.  TCSMKHVPGFIEKAEELK-SKGVDEIICFSVNDPFVMKAWGKTYPENK--HVKFVADGSG 106
PM20_YEAST   TCTVSHIPGYINYLDELVKEKEVDQVIVVTVDNPFANQAWAKSLGVKDTTHIKFASDPGC 120
PM20_ASPFU   VCSARHVPEYIEKLPEIR-AKGVDVVAVLAYNDAYVMSAWGKANQVTG-DDILFLSDPDA 117
             *:  *:* :::   :  * .: :  .: :: :.  *.::       .  :*

hTPx VI      AFGKETDLLLDDSLVSIFGNRRLKRFSMVVQDGIVKALNVEP-DGTGLTCSLAPNIISQL 162
AOPP_MOUSE   AFGKATDLLLDDSLVSLFGNRRLKRFSMVIDNGIVKALNVEP-DGTGLTCSLAPNILSQL 162
AAD28242.1.  EYTHLLGLELDLKDKGLG--VRSRRFALLLDDLKVTVANVES-GGE-FTVSSADDILKAL 162
PM20_YEAST   AFTKSIGFELAVGDGVYW----SGRWAMVVENGIVTYAAKETNPGTDVTVSSVESVLAHL 176
PM20_ASPFU   RFSKSIGWADEEGR--------TKRYALVIDHGKITYAALEP-AKNHLEFSSAETVLKHL 168
             : :  .                *:::::. :.  *.   . * . :: *
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**Fig. 10.** Sequence alignment of Trx-linked 1-Cys TSA members. Asterisk (*), colon (:), and dot (.) indicate perfectly conserved, well conserved, and conserved positions, respectively. The highly conserved N-terminal cysteine residues are underlined bold characters. The consensus sequences surrounding the N-terminal Cys are underlined. Putative peroxisomal signal sequences are boxed. Abbreviations: hTSA VI, a human TSA VI (accession no AJ249483.); AOPP_Mouse (P99029), mouse putative peroxisomal antioxidant enzyme; AAD282452.1 (AF121355), *arabidopsis thaliana* peroxiredoxin TSA1 mRNA gene product; PM20_Yeast (P38013), *Saccharomyces cerevisiae* probable peroxisomal membrane protein PMP 20; PM20_ASPFU (O43099), *Aspergillus fumigatuss* probable peroxisomal membrane protein PMP 20.

results, together with the previous reports mentioned above, suggest that the functional free SH of Cys47 probably exists as sulfenic acid in an oxidation condition.

## Discussion

The AhpC/TSA family is a large one of newly discovered peroxidases from prokaryotes to eukaryotes [4-6]. Instead of an active site of selenocysteine within glutathione peroxidase, they have one cysteine within TSA as a primary site of catalysis. In mammalian tissue five types of TSA isoenzyme were identified (Lim *et al.*, 1994; Chae and Rhee, 1994). TSA family members can be divided into two subgroups, such as 1-Cys and 2-Cys. Four human TSA isoenzymes (2-Cys TSA except ORF6) exist as a homodimer and contain two essential cysteine residues (VCP 1 region and VCP 2 region) (Fig. 2). The ORF6 is a 1-Cys TSA that contains one conserved Cys in the VCP 1 region (see Fig. 2). *In vivo* Trx does not support the catalyzing of the reduction of peroxidase by ORF6 (Kang *et al.*, 1990). The dendrogram of the alignment of six human TSAs (data not shown) shows that the amino acid sequence of hTSA VI is very different from those of other human TSAs, including human ORF6. The sequence identity among six

human TSAs is <17.2%. The sequence identity among four 2-Cys TSA (i.e., PAG, TSA, Mer5, AOE372) (see Fig. 2) is above 50%; whereas, the sequence identity between two 1-Cys TSAs (hTSA VI and ORF6) is 15.8%. When an amino acid sequence homology search for hTSA VI is made, the hTSA VI homologous proteins are discovered only from eukaryotes, including yeast (Fig. 10). This indicates that TSA VI is an eukaryotic TSA. The amino acid sequence identity among the hTSA VI homologous proteins is >30%. In addition to the big difference in the number of amino acid residues between the known 1-Cys TSA (ORF6) and the hTSA VI homologous proteins (Fig. 2 and Fig. 10), the amino acid sequence identity between the 1-Cys TSA and hTSA VI homologues is <20%. Therefore, the analysis of the sequence homology, together with the catalytic capability of hTSA VI to accept reducing power form Trx, suggests that hTSA VI homologues is a novel type of the TSA subfamily that is different than the known 1-Cys TSA (Kang *et al.*, 1990; Cho *et al.*, 1998). The consensus sequences surrounding the unique conserved Cys among the TSA VI subfamily (PGAFTPGCS K) (Fig. 10) strengthens the possibility that the functional Cys 47 exists as a free SH form.

In this paper, we demonstrate that hTSA VI is a new type of mammalian thiol peroxidase containing one functional cysteine. In contrast to human ORF6 (Kang *et al.*, 1990), the hTSA VI uses the reducing power given by Trx for catalyzing the reduction of peroxides, such as $H_2O_2$ and alkyl hydroperoxide. This suggests its *in vivo* catalytic function as a thioredoxin-linked thiol peroxidase. On the basis of our results, we propose a reaction mechanism of the hTSA VI that catalyzes the reduction of peroxides *via* a cysteine sulfenic acid (Cys-SOH). The cysteinyl sulfenic acid is reduced to Cys-SH by the catalyzing action of Trx. The reaction mechanism is similar to that of the selenium (Se)-dependent GSH peroxidase, which catalyzes the reduction of peroxides *via* Cys-SeOH. The sulfenic acid form of Cys-SeH is reduced to Cys-SeH by GSH (Ladenstein *et al.*, 1979). Several lines of our experimental evidence support our proposed reaction mechanism of the hTSA VI. We suggested that the functional Cys47 of hTSA VI exists as the sulfenic acid form of Cys-SH in an oxidation condition. In contrast to 2-Cys TSA, the hTSA VI exists as a monomer in the oxidation condition. The proposed mechanism of hTSA VI is summarized as follows:

$$TSA\text{-}SH^{47}+H_2O_2 \rightarrow TSA\text{-}Cys\text{-}S\text{-}OH+H_2O$$
$$TSA\text{-}S\text{-}OH+HS\text{-}Trx\text{-}SH \rightarrow TSA\text{-}S\text{-}S\text{-}Trx\text{-}SH \rightarrow$$
$$TSA\text{-}SH^{47}+oxidized\ Trx$$

In mammalian tissue, six types of TSA isoenzyme exist. Based on their cellular compartmentalization and tissue distribution, there is value in investigating the physiological role of each TSA isoenzyme. Mammalian Mer5 TSA is known to be a unique TSA, which is compartmentalized in the mitochondria (Araki *et al.*, 1999). The hTSA VI carries the C-terminal peroxisomal-like sorting sequence (SQL) that is similar to the putative S(A)K(H/R)L (I) (Gould *et al.*, 1988) (Fig. 10). Therefore, it may be helpful for understanding the physiological role of hTSA VI to investigate whether or not it can be translocated into the peroxisome. Previously, we confirmed the existence of a TSA VI homologue (PM20_Yeast) in the cytosol. However, it was not in the peroxisome of *Saccharomyces cerevisiae* despite the presence of a putative peroxisomal signal sequence in its C-terminal region, $^{174}$AHL$^{176}$ using immunoblot and compartmentalization studies (our unpublished data). This result may raise the possibility that TSA VI homologues are cytoplasmic TSA, not peroxisomal TSA, despite the presence of putative peroxisomal signal sequences in their C-terminal regions (see Fig. 10).

In conclusion, first our data demonstrates that hTSA is Trx-linked 1-Cys TSA. The hTSA exists ubiquitously in an eukaryote from human to single cell eukaryotes, such as yeast. The classical GSH peroxidase, found in the cytoplasm of various eukaryotic cells, reduces $H_2O_2$ and organic hydroperoxides such as t-butylhydroperoxide. Therefore, hTSA VI has a general hydroperoxidase activity that reduces $H_2O_2$ and organic hydroperoxides such as GSH peroxidase. However, unlike selenium-dependent peroxidase (GSH peroxidase) (Marinho *et al.*, 1997), hTSA VI utilizes the Trx system as an *in vivo* electron donor to the reduction reaction of peroxides. The different electron-donor utility of hTSA VI from that of GSH peroxidase may imply the physiological significance of a thioredoxin-dependent 1-Cys TSA subfamily.

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## References

Araki, M., Nanri, H., Ejima, K., Murasato, Y., Fujiqara, T., Nakashru, Y. and Ikeda, M. (1999) Antioxidant function of the mitochondrial protein SP-22 in the cardiovascular system. *J. Biol. Chem.* **274**, 2271-2278.

Cha, M. K. and Kim, I. H. (1995) Thioredoxin-linked peroxidase from human red blood cells: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cells. *Biochem. Biophys. Res. Commun.* **217**, 900-907.

Cha, M. K., Kim, H. K. and Kim, I. H. (1995) Thioredoxin-linked thiol peroxidase from periplasmic space of *Escherichia coli*. *J. Biol. Chem.* **270**, 28635-28641.

Cha, M. K., Kim, H. K. and Kim, I. H. (1996) Mutation and mutagenesis of thiol peroxidase of *Escherichia coli* and a new type of thiol peroxidase family. *J. Bacteriol.* **178**, 5610-5614.

Chae, H. Z. and Rhee, S. G. (1994) A thiol-specific antioxidant and sequence homology to various proteins of unknown function. *Biofactors* **4**, 177-180.

Chae, H. Z., Kang, S. W. and Rhee, S. G. (1999) Isoforms of mammalian peroxiredoxin that reduce peroxide in the presence of thioredoxin. *Methods Enzymol.* **300**, 219-226.

Chae, H. Z., Kim, I. H., Kim, K. and Rhee, S. G. (1993) Cloning, sequencing, and mutation of thiol-specific

antioxidant gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 16815-16821.

Chae, H. Z., Uhm, T. B. and Rhee, S. G. (1994) Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. *Proc. Natl. Acad. Sci. USA* **91**, 7022-7026.

Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G. and Rhee, S. G. (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain. *Proc. Natl. Acad. Sci. USA* **91**, 7017-7021.

Cho, H. J., Kang, S. W., Yang, C. H., Rhee, S. G. and Ryu, S. E. (1998) Crystal structure of a novel human peroxidase enzyme at 2.0 Å resolution. *Nature Struc. Biol.* **5**, 400-406.

Fridovich, I. (1978) The biology of oxygen radicals. *Science* **202**, 875-880.

Gould, S. J., Keller, G. A. and (1998) Subramani, S. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell. Biol.* **107**, 897-905.

Jacobson, F. S., Morgan, R. W., Christman, M. F. and Ames, B. N. (1989) An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. *J. Biol. Chem.* **264**, 1488-1496.

Jeong, J. S., Kwon, S. J., Kang, S. W., Rhee, S. G. and Kim, K. (1999) Purification and characterization of a second type thioredoxin peroxidase (type II TSA) from *Saccharomyces cerevisiae*. *Biochemistry* **38**, 776-783.

Kang, S. W., Naines, I. C. and Rhee, S. G. (1990) Characterization of a mammalian peroxiredoxin that contains one conserved cysteine. *J. Biol. Chem.* **273**, 6303-6311.

Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) Characterization of a mammalian peroxiredoxin that contains one conserved cysteine *J. Biol. Chem.* **273**, 6303-6311.

Kim, K., Kim, I. H., Lee, K. Y., Rhee, S. G. and Stadtman, E. R. (1988) The isolation and purification of a thiol-specific protector protein, which inhibits enzyme inactivation. *J. Biol. Chem.* **263**, 4707-4711.

Kim, I. H., Kim, K. and Rhee, S. G. (1989) Induction of an antioxidant protein of Saccharomyces cerevisiae by $O_2$, $Fe^{+3}$, or 2-mercaptoethanol. *Proc. Natl. Acad. Sci. USA.* **86**, 6018-6022.

Kwon, S. J., Park, J. W., Choi, W. K., Kim, I. H. and Kim, K. (1994) Inhibition of metal-catalyzed oxidation system by a yeast protector protein in the presence of thioredoxin. *Biochem. Biophys. Res. Commun.* **201**, 8-15.

Ladenstein, R., Epp, O., Bartels, K., Jones, A., Huber, R. and Wendel, A. (1979) Structure analysis and molecular model of the selenoenzyme glutathione peroxidase at 2.8 Å resolution. *J. Mol. Biol.* **134**, 199-218.

Lim, Y. S., Cha, M. K., Kim, H. K. and Kim, I. H. (1994) The thiol-specific antioxidant protein from human brain: gene cloning and analysis of conserved cysteine region. *Gene* **140**, 279-284.

Lim, Y. S., Cha, M. K., Yun, C. H., Kim, H. K., Kim, K. and Kim, I. H. (1994) Purification and characterization of thiol-specific antioxidant protein from human red blood cells. *Biochem. Biophys. Res. Commun.* **199**, 199-206.

Lundblad, R. L. and Noyes, C. M. (1984) *Chemical Reagents for Protein Modification* 1, p.55-93, CRC Press.

Marinho, H. S., Antunes, F. and Pinto, R. E. (1997) Role of glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase in the reduction of lysophospholipid hydroperoxides. *Free Radical Biol. & Med.* **22**, 871-883.

Parsonage, D., Miller, H., Ross, R. P. and Claiborne, A. (1993) Purification and analysis of streptococcal NADH peroxidase expressed in *Escherichia coli*. *J. Biol. Chem.* **268**, 3161-3167.

Sauri, H., Butterfield, L., Kim, A. and Shau, H. (1995) Antioxidant function of recombinant human natural killer enhancing factor. *Biochem. Biophys. Res. Commun.* **208**, 964-969.

Scandalios, J. G. (1997) *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sies, H. (1993) Strategies of antioxidant defense. *Eur. J. Biochem.* **215**, 213-219.

Stadtman, E. R. and Oliver, C. N. (1991) Metal-catalyzed oxidation of proteins. Physiological consequences. *J. Biol. Chem.* **266**, 2005-2008.

Wolff, S. P. (1994) Ferrous ion oxidation in presence of ferric ion: indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* **233**, 182-189.