Selenoprotein W is a glutathione-dependent antioxidant in vivo

Dae-wonJeong[a], Tae Soo Kim[a], Youn Wook Chung[a], Byeong Jae Lee[b], IckYoung Kim[a,*]

[a] *Laboratory of Cellular and Molecular Biochemistry, Graduate School of Biotechnology, Korea University, Seoul 136-701, South Korea*
[b] *Laboratory of Molecular Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, South Korea*

Received 15 February 2002; accepted 19 March 2002

First published online 29 March 2002

Edited by Barry Halliwell

Abstract The function of selenoprotein W (Se-W) was investigated by cloning the corresponding cDNA from mouse brain and expressing it in CHO cells and H1299 human lung cancer cells. Overexpression of Se-W markedly reduced the sensitivity of both cell lines to H_2O_2 cytotoxicity. The intracellular peroxide concentration of the transfected cells was lower than that of the parental cells in the absence or presence of extracellular H_2O_2. The resistance to oxidative stress conferred by Se-W was dependent on glutathione. Expression of Se-W mutants in which selenocysteine-13 or cysteine-37 was replaced by serine did not confer resistance to H_2O_2, implicating these residues in the antioxidant activity of Se-W in vivo.

Key words: Selenoprotein W; Selenocysteine; Glutathione; Hydrogen peroxide; Antioxidant

1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), the superoxide anion radical ($O_2^{\bullet-}$), and the hydroxyl radical ($OH^{\bullet}$) are toxic by-products of cellular oxygen metabolism [1]. The intracellular concentration of ROS has been implicated as a determinant of apoptosis, aging, cancer, and cell cycle arrest [2,3]. The direct reduction of H_2O_2 by glutathione peroxidase (GPx) and catalase as well as the scavenging of superoxide radicals by superoxide dismutase contribute to homeostasis of the intracellular redox state [4–6].

Two selenoproteins, GPx and thioredoxin reductase, serve to protect cells from oxidative stress. Whereas GPx catalyzes the reduction of peroxides that might damage cells and tissues [4], thioredoxin reductase provides reducing equivalents for various biochemical processes and antioxidant defenses [7]. Selenoprotein P has also been proposed to function in extracellular fluid as a glutathione-dependent peroxidase for phospholipid hydroperoxides [8].

Selenoprotein W (Se-W) contains both a selenocysteine (SeCys) residue that is encoded by a UGA codon in the open reading frame of the mRNA as well as a bound glutathione (GSH) molecule at residue Cys^{37} [9,10]. The protein is localized predominantly in the cytoplasm, but a small proportion of total Se-W is associated with the cell membrane [11,12]. Se-W was expressed in all tissues examined in selenium-supplemented animals, including muscle, heart, testis, spleen, kidney, intestine, tongue, brain, lung, and liver [10,13–15], and its abundance in various tissues was increased by selenium supplementation. Expression of Se-W in the brain was preserved even under conditions of selenium deficiency [14]. Given the presence of the bound GSH moiety, Se-W is thought to function in oxidation–reduction catalysis [16], and it may play a role in selenium deficiency disorders such as white muscle disease of sheep and Keshan disease in humans [12,17]. However, Se-W has not been shown directly to function as an antioxidant.

To investigate the possible role of Se-W as a ROS scavenger in vivo, we have isolated the corresponding cDNA from a mouse brain library and expressed the encoded protein in cell lines. We show that ectopic expression of Se-W renders cells resistant to H_2O_2 and that this resistance is dependent on GSH.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) *dhfr*$^-$ cells, which are deficient in dihydrofolate reductase, were cultured in Ham's F-12 nutrient medium (Gibco-BRL) containing L-glutamine and 14 mM $NaHCO_3$ and supplemented with 10% fetal bovine serum and antibiotics. Human lung cancer H1299 cells were cultured in RPMI 1640 medium (Gibco-BRL) containing L-glutamine and 24 mM $NaHCO_3$ and supplemented with 5% fetal bovine serum and antibiotics. Both cell lines were maintained at 37°C under an atmosphere of 95% air and 5% CO_2 in a humidified incubator.

2.2. Cloning of the Se-W cDNA and construction of stable cell lines

The Se-W cDNA was cloned from a mouse brain cDNA library in the Lambda ZAP II vector (Stratagene) by screening with a specific internal antisense oligonucleotide probe (5′-TCCACRTAGCCATCRCC-3′, where R indicates A or G; based on the sequences of mouse and rat cDNAs) with the use of ECL 3′-oligonucleotide labeling and detection systems (Amersham Pharmacia Biotech). The isolated full-length cDNA was incorporated into the pBluescript SK$^-$ plasmid by in vivo excision with helper phage (Stratagene). The cDNA was confirmed by sequencing with an Applied Biosystems model ABI 373A automated DNA sequencer (Pharmacia Biotech). The DNA fragment (containing the cDNA) released by digestion of the modified pBluescript SK$^-$ plasmid with *Bam*HI and *Xho*I was then subcloned into the mammalian expression vector pcDNA3.1+ (Invitrogen), yielding pcDNA3.1+/*selW*.

The $SeCys^{13}$ residue of Se-W was changed to Cys or Ser by site-specific mutagenesis of the cDNA with the use of the polymerase chain reaction (PCR). The PCR primers for mutagenesis were 5′-GCG*GAATTC*ATGGCGCTCGCCGTTCGAGTCGTGTATTGTGGAGCTTGC(TCA)GGCTAT-3′ (sense) and 5′-CCC*TCTAGA*CAG-

*Corresponding author. Fax: (82)-2-3290 3449.
E-mail address: ickkim@korea.ac.kr (I.Y. Kim).

Abbreviations: ROS, reactive oxygen species; GPx, glutathione peroxidase; Se-W, selenoprotein W; SeCys, selenocysteine; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; BSO, L-buthionine-[*S*,*R*]-sulfoxide; DCFH-DA, 2′,7′-dichlorofluorescin diacetate

0014-5793/02/$22.00
PII: S0014-5793(02)02628-5

CAGAATCCATC-3′ (antisense), in which the underlines indicate the substituted Cys (or Ser) codon and the italics indicate *Eco*RI and *Xba*I sites, respectively. The DNA fragments released by digestion of the PCR products with *Eco*RI and *Xba*I were ligated with pcDNA3.1+ that had been digested with the same restriction enzymes. The resulting mutants were designated SeC13C and SeC13S, respectively. Site-directed mutagenesis of Cys^{37} to Ser was also performed with a Quickchange kit (Stratagene); the mutagenic synthetic primer was 5′-GCCTGGACATTTCTGGCGAGGGGAC-3′, in which the underline indicates the substituted Ser codon. The resulting mutant was designated C37S.

Plasmids containing the wild-type or mutant Se-W cDNAs were introduced into H1299 or CHO *dhfr*$^-$ cells by transfection with the use of Lipofectamine (Gibco-BRL), and the resulting stable cell lines were selected by culture in the presence of 500 μM G418 (Gibco-BRL) for 14 days.

2.3. Cell viability after H_2O_2 exposure

The viability of cells after exposure to H_2O_2 was determined on the basis of trypan blue exclusion. Cells (2×10^5) were seeded on 60-mm dishes and incubated for 24 h, after which the medium was replaced and the cells were incubated for an additional 12 h before exposure to H_2O_2. The numbers of viable and non-viable cells at various times after exposure to H_2O_2 were counted with the use of a light microscope, and the percentage of cells excluding trypan blue was taken as an index of cell viability.

2.4. Depletion of GSH with BSO and measurement of total GSH

Cells (2×10^5) were incubated in 60-mm dishes for 24 h before exposure to fresh medium containing 300 μM L-buthionine-[*S*,*R*]-sulfoxide (BSO), an inhibitor of de novo GSH synthesis, for 16 h. The concentrations of both GSH and GSSG were determined by monitoring spectrophotometrically the GSH-dependent formation of 5-thio-2-nitrobenzoate from 5,5′-thiobis(2-nitrobenzoate) at 412 nm, as described previously [18].

2.5. Preparation of a monoclonal antibody to Se-W

A monoclonal antibody to Se-W was prepared by immunizing mice with purified Se-W (SeC13C mutant). Purification of Se-W was facilitated by fusing the protein to maltose-binding protein by ligation of a DNA fragment containing the mutant cDNA with the expression vector pMAL-cRI (New England Biolabs). The recombinant fusion protein was expressed in *Escherichia coli* BL21 (DE3) and then purified with the use of an amylose resin (New England Biolabs). Six-week-old male BALB/c mice were injected with 0.2 ml of a suspension containing 50 μg of antigen mixed with an equal volume of complete Freund's adjuvant. The mice were subsequently injected at 2-week intervals with antigen mixed with incomplete Freund's adjuvant or antigen alone, respectively. Monoclonal antibodies specific for Se-W were then generated as described previously [19]. Ascitic fluid was collected about 1 week after injection of hybridoma, and antibody titer was determined by immunoblot analysis.

3. Results and discussion

3.1. Cloning of Se-W cDNA and construction of stable cell lines overexpressing Se-W

The Se-W cDNA was cloned from mouse brain and sequenced (GenBank accession number AF241527). The cDNA sequence is 100% identical to those previously determined for Se-W cDNAs isolated from mouse skeletal muscle, mouse fetus, and rat skeletal muscle (GenBank accession numbers U67890, AF015284, and U25264, respectively), and includes a SeCys residue encoded by TGA at codon 13. The cloned cDNA was introduced by transfection into H1299 and CHO *dhfr*$^-$ cells, and the resulting stable cell lines were designated H1299/*selW* and CHO *dhfr*$^-$/*selW*, respectively.

The expression of the Se-W cDNA at the mRNA and protein levels was confirmed by reverse transcription-PCR analysis with specific primers (Fig. 1A) and by the incorporation of ^{75}Se into a protein of ~10 kDa (Fig. 1B), respectively. The Se-W mRNA and protein were detected in stable transfectants but not in the parental cells.

3.2. GSH-dependent resistance of transfected cells to H_2O_2

Preliminary studies revealed that H1299 cells were more sensitive to H_2O_2 than were CHO *dhfr*$^-$ cells. H1299 and CHO cells were therefore exposed to 50 and 200 μM H_2O_2, respectively, to investigate the effect of Se-W overexpression on sensitivity to this agent. The viability of stable transfectants of either cell line after treatment with H_2O_2 was greater than that of parental cells (Fig. 2A,B), indicating that expression of Se-W protects cells from oxidative damage induced by H_2O_2.

To determine whether the GSH moiety that is bound to

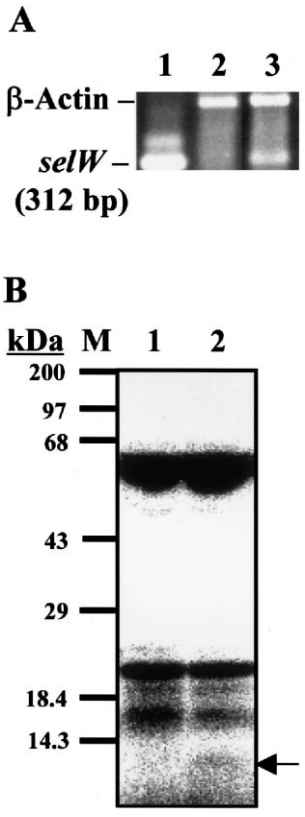

Fig. 1. Expression of Se-W cDNA in stable transfectants. A: Reverse transcription-PCR analysis of Se-W mRNA. Analysis was performed with primers specific for Se-W cDNA (Oligo 1, 5′-GGGGAATTCATGGCGCTCGCCGTT-3′; Oligo 2, 5′-CCCTCTAGATGCCTCTAGGGTCA-3′) and for β-actin cDNA (oligo 1, 5′-GATGACGATATCGCTGCGCT-3′; oligo, 2, 5′-GATCATTGCCGATAGTGATGACCT-3′). Lane 1, the PCR product of pcDNA3.1+/*selW* (control); lane 2, CHO *dhfr*$^-$ cells; lane 3, CHO *dhfr*$^-$/*selW* cells. The positions of the PCR products derived from β-actin cDNA and Se-W cDNA (312 bp) are indicated. B: Labeling of Se-W with ^{75}Se. H1299 (lane 1) and H1299/*selW* (lane 2) cells were grown to 60% confluence and then labeled with sodium [^{75}Se]selenite (2 μCi/ml; neutral pH) for 3 days. The cells were harvested, washed with phosphate-buffered saline, and lysed by gentle agitation in ice-cold RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at $12\,000\times g$, and the resulting supernatants (100 μg of protein) were fractionated by SDS–polyacrylamide gel electrophoresis through a 12% gel. The gel was then dried and analyzed with an imager (Fujifilm BAS-2500). Lane M, protein size markers (in kDa). Arrow indicates the 10-kDa band corresponding to Se-W.

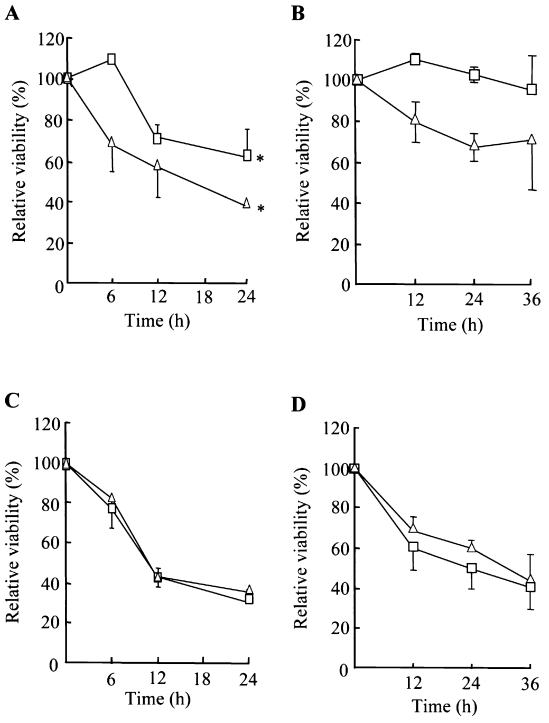

Fig. 2. Effects of Se-W expression and BSO on the sensitivity of cells to H_2O_2. A,B: Parental H1299 (A) or CHO *dhfr*$^-$ (B) cells (triangles) and the corresponding stable transfectants expressing Se-W (squares) were incubated for the indicated times in the presence of 50 μM (A) or 200 μM (B) H_2O_2. C,D: Cells were treated as in A and B, respectively, with the exception that they were preincubated for 16 h in the presence of 300 μM BSO before exposure to H_2O_2. Cell viability was expressed as a percentage of cells excluding trypan blue in the indicated times, and data are means ± S.D. of values from five experiments. *$P < 0.05$ versus the corresponding value for parental cells (Student's *t*-test).

Cys^{37} of Se-W [12,13] contributes to the observed antioxidant activity of this protein, we pretreated cells for 16 h with 300 μM BSO, an inhibitor of γ-glutamylcysteinyl synthetase, the first enzyme in the GSH biosynthetic pathway [20]. During the 16-h incubation with BSO, the total GSH content of H1299 cells decreased from 38.52 ± 3.34 to 2.74 ± 0.40 nmol/mg of protein and that of CHO *dhfr*$^-$ cells declined from 30.32 ± 2.85 to 3.20 ± 0.51 nmol/mg. BSO treatment of stable transfectants increased their sensitivity to H_2O_2 to levels similar to those apparent for non-treated parental cells (Fig. 2C,D). Consistent with previous observations [21], the sensitivity of GSH-depleted parental cells to H_2O_2 was greater (by ~20%) than that of the corresponding GSH-replete cells. BSO treatment alone did not affect cell viability during subsequent incubation of cells for 24 or 36 h in the absence of H_2O_2 (data not shown).

The loss of the protective effect of Se-W against H_2O_2-induced cytotoxicity in cells treated with an inhibitor of GSH synthesis indicates that Se-W is a GSH-dependent antioxidant in vivo. GSH and its redox cycle play a critical role in catabolizing H_2O_2 and other peroxides through enzymatic coupling reactions. Furthermore, GSH is important for the detoxification of electrophiles and for protection of the thiol groups of proteins from oxidation [22]. GSH is also required for regeneration of the GPx and glutaredoxin system. Measurement of GPx activity in lysates of parental cells and stable transfectants by monitoring spectrophotometrically (at 340 nm) the

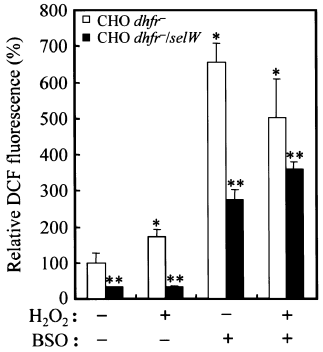

Fig. 3. Effects of Se-W expression and BSO on the intracellular peroxide concentration. CHO *dhfr*$^-$ and CHO *dhfr*$^-$/*selW* cells (2.5×10^5) in 60-mm dishes were incubated for 24 h before exposure to 300 μM BSO for 16 h. The cells were then incubated in the absence or presence of 200 μM H_2O_2 for 90 min before loading with 20 μM DCFH-DA (Sigma) for 30 min. The cells were washed with phosphate-buffered saline, detached from the dish by exposure to trypsin, and fixed with 1 ml of phosphate-buffered saline containing 2 mM EDTA, 5 mM dextrose, and 0.1% gelatin [23]. The fluorescence of 2′,7′-dichlorofluorescein (DCF) in samples containing 1×10^4 cells was then determined by flow cytometry with a FACSCalibur instrument (Becton Dickinson). DCF fluorescence, which reflects the intracellular peroxide concentration, was expressed as a percentage of the value for untreated parental cells. Data are means ± S.D. of values from three experiments. *$P < 0.005$, **$P < 0.0001$ versus value for untreated parental cells (Student's *t*-test).

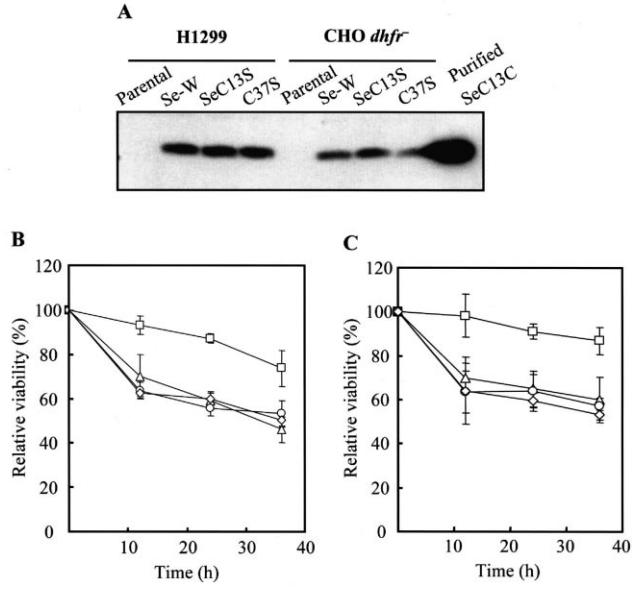

Fig. 4. Effects of expression of SeC13S or C37S mutants of Se-W on the sensitivity of cells to H_2O_2. A: Immunoblot analysis, with a specific monoclonal antibody (1:1000 dilution) to Se-W, of the abundance of wild-type and mutant Se-W proteins in lysates of H1299 and CHO *dhfr*$^-$ cell transfectants. The purified SeC13C mutant used as the antigen for preparation of the monoclonal antibody is shown in the right-most lane. B,C: Parental H1299 (B) and CHO *dhfr*$^-$ (C) cells (triangles) and the corresponding stable transfectants expressing either wild-type Se-W (squares) or the SeC13S (circles) or C37S (diamonds) mutants were exposed to 50 μM (B) or 200 μM (C) H_2O_2 for the indicated times, after which cell viability was analyzed by trypan blue exclusion. Data are expressed as a percentage of the value of H_2O_2-untreated cells and are means ± S.D. of values from three experiments.

decrease in NADPH concentration revealed that expression of Se-W did not affect GPx activity (data not shown).

3.3. GSH-dependent removal of intracellular peroxide by Se-W

Given that stable transfectants were less sensitive to oxidative stress than were parental cells, we next examined the effect of H_2O_2 treatment on the intracellular concentration of ROS. The intracellular peroxide (H_2O_2) concentration in CHO *dhfr*$^-$ cells and CHO *dhfr*$^-$/*selW* cells was determined by flow cytometry after loading of the cells with the oxidant-sensitive dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The basal peroxide concentration of the stable transfectants was lower than that of the parental cells (Fig. 3). Treatment with H_2O_2 resulted in an increase in the intracellular peroxide concentration in the parental cells but not in the stable transfectants. The intracellular peroxide concentrations of both CHO *dhfr*$^-$ and CHO *dhfr*$^-$/*selW* cells (exposed or not to H_2O_2) were increased markedly by pretreatment with BSO. Similar results were obtained with H1299 and H1299/*selW* cells (data not shown). These results thus indicate that Se-W mediates the removal of H_2O_2 from cells and that this function is dependent on GSH.

3.4. Role of $SeCys^{13}$ and Cys^{37} in the antioxidant activity of Se-W

To determine the contributions of $SeCys^{13}$ and Cys^{37} to the antioxidant activity of Se-W, we transfected H1299 and CHO *dhfr*$^-$ cells with expression vectors encoding mutant Se-W proteins in which these residues had been changed individually to Ser. Expression of the mutant proteins was confirmed by immunoblot analysis of cell lysates with a specific monoclonal antibody prepared as described in Section 2 (Fig. 4A). In contrast to the H_2O_2 resistance apparent in cells expressing wild-type Se-W, cells expressing the mutant proteins exhibited a sensitivity to H_2O_2 similar to that of parental cells (Fig. 4B,C). These results thus suggest that both $SeCys^{13}$ and Cys^{37} residues are required for the antioxidant function of Se-W in vivo.

Acknowledgements: We thank T.C. Stadtman (NIH, Bethesda, MD, USA) for helpful discussion. This work was supported by a grant (KOSEF 2000-2-20900-008-5) to I.Y.K. from the Korea Science and Engineering Foundation and by a grant from Korea University.

References

[1] Berlett, B.S. and Stadtman, E.R. (1997) J. Biol. Chem. 272, 20313–20316.
[2] Stadtman, E.R. (1992) Science 257, 1220–1224.
[3] Alsina, B., Corominas, M., Berry, M.J., Baguna, J. and Serras, F.J. (1999) J. Cell Sci. 112, 2875–2884.
[4] Epp, O., Ladenstein, R. and Wendel, A. (1983) Eur. J. Biochem. 133, 51–69.
[5] Bai, J., Rodriguez, A.M., Melendez, J.A. and Cederbaum, A.I. (1999) J. Biol. Chem. 274, 26217–26224.
[6] Fridovich, I. (1997) J. Biol. Chem. 272, 18515–18517.
[7] Prinz, W.A., Aslund, F., Holmgren, A. and Beckwith, J. (1997) J. Biol. Chem. 272, 15661–15667.
[8] Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E. and Takahashi, K. (1999) J. Biol. Chem. 274, 2866–2871.
[9] Beilstein, M.A., Vendeland, S.C., Barofsky, E., Jensen, O.N. and Whanger, P.D. (1996) J. Inorg. Biochem. 61, 117–124.
[10] Gu, Q.P., Beilstein, M.A., Barofsky, E., Ream, W. and Whanger, P.D. (1999) Arch. Biochem. Biophys. 361, 25–33.
[11] Vendeland, S.C., Beilstein, M.A., Chen, C.L., Jensen, O.N., Barofsky, E. and Whanger, P.D. (1993) J. Biol. Chem. 268, 17103–17107.
[12] Yeh, J.Y., Beilstein, M.A., Andrews, J.S. and Whanger, P.D. (1995) FASEB J. 9, 392–396.
[13] Yeh, J.Y., Gu, Q.P., Beilstein, M.A., Forsberg, N.E. and Whanger, P.D. (1997) J. Nutr. 127, 394–402.
[14] Yeh, J.Y., Vendeland, S.C., Gu, Q., Butler, J.A., Ou, B.R. and Whanger, P.D. (1997) J. Nutr. 127, 2165–2172.
[15] Allan, C.B., Lacourciere, G.M. and Stadtman, T.C. (1999) Annu. Rev. Nutr. 19, 1–16.
[16] Sun, Y., Gu, Q.P. and Whanger, P.D. (1998) FASEB J. 12, A824 (abstract).
[17] Stadtman, T.C. (1975) in: Advances in Enzymology and Related Areas of Molecular Biology, Vol. 48 (Meister, A., Ed.), pp. 1–27, John Wiley and Sons, New York.
[18] Anderson, M. (1985) Methods Enzymol. 113, 548–555.
[19] Yamamoto, H., Satoh, T., Kiyohara, T., Totsuka, A. and Moritsugu, Y. (1997) Biologicals 25, 373–380.
[20] Chen, K.H., Reece, L.M. and Leary, J.F. (1999) Free Radic. Biol. Med. 27, 100–109.
[21] Efferth, T., Fabry, U., Glatte, P. and Osieka, R. (1995) J. Mol. Med. 73, 47–49.
[22] Lu, S.C. (1999) FASEB J. 13, 1169–1183.
[23] Robinson, J.P. (1994) Methods Cell Biol. 41, 437–441.