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Protection of Mitochondrial Integrity From Oxidative Stress by Selenium-Containing Glutathione Transferase

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Abstract

The antioxidant activity of a novel artificial glutathione peroxidase–like enzyme, selenium-containing glutathione 5-transferase from *Lucilia cuprina* (seleno-LuGST1-1), was studied by using a ferrous sulfate/ascorbate-induced mitochondrial damage model system. Swelling of mitochondria, lipid peroxidation, and cytochrome-*c* oxidase activity were selected to evaluate the preservation of mitochondrial integrity in this system. Seleno-LuGST1-1 could effectively protect the mitochondria against oxidative damage in a dose-dependent manner and exhibited both higher catalytic activity and greater antioxidant ability than the classic mimic, 2-phenyl-1,2-benziososelenazol-3(2H)-one (Ebselen). This novel artificial biocatalyst therefore may have great potential for pharmacologic application in the treatment of reactive oxygen species-related diseases.

Index Entries: Antioxidant activity; artificial enzymes; mitochondria; oxidative stress; glutathione peroxidase; selenium-containing glutathione transferase.

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Introduction

Reactive oxygen species (ROS) *(1,2)* such as oxygen-free radicals (the superoxide radical anion, singlet oxygen), H_2O_2, nitric oxide, and the derived peroxynitrite radical *(3)* are produced continuously at a high rate as the byproducts of aerobic metabolism. Their threat to an organism is underscored by the existence of an impressive array of cellular defenses, including a nonenzymatic defense system (vitamine E, ascorbate, glutathione [GSH], and uric acid) and an enzymatic defense system (superoxide dismutase, catalase, and glutathione peroxidase [GPx]). However, if ROS loading overwhelms the antioxidative defenses, oxidative stress would damage cellular components and result in ROS-mediated diseases, such as ischemia-reperfusion injury, inflammation, age-related diseases, neuronal apoptosis, cancer, and cataracts *(4–6)*. Enzyme therapy has been accepted as one of the promising routes for the treatment of these diseases. GPx (EC 1.11.1.9) is an important selenoenzyme that can effectively protect cells against oxidative stress by catalyzing the reduction of hydroperoxide using GSH as reducing substrate. Because of the limitations of natural GPx associated with enzyme therapies (solution instability, short half-lives, costs of production, and proteolytic digestion), considerable efforts have been made to generate GPx-like biocatalysts *(7–9)*. Although some classic mimics have been extensively studied and their ability to scavenge ROS has been verified *(10)*, their pharmacological application is limited owing to the low GPx activity and water insolubility.

Intensive studies of protein structures have revealed that the evolution of proteins for novel functions is based largely on the redesign of existing protein frameworks in nature *(11,12)*. This principle has been exploited in the generation of novel GPx-like biocatalyst by redesigning existing protein scaffolds that are similar to that of natural GPx *(13,14)*. By replacing the active-site serine 9 with a cysteine and then substituting it with selenocysteine in a cysteine auxotrophic system, catalytically essential residue selenocysteine was bioincorporated into GSH-specific binding scaffold and, thus, glutathione 5-transferase (GST) from *Lucilia cuprina* was successfully converted into a selenium-containing enzyme, seleno-LuGST1-1, by genetic engineering. Because of the important structural similarities between seleno-LuGST1-1 and naturally occurring GPx, the generated selenoenzyme displayed a significantly high efficiency for catalyzing the reduction of H_2O_2 by glutathione, which was comparable with the efficiency of natural GPx.

In the present study, we evaluated the biologic effect of seleno-LuGST1-1 for protecting mitochondria against oxidative stress. Swelling of mitochondria, lipid oxidation, and cytochrome-*c* oxidase (COX) activity were selected to characterize the functional integrity of mitochondria. Seleno-LuGST1-1 was found to function more effectively as antioxidant than Ebselen and, therefore, exhibited great potential for pharmacological application.

Materials and Methods

Generation and Assay of Enzymatic Property of Seleno-LuGST1-1

The expression vector pSM3 containing the mutant of *gst* gene has been constructed previously *(13)*. In this gene, serine 9 was mutated to cysteine so that it could be further substituted by Sec in a cysteine auxotrophic strain when Sec was present in the medium instead of cysteine *(15)*. The other two cysteines in the gene, Cys 86 and Cys 200, were mutated to serines to realize site-directed substitution of Cys by Sec in the expression of selenoenzyme. pSM3 was then transformed into the cysteine auxotrophic strain BL21cysE51 and expressed the selenoenzyme, seleno-LuGST1-1, in a modification of M9 medium *(13)*. Analysis of 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that the expressed seleno-LuGST1-1 was about 13% of total bacterial proteins. This target protein was purified by affinity chromatography on glutathione Sepharose 4B *(16)*. The GPx activity of this protein was examined by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GSH reductase, which catalyzes the reduction of oxidized GSH formed in the catalytic cycle. Both sample and control cuvets contained 50 m*M* potassium phosphate buffer, pH 7.0; 1 m*M* EDTA; 1 m*M* sodium azide; 1 m*M* GSH; and 1 U of GSH reductase in a total volume of 0.5 mL. An aliquot of seleno-LuGST1-1 was added only to the sample cuvet. The reaction was started by adding 0.5 m*M* hydroperoxide to both cuvets, followed by a decrease in NADPH absorption at 340 nm. The activity was corrected for the control experiment. One unit of enzyme activity was defined as the amount of mimic that utilized 1 μmol of NADPH/min.

Preparation of Mitochondria

Bovine heart mitochondria were isolated from fresh bovine heart *(17)*; suspended in 25 m*M* HEPES/NaOH buffer, pH 7.4, containing 0.25 *M* sucrose and 10 m*M* EDTA; and maintained at 0°C. The concentration of the mitochondrial protein was determined by Coomassie brilliant blue *(18)* using bovine serum albumin as the standard.

Biologic Analysis of Seleno-LuGST1-1 Against Ferrous Sulfate/Ascorbate-Induced Mitochondrial Damage

The incubation mixture consisted of 0.125 *M* KCl, 1 m*M* $MgCl_2$, 5 m*M* glutamate, mitochondria (0.04 mg of protein/mL), 1 m*M* GSH, and appropriate enzyme mimic in 10 m*M* potassium phosphate buffer (pH 7.4, 37°C). Swelling of mitochondria, thiobarbituric acid-reactive substances (TBARS), and COX activity were determined at various intervals after the addition of 2 m*M* ascorbate and 50 μ*M* ferrous sulfate. Damage experiments were performed without seleno-LuGST1-1; control experiments were performed without enzyme mimic, ascorbate, and ferrous sulfate.

Swelling of mitochondria was assayed as described by Hunter et al. *(19)*. It was measured as the decrease in turbidity of the reaction mixture at 520 nm.

The content of TBARS in ferrous sulfate/ascorbate-treated mitochondria was analyzed as described previously *(20)*. In this assay, thiobarbituric acid (TBA) reacts with malonaldehyde and/or other carbonyl byproducts of free-radical-mediated lipid peroxidation to give 2:1 (mol/mol) colored conjugates. Before and during incubation with the different concentrations of seleno-LuGST1-1, a 1.0-mL aliquot was taken and vortex mixed with 1 mL of 75% (w/v) trichloroacetic acid and 1 mL of 0.5% (w/v) TBA in water. The assay mixtures were heated for 40 min at 80°C. After cooling and centrifuging, the A_{532} of the supernatants was recorded.

An aliquot of incubation mixture from the damage group or control group was taken at different time intervals and centrifuged (10,000*g*, 4°C, 2 min). The pellet was washed with 10 m*M* potassium phosphate buffer, pH 7.4, containing 125 m*M* KCl, 1 m*M* $MgCl_2$, and 5 m*M* glutamate. Then it was suspended in a small amount of 100 m*M* potassium phosphate buffer, pH 7.0, and an aliquot was taken for assay of COX activity *(21)* in a 2-mL reaction system containing 15 μ*M* cytochrome-*c*. The absorbance intensity, A_t, of cytochrome-*c* was recorded at various intervals during the reaction, and its decrease accompanied the oxidation of cytochrome-*c* in the sample cell. Five microliters of 10 m*M* $K_3Fe(CN)_6$ was added to oxidize cytochrome-*c* thoroughly after completion of the reaction. The absorbance intensity at this time was recorded as A_∞, and $\ln(A_t - A_\infty)$ vs time was plotted. The absolute value of the slope of the line represented the apparent rate constant, k_{app}, of cytochrome-*c* oxidation and was used to express COX activity.

Results

Enzymatic Activity of Seleno-LuGST1-1

Table 1 gives the GPx activities of seleno-LuGST1-1 and other species. The GPx activity of seleno-LuGST1-1 for the reduction of H_2O_2 by GSH was determined to be 2957 U/μmol, indicating that seleno-LuGST1-1 was much more efficient than most of the other GPx mimics. For instance, it is at least 500- and 2900-fold more efficient than the first semisynthetic selenoenzyme (Fig. 1), selenosubtilisin (4.6 U/μmol), and the well-known model compound Ebselen (0.99 U/μmol). Its activity is even comparable with those of some native GPxs, such as rabbit liver GPx, bovine liver GPx, human hepatoma HepG 2 cell giGPx, and human plasma pGPx *(22)*.

Effect of Seleno-LuGST1-1 on Swelling of Damaged Mitochondria

As shown in Fig. 2, the absorbance at 520 nm for the control group was basically constant, whereas the absorbance for the damage group decreased considerably with time. The decrease in the absorbance indicates an increase in mitochondrial swelling and degradation of mitochondrial integrity owing to the occurrence of oxidative damage after the addition of

Table 1
Comparison of GPx Activities
of Seleno-LuGST1-1-Catalyzed Reduction of H_2O_2
by GSH and Other Species

Catalyst	Activity (U/μmol)
Wild-type GST	ND
Seleno-LuGST1-1[a]	2957 (30)
Selenosubtilisin	4.6 (0.2)
Ebselen	0.99
Native (GPx, rabbit liver)	5780

[a]Reactions were carried out at 37°C in 500 μL of 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* EDTA, 1 m*M* GSH, 1 U of glutathione reductase, and 0.5 m*M* H_2O_2. One unit of enzyme activity was defined as the amount of mimic that utilized of 1 μmol of NADPH/min. All values are the means of five reactions, and standard deviations are given in parentheses.

ND, not detected.

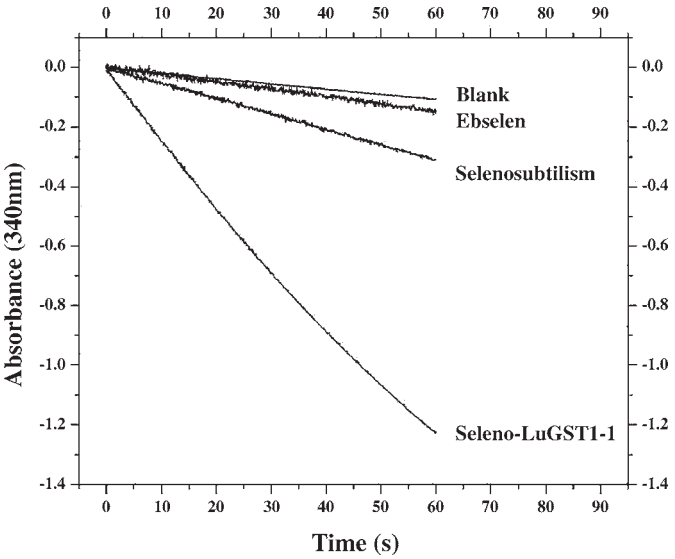

Fig. 1. Catalytic curves of seleno-LuGST1-1 and other catalysts. The reactions were carried out at 37°C in 500 μL of 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* EDTA, 1 m*M* GSH, 1 U of glutathione reductase, and 0.5 m*M* H_2O_2. The decrease in the absorption at 340 nm was caused by the decrease in NADPH along with the reducing reaction catalyzed by GPx mimics. Therefore, the slope of the catalytic curves was used to determine the initial rate of the reaction. The catalytic curves of blank (background), Ebselen (13.7 μ*M*), selenosubtilisin (14.7 μ*M*), and seleno-LuGST1-1 (0.138 μ*M*) are shown. The curve of the wild-type LuGST1-1 was identical with that of the blank and is not shown.

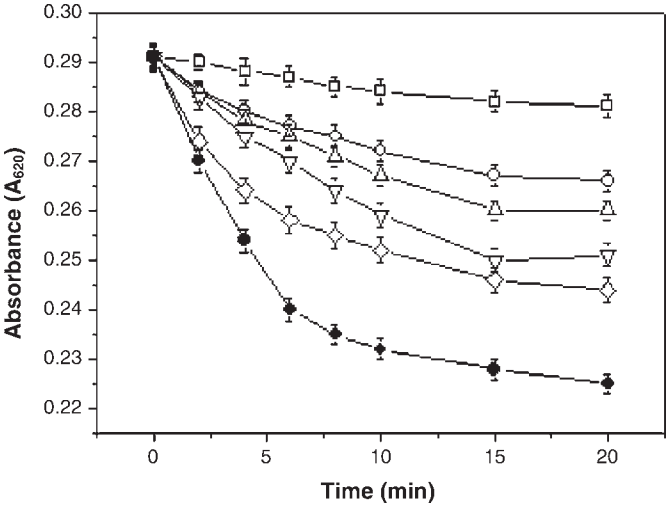

Fig. 2. Effect of seleno-LuGST1-1 on swelling of mitochondria: (□) control; (○) damage + 0.41 μ*M* seleno-LuGST1-1; (△) damage + 0.27 μ*M* seleno-LuGST1-1; (▽) damage + 0.13 μ*M* seleno-LuGST1-1; (◇) damage + 8 μ*M* Ebselen; (●) damage. The damage condition is explained in Materials and Methods. Data are presented as the mean ± SD (n = 3).

ferrous sulfate and ascorbate. Compared with the damage group, mitochondrial swelling was apparently inhibited by the addition of seleno-LuGST1-1 in a dose-dependant manner. Seleno-LuGST1-1 displayed a higher efficiency (even at a concentration as low as 0.13 μ*M*) for the inhibition of mitochondrial swelling than the well-studied antioxidant Ebselen (8 μ*M*).

Inhibition of Lipid Peroxidation by Seleno-LuGST1-1

Mitochondrial lipid peroxidation was assessed by the formation of TBARS from membrane lipids (Fig. 3). The amount of TBARS accumulated during damage of the mitochondria was considerably reduced in the presence of seleno-LuGST1-1 and was further reduced with an increase in concentration of seleno-LuGST1-1. After the addition of 0.41 μ*M* seleno-LuGST1-1, TBARS content over 50 min was only 40.8% of that of the damage group. Under similar conditions, when 8 μ*M* Ebselen and 0.13 μ*M* seleno-LuGST1-1 were added as antioxidant, TBARS content was 76.3 and 84.5% of that of the damage group, respectively, indicating that seleno-LuGST1-1 could protect mitochondria more effectively even when its concentration was approx 60-fold less than that of Ebselen.

Protection of COX Activity in Damaged Mitochondria

When mitochondria were exposed to Fe^{2+}/ascorbate-induced oxidative stress, COX activity was decreased, owing to the destruction of the integrity of the mitochondrial membrane. As shown in Fig. 4, higher COX

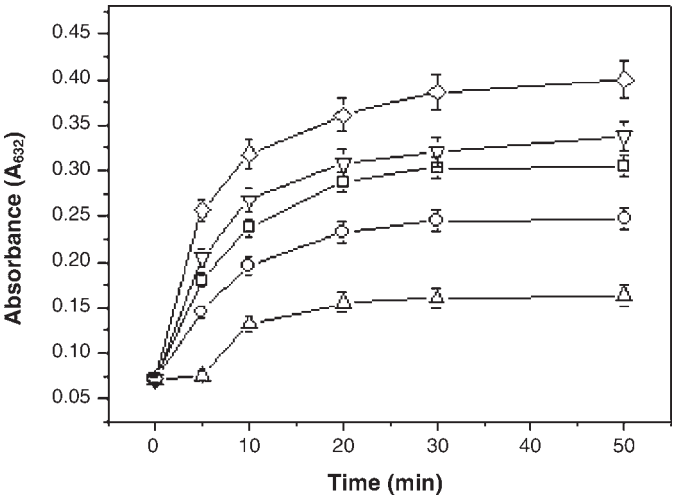

Fig. 3. Effect of seleno-LuGST1-1 on accumulation of TBARS during damage of mitochondria: (◇) damage; (▽) damage + 8 µ*M* Ebselen; (□) damage + 0.13 µ*M* seleno-LuGST1-1; (○) damage + 0.27 µ*M* seleno-LuGST1-1; (△) damage + 0.41 µ*M* seleno-LuGST1-1. The damage condition is explained in Materials and Methods. The absorbance values represent TBARS equivalents and are the mean ± SD (n = 3).

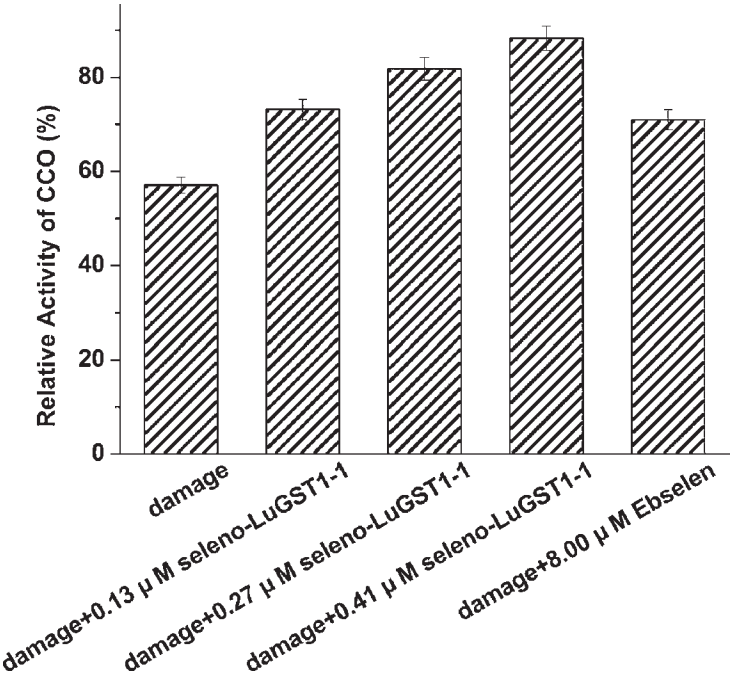

Fig. 4. Effect of seleno-LuGST1-1 on COX activity of mitochondria. Mitochondria were incubated for 60 min in the damage conditions (as explained in Materials and Methods). Data are presented as the mean ± SD (n = 3).

activity could be observed when seleno-LuGST1-1 was added, and the protection of COX activity was enhanced by increasing the concentration of seleno-LuGST1-1. Over 60 min, 88.3 ± 2.6% of COX activity could be retained by adding 0.41 μ*M* seleno-LuGST1-1, whereas COX activity was only 71 ± 2.1% in spite of adding Ebselen with a much higher concentration (8 μ*M*).

Discussion

An imbalanced production of ROS plays a role in the pathogenesis of a number of human diseases and the process of aging. Intensive studies have shown that mitochondria are the major source of ROS that cause oxidative lesions. Mitochondria are also particularly susceptible to ROS, and its deficits owing to oxidative damage are likely to be the major contributors to aging and numerous human diseases *(23,24)*. To evaluate the protection of mitochondria from oxidative damage by artificial enzyme, mitochondria were exposed to ferrous sulfate/ascorbate to mimic the natural oxidative damage occurring in vivo. The reactions catalyzed are described as follows:

$$\text{Ascorbic acid} + O_2 \rightarrow \text{dehydroascorbic acid} + H_2O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (2)$$

$$\text{Ascorbic acid} + 2Fe^{3+} \rightarrow \text{dehydroascorbic acid} + 2Fe^{2+} + 2H^+ \quad (3)$$

The production of H_2O_2 accompanies the oxidation of ascorbic acid to dehydroascorbic acid, and then the generated H_2O_2 reacts with Fe^{2+} to release hydroxyl radical via Fenton reaction. Hydroxyl radical is suggested to be the reactive oxidant that attacks mitochondrial components such as lipids, proteins, and DNA. The swelling of mitochondria, lowering of COX activity, and formation of TBARS illustrated changes in structure, morphology, and function of mitochondria caused by these oxidative damages.

As a significant antioxidant, GPx could effectively catalyze the reduction of H_2O_2 using GSH and, therefore, protect mitochondria from oxidative damage by blocking the production of hydroxyl radical. Because catalase is not abundantly expressed in many mitochondria, GPxs including cGPx and PHGPx play an important role in protecting mitochondria against oxidative damage. The engineered selenoenzyme seleno-LuGST1-1 exhibited significantly high GPx activities and, therefore, could serve as an antioxidant, as evidenced by the ferrous sulfate/ascorbate-induced mitochondrial damage model. Studies on protection group showed that even the low-concentration seleno-LuGST1-1 could evidently inhibit swelling of mitochondria induced by oxidative stress. These results suggest that protein oxidation, which caused changes in the permeability of transition pore *(25)*, was effectively controlled, and that the integrity of the mitochondria was preserved. The decrease in both the maximal level and the slope of rapid phase of TBARS accumulation in the presence of seleno-LuGST1-1

demonstrated that lipid peroxidation was effectively inhibited. Because COX functions as the terminal electron acceptor of the electron transport chain of mitochondria, the decrease in its activity will lead to a rise in oxygen concentration and a reduced state of one-electron donors in the respiratory chain *(26)*, which, consequently, increase ROS production in the respiratory chain and enhance oxidative stress. The diminution of COX activity is directly related to the decrease in the major lipid component of the inner mitochondrial membrane, cardiolipin, owing to oxidative damage *(27)*. Therefore, the inhibition of a decrease in COX activity by seleno-LuGST1-1 provided additional evidence of the prevention of lipid peroxidation in the protection group.

The outstanding antioxidant activity of seleno-LuGST1-1 could be attributable to its significantly high GPx activity as a result of structural similarities between seleno-LuGST1-1 and natural GPx. GPx and seleno-LuGST1-1 adopt a common thioredoxin fold *(28–30)* including a specific GSH-binding site. Their active-site residues, selenocysteines, which interact with the thiol group of the substrate glutathione, hold similar positions in their thioredoxin fold *(13)*. Therefore, seleno-LuGST1-1 could strongly bind reductant, GSH, and catalyze the reduction of H_2O_2 efficiently. Because the GPx activity of seleno-LuGST1-1 was about 2900-fold that of Ebselen, it is not surprising that seleno-LuGST1-1 could scavenge H_2O_2 and inhibit the production of hydroxyl radical more efficiently.

Conclusion

The biologic antioxidative effect of seleno-LuGST1-1 was evaluated by the ferrous sulfate/ascorbate-induced mitochondria damage model. Compared with the classic antioxidant Ebselen, seleno-LuGST1-1 exhibited more effective antioxidant properties for the protection of mitochondria against oxidative stress in this model. This selenium-containing artificial enzyme may therefore potentially be of high value for the treatment of ROS-related diseases, such as cancer, cataracts, cardiovascular disease, and chronic inflammation. However, the difficulties of administering an enzyme in an active state make it difficult to evaluate fully the antioxidant effect of an enzyme. More detailed studies still need to be conducted to investigate further the therapeutic potential of this novel GPx-like enzyme.

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