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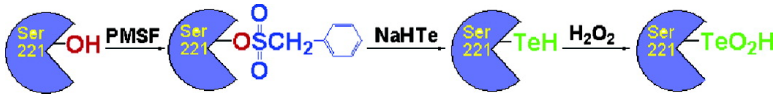

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Semisynthetic Tellurosubtilisin with Glutathione Peroxidase Activity

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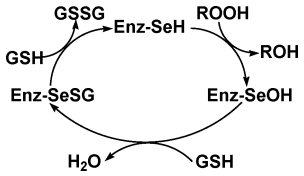
There has been increasing interest in mimicking the functions of an antioxidative selenoenzyme, glutathione peroxidase (GPx, EC 1.11.1.9), which catalyzes the reduction of a variety of hydroperoxides (ROOH) using glutathione (GSH) as the reducing substrate (Scheme 1), and thus protects cells from oxidative damage.[1] Owing to its biological importance, considerable efforts have been devoted to produce organoselenium compounds which mimic the properties of GPx in recent years.[2]

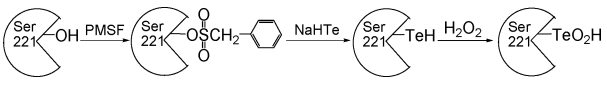
Scheme 1. Catalytic Cycle for GPx



Besides molecular biology techniques for gene manipulation and functional selection toward enzyme design, an alternative strategy for generating an efficient enzyme model is chemical incorporation of catalytic groups into naturally existing or artificially generated substrate binding scaffolds.[3] This strategy has been demonstrated very efficiently in the construction of GPx models by chemically introducing catalytic selenocysteine into thiol glutathione-specific antibody and bioimprinted protein by our group.[4] Natural proteins also provide versatile templates for chemical manipulation. Following the first semisynthetic thiol subtilisin,[5] Hilvert and coworkers developed a methodology to introduce selenium into the binding pocket of subtilisin to yield selenosubtilisin.[6] This hybrid protein combines the intrinsic chemistry of selenium to show GPx activity with the binding specificity of subtilisin for aromatic groups. Recent studies showed that organotellurium compounds also exhibit similar catalytic actions.[7] To our knowledge, however, tellurium has not been found in wild-type proteins, although tellurium had been bioincorporated to protein for structure analysis.[8] Herein, we reported the preparation of the first semisynthetic telluroenzyme, tellurosubtilisin, which was generated by chemically introducing tellurium into the binding site of subtilisin and then acted as an excellent GPx mimic.

Subtilisin is a bacterial serine protease [EC 3.4.21.14] that is chemically and structurally well-studied.[9] Along with the successful chemical modification of subtilisin to thiol/selenosubtilisin,[5,6] the semisynthetic GPx mimic, tellurosubtilisin, was prepared via a three-step protocol starting from subtilisin Carlsberg (Scheme 2). The hydroxyl group of serine-221 of subtilisin (150 mg in 10 mL of 50 mM 1,4-piperazinediethanesulfonic acid (PIPES) buffer containing 10 mM $CaCl_2$, pH 7.0) was specifically activated by reaction with

Scheme 2. Chemical Conversion of Subtilisin into Tellurosubtilisin



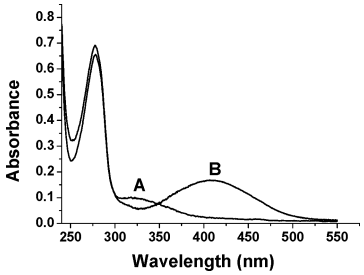


Figure 1. (A) UV spectra of isolated Enz-TeSAr in 50 mM PIPES buffer containing 10 mM $CaCl_2$, pH 7.0 (ϵ_{326} = 7600 M^{-1} cm^{-1}). (B) After addition of excess DTT (for ArSH, pH 7.0, ϵ_{410} = 13 600 M^{-1} cm^{-1}).

phenylmethanesulfonylfluoride (PMSF, 150 μL of a 20 mg mL^{-1} solution in acetonitrile) for 1 h at 25 °C. The sulfonylated enzyme solution was treated with 0.5 mL of 1 M sodium hydrogen telluride (NaHTe) solution, prepared according to the procedure of Akiba et al.[10] in a well-ventilated hood for safety, for 36 h at 40 °C under nitrogen. The resulting telluroprotein was aerially oxidized and then purified by gel filtration on Sephadex G-25 and affinity chromatography on thiopropyl-Sepharose 6B. The generated protein was homogeneous, as judged by polyacrylamide gel electrophoresis,[11] and the yield of telluroprotein was typically 10%, based on the amounts of the starting protein. As a reference experiment, subtilisin was directly treated by NaHTe without initial PMSF activation, and no detectable protein was observed on thiol-mediated affinity chromatography, indicating that the tellurium substitution took place at active serine-221 of subtilisin, similar to that previously reported for thiol/selenosubtilisin.[5,6]

The molecular weight (M_r) value was found to be 27 432 ± 5 by MALDI-TOF-MS, which was consistent with that of the expected tellurinic acid form of the enzyme (Enz-TeO_2H, calculated M_r = 27 432). The oxidized enzyme reacted with 3 equiv of 3-carboxy-4-nitrobenzenethiol (ArSH) to produce the tellurenyl sulfide intermediate (Enz-TeSAr) that can be reduced by dithiothreitol (DTT) to release an equivalent of ArSH (Figure 1), in analogy to the reduction of tellurocyclodextrin by ArSH,[7f] also indicating that it exists as the tellurinic acid form. About 1 equiv (0.97 ± 0.4) of tellurium per molar of subtilisin was incorporated, as judged by anaerobic titration of the reduced enzyme with 5,5′-dithiobis(2-nitrobenzoic acid).[12] One might expect that the tellurium substitution of the hydroxyl group of serine-221 should have no effect on the structure of subtilisin, and the identical behaviors of circular dichroism (CD) spectra for subtilisin and tellurosubtilisin verified this speculation.[11]

Like natural GPx, tellurosubtilisin can catalyze the reduction of ROOH by thiols. The catalytic activity was assessed in detail according to a modified method reported by Hilvert et al.[6] using ArSH as the GSH alternative. The initial rates for the reduction of H_2O_2 by ArSH in 100 mM 4-morpholineethanesulfonic acid (MES) buffer (pH 5.5) in the presence of tellurosubtilisin were determined at 25 °C by monitoring the disappearance of ArSH at 410 nm. To

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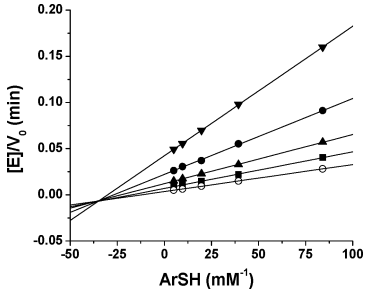

Figure 2. Lineweaver−Burk plots for reduction of H_2O_2 by ArSH catalyzed by 1 μM tellurosubtilisin at 25 °C and pH 5.5 (100 mM MES buffer, 10 mM $CaCl_2$, 1 mM EDTA) at various concentrations of the substrate H_2O_2: (▼) 1.0 mM, (●) 2.0 mM, (▲) 4.0 mM, (■) 8.0 mM, and (○) 25.0 mM. Each point was measured in triplicate, and standard error was less than 5%.

gauge the catalytic ability of tellurosubtilisin, we compared its catalytic efficiency with that of selenosubtilisin and diphenyl diselenide (PhSeSePh). At 25 °C and pH 5.5, the initial rates for the reduction of H_2O_2 (250 μM) by ArSH (100 μM) in the presence of 1 μM enzyme are $(3.9 \pm 0.2) \times 10^{-6}$ and $(2.1 \pm 0.1) \times 10^{-6}$ M min^{-1} for tellurosubtilisin and selenosubtilisin, respectively. Under similar conditions, but with 500 μM diphenyl diselenide as the catalyst, the initial rate is only $(0.95 \pm 0.05) \times 10^{-7}$ M min^{-1}. Thus, under the above conditions, the tellurosubtilisin was approximately 2 times and 20 000 times more efficient than selenosubtilisin and PhSeSePh for GPx activity. The maximal catalytic rate of tellurosubtilisin was observed below pH 6.0.[11] Furthermore, importantly, the tellurosubtilisin, stored in PIPES buffer (pH 7.0) at 4 °C, was particularly stable, and its GPx activity remained unchanged for several months.

The kinetic experiments were carried out in MES buffer (pH 5.5) by varying one substrate's concentration while keeping the other constant. Saturation kinetics was observed for the peroxidase reaction at all the individual concentrations of ArSH and H_2O_2 investigated. Double-reciprocal plots of initial rate versus substrate concentration, as shown in Figure 2, yielded a series of linear plots that all intersect at a point in the third quadrant, and it fits the typical sequential kinetics reaction well, just like the behavior of selenosubtilisin BPN′,[13] rather than the ping-pong mechanism of native GPx,[1a] indicating the formation of a ternary complex between enzyme, thiol, and hydroperoxide prior to product release.[14]

In light of sequential kinetic reaction, the following equation for the initial rate, which depends on [ArSH] and $[H_2O_2]$, accounts for these plots.

$$\frac{V_0}{[E]} = \frac{k_{max}[ArSH][H_2O_2]}{K_{ArSH\ H_2O_2} + K_{ArSH}[H_2O_2] + K_{H_2O_2}[ArSH] + [ArSH][H_2O_2]}$$

where V_0 is the initial rate of the enzymatic reaction, and [E] stands for the total concentration of the enzyme; k_{max} is a pseudo-first-order rate constant, and K_{ArSH} and $K_{H_2O_2}$ are the Michaelis constants for ArSH and H_2O_2, respectively. The constant, $K_{ArSH\ H_2O_2}$, has no easily grasped physical meaning. From the plots, we can get $k_{max} = 518.1 \pm 23.2$ min^{-1}, $K_{H_2O_2} = 20.9 \pm 1.1$ mM, $K_{ArSH} = 128 \pm 6$ μM, and $K_{ArSH\ H_2O_2} = 0.596 \pm 0.031$ mM^2. The k_{max} value represents the turnover number, about 500 molecules of H_2O_2 degraded per minute per molecule of tellurosubtilisin saturated with substrates. The $K_{H_2O_2}$ and K_{ArSH} values of tellurosubtilisin obtained are obviously smaller than that of selenosubtilisin ($K_{H_2O_2} > 0.3$ M and $K_{ArSH} > 400$ μM).[15] Although the k_{max} for tellurosubtilisin is apparently lower than that for selenosubtilisin ($k_{max} > 8000$ min^{-1}),[15] the second-order rate constants ($k_{max}/K_{H_2O_2} = 2.48 \times 10^4$ M^{-1} min^{-1}, and $k_{max}/K_{ArSH} = 4.05 \times 10^6$ M^{-1} min^{-1}) induced from the above data are almost identical with that of selenosubtilisin ($k_{max}/K_{H_2O_2} = 1.37 \times 10^4$ M^{-1} min^{-1}, and $k_{max}/K_{ArSH} = 1.1 \times 10^7$ M^{-1} min^{-1}).[15] The observation revealed that the tellurosubtilisin also favors the aromatic thiol substrate in the evolved specific binding site of subtilisin. To further prove this, we compared the catalytic efficiency of tellurosubtilisin with PhSeSePh in the classical-coupled enzyme assay using GSH as a thiol substrate and found that the former is only 8 times more efficient than the latter for GPx activity.[11]

In summary, we have reengineered the active site of subtilisin by chemical conversion of the catalytically essential serine into a tellurocysteine and produced the semisynthetic telluroprotein, tellurosubtilisin. It can catalyze the reduction of hydroperoxide by an aryl thiol with high catalytic efficiency, but it exhibits kinetic properties substantially different from those of selenosubtilisin. It is anticipated that tellurosubtilisin will present an ideal model for further studies of tellurium chemistry in protein.

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Supporting Information Available: Synthesis and full characterization of the tellurosubtilisin, experimental details for measurements of the GPx catalytic activity and hydrolytic activity, and for the inhibition of GPx activity. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Flohé, L.; Loschen, G.; Günzler, W. A.; Eichele, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 987. (b) *Selenium in Biology and Human Health*; Burk, R. F., Ed.; Springer-Verlag: New York, 1994. (c) Tappel, A. L. *Curr. Top. Cell. Regul.* **1984**, *24*, 87.

(2) (a) Sies, H.; Masumoto, H. *Adv. Pharmacol.* **1997**, *38*, 2229. (b) Mugesh, G.; Du Mont, W. W.; Sies, H. *Chem. Rev.* **2001**, *101*, 2125. (c) Mugesh, G.; Singh, H. B. *Acc. Chem. Res.* **2002**, *35*, 226. (d) Back, T. G.; Moussa, Z. *J. Am. Chem. Soc.* **2003**, *125*, 13455. (e) Iwaoka, M.; Tomoda, S. *J. Am. Chem. Soc.* **1994**, *116*, 2557. (f) Luo, G.; Ren, X.; Liu, J.; Mu, Y.; Shen, J. *Curr. Med. Chem.* **2003**, *10*, 1151.

(3) (a) Qi, H.; Tann, C.-M.; Haring, D.; Distefano, M. D. *Chem. Rev.* **2001**, *101*, 3081. (b) DeSantis, G.; Jones, J. B. *Curr. Opin. Biot.* **1999**, *10*, 324.

(4) (a) Ren, X.; Gao, S.; You, D.; Huang, H.; Liu, Z.; Mu, Y.; Liu, J.; Zhang, Y.; Yan, G.; Luo, G.; Yang, T.; Shen, J. *Biochem. J.* **2001**, *359*, 369. (b) Liu, J.; Luo, G.; Gao, S.; Zhang, K.; Chen, X.; Shen, J. *Chem. Commun.* **1999**, 199.

(5) (a) Polgár, L.; Bender, M. L. *J. Am. Chem. Soc.* **1966**, *88*, 3153. (b) Neet, K. E.; Koshland, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 1606.

(6) Wu, Z.-P.; Hilvert, D. *J. Am. Chem. Soc.* **1990**, *112*, 5647.

(7) (a) Engman, L.; Stern, D.; Cotgreave, I. A.; Andersson, C.-M. *J. Am. Chem. Soc.* **1992**, *114*, 9737. (b) Engman, L.; Stern, D.; Pelcman, M.; Andersson, C. M. *J. Org. Chem.* **1994**, *59*, 1973. (c) Kanda, T.; Engman, L.; Cotgreave, I. A.; Powis, G. *J. Org. Chem.* **1999**, *64*, 8161. (d) McNaughton, M.; Engman, L.; Birmingham, A.; Powis, G.; Cotgreave, I. A. *J. Med. Chem.* **2004**, *47*, 233. (e) You, Y.; Ahsan, K.; Detty, M. R. *J. Am. Chem. Soc.* **2003**, *125*, 4918. (f). Dong, Z.; Liu, J.; Mao, S.; Huang, X.; Yang, B.; Ren, X.; Luo, G.; Shen, J. *J. Am. Chem. Soc.* **2004**, *126*, 16395.

(8) (a) Boles, J. O.; Lewinski, K.; Kunke, M.; Odom, J. D.; Dunlap, B. R.; Lebioda, L.; Hatada, M. *Nat. Struct. Biol.* **1994**, *1*, 283. (b) Budisa, N.; Karnbrock, W.; Steinbacker, S.; Humm, A.; Prade, L.; Neuefeind, T.; Moroder, L.; Huber, R. *J. Mol. Biol.* **1997**, *270*, 616.

(9) (a) Markland, F. S., Jr.; Smith, E. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. III, pp 561−608. (b) Kraut, J. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. III, pp 547−560.

(10) Akiba, M.; Cava, M. P. *Synth. Commun.* **1984**, *14*, 1119.

(11) For the details, see Supporting Information.

(12) Cavallini, D.; Graziani, M. T.; Dupre, S. *Nature* **1966**, *212*, 294.

(13) Peterson, E. B.; Hilvert, D. *Biochemistry* **1995**, *34*, 6616.

(14) (a) Cleland, W. W. *Adv. Enzymol.* **1977**, *45*, 273. (b) Rudolph, F. B.; Fromm, H. J. *Methods Enzymol.* **1979**, *63*, 138.

(15) Bell, I. M.; Hilvert, D. *Biochemistry* **1993**, *32*, 13969.

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