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Study of 8 Types of Glutathione Peroxidase Mimics Based on β-Cyclodextrin

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Abstract: Glutathione peroxidase is key for the removal of H_2O_2 and other hydroperoxides and therefore, it has an important role in the maintenance of the reactive oxygen species (ROS) metabolic balance in vivo. The native enzymes of the glutathione peroxidase family (GPx) have many defects, such as instability in vitro and poor availability. GPx mimetics has become a topic of considerable interest in artificial enzyme research. Many forms of GPx mimics have been synthesized, by including selenium and tellurium (double-bridged and single-bridged, 2-substituted and 6-substituted) in a mother molecule but differences the GPx mimics enzymatic activity have rarely been compared. We designed and synthesized eight cyclodextrin derivatives and used two types of enzyme assays to determine their activities. The results show that: (a) tellurium-containing GPx mimics have higher activity than that of selenium-containing GPx mimics; (b) dual-bridged mimics have higher activity than bis-bridged mimics; and (c) 2-position modified cyclodextrin has higher activity than 6-position modified cyclodextrin.

Keywords: glutathione peroxidase; mimic enzyme; cyclodextrin derivatives; enzyme activity; kinetics

1. Introduction

Glutathione peroxidase (GPx, EC 1.11.1.9) is a key oxidoreductase of H_2O_2 and other hydroperoxides. Thus, it plays an important role in the maintenance of the reactive oxygen species (ROS) metabolic balance in vivo [1]. However, the native GPx shortcomings such as enzyme instability, antigenicity, and poor availability have limited its therapeutic use and encouraged the development of GPx artificial mimics [2,3]. The catalytic functional group of natural GPx is selenocysteine (Sec). It is extremely difficult to synthesize selenium-containing proteins by traditional recombinant DNA methods [4]; therefore considerable effort has been put into the development of other synthetic routes. Progress in GPx mimetics research has led not only to the increase of the knowledge on catalytic mechanisms but also to the production of GPx mimics with potential pharmaceutical application [4,5]. The introduction of selenium or tellurium into GPx mimic enzymes has become a topic of considerable interest in artificial enzyme research.

Typical host systems include cyclodextrins, catalytic antibodies, and natural enzymes [6–8]. Cyclodextrins (CDs) are well known, naturally-occurring, cyclic oligosaccharides composed of at least six α-1-4-linked α-D-glucopyranoside units. The outer surface of CDs is hydrophilic, but they possess an axial open cavity, which is hydrophobic and capable of encapsulating other apolar molecules (or their moiety) [9]. CDs have extensively been exploited as enzyme models [10]. Recently, a series of cyclodextrin-derived organoselenium and organotellurium compounds have been developed as GPx mimics with high substrate specificity [11–15]. Natural cyclodextrins include α-CD, β-CD, and γ-CD. In particular, β-CD has become widely used due to its ease of availability. At present, there are many forms of GPx mimics developed using β-CD as the mother molecule [16,17] and selenium replacement

of its hydroxyl groups at the 2- or 6-position. However, the selenium of selenium-containing GPx mimics have also been replaced tellurium.

Although there are many GPx mimetic enzymes which use β-CD as the mother, the difference between their activities is not yet clear. In order to assess the effects of element (Se/Te), position of hydroxyl group substitution (2/6) and type of bridge (dual bridged/bis-bridged) on enzyme activity and steady-state kinetics of GPx mimics, we synthesized eight small GPx mimics. The four types of selenium-containing mimics were the 2,2′-seleno-bis(2-deoxy- β-cyclodextrin) (2-SediCD), the 2,2′-seleno-(2-deoxy-β-cyclodextrin) (2-SeCD), the 6,6′-seleno-bis(6-deoxy-β-cyclodextrin) (6-SediCD) and the 6,6′-seleno-(6-deoxy-β-cyclodextrin) (6-SeCD). The four tellurium-containing mimics were the 2,2′-telluro-bis(2-deoxy-β-cyclodextrin) (2-TediCD), the 2,2′-ditellurobis-(2-deoxy-β-cyclodextrin) (2-TeCD), the 6,6′-telluro-bis(6-deoxy-β-cyclodextrin) (6-TediCD), and the 6,6′-ditellurobis-(6-deoxy-β-cyclodextrin) (6-TeCD). We determine their activities by two methods, and explore the reason that the enzymatic activity showed a greater vitality by different steady-state kinetics methods.

2. Results and Discussion

2.1. Determination of Enzymatic Activity the Eight Types of GPx Mimics

Firstly, we determined the activity of the newly synthetized GPx mimics using a coupled reductase assay where 1 U of GPx activity was defined as the amount of enzyme required to consume 1 μM NADPH per minute at 37 °C. Among the four selenium-containing GPx mimics, enzyme activity decreased, by different degrees, in the following order: 2-SeCD > 6-SeCD > 2-SediCD > 6-SediCD. Among the four tellurium-containing GPx mimics, enzyme activity decreased, by different degrees, in the following order: 2-TeCD > 6-TeCD > 2-TediCD > 6-TediCD. The results show that the instability (biodegradation) of the dual-bridged cyclodextrin derivatives is higher than that of the bis-bridged ones. Independently of the type of bridge, biodegradation of the 2-substitute modified cyclodextrin derivatives is higher than that of the 6-substitute ones. In addition, the activity of the tellurium-containing mimics is higher than that of selenium-containing mimics (2-TeCD > 2-SeCD, 6-TeCD > 6-SeCD, 2-TediCD > 2-SediCD, 6-TediCD > 6-SediCD).

Secondly, we determined the activity of the GPx mimics with a TNB reaction assay, where 1 U of GPx activity was defined as the amount of enzyme required to consume 2 μM TNB per minute at 37 °C. The enzymatic activity value for each GPx mimic is shown in Table 1.

Table 1. Enzyme activity was measured by a coupled reductase assay and TNB reaction assay.

GPx Mimics	Activity (U/μmol) by Coupled Reductase Assay	Activity (U/μmol) by TNB
β-CD	<0.1	≈0
2-SediCD	0.1 ± 0.1	90 ± 3.2
2-SeCD	7.4 ± 0.2	250 ± 10.2
6-SediCD	0.1 ± 0.1	41 ± 3.5
6-SeCD	4.2 ± 0.1	181 ± 10.1
2-TediCD	0.5 ± 0.1	502 ± 22.1
2-TeCD	46.7 ± 0.4	1155 ± 39.4
6-TediCD	0.3 ± 0.1	304.5 ± 14.1
6-TeCD	5.8 ± 0.2	858 ± 15.2

2.2. Determination of Enzyme Kinetics of the Eight Types of GPx Mimics

The two enzymatic assays used for the current study, are based on a dual substrate reaction were $[E]_0/v_0$ is determined by dual reciprocal mapping of substrate readings. The reactions where 6-TediCD is the catalyst, either in the GSH system (Figure 1) or in the TNB system (Figure 2) produced H_2O_2 reduction data points which could be arranged as parallel lines. The kinetic results, as obtained

from 6-TediCD catalyst reactions, match a Ping-Pong kinetic mechanism, which might be also used to represent the kinetics of the other seven GPx mimics and of the natural GPx enzyme.

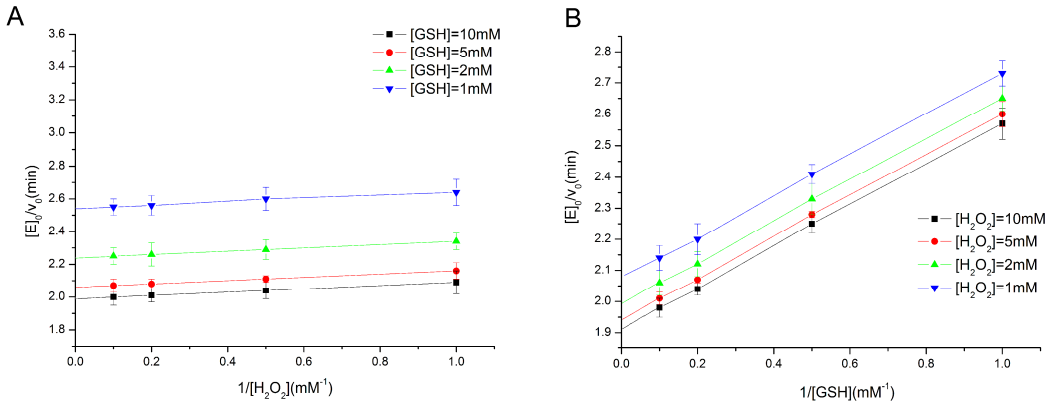


Figure 1. Double-reciprocal plots for the reduction of H_2O_2 catalyzed by 6-TediCD in the GSH system. (**A**) $[E]_0/v_0$ vs. $1/[H_2O_2]$ (mM^{-1}). (**B**) $[E]_0/v_0$ (min) vs. $1/[GSH]$ (mM^{-1}).

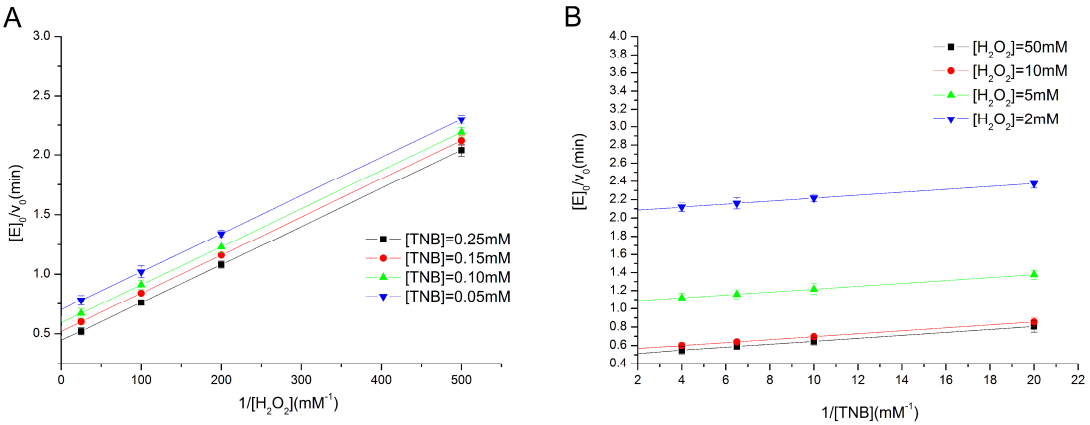


Figure 2. Double-reciprocal plots for the reduction of H_2O_2 catalyzed by 6-TediCD in the TNB system. (**A**) $[E]_0/v_0$ vs. $1/[H_2O_2]$ (mM^{-1}). (**B**) $[E]_0/v_0$ (min) vs. $1/[TNB]$ (mM^{-1}).

$[E]_0/v_0$ values were deduced from the equation below (Equation (1)) of a Ping-Pong kinetic mechanism.

$$\frac{v_0}{[E]_0} = \frac{k_{max}[RSH][H_2O_2]}{K_{H_2O_2}[RSH] + K_{RSH}[H_2O_2] + [RSH][H_2O_2]} \quad (1)$$

Here, v_0 is the initial reaction rate, $[E]_0$ is the initial concentration of the GPx mimic, k_{max} is the pseudo-first order reaction rate constant, $K_{H_2O_2}$ and K_{RSH} are the Michaelis–Menten constants of H_2O_2 and RSH. RSH is either GSH or TNB. The rate of k_{max}/K_{RSH} and the rate of $k_{max}/K_{H_2O_2}$ are the slopes of graphs A and B, respectively (Figure 2). The k_{max}/K_{RSH} and $k_{max}/K_{H_2O_2}$ are taken into Equation (1), which allows the k_{max}, K_{RSH}, and $K_{H_2O_2}$ values to be determined, independently of substrate concentrations. A different set of kinetic parameters were obtained from each enzyme kinetic assay system used.

As seen in Table 2, the values of k_{a1} ($k_{a1} = k_{max}/K_{H_2O_2}$) for the four dual-bridged GPx mimics 2-SeCD, 6-SeCD, 2-TeCD, and 6-TeCD were similar. The k_{a1} of the four bis-bridged GPx mimics 2-SediCD, 6-SediCD, 2-TediCD, and 6-TediCD were also similar but of a lower order of magnitude than those of the dual-bridged GPx mimics. A lower k_{a1} also shows the enzyme activity of the bis-bridged mimics was lower than that of the dual-bridged mimics. Diselenide reacts directly with the thiol substrate [18]. However, the monoselenides must be react first with the peroxide [19]. A study by

Dong et al. had also showed that 2-TeCD reacts directly with the thiol substrate [20]. It can explain why the activity of dual-bridged mimic enzymes was higher than that of single-bridged mimetic enzymes. The apparent rate constant of the GPx mimics-catalyzed GSH k_{b1} ($k_{b1} = k_{max}/K_{GSH}$) of bis-bridged mimics was 10 times higher than that of the dual-bridged mimics.The kinetic parameters can also be seen that the double bridging mimics have stronger affinity for the substrate, and their k_a and k_b values are significantly higher than those of single bridge mimetics.

Table 2. Enzyme kinetic parameters were measured by coupled reductase assay and TNB.

GPx Mimics	$k_{max}/K_{H_2O_2}$ (M·min^{-1})		k_{max}/K_{GSH} (M·min^{-1})	k_{max}/K_{TNB} (M·min^{-1})
	Coupled Reductase Assay	TNB		
2-SediCD	$(6.5 \pm 0.7) \times 10^3$	$(7.2 \pm 0.8) \times 10^2$	$(7.3 \pm 0.4) \times 10^2$	$(9.3 \pm 0.8) \times 10^4$
2-SeCD	$(5.2 \pm 0.6) \times 10^4$	$(5.6 \pm 0.5) \times 10^3$	$(2 \pm 0.4) \times 10^3$	$(8.4 \pm 0.9) \times 10^5$
6-SediCD	$(3.3 \pm 0.7) \times 10^3$	$(3.0 \pm 0.6) \times 10^2$	$(6.8 \pm 0.3) \times 10^2$	$(5.2 \pm 0.4) \times 10^4$
6-SeCD	$(3.3 \pm 0.4) \times 10^4$	$(4.3 \pm 0.3) \times 10^3$	$(1.8 \pm 0.2) \times 10^3$	$(6.1 \pm 0.6) \times 10^5$
2-TediCD	$(7 \pm 0.39) \times 10^3$	$(8.7 \pm 0.3) \times 10^2$	$(1.6 \pm 0.4) \times 10^3$	$(7 \pm 0.4) \times 10^6$
2-TeCD	$(8.2 \pm 0.9) \times 10^4$	$(6 \pm 0.5) \times 10^3$	$(6.2 \pm 0.7) \times 10^4$	$(1.1 \pm 0.6) \times 10^7$
6-TediCD	$(5.4 \pm 0.8) \times 10^3$	$(8.2 \pm 0.2) \times 10^2$	$(1.3 \pm 0.2) \times 10^3$	$(4.8 \pm 0.5) \times 10^6$
6-TeCD	$(3.7 \pm 0.7) \times 10^4$	$(1.9 \pm 0.3) \times 10^3$	$(1.6 \pm 0.1) \times 10^4$	$(8.6 \pm 0.7) \times 10^6$

The apparent velocity constant of the GPx mimics-catalyzed H_2O_2 as measured by the TNB assay was defined as k_{a2} ($k_{a2} = k_{max}/K_{H_2O_2}$). The apparent velocity constant of the GPx mimics-catalyzed TNB was defined as k_{b2} ($k_{b2} = k_{max}/K_{TNB}$). As measured by the TNB assay, the relationship between k_{a2} and k_{b2} remained the same among the eight GPx mimics. In order to further assess differences between the two enzyme systems, the k_{a1} of the GPx mimics was compared with k_{a2}, k_{b1}, and k_{b2}. Although for the same GPx mimic, the k_{a1} was about one order of magnitude higher than k_{a2}, its k_{b1} was three orders of magnitude smaller than k_{b2}. Therefore, the activity of the GPx mimics as measured by the coupled reductase assay was several times smaller than that measured by the TNB assay.

The k_{b1} of selenium-substituted enzymes was significantly lower than that of tellurium-substituted enzymes. As anticipated, the more sensitive oxidative-reductive property of tellurium compared with selenium endows enzyme models 2-TediCD, 6-TediCD, 2-TeCD, and 6-TeCD with higher catalytic activity than that of 2-SediCD, 6-SediCD, 2-SeCD, and 6-SeCD, respectively [21]. The k_{b1} of the 2-substitute mimics was significantly higher than that of the 6-substitute mimics, which also might explain why the activity of 2-substitute mimics was higher than that of the 6-substitute mimics, may be due to the fact that substrate GSH preferentially binds into the secondary face of β-CD [21].

The results summarized in Table 3 show that the values for the Michaelis–Menten constant K_{RSH} of the tellurium mimics were generally greater than those of the seleniumase mimics, which suggests that there is a stronger substrate affinity between tellurium GPx mimics and the RSH substrate. Also, GPx mimics with selenium/tellurium linked to cyclodextrin at the 2-position exhibited higher affinity for the substrate H_2O_2 compared to GPx mimics with selenium/tellurium linked to cyclodextrin at the 6-position. The affinity of the dual-bridged mimics to both substrates (H_2O_2 and RSH) was higher than that of the bis-bridged mimics. These results all confirm the above conclusions.

Table 3. Kinetic parameters for the mimics-catalyzed reduction of hydroperoxides by GSH and TNB.

GPx Mimics	$K_{H_2O_2}$ (μM^{-1})		K_{GSH} (μM^{-1})	K_{TNB} (μM^{-1})	K_{max} (min^{-1})	
	GSH	TNB			GSH	TNB
2-SediCD	15.64 ± 1.37	12.67 ± 1.21	139.32 ± 5.97	98.95 ± 3.49	0.101 ± 0.03	91.22 ± 3.59
2-SeCD	236.57 ± 12.54	46.74 ± 1.57	615.89 ± 31.33	91.62 ± 5.24	12.30 ± 0.98	261.76 ± 19.92
6-SediCD	30.94 ± 2.49	115.31 ± 4.92	150.17 ± 7.42	65.29 ± 5.13	0.102 ± 0.03	34.59 ± 2.63
6-SeCD	171.7 ± 9.32	43.5 ± 2.66	314.99 ± 19.65	69.85 ± 3.22	5.666 ± 1.07	189.01 ± 9.53
2-TediCD	74.83 ± 4.56	136.36 ± 5.33	327.4 ± 22.38	109.57 ± 6.84	0.523 ± 0.15	1186.99 ± 43.36
2-TeCD	719.2 ± 23.48	238.42 ± 15.29	951.2 ± 63.12	130.04 ± 5.39	58.97 ± 2.49	1430.52 ± 38.94
6-TediCD	57.52 ± 3.42	90.74 ± 8.31	238.93 ± 15.27	100.91 ± 7.27	0.310 ± 0.08	484.41 ± 21.04
6-TeCD	168.12 ± 5.35	120.23 ± 7.26	388.78 ± 25.58	141.7 ± 10.74	6.220 ± 1.84	1218.68 ± 49.81

3. Materials and Methods

3.1. General Procedure

β-cyclodextrin (β-CD), acetone, methanol, ethanol, and chloroform were obtained from the Beijing Chemical Works. Glutathione reductase (Gr), reduced coenzyme II tetrasodium salt, and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (Shanghai, China). Selenium, tellurium, and hydrogen peroxide (H_2O_2) were obtained from Aladdin. Sephadex G-25 and GSH were obtained from Pharmacia. p-toluene sulfonyl chloride (p-TsCl) was obtained from the Tianjin Chemical Reagent Factory. Sodium borohydride ($NaBH_4$) was obtained from the Tianjin Institute of Fine Chemical Industry.

For the synthesis of the GPx mimics, β-CD was initially ground to a powder and placed in an oven to vacuum dry at 60 °C. β-CD was recrystallized three times.

3.2. Synthesis of Eight Types of GPx Mimics

The synthesis route of the eight types of GPx mimics is shown in Scheme 1.

The regiospecific monotosylation of the 2-substituent hydroxyl group of cyclodextrin was carried out according to Wang et al. [22]. 0.96 g of NaOH was dissolved in 160 mL of water and 4 g of recrystallized β-CD was weighed. While stirring, the acetone solution of p-TsCl (4 g/10 mL) was slowly added dropwise and the pH of the solution was kept slowly at pH = 12.5. About 4 h drip finished, continue to react 1 h. The pH of the solution was adjusted to 7.0 with 1 M HCl, 200 mL of methanol was added and the insoluble material was removed by suction filtration. The solution was distilled off to about 50 mL under reduced pressure, 50 mL of methanol was added, and the mixture was allowed to stand at 4 °C for one week and the precipitate was filtered off. Sephadex G-25 column chromatography, H_2O as eluent, collecting the first peak. The powder after lyophilization is 2-OTs-β-CD.

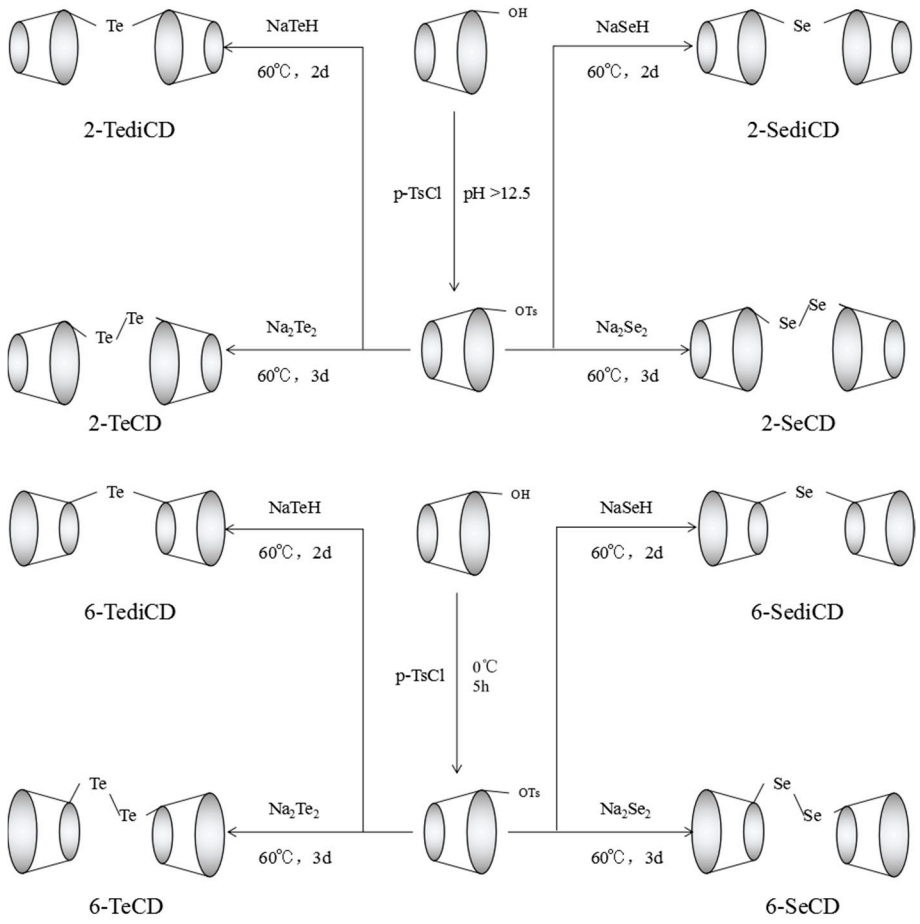
The regiospecific monotosylation of the 6-substituent hydroxyl group of cyclodextrin was carried out according to Matsui et al. [23]. 6.0 g of NaOH was dissolved in 200 mL of water, 10 g of recrystallized β-CD was added, ice bath and stirred. A solution of p-TsCl in acetone (3.36 g/10 mL) was slowly added and the reaction was carried out at about 0 °C for 5 h. The insoluble matter was removed by suction filtration and the pH of the solution was adjusted to 7.0 with 1 M HCl. The solution was allowed to stand in boiling water and the insoluble material was removed by suction filtration through a Buchner funnel and a suction filter bottle preheated at 100 °C. The filtrate was allowed to stand at room temperature and then cooled at 4 °C overnight. The purification step was repeated three times. The precipitate was finally collected and dried in vacuo at 60 °C in an oven.

NaHSe and Na_2Se_2 were prepared according to Klayman et al. [24]. 10 mL H_2O was added into the vials, 200 mg $NaBH_4$ (synthesis of Na_2Se_2 was 106 mg) and 110 mg Se powder (synthesis of Na_2Se_2 was 220 mg) was added and covered the plug and continued to bubble through with pure nitrogen. Placed on a magnetic stirrer to stir the reaction until the selenium powder is completely reduced, the solution was colorless with a small amount of white precipitated (the solution of Na_2Se_2 was yellow).

NaHTe and Na_2Te_2 were prepared according to Anslyn et al. [25]. 10 mL H_2O was added into the vial, bottle A, bubbled through with pure nitrogen gas for 15 min then sealed. Then another vial, bottle B, was made by adding 160 mg Te (synthesis of Na_2Te_2 was 84 mg) and 300 mg $NaBH_4$ (synthesis of Na_2Te_2 was 330 mg), bubbled through with pure nitrogen gas for 15 min, 10 mL H_2O solution was removed with a syringe into bottle B. Bottle B was then placed into the heating stirrer at 60 °C water bath stirring, the reaction process continued through the nitrogen. Tellurium powder reaction completely sealed, the solution was colorless (the solution of Na_2Te_2 was deep purple).

The synthetic process was carried out according to Breslow and Liu et al. [26,27]. 2-OTs-β-CD or 6-OTs-β-CD 330 mg was dissolved in 10 mL H_2O (two drops of DMF was added to 6-OTs-β-CD reaction mixture). The reaction mixture was then bubbled through with pure nitrogen gas for 20 min, and then 1 mL NaHSe/NaHTe (synthesis of dual-bridged mimics was Na_2Se_2/Na_2Te_2) was added. Next the mixture was bubbled through with pure nitrogen gas for 15 min then sealed and stirred for

48 h (synthesis of dual-bridged mimics was 72 h) at 60 °C. The reaction solution was removed and centrifuged at 5000 rpm for 20 min. The precipitate was discarded and twice the volume of absolute ethanol was added. Was centrifuged at 5000 rpm for 20 min, collected precipitation. Was dissolved in 5 mL H_2O, separated by Sephadex G-25 column chromatography, and H_2O was used as eluent. The first peak was collected and lyophilizated.



Scheme 1. The synthesis route of 8 types of GPx mimics.

We used ^{13}C-NMR, ESI-MS, and elemental analysis to characterize the newly synthesized mimics.

2-SediCD. The 2-SediCD was obtained in 32.01% yield as a white with a little yellow powder. ^{13}C-NMR (D_2O): δ 103.3, 101.3, 82.1, 80.1, 73.4, 72.9, 69.8, 60.4; MALDI-MS: Calcd 2366.9, Found 2367.2; Anal. Calcd for $C_{84}H_{138}O_{69}Se{\cdot}2H_2O$: C 43.58, H 6.01. Found: C 43.44, H 6.12.

2-SeCD. The 2-SeCD was obtained in 40.60% yield as a light yellow powder. ^{13}C-NMR (D_2O): δ 103.2, 101.8, 82.7, 80.3, 73.9, 72.8, 70.5, 61.9; MALDI-MS: Calcd 2517.7, Found 2518.2; Anal. Calcd for $C_{84}H_{138}O_{69}Se_2{\cdot}6H_2O$: C 40.32, H 5.96. Found: C 40.14, H 6.00.

6-SediCD. The 6-SediCD was obtained in 31.85% yield as a white with a little yellow powder. ^{13}C-NMR (D_2O): δ 102.3, 82.1, 73.5, 72.8, 72.3, 61.7, 59.3; MALDI-MS: Calcd 2366.9, Found 2367.4; Anal. Calcd for $C_{84}H_{138}O_{69}Se{\cdot}2H_2O$: C 43.45, H 6.13. Found: C 43.58, H 6.07.

6-SeCD. The 6-SeCD was obtained in 39.21% yield as a light yellow powder. ^{13}C-NMR (D_2O): δ 103.7, 81.6, 73.2, 72.5, 71.8, 63.5, 59.8; MALDI-MS: Calcd 2518.0, Found 2516.9; Anal. Calcd for $C_{84}H_{138}O_{69}Se_2{\cdot}6H_2O$: C 40.32, H 5.99. Found: C 40.38, H 5.87.

2-TediCD. The 2-TediCD was obtained in 25.37% yield as a yellow powder. ^{13}C-NMR (D_2O): δ 104.3, 102.2, 82.5, 79.8, 73.5, 72.3, 69.9, 61.3; MALDI-MS: Calcd 2381.6, Found 2382.2; Anal. Calcd for $C_{84}H_{138}O_{68}Te{\cdot}H_2O$: C 42.69, H 5.88. Found: C 42.62, H 5.95.

2-TeCD. The 2-TeCD was obtained in 33.41% yield as an orange powder. ^{13}C-NMR (500 MHz, D_2O), δ 101.8, 98.5, 80.1, 75.9, 72.3, 70.9, 68.3, 59.5. MALDI-MS: Calcd 2599.2, Found 2598.3; Anal. Calcd for $C_{84}H_{138}O_{68}Te_2{\cdot}6H_2O$: C, 38.32; H, 5.58. Found: C, 37.87, H, 5.65.

6-TediCD. The 6-TediCD was obtained in 28.48% yield as a yellow powder. ^{13}C NMR (500 MHz, D_2O): δ 102.6, 81.3, 72.9, 72.1, 71.8, 60.2; MALDI-MS: Calcd 2579.7, found 2579.9; Anal. Calcd for $C_{84}H_{138}O_{68}Te{\cdot}12H_2O$: C, 39.07; H, 6.28. Found: C, 38.93; H, 6.05.

6-TeCD. The 6-TeCD was obtained in 33.41% yield as an orange powder. ^{13}C NMR (500 MHz, D2O): δ 102.4, 81.5, 73.6, 72.5, 72.3, 60.5; MALDI-MS: Calcd 2689.4 Found 2688.3; Anal. Calcd for $C_{84}H_{138}O_{68}Te_2{\cdot}11H_2O$: C, 36.91; H, 5.95. Found: C, 36.75; H, 5.68.

The enzyme activity was measured at different pH (4–12) and at different temperatures, and the results show that the optimum pH and optimum temperature are 2-SediCD (pH 8.0, 55.3 °C), 2-SeCD (pH 7.8, 52.5 °C), 6-SediCD (pH 8.2, 58.2 °C), 6-SeCD (pH 7.9, 54.1 °C), 2-TediCD (pH 8.2, 52.3 °C), 2-TeCD (pH 7.9, 49.6 °C), 6-TediCD (pH 8.3, 54.6 °C), 6-TeCD (pH 8.1, 52.8 °C), respectively.

3.3. Determination of Activity and Steady-State Kinetics of GPx Mimics by a Coupled Reductase Assay

The GPx activity of the mimetic enzymes was determined by Wilson's dual enzyme coupling assay [28]. The equations for the reaction are described below (Equations (2) and (3)). The sample and control reaction tubes both contained 500 μL PBS (50 mM, pH 7.0), 100 μL EDTA (1 mM), 100 μL GSH (10 mM), 100 μL Gr (10 U/mL), 100 μL NADPH (2.5 mM), and 100 μL H_2O_2 (5 mM). The GPx-catalyzed reaction between glutathione and H_2O_2 consisted of a two-substrate enzymatic reaction. In order to determine the enzyme kinetic constants of the GPx mimics, we have fixed one of the substrate concentrations and change the other substrate concentration.

$$\mathrm{ROOH} + 2\mathrm{GSH} \xrightarrow{\mathrm{GPx}} \mathrm{ROH} + \mathrm{GSSH} + \mathrm{H_2O} \quad (2)$$

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{\mathrm{Gr}} 2\mathrm{GSH} + \mathrm{NADP^+} \quad (3)$$

3.4. Determination of Activity and Steady-State Kinetics of GPx Mimics by a TNB Reduction Assay

The synthesis reaction of TNB was performed according to Silver et al. [29]. The enzyme activity was determined according to Bell et al. [30]. Where the reaction equation is described below (Equation (4)). The sample and control reaction tubes both contained PBS (50 mM, pH 7.0), EDTA (1 mM), TNB (200 μM), and H_2O_2 (5 mM). The GPx-catalyzed reaction of TNB with H_2O_2 is a single enzyme reaction. In order to determine the enzymatic kinetic constants of the GPx mimics, we have fixed one of the substrate concentrations and change the other substrate concentration.

$$\mathrm{ROOH} + 2\mathrm{TNB} \xrightarrow{\mathrm{GPx}} \mathrm{ROH} + \mathrm{DTNB} + \mathrm{H_2O} \quad (4)$$

4. Conclusions

In summary, comparison among GPx mimics indicated that: (a) the activity of tellurium GPx mimics is higher than that of selenium GPx mimics; (b) the activity of dual-bridged mimic enzymes was higher than that of single-bridged mimetic enzymes; (c) selenium/tellurium introduced at the 2-position of the cyclodextrin produces enzyme mimics with higher activity than that of those with selenium/tellurium introduced at the 6-position.

The apparent velocity constant of Tellurium GPx mimics, the RSH constant (k_b), and the Michaelis–Menten constant for RSH (K_{RSH}) were higher than those of the selenium enzyme, which may

be due to the fact that tellurium atoms are more active than selenium atoms which would also contribute to higher activity levels of tellurium GPx mimics compared to those of the selenium GPx mimics.

The reason why the dual-bridge mimic enzymes' activity is higher than that of single-bridge mimic enzymes may be the fact that the dual-bridge mimetic enzyme might have a different identity from the single-bridge one. The kinetic parameters also show that the dual-bridge GPx mimics have stronger affinity for the substrate, and their k_a and k_b values are significantly higher than those of the single-bridge GPx mimics.

The activity of the selenium/tellurium 2-substitute mimics was higher than that of the 6-substitute mimics. Thus, GPx mimics mainly differed in their hydrogen peroxide apparent velocity constant (k_a).

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