Antioxidant Enzyme Mimics with Synergism

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Abstract: The antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase contribute dominatingly to enhance cellular antioxidant defense against oxidative stress. They act cooperatively to scavenge reactive oxygen species, and not one of them can singlehandedly clear all forms of reactive oxygen species. On the basis of the structural understanding for these natural enzymes, many mimics with multifunctional activities had been obtained by chemical synthesis, biosynthesis, and protein fusion techniques. Some of them display remarkable antioxidant cooperative effect in living model which possess potential application in medicine as potent antioxidants. This review summarizes aspects of some multifunctional mimics which have been reported so far.

Keywords: Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GST), reactive oxygen species (ROS), antioxidant enzymes, enzyme mimics, synergism.

I. INTRODUCTION

Reactive oxygen species (ROS), the by-products of oxygen consumption, are involved in the cell growth, differentiation, progression, and death. Higher amounts of ROS play a role in the aging process as well as in a number of human disease states, including cancer, ischemia, and failures in immunity and endocrine functions [1, 2]. Some ROS, such as superoxide radical anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), are formed in biological systems by the partial reduction of molecular oxygen. Oxygen (O_2) is partially reduced by either one electron to form the superoxide radical anion ($O_2^{-\bullet}$) or two electrons to generate hydrogen peroxide (H_2O_2). In addition, further reduction of $O_2^{-\bullet}$ and H_2O_2 produces other reactive species. H_2O_2 can be reduced to form the hydroxyl radical ($HO^{\bullet}$), another extremely reactive species that readily oxidizes all cellular macromolecules, including proteins, sugars, lipids and DNA. $O_2^{-\bullet}$ readily reacts with NO to form peroxynitrite ($ONOO^-$), which is unstable at physiologic pH and readily decomposes into potent oxidizing and nitrating species. Antioxidants could detoxify these reactive species to water (Fig. (**1**)) [3].

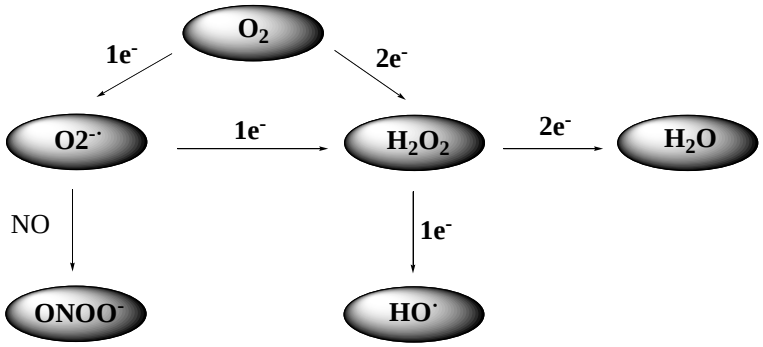

Fig. (1). The generation of ROS.

The antioxidant enzymes, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6)), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), and glutathione S-transferases (GST, EC 2.5.1.18) contribute dominatingly to enhance cellular antioxidant defense against oxidative stress. SOD catalyzes the destruction of the free radical $O_2^{-\bullet}$ by converting it to H_2O_2 and molecular oxygen [4]. CAT catalyzes the decomposition of H_2O_2 to water and molecular oxygen [5]. GPx, being the first

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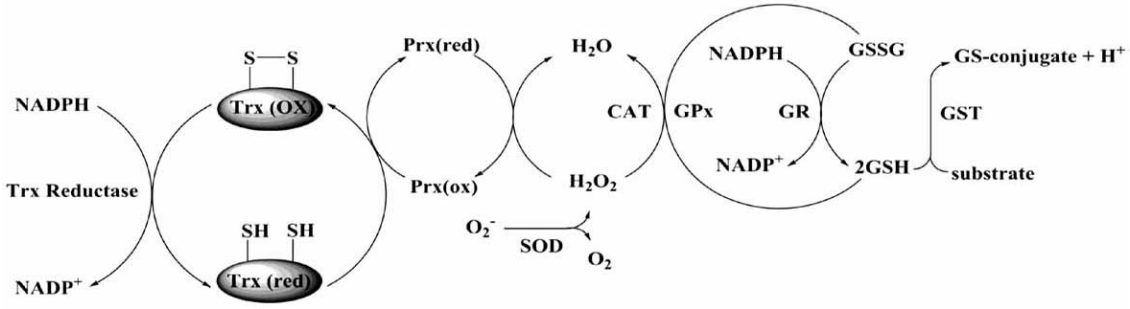


Fig. (2). Schematic summary of the antioxidant system.

identified selenium containing enzyme, catalyzes the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using glutathione (GSH) as substrate [6]. As a detoxification enzyme, GST is a versatile enzyme family whose main role is to inactivate a wide range of exogenous/endogenous toxic molecules and to turn them into water-soluble compounds [7]. These enzymes play crucial roles in balancing the production and decomposition of ROS in living organisms, which act cooperatively and synergistically to scavenge ROS, as not one of them can singlehandedly clear all forms of ROS. Superoxide anions ($O_2^{-\bullet}$) are reduced by SOD to form molecular oxygen and hydrogen peroxide (H_2O_2), then, H_2O_2 is reduced by CAT to form H_2O or by GPx to form H_2O through oxidation of two molecules of GSH, forming glutathione disulfide (GSSG) that subsequently can be reduced by GR under consumption of NADPH. GST catalyzes the conjugation of glutathione with other biomolecules (Fig. (**2**)). Therefore, to obtain novel antioxidant medicines, a great deal of effort has been devoted to the artificial imitation of the enzymes with cooperative multifunctional activities. In the past 20 years, on the basis of the structural understanding for SOD, GST, GPx, many enzyme mimics with bifunctional or trifunctional activities bad been obtained by chemical synthesis, biosyntheis, protein fusion techniques. Some of them display remarkable antioxidant cooperative effect in living model which possess potential application in medicine as potent antioxidants. This review summarizes the aspects of some bifunctional and trifunctional mimics which has been reported so far. To our knowledge, this is the first review on multifunctional antioxidant enzyme mimics.

II. SYNTHETIC MULTIFUNCTIONAL ENZYME MODELS WITH LOW MOLECULAR WEIGHT

(A) EUK Compounds, a Class of Multifunctional Antioxidant

SOD catalyzes the conversion of $O_2^{-\bullet}$ into H_2O_2 which is subsequently reduced to H_2O by CAT. Thus, SOD constitutes one of the main cellular protective systems against free oxygen radicals. Administration of either native or polymer-supported SOD has been shown to have beneficial effects against a number of conditions associated with increased production of oxygen free radicals, and the search for stable, low molecular weight SOD mimics has accordingly emerged as an area of active research.

EUK is a class of the manganese(III)-containing salen complexes with multifunctional activities. These complexes are perceived by us to hold some interesting features with respect to their potential as SOD mimics, such as stable, covalently bound manganese centers, manganese in a valence III resting state and well-established sites for oxygen binding [8, 9].

EUK-8 [manganese N, N'-bis(salicylidene)ethylenediamine chloride] is the originally classic EUK class with demonstrated SOD activity [10]. In addition, this compound also catalyzes the conversion of H_2O_2 to molecular oxygen, as detected by the generation of a stoichiometric excess of oxygen in the presence of hydrogen peroxide. It indicated that EUK-8 has CAT activity as well [11].

a. The Structure and Catalytic Mechanism of EUK Series

EUK series were manganese-salen complexes (Mn-salen) (Fig. (**3**)). The Mn(III) that is present at the center of the molecule confers it solubility in water, whereas its aromatic rings make it a very lipophilic molecule that easily enters through cellular membranes. This structure is similar to that of mitochondrial Mn-SOD, in which manganese is associated with four ligands, three of which are imidazole rings [12]. The Mn(III) is the SOD catalytically active center, however, the Mn(III) of the salen compound is coordinated by four axial ligands of oxygen and nitrogen, that is important in scavenging a wide variety of ROS. The mechanism for the dismutation of $O_2^{-\bullet}$ involves the reduction of Mn(III) to Mn(II) by $O_2^{-\bullet}$, which is oxidized to O_2 (Eqn. **1**). Then this Mn(II) is subsequently oxidized back to Mn(III) by another molecule of $O_2^{-\bullet}$, yielding H_2O_2 (Eqn. **2**). This mechanism is also very similar to that of Mn-SOD [13].

$$Mn(III) + O_2^- \longrightarrow Mn(II) + O_2 \qquad (1)$$

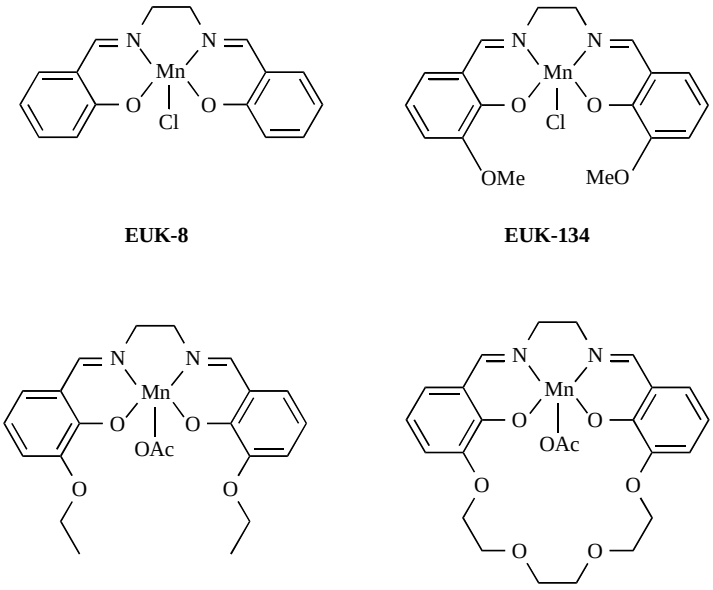
$$2H^+ + Mn(II) + O_2^- \longrightarrow Mn(III) + H_2O_2 \qquad (2)$$

The mechanism by which the Mn-Salen acts a CAT mimetic involves the oxidation of Mn-Salen to an oxomanganese-salen complex (oxoMn-Salen) by H_2O_2, releasing water (Eqn. **3**). The oxoMn-Salen is then reduced by another molecule of H_2O_2 to regenerate the Mn-Salen and generate water and O_2 (Eqn. **4**).

$$Mn(III) + H_2O_2 \longrightarrow Mn(V)O^{2-} + H_2O \qquad (3)$$

$$Mn(V)O^{2-} + H_2O_2 \longrightarrow Mn(III) + H_2O + O_2 \qquad (4)$$

$$\text{Mn(III)-Salen} + NO_2^- + H_2O_2 + AH_2 \longrightarrow \text{Mn(III)-Salen} + A + 2H_2O \qquad (5)$$



EUK-8 **EUK-134**

EUK-189 **EUK-207**

Fig. (3). The structure of EUK compounds.

EUK-8 also exhibits peroxidase (POD) activity [14]. The mechanism can be described by the following Eqn. **5**, where AH_2 is an oxidizable substrate such as ABTS [2, 2'-azino-bis (3-ethlbenz-thiazoline-6-sulfonic acid)]:

Many disease states are associated with an overproduction of ROS, $O_2^{-\bullet}$, H_2O_2 and OCl^-, and RNS, NO and $ONOO^-$. Sharpe *et al.* also demonstrated that Mn-Salens catalyse the removal of both OCl^- and some reactive nitrogen oxides (RNS), such as $ONOO^-$ and NO [15]. The new mechanism was elucidated as follow (Eqn. **6-11**):

$$\text{Mn(III)} + OCl^- \longrightarrow \text{Mn(V)}O^{2-} + Cl^- \qquad (6)$$

$$\text{Mn(III)} + ONOO^- \longrightarrow \text{Mn(V)}O^{2-} + NO_2^- \qquad (7)$$

$$\text{Mn(V)}O^{2-} + NO \longrightarrow \text{Mn(III)} + NO_2 \qquad (8)$$

$$NO_2 + NO \longrightarrow N_2O_3 \qquad (9)$$

$$N_2O_3 + H_2O \longrightarrow 2HNO_2 \qquad (10)$$

$$\text{Mn(V)}O^{2-} + NO_2^- \longrightarrow \text{Mn(III)} + NO_3^- \qquad (11)$$

b. Biological Effects of the EUK Series

In support of the potential clinical value of this multifunctional catalytic ROS scavenger, EUK compounds such as EUK-8 have protective effects in numerous pharmacological models for ROS-associated tissue damage. For example, EUK-8 could protect against lipopolysaccharide (LPS)-induced acute lung injury [11]. However, monofunctional SOD and SOD mimics could not have effect on pulmonary or systemic manifestations of acute endotoxicosis because H_2O_2 is the main toxic moiety in LPS-induced acute lung injury which is more important than $O_2^{-\bullet}$ [16]. To further prove oxygen free radicals mediate amyloid peptide (βAP)-induced neurotoxicity, Bruce *et al.* evaluated the effect of EUK-8 in organotypic hippocampal cultures on βAP toxicity. The results not only demonstrate ROS play a critical role in βAP toxicity but also highlight the therapeutic potential of synthetic radical scavenger in Alzheimer disease [17]. However, these studies did not demonstrate EUK-8 interacts directly with ROS. Using electron spin resonance (ESR) spectroscopy, Tanguy *et al.* showed that EUK-8 exhibited marked antioxidant activities (SOD and CAT activities) in the presence of ROS-generating system. Then they demonstrated the ability of EUK-8 to directly protect cardiomyocytes against ROS [18]. Apoptosis-inducing factor (AIF) is a highly conserved flavoprotein with pyridine nucleotide-disulphide oxidoreductase and DNA binding domains which were identify as a cardiac mitochondrial antioxidant. EUK-8 could protect the AIF-deficient myocardium against pressure overload in harlequin mouse and it may be useful as a novel therapeutic tool in the treatment of human heart failure [19].

EUK-134 [manganese 3-methoxy N, N'-bis(salicylidene) ethylenediamine chloride], a 3, 3'-methoxy salen ring-disubstituted EUK-8 analogue with equivalent SOD activity but enhanced catalase activity, was described and shown to be more neuroprotective than EUK-8 in a rodent stroke model [20]. It generally is accepted that oxidative stress increases the production of not only ROS, but also nitric oxide ($NO^{\bullet}$), thus resulting in formation of the toxic peroxynitrite ions. The experiment that EUK-134 protected hippocampal

neurons and suppressed several indicators of stress and tissue damage in a kainic acid-induced seizure model in rat indicates the interaction of EUK with $NO^{\bullet}$ or the generation of nitric oxide synthase (NOS) because NOS inhibitor protects kainite-induced neuronal damage [21]. The free radical $NO^{\bullet}$ could readily interact with $O_2^{-\bullet}$ to produce peroxynitrite ($ONOO^-$), a potent oxidant and nitrating agent and a mediator of nigrostriatal damage in Parkinson's disease (PD). As a catalytic scavenger of $O_2^{-\bullet}$ and H_2O_2, EUK-134 prevented the nitration of tyrosine hydroxylase and neurotoxicity in cultured dopaminergic neurons [22]. These results indicate that not only EUK-134 could treat PD, but also interacts with $ONOO^-$, $NO^{\bullet}$, or with NOS generation. Prevention of ethanol-induced limb malformations by EUK-134 illustrates that antioxidants can significantly improve the adverse developmental outcome that results from ethanol exposure in utero [23]. Endogenous oxidative stress is a major determinant of the rate of aging. Both EUK-8 and EUK-134 extended the lifespan in the nematode worm caenorhabditis elegans [24]. Subsequently, EUK-8 and EUK-134 were shown to prolong survival and attenuate indicators of spinal cord oxidative stress in a mouse model for familial amyotropic lateral sclerosis [25] and tree EUK complexes, EUK-8, EUK-134, EUK-189, were found to prolong survival and rescue oxidative pathologies in mice lacking manganese SOD [26]. Oxidative stress also implicated in cognitive impairment in both old experimental animals and aged humans. Baudry's group reversed age-related learning deficits and brain oxidative stress in mice with EUK-189 and EUK-207, another EUK compound [27]. Yet, the cooperative bifunctional activities of SOD and CAT may lead to adverse result, because some product like H_2O_2 also has a potential beneficial effect. For example, H_2O_2 could enhance the postischemic reperfusion and myocardial contractile function. Prevention of mouse heart from superoxide-induced reperfusion injury with monofunctional SOD mimetics is better than with EUK-134, because EUK-134 reduces H_2O_2 to O_2 and H_2O, giving a less increment in blood reperfusion and less reinforcement in heart function compared with use of monofunctional SOD mimetics [28].

In summary, the EUK complexes, in which the Mn moiety of the salen compounds is coordinated by four axial ligands, could vary in ability to protect against a wider range of ROS, and this ability is greatly influenced by salen ring alkoxy substitution and aromatic bridge modifications. As mentioned above, these complexes also react with other reactive species such as RNS that are formed from the reaction of ROS with biological molecules and prevent the nitrosative stress. Thus these Mn(III)-salen compounds have the potential to resist organism damage caused by both oxidant and nitrosative stresses by the catalytic breakdown of $O_2^{-\bullet}$, H_2O_2, $ONOO^-$ and NO to benign species: O_2, H_2O, NO_2^- and NO_3^-. However, the non-selectivity of this class of antioxidants causes the complexity of the mechanisms of action. They can protect against oxidative stress by neutralizing ROS *in vivo*, even some of the EUK compounds possibly act via an additional or alternative mechanism which does not involve ROS. Although the mechanism of action of EUK series as multifunctional free radical scavengers has not been completely elucidated, the low molecular weight and the potent antioxidant ability make them have broad clinical applicability.

B. Other Different Manganese Complex Mimics

a. SOD and CAT Mimics

In addition to EUK compounds, some other Mn-containing complexes with multifunctional enzyme mimics have been reported. Asayama *et al.* synthesized a bifunctional Mn-porphyrin-catalase conjugate with the activities of SOD and CAT (Fig. (**4**)). In contrast to EUK series, which is coordi-nated to oxygen and nitrogen atoms, Mn-porphyrin is only coordinated to nitrogen atoms. This Mn-porphyrins-catalase conjugate could overcome the shortcoming of the previous compounds with monofunctional SOD activity, which is unable to eliminate the reactive products of SOD catalysis such as H_2O_2 [29]. Moreover, this conjugate also shows carbohydrate recognition, which is expected to offer unique antioxidants to actively target to ROS overproducing sites.

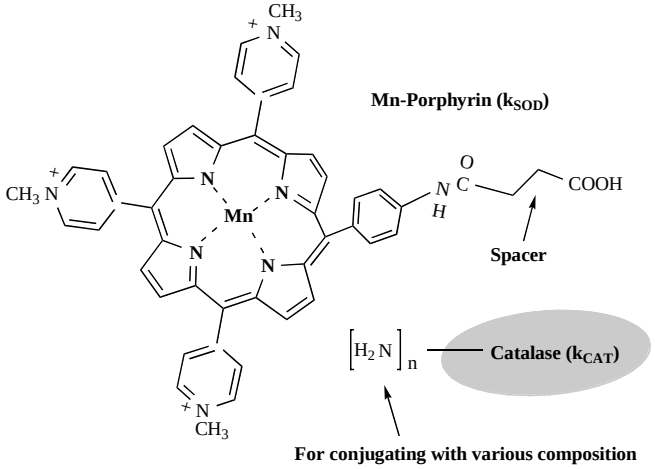
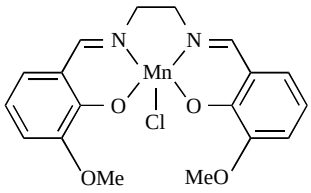


Fig. (4). Design of Mn-porphyrin-catalase conjugates.

Recently, W Park and D Lim reported the new synthesis of oligo(ethylene glycol)-modified manganese salen complexes (Fig. (**5**)) and their antioxidant activity. Their SOD



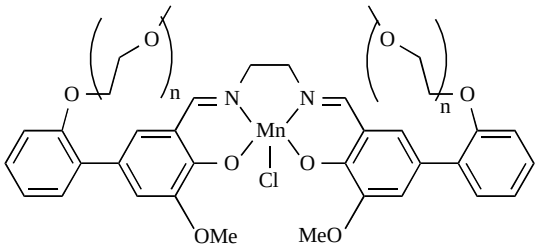


Fig. (5). Structures of manganese salen complex and its oligo(ethylene glycol) derivatives.

activities are similar to that of EUK-134 and some of them are more potent than EUK-134. But their CAT activity is

lower than that of EUK-134. However, compare to the EUK-8, the advantage of these complexes is possibly its low binding affinity to DNA strands due to the 3-dimensionally located OEG appendage. EUK-8 have pro-oxidant activity in the presence of H_2O_2, damaging free DNA after the intercalation between DNA strand [30].

b. SOD and GPx Mimics

In living organisms the antioxidant enzymes, SOD, CAT, and GPx contribute dominatingly to increase of cellular antioxidant defense against oxidative stress. SOD is a metal-loenzyme that catalyzes the dismutation of $O_2^{-\bullet}$ to H_2O_2 and dioxygen, and GPx, a selenium-containing enzyme, functions to catalyze the reduction of H_2O_2 and other harmful peroxides by thiols. In these antioxidant defense systems, living cell evolved to use GPx and/or CAT to detoxify H_2O_2 produced by dismutation of $O_2^{-\bullet}$ through SOD catalysis (Fig. (**6**)).

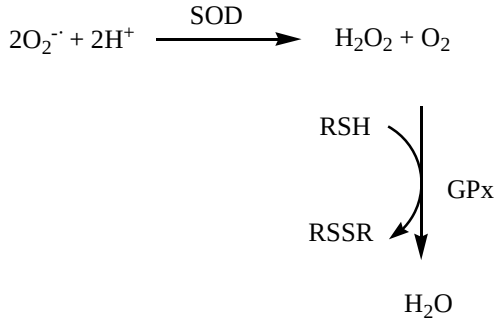


Fig. (6). Catalytic reactions of cooperative action of SOD and GPx.

Liu's group synthetized a bifunctional enzyme with both SOD and GPx activities, in which the ally 3-hydroxyproxyl selenide was linked to meso-tetrakis (4-carboxyphenyl) porphyrin (TCPP), then metal manganese was chelated to gain a Se-Mn(III)-TCPP complex (Fig. (**7**)) [31].

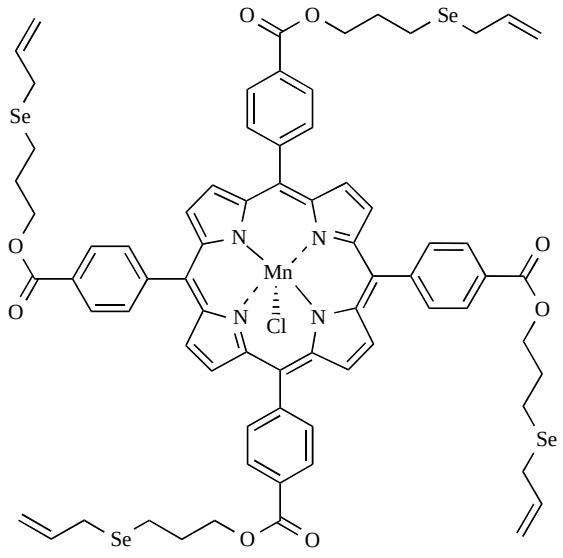


Fig. (7). Se-Mn(III)-TCPP.

Recently, a sodium salicylaldehyde-5-sulfonate was made as the parent of the trifunctional derivative synthesis, then the catalytic group of GPx, -SeH, was introduced into the parent to obtain a salicylaldehyde schiff base compound, which was chelated with Mn and oxidized in air to obtain the final product $Mn(III)_2$(L-Se-SO_3Na) (Fig. (**8**)). The product was demonstrated to have SOD, CAT and GPx activities at the same time, good solubility in water and strong ability to resist oxidative damage. Notably, $Mn(III)_2$(L-Se-SO_3Na) displays a better ability of inhibition of thiobarbituric acid reactive substances (TBARS) content and decreased mitochondrial swelling than that of mono-functional GPx mimic. It indicated that the cooperation of trifunctional enzyme maybe play an important role in protection of myocardial mitochondria oxidative damage [32].

Compared with the mimetics with combined SOD and CAT functions, design of manganese-containing low molecular weight antioxidant enzyme models with SOD and GPx activities has been rarely reported. This kind of models was designed by incorporation of manganese(III) and selenium, which are the active centers of SOD and GPx, respectively, and was expected to be a basic link between SOD and GPx activities, and might lead to a final removal of excess ROS due to cooperative function of the bifunctional enzyme mimic. However, the active site of natural GPx include not only selenium atom (selenocysteine residue) but also a hydrophobic cavity for thiol GSH (substrate) binding site. In the catalytic processes of enzymes, they must first recognize and bind their substrates to set up the correct geometry. The poor substrate binding ability may result in poor binding of manganese complex mimics to the GSH and, thereby limit their enzyme activities.

(C) Cyclodextrin-Based Enzyme Models

Cyclodextrins are cyclic oligosaccharides consisting of 6-8 glucose units linked together by 1, 4-glycosidic linkages. They have been wildly investigated as enzyme models and molecular receptors since they have a hydrophilic exterior and a hydrophobic interior cavity lining, which is capable of binding small organic molecules [33].

Liu synthesized a series of water soluble β-cyclodextrin derivatives containing a 1, 2-benzisoselenazol-3(2H)-one moiety (Fig. (**9**)) [34]. These compounds display both GPx and SOD activities. β-cyclodextrin itself and oligoamino-modified β-cyclodextrin including **7** and **8** do not show SOD and GPx activities at all, which indicates that the β-cyclodextrin cavity acts only as substrate-binding site and the organoselenium moiety is the catalytic group. All of the mimics **1**-**6** with the oranoselenium moiety at the primary rim of β-cyclodextrin display good SOD activities in the range of 121-330 U/mg. The SOD activity of the best mimic **2** is one tenth of that of natural bovine erythrocytes SOD. The mimic **2** has highest SOD activity probably due to its suitable length/flexibility of the oligoamino chain and thus there may exist the best cooperation between the catalytic group, 1,2- benzisoselenazol (2H)-one moiety, and the substrate-binding site, β-cyclodextrin cavity. The GPx activities of these mimics for reduction of H_2O_2 by GSH are in the range of 0.34-0.86 U/μM and lower than that of ebselen (0.99 U/μM), although the most important part of ebselen in some of them was modified. Low catalytic efficiency for reduction of H_2O_2 by GSH indicates that as thiol substrate, hydrophilic compound GSH does not suit the binding cavities of cyclodextrin, since cyclodextrins have a preference for the hydrophobic aryl groups rather than for the hydrophilic compound GSH.

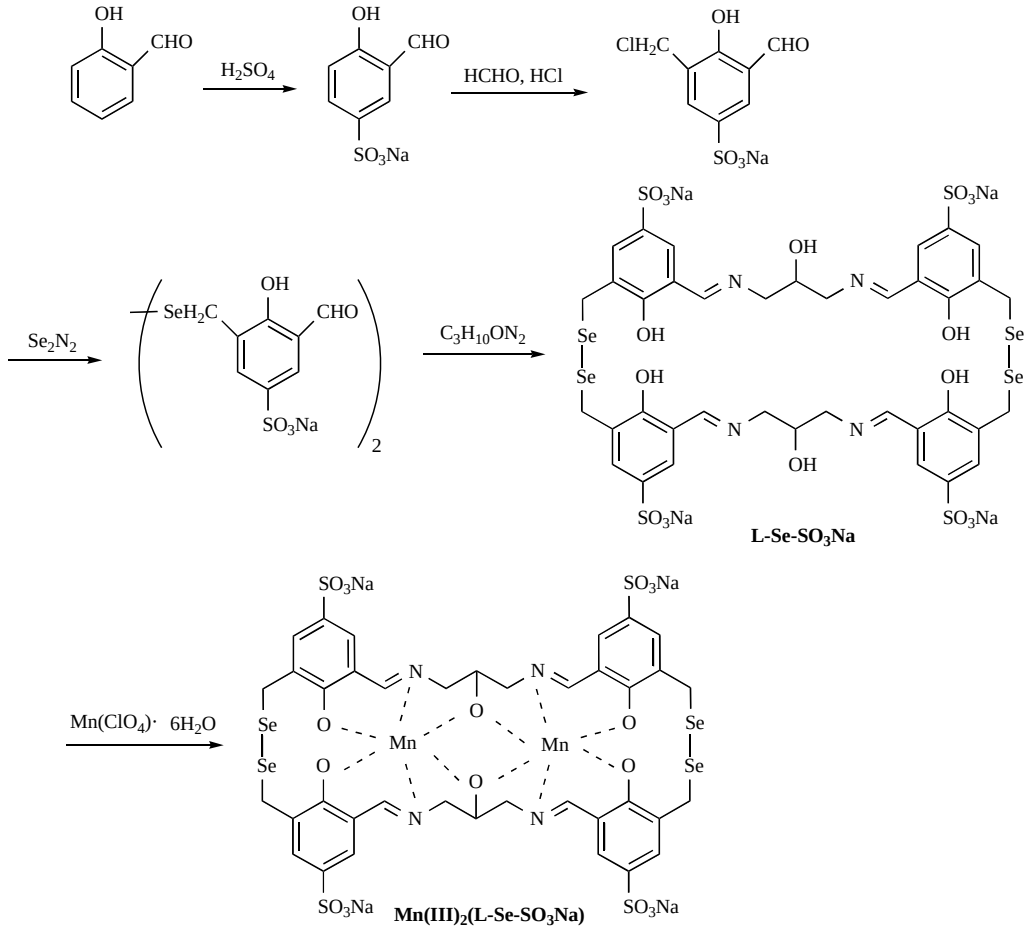
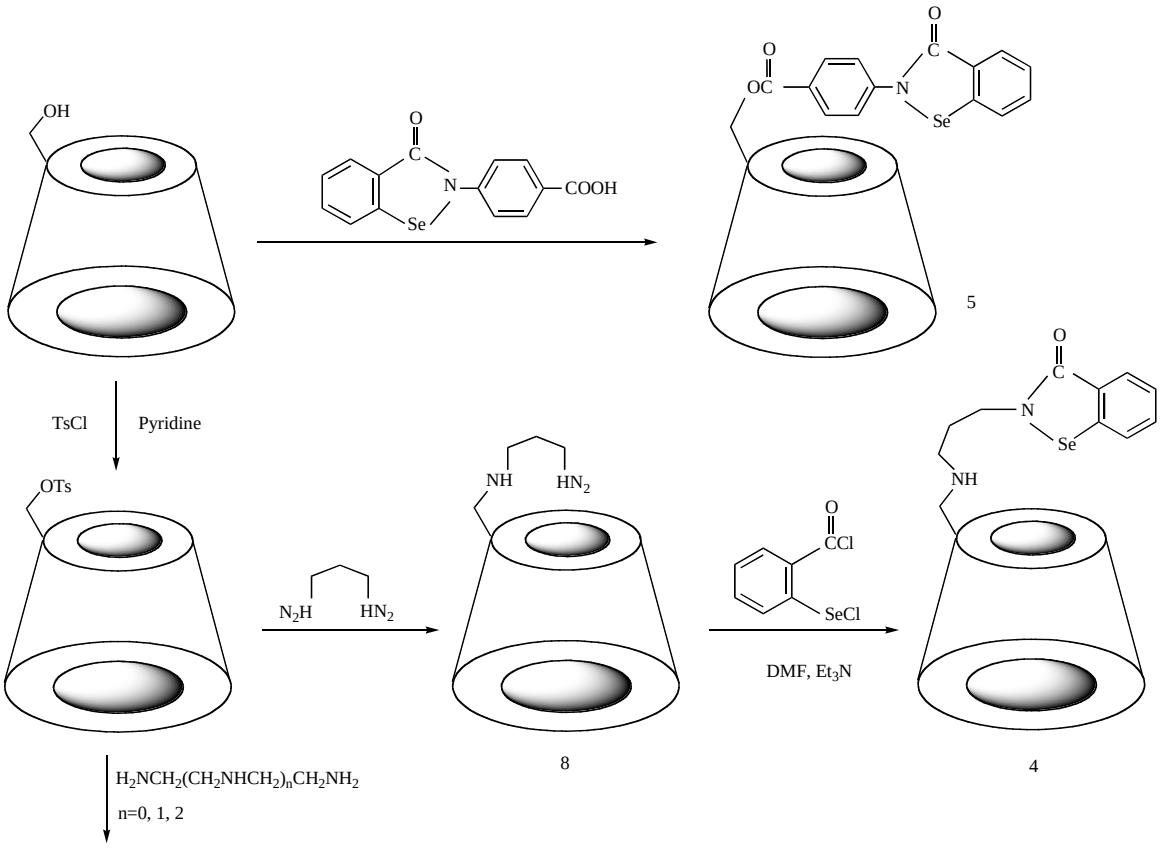


Fig. (8). The route of the synthesis of $Mn(III)_2$(L-Se-SO_3Na).



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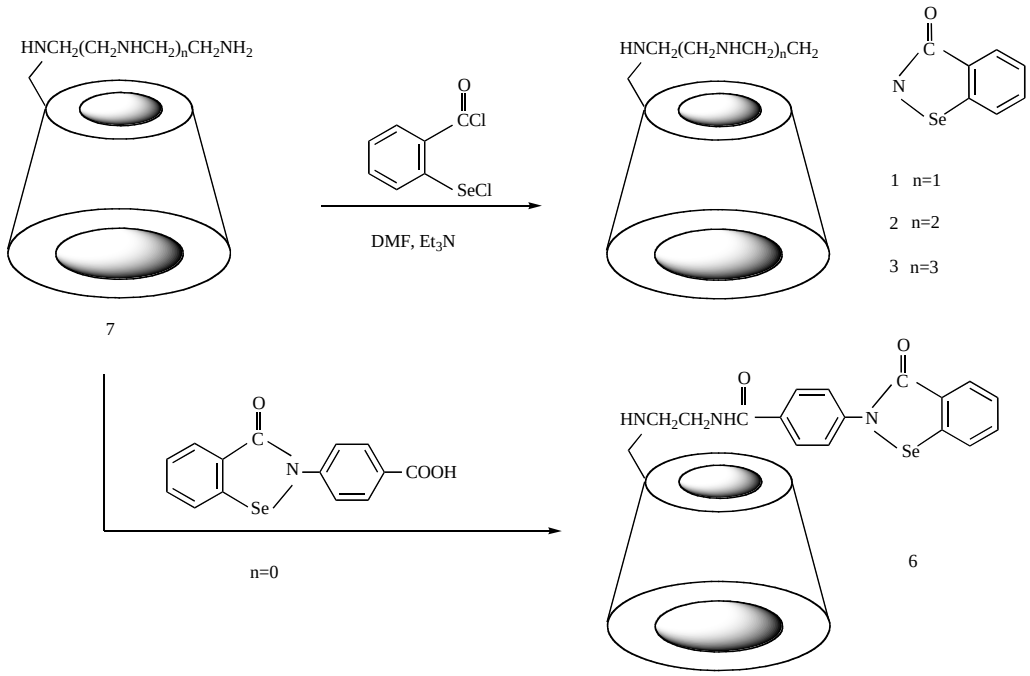


Fig. (9). The route of the synthesis of Liu's β-cyclodextrin derivatives.

Cyclodextrin is capable of forming highly stable inclusion complexes with adamantine derivatives in aqueous solution. In addition, cyclodextrin receptors chemisorbed on the surface of gold nanoparticles maintain the host properties that they exhibit in homogeneous aqueous solution. Villalonga *et al.* reported a bienzymatic supramolecular nanoassembly containing catalase and Cu, Zn-superoxide dismutase [35]. Beef liver CAT (10.3 U/g) was hydro-

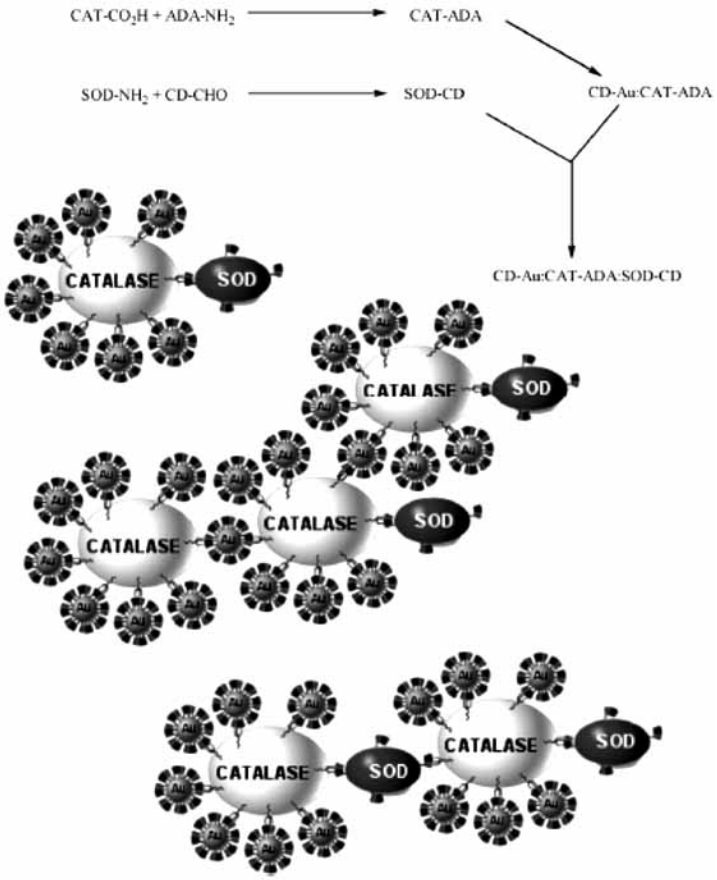


Fig. (10). Supramolecular assemblies of CAT and SOD on β-cyclodextrin-coated gold nanospheres.

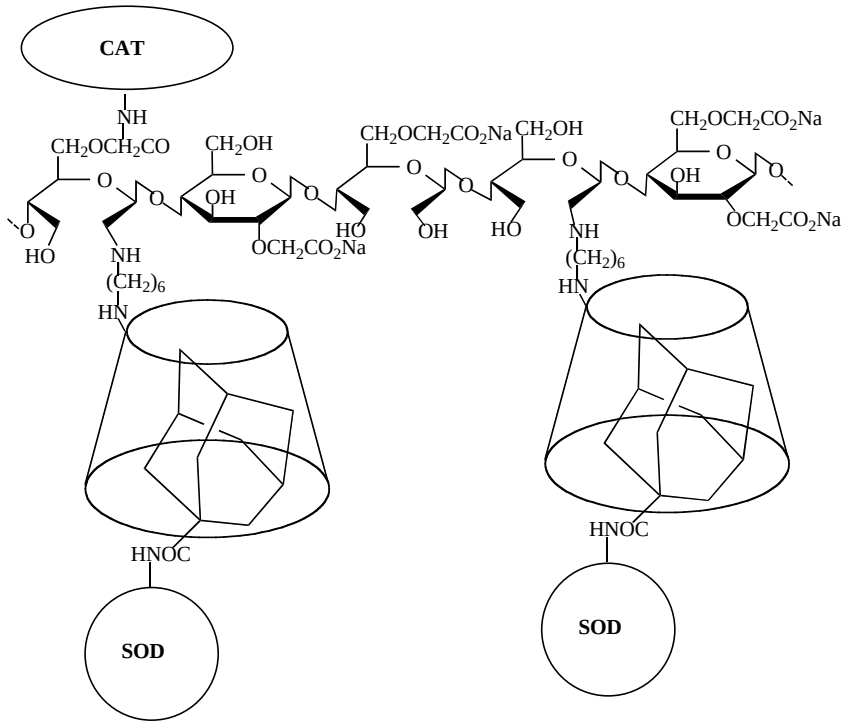
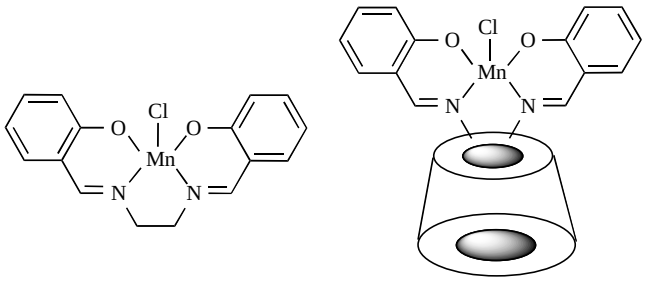


Fig. (11). The supramolecular complex with SOD and CAT activities.

phobically modified with 1-adamantanecarboxylic acid and then immobilized on β-cyclodentrin-coated gold nanospheres *via* supramolecular associations. The bienzymatic nanocatalyst was further prepared by co-immobilization of β-cyclodextrin-modified bovine erythrocytes SOD (3500 U/mg) (Fig. (**10**)). The CAT and SOD activities of this supramolecular association of bienzyme on the cyclodextrin-capped nanoparticles retained 73% and 35% of their initial specific activity, respectively. As we known, SOD has been proposed as potential enzyme drug for several diseases caused by overproduction of ROS. However, the pharmacological activity of this scavenger enzyme is limited by its rapid clearance through the kidney and inactivation by H_2O_2, its own reaction product. The half-life of SOD of this bienzyme was increased from 13 min to 19.5 h, demonstrating that co-immobilization of SOD with CAT on gold nanoparticles conferred significant stabilization of this enzyme. This suggests that inactivation of SOD by H_2O_2 is prevented by the presence of CAT in the context of the bienzymatic nanoparticle.

Soon after, Villalonga reported an another supramolecular approach to preparing bienzyme as antioxidant drug based on the co-immobilization of 1-adamantane modified SOD derivative with CAT chemically modified with β-cyclodextrin branched O-carboxymethylcellulose (Fig. (**11**)). In order to determine the anti-inflammatory activity of SOD, the carrageenan-induced paw oedema test was employed. The results show that after supramolecular association with the modified CAT form, the SOD could remarkably resist inactivation by H_2O_2 and its anti-inflammatory activity was increased by 4.5-fold, indicating that this bienzymatic supramolecular assembly with bifunctional activities could contribute to the improved anti-inflammatory activity, especially, the pharmacological activity of SOD could be improved through host-guest based strategy [36].

On above section, we have described EUK, the famous class of low molecular synthesis artificial enzyme model with both SOD and CAT activities, in detail. Vecchio synthesized a manganese (III) complex of the 6A, 6B-dideoxy-6A, 6B-di[(N-salicylidene)amino]-β-cyclodextrin [Mn(ABCDSA)Cl] (Fig. (**12**)). This cyclodextrin complex shows a larger solubility than EUK-8 and good SOD and CAT activity [37]. The conjugation of the cyclodextrin with salen-type ligands could increase the water solubility in comparison to the simple salen derivatives and water solubility is an important requirement for SOD mimic. In addition, cyclodextrins are able to tune the $HO^{\bullet}$ radicals and thus their appropriate metal complexes could act against a cocktail of radicals.



EUK-8 **Mn(ABCDSA)Cl**

Fig. (12). Comparison of structure of EUK-8 and Mn(ABCDSA)Cl.

Recently, Vecchio reported the synthesis of two novel conjugates of salen-type ligands tith the β-cyclodextrin [38]. These complexes show enhanced SOD activity, and also show CAT and POD activities, which are higher than that of

the simple salen complexes EUK 113 and EUK 108 (Fig. (**13**)). Cyclodextrin is widely used in pharmaceutical preparations, it could improve certain features of the salen moiety. Cyclodextrin, in fact, is used as complexing agents for drugs, and, in some cases, the cyclodextrins have been covalently conjugated to the drugs to enhance their stability, solubility and sitospecificity. Cyclodextrin-drug bioconjugates are reported in the literature as site specific carriers for the colon, being very promising as anti-inflammatory prodrugs for some diseases, such as colon [39]. Some results indicate that ROS as $O_2^{-\bullet}$, and $HO^{\bullet}$ have a role in mediating intestinal damage in inflammatory bowel diseases [40]. Thus, the bioconjugation of a salen type manganese(III) complex with cyclodextrins could be an interesting approach to carry an $O_2^{-\bullet}/H_2O_2$ scavenger in the colon.

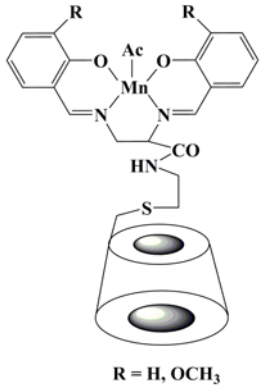


Fig. (13). The new conjugates of SOD/CAT mimics with cyclodestrins.

In a word, the high catalytic efficiency, molecular recognition and selectivity, together with water-soluble and thermal stability give these cyclodextrin-based enzyme models a real advantage compared to other small mimics.

III. MACROMOLECULAR PROTEIN-BASED ENZYME MODELS

(A) Generation of Conjugates by Chemical Cross-Linking of SOD and CAT

SOD has been championed as an effective antioxidant for the treatment of ischemia-reperfusion injury in a wide variety of tissues. However, while it dismutates a reactive free radical ($O_2^{-\bullet}$) it also produces a highly reactive oxidizing agent H_2O_2 which in the presence of iron results in the production of the very toxic $HO^{\bullet}$ *via* the Fenton reaction (Eqn. **12-14**). Poznansky reported a SOD-CAT covalent conjugate which could inhibit the Fenton reaction in the presence of CAT conjugated to SOD in an *in vitro* system and offer much greater protection in heart model of ischemia-reperfusion injury. This conjugation of CAT to SOD appears to ensure that as soon as a superoxide dismutation reaction occurs, the resultant H_2O_2 is removed by the immediate proximity of the catalase molecule [41].

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \quad (12)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (13)$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet} \quad (14)$$

(B) Preparation of Bifunctional Enzyme by Using Chemical Mutation

As we known, GPx is the one of the important antioxidant enzyme family, which catalyzes the reduction of hydroperoxides and organic peroxides by GSH and selenocysteine (Sec) residue is the catalytic functional group of GPx. Our group generated a GPx-SOD mimic by using chemical modification of native SOD from pig blood [42]. 14 serine (Ser) residues in two subunits of pig blood SOD were converted into Sec (Fig. (**14**)) [43]. Since the Ser residues are not essential for SOD catalysis, the SOD activity did not change greatly by the chemical mutation of Ser. However, the modified SOD displayed low GPx activity because the SOD is not capable of binding the GSH, the substrate of GPx (Table **1**). It indicates that substrate recognition and catalysis are utmost important in enzyme catalytic efficiency and play a key role in enzyme imitation.

(C) Design of New Bifunctional Biocatalysts Based on Naturally Occurring Scaffolds

Reformation of naturally occurring enzymes is one of the general strategies for redesigning new enzyme function. GST is another antioxidant enzyme which catalyzes the reaction of GSH conjugation to a wide range of electrophilic metabolites of xenobiotics, and also possess natural GSH binding site like GPx [44]. Liu *et al*. converted the GST from *Lucilia cuprina* (LuGST1-1) into a selenium-containing enzyme (seleno-LuGST1-1) by replacing the active site Ser 9 with a cysteine (Cys) and then substituting it with Sec in a Cys auxotrophic system [45]. Thus, catalytically essential residue Sec was bioincorporated into GSH-specific binding scaffold. Seleno-LuGST1-1 displays a significantly high efficiency for catalyzing the reduction of H_2O_2 by GSH, which is in the same order of magnitude compared with natural GPx. It is very interesting that Seleno-LuGST1-1 exhibits the GST activity when 1-chloro-2, 4-dinitrobenzene is used as the substrate, although the activity is one order of magnitude lower than that of wild type LuGST1-1 (Table **1**). 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 ebselen [46].

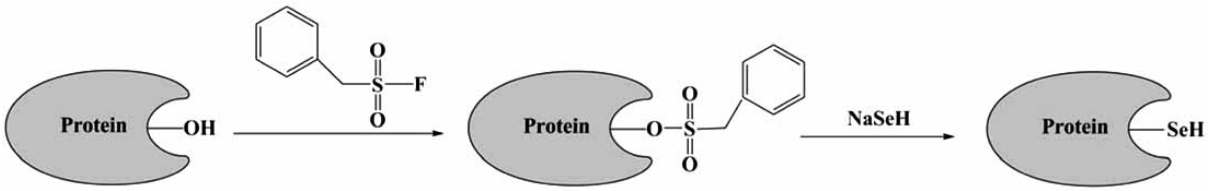


Fig. (14). The serine residues of the proteins studied were activated with phenylmethanesulfonyl fluoride to produce a sulfonylester of the serine hydroxyl groups. The sulfonate was then replaced with hydrogen selenide to generate selenoproteins.

Table 1. The SOD, GPx and GST Activities of Some Macromolecular Protein-Based Enzyme Mimics

Catalysts	SOD (U/μM)	GPx (U/μM)		GST (U/μM)		
	$O_2^{-\bullet}$	H_2O_2	CuOOH	CDNB	1-Bromo-4-nitrobenzene	CFA
Mutated SOD	6.38×10^5	29	ND	ND	ND	ND
Seleno-LuGST1-1	ND	2957	ND	23.5	ND	ND
Se-hGSTZ1-1	ND	8602	ND	ND	ND	0.103
seleno-GST-SOD	12813	286	ND	ND	ND	ND
scFv-2F3	ND	ND	27	29	108	ND
Se-SjGST-p35-Cu(II)	1708	2062	ND	2209	ND	ND

ND, no detectable SOD, GPx or GST activities.

Recently, we have taken advantage of a new scaffold, hGST 1-1, in which there are two Ser residues in the activity site, to achieve both high thiol selectivity and highly catalytic efficiency [47]. The crystal structure of hGST1-1 shows that its active site is located in a deep crevice between the N- and C-terminal domains and contains three very highly conserved residues (Ser14-Ser15-Cys16) to form the characteristic zeta class SSC motif [48]. Ser 14 and Ser 15 are located close to the thiol of GSH bound in the active site, thus, they were easily chemically modified to Sec after activation with phenylmethyl sulfonylfluoride (PMSF). The active site of hGSTZ1-1 is very accessible and allows ready access of both GSH and H_2O_2. The efficiency for catalyzing the reduction of H_2O_2 by GSH of Se-hGSTZ1-1 is about 1.5 times that of rabbit liver GPx, and the low GST activity with chlorofluorocaetic acid (CFA) as substrate was also measured (Table **1**). Up to present we have not a powerful proof to explain the origin of the GST activity, however, it may be that GPx and GST are consanguineous and they have a "GSH-binding protein" ancestor based on the similarities in their overall structures and the positioning of their important active site residues despite their functional differences and low sequence identity. It is very possible that they evolved from the same ancestor and separately developed different functions to accommodate to the constantly changing environment.

The remarkably high GPx activity of these selenium-containing GST could be ascribed to its special enzyme scaffold. Studies on crystal structure of GST and natural GPx [49] reveal that there are some important structure similarities between these two enzymes. Both of two enzymes include specific GSH binding sites and thus could both strongly bind to their common specific substrate GSH. Moreover, GPx and GST adopt a common thioredoxin fold, even though they share low sequence identity [50]. Their active site residues locate in the relatively same positions of their thioredoxin folds. Furthermore, on alignment of their structures, their substrate GSH is apparently bound at the same accessible point by an interaction with the conserved hydroxyl moiety of active residue Ser in GST and with the selenol group of the catalytic Sec in GPx, respectively. Therefore, the enzyme scaffold of selenium-containing GST seems to provide its catalytic Sec with an optimal geometric conformation even comparable with that of naturally occurring GPx. In addition, the secondary structure adjacent to the active site in this enzyme model also seems to have a considerable contribution to enzyme catalysis. In the thioredoxin fold, the active site residue (Ser in GST or Sec in GPx) is positioned at the N-terminal end of helix α_1 of a $\beta\alpha\beta$ structure (Fig. (**15**)) [49].

The other example, single chain antibody 2F3 (scFv-2F3) was elicited against the hapten GSH-S-DN_2phBu (a GSH

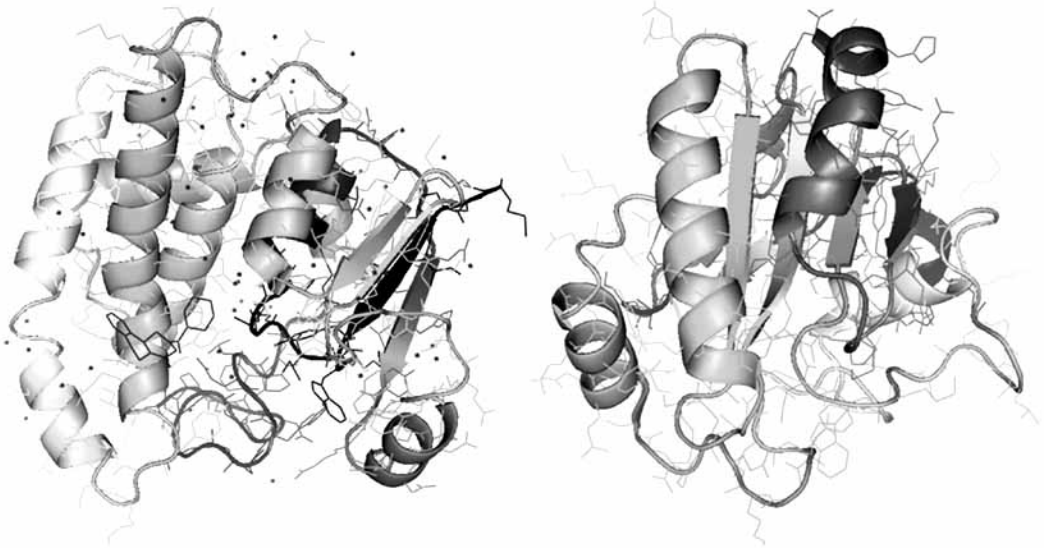


Fig. (15). Comparison of structure of GST and GPx. Left: natural Mu type GST. Right: natural cGPx.

derivate), which also is a GSH binding protein. scFv-2F3 not only display GST activities with variety substrates, but also show low GPx activity for reduction of CuOOH by GSH, despite no GPx activity to H_2O_2. It is notable that after scFv-2F3 converted into Se-scFv-2F3 by chemical mutation, Se-scFv-2F3 did not display any GST activity but exhibited a high GPx activity to CuOOH or H_2O_2 (Table **1**) [51].

Hilvert [52] and Liu [53] reported one of the smallest members of the thioredoxin (Trx) family, *E. coli* glutaredoxin (Grx) [54], as a template for creating artificial GPx. These two natural enzymes, one of which is prepared by chemical ligation and the other by biosynthesis, both display GPx and Grx activities. The structurally well-characterized protein Grx is monomeric and only 85 amino acids long. It possesses a redox-active disulfide at a position that is analogous to the Sec in GPx as well as a well-defined binding site for GSH. The redox-active disulfide was replaced with a Sec (C11U/C14S) by native chemical ligation from three fragments. This Seleno-Grx show weak GPx activity. Grx catalyze the reduction of protein as well as of low molecular weight mixed disulfides with GSH and are themselves reduced by GSH via this specific binding site. As antioxidant, Grx may catalyze formation of mixed disulfides from GSSG, which may be of a protective function to avoid oxidation of a thiol to higher oxidation states under oxidative stress (Eqn. **15-19**).

$$\text{Grx-S}_2 + 2\text{GSH} \rightleftharpoons \text{Grx-(SH)}_2 + \text{GSSG} \quad (15)$$

$$\text{Grx-(SH)}_2 + \text{Protein-S-SG} \rightleftharpoons \underset{\text{SH}}{\text{Grx-S-SG}} + \underset{\text{SH}}{\text{Protein-(SH)}_2} \quad (16)$$

$$\underset{\text{SH}}{\text{Grx-S-SG}} + \text{GSH} \rightleftharpoons \text{Grx-(SH)}_2 + \text{GSSG} \quad (17)$$

$$\text{Grx-(SH)}_2 + \text{Protein-S}_2 \rightleftharpoons \text{Grx-S}_2 + \text{Protein-(SH)}_2 \quad (18)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+ \quad (19)$$

There are no direct evidences for biological effect to prove that the bifunctional antioxidant enzyme activities of these mimics based on naturally occurring scaffolds participate in scavenging hydroperoxides and protecting against oxidative damage, however, there are powerful dominances of substrate specificity and highly catalytic efficiency to provide us a novel idea for designing efficient antioxidant.

(D) Generation of the Fusion Protein with Bifunctional or Multifunctional Antioxidant Enzyme Activities by Gene Engineering

In living organisms, the balance between the main antioxidant enzyme including SOD, GPx, CAT, GST and GR was believed to be more important than any single one. Lots of SOD/CAT enzymes have been proved to have better antioxidant ability than either single enzyme, and one important reason is that the harmful hydroperoxides H_2O_2, the catalytic product of SOD, could be detoxified to water and O_2 by CAT. In addition, Cu/Zn-SOD could be inactivated by high concentration of H_2O_2, resulting in inactivation and fragmentation of the enzyme, which cause oxidative injury. Liu's group generated a bifunctional enzyme (Seleno-GST-SOD) with both GPx and SOD activities by combining traditional GST fusion protein technology with Cys auxotrophic expression system [55]. The resistance to inactivation by H_2O_2 was detected using native *sweet potato* SOD (SW-SOD) and seleno-GST-SOD to further confirm the cooperation of GPx and SOD activities of the dual-functional enzyme (Table **1**). The experiment results suggested that the non-enzymatical reduction of H_2O_2 by GSH might not be enough to eliminate all the H_2O_2 quickly. But things became different in the case of seleno-GST-SOD. On one hand, the GPx activity of its GPx domain could accelerate the reduction of H_2O_2 so that it would decrease the concentration of the oxidant in the system and inhibit the inactivation of the adjacent SOD domain more effectively. On the other hand, the immediate proximity of the GPx active center allowed efficient and timely removal of H_2O_2 molecules that approached SOD domain of the fusion protein. Therefore, the cooperation of the two activities in the fusion protein facilitated the protection of the two active sites by each other and the enhancement of their antioxidant ability (Fig. (**16**)). The biological effect of seleno-GST-SOD was evaluated by the protection of mitochondria against oxidative damage. In the

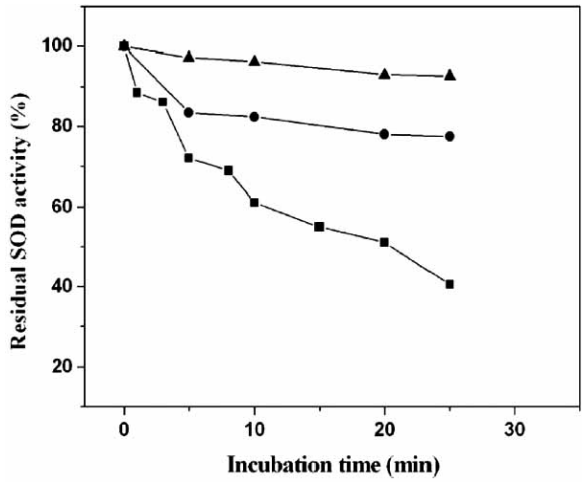


Fig. (16). Time course of the SOD activity of SW-SOD and seleno-GST-SOD in enzyme/H_2O_2 system at 37 °C. (■) SW-SOD was reacted with 1.0 mM H_2O_2 in 100 mM PBS (pH 7.5); (●) SW-SOD was reacted with 1.0 mM H_2O_2 in 100 mM PBS (pH 7.5) containing 2 mM GSH; (▲) seleno-GST-SOD was reacted with 1.0 mM H_2O_2 in 100 mM PBS (pH 7.5) containing 2 mM GSH.

free-radical-damage system (Xanthine/XOD), The XOD-catalyzed reaction is known to initially produce superoxide anions, which are spontaneously dismutated to H_2O_2 and then transformed to $HO^{\bullet}$, in the presence of Fe^{2+}. Therefore, there exist simultaneously superoxide anions, H_2O_2 and $HO^{\bullet}$, in this system. Taking mitochondrial swelling and lipid peroxidation as standards (Fig. (**17**)), it was found that a high concentration of single SW-SOD could enhance damages of mitochondria. Seleno-LuGST1-1 could catalyze the reduction of H_2O_2 and reduce the oxidative damage. But it could not remove superoxide anions, which could still act as oxidant in the system. When seleno-GST-SOD was added, the cooperation of GPx and SOD activities of the bifunctional enzyme could not only scavenge all kinds of ROS in the system, but also protect each other from the inactivation by ROS efficiently and further enhance the antioxidant

ability. Obviously, it has more advantages as an antioxidant and a potential therapeutic agent than either single GPx or SOD.

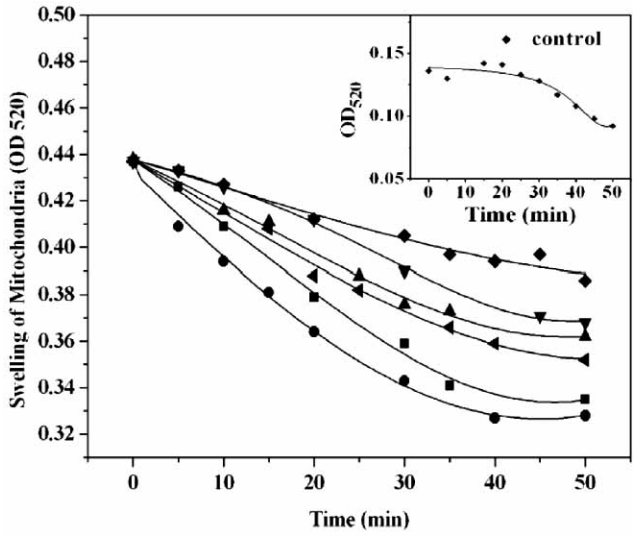


Fig. (17). Effects of different enzymes on the swelling of mitochondria. (♦) damage + 0.1 U/mL of seleno-GST-SOD; (▼) damage + 0.05 U/mL of seleno-GST-SOD; (▲) damage + 0.025 U/mL of seleno-GST-SOD; (◄) damage + 0.05 U/mL of seleno-LuGST1-1; (■) damage; (●) damage + 129 U/mL of SW-SOD. The damage condition was shown in Materials and methods. And the activity of seleno-GST-SOD was its GPx activity. Data are means ± SD for three separate experiments.

Our group also generated a fusion protein that had SOD, GPx and GST activities [56]. We inserted a DNA sequence encoding the 35-mer polypeptide, which link the 17-mer-SOD and 15-mer-GPx with a 3 amino acid linker, to the pGEX-2T vector at the C-terminus of the gene encoding *Schistosoma japonicum* GST (SjGST). After incorporation of copper ion and selenohydryl, the expression of fusion protein demonstrated activities of three enzymes (Table **1**). SOD, GPx and GST, in conjugation with GR, form a system to protect the organism from ROS injury. Two $O_2^{-\bullet}$ are reduced by SOD to form molecular oxygen and H_2O_2, then, H_2O_2 is reduced by GPx by oxidation of two molecules of GSH, forming GSSG that subsequently can be reduced by GR under consumption of NADPH. GST catalyzes the conjugation of GSH with other biomolecules. However, we had not explained how the trifunctional enzyme would be useful to scavenge ROS in a biological setting, and the investigation for 35-mer peptide activities. Experiments are now in progress to further demonstrate the cooperation of the trifunctional mimic, and to prepare the 35-mer peptide enzyme with GPx and SOD, which would be a low-molecular, lack of immunogenic response, and have a longer half-life in the blood, good cellular permeability and clear structure-functional relationship.

The macromolecular protein-based enzyme models such as abzymes could potentially be of high value for the treatment of ROS-related diseases, such as cancer, cataracts, cardiovascular disease, and chronic inflammation. However, more detailed studies still need to be conducted to investigate further the therapeutic potential of this enzyme models, for instance, how the macromolecular multifunctional proteins enter the cells and keep stable *in vivo* to execute their synergism.

(E) Bifunctional Peptide Mimics

Not long ago, a selenium and copper containing 32-mer peptide (Se-Cu-32P), which combined the previous 17-mer peptide (17CuP) with SOD activity and the 15-mer peptide (15SeP) with GPx activity, was synthesized by our groups [57]. The bifunctional Se-Cu-32P was demonstrated to be able to better protect vero cell from the injury induced by the xanthine oxidase (XOD)/xanthine/Fe^{2+} damage system than its parents (Fig. **(18)**). Significantly, the short peptide showed more lower activities and smaller value for steady-state kinetics parameters than that of natural enzyme. Therefore, it is important to consider not only catalytic groups assembly but also correct position of the catalytic group around the substrate binding site in designing efficient catalysts. Consequently, we want to adjust some amino acid sequences and redesign another new peptide, which possesses well defined three dimensional structures and high selectivity, by means of structure prediction and optimization of molecular dynamics, then, obtain the peptide mimics by using fusion protein expression technology. Compared with chemical synthesis, the approach to biosynthesis may be more potent in producing bifunctional peptide mimic. To construction of the enzyme model with low molecular weight, steady structure, clear active sites, definite catalytic mechanism may become the mainstream of future development trends.

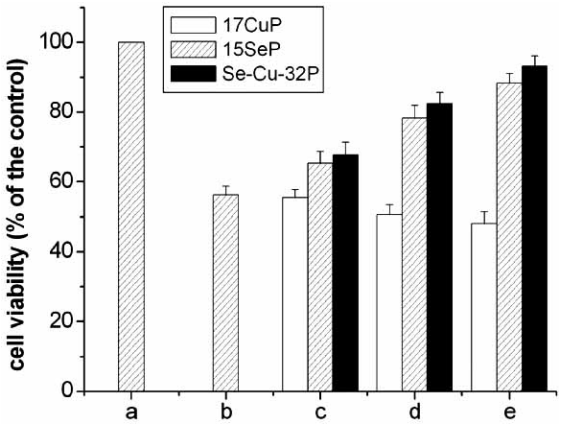


Fig. (18). Effects of Se-Cu-32P, 15SeP, and 17CuP on the viability of vero cells induced by XOD/xanthine/Fe^{2+} injury. The cells were treated as indicated in Materials and Methods. (**a**) Control; (**b**) XOD/xanthine/Fe^{2+} injury; (**c**) XOD/xanthine/Fe^{2+} +50 nM Se-Cu-32P(15SeP or 17CuP); (**d**) XOD/xanthine/Fe^{2+} + 100 nM Se-Cu-32P (15SeP or 17CuP); (**e**) XOD/xanthine/Fe^{2+} + 200 nM Se-Cu-32P (15SeP or 17CuP). Percentage of viable cells was counted by MTT assays. Data are expressed as mean ± SD for 3 independent preparations.

CONCLUSION

Oxidative stresses caused by the ROS are inevitable stresses of biological systems. In biological cells, antioxidant enzyme, such as SOD, GPx, CAT, GST and so on, are known to eliminate the ROS to protect the cells from oxidative stresses. There is a balance between both the

activities and the intracellular levels of these antioxidants that are essential for the survival of organisms and their health. These enzymes act cooperatively and synergistically to scavenge ROS, since not one of them can singlehandedly clear all forms of ROS and other reactive species which are formed from the reaction of ROS. However, natural antioxidant enzymes are known to subject to some limitations such as singleness in function, instability under biological environment, poor availability, large molecular weight, complicated structure. So much effort has been devoted to the design and development of artificial antioxidant enzymes with synergism in different models. Our review introduced some representative multifunctional mimics prepared by chemical synthesis, biosynthesis, semisynthesis, and some of them exhibited better synergistic biological effect than that of mono-functional enzymes. Of cause, the further study of the structure, cooperative mechanism and demonstration of the biological effect for these mimics is still needed. We anticipate that the antioxidant enzyme mimics with synergism may hold promise for the treatment of human diseases and possession of potential applications in medicine as potent antioxidants. Design and generation of multifunctional mimics will become main current for development of artificial enzyme field.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AIF = Apoptosis-Inducing Factor

βAP = β-Amyloid Protein

CAT = Catalase

CDNB = 1-chloro-2, 4-nitrobenzene

CFA = Chlorofluorocaetic Acid

CuOOH = Cumene hydroperoxide

Cys = Cystein

ESR = Electron Spin Resonance

GPx = Glutathione Peroxidase

GR = Glutathione Reductase

Grx = Glutaredoxin

GSH = Glutathione

GSSG = Glutathione disulfide

GST = Glutathione S-Transferase

hGST = Human Glutathione S-Transferase

LPS = Lipopolysaccharide

LuGST = *Lucilia cuprina* Glutathione S-Transferase

NADPH = β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form

NOS = Nitric Oxide Synthase

PD = Parkinson's Disease

PMSF = Phenylmethyl Sulfonylfluoride

POD = Peroxidase

RNS = Reactive Nitrogen oxides

ROS = Reactive Oxygen Species

ScFv = Single-Chain Fv fragment

Sec = Selenocysteine

Ser = Serine

SjGST = *Schistosoma japonicum* Glutathione S-Transferase

SOD = Superoxide Dismutase

SW-SOD = *Sweet potato* Superoxide Dismutase

TBARS = Thiobarbituric Acid Reactive Substances

Trx = Thioredoxin

XOD = Xanthine Oxidase

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