Pure Appl. Chem., Vol. 84, No. 12, pp. 2641–2651, 2012.
http://dx.doi.org/10.1351/PAC-CON-11-12-08

Bifunctional antioxidant enzyme mimics of albumin-binding salphen Schiff-base metal complexes*

Xiao-Chun Yin, Xiao-Xiao Li, Rong-Min Wang‡, Gang Li, and Yu-Feng He

Key Laboratory of Eco-Environment-Related Polymer Materials of Ministry of Education, Key Laboratory of Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China

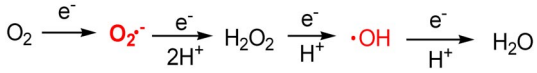
Abstract: New kinds of bifunctional antioxidant enzyme mimics were prepared, and their superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical (•OH) scavenging activity was investigated. These conjugates were prepared by binding insoluble salphen [*N*,*N*-(phenylene)salicylidene] Schiff-base metal complexes (HO-salphen-M, M = Co, Mn, Cu) with bovine serum albumin (BSA). They were characterized by UV–vis spectra, circular dichroism (CD), and native polyacrylamide gel electrophoresis (PAGE). It showed that the binding mode was an axial coordination between HO-salphen-Co and amino acid residue of BSA. The structure of BSA was maintained when the binding amount of HO-salphen-Co was less than 10. After combining HO-salphen-Co into BSA, the low solubility of HO-salphen-Co was overcome, and the $O_2^{\bullet-}$ and •OH scavenging activity of BSA was improved two orders of magnitude. In similar inhibitory value, the scavenging rate of salphen-Co20@BSA was far higher than others. The scavenging activity of different proportion salphen-Co@BSA was salphen-Co20@BSA > salphen-Co10@BSA > salphen-Co5@BSA > salphen-Co2@BSA. But salphen-Cu@BSA and salphen-Mn@BSA did not show •OH scavenging activity.

Keywords: bifunctional antioxidant enzyme mimics; biopolymer metal complexes; bovine serum albumin; hydroxyl radical scavenging; Schiff-base metal complexes; superoxide anion radical scavenging.

INTRODUCTION

Free radicals are a chemical species, capable of an independent existence, that contain one or more unpaired electrons [1]. They have been the target of considerable research since the last decade, with special attention given to reactive oxygen species (ROS). ROS was produced as a consequence of normal metabolism, which appeared together with aerobic life. It was formed during the consecutive univalent reduction of oxygen (eq. 1). Unstable free radicals could damage cellular proteins and lipids or form DNA adducts that may promote carcinogenic activity. They have been associated with carcinogenesis, coronary heart disease, and many health problems related to advancing age [2–4].

Pure Appl. Chem.* **84, 2499–2675 (2012). A collection of invited papers based on presentations at the 7th International Conference on Novel Materials and their Synthesis (NMS-VII) and the 21st International Symposium on Fine Chemistry and Functional Polymers (FCFP-XXI), Shanghai, China, 16–21 October 2011.
‡Corresponding author: Tel.: +86-931-7970358; E-mail: wangrm@nwnu.edu.cn



(1)

Superoxide anion radicals ($O_2^{\bullet-}$) were the first oxygen radicals produced in the reduction of molecular oxygen. Owing to the successive formations, $O_2^{\bullet-}$ could transform into other ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), while •OH was one of the most damaging oxidizing radicals and had tremendous potential for causing biological damage in skin and was an assumed pathogenic factor in a variety of diseases.

Epidemiological and clinical investigation indicated effective antioxidants could protect the human body from free radicals and retard the progress of many chronic diseases [5,6]. Thus, many ROS scavenging materials were used to reduce damage to the human body, which included natural and synthetic antioxidants [7,8]. Schiff-base metal complexes, one of the synthetic antioxidants, had been studied as effective scavengers of ROS [9–12]. However, the majority of Schiff-base metal complexes were insoluble in aqueous solution, which limited their application.

Proteins play an essential role in biology. Whether in catalysts or molecular recognition, proteins set a golden standard of efficiency and selectivity that few other natural or artificial molecules can match. For this reason, the study of protein function has been the focus of many years of research. Serum albumin was a versatile protein, principally characterized by its remarkable ability to bind a wide range of insoluble endogenous and exogenous compounds [13,14], such as nonesterified fatty acids, hemin, bilirubin, bile acids, thyroxine, and many kinds of drugs [15,16]. Because it was considered to be nonantigenic, biodegradable [17], and readily available, albumin has been used as a kind of biomaterial, such as drug delivery [18,19] and novel hydrophilic carriers [20,21]. But it was less often used as ROS scavenging material because its ROS scavenging activity was very low [22]. On another note, it was found that combining small synthetic molecules of metal complexes with macromolecules was one of the effective ways to improve their bioactivity [23]. In order to increase the dissolution and bioactivity of HO-salphen-M [salphen = *N*,*N*-(phenylene)salicylidene], we combined HO-salphen-M with BSA. Here, BSA was used as supporter for binding HO-salphen-M. Novel water-soluble metalloprotein conjugates were prepared. Their superoxide dismutase (SOD)-like activity was determined via nitrobule tetrazolium (NBT) assay and compared with that of natural SOD. The •OH scavenging activity of conjugates was also measured. The specific affinity between small molecule and protein has been proved to be one of the most straightforward and applicable approaches in the field of enzyme mimics.

MATERIALS AND METHODS

BSA was purchased from Shanghai Sangon Biological Engineering Technology and Services Co. Natural SOD (>90 %) was purchased from Shanghai Yuhua Life Science and Technology Development Co., Ltd. 4-Hydroxyl salicylaldehyde, *o*-phenylenediamine, *n*-propyl alcohol, $Co(OAc)_2{\cdot}4H_2O$, $Mn(OAc)_2{\cdot}4H_2O$, $Cu(OAc)_2{\cdot}H_2O$, and dimethylsulfoxide (DMSO) were obtained commercially, and used without further purification. Schiff-base solutions were prepared in DMSO. BSA solutions were prepared in sodium phosphate buffer solution (PBS pH = 7.4). Double-distilled water was used throughout.

Infrared spectra were recorded from KBr pellet (4000–400 cm^{-1}) on a Digilab FTS 3000 FI-IR spectrophotometer. The UV–vis absorption spectra were obtained using an Agilent 8453 UV–vis spectrophotometer with an Agilent temperature control unit. CD spectra of BSA solutions were recorded in 200–260 nm at 25 °C using a JASCO-820 spectropolarimeter with a band width of 1 nm and sample cell of 1 cm. Native polyacrylamide gel electrophoresis (PAGE) was performed on 5 % stacking gel and 8 % separating gel.

EXPERIMENTAL

Preparation of salphen Schiff-base ligand and its complexes

N,*N*'-bis(4-hydroxylsalicylidene)-*o*-phenylenediamine (HO-salphen) was synthesized by condensing of *o*-phenylenediamine with the 4-hydroxyl salicylaldehyde in a molar ratio of 1:2, which was described in the literature [24], using *n*-propyl alcohol as a solvent. The reaction mixture was stirred at 70 °C for 5 h. After cooling to room temperature (rt), the products were filtered off and washed with *n*-propyl alcohol 3 times. Then HO-salphen was dried under vacuum and kept dry in a desiccator over anhydrous calcium chloride. Elemental analysis ($C_{20}H_{16}N_2O_4$): C, 68.71 % (68.96); H, 4.98 % (4.63 %); N, 8.21 % (8.04 %). IR (KBr): 1612 cm^{-1} ($\nu_{C=N}$), 1201 cm^{-1} (ν_{Ph-O}), 3325 cm^{-1} (ν_{H-O}), 437 cm^{-1} (ν_{Co-O}), 662 cm^{-1} (ν_{Co-N}). ^{1}H NMR (acetone): 6.4–7.3 (10H, m, Ar–H), 8.363 (2H, s, –CH=N).

N,*N*'-bis(4-hydroxylsalicylidene)-*o*-phenylenediaminato cobalt(II) complex (HO-salphen-Co) was prepared as follows: Protected with N_2, the above reaction mixture was stirred at 70 °C for 0.5 h. Then the aqueous solution of $Co(OAc)_2$ was added with a molar ratio of 1:10. The reaction mixture was stirred at 70 °C for 5 h under nitrogen atmosphere. After cooling to rt, the products were filtered off and washed with *n*-propyl alcohol 3 times. Then HO-salphen-Co was dried under vacuum and kept dry in a desiccator over anhydrous calcium chloride, yield 80 %. The purity of the compounds was checked by UV–vis spectra and IR spectra. UV–vis spectra: 216 nm (K band), 320 nm (R band). IR (KBr): 1604 cm^{-1} ($\nu_{C=N}$), 1199 cm^{-1} (ν_{Ph-O}), 437 cm^{-1} (ν_{Co-O}), 501 cm^{-1} (ν_{Co-N}). The IR and UV–vis spectra measured for the obtained products were consistent with the corresponding Schiff-base formula.

The preparation of HO-salphen-Mn and HO-salphen-Cu was similar to that of HO-salphen-Co. In HO-salphen-Mn: (yield 75 %), IR (KBr): 1564 cm^{-1} ($\nu_{C=N}$), 1207 cm^{-1} (ν_{Ph-O}), 409 cm^{-1} (ν_{Mn-O}), 505 cm^{-1} (ν_{Mn-N}). HO-salphen-Cu (yield 60 %), IR (KBr): 1605 cm^{-1} ($\nu_{C=N}$), 1200 cm^{-1} (ν_{Ph-O}), 409 cm^{-1} (ν_{Cu-O}), 498 cm^{-1} (ν_{Cu-N}).

Preparation of BSA binding Schiff-base complex (salphen-M@BSA)

Firstly, BSA was dissolved in a PBS solution (pH = 7.4, 10 mmol·L^{-1}) and HO-salphen-Co was dissolved in DMSO. In 3.0 mL of PBS solution, 0.5 mL (1 mmol·L^{-1}) of BSA solution and 2.28 mL of HO-salphen-Co/DMSO (4.4 mmol·L^{-1}) were mixed (HO-salphen-Co: BSA = 20:1) and incubated for 12 h with rotation in the darkness at rt. The complex was dialyzed in PBS (pH 7.4) at 5–10 °C for removing DMSO, then obtained salphen-Co20@BSA. The ratio of HO-salphen-Co to BSA could be changed in preparation of BSA-binding salphen Schiff-base complexes (salphen-Co10@BSA, salphen-Co5@BSA, salphen-Co2@BSA).

The preparation of salphen-Cu@BSA, salphen-Mn@BSA was similar to that of salphen-Co@BSA.

Scavenging ability for hydroxyl radical (•OH)

The scavenging activity for •OH was measured with the phen-Fe^{2+} reaction [25]. Their scavenging activity was calculated according to eq. 2.

$$\text{Scavenging activity } \% = (A_2 - A_1 / A_0 - A_1) \times 100\ \% \quad (2)$$

Where A_2 is the absorbance in the presence of the tested compound; A_0 is the absorbance in the absence of the tested compound; A_1 is the absorbance in the absence of the tested compound, phen-Fe^{2+}, H_2O_2.

Scavenging ability for superoxide anion radicals ($O_2^{\bullet-}$)

Scavenging activity of superoxide anion radicals ($O_2^{\bullet-}$) was assayed by the inhibition of NBT reduction. Riboflavin luminescence method was used as the source of $O_2^{\bullet-}$. The formation of blue formazan was monitored at 560 nm (phosphate buffer), the inhibition rate (F %) of $O_2^{\bullet-}$ was calculated according to eq. 3.

$$F\ \% = (\Delta_0 - \Delta)/\Delta_0 \times 100\ \% \quad (3)$$

where Δ is the absorbance in the presence of the tested compound; Δ_0 is the absorbance in the absence of the tested compound.

The action mixture was mixed solution of riboflavin (3.4×10^{-6} mol·L^{-1}), methionine (0.01 mol·L^{-1}), NBT (4.66×10^{-5} 10^{-6} mol·L^{-1}). All of the action component was confecting with PBS (pH 7.80, 0.05 mol·L^{-1}). The action mixture was saturated under 25 °C and 0.5 h condition. In testing, 3 mL of action mixture and a different quantity of antioxidant was illuminated, light intensity was 4000 (±100) Lux, the absorbance of the action mixture in 560 nm was measured (time interval was 30 s). The SOD-like activity of studied complexes was compared with native Cu,ZnSOD.

RESULTS AND DISCUSSION

UV–vis spectra of salphen-M@BSA

UV–vis spectrum was used to check the binding between BSA and HO-salphen-Co. The characteristic absorption peak of BSA appears in 280 nm, which was obtained from some amino acid residues (e.g., tryptophan). The characteristic peak of HO-salphen-Co appeared in 264 nm (B band) and 300–500 nm (A band), respectively. The UV–vis spectra of salphen-Co@BSA appeared in 280 and 300–500 nm simultaneously. The B band of HO-salphen-Co shifted from 264 to 268 nm. It indicated that salphen-Co@BSA was prepared (Fig. 1).

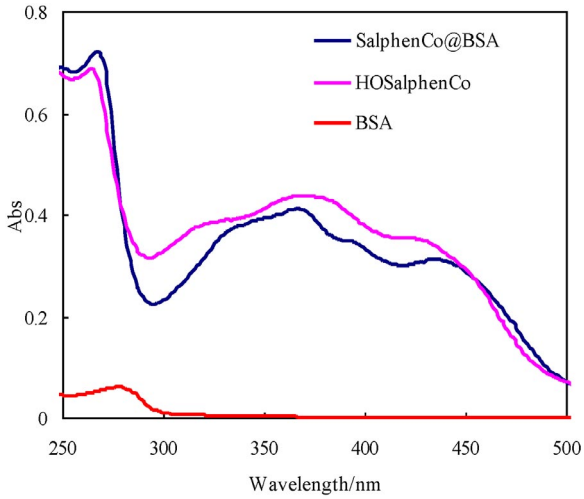


Fig. 1 UV–vis spectra of BSA, HO-salphen-Co, and salphen-Co@BSA.

In order to measure the binding mode between BSA and HO-salphen-Co, the model reaction using 1-methylimidazole (MeIm) as probe molecule was measured by UV–vis spectra (Fig. 2). When the axial ligand (MeIm) was added into the HO-salphen-Co solution in acetone, the B band of

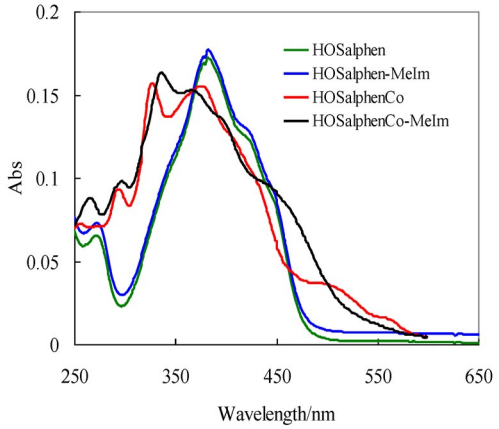


Fig. 2 UV–vis spectra of the model reaction ([HO-salphen-Co]: 10^{-6} mol/L, [HO-salphen]: 2×10^{-6} mol/L).

HO-salphen-Co shifted from 327 to 337 nm. However, the curve of salen ligand (HO-salphen) did not change if MeIm was added. It proved that the MeIm coordinated with MeIm. In UV–vis spectra of salphen-Co@BSA, the B band of HO-salphen-Co also shifted as the model reaction. It suggested that the binding mode between BSA and HO-salphen-Co was axial coordination between Co(II) and N in amino acid residues of BSA.

The binding amount of HO-salphen-Co to BSA was measured by UV–vis spectra because the absorption band near 366 nm of HO-salphen-Co did not change after binding to BSA. Therefore, λ_{366} nm was employed to calculate the binding amount of HO-salphen-Co to BSA. It was measured that the units of HO-salphen-Co in salphen-Co20@BSA, salphen-Co10@BSA, salphen-Co5@BSA, and salphen-Co2@BSA were 19, 10, 5, and 2, respectively. This means that almost all Co complexes could be bound to BSA if the ratio of HO-salphen-Co:BSA was lower than 20:1.

We found that other metal complexes (HO-salphensCu, HO-salphen-Mn) were different with HO-salphen-Co when they conjugated with BSA. BSA was denaturalized and precipitated if HO-salphensCu was added. The content of HO-salphen-Mn in BSA was found to be very low, because Mn(II) was difficult to bind with BSA and partly soluble in water. Therefore, it was very easy to move out when the mixture was dialyzed. Thus, the SOD-like activity of salphen-Cu@BSA and salphen-Mn@BSA was very low and did not show •OH scavenging activity in further radical scavenging ability test. In measures of salphen-M@BSA that followed, salphen-Co@BSA was mainly studied.

CD spectra of salphen-Co@BSA

BSA in PBS showed negative absorption bands with maxima at around 216 and 208 nm. The intensity of this double minimum reflects the amount of α-helical of BSA [26]. Schiff-base complexes did not show the CD signal in this region. So the far-UV region of CD spectroscopy was used to investigate the interaction of Schiff-base complexes with BSA [27,28].

Compared with BSA, the intensity of the characteristic CD signal in salphen-Co@BSA did not obviously decrease when the ratio of HO-salphen-Co to BSA was less than 10 (Fig. 3). But the CD signal in salphen-Co20@BSA (HO-salphen-M:BSA = 20:1) obviously decreased. It indicated that the amount of α-helical in salphen-Co@BSA (BSA:HO-salphen-M = 1:2, 1:5, 1:10) did not evidently change. So it showed that the secondary structure of BSA was maintained when the ratio of HO-salphen-Co to BSA was less than 10. In other words, the binding of 20 times HO-salphen-Co would induce the conformation of BSA (Fig. 3), which was consistent with what was reported in the literature on the interaction of albumin with small molecules [29].

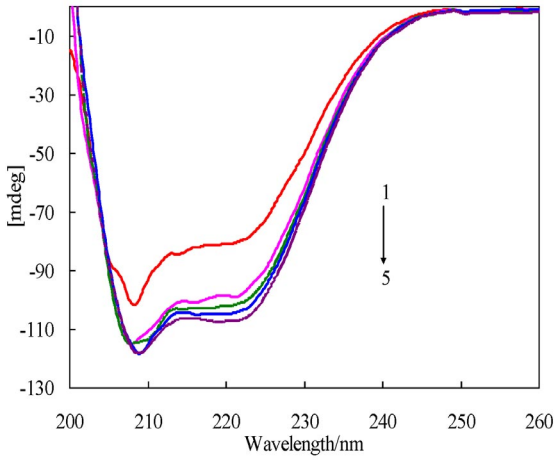


Fig. 3 CD spectra of BSA and salphen-Co@BSA in PBS (pH: 7.4, [BSA]: 1×10^{-6} mol/L, 1: salphen-Co20@BSA, 2: salphen-Co10@BSA, 3: salphen-Co5@BSA, 4: salphen-Co2@BSA, 5: BSA).

Gel electrophoresis

The salphen-Co@BSA was analyzed by sodium dodecyl sulfate (SDS) PAGE. It indicated that HO-salphen-Co did not cause change for the molecular weight of BSA. The salphen-Co@BSA conjugate was also analyzed by native PAGE (Fig. 4). It showed that the electrophoresis bands of salphen-Co1@BSA and salphen-Co2@BSA were similar to BSA. Salphen-Co5@BSA and salphen-Co10@BSA had a small difference with BSA, and salphen-Co20@BSA had a difference with BSA, obviously. In summary, compared to BSA, the more HO-salphen-Co combined to BSA, the longer the electrophoresis bands become. As HO-salphen-Co changed the charge density of BSA, the more Schiff-base metal complexes combined to BSA, the more change occurred. The results were agreed with UV–vis and CD spectra.

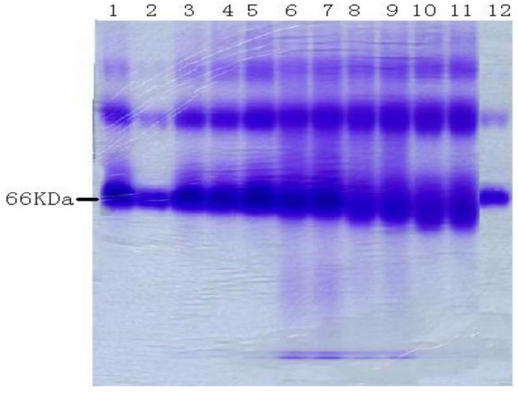


Fig. 4 Native PAGE of BSA and salphen-Con@BSA (n = 1, 2, 5, 10, 20). [(1,3): salphen-Co1@BSA; (4,5): salphen-Co2@BSA; (6,7): salphen-Co5@BSA; (8,9): salphen-Co10@BSA; (10,11): salphen-Co20@ BSA; (2,12): BSA].

Radical scavenging ability

Superoxide anion radical ($O_2^{\bullet-}$) scavenging ability of salphen-Co@BSA

BSA conjugates showed excellent activity for scavenging superoxide anion radicals. In the salphen-Co@BSA system, salphen-Co20@BSA had excellent scavenging activity, and the scavenging activity was dependent on concentration. The SOD-like activity of a different proportion of salphenCo@BSA was salphen-Co20@BSA > salphen-Co10@BSA > salphen-Co5@BSA > salphen-Co2@BSA (Fig. 5). Compared with BSA, the $O_2^{\bullet-}$ scavenging activity of salphen-Co20@BSA was 287 times of BSA (Fig. 6).

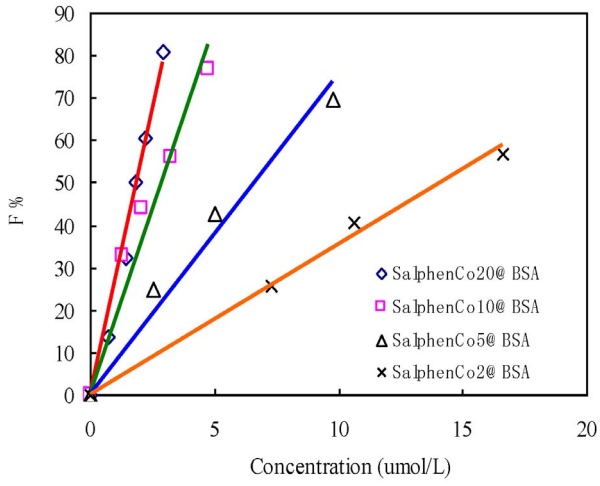


Fig. 5 The $O_2^{\bullet-}$ scavenging activity of salphen-Con@BSA (n = 2, 5, 10, 20).

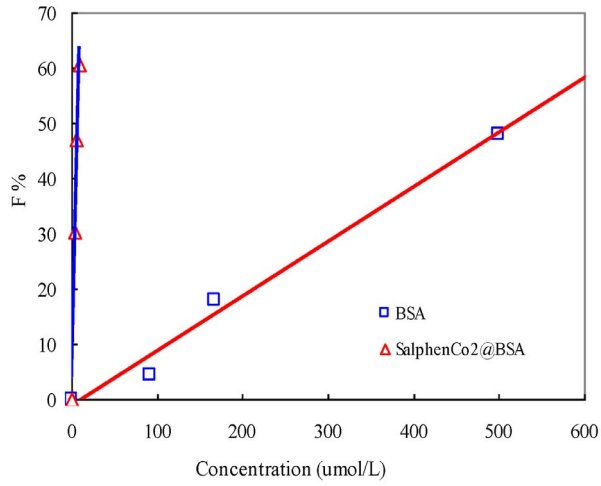


Fig. 6 The $O_2^{\bullet-}$ scavenging activity of salphen-Co@BSA and BSA.

The EC_{50} value of different conjugates was compared (datum were not shown). For antioxidant scavenger, the smaller EC_{50} value it had, the better scavenging activity it owned [30]. For salphen-Co@BSA conjugates, the EC_{50} of salphen-Co20@BSA was 1.80 μmol/L.

The $O_2^{\bullet-}$ scavenging activity of natural SOD was measured via NBT assay, the EC_{50} of natural SOD was 0.041 μmol/L. Salphen-Co20@BSA possessed lower EC_{50} than the standard compound M-40403 [31]. The analog quantity reached 2.28 % compared with natural Cu,ZnSOD.

Hydroxyl radical (•OH) scavenging ability of salphen-Co@BSA

The hydroxyl radical scavenging activity of salphen-Co@BSA was measured (Fig. 7). It showed that its hydroxyl radical scavenging activity was high. The •OH scavenging activity was dependent on concentration. Salphen-Co20@BSA showed better antioxidant activity than salphen-Co10@BSA. The scavenging values of salphen-Co20@BSA were far higher than that of others. The intensity of scavenging activity of different-proportion salphen-Co@BSA was salphen-Co20@BSA > salphen-Co10@BSA > salphen-Co5@BSA > salphenCo2@BSA.

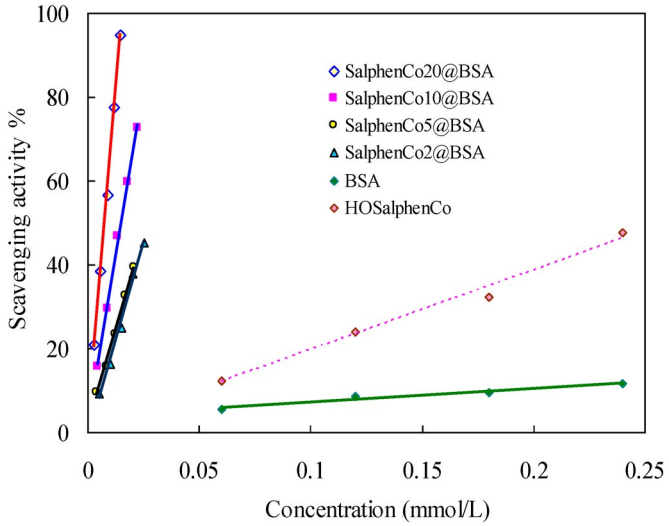


Fig. 7 Hydroxyl radical scavenging activity of salphen-Co*n*@BSA (n = 2, 5, 10, 20).

The •OH scavenging activities of BSA and HO-salphen-Co were measured. Their •OH scavenging activities were very low (datum were not shown). When BSA was added to 0.95 mmol/L, its scavenging effect reached 41 % only. HO-salphen-Co did not show •OH scavenging activity (Fig. 7).

Natural antioxidant enzymes such as SOD and catalase (CAT) could scavenge $O_2^{\bullet-}$ and •OH with high efficiency, but the specificity limits their application. Conjugates salphen-Co@BSA, which we prepared in this paper, had the ability of scavenging $O_2^{\bullet-}$ and •OH radical simultaneously, it was a bifunctional antioxidant enzyme mimic and could be used as ROS scavenger.

The mechanism of $O_2^{\bullet-}$ scavenging by salphen-Co@BSA

BSA had the ability to scavenge free radicals, but it was very weak, while BSA-Schiff-base complexes had more strongly antioxidant activity when they appeared in a small quantity. The results showed that the more Schiff-base complexes combined, the better antioxidant activity BSA-Schiff-base complexes possessed. So metal in conjugates was thought to act as important functions in ROS scavenging.

During hydroxyl radical scavenging, antioxidants were hydrogen donator and free radical quenchers. Hydroxyl radical is a brisk radical and accepts an electron or hydrogen radical to become a

stable molecule, UV–vis spectra showed metal of the Schiff-base complexes made axial coordination with the nitrogen atom of histidine, tryptophan, and abundance-dissociated amidocyanogen. It showed that the axial coordination could increase the antioxidant activity of BSA by making the hydrogen of the hydroxyl groups, which joined with the nitrogen atom, more easy to leave. Then, dissociated hydrogen combined with hydroxyl radical and stopped the next reaction of the hydroxyl radical.

The mechanism of $O_2^{\bullet-}$ scavenging was different from that of scavenging •OH. Fridorich and coworkers showed that M^n was the metalloenzyme in the reduced state and M^{n+1} was the enzyme in the oxidized state. Zhou found that arginine was indeed essential for high SOD activity and could steer the $O_2^{\bullet-}$ substrate to the metal ion. On the basis of our results and previously suggested catalytic mechanism [32–34], a possible mechanism for $O_2^{\bullet-}$ scavenging of salphen-Co@BSA was proposed: (1) a superoxide anion was attracted by the Co(II) ion under the orienting influence of the guanidyl cation; (2) the super oxide anion binding directly to the Co(II) ion could rapidly exchange between the axial and the planar position of the distorted square pyramid, which induced it to give up its electron and form an O_2 molecule; (3) the electrically neutral oxygen molecule left (4) a second super oxide anion was attracted by the Co(I) ion still under the orienting influence of the guanidyl cation; (5) the super oxide anion bound again to the Co(I) ion to accept an electron and a proton from the buffer. Since the proton exchange between the substrates and buffer was a rapid process, the superoxide anion further combines another proton from the solution to form a H_2O_2 molecule. Finally, electrically neutral H_2O_2 leaves, completing a catalytic cycle (Fig. 8).

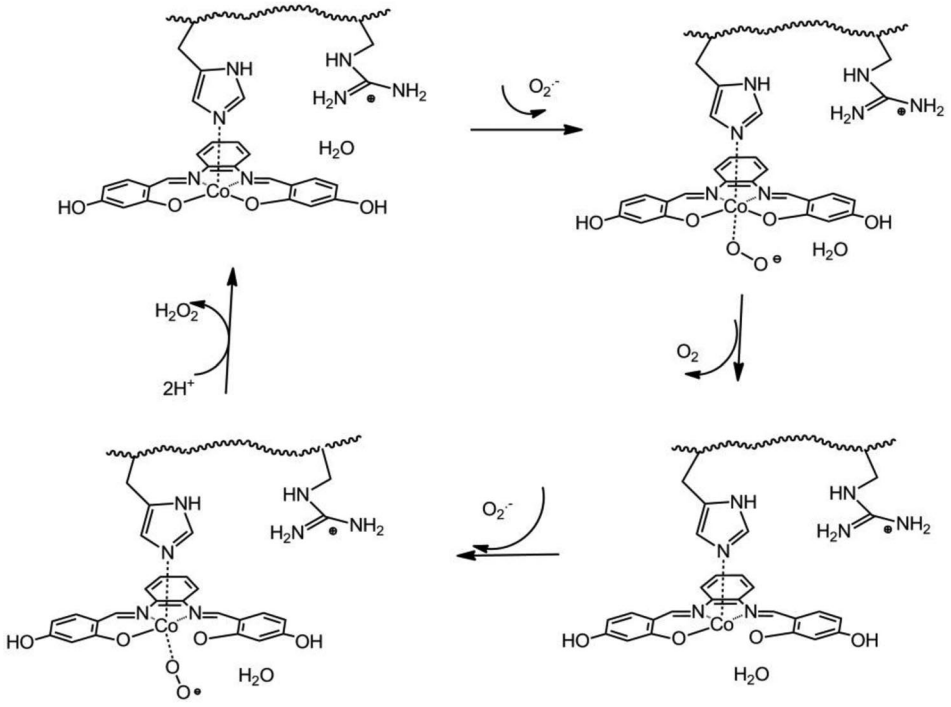


Fig. 8 Mechanism of $O_2^{\bullet-}$ scavenging.

CONCLUSIONS

BSA/Schiff-base metal complex conjugates had significant antioxidant activity. In the salphen-Co@BSA system, BSA acted as scaffold and the Schiff-base metal complexes acted as the catalytic center. When Schiff-base metal complexes bound with BSA, the poor water-solubility of the Schiff-base metal complexes could be well solved and the oxidative radical scavenging activity increased obviously. Salphen-Co@BSA conjugate was an excellent biopolymer ROS scavenger, while salphen-Cu@BSA and salphen-Mn@BSA showed lower SOD-like activity than that of salphen-Co@BSA, and salphen-Cu@BSA and salphen-Mn@BSA did not show activity. Salphen-Co@BSA had excellent ability in scavenging •OH and $O_2^{\bullet-}$, as a bifunctional mimic of antioxidant enzymes, which is worth further research as a good drug to scavenge free radicals.

ACKNOWLEDGMENTS

The project was supported by NSFC (No. 20964002), the Gansu Science and Technology Support Project (1011GKCA017), and Funda Res Funds Gansu Univ (2010-176).

REFERENCES

1. B. Halliwell, J. M. C. Gutteridge. In *Free Radicals in Biology and Medicine*, p. 543, Oxford University Press, New York (1989).
2. H. M. Qi, Q. B. Zhang, T. T. Zhao, R. Chen, H. Zhang, X. Z. Niu, Z. Li. *Int. J. Biol. Macromol.* **37**, 195 (2005).
3. K. Uehida. *Free Radical Biol. Med.* **28**, 1685 (2000).
4. E. Cadenas, K. J. Davies. *Free Radical Biol. Med.* **29**, 222 (2000).
5. J. E. Kinsella, E. Frankel, B. German, J. Kanner. *Food Technol*. **4**, 85 (1993).
6. N. Singh, P. S. Rajini. *Food Chem*. **85**, 611 (2004).
7. T. Shibata, K. Ishimaru, S. Kawaguchi, H. Yoshikawa, Y. Hama. *J. Appl. Phycol.* **20**, 705 (2008).
8. T. Ohse, S. Nagaoka, Y. Arakawa, H. Kawakami, K. Nakamura. *J. Inorg. Biochem.* **85**, 201 (2001).
9. J. Vanco, O. Svajlenova, E. Ramanska, J. Muselik, J. Valentova. *J. Trace Elem. Med. Biol.* **18**, 155 (2004).
10. L. Lacikova, J. Muselik, I. Masterova, D. Grancai. *Molecules* **12**, 98 (2007).
11. M. L. Pires dos Santos, A. Faljoni-Alário, A. Sálvio Mangrich, A. M. da Costa Ferreira. *J. Inorg. Biochem.* **71**, 71 (1998).
12. M. T. H. Tarafder, K. A. Crouse, A. M. Ali, B. M. Yamin, H. K. Fun, A. Kasbollah. *Polyhedron* **20**, 2363 (2001).
13. T. Peters Jr. *All about Albumin: Biochemistry Genetics and Medical Applications*, p. 432, Academic Press, San Diego (1995).
14. P. Kurtzhals, S. Havelund, I. Jonassen, B. Kiehr, U. Ribel, J. Markussen. *J. Pharm. Sci.* **85**, 304 (1996).
15. S. Curry, H. Mandelkow, P. Brick, N. Franks. *Nat. Struct. Biol.* **5**, 827 (1998).
16. M. Wardell, Z. Wang, J. X. Ho, J. Robert, F. Ruker, J. Ruble, D. C. Carter. *Biochem. Biophys. Res. Commun.* **291**, 813 (2002).
17. P. K. Gupta, C. T. Hung, D. G. Perrier. *Int. J. Pharm.* **33**, 137 (1986).
18. P. Kurtzhals, S. Havelund, I. Jonassen, B. Kiehr, U. D. Larsen, U. Ribel, J. Markussen. *Biochem. J.* **312**, 725 (1995).
19. W. P. Sheffield, L. J. Eltringham-Smith, S. Gataiance, V. Bhakta. *BMC Biotechnol.* **9**, 15 (2009).
20. R. M. Wang, T. Komatsu, A. Nakagawa, E. Tsuchida. *Bioconjugate Chem.* **16**, 23 (2005).

21. T. Komatsu, R. M. Wang, P. A. Zunszain, S. Curry, E. Tsuchida. *J. Am. Chem. Soc.* **128**, 16297 (2006).
22. E. C. Moran, A. S. Kamiquti, J. C. Cawley, A. R. Pettitt. *Br. J. Haematol.* **116**, 316 (2002).
23. P. Thordarson, B. Le Droumaquet, K. Velonia. *Appl. Microbiol. Biotechnol.* **73**, 243 (2006).
24. P. Pfeiffer, E. Breith, E. Lubbe, T. Tsumaki. *Justus Liebigs Ann. Chem.* **503**, 84 (1933).
25. Z. Guo, H. Liu, X. Chen, X. Ji, P. Li. *Bioorg. Med. Chem. Lett*. **16**, 6348 (2006).
26. S. Y. Venyaminov, K. S. Vassilenko. *Anal. Biochem*. **222**, 176 (1994).
27. N. S. Quiming, R. B. Vergel, M. G. Nicolas, J. A. Villanueva. *J. Health Sci.* **51**, 8 (2005).
28. Q. Xiao, S. Huang, Y. Liu, F. F Tian, J. C. Zhu. *J. Fluoresc*. **19**, 317 (2009).
29. S. M. T. Shaikh, J. Seetharamappa, S. Ashoka, P. B. Kandagal. *Dyes Pigments* **73**, 211 (2007).
30. Y. V. Yuan, D. E. Bone, M. F. Carrington. *Food Chem.* **91**, 485 (2005).
31. H. Lee, W. Park, D. Lim. *Bioorg. Med. Chem. Lett.* **20**, 2421 (2010).
32. P. J. Hart, M. M. Balbirnie, N. L. Ogihara, A. M. Nersissian, M. S. Weiss, J. S. Valentine, D. Eisenberg. *Biochemistry* **38**, 2167 (1999).
33. L. M. Ellerby, D. E. Cabelli, J. A. Graden, J. S. Valentine. *J. Am. Chem. Soc.* **118**, 6556 (1996).
34. Y. H. Zhou, H. Fu, W. X. Zhao, W. L. Chen, C. Y. Su, H. Sun, L. N. Ji, Z. W. Mao. *Inorg. Chem*. **46**, 734 (2007).

