

Research Article

Preparation of Keratin-Glycine Metal Complexes and Their Scavenging Activity for Superoxide Anion Radicals

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To address the problem of limited application of natural SOD, the development of SOD mimic enzymes is of great importance for bioantioxidation. Herein, we report on a new type of biopolymer antioxidant with excellent scavenging activity for $O_2^{\cdot-}$, keratin-glycine metal complexes (FK-GlyM, M = Zn, Cu, Mn, Ni). They are prepared by feather keratin firstly combined with glycine and then metal ions. Using FT-IR, TG, CD, and SEM, the performance of the obtained complexes (FK-GlyM) for scavenging $O_2^{\cdot-}$ is analysed and investigated. Importantly, the scavenging activity of FK-GlyCu is excellent in all FK-GlyM, and FK-GlyCu₆₀ has the most excellent anti- $O_2^{\cdot-}$ activity in all FK-GlyCu_x, of which EC₅₀ and degree of simulation were, respectively, up to $4.5 \times 10^{-3} \pm 0.0012 \mu\text{mol/L}$ and 911.1% compared with nature Cu, Zn-SOD. Finally, its mechanism was also discussed. In summary, this method about the simulation strategies will provide a novel idea for exploiting new-type biocompatible and highly reactive antioxidants.

1. Introduction

In vivo, excess oxygen free radical is harmful to the body [1]. Under normal circumstances, the production and the elimination of oxygen radicals are balanced [2]. However, due to the impact of the disease, age, and the external environment, excess oxygen radicals would be accumulated in the cells when the physiological balance was broken [3, 4], leading to host tissue be damaged [5], such as the damage of protein, lipid peroxide, DNA mutation, and enzyme inactivation. However, superoxide dismutase (SOD) as antioxidant is important to protect cells from oxidative damage [6], which can catalyze the disproportion action of superoxide anion radicals ($O_2^{\cdot-}$) and further balance $O_2^{\cdot-}$ *in vivo*. Although the natural SOD has the excellent activity for scavenging $O_2^{\cdot-}$, many disadvantages still exist [7], such as complex extract process, high cost, big molecular weight, easy deactivation, and not easily entering the cell membrane, which limited its application in medicine and other fields [8–10]. Therefore, the preparation of SOD mimic enzyme is becoming more and more popular [11, 12].

Currently, a lot of enzyme simulations for scavenging $O_2^{\cdot-}$ have been synthesized, including porphyrin metal complexes [7, 13], macrocyclic metal complexes [14], and Schiff-base metal complexes [15]. However, due to the low solubility in water, large side effects, and so on, the widespread applications of these SOD mimics based on micromolecule were still limited. Therefore, SOD mimics based on macromolecule become the new direction for simulation of SOD. In SOD mimic based on polymer, there are mainly cyclodextrins [16] and proteins [17–19]. For proteins, they can form directly complexes with metal ions since they contain a large number of amino and carboxyl functional groups with complex function. In general, those metals as SOD activity center mainly include Cu [20], Co [21], Mn [22, 23], and Fe [24]. Ibrahim et al. [25] reported that Ovotransferrin (OTF) had function of scavenging $O_2^{\cdot-}$ when it combined with Cu and Mn. Besides, there is an important complement oxygen free radical scavenger *in vivo*: Metallothionein (MT). It is a class of protein containing low molecular weight and abundant thiol groups, which contribute to scavenging $O_2^{\cdot-}$ by combining with metal ions [26].

Keratin widely exists in hair, feathers, wool, nails, and horns [27]. Due to its special molecular structure, it is hard to be used by humans and casually abandoned, which caused the waste of resources and bad environmental pollution [28]. However, they have a large number of disulfide bonds [29, 30]. If thiol groups produced by the reduction of disulfide bonds combine with metal, the structural system similar to metallothionein will be generated. In this paper, feather keratin (FK) and glycine (Gly) firstly were combined to generate FK-Gly in an aqueous solution, then the transition metal ions (Zn^{II} , Cu^{II} , Mn^{II} , and Ni^{II}) were combined with FK-Gly to generate keratin-based biopolymer metal complexes (FK-GlyM, M = Zn, Cu, Mn, Ni) and further studied its property of scavenging $O_2^{\cdot-}$ and seriously explored its mechanism.

2. Experiments

2.1. Materials. Glycine (Gly) was purchased from Shanghai Institute of Biochemistry. $Ni(OAc)_2 \cdot 4H_2O$, $Mn(OAc)_2 \cdot 4H_2O$, $Zn(OAc)_2 \cdot 4H_2O$, and $Cu(OAc)_2 \cdot H_2O$ were all obtained commercially. Both Chlorinated Nitro blue tetrazolium (NBT) and DL-Methionine are biochemicals, which are products of Zhongqin Chemical Reagent Co., Ltd., in Shanghai and Tianjin Guangfu Fine Chemical Research Institute. Feather keratin (FK) is self-made in our laboratory. Unless otherwise stated, all other chemicals were of analytical grade and used without further purification.

2.2. Equipment for Characterization. Fourier transform infrared spectra (FT-IR) were measured by an FT-IR spectrometer (FTS 3000) of DigiLAB, and the transmittance spectra were collected in the range of $4000\text{--}400\text{ cm}^{-1}$ at a resolution of 0.008 cm^{-1} . Thermogravimetric analyses of products were carried out on a PerkinElmer Pyris Diamond thermogravimetry (TG)/dynamic thermal analyzer at a heating rate of $10^\circ\text{C}/\text{min}$ under a nitrogen atmosphere. Circular Dichroism (CD) was recorded by a Jasco J-810 spectropolarimeter (1 cm cell). The wavelength of scanning was from 200 to 250 nm, the speed was $200\text{ nm}/\text{min}$, and a response time was of 1 s. The morphologies of product were studied by scanning electron microscopy (SEM) (Zeiss ULTRA Plus microscope, Germany).

2.3. Preparation of Keratin-Glycine Metal Complexes (FK-GlyM). Four kinds of metal salt solution [$M(OAc)_2$] were prepared as follows: $Ni(OAc)_2$, $Mn(OAc)_2$, $Zn(OAc)_2$, and $Cu(OAc)_2$ were individually dissolved in water and set aside. Glycine solution (0.02 mM) was prepared in a phosphate buffer solution (PBS, 100 mL).

2.3.1. Preparation of Keratin-Glycine Complexes (FK-Gly). Firstly, certain keratin, $Na_2S_2O_3$, and glycine were dispersed into water, and then certain concentration of NaOH solution was added to adjust the pH up to 7-8. Finally this mixture was stirred 6-12 h in the dark at room temperature. The keratin-glycine complex (FK-Gly) was successfully prepared.

2.3.2. Preparation of Keratin-Glycine Metal Complexes (FK-GlyM_x). The preparation process of FK-GlyZn₅ is as follows:

$Zn(OAc)_2$ and FK-Gly were added to simple tube ($M_{FK-Gly} : M_{Zn} = 1 : 5$). After being magnetic stirred 12 h in the dark, FK-GlyZn₅ was obtained and set aside in the dark at $0\text{--}4^\circ\text{C}$. The concentration of FK-GlyZn₅ was calculated according to the amount of FK.

The preparations of FK-GlyCu₅, FK-GlyMn₅ and FK-GlyNi₅ are similar to that of FK-GlyZn₅.

By changing the ratio of $Cu(OAc)_2$ being added to the mixture, keratin-based metal copper complex with different ratio was also obtained, namely, FK-GlyCu_x ($x = 5, 10, 20, 30, 40, 50, 60, 80, 100$).

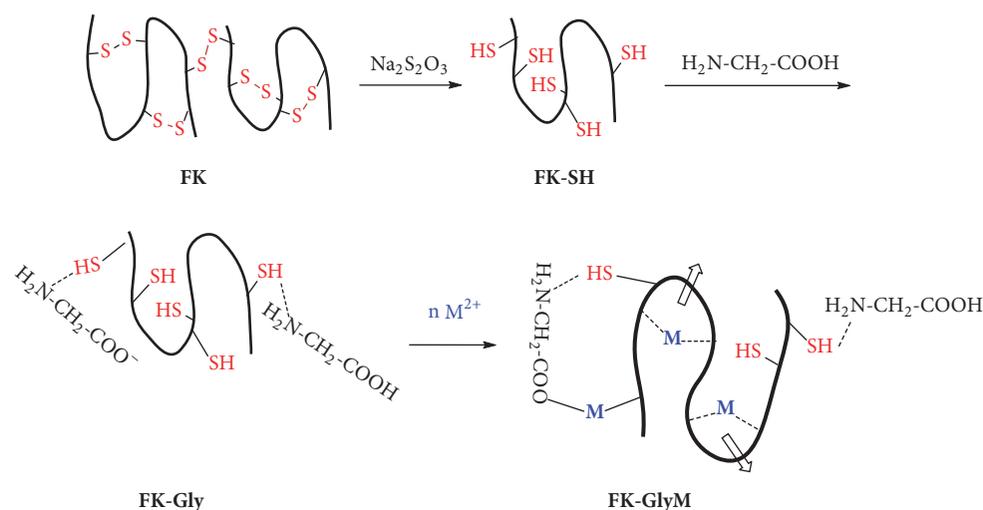
3. Results and Discussion

The preparation route of FK-GlyM was shown in Scheme 1, where the FK and FK-SH were the partially crosslinking protein and reduced protein. FK-Gly was modified by Gly and FK-GlyM was keratin-glycine metal complex. Firstly, the disulfide bonds (-S-S-) in keratin firstly were reduced to thiol groups (-SH) by strong reductant, and then these thiol groups reacted with the amino of glycine to generate keratin-glycine complex. Finally, keratin-glycine complex reacted with metal to give keratin-glycine metal complex.

3.1. Structure Characterization of FK-GlyM. The obtained FK-GlyM was characterized by FT-IR spectra, TG, CD, and SEM. Moreover, we also discussed the mechanism of anti-oxidation activity.

3.1.1. FT-IR Spectra. The prepared keratin-glycine Cu complex (FK-GlyCu₆₀) was frozen in the refrigerator at -20°C for 24 h. After being lyophilized, the products and KBr were mixed with 1:100 and made into transparent tablet for measuring FT-IR spectra. The result was shown in Figure 1. In FT-IR spectrum of FK, some characteristic absorption peaks at 1647 cm^{-1} , 1522 cm^{-1} , and 1236 cm^{-1} were, respectively, assigned to amide I, II, III band of FK, which indicated that the secondary structure of FK was mainly free coil. However, for FK-GlyCu₆₀, the characteristic absorption peaks of amide I, II, III band demonstrated that the secondary structure of FK-GlyCu₆₀ was mainly free coil and β -sheet structure. Therefore, the secondary structure of FK had been changed, and some random coil structures also had been into β -sheet structure in the preparation process of FK-GlyM. Besides, characteristic absorption peaks of S-S bonds had happened red shift from 580 cm^{-1} to 546 cm^{-1} , which illustrated that S-S bonds had become less stable in the preparation process. It could be conjectured that some S-S bond had shifted and might generate -SH or sulfur anion in preparation process.

3.1.2. TG Analysis. TG curves of FK and FK-GlyCu₆₀ were presented in Figure 2. It could be seen that FK gave three weight losses within total temperature range, which could be assigned to the weight loss of adsorbed water at about 100°C and keratin decomposition at 200°C and 375°C . Meanwhile, FK-GlyCu₆₀ also gave three weight losses within total temperature range, caused by the decomposition of FK at 200 and 375°C as well as no weight loss after 575°C . In summary, compared with feather keratin, the thermodynamic



SCHEME 1: The preparation process of FK-GlyM ($M = \text{Zn}^{\text{II}}, \text{Cu}^{\text{II}}, \text{Mn}^{\text{II}}, \text{Ni}^{\text{II}}$).

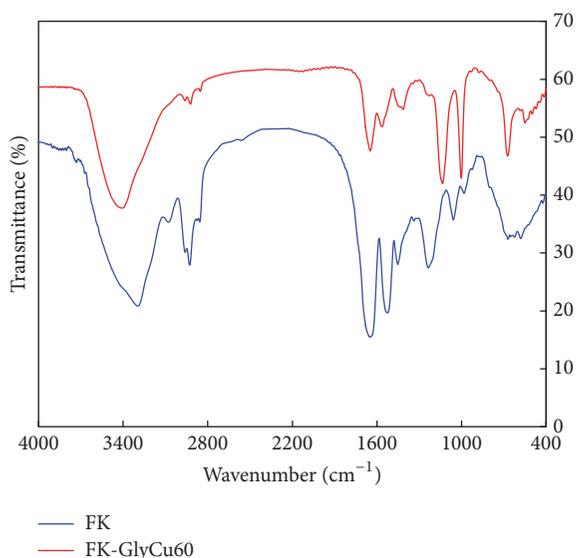


FIGURE 1: The IR spectra of FK-GlyCu₆₀ complex.

stability of the obtained keratin-glycine metal complexes was reduced.

3.1.3. CD Analysis. Circular Dichroism (CD) of FK-GlyCu_x (0.005 mM) was measured at the wavelength of 190–300 nm and room temperature. The results were shown in Figure 3. The characteristic peaks of random coil structure of FK appeared at near 195 nm while the characteristic peaks of β -sheet layer structure of FK-GlyCu_x complex appeared in the vicinity of 200 nm. Therefore, it was showed that the secondary structures of the prepared FK-GlyCu_x were mainly β -sheet and random coil, which could be attributed to the fact that the part random coil of secondary structure of the FK-GlyCu_x complex had transformed into β -sheet compared with pure FK. The result was the same as FT-IR spectrum analysis.

TABLE 1: EC₅₀ of FK-Gly and FK.

FK-GlyCu ₅	EC ₅₀ ($\mu\text{mol/L}$)	Degree of simulation (%)
FK	0.85 ± 0.11	4.8
FK-Gly	0.44 ± 0.08	9.3
FK-GlyCu ₅	0.067 ± 0.004	61.2
FK-GlyCu ₆₀	0.0045 ± 0.0012	911.1

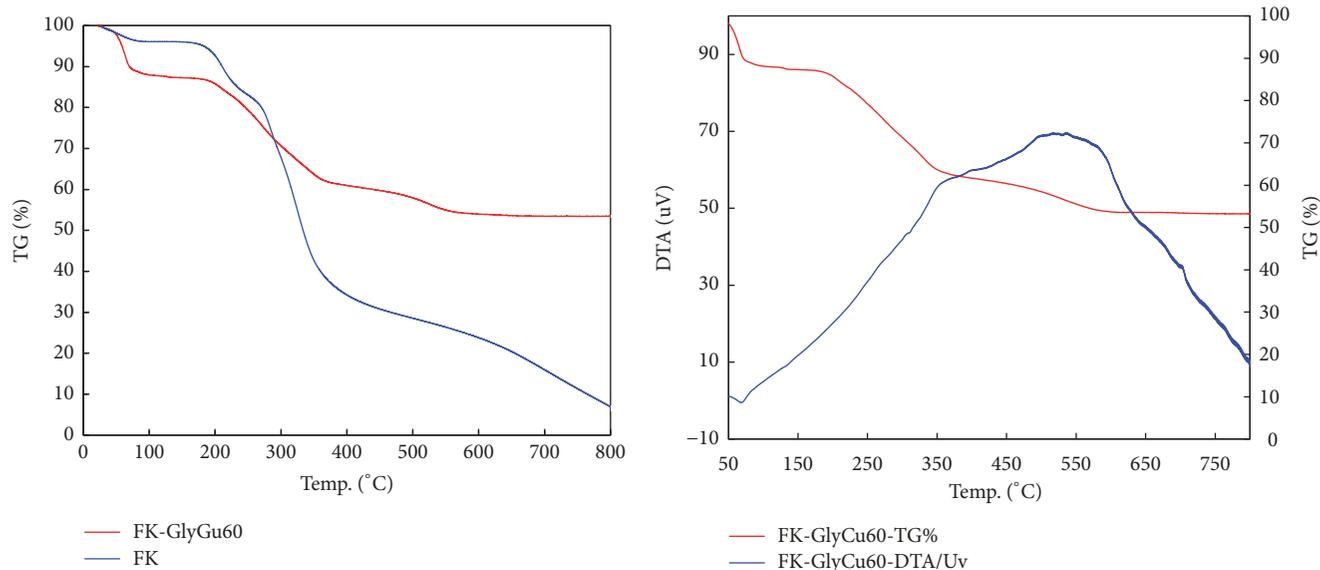
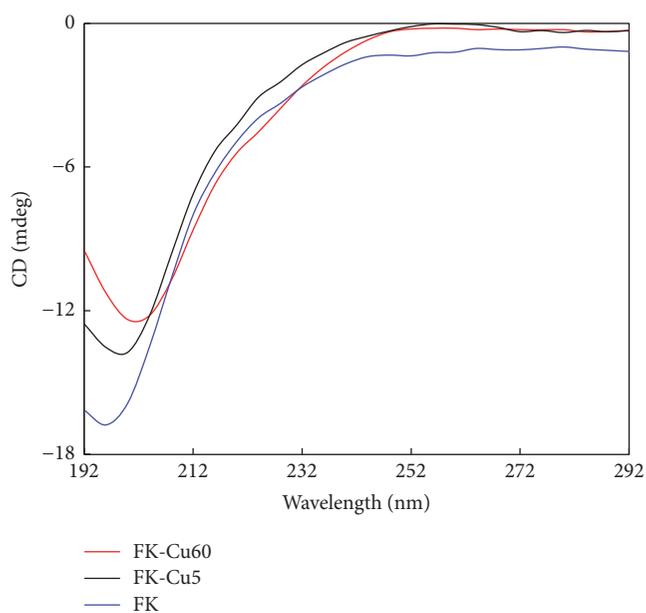
Cond: [FK] = 5 $\mu\text{mol/L}$, n (FK) : n (Gly) = 1 : 0.2; $T = 20^\circ\text{C}$.

3.1.4. SEM Images. After being lyophilized, the microstructure of dry FK and FK-GlyCu₆₀ was characterized by scan electron microscopy (SEM). As showed in Figure 4, in contrast to FK, it was found that they had the porous support structure and the pore size was 0.2–2 μm in FK-GlyCu₆₀. These porous structures would facilitate the adsorption of small molecules and macromolecular compounds.

3.2. O₂^{•-} Scavenging Activity of FK-GlyM. Using NBT photochemical reduction method to investigate scavenging superoxide anion radicals (O₂^{•-}) activity of keratin (FK) and the keratin-glycine complex (FK-Gly), the results were shown in Table 1 and Figure 5. According to the results, FK and FK-Gly ([FK] = 5 $\mu\text{mol/L}$) both presented some anti-O₂^{•-} activity, and the scavenging activity of FK-Gly was better than FK. Compared with FK, the activity of FK-Gly was doubled.

3.2.1. O₂^{•-} Scavenging Activity of FK-Gly under Different Environment. In order to get better FK-Gly, the effect factors on its scavenging activity, such as temperature, pH, and Gly content, were investigated.

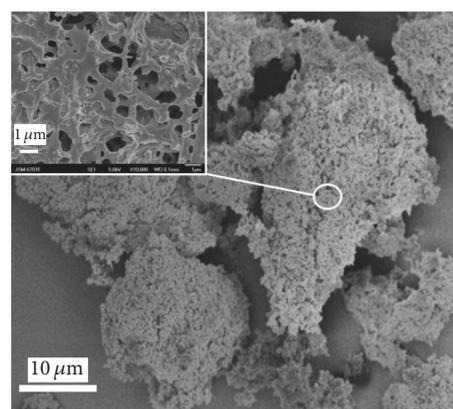
At Different Temperature. Under the condition of the same ratio, the effects of temperature on the scavenging activity of FK-Gly for O₂^{•-} were investigated. The results were shown in Table 2 and Figure 6, turning out that FK-Gly had the best scavenging activity for O₂^{•-} at 20°C and EC₅₀ and degree of

FIGURE 2: The TG of FK and FK-GlyCu₆₀.FIGURE 3: The CD spectra of FK-GlyCu_x complexes.TABLE 2: EC₅₀ of FK-Gly at different temperatures.

T (°C)	EC ₅₀ (μmol/L)	Degree of simulation (%)
10	0.61 ± 0.10	6.7
20	0.58 ± 0.03	7.1
30	0.65 ± 0.08	6.3
40	0.60 ± 0.11	6.8

Cond: [FK] = 5 μmol/L, n (FK) : (Gly) = 1 : 0.2.

simulation could, respectively, reach 0.58 ± 0.01 μmol/L and 7.1%.

FIGURE 4: The SEM images of FK-GlyCu₆₀.TABLE 3: EC₅₀ of FK-Gly at different pH values.

pH	EC ₅₀ (μmol/L)	Degree of simulation (%)
12.6	0.55 ± 0.11	7.5
11.0	0.25 ± 0.05	16.4
10.0	0.28 ± 0.07	14.6
8.7	0.42 ± 0.03	9.8
7.3	0.23 ± 0.10	17.8

Cond: [FK] = 5 μmol/L, n (FK) : (Gly) = 1 : 0.2, T = 20°C.

At Different pH Value. Under the condition of the same ratio and temperature, the effects of different pH on the scavenging activity of FK-Gly for O₂^{•-} were investigated. The results were shown in Table 3 and Figure 7, which indicated that the scavenging activity of FK-Gly was best when the pH was 7-8 and EC₅₀ and degree of simulation could, respectively, up to 0.23 ± 0.03 μmol/L and 17.8%.

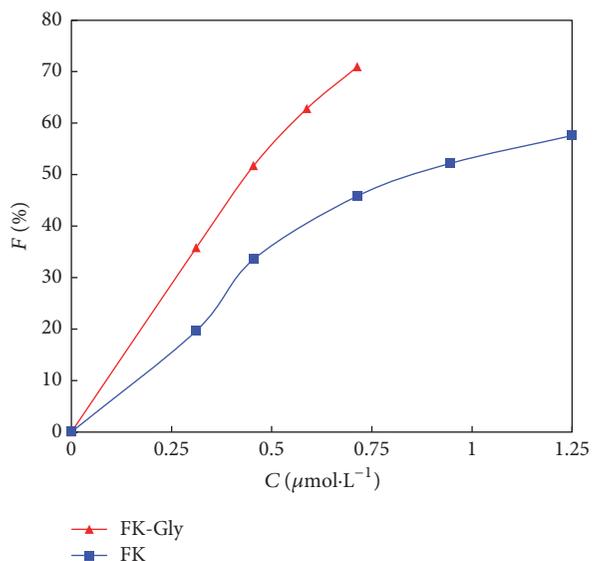


FIGURE 5: O₂^{•-} scavenging activity of FK-Gly and FK.

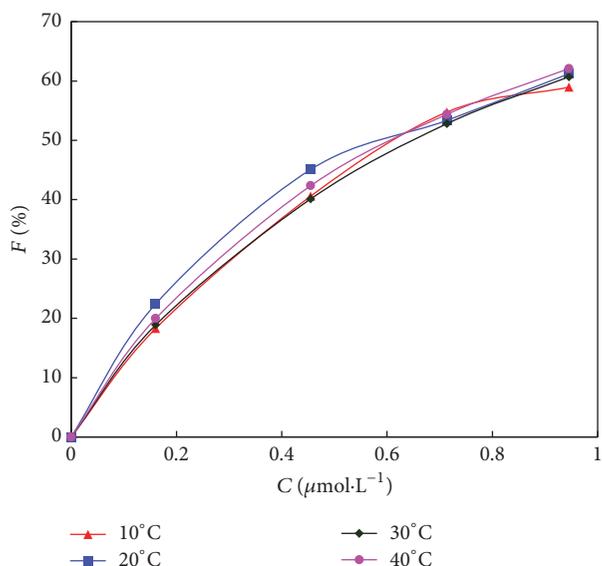


FIGURE 6: The comparison of O₂^{•-} scavenging activity of FK-Gly at different temperatures.

TABLE 4: EC₅₀ of FK-Gly at different mounts of Gly.

n (FK) : n (Gly)	EC ₅₀ (μmol/L)	Degree of simulation (%)
1:0.2	0.23 ± 0.07	17.8
1:0.4	0.23 ± 0.07	17.8
1:0.6	0.21 ± 0.12	19.5
1:0.8	0.50 ± 0.10	8.2
1:1	0.76 ± 0.11	5.4

Cond: [FK] = 5 μmol/L, T = 20°C, pH = 7-8.

At Different Gly Content. Under the condition of the same temperature and pH, the effects of different Gly content on the scavenging activity of FK-Gly for O₂^{•-} were investigated,

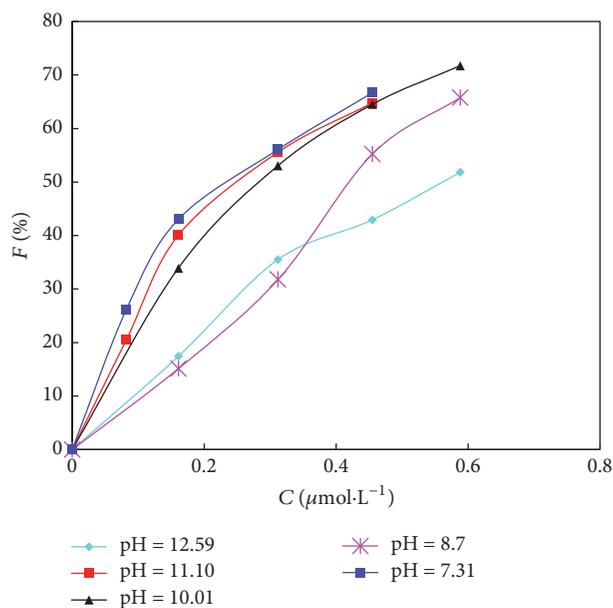


FIGURE 7: The comparison of O₂^{•-} scavenging activity of FK-Gly at different pH values.

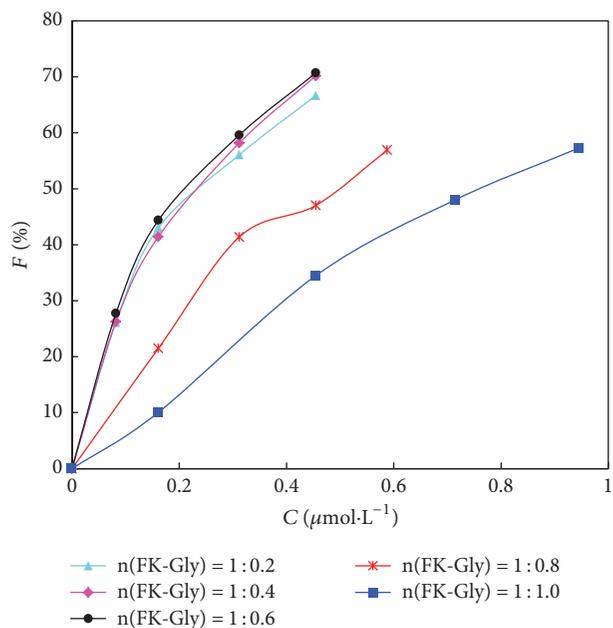


FIGURE 8: The comparison of O₂^{•-} scavenging activity of FK-Gly at different amounts of Gly.

and the results were shown in Table 4 and Figure 8. It was found that the scavenging activity of FK-Gly was best and EC₅₀ and degree of simulation could, respectively, reach 0.21 ± 0.06 μmol/L and 19.5% when the ratio of FK and Gly was 1:0.6 though the scavenging activity was similar when the ratios of FK and Gly were 1:0.2–1:0.6.

3.2.2. O₂^{•-} Scavenging Activity of FK-GlyM. Similarly, adopting NBT photochemical reduction method investigated anti-O₂^{•-} activity of FK-GlyM₅ (M = Zn, Cu, Mn, Ni). Although

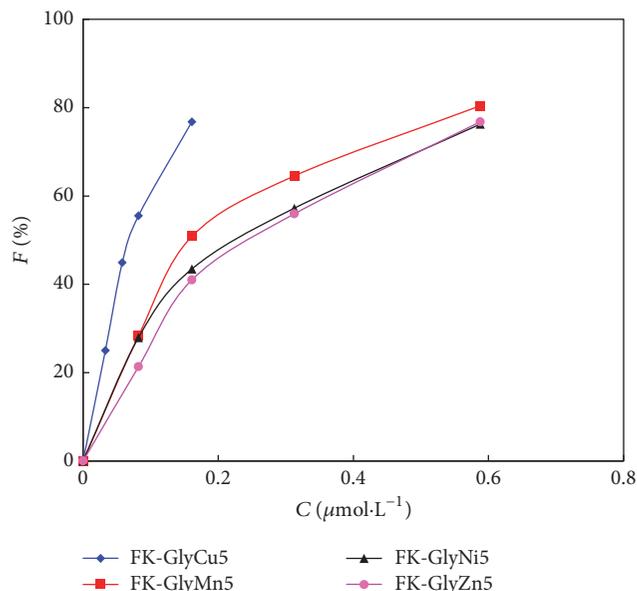


FIGURE 9: $O_2^{\bullet-}$ scavenging activity of FK-GlyM₅.

TABLE 5: EC₅₀ of FK-GlyM₅.

FK-GlyM ₅	EC ₅₀ (μmol/L)	Degree of simulation (%)
FK-GlyZn ₅	0.24 ± 0.01	17.1
FK-GlyCu ₅	0.067 ± 0.011	61.2
FK-GlyMn ₅	0.157 ± 0.008	26.1
FK-GlyNi ₅	0.22 ± 0.05	18.6

Cond: [FK] = 0.005 mmol/L, n (FK) : n (M (OAc)₂) = 1 : 5 (M = Zn, Cu, Mn, Ni).

they all had certain anti- $O_2^{\bullet-}$ activity, the different metal complexes had different activity. To compare anti- $O_2^{\bullet-}$ activity and EC₅₀ of different FK-GlyM in Table 5 and Figure 9, the results revealed that FK-GlyCu₅ had the most active for scavenging superoxide anion radicals, of which EC₅₀ was 0.067 ± 0.0014 μmol/L and degree of simulation was 61.2% comparing with nature Cu, Zn-SOD. However, the activity of FK-GlyZn₅ was most weak and its EC₅₀ was only 0.24 ± 0.08 μmol/L and degree of simulation was 17.1% comparing with nature Cu, Zn-SOD. To sum up, the scavenging activity for $O_2^{\bullet-}$ of four kinds of keratin-glycine metal complexes was FK-GlyCu₅ > FK-GlyMn₅ > FK-GlyNi₅ > FK-GlyZn₅.

Further, the effect of different ratios of FK-GlyCu_x on scavenging activity for $O_2^{\bullet-}$ was investigated in order to get the best FK-GlyCu. Firstly, the following complexes were prepared: FK-GlyCu₅, FK-GlyCu₁₀, FK-GlyCu₂₀, FK-GlyCu₃₀, FK-GlyCu₄₀, FK-GlyCu₅₀, FK-GlyCu₆₀, FK-GlyCu₇₀, FK-GlyCu₈₀, FK-GlyCu₉₀, and FK-GlyCu₁₀₀, and then they contrasted their anti- $O_2^{\bullet-}$ activity in Table 6 and Figure 10. The results indicated that the amount of metal ions (Cu^{II}) had important effect for anti- $O_2^{\bullet-}$ activity of FK-GlyCu_x complex. With the increase of metal ion Cu^{II}, the anti- $O_2^{\bullet-}$ activity of FK-GlyCu_x was first increased and then decreased. The scavenging activity of FK-GlyCu₆₀ for $O_2^{\bullet-}$ was strongest in Table 6 and Figure 10. Compared with the literature [31],

TABLE 6: The effect of the amount of Cu^{II} for $O_2^{\bullet-}$ scavenging activity of FK-GlyCu_x.

FK-GlyCu _x	EC ₅₀ (μmol/L)	Degree of simulation (%)
FK-GlyCu ₅	0.115 ± 0.010	35.7
FK-GlyCu ₁₀	0.055 ± 0.007	74.5
FK-GlyCu ₂₀	0.016 ± 0.012	256.3
FK-GlyCu ₃₀	0.0163 ± 0.0011	251.5
FK-GlyCu ₄₀	0.013 ± 0.005	315.4
FK-GlyCu ₅₀	0.0078 ± 0.0006	525.6
FK-GlyCu ₆₀	0.0045 ± 0.0012	911.1
FK-GlyCu ₇₀	0.007 ± 0.001	585.7
FK-GlyCu ₈₀	0.02 ± 0.01	205.0
FK-GlyCu ₉₀	0.025 ± 0.009	167.4
FK-GlyCu ₁₀₀	0.019 ± 0.002	221.6

of which EC₅₀ and degree of simulation, respectively, were 0.038 ± 0.003 μmol/L and 107%; here EC₅₀ of prepared FK-GlyCu₆₀ was far below it and the degree of simulation was much bigger than it. The values, respectively, were up to 0.0045 ± 0.0012 μmol/L and 911.1%.

3.3. Suggested Mechanism of Active Center. According to our reported results, the active center and mechanism of FK-GlyM for scavenging $O_2^{\bullet-}$ were suggested. There exist possibly three active reaction centers (Scheme 2). (1) Metal ion and N from the histidine and amino acid chain of FK residues formed coordination bonds, obtaining a six-member ring structure which provided $O_2^{\bullet-}$ reaction centers. (2) Metal ions and N, O in amino acid chain of FK coordinated, respectively, to form a plurality of annular reaction center, and the synergy of multiple active centers improved the scavenging activity of FK-GlyM for $O_2^{\bullet-}$. Meanwhile, due to the introduction of glycine, the aggregation of polymer chains in keratin became difficult and further increased the solubility of FK-GlyM. (3) For FK-GlyM, the partial COO⁻ of glycine and M²⁺ occurred to electrostatic attraction that led to form new reactive center. Moreover, the thiol (-SH) from S-S had active hydrogen atom which could cause the free radical of chain growth to become less active and further generate the free radical with poor reactivity or extremely stable inhibitor free radical via providing active hydrogen. In either of these two cases, the inhibitor slowed down the original radical reaction and even stopped the reaction carrying out.

4. Conclusion

In summary, a simple biopolymer antioxidant (FK-GlyM) with excellent scavenging activity has been gained via simply combining FK with Gly and metal ions. It was found that the scavenging activity of FK-GlyM for $O_2^{\bullet-}$ was prior to FK-Gly and FK. After exploring the effect of temperature, pH, and ratios on scavenging activity, the results proved that the optimal conditions were 20°C, pH = 7-8, and n (FK) : n (FK-Gly) = 1 : 0.6, where the scavenging capacity of FK-Gly for $O_2^{\bullet-}$ was

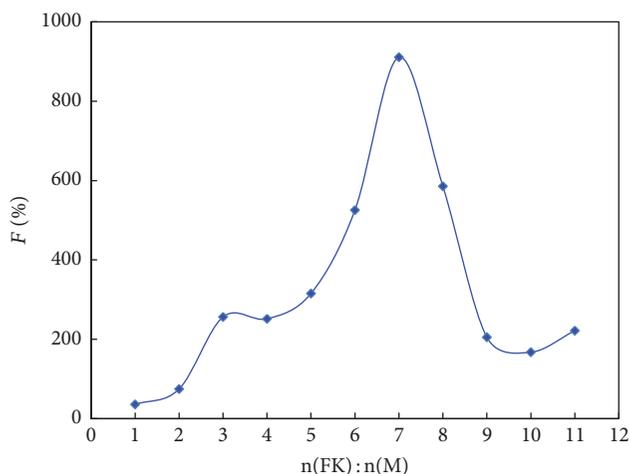
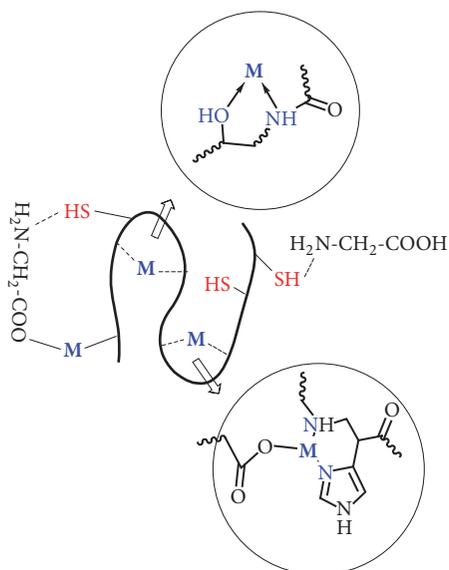


FIGURE 10: $O_2^{\cdot-}$ scavenging activity of FK-GlyCu_x with different ratios.



SCHEME 2: The active center for $O_2^{\cdot-}$ scavenging of FK-GlyM.

best. In four kinds of FK-GlyM, it turned out that the order of scavenging activity was FK-GlyCu₅ > FK-GlyMn₅ > FK-GlyNi₅ > FK-GlyZn₅. And FK-GlyCu₆₀ was proved to have the most outstanding anti- $O_2^{\cdot-}$ activity in all FK-GlyCu_x, of which EC₅₀ and degree of simulation separately reached $4.5 \times 10^{-3} \pm 0.0012 \mu\text{mol/L}$ and 911.1% compared with nature Cu, Zn-SOD. The reutilization of discarded feather keratin to prepare biopolymer antioxidant points out a new idea for both the problem of resources environment and the research on biopolymer antioxidant to us.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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