Binding Characteristics and Superimposed Antioxidant Properties of Caffeine Combined with Superoxide Dismutase

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ABSTRACT: The binding characteristics and superimposed antioxidant properties of caffeine combined with copper/zinc superoxide dismutase (SOD) were studied. The superimposed antioxidant activity of caffeine with SOD was investigated by detecting the concentration of malondialdehyde (MDA) present in cells, which was induced by hyperthermia and heavy metal exposure. The interactions between the SOD enzyme and caffeine were researched by ultraviolet spectrum, fluorescence spectrum, and molecular computation. The relative amounts of MDA contents of caffeine (0.1 mmol/L), SOD (0.1 mg/L), and caffeine (0.1 mmol/L) and SOD (0.1 mg/L) to water in cells were 0.70, 0.72, and 0.54, respectively, indicating that the antioxidant properties of caffeine combined with SOD may be superimposed. The fluorescence spectroscopy and molecular computation results show that the mixture of caffeine and SOD can result in the formation of a 1:1 complex through hydrogen bond and van der Waals forces spontaneously. The binding constant (Kb) of caffeine with SOD at five different temperatures are 4.41 × 10^4, 3.30 × 10^4, 2.29 × 10^4, 1.71 × 10^4, and 1.17 × 10^4 L/mol. The changes of Gibbs-free energy (ΔG) are −26.50, −26.21, −25.71, −25.12, and −24.29 KJ/mol and the ΔG of molecular docking calculation is −26.95 KJ/mol. The experimental results are in accordance with the results of theoretical calculations. The combination of caffeine with SOD can change the conformation and microenvironment of SOD but does not change the activity of SOD. In addition, the combination can superimpose the antioxidant activity of caffeine and SOD.

1. INTRODUCTION

Caffeine, which is a type of methyl xanthine alkaloid, can improve mood and combat fatigue, and it has been widely used in drugs, foods, and cosmetics.1 Caffeine can act directly on the human cerebral cortex, and it has many other effects on human physiology, such as exciting the central nervous system and vasomotor center,5 improving cognitive ability,6 and boosting circulation.5 Caffeine also affects the digestive, respiratory, and endocrine systems. Because caffeine has a long half-life and less-toxic side effects compared to theophylline and amphetamine,6 it is widely used. Therefore, the number of studies on caffeine have gradually increased over time.

Antioxidation is an important physiological function of caffeine.7,8 Many studies have shown that caffeine can scavenge free radicals3–14 and increase the activity of superoxide dismutase (SOD) in vivo.15,16 Caffeine can also stop the cross-linking of DNA and reduce oxidative stress.17,18 Many diseases are closely related to oxidative stress, antioxidation, and the metabolism of free radicals. Reactive oxygen species (ROS) are the most important free radicals that exist in organisms. ROS, including superoxide anion free radicals (O−2·), hydroxyl free radicals (·OH),19 hydrogen peroxide free radicals (HOO·), hydrogen peroxide (H2O2), lipid peroxide (LPO), and so on, are substances produced by organisms under normal and abnormal conditions. Oxygen free radicals are closely related to many diseases, such as antioxidant systems, such as glutathione peroxidise, SOD, and catalase have great effects on organisms. These oxidation systems are responsible for scavenging free radicals so that the structure and function of the cell membrane is protected.

SOD is the only enzyme that utilizes superoxide anion free radicals as a substrate;10,21 SOD plays an important role in the metabolism of ROS and can stop the damage caused by superoxide anion free radical. Caffeine is a good scavenger of ·OH and ·OCH3 but not superoxide anion free radical.12 However, Petrucci and Rita reported that caffeine might not be considered as an antioxidant.13 However, the combination of caffeine with SOD may change this result. Many experiments have shown that caffeine can enhance the oxidative activity of SOD and reduce oxidative stress in vivo.15,16,22–24 However, the interaction between caffeine and SOD at the molecular level has not been studied. The interactions between small
molecule drugs and proteins have been one of the most important research fields in molecular biology.25–32

In this paper, the antioxidant activities of caffeine, SOD, and caffeine combined with SOD were studied by pyrogallol autoxidation, and the concentration of malondialdehyde (MDA) present in cells was detected. The interaction between caffeine and SOD was investigated with ultraviolet spectroscopy, fluorescence spectroscopy, and molecular computation. The binding mechanism, binding constants, and binding sites were obtained. Molecular simulation was used to simulate the bonding between caffeine and SOD. Additionally, the effect of caffeine on the conformation of SOD was examined by fluorescence spectroscopy and molecular simulation. Furthermore, the effect of caffeine on the activity of SOD was also investigated. This work may provide useful information regarding the pharmacology of caffeine.

2. RESULTS AND DISCUSSION

2.1. Analysis of MDA Levels in Cell. MDA, which is the end-product of membrane lipid peroxidation, can be used as an indicator of membrane lipid peroxidation, and the concentration of MDA can indicate the degree of cell peroxidation.36 The concentration of MDA in cells changes because of hyperthermia and heavy metal exposure. To study the antioxidant properties of caffeine, the change in the MDA concentration in cells resulting from heating or heavy metal exposure was measured. The effects of different kinds of heavy metal ions (Ni^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}) on the concentration of MDA in cells were studied. Under the same conditions, Hg^{2+} causes the most serious damage to cells. Therefore, Hg^{2+} was selected to explore the antioxidant activity of MDA under the conditions caused by heavy metals. Because different batches of spinach leaves have different levels of MDA, the relative value is calculated using water as a reference. Figure 1 shows the relative amount of MDA after exposure to different concentrations of caffeine for 1 h.

Over a certain range, the relative amount of MDA decreased with increasing concentrations of caffeine. The best response of caffeine on MDA occurs at 0.08 mM, but when the concentration of caffeine is 0.1 mM, the concentrations of MDA tend to stabilize.

The amounts of MDA resulting from hyperthermia and heavy metal exposure in the presence of caffeine for different mixing times were examined, as shown in Figure 2. It can be seen from Figure 2A that the concentration of MDA decreased with increasing exposure times and reached the lowest concentration after 2 h. The amount of MDA of H_{2}O changed little (1, 1.02, 1.05, 1.04, 0.95, 0.94) relative to amount of MDA on adding caffeine (1, 0.86, 0.76, 0.68, 0.64, 0.76). As shown by Figure 2B, as the mixing time increased, the antioxidative effect of caffeine was obviously enhanced. The results show that caffeine can inhibit the production of free radicals and has a high antioxidant activity.

SOD is an antioxidant enzyme that scavenges and removes free radicals in organisms and protects organisms from damage caused by superoxide anion free radicals. Caffeine can inhibit hydroxyl and alkyl radicals. Therefore, SOD and caffeine were combined to determine the antioxidation effect, and the results are shown in Figure 3. The relative amount of MDA contents of caffeine, SOD, caffeine and SOD to water in cells were 0.70, 0.72, 0.54, respectively; 0.54 is much less than 0.70 or 0.72, and close to 0.504 (0.7 × 0.72), so the complexes of SOD with caffeine can maintain their respective antioxidant activity.

Determining whether caffeine and SOD can form a complex and whether the antioxidative activity of the complex can be superimposed is important. Next, the effect of caffeine on the antioxidation activity of SOD and the interaction between caffeine and SOD were investigated.

2.2. Effect of Caffeine on the Activity of Cu/Zn-SOD. The determination methods of SOD activity can be divided into direct and indirect methods. The direct determination method such as pulse radiolysis and stopped flow technique have high accuracy, but they need complex equipment and tedious experimental methods. Pyrogallol assay37 is an indirect method. It is simple and sensitive, and has been widely used. Since the establishment of the methodology in 1974, it has been cited more than 5000 times.38 Pyrogallol autoxidation was used to detect the antioxidation activity of SOD when it was mixed with different concentrations of caffeine. Figure 4a,b shows the inhibition of superoxide anion free radicals by caffeine and the caffeine and SOD mixture, respectively. The concentration of superoxide anions is the key to pyrogallol autoxidation. The changes shown in Figure 4 are not very obvious and show that caffeine had no significant effect on pyrogallol autoxidation, which indicates that caffeine could not scavenge the O-2^* free radicals. In addition, the result is
consistent with the literature report. Figure 4b also shows that the antioxidant effect of SOD mixed with caffeine does not change. The results show that the complexation of caffeine with SOD has no influence on the activity of SOD.

2.3. Ultraviolet Absorption Spectroscopic Studies.

The ultraviolet absorption spectra of proteins vary with the chromophore environment of proteins. The complexation of a protein with a small molecule will cause either a red shift or a blue shift in the absorption spectrum.32,39,40 A red shift at 223 nm is caused by a \( \pi - \pi^* \) transition of the peptide bond, which is related to the \( \alpha \)-helix of protein, and a blue shift is caused by a \( \pi - \pi^* \) transition. The UV absorption in the 250–280 nm range for the SOD solution occurs because of the absorption by tryptophan, phenylalanine, and tyrosine residues. Figure 5 shows the ultraviolet absorption spectra of the SOD solution with and without caffeine at 303 K. The absorption peak at 223 nm decreased after the red shift, whereas the peak at 275 nm increased. The results show that caffeine and SOD can form a complex, and the complexation can change the \( \alpha \)-helix of protein.

2.4. Effect of Caffeine on the Fluorescence of Cu/Zn-SOD.

2.4.1. Analysis of the Influence of Different Concentrations on Fluorescence Spectra.

Figure 5 shows the ultraviolet absorption spectra of the SOD solution with and without caffeine (pH = 8.2, \( T = 303 \) K). \( c(\text{SOD}) = 2.4 \times 10^{-3} \) g L\(^{-1} \); \( c(\text{caffeine})/(10^{-5} \) mol L\(^{-1} \)), 1–7: 0, 0.6, 1.1, 2.3, 3.4, 4.6, and 5.7, respectively.
spectroscopy is an important method used to study the interaction between proteins and small molecules.\textsuperscript{41,42} The fluorescence of proteins mainly derives from tryptophan and tyrosine residues.\textsuperscript{43,44} The fluorescence lifetime of SOD is related to its structure and microenvironment. Changes in the fluorescence lifetime of SOD mixed with different concentrations of caffeine at different temperatures are shown in Figure 6. The fluorescence lifetime of SOD increased with an increase in the concentration of caffeine. The results show that the interaction between caffeine and SOD can reduce the nonradiative transition and improve the efficiency of energy transfer.\textsuperscript{45}

At an excitation wavelength of 275 nm, a maximum emission peak was observed at 309 nm for the SOD solution.\textsuperscript{44} The maximum emission wavelength of most proteins is 280 nm. The maximum excitation wavelength of SOD is 275 nm, which is because compared with other proteins, there are fewer tryptophan residues in SOD. The influence of different caffeine concentrations on the fluorescence spectra of the SOD solution is shown in Figure 7. As the concentration of caffeine increased, the fluorescence intensity of the SOD solution decreased, and a shift was not observed. This is characteristic of endogenous fluorescence quenching.

2.4.2. Mechanism of SOD Fluorescence Quenching by Caffeine. Dynamic quenching and static quenching are two main theories used to explain the mechanism of fluorescence quenching. The changes of fluorescence quenching parameters between proteins and small molecules at different temperatures can be used to distinguish between dynamic quenching and static quenching.\textsuperscript{39} Dynamic quenching is a process with shortened excited state lifetimes, which should follow the Stern–Volmer equation.\textsuperscript{32,39}

\[ F_0/F = 1 + K_{SV}[Q] \]  

\[ F_0 \]—the steady-state fluorescence intensities without quencher;  
\[ F \]—the steady-state fluorescence intensities with quencher;  
\[ K_{SV} \]—the Stern–Volmer quenching constant;  
\[ [Q] \]—the concentration of the quencher; if a protein forms a complex with small molecules through noncovalent bonds, static quenching will occur. The binding constant \( K_A \) (L/mol) and the binding sites \( n \) can be calculated from eq 2.

\[ 1/(F_0 - F) = 1/F_0 + 1/K_A [Q] \]  

(2)

When the binding constants of caffeine and SOD at different temperatures are calculated, the thermodynamic parameters at different temperatures can be calculated by thermodynamic formulae.

Figure 8 shows the Stern–Volmer plot of SOD by caffeine at different temperatures. The slope decreased as the temperature increased. The results indicate that fluorescence quenching of SOD by caffeine decreased as the temperature increased, which is consistent with static quenching. Because noncovalent bonds are sensitive to temperature, the increase of the temperature is not conducive to the complex stability.

Figure 9 shows the double reversal diagram of the fluorescence quenching of SOD with caffeine at different concentrations and different temperatures. According to the slope and intercept of the standard equation, the binding sites...
and the binding constant for the complex of SOD and caffeine were obtained, as shown in Table 1. It can be seen from Figure 9 and Table 1 that, when the temperature is either 298, 303, 308, 310, or 312 K, the linear correlation of 1/(F0 − F) to 1/[Q] is good, and the correlation coefficient R is greater than 0.99. The SOD and caffeine binding sites are close to 1, which indicates that SOD and caffeine form complexes at stoichiometric ratios of 1:1 and also that the temperature has little effect on the binding ratio between SOD and caffeine. These results also show that the fluorescence quenching of caffeine with SOD is static.

2.4.3. Analysis of Binding Modes and Thermodynamic Parameters. Hydrophobic effect, hydrogen bond, van der Waals force, and electrostatic force are the main forces existing between small molecules and macromolecules.46 Ross reported that, when the change in the enthalpy and entropy of a system is greater than zero (ΔH > 0 and ΔS > 0), the main force between proteins and small molecules forming a complex is caused by hydrophobic effects; when ΔH < 0 and ΔS < 0, hydrogen bonds and van der Waals forces are the main forces that cause the formation of small molecule and protein complexes; when ΔH < 0 and ΔS > 0, electrostatic forces are the main force promoting the formation of a complex.47,48 Whereas the binding constants of SOD and caffeine at different temperatures were obtained, the thermodynamic parameters under the corresponding temperature conditions can be obtained. The results are shown in Table 1.

\[ \ln(K_{A2}/K_{A1}) = \Delta H(1/T_1 - 1/T_2)/R \]  \tag{5}

\[ \Delta S = (\Delta H - \Delta G)/T \]  \tag{6}

As shown in Table 1, the change in the enthalpy and entropy of the system is less than zero. Therefore, hydrogen bond and van der Waals force play the main role in the interaction between SOD and caffeine. In addition, ΔG < 0, which indicates that the complexation of caffeine and SOD is spontaneous. These results show that SOD complexes easily with caffeine, and high temperatures are not conducive to the formation of the caffeine–SOD complex.

2.5. Computational Modeling Studies. A caffeine molecule was positioned in the binding site, as shown in Figure 10, and the binding site was located at the largest cavity in the protein structure. Because of their remote distances, the metal sites were not affected, and therefore, the activity of Cu/Zn-SOD complexed with the caffeine molecule changed little, as exhibited by the previous experiment. The optimized result was chosen as the model. The calculated interaction energy was −26.9489 kJ/mol, which also confirmed the previous experiment. The goal was to bind a small molecule to SOD to form a complex.

Figure 10 shows the solid surface of the hydrogen bond interaction between caffeine and Cu/Zn-SOD. A small molecule was bound to the cavity of Cu/Zn-SOD, and interactions, such as conventional hydrogen bonds, carbon hydrogen bonds, and alkyl and π–alkyl interactions, occurred between the amino acid residues and the caffeine molecule. In the caffeine–SOD complex, hydrogen bonds were formed because of interactions between caffeine and the residues in two protein subdomains via Val7. A, Asn51. A, Lys9. B, Asn51. B, and Val146 B. Carbon hydrogen bonds were formed with Cys144. A, Gly145. B, Val7. B, Asn51. A, and Lys9. B. π–alkyl interactions were formed with Val146. A, Lys9. A, and Val7. B. The caffeine–SOD complex is illustrated in Figure 10. The abovementioned interactions lowered the energy of the complex.

3. RESULTS

In conclusion, the antioxidant activities of caffeine, SOD, and caffeine combined with SOD were studied by pyrogallol autoxidation and detecting the concentration of MDA present in cells. The experimental results show that caffeine has a good inhibitory effect on MDA content under the action of heating and heavy metals, but caffeine cannot eliminate superoxide radicals. The complexation of SOD with caffeine causes an superimposed effect on its antioxidant ability without changing the activity of Cu/Zn-SOD. The interaction between SOD and caffeine was studied by fluorescence spectroscopy, UV absorption spectroscopy, and molecular modelling. The UV–vis absorption spectroscopy shows that caffeine can cause the red shift and decrease of UV-absorption of SOD, suggesting

![Figure 9. Lineweaver–Burk plots for the quenching of Cu/Zn-SOD by caffeine at 298, 303, 308, 310, and 312 K (pH = 8.2).](image-url)
that the complexation of SOD with caffeine can change the secondary structure of SOD. The fluorescence spectroscopy shows that the fluorescence quenching mechanism of SOD combined with caffeine is static. The binding constants, binding sites, and thermodynamic parameters of the complexation of SOD with caffeine at five different temperatures indicate that SOD and caffeine can form a 1:1 complex through the spontaneous formation of hydrogen bonds and van der Waals force. The experimental results are in accordance with the results of theoretical calculations. UV–vis spectroscopy and fluorescence spectroscopy suggest that the complexation of SOD with caffeine can change the conformation and microenvironment of SOD. The pyrogallol autoxidation and molecular modeling show that the complexation of SOD with caffeine has no effect on the active center of SOD. All the results show that the complexation of SOD with caffeine did not affect the SOD active sites and that the antioxidant ability is superimposed. All the abovementioned results indicate that SOD can be used as an effective carrier of caffeine.

4. MATERIALS AND METHODS

4.1. Reagents. Cu/Zn-SOD was purchased from Doulai Biotechnology Co., Ltd. Caffeine (98% purity) was purchased from Tokyo Chemical Industry. Cu/Zn-SOD was diluted with distilled water to obtain a 4 mg/mL solution, and the resulting solution was stored at 4 °C. Caéine was dissolved with distilled water to prepare a 2 mmol/L solution. A 0.05 M Tris-HCl buffer solution with pH = 8.2 was prepared. All other reagents are analytically pure.

4.2. Measurements of the MDA Content. MDA produced by peroxidation is the most common indicator of lipid peroxidation. The improved thiobarbituric acid (TBA)–MDA assay can satisfactorily detect the concentration of MDA. Under acidic conditions or at high temperatures, MDA reacts with TBA to form 3,5,5-trimethyl oxazole-2,4-ketone 3,5,5-trimethylloxazolidine-2,4-dione, which has a maximum absorption at 532 nm and a minimum absorption at 600 nm. A saccharide and RBA complex also has a minimum absorption at 532 nm and a maximum absorption at 450 nm. To eliminate the interference of the saccharide complex, dual-wavelength spectrophotometry was applied. The concentration of MDA was calculated by formulas 7 and 8.

\[ C_1 \text{ (mmol/L)} = 11.71A_{450} \]  

\[ C_2 \text{ (μmol/L)} = 6.45(A_{532} - A_{600}) - 0.56A_{450} \]  

C₁— the concentration of the carbohydrate; C₂— the concentration of MDA; A_{450}— the absorbance values at 450 nm; A_{532}— the absorbance values at 532 nm; A_{600}— the absorbance values 600 nm.

The same part of spinach leaves without stems were suspended in 5 mL of 10% trichloroacetic acid, and the resulting mixture was mixed with different concentrations of caffeine, SOD, metal ions, SOD combined with caffeine, and SOD combined with metal ions. Then, to the mixture, 0.6% thiobarbituric acid (5 mL) was added. The homogenates without metal ions were heated at 373 K for 15 min, cooled quickly, and centrifuged at 8000 rpm for 20 min. The homogenates with metal ions were centrifuged without heating. The absorbances of all assay mixtures were recorded at 450, 532, and 600 nm.

4.3. Determination of the Cu/Zn-SOD Activity. A pyrogallol autoxidation method was chosen to analyze the antioxidant activity of Cu/Zn-SOD. Solutions (3.5 mL) containing SOD and caffeine (with concentrations ranging from 0 to 1 μM) were prepared using a 0.05 M Tris-HCl buffer with pH = 8.2.; before the test, 0.5 μM pyrogallol was rapidly added to the solutions containing SOD and caffeine. A caffeine solution prepared with the buffer at the same concentration was used as the blank control. The absorption was measured as a function of time at 325 nm and 303 K.

4.4. Ultraviolet Absorption Measurements. At 25 °C, the ultraviolet absorption spectra of Cu/Zn-SOD with and without caffeine were obtained from 190 to 400 nm using an 8000 A spectrophotometer which was made by Beijing Purkinje General Corporation. The mixtures of Cu/Zn-SOD with different concentrations of caffeine in 3.5 mL Tris-HCl (0.05 M) buffer at pH = 8.2 was equilibrated for 20 min before their use. Using the solution of caffeine and buffer at the same concentration as reference to reduce the impacts of caffeine.

4.5. Fluorescence Measurements. A FluoroMax-4 fluorescence spectrophotometer (Horibajy, France) equipped with a 1 cm quartz cell and a thermostat bath was applied to detect the fluorescence spectrometry. The caffeine and SOD solutions were mixed and stored at a constant temperature for 30 minutes prior to their use. Furthermore, the Tris-HCl (0.05 M) buffer at pH = 8.2 was also used in fluorescence measurements. The excitation and emission slits were fixed at 10 nm, and the excitation wavelength was 275 nm. The fluorescence lifetimes were measured by the time-correlated single-photon counting method. The emission spectra were collected from 290 to 400 nm and recorded at 298, 303, 308, 310, and 312 K.

4.6. Molecular Modeling Study. Receptor—ligand interaction of caffeine with Cu/Zn-SOD was carried out via Discovery Studio 2018 soft platform (DS 2018). The software also specializes in molecular docking, including calculation about molecular interaction between macromolecules and small molecules. The structure of caffeine was drawn, prepared, and minimized to give two conformations with small molecule tools of DS 2018 as optimized ligand. Bovine SOD

Figure 10. Surface and cartoon representations of caffeine in SOD.
was chosen as protein model (PDB ID: 1CBJ). By macromolecules tools, all water molecules of the SOD were removed, hydrogens were added, and amino acid residues were completed. Receptor–ligand tools (CDDOCKER) were used to analyze the interaction of caffeine and macromolecule, with CHARMM force field, 10 orientations to refine. Other parameters were set as default.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

SOD, superoxide dismutase; Cu/Zn-SOD, copper/zinc superoxide dismutase; MDA, malondialdehyde; Val, valine; Lys, lysine; Asn, asparagine; Cys, cysteine; TBA, thio-barbituric acid

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