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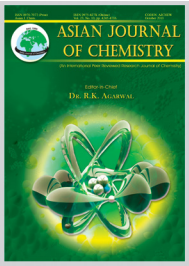

Preparation and Coordination Characterization of Glutathione-Zn^{2+} Complex

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Glutathione (GSH)-Zn^{2+} complex was prepared using a dialysis-lyophilization method, the coordination ratio of the prepared complex was determined to be: GSH:Zn^{2+} ≈ 2:1. IR, ^{1}H NMR and atomic force microscope tests indicated that the binding sites between glutathione and Zn^{2+} are the carbonyl oxygen in the amide group (Cys residue) and sulfur atom in the thiol groups. A coordination structure of the complex was proposed according to the coordination ratio and the bonding sites of glutathione. Further investigation demonstrated that the capability of GSH-Zn^{2+} complex to restrain hydroxyl and super-oxide radicals is stronger than that of free glutathione molecule.

Key Words: Glutathione, GSH-Zn^{2+} complex, Coordination mechanism, Antioxidation.

INTRODUCTION

Glutathione (GSH) is a non-protein tripeptide of the sequence γ-Glu-Cys-Gly and the most abundant non-protein thiol compound and major antioxidant in living plants or animal cells. Glutathione participates in many significant biological processes[1,2] and exhibit remarkable biomedical functions such as detoxicate xenobiotics and heave metals[3], maintaining the integrity of red blood cell (RBC) membrane, protecting the function of mitochondria and kidney,[4-6] deactivating free radicals and other poisons, controlling metal content and metallothionein and participating in the synthesis of DNA, RNA, protein and other macromolecules[7,8].

Glutathione molecules have multiple potential binding sites that can coordinate with metals. It has been reported that glutathione forms complexes with various metals and in particular its thiolate sulfur atom has a high affinity for soft metals. The investigation of glutathione-metal coordination mechanism is very essential since it can yield basic information on the function of glutathione and metal ions in many biological processes. The importance of this issue has triggered numerous metal binding studies of glutathione to cadmium, zinc, copper, lead, mercury and nickel, *etc*.[9-13] Among all these metals, Zn^{2+} has a high affinity with glutathione and the complexation of Zn^{2+} with glutathione is very important in the biological chemistry of Zn^{2+}. It has been determined by ^{1}H NMR that Zn^{2+} is coordinated by glutathione in intact human erythrocytes[14]. The study by Mills *et al*.[15] indicated that the decrease in blood glutathione levels was a specific result of zinc deficiency. Tang *et al*.[16] found that glutathione plays a role in the protection of zinc against Cd metallothionein (CdMT) nephrotoxicity. Accordingly, the binding of glutathione with Zn has been studied earlier also. Various pH-dependent zinc complex species of glutathione were observed, but none was isolated. Fuhr *et al*.[17] investigated the coordination mechanism between Zn^{2+} and glutathione in D_2O solution using ^{13}C NMR, they concluded that Zn^{2+} can coordinate with the thiol group or glutamyl amino group depending on the pH value of the solutions. The followed studies confirmed the involvement of the thiol function in Zn^{2+} coordination in the entire pH range of the binding, the sulfur is accompanied by nitrogen and oxygen atoms in the peptide bonds depending on pH and molar ratio[18-21]. While the nitrogen atoms in the amino group rarely participate in metal coordination due to the spatial constraints, the nitrogen atoms in the peptide bonds only coordinate with metal at high pH, where they deprotonate in aqueous solution. Diaz-Cruz *et al*.[22] studied the complexation between Zn^{2+} and glutathione using differential pulse polarography (DPP) in a borate buffer medium at a fixed pH and variable GSH-Zn^{2+} concentration ratios. A structure model for a 2:2 GSH:Zn^{2+} complex was proposed. Isothermal titration calorimetry was employed by Chekmeneva *et al*.[23] to investigate the binding of Zn^{2+} by glutathione. With different buffers and titration modes, different stoichiometries between glutathione and Zn^{2+} from 0.5 to 2 were obtained. Therefore, the complexation model between glutathione and Zn^{2+} is still lack of agreement due to the multiple binding sites in glutathione.

In this study, a dialysis-lyophilization method was used to prepare GSH-Zn^{2+} complex. Before, the common method to study GSH-Zn^{2+} complex is to simply dissolve Zn^{2+} into

glutathione solution with a fixed metal/glutathione ratio, which is easy and convenient. However, the uncoordinated Zn^{2+} in the system could affect the stability of the complex. In the dialysis-lyophilization method, excess Zn^{2+} solution is mixed with glutathione solution in a dialysis bag of a specific molecular weight cut-off (MWCO), the dialysis bag is put into a pool of circulating pure water. The uncoordinated Zn^{2+} can pass through the dialysis membrane into the pure water. By the method, one can finally acquire a stable complex solution. The prepared GSH-Zn^{2+} complex are very suitable for physical and chemical studies. Moreover, the coordination ratio can be determined from the amount of Zn^{2+} and glutathione molecules left in the dialysis bag before lyophilization.

The aim of this paper is to study the coordination mechanism of GSH-Zn^{2+} complex prepared using the dialysis-lyophilization method. IR analysis, ^{1}H NMR and AFM were used to clarify the binding sites of glutathione with Zn^{2+}. A possible coordination structure was finally proposed according to the coordination ratio and binding sites. At the final section, the antioxidation capability of GSH-Zn^{2+} complex was investigated and compared with that of free glutathione.

EXPERIMENTAL

Reduced glutathione (biochemical reagent grade) was purchased from Shanghai Qiude Biochemical Engineering Co. (Shanghai, China). $ZnSO_4 \cdot 7H_2O$ (analytical reagent grade) was purchased from Beibei Chemical Reagent Plant (Chongqing, China). Reagent boxes for all the antioxidation tests were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China).

Preparation of GSH-Zn^{2+} complex: $ZnSO_4 \cdot 7H_2O$ was first dissolved in double deionized water and then a specific amount of reduced glutathione was added. The solution is thoroughly stirred until $ZnSO_4$ and glutathione are totally dissolved. After filtered using a membrane filter (pore size, 0.22 μm), 20 mL solution was filled into a dialysis bag with molecular weight cut-off of 100 Da. The dialysis bag was then put into a tank containing 2000 mL double deionized water and dialysed under stirring with a magnetic stirrer (Fig. 1). The liquid outside the dialysis bag was regularly sampled to measure Zn^{2+} concentration and pH. According to the Zn^{2+} concentration outside the bag, the dialysed quantity of Zn^{2+} was calculated and plotted versus time. After the dialysis reached equilibrium, the liquid inside the dialysis bag was put into a lyophilization bottles at -55 ºC to prepare the GSH-Zn^{2+} complex. The bottles containing the complex were then sealed with aluminum covers and stored in a freezer at 4 ºC before being used.

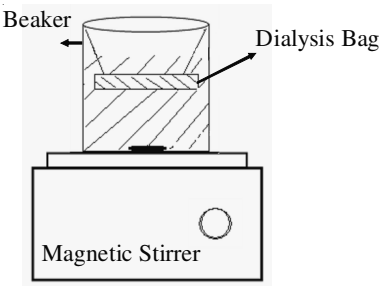

Fig. 1. Scheme of the dialysis set-up to prepare GSH-Zn^{2+} complex

Complex characterization

IR analysis: The IR spectrum of he GSH-Zn^{2+} complex was recorded on a MAGMA-IR550 spectrometer (Nicolet Company, USA) using KBr pressing pellet method.

^{1}H NMR experiments: ^{1}H NMR spectrum of GSH-Zn^{2+} complex dissolved in $CDCl_3$ was analyzed using a Bruker AV50 NMR spectrometer (Switzerland).

Atomic force microscopic morphology imaging of GSH-Zn^{2+} complex: GSH-Zn^{2+} solution in double deionized water was carefully spotted onto a clean super-flat gold foil and dried at 60 ºC, then tested using an IPC-208B Atomic Force Microscope (IPC-208B, Hengrui Company, Chongqing, China).

Measurement of Zn^{2+} concentration: Zn^{2+} concentration was measured using an atomic absorption spectrophotometer (Z-8000 Atomic Absorption Spectrophotometer, Hitachi Co.Ltd., Japan).[24]

Determination of antioxidation capability

Determination of the capability to restrain superoxide radicals: The capability for the GSH-Zn^{2+} complex to restrain superoxide radicals was measured using reagent boxes. The nitroblue tetrazolium (NBT) in the boxes can be reduced to an unsolvable dark-blue substance by the superoxide radicals. Therefore, the quantity of the superoxide radicals can be measured using colourimetric method. The capability for the samples to restrain superoxide radicals can be determined by comparing the quantity of superoxide radicals before and after adding the testing samples. The testing procedures are: the capability for the dilute solution of pig blood plasma to restrain superoxide radicals was first measured using the reagent boxes. Then, 3 mL dilute solution of pig blood plasma was mixed with 0.5 mL GSH-Zn^{2+} complex solution of 10 mmol/L, stirred thoroughly using a vortex agitator. The capability for the mixture to restrain superoxide radicals were measured and the capability of GSH-Zn^{2+} complex to restrain superoxide radicals were determined by comparing the capability of the dilute solution of pig blood plasma with that of the mixtures. The purpose to use the solution of pig blood plasma is to test the function of glutathione in a biological environment.

Determination of the capability to restrain free hydroxyl radicals: The procedures to determine the capability for the complex to restrain hydroxyl radicals are same as that to measure the capability to restrain superoxide radicals except using different reagent boxes. The mechanism for the reagent is the salicylic acid in the boxes can react with hydroxyl radicals to generate 2,3-dihydroxy benzoic acid, whose quantity can be measured using colourimetric method.

Determination of the total anti-oxidation capability: The total antioxidation of GSH-Zn^{2+} was also determined using reagent boxes. The Fe^{3+} ions in the boxes can be reduced to Fe^{2+} by the antioxidation groups in the testing samples and the Fe^{2+} can form stable complexes with Felons. Therefore, one can use colourimetric method to measure the quantity of Fe^{2+}, thus the antioxidation capability of the testing samples.

Preparation of dilute solution of pig blood plasma: Alsever antifreezing liquid of equal volume was added into fresh pig blood collected in a beaker and thoroughly mixed.

The liquid mixture was then centrifugalized for 10 min at 4000 rpm and the solution of pig blood plasma was then obtained by collecting the supernatant. The collected pig blood plasma solution was then diluted to 10 % of the original concentration using double distillated water and used in the study. It has to be noted that the solution must be fresh and be prepared just before it would be used.

RESULTS AND DISCUSSION

Dialysis curve of GSH-Zn^{2+} complex: The molecular structure of the reduced glutathione is shown in Fig. 2, which has eight potential binding sites that can coordinate with Zn^{2+}: *i.e*. three nitrogen atoms in amino and amide groups, four carbonyl oxygen atoms in amide and carboxylic groups and a sulfur atom in the thiol group.

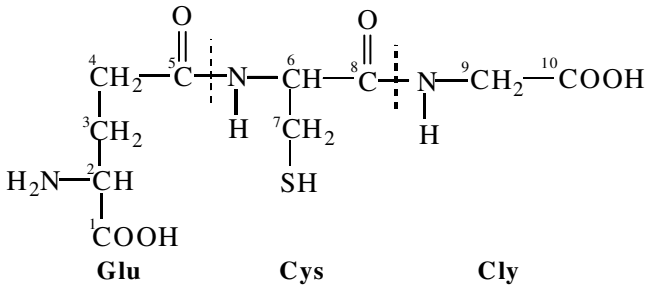

Fig. 2. Molecular structure of the reduced glutathione

In this study, a dialysis-lyophilization method was used to prepare GSH-Zn^{2+} complex with the dialysis set-up as Fig. 2. The molecular weight cut-off of the dialysis membrane is 100, glutathione, whose molecular weight is 307, can not permeate the dialysis membrane. Although free Zn^{2+} ions (m.w. = 64) can freely permeate the membrane, Zn^{2+} ions that form stable coordination complex with glutathione can not pass through the membrane. Thus, dialysis experiment can shed light on the coordination interaction between glutathione and Zn^{2+}. Fig. 3a shows the dialysis curve of 20 mL $ZnSO_4$ solution that contains 4 mmol Zn^{2+} ions (0.2 M). Since the speed for SO_4^{2-} ions (m.w. = 96 Da) to pass through the membrane is much slower than that of Zn^{2+}, more Zn^{2+} ions dialyses into outer liquid than SO_4^{2-} during the dialysis process. Consequently, the pH of the inner liquid decrease gradually and the pH of the outer liquid increases gradually, leading to the formation of $Zn(OH)_2$ precipitation in the outer liquid, when the Zn^{2+} dialysis reaching equilibrium after 28 h, the concentration of Zn^{2+} of both the inner and outer liquid is 1.74 mmol/L with 3.965 mmol Zn^{2+} ions dialysed outside the dialysis bag. However, at this stage, the SO_4^{2-} has not reached the equilibrium and pH of the inner liquid is lower than that of the outer liquid.

Fig. 3b shows the dialysis curve of the mixed solution that originally contains 0.2 mmol glutathione and 4 mmol $ZnSO_4$. Three equilibrium states appears on the dialysis curve at the range of 36-48 h, 76-106 h and 126-144 h, respectively. Since pH of the inner liquid decreases during the dialysis process, the three equilibrium states correspond to three different binding situations between glutathione and Zn^{2+} at different pH, which confirmed the previous reports that the coordination sites involved in the binding between glutathione

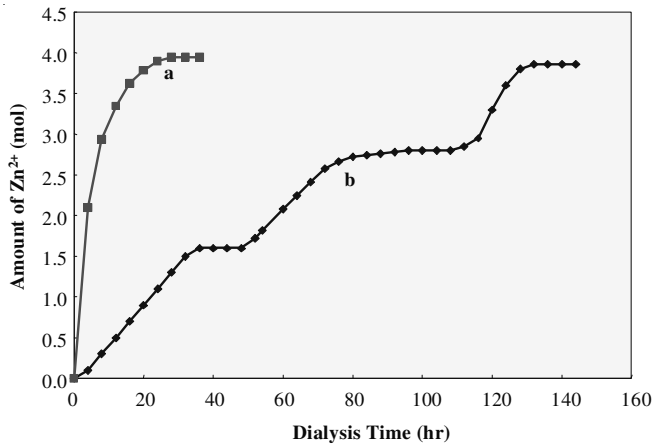

Fig. 3. Dialysis curves of (a) $ZnSO_4$ solution and (b) GSH + $ZnSO_4$ Solution

and Zn^{2+} is dependent on pH. In this study, the inner liquid after 140 h dialysis, when the pH of the inner liquid is 5.3, was lyophilized to prepare the final GSH-Zn^{2+} complex, whose coordination mechanism was discussed in the following sections. It is believed that the prepared complex is the most stable one among the three found in the dialysis process.

After 140 h dialysis, total amount of 3.86 mmol Zn^{2+} ions (including free Zn^{2+} and $Zn(OH)_2$ precipitation) dialysed from inner fluid into outer liquid. Among the 0.14 mmol Zn^{2+} ions left inside the dialysis membrane, free Zn^{2+} is about 0.036 mmol for the equilibrium concentration of free Zn^{2+} in both the inner liquid and the outer liquid is 1.81 mmol/L. Thus, the amount of the coordinated Zn^{2+} ions is 0.104 mmol and the coordination ratio of the final coordination complex is: GSH:Zn^{2+} = 0.2:0.106-2:1 (mol/mol).

IR characterization of GSH-Zn^{2+} complex: In this study, we focused on the light adsorption peaks of amino group, thiol group and amide groups since these groups contain the atoms that possible coordinate with glutathione. Fig. 4 compared the IR spectrum of free glutathione with that of GSH-Zn^{2+} complex. First, the peak at 2524 cm^{-1} corresponding to the thiol group ν(SH) in glutathione spectrum shifts to 2553 cm^{-1} with much weaker intensity in the spectrum of GSH-Zn^{2+}. In the spectrum of glutathione, there are two adsorption peaks at 1659 cm^{-1} and 1631 cm^{-1} corresponding to the amide groups, which become

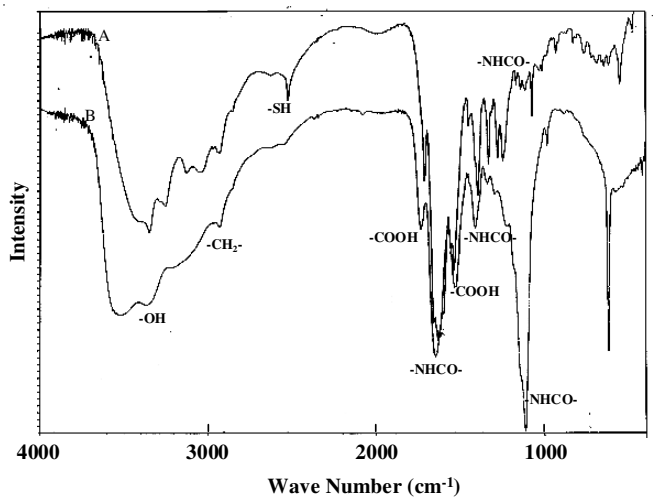

Fig. 4. IR analysis of glutathione (A) and GSH-Zn2+ complex (B)

one in GSH-Zn^{2+} spectrum at 1647 cm^{-1}. Furthermore, the peaks at 1115 and 1074 cm^{-1} in glutathione spectrum corresponding to amide groups merge into a very strong peak at 1113 cm^{-1}, while the peak at 1396 cm^{-1} and 1334 cm^{-1} belong to amide groups shift to 1412 cm^{-1} and 1340 cm^{-1} respectively with weaker intensity. Since both thiol group and amide groups do not carry charge at acidic conditions, the binding between them with Zn^{2+} can only be coordination bonds. The comparison between two spectra also shows that the peak ν(C=O) corresponding to carboxylic groups at 1712 cm^{-1} shifts to 1753 cm^{-1} and peaks corresponding to ν_{as}(COO) at 1557 cm^{-1} and δ_s (COO) at 1631 cm^{-1} all disappeared in the spectrum of GSH-Zn^{2+}, which indicated that Zn^{2+} also forms bonds with glutathione at carboxylic groups. Moreover, the IR spectrum does not show evidence that glutathione forms bonds with Zn^{2+} at the amino groups.

^{1}H NMR spectra of GSH-Zn^{2+} complex: ^{1}H NMR has been proven to be a powerful technique for studying the binding of metal ions with biological molecules. From ^{1}H NMR spectrum, one can analyze the binding information for the atoms or atom groups that connect with hydrogen atoms. Fig. 5 compared the ^{1}H NMR spectrum of glutathione with that of GSH-Zn^{2+}. Both spectra have 6 adsorption peaks corresponding to 6 groups of hydrogen atoms connected to carbon atoms. The most obvious difference between two spectra is the shift of the peaks corresponding to C_6 hydrogen (Cys H_α), the others are the shift and intensity attenuation of peaks corresponding to C_2 hydrogen (Glu H_α) and intensity attenuation of peaks corresponding to C_7 hydrogen of (Cys H_β). Since carbons cannot form bonds with Zn^{2+}, the changes of C_6 hydrogen indicates the possible coordination bonding between carbonyl oxygen or the imino nitrogen in the two amide groups with Zn^{2+}, the change of C_7 hydrogen indicates the possible coordination bonding of sulfur atom in the thiol group with Zn^{2+}. While the change of C_2 hydrogen indicates the possible coordination binding between amino nitrogen of Glu residue or the electrostatic bonding between carboxyl group with Zn^{2+}. Among the several possible binding sites, it is very difficult for the imino nitrogen of Cys residue to coordinate with Zn^{2+} due to the steric hindrance of carboxyl oxygens and coplanar hydrogen atoms[21], only possible coordination sites are the carbonyl atoms (Cys residue) in the amide groups and sulfur atoms in the thiol groups.

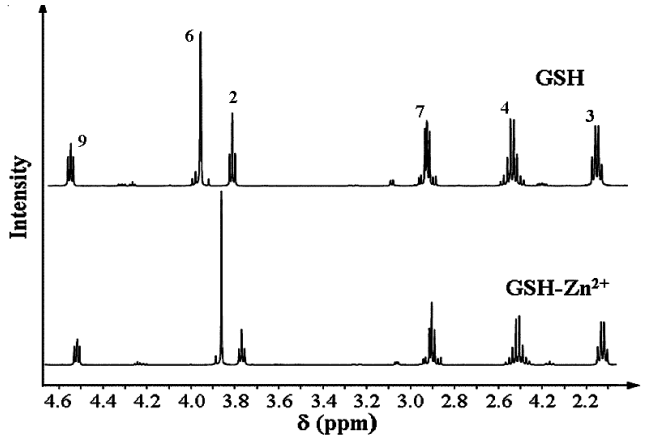

Fig. 5. ^{1}H NMR spectra of glutathione and GSH-Zn2+. The peak numbers are corresponding to the sequence of the carbon atoms in Fig. 2

Atomic force microscopic observation of GSH-Zn^{2+} complex: Fig. 6 shows AFM images of glutathione (Fig. 6a) and GSH-Zn^{2+} complex (Fig. 6b). Compared with that of glutathione, the morphological image of GSH-Zn^{2+} exhibits a large amount of ring structures and these ring structures are mainly six-membered or seven-membered rings. It is known from the previous IR and NMR analysis that the carbonyl oxygen atoms (Cys residue) and sulfur atoms in the thiol group are the possible coordination sites between Zn^{2+} and glutathione. Also considering that the coordination ratio between glutathione and Zn^{2+} is 2:1, the coordination mechanism of GSH-Zn^{2+} complex is proposed as Fig. 7.

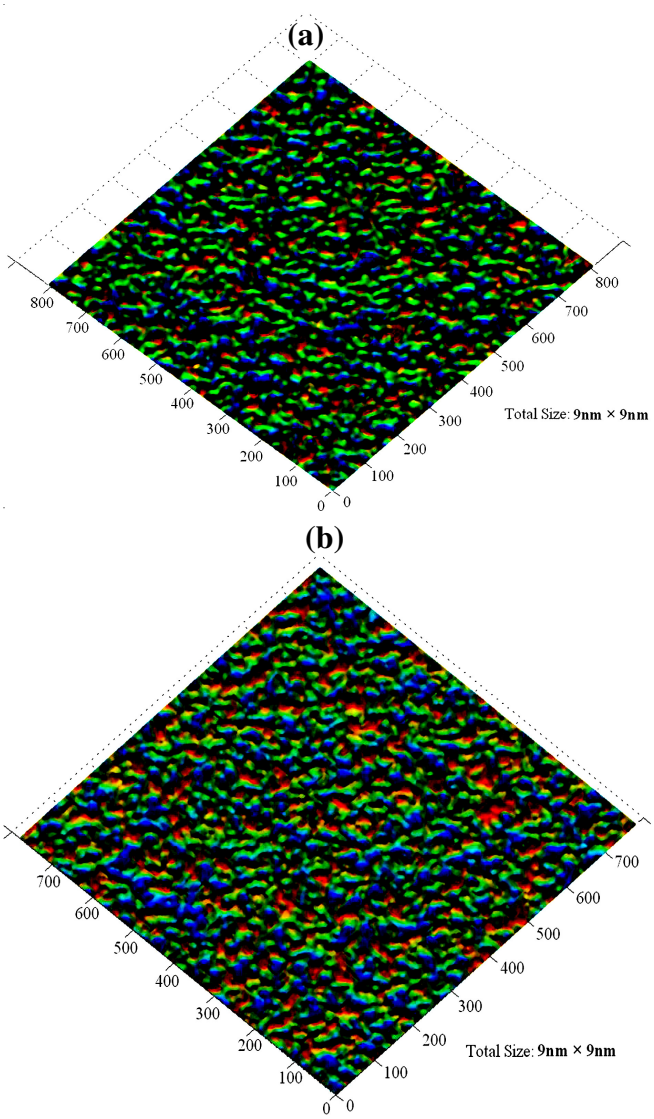

Fig. 6. AFM image of glutathione (a) and GSH-Zn^{2+} (b)

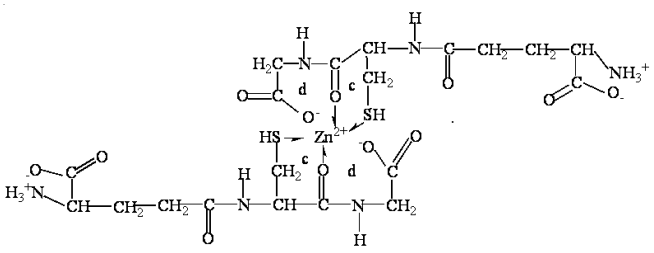

Fig. 7. Proposed structure of the GSH-Zn^{2+}

In Fig. 7, a single six-membered ring (c) was formed when the carbonyl oxygen and sulfur atom in a glutathione molecule coordinate with a Zn^{2+} ions, while a double six-membered ring was formed when the carbonyl oxygen and sulfur atoms in two glutathione molecules coordinate with a Zn^{2+} ions. In order to neutralize the positive charge of Zn^{2+}, the deprotoned C_{10} carboxylic group may form ionic bonds with Zn^{2+}, forming a seven-membered ring.

Antioxidation capability of GSH-Zn^{2+} complex: In biological systems, oxidation and reduction reactions frequently occur and the main oxidative agents are active oxygen atoms and radicals; the latter includes hydroxyl and superoxide radicals. Glutathione molecules have very good antioxidation function due to the ability of thiol groups to participate in the oxidation and reduction reactions, which enables glutathione molecules to take functions in oxidation and reduction reactions as both hydrogen donor and/or acceptor.

In this study, we compared the total antioxidation capability of GSH-Zn^{2+} complex with that of free glutathione. The results in Table-1 show that the total anti-oxidation capability of GSH-Zn^{2+} complex is about one eighth of that of free glutathione due to the participation of the active thiol group in the coordination with Zn^{2+}. However, Table-1 showed that GSH-Zn^{2+} has better restraining capability towards hydroxyl and super-oxide radicals than free glutathione, whose mechanism is worthy of special investigation, although free Zn^{2+} ions induce hydroxyl and super-oxide radicals indicated by the negative numbers.

TABLE-1
ANTIOXIDATION CAPABILITY OF GLUTATHIONE AND GSH-Zn^{2+} COMPLEX

Functions (U/mL)	Zn^{2+}	Glutathione	GSH-Zn^{2+}
Hydroxyl radical inhibited	-32.89	34.55	49.21
Superoxide radical inhibited	-32.58	105.28	155.73
Total antioxidation	-32.07	3169.7	386.04

Conclusion

GSH-Zn^{2+} complex were prepared using a dialysis-lyophilization method. Three equilibrium states appear in the dialysis curve, which indicates three different binding situations between glutathione and Zn^{2+}. This result agrees with the previous studies that the coordination chemistry between glutathione and Zn^{2+} is dependent on pH. In this study, the GSH-Zn^{2+} complex were prepared at the third equilibrium state and the coordination ratio of the prepared complex was determined to be: GSH:Zn^{2+}-2:1. The binding sites between glutathione and Zn^{2+} were demonstrated to be the carbonyl oxygen in the amide group (Cys residue) and sulfur atom in the thiol group according to the results of IR, ^{1}H NMR and AFM. Accordingly, a coordination structure was proposed based on the coordination ratio and sites. At the final section of the paper, the antioxidation capability of the prepared GSH-Zn^{2+} complex was studied and compared with that of glutathione. The result showed that the total anti-oxidation capability of GSH-Zn^{2+} is weaker, whereas its capability to restrain hydroxyl and super-oxide radicals is stronger than that of free glutathione molecules.

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