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ORIGINAL PAPER

Preparation, characterization, and antibacterial activity evaluation of collagen–Zn complex

Huiyun Liu · Shizheng Wang · Yu Xi · Pu Liu

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Abstract The different kinds of collagen–Zn complexes were prepared by zinc acetate, zinc chloride, zinc nitrate, and zinc sulfate reacted with collagen protein. Their antibacterial activities have been investigated by MIC method. It was found that the antibacterial activity of collagen–$ZnSO_4$ complex is better than that of others. To obtain a better antibacterial activity, collagen–$ZnSO_4$ complexes with different zinc amount were prepared using zinc sulfate as starting material. These complexes were characterized by FT-IR, XRD, and atomic absorption spectrometry. The results showed that zinc ion could chelate with N–H, C–O, and C=O group in collagen to form the stable complex. Antibacterial activities of collagen–$ZnSO_4$ complexes containing different Zn amount were evaluated against *Escherichia coli* and *Staphylococcus aureus*. The results suggested that antibacterial activity increases with the increase of zinc amount.

Keywords Collagen protein · Complex · Characterization · Antibacterial activity

Introduction

Collagen (CG) is the most abundant protein in animal organisms, where it provides the principal structural and mechanical support. It exists in various forms from skin, tendon and bone to cornea and basement membrane of the capillaries [1]. Over the past several decades, 19 different collagens have been identified, although the major types are the fibrous types I, II, and III and the non-fibrous type IV of basement

H. Liu · P. Liu (✉)
Department of Chemistry, Zhengzhou University, Zhengzhou 450001, China
e-mail: liupu@zzu.edu.cn

S. Wang · Y. Xi
Department of Bioengineering, Zhengzhou University, Zhengzhou 450001, China

membrane. They all possess the basic triple helix based on multiple repeats of the simple tri-peptide Gly-X-Y. The triple helix is composed of proteins that are called collagen α-chains. As the smallest of amino acids, Glycine is essential for the formation of triple helix because it is the only one that fits into the central core of helix. X and Y can represent any of amino acids, but a proline residue in collagen is often in position X and a post-translationally hydroxylated proline residue is often in position Y [2].

Collagen possesses many interesting properties that make it a notable material for biomedical applications. It has low antigenicity [3], low inflammatory and cytotoxic responses [4], and good hemostatic properties [5], promotes cell growth [6] and shows interesting biological performances in contact with cells [7]. This biopolymer has been investigated for a variety of biomedical and biotechnological applications including drug delivery, burn, or wound cover dressings [8] or as a substrate for tissue engineering [9, 10]. Other notable properties include its high tensile strength, controllable biodegradability [11], and simple processability [12].

Metals have been used as bactericidal and bacteriostatic agents for centuries. Each metal bactericidal and bacteriostatic agent has different property and spectrum of activity [13]. Among these, silver, gold, and zinc are often used. Literature survey shows that metal ion can react with collagen fiber to form Fe(III)-immobilized collagen fiber [14]. There is no report on the antibacterial activity of collagen–metal materials. In this study, we report the results about preparation, characterization, and antibacterial activity evaluation of collagen–Zn complexes.

Experimental

Materials

Collagen protein was kindly provided by Professor Keyong Tang (College of Materials Science and Engineering, Zhengzhou University), average molecular weight was 1.0×10^4 Da. Beef extract and peptone were purchased from Beijing Chemical Agent Co. (Beijing, China). The other chemicals used were all analytical-grade reagents.

The tested strains *Escherichia coli* (*E. coli*, ATCC29522) and *Staphylococcus aureus* (*S. aureus*, ATCC6538) were obtained from Henan Provincial Center for Disease Control and Prevention.

Characterization

FT-IR spectra were recorded with KBr discs in the range of 4,000–400 cm^{-1} on Perkin Elmer FT-IR1750 spectrophotometer. Metal content was measured by a Hitachi 180-80 atomic absorption spectrometry. X-ray diffraction (XRD) was recorded by Philips PW3040/60 diffractometer (using Cu Kα radiation) with scanning scope of 5–70 degrees and scanning speed of 4°/min.

Preparation of different zinc salt complexes

The reaction mixture was prepared by mixing 5.0 g of collagen protein in 18 mL distilled water and a certain amount of zinc salt (zinc acetate, zinc chloride, and zinc nitrate) in 4 mL distilled water, stirring at 30 °C for 7 h. After finished, the mixture was cooled to room temperature, then poured into 300 mL of ethanol to form a white precipitate. The white precipitate was filtered and washed with ethanol, and then dried under vacuum to obtain collagen–zinc salt complex.

Preparation of collagen–$ZnSO_4$ complex

5.0 g of collagen protein and a certain amount of $ZnSO_4 \cdot 7H_2O$ (Zn ion theoretical content were 1.0, 2.0, 4.0, 8.0, and 15.0 wt%) were dissolved in 18 and 4 mL of distilled water to get A and B, respectively. The reaction mixture was prepared by mixing A and B, and then reacted at 30 °C for 7 h with stirring. The mixture was cooled to room temperature, and then poured into 300 mL of ethanol to form a white precipitate. The white precipitate was filtered and washed with methanol, and then dried under vacuum to obtain collagen–$ZnSO_4$ complex (CG–Zn).

Evaluation of antibacterial activity

Minimum inhibitory concentration (MIC) was determined by the double dilution. That is, to prepare a series of solution about the different concentration of CG–Zn by using nutrient medium (beef extract 3 g, sodium chloride 5 g, peptone 10 g to 1,000 mL distilled water, pH 7.0–7.2, autoclaved at 121 °C for 30 min) as dilution [15], the final concentration of CG–Zn is 0.025, 0.125, 0.00625, 0.0031, 0.0016, 0.0008, 0.0004, and 0.0002 g/mL, respectively. Nutrient medium was used as a control. The final concentration of bacterial suspension was diluted to 10^5–10^6 CFU/mL by sterile saline water. 0.1 mL of bacterial suspension of 10^5–10^6 CFU/mL was added to the nine test tubes, respectively. After inoculation, the test tubes were incubated at 37 °C for 24 h with 180 rpm. All the experiments were carried out in triplicate. The bacteria growth is observed by the turbidity with the naked eye. If the culture medium becomes turbid as "+," which indicates that the bacteria growth is not inhibited. If the culture medium is still clear as "−," which indicates that the bacteria growth is inhibited. If some phenomena are difficult to observe with the naked eye to determine its turbidity, then plate culture was carried out to observe the bacteria growth. Growth sterile minimum concentration of complex is the corresponding strains MIC [16].

Results and discussion

Antibacterial activity evaluation of collagen–Zn complexes

The results on the antibacterial activity evaluation of different zinc salt complexes are listed in Table 1.

Table 1 Inhibitory effect of different zinc salt complex on *E. coli* and *S. aureus*

Complex	Bacteria	Complex concentration (g/mL)								Control
		0.025	0.0125	0.0063	0.0031	0.0016	0.0008	0.0004	0.0002	
Zinc acetate	*E. coli*	−	−	−	−	+	+	+	+	+
	S. aureus	−	−	−	−	+	+	+	+	+
Zinc chloride	*E. coli*	−	−	−	+	+	+	+	+	+
	S. aureus	−	−	−	−	+	+	+	+	+
Zinc nitrate	*E. coli*	−	−	−	+	+	+	+	+	+
	S. aureus	−	−	−	+	+	+	+	+	+
Zinc sulfate	*E. coli*	−	−	−	−	+	+	+	+	+
	S. aureus	−	−	−	−	−	+	+	+	+

Actual content % (Zn^{2+}): 2.0%

It can be observed that collagen–zinc sulfate complex has a better antibacterial activity comparing with collagen–zinc acetate, collagen–zinc chloride, and collagen–zinc nitrate. In the next experiment, a series of collagen–zinc sulfate complexes with different zinc content were prepared, and characterized by FT-IR, XRD, and atomic absorption spectrometry. Their antibacterial activities were tested.

Characterization of collagen–$ZnSO_4$ complex

Zn percentages in collagen–$ZnSO_4$ complexes are showed in Table 2. The results showed that Zn percentage increases with increasing Zn theoretical content used in the reaction. When Zn theoretical content is 8.0%, Zn actual content in complex arrives to 8.91%. When Zn theoretical content increases from 8.0 to 15.0%, Zn actual content in complex only increases 0.10%. This showed that Zn maximum percentage in complex is approximately 8.91%.

XRD analysis

XRD of CG, CG–Zn (d), and $ZnSO_4{\cdot}7H_2O$ are lay out in Fig. 1. The diffraction spectra of $ZnSO_4{\cdot}7H_2O$ exhibits two major crystalline peaks at 18° and 20°, while CG consists of two major peaks at 10° and 21°. The complex CG–Zn (d) has two peaks at 10° and 21°, which is as same as CG, there is no new noticeable diffraction

Table 2 Zn percentage in collagen–$ZnSO_4$ complexes

Complex	Theoretical content (wt%)	Actual content (wt%)
CG–Zn (a)	1.0	1.12
CG–Zn (b)	2.0	2.06
CG–Zn (c)	4.0	4.05
CG–Zn (d)	8.0	8.91
CG–Zn (e)	15.0	9.01

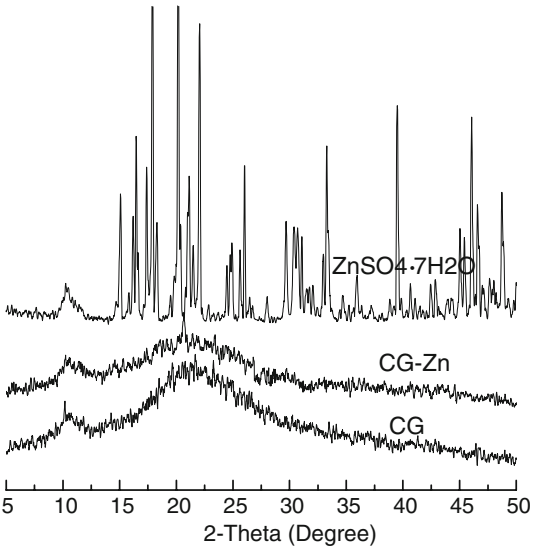

Fig. 1 XRD of CG, CG–Zn (d), and $ZnSO_4 \cdot 7H_2O$

peak and $ZnSO_4 \cdot 7H_2O$ diffraction peak. These results showed that zinc sulfate can be chelated with collagen and CG in CG–Zn complex keeps the stable triple helix structure.

FT-IR analysis

FT-IR spectrum of CG–Zn (d) exhibits many changes comparing with that of CG (Fig. 2).

The wide absorb peak at 3,396 cm^{-1}, which is the stretching vibration of N–H groups and O–H groups in collagen, shifted to lower frequency (3,313 cm^{-1}) in

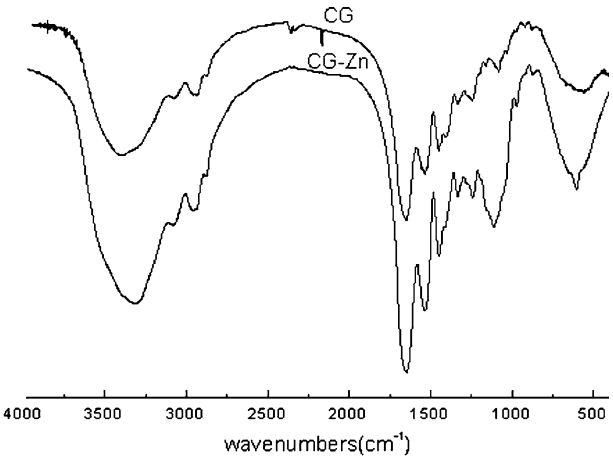

Fig. 2 FT-IR spectra of CG and CG–Zn (d)

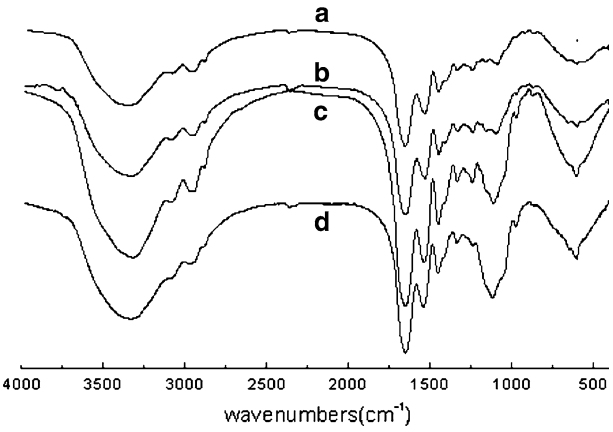

Fig. 3 FT-IR spectra of CG–Zn complexes with different zinc content. a, CS–Zn (a); b, CS–Zn (b); c, CS–Zn (c); and d, CS–Zn (d)

CG–Zn (d). The weak absorb band at 3,076 cm^{-1}, which is the stretching vibration of N–H groups in collagen, shifted to higher wavenumber (3,079 cm^{-1}) in CG–Zn (d). The absorb band at 1,656 cm^{-1}, assigned to the stretching vibration of C=O groups in collagen, shifted to lower wavenumber 1,649 cm^{-1} in CG–Zn (d). The absorb band at 1,539 cm^{-1}, assigned to C–N stretchings or N–H bending vibrations in collagen, showed a shift to lower wavenumber (1,534 cm^{-1}) in CG–Zn (d) absorb band at 1,164 cm^{-1}, assigned to the stretching vibrations of C–O groups in collagen, shifted to higher wavenumber (1,175 cm^{-1}) in CG–Zn (d). FT-IR spectra of CG–Zn complexes with the different Zn content are presented in Fig. 3. The absorb peak about the stretching vibration of N–H group and O–H group shifted gradually to lower wavenumber with the increase of Zn content in the complexes. When Zn theoretical percentage is 1.0, 2.0, 4.0, and 8.0%, respectively, the absorb peak about the stretching vibrations of N–H groups and O–H groups is 3,337, 3,322, 3,319, and 3,313 cm^{-1}, respectively. The absorb peak about the stretching vibrations of C=O group change very little with the increase of Zn content in the complexes and keep at 1,651–1,649 cm^{-1}. The absorb peak about the stretching vibration of C–O group shifted gradually to higher wavenumber with the increase of Zn content in the complex. When Zn percentage is 1.0, 2.0, 4.0, and 8.0%, respectively, the wavenumber is 1,168, 1,170, 1,173, and 1,175 cm^{-1}, respectively. A new absorb peak at 974 cm^{-1} is also observed in the spectra of CG–Zn complex and due to the stretching vibrations of S–O bonds [17]. The absorb peak of S–O bonds became stronger with the increases of Zn percentage. It revealed that the content of SO_4^{2-} increase in the complexes with increasing Zn theoretical content. The phenomena consisted with the results of elemental analysis.

All these infrared peak changes indicated that zinc ion can be chelated with C=O, C–O, and N–H in collagen to form a collagen–Zn complex. Based on FT-IR study, the basic chelate cells of CG–Zn were showed in Fig. 4 [18]. As a kind of d^{10} ions, Zn ion usually adopts a tetracoordinate mood with ligands. The reasonable chelate structure of CG–Zn can be assembled by a and b in Fig. 4.

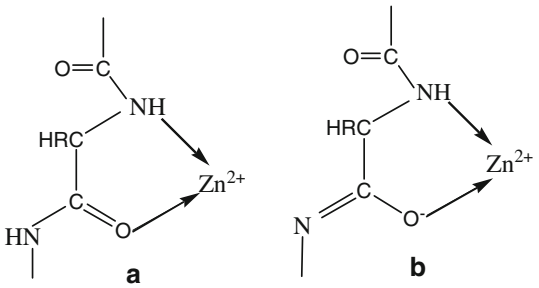

Fig. 4 Basic chelate cells of CG–Zn

Antibacterial activity

The pre-research result on the antibacterial activities of CG showed that collagen can promote the growth of *S. aureus* and *E. coli*. The antibacterial activities of CG–Zn complexes against *E. coli* and *S. aureus* were listed in Tables 3, 4, respectively. It can be observed that these complexes show antibacterial activities against *S. aureus* than against *E. coli* that depend from the increase of the Zn concentration in every CG–Zn complex. These results arise from the nature of bacteria: *S. aureus* is a Gram-positive bacterium and *E. coli* is a Gram-negative one. In general, Gram-negative bacteria are stronger than Gram-positive bacteria [19].

Table 3 Inhibitory effect of CG–Zn on *E. coli*

Complex	Complex concentration (g/mL)								Control
	0.025	0.0125	0.0063	0.0031	0.0016	0.0008	0.0004	0.0002	
a	−	−	−	+	+	+	+	+	+
b	−	−	−	+	+	+	+	+	+
c	−	−	−	−	+	+	+	+	+
d	−	−	−	−	−	+	+	+	+
e	−	−	−	−	−	+	+	+	+
$ZnSO_4 \cdot 7H_2O$	−	−	−	−	−	−	+	+	+

Table 4 Inhibitory effect of CG–Zn on *S. aureus*

Complex	Complex concentration (g/mL)								Control
	0.025	0.0125	0.0063	0.0031	0.0016	0.0008	0.0004	0.0002	
a	−	−	−	−	+	+	+	+	+
b	−	−	−	−	−	+	+	+	+
c	−	−	−	−	−	+	+	+	+
d	−	−	−	−	−	−	+	+	+
e	−	−	−	−	−	−	+	+	+
$ZnSO_4 \cdot 7H_2O$	−	−	−	−	−	−	−	+	+

Table 5 The antibacterial activity of CG–Zn (d) and $ZnSO_4 \cdot 7H_2O$

Bacteria	Complex ($\times 10^{-3}$ g/mL)		$ZnSO_4 \cdot 7H_2O$ ($\times 10^{-3}$ g/mL)	
	CG–Zn (d)	(Zn^{2+})	($ZnSO_4 \cdot 7H_2O$)	(Zn^{2+})
E. coli (ATCC29522)	1.60	0.12	0.80	0.18
S. aureus (ATCC6538)	0.8	0.06	0.40	0.09

The results about the antibacterial activities against *S. aureus* and *E. coli* of CG–Zn (d) are summarized in Table 5. As can be seen from Table 5, when *E. coli* was inhibited by CG–Zn (d) and $ZnSO_4 \cdot 7H_2O$, their MIC values were 0.12 and 0.18 Zn^{2+} mg/mL, respectively. When *S. aureus* was inhibited by CG–Zn (d) and $ZnSO_4 \cdot 7H_2O$, their MIC values were 0.06 and 0.09 Zn^{2+} mg/mL, respectively. These results suggest that CG–Zn complex has better antibacterial activity than $ZnSO_4 \cdot 7H_2O$.

Conclusion

Collagen–Zn complex can be easily prepared by the reaction of $ZnSO_4 \cdot 7H_2O$ with collagen. This complex has not only better antibacterial activity than $ZnSO_4 \cdot 7H_2O$, but also the advantages of collagen (for example, easy biodegrade, low-antigen activity, low cytotoxicity, and being easy to form membrane, etc.). By using collagen as support to form CG–Zn complex, $ZnSO_4 \cdot 7H_2O$ which is widely used as an external antibacterial material, will have wider applications.

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