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Preparation, Identification and Antioxidant Properties of Black-Bone Silky Fowl (*Gallus gallus domesticus* Brisson) Iron(II)-Oligopeptide Chelate

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Summary

Black-bone silky fowl iron(II)-oligopeptide chelate was synthesized from iron(II) solution and the black-bone silky fowl oligopeptide, which was extracted from the muscle protein of black-bone silky fowl (*Gallus gallus domesticus* Brisson). Orthogonal array analysis was used to determine the optimal conditions for the iron(II)-oligopeptide chelate preparation. Ultraviolet-visible (UV-Vis) spectroscopy, electron microscopy, and Fourier transform infrared (FTIR) spectroscopy were used to identify the structure of iron(II)-oligopeptide chelate. 2-Diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical scavenging assays were performed to compare the antioxidant abilities of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate. The optimal conditions for iron(II)-oligopeptide chelate preparation were 4 % of the black-bone silky fowl oligopeptide and a ratio of the black--bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 5:1 at pH=4. Under these conditions, the chelation rate was (84.9±0.2) % ($p<0.05$), and the chelation yield was (40.3±0.1) % ($p<0.05$). The structures detected with UV-Vis spectroscopy, electron microscopy and FTIR spectra changed significantly after chelation, suggesting that Fe(II) ions formed coordinate bonds with carboxylate (-$RCOO^-$) and amino (-NH_2) groups in the oligopeptides, confirming that this is a new oligopeptide-iron chelate. The iron(II)-oligopeptide chelate had stronger scavenging activity towards DPPH and superoxide radicals than did the black-bone silky fowl oligopeptide.

Key words: black-bone silky fowl oligopeptide, chelation, iron(II), structure identification, antioxidant activity, fortified food

Introduction

Iron is an essential trace element in the human diet. Iron deficiency affects the synthesis of haemoglobin, which results in iron deficiency anemia (IDA). IDA is one of the most common nutrient deficiencies in the world. Approximately 700 million people suffer from iron deficiency of different degrees, many of whom are women, children and the elderly (*1*). In developing countries, the situation is worse, 97 % of deaths caused by IDA occur in low- and middle-income countries (*2*). Currently, foods

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fortified with iron are the primary means of prevention and treatment of IDA. Although iron(II) sulfate and other non-haeme forms of iron are inexpensive, they tend to form hydrophobic complexes with phytic acid, polyphenols and other ingredients in food. Additionally, their absorption rates are lower, and they form endogenous free radicals leading to lipid peroxidation (*3*). The utilization efficiency of haeme iron is higher than of non-haeme iron but its highest absorption rate is only about 25 % (*4*). The utilization efficiency of iron-amino acid chelate is nearly double the efficiency of $FeSO_4$ in animals (*5*). According to Layrisse *et al.* (*6*), the iron absorption efficiency of fortified corn flour supplemented with $FeSO_4$ was 5.1 % and that supplemented with Ferrochel® (iron(II) bis(aminoacetate)) was 7.9 %. Studies have reported that metal-amino acid chelates can be absorbed by the small intestine in the same way as short peptides, and thus directly transferred to the body tissues (*7*).

Active peptide iron chelate is synthesized from iron(II) and a peptide extracted from a protein hydrolysate. Compared with amino acids, peptides are difficult to saturate with metals, although their absorption efficiency is higher (*8*). The peptide transporter 1 (PepT1), found in the small intestine, promotes the absorption of peptides (*9*). However, most studies on fortified food have focused on amino acid-metal chelates or metal salts, whereas only a few reports have focused on peptide-metal chelates. With higher absorption efficiency and physiological effects, iron supplements that incorporate active peptides are of increasing interest (*10*).

Black-bone silky fowl (*Gallus gallus domesticus* Brisson) is a unique Chinese breed rich in essential nutrients like amino acids and proteins. Essential amino acids make up 50.04 % of its total free amino acid content (*11*). Liu *et al.* (*12*) produced the black-bone silky fowl oligopeptides by treatment with composite enzymes. According to Lu *et al.* (*13*), 75.70 % of their molecular masses is distributed between 140 and 500 Da, with most being dipeptides and tripeptides that can be easily absorbed by the body. Thus, black-bone silky fowl oligopeptide-iron chelate may possess immunomodulatory and antioxidative properties as well as higher mineral absorption, all characteristics of bioactive peptides.

Herein, we report the determination of the optimal conditions required for the formation of the black-bone silky fowl oligopeptide-iron(II) chelate using $FeCl_2$ as a source of iron. The results of UV-Vis spectroscopy, electron microscopy and Fourier transform infrared (FTIR) spectroscopy were used to compare the structures before and after the chelation in an effort to explore the chelation mechanism. In addition, 2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical scavenging activities were investigated to compare the antioxidant abilities of the fowl oligopeptide and the obtained chelate.

Materials and Methods

Materials

Black-bone silky fowl oligopeptide powder was purchased from Zhonghaishi Bio Ltd. (Beijing, PR China). Tris was provided by Amresco Ltd. (Solon, OH, USA). DPPH was provided by TCI Ltd. (Tokyo, Japan). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, PR China). The reagents were of chemical purity.

Determination of optimal conditions for preparation of iron(II)-oligopeptide chelate

To define the optimal method for the iron(II)-oligopeptide chelate preparation, a 3^3 orthogonal array analysis was adopted (*14*). The black-bone silky fowl oligopeptide powder was dissolved in 100 mL of distilled water, then $FeCl_2$ was added, and the pH was adjusted with HCl or NaOH to the desired value. The closed reaction flask was incubated in a water bath at 30 °C for 30 min. A total of four volumes of absolute ethanol were added to precipitate the product for 1 h, then the products were filtered and the solvent was evaporated at room temperature for 12 h (*15*).

The *o*-phenanthroline method reported by Fortune and Mellon (*16*) with modifications was adopted to determine the content of iron in the chelate. A standard iron solution of 10 μg/mL was prepared from iron(II) ammonium sulfate. A total of 2 mL of 10 % ascorbic acid and 3 mL of *o*-phenanthroline solution were added to the standard iron solution (0, 2, 4, 6, 8 or 10 mL) or 1 mL of 0.1 % iron(II)-oligopeptide chelate solution, and then distilled water was added to a total volume of 50 mL, followed by incubation at 37 °C for 60 min. The content of iron in the chelate was calculated as follows:

$$w(\mathrm{Fe})/(\mathrm{mg/g}) = \frac{m(\mathrm{Fe})_{\mathrm{standard}}}{m_{\mathrm{sample}} \cdot V_{\mathrm{sample}} / V_{\mathrm{incubator}}} \qquad /1/$$

where $m(\mathrm{Fe})_{\mathrm{standard}}$ is the corresponding iron mass in the sample (mg) obtained from the standard curve, m_{sample} is the mass of the sample (g), V_{sample} is the volume of the sample added to the incubator (mL), and $V_{\mathrm{incubator}}$ is the constant volume of incubator (mL).

Chelation rate represents the efficiency of the iron chelation, while chelation yield represents the efficiency of iron(II)-oligopeptide chelate formation. The chelation rate and chelation yield are as follows:

$$\text{Chelation rate} = (m_{\mathrm{iron(II)}} / m_{\mathrm{iron(total)}}) \cdot 100 \qquad /2/$$

where $m_{\mathrm{iron(II)}}$ is the iron mass (mg) in the chelate and $m_{\mathrm{iron(total)}}$ is the total iron content (mg) that was added to the reaction system.

$$\text{Chelation yield} = (m_{\mathrm{chelate}} / m_{\mathrm{total}}) \cdot 100 \qquad /3/$$

where m_{chelate} is the mass of iron(II)-oligopeptide chelate and m_{total} is the mass of the black-bone silky fowl oligopeptide and $FeCl_2$ that are added to the reaction system.

Scanning electron microscopy

The microstructures of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate were scanned with electron microscope as described by Lorenzen *et al.* (*17*) and Zhao and Zheng (*18*), with modifications. The black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate powders were smeared on the sample trays using the double-sided adhesive for spraying and coating. Then the samples were processed using an S-3400N

scanning electron microscope (Hitachi Ltd., Tokyo, Japan) under vacuum. Prior to collection, the beam spot size was adjusted until the images were in focus and then they were taken at 1000× and 500× magnification.

Ultraviolet-visible spectroscopy

In order to determine the concentrations of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate, UV-Vis spectroscopy was used. The solutions of black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate were confirmed to be of the same concentration by scanning between 190 and 400 nm with a 752 UV-Vis spectrophotometer (Mapada Co. Ltd., Shanghai, PR China) according to the method used by Zhou *et al.* (*19*).

Fourier transform infrared spectroscopy

A total of 5 mg of iron(II)-oligopeptide chelate and 5 mg of black-bone silky fowl oligopeptide powders were purified by absolute ethanol precipitation together with 500 mg of dry spectroscopically pure KBr matrix and placed in an agate mortar. The mixture was ground under an infrared lamp. The mixed powder was then added to a tablet mould to make a transparent KBr tablet. A Spectrum GX type Fourier transform infrared spectrometer (Perkin Elmer Co. Ltd., Waltham, MA, USA) was used for qualitative analysis of the two samples in the spectral range of 4000–400 cm^{-1}, at a resolution of 4 cm^{-1}. The cumulative frequency of the scanning signal was 32 times, and the interference from water and carbon dioxide was subtracted during scanning (*20*).

Determination of DPPH scavenging ability

The method used to determine DPPH scavenging ability was adopted from Tang *et al.* (*21*) with some modifications. First, a total of 1.5 mL of black-bone silky fowl oligopeptide or iron(II)-oligopeptide chelate and 1.5 mL of DPPH solution (0.1 mmol/L) were mixed, left to stand for 5 min and then the absorbance (A_{sample}) was measured at 517 nm. At the same time, the absorbance of the mixture of 1.5 mL of DPPH solution (0.1 mmol/L) and 1.5 mL of ethanol (A_{blank}) and the absorbance of the mixture of 1.5 mL of the sample and 1.5 mL of ethanol ($A_{control}$) were measured at 517 nm. Black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate samples were diluted to 0.5, 1, 2, 5, 10, 15 and 20 mg/mL. As a strong antioxidant substance, ascorbic acid was used as a positive reference, and it was diluted to 2, 4, 6, 8 and 10 μg/mL. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging rate}=\left(1-\frac{A_{sample}-A_{blank}}{A_{control}}\right)\cdot 100 \quad /4/$$

Determination of superoxide radical scavenging ability

The method used to determine superoxide radical scavenging ability was adopted from Gao *et al.* (*22*) with some modifications. A volume of 4 mL of 50 mmol/L of Tris-HCl buffer (pH=8.2) and 0.5 mL of distilled water were combined together, and the mixture was preheated at 25 °C for 10 min, followed by the addition of 0.5 mL of sample (black-bone silky fowl oligopeptide or iron(II)-oligopeptide chelate). Next, 3 mmol/L of pyrogallol (used as control), which had been prepared in 10 mmol/L of hydrochloric acid preheated to 25 °C, were added. Then, the absorbance of the sample was measured after 1 min at 325 nm at 30-second intervals. Black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate samples were diluted to 2, 5, 10, 15 and 20 mg/mL. Ascorbic acid, used as a positive reference, was diluted to 0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL. Superoxide radical scavenging activity was calculated using the following equation:

$$\text{Superoxide radical scavenging activity}=\left(\frac{(\mathrm{D}A_{control}/\mathrm{D}t-\mathrm{D}A_{sample}/\mathrm{D}t)}{\mathrm{D}A_{control}/\mathrm{D}t}\right)\cdot 100 \quad /5/$$

where $DA_{control}/Dt$ is the autoxidation reaction rate of pyrogallol, while DA_{sample}/Dt is the autoxidation reaction rate of pyrogallol after the addition of the sample.

Statistical analysis

The results were analyzed by SPSS v. 20.0 software (SPSS Inc., Chicago, IL, USA) with the mean values of three independent experiments together with their corresponding confidence intervals at a 95 % level ($p<0.05$). Chelation rate, chelation yield and scavenging rate are presented as mean value±S.E. (standard error).

Results and Discussion

The optimal method of iron(II)-oligopeptide chelate preparation

The results of single factor analysis of black-bone silky fowl oligopeptide mass per volume ratio, mass ratio of the black-bone silky fowl oligopeptide to $FeCl_2{\cdot}4H_2O$ and pH are shown in Fig. 1.

The main factors that affect iron-peptide chelation are the mass per volume ratio of peptide, pH, temperature and reaction time (*23*). Generally, chelation happens quickly at room temperature so the temperature and reaction time do not influence the results significantly (*24*). Thus, the single factor tests were designed to examine three relevant factors: black-bone silky fowl oligopeptide mass per volume ratio, its mass ratio to $FeCl_2{\cdot}4H_2O$ and pH.

Mass per volume ratio of reactants is an important factor in the chelation process. In reaction kinetics, higher mass per volume ratio of the reactants can stimulate the reaction and increase the product yield. However, when the mass per volume ratio of reactants increases to a certain level, the yield may not increase further (*25*). In fact, the use of high mass per volume ratio of reactants may result in them being wasted if not converted to product. Fig. 1a shows that the chelation rate and yield were the highest when the black-bone silky fowl oligopeptide mass per volume ratio was 4 %.

The mass ratio of black-bone silky fowl oligopeptide to $FeCl_2{\cdot}4H_2O$ is another important factor in determining the success of the chelation process. When the ratio is too small, it is hard to form a stable cyclic structure, and as a result the chelate is unstable. When the ratio is too high, it results in the waste of black-bone silky fowl oligopeptide.

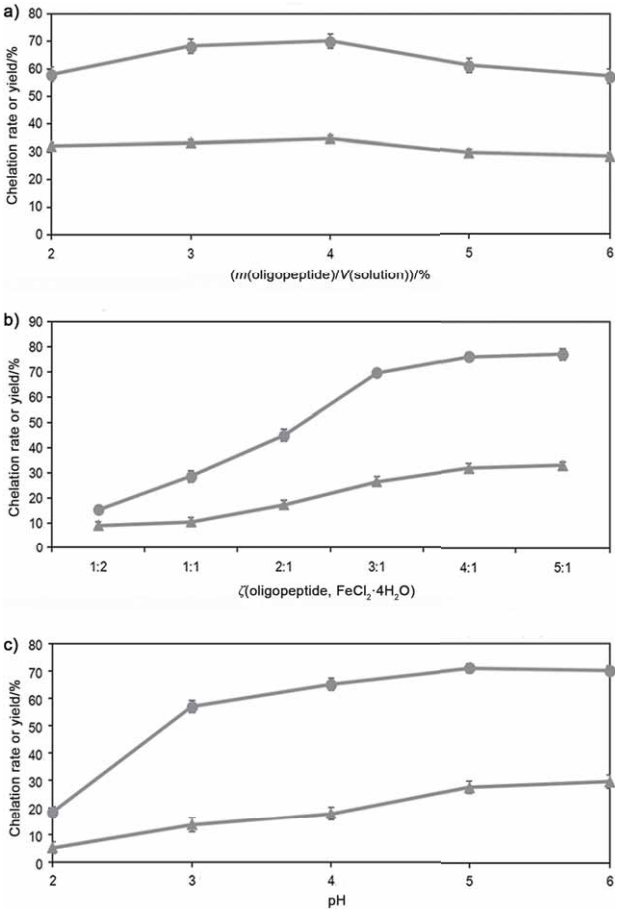

Fig. 1. Effects of: a) black-bone silky fowl oligopeptide mass per volume ratio (2, 3, 4, 5 and 6 %), b) mass ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ (1:2, 1:1, 2:1, 3:1, 4:1 and 5:1), and c) pH value (pH=2, 3, 4, 5 and 6) on the chelation rate (—■—) and chelation yield (—▲—)

Fig. 1b shows that with an increase in the ratio of black--bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$, the chelation rate and yield improved. When the ratio reached 4:1, the yield levelled off. Thus, a mass ratio of the black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 4:1 was sufficient for black-bone silky fowl oligopeptide to chelate most of the iron(II).

The pH is a key factor in the chelation of most peptides and trace metal elements. Under acidic conditions, proton (H^+) can compete as an electron-donating group with the metal ion, which is not likely to form a chelate. Under alkaline conditions, hydroxyl group (-OH) can also compete as an electron-donating group with the metal ion and form a hydroxide precipitate, which hinders the formation of chelates (*26*,*27*). As shown in Fig. 1c, the chelation rate gradually increased as the pH increased, resulting in an increase in chelation yield.

According to the results of singe factor tests, black--bone silky fowl oligopeptide mass per volume ratio of 3, 4 and 5 % (main factor A), mass ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 3:1, 4:1 and 5:1 (factor B), and pH=4, 5 and 6 (factor C) were chosen as the conditions in the orthogonal array test. The design and the results of the orthogonal array are shown in Table 1. A total of nine conditions were tested in the experiment. From

Table 1. Results of the orthogonal array analysis/L_9 (3^3)

Number	A	B	C	Chelation rate/% (*N*=3)	Chelation yield/% (*N*=3)
1	1	1	1	70.0±0.1	32.4±0.1
2	1	2	2	65.9±0.2	35.4±0.2
3	1	3	3	64.4±0.2	33.9±0.2
4	2	1	2	84.9±0.1	40.3±0.1
5	2	2	3	66.9±0.1	37.9±0.2
6	2	3	1	68.7±0.2	34.9±0.2
7	3	1	3	76.0±0.2	39.0±0.1
8	3	2	1	81.4±0.1	37.6±0.1
9	3	3	2	60.9±0.1	35.7±0.2

A (*m*(oligopeptide)/*V*(solution)): 1=3 %, 2=4 %, 3=5%; B (ζ(oligopeptide, $FeCl_2 \cdot 4H_2O$): 1=5:1, 2=4:1, 3=3:1; and C (pH): 1=4, 2=5, 3=6. Chelation rate and yield are presented as mean value±standard error

the range analysis of the chelation rate, it was inferred that among the three factors, the degree of influence was: B>A>C, indicating that the mass ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ was the most important factor for determining the chelation rate, followed by mass per volume ratio of black-bone silky fowl oligopeptide, while pH had a minimal effect. Thus, the optimal group for the chelation rate was $A_2B_1C_1$ (4 % of black-bone silky fowl oligopeptide, ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 4:1 and pH=4).

From the range analysis of the chelation yield, it was inferred that among the three factors, the degree of influence was: A>C>B, indicating that mass per volume ratio of black-bone silky fowl oligopeptide was the most important factor for determining the chelation yield, followed by pH, while the mass ratio of the black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ had minimal effect. Thus, the optimal group for the chelation yield was $A_2B_1C_2$ (4 % of black-bone silky fowl oligopeptide, ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 5:1 and pH=5).

It was also noted that prior to any adjustments, the pH of the solution was close to 4. To save time and processing costs, 4 % of black-bone silky fowl oligopeptide, a ratio of black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 5:1 and pH=4 were adopted as the most suitable conditions. Under these conditions, the chelation rate was (84.9± 0.2) %, and the chelation yield was (40.3±0.1) %.

Structure identification

Fig. 2 shows the images of black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate powder particles obtained by the scanning electron microscope at 500× and 1000× magnification. Black-bone silky fowl oligopeptide powder had particles of distinctive shape as uniform spheres with pores, which differed from iron(II)--oligopeptide chelate particles. Additionally, the particle surfaces of the two samples were different; the surface of the black-bone silky fowl oligopeptide was smoother than that of the chelate. It was similar to the structure of the chelates of Atlantic salmon (*Salmo salar* L.) peptides with

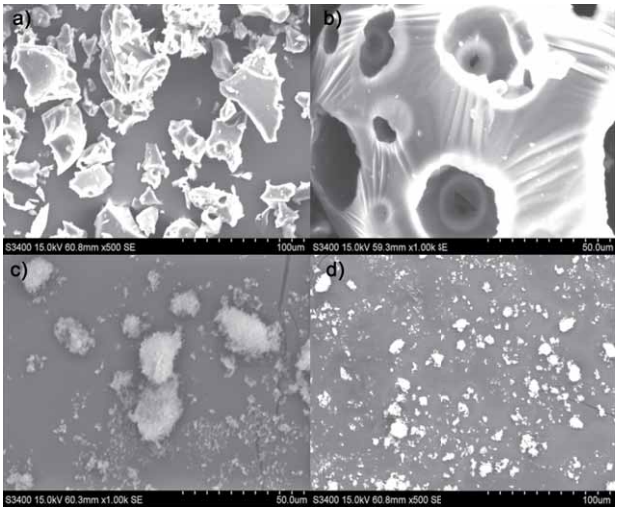

Fig. 2. Electron microscopy scans of black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate at magnification of 500× (a and b, respectively) and 1000× (c and d, respectively)

calcium, determined by scanning electron microscope (*28*). The images show that after chelation, the particle properties of the black-bone silky fowl oligopeptide were obviously changed, supporting the supposition that black--bone silky fowl oligopeptide and iron(II)-oligopeptide chelate are two different substances.

Fig. 3 shows the absorption of light by the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate in the UV-Vis region. From Fig. 3a it can be concluded that the highest absorption peak of black-bone silky fowl oligopeptide was at 225 nm, which was the characteristic absorption peak of the peptide bond and the carboxyl group (*19*,*28*). Fig. 3b shows that after black-bone silky fowl oligopeptide chelated with iron(II), the absorption peak moved to 217 nm, and the shape of the peak was markedly different from that of black-bone silky fowl oligopeptide. The generation of chelates typically affects the light absorption properties of the ligands (*29*). The difference observed in the absorption peak in the range from

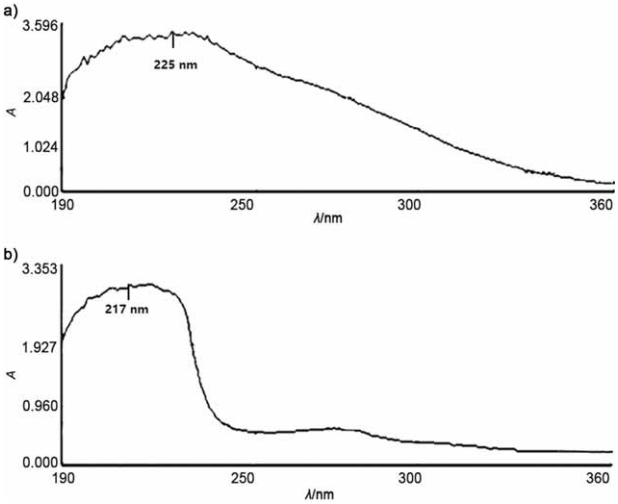

Fig. 3. Ultraviolet-visible (UV-Vis) scans of: a) black-bone silky fowl oligopeptide and b) iron(II)-oligopeptide chelate

225 to 217 nm was the result of the change of the atomic valence caused by electron transition after the black-bone silky fowl oligopeptide formed the chelate bond with iron. The change of the absorption peak confirmed that chelating reaction had occurred.

Using FTIR spectroscopy, iron(II)-oligopeptide chelate was shown to have significant differences in either intensity or position of its peaks compared with the black-bone silky fowl oligopeptide. In the present study, detection of the metal-amino acid chelates or metal-peptide chelates was confined to the higher frequency region to observe the internal vibrations of the ligands related to the chelating agents (*30*). As shown in Fig. 4, at 3030 cm^{-1} the absorption peak of iron(II)-oligopeptide chelate characteristic for the amino group disappeared, while there was a peak at 1046 cm^{-1}, indicating that there was a strong association of the amino group with Fe^{2+}. In the FTIR spectra of the black-bone silky fowl oligopeptide, there was a wide absorption peak at 3385 cm^{-1}, where O-H and N-H stretching vibration frequencies overlapped. Simultaneously, there was an absorption peak at 924 cm^{-1}, confirming the presence of free carboxyl group (-COOH) (*31*). Compared with the black-bone silky fowl oligopeptide, in the FTIR spectra of iron(II)-oligopeptide chelate the absorption peak at 3385 cm^{-1} became narrow, and there was no absorption peak from 955 to 915 cm^{-1}, indicating that there was no -COOH group in the iron(II)-oligopeptide chelate. Thus, we assumed that the -COOH group is covalently bound to Fe^{2+}.

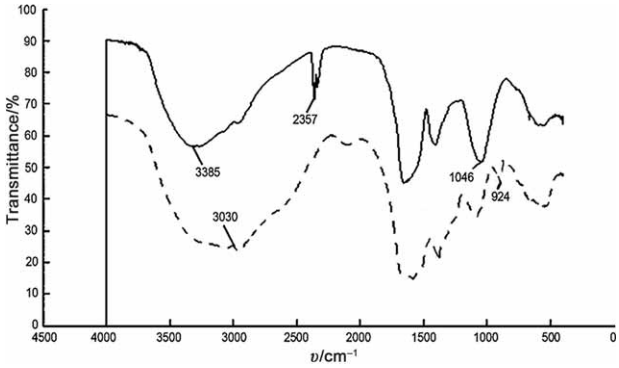

Fig. 4. FTIR spectra of black-bone silky fowl oligopeptide (dashed line) and iron(II)-oligopeptide chelate (solid line)

Furthermore, in the FTIR spectra of iron(II)-oligopeptide chelate, there was a high absorption peak at 2357 cm^{-1}, which is characteristic for cyano group (C≡N) (*31*). Comparatively, the absorption peak of nitrile (-CN) in the black-bone silky fowl oligopeptide was at 1046 cm^{-1}, and the number of very small peaks reduced. Thus, it was concluded that the number and structure of -CN changed after chelation. We hypothesised that during the chelation, Fe^{2+} ions bind to the amino ($-NH_2$) and carboxylate ($-RCOO^-$) groups in the black-bone silky fowl oligopeptide to form the novel peptide-iron chelate.

Antioxidant properties of oligopeptide and oligopeptide chelate

Figs. 5a and b show the DPPH radical scavenging activity of the black-bone silky fowl oligopeptide and

iron(II)-oligopeptide chelate compared to ascorbic acid used as a positive reference. It can be seen that at the concentrations of 0–20 mg/mL, the DPPH radical scavenging activity of both the oligopeptide and iron(II)-oligopeptide chelate increased as their concentrations increased. At low concentrations (0.5–2.0 mg/mL), the chelate had a scavenging rate of more than 70 %, while that of the oligopeptide was lower. When the concentration reached 5 mg/mL, the DPPH radical scavenging rate of iron(II)-oligopeptide chelate was close to 100 %.

According to the trend over the range of 0–5 mg/mL of oligopeptide and the chelate, the DPPH scavenging rate of the chelate fitted a logarithmic function model according to the formula:

$$y=11.949\cdot\ln(x)+0.0032 \quad (R^2=0.8691) \qquad /6/$$

In the range of 0–15 mg/mL of oligopeptide and the chelate, the radical scavenging rate of the black-bone silky fowl oligopeptide fitted a power function model according to the formula:

$$y=8.8507\cdot x^{0.8061} \quad (R^2=0.9783) \qquad /7/$$

DPPH is a stable free nitrogen radical that can be scavenged when it encounters a proton-donating substance like an antioxidant, causing a reduction in the absorbance (*32*,*33*). The IC_{50} values of DPPH radical scavenging of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate were 8.600 and 0.065 mg/mL, respectively, indicating that the DPPH radical scavenging ability of iron(II)-oligopeptide chelate was stronger than of the black-bone silky fowl oligopeptide.

Figs. 5c and d present the superoxide radical scavenging activity of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate compared to ascorbic acid. In our studies, during the initial stages of the reaction, the signal was variable, becoming stable after 3 min. Thus, the absorbance of the three substances was measured after 3 to 5 min. As it is shown, in the range of 2–20 mg/mL of oligopeptide and the chelate, the superoxide radical scavenging activity of iron(II)-oligopeptide chelate was stronger than that of the black-bone silky fowl oligopeptide.

Superoxide radicals have strong cellular oxidation abilities, causing damage to DNA and cell membranes. They produce singlet oxygen, hydroxyl radical, hydrogen peroxide and other strong oxidizing substances (*2*,*34*). The IC_{50} values of superoxide radical scavenging of the black-bone silky fowl oligopeptide and iron(II)-oligopeptide chelate were 20 and 3 mg/mL, respectively, while the IC_{50} of ascorbic acid was 0.05 mg/mL. Therefore, iron(II)--oligopeptide chelate can be used as an antioxidant. Moridani *et al.* (*35*) suggested that the iron complex may activate the iron centre to perform a double synergistic action, thus having a stronger ability to scavenge the superoxide radicals.

It has been reported that iron(II) complexes with amino acids or protein hydrolysates exhibit strong antioxidant activity (*36*,*37*). The antioxidant properties of peptides and iron-peptide chelates are related to their molecular structure. Transfer of hydrogen atom and electron donation are two main mechanisms that contribute to antioxidant processes (*38*). Iron chelation could increase the antioxidant activity of the compounds with low scavenging activity (*39*). The IC_{50} values of the black-bone silky fowl oligopeptide DPPH and superoxide scavenging activities obtained in this work were similar to that in reported literature (*12*). As the antioxidant activity of the black-bone silky fowl oligopeptide was still far from that of ascorbic acid, it could be regarded as low scavenging compound. However, iron(II)-oligopeptide chelate had stronger antioxidant activity than the black-bone silky fowl oligopeptide.

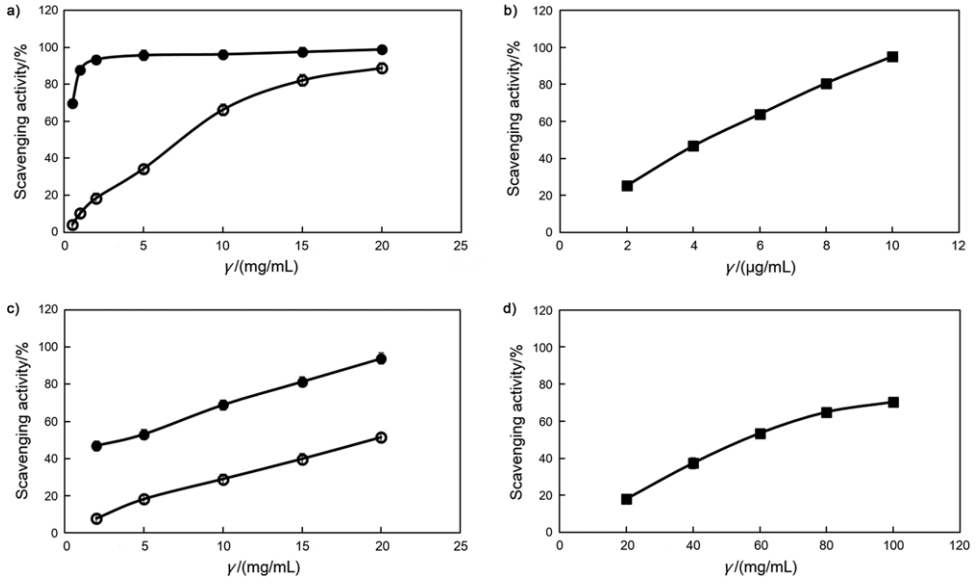

Fig. 5. Scavenging activity of: a) different concentrations (0.5, 1, 2, 5, 15 and 20 mg/mL) of iron(II)-oligopeptide chelate (—●—) and the black-bone silky fowl oligopeptide (—○—) on 2-diphenyl-1-picrylhydrazyl (DPPH) radical, compared to the b) scavenging activity of different concentrations (2, 4, 6, 8 and 10 μg/mL) of ascorbic acid (—■—), and c) scavenging activity of different concentrations (2, 5, 10, 15 and 20 mg/mL) of iron(II)-oligopeptide chelate (—●—) and the black-bone silky fowl oligopeptide (—○—) on superoxide radical, compared to d) the scavenging activity of different concentrations (20, 40, 60, 80 and 100 μg/mL) of ascorbic acid (—■—)

Conclusions

The optimal conditions for the preparation of iron(II)--oligopeptide chelate were using a black-bone silky fowl oligopeptide mass per volume ratio of 4 %, and a ratio of the black-bone silky fowl oligopeptide to $FeCl_2 \cdot 4H_2O$ of 5:1 at pH=4. Under these conditions, the chelation rate was (84.9±0.2) % ($p<0.05$), and the chelation yield was (40.3±0.1) % ($p<0.05$). The structure analysis indicated that it changed significantly after chelation. Iron(II) ions and $-NH_2$ and $-RCOO^-$ groups in the oligopeptides formed coordinate bonds, confirming that iron(II)-oligopeptide chelate is a new peptide-iron chelate. Radical scavenging activity analysis showed that iron(II)-oligopeptide chelate had stronger scavenging activity towards the DPPH and superoxide radicals than the black-bone silky fowl oligopeptide. The results of this study indicate that the preparation of iron(II)-oligopeptide chelate can be applied in the commercial production of a new kind of dietary nutrient that would have bioactive functions of the black-bone silky fowl oligopeptide and serve as an iron supplement with antioxidant properties.

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