Isolation and Structural Identification of Iron (II)-Chelated Oligopeptides from Black-Bone Silky Fowl

LIU Wen-ying[1], LU Jun[1], SONG Sha-sha[2], GU Rui-zeng[1], REN Di-feng[2], CAI Mu-yi[1]

(1.Beijing Engineering Research Center of Protein and Functional Peptides, China National Research Institute of Food and Fermentation Industries, Beijing 100015, China) (2.College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China)

Abstract: Black-bone silky fowl oligopeptides (BSFOP) were prepared by enzymatic hydrolysis of muscle from black-bone silky fowls and then reacted with iron (II) to yield iron (II)-chelated black-bone silky fowl oligopeptides (BSFOP-Fe). The iron-chelating capacity of BSFOP was 84.76±0.12%. BSFOP-Fe had a high protein content (54.64 ± 1.03%) and low molecular weight; 85.50% of peptides were less than 1000 u. Scanning electron microscopy, ultraviolet (UV) wavelength scanning, and infrared spectra were used to analyze the structure of BSFOP-Fe, and the results showed that BSFOP-Fe was a new type of iron-chelated compound. An *in vitro* stability study indicated that BSFOP-Fe maintained certain stability against temperature, pH, and *in vitro* gastric protease digestion. BSFOP-Fe was separated and purified by reverse-phase high performance liquid chromatography. One main fraction was collected and analyzed by mass spectrometry. One pentapeptide was identified from the main fraction of BSFOP-FE, and its amino acid sequence was Thr-Ser-Gly-Met-Pro. BSFOP-Fe could be applied as a food additive, to dietary nutrients, and to pharmaceutical products as an iron supplement.

Key words: black-bone silky fowl; characteristic; low-molecular-weight peptides; iron-chelated peptides; structure identification

Article NO.: 1673-9078(2017)8-95-102 DOI: 10.13982/j.mfst.1673-9078.2017.8.015

乌鸡低聚肽亚铁螯合物的分离纯化与结构鉴定

刘文颖[1]，鲁军[1]，宋莎莎[2]，谷瑞增[1]，任迪峰[2]，蔡木易[1]

（1.中国食品发酵工业研究院，北京市蛋白功能肽工程技术研究中心，北京 100015）

（2.北京林业大学生物科学与技术学院，北京 100083）

摘要：从乌鸡中酶解得到乌鸡低聚肽，然后与亚铁螯合制备乌鸡低聚肽亚铁螯合物，螯合率为 84.76±0.12%。乌鸡低聚肽亚铁螯合物具有较高的蛋白质含量，高达 54.64±1.03%。并且分子量较低，其中分子量小于 1000 u 的占 85.50%。通过扫描电镜、红外光谱对乌鸡低聚肽亚铁螯合物的结构进行分析，结果表明乌鸡低聚肽亚铁螯合物是一种新型的铁螯合物。体外稳定性研究表明乌鸡低聚肽亚铁螯合物具有一定的热稳定性、酸碱稳定性和体外消化稳定性。通过反相高效液相色谱对乌鸡低聚肽亚铁螯合物进行分离纯化，选择一个主要的组分进行收集，然后利用质谱仪进行质谱分析。从乌鸡低聚肽亚铁螯合物的主要组分中鉴定出一个五肽，氨基酸序列为 Thr-Ser-Gly-Met-Pro。乌鸡低聚肽亚铁螯合物可作为一种铁补充剂用于食品添加剂、营养物及医药制品中。

关键词：乌鸡；特性；低聚肽；肽铁螯合物；结构鉴定

1 Introduction

Iron is an essential element in human nutrition. It participates in many biochemical processes, including electron transfer reactions, gene regulation, binding and transport of oxygen, and cell growth and differentiation[1]. Iron deficiency may lead to diseases, such as anaemia, glossitis, angular stomatitis, koilonychia, blue sclera, and oesophageal webbing[1]. Other physiological manifestations of iron deficiency include pregnancy complications, pica, increased absorption of lead and cadmium, alteration in drug metabolism, increased insulin sensitivity and impaired immune and mental function, physical performance and thermoregulation[1].

收稿日期：2017-01-18

基金项目：国家十三五重点研发计划（2016YFD0400604）；国家高技术研究发展计划（863 计划）项目（2013AA102205-02）

作者简介：刘文颖（1984-），女，硕士，工程师，研究方向：食源性低聚肽研究

通讯作者：任迪峰（1973-），女，博士，教授，研究方向：食品营养与生物技术；蔡木易（1962-），男，教授级高级工程师，研究方向：功能肽研究与产业化

However, iron may generate reactive oxygen species (ROS) and be implicated in cardiovascular and neurological diseases[2]. Furthermore, ROS may have a negative impact on flavour, texture, nutritive value and shelf life of food products[3]. Therefore, chelating agents that decrease free iron and favour iron bioavailability may possess therapeutic potential and prevent its pro-oxidant effects.

Some dietary compounds, such as reducing components, stearic acids, certain amino acids (His, Glu, Asp, and Cys), peptides released during proteolytic digestion and the so called “meat factor”, enhance iron absorption[4]. These compounds may bind iron, forming soluble complexes and improving iron bioavailability[5]. Different studies have shown the beneficial effect on iron absorption of peptides produced by enzymatic hydrolysis of proteins, such as soybean and chickpea[6,7].

Black-bone silky fowl (*Gallus gallus domesticus* Brisson), a unique breed of chicken of China, lives up to its name with snow-white silky feather but black skin, meat and bones. Compared with ordinary chicken, black-bone silky fowl is well-known for its health functions, such as improving immunity, treating diabetes and anemia, curing menoxenia and postpartum complications[8,9]. Therefore, this breed has a time-honored place in traditional Chinese medicine. With the expansion of black-bone silky fowl breeding, its effective development and utilization are emergent. As for the innate function of supplementing iron and improving anemia conditions, the effect of black-bone silky fowl may be strengthened by chelating iron. Regarding black-bone silky fowl peptides, to the best of our knowledge, there are only a few reports dealing with iron-chelating black-bone silky fowl peptides. Information is lacking on the iron-chelating capacity and the structure of iron-chelating peptides derived from black-bone silky fowl.

In the present study, black-bone silky fowl oligopeptides (BSFOP) were prepared by hydrolyzing black-bone silky fowl and iron-chelating black-bone silky fowl oligopeptides (BSFOP-Fe) was obtained by iron-chelating peptides reaction from BSFOP. The iron-chelating capacity, chemical composition, amino acid composition, molecular weight distribution and characterization of BSFOP-Fe were determined. Then, BSFOP-Fe was separated by reversed-phase high performance liquid chromatography (RP-HPLC), and subjected to mass spectrometer to identify the peptides.

2 Materials and methods

2.1 Materials and reagents

Black-bone silky fowl (70~90 d old, half male and half female) was provided by Taihe Black-Bone Silky Fowl Industrial Co., Ltd. (Jiangxi, China). Acalase 2.4 L, papain, pepsin and trypsin were purchased from Novozymes Biological Co. (Tianjin, China). Ferrous chloride, ethanol, ascorbic acid, phenanthroline, ammonium ferrous sulfate, hydrochloric acid and sodium hydroxide were bought from Beijing Chemical Reagent Co. (Beijing, China). Acetonitrile was bought from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) was obtained from Alfa Aesar. All other reagents were of analytical or guaranteed reagent grade.

2.2 Preparation of BSFOP-Fe

Black-bone silky fowl muscle was rinsed with water and minced. Then it was stirred with a homogenizer (Donghua Homogenizer Factory, Shanghai, China) at 20,000 g for 10 min. The protein slurry was first hydrolyzed with Alcalase 2.4 L (1:100, *m/m*) for 3 h at pH 8.5 and 60 ℃. The resulting supernatant was further hydrolyzed with papain (1:100, *m/m*) for 2 h at pH 7.0 and 60 ℃. Hydrolysis was carried out in a thermostatically stirred-batch reactor (Dongding Machinery Co., Wenzhou, China). The pH of the reaction was kept constant during hydrolysis by addition of 1 M NaOH, and the hydrolysis was stopped by heating at 95 ℃ for 10 min.

The resulting hydrolysate was centrifuged at 3,000 g for 15 min (LG10-2.4A, Beijing LAB Centrifuge Co. Ltd, China). The supernatant was filtered with 10 and 1 ku molecular weight cut-off ceramic membranes successively to obtain the permeate liquid with low-molecular-weight peptides. Then, the permeate liquid was dialysed through a 100 u molecular weight cut off (MWCO) nanofilter membrane (Filter and Membrane Technology Co. Ltd, Fujian, China) at pH 7.0±0.5 to remove residual salt. Afterwards, the retentate was

evaporated by an R-151 rotavapor (BUCHI Co. Ltd, Switzerland) at 50 ℃ until the solid content of the concentrated liquid reached 30~40%. Finally, the concentrated liquid was spray dried using an L-217 Lab spray dryer (Beijing Laiheng Lab-Equipments Co. Ltd, China). The BSFOP powder obtained was stored in a desiccator until further analysis.

The BSFOP powder (8 g), $FeCl_2 \cdot 4H_2O$ (2 g) and ascorbic acid (2 g) were dissolved in 200 mL distilled water and incubated for 1 h at 50 ℃ in a water bath. After the incubation, the mixture was added to 800 mL、95% ethanol and left to stand for 1 h at room temperature. Then, the precipitate was collected by suction filtration and dried at room temperature. The BSFOP-Fe obtained was stored in a desiccator for further analysis. The yield of BSFOP-Fe was 40.27±0.15%.

The iron-chelating capacity of BSFOP-Fe was determined using the method of phenanthroline colorimetry. Briefly, ammonium ferrous sulfate was prepared into 10 μg/mL iron ion standard solution. Next, 2.0 mL、10% ascorbic acid solution and 3.0 mL phenanthroline solution were added into a volume of 0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL of iron ion standard solution, respectively, and then the mixture was diluted with distilled water to the volume of 50 mL. After reacted for 60 min at 37 ℃, the absorbance of the resulting solution was measured spectrophotometrically at 510 nm, and the standard curve was drawn. Then, 0.05 g of BSFOP-Fe was dissolved in 1 mL hydrochloric acid, and prepared into 0.1% BSFOP-Fe solution, and its absorbance was measured following the instructions of standard curve above. The iron-chelating capacity was calculated using $m_1/m_0 \times 100\%$, where m_1 was the iron content of BSFOP-Fe (mg) and m_0 was the total iron content of reaction system (mg)[10].

2.3 Chemical composition and amino acid composition assay

Crude protein content is expressed as total nitrogen (N)×6.25, of which the nitrogen content was determined by the Kjeldal method[11]. Amino acid composition was determined by an 835-50 automatic amino acid analyzer (Hitach, Ltd., Tokyo, Japan) according to the method of Yang, Tao, Liu, & Liu[12].

2.4 Determination of molecular weight distribution

Molecular weight distribution was evaluated using a LC-20A high performance liquid chromatography (HPLC) system (Shmadzu, Kyoto, Japan) equipped with a TSK gel G2000 SWXL 300×7.8 mm column (Tosoh, Tokyo, Japan). The mobile phase used was acetonitrile/water (45:55, *V/V*) containing 0.1 % (*V/V*) trifluoroacetic acid. Samples were eluted at a flow rate of 0.5 mL/min and monitored at 220 nm at 30 ℃. Tripeptide GGG (Mr 189), tetrapeptide GGYR (Mr 451), bacitracin (Mr 1450), aprotinin (Mr 6500) and cytochrome C (Mr 12500) (Sigma Chemical Co., St. Louis, USA) were used as molecular weight standards[13].

2.5 Characterization of BSFOP-Fe

2.5.1 Scanning electron microscope of BSFOP and BSFOP-Fe

The microstructures of BSFOP and BSFOP-Fe were analyzed by S-3400N electron scanning microscope (Hitach, Ltd., Tokyo, Japan) under 500 and 1000 times magnifications to observe the differences of the microstructures of BSFOP and BSFOP-Fe[14].

2.5.2 Infrared spectra of BSFOP and BSFOP-Fe

Nicolet 6700 fourier infrared spectrometer (Perkin Elmer Co., Massachusetts, USA) was used to analyze BSFOP or BSFOP-Fe. The spectrograms were scanned under the condition of 4000~500 cm^{-1}[14].

2.6 Stability of BSFOP-Fe

BSFOP-Fe solutions (2 mg/mL) were incubated at various temperatures, 20, 40, 60, and 80 ℃ for 2 h. The solutions were also incubated at 37 ℃ and pH values of 2, 3, 5, 7, and 9 for 2 h. After the solutions were acclimated to room temperature, the molecular weight distribution and iron content were determined[15].

Stability against *in vitro* pepsin was assessed by treating 2 mg/mL BSFOP-Fe solution in 0.1 M HCl-KCl buffer (pH 2.0), with 3% (*m/m*) pepsin for 3 h in a water bath at 37 ℃, stopped by boiling for 10 min. For stability to trypsin digestion, 2 mg/mL BSFOP-Fe solution in 0.1 M KH_2PO_4-NaOH buffer (pH=6.8) was digested by 3%

(*m*/*m*) trypsin for 3 h at 37 ℃. The digestion was stopped by boiling for 10 min. For stability to *in vitro* pepsin digestion plus further trypsin digestion, 2 mg/mL BSFOP-Fe solution was first digested by 3% (*m*/*m*) pepsin for 3 h and adjusted to pH 6.8 with addition of 1 N NaOH solution. The solution was digested further by 3% (*m*/*m*) trypsin at 37 ℃ for 3 h. The enzyme was inactivated by boiling for 10 min. After the solutions were acclimated to room temperature, the molecular weight distribution and iron content were determined[15]. The iron content was determined using the method of phenanthroline colorimetry[10].

2.7 Separation of BSFOP-Fe with RP-HPLC

BSFOP-Fe was separated with a RP-HPLC system (Shmadzu, Kyoto, Japan) equipped with an XBridge BEH130 C18 column (4.6×250 mm, Waters, USA). Gradient elution was eluent A (Milli-Q water containing 0.1% (*V*/*V*) TFA) and eluent B (80% (*V*/*V*) acetonitrile containing 0.1% (*V*/*V*) TFA). The separation was performed at a flow rate of 0.6 mL/min with a nonlinear gradient as follows: 0~20 min, 0~5% B; 20~30 min, 5~5% B; 30~80 min, 5~25% B; 80~90 min, 25~80% B; 90~100 min, 80~80% B. The effluent was monitored at 220 nm. Sample concentration was 10 mg/mL and the injection volume was 10 μL. The separation procedure was repeated successively for ten times. The fractions from the RP-HPLC system were freeze-dried for further analysis[16].

2.8 Identification of active peptides

Molecular mass and amino acid sequence of peptides were analyzed using a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass Co., Manchester, UK) equipped with an electrospray ionisation (ESI) source[17]. Firstly, the sample was subjected to primary mass spectrometry to obtain ESI-MS spectrogram. Ions to be tested were selected from the ESI-MS spectrogram, and were analyzed by ESI-MS/MS. Then, the spectrogram was transformed by MaxEnt 3 of the Q-TOF2 mass spectrometer, and the peptide sequences were deduced by Peptide Sequencing. N_2 was used as atomizing gas, and Ar was acted as collision gas. Capillary voltage was 800V.

2.9 Statistical analysis

All assays were carried out in triplicate. Data were expressed as means±standard deviations.

3 Results and discussion

3.1 Chemical composition and amino acid composition of BSFOP-Fe

In the preparation of BSFOP, 5 kg of black-bone silky fowl muscle was used as the raw material, and 1 kg of BSFOP powder was obtained. Then, 8 g of BSFOP powder was used to prepare BSFOP-Fe, and the yield of BSFOP-Fe was 40.27±0.15%. The iron-chelating capacity of BSFOP-Fe was 84.76±0.12%. Chemical composition analysis showed that BSFOP and BSFOP-Fe contained 88.34±1.08% and 54.64±1.03% protein (on a dry basis), respectively.

Table 1 Amino acid composition of BSFOP and BSFOP-Fe (%, *m*/*m*)[a]

Aminoacid	BSFOP	BSFOP-Fe
asparticacid[b]	9.51±0.23	9.56±0.29
glutamineacid[c]	17.21±0.37	15.06±0.43
serine	2.84±0.06	2.10±0.07
histidine	2.49±0.06	1.98±0.06
glycine	5.63±0.17	3.86±0.13
threonine	3.57±0.07	1.80±0.04
arginine	5.19±0.12	3.34±0.10
alanine	4.29±0.08	2.11±0.09
tyrosine	2.23±0.07	0.76±0.02
valine	3.74±0.09	0.99±0.03
methionine	1.62±0.05	0.31±0.01
phenylalanine	1.70±0.06	1.08±0.05
isoleucine	3.47±0.08	0.74±0.03
leucine	5.91±0.15	0.89±0.04
lysine	7.74±0.20	5.13±0.18
proline	3.43±0.09	1.58±0.05
NH_3	0.95±0.03	0.92±0.03

Note: [a]Reported values are means±SD (n=3), [b]Aspartic acid＋asparagine, [c]Glutamine acid＋glutamine.

The amino acid compositions of BSFOP and BSFOP-Fe were listed in Table 1. BSFOP was rich in glutamine acid, aspartic acid, lysine and leucine, and low in methionine, phenylalanine and histidine, which was similar to that reported in the literature[8]. The content of aspartic acid, glutamine acid, isoleucine and leucine were

9.51±0.23%, 17.21±0.37%, 3.47±0.08%, and 5.91±0.15%, respectively. After chelating iron, the content of these amino acids in BSFOP-Fe were 9.56±0.29%, 15.06±0.43, 0.74±0.03% and 0.89±0.04%, respectively. The result indicated that the iron-chelating capacity of aspartic acid and glutamine acid was better than isoleucine and leucine. It may be due to the carboxyl groups on the side chain which could exert stronger iron-chelating capacity during the reaction.

3.2 Molecular weight distribution of BSFOP-Fe

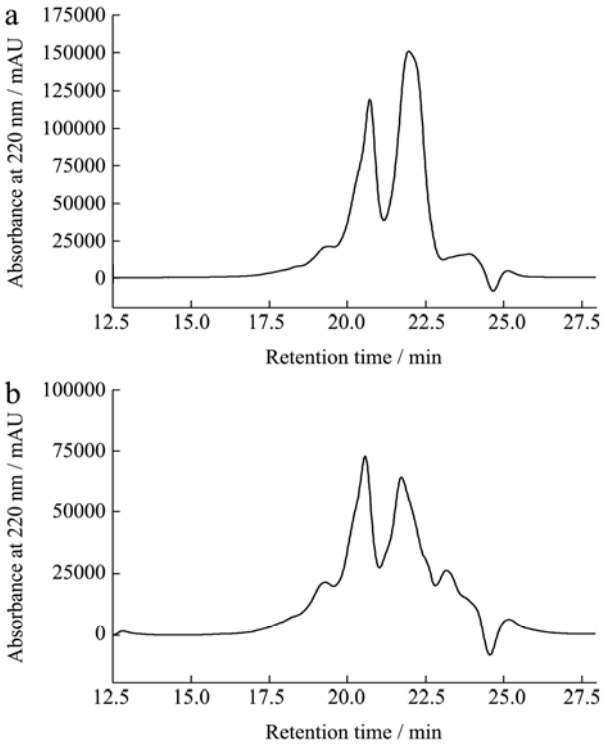


Fig.1 Size exclusion chromatography showing the molecular weight distribution of BSFOP (a) and BSFOP-Fe (b)

Note: The chromatography was carried out on a TSK-GEL G2000SWXL column (7.8×300 mm) eluted in 45% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min.

The molecular weight distribution of BSFOP and BSFOP-Fe were analyzed by size exclusion chromatography with an HPLC system. The result (Figure 1) showed that BSFOP and BSFOP-Fe were dominated by peptides below 1000 u (89.79% and 85.50%, respectively). The range of 500~140 u was the main molecular weight interval for both of BSFOP and BSFOP-Fe, which accounted for 61.36% and 52.83%, respectively. These peptides were mainly di- and tri-peptides. It has been reported that di- and tri-peptides are actively transported via a specific peptide transporter in the intestinal epithelial cells, and amino acid residues are absorbed more rapidly from di- and tri-peptides than from free amino acids[18]. The average molecular weight distributions of BSFOP and BSFOP-Fe were 421.50 u and 433.20 u, respectively. The results showed that after chelating iron, the molecular weight distribution of BSFOP-Fe was a little higher than that of BSFOP, which indicated that as for peptides below 1000 u, relatively larger peptides might have stronger iron-chelating capacity than low molecular weight peptides.

3.3 Characterization of BSFOP-Fe

3.3.1 Scanning electron microscope of BSFOP and BSFOP-Fe

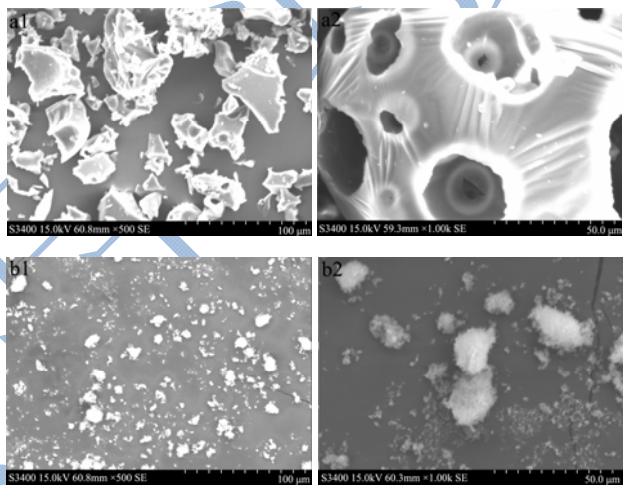


Fig.2 Scanning electron microscope of BSFOP (a) and BSFOP-Fe (b) under 500 (left) and 1000 (right) times magnifications

As depicted in Figure 2, the size, shape, and surface traits of the powder particles of BSFOP and BSFOP-Fe showed different states. The microstructure of BSFOP was spherical shaped with holes, while the microstructure of BSFOP-Fe was mainly irregular clump shaped and its surface was roughness. Therefore, by contrast, it was confirmed that after chelation reaction BSFOP and BSFOP-Fe were two completely different substances. The scanning electron microscope showed that after chelating Fe^{2+}, BSFOP and Fe^{2+} were generated into groups made up of small agminated granule, by ionic bonds and coordinate bonds. The structure of BSFOP-Fe formed was analogous to salts[19].

3.3.2 Infrared spectra of BSFOP and BSFOP-Fe

Figure 3 showed the infrared spectra of BSFOP and BSFOP-Fe. Compared with BSFOP, the peak in the

infrared spectrum of BSFOP-Fe at 3066 cm^{-1} disappeared, while an absorption peak at 1046 cm^{-1} appeared. It indicated that there is strong interaction between Fe^{2+} and amidogen. In the infrared spectrum of BSFOP, there was a wide absorption peak at 3385 cm^{-1}, where the stretching vibration frequencies of -OH and -NH overlapped. Besides, there was an absorption peak at 924 cm^{-1}, which indicated that there were free -COOH contained in BSFOP. In the infrared spectrum of BSFOP-Fe, the wide absorption peak was narrowed and there was no absorption peaks at 955~915 cm^{-1}, which indicated that there were not -COOH in the infrared spectrum of BSFOP-Fe. There were not absorption peaks near 1740 cm^{-1}, which indicated that there was no free carboxyl. Thus, it was speculated that carboxyl was combined with Fe^{2+} in the form of covalent bond.

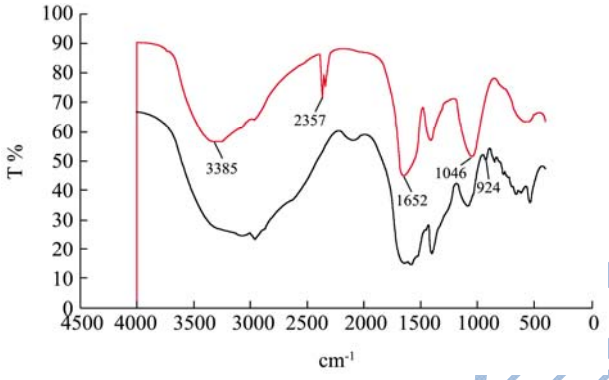


Fig.3 Infrared spectra of BSFOP (lower line) and BSFOP-Fe (upper line)

Note: The spectrograms were scanned under the condition of 4000~500cm^{-1}.

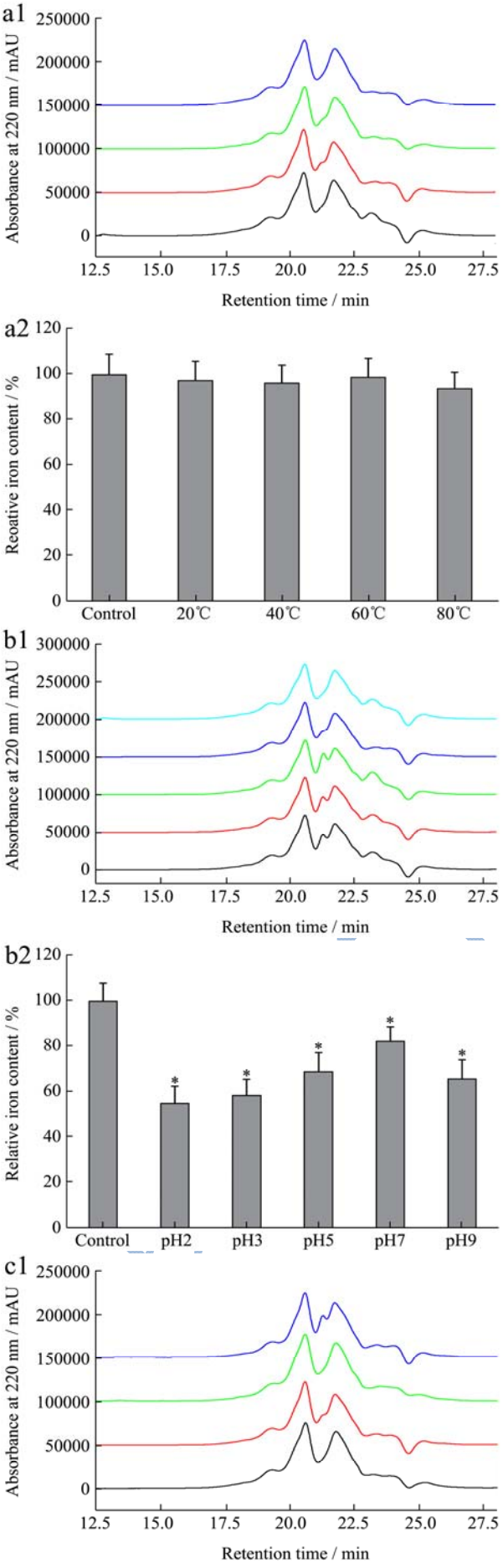
Besides, the strong absorption peaks at 2357 cm^{-1} in the infrared spectrum of BSFOP-Fe were characteristic absorption peaks of C≡N. There appeared strong absorption peaks of C-N bond at 1046 cm^{-1} and small peaks decreased, which indicate that the number and structure of N-H changed. The shared electrons of nitrogen atoms made a contribution to Fe^{2+} which led to the increasing of dipolarity of CN bonds. The results indicated that there was formation of a new type of black-bone silky fowl peptide chelated iron.

3.4 Stability of BSFOP-Fe

With the great interest in preparing foods with these "functional ingredients", it is important to test their processing stability. As shown in Figure 4a, these peptides retained molecular weight distribution and iron content after various temperature treatments, which indicate that BSFOP-Fe has satisfactory heat stability.

After various pH treatments, no distinct changes of peak height were observed on the size exclusion chromatograms of BSFOP-Fe (Figure 4b). In the acidic and alkaline conditions (pH 2, 3, pH 5 and pH 9), the molecular weights below 1000u of BSFOP-Fe were slightly elevated, but the content did not exceed 6%. It may be because the macromolecular ring structures formed by the peptides and iron were broken to micromolecular structure by alkali or acid. The result indicated that BSFOP-Fe showed certain stability to pH resistance in the respect of molecular weight. After digested by different pH values, the iron contents of BSFOP-Fe decreased and were in the range of 55.2±7.3% to 82.4±6.1%, compared to the control. The iron content decreased significantly under the conditions of strong acid or strong alkaline while it changed little in the neutral condition. The iron remaining chelating peptides could be absorbed rapidly in the intestinal tract with the help of small peptides[10].

For stability to *in vitro* gastric proteases digestion, neither distinct new peak nor no distinct changes of peak height were observed on the size exclusion chromatograms of BSFOP-Fe (Figure 4c). After digested by different proteases digestion modes, the molecular weights below 1000 u of BSFOP-Fe were slightly elevated, but the content did not exceed 7%. Compared to the control, after treated by proteases digestions, the molecular weights in the range of 140~500 u and <140 u were slightly elevated indicating that peptides with larger molecular weights could be digested to smaller peptides or amino acids. Previous reports have also shown that small peptides have low susceptibility to hydrolysis by gastric proteases[15,20]. The result suggested that BSFOP-Fe may be resistant to digestion in the gastrointestinal tract. BSFOP-Fe showed certain stability to proteases digestion by pepsin and trypsin in respect of molecular weight. After digested by pepsin digestion, trypsin digestion, and pepsin digestion plus further trypsin digestion, the iron contents of BSFOP-Fe decreased (p<0.05) and were 57.1±7.2%, 50.2±7.4% and 37.6±8.2%, respectively, compared to the control.



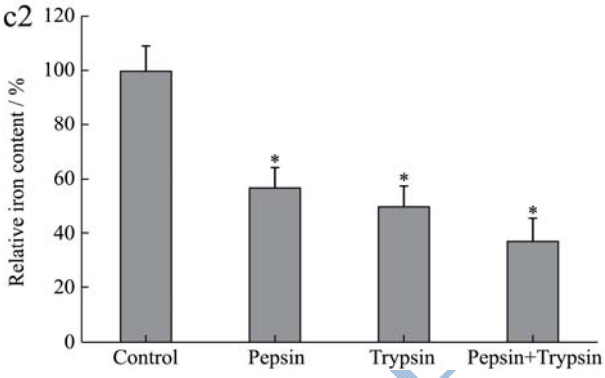


Fig.4 Stability of BSFOP-Fe after 3 h incubation at various temperature (a), pH (b) and proteases digestion (c) treatments.

Note: The line graphs were size exclusion chromatography showing the molecular weight distribution of BSFOP-Fe (a, treated at 20, 40, 60, and 80 ℃ from bottom to top; b, treated at pH 2, 3, 5, 7 and 9 from bottom to top; c, treated by pepsin digestion, trypsin digestion, and pepsin digestion plus further trypsin digestion from bottom to top). The figures in the right were relative iron content of BSFOP-Fe. The relative iron content was calculated as the ratio of iron content between the control and the treatments. *Asterisks* indicate significant differences compared to corresponding control (*p<0.05).

The structure of BSFOP-Fe was the important factor which could influence its iron content. As shown in the results of pH and proteases digestion treatments, small changes of molecular weight (no more than 6% and 7%, respectively) could lead to great changes of iron content.

3.5 Purification and identification of peptides from BSFOP-Fe

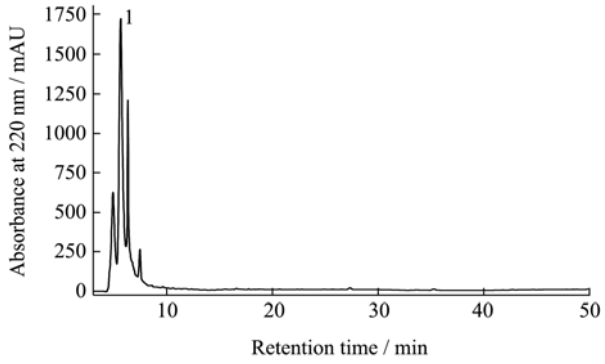


Fig.5 Elution profile of BSFOP-Fe by RP-HPLC C18 column

Note: Separation was carried out using a nonlinear gradient of eluent A (0.1% TFA in Milli-Q water, *V/V*) and eluent B (80% acetonitrile with 0.1% TFA, *V/V*) at a flow rate of 0.6 mL/min. Collected fraction is designated as fraction 1. The fraction was

collected according to the elution time, condensed, and free-dried.

In order to identify the peptides from BSFOP-Fe, it was subjected to fractionation with a RP-HPLC column. The elution profile of BSFOP-Fe was displayed in Figure 5. As shown in this figure, many peaks were detected. One major fraction, designated as fractions 1, was selected for further identification of active sequences. These fractions were pooled, freeze-dried, and subjected to a Q-TOF2 mass spectrometer. Overall, one peptide was identified from BSFOP-Fe. It was a pentapeptide, and the amino acid sequence was Thr-Ser-Gly-Met-Pro, with the molecular weight of 491.56 u.

4 Conclusions

BSFOP-Fe was prepared by hydrolyzing black-bone silky fowl prior to chelating iron, which was with high protein content and low molecular weights. Scanning electron microscope, UV wavelength scanning, and infrared spectra showed that BSFOP-Fe was a new type of black-bone silky fowl peptide chelated iron. BSFOP-Fe maintained certain stability against temperature, pH and in vitro gastric proteases digestion. BSFOP-Fe was purified and the amino acid sequences of the peptides were identified. Based on these results, it could be concluded that the production of BSFOP-Fe is a practice way to utilize black-bone silky fowl. This study suggested that BSFOP-Fe might be useful as food additives, dietary nutrients and pharmaceutical agents which could promote iron absorption. Further studies should be carried out to evaluate the iron-absorption efficiency *in vitro* and *in vivo* of BSFOP-Fe and investigate the iron-peptide chelating reaction using the purified peptide identified from BSFOP-Fe.

References

[1] Beard J L. Iron biology in immune function, muscle metabolism and neuronal functioning [J]. The Journal of Nutrition, 2001, 131(2): 568S-580S

[2] Blat D, Weiner L, Youdim M B H, et al. A novel iron-chelating derivative of the neuroprotective peptide NAPVSIPQ shows superior antioxidant and antineurodegenerative capabilities [J]. Journal of Medicinal Chemistry, 2007, 51: 126-134

[3] Chung Y C, Chang C T, Chao W W, et al. Antioxidative activity and safety of the 50 ethanolic extract from red bean fermented by bacillus subtilis IMR-NK1 [J]. Journal of Agricultural and Food Chemistry, 2002, 50(8): 2454-2458

[4] Swain J H, Tabatabai L B, Reddy M B. Histidine content of low-molecular-weight beef proteins influences nonheme iron bioavailability in Caco-2 cells [J]. The Journal of Nutrition, 2002, 132(2): 245-251

[5] Storcksdieck S, Bonsmann G, Hurrell R F. Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from *in vitro* digestion of different meat sources [J]. Journal of Food Science, 2007, 72: S19-29

[6] Murray-Kolb L E, Welch R, Theil E C, et al. Women with low iron stores absorb iron from soybeans [J]. The American Journal of Clinical Nutrition, 2003, 77: 180-184

[7] Torres-Fuentes C, Alaiz M, Vioque J. Iron-chelating activity of chickpea protein hydrolysate peptides [J]. Food Chemistry, 2012, 134(3): 1585-1588

[8] Liu J H, Tian Y G, Wang Y, et al. Characterization and in vitro antioxidant of papain hydrolysate from black-bone silky fowl (Gallus gallus domesticus Brisson) muscle and its fractions [J]. Food Research International, 2011, 44: 133-138

[9] Tian Y, Xie M, Wang W, et al. Determination of carnosine in black-bone silky fowl (*Gallus gallus* domesticus Brisson) and common chicken by HPLC [J]. European Food Research and Technology, 2007, 226: 311-314

[10] Song S, Gao F, Ren D, et al. Preparation and infrared spectrum identification of black-bone silky fowl oligopeptide-iron (Ⅱ) chelates [J]. Food and Fermentation Industries, 2013, 39(6): 13-17

[11] AOAC, Official methods of analysis. Association of official analytical chemist EUA [S]. 2001

[12] Yang Y, Tao G, Liu P, et al. Peptide with angiotensin I-converting enzyme inhibitory activity from hydrolyzed corn gluten meal [J]. Journal of Agricultural and Food Chemistry, 2007, 55: 7891-7895

[13] Gu R Z, Li C Y, Liu W Y, et al. Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from *Atlantic salmon* (*Salmo salar L.*) skin [J]. Food Research International, 2011, 44(5): 1536-1540

[14] Liu W Y, Lu J, Gao F, et al. Preparation, characterization and identification of calcium-chelating *Atlantic salmon* (*Salmo salar* L.) ossein oligopeptides [J]. European Food Research and Technology, 2015, 241(6): 851-860

[15] Wu J, Ding X. Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides [J]. Food Research International, 2002,

35(4): 367-375

[16] Gu R Z, Liu W Y, Lin F, et al. Antioxidant and angiotensin I-converting enzyme inhibitory properties of oligopeptides derived from black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle [J]. Food Research International, 2012, 49(1): 326-333

[17] Liu W, Gu R, Lin F, et al. Isolation and identification of antioxidative peptides from pilot-scale black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle oligopeptides [J]. Journal of the Science of Food and Agriculture, 2013, 93(11): 2782-2788

[18] Adibi S A, Phillips E. Evidence for greater absorption of amino acids from peptide than from free form in human intestine [J]. Clinical Research, 1968, 16: 446-448

[19] Zhu S Y, Li Y C, Jin L Q, et al. Research on combination between collagen polypeptide and calcium [J]. China Leather, 2005, 34(3): 26-29

[20] Grimble G K, Rees R G, Keohane P P, et al. Effect of peptide chain length on absorption of egg protein hydrolysates in the normal human jejunum [J]. Gastroenterology, 1987, 92: 136-142

现代食品科技