Preparation of corn ACE inhibitory peptide-ferrous chelate by dual-frequency ultrasound and its structure and stability analyses

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A R T I C L E   I N F O

Keywords:
Corn ACE inhibitory peptides
Peptide-ferrous chelate
Ultrasound
Structural characteristics
ACE inhibition activity

A B S T R A C T

In order to improve iron chelating ability and retain the activity of functional peptide, corn peptide was chelated with iron to form corn ACE inhibitory peptide-ferrous chelate (CP-Fe) treated by dual-frequency ultrasound. Furthermore, the chelating mechanism was revealed by analyzing various structural changes, and the stability was further evaluated. Under this study condition, the iron-binding capacity of corn ACE inhibitory peptide (CP) and chelate yield reached 66.39% and 82.87%, respectively. Ultrasound-treated CP exhibited a high iron chelating ability, meanwhile, chelation reaction had no significant effect on the ACE inhibition activity (82.21%) of the peptide. CP-Fe was formed by binding the peptides amino, carbonyl and carboxyl groups with Fe2+ demonstrated by Ultra-violet spectroscopy, Fourier transform infrared characterization, X-ray diffraction, energy dispersion spectrum, zeta potential, amino acid composition and other multi-angle analyses. Moreover, ultrasound-treated CP-Fe chelate exhibited porous surface and uniform nanoparticle shape. Furthermore, ultrasound-treated CP-Fe chelate exhibited an excellent stability towards various pH (retention rate ≥ 95.47% at pH 6–10), temperatures (retention rate ≥ 85.10% at 25–70 °C), and gastrointestinal digestion (retention rate 79.18%). Overall, ultrasound-treated CP-Fe chelate possessed high iron-chelating ability, ACE inhibition activity and stability. This study provides a novel synthesis method of the Iron-chelating corn ACE inhibitory peptide, which is promising to be applied as iron supplements with high efficiency, bioactivity, and stability.

1. Introduction

Iron is an important trace element, which has a certain influence on physiological activities such as participating in the transport of oxygen, maintaining normal hematopoietic function and the immune skills of the body [1]. However, the iron absorption efficiency in the intestine is quite low, ranging between 5% and 15% [2]. Moreover, trace metal elements generally exist in the form of inorganic salts, are easily affected by phytic acid and phosphoric acid, which have a low bioavailability [3]. The lack of iron in the body not only cause iron-deficiency anemia, but also lead to metabolic disorders [4]. Therefore, it is necessary to prepare good iron supplements to reduce the adverse symptoms caused by the iron deficiency.

Peptides are composed of modifiable group molecules, which can self-assemble with other structural units to form supramolecular structures through coordination bonds, ionic bonds, π–π stacking, hydrophilic, hydrophobic and electrostatic interactions [5]. Therefore, peptide-ferrous chelates can also be synthesized by self-assembly of ferrous ions and functional groups of peptides, which may carry both ferrous ion and active peptide functions and need to be deeply explored. On the one hand, peptide-ferrous chelates can improve the biological utilization of ferrous ions through peptide channels to promote multi-channel absorption of iron. On the other hand, it can effectively avoid the adverse effects of phytic acid and fiber on the utilization of free iron ions through its existence form of metal-chelating peptide. The improvement of peptide-ferrous chelates in iron bioavailability in vivo has been verified by several reports [6–7]. In addition, the unique physiological function of the peptide itself may not disappear after chelating with ferrous ions. Peptide-ferrous chelates may not only promote iron bioavailability, but also carry the functional properties of peptides. Hence, the peptide-ferrous chelate may have the dual nutritional effects of functional peptide activity and iron replenishing. However, there are no reports about more functional features of the peptide-ferrous chelates at present. Therefore, it is of great significance to explore this new type of biological nutrition agent.

As a by-product in corn starch wet processing, corn gluten meal has a
high surplus but is often discarded without any reutilization treatment, which results in a waste of corn resource. It is a potential raw material for the preparation of functional corn ACE inhibitory peptide because of its high protein content (~60%) and a high proportion of hydrophobic amino acids [8]. Corn ACE inhibitory peptide plays an important physiological role in the antihypertensive and anti-inflammatory activities [8–9], which has great research value and application prospects. Ultrasound has been found that it can be used to increase the yield and ACE inhibition activity of corn ACE inhibitory peptides [8]. Ultrasound can generate more cavitation, which can alter protein structure and improve enzymatic hydrolysis efficiency [8–9]. Hence, the peptide yield and activity can be increased by ultrasound treatment. However, there are no reports about the preparation of ferrous chelating corn ACE inhibitory peptide at present.

Therefore, our work focused on preparing a new ferrous chelating corn ACE inhibitory peptide (CP-Fe) treated by ultrasound to demonstrate its improved iron-binding capacity and ACE inhibition activity. Meanwhile, the structural properties of ultrasound-treated CP-Fe were investigated compared to the ultrasound-treated CP. Accordingly, stability testing of ultrasound-treated CP-Fe was measured in order to conduct a thorough assessment of the development prospects for iron supplements.

2. Materials and methods

2.1. Materials and reagents

Corn gluten meal was provided by Jiahui Feed Enterprise Store (Hebei, China). Neutrase with the activity of 1.61 × 10^5 U/mL and Alcalase with the activity of 2.69 × 10^5 U/mL were ordered from Nanjing Novozymes Biotechnology Co., Ltd. (Jiangsu, China). Other reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Preparation and purification of ultrasound-treated corn ACE inhibitory peptide

Ultrasound-treated corn ACE inhibitory peptide (CP) was prepared and purified by an enzymatic membrane reactor with the online dual-frequency ultrasound, gradient dilution feeding, and ultrafiltration membrane purification working-mode. The reaction system was developed by our research team and shown in Fig. 1.

(1) Corn gluten meal solution (80 mg/mL, pH 7.0) was pre-enzymatic hydrolyzed by Neutrase (ratio of Neutrase to corn gluten meal 2000 U/g) at 50 °C for 2.5 h. Subsequently, the solution was purified by an ultrafiltration device with the membrane size of 30 kDa (Pellicon 2 mini, Millipore Corporate, USA) to remove large molecule impurities and then the hydrolysate A was obtained.

(2) The hydrolysate A (volume 1.5 L, concentration 10.86 mg/mL, pH 8.5) was further hydrolyzed by Alcalase (ratio of Alcalase to hydrolysate A 7329 U/g) in the enzymatic hydrolysis reactor (N’1 in Fig. 1) at 40 °C for 5 h under an online dual-frequency ultrasound, gradient dilution feeding, and ultrafiltration membrane purification working-mode. Subsequently, the ultrasound-treated CP (purity ~ 95%) in the peptide collector (N’6 in Fig. 1) was collected and dried by a vacuum freeze dryer. In this working-mode, an online dual-frequency ultrasound in the ultrasonic reactor 8(N’11 in Fig. 1) was carried out in a continuous cycle using a peristaltic pump (N’3 in Fig. 1), as indicated by the red arrow. The ultrasonic probe operating parameters set by the ultrasonic generators (N’12 and 13 in Fig. 1) and the ultrasonic controller (N’14 in Fig. 1) were, notably, 150 W/L of power density, 20/28 kHz of synchronous dual-frequency, 66.7% of pulsed duty cycle, and four ultrasound cycles (work time 40 min, stop time 20 min). An online gradient dilution feeding working-mode was carried out following the green arrow’s instructions. The isothermal water and hydrolysate A (10.86 mg/mL) were pumped from water and substrate basins (N’9 and 10 in Fig. 1) into the N’1 using an automated batching and feeding device (N’8 in Fig. 1) programmed with a concentration gradient reduction of hydrolysate A from 10.86 mg/mL to 0. An online ultrafiltration membrane purification working-mode was carried out in a continuous cycle through an ultrafiltration device (N’5 in Fig. 1) with the membrane size of 3 kDa, as shown by the blue arrow. Additionally, to maintain volume equilibrium of the reaction system, the feeding speed of water and hydrolysate A and the outflow speed of ultrasound-treated CP were both set at 7 mL/min by adjusting the automatic batching and feeding device (N’8 in Fig. 1) and the pressure difference between the inlet and outlet pressure gauges (N’4 and 7 in Fig. 1). In addition, the temperature of the enzymatic hydrolysis cycle was controlled at 40 °C by circulating water in the water bath (N’2 in Fig. 1), as indicated by the dark purple arrow.

2.3. Preparation of ultrasound-treated CP-Fe

Ultrasound-treated CP-Fe chelate was prepared through the chelating reaction between the ultrasound-treated CP (concentration 10 mg/mL, pH 10) and the FeCl₂·4H₂O solution (ratio of CP to Fe 5:1, w/w)
protected by 20 mg/mL L-ascorbic acid at 20 °C for 40 min based on our previous optimization conditions. Subsequently, the reaction solution was centrifuged at 4000 × g for 10 min. The supernatant was mixed with four times the volume of absolute ethanol to precipitate for 60 min. The precipitate was collected after centrifugation at 4000 × g for 15 min, and dried by a vacuum freeze dryer. The ferrous content, chelate yield and ACE inhibition activity were determined.

2.4. Measurements of ferrous content and chelate yield

The ferrous ion mass in the ultrasound-treated CP-Fe was determined by the method reported by Zhang et al. [10] with some modifications. 1 mL of sample solution was mixed with 1 mL of 1 mol/L HCl solution, 1 mL of 100 mg/mL hydroxylamine hydrochloride, 1 mL of 1.2 mg/mL ferrozine and 5 mL of 100 mg/mL sodium acetate. The mixture reacted at 20 °C for 30 min, and the absorbance (y) was measured at 510 nm using a UV spectrometer. The ferrous ion mass (x, μg) in the sample was calculated according to the standard curve \( y = 0.004x - 0.0004 \). The ferrous content and chelate yield were calculated using Eq. (1) and (2).

\[
\text{ferrous content (\%)} = \frac{m_1}{m_0} \times 100
\]

\[
\text{chelate yield (\%)} = \frac{m_2}{m_1} \times 100
\]

where, \( m_0 \) is the ferrous ion mass added to the reaction system (μg), \( m_1 \) is the ferrous ion mass in the chelate (μg), \( m_2 \) is the chelate mass (μg), and \( m_3 \) is the mass of ultrasound-treated CP and FeCl\(_2\)-4H\(_2\)O added to the reaction system (g).

2.5. Measurement of ACE inhibition activity

The ACE inhibition activities of ultrasound-treated CP and CP-Fe were determined according to the method described by Qu et al. [11], directly relative to the antihypertensive activity. Each sample was analyzed three times in the HPLC test.

2.6. Measurement of Ultra-violet (UV) spectra

The ultrasound-treated CP and CP-Fe solutions with a 0.25 mg/mL concentration were dispersed and homogenized for 10 min, and the zeta potential distribution was determined by the laser particle size analyzer with the omega II sample pool. The experimental environment temperature is 25 °C.

2.10. Measurement of atomic force microscopy (AFM)

The ultrasound-treated CP and CP-Fe solutions (15 μL, 100 μg/mL) were dropped on the bubble-free and crack-free mica-sheet and dried for 12 h at 25 °C. The mica sheet was glued to a circular magnetic sheet (15 × 0.3 mm). The sample surface topography was observed and photographed by AFM device (Multimode 8, Bruker Corporation, Germany), the image was captured in PeakForce QNM mode, and the scanning range was 5 μm.

2.11. Measurement of particle size distribution

The ultrasound-treated CP and CP-Fe solutions with a 1 mg/mL concentration were dispersed and homogenized for 10 min. The particle size distribution was measured by a particle size laser (Litesizer 500, Anton Paar Corporation, Austria). The experimental environment temperature is 25 °C.

2.12. Measurement of zeta potential

The ultrasound-treated CP and CP-Fe solutions with a 0.5 mg/mL concentration were dispersed and homogenized for 10 min, and the zeta potential distribution was determined by the laser particle size analyzer with the omega II sample pool. The experimental environment temperature is 25 °C.

2.13. Measurement of amino acid composition

80 mg of ultrasound-treated CP and CP-Fe powders were hydrolyzed in 10 mL of 6 mol/L HCl (containing 0.1% (w/v) phenol) at 110 °C for 22 h. The hydrolysate was filtered with qualitative filter paper and diluted to 50 mL. Subsequently, 1 mL of diluted hydrolysate was concentrated twice using a vacuum centrifugal concentrator (5430 R, Eppendorf Corporation, Germany) at 48 °C and dissolved in 1 mL distilled water. The sample solution was filtered with a 0.45 μm filtration membrane to determine amino acid composition using an amino acid analyzer (S-433(D), Sykam Corporation, Germany).

2.14. Measurement of thermal stability

3 mg of ultrasound-treated CP and CP-Fe powders were weighed and measured by a thermal analyzer (STA449C, NETZSCH Corporation, German). Under N\(_2\) protection (flow rate 70 mL/min), the temperature was increased at 10 °C/min, and the test range was from 25 to 600 °C.

2.15. Measurement of the stability of ultrasound-treated CP-Fe to acidic and alkaline conditions

The pH of ultrasound-treated CP-Fe solution with a concentration of 5 mg/mL was adjusted to 2, 4, 6, 8 and 10, respectively, placed at a room temperature for 2 h, and then centrifuged at 4000 × g for 15 min. The supernatant was mixed with eight times the volume of absolute ethanol to precipitate for 1 h, and then centrifuged at 4000 × g for 15 min. The ferrous ion mass in the precipitate was determined using the method in section 2.4. The retention rate of ferrous iron was calculated using the Eq. (3):

\[
\text{retention rate (\%)} = \frac{m_1}{m_3} \times 100
\]

where, \( m_1 \) is ferrous ion mass in the precipitate (μg) and \( m_3 \) is ferrous ion mass in the ultrasound-treated CP-Fe solution (μg).
2.16. Measurement of the stability of ultrasound-treated CP-Fe to temperature

The ultrasound-treated CP-Fe solution with a 5 mg/mL concentration was respectively heated at 25, 40, 55, 70 and 85 °C for 2 h and then centrifuged at 4000 × g for 15 min. The supernatant was conducted with the same operation as in section 2.15, and the RC was determined using the same method.

2.17. Measurement of in vitro digestion stability of ultrasound-treated CP-Fe

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared by the method reported by Minekus et al. [12]. 10 mL of ultrasound-treated CP-Fe solution with a 10 mg/mL concentration was mixed with 8 mL of SGF, and the pH was adjusted to 3.0. The pepsin (0.134 g) and 5 μL CaCl₂ (0.3 mol/L) were added to the mixture and then reacted at 37 °C for 2 h. After SGF digestion, the pH was adjusted to 7.0, and the solution further reacted with 16 mL SIF, 0.2724 g porcine bile salt, 0.1 g trypsin and 40 μL CaCl₂ (0.3 mol/L) at 37 °C for 2 h. The ultrasound-treated CP-Fe solution after SGF-SIF digestion was centrifuged at 4000 × g for 15 min after inactivating the enzyme at 90 °C for 15 min. The supernatant was conducted with the same operation as in section 2.15, and the RC was determined using the same method. Moreover, the FeCl₂·4H₂O solution with the same ferrous content as the ultrasound-treated CP-Fe solution was used as the control.

2.18. Statistical analysis

All experiments were performed in at least triplicate, and the results were expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Significance was calculated using Duncan’s test. The significance level was established at p < 0.05.

3. Results and discussion

3.1. Preparation results

Three preparation experiments of ultrasound-treated CP-Fe chelate were carried out at the same conditions of 10 mg/mL CP, pH 10, ratio of CP to Fe 5:1, 20 mg/mL L-ascorbic acid, 20 °C and 40 min based on our previous optimization experiments. The measurements results of ultrasound-treated CP and CP-Fe are shown in Table 1. It was determined that the chelate ferrous content was 66.39 ± 1.49%, and the chelate yield was 82.87 ± 1.27%. In terms of the ferrous chelating content, ultrasound-treated CP-Fe was superior to some previously reported ferrous chelating peptides, such as mung bean peptide with the ferrous chelating activity of 19.24 μg/mg [13] and Pacific cod skin gelatin peptide with the ferrous chelating rate of 58.1 ± 0.4% [14]. This test showed that ultrasound-treated CP had a high ferrous chelating capacity. It was because that ultrasound generate more cavitation, which caused more active sites of the peptide to be exposed [8-9] and improved ferrous chelating capacity. Hence, the chelate ferrous content and yield were increased by ultrasound treatment. The ACE inhibition activity (82.21 ± 0.25%) of ultrasound-treated CP-Fe chelate had no significant difference compared to that (86.38 ± 0.35%) of ultrasound-treated CP under the same concentration of peptide (p > 0.05). It was concluded that ultrasound-treated CP had a high chelating ability with Fe²⁺; meanwhile, the iron-chelating reaction had no significant effect on the ACE inhibition activity of functional peptides. However, there are currently no reports about the effect of chelation reaction on peptide activity. As a result, our research is very valuable. It is of great significance to explore this new type of multifunctional metal-chelating peptide product. Meanwhile, since biological action is often dependent on the structure of the substance, later investigations studied the structure of ultrasound-treated CP-Fe chelate.

3.2. UV spectra analysis

UV absorption spectra of ultrasound-treated CP and CP-Fe chelate are shown in Fig. 2A. It was observed that ferrous ion chelating changed the UV absorption spectra. The maximum absorption peak of ultrasound-treated CP appeared at 200 nm, which corresponded to the n → π⁺ transition of C = O in the peptide bond [15]. While the maximum absorption peak of ultrasound-treated CP-Fe appeared at 196 nm with a significant blue shift, and its absorption intensity was significantly increased compared to those of ultrasound-treated CP. The blue-shift of the peak was due to the fact that the original structure of C = O changed after the ferrous ion chelating with C = O in the peptide, and so the n → π⁺ transition in the ultrasound-treated CP-Fe chelate was enhanced. In addition, the absorption intensity of ultrasound-treated CP-Fe at 275 nm was significantly increased by ferrous ion chelating compared to that of ultrasound-treated CP. The reason for the increased peak intensity at 275 nm was that the π → π⁺ transitions of the aromatic amino acid residues in the peptide were increased by the iron-chelating, resulting in a significant difference in UV absorption between ultrasound-treated CP and CP-Fe chelate. Similarly, Wang et al. [16] found a blue shift of the maximum absorption from 215 to 203 nm and an increased absorption around 250–300 nm in the UV spectrum after adding calcium in cucumber seed peptide. The shifting and intensity changes of peptide peaks in UV spectra indicated that ultrasound-treated CP interacted with ferrous ions through metal-binding sites, such as –CO–NH₂, –NH₂, –COOH and so on, to shape a new structure for CP-Fe chelate.

3.3. XRD spectra analysis

XRD is a technique to characterize the changes in the molecular crystallinity of substances. It can be seen from Fig. 2B that the ultrasound-treated CP displayed a characteristic diffraction peak at 2θ = 19.91° with a state of dispersion. It was indicated that the ultrasound-treated CP was in an amorphous state and had an irregular amorphous structure. Similarly, the octopus scraps peptides (OPS) in the study of Lin et al. [17] exhibited a strong characteristic diffraction peak at the 20 angle of 20.52°. After the chelation of ultrasound-treated CP and iron, the molecular crystallinity of ultrasound-treated CP-Fe chelate changed to 2θ = 12.22° with a narrow base, which indicated the formation of a new crystalline structure [17-19]. XRD is an analytical technique in the crystalline structure of the sample and hence the results of this study indicated the formation of a novel crystalline structure for ultrasound-treated CP-Fe chelate, different form the original CP.

3.4. FTIR spectra analysis

FTIR spectra of ultrasound-treated CP and CP-Fe chelate are shown in Fig. 2C. There was a clear difference in the infrared spectra, which indicated that some groups of amino acids reacted after chelating reaction between the peptides and the ferrous ions. The vibration frequency changed, resulting in the difference in absorption peak. Ultrasound-treated CP represented an absorption peak at 3350.35 cm⁻¹, corresponding to the stretching vibration of N–H band. It was found that the absorption peak of ultrasound-treated CP-Fe chelate shifted to 3339.24 cm⁻¹ with a redshift of 11.11 cm⁻¹ compared to that

| Table 1 | Measurements results of ultrasound-treated corn ACE inhibitory peptide (CP) and corn ACE inhibitory peptide-ferrous chelate (CP-Fe). |
|---------|---------------------------------------------------------------|
| Sample | Ferrous content (%) | Chelate yield (%) | ACE inhibition activity (%) |
| CP     | –  | –  | 86.38 ± 0.35%* |
| CP-Fe  | 66.39 ± 1.49 | 82.87 ± 1.27 | 82.21 ± 0.25%* |

* Different letters indicate significant differences (p < 0.05).
of ultrasound-treated CP. It was indicated that the N–H band of amino acids (such as N-terminal, Lys, Arg residues) participated in the ferrous chelation reaction; therefore, the stretching vibration intensity in the ultrasound-treated CP-Fe chelate became weaker. A similar study reported that the absorption peak of N–H band in the wheat germ peptide was redshifted after chelating with calcium [19]. The absorption peak of ultrasound-treated CP at 1667.04 cm\(^{-1}\), corresponding to the amide I band (1700–1600 cm\(^{-1}\)), was attributed to the stretching vibration of C = O [16]. Compared with ultrasound-treated CP, the absorption peak of the ultrasound-treated CP-Fe chelate was blueshifted from 1667.04 cm\(^{-1}\) to 1716.02 cm\(^{-1}\) after iron chelating. It was indicated that the vibration intensity of C = O in the ultrasound-treated CP-Fe chelate was increased, which was consistent with the conclusion of UV spectra (Section 3.2). The increased C = O bond vibration meant that the intramolecular hydrogen bond forces of the ultrasound-treated CP-Fe chelate was reduced, and more C = O provided the coordination site of ferrous ions. Malison et al. [20] also reported this migration phenomenon about the amide I band in the peptide-calcium chelate from chicken foot broth by-product. The absorption peak of ultrasound-treated CP at 1597.43 cm\(^{-1}\), corresponding to the amide II band (1600–1500 cm\(^{-1}\)), was attributed to the bending vibration of N–H [16]. The absorption peak of the ultrasound-treated CP-Fe showed a blue shift from 1597.43 cm\(^{-1}\) to 1601.14 cm\(^{-1}\), so N–H provided the coordination site of ferrous ions, which showed a pattern of change similar to the vibration intensity of C = O. Chen et al. [21] also found that the amide II band in the calcium-chelating Auxis thazard peptide was redshifted by the calcium chelation. The characteristic peak of the –COO- group in the ultrasound-treated CP-Fe redshifted from 1405.40 cm\(^{-1}\) to 1385.50 cm\(^{-1}\), indicated that the iron chelation site was also related to C-terminal carboxyl, Glu and Asp residues, which was similar to the results of Zhang et al. [10]. These results confirmed that –C = O, –NH–, –COOH groups were involved in the iron chelation reaction. In addition, the two absorption peaks of ultrasound-treated CP-Fe blueshifted from 1079.88 and 1028.47 cm\(^{-1}\) to 1166.53 and 1038.30 cm\(^{-1}\) after iron chelating, indicated that the stretching vibration intensity of C-O bond was enhanced by iron chelating [22]. This proved the formation of more ring structures of –COO-Fe- bond in ultrasound-treated CP-Fe. A similar blue-shift about C-O bond was found in mung bean peptide bound with iron [10]. Overall, FTIR spectra confirmed that ultrasound-treated CP interacted with iron to form a new chemical compound CP-Fe, which was different from the original CP. It was also confirmed that the carboxyl, carbonyl, and amino groups were the main chelating reaction sites of ultrasound-treated CP with ferrous ion. Similar studies reported that the principal binding sites of peptides were the carboxyl, carbonyl and amino groups in the oyster protein hydrolysates-zinc complex and whey peptide-calcium chelate [23–24].

### 3.5. SEM and EDS analyses

Fig. 3A and Fig. 3B showed the surface morphology of the
ultrasound-treated CP and CP-Fe. The surface of ultrasound-treated CP was relatively smooth, revealing a sheet-like structure (Fig. 3A). In contrast, the surface of ultrasound-treated CP-Fe (Fig. 3B) showed an apparent porous structure that was obviously different from that of ultrasound-treated CP, and similar to the surface microstructure characteristics of the calcium-chelating Auxis thazard peptide [21]. It was speculated to be related to the cyclic structure formed by the chelation reaction between the ultrasound-treated CP and the ferrous ion, which had been confirmed by FTIR spectra (Section 3.4). The ferrous ion was chelated with the peptide through the carboxyl, carbonyl and amino groups to form more cyclic structures; therefore, the surface microstructure of ultrasound-treated CP-Fe presented a large number of obvious pores [20]. Scanning electron microscopy (SEM) revealed the otherness between the peptide and peptide-ferrous chelate. The SEM difference of ultrasound-treated CP and CP-Fe indicated that the formation of peptide-ferrous chelate. Similarly, after chelating with zinc, oyster peptide-zinc nanocomposite and peptide revealed surface morphology differences [23].

The elemental composition of ultrasound-treated CP and CP-Fe were analyzed by EDS as shown in Fig. 3C and Fig. 3D. It can be clearly found that there was an iron peak in the ultrasound-treated CP-Fe, which confirmed that ferrous ion does indeed interact with the ultrasound-treated CP. Similarly, the appearance of the metal peak was also reflected in the EDS results of Fang et al. [22] and Wang et al. [16] in walnut meal hydrolysate-calcium chelate and cucumber seed peptide-calcium chelate. SEM and EDS are the analytical technique in the morphological characteristics and element composition of the sample and hence the results of this study confirmed that ultrasound-treated CP was bound to iron to form more porous CP-Fe chelate, which was similar to the results of UV, FTIR and XRD (Sections 3.2-3.4).

3.6. AFM analysis

The two-dimensional and three-dimensional images of ultrasound-treated CP and CP-Fe are shown in Fig. 4A and Fig. 4B. Their height and diameter were analyzed using the particle analysis function of Nanoscope-Analysis software. Fig. 4A clearly showed that the particles of ultrasound-treated CP were dispersed, and the particle size was relatively large with an average diameter and height of 6.60 and 191.80 nm, respectively. In contrast, the total number of particles of ultrasound-treated CP-Fe (Fig. 4B) was significantly increased, and the molecular size was significantly decreased (average diameter and height of 1.90 and 126.20 nm). It was due to the combination of ferrous ions with the peptides, resulting in the structural change and more cyclic structure formed (confirmed by the results of FTIR and SEM in sections 3.4 and 3.5), which caused an increase in the number of molecules and a smaller particle size of ultrasound-treated CP-Fe. AFM is an important method to measure surface morphology and size of particles to reflect the dispersion and aggregation tendency of the particles and hence the results of this study revealed that the chelation reaction made the surface morphology of the ultrasound-treated CP-Fe smaller and the number of particle aggregates increase.

3.7. Particle size distribution analysis

The particle size distribution curves of ultrasound-treated CP and CP-Fe are shown in Fig. 4C. It was found that compared with the particle size range (360.84–2520.77 nm) of ultrasound-treated CP with a hydrodynamic diameter of 1046.82 nm, the particles of ultrasound-treated CP-Fe were obviously concentrated in a smaller diameter range (91.06–586.63 nm) with the hydrodynamic diameter of 272.63 nm. This result indicated that the particle size of the ultrasound-treated CP-Fe
became smaller after the iron chelation reaction, which was complementary to the results of AFM (Section 3.6). The reason for the decreased diameter after iron-chelating was that the structural changes of the peptide occurred during the chelation of the peptide with the ferrous ion (confirmed by the results of UV and FTIR spectra in sections 3.2 and 3.4), which was in line with the research of Zhang et al. [23]. They found that zinc ions can bind to the zinc finger of oyster peptides, resulting in the folding of peptide structure during the chelation reaction. In addition, the polydispersity index (PDI) of ultrasound-treated CP and CP-Fe were 0.25 and 0.24 which were both < 0.3, indicating that the particle size distribution was uniform and stable [22]. The particle size distribution is an important parameter to measure the physical properties of composites and hence the results of this study revealed that ultrasound-treated CP-Fe showed a uniform monodispersity.

3.8. Zeta potential analysis

In order to further study the electrostatic interaction, the zeta potential analyses of ultrasound-treated CP and CP-Fe were performed and the results are shown in Fig. 4D. Compared with ultrasound-treated CP, the zeta potential of the ultrasound-treated CP-Fe was significantly changed from −17.24 mV to −2.65 mV (p < 0.05), reflecting the reduction of the negative charge on the surface of the chelate particles. This was similar to what was found by Sun et al. [25] that the zeta potential of the Antarctic krill peptides-zinc chelate changed from −29.20 to −10.20 mV. Studies have shown that the surface charge of the protein was related to the ionization of different amino acid residues in the polypeptide chain [26]. Athira et al. [27] also confirmed that the negative charge was due to the presence of acidic amino acids (Asp and Glu). The FTIR results (Section 3.4) revealed that acidic amino acids of ultrasound-treated CP were involved in the iron chelation reaction, and thereby, the zeta potential of the ultrasound-treated CP-Fe was
increased. Zeta potential is helpful to explore the potential mechanism of the electrostatic interaction between iron and peptides by reflecting the surface charge state of particles and hence the results of this study inferred that the oxygen atoms of the carboxy group and the nitrogen atoms of the amino group could interact with iron by donating electron pairs, and the electrostatic interaction was also responsible for the formation of the ultrasound-treated CP-Fe chelate.

3.9. Amino acid composition analysis

The amino acid compositions of ultrasound-treated CP and CP-Fe are shown in Table 2. The binding capacity of the peptide with metal is closely related to the types of amino acids. After chelating with ferrous ion, the contents of bound Asp, Glu and Lys in the ultrasound-treated CP-Fe were significantly increased. The contents of Asp and Glu in ultrasound-treated CP-Fe were greatly increased by 7.96% and 11.01% compared to those in ultrasound-treated CP. It was indicated that acidic amino acid residues played an important role in the metal chelation reaction, which could provide more active side chain groups, such as carboxyl groups that had metal-binding finger through electrostatic interaction or coordination [28–31]. Zhang et al. [23] also found that the contents of Asp and Glu increased in oyster peptide-zinc chelate. Moreover, the content of Lys in ultrasound-treated CP-Fe was greatly increased by 7.16% after the iron chelation. Zhang et al. [23] also found that the contents of Asp, Glu and Lys increased when preparing oyster peptide zinc chelate, and it was consistent with the conclusion obtained by Lee et al. [22] that higher contents of Lys, Asp and Glu in non-peptide have higher iron-binding capacity. Therefore, it appeared that Asp, Glu, and Lys residues were the major amino acids supporting the iron-binding. The results of the amino acid composition further confirmed the FTIR and zeta potential conclusions (Sections 3.4 and 3.8), in which carboxyl and amino groups played an important role in the iron chelation reaction and provided more active metal-binding finger through electrostatic interaction or electronic coordination. The amino acid composition plays an important role in peptide functions and hence the results of this study indicated that ultrasound-treated CP-Fe had high nutritional values and is suitable for functional food development.

3.10. Thermal stability analysis

The curves of thermogravimetric analysis (TGA) and derivative thermogravimetric (DTG) of ultrasound-treated CP and CP-Fe are shown in Fig. 5A and Fig. 5B. The melting point changes and chemical structure differences of the ultrasound-treated CP and CP-Fe can be determined by analyzing the pyrolysis rate in different temperature environments. Both ultrasound-treated CP and CP-Fe showed a certain degree of weight loss during the initial heating stage (25–130 °C), and the weight loss rate of the ultrasound-treated CP and CP-Fe at this stage was not significantly different, which was mainly due to the evaporation of free water in the substance. The weight loss rates of the ultrasound-treated CP and CP-Fe at this stage were similar and it reached the maximum at 65 °C. As the temperature continued to rise, ultrasound-treated CP and CP-Fe began to decompose rapidly. During the organic degradation process, the non-covalent bonds, including inter/intra-molecular hydrogen bonds, electrostatic and hydrophobic interactions, would go through some splitting. In addition, the covalent bonds in the amino acid such as C-N, C (O)-NH and C (O)-NH₂ would be broken with the increase in temperature. It was seen from the DTG curves that the ultrasound-treated CP had a significant weight loss at 157 °C and then reached the maximum decomposition rate at 279 °C, while the ultrasound-treated CP-Fe reached the first weight loss at 195 °C and was more stable after that. This result indicated that the thermostability of the ultrasound-treated CP-Fe was better than that of ultrasound-treated CP. The difference in heat resistance was due to the spatial conformation change of the chelate formation (confirmed by FTIR in section 3.4). Similarly, Lin et al. [17] found the thermostability of the OSP-Zn was better than that of OSP (Octopus scraps peptides).

3.11. The stability of ultrasound-treated CP-Fe to acidic and alkaline conditions

The stability of ultrasound-treated CP-Fe to acidic and alkaline conditions is shown in Fig. 5C. The retention rates of ferrous ion in the ultrasound-treated CP-Fe chelate at different pH reflected the obvious difference in the stability at acidic and alkaline conditions. In weak acidic and alkaline environments (pH 6–10), the ultrasound-treated CP-Fe chelate exhibited excellent stability, and the retention rates of iron were 95.47% and 95.71% at pH 6 and 10. However, in a strongly acidic environment (pH 2), the ultrasound-treated CP-Fe was unstable with an iron retention rate of 10.18%. Wu et al. [33] also reported a decrease in the calcium retention rate from 92.97% at pH 9 to 28.62% at pH 2 for the pig bone collagen peptide-calcium chelate. It was because that ferrous ion has a positive charge in an acidic environment, and H⁺, also positively charged, would compete with Fe²⁺ for the chelating site on the ultrasound-treated CP, resulting in the loss of part of the chelated Fe²⁺ [10]. In a strong alkaline condition, the negatively charged OH⁻ will bind to Fe²⁺, which will also cause iron levels to drop. In general, ultrasound-treated CP-Fe exhibited high stability under acidic and alkaline conditions (pH 6–10) with a higher retention rate of ferrous ion. The pH system of most common foods is between 5 and 9, which has little effect on the stability of ultrasound-treated CP-Fe chelate. Therefore, the ultrasound-treated CP-Fe in this study is applicable as an iron supplements and bioactive foods.

3.12. The stability of ultrasound-treated CP-Fe to temperature

Fig. 5D shows the stability of ultrasound-treated CP-Fe at different temperatures. In the range of 25–55 °C, the ultrasound-treated CP-Fe chelate exhibited excellent stability, and the retention rate of the iron was higher than 92%. Even at a high temperature of 70 °C, the ultrasound-treated CP-Fe still can maintain a chelated iron content of 85.10%. This stability change trend was similar to the result obtained by Xiao et al. [34] in the peanut peptide-ferrous chelator, which possesses good thermal stability within 10–60 °C and significantly lower ferrous chelation rate at a temperature higher than 60 °C. It was because the structure of ultrasound-treated CP-Fe was destroyed at a very high temperature, which led to the destruction of the binding site of the peptide with iron, resulting in worse stability. According to the above

Table 2

| Amino acids | CP (%) | CP-Fe (%) | Amino acids | CP (%) | CP-Fe (%) |
|-------------|--------|-----------|-------------|--------|-----------|
| Thr         | 3.96 ± 0.02 | 3.38 ± 0.01 | Ser         | 4.88 ± 0.01 | 5.24 ± 0.01 |
| Ala         | 7.37 ± 0.12 | 5.42 ± 0.02 | Glu         | 17.42 ± 0.12 | 28.43 ± 0.13 |
| Val         | 4.76 ± 0.01 | 2.72 ± 0.01 | Gly         | 3.93 ± 0.01 | 5.88 ± 0.02 |
| Met         | 2.49 ± 0.07 | 0.81 ± 0.01 | Cys         | 1.55 ± 0.01 | 2.06 ± 0.01 |
| Ile         | 4.18 ± 0.02 | 1.67 ± 0.01 | Tyr         | 4.87 ± 0.01 | 1.68 ± 0.03 |
| Leu         | 12.52 ± 0.13 | 3.01 ± 0.01 | His         | 6.11 ± 0.03 | 4.55 ± 0.01 |
| Phe         | 5.74 ± 0.01 | 1.94 ± 0.01 | Lys         | 3.17 ± 0.01 | 3.03 ± 0.01 |
| Pro         | 6.19 ± 0.04 | 3.05 ± 0.02 | Arg         | 3.23 ± 0.01 | 4.24 ± 0.01 |
| Asp         | 7.63 ± 0.06 | 15.59 ± 0.11 | Arg         | 0.92 ± 0.01 | 0.87 ± 0.01 |

*The values in parenthesis are the change of CP-Fe compared to CP.
results, it was concluded that the ultrasound-treated CP-Fe could maintain high heating stability (retention rate $\geq 85.10\%$) in a wide temperature range (25–70 $^\circ$C).

### 3.13. Digestion stability of ultrasound-treated CP-Fe in vitro

The retention rates of ferrous ion in the ultrasound-treated CP-Fe chelate after digesting by gastrointestinal enzymes are shown in Fig. 5E. In the in vitro gastrointestinal digestion process, ultrasound-treated CP-Fe contained significantly high digestion stability with a ferrous retention rate of 79.18% compared to the FeCl$_2$⋅4H$_2$O control (ferrous retention rate 23.11%) with an equivalent amount of ferrous ions ($p < 0.05$). It was indicated that the ultrasound-treated CP-Fe had a great stability in the gastrointestinal environment. This was consistent with the results obtained by Caetano-Silva et al. [28] that the iron-whey peptide complex had good stability to gastric digestion conditions. These results are also important because stable chelates potentially prevent the side effects related to free iron [35] and protect the gastrointestinal mucosa from damage caused by free iron [36].

### 4. Conclusions

In this study, corn ACE inhibitory peptide-ferrous chelate (CP-Fe) treated by dual-frequency ultrasound was successfully prepared and characterized to improve iron accessibility and ACE inhibition activity. Under this study condition, the ferrous content, yield, and ACE inhibition activity of the chelate were 66.39%, 82.87%, and 82.21%, respectively. Ultrasound-treated CP-Fe exhibited a high iron chelating capacity and ACE inhibition activity. Various structure analyses confirmed that the ultrasound-treated CP-Fe was formed by binding the peptides amino, carbonyl and carboxyl groups with ferrous ions. Ultrasound-treated CP-Fe showed high porous structure, uniform nanoscale particle size, and low negative charge properties. In terms of stability, the ultrasound-treated CP-Fe exhibited high stability towards various acidic and alkaline conditions (retention rate $\geq 95.47\%$ at pH 6–10), wide temperature ranges (retention rate $\geq 85.10\%$ at 25–70 $^\circ$C), and in vitro...
gastrointestinal digestion (retention rate 79.18%). The CP-Fe chelates in the present study are promising to be applied as iron supplements and bioactive peptide. It is necessary to test the absorption properties of the mineral-chelated peptides in vivo and explore novel bioactivities in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to extend their appreciation to the National Natural Science Foundation-China (31872892).