International Food Research Journal 30(2): 386 - 397 (April 2023)
Journal homepage: *http://www.ifrj.upm.edu.my*

Collagen peptide chelated zinc nanoparticles from tilapia scales for zinc supplementation

[1,2]*Guo, H. H., [1,2]Hong, Z. and [2]Yan, G. Y.

[1]*Technical Innovation Center for Utilization of Marine Biological Resources, Third Institute of Oceanography, Ministry of Natural Resources, No. 184, Daxue Road, Xiamen 361005, Fujian Province, China*
[2]*Xiamen Ocean Vocational College, No. 4566, Hongzhong Avenue, Xiamen 361100, Fujian Province, China*

Article history

Received:
14 October 2020
Received in revised form:
21 August 2021
Accepted:
16 November 2022

Keywords

tilapia scales, nanoparticle, collagen peptide, zinc supplementation, cytotoxicity

Abstract

Collagen peptide chelated zinc (CPCZ) nanoparticles were prepared using collagen peptide hydrolysate from tilapia scales. The nano-chelating ability of collagen peptide hydrolysates treated with ten enzymes was evaluated. Among these collagen peptide hydrolysates, trypsin hydrolysate exhibited the best nano-chelating ability. The molecular weight distribution of trypsin hydrolysate that produced CPCZ nanoparticles indicated that most of the peptides were less than 1,000 Da. These small molecular peptides with excellent nanozinc-chelating ability were chelated with zinc ions to form CPCZ nanoparticles. CPCZ nanoparticles were nearly spherical with an average diameter of approximately 100 nm, and a zinc content of 13.2%. Transmission electron microscopy coupled with energy dispersive spectroscopy and Fourier transform infrared spectrometry was used to measure the physicochemical properties of the CPCZ nanoparticles. Their cytotoxicity was also estimated by BHK21 cells. Result indicated that the CPCZ nanoparticles were non-toxic to BHK-21, and such nanoparticles significantly enhanced the survival of cells. The present work suggested that CPCZ nanoparticles could be used as zinc supplementation in the food and pharmaceutical industries.

DOI
https://doi.org/10.47836/ifrj.30.2.10

Introduction

Zinc is a crucial micronutrient in humans and animals (Zhu *et al.*, 2015). As an important bivalent element, zinc is a catalytic component of numerous enzymes, and it has structural and biological contributions for the normal development and maintenance of immune functions (Gatiatulina *et al.*, 2020). Zinc deficiency leads to many pathological problems such as growth defects, hypogonadism, dermal immune alterations, and neurological dysfunctions (Wang *et al.*, 2014). Zinc intake can be supplied from daily food. For example, marine products, cereals, and legumes contain abundant zinc. However, several factors can inhibit zinc absorption. For instance, fibres and phytates, which widely exist in many staple foods, can form insoluble complexes with zinc in the gastrointestinal tract (Chen *et al.*, 2013; Guo *et al.*, 2014). Therefore, the complexes cannot be absorbed and impair zinc absorption.

Many zinc supplements have been developed to avoid zinc deficiency. Zinc can be supplied in inorganic salts such as zinc sulphate. However, zinc salts are unsuitable for long-term intake because of their low bioavailability and gastrointestinal side effects (Jiang *et al.*, 2014). Recent studies have indicated that the chelates formed by peptides and zinc can enhance stability, zinc absorption, and bioavailability (Zhu *et al.*, 2013; Wang *et al.*, 2014). Collagen peptides are hydrolysed from collagen, contributing to the unique physiological functions of tissues in skins, tendons, bones, and cartilages (Ab-Aziz *et al.*, 2020; Salvatore *et al.*, 2020; Sungperm *et al.*, 2020). When compared with collagen, collagen peptides are more easily absorbed by humans because of their lower molecular weight (Fan *et al.*, 2016). Moreover, collagen peptides serve various physiological functions such as stimulating osteoblast growth and differentiation, reducing osteoclast differentiation, and promoting cell attachment and

*Corresponding author.
Email: hhguo@tio.org.cn

proliferation (Fan *et al.*, 2013). Fish by-products, including fish scales and skins, are an excellent raw material of collagen peptides because of their low cost and considerable collagen content. Collagen peptides obtained from fish by-products such as tilapia skin/scales (Chen *et al.*, 2014; 2017), Alaska pollock skin (Guo *et al.*, 2015b), and Pacific cod bone (Peng *et al.*, 2017) promote mineral absorption.

In recent years, nanoparticles have attracted considerable attention because of their unique physicochemical properties (Liang *et al.*, 2017; Tugarova and Kamnev, 2017). They have been widely used and shown great potential in biomedical sciences, engineering, agriculture, and food industry (Nguyen *et al.*, 2017). In the food industry, nutritional supplementation combined with nanotechnology can enhance the intestinal permeability and absorption of micronutrients (Wang *et al.*, 2020). Nanosupplements are more effective than common supplements because their small size makes them easily absorbed by human cells (Patra and Lalhriatpuii, 2020). In the present work, novel collagen peptide chelated zinc (CPCZ) nanoparticles were prepared. To the best of our knowledge, this could be the first work to investigate CPCZ nanoparticles from tilapia scales. Only collagen peptide hydrolysate treated with trypsin can be used to prepare CPCZ nanoparticles. The structure and cytotoxicity of the collagen peptide hydrolysate and CPCZ nanoparticles were also evaluated. The present work could provide a reference for the development of nanochelate-based nutritional supplementation.

Materials and methods

Materials

Collagen peptides from tilapia scales were purchased from Hainan Huayan Biotech Co. Ltd. (Hainan, China) without further purification. The composition was as follows: protein (94.53%), ash (0.32%), and moisture (5.15%). Papain (2×10^6 U/g), pepsin (4000 U/g), Alcalase (10^5 U/g), Flavourzyme (10^4 U/g), trypsin (4000 U/g), bromelain (10^5 U/g), neutral protease (10^5 U/g), acid protease (10^4 U/g), seafood hydrolase (10^5 U/g), and fish scale hydrolase (10^5 U/g) were obtained from Pangbo Biological Engineering Co. Ltd. (Nanning, China). Zinc sulphate was purchased from Xilong Scientific Co. Ltd. (Shantou, China). All chemicals used were of analytical reagent grade. Superdex peptide 10/300 GL and membrane elements were purchased from GE Company (Boston, USA). Standard proteins were purchased from Sigma (St. Louis, MO, USA).

Enzymolysis of collagen peptides

The purchased collagen peptides from tilapia scales were hydrolysed by ten enzymes namely papain, pepsin, Alcalase, Flavourzyme, trypsin, bromelain, neutral protease, acid protease, seafood hydrolase, and fish scale hydrolase. The degree of hydrolysis (DH) and metal-chelating ability are critical indicators. Briefly, 1 g of collagen peptides was suspended in 100 mL of ultrapure water. Then, the heating temperature and solution pH were adjusted to the reference value of each enzyme. One kind of enzyme was added (4,000 U/100 mL), and enzymolysis was continued for 6 h. The temperature of collagen peptide solution was elevated to 80°C, and retained for 20 min to terminate enzymolysis.

Degree of hydrolysis

DH was related to the cleavage of free amino groups from proteins, which can be determined by the ratio of α-amino nitrogen to total nitrogen (Wei *et al.*, 2018). Formaldehyde titration was used to determine the concentration of α-amino nitrogen (Chinese Standard, 2016; Noman *et al.*, 2018). Briefly, 5 mL of collagen peptide hydrolysate was added to 60 mL of ultrapure water. The pH was adjusted to 8.2. Then, 20 mL of neutralised formaldehyde was added, and the mixture was titrated with 0.1 mol/L of standard NaOH solution until pH 9.2. The titrated volume of NaOH solution was recorded, and the concentration of α-amino nitrogen $C_{\alpha\text{-amino nitrogen}}$ (mmol/L) was calculated using Eq. 1:

$$C_{\alpha\text{-amino nitrogen}} = [C_{NaOH} \times V_{NaOH}] / V_{sample} \times 10^3 \quad \text{(Eq. 1)}$$

where, C_{NaOH} = concentration of standard NaOH solution (0.1 mol/L), V_{NaOH} = titrated volume of standard NaOH solution (mL), and V_{sample} = used volume of collagen peptide hydrolysate (5 mL).

Metal-chelating ability

The metal-chelating ability of the collagen peptide hydrolysate was determined using the ferrous ion chelating assay described by Decker and Welch (1990) and Zhang *et al.* (2018). The collagen peptide hydrolysate was first diluted ten times with ultrapure water. Then, 100 μL of 2 mmol/L $FeSO_4$ and 200 μL of 5 mmol/L ferrozine were added to 5 mL of the

diluted collagen peptide hydrolysate. The mixture was shaken on a vortex mixer for 10 min. The absorbance of the mixture was obtained at 562 nm on a UH5300 spectrophotometer (Hitachi, Tokyo, Japan). The determination of each sample was performed in triplicate. The metal-chelating ability was calculated using Eq. 2:

$$\text{Metal chelating ability (\%)} = [1 - A_1 / A_0] \times 100 \quad \text{(Eq. 2)}$$

where, A_1 = absorbance of the sample at 562 nm, and A_0 = absorbance of the control at 562 nm.

Molecular weight distribution

The molecular weight distribution of collagen peptide hydrolysates was determined through gel filtration chromatography using an ÄKTA pure 150 system (General Electric [GE] Company, Boston, USA). A GE column, superdex peptide10/300GL, was equilibrated with 0.05 mol/L of phosphate solution (pH 7.0) at a flow rate of 0.5 mL/min at room temperature. Detection was conducted at the wavelength of 214 nm (Guo *et al.*, 2015a; Chen *et al.*, 2017). The standard proteins were cytochrome C (12,384 Da), aprotinin (6512 Da), bacitracin (1423 Da), $(Gly)_3$ (189 Da), and Gly (75 Da).

Preparation of CPCZ nanoparticles

The collagen peptide hydrolysate treated with trypsin was used to prepare CPCZ nanoparticles. The pH of the above-mentioned trypsin hydrolysate was adjusted to 4.0 by diluting H_2SO_4 solution. Zinc sulphate solution was then added dropwise into the trypsin hydrolysate to obtain CPCZ nanoparticles, and the molar ratio of trypsin hydrolysate to zinc was 1:1. The mixed solution was reacted at 70°C, and continuously stirred for 2 h. The as-prepared CPCZ nanoparticles were purified through a nanofiltration membrane with a molecular weight cut-off of 700 Da to remove impurities such as inorganic salts and unreacted small peptides.

Single-factor experiment

Single-factor experiments were conducted to optimise the preparation conditions of CPCZ nanoparticles (Fan *et al.*, 2016). The effects of four independent variables (reaction pH, reaction temperature, reaction time, and molar ratio of trypsin hydrolysate to zinc) on zinc content of CPCZ were investigated. During the experiment, only one factor was changed, whereas the three other factors were fixed.

Characterisation of CPCZ nanoparticles

The crude protein content of CPCZ was measured using the method of Kjeldahl (1883). Zinc content was determined using a TAS-990 flame atomic absorption spectrometer (Persee General Instrument Co. Ltd., Beijing, China) after the sample was digested by nitric/perchloric acids (4:1, v/v) (Guo *et al.*, 2015a). The morphology and composition were detected by transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Tokyo, Japan) with energy dispersive spectroscopy (EDS). Only 1 μL of sample solution was pipetted to a copper grid and air-dried at room temperature for TEM characterisation. Particle size distribution was measured by a Malvern laser particle size analyser (Nano-zs&MPT-2, Malvern Instrument Co. Ltd., Melvin, England). The infrared (IR) spectra of trypsin hydrolysate and CPCZ nanoparticles were performed using a Fourier transform infrared spectrometer (VER TEX70, Bruker Corporation, Karlsruhe, Germany). The test samples were prepared using the KBr-disk method. The mass ratio of sample to KBr was 1:50.

Cytotoxicity of CPCZ nanoparticles

Cytotoxicity test was performed according to Zhu *et al.* (2013) with slight modifications. The lyophilised samples of trypsin hydrolysate and CPCZ nanoparticles were first dissolved in minimal essential medium (MEM) to the concentration of 100 μg/μL, and then filtered through a 0.22 μm filter under sterilised conditions. Baby hamster kidney cells (BHK-21) were inoculated into 96-well culture plates at a density of 1.0×10^4 cells/mL (200 μL/well) in a CO_2 (5%) incubator at 37°C. After incubation for 24 h, the medium was discarded carefully. The sample solution was diluted 10^3 times, and 200 μL of the diluted sample was added to each test well. The control well was added with the normal cells without samples. After incubation for 24 and 48 h, the cell counting kit-8 (CCK-8) was added to each well, and the volume ratio of CCK-8 to MEM was 1:10. The absorbance of the solution was determined at 490 nm by using a microplate reader (MODEL550, Bio-Rad, Hercules, USA). The relative cell growth (%) was calculated using Eq. 3:

$$\text{Relative cell growth} = [OD]_{test} / [OD]_{control} \times 100 \quad \text{(Eq. 3)}$$

where, $[OD]_{test}$ = absorbance of the test well at 490 nm, and $[OD]_{control}$ = absorbance of the control well at 490 nm. The values were expressed as mean ± standard deviation (n = 3).

Statistical analysis

All experiments were performed in triplicate, and the results were presented as mean ± standard deviation (S.D.). All the data were analysed using SPSS 16.0. The level of significance was set at $p < 0.05$ for all statistical tests.

Results and discussion

Characterisation of peptides hydrolysed by different enzymes on degree of hydrolysis and metal-chelating ability

Collagen peptides from tilapia scales were hydrolysed by papain, pepsin, Alcalase, Flavourzyme, trypsin, bromelain, neutral protease, acid protease, seafood hydrolase, and fish scale hydrolase. The effects of enzymatic hydrolysis on $C_{\alpha\text{-amino nitrogen}}$ and metal-chelating ability are shown in Figure 1. In Figure 1, the $C_{\alpha\text{-amino nitrogen}}$ values of the collagen peptide hydrolysates treated with Flavourzyme and trypsin are higher than those of the collagen peptide hydrolysates treated with other enzymes. The collagen peptide hydrolysates treated with Alcalase and trypsin exhibited stronger metal-chelating abilities than the collagen peptide hydrolysates treated with other enzymes. Although the collagen peptide hydrolysate treated with Flavourzyme showed the highest $C_{\alpha\text{-amino nitrogen}}$, the metal-chelating ability decreased. Flavourzyme is a typical exopeptidase that can release free amino acids from peptides, which may decrease the metal chelating ability (Zhu *et al.*, 2015). However, Alcalase and trypsin are endopeptidases. Trypsin prefers to cleave at the C-terminal end of Lys and Arg residues (Wang *et al.*, 2014), and Alcalase prefers to catalyse the hydrolysis of residues with hydrophobic amino acids or aromatic acids at the C-terminal (Zhu *et al.*, 2015). Those residues cleaved by Alcalase and trypsin have high affinities to metal ions.

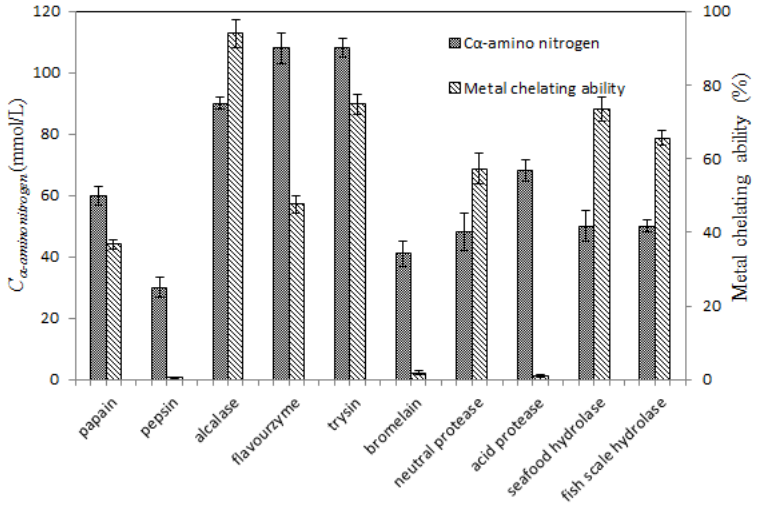

Figure 1. $C_{\alpha\text{-amino nitrogen}}$ and metal-chelating ability of collagen peptide hydrolysates treated with papain, pepsin, Alcalase, Flavourzyme, trypsin, bromelain, neutral protease, acid protease, seafood hydrolase, and fish scale hydrolase. Data are mean ± S.D. of triplicate determinations.

Molecular weight distribution

The molecular weight distribution of five standard proteins is shown in Figure 2A. On the basis of the relationship between the elution volume of peaks and molecular weights of standard proteins, the standard curve was plotted, and the molecular weight standard equation "$y = -0.23x + 6.4902$" was obtained, as shown in the inset of Figure 2A, where y was the lgM (M: molecular weight), and x was the elution volume. Based on the standard equation, the molecular weight of peptides can be calculated by the elution volume. The molecular weight distribution of the purchased collagen peptides from tilapia scales (Figure 2B) and the collagen peptide hydrolysates treated with Flavourzyme (Figure 2C), Alcalase (Figure 2D), and trypsin (Figure 2E) are also presented. The chromatogram indicates that the molecular weight of collagen peptides from tilapia

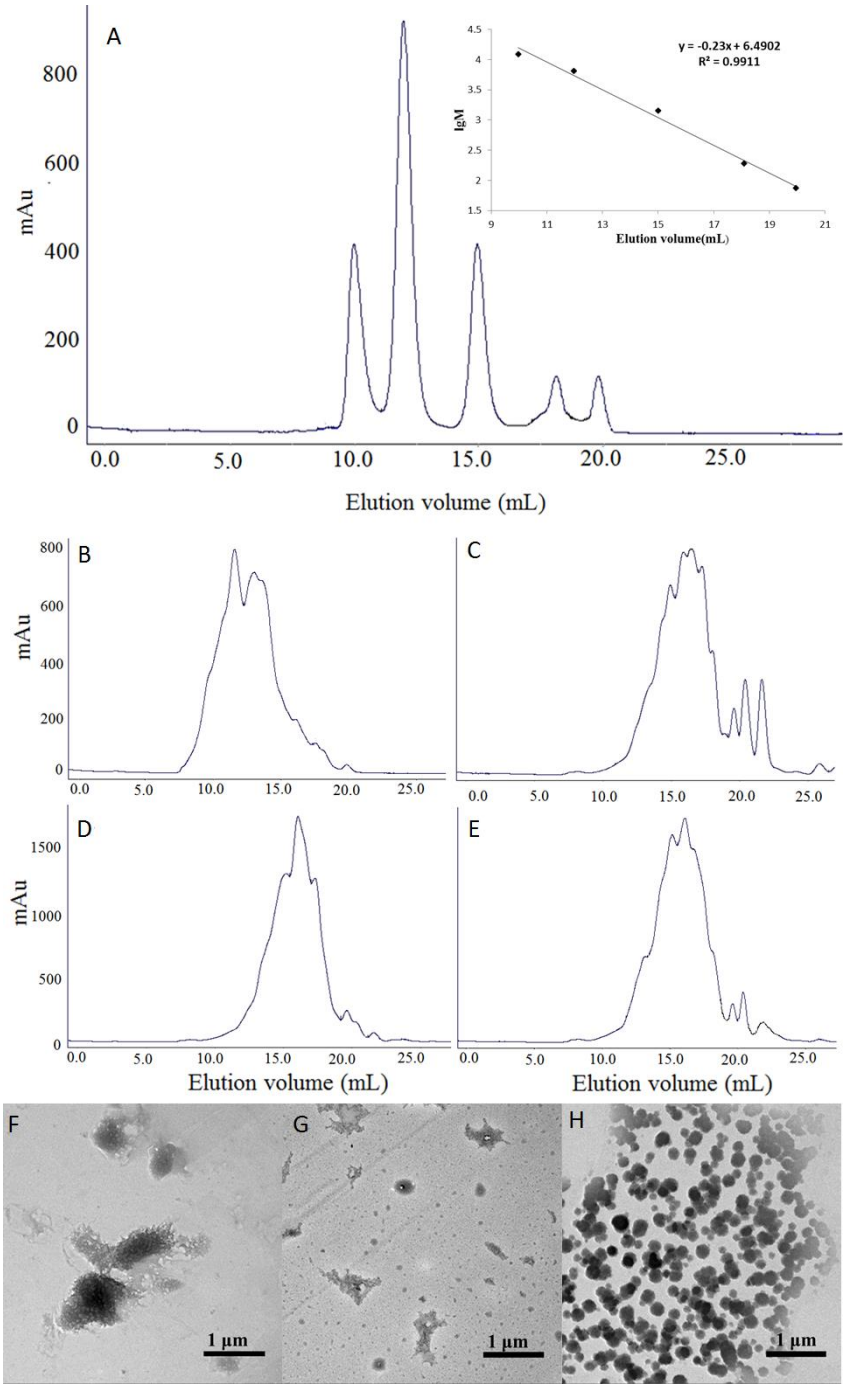

Figure 2. (A) Molecular weight distribution of five standard proteins (from left to right: cytochrome C, aprotinin, bacitracin, $(Gly)_3$, and Gly). Inset: standard curve between the elution volume of peaks and the molecular weights of standard proteins; molecular weight distribution of the purchased collagen peptides from tilapia scales **(B)** and collagen peptide hydrolysates treated with Flavourzyme **(C)**, Alcalase **(D)**, and trypsin **(E)**; TEM images of zinc chelates with collagen peptide hydrolysates treated with Flavourzyme **(F)**, Alcalase **(G)**, and trypsin **(H)**.

scales ranged from 75 Da to 10 kDa before hydrolysis, and most of the collagen peptides were between 200 and 7,000 Da. After hydrolysis, the molecular weight of the collagen peptide hydrolysates treated with Flavourzyme, Alcalase, and trypsin decreased, and most of the peptides were less than 1,000 Da. Moreover, Figure 2C shows that a small portion of the collagen peptide hydrolysate treated with Flavourzyme had molecular weight lower than 200 Da, which should be identified as free amino acids. Flavourzyme can consistently release free amino acids from peptides. The molecular weight distribution of the collagen peptide hydrolysate treated with pepsin ranged from 300 Da to 7,000 Da, which was much larger than other enzymes, thus indicating poor metal-chelating ability (Figure 1). The results indicated that molecular weight distribution was closely related to metal-chelating ability (Chen *et al.*, 2017). Low-molecular-weight peptides exhibit better chelating ability than large molecular peptides (Guo *et al.*, 2015b; Shakila *et al.*, 2016). As shown in Figures 2D and 2E, the collagen peptide hydrolysates treated with Alcalase and trypsin with high metal chelating ability were primarily composed of low-molecular-weight peptides less than 1,000 Da, thus indicating that they contained a large amount of peptides with ten or less amino acids (Sun *et al.*, 2015). Smaller molecular peptides contain more exposed side chains and higher surface charges, which provide more binding sites (Chen *et al.*, 2020). Metal-chelating abilities are influenced by the presence of suitable metal-binding sites in the peptides. Apart from molecular weight, metal-chelating abilities can also be affected by the enzyme used for hydrolysis as a result of the specific cleavage positions of enzymes on peptide chains. Different from Alcalase and Flavourzyme, trypsin showed a narrow substrate specificity. However, many studies have proven that trypsin was the most suitable enzyme to prepare a metal-chelating peptide primarily because of the high efficiency of trypsin and the good metal-chelating activity of trypsin hydrolysates (Wu *et al.*, 2017).

TEM characterisation

The collagen peptide hydrolysates treated with Flavourzyme, Alcalase, and trypsin were chelated with zinc to prepare CPCZ. TEM images of the zinc chelates with collagen peptide hydrolysates treated with Flavourzyme (Figure 2F), Alcalase (Figure 2G), and trypsin (Figure 2H) are presented in Figure 2. As shown in Figure 2H, the zinc chelate with collagen peptide hydrolysate treated with trypsin had a spherical shape and a uniform distribution, whereas the other zinc chelates showed a diffuse shape and no uniform particles. Therefore, only trypsin hydrolysate could form CPCZ nanoparticles. The trypsin hydrolysate may contain many Lys and Arg residues, which are all basic amino acids with nitrogen-rich groups (Peng *et al.*, 2017). Zinc ions with positive charges prefer to bind with peptide chains with electron-donating groups, and probably induce them to aggregate (Chen *et al.*, 2014). The low-molecular-weight trypsin hydrolysate may mediate the formation of CPCZ nanoparticles (Wu *et al.*, 2013).

Optimisation of the CPCZ nanoparticles formation/factors influencing the CPCZ nanoparticles formation

pH

Single-factor experiments were conducted to investigate the selected operational parameters that would affect the zinc content of the prepared CPCZ nanoparticles. The effects of different conditions on zinc content are shown in Figure 3. The effect of different pH levels (3, 4, 5, 6, and 7) on the zinc content was determined. As shown in Figure 3A, the zinc content significantly increased from 10.8 to 13.2% when the pH was increased from 3 to 4. Then, the zinc content gradually decreased when the pH was further increased to 7. However, the content evidently decreased when the pH value was adjusted to 8. Such a decrease in zinc content probably resulted from the increase in hydroxide ions, which led to zinc hydroxide precipitation. Moreover, pH was the most important factor affecting the formation and size of CPCZ nanoparticles. Weakly acidic and neutral pH values favour nanoparticle formation (Chen *et al.*, 2014). When the pH was increased from 4 to 7, CPCZ changed from a spherical and uniform shape to an irregular shape. At pH 8, no uniform particles were observed, and the CPCZ showed a diffuse shape. Therefore, the optimum reaction pH was 4.

Reaction temperature

As shown in Figure 3B, the zinc content of CPCZ increased from 11.5 to 13.1% as the reaction temperature was increased from 40 to 70°C. The increase in temperature possibly increased the activity of the reactants and the rate of the reaction (Fan *et al.*, 2016). However, the zinc content

decreased from 13.1 to 12.6% when the reaction temperature was further increased to 80°C. High temperature above 80°C did not facilitate the formation of CPCZ nanoparticles. Therefore, the optimal reaction temperature was 70°C.

Reaction time

The effect of different reaction times at 0.5, 1, 2, 4, 6, and 8 h on the zinc content was assessed to determine the optimum reaction time. As shown in Figure 3C, the zinc content gradually increased within the first 2 h. Then, the zinc content reached a plateau. The reason might be as follows: in the initial stage of chelation reaction, the nuclei of the CPCZ nanoparticles began to grow. Then, the CPCZ nanoparticles formed as the reaction time was prolonged. Finally, the chelation reaction reached dynamic equilibrium, and the zinc content did not increase with the extension of reaction time. On the basis of the obtained results, 2 h was selected as the suitable reaction time.

Molar ratio of trypsin hydrolysate to zinc

Different molar ratios of trypsin hydrolysate to zinc (1:3, 1:2, 1:1, 2:1, and 3:1) were set. As shown in Figure 3D, the zinc content increased as the molar ratio was increased from 1:3 to 1:1. The maximum zinc content was reached at the molar ratio of 1:1. However, the zinc content decreased when the molar ratio of trypsin hydrolysate to zinc was beyond 1:1. These results indicated that CPCZ nanoparticles formed when the molar ratio of trypsin hydrolysate to zinc was 1:1. Therefore, aside from the functional groups in trypsin hydrolysate, water might serve as a ligand participating in the coordination with zinc ion (Wang *et al.*, 2014).

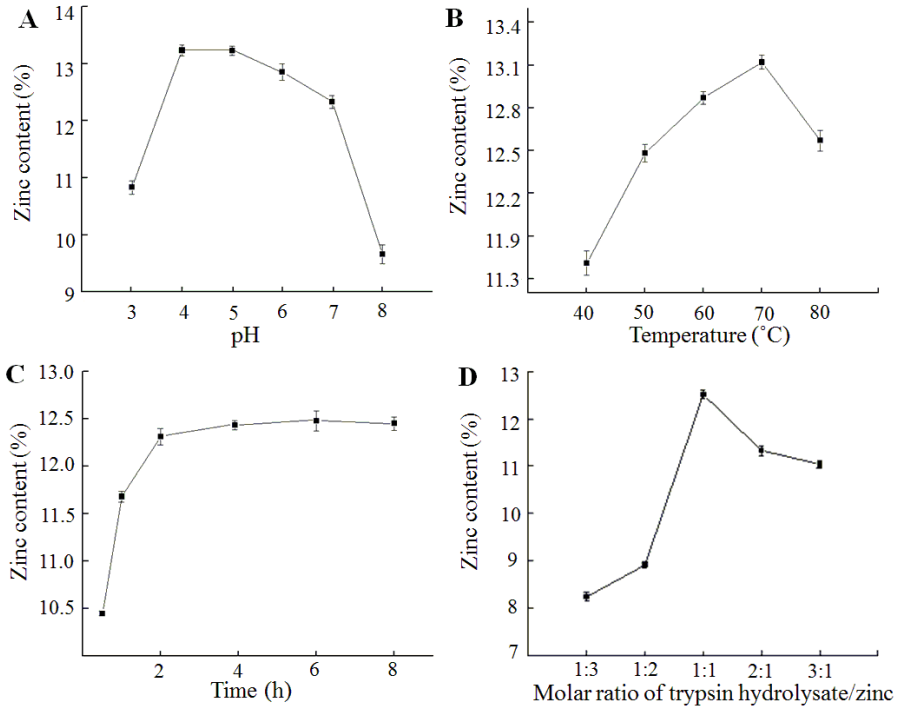

Figure 3. Effects of different conditions on zinc content: **(A)** pH, **(B)** temperature, **(C)** time, and **(D)** molar ratio of trypsin hydrolysate to zinc. Data are mean ± S.D. of triplicate determinations.

Characterisation of CPCZ nanoparticles

TEM and size distribution

TEM images of CPCZ nanoparticles in magnification of 2,000 and 10,000 are given in Figure 4. As shown in Figure 4, most of the CPCZ nanoparticles were nearly spherical with an average diameter of approximately 100 nm, and were well dispersed. The EDS pattern (Figure 4B inset) revealed that the CPCZ nanoparticles existed in an amorphous structure. Figure 4C shows the EDS pattern of a single CPCZ nanoparticle. This result indicated that the trypsin hydrolysate combined with zinc ions. Figure 4D shows the size distribution of the CPCZ nanoparticles, with an average size of approximately 110 nm. This result was almost consistent with the TEM images. The hydrated radius of the particles measured with a particle size analyser was slightly larger than that of the particles measured with an electron microscope (Guo *et al.*, 2015b).

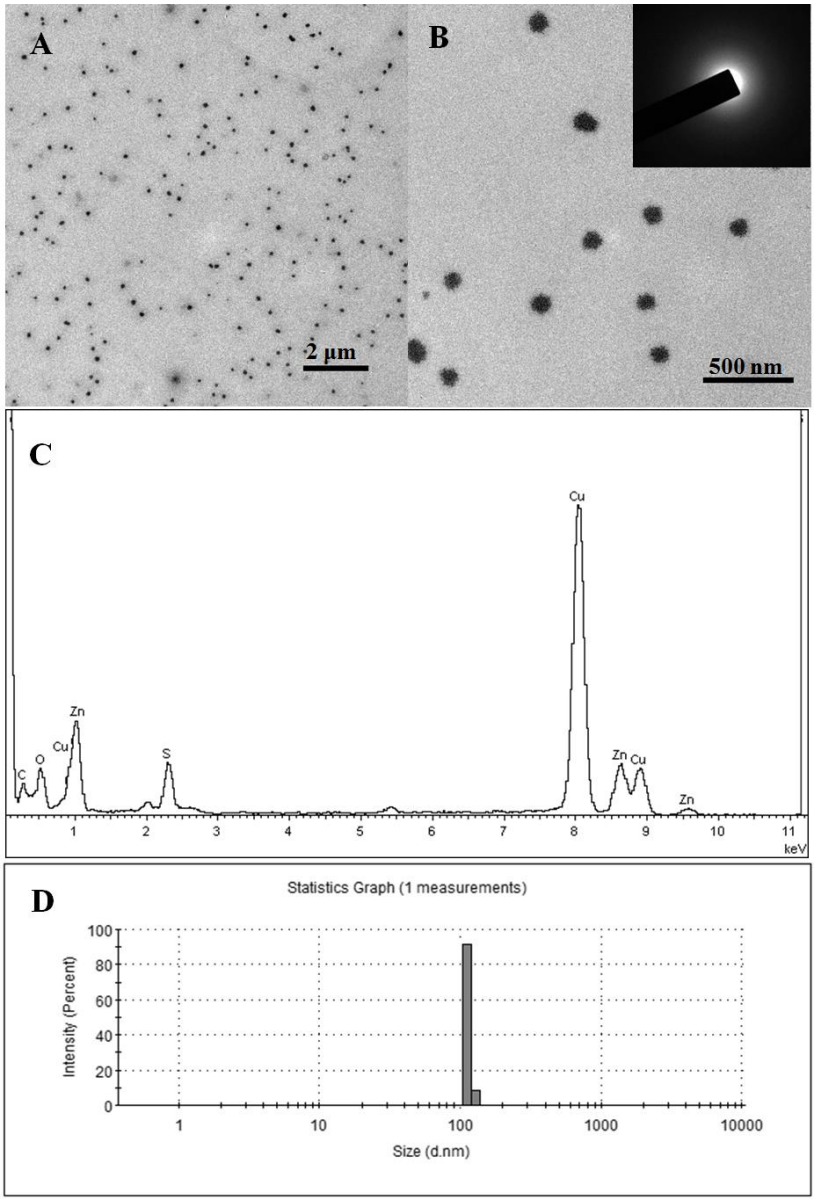

Figure 4. TEM images of CPCZ nanoparticles. **(A)** CPCZ nanoparticles at 2,000× magnification; **(B)** CPCZ nanoparticles at 10,000× magnification; Inset: electron diffraction pattern of a single CPCZ nanoparticle; **(C)** EDS pattern of a single CPCZ nanoparticle; and **(D)** size distribution of CPCZ nanoparticles.

Infrared analysis

The IR spectra of the trypsin hydrolysate and CPCZ nanoparticles are shown in Figure 5A. IR absorption peak changes could provide important information on the interaction between the ligand groups of the peptides and zinc ions. As shown in Figure 5A, after chelation with zinc, the absorption peak at 3416.4 cm^{-1} in peptide, attributed to the N-H stretching vibration (Wang and Xiong, 2018; Fang *et al.*, 2019), shifted to 3429.4 cm^{-1}, which was influenced by the hydrogen binding and coordination of the nitrogen to zinc (Zhu *et al.*, 2013). The absorption peak of peptide at 1651.2 cm^{-1} was characterised as an amide I band, and assigned to the vibrations of carbonyl groups of amide (Chen *et al.*, 2013). After chelation, it shifted to a lower frequency (1636.8 cm^{-1}), and the band at 1451.4 cm^{-1} corresponding to the carboxyl group (COO-) also moved to a lower frequency (1410.1 cm^{-1}) (Wang *et al.*, 2014; Peng *et al.*, 2017; Udechukwu *et al.*, 2018). The evident shift demonstrated that the C=O bonds in the peptide chain were weakened by the coordination

between C=O bonds and zinc ions. In addition, the absorption peak of peptide at 1396.2 cm^{-1} disappeared. The absorptions at 1400 - 1200 cm^{-1} was attributable to the C-N stretching vibration and N-H bending vibrations from amide linkages (Chen *et al.*, 2017). Therefore, the hydrogen bonds in the trypsin hydrolysate decreased after chelation. The N-H and C-N groups might participate in the coordination of Zn and peptide (Zhu *et al.*, 2013; Chen *et al.*, 2017). The trypsin hydrolysate might contain amounts of nitrogen-rich groups, thus contributing to zinc binding.

Cytotoxicity

The toxicity of trypsin hydrolysate and CPCZ nanoparticles was measured by cell experiments. Cytotoxicity test could rapidly and generally reveal a good correlation with other viability tests and *in vivo* results (Zhu *et al.*, 2013; Fan *et al.*, 2016). The results of cytotoxicity test are shown in Figure 5B. The trypsin hydrolysate and CPCZ nanoparticles did not promote BHK-21 proliferation at 24 h. However, at 48 h, the CPCZ nanoparticles increased the number of BHK-21 significantly, and showed an improved ability to promote cell proliferation when compared with the trypsin hydrolysate. Therefore, after chelation, the CPCZ nanoparticles were non-toxic to BHK-21 which significantly enhanced the survival of cells. These results indicated that CPCZ nanoparticles could be applied in zinc supplementation to avoid the deficiency of metal trace elements.

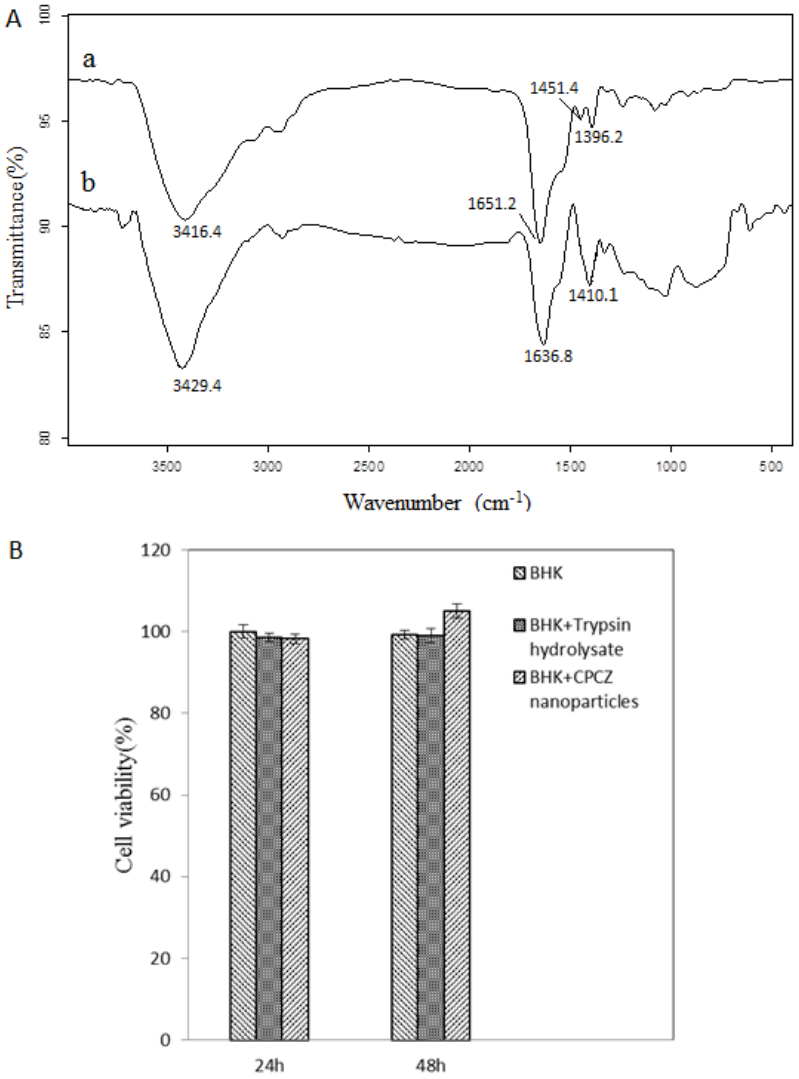

Figure 5. (A) Infrared spectra of trypsin hydrolysate (a) and CPCZ nanoparticles (b); **(B)** cell viability in trypsin hydrolysate and CPCZ nanoparticles after being cultured for 24 and 48 h. Data are mean ± S.D. of triplicate determinations.

Conclusion

In the present work, ten enzymes were used to hydrolyse collagen peptides from tilapia scales. Only trypsin hydrolysate could be used to form CPCZ nanoparticles. The molecular weight distribution of trypsin hydrolysate was less than 1,000 Da. These small molecular peptides could provide more suitable metal-binding sites than large molecular peptides. Apart from molecular weight, the specific cleavage position of trypsin was also the main influencing factor. The amino acid residues of trypsin hydrolysate had stronger nanozinc-chelating ability than other enzymes. A variety of amino acid residues in small molecular trypsin hydrolysate caused the formation of CPCZ nanoparticles. The optimum conditions of the formation of CPCZ nanoparticles were as follows: pH 4, 70°C for 2 h, and molar ratio of trypsin hydrolysate to zinc of 1:1. The IR spectra suggested that the C=O, N-H, and C-N groups were involved in zinc coordination. The cytotoxicity test showed that the trypsin hydrolysate and CPCZ nanoparticles were non-toxic to the BHK-21 cells. Moreover, the CPCZ nanoparticles could significantly enhance cell survival. The results indicated that the CPCZ nanoparticles would be a suitable option for zinc supplementation, which might be applied in functional foods or pharmaceutical preparations.

Acknowledgement

We are grateful for the financial support received from Xiamen Southern Oceanographic Center (22CZP008HJ10), Science and Technology Program of Fujian Province (2023), and Teaching Innovation Team Project of Xiamen Ocean Vocational College.

References

Ab-Aziz, N. A., Salim, N., Zarei, M., Saari, N. and Yusoff, F. M. 2020. Extraction, anti-tyrosinase, and antioxidant activities of the collagen hydrolysate derived from *Rhopilema hispidum*. Preparative Biochemistry and Biotechnology 51: 44-53.

Chen, D., Liu, Z., Huang, W., Zhao, Y., Dong, S. and Zeng, M. 2013. Purification and characterization of a zinc-binding peptide from oyster protein hydrolysate. Journal of Functional Foods 5: 689-697.

Chen, D., Mu, X. M., Huang, H., Nie, R. Y., Liu, Z. Y. and Zeng, M. Y. 2014. Isolation of a calcium-binding peptide from tilapia scale protein hydrolysate and its calcium bioavailability in rats. Journal of Functional Foods 6: 575-584.

Chen, J., Qiu, X. J., Hao, G. X., Zhang, M. and Weng, W. Y. 2017. Preparation and bioavailability of calcium-chelating peptide complex from tilapia skin hydrolysates. Journal of the Science of Food and Agriculture 97(14): 4898-4903.

Chen, L., Shen, X. R. and Xia, G. H. 2020. Effect of molecular weight of tilapia (*Oreochromis niloticus*) skin collagen peptide fractions on zinc-chelating capacity and bioaccessibility of the zinc-peptide fractions complexes in vitro digestion. Applied Sciences 10: 2041.

Chinese Standard. 2016. GB 5009.235-2016 - Determination of amino nitrogen in foods. China: Chinese Standard.

Decker, E. A. and Welch, B. 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. Journal of Agricultural and Food Chemistry 38: 674-677.

Fan, L. H., Cao, M., Gao, S., Wang, T., Wu, H., Peng, M., ... and Nie, M. 2013. Preparation and characterization of sodium alginate modified with collagen peptides. Carbohydrate Polymers 93: 380-385.

Fan, L. H., Tong, J., Tang, C., Wu, H., Peng, M. and Yi, J. Y. 2016. Preparation and characterization of carboxymethylated carrageenan modified with collagen peptides. International Journal of Biological Macromolecules 82: 790-797.

Fang, Z., Xu, L. Z., Lin, Y. L., Cai, X. X. and Wang, S. Y. 2019. The preservative potential of octopus scraps peptides−zinc chelate against *Staphylococcus aureus*: Its fabrication, antibacterial activity and action mode. Food Control 98: 24-33.

Gatiatulina, E. R., Sheina, E. A., Nemereshina, O. N., Popova, E. V., Polyakova, V. S., Agletdinov, E. F., ... and Tinkov, A. A. 2020. Effect of Zn supplementation on trace element status in rats with diet-induced non-alcoholic fatty liver disease. Biological Trace Element Research 197: 202-212.

Guo, H. H., Hong, Z. and Yi, R. Z. 2015a. Core-shell collagen peptide chelated calcium/calcium alginate nanoparticles from fish scales for

calcium supplementation. Journal of Food Science 80(7): 1595-1601.

Guo, L. D., Harnedy, P. A., Li, B. F., Hou, H., Zhang, Z. H., Zhao, X. and FitzGerald, R. J. 2014. Food protein derived chelating peptides: Biofunctional ingredients for dietary mineral bioavailability enhancement. Trends in Food Science and Technology 37: 92-105.

Guo, L. D., Harnedy, P. A., O'Keeffe, M. B., Zhang, L., Li, B. F., Hou, H. and FitzGerald, R. J. 2015b. Fractionation and identification of Alaska pollock skin collagen-derived mineral chelating peptides. Food Chemistry 173: 536-542.

Jiang, L. P., Wang, B., Li, B., Wang, C. and Luo, Y. K. 2014. Preparation and identification of peptides and their zinc complexes with antimicrobial activities from silver carp (*Hypophthalmichthys molitrix*) protein hydrolysates. Food Research International 64: 91-98.

Kjeldahl, J. 1883. New method for the determination of nitrogen in organic substances. Analytical and Bioanalytical Chemistry 22: 366-382.

Liang, J., Yan, H., Puligundla, P., Gao, X. L., Zhou, Y. B. and Wan, X. C. 2017. Applications of chitosan nanoparticles to enhance absorption and bioavailability of tea polyphenols: A review. Food Hydrocolloids 69: 286-292.

Nguyen, T. H. D., Vardhanabhuti, B., Lin, M. S. and Mustapha, A. 2017. Antibacterial properties of selenium nanoparticles and their toxicity to Caco-2 cells. Food Control 77: 17-24.

Noman, A., Xu, Y. S. and Al-Bukhaiti, W. Q. 2018. Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and functional properties of protein hydrolysate obtained from Chinese sturgeon (*Acipenser sinensis*) by using papain enzyme. Process Biochemistry 67: 19-28.

Patra, A. and Lalhriatpuii, M. 2020. Progress and prospect of essential mineral nanoparticles in poultry nutrition and feeding—A review. Biological Trace Element Research 197: 233-253.

Peng, Z., Hou, H., Zhang, K. and Li, B. F. 2017. Effect of calcium-binding peptide from Pacific cod (*Gadus macrocephalus*) bone on calcium bioavailability in rats. Food Chemistry 221: 373-378.

Salvatore, L., Gallo, N., Natali, M.L., Campa, L., Lunetti, P., Madaghiele, M., ... and Sannino, A. 2020. Marine collagen and its derivatives: Versatile and sustainable bio-resources for healthcare. Materials Science Engineering C 113: 110963.

Shakila, R. J., Fathiraja, P., Jeyasekaran, G. and Rajendran, S. 2016. Antioxidative properties of squid protein hydrolysates prepared using seer fish visceral enzymes in comparison with commercial enzymes. Journal of Aquatic Food Product Technology 25(7): 986-1000.

Sun, H., Yao, X. H., Wang, X., Wu, Y. F., Liu, Y., Tang, J. W. and Feng, J. 2015. Chemical composition and *in vitro* antioxidant property of peptides produced from cottonseed meal by solid-state fermentation. CyTA - Journal of Food 13(2): 264-272.

Sungperm, P., Khongla, C. and Yongsawatdigul, J. 2020. Physicochemical properties and angiotensin I converting enzyme inhibitory peptides of freshwater fish skin collagens. Journal of Aquatic Food Product Technology 29: 650-660.

Tugarova, A. V. and Kamnev, A. A. 2017. Proteins in microbial synthesis of selenium nanoparticles. Talanta 174: 539-547.

Udechukwu, M. C., Downey, B. C. and Udenigwe, C. 2018. Influence of structural and surface properties of whey-derived peptides on zinc-chelating capacity, and *in vitro* gastric stability and bioaccessibility of the zinc-peptide complexes. Food Chemistry 240: 1227-1232.

Wang, C., Wang, C., Li, B. and Li, H. X. 2014. Zn (II) chelating with peptides found in sesame protein hydrolysates: Identification of the binding sites of complexes. Food Chemistry 165: 594-602.

Wang, Q. L. and Xiong, Y. L. 2018. Zinc-binding behavior of hemp protein hydrolysates: Soluble versus insoluble zinc-peptide complexes. Journal of Functional Foods 49: 105-112.

Wang, T. T., Lin, S. Y., Cui, P. B., Bao, Z. J., Liu, K. X., Jiang, P. F., ... and Sun, N. 2020. Antarctic krill derived peptide as a nanocarrier of iron through the gastrointestinal tract. Food Bioscience 36: 100657.

Wei, C. K., Thakur, K., Liu, D. H., Zhang, J. G. and Wei, Z. J. 2018. Enzymatic hydrolysis of flaxseed (*Linum usitatissimum* L.) protein and

sensory characterization of Maillard reaction products. Food Chemistry 263: 186-193.

Wu, H., Liu, Z., Dong, S., Zhao, Y., Huang, H. and Zeng, M. 2013. Formation of ferric oxyhydroxide nanoparticles mediated by peptides in anchovy (*Engraulis japonicus*) muscle protein hydrolysate. Journal of Agriculture and Food Chemistry 61(1): 219-224.

Wu, W. F., Li, B. F., Hou, H., Zhang, H. W. and Zhao, X. 2017. Identification of iron-chelating peptides from pacific cod skin gelatin and the possible binding mode. Journal of Functional Foods 35: 418-427.

Zhang, Z. R., Zhou, F. B., Liu, X. L. and Zhao, M. M. 2018. Particulate nanocomposite from oyster (*Crassostrea rivularis*) hydrolysates *via* zinc chelation improves zinc solubility and peptide activity. Food Chemistry 258: 269-277.

Zhu, C. H., Sun, Y., Wang, Y. Y., Luo, Y. and Fan, D. D. 2013. The preparation and characterization of novel human-like collagen metal chelates. Materials Science Engineering C 33: 2611-2619.

Zhu, K. X., Wang, X. P. and Guo, X. N. 2015. Isolation and characterization of zinc-chelating peptides from wheat germ protein hydrolysates. Journal of Functional Foods 12: 23-32.