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Purification and characterisation of a zinc-binding peptide from oyster protein hydrolysate

Da Chen[1], *Zunying Liu*[1], *Wenqian Huang*, *Yuanhui Zhao*, *Shiyuan Dong*, *Mingyong Zeng*[*]

College of Food Science and Engineering, Ocean University of China, 5 Yushan Road, Qingdao, Shandong Province 266003, China

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ABSTRACT

A novel zinc-binding peptide produced from oyster protein hydrolysis using pepsin was purified and characterised. The hydrolysate was fractionated by immobilised metal ion affinity chromatography (IMAC-Zn^{2+}). The zinc-binding peptide identified by reverse-phase high-pressure liquid chromatography (RP-HPLC) and sequenced by liquid chromatography (LC/LTQ) mass spectrometry (sequence from N to C terminal) had a molecular weight of 1882.0 Da. The zinc-binding capacity of the peptide (HLRQEEKEEVTVGSLK) was 6.56 μg mg^{-1} and it was preserved at 85.98% of its original level upon *in vitro* simulated digestion. The UV–vis and FTIR spectra demonstrate that the amino nitrogen atoms and the oxygen atoms belonging to the carboxylate groups are the primary binding sites for Zn^{2+}. The results provide a feasible approach to isolate zinc-binding peptides and contribute to clarification of binding mechanism between zinc and peptides.

1. Introduction

As one of the most important micronutrients, zinc is involved in multiple functions related to the normal growth and survival of mammals. This is an essential cofactor for numerous metalloenzymes, which regulate gene expression, cellular proliferation and the resistance of the immune system (Anuraj & Ananda, 1998). The main source of zinc is provided by nutrition. However, zinc deficiency has become more and more widespread as a result of the presence of several zinc absorption inhibitors in food, such as phytates and fibre (Hambidge, 2000).

The bioavailability of zinc largely depends on the amount of soluble zinc in the duodenum (Salovara, Sandberg, & Andlid, 2002), where most of the zinc absorption occurs. Consequently, zinc binding to complexes such as proteins or polysaccharides is likely the predominant form that can increase the amount of soluble zinc (Camara & Amaro, 2003). The formation of stable complexes with unique characteristics could enhance the efficiency of zinc absorption. The zinc concentration in the tibia ash of broiler chickens whose feed was supplemented with a mixture of amino acids and $ZnSO_4$ was higher than those supplemented only with $ZnSO_4$ (Gheisari, Fathkoohi, Toghyani, & Gheisari, 2011). Similarly, the intestinal absorption of zinc bound to soluble caseinophosphopeptide (CPP) was significantly higher than the absorption of $ZnSO_4$ alone (Peres et al., 1998), which partly because CPP-zinc complex could either eliminate the inhibition of zinc absorption caused by other mineral elements such as Fe or Ca, or share the absorption channel with peptides or proteins, or prevent zinc precipitation induced by precipitant when passing through intestine (Ashmead, 1993; Peres et al., 1998). In contrast to Zn^{2+}, the zinc–peptides complexation in a yak casein hydrolysate improved its solubility and dialysability under *simulated* intestinal conditions and contributed to a higher bioavailability (Wang, Zhou, Tong, & Mao, 2011).

[*] *Corresponding author.* Tel.: +86 532 8203 2783; fax: +86 532 8203 2400.
E-mail address: mingyz@ouc.edu.cn (M. Zeng).

[1] These authors contributed equally to the work and should be considered joint first authors.

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Oyster is one of the most readily available and widely distributed marine biological resources worldwide whose annual production in 2010 was approximately 4.59 million tonnes according to the statistics of Food and Agriculture organisation of the United Nations (FAO) (Food & Agriculture Organization (FAO), 2012). Oysters contain considerable levels of proteins and mineral elements, especially zinc, which can bind to ligands such as proteins or amino acids in their soft tissue (Sharama, 1983). Glutamic (Glu), leucine (Leu), arginine (Arg) and aspartic acid (Asp), which are present in high proportions in oyster meat (Dong et al., 2010), might exhibit strong metal binding affinities (Lee & Song, 2009; Lv, Bao, Tang, Yang, & Guo, 2009). Despite the widely investigation of bioactive peptides derived from marine organisms in recently years (Harnedy & Fitzgerald, 2012; Kim & Wijesekara, 2010), the zinc-binding peptide production from oyster protein hydrolysis and its further extraction has never been investigated. The aim of this study was to isolate and identify a novel zinc-binding peptide in oyster protein hydrolysates using immobilised metal ion affinity chromatography (IMAC-Zn^{2+}), reverse-phase high-performance liquid chromatography (RP-HPLC) and liquid chromatography coupled to mass spectrometry (LC/LTQ). The change in the zinc-binding capacity of the peptide under *in vitro* simulation of gastrointestinal digestion was also investigated in this work. Furthermore, the interaction between identified peptide and zinc was studied by spectrometry of zinc–peptide complexation. This work could provide a reference for the development of oyster protein-based zinc supplements.

2. Materials and methods

2.1. Materials

Live oysters were obtained from a local aquatic product market (Qingdao, China). Pepsin (porcine gastric pepsin, activity 20 units mg^{-1} protein) was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Trypsin (powder, porcine 1:250 >250 USP units mg^{-1}), α-chymotrypsin (from bovine pancreas, >40 units mg^{-1}), and crystalline bovine albumin were purchased from Sigma Chemical Co. (Sigma–Aldrich Inc., St. Louis, MO, USA). Sepharose 6B was offered by Pharmacia (Uppsala, Sweden). Bio-Gel P-2 gel was obtained from BIO-RAD laboratories Co., Ltd. (Hercules, CA, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of oyster protein hydrolysates

The soft tissue removed from the oyster was cut up and homogenised with deionised water (the conductivity was about 1–1.5 μS cm^{-1}) at the ratio of 1:3 (soft tissue: water, w/w). Then, the homogenate was denatured in a boiling water bath to inactivate the endogenous enzymes and the crude protein content of it was determined by Kjeldahl method (Kjeldahl, 1883). Thereafter, the homogenate was adjusted to pH 1.8 with 2 M HCl and hydrolysed using pepsin at a ratio of 1.5:100 (enzyme: substrate, w/w, protein basis) at 40 °C for 5 h. At various time of hydrolysis, an aliquot (mL) hydrolysate was collected for zinc-binding capacity determination. Pepsin was inactivated by boiling for 10 min, and the sample was kept at room temperature for 30 min. Afterwards, the mixture was neutralised to pH 7.0 using 2 M NaOH and centrifuged at 3000*g* for 20 min at 4 °C. Supernatants from hydrolysate aliquots collected at various time of hydrolysis (between 0 and 300 min) were filtered through 0.45 μm Millipore filters to collect soluble peptide and were lyophilised. The experiment was triplicated.

2.3. Degree of hydrolysis

The degree of hydrolysis (DH) at different hydrolysis times was monitored by reacting the free amino acid groups with o-phthaldialdehyde (OPA) in the presence of beta-mercaptoethanol. Then, the optical density (OD) of the hydrolysate was determined at 340 nm (Nielsen, Petersen, & Dambamann, 2001). The total amount of amino acids in the homogenate was determined by completely hydrolysing the sample with 6 N HCl for 24 h at 120 °C prior to analysis and the OD total was determined. Deionised water was used as the blank. The DH was calculated using the formula shown below:

$$\mathrm{DH}\ (\%) = (\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{blank}})/(\mathrm{OD}_{\mathrm{total}} - \mathrm{OD}_{\mathrm{blank}} \times 100\%) \quad (1)$$

2.4. Zinc-binding peptide extraction and characterisation

2.4.1. IMAC-Zn^{2+}

IDA-Sepharose 6B was prepared by the method of Porath and Olin (1983). A mixture of 2 g wet weight of IDA-Sepharose 6B and 20 mL of 50 mM $ZnSO_4$ was shaken at 130 rpm for 30 min at room temperature. Then, the gel was packed into a column (1 × 13 cm) and washed with 10 column volume (CV) elution buffer (20 mM sodium acetate/500 mM NaCl, pH 4.0), to remove the unbound and weakly bound zinc. The column was then equilibrated with 5 CV binding buffer (20 mM sodium phosphate/500 mM NaCl, pH 7.2). Afterwards, 0.5 g of lyophilised pepsin hydrolysate dissolved in 10 mL of pH 7.2 binding buffer was loaded into the IMAC column and incubated at 30 °C overnight. Finally, the zinc-binding peptides were isolated with step elution. The pH of 20 mM sodium phosphate was reduced stepwise from pH 7.2 (15 CV) to pH 6.0 (10 CV) to finally reach pH 4.0 (10 CV). A solution of 50 mM ethylene-diamine-tetra-acetic acid (EDTA) was used for gel regeneration.

2.4.2. RP-HPLC

Peptides tightly bound to Zn^{2+}-IMAC phase and eluted using 20 mM sodium acetate (pH 4.0) were dissolved in deionised water and filtered through a 0.22 μm membrane (Millipore, MA, USA). Then, 250 μL sample was applied to the RP-HPLC system equipped with a ZORBAX SB-C18 column (5 μm, 9.4 × 240 mm, Agilent Technologies, Santa Clara, CA, USA) for further separation. Mobile phases A and B were Milli-Q water and acetonitrile, respectively, and both contained 0.1% TFA. Linear gradient elution with a flow rate of 1.5 mL min^{-1} was performed for the first 15 min from 95/5 (A/B) to 80/20 before changing to 55/45 over the last 45 min. The absorbance of the eluent was monitored at 220 nm. Fractions corresponding to major peaks were collected and lyophilised immediately.