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Contributions of molecular size, charge distribution, and specific amino acids to the iron-binding capacity of sea cucumber (*Stichopus japonicus*) ovum hydrolysates

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ABSTRACT

This study investigated the contributions of molecular size, charge distribution and specific amino acids to the iron-binding capacity of sea cucumber (*Stichopus japonicus*) ovum hydrolysates (SCOHs), and further explored their iron-binding sites. It was demonstrated that enzyme type and degree of hydrolysis (DH) significantly influenced the iron-binding capacity of the SCOHs. The SCOHs produced by alcalase at a DH of 25.9% possessed the highest iron-binding capacity at 92.1%. As the hydrolysis time increased, the molecular size of the SCOHs decreased, the negative charges increased, and the hydrophilic amino acids were exposed to the surface, facilitating iron binding. Furthermore, the Fourier transform infrared spectra, combined with amino acid composition analysis, revealed that iron bound to the SCOHs primarily through interactions with carboxyl oxygen of Asp, guanidine nitrogen of Arg or nitrogen atoms in imidazole group of His. The formed SCOHs-iron complexes exhibited a fold and crystal structure with spherical particles.

1. Introduction

Iron is an essential microelement that constitutes the spatial structure of hemoglobin, cytochromes, and enzymes, and is involved in a wide variety of biological functions, including respiration, energy metabolism regulation and cell proliferation (Puig, Askeland, & Thiele, 2005). However, iron deficiency affects more than two billion people worldwide and remains the top cause of anemia (McLean, Cogswell, Egli, Wojdyla, & De Benoist, 2009). The reported prevalence of iron deficiency in the absence of dietary fortification is approximately 40% in preschool children, 30% in menstruating girls and women, and 38% in pregnant women (Stevens et al., 2013). These rates reflect the increased physiological need for dietary iron during specific life stages and according to sex (Camaschella, 2015).

Dietary iron is available mainly in two forms: heme and non-heme iron. Heme iron, which only comes from haemoglobin and myoglobin in animal-based food, can be absorbed in the duodenum with a 15–35% absorption rate (Pereira & Vicente, 2013). The absorption rate of dietary non-heme iron is only below 10% (Martınez-Navarrete, Camacho, Martınez-Lahuerta, Martınez-Monzó, & Fito, 2002). The poor absorption of non-heme iron is attributed to its poor solubility at near-neutral pH and its interactions with food components, mainly phytic acid, polyphenols, and fibres (Hurrell & Egli, 2010). It is known that some dietary compounds, such as certain amino acids (His, Glu, Asp, and Cys) and peptides released during proteolytic digestion enhance iron absorption (Storcksdieck, Bonsmann, & Hurrell, 2007). These compounds may bind with iron, forming soluble complexes and thus improving iron absorption.

Various studies have revealed the beneficial effect on iron binding by peptides produced via enzymatic hydrolysis of various proteins, such as casein, whey protein, muscle protein and soy protein (O'Loughlin, Kelly, Murray, FitzGerald, & Brodkorb, 2015; Storcksdieck et al., 2007; Wang, Li et al., 2011; Wu, Liu, Zhao, & Zeng, 2012; Zhang, Huang, & Jiang, 2014). Enzymatic hydrolysis is a commonly used method for producing bioactive peptides (Sun et al., 2016). However, the choice of proteolytic enzyme seems to be crucial to the iron-binding capacity, because its speci-

Abbreviations: SCO, sea cucumber ova; SCOHs, sea cucumber ovum hydrolysates; DH, degree of hydrolysis; HPLC, High Performance Liquid Chromatography; UV–Vis, UV–visible; FTIR, Fourier transform infrared spectroscopy; Asp, aspartic acid; Arg, arginine; His, histidine.

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fic action will influence the final composition of the hydrolysis products, mainly the average peptide length and exposure of the side chains (Adler-Nissen, 1986). Therefore, it is known that enzyme type and degree of hydrolysis will significantly influence the iron-binding capacity of the hydrolysates (Wang, Li et al., 2011; Zhang et al., 2014; Zhou et al., 2012). However, the change in peptide size, charge distribution, and polar groups during the process of hydrolysis, as well as their relationships with the iron-binding capacity of the hydrolysates, remain to be clarified. Regarding the iron-binding sites of peptides, studies with iron-peptide complexes have revealed that the major iron-binding sites correspond primarily to the carboxyl groups of Asp and Glu (Huang, Ren, & Jiang, 2011; O'Loughlin et al., 2015). Sulphydryl groups and nitrogen-rich groups, such as the amino group of Lys, imidazole of His, and guanidine of Arg may also be involved in iron binding (Nicholson et al., 1997; Torres-Fuentes, Alaiz, & Vioque, 2012).

Sea cucumber (*Stichopus japonicus*) is one of the most important holothurian species in coastal fisheries. The aquaculture of sea cucumber has increased rapidly in Asia in recent decades (Anderson, Flemming, Watson, & Lotze, 2011). The total output of sea cucumber has exceeded 200,000 tons in China (China Fishery Statistical Yearbook, 2015). Sea cucumber ova are deemed as low-value by-products generated during industrial processing, and remain to be fully utilized as value-added materials. In our previous work, we found that defatted sea cucumber ova are rich in protein (80.9 ± 2.2% protein content) and that the relative Asp and Glu contents could reach 23% (unpublished data). It is speculated that sea cucumber ova may be a good source of metal-binding peptides. Hence, the aim of this study was to produce iron-binding peptides from sea cucumber ova though enzymatic hydrolysis and to investigate the dynamic variations in molecular size, charge distribution, and exposure of specific amino acids during the hydrolysis process to clarify their contributions to the iron-binding capacity of the hydrolysates. Further, the structural and physical characteristics of complexes of sea cucumber ovum hydrolysates and iron were investigated. This may aid immensely in the understanding of the nature of iron-binding peptides and associated iron binding mechanisms.

2. Materials and methods

2.1. Materials

Sea cucumber ova (SCO) were obtained from the Shangpintang Marine Biology Co., Ltd. (Dalian, China). Alcalase 2.4 L and neutrase 2.4 L were donated by Novozyme (Bagsvaerd, Denmark). Trypsin (from porcine pancreas) was purchased from Bio Basic Inc. (Toronto, Canada). Papain (a cysteine protease from papaya latex) and flavourzyme were obtained from Sangon Biotech Co. Ltd (Shanghai, China) and Pangbo Biological Engineering Co. Ltd (Nanning, Guangxi, China), respectively. All other chemicals and reagents used in this study were of analytical grade and commercially available.

2.2. Generation of sea cucumber ovum hydrolysates (SCOHs)

Sea cucumber ova were defatted with hexane/ethanol (3:1 v/v) at 50 °C for 6 h, filtered and naturally air-dried at room temperature. The defatted sea cucumber ova (SCO) powder was mixed with Milli-Q (Millipore, Bedford, MA) water at a ratio of 1:50 (w/v). The mixture was heated in boiling water for 10 min, and then hydrolyzed with trypsin (37 °C, pH 8.0), alcalase (50 °C, pH 8.5), neutrase (50 °C, pH 7.0), papain (50 °C, pH 7.0), or flavourzyme (37 °C, pH 8.0) at a dose of 3000 U/g protein. After 3 h of hydrolysis, the enzyme was inactivated by heating at 95 °C for 10 min. The suspension was centrifuged at 12,000*g* for 20 min at 4 °C and the precipitate was discarded. The supernatant was freeze-dried and stored at −20 °C for later investigation.

2.3. Determination of degree of hydrolysis

The determination of degree of hydrolysis (DH), defined as the ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in a protein substrate (h_{tot}), was carried out using the pH-stat method (Adler-Nissen, 1986). The formula for the calculation was as follows:

$$DH = \frac{h}{h_{tot}} \times 100\% = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\% \quad (1)$$

B is the volume (ml) of NaOH consumed to keep the pH constant during the enzymatic reaction, N_b is the normality of NaOH, MP is the mass of the protein (g), and h_{tot} is the total number of peptide bonds in the substrate (mmol/g protein). α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis and was calculated as follows:

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \quad (2)$$

The values of pH and pK were the values at which the proteolysis was conducted.

2.4. Iron-binding capacity assay

Iron-binding capacity was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to the method of O'Loughlin et al. (2015). At room temperature, 1 mg/ml samples (1 ml) were mixed with 1.35 ml of Milli-Q water and 50 μl of 2 mM $FeCl_2$ for 10 min. After the reaction, 100 μl of 5 mM ferrozine was added and gently mixed for 10 min. The absorbance at 562 nm was determined using a microplate reader (Tecan Infinite 200 PRO, Männedorf, Switzerland). The experiment was performed in triplicate and values are expressed as mean ± standard deviation (SD).

2.5. Preparation of sea cucumber ovum hydrolysates-iron complexes

Lyophilized SCOHs were dissolved in Milli-Q water at a concentration of 30 mg/ml, and $FeCl_2{\cdot}4H_2O$ was subsequently added to be a final concentration of 50 mM. The reaction was carried out in a shaker at 25 °C and pH 7.0 for 30 min, and then ethanol was added to the reactant to a final concentration of 85% to remove free iron. After centrifugation at 12,000*g* for 5 min, the precipitate was collected, freeze-dried, and labelled as SCOHs-iron.

2.6. Determination of molecular weight distribution

The molecular weight (MW) distribution profiles of the SCOHs treated with alcalase for different hydrolysis times were determined using gel-permeation chromatography on a Superdex Peptide 10/300 GL column (300 × 10 mm, GE Healthcare Co., Little Chalfont, Buckinghamshire, UK) with an Elite P230 high-performance liquid chromatography (HPLC) system (Elite Analytical Instruments Co., Ltd., Dalian, China) according to the method of Wu et al. (2016) with some modifications. Ten microlitres of SCOH solution (2 mg/ml, filtered through 0.45 μm filter) were loaded onto the HPLC (mobile phase: acetonitrile/water/trifluoroacetic acid = 30:70:0.1 v/v/v). The sample was eluted at a flow rate of 0.4 ml/min and monitored at 220 nm by an ultraviolet (UV) detector. The standards used were cytochrome *c* (12,500 Da), aprotinin (6512 Da), β-amyloid (4514 Da), vitamin B_{12} (1355 Da), MOG