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Enzymatic preparation and characterization of iron-chelating peptides from anchovy (*Engraulis japonicus*) muscle protein

Haohao Wu, Zunying Liu, Yuanhui Zhao, 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

Anchovy (*Engraulis japonicus*) is a small, low-value, marine fish and is mainly targeted for nonfood uses. In this study, the enzymatic preparation and properties of iron-chelating peptides from anchovy muscle protein were evaluated. The results showed that the trypsin hydrolysate possessed the greatest iron-chelating activity, and the iron-chelating activity was governed by the degree of hydrolysis (DH) and the iron-chelating conditions (temperature and pH). Among the gel-filtration fractions, Fraction 1 and Fraction 3 exhibited higher iron-chelating activities, and the IC_{50} values were 0.048 and 0.086 mg/ml, respectively. A *novo* trypsin-resistant, large-molecular-weight, iron-chelating fraction was purified from Fraction 1, and was identified to be homologous with the conserved domain of the coiled-coil myosin heavy-chain tail region. Fraction 3 yielded two oligopeptides, Ser-$(Gly)_7$-Leu-Gly-Ser-$(Gly)_2$-Ser-Ile-Arg and Ile-$(Glu)_2$-Leu-$(Glu)_3$-Ile-Glu-Ala-Glu-Arg. We thus conclude that anchovy might be beneficial as a bioresource to produce anti-anemia compounds which can be used in functional foods.

1. Introduction

Anchovy, the global capture of which has fluctuated narrowly around 9 million tons in the last decade, is one of the most harvested fish species in the world today (FAO, 2012). The annual catches of anchovy amounted to more than 500,000 ton in 2010 in China alone (Ministry of Agriculture of China, 2011). However, anchovy is mainly used for non-food purposes owing to some difficulties in traditional food handling and processing, such as rapid deterioration and high labor intensity. Tacon and Metian (2009) proposed that the redirection of small, pelagic forage fish for direct human consumption would be a global trend in the future. As a large and cheap protein source, anchovy is thus an attractive resource to produce bioactive peptides that could be used in functional food (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

Iron deficiency anemia is a public health problem in both developing and developed countries and has many kinds of adverse health effects. The promotion of non-heme iron absorption seems to be a practical way of combating iron deficiency anemia. Low intestinal solubility is the primary factor hindering food-derived non-heme iron absorption and the presence of iron absorption inhibitors, such as phytic acid, polyphenols and certain fibers, render the non-heme iron in plant food even more poorly absorbed (Hurrell & Egli, 2010). Caseinophosphopeptides (CPPs), a group of milk-derived phosphorylated peptides, have proved to be effective in promotion of non-heme iron absorption and are now widely used as mineral supplement (Ait-Oukhatar et al., 2002). Meat and fish has also long been considered to promote non-heme iron absorption possibly due to the release of iron-binding peptides during muscle-tissue digestion (Cook & Monsen, 1976; Storcksdieck, Bonsmann, & Hurrell, 2007). Iron-binding peptides, such as CPPs and muscle-derived peptides, can form soluble iron chelates through various donor coordination groups in their backbones or side chains, and soluble iron chelates, such as CPPs-bound iron and sodium iron (III) ethylenediaminetetraacetate (NaFeEDTA), are reported to possess good bioavailability, regardless of iron absorption inhibitors in plant food (Ait-Oukhatar et al., 2002; Zhu, Glahn, Yeung, & Miller, 2006). Anchovy muscle protein, which is cheaper than milk protein and many other animal proteins, is thus a potential resource to produce iron-chelating peptides in large quantities. The aim of this study was to produce, isolate, purify and characterize iron-chelating peptides from enzymatic hydrolysis of anchovy muscle protein.

2. Materials and methods

2.1. Materials

Frozen anchovy (*Engraulis japonicus*), 5–8 cm in length, were provided by Allen Ship Service Co. Ltd. (Shandong, China) and stored at −80 °C before use. Pepsin (a serine endoproteinase from porcine stomach mucosa with a declared activity of 1200 U/g), papain (a cysteine

* Corresponding author. Fax: +86 532 82032400.
E-mail address: mingyz@ouc.edu.cn (M. Zeng).

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protease from papaya latex with a declared activity of 3500 U/mg) and trypsin (a serine endoproteinase from bovine pancreas with a declared activity of 250 U/mg) were purchased from Sinopharm Chemical Co. Ltd. (Shanghai, China). Alcalase 2.4L® (a serine endoprotease from *Bacillus licheniformis* with a declared activity of 2.4 AU/g and a density of 1.18 g/mL), Neutrase 0.8L® (a neutral metallo-endoprotease from *Bacillus amyloliquefaciens* with a declared activity of 0.8 AU/g and a density of 1.26 g/mL) and Flavourzyme 500 MG® (a exoprotease from *Aspergillus oryzae* with a declared activity of 500 LAPU/g) were obtained from Novozymes A/S (Bagsvaerd, Denmark). Ferrozine (3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-p, p′-disulfonic acid monosodium salt hydrate) and iminodiacetic acid (IDA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sepharose 6B was obtained from GE Healthcare (Piscataway, NJ, USA). Other chemical reagents used in this study were of analytical grade and commercially available.

2.2. Preparation of enzymatic hydrolysates

After thawing in ice water, the fish were beheaded, eviscerated, deboned, washed and drained to obtain anchovy meat. One portion of the anchovy meat was homogenized with three portions of water (w/v), and the homogenates were then hydrolyzed with commercial enzymes at their optimal temperatures and pH (Table 1) with continuous stirring. The reaction mixture was heated in a boiling water bath for 10 min and then centrifuged at 10,000 ×*g* for 20 min. The supernatants were filtered through a 0.22 μm cellulose acetate membrane before use.

2.3. Determination of the degree of hydrolysis (DH)

The DH of anchovy muscle protein was determined using the o-phthaldialdehyde (OPA) method described by Nielsen, Petersen, and Dambmann (2001). Briefly, 400 μl sample solution was added into 3 ml OPA reagents and mixed for 5 s; after exactly 2 min, the mixture was read at 340 nm in the spectrophotometer. The total number of peptide bonds in the sample was determined after acid hydrolysis using 6 N HCl at 120 °C for 24 h. The DH was calculated as $(OD_{sample} - OD_{blank})/(OD_{total} - OD_{blank}) \times 100\%$.

2.4. Measurement of iron-chelating ability

A modified colorimetric assay was used to measure the iron-chelating abilities of anchovy muscle protein hydrolysates according to the method of Dinis, Madeira, and Almeida (1994). All the hydrolysates were adjusted to pH 5.5 and filtered through a 0.22 μm cellulose acetate membrane before testing. A 2-(N-morpholino) ethanesulfonic acid buffer (MES, 10 mM, pH 5.5) was used to dilute the hydrolysates to different peptide concentrations. Then, 20 μl of 0.2 mM $FeSO_4$ was added to 140 μl of anchovy muscle protein hydrolysates with different peptide concentrations in 96-well plates. The plates were then incubated in a thermostat chamber at 37 °C for 3 h. After incubation, 40 μl of 5 mM ferrozine solution was added to end the reaction. The plates were left at room temperature for 10 min, and the absorbance at 562 nm was then read by a microplate reader. The percentage inhibition of ferrozine–Fe^{2+} complex formation or the chelating ratio was calculated as $[(A_0 - A_s)/A_0] \times 100$, where A_0 was the absorbance of the blank and A_s was the absorbance of the sample. The IC_{50} was defined as the peptide concentration to inhibit 50% of ferrozine–Fe^{2+} complex formation. Caseinophosphopeptides (CPPs), prepared from trypsin digestion of casein, were used as a positive control.

2.5. Intrinsic fluorescence

Fluorescence experiments were performed to monitor metal-induced conformational changes in peptides using a Hitachi F-4600 fluorescence spectrophotometer (Hitachi Co. Ltd., Japan). A fluorescence quartz cuvette with a path length of 1.0 cm was used. All fluorescence experiments were conducted at room temperature, and an excitation wavelength of 280 nm or 295 nm was used. During a wavelength-scan experiment, emissions at wavelengths from 310 nm to 400 nm were recorded at intervals of 0.2 nm. In a time-scan experiment, the emission at 370 nm was recorded at intervals of 5 s.

2.6. Preparative size-exclusion chromatography

Ten milliliters of trypsin hydrolysate was loaded onto a water-equilibrated, Sephadex G-15 column (2.5 × 60 cm) and eluted with Milli-Q water at a flow rate of 0.5 ml/min. The eluent was monitored at 220 nm, and the peaks were collected manually. Eight fractions, Fraction 1 to Fraction 8 (F_1 to F_8), were obtained, and their iron-chelating abilities were determined. The highest iron-chelating fractions, F_1 and F_3, were further purified by immobilized-metal affinity chromatography (IMAC) and reverse-phase HPLC (RP-HPLC), respectively.

2.7. IMAC-Fe^{3+} purification

An Fe(III)-IDA-Sepharose bead was prepared by procedures described previously (Matsumoto, Mizuno, & Seno, 1979). The gel was packed in a laboratory column (10 × 120 mm) and equilibrated with acetate buffer (10 mM, pH 5.0) before use. After 20 ml of F_1 (4 mg/ml, pH 5.0) was loaded, the column was left at room temperature for 3 h. Unbound and weakly bound peptides were washed out with equilibration buffer and MES buffer (10 mM, pH 6.5), respectively. The retained peptides (represented as F_{IMAC}) were eluted with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 8) and pooled.

2.8. RP-HPLC purification

F_3 was further purified using a semipreparative RP-HPLC column (Zorbax SB-C18, 9.4 × 250 mm). Elution was performed using a linear gradient of 5–25% acetonitrile in water at a flow rate of 4 ml/min over 50 min. Two major peaks were pooled, and their iron-binding abilities were determined. The highest iron-binding fraction from the RP-HPLC column was used for peptide sequencing.

Table 1
Enzymatic hydrolysis of anchovy muscle protein treated with six proteases and their DH and iron-chelating abilities.

Materials	Enzyme	Parameters for enzymatic hydrolysis				DH* (%)	IC_{50}* (mg/ml)
		pH	Temperature (°C)	E/S (w/w)	Time (h)		
AMP	Flavourzyme	7.0	50	4/100	4	23.35 ± 0.09^{a}	1.56 ± 0.023^{a}
	Pepsin	2.0	37	4/100	4	16.63 ± 0.06^{b}	2.72 ± 0.038^{b}
	Neutrase	7.0	50	4/100	4	24.04 ± 0.04^{c}	1.15 ± 0.016^{c}
	Papain	7.0	55	4/100	4	15.96 ± 0.12^{d}	1.99 ± 0.050^{d}
	Trypsin	8.0	37	4/100	4	31.57 ± 0.17^{e}	0.13 ± 0.001^{e}
	Alcalase	8.0	55	4/100	4	25.50 ± 0.10^{f}	2.63 ± 0.032^{f}
Casein	Trypsin	8.0	37	4/100	4	33.00 ± 0.16^{g}	0.16 ± 0.003^{g}

* Expressed as means ± SD of triplicates. Values followed by a different letter in the same column are significantly different ($P<0.05$) according to a paired-samples *T*-test.