Food Chemistry 125 (2011) 334–341

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate

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ARTICLE INFO

Article history:
Received 28 June 2010
Received in revised form 30 July 2010
Accepted 25 August 2010

Keywords:
Squid gelatin
Antioxidant peptides
ACE-inhibitory peptides
Mass spectrometry

ABSTRACT

Squid gelatin obtained from inner and outer tunics was hydrolysed with Alcalase to isolate antioxidant peptide sequences. The ACE-inhibitory activity of the isolated peptides was also evaluated. After fractionation by ultrafiltration and size-exclusion chromatography into four fractions, the antioxidant activity of the peptide fractions was determined by radical scavenging ability and ferric reducing power. Fraction FIII showed the highest antioxidant activity, although slight differences could be expected in the antioxidant activity of the different fractions based on the amino acid composition. FIII was subjected to liquid chromatography and tandem mass spectrometry (LC–MS/MS) and two major compounds were identified: the compound with m/z 952.42, which could be mostly comprised by the carbohydrate fucose, and the peptide with m/z 1410.63. Three possible sequences were proposed and synthesised for this peptide, and the contribution of Leu or Hyp residues to the antioxidant and ACE-inhibitory activities of the resulting sequence was evaluated. The presence of Leu residues in the peptide sequence in replacement of Hyp seems to play an important role in the antioxidant and ACE-inhibitory activity.

1. Introduction

Giant squid (*Dosidicus gigas*) is widely distributed in the Pacific Ocean, and is especially abundant near the coasts of Peru and Mexico. The big size of the mantle together with the fact that it is usually marketed as cleaned and peeled mantle, imply the generation of important amounts of collagenous residues (inner and outer tunics), which could yield high value-added products. Squid skin gelatins have been reported to give biologically active peptides with high antioxidant activity, due to their radical scavenging activities, chelating effects on metal ions, reducing power or lipid peroxidation (Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Mendis, Rajapakse, Byun, & Kim, 2005; Rajapakse, Mendis, Byun, & Kim, 2005). However, to the best of our knowledge, ACE-inhibitory capacity has not been described in squid skin gelatin hydrolysates, although this activity has been reported in collagen and gelatin hydrolysates from other marine species (Fahmi et al., 2004; Zhao et al., 2007).

Lipid oxidation leads to the loss of food quality and shortening of shelf-life, as well as the production of potentially toxic reaction products. Furthermore, oxidative damage is related to numerous health disorders such as diabetes, cancer, neurodegenerative and inflammatory diseases (Pryor, 1982).

Hypertension is a worldwide problem of epidemic proportions that affects 15–20% of all adults. Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension because catalyzes the conversion of inactive angiotensin-I into angiotensin-II, a potent vasoconstrictor and inactivates bradykinin, a potent vasodilator (Murray & FitzGerald, 2007). Synthetic inhibitors of ACE are often used to treat hypertension and other cardio-related diseases. However, these compounds can cause adverse side effects (Atkinson & Robertson, 1979) and, therefore, interest for natural inhibitor has increased.

Biological activities of protein hydrolysates are related to the amino acid composition and sequence, size and configuration of peptides. For example, the presence of certain amino acids, such as His, Trp, Tyr, Phe, Met, Leu, Gly or Pro has been reported to enhance the scavenging activities of peptides (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Li, Chen, Wang, Ji, & Wu, 2007; Mendis, Rajapakse, & Kim, 2005; Park, Jung, Nam, Shahidi, & Kim, 2001). Regarding the ACE-inhibitory activity, binding to ACE is strongly influenced by the C-terminal tripeptide sequence. Although the structure–activity relationship of food derived ACE-inhibitory peptides has not yet been fully established, ACE prefers inhibitors containing hydrophobic amino acid residues at each of the three C-terminal positions (Murray & FitzGerald, 2007).

The objective of this study was the isolation and identification of peptides sequences with antioxidant activity from an enzymatic

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[1] This centre has implemented and maintains a Quality Management System which fulfils the requirements of the ISO standard 9001:2000.

0308-8146/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2010.08.058

hydrolysate of squid gelatin. Furthermore, the ACE-inhibitory activity of the isolated peptides was evaluated, as well as the contribution of Leu and Hyp residues to both biological activities.

2. Materials and methods

2.1. Gelatin hydrolysate

Frozen inner and outer tunics of jumbo flying squid (*D. gigas*) were kindly provided by PSK Océanos, S.A. (Vigo, Spain). Gelatin was extracted using the method described by Giménez et al. (2009). Dry gelatin (2.5% w/v) was dissolved in MiliQ water and subjected to enzymatic hydrolysis using Alcalase® (EC 3.4.21.14, 2.4L, 2.64 AU/g, Sigma–Aldrich, Inc., St. Louis, Mo., USA) with an enzyme-substrate ratio of 1:20 (w:w) in optimal conditions for enzymatic activity (pH 8, 50 °C) for 3 h. The pH of the reaction was kept constant by addition of 1 N NaOH solution to the reaction medium using a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). The enzyme was inactivated by heating at 90 °C for 10 min. The supernatant after centrifugation at 3000*g* for 15 min was taken as the gelatin hydrolysate.

The degree of hydrolysis (DH), calculated according to Adler-Nissen (1977), was 30.5%.

2.2. Fractionation of the hydrolysate

2.2.1. Ultrafiltration

The gelatin hydrolysate was fractionated by ultrafiltration with a molecular weight cut-off (MWCO) membrane of 10 kDa (Omega 10 kD Centramate™ cassette medium screen channel, Pall Corporation, Madrid, Spain). The hydrolysate permeate fraction was freeze-dried and designed as UF-10.

2.2.2. Size-exclusion chromatography (SEC)

The UF-10 was dissolved in MiliQ water at 10 mg/ml and loaded onto a gel filtration column (HiLoad™ 26/60 Superdex 30 pg, GE Healthcare, Barcelona, Spain), with a fractionation range below 10,000 Da. The injection volume was 2 ml and the flow rate 0.5 ml/min using MiliQ water as mobile phase. Peptides monitored at 215 nm were collected, freeze-dried and stored at −80 °C for further assay. Blue dextran (2000 kDa, Sigma Aldrich, Madrid, Spain), vitamin B12 (1355 Da, Sigma Aldrich, Madrid, Spain) and 6.7-dimethoxy-2-(4-methyl-1.4-diazepan-1-yl) quinazolin-4-amine (317 Da, from Lera's group, University of Vigo, Spain) were used as molecular weight standards. The elution times for these standards were 49, 155, and 211 min, respectively.

2.3. Amino acid composition

The different fractions isolated by SEC were dissolved at 1 mg/ml in MiliQ water. An amount of 50 μl of sample were dried and hydrolysed in vacuum-sealed glass tubes at 110 °C for 24 h in the presence of continuously boiling 6 N HCl containing 0.1% phenol with norleucine (Sigma–Aldrich, Inc., St. Louis, MO, USA) as internal standard. After hydrolysis, samples were again vacuum-dried, dissolved in application buffer, and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain).

2.4. Antioxidant activities

2.4.1. FRAP assay

FRAP (Ferric Reducing Ability of Plasma) is a measure of the reducing power of samples and was performed according to the method described by Benzie and Strain (1996) with some modification. An amount of 60 μl of dissolved sample (UF-10, peptide fractions, synthetic peptides) was incubated (37 °C) with 60 μl of MiliQ water and 900 μL of FRAP reagent containing 10 mM of TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mM of $FeCl_3$. Absorbance values were read at 595 nm after 30 min. Results were expressed as μmol Fe^{2+} equivalents/g of protein determined from the amino acid analysis, based on a standard curve of $FeSO_4{\cdot}7H_2O$. All determinations were performed at least in triplicate.

2.4.2. ABTS assay

The ABTS radical [2,2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulphonic acid)] scavenging capacity was carried out according to the method of Re et al. (1999). The stock solution of ABTS radical consisted of 7 mM ABTS in potassium persulphate 2.45 mM, kept in the dark at room temperature for 16 h. An aliquot of stock solution was diluted with MiliQ water in order to prepare the working solution of ABTS radical with absorbance at 734 nm of 0.70 ± 0.02. A 20 μl aliquot of sample (UF-10, peptide fractions, synthetic peptides) was mixed with 980 μl of ABTS reagent. The mixture was then left to stand in the dark at 30 °C for 10 min and absorbance values were read at 734 nm. Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of protein determined from the amino acid analysis, based on a standard curve of vitamin C. All determinations were performed at least in triplicate.

2.5. Purification of the antioxidative peptides

2.5.1. General

The fraction with the highest antioxidant activity was analysed on-line by LC-ESI-IT-MS/MS using a LC system model Surveyor (Thermo-Finnigan, San Jose, CA, USA) coupled with a linear IT mass spectrometer model LTQ (Thermo-Finnigan). Peptides were concentrated onto a RP trap column (PepMap C18 μ-precolumn 300 mm id × 1 mm; Dionex, Amsterdam, The Netherlands) for 5 min and eluted online onto a 75 μm × 100 mm Biobasic-C18 RP analytical column (PicoFrit column, New Objective, Ringoes, NJ, USA) at a flow rate of 200 ml/min. Water containing 0.1% of formic acid and acetonitrile containing 0.1% of formic acid, were used as solvents A and B, respectively, using the following gradient: 5% B for 5 min, 5–70% B in 60 min, and 95% B for 10 min. The mass spectrometer was operated in the data-dependent mode to automatically switch between full MS and MS/MS acquisition. The parameters for ion scanning were the following: full-scan MS (400–1800 *m*/*z*) plus top 12 peaks zoom and MS/MS scans (isolation width 2 *m*/*z*), normalised collision energy 35%. The scanning was performed using a dynamic exclusion list (20s exclusion list size of 50).

De novo peptide sequencing of the major peaks was performed by manual interpretation of the ion series in the spectra. BLAST program was used for homology searches between manual obtained sequences and those in the NCBInr database.

2.5.2. MALDI-TOF MS Analysis

The fraction with the highest antioxidant activity was also analysed by MALDI-TOF MS. One microliter was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive reflector mode, with an accelerating voltage of 20,000 V.