Fish & Shellfish Immunology 34 (2013) 1078–1084

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Purification of a novel peptide derived from *Mytilus coruscus* and in vitro/in vivo evaluation of its bioactive properties

Eun-Kyung Kim [a,f], Hyun-Jung Oh [b], Yon-Suk Kim [b], Jin-Woo Hwang [b], Chang-Bum Ahn [c], Jung Suck Lee [d], You-Jin Jeon [e], Sang-Ho Moon [f], Si Heung Sung [f], Byong-Tae Jeon [f], Pyo-Jam Park [b,f,*]

[a] Department of Natural Science, Konkuk University, Chungju 380-701, Republic of Korea
[b] Department of Biotechnology, Konkuk University, Chungju 380-701, Republic of Korea
[c] Department of Food Science and Nutrition, Chonam National University, Yosu 550-749, Republic of Korea
[d] Industry-Academic Cooperation Foundation, Jeju National University, Jeju 690-756, Republic of Korea
[e] Faculty of Applied Marine Science, Jeju National University, Jeju 690-756, Republic of Korea
[f] Korea Nokyong Research Center, Konkuk University, Chungju 380-701, Republic of Korea

ARTICLE INFO

Article history:
Received 7 November 2012
Received in revised form
9 January 2013
Accepted 20 January 2013
Available online 9 February 2013

Keywords:
Mytilus coruscus
Enzymatic hydrolysis
Inflammatory response
Antioxidant peptide
Anti-inflammatory

ABSTRACT

Excess oxidant can promote inflammatory responses. Moreover, chronic inflammation accompanied by oxidative stress is connected various steps involved in many diseases. From the aspect, we investigated an antioxidant peptide to prevent inflammatory response against oxidant overexpression. To prepare the peptide, eight proteases were employed for enzymatic hydrolysis, and the antioxidant properties of the hydrolysates were investigated using free radical scavenging activity by electron spin resonance (ESR) spectrometry. Papain hydrolysates, which showed clearly superior free radical scavenging activity, were further purified using consecutive chromatographic methods. Finally, a novel antioxidant peptide was obtained, and the sequence was identified as Ser-Leu-Pro-Ile-Gly-Leu-Met-Ile-Ala-Met at N-terminal. Oral administration of the peptide to mice effectively inhibited malondialdehyde (MDA) levels in a thiobarbituric acid reactive substances (TBARS) assay, and we also confirmed the antioxidative enzyme activities in superoxide dismutase (SOD) and glutathione-s-transferase (GST) assays. This is the first report of an antioxidant peptide derived from the hydrolysate of *Mytilus coruscus*, and also these results suggest that the peptide possesses potent antioxidant activity, and potential to enhance anti-inflammatory response.

1. Introduction

Although reactive oxygen species (ROS) play an important role in host defense against microbial infection, their overexpression and residual ROS can cause cellular damage [1,2], and are implicated in many inflammatory conditions [3]. Mitochondria are the major organelles that produce ROS and the main target of ROS-induced damage, as observed in various pathological states including aging, malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer, and gastric ulcer [4,5]. Major cellular defenses against ROS include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST) [6]. Therefore, the development and utilization of effective antioxidants that enhance the activity of the antioxidative enzymes are desired.

Meanwhile, acute inflammation is part of the defense response, but chronic inflammation has been found to mediate a wide variety of disease, including cardiovascular disease, cancers, diabetes, arthritis, Alzheimer's disease, pulmonary disease, and autoimmune disease. Moreover, ROS production by H_2O_2 activates the inflammasome which can promote inflammatory responses [7]. ROS may either directly trigger inflammasome assembly or be indirectly sensed through cytoplasmic proteins that modulate inflammasome activity [7]. From the view point of cellular biology, accordingly, chronic inflammation accompanied by oxidative stress is linked to various steps involved in many diseases mentioned above [8]. Therefore many antioxidants also have a natural anti-inflammatory action [3].

Peptides generated by digesting various proteins, including animal and plant sources, possess biofunctional activity. These peptides are inactive within the sequences of their parent proteins but are released during gastrointestinal digestion or food

* Corresponding author. Department of Biotechnology, Konkuk University, #518, Sang-Heo Research Building, Chungju 380-701, Republic of Korea. Tel.: +82 43 840 3588; fax: +82 43 852 3616.
E-mail address: parkpj@kku.ac.kr (P.-J. Park).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.01.013

processing [9]. Once such bioactive peptides are liberated, depending on their structural, compositional, and sequential properties, they may exhibit various biofunctional activities. The functional properties of a protein can be also improved by enzymatic hydrolysis under controlled conditions [10]. Several studies have reported antioxidant peptides generated from seafood sources and their potential for use as alternative antioxidants [11–15]. However, few studies have evaluated antioxidant properties of peptides derived from enzymatic hydrolysates of *Mytilus coruscus*.

The hard-shelled mussel, *M. coruscus*, belongs to the family Mytiloidae, is one of the most important marine shellfish species, and is widely cultured throughout coastal areas of the Bohai Sea, Yellow Sea, and East Sea in Korea and China [16]. Although it is an important commercial shellfish species, little is known about its antioxidant activity. Therefore, the aim of this study was to identify new antioxidant peptide from *M. coruscus*, and characterize its antioxidant properties in vitro and in vivo as an anti-inflammatory.

2. Materials and methods

2.1. Materials

Fresh *M. coruscus* was obtained from a local market (Jeonnam, Korea). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), (4-pyridyl-1- oxide)-N-tert-butylnitrone (4-POBN), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylatedhydroxytoluene (BHT) and four enzymes including papain, pepsin, α-chymotrypsin, and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and the other four enzymes including Flavourzyme, Neutrase, Protamex, and Alcalase were donated from Novozyme Co. (Bagsvaerd, Denmark). All other reagents were of the highest grade commercially available.

2.2. Preparation of enzymatic hydrolysates from M. coruscus

Prior to enzymatic hydrolysis, *M. coruscus* were pulverized into a powder using a grinder (FM-909T, Hanil Co., Korea), and the enzymatic hydrolysates were obtained according to the method described by Park et al. [17]. The optimum pH, temperature and characterization of various enzymes are summarized in Table 1. Briefly, one hundred milliliter of buffer solution was added to 2 g of the dried sample, and then 40 μL (or mg) of each enzyme was added after pre-incubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 h to achieve an optimum hydrolytic level, and followed by immediate heating at 100 °C for 10 min to inactivate the enzyme. Finally, the enzymatic hydrolysates were rapidly cooled to 20–25 °C in an ice bath, filtered, lyophilized, and stored at −20 °C until use.

2.3. Measurement of free radical scavenging activity

2.3.1. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the iron-catalyzed Haber–Weiss reaction (Fenton-driven Haber–Weiss reaction), and the hydroxyl radicals generated reacted rapidly with nitrone spin-trap DMPO. The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Briefly, 0.2 ml of each enzymatic hydrolysate with various concentrations was mixed with 0.2 ml DMPO (0.3 M), 0.2 ml $FeSO_4$ (10 mM), and 0.2 ml H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and then transferred to a 100 μL Teflon capillary tube. After 2.5 min, the ESR spectrum was recorded using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan). The hydroxyl radical scavenging activity was expressed as IC_{50} value, which means concentration for scavenging 50% of hydroxyl radicals. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 and temperature, 298 K.

Table 1
Optimum hydrolysis conditions and apoptosis rate of various enzymatic extracts from *M. coruscus*.

Enzyme	Optimum conditions		Buffer	Description
	pH	Temperature		
Flavourzyme	7.0	50 °C	Phosphate	Endo- and exo-peptidase activities
Neutrase	7.0	50 °C	Phosphate	An endoprotease
Protamex	7.0	50 °C	Phosphate	Hydrolysis of food proteins
Alcalase	7.0	50 °C	Phosphate	A endoprotease
Papain	6.0	37 °C	Phosphate	Endolytic cysteine protease
Pepsin	2.0	37 °C	Glycin-HCl	From porcin gastric mucosa
α-Chymotrypsin	7.0	37 °C	Phosphate	From bovine pancrease
Trypsin	7.0	37 °C	Phosphate	A serine protease

2.3.2. DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. [18]. Briefly, 60 μL of various concentrations of each enzymatic hydrolysate was added to 60 μL of DPPH (60 μM) in methanol solution. After mixing vigorously for 10 s, the solution was transferred to a 100 μL Teflon capillary tube, and the scavenging activity of each enzymatic hydrolysate on the DPPH radical was measured using an ESR spectrometer. The spin adduct was measured on an ESR spectrometer exactly 2 min later. The DPPH radical scavenging activity was expressed as IC_{50} value, which means concentration for scavenging 50% of DPPH radicals. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 and temperature, 298 K.

2.3.3. Superoxide radical scavenging activity

Superoxide radicals were generated by UV irradiation of a riboflavin/ethylenediaminetetra-acetic acid solution. The reaction mixtures, containing 0.1 ml of 0.8 mM riboflavin, 0.1 ml of 1.6 mM EDTA, 0.1 ml of 800 mM DMPO and 0.1 ml of various concentrations of peptide, were irradiated for 1 min under a UV lamp at 365 nm. The mixtures were transferred to a 100 μL quartz capillary tube of the ESR spectrometer for measurement. The superoxide radical scavenging activity was expressed as IC_{50} value, which means concentration for scavenging 50% of superoxide radicals. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5 and temperature, 298 K.

2.3.4. Peroxyl radical scavenging activity

Peroxyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures containing 0.1 ml of 10 mM AAPH, 0.1 ml of 10 mM 4-POBN, and 0.1 ml of the indicated concentrations of the tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on an ESR spectrometer. The peroxyl radical scavenging activity was expressed as IC_{50} value, which means concentration for scavenging 50% of peroxyl radicals. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 and temperature, 298 K.

2.4. Purification of antioxidant peptides from M. coruscus

2.4.1. Fractions from a tangential flow filtration (TFF) system

The enzymatic hydrolysates of *M. coruscus* were fractionated through ultrafiltration (UF) membranes with a range of MWCO of