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Purification and characterization of antioxidant peptides from enzymatically hydrolyzed ark shell (*Scapharca subcrenata*)

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ABSTRACT

Information of bioactive peptides derived from ark shell is seldom reported. In this study, ark shell was hydrolyzed by pepsin with enzyme to substrate (E/S) ratios of 1:100 and 1:500 for 1–4 h. Ark shell hydrolysates (ASHs) were subjected to evaluate their antioxidant activities by free radical scavenging and oxygen radical absorbance capacity (ORAC) assays. Antioxidant peptides (P) were purified and identified as MCLDSCLL (P1) and HPLDSLCL (P2). P1 and P2 showed potent antioxidant activities with IC_{50} value of 19.47 ± 0.35 and 74.36 ± 0.54 μM, and glutathione (GSH), as positive control, showed 19.31 ± 0.36 μM against DPPH radical. P1 and P2 exhibited 678.36 ± 5.33 μM trolox equivalent (TE)/mM P1 and 778.86 ± 9.64 μM TE/mM P2 against $ABTS^+$ radical scavenging, and ORAC values of 333.91 ± 26.05 μM TE/mM P1 and 209.81 ± 21.74 μM TE/mM P2. P1 and P2 also showed strong reducing power. In addition, P2 strongly inhibited copper-catalyzed human LDL oxidation.

1. Introduction

Metabolic pathways in living organisms produce various kinds of reactive oxygen species (ROS) and free radicals, which are biologically unavoidable sequences. In our body, the produced ROS and free radicals are readily detoxified by antioxidant defense system, in which endogenous antioxidants include superoxide dismutase, catalase, glutathione peroxidase, glutathione etc., and food-derived antioxidant molecules such as vitamins, polyphenols, and anthocyanins etc. are exogenous antioxidants [1]. Oxidative stress caused by an imbalance between the amounts of ROS and antioxidant defense ability results in cellular injury. Oxidative stress is deemed to play crucial roles in the etiology of human diseases, including cancer, atherosclerosis, diabetes, and asthma, by altering structure of DNA, proteins, and lipids and effecting on signal transduction [1,2]. Thus, antioxidant intervention as a route of preventing oxidative stress-mediated damage would seem to provide a reasonable and practical approach *in vivo*. Moreover, oxidative deterioration in food products is a main concern in food industry due to production of off-flavors and undesirable secondary lipid peroxidation products that can negatively affect body functions [3].

Dietary proteins are well recognized for their nutritional and functional properties. Protein hydrolysates or bioactive peptides from dietary proteins have health-promoting functions such as antioxidant, blood pressure reduction, and anti-cancer functions [4–6]. Therefore, the bioactive peptides have attracted consumer interest because it can be utilized as functional food ingredients aimed a health maintenance. In recent years, numerous bioactive peptides were isolated from food proteins after enzymatic hydrolysis and were evaluated to their real bioactivities, which depend on their amino acid sequence, composition, and molecular weight (MW) [3].

Ark shell (*Scapharca subcrenata*), a shellfish, is widely cultured and consumed as a foodstuff in Korea. Recently, several studies have reported that enzymatic hydrolysis of ark shell yielded peptides and hydrolysates that possess osteogenic and anti-obesity activities [7,8]. However, other bioactive peptides or hydrolysates with different bioactivities from ark shell have been seldom reported until now. Therefore, this study was aimed at isolation and identification of antioxidant peptides from ark shell protein hydrolysates by peptic hydrolysis. The isolated peptides were evaluated their antioxidant activities by measuring free radical and ROS scavenging activities, and inhibition ability of human LDL oxidation.

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2. Materials and methods

2.1. Materials

Ark shell was purchased at a local market (Sunchen, Korea). Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), trolox, fluorescein sodium salt, AAPH (2,2'-azobis(2-amidino-propane)dihydrochloride) and GSH were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). Pepsin (1:10,000) was obtained from Junsei Chemical Co. (Tokyo, Japan). All other reagents used were of analytical grade and commercially available.

2.2. Preparation of ark shell hydrolysates (ASH)

Protein content of ark shell was 68.24% (dry basis)by Kjeldahl method. Ark shell was wahsed using tap water and then lyophilized after boiling at 100 °C for 10 min in order to inactivate internal proteolytic enzymes. The pulverized ark shell was hydrolyzed using pepsin with E/S ratios of 1 :100 and 1 :500 for 1–4 h at pH 2 and 37 °C. Pepsin was inactivated by boiling at 100 °C for 10 min. The supernatant was lyophilized and evaluated antioxidant activities.

2.3. DPPH scavenging assay

DPPH scavenging activity of ASHs was measured using the method described by Ahn, Kim and Je [9] with a slight modification. Briefly, a 70 μL of ASH was mixed with 70 μL of DPPH solution (150 μM in MeOH) followed by standing room temperature for 30 min in the dark. The optical density was measured by microplate reader (Tecan GENios, Austria GmbH, Austria) at 517 nm.

2.4. $ABTS^+$ radical scavenging assay

$ABTS^+$ radical stock solution was prepared according to the method described by Ahn, Kim and Je [9], and the working solution (absorbance with 1.5 at 414 nm) was prepared by dilution. A 50 μL of sample and 150 μL of $ABTS^+$ were mixed, stand for 10 min, and determined the optical density at 414 nm. The scavenging activity was expressed by μM trolox equivalent (TE)/mg ASH or mM peptide using standard curve made by using trolox (1–150 μM).

2.5. ORAC assay

ORAC value of sample was measured by the method described by Zulueta, Esteve and Frígola [10] with a slight modification. Briefly, a 50 μL of sample in sodium phosphate buffer (75 mM, pH 7) and fluorescein (78 nM) was added into 96-well black plate and then incubated at 37 °C for 15 min. After addition of 25 μL of AAPH (221 mM), fluorescence was measured every 5 min for 1 h at E × 485 nm and Em 582 nm (Tecan GENios). ORAC value was expressed as μM trolox equivalent (TE)/mg ASH or mM peptide.

2.6. Reducing power

Reducing power of sample was measured by the method described by Oyaizu [11] with a slight modification. Briefly, a 200 μL of ASH, 300 μL of sodium phosphate buffer (0.1 M, pH 6.6) and 500 μL of potassium ferricyanide (1% w/v) were mixed and then incubated at 50 °C for 20 min A 500 μL of 10% TCA (w/v) was added into the mixture and then 100 μL of the upper layer was mixed with 100 μL of distilled water and 20 μL of $FeCl_3$ (0.1% w/v). Absorbance was then measured at 700 nm A higher absorbance indicates a higher reducing power.

2.7. Inhibition of copper-catalyzed human LDL oxidation

Inhibitory effect of peptides on copper-catalyzed human LDL oxidation was evaluated by using thiobarbituric acid reactive substances (TBARS) assay. Briefly, human LDL (100 μg/mL) was incubated with 10 μM $CuSO_4$ in the presence or absence of peptides at 37 °C for 18 h. EDTA (30 μL of 1 mM) was added immediately to terminate any further oxidation. TCA (20% w/v, 200 μL) and TBA (1% w/v in 0.3% NaOH, 200 μL) were added into the reaction mixture followed by boiling at 92 °C for 20 min After centrifugation (4000 rpm, 15 min), the red pigment in the supernatant was estimated by absorbance at 532 nm. TBARS was expressed as μM MDA using calibration curve prepared with an MDA standard.

2.8. Purification of antioxidant peptides

ASH was separated by using a Superdex™ Peptide 10/300 G L column (GE Healthcare, Uppsala, Sweden) equilibrated with distilled water containing 0.05% TFA at a flow rate of 0.7 mL/min. Pooled fractions were subjected to DPPH scavenging assay after lyophilization. The active fraction was further separated by RP-HPLC equipped with ODS C_{18} column (Hypersil Gold, 4.6 × 250 mm, 5 μm, Thermo Scientific, PA, USA). The elution was conducted by a linear gradient of acetonitrile (0–60% in 30 min) containing 0.05% TFA at a flow rate of 0.7 mL/min. After DPPH scavenging assay, the active fraction was further purified using the same column with a linear gradient of acetonitrile (20–38% in 30 min) containing 0.05% TFA at a flow rate of 0.5 mL/min.

2.9. Identification of antioxidant peptides by Q-TOF LC–MS/MS

Peptide identification was done using an ultra high resolution Q-TOF LC–MS/MS coupled with ESI source (maXis–HD™, Bruker Daltonics, Bremen, Germany). Peptide was eluted onto a Acclaim RSLC 120 C_{18} column (2.1 × 100 mm, 2.2 μm, Dionex) at a flow rate of 200 μL/min. The mobile phase was consisted of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B). The solvent gradient was performed as follows: 0–5 min, 5% B; 5–7 min, 5–10% B; 7–48 min, 10–30% B; 48–55 min, 30–50% B; 55–65 min, 95% B; 65–66 min, 95–5% B; 66–71 min, 5% B. Mass spectra were acquired in positive ion mode by scanning *m/z*-range from 50 to 2000. *De novo* peptide sequencing by MS/MS spectrometry was performed and MS/MS spectra was also analyzed uisng the Biotools 3.2 software (Bruker Dantonics).

2.10. Synthesis of antioxidant peptides

The identified peptides were synthesized by Fmoc SPPS (solid phase peptide synthesis) using PSI 300 synthesizer (NY, USA) from BioStem (Ansan, Korea) and purified by RP-HPLC using a kromasil C_{18} column (4.5 × 250 mm, 5 μm). MS of the synthetic peptide was confirmed using LC/MS (Shimadzu LC-MS2020, Kyoto, Japan). The purities of synthesized peptides were over 95%.

2.11. Statistical analysis

All results are expressed as mean ± S.D. with three determinations. Differences between means of each group were assessed by one-way analysis of variance followed by Duncan's test using PASW Statistics 19.0 software (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Preparation and antioxidant activities of ASH

In order to extract antioxidant peptides from ark shell, different E/S