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Short communication

Characterization of a novel antioxidative peptide from the sand eel *Hypoptychus dybowskii*

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ABSTRACT

Protein derived from the sand eel, *Hypoptychus dybowskii,* was hydrolyzed using different proteases (alcalase, neutrase, α-chymotrypsin, papain, pepsin, and trypsin) to produce an antioxidant peptide. Antioxidant activity of hydrolysates was evaluated using DPPH radical scavenging activity. The papain hydrolysate exhibited the highest antioxidative activity compared to other hydrolysates. The free radical scavenging activity of papain hydrolysate was 77.4% at 1.0 mg/ml. The peptide demonstrating the strongest antioxidative activity was isolated from the hydrolysate using consecutive chromatography. The amino acid sequences of purified peptide was identified as Ile–Val–Gly–Gly–Phe–Pro–His–Tyr–Leu (1189 Da), and the EC_{50} value of antioxidant peptide was 22.75 μM. The purified peptide exhibited an inhibitory effect against DNA oxidation induced by hydroxyl radical. The results of this study suggest that sand eel protein hydrolysate is a good source of natural antioxidants.

1. Introduction

Antioxidant activity is particularly important as oxidation is a common reaction that occurs in all living organisms [1]. Reactive oxygen species (ROS), in particular the superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), are unwanted metabolic byproducts of normal aerobic metabolism. Oxidation of biomolecules has been identified as a free radical mediated process, which results in numerous unfavorable impacts on food and biological systems [2]. In aerobic organisms, harmful radicals that inevitably form during the metabolism of oxygen are associated with the occurrence of several disease conditions including atherosclerosis, inflammation, and cancer [2]. Cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and some food-derived nutritional antioxidants, protect tissues from free radical mediated oxidative injuries [3]. In foods, development of rancid flavor and undesirable chemical compounds are the result of free radical mediated oxidation of fatty acids and lipids. Furthermore, oxidation of food lipids leads to the deterioration of food quality and shortens the shelf life. Even though the use of some synthetic antioxidants to overcome these problems in the food industry is common, it is under strict regulation because of potential health hazards. Since some artificial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and t-butylhydroquinone (TBHQ) may pose potential health hazards, many studies have been initiated to develop novel, safe and natural antioxidants [4]. Therefore, there is a growing interest to identify antioxidative properties in many natural sources including some dietary protein compounds. At the present, natural antioxidants such as vitamin C, α-tocopherol and phenolic compounds in vegetables, fruits and seeds possess the ability to reduce oxidative damage associated with many diseases.

Recently, enzymatic hydrolysis with proteases has garnered much attention. Protein hydrolysates or peptides affect health-related functions such as blood pressure and antioxidant function [5]. In this study, sand eel which is commonly used as food was evaluated in terms of its bioactivities after hydrolysis. A large number of proteolysis products including peptides and amino acids have been correlated with specific bioactivity [5].

Moreover, the researchers used various proteases to produce hydrolysates and to determine their functional activities. Many studies on purification of the antioxidative peptides from hydrolysates were done by gel filtration or ultrafiltration to determine the molecular weight distribution [6–8]. Protein hydrolysates of fish have been reported to possess antioxidative, antihypertensive, antimicrobial and immunomodulatory properties [9,10]. Even though the quantity of marine derived antioxidant is less when compared with the antioxidant peptide derived from artificial materials, the vast potential in terms of bioactivity of these marine derived antioxidant peptides has led to an emphasis on this field of research. Numerous studies have reported antioxidant activity of peptides derived from protein hydrolysates prepared from various fish sources such as capelin [11], yellowfin sole [12],

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Alaska Pollack [13], hoki [14], conger eel [15], scad muscle [16] and mackerel [8].

The sand eel, *Hypoptychus dybowskii* was most representative of the fish species in East Sea. In Korea, it is a traditional food that is a good source of calcium, essential amino acids, n-3 polyunsaturated fatty acids, and vitamins. Although there are many reports on the health-related activities of fish muscle hydrolysates to the level of peptide identification, no studies thus far have reported the characteristics of sand eel hydrolysates.

In the present study, we investigated the radical scavenging activity of enzymatically prepared sand eel protein hydrolysate and isolated a potent antioxidant peptide. Moreover, the protective effect of the purified peptide against DNA oxidation induced by the hydroxyl radical was tested.

2. Materials and methods

2.1. Materials

The sand eel, *H. dybowskii* was purchased in the local market in Gangneung, Korea. The sand eel whole body was grinded, and stored at −80 °C until used. Various enzymes, such as α-chymotrypsin (from bovine pancrease, type II), papain, pepsin, trypsin (from bovine pancrease, type II) were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase and neutrase were purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was also purchased from Wako Chemical Co. All other reagents used in this study were reagent grade chemicals.

2.2. Preparation of sand eel hydrolysates

To prepare sand eel hydrolysates, enzymatic hydrolysis was performed using various enzymes (Alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin) at their optimal conditions. The sand eel was hydrolyzed separately using various enzymes with a substrate to enzyme ratio of 1:100 for 12 h, under optimum pH and temperature conditions. After the reaction, enzyme activity was terminated by boiling at 100 °C for 10 min. An aliquot from each hydrolysate was centrifuged at 6000 rpm for 15 min and the supernatant was lyophilized for antioxidant activity analysis. Yield of hydrolysate from sand eel was calculated as follows:

$$\text{Yield}\,(\%) = \frac{\text{weight of the sand eel hydrolysates}}{\text{weight of the sand eel}} \times 100$$

2.3. Determination of DPPH radical scavenging activity

DPPH radical scavenging activity (RSA) was estimated using the method of Yen and Hsieh [17] with slight modification. The sample (40 μl) was mixed with 120 μl of methanol and then added to 40 μl of 0.15 mM DPPH in methanol. The mixture was allowed to stand at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a spectrophotometer (JASCO, Japan). The control was conducted in the same manner where methanol was used instead of sample. DPPH radical scavenging activity was calculated as follows: RSA (%) = $(A_{control} - A_{sample})/A_{control} \times 100$, where A_{sample} is the absorbance of sample and $A_{control}$ is the absorbance of the control. The EC_{50} value was defined as an effective concentration of peptide that is required to scavenge 50% of radical activity.

2.4. Purification of antioxidative peptides

The antioxidative hydrolysate obtained from sand eel was dissolved in distilled water and loaded onto a Sephadex G-25 gel filtration column (2.5 cm × 70 cm) which had been previously equilibrated with distilled water. The column was then eluted with the distilled water at a flow rate of 1.5 ml/min (fraction volume 7.5 ml), while separated fractions showing antioxidative activity were pooled and lyophilized. The antioxidative fraction was separated by reversed-phase HPLC on a Grom-sil 120 ODS-5 ST column (5 μm, 10 mm × 250 mm) using a linear gradient of acetonitrile (0–50%, v/v, 50 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min.

2.5. Determination of molecular weight and amino acid sequence

Molecular weight and amino acid sequence of purified peptide from sand eel protein were determined by Q-TOF mass spectrometry (Micromass, Altrincham, UK) coupled with electrospray ionization (ESI) source. The purified peptide dissolved in methanol/water (1:1, v/v) was infused into the ESI source and molecular mass was determined by doubly charged $(M + 2H)^{2+}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation and sequence information was obtained by tandem MS analysis.

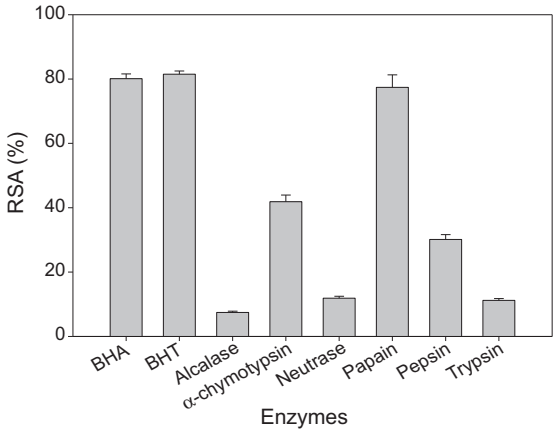

Fig. 1. DPPH radical scavenging activity (RSA) of sand eel hydrolysates prepared by various enzymes. Scavenging activity was measured at a concentration of 1.0 mg/ml.

2.6. Protection potential of induced DNA damage by hydroxyl radical

To evaluate the protective effects of the hydrolysate against DNA damage caused by hydroxyl radicals, a reaction was induced by placing the following reagents in an Eppendorf tube: 5 μl of genomic DNA (RAW 264.7 cell line), 2 mM $FeSO_4$, and various concentrations of the purified peptide. The mixture was then incubated at 37 °C for 30 min, followed by the addition of 4 μl of 10 mM H_2O_2 [18]. Next, the mixture was subjected to 1.0% agarose gel electrophoresis, after which the DNA bands were stained with ethidium bromide.

2.7. Statistical analysis

All experiments were performed in triplicate. All results were expressed as means ± standard errors of measurement.

3. Results and discussion

3.1. The sand eel protein hydrolysates and their antioxidant activity

The sand eel protein was hydrolyzed with Alcalase, α-chymotrypsin, neutrase, papain, peptide and trypsin in a batch reactor. The yield obtained were 79.95%, 73.38%, and 71.93% for trypsin, papain, and neutrase, respectively (data not shown). The antioxidant activity of the hydrolysates was evaluated using a DPPH radical, the strongest free radical scavenging. Among the hydrolysates resulting from various enzymes, the highest antioxidative activity was observed in the papain hydrolysate, and the activity was 77.4% at 1.0 mg/ml (Fig. 1). However, DPPH radical scavenging activity of papain hydrolysate was lower than that of synthetic antioxidants BHA and BHT. Antioxidant peptides are obtained by enzymatic hydrolysis of various proteins derived from marine organisms. Je et al. [19] reported that tuna backbone protein was separately hydrolyzed by six enzymes (alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin) for production of antioxidative peptides. Similarly, papain hydrolysate from tuna backbone had antioxidant activity.

3.2. Purification of antioxidant peptide

Considering the antioxidative effect on DPPH free radical scavenging activity, papain hydrolysate was employed for the purification and identification of antioxidant peptide. Initially, the papain hydrolysate was dissolved in distillated water and loaded on a Sephadex G-25 column using open column equipment and fractionated according to molecular weight using chromatography. Sephadex G-25 gel chromatography is a method that makes possible the separation of substances with different molecular dimensions, and it has been used for desalting protein solutions,

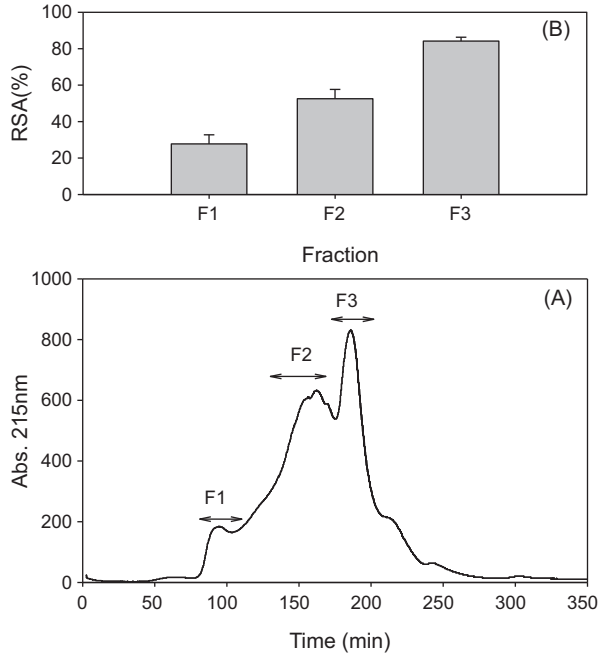

Fig. 2. Separation of antioxidant peptides from sand eel protein by Sephadex G-25 gel chromatography (A), and antioxidative activity of the fractions measured by DPPH radical scavenging activity (B). Elution was performed at flow rate of 1.5 ml/min with distilled water for 350 min.

protein separation from low molecular weight substances. It has also been applied for group separation of protein hydrolysates and biological extracts. As shown in Fig. 2, three fractions were isolated, among which, fraction F3 was identified to have the highest DPPH radical scavenging activity. The fraction F3 from Sepahadex G-25 was separated by reversed-phase HPLC using an ODS column and fractionated to six fractions (A–F) (Fig. 3(A)). The purified fraction A on HPLC possessed the highest antioxidant activity among the fractions. Next, fraction A was purified by reversed-phase HPLC using the C18 analytical column. Finally, three fractions (A1–A3) were obtained, and fraction A−1 had the higher antioxidative activity and its EC_{50} value was 24.39 μg/ml (Fig. 3(B)).

3.3. Characterization of purified antioxidant peptide

The purified fraction A−1 was analyzed by ESI–MS for molecular mass determination and ESI–MS/MS for the characterization of peptide. Amino sequences of the purified peptide was identified as Ile–Val–Gly–Gly–Phe–Pro–His–Tyr–Leu (M.W.; 1189 Da), and the EC_{50} value of the purified antioxidant peptide was 22.75 μM (Fig. 4). During hydrolysis, peptide bond cleavage allows the release of active peptides capable of sequestering oxygen radicals, chelating prooxidant metal ions and inhibiting lipid peroxidation in food systems [20]. The results further confirm the general finding that short peptides with 2–10 amino acids exhibit greater antioxidant activity and other bioactive properties than their parent native proteins or large polypeptides [21]. This indicated that it is possible to obtain antioxidative peptide from sand eel by enzymatic hydrolysis. Bougatef et al. [22] reported that antioxidant peptides were exhibited from sardinelle, such as Leu–Ala–Arg–Leu, Gly–Gly–Glu, Leu–His–Tyr and Gly–Ala–Leu–Ala–Ala–His. DPPH radical scavenging activity was 51 ± 1.31%, 38 ± 1.27%, 63 ± 1.5%, and 54 ± 1.38%, respectively. The antioxidant peptides from sardinelle displayed strong radical scavenging activity, which may be due to the presence of both histidine and tyrosine residues in this sequence [22]. In our results, we speculate that the His–Tyr sequence plays an important role in radical scavenging potency. Li et al. [21] reported that the antioxidative activity of histidine containing peptides was attributed to the proton-donation ability of the histidine imidazole group [22]. As well, histidine and proline participate in the antioxidative activity of designed peptides tests, among which Pro–His–His exhibited the greatest antioxidative activity [23]. Dávalos et al. [24] reported that among the amino acids, tyrosine, tryptophan and methionine showed the highest antioxidant activity, followed by histidine, cysteine and phenylalanine. The antioxidant activity of histidine containing peptides has been attributed to the chelating and lipid radical-trapping ability of the imidazole ring [25,26]. The amino acids of purified antioxidant peptide from sand eel had exists histidine, phenylalanine, tyrosine and phenylalanine.

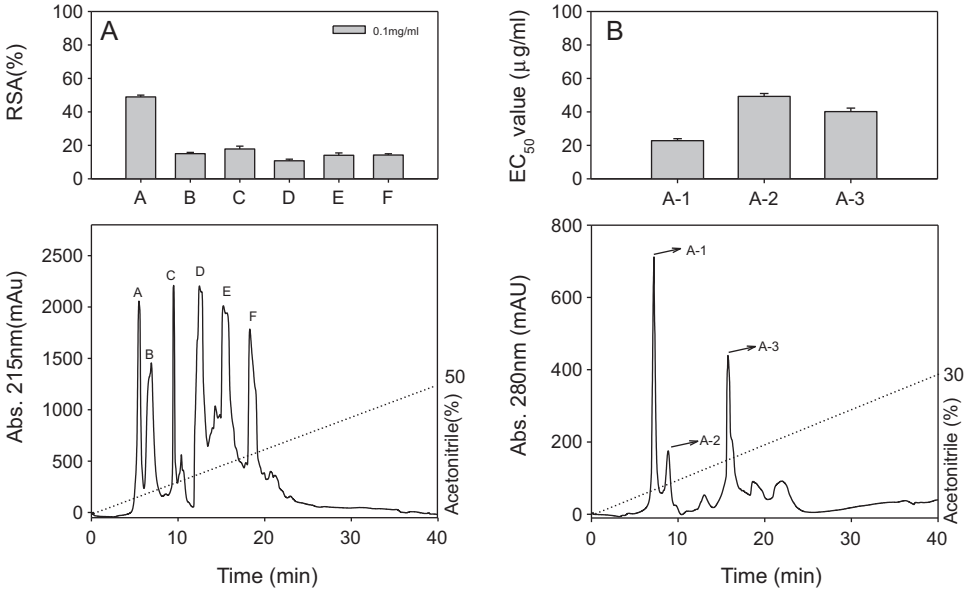

Fig. 3. (A) Reversed-phase HPLC pattern on a Grom-sil 120 ODS-5 ST column of active fraction F3 from Fig. 2, and antioxidative activity of the fractions measured by DPPH radical scavenging activity. HPLC operation was carried out with 50% acetonitrile as mobile phase at flow rate of 1.0 ml/min using UV detector at 215 nm. (B) Reversed-phase HPLC pattern on ODS C18 column of active fraction A, and antioxidative activity of the fractions measured by DPPH radical scavenging activity. HPLC operation was performed with 30% acetonitrile as mobile phase at flow rate of 0.5 ml/min using UV detector at 280 nm.

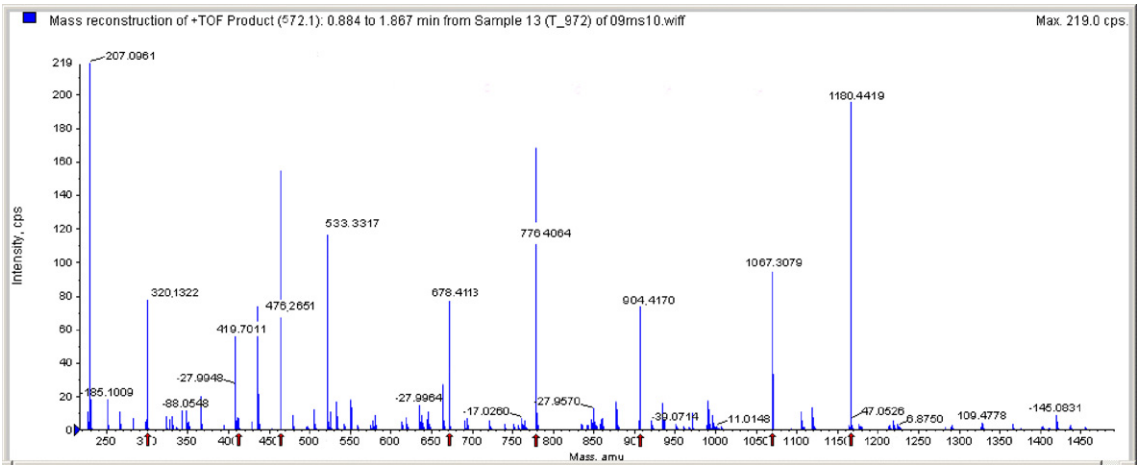

Fig. 4. Amino acid sequence of fraction A−1 of peptide purified from sand eel MS/MS spectrum from nanoflow-ESI/QTOF–MS fragment ion (observed mass 517.4ES + 2/expected mass 1189).

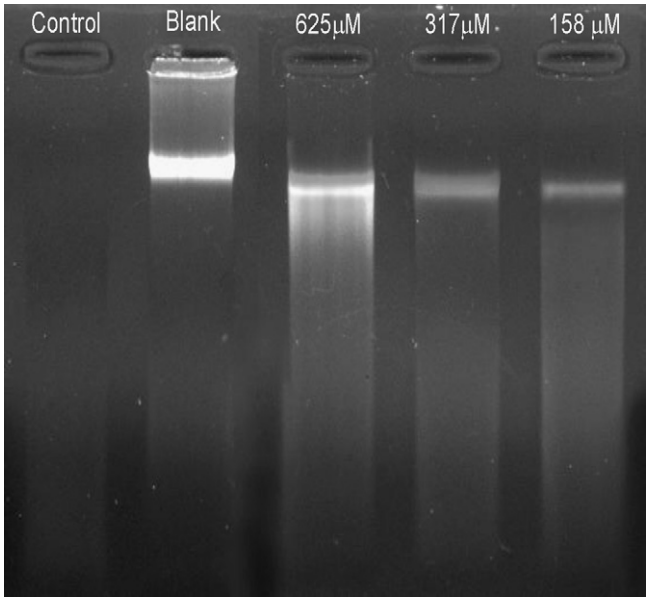

Fig. 5. Protective effect of purified peptide on hydroxyl radicals induced oxidation of genomic DNA. Control: distilled water instead of sample and treated H_2O_2. $FeSO_4$, blank: distilled water instead of sample and untreated H_2O_2, $FeSO_4$, sample; treated sample, H_2O_2, and $FeSO_4$.

3.4. Prevention of the purified peptide on oxidation-induced DNA damage

As shown in Fig. 5, the purified peptide had a protective effect against DNA oxidation induced by hydroxyl radical with increasing peptide concentrations ranging from 158 to 625 μM. These results indicated that purified peptides exerted adequate protective effects on radical-mediated DNA damage. Our results clearly explain that purified peptide can prevent oxidative damage to DNA when DNA was exposed to OH radical generated by Fe(II)/H_2O_2. Fe^{2+}-catalyzed conversion of H_2O_2 is a major route to the synthesis of OH radical in physical systems. The OH radical highly reacted with all components of the DNA molecule, leading to damage of both the purine and pyrimidine base, and also deoxyribose backbone lesion for DNA [27]. DNA is another sensitive bio-target for ROS-mediated oxidative damage [28]. DNA damage by ROS is known to initiate carcinogenesis or affect the pathogenesis for neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease; among ROS, the hydroxyl radical is recognized as a DNA-damaging agent of physiological significance [29].

4. Conclusion

The sand eel is the most abundant of the fish species in East Sea. In this study, sand eel protein was hydrolysed using enzymatic hydrolysis with various enzymes, and antioxidant activity peptide was determined and peptides were purified using chromatography. Finally, antioxidant peptide with nine-amino acids from papain hydrolysate of sand eel protein was purified. We suggest that hydrolysate from sand eel protein could be used as natural antioxidant to enhancing antioxidant properties of functional foods and to preventing oxidation reactions in food processing. In addition, it is expected that this will contribute to a developing interest in basic research in the potential application of bioactive peptides.

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