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Characterization of antioxidative peptide purified from black eelpout (*Lycodes diapterus*) hydrolysate

Jung Kwon Lee[1,2] and Hee-Guk Byun[1*]

Abstract

The functional peptides from protein hydrolysates of various fishery sources have been identified such as antioxidant activity. The main intention of this study was purification and characterization of antioxidative peptide from black eelpout muscle. The antioxidative peptides were purified from black eelpout (*Lycodes diapterus*) muscle using different proteases. Antioxidant activity of black eelpout hydrolysates was evaluated using DPPH radical scavenging activity. Among six hydrolysates, the pepsin hydrolysate had the highest antioxidant activity compared to the other hydrolysates. Therefore, it was further purified and a peptide with seven amino acid residues of DLVKVEA (784 Da) was identified by amino acid sequence analysis. The EC_{50} value for scavenging DPPH radicals by purified peptide was 688.77 μM. Additionally, the purified peptide exhibited protective effect against DNA damage induces by oxidation in mouse macrophages (RAW 264.7 cells). The results of this study suggest that black eelpout muscle protein hydrolysate could potentially contribute to development of bioactive peptides in basic research.

Keywords: Antioxidant, DPPH radical scavenging, Peptide, Hydrolysates, Pepsin, Black eelpout muscle

Background

Free radicals are highly reactive species with their single and unbalanced electrons. The oxidation by free radicals in the body may cause many chronic diseases such as cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders (Dong et al. 2008). Fatty acids and lipids oxidation induced by free radicals deteriorate the food quality (Liceaga-Gesualdo and Li-Chan 1999).

Reactive oxygen species (ROS) (O_2^- (superoxide anion), •OH (hydroxyl radical), and H_2O_2 (hydrogen peroxide)) are metabolic by-products of normal aerobic metabolism (Castro and Freeman 2001). Nevertheless, the body is supported with several antioxidant defense systems where they can scavenge and transform ROS or free radicals into harmless species (Yeung et al. 2002). The antioxidant defense system includes catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and glutathione reductase (GR). Enzymatic and non-enzymatic antioxidants team up to scavenge and eradicate the oxidative stress (McCord 1993). Steady-state maintenance of ROS/antioxidant ratio is vital for avoiding oxidative stress (Somani and Rybak 1996). Synthetic antioxidants (butylated hydroxyanisole (BHA), tbutylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and propyl gallate) have been widely used as food preservatives as they delay the discoloration and deterioration caused by oxidation (Wanita and Lorenz 1996). So, the use of these synthetic antioxidants has been limited in some countries due to their potential health hazard (Becker 1993).

Recently, enzymatic hydrolysis with proteases has garnered much attention. Protein hydrolysates or peptides affect health-related functions such as antioxidant function (Clemente 2000). Therefore, various antioxidant peptides have been isolated from marine organisms through enzymatic hydrolysis, including abalone muscle (*Haliotis discus hannai* Ino) and scallop (*Patinopecten yessoensis*) (Zhou et al. 2012), threadfin bream surimi (Wiriyaphan et al. 2012), croaker (*Otolithes ruber*) muscle (Nazeer et al. 2012), sand eel (Lee et al. 2011a, 2011b), sardinelle (*Sardinella aurita*) (Bougatef et al. 2010), tuna liver (Je et al. 2009), marine rotifer (Byun et al. 2009), and algae protein waste (Sheih et al. 2009).

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Enzymatic hydrolysates exhibited several advantages when incorporated into foods, by improving water-binding ability, solubility of protein, emulsifying stability, heat stability of myofibrillar protein, and the nutritional quality of foods. Thus, enzymatic hydrolysis has become an appreciated tool for modifying the applicability of proteins (Korhonen et al. 1998). Normally, bioactive peptides remain inactive within the parent protein molecule until they are released by hydrolysis. Most of bioactive peptides are composed with 2–20 amino acids. Amino acids arrangement of the peptides plays a critical role in its bioactivity (Himaya et al. 2012).

The black eelpout, *Lycodes diapterus*, is distributed in the Northwest Pacific/North of central East Sea of Korea and the Sea of Okhotsk and inhabits sand and mud bottoms in deep water of 150–200 m depth. Black eelpout is a traditional food that is rich in protein, essential amino acids, omega-3 polyunsaturated fatty acids, and vitamins. In the present study, we investigated the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity of enzymatically prepared black eelpout muscle protein hydrolysate to isolate a potent antioxidant peptide. And the protective effect of the purified peptide against deoxyribonucleic acid (DNA) oxidation induced by the hydroxyl radical was verified further.

Materials and methods

Materials

Fresh samples of black eelpout (*Lycodes diapterus*) were obtained from East Sea Fisheries Research Institute, Gangneung, South Korea. The bones and viscera were removed from the black eelpout. Then the separated muscle was stored at − 80 °C until use. Several commercial enzymes, such as α-chymotrypsin, papain, pepsin, and trypsin, were obtained from Sigma Chemical Co. (St. Louis, MO). Alcalase and Neutrase enzymes were obtained from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). DPPH was obtained from Wako Chemical Co. All other reagents used in this study were reagent grade chemicals.

Analysis of proximate compositions

Crude protein content of black eelpout was determined by the Kjeldahl method (Auto Kjeldahl system, Buchi B-324/435/412, Switzerland). Ether extraction method was used to determine the crude lipid content. Moisture content was determined by oven drying at 105 °C for 24 h. Ash content was determined by a muffler furnace at 550 °C for 4 h (Association of Official Analytical Chemist (AOAC) 2000). Amino acids were analyzed using an automatic analyzer (Hitachi Model 835-50, Japan) with a C18 column (5 μm, 4.6 × 250 mm, Watchers, MA). The reaction was carried out at 38 °C, with the detection wavelength at 254 nm and a flow rate of 1.0 mL/min. All analyses were carried out in triplicate.

Preparation of black eelpout muscle hydrolysates

To prepare black eelpout muscle hydrolysates, enzymatic hydrolysis was performed using various enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin) at their optimal conditions. Black eelpout muscle was hydrolyzed separately using various enzymes with a substrate to enzyme ratio of 1:100 for 6 h, under optimum pH and temperature conditions (Table 1). At the end of 6 h, hydrolysates were filtered by glass filter and lyophilized and stored at − 80 °C until use. The yield of hydrolysate from black eelpout muscle was calculated as follows:

$$\text{Yield}\ (\%) = \frac{\text{weight of the black eelpout hydrolysates}}{\text{weight of the black eelpout}} \times 100$$

Determination of DPPH radical scavenging activity

DPPH radical scavenging activity (RSA) was assessed by using the method of Yen and Hsieh (1995) with minor modifications. The sample was mixed with 120 μL of methanol and 40 μL of 0.15 mM DPPH in methanol was added. The mixture was incubated 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 sample was prepared in the same manner where methanol was used instead of the 40 μL sample volume. DPPH radical scavenging activity was calculated as follows:

$$\text{RSA}\ (\%) = \frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{control}}} \times 100$$

where A_{sample} is the absorbance of sample and A_{control} is the absorbance of the control. The EC_{50} value is defined as an effective concentration of peptide that is required to scavenge 50% of radical activity.

Table 1 Optimal conditions for enzymatic hydrolysis of various enzymes

Enzyme	Buffer	pH	Temperature (°C)
Alcalase	50 mM Na_2HPO_4-NaH_2PO_4	7.0	50
α-Chymotrypsin	50 mM Na_2HPO_4-NaH_2PO_4	7.0	37
Neutrase	50 mM Na_2HPO_4-NaH_2PO_4	7.0	50
Papain	50 mM Na_2HPO_4-NaH_2PO_4	7.0	37
Pepsin	20 mM HCl	2.0	37
Trypsin	50 mM Na_2HPO_4-NaH_2PO_4	7.0	37

Purification and identification of antioxidant peptides

The black eelpout muscle hydrolysate was dissolved in distilled water and loaded onto a Sephadex G-25 gel filtration column (2.5 × 70 cm) which had been previously equilibrated with distilled water. The column was then eluted with distilled water at a flow rate of 1.5 mL/min (fraction volume 7.5 mL) and separated fractions were monitored at 215 nm, collected at a volume of 7.5 mL, and measured for DPPH radical scavenging activity. Highest active fraction was injected into a preparative reverse-phase high performance liquid chromatography (RP-HPLC) column (Grom-Sil 120 ODS-5ST, ø 10 × 250 mm, 5 μm, Grom™, Germany) and was separated using linear gradient of acetonitrile (0–20% v/v) containing 0.1% trifluoroacetic acid (TFA) on an RP-HPLC system (Agilent Technologies, USA). Elution peaks were monitored at 280 nm on diode array detector (DAD). The purified fractions from preparative column were monitored at 280 nm and purified by RP-HPLC on a C18 analytical column (ø 4.6 × 250 mm, 5 μm, Waters, Milford, MA, USA) using an acetonitrile gradient of 5–30% (v/v) at a flow rate of 0.5 mL/min for 40 min. Finally, the fraction with the highest DPPH radical scavenging activity was collected and lyophilized followed by the amino acid sequence identification.

Determination of molecular weight and amino acid sequence

Molecular weight and amino acid sequence of purified peptide from black eelpout muscle protein were determined by quadrupole time-of-flight (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 the 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.

Protective potential by the hydroxyl radical-induced DNA damage

To assess the protective effects of the hydrolysate against DNA damage caused by hydroxyl radicals, the 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 from black eelpout hydrolysate. The mixture was then incubated at 37 °C for 30 min, followed by the addition of 4 μL of 10 mM H_2O_2 (Dávalos et al. 2004). Finally, the mixture was subjected to 1.0% agarose gel electrophoresis and DNA bands were stained with ethidium bromide.

Statistical analysis

Data were analyzed for statistical significance using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with statistical package for the social sciences (SPSS) software (version 14). All values obtained from three different experiments were expressed as the mean value ± standard deviation (SD).

Results and discussion

Proximate composition of black eelpout muscle

Proximate composition of black eelpout muscle showed the 20.81% moisture content, 8.63% lipid content, 4.09% ash, 2.46% carbohydrate, and 64.02% protein content (Table 2). The protein content was the highest among all the composition contents. However, the low lipid and ash content suggests that the extraction processes by enzymatic hydrolysis of biofunctional peptide is effective. The most abundant amino acids in black eelpout muscle were glycine, alanine, lysine, and leucine which accounted for 20.82%, 17.13%, 8.1%, and 6.24%, respectively (Table 3). Generally, fish and other mammalian skin have higher percentage of Gly, Leu, and Pro compared to muscle proteins (Gomez-Guillen et al. 2002).

Antioxidant activity of black eelpout muscle hydrolysates

Black eelpout muscle protein hydrolysates were prepared by using commercial proteases including Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin. The hydrolysis yields were 68.28%, 66.85%, 66.14%, and 58.76% for papain, Alcalase, pepsin, and trypsin, respectively (Table 4). Among six hydrolysates, pepsin hydrolysate exhibited the greatest DPPH radical scavenging activity relative to the other hydrolysates. In terms of the DPPH radical scavenging activation (Fig. 1), the lowest EC_{50} value was exhibited by the pepsin hydrolysate at 0.83 mg/mL. Thus the pepsin hydrolysate may contain bioactive compounds that could react with free radicals to transform them into more stable products and terminate the radical chain reaction. Peptides with antioxidative activity have been obtained by enzymatic hydrolysis of various marine organisms (Je et al. 2007). Several studies have suggested that the variation of antioxidant activity of a peptide is due to its amino acid sequence and length (Kim et al. 2001). However, DPPH radical scavenging activity of pepsin hydrolysate was

Table 2 Proximate compositions of black eelpout muscle

Components	Content (%)
Moisture	20.81
Protein	64.02
Lipid	8.63
Ash	4.09
Carbohydrate	2.46

Table 3 Amino acid contents of black eelpout muscle

Amino acids	Contents (%)
Tau	0.78
Asp	6.08
Thr	4.07
Ser	3.93
Glu	2.21
Gly	20.82
Ala	17.13
Val	3.61
Cys	0.34
Met	1.96
Ile	2.43
Leu	6.24
Try	1.71
Phe	2.33
Lys	8.10
His	1.80
Arg	5.13
Total	100.00

lower than that of synthetic antioxidants BHA and BHT. The next stage in analysis required the use of HPLC for purifying the antioxidant peptide from pepsin hydrolysate of black eelpout muscle.

Purification of antioxidant peptide

To identify the antioxidant peptide from pepsin hydrolysate of black eelpout muscle, the use of different chromatographic techniques is required. As shown in Fig. 2, chromatographic profiles were obtained during different purification steps of black eelpout muscle hydrolysate. In the first step, pepsin hydrolysate was separated into four fractions (A–D) on a Sephadex G-25 chromatography column (Fig. 2I). Among separated fractions, the B fraction had the highest DPPH radical scavenging activity at 0.65 mg/mL (Fig. 2I). Sephadex G-25 column chromatography separates according to molecular-size, where the primary fractions contain large-molecular-size peptides, and

Table 4 Yields of various hydrolysates from black eelpout muscle

Hydrolysates	Yields (%)
Alcalase	66.85
α-Chymotrypsin	51.30
Neutrase	39.47
Papain	68.28
Pepsin	66.14
Trypsin	58.76

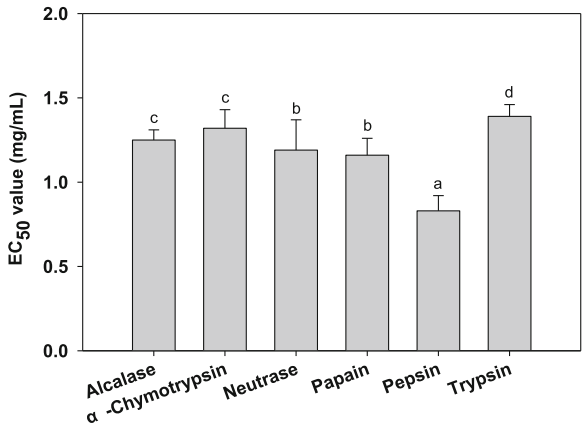

Fig. 1 EC_{50} values for DPPH radical scavenging activity of black eelpout muscle hydrolysates. Statistical significance was determined by ANOVA

secondary fractions contain small-molecular-size peptides. According to Pihlanto (2000), numerous bioactive peptides are found between 2 and 20 amino acids in length with a small-molecular-size. Therefore, the secondary fractions were assumed to have the greatest potential bioactivity. Fraction B was further separated by RP-HPLC using an ODS column and subsequently fractionated into three fractions (F1–F3) (Fig. 2II). Among separated fractions, the fraction F1 showed the highest DPPH radical scavenging activity with the EC_{50} value of 87.45 μg/mL (Fig. 2II). Fraction F1–1, with the strongest DPPH radical scavenging activity was purified further by using RP-HPLC on the C18 analytical column a linear gradient of acetonitrile (5–30%) for 40 min at a flow rate of 0.5 mL/min (Fig. 2III). The EC_{50} value of the purified peptide was 51.12 μg/mL, 16.24–fold compared to the pepsin hydrolysate (0.83 mg/mL) using the three-step purification procedure (Table 5). A single peptide fraction that demonstrated DPPH radical scavenging activity was purified on an analytical HPLC column and their amino acid sequences were determined by N-terminal sequencing analysis.

Characterization of purified antioxidant peptide

The purified fraction F1–1 was analyzed by electrospray ionization mass spectrometry (ESI-MS) for molecular mass determination and ESI-MS/MS for the peptide characterization. Amino acid sequence of purified antioxidant peptide was identified as Asp-Leu-Val-Lys-Val-Glu-Ala with EC_{50} value and molecular weight of 688.77 μM and 784 Da, respectively (Fig. 3). These results support for the general finding that short peptides with 2–10 amino acids demonstrate greater bioactive properties such as antioxidant activity compared to their parent native proteins or large polypeptides (Li et al. 2007). In this study, the purified antioxidant peptide was found to have a similar sequence with the other reports, including the sardinelle (*Sardinellaaurita*)

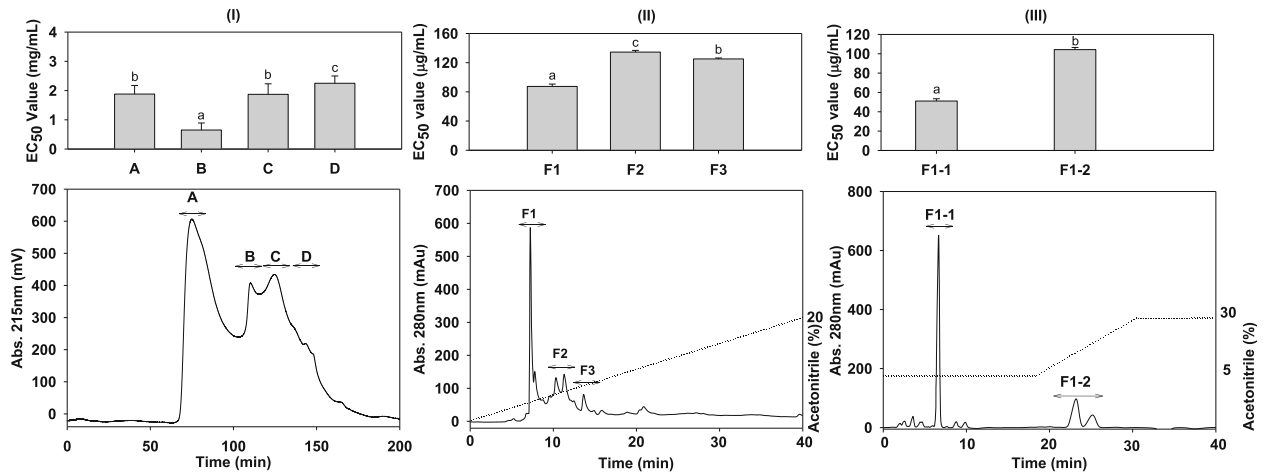

Fig. 2 Steps for the purification of DPPH radical scavenging activity peptide from black eelpout muscle hydrolysate. **I** Sephadex G-25 Gel filtration chromatogram of hydrolysates. Gel filtration chromatogram of hydrolysates prepared with black eelpout muscle. Separation was performed with 1.5 mL/min and collected at a fraction volume of 7.5 mL. The fractions isolated by Sephadex G-25 Gel column were separated (A–D) and DPPH radical scavenging activity was determined as upper panel. **II**, **III** Reverse phase-HPLC chromatograms of the potent DPPH radical scavenging activity fractions from the previous steps. The lower panels of each pair show the chromatography results of separated fractions while the top panels of each pair represent the DPPH radical scavenging activity of separated fractions in terms of their EC_{50} values expressed in mg/mL (**I**) or μg/mL (**II**, **III**). Statistical significance was determined by ANOVA

(Gly-Ala-Trp-Ala, RSA = 52 ± 1.44% at 150 μg/mL) (Bougatef et al. 2010), Nile tilapia (*Oreochromis niloticus*) (Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe, IC_{50} = 8.82 μM) (Ngo et al. 2010), black pomfret (*Parastromateus niger*) (Ala-Met-Thr-Gly-Leu-Glu-Ala, RSA = 78.6%) (Jai Ganesh et al. 2011), and croaker (Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala) (Samaranayaka and Li-chan 2011) (Lee et al. 2011a, 2011b). According to previous reports, the antioxidant peptides possess some metal chelation or hydrogen/electron donating activity, thereby allowing them to interact with free radicals and to terminate the radical chain reaction or prevent their formation (Ren et al. 2008; You et al. 2010). Amino acid constituents and sequence of peptides are vital for their antioxidant activity. Hydrophobic amino acids and one or more residues of cysteine, methionine, histidine, tyrosine, tryptophan, proline, and phenylalanine have been identified to enhance the activities of the antioxidant peptides (Ren et al. 2008; Je et al. 2007; You et al. 2010). As it has been confirmed, functional peptides rely on amino acid sequence and structure (Elias et al. 2008). Li et al. (2007) reported that the antioxidant activity of histidine-containing peptides was accredited to the proton-donation ability of the histidine imidazole group. Also, histidine and proline take part in the antioxidant activity of designed peptides tests, among Pro-His-His exhibited the greatest antioxidant activity (Tsuge et al. 1991). As reported by Dávalos et al. (2004), among amino acids, tyrosine, tryptophan, and methionine exhibited the highest antioxidant activity, followed by histidine, cysteine, and phenylalanine. The antioxidant activity of peptides containing histidine has been accredited to the chelating and lipid radical-trapping ability of the imidazole ring (Murase et al. 1993; Park et al. 2001). However, the active peptide in our study did not have hydrophobic amino acids. Since, our peptide yielded larger EC_{50} values.

Table 5 Purification of antioxidant peptide from black eelpout muscle hydrolysate by pepsin treatment

Purification step	EC_{50} value (μg/mL)	Purification fold[a]
Pepsin hydrolysate	830.01 ± 0.05	1.00
Sephadex gel filtration (B)	650.32 ± 0.14	1.28
RP–HPLC (F1)	87.45 ± 0.05	9.49
Purified peptide (F1–1)	56.12 ± 0.01	16.24

[a]Relative value of reciprocal of DPPH radical scavenging activity by EC_{50}

Prevention of oxidation-induced DNA damage by a black eelpout peptide

We evaluated the protective activity of purified antioxidant peptide against hydroxyl radical-induced DNA damage in in vitro studies by using RAW 264.7 cell line. As shown in Fig. 4, the purified peptide had a protective effect against DNA oxidation induced by hydroxyl radical with increasing peptide concentrations ranging from 50 to 200 μM. These results indicate that black eelpout peptide purified, exerted adequate protective effects on radical-mediated DNA damage. Furthermore, our results clearly explain the fact that purified peptide can inhibit oxidative damage to DNA when exposed to OH radical generated by Fe(II)/H_2O_2. Fe^{2+} catalyzes the conversion of H_2O_2 to OH radical in physical systems. The OH radical highly reacted leading to damage of both the purine and pyrimidine base and also

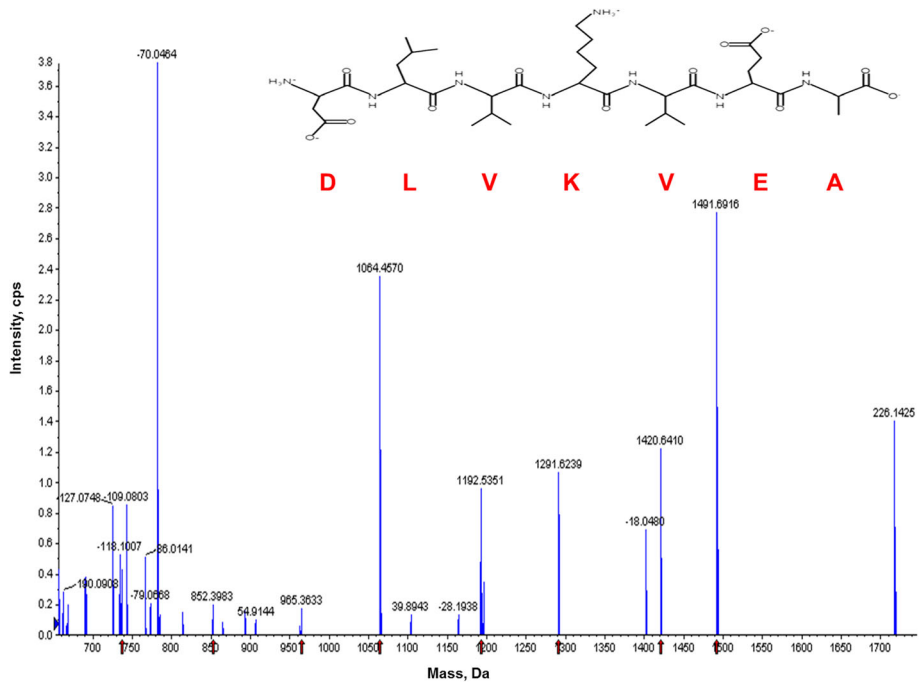

Fig. 3 Identification of molecular mass and amino acid sequence of the purified peptides from black eelpout muscle hydrolysate by HPLC. MS/MS experiments were performed on a Q-TOF tandem mass spectrometer equipped with a nano-ESI source

deoxyribose backbone lesion for DNA (Ngo et al. 2009). DNA is another sensitive bio-target for ROS-mediated oxidative damage (Martinez et al. 2003) as it is known to initiate carcinogenesis or pathogenesis in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Therefore, ROS, a hydroxyl radical, has been recognized as a DNA-damaging agent of physiological significance (You et al. 2002). Bioactive peptides with various biological activities such as antioxidative activity can be utilized in order to develop pharmaceutical and nutraceutical products in industrial scale (Abuine et al. 2019).

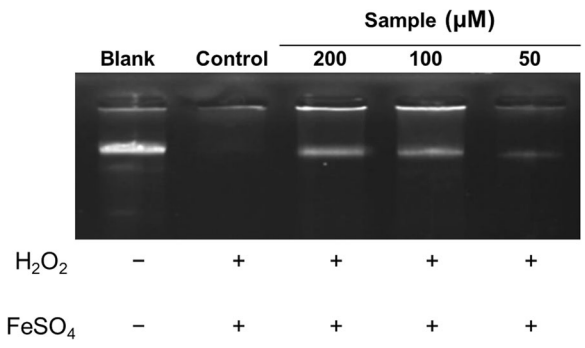

Fig. 4 Protective effect on oxidation-induced DNA damage of purified peptide from black eelpout at various concentrations. Blank: untreated sample and H_2O_2, $FeSO_4$. Control: distilled water instead of sample. Sample: Treated sample, H_2O_2, and $FeSO_4$. (+, treatment; −, not treatment)

Conclusion

In this study, black eelpout muscle protein was hydrolyzed using enzymatic hydrolysis with various enzymes. The antioxidant activity of the different enzyme hydrolysates was determined and compared. Pepsin hydrolysate showed the highest antioxidant activity and thus it was further purified using chromatography. A seven-amino acid residue peptide with antioxidant activity was identified from the pepsin hydrolysate of black eelpout muscle. Collectively, the results of this study suggest that black eelpout muscle protein hydrolysate could potentially contribute to development of bioactive peptides in basic research.

Abbreviations

ANOVA: Analysis of variance; BHA: Butylated hydroxyanisole; BHT: Butylated hydroxytoluene; CAT: Catalase; DAD: Diode array detector; DNA: Deoxyribonucleic acid; DPPH: 2,2-Diphenyl-1-picryl-hydrazyl-hydrate; ESI-MS: Electrospray ionization mass spectrometry; GR: Glutathione reductase; GSH-Px: Glutathione peroxidase; H_2O_2: Hydrogen peroxide; O_2−: Superoxide anion; •OH: Hydroxyl radical; Q-TOF: Quadrupole time-of-flight; ROS: Reactive oxygen species; RP-HPLC: Reverse-phase high performance liquid chromatography; SEM: Scanning electron microscope; SOD: Superoxide dismutase; SPSS: Statistical package for the social sciences; TBHQ: Tbutylhydroquinone; TFA: Trifluoroacetic acid

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Authors' contributions

HGB and LJK conceived and designed the study and helped to draft the manuscript and revised the manuscript. LJK performed the experiments,

analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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