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Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems☆

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Abstract

Low molecular weight peptides obtained from ultrafiltration (UF) of giant squid (*Dosidicus gigas*) muscle protein were studied for their antioxidative effects in different in vitro oxidative systems. The most potent two peptides, Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) and Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da), exhibited their antioxidant potential to act as chain-breaking antioxidants by inhibiting radical-mediated peroxidation of linoleic acid, and their activities were closer to highly active synthetic antioxidant, butylated hydroxytoluene. Addition of these peptides could enhance the viability of cytotoxic embryonic lung fibroblasts significantly ($P < .05$) at a low concentration of 50 μg/ml, and it was presumed due to the suppression of radical-induced oxidation of membrane lipids. Electron spin trapping studies revealed that the peptides were potent scavengers of free radicals in the order of carbon-centered (IC_{50} 396.04 and 304.67 μM), hydroxyl (IC_{50} 497.32 and 428.54 μM) and superoxide radicals (IC_{50} 669.34 and 573.83 μM). Even though the exact molecular mechanism for scavenging of free radicals was unclear, unusually high hydrophobic amino acid composition (more than 75%) of giant squid muscle peptides was presumed to be involved in the observed activities.

Keywords: Lipid peroxidation; Free radical; Cytotoxicity; Antioxidant

1. Introduction

Lipid oxidation, the reaction of oxygen with unsaturated lipids in biological systems and foods, has attracted a considerable research interest during the past few decades. Increasing evidences revealed that uncontrolled lipid peroxidation is involved in the occurrence of numerous chronic diseases [1,2]. In foods, lipid peroxidation is a major course of quality changes that affects the flavor, texture and appearance. In addition, this affects the nutritive value of foods and may cause disease conditions following consumption. Therefore, lipid oxidation and potential food-derived antioxidants that retard lipid peroxidation are important concerns in the study areas of human nutrition and biochemistry.

During last few decades, it has become evident that other than the major nutritional effects of animal and plant proteins, they can be used as a source to produce biologically active peptides to act as antioxidants. These peptides are inactive within the sequences of parent protein and can be released during gastrointestinal digestion or food processing [3]. Once bioactive peptides are liberated, depending on their structural, compositional and sequential properties, they may exhibit various bioactivities such as antioxidative [4,5], antihypertensive [6,7] and immunomodulatory effects [8,9]. Several studies have reported in vitro formation of antioxidative peptides from marine food sources and their potentials to use as alternative antioxidants [10,11]. In our previous studies on fish skin gelatin peptides, we could observe that they were more potent antioxidants compared to muscle protein-derived peptides [11].

Giant squid (*Dosidicus gigas*) is a cephalopod of the *Ommastrephes* genus and found in abundance in some parts of the world. This species is not much studied in depth due to its large soft mantel and low economic value. However, some special characteristics such as unusual amino acid composition compared to other fish and cephalopod species have attracted the interest to carry out research on this species [12]. Although the specific mechanism of antioxidative

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peptides is not clearly understood, it is believed that they act as scavengers of free radicals [4]. Therefore, in the present study, our interest was focused to isolate antioxidative peptides from giant squid protein sequences and to assess their antioxidative activities in different cellular and noncellular oxidative systems.

2. Methods and materials

2.1. Materials

Giant squid (*D. gigas*) was purchased from Charmson Co., Busan, South Korea. Digestive proteases, SP-Sephadex C-25 and Sephadex G-25 were products of Sigma (St. Louis, MO). All radical testing chemicals including 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were also purchased from Sigma. Human embryonic lung fibroblasts, MRC-5 (ATCC CCL-171), were obtained from American Type Culture Collection (Manassas, VA). Cell culture media and all the other materials required for culturing were obtained from Gibco, Life Technologies (USA). Other chemicals and reagents used were of analytical grade commercially available.

2.2. Enzymatic hydrolysis and amino acid composition of squid muscle protein

Giant squid skin was manually removed and the muscle was cut into small pieces. The muscle protein was separately hydrolyzed using pepsin, trypsin and α-chymotrypsin with substrate to enzyme ratio of 1:100 for 6 h, under optimum pH and temperature conditions. After the reaction, enzyme activity was terminated by boiling at 100°C for 10 min. Hydrolysates were analyzed for protein content by the method of Lowry et al. [13] and degree of hydrolysis (DH) was calculated. An aliquot from each hydrolysate was centrifuged at 12,000×*g* for 10 min and the supernatant was lyophilized for activity testing.

For the determination of amino acid composition, lyophilized squid muscle (20 mg) was hydrolyzed in 6 N HCl containing 0.1% thioglycolic acid at 110°C for 24 h under a vacuum. Amino acids derived with phenylisothiocyanate were identified and quantified using an automatic amino acid analyzer (Biochrom 20, Pharmacia Biotech, UK).

2.3. In vitro lipid peroxidation inhibition assay

Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidative activity following the method of Osawa and Namiki [14] with slide modifications. Briefly, peptide sample was dissolved in 5 ml of 50 mM phosphate buffer (pH 7.0) and added into a mixture of 99.5% ethanol (5 ml) and linoleic acid (0.065 ml) in which the final volume was adjusted to 12.5 ml with distilled water. In a parallel experiment, sample was replaced with a standard antioxidant α-tocopherol or butylated hydroxytoluene (BHT) as a positive control. The mixed solution in a slightly sealed screw-cap conical tube was incubated at 40°C in the dark. The degree of linoleic acid oxidation was measured at 24-h intervals by ferric thiocyanate method described by Mitsuda et al. [15]. Aliquot (0.1 ml) of reaction mixture was mixed with 75% ethanol (4.7 ml), 30% ammonium thiocyanate (0.1 ml) and 2×10^{-2} M ferrous chloride (0.1 ml) in 3.5% HCl. After 3 min, degree of color development that represents linoleic acid oxidation was measured spectrophotometrically at 500 nm.

2.4. Purification of antioxidative peptides

Tryptic protein hydrolysate was fractionated according to the molecular size using an ultrafiltration (UF) membrane reactor system (Millipore Minitan system, Millipore, Bedford, MA) separately with three different molecular weight cutoff (MWCO) membranes (10, 5 and 3 kDa) and lyophilized. Low molecular weight peptide fraction with a higher antioxidative activity was applied to an SP-Sephadex C-25 ion exchange column (∅ 3.5×30 cm) and washed with sodium acetate buffer (pH 4). Adsorbed peptides were eluted (1 ml/min) with a linear gradient of NaCl (0–2 M) in the same buffer and 4 ml fractions were collected. Peptide fractions were pooled monitoring the absorbance at 215 nm and desalted using a microacylizer, model G3 (Asahi Kasei, Kanagawa, Japan). Following the lipid peroxidation inhibition assay, the most active peptide group was further separated using a Sephadex G-25 column (∅ 2×75 cm) with distilled water at a flow rate of 0.5 ml/min, and eluted fractions (4 ml) were pooled after spectrophotometric measurements. The active fraction pool was injected into a capcell pack C_{18} UG120 (∅ 2×25 cm) preparative reverse-phase HPLC column (Shiseido Fine Chemicals, Tokyo, Japan) and eluted with a linear gradient of acetonitrile (0–60% v/v, in 60 min) at 3.5 ml/min flow rate. Peaks representing higher antioxidative activities were finally purified on a Zorbax SB C_{18} (∅ 0.46×25 cm) reverse-phase HPLC column (Agilent Technologies, USA) with a linear gradient of acetonitrile and 0.5 ml/min flow rate.

2.5. Radical-induced in vitro cytotoxicity assay

Human embryonic lung fibroblasts, MRC-5 (ATCC CCL-171), were grown as monolayers at 5% CO_2 and 37°C humidified atmosphere using EMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin–streptomycin.

To determine the radical-induced cytotoxicity, cells were seeded in a 96-well plate at a concentration of 1.3×10^4 cells/well and incubated with serum-free EMEM medium until 90% of confluence. Cells were treated with various concentrations of antioxidant peptide and incubated for 10 h. Cellular oxidation was accelerated exposing cells to 200 μM *t*-butylhydroperoxide (t-BHP), and after 2 h, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) method [16] that assesses the ability of succinate dehydrogenase to convert MTT into visible formazan crystals. For each well, 250 μl of MTT (0.5 mg/ml final concentration) was added and incubated (37°C) in the dark for 1 h. The formazan crystals formed were solubilized in DMSO and the optical density was measured at 570 nm by using an Emax microplate reader (Molecular Devices, Sunnyvale, CA). Relative cell viability was determined using the amount of MTT converted into formazan salt, and data were expressed as mean percentage of viable cells compared to the respective control culture.

2.6. Electron spin resonance determination of scavenging of free radicals

2.6.1. Hydroxyl radical scavenging assay

Fenton reaction was performed by reacting 50 μl of 10 mM $FeSO_4$ and 50 μl of 10 mM H_2O_2 to generate hydroxyl radicals [17]. Generated radicals were trapped by 50 μl of 0.3 M DMPO in the presence of peptide solution (50 μl) or same volume of phosphate buffer (pH 7.4) as a control. After 2.5 min, the reaction mixture was transferred to a sealed capillary tube and DMPO-OH adduct was recorded using a JES-FA ESR spectrometer (JEOL, Tokyo, Japan). Spectrometer conditions were controlled as follows: modulation frequency, 100 kHz; microwave power, 1 mW; microwave frequency, 9442 MHz; magnetic field, 336.5± 10 mT and sweep time, 30 s.

2.6.2. Carbon-centered radicals scavenging assay

For the generation of carbon-centered radicals, 20 μl of phosphate-buffered saline (pH 7.4), AAPH (40 mM), 4-POBN (40 mM) and peptide solution were mixed and incubated at 37°C for 30 min [18]. Reaction mixture was transferred to a sealed capillary tube, and POBN/carbon-centered radical adduct was recorded with the same spectrometer setting as described above except the microwave power (4 mW).

2.6.3. Superoxide radical scavenging assay

Superoxide anion radicals were generated by UV-irradiated riboflavin/EDTA system [19]. The reaction mixture containing 0.3 mM riboflavin, 5.0 mM EDTA, 0.1 M DMPO and different concentrations of peptide solution was irradiated for 1 min under UV lamp at 365 nm. The reaction mixture was transferred to the cavity of the ESR spectrometer using a sealed capillary tube, and the spin adduct was recorded under abovementioned spectrometric settings.

2.7. Characterization of antioxidative peptides

Accurate molecular mass and amino acid sequence of the most potent peptides were determined by Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with ESI source. The purified peptides were separately infused into the electrospray source following dissolve in methanol/water (1:1, v/v), and molecular mass was determined by doubly charged $(M+2H)^{+2}$ state in the mass spectrum. Following molecular mass determination, peptide was automatically selected for fragmentation, and sequence information was obtained by tandem MS analysis.

2.8. Statistical analysis

Data were expressed as mean±standard error of the mean ($n=3$). Student's t test was used to determine the level of significance at $P<.05$.

3. Results

3.1. Enzymatic preparation of peptides

Squid muscle was separately hydrolyzed with three digestive enzymes, pepsin, trypsin and α-chymotrypsin, to compare the lipid peroxidation inhibition ability of the resulted peptides. After 7 days of oxidative reaction, oxidation of linoleic acid could be reduced by 67% and 53% in the presence of trypsin- and α-chymotrypsin-derived peptides, respectively. These activities were closer to that of natural antioxidant α-tocopherol (vitamin E), and in contrast, activity of pepsin-derived peptides was comparatively much lower. Trypsin and α-chymotrypsin were found to hydrolyze squid muscle protein significantly (78% and 62%, respectively) compared to that of pepsin, measured by means of DH (data not shown). Amino acid composition of squid muscle protein was compared with that of different fish and shellfish species (Table 1). Glycine represented nearly two thirds (74.5 mol%) of total amino acids in squid muscle protein, and it was even higher to that of fish skin gelatin where glycine is abundant. In contrast, percentage composition of proline and arginine was more than 50% lower to that of shellfish and fish proteins.

3.2. Isolation and purification of antioxidative peptides

Considering the inhibitory effect on lipid peroxidation and DH, tryptic hydrolysate was employed for the identification of antioxidative peptides. Initially, tryptic hydrolysate was fractionated into four different molecular weight peptide pools employing UF with three different MWCO membranes (MWCO 10, 5 and 3 kDa). As shown in Table 2, a greater inhibition of lipid peroxidation (67.39%) was observed with low molecular weight peptides and the lowest molecular weight peptide pool (below 3 kDa) accounted for 64.68% of total peptides. Molecular weight distribution profiles of each peptide pool revealed that majority of peptides scattered within MWCO range of the membrane.

The lowermost molecular weight peptides were subjected to purification and characterization of their antioxidative properties. Eighteen grams of peptides below 3 kDa were applied onto a SP-Sephadex C-25 column, and

Table 1
Amino acid composition of giant squid protein and some other marine species

Amino acid	Residues/100 residues						
	Squid[a] muscle	Yellowfin[b] muscle	Eel[c] muscle	Mussel[d] muscle	Oyster[e] muscle	Hoki[f] gelatin	Squid[a] gelatin
Aspartic acid	3.24	12.32	8.73	8.58	9.46	3.80	7.06
Threonine	1.53	2.45	4.29	4.81	4.62	2.07	2.60
Serine	1.73	3.16	4.11	5.67	5.59	4.25	4.05
Glutamic acid	3.90	12.03	13.64	10.15	12.69	5.70	7.72
Proline	1.51	4.85	4.01	4.43	7.15	7.77	9.26
Glycine	74.47	11.10	10.50	16.83	13.49	47.63	36.48
Alanine	2.48	7.13	10.35	7.63	9.14	9.29	8.07
Cystine	0.23	1.56	0.35	1.14	0.70	0.16	0.26
Valine	1.07	7.85	5.78	5.05	4.03	1.71	2.00
Methionine	0.74	1.77	1.07	2.14	2.26	1.28	1.10
Isoleucine	0.90	5.91	4.76	4.86	3.60	1.09	1.10
Leucine	2.08	8.27	9.03	6.77	6.29	1.78	2.57
Tyrosine	0.77	7.72	2.67	2.29	2.42	0.19	0.73
Phenylalanine	0.99	6.41	3.96	3.29	3.76	1.19	1.25
Histidine	0.48	4.14	2.94	4.00	4.14	1.03	0.44
Lysine	2.37	1.77	12.27	7.72	6.40	2.11	2.04
Arginine	1.52	1.56	1.55	4.62	4.25	3.85	5.24
Hydroxyproline	–	–	–	–	–	4.47	7.34
Hydroxylysine	–	–	–	–	–	0.61	0.70

Skin gelatin from hoki (*Johnius belengerii*) and giant squid (*Dosidicus gigas*) was extracted as described by Kim et al. [11]

[a] *Dosidicus gigas*.
[b] *Limanda aspera*.
[c] *Conger myriaster*.
[d] *Mytilus edulis*.
[e] *Crassostrea gigas*.
[f] *Johnius belengerii*.

cationic peptides were eluted with a gradient of NaCl. As shown in Fig. 1, nine peptide fractions were isolated, and among them, fraction 8 was identified to be highly potent. It was further fractionated according to the molecular size using Sephadex G-25 column. Following activity test, the most potent peptides were purified with RP-HPLC on a capcell pack C_{18} UG120 column (Fig. 2). Seven clear peptide peaks (I–VII) were collected separately and compared for their antioxidative activity in free radical-mediated oxidative systems.

3.3. Inhibitory effects of peptides on lipid peroxidation

To assess the inhibitory effects of peptides on lipid peroxidation, linoleic acid was oxidized in an emulsified model system as described above. Except peptides I and VII, all the other peptides could retard lipid peroxidation efficiently than that of α-tocopherol (Fig. 2). Among them, peptides II and V exhibited the highest activities and their antioxidative patterns were closer to that of BHT during 7 days of oxidative reaction (Fig. 3). Further, peptides II and V contributed 87.7% and 95.8%, respectively, to the overall inhibition of lipid peroxidation after 7 days. In contrast, the contribution of peptides I and VII was significantly ($P<.01$) lower than that of peptides II and V.

Table 2
Ultra filtration separation of giant squid peptides and their in vitro antioxidative activities

Molecular weight range (kDa)	Yield recovered (%)	Distribution range of major peptides[a] (kDa)	Inhibition of lipid peroxidation[b] (%)
Above 10	2.18	12–15	18.27
10–5	8.73	7.2–5	23.32
5–3	24.41	3–4.5	48.45
Below 3	64.68	0.5–3	67.39

[a] Molecular weight distribution of peptides in each fraction obtained following ultra filtration was determined using gel-filtration HPLC column (OHpack SB-803, Showa Denko, Tokyo, Japan) and standard molecular weight markers.

[b] Inhibition of lipid peroxidation in the linoleic acid model system was determined as described in the text after 7 days.

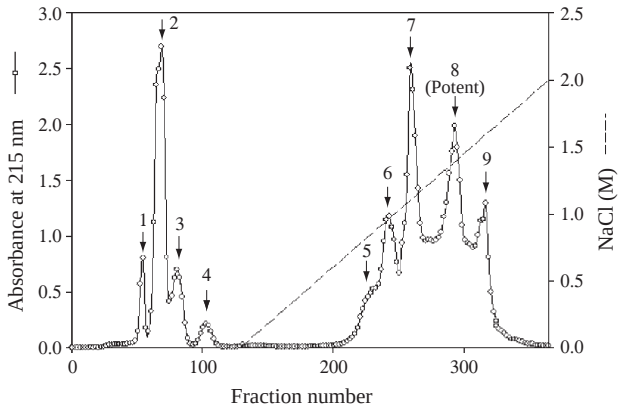

Fig. 1. Column chromatogram of giant squid muscle peptides on SP-Sephadex C-25. Bound peptides were eluted with a linear gradient of 0–2 M NaCl and peaks were collected at 215 nm optical density. Potent peak (8) with the highest lipid peroxidation inhibition activity was employed for further purification.

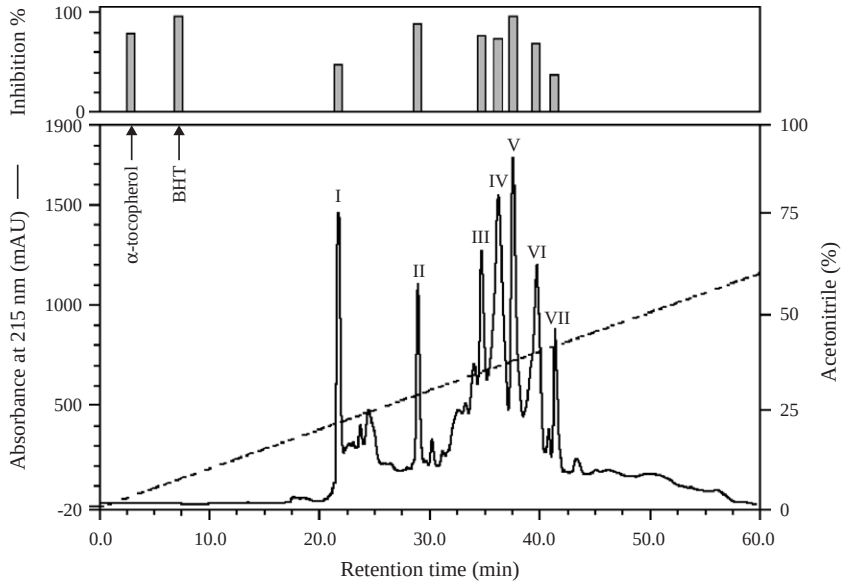

Fig. 2. A chromatogram of a reversed-phase HPLC (capcell pack C_{18} UG120 column) for the purification of antioxidative peptides. Peptides II (Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala) and V (Asn-Gly-Leu-Glu-Gly-Leu-Lys) were found to have the highest antioxidative activities and further characterized in different in vitro systems.

3.4. Effect of peptides on t-BHP-induced cytotoxicity

To assess the protective effects of squid muscle peptides on radical-mediated cellular injuries and death, cell culture experiment was performed using human embryonic lung fibroblasts. Cellular generation of alkoxyl and peroxyl radicals were simulated by exposing t-BHP for 2 h, and viability of cells were determined by MTT assay. According to the results of MTT assay, squid antioxidative peptides did not exhibit any cytotoxic effect to the lung fibroblasts at tested concentrations. Therefore, nontoxic concentrations of peptides (0–100 μg/ml) were used for the experiment, and peptides II and V significantly ($P<.05$) enhanced the viability of t-BHP-induced cytotoxicity up to 74% and 83% at the concentration of 75 μg/ml (Fig. 4).

3.5. Direct scavenging effects on free radicals

Direct scavenging effects of peptides II and V on hydroxyl, superoxide and carbon-centered radicals were determined using electron spin resonance spectroscopy (Table 3). Two peptides exerted varying scavenging potencies on free radicals and they were calculated based on the amplitude comparison of ESR spectra with their standard controls. However, both peptides could attenuate amplitudes of free radical spectra in the order of carbon-centered, hydroxyl and superoxide radicals. IC_{50} (concentration of peptide to

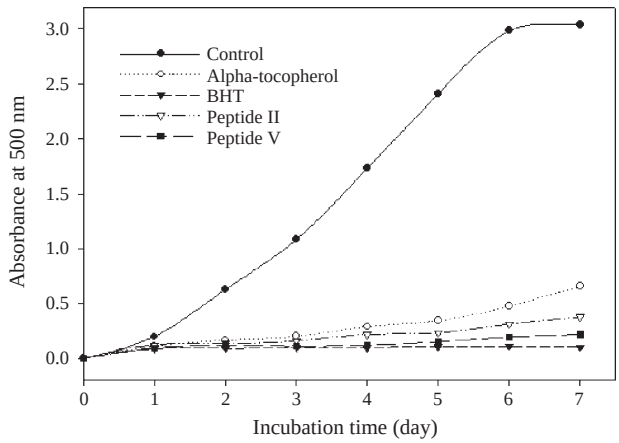

Fig. 3. In vitro lipid peroxidation inhibition activity of peptides II and V. The activity was measured in an emulsified linoleic acid model system as described in the text for 7 days using α-tocopherol and BHT as positive controls. Higher absorbance at 500 nm represents higher lipid peroxidation ability.

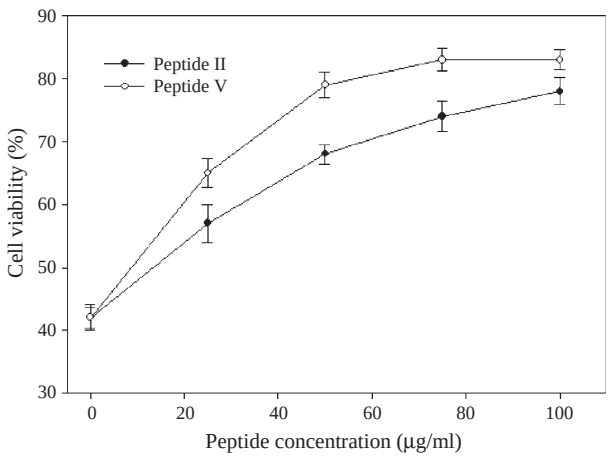

Fig. 4. Effect of peptides II and V on t-BHP-induced cytotoxicity. Human embryonic lung fibroblasts were cultured in EMEM medium, and cellular oxidative stress was artificially induced. Viability of cells treated with different concentrations of peptides was determined by MTT assay ($n=3$).

Table 3
Free radical scavenging activities of potent peptides

Peptide	IC_{50} (μM)		
	Carbon-centered radicals	Hydroxyl radical	Superoxide radical
II	396.04	497.32	669.34
V	304.67	428.54	573.83

scavenge 50% of radical activity) values of peptide II and V on highly scavenging carbon-centered radicals were found to be 396.04 and 304.67 μM, respectively. In addition, increased concentrations of all seven peptides exhibited dose-dependent scavenging activities with varying capacities (data not shown).

3.6. Characterization of antioxidative peptides

Among the seven peptides, peptides II and V were observed to be the most potent for their inhibitory effects on radical-mediated oxidative systems. Purity of above two peptides was further confirmed with analytical RP-HPLC Zorbax SB C_{18} column, and molecular mass together with amino acid sequence was analyzed using ESI/MS. Purified peptides II and V were identified to be Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) and Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da), respectively. *N*-terminals of both peptides were represented by asparagines residue and peptide profiles were rich in -Gly-Leu- sequence. In addition, these peptides represented the lowest molecular weight peptide fraction separated using 3 kDa MWCO UF membrane during initial stage of purification.

4. Discussion

Free radical-mediated oxidation of polyunsaturated fatty acids (PUFAs) is commonly observed in living tissues and foods. These PUFAs, characterized by one or more –CH=CH–CH_2–CH=CH– structural elements, are highly susceptible to attack by free radicals, and CH_2 groups localized between two double bonds are easily amenable to oxidation. Removal of a hydrogen radical from this CH_2 group requires much less energy than removal of hydrogen atom from other bonds, and it gives rise to series of different reactive oxygen species. Formation of reactive oxygen species contributes to lower the nutritive and other qualitative values of foods by uncontrolled oxidation of PUFAs. Further, these free radicals cause significant alteration in the structure of biological membranes that interferes with cellular integrity and metabolism leading to cellular toxicity, for which antioxidants could be of therapeutic value [1,2,20].

Several studies have already observed that some peptides derived from different protein sources possess antioxidative effects [4,5,11,21]. Our previous results revealed that fish skin gelatin-derived peptides were more potent antioxidants than peptides derived from other fish proteins, and one possible reason for this can be the unique amino acid composition. Generally, fish and other mammalian skin gelatins possess higher percentage of glycine and proline than that of their muscle proteins [22]. Surprisingly, glycine content of giant squid muscle was higher than the other fish gelatins, and proline content was considerably lower than that of other fish muscle proteins. In addition, most of the other amino acid contents closely resembled the amino acid composition of fish gelatin, but not fish or shellfish muscle proteins (Table 1). This unusual amino acid composition stimulated our curiosity to identify the antioxidative effects of squid muscle peptides in different in vitro model systems by artificial induction of free radicals or lipid peroxidation.

We utilized three main gastrointestinal proteases to extract antioxidative peptides, and throughout the peptide isolation, activity was evaluated in linoleic acid model system as described previously. The observed higher DH of trypsin- and α-chymotrypsin-derived hydrolysates (compared to that of pepsin-derived hydrolysate) could be due to the amino acid composition and the cleavage specificity of enzymes. Many researches report that low molecular weight peptides are more potent as bioactive peptides [6,8,21]. Considering both DH and antioxidative effects, trypsin-derived hydrolysate was firstly fractionated using UF membranes with different MWCOs. Our results agree with the results of above reports and exhibited a significant ($P<.001$) antioxidative activity in the lowermost molecular weight peptide fraction (below 3 kDa). Further, more than 50% of peptides could be recovered as low molecular weight peptides confirming higher DH by trypsin (Table 2). Following different chromatographic purification, we could isolate seven antioxidative peptides on reversed phase HPLC with milligram scale.

Two in vitro experiments were performed in different model systems to determine the significance of antioxidative activity. In the first model system, a well-known PUFA, linoleic acid was oxidized in an ethanol/water emulsion where transition metal ion, Fe^{2+}, accelerated lipid peroxidation. Chemistry of this process involves generation of peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals form preexisting lipid peroxide (LOOH) to initiate lipid peroxidation [23]. Cheng et al. [24] reported that phenolic compounds in a similar model system could perform antioxidative activity by scavenging lipid-derived radicals ($LOO^{\bullet}$ or $LO^{\bullet}$) and thereby breaking free radical chain reaction of lipid peroxidation. In another study, Tong et al. [25] revealed that high molecular weight fraction of whey protein was able to inhibit lipid peroxidation via scavenging of free radicals. As observed in this research, amino acid composition of giant squid muscle peptides was unique and rich in hydrophobic amino acids (more than 75%) to make better affinity for linoleic acid. Moreover, hydrophilic amino acids such as, proline, serine, threonine, cystine, aspartic acid and glutamic acid were lower in composition compared to the other fish and shellfish proteins, and supposed to improve its solubility in the emulsion (Table 1). This facilitates

scavenging of lipid radicals by peptides keeping close contact with lipid molecules and donating protons to lipid-derived radicals. Surprisingly, except glycine, contents of all the other hydrophobic amino acids were lower in squid muscle protein. Therefore, considering the amino acid composition of crude protein hydrolysate, glycine was presumed to play an important role for observed retardation (67%) of linoleic acid oxidation. Except peptide I and VII, all the other peptides exhibited a higher antioxidative activity than standard antioxidant α-tocopherol, indicating that these peptides can act as better chain-breaking antioxidants (Fig. 2). Moreover, peptides II and V had very closer inhibitory activity to strong synthetic reducing agent BHT (Fig. 3), and sequence analysis studies revealed that they are rich in hydrophobic amino acids such as glycine, leucine, and alanine. Furthermore, -Gly-Leu- sequence was frequently observed in both sequences. Therefore, this hydrophobic diamino acid sequence that represents approximately 50% of residues in the profiles of both peptides was expected to favor oxidation inhibition. Since our interest was focused to study on these -Gly-Leu-rich peptides in different in vitro oxidation systems, their sequence contribution for the activity was not identified in detail by making synthetic mimics.

In the second model system, lipid-derived radicals were generated in an in vitro cellular environment by exposing human embryonic lung fibroblasts to a membrane permeable oxidant, t-BHP. t-BHP can be easily metabolized into free radical intermediates and forms $LOO^{\bullet}$ and $LO^{\bullet}$ that initiate peroxidation of membrane lipids [26]. Peroxidation of membrane lipids affects cell integrity and forms covalent bonds with cellular molecules resulting in t-BHP-induced toxicity and cell death. The capacity of giant squid muscle peptides II and V to overcome oxidation-induced cytotoxicity had marked increment dose dependently, up to 75 μg/ml concentration (Fig. 4). The ability of peptide II to scavenge lipid-derived radicals was much lower than that of peptide V at the same concentration. Although at higher concentrations above 75 μg/ml, peptides could not increase their activity, our results clearly demonstrated the considerable ability of peptide II and V to decrease t-BHP-mediated cell death. And this was presumed to be due to their ability to scavenge lipid-derived radicals that would otherwise oxidize PUFAs in cell membranes. This effect was predicted based on the ability of a common lipid soluble antioxidant, α-tocopherol, to inhibit radical-mediated membrane lipid oxidation in cultured human umbilical endothelial cells [27]. Further, the higher activity of peptide V can be attributed to the lower molecular weight that improves contact ability with membrane lipids and/or permeability. Chu et al. [28] studied the antioxidative activity of capillarisin (an antioxidative compound extracted from *Artemisia capillaris*) against t-BHP-induced oxidative damage in rat primary hepatocytes and revealed that one of the reasons for its activity is scavenging of lipid-derived free radicals. Moreover, results of first model system further strengthen the ability of giant squid muscle peptides to reduce t-BHP-induced cell death by scavenging lipid-derived radicals in an in vitro cellular environment.

When linoleic acid is oxidized in an emulsified model system, types of radicals present in the oxidation process can be varied depending on the light/dark conditions [29]. It is known that superoxide anion (O_2^{-}) and hydrogen peroxide are formed during the light exposure, and hydroxyl radicals ($^{\bullet}OH$) are generated according to Haber-Weiss reaction [30]. Therefore, oxidation of linoleic acid can be accelerated by exposing to light where, $^{\bullet}OH$ and superoxide radicals (O_2^{-}) play a major role. To identify the direct scavenging effect of above radicals, they were generated separately, and scavenging was studied using electron spin trapping technique. Both peptides II and V could scavenge the tested radicals in the order of carbon-centered, hydroxyl and superoxide radicals. Carbon-centered radicals that represent $LOO^{\bullet}$, $LO^{\bullet}$ and $L^{\bullet}$ could be scavenged efficiently by peptide V than peptide II (Table 3). It is believed that aromatic amino acid and histidine act positively as direct radical scavengers within peptide sequences. But neither of these peptides consisted of above amino acids in their sequences. Therefore, it can be speculated that difference in scavenging activity could be due to the molecular weight or the specific arrangement of glycine and other hydrophobic residues in the sequence. Although the exact mechanism that underlie higher scavenging of carbon-centered radicals is not well understood, these results agree with the results of the above two in vitro model systems. Therefore, summarizing the results of antioxidative activity tested in in vitro model systems, it can be presumed that squid muscle peptides are potent antioxidants to scavenge lipid-derived radicals due to unique high content of hydrophobic amino acid residues in their sequences.

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