魚介類由来の生理活性ペプチドに関する研究(5) ワカメジペプチド誘導体の抗酸化活性について

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Studies on Biologically Active Peptide Derived from Fish and Shellfish － V Antioxidant Activities of *Undaria pinnatifida* Dipeptide Derivatives*1

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The antioxidant activity of *Undaria pinnatifida* dipeptides was firstly evaluated by the ferric thiocyanate method. Among the synthetic dipeptides containg Lys, three dipeptides had strong activity and their antioxidant activity evaluated by the induction period was 32.1 days for Lys-His, 26.1 days for Lys-Met and 25.0 days for Met-Lys. Furthermore, the IC_{50} value for superoxide anion-scavenging activity by XOD-XTT method was 2.52 mM for Lys-His, 10.5 mM for Lys-Met and 11.9 mM for Met-Lys. The IC_{50} value for hydroxyl radical-scavenging activity by the ESR method was 1.02 mM for Lys-His, 2.66 mM for Lys-Met and 2.86 mM for Met-Lys.

1 Introduction

Active oxygen species will attack some biologically relevant molecules such as proteins, lipids and nucleic acids. To prevent from the damage caused by free radicals, it is necessary to investigate antioxidants in various foods. The mechanism of the antioxidant effects of nitrogen-containing molecules still remains unclear.[1, 2] Recently, we found that the proteolytic digests of dried bonito[3] and prawn muscle[4] had the most potent antioxidant activity among several marine resources digests tested. In these studies, all antioxidant peptides obtained contained lysine in the sequence. On the other hand, many angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides have been derived from enzymatic protein hydrolysates to prepare functional food material available for antihypertension. The Lys-containing dipeptides having the potent ACE inhibitory activity were obtained from *Undaria pinnatifida* protein hydrolysate by protease, and the relationship between the ACE inhibitory activity and the structure was discussed in the previous paper.[5]

In the present study, we describe the antioxidant Lys-containing dipeptides synthesized, and further comparative active oxygen-scavenging activities (AOSA) of the *Undaria pinnatifida* dipeptides have been investigated.

2 Materials and Methods

2.1 Peptide synthesis

Peptides were synthesized by a solid-phase method using a 430A automated pep-

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tide synthesizer (Applied Biosystems Co., California), followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. The final products were homogeneous in high-resolution reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 150mm, Nomura Chemical Co.), using a gradient elution from 0.05% trifluoroacetic acid (TFA) to 16.7% acetonitorile (MeCN) in 0.05% TFA for 2 hr at a flow rate of 0.8 mℓ/min. The eluate was monitored by UV absorption at 215 nm with a UVIDEC 220E spectrophotometer (Nihonbunko Co., Tokyo).

2.2 Measurement of antioxidant activity

Antioxidant activity was determined according to the ferric thiocyanate method.[6] The mixture of two kinds of amino acids (2 mM each) or the synthetic dipeptide (0.2 mM each) was added to the following oxidation system. To each sample solution in the test tube (10 mℓ volume) with a screw cap, linoleic acid (18.6 mM), 4 mℓ of 0.1 M phosphate buffer (pH7.0), and 2 mℓ of water were added. Tubes were sealed tightly with silicon rubber caps and kept at 60℃ in the dark. Every 24 h, aliquots of the reaction mixtures were sampled with a microsyringe for determination of oxidation levels. To 0.1mℓ of the reaction mixture were added 9.7mℓ of 75% ethanol, 0.1mℓ of 30% ammonium thiocyanate, and 0.1mℓ of 20 mM ferrous chloride solution in 3.5% HCl. After 3 min, absorbance of the solution at 500 nm was measured with a UVIDEC 220E spectrophotometer (Nihonbunko Co., Tokyo). The number of days required to attain an absorbance of 0.3 was defined as the induction period. The induction period referred to the antioxidant activity of the samples.

2.3 Measurment of active oxygen-scavenging activity (AOSA)

The superoxide anion-scavenging activity of the synthetic dipeptide was measured by XOD-XTT method.[7] Into 2.5mℓ of 50 mM sodium carbonate buffer (pH 10.2) were added 0.1mℓ each of 3 mM xanthine, 3 mM ethylenamine-tetraacetic acid (EDTA), 0.75 mM solution, and sample solution or water. The reaction was initiated by the addition of 56 mU/mℓ XOD solution (0.1mℓ). The absorbance change at 470 nm for 20 minutes was monitered with a UVIDEC 220E spectro-photometer (Nihonbunko Co., Tokyo) thermostated at 25℃. The concentration of the test compound required to reduce the produced superoxide anion to one-half (IC_{50}) was calculated.

The activity of the synthetic dipeptide to scavenge the hydroxyl radical produced was examined by the spin trapping method[8] using 5,5-diethyl-1-pyrroline-N-oxide (DMPO) as the spin trapping agent. Seventy-five microliters of 1 mM $FeSO_4$ in 0.1 mM sodium phosphate buffer (pH7.8) containing EDTA, 50 μℓ of tested compound solution, and 10 μℓ of 0.9 M DMPO were successively put into a test tube, and stirred after the addition of 75 μℓ of 10 mM hydrogen peroxide (H_2O_2) to initiate the reaction. The reaction mixture was then transferred to a quartz cell used for the measurement of a JES-FR 30 electron spin resonance (ESR) spectrometer (JEOL). After one minute of the initial reaction, formed DMPO-OH was measured, and IC_{50} values (the concentrations of the test compounds required to reduce the produced hydroxyl radical to one-half) were calculated. The conditions for the ESR measurement were as follows: magnetic field, 336±5 mT; output, 10 mW; modulating, 100 kHz with 0.1 mT; response time, 0.1 second; sweeping time, 1 minute (interval of 10 mT); and amplification raito, 125.

Table 1. Antioxidant activity of amino acid mixture

Amino acid mixture	Induction period (Days)
Lys + Met	64
Lys + Tyr	20
Lys + Gly	20
Lys + His	19
Lys + Val	19
Lys + Thr	18.5
Lys + Arg	18.5
Lys + Asp	17.5
Lys + Phe	17.5
Lys + Leu	17
Lys + Ile	16.5
Lys + Pro	16.5
Lys + Ser	16
Lys + Ala	15
Lys + Glu	14
Lys + Trp	13.5

All amino acids (2 mM each) are of the L-configuration.

3 Results and Discussion

Kawashima et al.[9] investigated the effects of many synthetic peptides on lipid oxidation and found that some peptides having branched-chain amino acids showed antioxidant activity. Yamaguchi et al.[10] reported that the dipeptides consisting of Tyr and Trp at the N-terminal, and His and Met at the C-terminal showed stronger antioxidant activity than the constituent amino acid mixtures in an aqueous system.

The antioxidant activity of the mixture of Lys and other amino acids was shown in Table 1. The mixture of Lys and Met had the strongest antioxidant activity (Induction period; 64 days), which was more than three times of other amino acid mixtures. The antioxidant activities of artificial synthesized Lys-conatining dipeptides were shown in Table 2. The N-terminal Lys-dipeptides consisited of Trp, Ile, Ser, Pro, Arg, Thr and Phe showed more antioxidant activities than those at C-terminal. The C-terminal Lys-dipeptides consisted of His, Met, Gly, Tyr, Ala and Leu showed more antioxidant activities than those at N-terminal. The induction period of Lys-Met was 26.1 days and that of Met-Lys was 25 days. The dipeptides with the strongest antioxidant activity was Lys-His;32.5 days, but that of His-Lys (9.1 days) was not strong. As shown in this study, the potent antioxidant peptides contained His and Met residues in the sequence.

Table 2. Antioxidant activity of Lys-containing dipeptide

Lys antero-dipeptide COOH-terminal	Induction period (Days)	Lys retro-dipeptide NH_2-terminal	Induction period (Days)
Lys-His	32.5	Met-Lys	25
Lys-Met	26.1	Trp-Lys	17.5
Lys-Gly	15.5	Ile-Lys	17
Lys-Tyr	15	Ser-Lys	17
Lys-Asp	14	Pro-Lys	16.5
Lys-Ala	13	Arg-Lys	15.5
Lys-Trp	13	Thr-Lys	15.5
Lys-Ser	12.5	Phe-Lys	15
Lys-Phe	12	Asp-Lys	15
Lys-Leu	11	Lys-Lys	14.5
Lys-Thr	10	Val-Lys	14
Lys-Arg	10	Gly-Lys	12
Lys-Val	7.5	His-Lys	9.1
Lys-Cys	7.0	Tyr-Lys	7.5
Lys-Ile	6.5	Ala-Lys	7.5
Lys-Glu	6	Leu-Lys	7.5
Lys-Asn	5.5	Asn-Lys	6.5
Lys-Gln	5	Cys-Lys	5.5
Lys-Pro	4.5	Glu-Lys	5
		Gln-Lys	4.6

All amino acids (0.2 mM) are of the L-configuration.

Chang and Lim[11] have reported that the mixture of linolic acid salts and basic amino acids (Lys or Arg) show unusual stability toward the oxidation of linolic acid. Karel et al.[12] have reported that His, β-amino-butyric acid, Lys and Cys, have substantial antioxidant activity. Although the structure-activity relationship of antioxidant Lys-containing peptides has not been well clarified yet, it is considered that Lys may play as antioxidant only at the initial stage of the oxidation, and the Lys-

Table 3. Superoxide anion-scavenging activity of Lys-containing dipeptides

Sample	IC_{50} value (mM)
Lys-His	2.5
Lys-Met	10.5
Met-Lys	11.9
L-carnosine*	23.3

* Control.

Table 4. Hydroxyl radical-scavenging activity of Lys-containing dipeptides

Sample	IC_{50} value (mM)
Lys-His	1.02
Lys-Met	2.66
Met-Lys	2.86
L-carnosine*	0.654

* Control.

containing peptides showed their antioxidant ability mainly in the chelating ability of metal ions. Recently, the antioxidant activity of carnosine, His-containing dipeptide, was reviewed extensively.[13] The antioxidant activity is attributed to hydrogen-donating ability, lipid peroxy radical trapping, or the metal ion-chelating ability of the imidazole group. Furthermore, Tsuge et al.[14] reported the isolation of a potent antioxidant tripeptide, Ala-His-Lys, from the hydrolysate of egg white albumin, in which neither His-Lys nor a mixture of constituent amino acids had any activity, but Ala-His was as potent as the tripeptide.

On the other hand, there has been strong interest in the inhibition of lipid peroxidation, not only for extending the self-life of foods, but also for defending living cells against oxidative damage, because lipid peroxides and their decomposed products are associated with aging and carcinogenesis. Active oxygen in the forms superoxide anion, hydrogen peroxide and hydroxyl radical is a byproduct of normal metabolism and attacks biological molecules, leading to cell or tissue injury.[1, 2] Few recent *in vitro* studies have demonstrated the peptide having active oxygen-scavenging activity (AOSA) as a free radical scavenger.

Table 3 showed the superoxide anion-scavenging activity of potent antioxidant peptides; Lys-His, Lys-Met, and Met-Lys. From this result, Lys-His with IC_{50} value of 2.5 mM was most effective among them in superoxide anino-scavenging activity, and Lys-Met with IC_{50} value of 10.5 mM and Met-Lys with IC_{50} value of 11.9 mM were better than carnosine (IC_{50} value; 23.3 mM). Table 4 shows the hydroxyl radical-scavenging activity of potent antioxidant peptides. The Lys-His with IC_{50} value of 1.02 mM was more effective than Lys-Met with IC_{50} value of 2.66 mM and Met-Lys with IC_{50} value of 2.86 mM. However, these dipeptides (Lys-His, Lys-Met and Met-Lys) were less than carnosine (IC_{50} value; 0.654 mM).

Trelstad et al.[15] have reported that reduced oxygen derivatives can hydroxylate both free and polypeptide-bound Pro and Lys, and that scavengers of hydroxyl radicals suppress, but do not completely inhibit this reaction. Gardner[16] found that Lys may remove free radicals when it reacted with lipid peroxides. In our experiments, Lys-His had the significant antioxidant and radical scavenging capacity. Thus, based on the results obtained in the present investigation, it would be of value to search for more potent antioxidant and radical scavenging activity. Comparative studies of Lys-His analogues are currently underway.

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魚介類由来の生理活性ペプチドに関する研究—V
ワカメジペプチド誘導体の抗酸化活性について

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ワカメジペプチド誘導体を合成し，最初にそれら抗酸化活性をロダン鉄法で測定した結果，強い活性を示したリジン含有ジペプチドはLys-Hisでの誘導期間が32.1日，Lys-Metで26.1日およびMet-Lysで25.0日であった。続いて抗酸化活性の強いこれらリジン含有ジペプチドについて，活性酸素消去能としてのスーパーオキシドアニオン消去活性をXOD-XTT法で測定した結果，Lys-HisでのIC$_{50}$値が2.52 mM，Lys-Metで10.5 mMおよびMet-Lysで11.9 mMであった。また，ESR法によるヒドロキシルラジカル消去活性は，Lys-HisでのIC$_{50}$値が1.02 mM，Lys-Metで2.66 mMおよびMet-Lysで2.86 mMであった。