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Note

Water Soluble Proteinous Substances as Free Radical-Scavengers in Fish

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The role of high molecular weight compounds in the radical-scavenging activity of horse mackerel muscle was evaluated. After denaturation by boiling or by adding TCA solution, 30% of the total activity remained in the water extract. Fractions containing high molecular weight compounds showed both 1,1-diphenyl-2-picrylhydrazyl and peroxy radical-scavenging activity. These results indicate that water-soluble protein in fish muscle is responsible for the radical-scavenging activity.

Keywords: radical-scavenging activity, fish, protein

Introduction

Recent studies show that fish and fishery products have a high radical-scavenging activity, comparable to that of some vegetables (Khanum *et al.,* 1999; Mansur *et al.,* 2003; Bhadra *et al.,* 2004). The active components responsible for such high activity are not yet well understood. Previously, we evaluated the free radical-scavengers of fish and determined their contribution to the total activity (Bhadra *et al.,* 2004). The role of compounds such as ascorbic acid, tocopherol, glutathione and uric acid in the radical-scavenging activity of fish muscle was not considered significant. As the activity of fish tissue was found mainly in the water extracts by both 1,1-diphenyl-2-picrylhydrazyl (DPPH)-HPLC method (Yamaguchi *et al.,* 1998) and 2′-deoxyguanosine (2′-dG) oxidation method (Sakakibara *et al.,* 2002), water-soluble proteinous substances may contribute to the scavenging activity of fish.

Antioxidative activity of peptides produced from protein hydrolysis has been reported by numerous studies (Srinivas *et al.,* 1992; Chen *et al.,* 1995; Park *et al.,* 2001). Important physiological functions of protein hydrolysate have also been reported (Suetsuna and Osajima, 1986; Ohta *et al.,* 1997; Katayama *et al.,* 2003). However, few reports have addressed the antioxidative activity of proteins. Okada and Okada (2000) isolated water-soluble protein from broad beans (*Vicia faba*). Wayner *et al.* (1987) reported 10–50% contribution of human blood plasma protein to total radical trapping antioxidant parameter. Carp blood plasma protein also showed strong antioxidative activity (Xue *et al.,* 1998). In this paper, we evaluated proteinous substances of fish muscle extract as free radical-scavengers.

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Materials and Methods

Reagents 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and ODS (Cosmosil 140C_{18}) resin were obtained from Nacalai Tesque Inc. (Kyoto, Japan). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), and 2′-deoxyguanosine (2′-dG) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Sephadex™ G-25 was purchased from Amersham Biosciences (Uppsala, Sweden). Dye reagent concentrate for protein concentration was obtained from Bio-Rad Laboratories (Hercules, CA, USA)

Sample Skinless muscle from horse mackerel (*Trachurus japonicus,* length: 19.8±1.1 cm, weight: 66.7±11.7 g; September 2002, Tottori, Japan) was used for the experiment.

Sample preparation A minced fish sample (10 g) was homogenized for 50–60 s using a homogenizer (Kinematica Polytron Homogenizer PT-MR2000) in 20 mL of ultrapure water. The homogenate was centrifuged at 27,000×g for 20 min at 4℃. The supernatant was filtered through a 0.45-μm filter (Cosmonice Filter W, 13 mm; Nacalai Tesque Inc.). The filtrate was used as water extract for various analyses. No significant amount of oil remained in the water extract.

Water extract was deproteinized by using trichloroacetic acid (TCA) as a protein-denaturing agent. TCA solution (10%) was added to the sample solution at a ratio of 1: 1. The mixture was kept for 2 min at room temperature and was centrifuged at 12,000×g for 10 min at 4℃. The supernatant was filtered and used for analysis.

Another sample of deproteinized water extract was prepared by heating the water extract for 7 min in a boiling water bath. After centrifugation at 1,800×g for 5 min at 4℃, the supernatant was filtered again to get protein-free extract.

Contribution of peptide to radical-scavenging activity of water extract Samples deproteinized by boiling were then lyophilized and dissolved in 5 mL of 5% acetonitrile containing 0.1% trifluroacetic acid (TFA) and then adsorbed to ODS (C_{18}) resin pre-equilibrated with the same solution. The sample was first eluted by 5% acetonitrile containing 0.1% TFA. This fraction contained non-peptide hydrophilic compounds such as ascorbic acid and uric acid. The bound compounds, which are comprised with peptides and other hydrophobic compounds, were eluted by 80% acetonitrile containing 0.1% TFA. The 5% and 80% acetonitrile fractions were further lyophilized and dissolved in ultrapure water. These fractionated samples were then subjected to HPLC analysis for DPPH and peroxy radical-scavenging activity.

DPPH radical-scavenging activity Radical-scavenging activity was measured according to the DPPH-HPLC method of Yamaguchi *et al.* (1998). Briefly, an aliquot of sample solution (200 μL) was mixed with 100 mM of Tris-HCl buffer (pH 7.4, 800 μL) then 1 mL of 500 μM DPPH in ethanol was added. The mixture was vigorously shaken and incubated for 20 min at room temperature in the dark. After 20 min, the mixture was injected for HPLC analysis.

The HPLC analysis was carried out on a TSKgel Octyl-80Ts column (4.6×150 mm; Tosoh, Tokyo, Japan) equipped with a Shimadzu LC-6A pump, a Rheodyne injector fitted with a 20-μL loop and a Shimadzu SPD-10AV UV-VIS detector set at 517 nm at ambient temperature. The mobile phase consisted of methanol/water (70: 30, v/v), and the flow rate was 1 mL/min. As the control standard, 200 μL of 500 μM Trolox solution in ethanol was assayed in a similar manner during each run.

The DPPH radical-scavenging activity was evaluated by the difference in peak area decrease of the DPPH radical between a blank and a sample. The activity was expressed as mmol Trolox equivalent per 100 g of fresh weight of fish sample.

Peroxy radical-scavenging activity using 2′-dG oxidation method Suppression of 8-hydroperoxy-2′-deoxyguanosine (8-OOHdG) formation was measured according to the method of Sakakibara *et al.* (2002). Briefly, 500 μL of 1 mM 2′-dG solution was mixed with sample solution (10 μL) and the reaction was started by adding of 50 mM AAPH solution (500 μL). The reaction mixture was incubated at 37℃ for 1-h. After 1-h incubation, 10 μL of the reaction mixture was subjected to HPLC analysis. The HPLC analysis was carried out on a Capcell pak C18 UG120 (5 μm mesh, 4.6×250 mm) (Shiseido, Tokyo, Japan), equipped with a Shimadzu LC-6A pump, and a Shimadzu SPD-10AV UV-VIS detector set at 254 nm at ambient temperature. The mobile phase consisted of 6.5% methanol and 93.5% 20 mM potassium phosphate buffer (pH 4.5) containing 0.1 mM ethylenediaminetetraacetate (EDTA) disodium salt. The flow rate was 1.0 mL/min.

The radical-scavenging activity was evaluated by the difference in suppression of 8-OOHdG formations between a blank and a sample. The activity was expressed as mmol Trolox equivalent per 100 g of fresh weight of fish sample.

Protein concentration Protein concentration of the water extract was estimated by the method of Bradford (1976).

Autolysis of fish muscle To test the protein hydrolysis due to enzymes present in fish muscle during experiment, a minced fish sample (10 g) was homogenized for 50–60 s using a homogenizer (Kinematica Polytron homogenizer PT-MR2000) in 20 mL of ultra pure water. The homogenized sample was kept for 5 h at room temperature (25℃). DPPH radical-scavenging activity was measured at 1-h intervals.

Gel filtration chromatography Water extract of fish muscle (3 mL) was fractionated by gel filtration chromatography on a Sephadex G-25 column (2.25×30 cm) equilibrated and eluted with 0.1 M KCl. Each fraction of 2 mL was collected at a flow rate of 10 mL/h by a fraction collector. Absorbance at 215 nm and radical-scavenging activity of all fractions were measured by using a spectrophotometer (Shimadzu UV-2100PC; Shimadzu). Peroxy radical-scavenging activity of each fraction was measured by 2′-dG oxidation method as described above. Blue dextran, oxidized glutathione and ascorbic acid were used as the molecular markers.

Results and Discussion

Radical-scavenging activity of fish muscle DPPH and peroxy radical-scavenging activities of water extract, deproteinized extract by boiling or by TCA precipitation, and 5% and 80% acetonitrile fractions of the boiled sample are presented in Table 1. Radical-scavenging activity of water extract was 316.5 μmol Trolox eq./100 g and 1098.0 μmol Trolox eq./100 g in DPPH and 2′-dG oxidation method, respectively. After deproteinization by boiling or by adding 10% TCA solution, 22% and 12% of original activity remained in deproteinized sample, respectively. However, activity was trace and below detection level in 2′-dG oxidation method. These results showed that around 80% of the activity of fish extract are contributed by the proteinous substances, which can be denatured by boiling or by TCA precipitation. Activity remaining in the boiled sample appears to be derived from water soluble peptides and other small molecular weight compounds such as

Table 1. Changes in radical-scavenging activity of water extract during different treatments.

Treatment	DPPH radical-scavenging activity	Peroxy radical-scavenging activity
	(μmol Trolox eq/100 g)	
Water extract	316.5±19.6	1098.0±87.5
After 7-min boiling	70.9±2.9	ND*
After TCA precipitation	37.0±13.7	ND
5% Acetonitrile fraction	29.0±7.6	ND
80% Acetonitrile fraction	11.5±1.5	ND

Data are means±SD of three determinations.
ND, not detected.

Table 2. Changes in radical-scavenging activity of fish muscle during storage.

Storage time (h)	DPPH radical-scavenging activity
	(μmol Trolox eq/100 g)
0	615.6±21.6
1	516.5±31.0
2	567.1±0.2
3	547.9±5.7
4	519.6±23.2
5	510.7±35.3

Data are means±SD of three determinations.

ascorbic acid, glutathione, and uric acid. Boiled sample was fractionated through ODS resin in 5% and 80% acetonitrile to assume the contribution of peptides to radical-scavenging activity of the water extract. In 5% and 80% acetonitrile fractions of the boiled sample, 41.0% and 16.0% of the DPPH radical-scavenging activity remained, respectively. The activity was 9.0% and 3.5% of original water extract activity. In 80% acetonitrile fraction of the boiled sample, contained 7.8% of the peroxy radical-scavenging activity of water extract remained. Activity was below detection limit in the 5% acetonitrile fraction. Contribution of the peptides to DPPH and peroxy radical-scavenging activity was found to be very low.

Radical-scavenging activity of fish muscle after autolysis Changes in DPPH radical-scavenging activity of fish muscle homogenate during storage at room temperature (25℃) up to 5 h are shown in Table 2. The initial activity of fish muscle was 615.6 μmol Trolox eq/100 g, which decreased by 17% after 5 h of storage at room temperature. The action of proteolytic enzymes in fish muscle homogenate was expected to form peptides resulting in an increase in radical-scavenging activity. However, activity did not increase, suggesting that insignificant autolysis of muscle proteins occurred.

Gel filtration chromatography In order to evaluate the molecular size of components having radical-scavenging activity, gel filtration of fish muscle extract was carried out as shown in Fig. 1. DPPH radical-scavenging activity was found to be concentrated to the high molecular weight fractions. This result indicates that main components having radical-scavenging activity in fish muscle are proteinous substances and are not peptides as suggested in Table 1. The total activity of high molecular weight fractions was about 50% of that of muscle extract before gel filtration.

Peroxy radical-scavenging activity of fractions after gel filtration of fish muscle extract was measured as shown in Fig. 2. The activity was also concentrated to the high molecular weight fractions as shown in Fig. 1. Contribution of high molecular weight fractions was about 85% of the activity of fish muscle extract (data not shown). From the results of the above experiment, it can be concluded that water-soluble protein of fish muscle has strong free radical-scavenging activity.

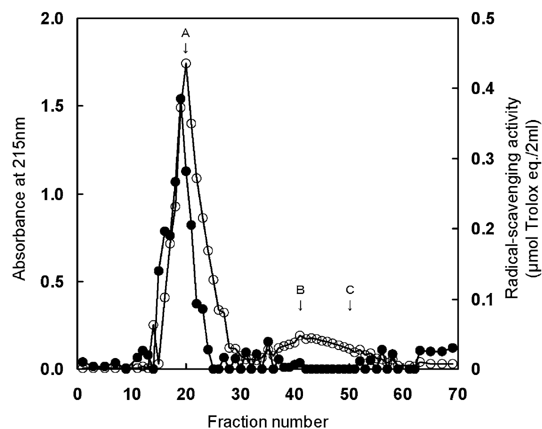

Fig. 1. Fractionation through gel filtration chromatography of high molecular weight compounds and DPPH radical-scavenging activity in fish muscle extract. Average of three replicates are presented. ○, Absorbance at 215 nm; ●, radical-scavenging activity. A, void volume; B, GSSG; C, ascorbic acid.

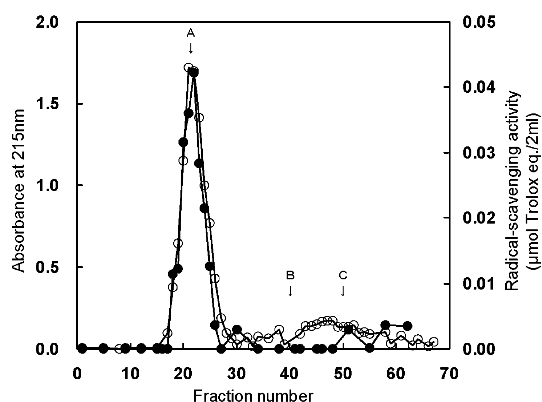

Fig. 2. Fractionation through gel filtration chromatography of high molecular weight compounds and peroxy radical-scavenging activity in fish muscle extract. Average of three replicates are presented. ○, Absorbance at 215 nm; ●, radical-scavenging activity. A, void volume; B, GSSG; C, ascorbic acid.

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