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Antioxidative Constituents from Fermented Sardine with Rice-Bran

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Information on antioxidative constituents from fermented sardine with rice-bran (FSR, "*Iwashinukazuke*") was obtained by fractionating the hot water soluble fraction of fish meat in FSR. Antioxidative activity of each fraction was measured in the model system for linoleic acid oxidation. Chemical composition of the fraction showing the strongest oxidative activity was analyzed and separated by size exclusion chromatograpy. Antioxidative activities of hot water soluble fraction of FSR increased with ripening. The strongest antioxidative fraction from FSR was estimated to be about 3900 Da and was composed of peptides consisting of mainly Asp, Glu, Gly, and Lys. It is suggested that the increase of antioxidative activity during the ripening of FSR is attributable to the production of the antioxidative peptides from the meat.

Keywords: Antioxidants, antioxidative constituents, sardine, *Etrumeus micropus*, fermented sardine, iwashi, nukazuke, fermented sardine with rice-bran

Fermented sardine with rice-bran (FSR, "*Iwashinukazuke*") is a Japanese traditional fermented fish product from Ishikawa Prefecture. It is produced by fermenting sardine with rice-bran in a barrel from 6 months to a year. During the ripening FSR is produced by adding the brine, "*Shiojiru*" to the fermenting barrel at suitable intervals, so that it percolates through the sardine body during salting and it then percolates. We reported earlier that the brine acted as a protease source (Yatsunami & Takenaka, 1996), and the warmed brewing of FSR accelerated hydrolysis (Yatsunami & Takenaka, 1995). Protein hydrolysates of sardine and capelin have been found to show antioxidative activity (Hatate *et al.*, 1990; Amarowicz & Shahidi, 1997). FSR scarcely underwent oxidative rancidity, although sardine lipid is highly susceptible to oxidation. The oxidative stability of the lipids in FSR may be caused by the antioxidative substances in fish meat (Chang *et al.*, 1992), however there are few reports on the antioxidative constituents of FSR.

The oxidative stability of the lipids in commercial FSR was studied here or to identify the antioxidative constituents. The hot water soluble fraction of fish meat in FSR was separated by gel filtration and its chemical composition and amino acid composition analyzed.

Materials and Methods

Materials Fresh sardine (*Etrumeus micropus*) and FSR which had been fermented at ambient temperature more than 6 months were purchased from the fish processing plant in Mikawa-Machi, Ishikawa prefecture. They were used for the experiment of oxidative stability of the lipids and fractionation of antioxidant. The FSR samples used for measurement of antioxidative activity during the ripening were obtained from the above fish processor each month.

Lipid extraction and measurement of lipid oxidation The lipid of raw sardine and FSR was extracted according to the method of Hatano *et al.* (1993). The lipid oxidation was judged from the peroxide value (POV), thiobarbituric acid (TBA) value, carbonyl value (COV), and acid value(AV). POV was measured according to AOAC procedures (Association of Official Analytical Chemists[AOAC], 1990). TBA value was determined by the official method of the Pharmaceutical Society of Japan(1990), and the TBA value was calculated as mg of malonaldehyde per kg lipid. COV and AV were determined in accordance with the official methods (The Japan Oil Chemists' Society).

Fractionation of antioxidant from FSR The fractionation (F1-F7) of antioxidant from FSR was carried out as shown in Fig. 1. The edible portions of FSR were homogenized with 1.5 volumes of distilled water. The homogenate was heated at 98°C for 15 min and filtered with Toyo filter paper (No.2, Toyo Roshi Co.) and then the residue was collected as F1. The filtrate (F2) was defatted with *n*-hexane and the oil layer was collected as F3. The aqueous layer was separated into the retentate (F4) and the dialyzate by dialysis (molecular weight exclusion limit: 1.2×10^4 Da). The dialyzate was applied to AG50W-X4 column (5×20 cm, Bio Rad). After washing with water, it was eluted with 2 N NH_4OH and then concentrated under reduced pressure. The concentrate (F5) was lyophilized and separated into the retentate (F6) and the dialyzate (F7) by ultrafiltration (the exclusion limit: 2×10^3 Da). Each fraction was collected and lyophilized.

Measurement of antioxidative activity Antioxidative activity was measured in the linoleic acid model system (Osawa & Namiki, 1981). Each sample (1 mg) was added to a mixed solution of linoleic acid (0.13 ml), 99.0% ethanol (10 ml), and 50 mM phosphate buffer (pH 7.0, 10 ml); the total volume was made up to 25 ml with distilled water. The mixed solution in a screw-capped dark bottle was kept at 40°C in the dark. During incubation, the degree of oxidation was measured by the thiocyanate method (Mitsuda *et al.*, 1966).

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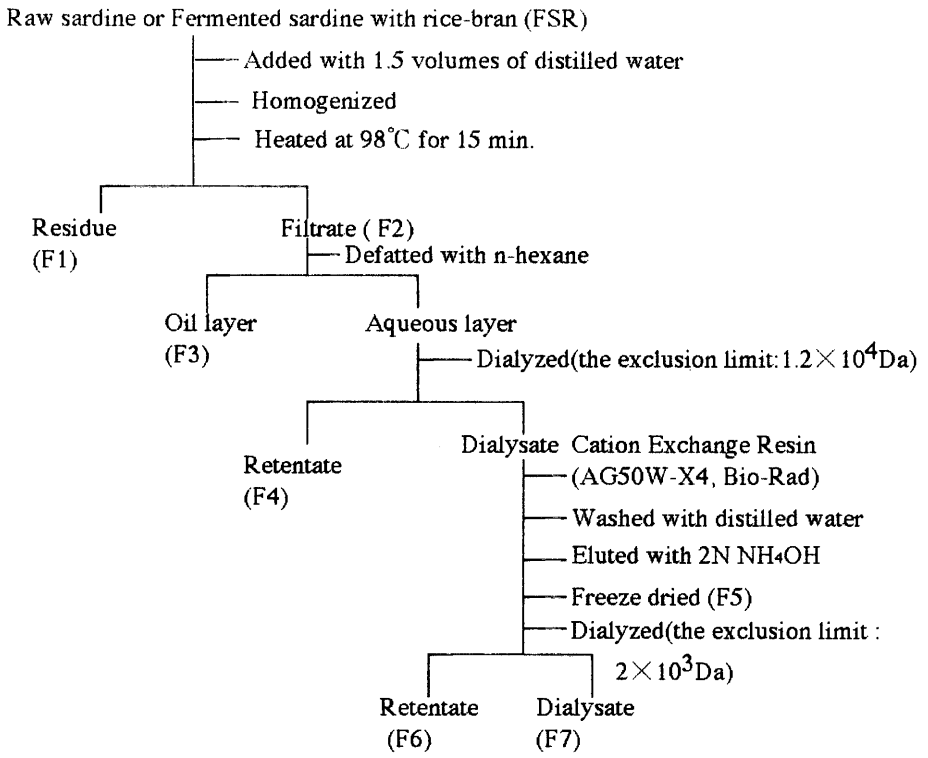

Fig. 1. Fractionation of antioxidant from raw sardine and fermented sardine with rice-bran.

Table 1. POV, TBA, COV, and AV of the lipids in raw sardine and commercial fermented sardine with rice-bran (FSR).

	Raw sardine		FSR	
	Analysis	References	Analysis	References
POV (meq/kg lipid)	9.9±4.1	7.6-42.6[a),b)]	11.2±2.7	11.9-60.2[d),e)]
TBA (mg malonaldehyde/kg lipid)	0.53±0.07	83±6.5[a)]	15.43±2.03	63.0-82.1[d)]
COV (meq/kg lipid)	0.62±0.07	0.5-4.1[c)]	6.50±0.69	2.5-16.8[d),e)]
AV (mg/g lipid)	18.8±1.3	0.6-19[b),c)]	83.4±0.8	53.4-59.6[d),e)]

Analytical values were the mean±SD (n=5).
[a)] Chang *et al.*(1992), [b)] Tsukuda (1978), [c)] Shibata (1980), [d)] Chang *et al.* (1991), [e)] Izumi *et al.*(1989).

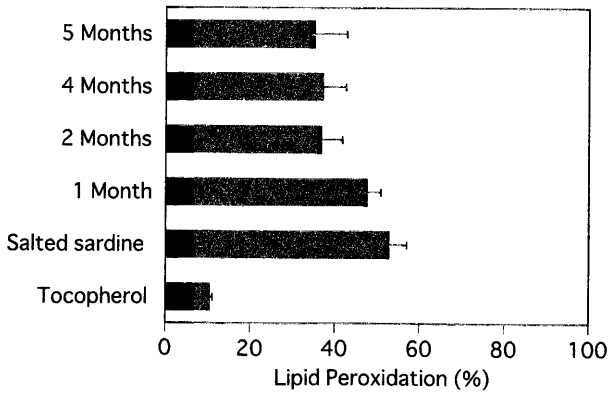

Fig. 2. Antioxidative activities of hot water soluble extract (1000 μg) of the fermented sardine with rice-bran during the ripening in the linoleic acid model system. Oxidation was performed at 40°C for 4 days. Tocopherol (200 μg) was used for the standard sample. The control (ΔO.D.=0.57) was considered to have 100% lipid peroxidation, with other values being reported as a percentage peroxidation in relation to the control. Reported values was the mean±SD (n=3).

The relative antioxidant activity (R.A.A.) of each fraction was calculated as follows, based on the data at 6 days after start of the incubation: R.A.A.=$\{(\Delta Dc-\Delta Ds)/\Delta Dc\}/\{(\Delta Dc-\Delta DE)/\Delta Dc\}$, where ΔDc is the $\Delta O.D.$ for the control (without antioxidants), ΔDs is that for reaction mixture containing the sample, ΔDE is that for reaction mixture containing the most effective fraction.

Chemical analysis Moisture and NaCl content were measured by AOAC procedures (AOAC, 1980). Total nitrogen was determined by micro-Kjeldal method and total sugar was determined by phenol-sulfuric acid method.

Elution profile by size exclusion chromatograpy The molecular weight was determined using TSK-GEL Toyopearl HW-40 column (2.5×72 cm) preliminarily equilibrated with distilled water. Each fraction (4 ml) was collected and the elution was monitored at 280 nm. The antioxidative activities of 1 mg of each peak fraction were measured in the linoleic acid model system.

Amino acid analysis Amino acids were determined by HPLC (Amino acid analytical system LC10A, Shimadzu, Kyoto). The content of each amino acid from a peptide was calculated to subduct the content of free amino acid from the content of amino acid which was measured after hydrolyzing with 6 N HCl at 110°C for 24 h.

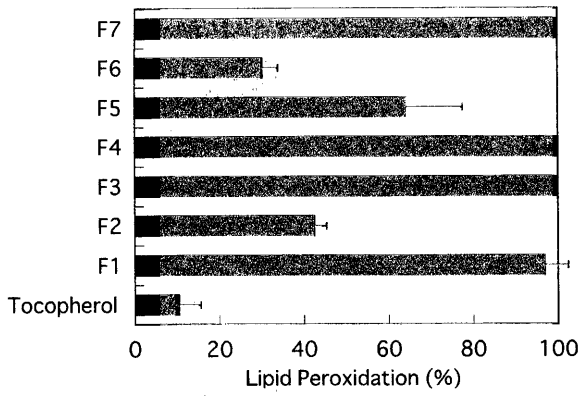

Fig. 3. Antioxidative activities of fractions (1000 μg) separated from fermented sardine with rice-bran in the linoleic acid model system. Oxidation was performed at 40°C for 4 days. Tocopherol (200 μg) was used for the standard sample. The control (ΔO.D.=0.46) was considered to have 100% lipid peroxidation, with other value being reported as a percentage peroxidation in relation to the control. Reported values was the mean±SD (n=3).

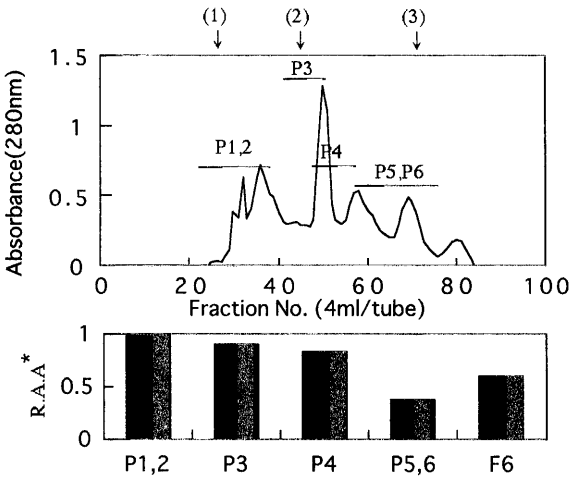

Fig. 4. Elution profile and antioxidant activity of F6 fraction by size exclusion chromatography on TSK-GEL Toyopeal HW40. The column (2.5× 72 cm) was equilibrated and eluted with distilled water. The numbers deboted elution positions of the following compounds: (1) Insulin (M.W. 5790); (2) bacitracin (M.W. 1411); (3) tyrosine (M.W. 181). Each peak was used to determine the antioxidative activity. R.A.A*: Relative antioxidative activity. For the calculation, refer to measurement of antioxidative activity in methods.

Table 2. Yields of each fraction from raw sardine and the fermented sardine with rice-bran (FSR).

Fraction	Yield (g/100 g dry matter)	
	Raw sardine	FSR
F1	79.1	57.0
F2	20.8	42.9
F3	0.6	0.6
F4	5.2	12.0
F5	2.4	10.2
F6	0.01	0.38
F7	1.0	5.7

Table 3. Chemical analysis of F6 fraction from the fermented sardine with rice-bran.

	Contents (%)
Moisture	13.5
Amino acid from peptide	71.89
Free amino acid	3.31
Total sugar (as glucose)	9.3

Table 4. Amino acid analysis of each peak from F6 fraction.

Amino acid	Amino acid from peptides (g/100g)			
	P1,2	P3	P4	P5,6
Asp	13.84	5.17	0.94	0.36
Thr	4.40	0.93	0.30	0.17
Ser	4.43	0.93	0.34	0.13
Glu	17.69	6.37	1.31	0.49
Pro	5.50	0.98	0.47	0.18
Gly	9.90	1.74	0.66	0.30
Ala	4.74	1.10	0.42	0.23
Cys	0	0.13	0.08	0
Val	3.46	0.65	0.31	0.16
Met	0	0.05	0.15	0.04
Ile	1.10	0.41	0.31	0.11
Leu	2.54	0.81	0.53	0.22
Tyr	1.92	0.46	0.21	0.18
Phe	0	0	0.29	0.27
His	4.19	1.46	0.53	0.19
Lys	12.03	0	1.25	0.67
Arg	5.60	1.32	0.48	0.29
Total	91.34	22.51	8.58	3.99

Results and Discussion

Table 1 presents POV, TBA, COV, and AV values of the lipids in raw sardine and the commercial FSR. POV, COV and AV values of raw sardine were almost the same as those of references: Chang *et al.*, 1992; Tsukuda, 1978; Shibata, 1980. TBA value of raw sardine from the references was apparently higher than that of our analysis. Consequently, it was evaluated that the lipid in raw sardine in this study underwent little oxidative rancidity. During the ripening the slight increase of TBA, COV, and AV values of the our FSR analysis was observed, these values except AV were also rather low compared with the references: Chang *et al.*, 1991; Izumi *et al.*, 1989. Therefore, the lipid in FSR was fairly stable, although sardine lipid was susceptible to oxidation. Chang *et al.*(1992) reported that the lipid in FSR was oxidatively stable, but there are few reports about the antioxidative constituents formed during the ripening. The antioxidative activity increased during the ripening of FSR processing. We reported on the fish meat protein decomposition during the ripening (Yatsunami & Takenaka, 1996) suggesting that a protein hydrolysate formed which had antioxidative activity. Figure 2 shows the changes in antioxidative activities of the hot water soluble fraction of FSR during the ripening: it increased almost 40%. As the antioxidative activity did not decrease after decolorization by activated carbon, browning substance was not the cause of the activity. Figure 3 shows the result of antioxidative activities of the fractions of FSR described in Fig. 1 in the linoleic acid system. F6 and F2 fractions showed positive activities with F6

fraction being the stronger. F1, F3, F4, and F7 fractions were not active. Table 2 indicates the yield of each fraction from FSR and raw sardine, that is, the antioxidative activity of each fraction from FSR to elucidate the oxidative constituents from FSR. The yield of F1 in raw sardine was higher than in FSR, and the yields of F2, F4, F5, F6, and F7 in FSR were higher than raw sardine. This showed an increase of the extract from FSR, especially an increase of the low molecular weight components which were absorbed by cation exchange resin during the ripening. The yield of F6 in FSR was almost 40 times higher than that of F6 from raw sardine, so the increase in antioxidative activity might be due to an increase in the constituents of F6 fraction. The increase of antioxidative activity during the ripening seemed to result in the formation of antioxidative peptides. Table 3 presents the results of chemical analysis of F6 fraction which contains the components with relation to the increase of antioxidative activity during the ripening. This also shows that the F6 fraction had a higher content of nitrogen compounds such as peptide and less total sugar and free amino acid content. F6 fraction had maximum absorption at 220 nm and showed positive reaction of ninhydrin. F6 fraction was separated into six peaks (P1-P6), and P1 and P2 were estimated to be about 4700 Da and 3600 Da by the molecular weight standard shown in Fig. 4. The antioxidative activity of each peak from this fraction was observed and measured in P1,2 >P3 >P4> P5,6. Table 4 lists the amino acid analysis of each fraction. The peptide content was also observed in P1,2 >P3 >P4> P5,6. The increase in antioxidative activity of a sample must be caused by the increase in its peptide content. The peptides of P1,2 fraction consisted mainly of Asp, Glu, Gly, and Lys, and in those containing Lys the antioxidative activity was mostly caused by their chelating activity (Suetsuna, 1999); Lys was reported to have radical scavenging activity (Gardner, 1979). The peptides containing Lys may be attributable to the development of antioxidative activity of the P1,2 fraction. However, P3-P5,6 fractions showed the antioxidative activity even though the peptide contents were rather low. P3-P5,6 fractions contained a large amount of free amino acids data not shown. Thus, it was considered that the antioxidative peptide in F6 fraction affected the antioxidative activity observed in the meat of FSR. In conclusion, one reason the lipids in FSR were oxidatively stable may be the increase in the content of antioxidative peptides in the meat of FSR during the ripening.

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