Antioxidative properties of aqueous and aroma extracts of squid *miso* prepared with *Aspergillus oryzae*-inoculated *koji*

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ABSTRACT

To evaluate the antioxidative properties of a newly developed fermented paste (squid *miso*) prepared from squid mantle flesh with *Aspergillus oryzae*-inoculated *koji*, we used *in vitro* models, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, nitric oxide (NO) and carbon-centered radical-scavenging activity (RSA), reducing power ability (RPA), and linoleic acid oxidation. The antioxidant activity of volatile compounds, which were extracted from squid *miso* by dichloromethane, was confirmed based on the results obtained by the oxidation models consisting of carotene linoleate and hexanal. The RSA of the *miso* against several radicals, including DPPH, hydroxyl, nitric oxide and carbon-centered radicals measured by ESR spectrometry increased gradually through the fermentation period. On the other hand, the RPA increased rapidly in early stages of fermentation. Proteins were hydrolyzed to peptides and amino acids during fermentation. Certain peptides of low molecular mass significantly contributed to RSA. 4-Ethylguaiacol in the RSA active fraction, which was obtained by column chromatography, was the most potent antioxidative in the volatile extract. Analysis of phenolics in ethyl acetate fraction clearly suggested that 4-ethylguaiacol in the squid *miso* is possibly a bioconversion product of ferulic acid derived from *koji*. However, the aqueous extracts containing antioxidative peptides contributed more remarkably to radical scavenging than heterocyclic volatiles and phenolics.

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1. Introduction

In traditional Japanese food industries, *Aspergillus oryzae*, *A. sojae*, and *A. awamori* are frequently used to prepare *koji* (fermented rice malt), using rice, wheat, and soybean to produce various seasonings, alcohols, and foods such as sake (rice alcohol), soy sauces, *miso*, and mirin (a kind of seasoning). (Bennett & Klich, 1992). *Miso* has been used as a traditional daily seasoning for several centuries. It has beneficial physiological properties, including suppression of hypertension (Kanda, Hoshiyama, & Kawaguchi, 1999), colonic aberrant crypt foci (Ohara et al., 2002), cerebrovascular disease (Kanazawa et al., 1995), and elevated plasma cholesterol (Horii, Ide, Kawashima, & Yamamoto, 1990). Heterocyclic compounds like 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone and its derivatives found in fermented soy *miso* were reported to contribute several bioactive properties (Sugawara, Hashimoto, Sakurai, & Kobayashi, 1994). Amadori rearrangement products, melanoidins, modified protein

and its hydrolysate, brown pigments isolated from soy *miso* also exhibited strong scavenging activity against hydroxyl radical and superoxide anion (Chuyen, Ijichi, Umetsu, & Moteki, 1998). Certain phytochemicals found in fermented soy *miso* products appear to be the active compounds resulting many beneficial health effects (Dixon, 2004). Soy isoflavones in particular, a group of natural heterocyclic phenols comprising mainly of aglycones and glucosides are of great importance due to their pharmacological and antioxidative properties (Wiseman, 2006). By evaluating several *koji* fermented products, Lee, Hung, and Chou (2008) reported that production of certain phenolic compounds and anthocyanins contents might lead to increase antioxidant activities of black bean *koji*. Zhu, Cheng, Wang, Fan, and Li (2008) investigated the antioxidative activity of Meitauza *koji* (Meitauza, a Chinese traditionally fermented okara) okara fermented by *A. oryzae*, *Rhizopus oligosporus*, *Actinomucor elegans* and *Bacillus subtilis* B2 as *koji* and suggested the potentiality for developing traditional functional food enriched with bioactive peptides. There have been attempts to ferment meat-based products with *Aspergillus* in a search for novel properties. Yin, Tong, and Jiang (2005) reported that *A. oryzae* produces multiple enzymes and is able to hydrolyze minced mackerel. We produced fish *miso* from trash fishes, including squid, using *Aspergillus*-inoculated *koji*. There are previous reports on the nutritional value, taste, and aroma of fish *miso* (Giri, Osako, & Ohshima, 2009a,b, 2010), but none on its antioxidative properties. The analyses of several physico-chemical parameters of the finished

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products, including free amino acid, oligopeptide, organic acid, and mineral content, also revealed the potential utility of both rinsed and unrinsed squid meat for the production of *miso*-like fermented fish pastes (Giri et al., 2009a,b, 2010).

We investigated antioxidant activity and free radical-scavenging capacity in squid *miso* (prepared from squid mantles) through the fermentation process. Aqueous, dichloromethane, and ethyl acetate extracts of squid *miso* were analyzed for antioxidative compounds. We also determined the effects on antioxidant properties of preparatory rinsing of raw squid mantles before fermentation. Changes in the contents and molecular distributions of peptides, profiles of volatile compounds, and contents of phenolic compounds are presented.

2. Materials and methods

2.1. Materials

Common squid (*Todarodes pacificus*) with a mean body weight of 311 ± 18 g and mean mantle length of 24.7 ± 0.5 cm were net caught offshore near Nagasaki. Animals were stored at -50 °C for a month prior to use. A pure strain of *koji* mold [*A. oryzae* (M1 mold)] was purchased from Nihon Jozo Kogyo (Tokyo, Japan).

2.2. Chemicals

All authentic standards of volatile compounds used for identification and other analysis purposes were GC-analytical grade and purchased from Tokyo Chemical Industry, Tokyo, Japan.

2.3. Preparation of fermented squid meat paste (squid miso)

Polished rice was soaked in two volumes of fresh water for 12 h at room temperature and then steamed at 90 °C for 1 h. After cooling at room temperature to 35 °C, the rice was inoculated with *koji* mold, incubated at 35 °C for 48 h; malt–rice thus obtained was used as *koji*, a starter for fermentation. After grinding fresh squid mantle with a model M-22 grinder (Nantsune Tekko, Osaka, Japan), the ground meat was transferred to an aluminum-coated heat-stable polyvinylchloride pouch, vacuum-sealed, and then steamed at 90 °C for 1 h. Portions were then pressed at 2 MPa with a model KS-1 filter press (Komagata Kikai Seisakusho, Tokyo, Japan) to achieve a moisture contents between 50 and 55%. Moisture content of squid meat was measured by taking a portion into an oven maintained at 110 °C for around 6 h until the stabilization of weight.

The dehydrated meat thus obtained was termed "unrinsed." Other portions were rinsed thrice in five volumes of fresh water before pressing to obtain "rinsed" meat. Squid meat, *koji*, and sodium chloride in a ratio of 5:5:1 by wet weight were mixed in a grinder. About 3 kg of paste were packed and fermented at 25–30 °C for 365 days. The contents of each container were mixed thoroughly once a month. The products were sampled for analysis on days 0, 15, 30, 60, 90, 135, 180, 270, and 365.

2.4. Water extraction of squid miso

Two grams of sampled *miso* were mixed thoroughly in 20 mL of 10 mM phosphate buffer saline (pH 7.4) and centrifuged at 1300 rpm for 10 min. Supernatant (44.1 ± 3.8 mg dry matter/mL) lyophilized extracts were used for further analysis. Preliminary observations clearly indicated that the dehydration occurred during the long
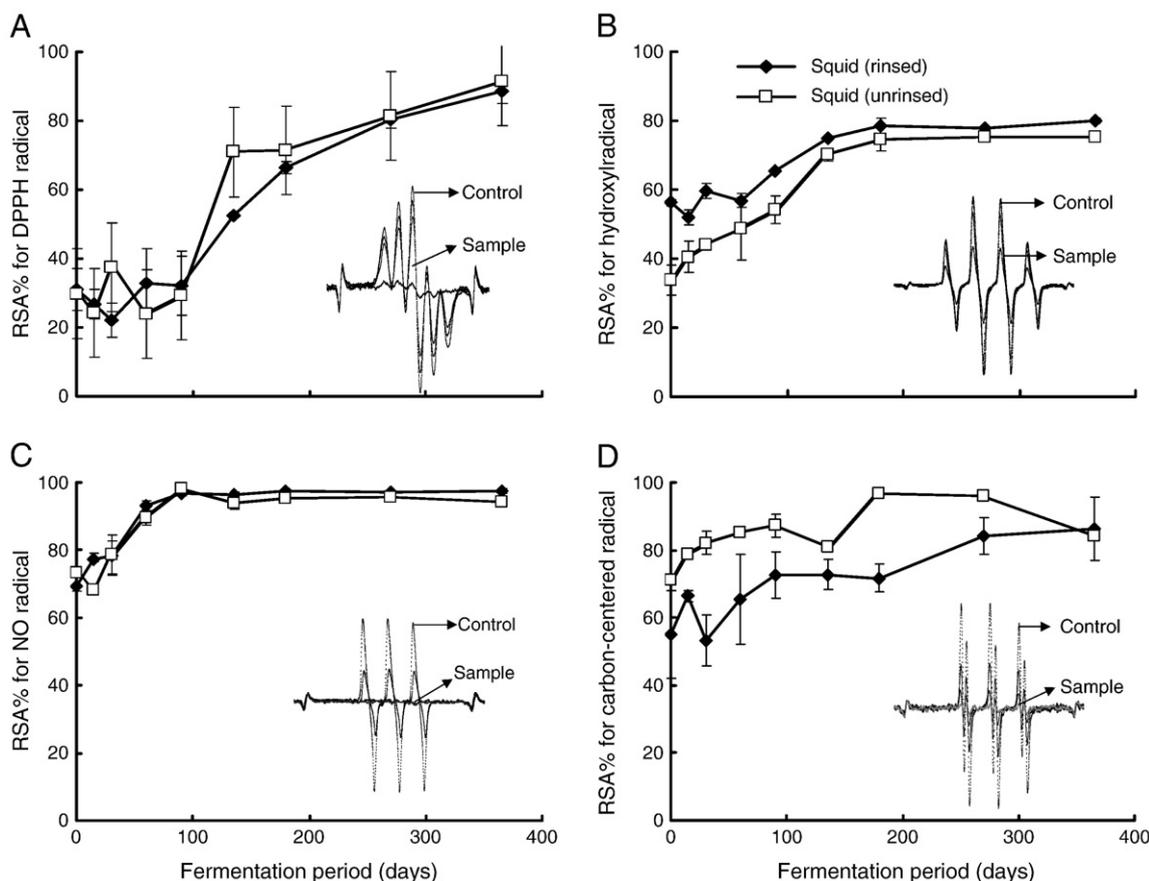

Fig. 1. Changes of DPPH (A), hydroxyl (B), NO (C) and carbon-centered (D) radical-scavenging activity of the *miso* prepared from rinsed and unrinsed squid meat at different fermentation period (ESR signal patterns for control and added with sample are presented inset for all the types of radicals).

fermentation period and moisture contents reduced maximum of 10–15%. Keeping that in mind we have expressed the analytical findings based on dry weight basis

2.5. Radical-scavenging assay

2.5.1. DPPH radical-scavenging assay

DPPH radical-scavenging activity was measured following Nanjo et al. (1996), with some modifications. In brief, a 50 µL portion of squid *miso* extract was added to 50 µL of DPPH solution (100 µM in 85% ethanol) and thoroughly vortexed. After 15 min, the mixture was transferred into a 50 µL Pyrex NMR capillary tube, and DPPH radical spin resonance was measured. Conditions for JES-TE300 ESR (JEOL Ltd., Tokyo, Japan) spectrometry were controlled as follows: microwave power, 1.2 mW; microwave frequency, 9149.3 MHz; magnetic field, 325.5 ± 25 mT; and sweep time, 30 s.

2.5.2. Hydroxyl radical-scavenging assay

Hydroxyl radicals were generated by the Fenton reaction (Rosen & Rauckman, 1984). A 50 µL portion of squid *miso* extract, or 50 µL of 10 mM phosphate buffer (pH 7.4) as a control, was added to 50 µL of 0.3 M DMPO and 50 µL of 10 mM $FeSO_4$. The reaction was initiated by adding 50 µL of 10 mM H_2O_2. The reaction mixture was transferred to a 50 µL Pyrex NMR capillary tube; DMPO-OH adduct was recorded after 2.5 min using a JES-TE300 ESR spectrometer with the conditions described in 2.5.1.

2.5.3. Nitric oxide radical-scavenging assay

Fifty-micromolar nitric oxide radical was prepared by dissolving potassium nitrosodisulfonate {Fremy's salt, $K_2NO(SO_3)_2$} in a saturated solution of Na_2CO_3; 50 µL of this solution were mixed with 50 µL of the *miso* extract and left to stand for 5 min. The reaction mixture was transferred to a Pyrex NMR capillary tube; spin adduct was recorded as described in 2.5.2.

2.5.4. Carbon-centered radicals scavenging assay

Carbon-centered radicals were generated with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, Hiramoto, Johkoh, Sako, & Kikugawa, 1993). Fifty microliters of *miso* extract were mixed with 50 µL of 40 mM 4-POBN and 50 µL of 40 mM AAPH, followed by incubation at 37 °C for 30 min. The reaction mixture was transferred to a 50 µL Pyrex NMR capillary tube; spin adduct was recorded as in 2.5.2.

ESR signal intensities obtained for different radicals were quantified as the sum of signal intensity (sum of area under the signal curve) calculated with Mathematica 6.0 software (Wolfram Research, Inc. Champaign, USA).

2.6. Reducing power assay

Reducing power was determined according to Oyaizu (1986). A 2.5 mL portion of squid *miso* extract was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid (w/v) were added, and the mixture was centrifuged at 650 rpm for 10 min. A 5 mL portion of the supernatant was mixed with 5 mL of deionized water and 1 mL of 0.1% of ferric chloride. Absorbance was measured at 700 nm. Ascorbic acid sodium salt, tert-butylhydroquinone (TBHQ), and butylated hydroxyanisole (BHA) were used as reference standards.

2.7. Assessment of antioxidative activity by inhibition of lipid peroxidation

Considering higher scavenging activity obtained from one year old fermented squid *miso* product, we decided to elucidate the antioxidative capacity of the aromatic compounds in the extract prepared

from squid *miso*. Antioxidative activity of squid *miso* against linoleic acid was evaluated following Osawa and Namiki (1985), with a slight modification. Briefly, 1 mL of the extract of mature *miso* was added to a mixture of 5 mL of 99.5% ethanol, 65 µL of purified linoleic acid, and 4 mL of distilled water, followed by vigorous shaking. Linoleic acid was used after further purification by using an open silica gel column (2.4×20 cm) equilibrated by *n*-hexane and eluted by *n*-hexane and diethylether consecutively to eliminate oxidation products generated during storage and transportation of the chemical. Further TLC on silica gel was carried out to determine its purity. One milliliter of 500 ppm authentic BHA was a positive control, and 1 mL of 10 mM PBS (pH 7.4) was a negative control (replacing squid *miso* extract). The mixed solutions were sealed tightly in 22-mL glass vials with polytetrafluoroethylene/silicone coated aluminum caps (Supelco, Bellefonte, PA, USA) and incubated at 40 °C in the dark. Degree of linoleic acid oxidation was measured at 48 h intervals by total hydroperoxide content and absorbed oxygen as oxidation indices.

Total hydroperoxides were analyzed in a flow-injection analysis (FIA) system equipped with a fluorescence detection mechanism using DPPP as a fluorescent reagent as described by Sohn, Taki, Ushio, and Ohshima (2005). Oxygen uptake was measured by gas chromatography following Ohshima, Li, and Koizumi (1993).

2.8. Online HPLC–DPPH method for monitoring development of radical-scavenging peptides and their molecular weight distribution

An online HPLC–DPPH method was developed using an 85% ethanolic solution of free DPPH radical for screening radical-scavenging compounds in the *miso* extract. HPLC separated analytes reacted with DPPH at a concentration of 100 µM/L in the post-column reaction. DPPH solution was pumped with a LC-10AD HPLC pump (Shimadzu, Japan) at a flow rate of 0.2 mL/min; induced bleaching intensity was detected photometrically at 517 nm as a negative peak. The length of reaction tubing used for the post-column reaction was adjusted to achieve a reaction time of 90 s. Antioxidative components were separated in a HPLC system (Shimadzu, Kyoto, Japan) comprising a LC-10AD HPLC pump, a DGU-4A degasser, a CTO-10 AC column oven coupled with a SPD-M10A photodiode array detector for pre-reaction detection and a SPD-M10AVP photodiode array detector for post-reaction detection. The samples were loaded into a TSK gel G2000 SWXL column (7.8 i.d.×300 mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 mL/min and monitored at 220 nm. A calibration curve for molecular mass was obtained using the following protein standards: cytochrome C (12,500 Da) and aprotinin (6500 Da) from
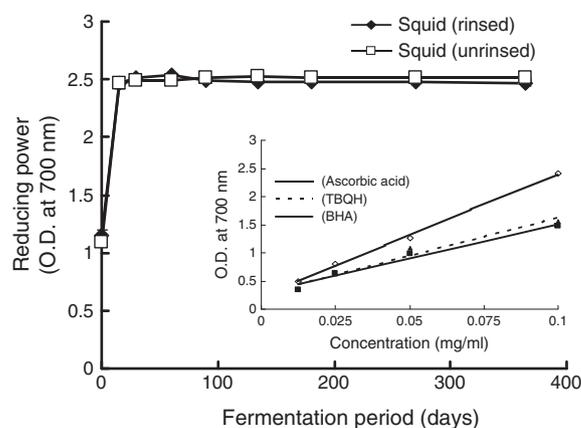
Fig. 2. Changes of reducing power ability of the fish *miso* prepared from rinsed and unrinsed squid meat at different fermentation period (calibration curve of standard compounds is inset).

Sigma and bacitracin (1450 Da), tetrapeptide GGYR (451 Da) and tripeptide GGG (189 Da) from the Peptide Institute (Osaka, Japan).

2.9. Quantitative analysis of free amino acids and amino acids of peptides (including oligopeptides)

The concentrations of free amino acids in squid *miso* extracts were determined with a HPLC series (Shimadzu, Kyoto, Japan) equipped with a LC-10AT VP, a Shim-pack-Amino-Li column (6.0 mm i. d.×100 mm), and a RF-10A XL fluorescence detector. The CTO-10A VP column oven temperature was set at 60 °C. Amino acid mixture standard solution types AN II and B (Wako Pure Chemical industries, Ltd. Japan) were used for identification and quantification. To obtain the oligopeptide fraction the *miso* solution was filtered through a 0.45-μm filter, and then filtered again through Amicon ultra-filters (Millipore Corporation, Bedford, MA) with a molecular weight cut-off of 500 Da. The filtrate thus obtained was further hydrolyzed by 6 M HCl at 110 °C for 22 h and subsequently neutralized with a NaOH solution. After filtering, the samples were analyzed by HPLC, as described above.

2.10. Extraction of volatile active compounds by dichloromethene

To extract volatile compounds from different *miso* products, 10 g portions were dissolved in 100 mL of deionized water and mixed thoroughly. The solutions were then filtered, and the filtrates were extracted three times with 100 mL of dichloromethane using a liquid–liquid continuous extractor for 6 h. The dichloromethane layer extract was dried over anhydrous sodium sulfate overnight at 4 °C. The dichloromethane extract thus obtained was concentrated to 1 mL with an evaporator and, subsequently, to 0.5 mL in a nitrogen stream of high purity.

2.11. Determination of volatile extract antioxidant activity using a β-carotene linoleate model system

Antioxidant activity was measured following Jayaprakasha, Singh, and Sakariah (2001), with a slight modification. Three milligrams of β-carotene in 1 mL of chloroform, 40 mg of linoleic acid, and 400 mg of Tween-20 were thoroughly mixed. After mixing, chloroform was removed at room temperature under vacuum in a rotary evaporator. The resulting mixture was immediately diluted with 5–10 mL of distilled water and mixed well. The emulsion was made up to 200 mL with 0.01 M hydrogen peroxide; a 5 mL portion was transferred to a test tube containing 50 μL of volatile extract. BHA was used for comparative purposes in this experiment. A 5 mL portion of the emulsion without additives was used as a control. The tubes containing reaction mixtures were placed in a water bath at 50 °C. Absorbances at 470 nm were measured at the start and every 20 min thereafter until the color of β-carotene disappeared from the control. A mixture without β-carotene was used as a blank.

2.12. Fractionation of samples by column chromatography

Volatile compounds in the squid *miso* extracts of one year old (prepared from unrinsed meat) with highest antioxidative activity were further fractionated by column chromatography. Extracts were transferred to an open column (15 cm×1 cm i.d.) packed with silica gel (spherical, 40–50 μm; Cica-Reagent, Tokyo, Japan) using *n*-pentane. Volatile compounds in the extracts were eluted sequentially with 100 mL of solvent mixture containing different ratios of *n*-pentane and ethyl acetate (100:0, 80:20, 50:50, 20:80, and 0:100 v/v), and finally with 200 mL of acetone. The six fractions thus obtained were each concentrated to 1 mL in a rotary evaporator, and subsequently to 0.5 mL in a purified nitrogen stream.

2.13. Antioxidative test for volatile extract fractions with a hexanal oxidation model system

Antioxidative properties of volatile extracts were examined by quantification of their inhibitory effect on oxidative conversion of hexanal to hexanoic acid following Fuster, Mitchell, Ochi, and Shibamoto (2000). Each 50 μL extract was added to a vial containing 2 mL of a dichloromethane solution of hexanal (3 mg/mL) containing 0.2 mg/mL of *n*-dodecane as an internal standard; vials were then sealed with a Teflon® cap. Mixtures without extracts were prepared as controls. Fifty-microliter portions of solutions containing 100 μg/mL of BHT were included as samples of a known antioxidant. Oxidation of sample solutions was initiated by heating at 60 °C in sealed vials for 10 min. The headspaces of vials were purged with pure air for 2 s every 2 days during the first 10 days. Decrease in hexanal was monitored by gas chromatography at 5-day intervals, using a Shimadzu model 14A gas chromatograph (Kyoto, Japan) equipped with a Supelcowax™-10 fused silica capillary column (0.32 mm i. d.×60 m, 0.25 μm film thickness) and a flame ionization detector (FID) to monitor relative amounts of hexanal in the samples. The temperature of the injector and detector was 250 °C. The oven temperature was held at 60 °C for 5 min and then programmed to 120 °C at 6 °C/min.

2.14. Identification and quantification of volatile compounds in dichloromethane crude extract and active fractions

A Hewlett Packard 5890 Series II gas chromatograph (Supelco-wax™-10 fused silica capillary polar column: 0.25 mm i.d.×60 m, 0.25 μm film thickness, Supelco) equipped with a Tekmar 7000 headspace auto-sampler (Cincinnati, OH, USA) and an Automass mass spectrometer (JEOL, Japan) were used for identification and quantification of volatile compounds in dichloromethane solutions of crude extract and active fractions. Ionization energy, scan range, scan rate, and scanning speed for the analysis were 70 eV, 40–350 m/z, and 500 amu/s, respectively. Column temperature was initially held at 40 °C for 3 min, and subsequently programmed to 200 °C at a rate of 3 °C/min. The quantification of volatile compounds were carried out using the addition of 2,4,6-trimethylpyridine as an internal standard to normalize the volatile isolation process and relating calibration curves were obtained by using authentic volatile compounds detected in the aroma fractions of the *miso*. The volatiles were identified by comparing their mass spectra and linear retention indices (LRI) with those obtained from authentic compounds.

2.15. Identification and quantification of phenolic compounds in squid miso

The content of phenolics in squid *miso* extract was quantitatively determined following Onjia, Vasiljevi, Oke, and Milalau (2002), with a slight modification. Briefly, a 300 mg portion of lyophilized extract were dissolved in 20 mL of distilled water, and phenolic compounds were extracted three times for 6 h in 20 mL of ethyl acetate. The ethyl acetate fraction was separated, dried, and then dissolved in 1 mL of methanolic solution (1:1 v/v). Analysis of phenolic compounds was performed with a model LCMS-2010EV high-performance liquid chromatograph mass spectrometer (Shimadzu) equipped with a *L*-column ODS (4.6 mm i.d.×250 mm, Chemical Inspection and Testing Institute, Tokyo, Japan). The mobile phase comprising methanol, water, and acetic acid (40:59:1, v/v) was applied at a flow rate of 0.175 mL/min. Identifications were made by comparing retention times, UV spectra, and m/z ([M+H]$^+$) with those of authentic standards. For quantification, calibration curves were obtained for different concentrations of authentic phenolic standards.
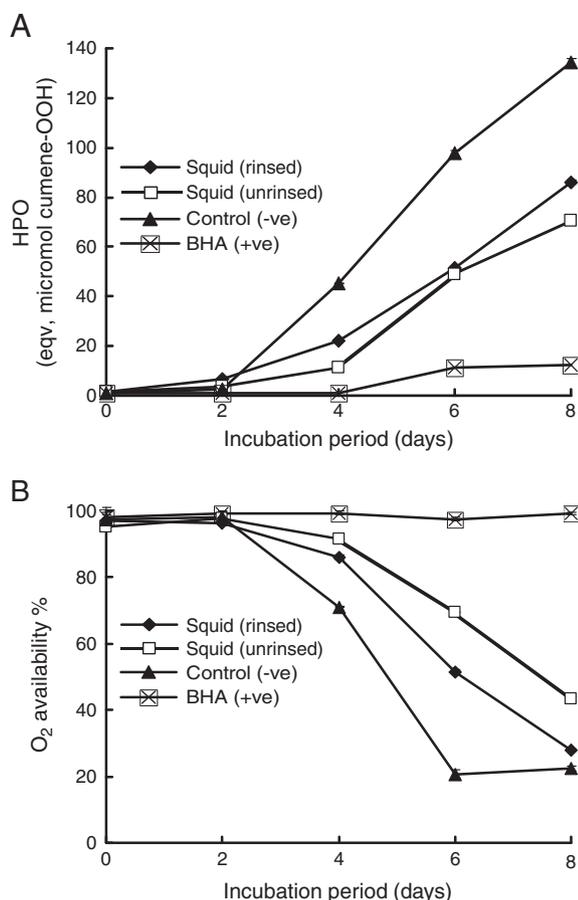
Fig. 3. Changes of HPO (A) and O_2 consumption (B) in the linoleic acid model oxidation system added fish *miso* extracts and known standards during incubation period. −ve; negative control; +ve: positive control.

2.16. Statistical analysis

Student's *t*-tests were used to determine statistical significance of differences between mean values (n=3). Correlation coefficients were calculated using Microsoft Excel software. The level of significance was set at $P<0.05$.

3. Results and discussion

3.1. Radical-scavenging activity and antioxidative properties of aqueous squid miso extracts

Scavenging activity of aqueous squid *miso* extracts against DPPH, hydroxyl, NO, and carbon-centered radicals increased with duration of fermentation (Fig. 1). *Miso* prepared from rinsed and unrinsed squid meat behaved similarly for all radicals tested. These time course observations indicated that the substrate responsible for radical scavenging developed during the process of fermentation. Responsible substrates donate hydrogen to free radicals, forming non-toxic species and thereby inhibiting the propagation phase of lipid oxidation. There was variation in the stoichiometry of reactions between antioxidant compounds in squid *miso* extracts and different radical systems (DPPH, hydroxyl, nitric oxide, and carbon-centered radicals); this variation is often used to explain differences in scavenging potential of compounds tested.

The reducing powers of *miso* extracts sampled at different fermentation and storage stages are depicted in Fig. 2. In this assay, reducing power ability increased rapidly in the very early stages (15 days) of fermentation, and remained stable thereafter through 365 days. Hence, the substrate responsible for reducing power developed during the initial fermentation period.

We compared antioxidant properties of crude extract from matured squid *miso* with those of the known antioxidant BHA by hydroperoxide formation and O_2 consumption in a linoleic acid o/w emulsion system (Fig. 3A and B, respectively). Oxidation of linoleic acid was markedly inhibited by addition of crude squid *miso* extracts. The extract from unrinsed squid *miso* had a higher potential for preventing lipid oxidation.
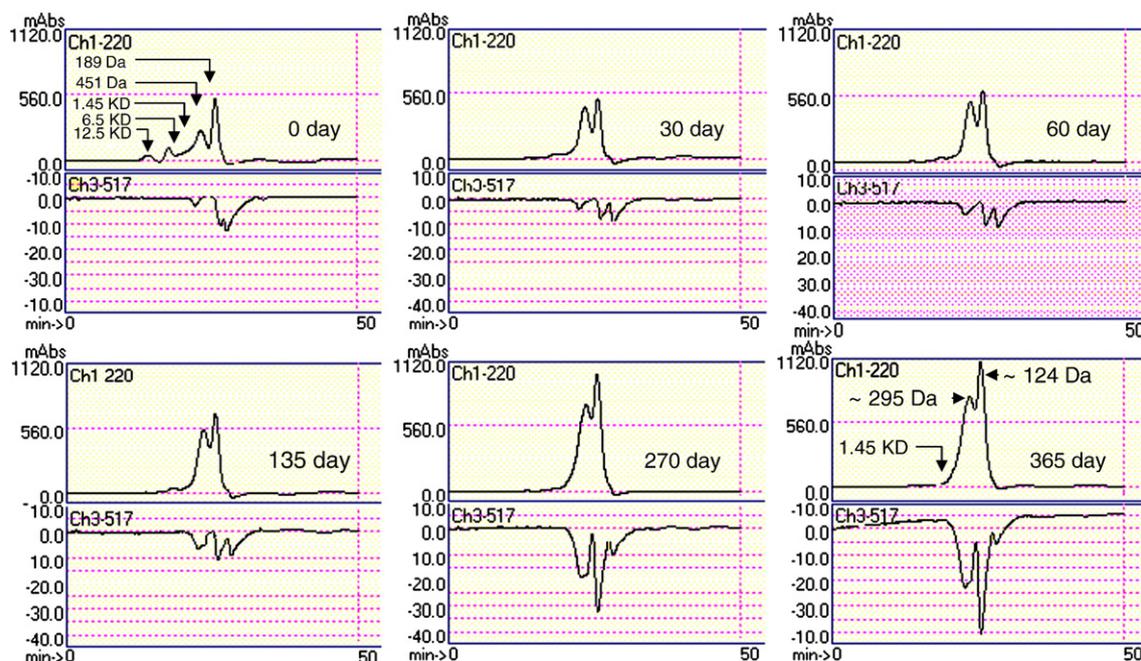

Fig. 4. Changes in molecular mass distribution of peptides and radical-scavenging capacity of the *miso* extracts prepared from unrinsed squid meat at different fermentation period measured with an online HPLC–DPPH system.

Table 1
Free amino acids and related compounds (mg/g dry extract) and mol % (in parenthesis) in squid *miso* during fermentation period.

Amino acids	0 day		30 days		60 days		135 days		270 days		365 days	
Taurine	38.05[a]	(25.74)	27.56[b]	(10.42)	24.08[b]	(8.66)	22.34[b]	(7.64)	20.99[c]	(7.35)	17.49[c]	(6.23)
Aspartic acid	4.07[d,e]	(2.59)	21.70[b]	(7.71)	30.67[a,b]	(10.37)	39.80[a]	(12.80)	42.63[a]	(14.03)	45.07[a]	(15.09)
Threonine	2.25[d,e]	(1.59)	7.18[c,d]	(2.85)	7.73[d]	(2.92)	8.16[d]	(2.93)	7.93[d]	(2.91)	7.31[d]	(2.73)
Serine	3.51[d,e]	(2.83)	12.25[c]	(5.51)	13.26[c]	(5.68)	14.15[c,d]	(5.76)	12.58[c,d]	(5.24)	10.41[c,d]	(4.41)
Glutamic acid	3.52[d,e]	(2.02)	24.50[b]	(7.87)	29.58[a,b]	(9.05)	36.88[a]	(10.73)	34.91[b]	(10.39)	27.53[b]	(8.34)
Proline	14.50[c]	(10.66)	14.07[c]	(5.78)	15.57[c]	(6.09)	17.73[c]	(6.59)	18.18[b,c]	(6.92)	14.68[c]	(5.68)
Glycine	3.17[d,e]	(3.58)	6.24[d]	(3.93)	7.22[d]	(4.33)	8.11[d]	(4.62)	8.02[d]	(4.68)	7.16[d]	(4.25)
Alanine	7.84[d]	(7.45)	22.31[b,c]	(11.84)	25.21[b]	(12.74)	27.56[b]	(13.24)	27.30[b]	(13.42)	25.15[b]	(12.58)
Valine	3.49[d,e]	(2.51)	10.34[c]	(4.17)	11.17[c,d]	(4.29)	12.06[c,d]	(4.40)	13.10[c,d]	(4.90)	12.35[c,d]	(4.70)
Cysteine	4.27[d,e]	(2.98)	2.86[d,e]	(1.11)	2.72[e]	(1.01)	2.68[e]	(0.94)	3.58[e]	(1.29)	15.72[c]	(5.78)
Methionine	2.52[d,e]	(1.42)	6.53[d]	(2.07)	5.83[e]	(1.75)	5.24[e]	(1.50)	5.94[d,e]	(1.74)	9.52[c,d]	(2.84)
Isoleucine	2.28[d,e]	(1.46)	6.65[d]	(2.39)	7.02[d,e]	(2.41)	7.25[d,e]	(2.36)	7.95[d,e]	(2.65)	9.47[c,d]	(3.21)
Leucine	28.00[b]	(18.01)	40.89[a]	(14.69)	38.05[a,b]	(13.01)	34.94[a]	(11.36)	25.42[b,c]	(8.45)	24.55[b]	(8.31)
Tyrosine	3.65[d,e]	(1.70)	6.73[d]	(1.75)	7.00[d]	(1.74)	7.17[d]	(1.69)	7.64[d]	(1.84)	8.00[d]	(1.96)
Phenylalanine	3.01[d,e]	(1.54)	6.38[d]	(1.82)	6.23[d]	(1.69)	6.44[d]	(1.67)	7.58[d]	(2.01)	6.65[d,e]	(1.79)
ß-Alanine	0.13[e]	(0.12)	0.13[e]	(0.07)	0.12[f]	(0.06)	0.11[f]	(0.05)	0.09[f]	(0.04)	0.09[f]	(0.04)
4-Aminobutyric acid	2.27[d,e]	(1.86)	1.74[d,e]	(0.79)	1.54[e,f]	(0.67)	1.43[e,f]	(0.59)	1.47[e,f]	(0.62)	1.21[e]	(0.52)
Histidine	1.63[d,e]	(0.88)	2.27[d,e]	(0.69)	2.11[e,f]	(0.61)	1.85[e,f]	(0.51)	1.48[e,f]	(0.41)	1.39[e]	(0.40)
Ornithine	0.33[e]	(0.21)	0.35[e]	(0.12)	0.29[f]	(0.09)	0.84[f]	(0.27)	1.98[e,f]	(0.65)	4.05[d,e]	(1.36)
Lysine	8.06[c]	(4.15)	24.39[b]	(7.03)	24.35[b]	(6.68)	21.95[b,c]	(5.72)	16.06[c]	(4.28)	12.83[c,d]	(3.48)
Arginine	13.67[c]	(6.64)	26.85[b]	(7.29)	23.53[b]	(6.08)	18.51[c]	(4.55)	24.35[b,c]	(6.12)	24.32[b]	(6.22)

Different letters (a–f) represent significant differences at p<0.05.

Table 2
Amino acids and related compounds in oligopeptides (mg/g dry extract) and mol% (in parenthesis) in squid *miso* during fermentation period.

Amino acids	0 day		30 days		60 days		135 days		270 days		365 days	
Taurine	7.24[c]	(3.78)	10.03[d]	(3.32)	13.33[d]	(3.97)	13.52[d]	(4.01)	9.60[d,e]	(2.60)	12.66[e]	(3.17)
Aspartic acid/asparagine	13.69[b]	(6.73)	17.08[c,d]	(5.33)	14.91[d]	(4.18)	9.71[d,e]	(2.70)	10.49[d,e]	(2.67)	10.56[e]	(2.49)
Threonine	6.26[c]	(3.43)	10.46[d]	(3.64)	12.50[d]	(3.91)	13.39[d]	(4.17)	13.45[d]	(3.83)	14.05[e]	(3.70)
Serine	8.16[b,c]	(5.08)	9.23[d]	(3.65)	10.68[d,e]	(3.79)	11.23[d,e]	(3.96)	11.12[d,e]	(3.59)	12.89[e]	(3.85)
Glutamic acid/glutamine	38.53[a]	(17.13)	64.25[a]	(18.14)	72.05[a]	(18.27)	72.70[a]	(18.34)	77.17[a]	(17.81)	88.60[a]	(18.90)
Proline	8.63[b,c]	(4.90)	15.10[c]	(5.45)	12.33[d]	(3.99)	17.17[d]	(5.53)	27.82[c]	(8.20)	29.19[c]	(7.96)
Glycine	16.45[b]	(14.34)	21.65[b,c]	(11.98)	22.69[c]	(11.28)	23.19[c,d]	(11.46)	20.88[c,d]	(9.44)	22.18[d]	(9.27)
Alanine	10.63[b,c]	(7.80)	13.86[d]	(6.46)	15.10[d]	(6.32)	15.14[d]	(6.31)	14.76[d]	(5.62)	18.44[d,e]	(6.49)
Valine	6.72[c]	(3.75)	12.72[d]	(4.51)	15.30[d]	(4.87)	16.86[d]	(5.34)	18.37[d]	(5.32)	21.19[d,e]	(5.67)
Cysteine	5.48[c]	(2.95)	15.53[d]	(5.32)	14.78[d]	(4.55)	4.02[e,f]	(1.23)	12.85[d,e]	(3.60)	2.99[f]	(0.77)
Methionine	1.55[d]	(0.68)	6.98[e,f]	(1.94)	11.77[d,e]	(2.94)	4.61[e,f]	(1.14)	1.76[f]	(0.40)	5.54[f]	(1.16)
Isoleucine	5.77[c]	(2.88)	12.18[d]	(3.85)	15.16[d]	(4.31)	17.33[d]	(4.90)	19.70[d]	(5.10)	20.31[d]	(4.86)
Leucine	16.02[b]	(7.96)	19.24[b,c]	(6.06)	5.98[e]	(1.69)	12.01[d,e]	(3.38)	11.30[d,e]	(2.91)	12.16[e]	(2.89)
Tyrosine	8.61[b,c]	(3.11)	13.58[d]	(3.11)	27.40[c]	(5.64)	29.04[c]	(5.95)	32.81[c]	(6.14)	46.34[b]	(8.02)
Phenylalanine	6.33[c]	(2.50)	1.86[e]	(0.46)	10.27[d,e]	(2.32)	8.40[e]	(1.88)	10.97[d,e]	(2.25)	11.50[e]	(2.18)
ß-Alanine	3.55[c,d]	(2.60)	7.30[d,e]	(3.40)	8.47[e]	(3.55)	9.54[d,e]	(3.97)	10.86[d,e]	(4.13)	11.48[e]	(4.04)
4-Aminobutyric acid	0.31[e]	(0.19)	0.56[f]	(0.22)	0.99[f]	(0.35)	1.39[f]	(0.50)	2.22[f]	(0.73)	3.11[f]	(0.94)
Histidine	3.22[c,d]	(1.35)	6.73[e,f]	(1.80)	7.31[e]	(1.75)	7.39[d]	(1.76)	5.93[e,f]	(1.29)	5.20[f]	(1.05)
Ornithine	3.94[c,d]	(1.94)	4.19[e,f]	(1.31)	4.42[e,f]	(1.24)	4.78[d,e]	(1.34)	11.65[d,e]	(2.99)	7.66[e,f]	(1.81)
Lysine	14.52[b]	(5.78)	31.72[b]	(8.02)	37.61[b]	(8.55)	40.71[b]	(9.20)	45.75[b]	(9.46)	47.81[b]	(9.14)
Arginine	2.81[c,d]	(1.05)	8.29[d]	(1.97)	11.50[d,e]	(2.46)	13.33[d]	(2.84)	9.42[d,e]	(1.83)	8.61[e]	(1.55)

Different letters (a–f) represent significant differences at p<0.05.

3.2. Development of peptides with radical-scavenging activity

Using the online HPLC–DPPH method, we followed development of peptides with radical-scavenging activity and their molecular mass distributions (Fig. 4). On day 0, peptides with high molecular mass >6.5 KD were detected; these seemed hydrolyze rapidly during early stages of fermentation. The high molecular mass peptides did not have any radical-scavenging activity. As fermentation proceeded (>60 days), peptides with low molecular mass (1.45 KD) and radical-scavenging ability developed indicating the involvement of those peptides in squid *miso* resulted to improve antioxidative properties. A high positive correlation between RSA peak area and peptide peak area was obtained (not shown here). Scavenging activity increased remarkably after 270 days due to the development of peptides with molecular masses of ca. 295 Da and 124 Da.

Changes in free amino acids contents of squid *miso* prepared from unrinsed meat are presented in Table 1. Content of free amino acids, including Asp/Asn, Glu/Gln, Ala, and Leu, increased rapidly through 270 days of fermentation. Amino acids of oligopeptides (Table 2) also
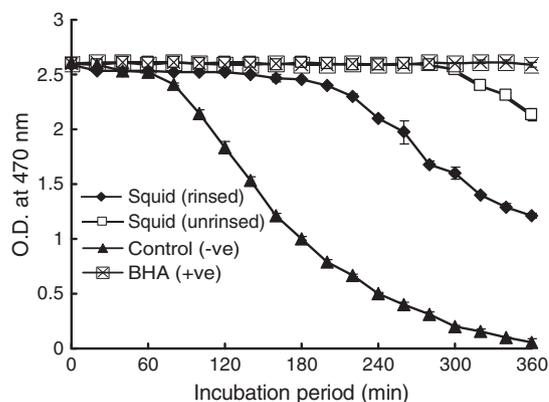

Fig. 5. Rate of *beta*-carotene bleaching in *beta*-carotene–linoleate model system added with aroma extracts of fish *miso* prepared from rinsed and unrinsed squid meat *miso* and antioxidants during incubation period. −ve; negative control; +ve: positive control.
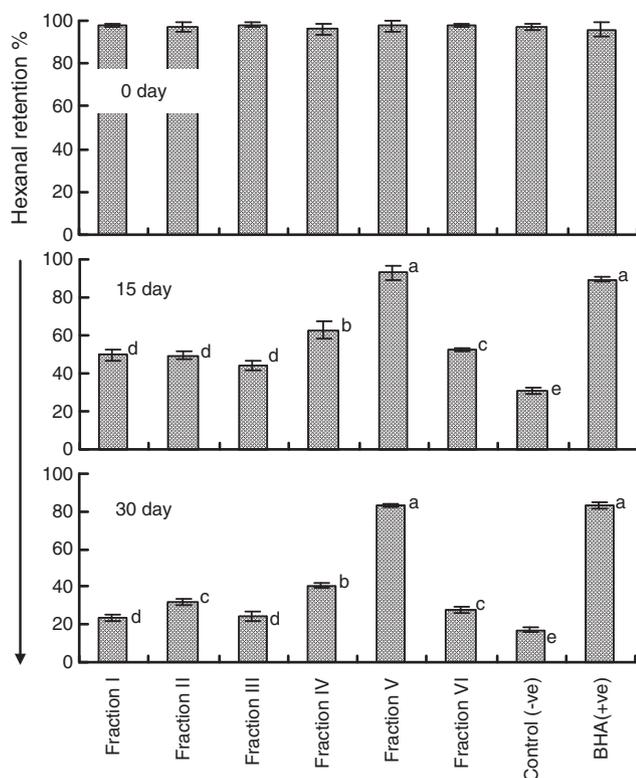
Fig. 6. Rate of hexanal depletion in hexanal oxidation model system added with aroma fractions (I–VI) obtained from fish *miso* aroma extracted prepared from unrinsed squid *miso* and known antioxidants during incubation period. Different letters (a–e) represent significant differences at p<0.05. −ve; negative control; +ve: positive control.

increased in this time period, demonstrating the development of low molecular weight peptides as fermentation proceeded. The oligopeptides comprised mainly Glu/Gln, Pro, Gly, Ala, Val, Lys, and Leu.

The presence of peptides with enhanced hydrophobicity (derived from many protein sources) is linked to antioxidative properties (Chen, Muramoto, & Yamauchi, 1995). An increase in hydrophobicity increases their affinity to lipids and therefore enhances their antioxidative property (Rajapakse, Mendis, Byun, & Kim, 2005) and thus suggesting that hydrophobic amino acids such as Leu, Val, and Ala present in the sequences of squid *miso* peptides might have

promoted radical-scavenging properties. These amino acids are effective as inhibitors of linoleic acid oxidation in the o/w emulsion model system (Marcuse, 1962). In addition, amino acid sequences of peptides strongly affect antioxidative activity; correct positioning of Glu/Gln, Leu, and His in antioxidative peptide sequences improves radical-scavenging activities (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Decomposition of the imidazole ring in histidine is responsible for the strong radical-scavenging activity of this amino acid. We assume that radical-scavenging activity of squid *miso* results from biophysical properties of some of the peptides developing during fermentation.

3.3. Antioxidant activity of volatile active compounds extracted with dichloromethane

Miso extracts prepared from rinsed and unrinsed squid meat were remarkably effective in preventing bleaching of β-carotene (Fig. 5). Squid *miso* extract prepared from unrinsed meat had higher anti-bleaching activity. The dichloromethane volatile extract from squid *miso* prepared from unrinsed meat was further fractionated by column chromatography; fractions obtained were tested for antioxidative activity with the hexanal oxidation procedure. Fraction-V had significantly higher antioxidative activity than other fractions through 30 days of incubation (Fig. 6). Comparative GC analyses of crude volatile extract and fraction-V (Fig. 7) demonstrated that a number of heterocyclic compounds present in the active fraction were responsible for antioxidative activity (Table 3). It is quite difficult to match the individual activity of the compounds to the total contribution since the synergistic effects were observed in case of mixing the antioxidants in most of the cases. In the present study, therefore, we decided to identify the major active compounds in the most active fraction-V. Among the heterocyclic compounds, 4-ethylguaiacol (4-EG) had high DPPH radical-scavenging activity and was the main contributor to antioxidant activity of fraction-V. Antioxidative properties of 4-EG and related compounds are broadly accepted (Bortolomeazzi, Sebastianutto, Toniolo, & Pizzariello, 2007).

The squid *miso* was prepared by mixing squid meat and rice *koji* at a ratio of 1:1 (w/w). As a result rice *koji* contributed as major source of phenolics in squid *miso*. During the squid *miso* fermentation process several carbohydrate cleaving/hydrolyzing enzyme activity promoted the release of several phenolic compounds in the squid *miso*. Thus, a significant amount of phenolic compounds, including kojic acid, ferulic acids, protocatechuic acid etc. were resulted from the *koji* in fish *miso*. Phenolic compounds in the ethyl acetate fraction of squid
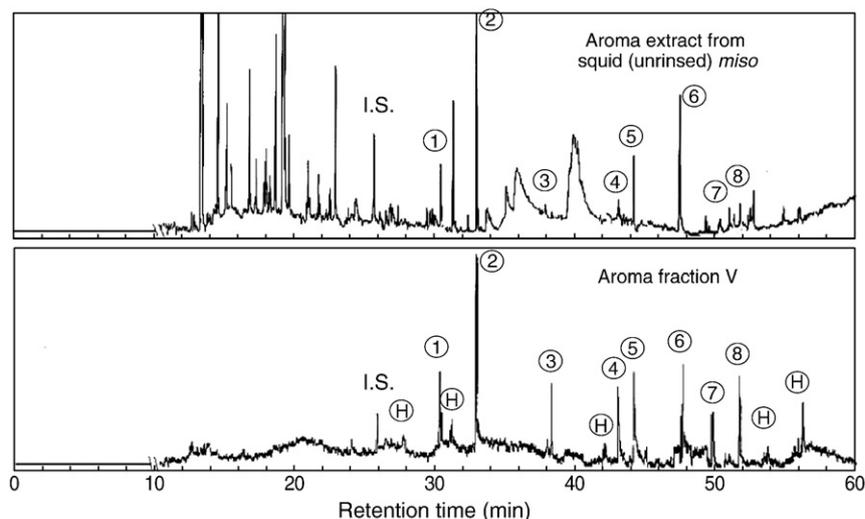

Fig. 7. Gas chromatogram of the volatile profile of aroma extract of the squid *miso* prepared from unrinsed squid meat and volatile fraction-V separated by column chromatography on open silica gel column. Peak indicated as 1–8 are listed in Table 3. I.S. and H indicate internal standard and hydrocarbon respectively.

Table 3
Major volatile compounds identified in highest antioxidative fraction-V of fish *miso* aroma extract prepared from unrinsed meat of squid.

Peak no. indicated in Fig. 7	Volatile compounds	Retention index[a]	Concentration in aroma extract (µg/mL)	Concentration in fraction-V (µg/mL)	DPPH radical-scavenging activity (IC50 value)
1	2-Furfuraldehyde	1484	7.0	2.1	11.95 mg/mL
2	Benzaldehyde	1544	99.0	61.9	Low RSA
3	Furfuryl alcohol	1689	2.1	3.5	6.96 mg/mL
4	Ethylphenyl acetate	1799	3.3	2.1	Low RSA
5	2-Phenylethyl acetate	1834	4.2	3.0	Low RSA
6	Phenylethyl alcohol	1931	171.0	111.1	Low RSA
7	2-Acetylpyrrole	1993	23.0	9.2	7.32 mg/mL
8	4-Ethylguaiacol	2046	61.0	49.0	19.53 µg/mL

[a] Kovats index on Spelcowax™-10 column.

Table 4
Identification of phenolic components in squid *miso* and their changes during fermentation.

Peak no.	Phenolic compounds	UV max (nm)[a]	MW	$[M+H]^+$ m/z	RSA (IC$_{50}$) µg/mL	Concentration (µg/g dry extract)					
						0 day	30 days	60 days	135 days	270 days	365 days
1	Unknown[b]	249	–	–	–	163.8 ± 6.5	158.8 ± 0.6	172.2 ± 18.7	163.8 ± 11.2	108.8 ± 7.4	97.9 ± 2.1
2	Gallic acid	271	170	171	19	1.9 ± 0.0	3.2 ± 0.1	4.4 ± 0.0	3.6 ± 0.1	5.1 ± 0.2	6.8 ± 0.5
3	Protocatechuic acid	260, 295	154	155	58	5.0 ± 0.0	5.9 ± 0.1	6.2 ± 0.0	6.7 ± 0.1	28.1 ± 0.1	37.3 ± 0.2
4	Chlorogenic acid	326, 241	354	355	29	5.2 ± 0.0	5.2 ± 0.0	5.4 ± 0.0	5.7 ± 0.0	7.0 ± 0.1	8.0 ± 0.3
5	*p*-Hydroxy benzoic acid	256	138	139	786	8.2 ± 0.1	7.8 ± 0.1	8.6 ± 0.1	9.0 ± 0.3	12.2 ± 0.2	12.6 ± 2.3
6	Vanillic acid	262, 295	168	169	221	1.1 ± 0.0	1.5 ± 0.0	2.0 ± 0.0	2.3 ± 0.1	5.3 ± 0.0	7.7 ± 0.1
7	Ferulic acid	322, 236	194	195	79	88.8 ± 0.5	59.8 ± 0.1	48.5 ± 0.5	34.4 ± 0.0	1.8 ± 0.2	1.4 ± 0.2

[a] Where there is more than one absorption band, the most intense peak is underlined and used for peak area calculation.
[b] Concentration is based on gallic acid equivalent.

miso prepared from unrinsed meat increased in abundance as fermentation proceeded (Table 4). In contrast, ferulic acid, which forms in early stages of *koji* preparation (Saigusa & Ohba, 2007), decreased in abundance through the fermentation period and was negatively correlated with 4-EG. The 4-EG content of squid *miso* increased with decreasing ferulic acid content, suggesting that 4-EG was a bioconversion product of ferulic acid (Fig. 8). A hypothetical pathway for the bioconversion of ferulic acid to 4-EG is supported by the work of Suezawa and Suzuki (2007).

3.4. Radical-scavenging activities of aqueous, dichloromethane, and ethyl acetate extracts of squid miso

Aqueous extracts contributed greater radical-scavenging activity than dichloromethane and ethyl acetate extracts (Fig. 9). Elevated activity in aqueous extracts reflects the radical-scavenging characteristics of peptides. The key role of peptides as scavengers of free radicals has been emphasized previously (Marcuse, 1962; Yamashoji, Yoshida, & Kajimoto, 1979). Antioxidant activities of heterocyclics in non-polar dichloromethane and phenolics in ethyl acetate extracts may also be attributable to peptides, since they were present in these extracts. Moreover, radical-scavenging activity is one of various mechanisms contributing to overall activity; there is an overall synergy of effects.

We fermented squid meat with *A. oryzae*-inoculated *koji*, which not only enhances antioxidative activity, but also positively improves the flavor of squid *miso* without development of an ammoniacal fishy flavor (Giri et al., 2010). Therefore, *A. oryzae* could well be a suitable microorganism for producing squid *miso* products with good flavor and functionality. In addition, incorporation of protein hydrolysate into foods may confer desirable nutritional and functional properties (Kim, Je, & Kim, 2007). Amino acid sequences of highly antioxidative peptides in squid *miso* fermented with *A. oryzae* have not been determined. However, it is well understood that certain peptides with characteristic amino acid sequences contribute to radical-scavenging activity (Chen et al., 1996). Peptides with demonstrably high antioxidative activity in our study may well have particularly functional amino acid sequences.
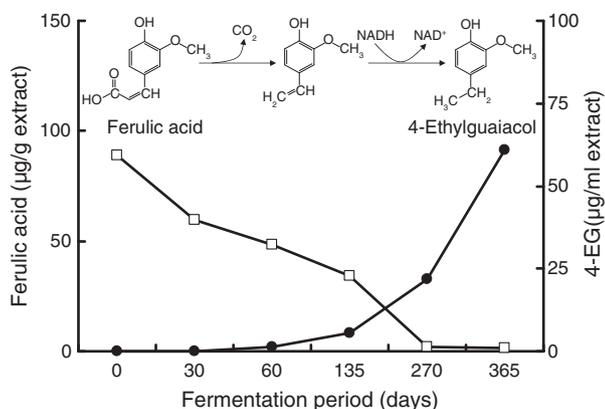

Fig. 8. Changes of ferulic acid and 4-ethylguaiacol in the squid *miso* during the fermentation period. Inset: proposed mechanism for conversion of ferulic acid to 4-ethylguaiacol.
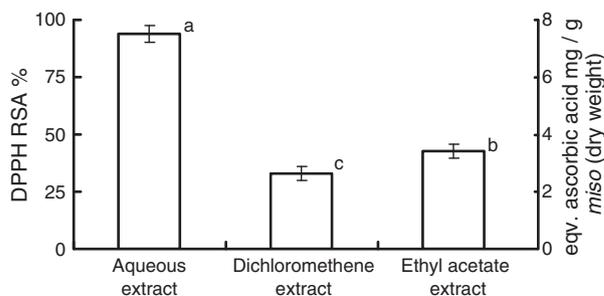

Fig. 9. Comparison of DPPH radical-scavenging activities of the aqueous, dichloromethane and ethyl acetate extracts of the fish *miso* prepared from unrinsed squid meat.

4. Conclusions

Enhanced antioxidant activity with improved nutritional quality in squid *miso* can be obtained after fermentation with *A. oryzae*-inoculated *koji*. Antioxidative peptides (ca. 500 Da) were identified as major contributors to radical-scavenging activity and inhibitors of linoleic acid oxidation. In addition, during the fermentation process, there was a synergistic contribution of antioxidative activity from compounds extracted with dichloromethane and ethyl acetate. Squid *miso* fermented with *A. oryzae*-inoculated *koji* had high antioxidant activity and good flavor.

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