Antioxidant Activity and Isolation from Soybeans Fermented with *Aspergillus* spp.

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Steamed soybeans (SSB) were fermented with 30 kinds of *Aspergillus* strains which have been used in manufacturing Japanese fermented foods and beverages. Methanol extracts from the fermented soybeans were tested for antioxidative activity. Extract from soybeans fermented with *Aspergillus saitoi*, which had the strongest activity, was subjected to a silica gel column followed by preparative HPLC using ODS columns. Antioxidative principle (AS-4) was isolated and identified as 2,3-dihydroxybenzoic acid (2,3-DHBA) by UV, EI-MS, and ^{1}H- and ^{13}C-NMR techniques. Among the six DHBA isomers tested in both oil and lipid/aqueous systems, 2,3-, 2,5-, and 3,4-dihydroxybenzoic acids exhibited the greatest antioxidative activities. Since 2,3-DHBA was not found in unfermented soybeans (SSB), it was produced during the incubation with *A. saitoi*.

Keywords: *Fermented soybeans; Aspergillus spp.; Aspergillus saitoi; antioxidant; 2,3-dihydroxybenzoic acid*

INTRODUCTION

Soybean seeds have a good nutritional quality because they are rich in protein and oil. Most East Asian countries, including Japan, have a long history of utilizing soybeans in great quantities as essential protein foods for humans.

In the traditional Japanese food fermentation industry, koji molds (*Aspergillus oryzae*, *A. sojae*, *A. tamarii*, *A. awamori*, *A. saitoi*, *A. kawachi*, etc.) have been widely used for over a thousand years as the starter for the preparation of koji. Koji, which is a solid culture of koji-mold strain grown in either steamed rice, barley, soybeans, or a mixture of wheat and soybeans, performs a saccharifying and diastatic function in the manufacture of fermented beverages and foods, e.g., sake (rice wines), shochu (Japanese spirits), miso (soybean paste), shoyu (Japanese-style fermented soy sauce), etc. Koji also contributes to producing physiologically functional constituents such as ferulic acid and its analogues (Yoshizawa et al., 1970) in fermented products, in addition to color, flavor and aroma (Koseki et al., 1994). There are several reports on the physiological functions such as antitumor, antioxidative, antimicrobial, and hypotensive actions of miso (Ebine, 1990; Yamaguchi, 1992), shoyu (Ohshita, 1990), sake (Takahashi, 1990; Ohta et al., 1992), etc.

We have done research on fermented soybean products, especially from the viewpoint of antioxidative activities (Esaki et al., 1994, 1996), which have the possibility of inhibiting carcinogenesis and retarding aging (Jang et al., 1997; Ames, 1983). In this study, steamed soybeans were incubated with 30 kinds of *Aspergillus* strains which have been used for manufacturing fermented beverages and foods. The screening test for antioxidative activity was carried out using the methanol extracts of these fermented soybeans. An antioxidative substance was isolated from the soybeans fermented with *A. saitoi* and identified as 2,3-dihydroxybenzoic acid, and its activity was studied in various antioxidative model systems.

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MATERIALS AND METHODS

Materials. Soybeans were obtained from Ichibiki Co. Ltd., Aichi, japan. The beans were grown in kokuryu-shyo in China. Methyl linoleate was purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo. Egg lecithin, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), butylated hydroxytoluene (BHT), *d*-methanol, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dihydroxybenzoic acids (DHBA), α-tocopherol (α-Toc), and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries, Ltd., Osaka. 2,3-Dihydroxybenzoic acid (2,3-DHBA) was purchased from Kanto Chemical Co., Inc., Tokyo.

Preparation of Fermented Soybeans. Fermented soybeans with 30 kinds of *Aspergillus* strains were prepared in Bio'c Co. Ltd. (Aichi, Japan), which is a maker of tane-koji (conidia of *Aspergillus* strains). Whole soybeans (2 kg) were soaked in water at room temperature for 6 h and then steamed at 121 °C for 20 min. Split 120 g quantities of the steamed soybeans (SSB), cooled and mixed with 3 g of baked barley flour, were inoculated with individual tane-koji. The rate of inoculation was 1×10^6 conidia per gram of raw soybeans. The incubation was carried out at 25 °C for 4 days, at which stage the individual fungal mycelia were completely grown. The resulting fermented soybeans, in addition to non-inoculated steamed soybeans (SSB), were lyophilized and ground to a powder with a Sibata scissor type I mill, 40 mesh. In addition, miso, natto, and tempeh were also lyophilized, powdered, and analyzed. Miso and natto were provided by Ichibiki Co. Ltd. and Maruai-Natto Co. Ltd. (Aichi, Japan), respectively. Tempeh was prepared as in a previous report (Esaki et al., 1996) in our laboratory by using the strain of *Rhizopus oligosporus* IFO 32002. In later studies, 500 g quantities of the steamed soybeans, which were treated in a similar procedure, were inoculated with *A. saitoi* IAM 2210 at the same inoculation rate (1×10^6 conidia per gram). The inoculated soybeans were split into four 120 g subsamples and placed in four stainless steel trays. Each subsample was then incubated at 25 °C. The first tray was removed from the incubator at 1 day, the second at 2 days, the third at 3 days, and the other at 4 days. Each sample was lyophilized immediately, and stored at −30 °C until analysis. Large

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quantities (1.2 kg) of the steamed soybeans were also incubated with *A. saitoi* for 4 days in the same stainless steel trays. The resulting fermented soybeans were lyophilized and used for the isolation of antioxidant.

Screening Test for Antioxidative Activity of Fermented Soybeans. Each individual sample (250 mg) of the 30 fermented soybeans, SSB, miso, natto, and tempeh was extracted with two 4.0 mL portions of methanol. Antioxidative activities of the methanol extracts were assayed by using the weighing method (Fukuda et al., 1985) as follows: each aliquot (400 μL) of individual extract was mixed with 400 mg of methyl linoleate, which had been measured into a tared Petri dish (i.d. 27 mm). The solvent was removed under reduced pressure, and each dish was incubated at 40 °C in the dark. The cover of each dish was opened daily to introduce air into the oil. At intervals during incubation, the degree of oxidation was monitored by measuring the weight gain from oxygen. The induction period (IP), defined as the days required to increase the weight of methyl linoleate by 0.5%, was used for the evaluation of the antioxidative activity of each sample.

Antioxidative Assay of Fermented Soybeans with *A. saitoi*. Individual samples (2.0 g) of the lyophilized fermented soybeans incubated with *A. saitoi* for 4 days were extracted with a different solvent, i.e., with 6.0 mL of hexane, chloroform, ethyl acetate, and methanol individually. Each extract (100 μL) was used for the determination of antioxidative activity according to the weighing method described above. In addition, the antioxidative activity of fermented soybeans with different fermentation periods was also assayed according to the method of Esaki et al. (1996). In this method, the lyophilized materials (500 mg) obtained from the incubation with *A. saitoi* for 0, 1, 2, 3, and 4 days were extracted with methanol (5.0 mL). The antioxidative analysis by high-performance liquid chromatography (HPLC) using methyl linoleate was carried out as follows: Each extract (1 mL) was diluted with methanol until the total volume of solution was 6 mL, and then each diluted sample (100 μL) was added to 89 mg (100 μL) of methyl linoleate in a test tube (i.d. 14 mm). After removal of the solvent under reduced pressure, the tubes, open to the air, were incubated at 40 °C for 18 h in the dark. The autoxidation of methyl linoleate was stopped by the addition of 5.0 mL of 0.08% BHT/hexane solution. Total peak areas of the 13- and 9-hydroperoxides from methyl linoleate, to which each sample was added or not, were determined by HPLC. The total peak area obtained from the control containing no added samples was defined as 100% lipid peroxidation. A lipid peroxidation rate (%) of each sample was evaluated as the ratio to the total peak areas of the control. The HPLC was carried out by using a Develosil silica 60-5 column (Nomura Chemical Co. Ltd., Japan, 4.6 mm i.d. × 250 mm) with a spectrophotometric detector (UV-8010, TOSOH) with UV (235 nm) and hexane/1,4-dioxane/2-propanol (98:1:1 v/v/v) as solvent at a flow rate of 1.0 mL/min.

Isolation of the Antioxidant from Fermented Soybeans with *A. saitoi*. The lyophilized material (500 g), incubated with *A. saitoi* for 4 days, was shaken occasionally with three 1.5 L portions of hexane for 3 days. The defatted residue was extracted in three steps with 1.5 L aliquots of methanol to obtain the brownish syrup (66 g) after solvent removal. The methanol extract was chromatographed on a low-pressure silica gel (Wakogel C-200) column (5.2 cm i.d. × 40 cm) with each 3.0 L of a solvent system of *n*-hexane/ethyl acetate (3:1 v/v; 1:1 v/v; and 1:3 v/v), followed by ethyl acetate, ethyl acetate/methanol (1:1 v/v), and methanol. The eluate collected in each fraction was concentrated to remove solvent under reduced pressure, and then 200 μg of each sample was assayed according to the antioxidative analysis by HPLC using methyl linoleate described above. The eluate from *n*-hexane/ethyl acetate (1:1 v/v) was purified by repeating preparative HPLC. The first HPLC was carried out by using a Develosil Lop-ODS (Nomura, 50 mm i.d. × 300 mm) with a UV−vis detector (875-UV, JASCO) with UV (254 nm) and methanol−water (45:55 v/v) as solvent at a flow rate of 26 mL/min. The active fraction of these eluates (methanol−water, 45:55 v/v) was further purified by using a Develosil ODS-15/30 (50 mm i.d. × 500 mm) under the same HPLC conditions except for the use of methanol-water (30:70 v/v) as solvent. The last HPLC was performed by using a Develosil ODS-10 (20 mm i.d. × 250 mm) with a variable wavelength UV monitor (Hitachi, 655A-2) with UV (250 nm) and methanol−water (30:70 v/v) containing 0.1% (v/v) trifluoroacetic acid (TFA) as solvent at a flow rate of 6.0 mL/min. The antioxidant (AS-4) was obtained in a total yield of 8.0 mg from extraction/purification of 500 g of fermented soybean powder. The purity of AS-4 was confirmed by using three-dimensional HPLC [Develosil ODS-HG-5 column, 4.6 mm i.d. × 250 mm, methanol−water (30:70 v/v) containing 0.1% (v/v) TFA, 0.7 mL/min, photodiode array UV−vis detector; Shimadzu].

Instrumental Analysis of Isolated Compound (AS-4). Isolated AS-4 ws dissolved in methanol containing 0.1% (v/v) TFA, and the UV absorption spectrum was measured with a Shimadzu UV-160A spectrophotometer. The electron impact mass spectra (EI-MS) were recorded on a JEOL JMS-DX-705L mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained by a Bruker ARX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) in *d*-methanol.

Antioxidative Assay of 2,3-DHBA and Other Isomers. 2,3-DHBA and the other isomers of dihydroxybenzoic acid were dissolved in methanol at a constant molar concentration. The antioxidative activities of these dihydroxybenzoic acids were also assayed in a liposome system (Tsuda et al., 1993), in addition to the antioxidative analysis by HPLC using methyl linoleate as described above.

Liposome System. Egg lecithin (100 mg) was dissolved in 2 mL of chloroform, and the solvent was removed by rotary evaporator to form a thin film. The film was sonicated in an ultrasonic cleaner (Branson model B-12) with a 10 mM phosphate buffer (pH 7.4, 10 mL). The resulting multilamellar vesicles (MLV) were sonicated in a cup−horn-type sonicator (Insonator model 200M, Kubota, Japan) at 180 W for 20 min, by which process small unilamellar vesicles (SUV) were obtained. The SUV solution (10 mg of liposome/mL), AAPH, with a phosphate buffer (pH 7.4), and each antioxidant (2,3-DHBA and the other isomers) were mixed to contain a final concentration of 1 mg of liposome/mL, 4 mM AAPH, 1 mM phosphate buffer, and 0.1 mM antioxidant, respectively. The reaction mixture was incubated at 37 °C for 2 h. The quantity of thiobarbituric acid (TBA)-reacting substance (TBARS) was determined at 535 nm after coloration with thiobarbituric acid (Buege and Aust, 1978).

Quantitative Analyses of 2,3-DHBA in Fermented Soybeans with Different Fermentation Periods. The amounts of 2,3-DHBA in fermented soybeans with different fermentation periods were determined by using HPLC. Each extracted solution, which had been previously prepared for an antioxidative assay of fermented soybeans with different fermentation periods, was also used in this experiment. The HPLC was performed by using a Develosil ODS-7 column (Nomura, 4.6 mm i.d. × 250 mm) with a variable wavelength UV monitor (Hitachi, 655A-2) with UV (250 nm) and methanol−water (25:75 v/v) containing 0.1% (v/v) TFA as solvent at a flow rate of 0.7 mL/min.

Statistics. The assays throughout this study were repeated twice, and each sample was tested in triplicate. Statistical analysis was performed by using Student's *t*-test.

RESULTS AND DISCUSSION

Screening for Antioxidative Activity of Fermented Soybeans Incubated with *Aspergillus* Strains. The 30 kinds of *Aspergillus* strains used for the incubation of fermented soybeans are shown in Table 1. Most of these strains are now utilized in the production of Japanese fermented beverages and foods. Antioxidative activities of the methanol extracts from fermented soybeans with *Aspergillus* strains were assayed against methyl linoleate by using the weighing method. In addition, the methanol extracts of miso, natto, and tempeh, which are representative fermented soybean products, were also determined with the same

Table 1. *Aspergillus* Strains Used in the Screening Test

Aspergillus strains		
A. oryzae	(soy sauce)	No. 1, 2, 3, 4, and 5
A. oryzae	(miso)	No. 6, 7, 8, 15, 17, and 18
A. oryzae	(sake)	No. 9, 10, 11, 12, 13, and 14
A. oryzae	(amazake)	No. 16
A. oryzae	(RIB 40)[a]	No. 19
A. oryzae	(RIB 128)	No. 20
A. oryzae	(RIB 642)	No. 21
A. sojae	(soy sauce)	No. 22
A. sojae	(RIB 401)	No. 23
A. tamarii	(RIB 3005)	No. 24
A. tamarii	(RIB 3007)	No. 25
A. usamii	(RIB 2001)	No. 26
A. shirousamii	(RIB 2501)	No. 27
A. awamori	(RIB 2803)	No. 28
A. saitoi	(IAM 2210)[b]	No. 29
A. kawachi	(IAM 2063)	No. 30

[a] RIB, National Research Institute of Brewing. [b] IAM, Institute of Applied Microbiology in Tokyo University.

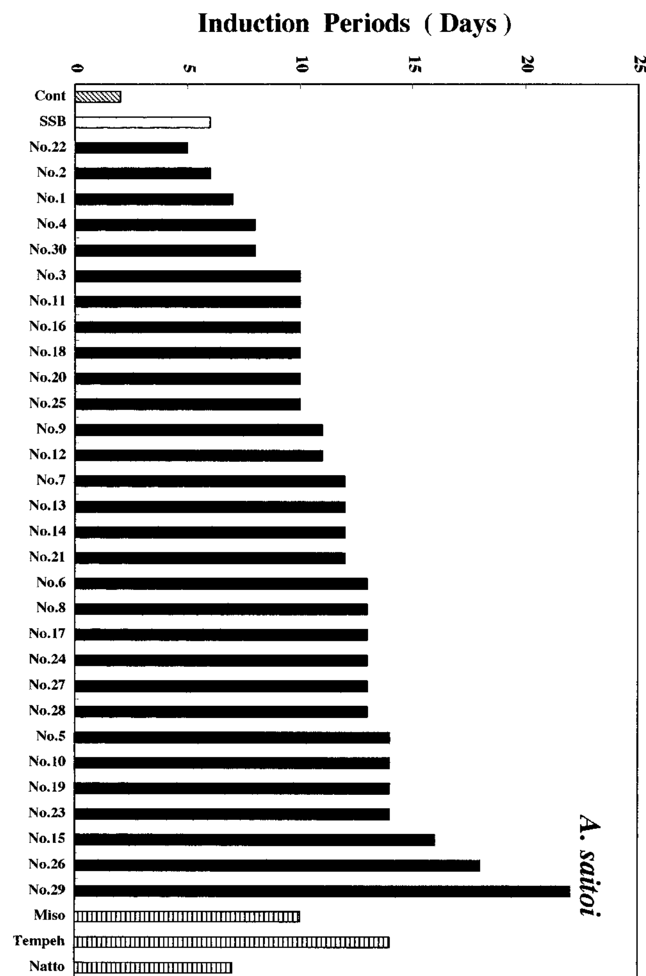

Figure 1. Antioxidative activities of the methanol extracts from SSB (unfermented soybeans) and various fermented soybeans incubated with 30 kinds of *Aspergillus* strains. Each fungal number is represented in Table 1. A control contained methanol alone instead of the methanol extract. Induction period of the weighing method was measured at 40 °C with methyl linoleate as substrate. Values in this figure are the averages of duplicates.

procedure. The results of the screening test are shown in Figure 1. The majority (28 kinds) of the fermented soybeans exhibited stronger antioxidative activity than that of unfermented steamed soybeans (SSB). Further, the fermented soybeans incubated with *A. saitoi* IAM 2210 (No. 29) possessed the strongest antioxidative activity (IP, 22 days) among the 30 kinds of fermented soybeans. Its activity was higher than that of miso, natto, or tempeh. Therefore, we focused on the fermented soybeans incubated with *A. saitoi*.

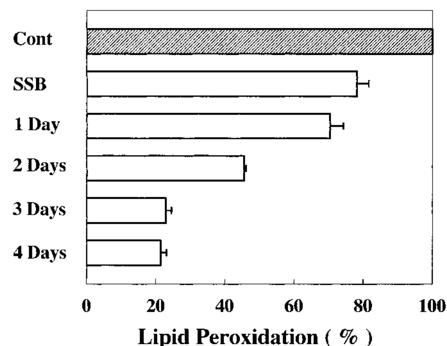

Figure 2. Antioxidative activities of the methanol extracts from SSB and various fermented soybeans incubated for 1, 2, 3, and 4 days, as measured by the antioxidative analysis by HPLC using methyl linoleate. Values in this figure are the mean ± SD ($n = 3$). The value for a control containing no added samples represents 100% lipid peroxidation.

Antioxidative Activities of Fermented Soybeans with *A. saitoi*. Antioxidative activities of crude extracts from the fermented soybeans incubated with *A. saitoi* for 4 days with different solvents were assayed according to the weighing method with methyl linoleate. The methanol extract had the strongest antioxidative activity (IP, 28 days), compared with that of hexane (IP, 5 days), chloroform (IP, 6 days), and ethyl acetate (IP, 7 days) extract. These results suggested that the potent antioxidants, which were produced during the incubation of fermented soybeans with *A. saitoi*, are soluble in methanol.

In further studies, the fermented soybeans with different fermentation periods (0, 1, 2, 3, and 4 days) were extracted with methanol. Each extract was assayed according to the antioxidative analysis by HPLC using methyl linoleate, which is able to quickly evaluate the antioxidative activity by the determination of hydroperoxide from methyl linoleate by using HPLC. As shown in Figure 2, the antioxidative activity gradually increased during 4 days in the fermentation of *A. saitoi*.

From these results, the isolation of an active principle was started using the methanol extract from fermented soybeans incubated with *A. saitoi* for 4 days.

Isolation and Identification of Antioxidative Compound. A methanol extract prepared from a defatted fermented soybeans was fractionated by silica gel column chromatography. From the results (data not shown) of antioxidative analysis in each fraction, *n*-hexane/ethyl acetate (1:1 v/v; and 1:3 v/v) and ethyl acetate eluates had the stronger antioxidative activity. By repeating the preparative HPLC of the *n*-hexane/ethyl acetate (1:1 v/v) fraction, we were able to isolate a strong antioxidative substance (AS-4). Three-dimensional HPLC showed that its purity was more than 99.9%. The data on the instrumental analyses of AS-4 were presented as follows: UV λ_{max} in methanol containing 0.1% (v/v) TFA (nm) 248 (log $\epsilon = 3.89$), 319 (log $\epsilon = 3.55$); EI-MS (*m/z*) 154 (M^+), 137 ($M^+ - OH$), 108, 80; ^1H NMR (in *d*-methanol) δ 6.73 (1H, dd, $J = 7.8$, 7.9 Hz, H-5), 7.00 (1H, dd, $J = 7.8$, 1.5 Hz, H-4), 7.35 (1H, dd, $J = 7.9$, 1.5 Hz, H-6); ^{13}C-NMR (in *d*-methanol) δ 173.9 (C=O), 151.8 (C2), 146.9 (C3), 121.8 (C4), 121.5 (C6), 119.7 (C5), 114.3 (C1). From these analytical data, AS-4 was identified as 2,3-dihydroxybenzoic acid (2,3-

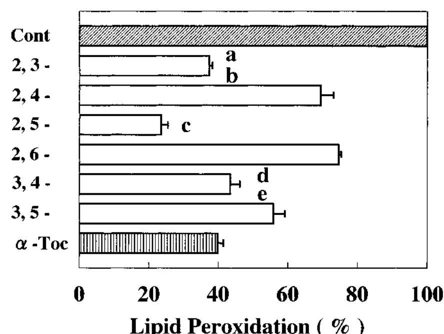

Figure 3. Antioxidative activities of 2,3-dihydroxybenzoic acid and the other isomers (final concentration, 1 mM) in methyl linoleate. Values in this figure are the mean ± SD ($n = 3$). The value for a control containing no added samples represents 100% lipid peroxidation. (a) $P < 0.01$, compared with 2,4-, 2,5-, 2,6-, and 3,5-DHBA. (b) $P < 0.05$, compares with 3,4-DHBA. (c) $P < 0.01$, compared with 2,3-, 2,4-, 2,6-, 3,4-, and 3,5-DHBA and α-tocopherol (α-Toc). (d) $P < 0.01$, compared with 2,4-, 2,5-, and 2,6-DHBA. (e) $P < 0.05$, compared with 3,5-DHBA.

DHBA), which was confirmed by direct comparison with an authentic sample.

Antioxidative Activities of Isolated Compound. *Comparison of 2,3-DHBA with Other Isomers of Dihydroxybenzoic Acid.* Some natural antioxidants of microbial origin have been isolated and identified. 3,4-Dihydroxybenzoic acid (protocatechuic acid) was isolated from the culture filtrate of *Penicillium* sp. A-257 (Aoyama et al., 1982). This compound is known to be a fermentation product of filamentous fungus (Haslam et al., 1961). Recently, 2,3-dihydroxybenzoic acid was also reported to be an antioxidant from the culture broth of *P. roquefortii* IFO 5956 (Hayashi et al., 1995). However, the antioxidative assays of these compounds were not determined in detail. In this study, the antioxidative comparisons of 2,3-DHBA with the other isomers were done in both oil and lipid/aqueous systems. The antioxidative effects of 2,3-DHBA and the other five isomers of dihydroxybenzoic acid at the same concentration (final concentration, 1 mM) on the peroxidation of methyl linoleate were first studied and compared with α-tocopherol by the antioxidative analysis by HPLC. As shown in Figure 3, 2,3-DHBA exhibited the same significant activity as α-tocopherol. Among the six isomers of dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid (2,5-DHBA), which has *p*-dihydroxy groups in the molecule of benzoic acid, showed the strongest ($P < 0.01$) activity. 2,3- and 3,4-dihydroxybenzoic acid (2,3-DHBA and 3,4-DHBA), which are both *ortho*-type dihydroxybenzoic acids, showed stronger activity than that of 3,5-, 2,4-, or 2,6-dihydroxybenzoic acid (3,5-, 2,4-, or 2,6-DHBA). It is well-known that the *o*-dihydroxybenzoic acid structure is very important for the development of strong antioxidative activity. In addition, Takizawa et al. (1980) reported that *p*-dihydroxybenzene showed a marked activity compared with *o*- or *m*-dihydroxybenzene. They also reported that vicinal carboxylic acid of phenol (2-hydroxybenzoic acid) had the strongest antioxidative activity among the three isomers, and it was suggested that these results are adequately applicable to the structure of dihydroxybenzoic acid isomers. In this experiment, 2,3- and 2,5-DHBA, which have vicinal carboxylic acid, exhibited stronger antioxidative activity.

A main target of free radical damage in the body is the cellular membrane which contains abundant unsaturated lipids; promotion of lipid peroxidation is closely related to aging and carcinogenesis (Jang et al., 1997; Ames, 1983). Liposomes have been used extensively as cellular models for *in vitro* lipid peroxidation studies. Therefore, liposomes (SUV) were also used for the antioxidative evaluation of 2,3-DHBA, as the lipid/aqueous system. The results of antioxidative activities of 2,3-DHBA and the other five isomers in the liposome system (final concentration, 0.1 mM) are shown in Figure 4. 2,3-, 2,5-, and 3,4-DHBA exhibited strong antioxidative activities among the six isomers in the lipid/aqueous system as well. Specifically, the activities of 2,3-DHBA and 2,5-DHBA were a little higher ($P < 0.01$ or $P < 0.05$) than that of α-tocopherol or BHT in this system. Comparing the results in Figure 4 with that in Figure 3, the antioxidative activities of 2,3-, 2,5-, and 3,4-DHBA in a lipid/aqueous system were stronger than those in the oil system.

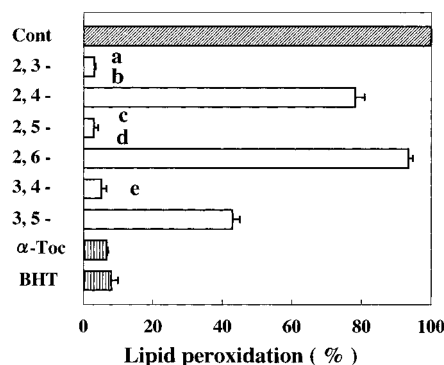

Figure 4. Antioxidative activities of 2,3-dihydroxybenzoic acid and the other isomers (final concentration, 0.1 mM) in the liposome system. Lipid peroxidation was induced by 4 mM AAPH. Values in this figure are the mean ± SD ($n = 3$). The value for a control containing no added samples represents 100% lipid peroxidation. (a) $P < 0.01$, compared with 2,4-, 2,6-, and 3,5-DHBA and α-tocopherol (α-Toc). (b) $P < 0.05$, compared with BHT. (c) $P < 0.01$, compared with 2,4-, 2,6- and 3,5-DHBA. (d) $P < 0.05$, compared with α-Toc and BHT. (e) $P < 0.01$, compared with 2,4-, 2,6-, and 3,5-DHBA.

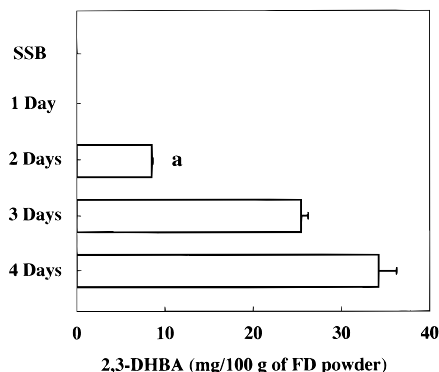

Figure 5. Amount of 2,3-dihydroxybenzoic acid in SSB and various fermented soybeans incubated for 1, 2, 3, and 4 days. Values in this figure are the mean ± SD ($n = 3$). (a) $P < 0.01$, compared with 3 and 4 days.

2,3-DHBA Contents in Fermented Soybeans with *A. saitoi*. From the antioxidative assays above, 2,3-DHBA exhibited strong antioxidative activity in both oil and lipid/aqueous systems. This suggested that 2,3-DHBA is one of the potent antioxidants in fermented soybeans incubated with *A. saitoi*. To confirm this suggestion, 2,3-DHBA contents in the fermented soybeans with different fermentation periods were assayed. The results of quantitative analyses of 2,3-DHBA by using HPLC are shown in Figure 5. 2,3-DHBA was not detected in fermented soybeans incubated for 1 day or in unfermented steamed soybeans (SSB). However, it

appeared at 2 days and significantly ($P < 0.01$) increased from 2 days to 3 or 4 days, which had the strongest antioxidative activity among the fermented soybeans with different fermentation periods (Figure 2). The molar concentration of 2,3-DHBA at 4 days was more than 2 mM.

Conclusions. Lipid peroxidation causes oxidative damage not only in food systems but also in human body. Recently, an intensive search for novel natural antioxidants has been carried out in numerous plant materials, including those used as foods (Osawa et al., 1990; Nakatani, 1990). Some microorganisms, especially fungi and yeasts, are known to produce antioxidative substances (Aoyama et al., 1982; Hayashi et al., 1995; Meisinger et al., 1959). *Aspergillus* strains used in manufacturing traditional fermented foods and beverages are safe, because those microbes have been eaten in safety by many people over a long term of years (Barbesgaard et al., 1992). In this study, we enhanced the antioxidative activity of soybeans by fermentation with *Aspergillus* strains. The fermented soybeans incubated with *A. saitoi*, which has been utilized for manufacturing "awamori" (millet spirits) in the Okinawa area, had the most potent antioxidative activity among the 30 kinds of fermented soybeans. One of the potent antioxidants was isolated and identified as 2,3-DHBA, which showed antioxidative activities in both oil and lipid/aqueous systems. This antioxidant was produced during the incubations of *A. saitoi* but was not detected in fermented soybean products such as miso (rice/soybean, barley/soybean, and soybean), shoyu, natto, and tempeh (data not shown). In further research, we would like to design the utilization of fermented soybeans as potent antioxidative food materials in addition to finding other new antioxidative substances in them.

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