New Antioxidant Isolated from Tempeh

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Dried tempeh is known to be significantly stable to lipid oxidation compared with unfermented soybeans (BS). A new antioxidant was isolated from the methanol extract of tempeh and identified as 3-hydroxyanthranilic acid (HAA) by UV, IR, EI-MS, and ^{1}H-, ^{13}C-, HMQC-, and HMBC-NMR techniques. HAA was effective in preventing autoxidation of soybean oil and soybean powder, while 6,7,4′-trihydroxyisoflavone (a well-known antioxidant) was not. HAA also exhibited strong antioxidative activity in both water/ethanol and rabbit erythrocyte membrane ghost systems. HAA was not found in BS, but was produced during the incubations with *Rhizopus oligosporus* IFO 32002 and 32003. The amount of HAA reached a maximum at the stage of 2 days (acceptable), which had the strongest antioxidative activity.

Keywords: *Tempeh; fermented soybean product; antioxidant; 3-hydroxyanthranilic acid*

INTRODUCTION

Antioxidants in foods have recently attracted special interest because they can protect the human body from free radicals, which may cause various diseases (including carcinogenesis) and aging (Osawa et al., 1987; Cutlar, 1992). These antioxidants can also retard oxidative rancidity caused by atmospheric oxidation and thus protect oils, fats, and fat-soluble components such as vitamins, carotenoids, and other nutritive ingredients in foods.

With this background, we began antioxidative studies of traditional Asian fermented soybean products (Esaki et al., 1994). Miso, natto, and tempeh, which were incubated with *Aspergillus oryzae*, *Bacillus natto*, and *Rhizopus oligosporus*, respectively, were more stable against lipid peroxidation than unfermented steamed soybeans. Tempeh and miso showed remarkably stronger antioxidative activity than natto.

In regard to tempeh, it has already been reported that this fermented soybean product is very stable to rancidity development, and the antioxidative 6,7,4′-trihydroxyisoflavone was isolated and identified (György et al., 1964). This isoflavone was shown to be a potent antioxidant in lipid/aqueous systems (Ikehata et al., 1968; Pratt and Birac, 1979). However, it was not effective in preventing autoxidation of soybean oil or soybean powder (Ikehata et al., 1968).

The crude tempeh oil extracted with hexane/alcohol (1:2) has also been reported to be more stable to oxidation compared with the oil from unfermented soybeans (György et al., 1974; Murakami et al., 1984). This tempeh oil showed its antioxidative effect when added to soybean, cottonseed, corn, and safflower oils and lard (György et al., 1974).

It is noteworthy that the crude tempeh oil does exert its antioxidant effect when mixed with soybean oil, while purified 6,7,4′-trihydroxyisoflavone was not effective in preventing autoxidation of soybean oil. This finding suggests that other antioxidants must be present in the crude tempeh oil. In this paper, we report the isolation and identification of a potent antioxidant effective on soybean oil, in addition to its antioxidative activity in various model systems.

MATERIALS AND METHODS

Materials. Dehulled soybeans were obtained from Ichibiki Co. Ltd., Aichi, Japan. The beans were grown in Kokuryu-shyo in China. The strains of *R. oligosporus* IFO 32002 and 32003, which are authorized by the Indonesian government, were obtained from the Institute for Fermentation, Osaka (IFO), Japan. Methyl linoleate and an authentic sample of 3-hydroxyanthranilic acid were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo. Soybean oil, linoleic acid, trifluoroacetic acid (TFA), genistein, α-tocopherol, and butylated hydroxytoluene (BHT) were obtained from Wako Pure Chemical Industries, Ltd., Osaka.

Preparation of Tempeh. Tempeh with different fermentation periods was prepared in our laboratory by modifying the method of Steinkraus et al. (1965). Dehulled soybeans were soaked and boiled at 105 °C in 0.14% acetic acid for 30 min. Boiled soybeans (BS), drained and cooled to 37 °C, were mixed with a suspension of spores of *R. oligosporus* IFO 32002. The 150 g quantity of the inoculated soybeans was placed in three 1.0 L Erlenmeyer flasks and incubated at 37 °C. One flask was drawn at 1 day, another at 2 days, and the other at 4 days. Large quantities of tempeh fermented for 2 days were also made in 1.0 L Erlenmeyer flasks. Tempeh with the three different fermentation periods, BS, and large quantities of the tempeh incubated for 2 days were lyophilized and ground to a powder by Sibata scissor type I mill, 40 mesh.

Antioxidative Assay of Crude Extracts with Different Solvents. Individual 1.0 g samples of the lyophilized tempeh powder fermented for 2 days and BS powder were extracted, each with a different solvent, i.e., with 3.0 mL of hexane, chloroform, ethyl acetate, or methanol. The antioxidative activities of crude extracts were assayed by using the weighing method (Olcott and Einset, 1958) as follows: Each aliquot (100 μL) of individual extract was mixed with 400 mg of a tocopherol-free soybean oil (Mohri et al., 1983), which had been measured into a Petri dish (i.d. 27 mm). The solvent was removed with a vacuum pump, and each dish was incubated at 40 °C in the dark. The cover of each dish was daily opened to introduce air into the oil. At intervals during incubation, the degree of oxidation was monitored with A&N ER-182A electronic balance (±0.1 mg) by measuring the weight gain from oxygen. The induction period (IP), which was defined

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0021-8561/96/1444-0696$12.00/0

as the days required to increase the weight of oil by 0.5%, was used for the evaluation of the antioxidative activity of each sample.

Antioxidative Assay of Tempeh with Different Fermentation Periods. The lyophilized tempeh powders (1.0 g) fermented for 0, 1, 2, and 4 days were extracted with methanol (3.0 mL). Each extract (200 μL) was used for the determination of antioxidative activity according to the weighing method described above. In addition, the antioxidative activity of tempeh with different fermentation periods was also assayed by reference to the method of Terao and Matsusita (1986). The antioxidative analysis using methyl linoleate was conducted as follows: Each extract was diluted with 7 volumes of methanol, and then each diluted sample (50 μL) was added to 100 mg of methyl linoleate in a test tube (i.d. 14 mm). After removal of the solvent, 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. The hydroperoxide from methyl linoleate was determined by high-performance liquid chromatography (HPLC). The HPLC was carried out by using a Develosil silica 60-5 column (Nomura Chemical Co. Ltd., Japan, 4.6 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) as solvent at a flow rate of 1.0 mL/min.

Isolation of the Antioxidant from Tempeh. The lyophilized tempeh powder (1130 g), which had been fermented for 2 days in large quantities, was extracted with 3.4 L of methanol three times to obtain the yellowish syrup (150 g). The methanol extract was chromatographed on a low-pressure silica gel column (6.0 i.d. × 51.0 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 from ethyl acetate was further purified by preparative HPLC. The HPLC was carried out by using a Develosil Lop-ODS (Nomura, 50 i.d. × 300 mm) with an intelligent UV−vis detector (875-UV, JASCO) with UV (298 nm) and 0.1% TFA/5% acetonitrile as solvent at a flow rate of 30 mL/min. The antioxidative compound (HAA) was obtained in a total yield of 52.7 mg from extraction/purification of 1130 g of tempeh powder. The purity of HAA was confirmed by using three-dimensional HPLC (Develosil ODS-HG-5 column, 4.6 i.d. × 250 mm, 0.1% TFA/5 % acetonitrile, 0.7 mL/min, photodiode array UV−vis detector; Shimadzu).

Instrumental Analysis of Isolated Compound (HAA). HAA was dissolved in methanol and 0.1% TFA/methanol, and the UV absorption spectra were measured with a Shimadzu UV-160A spectrophotometer. IR spectra were taken on a JASCO FT/IR-8000 spectrophotometer by using KBr method. The electron impact mass spectra (EI-MS) were recorded on a JEOL JMS-DX-705L mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained by a Brucker ARX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) in dimethyl-*d* sulfoxide (DMSO-*d*) containing tetramethylsilane (TMS) as an internal standard for all samples.

Antioxidative Activities of HAA. HAA was dissolved in methanol unless otherwise noted, and the antioxidative activities were assayed in various model systems.

In Oil System. HAA solution (200 μL) in different concentrations was mixed with 400 mg of soybean oil. The antioxidative activities were measured according to the weighing method described above. α-Tocopherol, BHT, and genistein were used as antioxidative controls. In addition, the antioxidative activity of HAA against methyl linoleate was also assayed by the antioxidative analysis using methyl linoleate as described before.

In Water/Ethanol System. Autoxidation of linoleic acid in water/ethanol systems was carried out by using the method of Osawa and Namiki (1981). HAA (6, 12.5, 25, 50, and 100 μg) was added to a solution mixture of purified linoleic acid (0.13 mL) and ethanol (10 mL), with a 0.2 M phosphate buffer (pH 7.0, 10 mL); the total volume was adjusted to 25 mL with distilled water. The reaction mixture in a sealed conical flask was incubated at 40 °C in the dark. At intervals during incubation, the hydroperoxide from linoleic acid was determined by using the thiocyanate method (Mitsuda et al., 1966). An aliquot (200 μL) of the reaction mixture was mixed with 75% ethanol (9.4 mL), 30% ammonium thiocyanate (200 μL), and 20 mM iron(II) chloride (200 μL), and the absorbance of this coloring solution was measured at 500 nm. The induction period was defined as the days required for the absorbance at 500 nm by the thiocyanate method to reach 0.3.

In Rabbit Erythrocyte Membrane Ghost System. Commercially available rabbit blood (100 mL) was obtained from Japan Biotest Institute Co. Ltd. and diluted with 100 mL of isotonic buffer solution (10 mM phosphate/152 mM NaCl, pH 7.4). The diluted blood solution was centrifuged at 1500*g* for 20 min, and the red blood cells were collected and washed three times with 100 mL of the isotonic buffer solution and lysed in 10 mM phosphate buffer (pH 7.4). Erythrocyte membrane ghosts were pelleted by centrifugation (2000*g*, 40 min), and the precipitate was diluted to give a suspension (1.0 mg of protein/mL) (Osawa et al., 1987). Peroxidation of the erythrocyte membrane ghosts induced by *tert*-butylhydroperoxide (*t*-BH) was carried out according to the method of Ames et al. (1981). HAA and α-tocopherol were dissolved in dimethyl sulfoxide, and the sample solution (100 μL) was mixed with the ghost suspension (850 μL) and 24 mM *t*-BH solution (50 μL). After incubation at 37 °C for 20 min, 1 mL of 2.0 M TCA/1.7 M HCl and 2 mL of 0.67% thiobarbituric acid (TBA) solution were added to stop the reaction. The quantity of TBA reactive substance (TBARS) was determined at 532 nm according to the method described by Wilbur et al. (1949).

Antioxidative Effect of HAA on Soybean Powder. One gram of lyophilized soybean powder (BS) was mixed with 1, 5, and 10 mg of HAA dissolved in 2 mL of methanol, and the methanol was removed by rotary evaporator followed by vacuum pump. Another 1 g of BS was mixed with 2 mL of methanol alone as control. Each soybean powder absorbed with HAA was incubated at 40 °C in the dark. The extent of lipid peroxidation was monitored at regular intervals according to the thiocyanate method (Mitsuda et al., 1966). This experiment was duplicated.

Quantitative Analyses of HAA in Tempeh with Different Fermentation Periods. HAA was extracted with 0.1% TFA/50% methanol from the lyophilized tempeh powder with different fermentation periods. The amounts of HAA in tempeh were determined by using HPLC. The HPLC was performed by using a Develosil ODS-7 column (Nomura, 4.6 i.d. × 250 mm) with a variable wavelength UV monitor (Hitachi, 655A-2) with UV (298 nm) and 0.1% TFA/5% acetonitrile as solvent at a flow rate of 0.7 mL/min.

RESULTS AND DISCUSSION

Preparation of Tempeh. Tempeh fermentation was conducted in Erlenmeyer flasks to prevent contamination by other microorganisms. The mycelial growth became visible on the soybeans after 1 day. After 2 days of incubation, the mycelia became dense and beans were completely overgrown with white mold and knitted into a compact cake. The tempeh product after 2 days is acceptable as a tempeh cake.

The mycelia were found to undergo sporulation after 3−4 days, and the tempeh cake turned black.

Antioxidative Activity of Crude Extracts against Soybean Oil. Antioxidative activities of crude extracts from tempeh and BS with different solvents were assayed against soybean oil. The results from the weighing method are shown in Figure 1. There were no significant differences in the antioxidative activities of the hexane, chloroform, and ethyl acetate extracts of tempeh and BS. On the other hand, the methanol extract from tempeh showed much stronger antioxidative activity (IP, 15 days) compared with that of BS (IP, 5 days). These results suggest that the potent antioxidants effective against soybean oil, which were produced during fermentation of tempeh, tend to be dissolved in methanol.

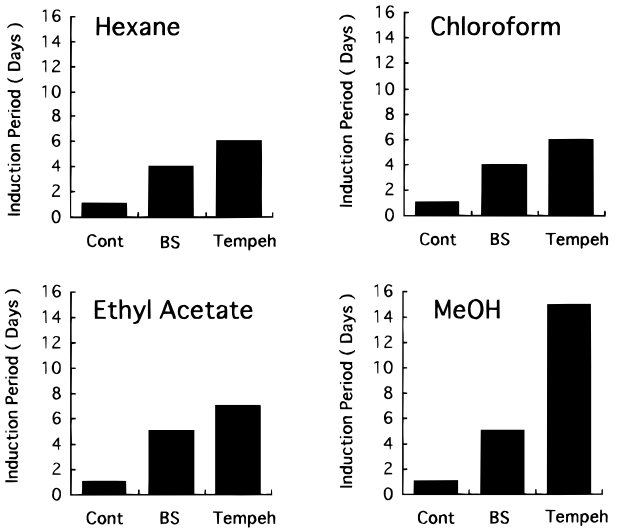

Figure 1. Antioxidative activities of the crude hexane, chloroform, ethyl acetate, and methanol extracts of tempeh and BS (unfermented soybeans). Each control contained the respective solvent alone instead of each extract. Induction periods of the weighing method were measured at 40 °C with soybean oil as substrate. Values in this figure are the average of duplicates.

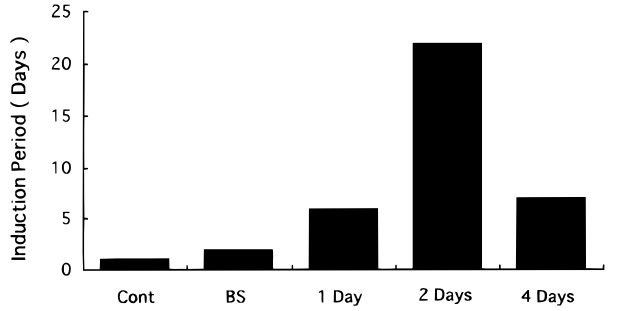

Figure 2. Antioxidative activities of the methanol extracts from BS and various tempehs fermented for 1, 2, and 4 days (weighing method). A control contained methanol alone instead of the methanol extract. Values in this figure are the average of duplicates.

Antioxidative Activity of Tempeh with Different Fermentation Periods. The tempeh with different fermentation periods (0, 1, 2, and 4 days) was extracted with methanol. Antioxidative activities of the extracts were determined according to the weighing method with soybean oil. As shown in Figure 2, the antioxidative activity had progressively increased during fermentation of tempeh, and the tempeh fermented for 2 days had the strongest activity. However, the antioxidative activity in the 4 day fermentation extract was dropped dramatically. It is therefore presumed that the antioxidative components including HAA in tempeh are decomposed by the 4 day fermentation. Further research will be necessary to determine the antioxidative activities of tempehs fermented for 1.5, 2.5, and 3 days. In addition, the methanol extracts were also assayed by the antioxidative analysis using methyl linoleate, which is able to evaluate the antioxidative activity by the determination of hydroperoxide from methyl linoleate by using HPLC. These results (data not shown) were in good agreement with those obtained by the weighing method with soybean oil.

Therefore, we focused on the tempeh fermented for 2 days, and the isolation of potent antioxidants effective on soybean oil was carried out using the methanol extract.

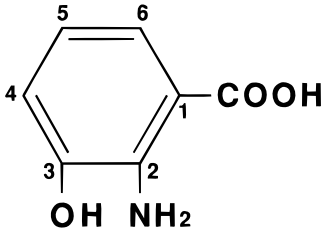

Figure 3. Chemical structure of 3-hydroxyanthranilic acid (HAA) isolated from tempeh.

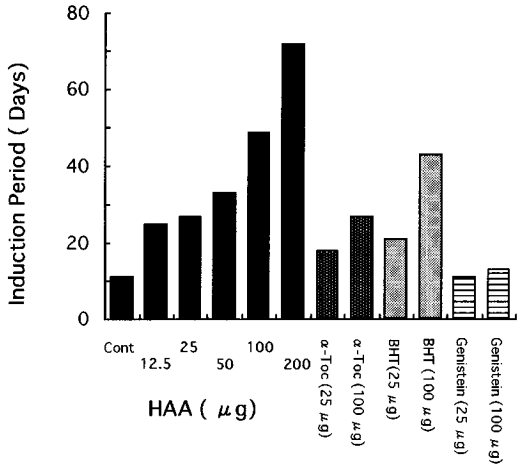

Figure 4. Antioxidative effect of HAA on soybean oil. HAA (12.5, 25, 50, 100, and 200 μg), α-tocopherol (25 and 100 μg), BHT (25 and 100 μg), and genistein (25 and 100 μg) were added to 400 mg of soybean oil, and the antioxidative activity of each was measured by the weighing method. Values in this figure are the average of duplicates.

Isolation and Identification of Antioxidative Compound. A methanol extract prepared from a large amount of the tempeh powder was fractionated by silica gel column chromatography. The eluate collected in each fraction was concentrated to remove solvent under reduced pressure, and then each 200 μg sample was assayed by the antioxidative analysis using methyl linoleate. From the most active ethyl acetate fraction, a strong antioxidative compound (HAA) was isolated by repeating preparative HPLC. Three-dimensional HPLC showed that its purity was more than 99.9%. Identification of HAA was performed. HAA exhibited the following properties: UV λ_{max} in methanol (nm) 340 (log $\epsilon = 3.64$), 249 (shoulder); UV λ_{max} in 0.1% TFA/methanol (nm) 298 (log $\epsilon = 3.50$), 340 (log $\epsilon = 2.71$); IR ν_{max} in KBr (cm^{-1}) 2839, 2646, 1653, 1617, 1300, 1222, 826, 785, 764; EI-MS (*m/z*) 153 (M^+), 135, 107, 79; ^{1}H-NMR (in DMSO-*d*) δ 6.37 (1H, dd, $J = 7.7$, 8.0 Hz, H-5), 6.81 (1H, dd, $J = 7.7$, 1.3, H-4), 7.21 (1H, dd, $J = 8.0$, 1.3, H-6); ^{13}C-NMR (in DMSO-*d*) δ 169.7 (C=O), 144.4 (C3), 141.1 (C2), 121.3 (C6), 116.6 (C4), 113.9 (C5), 109.9 (C1). From these analytical data and considering two-dimensional NMR spectra, HMQC, and HMBC (data not shown), HAA was identified as 3-hydroxyanthranilic acid (Figure 3), which was confirmed by direct comparison with an authentic sample.

Antioxidative Activities of Isolated Compound. The antioxidative effect of HAA on soybean oil was first studied by the weighing method. As shown in Figure 4, HAA had appreciable antioxidative activity in soybean oil system. Its activity increased proportionally with its concentration. The activity of HAA was stronger than that of α-tocopherol and BHT at the same concentration. Murakami et al. (1984) reported that the main isoflavones responsible for the antioxidative activ-

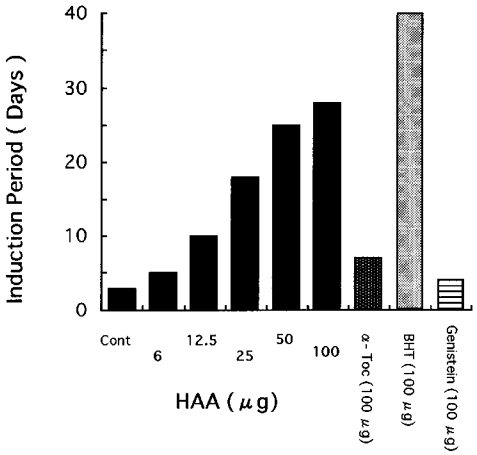

Figure 5. Antioxidative activities of HAA in a water/ethanol system with linoleic acid. Values in this figure are the average of duplicates.

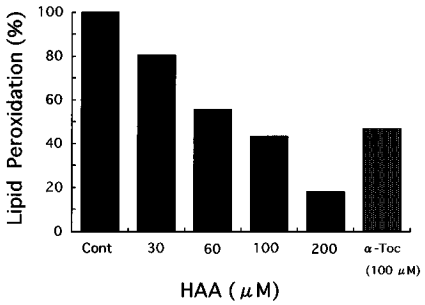

Figure 6. Antioxidative activities of HAA and α-tocopherol in the rabbit erythrocyte membrane ghost system. Details are given under Materials and Methods. Values in this figure are the average of duplicates.

ity in tempeh were genistein and daidzein, which are liberated from genistin and daidzin in soybeans by *β*-glucosidase from *R. oligosporus* during fermentation. These free isoflavones, in addition to 6,7,4′-trihydroxyisoflavone which was isolated from tempeh, are known to exert antioxidative activity in aqueous solution (Ikehata et al., 1968; Pratt and Birac, 1979). The main one of these isoflavones, genistein, was assayed in this system. As shown in Figure 4, genistein possessed very little antioxidative activity in the soybean oil system.

The antioxidative activity of HAA in a water/ethanol system with linoleic acid was measured according to the thiocyanate method. The results are shown in Figure 5. HAA also exhibited strong activity in the water/ethanol system. The extent of activity of HAA was stronger than that of α-tocopherol at the same concentration, although slightly weaker than that of BHT. The antioxidative activity of genistein was very low compared with that of HAA.

Most biomembranes in our bodies are subjected to lipid peroxidation because of the presence of high concentrations of polyunsaturated fatty acids. The antioxidative activity of HAA in a biological system *in vitro* was evaluated using the red blood cell membrane of rabbit. The antioxidative effect of HAA on erythrocyte membrane was observed to be as strong as that of α-tocopherol at the same concentration as shown in Figure 6.

Antioxidative Effect of HAA on Soybean Powder. The lyophilized tempeh powder has proven to be more stable against lipid peroxidation than unfermented soybean powder (Ikehata et al., 1968; Esaki et al., 1994). This result indicates that antioxidative compounds can

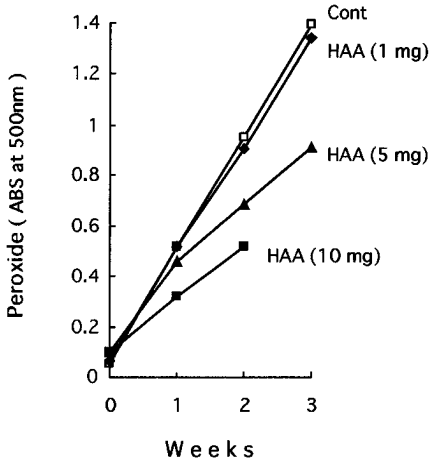

Figure 7. Antioxidative effect of HAA on soybean (BS) powder. HAA (1, 5, and 10 mg) was added to 1 g of BS powder. A control contained no additive. After incubation at 40 °C, lipid peroxidation was determined by the thiocyanate method (500 nm). This figure is a representative result of duplicates.

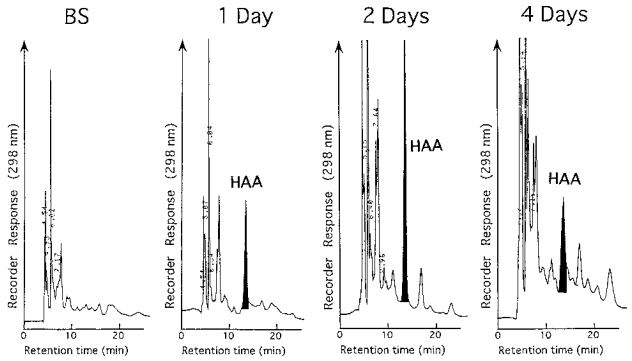

Figure 8. HPLC chromatograms of the 0.1% TFA/50% methanol extracts from BS and different tempehs fermented for 1, 2, and 4 days. HAA was identified from retention time and UV spectrum by three-dimensional HPLC. HPLC conditions are given under Materials and Methods. This figure is a representative result of three replicates.

be produced by fermentation. The 6,7,4′-trihydroxyisoflavone isolated from tempeh was not effective in preventing autoxidation of soybean powder (Ikehata et al., 1968). HAA was added to the lyophilized BS powder, and the antioxidative effect of HAA on soybean powder was determined. As shown in Figure 7, the soybean powder with 1 mg of HAA/g of BS powder showed a slight retardation of lipid peroxidation of the soybean oil. Definite retardation of peroxidation could be recognized by increasing the added amount of HAA. It was confirmed that the antioxidative effect of HAA was reproducible on repeated runs.

Comparison of Antioxidative Activity with HAA Content. From this background, HAA is presumed to be a potent antioxidant responsible for the autoxidative stability of dried tempeh powder. To confirm this reasoning, HAA content in tempeh with different fermentation periods was assayed and compared with the antioxidative activity of each sample. Preliminary experiments revealed that HAA was effectively extracted with 0.1% TFA/50% methanol. By using this solvent, HAA was extracted from the tempeh and determined by using HPLC. The chromatograms of BS and each tempeh are shown in Figure 8. Each peak of HAA was identified from the retention time, in addition to the UV spectrum obtained from three-dimensional HPLC. HAA was not detected in unfermented soybeans (BS). It was increased during the fermentation of

tempeh and reached a maximum content at the stage of 2 days, which had the strongest antioxidative activity among the tempehs with different fermentation periods (Figure 2). The amounts of HAA in tempehs that were fermented for 1, 2, and 4 days were 22.2, 50.2, and 33.5 mg/100 g of lyophilized powder, respectively. This antioxidant, HAA, was also produced by the fermentation of *R. oligosporus* IFO 32003. The amount of HAA was 44.1 mg/100 g of lyophilized powder at the stage of 2 days.

Conclusion. Tempeh, a traditional fermented soybean product of Indonesia, has been popularized throughout the world. Dried tempeh powder has been sometimes used as an antioxidant by covering freshly caught fish with it (György et al., 1974). György et al. (1964) reported tempeh to be very stable to rancidity development and identified 6,7,4′-trihydroxyisoflavone as an antioxidant from tempeh. However, this isoflavone did not show any protective effect when it was added to soybean oil and soybean powder (Ikehata et al., 1968).

In this study, we isolated a new potent antioxidant (HAA) from tempeh. It was identified as 3-hydroxyanthranilic acid and possessed an antioxidative effect on both soybean oil and soybean powder. HAA was increased during *Rhizopus* fermentation and reached a maximum content (ca. 50 mg/100 g of dried matter) after 2 days of fermentation, which showed the strongest antioxidative activity. Genistein, which is released during *Rhizopus* fermentation and exerts antioxidative activity in lipid/aqueous solution, possessed very little antioxidative effect on soybean oil. From these results, it is concluded that HAA is the principal antioxidant responsible for the autoxidative stability of dried tempeh.

ACKNOWLEDGMENT

We thank Ichibiki Co. Ltd. in Aichi Prefecture for generously providing the dehulled soybeans and T. Oya, K. Hasegawa, N. Hayashi, and H. Akai for technical assistance.

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Received for review July 18, 1995. Revised manuscript received December 11, 1995. Accepted December 31, 1995.[⊗]

JF950454T

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1996.