Antioxidative Stability of Tempeh and Liberation of Isoflavones by Fermentation

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Dried tempeh is known to be remarkably stable to lipid oxidation. An isoflavone has been isolated and is considered to be one of the antioxidants in tempeh. However, the true origin of the antioxidative activity of tempeh is still obscure. In this paper, isoflavones and their glucosides in tempeh were analyzed by HPLC, and the liberation of isoflavones from glucosides occurred during fermentation was made clear. The main isoflavones responsible for the antioxidative activity in tempeh were deduced to be daidzein and genistein.

Tempeh is a fermented soybean food, which is indigenous to Indonesia. Dried tempeh is known to be remarkably stable to lipid oxidation compared with unfermented soybeans. A powerful antioxidant has been isolated from tempeh and has been recognized as 6,7,4'-trihydroxyisoflavone.1,2) This compound has been proven to be one of the most active antioxidants among natural flavonoids in aqueous suspension of linoleic acid. However, this new trihydroxyisoflavone did not prevent autoxidation of soybean oil and soybean meal when the synthesized compound was added.3) It showed little hemolysis-preventing activity in rats fed a vitamin E-deficient diet in vivo. On the other hand, extracted tempeh oil showed effective antioxidative activity when added to refined oils.4) The true cause of the antioxidative stability of tempeh is still obscure.

The stability of tempeh to autoxidation increased during fermentation.5) Generally, antioxidative activity is shown by phenols or amines. Tocopherols and isoflavonoids are the main phenols found in soybeans. Among these compounds, tocopherols might not be modified by the fermentation process, but lipophilic aglycone of isoflavonoids is liberated by β-glucosidase during fermentation.5) Saponins show antioxidative activity,6) but it is not clear that sapogenins are liberated during fermentation. In this paper, our attention is focused only on isoflavones.

Soybeans are known to contain several isoflavones and their glucosides.7,8) These compounds were proved to have antioxidative activity.9) The quantitative determination of soybean isoflavones has been performed using gas liquid chromatography (GLC)10) and high performance liquid chromatography (HPLC).11–16) Determination of isoflavones in soybean meal, protein concentrates and protein isolates has also been reported. In this study, isoflavones in tempeh were analyzed by HPLC and identified with the aid of gas chromatograph-mass spectrometry (GC/MS). The antioxidative stability of tempeh oil is discussed in relation to the appearance of isoflavones.

MATERIALS AND METHODS

Materials. Tempeh (fermented for 40 hr with Rhizopus oligosporus) and soybeans (dehusked and steamed) were obtained from Marusanai Co. (Okazaki). A part of these samples was freeze-dried for stability tests and another part was dried by blowing warmed air at 50°C through it for extraction of isoflavones. Both dried samples were powdered by a coffee mill. Heated and dried meal was defatted with hexane. Well-fermented and spray-dried
tempeh meal was provided by Marusanai Co. This preparation was also defatted with hexane. \( n \)-Butyrophenone and TMS-HT (hexamethyldisilazane and trimethylchlorosilane) were purchased from Tokyo Kasei Co.

**Incubation of tempeh and soybean meals.** Freeze-dried tempeh and soybean meals (100 g) were placed separately in 18 cm diameter culture dishes and were incubated at 37°C in the dark. Lipids were extracted from 10 g of the samples with ethyl ether every two weeks. The yield of lipids was about 2.1 g. The PV of the extracted lipids were determined.

**HPLC of isoflavones.** Isoflavones and their glucosides were extracted twice from soybean and tempeh meal (3 g) with 80% methanol (30 ml). The extracts were dried by vacuum distillation. The residue was dissolved in 5 ml of 80% methanol and was placed in a refrigerator. The white precipitate was filtered off, and the clear filtrate was made up to 10 ml with 80% methanol.

HPLC was carried out with a Shimadzu LC-4A, equipped with a stainless column (6 x 150 mm) of YMC-Pack A-312 containing a precolumn (4 x 10 mm). A UV Shimadzu SPD-2A variable wave length UV detector was used to monitor the eluent at 262 nm. The solvent flow rate was 1 ml/min. Separations were carried out with a linear gradient of methanol in water from 20 to 60% as shown in Fig. 2. \( n \)-Butyrophenone was added as the internal standard.

**RESULTS**

**Stability of tempeh oil**
Tempeh and soybean meals were incubated to test the oxidative stability of their oils. As shown in Fig. 1, oils in tempeh were very stable to oxidation.

**Determination of isoflavones**
Eighty % methanol extracts of soybean and tempeh were analyzed with HPLC. The isoflavone glucosides having fewer hydroxyl groups are eluted faster than the glucosides having more hydroxyl groups. And isoflavone glucosides are eluted faster than free isoflavones. The results are shown in Figs. 2, 3 and 4. Soybean extract (Fig. 2) contained large amounts of isoflavone glucosides, but tempeh extract (Fig. 3) showed a decrease of glucosides and an increase of isoflavones. Glucosides a, b and c and aglycones a', b' and c' were able to be assigned to daidzin, glycitein-7-O-glucoside and genistin and daidzein, glycine and genistein on the basis of several references and of the following GC/MS experiments. Well fermented tempeh (Fig. 4) contained no isoflavone glucosides but large amounts of isoflavones.

**GC/MS of isoflavones**
Isolavone fractions were analyzed by GC/MS. From derivatization procedures, two peaks, A and B, were found to correspond to trimethylsilylated derivatives of isoflavones. The mass spectrum A (Fig. 5) showed fragment ions at m/z 398 (M⁺) and 383 (M⁺-CH₃). From the spectrum, this compound was identified as daidzin, glycitein-7-O-glucoside and genistin and daidzein, glycine and genistein on the basis of several references and of the following GC/MS experiments. Well fermented tempeh (Fig. 4) contained no isoflavone glucosides but large amounts of isoflavones.

**FIG. 1.** Changes of Peroxide Value of the Oils Extracted from Tempeh and Soybean Meals.
Isoflavones in Tempeh

Fig. 2. HPLC Elution Diagram of an Aqueous Methanolic Extract (80%) of Defatted Soybean Meal. The solution, 200 μl, was injected onto the column. Internal standard, 20 μg, was added to the sample. The amount of isoflavones and their glucosides are estimated from their UV absorptions to be roughly 30 and 320 mg in 100 g defatted soybean meal. a, daidzine; b, glycitein-7-O-glucoside; c, genistin; a', daidzein; b', glycitein; c', genistein; s, internal standard.

Fig. 3. HPLC Elution Diagram of an Aqueous Methanolic Extract (80%) of Defatted Tempeh Meal. The amount of isoflavones and their glucosides are estimated to be roughly 130 and 220 mg in 100 g defatted tempeh meal.

Fig. 4. HPLC Elution Diagram of an Aqueous Methanolic Extract of Defatted Well-fermented Tempeh Meal.
boring hydroxyl groups may be difficult due to steric hindrance except a trace amount is completely silylated. Therefore, this compound is considered to contain three TMS groups, but one of them is not appreciably trimethylsilylated by TMS-HT. Trimethylsilylation with N,O-bis-(TMS)-acetamide and TMS-imidazole also showed the difficulty of complete silylation of three OH groups. From these considerations, this compound can be attributed to genistein.

DISCUSSION

Tempeh oil has been reported to be stable for oxidation. The sample used in this experiment was also quite stable for oxidation as shown in Fig. 1. This phenomenon was considered to depend on the existence of antioxidants. One of them has been isolated and identified as 6,7,4'-trihydroxy isoflavone.1,2 Murata et al.3,5 considered that the antioxidative stability of tempeh oil depends on the liberation of lipophilic aglycons from isoflavone glucosides which exist originally in soybeans and proved the appearance of β-glucosidase in tempeh during fermentation.

To clarify this phenomenon, isoflavones and their glucosides in tempeh and nontreated soybeans were analyzed by HPLC in this paper. There are several studies11–16 on HPLC analysis of isoflavones and their glucosides. Use of 80% methanol seems to be recommended for the extraction.16 As shown in Figs. 2 and 3, soybean contained large amounts of isoflavone glucosides but tempeh lost them and isoflavones increased significantly. In the case of well fermented tempeh (Fig. 4), almost all glucosides disappeared and changed to aglycones. Three components involving daidzein and genistein were found to be major aglycones resulting from the fermentation process of tempeh.

Our HPLC data of tempeh extract support the explanation by Murata3 that the stability of tempeh to oxidation is generated by the liberation of lipophilic isoflavones from glucosides by β-glucosidase. However, the main components responsible for the stability may be genistein and daidzein. These isoflavones are known to possess antioxidative activity.4

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