SHORT COMMUNICATION

Structure of an Antioxidant from Fermented Soybeans (Tempeh)

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ABSTRACT: In an exhaustive investigation of the antioxidative properties of tempeh constituents, the substance at R_f 0.58 (cyclohexane/ethyl ether, 9:1) was isolated and purified. Until now, only the ultraviolet and fluorescence data of the substance were known, and the presence of an OH group was indicated. In the present paper, the structure of the substance at R_f 0.58 was elucidated by the application of spectroscopic methods and found to be 5-(δ-tocopheroxy)-δ-tocopherol. That previous researchers did not confirm antioxidant activity in tempeh oil has been related to the way the tempeh oil was prepared. Previous suggestions regarding the substance at R_f 0.58 as being one of the main tempeh antioxidants could not be proved. The antioxidative effect of tempeh oil seems to be the result of a synergistic effect of tocopherols (present in the soybeans) and amino acids (liberated during the fermentation process with *Rhizopus oligosporus*).
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KEY WORDS: Antioxidants, soybeans (fermented), 5-(δ-tocopheroxy)-δ-tocopherol.

Tempeh is an indigenous food in southeast Asia, especially Indonesia. Owing to its nutritive qualities and sensory acceptability, it is gradually becoming popular in the West. Tempeh has several beneficial characteristics, antioxidative activity being the most important of them. For more than 10 yr, we have been working on the isolation and structural elucidation of tempeh constituents and investigating their physiological activities. The antioxidative property of tempeh is caused by the synergistic effect of some of the tempeh constituents on the tocopherols present. One of the antioxidants present in tempeh is the substance R_f 0.58 (cyclohexane/ethyl ether, 9:1), which was isolated by Fukuyama *et al.* (1). This paper describes the isolation, purification, and elucidation of the structure of this substance and suggests reasons for deviation in the results of Stahl and Sims (2) and of Murata (3) concerning the antioxidative property of tempeh.

MATERIALS AND METHODS

All organic solvents used were of analytical or higher grade and were obtained from Merck (Darmstadt, Germany). Silica Gel 60 (mesh 15–40 nm) and florisil (mesh 150–250 nm) for column chromatography, and thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) plates Si 60 were from Merck. The freeze-dryer was from Edwards (Crawley, England). Ultraviolet (UV) spectra were measured with a UV-visible recording spectrophotometer (model UV-160; Shimadzu, Kyoto, Japan). Samples were measured in *n*-hexane. Infrared (IR) spectra were measured with a Perkin Elmer 157 G grating IR spectrophotometer (Beaconsfield, England). Fluorescence spectra were recorded on a Perkin Elmer MPF 4 fluorescence spectrophotometer.

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Bruker AMX 500 (Karlsruhe, Germany). Mass spectra were measured on a VG Analytical ZAB HF mass spectrometer (Manchester, England) with inverse geometry, in which a fast atom bombardment (FAB) gun is implanted.

Thin-layer chromatography (TLC). Analytical TLC was performed in a Desaga horizontal TLC chamber 5×5 cm with cyclohexane/ethyl ether, 9:1 (vol/vol). Antioxidative substances were detected with Emmerie-Engel reagent (4) (0.25 g 2,2′-bipyridyl and 0.1 g $FeCl_3$ in 100 mL absolute ethanol).

Preparative TLC was performed on 0.25-mm TLC plates (20×20 cm) on which the mixture was spotted in a line. Chloroform was used as the solvent for developing the thin-layer chromatogram and for extracting the scraped-off silica gel.

Preparation of tempeh and tempeh extract. Canadian soybeans (*Glycine max* L. Merr) were cooked for 10 min, soaked overnight in lactic acid solution at pH 5.5, dehulled manually, cooked again for 45 min, cooled, and dried with an aspirator for 30 min. The prepared soybeans (433 g) were inoculated with 0.25% of an inoculum, containing *Rhizopus oligosporus,* from Koperasi Bina Kimia-Lipi (Bandung, Indonesia), and fermented for 30 h at 32°C. The tempeh (415 g) was cut into slices, frozen at –24°C, and then lyophilized at room temperature. The dried tempeh (184 g) was extracted three times with *n*-hexane/ethanol, 2:1 (vol/vol), with an Ultra Turrax (IKA, Staufeni. Br., Germany) for 5 min. The slurry was filtered through a Buchner funnel. The eluant was evaporated under reduced pressure at 35°C, and 46 g of crude tempeh extract was obtained. The details of the isolation and purification of the substance at R_f 0.58 are described later.

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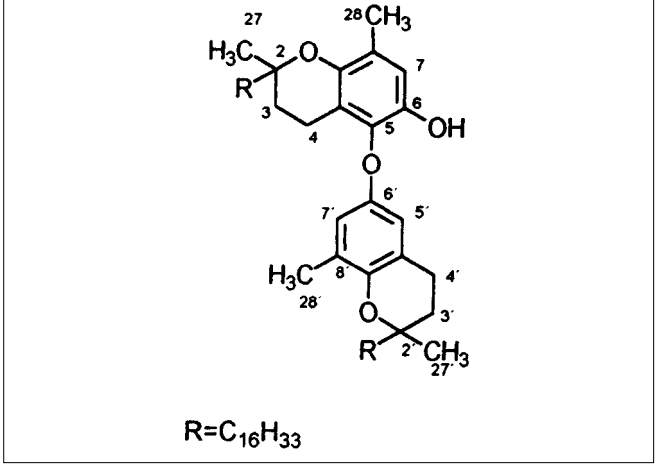
Synthesis of the dimer of δ-tocopherol (5). δ-Tocopherol (50 mg) was dissolved in 2.5 mL petroleum (b.p. 60–80°C). To this mixture was added 1 mL of a 0.2 N sodium hydroxide solution that contained 0.1 g $K_3Fe(CN)_6$, and the reaction mixture was stirred at 45°C for 45 min. The aqueous phase was extracted three times with petroleum. The combined petroleum phases were washed five times with water, dried over sodium sulfate, and evaporated at 20°C under reduced pressure. The reaction mixture was separated with preparative TLC as just described.

RESULTS AND DISCUSSION

The crude tempeh extract was dissolved in 200 mL chloroform, and 80 g of Silica Gel Si 60 (mesh 15–40 nm) was added. The solvent was removed under reduced pressure at 40°C. The silica gel–extract mixture was placed in a silica gel column (length 40 cm, width 4 cm), filled with cyclohexane, and eluted with cyclohexane; cyclohexane/chloroform, 100:5, 10:1, 10:3, 1:1 (200 mL each); and chloroform (1 L). Fifty-milliter fractions were collected. The substance with the R_f value of 0.58 on TLC in cyclohexane/ethyl ether, 9:1 (vol/vol) was found in fractions 16–24. Combination of these fractions yielded 5 g of residue after evaporation under reduced pressure at 40°C. This residue was chromatographed on a florisil column (length 40 cm, width 4 cm) with *n*-hexane, and 2 mL fractions were collected. The substance with R_f of 0.58 was obtained, together with other substances, in fractions 32–55. After evaporation of these combined fractions under reduced pressure at 40°C, a residue of 50 mg was obtained. This residue was separated on preparative TLC plates with chloroform as solvent. After localization of the substance R_f 0.58 with the help of Emmerie-Engel reagent (4), the silica gel was scraped from the plate and eluted with chloroform. The pure substance (1.2 mg) was obtained, which was analyzed by UV, fluorescence, and IR spectroscopy. The isolated substance had the same properties as reported earlier (1).

Afterward, the substance was analyzed by FAB mass spectroscopy, and peaks were detected at *m/z* 802, 577, and 402. The mass peaks 802 and 402 suggest that the isolated product is a dimer of δ-tocopherol.

By NMR spectroscopy (^{1}H and CH correlation), the positions of the protons could be assigned as follows: ^{1}H NMR δ (ppm): δ 2.15 + 2.12 (2 *s*, 28H + 28′H), 2.49 + 2.63 (*m*, 4H + 4′H), 4.85 (*s*, ArOH), 6.35 (*d, J* = 3 Hz, 5′, Ar-H), 6.55 (*d*, *J* = 3 Hz, 7′ Ar-H), 6.71 (*s*, 7 Ar-H). ^{13}C NMR δ (ppm): 15.9; 16.3 (28,28′ $ArCH_3$), 17.8–40.4 (3,11–27, 3′,11′–27′ CH,CH_2,CH_3) 17.8; 21.1 (4,4′ Ar-CH_2), 75.4; 75.8 (2,2′ C), 111.8 (5′ Ar-H), 115.1 (7′ Ar-H), 115.7 (7, Ar-H). CH correlation δ^1H/δ^{13}C:2.12, 2.15/15.9, 16.3; 0.9–1.8/17.8–40.3; 2.49, 2.63/17.8, 21.1; 6.35/111.8; 6.55/115.1; 6.71/115.7. Furthermore, the identity of the substance was established by cochromatography with the synthesized dimer (5) of δ-tocopherol and by comparison of their FAB mass spectra. The structure of 5-(δ-tocopheroxy)-δ-tocopherol is shown in Scheme 1.



SCHEME 1

The antioxidative ability of the tocopherol dimer was analyzed by the weight gain method (6), and a prolongation of the lag phase of about 80% over that of the monomer, δ-tocopherol, was observed when used in the same molar concentrations (0.25×10^{-6} mol/g lard). According to this result, the R_f 0.58 substance did not seem to be the strong antioxidative inhibitor of tempeh reported earlier (7,8). Further studies on the antioxidants of tempeh carried out in our laboratory showed that the antioxidative effect of tempeh is a result of synergistic effects (9) of the tocopherols present in the soybeans with amino acids that are liberated during fermentation with the *R. oligosporus.*

Stahl and Sims (2) used the oxygen uptake method to measure the antioxidative effect of tempeh oil and found a prooxidative effect. They suggested that the antioxidative effect of tempeh oil reported by other groups (7,8) was found only because they used the peroxide value method. A favorable method, in which free fatty acid peroxides decompose quickly to aldehydes, was not included in the test. Because of the lipase activity of *R. oligosporus,* the free fatty acid level was higher in tempeh than in soybeans. Possibly, the prooxidant effect of tempeh oil was a result of the following two causes. First, they used a Soxhlet extractor with *n*-hexane for extracting the tempeh. Although the time for the extraction period was not mentioned, it generally takes several hours to extract in a Soxhlet apparatus. The method cited for extracting tempeh with *n*-hexane was that of Murakami *et al.* (7), who did not mention the use of a Soxhlet nor the temperature and time of extraction. They only wanted to remove the oil and fat so that they could extract the residue with a polar solvent to obtain the isoflavones. Because the boiling point of *n*-hexane is 68°C, some lipid peroxides may have been formed during the extraction period, which catalyzed further oxidation of the oil. Second, most of the polar synergists of tocopherol were not extracted with *n*-hexane. If Stahl and Sims had used a more polar extraction medium, such as *n*-hexane/ethanol, 2:1 (8) or, even better, methanol (9), and extracted the tempeh by using an Ultra Turrax at room tem-

perature, they also might have observed the antioxidative effect of tempeh extract, regardless of the method of estimation used.

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