Effect of Indonesian Fermented Soybean Tempeh on Iron Bioavailability and Lipid Peroxidation in Anemic Rats

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The effects of an Indonesian non-salted fermented soybean product, tempeh, on iron bioavailability and lipid peroxidation are suspected to contribute to iron deficiency. Three groups of iron-deficient Wistar-strain rats were fed a diet of casein (C), unfermented soybean (U), or tempeh (T) for 11 days. Liver iron concentration and superoxide dismutase (SOD) activity in rats fed the T diet was significantly higher than in rats fed the U diet. The concentration of liver thiobarbituric acid-reactive substances (TBARS) was significantly higher in rats fed the U diet than in rats fed the C diet, but did not significantly differ between rats fed the C and T diet. The results of this study confirmed that the fermented soybean tempeh increased liver iron, compared with unfermented soybean, without promoting lipid peroxidation in iron-deficient anemic rats.

Keywords: *Iron deficiency anemia; lipid peroxidation; soybean; tempeh*

INTRODUCTION

Nutritional anemia caused mainly by iron deficiency is one of the most important nutritional problems in developing countries as well as in affluent societies. It is particularly prevalent among infants, young children, and pregnant and lactating women (Stephenson, 1995). Although some plant foods, especially soybeans, are potentially rich sources of iron in the diet, this iron is poorly absorbed due to the high fiber content. Another factor commonly suspected to depress the availability of iron in cereal grains and legumes is phytate (Jaffe, 1981; Plat and Clydesdale, 1984). Oral iron supplementation programs are the most common public health intervention against iron deficiency and have been underway in nearly all countries for decades (Stephenson, 1995).

Lipid peroxidation is promoted when free iron is increased excessively in the liver. This toxicity may be due to the formation of the extremely reactive hydroxyl radical, following the interaction of iron with hydrogen peroxide (Halliwell and Gutteridge, 1986). However, many antioxidant enzymes prevent lipid peroxidation in the body. Superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide (Donnelly, 1989), while glutathione peroxidase (GSHPx) and catalase convert hydrogen peroxide to water (Halliwell and Gutteridge, 1986).

Tempeh is a non-salted fermented soybean product originally developed in Central Java, Indonesia. A white cottony mycelia of *Rhizopus oligosporus* or *Rhizopus oryzae* is produced on the surface of the fermented soybean cake. Tempeh has been known to reduce the level of phytic acid (Sudarmadji and Markakis, 1977; Astuti, 1994). In addition, β, γ, and δ tocopherol (vitamin E) concentrations are increased during fermentation (Astuti, 1994). Although α tocopherol is considered the main source of free radical scavenging activity, γ tocopherol prevents lipid peroxidation longer than α tocopherol does *in vitro* (Gloor et al., 1966). Tempeh also contains isoflavone, which is a factor contributing to the high antioxidative activity *in vivo* (Ikehara et al., 1968).

The aim of the present study was to examine the effect of the non-salted fermented soybean product tempeh on iron bioavailability and its prevention of lipid peroxidation in iron-deficient anemic rats. Hemoglobin, serum iron, and iron concentration in liver were measured, as were TBARS as an index of lipid peroxidation, and the activity of antioxidant enzymes.

MATERIALS AND METHODS

Preparation of Fermented Soybean Tempeh. Tempeh and unfermented soybean were sent from Gadjah Mada University, Yogyakarta, Indonesia. Tempeh was prepared by the method of Astuti et al. (1994). Wilis variety soybeans grown in Yogyakarta, Indonesia, were washed and boiled in distilled, deionized water (DD water) for 30 min. The soybeans were then soaked for 24 h at room temperature, at a ratio of 3 parts DD water to 1 part soybeans. Following soaking, the DD water was discarded and the beans were dehulled by hand and then soaked in DD water for 24 h at room temperature. Again the DD water was discarded and the soybeans were boiled for 60 min. The excess DD water following boiling was drained off. As soon as the temperature reached 30 °C the cooked beans were inoculated with tempeh inoculum powder (NRRL2710, originally supplied by the Northern Regional Research Lab., Peoria, IL) at 0.2 g/100 g of dried soybeans. The inoculated soybeans were packed in Petri dishes and incubated at 30 °C for 48 h. The fermented soybean tempeh was steamed for 5 min, and then minced, freeze-dried for 48 h, ground, and filtered through a 35 mesh sieve. Unfermented soybeans were also prepared from Wilis variety soybeans grown in Yogyakarta. They were steamed for 5 min, and then minced, freeze-dried for 48 h, ground, and filtered through a 35 mesh sieve, as for the tempeh preparation.

General Treatment of Animals. The study was approved by the Tokyo University of Agriculture Animal Use Committee,

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Table 1. Composition of Experimental Diets, in Grams per 100 g of Diet

ingredient	casein	unfermented soybean	tempeh
casein	22	–	–
unfermented soybean	–	35[a]	–
tempeh	–	–	35[b]
corn starch[c]	29	25	24
soybean oil[d]	8	–	1
sucrose	30	30	30
cellulose	5	4	4
mineral mixture[e]	2	2	2
vitamin mixture[f]	4	4	4
iron content (ppm)	54	33	34

[a] 35 g as unfermented soybean powder was added in order to adjust the protein level to that of the casein diet. [b] 35 g of tempeh powder was added in order to adjust the protein level to that of the casein diet. [c] Starch was adjusted to the same level. [d] Soybean oil was adjusted to the same level. [e] Harper mineral mixture. [f] Takeda panvitan powder (Takeda Chemical Co., Tokyo, Japan).

and animals were maintained in accordance with the guidelines for the care and use of laboratory animals, Tokyo University of Agriculture. Male rats of the Wistar strain (CLEA Japan Co., Tokyo, Japan) were housed individually in suspended wire-bottom, stainless-steel cages and maintained at 25 ± 2 °C and 60% relative humidity in a room with a 12-h light/dark cycle (lights on between 0800 and 2000 h).

Hemoglobin Repletion Assay. The effect of the three types of diets on iron bioavailability in anemic rats was assessed using the hemoglobin (Hb) repletion assay (Forbes et al., 1989). Male 21 day-old rats were all made anemic by feeding them an iron-deficient diet (11 mg iron/kg diet) for 14 days. The compositions of the experimental diets are shown in Table 1. We used the following Harper mineral mixture (1959) (mg/kg diet): Ca, 5898; P, 3946; K, 4929; Na, 4927; Cl, 7609; Mg, 492; S, 666; Zn, 5; Cu, 22; I, 0.2; Mo, 0.1. Except for group C, iron was excluded from the mineral mixture. At the end of the iron depletion period, blood was obtained from the tail vein, and Hb and hematocrit were determined for each rat. Rats were divided into three groups ($n = 7$) on the basis of Hb and body weight. One group was fed the casein (C) diet, and the other groups were fed the unfermented soybean (U) diet or tempeh (T) diet. At the end of the iron repletion period, blood was taken from the tail vein and Hb, hematocrit, and unsaturated iron-binding capacity (UIBC) were analyzed. The liver was immediately removed, and perfused by cold 0.9% sodium chloride solution. The concentrations of iron in liver, serum, and diet were determined using the atomic absorption/flame emission spectrophotometer (Shimadzu, Co., Tokyo, Japan) (Uehara et al., 1988).

Analysis of Hematological Indices. Hb was measured using Cyanmethemoglobin methods (Beutler, 1975). Packed red cell volumes (hematocrit) were determined by centrifugation in a capillary tube system. UIBC was determined by use of a commercial diagnostic kit (Wako Pure Chemical Industries, Osaka, Japan) based on the reaction of free iron with ferritin. Hb-iron gain was calculated for each rat as the difference between Hb-iron at the end of the repletion period and that at the start of repletion. For calculating the initial and final Hb-iron concentration, blood was assumed to be 7.5% (Whittaker et al., 1984) of body weight and Hb was assumed to contain 3.55 mg/g (Zhang et al., 1985). The iron intake of each rat was calculated from food intake and the analyzed iron content of the diet. The hemoglobin regeneration efficiency (HRE) was calculated as the percentage of iron consumed that was retained in circulating Hb (Forbes et al., 1989).

Preparation of Hepatic Cytosol Fraction. Two grams of liver tissue was homogenized with 10 mL of 0.25 M sucrose. The homogenates were centrifuged at 9000*g* for 20 min, and then the supernatant solution recentrifuged at 105000*g* for 60 min. The supernatant was used for the cytosol fraction of the enzymatic assay (Uehara et al., 1988). The protein concentration was determined according to the method of Lowry et al. (1957), using bovine albumin as a standard.

Table 2. Final Body Weight, Food Intake, and Liver Weight in Rats Fed the Respective Test Diets[a]

diets	final body weight (g)	food intake (g/11 days)	food efficiency (g/100 g diet)	liver weight (g)
casein	184.5 (2.7)	188.0[a] (2.7)	38.5[b] (1.1)	9.13[a] (0.47)
unfermented soybean	181.3 (3.9)	201.5[b] (4.5)	36.3[a,b] (1.7)	11.03[b] (0.45)
tempeh	182.0 (2.1)	208.1[b] (4.7)	32.2[a] (1.3)	10.28[a,b] (0.45)

[a] Values are the mean where $n = 7$, with standard errors (SE) in parentheses. Values not sharing a common superscript letter were significantly different ($P < 0.05$) when analyzed by the Duncan new multiple range test. Average initial body weight, 110 g (range: 108−115 g).

Table 3. Hematological Indices, Iron Absorption, Liver Iron, and Serum Iron in Rats Fed the Respective Test Diets[a]

diet	hemoglobin (g/100 mL)	iron (%)		
		hematocrit	absorption	HRE[b]
casein	14.6[b] (0.5)	45.0[b] (0.6)	65 (3)	42.0 (0.9)
unfermented soybean	11.4[a] (0.3)	39.1[a] (1.5)	64 (3)	43.6 (3.3)
tempeh	12.1[a] (0.6)	38.5[a] (0.9)	63 (3)	41.2 (2.7)

diet	liver iron (μg/g tissue)	serum iron (μg/100 mL)	UIBC[c]
casein	435[c] (17)	492[b] (34)	368[a] (42)
unfermented soybean	127[a] (8)	326[a] (1)	708[b] (41)
tempeh	186[b] (12) ($n = 5$)	280[a] (10)	679[b] (46)

[a] Values are the mean ($n = 7$), with SE in parentheses. Values not sharing a common superscript letter were significantly different ($P < 0.05$), when analyzed by the Duncan new multiple range test. [b] HRE, hemoglobin regeneration efficiency. [c] UIBC, unsaturated iron binding capacity.

Table 4. Xanthine Oxidase Activity in the Cytosol Fraction in Liver of Rats Fed the Respective Test Diets[a]

diet	xanthine oxidase activity (μg/mg of protein)
casein	6.30[b] (0.87)
unfermented soybean	4.83[ab] (0.39)
tempeh	3.64[a] (0.47)

[a] Values are the mean ($n = 7$), with SE in parentheses. Values not sharing a common superscript letter were significantly different ($P < 0.05$) when analyzed by the Duncan new multiple range test.

Assays of Hepatic Enzyme Activity. Xanthine oxidase (XOD) activity in the cytosol fraction was determined by the method of Bergmeyer et al. (1974). Superoxide dismutase (SOD) in the cytosol fraction was evaluated using the nitrite method (Oyanagi, 1984). This method is based on the inhibition of nitrite formation from hydroxylamine in the presence of O^{2-} generators. One activity unit (NU) corresponds to the amount of SOD contained in a 3.0 mL assay volume that reduces nitrite formation. Glutathione peroxidase (GSHPx) activity in the cytosol fraction was assayed by the Jensen and Clausen (1981) method. A UV-240 spectrophotometer (Shimadzu, Co., Tokyo, Japan) set at 340 nm was employed in the analysis for reduction of NADPH. Catalase activity was assayed by the method of Aebi (1974). This method is based on the decomposition of hydrogen peroxide (H_2O_2) with catalase.

Assay of Thiobarbituric Acid-Reactive Substances in Liver. Levels of TBARS in the liver were measured according to the method of Uchiyama and Mihara (1978), and was calculated as the difference in optical density at 535 and 520 nm in the butanol layer after centrifugation of liver homogenate.

Statistical Analysis. Data were evaluated by ANOVA and subsequently by the Duncan new multiple range test using

Table 5. Antioxidant Enzyme Activities in Liver of Rats Fed the Respective Test Diets[a]

diet	SOD[b] (NU/mg of protein)	GSHPx[c] [nmol of NADPH oxidized/min/(mg of protein)]	catalase [μmol of H_2O_2 decomposed/min/(mg of protein)]
casein	350.2[c] (16.7)	375.5 (23.7)	389.3 (26.9)
unfermented soybean	80.5[a] (4.2)	346.8 (21.5)	322.2 (28.7)
tempeh	291.1[b] (18.9)	329.1 (23.5)	298.3 (45.7)

[a] Values are the mean ($n = 7$), with SE in parentheses. Values not sharing a common superscript letter were significantly different ($P < 0.05$), when analyzed by the Duncan new multiple range test. [b] SOD, superoxide dismutase. [c] GSHPx, glutathione peroxidase.

the statistical package software, Super ANOVA from Abacus Concepts Inc. (Berkeley, CA). The significance of relationships between data was established by linear regression analysis with StatView (Abacus Concepts Inc. Berkeley, CA). Values were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Tempeh on Iron Bioavailability. Table 2 shows final body weight, total food consumption, food efficiency, and liver weight in rats fed the respective test diets for 11 days. Final body weight did not significantly differ between the groups. Total food consumption was lower in rats fed the C diet than in rats fed the U and T diets. There were no significant differences in total food consumption, food efficiency, or liver weight between the rats fed the U and T diets. Table 3 shows the effects of the respective test diets on Hb, hematocrit, serum iron concentration, UIBC, iron concentration in the liver, apparent absorption of iron, and HRE in rats. There were no significant differences in iron absorption or HRE among the groups. Levels of Hb, hematocrit, serum iron and UIBC in rats fed the C diet were significantly higher than in those fed the U and the T diets. There were no significant differences in these parameters between the U and T diet groups. However, liver iron concentrations in rats fed the T diet were significantly higher than in rats fed the U diet but were significantly lower than in rats fed the C diet. Zinc levels in the livers of rats fed the respective C, U, and T test diets were 117.3, 65.8, and 70.7 μg/g liver. Although zinc levels in the livers of rats fed the C diet were significantly higher than in those of rats fed the other two test diets, there was no significant difference between the U and T diet groups. Table 4 shows XOD activity in the cytosol fraction in liver of rats fed the respective test diets. Activity in rats fed the T diet was significantly lower than in rats fed the C diet, but not in rats fed the U diet. The XOD activity of rats fed the U diet was about 1.3 times higher than that of rats fed the T diet.

There are two stages in iron deficiency indices anemia. One is expressed as depressed blood iron, especially Hb levels. The other stage is a moderate iron deficiency anemia. In the latter stage, the Hb in blood has not yet been depressed, whereas ferritin in the liver is decreased (Hunter, 1978). We focused on moderately anemic rats in this study. Indeed, the Hb level after the depletion period was about 9.0 g/100 mL, which was slightly higher than values obtained in other studies (Moeljopawiro et al., 1987; Kim and Atallah, 1992; Margareth and Sgardieri, 1992); one possible reason for this was that there were no significant differences in hematological indices and iron absorption between the U and T diets. On the other hand, Topham et al. (1982) reported that XOD activity is induced in the livers of rats fed an iron-deficient diet. In this regard, we might suggest that iron in the liver of rats fed the U diet was used to maintain the Hb level in the serum before being stored in the form of ferritin.

Table 6. TBARS in Liver of Rats Fed the Respective Test Diets[a]

diet	nmol of TBARS/g of liver	nmol of TBARS/mg of protein
casein	111.5[a] (9.1)	531.7[a] (55.2)
unfermented soybean	227.1[b] (33.0)	1178.7[b] (165.8)
tempeh	163.8[ab] (50.3)	811.5[ab] (285.7)

[a] Values are the mean ($n = 7$), with SE in parentheses. Values not sharing a common superscript letter were significantly different ($P < 0.05$), when analyzed by the Duncan new multiple range test.

Effect of Tempeh on Lipid Peroxidation. The activities of SOD, GSHPx, and catalase in the liver of rats fed the respective test diets are shown in Table 5. Although GSHPx and catalase activities did not significantly differ among the groups, SOD activity in rats fed the T diet was about 3.6 times higher than that in rats fed the U diet, and significantly lower than in rats fed the C diet. There was a positive correlation between the zinc level and SOD activity in the liver ($Y = 1.9X + 78.2$, $R = 0.5029$, $P = 0.0201$). Table 6 shows the effect of the respective test diets on TBARS in liver. TBARS concentration was significantly greater in rats fed the U diet than in rats fed the C diet. There was no significant difference between rats fed the T and C diets. Although TBARS concentration of liver in rats fed the T diet did not differ significantly from that in rats fed the U diet, the level in rats fed the T diet was reduced by about 72% compared with that in rats fed the U diet. There was a negative correlation between SOD activity and TBARS ($Y = -0.45X + 275.8$, $R = 0.555$, $P = 0.0088$).

Tempeh contains isoflavone, which is a factor contributing to high antioxidative activity *in vivo* (Ikehara et al., 1968). In addition, β, γ, and δ tocopherols (vitamin E) were increased during fermentation (Astuti, 1994). Although α tocopherol is considered the main source of free radical scavenging activity, γ tocopherol prevents lipid peroxidation longer than α tocopherol does *in vitro* (Gloor et al., 1966). Our results suggest that SOD activity is one cause of the antioxidative effect of fermented soybean Tempeh.

In conclusion, the results indicate that the fermented soybean tempeh increased liver iron, compared with unfermented soybean, without promoting lipid peroxidation in iron-deficient anemic rat.

ABBREVIATIONS USED

GSHPx, glutathione peroxidase; Hb, hemoglobin; HRE, hemoglobin regeneration efficiency; TBARS, thiobarbituric acid-reactive substances; UIBC, unsaturated iron binding capacity; XOD, xanthine oxidase.

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