Inhibition of Cholesterol Autoxidation by the Nonsaponifiable Fraction in Rice Bran in an Aqueous Model System

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ABSTRACT: The inhibition of cholesterol autoxidation by nonsaponifiable fraction from rice bran oil (700, 1400, and 2100 ppm) was studied in an aqueous model system for 16 h at pH 5.5 and 80°C. Antioxidant effectiveness was investigated by following the loss of cholesterol and the formation of 7-ketocholesterol. The changes in levels of vitamin E vitamers and γ-oryzanol in the system were determined during cholesterol autoxidation. The 2100 ppm treatment produced a 92% reduction of 7-ketocholesterol, 1400 ppm an 82% reductions and 700 ppm a 64% reduction after 16 h, whereas without the nonsaponifiable fraction, the samples showed almost complete degradation of cholesterol. Vitamin E vitamers decreased ($P < 0.05$) in all treatments, but γ-oryzanol was not significantly ($P > 0.05$) reduced in the 2100 ppm treatment.

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KEY WORDS: Cholesterol autoxidation, 7-ketocholesterol, nonsaponifiable fraction, γ-oryzanol, vitamin E vitamers.

Crystalline cholesterol and aqueous cholesterol dispersions readily undergo oxidation at relatively mild temperatures when exposed to air, with the oxidation reactions producing a variety of products (1–6). Some cholesterol oxidation products (COP) have been shown to be cytotoxic, atherogenic, mutagenic, and carcinogenic when ingested by laboratory animals (1–6). These COP are formed in foods containing cholesterol during the cooking process or during prolonged storage (1). Eight common autoxidation products of cholesterol have been identified: 25-hydroxycholesterol, cholestanetriol, 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, the α- and β-epoxides of cholesterol, and cholesta-3,5-dien-7-one, the last compound being an artifact derived from 7-ketocholesterol (1–6). The 7-ketone derivative of cholesterol, 7-ketocholesterol, is a COP found at high concentration in certain foods like muscle tissue (1).

Addis (7) suggested that, since there appears to be no practical method to lower the cholesterol in certain foods like muscle tissue, research should be directed toward protecting against cholesterol oxidation in food. Thus, techniques that lower the cholesterol oxide content of food products should be considered. There would be two practical approaches to accomplish this objective in meat products. The first would be to increase the levels of endogenous antioxidants in muscle cell membranes by dietary supplementation of antioxidant compounds, primarily vitamin E. The higher levels of vitamin E in the muscle would theoretically reduce the susceptibility of cholesterol to oxidation. Vitamin E supplementation of animal diets has been shown to be effective in reducing cholesterol oxide levels in cooked pork (8), raw and cooked veal (9), and vacuum-packaged cooked beef steaks (10). However, dietary supplementation with vitamin E or other antioxidant compounds incurs greater feed costs, which may not be acceptable to animal producers. A widely practiced alternative would be to add antioxidant compounds directly to meat products, although concern for negative consumer attitudes toward food additives has modulated this practice. Our concept has been to combine food products that may have complementary nutritional and functional properties, such as beef and rice bran (11).

Rice bran oil contains potentially important cholesterol oxidation-lowering agents in the nonsaponifiable fraction (4.2% of oil by weight) that includes tocopherols (T), tocotrienols (T3) (0.08%), and especially γ-oryzanol (1.6%) (12). γ-Oryzanol, hereafter referred to as oryzanol, was initially thought to be a single compound but is now known to be a mixture of ferulate esters with sterols and triterpene alcohols, predominantly campesterol, 24-methylenecycloartanol, and cycloartenol (13–16). Compared with other cereals, rice bran has a high level (3500 mg/kg bran) of oryzanol (13).

Currently, in the food industry there is a trend to use natural antioxidant compounds rather than synthetic antioxidants. Thus, inclusion of the purified nonsaponifiable fraction of rice bran oil into food products may be a compelling way to obtain natural antioxidants.

An emulsion of fatty acids and aqueous buffer was used as a relatively simple model system for studying lipid oxidation of muscle-based foods (5,17). Systems containing biological membranes such as red blood cells are quite complex, but micellar systems are relatively simple, uniform, and well understood, although heterogeneous (5). In addition, colloidally dispersed cholesterol may be a more suitable model of the state of cholesterol in foods and in the aqueous fluids of animal tissues (17). For this reason, the incorporation of natural antioxidants into a simple model system that represents the state of cholesterol in food and human tissue may have utility

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in studying the effectiveness of natural antioxidants in inhibiting cholesterol autoxidation. The objectives of this research were (i) to study the change of vitamin E vitamers and oryzanol in a cholesterol dispersion containing various levels of rice bran oil nonsaponifiable fraction, (ii) to study, quantitatively, the disappearance of cholesterol and the formation of 7-ketocholesterol in a model system, and finally (iii) to determine the efficacy of nonsaponifiable fraction of rice bran oil in suppressing the formation of 7-ketocholesterol during cholesterol autoxidation in a model system.

EXPERIMENTAL PROCEDURES

Purified nonsaponifiable component extraction. A solid-phase extraction (SPE) method reported by Shin and Godber (18) was adapted to extract a purified nonsaponifiable oil fraction directly from rice bran using C18-SPE tubes. Rice bran (500 mg) was placed in a 15-mL test tube with 5 mL of ethanol and 0.1 g of ascorbic acid. The test tube was incubated in an 80°C water bath for 10 min, and then 0.2 mL of 80% KOH (wt/vol in water) was added. The rice bran mixture was left for 15 min, and 2 mL of 15% acetic acid (vol/vol in water) was then added to the sample tube and the tube was centrifuged at 537 × *g* for 6 min. A 3-mL Supelclean LC-18 tube (Supelco, Inc., Bellefonte, PA) was washed with 2 mL of methanol followed by 2 mL of 1% acetic acid (vol/vol in water) prior to use. A reservoir (20 mL) was connected to the SPE tube. The supernatant in the test tube was transferred to the SPE reservoir. Ethanol and acetic acid were added to the test tube and mixed with a vortex mixer for 30 s. The test tube was centrifuged, and the supernatant was transferred to the reservoir as above. This procedure was repeated one more time. The extract was allowed to pass through the SPE tube at a rate of less than 2 mL/min using an SPE vacuum manifold. The solution eluted from the SPE tube was discarded. The tube was washed sequentially with 2 mL of water and 2 mL of 1% acetic acid (vol/vol in water) and methanol/water (50:50, vol/vol) to remove unwanted and unretained materials. The packing in the tube was dried thoroughly under high-purity nitrogen, and compounds of interest were recovered by eluting the SPE tube with 0.5 mL of a mixture of ethyl acetate and hexane (20:80, vol/vol) three times. We have obtained recovery values for SPE of 97, 99, 96, and 100% for α-, β-, γ-, and δ-tocopherol, respectively, 98, 99, and 100% for α-, γ-, and δ-tocotrienol, respectively, and 100% for oryzanol (19).

Cholesterol dispersion preparation. The dispersion system was prepared by using a modification of methods described by Rankin and Pike (2) and Maerker and Bunick (5). The buffer used for making the aqueous dispersion was 0.025 M histidine buffer prepared with deionized water. The buffer solution (312 mL) was heated to 80°C, and approximately 312 mg of sodium dodecyl sulfate (SDS), previously dissolved in absolute ethanol (5 mL), was added to the vigorously stirred solution. Cholesterol (500 ppm) (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol (5 mL) and the dried nonsaponifiable fraction at different concentrations (0, 700, 1400, and 2100 ppm) were added to the mixture of SDS and buffer solution. The pH was adjusted to 5.5 [the typical pH of meat products] (2) with 2 N HCl at room temperature (~24°C). The dispersion was incubated at 80°C to increase the rate of cholesterol autoxidation over time (0, 4, 8, 12, and 16 h). Additionally, 50 μL of copper(II) sulfate (0.005 M) was added to promote autoxidation. An aliquot of cholesterol dispersion (5 mL) was pipetted into a 15-mL test tube, which was then covered with a stopper, and the samples were incubated in a 80°C water bath. Concentrations of 7-ketocholesterol and cholesterol were determined to compare the degree of cholesterol oxidation over the 16-h period.

Sample extraction and preparation. A 5-mL aliquot of cholesterol dispersion was extracted three times with 10 mL of diethyl ether by liquid–liquid extraction and was washed with 5 mL of water twice to yield sterol compounds such as cholesterol and 7-ketocholesterol, vitamin E vitamers, and oryzanol. Extraction recovery for cholesterol and 7-ketocholesterol was quantitatively determined by the percentage recovery from a 5-mL aliquot of cholesterol dispersion in which the sample extraction and preparation procedure was performed after being spiked with known sterol standards (Sigma Chemical Co.) 20, 50, and 100 ppm. The extraction recovery for cholesterol was 76.4 ± 1.28, 80.26 ± 3.75, and 83.47 ± 1.95% for 20, 50, and 100 ppm spike, respectively, with a mean of 80% (n = 3). The extraction recovery for 7-ketocholesterol was 87.5 ± 1.5, 85.9 ± 2.7, and 88.9 ± 1.6% for 20, 50, and 100 ppm spike, respectively, with a mean of 87% (n = 3). Collected extracts were filtered through anhydrous sodium sulfate. These extracts were evaporated completely under a stream of nitrogen at 45°C and then diluted in 1 mL mobile phase before high-performance liquid chromatography (HPLC) analysis.

HPLC. HPLC was performed using a Waters (Milford, MA) system consisting of a model 510 pump, model 680 automated gradient controller, model 715 Ultra WISP injector, and model 470 scanning fluorescence detector with excitation wavelength at 290 nm and emission wavelength at 330 nm for vitamin E vitamers and oryzanol and UV-VIS variable-wavelength detector (Hewlett- Packard 1050; Palo Alto, CA) at 230 nm for 7-ketocholesterol and 211 nm for cholesterol. A Supelcosil™ (Supleco) LC-Si, 5 μm, 25 cm × 4.6 mm i.d. normal-phase column was used for vitamin E vitamers and oryzanol, and the mobile phase was hexane/ethyl acetate/acetic acid/dimethoxy-propane (DMP) (98.9:0.5:0.5:0.1). The mobile phase was pumped at 1.8 mL/min for the first 9 min and at 2.4 mL/min from 10 to 26 min with the use of a gradient controller. Quantification was accomplished using a Maxima chromatography workstation (Waters) with external standard curves obtained using vitamin E vitamers purified as described by Shin and Godber (18). Tocopherol and tocotrienol standards were prepared from natural sources (18), and oryzanol was isolated from crude rice bran oil (14). The same column was used for 7-ketocholesterol, whereas a C18 5 μm 100A reversed-phase column (Rainin Instrument Company, Inc., Woburn, MA) was used for cholesterol. The mobile phase con-

sisted of hexane/isopropyl alcohol (97.5: 2.5) for 7-ketocholesterol and methanol/acetonitrile (7:3) for cholesterol. The mobile phase was pumped at 1.5 mL/min for the first 5 min and then at 2.5 mL/min from 6 to 14 min for 7-ketocholesterol and at 1.3 mL/min for 14 min for cholesterol. Quantification was accomplished using the same program as described above to calculate the concentration of sterol components from external standard curves. The standards of cholesterol and 7-ketocholesterol were obtained from the Sigma Chemical Co.

Experimental design and statistical analysis. Three separate replications were performed in a randomized complete block design with treatments assigned in a 4 × 5 factorial arrangement. Replications ($n = 3$) were blocked and the nonsaponifiable fraction treatment at different levels (0, 700, 1400, and 2100 ppm) and the oxidation time (0, 4, 8, 12, and 16 h) were the main treatment factors. Each treatment combination had duplicate samples for each replicate determination. The General Linear Model (GLM) procedure was applied to the data with a level of $P < 0.05$ for statistical analysis (20), and Least Significance Difference (LSD) was used to compare the mean differences among treatment combinations.

RESULTS AND DISCUSSION

Changes of vitamin E vitamers and oryzanol during oxidation. The approximate concentrations of vitamin E vitamers and oryzanol in the nonsaponifiable fraction (in μg/g) were: α-T, 3.8; β-T, 0.21; γ-T, 1.5; δ-T, 0.03; α-T3, 1.70; γ-T3, 4.30; δ-T3, 0.14; and oryzanol, 363.4. The changes of total vitamin E vitamers and oryzanol in a model system with cholesterol and the nonsaponifiable fraction from rice bran during oxidation are shown in Table 1. With increased incubation times, there was a reduction ($P < 0.05$) in total vitamin E vitamers in the aqueous model system. The loss of total vitamin E vitamers in the dispersions during 16 h of cholesterol oxidation varied with the concentration of the nonsaponifiable fraction in the order 700 ppm (97.59 %) > 1400 ppm (95.66 %) > 2100 ppm (87.44%) (Table 1). Vitamin E vitamers are stable at elevated temperature in the absence of oxygen. The rate of oxidation of vitamin E vitamers, however, is accelerated by heat under aerobic conditions (21). Vitamin E vitamers are lost if peroxidizing lipids are present (21). Therefore, vitamin E vitamers may be oxidized by the co-oxidation reaction of cholesterol in an aqueous dispersion system. Table 2 shows the time course for the loss of individual vitamin E isomers. The order of decomposition of vitamin E isomers in the dispersions during 16 h incubation was γ-T3 (88.65%) > α-T (87.75%) > α-T3 (86.24%) = γ-T (86.16%) > β-T (80.67%) > δ-T3 (78.63%) > δ-T (77.14%). The decomposition rates of vitamin E vitamers differ with the amount of antioxidants, heating time, heating method, and food composition. In our study, the decomposition of vitamin E vitamers showed a similar trend during 16 h cholesterol autoxidation in a model system. The exact order of antioxidant activity or stability of vitamin E vitamers in a food system may be influenced by the concentration of antioxidant rather than the absolute antioxidant activity or stability in a pure system. Koskas *et al.* (22) reported the antioxidant effect of α-, γ-, and δ-tocopherols during autoxidation of linoleic acid dispersed in an aqueous medium. α-T was completely lost, regardless of its initial concentration, during linoleic autoxidation, whereas γ-T underwent partial oxidation and δ-T was practically unchanged.

Oryzanol was lost slowly at the higher concentrations of the nonsaponifiable fraction (Table 1). The loss of oryzanol from 0 to 16-h incubation period varied with the concentration of the nonsaponifiable fraction in the order 700 ppm (71.10%) > 1400 ppm (57.10%) > 2100 ppm (28.58%), which indicates less relative loss at a higher concentration of the nonsaponifiable fraction from rice bran. The concentration of oryzanol was significantly changed at both the 700 and the 1400 ppm nonsaponifiable fraction treatment. At the higher concentration of 2100 ppm of the nonsaponifiable fraction, the total weight percentage of vitamin E and oryzanol remained constant after 4 h of oxidation compared with those of the other two concentrations even though there was a significant decrease in total vitamin E during oxidation (Table 1). This may be due to the relatively high concentration of oryzanol, which is more heat-stable (16), and a sacrificial consumption of vitamin E vitamers during the initial stage of oxidation. The molar concentration of oryzanol in rice bran oil is about five times higher than total vitamin E (19), and the nonsaponifiable fraction (4.2%) present in rice bran oil has 0.08% total vitamin E vitamers and 1.6% oryzanol (13).

An antioxidative function of oryzanol has been previously

TABLE 1
Changes of Levels of Vitamin E Vitamers and Oryzanol in a Model System During a 16-h Incubation at 80°C

	Nonsaponifiable fraction, vitamin E vitamers[a] (ppm)			Nonsaponifiable fraction, oryzanol[a] (ppm)		
Time (h)	700 ppm	1400 ppm	2100 ppm	700 ppm	1400 ppm	2100 ppm
0	8.30[a]	17.06[a]	24.60[a]	255.00[a]	523.47[a]	739.88[a]
4	5.36[b]	10.76[b]	18.53[b]	168.17[b]	401.73[b]	618.61[b]
8	1.61[c]	4.16[c]	10.10[c]	118.12[c]	311.35[c]	613.18[b]
12	0.47[d]	1.34[d]	6.30[d]	92.46[d]	234.63[d]	599.92[b]
16	0.20[d]	0.74[d]	3.09[d]	73.70[d]	224.65[d]	528.23[b]

[a]Each value represents mean of replication ($n = 3$) with duplicates of each sample. For each column, means with different letters are significantly different ($P < 0.05$).

TABLE 2
Retention of Vitamin E Vitamers Remaining in 2100 ppm Nonsaponifiable Fraction Dispersion During 16 h Incubation at 80°C[a]

Time (h)	α-T	α-T3	β-T	γ-T	γ-T3	δ-T	δ-T3
0	8.00	3.54	0.45	3.25	8.99	0.07	0.29
4	6.17	2.27	0.38	2.47	7.03	0.06	0.15
8	3.00	1.07	0.17	1.15	4.56	0.05	0.10
12	1.75	0.64	0.10	0.66	3.01	0.02	0.07
16	0.98	0.47	0.09	0.45	1.02	0.02	0.06
%[b]	12.25	13.75	19.33	13.84	11.35	22.86	21.37

[a]Values are in units of ppm. Each value represents mean of replication (n = 3) with duplicates of each sample. T, tocopherol; T3, tocotrienol.
[b]Percentage of retention of vitamin E vitamers after 16 h.

reported (15). It has been suggested that the phenolic hydroxyl group in the ferulate esters of oryzanol might be responsible for its antioxidative function. Generally, phenolic antioxidants inhibit lipid oxidation by trapping the peroxy radical to yield the hydroperoxide, thereby preventing the peroxy radical from reacting to produce a lipid radical and propagate a free-radical chain reaction. When the rates of termination reactions of aroxyl radicals, which are produced after phenolic acid donates a proton to a peroxy radical, exceed the rates of reactions that produce free radicals, inhibition of oxidation by the phenolic compounds will occur.

Cholesterol autoxidation in a model system. Table 3 lists the amounts of cholesterol lost and 7-ketocholesterol formed during a 16-h incubation at 80°C. Cholesterol was significantly autoxidized in all of the dispersion samples during the 16-h incubation at 80°C. Based on the initial concentration of cholesterol (500 ppm) the extent to which the cholesterol oxide product 7-ketocholesterol was formed during the 16-h incubation decreased from 41.56% in the absence of the nonsaponifiable fraction to 15.34% for the 700 ppm treatment and decreased further with an increase in the concentration of the nonsaponifiable fraction (7.56% and 3.45% for the 1400 and 2100 ppm treatments, respectively).

Cholesterol oxidation is initiated by hydrogen abstraction, predominantly at C-7, which is most sensitive to molecular attack by oxygen. As a result, 7-hydroperoxides, which are unstable thermodynamically, are converted to 7-ketocholesterol and 7-hydroxycholesterols, the ratio of 7-ketone to combined hydroxy compounds being 2:1 (6). Thus, 7-ketocholesterol is the principal oxidation product (1–6).

Cholesterol undergoes rapid autoxidation when dispersed in the liquid phase, and the autoxidation of cholesterol has been reported as an air/water interface phenomenon (17). Maerker and Bunick (5) reported that 7-ketocholesterol and 5,6-epoxides were the major oxidation products in aqueous dispersion with sodium stearate or Triton surfactants regardless of pH or temperature. As our results showed, 7-ketocholesterol gradually increased as the oxidation time increased (Table 3). 7-Ketocholesterol formed during 12 h incubation was at the level of 60–70% of the total amount formed during 16 h incubation in samples treated with the nonsaponifiable fraction. During 16 h oxidation at 80°C, 67–69% of the cholesterol was lost in nonsaponifiable fraction treatment samples. However, the loss of cholesterol in the dispersion samples without the nonsaponifiable fraction was around 92% after 16 h oxidation (Table 3).

At 16 h, without the nonsaponifiable fraction treatment (Table 3), the amount of 7-ketocholesterol decreased compared with that at 12 h cholesterol oxidation, which caused an interaction effect between incubation time and the nonsaponifiable fraction treatment. The decrease in 7-ketocholesterol may be due to inherent thermal instability of cholesterol oxide products and pH change resulting from extended cholesterol autoxidation under high temperature. It is known that the 7-ketocholesterol is highly sensitive to alkaline pH and is subject to thermal dehydration yielding 3,5-cholestadien-7-one. Kim and Nawar (3) investigated the effects of temperature, time, presence of water, pH, type of buffer, and form of substrate used on cholesterol oxidation. 7-Ketocholesterol/7-hydroxycholesterol dropped significantly with in-

TABLE 3
Time Course of Autoxidation of Cholesterol in Aqueous Dispersions with Nonsaponifiable Fraction of Rice Bran Oil at 80°C and pH 5.5[a]

Time	Treatment							
	0 ppm		700 ppm		1400 ppm		2100 ppm	
(h)	Cholesterol	7-Ketocholesterol	Cholesterol	7 Ketocholesterol	Cholesterol	7-Ketocholesterol	Cholesterol	7-Ketocholesterol
0	500.00 ± 0.00[a]	0.00 ± 0.00[a]	500.00 ± 0.00[a]	0.00 ± 0.00[a]	500.00 ± 0.00[a]	0.00 ± 0.00[a]	500.00 ± 0.00[a]	0.00 ± 0.00[a]
4	277.32 ± 7.68[b]	14.09 ± 2.82[a]	442.06 ± 9.63[b]	2.33 ± 0.26[a]	459.95 ± 3.10[a,b]	2.23 ± 0.71[a]	477.83 ± 6.85[a,b]	0.03 ± 0.02[a]
8	161.22 ± 5.62[c]	52.91 ± 2.27[a]	231.16 ± 6.42[c]	12.91 ± 1.43[b]	427.63 ± 5.79[b]	11.23 ± 1.43[b]	448.55 ± 10.95[b]	2.13 ± 0.61[a]
12	97.76 ± 2.20[d]	223.59 ± 6.70[b]	182.64 ± 5.82[d]	53.88 ± 1.59[c]	191.86 ± 4.14[c]	24.59 ± 1.23[c]	307.90 ± 5.06[c]	10.68 ± 1.61[b]
16	36.66 ± 1.67[e]	207.78 ± 6.16[b]	155.94 ± 2.81[d]	76.69 ± 1.47[d]	155.34 ± 2.03[c]	37.80 ± 1.27[d]	168.46 ± 3.15[d]	17.23 ± 1.60[c]

[a]Values are in units of ppm. Each value refers to mean ± standard error of replication (n = 3) with duplicates of each sample. For each column, means with different letters are significantly different ($P < 0.05$).

creasing pH, but 7-ketocholesterol underwent further decomposition during heating. In addition, in aqueous suspensions, dehydration of the 7-hydroperoxide to yield 7-ketocholesterol occurred readily at lower pH, whereas the 7-ketocholesterol was particularly sensitive to alkaline conditions (1–6). Our aqueous dispersion model system had conditions favorable to oxidation. These conditions included oxygen saturation, high temperature, and a copper catalyst, which accelerated cholesterol autoxidation within 16 h.

The addition of natural antioxidants, such as the nonsaponifable fraction in rice bran oil, at high levels into a food system may bring several benefits. It may increase the nutritional value of the food and allow it to play a role as a functional food as well as inhibit cholesterol oxidation. Though this study shows an antioxidative effect of nonsaponifiable fraction from rice bran oil in suppressing cholesterol autoxidation, it does not indicate the antioxidant effects of the individual vitamin E isomers and oryzanol. The effects of each compound on cholesterol autoxidation require further research in order to understand more completely how they inhibit the formation of COP. In addition, it would also be helpful to establish the actual concentrations of each compound that are needed to prevent cholesterol autoxidation.

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