Antioxidant Activity of Tocopherols, Tocotrienols, and γ-Oryzanol Components from Rice Bran against Cholesterol Oxidation Accelerated by 2,2′-Azobis(2-methylpropionamidine) Dihydrochloride†

Zhimin Xu, Na Hua, and J. Samuel Godber*

Department of Food Science, Louisiana Agricultural Experimental Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803

The antioxidant activities of vitamin E (α-tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol) and γ-oryzanol components (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate) purified from rice bran were investigated in a cholesterol oxidation system accelerated by 2,2′-azobis(2-methylpropionamidine) dihydrochloride. All components exhibited significant antioxidant activity in the inhibition of cholesterol oxidation. The highest antioxidant activity was found for 24-methylenecycloartanyl ferulate, and all three γ-oryzanol components had activities higher than that of any of the four vitamin E components. Because the quantity of γ-oryzanol is up to 10 times higher than that of vitamin E in rice bran, γ-oryzanol may be a more important antioxidant of rice bran in the reduction of cholesterol oxidation than vitamin E, which has been considered to be the major antioxidant in rice bran. The antioxidant function of these components against cholesterol oxidation may contribute to the potential hypocholesterolemic property of rice bran.

Keywords: *Cholesterol; oxidation; tocopherol; tocotrienol; oryzanol*

INTRODUCTION

Oxidation products of cholesterol are considered to be mutagenic and carcinogenic compounds (*1*−*5*). These products also are harmful to many cells in blood vessels, such as macrophage and endothelial cells, and contribute to plaque formation, which results in a variety of cardiovascular and pulmonary diseases (*6*−*9*). Studies have suggested that the antioxidant vitamin E lowers the risk of cancer formation and coronary heart diseases (*10*, *11*).

Rice bran is a rich natural source of vitamin E, containing up to 300 mg/kg (*12*). The major components of vitamin E in rice bran are α-tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol. Rice bran also possesses ∼3000 mg/kg γ-oryzanol, which is a mixture of 10 ferulate esters of triterpene alcohol (*12, 13*). Also, γ-oryzanol has been reported to possess the capability of lowering cholesterol levels in serum (*14*−*16*). γ-Oryzanol components may have antioxidant functions because their structure includes ferulic acid, a strong antioxidant (*17*). However, antioxidant activities of vitamin E and γ-oryzanol in rice bran against cholesterol oxidation have not been studied. Investigation of their antioxidant function in preventing cholesterol oxidation may improve our understanding of the hypocholesterolemic property of rice bran.

The mechanism of cholesterol oxidation is similar to lipid oxidation (*18*−*21*). Cholesterol oxidation is initiated by free radicals to produce hydroperoxides, peroxides, and other degradation products. 7-Hydroperoxycholesterol (7-OOH) is the primary cholesterol oxidation product produced in the free radical chain reaction with 5,6-epoxycholesterol (5,6-EP), 7-ketocholesterol (7-keto), and 7-hydroxycholesterol (7-OH) as secondary oxidation products produced after 7-OOH is epoxidized, dehydrated, and reduced. Also, 7-keto can be produced after dehydrogenation of 7-OH. Seven cholesterol oxidation products, 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 7-keto, 7α-hydroperoxycholesterol (7α-OOH), 7β-hydroperoxycholesterol (7β-OOH), 7α-hydroxycholesterol (7α-OH), and 7β-hydroxycholesterol (7β-OH), were found during heating of cholesterol in 30 min (*21*). In a kinetic model study of cholesterol oxidation during heating at 150 °C, the highest generation rate among oxidation products was for 7-hydroperoxide cholesterol followed by epoxidation, dehydration, reduction, and dehydrogenation (*21*). These results could indicate changes in cholesterol oxidation products in food during cooking. Because higher temperatures are used as the means to accelerate oxidation, the model may not be appropriate to predict cholesterol oxidation status in the human body, which is dominated by free radical chain reactions. However, extremely long oxidation times would be necessary for the cholesterol oxidation products to reach significant levels without an accelerating factor. In this study, 2,2′-azobis(2-methylpropionamidne) dihydrochloride (AAPH), an aqueous free radical reaction initiator, was used to accelerate cholesterol oxidation with a reaction temperature of 37 °C. Thus, it could be more appropriate than the high-temperature oxidation model to evaluate antioxidant

* Corresponding author [e-mail fsgodb@lsu.edu; telephone (225) 388-5192; fax (225) 388-5300].

† Approved for publication by the Director of the Louisiana Agricultural Experiment Station as Manuscript 00-21-0628.

10.1021/jf0012852 CCC: $20.00
Published on Web 03/01/2001

Table 1. Concentration of Each Treatment in the Reaction Solution in Mass Units

treatment	concn (mg/mL)
α-tocopherol	0.216
γ-tocopherol	0.213
α-tocotrienol	0.209
γ-tocotrienol	0.206
cycloartenyl ferulate	0.235
24-methylenecycloartanyl ferulate	0.242
campesteryl ferulate	0.236

activity for compounds of interest against cholesterol oxidation at relatively similar conditions to the human body.

MATERIALS AND METHODS

Chemicals. Cholesterol, 5,6α-EP, 5,6β-EP, 7-keto, 7α-OOH, 7β-OOH, 7α-OH, 7β-OH, AAPH, and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Hexane and acetic acid were from Fisher Chemicals (Fair Lawn, NJ), and 2-propanol was from Mallinckrodt Co. (Paris, KY). Ethyl acetate was from EM Science (Gibbstown, NJ).

Extraction and Purification of Vitamin E and γ-Oryzanol Components from Rice Bran. Rice bran was obtained from the Riviana Rice Mill (Abbeville, LA). Lipids containing vitamin E and γ-oryzanol components were extracted using hexane (*13*, *22*). α-Tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol occupy ~90% of total vitamin E in rice bran (*22*). Also, ~80% of γ-oryzanol in rice bran is cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate (*13*). α-Tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol in the extracted rice bran lipid were purified using normal phase HPLC with a silica column (Supelcosil LC_SI, 25 cm × 4.6 mm, Supelco, Bellefonte, PA) (*22*). The mobile phase was hexane, ethyl acetate, and acetic acid (99, 0.5, and 0.5%, respectively). The flow rate was 1.8 mL/min. The excitation and emission wavelengths of the fluorescence detector were set at 290 and 330 nm, respectively. Ten γ-oryzanol components were separated using reversed phase HPLC (*13*). The C18 column was obtained from Rainin Instrument Co. (Woburn, MA). The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50, 44, 3, and 3%, respectively), and the flow rate was 1.4 mL/min. The wavelength of the UV detector was 330 nm. The three major components of γ-oryzanol, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate, were used in this study due to insufficient quantities of the other seven components. The high-purity components were obtained by collecting their fractions during HPLC (*13*, *22*). The mobile phase of the fraction was evaporated under an ultrapure nitrogen flow. Ten milliliters of mobile phase was collected without loading any sample on the HPLC, which was used as a blank to account for possible effects of residual chemicals in the mobile phase and column packing materials on cholesterol oxidation.

Reaction System and Sample. Cholesterol stock solution was 12 mM, made by dissolving 1.161 g of cholesterol in 150 mL of hexane, 93 mL of 0.05 M phosphate buffer (pH 6.9), and 7 mL of Tween 20. AAPH solution (3, 30, and 60 mM) was freshly prepared with 0.05 M phosphate buffer. The reaction solution was a mixture of 10 mL of cholesterol stock solution and 2 mL of AAPH solution for treatment or 2 mL of phosphate buffer. The concentration of cholesterol in the reaction solution was 10 mM. The AAPH in the reaction solution was 0.5, 5, or 10 mM corresponding to the addition of 3, 30, or 60 mM AAPH solution. The concentration of test component was 0.5 mM in the 12 mL reaction solution. The concentration in mass units of test component in the reaction solution is listed in Table 1. The reaction solution was added to a 100-mL bottle (diameter = 56 mm) with a Teflon seal disk cap. The bottle was placed in a 37 °C water bath (PolyStat model 12050, Cole-Parmer Instrument Co., Chicago, IL) and shaken at 200 rpm. A 1.5-mL aliquot of reaction solution was centrifuged at 4000*g* to separate the hexane layer from the solution. The hexane layer was eluted through a funnel filled with Na_2SO_4 and then was transferred to an HPLC injection vial. A recovery study was performed by comparing the known concentrations of oxidation products spiked in the reaction solution to their final concentrations obtained in the extracted hexane layer. This indicated that the oxidation products in the solution were extracted in the hexane layer after centrifuging.

Cholesterol oxidation products were analyzed using an HPLC method (*21*). The system included a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium chromatography station (Milford, MA). The mobile phase was a mixture of hexane and 2-propanol (99 and 1%, respectively) at 1.5 mL/min flow rate. Detection was at 234 nm.

Statistical Analysis. The experiment for each reaction condition and test component was replicated three times. The experimental data were analyzed by ANOVA using the General Linear Model procedure (SAS system, SAS Institute Inc., Cary, NC) with significant differences between means separated using Tukey's test at $P < 0.05$.

RESULTS AND DISCUSSION

Cholesterol Oxidation Product Analysis. The retention times of the seven cholesterol oxidation products in our HPLC chromatograph are similar to those found by Chien et al. (*21*) when standards were tested. Figure 1 is the chromatogram of cholesterol oxidation products after 24 h of oxidation with 5 mM AAPH. However, no peaks were observed in the chromatograph at the retention times for 7α-OOH, 7α-OH, and 7β-OH. The four major cholesterol oxidation products, 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH, were detected after cholesterol was oxidized by the free radical chain reaction accelerated using AAPH. Similar to the chromatogram of Chien et al. (*21*), cholesterol and its degradation components have shorter retention times (<5 min) than these cholesterol oxidation products. Without AAPH, the only cholesterol oxidation product detected was 7β-OOH at very low concentration (0.05 μg/mL) after 24 h of incubation.

Cholesterol Oxidation Products during AAPH Acceleration. The concentration of each cholesterol oxidation product with 0, 0.5, 5, and 10 mM AAPH is shown in Figure 2. Parts a, b, c, and d of Figure 2 represent the concentrations of 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH, respectively, at different reaction times. In the 0 mM AAPH system, 5,6α-EP, 5,6β-EP, and 7-keto were not detected, and 7β-OOH did not increase after 24 h of incubation at 37 °C. In 0.5, 5, and 10 mM AAPH systems, each oxidation product concentration significantly increased within 24 h. The higher the concentration of AAPH, the greater the increase in the rate of oxidation product production. At 10 mM AAPH, the concentrations of 5,6-EP (5,6α-EP and 5,6β-EP), 7-keto, and 7-OOH (only 7β-OOH) increased to 0.46, 0.32, and 2.16% of cholesterol, respectively, in the 24-h oxidation period. In a study of cholesterol oxidation during heating, seven cholesterol oxidation products were observed by Chien et al. (*21*). The percentages of oxidation product were approximately 8% (5,6-EP), 6% (7-keto), and 0.25% (7-OOH) of cholesterol in 30 min of heating at 150 °C. The concentration of cholesterol oxidation products was much higher than was found in this study, except for 7-OOH. During heating at higher temperatures, 7-OOH may not be stable and readily decomposed or transformed to 7-OH and 7-keto. These results suggest that heating is a more aggressive acceleration method compared with AAPH for choles-

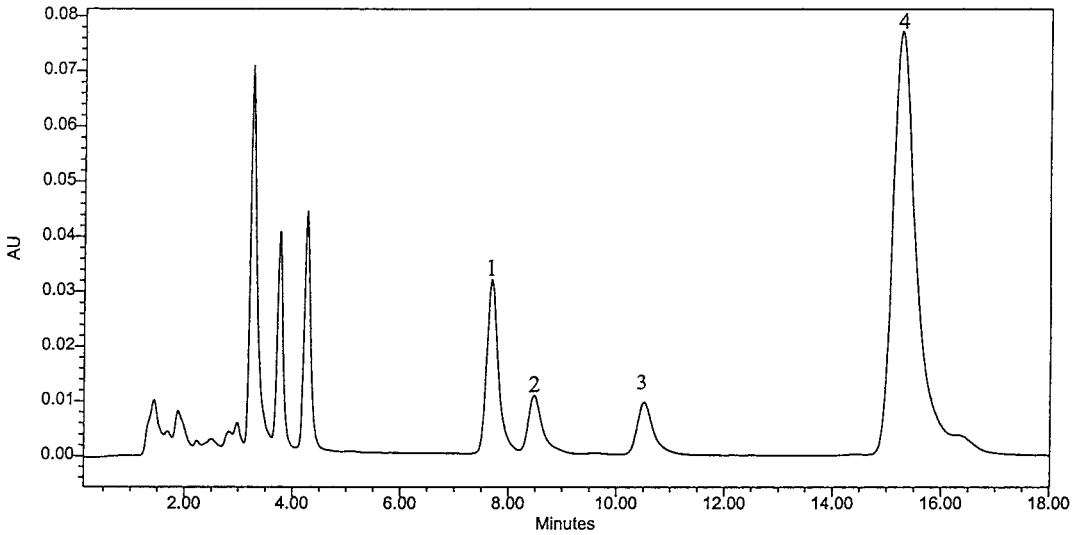

Figure 1. Chromatography of cholesterol oxidation products after 24 h of oxidation with 5 mM AAPH and 10 mM cholesterol: (peak 1) 5,6α-EP; (peak 2) 5,6β-EP; (peak 3) 7-keto; (peak 4) 7β-OOH.

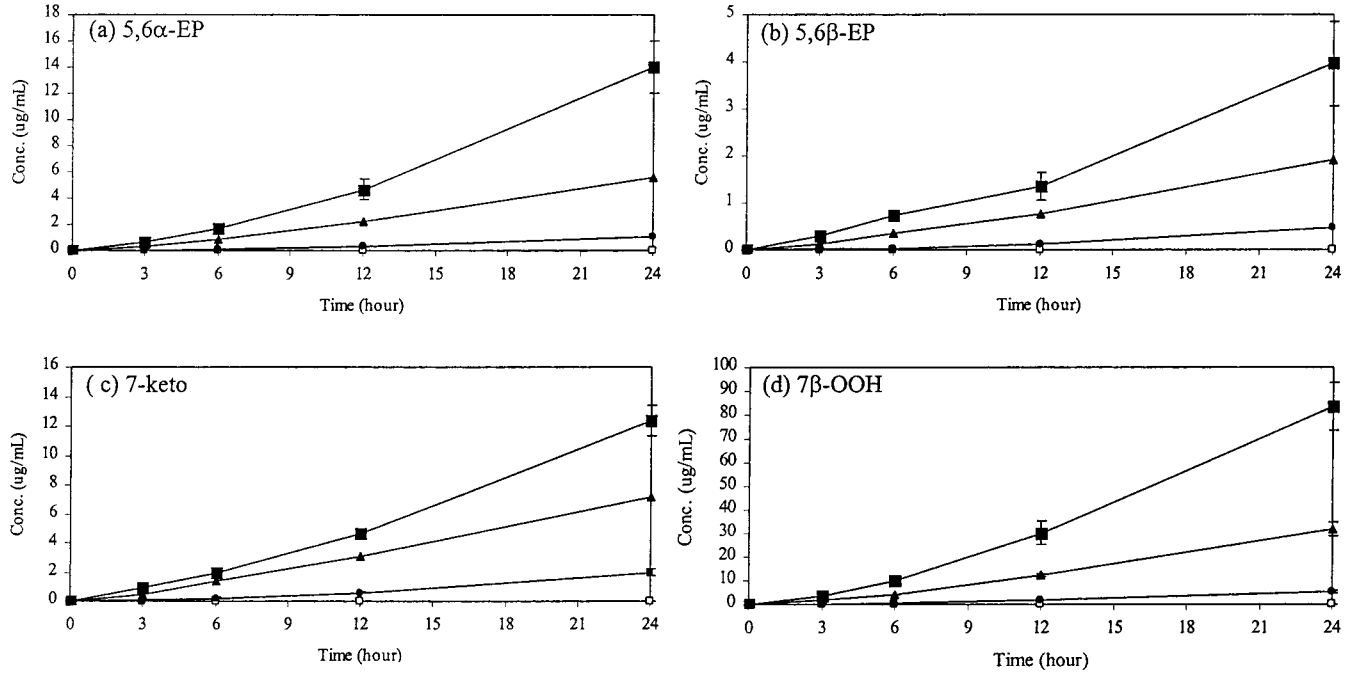

Figure 2. Concentration of cholesterol oxidation products with different concentrations of AAPH and 10 mM cholesterol at different oxidation times: (□) 0 mM AAPH; (●) 0.5 mM AAPH; (▲) 5 mM AAPH; (■) 10 mM AAPH.

terol oxidation. However, the production of 7-OOH and 7-OH remained constant after 30 min at 150 °C (*21*). In the AAPH system, the formation rate of 7β-OOH was the highest and the concentrations of oxidation products increased continually with no tendency to reach a plateau during 24 h of AAPH oxidation. The cholesterol oxidation accelerated by AAPH is relatively mild but persistent, compared with heating. 7-Keto had the second highest concentration among the four cholesterol oxidation products in the AAPH system. Besides dehydration from 7-OOH, another formation pathway of 7-keto is from dehydrogenation of 7-OH (*23*, *24*). As 7-OH was not found in the AAPH system, 7-keto formation may largely depend on the dehydration of 7β-OOH. The occurrence of cholesterol epoxidation during oxidation is related to the concentration of 7-OOH (*18*, *25*). The concentration of 5,6-EP (5,6α-EP and 5,6β-EP) increased along with the concentration of 7-OOH (7α-OOH and 7β-OOH) (*25*). The total concentration of 5,6α-EP and 5,6β-EP was close to that of 7-keto in this study, although that of 5,6β-EP was low.

Antioxidant Activity of Major Vitamin E and γ-Oryzanol Components from Rice Bran. Figure 3 shows concentration changes of four oxidation products, 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH, with different components and the blank obtained after evaporation of the mobile phase at 5 mM AAPH. The reaction system with each component demonstrated significantly lower concentrations of the four cholesterol oxidation products within 12 h, compared with the blank. The oxidation products in each treatment of γ-oryzanol component were also significantly lower than in each treatment of vitamin E. After 24 h of oxidation, the concentrations of 5,6α-EP, 5,6β-EP, and 7-keto with α-tocopherol and α-tocotrienol were not significantly different from the blank. However, the four oxidation products in the

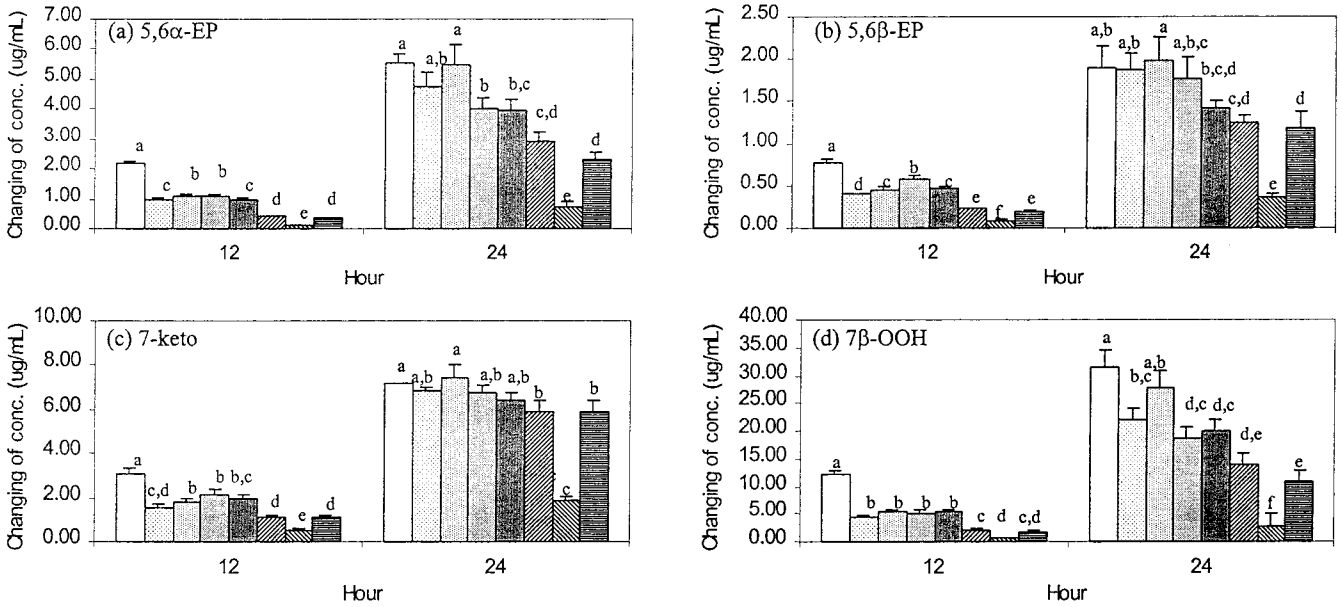

Figure 3. Concentration changes of cholesterol oxidation products in reaction solution of 10 mM cholesterol, 5 mM AAPH, and 0.5 mM test compound at different oxidation times: (bars represent from left to right in each grouping) absence of testing compound; α-tocopherol; α-tocotrienol; γ-tocopherol; γ-tocotrienol; cycloartenyl ferulate; 24-methylenecycloartanyl ferulate; campesteryl ferulate. Significant differences in concentration ($P < 0.05$) are expressed by a different letter in each oxidation time.

reaction system of 24-methylenecycloartanyl ferulate, campesteryl ferulate, and cycloartenyl ferulate were still significantly lower than the blank. The concentration of 7β-OOH in each system with test component remained significantly lower than the blank, except with α-tocotrienol. From these results, significantly higher antioxidant activities in preventing cholesterol oxidation were observed for the three γ-oryzanol components than the four vitamin E components, with 24-methylenecycloartanyl ferulate possessing a much higher antioxidant activity.

The four vitamin E components showed similar antioxidant activities in this system, indicating that differences in structure between vitamin E components do not appear to be a significant factor related to antioxidant activity toward cholesterol oxidation in this system. α-Tocopherol showed the highest antioxidant effect, followed by γ-tocopherol and δ-tocopherol in the chlorophyll-photosensitized oxidation of soybean oil (*26*). However, in an oxidation mixture that contained 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and *cis*-parinaric acid in hexane, the antioxidant activities of tocopherols and tocotrienols were similar (*27*).

The antioxidant function of γ-oryzanol components may depend on a phenolic hydroxy group in the ferulate portion of their structure. Ferulic acid was reported as a phenolic acid type of antioxidant due to the hydroxy group on the phenolic ring (*28, 29*). In this study, γ-oryzanol components possessed significantly higher antioxidant activity than vitamin E components. The higher antioxidant activities of γ-oryzanol components may be due to their structure, which is very similar to that of cholesterol (Figure 4). The analogous structure of γ-oryzanol components and cholesterol leads to similar chemical characteristics in the emulsion system. The γ-oryzanol components may have greater ability to associate with cholesterol in the small droplets of the emulsion and become more efficient in protecting cholesterol against free radical attack. Because 24-methylenecycloartanyl ferulate had a significantly higher antioxidant activity among the three γ-oryzanol components, this might indicate that differences in structure among these components affect their antioxidant activities in this model. The unique structure of 24-methylenecycloartanyl ferulate that is different from both cycloartenyl ferulate and campesteryl ferulate is a methylene group on C-24. The methylene group is attached to two alkyl groups, which may confer greater

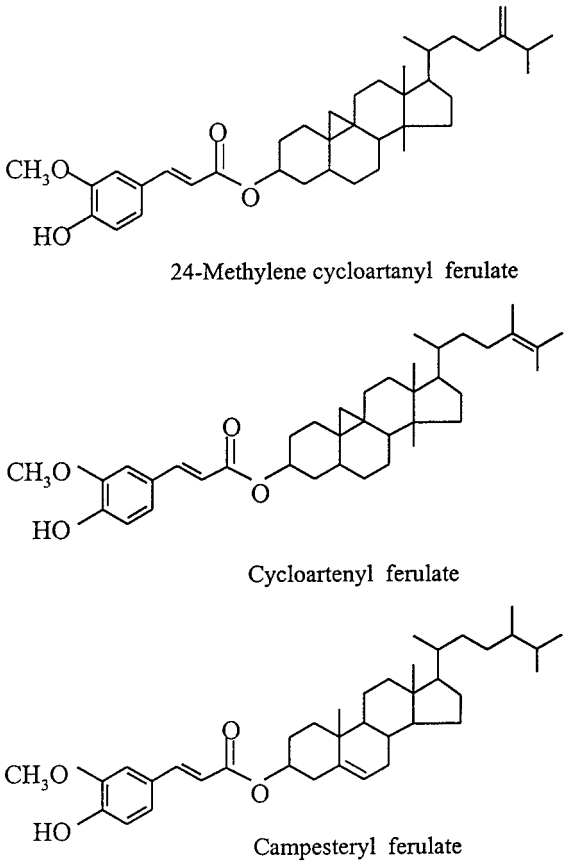

Figure 4. Structures of the three major γ-oryzanol components.

antioxidant activity than the alkene on the C-24 of cycloartenyl ferulate in the emulsion system.

The antioxidant activity of vitamin E and γ-oryzanol components reduces the production of toxic cholesterol oxidation products that may support the potential hypocholesterolemic property of rice bran. Vitamin E has normally been considered to be the most important antioxidant in rice bran; however, in this study, the activity of γ-oryzanol components was significantly higher than that of each of the vitamin E components. Furthermore, the level of γ-oryzanol is ~10 times higher than that of vitamin E in rice bran. Thus, the potential of γ-oryzanol as an inhibitor of cholesterol oxidation is quite compelling and requires further investigation.

LITERATURE CITED

(1) Ansari, G. A. S.; Walker, R. D.; Smart, V. B.; Smith, L. L. Further investigations of mutagenic cholesterol preparation. *Food Chem. Toxicol.* **1982**, *20*, 35−41.

(2) Gerhardt, A. L.; Gallo, N. B. Full-fat rice bran and oat bran similarly reduce hypercholesterolemia in humans. *J. Nutr.* **1998**, *128*, 865−869.

(3) Paniangvait, P.; King, A. J.; Jones, A. D.; German, B. G. Cholesterol oxides in foods of animal origin. *J. Food Sci.* **1995**, *60*, 1159−1165.

(4) Watanabe, K.; Nakamura, R.; Hosono, A. Mutagenic activity of heat-induced cholesterol-degradation products. *J. Food Sci.* **1988**, *38*, 119−123.

(5) Woods, J. A.; O'Brien, N. M. Investigation of the potential genotoxicity of cholesterol oxidation products in two mammalian fibroblast cell lines. *Nutr. Cancer* **1998**, *31*, 192−198.

(6) Lyon, M. A.; Brown, A. J. 7-Ketocholesterol. *Int. J. Biochem. Cell Biol.* **1999**, *31*, 369−375.

(7) Kumar, N.; Singhal, O. P. Cholesterol oxides and atherosclerosis: a review. *J. Sci. Food Agric.* **1991**, *55*, 497−510.

(8) Morel, D. W.; Lin, C. Y. Cellular biochemistry of oxysterols derived from the diet or oxidation *in vivo*. *J. Nutr. Biochem.* **1996**, *7*, 495−506.

(9) Wilson, A. M.; Sisk, R. M.; O'Brien, N. M. Modulation of cholestan-3β,5α,6β-triol toxicity by butylated hydroxytoluene, α-tocopherol and β-carotene in newborn rat kidney cells in vitro. *Br. J. Nutr.* **1997**, *78*, 479−492.

(10) Bellizzi, M. C.; Franklin, M. F.; Duthie, G. G.; James, W. P. T. Vitamin E and coronary heart disease: the European paradox. *Eur. J. Clin. Nutr.* **1994**, *48*, 822−831.

(11) Morrissey, P. A.; Quinn, P. B.; Sheehy, P. J. A. Newer aspects of micronutrients in chronic disease: vitamin E. *Proc. Nutr. Soc.* **1994**, *53*, 571−582.

(12) Shin, T.; Godber, J. S.; Martin, D. E.; Wells, J. H. Hydrolytic stability and changes in E vitamers and oryzanol of extruded rice bran during storage. *J. Food Sci.* **1997**, *62*, 704−708.

(13) Xu, Z.; Godber, J. S. Purification and identification of components of γ-oryzanol in rice bran oil. *J. Agric. Food Chem.* **1999**, *47*, 2724−2728.

(14) Guardiola, F.; Codony, R.; Addis, P. B.; Rafecas, M.; Boatella, J. Biological effects of oxysterols: current status. *Food Chem. Toxicol.* **1996**, *34* (2), 193−211.

(15) Seetharamaiah, G. S.; Chandrasekhara, N. Studies on hypocholesterolemic activity of rice bran oil. *Atherosclerosis* **1989**, *78*, 219−235.

(16) Sugano, M.; Tsuji, E. Rice bran oil and cholesterol metabolism. *J. Nutr.* **1997**, *127*, 521S−524S.

(17) Miller, N. J.; Rice-Evans, C. A. Cinnamates and hydroxybenzoates in the diet: antioxidant activity assessed using the $ABTS^{\bullet+}$ radical cation. *Br. Food J.* **1997**, *99*, 57−61.

(18) Smith, L. L. Cholesterol autoxidation. *Chem. Phys. Lipids* **1987**, *44*, 87−125.

(19) Maerker, G. Cholesterol autoxidation—current status. *J. Am. Oil Chem. Soc.* **1987**, *64*, 388−392.

(20) Yan, P. S.; White, P. J. Cholesterol oxidation in heated lard enriched with two levels of cholesterol. *J. Am. Oil Chem. Soc.* **1990**, *67*, 927−931.

(21) Chien, J. T.; Wang, H. C.; Chen, B. H. Kinetic model of the cholesterol oxidation during heating. *J. Agric. Food Chem.* **1998**, *46*, 2572−2577.

(22) Shin, T.; Godber, J. S. Isolation of four tocopherols and four tocotrienols from a variety of natural sources by semipreparative high-performance liquid chromatography. *J. Chromatogr. A* **1994**, *678*, 49−58.

(23) Kim, S. K.; Nawar, W. W. Parameters influencing cholesterol oxidation. *Lipids* **1993**, *28*, 917−922.

(24) Nielsen, J. H.; Olsen, C. E.; Skibsted, L. H. Cholesterol oxidation in a heterogeneous system initiated by water soluble radicals. *Food Chem.* **1996**, *56*, 33−37.

(25) Maerker, G.; Bunick, F. J. Cholesterol oxides II. Measurement of the 5,6-epoxides during cholesterol oxidation in aqueous dispersion. *J. Am. Oil Chem. Soc.* **1986**, *63*, 771−777.

(26) Yang, W. T.; Min, D. B. Chemistry of singlet oxygen oxidation of foods. *ACS Symp Ser.* **1994**, *No. 558*, 15−29.

(27) Serbinova, E. A.; Packer, L. Antioxidant properties of α-tocopherol and α-tocotrienol. *Methods Enzymol.* **1994**, *234*, 355−366.

(28) Cuvelier, M. E.; Richard, H.; Berset, C. Comparison of the antioxidative activity of some acid-phenols: structure−activity relationship. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 324−330.

(29) Marinova, E. M.; Yanishieva, N. V. Effect of lipid unsaturation on the antioxidative activity of some phenolic acids. *J. Am. Oil Chem. Soc.* **1994**, *71*, 427−434.

Received for review October 24, 2000. Revised manuscript received January 25, 2001. Accepted January 25, 2001.

JF0012852