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Antioxidative Activity of Microencapsulated γ-Oryzanol on High Cholesterol-Fed Rats

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The effectiveness of microencapsulated γ-oryzanol (M-γ-OZ) was evaluated as an antioxidant in Sprague−Dawley rats. Lard containing 100 ppm of γ-OZ (HCD III) or 100 ppm of M-γ-OZ (HCD IV) was heated in an oven for 7 days, and the heat-treated lard as an ingredient in a high cholesterol diet (HCD) formulation was tested for analyzing in vivo cholesterol and lipid profiles. The HCDs containing fresh lard (HCD I) and heat-treated lard (HCD II) were fed to the rats for 4 weeks as control groups A and B, respectively, in this experiment. The liver thiobarbituric acid reactive substances values of group C (fed with HCD III) and group D (with HCD IV) were significantly lower ($p < 0.05$) than that of negative control, group B. One of the cholesterol oxidation products, 7-ketocholesterol, was not detected from group D, indicating that microencapsulation preserved antioxidative activity effectively. The levels of serum total cholesterol and lipoproteins, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein were also affected by heat-induced lipid oxidation.The M-γ-OZ evidently decreased LDL-cholesterol content and increased HDL-cholesterol in blood samples of tested rats. These results suggested that the M-γ-OZ was not only effective in inhibiting the hypercholesterolemia of serum and liver but also reduced the oxidation degree of lipids and cholesterol. Therefore, this microencapsulation can be a good potential technique to protect the antioxidant activity of γ-OZ from heat-induced lipid oxidation.

KEYWORDS: γ-Oryzanol (γ-OZ); microencapsulation; 7-ketocholesterol; TBARs (thiobarbituric acid reactive substances); LDL-cholesterol

INTRODUCTION

A component of rice bran extract, γ-oryzanol (γ-OZ), was of substantial commercial significance in Japan as food and medical antioxidants. In recent years, there has been a trend to use natural antioxidant compounds rather than synthetic antioxidants in the food industry (*1*). The γ-OZ was known to be a powerful inhibitor of iron-driven hydroxyl radical formation, and it was also reported to possess antioxidant activity in stabilizing lipids (*2*, *3*). The γ-OZ that is a mixture of ferulic acid esters of triterpene alcohols and sterols has been investigated for its hypocholesterolemic activity in rats (*4*).

Cholesterol is a compound that is susceptible to oxidation even at ambient temperature when exposed to air. Cholesterol oxidation products (COPs) may be toxic to cells and possibly be involved in cardiovascular disease (*5*). 7-Ketocholesterol is one of the most common COPs found in muscle foods containing cholesterol during the heating process or during prolonged storage (*3*, *6*). Because both polyunsaturated fatty acid (PUFA) and cholesterol oxidations proceeded by the same free radical mechanism, the chemicals that inhibit PUFA oxidation may also inhibit cholesterol oxidation (*7*). The crystalline form and the aqueous dispersion of cholesterol readily undergo oxidation at relatively mild temperatures when exposed to air, producing a variety of oxidized products (*3*, *8*, *9*). Certain COPs have been shown to be cytotoxic, atherogenic, mutagenic, and carcinogenic, when ingested by laboratory animals (*10*, *11*). Those are known to be toxic sterols and potent inhibitors of cholesterol biosynthesis, although these are essential for cell functions (*5*).

Microencapsulation is a technique with which solid, lipid droplets harboring core materials are packaged in a continuous film that can release the microencapsulated core materials at controlled rates under the desired conditions. Microencapsulation has been widely applied in the food industry for safely delivering vitamins, minerals, and other sensitive ingredients. Microencapsulation provides several advantages in the food industry, mainly by protecting the core materials from their environments (*12*). Thus, the microcapsules offer the food processors a mean to protect sensitive food components. Various techniques are

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now available for generating microcapsules as reported previously (*13*). In this study, the microencapsulating method applied was based on the chemical reaction between the water soluble agar and the waxy corn starch to form the water insoluble γ-OZ. Using this encapsulating technique, we investigated the effectiveness of microencapsulated γ-OZ (M-γ-OZ) as an antioxidant on high cholesterol-fed rats.

MATERIALS AND METHODS

Materials. Waxy corn starch and γ-OZ were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), respectively. Agar was obtained from Duksan Pure Chemical Co. (Seoul, Korea). Emulsifiers such as Tween 20, Tween 80, and Span #20 (Sorbitan Monalaurate) were obtained from DaeJung Chemicals & Metals Co. Ltd. (Seoul, Korea). Thiobarbituric acid reactive reagent, 1,1,3,3-tetramethoxy propane, and trichloroacetic acid reagent were purchased from Sigma Chemical Co.. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) kits were supplied by Shin Yang Chemical Co. (Tokyo, Japan). The solvents used in this study were high-performance liquid chromatography (HPLC) grade.

Microencapsulation. With the 2:1 (w/w) mixture of agar and waxy corn starch as a coating material, γ-OZ was emulsified and microencapsulated using an extrusion spraying technique. The method reported by Chang et al. (*14*) was used with some modifications for microencapsulation. Briefly, agar and waxy corn starch were mixed and dissolved in distilled water at 78 °C. Then, the Tween 20 (sorbitan laurate + ethylene oxide, HLB = 16) was added from 0 to 0.8% (v/v), and the mixture was dispersed with a homogenizer (T25 basic, IKA. Labortechnik Staufen, Germany) for 30 s at 9000*g*. The O/W emulsion was dispersed into 200 mL of distilled water using a spray gun (Wagner 300, Wagner Spraytech Ltd., Germany). The final conditions for the microencapsulation of the γ-OZ were a ratio of core [C_1m] to coating material [C_2m] of 4.8:5.2 (w/w), a temperature of dispersion fluid of 24.99 °C, and an emulsifier concentration of 0.38%.

Experimental Diets. The basic high cholesterol diet (HCD) was composed of 66.3% corn starch, 15% casein, 10% lard, 4% AIN-93 mineral mixture, 2% cellulose, 1% AIN-93 vitamin mixture, 1% cholesterol, 0.5% cholic acid, and 0.2% choline chloride. HCD I consisted of the basic diet with fresh lard; HCD II contained heat-treated lard; HCD III contained lard plus 100 ppm of γ-OZ; HCD IV contained lard plus 100 ppm of M-γ-OZ. The lards with γ-OZ and M-γ-OZ in HCDs III and IV, respectively, were heat-treated at 180 °C in an oven for 7 days before they were mixed into the basic HCD diet.

Male Spraque−Dawley (SD) Strain Rats. Forty male SD rats weighing between 75 and 100 g were used for this experiment. The SD rats (Samtaco Inc., Ansan, Korea) were initially fed a commercial diet with tap water for a 1 week period of acclimatization. The acclimated rats were randomly assigned to four groups of ten. Group A (control) was fed HCD I, and groups B (negative control), C, and D were fed with experimental diets HCD II, III, and IV, respectively, for 4 weeks. The diets and water were uptaken by the rats without any restrictions. The air-conditioned incubating room was maintained at 23 ± 2 °C, and the relative humidity was controlled at 50 ± 5%. The rats were nurtured individually in screen-bottomed cages under the 12 h light−dark cycles. The body weight of rats and the amount of diet intake by rats were measured and recorded with a weekly basis. At the end of the 4 week feeding period, the rats were fasted overnight and the blood was drawn by cardiac puncture. The blood-drawn rats were sacrificed by chloroform anesthesia. At that point, the liver samples were excised, washed in prechilled saline solution, and stored at −80 °C until further analysis.

Thiobarbituric Acid Reactive Substances (TBARs) Analysis. TBARs were determined, following the method of Tarladgis et al. (*15*) with some modifications by Ohkawa et al. (*16*), to measure the level of lipid peroxides in the liver. The liver sample was added to a 10-fold volume of a 10 mM phosphate buffer (pH 7.4), and the buffered sample was homogenized. The sample preparation was mixed with the same volume of 0.6% (w/v) thiobarbutric acid solution containing 17.5% (w/v) trichloroacetic acid (TCA). The sample mixture was heated at 95 °C for 15 min and was placed in an ice bath. Then, it was added to 1 mL of TCA solution and was placed at room temperature for 20 min. The mixture was centrifuged at 4000*g* for 10 min, and the absorbance of the supernatant at 534 nm was read. The TBARs was determined from the standard curve that was constructed using 1,1,3,3-tetramethoxy propane (TMP) and was expressed as ng/mg protein. The amount of protein was determined by the Bradford method (*17*).

Cholesterol and 7-Ketocholesterol Analysis. The lipid components were extracted from the liver by the method of Folch et al. (*18*). Liver samples were homogenized in chloroform to methanol (2:1) to extract tissue lipids using a tissue homogenizer with a loose fitting pestle. After filtration through a funnel, 1% saturated potassium chloride was added to the extract, and the mixture was separated in a separating funnel to remove chloroform from the bottom layer. The separated chloroform layer was evaporated in a vacuum flash evaporator until about 5 mL remained in a measuring jar. The resulting solution was saponified, and the unsaponifiable fraction was used for analysis of cholesterol and 7-ketocholesterol by HPLC as described previously (*19*).

Cholesterol, Lipoprotein, and TG Profiles in Blood. Blood samples were collected in the heparin-treated tubes containing EDTA (1.5 mg/mL) and centrifuged at 2000*g* for 30 min to separate the blood plasma. The plasma samples were labeled and stored at −80 °C until further analysis. The TC, HDL cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and TG were analyzed from serum samples in assistance with enzymatic analysis. An enzymatic test kit (Shin Yang Chemical Co., United States) was used for the quantitative analyses of TG, TC, and HDL-C. The amounts of LDL-C and very low-density lipoprotein cholesterol (VLDL-C) were determined following the method of Friedwald et al. (*20*). Briefly, the amount of LDL-C was calculated by subtracting TG/5 and HDL-C from TC, and the VLDL-C was obtained by subtracting HDL-C and LDL-C from TC.

Statistical Analysis. A randomized complete block design was used with 44 factorial arrangements. A group ($n = 10$) was blocked, and the dietary treatment was the main treatment factor. The general linear model procedure was applied to the data with the level of $p < 0.05$ for statistical analysis (*21*), and the analysis of variance was used to compare the mean differences among the treatment groups.

RESULTS AND DISCUSSION

Food Efficiency Ratio and Liver Index. The food efficiency ratio and liver index of the SD rats during the 4 week intake period of the formulated diet were summarized in **Table 1**. The weight gain and the amount of diet intake were not significantly different among the groups B, C, and D, while the diet intake of group A is relatively greater than those of other groups. When the food efficiency ratio was compared, it was clearly shown that there was no significant difference in the diet intake per unit amount of body weight gain among the four groups. It indicated that the amount of consumed diet did not affect the growth rate of the rats. Group A displayed the highest liver weight after the 4 week diet program, and group D showed the lowest. The liver indexes of groups C and D (5.39 and 5.14 g/100 g of body weight, respectively) were significantly smaller than groups A and B containing no γ-OZ, which means that the γ-OZ added to the diet substantially decreased the liver weight of the rats. It was evident that there existed no apparent effect of encapsulation on the liver weight change, because the difference in the liver weight was not significant between groups C and D.

TBARs in Liver Tissue. The TBARs was measured to determine the level of lipid oxidation in the liver of 4 week nurtured SD rats, and the result was shown in **Table 2**. Because the lard in group B was oxidized by heating in this experiment, it was expected that group B had a larger TBARs value than group A. When the γ-OZ was included in the meals, the value

Table 1. Body Weight Gain and Diet Intake of the SD Rats Nurtured with HCDs

group[a]	weight gain (g/4 weeks)	diet intake (g/day)	food efficiency ratio[b]	liver weight (g)	liver index[c] (g/100 g of body weight)
A	115.44 ± 43.66[d] a[e]	18.83 ± 1.75 a	0.163NS[f]	12.68 ± 2.59 a	8.74 ± 0.95 a
B	94.22 ± 36.96 b	14.64 ± 3.88 b	0.155	11.67 ± 2.71 ab	6.67 ± 0.42 b
C	135.44 ± 23.26 ab	15.34 ± 2.68 b	0.114	10.00 ± 1.63 bc	5.39 ± 0.94 bc
D	122.44 ± 22.28 ab	15.35 ± 3.42 b	0.125	8.820 ± 1.39 c	5.14 ± 0.15 c

[a] Group A (control) was fed a HCD I, and groups B (negative control), C, and D were fed with experimental diets HCD II, III, and IV, respectively, for 4 weeks. The recipe of each HCD was described in the Materials and Methods. [b] Food efficiency ratio = diet intake (g)/weight gain (g). [c] Liver index = the weight of liver/the weight of body. [d] Mean ± SD. [e] The values with different letters within the same column were significantly different among the groups at the level of $p = 0.05$ by least significance difference (LSD). [f] NS means no significant difference by LSD.

Table 2. Effects of γ-OZ and M-γ-OZ on TBARs in the SD Rats Nurtured with HCDs

group[a]	TBARs (ng/mg protein)
A	3.09 ± 0.11[b,c] c
B	5.36 ± 0.18 a
C	3.94 ± 0.08 b
D	3.99 ± 0.08 b

[a] The nature of groups A, B, C, and D was presented as a footnote in **Table 1**. [b] Mean ± SD. [c] Values with different letters were significantly different among the groups at the $p = 0.05$ level by LSD.

Table 3. Effects of γ-OZ and M-γ-OZ on Cholesterol and 7-Ketocholesterol Levels in the HCD-Nurtured SD Rats

group[a]	cholesterol (mg/g)	7-ketocholesterol (μg/g)
A	5.16 ± 0.51[b,c] b	ND[d]
B	6.03 ± 0.10 a	0.47 ± 0.03 a
C	5.10 ± 0.04 b	0.23 ± 0.02 b
D	5.14 ± 0.08 b	ND

[a] The nature of groups A, B, C, and D was presented as a footnote in **Table 1**. [b] Mean ± SD. [c] Values with different letters within the same column were significantly different among the groups at the $p = 0.05$ level by LSD. [d] ND, not detected.

was smaller than group B but larger than group A. The differences in TBARs values between γ-OZ-treated groups C and D were not statistically different. From these results, it was suggested that the encapsulation preserved the antioxidant activity of γ-OZ and prevented lipid oxidation effectively.

Cholesterol and 7-Ketocholesterol in Liver Tissue. The cholesterol level in the liver tissue was compared among the groups, and the results indicated that they were significantly higher ($p < 0.05$) in the negative control, group B, as compared to other groups (**Table 3**). However, the differences in the cholesterol levels were not statistically different among the control group A, C, and D.

7-Ketocholesterol was not detected in groups A and D, while a significantly larger amount of 7-ketocholesterol was detected in group B ($p < 0.05$) than any other groups. The repression of oxidation reaction by γ-OZ in groups C and D resulted in a significant decrease in the amounts of cholesterol and 7-ketocholesterol in the liver, of which levels were similar to that of group A (that was the HCD containing fresh lard). Moreover, 7-ketocholesterol was not detected in the rats fed with the HCD containing M-γ-OZ (group D), indicating that the γ-OZ release control of encapsulation positively affected the lipid metabolism in the rats. These results suggested that the M-γ-OZ was not only effective in inhibiting the hypercholesterolemia but also reducing cholesterol oxidation.

Cholesterol, Lipoprotein, and TG Profiles in Blood. The cholesterol, TG, and lipoprotein profiles in **Table 4** indicated that there was a significantly larger amount of serum TC in group B at the level of $p < 0.05$ when compared to other groups. The differences in the amount of TC among groups A, C, and D were not statistically different. The TC levels of the groups C and D, fed with the γ-OZ-added lards, were comparable to that fed with fresh lard (group A), but group B with the oxidized lard seemed to increase the cholesterol level in the blood. The TC level of group D was even lower than that of group C. The TG levels were not statistically different among the groups tested except for group D. The amount of serum TG in group D was even lower than that in the control group, A. It was clearly shown in **Table 4** that the HDL and LDL levels were both affected by heat-induced oxidation of lipids. The lipid oxidation resulted in more than three times larger LDL-C levels, which in turn displayed the decrease in the HDL-C level significantly. When the γ-OZ was added to the diet containing heated lard, the amount of TC in the blood decreased substantially as shown from groups C and D. However, there was a noticeable difference in change patterns in LDL-C and HDL-C levels. In other words, both LDL-C and HDL-C in group C decreased when compared to group B, whereas a drastic decrease in LDL-C and a substantial increase in HDL-C were observed from group D. When the VLDL-C level was compared among the HCD-treated groups, the smallest amount of VLDL-C was detected from group D while a larger amount than any other group was observed from group B. These results suggested that the M-γ-OZ was not only effective in inhibiting the hypercho-

Table 4. Effects of γ-OZ and M-γ-OZ on Cholesterol, Lipoprotein, and TG Profiles in the Blood of HCD-Nurtured SD Rats

	mg/dL				
group[a]	TC	TG	HDL-C	LDL-C	VLDL-C
A	141.19 ± 10.26[b,c] b	134.13 ± 5.47 a	73.76 ± 4.13 b	63.25 ± 0.85 b	25.40 ± 2.42 bc
B	230.40 ± 58.91 a	168.43 ± 12.74 a	29.04 ± 2.37 c	198.28 ± 1.52 a	32.08 ± 2.58 a
C	166.83 ± 17.99 b	141.72 ± 14.67 a	13.74 ± 2.06 c	89.59 ± 1.20 b	27.57 ± 2.54 ab
D	120.07 ± 15.58 b	99.96 ± 5.68 b	90.94 ± 2.18 a	20.06 ± 0.21 c	19.92 ± 1.85 c

[a] The nature of groups A, B, C, and D was presented as a footnote in **Table 1**. [b] Mean ± SD. [c] Values with different superscripts within the column were significantly different among the groups at the $p = 0.05$ level by LSD.

Table 5. Correlation Coefficients for Lipoproteins and 7-Ketocholesterol in the HCD-Nurtured SD Rats

	correlation coefficients		
	LDL-C	VLDL-C	7-ketocholesterol
7-ketocholesterol	0.93988[a]	0.89614[a]	
LDL-C		0.95278[a]	0.93988[a]
VLDL-C	0.95278[a]		0.89614[a]

[a] Values were significantly different among samples at the $p = 0.05$ level.

lesterolmia in serum but also regulating serum lipoprotein profiles in a positive direction.

Correlation of TBARs and 7-Ketocholesterol. The quantity of oxidized lipids in the diet was directly related to the level of oxidized lipids in serum postprandial chylomicrons (*22*, *23*), which provided a mechanism by which dietary oxidized lipids can affect the oxidative states of endogenous lipoproteins (*24*). Previously, it was hypothesized that the oxidation mechanism of lipids was similar to that of cholesterol (*8*, *9*, *10*, *25*). When the statistical analysis was performed to find a possible correlation between these two components, the change in TBARs values was highly correlated to the cholesterol oxidation level with $r^2 = 0.86338$ at the significance level of $p < 0.05$. This result indicated that the oxidation level of lipid components in the liver tissue was closely related to the occurrence of the oxidized sterol, 7-ketocholesterol. Thus, the TBARs values could be used as the indirect indication of the oxidation level of sterols. This high correlation was also found among 7-ketocholesterol and lipoproteins, as shown in **Table 5**. The increase in 7-ketocholesterol level was positively related to the levels of both LDL-C and VLDL-C while the highest correlation was observed between LDL-C and VLDL-C levels.

Consequently, physically reinforced γ-OZ by microencapsulation displayed the similar biological functions as effective as naked γ-OZ in terms of inhibiting the hypercholesterolemia and reducing oxidation degree of lipids. Even better positive effects were observed in the blood cholesterol composition from the M-γ-OZ-treated group, probably due to the physical protection and controlled release mechanism of γ-OZ. Therefore, this microencapsulation can be a good potential technique to preserve the antioxidant effect of γ-OZ when ingested into in vivo system.

ABBREVIATIONS USED

HCD, high cholesterol diet; γ-OZ, γ-oryzanol; M-γ-OZ, microencapsulated γ-oryzanol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; TG, triglycerides; TBARs, thiobarbituric acid reactive substances.

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