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Physicochemical and Hypocholesterolemic Characterization of Oxidized Oat β-Glucan

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2,2,6,6-Tetramethyl-1-piperidine oxoammonium ion (TEMPO)-mediated oxidation was applied to oat β-glucans, and the physicochemical and hypocholesterolemic properties of the resulting derivatives were investigated. The ^{13}C NMR spectra revealed that C6 primary alcohol groups were selectively oxidized into carboxyl groups. The oxidized derivatives exhibited enhanced water solubility and improved in vitro bile acid binding capacity. When hypercholesterolemic rats were fed diets containing the oxidized β-glucan, the levels of triglyceride, total cholesterol, LDL-C, and VLDL-C in the rats significantly decreased ($p < 0.05$), consequently improving the serum lipid profiles. Dietary supplementation with β-glucans reduced also the total cholesterol level in liver. Furthermore, more fecal eliminations of total cholesterol and triglyceride were observed, which were favorably correlated to their reduced levels in the serum and liver. As a result, oxidized oat β-glucan exhibits potential use as an active cholesterol-lowering ingredient.

KEYWORDS: Oxidation; oat; β-glucan; bile acid binding capacity; hypocholesterolemic effect

INTRODUCTION

Chemical modification of polysaccharides has been widely carried out for commercial as well as scientific interests. Substitution of hydroxyl groups with new functional groups can impart new functionalities to the polysaccharides or improve their intrinsic properties. In particular, oxidation has been often applied to various polysaccharides whereby carboxylic groups are grafted on the backbone of the polysaccharides. The introduction of the carboxylic groups gives enhanced interactions with cationic materials and increased water solubility to the corresponding polymers. This may have advantages in pharmaceutical and food applications because oxidation plays an important role in synthesizing chemicals such as fragrances or food additives (*1*). Ever since the primary alcohol groups of cellulose were preferentially oxidized to carboxyl groups by using NO_2 (*2*), the oxidation reaction has been often applied to obtain polyuronic acids from polysaccharides.

2,2,6,6-Tetramethyl-1-piperidine oxoammonium ion (TEMPO) is a representative radical reagent for the oxidative reaction and is remarkably stable under ambient conditions, compared to other reagents (*3*). The use of TEMPO in combination with sodium hydrochlorite and sodium bromide provides highly selective and efficient conversion of C6 primary hydroxyl groups to carboxyl groups, producing high-yield polyuronic acids (*4*−*6*). Moreover, serious depolymerization of polysaccharides, which was a critical problem in the oxidation with nitrogen dioxide, does not take place through the TEMPO-mediated oxidation (*7*).

Therefore, in the recent decade, TEMPO-mediated oxidation has been often used in polysaccharide chemistry; it was first applied to water-soluble glucans such as potato starch, amylodextrin, and pullulan (*8*). Thaubret et al. (*9*) oxidized maltodextrin and D-glucose and investigated the pH effect on the selectivity and sequestering ability of the resulting derivatives. In addition, the viscosity and biodegradability of oxidized chitin (*10*) and the immunostimulatory properties of oxidized β-glucan from *Saccharomyces cerevisiae* (*11*) were tested. More recent studies investigated the water solubility and bile acid binding capacity of the oxidized chitosan (*6*) and the wet strength of the sheets prepared with TEMPO-oxidized cellulose (*5*). However, there is still a lack of information on the effect of TEMPO-mediated oxidation on the biological properties of carbohydrates. Even so, their in vivo tests involving hypocholesterolemic effects have not been carried out yet.

In this study, β-glucan derived from oats was subjected to TEMPO-mediated oxidation and the hypocholesterolemic effects of the corresponding derivative were evaluated in vivo and also correlated to its in vitro bile acid binding capacity.

MATERIALS AND METHODS

Materials. The oats (*Avena sativa* L.) used in this study were obtained from the Korean National Institute of Crop Science (Suwon, Korea). Oat β-glucan was extracted according to the previous method by Shin et al. (*12*), which was mainly based on enzyme treatment (α-

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amylase and amyloglucosidase) and centrifugal fractionation after ethanol addition. TEMPO and sodium hypochlorite (NaOCl) solution were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Junsei Chemical Co. (Tokyo, Japan), respectively. Other chemicals used in this study were of analytical reagent grade.

Preparation of Oxidized Oat β-Glucans. According to the method of Chang and Robyt (*13*), TEMPO (1.52 mg), sodium bromide (NaBr, 0.46 g), and NaOCl (0.16 g) were added into β-glucan solution (0.162 g in 30 mL of DW) and reacted at 25 °C, pH 10.8, with 1 N hydrogen chloride (HCl). The resulting solution was oxidized at pH 10.8 with 0.5 N sodium hydroxide (NaOH), and the degree of oxidation was determined from the amount of NaOH consumed. The reaction was stopped by adding 10 mL of ethanol and neutralizing with 4 N HCl. The oxidized glucan was precipitated by adding 3 volumes of acetone and then vacuum-dried at 55 °C for 24 h.

Structural Analysis. For the structural analysis of the β-glucan derivative, its NMR spectra were recorded on a Varian spectrometer (Unit INOVA, Varian Co., Palo Alto, CA) operating at a carbon NMR frequency of 300 MHz. The samples (80 mg) were dissolved in D_2O (2 mL), and TMS was used as internal standard.

Water Solubility. Water solubilities of native and oxidized β-glucans were determined according to the method of Chang and Cho (*14*). The β-glucan dispersion (25 mg/mL for the native; 100 mg/mL for the oxidized) was agitated at 25 °C for 24 h. The resulting dispersion was centrifuged at 6000*g* for 15 min, and the supernatant was freeze-dried. The solubility was calculated using the following equation:

$$\text{solubility (\%)} = \frac{\text{wt of } \beta\text{-glucan dissolved in water}}{\text{wt of } \beta\text{-glucan initially added to water}} \times 100$$

In Vitro Bile Acid Binding Capacity. In vitro bile acid binding capacities of the native and oxidized glucans were measured according to previous procedures with modification (*15*, *16*). The dry β-glucan samples were added to 0.01 M sodium phosphate buffer (pH 7.0) containing 200 μM bile acid at a concentration of 2.5 mg/mL, stirred at 37 °C for 2 h, and then filtered (0.2 μm syringe filter, Waters Co., Milford, MA). The resulting solutions (0.2 mL) were treated with 70% sulfuric acid (H_2SO_4, 1 mL) for 5 min, and 25% furfural (C_4H_3OCHO, 0.2 mL) was then added. After 1 h, the absorbance was measured at 510 nm.

Animal Experiment. Four-week-old Wistar male rats were purchased from Central Laboratory Animal Inc. (Seoul, Korea) and acclimated for a week on standard rodent chow (Samyang, Seoul, Korea) that consisted of protein 22.0%, fat 4.5%, fiber 6%, ash 8%, calcium 0.7%, and phosphorus 0.5%. Then, the animals stratified by weight were divided into four groups ($n = 8$ per group). For 4 weeks, the control group (CON) was fed a high-cholesterol diet, and the experimental groups were fed a high-cholesterol diet containing native β-glucan (BG), 100% oxidized β-glucan (OXI), and their mixture (BO) (**Table 1**). The animals were housed in cages with wire-mesh bottoms in conditioned rooms (24 °C; relative humidity = 55%), and food and water were provided ad libitum during the 4 week period. Food intakes and body weights were measured every other day. The care and treatment of rats were approved by the Hanyang University Laboratory Animal Care Committee and were in accordance with the Korean Guide for the Care and Use of Laboratory Animals.

After food was removed at the end of the experimental term, the animals were fasted for 24 h. They were then anesthetized with dry ice and dissected. Serum was obtained from the blood by centrifugation at 300*g* for 15 min at 4 °C. The concentrations of triglyceride and cholesterols in the serum were measured by using an enzymatic colorimetric kit (Asan Pharmaceutical Co., Seoul, Korea). For the liver lipid analysis, the liver that was excised, frozen in liquid nitrogen, and kept at −70 °C, was treated with chloroform/methanol (2:1, v/v) to extract total lipids (*17*). Then, triglyceride and total cholesterol were analyzed by using an enzymatic colorimetric kit (Asan Pharmaceutical Co.).

The feces collected during three consecutive days before sacrifice were dried at 55 °C in a forced-air oven, ground, and stored at −70 °C. The fecal lipid profile was measured using the same procedure as that used for the liver sample. Bile acid was extracted according to the modified method of Uchida (*18*) and determined with a bile acid assay kit (Wako Chemical, Osaka, Japan).

Table 1. Compositions (Grams per Kilogram of Diet) of the Experimental Diets

	group[a]			
ingredient	control	BG	BO	OXI
casein	200	200	200	200
L-cysteine	3	3	3	3
corn starch	517.48	467.48	467.48	467.48
sucrose	100	100	100	100
cellulose	50	50	50	50
soybean oil	70	70	70	70
tert-butylhydroquinone	0.014	0.014	0.014	0.014
mineral mix S10022G	35	35	35	35
vitamin mix V10037	10	10	10	10
choline bitartrate	2.5	2.5	2.5	2.5
cholestrol	10	10	10	10
cholic acid	2	2	2	2
oat β-glucan		50	25	
100% oxidized β-glucan			25	50

[a] Control, high-cholesterol diet, 1% cholesterol modified AIN-96G purified rodent diet (Dyets Inc., Bethlehem, PA); BG, high-cholesterol diet containing native β-glucan; BO, high-cholesterol diet containing native and 100% oxidized β-glucans; OXI, high-cholesterol diet containing 100% oxidized β-glucan.

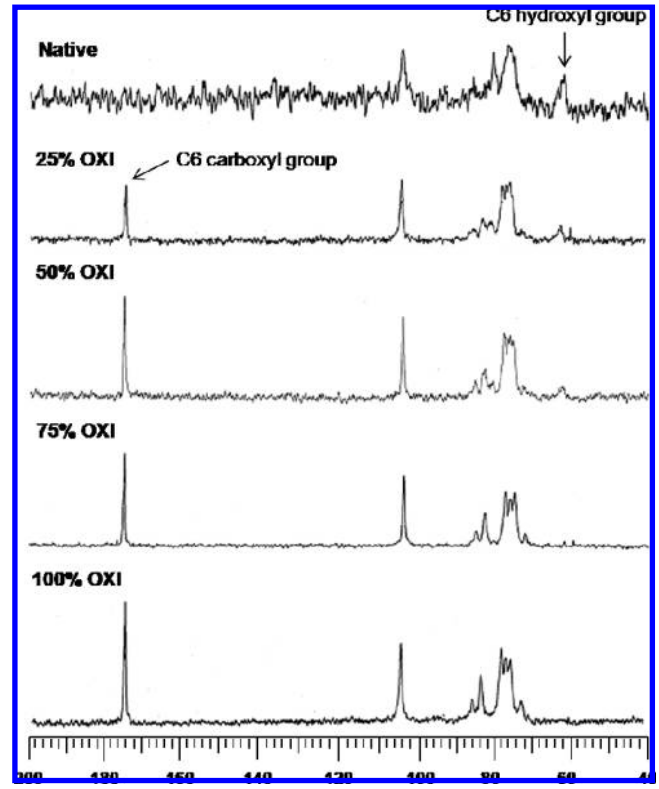

Figure 1. NMR spectra of native and oxidized oat β-glucans.

Statistical Analysis. All experiments were carried out in triplicate. For statistical analysis, Statistical Package for the Social Science (SPSS, version 12.0, 2004, SPSS Inc., Chicago, IL) was used. The results were subjected to analysis of variance (ANOVA), followed by Duncan's multiple-range test for mean comparison at the level of 0.05.

RESULTS AND DISCUSSION

Physicochemical Properties of Oxidized β-Glucan. The ^{13}C NMR spectra of oxidized oat β-glucans were obtained and are shown in **Figure 1**. The resonance signals of C6 hydroxyl groups in native β-glucan were detected at 60.2 ppm, which could be favorably compared with those in the oxidized

Table 2. Water Solubility of Native and Oxidized Oat β-Glucans

sample	water solubility[a] (%)
native β-glucan	36.20 ± 3.53a
25% oxidized β-glucan	56.84 ± 7.56b
50% oxidized β-glucan	71.12 ± 3.58c
75% oxidized β-glucan	80.41 ± 1.62d
100% oxidized β-glucan	87.81 ± 1.58e

[a] Values with different letters are significantly different among samples at the α = 0.05 level by Duncan's multiple-range test.

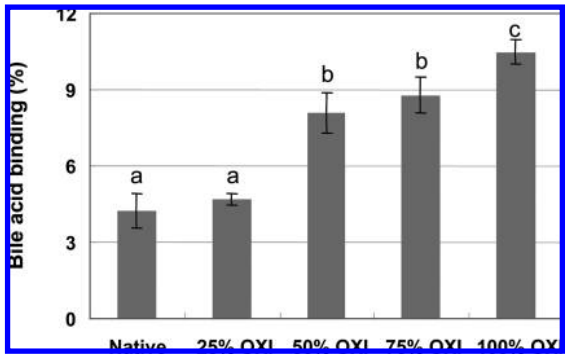

Figure 2. In vitro bile acid binding capacities of native and oxidized oat β-glucans.

hyaluronan and water-soluble starch (*4, 19*). As can be seen in **Figure 1**, it is interesting to note that the signal intensity of the C6 hydroxyl groups gradually decreased as the oxidation proceeded. In turn, a new signal at 175.2 ppm appeared in the NMR spectra of the oxidized samples, and its intensity increased with oxidation. It seemed that the new peak corresponded to C6 carboxyl groups (*13*). Therefore, the increase in the signal intensity at 175.2 ppm presented the successful oxidation of hydroxyl groups at C6 to carboxyl groups. Also, it was likely that the oxidation occurred mainly in the primary alcohol group because no resonance in the range between 198 and 205 ppm (no formation of ketone group) was observed (*19*).

The effect of oxidation on the water solubility of oat β-glucans was investigated. As also shown in **Table 2**, the water solubility is shown to be significantly ($p < 0.05$) responsive to the degree of oxidation. The water solubility, which was about 37% for the native β-glucan, increased up to about 88% when 100% of the primary alcohol groups in β-glucan were oxidized. The anionic characteristics by the introduction of carboxyl groups along the polymer chain would be responsible for this increase in the water solubility of the oxidized β-glucans. Also, it was previously reported that oxidation led to an increase in the water solubility of various polysaccharides (*14*). It is recognized that the improved water solubility is desirable for pharmaceutical applications because it provides ease of administration as well as enhanced physiological activities such as anticoagulant and antitumor properties (*20, 21*). Also, food applications can benefit from the increased water solubility in terms of blending and stability.

In Vitro Bile Acid Binding Capacity of Oxidized β-Glucan. The changes in the in vitro bile acid binding capacity of oat β-glucans by oxidation were investigated (**Figure 2**). There was a general increase in the in vitro bile acid binding capacity of the β-glucan with progressively increasing degree of oxidation. Compared to native β-glucan, the bile acid binding capacity of 100% oxidized β-glucan was increased by 2.5-fold. Thus, it may be correlated to the enhanced cholesterol-lowering effects of the oxidized β-glucan because the binding and excretion of bile acid aid in reducing blood cholesterol levels (*22, 23*). In addition, it is reported that water-soluble dietary fibers are more effective in lowering cholesterol levels than water-insoluble dietary fibers (*24, 25*). Thus, water solubility appears to be involved in biological activities such as bile acid binding capacity (*6*). Therefore, better functionality of oxidized β-glucans to bind bile acids in vitro might be partly explained by their improved water solubility. Thereby, because the 100% oxidized β-glucan exhibited great in vitro bile acid binding capacity, it was selected for in vivo study on the effect of oxidation on hypocholesterolemic effect in rats.

Table 3. Body Weight and Feed Efficiency of Rats Fed High-Cholesterol Diet with Native and Oxidized Oat β-Glucans for 4 Weeks[a]

group[b]	initial wt (g)	wt gain (g)	food intake (g)	feed efficiency[c]
CON	134.25 ± 3.16[d]	131.75 ± 7.52	528.6 ± 24.00[d]	0.25 ± 0.20[d]
BG	136.13 ± 2.28	147.00 ± 6.35	542.25 ± 4.57	0.27 ± 0.01
BO	136.25 ± 2.17	124.25 ± 7.65	526.88 ± 17.14	0.24 ± 0.02
OXI	136.25 ± 2.28	128.50 ± 7.63	562.88 ± 9.36	0.23 ± 0.01

[a] Values are means ± SD. [b] CON, high-cholesterol diet; BG, high-cholesterol diet containing native β-glucan; BO, high-cholesterol diet containing native and 100% oxidized β-glucans; OXI, high-cholesterol diet containing 100% oxidized β-glucan. [c] Feed efficiency = mean body wt gain (g)/mean food consumption (g). [d] Not significantly different among samples at the α = 0.05 level by Duncan's multiple-range test.

Hypocholesterolemic Effect of Oxidized β-Glucan. The effect of oxidation on the cholesterol-lowering effect of oat β-glucan was determined on hypercholesterolemic rats. **Table 3** presents the body weight, weight gain, food intake, and feed efficiency of the rats, which were not significantly different among the experimental groups. The serum lipid profiles of the rats are shown in **Table 4**. Overall, the rats fed the diets containing native and oxidized β-glucans exhibited lower levels of total cholesterol, although significant differences between the BG/BO and CON groups were not detected. Specifically, the total cholesterol level in the OXI group was significantly lower by 20% than that in the control group. Also, the consumption of the diet with oxidized β-glucan was efficient in significantly reducing the serum triglyceride level in the rats. Also, LDL-C and VLDL-C had trends similar to that of triglyceride. However, there were no noticeable differences in HDL-C. Thus, the diets containing oxidized β-glucan seemed to be more effective at improving the serum lipid profile of the rats, compared to native β-glucan.

Table 5 exhibits the changes in the levels of total cholesterol and triglyceride in the liver of rats that consumed the diets with oxidized β-glucans. The contents of total cholesterol and triglyceride in liver showed tendencies similar to those in serum. With respect to total cholesterol, the groups fed the diets containing oxidized β-glucan had significantly lower values than the control group. However, statistically, the same triglyceride level was observed among the groups even though the groups fed the diets with oxidized β-glucan exhibited lower mean values. It is widely recognized that the consumption of β-glucan leads to an elevated intestinal viscosity that prevents the absorption of bile acids and promotes their excretion (*23*). Therefore, the fecal excretion of bile acids can play an important role in reducing the cholesterol level in the body because a great proportion of cholesterol is used for bile acid synthesis.

Overall, the rats that were fed the diets supplemented with the β-glucan derivative excreted more fecal matter as shown in **Table 6**. This is likely explained by one of the well-known functionalities of β-glucan as a soluble fiber, which is a laxative effect by proliferation of fecal microorganisms, consequently promoting an increase in stool weight. Hence, it seems plausible to assume that the oxidation did not have a negative effect on

Table 4. Serum Lipid Profile of Rats Fed High-Cholesterol Diet with Native and Oxidized Oat β-Glucans for 4 Weeks[a]

group[b]	triglyceride (mg/dL)	total cholesterol (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)
CON	59.60 ± 5.36 b	176.84 ± 14.13 b	28.70 ± 4.05 ns	136.22 ± 14.78 b	11.92 ± 1.07 b
BG	49.31 ± 5.67 ab	169.01 ± 8.26 ab	29.08 ± 1.77	130.06 ± 8.03 a	9.86 ± 1.13 ab
BO	43.05 ± 2.81 a	162.78 ± 11.01 ab	30.57 ± 3.07	123.60 ± 11.53 a	8.61 ± 0.56 a
OXI	41.15 ± 5.56 a	139.89 ± 11.35 a	26.14 ± 1.83	105.52 ± 12.63 a	8.23 ± 1.11 a

[a] Values are means ± SD. Values with different letters within the same column are significantly different among samples at the $\alpha = 0.05$ level by Duncan's multiple-range test. ns, not significantly different among samples at the $\alpha = 0.05$ level by Duncan's multiple-range test. [b] CON, high-cholesterol diet; BG, high-cholesterol diet containing native β-glucan; BO, high-cholesterol diet containing native and 100% oxidized β-glucans; OXI, high-cholesterol diet containing 100% oxidized β-glucan.

Table 5. Liver Lipid Profile of Rats Fed High-Cholesterol Diet with Native and Oxidized Oat β-Glucans for 4 Weeks[a]

group[b]	liver wt (g)	triglyceride (mg/g of liver)	total cholesterol (mg/g of liver)
CON	14.09 ± 0.53 ab	10.01 ± 0.94 ns	14.02 ± 1.43 c
BG	14.80 ± 0.60 b	9.42 ± 0.57	11.02 ± 0.75 c
BO	12.76 ± 0.39 a	9.13 ± 0.99	8.78 ± 0.72 ab
OXI	12.79 ± 0.53 a	8.36 ± 0.84	7.31 ± 0.66 a

[a] Values are means ± SD. Values with different letters within the same column are significantly different among samples at the $\alpha = 0.05$ level by Duncan's multiple-range test. ns, not significantly different among samples at the $\alpha = 0.05$ level by Duncan's multiple-range test. [b] CON, high-cholesterol diet; BG, high-cholesterol diet containing native β-glucan; BO, high-cholesterol diet containing native and 100% oxidized β-glucans; OXI, high-cholesterol diet containing 100% oxidized β-glucan.

Table 6. Fecal Weight, Lipid Profile, and Bile Acid Excretion in Feces of Rats Fed High-Cholesterol Diet with Native and Oxidized Oat β-Glucans for 4 Weeks[a]

group[b]	fecal wt (g/day)	triglyceride (mg/day)	total cholesterol (mg/day)	bile acid (mmoL/day)
CON	1.76 ± 0.09 a	0.38 ± 0.07 a	13.24 ± 1.93 a	4.10 ± 0.27 ab
BG	1.93 ± 0.14 ab	0.74 ± 0.10 b	18.39 ± 1.69 b	5.23 ± 0.54 b
BO	2.13 ± 0.06 b	0.88 ± 0.08 b	21.18 ± 1.20 b	4.86 ± 0.34 b
OXI	1.97 ± 0.15 ab	0.97 ± 0.09 b	21.55 ± 1.50 b	3.17 ± 0.23 a

[a] Values are means ± SD. Values with different letters within the same column are significantly different among samples at the $\alpha = 0.05$ level by Duncan's multiple-range test. [b] CON, high-cholesterol diet; BG, high-cholesterol diet containing native β-glucan; BO, high-cholesterol diet containing native and 100% oxidized β-glucans; OXI, high-cholesterol diet containing 100% oxidized β-glucan.

the laxative property of β-glucan. Total cholesterol and triglyceride contents in feces showed the same tendency, exhibiting more excretion in rats fed the diets containing β-glucan samples regardless of oxidation. More fecal eliminations of total cholesterol and triglyceride were favorably correlated to their reduced levels in the serum and liver as shown in **Tables 4** and **5**. The possible cause of greater fecal excretion of cholesterol and triglyceride would be related to their laxative activity, consequently causing greater weight of fecal matter that contained more cholesterol and triglyceride.

In conclusion, β-glucans derived from oats were subjected to chemical modification, specifically 2,2,6,6-tetramethyl-1-piperidine oxoammonium ion-mediated oxidation whereby C6 primary hydroxyl groups were selectively oxidized into carboxyl groups. The results showed that the oxidation gave rise to increased water solubility of oat β-glucans. In addition, when the lipidemic responses in rats were studied, the rats fed the diets containing the oxidized samples had enhanced lipid profiles in serum and liver, which were favorably correlated to greater fecal excretion.

As the first in vivo study of oxidized oat β-glucan, this study shows the potential possibility of the use of oxidized oat β-glucan as an active cholesterol-lowering ingredient. Thus, the expanded use of β-glucans derived from cereal sources may be taken into account for better health improvement.

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