Reactivity of Milk Cholesterol with Bacterial Cholesterol Oxidases

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We tested three bacterial cholesterol oxidases on cholesterol in milk and milk fractions to probe cholesterol reactivity. *Pseudomonas fluorescens* cholesterol oxidase oxidized cholesterol rapidly in homogenized/pasteurized milk or sonicated raw milk but catalyzed cholesterol oxidation only slowly in intact raw milk. Pasteurization had little influence on oxidation rates. Oxidases from *Streptomyces* and *Rhodococcus* species were significantly less active in milk than the *Pseudomonas* enzyme. The *Pseudomonas* oxidase oxidized cholesterol in all raw milk fractions tested, providing the fractions were first disrupted by sonication or treatment with lipase. Cholesterol oxidation in homogenized milk depended on temperature and enzyme concentration. A maximum of 85% of the initial whole milk cholesterol (initially 100–138 μg/mL) was oxidized in 96 h, giving final cholesterol concentrations of 20–30 μg/mL. Sonicated skim milk cholesterol concentrations (initially 17–20 μg/mL) were reduced to 4–6 μg/mL.

I. INTRODUCTION

Various physical, chemical, and biological methods have been proposed for reducing cholesterol in milk because of the perceived association of dairy products with cardiovascular disease (Deeth, 1983). The physical/chemical methods include skimming, blending vegetable oils into skim milk (Reiser, 1969; Durkley, 1982), extracting milk fat with solvents (supercritical CO_2; Shishikura et al., 1986; Kankare and Antila, 1989), or adsorbing milk fat with agents that form cholesterol complexes (Micich, 1990). Physical/chemical methods tend to be relatively nonselective, removing flavor and nutritional components with the cholesterol. Skim milk has reduced fat and cholesterol, but its altered appearance, texture, and taste make it less desirable than whole milk for consumption or conversion to other dairy products such as cheese.

Bacterial and enzyme methods offer potential cost and selectivity advantages. *Eubacterium* can anaerobically reduce cholesterol to coprostanol, which is absorbed poorly by the human gastrointestinal tract (Eyssen et al., 1973; Brinkley et al., 1982). *Rhodococcus equi* oxidizes cholesterol to carbon dioxide and water (Aihara et al., 1988a) and was used (as cell suspensions or crude extracts) to treat egg yolk and lard (Aihara et al., 1988b; Watanabe et al., 1989; Johnson and Somkuti, 1990). Potentially harmful oxidation products (Bischoff and Bryson, 1977; Peng et al., 1979) apparently did not accumulate in cell suspension treated egg yolk or lard. *R. equi* produces a soluble cholesterol oxidase which catalyzes the first step in cholesterol oxidation (Watanabe et al., 1989). Unless whole milk is to be fermented, bacterial cells or crude enzyme extracts cannot be used. In this paper, we describe experiments with cholesterol oxidase to assess milk cholesterol reactivity.

II. MATERIALS AND METHODS

Materials. Cholesterol oxidase (EC 1.1.3.6) from *Pseudomonas fluorescens*, *Streptomyces* sp., and *Rhodococcus erythropolis* and *Pseudomonas* cholesterol esterase (EC 3.1.1.13), *Pseudomonas* lipase (EC 3.1.1.3), *Clostridium welchii* phospholipase C (EC 3.1.4.3), protease type XXV (a mixture of proteases from *Streptomyces griseus*), catalase (EC 1.11.1.6), and horseradish peroxidase (EC 1.11.1.7) were purchased from Sigma Chemical Co., St. Louis, MO. The cholesterol oxidases from *Pseudomonas* and *Streptomyces* were dissolved in 20 mM potassium phosphate buffer (pH 7.0, 50 units/mL) before use. We added *Rhodococcus* oxidase suspension directly to incubation mixtures to the final enzyme concentrations.

Cholesterol, 4-cholesten-3-one, and 4-aminoantipyrine were purchased from Sigma. Cow's milk was used for all of the experiments described here. Homogenized, pasteurized milk, certified raw (unpasteurized) nonfat milk, and certified raw (unpasteurized) milk were purchased from local retail vendors.

Milk Fractions. Milk fractions (milk fat, globule membranes, and skim milk) were prepared from raw milk by centrifuging the raw milk and separating the cream (top layer) and skim milk (liquid) fractions.

Raw milk (400 mL, unpasteurized) was centrifuged at 16000*g* (4 °C) for 1 h. The cream layer was collected and the liquid phase again centrifuged at 16000*g* for 1 h. The second cream layer was removed and discarded. The liquid (skim milk) fraction was stored on ice until used (within 24 h).

Cream (approximately 20 mL) from the first centrifugation was used for preparing the milk fat and globule membrane fractions. The cream was washed once by suspending it in 40 mL of 20 mM potassium phosphate (pH 7.0, phosphate buffer) containing 0.85% (w/v) NaCl followed by centrifugation at 30000*g* for 1 h. The cream layer, after centrifugation, was separated from the liquid phase and frozen in a dry ice–acetone bath to disrupt the milk globule membranes. The cream was stored frozen for at least 24 h. After storage, the cream was thawed, resuspended in 40 mL of phosphate buffer, and centrifuged at 30000*g* for 1 h. The milk fat (top layer), liquid phase, and pellet from the centrifugation were separated, and the liquid phase was discarded. The milk fat layer was resuspended in 40 mL of phosphate buffer by ultrasonic treatment (milk fat fraction). The pellet was washed three times with 10 mL of phosphate buffer followed by centrifugation at 30000*g* for 20 min. The final pellet (membrane fraction) was resuspended in 10 mL of phosphate buffer and stored on ice until used. The protein concentration of the membrane fraction was 970 μg/mL.

Assays. All assays were in triplicate. Protein was assayed according to the dye-binding method of Bradford (1976) using bovine serum albumin (Sigma, fraction V) as a standard. Normally, 20 mM phosphate buffer was used as the diluent. Protein in milk membranes was also assayed according to the Bradford method, but 0.1 N NaOH was used as the diluent for the bovine serum albumin standards and membrane samples (to solubilize the lipid).

We used the colorimetric assay of Smith and Brooks (1976) to assay cholesterol oxidase activity in our stock enzyme

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preparations. The assay depended on horseradish peroxidase, 4-aminoantipyrine, phenol, and hydrogen peroxide for color development. We monitored absorbances using a Gilford Model 260 constant-temperature recording spectrophotometer (Ciba Corning Diagnostics Corp., Oberlin, OH).

Cholesterol in milk and cholesterol oxidase activity in milk samples were assayed according to the *o*-phthalaldehyde procedure (Rudell and Morris, 1973) after the milk samples were saponified to eliminate interference by triglycerides (Bachman and Lin, 1975). We carried out the saponifications on 1-mL samples of milk. The samples were saponified in 3 mL of 50% (w/v) potassium hydroxide plus 3 mL of 95% ethanol at 60 °C for 15 min. The saponified samples were extracted for 1 h by shaking with 5 mL of hexane plus 1 mL of distilled water. Aliquots (1 mL) of the hexane extract were evaporated to dryness under N_2 and then treated with 2 mL of a solution of *o*-phthalaldehyde (500 μg/mL) in glacial acetic acid. After 10 min, concentrated H_2SO_4 (1 mL) was added and the mixture vortexed. We read absorbances at 590 nm after 10 min of color development. Estimates of cholesterol concentration were based on standards containing 100 μg of pure cholesterol (1 mg/mL in methanol), carried through the saponification and extraction steps.

When anticipated cholesterol concentrations were low, as in skim milk fractions, we modified the assay procedure by saponifying 2 mL of milk (instead of 1 mL) and by evaporating 3 mL of the hexane extract (instead of 1 mL). These modifications increased the sensitivity of the *o*-phthalaldehyde assay 6-fold.

From 85 to 90% of the cholesterol in milk is free cholesterol; only 5–10% is present as esters (Wood and Bitman, 1986). Cholesterol esters do not react with cholesterol oxidase (Smith and Brooks, 1976) and would appear as unreacted cholesterol in *o*-phthalaldehyde assays of oxidase-treated milk.

Treatment with Enzymes, Pasteurization, and Sonication. All assays and enzyme incubations were conducted on triplicate samples. Enzymes were added to 10 mL of milk samples or milk fractions to a final concentration of 0.2 unit/mL, and milk samples and enzymes were incubated with shaking (150 rpm) in 125-mL flasks in a water bath at 37 °C unless indicated otherwise. When incubation times were longer than 180 min, streptomycin sulfate (200 μg/mL final concentration) and chloramphenicol (50 μg/mL) were added to suppress bacterial growth.

Milk fat fractions were diluted 1:5 in 20 mM potassium phosphate (pH 7.0, 87.6 μg/mL final cholesterol concentration) before cholesterol oxidase was added. Membrane fractions (950 μg/mL protein in 20 mM potassium phosphate, pH 7.0) were in a volume of 2 mL.

For some experiments, raw skim milk (100 mL volume) was pretreated with protease (0.2 unit/mL final concentration of Sigma Pronase E) at 30 °C for 24 h before other enzymes were added. Streptomycin sulfate (200 μg/mL) and chloramphenicol (50 μg/mL) were added to suppress bacterial growth. After incubating with protease, 10 mL of protease inhibitor (phenylmethanesulfonyl fluoride) was added to a final concentration of 1 mM. The protease-treated milk was used for enzyme experiments in the same way as other milk samples.

Raw milk samples were pasteurized by holding them at 61 °C for 30 min. The samples were then cooled on ice.

Sonication treatments of milk and milk fractions were carried out by pulsing 50 mL of sample with a Cole-Parmer Model 4710 ultrasonic homogenizer for 30 min. Samples were cooled on ice between bursts of ultrasonic frequencies. The mean particle size after treatment was approximately 2 μm, estimated visually by phase contrast microscopy.

Cholesterol Oxides. Formation of 7-ketocholesterol and 7-β-hydroxycholesterol was monitored by high-performance liquid chromatography (HPLC) using the methods of Tsai and Hudson (1981) and Park and Addis (1985). Triplicate commercial homogenized/pasteurized milk samples (35 mL) were extracted with 100 mL of chloroform/methanol (2:1 v/v). The extracts were collected by centrifugation at 2000*g* for 5 min and then evaporated to dryness under N_2. The residue was dissolved in 5 mL of 5% ethyl ether/95% hexane (v/v) and adsorbed onto 1 g of silica (Sigma, type H). The silica was washed with 30 mL of 10% (v/v) ether in hexane to remove cholesterol and fat. After washing, the cholesterol oxides were eluted with 20 mL of 37.5% (v/v) ether in hexane. The eluted solvent (100 μL) was injected into an Isco HPLC system with a silica column (0.46 by 25 cm, 5-μm bead size) and chromatographed isocratically using 7% (v) 2-propanol in hexane as the mobile phase. The flow rate was 1 mL/min, and peaks were detected by their absorbances at 210 nm.

Table I. Oxidation of Cow's Milk Cholesterol by Bacterial Cholesterol Oxidases

enzyme source	milk[a]	cholesterol,[b] μg/mL 0 min	180 min	oxidized, %
P. fluorescens	R	115 (2.5)	96.1 (5.5)	16.4
	H	108 (2.1)	24.2 (0.5)	77.6
+*Pseudomonas* lipase	H	119 (1.5)	35.3 (1.9)	70.3
R. erythropolis	R	105 (5.3)	103 (2.5)	1.9
	H	103 (1.5)	82.4 (2.7)	20.0
+*Pseudomonas* lipase	H	113 (2.1)	106 (2.0)	6.2
Streptomyces species	R	110 (0.6)	111 (2.1)	0
	H	98.9 (1.3)	83.0 (5.9)	16.1
+*Pseudomonas* lipase	H	112 (0.6)	66.8 (4.0)	40.4

[a] R, certified raw milk; H, homogenized and pasteurized milk. [b] Parentheses indicate standard deviations.

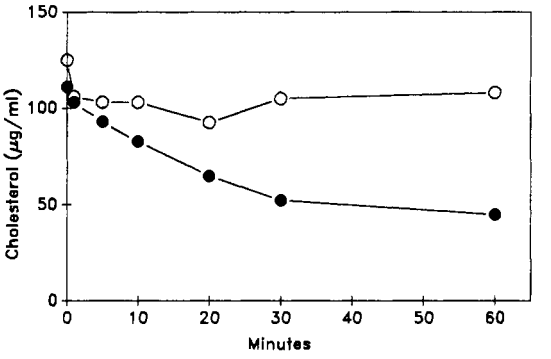

Figure 1. Oxidation of homogenized and raw milk by cholesterol oxidase: raw milk (○); homogenized milk (●).

III. RESULTS AND DISCUSSION

Milk Cholesterol Oxidation. We tested commercial cholesterol oxidases from *P. fluorescens*, *R. erythropolis* and *Streptomyces* species at 37 °C on raw milk and commercial homogenized/pasteurized milk (Table I). None of the enzymes oxidized more than 17% of the cholesterol in raw milk in 3 h. In homogenized/pasteurized milk, the *P. fluorescens* oxidase oxidized 78% of the initial cholesterol in 3 h, compared with 20% for the *Rhodococcus* enzyme and 16% for the *Streptomyces* enzyme. We used the *Pseudomonas* (*P. fluorescens*) cholesterol oxidase for subsequent experiments.

If *Pseudomonas* lipase was added at the same time as cholesterol oxidases to milk, cholesterol oxidation increased during 3 h for the *Streptomyces* oxidase but not for the *P. fluorescens* or *Rhodococcus* oxidases. This suggests that the three enzymes exhibit real differences in their cholesterol-oxidizing activities with milk and that the *Pseudomonas* oxidase activity is not the result of contaminating lipase.

Figure 1 shows a time course for cholesterol oxidation by 0.2 unit/mL of *Pseudomonas* cholesterol oxidase in raw and commercial homogenized/pasteurized whole milks. Much of the cholesterol oxidation (approximately 50%) in homogenized milk occurred within 60 min at 37 °C. Little or no cholesterol was oxidized in 60 min in raw milk samples. After extended incubation (28 h; data not shown), 76% of the initial homogenized/pasteurized milk cholesterol and 50% of the raw milk cholesterol were oxidized.

Table II. Oxidation of Raw Milk Cholesterol by *P. fluorescens* Cholesterol Oxidase

milk	cholesterol,[a] μg/mL, after incubation of 0 min	30 min	60 min	180 min
whole milk	134 (5.0)	136 (0.6)	133 (1.7)	135 (2.6)
pasteurized	138 (0.6)	133 (8.0)	132 (2.3)	121 (2.1)
sonicated	135 (1.2)	47 (3.1)	30 (1.7)	22 (3.1)
milk fractions				
milk fat	438 (26.5)			302 (29.0)
membrane	15.7 (1.25)			7.7 (0.8)
skim milk	17.8 (0.8)			16.8 (0.7)
sonicated	17.0 (1.1)			6.1 (0.4)
nonfat	28.3 (0.4)			26.0 (0.3)
sonicated	28.9 (1.7)			4.4 (0.2)

[a] Parentheses indicate standard deviations.

We observed a maximum of 85% of initial cholesterol oxidized in homogenized/pasteurized milk after incubation for 96 h at 37 °C with streptomycin sulfate (data not shown). If *Pseudomonas* cholesterol esterase (0.2 unit/mL) was included with *P. fluorescens* cholesterol oxidase in homogenized/pasteurized milk, the rate or extent of cholesterol oxidation within 24 h did not seem to increase compared to cholesterol oxidase alone.

Table II shows that pasteurization of raw milk at 61 °C had only a slight effect on cholesterol oxidation by the *P. fluorescens* oxidase after incubation for 3 h with enzyme compared with untreated raw milk. Sonication treatment of the unpasteurized raw milk, however, had a marked effect; approximately 84% of the initial cholesterol was oxidized in 3 h. The activity of the *P. fluorescens* oxidase apparently depended on mechanical disruption of the milk globules by sonication and did not result from destruction of an inhibitor by pasteurization.

Previous work indicates that cholesterol is distributed throughout the various milk fractions. From 67 to 95% of the cholesterol is dissolved in the milk globule triglycerides (Mulder and Zuidhoff, 1958; Huang and Kuksis, 1967). Electron microscopic evidence allowed visualization of cholesterol in the globule fat and globule membranes (Martin, 1989). Keenan et al. (1983) reported that homogenization disrupted the milk globule membranes but that some membrane material remained associated with the globules afterward. The differences in cholesterol reactivity between raw milk and homogenized milk probably reflect the condition of the milk globule membranes, because cholesterol oxidase is known to be noncatalytic toward cholesterol in undisturbed cell membranes (Gottlieb, 1977; Patzer et al., 1978; Lange et al., 1984; Thurnhofer et al., 1986).

Raw milk fractions showed varying susceptibilities to cholesterol oxidation by the *Pseudomonas* oxidase. After incubation for 3 h at 37 °C with 0.2 unit/mL of enzyme, only 31% of the cholesterol in the milk fat fraction was oxidized, probably because the milk fat droplets tended to melt and coalesce during incubation, reducing the surface area for cholesterol oxidation.

The milk membrane fraction was also susceptible to cholesterol oxidation; 51% of the cholesterol was oxidized even though the initial cholesterol concentration was quite low (15.7 μg/mL). The susceptibility to cholesterol oxidase probably resulted from disruption by freezing and thawing during preparation.

The skim milk fraction was resistant to oxidation. The initial cholesterol concentration (17.8 μg/mL) was similar to that of the membrane fraction. Only 5.6% of the skim milk cholesterol was oxidized in 3 h. Cholesterol in the skim milk fraction (Table II) was oxidized by cholesterol oxidase if the skim milk was first sonicated for 25 min. This resembled the behavior we observed for whole raw milk.

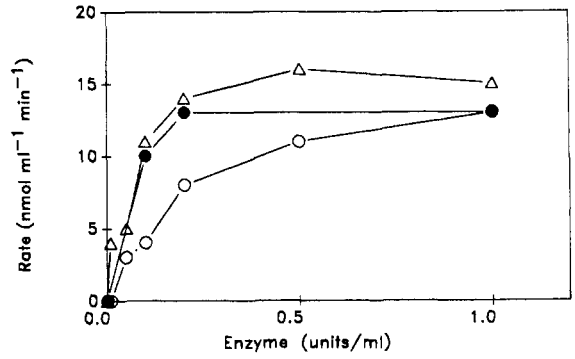

Figure 2. Effect of *P. fluorescens* cholesterol oxidase concentration and temperature on rates of cholesterol oxidation in milk. Temperatures: 35 (○), 45 (●), and 55 °C (△).

The cholesterol in the commercial nonfat milk, as in our skim milk fraction and whole raw milk, was not oxidized by cholesterol oxidase unless the milk was sonicated first. In the skim and commercial nonfat milks, the cholesterol concentrations after oxidase treatment (4–6 μg/mL) fell below the final cholesterol concentrations achieved in whole milk (20–30 μg/mL) incubated for extended times with oxidase; they show that whole milk cholesterol oxidation was not limited entirely as a result of low cholesterol concentrations.

Oxidase Concentration and Temperature. Rates of cholesterol oxidation in commercial homogenized/pasteurized milk increased with increasing temperature and *P. fluorescens* oxidase concentration as expected (Figure 2). We observed a slow oxidation of cholesterol at 4 °C and 0.05 unit/mL of enzyme; the cholesterol oxidase oxidized 17.3% of the cholesterol in 24 h and 51.8% after 7 days compared with 64.1% of the milk cholesterol in 90 min at 37 °C (initial cholesterol concentration, 113 μg/mL).

We obtained oxidation rates at 25 °C (0.2 unit/mL of *Pseudomonas* cholesterol oxidase) of 2.7 nmol/min [61% of the initial cholesterol (104 μg/mL) was oxidized in 3 h]. This was nearly 27% of the rate at 35 °C with the same enzyme concentration (Figure 2). We observed maximum oxidation rates at 55 °C at 1 unit/mL of enzyme (the highest enzyme concentration tested). Oxidation rates at 45 and 55 °C became maximal at 0.2 unit/mL of added enzyme or greater. The rates tended to increase nonlinearly with enzyme concentration, suggesting that the active sites were not saturated with cholesterol. These rates are much slower than would be expected from the measured values for the K_m (25 μM or 9.7 μg/mL) and V_{max} of *P fluorescens* cholesterol oxidase (Cheillan et al., 1989).

Treatment with Lipase and Protease. *Pseudomonas* lipase treatments (Table III) were as effective as sonication treatments in stimulating raw milk or raw skim milk cholesterol oxidation by *Pseudomonas* cholesterol oxidase. Phospholipase C, which should attack membranes, stimulated cholesterol oxidation, but stimulation was not as great as that of *Pseudomonas* lipase. These results suggest that raw milk cholesterol is protected by lipid milk components and exposed during hydrolysis of the lipids by lipases.

Lipase treatment of homogenized milk did not increase cholesterol oxidation by the *Pseudomonas* or *Rhodococcus* cholesterol oxidase but did result in a small increase in *Streptomyces* cholesterol oxidase oxidation. The differences in the three oxidase activities probably reflect

Table III. Effect of Lipase and Protease Treatments of Milk on *P. fluorescens* Cholesterol Oxidase Activity[a]

addition to milk	cholesterol μg/mL 0 min	cholesterol μg/mL 180 min	oxidized, %
raw milk			
lipase + PC	113 (0.5)	119 (9.1)	0
PCO	129 (1.2)	104 (8.5)	19.4
PCO + lipase	128 (2.5)	43.3 (1.2)	66.2
PCO + PC	144 (14.8)	79.3 (7.5)	44.9
PCO + lipase + PC	138 (7.6)	48.0 (1.0)	65.2
skim milk			
PCO	20.0 (0.5)	18.4 (1.0)	8.0
PCO + lipase	20.5 (1.5)	11.8 (0.7)	42.4
PCO + PC	19.9 (0.2)	19.3 (1.2)	3.0
protease	25.7 (1.5)	21.6 (1.0)	16.0
PCO + protease	21.6 (1.6)	10.5 (0.36)	51.4
PCO + protease + lipase	21.6 (1.0)	7.7 (0.43)	64.4

[a] PCO, *Pseudomonas* cholesterol oxidase; lipase, *Pseudomonas* lipase; PC, *C. welchii* phospholipase C; protease, Sigma Pronase E. Parentheses indicate standard deviations.

real differences in the enzymes and not differences in possible contaminating lipases.

Pretreatment of raw skim milk with protease (Pronase E) also increased cholesterol oxidation compared to that in untreated skim milk. The protease treatment was more effective when combined with lipase treatment than protease or lipase treatment alone, suggesting that milk protein components also limited cholesterol oxidation, possibly by binding specifically or nonspecifically to cholesterol.

Cholesterol Oxides. Cholesterol slowly autoxidizes in air to form some 80 different oxidation products. Cholesterol oxides are considered toxic and a potential health problem with any food treatment method that exposes cholesterol to air. We looked for 7-keto- and 7-β-hydroxycholesterol formation in milk samples (35 mL) incubated for 48 h at 30 °C with *P. fluorescens* cholesterol oxidase. Control samples consisted of untreated milk refrigerated for 48 h, untreated milk incubated at 30 °C with shaking, and milk samples treated with cholesterol oxidase (*P. fluorescens*, 0.2 unit/mL final concentration) plus catalase (56 units/mL final concentration). We observed no peaks by HPLC that corresponded to our 7-keto- or 7-β-hydroxycholesterol standards in cholesterol oxidase treated milk or controls.

Using Enzymes To Treat Milk. Our experiments show that enzyme methods might be used to reduce cholesterol levels in milk if a mechanical or enzyme pretreatment step is included. Although we used cholesterol oxidase as an enzyme source, our results are probably applicable to most enzyme systems. Enzyme treatments for cholesterol address only the problem of cholesterol and not the related health problem of saturated fats (Watts, 1989).

Cholesterol oxidase (by itself) or bacteria that produce it might not be suitable for removing food cholesterol because the hydrogen peroxide formed might generate toxic compounds or undesirable flavor components. However, it might be possible to control hydrogen peroxide formation with catalase, peroxidase, or reducing agents.

4-Cholesten-3-one, a principal product of cholesterol oxidase, is believed to be toxic because a 5% solution in dimethyl sulfoxide, instilled intrarectally into mice, caused nuclear aberrations in mouse tissue (Suzuki et al., 1986) and because it was moderately cytotoxic in in vitro toxicity tests with cultured aortic tissue cells (Peng et al., 1979). Eukaryotic enzyme systems might be used to remove 4-cholesten-3-one by conversion to cholestanol (Bjorkhem and Karlmar, 1974). However, the relevance to health of in vitro toxicity tests was recently questioned, and pitfalls in many in vivo toxicity tests were discussed (Smith and Johnson, 1989). 4-Cholesten-3-one is nonmutagenic in the Ames test for carcinogens (Suzuki et al., 1986). Clearly, more work is needed to establish the safety of steroids, especially dietary steroids, generated by treatment of foods.

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