Cholesterol Removal from Homogenized Milk with β-Cyclodextrin

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ABSTRACT

This study was carried out to determine optimum conditions of five different factors (β-cyclodextrin concentration, mixing temperature, mixing time, centrifugal force, and centrifugation time) in reduction of cholesterol in 3.6% fat, homogenized milk by application of β-cyclodextrin. β-Cyclodextrin at 0.5 to 1.5% provided 92.2 to 95.3% removal of cholesterol when mixed at 10°C for 10 min. Among other factors, mixing time (5 to 20 min) did not significantly affect cholesterol removal. Removal was enhanced with increasing centrifugal forces up to 166 $\times$ g (95.9%) but decreased thereafter. Various centrifugation times (5 to 20 min) did not have significant effects. Based on these results, we suggest that the optimum conditions for the process are addition of 1.5% β-cyclodextrin, mixing temperature of 10°C, 10-min mixing time, and centrifugation at 166 $\times$ g for 10 min.
(**Key words:** cholesterol removal, β-cyclodextrin, homogenized milk)

Abbreviation key: β**-CD** = β-cyclodextrin.

INTRODUCTION

Experiments on animals and human have shown that plasma cholesterol can be raised by increased intake of cholesterol and saturated fat (2, 5, 16, 19). Most consumers are concerned about excessive intakes of cholesterol and fat in their daily diets because of the risk of coronary heart disease (4, 5). There have been dramatic increases in no-, low-, and reduced-cholesterol products in the market place (18).

Food companies have developed many methods to reduce cholesterol by using various physical, chemical, and biological methods. Some examples are blending in of vegetable oils (3), extraction by organic solvent (7), adsorption with saponin and digitonin (10) to form cholesterol complexes, degradation of cholesterol by cholesterol oxidases (23), and removal by supercritical carbon dioxide extractions (15). However, most of these methods are relatively nonselective and remove flavor and nutritional components when cholesterol is removed. Moreover, some methods require high investment and operation costs.

β-cyclodextrin (β**-CD**) is a cyclic oligosaccharide composed of α-(1–4) linkages of seven glucose units. It has a cavity at the center of its molecular arrangement, which forms an inclusion complex with various compounds including cholesterol (21). The β-CD is also nontoxic, edible, nonhygroscopic, and chemically stable and is easy to separate from the complex (11). Thus, β-CD provides advantages when used for removal of cholesterol from various foods.

Several studies have reported that cholesterol is effectively removed from animal fats with β-CD (6, 8, 13, 22). In our preliminary study (unpublished data), only 25 to 30% of cholesterol was removed from unhomogenized milk using 1% β-CD, at 7°C and mixing of 10 min. This result might have been due to large fat globules, including cholesterol molecules, in unhomogenized milk, which could not interact with β-CD. Therefore, the objective of this study was to find optimum conditions for cholesterol removal from homogenized milk by addition of β-CD.

MATERIALS AND METHODS

Materials

Commercial milk (3.6% milk fat) was purchased from a retail store as needed, and β-CD (purity 99.1%) was obtained from Nihon Shokuhin Cako Co. LTD. (Osaka, Japan). Cholesterol and 5α-cholestane were purchased from Sigma Chemical Co. (St. Louis, MO), and all solvents were gas-chromatographic grade.

Cholesterol Removal

To study the effects of five different factors, 50 g of milk was placed in a 1000-ml beaker, and different concentrations of β-CD (0.5, 1.0, 1.5, or 2.0%) were added. The mixture was stirred at 800 rpm with a blender (Tops; Misung Co., Seoul, Korea) in a temperature-controlled water bath with different mixing temperatures (4, 10, 15, 20, or 25°C) and mixing times (5, 10, 15, 20, or 25

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min). The mixture was centrifuged (HMR-220IV; Hanil Industrial Co., Seoul, Korea) with different centrifugal forces (55, 111, 166, 222, or 278 × *g*) for 5, 10, 15, 20, or 25 min.

For each treatment after centrifugation, the supernatant fraction containing cholesterol-reduced milk was decanted and was used for cholesterol determination. All treatments were duplicated.

Extraction and Determination of Cholesterol

For the extraction of cholesterol from milk, 1 g of the β-CD-treated milk was placed in a screw-capped glass tube (15 mm × 180 mm), and 1 ml of 5α-cholestane (1 mg/ml) was added as an internal standard. The sample was saponified at 60°C for 30 min with 5 ml of 2 *M* ethanolic potassium hydroxide solution (1). After cooling to room temperature, cholesterol was extracted with 5 ml of hexane (1). The process was repeated four times. The hexane layers were transferred to a round-bottomed flask and dried under vacuum. The extract was redissolved in 1 ml of hexane and was stored at −20°C until analysis.

Total cholesterol was determined on a silica-fused capillary column (HP-5, 30-m × 0.32-mm i.d. × 0.25-μm thickness) using a gas chromatograph (5890A; Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector. Temperatures of the injector and detector were 270 and 300°C, respectively. Oven temperature was programmed to increase from 200 to 300°C, at 10°C/min, and then was constant for 20 min. Nitrogen was used as carrier gas at a flow rate of 2 ml/min. The sample injection volume was 2 μl with a split ratio of 1/50. Quantitation of cholesterol was done by comparing sample peak areas with the response of an internal standard.

The percentage of cholesterol reduction was calculated as follows: cholesterol reduction (%) = amount of cholesterol in β-CD-treated milk × 100/amount of cholesterol in untreated milk (control). Cholesterol determination for a control was done with each treatment batch.

Statistical Analysis

Data from each experiment were analyzed by ANOVA with SAS software (17) (SAS Institute Inc., Cary, NC). The significance of the results was analyzed by least significant difference (LSD) test. Differences of $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Effect of β-CD Concentration

The effect of β-CD concentration on cholesterol removal from milk is shown in Table 1. The average cholesterol content of the milks (control) was 13.14 ± 2.2 mg/100 g. The β-CD (0.5, 1.0, or 1.5%) removed 92.2 to 95.3% of the cholesterol (when mixed at 10°C for 10 min). Addition of β-CD at 2.0% showed the least efficiency of removal (88.6%). Similar studies (10, 14) using saponin and digitonin for cholesterol adsorption indicated that above certain concentrations, saponin and digitonin showed a decrease of cholesterol removal from milk and butter oil, respectively. These authors have suggested that an excess of β-CD could compete with itself to bind to cholesterol molecules, resulting in reduced cholesterol adsorption. Therefore, it appears that 0.5% β-CD may be sufficiently effective to remove greater than 90% of cholesterol from homogenized milk.

TABLE 1. Effect of various β-cyclodextrin concentrations on cholesterol removal from milk.[1,2]

Concentration of β-cyclodextrin	Cholesterol removal	SD
(%)		
0.5	92.2[a]	1.2
1.0	94.0[a]	2.6
1.5	95.3[a]	1.1
2.0	88.6[b]	0.7

[a,b]Means within a column with different superscript letters differ ($P < 0.05$).

[1]Means of duplicates.

[2]Other experimental factors included mixing speed, 800 rpm; mixing temp, 10°C; mixing time, 10 min; centrifugation force, 111 × *g*; and centrifugation time, 10 min.

Oakenfull and Sihdu (12) reported that addition of 1% β-CD to milk resulted in reduction of cholesterol by 77.1%, whereas 2% addition reduced cholesterol by 90.8% with 10 min of mixing at 4°C. Another study (24) has shown that removal of cholesterol from lard was highly correlated with the concentration of added β-CD. In that study, about 90 to 95% cholesterol was removed by stirring with 10% β-CD for 30 min (24).

In our laboratory, 95% of cholesterol has been successfully removed with 10% β-CD from commercial heavy cream containing 36% milk fat (6). Another study (12) has indicated that 79% of cholesterol was removed in cream containing 18% milk fat when it was treated with 2% β-CD at 40°C.

Effect of Mixing Temperature

No difference was found in cholesterol removal at 4, 10, 15, or 20°C Mixing at 10 and 25°C tended to increase cholesterol removal compared with mixing at 4, 15, or 20°C (Table 2). The rate of cholesterol removal was in the range of 93.0 to 95.2%.

Another report (12) disagreed with our result; in that report removal of cholesterol from milk with β-CD was

TABLE 2. Effect of various mixing temperatures on cholesterol removal from milk.[1,2]

Mixing temperature	Cholesterol removal	SD
(°C)	(%)	
4	93.7[a]	0.9
10	94.6[ab]	1.3
15	93.0[a]	1.2
20	93.5[a]	0.7
25	95.2[b]	0.3

[a,b]Means within a column with different superscript letters differ ($P < 0.05$).

[1]Means of duplicates.

[2]Other experimental factors included β-cyclodextrin added, 1%; mixing speed, 800 rpm; mixing time, 10 min; centrifugation force, 111 × g; and centrifugation time, 10 min.

TABLE 4. Effect of various centrifugal forces on cholesterol removal from milk.[1,2]

Centrifugation speed	Cholesterol removal	SD
(× g)	(%)	
55	86.7[a]	3.3
111	94.9[b]	0.9
166	95.9[b]	1.1
222	91.5[ab]	3.1
278	87.3[a]	2.2

[a,b]Means within a column with different superscript letters differ ($P < 0.05$).

[1]Means of duplicates.

[2]Other experimental factors included β-cyclodextrin added, 1%; mixing speed, 800 rpm; mixing temp, 10°C; mixing time, 10 min; and centrifugation time, 10 min.

markedly influenced by temperature. In that study, higher rate of removal was found at lower temperatures (i.e., 77, 63, and 62% cholesterol were removed when treated with β-CD at 4, 8, and 40°C, respectively, with 1.0% β-CD during 10 min of mixing). Yen and Tsui (24) reported that removal of cholesterol with β-CD from lard stirred at 50°C was greater than when mixed at 27 or 40°C.

Effect of Mixing Time

Cholesterol removal was not significantly affected by mixing time between 5 and 20 min (Table 3). However, it was decreased at 25 min of mixing. These data suggest that 5 min of mixing with 1.0% β-CD at 800 rpm could be sufficient for greater than 90% reduction of cholesterol in milk. Another study (12) showed that 10 and 20 min of mixing resulted in 90.2 and 92.9% reduction, respectively (12).

In lard, cholesterol reduction dramatically increased up to 30 min of mixing at all temperatures and plateaued thereafter up to 2 h (24). About 90 to 95% of the cholesterol from lard was removed with 10% β-CD with 30 min of mixing. However, cholesterol removal was slightly decreased when samples were stirred for 2 h. This finding may be due to the instability of an inclusive complex between β-CD and cholesterol during longer mixing times (24). Makoto et al. (9) reported that 91.1 and 94.6% of cholesterol was removed from cheese by mixing with 10% β-CD at 45°C for 20 and 30 min, respectively. Therefore, the optimum mixing time might vary with different samples.

TABLE 3. Effect of various mixing times on cholesterol removal from milk.[1,2]

Mixing time	Cholesterol removal	SD
(min)	(%)	
5	93.2[a]	1.3
10	93.9[a]	0.9
15	92.1[a]	0.7
20	92.5[a]	1.1
25	90.3[b]	0.6

[a,b]Means within a column with different superscript letters differ ($P < 0.05$).

[1]Means of duplicates.

[2]Other experimental factors included β-cyclodextrin added, 1%; mixing speed, 800 rpm; mixing temp, 10°C; centrifugation force, 111 × g; and centrifugation time, 10 min.

Effect of Centrifugal Force

When the β-CD-cholesterol complex is formed, it can be precipitated from milk on cooling at 5°C or lower and by centrifugation (12). When one of five centrifugal forces (55, 111, 166, 222, or 278 × g) was applied with 1.0% β-CD, 10 min of mixing, at a mixing temperature of 10°C, and 10 min of centrifugation, cholesterol removal increased at 166 × g (95.9%) compared with 55 × g (86.7%). However, cholesterol removal decreased to 87.3% at 278 × g (Table 4). The lower cholesterol reduction at lower centrifugal force could have been due to the β-CD-cholesterol complex not being completely precipitated at a lower centrifugal force (below 55 × g). In our preliminary study, the cholesterol and fat contents of homogenized milk were dramatically reduced because of fat separation (data not shown) when a centrifugal force of 442 × g was applied.

Smith et al. (20) suggested that centrifugal force is another important factor affecting cholesterol reduction in eggs. The β-CD remaining in milk after centrifugation was not considered in this study, but it is an important factor affecting process acceptability.

Effect of Centrifugation Time

The effect of centrifugation time appeared to be marginal between 5 and 20 min, although the cholesterol

TABLE 5. Effect of various centrifugation times on cholesterol removal from milk.[1,2]

Centrifugation time	Cholesterol removal	SD
(min)	(%)	
5	91.9[a]	1.2
10	94.6[a]	1.7
15	94.1[a]	1.1
20	92.9[a]	0.7
25	88.3[b]	1.3

[a,b]Means within a column with different superscript letters differ ($P < 0.05$).

[1]Means of duplicates.

[2]Other experimental factors included β-cyclodextrin added, 1%; mixing speed, 800 rpm; mixing temp, 10°C; mixing time, 10 min; and centrifugation force, $111 \times g$.

reduction was slightly improved with 10 and 15 min of centrifugation time (Table 5). No statistical significant difference was found with centrifugation times of 5 to 20 min ($P > 0.05$). Our data suggest that centrifugation for 10 min may be sufficient to precipitate most of the β-CD-cholesterol complex.

CONCLUSIONS

This study suggested that the optimum conditions for cholesterol removal from 3.6% fat, homogenized milk were addition of 1.5% β-CD, 10°C mixing temperature, 10 min of mixing time, and centrifugation at $155 \times g$. As a result, an average of 94.3% of cholesterol was removed from homogenized milk. Because no difference was found among additions of β-CD at 0.5, 1.0, or 1.5%, which was a most effective factor, an addition of 0.5% could be used for economical and environmental reasons. Use of refrigerated-mixing temperature (4 to 10°C) in the removal process was also important for quality maintenance. Another finding was that use of homogenized milk increased the rate of cholesterol removal by β-CD compared with that using unhomogenized milk. Therefore, effect of homogenizing pressure on cholesterol removal and use of cholesterol-removed milk in other dairy-products may require further study.

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