Cholesterol Removal from Homogenized Milk with Crosslinked β-cyclodextrin by Adipic Acid

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ABSTRACT : The present study was carried out to develop crosslinking of β-cyclodextrin (β-CD) using adipic acid, and to determine the optimum conditions of different factors (β-CD concentration, mixing temperature, mixing time and mixing speed) on cholesterol reduction from homogenized milk. Crosslinked β-CD was prepared with adipic acid. When the milk was treated with different conditions, the cholesterol removal rate was in the range of 92.1 to 93.1% with 1% β-CD addition, which were not significantly different among treatments. After cholesterol removal from milk, the used crosslinked β-CD was washed for cholesterol dissociation and reused. For recycling study, the cholesterol removal rate in the first trial was 92.5%, which was mostly same as that using new crosslinked β-CD. With repeated ten time trials using same sample, 81.4% of cholesterol was removed from milk. Therefore, the present study indicated that the optimum conditions for cholesterol removal using crosslinked β-CD were 1% β-CD addition and 10 min mixing with 400 rpm speed at 5°C with over 90% cholesterol removal. In addition, crosslinked β-CD made by adipic acid resulted in the effective recycling efficiency. *(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1794-1799)*

INTRODUCTION

Experiments on animal and human have shown that plasma cholesterol can be raised by increased intake of cholesterol and saturated fat (Pyorala, 1987; Carleton et al., 1991; Gurr, 1992; Sieber, 1993). Most consumers are concerned about excessive intakes of cholesterol and fat in their daily diets because of the risk of coronary heart disease (Grundy et al., 1982; Gurr, 1992). There have been dramatic increases in no-, low-, and reduced-cholesterol products in the market place (Schroder and Baer, 1990).

Nowadays, a number of studies have indicated that cholesterol removal from dairy products was most effectively achieved by powder β-cyclodextrin (β-CD) (Oakenfull and Sihdu, 1991; Makoto et al., 1992; Kwak et al., 2002; Kwak et al., 2003; Shim et al., 2003; Shim, 2004; Hwang et al., 2005; Kim et al., 2005; Kwak et al., 2005; Lee et al., 2005). 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 (Szejtli, 1982). The β-CD is also nontoxic, edible, nonhygroscopic and chemically stable, and is easy to separate from the complex (Nagamoto, 1985). Thus, β-CD provides advantages when used for removal of cholesterol from various foods. While this method allows cholesterol removal in milk (about 90%), using β-CD powder is an ineffective way for separation from food

systems and recovery. Also, most of these methods are relatively nonselective and remove flavor and nutritional components along with cholesterol. Moreover, some methods require high investment and operation costs.

For example, using powder β-CD has been recognized as one of the effective ways to remove cholesterol from dairy products. While this method allows an effective removal of cholesterol (90%), lots of β-CD was consumed for this process due to ineffective recovery from dairy products. Another method to overcome these problems could be an immobilization of β-CD on solid support. However, using this method, the highest cholesterol removal rate was about 40-50% in milk (Kwak et al., 2004). When we tried to crosslink β-CD by apichlorohydrin in previous study, cholesterol removal rate was in the range of 79.4 to 83.3% and recycling rate reached almost 100% (Kim et al., 2004). In their study, crosslinked β-CD made by epichlorohydrin did not result in over 90% for cholesterol removal.

Crosslinking is a commonly used derivatization technique for manipulating starch functionality, and epichlorohydrin and adipic anhydride have been extensively used to produce crosslinked starches, which inter- or intramolecular mono- and diethers are formed with hydroxyly groups of starch (Hamerstrand et al., 1959). This modification produces important changes in the starch functional properties such as an increase or decrease in viscosity (Whistler and Daniel, 1990). Crosslinked starches are employed mainly as thickening agents and stabilizers in most food systems such as sauces and dressings for pizzas, spaghetti, jams and pie fruit fillings (Fleche, 1985; Luallen,

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1985; Whistler and Daniel, 1990). Modification of the starch by crosslinkage formation has been proposed to provide the pastes with more stability when overheated, more mechanical power and pH changes, which are indispensable for food manufacture (Hosney, 1986). However, no information is available about the efficiency of crosslinked β-CD by adipic acid on cholesterol removal, therefore, the objective of this study was to find the optimum conditions on cholesterol removal and recycling efficiency from homogenized milk using crosslinked β-CD made by adipic acid.

MATERIALS AND METHODS

Materials

Commercial homogenized 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, USA), and all solvents were gas-chromatographic grade.

Preparation of crosslinked β-CD

A sample of 100 g β-CD was prepared in a 80 mL distilled water and placed in a stirrer at room temperature with constant agitation for 2 h. Then 5 g of 5% adipic acid was incorporated with 100 g β-CD and pH was adjusted to pH 10 with 1 N NaOH. The β-CD solution was stirred at room temperature for 90 min and then readjusted to pH 5 with acetic acid. β-CD was recovered by filtering with Whatman paper No. 2 and washed three times with 150 mL of distilled water. The product was dried at 60°C in a Lab-line mechanical convection oven for 20 h and passed through a 100 mesh.

Cholesterol removal

To study the effects of four different factors, 5 g of milk was placed in a 1,000 mL beaker and different concentrations of β-CD (0.5, 1.0, 1.5, 2.0 or 2.5%) 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 (0, 5, 10, 15 or 20°C), mixing speeds (400, 600, 800, 1,000 or 1,200 rpm) and mixing times (1, 5, 10, 15 or 20 min). The mixture was centrifuged (HMR-220IV; Hanil Industrial Co., Seoul, Korea) with 166×g for 10 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, 1 g of milk sample 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 (Adams et al., 1986). After cooling to room temperature, cholesterol was extracted with 5 mL of hexane (Adams et al., 1986). 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 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 Hewlett-Packard 5890A gas chromatography (Palo Alto, CA, USA) equipped with a flame ionization detector. The temperatures of injector and detector were 270 and 300°C, respectively. The oven temperatures were programmed from 200 to 300°C at 10°C/min and hold for 20 min. Nitrogen was used as a carrier gas at a flow rate of 2 mL with a split ratio of 1/50. Quantitation of cholesterol was done by comparing the peak areas with a response of an internal standard.

The percentage of cholesterol reduction was calculated as followed: cholesterol reduction (%) = 100-(amount of cholesterol in β-CD-treated butter×100/amount of cholesterol in control). Cholesterol determination for control was averaged with each batch of treatments.

Recycling of β-CD

The cholesterol-crosslinked β-CD complex was soaked in glass tube in acetic acid : butanol = 3:1 (v/v) with 100 rpm stirring speed for 2 h at 50°C (Kwak et al., 2001) and the ratio of complex to solvent was 6:1. Then, the sample was cooled to room temperature and centrifuged at 630×g for 5 min. β-CD was then precipitated and dried at 50°C in dry oven for 6 h and reused for recycling study.

Statistical analysis

Data from the determination of optimum conditions of milk, one-way ANOVA (SAS, 1985) was used. The significance of the results was analyzed by the least significant difference (LSD) test. Difference of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

In the present study, the optimum conditions of four different factors (β-CD concentration, mixing time, mixing temperature and mixing speed) were examined in reduction

Table 1. Effect of different concentrations of crosslinked β-CD on cholesterol removal in homogenized milk (%)

β-CD	Cholesterol removal[1]
0.5	85.05[b]
1.0	93.13[a]
1.5	93.02[a]
2.0	92.64[a]
2.5	92.58[a]
SEM	1.00

[1] Means within column by the same letter are not significantly different (p<0.05).

Factors of cholesterol removal; mixing temp.; 10°C, mixing speed; 800 rpm, mixing time; 10 min, milk fat in milk; 3.6%.

of cholesterol in milk using crosslinked β-CD.

Effects of crosslinked β-CD concentration

The effect of crosslinked β-CD concentration on the cholesterol removal from milk is shown in Table 1. The average cholesterol content of the milk was 13.4 mg/ 100 g. The β-CD (0.5, 1.0, 1.5, 2.0 or 2.5%) removed 85.1 to 93.1% of the cholesterol (when mixed at 10°C for 10 min). Addition of β-CD at 0.5% showed the least efficiency of removal (85.1%), while 1.0% addition resulted in the highest removal rate as 93.1%.

Several studies (Lee et al., 1999; Kwak et al., 2002; Shim et al., 2003; Hwang et al., 2005; Kim et al., 2005; Kwak et al., 2005; Lee et al., 2005) performed in our laboratory shown that powder β-CD at 0.5 to 1.5% provided 92.2 to 95.3% removal of cholesterol when mixed at 10°C for 10 min (Lee et al., 1999). Another trial was β-CD immobilization, which resulted in at most 40-50% cholesterol removal (Kwak et al., 2004). In addition, crosslinked β-CD by epicholorohydrin showed in the range of 79.4 to 83.3% removal rate (Kim et al., 2004).

Other studies (Micich, 1990; Oh et al., 1998) 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 itself to bind to cholesterol molecules, resulting in reduced cholesterol adsorption. Therefore, the present study indicated that 1.0% β-CD may be sufficiently effective to remove greater than 90% of cholesterol from homogenized milk.

Oakenfull and Sihdu (1991) 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 (Yen and Tsui, 1995) 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

Table 2. Effect of different mixing temperatures on cholesterol removal in homogenized milk

Mixing temperature (°C)	Cholesterol removal[1] (%)
0	85.14b
5	85.33b
10	92.38a
15	92.56a
20	93.08a
SEM	1.00

[1] Means within column by the same letter are not significantly different (p<0.05).

Factors of cholesterol removal; crosslinked β-CD; 1%, mixing speed; 800 rpm, mixing time; 10 min, milk fat in milk; 3.6%.

Table 3. Effect of different mixing times on cholesterol removal in homogenized milk

Mixing time (min)	Cholesterol removal[1] (%)
1	86.94[c]
5	92.48[a]
10	92.01[ab]
15	90.44[b]
20	90.42[b]
SEM	1.00

[1] Means within column by the same letter are not significantly different (p<0.05).

Factors of cholesterol removal; β-CD concentration; 1%, mixing speed; 800 rpm, mixing temp.; 10°C, milk fat in milk; 3.6%.

stirring with 10% β-CD for 30 min (Yen and Tsui, 1995).

Effects of mixing temperature

No difference was found in cholesterol removal at 10, 15 or 20°C. However, mixing at 0 and 5°C tended to decrease cholesterol removal compared with mixing at higher temperature (Table 2). The rate of cholesterol removal in 10, 15 or 20°C was in the range of 92.4 to 93.1%.

Another report (Oakenfull and Sidhu, 1991) disagreed with our result; in that report removal of cholesterol from milk with β-CD was 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 (1995) reported that removal of cholesterol with β-CD from lard stirred at 50°C was greater than when mixed at 27 or 40°C.

However, our previous studies indicated that mixing temperature did not affect significantly in cholesterol removal in milk when powder β-CD and crosslinked β-CD by epicholorohydrin were used (Kim et al., 2004). In the case of immobilized β-CD, no difference was found in cholesterol removal at 5, 10, or 15°C. The rate of cholesterol removal was in the range of 39.6 to 40.2% when milk was mixed 1:1 with beads in 7 cm tube size (i.d.) with

Table 4. Effect of different mixing speeds on cholesterol removal in homogenized milk

Mixing speed (rpm)	Cholesterol removal[1] (%)
400	92.05[a]
600	91.63[a]
800	90.81[a]
1,000	88.72[b]
1,200	87.22[b]
SEM	1.00

[1] Means within column by the same letter are not significantly different ($p < 0.05$).

Factors of cholesterol removal; β-CD concentration; 1%, mixing temp.; 10°C, mixing time; 10 min, milk fat in milk; 3.6%.

140 rpm for 6 h (Kwak et al., 2004).

Effects of mixing time

Cholesterol removal was not significantly affected by mixing time between 10 and 20 min (Table 3). However, it was significantly higher at 5 min of mixing (92.48%). These data suggested that 5 min of mixing with 1.0% crosslinked β-CD at 800 rpm could be sufficient for greater than 90% reduction of cholesterol in milk.

Another study (Oakenfull and Sidhu, 1991) using 1% powder β-CD showed that 10 and 20 min of mixing resulted in 90.2 and 92.9% reduction, respectively. When 1% crosslinked β-CD by epicholorohydrin was added, 83.3% of cholesterol was removed from milk with for 10 min treatment at 400 rpm at 10°C.

In lard, cholesterol reduction dramatically increased up to 30 min mixing at all temperatures and plateaued thereafter up to 2 h (Yen and Tsui, 1995). 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 founding may be due to the instability of an inclusive complex between β-CD and cholesterol during longer mixing times (Yen and Tsui, 1995). Makoto et al. (1992) 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.

Effect of mixing speed

The removal rate was in the range of 87.2 to 92.1% when milk was mixed with 1% crosslinked β-CD at 10°C for 10 min (Table 4). The mixing speed might not an effective on cholesterol removal using crosslinked β-CD by adipic acid below 1,000 rpm mixing, however, cholesterol removal decreased with above 1,000 rpm mixing.

Lee et al. (1999) reported that stirring time affected cholesterol removal in milk. In cream, 83% of cholesterol was removed when powder β-CD was applied for 120 min
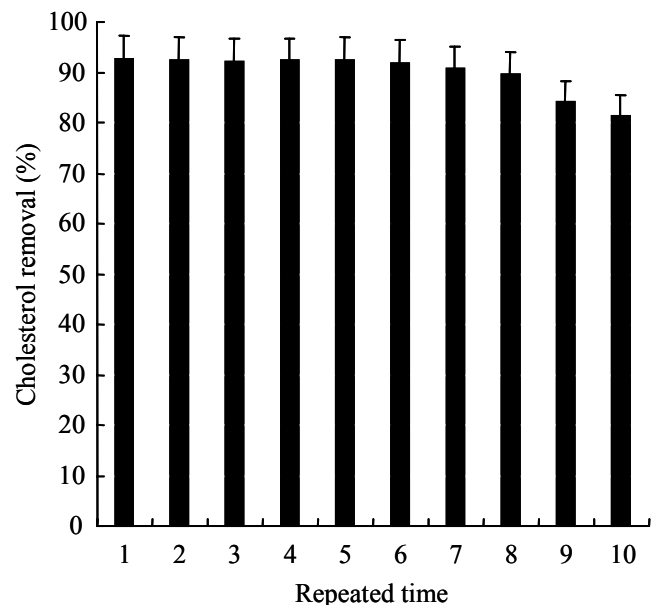
Figure 1. Change of cholesterol removal rate using crosslinked β-CD with repeated time in homogenized milk.

(Lee et al., 1993). In lard and cheese, cholesterol removal increased with stirring time at different temperatures (Makoto et al., 1992; Yen and Tsui, 1995).

Recycling of β-CD

Since the optimum conditions were determined for recycling β-CD by the previous study (Kwak et al., 2001), we examined how effective the recycled crosslinked β-CD would be in cholesterol removal rate. For the recycling study, the crosslinked β-CD was applied to homogenized milk 10 times repeatedly and results are shown in Figure 1. The recycled crosslinked β-CD showed a similar cholesterol removal rate as that of unused crosslinked β-CD (Figure 1). The cholesterol reduction existed between 81.4 to 92.5%. When crosslinked β-CD was used until 8th time, relatively high cholesterol removal rate was found in the range of 92.4 to 89.5%, and no significantly difference was found among them. However, a slightly lower rate was found in 9 and 10th (81.4 to 84.02%). Therefore, the present study provided possibility for applying crosslinked β-CD repeatedly in homogenized milk.

In similar recycling study (Kwak et al., 2001), recycled powder β-CD showed 75.07% of cholesterol removal in cream, while the mixture of recycled to unused powder β-CD with the ratio of 6 to 4 increased cholesterol removal to 95.59%. Their study indicated that only recycled powder β-CD may not effective as much as unused β-CD. Therefore, the present study indicated that crosslinked β-CD could be applied into milk on cholesterol removal process with an effective reproductivity.

CONCLUSIONS

Crosslinked β-CD was prepared with adipic acid. When milk was treated with different conditions (mixing temperature, time and speed), the cholesterol removal rate was in the range of 92.1 to 93.1% with 1% crosslinked β-CD addition. Not much difference was found among other conditions. Interestingly, in recycling study, the cholesterol removal rate in first trial was 92.5%, which was mostly same as that using new crosslinked β-CD. Up to 10th trial repeatedly, the mean cholesterol removal rate was 81.4%. Therefore, the present study indicated that the optimum conditions of cholesterol removal using crosslinked β-CD by adipic acid were 10 min mixing with 400 rpm speed at 10°C with over 90% cholesterol removal. In addition, this study showed a first evidence of possibility for applying crosslinked β-CD by adipic acid in dairy food, and further study would be needed in future.

ACKNOWLEDGEMENTS

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