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# Crosslinking of β-Cyclodextrin on Cholesterol Removal from Milk

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This study was designed to develop crosslinking of β-cyclodextrin (β-CD), and determine the optimum conditions of different factors (mixing time, mixing temperature, and mixing speed) on cholesterol reduction from milk. Crosslinked β-CD was prepared with epichlorohydrin. When milk was treated with different conditions, the cholesterol removal rate was in the range of 79.4 to 83.3% with 1% crosslinked 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 first trial was 81.8%, which was mostly same as that using new crosslinked β-CD. With five trials repeatedly using the same sample, the mean cholesterol removal rate was 81.2%. The present study indicated that the optimum conditions on cholesterol removal using crosslinked β-CD were 10 min mixing with 400 rpm speed at 5°C with about 80% cholesterol removal. In addition, crosslinked β-CD resulted in the effective recycling efficiency almost 100%.

**Key words:** Crosslinked β-CD, Cholesterol removal, Milk, Recycling

## INTRODUCTION

Since a strong positive correlation exists between increased serum cholesterol concentrations and risk of coronary heart disease, most consumers are concerned about excessive intake of cholesterol (Grundy *et al.*, 1982; Gurr, 1992). Therefore, physical, chemical, and biological methods to reduce cholesterol have been studied in foods, including dairy products (Szjetli, 1988; Ahn and Kwak, 1999; Lee *et al.*, 1999; Kwak *et al.*, 2001).

Food companies have developed many methods to reduce cholesterol, 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. 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; Ahn and Kwak, 1999, Lee *et al.*, 1999; Kwak *et al.*, 2001). Beta-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 arrangements, which forms an inclusion complex with various compounds including cholesterol (Szejtli, 1982). Also, β-CD is nontoxic, edible, nonhygroscopic, chemically stable, and easy to separate from the complex (Nakamoto, 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 system and recovery.

While this method allows an effective removal of cholesterol (90%), lots of β-CD was consumed for this process due to an ineffective recovery from dairy products. One method to overcome these problems could be an immobilization of β-CD on solid support. However, using this method, the highest cholesterol rate was about 40-50% in milk (Kwak *et al.*, 2004). Therefore, we postulated the possibility of crosslinked β-CD to increase the cholesterol removal rate and to recover the used β-CD for recycling.

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 hydroxyl groups of starch (Hamerstrand *et al.*, 1959). This modification produces important changes in the starch functional properties as increase or decrease in viscosity (Whistler and Daniel,

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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, 1985; Whistler and Daniel, 1990). Modification of the starch by cross-linkage 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 on cholesterol removal, therefore, the objective of this study was to find the optimum conditions on cholesterol removal and recycling efficiency from milk using crosslinked β-CD.

## MATERIALS AND METHODS

### Materials

Commercial milk (3.6% milk fat) was purchased from a retail store as needed, and powder β-CD (purity 99.1%) was obtained from Nihon Shokunin 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 of β-CD was prepared in a 166 mL distilled water and placed in a stirrer at room temperature with constant agitation. Then, 2 g of epichlorohydrin was incorporated and pH was adjusted to 10 with 1 M NaOH. The β-CD solution was stirred at room temperature for 2 h, and then readjusted to pH 5.5 with acetic acid. β-CD was recovered by filtering with Whatman paper No 2, and washed two times with 150 mL of distilled water and once with 15 mL of 95% ethanol. The product was dried at 40 °C in a Lab-line mechanical convection oven and passed through a 100 mesh sieve.

### Scanning electron microscopy (SEM)

Morphology of the crosslinked β-CD was examined at a magnification of 2,000×using a scanning electron microscope (JSM 5410LV, Jeol, Tokyo, Japan) at 15 kV and 15 tilt.

### Cholesterol removal rate

To study the effects of three different factors (mixing time, mixing temperature, and mixing speed), 100 mL of milk was placed in a 100 mL tube in a temperature-controlled waterbath. Three factors were applied into the cholesterol removal process, namely, the mixing time, mixing temperature, and mixing speed. After mixing, 1 mL of milk was used for cholesterol quantitative determination.

### Extraction and determination of cholesterol

For the extraction of cholesterol from milk, 1 mL of the crosslinked β-CD-treated milk was placed in a screw-capped glass tube (15 mm×180 mm), and 1 mL of the 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. 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, USA) equipped with a flame-ionization detector. Temperatures of the injector and detector were 170 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. Quantification 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 crosslinked β-CD-treated milk × 100 / amount of cholesterol in untreated milk (control). Cholesterol determination for a control was done with each treatment batch.

### Recycling of β-CD

The study how effective the recycled crosslinked β-CD was for cholesterol reduction was carried out. The recycled crosslinked β-CD were soaked in glass tube in acetic acid : butanol = 3:1 (v/v) (Kwak *et al.*, 2001) for 24 h at room temperature. The recycled crosslinked β-CD were dried at room temperature and reused for recycling study.

### Statistical analysis

Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (SAS Inc., 1985) and differences among treatments were determined by Duncan's multiple test at $p < 0.05$, unless otherwise stated.

## RESULTS AND DISCUSSION

In the past two decades, a number of studies have indicated the importance of cholesterol reduction in dairy products, therefore, using powder β-CD was 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.

As a simple method, immobilized β-CD glass beads were applied to remove cholesterol in dairy products, however, the highest cholesterol removal rate was only 41% (Kwak *et al.*, 2004) with high recycling rate. Therefore, the crosslinking method was introduced to prepare the β-CD for cholesterol removal process. In the present study, the optimum conditions of three different factors (mixing time, mixing temperature, and mixing speed) were examined in reduction of cholesterol in milk using crosslinked β-CD.

### Crosslinking morphology

Granular morphology of the crosslinked and native β-CD is presented in Fig. 1. The morphology of the cross-linked β-CD was different from that of the powder β-CD. Crosslinked-β-CD granules were observed to be polygonal and angular in shape, with slight variation in sizes. Granules was found to have the narrowest range in size of 10-20 μm.

### Effect of mixing time

The effect of mixing time on cholesterol removal from milk is shown in Table I. Cholesterol removal rate was not significantly affected by mixing time between 5 and 15 min. The average cholesterol content of the milks (control) was 13.14 mg/100 g. The 83.3% cholesterol was removed from milk for 10 min when treated with 1% crosslinked β-CD at 400 rpm at 10°C. More time for mixing did not enhance the cholesterol removal rate. These data suggest that 5 min of mixing with 1% crosslinked β-CD at 400 rpm could be sufficient for greater than 80% reduction of cholesterol in milk. Similar study using powder β-CD (Lee *et al.*, 1999) showed the higher reduction rate as 93.2% with 5 min of mixing.

In lard, cholesterol reduction dramatically increased up to 30 min of mixing at all temperatures and plauteaued 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 finding may be due to the instability if an inclusive complex between β-CD and cholesterol during longer mixing with 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.

A B

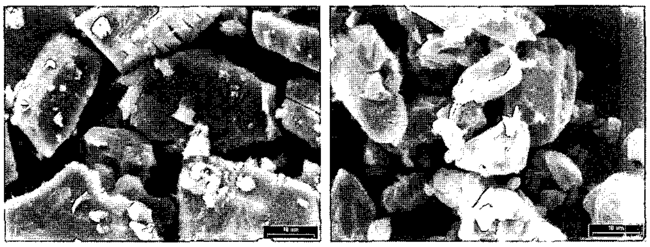

**Fig. 1.** Scanning electron microscope (SEM) of crosslinked β-cyclodextrin by epichlorohydrin. A, Powder β-cyclodextrin 15 kV × 2000; B: Crosslinked β-cyclodextrin 15 kV × 2000

**Table I.** Effect of various mixing times of crosslinked b-cyclodextrin on cholesterol removal from milk[1]

| Mixing time (min) | Cholesterol removal (%) |
|---|---|
| 5 | 81.7[a] |
| 10 | 83.3[a] |
| 15 | 81.0[a] |

[1] Means within column by same letter are not significantly different ($P<0.05$).
Other experimental factors included crosslinked β-cyclodextrin added, 1%; mixing temp, 10°C; and mixing speed, 400 rpm

### Effect of mixing temperature

No difference was found in cholesterol removal at 5, 10, or 15°C (Table II). The rate of cholesterol removal was in the range of 80.2 to 81.3% when milk was treated with 1% crosslinked β-CD at 400 rpm for 10 min.

Another study (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 mixing). Yen and Tusi (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.

### Effect of mixing speed

Similar to mixing time and temperature, no significant difference was found with different mixing speeds (Table

**Table II.** Effect of various mixing temperatures of crosslinked β-cyclodextrin on cholesterol removal from milk

| Mixing temp. (°C) | Cholesterol removal[1)] (%) |
|---|---|
| 5 | 80.2[a] |
| 10 | 81.3[a] |
| 15 | 81.2[a] |

[1] Means within column by same letter are not significantly different ($P<0.05$).
Other experimental factors included crosslinked β-cyclodextrin added, 1%; mixing time, 10 min; and mixing speed, 400 rpm

**Table III.** Effect of various mixing speeds of crosslinked β-cyclodextrin on cholesterol removal from milk

| Mixing speed (rpm) | Cholesterol removal[1] (%) |
|---|---|
| 200 | 79.4[a] |
| 400 | 81.6[a] |
| 600 | 81.2[a] |

[1]Means within column by same letter are not significantly different (P<0.05).
Other experimental factors included crosslinked β-cyclodextrin added, 1 %; mixing time, 10 min, and mixing temp, 10°C.

**Table IV.** The effect of recycling repeatability on immobilized β-cyclodextrin on cholesterol removal rate from milk[1]

| Number of repeated recycling | Cholesterol removal (%) |
|---|---|
| 1st | 81.8[a] |
| 2nd | 81.5[a] |
| 3rd | 80.5[a] |
| 4th | 81.6[a] |
| 5th | 80.7[a] |

[1]Means within a column with different superscript letters differ (p < 0.05). Means of triplicated cholesterol extraction.
Condition for recycling were acetic acid : butanol = 3 : 1, solvent : crosslinked β-cyclodextrin = 6 : 1 for 24 h in room temperature.
Other experimental factors included crosslinked β-CD added, 1%; mixing time, 10 min; mixing temp, 10°C; and mixing speed, 400 rpm.

III). The removal rate was in the range of 79.4 to 81.6% when milk was mixed with 1% crosslinked β-CD at 10°C for 10 min. The mixing speed might not an effective factor on cholesterol removal using crosslinked β-CD.

A number of studies have indicated that the removal of cholesterol from milk and cream was effectively conducted by treatment with powder β-CD (Ahn and Kwak, 1999; Lee *et al.*, 1999). In our laboratory, over 90% of the cholesterol was removed from commercial milk at refrigerated temperature with 1% β-CD (Lee *et al.*, 1999).

Several other studies also reported that cholesterol removal was closely related to powder β-CD concentration (Oakenfull and Sihdu, 1991; Lee *et al.*, 1993, Yen and Tsui, 1995). Oakenfull and Sidhu (1991) reported that the addition of 2% β-CD resulted in a 90.8% cholesterol reduction in milk, while 1% addition reduced it by 77.1%. In another study, about 90 to 95% cholesterol in lard and water mixture was removed by stirring with a 10% β-CD concentration for 30 min (Yen and Tusi, 1995).

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 (Lee *et al.*, 1993). In lard and cheese, cholesterol removal increased with stirring time at different temperatures (Makoto *et al.*, 1992; Yen and Tusi, 1995). Also, 99 and 95% of cholesterol have been successfully removed with 1 and 10% powder β-CD from commercial milk and heavy cream containing 36% milk fat, respectively, in our laboratory (Ahn and Kwak, 1999; Lee *et al.*, 1999).

### Recycling of β-CD

Since the optimum conditions were chosen 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 test, the crosslinked β-CD was applied to milk 5 times repeatedly and results are shown in Table IV. The recycled crosslinked β-CD showed exactly the same cholesterol removal rate as that of unused crosslinked β-CD (Table IV). The cholesterol reduction existed between 80.5 to 81.8%, which almost identical with that when applying unused one (p < 0.05).

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.

## CONCLUSION

Cholesterol has been removed from dairy, meat, and egg products because most consumers are concerned about the excessive intake of cholesterol causing coronary heart disease (Grundy *et al.*, 1982; Gurr, 1992). In present, even though lots of results are reported the effective cholesterol removal by using powder β-CD, commercial β-CD is expensive and waste in the process resulting in an environmental problem. Therefore, the present study examined the possibility of crosslinked β-CD application on cholesterol removal from milk.

Crosslinked β-CD was prepared with epichlorohydrin. When milk was treated with different conditions, the cholesterol removal rate was in the range of 79.4 to 83.3% with 1% crosslinked β-CD addition. No significant difference was found among different conditions. However, in recycling study, the cholesterol removal rate in first trial was 81.8%, which was mostly same as that using new crosslinked β-CD. Upto 5th trial repeatedly, the mean cholesterol removal rate was 81.2%. The present study indicated that the optimum conditions of cholesterol removal using crosslinked β-CD were 10 min mixing with 400 rpm speed at 5°C with about 80% cholesterol removal. In addition, crosslinked β-CD resulted in the effective recycling efficiency almost 100%. In addition, this study showed a first evidence of possibility using crosslinked β-CD in food, and further study would be needed in future.

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