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Optimization of Cholesterol Removal by Crosslinked β-Cyclodextrin in Egg Yolk

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Abstract Optimum conditions for cholesterol removal in egg yolk were evaluated based on ratio of egg yolk-to-water, crosslinked β-cyclodextrin (β-CD) concentration, and mixing temperature, time, and speed by adding crosslinked β-CD treated with adipic acid. Cholesterol removal in egg yolk-water mixture increased with increasing β-CD level (10-25%). About 95% was removed by 25% β-CD at 1:1 ratio of egg yolk-to-water and 800 rpm mixing at 40°C for 30 min. In recycling study, removal rates were measured using ten times recycled crosslinked β-CD in egg yolk, and 85% cholesterol removal was observed with eight times reuse. These results indicated that over 90% cholesterol was removed at 1:1 ratio of egg yolk-to-water, 20% crosslinked β-CD addition, and 30 min mixing with 600 rpm at 40°C.

Key words: crosslinked β-CD, cholesterol removal, egg yolk, recycling

Introduction

Studies on the relationship between dietary fat and the development of atherosclerosis have led to numerous publications encouraging changes in the human diet, such as reduction of total fat content, ratio of saturated to unsaturated fatty acids, and total cholesterol intake of less than 300 mg/day (1). Following these recommendations, there have been dramatic increases in no-, low-, and reduced-cholesterol products in the market place (2).

Article in the Wall Street Journal shows the impact of "cholesterol-free" advertising on sales of animal-based products, basic distrust of food industry and a marked fear for food fats (3, 4); sixty-one percent of consumers surveyed judged fats to be a serious health hazard and 35% thought fat in the diet to be somewhat of a health hazard. In addition, 59% thought cholesterol in the diet was a serious health hazard, and 35% thought it was somewhat of a hazard. Thus, removal of cholesterol from products, such as milk fat, without altering the composition or properties of fat would increase consumers' preference (3).

Each egg contains about 200-250 mg cholesterol, and the perception of eggs by the public as a major source of dietary cholesterol, although with questionable scientific basis, is seen as a significant factor contributing to the overall decline in their consumption (5). Nevertheless, the poultry industry clearly believes that 'low cholesterol eggs' would have significant competitive advantage.

Responding to the consumers' demand, attempts are made by the food and pharmaceutical industries to reduce fat and cholesterol in a wide variety of foods, and different approaches have been investigated to reduce cholesterol in eggs (6) such as hypocholesterolemic agent administration (7, 8), extraction with solvents (9, 10) or oils (11), and supercritical fluid extraction (12). The removal of cholesterol by adsorption with β-cyclodextrin (β-CD) is an alternative approach (13). β-Cyclodextrins are cyclic oligosaccharides, consisting of seven glucose units arranged in a donut-shaped ring. In addition, use of CD to remove cholesterol from egg yolk has been described (14-16). A reduced cholesterol egg product (80% cholesterol free), based on the patent of Cully and Vollbrecht (15), was marketed briefly in the US.

Although efficient cholesterol removal of β-CD has been achieved by several studies (14, 17-24), use of powder β-CD was a costly process due to its ineffective recovery from dairy products. In our previous study, crosslinked β-CD made with adipic acid showed over 90% cholesterol removal rate and highly efficient recycling rate in milk and cream (25). Crosslinking is a commonly used derivatization technique for manipulating starch functionality, and epichlorohydrin and adipic anhydride have been extensively used to produce crosslinked starches, in which inter- or intramolecular mono- and diethers are formed with hydroxyly groups of starch (26). Therefore, the objective of this study was to examine the effect of crosslinked β-CD on cholesterol removal from egg yolk and its recycling efficiency.

Materials and Methods

Materials Egg was purchased from retail store as needed, and egg yolk was separated. 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 of gas-chromatographic grade.

Preparation of crosslinked β-CD β-CD sample (100 g) was added to 80 mL distilled water and stirred at room temperature with constant agitation for 2 hr. Two grams of adipic acid was then added to the β-CD solution, which was adjusted to pH 10 with 1 N NaOH. The mixture was stirred at room temperature for additional 16 hr, and readjusted to pH 5 with acetic acid. β-CD was recovered

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by filtering with Whatman paper No. 2, washed three times with 150 mL distilled water, dried at 60°C in a Lab-line mechanical convection oven for 17 hr, and passed through a 100-mesh sieve.

Cholesterol removal To study the effects of six different factors, 50 g egg yolk was diluted to 0.5-, 1-, 1.5-, 2-, or 3-fold with distilled water and placed in a 500-mL beaker. Subsequently, different concentrations of β-CD (10, 15, 20, 25, or 30%) were added. The mixture was stirred in a blender (Tops; Misung Co., Seoul) in a temperature-controlled water bath at different mixing temperatures (30, 35, 40, 45, or 50°C), speeds (400, 600, 800, 1,000, or 1,200 rpm), and times (10, 20, 30, 40 or 50 min), and centrifugal speeds (2,210, 2,890, 3,660, 4,520, or 5,470 × g). The mixture was centrifuged (HMR-220IV; Hanil Industrial Co., Seoul) for 10 min at 20°C.

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

Extraction and determination of cholesterol For the extraction of cholesterol, 1 g supernatant was placed in a screw-capped glass tube (15 mm × 180 mm), and 1 mL 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 (27). After cooling to room temperature, cholesterol was extracted with 5 mL hexane (27). The process was repeated four times. The hexane layer was transferred into a round-bottomed flask and dried under vacuum. The extract was redissolved in 1 mL 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 mm thickness) using Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector. The temperatures of injector and detector were 270 and 300°C, respectively. The oven temperature was programmed from 200 to 300°C at 10 °C/min and held for 20 min. Nitrogen was used as a carrier gas at a flow rate of 2 mL/min with a split ratio of 1/50. Quantitation of cholesterol was done by comparing the peak areas with an internal standard.

The percentage of cholesterol reduction was calculated as follows: 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 recycling rate was determined based on the cholesterol dissociation rate from cholesterol-crosslinked β-CD complex. The cholesterol-crosslinked β-CD complex was soaked in glass tube in acetic acid: butanol = 3:1 (v/v) at 100 rpm stirring speed for 2 hr at 50°C (28), and the ratio of complex to solvent was 6:1. The sample was then cooled to room temperature and centrifuged at 6,300 × g for 5 min. β-CD was precipitated and dried at 50°C in a dry oven for 6 hr and reused for the recycling study.

Statistical analysis

Analysis of variance (29) was performed to determine the difference ($p < 0.05$) in cholesterol removal rate among treated samples. When differences were found among samples, least significant difference (LSD) test was performed to separate the means.

Results and Discussion

Effect of ratio of egg yolk-to-water Cholesterol removal in egg yolk by crosslinked β-CD was markedly related to the ratio of egg yolk-to-water (Table 1). The highest cholesterol removal (94.6%) was found at 1:1 ratio of egg yolk-to-water in the sample treated with 20% crosslinked β-CD at 800 rpm for 30 min (Table 1), and addition of water had no significant effect ($p > 0.05$). However, significantly lower rate of cholesterol removal was found in the 1:0.5 ratio of egg yolk-to-water compared with others ($p < 0.05$). This may be due to the fact that higher water content with a certain limit would reduce the inclusion between β-CD and cholesterol.

Low cholesterol could be removed by β-CD without dilution with water, because the increased viscosity may result in decreased inclusion capacity. Therefore, adequate dilution during operation is necessary. According to Courregelongue *et al.* (30), only 18% cholesterol in lard was removed by 5% β-CD treatment. However, other study reported 90% cholesterol removal at the same concentration of β-CD when lard was diluted with distilled water at 1:1 ratio (31).

In most studies, egg yolk products were diluted with water or other solutions to increase the cholesterol removal rate when treated with β-CD (13, 16, 32). For example, study by Chiu *et al.* (32) showed 92% cholesterol removal with 30-fold diluted yolk solution using 1% immobilized β-CD in chitosan beads. In addition, 89.2% cholesterol was removed when egg yolk was diluted to a water:solid ratio of 2.9 at a CD:cholesterol molar ratio of 4.0 (13). The present study showed over 90% cholesterol removal only with 2-fold dilution. Previous study has shown that lower dilution could provide more effective and useful application in egg yolk process (13). Therefore, our result indicated that crosslinked β-CD could be effective for removing cholesterol in liquid egg yolk.

Table 1. Effect of ratio of egg yolk-to-water (w/v) on cholesterol removal in egg yolk using crosslinked β-CD

Egg yolk-to- water (w/v)	Cholesterol removal[1)](%)
1 : 0.5	76.5[b]
1 : 1.0	94.6[a]
1 : 1.5	93.2[a]
1 : 2.0	93.5[a]
1 : 3.0	84.6[ab]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p < 0.05$).
Conditions for cholesterol removal; crosslinked β-CD; 20%, mixing temp.; 40°C, mixing speed; 800 rpm, mixing time; 30 min, centrifugal speed; 2,890 × g.

Effect of crosslinked β–CD concentration The effect of crosslinked β-CD concentration on the cholesterol removal in egg yolk is shown in Table 2. The crosslinked β-CD (10, 15, 20, 25, or 30%) removed 78.5 to 95.8% cholesterol when mixed at 40°C for 30 min. No significant difference was found among the three samples treated with 20, 25, or 30% β-CD, and cholesterol removal rates were in the range of 93.3 to 95.8% ($p < 0.05$). Therefore, the present study indicated that 20% crosslinked β-CD may be a sufficient amount to remove cholesterol effectively in egg yolk.

Several studies have been performed on removal of cholesterol in various foods using β-CD. Powder β-CD at 0.5 to 1.5% showed 92.2 to 95.3% cholesterol removal in milk when mixed at 10°C for 10 min (18). Use of immobilized β-CD resulted in up to 40-50% removal rate (33), and crosslinked β-CD made by epicholorohydrin showed 79.4 to 83.3% removal rate (34). In liquid egg yolk sample, Smith *et al.* (13) using powder β-CD and Chiu *et al.* (32) using immobilized β-CD reported 89.2 and 92% cholesterol removal rates, respectively, which are slightly lower than our result. In other studies (35, 36), when saponin and digitonin were used for cholesterol adsorption at above certain concentration, decreased cholesterol removal rates were observed in milk and butter oil, and suggested that excess β-CD could compete with itself to bind to cholesterol molecule, thereby resulting in reduced cholesterol adsorption.

Oakenfull and Sihdu (14) reported that addition of 1% β-CD to milk resulted in 77.1% cholesterol reduction, whereas 2% addition reduced cholesterol by 90.8% with 10 min of mixing at 4°C. A different study showed that cholesterol removal from lard was highly correlated with the concentration of added β-CD; about 90 to 95% cholesterol was removed by stirring with 10% β-CD for 30 min (31).

Effect of mixing temperature No differences were found in cholesterol removal rates at 30, 35, 40, 45, and 50°C, ranging 88.0 to 90.1% (Table 3). On the other hand, a different study (14) found that removal of cholesterol in 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 at 10 min of mixing). In addition, Yen and Tsui (31) reported that cholesterol removal with β-CD from lard stirred at 50°C was greater than when mixed at 27 or 40°C. However, our previous studies showed that mixing temperature did not significantly affect cholesterol removal in milk when powder β-CD (18), crosslinked β-CD by epicholorohydrin (34) or immobilized β-CD (33) was used.

Effect of mixing time Cholesterol removal was not significantly affected by mixing time between 10 and 40 min (Table 4), whereas was significantly lower at 50 min of mixing (78.9%). This result suggested that 30 min of mixing with 20% crosslinked β-CD at 800 rpm could sufficiently remove over 90% cholesterol in egg yolk.

Another study (14) using 1% powder β-CD showed that 10 and 20 min of mixing resulted in 90.2 and 92.9% reductions in milk, respectively. When 1% crosslinked β-CD made with epicholorohydrin was added, 83.3% cholesterol was removed from milk when treated at 400 rpm and 10°C for 10 min.

In lard, cholesterol reduction dramatically increased up to 30 min mixing at all temperatures and plateaued thereafter up to 2 hr (31). About 90 to 95% cholesterol was removed from lard with 10% β-CD after 30 min of mixing. However, cholesterol removal slightly decreased when samples were mixed for 2 hr. This finding may be due to the instability of an inclusive complex between β-CD and cholesterol during longer mixing time (31). Other study by Makoto *et al.* (17) reported 91.1 and 94.6% cholesterol removals from cheese by mixing with 10% β-CD at 45°C for 20 and 30 min, respectively, suggesting

Table 2. Effect of various crosslinked β-CD concentrations on cholesterol removal in egg yolk using crosslinked β-CD

β-CD concentration (%)	Cholesterol removal[1)] (%)
10	78.5[b]
15	82.7[b]
20	93.3[a]
25	95.8[a]
30	93.7[a]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p < 0.05$).
Conditions for cholesterol removal; mixing temp.; 40°C, mixing speed; 800 rpm, mixing time; 30 min, egg yolk:water = 1:1, centrifugal speed; 2,890 × g.

Table 3. Effect of various mixing temperatures on cholesterol removal in egg yolk using crosslinked β-CD

Mixing temperature (°C)	Cholesterol removal[1)] (%)
30	82.7[b]
35	88.0[ab]
40	90.1[a]
45	88.1[ab]
50	88.2[ab]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p < 0.05$).
Conditions for cholesterol removal; crosslinked β-CD; 20%, mixing speed; 800 rpm, mixing time; 30 min, egg yolk:water = 1:1, centrifugal speed; 2,890 × g.

Table 4. Effect of various mixing times of crosslinked β-CD on cholesterol removal in egg yolk using crosslinked β-CD

Mixing time (min)	Cholesterol removal[1)] (%)
10	84.5[ab]
20	88.1[ab]
30	91.5[a]
40	91.3[a]
50	78.9[b]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p < 0.05$).
Conditions for cholesterol removal; crosslinked β-CD; 20%, mixing speed; 800 rpm, mixing temp.; 40°C, egg yolk:water = 1:1, centrifugal speed; 2,890 × g.

optimum mixing time might vary depending on sample types.

Effect of mixing speed The removal rate was in the range of 75.9 to 91.6% when egg yolk was mixed with 20% crosslinked β-CD at 40°C for 30 min (Table 5). Although mixing speed might not be an important factor on cholesterol removal using crosslinked β-CD at below 1,000 rpm mixing speed, cholesterol removal decreased at higher mixing speed. The optimum mixing speed also may vary with different food products and other factors as well as mixing time.

Effect of centrifugal speed Centrifugal speed at 2,210 to 5,470 × g had no significant effect on the removal rate; however, over 5,470 × g centrifugal speed resulted in lower cholesterol removal with 20% crosslinked β-CD when mixed at 800 rpm for 30 min (Table 6). These results showed that centrifugal speed may not play an important role on the cholesterol removal rate in egg yolk.

Recycling of β-CD Based on the optimum conditions determined for recycling of β-CD in a previous study (28), we examined the effectiveness of recycled crosslinked β-CD in removing cholesterol in egg yolk sample. For recycling experiment, the crosslinked β-CD was repeatedly applied to egg yolk 10 times, and the results are shown in Fig. 1. When unused crosslinked β-CD was used, 92.7% cholesterol was removed, decreasing to 84.9 % with once used β-CD. However, over 80% cholesterol removal rate was maintained with up to 8 times used β-CD, and no significant differences were observed depending on how many times the crosslinked β-CD were recycled ($p > 0.05$). When 9 and 10 times used crosslinked β-CD were applied, cholesterol removal significantly decreased to 78.7 and 75.7%, respectively.

In a similar recycling study (28), recycled powder β-CD showed 75.1% cholesterol removal in cream, while 6 to 4 ratio of unused powder to recycled β-CD enhanced cholesterol removal to 95.6%; in addition, 100% recycled powder β-CD was not as effective as unused powder β-CD. Comparatively, the present study showed that even eight-times used crosslinked β-CD may remove over 85% cholesterol in egg yolk.

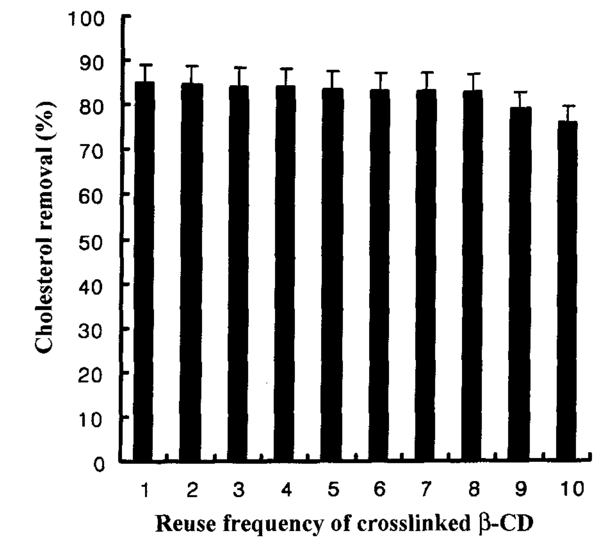

Fig. 1. Cholesterol removal rate in egg yolk by reuse frequency of crosslinked β-CD. Conditions for cholesterol removal: egg yolk:water, 1:1; crosslinked β-CD; 20%, mixing temp.; 40°C, mixing speed; 800 rpm, mixing time; 30 min, centrifugal speed;

Table 5. Effect of various mixing speeds on cholesterol removal in egg yolk using crosslinked β-CD

Mixing speed (rpm)	Cholesterol removal[1)] (%)
400	85.1[a]
600	90.5[a]
800	91.6[a]
1,000	88.6[a]
1,200	75.9[b]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p > 0.05$).
Conditions for cholesterol removal; crosslinked β-CD; 20%, mixing temp.; 40°C, mixing time; 30 min, egg yolk:water = 1:1, centrifugal speed; 2,890 ×g.

Table 6. Effect of various centrifugal speeds on cholesterol removal in egg yolk using crosslinked β-CD

Centrifugal speed (x g)	Cholesterol removal[1)] (%)
2,210	89.6[ab]
2,890	90.9[a]
3,660	89.3[ab]
4,520	89.3[ab]
5,470	87.9[b]
SEM	1.0

[1)]Means with same letter within column are not significantly different ($p > 0.05$).
Conditions for cholesterol removal; crosslinked β-CD; 20%, mixing speed; 800 rpm, mixing temp.; 40°C, mixing time; 30 min, egg yolk: water = 1:1.

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