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Optimization of the Conditions for Removing Cholesterol from Cod Liver Oil by β-Cyclodextrin Crosslinked with Adipic Acid

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Abstract This study was conducted to find the optimum conditions for β-cyclodextrin (β-CD) crosslinked by adipic acid to remove cholesterol from cod liver oil. The cholesterol content of the non-treated cod liver oil was 554.51 mg/100 g oil. The different factors considered were concentrations of crosslinked β-CD, mixing temperature, ratio of cod liver oil to distilled water, mixing time, and mixing speed. The optimum conditions for cholesterol removal from cod liver oil using crosslinked β-CD were a 1:2 ratio of cod liver oil to distilled water, 25% (crosslinked β-CD/distilled water, w/v) crosslinked β-CD concentration, 20 min mixing time, 400 rpm mixing speed and 60 °C mixing temperature with about 87% cholesterol removal. In a recycling study, cholesterol removal from the cod liver oil with recycled crosslinked β-CD in the first recycling trial was 85.09%, which was slightly lower than that with new crosslinked β-CD (87.27%). Up to three time trials, more than 82% cholesterol removal was observed.

Keywords Cholesterol removal · Cod liver oil · Crosslinked β-cyclodextrin · Recycling

Introduction

Cod liver oil, consisting of n-3 polyunsaturated fatty acids (commonly known as omega-3 fatty acids), comprising high levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), triacylglycerols, mono- and di-acylglycerols, free fatty acids, and vitamins A, D and E, has been used as a nutritional supplement in the world [1–4]. Brunborg et al. [5] studied the effects of short-term oral administration of cod liver oil on inflammatory bowel disease-associated joint pain in the patients with the pain. The authors observed an improvement in the joint pain parameters (i.e., swollen joint count, tender joint count, joint pain intensity and back pain intensity) after cod liver oil administration. Moreover, cod liver oil contributes to faster wound healing and an improvement in skin quality [6].

There are several studies on animals and humans that plasma cholesterol can be increased with elevating the intake of cholesterol and saturated fat [7, 8]. Due to the risk of coronary heart diseases, most consumers are concerned about excessive intakes of cholesterol in their daily diets [7, 9, 10]. Therefore, in recent years, the no-, low-, and reduced-cholesterol products in the market place have increased remarkably [11, 12].

In our previous studies, crosslinked β-cyclodextrin (CD) made with adipic acid exhibited slightly more than 90% cholesterol removal and a highly efficient recycling rate in cream cheese [10], butter [12], blue cheese [13], squid liver oil [14], egg yolk [15], cream [16], lard [17], milk [18], yogurt [19, 20], ice cream [21], and processed cheese spread [22]. According to Guillén et al. [2], several cod liver oil samples had a cholesterol content from 4.9 to 12.8 mg/mL of oil. Therefore, it is hypothesized in the present study that removing the cholesterol from the cod liver oil can be a great way of enhancing the health benefits, and there is a possibility of producing nutraceutical food products including cholesterol-free cod liver oil. However, no information is available on the efficiency of β-CD crosslinked with adipic acid on cholesterol removal from cod liver oil. Therefore, the objective of the present study was to investigate the optimum conditions for

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cholesterol removal from cod liver oil using β-CD crosslinked with adipic acid and the recycling efficiency.

Experimental Procedures

Materials

Commercial cod (*Gadus morhua*) liver oil was obtained from Denomega Nutritional Oils (Sarpsborg, Norway) and stored at −20 °C until needed. Commercial β-CD (purity 99.1%) was purchased from Nihon Shokuhin Cako Co. Ltd. (Osaka, Japan). Cholesterol and 5α-cholestane were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Adipic acid (purity 99%) was provided by Shinyo Pure Chemicals Co. Ltd. (Tokyo, Japan), and all solvents were gas-chromatographic grade.

Preparation of Crosslinked β-CD

A 100-g sample of β-CD was dissolved in 80 mL of distilled water and placed on a stirrer at room temperature with constant agitation for 2 h. Two grams of adipic acid were then incorporated into the β-CD solution and the pH was adjusted to pH 10.0 with 1 N NaOH. The β-CD solution was stirred at room temperature for 90 min and then readjusted to pH 5.0 with 0.5% acetic acid. The β-CD was recovered by filtering through Whatman no. 2 filter paper and washing three times with 150 mL of distilled water. The product was dried at 60 °C in a Lab-Line mechanical convection oven (O-Sung Scientific Co., Seoul, Korea) for 20 h and passed through a 100-mesh sieve [12].

Cholesterol Removal

To study the effects of five different factors, distilled water was added to 50 mL of cod liver oil to make mixtures with the ratios 1:1, 1:2, 1:3 or 1:4 of cod liver oil to distilled water (v/v) and placed in 500-mL beakers. Subsequently, different concentrations, 15, 20, 25 or 30% (crosslinked β-CD/distilled water, w/v) of β-CD, were added. The mixture was stirred in a blender (Tops: Misung Co., Seoul, Korea) in a temperature-controlled water bath at different mixing speeds (200, 400, 800 or 1,200 rpm), different mixing temperatures (40, 50, 60 or 70 °C) and different mixing times (5, 10, 20 or 30 min). To prevent the oxidation of cod liver oil during the cholesterol removal from the oil, all the experiments were performed under nitrogen. The mixture was centrifuged (HMR-220IV, Hanil Industrial Co., Seoul, Korea) at 1,157$\times g$ for 15 min, and the supernatant, the cholesterol-reduced cod liver oil, was collected for future study. All treatments were carried out in triplicate.

Determination of Cholesterol

For the determination of the cholesterol concentration in cod liver oil, 1 g of a 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 [23]. 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 a vacuum. The extract was re-dissolved in 1 mL of hexane and was stored at −20 °C until analysis.

The cholesterol was determined on a silica fused capillary column (HP-5, 30 m × 0.32 mm I.D. × 0.25 μm thickness) using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector. The injector and detector temperatures 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 50:1. Quantification of cholesterol was done by comparing the peak areas with the response of an internal standard.

The percentage of cholesterol reduction was calculated as follows: cholesterol reduction (%) = 100 − [(the amount of cholesterol in β-CD treated cod liver oil × 100)/the amount of cholesterol in the untreated cod liver oil (control)].

Cholesterol determination for all the samples was averaged with each batch of treatments.

Acid Value

Acid values of each sample were determined by the procedure described by Watson [24].

Recycling of β-CD

To study how effective the recycled crosslinked β-CD was for cholesterol reduction, the following process was carried out. The cholesterol-crosslinked β-CD complex was soaked in a glass tube in chloroform:methanol = 1:1 (v/v) at 100 rpm stirring speed for 2 h at 50 °C. The sample was then cooled to room temperature and centrifuged (HMR-220IV, Hanil Industrial Co., Seoul, Korea) at 6,300$\times g$ for 5 min. After centrifuging, the supernatant was decanted. The sediment (β-CD) was obtained, then dried at 50 °C in a drying oven for 6 h and reused for the recycling study.

Statistical Analysis

All statistical analyses were performed using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed using the general linear models (GLM) procedure to determine significant differences among the samples. Means were compared by using Fisher's least significant difference (LSD) procedure. Significance was defined at the 5% level.

Results and Discussion

Effects of Crosslinked β-CD Concentration

Table 1 lists the effects of different concentrations of crosslinked β-CD on cholesterol removal and acid value in cod liver oil. The cholesterol content and acid value of the control cod liver oil were 554.51 mg/100 g cod liver oil and 0.42 (data not shown). The finding on the acid value of cod liver oil was consistent with Fuse et al. [25], who reported the acid value (0.45) of cod liver oil determined by a conventional titration using phenolphthalein as an indicator. In the present study, after mixing cod liver oil with crosslinked β-CD (15–30%, w/v) at 50 °C for 20 min, the acid values of the oil slightly increased from 0.42 to 0.56.

The range of cholesterol (73.38–85.60%) was removed from cod liver oil by mixing with crosslinked β-CD (15–25%, w/v) at 50 °C for 20 min. In our laboratory, several studies on the cholesterol removal from different food products including squid liver oil [14], egg yolk [15], cream [16], lard [17], and milk [18] by crosslinked β-CD have been performed. In squid liver oil, the range of 80.04–87.08% cholesterol was removed by mixing with crosslinked β-CD (15–25%, w/v) at 55 °C for 20 min [14]. The crosslinked β-CD at 1–20% (w/v) provided cholesterol removal from 81.73 to 90.72% in cream when mixed at 40 °C for 30 min [16]. The crosslinked β-CD (10–30%, w/v) removed 78.5–95.8% cholesterol from egg yolk when mixed at 40 °C for 10 min [15]. In lard, the range of 64–93% cholesterol was removed by stirring with 1–9% (w/v) crosslinked β-CD at 40 °C for 1 h [17]. The cholesterol (85–93.1%) was removed when the crosslinked β-CD (0.5–2.5%, w/v) was mixed with milk at 10 °C for 10 min [18]. Accordingly, the optimum concentration of crosslinked β-CD for cholesterol removal could vary with different food products.

Table 1 Effects of various crosslinked β-cyclodextrin concentrations on cholesterol removal from cod liver oil

β-Cyclodextrin (%, w/v)	Cholesterol removal (%)
15	73.38 ± 0.54[c]
20	80.14 ± 0.57[b]
25	85.60 ± 1.00[a]
30	80.45 ± 0.54[b]

Values with *different letters* within the same column differ significantly ($P < 0.05$)

Factors of cholesterol removal: mixing temperature, 50 °C; mixing speed, 800 rpm; mixing time, 20 min; ratio of cod liver oil to distilled water, 1:3

Acid values in all cod liver oils after cholesterol removal under different conditions are 0.56

In the present study, an increase in the concentrations of crosslinked β-CD from 15 to 25% (w/v) led to a significant increase in the cholesterol removal from cod liver oil from 73.38 to 85.60%, but thereafter significantly decreased up to 30% (w/v) of crosslinked β-CD (80.45%). According to Lee et al. [14] evaluating the optimum concentration of crosslinked β-CD for cholesterol removal from squid liver oil, the optimum concentration of crosslinked β-CD was 25% (w/v). Other studies using digitonin [26] and saponin [27] for cholesterol adsorption indicated that above certain concentrations, saponin and digitonin showed a decrease in cholesterol removal from milk and butter oil, respectively. The authors reported that an excess of β-CD could compete with itself to bind to cholesterol molecules, thereby leading to decreased cholesterol adsorption. Therefore, it is suggested in the present study that crosslinked β-CD (25%, w/v) can be sufficiently effective to remove more than about 85% of cholesterol from cod liver oil.

Effects of the Ratio of Cod Liver Oil to Distilled Water

Cholesterol removal from cod liver oil by 25% (w/v) crosslinked β-CD was significantly associated with the ratio of cod liver oil to distilled water (Table 2). The acid values of cod liver oil were not significantly changed after cholesterol removal from the cod liver oil with different ratios of cod liver oil to distilled water, as shown in Table 2. Elevating the ratio of cod liver oil to distilled water from 1:1 to 1:2 (v/v) significantly increased the cholesterol removal from 82.80 to 86.35%. In other words, a smaller amount of cholesterol was removed from cod liver oil by crosslinked β-CD with a lower addition of water. According to Lee et al. [14], increasing the ratio of squid liver oil to distilled water up to 1:3 (v/v) showed the maximum cholesterol removal from squid liver oil. Jung et al. [15] reported that a small amount of cholesterol was removed from egg yolk by crosslinked β-CD without dilution with water because the increased viscosity may lead to decreased inclusion capacity.

In the present study, there was a significant reduction in the cholesterol removal rate from 85.65 to 80.35% on

Table 2 Effects of the cod liver oil to distilled water ratio (v/v) on cholesterol removal from cod liver oil using crosslinked *β*-cyclodextrin

Cod liver oil to distilled water (v:v)	Cholesterol removal (%)
1:1	82.80 ± 0.62[b]
1:2	86.35 ± 0.48[a]
1:3	85.65 ± 0.73[a]
1:4	80.35 ± 0.83[c]

Values with *different letters* within the same column differ significantly ($P < 0.05$)

Factors of cholesterol removal: crosslinked *β*-cyclodextrin, 25% (w/v); mixing temperature, 50 °C; mixing speed, 800 rpm; mixing time, 20 min

Acid values in all cod liver oils after cholesterol removal under different conditions are 0.56

increasing the ratio of cod liver oil to distilled water from 1:3 to 1:4. Jung et al. [15] showed that increasing the ratio of egg yolk to water from 1:2 to 1:3 significantly reduces the cholesterol removal rate from egg yolk with crosslinked *β*-CD. The results obtained from the present study show that an adequate addition of water, but not too much water, during operation is necessary.

Effects of Mixing Temperature

To investigate effect of temperature on cholesterol removal from cod liver oil, four different mixing temperatures (40, 50, 60 or 70 °C) were employed and the acid values of the cod liver oil were not remarkably affected by the mixing temperatures used in the present study (Table 3). Cholesterol removal from cod liver oil significantly increased from 64.36 to 87.36% when the mixing temperature was increased from 40 to 60 °C and significantly reduced thereafter up to 70 °C. The observation (on the increase in cholesterol removal from cod liver oil with increasing mixing temperature) was consistent with the findings of Lee et al. [14], Kim et al. [17], and Han et al. [16]. Kim et al. [17] determined the effects of mixing temperatures on cholesterol removal from lard, and they reported that increasing mixing temperature from 20 to 60 °C significantly increased the cholesterol removal rate from 90.11 to 92.07%. Lee et al. [14] noted that cholesterol removal from squid liver oil significantly increased from 63.10 to 86.57% when the mixing temperature was increased from 25 to 55 °C and was stable thereafter up to 70 °C. In milk, the cholesterol removal was significantly increased from 85.14 to 92.38% with increasing the mixing temperature from 0 to 10 °C when milk was mixed with 1% (w/v) crosslinked *β*-CD at 800 rpm for 10 min [28].

Table 3 Effects of various mixing temperatures on cholesterol removal in cod liver oil using crosslinked *β*-cyclodextrin

Mixing temperature (°C)	Cholesterol removal (%)
40	64.36 ± 0.44[d]
50	85.77 ± 0.42[b]
60	87.36 ± 1.33[a]
70	73.67 ± 1.25[c]

Values with *different letters* within the same column differ significantly ($P < 0.05$)

Factors of cholesterol removal: crosslinked *β*-cyclodextrin, 25% (w/v); mixing speed, 800 rpm; mixing time, 20 min; ratio of cod liver oil to distilled water, 1:2

The acid value in all cod liver oils after cholesterol removal under different conditions was 0.56

Effects of Mixing Speed

The acid value of cod liver oil did not vary after cholesterol removal from the oil, and the cholesterol removal from cod liver oil was in the range of 82.13–87.14% when cod liver oil was mixed at different mixing speeds (400–1,200 rpm) with 25% (w/v) crosslinked *β*-CD at 60 °C for 20 min (Table 4). Elevating mixing speed from 200 to 400 rpm resulted in a significant increase in cholesterol removal from cod liver oil from 83.72 to 87.14%, and thereafter the cholesterol removal was not significantly changed up to 800 rpm. On the other hand, cholesterol removal from cod liver oil significantly decreased when increasing the mixing speed from 800 to 1,200 rpm. Accordingly, it is suggested in the present study that the optimum mixing speed to obtain the maximum cholesterol removal from cod liver oil was 400 rpm.

Cholesterol reduction (90.81–92.05%) from milk by crosslinked *β*-CD was not significantly affected by increasing the mixing speed from 400 to 800 rpm and

Table 4 Effects of various mixing speeds on cholesterol removal in cod liver oil using crosslinked *β*-cyclodextrin

Mixing speed (rpm)	Cholesterol removal (%)
200	83.72 ± 0.45[b]
400	87.14 ± 0.68[a]
800	86.95 ± 0.89[a]
1,200	82.13 ± 0.74[c]

Values with *different letters* within the same column differ significantly ($P < 0.05$)

Factors of cholesterol removal: crosslinked *β*-cyclodextrin, 25% (w/v); mixing temperature, 60 °C; mixing time, 20 min; ratio of cod liver oil to distilled water, 1:2

The acid value in all cod liver oils after cholesterol removal under different conditions was 0.56

decreased thereafter up to 1,200 rpm [28]. In lard, even though the mixing speed might not be an important factor on cholesterol removal using crosslinked β-CD at mixing speeds over 150 rpm, cholesterol removal was significantly lower below a 100-rpm mixing speed [17].

Effects of Mixing Time

The effects of various mixing times on the cholesterol removal and acid value in cod liver oil using crosslinked β-CD are presented in Table 5. Increasing the mixing time did not influence the acid values of cod liver oil. Cholesterol removal from cod liver oil significantly increased from 68.81 to 87.26% when mixing time increased from 5 to 20 min. After mixing time of 30 min, cholesterol removal from cod liver oil was significantly reduced up to 78.69%. Thus, the observation indicated that 20 min of mixing time with 25% (w/v) crosslinked β-CD at 60 °C could be sufficient for greater than about 87% reduction of cholesterol from cod liver oil. The decrease in cholesterol removal rate after 30 min of mixing time could be related to the instability of an inclusive complex between β-CD and cholesterol during longer mixing time [16, 17].

Recycling of β-CD

After the optimum conditions were found for cholesterol removal from cod liver oil, we examined whether the crosslinked β-CD could be recycled and still be able to remove cholesterol effectively or not. The β-CD for recycling was applied to cod liver oil four times, and the results are shown in Fig. 1. When crosslinked β-CD was used for up to three times, relatively higher cholesterol removal was found in the range of 83.09–87.27%, but thereafter began to decline (76.95%). Lee et al. [14] reported that over 70% of cholesterol in squid liver oil with crosslinked β-CD was removed after three times of recycling. Therefore, the present study demonstrated the possibility for applying crosslinked β-CD repeatedly (i.e., up to three times) in cod liver oil.

In a similar recycling study [29], recycled β-CD showed 75.07% cholesterol removal from cream, while the mixture of recycled to unused powdered β-CD with the ratio of 6–4 increased cholesterol removal to 95.59%. Therefore, the present study suggested that to improve the recycling efficiency of the recycled β-CD from cod liver oil, proper amounts of unused β-CD could be combined with the recycled β-CD.

Table 5 Effects of various mixing times on cholesterol removal in cod liver oil using crosslinked β-cyclodextrin

Mixing time (min)	Cholesterol removal (%)
5	68.81 ± 0.66[d]
10	75.57 ± 1.54[c]
20	87.26 ± 0.95[a]
30	78.69 ± 0.43[b]

Values with *different letters* within the same column differ significantly ($P < 0.05$)

Factors of cholesterol removal: crosslinked β-cyclodextrin, 25% (w/v); mixing temperature, 60 °C; mixing speed, 400 rpm; ratio of cod liver oil to distilled water, 1:2

The acid value in all cod liver oils after cholesterol removal under different conditions was 0.56

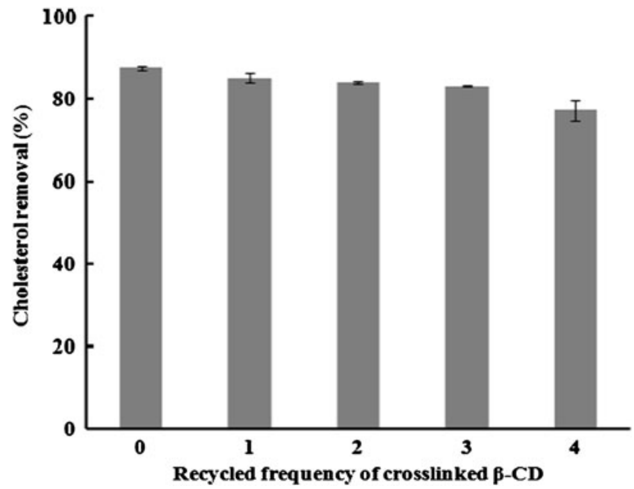

Fig. 1 Cholesterol removal rate in cod liver oil by reuse frequency of crosslinked β-cyclodextrin (*β-CD*). Factors of cholesterol removal: ratio of cod liver oil to distilled water, 1:2; crosslinked β-CD, 25% (w/v); mixing temperature, 60 °C; mixing speed, 400 rpm; mixing time, 20 min

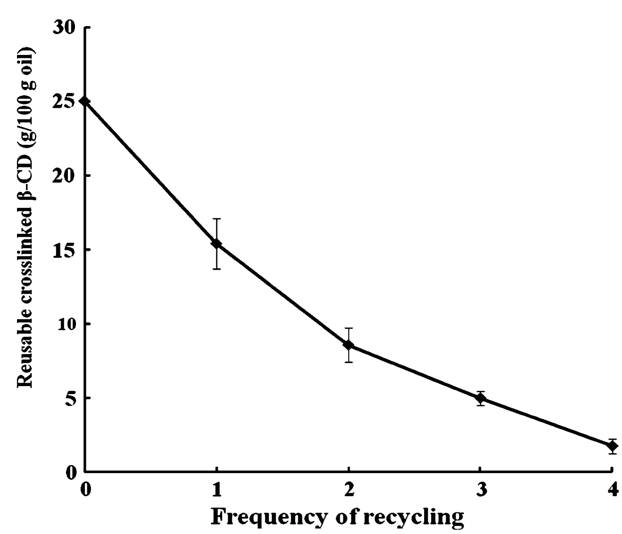

Fig. 2 The amounts of reusable crosslinked β-cyclodextrin (*β-CD*) used for cholesterol removal from cod liver oil after repeated recycling

The amounts of reusable crosslinked β-CD used for cholesterol removal from cod liver oil after repeated times of recycling are shown in Fig. 2. The amounts of reusable crosslinked β-CD decreased with increasing the frequency of recycling. This finding could be explained by the destruction or loss of β-CD during the separation of the cholesterol-crosslinked β-CD complex.

In conclusion, based on the findings in the present study, optimized conditions for cholesterol removal from cod liver oil were a 1:2 ratio of cod liver oil to distilled water, 25% (w/v) crosslinked β-CD concentration, 20 min mixing time, 400 rpm mixing speed and a mixing temperature of 60 °C, resulting in the removal of over about 87% of cholesterol. It is also indicated that β-CD crosslinked with adipic acid was effectively recyclable. The present study showed the first evidence of the possibility of applying crosslinked β-CD to reduce cholesterol from cod liver oil.

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