J Am Oil Chem Soc (2010) 87:233–238
DOI 10.1007/s11746-009-1487-z

ORIGINAL PAPER

Cholesterol Removal from Squid Liver Oil by Crosslinked β-Cyclodextrin

Jeong Eun Lee · Mi Hwa Seo · Yoon Hyuk Chang · Hae-Soo Kwak

Received: 20 July 2009 / Revised: 20 September 2009 / Accepted: 8 October 2009 / Published online: 27 October 2009

Abstract The present study was designed to optimize the different conditions for β-cyclodextrin (β-CD) crosslinked by adipic acid on cholesterol removal from squid liver oil. Different factors were concentrations of crosslinked β-CD, mixing temperature, ratio of squid liver oil to distilled water, mixing time and mixing speed. It was found that cholesterol removal from squid liver oil was significantly affected by concentrations of crosslinked β-CD, mixing temperature, ratio of squid liver oil to distilled water, mixing time and mixing speed. In a recycling study, cholesterol removal from the squid liver oil with recycled crosslinked β-CD in the first recycling trial was 81.05%, which was slightly lower than that with new crosslinked β-CD (87.06%). In up to three time trials, over 70% of cholesterol removal was observed. The present study indicated that the optimum conditions for cholesterol removal from squid liver oil using crosslinked β-CD were a 1:3 ratio of squid liver oil to distilled water, 25% (crosslinked β-CD/distilled water, w/v) crosslinked β-CD concentration, 20 min mixing time, 800 rpm mixing speed and 55 °C mixing temperature with about 87% cholesterol removal.

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

J. E. Lee · M. H. Seo · Y. H. Chang · H.-S. Kwak (✉)
Department of Food Science and Technology,
Sejong University, Seoul 143-747, Korea
e-mail: kwakhs@sejong.ac.kr

Introduction

In recent years, a number of studies have indicated that cholesterol removal from food products such as milk, cream, butter, lard, egg yolk and cheese was most effectively achieved by powdered β-cyclodextrin (β-CD) treatment [1–9]. β-CD is a cyclic oligosaccharide composed of α(1→4) linkages of seven glucose units. It has a hydrophobic cavity at the center of its molecular arrangement, which forms an inclusion complex with various non-polar molecules including cholesterol. β-CD is also nontoxic, edible, non-hygroscopic, chemically stable, and easily separated from the complex [10, 11].

Even though the powdered β-CD treatment allows an effective removal of cholesterol (more than 90%) from milk and cream, considerable β-CD was consumed for this process due to the ineffective recovery [5, 12]. To overcome the problem of the powdered β-CD, the crosslinking of β-CD has been investigated [2, 3, 5–8]. 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 hydroxyl groups of starch [13]. In our previous studies, crosslinked β-CD made with adipic acid exhibited over 90% cholesterol removal and highly efficient recycling rate in egg yolk [3], cream [5], lard [6] and milk [12].

Squid liver oil extracted from fresh squids is a product containing considerable amounts of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), highly unsaturated ω-3 fatty acids, which are essential for growth of marine fishes. That is, the weight gain, feed efficiency and protein efficiency ratio of fish fed the diets containing squid liver oil were significantly higher than those fed the diets containing lauric acid, corn oil or linseed oil as the sole lipid source [14]. However, it has been generally known that the squid liver oil contains a considerable amount of cholesterol. Therefore, removing the cholesterol from the squid liver oil can be a great way to enhance the health benefits, and there is a possibility of producing nutraceutical food products including cholesterol-free squid liver oil. However, no information is available on the efficiency of β-CD crosslinked with adipic acid on cholesterol removal from squid liver oil. Therefore, the objective of the present study was to investigate the optimum conditions for cholesterol removal and recycling efficiency from squid liver oil using β-CD crosslinked with adipic acid.

Experimental Procedures

Materials

Commercial squid (*Todarodes pacificus*) liver oil was obtained from Dongnam Fishery Co. (Samchuk, Korea) 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 purchased from Sigma Chemical Co. (St. Louis, MO, USA), 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 in a stirrer at room temperature with constant agitation for 2 h. Two grams of adipic acid (purity 99%, Shinyo Pure Chemicals Co. Ltd., Tokyo, Japan) 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 a Whatman No. 2 filter paper and washing three times with 150 mL 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 squid liver oil to make mixtures with the ratios 1:1, 1:2, 1:3 or 1:4 of squid 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 1,000 rpm), different mixing temperatures (25, 40, 55 or 70 °C) and different mixing times (5, 10, 20 or 30 min). To prevent the oxidation of squid 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×*g* for 15 min, and the supernatant, the cholesterol-reduced squid liver oil, was collected for the future study. All treatments were carried out in triplicate.

Determination of Cholesterol

For the determination of cholesterol concentration from squid 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 [15]. 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 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 chromatography (Palo Alto, CA, USA) equipped with a flame ionization detector. The injector and detector temperature were 270 and 300 °C, respectively. The oven temperatures were programmed from 200 to 300 °C at 10 °C/min and held for 20 min. Nitrogen was used as the 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 squid liver oil × 100/the amount of cholesterol in the untreated squid liver oil (control)].

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

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 = 3:1 (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×*g* for 5 min. β-CD was precipitated and dried at 50 °C in a dry 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 Discussions

Effects of Crosslinked β-CD Concentration

The effects of different concentrations of crosslinked β-CD on cholesterol removal from squid liver oil are presented in Table 1. The cholesterol content of the control squid liver oil was 1,640 mg/100 g squid liver oil (data not shown). The crosslinked β-CD (15–30%, w/v) removed the cholesterol from 80.04 to 87.08% when mixed with squid liver oil at 55 °C for 20 min. Several studies on the cholesterol removal from different food products such as egg yolk [3], cream [5], lard [6] and milk [12] by crosslinked β-CD have been performed in our laboratory. 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 [5]. The crosslinked β-CD (10–30%, w/v) removed 78.5 to 95.8% cholesterol from egg yolk when mixed at 40 °C for 10 min [3]. In lard, the range of 64–93% cholesterol was removed by stirring with 10% (w/v) crosslinked β-CD for 1 h [6]. 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 [12]. Therefore, the optimum concentration of crosslinked β-CD for cholesterol removal could vary with different food products.

In the present study, elevating the concentrations of crosslinked β-CD from 15 to 25% (w/v) significantly increased the cholesterol removal from squid liver oil from 81.76 to 87.08%, but thereafter significantly reduced up to 30% (w/v) of crosslinked β-CD (80.04%). Other studies using digitonin [16] and saponin [17] 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 87% of cholesterol from squid liver oil.

Effects of Mixing Temperature

To evaluate conditions that could influence cholesterol removal from squid liver oil by temperature, four different mixing temperatures (25, 40, 55 or 70 °C) were employed (Table 2). Cholesterol removal from squid liver oil significantly increased from 63.10 to 86.57% when mixing temperature increased from 25 to 55 °C and was stable thereafter up to 70 °C. The finding (on the increase in cholesterol removal from squid liver oil with increasing mixing temperature) was consistent with Kim et al. [6], who studied the effects of mixing temperatures on cholesterol removal from lard. They reported that increasing mixing temperature from 20 to 60 °C significantly increased the cholesterol removal rate from 90.11 to 92.07%. Furthermore, 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.

Effects of the Ratio of Squid Liver Oil to Distilled Water

Cholesterol removal from squid liver oil by 25% (w/v) crosslinked β-CD was significantly associated with the ratio of squid liver oil to distilled water (Table 3). The

Table 1 Effects of various crosslinked β-cyclodextrin concentrations on cholesterol removal in squid liver oil

β-cyclodextrin (%, w/v)	Cholesterol removal (%)
15	81.76 ± 0.69^{b}
20	82.56 ± 1.19^{b}
25	87.08 ± 0.45^{a}
30	80.84 ± 0.81^{c}

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

Factors of cholesterol removal: mixing temperature, 55 °C; mixing speed, 800 rpm; mixing time, 20 min; ratio of squid liver oil to distilled water, 1:3

Table 2 Effects of various mixing temperatures on cholesterol removal in squid liver oil using crosslinked β-cyclodextrin

Mixing temperature (°C)	Cholesterol removal (%)
25	63.10 ± 0.51^{c}
40	69.54 ± 0.58^{b}
55	86.57 ± 1.20^{a}
70	88.37 ± 1.03^{a}

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 squid liver oil to distilled water, 1:3

lowest removal (83.04%) of cholesterol from squid liver oil occurred at the ratio of squid liver oil to distilled water (1:1), and the highest removal (86.86%) of cholesterol was found at the ratio of 1:3. Otherwise, a small amount of cholesterol was removed from squid liver oil by crosslinked β-CD with less addition of water. Jung et al. [3] reported that low 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 86.86 to 80.92% with increasing the ratio of squid liver oil to distilled water from 1:3 to 1:4. Jung et al. [3] showed that the cholesterol removal rate from egg yolk with crosslinked β-CD was significantly decreased with more than 1:3 ratio of egg yolk to water. The results obtained from the present study show that that an adequate addition of water during the operation is necessary.

Effects of Mixing Time

Cholesterol removal from squid liver oil significantly increased from 75.99 to 86.86% when mixing time increased from 5 to 20 min. After mixing time of 30 min, cholesterol removal from squid liver oil was significantly decreased (80.92%, Table 4). These data suggested that 20 min of mixing time with 25% (w/v) crosslinked β-CD at 55 °C could be sufficient for greater than about 86% reduction of cholesterol from squid liver oil. The decrease in the 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 the longer mixing time [5, 6].

Effects of Mixing Speed

Cholesterol removal was in the range of 80.97–87.16% when squid liver oil was mixed with 25% (w/v) crosslinked β-CD at 55 °C for 20 min (Table 5). Elevating mixing speed from 200 to 800 rpm significantly increased cholesterol removal from 83.03 to 87.16%. However, cholesterol removal significantly decreased with mixing speeds higher than 1,000 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 decreased thereafter up to 1,200 rpm [12]. In lard, even though mixing speed might not be an important factor for cholesterol removal using crosslinked β-CD at mixing speeds over 150 rpm, cholesterol removal was significantly lower below a 100-rpm mixing speed [6].

Table 3 Effects of ratio of squid liver oil to distilled water (v/v) on cholesterol removal in squid liver oil using crosslinked β-cyclodextrin

Squid liver oil to distilled water (v:v)	Cholesterol removal (%)
1:1	83.04 ± 0.60[c]
1:2	85.15 ± 0.62[b]
1:3	86.86 ± 0.74[a]
1:4	80.92 ± 0.59[d]

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, 55 °C; mixing speed, 800 rpm; mixing time, 20 min

Table 4 Effects of various mixing times on cholesterol removal in squid liver oil using crosslinked β-cyclodextrin

Mixing time (min)	Cholesterol removal (%)
5	75.99 ± 0.77[c]
10	83.47 ± 0.79[b]
20	86.86 ± 1.33[a]
30	80.92 ± 0.59[d]

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, 55 °C; mixing speed, 800 rpm; ratio of squid liver oil to distilled water, 1:3

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

Mixing speed (rpm)	Cholesterol removal (%)
200	83.03 ± 0.71[c]
400	84.17 ± 0.51[b]
800	87.16 ± 0.88[a]
1,000	80.97 ± 0.69[d]

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, 55 °C; mixing time, 20 min; ratio of squid liver oil to distilled water, 1:3

Recycling of β-CD

Since the optimum conditions were chosen for cholesterol removal from squid liver oil, we tried to examine whether the recycled crosslinked β-CD could remove cholesterol effectively or not. The β-CD for recycling was applied to squid liver oil six 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 71.85–87.06%, but thereafter began to decline (61.03–63.54%). Therefore, the present study demonstrated the possibility for applying crosslinked β-CD repeatedly (i.e. up to three times) in squid liver oil.

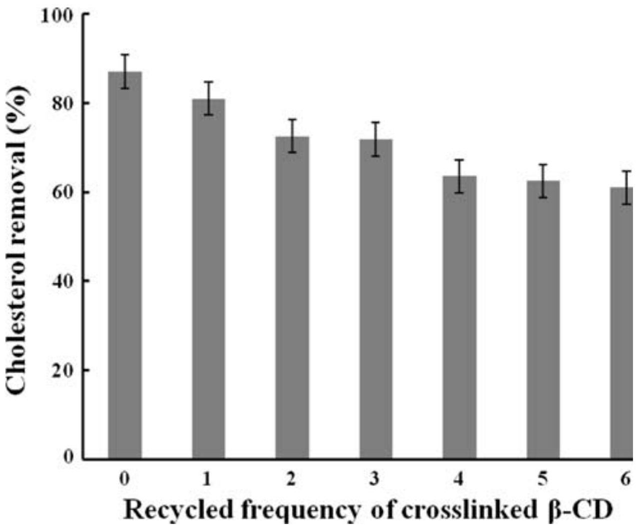

Fig. 1 Cholesterol removal rate in squid liver oil by reuse frequency of crosslinked β-cyclodextrin (β-CD). Factors of cholesterol removal: ratio of squid liver oil to distilled water; 1:3, crosslinked β-CD; 25% (w/v), mixing temperature; 55 °C, mixing speed; 800 rpm, mixing time; 20 min

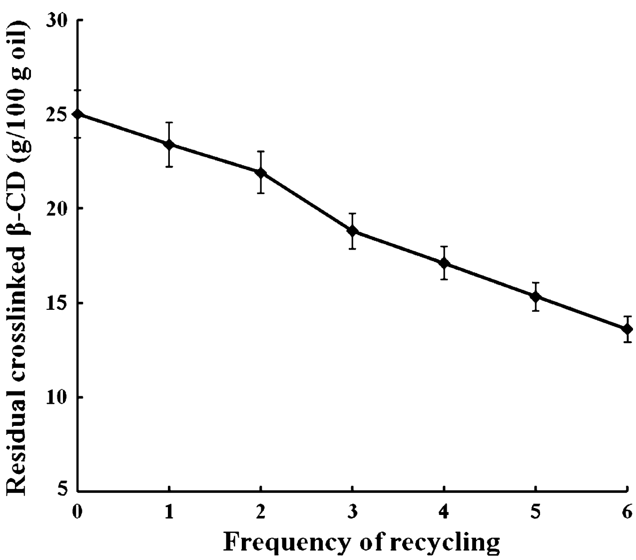

Fig. 2 The amounts of reusable crosslinked β-cyclodextrin (β-CD) used for cholesterol removal from squid liver oil after repeated recycling

In a similar recycling study [18], 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 to 4 increased cholesterol removal to 95.59%. Therefore the present study suggested that to improve the recycling efficiency of recycled β-CD from squid liver oil, proper amounts of unused β-CD could be combined with the recycled β-CD.

The amounts of reusable crosslinked β-CD used for cholesterol removal from squid liver oil after repeated times of recycling are shown in Fig. 2. The amounts of reusable crosslinked β-CD were very slightly decreased (21.92–23.41 g/100 mL oil) up to being used two time, however, thereafter it decreased considerably (13.62–18.82 g/100 mL oil). This finding could be explained by the destruction or loss of β-CD during separation of the cholesterol-crosslinked β-CD complex.

In conclusion, based on the findings in the present study, optimized conditions for cholesterol removal from squid liver oil were 1:3 ratio of squid liver oil to distilled water, 25% (w/v) crosslinked β-CD concentration, 20 min mixing time, 800 rpm mixing speed and 55 °C mixing temperature, resulting in the removal of over about 87% of the cholesterol.

Acknowledgment The present study was supported by a grant of the Brain Korea 21 Project in Seoul, Republic of Korea.

References

1. Kim SH, Ahn J, Kwak HS (2004) Crosslinking of β-cyclodextrin on cholesterol removal from milk. Arch Pharm Res 11:1183–1187
2. Kim SH, Han EM, Ahn J, Kwak HS (2005) Effect of crosslinked β-cyclodextrin on quality of cholesterol-reduced cream cheese. Asian Aust J Anim Sci 18:584–589
3. Jung TH, Park HS, Kwak HS (2005) Optimization of cholesterol removal by crosslinked β-cyclodextrin in egg yolk. Food Sci Biotechnol 14:793–797
4. Kim JJ, Jung TH, Ahn J, Kwak HS (2006) Properties of cholesterol-reduced butter made with β-cyclodextrin and added evening primrose oil and phytosterols. J Dairy Sci 89:4503–4510
5. Han EM, Kim SH, Ahn J, Kwak HS (2007) Optimizing cholesterol removal from cream using β-cyclodextrin cross-linked with adipic acid. Int J Dairy Technol 60:31–36
6. Kim SH, Kim HY, Kwak HS (2007) Cholesterol removal from lard with crosslinked β-cyclodextrin. Asian Aust J Anim Sci 20:1368–1472
7. Lee SJ, Hwang JH, Lee S, Ahn J, Kwak HS (2007) Property changes and cholesterol-lowering effects in evening primrose oil-enriched and cholesterol-reduced yogurt. Int J Dairy Technol 60:22–30
8. Kim HY, Bae HY, Kwak HS (2008) Development of cholesterol-reduced Blue cheese made by crosslinked β-cyclodextrin. Michwissenschaft 63:53–56
9. Alonso L, Cuesta P, Gilliland SE (2009) Effect of β-cyclodextrin on *trans* fats, CLA, PUFA and phospholipids of milk fat. J Am Oil Chem Soc 86:337–342
10. Nagamoto S (1985) Cyclodextrin-expanding the development of their functions and applications. Chem Econ Eng Rev 17:28–34
11. Astray G, Gonzalez-Barreiro C, Mejuto JC, Rial-Otero R, Simal-Gándara J (2009) A review on the use of cyclodextrins in foods. Food Hydrocolloid 23:1631–1640
12. Han EM, Kim SH, Ahn J, Kwak HS (2005) Cholesterol removal from homogenized milk with crosslinked β-cyclodextrin by adipic acid. Asian Aust J Anim Sci 18:1794–1799
13. Cardwell CG (1952) Industrial starch research. Chem Eng News 30:514–517
14. Lee SM, Cho SH (2009) Influences of dietary fatty acid profile on growth, body composition and blood chemistry in juvenile fat cod (*Hexagrammos otakii* Jordan et Starks). Aquacult Nutr 15:19–28

15. Adams ML, Sullivan DM, Smith RL, Richer EF (1986) Evaluation of direct saponification method in determination of cholesterol in meats. J Assoc Anal Chem 69:844–846
16. Micich TJ (1990) Behaviors of polymer-supported digitonin with cholesterol in the absence and presence of butter oil. J Agric Food Chem 38:1839–1843
17. Oh HI, Jang EJ, Kwak HS (1998) Conditions of the removal of cholesterol from milk by treatment with saponin. Korean J Dairy Sci 20:253–260
18. Kwak HS, Suh HM, Ahn J, Kwon HJ (2001) Optimization of β-cyclodextrin recycling process for cholesterol removal in cream. Asian Aust J Anim Sci 14:548–552