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Immobilized β-Cyclodextrin as a Simple and Recyclable Method for Cholesterol Removal in Milk

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This study was designed to determine the optimum conditions of three different factors (mixing time, mixing temperature, and tube size) in reduction of cholesterol in milk using immobilized β-CD beads. Immobilized β-CD glass beads were prepared at different conditions of silanization and β-CD immobilization reactions. In result, the glass beads (diameter 1 mm) at 20 mM 3-isocyanatopropyltriethoxysilane and 30 mM β-CD without base showed the highest cholesterol removal rate as 41%. Using above immobilized β-CD glass beads, the cholesterol removal rate was 40.2% with 6 h of mixing time in 7 mm diameter tube at 10°C. After cholesterol removal from milk, the glass beads were washed for cholesterol dissociation and reused. In recycling study, the cholesterol removal rate was 41%, which was mostly same as that using new glass beads. These results indicated that cholesterol removal rate was about 40% with β-CD immobilized glass beads, however, the recycling efficiency was almost 100%.

Key words: β-CD immobilization, Cholesterol removal, 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).

Experiments on animals and human have shown that plasma cholesterol can be raised by increased intake of cholesterol and saturated fat (Gurr, 1982; Pyorala, 1987; Sieber, 1990; Carleton *et al.*, 1991). 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).

Food companies have developed many methods to reduce cholesterol by using various physical, chemical, and biological methods. Some examples are blending in vegetable oils (Durkley, 1982), extraction by organic solvent (Larsen and Froning, 1981), adsorption with saponin and digitonin (Micich, 1990) to form cholesterol complexes, degradation of cholesterol by cholesterol oxidases (Watanabe *et al.*, 1989), and removal by supercritical carbon dioxide extractions (Ong *et al.*, 1990). 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 milk, cream, and Mozzarella cheese was most effectively achieved by β-CD treatment (Oakenfull and Sihdu, 1991; Makoto *et al.*, 1992; Ahn and Kwak, 1999; Lee *et al.*, 1999; Kwak *et al.*, 2001). β-cyclodextrin (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 arrangements, which forms an inclusion complex with various compounds including cholesterol (Szejtli, 1982). Also, β-CD is 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

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powder is an ineffective way for β-CD recovery. Therefore, the objective of this study was to examine the possibility of β-CD immobilization on solid support. β-CD on solid support would allow easy and complete recovery of β-CD-cyclodextrin from milk and additionally, to be recycled easily.

MATERIALS AND METHODS

Materials

Commercial milk (3.6% milk fat) was purchased from a retail store as needed, and 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.

Preliminary study

To test the degree of β-CD immobilization, four different immobilized β-CD glass beads were made at different conditions of silanization and β-CD immobilized reaction. Then, beads prepared at different conditions were shaken with same amount of milk (100 g beads/100 g milk) for 3 h at 140 rpm. The cholesterol removal rate was measured by gas chromatography.

Preparation of β-CD immobilized glass beads

To a 100 g of glass beads in 2.5 mm diameter, which was previously washed with 100 mL of distilled water three times, was poured 160 mL of piranha solution (H_2SO_4 : H_2O_2 = 3 : 1, v/v) and stirred for 30 min. After piranha solution was removed from glass beads, 100 mL of distilled water was poured into glass beads and sonicated for 5 min. Then distilled water was removed from glass beads and the glass beads were dried at room temperature for 12 h. After the glass beads were dried, 100 mL of 20 mM 3-isocyanatopropyltriethoxysilane in dried toluene was poured to glass beads and stirred for 4 h. Then the isocyanatopropyltriethoxysilane solution was removed from glass beads and the glass beads were washed with 100 mL of dried toluene three times. These glass beads were added into 100 mL of 30 mM β-CD solution in anhydrous dimethylsulfoxide (DMSO) and heated for 100°C overnight. The glass beads were filtered from the β-CD solution, washed three times with 100 mL of ethanol and dried at reduced pressure.

Cholesterol removal rate

To study the effects of three different factors, 100 mL of milk was placed in a 100-mL tube in a temperature-controlled water bath. Three factors were applied into the cholesterol removal process, namely, the amount of immobilized β-CD, mixing time, mixing temperature, and tube size. After mixing, 1 mL of milk was used for cholesterol determination.

Extraction and determination of cholesterol

For the extraction of cholesterol from milk, 1 mL of the immobilized 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. Quantitation 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 immobilized 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 β-CD was for cholesterol reduction was carried out. The used glass beads 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 beads 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 (Cary, NC, 1985) and differences among treatments were determined by Duncans multiple test at $p < 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

Immobilization of β-CD

In the past two decades, evidence has being gathered to suggest that an excess of cholesterol might be deleterious. Since a number of studies have indicated the importance of cholesterol reduction in dairy products, cholesterol has been removed from milk and dairy products by a β-CD-based process, and the resulting low-cholesterol butter and cheese appeared to be indistinguishable from conventional products. The possibility of fat or fat-soluble vitamin removal by β-CD was reported (Szejtli, 1982), however, our previous studies indicated that little difference was found in fat composition even cheeses, high fat product (Kwak *et al.*, 2002; Kwak *et al.*, 2003). Therefore, the removal of other nutrients is not likely to be the primary concern in this application.

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. Even though there are other methods known for the recovery of β-CD such as utilizing hydrogen bond inhibitor (Wen Shieh, 1994), heating (Mentink *et al.*, 1990) and sodium chloride (Weu Shich *et al.*, 1995), simpler methods are still required for the effective process.

One method to overcome these problems is immobilization of β-CD on solid support. β-CD on solid support would allow easy and complete recovery of β-CD from dairy products. In addition, β-CD on solid support can be recycled easily. β-CD is composed of 7 glucoses, which form a ring with 21 hydroxyl groups. Therefore, selective introduction of other functional group, which is needed to react with solid support, to the β-CD was challenging. Pretreatment of β-CD for the introduction of new functional groups to the β-CD would required several steps of chemical reactions which require high cost and long time (Takahashi *et al.*, 1984; Cornwell *et al.*, 1995). Without pretreatment of β-CD, the immobilization process would be much easier and save a lot of cost and time. Therefore, we pursued a process, which do not require pretreatment of β-CD.

The degree of β-CD immobilization was tested by degree of cholesterol extraction from milk. The degree of β-CD immobilization should be affected by the conditions of silanization and β-CD immobilization reactions. Therefore, we have prepared the β-CD immobilized glass bead at different conditions of silanization and β-CD immobilization reactions. Then these glass beads prepared at different conditions were shaken with same amount of milk (100 g beads/100 g milk) for 3 h at 140 rpm. Then the amount of cholesterol left in the milk was measured by gas chromatography. The results are shown in Table I. The glass beads prepared at 20 mM 3-isocyanatopropyltriethoxysilane and 30 mM β-CD without base showed the best results.

To develop one step preparation of β-CD immobilized glass bead as a simple method, we imagined the reaction between isocyanate modified glass bead surface and primary hydroxyl groups in β-CD. They would react well at elevated temperature and no modification of functional groups in the β-CD would be necessary. After the reaction, β-CD would be immobilized on the glass bead surface with robust carbamate linkage.

Table I. The extraction rate of cholesterol from milk with β-cyclodextrin immobilized glass beads prepared at different conditions

Condition	Concentration of 3-isocyanatopropyltriethoxy (mM)	Concentration of β-cyclodextrin (mM)	Cholesterol removal (%)
1	4	30	25.3
2	4	30, with 2 eq. NaOH	30.2
3	20	30	41.0
4	20	30, with 2 eq.NaOH	25.8

The isocyanate modified glass beads were easily obtained from the reaction between 3-isocyanatopropyltriethoxysilane and glass beads which were previously treated with piranha solution (H_2SO_4 : H_2O_2 = 3 : 1, v/v). Then the β-cyclodextrin modified glass beads were prepared from the reaction of β-CD and the isocyanate modified glass beads at 100°C in dried dimethylsulfoxide for 24 h (Fig. 1). Results suggested that the effectiveness of cholesterol adsorption by immobilized β-CD was dependent on several factors including the immobilized adsorbent concentration, bead size, stirring tube size, stirring time and stirring temperature.

Optimimum conditions of cholesterol removal effect of mixing time

To examine conditions that might affect cholesterol removal by time, three different mixing times were tested. Cholesterol removal was not significantly affected by mixing time after 6 h mixing treatment (Table II). The β-CD immobilized beads removed 41.1% of the cholesterol for 6 h when mixed in 7 cm tube size (i.d.) with 140 rpm at

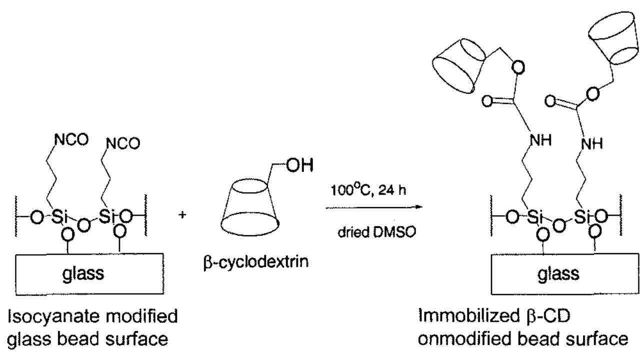

Fig. 1. One step immobilization of β-cyclodextrin on glass bead surface for using cholesterol removal

Table II. Effect of different mixing times on cholesterol removal using immobilized β-cyclodextrin in milk[1]

Mixing time (h)	Cholesterol removal (%)
3	36.7[b]
6	41.1[a]
9	40.1[a]

[1]Means within a column with different superscript letters differ ($p < 0.05$). Means of triplicated cholesterol extraction.
Other experimental factors included the ratio of sample and immobilized β-CD, 1:1; mixing temp., 10°C; mixing speed, 140 rpm; and tube size, I.D. 7 cm.

10°C with 1:1 ratio (beads to milk). More time of mixing did not enhance the cholesterol removal rate. Therefore, it appears that 6 h of mixing may be sufficiently effective to remove cholesterol from milk.

Effect of mixing temperature

To examine conditions that might affect cholesterol removal by temperature, three different mixing temperatures were tested. No difference was found in cholesterol removal at 5, 10, or 15°C (Table III). 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 140 rpm for 6 h.

Effect of tube size

Similar to mixing time and temperature, no significant difference was found with different tube sizes (Table IV). The removal rate was in the range of 37.9 to 40.2% when milk was mixed 1:1 with beads in 7 cm tube size (i.d) with 140 rpm for 6 h.

Recycling of β-CD

Since the optimum conditions were chosen for collecting recycled β-CD, we decided to examine how effective the recycled immobilized β-CD would be in cholesterol removal. The β-CD immobilized glass beads for recycling were applied to milk 5 times repeatedly, as shown in Table V. As results, the recycled immobilized β-CD showed exactly same cholesterol removal rate as that of unused immobilized β-CD (Table V). The cholesterol reduction existed between 39.8 to 40.6%, which almost identical with that when applying unused one ($p < 0.05$).

Table III. Effect of different mixing temperatures on cholesterol removal using immobilized β-cyclodextrin in milk[1]

Mixing temperature (°C)	Cholesterol removal (%)
5	39.6[a]
10	39.7[a]
15	40.2[a]

[1]Means within a column with different superscript letters differ ($p < 0.05$). Means of triplicated cholesterol extraction.
Other experimental factors included the ratio of sample and immobilized β-CD, 1:1; mixing time, 6 h; mixing temp., 10°C; mixing speed, 140 rpm; and tube size, I.D. 7 cm.

Table IV. Effect of different tube sizes on cholesterol removal using recycled immobilized β-cyclodextrin for in milk[1]

Tube size (I.D., cm)	Cholesterol removal (%)
5	38.4[a]
7	40.2[a]
9	37.9[a]

[1]Means within a column with different superscript letters differ ($p < 0.05$). Means of triplicated cholesterol extraction.
Other experimental factors included the ratio of sample and immobilized β-CD, 1:1; mixing time, 6 h; mixing temp., 10°C; and mixing speed, 140 rpm.

CONCLUSION

A number of studies have indicated that the removal of cholesterol from milk and cream was effectively conducted by treatment with powder β-CD (Makoto *et al.*, 1992;Yen and Tsui, 1995; Ahn and Kwak, 1999; Lee *et al.*, 1999). In our laboratory, over 90% of cholesterol was removed from commercial milk at refrigerated temperature with 1% β-CD. Even though lots of results are reported the effective cholesterol removal by using powder β-CD, little information is available using immobilized β-CD applying in milk nowadays.

The present study showed that immobilized β-CD glass beads prepared by silanization and β-CD immobilization reaction resulted in 41% of cholesterol removal rate in milk. Therefore, we may suggest that simpler method of cholesterol removal using immobilized β-CD glass beads was found and can be applied in milk or other dairy

Table V. The effect of recycling repeatability on immobilized β-cyclodextrin for cholesterol removal rate in milk[1]

Number of repeated recycling	Cholesterol removal (%)
1st	40.1[a]
2nd	39.8[a]
3rd	40.6[a]
4th	40.0[a]
5th	39.9[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 : immobilized β-cyclodextrin = 1 : 1 for 24 h in room temperature.
Other experimental factors included the ratio of sample and immobilized β-CD, 1:1; mixing time, 6 h; mixing temp., 10°C; mixing speed, 140 rpm, tube size, I.D. 7 cm.

products. More interesting thing is that immobilized β-CD glass beads was as effective as new powder β-CD after recycling. More profound study would be needed in future.

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