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Morphological Change of Crosslinked β-Cyclodextrin after Recycling

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ABSTRACT

The present study was carried out to examine the effect of crosslinked β-cyclodextrin(β-CD) made by adipic acid on cholesterol removal rate and find the structural change after recycling on SEM observation. The size reduction and morphological changes were found during the recycling process and the profound changes were observed at 8th time reuse. After cholesterol removal in milk, the used crosslinked β-CD was washed for cholesterol dissociation and reused. In recycling study, the cholesterol removal rate at first trial was 92.5% in milk, which was mostly same as that using new crosslinked β-CD(92.4%). With repeated 10th reuse of crosslinked β-CD resulted in 81.4% of cholesterol removal in milk. Similar trend was found in cream and cholesterol removal was 91.5% at 1st trial and 83.4% at 10th trial. In both milk and cream samples, the removal rate at 1st reuse was not significantly different from that at 6th reuse($p>0.05$). The present study indicated that crosslinked β-CD made by adipic acid resulted in the effective recycling efficiency, especially up to 6th reuse and morphological modifications were not distinguishable up to 8th reuse.

(Key words : recycling efficiency, crosslinked β-CD, cholesterol removal, milk, cream)

INTRODUCTION

Experiments on animal and human have shown that plasma cholesterol can be raised by an increased intake of cholesterol and saturated fat(Pyorala, 1987; Carleton *et al.*, 1991; Gurr, 1992; Sieber, 1993). 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).

β-cyclodextrin(β-CD) is a cyclic oligosaccharide composed of α(1-4) linkage of seven glucose units. It has a cavity at the center of its molecular arrangement, which forms an inclusion complex with various compounds including cholesterol(Szejtli, 1982). In addition, 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 systems and recovery. Also, most of these methods are relatively non-selective and remove flavor and nutritional components along with cholesterol. Moreover, some methods require high investment and operation costs. Therefore, crosslinked β-CD was developed and its utility needed to be found.

Crosslinking is a commonly used derivatization technique for manipulating starch functionality, and epichlorohydrin and adipic anhydride have been extenively used to produce crosslinked starches, which inter- or intramolecular mono- and diethers are formed with hydroxyly groups of starch(Hamerstrand *et al.*, 1959). This modification produces important changes in the starch functional properties an increase or decrease in viscosity(Whistler and Daniel, 1990).

Scanning electron microscopy(SEM) is intrinsically a non-destructive 3-dimensional imaging tool that is of great importance when specimens with elaborate surface ornamentations have to be examined(Bazzola and Russell, 1999). The large depth of field and small beam size make it possible to image far below the top layer of specimens. SEM can be suitable for the structural examination in this study, since it can provide visual information on morphological changes of crosslinked β-CD after the recycling process. No information is available about the recycling rate

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and morphological change of β-CD during recycling process, therefore, the objective of this study was to examine the efficiency of reused crosslinked β-CD on cholesterol removal in milk and cream when used 10 times repeatedly.

MATERIALS AND METHODS

1. Materials

Commercial homogenized milk(3.6% milk fat) and cream (36% milk fat) were purchased from a retail store as needed, and 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 gas-chromatographic grade.

2. Preparation of Crosslinked β-CD

A sample of 100g of β-CD was prepared in a 80mL distilled water and placed in a stirrer at room temperature with constant agitation for 2h. Then 5g of 5% adipic acid was incorporated with 100g β-CD and pH was adjusted to pH 10 with 1N NaOH(Hamerstrand *et al.*, 1959). The β-CD solution was stirred at room temperature for 90min, and then readjusted to pH 5 with acetic acid. β-CD was recovered by filtering with Whatman paper No. 2, and washed three times with 150 mL of distilled water. The product was dried at 60℃ in a Lab-line mechanical convection oven for 20h and passed through a 100mesh.

3. Extraction and Determination of Cholesterol

For the extraction of cholesterol, 1g of milk or cream sample was placed in a screw-capped glass tube(15mm×180mm), and 1mL of 5α-cholestane(1mg/mL) was added as an internal standard. The sample was saponified at 60℃ for 30 min with 5mL of 2M ethanolic potassium hydroxide solution(Adams *et al.*, 1986). After cooling to room temperature, cholesterol was extracted with 5mL of hexane(Adams *et al.*, 1986). 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 1mL of hexane and was stored at −20℃ until analysis.

Total cholesterol was determined on a silica fused capillary column(HP-5, 30m×0.32mm I.D.×0.25μm thickness) using Hewlett - Packard 5890A gas chromatograpyh(Palo Alto, CA, USA) equipped with a flame ionization detector. The temperatures of injector and detector were 270 and 300℃, respectively. The oven temperatures were programmed from 200 to 300℃ at 10℃/min and hold for 20min. Nitrogen was used as a carrier gas at a flow rate of 2mL with a split ratio of 1/50. Quantitation of cholesterol was done by comparing the peak areas with a response of an internal standard.

The percentage of cholesterol reduction was calculated as followed: cholesterol reduction(%)=100−(amount of cholesterol in β-CD-treated sample×100/amount of cholesterol in control). Cholesterol determination for control was averaged with each batch of treatments.

4. SEM Observations

Crosslinked β-CD was mounted on a brass stub(10mm in diameter) using two-sided adhesive tape. The stub surface was gently blown to remove unattached crosslinked β-CD powders using a hand-held blower. The specimens were then made electrically conductive by coating under an argon atmosphere with a thin layer(approximately 30nm in thickness) of gold using a sputter-coater(JFC-1100E; JEOL Ltd., Tokyo, Japan). The specimens were examined with a scanning electron microscope(JSM-5410LV; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20kV(Baboota and Agarwal, 2005).

5. Measurement of β-CD Molecule

One milligram of reused crosslinked β-CD was dissolved in 100mL distilled water. The solution was filtered and 50μL of sample was injected into HPLC(Kant *et al.*, 2004). Beta-CD was analyzed on GROM-SIL 100 ODS-S FE column(4.6mm× 250mm), and a HPLC(Waters, Plymouth, MN, USA) equipped with Differential refractometer was used. Flow rate was 0.8 mL/min and mobile phase was distilled water : methanol(95 : 5, v/v). All samples were analyzed in triplicate.

6. Recycling Efficiency of β-CD

To study how effective the recycled crosslinked β-CD was in cholesterol reduction of milk or cream sample, the following process was carried out. The cholesterol-crosslinked β-CD complex was soaked in glass tube in acetic acid : butanol=3 : 1 (v/v) with 100rpm stirring speed for 2h at 50℃(Kwak *et al.*, 2001) and the ratio of complex to solvent was 6 : 1. Then, the sample was cooled to room temperature and centrifuged at 630 ×g for 5min. β-CD was then precipitated and dried at 50℃ in dry oven for 6h and reused for recycling study.

7. Statistical Analysis

Data from the determination of optimum conditions of butter,

Table 1. The efficiency of recycled crosslinked β-cyclodextrin on cholesterol removal in milk and cream[1]

Number of reuse	Cholesterol removal(%)	
	Milk	Cream
Unused	92.45[a]	91.82[a]
2[nd]	92.22[ab]	91.35[a]
4[th]	92.18[ab]	90.85[ab]
6[th]	91.52[ab]	90.95[ab]
8[th]	89.37[b]	89.76[b]
10[th]	83.28[c]	85.47[c]

[1] Means within column by the same letter are not significantly different(p <0.05).

Recycled crosslinked β-cyclodextrin was treated by following factors; Acetic acid : isopropanol = 3 : 1, solvent : crosslinked β-CD = 6 : 1, mixing time: 2hr, mixing temp.: 50℃, mixing speed: 100rpm, centrifugal force: 630×g, and centrifugal time: 5min.

one-way ANOVA(SAS, 1985) was used. The significance of the results was analyzed by the least significant difference (LSD) test. Difference of p<0.05 was considered to be significant.

RESULTS AND DISCUSSION

1. Morphological Changes by SEM Observation

The gradual reductions in size and associated morphological changes that had occurred during the recycling process were revealed by SEM observations(Fig. 1). After the first recycling process, highly agglomerated β-CD molecules were prevalent. The crosslinked β-CD appeared as irregular-shaped pieces of diverse size. There was no significant difference in size and morphology of β-CD after the initial several times of recycling processes(No. 2 to 7). The most striking difference in particle size and morphology was found after the eight times of recycling process(No. 8). A rather drastic reduction in the shape of the β-CD was observed thereafter up to ten times of recycling process. Many small-adhered particles were found on the surface of large particles. Discrete individual particles with irregular forms were detected.

SEM observations revealed the external morphological changes of crosslinked β-CD molecule after recycling processes. It appeared that the agglomerated particles after the initial recycling processes were attributed to the crosslinking of β-CD. Compared with the particles after the initial recycling processes, the most striking difference in particle size and morphology was found after the eight times of recycling process in this study. These results were consistent with those from the

Fig. 1. Scanning electron micrographs of crosslinked β-cyclodextrin after recycling process. The gradual reductions in size and associated morphological changes after recycling process were revealed by SEM observations. 1. After one time reuse, 2. After two time reuse, 3, After three time reuse, 4. After four time reuse, 5. After five time reuse, 6. After six time reuse, 7. After seven time reuse, 8. After 8 time reuse, 9. After 9 time reuse, and 10. After ten time reuse. Scale bars = 20 μm.

quantification of crosslinked β-CD after recycling process, showing the gradual reductions in size after around eight times of recycling. It is possible that the crosslinked β-CD undergo a significant fragmentation to individual subunits after around eight times of recycling.

2. Amount of β-CD Molecule after Recycling

Since the cholesterol removal rate of recycled crosslinked β-CD was maintained up to 6 time reuse, we tried to find out

whether amount of β-CD molecules was related closely to above aspects or not. Therefore, amount of β-CD molecule regardless of powder or crosslinked type was measured by HPLC and the results are shown in Fig. 2. At initial(unused crosslinked β-CD), 99.8ppm of β-CD out of 100ppm was existed and the amount decreased slowly up to 8 time reuse as 96.1ppm, which was not significantly different($p<0.05$). From 9 time reuse, the crosslinked β-CD remaining were 93.3 and 90.4ppm. This result may not clearly explain the cholesterol removal rate described above, however, it could be indicated that the decreased amount of crosslinked β-CD molecule could be one reason of the lower cholesterol removal rate with reuse.

3. Recycling Efficiency of Crosslinked β-CD

For the recycling study, the crosslinked β-CD was applied to homogenized milk 10 times repeatedly and results are shown in Table 1. In milk, the recycled crosslinked β-CD showed a similar cholesterol removal as that of unused crosslinked β-CD. The cholesterol reduction was in the range of 83.28 to 92.45%. Up to 6 times of crosslinked β-CD reuse, cholesterol removal was slightly but not significantly higher than that in 8 time reuse, whereas it was significantly higher than in 10 time reuse($p<0.05$). Therefore, the present study provided possibility for applying crosslinked β-CD repeatedly in homogenized milk. In cream, the cholesterol removal was between 85.47 to 91.82% with 10 time reuse. When crosslinked β-CD was used up to 6 times, cholesterol removal was over 90%, and

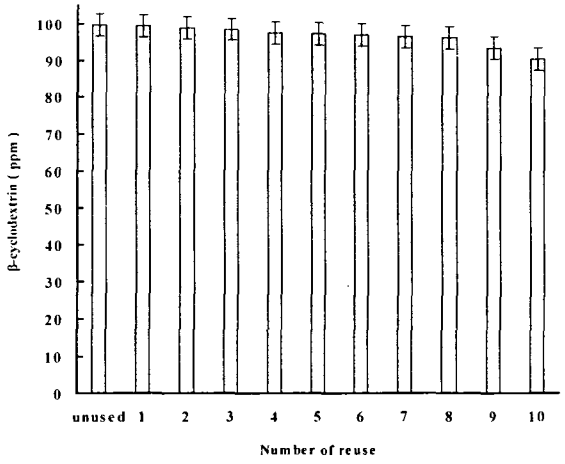

Fig. 2. The changes of amount of β-CD molecules after recycling process.

significantly higher compared with that in 10 time reuse($p<0.05$). Therefore, the present study provided an advantage for using recycled crosslinked β-CD on cholesterol removal in milk and cream, which showed an almost identical result with unused crosslinked β-CD applying.

The present results were in accordance to our previous recycling studies using crosslinked β-cyclodextrin(Han *et al.*, 2005; Han *et al.*, 2007). In addition, recycled powder β-CD showed 75.1% 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%(Kwak *et al.*, 2001). 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 and cream.

Crosslinked β-CD was prepared with adipic acid. In recycling study, the cholesterol removal rate in second trial was 92.45%, which was mostly same as that using new crosslinked β-CD. In addition, this study showed a first evidence of possibility for applying crosslinked β-CD by adipic acid in dairy food, and further study would be needed in future. Therefore, we may conclude that crosslinked β-CD could be replaced the powder β-CD for cholesterol removal process in food industry because of the recycling efficiency and operation cost.

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