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Recovery of Cholesterol from the β-Cyclodextrin-Cholesterol Complex Using Immobilized Cyclomaltodextrinase of Alkalophilic *Bacillus* sp. KJ 133

KWON, HO JEONG[1]*, HYE JIN JUNG[1], AND HAE SOO KWAK[2]

[1]Department of Bioscience and Biotechnology and [2]Department of Food Science, Sejong University, Seoul 143-747, Korea

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Abstract A new combined method including the enzymatic hydrolysis of β-cyclodextrin (β-CD) and solvent extraction of cholesterol from the hydrolyzed mixture was developed to recover cholesterol from a β-CD-cholesterol complex prepared from dairy products, such as cream, milk, and cheese. Cyclomaltodextrinase (cyclomaltodextrin dextrin hydrolase, EC 3.2.1.54, CDase) prepared from alkalophilic *Bacillus* sp. KJ 133 hydrolyzed the β-CD of the β-CD-cholesterol complex, and then, free cholesterol was efficiently extracted from the hydrolyzed mixture by a nonpolar solvent such as ethyl acetate. To increase the stability of free CDase, immobilized CDase was developed using sodium alginate as a carrier. The immobilized CDase showed a high recovery yield of cholesterol in a time-dependent manner compared to the free CDase. A gas chromatography analysis showed that more than 70% of cholesterol was recovered from the β-CD-cholesterol complex of cream by the immobilized CDase, whereas only 3% and 29% of cholesterol were recovered when the solvent extraction and free CDase treatment were used, respectively. The cholesterol recovered can be used as a raw material for steroid synthesis. Furthermore, this method can be an efficient way to recover cholesterol or other organic compounds that are bound in a β-CD-cholesterol or -organic compound complex.

Key words: CDase, β-CD, cholesterol, immobilized enzyme

Several chronic diseases, such as arteriosclerosis, cardiac insufficiency, or hyperpiesia, are known to be caused by the excessive intake of cholesterol [5, 16]. Numerous efforts have been made to develop efficient methods for removing cholesterol from dairy products with high cholesterol content [10, 18]. β-cyclodextrin (β-CD), a cyclic nonreducing maltooligosaccharide, has been used for this purpose since its hydrophobic interior cavity structure adsorbs nonpolar cholesterol or organic compounds [6, 14, 15].

Meanwhile, cholesterol, a representative sterol and a component of all eukaryotic plasma membranes, is essential for the growth and viability of higher organisms [2, 4]. It is also an important precursor of bile salts, that degrade lipid, and steroid hormones such as aldosterone, estrogen, and testosterone [3, 17]. In addition, cholesterol helps to transport lipids into the blood and neutralizes their harmful effects *in vivo* [9, 19]. Since cholesterol can be utilized as a synthetic precursor of steroid compounds or other bio-active materials, the cholesterol removed from dairy products can be a good source for this purpose.

This study aimed to develop an efficient method for cholesterol recovery from the β-CD-cholesterol complex using cyclomaltodextrinase (cyclomaltodextrin dextrin hydrolase, EC 3.2.1.54, CDase), which can hydrolyze the β-CD, thereby releasing cholesterol from the complex [7, 8].

As the source of CDase, an alkalophilic *Bacillus* sp. KJ 133 was used, based on a previous study that it produced the enzyme [16]. The enzyme was prepared from a culture of *Bacillus* sp. KJ 133 in a medium consisting of 1.0% soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.1% KH_2PO_4, and 0.02% $MgSO_4 \cdot 7H_2O$; the pH was adjusted to 10.6 with 1.5% Na_2CO_3 after autoclaving [1]. The strain was grown aerobically in conical baffled flasks at 37°C with shaking (250 rpm).

The enzyme purification was carried out at 4°C in 0.05 M Na-phosphate buffer (pH 6.0). Briefly, the cultured cells were harvested and washed twice with the buffer and disrupted by an ultrasonic oscillator (for 15 sec with 3 intervals) on ice. The soluble cell-free extract was recovered by centrifugation and used as crude CDase.

The CDase activity was determined by measuring the degree of the color development of phenolphthalein released from the β-CD-phenolphthalein complex. The reaction mixture was incubated at 40°C for 30 min and 0.1 M

*Corresponding author
Phone: 82-2-3408-3640; Fax: 82-2-3408-3334;
E-mail: kwonhj@sejong.ac.kr

Na_2CO_3 was added to stop the reaction. The absorbance of the phenolphthalein was measured in a spectrophotometer at 550 nm. The enzyme activity unit was determined by multiplying by 100 times the UV absorbance at 550 nm, which was resulted from phenolphthalein released from the β-CD-phenolphthalein complex. The activity of the enzyme and the cell growth showed maximal levels after 48 h of cultivation. The pH did not significantly change during the cultivation [11]. The CDase production pattern and activity of alkalophilic *Bacillus* sp. KJ 133 were similar with those of alkalophilic *Bacillus* sp. no. 199 that was known to produce CDase (data not shown). Consequently, a crude CDase preparation was prepared from the cells after 48 h of cultivation.

The crude CDase was also immobilized in alginic acid, a marine polysaccharide, to increase the stability of the enzyme [12, 13]. Briefly, 2.5% sodium-alginate and crude CDase solutions were mixed at 1:1 ratio, and a 0.1 M $CaCl_2$ solution was then added for gelatinization. The resulting translucent spherical gel was left for 15 min at room temperature, then stored at 4°C and used as immobilized CDase.

The soluble and immobilized CDase were used individually with a 1% β-CD-cholesterol complex in 0.05 M Na-phosphate (pH 6.0) prepared from cream to recover the cholesterol from the complex. The reaction mixture was incubated at 40°C with shaking (200 rpm), then 5 ml of the products were removed after 1, 3, and 5 h, respectively. Ethyl acetate (10 ml) was then added to the products which were mixed thoroughly. The ethyl acetate layer was collected and concentrated *in vacuo* and 500 μl of methanol was added to solublize the cholesterol. Ten ml of an internal standard material (1 mg/ml 5α-cholestane in 99.8% ethanol) and 10 ml of 2 M ethanolic KOH were mixed with the extracts which were then saponified at 80°C for 2 h and cooled to room temperature. The cholesterol was recovered by extraction using 5 ml of hexane and 2.5 ml of distilled water. Finally, the extract was dissolved in 200 μl of hexane after evaporation. Five μl of the extract was injected onto a gas chromatography (GC) column, then the amount of cholesterol was analyzed quantitatively. Table 1 shows the instrument and working conditions of the GC used for the cholesterol analysis.

Table 1. Instrument and working conditions of GC for analysis of cholesterol.

Instrument	HP Model 5890 series (Hewlett-Packard Co., Palo Alto, CA, U.S.A.)
Column	HP-5 (30 m×0.32 mm I.D.×0.25 μm film thickness)
Carrier gas	N_2 (2 ml/min)
Oven temp.	200°C→10°C/min→300°C (20 min)
Injector temp.	270°C
Detector temp.	300°C
Detector	FID

Figure 1 shows the gas chromatogram pattern of the cholesterol recovered from the β-CD-cholesterol complex by the CDase treatment. The cholesterol and cholestane were identified by their specific retention time and quantified based on the peak area. Using this gas

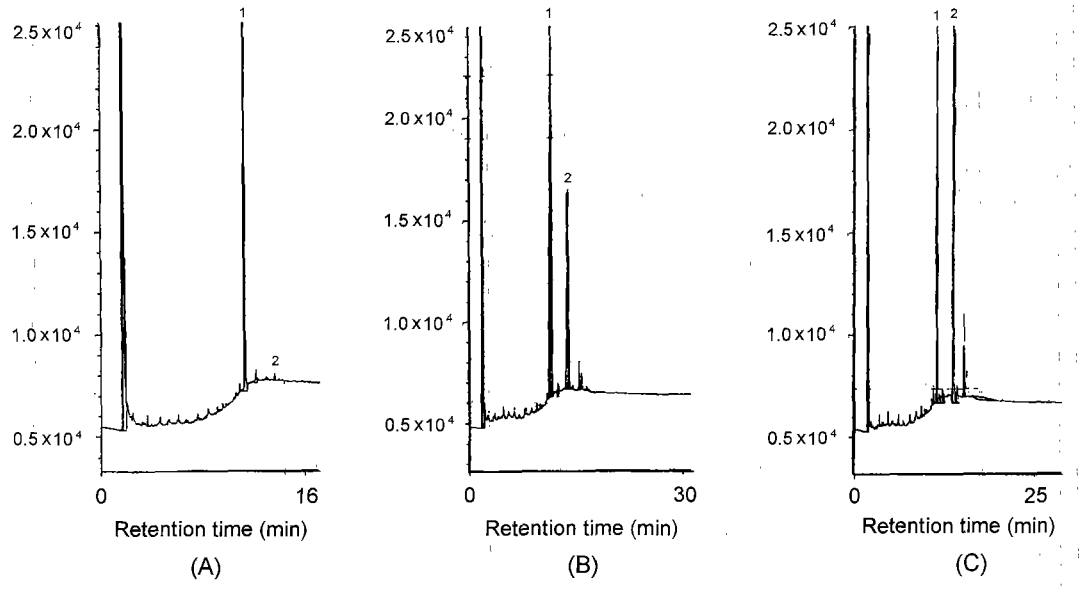

Fig. 1. Gas chromatogram patterns of cholesterol recovered from the β-CD-cholesterol complex under different conditions.
(A) Control (solvent extraction only), (B) Soluble CDase treatment, (C) Immobilized CDase treatment. Each peak is identified as follows: 1, 5α-cholestane; 2, cholesterol. Column: Hewlett-Packard cross-linked 5% phenyl methyl silicone fused capillary column (30 m×0.32 mm I.D.× 0.25 μm film thickness); Oven temperature: 200°C for 1 min, 10°C/min to 300°C, hold for 20 min; N_2 (carrier gas): 2 ml/min. H_2: 30 ml/min. air: 300 ml/min. auxiliary gas (N_2): 28 ml/min, split ratio: 1: 50; Detector: FID.

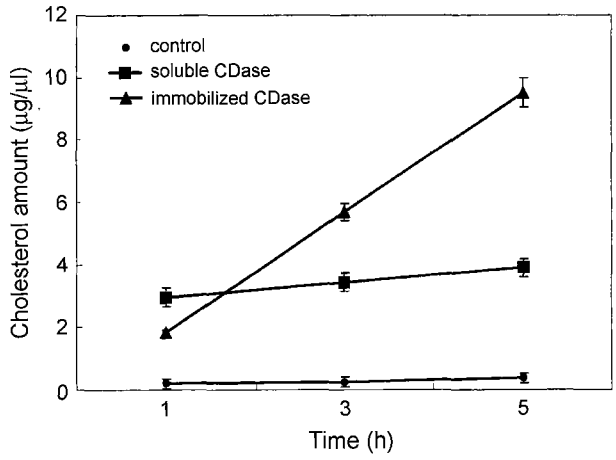

Fig. 2. Cholesterol amount released from the β-CD-cholesterol complex under different conditions.
A 1% β-CD-cholesterol complex in 0.05 M Na-phosphate (pH 6.0) was used as the substrate. The reaction conditions were as follows: control (no enzyme); 11.5 ml of buffer, 8.5 ml of substrate, soluble CDase; 10 ml of buffer, 1.5 ml (120 units) of enzyme, 8.5 ml of substrate, immobilized CDase; 10ml of buffer, 1.5 ml (120 units) of enzyme, 8.5 ml of substrate. Each reaction was carried out at 40°C in a shaking incubator (200 rpm), then the cholesterol was extracted from 5 ml of the products using ethyl acetate after 1, 3, and 5 h, respectively.

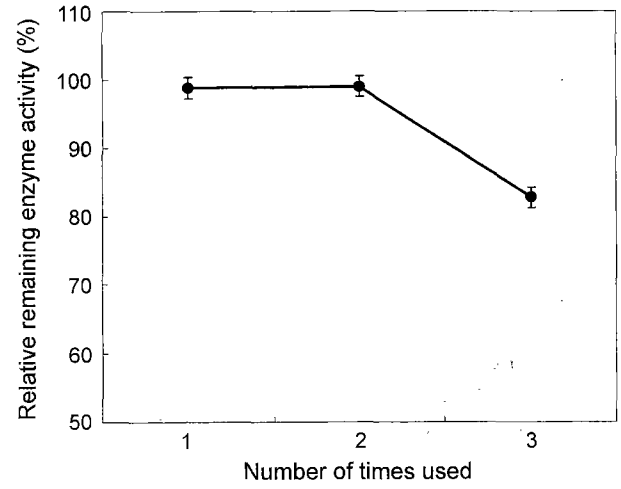

Fig. 3. Residual activity of immobilized CDase with repeated reaction.
Four ml of immobilized CDase was mixed with 1.7 ml of a substrate (0.5% β-CD, 0.002% phenolphthalein, and 0.05 M Na-phosphate buffer (pH 6.0)), and incubated for 1 h at 40°C in a shaking incubator (200 rpm). The used immobilized CDase was reacted again with 1.7 ml of a new substrate under the same conditions. This whole treatment was repeated three times.

chromatography, the amount of cholesterol recovered under different conditions was investigated. We first investigated the efficiency of cholesterol recovery from the β-CD-cholesterol complex using solvent alone or the combination of solvent with soluble or immobilized enzyme treatment. An equal amount of enzyme (120 units) was used for soluble and immobilized enzymes. As shown in Fig. 2, the amount of cholesterol recovered from the solvent extraction alone was minimal in the absence of CDase. Treatment by soluble CDase showed cholesterol content to be higher than that of solvent extraction in the early incubation stage. However, the amount of cholesterol increased slowly due to enzyme instability. In contrast, treatment with immobilized CDase showed a significant increase in the cholesterol content due to the increased stability of the enzyme in the immobilized beads. As a result, 70% of cholesterol was recovered with the immobilized CDase treatment, whereas only 3% and 29% of cholesterol were recovered with the solvent extraction and free CDase treatments, respectively (Table 2). Even with the immobilized CDase treatment, not all the cholesterol was recovered, however, all the β-CD seemed to be hydrolyzed by the immobilized CDase treatment, because there was no precipitation found after the reaction (data not shown). Further studies on the optimum extraction conditions for cholesterol from a reaction mixture are currently underway.

Table 2. Yield of cholesterol recovery when treated with soluble and immobilized CDase.

Sample	Cholesterol content (μg/g of cream)	Yield (%)
β-CD-cholesterol complex	13.6	
Control (solvent extraction only)	0.371	3
Soluble CDase	3.907	29
Immobilized CDase	9.520	70

Next, the stability of immobilized CDase was investigated in repeated experiments. The used immobilized CDase was again reacted with a new substrate under the same conditions up to three times. The enzyme activity remained at more than 80% even after being used three times, thereby implying an increased stability of the enzyme due to the immobilization (Fig. 3).

In conclusion, a new cholesterol recovery method was developed using immobilized CDase that hydrolyzed the β-CD in a β-CD-cholesterol complex from dairy products. The cholesterol recovered can then be used as a raw material for steroid synthesis, and this method can be an efficient way to recover cholesterol or other organic compounds, such as aromatic, lipidic, or pigmental compounds, from a β-CD-cholesterol or -organic compound complex.

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