# Enzymatic Synthesis of Steryl Esters of Polyunsaturated Fatty Acids

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**ABSTRACT:** Steryl esters of long-chain fatty acids have water-holding properties, and polyunsaturated fatty acids (PUFA) have various physiological functions. Because steryl ester of PUFA can be expected to have both features, we attempted to synthesize steryl esters of PUFA by enzymatic methods. Among lipases used, *Pseudomonas* lipase was the most effective for the synthesis of cholesteryl docosahexaenoate. When a mixture of cholesterol/docosahexaenoic acid (3:1, mol/mol), 30% water, and 3000 units/g of lipase was stirred at 40°C for 24 h, the esterification extent attained 89.5%. Under the same reaction conditions, cholesterol, cholestanol, and sitosterol were also esterified efficiently with docosahexaenoic, eicosapentaenoic, arachidonic, and γ-linolenic acids.

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**KEY WORDS:** Esterification, lipase, polyunsaturated fatty acid, *Pseudomonas,* steryl ester.

Steryl esters of fatty acids have water-holding properties and are widely used as ingredients of cosmetics and bath additives (1). Furthermore, cholesteryl esters were reported to be very important as intercellular lipids (2). On the other hand, polyunsaturated fatty acids (PUFA) are used as pharmaceutical substances, ingredients of cosmetics, and in health foods and food materials. Actually, the ethyl ester of eicosapentaenoic acid (EPA; 20:5n-3) has been used in the treatment of arteriosclerosis obliterans and hyperlipemia (3). Tuna oil containing docosahexaenoic acid (DHA; 22:6n-3) and borage oil containing γ-linolenic acid (GLA; 18:3n-6) have been used as components in infant formulas, health foods, and food materials (4,5). In addition, a single-cell oil containing arachidonic acid (AA; 20:4n-6) has been desired as a component in infant formulas, because it shows an effect on the growth acceleration of infants as well as does DHA (6,7). Thus, we attempted to synthesize steryl esters of PUFA which are expected to have both features.

Because PUFA are very sensitive to heat and oxidation, enzymatic catalysis, which proceeds efficiently under mild conditions, is attractive for the synthesis of steryl esters of PUFA. It has already been reported that steryl esters of long-chain fatty acids were synthesized with *Candida rugosa* lipase (1), while steryl esters of PUFA have not been synthesized. This paper deals with the synthesis of steryl esters of PUFA with a lipase from *Pseudomonas* sp.

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## MATERIALS AND METHODS

*Chemicals*. Sterols and linoleic acid were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). DHA and EPA were products of Maruha Corp. (Tokyo, Japan), and their purities were 95%. GLA (purity, 97%) and AA (92%) were gifts from Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan) and Suntory Co. (Osaka, Japan), respectively. The molar amount of fatty acid was calculated on the basis of its acid value.

*Lipases.* Lipases were gifts from the following companies: *Pseudomonas aeruginosa* lipase (LPL; Toyobo Co. Ltd., Osaka, Japan); *Pseudomonas* sp. lipase (LIPOSAM; Showa Denko, K.K., Tokyo, Japan); *Pseudomonas* sp. lipases (Lipase-AK, and Lipase-PS; Amano Pharmaceutical Co. Ltd., Aichi, Japan), *C. rugosa* lipase (Lipase-OF; Meito Sangyo Co. Ltd., Aichi, Japan) and *Rhizopus delemar* lipase (Ta-lipase; Tanabe Seiyaku Co. Ltd., Osaka, Japan). Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Ind. Co., Osaka, Japan) with 50 mM KOH as described previously (8). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

*Reaction*. A reaction mixture of sterol, PUFA, water, and lipase was stirred at 500 rpm in a 50-mL screw capped vial sealed with nitrogen gas. The esterification extent was calculated from the amount of fatty acid consumed during the reaction.

*Silica gel column chromatography*. Steryl ester and sterol were extracted with 150 mL *n*-hexane after adding 70 mL of 0.5 N KOH (30% ethanol solution) to 10–15 g of reaction mixture. The extracts (*ca*. 10 g) were applied to a silica gel 60 column (30 × 250 mm; Merck, Darmstadt, Germany), and steryl ester was eluted with a mixture of *n*-hexane/ethyl acetate (98:2, vol/vol).

*Analysis*. Fatty acids in cholesteryl ester were methylated in methanol using sodium methylate as a methylating reagent, and were analyzed with a Hewlett-Packard 5890 *plus* gas

chromatograph (Avondale, PA) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA). The column temperature was raised from 190 to 290°C at a rate of 5°C/min, and then raised to 320°C at a rate of 10°C/min. The temperatures of the injector and detector (FID, flame-ionization detector) were set at 245 and 340°C, respectively. The carrier gas was helium at a flow rate of 25 cm/s. Thin-layer chromatography was performed by developing substances on a silica gel plate 60 (Merck) with a mixture of *n*-hexane/ethyl acetate/acetic acid (80:20:1, vol/vol/vol). Infrared (IR) spectrum was measured with a Shimadzu FTIR-8100M spectrophotometer (Kyoto, Japan). Nuclear magnetic resonance (NMR) spectrum was measured in $CDCl_3$ using tetramethylsilane as the internal standard with a UNITY 300 spectrometer (Varian, CA). The molecular mass was analyzed by field desorption mass spectrometry (FD-MS) performed on a JMS-DX303HF (JEOL Ltd., Tokyo, Japan).

## RESULTS AND DISCUSSION

*Screening for suitable lipase for synthesis of cholesteryl esters of PUFA.* Because lipases act generally on DHA most weakly among PUFA, we screened for a suitable lipase for the synthesis of cholesteryl docosahexaenoate (ChDHA) (Table 1). *Rhizopus delemar* lipase did not synthesize ChDHA, because the lipase is a 1,3-positional specific lipase and does not act on secondary alcohol (9). *Candida rugosa* lipase is nonspecific toward ester bonds of triglyceride and acts on secondary alcohol (10). The enzyme synthesized cholesteryl linoleate (esterification extent, 73.7%), but not ChDHA. This result was shown to be due to the very weak activity on DHA. Several *Pseudomonas* lipases act on secondary alcohol, and also on DHA somewhat (11,12). *Pseudomonas* sp. lipase (LIPOSAM) was the most effective among the enzymes tested. Thus the lipase was selected for the following experiments.

*Several factors affecting esterification of cholesterol with DHA.* Cholesterol was esterified with two molar equivalents of DHA in a mixture containing 20% water, by weight, using various amounts of lipase (Table 2). The esterification extent of cholesterol increased with increasing amounts of the lipase, but did not increase significantly once more than 2000 U/g-reaction mixture of the lipase was used.

Effect of water content on the synthesis of ChDHA was examined (Fig. 1). The highest esterification extent was obtained in mixtures containing 30 to 50% water. In general, esterification proceeded efficiently in the mixture with smaller amount of water. In this reaction, the enzyme exists in the water phase in the reaction mixture. Therefore, a smaller amount of water reduces the contact of the enzyme with the substrates and results in low esterification extent.

Figure 2 shows the effect of the amount of DHA on esterification of cholesterol. An increase in the extent of esterification was not observed even though more than three molar

**TABLE 1**
**Suitable Lipases for Synthesis of Cholesteryl Docosahexaenoate[a]**

| Lipase | Esterification (%)[b] |
|---|---|
| *Pseudomonas aeruginosa* | 38.3 |
| *Pseudomonas* sp.[c] | 62.5 |
| *Pseudomonas* sp.[d] | 14.0 |
| *Pseudomonas* sp.[e] | 10.0 |
| *Candida rugosa* | 3.0 |
| *Rhizopus delemar* | N.D.[f] |

[a]A mixture of 2.4 g docosahexaenoic acid (DHA)/cholesterol (=2:1, mol/mol), 0.6 g water and 4500 units (U) lipase was incubated at 30°C for 20 h with stirring at 500 rpm.
[b]Esterification extent of cholesterol.
[c]LIPOSAM (Showa Denko, K.K., Tokyo, Japan).
[d]Lipase-AK (Amano Pharmaceutical Co. Ltd., Aichi, Japan).
[e]Lipase-PS (Amano Pharmaceutical Co. Ltd.)
[f]N.D., Not detected.

**TABLE 2**
**Effect of Lipase Amount on Synthesis of Cholesteryl Docosahexaenoate[a]**

| Lipase amount (U/g)[b] | Esterification (%)[c] |
|---|---|
| 400 | 6.4 |
| 700 | 24.2 |
| 1000 | 39.7 |
| 2000 | 75.3 |
| 4000 | 80.0 |

[a]A mixture of 2.4 g of DHA/cholesterol (2:1, mol/mol) and 0.6 g water was stirred at 30°C for 20 h with various amounts of *Pseudomonas* lipase (LIPOSAM).
[b]Lipase amount per 1 g of reaction mixture.
[c]Esterification extent of cholesterol. See Table 1 for abbreviations and company source.

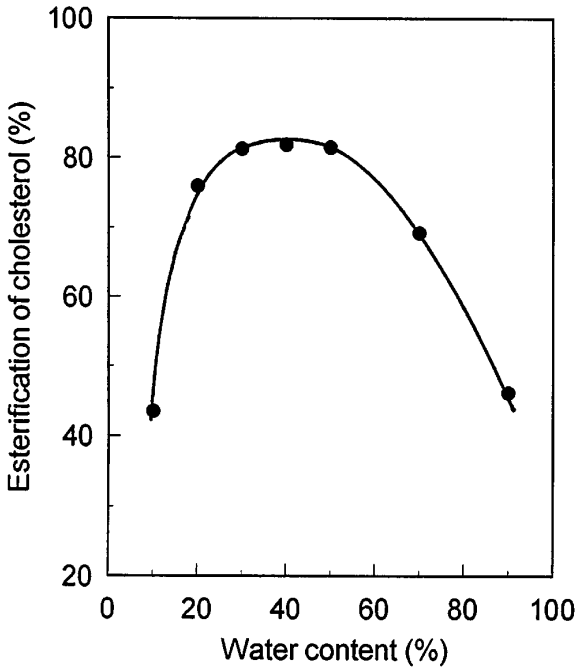


**FIG. 1.** Effect of water content on esterification of cholesterol with docosahexaenoic acid (DHA). A mixture (3.0 g) of DHA/cholesterol (=2:1, mol/mol) and various amounts of water was stirred at 30°C for 20 h with 3000 units (U)/g-reaction mixture of *Pseudomonas* lipase (LIPOSAM; Showa Denko, K.K., Tokyo, Japan). Percentages of water are expressed on a weight basis.

equivalents of DHA was added. The effect of temperature is shown in Figure 3. The esterification extent at the early stage of reaction (after 4 h) increased with raising temperature, but the extent after 20 h was constant above 30°C.

On the basis of the above results, the reaction conditions were set as follows: A reaction mixture containing DHA/cholesterol (3:1, mol/mol), 30% water, and 3000 U/g-reaction mixture of *Pseudomonas* lipase was incubated at 40°C with stirring at 500 rpm. The reaction period was extended to over 24 h under these conditions, but the esterification extent did not increase. Thus the reaction period was fixed at 24 h.

*Structure of synthesized product*. Cholesterol (2.5 g) was esterified with DHA under the above conditions (esterification extent, 93.5%). The reaction product was extracted with *n*-hexane under alkaline conditions, and purified by silica gel column chromatography (purified product, 2.7 g). The product was methylated and then analyzed by gas chromatography. As a result, cholesterol and methyl docosahexaenoate were detected at the molar ratio of 50:48, which showed that the synthesized product consisted of equal amounts of cholesterol and DHA.

The absorption maxima of the IR spectrum of the synthesized product were observed at the following position: 3013, 1736 (C=O), 1466, and 1171 (C–O–) $cm^{-1}$. The absorbance of hydroxy group was observed in cholesterol and DHA, but not in the synthesized product. The absorbance of the carbonyl group in the product shifted to a shorter wavelength by 23 $cm^{-1}$, indicating the formation of ester bond.

The synthesized product was identified by $^{1}H$ and $^{13}C$ NMR, and mass spectroscopies. $^{1}H$-NMR $\delta$ = 0.68 (3H, *s*, $CH_3$), 0.86 (3H, *d*, *J* = 6.6 Hz, $CH_3$), 0.87 (3H, *d*, *J* = 6.6 Hz, $CH_3$), 0.92 (3H, *d*, *J* = 6.6 Hz, $CH_3$), 0.97 (3H, *t*, *J* = 7.5 Hz, $CH_3$), 1.01 (3H, *s*, $CH_3$), 1.07–1.70 (22H, *m*), 1.78–1.91 (2H, *m*), 1.92–2.13 (4H, *m*), 2.26–2.42 (6H, *m*), 2.78–2.90 (10H, *m*), 4.53–4.67 (1H, *m*, >C<u>H</u>–O–CO–), 5.18–5.47 (13H, *m*, –<u>H</u>C=C<u>H</u>– and >C=C<u>H</u>–); $^{13}C$-NMR $\delta$ = 11.8, 14.2, 18.7, 19.3, 20.5, 21.0, 22.5, 22.8, 22.9, 23.8, 24.3, 25.5, 25.6 × 4, 27.8, 28.0, 28.2, 31.8, 31.9, 34.5, 35.8, 36.2, 36.6, 37.0, 38.1, 39.5, 39.7, 42.3, 50.0, 56.1, 56.7, 73.9, 122.6, 127.0, 127.8, 128.0, 128.05, 128.1 × 2, 128.16 × 2, 128.2, 128.5, 129.1, 132.0, 139.6, 172.4. Signals from the carbonyl carbon of DHA and the secondary alcohol carbon at C-3 of cholesterol were observed at 179.6 and 71.7 ppm, respectively. These signals of the synthesized product shifted to 172.4 and 73.9 ppm, respectively. It was therefore shown that DHA was bound at the hydroxy group at C-3 of cholesterol. The molecular weight ion was observed at *m/z* 696 ($M^+$) on FD-MS. These results show that the synthesized product is ChDHA.

*Synthesis of steryl esters of PUFA*. To show that the enzymatic method is effective for the synthesis of steryl esters of PUFA, several sterols were esterified with PUFA under the same conditions described before (Table 3). All the sterols were efficiently esterified with fatty acids used as substrates. The reaction mixture contains 30% water, but the esterification extents of all reactions were more than 85%. When cholesteryl, cholestanyl, and sitosteryl docosahexaenoates were stirred at 40°C for 24 h in a reaction mixture containing 30% water, their hydrolysis extents were only 15.9, 18.5, and 11.8%, respectively. This result showed that the equilibrium of the reaction was shifted to esterification because steryl esters were poor substrates of the lipase.

*Superiority of reaction presented here*. In general, esterification reactions using lipases are disturbed by water gener-

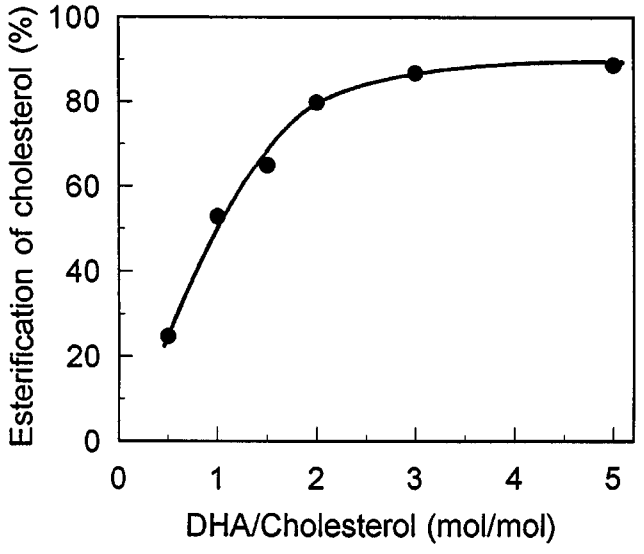


**FIG. 2.** Effect of amount of DHA on esterification of cholesterol. Cholesterol was esterified at 30°C for 20 h with various amounts of DHA in a mixture containing 30% water and 3000 U/g-reaction mixture of *Pseudomonas* lipase (LIPOSAM). The amount of DHA was expressed as a molar ratio to that of cholesterol. See Figure 1 for abbreviations and company source.

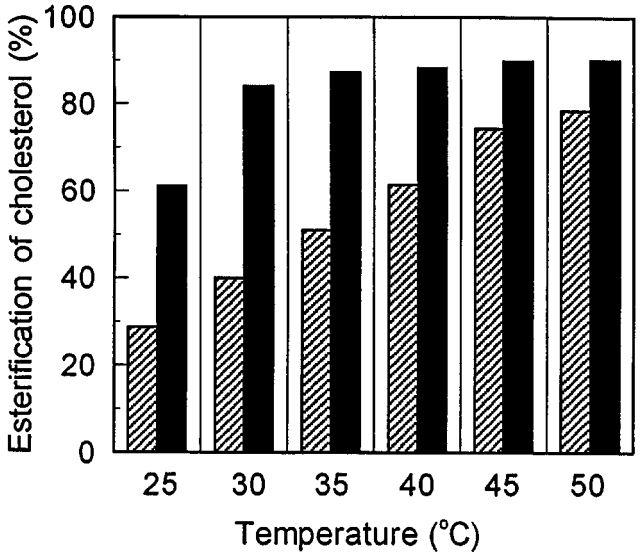


**FIG. 3.** Effect of temperature on esterification of cholesterol with DHA. A mixture containing 2.1 g of DHA/cholesterol (3:1, mol/mol), 0.9 g water and 9000 U *Pseudomonas* lipase (LIPOSAM) was stirred for 4 h (hatched box) or 20 h (solid box) at a range of 20 to 50°C. See Figure 1 for abbreviations and company source.

**TABLE 3**
**Esterification of Cholesterol, Cholestanol, and Sitosterol with Polyunsaturated Fatty Acids[a]**

| Sterol | Fatty acid | Esterification (%)[b] |
|---|---|---|
| Cholesterol | Docosahexaenoic acid | 89.5 |
| | Eicosapentaenoic acid | 88.0 |
| | Arachidonic acid | 92.1 |
| | γ-Linolenic acid | 89.5 |
| | Linoleic acid | 91.9 |
| Cholestanol | Docosahexaenoic acid | 87.3 |
| | Eicosapentaenoic acid | 86.9 |
| | γ-Linolenic acid | 90.6 |
| | Linoleic acid | 91.7 |
| Sitosterol | Docosahexaenoic acid | 92.7 |
| | Eicosapentaenoic acid | 85.3 |
| | γ-Linolenic acid | 91.5 |
| | Linoleic acid | 91.2 |

[a]A mixture of fatty acid/sterol (3:1, mol/mol), 30% water, and 3000 U/g-reaction mixture of *Pseudomonas* lipase (LIPOSAM) was stirred at 40°C for 24 h.
[b]Esterification extent of sterol. See Table 1 for abbreviations and company source.

ated through the reaction. To remove the water, methods of using a large amount of *n*-hexane (13,14), of adding molecular sieves (15), and of removing water with vacuum pump (16) have been proposed. The use of organic solvent requires a large-scale reactor and involves a risk of explosion. The addition of molecular sieves or the use of vacuum pump is not suitable for the industrial production because of high cost. The method described here may be effective for a large-scale production of steryl esters of PUFA, because the high esterification extent can be obtained even though the reaction mixture contains water.

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