Enzymatic Conversion of Steryl Esters to Free Sterols

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ABSTRACT: Conversion of oleic acid phytosteryl esters (OASE) to free phytosterols (referred to as sterols) by an enzymatic process was attempted. Enzymatic hydrolysis of OASE reached a steady state at 55–60% hydrolysis, but addition of methanol (MeOH) significantly accelerated the conversion of OASE to sterols. Screening of commercially available enzymes indicated that *Pseudomonas aeruginosa* lipase was most effective for the conversion. Based on the study of several factors affecting the reaction, the optimal conditions were determined as follows: ratio of OASE to MeOH, 1:2 (mol/mol); water content, 10 wt%; lipase amount, 20 U/g by weight of reaction mixture; temperature, 30°C. When the reaction was conducted for 48 h with stirring, the conversion reached 98%. FAME accumulated in the reaction mixture, but FFA did not, indicating that the FAME was poorly recognized as a substrate in the reverse conversion of sterols to OASE but the FFA was easily recognized as a substrate. The high conversion of OASE to sterols was therefore due to elimination of FFA from the reaction system. After the enzymatic reaction, the oil layer was fractionated at –20°C with 5 vol parts of *n*-hexane. Sterols were efficiently purified in the resulting precipitate (92% recovery, 99% purity).

Paper no. J10373 in *JAOCS 80*, 243–247 (March 2003).

KEY WORDS: Enzymatic conversion, lipase, *Pseudomonas aeruginosa*, solvent fractionation, sterol, steryl ester.

The cholesterol-lowering effect of phytosterols (referred to as sterols) has been studied since the 1950s (1–5) and is believed to be caused by an inhibition of cholesterol absorption due to the higher solubility of sterols than of cholesterol in bile salt micelles (4,5). Steryl esters show the same physiological effect as free sterols (6–8), which has resulted in development of several functional foods, such as salad oil and dressing with addition of sterols, and margarine blended with steryl esters.

Natural tocopherols are purified from vegetable oil deodorizer distillate; sterols are also recovered as by-products, although the yield is low (*ca*. 50%). In the process, the yield of tocopherols can be significantly increased by an enzymatic conversion of sterols to their esters, so sterols were recovered at >90% purity of steryl esters in >90% yield (9). The steryl esters were suitable for use in food materials, but free sterols were not recovered through the process. Because free sterols are also important food additives, we attempted to produce sterols from high-purity steryl esters. This paper shows that steryl esters can be efficiently converted to free sterols by a lipase-catalyzed reaction in the presence of water and methanol (MeOH). In addition, *n*-hexane fractionation is shown to be effective for purification of sterols from the reaction mixture.

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MATERIALS AND METHODS

Materials. Oleic acid steryl ester (OASE; purity, 92.6%) was a commercial product of The Nisshin OilliO, Ltd., Tokyo, Japan. The composition of constituent sterols of OASE was 4.0 wt% brassicasterol, 28.3 wt% campesterol, 17.0 wt% stigmasterol, and 50.7 wt% β-sitosterol; the FA composition of OASE was 0.9 wt% palmitic acid, 3.1 wt% stearic acid, 90.0 wt% oleic acid, and 4.4 wt% linoleic acid. Fatty alcohols (C_1 to C_{12}, and oleyl alcohol) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Geotrichum candidum lipase was prepared according to Tsujisaka *et al*. (10). The strain was aerobically cultivated at 28°C for 30 h in a medium of 5% corn steep liquor, 1% soybean oil, and 0.5% ammonium nitrate (pH 6.0). After cultivation, ammonium sulfate was added to the culture filtrate to give 80% saturation, and the resulting precipitates were dialyzed against water. Other lipases were gifts from the following companies: *Candida rugosa*, *Pseudomonas stutzeri*, and *Burkholderia cepacia* lipases (Meito Sangyo Co. Ltd., Aichi, Japan); *P. aeruginosa* lipase (LPL; Toyobo Co. Ltd., Osaka, Japan); *B. glumae* lipase (Asahi Chemical Industry Co. Ltd., Tokyo, Japan); *Pseudomonas* sp. lipase (Lipase-PS; Amano Enzyme Inc., Aichi, Japan). Lipase activity was measured by titrating FA liberated from olive oil (Wako Pure Chemical Co. Ltd., Osaka, Japan) with 0.05 N KOH, according to our previous paper (11). In brief, the reaction was performed at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase was defined as the amount that liberated 1 μmol FA/min.

Reaction. Hydrolysis of OASE was performed at 30°C in a 50-mL vessel containing 2.5 g OASE, 2.5 mL water, and 500 U lipase with stirring at 500 rpm. Conversion of OASE to sterols in the presence of MeOH and water was conducted with stirring at 30°C in a mixture of 4.5 g OASE/MeOH (1:2, mol/mol), 0.5 mL water, and 100 U lipase. Hydrolysis and conversion degrees were determined from the degree of reduction of OASE during the reactions.

n-*Hexane fractionation*. The reaction mixture was heated at 90°C to separate it into oil and water layers. After 5 vol parts of *n*-hexane had been added in the oil layer, the mixture was kept at −20°C for 2 h with occasional agitation. The precipitate was recovered by centrifugation (9800 × *g*, 10 min) at 0°C and was then dried overnight under reduced pressure at 3 mm Hg.

Analysis. Contents of sterol, steryl ester, FFA, and FAME were determined with a Hewlett-Packard 5890 gas chromatograph connected to a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific, Folsom, CA). For the analysis, the column temperature was raised from 120 to 280°C at 15°C/min and from 280 to 370°C at 10°C/min, and then maintained for 1 min. The injector and detector (FID) temperatures were set at 370 and 390°C, respectively. The sterol moieties of OASE were β-sitosterol, campesterol, stigmasterol, and brassicasterol, and the sterols were liberated from OASE by lipase-catalyzed reactions. The sterol content was expressed as their total content. FFA and FAME were mainly oleic acid and its methyl ester, respectively. The peaks of FFA and FAME did not separate completely under the conditions. Hence, total content of FFA and FAME was measured by GC, and FFA content was determined by alkali titration. The titration was conducted with KOH solution after adding 20 mL chloroform/ethanol (1:1, vol/vol) to 1–5 g of reaction mixture.

RESULTS AND DISCUSSION

Hydrolysis of OASE. OASE were hydrolyzed at 30°C in a mixture containing 50% water with 100 U/g-reaction mixture of several lipases that recognize all ester bonds of TAG (nonspecific enzymes) (Table 1). All lipases tested hydrolyzed OASE, especially *C. rugosa, P. aeruginosa, P. stutzeri,* and *B. cepacia* lipases, and achieved >55% hydrolysis after the 72-h reaction. The hydrolysis degree, however, did not exceed 60%, even though the reaction was conducted for 72 h with 500-U/g reaction mixture of these lipases. Equilibrium of the reaction was apparently 55–60% hydrolysis. To attain higher conversion of OASE to free sterols, we attempted alcoholysis.

Suitable lipase for conversion of OASE to sterols in the presence of MeOH. A mixture of 4.26 g OASE, 0.24 g MeOH (1.2 mol MeOH/mol OASE), 0.5 mL water, and 100 U lipase was incubated with stirring at 30°C (Table 2). Among the lipases tested, *P. aeruginosa* and *P. stutzeri* lipases were effective for conversion of OASE to sterols. The composition of free sterols (brassicasterol, campesterol stigmasterol, and β-sitosterol) coincided with that of sterol constituents of OASE, indicating that the two lipases did not show selectivity for the constituent sterols. After the 72-h reaction with *P. aeruginosa* lipase, 95% of the OASE were converted to sterols, and the ratio of FAME to the total amount of FFA and FAME was 89 mol%. Based on these results, *P. aeruginosa* lipase was selected for subsequent experiments.

TABLE 1
Hydrolysis of Oleic Acid Steryl Ester (OASE) with Several Lipases[a]

Lipase	Hydrolysis (%) 24 h	72 h
Candida rugosa	52.5	56.2
Geotrichum candidum	31.5	44.8
Pseudomonas aeruginosa	41.9	55.3
Pseudomonas stutzeri	42.5	55.8
Pseudomonas sp.[b]	12.2	22.5
Burkholderia glumae	31.2	50.0
Burkholderia cepacia	54.6	56.6

[a]A mixture of 2.5 g OASE, 2.5 mL water, and 500 U lipase was stirred at 30°C. Hydrolysis degree was calculated from the reduced amount of OASE.
[b]Lipase-PS (Amano Enzyme Inc., Aichi, Japan).

The effect of the amount of lipase on the reaction was studied. The reaction was conducted at 30°C in a mixture of 4.26 g OASE, 0.24 g MeOH, 0.5 mL water, and different amounts of *P. aeruginosa* lipase. The conversion after 24 h depended on the amount of lipase, and the conversion after 72 h using >20-U/g reaction mixture of the lipase reached an equilibrium state (94% conversion; results not shown).

Suitable alcohol for conversion of OASE to sterols. Fatty alcohols (C_1 to C_{12}, and oleyl alcohol) were screened. Mixtures containing 4.5 g OASE/alcohol (1:1.2, mol/mol), 0.5 mL water, and 100 U *P. aeruginosa* lipase were stirred at 30°C, and conversions after 24 and 72 h were measured. MeOH, butanol, octanol, and decanol were most effective (93–95% conversion after 72 h). When ethanol, propanol, and hexanol were used as substrates, 80% conversion was attained after 72 h. Dodecanol and oleyl alcohol were not effective for the conversion (conversion after 72-h reaction with dodecanol, 40%; with oleyl alcohol, 11%). MeOH was selected for conversion of OASE to sterols.

Effect of water and MeOH contents on conversion of OASE to sterols. The reaction mixture was composed of a water layer and an oil layer, with lipase and MeOH dissolved in the water layer. Generally, when a small amount of water is added to such a reaction mixture, lipase molecules are exposed to high concentrations of MeOH. Short-chain alcohols, such as MeOH and ethanol, inactivate the enzymes or inhibit their activities (12–14). Hence, we first studied the effect of water on conversion of OASE to sterols in the presence of MeOH.

A 5-g mixture of OASE/MeOH (1:1.2, mol/mol), different amounts of water, and 100 U *P. aeruginosa* lipase was stirred at 30°C (Table 3). Maximal conversion of OASE was obtained in the presence of 10% water by the weight of reaction mixture; the degrees of conversion after 24 and 72 h were 87 and 95%, respectively. A small amount (2 wt%) of water significantly depressed conversion of OASE to sterols, and a large amount of water (>10 wt%) increased the degree of conversion. Thus, a decrease in MeOH concentration by water addition was effective in achieving a high conversion of OASE. Meanwhile, larger amounts of water slightly increased FFA content in the reaction mixture and decreased the FAME content.

The effect of the molar ratio of MeOH to steryl ester on the conversion of OASE to sterols was investigated (Table 4). The reaction was conducted at 30°C in a mixture of 4.5 g

TABLE 2
Conversion of OASE to Sterols in the Presence of MeOH with Several Lipases[a]

	Composition after 24-h reaction (wt%)				Composition after 72-h reaction (wt%)			
Lipase	FFA[b]	FAME[c]	Sterol	OASE	FFA	FAME	Sterol	OASE
None	ND	ND	3.9	92.6	—	—	—	—
Candida rugosa	0.1	0.3	4.4	92.2	0.2	0.8	5.1	90.4
Geotrichum candidum	ND	ND	4.0	92.8	ND	0.2	4.0	92.3
Pseudomonas aeruginosa	5.3	29.9	51.2	12.6	3.8	33.5	56.9	4.9
Pseudomonas stutzeri	3.5	26.6	43.9	23.9	3.7	32.5	54.8	7.9
Pseudomonas sp.[d]	0.2	2.7	6.9	85.8	0.2	6.1	12.2	78.6
Burkholderia glumae	2.8	9.1	20.7	65.9	2.2	18.0	32.1	47.1
Burkholderia cepacia	2.5	20.2	32.5	42.0	3.0	28.9	47.1	19.8

[a]Reaction was conducted at 30°C in a mixture of 4.5 g OASE/MeOH (1:1.2, mol/mol), 0.5 mL water, and 100 U lipase with stirring. ND, not detected.
[b]Main component was oleic acid.
[c]Main component was oleic acid methyl ester.
[d]Lipase-PS. See Table 1 for abbreviations and for manufacturer.

TABLE 3
Effect of Water Content on Conversion of OASE to Sterols in the Presence of MeOH Using *Pseudomonas aeruginosa* Lipase[a]

	Composition after 24-h reaction (wt%)				Composition after 72-h reaction (wt%)			
Water content (wt%)	FFA	FAME	Sterol	OASE	FFA	FAME	Sterol	OASE
2	2.3	16.8	28.0	51.3	3.6	24.8	40.7	30.2
10	4.1	30.6	52.3	11.9	5.2	33.0	56.6	4.4
20	5.7	28.9	51.4	13.0	6.4	30.9	55.5	6.2
50	7.6	26.6	50.7	14.0	7.6	29.4	54.6	7.1
75	9.6	24.4	49.2	15.9	10.7	26.1	53.4	8.7

[a]A 5-g mixture of OASE/MeOH (1:1.2 mol/mol), water, and 100 U *P. aeruginosa* lipase was stirred at 30°C. For abbreviation see Table 1.

OASE/MeOH, 0.5 mL water, and 100 U *P. aeruginosa* lipase. The conversion was significantly increased by adding an equimolar amount of MeOH relative to OASE, and reached a constant value when >2 molar amounts of MeOH were added. The conversion after 72-h reaction with 2 mol of MeOH was 98%.

Effect of temperature on conversion of OASE to sterols. A mixture of 4.5 g OASE/MeOH (1:2, mol/mol), 0.5 mL water, and 100 U *P. aeruginosa* lipase was stirred over a range of temperatures from 20 to 50°C. The conversion after 72 h showed the highest value at 30°C, although the conversion at 40°C (97%) was almost the same as that at 30°C (98%) (results not shown).

Time course of conversion of OASE to sterols. Based on the above results, the reaction conditions were determined as follows: ratio of OASE to MeOH, 1:2 (mol/mol); water content, 10 wt%; lipase amount, 20 U/g by weight of the reaction mixture; reaction temperature, 30°C. Figure 1 shows a typical time course under these conditions. The decrease in OASE content correlated with the increase in sterol, and the reaction proceeded rapidly during the first 5 h and gradually thereafter. The content of FAME increased parallel to that of sterol, and

TABLE 4
Conversion of OASE to Sterols as a Function of the Molar Ratio of MeOH and OASE Using *P. aeruginosa* Lipase[a]

	Composition after 24-h reaction (wt%)				Composition after 72-h reaction (wt%)			
MeOH/OASE (mol/mol)	FFA	FAME	Sterol	OASE	FFA	FAME	Sterol	OASE
0:1	15.3	0	24.9	58.6	21.5	0	33.1	43.6
1:2	10.1	18.3	41.8	27.9	14.5	16.6	46.8	20.4
1:1	5.6	29.1	50.7	13.5	6.9	30.3	54.3	7.4
2:1	1.6	34.0	53.2	10.4	1.9	37.7	57.6	1.9
3:1	1.7	34.3	52.9	10.1	1.8	37.4	58.1	1.8

[a]A mixture of 4.5 g OASE/MeOH, 0.5 mL water, and 100 U *P. aeruginosa* lipase was stirred at 30°C. For abbreviations see Table 1.

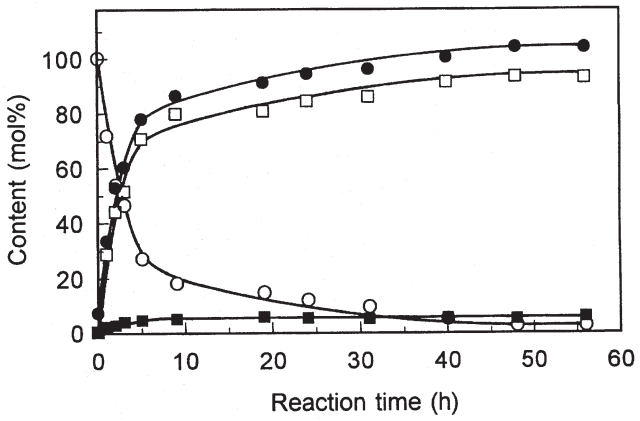

FIG. 1. Time course of conversion of oleic acid steryl ester (OASE) to sterols with *Pseudomonas aeruginosa* lipase. A 10-g mixture of OASE/MeOH (1:2, mol/mol), 10 wt% water, and 20-U/g reaction mixture of the lipase was stirred at 30°C. The contents of OASE, sterols, FAME, and FFA were expressed as mol% of the initial amount of OASE. ○, OASE; ●, sterols; □, FAME; ■, FFA.

little accumulation of FFA in the reaction mixture was observed. After the 48-h reaction, the conversion of OASE reached 98%, and the molar ratio of FAME to total amount of FFA and FAME was 95%.

From the time course of the reaction, the following four hypotheses can be made regarding conversion of OASE to sterols in the presence of MeOH and water: (i) Hydrolysis and methanolysis of OASE occurred independently. (ii) FFA were released by hydrolysis of OASE and FFA were subsequently esterified with MeOH faster than FAME were hydrolyzed. (iii) FAME were generated by methanolysis of OASE, and the FAME were hydrolyzed. However, because FAME were poorly hydrolyzed, the degree of hydrolysis reached the equilibrium state at *ca.* 5% in the presence of 10% water. (iv) Conversion of OASE to sterol by hydrolysis and methanolysis, conversion of sterol to OASE by esterification with FFA and acidolysis of FAME, esterification of FFA with MeOH, and hydrolysis of FAME occurred concomitantly.

Analysis of the equilibrium state of the conversion of OASE showed several findings. Hydrolysis of OASE with *P. aeruginosa* reached steady state at 55–60% (Table 2), showing that the lipase recognized OASE, sterols, and FFA. Treatment of OASE with lipase in the presence of MeOH and water generated FAME, which were poorly recognized as a substrate of the lipase. FAME were thus hydrolyzed only very weakly, even in the presence of water (Fig. 1). The lipase recognized FFA, OASE, and sterols as substrates, but the FFA content in the reaction mixture was very low. Hence, conversion of OASE to sterols shifted significantly toward the accumulation of sterols.

Purification of sterols from the reaction mixture. Sterols produced from OASE were purified by *n*-hexane fractionation (Table 5). A mixture of 9.0 g OASE/MeOH (1:2, mol/mol), 1.0 mL water, and 200 U *P. aeruginosa* lipase was first incubated at 30°C for 48 h in a 50-mL vessel with stirring. The reaction was conducted simultaneously in 11 vessels (total reaction mixture, 110 g; total OASE, 90.4 g). After the reaction, the reaction mixtures were heated at 90°C to produce fluidity, and all mixtures were then combined. The mixture separated into oil and water layers, and the oil layer was recovered (86.9 g; average conversion, 98.1%).

TABLE 5
Purification of Sterols from OASE by a Process Comprising Enzymatic Reaction and *n*-Hexane Fractionation

	Composition (g)				
Step	Total	FFA	FAME	Sterol	OASE
Enzyme reaction[a]					
Before	90.4	0	0	3.5	83.7
After (oil layer)	86.9	1.7	31.3	51.1	1.6
Hexane fractionation[b]					
Supernatant	36.6	1.7	30.1	2.1	1.6
Precipitate	47.6	<0.05	<0.05	47.1	<0.05

[a]A mixture of OASE/MeOH (1:2, mol/mol), 10 wt% water, and 20-U/g reaction mixture of *P. aeruginosa* lipase was stirred at 30°C for 72 h.
[b]After separation of the reaction mixture into oil and water layers, *n*-hexane (450 mL) was added to the oil layer (86.9 g), and the suspension was kept at –20°C for 2 h with occasional agitation. The supernatant and precipitate were separated by centrifugation at 0°C. *n*-Hexane was removed under reduced pressure. For abbreviation see Table 1.

We next attempted to purify sterols from the resulting oil layer. OASE, FFA, and FAME are soluble in *n*-hexane but sterols are poorly soluble. Although *n*-hexane was added to the oil layer and the mixture was allowed to stand on ice, 16% of the sterols still remained in the soluble fraction. Hence, 450 mL of *n*-hexane was added to the resulting oil layer, and the mixture was kept at –20°C for 2 h with occasional agitation. The precipitate was recovered by centrifugation at 0°C and was then dried under reduced pressure. As shown in Table 5, this solvent fractionation step recovered 47.6 g of purified sterols of 98.9% purity, showing that *n*-hexane fractionation was effective for purification of sterols from the reaction mixture obtained by enzymatic conversion of steryl esters to sterols.

ACKNOWLEDGMENTS

We gratefully thank Yomi Watanabe at Osaka Municipal Technical Research Institute for her valuable discussions.

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[Received July 2, 2002; accepted November 19, 2002]