Purification of Tocopherols and Phytosterols by a Two-Step *in situ* Enzymatic Reaction

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ABSTRACT: The purification of tocopherols and phytosterols (referred to as sterols) from soybean oil deodorizer distillate (SODD) was attempted. Tocopherols and sterols in the SODD were first recovered by short-path distillation, which was named SODD tocopherol/sterol concentrate (SODDTSC). The SODDTSC contained MAG, DAG, FFA, and unidentified hydrocarbons in addition to the two substances of interest. It was then treated with *Candida rugosa* lipase to convert sterols to FA steryl esters, acylglycerols to FFA, and FFA to FAME. Methanol (MeOH), however, inhibited esterification of the sterols. Hence, a two-step *in situ* reaction was conducted: SODDTSC was stirred with 20 wt% water and 200 U/g mixture of *C. rugosa* lipase at 30°C, and 2 moles of MeOH per mole of FFA was added to the reaction mixture after 16 h. The lipase treatment for 40 h in total achieved 80% conversion of the initial sterols to FA steryl esters, complete hydrolysis of the acylglycerols, and a 78% decrease in the initial FFA content by methyl esterification. Tocopherols did not change throughout the process. To enhance the degree of steryl and methyl esterification, the reaction products, FA steryl esters and FAME, were removed by short-path distillation, and the resulting fraction containing tocopherols, sterols, and FFA was treated with the lipase again. Distillation of the reaction mixture purified tocopherols to 76.4% (recovery, 89.6%) and sterols to 97.2% as FA steryl esters (recovery, 86.3%).

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KEY WORDS: *Candida rugosa* lipase, esterification, methyl esterification, short-path distillation, phytosterol, soybean oil deodorizer distillate, tocopherol.

Tocopherols are natural antioxidants present in oilseeds. α-Tocopherols are used as pharmaceuticals and cosmetics, and a mixture of α-, γ-, and δ-tocopherols is added to various foods including fats and oils. Phytosterols (referred to as sterols) are also contained in oilseeds, and these are known to interfere with the intestinal absorption of cholesterols and to reduce the cholesterol content in the blood (1–4). This useful physiological activity has resulted in the development of several nutraceutical foods, such as salad oils and dressings containing added sterols, as well as margarine blended with FA steryl esters.

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Tocopherols and sterols are industrially purified from vegetable oil deodorizer distillate (VODD), which is a by-product of the deodorization step of vegetable oil refining. VODD includes acylglycerols, FFA, FA steryl esters, and hydrocarbons in addition to tocopherols and sterols. The tocopherols in VODD have been purified by a combination of chemical methylation, molecular distillation, solvent fractionation, and ion-exchange chromatography, and the sterols have been purified by solvent fractionation of a by-product of the tocopherol purification process. However, a further improvement of the process has been strongly desired to achieve higher purity and recovery.

Shimada *et al.* (5) successfully converted sterols in soybean oil deodorizer distillate (SODD) to FA steryl esters and completely hydrolyzed acylglycerols by applying lipase reactions to the purification of tocopherols and sterols resulting in an efficient fractionation of tocopherols and sterols as FA steryl esters by short-path distillation. However, the process included the drawback that FFA and tocopherols were not efficiently fractionated because the b.p. of the two substances were close. This problem could be solved by conversion of the FFA to their methyl esters. We thus attempted to develop a reaction system in which the methyl esterification of FFA proceeded simultaneously with the conversion of sterols to FA steryl esters and the hydrolysis of acylglycerols.

This paper deals with a two-step *in situ* reaction system with *Candida rugosa* lipase. In the first step, the esterification of sterols with FFA and the hydrolysis of acylglycerols were carried out; in the second step, the methyl esterification of FFA was conducted. In addition, short-path distillation of the reaction mixture was shown to be effective for the purification of tocopherols and of sterols as their esters.

MATERIALS AND METHODS

Preparation of the starting material. SODD (Yashiro Co. Ltd., Osaka, Japan), produced in the deodorization step of soybean oil refining, was used as a starting material without further processing. The deodorizer distillate was distilled at 240°C and 0.02 mm Hg to remove high b.p. substances such as FA steryl esters, DAG, and TAG. The resulting distillate, named SODD tocopherol/sterol concentrate (SODDTSC), was used for this study. Its acid value was 95 mg KOH/g.

Reagents. Candida rugosa lipase (Lipase-OF) was purchased from Meito Sangyo Co. (Aichi, Japan). The powdered

preparation was dissolved in deionized water at a concentration of 0.2 g/mL [16,200 units (U)/mL], and the lipase solution was added to a reaction mixture. The activity was measured by titrating FA liberated from olive oil (Wako Pure Chemical Industry, Co., Osaka, Japan) with 50 mM KOH as described previously (6). One unit was defined as the amount of enzyme that liberated 1 μmol FFA per minute.

Methanol (MeOH) and tricaproin were obtained from Wako Pure Chemical Industry Co. and Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), respectively. Other chemicals were of analytical grade.

Lipase reactions and distillation. In a previous experiment (5), water content was found to exert negligible effects on the esterification of sterols and FFA in SODDTSC with *C. rugosa* lipase, since the enzyme strongly recognized sterols and FFA as substrates and acted poorly on the esterification product, FA steryl esters. Because addition of 20 wt% water to SODDTSC slightly increased the fluidity of the reaction mixture, all lipase treatments were conducted in the presence of 20 wt% water.

A small-scale reaction was performed in a 50-mL vessel overlain with nitrogen gas. The reaction mixture, which consisted of 4.0 g SODDTSC, 1.0 g water, and 100–700 U/g reaction mixture of *C. rugosa* lipase, was incubated at 30°C with stirring at 500 rpm. After incubation for 5, 16, and 24 h, MeOH was added to the mixture.

A large-scale reaction was conducted in a 1- or 5-L reactor (KMJ-1C or KMJ-5C; Mitsuwa Co. Ltd., Osaka, Japan) overlain with nitrogen gas. A mixture of 0.5–3.56 kg SODDTSC, 20 wt% water, and 200 U/g reaction mixture of *C. rugosa* lipase was agitated at 30°C and 250 rpm. After a 16-h incubation, 2 moles of MeOH per mole of FFA was added to the reactor and the reaction was continued for a further 6–10 h (total reaction time, 22–26 h).

The oil layer was recovered from the first lipase treatment and dehydrated at 80°C and 5 mm Hg for 60 min (water content, <100 ppm). The dehydrated oil layer was distilled at 160°C and 0.2 mm Hg using a distillation apparatus, Wiprene type 2-03 (Shinko Pantec Co. Ltd., Hyogo, Japan), mainly to remove the FAME in the distillate. The resulting residue (distilland) was redistilled at 240°C and 0.02 mm Hg to isolate FA steryl esters in the residue. The resulting distillate (0.2–1.62 kg) containing tocopherols, sterols, and FFA was agitated together with 20 wt% water and 200 U/g mixture of *C. rugosa* lipase in a 1- or 5-L reactor under the same conditions as the first lipase treatment. After a 16-h incubation, 2 moles of MeOH per mole of FFA was added and the reaction was continued for a further 6–10 h (total reaction time, 22–26 h). The oil layer recovered from the second lipase treatment was dehydrated and then distilled stepwise: step 1, at 160°C and 0.2 mm Hg; step 2, at 175°C and 0.2 mm Hg; step 3, at 240°C and 0.02 mm Hg.

Analyses. A sample of each reaction mixture (*ca.* 5 mL) was heated at 90°C until it separated into two layers. The oil (upper) layer was recovered and used for analyses. The contents of sterols, tocopherols, partial acylglycerols, and FA steryl esters were determined with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) connected to a DB1-ht capillary column (0.25 mm × 5 m; J&W Scientific, Folsom, CA). The column temperature was held for 0.5 min at 120°C. It was then increased at 15°C/min from 120 to 280°C and at 10°C/min from 280 to 370°C, and finally held for 1 min at 370°C. The injector and detector temperatures were set at 370 and 390°C, respectively.

FAME content was determined with a gas chromatograph connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific) using tricaproin as an internal standard. The column temperature was controlled as follows: 0.5 min at 190°C; 4°C/min to 210°C; 10°C/min to 290°C; 5°C/min to 310°C; and 1 min at 310°C. The injector and detector temperatures were 245 and 350°C, respectively.

Chemical methylation of FA in the SODDTSC was conducted by heating 0.1 g of sample in 3.0 mL MeOH containing 0.5% boron trifluoride (Wako Pure Chemical Industry) at 75°C for 5 min. FA in the FA steryl esters were methylated by heating 0.1 g of sample in 4.0 mL MeOH containing 5.9% sodium methylate (Wako Pure Chemical Industry) at 75°C for 30 min. FAME were extracted by 3.0 mL *n*-hexane and analyzed with a Hewlett-Packard 5890 gas chromatograph connected to a DB-23 capillary column (0.25 mm × 10 m; J&W Scientific). The column temperature was set as follows: 0.5 min at 150°C; 4°C/min to 170°C; 5°C/min to 195°C; 10°C/min to 215°C; and 11 min at 215°C. The injector and detector temperatures were set at 245 and 250°C, respectively.

Acid values of the SODDTSC and other samples were measured by titration of the FFA with 0.1–1.0 N KOH. The FFA content was determined based on the acid value of soybean FFA (198.8 mg KOH/g). Water content in the oil layer after dehydration was measured by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

All analyses were conducted three to five times under the same experimental conditions. The relative SD were less than ±11.1% for the average value of <1%, less than ±3.1% for the value of 1–3%, less than ±2.1% for the value of >3–10%, and less than ±1.2% for >10%.

RESULTS AND DISCUSSION

The SODDTSC contained 47.4 wt% FFA, 4.8 wt% partial acylglycerols (estimated value; see below), 3.6 wt% campesterol, 2.7 wt% stigmasterol, 5.7 wt% β-sitosterol, 1.5 wt% α-tocopherol, 11.4 wt% γ-tocopherol, 4.3 wt% δ-tocopherol, and 18.6 wt% unknown hydrocarbons. The treatment of SODDTSC with *C. rugosa* lipase in the presence of 20 wt% water led to the simultaneous esterification of sterols and hydrolysis of acylglycerols (5). Meanwhile, a preliminary study in our laboratory revealed that the lipase catalyzed methyl esterification of the FFA even in the presence of water (data not shown). Based on these facts, we attempted to conduct three reactions (the hydrolysis of acylglycerols, the esterification of sterols with FFA, and the methyl esterification of FFA) in a reaction system with one lipase.

Effect of MeOH on the lipase treatment of SODDTSC. A mixture of 4.0 g of SODDTSC, 1.0 g of water, and 100 U/g of

TABLE 1
Effect of MeOH Addition on the Esterification of Sterols and the Methyl Esterification of FFA[a]

Time of MeOH addition (h)	Reaction time (h)	Content (wt%)				
		FFA	FAME	Sterols	FA steryl esters	Tocopherols
0	0	47.4	0	12.0	ND[b]	17.2
	24	15.4	36.7	9.6	4.1	16.5
5	5	47.8	0	6.0	10.1	17.2
	29	14.2	35.6	6.3	9.4	17.5
16	16	46.6	0	2.5	15.5	16.1
	40	18.7	28.2	2.8	15.2	17.3
24	24	44.9	0	2.4	15.8	16.9
	48	20.1	26.4	2.3	15.9	16.8

[a]Soybean oil deodorizer distillate (SODD) was distilled at 240°C and 0.02 mm Hg. The resulting distillate was named SODD tocopherol/sterol concentrate (SODDTSC). A mixture of 4.0 g of SODDTSC, 1.0 g of water, and 100 U/g of the reaction mixture of *Candida rugosa* lipase was stirred at 30°C. After incubation for 0, 5, 16, and 24 h, 2 moles of methanol (MeOH) per mole of FFA was added. The reaction was continued for 24 h (24–48 h in total).
[b]Not detected (<0.2 wt%).

the reaction mixture of *C. rugosa* lipase was stirred at 30°C with 2 moles of MeOH per mole of FFA (Table 1). After 24 h, a mixture containing 15.4 wt% FFA, 36.7 wt% FAME, 9.6 wt% sterols, and 4.1 wt% FA steryl esters was obtained. Although the FFA content decreased by 67.5% of the initial content, the decrease in sterols was only 20.0%.

In other reactions, the treatment of SODDTSC was started without the addition of MeOH. DAG (initial content, 1.6 wt%) were not detected after 5 h, strongly suggesting that MAG in the SODDTSC also were hydrolyzed completely. When the reaction was conducted for 5, 16, and 24 h without addition of MeOH (Table 1), the total contents of FFA and FA in the FA steryl esters were 52.0, 53.0, and 51.5 wt% (average, 52.2 wt%). As the content of FFA before the treatment was 47.4 wt%, an increase in the total content of FA was assumed to be due to the hydrolysis of acylglycerols; the content of FA originating from acylglycerols was estimated to be 4.8 wt% (= 52.2 – 47.4).

After incubation for 5, 16, and 24 h, 2 moles of MeOH per mole of FFA was added to the mixtures, and the reactions were continued for a further 24 h (Table 1). The content of FAME in the final reaction mixture decreased as the time of MeOH addition was delayed. Meanwhile, the content of FA steryl esters after 24 h (29, 40, and 48 h in total) increased with the delay of MeOH addition. Approximately 80% of the sterols were converted to their esters when MeOH was added after ≥16 h.

Esterification of the sterols and methyl esterification of the FFA would improve the efficiency of tocopherol separation by short-path distillation. Because contamination with sterols makes it very difficult to obtain high-purity tocopherols by distillation, FA esterification of the sterols should be carried out prior to methyl esterification of the FFA. Table 1 shows that the inhibition of steryl esterification is eliminated by adding MeOH after ≥16 h. Therefore, we decided to add MeOH to the reaction mixture after the conversion of sterols to FA steryl esters had reached a steady state.

The SODDTSC contained 3.6 wt% campesterol, 2.7 wt% stigmasterol, and 5.7 wt% β-sitosterol. The ratio of the three in the FA steryl esters was the same as that in the SODDTSC, showing that *C. rugosa* lipase acted equally on the three sterols. The sterol content was therefore expressed as the total content of the three. The tocopherol content decreased very little in any reaction (Table 1). The ratios of the three tocopherols, α-, γ-, and δ-tocopherol, in the final reaction mixtures were the same as that in the SODDTSC; thus, the tocopherol content was also expressed as the total contents.

Effect of the amounts of enzyme and MeOH on the two-step in situ *reaction of SODDTSC.* A two-step *in situ* reaction was conducted as follows: A mixture of 4.0 g SODDTSC, 1.0 g water, and various amounts of *C. rugosa* lipase was stirred at 30°C for 16 h. Different amounts of MeOH were then added to the mixture and incubated for 24 h (40 h in total) (Fig. 1). The sterol content decreased from 12.0 to 2.5 wt%, and the FA steryl ester content attained 15.5 wt% regardless of the amounts

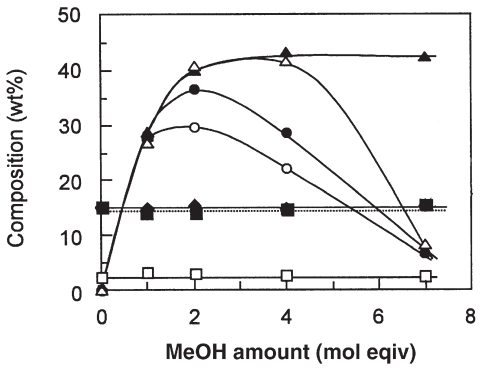


FIG. 1. Effect of the amounts of enzyme and methanol (MeOH) used on the two-step *in situ* reaction of soybean oil deodorizer distillate tocopherol/sterol concentrate (SODDTSC) with *Candida rugosa* lipase. A mixture of 4.0 g SODDTSC and 1.0 g water was stirred at 30°C with various amounts of *C. rugosa* lipase. After 16 h of incubation, 1, 2, 4, or 7 moles of MeOH per mole of FFA was added and the reaction was continued for 24 h (40 h in total). Content of FAME in the reaction mixture containing (○)100, (●) 200, (△) 400, or (▲) 700 U/g mixture of the lipase. Content of (□) sterols, (■) FA steryl esters, and (◆) tocopherols in the reaction with 100 U/g mixture of the lipase. The contents of sterols, FA steryl esters, and tocopherols in the mixture containing 200–700 U/g of the lipase were the same as those in the mixture containing 100 U/g of the lipase.

TABLE 2
Stepwise Addition of MeOH for the Methyl Esterification of FFA[a]

Times of MeOH addition	Reaction time (h)	MeOH amount (mol eqiv)	Content (wt%)				
			FFA	FAME	Sterols	FA steryl esters	Tocopherols
	0		47.4	0	12.0	ND	17.2
1[b]	16	2	45.7	0	2.7	15.3	17.1
	40	—	17.0	30.1	2.8	15.7	16.8
	64	—	16.5	30.7	2.8	15.7	16.3
2[c]	16	1	45.7	0	2.7	15.3	16.8
	40	1	18.7	27.3	3.5	14.2	17.6
	64	—	15.1	32.3	3.0	14.7	16.4
4[d]	16	0.5	45.7	0	2.7	15.3	16.6
	24	0.5	28.9	17.7	2.9	15.0	17.4
	40	0.5	19.0	28.2	3.0	14.9	16.8
	48	0.5	14.8	32.6	3.2	14.7	16.5
	64	—	13.2	34.4	3.2	14.6	16.4

[a]A mixture of 4.0 g of SODDTSC and 1.0 g of water was stirred at 30°C with 100 U/g mixture of *C. rugosa* lipase.
[b]After incubation for 16 h, 2 moles of MeOH per mole of FFA was added and incubated for 48 h.
[c]After incubation for 16 and 40 h, 1 mole of MeOH per mole of FFA was added and incubated for 24 h.
[d]After incubation for 16, 24, 40, and 48 h, 0.5 mole of MeOH per mole of FFA was added and incubated for 16 h at 30°C.
See Table 1 for abbreviations.

of lipase and MeOH used. The tocopherol content did not change in any of the reactions. In the reactions with 100 and 200 U/g mixture of the lipase, the content of FAME reached a maximum (29.9 and 36.2 wt%, respectively) at 2 moles of MeOH per mole of FFA. The reaction with 400 U/g mixture of the lipase led to the synthesis of *ca.* 40 wt% FAME in the presence of 2–4 moles of MeOH, and the use of 700 U/g mixture of the lipase maintained *ca.* 40 wt% FAME even at 7 moles of MeOH. Increasing the amount of lipase enhanced the methyl esterification of FFA in the presence of >2 moles of MeOH. This result may be due to inactivation of the lipase by larger amounts of MeOH.

Stepwise addition of MeOH. Preventing lipase inactivation caused by MeOH may enhance the conversion of FFA to FAME. Earlier, we reported that the inactivation of a lipase by a short-chain alcohol can successfully be avoided by the stepwise addition of the required amounts of alcohol (7–9). Therefore, after the reaction was conducted without MeOH for 16 h, an equimolar or a half-molar amount of MeOH was added at appropriate time intervals.

As a control reaction, a mixture of 4.0 g SODDTSC, 1.0 g water, and 100 U/g mixture of *C. rugosa* lipase was stirred at 30°C, and 2 moles of MeOH per mole of FFA was added at 16 h (Table 2). After a full 64 h of incubation, 30.7 wt% FAME and 15.7 wt% FA steryl esters were generated. Addition of an equimolar amount of MeOH at 16 and 40 h increased the FAME content to 32.3 wt% after 64 h, but the FA steryl ester content decreased to 14.7 wt%. When a half-molar amount of MeOH was added at 16, 24, 40, and 48 h, the contents of FAME and FA steryl ester after 64 h were 34.4 and 14.6 wt%, respectively. The stepwise addition of MeOH slightly increased the degree of methyl esterification of FFA, but a smaller amount of FA steryl esters was formed from the free sterols. Because the conversion of sterols to FA steryl esters should be carried out prior to the synthesis of FAME to achieve the best yield of tocopherols, we abandoned the stepwise addition of MeOH.

Based on the aforementioned results, the reaction conditions were determined to be as follows: A mixture of SODDTSC, 20 wt% water, and 200 U/g mixture of *C. rugosa* lipase was stirred at 30°C for 16 h, and then 2 moles of MeOH per mole of FFA was added.

Repeated two-step in situ *reaction of SODDTSC.* The conversion of sterols to FA steryl esters did not exceed 80% in a single reaction. We thus attempted to repeat the lipase treatment to further increase the conversion.

The first reaction was conducted as follows: A mixture of 500 g SODDTSC, 125 g water, and 200 U/g mixture of *C. rugosa* lipase was agitated at 30°C. The time course of the reaction is shown in Figure 2. The sterol content decreased rapidly, and FA steryl ester content increased. The conversion of sterols to steryl esters reached nearly steady state after 10 h, and the degree of conversion at 16 h was 80.1%. DAG were not

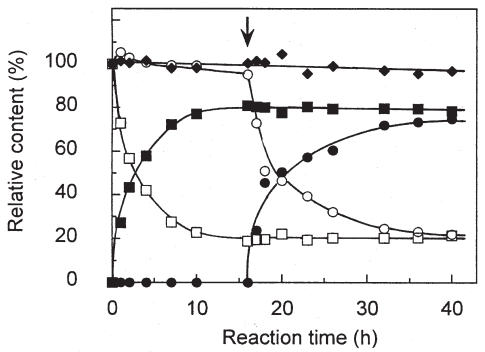


FIG. 2. Time course of the first two-step reaction of SODDTSC with *C. rugosa* lipase. A mixture of 500 g of SODDTSC, 125 g of water and 200 U/g mixture of the lipase was stirred at 30°C. After 16 h (indicated with an arrow), 2 moles of MeOH per mole of FFA was added and the reaction was continued for 24 h (40 h in total). Molar contents of FFA and FAME were expressed relative to the initial FFA content, and those of sterols and FA steryl esters were expressed relative to the initial sterol content. The content of tocopherols was expressed relative to the initial content. Contents of (○) FFA, (●) FAME, (□) sterols, (■) FA steryl esters, and (◆) tocopherols. See Figure 1 for abbreviations.

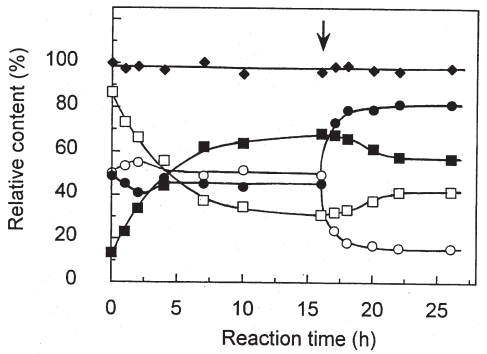


FIG. 3. Time course of the second reaction with *C. rugosa* lipase. An oil layer, which was recovered from the first reaction step (Fig. 2), was distilled and separated into three fractions as detailed in the text. The distillate at 240°C (19.5 wt% FFA, 19.8 wt% FAME, 36.4 wt% tocopherols, 4.7 wt% sterols, and 1.2 wt% FA steryl esters) was used as a substrate for the second treatment. A mixture of 200 g of the distillate, 50 g of water, and 200 U/g mixture of *C. rugosa* lipase was stirred at 30°C. After 16 h (indicated with an arrow), 2 moles of MeOH per mole of FFA was added and the reaction was continued for 10 h (26 h in total). The molar contents of FFA and FAME were expressed relative to the total FFA content in the substrate, and those of sterols and steryl esters were expressed relative to the total sterol content in the substrate. The content of tocopherols was expressed relative to the initial content in the substrate. Content of (○) FFA, (●) FAME, (□) sterols, (■) FA steryl esters, and (◆) tocopherols. See Figure 1 for abbreviations.

detectable after 2 h (data not shown), indicating that the hydrolysis of acylglycerols occurred concomitantly. After 16 h of incubation, 2 moles of MeOH per mole of FFA was added to the mixture. The relative content of FFA decreased to 22.1%, and that of FAME increased to 74.9% after 40 h in total. The tocopherol content scarcely changed during the reaction.

FAME in the oil layer (490 g) that had separated from the reaction mixture were first removed by molecular distillation at 160°C and 0.2 mm Hg. The distillate (180 g) contained 7.2 wt% FFA, 69.0 wt% FAME, and 0.4% sterols. The resulting residue was then redistilled at 240°C and 0.02 mm Hg to distill other compounds away from the FA steryl esters: The distillate (225 g) contained 36.4 wt% tocopherols (recovery, 95.2%), 4.7 wt% sterols, 1.2 wt% FA steryl esters, 19.5 wt% FFA, and 19.8 wt% FAME. The residue (71 g) contained 97.1 wt% FA steryl esters (recovery as sterols, 70.1%), 0.6 wt% FFA, 0.3 wt% FAME, and 0.9 wt% tocopherols.

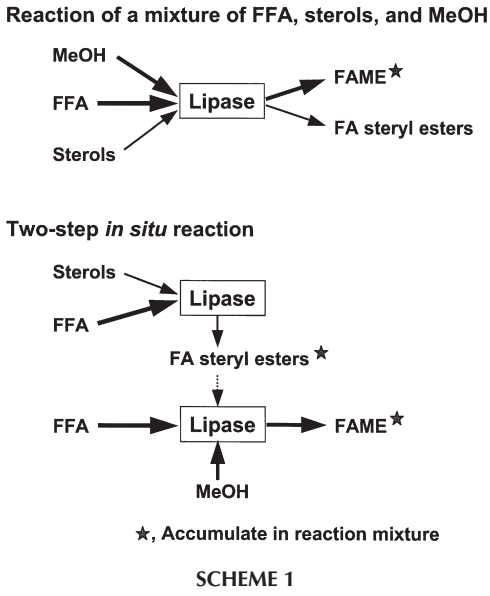
The distillate containing tocopherols, which was obtained by the second distillation at 240°C, was then used as the substrate for a second lipase treatment. The reaction was conducted at 30°C in a reactor containing 200 g of the distillate, 20 wt% water, and 200 U/g mixture of the *C. rugosa* lipase (Fig. 3). After conversion of the sterols to FA steryl esters had reached a steady state (16 h; sterols/steryl esters = 31.1:68.9, mol/mol), 2 moles of MeOH per mole of FFA was added to the reaction mixture. The tocopherol content scarcely changed during the second reaction. FFA were rapidly converted to their methyl esters after the addition of MeOH, and the esterification reached a steady state after 4 h (total reaction time, 20 h). The FAME content at 26 h in total was 81.8 mol% based on the total content of FFA and FAME. A decrease in the FA steryl ester content, however, took place after the addition of MeOH. This indicated that some hydrolysis and/or methanolysis of the FA steryl esters had occurred. This phenomenon might be due to the small amounts of FFA in the substrate used for the second reaction compared with the amount in the SODDTSC. We previously reported that a *Pseudomonas* lipase efficiently catalyzed the conversion of FA steryl esters to sterols in a mixture of steryl esters/MeOH (1:2, mol/mol) and 50 wt% water (10).

Large-scale purification of tocopherols and FA steryl esters

TABLE 3
Purification of Tocopherols and Sterols from SODDTSC by a Process Comprising Enzymatic Treatment and Molecular Distillation

	Weight	FFA	FAME	Tocopherols		Sterols		FA steryl esters	
Step	(g)	(wt%)	(wt%)	(wt%)	(g)	(wt%)	(g)	(wt%)	(g)
SODDTSC	3560	47.4	0	17.2	612	12.0	427	<0.2	—
Lipase reaction 1[a]	3560	10.4	37.0	17.3	616	2.5	89.0	15.6	555
Oil layer 1	3520	11.6	36.8	17.0	598	2.5	88.0	15.3	539
Distillation 1									
Distillate 1-1[b]	1320	7.7	68.2	<0.2	—	<0.2	—	<0.2	—
Distillate 1-2[c]	1620	17.8	22.2	36.1	585	5.9	95.6	0.5	8.1
Residue 1-2[c]	522	0.2	<0.2	0.7	3.4	<0.2	—	97.6	509
Lipase reaction 2[d]	1620	5.7	32.4	35.9	582	2.5	40.5	6.1	98.2
Oil layer 2	1570	5.7	32.4	35.7	561	2.6	40.8	6.2	97.3
Distillation 2									
Distillate 2-1[b]	684	10.1	70.3	<0.2	—	<0.2	—	<0.2	—
Distillate 2-2[e]	94.6	10.3	26.2	34.8	32.9	1.1	1.0	<0.2	—
Distillate 2-3[c]	675	1.3	<0.2	76.4	515.7	5.4	36.5	0.2	1.3
Residue 2-3[c]	99	0.2	<0.2	2.8	2.8	1.0	1.1	95.8	94.8

[a]SODDTSC was incubated at 30°C with 20 wt% water and 200 U/g mixture of *C. rugosa* lipase. After 16 h, 2 moles of MeOH per mole of FFA was added and incubated for 24 h.
[b]Distilled at 160°C and 0.2 mm Hg.
[c]Distilled at 240°C and 0.02 mm Hg.
[d]Distillate 1-2 was incubated at 30°C with 20 wt% water and 200 U/g mixture of *C. rugosa* lipase. After 16 h, 2 moles of MeOH per mole of FFA was added and incubated for 6 h.
[e]Distilled at 175°C and at 0.2 mm Hg.



SCHEME 1

from SODDTSC. The purification of tocopherols and of sterols as FA steryl esters by a combination of a repeated two-step *in situ* reaction and molecular distillation was carried out (Table 3). The first reaction was conducted as follows: SODDTSC (3.56 kg) and water (0.89 kg) were agitated with 200 U/g mixture of *C. rugosa* lipase for 16 h, after which 2 moles of MeOH per mole of FFA was added to the reaction mixture. After 24 h (40 h in total), the contents of sterols and FFA in the SODDTSC decreased to 2.5 and 10.4 wt%, respectively. To decrease their contents further, the reaction products (FA steryl esters and FAME) were removed by short-path distillation. The first distillation was conducted at 160°C and 0.2 mm Hg, and a large part of the FAME was removed in distillate 1-1. The residue was subjected to a second distillation at 240°C and 0.02 mm Hg, and most of the FA steryl esters were concentrated in residue 1-2. Tocopherols were almost completely recovered in distillate 1-2 (95.6% recovery; content, 36.1 wt%), which contained 5.9 wt% sterols, 17.8 wt% FFA, and 22.2 wt% FAME.

The second reaction was conducted as follows: A mixture of 1.62 kg of distillate 1-2, 0.41 kg of water, and 200 U/g mixture of *C. rugosa* lipase was agitated for 16 h, and 2 moles of MeOH per mole of FFA was added to the reaction mixture. After 6 h (22 h in total), the content of sterols was reduced from 5.9 to 2.5 wt%, and the content of FFA was decreased from 17.8 to 5.7 wt%. Tocopherols in the reaction mixture were purified by a three-step distillation process. The first distillation step was conducted at 160°C and 0.2 mm Hg to remove a large part of the FAME in distillate 2-1. To completely remove the remaining FAME and FFA, a second distillation step was conducted at 175°C and 0.2 mm Hg. Although distillate 2-2 contained 34.8 wt% tocopherols, the FAME and FFA contents of the residue were 1.3 and 0.2 wt%, respectively. The third distillation step was conducted at 240°C and 0.02 mm Hg. The greater part of the tocopherols was successfully recovered in distillate 2-3, with 84.3% recovery of the initial content in the SODDTSC (purity, 76.4%). The recovery of tocopherols by a series of purifications was calculated to be 89.6% of the initial content of SODDTSC, because distillate 2-2 could be added back for a subsequent distillation step. The contents of tocopherols in distillate 2-3 were 7.2 wt% α-tocopherol, 46.8 wt% γ-tocopherol, and 22.4 wt% δ-tocopherol. The ratio of the three did not change during the purification.

Constituent FA in FA steryl esters. When residues 1-2 and 2-3 (Table 3) were combined as the FA steryl ester fraction (purity, 97.2%), recovery of the constituent sterols was 86.3% of the initial content in the SODDTSC. The compositions of the constituent sterols and FA in the FA steryl esters were analyzed. The composition of sterols was 28.1 wt% campesterol, 23.0 wt% stigmasterol, and 48.9 wt% β-sitosterol, which was consistent with the original sterol composition in the SODDTSC. This result indicated that *C. rugosa* lipase had almost the same activity on the three sterols.

On the other hand, the FA composition in the FA steryl esters was 12.0 wt% palmitic acid (PA), 2.1 wt% stearic acid (SA), 26.9 wt% oleic acid (OA), 48.6 wt% linoleic acid (LnA), and 9.0 wt% α-linolenic acid (ALA), which was different from the composition of FFA in the SODDTSC: 17.9 wt% PA, 4.9 wt% SA, 26.3 wt% OA, 44.0 wt% LnA, and 5.8 wt% ALA. Activity of the lipase on each FA can be expressed by the ratio of F_{SE}/F_{FFA}, where F_{SE} and F_{FFA} are the contents of a particular FA in the FA steryl esters and in the SODDTSC, respectively. Activities on PA, SA, OA, LnA, and ALA were 0.67, 0.41, 1.0, 1.1, and 1.6, respectively. This result showed that the activities of *C. rugosa* lipase on FA during the synthesis of FA steryl esters were in the order of ALA > LnA > OA > PA > SA.

Features of the two-step in situ *reaction*. Scheme 1 shows an outline of the reaction of FFA, sterols, and MeOH using *C. rugosa* lipase. In a mixture of FFA, sterols, and MeOH, the lipase recognized FFA and MeOH more strongly than the sterols and acted poorly on the FAME and FA steryl esters; thus, the reaction synthesized FAME in preference to FA steryl esters. On the other hand, in the two-step reaction, FA steryl esters were first synthesized from the FFA and sterols. Because the lipase acted poorly on the FA steryl esters, addition of MeOH to a mixture containing FFA and FA steryl esters led only to esterification of the FFA.

The two-step *in situ* reaction of SODDTSC with *C. rugosa* lipase successfully achieved hydrolysis of the acylglycerols, esterification of the sterols with FA, and methyl esterification of the FFA. A combination of the enzyme reaction and short-path distillation enriched the tocopherols from 17.2 to 76.4% (recovery, 89.6%) and purified the sterols to 97.2% as FA steryl esters (recovery, 86.3%).

REFERENCES

1. Peterson, D.W., Effect of Soybean Sterols in the Diet on Plasma and Liver Cholesterol in Chicks, *Proc. Soc. Exp. Biol. Med. 78*:219–225 (1951).
2. Lees, A.M., H.Y. Mok, R.S. Lees, M.A. McCluskey, and S.M. Grundy, Plant Sterols as Cholesterol-Lowering Agents: Clinical Trials in Patients with Hypercholesterolemia and Studies of

Sterol Balance, *Atherosclerosis 28*:325–338 (1977).
3. Heinemann, T., G.-A. Kullak-Ublick, B. Pietruck, and K. von Bergmann, Mechanisms of Action of Plant Sterols on Inhibition of Cholesterol Absorption, *Eur. J. Clin. Pharmacol. 40*:S59–S63 (1991).
4. Ikeda, I., K. Tanaka, M. Sugano, G.V. Vahouny, and L.L. Gallo, Discrimination Between Cholesterol and Sitosterol for Absorption in Rats, *J. Lipid. Res. 29*:1583–1591 (1988).
5. Shimada, Y., S. Nakai, M. Suenaga, A. Sugihara, M. Kitano, and Y. Tominaga, Facile Purification of Tocopherols from Soybean Oil Deodorizer Distillate in High Yield Using Lipase, *J. Am. Oil Chem. Soc. 77*:1009–1013 (2000).
6. Sugihara, A., M. Ueshima, Y. Shimada, S. Tsunasawa, and Y. Tominaga, Purification and Characterization of a Novel Thermostable Lipase from *Pseudomonas cepacia, J. Biochem. 112*: 598–603 (1992).
7. Shimada Y., Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, and Y. Tominaga, Conversion of Vegetable Oil to Biodiesel Using Immobilized *Candida antarctica* Lipase, *J. Am. Oil Chem. Soc. 76*:789–793 (1999).
8. Watanabe, Y., Y. Shimada, A. Sugihara, H. Noda, H. Fukuda, and Y. Tominaga, Continuous Production of Biodiesel Fuel from Vegetable Oil Using Immobilized *Candida antarctica* Lipase, *Ibid. 77*:354–360 (2000).
9. Watanabe, Y., Y. Shimada, A. Sugihara, and Y. Tominaga, Stepwise Ethanolysis of Tuna Oil Using Immobilized *Candida antarctica* Lipase, *J. Biosci. Bioeng. 88*:622–626 (1999).
10. Shimada, Y., T. Nagao, Y. Watanabe, Y. Takagi, and A. Sugihara, Enzymatic Conversion of Steryl Esters to Free Sterols, *J. Am. Oil Chem. Soc. 80*:243–247 (2003).

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