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Improvement of a process for purification of tocopherols and sterols from soybean oil deodorizer distillate

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Abstract

Soybean oil deodorizer distillate (SODD) is a useful material for purification of tocopherols and phytosterols (referred to as sterols). The SODD was first distilled, and the two substances were enriched. The preparation, which mainly contained free fatty acids (FFAs), sterols, and tocopherols, was named SODD tocopherols/sterols concentrate (SODDTSC). If sterols are converted to steryl esters and FFAs are converted to fatty acid methyl esters (FAMEs), relatively easy purification of tocopherols and steryl esters can be achieved because the boiling points of FAMEs, tocopherols, and steryl esters are different significantly. Hence, the development of a new two-step in situ reaction system was tried out for esterification of sterols with FFAs (first step) and esterification of FFAs with methanol (MeOH) (second step). A mixture of SODDTSC/water (95:5, w/w) and 250 units (U)/g-mixture of *Candida rugosa* lipase was prepared beforehand for the first-step reaction, and was agitated at 40 °C for 24 h with dehydration at 20 mmHg. Sterols were efficiently esterified, and the degree of esterification reached 95%. To the reaction mixture were added 7 M amounts of MeOH against unreacted FFAs, 20 wt.% water, and 25 U/g-mixture of *Alcaligenes* sp. lipase. The second-step reaction was then conducted at 30 °C for 20 h. Consequently, 95% FFAs were converted to FAME, and steryl esters synthesized by the first-step reaction were not reconverted to free sterols. Finally, SODDTSC (1.5 kg) was subjected to this two-step in situ reaction, and tocopherols and steryl esters were purified from the reaction mixture by short-path distillation. Tocopherols were purified to 72% (yield, 88%) and steryl esters were purified to 97% (yield, 97%).

Keywords: *Alcaligenes*; *Candida rugosa*; Lipase; Phytosterols; Phytosteryl esters; Short-path distillation; Soybean oil deodorizer distillate; Tocopherols; Two-step in situ reaction

1. Introduction

Tocopherols are natural antioxidants present in oil seeds. α-Tocopherol is used as pharmaceuticals and cosmetics, and a mixture of α-, γ-, and δ-tocopherols is added to various oils- and fats-based foods. Oil seeds also contains phytosterols (brassicasterol, campesterol, stigmasterol, β-sitosterol, etc; referred to as sterols) and their fatty acid esters (steryl esters), which are known to have choresterol-lowering effect [1–4]. This physiological activity has led to the development of several foods for specified health uses in Japan; such as salad oils with addition of sterols and steryl esters, and a margarine and mayonnaise blended with steryl esters.

Tocopherols are purified industrially from vegetable oil deodorizer distillate, which is a by-product in the final deodorization step of vegetable oil refining, by some processes composed of chemical methylation, shot-path distillation, solvent fractionation, and/or ion-exchange chromatography. In addition, sterols are purified by solvent fractionation of a by-product in the tocopherol purification process. However, a more efficient process has been desired strongly to achieve higher purity and yield.

Our laboratory reported to date that not only tocopherols, but also sterols (as steryl esters) can be purified with good yields by converting sterols to steryl esters and by converting free fatty acids (FFAs) to fatty acid methyl esters (FAMEs) [5]. The two conversions were achieved in one-batch reaction with *C. rugosa*

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lipase: Esterification of sterols with FFAs was conducted, and then methyl esterification of FFAs was conducted successively by addition of methanol (MeOH) to the reaction mixture. However, this two-step in situ reaction converted only 80% sterols to steryl esters and 78% FFAs to FAMEs [5]. These degrees of the two reactions are not enough for efficient purifications of tocopherols and sterols as steryl esters. Hence, the two-step in situ reaction had to be conducted twice to obtain higher degrees of the two reactions. This paper describes about a new two-step in situ reaction, which can attain high degrees of conversions of sterols to steryl esters and of FFAs to FAMEs in its single reaction.

2. Materials and methods

2.1. Pretreatment of soybean oil deodorizer distillate (SODD)

When a starting material does not include any components of which molecular weights are larger than that of sterols, sterols can be purified easily as steryl esters by converting sterols to steryl esters. Hence, SODD (Yashiro Co. Ltd., Osaka, Japan), which is a by-product in the deodorization step of soybean oil refining, was first distillated at 240 °C/0.02 mmHg to remove high boiling point substances, such as steryl esters, diacylglycerols, and triacylglyceols. The resulting distillate, named SODD tocopherols/sterols concentrate (SODDTSC), was used for this study. The SODDTSC was composed of 52.8 wt.% FFAs, 1.4 wt.% partial acylglycerols, 2.3 wt.% campesterol, 2.4 wt.% stigmasterol, 3.7 wt.% β-sitosterol, 1.7 wt.% α-tocopherol, 13.4 wt.% γ-tocopherol, 5.7 wt.% δ-tocopherol, 1.7 wt.% steryl esters, and 14.9 wt.% unknown hydrocarbons. SODDTSC used in this study included 6500 ppm water.

2.2. Lipases and chemicals

Lipases were obtained from the following companies: *Aspergillus niger* lipase (Lipase AP), *Rhizopus oryzae* lipase (Lipase T), *Candida rugosa* lipase (Lipase AY), and *Burkholderia cepacia* lipase (Lipase PS) were from Amano Enzyme Inc. (Aichi, Japan); *C. rugosa* lipase (Lipase OF), *Alcaligenes* sp. lipase (Lipase QLM) and *B. cepacia* lipase (Lipase SL), *Pseudomonas stutzeri* lipase (Lipase TL) were from Meito Sangyo Co. Ltd. (Aichi, Japan); *P. aeruginosa* lipase (Lipase LPL) was from Toyobo Co. Ltd. (Osaka, Japan); *Thermomyces lanuginosa* lipase (Lipozyme TL) was from Novozymes (Bagsvaerd, Denmark); *B. glumae* was from Asahi Chemical Industry Co. Ltd. (Tokyo, Japan). Unless otherwise specified, a powdered preparation was dissolved in deionized water and the lipase solution was added to the reaction mixture. The lipase activity was measured by titrating fatty acids (FAs) liberated in hydrolysis from olive oil (Wako Pure Chemical Industry, Co., Osaka, Japan) with 50 mM KOH as described previously [6]. One unit (U) was defined as the amount of enzyme that liberated 1 μmol FA per minute. Methanol (MeOH) and tricaproin were purchased from Wako Pure Chemical Industry and Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), respectively. Other chemicals were of analytical grade.

2.3. Reactions

The reaction is a two-step in situ reaction consisting of esterification of sterols with FFAs (first step) and methyl esterification of FFAs (second step). A standard mixture for the first-step reaction was composed of SODDTSC/water (95:5, w/w) and 250 U/g-mixture of *C. rugosa* lipase. The reaction was conducted at 30 °C with dehydration at 5 or 20 mmHg. The dehydrations at 5 and 20 mmHg were performed using an oil rotary vacuum pump (GVD-050A; pumping speed, 60 L/min; ULVAC Kiko Inc., Kanagawa, Japan) and a diaphragm vacuum pump (DAH-20C; pumping speed, 24 L/min; ULVAC Kiko Inc.), respectively. After the first-step reaction, 7 M amounts of MeOH for FFA, 20 wt.% water, and 25 U/g-mixture of *Alcaligenes* lipase were added to the reaction mixture. The second-step reaction was then conducted at 30 °C in the reactor overlain with nitrogen gas. A small-scale reaction was conducted in a 50-mL vessel with stirring at 500 rpm, and a large-scale reaction was conducted in a 1- or 3-L four-necked round-bottomed flask with agitating at 200 rpm.

The degree of esterification of sterols was expressed as a ratio (mol%) of the amount of steryl esters to total amount of sterols and steryl esters. The degree of methyl esterification of FFAs was expressed as a ratio (mol%) of the amount of FAMEs to total amount of FFAs and FAMEs. The molecular weights used were 410 and 278 for sterols and FFAs, respectively, which were average values of the components in SODDTSC. In addition, the molecular weights of 670 and 292 were used for steryl esters and FAMEs, respectively.

2.4. Distillation

The reaction mixture was heated at 80 °C under nitrogen atmosphere and was separated into the oil and water layers by standing. The oil layer was dehydrated at 80 °C/5 mmHg for 30 min (water content, <100 ppm). To remove insoluble materials in the oil layer, 2 wt.% Hyflo Super-Cell (Celite Corp., CA, USA) was added to the oil layer and the mixture was then filtrated with filter paper. The resulting oil layer was distilled stepwise using a distillation apparatus (Wiprene type 2-03; Kobelco Eco-Solutions Co. Ltd., Hyogo, Japan): Step 1, at 160 °C/0.2 mmHg; step 2, at 175 °C/0.2 mmHg; step 3, at 230 °C/0.02 mmHg; step 4, at 240 °C/0.02 mmHg.

2.5. Analyses

A sample of each reaction (3–5 mL) was centrifuged at 6500 × *g* for 5 min, and the oil layer was used for analyses. All analyses were conducted as described previously [5]. In brief, the contents of FFAs, FAMEs, sterols, tocopherols, partial acylglycerols, and steryl esters were determined with a Shimadzu GC-18A gas chromatograph (Kyoto, Japan) connected to a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific, CA, USA), using tricaproin as an internal standard. FFAs in SODDTSC

were methylated in 3 mL MeOH containing 0.5% BF_3, and FAs in steryl esters were methylated in 4 mL MeOH containing 5.9% sodium methylate. The resulting FAMEs were analyzed with a Agilent 6890N gas chromatograph (Palo Alto, CA, USA) connected to a DB-23 capillary column (0.25 mm × 10 m; J&W Scientific). The content of FFAs was calculated from the acid value, and that of water in the oil layer was measured by Karl Fisher titration (Moisture Meter CA-07; Dia Instruments Co. Ltd., Kanagawa, Japan).

3. Results and discussion

When a mixture of sterols/FFAs/MeOH was stirred with *C. rugosa* lipase, esterification of FFAs with MeOH proceeded efficiently and esterification of sterols with FFAs proceeded scarcely [5]. It was therefore planed that sterols are esterified with FFAs (first step) and the unreacted FFAs are then esterified with MeOH (second step).

3.1. First step: effect of dehydration on esterification of sterols

It is well known that *C. rugosa* lipase catalyzes esterification of sterols with FFAs, and that the esterification attains about 80% in the presence of 20 wt.% water [7,8]. However, an efficient purification of tocopherols from SODDTSC demands the conversion of >95% sterols to steryl esters [5,8]. As the lipase catalyzes hydrolysis of steryl esters as well as esterification of sterols with FFAs, the degree of esterification may be raised by repression of the hydrolysis. Hence, an effect of dehydration on esterification of sterols with FFAs was first studied. In this paper, *C. rugosa* lipase is Lipase OF from Meito Sangyo, unless otherwise specified.

C. rugosa lipase (powdered preparation) did not act in a mixture without addition of water; thus, a mixture of SODDTSC/water (4:1, w/w) and 250 U/g-mixture of the lipase was prepared beforehand and was stirred with dehydration at 5 and 20 mmHg (Table 1). A control reaction was conducted without dehydration. After 20 h, the reaction converted 80% sterols to steryl esters. Meanwhile, the reaction with dehydration at 20 mmHg reached 95% esterification of sterols after 20 h, and the reaction with dehydration at 5 mmHg attained only 26% esterification. These results showed that moderate dehydration is effective for an increase in the degree of esterification of sterols, and that the content of water in the reaction mixture may be an important factor.

Table 1
Effect of dehydration on esterification of sterols with FFAs in SODDTSC

Reaction	Content (wt.%)				Esterification of sterol (%)
	FFA	Sterol	Steryl ester	Tocopherol	
Before[a]	52.5	8.4	1.7	20.8	–
After					
Without dehydration[b]	49.7	1.7	12.7	20.5	79.8
With dehydration[b]					
At 20 mmHg	48.5	0.4	15.4	20.3	95.4
At 5 mmHg	51.4	6.2	5.3	21.1	26.2

[a] The contents of components in SODDTSC.
[b] A 5-g mixture of SODDTSC/water (4:1, w/w) and 250 U/g-mixture of *C. rugosa* lipase (Lipase OF) was stirred at 40 °C for 20 h without dehydration or with dehydration at 5 and 20 mmHg.

SODDTSC/water (4:1, w/w) was agitated with dehydration at 5 and 20 mmHg using 250 and 1000 U/g-mixture of *C. rugosa* lipase, and an effect of the content of water in the reaction mixture on esterification of sterols was studied (Fig. 1). The reaction at 20 mmHg with 250 U/g-mixture of the lipase reached steady state after 20 h, and the degree of esterification of sterols was 95% (Fig. 1A). The content of water in the reaction mixture decreased and the contents at 20 and 24 h were 800 and 510 ppm, respectively. The reaction at 5 mmHg with 250 U/g-mixture of the lipase ceased at 20% esterification (Fig. 1B), but the reaction at 5 mmHg with 1000 U/g-mixture of the lipase increased the degree of esterification to 77% (Fig. 1C). The two reactions ceased at about 500 ppm water. These results showed that the lipase did not act when the content of water in the reaction mixture was <500 ppm.

Water was added to the 24-h reaction mixture in Fig. 1B and C to give 20 wt.%, and the reactions were continued for further 24 h without dehydration. Surprisingly, esterification of sterols did not increased, showing that the activity once lost does not

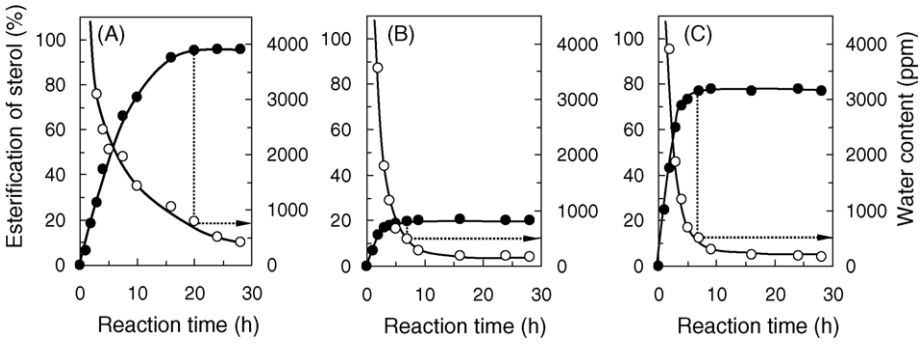


Fig. 1. Effect of the content of water in the reaction mixture on esterification of sterols. A 300-g mixture of SODDTSC/water (4:1, w/w) and 250 or 1000 U/g-mixture of *C. rugosa* lipase was prepared beforehand, and was agitated at 40 °C with dehydration at 5 and 20 mmHg. (A) Reaction at 20 mmHg with 250 U/g-mixture of the lipase. (B) Reaction at 5 mmHg with 250 U/g-mixture of the lipase. (C) Reaction at 5 mmHg with 1000 U/g-mixture of the lipase. Closed circles, the degree of esterification of sterols; open circles, the content of water in the reaction mixture.

restore at least in this reaction system. It is presently unclear why this phenomenon was observed; although it may be assumed that water molecule(s) participates in expression of the lipase activity.

3.2. First step: reaction conditions for achieving enough esterification of sterols

In the mixture of SODDTSC and *C. rugosa* lipase, >500 ppm water was necessary for maintenance of the activity (Fig. 1). Meanwhile, the starting material, SODDTSC, included 6500 ppm water, which is enough to express the activity. But when powdered *C. rugosa* lipase was added to SODDTSC, the lipase scarcely catalyzed esterification of sterols with FFAs. This inconsistency can be explained by assuming that moderate amounts of water are necessary for full expression of the activity, and that the activated enzyme maintains the activity in the presence of >500 ppm water. Because this hypothesis shows that excess amounts of water is necessary at the beginning of the reaction, the effect of initial water content on esterification of sterols was studied.

A mixture of SODDTSC and 0–20 wt.% water was stirred at 20 mmHg for 20 h with 250 U/g-mixture of *C. rugosa* lipase, and the degree of esterification of sterols and the content of water in the reaction mixture were plotted in Fig. 2A. The reaction without addition of water scarcely catalyzed esterification of sterols with FFAs, and the increase in initial content of water raised the degree of esterification. The degree maintained 95% when the reaction was started in a mixture containing 3–20 wt.% water. Furthermore, the contents of water in all reaction mixtures reached almost the same value (3900–4600 ppm) at 2 h and were 720–890 ppm after 20 h. These results showed that >3 wt.% water is necessary at the beginning of the reaction for an efficient esterification of sterols and FFAs in SODDTSC.

A small amount of enzyme requires long time to reach the equilibrium state. If the content of water in the reaction mixture becomes <500 ppm before achievement of the equilibrium, the reaction will cease and a high degree of esterification can not be obtained. To know a minimum amount of enzyme for achieving 95% esterification, a mixture of SODDTSC/water (95:5, w/w) was stirred at 20 mmHg for 20 h with various amounts of *C. rugosa* lipase (Fig. 2B). The content of water after 20 h was 750–810 ppm in all reactions, and reactions with 250 U/g-mixture or more of lipase achieved 95% esterification.

Based on these results, the conditions for the first-step esterification were fixed as follows: The ratio of SODDTSC/water, 95:5 (w/w); dehydration, 20 mmHg; and temperature, 40 °C. A 300-g scale of reaction was conducted for 24 h under the conditions fixed. After the reaction, small amounts of insoluble materials appeared in the reaction mixture, which were predicted to come from the lipase preparation, and the lipase activity was observed only very little in the reaction mixture. However, to completely eliminate an effect of *C. rugosa* lipase, the insoluble materials were removed by filtration, and the filtrate was then heated at 90 °C for 30 min. The resulting mixture was composed of 48.2 wt.% FFAs, 0.4 wt.% sterols, 15.2 wt.% steryl esters, 20.4 wt.% tocopherols, and 15.8 wt.% unknown hydrocarbons. This mixture (referred to as Mix-1st) was used as a substrate for the second-step reaction in which FFAs are esterified with MeOH.

3.3. Second step: suitable lipase for methyl esterification of FFAs

FFAs in Mix-1st were esterified with 2 M amounts of MeOH for FFAs in the presence of 20 wt.% water using various lipases (Table 2). All lipases catalyzed efficiently methyl esterification of FFAs, and achieved >75% esterification. Lipases from *P. stutzeri*, *P. aeruginosa*, and *B. cepacia* (Lipase-SL) converted moderate amounts of steryl esters, which were synthesized in the first-step reaction, to free sterols, and *C. rugosa* lipase (Lipase-AY) converted small amounts of steryl esters to sterols. Meanwhile, lipases from *Alcaligenes* sp., *B. cepacia* (Lipase-PS), and *R. oryzae* did not convert steryl esters to sterols and achieved >90% methyl esterification of FFAs. Among the three enzymes, *R. oryzae* lipase was unstable in the presence of >3 M amounts of MeOH (data not shown). In addition, *Alcaligenes* lipase has been used more preferably in the food industry than *B. cepacia* lipase. Hence, *Alcaligenes* lipase was selected as a catalyst for the second-step reaction.

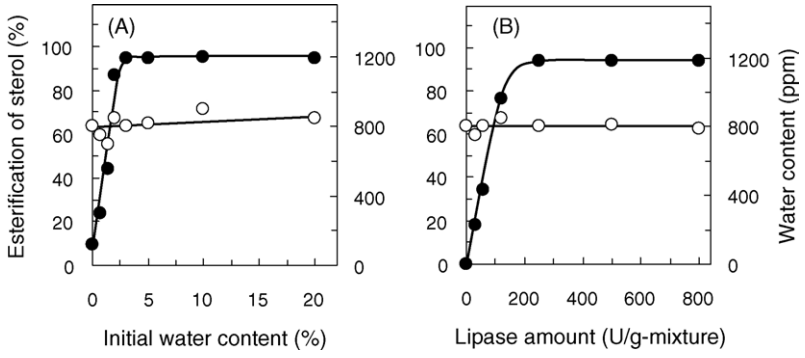


Fig. 2. Effects of initial water content and lipase amount on esterification of sterols and FFAs in SODDTSC. (A) Effect of initial water content. A 5-g mixture of SODDTSC and different concentrations of water was stirred at 40 °C/20 mmHg for 20 h with 250 U/g-mixture of *C. rugosa* lipase. (B) Effect of lipase amount. A 5-g mixture of SODDTSC/water (95:5, w/w) was stirred at 40 °C/20 mmHg for 20 h with different amounts of *C. rugosa* lipase. Closed circles, the degree of esterification of sterols; open circles, the content of water in the reaction mixture.

Table 2
Methyl esterification of FFAs in the first-step reaction mixture using various lipases

Lipase	Content (wt.%)				Esterification of FFA (%)
	FFA	FAME	Sterol	Steryl ester	
None	48.2	0	0.4	15.2	–
Alcaligenes sp.	4.6	45.8	0.4	15.1	90.5
P. stutzeri	6.3	46.5	3.8	9.3	87.5
P. aeruginosa	5.2	48.2	4.9	7.5	89.8
B. glumae	5.6	44.5	0.6	14.8	88.3
B. cepacia[a]	8.5	43.5	3.1	10.6	83.0
B. cepacia[b]	4.7	45.9	0.5	14.9	90.3
C. rugosa[c]	11.0	38.9	0.4	15.2	77.1
C. rugosa[d]	11.7	38.5	0.9	14.2	75.8
A. niger	9.5	40.5	0.4	15.0	80.2
T. lanuginosa	6.4	43.9	0.4	15.4	86.7
R. oryzae	4.7	45.7	0.4	15.2	90.3

The reaction was conducted at 30 °C for 20 h in a mixture of 3.6 g Mix-1st, 0.4 g MeOH (2 M amounts for FFAs in Mix-1st), 1.0 mL water, and 200 U/g-mixture of lipase. Mix-1st is a first-step reaction mixture that was heated at 90 °C for 30 min.

[a] Lipase SL from Meito Sangyo.
[b] Lipase PS from Amano Enzyme.
[c] Lipase OF from Meito Sangyo.
[d] Lipase AY from Amano Enzyme.

3.4. Second step: reaction conditions for achieving high degree of methyl esterification of FFAs

Methyl esterification of FFAs is presumed to be affected by the amounts of MeOH and water in the reaction mixture. Hence, at first a mixture of Mix-1st and different amounts of MeOH was stirred at 30 °C for 20 h using 50 U/g-mixture of *Alcaligenes* lipase in the presence of 20 wt.% water (Fig. 3A). Five molar or more amounts of MeOH achieved >95% methyl esterification of FFAs, and 10 M or more amounts of MeOH reconverted only a small amount of steryl esters to free sterols. Based on these results, the amount of MeOH was fixed at 7 M amounts against FFAs in Mix-1st.

Effect of water content on the methyl esterification was next studied. A mixture of Mix-1st, 7 M amounts of MeOH, and different amounts of water was stirred at 30 °C for 20 h using 50 U/g-mixture of *Alcaligenes* lipase (Fig. 3B). The lipase scarcely catalyzed the methyl esterification in the presence of 5 wt.% or less concentration of water and efficiently catalyzed at 10 wt.% or more concentration of water. The highest degree of esterification (95%) can be achieved at 20 wt.% of water. Reconversion of steryl esters to free sterols was not observed in all reactions. Based on these results, the amount of water was fixed at 20 wt.%.

The second-step reaction was conducted in a mixture of Mix-1st, 20 wt.% water, and different amounts of *Alcaligenes* lipase to determine the amount of enzyme. The results showed that >12 U/g-mixture of the lipase achieves >95% methyl esterification of FFAs within 20 h. The amount of the lipase used in the second-step reaction was therefore fixed at 25 U/g-mixture in the following reactions.

3.5. Two-step in situ reaction

Only very little activity of *C. rugosa* lipase was detected even though water was added to the mixture after the first-step reaction; thus, the second-step reaction was preliminarily conducted using the reaction mixture without heating as a substrate. The time course was the same as that of the reaction conducted using Mix-1st, which had been treated by heating. Hence, the second-step reaction was conducted successively after the fist-step reaction.

A mixture of SODDTSC/water (95:5, w/w) and 250 U/g-mixture of *C. rugosa* lipase was agitated at 40 °C with dehydration at 20 mmHg. After 24 h, 20 wt.% water, 7 M amounts of MeOH, and 25 U/g-mixture of *Alcaligenes* lipase were added to the reaction mixture, and the second-step reaction was conducted at 30 °C without dehydration. A typical time course is shown in Fig. 4. The first-step reaction hydrolyzed small amounts of acylglycerols almost completely in the early stage of reaction, and 95% sterols were converted to steryl esters after 20 h. The

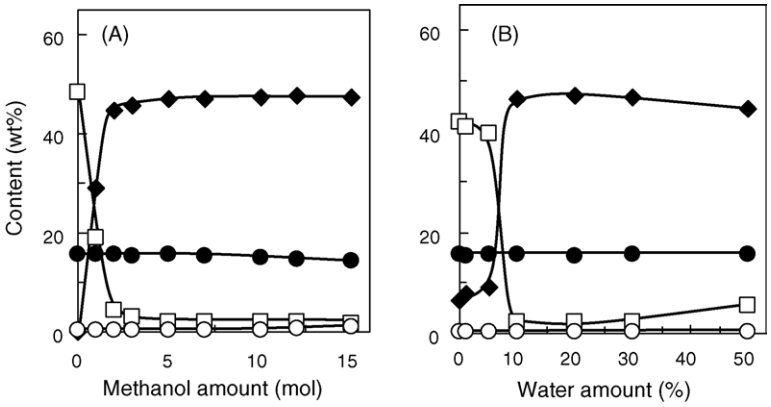


Fig. 3. Effects of the amounts of MeOH and water on methyl esterification of FFAs. Reaction mixture after the first-step reaction was heated at 90 °C for 30 min, and the resulting mixture (Mix-1st) was used as a substrate. (A) Effect of MeOH amount. A 5-g mixture of Mix-1st, 0–15 M amounts of MeOH for FFAs was stirred at 30 °C for 20 h using 50 U/g-mixture of *Alcaligenes* lipase in the presence of 20 wt.% water. (B) Effect of water amount. A 5-g mixture of Mix-1st, 7 M amounts of MeOH for FFAs, and 0–50 wt.% water was stirred at 30 °C for 20 h using 50 U/g-mixture of *Alcaligenes* lipase. Open circles, the content of sterols; closed circles, steryl esters; open squares, FFAs; closed diamonds, FAMEs.

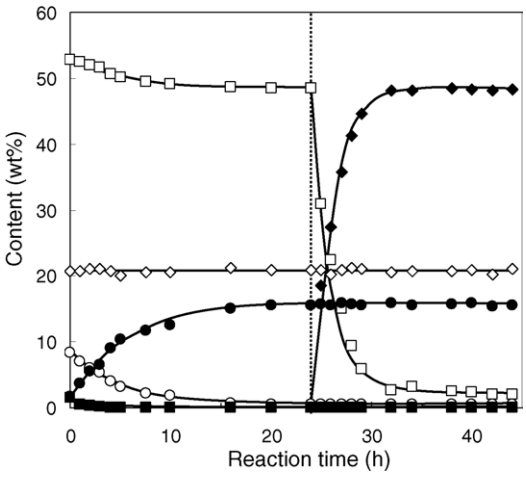
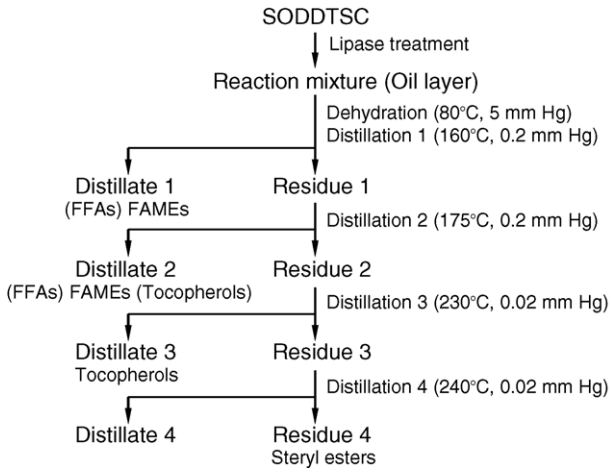


Fig. 4. Time course of two-step in situ reaction. The reaction was conducted at 40 °C with agitation at 200 rpm in 1-L round-bottomed flask containing 285 g SODDTSC, 15 g water, and 250 U/g-mixture of *C. rugosa* lipase, with dehydration at 20 mmHg. After 24 h indicated with a dotted line, 20 wt.% water, 7 M amounts of MeOH for FFAs, and 25 U/g-mixture of *Alcaligenes* lipase were added to the reaction mixture, and the second-step reaction was performed at 30 °C with agitation at 200 rpm without dehydration. Open circles, the content of sterols; closed circles, steryl esters; open squares, FFAs; closed squares, acylglycerols; open diamonds, tocopherols; closed diamonds, FAMEs.



Scheme 1. Process comprising two-step in situ reaction and distillation for purification of tocopherols and sterols as steryl esters from SODDTSC.

second-step reaction efficiently converted FFAs to FAMEs, and the degree of methyl esterification of FFAs reached 95% after 8 h (32 h in total). During the second-step reaction, steryl esters synthesized by the first-step reaction were not reconverted to free sterols. In addition, the content of tocopherols was constant during this two-step in situ reaction.

3.6. Large-scale purification of tocopherols and steryl esters

SODDTSC (1.5 kg) was subjected to the two-step in situ reaction, and 95% of sterols were converted to steyl esters and 96% FFAs were converted to FAMEs/steryl esters. Main components in the reaction mixture were FAMEs, tocopherols, and steryl esters of which boiling points are different significantly. Hence, the desired components (tocopherols and steryl esters) were fractionated by short-path distillation. The procedure is shown in Scheme 1, and the result is summarized in Table 3. The oil layer recovered by standing the reaction mixture was dehydrated at 80 °C/5 mmHg for 30 min. After insoluble materials in the dehydrated oil layer were removed by filtration, the resulting oil layer was distilled at 160 °C/0.2 mmHg. Almost FAMEs were removed in distillate 1, but small amounts of FAMEs and FFAs were remained in residue 1. The residue was therefore distilled at 175 °C/0.2 mmHg to remove them in distillate 2. The contents of FAMEs and FFAs in residue 2 reduced to <0.1 and 0.8 wt.%, respectively. To purify tocopherols, residue 2 was distilled at 230 °C/0.02 mmHg. Tocopherols were consequently recovered in distillate 3 (the purity, 72%; the recovery, 88%). Residue 3 was steryl ester fraction, but the purity was 87 wt.% because of contamination of low boiling point substances including tocopherols. Hence, residue 3 was finally distilled at 240 °C/0.02 mmHg, and steryl esters were recovered in residue 4, in which the purity of steryl esters increased to 97 wt.%. A series of procedures recovered 97% of sterols/steryl esters in SODDTSC as steryl esters. The purities of tocopherols and steryl esters were almost the same as those in previous process [5]. Also, the recovery of tocopherols was improved a little compared with that in previous process, and the recovery of steryl esters was better than that in previous process by >10%.

Table 3
Purification of tocopherols and sterols by a process comprising lipase treatment and distillation

	Weight (g)							
	Total	FFA	FAME	Acylglycerol	Tocopherol	Sterol	Steryl ester	Others[a]
SODDTSC	1500	792	0	22	307	126	26	227
Lipase treatment	1480	29	739	ND	303	6.2	232	171
Distillate 1	737	18	696	ND	2.1	ND	ND	20
Distillate 2	90	5.3	35	ND	15	ND	ND	34
Distillate 3	372	2.6	ND	ND	269	4.3	0.7	95
Distillate 4	26	0.2	0	ND	12	0.9	3.1	10
Residue 4	231	0.4	0	ND	1.8	ND	225	3.6

Purification was performed according to Scheme 1. ND: not detected (<0.1 wt.%).
[a] Main components were unknown hydrocarbons.

3.7. Compositions of FAs and sterols in purified steryl esters

FA specificity of *C. rugosa* lipase are in the order of α-linolenic acid (ALA) > linoleic acid (LnA) > oleic acid (OA) > palmitic acid (PA) > stearic acid (SA) [5]. Because FAs esterified with sterols in the first-step reaction were about 10 wt.% of total FA in the reaction mixture, FAs should be esterified with sterols according to the FA specificity of the lipase. FA composition of steryl esters (residue 4 in Table 3) was analyzed to be 8.6 wt.% PA, 2.0 wt.% SA, 31.3 wt.% OA, 48.2 wt.% LnA, 8.8 wt.%, and 1.0 wt.% other FAs. FA composition of FFAs in SODDTSC was 12.9 wt.% PA, 4.7 wt.% SA, 30.9 wt.% OA, 44.1 wt.% LnA, 5.9 wt.% ALA, and 1.5 wt.% other FAs. Relative activity of the lipase on each FA can be expressed by a ratio of F_{se}/F_{ffa}, where F_{se} and F_{ffa} are the contents of a particular FA in the steryl esters and in the SODDTSC, respectively. The ratio of each FA was calculated, showing that the relative activity on PA, SA, OA, LnA, and ALA were 0.67, 0.43, 1.01, 1.09, and 1.51, respectively. This result coincided with FA specificity of *C. rugosa* lipase.

On the other hand, as 95% sterols were esterified with FFAs, the composition of sterols in purified steryl esters was expected to be the same as that in SODDTSC. The composition of sterols in steryl esters was 27 wt.% campesterol, 29 wt.% stigmasterol, and 44 wt.% β-sitosterol, which was coincided with that in SODDTSC.

4. Conclusion

Our previous study showed that a two-step in situ reaction with *C. rugosa* lipase was effective for purification of tocopherols and sterols in SODDTSC [5]. But the single reaction achieved only 80% esterification of sterols and 80% methyl esterification of FFAs. Hence, the reaction had to be conducted twice to achieve >95% esterification in the two reactions. In this study, esterification of sterols with FFAs using *C. rugosa* lipase was improved by removing water, and methyl esterification of FFAs was improved by selecting *Alcaligenes* lipase and by conducting the reaction in a mixture of a large amount of MeOH (7 M amounts for FFAs). These improvements achieved 95% of esterification of sterols to steryl esters and 96% of esterification of FFAs to FAMEs/steryl esters in the single reaction. When a desired material is produced industrially, the number of process affects the cost of production. In this study, the reduction of the number of process was achieved, showing that the process comprising two-step in situ reaction and short-path distillation may be effective as an industrial process not only for purification of tocopherols, but also for recovering sterols as high purity of steryl esters in a very good yield.

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