Facile Purification of Tocopherols from Soybean Oil Deodorizer Distillate in High Yield Using Lipase[1]

Yuji Shimada[a,*], Seiichi Nakai[b], Masaharu Suenaga[b], Akio Sugihara[a], Motohiro Kitano[b], and Yoshio Tominaga[a]

[a]Osaka Municipal Technical Research Institute, Osaka 536-8553, Japan, and [b]Yashiro Co. Ltd., Osaka 547-0003, Japan

ABSTRACT: Tocopherols have been purified from deodorizer distillate produced in the final deodorization step of vegetable oil refining by a process including molecular distillation. Deodorizer distillate contains mainly tocopherols, sterols, and free fatty acids (FFA); the presence of sterols hinders tocopherol purification in good yield. We found that *Candida rugosa* lipase recognized sterols as substrates but not tocopherols, and that esterification of sterols with FFA could be effected with negligible influence of water content. Enzymatic esterification of sterols with FFA was thus used as a step in tocopherol purification. High boiling point substances including steryl esters were removed from soybean oil deodorizer distillate by distillation, and the resulting distillate (soybean oil deodorizer distillate tocopherol concentrate; SODDTC) was used as a starting material for tocopherol purification. Several factors affecting esterification of sterols were investigated, and the reaction conditions were determined as follows: A mixture of SODDTC and water (4:1, w/w) was stirred at 35°C for 24 h with 200 U of *Candida* lipase per 1 g of the reaction mixture. Under these conditions, approximately 80% of sterols was esterified, but tocopherols were not esterified. After the reaction, tocopherols and FFA were recovered as a distillate by molecular distillation of the oil layer. To enhance further removal of the remaining sterols, the lipase-catalyzed reaction was repeated on the distillate under the same reaction conditions. As a result, more than 95% of the sterols was esterified in total. The resulting reaction mixture was fractionated to four distillates and one residue. The main distillate fraction contained 65 wt% tocopherols with low contents of FFA and sterols. In addition, the residue fraction contained high-purity steryl esters. Because the process presented in this study includes only organic solvent-free enzymatic reaction and molecular distillation, it is feasible as a new industrial purification method of tocopherols.

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KEY WORDS: *Candida rugosa*, esterification, lipase, molecular distillation, soybean oil deodorizer distillate, sterol, tocopherol.

Natural tocopherols are useful antioxidants present in oil seeds. The α-isomer is used as a pharmaceutical substance, an ingredient of cosmetics, and a health food. A mixture of α-, γ-, and δ-isomers containing 60 wt% tocopherols is widely utilized as an additive to various kinds of foods. Tocopherols have been purified from deodorizer distillate, produced in the final deodorization step of vegetable oil refining, by a combination of molecular distillation, ethanol fractionation, chemical alcoholysis, and ion exchange chromatography. Sterols are one of the major components of deodorizer distillate and cannot be removed by molecular distillation because their molecular weights are similar to those of tocopherols. Since sterols are insoluble in cold ethanol, but tocopherols are soluble, industrial purification of tocopherols adopts ethanol fractionation for the removal of sterols. However, the recovery of tocopherols is less than 75%, because a part of tocopherols coprecipitates with sterols.

Recently, it was reported that lipase reactions are very effective for enrichment or purification of useful components in oils, e.g., polyunsaturated fatty acids (PUFA); (i) production of PUFA-rich oil by selective hydrolysis (1–3); (ii) purification of free PUFA by selective esterification (4,5); and (iii) purification of PUFA ethyl esters by selective alcoholysis (6,7). Furthermore, sterols are efficiently esterified with fatty acids in organic solvent-free systems using *Candida rugosa* lipase (8,9) and *Pseudomonas* sp. lipase (9). Hence we attempted to develop a new process, in which sterols are removed after being converted to their esters with an enzyme. The process is applicable not only to purification of the tocopherols but also to the purification of steryl esters, which have been used as ingredients of cosmetics and health foods.

The present paper describes how the total content of tocopherols is raised to 65 wt% in nearly 90% yield by esterification of sterols with free fatty acids (FFA) in soybean oil deodorizer distillate using *Candida* lipase, followed by molecular distillation of the reaction mixture. Steryl esters can be recovered as by-products in good yield and high purity.

MATERIALS AND METHODS

Preparation of soybean oil deodorizer distillate without high boiling point substances. Soybean oil deodorizer distillate (Yashiro Co. Ltd., Osaka, Japan) was distilled at 250°C and 0.2 mm Hg to remove high boiling point substances containing steryl esters, and the distillate was used as a starting material. Almost all tocopherols were recovered in the distillate,

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*To whom correspondence should be addressed at Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536-8553, Japan. E-mail: shimaday@omtri.city.osaka.jp

which we called soybean oil deodorizer distillate tocopherol concentrate (SODDTC) in the present study.

Lipases. Candida rugosa lipase (Lipase-OF) was purchased from Meito Sangyo Co. (Aichi, Japan). *Pseudomonas aeruginosa* lipase (LPL), *Pseudomonas* sp. lipase (LIPOSAM), and *Pseudomonas* sp. lipase (Lipase-PS) were gifts from Toyobo Co. Ltd. (Osaka, Japan), Showa Denko K.K. (Tokyo, Japan), and Amano Pharmaceutical Co. Ltd. (Aichi, Japan), respectively. Lipases from *Serratia marcescens* and *Rhizopus delemar* were donated by 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 (10). One unit (U) of lipase activity was defined as the amount of enzyme which liberated 1 μmol fatty acid/min.

Monoglycerides. Monoglycerides were purified from 20 g of monoolein (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) by silica gel 60 column chromatography (Merck, Darmstadt, Germany; column size, 30 × 260 mm). Monoglycerides were eluted with 400 mL of *n*-hexane/ethyl acetate (6:4, vol/vol) after eluting diglycerides with 500 mL of *n*-hexane/ethyl acetate (8:2, vol/vol). The purity of the resulting monoglycerides (12.6 g) was 97%, and the composition of main fatty acids was 4.8 wt% palmitic acid, 6.6 wt% stearic acid, 85.3 wt% oleic acid, and 2.8 wt% linoleic acid.

Lipase reaction. A small-scale reaction was performed in a 50-mL vessel. The standard reaction mixture contained 4 g SODDTC, 1 g water, and 1000 U *Candida* lipase, and the reaction was conducted at 35°C for 24 h with stirring at 500 rpm. Factors affecting the lipase-catalyzed reaction were investigated by changing only one factor. To confirm concomitant hydrolysis of monoglycerides, a mixture of 3.6 g SODDTC, 0.4 g monoglyceride, 1.0 g water, and 1000 U *Candida* lipase was incubated at 35°C for 24 h with stirring. A large-scale reaction was performed at 35°C for 24 h in a 30-L reactor (Mitsuwa Co. Ltd., Osaka, Japan) containing 8 kg SODDTC, 2 kg water, and 2×10^6 U *Candida* lipase with agitation at 200 rpm.

Distillation. Dehydration of the oil layer obtained after the lipase-catalyzed reaction was carried out at 80°C under vacuum (5 mm Hg) for 60 min before applying molecular distillation. The water content was reduced to less than 100 ppm by the dehydration step. The purification of tocopherols and steryl esters from the dehydrated oil layer was performed with molecular distillation apparatus, Wiprene type 2-03 (Shinko Pantec Co. Ltd., Hyogo, Japan), by stepwise distillation under four different conditions: at 160°C and 0.2 mm Hg, at 200°C and 0.2 mm Hg, at 230°C and 0.04 mm Hg, and at 255°C and 0.03 mm Hg.

Analysis. The contents of sterols and tocopherols were determined with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA) using tricaproin (Tokyo Kasei Kogyo) as an internal standard. The column temperature was raised from 190 to 290°C at 10°C/min and from 290 to 320°C at 5°C/min, and was then maintained for 3 min. The injector temperature was 250°C, and the detector temperature was set at 330°C because the same results were obtained at the temperature range of 320 to 380°C. SODDTC includes campesterol, stigmasterol, and β-sitosterol. Because the ratio of their contents scarcely changed during the purification process, the sterol content was expressed as their total content. For the same reason, the tocopherol content was also expressed as the total content of α-, γ-, and δ-tocopherols. The fatty acid composition was analyzed by gas chromatography with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific) after methylation with Na-methylate or boron trifluoride as described previously (11). Thin-layer chromatography (TLC) was visualized with sulfuric acid after developing a sample on silica gel 60 plate (Merck) with a mixture of *n*-hexane/ethyl acetate/acetic acid (90:10:1, vol/vol/vol). The contents of monoglycerides and FFA were determined by a TLC/flame-ionization detector analyzer (Iatroscan TH-10; Iatron Co., Tokyo, Japan) after development with a mixture of *n*-hexane/ethyl acetate/acetic acid (85:15:1, vol/vol/vol). Water content in the oil layer after dehydration was measured by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

RESULTS AND DISCUSSION

Composition of SODDTC. The contents of sterols, tocopherols, and FFA in SODDTC prepared by distillation of soybean oil deodorizer distillate are shown in Table 1. SODDTC contained 4.1 wt% monoglycerides, but di- and triglycerides were absent. The other components were unknown hydrocarbons. The acid value and saponification value of SODDTC were 83 and 90 mg KOH/g, respectively.

Treatment of SODDTC with several lipases. Table 2 shows the contents of sterol and tocopherol before and after the reactions with several lipases. Because the reaction mixture contained 20% water, hydrolysis could occur during incubation, in addition to intended esterification. Hence the acid value of the reaction mixture was also measured as an index to follow the total reactions. None of the lipases changed the tocopherol content, and *Candida* lipase and *Pseudomonas* lipase (LIPOSAM) efficiently reduced the sterol content. TLC

TABLE 1
Composition of Soybean Oil Deodorizer Distillate Tocopherol Concentrate (SODDTC)[a]

Component	Content (wt%)	Component	Content (wt%)
Sterol		Free fatty acid	
Campesterol	3.7	16:0	4.9
Stigmasterol	3.0	18:0	2.0
β-Sitosterol	6.4	18:1	10.8
Tocopherol		18:2	19.3
α-Tocopherol	2.3	18:3n-3	2.6
γ-Tocopherol	9.9	Others	2.0
δ-Tocopherol	4.3	Unknown	24.7
Monoglycerides	4.1		

[a]SODDTC was prepared by distillation of soybean oil deodorizer distillate at 250°C and 0.2 mm Hg.

TABLE 2
Sterol Content, Tocopherol Content, and Acid Value of Reaction Mixture Obtained by Incubation of SODDTC with Several Lipases[a]

Lipase	Sterol content (wt%)	Tocopherol content (wt%)	Acid value (mg KOH/g)
None	13.0	16.5	83.1
Candida rugosa	2.6	16.4	75.1
Pseudomonas aeruginosa	12.5	16.3	86.4
Pseudomonas sp.[b]	3.6	16.3	75.6
Pseudomonas sp.[c]	12.9	16.4	88.2
Serratia marcescens	12.7	16.3	83.7
Rhizopus delemar	12.9	16.5	89.7

[a]A mixture of 4.0 g SODDTC and 1.0 g water was stirred at 35°C for 24 h with 1000 U of several lipases.
[b]LIPOSAM (Showa Denko K.K., Tokyo, Japan).
[c]Lipase-PS (Amano Pharmaceutical Co., Aichi, Japan). See Table 1 for abbreviation.

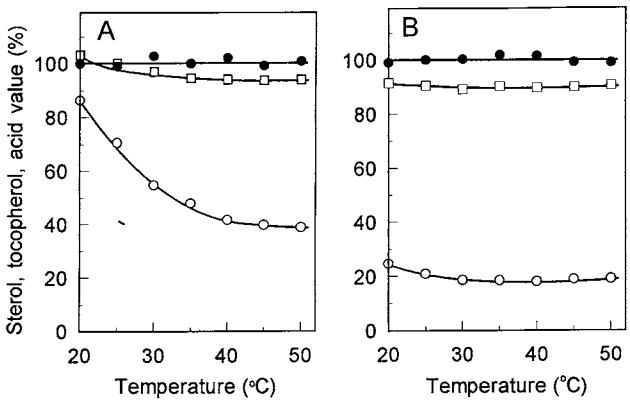

FIG. 1. Effect of temperature on enzymatic treatment of soybean deodorizer distillate tocopherol concentrate (SODDTC). The reaction was performed by stirring a mixture of 4.0 g SODDTC, 1.0 g water, and 1000 U *Candida rugosa* lipase at 20 to 50°C for 3.5 h (A) and 24 h (B). The contents of sterols (○) and tocopherols (●) were expressed relative to their initial contents. Acid value (□) was expressed as a percentage of the initial value.

analysis of the reaction mixture showed a decrease in sterols and an increase in steryl esters, indicating that sterols were converted to their fatty acid esters by the lipases. Because *Candida* lipase can be used for food processing, the lipase was selected for further experiments.

Concomitant hydrolysis of monoglycerides. The treatment of SODDTC with *Candida* lipase reduced the sterol content from 13.0 to 2.6 wt% (Table 2). This result indicates that 10.4 wt% (0.25 mmol/g) sterols was converted to esters, and that the amount of FFA consumed by the reaction corresponds to 14 mg KOH/g acid value. Hence, the acid value after the reaction should decrease from 83 to 69 mg KOH/g, but the observed value was 75 mg KOH/g (Table 2). Monoglycerides (4 wt%) present in SODDTC were not detected in the mixture after the reaction, indicating that *Candida* lipase hydrolyzed monoglycerides concomitantly with esterifying sterol. The monoglyceride content in soybean oil deodorizer distillate can reach nearly 10 wt%. Thus, SODDTC with 10% monoglycerides added was used as a substrate for the lipase-catalyzed reaction. The content of monoglycerides decreased from 13.5 to 0.8 wt%, and the acid value increased from 75 to 89 mg KOH/g. The sterol content decreased from 11.7 to 2.4 wt%, while the tocopherol content did not change during the reaction. These results confirmed that monoglycerides were hydrolyzed by the lipase during esterification of sterols. In addition, similar reactions with di- and triglycerides indicated that di- and triglycerides were also hydrolyzed concomitantly (data not shown).

Several factors affecting lipase treatment of SODDTC. SODDTC was treated in mixtures containing 2–80% water to investigate the effect of water content on the reaction. The fluidity of the reaction mixture increased slightly when more than 20% water was added. When the reaction was performed in a mixture containing 2% water, the sterol content and the acid value decreased to 2.9 wt% and 72 mg KOH/g, respectively. Experiments for which water was added at 5% or more all yielded the same esterification of sterol and change in the acid value: the sterol content fell to 2.6 wt%, and the acid value decreased from 83 to 75 mg KOH/g. In addition, the tocopherol content did not change, showing that tocopherol was not esterified in the reaction at any concentration of water. Thus, the water content exerted negligible effects on the treatment of SODDTC with *Candida* lipase, in agreement with our previous result that the water content did not affect the esterification extent of sterols because the lipase did not recognize steryl esters but sterols as substrates (9).

Figure 1 shows the effect of temperature on the reaction rate and the equilibrium. The sterol content after 3.5-h reaction decreased with increasing temperature, and the decrease of the acid value was associated with decreasing sterol content. The optimal temperature was nearly 50°C. When the reaction was performed at 25–50°C, the sterol content decreased to 2.4 wt% after 24 h. The tocopherol content did not change over the temperature range tested.

The effects of enzyme dose were investigated next (Fig. 2). When the reaction was carried out with 50 U/g of the lipase, the decrease of the acid value was associated with the decrease of the sterol content. After 40 h of reaction, the sterol content had decreased to 3.3 wt%, and the acid value was 76 mg KOH/g. The reaction with 200 U/g of the lipase attained equilibrium after 24 h, and the sterol content and the acid value were 2.4 wt% and 74 mg KOH/g, respectively. When the amount of the enzyme was increased to 800 U/g, the reaction reached equilibrium after 7 h. The tocopherol content did not change, even though the amount of lipase was increased and the reaction period was extended.

On the basis of these results, the reaction conditions were set as follows: SODDTC was incubated at 35°C for 24 h in a mixture of 20% water and 200 U/g mixture of *Candida* lipase with stirring.

Purification of tocopherol by a combination of sterol esterification and molecular distillation. Tocopherol purification from 8.0 kg SODDTC is summarized in Table 3. The sterol content was reduced to 2.2 wt% by treating SODDTC

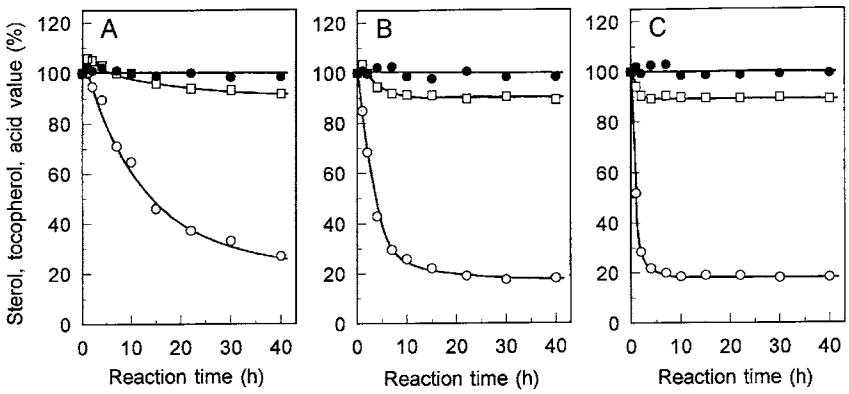

FIG. 2. Time course of treatment of SODDTC with different amounts of *Candida* lipase. A mixture of 4.0 g SODDTC and 1.0 g water was stirred at 35°C with 50, 200, and 800 U/g-mixture of the lipase. (**A**), 50 U/g; (**B**), 200 U/g; and (**C**), 800 U/g. The contents of sterols (○) and tocopherols (●) were expressed relative to their initial contents. Acid value (□) was expressed as a percentage of the initial value. See Figure 1 for abbreviation.

with *Candida* lipase under the conditions described above (lipase reaction 1). To further decrease the sterol content, the reaction products, steryl esters, were removed from the reaction mixture by distillation, and the enzyme reaction was then repeated under the same conditions. The distillation almost completely recovered tocopherols in distillate 1, and treatment of the resulting distillate 1 (lipase reaction 2) reduced the sterol content of the oil layer from 2.5 wt% to less than the detectable amount (<0.5%).

Tocopherols in the oil layer after lipase reaction 2 were purified by stepwise distillation (distillation 2) at three different temperatures (Table 3). The distillation was first conducted at 160°C and 0.2 mm Hg to remove FFA, and 2.32 kg distillate 2-1 and 4.12 kg distilland (residue 2-1) were obtained. The acid value and tocopherol content of distillate 2-1 were 124 mg KOH/g and 1.2 wt%, respectively. Meanwhile, the acid value of residue 2-1 was still high (73 mg KOH/g), and the content of FFA was 36 wt%. Thus, the distillation of residue 2-1 was next performed at 200°C and 0.2 mm Hg, and 2.59 kg distillate 2-2 and 1.51 kg distilland (residue 2-2) were obtained. The distillation transferred 36 wt% of total tocopherols to distillate 2-2, but the acid value of residue 2-2 was reduced to 3 mg KOH/g. To recover tocopherol in residue 2-2, the final distillation was carried out at 230°C and 0.04 mm Hg, and 1.1 kg distillate 2-3 and 0.4 kg residue 2-3 were obtained. The tocopherol content in distillate 2-3 attained to

TABLE 3
Purification of Tocopherol and Steryl Esters from SODDTC by a Combination of Enzymatic Esterification and Distillation

	Weight	Acid value	Tocopherol		Sterol	
Step	(g)	(mg KOH/g)	(wt%)	(g)	(wt%)	(g)
SODDTC	8000	83.1	16.5	1318	13.0	1042
Lipase reaction 1[a]	8000	74.6	16.4	1312	2.2	180
Distillation 1						
Distillate 1[b]	6450	91.0	19.5	1257	2.5	159
Residue 1[b]	1520	1.1	2.3	36	1.3	19
Lipase reaction 2[c]	6450	90.4	19.4	1251	<0.5	—
Distillation 2						
Distillate 2-1[d]	2320	123.7	1.2	29	<0.5	—
Distillate 2-2[e]	2590	112.5	17.1	443	<0.5	—
Distillate 2-3[f]	1100	3.7	65.3	720	2.9	32
Residue 2-3[f]	404	1.5	6.7	27	0.5	2
Distillation 3						
Distillate 3[g]	67	2.5	23.8	16	1.9	1
Residue 3[g]	317	1.3	3.0	10	<0.5	—

[a]SODDTC was incubated at 35°C for 24 h in a mixture containing 20% water using 200 U/g-mixture of *Candida* lipase.
[b]Distilled at 250°C and 0.2 mm Hg.
[c]Distillate 1 was incubated at 35°C for 24 h in a mixture containing 20% water using 200 U/g *Candida* lipase.
[d]Distilled at 160°C and 0.2 mm Hg.
[e]Distilled at 200°C and 0.2 mm Hg.
[f]Distilled at 230°C and 0.04 mm Hg.
[g]Distilled at 255°C and 0.03 mm Hg. See Table 1 for abbreviation.

TABLE 4
Compositions of Fatty Acid Methyl Esters and Sterols After Methanolysis of a Mixture of Distillation Residues 1 and 3 with Na-methylate[a]

Fatty acid methyl ester	Content (mol%)	Sterol	Content (mol%)
16:0	5.5	Campesterol	12.5
18:0	2.3	Stigmasterol	10.4
18:1	12.4	β-Sitosterol	22.2
18:2	22.0		
18:3	2.9		

[a]A mixture of residues 1 and 3 (100 mg) obtained as described in Table 3 was heated at 75°C for 15 min in 10 mL methanol containing 2% Na-methylate, and the extracts with *n*-hexane were then analyzed by gas chromatography.

65.3 wt%, and the sterol content was only 2.9 wt%. The acid value of this fraction was 3.7 mg KOH/g, and the FFA content was 1.9 wt%. Because distillate 2-2 could be added back for a subsequent distillation step, the recovery of tocopherol by a series of purifications was calculated to be 88.2% of the initial content of SODDTC.

Purity of steryl esters recovered in distillation residues. After this distillation protocol, residue 2-3 contained 6.7 wt% tocopherol (Table 3). To recover this tocopherol, residue 2-3 was distilled at 255°C and 0.03 mm Hg to give 67 g distillate 3 and 317 g residue 3 (Table 3). Because high boiling point substances, steryl esters, should be recovered in residues 1 and 3 by this distillation protocol, both residues were combined and their compositions were analyzed to investigate the purity of steryl esters. The contents of FFA, sterol, and tocopherol of the combined residues were 0.6, 1.1, and 2.4 wt%, respectively. TLC analysis showed only one major spot, the mobility of which coincided with that of steryl ester. The combined residues were methanolyzed using Na-methylate as a catalyst, and the components were then analyzed by gas chromatography (Table 4). The content of fatty acid methyl esters and sterols reached 90 wt%, and the other components were FFA, tocopherols, and unknown hydrocarbons. The total contents of fatty acid methyl esters corresponded completely with the content of sterols, and the composition ratios of fatty acids and sterols also agreed with those in SODDTC (Table 1). In addition, 1042 g of total sterols in the starting SODDTC were calculated to be converted to 1697 g of their esters by the esterification with soybean fatty acids, which agreed well with the total amount of residues 1 and 3 (1834 g). These results indicated that the distillation residues were steryl esters of which the purity was approximately 90%.

Characteristics of the process proposed in this study. We have described an advanced process including lipase reaction for purification of tocopherols. The procedure has the following advantages: (i) This process does not require ethanol fractionation for removing sterols, and high-purity tocopherols can be obtained in nearly 90% yield. (ii) Because the presented process is composed of only enzymatic reaction and molecular distillation, denaturation of tocopherols can be strongly suppressed. (iii) Steryl esters are highly purified in good yield. (iv) Organic solvent is not necessary for the process. These distinctive characteristics indicate that the procedure presented in this study is applicable to industrial purification of tocopherols. Furthermore, a similar lipase-catalyzed reaction may be effective for the purification of the other useful compounds contained in oil seeds.

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