Enzymatic Enrichment of Astaxanthin from *Haematococcus pluvialis* Cell Extracts

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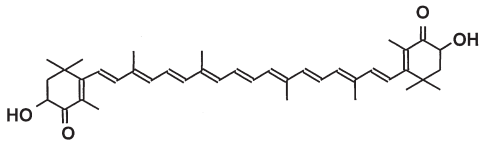
ABSTRACT: An industrially available preparation of astaxanthin (Ax) from *Haematococcus pluvialis* contained 41.6 wt% acylglycerols and 24.9 wt% FFA in addition to 14.6 wt% Ax, which was a mixture of free and FA ester forms (free Ax/Ax monoesters/Ax diesters = 4.9:80.3:14.8, by mol). Enrichment of Ax by a two-step process was attempted. The first step was hydrolysis of acylglycerols with *Candida rugosa* lipase: A mixture of 1.0 kg *H. pluvialis* cell extracts, 1.0 L water, and 50 U/g-reaction mixture of the lipase was agitated at 30°C for 42 h. The degree of hydrolysis of acylglycerols reached 94.4%, but Ax esters were not hydrolyzed. Removal of FFA from the resulting oil layer by molecular distillation enriched the content of Ax esters to 40.8 wt% (named Ax40). The second step was enzymatic conversion of Ax esters to free Ax, which successfully proceeded in the presence of ethanol (EtOH). When a mixture of 50.0 g Ax40, 8.2 g EtOH (5 molar equiv. against FA), 58.2 mL water, and 1500 U/g-mixture of *Pseudomonas aeruginosa* lipase was stirred at 30°C for 68 h, the free Ax content increased to 89.3 mol%. Free Ax was efficiently recovered by precipitation with *n*-hexane. The purity of Ax was thereby raised to 70.2 wt% with a 63.9% overall recovery of the initial content in the cell extracts.

Paper no. J10587 in *JAOCS 80*, 975–981 (October 2003).

KEY WORDS: Astaxanthin, *Candida rugosa*, enrichment, ethanolysis, *Haematococcus pluvialis*, hydrolysis, lipase, *Pseudomonas aeruginosa*.

Astaxanthin (Ax: 3,3′-dihydroxy-β,β-carotene-4,4′-dione; Scheme 1) is widely distributed in marine animals and has various physiological functions, such as a precursor of vitamin A (1), quenching free radicals and active oxygen species (2,3), anticancer activity (4,5), enhancement of the immune response (6), and inhibition of *Helicobacter pylori* infection (7). These activities have attracted a great deal of attention, and Ax has been used as a nutraceutical food, an ingredient in cosmetics, and a supplement for pigmentation of cultured fish and shellfish.

Several microorganisms, including *Phaffia rhodozyma* (8), *Agrobacterium aurantiacum* (9), and *Haematococcus pluvialis* (10,11), have been reported to produce Ax. Among these microorganisms, *H. pluvialis* shows the highest productivity, and >90 wt% of the total carotenoids were free Ax (mainly 3*S*,3′*S*-isomer) and Ax FA mono- and diesters (12). A commercially available product is prepared by acetone extraction of the cells, but the purity is low (14.6 wt%) and the preparation has an odor. A method for the purification of Ax and its FA esters from a microorganism has been reported. After crude Ax from *Chlorococcum* sp. was obtained by saponification of Ax esters, followed by extraction with organic solvents, Ax was purified by high-speed countercurrent chromatography with a two-phase solvent system composed of *n*-hexane/ethyl acetate/ethanol/water (13). Although the purity of Ax was raised to 97%, this process was not suitable for an industrial-scale purification procedure in light of cost. Hence, an industrially available process for enrichment of Ax from cell extracts has been strongly desired.



SCHEME 1

Ax is very unstable to oxidation. Accordingly, adoption of an enzymatic method for its enrichment is attractive because enzymes effectively catalyze the reaction even at ambient temperature and under nitrogen. This paper shows that a lipase-catalyzed two-step process is effective at increasing the purity of Ax to 70.2 wt% in a high yield.

MATERIALS AND METHODS

Ax preparation. The preparation was a commercial product of Fuji Chemical Industry Co. Ltd. (Toyama, Japan). *Haematococcus pluvialis* was cultivated using solar energy. The harvested cells were mechanically disrupted, and Ax and its FA esters were then extracted with acetone. The composition of the extracts is shown in the Results and Discussion section.

Lipases. Geotrichum candidum lipase was prepared according to Tsujisaka *et al*. (14). The cells were cultivated at 27°C for 24 h in a medium (pH 6.0) containing 5% corn steep liquor, 1% soybean oil, and 0.5% NH_4NO_3. The enzyme solution was prepared by ammonium sulfate fractionation, followed by dialysis against water. The other lipases were obtained from the following companies: lipases from *Candida rugosa*, *Alcaligenes*

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sp. (Lipase-QLM), *Burkholderia cepacia*, and *Pseudomonas stutzeri* were from Meito Sangyo Co. Ltd. (Aichi, Japan); *Rhizopus oryzae* lipase was from Tanabe Seiyaku Co. Ltd. (Osaka, Japan); *Thermomyces lanuginosa* lipase was from Novozymes (Bagsvaerd, Denmark); lipases from *Aspergillus niger* and *Pseudomonas* sp. were from Amano Enzyme Inc. (Aichi, Japan); *B. glumae* lipase was from Asahi Chemical Industry Co. Ltd. (Tokyo, Japan); and *P. aeruginosa* lipase was from Toyobo Co. Ltd. (Osaka, Japan). Lipases from *R. oryzae, T. lanuginosa,* and *A. niger* are 1,3-positionally specific, and the other lipases are nonspecific.

Lipase activity was measured by titration of FA liberated from olive oil (Wako Pure Chemical Ind. Ltd., Osaka, Japan) with 50 mM KOH as described previously (15). In brief, hydrolysis of olive oil was conducted at 30°C with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount that liberated 1 μmol of FA per minute.

Hydrolysis of acylglycerols in H. pluvialis *cell extracts*. A small-scale reaction was performed in a sealed 50-mL vessel under nitrogen gas. The standard reaction mixture contained 2.0 g of the cell extracts, 2.0 g water, and 200 U lipase, and the reaction was conducted at 30°C for 16 h with stirring at 500 rpm. Conditions in lipase-catalyzed reactions were studied by changing only one factor at a time. A large-scale reaction was performed at 30°C for 42 h in a 5-L reactor (MDL-500; Marubishi Bioengineering Co. Ltd., Tokyo, Japan) containing 1.0 kg of the cell extracts, 1.0 kg water, and 50 U/g-mixture of *C. rugosa* lipase with agitation at 500 rpm. The reaction was carried out in a sealed container under nitrogen gas.

Distillation. FFA in the reaction mixture were removed by molecular distillation. The reaction mixture was allowed to stand, and the resulting oil layer was dehydrated at 80°C and 5 mm Hg for 30 min with blowing nitrogen gas (water content, <100 ppm). The oil layer was applied to an apparatus (Wiprene type 2-03; Shinko Pantec Co. Ltd., Hyogo, Japan) and was stepwise distilled as follows: the first step, at 180°C and 0.2 mm Hg; the second step, at 200°C and 0.2 mm Hg.

Conversion of Ax esters to free Ax. A small-scale reaction was performed in a 50-mL vessel. The standard reaction mixture of 2.0 g Ax/ethanol (EtOH) mixture, 2.0 mL water, and 500 U/g-mixture of lipase was incubated at 30°C for 68 h with stirring at 500 rpm.

n-*Hexane fractionation.* Free Ax in the reaction mixture was purified by *n*-hexane fractionation. Five volume parts of *n*-hexane was added to the oil layer separated from the reaction mixture. The resulting precipitate was recovered by centrifugation (9800 × *g*, 10 min) at 20°C, and then dried overnight under reduced pressure at 3 mm Hg.

Analysis. The FFA content was determined based on the acid value analyzed by titration with 50 mM KOH. The content of Ax based on free form was measured from the absorbance at 480 nm using the absorption coefficient, ε (1 cm, 1 wt%) = 2100. The relative contents of free Ax, Ax monoesters, and Ax diesters were analyzed on a Develosil 300 DIOL-5 column (4.6 × 250 mm; Nomura Chemical Co. Ltd., Aichi, Japan) connected to an HPLC system (L-7000; Hitachi, Tokyo, Japan). The mobile phase of *n*-hexane/acetone (35:65, vol/vol) was used at a flow rate of 0.5 mL/min and 40°C, and the peaks of free Ax and Ax esters were detected at 480 nm. The FA composition was analyzed by GC (Hewlett-Packard 5890, Avondale, PA) with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) after methylation with Na-methylate as described previously (16). The contents of acylglycerols, FFA, and FA ethyl ester (FAEE) were determined by GC with a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific) using tricaproin (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) as an internal standard. The column temperature was raised from 120 to 270°C at 25°C/min and from 270 to 370°C at 8°C/min, and was then maintained for 1 min. The injector and detector temperatures were set at 370 and 390°C, respectively. The peaks of FFA and FAEE did not separate completely under these conditions. Hence, the total content of FFA and FAEE was measured by GC, and FFA content was determined based on the acid value.

RESULTS AND DISCUSSION

Composition of Ax from H. pluvialis *cell extracts*. The cell extracts of *H. pluvialis* contained 14.6 wt% of total Ax. In this paper, unless otherwise stated, Ax content is expressed as the total content (wt%) of free Ax and Ax esters. The composition of free Ax, Ax monoesters, and Ax diesters was 4.9, 80.3, and 14.8 mol%, respectively. The cell extracts contained 24.9 wt% FFA, 4.3 wt% MAG, 8.0 wt% DAG, 29.3 wt% TAG, and was contaminated with 18.9 wt% unknown substances. The composition of FA in acylglycerols was 21.0 wt% palmitic acid, 1.3 wt% stearic acid, 27.0 wt% oleic acid, 30.9 wt% linoleic acid, and 17.8 wt% linolenic acid.

Hydrolysis of acylglycerols in H. pluvialis *cell extracts*. We initially attempted to purify Ax from the cell extracts by molecular distillation. However, the content was increased to only 18.4 wt%, because acylglycerols in the extracts were not removed. Hydrolysis of the acylglycerols was therefore attempted to further increase Ax content.

Haematococcus pluvialis cell extracts were hydrolyzed at 30°C for 20 h in a mixture containing 50% water with 50 U/g-mixture of various lipases as catalysts (Table 1). Among the lipases tested, *C. rugosa* lipase was the most effective for hydrolysis of acylglycerols, and the FFA content was increased from 24.9 to 54.3 wt%. In these reaction conditions, the contents of free Ax and Ax esters were not changed.

To determine the reaction conditions for hydrolysis of acylglycerols in the cell extracts, the effects of water content, temperature, and lipase amount were studied. A 4.0-g mixture composed of the cell extract, various amounts of water, and 50 U/g-mixture of *C. rugosa* lipase was stirred at 30°C for 16 h (Fig. 1). Acylglycerols were scarcely hydrolyzed in the presence of 0.3% water. The hydrolysis was enhanced by an increase in the amount of water. Because a large amount of water in the reaction system requires a large reaction apparatus, the ratio of cell extracts/water was fixed at 1:1 (wt/wt) at which point acylglycerols are hydrolyzed effectively (Fig. 1).

TABLE 1
Hydrolysis of Acylglycerols in *Haematococcus pluvialis* Cell Extracts by Different Lipases[a]

	Content (wt%)				
			Ax[b]		
Lipase	FFA	Acylglycerol	Free	Mono	Di
None	24.9	41.6	0.5	11.3	2.8
Candida rugosa	54.3	11.4	0.5	11.6	2.9
Geotrichum candidum	45.0	21.6	0.5	11.3	2.9
Rhizopus oryzae	31.1	35.3	0.6	11.5	2.6
Thermomyces lanuginosa	37.9	28.1	0.5	11.3	2.8
Aspergillus niger	42.8	23.8	0.5	11.5	2.7
Alcaligenes sp.	36.5	30.6	0.4	11.3	2.9
Burkholderia glumae	40.9	25.6	0.5	11.5	2.9
B. cepacia	39.8	26.1	0.5	11.3	2.8
Pseudomonas sp.	35.4	31.6	0.5	11.4	2.8
P. aeruginosa	25.3	41.0	0.7	11.5	2.4
P. stutzeri	26.1	40.6	0.7	11.3	2.6

[a]A mixture of 2.0 g cell extracts and 2.0 g water was stirred at 30°C for 16 h with 50 U/g-mixture of lipase.
[b]Free: free astaxanthin (Ax); Mono: Ax monoesters; Di: Ax diesters.

Reactions conducted at 20, 30, 40, and 50°C for 16 h showed degrees of acylglycerol hydrolysis of 74.3, 84.8, 67.7, and 8.8%, respectively. To study the effect of the lipase amount on the hydrolysis of acylglycerols, the reaction was conducted with a mixture of 2.0 g cell extracts, 2.0 g water, and various amounts of *C. rugosa* lipase at 30°C for 40 h. The results showed that the degree of hydrolysis did not increase when >50 U/g-mixture of lipase was used.

Based on these results, reaction conditions were determined as follows: ratio of the cell extracts/water, 1:1 (wt/wt); temperature, 30°C; lipase amount, 50 U/g-reaction mixture. Figure 2 shows a typical time course under these conditions. FFA content increased with a decrease in acylglycerols, and the reaction reached nearly steady state after 40 h. The hydrolysis reached 96.2% after 72 h, and TAG, DAG, and MAG contents decreased to 0.6, 0.6, and 0.4 wt%, respectively. FFA content increased from 24.9 to 62.1 wt%. Ax content was raised slightly from 14.6 to 15.8 wt%. The phenomenon can be explained by removal of water-soluble substances (mainly glycerol generated by hydrolysis) in the water layer.

Recovery of Ax from the reaction mixture. A process for recovery of free Ax and Ax esters from the reaction mixture is summarized in Table 2. A mixture of 1.0 kg of cell extract, 1.0 L water, and 50 U/g-mixture of *C. rugosa* lipase was agitated at 30°C for 42 h (hydrolysis of acylglycerols, 94.4%; FFA content, 61.6 wt%). The reaction mixture was separated into oil and water layers, and the oil layer was then dehydrated at 80°C and 5 mm Hg for 30 min. FFA in the oil layer (537 g) was removed by two-step distillation at 180°C/0.2 mm Hg and 200°C/0.2 mm Hg. The first distillation removed 71.1% FFA, and the second distillation removed 24.2% FFA. Free Ax and Ax esters were not detected in distillates 1 and 2. The FFA content of residue 2 (331 g) was reduced to 3.0 wt%, and its Ax content increased to 40.8 wt% with a recovery of 92.5% of the initial content in the cell extracts. Hereafter, residue 2 is named Ax40.

Further purification of Ax by distillation was attempted, but contaminants in Ax40 could not be removed. A preliminary experiment showed that Ax esters were soluble in *n*-hexane but free Ax was only slightly soluble. We thus attempted further purification of Ax by conversion of Ax esters to free Ax, followed by *n*-hexane fractionation.

Lipase suitable for conversion of Ax esters to free Ax in the presence of EtOH. It was previously determined that enzymatic hydrolysis of oleic acid phytosteryl esters (OASE) reached a steady state at 55–60% hydrolysis, but addition of alcohol

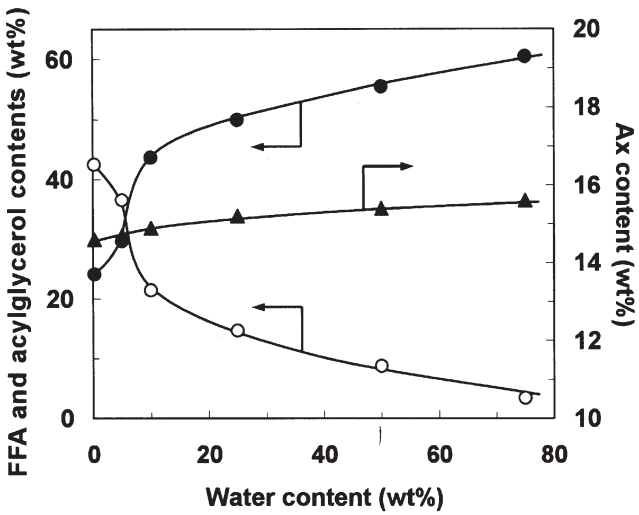

FIG. 1. Effect of water content on hydrolysis of acylglycerols in *Haematococcus pluvialis* cell extracts with *Candida rugosa* lipase. A 4.0-g mixture composed of cell extract and various amounts of water (0.3, 5, 10, 25, 50, and 75 wt%) was stirred at 30°C for 16 h with 50 U/g-mixture of lipase. After the separation of the reaction mixture to oil and water layers, the composition of the oil layer was analyzed. ○, Acylglycerols; ●, FFA; ▲, total content of astaxanthin (Ax) and its esters.

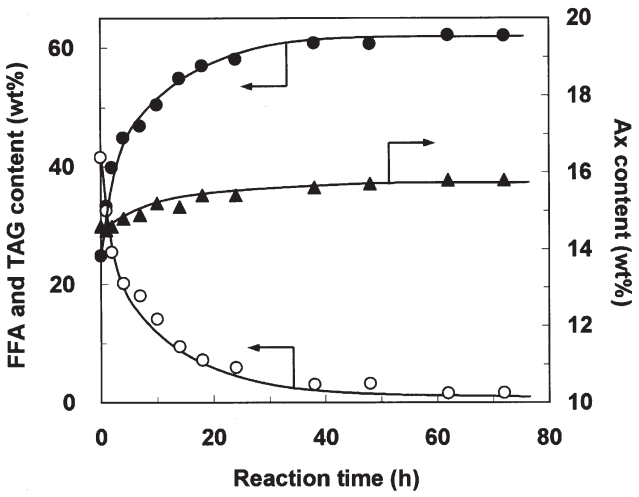

FIG. 2. Time course of hydrolysis of acylglycerols in *H. pluvialis* cell extracts with *C. rugosa* lipase. A mixture of 20.0 g cell extracts, 20.0 g water, and 50 U/g-mixture of lipase was stirred at 30°C. The reaction mixture was periodically withdrawn, and the composition of the oil layer recovered by centrifugation (9000 × *g*, 10 min) was analyzed. ○, Acylglycerols; ●, FFA; ▲, total content of Ax and its esters. See Figure 1 for abbreviation.

significantly accelerated the conversion of OASE to free sterols (degree of conversion, 98%) (17). A reaction system containing alcohol was therefore studied for the conversion of Ax esters to free Ax.

We first screened for a suitable lipase for the conversion of Ax esters. A mixture containing 1.88 g Ax40, 0.12 g EtOH (2 molar equiv. of EtOH against the total FA in Ax40), 2.0 mL water, and 2000 U lipase was incubated at 30°C for 68 h with stirring at 500 rpm, and the composition of free Ax and Ax esters in the oil layers was analyzed (Table 3). Lipases from *C. rugosa*, *R. oryzae*, and *T. lanuginosa* scarcely catalyzed the conversion of Ax esters to free Ax, but lipases from *P. aeruginosa* and *P. stutzeri* catalyzed the conversion efficiently. *Pseudomonas aeruginosa* lipase was the most effective, increasing the content of free Ax to 65.4 mol%. This lipase was therefore selected as the catalyst.

Several factors affecting conversion of Ax esters to free Ax. Fatty alcohols of different carbon lengths (C1 to C12) were screened to select a suitable alcohol. In the reaction, the amount of alcohol was fixed at 2 molar equiv against the total FA in Ax40. A mixture of 2.0 g Ax40/alcohol, 2.0 mL water, and 1000 U/g-mixture of *P. aeruginosa* lipase was stirred at 30°C for 68 h. Although the reaction with dodecanol converted only 47.8 mol% Ax esters to free Ax, the use of the other alcohols increased the degree of conversion to 71.1–74.8%. Since EtOH can be used in food processing, EtOH was selected as a substrate (degree of conversion, 74.3%).

Lipases are generally inactivated in the presence of short-chain alcohols, and the inactivation is often avoided by dilution of alcohols with a reaction mixture (18). We thus attempted to dilute the EtOH with water. A 4.0-g mixture composed of Ax40, 2 molar amounts of EtOH against FA in Ax40, different amounts of water, and 1000 U/g-mixture of *P. aeruginosa* lipase was stirred at 30°C for 68 h (Fig. 3). Ax esters did not convert to free Ax in the presence of <10% water. The degree of conversion was raised by an increase in the amount of water, and the FFA content in the reaction mixture increased. Since the degree of conversion reached nearly a steady state at 50% water, the following reactions were conducted in the presence of 50% water.

The effect of EtOH amount on the conversion of Ax esters was studied next. The reaction was conducted at 30°C for 68 h in a 4.0-g mixture composed of Ax40, various amounts of EtOH against FA in Ax40, 50 wt% water, and 1000 U/g-mixture of *P. aeruginosa* lipase (Fig. 4). When EtOH was not added to the reaction mixture, the content of free Ax was only 40.4 mol% (degree of conversion, 39.6%). The degree of conversion increased with increasing EtOH amount and reached a maximum at 3–5 molar amounts of EtOH against FA in Ax40 (free Ax content, 83.9–85.4 mol%; degree of conversion, 84.6–86.0%). Meanwhile, the degree of conversion decreased in the reaction mixture with >5 mol of EtOH, owing to the inactivation of lipase by large amounts of EtOH.

In a study of the effect of temperature, the reaction was conducted for 68 h in a mixture of Ax40, 5 mol of EtOH, 50 wt% water, and 1000 U/g-mixture of *P. aeruginosa* lipase in the

TABLE 2
Enrichment of Ax from *H. pluvialis* Cell Extracts by a Process Comprising Enzymatic Treatment and Distillation

				Ax[a]		
Step	Total (g)	FFA (g)	Acylglycerol (g)	Weight (g)	Content (wt%)	Yield (%)
Cell extracts	1000	249	416	146	14.6	100
Hydrolysis[b]	871	537	19	138	15.8	94.2
Distillation						
Distillate 1[c]	384	382	1	—	ND[e]	—
Distillate 2[d]	136	130	3	—	ND	—
Residue 2[d]	331	10	14	135	40.8	92.5

[a]Total of Ax and its FA esters.
[b]A mixture of the cell extracts/water (1:1, w/w) was agitated at 30°C for 42 h with 50 U/g-mixture of *C. rugosa* lipase.
[c]Distilled at 180°C and 0.2 mm Hg.
[d]Distilled at 200°C and 0.2 mm Hg. Residue 2 is also called Ax40.
[e]Not detected, <0.05 wt%. See Table 1 for abbreviations.

TABLE 3
Conversion of Ax Esters to Free Ax by Different Lipases[a]

Lipase	Ax composition (mol%)		
	Free	Mono	Di
None	4.9	80.3	14.8
Candida rugosa	9.3	84.1	6.6
Rhizopus oryzae	5.1	88.9	6.0
Thermomyces lanuginosa	7.7	85.8	6.5
Aspergillus niger	19.8	73.1	7.1
Alcaligenes sp.	15.3	78.6	6.1
Burkholderia cepacia	12.7	83.0	4.3
Pseudomonas sp.	19.9	74.9	5.2
P. aeruginosa	65.4	33.1	1.5
P. stutzeri	48.7	49.0	2.3

[a]Ax obtained by *C. rugosa* lipase-catalyzed treatment of *H. pluvialis* cell extracts (Ax40; residue 2 in Table 2) was used as a substrate. A mixture of 1.88 g Ax40, 0.12 g EtOH (2 molar equiv against the total FA in Ax40), and 2.0 mL water was stirred at 30°C for 68 h with 500 U/g mixture of several lipases. Ax composition was expressed as a molar percentage of the total Ax. See Tables 1 and 2 for abbreviations.

range of 20 to 50°C. The results showed that the highest conversion was obtained at 30°C (data not shown). The reaction was also conducted at 30°C in a mixture of Ax40, 5 mol of EtOH, 50 wt% water, and various amounts of the lipase. The

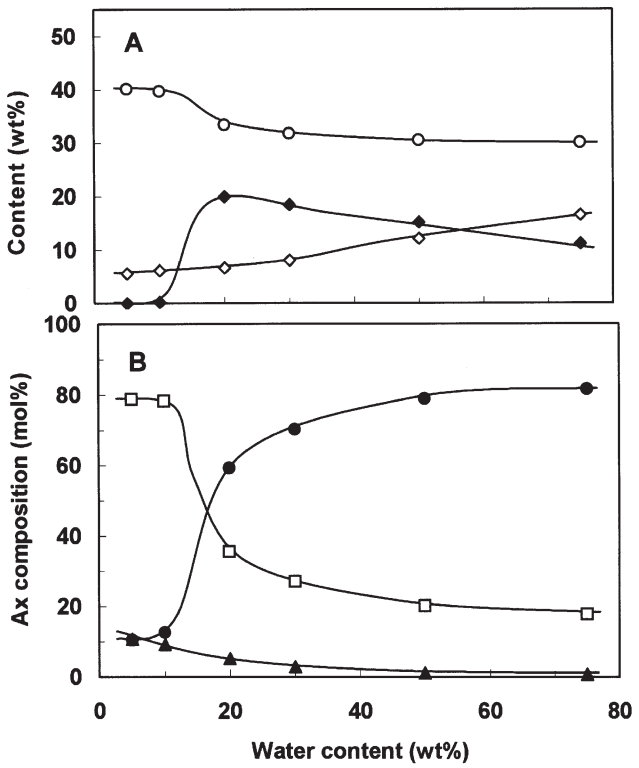

FIG. 3. Effect of water content on conversion of Ax esters to free Ax with *Pseudomonas aeruginosa* lipase. A 4.0-g mixture composed of Ax40 (residue 2 in Table 2), 2 molar equiv of ethanol (EtOH) against the total FA in the Ax40, various amounts of water, and 4000 U *P. aeruginosa* lipase was stirred at 30°C for 68 h. (A) Contents of total Ax (○), fatty acid ethyl esters (FAEE) (◆), and FFA (◇) in the reaction mixture. (B) Composition of Ax: free Ax (●), Ax monoesters (□), Ax diesters (▲). See Figure 1 for abbreviation.

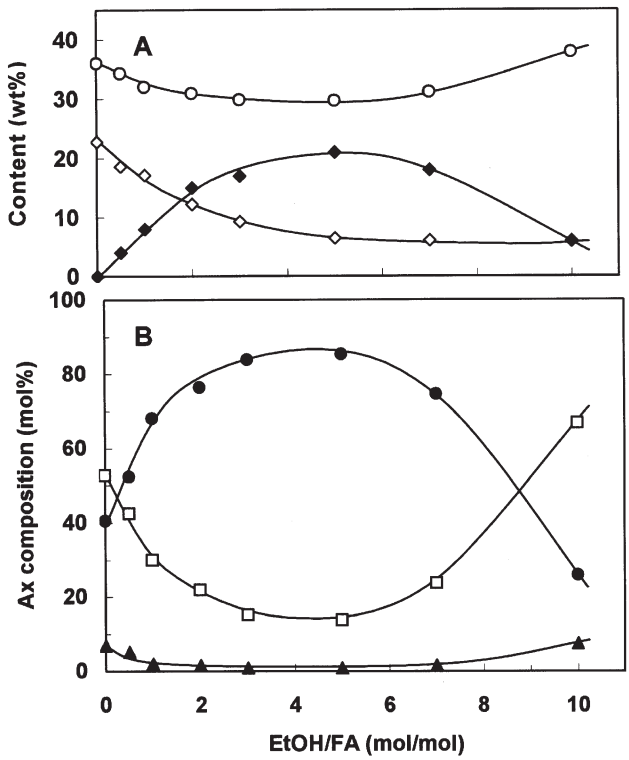

FIG. 4. Effect of EtOH content on conversion of Ax esters to free Ax with *P. aeruginosa* lipase. A 4.0-g mixture composed of Ax40, various amounts of EtOH, 2.0 g water, and 4000 U *P. aeruginosa* lipase was stirred at 30°C for 68 h. (A) Contents of total Ax (○), FAEE (◆), and FFA (◇) in the reaction mixture. (B) Composition of Ax: free Ax (●), Ax monoesters (□), Ax diesters (▲). The total EtOH added is expressed as molar amounts against FA in Ax40. See Figures 1 and 3 for abbreviations.

degree of conversion after 68 h reached 88.2% at a concentration of 1500 U/g-mixture of the lipase, and did not increase further when more lipase was used (data not shown).

Time course of conversion of Ax esters to free Ax. On the basis of the foregoing results, the reaction conditions were determined as follows: EtOH, 5 molar amounts against FA in Ax40; water content, 50 wt%; reaction temperature, 30°C; lipase, 1500 U/g-mixture. Figure 5 shows a typical time course. FA and FAEE were generated along with the reaction time, and the total Ax content (wt%) in the reaction mixture decreased (Fig. 5A). The decrease of Ax esters depended on the increase of free Ax, and the reaction proceeded rapidly during the first 10 h and gradually thereafter (Fig. 5B). The reaction reached nearly a steady state after 78 h, and the composition of free Ax, Ax monoesters, and Ax diesters was 87.7, 11.4, and 0.9 mol%, respectively (degree of conversion, 88.3%). The content of Ax before the reaction was 0.20 mmol/g-reaction mixture, and that after 78 h was 0.19 mmol/g. This result showed that Ax scarcely decomposed during the reaction. Total content of FFA and FAEE after the reaction was 0.46 mmol/g-reaction mixture, which was more than the total content of FA in FFA, acylglycerols, and Ax esters in Ax40 (0.34 mmol/g). This result

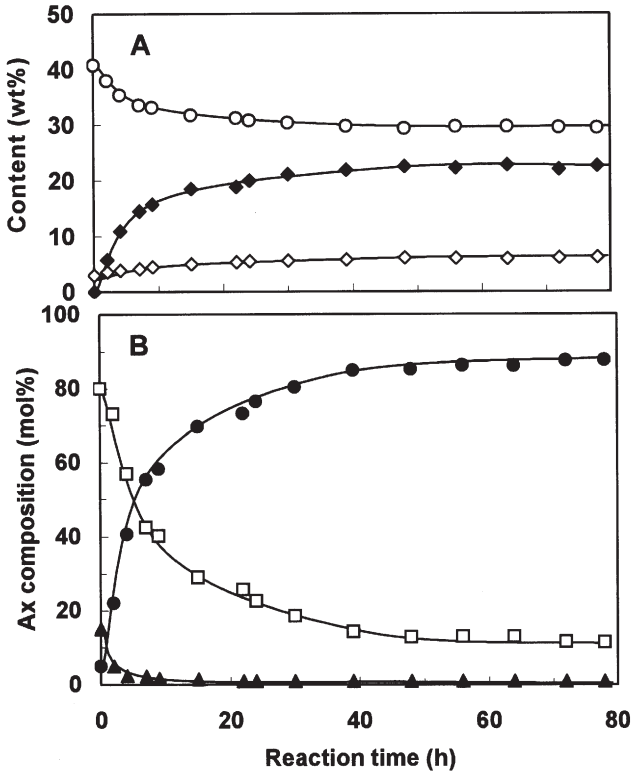

FIG. 5. Time course of conversion of Ax esters to free Ax using *P. aeruginosa* lipase. A mixture of 5.0 g Ax40, 0.8 g EtOH (5 molar equiv against FA in Ax40), 5.8 g water, and 1500 U/g of *P. aeruginosa* lipase was stirred at 30°C. (A) Contents of total Ax (○), FAEE (◆), and FFA (◇) in the reaction mixture. (B) Composition of Ax: free Ax (●), Ax monoesters (□), Ax diesters (▲). See Figures 1, 3, and 4 for abbreviations.

showed that unknown FA esters are also converted to their free forms during the reaction.

Conversion of Ax esters to free Ax is enhanced by addition of EtOH. This result is explained by to the fact that a high conversion of phytosteryl esters to free sterols was achieved by addition of methanol in the reaction with the same lipase as that used in this study (17). The high conversion of Ax esters to free Ax may be explained as follows. The lipase recognizes Ax esters, free Ax, and FFA; thus, hydrolysis of Ax esters with the lipase reached steady state at about 40%. Addition of EtOH decreases the content of FFA and increases the content of FAEE, indicating that the lipase poorly recognizes FAEE as a substrate. Because the FAEE are apparently excluded from the reaction system, the reaction shifts to accumulation of free Ax.

Purification of Ax from the reaction mixture. Purification of Ax from Ax40 is summarized in Table 4. A mixture of 50.0 g Ax40, 8.2 g EtOH (5 molar equiv), 58.2 mL water, and 1500 U/g-mixture of *P. aeruginosa* lipase was first stirred at 30°C for 68 h. After reaction, the oil layer was recovered (yield, 49.5 g). The content of free Ax, Ax monoesters, and Ax diesters was 89.3, 9.9, and 0.8 mol%, respectively.

To purify Ax from the oil layer, 250 mL *n*-hexane was added to the oil layer. The resulting precipitate was recovered by centrifugation at 20°C and dried overnight under reduced pressure. FFA and FAEE were completely removed in the supernatant fraction, and the Ax purity increased to 70.2 wt% in a yield of 14.2 g. The recovery of Ax based on the initial content in Ax40 was 69.1%, and the purified fraction contained 92.6 mol% free Ax, 6.9 mol% Ax monoesters, and 0.5 mol% Ax diesters. With the results obtained in Table 2, the recovery of Ax was calculated to be 63.9% of the initial content of *H. pluvialis* cell extracts.

Necessity of a two-step process for purification of Ax. Ax was purified from *H. pluvialis* cell extracts by a two-step process. The first step is hydrolysis of acylglycerols in the cell extracts with *C. rugosa* lipase and removal of FFA by molecular distillation. The second step is conversion of Ax esters to free Ax in a reaction system containing EtOH with *P. aeruginosa* lipase and recovery of free Ax in the precipitate by *n*-hexane fractionation. To simplify the purification process, we attempted to convert Ax esters in *H. pluvialis* cell extracts directly. The conversion, however, scarcely occurred. This result indicated that the excessive amount of FA in the cell extracts inhibited the conversion of Ax esters to free Ax. It was therefore concluded that removal of FA in the first step was necessary for efficient purification of Ax.

TABLE 4
Purification of Ax from Ax40 by a Process Comprising an Enzymatic Reaction and *n*-Hexane Fractionation

	Composition (g)						
		Ax					
Step	Total	Free	Mono	Di	FFA	FAEE	Acylglycerols
Lipase treatment[a]							
Before	50.0	0.7	15.8	3.9	1.5	0	2.1
After	49.5	12.7	2.2	0.2	3.2	10.1	ND[c]
n-Hexane fractionation[b]							
Supernatant	32.8	3.1	1.5	0.1	3.1	10.0	ND
Precipitation	14.2	9.1	0.7	0.1	ND	ND	ND

[a]A mixture of Ax40/EtOH (1:5, mol/mol), 50 wt% water, and 1500 U/g *P. aeruginosa* lipase was stirred at 30°C for 68 h.
[b]After separation of the reaction mixture to oil and water layers, *n*-hexane (250 mL) was added to the oil layer. The supernatant and precipitate were separated by centrifugation at 20°C. *n*-Hexane was removed under reduced pressure.
[c]ND, not detected, <0.05 g. See Tables 1 and 2 for abbreviations.

ACKNOWLEDGMENTS

We thank Yomi Watanabe of Osaka Municipal Technical Research Institute for her valuable discussion, and Masaki Hayakawa of Kinki University for his technical support.

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[Received March 10, 2003; accepted July 30, 2003]