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Enzymatic purification of dihomo-γ-linolenic acid from *Mortierella* single-cell oil

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Abstract

Purification of dihomo-γ-linolenic acid (20:3n−6; DGLA) from a single-cell oil containing 39 wt.% DGLA was attempted. The process comprised: (i) non-selective hydrolysis of the oil to prepare a mixture of free fatty acids (FFAs); (ii) urea adduct fractionation of the FFA mixture to remove saturated fatty acids; and (iii) repeated selective esterification of the resulting mixture with two kinds of lipases. In the first step, *Candida rugosa* lipase (Lipase-OF from Meito Sangyo Co. Ltd., Aichi, Japan) was the most effective for preparation of the FFAs from the oil; 99% hydrolysis was achieved by the reaction at 40 °C for 72 h. Urea adduct fractionation of the FFA mixture removed almost completely behenic and lignoceric acids, and the content of DGLA increased from 39 to 55 wt.%. The FFAs were esterified with 2 mol equivalent of lauryl alcohol (LauOH) using *C. rugosa* lipase (Lipase-AY from Amano Enzyme Inc., Aichi, Japan). In consequent, DGLA was enriched to 86 wt.% in the unesterified FFA fraction. To further increase the content of DGLA, the esterification was repeated using the same lipase. Accordingly, the content of DGLA increased to 91 wt.%, but the preparation was contaminated with 3.3 wt.% γ-linolenic acid. This contaminant was removed finally by selective esterification of the FFAs with 2 mol equivalent of LauOH using *Pseudomonas aeruginosa* lipase. A series of procedures purified DGLA to 95 wt.% in a yield of 51% of the initial content in the single-cell oil.
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Keywords: *Candida rugosa*; Dihomo-γ-linolenic acid; Esterification; Hydrolysis; Lipase; *Pseudomonas aeruginosa*

1. Introduction

Dihomo-γ-linolenic acid (20:3n−6; DGLA) is synthesized from γ-linolenic acid (18:3n−6; GLA), and is further converted to arachidonic acid (20:4n−6, AA) [1]. In addition, it is the immediate precursor of prostaglandin E_1, and inhibits the production of leukotriene B_4 [2,3]. This polyunsaturated fatty acid (PUFA) was therefore reported to exert anti-inflammatory [3], antiatherosclerotic [4], antihypertensive [5], and antiallergic actions [6]. But because the content of DGLA in natural oils and fats is only very low and an industrial production of DGLA is very difficult, the exact physiological activities have been unclear yet.

Recently, Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4 was reported to efficiently produce a single-cell oil containing DGLA [7], and an oil containing about 40% DGLA became also to be produced in an industrial scale [8]. However, high purity of DGLA is necessary to study its physiological activities. Furthermore, the single-cell oil of DGLA was contaminated with GLA which has the physiological activity of modulating immune and inflammatory responses [9]. In addition, there is a report that the activity of GLA may be induced by its conversion to DGLA in the body [10]. To eliminate this possibility, GLA-free DGLA preparation is strongly desired.

Lipase-catalyzed reactions were effective as one of the methods for purifying PUFAs [11]. Especially, docosahexaenoic acid (22:6n−3), GLA, AA, and conjugated linoleic acid (CLA) isomers (9*cis*,11*trans*- and 10*trans*,12*cis*-CLAs) were highly purified by processes including selective esterification with lauryl alcohol (LauOH) [12,13]. This paper deals with purification of

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DGLA from the single-cell oil from a Δ5-desaturase-defective mutant of *M. alpina* through an enzymatic process.

2. Materials and methods

2.1. Single-cell oil

An oil containing DGLA (referred to as TGD39 oil) was obtained from Suntory Ltd. (Osaka, Japan). The content of triacylglycerols in the oil was >99 wt.%. Main constituent fatty acids (FAs) were 17.9 wt.% palmitic acid (16:0), 8.2 wt.% stearic acid (18:0), 8.4 wt.% oleic acid (l8:1n−9), 8.2 wt.% linoleic acid (18:2n−6), 2.4 wt.% GLA, 38.9 wt.% DGLA, 3.1 wt.% behenic acid (22:0), and 8.2 wt.% lignoceric acid (24:0). Lauryl alcohol, urea, and 28% Na-methylate/methanol were purchased from Wako Pure Chemical Industry Co. (Osaka, Japan).

2.2. Lipases

The lipases were from the following companies: *Candida rugosa* lipase (Lipase-AY), *Rhizopus oryzae* lipase (Lipase-T), and *Burkholderia cepacia* lipase (Lipase-PS) were from Amano Enzyme Inc. (Aichi, Japan); *C. rugosa* lipase (Lipase-OF), *Alcaligenes* sp. lipase (Lipase-QLM), *B. cepacia* lipase (Lipase-SL), and *Pseudomonas stutzeri* lipase (Lipase-TL) were from Meito Sangyo Co. Ltd. (Aich, Japan); *Pseudomonas aeruginosa* lipase (Lipase-LPL) was from Toyobo Co. Ltd. (Osaka, Japan). One unit (U) of lipase activity was defined as the amount of lipase that liberated 1 μmol of FAs per minute in hydrolysis of olive oil (Wako Pure Chemical) as described previously [14].

2.3. Reactions

A small scale reaction was conducted in a 50 mL screw-capped vessel with stirring at 500 rpm. A large scale reaction was conducted in a 3 L or 200 mL three-necked round-bottomed flask with agitating at 200 rpm. All reactions were performed under a nitrogen atmosphere.

Non-selective hydrolysis of TGD39 oil was conducted at 40 °C in a mixture of oil/water (1:2, w/w) and 1200 U/g mixture of lipase. The degree of hydrolysis was calculated from the acid value of the reaction mixture and the saponification value (185 mg KOH/g) of TGD39 oil. Esterification of free fatty acids (FFAs) with LauOH was conducted under the following conditions: a mixture of FFAs/LauOH (1:2, mol/mol), 20 wt.% water, and required amounts of lipase was incubated at 30 °C with stirring at 500 rpm. The acid value was measured before and after the reaction by titrating with 0.5 M KOH, and the degree of esterification was calculated on the basis of the amount of FFAs consumed during the reaction.

2.4. Urea adduct fractionation of FFAs from TGD39

TGD39 oil was hydrolyzed non-selectively with *C. rugosa* lipase (OF) (degree of hydrolysis, 99%). The reaction mixture was centrifuged at 8000 × *g* for 10 min, and the oil layer was recovered. To remove saturated FAs, urea adduct fractionation was performed in a manner similar to that described previously [15]. In brief, the oil layer (200 g) was dissolved at 60 °C in a solution of 1 L methanol, 27 mL water, and 200 g urea. The temperature was decreased gradually to 5 °C over about 10 h with stirring. After the resulting precipitates were removed by filtration, 1 L of 0.1 M HCl was added to the filtrate and FFAs were then extracted with 2 L *n*-hexane. Finally, the organic solvent was removed by evaporation.

2.5. Recovery of FFAs from reaction mixture by n-hexane extraction

After the lipase-catalyzed esterification of FFAs with LauOH, FFAs were extracted from the reaction mixture with *n*-hexane in the aforementioned manner [16]. In brief, 0.5 M KOH/20% ethanol was added to the reaction mixture, and FA lauryl esters (FALEs) and LauOH were removed with *n*-hexane. FFAs in the water layer were then extracted with *n*-hexane after returning to an acidic pH (<pH 2) with HCl.

2.6. Analyses

FAs in TGD39 oil were methylated at 75 °C in 3 mL methanol containing 1% Na-methylate for 15 min. FFAs were methylated in 3 mL of 5% HCl/methanol at 75 °C for 10 min. The resulting FA methyl esters were analyzed with an Agilent Technologies 6890N gas chromatograph (Palo Alto, CA, USA) connected to a DB-23 capillary column (0.25 mm × 30 m; Agilent Technologies) as described previously [16]. The initial column temperature was 150 °C, which was increased by 4 °C/min to 170 °C, 5 °C/min to 195 °C, and 10 °C/min to 215 °C, followed by a hold at that temperature for 11 min. The injector and detector temperatures were set at 245 and 250 °C, respectively.

3. Results and discussion

3.1. Non-selective hydrolysis of TGD39

A mixture of TGD39 oil/water (1:2, w/w) was stirred at 40 °C with 1200 U/g mixture of several kinds of lipases (Table 1). *C. rugosa* lipase (AY) and *R. oryzae* lipase achieved low degree of hydrolysis even after 72 h, but the other lipases tested achieved >91% hydrolysis. In particular, *C. rugosa* lipase (OF) was the most effective for non-selective hydrolysis of TGD39 oil; 99% hydrolysis at 72 h.

3.2. Preparation of FFAs from TGD39 oil

A mixture of 500 g TGD39 oil and 1.5 L water was agitated at 40 °C for 72 h with 1200 U/g of *C. rugosa* lipase (OF) (degree of hydrolysis, 99%), and the oil layer was then recovered by centrifugation; it was named FFA-Hyd. FFA-Hyd was in a solid state at room temperature because of the high content of saturated FAs, such as behenic acid (3.1 wt.%) and lignoceric acid (8.3 wt.%). In general, lipase reactions proceed efficiently on liquid-state substrates, but not on solid-state ones. Hence,

Table 1
Hydrolysis of TGD39 with lipases[a]

Lipase	Degree of hydrolysis (%)		
	24 h	48 h	72 h
C. rugosa[b]	93.7	95.1	98.8
C. rugosa[c]	54.9	68.3	77.2
R. oryzae	41.3	53.5	66.1
Alcaligenes sp.	93.6	96.2	96.7
B. cepacia[d]	89.4	91.4	91.5
B. cepacia[e]	96.5	96.5	96.4
P. stutzeri	73.4	89.7	95.0
P. aeruginosa	89.0	94.7	94.6

[a] A mixture of 2 g TGD39 oil, 4 g water, and 7200 U lipase was stirred at 500 rpm under 40 °C.
[b] Lipase-OF from Meito Sangyo.
[c] Lipase-AY from Amano Enzyme.
[d] Lipase-PS from Amano Enzyme.
[e] Lipase-SL from Meito Sangyo.

urea adduct fractionation of FFA-Hyd was conducted according to Materials and methods section. The fractionation removed almost all behenic and lignoceric acids and a part of palmitic and stearic acids. The resulting FFA mixture was in a liquid state at room temperature; it was named FFA-urea. The composition of main FAs in FFA-urea were 12.0 wt.% palmitic acid, 2.0 wt.% stearic acid, 10.8 wt.% oleic acid, 11.8 wt.% linoleic acid, 3.4 wt.% GLA, and 53.9 wt.% DGLA.

3.3. FA specificity of several lipases in selective esterification

Selective esterification with LauOH was reported to be the most effective for enrichment of desired FAs in a FFA mixture [17,18]. Hence, FFA-urea was esterified under similar conditions as reported previously: FFAs/LauOH, 1:2 (mol/mol); water content, 20 wt.%; reaction temperature, 30 °C.

In order to study the activities of several lipases on FAs in FFA-urea, FFA-urea was esterified with 2 mol equivalent of LauOH in the presence of 20 wt.% water (Table 2). The degree of esterification of total FAs was controlled at about 30% by the amount of lipase and reaction time. The comparison of the degree of esterification of each FA with that of total FAs showed that *C. rugosa* lipases (OF and AY), *R. oryzae* lipase and *P. aeruginosa* lipase are effective for enrichment of DGLA, because they act on DGLA weakly and on the other FAs (palmitic, oleic, and linoleic acids) strongly. Meanwhile, *Alcaligenes*, *B. cepacia*, and *P. stutzeri* lipases acted on DGLA moderately; thus, they are not effective for enrichment of DGLA. In addition, *P. aeruginosa* lipase acted on GLA strongly and on DGLA weakly. This lipase, therefore, may be effective for removal of GLA which is assumed to become a major contaminant in the purified DGLA preparation.

3.4. Selective esterification of FFA-urea

Preliminary experiment of selective esterification of FFA-urea with eight lipases listed in Table 2 showed that *C. rugosa* lipases (OF and AY), *R. oryzae* lipase, and *P. aeruginosa* lipase increased the content of DGLA in the FFA fraction to >80 wt.%, but that the other lipases did not increase the content to >65 wt.%. Hence, FFA-urea was esterified with 2 mol equivalent of LauOH using fixed amounts of the four lipases for different hours. After the reaction, the FFA fraction was recovered by *n*-hexane extraction, and its FA composition was analyzed. Fig. 1 shows the relationship between the degree of esterification of total FAs (referred to as total esterification) and the contents of DGLA and GLA in the FFA fraction.

Table 2
Degree of esterification of FFA-urea with LauOH using lipases[a]

Lipase	Degree of esterification (%)					
	Total[b]	16:0[c]	18:1[c]	18:2[c]	18:3 (GLA)[c]	20:3 (DGLA)[c]
C. rugosa[d]	28.5	44.5	68.8	86.8	10.3	5.7
C. rugosa[e]	26.9	48.3	67.0	82.7	5.5	2.8
R. oryzae[f]	27.6	51.4	60.1	57.1	8.3	8.1
Alcaligenes sp.[g]	29.6	54.4	20.6	40.8	55.1	22.3
B. cepacia[h]	29.3	59.2	19.2	40.0	57.2	21.2
B. cepacia[i]	27.5	46.7	16.4	31.2	49.7	15.9
P. stutzeri[j]	32.1	59.8	37.2	43.0	66.9	25.3
P. aeruginosa[k]	29.0	66.4	40.5	48.7	71.5	9.2

[a] FFA-urea contained 13.6 mol% palmitic acid, 11.1 mol% oleic acid, 12.2 mol% linoleic acid, 3.6 mol% GLA, and 51.2 mol% DGLA. A mixture of 2 g FFA-urea/LauOH (1:2 mol/mol), 0.5 g water, and 25–600 U/g of lipase was stirred at 30 °C for 1–12 h.
[b] The degree of esterification of total FAs was calculated from the acid values of the reaction mixture before and after the reaction.
[c] Molar ratio of the amount of individual FA in the FALE fraction to that of the FA in FFA-urea.
[d] Lipase-OF. Reaction conditions: lipase amount, 400 U/g; reaction time, 4 h.
[e] Lipase-AY. Reaction conditions: lipase amount, 400 U/g; reaction time, 3.5 h.
[f] Reaction conditions: lipase amount, 600 U/g; reaction time, 4 h.
[g] Reaction conditions: lipase amount, 100 U/g; reaction time, 1 h.
[h] Lipase-PS. Reaction conditions: lipase amount, 50 U/g; reaction time, 1 h.
[i] Lipase-SL. Reaction conditions: lipase amount, 25 U/g; reaction time, 1 h.
[j] Reaction conditions: lipase amount, 200 U/g; reaction time, 12 h.
[k] Reaction conditions: lipase amount, 100 U/g; reaction time, 2 h.

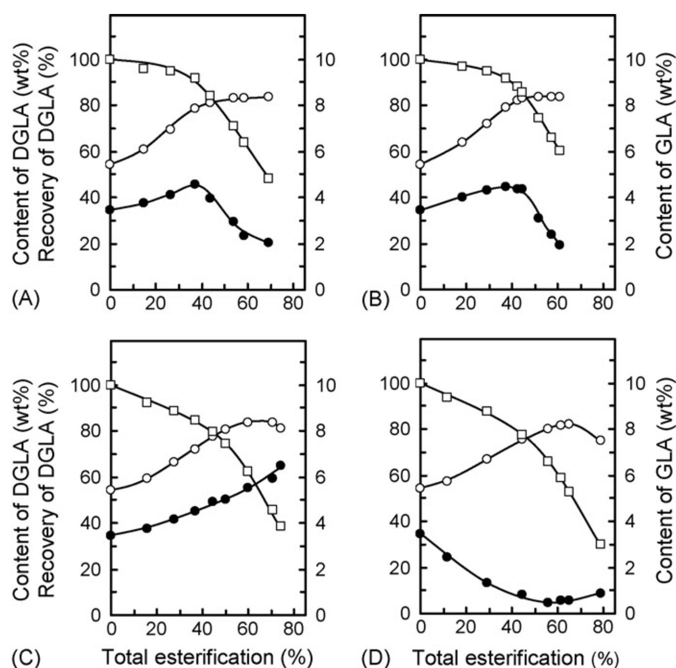

Fig. 1. Enrichment of DGLA by selective esterification of FFA-urea with LauOH using four lipases. A 2.5 g mixture of FFA-urea/LauOH (1:2, mol/mol), 20 wt.% water, and fixed amounts of lipase was stirred at 30 °C. (A) Esterification using 400 U/g *C. rugosa* lipase (OF). The reaction was conducted for 0, 2, 4, 16, 36, 72, 96, and 144 h. (B) Esterification using 400 U/g *C. rugosa* lipase (AY). The reaction was conducted for 0, 2, 4, 20, 24, 48, 72, 96, and 144 h. (C) Esterification using 600 U/g *R. oryzae* lipase. The reaction was conducted for 0, 2, 4, 7, 10, 16, 24, 48, and 72 h. (D) Esterification using 50 U/g *P. aeruginosa* lipase. The reaction was conducted for 0, 2, 4, 8, 13, 15, 17, and 24 h. Open circles, content of DGLA in the FFAs fraction; closed circles, content of GLA in the FFAs fraction; open squares, recovery of DGLA based on the content in FFA-urea.

Two *C. rugosa* lipases showed a similar tendency (Fig. 1A and B). The content of DGLA in the FFA fraction increased depending on increase of total esterification, and reached 83 wt.% when total esterification was 52–54%. The content of GLA in the FFA fraction increased with progress of the esterification, and decreased after total esterification reached about 40%. *R. oryzae* lipase increased not only the content of DGLA, but also that of GLA with increasing total esterification. *P. aeruginosa* lipase increased the content of DGLA and decreased the content of GLA. When the content of DGLA reached 80 wt.%, *C. rugosa* lipases achieved the higher recovery (about 90%) of DGLA in the FFA fraction than *R. oryzae* lipase (about 75%) and *P. aeruginosa* lipase (about 65%). Based on these results, *C. rugosa* lipase (AY) was selected for the first esterification. After the first esterification was conducted with the lipase (total esterification, 42%), FFAs were recovered by *n*-hexane extraction and used as a material for the second esterification. The mixture of FFAs was named FFA-Est1. The composition of main FAs in FFA-Est1 were 2.1 wt.% palmitic acid, 1.7 wt.% oleic acid, 1.6 wt.% linoleic acid, 4.3 wt.% GLA, and 82.5 wt.% DGLA.

3.5. Selective esterification of FFA-Est1

FFA-Est1 was esterified with 2 mol equivalent of LauOH for different hours using fixed amounts of *C. rugosa* lipase (AY) and *P. aeruginosa* lipase, which achieved the highest recovery of DGLA and removed GLA most efficiently, respectively. The contents of DGLA and GLA in the FFA fraction and the recovery of DGLA were plotted against total esterification (Fig. 2).

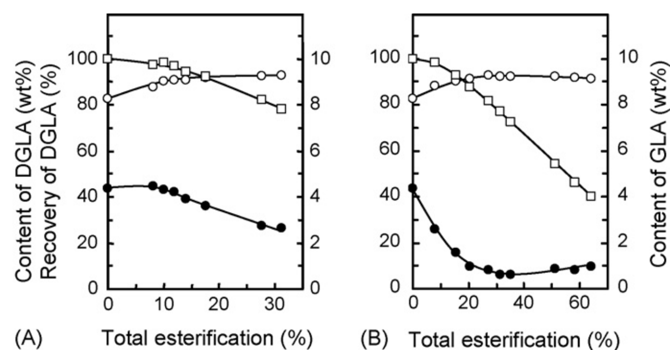

Fig. 2. Enrichment of DGLA by selective esterification of FFA-Est1 with LauOH using *C. rugosa* lipase (AY) and *P. aeruginosa* lipase. A 2.5-g mixture of FFA-urea/LauOH (1:2, mol/mol), 20 wt.% water, and fixed amounts of lipase was stirred at 30 °C. (A) Esterification using 400 U/g *C. rugosa* lipase (AY). The reaction was conducted for 0, 4, 8, 12, 16, 24, 51, and 72 h. (B) Esterification using 200 U/g *P. aeruginosa* lipase. The reaction was conducted for 0, 1, 2, 4, 6, 8, 9, 12, 16, 24 h. Open circles, content of DGLA in the FFAs fraction; closed circles, content of GLA in the FFAs fraction; open squares, recovery of DGLA based on the content in FFA-Est1.

The esterification with *C. rugosa* lipase increased the content of DGLA in the FFA fraction gradually and decreased the content of GLA after total esterification exceeded 10%. When total esterification was 17%, the contents of DGLA and GLA were 91 and 3.6 wt.%, respectively (recovery of DGLA, 92%). Meanwhile, the reaction with *P. aeruginosa* lipase increased the content of DGLA and decreased the content of GLA efficiently. The contents of DGLA and GLA at 21% total esterification were 91 and 0.9 wt.%, respectively (recovery of DGLA, 87%). The content of DGLA did not rise to >92 wt.% with each enzyme. The recovery of DGLA in the reaction with *C. rugosa* lipase was slightly better than that with *P. aeruginosa* lipase. Hence, FFA-Est1 was esterified with *C. rugosa* lipase (AY) (total esterification, 19%), and FFAs were then recovered by *n*-hexane extraction. The FFA mixture was named FFA-Est2, and used as a material for the third esterification. The compositions of main FAs in FFA-Est2 were 91.0 wt.% DGLA and 3.8 wt.% GLA.

3.6. Selective esterification of FFA-Est2

To remove GLA in FFA-Est2, *P. aeruginosa* lipase was assumed to be effective. FFA-Est2 was therefore esterified with 2 mol equivalent of LauOH for different hours using the lipase. The contents of DGLA and GLA in the FFA fraction and the recovery of DGLA were plotted against total esterification (Fig. 3). The content of DGLA increased from 91 to 95 wt.% with a 79% recovery when total esterification reached 24%, and the content of GLA decreased from 3.8 to 0.6 wt.%.

3.7. Purification of DGLA by a series of procedures

Purification of DGLA was performed using 50 g TGD39 oil as a starting material (Table 3). The oil was hydrolyzed

Table 3
Purification of DGLA from TGD39 oil

Step	Weight of FFAs (g)	FA composition (wt.%)[a]								Recovery of DGLA[b] (%)
		16:0	18:0	18:1	18:2	18:3	20:3	22:0	24:0	
TGD39 oil[c]	47.9[d]	17.9	8.2	8.4	8.2	2.4	38.9	3.1	8.2	100
Hydrolysis (FFA-Hyd)[e]	46.2	18.3	8.4	8.5	8.3	2.4	39.1	3.1	8.3	96.9
Urea adduct (FFA-urea)	31.2	11.9	1.7	11.3	11.7	3.4	55.4	ND[f]	ND	92.5
Esterification (FFA-Est1)[g]	17.9	1.8	1.1	1.5	1.5	4.4	84.4	ND	ND	80.8
Esterification (FFA-Est2)[h]	13.7	0.2	0.2	0.2	0.2	3.3	90.9	ND	ND	66.7
Esterification (FFA-Est3)[i]	10.1	ND	0.2	ND	ND	0.5	94.8	ND	ND	51.3

[a] FA composition in the FFA fraction.
[b] Recovery of DGLA based on the content in TGD39 oil.
[c] FA composition of TGD39 oil.
[d] The amount of FFAs contained in 50 g TGD39 oil.
[e] The degree of hydrolysis was 99.1%.
[f] Not detected (<0.2 wt.%).
[g] The degree of esterification was 40.9%.
[h] The degree of esterification was 16.3%.
[i] The degree of esterification was 22.4%.

at 40 °C for 72 h with *C. rugosa* lipase (OF). Because the oil was hydrolyzed almost completely (degree of hydrolysis, 99%), the oil layer was recovered by centrifugation and was used as a FFA mixture (FFA-Hyd). The recovery of DGLA in FFA-Hyd was 97%. Urea adduct fractionation of FFA-Hyd increased the content of DGLA from 39 to 55 wt.% with a 93% recovery based on the initial content of TGD39 oil. The resulting FFA mixture (FFA-urea) was then subjected to selective esterification with LauOH using *C. rugosa* lipase (AY), and unesterified FFAs (FFA-Est1) were recovered from the reaction mixture by *n*-hexane extraction. The content of DGLA was increased to 84 wt.%. To further increase the content of DGLA, FFA-Est1 was esterified using *C. rugosa* lipase (AY). The repeated esterification increased the content of DGLA in the FFA fraction (FFA-Est2) to 91 wt.%. Since this preparation was contaminated with 3.3 wt.% GLA, FFA-Est2 was esterified finally with *P. aeruginosa* lipase. A series of procedures purified DGLA to 95 wt.% with a 51% recovery based on the initial content of TGD39 oil. The content of GLA in this purified preparation was very low (0.5 wt.%).

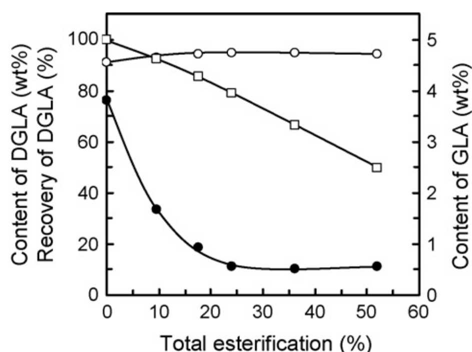

Fig. 3. Enrichment of DGLA by selective esterification of FFA-Est2 with *P. aeruginosa* lipase. A 2.5 g mixture of FFA-urea/LauOH (1:2, mol/mol), 20 wt.% water, and 400 U/g of the lipase was stirred at 30 °C for 0, 2, 5, 9, 16, and 21 h. Open circles, content of DGLA in the FFAs fraction; closed circles, content of GLA in the FFAs fraction; open squares, recovery of DGLA based on the content in FFA-Est2.

4. Conclusion

DGLA in TGD39 oil can be efficiently purified through a process comprising non-selective hydrolysis, urea adduct fraction, and repeated selective esterification. This process required only simple and conventional equipments; thus, could handle a large amount of material. Furthermore, since the purified preparation was contaminated with only small amounts of n−6 PUFAs other than DGLA, the preparation may be used for study of physiological activities of DGLA.

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