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

Toshihiro Nagao [a,*], Yomi Watanabe [a], Takashi Kobayashi [a], Motoo Sumida [b], Noriaki Kishimoto [c], Tokio Fujita [c], Yuji Shimada [a]

[a] *Osaka Municipal Technical Research Institute, 1-6-50 Morinomina, Joto-ku, Osaka 536-8553, Japan*
[b] *Institute for Health Care Science, Suntory Ltd., 5-2-5 Yamazaki, Shimamoto-cho, Mishima-gun, Osaka 618-0001, Japan*
[c] *Department of Agricultural Chemistry, Faculty of Agriculture, Kinki University, 3327-204, Nakamachi, Nara 631-8505, Japan*

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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

* Corresponding author. Tel.: +81 6 6963 8073; fax: +81 6 6963 8079.
E-mail address: nagao@omtri.city.osaka.jp (T. Nagao).

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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 (18:1n−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,

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