Hydrolysis of Fish Oil by Olive Oil-Induced Vibrio sp. Lipase

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Hydrolysis of Fish Oil by Olive Oil-Induced *Vibrio* sp. Lipase

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Keywords : Marine bacterium, Lipase, Fish oil, n-3 Polyunsaturated fatty acid.

Abstract

A potent marine bacterium of genus *Vibrio* for lipase production was isolated from the red tides and tentatively named VB-5.

The strain VB-5 showed the optimal production of lipase when cultivated at 20℃ for 48 hr in a medium composed of casitone, proteose peptone, yeast extract, natural seawater and olive oil. The lipase activity from the strain VB-5 was enhanced by adding 0.33% olive oil to the medium. The olive oil-induced lipase had an optimal pH of 10 for activity at 25℃. The lipase hydrolyzed all ester bonds of triacylglycerol regardless of their positions. The lipase from the strain VB-5 was capable of liberating icosapentaenoic and docosahexaenoic acids along with palmitic and oleic acids from fish oil.

Consumption of fish oil has been advocated for anti-atherogenic effects attributed to constituent n-3 polyunsaturated fatty acids (n-3 PUFA) such as icosapentaenoic (EPA) and docosahexaenoic (DHA) acids[1-3]. Methods used for the enrichment of n-3 PUFA concentration include urea adduct formation[4], separation on Y-Zeolite[5] and lipase reaction[6-11]. Since lipase reaction is advantageous with respect to stereochemical and positional specificities, and mild reaction condition, lipase is considered to be suitable for the production of n-3 PUFA-containing lipids which are labile by oxidation. Some available lipases from fungi and yeasts have been actually reported for this purpose[6-11]. The bacterial lipases, however, are limited to a few species of *Chromobacterium*[7,12-14], *Pseudomonas*[12] and *Staphylococcus*[12].

Marine fishes have been known to require n-3 PUFA and to contain n-3 PUFA at fairly high levels[15]. EPA-producing bacteria have recently isolated from the intestinal contents of marine fishes[16]. This situation strongly suggests the possibility that marine bacteria may produce some available lipases which have a strong specificity toward n-3 PUFA.

To develop a new lipase preparation, screening was carried out among 45 strains of marine bacteria which were isolated from red tides[17]. As a preliminary result, a strain of genus *Vibrio* which produced a lipase for the hydrolysis of n-3 PUFA-containing fish

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oil was isolated and tentatively named VB-5. The lipase activity from the strain VB-5 was significantly enhanced by adding olive oil to the medium. The olive oil-induced lipase from the strain VB-5 was capable of liberating EPA and DHA as free fatty acid (FFA) along with palmitic and oleic acids from fish oil.

The present paper deals with the cultivation conditions of the strain VB-5 and some properties of crude lipase preparation.

Materials and Methods

Cultivation conditions

A cultivation of genus *Vibrio*, tentatively named VB-5 whose characteristics were shown in Table 1, isolated from the red tides of Tanabe Bay in Wakayama Prefecture of Japan, was grown in a selected medium, CPY, containing 0.33% olive oil at 20℃ with rotary shaking (100 rpm) for 2 days. The CPY medium comprised 0.1% casitone, 0.05% proteose peptone, 0.1% yeast extract and 100mℓ of 75% natural seawater.

Table 1. Characteristics of VB-5

Shape	rod
Gram stain	−
Flagella	polar
Motility	+
Pigment	−
Oxidase	+
Catalase	++
OF test*	fermentative
Salt requirement	marine type
Hydrolysis of	
gelatin	+
DNA	+

* Oxidative or Fermentative test

The optimum concentration of olive oil for the lipase production was determined by cultivating the strain VB-5 in the presence of olive oil ranging 0.17 to 3.3%. For the determination of optimal temperature for the lipase production, the strain VB-5 was cultivated at a selected temperature in the range 10 to 30℃.

Lipase assay

The growth medium was filtered, and the cell-free broth was assayed for the lipase activity. The reaction mixture, composed of 50mg of triolein or triacylglycerol (TG) from fish oil, 5mℓ of McIlvaine's buffer (pH 10), 2.5mℓ of 0.05% sodium taurocholate

and 5mℓ of cell-free broth, was incubated at 25℃ for 20 hr with constant agitation (500 rpm) by a magnetic stirrer. Air in the reaction flask was replaced by argon gas. After incubation, 50mℓ of diethyl ether was added to the reaction mixture to stop the reaction and to extract the reaction products.

The effect of reaction time on the hydrolysis of TG was studied. The reaction system just described was set to pH 10 and the reaction was run at 25℃ for 1 to 20 hr. For the determination of optimal pH for the lipase activity, the reaction was carried out nine pHs in the range 4 to 12 using McIlvaine's buffer.

Quantitative estimation of reaction products

The hydrolysis products of TG were analyzed quantitatively by thin-layer chromatography (TLC). The TLC plates (Kieselgel 60, ready-made plate from Merck) were developed using *n*-hexane/ diethyl ether/ formic acid (75 : 25 : 2, by volume) and quantitated using a Cosmo F-808 densitometer. The spots of hydrolysis products were visualized by spraying a 3% copper acetate-8% phosphoric acid and heating at 150℃ for 10 min.

A portion of the hydrolysis products of TG was applied to preparative TLC. The TLC plate was sprayed with a Rhodamine 6G reagent, and TG, FFA, diacylglycerol (DG) and monoacylglycerol (MG) were detected under ultraviolet light. Each lipid class was eluted with diethyl ether and their fatty acid distributions were assayed by gas-liquid chromatography (GLC) after methylation with methanol containing anhydrous hydrogen chloride. GLC analysis was performed with a Hitachi 063 gas chromatograph equipped with a hydrogen flame ionization detector, using a glass column (3mm i. d. and 4m length) packed with Unisol 3000 on a Uniport C (80-100 mesh).

Protein assay

The protein content of cell-free broth was measured by the biuret method[18], using bovine serum albumin as a standard.

Results

pH and incubation time optima of lipase reaction

The effect of pH and incubation time on lipase reaction was studied at 25℃ using triolein as a substrate. Fig. 1 illustrates the results of the pH optimization experiment, in which the reaction was run for 20 hr. The crude lipase from the strain VB-5 had the maximum activity at pH 10. Fig. 2 shows the time course of hydrolysis of triolein by the lipase. At 20 hr of incubation time almost all of triolein was hydrolyzed to FFA (89%), DG (1.5%) and MG (9.2%) at pH 10. The lipase assay was run, consequently, at 25℃ for 20 hr at pH 10 unless otherwise noted.

The lipid composition obtained by the lipase-catalyzed hydrolysis of triolein is shown in Fig. 3. The hydrolysis percentage of triolein was proportional to the crude lipase solution. More than 80% of FFA was liberated from triolein by the crude lipase (5mg)

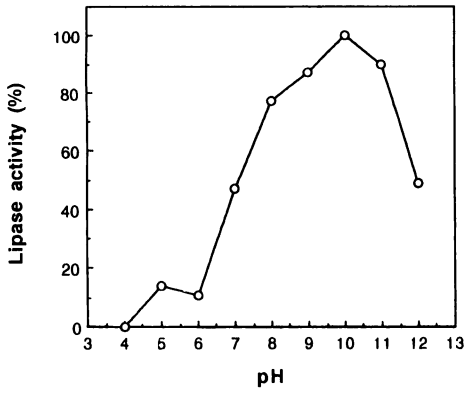

Fig. 1.
Effect of pH on the activity of VB-5 lipase.
The lipase reaction was carried out at 25℃ for 20 hr at various pH. The amount of VB-5 lipase was 5 mg as protein content. Lipase activity (%) was expressed as the decrement of triolein.

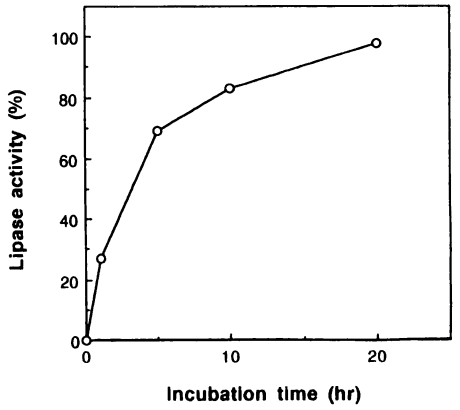

Fig. 2.
Time course of hydrolysis of triolein by VB-5 lipase.
The lipase reaction was carried out at 25℃ and pH 10. The amount of VB-5 lipase was 5 mg as protein content. Lipase activity (%) was expressed as the decrement of triolein.

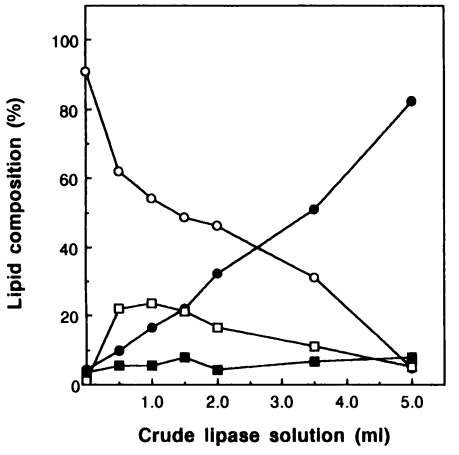

Fig. 3.
Changes in lipid composition of triolein hydrolyzed by VB-5 lipase.
The lipase reaction was carried out at 25℃ for 20 hr at pH 10 using various amount of crude lipase solution. The amount of VB-5 lipase was 5 mg as protein content per 5 mℓ of crude lipase solution.
○, TG; ●, FFA; □, DG; ■, MG.

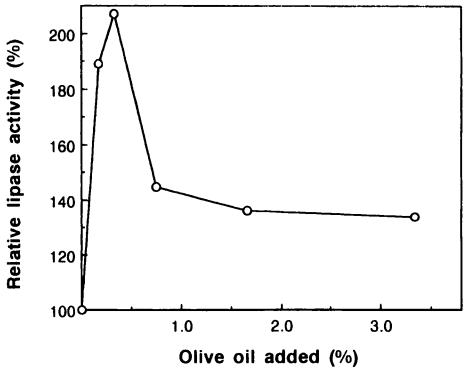

Fig. 4.
Effect of olive oil on the activity of lipase produced from the strain VB-5.
The strain VB-5 was cultivated at 20℃ for 48 hr in the CPY medium containing various amount of olive oil. The lipase reaction was carried out at 25℃ for 20 hr at pH 10. The amount of VB-5 lipase was 5 mg as protein content. Each lipase activity was expressed as percentage of that from VB-5 cultivated in the olive oil-free medium.

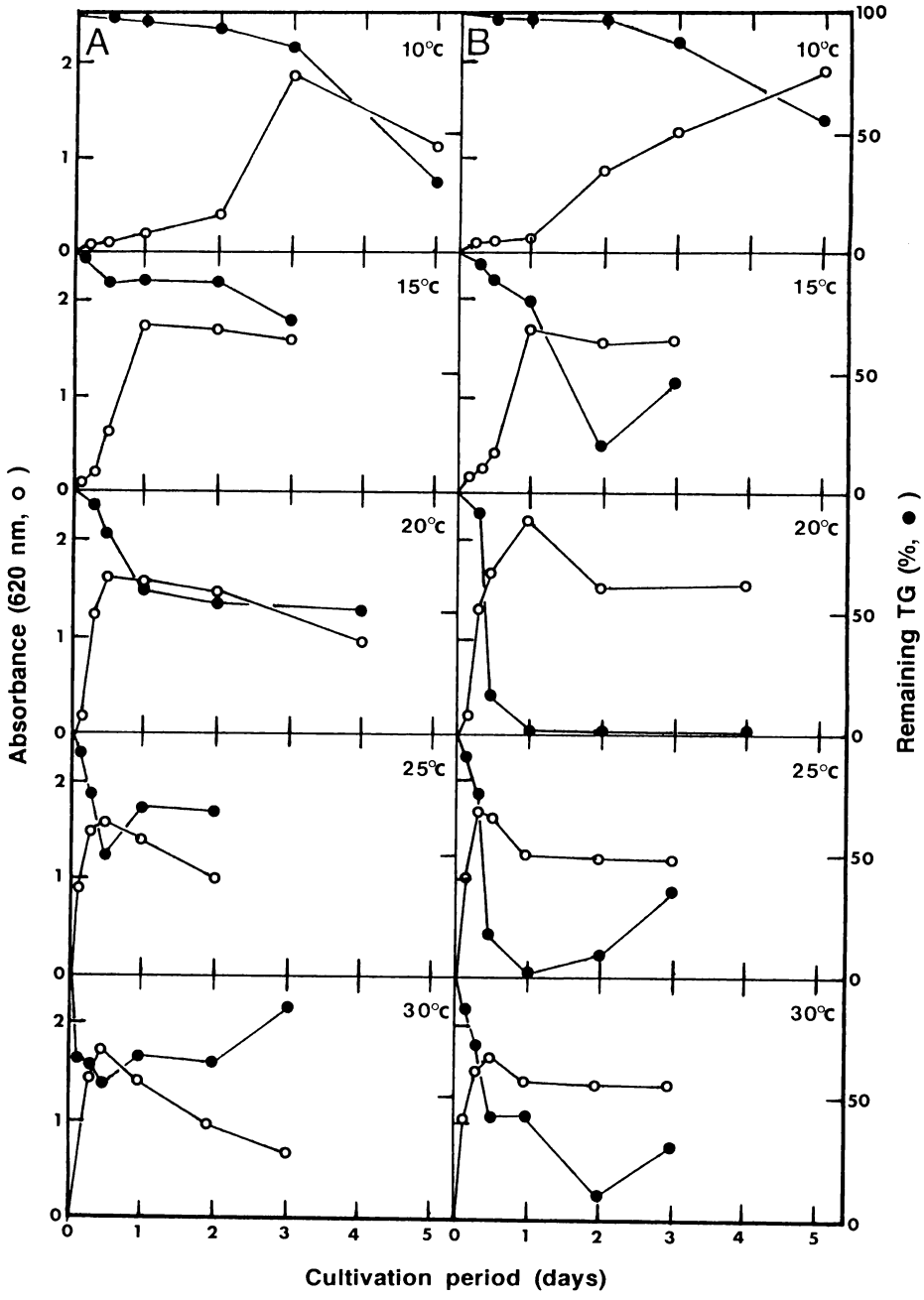

Fig. 5. Growth curve (○) and lipase activity (●) of the strain VB-5 cultivated at various temperatures in the olive oil-free (A) or olive oil added (0.33%, B) medium.

The lipase reaction was carried out at 25℃ for 20 hr at pH 10. The amount of VB-5 lipase was 5 ㎎ as protein content under the optimal condition of cultivation. Lipase activity (%) was expressed as the decrement of triolein.

from the strain VB-5.

Olive oil concentration and cultivation temperature optima for lipase production

The effect of olive oil on the activity of lipase produced from the strain VB-5 is shown in Fig. 4. The lipase activity of the strain VB-5 toward triolein was enhanced when cultivated in the presence of olive oil. The strain VB-5 was cultivated in CPY medium containing 0.33 % olive oil unless otherwise noted, since the highest lipase activity was found in the cell-free broth which was obtained from the strain VB-5 cultivated in the presence of 0.33% olive oil.

The growth and change of lipase activity of the strain VB-5 with period and temperature of cultivation are shown in Fig. 5. The activities of lipase from the strain VB-5 cultivated at various temperatures in the olive oil-free medium were undoubtedly low compared with those cultivated in the presence of olive oil, when assayed using triolein as a substrate. The strain VB-5 showed the highest lipase activity when cultivated in the CPY medium containing 0.33% olive oil at 20℃ for 48 hr, resulting a hydrolysis of triolein completely.

Hydrolysis of fish oil by cell-free broth of strain VB-5

The strain VB-5, which was cultivated at various temperatures for the optimal

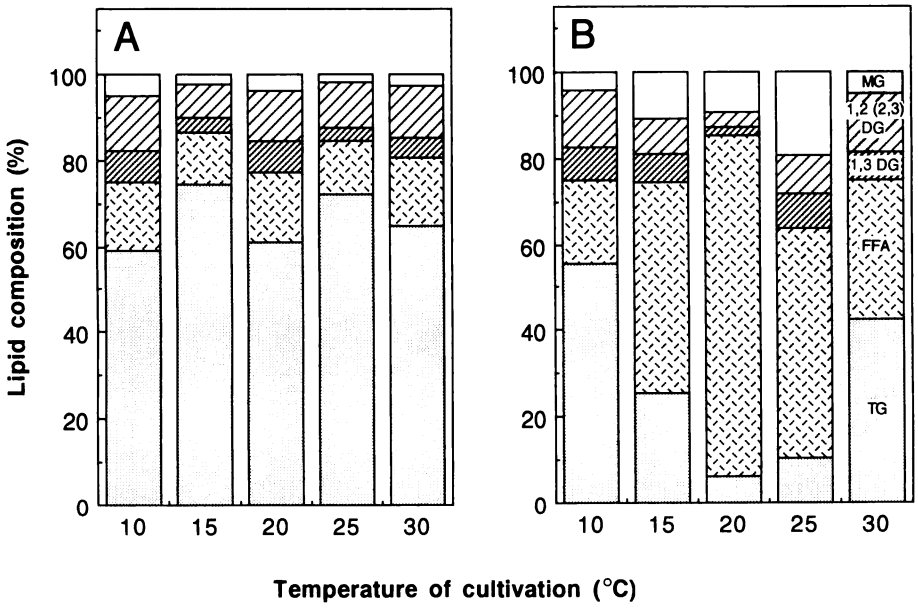

Fig. 6. Changes in lipid composition of n-3 PUFA-containing fish oil by VB-5 lipase.

The strain VB-5 was cultivated at various temperatures for optimal period (10℃ - 120 hr, 15℃ - 72 hr, 20℃ - 48 hr, 25℃ - 24 hr, 30℃ - 12 hr) in the olive oil-free (A) or olive oil added (0.33%, B) medium. The lipase reaction was carried out at 25℃ for 20 hr at pH 10. The amount of VB-5 lipase was 5 mg as protein content. TG, triacylglycerol; FFA, free fatty acid; 1, 3-DG, 1, 3-diacylglycerol; 1, 2 (2, 3)-DG, 1, 2 (2, 3)-diacylglycerol; MG, monoacylglycerol.

periods with or without olive oil, was further examined for the hydrolysis of fish oil. Fish oil, as well as triolein, was hydrolyzed by the cell-free broth from the strain VB-5 (Fig. 6). The highest lipase activity toward fish oil was also found in the cell-free broth, which was obtained from the strain VB-5 cultivated at 20℃ for 48 hr in the presence of 0.33% olive oil. With respect to hydrolyzates of fish oil by the action of lipase from the strain VB-5, both 1, 2 (2, 3)-DG and 1, 3-DG were detected.

The fatty acid distribution of each lipid class was calculated from the data on the compositions of lipid and fatty acid to examine whether the cell-free broth from the strain VB-5 was capable of hydrolyzing n-3 PUFA-containing fish oils. Saturated and monoenoic fatty acids were liberated easily from fish oil by the action of lipase from the strain VB-5, compared with n-3 PUFA (Fig. 7). At the optimal cultivation conditions for the lipase production, almost all of saturated and monoenoic fatty acids of fish oil were liberated as FFA, whereas more than 60% of n-3 PUFA was distributed as FFA.

Discussion

n-3 PUFA such as EPA and DHA are of considerable pharmaceutical interest due to their biomedical properties[1-3]. This leads to the attempts to produce n-3 PUFA-containing lipids by lipase reaction, because the lipase is expected to react mildly with n-3 PUFA which are labile by oxidation. Most of lipase used for this purpose are derived from fungi and yeasts which were separated from soil[6-11]. Taking into account the facts that marine fishes contain n-3 PUFA[15] and EPA-producing bacteria were isolated from the intestinal contents of marine fishes[16], the lipase from marine bacteria may have a high specificity toward n-3 PUFA. No report was, however, available for the lipases from marine bacteria.

In our preliminary screening[17], a strain of genus *Vibrio* which produced a lipase for the hydrolysis of n-3 PUFA-containing fish oil was isolated and tentatively named VB-5. The strain VB-5 was regarded as a typical marine bacterium which required seawater for growth (Table 1). The lipase from the strain VB-5 had some noticeable properties. The optimal pH of the lipase from the strain VB-5 was 10 (Fig. 1). This was distinct from the commercially available lipases whose optimal pH were roughly in the neutral range[7,9]. The lipase activity of the strain VB-5 was enhanced significantly when cultivated in the presence of olive oil, suggesting the lipase from the strain VB-5 was induced by olive oil (Fig. 4). The strain VB-5 showed the optimal production of lipase when cultivated in CPY medium containing 0.33% olive oil at 20℃ for 48 hr (Fig. 5). The olive oil-induced lipase was capable of hydrolyzing n-3 PUFA-containing fish oil as well as triolein. The percentage of TG was reduced to 6% by the olive oil-induced lipase, while those of FFA, DG and MG increased to 79, 6 and 9%, respectively (Fig. 6). With respect to hydrolyzates of fish oil by the action of olive oil-induced lipase, both 1, 2 (2, 3)-DG and 1, 3-DG were detected on the TLC plate. This result suggests that the lipase from the strain VB-5 hydrolyzes all ester bonds of TG regard-

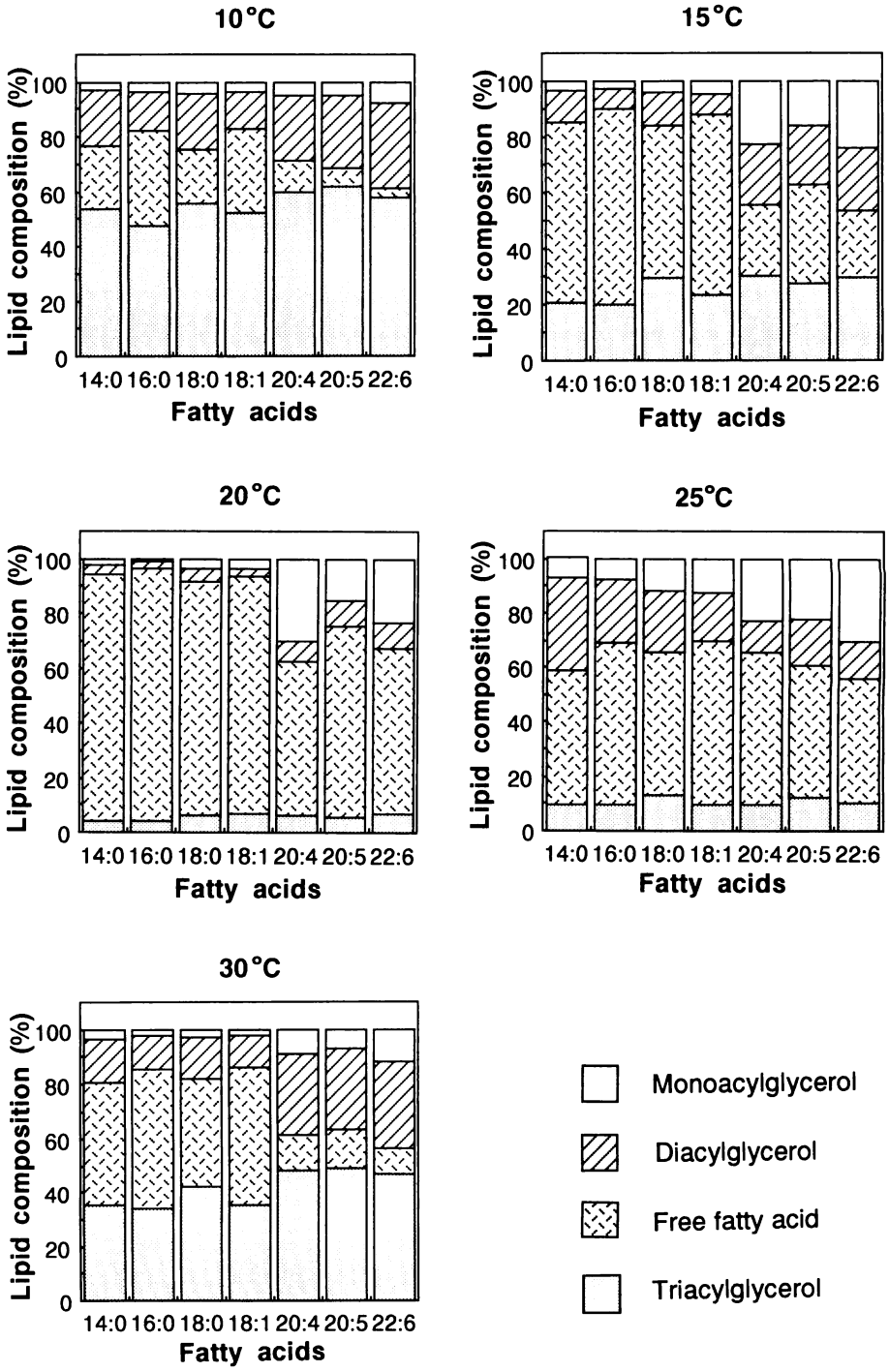

Fig. 7. Fatty acid distribution of each lipid class of n-3 PUFA-containing fish oil hydrolyzed by olive oil-induced lipase from the strain VB-5.
The cultivation condition of the strain VB-5 is referred to Fig. 6.

less of their positions. All fatty acids including saturated, monoenoic and n-3 PUFA were liberated from fish oil by the olive oil-induced lipase from the strain VB-5, suggesting that the lipase was not specific only for n-3 PUFA (Fig. 7). The activity of lipase from the strain VB-5 toward n-3 PUFA-containing fish oil was considered to be comparable to the commercially available lipases such as Lipase OF[7,9] and Lipase TOYO[7].

The lipase from the strain VB-5 thus was novel in respect of optimal pH and induction by olive oil. It is hoped that the olive oil-induced lipase from the strain VB-5 is capable of catalyzing the esterification reaction with n-3 PUFA, since the reversible reaction is known for lipase[19]. Further studies on the purification of the lipase from the strain VB-5 will be reported in the near future.

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