

LETTER

Hydrolysis and Synthesis of Icosapentaenoic Acid-Docosahexaenoic Acid Rich Oil by Lipase TOYO (*Chromobacterium viscosum*)

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The hydrolysis and synthesis of icosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) rich oil by Lipase TOYO (*Chromobacterium viscosum*) were conducted.

EPA and DHA were found to be released from the glycerol moiety at a constant rate, and were incorporated, by the reverse reaction, into the glycerol moiety using Lipase TOYO.

The optimum water content and enzyme amount for synthesis of triglyceride were 0.93% and 200 units respectively. Under these conditions, using pure EPA as well as DHA as the substrate, 91.3% of EPA and 94.9% of DHA were incorporated into the glycerol moiety.

It was pointed out by Lawson and Hughes¹⁾ that icosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are completely absorbed only as free fatty acids (FFA), moderately absorbed as triglyceride (TG) and poorly absorbed as ethyl esters. From this aspect, not only the composition of the fatty acid, but also the type of the lipid should be taken into consideration. FFA is usually unacceptable as food. Therefore, TG is considered to be the most desirable form.

This letter reports an application of Lipase TOYO (*Chromobacterium viscosum*) to the synthesis of TG rich in EPA as well as DHA. It is well known that the enzymatic reaction is usually reversible²⁾. As a preliminary study, hydrolysis of sardine oil was carried out prior to the glyceride synthetic reaction according to the method of Luddy *et al*³⁾. To the 50 mg of sardine oil (EPA 22.4%, DHA 6.7%), combined solution of 0.1 M phosphate buffer (pH 7.0, 10 mL) and bile salts (0.05%, 2.5 mL) were added. And it was preincubated for one minute at 37°C. Then, 10 mg of Lipase TOYO (Toyo Jyozo Co. Ltd., Tokyo) was added, and suspended by shaking the container vigorously at 37°C under nitrogen gas. The container used was a 50 mL volume glass stopper Erlenmeyer flask. It was incubated for 6 h at 37°C with a stroke speed of 10 m/min, 100 strokes/min. The hydrolytic reaction was stop-

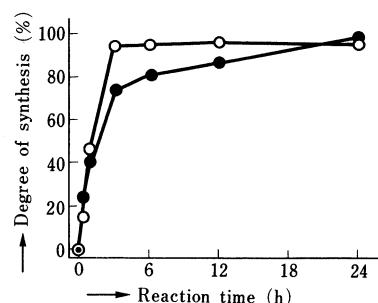
ped by adding 10 mL of ethanol followed by the addition of 10 mL 6N hydrochloric acid. Hydrolyzed lipids were extracted using diethyl ether and subjected to thin layer chromatography in order to analyse the lipid composition, and to recover the FFA fraction by scraping off the corresponding band. The fatty acid composition of FFA fraction was analysed by gas liquid chromatography subsequent to diazomethane methyl esterification. After hydrolysis of the sardine oil, the lipid composition changed from TG/DG/MG/FFA (100 : 0 : 0 : 0) to (10.4 : 10.4 : 3.4 : 75.6). EPA and DHA in the FFA fraction amounted to 23.0% and 3.0%, respectively. It has been widely accepted that the long-chain highly unsaturated fatty acids such as EPA and DHA are hard to be hydrolyzed. But it was borne out in this work that EPA and DHA are released from the glycerol moiety in a thorough rate by the use of Lipase TOYO. This alludes to a possibility in synthesizing TG from EPA and DHA rich FFA mixtures. The next step of this work was to verify the reaction *i.e.* synthesis of the EPA and DHA rich TG. Prior to this experiment, the optimum condition of TG synthesis by Lipase TOYO was determined using oleic acid as a substrate. The method of Tsujisaka *et al*⁴⁾, was introduced for this purpose. To the mixture of 4 mL glycerol and 0.3 mL oleic acid (70% purity, Wako Pure

Chemical Industries, Ltd., Osaka), Lipase TOYO solution was added (Water content and enzyme amount were varied from 2.21~9.79% and 20~400 unit, respectively). The container used was the same with the one used for hydrolysis, except that a glass stopper plug straight bore stopcock was used, and was degassed by a rotary vacuum pump for 2 min. During incubation, magnetic stirrer (1500 rpm) was used instead of the reciprocating shaker. It was stand for 1, 3, 6, 12, 24 h at 25°C. The reaction was stopped by the addition of 15 mL ethanol. And the rate of synthesis was determined by the decrease rate of the acid value⁴⁾. Under the optimum condition which was determined in this manner, the synthesis of EPA and DHA rich TG was carried out by using two substrate systems *i.e.* the pure EPA and DHA (99% purity, Idemitsu Petro Chemical Co. Ltd., Tokyo) system, and the FFA mixture obtained by the saponification⁵⁾ of sardine oil (EPA 14.2%, DHA 10.1%) system. After 24 h periodical reaction, the reaction was stopped in the same manner. And the lipid extraction, lipid composition analysis as well as fatty acid composition analysis were also done in the same manner as aforementioned.

The optimum water content and enzyme amount for synthesis of TG were 0.93% and 200 unit, respectively. Under this condition and by using pure EPA as well as DHA as substrate, 91.3% of EPA and 94.9% of DHA were incorporated into the glycerol moiety, though the lipid composition of the reaction product remained as TG/1,3-DG/1,2-DG/MG/FFA (26.1 : 19.0 : 18.7 : 15.5 : 20.7) for EPA, and (29.7 : 18.5 : 17.8 : 16.8 : 17.1) for DHA.

Fig.-1 shows the synthesis of TG from the FFA obtained by sardine oil saponification in comparison to the oleic acid as substrate. Though the reaction velocity is low in EPA and DHA rich substrate, it was demonstrated that the rate of synthesis is ultimately the same with oleic acid. After 24 h reaction, the synthetic rate of glycerides reached at a level of 97.7% (Oleic acid was 95.3%). And at this point, the lipid composition was TG/1,3-DG/1,2-DG/MG/FFA (44.9 : 13.9 : 11.4 : 14.3 : 15.1).

As shown in **Table-1**, synthesized TG contained 15.9% of EPA and 9.7% of DHA which are fairly comparable to the original sardine oil



Reaction was carried out by the batchwise method using Lipase TOYO (*Chromobacterium viscosum*).

○ : Oleic acid as substrate, ● : Free fatty acid mixture prepared from sardine oil as substrate.

Fig.-1 Time course of lipase reaction.

Table - 1 Rate of EPA and DHA in synthesized glycerides prepared from glycerol and free fatty acid mixture of sardine oil using Lipase TOYO (*Chromobacterium viscosum*)

Free fatty acid	Original sardine oil (%/total fatty acid)	After synthesis for 24 hours (%/total fatty acid)				
		TG	1,3-DG	1,2-DG	MG	FFA
EPA	14.17	15.85	15.94	15.97	8.65	5.17
DHA	10.14	9.72	10.57	8.88	6.42	9.50

TG.

The present work indicates that if highly concentrated EPA, DHA and ordinary fish oil are subjected to the Lipase TOYO acidolysis, EPA and DHA rich TG might be easily synthesized.

This will be demonstrated in the next paper.

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リパーゼ TOYO (*Chromobacterium viscosum*)
によるイコサペンタエン酸とドコサヘキサエ
ン酸含有油脂の加水分解と合成

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イコサペンタエン酸 (EPA), ドコサヘキサエニ酸
(DHA) を多く含む油脂の加水分解, 合成について検

討した。

Lipase TOYO (*Chromobacterium viscosum*) により,
いずれの反応も比較的容易に進行し, 本酵素によりこれ
らの脂肪酸を含む油脂の改質が可能であることが明らか
となった。

あらかじめオレイン酸とグリセリンによりトリグリセリド (TG) を合成するための至適条件を求めたところ,
反応系中の水分量が 0.93%, 酵素量が 200 Unit のとき
が最良であると判断され, また, 同条件下での EPA,
DHA とグリセリンからの TG の合成率は, EPA で
91.3%, DHA では 94.9% に達した。