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The Distribution and Position of Fatty Acids in Glycerides Hydrolyzed from Fish Oil by Lipase

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Abstract In order to determine the position and the content of fatty acids attached to glycerides and the migration degree of fatty acids in the migration reaction, fish oil was hydrolyzed with lipolase-100T which was derived from *Aspergillus oryzae*. The content of fatty acids in the glyceride mixture was analyzed and compared with that of fish oil. The amounts of fatty acid in a 2-position and the migration degree of the fatty acid in 2,3-DG (diglyceride) and 2-MG (monoglyceride) were carefully calculated. The results showed that approximately 95% (w/w) of DHA (docosahexaenoic acid) and 65% of EPA (eicosapentaenoic acid) were attached to the 2-position of glycerides in fish oil. Approximately 87% (w/w) of DHA and 75% of EPA remained in 2,3-DG, and 88% of DHA and 65% of EPA in 2-MG were not involved in the migration reaction.

Key words: Fatty acid, DHA, EPA, hydrolysis, migration, 1,3-positional specificity

The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been known to play important roles in the prevention or treatment of human diseases and disorders [1, 13]. The omega-3 fatty acids, however, must be obtained from marine products because they can not be synthesized *in vivo*. Therefore, an active research on the production of DHA has been conducted for a long time. The fish oil contains the highest concentration of DHA among various marine products. Therefore, it has been known as the most important source for DHA.

Since the omega-3 family of polyunsaturated fatty acid (PUFA) is very unstable on heat and oxygen, oxidation, *cis-trans* isomerization, and transposition of double bonds occur easily, and consequently they decompose to the *cis* n-3 structure of PUFA [3]. Thus, traditional chemical processes are not suitable for the concentration of DHA. Enzymatic processing of PUFA-containing oil have drawn much attention, since they proceed efficiently at ambient temperature and pressure [6]. It has been reported that glycerides containing EPA and DHA residues are more readily absorbed into human blood stream than the ethyl ester form [8]. When n-3 fatty acid are present as fatty acid residues in glycerides, their oxidative stability is greatly enhanced compared to the free fatty acid form [11]. Therefore, it is not only important to develop methods for the purification and concentration of PUFA in the glyceride form, but also to know the position of the attachment of each fatty acid and the amount of fatty acid at each position in the hydrolysis process.

There has been no report to quantitatively determine the amount of free fatty acid bonded to 2-position carbon of glycerides in fish oil and the migration degree of each kind of fatty acid in 2,3(1)-diglyceride and 2-monoglyceride. In our previous report, we determined the amount of free fatty acid produced during hydrolysis from triglyceride (TG), diglyceride (DG), or monoglyceride (MG) [5]. In this study, lipase derived from *Aspergillus oryzae* which is a 1,3-positional specific lipase, was used to hydrolyze fish oil. The percentage of weight of various fatty acids at the 2-glyceryl position of glycerides in fish oil and the migrated fraction of each type of fatty acid in 2,3(1)-DG and 2-MG were determined.

MATERIALS AND METHODS

Materials

Lipolase-100T from *Aspergillus oryzae* was purchased from NOVO Nordisk Corporation. Fish oil refined by LIPRO AS Corporation of Norway was used. The DHA content of fish oil was 8.6% (w/w) of the total fatty acid content.

Hydrolysis, Deacidification, and Esterification

The volumetric ratio in the reaction mixture of fish oil and sodium phosphate (pH 7.0, 0.05 M) was 4:1 and lipase was

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added at 4% [(w/v) oil]. The mixture was agitated at 200 rpm for 72 h at 30°C. Then, the tubes were immediately placed into boiling water to stop the enzyme activity. Deacidification was carried out by the method recommended by the Korea Standard Association [7]. After adding 3 ml each of acetone and hexane to 0.6 ml of the sample, the solution was titrated with 100 ml of 0.2 N aqueous NaOH solution to remove any trace of free fatty acid (FFA). The lower layer was removed while the upper layer was washed several times with distilled water. The glyceride mixture of triglyceride (TG), diglyceride (DG), and monoglyceride (MG) was obtained by evaporating the solvent in the upper layer, and esterified with acetyl chloride-methanol solution according to the method described by Lepage and Roy [9].

Analysis

The esterified mixture was analyzed by using a Hewlett-Packard 6890 gas chromatography equipped with a flame ionization detector (FID) and HP 19091J-413 capillary column. The column and oven temperature was raised from 150°C to 265°C at 7°C/min. The detector temperature was 300°C. Fatty acids were identified by comparing retention times with standards.

To determine the composition of TG, DG, MG, and FFA after the hydrolysis, the reaction mixture was analyzed by TLC-FID. One-hundred microliters of sample taken from the upper layer of the reaction products was dissolved in 100 ml chloroform. One microliter of the solution was spotted on a thin layer chromatograph [CHROMROD-SIII] and developed with a solvent that consisted of benzene, chloroform, and acetone (70:30:2, vol/vol/vol). Hydrogen gas velocity and air velocity were 160 ml/min and 20 l/min, respectively, and scan speed was 30 sec. The results were treated with IATROCODER TC-21 [IATRON, Japan].

Stoichiometric Analysis

Macrae [10] reported that triglycerides were hydrolyzed to 1,2(2,3)-diglycerides in the first step and the diglycerides to 2-monoglycerides in the second step with 1,3-specific lipase. In addition, 1,2(2,3)-diglycerides and 2-monoglycerides underwent acyl migration to produce 1,3-diglycerides and 1(3)-monoglycerides, respectively, by a slow chemical isomerization reaction. Based on the above results, the reaction mechanism of the hydrolysis of fish oil with 1,3-specific lipases could be proposed as shown in Fig. 1.

The stoichiometric equations for each variable in Fig. 1 were obtained by using the same method described in the previous paper [5]. In order to determine the content of each fatty acid in 2-position in the original fish oil, the following assumptions are made:

The total weight of fish oil is W, where x of TG is hydrolyzed to give y of 2,3-DG. Each species of fatty acid (in this paper, DHA is used as an example) in the *i*-position of TG and 2.3-DG is $(DHA_i)_0$ and $(DHA_i)_1$, respectively.

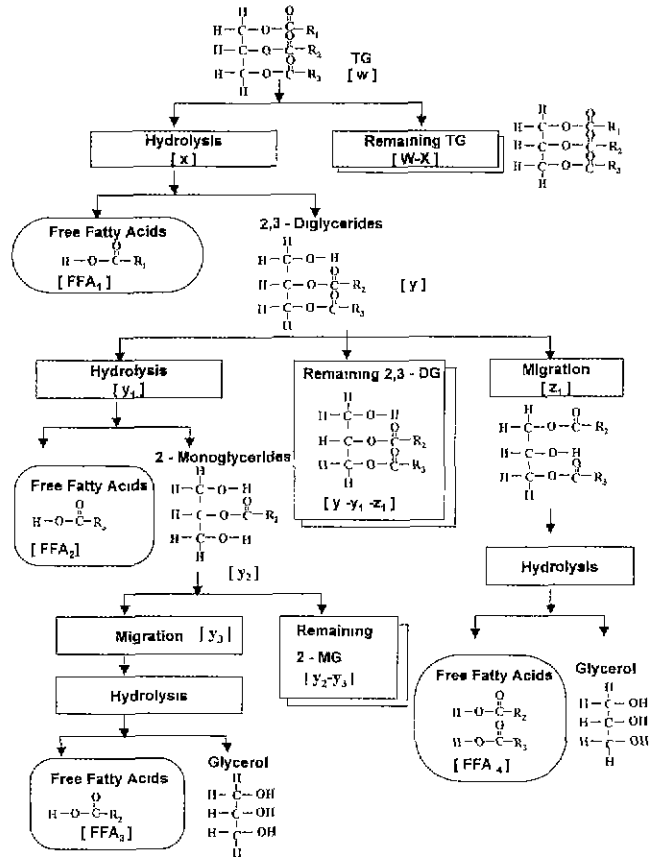

Fig. 1. Proposed hydrolysis mechanism of fish oil with lipase 100-T.

According to the material balance, the following relationships are established:

$$(DHA_i)_0=\frac{(MW)_{DHA\,i}}{(MW)_{TG}}\times W \tag{1}$$

$$(DHA_i)_1=\frac{(MW)_{DHA\,i}}{(MW)_{TG}}\times x \tag{2}$$

Equation (3) can be obtained from Eq. (1) and Eq. (2):

$$(DHA_i)_1=\frac{x}{W}\times(DHA_i)_0 \tag{3}$$

where i (i=1, 2, 3) represents the position of the glyceride to which DHA is attached.

The content of DHA in the remaining TG, DG, and MG can be obtained by the same method:

$$(DHA)_{RTG}=\frac{W-x}{W}[(DHA_2)_0+(DHA_3)_0] \tag{4}$$

$$(DHA)_{RDG}=\frac{y-y_1-z_1}{y}\cdot\frac{x}{W}[(DHA_2)_0+(DHA_3)_0] \tag{5}$$

$$(DHA)_{RMG}=\frac{y_2-y_3}{y_2}\cdot\frac{y_1}{y}\cdot\frac{x}{W}(DHA_2)_0 \tag{6}$$

As shown in Fig. 1, the amount of remaining MG is $y_2 - y_3$, and the weight percent of DHA in MG can be obtained from the experimental data. Therefore, the content of DHA in 2-position in fish oil can be calculated by Eq. (7):

$$(DHA_2)_0 = \frac{y_2}{y_1} \cdot \frac{y}{x} \cdot W \cdot P_{MG} \quad (7)$$

P_{MG} is the weight percentage of DHA in the remaining MG. As described in the previous paper [5]:

$$y_2 = \frac{M_{MG}}{M_{DG}} \cdot y_1 \quad (8)$$

$$y = \frac{M_{DG}}{M_{TG}} \cdot x \quad (9)$$

M_{MG}, M_{DG}, and M_{TG} represent the average molecular weight of MG, DG, and TG, respectively. The fraction of DHA in 2-position in fish oil can be calculated by Eq. (10):

Fraction of DHA in 2-position in fish oil

$$= \frac{(DHA_2)_0}{(DHA)_0} = \frac{P_{MG}}{P_{fish\ oil}} \cdot \frac{M_{MG}}{M_{TG}} \quad (10)$$

$P_{fish\ oil}$ is the weight percent of DHA in the fish oil and $(DHA)_0$ is the total amount of DHA in W g of the fish oil. The fraction of other fatty acids in 2-position can also be calculated using this method.

RESULTS AND DISCUSSION

Figure 2 shows the distribution of lipid components as TG, DG, MG, and FFA in the hydrolyzed reaction mixture. Since lipase was resistant to the DHA in fish oil, it was presumed that the TG would decrease and DHA-rich DG and MG would increase as the hydrolysis progressed. As shown in Fig. 2, the content of TG in the reaction mixture was 28% (w/w), which was much higher than those of DG and MG. This was probably because the saturated and monoenoic acids in TG that did not contain DHA were more easily hydrolyzed than those in TG that contained DHA. As a result, the level of TG concentration in the glyceride mixtures remained high. Tanaka *et al.* [12] suggested that the hydrolysis reaction proceeded in two steps. In the first step, TG without DHA was hydrolyzed and, in the second step, TG containing DHA hydrolyzed. The result is also consistent with the reports by Yadwad *et al.* [14].

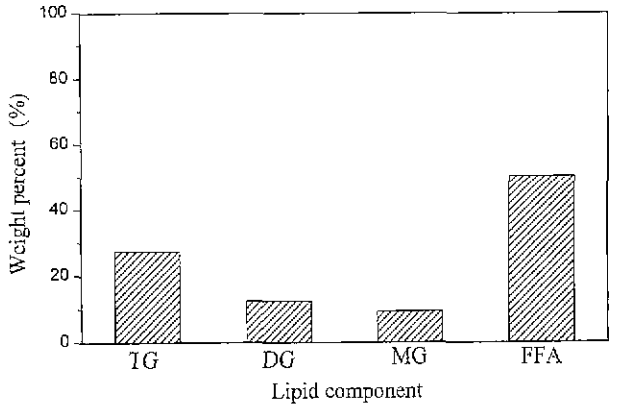

Fig. 2. The distribution of lipid components as TG, DG, MG, and FFA in the hydrolyzed mixture.

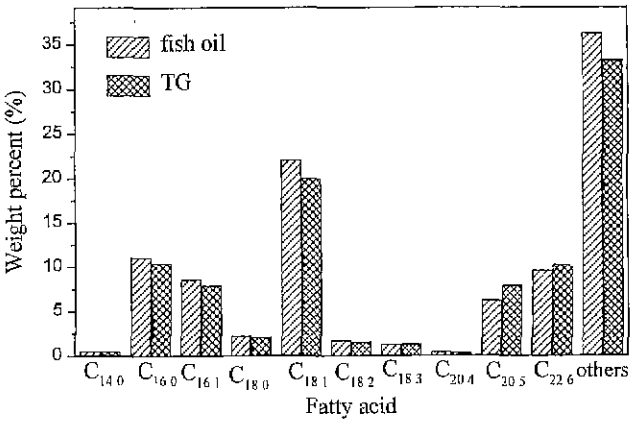

Fig. 3. Fatty acid compositions of TG and fish oil.

The compositions of fatty acid in TG, DG, MG, and FFA are shown in Figs. 3 to 6, and are compared with the compositions of the starting fish oil. Other fatty acids present in the fish oil were not quantified, because the fatty acid standards were not available. As shown in Figs. 3 to 5, the content of fatty acids, except EPA and DHA in the TG, DG and MG, are lower than in the fish oil. The weight percent of DHA in MG was 23.5%, which is about three times higher than in the fish oil. However, the content of DHA in FFA was lower than other fatty acids (Fig. 6). The reason could be that, because DHA is a poor substrate for the lipolase-100T, DHA is concentrated as the glyceride form in the reaction mixture.

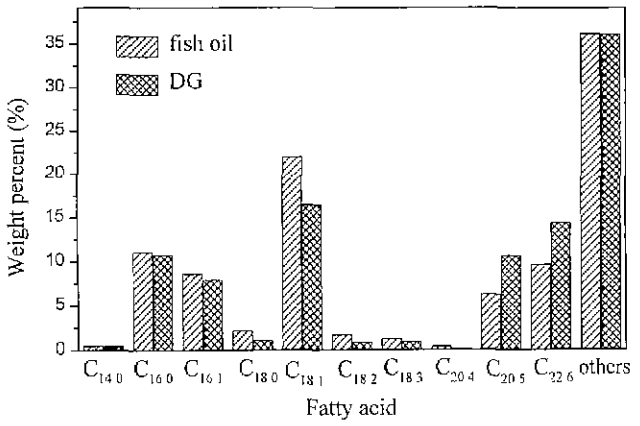

Fig. 4. Fatty acid compositions of DG and fish oil.

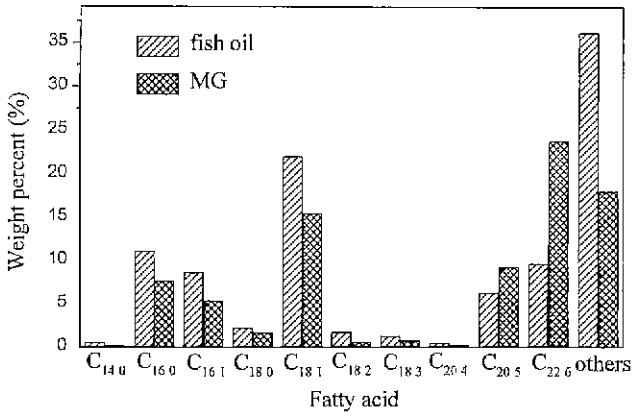

Fig. 5. Fatty acid compositions of MG and fish oil.

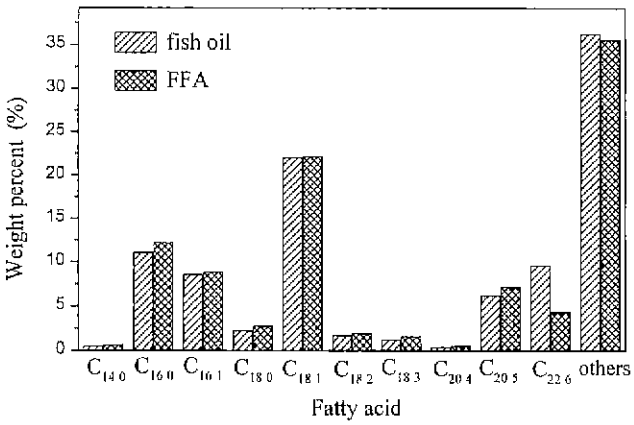

Fig. 6. Fatty acid compositions of FFA and fish oil

The compositions of EPA and DHA in each lipid component are shown in Fig. 7. The MG fraction in digestion mixtures contained 23.5% DHA, whereas fish oil contained only 8.6% DHA. In contrast, the EPA content of the different lipid components was lower in a range from 7.1 to 12.3%, compared with 16.2% in fish oil. This

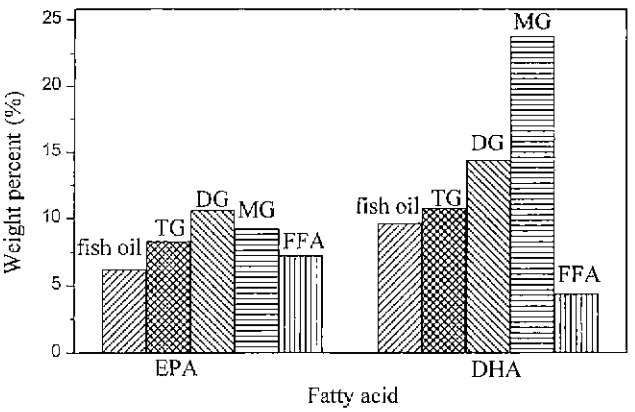

Fig. 7. Compositions of EPA and DHA in lipid components.

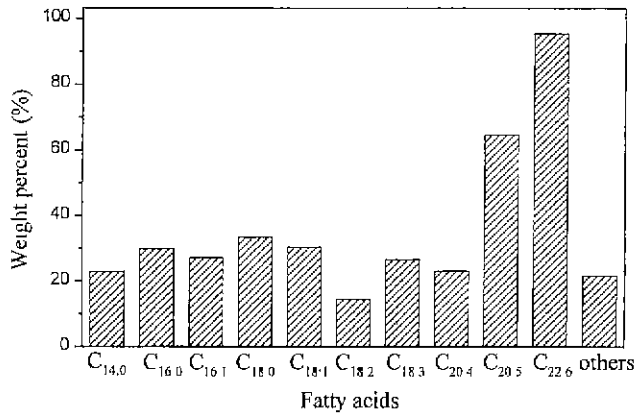

Fig. 8. Compositions of fatty acids attached to the 2-position in the fish oil.

indicates that EPA is more easily hydrolyzed than DHA. Tanaka *et al.* [12] reported that the lipase derived from *Candida cylindracea* preferentially hydrolyzed EPA residues over DHA residues when fish oil and tuna oil were employed as the substrates.

Figure 8 shows the weight percent of each fatty acid at the 2-position in the fish oil. Ninety five percent (w/w) of DHA in the fish oil was bonded to the 2-position of glycerides, while the composition of the other fatty acids, except DHA and EPA, was less than 40%. This indicates that the other fatty acids in the glycerides are evenly distributed in the fish oil. It is known that DHA is considerably more enriched at the 2-position on the glyceride [2]. Although acyl migration could occur from the 2-position to the 1- or 3-position [4], the predominance of DHA in the mid-position probably contributed to the observed enzymatic preference. Yadwad *et al.* [14] claimed that the retention of DHA in the monoglyceride fraction was due to a combination of two things: lower specificity towards DHA than other fatty acids, and the enrichment of DHA at the 2-glyceryl position of triglycerides in fish oil.

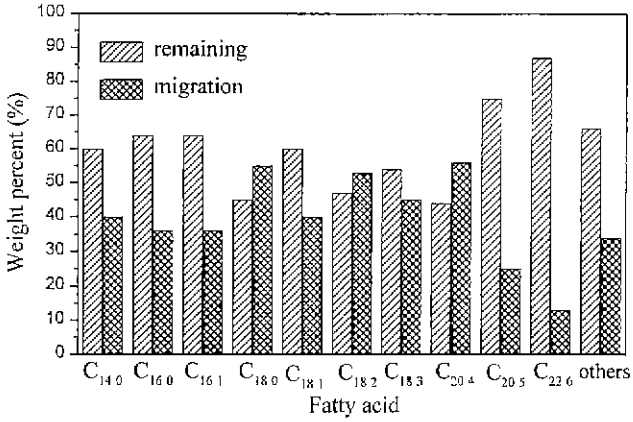

Fig. 9. The content of each kind of fatty acid migrated and remained in 2,3-DG.

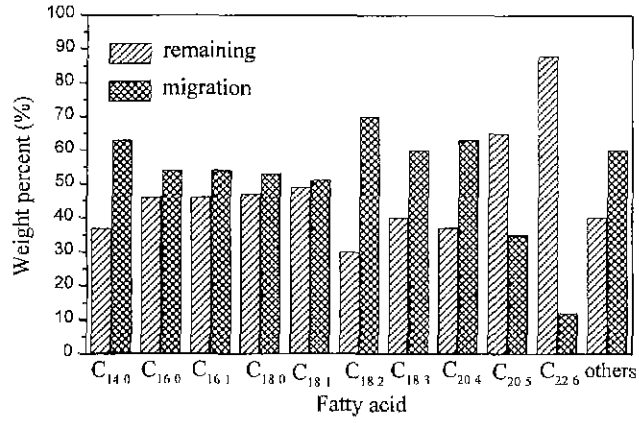

Fig. 10. The content of each kind of fatty acid migrated and remained in 2 -MG.

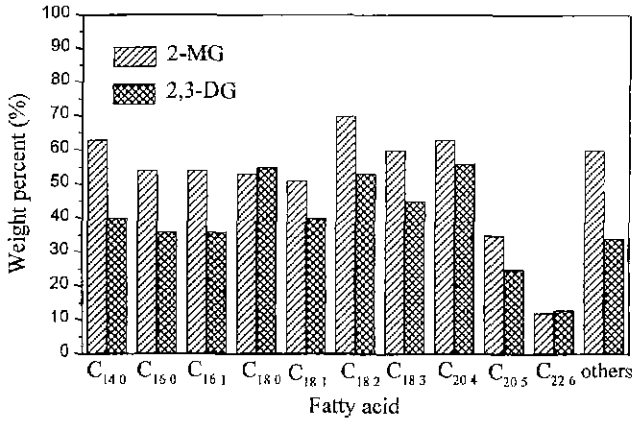

Fig. 11. Comparison of the weight percent of fatty acids between those migrated in 2,3-DG and that in 2-MG.

As shown in Fig. 1, 2,3-DG and 2-MG are the products of the hydrolysis reaction. It has been reported that both 2,3-DG and 2-MG are chemically unstable species and undergo acyl group migration to produce 1-MG and 1,3-DG, respectively [4]. However, there has been no reports to quantitatively determine the migration degree of each fatty acid in the acyl-rearranged reaction. Figure 9 shows the weight percent of fatty acids migrated and remained in 2,3-DG. The migration degree of each kind of fatty acid was different: 87% of the DHA and 75% of the EPA in 2,3-DG remained, which were higher than other fatty acids. It was discovered that 88% of the DHA and 65% of the EPA were kept in 2-MG (Fig. 10). These results indicate that DHA and EPA are very difficult to migrate and that the content of DHA and EPA will be increased in a glyceride form. Figure 11 compared the weight percent of each fatty acids for migration in 2,3-DG with that in 2-MG. Most fatty acids in 2-MG were easier to migrate than those in 2,3-DG.

Acknowledgments

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