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# Hydrolysis Mechanisms of Fish Oil by Lipolase-100T

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**Abstract** In order to investigate the position of various fatty acids attached to glycerol and the specificity of Lipolase-100T, hydrolysis of fish oil was carried out with Lipolase-100T derived from *Aspergillus oryzae*. The amounts of free fatty acids produced from triglyceride, 1,2(2,3)-diglyceride, 1,3-diglyceride, and 2-monoglyceride and conversion rates of 1,2(2,3)-diglyceride to 1,3-diglyceride and 2-monoglyceride to 1(3)-monoglyceride were also calculated. The ratio of 1,2-diglyceride content to 1,3-diglyceride was higher than 70 in the early period of hydrolysis. The fatty acid content of the glyceride mixture after 72 h of hydrolysis was compared with that of fish oil, and it was found that polyunsaturated fatty acids such as C16:4, C20:4 n-3, C20:5 n-3, C21:5 n-3, C22:5 n-3 and C22:6 n-3 were located in the 2-position of glycerol. Material balance of each component in the hydrolysis system was written to obtain a set of simultaneous linear equations. The theoretical quantity of free fatty acids produced from triglyceride, 1,2-diglyceride, 1,3-diglyceride, and monoglyceride, respectively, were calculated by solving the linear equation system. The conversion rate of 1,2(2,3)-diglyceride to 1,3-diglyceride and that of 2-monoglyceride to 1(3)-monoglyceride were also obtained. The results showed that the migration rate of 1,2(2,3)-diglyceride to 1,3-diglyceride was higher than the hydrolysis rate of 1,2(2,3)-diglyceride to 2-monoglyceride and the conversion rate of 2-monoglyceride to 1(3)-monoglyceride was extremely low.

**Key words:** Polyunsaturated fatty acid, 1,3-positional specificity, acyl chain specificity, hydrolysis, migration

The microbial lipases fall into several categories, including nonspecific lipases, 1,3-positional specific lipases, and acyl chain specific lipases [11, 10]. The specific lipases, which are lipases derived from *Corynebacterium acnes* [7] and *Staphylococcus aureus* [7], show no marked specificity, and both in regard the position of the glycerol molecule attacked and the nature of the fatty acid released. The 1,3-specific lipases from *Rhizopus javanicus* [1], *Rhizopus niveus* [11], and *Asperzillus niger* [10] possess a catalytic activity on both outer 1- and 3-positions of glyceride. The acyl chain specific lipases catalyze release of a particular type of fatty acid from glyceride molecules. They are mainly produced from *Mucor miehei* [6], *Candida cylindracea*, and *Rhizopus arrhizus* [8].

It is difficult to clearly define the specificities of lipases, because the reaction rate depends on the water-oil ratio, stirring method, shape of reactor, and the presence of surfactant in heterogeneous reactions such as the hydrolysis of fish oil. The hydrolysis resistant value (HRV) is used to define the acyl chain specificity, and the positional specificity index can be used to determine the 1,3-positional specificity [11]. Until now, many studies have been carried out to define specificities of lipases and also, in a special case, to determine the reaction rate of rearrangement of 2-monoglyceride to 1-monoglyceride [1, 7].

To the best of our knowledge, however, no studies have been conducted to determine the amount of free fatty acid produced during hydrolysis from either triglyceride, diglyceride, or monoglyceride, and also to develop a general method to calculate the quantity of acyl chain conversion of 1,2(2,3)-diglyceride to 1,3-diglyceride.

In this study, the specificity of *Aspergillus oryzae* lipase was investigated by using a ratio of 1,2(2,3)-diglyceride to 1,3-diglyceride in the glyceride mixture. Material balance on each component in the reaction system was written to obtain a set of simultaneous linear equations. The quantity of free fatty acid hydrolyzed from each glyceride in the reaction mixture and the amount of acyl chain conversion were calculated by solving the set of simultaneous linear equations.

## MATERIALS AND METHODS

### Materials

Lipolase-100T from *A. oryzae* was the product of NOVO Nordisk Corporation. It was kept in a desiccator at 4°C in a

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refrigerator. The enzyme activity was measured before hydrolysis. The lipase activity was measured according to the method described by Park, K. G. [personal communication]. Fish oil refined by LIPRO AS Corporation of Norway was used. The fish oil contained 17% EPA and 11% DHA.

**Hydrolysis, Deacidification, and Esterification**

The mixture of fish oil and distilled water (1:1 by v/v) was agitated at 38°C and at 250 rpm for 120 h. Samples were taken at 0.5, 2, 4, 7, 10, 15, 24, 48, 72, and 120 h of hydrolysis and placed into test tubes. Then, the tubes were immediately placed into boiling water to stop the enzyme activity. Deacidification was carried out by Korean Standard Association (KSM2731). After adding 3 ml of acetone and 3 ml 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 and 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 [5].

**Analysis**

The esterified mixture was analyzed by Hewlett-Packard 6,890 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 (2 min) to 265°C (2 min) at 7°C/min. The detector temperature was 300°C.

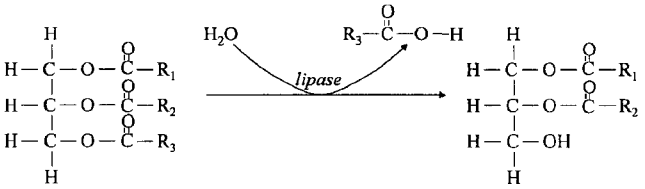
The composition of TG, DG, MG and FFA was analyzed by TLC-FID. One-hundred μl sample taken from the upper layer of the reaction products was dissolved in 100 ml chloroform. One μl of the solution was spotted on a thin layer chromatograph [CHROMROD-SIII] and developed in a solvent mixture consisted of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

**Stoichiometric Analysis**

Macrae [7] 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 conversion to give 1,3-diglycerides and 1(3)-monoglycerides, respectively, by a slow chemical isomerization reaction. Holmberg and Osterberg [3] postulated that when the 1,3-specific hydrolysis of palm oil was investigated, the rate of acyl group migration was found to be comparable to the rate of diglyceride to monoglyceride hydrolysis. Boswinkel *et al.* [1] strongly suggested from the results of Holmberg that the rearrangement reaction of 2-monoglyceride to 1(3)-monoglyceride was the rate-limiting step in the further hydrolysis of monoglycerides to glycerol by 1,3-specific lipases, and did not involve the enzyme-catalyzed step.

From the results stated above, the reaction mechanism of the hydrolysis of fish oil with 1,3-specific lipases could be proposed as shown in Fig. 1.

When triglyceride is hydrolyzed into 1,2-DG and FFA, the chemical equation states




that 1 mole of TG and 1 mole of $H_2O$ are used to form 1 mole of 1,2-DG and 1 mole of FFA. Therefore, in a case where x g of TG is hydrolyzed to give y g of 1,2-DG and $FFA_1$ g of FFA, the following relationships are established.

$$\frac{y}{x} = \frac{(\text{MW of 1,2-DG})(\text{MN of 1,2-DG produced})}{(\text{MW of TG})(\text{MN of TG hydrolyzed})} = \frac{\text{MW of 1,2-DG}}{\text{MW of TG}} = \frac{(M)_{1,2\text{-DG}}}{(M)_{TG}} \quad (1)$$

$$\frac{FFA_1}{x} = \frac{(M)_{FFA}}{(M)_{TG}} \quad (2)$$

The stoichiometric equations for each variable in Fig. 1 are obtained by using the same method as follows.

· Hydrolysis of TG, production of 1,2-DG and FFA, and remaining TG:

$$y = \frac{(M)_{1,2\text{-DG}}}{(M)_{TG}} x \quad (3)$$

$$FFA_1 = \frac{(M)_{FFA}}{(M)_{TG}} x \quad (4)$$

$$\text{remaining TG} = W - x \quad (5)$$

· Hydrolysis of 1,2(2,3)-DG and 1,3-DG, production of MG and FFA, and remaining 1,2(2,3)-DG and 1,3-DG:

$$W_1 = \frac{(M)_{MG}}{(M)_{1,2\text{-DG}}} Z_2 \quad (6)$$

$$FFA_2 = 2\frac{(M)_{FFA}}{(M)_{1,3\text{-DG}}} Z_1 \quad (7)$$

$$FFA_3 = \frac{(M)_{FFA}}{(M)_{1,2\text{-DG}}} Z_2 \quad (8)$$

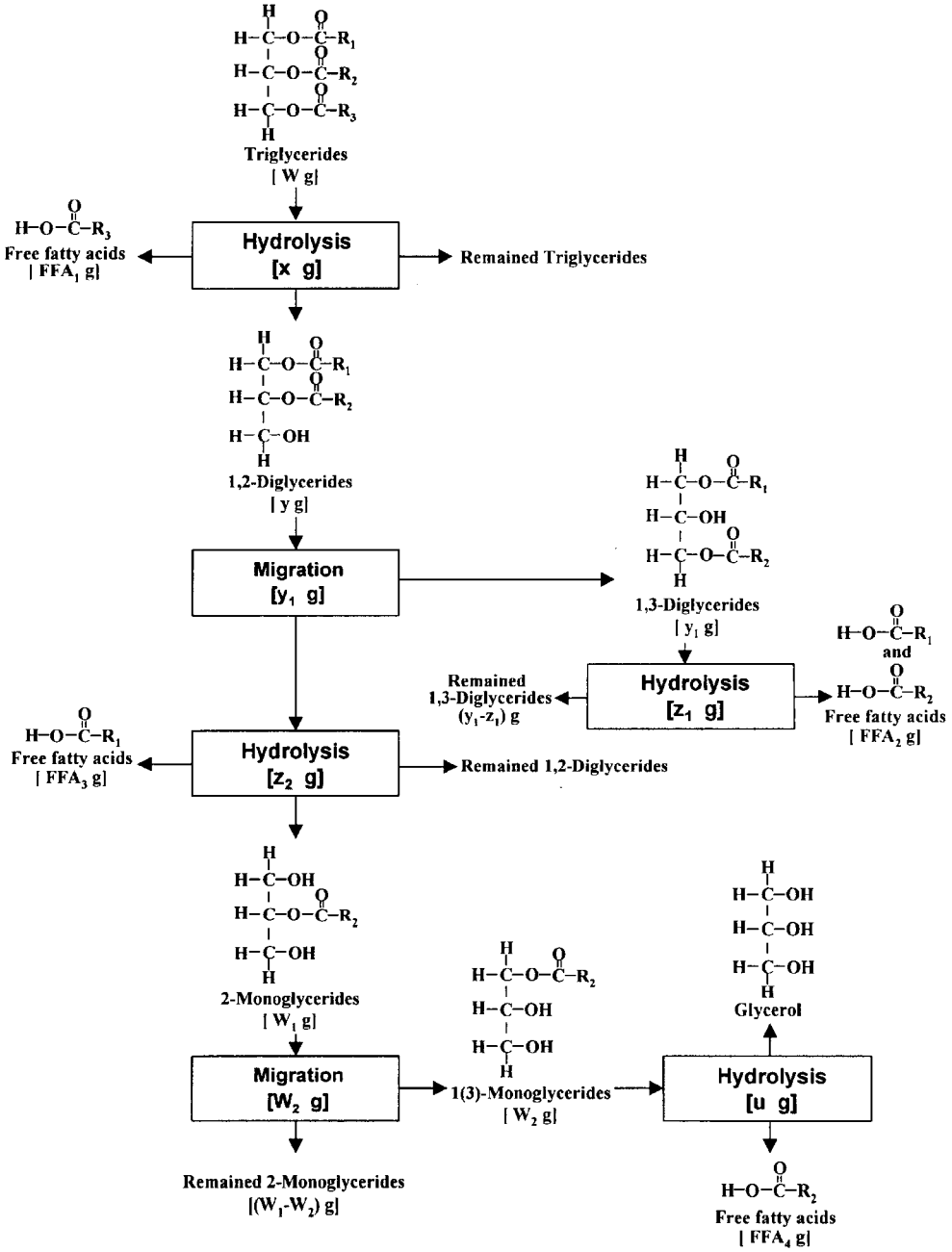



**Fig. 1.** Proposed hydrolysis mechanism of fish oil with Lipolase-100T derived from *Aspergillus oryzae.*

$$\text{remaining 1,3-DG} = y_1 - z_1 \tag{9}$$

$$\text{remaining 1,2-DG} = \frac{(M)_{1,2\text{-DG}}}{(M)_{TG}}x - y_1 - z_2 \tag{10}$$

· Migration of 2-MG to 1(3)-MG, hydrolysis of 1(3)-MG, and production of FFA:

$$FFA_4 = \frac{(M)_{FFA}}{(M)_{MG}}w_2 \tag{11}$$

$$\text{remaining 2-MG} = \frac{(M)_{MG}}{(M)_{1,2\text{-DG}}}z_2 - w_2 \tag{12}$$

· Total weight of reacted mixture without glycerol:

$$W_t = W + \left[\frac{(M)_{(1,2\text{-DG})}}{(M)_{TG}} + \frac{(M)_{FFA}}{(M)_{TG}} - 1\right]x + \left[2\frac{(M)_{FFA}}{(M)_{1,3\text{-DG}}} - 1\right]z_1 + \left[\frac{(M)_{MG}}{(M)_{1,2\text{-DG}}} + \frac{(M)_{FFA}}{(M)_{1,2\text{-DG}}} - 1\right]z_2 + \left[\frac{(M)_{FFA}}{(M)_{MG}} - 1\right]w_2$$

$$= W + \alpha x + \beta z_1 + \gamma z_2 + \delta w_2 \tag{13}$$

From the equations of (3) to (13), the weight fractions of triglyceride, 1,2-diglyceride, 1,3-diglyceride, monoglyceride, and a free fatty acid are obtained as follows.

$$(TG)_f=\frac{W-x}{W_t} \tag{14}$$

$$(1,2DG)_f=\frac{[(M)_{1,2\text{-}DG}/(M)_{TG}]x-y_1-z_2}{W_t} \tag{15}$$

$$(1,3DG)_f=\frac{y_1-z_1}{W_t} \tag{16}$$

$$(MG)_f=\frac{[(M)_{MG}/(M)_{1,2\text{-}DG}]z_2-w_2}{W_t} \tag{17}$$

$$(FFA)_f=\frac{[(M)_{FFA}/(M)_{TG}]x+2[(M)_{FFA}/(M)_{1,3\text{-}DG}]z_1}{W_t}+\frac{[(M)_{FFA}/(M)_{1,2\text{-}DG}]z_2+[(M)_{FFA}/(M)_{MG}]w_2}{W_t} \tag{18}$$

The system of five linear equations in a five unknown are finally derived from the equations of (13) to (18).

$$[\alpha(TG)_f+1]x+[\beta(TG)_f]z_1+[\gamma(TG)_f]z_2+[\delta(TG)_f]w_2=[1-(TG)_f]W$$

$$\left[\alpha(1,2\text{-}DG)_f-\frac{(M)_{1,2\text{-}DG}}{(M)_{TG}}\right]x+y_1+[\beta(1,2DG)_f]z_1+[\gamma(1,2DG)_f+1]z_2+[\delta(1,2DG)_f]w_2=-(1,2DG)_fW$$

$$[\alpha(1,3DG)_f]x-y_1+[\beta(1,3DG)_f+1]z_1+[\gamma(1,3DG)_f]z_2+[\delta(1,3DG)_f]w_2=-(1,3DG)_fW$$

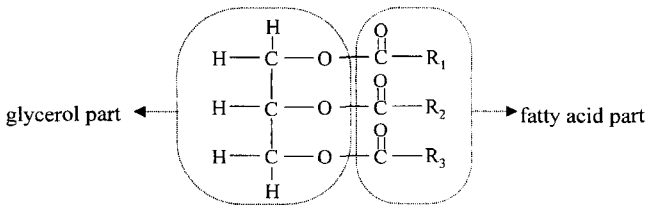
$$[\alpha(MG)_f]x+[\beta(MG)_f]z_1+\left[\gamma(MG)_f-\frac{(M)_{MG}}{(M)_{1,2\text{-}DG}}\right]z_2+[\delta(MG)_f+1]w_2=-(MG)_fW$$

$$\left[\alpha(FFA)_f-\frac{(M)_{FFA}}{(M)_{TG}}\right]x+\left[\beta(FFA)_f-2\frac{(M)_{FFA}}{(M)_{1,2\text{-}DG}}\right]z_1+\left[\gamma(FFA)_f-\frac{(M)_{FFA}}{(M)_{1,2\text{-}DG}}\right]z_2+\left[\delta(FFA)_f-\frac{(M)_{FFA}}{(M)_{MG}}\right]w_2=-(FFA)_fW \tag{19}$$

Molar weights of TG, 1,2(2,3)-DG, 1,3-DG, 2-MG, and FFA must be determined in order to solve the system (19). Fish oil has different kinds of fatty acids. Therefore, the present work used an average molar weight of fatty acid calculated by equation (20).

$$(M)_{FFA}=\frac{\Sigma W_{FFA,i}}{\Sigma(W_{FFA,i}/M_{FFA,i})} \tag{20}$$

$W_{FFA,i}$ is the weight of i-fatty acid composed of glyceride mixture and $M_{FFA,i}$ is the molar weight of i-fatty acid. The structural formula for triglyceride is




The weight of the glycerol part is 89 g/mole and that of fatty acid is $[(M)_{FFA}-17]\times3$. Therefore, the molar weight of TG, 1,2-DG, 1,3-DG, and MG can be calculated by the following equations.

$$(M)_{TG}=[(M)_{FFA}-17]\times3+89 \tag{22}$$

$$(M)_{1,2\text{-}DG}=[(M)_{FFA}-17]\times2+90 \tag{23}$$

$$(M)_{1,3\text{-}DG}=[(M)_{FFA}-17]\times2+90 \tag{24}$$

$$(M)_{MG}=[(M)_{FFA}-17]+91 \tag{25}$$

The equations of (20) and (22) to (25), and the analytical results of TLC for the reacted mixture were applied to the system (19), and the system was solved by using the Gauss elimination method.

## RESULTS AND DISCUSSION

### Characteristics of Hydrolysis

Figure 2 shows the hydrolysis of fish oil with *Aspergillus orzae* lipase. The amount of MG and 1,3-DG were much lower than that of 1,2-DG. FFA increased up to 16.5% and 1,2-DG to 13.8%, respectively, as hydrolysis progressed. The amount of 1,2-DG increased by hydrolysis at first, but decreased after 50 h of hydrolysis.

Therefore, the 1,2-DG content became lower than that of FFA after 55 h of hydrolysis. Figure 3 represents the ratios of 1,2-DG to 1,3-DG content in the glyceride mixture along with hydrolysis. The 1,2-DG content derived from hydrolysis with 0.05% Lipolase-100T was about 20 times higher than the 1,3-DG content, and that with 0.1% Lipolase-100T was about 70 times higher in the early period of hydrolysis. The ratios decreased with hydrolysis, and they were around 2 for both 0.05% and 0.1% Lipolase-100T after 120 h of hydrolysis. It is surmized that the

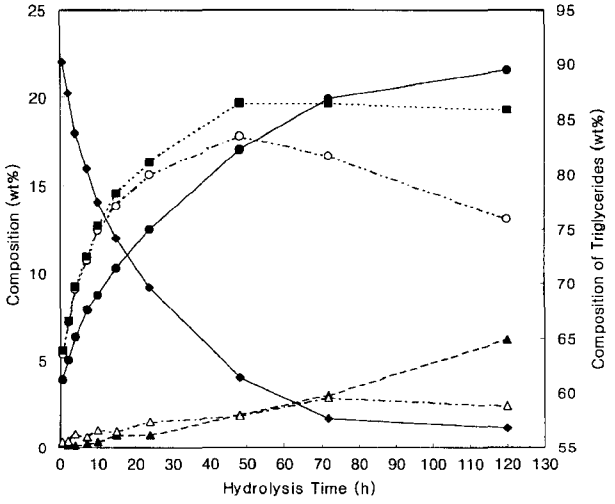



**Fig. 2.** Changes of weight percent of 1,3-DG (▲), 1,2-DG (○), MG (△), FFAs (●), total DG (■), and TG (◆) in the reacted mixture. Lipolase-100T; 0.1 wt% of fish oil.

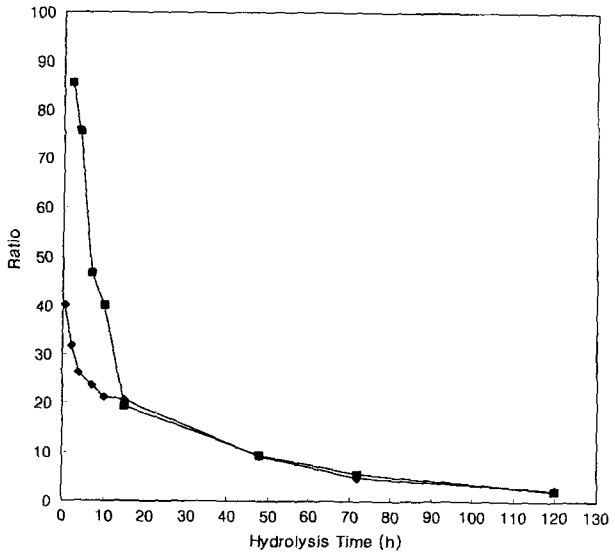



**Fig. 3.** The ratios of 1,2(2,3)-DG content to 1,3-DG in the glyceride mixture according to hydrolysis time.
The weight percents of Lipolase-100T were 0.05 (◆) and 0.1 (■).

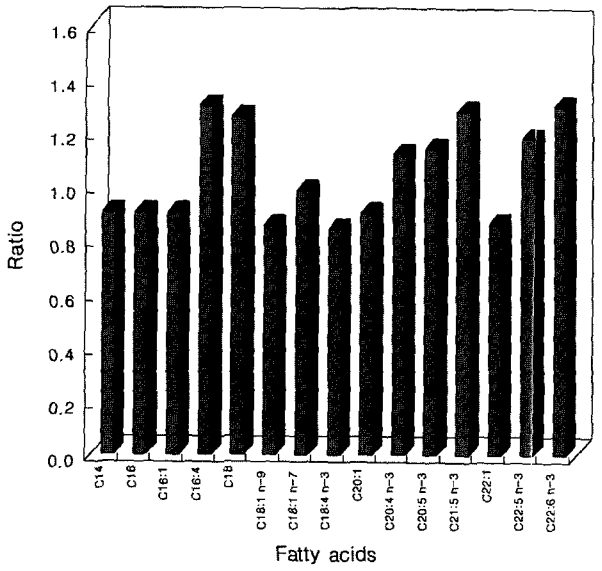



**Fig. 4.** The ratios between free fatty acid contents of the reacted mixture after 120 h of hydrolysis and those of fish oil.

specificity of Lipolase-100T was 1,3-position, based on the exceedingly high values of the ratios of 1,2-DG content to 1,3-DG, and that the hydrolysis took place at 1-position or 3-position at the beginning, and the acyl chain migration from 2-position to 1-position or 3-position occurred to increase the 1,3-DG content as hydrolysis progressed. These results are in good agreement with the reports of Macrae [7] and Boswinkel *et al.* [1] that the hydrolysis of oils and fats with 1,3-specific lipase produced 1,2(2,3)-DG and 2-MG, and acyl chain migrated from 2-position to 1-position or 3-position, because 1,2(2,3)-DG and especially 2-monoglycerides are chemically unstable. Jung *et al.* [4] reported that the hydrolysis of olive oil could be accelerated by the combined use of 1,3-specificity of Lipolase-100T and the non-specificity of Lipase-OF derived from *Candida cylindracea*.

Figure 4 shows the ratios between the fatty acid content composed of the glyceride mixture after 120 h of hydrolysis with 0.1 wt% Lipolase-100T and the fish oil before the hydrolysis. The ratio greater than a unit means that the content of fatty acid in the glyceride mixture is higher than that in the fish oil. Considering that Lipolase-100T has 1,3-positional specificity, the greater the ratio of fatty acid, the better the chance for the fatty acid to be located in the 2-position of the glyceride. The ratios of polyunsaturated fatty acids (PUFAs) such as C16:4, C20:4 n-3, C20:5 n-3, C21:5 n-3, C22:5 n-3, and C22:6 n-3 were greater than one unit, but those of saturated and monoenoic fatty acids were less than one unit (Fig. 4). However, C18 was a unique saturated fatty acid in which the ratio was greater than a unit.

These results are in support with the reports of Christie [2] that, because n-3 PUFAs were concentrated in the 2-position of TG and saturated fatty acids were placed in a 1,3-position, hydrolysis of fish oil with 1,3-specific lipase produced PUFAs-rich 2-MG and 1,2(2,3)-DG.

### Stoichiometric Analysis for the Formation of 1,2-DG, 1,3-DG, 2-MG, and FFA

Figure 5 shows the various weight fractions based on 1,2-DG produced from the initial TG when hydrolysis was performed with 0.1 wt% Lipolase-100T. The fraction of the 1,3-DG migrated from a 1,2-DG increased along with the hydrolysis of 1,2-DG to 2-MG, as hydrolysis progressed.

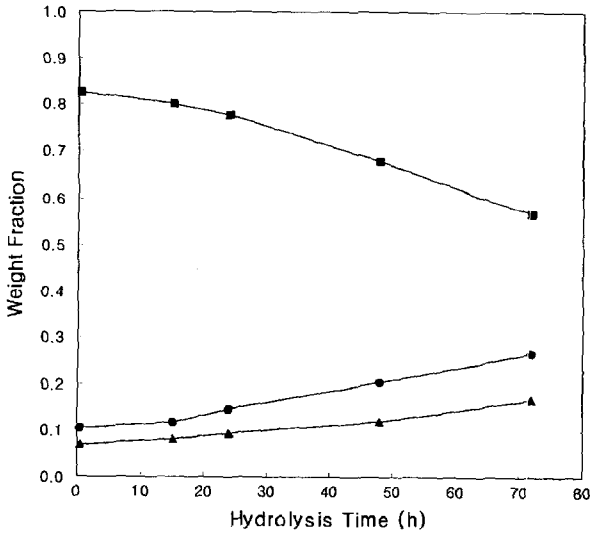



**Fig. 5.** The various weight fractions, such as those of 1,3-DG migrated from 1,2-DG (●), 2-MG produced from 1,2-DG (▲), and remaining 1,2-DG (■), based on the 1,2-DG produced from the initial TG.

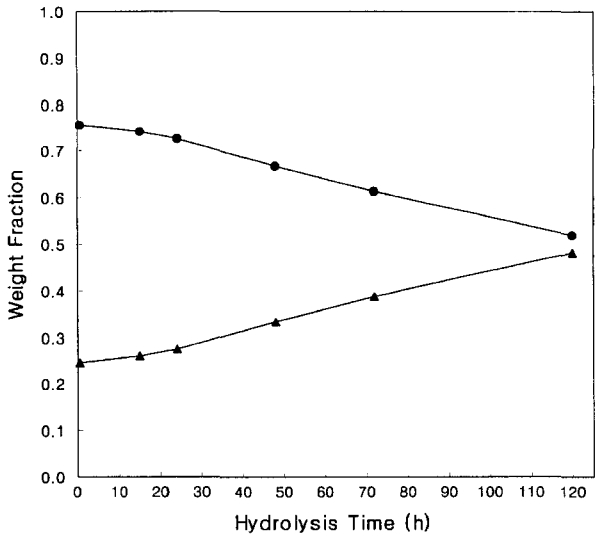



**Fig. 6.** The weight fraction of hydrolysis of 1,3-DG to FFA and glycerol (●), and that of the remaining 1,3-DG (▲).
The basis was the amount of 1,3-DG migrated from 1,2-DG.

However, after 72 h of hydrolysis, the fraction of the remaining 1,2-DG had the highest value among the three fractions. The result also indicated that the conversion rate of 1,2-DG to 1,3-DG was greater than the hydrolysis rate of 1,2-DG to 2-MG, and that the difference between the two rates became larger with hydrolysis. Until now, there has been no report on the quantitative comparison of the conversion rate of 1,2-DG to 1,3-DG to that of 1,2-DG to 2-MG during hydrolysis of fish oil with 1,3-specific lipase.

Figure 6 shows the hydrolysis rate of the 1,3-DG which was converted from 1,2-DG into FFA and glycerol. The hydrolysis rate of 1,3-DG also decreased with hydrolysis. However, the rate was higher than 0.6 after 72 h of hydrolysis. The results indicate that, in the early period of hydrolysis, most of 1,3-DG were hydrolyzed into FFA and glycerol, but in the late period, much of the 1,3-DG remained without hydrolysis. Therefore, it is presumed that, at the beginning of hydrolysis, the content of 1,3-DG is very low, corresponding with the result shown in Fig. 2.

Figure 7 represents the weight fractions of the 1(3)-MG migrated from 2-MG and the remaining 2-MG, based on the 2-MG produced from 1,2-DG. The fraction of the remaining 2-MG was higher than 95% from 0.5 h to 72 h of hydrolysis. On the other hand, the fraction of the 1(3)-MG migrated from 2-MG was very low. The results of Figs. 5 and 7 show that the migration of 2-MG to 1(3)-MG was very difficult, but that of 1,2(2,3)-DG to 1,3-DG was quite simple.

Figure 8 represents the weight fraction of $FFA_1$ to $FFA_4$. $FFA_1$ is the weight of FFA produced when TG was hydrolyzed to 1,2-DG. $FFA_2$ is that of FFA produced when 1,3-DG was hydrolyzed to FFA and glycerol. $FFA_3$ is that of FFA formed when 1,2-DG was hydrolyzed to 3-MG. According to the results of Fig. 8, most of FFA was

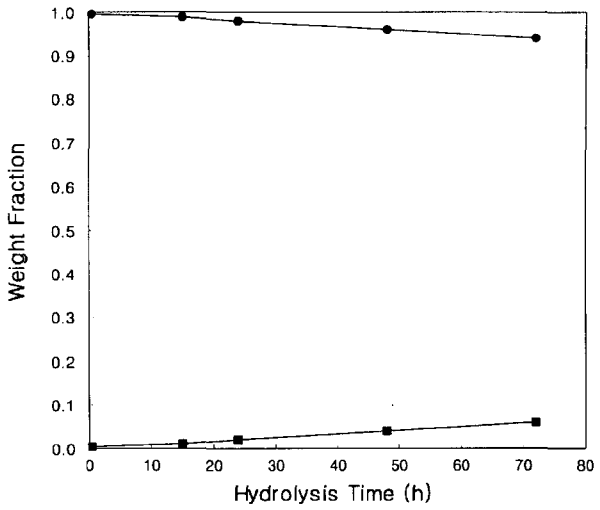



**Fig. 7.** The weight fractions, such as those of 2-MG migrated to 1(3)-MG (■) and remaining 2-MG (●), based on the 2-MG produced from 1,2-DG.

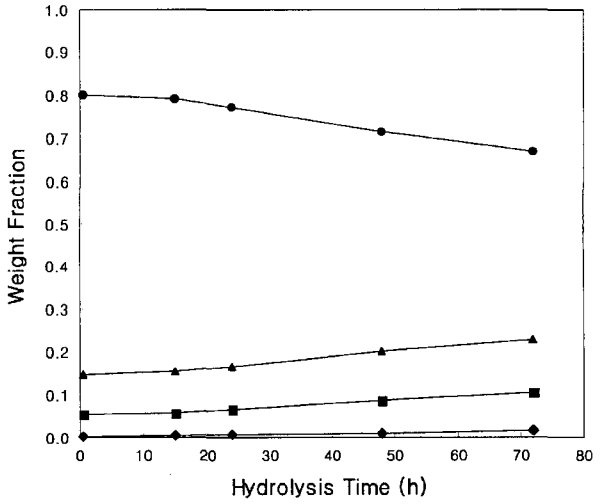



**Fig. 8.** The weight fractions of $FFA_i$, such as those of $FFA_1$ (●), $FFA_2$ (▲), $FFA_3$ (■), and $FFA_4$ (◆), based on the total weight of FFA produced from TG, DG, and MG.

produced by the hydrolysis of TG to 1,2-DG. In particular, at the beginning of hydrolysis, the content of $FFA_1$ was about 80%. $FFA_2$ and $FFA_3$ increased as the hydrolysis progressed. However, the sum of $FFA_2$ and $FFA_3$ contents was less than 35%. $FFA_2$ was also more than $FFA_3$ in all ranges of hydrolysis. The content of $FFA_4$ that was produced from 2-MG was extremely low because the migration of 2-MG to 1(3)-MG was very difficult (Fig. 7).

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