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Studies on Wax Esters in Marine Animals (1)

Lipid Composition of Mullet Roe Oil

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水產動物의 Wax Ester에 關한 硏究 (1)

숭어卵油의 脂質組成에 關하여

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Abstract

The total amount of lipid content in the mullet roe is 20.5%, and iodine value and unsaponifiable matters content are 118 and 38.7%, respectively.

The lipid composition of the muscle, roe and liver of the mullet, *Mugil cephalus*, shows differences. Triglyceride, wax esters, and free fatty acids are mainly contained in the lipids of the muscle, roe and liver, respectively.

The mullet roe lipids are mainly composed of 59.1% of wax esters with a trace of sterol esters, 26.9% of polar lipids with pigments, 9.0% of triglyceride plus a trace of free fatty alcohols and fatty acids, and 3.0% of sterol contaminated with a trace of fatty alcohols.

The major fatty acids of wax esters are C16 : 0, 47.5%, C18 : 1, 23.0%, C16 : 3, 6.5%, C20 : 5, 4.0%, those of triglyceride are C16 : 1, 25.1%, C18 : 1, 16.7%, C16 : 0, 16.3%, C22 : 1, 7.9%, C18 : 0, 5.5%, C22 : 6, 4.4%, and those of polar lipids are C16 : 0, 35.0%, C18 : 1, 24.7%, C16 : 1, 6.1%, C20 : 5, 5.3%, C22 : 6, 4.2%.

The major alcohols of wax esters are 51.0% of cetyl alcohol, 18.2% of palmitoleyl alcohol, and 10.7% of oleyl alcohol, and considerable amounts of odd-numbered alcohols such as C15 : 0, C15 : 1, C17 : 0, C17 : 1 and C19 : 1 are also found.

Introduction

Esters of fatty acids with long chain alcohol, the wax esters, occur in many marine animals, for example, Calanoid copepods,[1,2] small crustaceans which are the principal food of herrings, sardines, anchovies and other fishes.

In the course of the lipid research of marine animals in deep-sea, some workers indicated that certain marine animals contained special type of lipids, such as diacyl glyceryl ether[3~6] and wax esters.[9~11,30]

Sato[12,13] reported that seborrhea was observed in rats fed by wax ester in oil. Matsuo[14,15] indicated that seborrhea in animals was caused by the waxes composed of oleic acid esterified with straight chain alcohol with 12, 14, 16 or 18 carbon atoms.

According to Lee[2], wax esters increased rapidly in the copepod, *Calanus helgolandicus* when it was fed on diatoms containing no wax esters. Iyengar[16]

showed that wax esters were found in mullet lipids only after the mature of ovaries, presumably without any change in diet.

A few investigations of biosynthesis in wax ester have been carried out in fishes. Malins[17] showed that 1-C^{14}-palmitic acid was incorporated into free alcohols as well as into the alcohol and acid moieties of wax ester in the dogfish liver.

However, there are few reports on the enzyme catalyzing the synthesis of wax ester from alcohol and acid wide-spread in marine animal tissues. Friedberg & Grèene[18] showed that 1-hexadecanol was incorporated into wax esters by homogenates of whole dogfish liver by microsomal and supernatant fractions. Nevenzel & Kayama[19] working with lantern fishes which contain large amounts of wax esters, found that the usual lipid precursors were incorporated in vivo into both the long chain alcohol and fatty acid portions of the wax esters.

The present work is aimed to study the composition of mullet roe lipids prior to investigating the biosynthetic mechanism of wax ester during the maturity of mullet ovary.

Materials and Methods

Materials

Two mullets pregnant with egg were caught in the estuary of the Yeong San River, Samyang-Ri, Sam yang-Myen, Muan-Gun, Jeolla Nam-Do, Korea, on May 2, 1978. Being chilled with ice for 8 hours, they were transported to this laboratory and kept in the frozen state until use. Fishes containing roe were about 65 cm long, weighed 1,500 g, and had 850 g of roe. Of which 374.7 g was used in this experiment.

Extraction of Lipids

The total lipids were extracted from the roe with a mixture of chloroform-methanol (2 : 1, V/V) according to the procedure of Bligh & Dyer[20]. The chloroform layer was reduced under nitrogen flow by a vacuum rotary evaporator.

Lipid Analysis by Thin-Layer Chromatography (TLC)[21,22] and Column Chromatography[23].

The TLC plates(20×20 cm) coated with Wakogel B-5 (thickness 0.25 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were activated for 3 hours at 110°C and stored in a stock box until cooled. A small amount of total lipids dissolved in diethyl ether, were spotted at the site 3cm above the bottom, and developed with petroleum ether (p.e., bp. 30~70°C)-diethyl ether-acetic acid (80 : 20 : 1, V/V/V) until the front went up to the level 3cm below the top. The spots were checked by iodine vapor and identified by spraying 50% sulphuric acid or by running the standard materials, when needed.

A small quantity of the total lipids, 112 mg were adsorbed on 50 g of silicic aid (Mallinckrodt, 80~100 mesh) activated at 120°C for 5 hours, and were eluted with p.e., 1, 3, 7, 13, 20 and 30% of diethyl ether in p.e. (V/V), diethyl ether, and 25% chloroform-methanol (V/V), in sequence.

Analysis of Fatty Acids and Fatty Alcohols

A portion of triglyceride was saponified with a mixture of 10% KOH-ethanol and the recovered fatty acids were converted to methyl esters with diazomethane reagent[24].

The wax esters purely isolated from the roe lipids by column chromatography were saponified, and the fatty alcohols extracted with diethyl ether were converted to acetates by reaction with acetic anhydride and anhyhrous pyridine[25]. The fatty acids obtained from wax esters were methylated as mentioned above.

After interesterification of the polar lipids with 3% HCl-methanol[26] in water bath (80°C) for 2 hours, the fatty acid methyl esters were recovered by addition of p.e., and condensed with a vacuum rotary evaporator.

Gas-Liquid Chromatographic (GLC) Analysis of Fatty Acid Methyl Esters and Fatty Alcohol Acetates.

The GLC apparatus used in this study was a dual column Hitachi Model GC-2C with a flame ionization detector. The condition of GLC analysis for fatty acids and alcohols was as follows; the coiled stainless column (3 m×3 mm) packed with 10% DEGS on Chromosorb W, and the column operating temperature of 190°C, nitrogen flow rate of 30 ml/min. The identification of each peak on GLC of fatty acids and fatty alcohols were carried out by comparing the retention

times to those of standard fatty acids and fatty alcohols, and by equivalent chain length value[27]. When needed, they were hydrogenated and subjected to GLC.

Results and Discussion

As given in Table 1, 2, the total lipid content amounted to 20.5%, which corresponded to the results from Iyengar[16]. The roe lipids showed the following characteristics; specific weight D^{20}_{20} 0.9327, saponification value 144.3, iodine value 118, unsaponifiables 38.7%. Unusually high content of unsaponifiables strongly suggested the presence of wax ester. Kafuku and Hata[28], Tsujimoto[11], and Iizuka[29] reported nearly one-half of the oil was unsaponifiable lipid of mullet roe and they were mainly composed of alcohols.

By running the muscle lipids, roe lipids and liver lipids of mullet on TLC of silica gel(Wakogel B-5), it is evident that the composition of each lipid is strikingly different. The major component of muscle lipids, roe lipids is triglyceride, wax esters & sterol esters, and free fatty acids, respectively. After saponifying wax esters & sterol esters scraped off from the TLC of roe lipids, the unsaponifiables were run on TLC again but negligiable amounts of sterol were encountered. In the liver lipids, it was not colored in pink which was specific to sterol and hydrocarbon by spraying 50% sulphuric acid, although the TLC spot was corresponding to the wax ester spot. Small quantities of the liver lipids were not allowed to confirm this spot further. Iyengar[16] also indicated that the wax esters did not occur elsewhere except in the mullet roe.

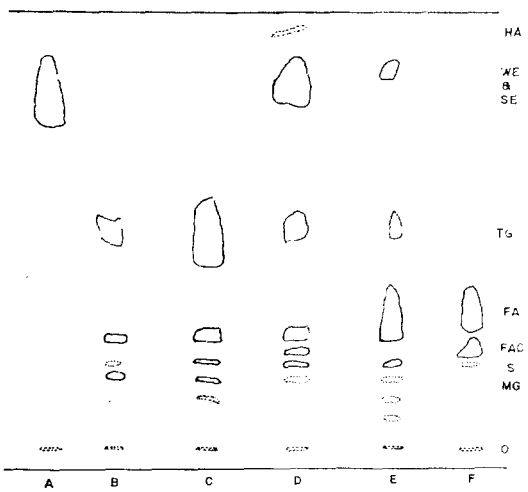

Fig. 1. Thin layer chromatogram of the roe lipids of mullet

Conditions: Wakogel B-5 with petroleum ether-ether-acetic acid (80:20:1) A: Palmitoleyl strearate, B: Pollack roe oil, C: Muscle lipids of mullet, D: Roe lipids of mullet, E: Liver lipids of mullet, F: Hydrolytic products of the spot on TLC of the roe lipids of mullet corresponding to wax esters and sterol esters, HA: Hydrocarbon, WE & SE: Wax ester & sterol ester, TG: Triglyceride, FA: Fatty acid, FAC: Fatty alcohol, S: Sterol, MG: Monoglyceride, O: Origin.

Table 1. Sampling site, date and sample weight

Sampling site	Sampling date	Sample weight (g)	Oil content
Samyang-Ri, Samyang-Myen, Muan-Gun, Jeolla Nam-Do, Korea	May 2, 1978	374.7	770.0 g 20.5 %

Table 2. Lipid properties of *Mugil cephalus* roe

Iodine value (Wij's method)	Saponification value	Specific weight D^{20}_{20}	Unsaponifiables (%)
118	144.3	0.9327	38.7

By column chromatography, the total lipids of the roe were separated into hydrocarbon (trace), 59.1% of wax esters & sterol esters, 9.0% of triglyceride with traces of free fatty acids & fatty alcohols, 3.0% of sterol with fatty alcohol, 2.0% of monoglyceride, eand 26.9% of polar lipids with pigments in the order of increasing solvent polarity. The results are summarized in Table 3.

The presence of wax esters in the muscle of teleost fishes appears to preclude their presence in the roe of that species; wax esters are the major lipid type in the muscle of "Kuro-ômatôdai", *Allocyttus verrucosus*[30], castor oil fishes, *Lepidocybium flavobrunneum*[31] and *Ruvettus pretiosus*[31~33], but are present only as a trace, if at all, in its eggs; the reverse situation prevails with the mullet.

As shown in Table 4, the fatty acid distribution in wax esters, triglyceride, and polar lipids fraction

Table 3. The lipid composition of *M. cephalus* roe

Eluent	Volume(ml)	Fractions	Weight(mg)	%
100% Petroleum ether(p.e.)	200	Hydrocarbon	trace	
1% Ether-p.e(V/V)	400	Wax ester, sterol ester	57.9	59.1
3% Ether-p.e(V/V)	200	Triglyceride, free fatty acid & fatty alcohol	8.8	9.0
7% Ether-p.e(V/V)	300			
13% Ether-p.e(V/V)	400	Free fatty alcohol & sterol	2.9	3.0
20% Ether-p.e(V/V)	300	Monoglyceride	2.0	2.0
30% Ether-p.e(V/V)	300			
Ether	400	Pigment & polar lipids	26.3	26.9
25% Chloroform-methanol(V/V)	600			
Methanol	300			
Total weight			97.9	

Total lipids 112.0 mg loaded 50 g of silicic acid, activated at 120°C for 5 hours.
Recovery: 87.4%

Table 4. Fatty acids in mullet roe lipids
(GLC area percent of methylesters)

Acid	ECL*	Wax Ester	Triglyceride	Polar lipid
C14:0		1.5	5.0	1.9
1		0.2		
C15:0		0.1	0.8	1.4
1		0.9	0.8	1.2
C16:0		1.5	16.3	35.0
1		47.5	25.1	6.1
2		1.1	2.8	1.5
3		6.5	trace	2.0
C18:0		1.9	5.5	2.1
1		23.0	16.7	24.7
2		0.9	trace	0.4
3		0.8	0.5	0.8
4		0.5	0.7	trace
C20:0		0.5	0.8	
1		0.8	0.9	1.4
2		0.8	0.8	1.2
3			1.3	0.5
4		1.2	1.0	2.5
5		4.0	2.4	5.3
C21:1		0.2		
C22:1		1.2	7.9	1.4
3		0.6	0.6	1.5
4		trace	1.5	1.1
5		1.8	0.9	2.0
6		1.7	4.4	4.2
C24:0		trace	0.4	0.2
1		0.2	0.6	0.4
Unknown	21.2	0.3		
Unknown	22.6		0.3	1.2

* Equivalent carbon chain length

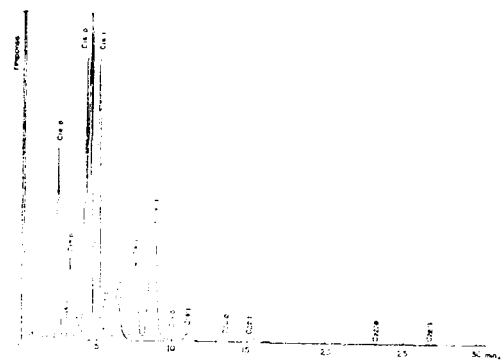

Fig. 2. Gas-liquid chromatogram of fatty alcohols

Stationary phase; 10% DEGS on Chromosorb W, Dector; FID, Column length; 3 m×3 mm (i.d., stainless steel), Column temperature; 190°C, Detector temperature; 250°C, N_2 flow rate; 30 ml/min.

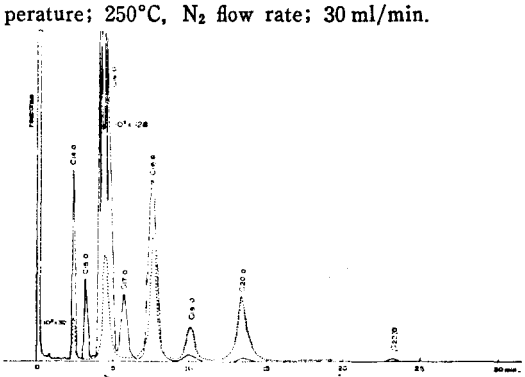

Fig. 3. Gas-liquid chromatogram of the hydrogenated fatty alcohols and the standard alcohols

——— : Hydrogenated fatty alcohols
····· : Standard fatty alcohols
GLC conditions are shown in Fig. 2

of this roe showed the differences. Wax esters contained much more monoethenoic acids and less saturated fatty acids than the others. The fatty acid moiety of wax esters is characterized by predominance with 47.5% of palmitoleic acid and 23.0% of oleic acid. This fact is indiscrepancy with that of Mori[34] (27.5% of palmitoleic acid, 21.3% of oleic acid), and Iyengar[16] (23.3% of palitoleic acid, 12.8% of oleic acid). The content of fatty acids with carbon chains longer than 16 in wax esters were slightly lower than that in triglyceride and polar lipids. Their amounts were much less than those of marine roe lipids[35~37] of which major components were not wax ester but glyceride, although polyunsaturated fatty acids such as C20:4, C20:5 and C22:6 were contained in each lipid fraction.

The alcohols were consisted of above 50% of cetyl alcohol, and no unsaturated alcohols other than monoethenyl alcohol were detected. Odd-numbered alcohols such as C15:0, C15:1, C17:0, C17:1 and C19:1, were found in considerable amounts. The results obtained are summarized in Table 5, and the GLC-grams are shown on Fig. 2 and 3.

From the reports[2,16,35] published up-to-date, it appears that polyunsaturated fatty alcohols are not detected in any wax esters which mainly constituted roe lipids except the wax esters of *Calanus helgo landicus*[2] and gouramis, *Trichogaster cosby*[38]. Thi tropical fresh-water fish contains polyunsaturate alcohols such as C18:3, C20:3, C20:4, C20:5, C22:: and C22:6.

Table 5. The composition of fatty alcohols
(% of acetates)

Alcohol	%
C14:0	6.1
1	0.1
C15:0	3.4
1	0.2
C16:0	51.0
1	18.2
C17:0	1.3
1	2.3
C18:0	5.4
1	10.7
C19:0	trace
1	0.8
C20:0	0.1
1	0.4
C22:0	trace
1	trace

Sand & Schlenk inferred from the structures of th polyunsaturated alcohols of roe wax esters and th good conversion of labelled fatty acids (includin C18:2, C18:3) into alcohols that the alcohols includin the polyunsaturated homologues were biosynthesize from the corresponding fatty acids in the gouramis i vivo. But there is no structural relationships sug gesting that the alcohols may arise by biological reduction from the corresponding acids in this work. Presumably, the combination of exclusively saturated and monounsaturated alcohols with a considerable percentage of polyunsaturated acids seems to empha size that saturated and monounsaturated acids are preferentially amenable to biological reduction of their carboxyl group to alcohol radical.

要 約

숭어 卵油의 脂質組成을 調査한 結果를 보면 다음과 같다.

1. 숭어의 各組織의 脂質成分은 相異하였다. 즉 筋肉, 卵 및 肝에서 抽出한 脂質主要成分은 各各 triglyceride, wax ester, 遊離脂肪酸이였다.

2. 숭어卵의 脂質含量은 約 20.5%였고, 그 脂質의 요-드價는 118, 不鹸化物의 量은 38.7%였다.

3. 숭어卵油의 重要脂質組成은 wax ester가 59.1%로 제일 많았고, 다음이 極性脂質로 26.9%, 그 다음이 triglyceride로 9.0%였으며, Sterol은 3.0%였다.

4. Wax ester의 重要한 脂肪酸組成을 보면 palmitic acid가 47.5%로 제일 많았으며, 그 다음이 oleic acid로 23.0%, hexadecatrienoic acid가 6.5%, eicosapentaenoic acid가 4.0%였다. 또 triglyceride의 組成을 보면 palmitoleic acid가 25.1%, oleic acid가 16.7%, palmitic acid가 16.3%, eicosamonoenoic가 7.9%, stearic acid가 5.5%였다. 極性脂質의 경우는 palmitic acid가 35.0%, oleic acid가 24.7%, palmitoleic acid가 6.1%, eicosapentaenoic acid가 5.3%였다.

5. Wax ester의 重要한 alcohal은 cetyl alcohol(C16:0)가 51.0%로 제일 많았고, 그 다음이 palmitoleyl alcohol(C16:1)로 18.2%, 그 다음이 oleyl alcohol

(C18:1)로 10.7%였다. 또 C15:0, C15:1, C17:0, C17:1, C19:1과 같은 奇數炭素 alcohol가 相當量 發見되었다.

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