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Separation of Eicosapentaenoic Acid-Enriched Triglycerides by Column Chromatography on Silicic Acid

Kenji HAYASHI* and Hideki KISHIMURA*

Abstract

Eicosapentaenoic acid (20 : 5n-3)-enriched triglycerides (TG) from crude lipids of scallop (*Patinopecten yessoensis*), sardine (*Sardinops melanosticta*), and squid (*Todarodes pacificus*) have been separated by column chromatography on silicic acid.

Five to six different TG fractions from original TG in the crude lipids were fractionated with 5% diethyl ether in *n*-hexane as the eluent, except for the final fraction, which was with 10% diethyl ether in *n*-hexane. Each fractionating TG showed various mobility in thin-layer chromatography according to differences in degree of unsaturation. Total unsaturation indices of these fractionating TG produced nearly linear plots. They corresponded to a decrease in saturated and/or monoenoic fatty acid percentages and to an increase in polyenoic ones. Finally, the isolating polyunsaturated TG, which contained considerable amounts of 20 : 5n-3 (47-58% for the scallop and 32-40% for the sardine and squid), yielded about 4-9% and 6-7% of the crude lipids, respectively. Silicic acid column chromatography used in this study is a rapid and simple method in separating highly polyunsaturated TG and applicable for large scale preparation of the compounds.

Introduction

Eicosapentaenoic acid (20 : 5n-3) is particularly effective in lowering serum triglycerides (TG) and inhibiting platelet aggregation and blood clotting, thereby reducing the risk of heart attacks[1]. In human subjects, it was also evidenced that absorption of 20 : 5n-3 in a TG form was more advantageous than in an ethyl ester form[2].

Natural TG, especially those in marine lipids, consist of a mixture of TG molecules with different fatty acids based on both chain-length and number of double bonds. It is obvious that the use of a low temperature recrystallization method will not permit separation of highly polyunsaturated TG from the marine lipids. For separating fish oil TG molecules according to degree of unsaturation, Dolev and Olcott[3] have used a column of silica gel impregnated with silver nitrate and Laakso et al.[4] utilized high-performance liquid chromatography with a silver ion column. Recently, we have demonstrated that silicic acid column chromatography could be used to derive the highly polyunsaturated TG from the marine lipids[5].

The present study was also expanded to include the preparative scale isolation of 20 : 5n-3-enriched TG from marine lipids with a variety of 20 : 5n-3 levels and to evaluate the utility of this method in separation of the highly polyunsaturated TG.

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Experimental

Materials

Samples of crude lipids from scallop (*Patinopecten yessoensis*) hepatopancreas, sardine (*Sardinops melanosticta*) tissues, and common squid (*Todarodes pacificus*) livers were prepared in the laboratory by the method of Bligh and Dyer[6].

Methods

Silicic acid column chromatography was used to separate TG molecules from the crude lipids according to differences in degree of unsaturation. An aliquot (ca. 4.0 or 5.0 g) of the crude lipid samples was dissolved in a small volume of *n*-hexane and applied to a column (160 or 200 g, $\phi 3.2 \times 40$ cm or $\phi 3.9 \times 43$ cm) of silicic acid (Wakogel C-200)-celite 545 (2 : 1 w/w, Wako Pure Chemical Industries Ltd., Tokyo)

Table 1. Elution sequence and fatty acid composition of triglyceride fractions obtained from scallop lipids (A) by silicic acid column chromatography.

	Original TG	Fraction*1					
		1	2	3	4	5	6
Yield mg		27	1593	990	549	439	67
%*2	72.9	0.5	32.1	19.9	11.1	8.8	1.3
%*3	100	0.7	43.5	27.0	15.0	12.0	1.8
Rf value*4	0.45	0.49	0.47	0.44	0.43	0.41	0.40
Fatty acid %*5							
14 : 0	4.1	3.2	5.0	4.3	3.4	2.1	0.9
16 : 0	13.7	24.2	19.3	12.7	7.7	3.0	1.6
18 : 0	2.2	4.3	2.7	1.7	1.5	0.8	1.1
16 : 1	12.3	12.3	16.4	12.3	8.7	5.2	2.3
18 : 1	8.7	20.2	13.3	6.8	3.6	1.3	0.8
20 : 1	2.4	7.3	3.4	0.8	1.7	0.4	1.1
18 : 4n-3	4.8	0.3	1.6	5.5	7.8	11.7	16.3
20 : 5n-3	30.4	5.4	17.3	34.3	43.2	53.1	51.6
22 : 6n-3	7.0	4.2	6.7	6.4	6.3	5.3	3.5
Saturates	22.4	34.1	30.0	21.8	15.0	7.7	4.6
Monoenes	25.7	51.1	36.2	21.8	16.2	9.6	7.4
Polyenes	51.8	14.5	33.6	56.4	68.7	82.6	88.0
Branched	0.1	0.3	0.2		0.1	0.1	
Total UI*6	269.1	119.3	194.1	284.4	339.6	397.3	411.9

*1 Each fraction contained triglycerides (TG) separated by column chromatography.
*2 % to the scallop lipids (A : 4,967 mg).
*3 % to the original TG in the scallop lipids.
*4 Data were obtained by TLC on silicic acid plates using the developing solvent of *n*-hexane/diethyl ether/acetic acid (80 : 20 : 1, v/v/v).
*5 Expressed as peak area percentage.
*6 Unsaturation index = fatty acid % × number of fatty acid double bonds.

which was activated for 5 h at 110°C and packed with *n*-hexane. Elutions were done with a solvent series of *n*-hexane (360 or 500 ml), 5% diethyl ether in *n*-hexane (1,800 or 2,500 ml), 10% diethyl ether in *n*-hexane (720 or 1,000 ml), diethyl ether (360 or 500 ml), and methanol (720 or 1,000 ml). Fractions of each of the 360 ml or 500 ml eluants were collected and identified by thin-layer chromatography (TLC). Flow rate of the eluent was ca. 3.3 ml/min and the column was kept at ambient temperature.

Lipid composition analysis by TLC and TLC-flame ionization detection, separation of original TG from the crude lipids by TLC, preparation of fatty acid methyl esters and determination of the compounds by gas-liquid chromatography were all performed as described in a previous report[7].

Results and Discussion

The scallop hepatopancreas obtained from three local varieties (A, B and C) contained 24.1, 8.2 and 6.8% crude lipids, which were characterized by relatively

Table 2. Elution sequence and fatty acid composition of triglyceride fractions obtained from scallop lipids (B) by silicic acid column chromatography.

	Original TG	Fraction*1					
		1	2	3	4	5	6
Yield mg		68	1180	896	563	591	336
%*2	86.5	1.6	28.1	21.3	13.4	14.1	8.0
%*3	100	1.9	32.5	24.6	15.5	16.3	9.2
Rf value*4	0.57	0.61	0.60	0.57	0.55	0.52	0.50
Fatty acid %*5							
14 : 0	5.0	3.2	5.1	5.0	4.3	3.9	1.9
16 : 0	17.0	21.8	22.6	16.6	13.0	8.4	2.5
18 : 0	2.0	4.0	3.0	1..9	1.6	1.1	0.6
16 : 1	12.6	11.7	14.6	13.3	10.1	8.4	4.1
18 : 1	10.6	18.5	16.8	10.9	7.7	4.4	1.1
20 : 1	2.4	6.4	4.2	2.4	1.6	0.4	
18 : 4n-3	3.4	0.6	0.6	2.8	4.9	6.1	8.6
20 : 5n-3	24.5	4.8	9.0	22.3	31.2	41.7	57.7
22 : 6n-3	10.4	10.5	10.8	12.0	11.4	10.4	8.4
Saturates	26.0	31.7	33.2	25.5	20.8	15.0	6.2
Monoenes	28.1	42.8	39.3	28.6	21.6	15.5	7.6
Polyenes	45.6	24.9	27.0	45.6	57.2	69.2	86.0
Branched	0.3	0.6	0.5	0.3	0.4	0.3	0.2
Total UI*6	248.9	158.3	169.9	247.8	294.6	345.8	420.2

*1 Each fraction contained triglycerides (TG) separated by column chromatography.
*2 % to the scallop lipids (B : 4,199 mg).
*3~6 See footnotes of Table 1.

high levels of TG (72.9, 86.5 and 56.7%) followed by phospholipids (15.0, 9.5 and 31.3%), respectively. The sardine tissues and common squid livers examined yielded 17.4 and 21.6% crude lipids, respectively. They predominantly consisted of TG (88.3 and 75.2%) with phospholipids (9.2 and 11.8%), respectively. Small amounts of steryl esters, diacyl glyceryl ethers, free fatty acids, sterols, and partial glycerides were also detected in the crude lipid samples. Percentages of 20 : 5n-3 in the original TG of the crude lipids ranged from 20.6% to 30.4% for scallop and from 14.2% to 17.4% for sardine and squid.

Using silicic acid column chromatography, the different TG fractions were separated from the crude lipids of scallop, sardine and squid according to differences in degree of unsaturation. The elution sequence and fatty acid composition of TG fractions from the scallop lipids (A), (B) and (C) are given in Tables 1, 2 and 3, respectively. The chromatographic results and fatty acid composition of the TG separated from the sardine and squid lipids are tabulated in Tables 4 and 5, respectively. Also included in Tables 1 to 5 are the fatty acid compositions of original TG

Table 3. Elution sequence and fatty acid composition of triglyceride fractions obtained from scallop lipids (C) by silicic acid column chromatography.

	Original TG	Fraction*1				
		1	2	3	4	5
Yield mg		766	901	520	540	181
%*2	56.7	14.9	17.6	10.1	10.5	3.5
%*3	100	26.3	31.0	17.9	18.6	6.2
Rf value*4	0.47	0.50	0.47	0.46	0.44	0.43
Fatty acid %*5						
14 : 0	2.0	4.4	4.7	3.5	3.1	1.6
16 : 0	15.1	21.0	18.2	13.4	9.5	3.1
18 : 0	3.4	4.9	3.0	2.3	1.8	1.0
16 : 1	11.1	14.6	16.6	12.5	9.7	5.0
18 : 1	14.8	18.8	14.2	9.8	6.5	2.0
20 : 1	4.6	6.3	3.4	2.6	1.6	0.3
18 : 4n-3	3.3	0.4	1.6	4.9	7.9	12.7
20 : 5n-3	20.6	5.2	15.9	26.6	34.2	47.1
22 : 6n-3	9.9	6.3	7.9	9.5	8.6	8.5
Saturates	22.7	34.0	28.4	21.4	16.4	7.0
Monoenes	31.5	46.3	36.0	26.7	19.9	10.0
Polyenes	45.2	18.5	35.2	51.7	63.4	82.9
Branched	0.6	1.2	0.4	0.2	0.3	0.1
Total UI*6	240.7	129.4	199.0	270.8	315.9	397.3

*1 Each fraction contained triglycerides (TG) separated by column chromatography.

*2 % to the scallop lipids (C : 5,132 mg).

*3~6 See footnotes of Table 1.

in the crude lipid samples. In all cases, from the original TG in the crude lipids five to six well-separated TG fractions were eluted with 5% diethyl ether in *n*-hexane as the eluent, except for the final TG fraction, which was with 10% diethyl ether in *n*-hexane. All of the isolated initial TG fractions from the crude lipid samples were accompanied by steryl esters and/or diacyl glyceryl ethers. In the chromatographic runs for the scallop lipids (Table 1) and squid lipids (Table 5), the final TG separated fractions were contaminated by relatively high levels of free fatty acids.

In silicic acid column chromatography of the different marine lipids examined, each TG fraction showed various mobility in TLC. As given in Tables 1 to 5, Rf values of TG in the fractions were slightly reduced in their order of separation. These TG were differentiated from each other in TLC by degree of unsaturation. This result was in agreement with that previously reported by Mangold and Malins[8].

The TG fractions were separated according to the number of double bonds in the fatty acyl residues by silicic acid column chromatography. As shown in Tables 1 to 5, the separated TG fractions from the original TG in the crude lipids of scallop,

Table 4. Elution sequence and fatty acid composition of triglyceride fractions obtained from sardine lipids by silicic acid column chromatography.

	Original TG	Fraction*1					
		1	2	3	4	5	6
Yield mg		91	1335	894	528	445	261
%*2	88.3	2.3	33.2	22.2	13.1	11.1	6.5
%*3	100	2.6	37.6	25.1	14.9	12.5	7.3
Rf value*4	0.41	0.47	0.44	0.42	0.41	0.40	0.37
Fatty acid %*5							
14 : 0	9.2	6.5	8.7	10.6	11.4	8.4	5.0
16 : 0	18.0	24.4	23.7	18.6	13.6	10.0	4.7
18 : 0	3.3	5.5	4.3	2.8	2.4	1.8	2.3
16 : 1	9.0	6.8	9.3	10.5	8.8	8.0	5.1
18 : 1	13.7	20.8	20.2	13.2	9.0	5.8	2.9
20 : 1	2.1	8.1	4.6	1.4	1.1	0.3	0.4
22 : 1	1.7	7.5	3.6	1.1	0.3	0.3	0.2
18 : 4n-3	1.9	0.1	0.4	1.6	3.4	4.3	5.9
20 : 5n-3	17.4	2.0	5.2	16.6	23.3	33.9	39.7
22 : 6n-3	7.4	8.2	7.6	7.7	6.9	7.0	5.5
Saturates	33.0	38.8	39.2	34.4	30.6	22.9	14.5
Monoenes	28.6	44.3	39.0	27.6	21.5	17.1	12.7
Polyenes	38.0	14.6	20.8	37.5	47.8	59.7	72.8
Branched	0.4	2.3	1.0	0.5	0.1	0.2	
Total UI*6	209.9	117.9	138.9	207.0	244.3	302.1	351.8

*1 Each fraction contained triglycerides (TG) separated by column chromatography.
*2 % to the sardine lipids (4,026 mg).
*3~6 See footnotes of Table 1.

sardine and squid were made up of much larger quantities of saturated and/or monoenoic fatty acids in fr. 1 and 2 and of polyenoic ones in fr. 5 and 6. In the isolating TG fractions, percentages of polyenoic fatty acids such as 20 : 5n-3 and 18 : 4n-3 increased in their order of separation, except for 22 : 6n-3. This indicated that 22 : 6n-3 may enter into such combination with saturated and/or monoenoic fatty acids in the TG molecules.

Percentages of saturated, monoenoic and polyenoic fatty acids and total unsaturation indices of the fractionating TG from the original TG in the crude lipids of scallop (A), sardine and squid are illustrated in Fig. 1. As shown in Fig. 1, the unsaturation indices of the TG in fractions produced nearly linear plots. They corresponded to a decrease in saturated and/or monoenoic fatty acid percentages and to an increase in polyenoic ones. Variations in percentages of 20 : 5n-3 and 22 : 6n-3 in the separating TG from the original TG in the crude lipids of scallops (A, B

Table 5. Elution sequence and fatty acid composition of triglyceride fractions obtained from squid lipids by silicic acid column chromatography.

	Original TG	Fraction*1					
		1	2	3	4	5	6
Yield mg		184	1406	744	400	226	42
%*2	75.2	4.6	35.2	18.6	10.0	5.7	1.1
%*3	100	6.1	46.8	24.8	13.3	7.5	1.4
Rf value*4	0.65	0.67	0.66	0.63	0.59	0.55	0.51
Fatty acid %*5							
14 : 0	5.0	2.9	4.7	6.1	4.6	4.3	2.3
16 : 0	19.6	19.4	22.5	18.8	14.6	11.1	5.1
18 : 0	2.4	3.5	3.2	1.7	1.2	1.0	0.8
16 : 1	5.8	4.6	5.4	7.0	5.8	5.6	3.5
18 : 1	17.8	20.0	21.8	16.0	11.5	8.7	4.7
20 : 1	6.3	12.2	9.1	3.6	1.8	0.9	0.7
22 : 1	3.0	7.3	4.3	1.2	0.7	0.7	
18 : 4n-3	0.9	0.1	0.2	0.8	2.2	4.2	7.4
20 : 4n-3	3.8	2.7	2.9	4.9	4.6	3.9	3.7
20 : 5n-3	14.2	5.4	7.6	15.7	25.0	31.8	38.1
22 : 6n-3	12.5	10.4	10.1	13.7	16.6	14.8	15.5
Saturates	28.4	27.0	31.8	28.5	22.0	17.9	9.6
Monoenes	34.1	46.8	41.9	29.2	21.1	17.5	11.5
Polyenes	36.2	22.1	24.5	41.7	56.7	64.2	78.5
Branched	1.3	4.1	1.8	0.6	0.2	0.4	0.4
Total UI*6	214.4	158.1	164.2	233.8	301.7	329.1	386.8

*1 Each fraction contained triglycerides (TG) separated by column chromatography.
*2 % to the squid lipids (3,994 mg).
*3~6 See footnotes of Table 1.

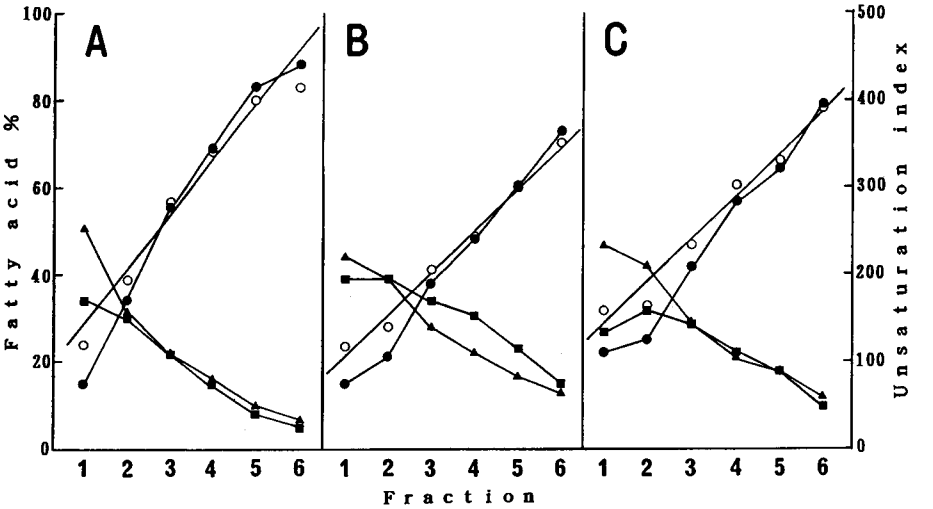

Fig. 1. Percentages of saturated, monoenoic and polyenoic fatty acids and total unsaturation indices of the TG separated from the crude lipids examined. A: Scallop lipids, B: Sardine lipids, C: Squid lipids. ■, Saturates; ▲, Monoenes; ●, Polyenes; ○, Total unsaturation index.

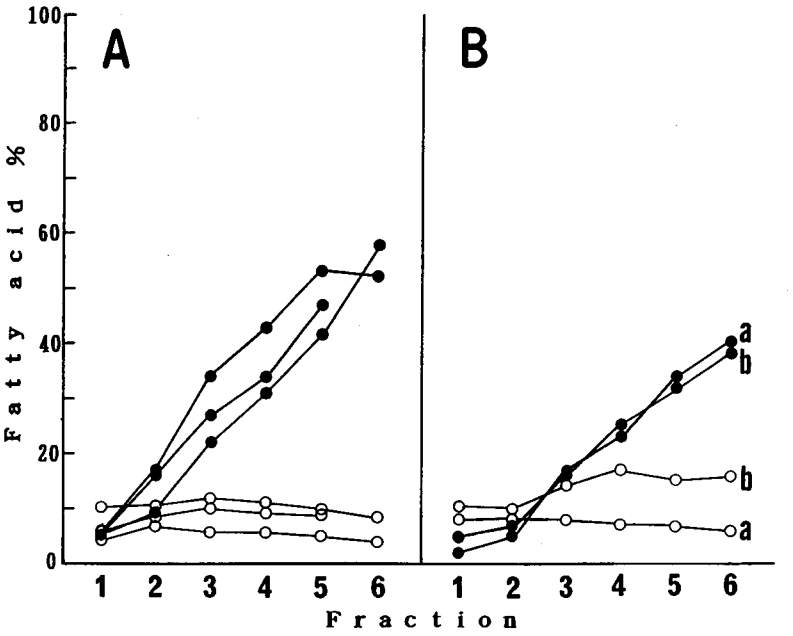

Fig. 2. Percentages of 20: 5n-3 and 22: 6n-3 in the TG separated from the crude lipids examined. A: Scallop lipids (three different samples), B: Sardine lipids (a) and squid lipids (b). ●, 20: 5n-3; ○, 22: 6n-3.

and C), sardine and squid examined are also shown in Fig. 2.

Finally, the isolated polyunsaturated TG, which contained considerable amounts of 20 : 5n-3 (47.1-57.7% for the scallop and 31.8-39.7% for the sardine and squid), yielded 3.5-8.8% and 5.7-6.5% of the crude lipid samples. Therefore, the 20 : 5n-3 in the TG was concentrated to 1.7-2.4-fold and 2.2-2.3-fold, respectively. The preparative scale isolation of 20 : 5n-3-enriched TG from the marine lipids were done by column chromatography on silicic acid. Silicic acid column chromatography used in this study is a rapid and simple method in separating highly polyunsaturated TG and applicable for large scale preparation of the compounds.

Acknowledgments

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