Fatty Chain Composition of Phospholipids from Muscle of Crayfish, *Procambarus clarkii*

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The lipids of the crayfish muscle, composed simply of phospholipids (78%) and free sterols (20%), were analyzed precisely, since furan fatty acids (F acids) had previously been found in the neutral lipids from hepatopancreas of the animal. The analysis yielded the following findings. (i) The main phospholipids were choline phosphoglycerides (CPG, 40.3%) and ethanolamine phosphoglycerides (EPG, 16.6%). (ii) The fatty acid analysis of EPG and CPG (mixtures of diacyl and ether-containing phosphoglycerides) showed that the predominant acyl chains at the *sn*-1 position were 18:1 in EPG and 16:0 in CPG, and that acyl chains at the *sn*-2 position were rich in polyunsaturated fatty chains. (iii) F acids were detected at the *sn*-2 position of EPG. The profile of F acids in the total phospholipid fraction was the same as that in the case of the hepatopancreas. (iv) EPG and CPG contained significant amounts of ether-containing phosphoglycerides (40% and 45%, respectively). (v) In the ether-containing phosphoglycerides, 1-*O*-alkenyl and 1-*O*-alkyl chains were composed of saturated, monounsaturated, and branched chains. Acyl chains at the *sn*-2 position consisted of polyunsaturated chains for the most part and of phytanic chains in a small amount.

Keywords crayfish; muscle; ether-containing phosphoglyceride; furan fatty acid; phytanic acid

Introduction

It is known that the lipids of the crustacean muscle and gills are particularly rich in phospholipids; they contain little neutral lipid except cholesterol.[1-3] The metabolic balances of phospholipids as membrane components during changes of environmental conditions such as temperature and salinity have been studied.[4-8] However, relatively little is yet known of the details of the lipid composition. Previously, we reported on the lipid composition of the crayfish hepatopancreas with emphasis on furan fatty acids (F acids).[9,10] This paper describes a study on the crayfish muscle phospholipids, which also contain F acids. We have already reported on the F acid-containing phospholipids in salmon roe in the previous article.[11]

Experimental

Extraction and Fractionation of Lipids from Crayfish Muscle Crayfish (*Procambarus clarkii*) were captured at Inbanuma, Chiba Prefecture, in October 1984. The animals (50—70 g) were kept alive in tap water at room temperature for at least two weeks. The lipids were extracted from the muscles by the method of Bligh and Dyer. The main phospholipids, ethanolamine phosphoglycerides (EPG) and choline phosphoglycerides (CPG), were isolated by silicic acid column chromatography.[11]

Analysis of Phospholipids The determination of phospholipid classes was carried out by the method described in the preceding paper.[11]

Separation and Determination of 1-*O*-(1-Alkenyl)-2-acyl (Alkenylacyl), 1-*O*-Alkyl-2-acyl (Alkylacyl), and 1,2-Diacyl EPG and CPG The polar head group of EPG and CPG was removed by treatment with phospholipase C (*Bacillus cereus*) by the method of Watanabe *et al.*[12] The resulting mixture was made less polar by acetylation according to the method of Sugiura *et al.*[13] The acetylated mixture (1,2-diradyl-3-acetylglycerols) was separated into alkenylacyl-, alkylacyl-, and diacyl-3-acetylglycerols according to the method of Sugiura *et al.*[13] The relative amounts of the three classes were estimated by determining the amounts of acyl residues in these classes using 22:0 methyl ester as an internal standard for gas liquid chromatographic (GLC) analysis.

Analysis of Fatty Acids Fatty acyl residues of lipids were obtained as their methyl esters by the use of 0.5 M CH_3ONa in methanol, followed by purification by thin layer chromatography (TLC) on silica gel G using hexane–benzene (2:1, v/v). The fatty acids were analyzed using GLC and GLC-mass spectrometry (GLC-MS). Fatty acid positional analysis of the total EPG and CPG fractions using phospholipase A_2 (*Naja naja* venom) was performed by the method of the previous paper.[11] The F acids were concentrated by the selective hydrogenation and urea fractionation of fatty acid methyl esters from the total phospholipid fraction.[11]

Analysis of 1-*O*-(1-Alkenyl) Chains The fatty aldehydes were liberated from 1-*O*-(1-alkenyl)-2-acyl-3-acetylglycerols by the use of concentrated HCl according to the method of Anderson *et al.*[14] The fatty aldehydes were purified by preparative TLC on silica gel G using hexane–benzene (2:1, v/v). The *N,N*-dimethylhydrazone derivatives of aldehydes were prepared according to the method of Johnson *et al.*[15] In order to determine the location of methyl branches, the fatty aldehydes were converted to the fatty acids using argentic oxide in tetrahydrofuran–water (9:1, v/v) at room temperature for 14 h according to the method of Corey *et al.*[16] The resulting fatty acids were heated at 100 °C for 3 h with 5% HCl in methanol to yield the corresponding methyl esters and analyzed by GLC.

Analysis of 1-*O*-Alkyl Chains 1-*O*-Alkyl-2-acyl-3-acetylglycerols were reduced to 1-*O*-alkylglycerols with Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] by the method of Snyder *et al.*[17] The isopropylidene derivatives of 1-*O*-alkylglycerols were prepared according to the method of Wood.[18] To determine the location of methyl branches, alkenylglycerols already identified were hydrogenated with Adams catalyst. The resulting alkylglycerols were converted to the corresponding isopropylidene derivatives, which were used as standards for GLC analysis.

GLC and GLC-MS (i) GLC: GLC was performed using a Hewlett Packard (HP) 5710 A instrument equipped with a model 3390 A reporting integrator and a fused silica capillary column (0.3 mm i.d. × 25 m) coated with Carbowax 20 M. On every capillary GLC analysis, the carrier gas was He at a flow rate of 1 ml/min and the split ratio was 25:1. To analyze fatty acid methyl esters, the column temperature was first kept at 160 °C for 5 min and then increased to 220 °C at 3 °C/min. To analyze long chain fatty aldehydes and *N,N*-dimethylhydrazone derivatives, the program of 160 to 220 °C at 2 °C/min was used. For the analysis of the isopropylidene derivatives of alkylglycerols, the program of 180 to 220 °C/min was required. The flow rate of carrier gas (He) was 2 ml/min. The split ratio was 25:1.

(ii) GLC-MS: GLC-MS was performed with an HP 5995A instrument controlled by a HP 9825 A desktop computer system. The fused silica capillary column used for GLC-MS analysis was the same one as used for GLC. In the mass spectrum of fatty aldehydes using the electron-impact (EI) technique with this instrument, no molecular ion was present, but characteristic hydrocarbon fragments were predominant. To compensate for the absence of the molecular ion in the mass spectrum, the *N,N*-dimethylhydrazone derivatives of aldehydes were analyzed (M^+ relative intensity: 17—23%). For analysis of the isopropylidene derivatives, the base peak *m/z* 101 and the $M^+ - 15$ fragment formed by the loss of CH_3 of these derivatives were monitored. To determine the locations of double bonds and methyl branches, the analysis of *N*-acyl pyrrolidide derivatives was performed using a fused silica capillary column (0.3 mm i.d. × 25 m) coated with Carbowax 20 M, programmed from 220 to 240 °C at 3 °C/min. The ion source temperature was 270 °C. All spectra were recorded at an ionization potential of 70 eV.

Results and Discussion

Composition of Phospholipids The lipids of the crayfish muscle were composed of phospholipids (77.6%) and ste-

rols (20%). Only a small amount of neutral lipids except sterols was observed. The major phospholipids were CPG and EPG (Table I). Both phospholipids contained a large amount of ether-containing phosphoglycerides (about 40%—45% in each case) (Table II). It has just been reported that ether-containing phosphoglycerides are also present in Pacific crabs, a species of crustaceans.[1]

TABLE I. Lipid Composition of Crayfish Muscle[a]

	% of total lipids
Phospholipids	
CPG	40.3±2.8
EPG	16.6±2.1
Serine phosphoglycerides	6.5±1.5
Inositol phosphoglycerides	3.3±0.6
Sphingomyelin	3.3±0.4
Cardiolipin	2.6±1.2
Phosphatidic acid	0.4±0.1
Methanol phosphoglycerides[b]	4.6±0.6
Neutral lipids	
Free sterols	20.0±1.5
Others[c]	2.0±2.5

a) Means (weight %)±S.D. of nine analyses. *b*) Artifact formed during extraction with methanolic solvent. *c*) Triacylglycerol and unknown pigments.

TABLE II. Class Composition of EPG and CPG of Crayfish Muscle

	EPG (N=3)	CPG (N=4)
1-*O*-(1-Alkenyl)-2-acyl	26.2±3.8	9.3±2.7
1-*O*-Alkyl-2-acyl	14.8±0.1	35.7±2.6
1,2-Diacyl	58.9±3.4	54.8±1.1

Data are presented as means (weight %)±S.D. of N analyses.

Positional Distribution of Fatty Acids TLC analysis was a necessary procedure to classify ether-containing phosphoglycerides. However, F acids which could be detected in the total fatty acids of the whole phospholipids disappeared due to decomposition during the process of TLC analysis. Consequently, it was necessary to employ column chromatography to separate the phospholipids into EPG, CPG, and the other fractions without segregating ether-containing lipids from diacyl type lipids. Positional analysis of fatty acids in the total EPG and CPG fractions (diacyl and ether-containing phosphoglycerides) was carried out by phospholipase A_2 hydrolysis to the *sn*-2 acyl chains, followed by CH_3ONa–MeOH methanolysis of the resulting lyso phospholipids, to the *sn*-1 acyl chains (Table III). The predominant acyl chains at the *sn*-1 position of only the diacyl phosphoglycerides were 18:1 in EPG and 16:0 in CPG. On the other hand, the major acyl chains at the *sn*-2 position of EPG (mixture of three classes) were 20:4 (*n*-6), 20:5 (*n*-3), and 22:6 (*n*-3), while those of CPG were 18:1, 18:2 (*n*-6), 20:4 (*n*-6), and 20:5 (*n*-3). The polyunsaturated fatty acids were predominantly seen at the *sn*-2 position. The disposition was marked in the EPG fraction. It is noteworthy that F acid (F_6) was detected at the *sn*-2 position of EPG, not at the *sn*-1 position. Small amounts of F acids were seen in the CPG, but their position could not be decided.

Fatty Chain Composition of Ether-Containing Phosphoglycerides The fatty chain composition of ether-containing (alkenylacyl and alkylacyl) phosphoglycerides of EPG and CPG, which were separated by TLC, are shown in Table IV. At the *sn*-1 position of every fraction, saturated chains such as 16:0 and 18:0 were predominant,

TABLE III. Fatty Acid Distribution of Total EPG and CPG Fractions[a] (Mixture of Diacyl and Ether-Containing Phosphoglycerides)

	EPG				CPG			
	Total (N=3)	Expected[b]	*sn*-1 (N=2)	*sn*-2 (N=2)	Total (N=3)	Expected[c]	*sn*-1 (N=2)	*sn*-2 (N=2)
14:0							1.5±0.4	
i 15:0							0.5±0.2	
15:0					1.0±0.2		1.9±0.1	
16:0	3.2±0.2	3.5	7.2±0.4	1.4±0.2	16.8±0.4	15.7	35.5±2.7	4.8±1.2
16:1 (*n*-7,5)	1.6±0.5	1.7	1.8±0.2	1.6±0.2	5.1±1.6	4.6	5.3±0.9	4.2±0.9
i 17:0			1.0±0.1		0.7±0.2		1.6±0.4	
a 17:0					0.5±0.2		0.8±0.3	
17:0	0.7±0.2		1.6±0.4		1.2±0.5		3.0±0.5	
17:1 (*n*-8)						0.9	0.8±0.5	0.9±0.7
18:0	4.0±0.7		9.1±0.5		2.8±0.2		7.1±0.3	
18:1 (*n*-9,7)	18.3±1.8	18.9	42.6±1.4	5.0±1.8	24.6±1.2	24.1	19.2±1.1	26.8±2.5
18:2 (*n*-6)	6.3±0.4	6.1	8.7±1.5	4.6±1.4	8.7±0.5	8.6	6.0±0.9	10.1±1.7
18:3 (*n*-3)	2.8±0.9	2.7	2.9±0.2	2.6±1.1	3.7±0.2	3.7	2.7±0.6	4.3±0.2
20:1 (*n*-9)			2.0±0.2		1.2±0.5	1.2	1.5±1.1	1.1±0.3
20:0 (*n*-6)	1.1±0.1		1.9±0.4		1.2±0.4	1.2	1.8±0.9	0.8±0.1
20:3 (*n*-6)								0.6±0.1
(*n*-3)						0.6	0.5±0.3	0.6±0.6
20:4 (*n*-6)	14.6±0.3	13.5	5.6±2.1	18.2±0.1	7.3±0.2	7.6	1.7±1.0	10.9±1.5
20:5 (*n*-3)	26.9±1.6	25.2	8.1±1.9	35.3±2.1	13.4±0.3	14.5	2.2±0.4	21.3±3.8
22:5 (*n*-6)	1.8±0.7			2.5±0.1	0.9±0.4			1.5±0.6
(*n*-3)	1.9±0.9	1.8	0.5±0.1	2.6±0.1	1.7±0.2			1.6±1.5
22:6 (*n*-3)	9.7±0.3	9.8	1.2±0.2	14.9±2.6	3.4±0.4	3.7	0.7±0.2	5.3±2.3
F_6	1.5±0.2			1.7±0.2	(0.4)			

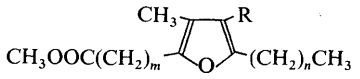
Abbreviations; i=iso, a=anteiso. *a*) Data are presented as the mean (>0.5%)±S.D. of N determinations. *b*) $\frac{58.9}{100+58.9}\times(\%)$ at *sn*-1 position + $\frac{100}{100+58.9}\times(\%)$ at *sn*-2 position. *c*) $\frac{54.8}{100+54.8}\times(\%)$ at *sn*-1 position + $\frac{100}{100+54.8}\times(\%)$ at *sn*-2 position.

TABLE IV. Fatty Chain Compositions of the Ether-Containing EPG and CPG

	1-*O*-Alkenyl				1-*O*-Alkyl			
	EPG		CPG		EPG		CPG	
	sn-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
i 14:0							0.7±0.4	
14:0		0.6±0.1	1.6±0.4	0.8±0.1	1.1±0.2		6.5±1.4	
14:1							1.3±2.5	0.7±0.5
i 15:0	2.3±0.6		5.6±0.8		4.7±0.4		9.8±2.0	
a 15:0			1.3±0.2		0.7±0.1		3.6±0.2	
15:0	1.4±0.6		2.4±0.5	0.6±0.1	1.8±0.4		4.1±0.2	
15:1				0.6±0.1			1.5±0.5	
i 16:0	1.1±0.2		1.5±0.4		1.0±0.5		1.1±0.4	
16:0	28.7±3.3	2.5±1.2	34.7±0.4	4.4±2.4	33.7±1.8	4.4±2.9	28.1±1.7	2.6±1.0
16:1	2.5±1.1	(*n*-7,5) 3.4±2.3	5.7±0.9	(*n*-7,5) 3.9±2.7	11.8±1.4	(*n*-7,5) 2.1±0.9	22.4±1.3	(*n*-7,5) 3.1±1.6
i 17:0	4.1±2.8		6.1±2.3		2.6±0.2		1.5±0.8	
a 17:0	2.1±0.9		3.1±2.0		0.9±0.3		0.5±0.4	
17:0	7.2±2.1		6.3±1.0	0.7±0.4	5.3±0.2		2.3±0.6	
Phy						0.6±0.1		1.4±0.9
17:1		(*n*-8) 0.7±0.2	0.8±0.5	(*n*-8) 0.9±0.3	2.4±0.6	(*n*-8) 0.6±0.1	1.5±0.2	
i 18:0	1.3±1.1		1.4±0.8		0.6±0.4			
18:0	36.3±2.9	0.6±0.1	16.5±2.5	1.2±0.9	23.4±2.1	3.7±0.9	8.7±1.1	0.7±0.3
18:1	9.1±2.6	(*n*-9,7) 10.0±2.9	10.1±0.6	10.4±2.1	6.3±0.4	(*n*-9,7) 15.7±5.0	3.5±1.3	(*n*-9,7) 11.1±4.2
18:2	1.0±0.3	(*n*-6) 5.7±0.5	1.0±0.4	(*n*-6) 5.2±0.6		(*n*-6) 5.7±2.3		(*n*-6) 6.6±1.6
18:3 (*n*-3)		3.8±0.3		3.9±0.9		2.2±0.4		4.6±1.6
19:0					1.9±0.2		1.0±0.7	
20:0					0.7±0.4		1.0±0.6	
20:1 (*n*-9)		0.5±0.3		0.5±0.3		0.9±0.4	0.8±0.9	0.5±0.3
20:2 (*n*-6)						0.9±0.3		0.5±0.2
20:4 (*n*-6)		10.9±1.7		15.2±2.1		11.6±1.9		14.3±3.0
20:5 (*n*-3)		34.7±1.9		39.1±3.4		19.8±3.4		38.7±4.6
22:5 (*n*-6)		1.4±0.5		0.6±0.1		2.3±1.3		1.0±1.3
(*n*-3)		2.0±0.4		0.8±0.5		3.4±0.8		1.0±0.8
22:6 (*n*-3)		19.5±4.9		8.4±0.4		22.8±6.3		10.9±1.5

Data are presented as the mean (>0.5%)±S.D. of three determinations done on the various purified phosphoglycerides.

TABLE V. F Acid Composition of Phospholipids from Crayfish Muscle



F acids[a]	CCL[b]	*m*	*n*	R	%[c]
F_1	16	8	2	CH_3	4.9
F_{13}	17	8	3	CH_3	0.6
F_2	18	8	4	H	13.9
F_{15}	18	10	2	H	0.7
F_3	18	8	4	CH_3	30.1
F_4	18	10	2	CH_3	10.8
F_{21}	19	9	4	CH_3	1.6
F_5	20	10	4	H	4.6
F_6	20	10	4	CH_3	29.9
F_{iso33}		Olefinic F_3[d]			0.9
F_{29}	22	12	4	H	1.4

a) See reference 10 for identities of numbered peaks. *b*) Carbon chain length. *c*) Weight (%) of the total F acids. *d*) F_3 methyl ester with one additional double bond, conjugated with the furan ring.

with smaller amounts of monounsaturated chains of 16:1 and 18:1. Odd-numbered chains and the "iso and anteiso" type chains as well were observed to some extent at the *sn*-1 position, but not at the *sn*-2 position in both EPG and CPG. At the *sn*-2 position, the main acyl chains were long chain fatty acids which were hightly unsaturated (over 20:4) and accounted for about 65% of the total. Furthermore, a small amount of phytanic acyl chain was detected only at the *sn*-2 position of alkylacyl phosphoglycerides. Phytanic acids have been generally considered to be present at the *sn*-1 position of diacyl phosphoglycerides as seen in the case of the Refsum's disease.[19]

We reported that F acids in salmon roe lipids were linked exclusively to the *sn*-1 position of the phosphoglycerides.[11] The phosphoglycerides, composed of only diacyl type, were different from those of crayfish (containing large amounts of ether type). As for F acids, Sand *et al.*[20] reported that labeled acetic acid was not incorporated into the furan ring of the acid in fish. The experiment means that F acids are not *de novo* synthesized in the body of fish but are taken from foods in the same manner as phytanic acid. Both F acids and phytanic acid, which are not regarded as ordinary physiological components, may be incorporated into phospholipids in a similar manner. From this point of view, it is supposed that F acids found at the *sn*-2 position are linked to the ether lipids like phytanic acid.

The profile of F acids in the muscle phospholipids concentrated by the combination of catalytic hydrogenation and urea fractionation was almost the same as that[10] in the sterol esters of the hepatopancreas (Table V).

The F acids detected in specific animals[10,21-23] and plants[24] have not yet been fully identified. In a forthcoming report, we will propose that the choline phosphoglycerides containing F acids have an antioxidant activity as one of their biological functions.

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