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Composition and Positional Distribution of Fatty Acids in Phospholipids Isolated from Pork Muscle Tissues[1,2]

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Abstract

The composition and positional distribution of fatty acids in phospholipids isolated from four locations of a hog carcass is presented. Variations in fatty acid composition of phospholipids were found depending upon the location in the carcass. The total unsaturated fatty acid content averaged 34.3 mole % for lecithin, 52.5 mole % for phosphatidylethanolamine, 40.3 mole % for phosphatidylserine and 41.3 mole % in sphingomyelin. The cephalins had a much higher percentage of polyunsaturated fatty acids than lecithin. The chief saturated fatty acid in lecithin and sphingomyelin was palmitic and in cephalins it was stearic. A snake venom enzyme preparation *(Crotalus adamanteus)* hydrolyzed primarily unsaturated fatty acids in phosphoglycerides and the higher the percentage of unsaturation within the fatty acid the higher percentage of hydrolysis occurred. The unsaturated fatty acids were found chiefly at the the β-position and the saturated fatty acids at the α-position in the phosphoglycerides.

Introduction

THE PHOSPHOLIPIDS of blood and of neural and organ tissue of many animals have been investigated relative to kind, composition and, in some cases, to positional location of the fatty acids. Relatively little attention has been given to the phospholipid content of skeletal tissue and especially to their presence in such tissues of meat animals, although it is suspected that phospholipids may be important in keeping quality and flavor. Hornstein et al. (4) determined the phospholipids, as general classes, in pork and beef, and Kuchmak and Dugan (1) determined the specific phospholipids in four locations in a hog carcass. Younathan and Watts (10) showed that the bound lipids in pork and Zipser et al. (11) showed the bound lipids in mullet were implicated in oxidative deteriorations.

This study was aimed first at elucidation of the fatty acid composition of the phospholipids of pork muscle tissue as influenced by carcass location and subsequently at the elucidation of the positional location of the fatty acids in the phosphoglyceride moieties.

Experimental

Materials. Silicic acid, cp, precipitated, was obtained from Fisher Scientific Company. Silicic acid was washed with methanol and reactivated at 120C. All solvents used were freshly redistilled.

Crotalus adamanteus venom, used as a source of phosphotidase A (lecithinase A), was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla.

Methyl esters of fatty acids, used as reference compounds in GLC, were obtained from the California Corp. for Biochemical Research and from Applied Science Laboratories.

Origin of Samples. Samples of muscle tissue were taken from the center belly section, a ham cross section, loin center cut and the 4–6 rib section of a hog of known breed and feeding history (Yorkshire-Hampshire castrate male, five months old, 190 lb standard growth feeding ration.)

Isolation of Phospholipids. Lipid extraction, silicic acid chromatography and procedures for establishment of identity of isolated phospholipid classes have been reported previously (1).

Enzymatic Hydrolysis of Lecithin. Enzymatic hydrolysis of lecithin with snake venom in diethyl ether solution and separation of hydrolysis products were achieved by the procedure of Tattrie (2). The precipitate which resulted after hydrolysis was removed by centrifuging and the ether solution was tested for the presence of phosphorus. If phosphorus was present in the ether solution, indicating the presence of unreacted lecithin, the enzymatic hydrolysis was repeated on another sample. When enzymatic hydrolysis was complete, the ether phase contained fatty acids only. Lysolecithin was recovered from the precipitate by dissolving it in chloroform and centrifuging out the protein. Complete enzymatic hydrolysis was achieved only with the lecithin sample from belly muscles and the best results on the samples from the other three locations on the hog carcass

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TABLE I

Fatty Acid Composition and Location in Lecithin

Acid	Belly			Ham			Loin			Ribs		
	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β
10:0	1.8	1.8	..	4.8	1.7	3.1	2.1	0.9	1.2	2.6	0.7	1.9
12:0	0.9	0.4	0.5	4.0	1.9	2.1	2.2	1.2	1.0	1.5	0.4	1.1
13:0	0.4	....	0.4	2.0	....	2.0	1.0	1.0	....	....	....	....
[a]	0.2	0.1	0.1	1.3	0.9	0.4	0.6	0.4	0.2	0.3	0.1	0.2
14:0	0.5	0.2	0.3	2.0	0.9	1.1	1.2	0.7	0.5	1.0	0.4	0.6
[b]	0.3	0.1	0.2	1.6	....	1.6	0.6	0.6	....	0.4	....	0.4
15:0	0.4	0.1	0.3	3.1	1.8	1.3	1.8	....	1.8	1.7	0.4	1.3
16:0	33.6	31.6	2.0	50.1	36.3	13.8	53.1	36.1	17.0	52.3	36.0	16.3
16:1	0.9	....	0.9	....	....	....	....	....	....	0.9	0.3	0.6
17:0	0.6	0.4	0.2	1.5	0.9	0.6	1.1	0.5	0.6	1.3	0.4	0.9
18:0	5.4	5.1	0.3	4.0	2.1	1.9	8.6	4.7	3.9	8.2	4.8	3.4
18:1	14.7	2.0	12.7	14.3	4.8	9.5	16.9	6.9	10.0	19.3	8.6	10.7
18:2	34.6	1.3	33.3	6.3	2.1	4.2	7.1	1.7	5.4	6.1	1.8	4.3
18:3	0.7	....	0.7	0.8	0.4	0.4	1.0	....	1.0	0.9	....	0.9
20:1	0.8	....	0.8	2.0	0.2	1.8	2.6	0.4	2.2	2.9	1.0	1.9
21:0	1.2	0.1	1.1	0.4	....	0.4	....	....	....	0.2	....	0.2
20:4	2.5	....	2.5	....	....	....	0.2	....	0.2	0.1	....	0.1
22:0	0.4	....	0.4	....	....	....	....	....	....	0.4	....	0.4
22:1	..	....	..	1.8	0.9	0.9	..	....	....	....	....	....

[a] r_x, 16:0 = 0.465.
[b] r_x, 16:0 = 0.647.
r_x, 16:00 = adjusted relative retention volume with palmitic acid as the standard = 1.

achieved between 95 and 97% enzymatic hydrolysis. The fatty acids were freed from the 3–5% of unhydrolyzed lecithin on silicic acid columns.

Enzymatic Hydrolysis of Cephalins. Enzymatic hydrolysis with snake venom of either phosphatidylethanolamine or phosphatidylserine was not successful when applying a procedure similar to that for lecithin hydrolysis in diethyl ether solution. Both cephalins were readily attacked by the enzyme in water solution at 37C. This procedure was similar to that described by Magee et al. (3) for enzymatic hydrolysis of lecithin by phospholipase A from human pancreas. Enzymatic hydrolysis of either cephalin was achieved with 1 mg of snake venom for each 100 mg cephalin. Cephalins were solubilized with sodium deoxycholate in glycyl-glycine buffer, pH 7.3. Products of enzymatic reaction were extracted with chloroform. Most of the chloroform was removed under reduced pressure with a rotary evaporator and evaporation of solvents was complete under a stream of nitrogen. This procedure was required to remove any water which may have dissolved in the chloroform and which would have adverse effects on subsequent separations to be made with the silicic acid columns used in the next step. The residue was taken up with a small volume of chloroform and put on a silicic acid column prepared in chloroform. Fatty acids were eluted from the column with chloroform. Unreacted cephalins, if present, were eluted with 20% methyl alcohol and the lysophosphatidyl cephalins were eluted with methanol. The elution pattern was followed by use of the ninhydrin test using equal volumes of solution from the column, ninhydrin and 2,4-lutidine. No unhydrolyzed cephalin was detected in five out of eight cephalin samples. These were the phosphatidylethanolamine from belly muscles and the phosphatidylserine from all four carcass locations. This indicated that in all these cases the enzymatic hydrolysis of cephalins was complete. The samples of phosphatidyl ethanolamine from ham, loin and rib muscles each showed about 4% of enzymatically unhydrolyzed cephalin.

Preparation of Methyl Esters. The isolated phosphoglycerides and their corresponding lysoderivatives, obtained by selective enzymatic hydrolysis, were saponified with methanolic potassium hydroxide. Fatty acids were freed with concn HCl and then were extracted with petroleum ether. This procedure was similar to that described by Hornstein et al. (4). Fatty acids obtained by alkaline or enzymatic hydrolysis were esterified on the basic ion-exchange resins with anhydrous methanol-HCl solution (5).

Since sphingomyelin is known to be resistant to alkaline hydrolysis (6), it was subjected to hydrolysis and esterification in one step with 5% anhydrous HCl in anhydrous methanol by gentle refluxing for five hr. After hydrolysis and esterification, the resulting methyl esters of the fatty acids were extracted with several successive portions of petroleum ether. The combined petroleum ether extract was washed with water until free of acid, dried over anhydrous sodium sulfate and evaporated under a stream of nitrogen to a volume suitable for GLC.

Gas-Liquid Chromatography. Gas-liquid partition chromatography was carried out on an F and M Scientific Corp. Model 500 gas chromatograph with thermal conductivity detector, using a bridge current of 150 mA, injection port temp 200C, column temp 215C and block temp 250C. Helium was used as the carrier gas. The chromatographic column was composed of copper tubing (0.25 in. internal diameter by 5 ft length) packed with chromosorb W impregnated with 20% diethylene glycol adipate.

The identities of all fatty acids except eicosenoic acid were established by direct comparison of adjusted retention volumes of the methyl esters with those of reference compounds. The assignment for eicosenoic acid was based on the plot of the log of retention volumes versus the number of carbon atoms for monounsaturated fatty acid methyl esters.

Numerical Presentation of Results. The areas of peaks on the GLC were measured with a planimeter and the area percentages for each ester, based on the total area for all esters on the chromatogram were computed. Quantitative relationship between areas for esters and mole percentage of acids present was established by running chromatograms with known wt of the reference compounds.

The sizes of the samples used for gas chromatography were not always evenly matched. Therefore for the distribution of fatty acids between α- and β-positions, the area percentage of fatty acids for each position in the phosphoglyceride was calculated and mole percentage of fatty acids in the unhydrolyzed compound was distributed between the α- and β-positions proportionally to their respective areas.

Results and Discussion

Fatty acid composition and location in lecithin isolated from muscle tissue from four locations on a hog carcass are presented in Table I. Palmitic acid

TABLE II

Fatty Acid Composition and Location in Phosphatidylethanolamine

Acid	Belly			Ham			Loin			Ribs		
	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β
10:0	2.0	1.2	0.8	2.9	2.9	....	5.0	5.0	....	3.0	2.6	0.4
12:0	1.7	1.0	0.7	2.7	1.2	1.5	2.4	1.1	1.3	3.1	1.5	1.6
13:0	0.8	0.5	0.3	....	....	....	0.9	0.5	0.4	1.5	0.7	0.8
[a]	0.3	0.3	....	1.3	0.5	0.8	0.4	0.2	0.2	0.7	0.3	0.4
14:0	0.6	0.3	0.3	0.9	0.3	0.6	0.7	0.4	0.3	1.1	0.5	0.6
[b]	0.8	0.5	0.3	....	....	....	0.5	0.3	0.2	0.9	0.4	0.5
15:0	2.3	1.2	1.1	1.6	0.7	0.9	0.8	0.5	0.3	2.0	1.0	1.0
16:0	3.1	1.6	1.5	5.7	3.3	2.4	7.9	4.8	3.1	6.6	3.6	3.0
16:1	1.1	0.6	0.5	1.5	0.8	0.7	1.1	0.7	0.4	1.9	1.0	0.9
17:0	0.8	....	0.8	1.8	0.8	1.0	1.2	0.6	0.6	1.7	0.9	0.8
18:0	17.9	15.3	2.6	30.6	28.2	2.4	28.9	24.0	4.9	32.8	27.5	5.3
18:1	8.7	4.2	4.5	12.7	3.6	9.1	14.2	5.0	9.2	14.5	5.5	9.0
18:2	36.6	5.6	31.0	28.2	9.2	19.0	24.7	6.0	18.7	20.3	5.9	14.4
18:3	1.4	....	1.4	..	....	....	..	....	....	....	....	....
20:1	1.4	0.8	0.6	1.7	0.7	1.0	3.1	1.7	1.4	3.4	1.5	1.9
21:0	3.3	....	3.3	1.0	0.2	0.8	0.5	0.1	0.4	....	....	....
20:4	17.4	....	17.4	5.3	1.3	4.0	3.0	0.8	2.2	2.3	0.4	1.9
22:0	....	....	....	1.0	0.5	0.5	2.2	1.0	1.2	2.1	1.1	1.0
22:1	....	..	....	1.0	0.6	0.4	2.3	1.7	0.6	2.3	1.4	0.9

[a] rx, 16:0 = 0.469.
[b] rx, 16:0 = 0.645.

was found to be the chief saturated fatty acid, ranging from 33.6 mole % in lecithin from belly muscles to 53.1 mole % in lecithin from the loin. The next major saturated fatty acid was stearic which was present in ca. one-seventh the quantity of palmitic acid. The remaining 7–11 mole % of saturated fatty acids was distributed among eight other identified fatty acids.

The main monounsaturated fatty acid in lecithin was oleic. This ranged from 14.3% in ham muscle lecithin to 19.3% in lecithin from loin. Linoleic acid was the major polyunsaturated fatty acid with content from 6.1 mole % in rib muscle lecithin to 34.6% in lecithin from belly muscles. The total content of unsaturated fatty acids in lecithins from hog carcass muscles varied from 25.2% for that in ham to 54.2% for that in belly muscle and averaged 34.3 mole % for lecithin from all four locations. The ratio between mono- and polyunsaturated fatty acids in lecithin was ca. 1:0.8, respectively.

Saturated fatty acids were found predominantly in the α-position. Approx 74% of palmitic acid and ca. two-thirds of the stearic acid was present in this position. Minor amt of other saturated fatty acids appeared rather randomly scattered between both positions in lecithin.

Unsaturated fatty acids were located predominantly in the β-position and the greater the unsaturation of the acid the greater the percentage of it which was found in the β-position. As an illustration, the average content of oleic, linoleic, linolenic and arachidonic acids in the β-position of lecithin was 66%, 87%, 88% and 100%, respectively, of the total of each acid present in lecithin.

Table II illustrates fatty acid composition and location in phosphatidylethanolamine. Phosphatidylethanolamine had a higher percentage of unsaturated acids than was found in lecithin. An average 52.5% of all acids in phosphatidylethanolamine were unsaturated, ranging from 44.5% in phosphatidylethanolamine from ribs to 66.6% in phosphatidylethanolamine from belly muscles. Polyunsaturated fatty acids in phosphatidylethanolamine were more than twofold greater than found in lecithin, with an average of 34.8%. These ranged from 22.6% in phosphatidylethanolamine from rib muscles to 55.4% in phosphatidylethanolamine from belly muscles. The ratio of mono- to polyunsaturated fatty acids in phosphatidylethanolamine was ca. 1:2, respectively. The main unsaturated fatty acid was linoleic, ranging from 20.3% for phosphatidylethanolamine in rib muscle to 36.6% phosphatidylethanolamine from belly muscle, with an average of 27.5 mole %. The average content of oleic acid was 12.5% and arachidonic 7.0 mole %.

The chief saturated fatty acid in phosphatidylethanolamine was stearic instead of palmitic which was dominant in lecithin. The average content of stearic acid was 27.5%. The amt of palmitic acid in phosphatidylethanolamine was only 5.8% and the ratio between palmitic and stearic acid was ca. 1:5, or close to the inverse ratio for the two acids in lecithin.

As was noted with lecithin, the enzymatic hydrolysis was more pronounced toward the unsaturated fatty acids with a greater percentage liberation of acid with greater unsaturation. An average 64% of oleic, 76% of linoleic and 91% of arachidonic acids present were liberated by the enzyme. Only 14% of stearic acid, which was the predominant saturated acid, was hydrolyzed from phosphatidylethanolamine.

Table III has data concerning fatty acids in phosphatidylserine. These results reval a lower level of total unsaturated and total polyunsaturated fatty acids than was found in phosphatidylethanolamine. The average content of unsaturated fatty acids in phosphatidylserine was 40.3 mole % and polyunsaturated 20.2 mole % or 23% and 42%, respectively, less than in phosphatidylethanolamine. The major unsaturated fatty acids of phosphatidylserine were oleic, linoleic and arachidonic in quantitative ratios of 1:1:0.6, respectively. The major saturated fatty acid in phosphatidylserine was stearic which varied from 23.5 mole % from that in rib muscles to 29.6 mole % from that in belly muscles.

The substantial differences in the content of unsaturated fatty acids between phosphatidylserine and phosphatidylethanolamine could be responsible for obtaining different IR absorption coefficients for the 5.8 μ and 9.8 μ bands, which were common for both cephalins (1).

The distribution of fatty acids between the two positions in phosphatidylserine was close to 1:1 with a max deviation of less than 3 mole %. The unsaturated fatty acids were predominant among those liberated by the enzyme as was also noted with phosphatidylethanolamine and lecithin. The variations in the content of major fatty acids of phosphatidylserine in different locations on the carcass were much less than in phosphatidylethanolamine or lecithin.

The fatty acid composition of sphingomyelin is presented in Table IV. The average total content of unsaturated and polyunsaturated fatty acids in sphingomyelin was higher than in lecithin and was rather

TABLE III

Fatty Acid Composition and Location in Phosphatidylserine

Acid	Belly			Ham			Loin			Ribs		
	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β	Mole %	Distribution α	Distribution β
10:0	....	....	....	7.2	4.4	2.8	8.9	4.1	4.8	4.5	4.5	
12:0	7.2	4.1	3.1	5.5	2.3	3.2	4.9	2.7	2.2	4.9	2.0	2.9
13:0	3.7	2.2	1.5	2.9	1.2	1.7	3.1	1.7	1.4	1.9	0.9	1.0
[a]	2.3	1.4	0.9	2.8	1.1	1.7	1.6	0.8	0.8	1.7	1.0	0.7
14:0	4.2	1.6	2.6	4.8	1.9	2.9	4.1	2.1	2.0	2.5	1.4	1.1
14:1	....	....	....	....	....	....	....	....	....	2.5	1.4	1.1
16:0	5.0	3.0	2.0	5.2	4.5	0.7	4.6	2.6	2.0	4.9	3.0	1.9
16:1	2.0	1.2	0.8	1.6	....	1.6	1.7	0.7	1.0	2.1	1.4	0.7
17:0	3.8	2.3	1.5	3.6	1.5	2.1	3.1	1.8	1.3	4.1	2.3	1.8
18:0	29.6	25.8	3.8	26.7	24.2	2.5	25.7	17.4	8.3	23.5	16.2	7.3
18:1	11.4	1.9	9.5	10.7	2.5	8.2	11.5	5.6	5.9	10.9	5.0	5.9
18:2	12.5	3.0	9.5	11.7	1.9	9.8	11.3	5.3	6.0	10.9	4.6	6.3
18:3	0.8		0.8	1.2	....	1.2	1.3	....	1.3	1.8	....	1.8
20:1	3.7	2.3	1.4	3.4	1.5	1.9	3.6	1.3	2.3	5.1	2.6	2.5
21:0	2.0	0.7	1.3	1.5	0.7	0.8	1.7	0.9	0.8	3.9	1.2	2.7
20:4	7.4	0.5	6.9	6.1	....	6.1	7.6	....	7.6	8.3	....	8.3
22:0	3.4	2.2	1.2	3.7	1.0	2.7	4.1	1.8	2.3	....	....	....
22:1	1.2	0.6	0.6	1.2	0.3	0.9	1.4	....	1.4	6.3	3.8	2.5

[a] rx, 16:0 = 0.460.

close to the values of those in phosphatidylserine. On the other hand, the major saturated fatty acid was palmitic and the relative quantities of the major fatty acids present more nearly resembled the distribution in lecithin than that of the other phosphoglycerides.

If the enzyme liberates fatty acids only from the β-position of phosphoglycerides, the mole % of acids in the α- and β-positions should be equal. Even by taking into consideration the possibilities of accumulating experimental errors in the steps involving fatty acid hydrolysis, separation, methyl ester preparation and their estimation on the chromatograms, an approximation of equal distribution of fatty acids between the α- and β-positions should be expected. The fatty acid distribution between the α- and β-position in phosphatidylethanolamine and lecithin from ham, loin and rib muscle deviated up to 5 mole %. All these phospholipid samples were hydrolyzed to between 95 and 97% by the enzymes and this also could contribute to any observed unequal distribution of fatty acids in the α- and β-positions. Regardless of these discrepancies, the overall picture of the fatty acid distribution is sufficiently clear to draw conclusions regarding their position.

On the other hand phosphatidylethanolamine and lecithin from belly muscle were entirely hydrolyzed since there was no unhydrolyzed compound detected. Instead of obtaining a closer 1:1 ratio in fatty acid distribution, there were found enzymatically hydrolyzed 56.7 mole % of the fatty acids in lecithin and 67.1 mole % in phosphatidylethanolamine. Both lecithin and phosphatidylethanolamine in belly muscle contain much higher percentages of unsaturated and particularly polyunsaturated fatty acids than corresponding samples in another carcass location. The total content of unsaturated fatty acids in belly muscle lecithin was 54.2 mole % and in phosphatidylethanolamine 66.6 mole %.

These results indicate that snake venom enzyme is not limiting its hydrolytic action to the fatty acids in one position on phosphoglycerides. The total unsaturated fatty acid content within the phospholipid molecule exceeding 50% of the total fatty acids present seems to facilitate enzymatic action in the α-position, although the enzyme is not hydrolyzing off exclusively the unsaturated fatty acids. This compares with the observations made by Uziel and Hanahan (7) from which they proposed the concept of a lysolecithin migratase and suggests the presence of more than one active enzyme in the snake venom.

Tattrie (2) proposed that phospholipase A hydrolyzed only the β-linked fatty acids from egg lecithin and then Hanahan et al. (8) concluded that the site of attack of lecithinase A is exclusively at the β-ester position in lecithin. Concurrently, de Haas et al. (9) arrived at an identical conclusion with regard to the *L*-isomers of lecithins and showed that *D*-isomers were resistant to attack by lecithinase A from *Crotalus adamanteus*. It seems reasonable to conclude that assignment of the enzymatically liberated fatty acids, mostly unsaturated in composition, to the β-position of lecithin from hog muscle tissues is appropriate. By analogy with the lecithins, it is assumed that similar criteria apply to the assignment of the major content of unsaturated fatty acids in phosphatidylserine and phosphatidylethanolamine to the β-position in these phosphoglycerides.

TABLE IV

Mole % of Fatty Acids in Sphingomyelin

Acid	Belly	Ham	Loin	Ribs
10:0	1.2	4.2	1.9	1.7
12:0	0.8	5.3	2.7	2.2
13:0	....	2.6	0.7	1.2
[a]	....	1.2	....	1.2
14:0	1.1	2.8	1.3	2.0
[b]	....	2.0	0.7	....
15:0	0.5	....	0.9	1.2
16:0	51.3	27.7	34.2	44.9
16:1	1.5	1.8	1.1	....
17:0	1.0	2.1	1.7	2.2
18:0	6.2	5.2	12.1	4.5
18:1	8.8	11.9	16.2	10.2
18:2	23.7	26.9	19.6	25.8
18:3	0.7	0.5	1.7	1.5
20:1	1.0	2.2	1.6	0.5
21:0	....	....	0.3	....
20:4	1.8	1.7	2.2	0.8
22:0	0.2	....	1.0	....
22:1	....	1.9	....	....

[a] rx, 16:0 = 0.469.
[b] rx, 16:0 = 0.642.

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