STRUCTURAL INVESTIGATIONS OF THE PHOSPHOLIPIDS OF SEEDS OF THE COTTON PLANT OF THE VARIETY "TASHKENT-1"

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The present paper gives the results of a structural investigation of the components present in largest amount in the combined phospholipids of the seeds of the cotton plant of variety "Tashkent-1" of the 1971 harvest. The combined phospholipids isolated from the defatted kernels of the cotton seeds by a known method [1, 2] were separated by the solubilities of the individual fractions in ethanol. The phospholipids of the ethanol-soluble and ethanol-insoluble fractions of the total were chromatographed on columns of silica gel. The phosphatidylcholines, phosphatidylinositols, and phosphatidylethanolamines were finally purified by preparative chromatography in a thin layer of silica gel. The individual fractions obtained in this way had the following constants.

Phosphatidylcholines: % N 1.59, P 3.31; N/P molar ratio 1.06, $[\alpha]_D$ +7.7° C (2.0 chloroform); IR spectrum, cm^{-1}: 3300-3600 (OH), 2940, 2870, 1480 (CH, CH_2, CH_3), 1740 (ester C=O), 1260 (P=O), 980 [$\overset{+}{N}(CH_3)_3$].

Phosphatidylethanolamines: % N 1.26, P 3.03; N/P molar ratio 0.91; $[\alpha]_D$ +6.1° (c 1.3; chloroform); IR spectrum, (cm^{-1}): 2960, 2940, 2860, 1460 (CH, CH_2, CH_3), 1730 (C=O), 1085, 1040, 1030 (P–O–C), 1240 (P=O).

Phosphatidylinositols: % P 3.43, no N; IR spectrum (cm^{-1}): 3500-3200 (OH), 2870, 2940 (CH, CH_2, CH_3), 1735 (C=O), 1060, 1120 (P–O–C), 1250 (P=O).

The structures of the homogeneous fractions shown were confirmed by the study of the products of their acid hydrolysis. Fatty acids were found in the hydrolyzates of all the phospholipids [2]. Among the water-soluble hydrolysis products from the phosphatidylcholines we found choline; from the phosphatidylethanolamines, ethanolamine; and from the phosphatidylinositols, inositol; the hydrolyzates of all the fractions contained glycerol. The polyols and amines were identified from their R_f values with markers in a thin layer of silica gel in the isopropanol – 25% ammonia – water (5 : 4 : 1) [3] and (49 : 7 : 14) [4] systems. The spots were revealed with Dragendorff's reagent, ninhydrin solution, a 0.5% solution of $KMnO_4$, and potassium metaperiodate – benzidine.

It is known from the literature that phospholipase A exhibits a specific action on the β-position of a phospholipid molecule. The fatty acids obtained in this process are mainly unsaturated. In order to investigate the position distribution of the fatty acids in the individual fractions we studied the products of the enzymatic hydrolysis of these components. As the source of phospholipase A we used the venom of the Azerbaidzhan kufi [5] in tris buffer at pH 9.6. In order to compare the results, the phosphatidylethanolamines were hydrolyzed by this enzyme in the presence of $CaCl_2$ [6], and it was found that the phosphatidylcholines are hydrolyzed completely in 4 h and the phosphatidylethanolamines in 22 h. When the reaction was performed in the presence of $CaCl_2$ (instead of the tris buffer) the reaction took place to the extent of 93-94% in 22 h. It was more difficult to hydrolyze the phosphatidylinositols; after 48 h ~80% of the phospholipid had been hydrolyzed. The hydrolysis products were separated by preparative TLC. The lyso compounds were subjected to alkaline hydrolysis and the fatty acids both from the α-position (split off from the lyso compounds) and from the β-position (obtained on enzymatic cleavage) were analyzed in the form of methyl esters by GLC (Table 1).

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TABLE 1. Position Distribution of the Fatty Acids in the Main Fractions of the Phospholipids of Seeds of the "Tashkent-1" Cotton Plant, %

Fatty acids	Symbol	Phosphatidylcholines			Phosphatidylethanolamines			Phosphatidylinositols		
		initial	position		initial	position		initial	position	
			α	β		α	β		α	β
$C_{10:0}$	C	—	—	—	2,46	3,00	—	—	—	—
$C_{12:0}$	La	—	—	—	2,18	2,43	—	—	—	—
$C_{14:0}$	M	1,22	1,91	1,71	1,78	2,42	1,44	2,35	—	2,45
$C_{16:0}$	P	18,18	35,08	3,20	22,11	49,15	4,54	31,91	52,60	6,00
$C_{16:1}$	Po	1,74	2,40	1,67	3,83	2,38	1,94	3,66	9,42	3,33
$C_{18:0}$	S	3,21	5,95	—	4,11	2,42	—	5,64	9,52	3,06
$C_{18:1}$	Ol	23,04	21,90	23,81	12,47	4,46	14,43	10,43	11,06	9,16
$C_{18:2}$	L	52,61	32,76	69,61	51,06	33,74	77,65	46,01	17,40	75,97
Total saturated acids	S	22,61	42,94	4,91	32,64	59,42	5,98	39,90	62,12	11,51
Total unsaturated acids	U	77,39	57,06	95,09	67,36	40,58	94,02	60,10	37,88	88,49

TABLE 2. Diglyceride Composition of the Phosphatidylcholines*

β-Positions of the fatty acids	Symbol	Taking position isomerization into account	β-Positions of the fatty acids	Symbol	Taking position isomerization into account
M 1,7	PM	0,351×1,7 0,6	Ol 23,8	MOl	0,019×23,8=0,5
	SM	0,059×1,7 0,1		POl	0,351×23,8=8,3
	OlM	0,219×1,7=0,4		PoOl	0,024×23,8=0,6
	LM	0,328×1,7 0,6		SOl	0,059×23,8=1,4
P 3,2	MP	0,019×3,2 0,1		OlOl	0,219×23,8=5,2
	PP	0,351×3,2=1,1		LOl	0,328×23,8=7,8
	PoP	0,024×3,2 0,1	L 69,6	ML	0,019×69,6= 1,3
	SP	0,059×3,2 0,2		PL	0,351×69,6=24,4
	OlP	0,219×3,2 0,7		PoL	0,024×69,6= 1,7
	LP	0,328×3,2 1,0		SL	0,059×69,6= 4,2
Po 1,7	PPo	0,351×1,7 0,6		OlL	0,219×69,6=15,3
	SPo	0,059×1,7 0,1		LL	0,328×69,6=22,8
	OlPo	0,219×1,7 0,4			
	LPo	0,328×1,7=0,5		Total	100,0

*Hence SS = 2.1%, and of these the monoacid compounds make up 1.1%; SU – 43.6%; and UU – 54.3%, and of which the monoacid compounds make up 28%.

It can be seen from Table 1 that in the three fractions studied there is a specific distribution of the saturated and unsaturated acids: in the β-positions the phosphatidylcholine molecules are esterified with unsaturated acids to the extent of 95.09%, the phosphatidylethanolamines to 94.02%, and the phosphatidylinositols to 88.49%; in the α-positions of the phosphatidylethanolamines and phosphatidylinositols saturated acids predominate (59.42 and 62%, respectively). The predominance of unsaturated fatty acids in the α-positions of the phosphatidylcholines is possibly explained by the more unsaturated nature of the fatty acids in the initial molecule.

The results of a determination of the position distribution of the fatty acids using Coleman's method of calculation [7] in the modification of A. L. Markman et al. [8] enabled us to calculate the diglyceride composition of the phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols as described previously [5] (Table 2-4).

EXPERIMENTAL METHOD

For chromatography we used silica gel of type KSK (up to 100 μ for thin-layer chromatography and 160-250 μ for column chromatography) and the following solvent systems: 1) chloroform–methanol–water (65:35:5) and 2) chloroform–methanol–25% ammonia (65:35:5). The IR spectra were taken on a UR-20

TABLE 3. Diglyceride Composition of the Phosphatidylcholines*

β-Positions of the fatty acids	Symbol	Taking position isomerization into account	β-Positions of the fatty acids	Symbol	Taking position isomerization into account
M 1,4	PM	0,491×1,4=0,7		COl	0,03×14,4=0,4
	OlM	0,045×1,4=0,1		LaOl	0,024×14,4=0,3
	LM	0,337×1,4=0,5		MOl	0,024×14,4=0,3
	CP	0,03×4,5=0,1		POl	0,491×14,4=7,2
	LaP	0,024×4,5=0,1	Ol 14,4	PoOl	0,024×14,4=0,3
	MP	0,024×4,5=0,1		SOl	0,024×14,4=0,3
P 4,5	PP	0,491×4,5=2,2		OlOl	0,045×14,4=0,7
	PoP	0,024×4,4=0,1		LOl	0,337×14,4=4,9
	SP	0,024×4,5=0,1		CL	0,03×77,6=2,3
	OlP	0,045×4,5=0,2		LaL	0,024×77,6=1,9
	LP	0,337×4,5=1,5		ML	0,024×77,6=1,9
			L 77,6	PL	0,491×77,6=38,3
Po 1,9	PPo	0,491×1,9=1,0		PoL	0,024×77,6=1,9
	OlPo	0,045×1,9=0,1		SL	0,024×77,6=1,9
	LPo	0,337×1,9=0,7		OlL	0,045×77,6=3,5
				LL	0,337×77,6=26,4
					Total 100,0

*Hence SS = 3.3%, of which the monoacid compounds make up 2.2%; SU – 58.2%; and UU – 38.5%, of which the monoacid compounds make up 27.1%.

TABLE 4. Diglyceride Composition of the Phosphatidylcholines*

β-Positions of the fatty acid	Symbol	Taking position isomerization into account	β-Positions of the fatty acids	Symbol	Taking position isomerization into account
M 2,5	PM	0,526×2,5=1,3	S 3,0	PS	0,526×3=1,6
	PoM	0,094×2,5=0,2		PoS	0,094×3=0,3
	SM	0,095×2,5=0,2		SS	0,095×3=0,3
	OlM	0,111×2,5=0,3		OlS	0,111×3=0,3
	LM	0,174×2,5=0,4		LS	0,174×3=0,5
P 6,0	PP	0,526×6=3,2	Ol 9,2	POl	0,526×9,2=4,8
	PoP	0,094×6=0,6		PoOl	0,094×9,2=0,9
	SP	0,095×6=0,6		SOl	0,095×9,2=0,9
	OlP	0,111×6=0,7		OlOl	0,111×9,2=1,0
	LP	0,174×6=1,0		LOl	0,174×9,2=1,6
Po 3,3	PPo	0,526×3,3=1,8	L 76,0	PL	0,526×76=39,9
	PoPo	0,094×3,3=0,3		PoL	0,094×76=7,2
	SPo	0,095×3,3=0,3		SL	0,095×76=7,2
	OlPo	0,111×3,3=0,4		OlL	0,111×76=8,4
	LPo	0,174×3,3=0,6		LL	0,174×76=13,2
					Total 100,0

*Hence SS = 7.2%, of which monoacid compounds make up 3.5%; SU – 59.2%; and UU – 33.6%, of which the monoacid compounds make up 14.5%.

instrument in the form of films. Gas–liquid chromatography was performed on a UKh-2 instrument at 197° C with poly(ethylene succinate) as the stationary phase.

Extraction of the Combined Phospholipids. The kernels (100 g) of cotton seeds of the variety "Tashkent-1" were defatted with acetone at room temperature. The phospholipids were extracted from the dried meal with a mixture of chloroform and methanol (2 : 1). The solvents were distilled off in vacuum under a current of nitrogen. The residue was dissolved in chloroform and the solution was filtered. The filtrate was concentrated to small volume, and the phospholipids were precipitated with acetone. The resultant flocculent precipitate was separated by centrifuging. From the ethereal solution of phospholipids the water-soluble substances were extracted with a 0.9% aqueous solution of NaCl. The yield of combined phospholipids was ~1.5 g, which corresponds to ~1.5% of the weight of the dry kernels.

Fractionation of the Combined Phospholipids with Ethanol. The combined phospholipids (1.35 g) in 7 ml of chloroform were precipitated with 50 ml of ethanol. The precipitate that deposited was separated

by centrifuging, dried, and weighed. The centrifugate was evaporated in vacuum under a current of nitrogen to dryness. The weight fo the ethanol-soluble fraction was 0.945 g and of the ethanol-insoluble fraction 0.4 g.

Column Chromatography of the Total Ethanol-Soluble Fraction. In a column containing 45 g of silica gel, 945 mg of the ethanol-soluble fraction of combined phospholipids was eluted with chloroform, mixtures of chloroform and ethanol in various ratios, and then with methanol. The following fractions were obtained:

I – chloroform: neutral lipids (20 mg);

II – chloroform – methanol (9 : 1) : 100 mg (neutral lipids and polyglycerophosphatides);

III – chloroform – methanol (4 : 1) : 450 mg (mixture of phosphatidylethanolamines and phosphatidylcholines);

IV – chloroform – methanol (7 : 3) : 90 mg (phosphatidylcholines and traces of phosphatidylinositols);

V – chloroform – methanol (2 : 1) : 180 mg (phosphatidylcholines and lysophosphatidylcholines); and

VI – methanol: 80 mg (phosphatidylcholines and lysophosphatidylcholines).

Then the fractions were separated by preparative TLC. The separation of the total ethanol-insoluble fraction of phospholipids was performed similarly, and phosphatidylinositols and phosphatidylethanolamines predominated in the eluates.

Acid Hydrolysis of the Individual Phospholipids. An individual phospholipid fraction (35-50 mg) in 3-4 ml of 3 N HCl in a sealed tube was boiled in the water bath for 24 h. Then the tube was opened, the fatty acids were extracted from the acid solution with petroleum ether (40-70° C), the residue was evaporated in vacuum and dissolved in water, and the hydrolysis products were analyzed by TLC. Glycerol, choline, ethanolamine, and inositol were used as markers.

Enzymatic Hydrolysis of the Phospholipids. A. Phosphatidylcholines. To 94 mg of the sample in 15 ml of ether was added 2.6 mg of kufi venom dissolved in 0.4 ml of 0.1 M tris buffer. The mixture was left at room temperature. After 4 h, the solvent was evaporated off, the residue was dissolved in chloroform – methanol (2 : 1), and the hydrolysis products were separated prepatatively in system 1; R_f 0.9 (iodine vapor) – fatty acids from the β-position, yield 37 mg; R_f 0.15 (iodine vapor and Dragendorff's and Vas'kovskii's reagents), yield 55 mg.

Lysosphosphatidylcholine. % N 3.10, P 5.93; N/P molar ratio 1.1.

B. Phosphatidylethanolamines. I. A sample (164 mg) was dissolved in 20 ml of ether, and 3.6 mg of snake venom in 0.5 ml of tris buffer (pH 9.6) was added. The reaction was complete after 22 h. Then the hydrolysis products were treated as in the case of the phosphatidylcholines. The yield of fatty acids was 50 mg and of lysophosphatidylethanolamines 105 mg.

The phosphatidylethanolamines (60 mg) were dissolved in 8 ml of ether and 0.3 ml of a solution of snake venom containing 4 mg of venom in 1 ml of water and 0.15 ml of 0.025 M $CaCl_2$ were added. The mixture was left at room temperature. After 22 h, the hydrolysis products were separated by preparative TLC. This gave fractions with R_f 0.9 – the fatty acids split off, weight 25 mg; 0.8 – unchanged phosphatidylethanolamines, 4 mg; and 0.45 – lysophosphatidylethanolamines, 27 mg.

Lysophosphatidylethanolamines. % N 2.36, P 5.6; N/P molar ratio 0.94. In a thin layer of silica gel in system 1, R_f 0.45 (I_2 vapor, solution of ninhydrin, Vas'kovskii's reagent, and 10 % H_2SO_4/CH_3OH).

C. Phosphatidylinositols. A solution of 94 mg of the sample in 15 ml of ether was mixed with 2.6 mg of snake venom in 0.4 ml of 0.1 M tris buffer. The mixture was left at room temperature for 48 h. Then the solvents were evaporated off, and the residue was dissolved in a mixture of chloroform and ethanol (2 : 1); on TLC, the mixture showed three spots, and these were separated preparatively in system 1. This gave fractions with R_f 0.9 – the fatty acids split off; 0.4 – unchanged phosphatidylinositols (19 mg); and 0.15 – lysophosphatidylinositols.

The lysophosphatidylinositols do not dissolve in alcohols and ether and are sparingly soluble in chloroform; they contain 6.5 % of P. In the isopropanol – formic acid – water (3 : 1 : 1) system [9], R_f 0.4.

Alkaline Hydrolysis of the Lyso Compounds. The lysophosphatidylcholines, lysophosphatidylethanolamines, and lysophosphatidylinositols (20-30 mg) were hydrolyzed in 0.1 M KOH/CH_3OH (1-1.5 ml) on the water bath under reflux for 20 min. Then the solvent was evaporated off, the residue was dissolved in water, the solution was acidified with 10 % HCl, and the fatty acids were extracted with petroleum ether (3 × 15 ml).

The combined petroleum ether solutions were washed with distilled water and dried over Na_2SO_4. The solvent was evaporated off in vacuum. The fractions obtained consisted of the fatty acids that had been present in the α-positions of the corresponding phospholipids. The fatty acids were methylated with a freshly prepared solution of diazomethane and analyzed by GLC.

SUMMARY

1. The results of the study of the products of acid hydrolysis confirm the structures of the phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols of the seeds of the cotton plant of the variety "Tashkent-1."

2. By the enzymatic hydrolysis of these fractions of phospholipids, the position distribution of the fatty acids in them have been determined and the lyso compounds have been characterized.

3. The diglyceride composition of the phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols have been calculated.

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