DETERMINATION OF THE POSITION DISTRIBUTION OF FATTY ACIDS IN NATURAL PHOSPHATIDYLCHOLINES

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It is known that phospholipase A acts specifically on the β ester position of phosphatidylcholines, splitting out the fatty acid and forming the lyso products 1-acyl-3-glycerylphosphorylcholines. The acids liberated are mainly unsaturated acids, the acids in the 1- position having a more saturated nature.

In the majority of investigations connected with the determination of the positions of the fatty acids in phosphatidylcholines of different origins, the source of phospholipase A used is the lyophilized snake venom of Crotalus adamanteus (rattlesnake) [1-5], and more rarely that of Naja naja (cobra) [6, 7]. In hydrolysis, buffer solutions with a pH close to 7 are most frequently used, for example, tris buffer with pH 7.4 [4, 5] or 7.45 [2], or a 0.005 M solution of $CaCl_2$ [1]. The amount of enzyme for cleavage varies between 0.33 and 2 mg per 50 mg of substrate, and the time of hydrolysis from 2 to 16 hours and more.

The present paper describes the determination of the position distribution of the fatty acids in natural phosphatidylcholines by enzymatic hydrolysis at pH 7.45 and 10.15 with the venom of Vipera lebetina obtusa (Azerbaidzhan blunt-nosed viper) dried in a desiccator over $CaCl_2$. This venom, which we obtained from the herpetology laboratory of the Institute of Zoology and Parasitology of the Academy of Sciences of the Uzbek SSR, served as the source of phospholipase A.

There is no information in the literature on the use of this type of snake venom for the cleavage of phosphatidylcholines, and therefore, to check the specificity of its action, as a standard substrate we used phosphatidylcholines (lecithins) of egg yolk, and as the sample under investigation a pure fraction of the phosphatidylcholines of Helianthus annuus (sunflower). Both samples of phosphatidylcholines were isolated from the combined phospholipids by column chromatography on silica gel [8].

Hydrolysis of the standard substrate at pH 7.45 showed the complete specificity of the action of the enzyme on the β ester position of egg lecithin, since the acids split off were mainly unsaturated acids (98.2%), while the fatty acids in the α position are saturated (Table 1). Our results agree well with the literature [4, 5].

In order to establish the position distribution of the fatty acids in the sunflower phosphatidylcholines, cleavage was carried out under the same conditions; it was found that 96.3% of the acids in the β position were unsaturated and only 71.6% of those in the α position (see Table 1). The dominance of unsaturated acids in both positions is due to the more unsaturated nature of the fatty acid composition of the initial phosphatidylcholines.

We then performed experiments on the enzymatic hydrolysis of both substrates at pH 10.15. The specificity of the action of the phospholipase A was also judged from the composition of the fatty acids split out under these conditions. As can be seen from Table 1, the compositions of the acids in the α and β positions were identical with those found at pH 7.45. Enzymatic hydrolysis at the higher pH value took place in a considerably shorter time, and therefore the activity of the phospholipase A was determined in

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TABLE 1. Position Distribution of the Fatty Acids in the Phosphatidylcholines of Egg Yolk and of Sunflower, %

Fatty acids	Symbol	Egg yolk, pH					Sunflower, pH				
		initial	7,45		10,15		initial [81]	7,45		H 10,15	
			α	β	α	β		α	β	α	β
$C_{14:0}$	M	0,2	0,5	0	0,4	0	0,5	0,5	0,7	0,4	0,7
$C_{16:0}$	P	34,4	61,8	1,8	62,1	1,8	10,5	18,8	2,0	18,7	2,1
$C_{16:1}$	Po	1,2	1,1	1,2	1,4	1,2	1,3	1,2	1,1	1,1	0,9
$C_{18:0}$	St	14.2	27,4	0	27,3	0	5,0	9,0	1,0	9,4	1,0
$C_{18:1}$	Ol	31,3	9,1	57,3	8,8	57,6	15,2	12,2	18,8	12,2	18,7
$C_{18:2}$	L	14,9	0	31,6	0	31,3	67,5	58,3	76,3	58,2	76,6
$C_{20:4}$	A	3,8	0	8,1	0	8,1	0	0	0	0	0
Total saturated	S	48,8	89,8	1,8	89,8	1,8	16,0	28,4	3,7	28,4	3,8
Total unsaturated	U	51,2	10,2	98,2	10,2	98,2	84,0	71,6	96,3	71,6	96,2
Ratio	S:U	1:1,04	8,8:1	1:54	8,8:1	1:54	1:5,2	1:2,5	1:26	1:2,5	1:26

TABLE 2. Determination of the Activity of Phospholipase A

Hydrolysis products	Time of hydrolysis, min						
	0,5	5	10	15	20	25	30
Amounts of substances formed, %							
Phosphatidylcholines	30	18	11	2	0	0	0
Lysophosphatidylcholines	70	82	89	98	100	100	100
Consumption of 0.1 N KOH, ml							
Fatty acids	0,10	0,14	0,17	0,20	0,20	0,20	0,20

the hydrolysis of the sunflower phosphatidylcholines under the same conditions. Its activity was estimated from the amount of hydrolysis products formed after definite intervals of time, namely from the contents of phosphorus in the lysophosphatidylcholines and in the unchanged phosphatidylcholines and also from the amount of 0.1 N KOH solution consumed in the titration of the fatty acids split out (Table 2).

It can be seen from Table 2 that even in the first minute, i.e., almost instantaneously, 70% cleavage had taken place, and then the process slowed down and was complete after 20 min.

The results of the investigation of the specificity and activity of the unpurified phospholipase A of the venom of the blunt-nosed viper give grounds for recommending it for the cleavage of natural phosphatidylcholines. This source of phospholipase A for the enzymatic hydrolysis of phosphatidylcholines at high pH values can be used for the accelerated determination of the position distribution of the fatty acids in their molecules.

The mean experimental results (Table 3) for the position distribution of the fatty acids present in sunflower phosphatidylcholines together with the principle of Coleman's method of calculation [9] in the variant of Markman et al. [10] (for triglycerides) enabled us to determine the diglyceride composition of the phosphatidylcholines (Table 4).

TABLE 3. Mean Figures for the Fatty-acid Composition of Sunflower Phosphatidylcholines, %

Position	Symbol					
	M	P	Po	S	Ol	L
α	0,4	18,8	1,1	9,2	12,2	58,3
β	0,7	2,6	1,0	1,0	18,8	76,5

TABLE 4. Diglyceride Composition of Sunflower Phosphatidylcholines, %

Fatty acids in the β position	Symbol	Taking position isomerism into account	Fatty acids in the β position	Symbol	Taking position isomerism into account
M 0,7	PM	0,188·0,7=0,2	Ol 18,8	MOl	0,004·18,8= 0,1
	OlM	0,122·0,7=0,1		POl	0,188·18,8= 3,5
	LM	0,583·0,7=0,4		PoOl	0,011·18,8= 0,2
P 2,0	PP	0,188·2,0=0,4		StOl	0,092·18,8= 1,7
	StP	0,092·2 0=0,2		OlOl	0,122·18,8= 2,3
	OlP	0,122·2,0=0,2		LOl	0,583·18,8=11,0
	LP	0,583·2,0=1,2			
Po 1,0	PPo	0,188·1,0=0,2	L 76,5	ML	0,004·76,5= 0,4
	StPo	0,092·1,0=0,1		PL	0,188·76,5=14,4
	OlPo	0,122·1,0=0,1		PoL	0,011·76,5= 0,8
	LPo	0,583·1,0=0,6		Stl	0,092·76,5= 7,0
S 1,0	PSt	0.188·1,0=0,2		OlL	0,122·76,5= 9,3
	StSt	0,092·1,0=0,1		LL	0,583·76,5=44,6
	OlSt	0,122·1,0=0,1			
	LSt	0,583·1,0=0,6	Total		100,0

Components with a concentration of less than 0.1% not considered.

On separately summing the saturated (S), unsaturated (U), and monosaturated-monounsaturated (SU) compounds we obtained the group diglyceride composition of the phosphatidylcholines (%): SS – 1.1, including 0.5 of monoacid compounds; SU – 30.0; UU – 68.9, including 46.9 of monoacid compounds.

This pattern of the diglyceride varieties of sunflower is explained by the ratio of S and U acids both in the initial phosphatidylcholines and in their position distribution.

EXPERIMENTAL

The solvents were purified by generally accepted methods [11]. Chromatography was performed with KSK silica gel (100-150 mesh for column chromatography and smaller than 150 mesh for thin-layer chromatography) which had been washed with hydrochloric acid, water, acetone, methanol, and chloroform. The fatty acid composition was determined by the gas chromatographic method on a UKh-2 chromatograph at 200° C with a column 2.5 m long. The stationary phase used was poly(ethylene succinate) [12].

Enzymatic Hydrolysis

1. At pH 7.45. A solution of 152 mg of phosphatidylcholines in 30 ml of moist diethyl ether was transferred to a 50-ml two-necked pear-shaped flask and treated with 2.7 mg of snake venom dissolved in 0.4 ml of 0.1 M tris · HCl buffer (pH 7.45); the flask was attached to a reflux condenser, after which a stirrer with a fractional-horsepower motor was passed through it and the contents were carefully stirred. The reaction mixture was kept at room temperature for 4 h with periodic stirring. Then the ethereal solution containing the bulk of the fatty acid split out was carefully decanted off; the residue in the reaction flask, consisting mainly of lysophosphatidylcholines and partly of fatty acids, and possibly unchanged phosphatidylcholines, was washed with diethyl ether (2 × 5 ml), dissolved in 2-3 ml of methanol, separated from protein by filtration or centrifuging, and evaporated to dryness in a current of nitrogen under reduced pressure (40-45° C). The residue was dissolved in 5 ml of chloroform, and the hydrolysis products were separated by column chromatography. The combined ethereal extracts were washed with distilled water (3 × 5 ml), dried over Na_2SO_4, and evaporated in a current of nitrogen. The fatty acid fraction was obtained.

2. Hydrolysis at pH 10.15. The substrates were cleaved by the method described above with the only difference that the snake venom was dissolved in 0.1 M tris(hydroxymethyl)aminomethane and the time of hydrolysis was shortened to 30 min.

Separation of the Hydrolysis Products. A suspension of 4 g of silica gel in 20 ml of chloroform was introduced into a glass column (15 × 450 mm). The column was washed with another 10 ml of the same solvent and the hydrolysis products were eluted: the fatty acids with 30 ml of chloroform–methanol (9 : 1); the unchanged phosphatidylcholines with 30 ml of chloroform–methanol (1 : 4); and the lysophosphatidylcholines with 50 ml of methanol.

The fatty acids isolated by column chromatography were combined with those obtained from the ethereal solutions. The fatty acids were liberated from the lysophosphatidylcholines by alkaline hydrolysis (0.5 N solution of KOH in methanol, 1.5 h, 70° C). Both fatty-acid fractions were methylated with diazomethane and analyzed by gas-liquid chromatography.

Determination of the Activity of the Phospholipase A. Solution A was made by dissolving 170 mg of sunflower phosphatidylcholines in 34 ml of diethyl ether, and solution B by dissolving 3 mg of snake venom in 0.44 ml of 0.1 M tris(hydroxymethyl)aminomethane with pH 10.15. Each of seven 10-ml conical flasks was charged with 4 ml of solution A and 0.05 ml of solution B and an eighth flask was charged with 4 ml of solution A and 0.05 ml of a 0.1 M solution of tris containing no snake venom (blank test). Each mixture was carefully stirred. Hydrolysis was stopped by the addition of 2 ml of methanol: in the first flask immediately after the addition of solution B and stirring, and in the others after hydrolysis for 5, 10, 15, 20, 25, and 30 min. The blank sample was kept for 30 min before the addition of methanol.

The contents of the flasks were evaporated to dryness in a current of nitrogen under reduced pressure (bath temperature 40-45° C). The residues were each dissolved in 2 ml of chloroform. An aliquot of each solution (0.1 ml) was deposited in the form of a strip 1 cm wide with a 1.5-cm interval between strips on two plates with dimensions of 18 × 24 cm for the chromatographic separation of the hydrolysis products in the chloroform–methanol–water (65 : 25 : 4) system.

After the chromatograms had been run, the plates were dried in the air and were then subjected to the action of iodine vapor, which showed up the spots of the fatty acids, the unchanged phosphatidylcholines, and the lysophosphatidylcholines with R_f 0.85, 0.40, and 0.16, respectively. The spots corresponding to the fatty acids were removed and, after the evaporation of the excess of iodine, dried in small flasks. For the blank, a pure section of silica gel of the same size was taken. The fatty acids were eluted with diethyl ether (4 × 3 ml). The eluates were evaporated to dryness, each residue was dissolved in 5 ml of a mixture of diethyl ether and ethanol (2 : 1), and the acids were neutralized with 0.01 N KOH solution.

The amounts of lysophosphatidylcholines and unchanged phosphatidylcholines were estimated from the amount of phosphorus in each of them as a fraction of the total amount of phosphorus in both.

For this purpose, after the removal of the spots of the fatty acids, the plates were sprayed with 50% H_2SO_4 and carbonized at 150° C for 40-50 min. The spots corresponding to given hydrolysis products were dried and treated as recommended by Dyatlovitskaya et al. [13]. The phosphorus content was determined by a modification of Tevekelov's micro method [14].

Qualitative thin-layer chromatography was carried out in the same way on plates with dimensions of 2.5 × 7.5 cm.

SUMMARY

It has been shown that the snake venom of Vipera lebetina obtusa can be used as a source of phospholipase A.

A method is given for the accelerated enzymatic hydrolysis of natural phosphatidylcholines in order to determine the position distribution of the fatty acids by bringing the pH of the medium to 10.15.

A technique for calculating the composition of the diglycerides of the phosphatidylcholines is given.

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