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HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

II. FATTY ACID CHAIN LENGTH PREFERENCE OF THE ENZYME

G. V. MARINETTI, J. ERBLAND AND ELMER STOTZ

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

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SUMMARY

Phospholipase A of snake venom shows chain length preference with respect to the hydrolysis of lecithin fatty acids. With pig heart, egg, and rat-liver lecithins the C-22, C-20 and C-18 acids are hydrolyzed in preference to the C-16 acids. It also appears that the C-22 and C-20 acids are hydrolyzed in preference to both the C-16 and C-18 acids.

INTRODUCTION

In a previous paper[1] the structure of the enzymically produced lysolecithins was investigated. Evidence was provided that snake venom phospholipase A hydrolyzes either the alpha or beta linked fatty acid on lecithin to yield a mixture of alpha and beta lysolecithins. However, the beta linked fatty acid is preferentially removed in

some cases. This paper will deal with the analysis of the fatty acids which are hydrolyzed by the enzyme. With pig heart, egg, and rat-liver lecithins the C-22, C-20 and C-18 acids are hydrolyzed in preference to the C-16 acids. It also appears that the C-20 and C-22 acids are removed in preference to both the C-16 and C-18 acids.

METHODS AND REAGENTS

The experimental procedures for the preparation of the lecithins and the hydrolysis of the lecithins by the phospholipase A are reported in previous papers[2,3]. The fatty acids were analyzed by paper chromatography on mineral oil impregnated paper[4]. The solvent was acetic acid–water (9 : 1)*. In the case of rat-liver lecithin the fatty acids were analyzed before and after reduction with hydrogen. The reduction was carried out in methanol over platinum oxide at 50 lbs. pressure. The lecithins and lysolecithins were also hydrolyzed with 1 *N* HCl for 1 h and the fatty acids obtained by ether extraction in the usual way were analyzed by paper chromatography.

The fatty acids on the chromatograms were detected in the following manner: The chromatograms were dried in air for several hours, immersed in a 0.001 % solution of Rhodamine 6G for about 2 min, and then washed by immersing in distilled water for 15 min. The chromatograms were dried in air and observed under u.v. light (366 mμ). The fatty acids appear as yellow staining spots. These spots can be markedly intensified by passing the chromatogram several times through a 0.2 % aqueous alkaline solution of brom thymol blue and washing off the excess dye with water. The fatty acids were also detected by the procedures given by ASHLEY AND WESTPHAL[5]. The unsaturated acids were detected by dipping the chromatogram in a 1 % solution of potassium permanganate and washing off the excess permanganate. Brown spots appear where unsaturated fatty acids occur.

RESULTS AND DISCUSSION

The chromatographic analysis of the fully reduced (saturated) fatty acids of egg lecithin, egg lysolecithin, pig heart lysolecithin, and the fatty acids liberated by phospholipase A from the reduced egg and pig heart lecithin are shown in Fig. 1. Also included for sake of reference is a standard mixture of myristic (C-14), palmitic (C-16), and stearic (C-18) acids. The fatty acids of reduced egg lecithin were found to be mainly C-18 and C-16 and smaller amounts of C-20 and C-22. The fatty acids of egg phosphatides are given elsewhere[6]. The egg lysolecithin fatty acids were found to be C-16 and C-18 with the former in higher amount. No C-20 and C-22 acids were detected in the lysolecithin**. Hence the higher chain fatty acids are completely removed by the phospholipase A. The fatty acids released by Crotalus adamanteus venom by hydrolysis of the reduced egg lecithin are predominantly C-18 and smaller amounts of C-20 and C-22. No detectable amounts of C-16 acids were released by the venom**.

* Whatman No. 1 filter paper was impregnated by passing once through a 10 % solution (v/v) of liquid petrolatum in benzene. The solvent system was acetic acid–water (9 : 1) saturated with liquid petrolatum. Chromatography was carried out by the ascending technique in cylinders (6″ dia. × 18″ in height) which were lined internally with Whatman No. 1 filter paper. Chromatography was carried out at 23° and required 24–30 h. The fatty acids were dissolved in chloroform at a concentration of 20–30 μg/10 μl. 10 and 20 μl were used for chromatography.

** These acids may occur to the extent of a few percent and not be detected by these procedures.

In the case of reduced pig heart lecithin (which was a mixture of the diester lecithin and plasmalogen in the ratio of 3 : 2) the fatty acids of the lysolecithins are a mixture of C-16, C-18, and C-20 and a small amount of C-22. The long chain monoether of glycerolphosphoric acid (GP-ether) also occurs in this fraction. The fatty acids released by the venom are predominantly C-20, a lesser amount of C-18, and a very small amount of C-22. No detectable amounts of C-16 acids were released by the venom**.

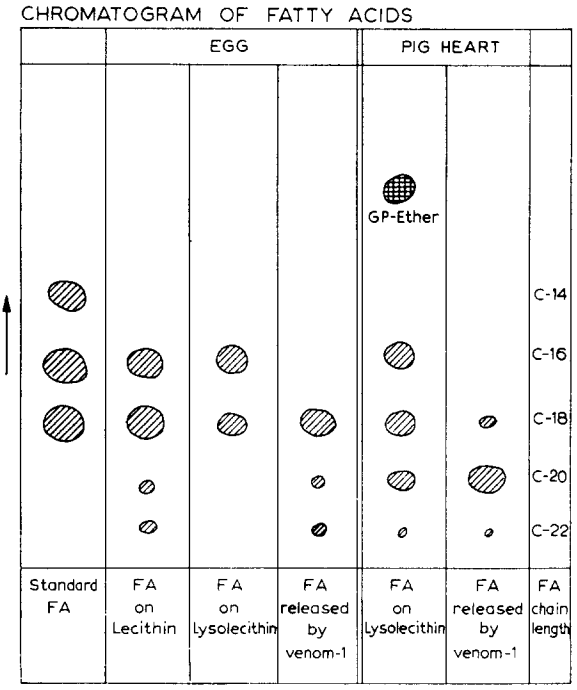

Fig. 1. Chromatograms of the fatty acids of reduced egg and pig heart lecithins and their corresponding enzymically produced lysolecithins. Chromatography was carried out as explained in the text. The standard fatty acids are myristic (C-14), palmitic (C-16) and stearic (C-18). GP-ether = the long chain ether of glycerolphosphate. This ether results by hydrolysis of the reduced plasmalogens which were found in the pig-heart lecithin. Venom —1 = *Crotalus adamanteus* venom. FA signifies fatty acid.

The paper chromatographic analysis of the fatty acids of rat-liver lysolecithin, and the fatty acids released by both *Naja naja* and *Crotalus adamanteus* are shown in Fig. 2. In this case the venoms were allowed to act on the unreduced lecithins. The fatty acids released by the venom are predominantly unsaturated C-18, C-20 and C-22. These acids gave a strong positive test with the permanganate reagent. The fatty acids of the rat-liver lysolecithins are predominantly saturated C-16 and C-18 (they fail to react with permanganate). When the fatty acids released by the venom were hydrogenated it became apparent that these acids were predominantly C-20 but that smaller amounts of C-18 and C-22 acids also occurred. Although the venoms liberated the C-20 acids in highest amount, in the case of *Naja naja* less C-18 and more C-20

** See footnote on page 535.

acids were released than was so with *Crotalus adamanteus*. Hence with three different lecithin preparations no detectable amount of C-16 acids were liberated by the phospholipase.

The fatty acids of the keto-lysolecithin produced by bromine oxidation[1] of egg lecithin and the fatty acids of the lysolecithin which resists oxidation by bromine were also investigated. Both lyso-compounds were found to contain C-16 and C-18 acids. The C-16 acids predominated. It is apparent from these studies that egg

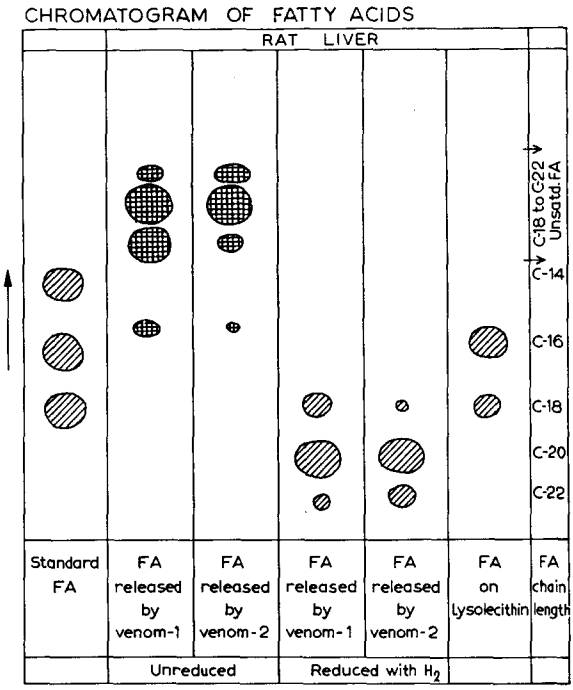

Fig. 2. Chromatogram of the fatty acids of rat-liver lecithin and its corresponding enzymically produced lysolecithin. Chromatography was carried out as given in the text. The fatty acids of rat-liver lecithin were chromatographed before and after reduction with hydrogen. The standard fatty acids are the same as those shown in Fig. 1. Venom —1 = *Crotalus adamanteus* venom; venom —2 = *Naja naja* venom. FA signifies fatty acid.

lecithin is heterogeneous with respect to the fatty acids and it is unlikely that lecithins containing both fatty acids of the same type (*i.e.* distearyl, dipalmityl, dioleyl, etc.) occur in egg in appreciable amount.

The work in recent years has implicated phosphatidic acids or diglycerides as intermediates in the synthesis of both phosphatides and glycerides[7-9]. If this finding is true, then it may be expected that the beta-linked and one of the alpha-linked fatty acids of triglycerides have the same distribution pattern as found in the glycerophosphatides. The fact that triglycerides contain less highly unsaturated fatty acids than the phosphatides[10] may mean therefore that the fatty acids of the triglycerides undergo reduction after they have been positioned on the molecule. On the other hand, the reverse situation may prevail, namely that the fatty acids of the phosphatides undergo dehydrogenation once they are positioned on the molecule. In this

regard it is noteworthy that the desaturation of fatty acids is restricted mainly to stearic to oleic, palmitic to palmitoleic and linoleic to arachidonic conversions[11,12]. If isomerization of the fatty acids occurs either on the phosphatides or triglycerides or if transesterification occurs between the various lipids the problem then becomes quite complex. Data relative to these latter processes are, however, yet forthcoming. It is possible also that the more unsaturated diglycerides are used primarily for the synthesis of phosphatides[13,14].

Since by our studies it appears that the venom phospholipase A shows preference in removing the beta-linked fatty acid on lecithins and that these fatty acids are predominantly unsaturated, it seems, in direct contrast to the assumptions of HANAHAN[15], that the unsaturated fatty acids occur to a greater extent on the beta position of these phosphatides*. Furthermore, the C-16 acids (in particular palmitic acid) are located primarily on the alpha position of glycerol. This conclusion can be made at least for rat-liver lecithin but whether it can be made for lecithins from other sources remains to be elucidated. This finding would be in harmony with the recent studies of MATTSON AND LUTTON[16] who have shown that in most animal fats (except lard from pig) the unsaturated fatty acids show some selection for the beta position. These observations would also be compatible with recent work which indicates that diglycerides are common intermediates in the synthesis of both triglycerides and phosphatides[7–9].

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* In a personal communication Dr. M. KATES of the National Research Council, Ottawa, Canada, has informed the authors that he and Dr. A. TATTRIE have confirmatory evidence that the snake venom phospholipase A preferentially liberates the β-linked fatty acids of egg lecithin and that these acids are predominantly unsaturated. This work is now in press (*J. Lipid Research*).