The Action of Cabbage-Leaf Phospholipase D upon Lysolecithin

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1. The action of the water-soluble phospholipase D from Savoy cabbage leaves upon an aqueous solution of lysolecithin has been studied. 2. Optimum conditions required the presence of Ca^{2+} ions and pH 5.8. 3. Equivalence was found between the amounts of lysolecithin hydrolysed and free choline released. The reaction was not accompanied by deacylation. 4. As the enzymic degradation proceeded, an opalescence developed, owing to the insolubility of the reaction product in the presence of Ca^{2+} ions. 5. Evidence has been obtained indicating the heterogeneity of this reaction product.

Davidson & Long (1958) described the partial purification from Savoy cabbage leaves of a water-soluble phospholipase D (phosphatidylcholine phosphatidohydrolase; EC 3.1.4.4), and studied its action upon ovolecithin and certain other phospholipid substrates. In confirmation of the work of Kates (1954), who used the insoluble plastid enzyme, the presence of diethyl ether in the reaction system was found to be necessary when water-insoluble substrates were used. Unlike the plastid enzyme, however, the water-soluble phospholipase D exhibited an absolute requirement for Ca^{2+} ions. Davidson & Long (1958) reported that lysolecithin acted as a substrate for the soluble phospholipase D, and that this reaction also required Ca^{2+} ions but was inhibited by the presence of ether.

In the present work, the enzymic decomposition of lysolecithin has been investigated in greater detail. Some of these results have already been published in a preliminary report (Long, Odavić & Sargent, 1962).

EXPERIMENTAL

Materials

Ovolecithin. This was isolated from egg yolk by the procedure of Rhodes & Lea (1957), with chromatography on columns of alumina and silicic acid.

Lysolecithin. A solution of ovolecithin in diethyl ether was treated with the venom of the black cottonmouth moccasin, *Agkistrodon piscivorus piscivorus*, under the conditions described by Long & Penny (1957). The precipitated lysolecithin was collected by centrifugation, dissolved in CHCl_3 -methanol (2:1, v/v) and washed once with 0.2 vol. of 0.1M-KCl (Folch, Lees & Sloane-Stanley, 1957). The

lower phase was evaporated to dryness under reduced pressure at a temperature below 40° . The residue was dissolved in CHCl_3 -methanol (47:3, v/v) and stored at -20° . To prepare an aqueous solution, a sample was evaporated to dryness in a current of N_2 , and the residue was dissolved in water to final concn. $20 \mu\text{moles/ml}$.

Phospholipase D. Several preparations of the enzyme, purified up to stage 3 (acetone-precipitation step), were made according to the method of Davidson & Long (1958). Since the enzyme is only about 15% as active with lysolecithin as with lecithin as substrate, these preparations were combined and the acetone-precipitation step was repeated. This gave a highly active enzyme, which was dissolved in water and stored in the frozen state at -20° . This solution contained in 1 ml. the enzyme extractable from 15 g. of cabbage leaves. Activity was constant for at least 18 months.

Methods

Phospholipase D reaction system. For kinetic studies, enzymic degradation was carried out in glass-stoppered 5 ml. centrifuge tubes, the total volume of the reaction mixture being 0.2 ml. This contained lysolecithin and phospholipase D, together with various concentrations of CaCl_2 and sodium acetate-acetic acid buffers of different pH values. The reaction mixtures were incubated at 38° for appropriate periods, enzymic activity invariably being indicated by the formation of an opalescence. Enzyme action was stopped by addition of 0.8 ml. of CHCl_3 -methanol (2:1, v/v). After mixing thoroughly and centrifuging, the lower chloroform-rich phase was transferred by teat pipette to a second tube. The upper phase was then washed twice in the same way with 0.5 ml. portions of CHCl_3 -methanol (17:3, v/v). The combined CHCl_3 -rich lower phases contained the unchanged lysolecithin and the final aqueous methanolic upper phase contained the free choline produced by enzymic decomposition. This upper phase was evaporated to dryness in a current of N_2 , and the residue was dissolved in water (0.4 ml.) for determination of choline.

In some experiments, the amount of unchanged lysolecithin was also determined. For this purpose, the solvent

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was removed from the combined CHCl_3 -rich lower phases and the residue was hydrolysed by heating with 0.2 ml. of N-HCl on a boiling-water bath for 1 hr. The cooled hydrolysate was extracted with CHCl_3 -methanol, as already described, to remove fatty acids. The final upper phase was evaporated to dryness, dissolved in water (0.4 ml.) and used for the determination of choline.

Determination of choline. The method used was similar to that described by Appleton, La Du, Levy, Steele & Brodie (1953), as modified by Webster (1956). Tapered 3 ml. centrifuge tubes were used. The sample containing choline (not more than $0.2 \mu\text{mole}$) in aqueous solution (0.4 ml.) was treated with 0.1 ml. of 25% (w/v) trichloroacetic acid containing $0.025 \mu\text{mole}$ of choline iodide, followed by 0.2 ml. of the iodine reagent (15.7 g. of I_2 and 20 g. of $\text{KI}/100 \text{ ml.}$). The tubes were left at 2° in a cold room for 30 min. and then centrifuged for 15 min. at 5000 g, also at 2° . The supernatant was removed carefully and completely by means of a fine suction pipette attached to a water pump. To each tube 0.3 ml. of CHCl_3 (A.R. grade; Hopkin and Williams Ltd., Chadwell Heath, Essex) was added and the solid choline enneaiodide was dissolved by holding the tube against a vibrating rod. The solution and washings from the tube were finally diluted to 10 ml. with CHCl_3 and the extinction was read at $365 \text{ m}\mu$. Standards (0.1 and $0.2 \mu\text{mole}$ of choline iodide) and blanks were run simultaneously. The addition of a constant small quantity of choline iodide in the trichloroacetic acid reagent, which was suggested to us by Dr G. H. Sloane-Stanley of this Department, slightly overcompensated for the solubility of choline enneaiodide in the aqueous medium. Thus after deduction of a small blank value (extinction about 0.05), a linear relationship between choline concentration and extinction was obtained.

Lysolecithin itself reacted with the iodine reagent to give an extinction at $365 \text{ m}\mu$ equivalent to about 0.62 of that obtained with the same molecular proportion of choline. It was for this reason that it was necessary to remove the unchanged lysolecithin from the reaction medium before proceeding to the determination of the free choline produced.

Total phosphorus. This was determined by the method of Berenblum & Chain (1938), as modified by Long (1943), on samples of material ashed with 72% perchloric acid.

Acyl ester. The hydroxamate method of Shapiro (1953) was used. When this procedure was applied directly to the product of the action of phospholipase D on lysolecithin, values lower than those expected were observed, i.e. there was an apparent disappearance of acyl ester groups. This result was traced to the insolubility of the calcium salt of the product of enzyme action. To overcome this difficulty, the calcium was first removed by treating the reaction mixture with m-citric acid (0.1 ml.). The lipids were then extracted with 1.2 ml. of CHCl_3 -methanol (2:1, v/v) followed by $2 \times 0.5 \text{ ml.}$ of CHCl_3 -methanol (17:3, v/v). The combined lower phases were evaporated to dryness in a current of N_2 before applying the hydroxamate procedure.

Total nitrogen. This was determined according to Long & Staples (1961).

pH values. These were measured with a pH meter (Beckman) and a glass electrode.

Thin-layer chromatography. This was carried out in the usual way with Camlab apparatus. The developing solvent was CHCl_3 -methanol-water (65:25:4, by vol.). Phospholipids were detected by spraying with a solution consisting

of sodium molybdate (6.85 g.), hydrazine sulphate (0.4 g.) and conc. H_2SO_4 (100 ml.), diluted with water to 1 l. This is the reagent proposed by Hahn & Luckhaus (1956) for the quantitative determination of inorganic orthophosphate. Phospholipids give blue spots on a colourless background; no heating is necessary. Low-molecular-weight water-soluble phosphorus compounds (inorganic orthophosphate, β -glycerophosphate, phosphorylcholine) do not react under these conditions; the presence of a long fatty chain seems to be required in addition to a phosphate group.

RESULTS AND DISCUSSION

General properties of the enzyme

The phospholipase D preparation used throughout these studies contained $3.6 \mu\text{g. atoms}$ of total phosphorus and $74.2 \mu\text{g. atoms}$ of total nitrogen/ml. All of the phosphorus was found to be due to contamination by inorganic orthophosphate. When $0.20 \mu\text{mole}$ of lysolecithin was used as substrate with 0.003 ml. of enzyme in a total volume 0.20 ml. , optimum conditions required the presence of $5 \mu\text{moles}$ of calcium chloride and pH 5.8; the buffer used was sodium acetate-acetic acid (final concn., 0.1 M). Incubation at 38° for 60 min. caused an average release of $0.132 \mu\text{mole}$ of choline; range 0.105 – 0.171 (7). These values correspond to about 0.7 unit of activity/ml. of enzyme, or a specific activity $0.11 \text{ unit/mg. of protein}$ (*Enzyme Nomenclature*, p. 7, 1965; Amsterdam: Elsevier Publishing Co.). The reaction rate was proportional to time and to enzyme concentration until about 80% of the substrate had disappeared, and thereafter decreased.

Fig. 1. Effect of pH on degradation of lysolecithin by phospholipase D. Reaction mixtures contained lysolecithin ($0.2 \mu\text{mole}$), enzyme (0.003 ml.), CaCl_2 ($2 \mu\text{moles}$) and acetate buffers of different pH values (final concn., 0.1 M). Total vol., 0.20 ml. Incubation was for 60 min. at 38° .

Effect of pH. Fig. 1 shows the relationship between pH and enzymic activity. There is a fairly broad optimum between pH 5.4 and 6.4, with maximum activity at pH 5.8. This optimum pH is similar to that found by Davidson & Long (1958), with ovoidlecithin as substrate. Values between 5 and 6 have also been reported for the enzyme from cottonseed (Tookey & Balls, 1956) and carrot (Einset & Clark, 1958), with soya-bean lecithin as substrate.

Effect of calcium ion concentration. This is shown in Fig. 2. In the absence of added calcium, the enzyme is inactive; maximum activity required the presence of about 5 $\mu\text{g. atoms}$ of calcium/0.20 ml., i.e. concentration 0.025 M.

Effect of substrate concentration. Fig. 3 shows the relationship between choline formation and lysolecithin concentration. Even at the lowest concentration used, the enzyme was almost saturated. A Lineweaver-Burk plot of the results gave a Michaelis constant about 1.5×10^{-4} M. There is some inhibition of activity by excess of substrate. With lecithin as substrate, much higher K_m values have been reported, e.g. 2.1×10^{-2} M for the cabbage enzyme (Tookey & Balls, 1956) and 1×10^{-2} M for the carrot enzyme (Einset & Clark, 1958); however, in view of the insolubility of this substrate, the significance of these values is doubtful.

Relationship between lysolecithin disappearance and release of free choline. In the experiment shown

in Fig. 4, both the amounts of choline released and residual lysolecithin were determined after different periods of incubation. It will be seen that the disappearance of lysolecithin is accompanied by the formation of an equivalent amount of free choline.

Absence of deacylation during the enzymic degradation. Experiments in which acyl ester was determined directly on the reaction products by the hydroxamic acid procedure of Shapiro (1953) indicated that there was some decrease in the amount during the course of the reaction. However, this result was traced to the insolubility of the calcium salt of the reaction product. When calcium was removed, as described in the Experimental section, the acyl ester value was found to be 1.05 μmoles after 2 hr. of incubation, compared with 1.00 μmole at zero time. During this same period, 78% of the lysolecithin had been degraded.

Product of enzyme action

Development of opalescence. Under the usual conditions of enzyme assay with a medium buffered at pH 5.8, an opalescence developed during the

Fig. 2. Effect of Ca^{2+} ions on the degradation of lysolecithin by phospholipase D. Reaction mixtures contained lysolecithin (0.2 μmole), enzyme (0.003 ml.), acetate buffer, pH 5.8 (0.1 M, final concn.) and various amounts of CaCl_2 . Total vol., 0.20 ml. Incubation was for 60 min. at 38° .

Fig. 3. Effect of substrate concentration on the degradation of lysolecithin by phospholipase D. Reaction mixtures contained enzyme (0.002 ml.), CaCl_2 (4 μmoles), acetate buffer, pH 5.8 (final concn., 0.1 M) and different amounts of lysolecithin. Total vol., 0.20 ml. Incubation was for 30 min. at 38° .

Fig. 4. Relationship between lysolecithin breakdown by phospholipase D and choline release. Reaction mixtures contained lysolecithin ($1\mu\text{mole}$), enzyme (0.010ml.), CaCl_2 ($20\mu\text{moles}$) and acetate buffer, pH 5.8 (final concn., 0.1M). Total vol., 0.25ml. Incubation was for 30, 60 or 120 min. at 38° . ●, Lysolecithin; ○, choline.

incubation and a white insoluble reaction product gradually separated out in granular form. When an unbuffered system was used, however, the opalescence proved to be stable, so that the course of the reaction could be followed by measuring the extinction of the reaction mixture. This is shown in Fig. 5.

Any procedure which severely inhibited enzymic activity, as measured by the release of free choline, e.g. unfavourable pH, absence of Ca^{2+} ions, presence of 0.001M -mercuric chloride etc., was unaccompanied by opalescence.

Solubility properties. Preliminary experiments suggested that the reaction product was heterogeneous in nature. Thus when lysolecithin (about $7\mu\text{moles}$) was incubated with appropriate amounts of enzyme and calcium chloride and the reaction mixture was centrifuged, about two-thirds of the material based on phosphorus content was present in insoluble form (A), the remaining one-third (B) being present in the supernatant. These proportions were found after making an allowance for the residual lysolecithin (about 10% of the starting

Fig. 5. Development of opalescence during the enzymic degradation of lysolecithin by phospholipase D. The unbuffered reaction mixture contained lysolecithin ($7\mu\text{moles}$), enzyme (0.105ml.), CaCl_2 ($105\mu\text{moles}$) and water to a total vol. of 2.1ml. The tube was incubated at 38° . At intervals, the extinction was measured in a photoelectric colorimeter (Evans Electroselenium Ltd.) with filter OB2 (max. transmission at $480\text{m}\mu$).

amount) based on determinations of bound choline, and for the phosphorus content of the enzyme. When these two fractions were partitioned between chloroform, methanol and water, in the proportions used in the procedure of Folch *et al.* (1957), the insoluble material (A) passed almost completely into the chloroform-rich phase, whereas the water-soluble product (B) remained entirely in the aqueous phase.

Thin-layer chromatography. An enzymic degradation of lysolecithin was conducted under optimum conditions and $10\mu\text{l.}$ samples of the reaction mixture were taken at different times for thin-layer-chromatographic examination, under the conditions described in the Experimental section. At zero time, only the lysolecithin spot ($R_f 0.10$) was seen. After 30 min., much of the lysolecithin had disappeared, and had been replaced by two spots, one remaining at the origin, and the other moving with R_f about $0.45\text{--}0.50$. After 180 min., all the lysolecithin had disappeared. The two fractions, designated A and B in the last section, were also examined by thin-layer chromatography. Fraction A had R_f about 0.50 , and fraction B remained at the origin. The chemical nature of these two

compounds is discussed in the next paper (Long, Odavić & Sargent, 1967).

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