Properties and Specificity of Pancreatic Phospholipase A

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The hydrolysis of lecithin by pancreatic juice and extracts has been the subject of many investigations. However, the results obtained by various authors are contradictory (see Ercoli, 1940): whereas some failed to find any activity, others found more or less active splitting of lecithin. The contradictions seem to be due partly to the various preparations used (fresh pancreas, partly autolysed tissue, pancreatic juice, pancreatin preparations, etc.) or to the inadequacy of analytical methods.

In more recent investigations, Schmidt, Hersman & Thannhauser (1945) isolated glycerylphosphorylcholine from autolysing ox pancreas, and Shapiro (1952) demonstrated the presence in acetone-dried powders of ox pancreas of two enzymes, which split lecithin and lysolecithin into glycerylphosphorylcholine. The lysolecithinase was purified to a high degree (Shapiro, 1953), but the mode of action of the lecithin-splitting enzyme remained unclear.

Hanahan (1952) reported that pancreatic phospholipase A could split lecithin in ether solution. This enzyme was found in extracts of fresh rat pancreas or in commercial pancreatin preparations. Working with fresh ox pancreas, we were unable to extract an active enzyme nor could any lecithinase activity be demonstrated in the homogenate of the fresh tissue. However, upon storage of the frozen pancreas for at least 2 weeks, phospholipase activity appeared. This activity was confined to the insoluble particles.

This paper deals with the purification of this enzyme, its properties and its specificity. The substrates tested were phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, which are generally regarded as substrates of phospholipase A (Zeller, 1950). In addition, phosphatidic acid, which was reported in a preliminary note (Rimon, Garcia & Shapiro, 1957) to be split by the pancreatic enzyme, was also included in this study.

EXPERIMENTAL

Materials. Phosphatidyl choline (PC) was prepared from egg yolk according to Hanahan, Turner & Jayko (1951). Phosphatidic acid (PA) was prepared enzymically from PC

according to Kates (1956). Phosphatidyl ethanolamine (PEA) and phosphatidyl serine (PS) were prepared from sheep brain by the method of Folch (1942, 1949), based on the differences in solubility of the phospholipids in various mixtures of chloroform and ethanol.

Reaction system. The test mixture was composed of 4–5 μ moles of substrate suspended in 0.5 ml. of water and 0.5 ml. of enzyme suspension at pH 6.5. A solution containing 30 μ moles of CaCl_2 (0.05 ml.) was added to the test mixture, except when PA served as substrate. The incubation was carried out in 10 mm. $\times$ 100 mm. test tubes at 37°. It was found that under these conditions the reaction velocity was linear until 80% of the substrate was decomposed.

Measurement of enzyme activity. The enzyme activity was measured by the decrease in concentration of acyl ester bonds after a period of incubation by the following procedure: The reaction was stopped by the addition of 4 ml. of ethanol-diethyl ether (3:1, v/v). The tubes were centrifuged for 3 min. at 1000 g. Samples of the supernatant (2 ml.) were transferred into 15 mm. $\times$ 150 mm. tubes and the volume was brought to 3 ml. by the addition of the ethanol-ether mixture and acyl ester-bond concentration was estimated by the hydroxamic acid method of Stern & Shapiro (1953). A unit of enzyme activity was defined as the amount of enzyme catalysing the splitting of μ equiv. of ester bonds in 1 hr. at 37°. Specific activity was expressed in units/mg. of protein N.

Preparation of enzyme

Step 1: homogenization of pancreas. Ox pancreas, which had been stored at -12° for at least 2 weeks, was used as a source of the enzyme. Such pancreas showed appreciable activity, which was preserved for many months if the tissue was kept frozen. Samples of the frozen pancreas were thawed and homogenized with twice their weight of water in a Waring Blendor for about 1 min. The homogenate was filtered by squeezing through a double layer of gauze. The filtrate is referred to as enzyme preparation I.

Step 2: separation from soluble proteins. Preparation I was centrifuged for 1 hr. at 20 000 g at $3-6^\circ$ and the turbid supernatant layer discarded. (With pancreas preparations which had been kept frozen for more than a month, longer centrifuging was needed.) The precipitate was resuspended by blending in the original volume of 0.05 M-sodium ethylenediaminetetra-acetate solution, pH 6.0, and centrifuged for 10 min. as before. The slightly turbid supernatant layer was again discarded and the precipitate resuspended in water and again recovered by centrifuging. The supernatant layer, which was usually practically clear, was discarded. The precipitate was again resuspended with the aid of a Waring Blendor in 0.067 M-Sørensen phosphate buffer, pH 6.5. This is referred to as enzyme preparation II.

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Table 1. *Substrate specificity and degree of purification of phospholipase A preparations from pancreas*

Activity was measured as described in the Methods section with lecithin as substrate. (A unit of enzyme action was defined as the amount of enzyme catalysing the splitting of 1 μ equiv. of ester bonds in 1 hr. at 37°.) Preparation I: crude homogenate; II: washed particles from the homogenate; III: heat-treated particles; IV: purified heat-treated particles.

Enzyme preparation	Total activity (units)	Specific activity (units/mg. of protein N)	Substrates decomposed by the preparation
I	156	0.02	PC; PA; lysolecithin; triglycerides; PEA; PS
II	84	0.15	PC; PA; lysolecithin; triglycerides; PEA; PS
III	63	0.26	PC; PA; PEA; PS
IV	21	0.87	PC; PA; PEA; PS

Fig. 1. Solubility of pancreatic phospholipase A in glycine buffer of various pH values. Enzyme preparation III (see Table 1 and text) was suspended in 0.1M-glycine-NaOH buffer, of various pH values, at room temp. The suspension was centrifuged for 10 min. at 3–6° at 20 000 g and phospholipase A activity was measured in the supernatant (●) and in the precipitate, which had been readjusted to pH 6.5 (○), by the method described with lecithin as substrate.

Quantitatively it contained particles from 1 g. of pancreas in 2 ml. of suspension.

Step 3: destruction of phospholipase B activity. Enzyme preparation II was heated at 70–75° for 10 min., cooled under tap water and centrifuged for 15 min. at 20 000 g at 3–6°. This treatment destroyed any phospholipase B activity present, and the activity of phospholipase A remained unchanged. The precipitate is referred to as preparation III.

Step 4: dissolving and reprecipitating. The solubility of the enzyme at various pH values in glycine-NaOH buffer is shown in Fig. 1. When dissolved at pH 9.8, addition of ethanol up to 32% (v/v) did not bring about precipitation of the enzyme, and higher concentrations destroyed it. Accordingly, preparation III was resuspended at room temperature in 0.1M-glycine-NaOH buffer, pH 9.8, which contained 32% (v/v) of ethanol. The undissolved material was removed by centrifuging for 10 min. at 13 000 g at 3–6°. The supernatant was adjusted to pH 6.5–7.0 with N-HCl. The precipitate that formed was collected by centrifuging for 20 min. at 20 000 g at 3–6° and resuspended in half the original volume of 0.067M-phosphate buffer, pH 6.5. This is referred to as enzyme preparation IV and contained, in 1 ml. of suspension, particles originating

Table 2. *Influence of calcium ions on the enzymic decomposition of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidic acid by pancreatic phospholipase A*

Enzyme preparation II (see Table 1 and text) was used in these experiments with the indicated concentrations of Ca^{2+} ions and 4 μ moles of substrates. Time of incubation with PC was 60 min., with PA 30 min. and with PEA and PS 5 min.

Concn. of Ca^{2+} ions present (M)	Enzymic decomposition (μ moles) of			
	PC	PEA	PS	PA
0	0.0	0.0	0.0	2.5
0.02	2.9	1.2	1.5	—
0.04	3.1	1.0	1.7	0.5

from 1 g. of pancreas. The specificity, state of purity and the yield of the four enzyme preparations is given in Table 1. It is evident that after the third step in the preparation only phospholipase A activity is present and that the four steps described yield at least a 40-fold purification, since the activities for preparations I and II given are too high, owing to the lysolecithinase activity which still prevails in them.

RESULTS

Influence of calcium ions upon enzyme activity. Previous observations, that the presence of Ca^{2+} ions is required for phospholipase A activity (Zeller, 1950; Hayaishi & Kornberg, 1954), could be verified for the action of the enzyme on PC, PEA or PS. On the other hand, when PA served as a substrate, Ca^{2+} ions had an inhibitory effect. Table 2 shows the influence of Ca^{2+} ions on the enzymic decomposition of PC, PEA, PS and PA.

Heat-stability of the enzyme. Various figures are given in the literature for the heat-stability of phospholipase A from various sources. A bacterial preparation was stable to 97° at pH 9 for 5 min. (Hayaishi & Kornberg, 1954). A phospholipase A from snake venom was stable up to 97° at pH 5.9 (Hughes, 1935). An enzyme extracted from pancreatin was 80% destroyed by heating it to 70° for 10 min. at pH 6.8 (Hanahan, 1952).

It was found that our enzyme preparations were stable up to about 80° when heated for 10 min. at

pH 6.5. When the temperature was raised above this point, inactivation started. However, the temperatures producing inactivation differed from preparation to preparation. This may be partly due to the amounts of Ca^{2+} ions retained in the preparation, since addition of Ca^{2+} ions lowered the temperature at which inactivation started. When 0.02M - Ca^{2+} ion was present during heating, temperatures between 70° and 90° caused inactivation when PC was subsequently used as the substrate, but not with PA, PEA or PS (Table 3).

Reactivation of the heat-inactivated enzyme. Enzymic preparations which had lost their activities towards PC through heating for 10 min. at 90° in the presence of Ca^{2+} ions, could be reactivated by pre-incubation at 37° for 2–4 hr. (Fig. 2). The presence of PC during the reactivation period did not influence the process of reactivation. Owing to this reactivation effect, complete inactivation could be demonstrated only when the incubation time of the tests was short (30–60 min.). With longer incubations, heat-inactivation became less and less pronounced.

Product of decomposition of phosphatidic acid. As PA has previously not been used as a substrate of phospholipase A, the identification of the reaction product was undertaken. In the reaction at 37° after 2 hr. the acyl ester-bond concentration was halved. No further decrease was observed when the incubation was continued for another 20 hr. Thus the reaction product was a monoacyl compound derived from PA by hydrolysis of one of the two fatty acids attached to the glycerol moiety.

The isolation of the product was based on its solubility properties, which resembled those of PA except that the sodium salt was insoluble in ether and gave a clear solution in water at 37° . The procedure used for isolation of the product was as

follows: 100 mg. of PA was suspended in 25 ml. of water; to this was added 50 ml. of enzyme preparation III and the mixture was incubated for 3 hr. at 37° . The reaction was stopped by the addition of 15 ml. of *N*-perchloric acid, and the whole was then extracted with ether (3×100 ml.). The combined ether extracts were concentrated *in vacuo* to the minimum volume which permitted a clear solution of the lipids therein, and 4 vol. of ethanol were added. Any precipitate that formed was discarded. To the clear ethanol-ether solution 40% (w/v) NaOH soln. was added in drops until

Fig. 2. Reactivation of heat-inactivated phospholipase A. Enzyme preparation II (see Table 1 and text) was heated for 10 min. at 90° in the presence of 0.02M - Ca^{2+} ions. Its activity was determined by incubation with PC as described. ▲, Unheated enzyme; ●, heated enzyme; ○, enzyme which had been pre-incubated for 4 hr. at 37° after heating.

Table 3. *Decomposition of phosphatidyl choline, phosphatidic acid, phosphatidyl ethanolamine and phosphatidyl serine by heat-treated phospholipase A*

Preparation II (see Table 1 and text) was used as the enzyme. The preparation was heated for 10 min. at 90° at pH 6.5 in the presence of 0.02M - Ca^{2+} ions. PC and PA were incubated at pH 6.5; PEA and PS at pH 7.0. Phospholipase A activity was measured as described in the text.

		Hydrolysis of μ equiv. of ester upon incubation with							
		Untreated enzyme				Heated enzyme			
		Time (min.)							
Expt.	Substrate	10	15	30	60	10	15	30	60
1	PC	—	—	0.7	1.4	—	—	0.2	0.6
	PA	—	—	1.0	1.4	—	—	0.9	1.1
	PEA	1.1	1.3	—	—	1.1	1.3	—	—
	PS	0.6	0.7	—	—	0.6	0.7	—	—
2	PC	—	—	—	1.0	—	—	—	0.0
	PA	—	—	1.5	—	—	—	1.5	—
	PC*	—	—	—	1.3	—	—	—	0.9
	PA*	—	—	1.2	—	—	—	0.9	—

* Ca^{2+} ions omitted.

a pH value of 6.0–6.5 was obtained. The precipitate which formed was collected by centrifuging and extracted with ether (2×10 ml.). The ether extracts were discarded. The ether-washed precipitate was dried and dissolved in water at 37°. Upon analysis it contained phosphorus and acyl ester bonds in a molar ratio of 1:1, and 90–95% of the theoretical values calculated as a disodium salt of monoacylglycerophosphate. Its structure was established according to the method of Hanahan (1954) by oxidation with potassium permanganate followed by acid hydrolysis and analysis of organic and inorganic phosphate. According to this method, the structure was of a β -acyl- α -glycerophosphate. The product was found to have haemolytic properties of a potency equalling that of lysolecithin (Rimon, 1958).

DISCUSSION

Lecithinase A was found to be inactive in fresh ox pancreas and to become activated during storage in the frozen state. The mechanism of this activation is not yet clear. Experiments conducted with the aim of activating the enzyme by autolysis at higher temperatures gave variable results. This may perhaps explain the variations in activities found by various authors working with different commercial-pancreatin preparations.

The enzyme, which is soluble at neutral pH values, was much more active towards phosphatidyl serine, phosphatidyl ethanolamine and phosphatidic acid than towards phosphatidyl choline. The choline group seems to be detrimental to the enzymic cleavage. The difference between the substrates is even more pronounced with the heat-treated enzyme. Heating to 90° in the presence of Ca^{2+} ions had almost no effect on the activity towards all the non-choline substrates but destroyed activity towards lecithin. Experiments to elucidate these special properties of the enzyme towards phosphatidyl choline are in progress.

The product formed by the action of phospholipase A on phosphatidic acid, β -acyl- α -glycerophosphate, has, as far as we are aware, not previously been isolated as the product of an enzymic reaction. Other monoacylglycerophosphate compounds have been prepared synthetically; thus Arnold (1938) synthesized α -acyl- α' -glycerophosphate and α -acyl- β -glycerophosphate, and Kabashima (1938) prepared α -palmitoyl- α' -glycerophosphate. It will be of interest to see whether these isomers resemble our product in haemolytic properties and what structural features are required for haemolysis.

SUMMARY

1. The preparation and purification of phospholipase A from aged ox pancreas is described. No phospholipase A activity could be demonstrated in fresh ox pancreas.
2. The enzyme preparation was insoluble at neutral pH and hydrolysed phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidic acid.
3. The rate of reaction was much slower with phosphatidyl choline than with the other substrates.
4. The product of the enzymic decomposition of phosphatidic acid was isolated and identified as β -acyl- α -glycerophosphate.
5. Ca^{2+} ions were required for the enzymic decomposition of all the substrates except phosphatidic acid.
6. By heating the enzyme preparation to 70–90° in the presence of Ca^{2+} ions the activity towards phosphatidyl choline was lost, whereas that with the other substrates remained unimpaired. The heat-inactivated enzyme could be reactivated by pre-incubation at 37° for several hours.

REFERENCES

- Arnold, H. (1938). *Ber. dtsh. chem. Ges.* **71**, 1505.
Ercoli, A. (1940). In *Handbuch der Enzymologie*, vol. 1, p. 487. Ed. by Nord, F. F. & Weidenhagen, R. Leipzig: Akademische Verlagsgesellschaft.
Folch, J. (1942). *J. biol. Chem.* **146**, 35.
Folch, J. (1949). *J. biol. Chem.* **177**, 427.
Hanahan, D. J. (1952). *J. biol. Chem.* **195**, 199.
Hanahan, D. J. (1954). *J. biol. Chem.* **207**, 879.
Hanahan, D. J., Turner, M. B. & Jayko, M. E. (1951). *J. biol. Chem.* **192**, 623.
Hayaishi, O. & Kornberg, A. (1954). *J. biol. Chem.* **206**, 647.
Hughes, A. (1935). *Biochem. J.* **29**, 437.
Kabashima, J. (1938). *Ber. dtsh. chem. Ges.* **71**, 1073.
Kates, N. (1956). *Canad. J. Biochem. Physiol.* **34**, 967.
Rimon, A. (1958). *Bull. Res. Coun. Israel*, **7A**, 106.
Rimon, A., Garcia, M. D. & Shapiro, B. (1957). *Bull. Res. Coun. Israel*, **6A**, 305.
Schmidt, G., Hershman, B. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 523.
Shapiro, B. (1952). *Nature, Lond.*, **169**, 29.
Shapiro, B. (1953). *Biochem. J.* **53**, 663.
Stern, I. & Shapiro, B. (1953). *Brit. J. clin. Path.* **6**, 158.
Zeller, E. A. (1950). In *The Enzymes*, vol. 1, p. 988. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.