Purification and Properties of a Lysolecithinase from Pancreas

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In a preliminary note (Shapiro, 1952) it was reported that two different enzyme systems which catalyse the formation of glycerophosphorylcholine can be extracted from acetone powders of ox pancreas. One enzyme, a lysolecithinase, catalyses the hydrolytic splitting of lysolecithin and is without effect on lecithin. The second system splits lecithin into glycerophosphorylcholine without any concomitant decrease in total-ester content, suggesting the transfer of the fatty acids from lecithin to an unknown carrier. The present work deals with the properties of the first system, the lysolecithinase, and gives procedures for the quantitative assay and for the purification of this enzyme.

EXPERIMENTAL

Test mixtures. For the estimation of enzyme activity these were made up as follows: 1 ml. of the test mixture contained 5 μ moles of lysolecithin, 40 μ moles of phosphate buffer, pH 6.0, and varying amounts of enzyme solution. With purified enzymes it was found necessary to add glycerol to a concentration of 25% (v/v) in order to avoid rapid inactivation (see Fig. 2). The mixtures were incubated at 37° until not more than 40% of the substrate was split. The activity was followed by estimation of the decrease in acyl-ester content or by the formation of glycerophosphorylcholine.

Unit of enzyme activity. This was defined as the amount of enzyme catalysing the splitting of 1 μ mole of lysolecithin in 1 hr. at 37°.

Specific activity. This was expressed in units/mg. protein.

Analytical methods

Acyl-ester content. A modified hydroxamic acid method was used (Stern & Shapiro, 1952). The reaction was stopped by the addition of 4 ml. of an ethanol-ether mixture (3:1). To 3 ml. of the filtrate 0.5 ml. of a 2M-hydroxylamine hydrochloride solution was added, followed by 0.5 ml. of a 3.5N-NaOH solution. After mixing, the tubes were allowed to stand for 20 min. at room temperature, 0.6 ml. of 3.3N-HCl was added and, after mixing, 0.5 ml. of a FeCl₃ solution (0.37M in 0.1N-HCl) was introduced and the colour was measured in a photometer with a filter, having a maximum transmission at 520 m μ .

Glycerophosphorylcholine. A modification of the method of Schmidt, Hecht, Strickler & Thannhauser (1949) was used, employing the precipitation of choline by iodine according to Roman (1930). Proteins and lysolecithin were precipitated from the reaction mixture by adding 0.1 ml. of a 5% serum-albumin solution and 4 ml. of trichloroacetic

acid (5%, w/v). Samples (2 ml.) of the filtrate were measured into a 15 ml. centrifuge tube, 0.1 vol. of 10N-HCl was added and the tube was heated in a boiling-water bath for 20 min. This treatment splits off the choline from the glycerophosphorylcholine but does not attack phosphorylcholine. After cooling, a drop of 0.1% phenolphthalein solution was introduced and conc. ammonia solution was added until a distinct red colour appeared. (NaOH cannot be used for neutralization, since large amounts of NaCl were found to precipitate the iodine in the later steps of the procedure and give erroneous results.) N-Acetic acid was now added until the red colour just disappeared. The solution was cooled in an ice bath and 0.8 ml. of iodine reagent (157 g. of iodine and 200 g. of KI dissolved in 1 l. of water) added. The tubes were mixed and allowed to stand in the ice bath for 10 min. They were then centrifuged at 2500 rev./min. for 10 min. The supernatant was carefully decanted and the precipitate was washed several times with ice-cold water. The washed precipitate was then dissolved in 2 ml. of CHCl₃ and titrated with vigorous shaking against 0.005N-Na₂S₂O₃: 1 ml. of the Na₂S₂O₃ solution is equivalent to 0.55 μ mole of choline.

Free choline. This was determined by the same procedure but without acid hydrolysis. Under these conditions, glycerophosphorylcholine did not react with the iodine reagent.

In the procedure used by Schmidt *et al.* (1949) mercury acetate was used to remove interfering substances, before the precipitation of the choline with reineckate. Whilst this treatment is necessary for the determination of choline in animal tissues and especially in autolysates, it did not change the results in our enzyme-substrate mixtures.

Protein. Protein in the enzyme solutions was determined spectrophotometrically by measurement of the light absorption at 280 and 260 m μ . with a correction for the nucleic acid content (Warburg & Christian, 1941).

Materials

Lysolecithin. This was prepared by incubating egg yolk with lecithinase A, made from commercial pancreatin according to Gronchi (1936), and was purified according to Belfanti (1941). The waxy mass obtained, dissolved easily in water at 37°. It precipitated on storage in the refrigerator but redissolved immediately on warming.

Lecithin. This was prepared from egg yolk according to Levine & Rolf (1927), suspended by shaking vigorously in hot water and then stabilized by the addition of 3% serum albumin.

RESULTS

Extraction and purification of enzyme

An acetone powder of ox pancreas was extracted twice with cold ether and dried in a desiccator. The dry powder was kept in a refrigerator. The lysolecithinase activity of this

powder remained unchanged for many weeks. The action on lecithin, however, was lost after prolonged storage.

Samples (10 g.) of this powder were extracted with 75 ml. of 50% (v/v) glycerol by grinding in a mortar for 15 min. at room temperature; 75 ml. of 0.1% NaHCO_3 solution were then added and the extraction continued for another 15 min. The suspension was centrifuged at 10 000 rev./min. for 15 min. The resulting glycerol extract kept its activity for several weeks when stored in the cold. This extraction yielded 118 ml. of solution with a total activity of 650 units and a specific activity of 0.15 unit/mg. protein.

Portions of the glycerol extract (110 ml.) were diluted with 440 ml. of water, and N -acetic acid was added with stirring to bring the solution to pH 5.0. The resulting precipitate was collected by centrifugation and was suspended in 100 ml. of 0.04M-phosphate buffer, pH 6.0. Total activity of this suspension was 450 units with a specific activity of 0.28 unit/mg. protein.

The suspension was stirred for 15 min. and the undissolved material, which possessed very low activity, was discarded. The solution was cooled in ice and $(\text{NH}_4)_2\text{SO}_4$ (35 g./100 ml. solution) was added in small portions over a period of 30 min. with continuous stirring. The precipitate was centrifuged in the cold and dissolved in 30 ml. of water. (Yield: 580 units total activity with a specific activity of 1.4 units/mg.) At this stage of purification the enzyme is unstable when kept in aqueous solution, but is relatively stable in the presence of $(\text{NH}_4)_2\text{SO}_4$.

The solution was dialysed for 1 hr. against ice-cold distilled water, with stirring. (Prolonged dialysis caused marked inactivation.) The dialysed solution was diluted with water to 80 ml. and acetic acid was added to bring the pH to 5.0. The resulting precipitate contained very little activity (in contrast to the results with the crude extracts). The clarified solution was refractionated with $(\text{NH}_4)_2\text{SO}_4$ by adding 20 g./100 ml. of the ice-cooled solution, with mechanical stirring. The precipitate was collected by centrifugation below 0° , yielding 86 units with a specific activity of 0.45 unit/mg. protein. To the supernatant, a further 5 g. $(\text{NH}_4)_2\text{SO}_4$ were added for each 100 ml. solution and another fraction was collected, containing 240 units with a specific activity of 1.4 units/mg. protein. By increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration by another 3 g./100 ml. a highly active fraction was obtained, containing 200 units with a specific activity of 4.0 units/mg. protein.

It was found that still higher activity could be obtained, although with much lower yield, by allowing the supernatant, obtained after the addition of 25 g. $(\text{NH}_4)_2\text{SO}_4$ /100 ml., to warm up to 25° and stand for 24 hr. at that temperature. A crystalline material separated out showing a specific activity of 6.5 units/mg. protein. The activity adhered to these crystals in spite of many recrystallizations by dissolving the material in water and adding $(\text{NH}_4)_2\text{SO}_4$ at 0° until a slight turbidity was obtained and then warming up to 25° . The specific activity did not change during these operations. The crystals had the form of small fibres. It was, however, difficult to obtain a satisfactory microscopic picture since they were very fragile.

The above procedure yielded a purification of about 25 times with a reasonable yield of 30–35%. A further purification to about 45 times, but giving low yields, could be obtained by the crystallization procedure.

Products of the reaction and specificity

The results summarized in Table 1 show that the reaction catalysed by the enzyme preparation is the following:

The decrease in acyl-ester content was nearly equivalent to the increase in choline liberated by acid hydrolysis (glycerophosphorylcholine). No free choline was formed. Phosphorylcholine is not determined by the procedures used, but it did not seem to be formed since the decrease in ester linkages was almost completely accounted for by glycerophosphorylcholine formation. Thus, the

Table 1. *Specificity of lysolecithinase action*

(1 ml. of the test mixture, containing 0.5 mg. enzyme protein (fraction obtained between 20–25% (w/v) $(\text{NH}_4)_2\text{SO}_4$) incubated for 60 min. at 37° .)

Substrate	Decrease in ester content (μ moles)	Increase (μ moles) of	
		Glycerophosphorylcholine	Free choline
Lysolecithin	1.85	1.68	0.03
Lecithin	0.02	0.03	0.02

Table 2. *Action of lysolecithinase on triolein*

(5 μ equiv. of triolein dispersed in 0.5 ml. 3% serum albumin + 0.2 ml. of 0.2M-phosphate buffer + 0.05 ml. of 0.05M- CaCl_2 + 3 mg. of enzyme protein, made up to 1 ml. with water and incubated at 37° for 1 hr. Specific activities measured with lysolecithin and values given in brackets.)

Enzyme preparation	pH	Decrease in ester content (μ equiv.)
Crude glycerol extract		
(0.15)	7.5	3.50
(0.15)	6.0	1.58
Acid precipitate		
(0.28)	7.5	0.54
(0.28)	6.0	0.18
$(\text{NH}_4)_2\text{SO}_4$ fraction 25–28% (w/v)		
(4.0)	7.5	0.04
(4.0)	6.0	0.00

enzyme was free from systems causing the further degradation of phosphorylcholine. Contrary to Contardi & Ercoli (1933), who claimed that lecithinase B splits both lecithin and lysolecithin, our preparations did not attack pure lecithin. Glycerol extracts of fresh acetone powders split both lecithin and lysolecithin (Shapiro, 1952); however, two different enzyme systems are involved. The lecithin-splitting system was inactivated on storage and was also dissociated from the lysolecithinase by the purification procedure given in this paper.

The lysolecithinase is not identical with ordinary lipase. Although crude preparations still contained a considerable lipase activity, this activity decreased with the purification concomitant with the increase in lysolecithinase activity. The purer preparations had no lipase activity (Table 2).

pH Optimum

Fig. 1 presents the pH-activity curve of our lysolecithinase preparation. The pH optimum, which lies around pH 6.0, also differentiates this enzyme from the lecithinase preparation, which has its pH optimum at pH 4.5 (Shapiro, 1952). No marked difference was found in the enzyme activity when various buffer materials, such as acetate, phosphate or phthalate were used.

Fig. 1. Effect of pH on lysolecithinase activity. Conditions as in 'test mixture' (see Experimental) except that various pH values and buffers were used. Incubation time, 60 min. at 37°; ●—●, acetate buffer; ○—○, phosphate buffer. The enzyme used was the first $(\text{NH}_4)_2\text{SO}_4$ precipitate.

Substrate and enzyme concentration

The dependence of activity upon substrate concentration is presented in Fig. 2. It is evident that high substrate concentrations inhibited the enzymic activity, as in many other esterases. In the region of substrate concentration of 3–8 $\mu\text{moles/ml.}$ the initial activities were independent of the initial concentration. In this region, the time-activity curve was linear at least up to 50% of cleavage of the total substrate. This was not the case when glycerol was omitted from the test mixture. In the absence of glycerol, the initial rate of the reaction was normal, but the activity disappeared after a short time (curve A). This effect of glycerol does not seem to be due to any impurities, which might serve as an activator, since the same result was obtained with many different brands of glycerol,

varying in their purity. Glycerol seems to act as a stabilizer of the enzyme.

When a substrate concentration of 5 $\mu\text{moles/ml.}$ was chosen it can be seen from Fig. 3 that a straight proportionality exists between enzyme concentration and reaction rate, provided that the extent of hydrolysis is not carried beyond 40% of the initial concentration. The conditions given in our test mixture were thus suitable for a quantitative estimation of the enzyme and the estimation of the decrease in ester content was found to be the easiest and most exact method.

Fig. 2. Progress curves of lysolecithin hydrolysis with different initial substrate concentrations. Conditions as in 'test mixture' except for varying substrate concentrations. Glycerol omitted in curve A. Enzyme as in Fig. 1; constant amount in all experiments.

Fig. 3. Rate of lysolecithin hydrolysis with varying enzyme concentrations. Conditions as in 'test mixture'; incubation 60 min. at 37°. Enzyme as in Fig. 1.

Addition of calcium, magnesium and potassium ions, as well as cysteine, had no effect on the reaction.

DISCUSSION

The enzyme isolated from ox pancreas is similar in its specificity to the enzyme isolated by Fairbairn (1948) from *Penicillium notatum*. In both cases no activity was discovered against lecithin. The pH optima are different, being 4.0 with the *Penicillium* enzyme and 3.5 for the enzyme found in *Aspergillus oryzae* by Contardi & Ercoli (1933). It was not established in this work whether the enzyme attacks lysocephalin, since no purified substrate was available. Our substrate of lysolecithin contained small amounts of lysocephalin, not in quantities large enough, however, to disturb significantly the equivalence between the ester splitting and the glycerophosphorylcholine formation. In the case of the *Penicillium* enzyme, Fairbairn (1948) showed that some ethanolamine could be demonstrated in the glycerophosphorylcholine fraction liberated by the enzyme. Thus, it is very likely that the enzymes discussed are lysophospholipases, rather than specific lysolecithinases.

It remains to be shown whether the crystalline preparation obtained is a pure lysolecithinase protein. The constancy of its specific activity, in

spite of repeated recrystallizations, tends to show that we are not dealing with a chance contamination. However, adequate proof for its homogeneity must await preparations on a larger scale than could be carried out up to now.

SUMMARY

1. Methods for the determination of lysolecithinase activity are described. These are based on the decrease in ester content, measured by a hydroxamic acid reaction, and on the formation of glycerophosphorylcholine, measured by the choline liberated by acid hydrolysis.

2. A procedure is described, by which the pancreas enzyme is purified 25 times with a reasonable yield (30 %).

3. A crystalline preparation was obtained in low yields, with a specific activity 40 times that of the original extract.

4. Optimum activity was found at pH 6.0 and at a substrate concentration varying from 3–8 μ moles/ml.

5. The enzyme does not attack lecithin and triolein.

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The 'Acid Polysaccharides' of Hog Gastric Mucosa

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Following earlier work by Levene & López-Suárez (1916), Meyer, Smyth & Palmer (1937) described a mucoitin monosulphuric acid as the 'acid polysaccharide' of hog gastric mucosa. They used an alkaline extraction of the commercial hog gastric mucin of Wilson Laboratories, Chicago, which is a preparation from whole gastric mucosal tissue. Wolfrom, Weisblat, Karabinos, McNeely & McLean (1943) prepared from the same source, and by a slightly modified procedure, a mucoitin sulphuric acid differing in rotation and analysis from the

former compound. Meyer & Smyth (1938) and Meyer (1938) described the acid calcium salt of a mucoitin sulphuric acid isolated without alkaline digestion. The sulphur content (9.4 %) indicated more sulphate groups than a mucoitin monosulphuric acid contains, and the optical rotation differed from the above compounds.

No criteria of purity were given for any of these preparations and they were undoubtedly mixtures of compounds. Wolfrom & Rice (1947) showed by electrophoretic analysis that the material produced