MULTIPLE FORMS OF PORCINE PANCREATIC PHOSPHOLIPASE A_2: ISOLATION AND SPECIFICITY

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Porcine pancreatic phospholipase A_2 was purified from commercial pancreatin by a method involving heat denaturation, trichloroacetic acid precipitation, and DEAE-cellulose chromatography. Assaying the eluate of the chromatography step by a new titrimetric method using vegetable lecithin-albumin emulsion as the substrate, several species of phospholipase A were found. Some of these went undetected when the conventional egg yolk emulsion assay was used. Two phospholipases A_2 were isolated in a homogeneous form and shown to have similar chemical and physical properties. Catalytic specificity of the two enzymes differs remarkably toward lecithins in different emulsified states.

Phospholipases are used in a variety of techniques ranging from phospholipid structure determination to probing the function of phospholipids in supramolecular structures such as membranes and lipoproteins. An implicit assumption underlying most of the studies using this strategy is that the enzyme hydrolyzes all available phospholipid molecules of the proper stereochemistry. In the case of phospholipase A_2, however, the most extensively studied of these enzymes, large rate differences can be observed with substrates having fatty acids of different chain length (1). Rates might also vary according to the physical state of the substrate dispersion (1) and the source of the enzyme (2). Since fatty acid chain length is an important determinant in the state of dispersion of lipid molecules, it is not clear whether differences in rate reflect enzyme specificity for molecular shape or for supramolecular structural organization. Phospholipase activity can also be critically dependent upon the alteration of the emulsified state of the substrate by interaction with amphipathic additives (3, 4). All of these experiments, which tested the effect of single enzymes on substrates in different physical states, have the disadvantage that changes in substrate structure lead to changes in state of dispersion, so that ambiguity still exists as to the determinants of enzyme specificity.

We have been able to take a different approach to this problem, and the results permit a more definite assessment of the importance of physical state of the substrate dispersion. In this paper we will show that two closely related forms of phospholipase A_2 can give identical hydrolytic rates on one substrate dispersion and vastly different ones on another, thereby showing that substrate specificity and physical state specificity are not related for these two enzymes.

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METHODS

Vegetable phosphatidylcholine was purified by column chromatography on alumina (5).

Dioctanoyl lecithin monohydrate was synthesized by the method of Cubero Robles and van der Berg (6). The compound was pure by criteria of tlc on Unisil silicic acid (chloroform-methanol-water; 65:35:5) and by elemental analysis. Calculated for $C_{24}H_{50}NO_9P$: C, 54.63; H, 9.55; N, 2.65; P, 5.87. Found C, 54.79; H, 9.53; N, 2.52; P, 5.92. The compound was completely hydrolyzed by both pancreatic and snake venom phospholipases A_2, and thus was optically pure.

Porcine pancreatin, USP, lot #X3252, was purchased from Schwartz-Mann.

Deoxycholic acid, Calbiochem, lot #72904 and sodium deoxycholate, Sigma, lot #82C–3060 were used.

Bovine plasma albumin (BPA), crystallized, was purchased from Metrix, Armour Pharmaceutical, lot #G71812.

Phospholipase activity was determined titrimetrically using either an egg yolk emulsion (7) or a vegetable lecithin emulsion as substrate.

The Egg Yolk Emulsion Assay

The egg yolk was separated from the white, washed several times with water, suspended in 100 ml of water and sonicated in an ice water bath under N_2 five times for 1 ml of 1 mM Tris-maleate buffer, pH 6.5, containing 2% BPA and 10 mM $CaCl_2$. The lecithin emulsion was prepared by evaporating 5 ml of diethyl ether containing 200 mg stock emulsion was mixed with 1 ml of 1.8×10^{-2} M $CaCl_2$ and the pH adjusted to 8.1 $\pm$ 0.2 with 1 N NaOH in the reaction vessel of a Radiometer Titrigraph at room temperature. An aliquot of the enzyme solution was then added and the liberated acid was titrated with 1.4×10^{-2} N NaOH as a function of time. One enzyme unit was defined as the amount of enzyme yielding a rate of 1μm of acid per minute under these conditions.

The vegetable lecithin emulsion assay was performed essentially in the same way, but the reaction mixture (at pH 6.8) consisted of 1 ml of lecithin emulsion mixed with 1 ml of T mM Tris-malate buffer, pH 6.5, containing 2% BPA and 10 mM $CaCl_2$. The lecithin emulsion was prepared by evaporating 5 ml of diethyl either containing 200 mg of purified lecithin. Twenty ml of water was then added and the mixture was stirred for 15 min under N_2 with a magnetic stirrer. Then the suspension was sonicated five times for one min at 75 watts, in an ice bath and under N_2.

Amino Acid Analysis

Samples were hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 hr. Composition was determined with a Durrum D-500 automatic amino acid analyzer, and corrected for decomposition by extrapolation to zero time.

RESULTS

In our search for a rich and readily available source of pancreatic phospholipase A_2, we measured the amount of this enzyme and its zymogen in several commercial, defatted,

pancreatin preparations. Although the zymogen–enzyme ratio is quite variable, depending on the source, we found that all pancreatins contained large amounts of the enzyme, and that the Schwartz-Mann USP Pancreatin consistently contained the fully activated form of phospholipase. Therefore, we chose the latter as our starting material in the preparation of porcine pancreatic phospholipase A_2. Our goal was to develop a reproducible procedure which could yield a stable product that could be stored for long periods of time.

The remarkable heat stability of porcine pancreatic phospholipase is well known (7, 8). Consistent with these observations, we found that the enzymatic activity can be recovered in good yield even after precipitation by 10% trichloroacetic acid (TCA), a harsh treatment resulting in the irreversible denaturation of most known proteins. For this reason, our purification is based on a TCA precipitation, preceded by heat denaturation and followed by ion exchange chromatography on DEAE-cellulose.

Purification Procedure

Seven hundred grams of pancreatin and 1 gm of Dow Corning antifoam AF emulsion were suspended in 1.75 liters of 0.15 N NaCl at room temperature and vigorously stirred for 1 min in a Waring blender. The resulting mixture was brought to pH 4.0 by slow addition of concentrated HCl, and heated to 70° for 3 min. After rapid cooling the mixture was centrifuged at 4° at 16,300 × g for 30 min in a Sorvall RC2–B. The supernatant was freed of fat by filtering through glass wool and then adjusted to pH 8.0 with 6 N KOH. The precipitate formed was eliminated by centrifugation as above, and the resulting supernatant was treated at room temperature with 50% TCA solution to a final concentration of 10% TCA. After 15 min of incubation the abundant precipitate was isolated by centrifugation for 40 min at 5,860 × g and 4°, and then repeatedly washed with a total volume of 0.5 liters of acetone on a sintered glass funnel at 4°. The powder was dried under vacuum, resuspended in 50 ml of water at room temperature, adjusted to pH 7.42 with Tris base, and centrifuged at 12,100 × g for 20 min at 4°. The supernatant was chromatographed at 4° on a DEAE–32 column (2.5 × 40 cm) equilibrated with 5 mM Tris-HCl, pH 8.0. Using first a shallow NaCl gradient between 0 and 0.0315 M followed by a steeper gradient between 0.0325 and 0.1 M, several protein peaks were eluted which coincided with enzymatic activity (see Fig. 1). If the peak fractions were immediately frozen and stored at −12°, less than 20% of activity was lost over a 2 month period. Table I summarizes the results of the purification.

Purity and Physical Properties

Analytical polyacrylamide disc-gel electrophoresis at pH = 8.9 (9, 10) of the eluted peaks revealed that at least two of the enzymatically active fractions contained single proteins of different R_f values and less than 3% impurity. The rest of our study will be restricted to these pure enzymes labeled I and II in Fig. 1.. The UV absorption spectra showed that the two enzymes were still contaminated by a low molecular weight UV absorbing material which was readily eliminated by gel filtration on Sephadex G–25. The values of A_{280}/A_{260} are: for enzyme I, 1.06 before and 1.74 after gel filtration (λ_{max} = 277 nm); and for enzyme II, 1.19 before and 1.63 after gel filtration (λ_{max} = 277 nm). The amino acid composition of both enzymes, based on a molecular weight of 14,000, is shown in Table II together with the analysis reported for porcine pancreatic phospholipase A_2 (11).

Estimation of molecular weight by SDS polyacrylamide disc-gel electrophoresis (12) gave approximately 14,000 for both enzymes and 15,000 for their reduced and carboxymethylated forms. The sedimentation velocity and diffusion coefficient were determined for enzyme I on a Beckman Model E Analytical Ultracentrifuge ($S_{20,w}$ = 1.86S; $D_{20,w}$ = 1.12×10^{-6} cm^2/sec). From these results a molecular weight of 13,900 was calculated.

Specificity

The reaction products were analyzed from the hydrolysis of a vegetable lecithin emulsion by our two pancreatic enzymes and by an authentic phospholipase A_2 from snake venom (Ancistrodon piscivorus piscivorus (13), Ross Allen's Reptile Institute, Inc.). The distribution of the 16:0, 18:0, 18:1, and 18:2 fatty acids between the fatty acid and the lysolecithin products was identical for all three enzymes, thus confirming that both pancreatic enzymes are A_2 phospholipases.

The specific activity, as measured with the vegetable lecithin assay at room temperature, was 350 U/mg for enzyme I and 590 U/mg for enzyme II. When activity was measured with the egg yolk lecithin assay at 40° (7), enzyme I was found to have 800 U/mg, quite comparable to the specific activity of the porcine pancreatic phospholipase A_2 described by de Haas et al. (7), whereas enzyme II was virtually inactive under the same conditions.

The specificity of these two enzymes toward the two lecithins was further compared under identical conditions (room temperature and 4 ml total volume). The results are presented in Table III. When sodium deoxycholate was used as the emulsifier, enzyme I displayed the same activity toward both substrates, whereas enzyme II reacted only with the vegetable lecithin. In the presense of BPA both enzymes were specific for vegetable

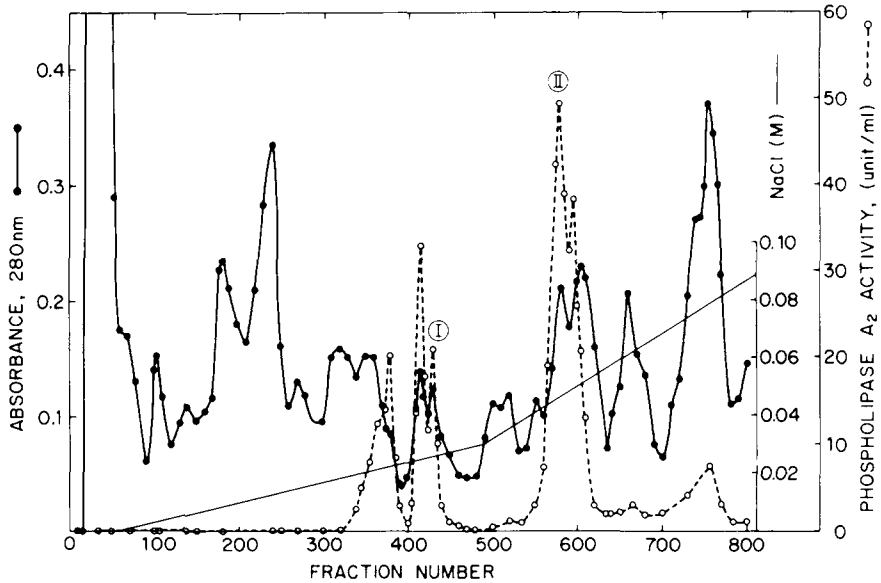

Fig. 1. DEAE–cellulose column chromatography of phospholipases. One gram of protein (24,000 enzyme units) was applied to the DE–32 column (see text). (●—●) Absorbance at 280 nm; (○--○), phospholipase A_2 activity measured with the lecithin-albumin emulsion; (—), NaCl (M). Phospholipase A_2 – I was collected from fraction No. 425–435; phospholipase A_2 – II was collected from fraction No. 575–590. Fraction volume, 4 ml.

lecithin. Finally the simultaneous presence of both deoxycholate and BPA showed the dominance of the deoxycholate effect.

The differences in the reactivity of the two enzymes toward the two lecithin emulsions offer the possibility of determining the relative abundance of the two enzymes in a mixture of the two, by measuring the activity of the mixture in the two standard assays. If the specific activity of the two enzymes toward the vegetable lecithin is denoted by SVI and SVII and toward the egg lecithin by SEI and SEII, then the experimentally measured rates by the two assays (V_v and V_e) yield the weight percentage of enzyme I by the equation.

$$\% \text{ I} = \frac{100 \text{ SVII}}{(\text{SVII} - \text{SVI}) + \text{SEI } (V_v/V_e)}$$

With the use of this method we found that the crude extract of pancreatin contains almost exclusively enzyme of type I in the active form. During the purification procedure the relative abundance of enzyme of type II appears to increase, beginning even before the heat denaturation step.

DISCUSSION

Two phospholipase A_2 enzymes can be isolated in pure form from porcine pancreatin by the procedure reported here. The presence of several other enzymes with activity toward lecithin emulsions was demonstrated, but their characterization would require further

TABLE I. Purification of Phospholipase A_2

Step	Total protein* (mg)	Total activity** (units)	Specific activity (units/mg protein)	Yield %	Purification factor
Homogenate	322,000	48,000	0.15	100	1
Heat treatment	43,000	57,000	1.3	100	9
Redissolved TCA precipitate	1,000	24,000	24	45	160
DE-32 Total	119	14,000	118	26	787
Phospholipase A_2-I	2.3	807	350	1.5	2340
Phospholipase A_2-II	5.6	3,300	590	6.0	3930
Sephadex G-25 Phospholipase A_2-I	2.0	720	360	1.3	2400
Phospholipase A_2-II	5.4	2,800	520	5.1	3450

*Determined by A_{260}/A_{280}

**Activity determined by the lecithin-albumin emulsion assay at room temperature.

purification. The physical and chemical properties of the two pure enzymes were similar and not markedly different from those of the porcine pancreatic phospholipase A_2 isolated from fresh pancreas (7). Significant differences in the amino acid composition suggest, however, that enzymes I and II are distinct protein species. Their separate identity is also shown by the specificity pattern they display.

It is clear that enzymes I and II are closely related as shown by the identical stereospecificity, the similar specific activity toward the vegetable lecithin-albumin substrate dispersion, and the almost identical molecular weight and amino acid composition. The enzymes behave in a markedly different way, however, when an egg yolk phospholipid dispersion is used, since enzyme II fails to show any appreciable activity toward this substrate. Earlier studies of pancreatic phospholipase would have failed to detect the second form of the enzyme, since the egg yolk assay was employed exclusively (7, 14). Enzyme II seems to be generated during the purification procedure and must be present in pancreatin in an inactivated or in an unactivated form. Our experience indicates that enzyme II is a potential contaminant in any conventional pancreatic phospholipase purification procedure. Yamaguchi et al. (14) have also demonstrated a multiplicity of phospholipases from porcine pancreatin, and it is possible that one of the enzymes isolated by us might correspond to their "protoplast bursting factor" fraction.

The lack of reactivity of enzyme II toward the egg yolk lecithin emulsion cannot be due to the particular fatty acid composition of this lipid, since vegetable lecithin and egg lecithin have a very similar distribution of fatty acids (5, 15) and the vegetable lecithin

TABLE II. Amino Acid Composition of Phospholipases A_2–I and A_2 –II

Amino acid	Phospholipase A_2 – I*	Phospholipase A_2 – II*	Reported in ref. (11)
Aspartic acid	17.3	18.3	23
Threonine**	4.8	5.7	6
Serine**	9.5	10.6	10
Glutamic acid	9.7	9.0	7
Proline	8.5	8.9	5
Glycine	13.3	15.6	6
Alanine	9.3	10.7	8
Half-cystine†	11.1	10.7	12
Valine	3.8	4.3	2
Methionine	2.0	2.1	2
Isoleucine	5.3	5.2	5
Leucine	7.7	6.5	7
Tyrosine	6.1	5.6	8
Phenylalanine	4.8	4.8	5
Histidine	3.1	2.3	3
Lysine	7.4	5.9	9
Arginine	3.6	4.1	4
Tryptophan ‡	1.0	1.0	1

*Calculations based on molecular weight = 14,000. Mean values from 9 analyses.

**Determined by extrapolation to zero time from the mean values of 24–, 48–, 72–hr hydrolysates.

†Determined as S-carboxymethylcysteine.

‡Determined spectrophotometrically.

TABLE III. Specific Activities of Enzymes I and II Toward Lecithin Emulsions*

Additive	Vegetable lecithin		Egg yolk	
	Enzyme I	Enzyme II	Enzyme I	Enzyme II
Na deoxycholate** (2.7×10^{-3} M)	270	60	250	< 3
Bovine plasma albumin† (1%)	280	360	< 3	< 3

*1 ml of lecithin emulsion, 1 ml of additive, 1 ml of water, 1 ml of 24 mM $CaCl_2$ solution, room temperature.

**pH 8.

†pH 7.

emulsion is readily hydrolyzed by enzyme II. The presence in the egg yolk of an inhibitor specific for enzyme II is equally unlikely, since enzyme II is inactive toward an emulsion of purified egg lecithin. The specificity pattern displayed by the two enzymes most likely originates in the different physical properties of the emulsions prepared with the two lecithins and the modification of these properties by interaction with the added emulsifier.

In order to explore further the differences between the two phospholipases, we have turned to a study of the action of these enzymes on substrate monolayers at the air–water interface. The results, which will be reported in detail elsewhere, indicate that enzyme II undergoes denaturation at the air–water interface, whereas enzyme I is relatively stable under the same conditions. Enzyme II may show similar instability at certain water–lipid interfaces, such as that in the egg yolk emulsion.

These results demonstrate that caution must be exercised in interpreting experiments in which phospholipases are used as probes of lipid function. Depending upon the orientation of lipids in membranes, lipoproteins, etc., one might observe quite different rates and degrees of hydrolysis with different enzyme preparations. Certainly conclusions that treatment with a phospholipase would always destroy all exposed phospholipids do not seem to be warranted.

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