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Novel Intestinal Phospholipase A_2: Purification and Some Molecular Characteristics†

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ABSTRACT: We purified to homogeneity a new phospholipase A_2 from pig ileum which hydrolyzes phosphatidylglycerol at least 200 times more rapidly than phosphatidylcholine. The method involved the following steps: (1) complete delipidation of ileal homogenates by solvent extraction; (2) fractionation and partition between *n*-butanol and $(NH_4)_2SO_4$ solution; (3) hydrophobic affinity chromatography on octyl-Sepharose; (4) adsorption chromatography on hydroxylapatite; (5) ion-exchange chromatography on carboxymethyl-Sepharose. Amino acid composition, molecular weight (15 000–16 000), *N*-terminal amino acid sequence to residue 48, and enzymatic activity on phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol were determined.

Lipolytic enzymes are present in nearly all tissues which have been investigated (Brockerhoff & Jensen, 1974; van den Bosch, 1980). However, the amount of enzyme activity can vary by a factor of 10^6 as, for example, between pancreatic exocrine glands and hepatocytes.

Phospholipases in intestinal mucosa were first described in 1931 by several laboratories (King, 1931; Epstein & Shapiro, 1957, 1959; Schmidt et al., 1957; Ottolenghi, 1964, 1967; Gallai-Hatchard & Thompson, 1965; Sarzala, 1969; Subbaiah & Ganguly, 1970; Bonnefis et al., 1975, 1977, 1978; Takagi & Sasaki, 1979) by using either endogenous phospholipids, exogenous phosphatidylcholine (PC),[1] or phosphatidylethanolamine (PE) as substrate. Unfortunately, these investigations were carried out with different types of crude intestinal preparations, and therefore contamination with enzymes from other origins (pancreas) cannot be ruled out. In 1974, when the first preparations of closed intestinal brush border vesicles became available in our laboratory (Louvard et al., 1973), we detected hydrolytic activity using monomolecular phospholipid films of phosphatidylglycerol (PG) that was 200 times greater than when PC was used. This initial observation prompted us to trace and isolate the enzyme responsible for this unusually selective phospholipase activity of the A type.

In a preliminary work, Mansbach et al. (1982) evaluated phospholipase activity in the intestine of rats and other species. In pancreatic juice diverted rats, mucosal and gut luminal phospholipase specific activity was greater than controls, thus assuring that enzyme activity was not due to pancreatic phospholipase. A bacterial origin of phospholipase activity was also eliminated by finding phospholipase activity in germ-free rats.

In the present paper, we describe the purification from porcine intestine of an acid-stable, small molecular weight phospholipase A_2 which hydrolyzes PG at least 200 times more rapidly than PC. We also detail several physicochemical and molecular characteristics of this enzyme.

Materials and Methods

Determination of Phospholipase Activity. When using the classical potentiometric egg yolk assay, designed for pancreatic phospholipases A_2 (Nieuwenhuizen et al., 1974), we could not detect any enzymatic activity due to the intestinal phospholipase using different taurodeoxycholate (0–6 mM) or calcium (0–15 mM) concentrations. Similarly, no measurable hydrolysis was found at pH 8.0 when short-chain dioctanoyl-PC was used at different NaCl concentrations. Due to the sharp substrate specificity of the intestinal phospholipase, we tried to set up a pH stat titration assay using several tissue homogenates or lipid extracts naturally rich in PG or acidic phospholipids.

We first checked, without success, *Escherichia coli* or lamb brain homogenates at pH 8.0 in 21 mM $CaCl_2$ and 1.2 mM taurodeoxycholate. Then we tried, also unsuccessfully, chloroform/methanol (2:1) extracts of *E. coli*, spinach leaves, and lamb brain as phospholipase substrates as well as purified *E. coli* PG in the presence of Triton X-100 (1%) or deoxycholate

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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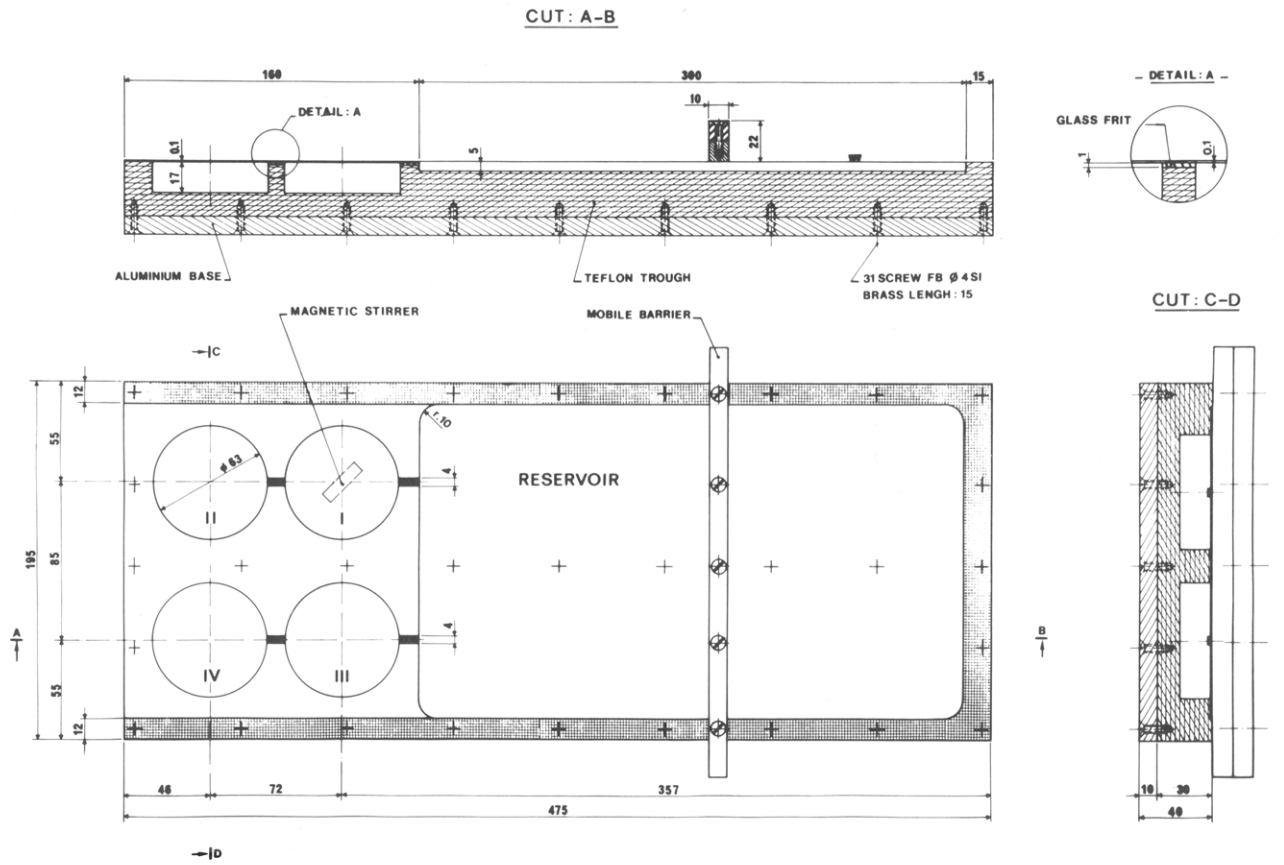


FIGURE 1: Special zero-order trough with four reaction chambers (volume, 51 mL; surface, 31 cm^2). The dimensions are expressed in millimeters.

(2.8 mM at pH 8.0 and 16 mM $CaCl_2$). However, when radioactively labeled PG was used, we were able to demonstrate hydrolysis of the ester bond at the *sn*-2 position of the PG (see Results).

Consistent with other known phospholipases A_2, the intestinal enzyme specifically requires calcium ions to express its activity. It is also well-known that calcium ions interact strongly with negatively charged phospholipids, forming macroscopic aggregates (Papahadjopoulos, 1971).

The insolubility of the acidic phospholipid–calcium salt complex could explain our inability, up to now, in finding a manageable titrimetric bulk assay for intestinal phospholipase. This situation led us to use the surface barostat method and didodecanoyl-PG monolayers spread over a calcium subphase (10 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA) to routinely evaluate intestinal phospholipase activity (Verger, 1980). However, in order to perform four enzyme assays in one run, we used a zero-order trough with four reaction chambers as shown in Figure 1. The PG monolayer was initially present over the reservoir compartment and the four reaction chambers. The first enzyme injection was performed in chamber 1 and the surface pressure kept constant by moving the mobile barrier placed over the reservoir compartment. At the end of this first kinetics, a small Teflon bar was put across the surface channel communicating between chambers 1 and 2. The contents of chamber 1 was then removed by aspiration. Subsequent assays were then performed by injecting the other phospholipase samples in chambers 2, 3, and 4 successively. One arbitrary phospholipase unit corresponds to the hydrolysis of 1.3 nmol/min of a PG film spread at 30 dyn/cm.

Amino Acid Analysis. Amino acid analyses were performed in duplicate in a Beckman automatic analyzer (Model 120 C) after 24, 48, and 72 h of hydrolysis at 115 °C (1.7 mg of protein in 1 mL, 5.7 M triple-distilled HCl in sealed tubes). The individual values obtained for each residue were averaged, except in the cases of Val and Ile for which the highest concentration determined after 72 h was used and of Ser and Thr for which linear extrapolations to zero time were made. Cys was estimated as cysteic acid after performic acid oxidation of duplicate samples. Trp was determined after NaOH hydrolysis according to Wilkinson et al. (1976).

Ultracentrifugation Analysis. Sedimentation equilibrium and the sedimentation coefficient were determined in an analytical ultracentrifuge (Beckman, Model E) according to Yphantis (1964).

Determination of the Partial Specific Volume by the Mechanical Oscillator Technique. Densities were evaluated with the aid of an Anton Parr Model DMA digital microdensimeter 02C which yielded five significant figures under optimal conditions.

N-Terminal Amino Acid Automated Sequence Analysis. Two analyses were performed, one on the native protein and the other on a reduced and carboxymethylated derivative: 125 nmol of unmodified lyophilized protein were taken up in 300 μL of 50% formic acid and analyzed in a Beckman Model 890 C sequencer by using the 0.1 M Quadrol program. The second analysis was performed on 100 nmol of reduced and carboxymethylated intestinal phospholipase A_2 in the presence of 3.8 mg of apocytochrome *c* as carrier (Bonicel et al., 1977). This sequence determination was made by using the DMBA buffer program on a Socosi Model PS 100 sequencer modified to be fully driven by a computer (Commodore 2001). The phenylthiohydantoin amino acids were identified by gas chromatography and high-pressure liquid chromatography (Waters Instruments; Bonicel et al., 1981). The initial yield was 50%, and repetitive yields averaged 94%. The automated sequential degradation gave, except at position 43, significant data up

Table I: Phospholipase Activity in Pig Intestine

gut segment[a]	enzyme act.[b]	intestinal luminal contents	villi[c]	crypts + remaining intestinal wall[c]
I	total units ($\times 10^{-3}$)	1.7	1.4	0.4
	units/mg of protein		3	2
II	total units ($\times 10^{-3}$)	4	1.7	0.3
	units/mg of protein		3	1
III	total units ($\times 10^{-3}$)	7	3.3	72
	units/mg of protein		7	46
cecum	total units ($\times 10^{-3}$)			41
	units/mg of protein			67

[a] One small intestine was divided into thirds, pylorus (I) to cecum (III). [b] Arbitrary units measured as described under Materials and Methods. Each number is an average of four determinations (±10%). [c] Delipidated (see Materials and Methods).

to residues 39 and 48 for the first and second analysis, respectively.

Lipids. 1,2-Didodecanoyl-PC was prepared as described by Bonsen et al. (1972) and was the generous gift of Dr. G. H. de Haas (Utrecht, The Netherlands). 1,2-Didodecanoyl-PE was prepared by Dr. H. M. Verheij (Utrecht, The Netherlands), and 1,2-didodecanoyl-PG was synthesized (Tocanne et al., 1974; Centre de Recherche de Biochimie et de Génétique Cellulaire, Toulouse, France). 1,2-Didodecanoyl-phosphatidylserine (PS) was the generous gift of Dr. A. J. Slotboom (Utrecht, The Netherlands). All these lipids were checked for purity by thin-layer chromatography. All lipids were spread from a chloroform solution by using a glass microliter syringe.

^{14}C-Labeled PG was prepared by transphosphatidylation (Lennarz et al., 1967) of a mixture of 20 mg of purified egg PC and 2.5 μCi of 1-palmitoyl-2-(oleoyl-*1*-^{14}*C*)-*sn*-3 phosphatidylcholine (New England Nuclear, reference 763, 50 mCi/mmol). We omitted the $BaCl_2$ precipitation and performed a pentane extraction of the acidified methanolic phase. ^{14}C-Labeled PG was purified on a preparative thin-layer chromatography plate (Merck, reference 5745) with a $CHCl_3$/MeOH/H_2O mixture (65:25:4 v/v/v). The labeled PG spot was localized by autoradiography and eluted from the silica gel.

Results and Discussion

Purification Procedure from Pig Ileum. During the present study we confirmed in the pig the observations of Mansbach et al. (1982) in rat intestine: phospholipase activity was found to be greater (165 times) in the distal than the proximal intestine and in cryptal cells (20 times) vs. villus tip cells (see Table I). For this reason we used porcine intestinal ileum freed of villi as the starting phospholipase source.

The most distal 3 m of ileum from 10 pigs were excised in the slaughterhouse immediately after death, placed in crushed ice, and kept no longer than 4 h. In the laboratory, the content of each ileal segment was flushed twice with 100 mL of 0.15 M NaCl. Adherent mesentery was removed as completely as possible. The intestine was passed once through the two rubber rollers of a hand-powered wringer to extrude the villi which were discarded. The remaining intestine was minced and then

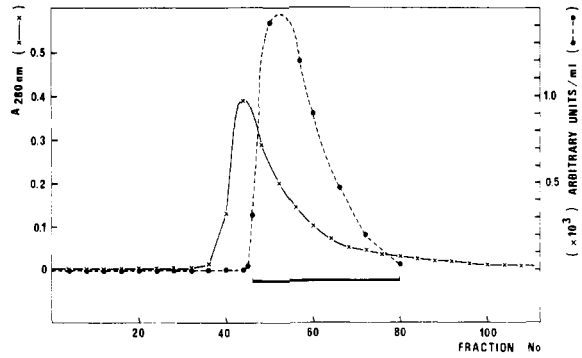


FIGURE 2: Hydrophobic affinity chromatography on octyl-Sepharose. The column (10 × 10.5 cm) was equilibrated with 50 mM acetate buffer (pH 5.0), 2 M NaCl, and 20 mM $CaCl_2$ at room temperature and eluted in the cold room with 50 mM acetate buffer (pH 5.0). Flow rate 1560 mL/h; volume of fractions 26 mL.

blenderized. The slurry obtained (900 g) was homogenized in an explosion-proof Waring blender of 4-L capacity, once with 2700 mL and once again with 1800 mL of acetone, 3 times with 2700 mL of a chloroform *n*-butanol (9:1 v/v) mixture, and finally twice with 1800 mL of diethyl oxide. After each solvent treatment, the suspension was filtered under vacuum on Whatman No. 1 paper. The residue after the last ether extraction was dried under vacuum in a rotatory evaporator. The resulting powder (1480 phospholipase units/g of powder, 165 g of powder/kg of fresh mucosa) could be stored for several months at −20 °C without any detectable inactivation.

Unless otherwise stated, all subsequent steps of the purification were performed at 0–4 °C. The delipidated intestinal powder (744 g) was constantly stirred with 7.44 L of 1 N HCl for 1 h to extract the phospholipase. After centrifugation (6000*g*, 30 min) the clear acidic extract (pH 0.3) was fractionated by a procedure first described by Melius & Simmons (1965). *n*-Butanol (50% v/v of acidic extract) and solid $(NH_4)_2SO_4$ 28.7% (w/v of acidic extract) were added to the solution. After 1 h of agitation, the mixture was centrifuged for 20 min at 27500*g*. The protein containing cream located at the butanol–water interface was collected by decanting and was suspended in 1 L of acetate buffer (pH 5.0, 50 mM acetate). The suspension was first dialyzed overnight against 10 L of this buffer and was centrifuged for 30 min at 27500*g*. The clear supernatant was then dialyzed once more for 24 h against 10 L of acetate buffer (pH 5.0, 50 mM acetate, 2 M NaCl, 20 mM $CaCl_2$). The clear solution obtained was ready for the subsequent series of chromatographic steps on octyl-Sepharose, hydroxylapatite, and carboxymethyl-Sepharose.

The enzyme solution containing 1.1×10^6 phospholipase units and 35 g of protein was maintained in an ice bath and placed on an octyl-Sepharose column kept at room temperature. The column was equilibrated with the acetate buffer (pH 5.0) for the second dialysis supplemented with 2 M NaCl. After the emergence of a nonretarded fraction containing 8% of the total phospholipase activity, the column was washed with a further 2000 mL of buffer. The elution was stopped, and the column was placed in a cold room. After 12 h for thermic equilibration, the phospholipase activity was eluted by acetate buffer (pH 5.0, 50 mM acetate) as shown in Figure 2. The active fractions, indicated by the horizontal bar in Figure 2, representing approximately 73% of the activity loaded on the column, were pooled, and dialyzed 3 times against 10 L of phosphate buffer (pH 6.8, 10 mM phosphate), and conductivity was measured in order to check for complete buffer equilibration.

Table II: Flowsheet of the Purification

steps	phospholipase units ($\times 10^{-3}$)	proteins (mg)	sp act. (units/mg)	yield in act. (%)
(1) 1 N HCl extract of 744 g of delipidated powder	1100	35000	31	100
(2) *n*-butanol/$(NH_4)_2SO_4$ fractionation and dialysis	890	2500	356	81
(3) hydrophobic affinity chromatography on octyl-Sepharose	650	250	2600	59
(4) hydroxylapatite chromatography	520	20	26000	47
(5) carboxymethyl-Sepharose chromatography	260	6	43330	24

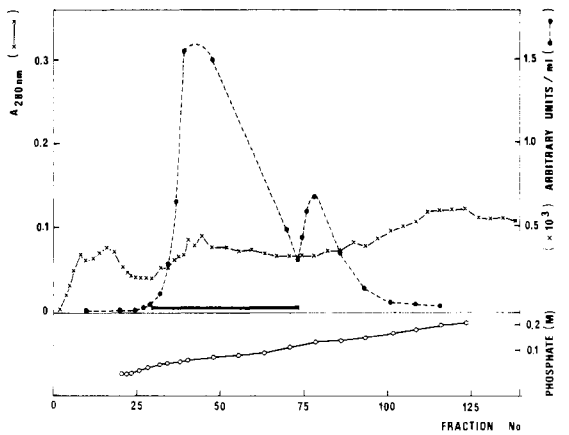


FIGURE 3: Adsorption chromatography on hydroxylapatite (Ultrogel HA, LKB 2204-110). The column (4 × 16 cm) was equilibrated with a 10 mM phosphate buffer (pH 6.8) and eluted by a linear increase of phosphate concentration (10–300 mM; total volume 3400 mL). Flow rate 480 mL/h; Volume of fractions 20 mL.

The dialyzed phospholipase solution (650 × 10^3 units, 250 mg of protein) was placed on an hydroxylapatite column equilibrated with the phosphate buffer used for the previous dialysis. The column was washed with 1100 mL of this buffer. The phospholipase was eluted consecutively by a linear concentration gradient between 10 and 300 mM phosphate buffer (pH 6.8). Figure 3 shows the elution profile of protein and phospholipase activity. We frequently observed two phospholipase activity peaks with recovery yields in enzyme units of greater than 100%.

The fractions indicated by the horizontal bar in Figure 3 were combined and freed of phosphate by dialyzing for 24 h against 15 L of 50 mM acetate (pH 5.0) by using three changes of buffer. Then the enzyme solution was dialyzed for 4 h against 15 L of a 50 mM Tris-HCl buffer (pH 9.0).

The phospholipase solution (520 × 10^3 phospholipase units, 20 mg of protein) was placed on a carboxymethyl-Sepharose column equilibrated with the buffer (pH 9.0) used for the last dialysis. The column was washed with 200 mL of the Tris buffer, then with 1300 mL of a 50 mM acetate buffer (pH 5.0), and finally with 50 mL of acetate buffer containing 0.4 M NaCl. Pure phospholipase was eluted by a linear salt gradient between 0.4 and 1 M NaCl as shown in Figure 4. The main steps of the purification procedures are summarized in Table II.

Comments on the Purification Procedure. It has been observed that lipolytic enzymes are often contaminated by tightly bound lipids. The complete removal of these lipids requires drastic conditions which, when applied to partly purified solutions, can cause extensive inactivation of the enzyme. Much better results were obtained in the past by delipidation of the tissue prior to the aqueous extraction of lipolytic enzymes (Verger et al., 1969). We adapted the delipidation method, used for pancreatic lipase, to intestinal phospholipase.

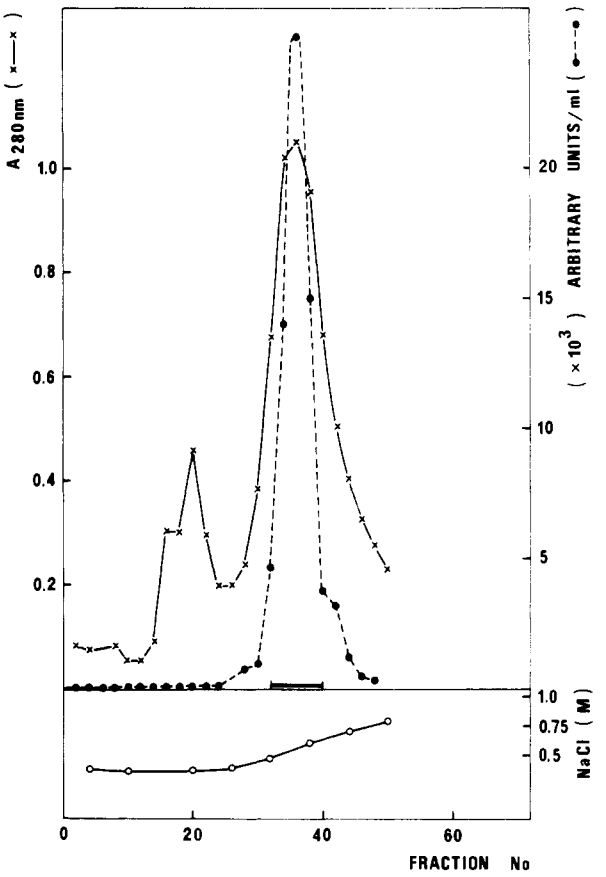


FIGURE 4: Ion-exchange chromatography on carboxymethyl-Sepharose (CM-Sepharose CL-6B Pharmacia). The column (1 × 12 cm) was equilibrated and loaded with a 50 mM Tris-HCl buffer (pH 9.0) and then washed with 50 mM acetate buffer (pH 5.0) and finally with the same buffer containing 0.4 M NaCl. The pure phospholipase was eluted by a linear concentration gradient of NaCl (0.4–1 M; total volume 80 mL). Flow rate 42 mL/h; volume of fractions 1.4 mL.

A 1 N HCl solution was used to extract phospholipase activity because of the well-known resistance to acid of all phospholipases A_2.

It was important to dialyze the protein-containing cream obtained at the butanol–$(NH_4)_2SO_4$ step against a low ionic strength buffer. This dialysis ensures maximal phospholipase solubilization. The second dialysis step (against a 2 M NaCl buffer) was found to be necessary in order to keep the activity soluble in the high ionic strength medium required for the hydrophobic adsorption step on the octyl-Sepharose column.

We chose the octyl-Sepharose derivative to perform hydrophobic affinity chromatography after several trials on hexyl-, nonyl-, and decyl-Sepharose gels. Octyl-Sepharose was found to be optimally suited for adsorption of phospholipase activity in a 2 M NaCl buffer solution at room temperature followed by an elution step in the cold with a low ionic strength buffer. We observed an identical chromatographic behavior

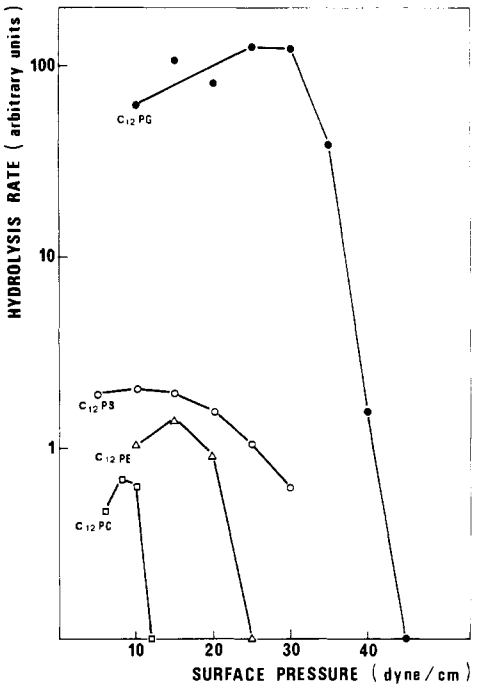


FIGURE 5: Variation with surface pressure of relative hydrolysis velocity of didodecanoylphosphatidylcholine (PC), -ethanolamine (PE), -serine (PS), and -glycerol (PG) monolayers by pure intestinal phospholipase A_2 (3.75, 2, 1, and 0.025 μg for PC, PE, PS, and PG, respectively) injected in the subphase of the special zero-order trough shown in Figure 1. Temperature 25 °C.

of the intestinal phospholipase either on commercially available or laboratory made octyl-Sepharose gels (Shaltiel, 1974). Less successful was performing the ion exchange on carboxymethyl-Sepharose before the hydroxylapatite chromatography. In the latter case we ended up with a very dilute phospholipase solution in a phosphate buffer preventing the further addition of calcium.

The identification and purification of this novel phospholipase A_2 illustrate an analytical application of the monolayer technique. In fact, the monolayer system may be best suited for detecting and tracing new lipolytic enzymes in future investigations, due to inherent technical difficulties of the bulk system, i.e., the insolubility of the Ca^{2+} salt of acidic phospholipids and hence an uncontrolled interfacial quality.

Substrate Specificity of the Intestinal Phospholipase. Figure 5 shows activity–surface pressure profiles obtained with four different dilauroylphospholipid classes, i.e., PC, PE, PS, and PG, using the pure intestinal enzyme. It is clear that the enzyme activity measured on PG films is the greatest of all other phospholipids evaluated. PS, another negatively charged phospholipid, was hydrolyzed 28 times more slowly than PG at all surface pressures tested. The very high enzymatic activity with PG had an optimal surface pressure of 25–30 dyne/cm. A similar pressure optimum as well as activity values were observed previously on PG monolayers with porcine pancreatic phospholipase A_2 (Verger et al., 1976; Hendrickson et al., 1981). However, when PC films were used, the level of enzyme activity with pancreatic phospholipase A_2 was around 15 times higher as compared with those obtained with the intestinal enzyme. Since this enzyme would not have been discovered if PG monolayers were not used as substrate, this emphasizes the importance of using a variety of substrates and techniques when searching for new lipolytic activities.

Positional Specificity of the Intestinal Phospholipase. It is obvious from the principle used in the monolayer assay, e.g., decrease (down to zero value) of the surface pressure of an insoluble phospholipid monolayer upon enzyme action, that we are dealing with an intestinal phospholipase of an A_1 or A_2 type. We used 1-palmitoyl-2-(oleoyl-*1*-^{14}C)-*sn*-3-phosphatidylglycerol to demonstrate hydrolysis of the ester bond at the *sn*-2 position of the radioactively labeled PG (data shown in Table III). We observed by thin-layer chromatography, using the intestinal as well as the pancreatic phospholipase A_2 taken as a reference, the appearance of a nonradioactive spot corresponding to the migration of lyso-PG and a radiolabeled spot on the solvent front, identified as free fatty acid.

Table III: Positional Specificity of the Intestinal Phospholipase[a]

incubation time (min)	spot migration reference	blank (dpm)	pancreatic phospholipase A_2 (dpm)	intestinal phospholipase (dpm)
30	FFA	0	2204	452
	PG	2860	1384	2786
	lyso-PG	0	31	227
60	FFA	0	2113	590
	PG	2352	1396	2052
	lyso-PG	13	96	17
90	FFA	92	3290	1086
	PG	2822	480	1594
	lyso-PG	0	109	25

[a] Incubation medium: 0.1 mL of a 10 mM Tris-acetate buffer, pH 6.0, 125 mM NaCl, and 5 mM $CaCl_2$ containing 50 μg of 1-palmitoyl-2-(oleoyl-*1*-^{14}C)-*sn*-3-phosphatidylglycerol (13 800 dpm) with no (blank) or 5 μg of pure phospholipase (pancreatic or intestinal). Incubation temperature 30 °C. The cloudy suspension of the Ca^{2+}–(PG) complex clarified during hydrolysis. Aliquots of the incubation media were directly dried on analytical thin-layer precoated plates (5 × 20 cm, Whatman K5) and chromatographed in a $CHCl_3$/MeOH/H_2O mixture (65:25:4 v/v/v). The plates were stained in iodine vapor and divided into three parts corresponding to the respective migration of free fatty acid (FF), PG, and lyso-PG taken as references. We scraped off the silica gel coat from the plates and determined the ^{14}C radioactivity using a Packard Tri-carb 2450, liquid scintillation spectrometer.

Physical Properties and Amino Acid Composition. Intestinal phospholipase was found to be homogeneous by Na-DodSO_4/polyacrylamide gel electrophoresis (Maizel, 1969). Furthermore, no sign of heterogeneity could be observed on the schlieren pattern when a 7 mg/mL phospholipase solution in acetate buffer (pH 5.0) in 0.1 M NaCl was centrifuged at 271025*g* for 36 min. The sedimentation coefficient of the enzyme dissolved at a 7.2 mg/mL concentration was 1.8 S. The isoelectric point, measured by electrofocusing, was found to be greater than 9.6. The extinction coefficient at 277 nm (pH 7.0) was found to be $E^{1\%}_{1cm}$ = 12.0 when the amount of protein was evaluated by amino acid analysis.

The partial specific volume extrapolated to zero protein concentration according to the mechanical oscillator technique was found to be $\bar{V}$ = 0.730 mL g^{-1} (25 °C in H_2O).

The molecular weight determined by sedimentation equilibrium was 14000 ± 500 when the experimentally determined $\bar{V}$ of 0.73 mL g^{-1} was used [0.6 mg/mL solution of intestinal phospholipase in 50 mM acetate buffer (pH 5.0) (0.1 M NaCl)]. The electrophoretic migration of intestinal phospholipase through a 15% polyacrylamide gel in the presence of 0.1% Na-DodSO_4 and mercaptoethanol was investigated. The intestinal phospholipase band was in the position expected for a protein with a molecular weight of about 16000 corresponding to a single polypeptide chain.

The amino acid composition of the intestinal phospholipase is given in Table IV. The compositions of horse, pig, and ox pancreatic phospholipases A_2 are also given for purposes of comparison. The basic character of the intestinal phospholipiase can be easily explained by the high ratio (1.33) of basic to acidic amino acid residues. This ratio is 0.53 for the pig

Table IV: Amino Acid Composition of Phospholipases Isolated from Horse, Pig, and Ox Pancreas and Pig Ileum

	pancreas			intestine (ileum) pig	
amino acid	horse[a]	pig[b]	ox[c]	exptl values	next integers
Asx	19	22	25	10.08	10
Thr	6	6	4	6.18	6
Ser	11	10	10	4.05	4
Glx	11	7	8	10.98	11
Pro	6	5	5	6.70	7
Gly	6	6	6	9.58	9-10
Ala	9	8	6	11.26	11
Cys	14	14	14	13.68	14
Val	5	2	4	5.86	6
Met	1	2	1	1.89	2
Ile	4	5	5	5.38	5
Leu	6	7	8	13.04	13
Tyr	7	8	7	6.88	7
Phe	6	5	4	5.29	5
Lys	8	9	11	15.85	16
His	1	3	2	1.98	2
Arg	4	4	2	9.77	10
Trp	1	1	1	1.04	1
total no. of amino acids	125	124	123		139-140
M_r	13928	13981	13783		15814 ± 39

[a] Evenberg et al. (1977). [b] Puyk et al. (1977). [c] Fleer et al. (1978).

pancreatic enzyme. Another striking difference between the two enzymes is the Ser and Asp content. It is worthwhile to note that both the pancreatic and intestinal phospholipases possess only one Trp and seven disulfide bridges.

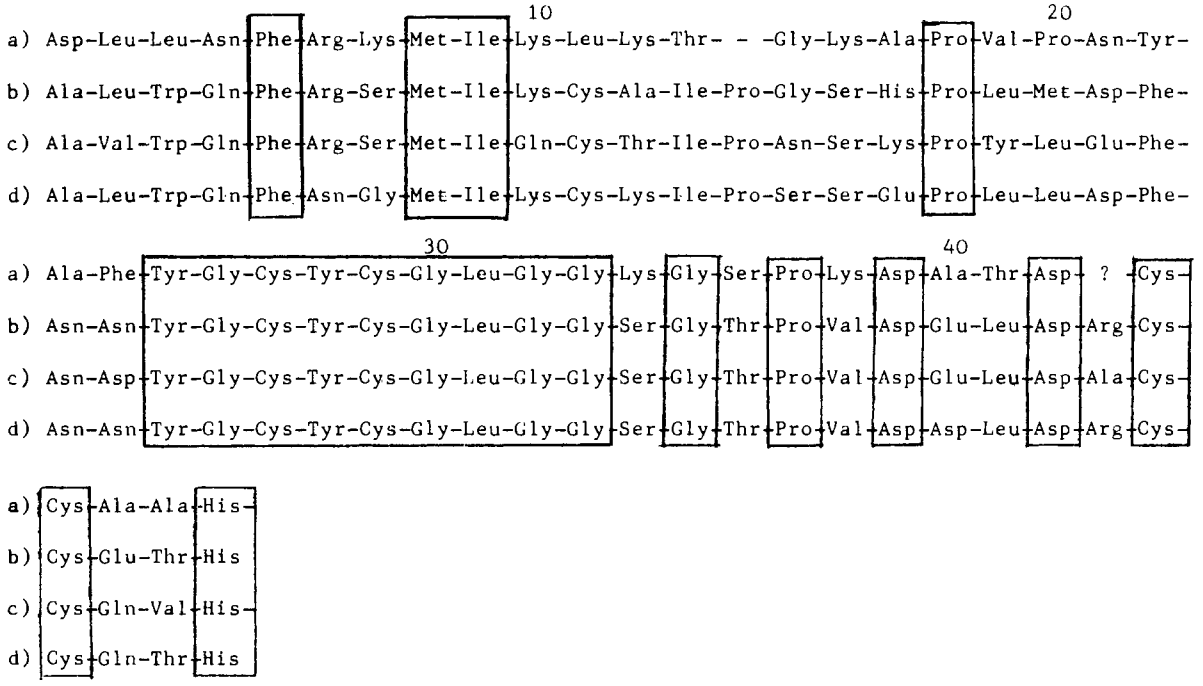
N-Terminal Amino Acid Sequence Analysis. Table V presents the *N*-terminal amino acid sequence comparison of porcine intestinal, porcine pancreatic, equine pancreatic, and bovine pancreatic phospholipases A_2. It is noteworthy that the invariant residues in all pancreatic phospholipases A_2 are conserved in the intestinal enzyme. One remarkable exception to this strong homology is the Asn residue 4 in the intestinal enzyme which replaces the Gln residue present in at least 32 phospholipases A_2 from pancreatic or venom origin (Verheij et al., 1981).

Van Scharrenburg et al. (1982) prepared a bovine pancreatic ε-amidinated phospholipase A_2 (AMPA) analogue in which Gln^4 was substituted by Nle^4. This substitution resulted in the almost complete loss of catalytic activity when assayed on micellar L-dioctanoylecithin. Furthermore no measurable affinity for micelles of *cis*-9-octadecenylphosphocholine could be detected. These findings strongly suggest the absence of the lipid binding site for micelles for Nle^4 AMPA which, however, still possesses about 25% of the catalytic activity of "native" AMPA toward monomeric bis(thiohexanoyl)lecithin. This "essential" Gln^4 residue is supposed to be hydrogen bonded to the α-amino group and thus part of the proton relay system of phospholipases A_2 (Dijkstra et al., 1981). It was of interest to know whether or not this remarkable substitution in intestinal phospholipase at position 4 was responsible for the unique phospholipid specificity of the enzyme, i.e., lack of activity toward PC compared to that of PG.

The $^1/_2$-Cys in position 11, present in all Elapidae, Hydrophidae, and pancreatic phospholipases A_2, is absent in the intestinal enzyme, suggesting the existence of a disulfide bridge, between the C-terminus and $^1/_2$-Cys number 50, only present in Viperidae and Crotalidae phospholipases A_2. This point is indicative of surprising similarities between the intestinal phospholipase A_2 and some basic venom enzymes (Verheij et al., 1981).

It is also worthwhile to note that the single Trp residue of the intestinal enzyme is not located at position 3, as in other pancreatic phospholipases, whereas the essential and invariant active site His 48 is conserved.

Table V: N-Terminal Amino Acid Sequence Comparison of Porcine Intestinal (a), Porcine Pancreatic (b), Equine Pancreatic (c), and Bovine Pancreatic (d) Phospholipases A_2[a]



[a] Gaps (–) have been introduced in order to get maximal alignments of half-cystine and maximal homology. Invariant residues in the four proteins are enclosed by boxes. (b) From Puyk et al. (1977); (c) from Evenberg et al. (1977); (d) from Fleer et al. (1978).

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