Reverse-phase high-performance liquid chromatographic assay of phospholipases: application of spectrophotometric detection to rat phospholipase A2 isozymes

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Abstract This paper appraises an HPLC method for assaying phospholipase A2 (PLA2). The procedure is based on heptane-isopropanol- $H_2$SO$_4$ extraction of fatty acids released by the enzyme in the presence of margaric acid as an internal standard, and precolumn derivatization with 9-anthryldiazomethane. The derivatives of naturally occurring long-chain fatty acids were accurately determined by reverse-phase HPLC with ultraviolet detection at 254 nm; the fatty acids were identical with margaric acid in terms of their extraction efficiency in the presence or absence of a bile salt, reactivity with the labeling reagent, and molar extinction coefficients of their derivatives. HPLC conditions were optimized so as to separate the derivatives of palmitic and oleic acids completely within 7 min. The use of the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol/cholate system as substrate proved useful for the sensitive detection of PLA2 activities in rat tissue homogenates. Distribution of immunoreactive pancreatic and group II phospholipases A2 was estimated from the degree of inhibition of enzyme activities by specific antibodies raised against either forms of phospholipase A2 isozymes. The results were consistent with those of immunoblot analyses.—Tojo, H., T. Ono, and M. Okamoto.


Supplementary key words pancreatic and group II phospholipases

A之所以有 A2 + phosphatidylglycerol + 9-anthryldiazomethane

Phospholipase A2 (EC 3.1.1.4; PLA2) catalyzes the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids. The enzymes play a key role in regulating a variety of cellular and extracellular reactions in phospholipid metabolism. Recent determination of primary structures of two PLA2 isozymes of mammalian origin in addition to known structures of well-characterized pancreatic PLA2s (1–3) have revealed the isozymic nature of PLA2. To explore the physiological significance of these isozymes, it is necessary to examine enzymatic properties in detail, e.g., positional specificity, and substrate specificities toward sn-1 radyl and sn-2 acyl moieties and polar head groups. For this, in addition to well-established radiometric assay methods (4), it may be helpful to develop a nonradiometric method that can use many kinds of commercially available synthetic highly purified phospholipids.

During the course of a study on PLA2 isozymes of rat spleen, we developed an assay based on reverse-phase high-performance liquid chromatographic separation of 9-anthrylbenzyl derivatives of fatty acids released by PLA2 as an alternative to the radiometric Dole assay (4); the substrate specificities of PLA2 isozymes could be compared with each other (5, 6). Since that time we have used this method extensively for studying the enzymatic properties of PLA2 from various sources, and the experimental protocol has already been published (5, 7). In the present study, we report an appraisal of the reliability of this assay. Furthermore, we previously demonstrated that mixed micelles of phosphatidylglycerol and cholate are the best substrate for both rat pancreatic and group II PLA2s (5, 6). This substrate system was found to be suitable for highly sensitive detection of PLA2 activity in rat tissue homogenates. We therefore examined the distri-
bution of Ca²⁺-dependent PLA₂ activities among various rat tissues using this substrate, and the contribution of the pancreatic and group II enzymes to the measured activities was roughly estimated by the extent of inhibition by specific antibodies. The results were verified by immunoblot analyses.

MATERIALS AND METHODS

Materials

9-Anthryldiazomethane (ADAM) was obtained from Funakoshi Co., Tokyo, and used without further purification. The following fatty acids were purchased from Nacalai Tesque, Inc.: myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and arachidonic acid (C20:4). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) were products of Avanti Polar Lipids, Inc.

Assay for PLA₂ activity

The assay method is a modified version of the assay based on extraction of fatty acids according to the method of Dole and Meinertz (8) followed by silicic acid treatment (Dole assay). The modifications included the derivatization of extracted fatty acids with ADAM, and HPLC quantitation of each derivatized fatty acid. Detailed procedures including the preparation of ADAM and substrate solutions have been described previously (7). The standard reaction mixtures contained either 0.8 mM POPG and 4.8 mM sodium cholate or 1 mM POPE and 6 mM sodium deoxycholate (DOC), 5 mM CaCl₂, 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.5), and the enzyme in a final volume of 50 μl. This volume can, if necessary, be increased to 120 μl. In that case the same assay procedures would be followed except for a proportional decrease in the volume of water added on extraction of fatty acids. The enzyme reaction was stopped by the addition of 200 μl of n-heptane-2-propanol-2 N sulfuric acid 10:40:1 (v/v). To the reaction mixture were added 120 μl of heptane, 70 μl water, and 5 nmol of C17:0 as an internal standard. Fatty acids were extracted into the upper heptane layer after vortexing the mixture for 20 sec, and the extracted fatty acids were derivatized and analyzed by HPLC as described below. As a control, CaCl₂ was replaced by EDTA (10 mM) or phospholipid was omitted.

For the inhibition of the PLA₂ activity by the antibody, the enzyme sample was incubated with 0.5 mg/ml antiserum group II PLA₂ IgG or 0.3 mg/ml anti-pancreatic PLA₂ IgG in 20 μl of 0.1 M Tris-HCl and 0.1 M NaCl, pH 8.5, for 2 h at 4°C. The remaining activity was then determined.

Derivatization of fatty acids with ADAM

Immediately before use, the 9-anthryldiazomethane was completely dissolved in 1 volume of ethyl acetate followed by the addition of 9 volumes of methanol. An ADAM concentration of 0.5 mg/ml (9) was used as described below. The methanolic mixture of fatty acids or the heptane solution after extraction (30–50 μl) was placed in a microvial (0.3 ml) and the solvent was evaporated using a water aspirator. Then, 50 μl of the 0.05% ADAM solution was added. The vial was incubated for a desired time at room temperature (25°C). Unless otherwise noted, termination of the reaction was unnecessary except when the time course for the reaction of ADAM with fatty acids and the concentration dependence of ADAM were examined. In these instances the reaction was stopped by the addition of 5 μl glacial acetic acid. An aliquot (3–10 μl) of the mixture was injected manually or from an autosampler onto a reverse-phase column.

HPLC of ADAM-derivatized fatty acids

The HPLC system consisted of a Gilson Model 302 liquid delivery module and a Gilson autosampler 231-401 equipped with a Rheodyne 7010 sample injection valve with a 20-μl sample loop. The effluents were monitored at 254 nm with a Gilson 116 detector or a UV monitor III model 1203 (Laboratory Data Control, Riviera Beach, FL). At the initial stage of the study, ADAM-derivatized fatty acids were separated on a Rainin Microsorb C18 or C8 column (4 × 100 mm) at room temperature. Later they were routinely separated on a Superspher RP-18 column (4 × 50 mm, Merck) at 20°C and at a flow rate of 1 ml/min. For rapid separation but with less resolution between C18:1 and C16:0 peaks, a Cosmosil 3C18 column (Nacalai Tesque, Inc., 2.1 × 50 mm), which was slurry-packed in this laboratory, was used at room temperature at a flow rate of 1 ml/min. Unless otherwise noted, the solvent systems used were: 96% acetonitrile for Superspher RP-18 and Microsorb C18, 96% acetonitrile for Cosmosil 3C18, and 88% acetonitrile for Microsorb C8.

Preparation of tissue homogenates

Rats weighing 150–200 g were anesthetized with pentobarbital and killed by drawing 9 ml of blood into a plastic syringe containing 1 ml of 3.8% sodium citrate from the abdominal aorta. The organs to be studied were removed and then homogenized with 20 volumes of 0.25 M sucrose containing 1 mM EDTA, 2 mM MgCl₂, and 3 mM Tris-HCl (pH 7.4). Platelet-rich plasma was prepared by centrifuging the blood at 300 g for 10 min at room temperature. The platelets were then pelleted by centrifugation at 2800 g for 10 min and resuspended with the buffer. The supernatant plasma was further centrifuged at 35,000 g for 25 min at 4°C. This supernatant was used as “plasma” for the PLA₂ assay; otherwise a large
ADAM and a mixture of fatty acids C18:3, C18:2, C18:1, and C18:0 (3 nmol each) including 5 nmol of C17:0 as an internal standard. When the reaction mixtures, after a 1-h incubation, were analyzed by HPLC, the UV responses of each of the derivatized fatty acids increased linearly with increasing the ADAM concentration and then leveled off at a concentration of about 0.05% (data not shown), confirming the results of Nimura and Kinoshita (9). This concentration was used to minimize HPLC peaks associated with the reagent and its degradation products while ensuring optimal derivatization of amounts of fatty acids (0.1-15 nmol) usually encountered in PLA2 assays.

We examined the time course for reactions of ADAM with saturated and unsaturated long-chain fatty acids that were extracted by the Dole and Meinertz method under the same conditions as in PLA2 assay. As representative of various fatty acids, reaction kinetics of C17:0 with ADAM is shown in Fig. 1A. The peak area of the C17:0 ester reached about 90% of the maximum during the initial 30 min, and thereafter there was a gradual increase in area for 5.5 h at room temperature. All fatty acids tested were derivatized with ADAM at the same rate as the internal standard (Fig. 1B), indicating that the nucleophilicity of the carboxyl groups of the fatty acids examined did not depend on the chain length and the number of double bonds. Therefore, a 15-min incubation at room temperature (25°C) was sufficient for quantitative analysis in the presence of the internal standard. Furthermore, these results indicated that it was unnecessary to stop the reactions.

amount of fibrin gel-like material was formed in assay mixtures containing a bile salt and calcium ions.

Other analytical methods

Protein concentrations were determined with the bicinechonic acid protein assay reagent (Pierce). Immunoblot analyses were performed with anti-pancreatic PLA2 and anti-splenic PLAP IgGs as reported previously (10). Samples were separated on 14% gels in the absence of 2-mercaptoethanol, because reduction of PLA2 leads to a significant decrease in its reactivity with the antibodies. Immunoreactive bands were visualized by an avidin-biotin-peroxidase method.

RESULTS AND DISCUSSION

Reaction of ADAM with long-chain fatty acids

The reactivity of the commercially available ADAM with fatty acids in ethyl acetate-methanol 1:9 (v/v) was tested with various concentrations (0.01-0.25%) of
Fig. 3. Quantitation of known amounts of oleic acid. Various concentrations of C18:1 in the standard assay mixture containing POPE minus PLA were analyzed as in Fig. 2.

Determination of fatty acids in assay mixtures by HPLC

The UV responses per mol of individual derivatized fatty acid, after separation by reverse-phase HPLC as described above, did not vary according to the chain length in the range of C14 to C18 or the degree of unsaturation. The ratios of UV responses (fatty acid/C17:0) were as follows (n = 5 each): C18:3, 1.02 ± 0.03; C18:2, 1.00 ± 0.05; C18:1, 1.03 ± 0.02; C18:0, 1.03 ± 0.03; C16:0, 1.00 ± 0.03; C15:0, 0.97 ± 0.02; and C14:0, 0.98 ± 0.01. This indicates that the ratio of the observed peak area for a particular fatty acid to that of C17:0 is directly proportional to the mole ratio. The UV response factors of 4-bromomethyl-7-methoxycoumarin esters of fatty acids are reported to depend on the chain length in a linear fashion (11); this is not the case with the ADAM esters.

Next, we examined whether extractability of fatty acids by the Dole and Meinertz method is different in the absence or presence of various concentrations of DOC, which is a component of substrate mixed micelles used in the standard PLA assay. The mixtures of saturated or unsaturated fatty acids, which were dissolved in the assay mixture containing various concentrations of DOC, were extracted and then the fatty acids were derivatized with ADAM. In the absence of detergent the UV response factors of the fatty acids were close to unity, in agreement with previous results obtained by an acid-titration study (8). The presence of the detergent may affect the partition of fatty acids between heptane and aqueous isopropanol phases; no difference was, however, observed for all fatty acids tested in the range of DOC concentration usually used in PLA assay (Fig. 2). Similar results were obtained with sodium cholate (data not shown).

These results indicate that the absolute quantity of each fatty acid in PLA assay mixtures can be measured directly from the integrated peak area on the basis of a known amount of C17:0 without correcting for recovery on extraction of fatty acids longer than or equal to C14:0. To confirm this, the standard PLA assay mixtures con-
taining known amounts of C18:1, ranging from 0.535 to 32.1 nmol, were extracted and analyzed according to standard procedures. The calibration line was highly linear (correlation coefficient of 0.997), and the slope (1.002) and the intercept (−0.012) were very close to one and zero, respectively (Fig. 3).

As to usual assays using pancreatic PLA₂ and POPE, ADAM derivatives of major fatty acids involved in the reaction mixture, i.e., C18:1, C16:0, C17:0, and C18:0, were completely separated in this order on a Superspher RP-18 column within 11.5 min with a simple acetonitrile–H₂O binary solvent system (Fig. 4A), although a previous report stated that the separation of C18:1 and C16:0 esters was somewhat difficult (12). The C16:0 and C18:0 esters that appeared on the chromatogram were derived mainly from environments such as pipet tips, tubes, and hands. This rapid separation could be achieved by using a short column packed with fine supports of 3 μm in average diameter and by operating it at a relatively low temperature (20°C). The temperature control was essential for good separation of C18:1 and C16:0 esters on this column. The separation of this pair of ADAM derivatives was important because 1-palmitoyl-2-oleoyl-glycerophospholipids with various polar head groups and 1-O-hexadecyl-2-oleoyl-PC are available from commercial sources and can readily be used to determine substrate specificities systematically. Samples were repeatedly injected onto the column every 10 min with the aid of an Autosampler without column washing after a run because the ADAM ester of C18:0 was the only major peak eluted later than the C17:0 ester and no other peak that interfered with the identification of fatty acids on the next run was observed (Fig. 4). In our experience, more than 5000 injections were possible without any deterioration of the resolution when an inline filter was connected just prior to the column. The use of a guard column was unnecessary. It is important to remember that fine-tuning of the elution conditions may be required to obtain an optimal separation, depending on experimental designs such as columns, types of acyl groups of phospholipids, and HPLC systems. For example, when a 2-arachidonoyl glycerophospholipid was used as a substrate, acetonitrile concentration 2–3% lower than in the case of the 2-oleoyl derivative was preferable.

PLA₂ activity measurements

The validity of this method of assaying PLA₂ activity was illustrated for rat pancreatic PLA₂ which has an absolute requirement for calcium ion for activity and is specific for hydrolysis at the sn-2 position. The time course of the hydrolysis of 1 mM POPE in mixed micelles with 6 mM DOC by rat pancreatic PLA₂ was followed for 10 min (Fig. 5). Release of C18:1 proceeded almost linearly for the initial 4 min and then gradually decreased. On the other hand, C16:0 (a product of hydrolysis at the sn-1 position) was not appreciably released, confirming the absolute specificity of the enzyme for the sn-2 position. Release of C18:1 was completely inhibited in the presence of 10 mM EDTA. Furthermore, a specific antibody directed against rat pancreatic PLA₂ almost completely inhibited C18:1 release (Fig. 4C) in agreement with our previous results (5). This clearly demonstrated that the release of C18:1 was indeed due to PLA₂ action. The rate of C18:1 release was proportional, at least in the enzyme concentration range of 1 to 100 ng/ml, when mixed micelles of 0.8 mM POPG and 4.8 mM cholate were used as substrate.

Figs. 4D–F show the HPLC analyses of the reaction products using rat lung homogenate as an enzyme source. Because crude samples contain appreciable amounts of free fatty acids and phospholipids, background levels of fatty acids derived from these endogenous compounds should be determined by measuring the reaction in the presence of EDTA in place of CaCl₂ (Fig. 4E). The hydrolytic rate of endogenous substrates alone could be estimated by monitoring the release of the fatty acid in question in the absence of exogenous substrate; the rate was usually quite slower than that of exogenous substrates. When POPG was used as substrate, the rates of the C18:1 release in various rat tissue homogenates in the absence of POPG were less than 5–6% of those in its presence (Fig. 4F). The hydrolyses of endogenous phospholipids can be observed by monitoring the time course of release of fatty acids other than acyl groups of exogenous phospholipids (Fig. 4D). We used substrate concentrations that gave near the maximal velocity under the standard conditions (5), which could minimize the errors in the measured hydrolytic rate caused by changes in substrate concentrations due to inclusion of endogenous substrates. The recoveries of oleic acid exogenously added to assay mixtures containing liver, lung, and spleen homogenates were 97.7%, 100%, and 102%, respectively.

Fluorescence detection of the ADAM esters is an order of magnitude more sensitive than UV detection (13).
Nevertheless, we used UV detection to monitor the ADAM esters in this study, because the sensitivity of this method was limited by the background levels of the same fatty acids esterified to exogenous phospholipids, but not by detection limits of a detector itself. In a pure sample the blank level of C18:1 was about 0.08 nmol per assay tube, while in crude samples it varied from sample to sample. A background level may be significantly lowered by using phospholipids with an unnatural acyl chain, although we have not pursued work along this line. Alternatively, the selection of the preferred substrates for given PLA$_{2s}$ can help in detecting the PLA$_2$ activity more sensitively as described below.

Because some portions of phospholipids were extracted into the heptane phase together with fatty acids by the Dole and Meinertz extraction procedures (4), the radioactive substrate should be separated from the released radioactive fatty acids by treatment of the heptane layer with silicic acid. This step was usually unnecessary in the present HPLC method, except when the POPG concentrations of greater than 1 mM were used (7). A modification of the Dole assay which eliminated silicic acid treatment has been reported (14). This method was based on preferential distribution of phosphatidylcholine (PC) into the aqueous Triton phase by extraction with hexane-1% Triton X-100.

The HPLC method can simultaneously monitor the release of products of the hydrolyses at the sn-1 and sn-2 positions of mixed-acyl phospholipids. This provides a clue to the positional specificity of phospholipase A action; precise positional specificity should be determined in conjunction with separate analyses of lysophospholipid molecular species (e.g., ref. 15), if hydrolysis at both positions is detected. In the radiometric assay the corresponding experiments require doubly labeled phospholipids. The specificity for sn-2 acyl moiety can be determined by this method using mixtures of phospholipids with different sn-2 acyl groups to normalize the contributions of interfacial properties of aggregated substrates to the hydrolytic rates (Fig. 4H).

**Distribution of PLA$_2$ activity in various rat tissues**

We previously reported (5, 6) that the general tendency of the substrate specificity of splenic group II PLA$_2$ was similar to that of pancreatic PLA$_2$: both isozymes preferred anionic substrates, i.e., phosphatidylglycerol and its mixed micelles with negatively charged bile salts, unlike venom PLA$_{2s}$ with a specificity for zwitterionic substrates, e.g., PC and PE and their mixed micelles with nonionic detergents (16). Although there were some differences between the two isozymes in the dependency of the specificity on the cholate/phospholipid molar ratio, a cholate/POPG molar ratio of 6 was found to be optimal for both isozymes. This substrate system provided the sensitive tool for detecting these PLA$_2$ activities. Table 1 shows the results of Ca$^{2+}$-dependent PLA$_2$ activities meas-

<table>
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<th>Tissues</th>
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<th>Specific Activity</th>
<th>Enzyme Activity</th>
<th>Anti-pancreatic PLA$_2$ IgG</th>
<th>Anti-group II PLA$_2$ IgG</th>
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<tr>
<td></td>
<td></td>
<td>nmol/min/mg</td>
<td>µmol/min/g Tissue</td>
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<td>-(102)$^i$</td>
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<td>Liver</td>
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Mixed micelles of 0.8 mM POPG and 4.8 mM sodium cholate were used as substrate. The values of enzyme activity are given as means ± SD; N, not determined; nd, not detectable.

$^i$Not inhibited, but rather enhanced in some cases. The reasons for this are unknown. The values in parentheses indicate the ratio (%) of activity in the presence of IgG to that in its absence.

$^i$nmol/min per ml.

**TABLE 1. Distribution of Ca$^{2+}$-dependent PLA$_2$ activity in various rat tissues and its inhibition by antibody directed against either rat pancreatic PLA$_2$ or splenic group II PLA$_2$.**
ured with 0.8 mM POPG and 4.8 mM cholate in various rat tissues. The activities were detectable in all tissues except for testis. Rich sources of PLA₂ are, in descending order, platelets, stomach, ileum, pancreas, spleen, colon, and lung. The specific activity values in Table 1 were about 4 times those when 1 mM POPE/6 mM DOC was used as a substrate. Moreover, those values were 20- to 200-fold greater than those reported previously with sonicated radioactive phospholipid dispersions (e.g., ref. 17). Cellular PLA₂ activity has usually been measured with sonicated dispersions of PC or PE as substrate; the phospholipids, especially PC, in this aggregate ion state were, however, poorly hydrolyzed by both pancreatic and group II PLA₂s. It is important to know the specificity of the PLA₂ under investigation toward the polar head groups and molecular state of substrate aggregation for increasing the sensitivity. Crude samples may contain lipolytic enzymes other than PLA₂, such as phospholipase A₁ and lysophospholipase. The combined action of these enzymes may make it difficult to judge which enzymes contribute to the observed release of sn-1 and sn-2 fatty acids. The majority of C18:1 release was inhibited by either anti-pancreatic PLA₂ antibody or anti-group II PLA₂ antibody in almost all tissues examined (Table 1). This suggests that either of the PLA₂ isozymes is mainly responsible for the measurable activity under the assay conditions used. In this study we separately determined the fatty acids released from the sn-1 and sn-2 positions of POPG. A small but significant amount of C16:0 was released in addition to the predominant C18:1 release (e.g., Fig. 4D). It cannot therefore be ruled out that a small portion of released C18:1 may be formed by the action of lysophospholipase in combination with phospholipase A₁, although the assay mixture contained sodium cholate which has been reported to minimize lysophospholipase activity (18). To determine the contribution of lipolytic enzymes involved towards the rate of C18:1 release quantitatively, it is necessary to establish the stoichiometry for the formation of all lipolytic products.

The respective contributions of the pancreatic and group II PLA₂s to the measurable activity were estimated from the degrees of its inhibition by anti-pancreatic PLA₂ and anti-splenic PLA₂ antibodies. Among all tissues examined only activities in the stomach and pancreas were significantly inhibited by anti-pancreatic PLA₂ antibody (Table 1) in agreement with our previous results (19). Because most of the pancreatic PLA₂ is stored in cells as its proenzyme, the difference between the activities in the stomach and pancreas homogenates does not necessarily reflect their protein contents. Indeed, the total contents of the PLA₂ and proenzyme of the gastric mucosa and pancreas were comparable as judged by immunoblot analyses (Fig. 6A, and ref. 19). The complexes of PLA₂ and proenzyme with sodium dodecyl sulfate show slightly different electrophoretic mobilities on a 14% gel under nonreducing conditions: the active enzyme is more abundant in gastric mucosa homogenate than in pancreas homogenate in agreement with the results of enzyme activity measurements (Fig. 6). On the other hand, PLA₂ activities were appreciably inhibited by anti-splenic PLA₂ antibody in almost all tissues examined, suggesting that group II-like PLA₂ is present ubiquitously in rat tissues and plasma, notably platelets, ileum, and spleen. This inhibition assay has an inherent limitation, that is, difficulty in detecting a minor inhibitable fraction in a predominant remaining activity; the results of the distribution of immunoreactive PLA₂ isoenzymes obtained by the method were therefore compared with those of immunoblot analyses as shown in Fig. 6. The results were virtually compatible with those of the former method. The methods used in the present study did not disclose the presence of pancreatic type PLA₂ in lung and spleen where its presence has been reported (5, 20). Recently, we succeeded in detecting pancreatic type PLA₂ in lung and spleen by a sensitive enzyme immunoassay (J. Higaki and H. Tojo, unpublished results).

Evidence for the difference between immuno-cross-reactivities of antibodies raised against platelet and liver group II PLA₂s has been reported in spite of a close similarity of their NH₂-terminal amino acid sequences. Anti-liver enzyme antibodies recognized the platelet enzyme (21), whereas anti-platelet enzyme antibodies were not at all crossreactive with the liver enzyme (17). The results of our previous (10) and present studies clearly show that anti-splenic PLA₂ antibody recognizes the platelet and liver enzymes as well, and thus support the former results. The reasons for the discrepancy are unknown at present.

**Fig. 6.** Immunoblot analyses of various rat tissues after electrophoresis on 14% gels in the absence of 2-mercaptoethanol. Staining was with anti-pancreatic PLA₂ IgG (A) and anti-splenic group II PLA₂ IgG (B). Lanes: 1, pancreatic proenzyme; 2, pancreatic PLA₂; 3, pancreas (40, 50); 4, gastric mucosa (40, 50); 5, liver (55, 65); 6, lung (45, 50); 7, colon mucosa (55, 50); 8, spleen (45, 35); 9, ileum mucosa (45, 35); 10, platelets (45, 35); 11, splenic group II PLA₂. The numbers in parentheses indicate amounts of protein (μg) in tissue homogenates (A) and the 100,000 g pellets of their post-120 g supernatants (B), respectively. As group II-like PLA₂ was enriched in the particulate fractions of tissue homogenates (7), the latter pellets were used in B.

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3Instead, this tissue had a relatively greater Ca²⁺-independent PLA₂ activity (2.69 ± 0.79 nmol/min per mg). Ileal mucosa also had a high Ca²⁺-independent PLA₂ activity.
In summary, reliability of the HPLC method of assaying PLA₂ activity was verified. This method is applicable to PLA₂ activity determinations in pure and crude samples. The selection of preferred substrates for a particular PLA₂ was crucial for sensitive detection. In addition, the present method can be easily adapted to assay various other lipolytic enzymes such as lipases and lysophospholipases without obvious modifications except for change in compositions of assay mixtures. In this laboratory we are routinely using the HPLC method for determinations of lipase activity with an emulsion of triolein and gum arabic as substrate for assays and of lysophospholipase as well as for PLA₂ activities.

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