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Detection of two phospholipase A_2(PLA_2) activities in leaves of higher plant *Vicia faba* and comparison with mammalian PLA_2's

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Abstract

Leaves of higher plant *Vicia faba* contains two Phospholipase A_2 (PLA_2) activities which are detected in cytosolic fractions. Based on a gel filtration column chromatography, two cytosolic PLA_2 activities migrated with molecular masses of 70 kDa and 14 kDa. The first (70 kDa peak) was optimally active at pH 4.5 and was not dependent on [Ca^{2+}] for its activity. In the presence of 5 mM $CaCl_2$, 'phospholipase B' activity was shown in the 70 kDa peak. The second (14 kDa peak) was optimally active in the pH range 9–10 and required millimolar concentrations of calcium for optimal activity. The two activities were not inhibited by dithiothreitol. Neither anti-pancreatic PLA_2 antiserum nor anti-(pig spleen 100 kDa cytosolic PLA_2) antiserum immunoprecipitated any activity of the two plant PLA_2's. The present results indicate that at least the 14 kDa form of the two PLA_2 enzymes detected in leaves of higher plants is biochemically and immunochemically different from the well characterized Ca^{2+}-dependent mammalian PLA_2's.

Key words: Plant phospholipase A_2; Free fatty acid; Lysophospholipid; *Vicia faba*

1. Introduction

Phospholipase A_2 (PLA_2) hydrolyzes the *sn*-2 fatty acyl ester bond of glycerophospholipids to produce lysophospholipids and free fatty acids. In animal cells, these enzymes play important roles in a number of fundamental cellular regulatory events through intracellular signaling. The two products of PLA_2activity have been involved in intracellular enzyme regulation, membrane channel opening, gene regulation, and growth [1–3]. In plants, it has become increasingly evident that the products of PLA_2activity may serve as second messengers in signal transduction processes [4]. Lysophospholipids, and/or free fatty acids, stimulate a number of plasma membrane enzymes of higher plants, including H^+-ATPase [5] and NADH oxidases [6,7] and protein kinases [8,9] which are implicated in growth stimulation. PLA_2 activation may also regulate levels of phosphatidylinositol phosphate (PIP) which activates plasma membrane ATPase in plants [10]. Biologically active auxins stimulate PLA_2 activity in vivo and in vitro, as indicated by the increases in lysophosphatidylcholine (lyso-PC) and lysophosphatidylethanolamine (lyso-PE) levels following the treatment of cultured soybean cells or zucchini hypocotyl membranes with the hormone [11]. A polyclonal antibody against an auxin-binding protein blocks the stimulation of PLA_2 activity [12]. Based on these results, a signal transduction chain was proposed leading from auxin stimulation to PLA_2 activation, to protein kinase activation, to H^+-ATPase activation, resulting in an enhanced growth response [12]. Despite their potential importance, plant PLA_2 activities and their characteristics remain largely unknown. In the present study, we report the identification and characterization of two kinds of PLA_2 from leaves of higher plant *Vicia faba*.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-[1-^{14}C]palmitoyl-glycerophosphocholine (-GPC) (55 mCi/mmol), 1,2-dipalmitoyl-glycerophospho-[*N*-methyl-^{14}C]choli ne (58 mCi/mmol), 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC (54 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]linoleoyl-GPC (54 mCi/mmol) and 1-acyl-2-[1-^{14}C]arachidonoyl-glycerophosphoethanolamine (-GPE) (54 mCi/mmol) were purchased from the radio-chemical Center, Amersham, UK. 1,2-dipalmitoyl-GPC, 1-stearoyl-2-arachidonoyl-GPC, 1-palmitoyl-2-linoleoyl-GPC, 1-palmitoyl-2-arachidonoyl-GPE, arachidonic acid, linoleic acid, palmitic acid, and lyso-PC were obtained from Sigma, St. Louis. Heparin-Sepharose CL-4B and Protein A-Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, Sweden. DEAE-cellulose (DE-52) was obtained from Whatman, Clifton, NJ.

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Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether) tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; Tris, Tris (hydroxymethyl) aminoethane; SDC, sodium deoxycholate; PLA_2, phospholipase A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HPLC, high performance liquid chromatography.

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2.2. Preparation of enzyme sources

Leaves of *Vicia faba* were cut and washed several times with buffer A (50 mM Tris-HCl, pH 9.0, 10 mM EDTA, 0.12 M NaCl). 20 g of the leaves was homogenized with 10 ml of buffer A using a Brinkmann homogenizer (Polytron, Model PCU 7). The debris and unlysed tissues were removed by centrifuging the homogenates at 300 × g at 4°C. The supernatants were then centrifuged at 100,000 × *g* for 60 min. The resulting supernatants and pellets were used as sources of PLA_2.

2.3. Assay for PLA_2

Each substrate (approximately 50 nmol) was dried under a nitrogen stream and resuspended in 0.1 ml of dimethylsulfoxide (DMSO). The standard incubation system (200 μl) for assay of PLA_2 activity contained 75 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 1 mg/ml bovine serum albumin and 5.0 nmol of radioactive phospholipids (approximately 65,000 cpm). The reactions were carried out at 37°C for 30 min and were stopped by adding 1 ml of chloroform/methanol (1:2, v/v). Lipids were extracted by the method of Bligh and Dyer [13]. Solvents were removed by a stream of nitrogen gas and the lipids were resuspended in chloroform/methanol (1:1, v/v). Phospholipids and neutral lipids were separated by double migration in a single direction on Silica-gel G plate, first in chloroform/methanol/water (60:30:5, by vol.) and after drying, in hexane/diethyl ether/acetic acid (80:20:1.5, by vol.). After drying, the plates were subjected to iodine vapour and the bands identified by their co-migration with authentic free fatty acids and lyso-PC. Products were quantified by scraping their corresponding spots into counting vials containing 10 ml of Aquasol II, and counting for radioactivity in a Packard Tri-Carb liquid scintillation spectrophotometer.

2.4. Gel filtration high performance liquid chromatography

The 100,000 × *g* supernatant of the leaves of *Vicia faba* was loaded onto a Heparin-Sepharose column pre-equilibrated with 50 mM Tris-HCl (pH 7.4) containing 0.12 M NaCl and 1 mM EDTA. The flow-through fractions were pooled and concentrated by 10-fold using Centricon 10 (Amicon, Denver, USA). The concentrated 100,000 × *g* supernatant (250 μl) was injected onto a TSK G 3000 SW column (Tosoh, Tokyo) pre-equilibrated with 50 mM Tris-HCl (pH 7.4) buffer containing 0.2 M NaCl and 1 mM EDTA. The column was eluted with the same buffer at a flow rate of 1 μl/min. 50 μl of each fraction (1 ml) was assayed for PLA_2 activity.

2.5. Immunoprecipitation of PLA_2 activity

Rabbit antiserum against pig spleen cytosolic 100 kDa PLA_2 was prepared as described previously [14]. Rabbit anti-pancreatic PLA_2 antiserum was purchased from UBI (Boston, MA). These antisera and rabbit normal serum (Sigma, St. Louis) were mixed with packed Protein A-Sepharose beads pre-equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mg/ml BSA) (1:1, v/v) and incubated for 24 h at 4°C with constant shaking. The beads were washed three times with buffer A. 50 μl of the packed beads was incubated with 0.5 ml of peak A and peak B fractions from TSK G 3000 SW HPLC for 4 h at 4°C with constant shaking. The beads were then pelleted and the supernatants were assayed for PLA_2 activity.

2.6. Analytical procedure

Protein concentration was measured with Bradford reagent [15] using bovine serum albumin as standard.

3. Results and discussion

We have detected two different PLA_2 activities in leaves of the higher plant, *Vicia faba*, and for the first time in plant systems, performed detailed characterization of PLA_2 activities. When the homogenates of leaves of *Vicia faba* (20 g) were fractionated by ultracentrifugation at 100,000 × *g* and the PLA_2 activity assayed using 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC as substrate, the enzyme activity was recovered primarily from the soluble cytoplasmic fraction; 250 nmol/h from 100 mg of total

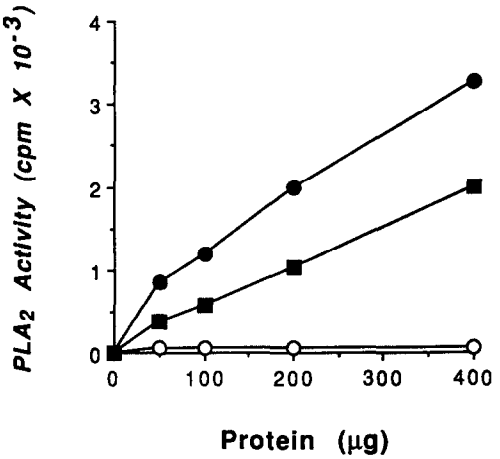

Fig. 1. Hydrolysis of radiolabelled phosphatidylcholine by 100,000 × *g* supernatant of leaves of *Vicia faba* PLA_2 activities were assayed using 25 mM 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC (●, ○) and 25 mM 1,2-dipalmitoyl-glycerophospho-[*N*-methyl-^{14}C]choline (B) as substrates. Following the reaction, [1-^{14}C]palmitate (●) and lyso-[1-^{14}C]palmitoyl-PC (○) from 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC, and lyso-[*N*-methyl-^{14}C]-PC (■) from 1,2-dipalmitoyl-glycerophospho-[*N*-methyl-^{14}C]choline were determined as described in section 2. Vehicle for the substrates, DMSO, did not affect the plant PLA_2 activities and non-enzymatic hydrolysis performed without enzyme yielded less than 50 cpm from the labelled substrates. Values are from a representative experiment which was reproduced three times with similar results.

cytoplasmic protein, and 30 nmol/h from 15 mg of total protein in the particulate fraction. Thus the major PLA_2 activity in leaves of *Vicia faba* was located in the cytoplasm. To test whether the phospholipase activity in the 100,000 × *g* supernatant is of 'A_2' type, we determined the formation of radiolabelled free fatty acid and lyso-PC hydrolyzed from 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC and 1,2-dipalmitoyl-glycerophospho-[*N*-methyl-^{14}C]choline, respectively. As shown in Fig. 1, when 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC was used as substrate, the release of free [1-^{14}C]palmitate was increased as the amount of added protein was increased, but no increase of lyso-2-[1-^{14}C]palmitoyl-PC was observed, indicating that the activity is the 'A_2' or 'B' type. To confirm this, we used 1,2-palmitoyl-glycerophospho-[*N*-methyl-^{14}C] choline as substrate. The production of lyso-[*N*-methyl-^{14}C]-PC, one of the products of the PLA_2 reaction, was dose-dependently increased, but less than that of free [1-^{14}C]palmitate released from 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC, indicating that the 100,000 × *g* supernatant contains 'B' type or 'lysophospholipase A_1' as well as 'A_2' type.

In animal cells, four PLA_2 isoforms, secretory pancreatic Group I [16,17] and non-pancreatic Group II PLA_2 [18–20], the high molecular mass form of cytosolic PLA_2 [21–25], and lysosomal PLA_2 [26,27], have been identified. These isoforms have different column chromatography profiles as well as biochemical properties. We compared enzyme activities and biochemical properties of

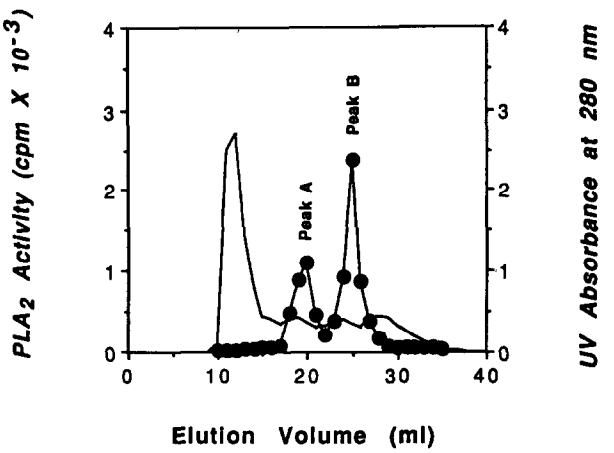

Fig. 2. TSK G 3000 SW gel filtration HPLC of heparin-non-binding PLA_2 activity. The flow-through fractions from a Heparin-Sepharose column were concentrated and injected into TSK G 3000 SW column as described in section 2. 50 μl of each fraction (1 ml) was assayed for phospholipase activity using 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC as substrate. The column was calibrated using standards (Sigma): blue dextran (6,000 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease (13.7 kDa). Closed circle: PLA_2 activity; solid line: protein.

PLA_2's in leaves of *Vicia faba* with properties of animal PLA_2's. The 100,000 × *g* supernatant of leaves of *Vicia faba* was loaded onto a Heparin-Sepharose column, which has been used to discriminate the mammalian high molecular mass form of cytosolic PLA_2 from the mammalian Group II PLA_2 [18]. The PLA_2 activity did not bind to the Heparin-Sepharose column pre-equilibrated with 50 mM Tris-HCl (pH 7.4) buffer containing 0.12 M NaCl and 1 mM EDTA. No PLA_2activity could be detected in the binding fractions eluted with the same buffer containing 2.0 M NaCl and 1 mM EDTA (data not shown), suggesting that in this respect, the profile of the enzyme activity was similar to that of mammalian high molecular mass form of cytosolic PLA_2 which does not bind to this column under the same conditions.

To identify the apparent molecular mass of the enzyme activity, we concentrated the heparin-non-binding fractions with Centricon 10 (Amicon, Denver, USA). More than 90% of total activity was recovered in the concentrate. When it was injected into a TSK G 3000 SW gel filtration column, phospholipase activity migrated as two peaks at an approximate molecular mass of 70 kDa (peak A) and an approximate molecular mass of 14 kDa (peak B) (Fig. 2). Whereas the mammalian pancreatic Group I and non-pancreatic (secretory) Group II enzymes have molecular mass of approximately 14 kDa, the mammalian cytosolic PLA_2 has relatively high molecular mass of more than 85 kDa. In this respect, phospholipase activity in peak A may be similar to high molecular cytosolic PLA_2 and that in peak B may be similar to the mammalian small molecular PLA_2's. To further examine peak A activity, active fractions from peak A were loaded onto a DEAE-cellulose column pre-equilibrated with 50 mM Tris-HCl (pH 7.4) containing 0.12 M NaCl and 1 mM EDTA. PLA_2 activity was recovered in non-binding fractions and no activity was detected in 0.12 to 1.0 M NaCl gradient fractions (data not shown). This result suggested that peak A PLA_2 is different from high molecular cytosolic enzyme which is bound to this column under the same conditions [18].

We tested the calcium requirements of the PLA_2 activities (Fig. 3). It is well known that many forms of mammalian PLA_2require Ca^{2+} for maximal activity; Group I and Group II PLA_2 require millimolar [Ca^{2+}] for their full enzymatic activity, and the high molecular cytosolic PLA_2 shows optimal activity with [Ca^{2+}] in the submicromolar range [21–25]. Some forms of PLA_2, however, are Ca^{2+}-independent [26–30] including lysosomal enzymes [26,27]. Whereas the enzyme activity of peak A could be detected regardless of [Ca^{2+}] and gradually increased by about two fold as [Ca^{2+}] was increased, that of peak B

Table 1
Positional specificity of the two PLA_2 activities from leaves of *Vicia faba*.

	Released [1-^{14}C]palmitic acid or Lyso-[*N*-methyl-^{14}C]PC (nmol/h/mg)		
	peak A		peak B
Products	5 mM $CaCl_2$	5 mM EGTA	5 mM $CaCl_2$
[1-^{14}C]Palmitic acid	36.5	20.8	8.2
Lyso-[*N*-methyl-^{14}C]PC	2.1	18.2	6.8

PLA_2 activities of peak A and peak B were assayed as described in section 2. Results are averages of three experiments with standard deviations less than 10%.

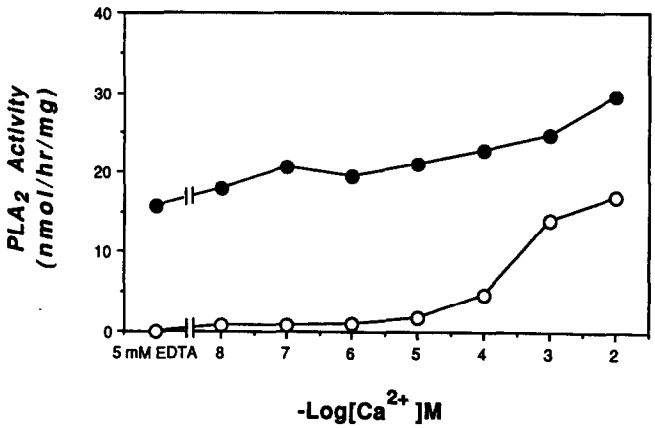

Fig. 3. Ca^{2+}-requirements for two PLA_2 activities isolated from leaves of *Vicia faba* by TSK G 3000 SW HPLC. PLA_2 activities of peak A (●) and peak B (○) were assayed as described in section 2 using 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC as substrate with the modifications in [Ca^{2+}] concentrations as indicated. The calcium concentrations were determined in EGTA/$CaCl_2$ buffers at pH 7.4 as described previously [33]. Values are from a single experiment which was reproduced three times with similar results.

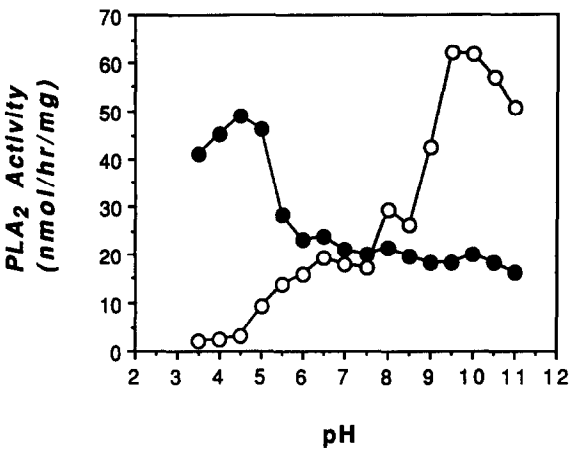

Fig. 4. Effect of pH on two PLA_2 activities isolated from leaves of *Vicia faba*. PLA_2activities of peak A (●) and peak B (○) were assayed with 5 mM $CaCl_2$ in 0.15 M-buffers as described in section 2 using 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC as substrate. Reactions were conducted in either sodium acetate/acetic acid buffer for pH 4.0–5.0, Tris/maleate buffer for pH 5.5–9.0, or glycine NaOH buffer for pH 9.5–11.0. Values are from a single experiment which was reproduced three times with similar results.

required millimolar [Ca^{2+}] for its optimal activity. We also determined the positional specificity using 1,2-dipalmitoyl-glycerophospho-[*N*-methyl-^{14}C]choline as well as 1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC as substrates. As shown in Table 1, whereas peak B showed PLA_2 activity, peak A exhibited 'B' type activities in the presence of [Ca^{2+}], but showed only 'A_2' activity in the calcium-free assay condition. Thus, the PLA_2 in peak B is similar to the mammalian secretory PLA_2's found in animal systems which require millimolar [Ca^{2+}] for their full activitites [16–20].

We determined the pH optima of the two PLA_2 activities in the presence of 5 mM $CaCl_2$ (Fig. 4). Whereas Ca^{2+}-dependent mammalian PLA_2's have pH optima in the alkaline pH range of 8.5–10.0, Ca^{2+}-independent lysosomal have optimal activity at pH 4–5. Activity of peak A was optimal at pH 4.5 and that of peak B was optimal in the range of pH 9–10, suggesting that the PLA_2 in peak A was similar to mammalian lysosomal PLA_2.

We determined the effect of a commonly used anionic detergent, sodium deoxycholate, on the PLA_2 activities (Table 2). We selected this detergent because it has been used to discriminate mammalian Group II from Group I PLA_2. Whereas its sensitivity for the PLA_2 activity of peak A was dependent on the added calcium ion, that of peak B was inhibited by the addition of exogenous detergent resulting in altered physical state of the substrate. This property of peak B is in contrast with that of mammalian Group I PLA_2, which usually requires that substrate phospholipids be incorporated into detergent micelles [31]. Since whereas dithiothreitol inhibited PLA_2 activities of small molecular weight forms, activity of high molecular cytosolic form is not affected, the reagent has been used to discriminate between the two forms of PLA_2. Neither peak A nor peak B activity was affected under the conditions where pancreatic and rat Group II PLA_2 activities were almost completely inhibited by the reagent (data not shown). This observation indicate that peak B activity is different from activity of small molecular weight form of PLA_2.

It has been known that whereas mammalian Group I and Group II PLA_2 as well as the lysosomal enzyme show no selectivity for acyl chain at *sn*-2 position, the high molecular cytosolic form selectively hydrolyzes their substrates containing arachidonoyl residue at *sn*-2 position. It is known that higher plants do not contain arachidonic acid [32]. However, to compare with the substrate specificity of mammalian PLA_2, that of *Vicia faba* PLA_2 for acyl chain at *sn*-2 position was determined. As shown in Table 3, these plant PLA_2 activities

Table 2
Effect of sodium deoxycholate on the two PLA_2 activities isolated from leaves of *Vicia faba*

Conditions	Released arachidonic acid (nmol/h/mg)	
	Peak A	Peak B
5 mM EGTA	13.5	ND
5 mM EGTA,10 mM SDC	5.1	ND
5 mM Ca^{2+}	28.9	16.9
5 mM Ca^{2+}, 10 mM SDC	131.0	5.3

ND; not detected
PLA_2 activities of peak A and peak B were assayed as described in section 2 using 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC as substrate. Substrates were added to the reaction mixture followed by addition of sodium deoxycholate. After vigorously vortexing for 30 s, reactions were initiated by adding enzyme preparations. Values represent averages of three experiments with standard deviations less than 10%.

Table 3
Substrate specificities of the two PLA_2 activities isolated from leaves of *Vicia faba*

Substrates	Released free fatty acids (nmol/h/mg)	
	peak A	peak B
1-palmitoyl-2-[1-^{14}C]palmitoyl-GPC	22.4	10.8
1-palmitoyl-2-[1-^{14}C]linoleoyl-GPC	28.2	22.7
1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC	14.5	21.1
1-acyl-2-[1-^{14}C]arachidonoyl-GPE	42.1	38.6

PLA_2 activities of peak A and peak B were assayed as described in section 2 using 25 mM of various substrates. Activity of peak A was determined in 75 mM Tris-HCl (pH 7.4) containing 5 mM EGTA and 1 mg/ml bovine serum albumin. Activity of peak B was assayed in 75 mM Tris-HCl (pH 9.0) containing 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. Results are averages of three experiments with standard deviations less than 10%.

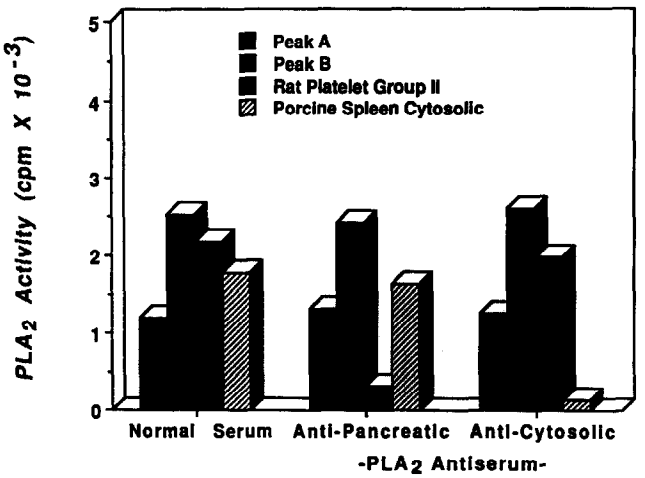

Fig. 5. Immunoprecipitation of two PLA_2 activities of *Vicia faba* leaves. Pig spleen 100 kDa cytosolic PLA_2 was partially purified as described previously [14] and rat platelet Group II PLA_2 was partially purified with a Heparin-Sepharose column previously described [18]. As described in section 2, after pelleting the Protein A beads, the supernatants were assayed for PLA_2 activity using 1-acyl-2-[1-^{14}C]arachidonoyl-GPE as substrate. Each point is average of duplicate measurements and representative of three independent experiments using different enzyme preparations.

do not show any marked selectivity for head group moieties or acyl chains at *sn*-2 position although both activities were highest when arachidonic acid was present in the *sn*-2 position.

Finally, immunochemical studies were performed for further characterization of the plant PLA_2 activities. Whereas almost complete precipitation was achieved when Group II rat platelet PLA_2 and pig spleen cytosolic PLA_2 preparations were incubated in the presence of anti-pancreatic PLA_2 antiserum and anti-(pig spleen 100 kDa cytosolic PLA_2) antiserum, respectively, any activity of the two plant PLA_2's was not absorbed with either anti-pancreatic PLA_2 antiserum or anti-(pig spleen 100 kDa cytosolic PLA_2) antiserum (Fig. 5).

In conclusion, the present study reports the identification and characterization of two PLA_2 enzymes in leaves of a higher plant, *Vicia faba*. One had an acidic pH optimum, was Ca^{2+}-independent and had a molecular mass of approximately 70 kDa. The other had an alkaline pH optimum, was Ca^{2+}-dependent, detergent-sensitive, dithiothreitol-insensitive, and had molecular mass of approximately 14 kDa. Although these enzyme entities should be purified to homogeneity and their primary structures analyzed for complete characterization, these results suggest that whereas the one enzyme is similar to a soluble lysosomal PLA_2 characterized previously in animal, the second is biochemically and immunochemically different from Ca^{2+}-dependent PLA_2's so far characterized in mammals. Although the possible mechanism by which the PLA_2 enzymes produce free fatty acids and lysophospholipids during plant cell stimulation remain to be elucidated, detection of the enzymes in higher plant may indicate their participation in the production of the lipid mediators which regulate a number of growth-related enzymes of higher plant.

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