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IDENTIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE A_2 IN OAT CELLS

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Abstract — The activity of phospholipase A_2 (PLA_2) was identified and characterized from cytosolic and membrane fractions of oat cells, respectively. PLA_2 activity was determined fluorometrically in the presence of serum albumin using phospholipids labeled at *sn*-2-acyl position with 10-pyrenyldecanoic acid. When the cell-free extracts of oat tissues were fractionated by ultracentrifugation at 100,000 × g and the PLA_2 activity was assayed, we found that most of the PLA_2 activity was revealed from the cytoplasmic fraction rather than from the membrane fraction. The activity of cytosolic PLA_2 was dependent on Ca^{2+} concentration and the optimum concentration of Ca^{2+} was found to be 100 μM. It was also found that PLA_2 could be translocated toward the membrane site from the cytosol upon increasing Ca^{2+} concentration. These results might suggest that an increased $[Ca^{2+}]_i$ by phytochrome action could promote the translocation of the cytosolic PLA_2 toward the membrane site.

INTRODUCTION

Phospholipase A_2(PLA_2)† catalyzes the hydrolysis of the *sn*-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids. In animal cells, PLA_2 plays a central role in diverse cellular processes including phospholipid metabolism,[1,2] host defense[3,4] and signal transduction.[5,6]

If the cleaved fatty acid is arachidonic acid, PLA_2 can provide precursors for eicosanoid generation.[1,7,8] When the *sn*-1 position of the phospholipid contains an alkyl ether linkage,[9] the platelet-activating factor can also be formed.

In plant cells, recent studies[10-15] suggest that the two products(free fatty acids and lysophospholipids) formed by the PLA_2 activation may serve as second messengers in signal transduction processes. There are a few reports[11,13,15] describing that biologically active auxins stimulate PLA_2 activity *in vivo* and *in vitro*, respectively. When the cultured soybean cells or *zucchini* hypocotyl membranes were treated with the biologically active auxins, the levels of lyso-PC and lyso-PE were shown to enhance substantially.[11] In higher plants, it was reported that free fatty acids and/or lysophospholipids stimulated important enzymes like protein kinases[10,12] and NADH oxidases,[13,16] which might be involved in the photomorphogenic reactions. Particularly, Chae *et al.* found that linolenic acid activated the protein kinase C in oat cells.[14]

These results make us speculate that red light can activate PLA_2 and its product linolenic acid activates the protein kinase C which might be the key enzyme in the phytochrome-mediated signal transduction processes.

Final goal of our study is to investigate an involvement of PLA_2 in the phytochrome-mediated signal transduction through the protein kinase C. However, this time we only report the initial results concerned with calcium dependent properties of PLA_2. An initial signal of phytochrome is strongly believed to be Ca^{2+} and thus it is very important to investigate the relationship between Ca^{2+} and PLA_2. In this report we demonstrate the calcium-induced translocation of PLA_2 from cytosol toward the plasma membrane in which substrate of PLA_2 exists.

MATERIALS AND METHODS

Materials: 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphatidylcholine ($PyC_{10}PC$), 10-pyrenyldecanoic acid (PyC_{10}), bee venom(*Apis mellifera*)PLA_2, bovine serum albumin and polyethylene glycol 3350 were purchased from Sigma. Dextran T-500 was purchased from Pharmacia Fine Chemicals. Oat seed (*Avena Sativa* L.cv.Gary) was purchased from Stanford seed Co., Buffalo, N.Y., USA. All chemicals and solvents were used

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† *Abbreviation* : PLA_2, phospholipase A_2

of analytical grade.

Oat seedling: 50 g of oat seed was soaked in water at 25°C for 24 h. in darkenss. The soaked seed was spread on wet vermiculite (100 g/tray) in aluminum trays (35 × 45 cm), and the trays were put in an oat-grown box made of wood. The oats were grown at 25°C for 5 days in complete darkenss.

Preparation of enzyme sources: 300-350 g of oat tissues was homogenized with a homogenizer in homogenization buffer containing 10 m*M* Tris-HCl (pH 7.5), 330 m*M* sucrose, 1 m*M* EDTA, 1 m*M* PMSF, 1 μg/ml leupeptin and 70 m*M* β-mercaptoethanol. The homogenate was subjeted to centrifugation at 8,000 × g for 15 min at 4°C. The supernatants were then centrifuged at 100,000 × g for 60 min at 4°C. The resulting supernatants (cytosolic fraction) and pellets (microsomal fraction) were used as sources of PLA_2. In the subcellular PLA_2 translocation experiments, oat tissues were homogenized in homogenation buffer containing different concentrations of free Ca^{2+}. The Ca^{2+} concentrations were controlled by using Ca^{2+}- EGTA buffers.

Homogenates were centrifuged at 100,000 × g for 60 min at 4°C and the pellets were resuspended in the same buffer used for the lysis.

Preparation of outside-out plasma membrane: The resulting microsomal pellet was resuspended in suspension buffer containing 5 m*M* potassium phosphate (pH 7.8), 0.33 *M* sucrose, and 4 m*M* KCl. The microsomal fraction was added to a two-phase system with a final weight of 36.0 g and a final composition of 6.5%(w/v) Dextran T500, 6.5%(w/w) polyethylene glycol 3350, 5 m*M* potassium phosphate (pH 7.8), 0.33 *M* sucrose and 4 m*M* KCl. After mixing by repeated inversions (30-35 times), the two-phase systems were separated by a 5 min centrifugation at 1,500 × g at 4°C. The upper phase, containing the right-side-out plasma membrane, was applied to the three-step batch procedure described previously by Michael *et al.*[17] The final upper phases containing the plasma membrane were diluted several-fold with inside-out buffer containing potassium phosphate (pH 7.8), 0.33 *M* sucrose, 50 m*M* KCl, 0.1 m*M* EDTA and 1 m*M* DTT. After a centrifugation at 100,000 × g for 60 min, the resulting pellet, which contained the purified plasma membrane, was resuspended in inside-out buffer.

Formation of Inside-out vesicles from right-side-out vesicles: The highly purified right-side-out plasma membrane vesicles were frozen (-195°C) and thawed (25°C) to produce a mixture of inside-out and right-side-out vesicles. Typically, 0.8 to 1 mL of the mixture was frozen in liqid N_2 and thawed in water at 25°C repeatedly four times. These mixtures of inside-out and right-side-out vesicle were centrifuged at 100,000 × g for 60 min at 4°C. Resulting pellets were resuspended in proper buffer system. The membranes were stored in liquid N_2 until further use.

Phospholipase A_2 assay: A sensitive and continuous fluorometric assay for PLA_2 was described in detail previously[18] and involved the following assay conditions. The fluorescent phospholipids were dissolved in ethanol at a concentration of 1 mg/mL. These stock solutions were kept at -20°C and could be used for at least 1 month. Vesicles were prepared by adding the fluorescent phospholipid dissolved in ethanol to an aqueous medium by sonication. The reaction solution contains 50 m*M* Tris-HCl (pH 7.5), 2 m*M* EGTA, 2 μ*M* $PyC_{10}PC$, 0.1% BSA and 2.1 m*M* $CaCl_2$. The fluorescence of reaction medium (blank) was recorded and the reaction was initiated by the addition of the phospholipase A_2. All fluoresence measurements were done with Hitachi F-3000 spectrofluorometer equipped with a Xenon lamp.

RESULTS AND DISCUSSION

The most common and sensitive method for assaying PLA_2 has been to use a radioisotope labeled substrate. However, many limitations in using isotopes prompted us to hire another method for assaying PLA_2 and thus we established a fluorometric assay procedure for PLA_2 *in vivo* and *in vitro*, using a substrate whose *sn*-2-acyl position was labeled with a fluorescent probe (10-pyrenyldecanoic acid ; PyC_{10}).[18,19] Using this fluorometric assay method, we succeeded in measuring the activity of PLA_2 in cytosolic and microsomal fractions of oat cells. The most critical point of this method is an excimer fluorescence caused by pyrene-pyrene interactions. In the case of phospholipids esterified at the *sn*-2-acyl position with insoluble fatty acid like pyrenyldecanoic acid, the fluorescent fatty acids produced by the enzymatic reaction remain in an insoluble form unless serum albumin, which binds these molecules, is present in the reaction medium. In order to continuously monitor the enzymatic reaction with the monomeric fluorescence of fluorescent fatty acid, we added serum albumin into the reaction medium.

Figure 1 shows the fluorescence emisson spectra of $PyC_{10}PC$ and PyC_{10} in an aqueous assay medium. The vesicular $PyC_{10}PC$ reveals intermolecular eximer emission at 480 nm as observed in other papers,[18] but monomer emission at 380 nm and 398 nm is absent. These observations indicate that the pyrene phospholipid derivative is in an aggregated state in aqueous solution. In addition, we observed two behaviors supporting that above results were true ; 1) the hydrolysis of the fluorescent phospholipid derivatives with bee venom PLA_2 caused the progressive disappearance of eximer emission at 480 nm. 2) the intensity of the monomer emission was very small in the absence of serum albumin, compared to that observed in the presence of serum albumin.

Figure 2 shows the enzymatic activity of PLA_2 from cytosolic fraction as a function of $PyC_{10}PC$ concentration. The homogenate of oat tissues was fractionated by ultracentrifugation at 100,000 × g and

PLA_2 activity was assayed using $PyC_{10}PC$ as substrate. The enzymatic activity was primarily recovered from the soluble cytoplasmic fraction (> 99 %). Thus the major activity of PLA_2 was located in the cytoplasm and it was not detected in the microsomal fraction.

However, we could measure the enzymatic of PLA_2 in the plasma membrane which was prepared from microsomal fraction. This result indicates that very small amount of PLA_2 is bound in the plasma membrane of oat cells. Considering that the substrates of PLA_2 are existing in the membranes, translocation of cytosolic PLA_2 toward the membranes could be assumed.

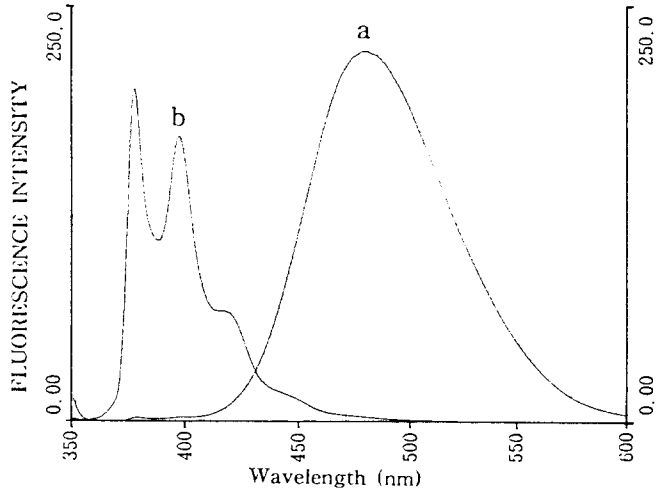

Figure 1. Fluorescence emission spectra of (a) $PyC_{10}PC$ and (b) PyC_{10} bound to serum albumin. Emission spectra of (a) $PyC_{10}PC$ and (b) PyC_{10} were recorded in 50 m*M* Tris-HCl, pH 7.5, containing 0.33 *M* sucrose, 2 m*M* EGTA and 1 mg/mL (0.1%) BSA.

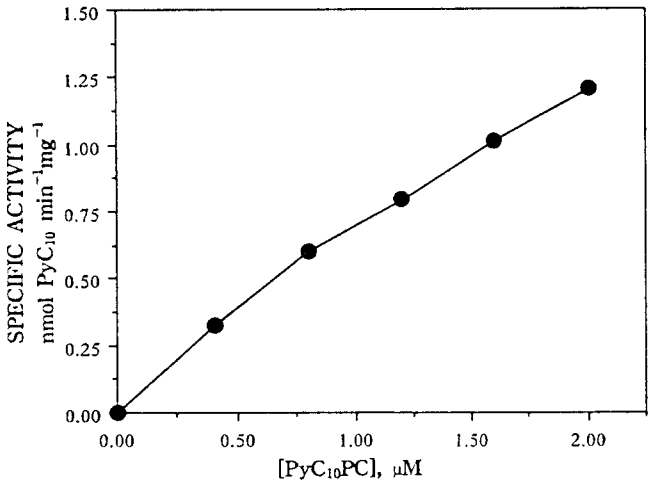

Figure 2. Enzyme activity of phospholipase A_2 in cytoplasm as a function of $PyC_{10}PC$ concentration. $PyC_{10}PC$ (0 *μM*-2 *μM*) was incubated in 50 m*M* Tris-HCl, pH 7.5, containing 0.33 *M* sucrose, 2 m*M* EGTA, 2.1 m*M* $CaCl_2$, 1 mg/mL (0.1%) serum albumin, and 0.1 mg of cytosolic protein. The monomeric fluorescence (excitation wavelength, 345 nm : emission wavelength, 398 nm) of $PyC_{10}PC$ was recorded.

Dependencies on the cytosolic protein concentrations (Fig. 3) and pH (Fig. 4) for the enzymatic activity of PLA_2 in cytosolic fraction were checked, respectively. The enzymatic activity was linearly dependent on the protein concentration up to 300 *μg*. Optimal pH was observed at 7.5-8.0 whose value was slightly higher than the one determined in animal

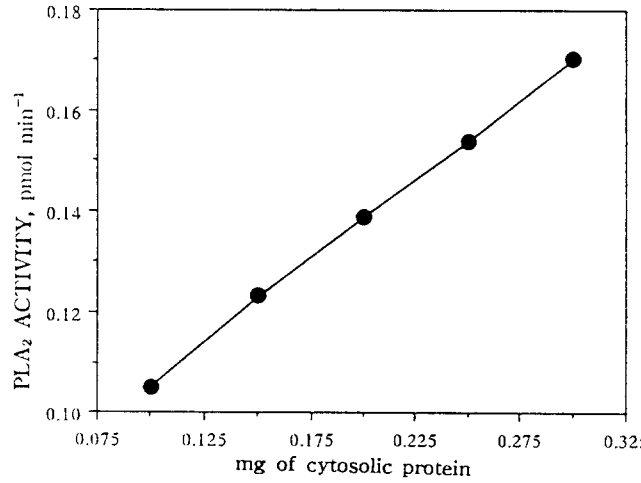

Figure 3. Phospholipase A_2 activity as a function of increasing cytosolic protein. The assay of phospholipase A_2 activity was performed under the same conditions as described in Figure 2, except for the using of a diverse amount of cytosolic protein.

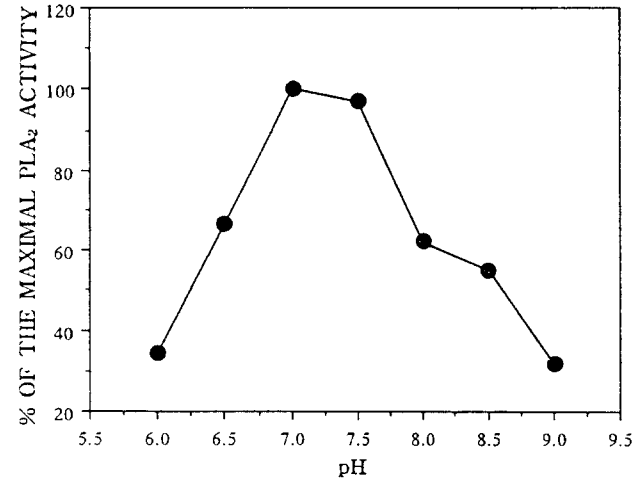

Figure 4. Effect of pH on phospholipase A_2 activities of cytosolic fraction. Phospholipase A_2 activities of cytoplasm were assayed under the same conditions as described in Figure 2, except for theusing of a proper buffer systems.

cells. PLA_2 activity in the cytosolic fraction was dependent on free calcium ion concentration. The calcium dependency was determined in Ca^{2+}-EGTA buffer at pH 7.5. The PLA_2 activity was detectable at 1 μM of free calcium and it was elevated nearly 5 fold under the free calcium concentration of 100 μM (Fig. 5). But the hydrolytic activity of this enzyme was inhibited upon elevation of calcium concentration from 100 μM to 1 μM. The fact that PLA_2 was activated by the sub-micromolar concentration of calcium implied that this enzyme could have its own role in the intracellular calcium concentration which can be generated by irradiation of red light on oat cells.[20,21,22]

The calcium concentration also regulated the translocation of soluble PLA_2 activity from cytosol to plasma membrane in oat cell. When the tissues were homogenized in a homogenation buffer in which free Ca^{2+} was buffered at various concentrations, the specific activity of PLA_2 in the microsomal fraction increased, directly dependent on the free Ca^{2+} concentration (0.1 μM to 10 μM) (Fig. 6). The calcium-dependent translocation of PLA_2 to membrane has been reported in human monocytic leukemic U937 cells[23] and rat liver macrophages.[24] In plant cell, this is the first report that cytosolic PLA_2 can be translocated toward the plasma membrane by regulating cytosolic free Ca^{2+} concentration.

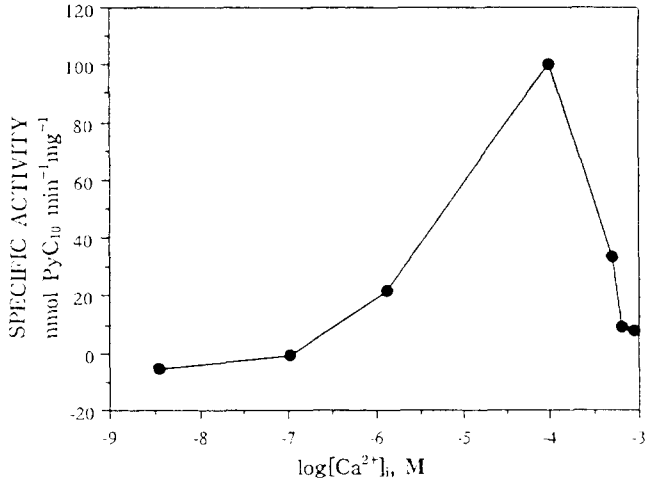

Figure 5. Calcium dependency of the phospholipase A_2 activity in cytosolic fraction. The assay for phospholipase A_2 was performed in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, containing 0.33 M sucrose, 1 mg/mL (0.1%) BSA, and 0.1 mg of cytosolic protein at free calcium concentration of 3.6 nM to 1 mM using Ca^{2+}-EGTA buffer.

Our previous studies[20,22] presented that phytochrome signal controlled the cytosolic free Ca^{2+} concentration and we also have been investigating the roles of Ca^{2+} in the next steps of signal transduction.

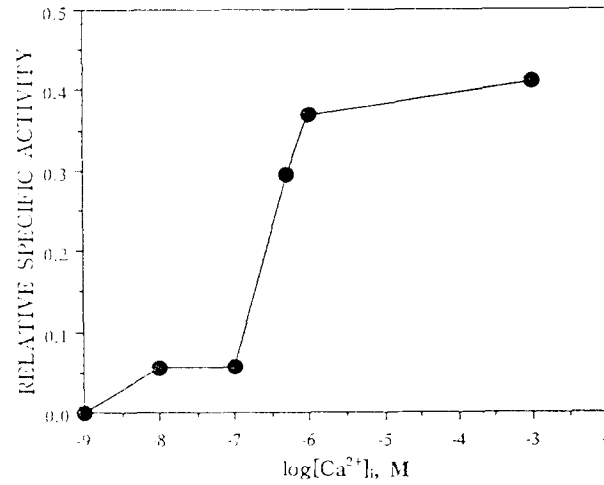

Figure 6. Effect of Ca^{2+} on the translocation of phospholipase A_2 activity from cytosolic fraction to membrane fraction. Oat tissues were homogenized in the presence of various calcium concentrations in Ca^{2+}-EGTA buffer. The assay of phospholipase A_2 activity was performed under the same conditions as described in Figure 2.

Upon summarizing our previous results concerned with the phytochrome-mediated signal transduction processes, the elevation of calcium concentration in cytoplasm of oat cell through phospholipase C activation by red-light may induce translocation of PLA_2 toward the plasma membrane. One other possibility for the involvement of PLA_2 in the phytochrome signal transduction is a participation of products of PLA_2 as second messengers in the signal transduction processes. As a conclusive suggestion, a possible model for phytochrome-mediated signal transduction *via* PLA_2 is proposed carefully (Fig. 7).

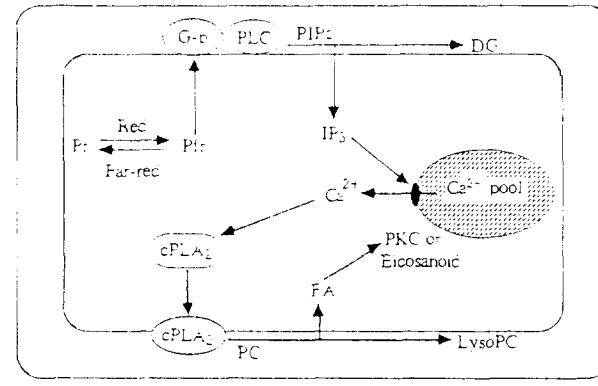

Figure 7. A possible model for the activation of cytosolic phospholipase A_2, in connection with the red light signal. This model is tentatively proposed after summarizing all our previous results concerning with phytochrome studies and the translocation behavior of cytosolic PLA_2 ($cPLA_2$) by variation of calcium concentration.

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