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Secretory phospholipase A_2-α from *Arabidopsis thaliana*: functional parameters and substrate preference

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Abstract

The secretory phospholipase A_2-α from *Arabidopsis thaliana* (AtsPLA$_2$-α), being one of the first plant sPLA$_2$s obtained in purified state, has been characterised with respect to substrate preference and optimum conditions of catalysis. The optima of pH, temperature, and calcium concentration were similar to the parameters of secretory PLA$_2$s from animals. However, substrate preferences markedly differed. In contrast to pancreatic PLA$_2$s, AtsPLA$_2$-α preferred zwitterionic phospholipids, and showed lower activity toward anionic phospholipids.

In substrates with two identical fatty acid chains, AtsPLA$_2$-α showed optimum activity toward phospholipids with decanoyl groups. In substrates with palmitoyl groups in *sn-1* position, acyl chains with higher degree of unsaturation in *sn-2* position were preferred, excluding arachidonic acid, showing the evolutionary adaptation of the enzyme to substrate composition in plants. K_m values for short chain phospholipids were comparable to sPLA$_2$s from animals, whereas k_{cat} values were much smaller and interfacial activation was less important.

Keywords: Secretory phospholipase A_2; *Arabidopsis thaliana*; Substrate preference; Interfacial activation; Short chain phospholipids

1. Introduction

Phospholipases A_2 (PLA$_2$s; EC 3.1.1.4) hydrolyse stereospecifically the 2-acyl ester bond of 1,2-diacyl-*sn*-3-phosphoglycerides and generate fatty acids and lysophospholipids. The released fatty acid, in animals most notably arachidonic acid (Burgoyne and Morgan, 1990), plays an important role in inflammation processes as a precursor of prostaglandins and leukotrienes (Soloff et al., 2000; Wijewickrama et al., 2006). The corresponding lysophospholipids are involved in the synthesis of platelet-activating factor and other physiologically important lipid mediators, such as lysophosphatidic acid which is a compound with potent mitogenic activity (Gardell et al., 2006).

Abbreviations: AtsPLA$_2$-α, secretory phospholipase A_2-α from *Arabidopsis thaliana*; Bis-Tris-propane, 1,3-Bis[tris(hydroxymethyl) methylamino]propane; Caps, 3-[cyclohexylamino]-1-propanesulfonic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, 1,2-diacyl-*sn*-glycero-3-phospho-(1-D-*myo*-inositol); PLA$_2$, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol); PS, phosphatidylserine; sPLA$_2$, secretory phospholipase A_2

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In plant cells, the release of linolenic acid probably plays a similar role as arachidonic acid in animal cells. Linolenic acid is assumed to be the precursor of jasmonic acid which is a potent and multifunctional growth regulator (Ishiguro et al., 2001) that modulates anther dehiscence, fruit ripening, root growth, tendril coiling, and plant resistance to insects and pathogens. Whereas the enzymes involved in jasmonic acid biosynthesis downstream of linolenic acid are well known, there is a great number of open questions concerning the source of linolenic acid in the plant cell.

Secretory PLA_2s ($sPLA_2$s) are one of the main classes within the PLA_2 superfamily and some of their members are very well characterised. Important common structural features of the small $sPLA_2$s are two well-conserved central α-helices with the catalytic His–Asp dyad and a hydrogen-bonding network connecting the interfacial binding site, the catalytic and the calcium-binding sites (Six and Dennis, 2000). $sPLA_2$s contain 5–8 disulphide bonds and are highly stable. $sPLA_2$s are widespread in nature and have so far been mainly found in the venoms of reptiles and insects, pancreatic juices and other extracellular fluids. Since recently, a steadily increasing number of $sPLA_2$s have been shown to be present in invertebrate animals, fungi, and plants.

In comparison to animal $sPLA_2$s, the knowledge on $sPLA_2$s from plants is still limited, even though within the last years some heterologously expressed plant enzymes have been characterised. The plant $sPLA_2$s belong to groups XIA and XIB (Six and Dennis, 2000; Mansfeld et al., 2006) which differ significantly in their primary structures and the requirement of Ca^{2+} ions. Despite the identity to $sPLA_2$s from animal sources of about 15% only, the known plant $sPLA_2$s show a significant degree of similarity with animal $sPLA_2$s in the active site and the calcium-binding loop regions. The plant $sPLA_2$s contain 12 cysteine residues being potentially able to form 6 disulphide bridges. Computer-modelling studies on the isoform $sPLA_2$-α from *A. thaliana* ($AtsPLA_2$-α) (accession number AY136317, Mansfeld et al., 2006) provided the prediction of at least four disulphide bridges.

In this paper, $AtsPLA_2$-α (group XIB) which was produced by heterologous expression in *E. coli* and renatured from inclusion bodies (Mansfeld et al., 2006) has been studied with respect to substrate preferences including different physical states of the substrates, calcium ion requirement, pH and temperature optima. The results are discussed in comparison with the properties of $sPLA_2$s from animal sources.

2. Materials and methods

Guanidine hydrochloride was from ICN (Eschwege, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), phosphatidic acid (PA) from soybean, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (POPG) were a gift of Lipoid GmbH (Ludwigshafen, Germany). 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids (Alabaster, USA). All other phospholipids, 1,4-dithiothreitol (DTT), the Bradford protein assay reagent, and all buffer substances were from Sigma (Taufkirchen, Germany). The BCA protein assay kit was from Pierce (Bonn, Germany). The NEFA C Kit was obtained from WAKO Chemicals (Neuss, Germany). The HiPrep® 26/10 Desalting, MonoQ® columns, and the low molecular weight marker kit were supplied by Amersham Biosciences (Freiburg, Germany). The SP 250/10 NUCLEOSIL 500-5 C3 PPN column was purchased from Macherey & Nagel, Düren, Germany. All other chemicals used were of the highest quality commercially available.

2.1. Expression and isolation of the protein

Expression in BL21(DE3) cells containing the $AtsPLA_2$-α gene in the vector pET-26b(+) was performed as described in Mansfeld et al. (2006). After cell disruption, inclusion bodies were collected, washed and subsequently solubilised in 100 mM Tris/HCl buffer, pH 8.3, containing 6 M guanidine hydrochloride, 100 mM DTT, and 1 mM EDTA. After renaturation, the enzyme was applied to a HiPrep® 26/10 Desalting column, and subsequently purified by anion-exchange chromatography on Mono Q® HR5.5 column. The protein was eluted with a linear NaCl gradient (0–0.5 M) in 50 mM Tris/HCl buffer, pH 8.5, 10 mM $CaCl_2$. Alternatively, the enzyme was purified by reversed-phase chromatography on a SP 250/10 NUCLEOSIL 500-5 C_3 PPN column (Solvent A: 0.1% (v/v) trifluoroacetic acid in H_2O, 5% acetonitrile (v/v); solvent B: 0.1% (v/v) trifluoroacetic acid, 70% (v/v) acetonitrile). Peak fractions were assayed for activity, and purity was proven by SDS-PAGE and mass spectrometry using a Bruker REFLEX mass spectrometer (Bruker, Daltonik, Bremen, Germany), upgraded with a gridless delayed extraction ion source (pulsed ion extraction).

2.2. Protein determination

Protein concentrations were determined by the Bradford (solubilised inclusion bodies) or the BCA protein

assay (purified protein) with bovine serum albumine as standard according to the instructions of the suppliers.

2.3. Activity measurements

PLA_2 activities were routinely determined with DOPC/SDS/Triton X-100 mixed micelles as substrate in adaptation to the method described by Kasurinen and Vanha-Perttula (1987). DOPC (10 mg, 12.7 μmol), dissolved in 200 μL of chloroform and 100 μL of methanol, was dried in vacuum. The lipid film was dissolved in 600 μL of buffer containing 0.5 M Tris/HCl, pH 8.5, 42 mM Triton X-100, 8 mM SDS, and 42 mM $CaCl_2$ by vortexing for 15 min. The enzyme (0.1 nmol) dissolved in 0.1 M Tris/HCl buffer, pH 8.5, was incubated for 5–30 min at 37 °C with 1.27 μmol of DOPC in a total volume of 100 μL. Aliquots (10 μL) were removed after defined times of incubation, and the reaction was stopped by addition of 10 μL of 0.2 M EDTA. Initial rates were determined from the increase of released fatty acids using the NEFA C Kit according to Hoffmann et al. (1986). Enzyme activities were linearly dependent on enzyme concentration up to 4 μM.

For the preparation of mixed micelles of substrates and Triton X-100 without SDS, the lipid film was dissolved in 600 μL of buffer containing 0.5 M Tris/HCl, pH 8.5, 84 mM Triton X-100, and 42 mM $CaCl_2$ by vortexing for 15 min.

For the determination of head group and acyl chain preferences, the corresponding phospholipids were used instead of DOPC. The use of mixed micelles of phospholipids with Triton X-100 and SDS or solely Triton X-100 is indicated in the text and the corresponding tables. All experiments were run in duplicate or triplicate.

To vary the molar ratio of Triton X-100 to phospholipid from 2:1, 4:1, and 16:1, the corresponding amounts of the phospholipid and Triton X-100 stock solutions were used for the preparation of the mixed micelles. In all cases, the phospholipid concentration in the assay was varied between 0 and 20 mM.

For the preparation of small unilamellar vesicles (SUVs) of phosphatidylcholine (PC) from soybean or DOPC containing different amounts of other phospholipids, stock solutions of the corresponding phospholipids (42.3 mM) were prepared in chloroform or chloroform/methanol (2:1, v/v), respectively. Varying mol% of other phospholipids in PC were obtained by using appropriate amounts of the corresponding stock solutions. Then, the organic solvent was removed by vacuum. The lipid film was subsequently dissolved in 600 μL of 50 mM Tris/HCl buffer, pH 8.5, by ultrasonic treatment with 300 Ws mL^{-1} using a 60 W Vibra Cell ultrasonic disintegrator (Avantec, France) with a microtip probe of 3 mm. Final concentration of phospholipids in the assay was 13 mM. Assay conditions and determination of initial rates of the fatty acid release were as described above.

For the preparation of micelles of short chain PCs (C_6–C_8), the corresponding lipid film was dissolved in 50 mM Tris/HCl buffer, pH 8.5, containing 10 mM $CaCl_2$.

All experiments were run in duplicate or triplicate.

2.4. Determination of kinetic constants

For the determination of the kinetic constants, initial reaction rates (v_0) were determined as described above at substrate concentrations between 1 and 40 mM and expressed as μmol of liberated fatty acid per minute and mg protein. v_0 was plotted versus substrate concentration, and K_M^{app} and V_{max}^{app} were obtained by nonlinear regression using the Michaelis–Menten function in Sigma-Plot 8.0. The standard deviations of the derived kinetic constants were ≤15%.

The kinetic constants for 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine were determined according to Berg and Jain (2002) assuming that above the critical micelle concentration (CMC) the bulk concentration of phospholipid present as micelles ($[S^*] = [S_T] - CMC$) determines the concentration of enzyme bound to the interface. $[S_T]$ represents the total substrate concentration.

2.5. Inactivation by p-bromophenacylbromide

The enzyme (6.5 μM) was incubated with 2 mM *p*-bromophenacylbromide at 25 °C in 0.05 M Tris/HCl buffer, pH 8.5, in the absence or presence of 10 mM $CaCl_2$. Aliquots of the reaction mixture were withdrawn at appropriate time intervals and immediately assayed for activity using the routine assay described above.

2.6. Determination of temperature optimum

For the determination of the temperature optimum, the enzyme (1 μM) was incubated with DOPC in mixed micelles with SDS and Triton X-100, prepared as described above, at temperatures between 20 and 90 °C for 6 min in 50 mM Tris/HCl buffer, pH 8.5, 10 mM $CaCl_2$. Product formation was quantified by the NEFA C kit. Activity is expressed as the amount of released fatty acid per minute and mg protein.

2.7. Determination of pH optimum

For the determination of the pH optimum the following buffers were used for the preparation of DOPC in mixed micelles with SDS and Triton X-100 and dilution of enzyme solution instead of the usual buffer: 75 mM acetate buffer (pH 4.5–5.5), 75 mM Mes buffer (pH 5.5–6.5), 75 mM Bis–Tris–propane buffer (pH 6.5–9.5), 75 mM Tris/HCl buffer (pH 7.0–8.5), 75 mM Caps buffer (pH 9.5–11). The enzyme (1 μM) was incubated with the substrate in the corresponding buffer and assayed as described above.

3. Results

3.1. Production of AtsPLA$_2$-α

Recombinant AtsPLA$_2$-α was obtained by expression in *E. coli*, renaturation from inclusion bodies and purification by anion-exchange chromatography as described in Mansfeld et al. (2006). The enzyme could also be purified by reversed-phase chromatography on a C_3 column without great loss of activity. The enzyme was homogeneous in SDS-PAGE (Fig. 1) and showed the correct mass (14,299.6 kDa, expected 14,299.9 kDa) in mass spectrometry.

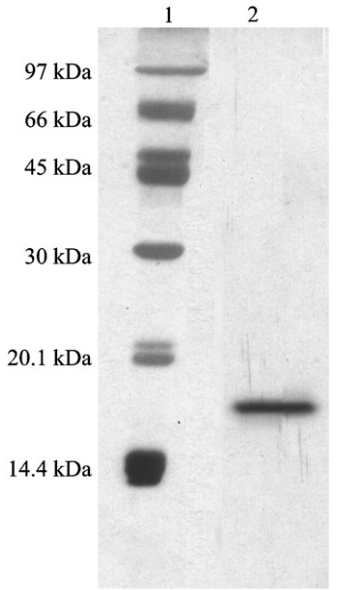

Fig. 1. SDS-PAGE analysis of purified AtsPLA$_2$-α. Lane 1 shows the molecular weight marker and lane 2 the purified enzyme.

3.2. Temperature optimum

The temperature optimum of AtsPLA$_2$-α, determined with mixed micelles of DOPC, Triton X-100, and SDS as substrate, was between 30 and 40 °C (Fig. 2A).

3.3. pH optimum

AtsPLA$_2$-α has no activity toward mixed micelles of DOPC, Triton X-100, and SDS in the pH range below 6.5 in the presence of 10 mM $CaCl_2$. The pH optimum is between pH 8.5 and 9 (Fig. 2B).

3.4. The effect of Ca^{2+} ions

AtsPLA$_2$-α requires millimolar amounts of Ca^{2+} ions and is not active in the absence of Ca^{2+} ions. Enzyme activity increases up to 10 mM $CaCl_2$ and reaches saturation at this concentration (Fig. 3A). The K_D value was determined to be 0.42 ± 0.06 mM. Calcium ions (10 mM) protect the enzyme partially from alkylation by 2 mM *p*-bromophenacylbromide (Fig. 3B). The inactivation progress curves can be fitted by a double-exponential decay function with half-lives of <1 and 142 min in the absence of calcium ions and 41 and 827 min in the presence of calcium ions. The fractional amplitudes for the two phases were 0.52 and 0.48 in the presence of $CaCl_2$; 0.49 and 0.52 in the absence of $CaCl_2$.

3.5. Activity of AtsPLA$_2$-α toward phospholipids with different head groups

As depicted in Table 1, zwitterionic PC with oleoyl groups in *sn-1* and *sn-2* position, DOPC, is cleaved with approximately the same rate as the corresponding phosphatidylethanolamine (PE) when the substrate was prepared as mixed micelles under standard assay conditions. The corresponding anionic 1,2-dioleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (DOPG), was converted with a 40.7% lower activity. AtsPLA$_2$-α did not accept phosphatidylserine (PS) and PA as substrate but it hydrolysed phosphatidylinositol (PI), with 17.2% activity in relation to DOPC. The absence of SDS in the mixed micelles did not change activities significantly (not shown) and also the variation of the molar ratio of Triton X-100 to phospholipid from 2:1 to 4:1 or 16:1 did not modify the observed trends. Selected analyses according to the concept of surface dilution kinetics (Carman et al., 1995) showed that neither V_{max} nor $K_{M(app)}$ are dramatically changed by the Triton X-100 content. Thus, V_{max} for DOPC was 26.7, 29.8, and 27.7 μmol min^{-1} mg^{-1} in mixed micelles with Triton X-100/DOPC ratios of 2:1, 4:1, and 16:1,

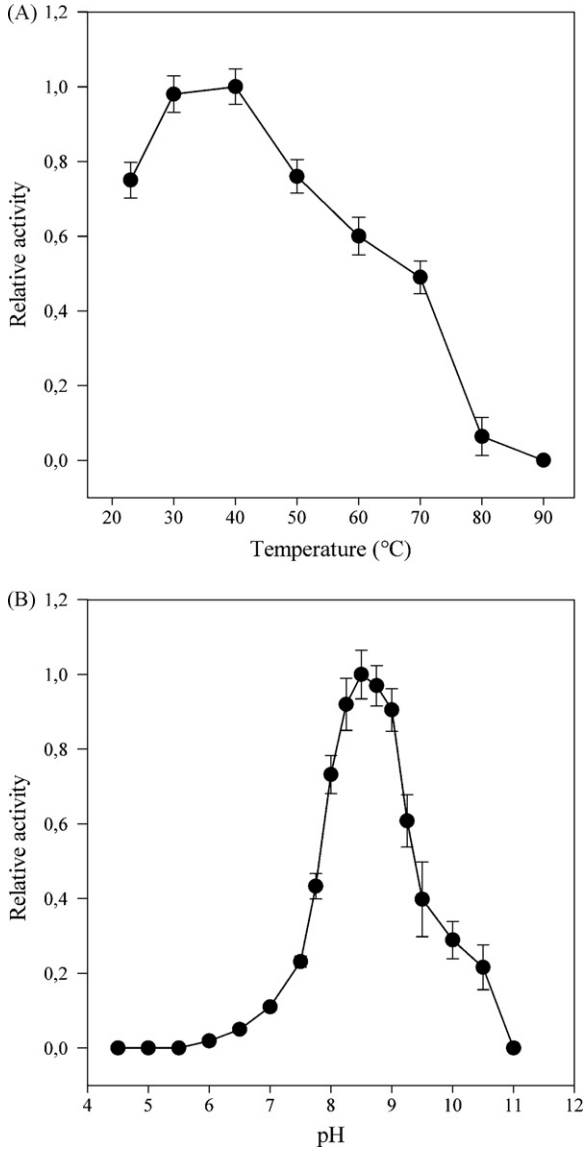

Fig. 2. (A) Temperature optimum of AtsPLA$_2$-α. The enzyme was incubated with mixed micelles of 12.7 mM DOPC/25 mM Triton X-100 and 4.8 mM SDS in 0.3 M Tris/HCl buffer, pH 8.5, 10 mM $CaCl_2$, and the amount of released product was determined as described in Section 2. Initial rates were determined and related to the maximum activity measured at 40 °C (17.1 μmol min^{-1} mg^{-1}). (B) pH optimum of AtsPLA$_2$-α. The enzyme was incubated with mixed micelles of 12.7 mM DOPC/25 mM Triton X-100 and 4.8 mM SDS in the corresponding buffer, containing 10 mM $CaCl_2$, at 37 °C and the amount of released product was determined as described in Section 2. Initial rates were determined and related to the maximum activity measured at pH 8.5 (16.7 μmol min^{-1} mg^{-1}).

while the corresponding K_M^{app} values were 5.4, 5.7, and 3.1 mM.

Activities toward phospholipids with different head groups were also determined in detergent-free SUVs.

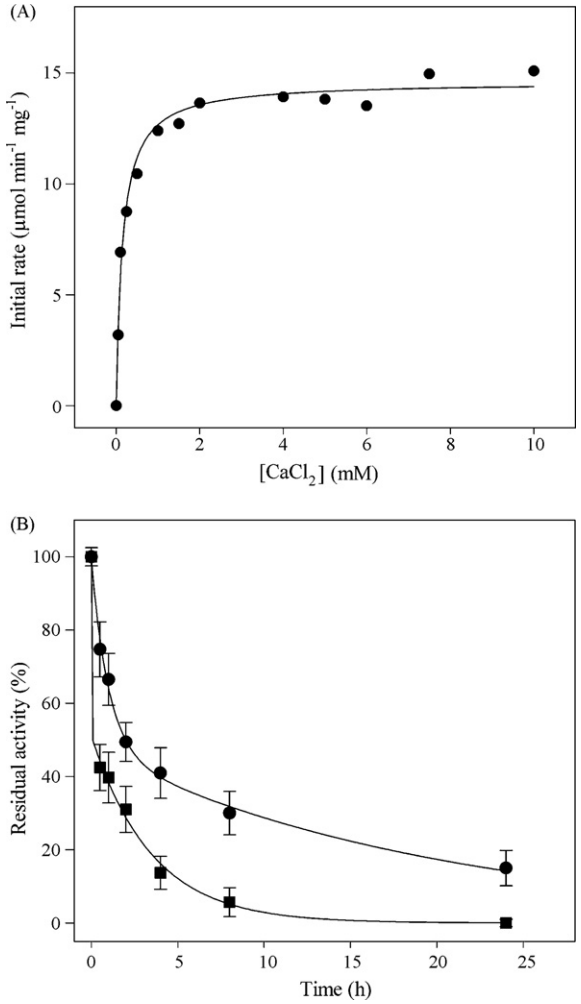

Fig. 3. (A) Hydrolytic activity of AtsPLA$_2$-α as a function of Ca^{2+} ion concentration. The enzyme was incubated with mixed micelles of 12.7 mM DOPC/25 mM Triton X-100 and 4.8 mM SDS in 50 mM Tris/HCl buffer, pH 8.5, containing varying amounts of $CaCl_2$ as described in Section 2. The curve was fitted by a hyperbolic function. (B) Inactivation of AtsPLA$_2$-α by 2 mM p-bromophenacylbromide [in the absence (■) and presence of 10 mM $CaCl_2$ (●)]. The enzyme was incubated with 2 mM *p*-bromophenacylbromide in 50 mM Tris/HCl buffer, pH 8.5 [without or with 10 mM $CaCl_2$]. Residual activities were determined using the routine activity assay. The curves were fitted by a double-exponential decay function.

These values showed the same trends concerning the head group preferences as the results obtained with mixed micelles. Thus, oleic acid was released from phosphatidylglycerol with 58% of the activity toward PC. The pure anionic PS could also not be converted when used as SUVs, and PA was not attacked, too. Surprisingly, however, the use of substrates containing an equimolar mixture of PC and PS resulted in an increased activity (1.3-fold) compared to PC alone. The

Table 1
Specific activities of AtsPLA$_2$-α toward glycerophospholipids with different head groups

Substrate	Activity (μmol min^{-1} mg^{-1})
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	16.7 ± 1.1
1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine[a]	15.3 ± 1.6
1,2-Dioleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol)	9.9 ± 0.5
1,2-Dioleoyl-*sn*-glycero-3-phosphoserine	0.0 ± 0.2
1,2-Diacyl-*sn*-glycero-3-phosphocholine from soybean	11.6 ± 1.4
1,2-Diacyl-*sn*-glycero-3-phospho-(1-D-myo-inositol) from soybean	2.0 ± 0.3
1,2-Diacyl-*sn*-glycero-3-phosphate from soybean	0.0 ± 0.2

Activities were determined in 0.3 M Tris/HCl, pH 8.5, 10 mM $CaCl_2$, using mixed micelles of the corresponding phospholipid (12.7 mM) with 25 mM Triton X-100 and 4.8 mM SDS as described in Section 2. The standard deviation was determined from at least 3 independent assays.
[a] Triton X-100 concentration was 50 mM due to poor solubility of the substrate.

activity increase observed with equimolar amounts of PC and PG in the SUVs was less pronounced (1.1-fold), whereas mixing of PC and PE had no effect in comparison to the pure phospholipids. Because of the activating effect of the anionic PS on PC hydrolysis, also the anionic PA was included into the measurements. In Fig. 4, the activation effects of PG, being itself a good substrate, and PA, being no substrate of AtsPLA$_2$α, are compared as function of their percentages in PC-SUVs. In both cases, reaction rates were increased considerably up to a maximum mole-percentage of 12.5%. At higher fractions of PG or PA, activities decreased again. In all cases, the reaction progress curves for the hydrolysis of PC showed no latency phase.

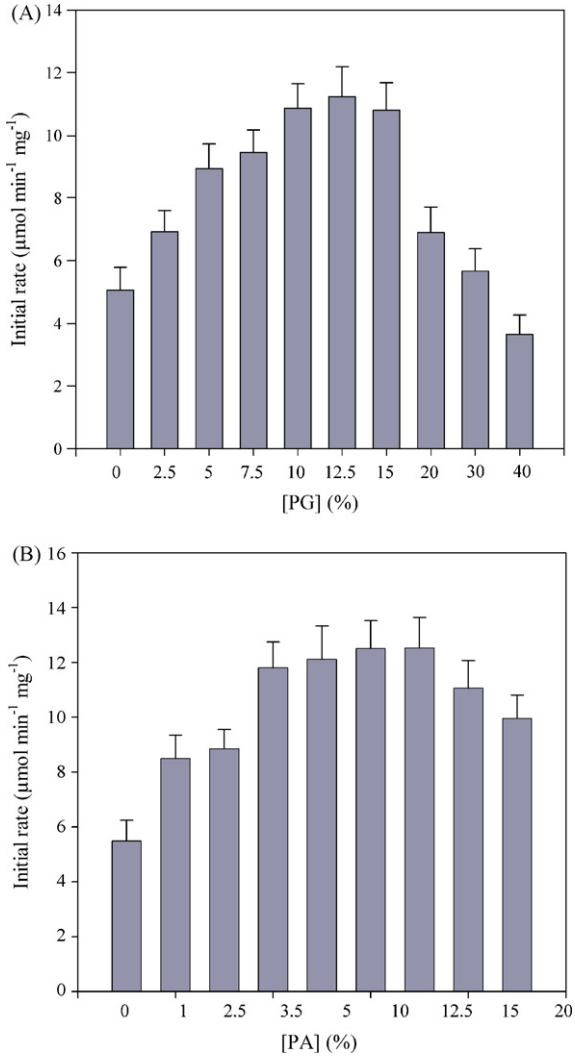

Fig. 4. Influence of anionic phospholipids on activity of AtsPLA$_2$-α toward PC in form of SUVs. (A) Influence of PG percentage in substrate preparation. Varying mol% of PG and PC were obtained by using appropriate amounts of the corresponding stock solutions. The phospholipid concentration in the assay was 12.7 mM. Activities were determined as described in Section 2. (B) Influence of PA percentage. Varying mol% of PA and PC were obtained by using appropriate amounts of the corresponding stock solutions. The phospholipid concentration in the assay was 12.7 mM. Activities were determined as described in Section 2.

3.6. Activity of AtsPLA$_2$-α toward phosphatidylcholine with different acyl chains

The specific activities of AtsPLA$_2$-α toward mixed micelles of PCs with homogeneous and heterogeneous fatty acid composition and with different chain lengths of the fatty acids (C_6–C_{22}) in *sn-1* and *sn-2* position have been determined (Table 2). When palmitic acid chains were present in *sn-1* position, AtsPLA$_2$-α preferred in *sn-2* position fatty acid residues with the sequence linoleoyl > palmitoyl > oleoyl (Table 2). PC with arachidonic acid in *sn-2* position was a very poor substrate (Table 2) and also in PE arachidonic acid could not be released (results not shown). With oleoyl chains in *sn-1* position, no differences were found between PCs with palmitoyl, stearoyl and oleoyl groups in *sn-2* position (Table 2). For PCs with identical acyl chains in *sn-1*

Table 2
Specific activities of AtsPLA$_2$-α toward different glycerophosphocholines in form of mixed micelles

Substrates with palmitoyl chains in *sn-1* position	Activity (μmol min^{-1} mg^{-1})
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	16.5 ± 1.4
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine	20.6 ± 0.7
1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine	25.3 ± 1.6
1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine	0.04 ± 0.03
Substrates with oleoyl chains in *sn-1* position	
1-Oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine	18.0 ± 0.6
1-Oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine	17.8 ± 0.9
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	16.7 ± 1.4
Substrates with identical acyl chains in *sn-1* and *sn-2* position	
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	16.7 ± 1.4
1,2-Dilinoleoyl *sn*-glycero-3-phosphocholine	19.5 ± 1.1
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine	20.6 ± 0.7
1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	27.3 ± 1.2
1,2-Dilauroyl-*sn*-glycero-3-phosphocholine	29.3 ± 1.4
1,2-Didecanoyl-*sn*-glycero-3-phosphocholine	35.5 ± 1.7
1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine	14.4 ± 0.8
1,2-diheptanoyl-*sn*-glycero-3-phosphocholine	5.9 ± 0.2
1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine	1.1 ± 0.03

Activities were determined in 0.3 M Tris/HCl, pH 8.5, 10 mM $CaCl_2$, using mixed micelles of the corresponding phospholipid (12.7 mM) with 25 mM Triton X-100 and 4.8 mM SDS as described in Section 2. The standard deviation was determined from at least three independent assays.

and *sn-2* position, a strong dependence on the chain length of the fatty acids was found (Table 2). Maximal activity of AtsPLA$_2$-α was found for PC with decanoic acid showing that 10 carbon atoms in the acyl chains are required for optimal activity. With increasing chain length, activity decreases significantly. Activities toward substrates with short acyl chains, such as hexanoyl, heptanoyl, and octanoyl chains were considerably lower than those toward substrates with longer acyl chains (Table 2).

3.7. Kinetic constants for substrates with short acyl chains in sn-1 and sn-2 position

Short chain phospholipids have found wide application as substrates for sPLA$_2$s (De Haas et al., 1971) because their critical micelle concentration is in the millimolar range in contrast to CMCs in the subnanomolar range for phospholipids with long acyl chains (e.g. 10^{-10} M for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Smith and Tanford, 1972)). Therefore, they can also be used as momomers and do not need surfactants to form micelles. Hence, we studied the activity of AtsPLA$_2$-α toward 1,2-dioctanoyl-, 1,2-diheptanoyl-, and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine as a function of their concentrations.

In the case of 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, a significant increase of activity was observed, when the CMC (10 mM, Table 3) was reached showing a preference of the enzyme toward the substrate in micellar form (Fig. 5). The complex kinetics can be described by the sum of two hyperbolic functions, below and above the CMC, yielding two apparent k_{cat} and K_M values (Table 3). Addition of NaCl, which drastically lowers the CMC (Berg et al., 1997) resulted in a significant change of the kinetics which could be described by a normal Michaelis–Menten fit (Fig. 5, Table 3).

In contrast, the initial rate of the hydrolysis of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine by AtsPLA$_2$-α as function of its concentration showed a normal Michaelis–Menten kinetics even in the absence of NaCl and was not influenced by the addition of 2 M NaCl. Table 3 shows the corresponding catalytic parameters and CMCs.

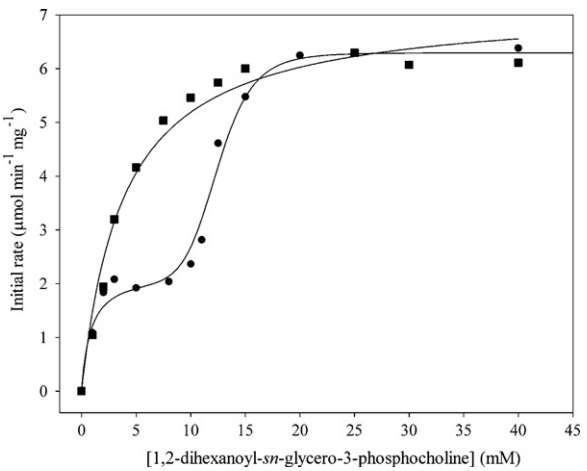

Fig. 5. Initial rates of the hydrolysis of 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine by AtsPLA$_2$-α in the absence (●) and presence of 2 M NaCl (■) as a function of the substrate concentration.

Table 3
Kinetic constants of phospholipids with short chains in *sn-1* and *sn-2* position

Substrate	CMC (mM)	k_{cat}^{app}(s^{-1})	K_m^{app}(mM)
1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine	0.17[a]	4.38 ± 0.20	0.12 ± 0.2
1,2-Diheptanoyl-*sn*-glycero-3-phosphocholine	2[b]	3.86 ± 0.18	3.92 ± 0.32
1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine	10[c]		
Below CMC		0.53 ± 0.06	0.94 ± 0.15
Above CMC		1.71 ± 0.21	13.85 ± 1.88
In 2 M NaCl		1.71 ± 0.18	3.85 ± 0.36

Kinetic constants were determined in 50 mM Tris/HCl, pH 8.5, 10 mM $CaCl_2$, as described in Section 2.
[a] Wells, 1974.
[b] Van Eijk et al., 1983.
[c] Plückthun and Dennis, 1981.

4. Discussion

AtsPLA$_2$-α (group XIB) (Lee et al., 2005; Mansfeld et al., 2006) is one of the first plant PLA$_2$s which were produced in amounts sufficient for biochemical characterisation. Despite low similarities between the amino acid sequences of AtsPLA$_2$-α and the well-known animal sPLA$_2$s, if considering the whole sequences, the cores of the proteins are similar. On the basis of the crystal structures of sPLA$_2$ from bovine or porcine pancreas (group I) and bee venom (group III), we have shown by homology modelling that the catalytic region with its two α-helices stabilised by two disulphide bonds as well as the Ca^{2+}-binding loop of AtsPLA$_2$-α are widely congruent with the corresponding regions in the animal sPLA$_2$s (Mansfeld et al., 2006). Interestingly, however, the catalytic His–Asp dyad typical of PLA$_2$ from animal sources does not exist in AtsPLA$_2$-α and other plant PLA$_2$s. In AtsPLA$_2$-α the Asp residue of the dyad is replaced by a Ser residue which is obviously involved in the catalytic mechanism (Mansfeld et al., 2006). However, the Ser residue does not represent a nucleophile in the sense of cytosolic PLA$_2$s and patatins because it is not part of a GXSXG(S) consensus sequence being typical of serine hydrolases. In the present study, we have focussed on a screening of similarities and differences in the enzymatic properties, particularly in substrate preferences, of AtsPLA$_2$-α and the well-known animal sPLA$_2$s. In nature, plant and animal sPLA$_2$s meet very different membrane compositions and fulfil different physiological functions. Hence, remarkable differences were expected.

Because phospholipases act on interfaces, their kinetic characterisation is not trivial. As the activity of PLA$_2$s strongly depends on the supramolecular structure of the substrate aggregates including charge and molecular dimensions of the phospholipids and surfactants used for micelle formation, apparent kinetic parameters are obtained, which are the result of the interplay of interface recognition and active-site specificity. Several approaches such as the model of the surface dilution kinetics by the group of Dennis (Carman et al., 1995) were developed to separate effects of the physical characteristics of the interface from true effects of enzyme kinetics. Although most of the results presented here are restricted to measurements of apparent activities and renounce a laborious accurate kinetic analysis, several general conclusions can be drawn.

4.1. Functional parameters

The temperature and pH optima of AtsPLA$_2$-α being between 30 and 40 °C and pH 8.5 and 9, respectively, under standard assay conditions (Fig. 2) show no striking differences neither in comparison with other plant sPLA$_2$s nor with animal sPLA$_2$s. The enzymes from *N. tabacum* (Fujikawa et al., 2005) and elm (Stahl et al., 1999) had pH optima in the more alkaline range (8–10 and 8–9, respectively) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as substrates, while the highest activity of the enzyme from *N. tabacum* was found between pH 6 and 7 with the poorer substrate POPG (Fujikawa et al., 2005). Similarly, the pH optima of sPLA$_2$s from porcine pancreas (de Haas et al., 1968) or bee venom (Shipolini et al., 1971) are reported to be around 8.0.

Temperature optima of sPLA$_2$s are seldom reported. In our laboratory, a similar temperature optimum (30–40 °C) was found for recombinant sPLA$_2$ from bee venom (Markert et al., 2007).

Like most sPLA$_2$s from animal sources, AtsPLA$_2$-α requires calcium ions in the millimolar range and is inactive in the absence of calcium ions (Fig. 3A). The

homology model (Mansfeld et al., 2006) shows that the calcium-binding loop in AtsPLA$_2$-α has a structure similar to sPLA$_2$s from animal sources. Correspondingly, the K_D value of AtsPLA$_2$-α for the dissociation of calcium ions (0.42 mM) is in the range of the K_D values of sPLA$_2$s from bovine (0.15 mM, Huang et al., 1996) and porcine (0.35 mM, Yu et al., 1993) pancreas. Like in most sPLA$_2$s from animal sources (Bayburt et al., 1993, Jain et al., 1991b), calcium ions are able to protect the enzyme from inactivation by *p*-bromophenacylbromide. Interestingly, AtsPLA$_2$-α contains only two histidine residues (His9 in the N-terminal region, His62 in the active site). The two-step inactivation (Fig. 3B) and the corresponding fractional amplitudes of about 0.5 each suggest that the fast step (half-life <1 min), which is decelerated by a factor of more than two orders of magnitude in the presence of Ca^{2+} ions, results from the alkylation of the catalytic His62, while the slower step (half-life 41 min) is caused by alkylation of His9 influencing the activity to a lower degree.

4.2. Head group preferences

The use of different substrate forms (mixed micelles containing Triton X-100 of different concentrations or in the presence of SDS; SUVs) allows to extract general trends for the acceptance of phospholipid head groups by AtsPLA$_2$-α. The results show that zwitterionic PCs are the best substrates (Table 1), independent of the aggregate type. Zwitterionic PEs are accepted as well, while AtsPLA$_2$-α shows a lower activity toward PG, a still lower activity to PI, and no activity to PS and PA, all of them being anionic. These trends were independent of the addition of neutral or anionic surfactants. Therefore, the results suggest that the active-site specificity plays a greater role than interfacial recognition. Moreover, the results lead to the presumption that the head group specificity is linked to the biological function of AtsPLA$_2$-α. Thus, the acceptance of PI as substrate might imply a possible involvement of AtsPLA$_2$-α in signal transduction pathways. On the other hand, the preference of PC is plausible because PC is the most abundant phospholipid in plants (http://www.lipidlibrary.co.uk).

Comparable studies on other plant PLA$_2$s, as far as available, showed similar results. Thus, recombinant sPLA$_2$ from *N. tabacum* was shown to prefer PC over PG and PE in mixed micelles with sodium cholate (Fujikawa et al., 2005) while a slight preference of PE over PC for AtsPLA$_2$-α and -β in mixed micelles with Triton X-100 was reported by Lee et al. (2005).

In contrast, the pancreatic PLA$_2$s analysed so far are characterised by a tighter binding to anionic phospholipids than to zwitterionic ones (Jain et al., 1991a, Tatulian, 2001, Pande et al., 2006). This might reflect the evolutionary adaptation of the pancreatic enzymes to the micromilieu in the digestive system where they mainly degrade phospholipids codispersed with bile salts.

PA which was not hydrolysed by AtsPLA$_2$-α and other anionic phospholipids such as PG or PS enhanced the initial reaction rates of hydrolysis of zwitterionic phospholipids (Fig. 4). However, enhancement was less pronounced for AtsPLA$_2$-α (2.3 with PA, 2.2 with PG, and 1.3-fold with PS, in comparison to 50-fold with PA for porcine pancreatic sPLA$_2$, Ghomashchi et al., 1991). The reaction progress curves for the hydrolysis of the zwitterionic PC showed no latency phase as observed with the enzymes from bee venom (Upreti and Jain, 1978), porcine pancreas (Apitz-Castro et al., 1982), and the human non-pancreatic enzyme (Bayburt et al., 1993). Altogether, these results point to a weaker electrostatic component of membrane binding of AtsPLA$_2$-α in comparison to most enzymes from animal sources.

4.3. Fatty acid preference

AtsPLA$_2$-α has been shown to release fatty acids in *sn-2* position while the *sn-1* ester bond in glycerophospholipids is not attacked (Mansfeld et al., 2006). With respect to the acyl chain specificity, however, the type of fatty acid in *sn-1* position was found to be important. With palmitic acid chains in *sn-1* position, a preference of linoleoyl over palmitoyl and palmitoyl over oleoyl chains for the *sn-2* position was found, whereas no differences were observed when an oleic acid chain was present in *sn-1* position (Table 2). Most interestingly, PCs or PEs with arachidonic acid in *sn-2* position which are extremely rare in plants (arachidonic acid is found for instance in mosses, ferns, liverworts, but in higher plants it is only known for *Agathis robusta* (http://www.lipidlibrary.co.uk)) were not cleaved. These results are in strong contrast to the fatty acid specificity of animal sPLA$_2$s, which easily hydrolyse arachidonic acid containing phospholipids, and suggest an evolutionary adaptation of enzyme specificity to the natural phospholipid composition. Phospholipids in *A. thaliana* contain mainly fatty acid chains with 16 carbon atoms in *sn-1* and preferably unsaturated fatty acid chains with 18 carbon atoms in *sn-2* position (linoleic and linolenic acid). Phospholipids with identical fatty acid chains in *sn-1* and *sn-2* position are also good substrates. Comparable to pancreatic PLA$_2$s which preferentially hydrolyse substrates with nine carbon atoms in the acyl chains (Pattus et al., 1979), AtsPLA$_2$-α has an optimum activity toward substrates with 10 carbon atoms in the acyl chains (Table 2).

4.4. Interfacial activation on short chain phospholipids

With AtsPLA$_2$-α, an activation above the CMC as typical for sPLA$_2$s could be obtained for 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (Fig. 5, Table 3). However, the activation was abolished by the addition of 2 M NaCl which decreases the CMC (Berg et al., 1997). Normal hyperbolic curves were obtained for 1,2-dioctanoyl- and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine. In contrast, with porcine pancreatic and cobra venom PLA$_2$, dramatic activity increases were observed when the concentration of zwitterionic substrates reached the CMC and aggregation into micelles occurred (Pieterson et al., 1974). Obviously, AtsPLA$_2$-α is less influenced by interfacial activation than these enzymes. Similarly, also sPLA$_2$ from bee venom is less sensitive toward the aggregation state of the substrate (Lin et al., 1988).

The comparison of the catalytic constants of AtsPLA$_2$-α for the short chain substrates (Table 3) with those of the animal sPLA$_2$s (references in Noel et al., 1991) shows that the k_{cat}^{app} values are lower for the plant sPLA$_2$ by more than 2 orders of magnitude, while the K_m^{app} values (e. g. 0.18–3.2 mM for 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine with animal PLA$_2$s in comparison to 0.12 mM with AtsPLA$_2$-α) are less different. Therefore, the low activity of AtsPLA$_2$-α seems to be mainly caused by a lower rate of the catalytic step. As concluded from the homology model for AtsPLA$_2$-α (Mansfeld et al., 2006), the replacement of the Asp residue in the common catalytic His–Asp dyad by a Ser residue might explain the lower catalytic efficiency in comparison to the enzymes from animal sources.

4.5. Concluding remarks

Although not based on an explicit kinetic analysis, the results of this study suggest that – in accordance to the similar core structures of AtsPLA$_2$-α and animal PLA$_2$s – these enzymes have many common features such as optimum conditions for pH, temperature and calcium-binding. However, there are obviously differences in the substrate preference with respect to the head group and the acyl chains of the glycerophospholipids. The deviations of AtsPLA$_2$-α correlate with the occurrence of the corresponding structures in plant phospholipids. Obviously, starting from the basic scaffold the enzyme was adapted by nature to meet the requirements of turnover and regulation. Accordingly, phospholipids with arachidonoyl residues in *sn-2* position are not cleaved by AtsPLA$_2$-α, whereas linoleic acid chains and presumably also linolenic acid chains are the preferred fatty acids in *sn-2* position of PC. The less abundant anionic phospholipids seem to be poorer substrates than the most abundant PC. Also the interfacial activation and the catalytic efficiency is presumably adapted to the enzyme function in plants.

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