Purification, characterization, molecular cloning and extracellular production of a phospholipase A₁ from Streptomyces albidoflavus NA297

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A R T I C L E   I N F O
Article history:
Received 8 September 2012
Received in revised form 25 September 2012
Accepted 25 September 2012

Keywords:
Streptomyces albidoflavus
Phospholipase A₁
Purification
Characterization
Expression

A B S T R A C T
A novel metal ion-independent phospholipase A₁ from Streptomyces albidoflavus isolated from Japanese soil has been purified and characterized. The enzyme consists of a 33-residue N-terminal signal secretion sequence and a 269-residue mature protein with a deduced molecular weight of 27,199. Efficient and extracellular production of the recombinant enzyme was successfully achieved using Streptomyces lividans cells and an expression vector. A large amount (25 mg protein, 14.7 kIU) of recombinant enzyme with high specific activity (588 U/mg protein) was purified by simple purification steps. The maximum activity was found at pH 7.2 and 50 °C. At pH 7.2, the enzyme preferably hydrolyzed phosphatidic acid and phosphatidylserine; however, the substrate specificity was dependent on the reaction pH. The enzyme hydrolyzed lysophosphatidylcholine and not triglyceride and the p-nitrophenyl ester of fatty acids. At the reaction equilibrium, the molar ratio of released free fatty acids (sn-1:sn-2) was 63:37. The hydrolysis of phosphatidic acid at 50 °C and pH 7.2 gave apparent Vₘₐₓ and Kₘ values of 1389 μmol min⁻¹ mg protein⁻¹ and 630 s⁻¹, respectively. The apparent Kₘ and kcat/Kₘ values were 2.38 mM and 265 mM⁻¹ s⁻¹, respectively. Mutagenesis analysis showed that Ser11 is essential for the catalytic function of the enzyme and the active site may include residues Ser216 and His218.

1. Introduction

Phospholipase A₁ (PLA₁) [EC 3.1.1.32] and A₂ (PLA₂) [EC 3.1.1.4] (PLAs) cleave glycerophospholipids into lysophospholipid and free fatty acids (FFAs). They are classified as PLA₁ or PLA₂ based on whether they cleave the sn-1 or sn-2 of FFAs, respectively. PLAs exist in various organisms, including microorganisms, snakes, bees, plants and mammals. Numerous PLAs have been identified and characterized (BRENDA database, http://www.brenda-enzymes.info/php/result_flat.php?ecno=3.1.1.32). PLAs are further divided into groups based on attributes including cellular location, calcium dependence and active site residues. PLAs appear to be essential components of bee and snake venoms. These enzymes were obtained primarily from bee and snake venoms or the porcine pancreas. Several PLAs have been found in microorganisms: PLA₁s from Aspergillus oryzae [1], Streptomyces platensis sp. [2] and Escherichia coli [3], and PLA₂s from E. coli [4], Streptomyces violaceoruber [5] and Pseudomonas aeruginosa [6]. Both PLAs of E. coli are membrane-bound enzymes. PLAs are metal ion-dependent enzyme. There is only one report describing a calcium-independent PLA₂ from the P388D1 macrophage-like cell line [7]. Besides A. oryzae PLA₁ and S. violaceoruber PLA₂, large-scale recombinant production of PLA₁ has not been developed, and its crystal structure and the catalytic mechanism have not been elucidated.

Here we report purification, characterization, gene cloning, and expression of a novel metal ion-independent PLA₁ from Streptomyces albidoflavus. We describe the kinetics for the hydrolytic reaction, substrate specificity and the positional specific hydrolysis of glycerophospholipids. Moreover, a predictive active site is discussed on the basis of a mutagenesis analysis.

Enzymes : phospholipase A₁ [EC 3.1.1.32]
Abbreviations: PLA₁, phospholipase A₁; PLA₂, phospholipase A₂; PLD, phospholipase D; SaPLA₁, phospholipase A₁ from Streptomyces albidoflavus; ECPLA, phospholipase A₁ from Escherichia coli; SaPLA₁, phospholipase A₁ from Serratia sp. y1; SMPLA, phospholipase A₁ from Serratia sp. MK1; SaExt, esterase of Streptomyces albus J1074; SoExt, esterase from S. scabies; CV, column volume; DLS, dynamic light scattering; TSB, tryptic soy broth; SBL, lecithin from soybean; EGGL, lecithin from egg yolk; PC, l-α-phosphatidylcholine; PS, l-α-phosphatidylinositol; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DMPA, 1,2-Dimyrystoyl-sn-glycero-3-phosphoethanolamine; DPPC, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; POPC, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPA, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine; POPS, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine; POPG, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; POPC, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phospho-rac(1-glycerol); LPC, 1-α-Lyso phosphatidylcholine; pNBP, p-nitrophenyl butyrate; pNPD, p-nitrophenyl dodecanoate; pNPL, p-nitrophenyl laurate; pNPP, p-nitrophenyl palmitate; pNPS, p-nitrophenyl steарате; FFA, free fatty acid

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http://dx.doi.org/10.1016/j.fob.2012.09.006
2. Results

2.1. Isolation of Streptomyces albidoflavus

Strain NA297 from a soil sample of Fukushima, Japan was assigned as *S. albidoflavus* by morphological, physiological and biochemical characterizations, as well as 16S rDNA sequence analysis. *S. albidoflavus* NA297 was deposited as NITE BP-1014 in the NPMD (Chiba, Japan).

2.2. Purification of PLA1 from *S. albidoflavus*

The enzyme was purified to electrophoretic homogeneity from the culture supernatant by ammonium sulfate precipitation, hydrophobic interaction chromatography and anion exchange chromatography. A summary of the purification of PLA1 is shown in Table 1. The purified PLA1 with a specific activity of 2873 U/mg-protein was obtained, and the total pure protein amount was 8.84 ng. The purified enzyme was subjected to SDS–PAGE analysis. A single band with an apparent molecular mass of ∼28 kDa was visualized by CBB staining (Fig. 1).

2.3. Properties of PLA1

We have examined the pH and temperature profile, effect of chemicals and inhibitors, and substrate specificity of the purified PLA1. As shown in Fig. 2, the enzyme exhibited a wide range of pH activity (5–8). The maximum activity was found at pH 7.2 and 50 °C (Fig. 2(A) and (B)). The apparent activation energy (Ea) for EGGL hydrolysis by the wild-type enzyme was 18.8 kJ mol⁻¹ in the reaction buffer of pH 5.6 (data not shown). The wild-type and recombinant enzyme was stable between pH 7.2 and 9 or pH 5.6 and 9, respectively (Fig. 2(C)), and at 40 °C (Fig. 2(D)). Table 2 summarizes the effects of the chemicals on the purified PLA1 activity against EGGL as the substrate. The enzyme activity was inhibited by Fe²⁺ and Fe³⁺ ions, >0.1 M Ca²⁺ ions and SDS; however, the enzyme was not inhibited by EDTA and DTT. Weak inhibition was observed for 2-mercaptoethanol, PMSF and >0.23% (wt/vol) Triton X-100. The effect of Triton X-100 concentration on the activity was investigated. As shown in Fig. 3(A), the enzyme activity was a minimum at 0.5% (wt/vol) Triton X-100 for the standard assay mixture. At pH 5.6, the enzyme exhibited the highest hydrolytic activity against PI, crude SBPC and SBL (Fig. 4). On the other hand, at pH 9, PS and PG were the preferred substrate over PC, especially crude PC.
### Table 1
Purification of PLA1 from *S. albidoflavus* NA297.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity a (U/ml)</th>
<th>Sample vol. (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55-h culture supernatant</td>
<td>1.51</td>
<td>355</td>
<td>1.06</td>
<td>377</td>
<td>1.42</td>
<td>536</td>
<td>100</td>
</tr>
<tr>
<td>80% ammonium sulfate</td>
<td>2.67</td>
<td>130</td>
<td>1.17</td>
<td>152</td>
<td>2.28</td>
<td>347</td>
<td>64.8</td>
</tr>
<tr>
<td>Phenyl-650M</td>
<td>4.78</td>
<td>52.8</td>
<td>0.257</td>
<td>13.6</td>
<td>18.6</td>
<td>252</td>
<td>47.1</td>
</tr>
<tr>
<td>HiTrap SP HP</td>
<td>9.13</td>
<td>11.4</td>
<td>7.20 × 10⁻³</td>
<td>82.1 × 10⁻³</td>
<td>1268</td>
<td>104</td>
<td>19.4</td>
</tr>
<tr>
<td>HiTrap Q HP</td>
<td>3.74</td>
<td>6.80</td>
<td>1.33 × 10⁻³</td>
<td>8.84 × 10⁻³</td>
<td>2873</td>
<td>25.4</td>
<td>4.74</td>
</tr>
</tbody>
</table>

a PLA1 activity was assayed using the reaction mixture containing 0.1 M Tris–HCl buffer (pH 8.0), 2.5% (wt/vol) EGGL, 0.005% (wt/vol) Triton X-100 and 25 mM EDTA at 37 °C.

### Table 2
Effect of various chemicals on the PLA1 activity for egg yolk lecithin (EGGL) hydrolysis.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Relative activity (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA free</td>
<td>100</td>
</tr>
<tr>
<td>25 mM EDTA</td>
<td>108</td>
</tr>
<tr>
<td>50 mM EDTA</td>
<td>100</td>
</tr>
<tr>
<td>75 mM EDTA</td>
<td>84.8</td>
</tr>
<tr>
<td>100 mM EDTA</td>
<td>85.2</td>
</tr>
<tr>
<td>10 mM CaCl₂</td>
<td>82.6</td>
</tr>
<tr>
<td>100 mM CaCl₂</td>
<td>45.2</td>
</tr>
<tr>
<td>200 mM CaCl₂</td>
<td>22.6</td>
</tr>
<tr>
<td>10 mM CoCl₂</td>
<td>104</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>109</td>
</tr>
<tr>
<td>10 mM MnCl₂</td>
<td>123</td>
</tr>
<tr>
<td>10 mM ZnCl₂</td>
<td>83.0</td>
</tr>
<tr>
<td>10 mM FeCl₂</td>
<td>10.7</td>
</tr>
<tr>
<td>10 mM FeCl₃</td>
<td>41.1</td>
</tr>
<tr>
<td>2 mM 2-mercaptoethanol</td>
<td>65.8</td>
</tr>
<tr>
<td>2 mM dithiothreitol</td>
<td>100</td>
</tr>
<tr>
<td>2 mM PMSF</td>
<td>78.0</td>
</tr>
<tr>
<td>2 mM sodium dodecyl sulfate</td>
<td>11.7</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>99.5</td>
</tr>
<tr>
<td>0.23% Triton X-100</td>
<td>59.2</td>
</tr>
<tr>
<td>1% Triton X-100</td>
<td>55.7</td>
</tr>
</tbody>
</table>

b The purified enzyme was assayed under standard assay conditions, 0.1 M sodium acetate buffer (pH 5.6), 2.5% (wt/vol) EGGL, 0.005% (wt/vol) Triton X-100 and 25 mM EDTA at 50 °C for 5 min. The enzyme was preincubated in the reaction mixture with each chemical at 50 °C for 5 min, and then assayed by incubation at 50 °C for 5 min.

c The activity was measured under the assay condition without EDTA.

d The relative activity is expressed as a percentage of the activity under the assay condition without EDTA.

### 2.4. Positional specificities of PLA1 and lysophosphocholine production

The SaPLA1 enzyme activity was detected by the EnzCheck® Phospholipase A1 assay kit; however, PLA2 activity was not detected by the Phospholipase A2 assay (data not shown). These results suggest that the SaPLA1 enzyme is PLA1. Gas chromatography (GC) analysis demonstrated that FFAs were released proportionally with the enzymatic reaction time from the sn-1 and sn-2 position of POPC (Fig. 5(A)). At an early reaction time point (5 min), the molar ratio of released FFAs was a sn-1/sn-2 ratio of 71.5:28.5 (Fig. 5(B)). In contrast, in the equilibrium mixture of the reaction, the molar ratio of released FFAs was a sn-1/sn-2 ratio of 63.3:37. The positional selectivity was almost equal to that of *A. oryzae* PLA1 (data not shown).

### 2.5. Cloning of the PLA1 gene

The partial nucleotide sequence of the gene encoding PLA1 (*pla*) was determined by a standard PCR using primer sets designed from the N-terminal and internal amino acid sequences. The 359-bp determined nucleotide sequence encoded a protein of 111 amino acids in length. The nucleotide sequence of the 5' upstream region of *pla* was determined by inverse PCR; however, only a few nucleotides of the 3' downstream region were determined (data not shown). The *pla* gene was then amplified using the 3' region nucleotide sequence of a secreted hydrolase of *S. albus* J1074 exhibiting 100% identity to the 359-bp determined nucleotide sequence of *S. albidoflavus*, and the
obtained PCR fragment was cloned into the pMD20 vector. Consequently, the nucleotide sequence of pla was determined from the sequence of the 1.18-kb PCR product. The ORF of pla consisted of 807 nucleotides encoding a 269-amino-acid protein with a deduced sequence of lipase (GXSXG) represented by the double underline. A putative signal sequence are indicated by rbs and the arrow, respectively. A consensus sequence of lipase (GXSXG) was found in the full ORF of pla. The molecular weight of the gene product without the signal sequence was calculated to be 27,199, which is in agreement with that of the purified enzyme estimated by SDS–PAGE and DLS analyses. The complete nucleotide sequence of pla has been deposited in the GenBank database under the accession number AB605634.

2.6. Expression, purification and characterization of PLA1

High efficiency extracellular production of S. albidoflavus PLA1 has been successfully achieved in Streptomyces lividans cells transformed with the expression vector pUC702/pla. The specific activity in the culture supernatant (46.4 U/mg) was about 30-fold higher than that (1.42 U/mg) of the wild-type strain. A large amount (25 mg-protein) of PLA1 with a high specific activity (588 U/mg) and total activity (14.7 kU) was purified to electrophoretic homogeneity from the cultured supernatant by simple purification steps (Table 3). As shown in Fig. 6, the recombinant enzyme preferably hydrolyzed POPC and PS at 50°C. The substrate specificity was in the following order: POPA, PS, PI > POPG (Fig. 7). The apparent Km values were 2.38 mM and 10.3% for POPC, POPG and POPG, respectively. As shown in Fig. 8, the enzyme exhibited much lower activities towards PI (Fig. 7(A) and (B)). For the following assay, the enzyme reaction was performed at pH 7.2 in a Tris–HCl buffer (pH 7.2). The apparent Vmax and turnover rate (kcat) were determined to be 1389 ± 138 μmol min⁻¹ mg⁻¹ protein⁻¹ and 630 s⁻¹, respectively. The apparent Km and kcat/Km values were 2.38 mM and 265 mM⁻¹ s⁻¹, respectively.

2.7. Steady-state kinetics of PLA1

Good linear regression analysis was achieved by a Lineweaver–Burk plot (Fig. 8). On the hydrolysis of POPA by the purified recombinant enzyme at 50°C and pH 7.2, the apparent Vmax and turnover rate (kcat) were determined to be 1389 ± 138 μmol min⁻¹ mg⁻¹ protein⁻¹ and 630 s⁻¹, respectively. The apparent Km and kcat/Km values were 2.38 mM and 265 mM⁻¹ s⁻¹, respectively.

2.8. Mutation analysis of PLA1

The mutants of S11A, S11D, S11E, S11T, S11Y, S216A and H218A exhibited no activity. The mutants S216D, S216E and H218R showed negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A showed about 10%–20% relative activity.
Table 3

Purification of the expressed PLA1.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (U/ml)</th>
<th>Sample vol. (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (kU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48-h culture supernatant</td>
<td>91.2</td>
<td>410</td>
<td>1.97</td>
<td>806</td>
<td>46.4</td>
<td>37.4</td>
<td>100</td>
</tr>
<tr>
<td>80% ammonium sulfate</td>
<td>328</td>
<td>80.0</td>
<td>2.79</td>
<td>223</td>
<td>118</td>
<td>26.3</td>
<td>70.3</td>
</tr>
<tr>
<td>Phenyl-650M</td>
<td>163</td>
<td>88.4</td>
<td>0.281</td>
<td>24.8</td>
<td>582</td>
<td>14.4</td>
<td>38.6</td>
</tr>
<tr>
<td>HiTrap Q HP</td>
<td>735</td>
<td>20.0</td>
<td>1.25</td>
<td>25.0</td>
<td>588</td>
<td>14.7</td>
<td>39.3</td>
</tr>
</tbody>
</table>

a PLA1 activity was assayed using the reaction mixture containing 0.1 M sodium acetate buffer (pH 5.6), 2.5% (wt/vol) EGG, 0.005% (wt/vol) Triton X-100, and 25 mM EDTA at 50 °C.

Fig. 7. Substrate specificity profiles of the purified recombinant PLA1. The enzyme activity was assayed by incubation at 50 °C for 5 min with 0.5% (wt/vol) phospholipids, soybean oil, olive oil, or 0.013% (wt/vol) pNP esters in 0.1 M Tris-HCl (pH 7.2) containing 1% (wt/vol) Triton X-100 and 25 mM EDTA or 10 mM CaCl2. Data are the average of experiments performed in triplicate. Error bars represent the standard deviation.

Table 4

Enzyme activity of the wild-type and recombinant enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
</tr>
<tr>
<td>S11A</td>
<td>0</td>
</tr>
<tr>
<td>S11E</td>
<td>0</td>
</tr>
<tr>
<td>S11T</td>
<td>0</td>
</tr>
<tr>
<td>S11Y</td>
<td>0</td>
</tr>
<tr>
<td>S216A</td>
<td>0</td>
</tr>
<tr>
<td>S216D</td>
<td>1.77</td>
</tr>
<tr>
<td>S216E</td>
<td>0.746</td>
</tr>
<tr>
<td>S216T</td>
<td>23.1</td>
</tr>
<tr>
<td>S216Y</td>
<td>8.24</td>
</tr>
<tr>
<td>H218A</td>
<td>0</td>
</tr>
<tr>
<td>H218R</td>
<td>1.31</td>
</tr>
</tbody>
</table>

a The activity was measured in the reaction mixture consisting of 0.1 M Tris- HCl buffer (pH 7.2), 2.5% (wt/vol) EGG, 0.005% (wt/vol) Triton X-100, and 25 mM EDTA at 37 °C.

b Relative activities were determined by defining the activity of the wild-type enzyme (specific activity, 55.4 U/mg-protein) as 100%.

3. Discussion

This is the first report of a PLA1 from actinomycetes. Known microbial PLA1s of A. oryzae [1], Serratia sp. [2] and E. coli [3] are calcium ion-dependent enzymes, whereas PLA1 of S. albidoflavus (SaPLA1) was a metal ion-independent enzyme. SaPLA1 was isolated to high purity and high specific activity (2873 U/mg-protein) was obtained by employing efficient purification steps. It has been reported that high specific activities of PLA1 from the venom of the social wasp Polybia paulista and recombinant PLA1 (SaPLA) from Serratia sp. xjF1 (SxPLA1) were 2898 and 202.3 U/mg-protein, respectively [8,9]. Thus, with respect to bacterial PLA1, we concluded that SaPLA1 has very high specific activity. The properties of metal ion-independent and the much higher specific activity of the enzyme from a non-pathogenic bacterium should be an advantage for industrial applications. In addition to this, we have successfully achieved the efficient extracellular production of the enzyme using S. lividans cells. SaPLA1 was inhibited in the presence of 10 mM Fe2+ and Fe3+ ions but was less sensitive to the other metal ions, suggesting that the inhibition results from the binding of Fe ions to the enzyme molecule, but not to the substrate interface. The enzyme molecule is also possibly inactivated because of metal ion-related denaturation. Iwai et al. reported that A. niger lipase was inhibited by low concentrations of Fe2+ [10]. PLA1s from
Mycobacterium phlei [11] and from Corticium centrifugum [12] were inhibited by Fe^{3+} and Fe^{2+} ions. SDS and high concentrations of Triton X-100 inhibited the enzyme activity of SaPLA₁. Moreover, the optimum concentration of Triton X-100 was dependent on the substrate molecule type, suggesting that enzyme activity could be affected with size and the form of the mixed micelle composed of the substrate and detergent.

The deduced amino acid sequence of mature SaPLA₁ exhibited 100% identity to an esterase SGNH (UniProt ID, D6BAL1) annotated in the genome of S. albus J1074. “Annotation of Streptomyces albus strain J1074.” has been submitted to the EMBL/GenBank/DDB databases (October, 2008); however, the esterase of S. albus J1074 (SaEst) was only predicted and not characterized. Moreover, SaPLA₁ exhibited no lipase and carboxylesterase activity. In addition, the deduced amino acid sequence of the mature enzyme of SaPLA₁ exhibited 68.6% and 63.4% identities to those of lipase Sct1 from S. coelicolor (Q9S2A5) and lipase SrLip from S. simulans (Q93MW7), respectively. SrLip has been reported to show lipase, phospholipase, esterase, thioesterase and Tweenase activities, and the preferred esters of the medium-chain acids (C₄-C₁₂), whereas Sct1 shows its highest activity towards a long-chain p-nitrophenyl ester (C₁₄) [13]. The sequence blocks I, II, III and V suggested to be characteristic for enzymes of the SGHN family could readily be identified in SaPLA₁. Although SrLip showed its highest activity for diheptanoyl glycerophosphocholine (1196 U/mg), it also exhibited low activities toward dioleoyl glycerophosphocholine (18 U/mg), triolein (171 U/mg) and pNPL (365 U/mg). These results indicate that substrate recognition of SaPLA₁ is essentially different from that of SrLip.

Interestingly, SaPLA₁ exhibited high activity over a broad pH range (between 5 and 8). The active pH range was similar to that of E. coli membrane-bound PLAP (EcPLA₁) [3]. The optimal pH of 5.6 for the purified native SaPLA₁ enzyme was different from that (pH 7.2) of the recombinantly expressed SaPLA₁, showing that the recombinant enzyme may be more stable than the native enzyme. This observation was supported by the results of the pH and thermal stability tests. The results of the thermal stability experiment showed that the half-life of the activities for the wild-type and recombinant enzymes were 48 and 62 °C, respectively. In addition, the recombinant enzyme maintained 100% activity over a period of 1.5 years at 4 °C in 20 mM Tris–HCl buffer (pH 9.0). Since the optimal pH of EcPLA₁ [3] and SxPLA₁ [9] are pH 8.4 and 9, respectively, these enzymes are alkaline PLA₁, whereas SaPLA₁ shows optimal activity at a more neutral pH value.

The maximum optimal temperature of activity of SaPLA₁ (i.e., 50 °C) is higher than SxPLA₁ (35 °C) [9]. However, SaPLA₁ appears to be unstable at 50 °C. Thus, the maximum temperature presumably results from physical effects such as fluidities of the substrate and the enzyme itself. That is, there is a trade-off between the catalysis of the enzyme and the thermal stability. The apparent activation energy, $E_a = 18.8$ kJ mol⁻¹, for EGGL hydrolysis by the wild-type SaPLA₁ differed to the value for the recombinant enzyme (i.e., 58.3 kJ mol⁻¹), indicating that the optimal pH may be at around pH 5.6. The $E_a$ of PLAP from cobra venom has been reported as 29.7 kJ mol⁻¹ for micelles of diheptanoyl-PC [14]. There is no report on the $E_a$ of PLAP from other organisms. It is known that when the $E_a$ changed from 28.2 to 41.9 kJ mol⁻¹, the $k_{cat}$ increased 4.5 × 10² times, indicating that SaPLA₁ has remarkably high catalytic efficiency. However, further studies are needed to fully understand the reason for such high efficiency.

The substrate specificity was affected by the reaction pH, suggesting that the specificity probably results from the ionization state of residues located in the active site as well as the ionization state of the head groups of the substrate. If the substrate specificity correlated with the ionization state of the head groups of the substrate, the enzymatic activity toward PE and DOPE or DPPC would have been similar, because the pKa value of the phosphate groups of these substrates is very similar. Therefore we conclude that it is changes in the ionization state of amino acid residues in the active site that are likely to be important in substrate specificity. On the other hand, at pH 7.2, the recombinant enzyme had a tendency to hydrolyze preferably POPA and PS. Scandella and Kornberg reported that a membrane-bound EcPLA₁ of E. coli can hydrolyze PC, PE, PG and cardiolipin at comparable rates [3]. To our knowledge, there is no report of substrate specificity of other bacterial PLA₁s. Rose and Prestwich have reported head group selectivity of PLA₂ from various organisms [15]. S. violaceoruber PLA₂ preferred the PC head group, followed by PG > PE > PS > PA. They also described that bacterial and mammalian PLA₁s, except for the venom and pancreatic enzymes, showed no or weak hydrolysis of PA. In contrast, SaPLA₁ showed the highest activity toward PA. Further studies are needed to elucidate the mechanism of head group specificity of SaPLA₁.

The apparent $K_m$ of SaPLA₁ was a somewhat higher value than those of EcPLA₁ [3], PLA₁ from Serratia sp. MK1 (SMPLA₁) [16] and SxPLA₁ [9]. The $k_{cat}$ value, 630 s⁻¹, of SaPLA₁ was much higher than that of SMPLA₁ [16]. We conclude that the binding affinity of SaPLA₁ toward the substrate is lower than those of other bacterial PLA₁s. Nevertheless the turnover rate is much higher than all known PLA₁s, as shown in the BRENDA database.

The EnzCheck® Phospholipase A₂ assay kit suggested that the SaPLA₁ enzyme is certainly not a PLA₂. However, SaPLA₁ may not be able to recognize the Phospholipase A₂ assay’s glycerophospholipid with the dye-labeled acyl chain as the substrate. GC analysis showed that SaPLA₁ hydrolyzed the sn-2 acyl ester bond as well as sn-1. The proportion of sn-2 hydrolysis by SaPLA₁ may be higher than that of other PLA₁ enzymes. The selectivity is lower than that of SMPLA₁ [2], but the analysis was carried out with non-pure enzyme. There is no evidence for the positional specificity of PLA₁ from A. oryzae [1]. GC analysis showed that the positional selectivity of PLA₁ from A. oryzae was almost equal to that of SaPLA₁. It was reported that acyl migration from the 2-position to the 1(3)-position or the opposite of diacylglycerol does occur [17]. Moreover, SaPLA₁ hydrolyzed LPC as well as diacylglycerophospholipids, suggesting that the transesterified acyl group may be hydrolyzed. However, no acyl migration would happen in our reaction time due to the low acyl migration rate. We conclude that SaPLA₁ is able to hydrolyze the sn-2 position of the acyl ester in glycerophospholipids. Further studies are needed to elucidate the positional specificity mechanism of SaPLA₁.

The results of the mutagenesis analysis showed that Ser11 is essential for the catalytic function of SaPLA₁, and the active site may be composed of S216 and H218, resembling that of SsEst (1ESC) with the active site composed of Ser14, Trp280 and His283. This postulation requires further analysis. We presently aim to determine the crystal structure to reveal structural features, metal ion-independency, the substrate binding mechanism and substrate recognition mechanism of SaPLA₁.

4. Experimental procedures

4.1. Materials

Tryptic soy broth (TSB) and BactoTryptone were from BD (NJ, USA). Lecithin (SBL) from soybean and olive oil were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Lecithin (EGGL) (L-α-phosphatidylcholine approx. 70% as phospholipids min. 99% from egg yolk, L-α-phosphatidylcholine (PC) from egg yolk (purity 98%, TLC) and soybean oil were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). L-α-phosphatidylcholine (SBPC) from soybean (Type IV-S, >30%) and L-α-phosphatidyl-L-serine (PS) from Glycine max (soybean), L-nitrophenyl butyrate (C₄) (pNPB), p-nitrophenyl octanoate (C₈) (pNPO), p-nitrophenyl decanoate (C₁₀) (pNPD), p-nitrophenyl laurate (C₁₂) (pNPL), p-nitrophenyl palmitate (C₁₆) (pNPMP), and p-nitrophenyl stearate (C₁₈) (pNPSt) were obtained...
from Sigma-Aldrich Co. LLC. (MO, USA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Dimyristoyl-sn-glycero-3-phosphate (DMPA), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphate (POPG), 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phospho-1-glycerol (POPG) and 1-Lysophosphatidylcholine (LPC) were from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). 1-Lysophosphatidylglycerol from egg (PG) was purchased from Funakoshi Co. (Tokyo, Japan). 1-L-α-Polyethyldiylinositol (Pl) from wheat ovule was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). TOYOPEARL Phenyl-650M was from Tosoh (Tokyo, Japan). HiTrap S HP and HiTrap Q HP were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). All other chemicals were of the highest grade.

4.2. Bacterial strains and culture conditions

Approximately 1500 strains were isolated from various soil samples of Fukushima, Japan using HV medium [18]. Among the isolates, strains exhibiting a clear halo on TSB plates containing lecinthin were selected [19]. Strain NA297 exhibiting high enzyme activity and good reproducible production of enzymes was selected and was identified as a strain related to S. albidoflavus based on morphological and the 16S rDNA sequence (DBD database under accession number AB738935). S. albidoflavus NA297 was deposited as NITE BP-1014 in the NITE Patent Microorganisms Depository (NPD) (Chiba, Japan).

Strain NA297 was maintained on 3% (wt/vol) TSB agar plates and kept at –80 °C as 10% (vol/vol) glycerol stocks for long-term storage. A loopful of colonies were scraped from a plate and inoculated into a test tube (18 mm, 180 mm) containing 5–ml seed medium of 3% (wt/vol) TSB. This culture was incubated with shaking (160 strokes per min) at 28 °C. After 48 h cultivation, a 1% (vol/vol) inoculum was transferred into a 500–ml flask containing 50-ml fermentation medium of 3% TSB supplemented with 1% (wt/vol) SBL and 0.1% (wt/vol) Tween 80 and cultivated with shaking (180 rpm) at 28 °C for 55 h. The cells were isolated from the culture by centrifugation at 18,800 × g for 20 min.

E. coli HST08 Premium competent cells (Takara Bio Inc., Shiga, Japan) were used as a host for recombinant plasmids. A plasmid T-Vector pMD20 (Takara Bio Inc.) was used as a cloning vector. E. coli HST08 was cultured in LB medium (pH 7.2) at 37 °C; if necessary, the medium was supplemented with ampicillin (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (0.5 mM) and X-Gal (0.005% (wt/vol)). S. lividans 1326 (NBRC15675) used as a host for the expression of PLA1 was obtained from the NITE Biological Resource Center (Chiba, Japan).

4.3. Purification of wild-type PLA1 from S. albidoflavus

All procedures were performed at 4 °C. The culture supernatant was obtained by centrifugation (18,800 × g for 20 min) after 55 h of culturing. The resultant supernatant was placed in a saturated ammonium sulfate solution ((NH₄)₂SO₄ mass fractionation = 80%) and was centrifuged at 18,800 × g for 20 min. The resultant precipitate was suspended in 20 mM Tris–HCl buffer (pH 9.0) and dialyzed for 2 d against the same buffer. The enzyme sample was adjusted to 1.5 M ammonium sulfate and loaded onto a TOYOPEARL Phenyl-650M column (2.5 × 4 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 1.5 M (NH₄)₂SO₄. The column was washed with three column volumes (CV) of the same buffer at a flow rate of 8 ml/min, and the protein was eluted with a linear gradient (15 CV) of 1.5 to 0 M (NH₄)₂SO₄ in the same buffer at 6 ml/min. The active fractions were pooled and the buffer changed to 20 mM MES-NaOH (pH 6.0) using Vivaspin 20–10 K (GE Healthcare UK Ltd., Buckinghamshire, England). This was followed by applying the sample to a Hitrap SP HP column (5 ml) equilibrated with the same MES buffer. The column was washed with three CV of the same buffer at a flow rate of 8 ml/min, and the protein was eluted with a linear gradient (10 CV) of 0 to 1 M NaCl in the same buffer at 2 ml/min. The active fractions were pooled. The buffer was exchanged with 20 mM Tris–HCl buffer (pH 9.0) using the same method mentioned above. The enzyme solution was applied to a HiTrap Q HP column (5 ml) equilibrated with the same Tris buffer. The column was washed with three CV of the same buffer at a flow rate of 8 ml/min and the protein was eluted with a linear gradient (10 CV) of 0 to 1 M NaCl at 2 ml/min. Fractions exhibiting high specific activity were pooled and used for investigation.

4.4. Enzyme activity assays

For PLA1 activity, the typical assay mixture, containing 10 μl of the enzyme solution, 50 μl of 0.2 M Tris–HCl buffer (pH 8.0), 25 μl of 10% (wt/vol) phospholipid/0.02% (wt/vol) Triton X-100, 10 μl of distilled water and 5 μl of 0.5 M EDTA was incubated at 37 °C for 5 min. The reaction was stopped by incubation at 100 °C for 5 min. The sample was subsequently centrifuged at 21,600 × g for 5 min and the supernatant collected. The FFAs released by the hydrolysis of phospholipids at the sn-1 and/or sn-2 position were quantified with the NEFA-C-Kit® (Wako Pure Chemical Industries, Ltd, Osaka, Japan), according to the instructions of the manufacturer. The rates of FFAs release from the enzyme reaction mixtures were calculated and one unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of FFA per minute. For the assay of the mutant enzyme, the enzyme activity was assayed at 37 °C for 5 min using of the cultured supernatant of the mutant. The reaction mixture (0.1 ml) contained 0.1 M Tris–HCl buffer (pH 7.2), 2.5% (wt/vol) EGGL, 0.005% (wt/vol) Triton X-100, 25 mM EDTA and the enzyme sample (10 μl). The reaction was stopped by incubation at 100 °C for 5 min. The enzyme activity was determined by the same method described above. Lipase activity assay mixture contained soybean oil or olive oil as a substrate instead of phospholipids. The enzyme activity was assayed at 50 °C for 5 min using of the purified wild-type enzyme. The reaction mixture (0.1 ml) contained 0.1 M Tris–HCl buffer (pH 7.2), 5% (wt/vol) soybean oil or olive oil, 1% (wt/vol) Triton X-100, 25 mM EDTA or 10 mM CaCl₂. The reaction was stopped by incubation at 100 °C for 5 min. The enzyme activity was determined by the same method described above. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of FFA per min. Esterase activity was determined spectrophotometrically by hydrolysis of different p-nitrophenyl esters [20]. The typical reaction mixture (0.15 ml) contained 0.0133% (wt/vol) pNPS, 20 mM Tris–HCl buffer (pH 7.2), 1% (wt/vol) Triton X-100 and 4.8 μl (45 ng) of the purified PLA1 (15 μl). The enzymatic reaction was performed at 50 °C and the hydrolysis was measured at 405 nm with an ε405 of 16,980 M⁻¹ cm⁻¹. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol per min under assay conditions. PLA1 and PLA2 activity assays were carried out using the EnzCheck® Phospholipase A2 Assay Kit and the EnzCheck® Phospholipase A2 Assay Kit (Life Technologies Corporation, California, USA). The assay kits are a simple, fluorometric method designed for continuous monitoring of PLA1 or PLA2 activity. The substrates are specific for each enzyme and are a dye-labeled glycerophosphoethanolamine and glycerophosphocholine with a BODIPY(R) FL dye-labeled acyl chain at the sn-1 or the sn-2 position. The results are a PLA1- or PLA2-dependent increase in BODIPY(R) FL fluorescence emission detected at approximately 515 nm. Specificity is imparted by the placement of the BODIPY(R) FL acyl chain in each position and by the incorporation of an acyl group with an enzymatic resistant (non-cleavable) ether linkage in each position. Each activity was determined according to the protocol outlined by the manufacturer.
4.5. Effect of pH, temperature and chemicals on PLA₁ activity

Each buffer (sodium acetate, BisTris–HCl, Tris–HCl and glycin-NaOH) was used to identify optimum pH and to determine pH stability. The optimum pH was examined by incubation at 37 °C for 5 min with 2.5% (wt/vol) EGGL in 0.12 M of each buffer containing 25 mM EDTA and 0.005% (wt/vol) Triton X-100. The pH stability was assayed by incubating the enzyme at 4 °C for 3 h in 50 mM of each buffer solution. The remaining activity was assayed under standard assay conditions, by incubation at 50 °C for 5 min with 2.5% (wt/vol) EGGL in 0.12 M sodium acetate (pH 5.6) containing 25 mM EDTA and 0.005% (wt/vol) Triton X-100. The PLA₁ activity was determined at each temperature by incubation (5 min) with 2.5% (wt/vol) EGGL in 0.12 M sodium acetate (pH 5.6) containing 25 mM EDTA and 0.005% (wt/vol) Triton X-100. The apparent activation energy (E_a) for EGGL hydrolysis was determined from the slope of the Arrhenius plot. The thermal stability was determined by incubating the enzyme in 0.2 M sodium acetate (pH 5.6) at each temperature for 30 min, and then the residual activity was measured by incubation at 50 °C for 5 min with 2.5% (wt/vol) EGGL in 0.12 M sodium acetate (pH 5.6) containing 25 mM EDTA and 0.005% (wt/vol) Triton X-100. The effect of chemicals such as metal ions and inhibitors on the enzyme activity was investigated. The enzyme activity was assayed by incubation at 50 °C for 5 min with 2.5% (wt/vol) EGGL in 0.1 M sodium acetate (pH 5.6) containing each concentration of the chemicals examined. The effect of the Triton X-100 concentration in the reaction mixture on the enzyme activity was examined. The enzyme activity was assayed by incubation at 50 °C for 5 min with 2.5% (wt/vol) EGGL, DPPC, or DMPA in 0.1 M sodium acetate (pH 5.6) containing 25 mM EDTA and each percentage of Triton X-100.

4.6. Protein analysis

Protein concentration was determined with the Pierce BCA protein assay kit (Takara Bio Inc., Japan) and BSA as the standard. Protein samples were analyzed by SDS–PAGE according to Laemmli [21]. The molecular mass of purified PLA₁ was estimated by gel filtration and dynamic light scattering (DLS) analysis. Gel filtration was performed using a TSK gel G3000SWXL column (Tosoh, 7.8-mm i.d. × 30-cm) at a flow rate of 1.0 ml/min with 50 mM potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl. The column was calibrated with a gel filtration calibration kit (GE Healthcare UK Ltd.) before and after the enzyme was subjected to a chromatography procedure. DLS measurement was performed on a Zetasizer NanoZ (Malvern Instruments, Malvern, UK) and analyzed by algorithms included in the Zetasizer Nano software.

4.7. Peptide sequencing

The purified protein was resolved by SDS–PAGE and then electrophoretically onto a PVDF membrane (Immobilon-P transfer membrane, Millipore Co., Billerica, MA, USA). The PVDF membrane was stained with Coomassie brilliant blue R-250 (CBB), and the transferred 28-kDa band was excised and subjected to N-terminal amino acid sequence analysis (Procise 494 HT Protein Sequencing System; Applied Biosystems, Foster City, CA, USA). For internal amino-acid sequencing, an SDS–PAGE gel was stained using CBB, the 28-kDa band was excised and then decolorized with 30% (vol/vol) acetonitrile containing 25 mM (NH₄)₂HCO₃. The in-gel digestion was performed by the method described by Shevchenko et al. [22]. Briefly, the excised 28-kDa band was digested with Trypsin (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, WI, USA) for 45 h at 4 °C. The fragments were analyzed with a nanoACQUITY UPLC Xevo TSQ of MS system (Waters Corp., Milford, MA, USA). The sample solution was transferred to an autosampler vial. One μl was chromatographed on a nanoAcquity column BEH130C18 (75 μm × 150 mm) using a nanoAcquity UPLC system (Waters Corp., Milford, MA, USA). The column was heated to 40 °C, and ultrapure water containing 0.1% (vol/vol) formic acid (A) and 0.1% (vol/vol) formic acid/acetonitrile (B) were employed. A typical 101-min sample run consisted of a gradient from 99% to 50% solvent A over 95 min, from 50% to 10% solvent A over 1 min, and maintaining 10% solvent A for 4 min followed by an increase of solvent A up to 99% over 1 min. A flow rate of 0.3 μl/min was used and the effluent was sprayed using Pre-cut PicTip Emitter (Waters, 360 μm OD × 20 μm ID; 10 μm tip; 6.35 cm length). The UPLC system was interfaced by electrospray ionization (ESI+) to a Waters Xevo QTOF-MS operated in data-dependent acquisition (DDA) mode with positive ionization. The capillary and sampling cone voltages were set to 3000 and 24 V, respectively. Source and desolvation temperatures were set to 90 and 200 °C, respectively, and the cone, desolvation and nanoflow gas flows were set to 30, 800 and 0.3 l/h, respectively. The collision argon gas energy was optimized to monitor the product ions of interest. To maintain mass accuracy, [Glu₁]-Fibrinopeptide B human (Sigma-Aldrich Co. LLC., MO, USA) as a lock mass (m/z 785.00 for positive ion mode) at a concentration of 500 fmol/μl in 0.1% (vol/vol) formic acid/50% (vol/vol) acetonitrile was used, and injected at a rate of 0.5 μl/min. Accurate mass LC-MS/MS DDA data were acquired in the centroid mode from 50 to 1990 m/z. Data acquisition was achieved with Masslynx version 4.1 SCN 712 (Waters Corp., Milford, MA, USA). De novo sequencing was performed with the ProteinLynx Global SERVER (Waters Corp., Milford, MA, USA).

4.8. Gas chromatography (GC) analysis

The positional specificity on the hydrolytic reaction was determined by capillary GC analysis. The purified wild-type enzyme of S. albidoflavus was used for the experiment. The enzymatic reaction containing 1% (wt/vol) POPA as a substrate was performed at 37 °C in 0.16 M Tris–HCl buffer (pH 9.0) containing 1% (wt/vol) Triton X-100 and 25 mM EDTA by the above-mentioned methods. The reaction was terminated by extracting with chloroform–methanol (2:1, vol/vol). One microliter of the extracts was injected with a split ratio of 50:1 into a Shimadzu GC-14B (Kyoto, Japan) chromatograph system equipped with a Nukol column (15 m × 0.53 mm × 0.50 μm; Sigma-Aldrich). The GC operation conditions: the GC column was heated at 8 °C/min from 110 to 220 °C and held for 15 min at 220 °C, the injector and detector temperature was 250 °C, and the flow rate of the He carrier gas was 25 ml/min. The released FFAs were separated.

4.9. Steady-state kinetics

The expressed and purified enzyme was used for steady-state kinetics. The enzymatic reaction containing POPA as a substrate was performed at 50 °C in 0.1 M Tris–HCl buffer (pH 7.2) containing 25 mM EDTA and 1% (wt/vol) Triton X-100 by the above-mentioned methods. The concentration of POPA ([POPA]) was calculated using a molecular weight of 696.92. The corresponding 1/Km, Vmax, and V/and intercepts of the regression lines, respectively, were determined by extrapolation using the Lineweaver–Burk plot by linear regression (KaleidaGraph, Synergy Software, PA, USA). The Km and Vmax were determined from the x- and y-intercepts of the regression line, respectively. The kcat was calculated using a molecular weight of 27,199 for monomeric protein and one catalytic site.

4.10. Cloning of the PLA₁ gene

Chromosomal DNA of S. albidoflavus was purified according to Kiersel et al. [23]. Oligonucleotides were synthesized based on the N-terminal (AACGVALGDS) and internal amino acid sequences (APSANVVF and FVSTEPGLPR) of the enzyme for use in PCR with the sense primer N 5'-gcgcgccggcgctacgtsgc-3' and antisense primer A1.
5′-saccasacattgccccwggcggc-3′ and A2 5′-cggcgcgctkgtggsctcactcg-3′. The PCR reaction mixture (20 μL × 10) contained: MightyAmp buffer, 12.5 pmol of each primer, 0.5 U of MightyAmp DNA polymerase (Takara Bio Inc., Japan) and −4.52 ng of S. albidoflavus chromosomal DNA as a template. The thermal cycling parameters were 98 °C for 2 min followed by 25 cycles of 98 °C for 10 s, 68°C for 1 min and 68°C for 1 min after the completion of the 25 cycles. The PCR fragment amplified using the sense primer N and antisense primer A2 was purified and cloned into the pMD20 vector (Takara Bio), and the resulting vector was called pPLA. Sequencing of the partial PLA1 gene on pPLA was performed as described above. The resulting constructs were verified by DNA sequencing. The nucleotide sequence of the PLA1 gene, designated pla, was deposited in the DDBJ database under the accession number AB605634.

4.12. Nucleotide and peptide sequence accession number

The nucleotide sequence of the PLA1 gene, designated pla, was deposited in the DDBJ database under the accession number AB605634.

4.13. Cloning and enzyme assay of mutant PLA1

The active site amino acids of SsEst are composed of Ser14, Trp280, His283 and the esterase hydrolyzes specific ester bonds in suberin, a wax-like lipid [25]. Amino acid residues that were deduced to be involved in the active center of PLA1 were replaced by different amino acids by site-directed mutagenesis using inverse-PCR amplification. PLA1 variants (S11A, S11D, S11E, S11T and S11Y; S216D, S216E, S216T and S216Y; H218A and H218R) were generated using a KOD Plus mutagenesis kit (Toyobo Co. Ltd., Tokyo, Japan) and pUC702/pla as a template. The mutant proteins were produced extracellularly by the transformed S. lividans. Clones exhibiting a clear halo were selected and clones having the highest activity were selected. The PLA1 produced by the transformed S. lividans was purified from a 48-h culture supernatant by ammonium sulfate precipitation, hydrophobic interaction chromatography and anion exchange chromatography.

Acknowledgement

We thank Associate Professor Chiaki Ogino (Kobe University, Japan) for providing the expression vector and Associate Professor Kazutaka Murayama (Tohoku University, Japan) for help with DLS analysis.

References


