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Purification, characterization, molecular cloning and extracellular production of a phospholipase A_1 from *Streptomyces albidoflavus* NA297

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ABSTRACT

A novel metal ion-independent phospholipase A_1 of *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 kU) 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 V_{max} and k_{cat} values of 1389 μmol min^{-1} mg $protein^{-1}$ and 630 s^{-1}, respectively. The apparent K_m and k_{cat}/K_m 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 A_1 (PLA_1) [EC 3.1.1.32] and A_2 (PLA_2) [EC 3.1.1.4] (PLAs) cleave glycerophospholipids into lysophospholipid and free fatty acids (FFAs). They are classified as PLA_1 or PLA_2 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.php4?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: PLA_1s from *Aspergillus oryzae* [1], *Serratia* sp. [2] and *Escherichia coli* [3], and PLA_2s 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 PLA_2 from the P388D1 macrophage-like cell line [7]. Besides *A. oryzae* PLA_1 and *S. violaceoruber* PLA_2, large-scale recombinant production of PLA_1 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 PLA_1 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 A_1 [**EC 3.1.1.32**]

Abbreviations: PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLD, phospholipase D; $SaPLA_1$, phospholipase A_1 from *Streptomyces albidoflavus*; $EcPLA_1$, phospholipase A_1 from *Escherichia coli*; $SxPLA_1$, phospholipase A_1 from *Serratia* sp. xjF1; $SMPLA_1$, phospholipase A_1 from *Serratia* sp. MK1; SaEst, esterase of *Streptomyces albus* J1074; SsEst, 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-phosphatidyl-L-serine; PG, L-α-phosphatidylglycerol; PI, L-α-phosphatidylinositol; DOPE, 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine; DMPA, 1,2-Dimyristoyl-*sn*-glycero-3-phosphate; DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; POPE, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPA, 1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphate; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); LPC, L-α-Lysophosphatidylcholine; pNPB, *p*-nitrophenyl butyrate; pNPO, *p*-nitrophenyl octanoate; pNPD, *p*-nitrophenyl decanoate; pNPL, *p*-nitrophenyl laurate; pNPP, *p*-nitrophenyl palmitate; pNPS, *p*-nitrophenyl stearate; FFA, free fatty acid

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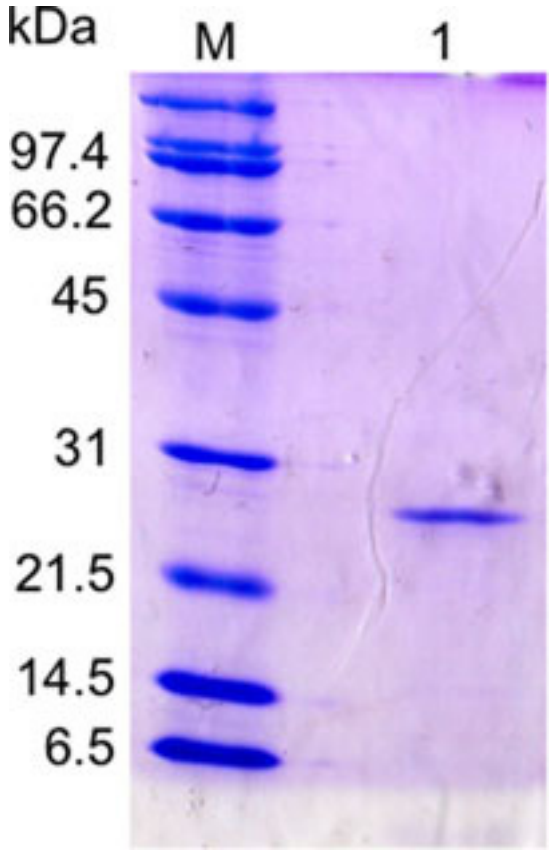

Fig. 1. SDS–PAGE analysis of purified PLA_1 from *S. albidoflavus*. Lane M, molecular marker; lane 1, purified PLA_1.

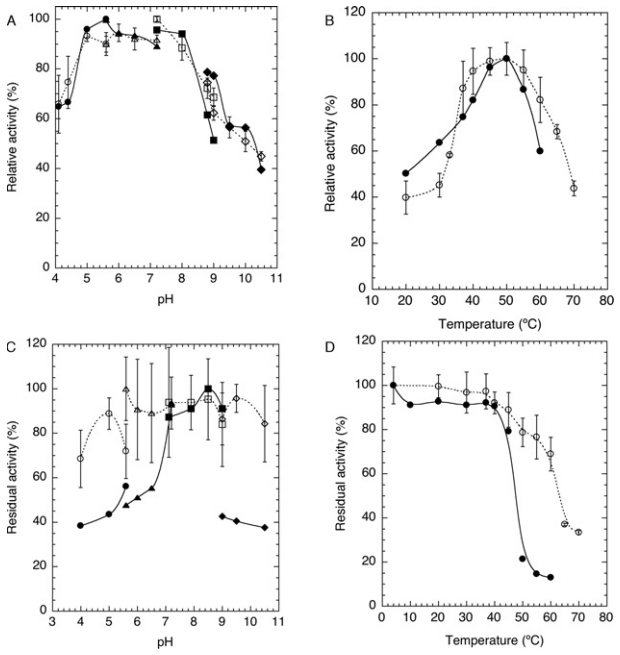

Fig. 2. Effect of pH and temperature on PLA_1 activity (A, B) and stability (C, D) of the wild-type (closed symbols) and recombinant enzyme (open symbols) for lecithin (EGGL) hydrolysis. (A) The enzyme activity was assayed at 37 °C for 5 min with 2.5% (wt/vol) EGGL in 0.12 M of each buffer containing 25 mM EDTA and 0.005% (wt/vol) Triton X-100. The buffers were: sodium acetate (pH 4.1–5.6), BisTris–HCl (pH 5.6–7.2), Tris–HCl (pH 7.2–9.0) and glycine–NaOH (pH 8.8–10.5). (B) The enzyme activity was assayed at each temperature in 0.12 M sodium acetate buffer (pH 5.6) for the wild-type enzyme and in 0.12 M Tris–HCl buffer (pH 7.2) for the recombinant enzyme. (C) The enzyme was incubated at 4 °C for 3 h in 50 mM of each buffer solution. The remaining activity was assayed by incubation at 50 °C for 5 min in 0.12 M sodium acetate buffer (pH 5.6) for the wild-type enzyme and in 0.12 M Tris–HCl buffer (pH 7.2) for the recombinant enzyme. (D) The enzyme was incubated at each temperature for 30 min in 0.2 M sodium acetate buffer (pH 5.6) for the wild-type enzyme and in 0.2 M Tris–HCl buffer (pH 7.2) for the recombinant enzyme. The residual activity was assayed by incubation at 50 °C for 5 min in 0.12 M sodium acetate buffer (pH 5.6) for the wild-type enzyme and in 0.12 M Tris–HCl buffer (pH 7.2) for the recombinant enzyme. Data are the means of experiments performed in triplicate. Error bars represent the standard deviation.

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 PLA_1 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 PLA_1 is shown in Table 1. The purified PLA_1 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). The determination of the molecular mass of the native enzyme by gel filtration chromatography or HPLC analysis was unsuccessful, due to non-specific binding to the gel matrix (data not shown). DLS analysis proved that PLA_1 was a monomeric protein and its molecular size agreed with the result of SDS–PAGE (data not shown).

2.3. Properties of PLA_1

We have examined the pH and temperature profile, effect of chemicals and inhibitors, and substrate specificity of the purified PLA_1. 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 (E_a) 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 PLA_1 activity against EGGL as the substrate. The enzyme activity was inhibited by Fe^{2+} and Fe^{3+} ions, >0.1 M Ca^{2+} 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 on EGGL hydrolysis. Since the critical micelle concentration of Triton X-100 is 0.24 mM (0.015% (wt/vol)), the interaction of micelles with the substrate may have influenced the enzyme activity. In contrast, the hydrolytic activity on DMPA and DPPC was maximal at 0.2%–1% and 1% (wt/vol) Triton X-100, respectively (Fig. 3(B)). Consequently, we selected 1% (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 PLA_1 from *S. albidoflavus* NA297.

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

[a] PLA_1 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 PLA_1 activity for egg yolk lecithin (EGGL) hydrolysis.[a]

Chemical	Relative activity (%)[c]
EDTA free[b]	100
25 mM EDTA[a]	108
50 mM EDTA	100
75 mM EDTA	84.8
100 mM EDTA	85.2
10 mM $CaCl_2$	82.6
100 mM $CaCl_2$	45.2
200 mM $CaCl_2$	22.6
10 mM $CoCl_2$	104
10 mM $MgCl_2$	109
10 mM $MnCl_2$	123
10 mM $ZnCl_2$	83.0
10 mM $FeCl_3$	10.7
10 mM $FeCl_2$	41.1
2 mM 2-mercaptoethanol	65.8
2 mM dithiothreitol	100
2 mM PMSF	78.0
2 mM sodium dodecyl sulfate	11.7
0.1% Triton X-100	99.5
0.23% Triton X-100	59.2
1% Triton X-100	55.7

[a] 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.
[b] The activity was measured under the assay condition without EDTA.
[c] The relative activity is expressed as a percentage of the activity under the assay condition without EDTA.

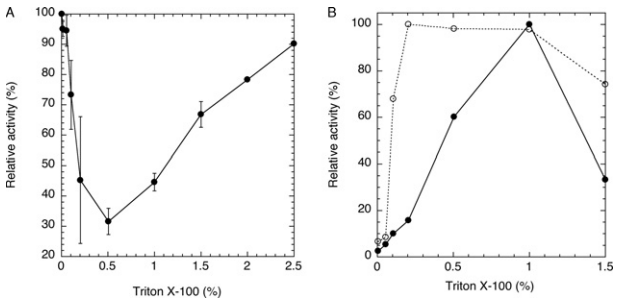

Fig. 3. The effect of the Triton X-100 concentration in the enzyme reaction mixture on the enzyme activity. The enzyme activity was assayed by incubation at 50 °C for 5 min with 2.5% (wt/vol) EGGL (A), DPPC (B, closed circle), or DMPA (B, open circle) in 0.1 M sodium acetate (pH 5.6) containing 25 mM EDTA and each percentage of Triton X-100.

2.4. Positional specificities of PLA_1 and lysophosphocholine production

The $SaPLA_1$ enzyme activity was detected by the EnzCheck® Phospholipase A_1 assay kit; however, PLA_2 activity was not detected by the Phospholipase A_2 assay (data not shown). These results suggest that the $SaPLA_1$ enzyme is PLA_1. Gas chromatography (GC) analysis demonstrated that FFAs were released proportionally with the enzymatic reaction time from the *sn*-1 and

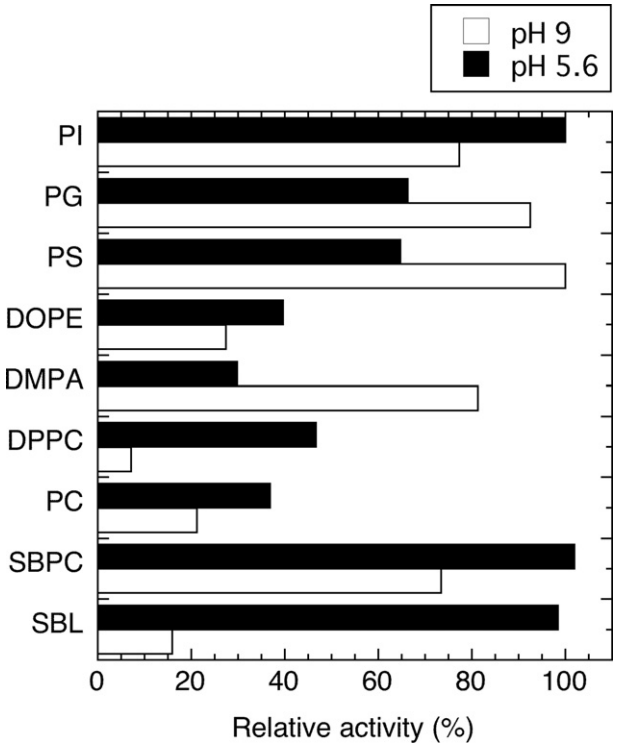

Fig. 4. Substrate specificity of the enzyme. Black and white bars represent for substrate profiles at pH 5.6 and 9.0, respectively. The enzyme activity was assayed by incubation at 50 °C for 5 min with 2.5% (wt/vol) of each substrate in 0.1 M sodium acetate (pH 5.6) or Tris–HCl (pH 9.0) containing 25 mM EDTA and 1% (wt/vol) Triton X-100.

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 63:37. The positional selectivity was almost equal to that of *A. oryzae* PLA_1 (data not shown).

2.5. Cloning of the PLA_1 gene

The partial nucleotide sequence of the gene encoding PLA_1 (*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_1 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

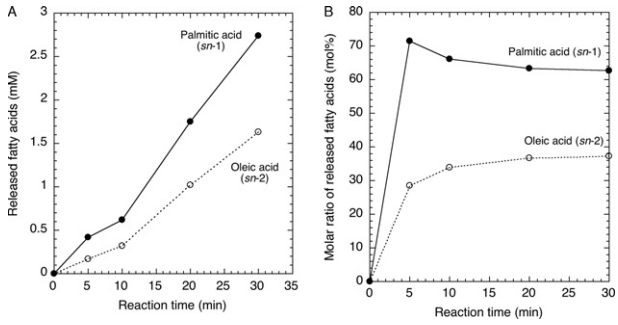

Fig. 5. GC analysis of the time course of the enzyme reaction. The enzyme reaction was carried out by incubation at 37 °C with 1% (wt/vol) POPA in 0.16 M Tris–HCl (pH 9.0) containing 25 mM EDTA and 1% (wt/vol) Triton X-100. (A) The released FFA concentration, and (B) the molar ratio of the released FFA.

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 molecular weight of 27,199 (Fig. 6). As shown in Fig. 6, the putative ATG translational start codon is preceded at a spacing of 4 bp by a potential ribosome binding site (ggagg). A possible promoter region was not found. The N-terminal sequence of the mature enzyme starts at Ala-34 of the deduced amino acid sequence, indicating that the preceding 33 residues are a signal sequence for secretion. A consensus sequence of lipase (GXSXG) was found in the 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 PLA_1

High efficiency extracellular production of *S. albidoflavus* PLA_1 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 PLA_1 with a high specific activity (588 U/mg-protein) and total activity (14.7 kU) was purified to electrophoretic homogeneity from the cultured supernatant by simple purification steps (Table 3). Although the pH and temperature profile of the recombinant enzyme was almost the same as that of the wild-type enzyme, the maximum activity of the recombinant enzyme was observed at pH 7.2 in the Tris–HCl buffer at 50 °C (Fig. 2(A) and (B)). For the following assay, the enzymatic reaction was performed at 50 °C in a Tris–HCl buffer (pH 7.2). The apparent E_a value for EGGL hydrolysis by the recombinant enzyme was 58.3 kJ mol^{-1} (data not shown). The recombinant enzyme was stable between pH 5.6 and pH 9 at 4 °C, and at 40 °C and pH 7.2 (Fig. 2(C) and (D)). Thermal and pH stabilities of the expressed PLA_1 were a little higher than those of the wild-type enzyme. No differences of the effects of the chemicals on the activity between the expressed enzyme and the wild-type protein were observed (data not shown). As shown in Fig. 7, the recombinant enzyme preferably hydrolyzed POPA and PS at 50 °C in Tris–HCl buffer (pH 7.2). The difference in substrate specificity between the wild-type and recombinant enzyme likely resulted from the influence of the reaction pH. The substrate specificity was in the following order: POPA, PS > PI > POPC, PG > POPE. PLA_1 hydrolyzed LPC as well as glycerophospholipids. Relative activity was 56.5 ± 6.3% for LPC and 44.6 ± 10.3% for POPC, with activity towards POPA set to 100%. The PLA_1 could not hydrolyze triglycerides such as soybean oil and olive oil, even in the presence of 10 mM $CaCl_2$. The PLA_1 exhibited much lower activities towards *p*-nitrophenyl esters of fatty acids even in the presence of 10 mM $CaCl_2$. These results prove that the enzyme is a phospholipase, but not a lipase as well as a carboxylesterase.

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cggtgagccg cgatgtcgtt ccgtgagaga agacgatggc ttcatgccga tggtctgcat     60
tggcgacttt gccccccagc gcgccggatg ccggacttca atgtggcgca gagctgccgg    120
gccgtcaaga tgcggagaga gtgtgcagat gcgggcggcc gtcccgcccg tctgaggcca    180
tgcgttcacg aacgagtggt tcgtgttatc ggaatgacca ggcgtcacat cgttgcccca    240
cctattgctt tcgcaaagag tgcagcgcag gcccctctct ccgcgcgtag atgttttggc    300
atgaacactt ccgaggctcc gtcgccgcgt gctaccaagt gcacgccgac cccccgcaga    360
tggaggcttc atg aaa ctg cgt aga acc gcg aca tat gtg acc tcg ctc        409
  rbs      Met Lys Leu Arg Arg Thr Ala Thr Tyr Val Thr Ser Leu
            1               5                  10
ctc gcc gcc gtc acc ctc gcc ctc acc ggg gcc agt gcc gca gcg gcc      457
Leu Ala Ala Val Thr Leu Ala Leu Thr Gly Ala Ser Ala Ala Ala Ala
    15                  20                  25
gcc gac gcc cag↓gcc gca ggc ggc tac gtc gcc ctg ggt gac tcc tac      505
Ala Asp Ala Gln Ala Ala Gly Gly Tyr Val Ala Leu Gly Asp Ser Tyr
30                  35                  40                  45
tcc tcc ggg gtc ggt gcc ggc tcc tac gac agc ggc agc ggc gac tgc      553
Ser Ser Gly Val Gly Ala Gly Ser Tyr Asp Ser Gly Ser Gly Asp Cys
                50                  55                  60
cgg cgc acc ccc aag gcg tac ccc gcc ctg tgg gcc gcc gcg aac tcg      601
Arg Arg Thr Pro Lys Ala Tyr Pro Ala Leu Trp Ala Ala Ala Asn Ser
            65                  70                  75
ccc gcc agc ttc gac ttc gtg gcc tgc tcc ggt gcg gtg acc agc gat      649
Pro Ala Ser Phe Asp Phe Val Ala Cys Ser Gly Ala Val Thr Ser Asp
        80                  85                  90
gtc ctc aac aag cag atg ggc ccg ctg aac tcc tcc acc agc ctc gtc      697
Val Leu Asn Lys Gln Met Gly Pro Leu Asn Ser Ser Thr Ser Leu Val
    95                  100                 105
tcg ctg acc atc ggc ggc aac gac gcc ggg ttc gcg gac gtc atg acc      745
Ser Leu Thr Ile Gly Gly Asn Asp Ala Gly Phe Ala Asp Val Met Thr
110                 115                 120                 125
acc tgc gtc ctc cag tcc gag gcc aac tgc atc gcc cgg gtc aac acg      793
Thr Cys Val Leu Gln Ser Glu Ala Asn Cys Ile Ala Arg Val Asn Thr
                130                 135                 140
gcc aag gcc ttc gtc gag agc acc ctg ccc ggc cgg ctc gac tcg gtc      841
Ala Lys Ala Phe Val Glu Ser Thr Leu Pro Gly Arg Leu Asp Ser Val
            145                 150                 155
tac tcg caa gtc cgc gcc aag gcc ccc tcg gcc aac gtc gtc gtc ctc      889
Tyr Ser Gln Val Arg Ala Lys Ala Pro Ser Ala Asn Val Val Val Leu
        160                 165                 170
ggc tac ccg cgg ttc tac aag ctc aac ggc acc tgc gtg gcc ggc ctg      937
Gly Tyr Pro Arg Phe Tyr Lys Leu Asn Gly Thr Cys Val Ala Gly Leu
    175                 180                 185
acc gag ggc gag cgc acc gcc atc aac ggc gcc gcc gac ctc ctc aac      985
Thr Glu Gly Glu Arg Thr Ala Ile Asn Gly Ala Ala Asp Leu Leu Asn
190                 195                 200                 205
tcc gtc atc tcc aag cgt gcc gcc gac cac ggc tac gcc tac ggg gac     1033
Ser Val Ile Ser Lys Arg Ala Ala Asp His Gly Tyr Ala Tyr Gly Asp
                210                 215                 220
atc gcc gcc gcc ttc acc ggc cac gag atc tgc tcc ggc gac tcc tgg     1081
Ile Ala Ala Ala Phe Thr Gly His Glu Ile Cys Ser Gly Asp Ser Trp
            225                 230                 235
cta cac agc gtc aag tgg acc ggc atc aac gac tcc tac cac ccg acg     1129
Leu His Ser Val Lys Trp Thr Gly Ile Asn Asp Ser Tyr His Pro Thr
        240                 245                 250
gcc gcc ggc cag tcc ggc ggc tac ctg ccg gtg ctg aac tcc aag gcc     1177
Ala Ala Gly Gln Ser Gly Gly Tyr Leu Pro Val Leu Asn Ser Lys Ala
    255                 260                 265
tga
Stop
270
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Fig. 6. Nucleotide and deduced amino acid sequence of *S. albidoflavus* PLA_1. Underlined regions of the amino acid sequences were determined by protein sequencing and nanoLC-MS/MS. The deduced ribosome binding domain and cleavage site by signal peptidase are indicated by rbs and the arrow, respectively. A consensus sequence of lipase (GXSXG) is represented by the double underline.

2.7. Steady-state kinetics of PLA_1

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 V_{max} and turnover rate (k_{cat}) were determined to be 1389 μmol min^{-1} mg-$protein^{-1}$ and 630 s^{-1}, respectively. The apparent K_m and k_{cat}/K_m values were 2.38 mM and 265 mM^{-1} s^{-1}, respectively.

2.8. Mutant analysis of PLA_1

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 S216Y showed about 10%–20% relative activity.

Table 3
Purification of the expressed PLA_1.

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

[a] PLA_1 activity was assayed using the reaction mixture containing 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.

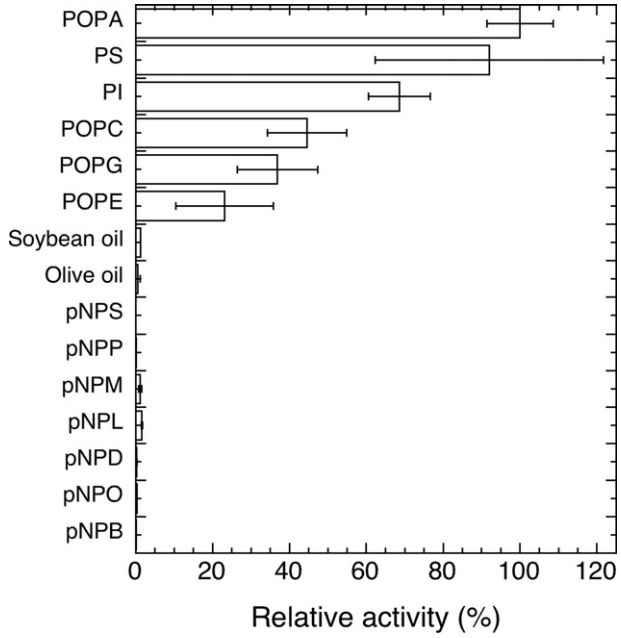

Fig. 7. Substrate specificity profiles of the purified recombinant PLA_1. The enzyme activity was assayed by incubation at 50 °C for 5 min with 0.5% (wt/vol) phospholipids, soybean oil, olive oil or 0.013% (wt/vol) pNP esters in 0.1 M Tris–HCl (pH 7.2) containing 1% (wt/vol) Triton X-100 and 25 mM EDTA or 10 mM$CaCl_2$. Data are the average of experiments performed in triplicate. Error bars represent the standard deviation.

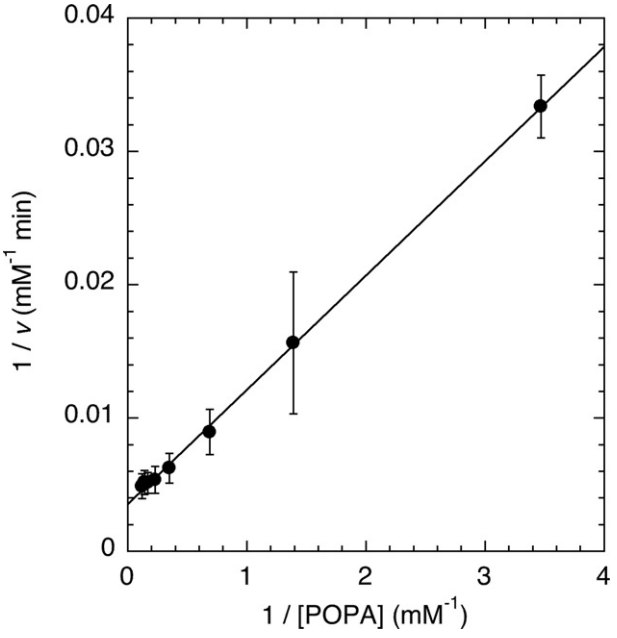

Fig. 8. Lineweaver–Burk plot of the steady-state kinetics of the recombinant PLA_1 activity. The initial rate of POPA hydrolysis by the purified recombinant PLA_1 was determined at various POPA concentrations and then plotted in a Lineweaver–Burk plot ($1/v$ vs.$1/[POPA]$). The initial rate of the enzyme reaction was assayed by incubation at 50 °C for 4 min with various POPA concentrations in 0.1 M Tris–HCl (pH 7.2) containing 25 mM EDTA and 1% (wt/vol) Triton X-100.

Table 4
Enzyme activity of the wild-type and recombinant enzymes.[a]

Enzyme	Relative activity (%)[b]
Wild type	100
S11A	0
S11D	0
S11E	0
S11T	0
S11Y	0
S216A	0
S216D	1.77
S216E	0.746
S216T	23.1
S216Y	8.24
H218A	0
H218R	1.31

[a] The activity was measured in the reaction mixture consisting of 0.1 M Tris – HCl buffer (pH 7.2), 2.5% (wt/vol) EGGL, 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 PLA_1 from actinomycetes. Known microbial PLA_1s of *A. oryzae* [1], *Serratia* sp. [2] and *E. coli* [3] are calcium ion-dependent enzymes, whereas PLA_1 of *S. albidoflavus* ($SaPLA_1$) was a metal ion-independent enzyme. $SaPLA_1$ 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 PLA_1 from the venom of the social wasp *Polybia paulista* and of recombinant PLA_1 from *Serratia* sp. xjF1 ($SxPLA_1$) were 2898 and 202.3 U/mg-protein, respectively [8,9]. Thus, with respect to bacterial PLA_1, we concluded that $SaPLA_1$ 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. $SaPLA_1$ was inhibited in the presence of 10 mM Fe^{2+} and Fe^{3+} 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 Fe^{2+} [10]. PLA_1s from

Mycobacterium phlei [11] and from *Corticium centrifugum* [12] were inhibited by Fe^{2+} and Fe^{3+} ions. SDS and high concentrations of Triton X-100 inhibited the enzyme activity of $SaPLA_1$. 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 $SaPLA_1$ 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/DDBJ databases (October, 2008); however, the esterase of *S. albus* J1074 (SaEst) was only predicted and not characterized. Moreover, $SaPLA_1$ exhibited no lipase and carboxylesterase activity. In addition, the deduced amino acid sequence of the mature enzyme of $SaPLA_1$ exhibited 68.6% and 63.4% identities to those of lipase Sc1 from *S. coelicolor* (Q9S2A5) and lipase SrLip from *S. rimosus* (Q93MW7), respectively. SrLip has been reported to show lipase, phospholipase, esterase, thioesterase and Tweenase activities, and the preferred esters of the medium-chain acids (C_8–C_{12}), whereas Sc1 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 SGNH family could readily be identified in $SaPLA_1$. 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 $SaPLA_1$ is essentially different from that of SrLip.

Interestingly, $SaPLA_1$ 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 PLA_1 ($EcPLA_1$) [3]. The optimal pH of 5.6 for the purified native $SaPLA_1$ enzyme was different from that (pH 7.2) of the recombinantly expressed $SaPLA_1$, 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 optimum pH of $EcPLA_1$ [3] and $SxPLA_1$ [9] are pH 8.4 and 9, respectively, these enzymes are alkaline PLA_1, whereas $SaPLA_1$ shows optimal activity at a more neutral pH value.

The maximum optimal temperature of activity of $SaPLA_1$ (i.e., 50 °C) is higher than $SxPLA_1$ (35 °C) [9]. However, $SaPLA_1$ 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 $SaPLA_1$ 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 PLA_2 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 PLA_1 from other organisms. It is known that when the E_a changed from 62.8 to 41.9 kJ mol^{-1}, the k_{cat} increased 4.5 × 10^7 times, indicating that $SaPLA_1$ 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 PI and PG or DOPE and 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 $EcPLA_1$ 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 PLA_1s. Rose and Prestwich have reported head group selectivity of PLA_2 from various organisms [15]. *S. violaceoruber* PLA_2 preferred the PC head group, followed by PG > PE >> PA. They also described that bacterial and mammalian PLA_2s, except for the venom and pancreatic enzymes, showed no or weak hydrolysis of PA. In contrast, $SaPLA_1$ showed the highest activity toward PA. Further studies are needed to elucidate the mechanism of head group specificity of $SaPLA_1$.

The apparent K_m of $SaPLA_1$ was a somewhat higher value than those of $EcPLA_1$ [3], PLA_1 from *Serratia* sp. MK1 ($SMPLA_1$) [16] and $SxPLA_1$ [9]. The k_{cat} value, 630 s^{-1}, of $SaPLA_1$ was much higher than that of $SMPLA_1$ [16]. We conclude that the binding affinity of $SaPLA_1$ toward the substrate is lower than those of other bacterial PLA_1s. Nevertheless the turnover rate is much higher than all known PLA_1s, as shown in the BRENDA database.

The EnzCheck® Phospholipase A_2 assay kit suggested that the $SaPLA_1$ enzyme is certainly not a PLA_2. However, $SaPLA_1$ may not be able to recognize the Phospholipase A_2 assay's glycerophospholipid with the dye-labeled acyl chain as the substrate. GC analysis showed that $SaPLA_1$ hydrolyzed the *sn*-2 acyl ester bond as well as *sn*-1. The proportion of *sn*-2 hydrolysis by $SaPLA_1$ may be higher than that of other PLA_1 enzymes. The selectivity is lower than that of $SMPLA_1$ [2], but the analysis was carried out with non-pure enzyme. There is no evidence for the positional specificity of PLA_1 from *A. oryzae* [1]. GC analysis showed that the positional selectivity of PLA_1 from *A. oryzae* was almost equal to that of $SaPLA_1$. It was reported that acyl migration from the 2-position to the 1(3)-position or the opposite of diacylglycerol does occur [17]. Moreover, $SaPLA_1$ hydrolyzed LPC as well as diacylglycerophospholipids, suggesting that the transesterificated 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 $SaPLA_1$ 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 $SaPLA_1$.

The results of the mutagenesis analysis showed that Ser11 is essential for the catalytic function of $SaPLA_1$, 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. For SsEst, the active site involves a Ser–His dyad and the carbonyl group of Trp280. 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 $SaPLA_1$.

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), *p*-nitrophenyl butyrate (C_4) (pNPB), *p*-nitrophenyl octanoate (C_8) (pNPO), *p*-nitrophenyl decanoate (C_{10}) (pNPD), *p*-nitrophenyl laurate (C_{12}) (pNPL), *p*-nitrophenyl palmitate (C_{16}) (pNPP), and *p*-nitrophenyl stearate (C_{18}) (pNPS) 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 (POPA), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (POPG) and L-α-Lysophosphatidylcholine (LPC) were from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). L-α-phosphatidylglycerol from egg (PG) was purchased from Funakoshi Co. (Tokyo, Japan). L-α-phosphatidylinositol (PI) 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 lecithin 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 (DDBJ database under accession number AB738935). *S. albidoflavus* NA297 was deposited as NITE BP-1014 in the NITE Patent Microorganisms Depositary (NPMD) (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 PLA_1 was obtained from the NITE Biological Resource Center (Chiba, Japan).

4.3. Purification of wild-type PLA_1 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 ($(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$. 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 $(NH_4)_2SO_4$ 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 PLA_1 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), 0.5% (wt/vol) soybean oil or olive oil, 1% (wt/vol) Triton X-100, 25 mM EDTA or 10 mM $CaCl_2$. 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 mU (45 ng) of the purified PLA_1 (15 μL). The enzymatic reaction was performed at 50 °C and the hydrolysis was measured at 405 nm with an ε_{405} of 16,980 M^{-1} cm^{-1}. One unit of activity was defined as the amount of enzyme releasing 1 μmol per min under assay conditions. PLA_1 and PLA_2 activity assays were carried out using the EnzCheck® Phospholipase A_1 Assay Kit and the EnzCheck® Phospholipase A_2 Assay Kit (Life Technologies Corporation, California, USA). The assay kits are a simple, fluorometric method designed for continuous monitoring of PLA_1 or PLA_2 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 PLA_1- or PLA_2-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 PLA_1 activity

Each buffer (sodium acetate, BisTris–HCl, Tris–HCl and glycine–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 PLA_1 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 PLA_1 was estimated by gel filtration and dynamic light scattering (DLS) analysis. Gel filtration was performed using a TSK gel $G3000SW_{XL}$ 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 electroblotted onto a PVDF membrane (Immobilon-P^{SQ} transfer membrane, Millipore Co., Billerica, MA). 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 terminal 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 $(NH_4)_2HCO_3$. 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 QTof 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 PicoTip 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, [Glu^1]-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 $1/v$ vs. $1/[POPA]$ plots were treated according to a Michaelis–Menten equation. Kinetic constants were determined by extrapolation using the Lineweaver–Burk plot by linear regression (KaleidaGraph, Synergy Software, PA, USA). The K_m and V_{max} were determined from the x- and y-intercepts of the regression line, respectively. The k_{cat} was calculated using a molecular weight of 27,199 for monomeric protein and one catalytic site.

4.10. Cloning of the PLA_1 gene

Chromosomal DNA of *S. albidoflavus* was purified according to Kieser et al. [23]. Oligonucleotides were synthesized based on the N-terminal (AAGGYVALGDS) and internal amino acid sequences (APSANVVV and FVESTLPGR) of the enzyme for use in PCR with the sense primer N 5′-gcsgcsggcggctacgtsgc-3′ and antisense primer A1

5′-sacsacsacattsgcsswsggsgc-3′ and A2 5′-cggccsggstkggtsswctcsacg-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 PLA_1 gene on pPLA was performed with the BigDye Terminator cycle sequencing kit (Life Technologies Corporation, California, USA) and analyzed in an ABI Prism 3100 genetic analyzer (Life Technologies Corporation). To reveal the complete sequence of the gene encoding PLA_1, inverse PCR was performed with the forward primer 5′-gggtacgccttgggggtg-3′ and the reverse primer 5′-cctgcgtcctccagtccg-3′. Genomic DNA was digested with restriction endonucleases (*Sph* I, *Nco* I, *Sac* I, *Sac* II, *Kpn* I, *Sau* 3AI and *Pvu* I). Digested DNA was circularized by self-ligation and used as templates for inverse PCR. The PCR reaction mixture (20 μL × 8) contained: MightyAmp buffer, 12.5 pmol of each primer, 0.5 U of MightyAmp DNA polymerase and 0.2 μl of the self-ligation solution as a template. The inverse PCR program was 98 °C for 2 min and followed by 30 cycles of 98 °C for 10 s, 70 °C for 4 min and 70 °C for 7 min after the completion of the 30 cycles. As above-mentioned, the DNA fragment was cloned into the pMD20 vector and sequenced. A database homology search revealed using BLAST that the deduced partial amino acid sequence of PLA_1 showed 100% identity to that of a secreted hydrolase of *S. albus* J1074 (DDBJ database under accession number D6BAL1). To clone the PLA_1 gene, reverse primer CRV (5′-tcaggccttggagttcagcac-3′) was designed based on the 3′ region of the secreted hydrolase gene of *S. albus* J1074, PCR was carried out using the sense primer N1 (5′-gccgcaggcggctacgtcgc-3′) and the reverse primer CRV. The PCR reaction mixture (20 μL × 10) contained: MightyAmp buffer, 12.5 pmol each of the sense primer N and the reverse primer CRV, 0.5 U of MightyAmp DNA polymerase, and ~4.52 ng of the *S. albidoflavus* chromosomal DNA as a template. The PCR program was 98 °C for 2 min and 20 cycles of 98 °C for 10 s followed by 68 °C for 1 min and a final step after the cycles of 68 °C for 5 min. The obtained PCR fragment was purified and cloned into the pMD20 vector and the resulting vector was called pPLA1. Sequencing of the PLA_1 gene on pPLA1 was performed as described above.

4.11. Expression and purification of PLA_1

S. lividans 1326 (NBRC15675) was obtained from the NITE Biological Resource Center (Chiba, Japan). *S. lividans* 1326, possessing no PLA_1 activity, was used as a host for PLA_1 expression. To replace the *Bgl* II site in the PLA_1 gene, the *S. albidoflavus* PLA_1 gene (*pla*) was amplified from chromosomal DNA by a two-step PCR. The first PCR was performed using the following primers: 5′-aaa*gctagc*gccgcaggcggctacgtcg-3′ (Nhe I-F1) containing the first codon (*Nhe* I, italic; Ala, underlined) of mature PLA_1 and 5′-gag*cagatttc*gtggccgg-3′ (*Bgl* II-repair RV1); 5′-accggccacg*aaatct*gctc-3′ (*Bgl* II-repair F2) and 5′-ata*agatct*tcaggccttggagttcagc-3′ (*Bgl* II-RV2; *Bgl* II, italic). The PCR reaction mixture (25 μL × 10) contained: 1 × buffer #1, 30 nmol of $MgSO_4$, 75 pmol of each primer set (*Nhe* I-F1 and *Bgl* II-repair RV1; *Bgl* II-repair F2 and *Bgl* II-RV2), 7.6 nmol of dNTP, 1 μL of DMSO, 0.5 U of KOD-Plus-DNA polymerase and 233 ng of the *S. albidoflavus* chromosomal DNA as a template. The thermal cycling parameters were 98 °C for 2 min, followed by 30 cycles of 98 °C for 15 s, 72 °C for 2 s and 74 °C for 25 s. At the end of the cycles, a final round of 74 °C for 10 s was applied. The second PCR was performed using each first-step amplification product as a template with the forward primer (*Nhe* I-F1) and the reverse primer (*Bgl* II-RV2). PCR was carried out in a reaction mixture (25 μl × 6) containing 1 × buffer #2, 30 nmol of $MgSO_4$, 75 pmol each primer, 7.6 nmol of dNTP, 1 μL of DMSO, 300 ng of the products (ca. 600 bp) amplified using the primer set of *Nhe* I-F1 and *Bgl* II-repair RV1, 338 ng of the products (ca. 100 bp) amplified using the primer set of *Bgl* II-repair F2 and *Bgl* II-RV2, and 0.5 U KOD-Plus-DNA polymerase. Amplification was performed under the above-mentioned conditions. The obtained fragment was purified and digested with *Nhe* I and *Bgl* II, and then subcloned into the *Nhe* I and *Bgl* II sites of pUC702 [24] carrying the promoter, signal sequence and the terminator region of the phospholipase D (PLD) ORF from *Streptoverticillium cinnamoneum*. This expression plasmid was sequenced and designated as pUC702/pla. The transformation techniques of Kieser et al. were followed for *S. lividans* [23]. Transformants were screened using lecithin-emulsified nutrient plates according to Kim and Rhee [19]. Clones exhibiting a clear halo were collected and clones having the highest activity were selected. The PLA_1 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.

4.12. Nucleotide and peptide sequence accession number

The nucleotide sequence of the PLA_1 gene, designated *pla*, was deposited in the DDBJ database under the accession number AB605634.

4.13. Cloning and enzyme assay of mutant PLA_1

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 PLA_1 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 cloud halo around their colony and no halo were screened and selected. PLA_1 activity of the transformants was assayed. The production of all resulting mutant proteins was verified by SDS–PAGE analysis. In addition, all the resulting constructs were verified by DNA sequencing.

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