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

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**Abstract**

A novel metal ion-independent phospholipase A₁ 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 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 $V_{\text{max}}$ and $k_{\text{cat}}$ values of 1389 μmol min⁻¹ mg protein⁻¹ and 630 s⁻¹, respectively. The apparent $K_{\text{m}}$ and $k_{\text{cat}}/K_{\text{m}}$ 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.

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**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], *Serratia* 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 macrophase-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*; ECPLA₁, phospholipase A₁ from *Escherichia coli*; SaPLA₂, phospholipase A₂ from *Serratia* sp. J1074; SMPLA, phospholipase A₁ from *Serratia* sp. MK1; SaEst, esterase of *Streptomyces albus* J1074; SaEst, 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, 1,2-Diacyl-sn-glycerol-3-phosphocholine; PS, 1,2-Diacyl-sn-glycerol-3-phosphatidylserine; LPC, 1--sn-glycerophosphate; LPE, 1- and 2-sn-glycerophosphonolipids; SL, 1- and 2-snglycerophospholipids; DOPE, 1,2-Diacyl-sn-glycerol-3-phosphoethanolamine; DMPA, 1,2-Dimyristoyl-sn-glycerol-3-phosphate; DPPC, 1,2-Dipalmitoyl-sn-glycerol-3-phosphocholine; POPE, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine; POPA, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphate; POPC, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPG, 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; LPC, 1-sn-Lyso phosphatidylcholine; pNPB, p-nitrophenyl butyrate; pNPD, p-nitrophenyl octanate; pNPP, p-nitrophenyl decanate; pNPL, p-nitrophenyl laurate; pNPQ, p-nitrophenyl palmitate; pNPS, p-nitrophenyl stearate; FFA, free fatty acid

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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 $\sim 28$ kDa was visualized by CBB staining (Fig. 1).

![Fig. 1. SDS–PAGE analysis of purified PLA1 from *S. albidoflavus*. Lane M, molecular marker; lane 1, purified PLA1.](image)

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 ($E_a$) 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 PLA1 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 PLA1 from *S. albidoflavus* NA297.

| Purification step          | Activitya (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 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.a

| Chemical                  | Relative activity (%) |
|---------------------------|-----------------------|
| EDTA freeb                | 100                   |
| 25 mM EDTAc               | 108                   |
| 50 mM EDTA                | 100                   |
| 75 mM EDTA                | 84.8                  |
| 100 mM EDTA               | 85.2                  |
| 10 mM CaCl₂               | 82.6                  |
| 100 mM CaCl₂              | 45.2                  |
| 200 mM CaCl₂              | 22.6                  |
| 10 mM MgCl₂               | 104                   |
| 10 mM MgCl₂               | 109                   |
| 10 mM MnCl₂               | 123                   |
| 10 mM ZnCl₂               | 83.0                  |
| 10 mM FeCl₃               | 10.7                  |
| 10 mM FeCl₃               | 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.

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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 63:37. 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 molecular weight of 27,199 (Fig. 6). As shown in Fig. 6, the putative 807 nucleotides encoding a 269-amino-acid protein with a deduced sequence of the 1.18-kb PCR product. The ORF 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) was observed at pH 7.2 in the enzymatic reaction was performed at 50°C in a Tris–HCl buffer at 50°C containing 25 mM EDTA and 1% (wt/vol) Triton X-100. The apparent molecular weight of the gene product without the signal sequence was calculated to be 27,199, which is in agreement with that of the recombinant 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.

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 5.0) 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.

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 Km value for EGGL hydrolysis by the recombinant enzyme was observed (data not shown). As shown in Fig. 7, the recombinant enzyme preferably hydrolyzed POPA and PC at 50°C in a Tris–HCl buffer (pH 7.2). The apparent E0 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(A) and (B)). Thermal and pH stabilities of 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 PC at 50°C in a Tris–HCl buffer (pH 7.2). The apparent E0 value for EGGL hydrolysis by the recombinant enzyme was observed (data not shown). The mutants of pla were virtually unstable at 50°C, and the mutants S11A, S11D, S11E, S11T, S11Y, S216A and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4). The mutants S216T and H218A exhibited negligible activity compared with the native enzyme (Table 4).
Table 3
Purification of the expressed PLA₁.

| Purification step | Activity (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 |

* PLA₁ 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 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.⁴

| Enzyme | Relative activity (%) |
|--------|-----------------------|
| 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 |

* 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.

* 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 of recombinant PLA₁ 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^{2+} and Fe^{3+} 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_SGHN (UniProt ID, D68AL1) annotated in the genome of *S. albus* J1074. "An 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 Sc1 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 (C8–C12), 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 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 PLA1 (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 optimum 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, \( E_a = 18.8 \text{ kJ mol}^{-1} \), 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 PLA2 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 PLA1 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 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 PI and PE or DOPE and DPPC would have been similar, because the pK\(_a\) 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 prefer the PC head group, followed by PG > PE > 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 that 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\(^{\text{®}}\) Phospholipase A\(_2\) assay kit suggested that the SaPLA1 enzyme is certainly not a PLA2. However, SaPLA1 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 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, 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 SaEst (1ESCl) with the active site composed of Ser14, Trp280 and His283. For SaEst, 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, metin-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) (1-α-phosphatidylcholine approx. 70% as phospholipids min. 99% from egg yolk, 1-α-phosphatidylcholine (PC) from egg yolk (purity 98%, TLC) and soybean oil were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 1-α-phosphatidylcholine (SBPC) from soybean (Type IV-S, ≥ 90%) and 1-α-phosphatidyl-t-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\(_{16}\)) (pNPMP), 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-Palmityl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-Palmityl-2-oleoyl-sn-glycero-3-phosphate (POPA), 1-Palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-Palmityl-2-oleoyl-sn-glycero-3-phospho-rac(1)-glycerol (POPG) and L-α-Lyso phosphatidylcholine (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-Phosphatidylglycerolinositol (PI) from wheat ovule was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). TOYOPEARL Pheny-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 (DBB 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 at 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) was used as a host for the expression of PLA₁, which 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 x 9 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 x 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 x 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). Following this was followed by applying the sample to a Hit trap 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), 0.5% (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 A405 of 16,980 M⁻¹ cm⁻¹. One unit of enzyme 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 A₁ 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 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 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 (Ea) 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 each concentration of the chemicals examined. The positional specificity on the hydrolytic reaction was determined by incubating at 50 °C for 5 min with 2.5% (wt/vol) EGGL in 0.1 M sodium acetate (pH 5.6) containing 25 mM EDTA and each percentage of Triton X-100.

4.7. Peptide sequencing

The purified protein was resolved by SDS–PAGE and then electroblotted onto a PVDF membrane (Immobilon-PSQ 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 (Pierce 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 (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 nanoAcquiUPLC 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 95% 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 desolation temperatures were set to 90 and 200 °C, respectively, and the cone, desolation 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 pmol/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 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 Kieser et al. [23]. Oligonucleotides were synthesized based on the N-terminal (AAGGYVALGDS) and internal amino acid sequences (AP-SANVVV and FVESTLPGR) of the enzyme for use in PCR with the sense primer N 5’-gcsgcgsggctagctsgc-3’ and antisense primer A1
ng of the buffer, 12.5 pmol of each primer, 0.5 U of MightyAmp DNA polymerase (Takara Bio Inc., Japan) and −4.52 ng of \textit{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 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 PLA1, inverse PCR was performed with the forward primer 5′-ggtacctgctctcctcgccggtg-3′ and the reverse primer 5′-cctctggcctcctcctcctcgccggtg-3′. Genomic DNA was digested with restriction endonucleases (Sph I, Nco I, Sac I, Sac II, Kpn I, Sau 3A I 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 40 s 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 amino acid sequence of S. albidoflavus PLA1 showed 100% identity to that of a secreted hydrodase of \textit{S. albus} J1074 (DDB database under accession number D68AL1). To clone the PLA1 gene, reverse primer CRV (5′-tccgcttgagctcagc-3′) was designed based on the 3′ region of the secreted hydrodase gene of \textit{S. albus} J1074, PCR was carried out using the sense primer N1 (5′-ggcgcgccgctatcgc-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 \textit{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 PLA1 gene on pPLA1 was performed as described above.

4.11. Expression and purification of PLA1

\textit{S. lividans} 1326 (NBRC15675) was obtained from the NITE Biological Resource Center (Chiba, Japan). \textit{S. lividans} 1326, possessing no PLA1 activity, was used as a host for PLA1 expression. To replace the \textit{Bgl II} site in the PLA1 gene, the \textit{S. albidoflavus} PLA1 gene (\textit{pla}) was amplified from chromosomal DNA by a two-step PCR. The first PCR was performed using the following primers: 5′-aagtcgaccgccggccgctacg-3′ (Nhe I-F1) containing the first codon (\textit{Nhe I}, italic; \textit{Ala}, underlined) of mature PLA1 and 5′-gaccgatctgcgcttcg-3′ (\textit{Bgl II}-repair RV1); 5′-agcgcggccgctacg-3′ (\textit{Bgl II}-repair F2) and 5′-ataagtcgaccgccggccgctacg-3′ (\textit{Bgl II}-RV2; \textit{Bgl II}, italic). The PCR reaction mixture (25 μL × 10) contained: 1 μL of DNA, 200 μM of dNTP, 1 μL of DMSO, 0.5 U of KOD-Plus-DNA polymerase and 233 ng of the \textit{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 (\textit{Nhe I}-F1) and the reverse primer (\textit{Bgl II}-RV2). PCR was carried out in a reaction mixture (25 μL × 6) containing 1 μL of buffer #2, 30 nmol of MgSO\textsubscript{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 S. lividans with \textit{Bgl II} and \textit{Bgl II}-repair RV1, 338 ng of the products (ca. 100 bp) amplified using the primer set of \textit{Bgl II} and \textit{Bgl II}-repair 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 \textit{Nhe I} and \textit{Bgl II}, and then subcloned into the \textit{Nhe I} and \textit{Bgl II} sites of pUC720 [24] carrying the promoter, signal sequence and the terminator region of the phospholipase D (PLD) ORF from \textit{Streptoverteccillum cinnaeum}. This expression plasmid was sequenced and designated as pUC720/pla. The transformation techniques of Kieser et al. were followed for \textit{S. lividans} [23]. Transformants were screened using lecithin-eminulsified nutrient plates according to Kim and Rhee [19]. Clones exhibiting a clear halo were collected and clones having the highest activity were selected. The PLA1 produced by the transformed \textit{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 PLA1 gene, designated \textit{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 pUC720/pla as a template. The mutant proteins were produced extracellularly by the transformed \textit{S. lividans}. Clones exhibiting a cloud halo around their colony and no halo were screened and selected. PLA1 activity of the transformed \textit{S. lividans} 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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