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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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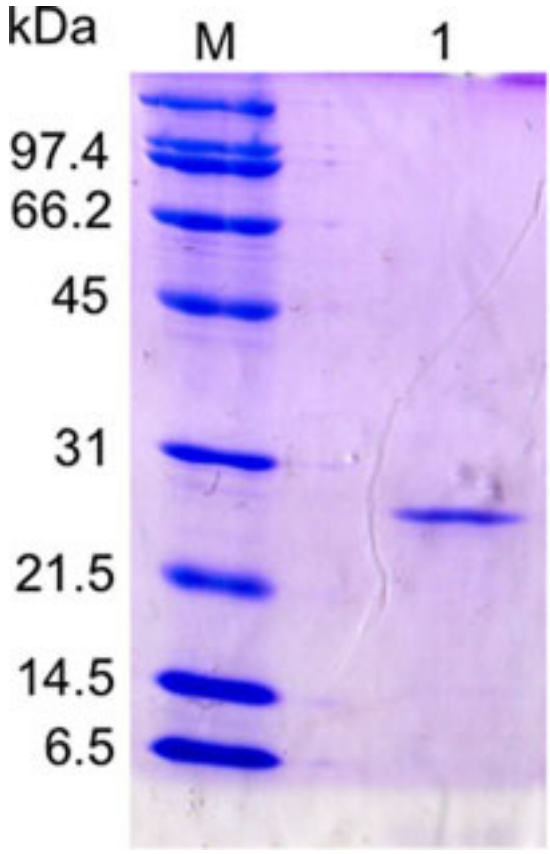

Fig. 1. SDS–PAGE analysis of purified PLA_1 from *S. albidoflavus*. Lane M, molecular marker; lane 1, purified PLA_1.

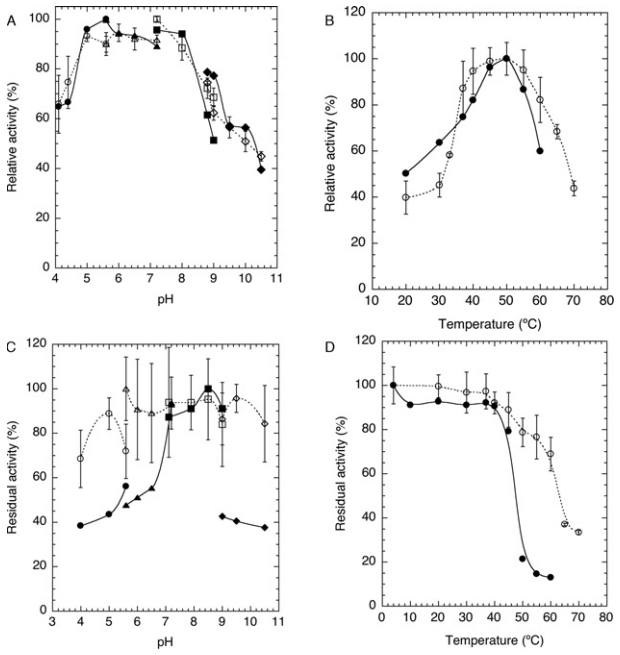

Fig. 2. Effect of pH and temperature on PLA_1 activity (A, B) and stability (C, D) of the wild-type (closed symbols) and recombinant enzyme (open symbols) for lecithin (EGGL) hydrolysis. (A) The enzyme activity was assayed at 37 °C for 5 min with 2.5% (wt/vol) EGGL in 0.12 M of each buffer containing 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.