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Biochimica et Biophysica Acta 1722 (2005) 331–342

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Regular paper

Identification of a key amino acid residue of *Streptomyces* phospholipase D for thermostability by in vivo DNA shuffling

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Received 23 July 2004; received in revised form 20 December 2004; accepted 4 January 2005
Available online 2 February 2005

Abstract

To isolate thermostability-related amino acid residues of *Streptomyces* phospholipase D (PLD), we constructed a chimeral genes library between two highly homologous *plds*, which exhibited different thermostabilities, by an in vivo DNA shuffling method using *Escherichia coli* that has a mutation of a single-stranded DNA-binding protein gene. To confirm the location of the recombination site, we carried out the restriction mapping of 68 chimeral *pld* genes. The recombination sites were widely dispersed over the entire *pld* sequence. Moreover, we examined six chimeral PLDs by comparing their thermostabilities with those of parental PLDs. To identify a thermostability-related amino acid residue, we investigated the thermostability of chimera C that was the most thermolabile among the six chimeras. We identified the thermostability-related factor Gly-188, which is located in the alpha-7 helix of PLD from *Streptomyces septatus* TH-2 (TH-2PLD). TH-2PLD mutants, in which Gly-188 was substituted with Phe, Val or Trp, exhibited higher thermostabilities than that of the parental PLD. Gly-188 substituted with the Phe mutant, which was the most stable among the mutants, showed an enzyme activity almost the same as that of TH-2PLD as determine by kinetic analysis.

Keywords: Phospholipase D; Thermostability; *Streptomyces*; Biocatalyst

1. Introduction

Phospholipase D (EC3.1.4.4., PLD), which is present in mammals, plants, and bacteria, catalyzes the hydrolysis of the ester bond between the phosphatidic acid and alcohol moieties of phospholipids. Almost all PLDs contain two separate copies of the highly conserved 'HxKxxxxD' motif, the HKD motif, in their structures. Recently, the deletion mutants of PLDs that contain only an N- or a C-terminal HKD domain has been reported to exhibit no catalytic activity in rat brain PLD1 and *Streptomyces* PLD. In addition, when the N- and C-terminal halves of PLD coexist, the two fragments physically associate and the catalytic activity of the enzyme is restored [1,2]. Therefore,

Abbreviations: PLD, phospholipase D; PCR, polymerase chain reaction; PpNP, phosphatidyl-*p*-nitrophenol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Amp, ampicillin; Kan, kanamycin; Tet, tetracycline; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; MinA, Minimal A; P-Gal, phenyl-β-D-galactopyranoside; CD, Circular dichroism; DSC, differential scanning calorimetry

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doi:10.1016/j.bbagen.2005.01.009

it is presumed that two HKD motifs are required for PLD activity. Moreover, this enzyme catalyzes the transphosphatidylation reaction in the presence of alcohols as a nucleophile donor. *Streptomyces* PLD with higher transphosphatidylation activity is a useful catalyst in the synthesis of phospholipids, including rare natural phospholipids and novel artificial phospholipids [3,4]. The transphosphatidylation reaction is usually performed in biphasic systems consisting of water-immiscible organic solvents. Thus, the stability of *Streptomyces* PLD is important when it is used as a biocatalyst in these systems.

DNA shuffling is performed using two distinct methods, in vitro [5,6] and in vivo [7,8]. These DNA shuffling methods may possibly open a new field of protein engineering that may lead to the improvement of enzyme activity, stability and substrate specificity by the recombination of several genes with high sequence homology. Furthermore, this methodology is also applied to laboratory-directed evolution techniques. The in vitro DNA shuffling method using polymerase chain reaction (PCR) is a very powerful tool for studies of molecular evolution. When this method is carried out to improve protein functions, one of the important factors is the establishment of a selection procedure. However, it is impossible to apply the in vitro DNA shuffling method to toxic proteins, such as PLD [9], because of its lethality to the host strains. On the other hand, in vivo DNA shuffling using a linear plasmid is performed by *rec*A-dependent recombination and the resulting chimeral proteins are limited in number and variability [7,8]. Therefore, we attempted to overcome these problems with a novel in vivo DNA shuffling method using an *Escherichia coli* strain possessing a mutation in the single-stranded DNA-binding protein (SSB) gene. The *E. coli ssb-3* allele, whose SSB protein has a mutation at amino acid number 15 at the N-terminus (Gly to Asp) [10], was shown to increase deletion rate 250-fold that of the wild-type *E. coli* [11]. It is assumed that inducing a deficiency in repair systems is effective for stabilizing heteroduplex DNA strands, which contain mismatches that are not stable in wild-type *E. coli*, and is the cause of the *ssb-3* mutant having a higher frequency of recombination in low-homology regions than the wild type [11].

Recently, we have isolated two *Streptomyces* species that produce PLDs with different thermostabilities [12]. PLD from *Streptomyces septatus* TH-2 (TH-2PLD) is relatively thermostable, and that from *Streptomyces halstedii* K1 (K1PLD) is thermolabile. We are interested in the thermostability-related amino acid residues of *Streptomyces* PLD for protein engineering, but these residues are unknown to date. Thus, to determine the relationship between the structure and thermostability of *Streptomyces* PLDs, we applied the in vivo DNA shuffling method using the *E. coli ssb-3* mutant.

In this experiment, we constructed a chimeral-gene library between thermolabile and thermostable PLDs, and characterized the resulting chimeral PLDs by comparing their thermostabilities. Furthermore, the most thermolabile chimera among the chimeral PLDs was selected to investigate the molecular basis of thermostability. We constructed TH-2PLD mutants with improved thermostabilities on the basis of the information obtained from a comparative study.

2. Materials and methods

2.1. Materials

Phosphatidyl-*p*-nitrophenol (PpNP) was prepared from soybean phosphatidic acid and *p*-nitrophenol, according to the method of D'Arrigo et al. [13]. The Hiprep 16/10 SP column (1.6×10 cm) and molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Amersham Biosciences. A protein assay kit and gels for SDS-PAGE were obtained from Bio-Rad. The chemicals used were commercially available products of the highest grade.

2.2. Strains and growth conditions

The *E. coli* strains EJ2848 [11] and EJ2885 were used for in vivo DNA shuffling. EJ2885 is a tetracycline-sensitive derivative of strain EJ2882 [11]. JM109 (TOYOBO) and DH5α (TOYOBO) were used as hosts for the recombinant plasmid. The *E. coli* strain BL21-Gold (DE3) (Stratagene) was used to express *pld* genes. Unless otherwise indicated, the cells were grown at 37 °C.

2.3. Medium

The LB liquid medium and LB solid agar medium used were commercially available. These media were used with the addition of appropriate antibiotics: 50 μg/ml ampicillin (Amp), 50 μg/ml kanamycin (Kan), 20 μg/ml tetracycline (Tet), 20 μg/ml chloramphenicol (Cm), 100 μg/ml streptomycin (Sm), and 40 μg/ml spectinomycin (Sp). The SOB and SOC, H and Minimal A (MinA) media were prepared as previously described [11]. The lactose minimal medium was the same as the MinA medium except for the addition of 0.2% lactose and the removal of phenyl-β-D-galactopyranoside (P-Gal).

2.4. Nucleic acid accession numbers

The sequences of *th-2pld* and *k1pld* genes have been submitted to DDBJ under accession nos. AB058783 and AB062136, respectively.

2.5. Construction of plasmids for DNA shuffling

A plasmid, pKT1002, for in vivo DNA shuffling, which contained the thermolabile *k1pld* gene and the relatively