Simultaneous Enhancement of Thermostability and Catalytic Activity of Phospholipase A₁ by Evolutionary Molecular Engineering

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The thermal stability and catalytic activity of phospholipase A₁ from Serratia sp. strain MK1 were improved by evolutionary molecular engineering. Two thermostable mutants were isolated after sequential rounds of error-prone PCR performed to introduce random mutations and filter-based screening of the resultant mutant library; we determined that these mutants had six (mutant TA3) and seven (mutant TA13) amino acid substitutions. Different types of substitutions were found in the two mutants, and these substitutions resulted in an increase in nonpolar residues (mutant TA3) or in differences between side chains for polar or charged residues (mutant TA13). The wild-type and mutant enzymes were purified, and the effect of temperature on the stability and catalytic activity of the enzymes was investigated. The melting temperatures of the TA3 and TA13 enzymes were increased by 7 and 11°C, respectively, compared with the melting temperature of the wild-type enzyme. Thus, we found that evolutionary molecular engineering was an effective and efficient approach for increasing thermostability without compromising enzyme activity.

Enzymes can be tailored for optimal performance in industrial applications by evolutionary molecular engineering, which is also called directed evolution or in vitro evolution (2, 26). Many successfully engineered proteins have been described, and enzymes with enhanced properties, such as sufficient stability (12, 38), a high level of activity (34), altered substrate specificity (36), and the ability to interact correctly with surfaces (9), have been developed for industrial applications (3). Judging from the recent successes of irrational design approaches, such as mutagenic PCR (6) and DNA shuffling (33) followed by screening for improved properties, directed evolution may be more efficient than rational design involving both iterative computer design and site-directed mutagenesis.

Thermostability is often a primary goal when workers try to improve the properties of an industrial enzyme, since high temperatures in industrial processes provide such benefits as increased substrate solubility, decreased viscosity of the medium, and a lower risk of microbial contamination. In other words, thermostable enzymes are of considerable biotechnological interest since their enhanced stability could greatly reduce enzyme replacement costs or permit processes to be carried out at high temperatures. Recently, many attempts have been made to understand the principles underlying the stability of proteins; introducing disulfide bonds (25), chemical cross-links (20), salt bridges (11), and metal binding sites (22) and increasing intramolecular hydrophobic packing (37) have all been proposed. However, the use of these techniques is limited to the enzymes whose three-dimensional structures have been determined. In the case of enzymes for which extensive information concerning structure and function is not available, directed evolution is a powerful tool for studying or engineering thermostability and catalytic activity of the enzymes.

Phospholipase A₁ hydrolyzes the 1-acyl group of a phospholipid to lysophospholipid and fatty acid. Recently, it has been reported that phospholipase A₁ and the well-characterized enzyme phospholipase A₂ play important biological roles in both phospholipidosis, a pathological condition in which phospholipids accumulate in lysosomes (24), and virulence factors for bacterial and fungal pathogenesis (15, 29). In addition to its physiological roles, phospholipase A₁ is of particular interest for industrial applications as it yields 2-acyl-lysophospholipids.

Lysophospholipids are excellent emulsifiers and are particularly suitable for use in many industrial applications, such as food technology and the cosmetics and pharmaceutical industries. Lysophospholipids are able to enhance emulsification in oil-water emulsions due to increased solubility in water, form emulsions which are more stable to changing pH and temperature conditions, and keep emulsions stable in the presence of magnesium or calcium ions. Lysophospholipids also have several physiological functions, including a role in platelet aggregation and a role as a signaling molecule (8). They also affect ripening and storage characteristics of fruits, leaves, and green plant tissue (21).

Phospholipase A₁ gene plaA of Serratia sp. strain MK1, encodes a 321-amino-acid monomer and has been cloned as described previously (32). Even though phospholipase A₁ has been found in various organisms (5, 7, 15, 23, 29), the use of phospholipase A₁ for phospholipid modification has been limited due to its low stability. Moreover, crystallographic and structural data for phospholipase A₁ have not been obtained yet. Since the evolutionary protein engineering technique was a more useful approach for modifying enzymes in the absence of such information, we attempted to use evolutionary molecular engineering to improve phospholipase A₁ for practical purposes. The evolutionary engineering technique used in this study involved preparing protein variants by using mutagenic PCR, expressing the protein, and then screening for mutants with improved thermostability. In this study, we enhanced the catalytic activity and the thermostability of phospholipase A₁ from Serratia sp. strain MK1 by the evolutionary technique. To do this, we developed a filter-based...
screening system with which we could identify both properties on a single processed filter; with this system the in which catalytic activity of a heat-treated mutant library could be assayed on a phosphatidylcholine-containing gel at a normal temperature. Below we describe two thermostable phospholipase A1 mutants which exhibited higher levels of activity at the temperatures examined than the wild type exhibited.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Escherichia coli XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdRI7 supE44 thi-1 [F' proAB lacIq ZA15 Tn5 (Tet)] (Strategene, San Diego, Calif.) was used as the host strain. Plasmid pMJ1 encoding phospholipase A1 gene plaa of Serratia sp. strain MK1 (32) was used as a template in PCR mutagenesis for evolutionary molecular engineering. Plasmid pSTV26 (Takara Shuzo, Shiga, Japan) was used to construct a mutant enzyme library. Recombinant strains were grown in Luria-Bertani (LB) medium or TYSN medium (20 g of Bacto Tryptone [Difco Laboratories] per liter, 10 g of yeast extract per liter, 5 g of NaHPO₄ per liter 10 g of KNO₃ per liter, 5 g of NaCl per liter). When necessary, antibiotics, such as ampicillin (100 μg/ml) or chloramphenicol (50 μg/ml), were added to the media.

DNA manipulation and sequencing. Standard recombinant DNA manipulation techniques were used for isolation of plasmid DNA, restriction digestion, ligation, and transformation into E. coli (28). All restriction enzymes, DNA-modifying enzymes, and related reagent mixtures were purchased from Sigma, New England Biolabs Inc., or Boehringer Mannheim. DNA fragments required for subcloning experiments were gel purified with a QIAGEN kit (QIAGEN, Hilden, Germany). DNA was sequenced by cycle sequencing by using an ABI PRISM BigDye Primer cycle sequencing kit and AmpilTag DNA polymerase (Perkin-Elmer, Foster City, Calif.).

Plasmid construction. Plasmids pTA3 and pTA13, which were isolated from the second random mutation library, were derivatives of plasmid pSTV26 (Takara Shuzo) containing the evolved phospholipase A1 gene. Plasmids containing six consecutive histidine-tagged (6× His-tagged) and evolved phospholipase A1 genes were generated from plasmids pTA3 and pTA13, respectively, by PCR amplification by using cloned Pho polymerase (Strategene) and a primer that included an 18-amino-acid 6× His tag-encoding sequence. Construction of a plasmid harboring the 6× His-tagged and native phospholipase A1 genes was identical to construction of thermostable variants, except that plasmid pMJ1 was used as a template. The derivatives were used for purification of wild-type phospholipase A1 and two thermostable phospholipase A1 variants.

Random mutagenesis and mutant library construction. Random mutagenesis of the plaa gene was carried out by performing mutagenic PCR. Two oligonucleotides flanked by EcoRI and BamHI restriction sites, 5′-CCGAATTCCTTGA GTC-3′ and 5′-GGCGATCCCATGAGGCGGCTTTC-3′, and 5′-GGCGATCCCATGAGGC CGC CATCGCCCTCC-3′, were used as forward and reverse primers, respectively. In order to obtain both the desired level of mutation (three or four amino acid substitutions) and base substitutions without mutational bias, the conditions used for PCR mutagenesis were optimized for DNA manipulation. The DNA was purchased from Sigma, New England Biolabs Inc., or Boehringer Mannheim.

RESULTS

Isolation of thermostable mutant phospholipase A1.

Enzyme purification. For purification of native phospholipase A1 and evolved thermostable phospholipase A1, a 6× His affinity tag was fused to the C terminus of each enzyme as described above. Each E. coli strain harboring recombinant wild-type or thermostable mutant phospholipase A1 was grown at 30°C in TYSN medium. Crude cell extract in a solution containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.05% Triton X-100, and 10% glycerol was purified with nickel-nitritofluoracetate acid agarose resin (QIA-GEN) and imidazole-containing buffer. After extensive washing, the bound protein was eluted with 250 mM imidazole-containing phosphate buffer. The active eluates were applied to a Sephacyr S-200 column (Amersham-Pharmacia). The buffer used was 75 mM CaCl2–300 mM NaCl in 50 mM sodium phosphate buffer. Fractions containing enzymatic activity were pooled and then concentrated by applying them to a centrifugal filtration device (Centricon Concentrators, Amicon Millipore). The protein was dialyzed against 50 mM phosphate (pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride protease inhibitor. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to determine the purity of the protein.

Enzyme activity. Free fatty acids released by catalysis of phospholipase A1 were measured by the pH stat titration method. The activity was measured at an assay temperature of 37°C with an autotitrator and an autoburette (Radiometer Copenhagen, Lyon, France). An aqueous emulsion containing 3.4 mM phosphatidylcholine, 10 mM CaCl₂, and 2.6 mM decycholeic acid was used as the substrate. Phospholipase A1 activity was expressed in micromoles of phosphatidylcholine hydrolyzed per minute. The protein concentration of each purified enzyme was determined by using the Bio-Rad protein assay reagent (Bio-Rad Life Science Group, Hercules, Calif.).

Thermal stability and kinetic parameters. Heat treatment of the purified enzymes was performed with a programmable temperature controller. An enzyme in 50 mM sodium phosphate (pH 8.0) was incubated at different temperatures and cooled immediately in an ice bath. The phospholipase A1 activity remaining after the heat treatment was measured by using the pH stat titration method described above. The Kₗ and kcat values were estimated from the intercepts of Lineweaver-Burk plots. The enzyme activity was measured as described above, except that the concentrations of the phosphatidylcholine substrate used were 200, 100, 67, 50, and 40 μM.

Plasmid pMJ1 containing the 960-bp plaa gene (GenBank accession no. U37262) was used as a template in the first mutagenic PCR in order to introduce random point mutations into the phospholipase A1-encoding region. After the first cycle of PCR mutagenesis and screening, eight positive clones containing thermostable phospholipase A1 variants were isolated from approximately 10,000 colonies. The phospholipase A1 activities of these mutants were detected on indicator plates (PCY plates) after heat treatment at 70°C for 2 h, although no halo was detected under the same conditions in the case of the wild-type enzyme (Fig. 1). Plasmids encoding each mutant phospholipase A1 were isolated from eight clones and pooled (equal amounts of all plasmids). A possible limitation of sequential engineering approaches is that a particular mutational pathway or its branches may be brought about by selecting only one highly enhanced mutant to parent subsequent generations. Therefore, we used this plasmid mixture as template DNA in...
the second mutagenesis step in order to obtain as many available evolutionary pathways to thermostability as possible. The resultant mutagenic PCR products were subcloned in order to prepare a mutant library for the second screening. After a heat treatment which involved incubating the second mutant library at 80°C for 3 h, we finally isolated two clones from which two plasmids (pTA3 and pTA13) were prepared. These two plasmids were sequenced to identify the locations of the substituted amino acid residues.

DNA sequences and thermal stabilities of wild-type phospholipase A1 and mutant phospholipase A1 variants. DNA sequences of the two thermostable phospholipase A1 genes were determined. Two phospholipase A1 mutants, TA3 and TA13, had seven and six amino acid substitutions, respectively (Fig. 2). Mutant TA3 had substitutions that resulted in amino acids which had uncharged and nonpolar side chains (Q32P, T39A, E105V, M153I, and S158I). On the other hand, substitutions in amino acids that had polar or charged side chains were found in mutant TA13 (E35Q, S101N, M153K, L157W, L280Q, and K290N).

We placed the 6× His tag at the C terminus of each protein in order to purify the wild type and the thermostable mutants (TA3 and TA13). Each recombinant strain which produced the 6× His-tagged proteins was cultured in TYSPN medium, and the wild-type phospholipase A1 and mutant phospholipase A1 variants were purified as described above. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the wild-type phospholipase A1 and evolved phospholipase A1 variants were purified to homogeneity (data not shown). The thermal stabilities of the purified enzymes were estimated by measuring the remaining activities at 40°C after heat treatment at various temperatures (Fig. 3). Figure 3 shows that the melting temperatures (Tm) of thermostable mutant enzymes TA3 and TA13 (Tm is the temperature at which 50% inactivation occurs after heat treatment for 20 min) were higher by 7 and 11°C, respectively, than the Tm of the wild-type enzyme.

Activities of thermostable mutant phospholipase A1 variants. The kinetic parameters of the wild-type and mutant enzymes are shown in Table 1. The wild-type phospholipase A1 and mutant phospholipase A1 variants exhibited typical Michaelis-Menten saturation kinetics when the initial velocity was plotted against the concentration of the substrate. The Michaelis constant (Km) of the mutant TA3 enzyme was slightly (8.2%) less than the Km of the wild-type enzyme. In contrast, the Km of the mutant TA13 enzyme was slightly
Amino acid changes generated by directed evolution might give extra stability to loops (35). Therefore, one possible explanation for the thermostability of the TA3 enzyme is that the amino acid changes generated by directed evolution might have caused the structure to change so that the hydrophobic core packing was tighter. In contrast to TA3, however, most of the mutations in TA13 resulted in changes in side chains in polar or charged residues. It has been proposed that creating charged and hydrogen bonding is important in the stabilization of proteins (1, 14, 16). Although the estimate obtained without a precise three-dimensional structure of phospholipase A1 is somewhat imperfect, stabilization in TA13 is believed to be achieved by the formation of additional hydrogen bonds or charged pairs. In recent years, workers have proposed a variety of theories to explain the enhanced thermostability of proteins in terms of amino acid composition (13, 27). Judging from a variety of the proposed mechanisms (1, 4, 13, 14, 16, 27, 31, 35), mutants TA3 and TA13 could have obtained enhanced thermal stability through different evolutionary pathways. In other words, it is thought that the pathway which results in greater thermostability in mutant TA3 is the pathway involved in the increase in internal hydrophobicity; on the other hand, the pathway in mutant TA13 is the pathway involved in evolution of additional hydrogen bonds or charged pairs. We, therefore, suggest that diverse enzymes that evolve from the different evolutionary pathways can be obtained, unless the available evolutionary pathways are restricted to thermostability pathways.

Although sometimes stabilization of an enzyme is achieved with a significant loss of catalytic activity (30), we obtained phospholipase A1 mutants that exhibited both increased activity and increased thermostability. Molecular flexibility of an enzyme is essential for both binding to substrates and catalysis. It has been proposed that thermophilic and mesophilic proteins exhibit similar degrees of flexibility at their respective optimum temperatures (17), although thermophilic proteins are thought to be less flexible than mesophilic proteins at lower temperatures. Moreover, the specific activity of a thermophilic enzyme at its optimum temperature is often comparable to the specific activity of a homologous enzyme from a mesophile at its optimum temperature (10). It has been reported frequently that thermostability and catalytic activity at low temperatures are not mutually exclusive, suggesting that activity and thermostability are at least partially independent properties and that these two properties are not incompatible in an enzyme (12, 38). Thus, if an evolutionary engineering experiment is not limited to one property (i.e., thermostability) but allows workers to determine both properties (i.e., thermostability and catalytic activity), as shown in our screening study, thermostability and catalytic activity can be enhanced simultaneously in an enzyme. When the catalytic activities were measured at a number of temperatures in order to verify this, our evolved thermostable phospholipase A1 variants not only were stable at the temperatures examined but also exhibited high levels of catalytic activity (data not shown). Thus, our results also indicate that evolution of molecular engineering is a powerful technique for enhancing the properties (i.e., thermostability and catalytic activity) of an enzyme that is to be used at a high temperature.

Now we are investigating the applied aspects of the engineered thermostable phospholipase A1. It is frequently noted that a higher temperature is used in enzyme reactors to produce useful phospholipid derivatives due to the insolubility of phospholipids at room temperature. Therefore, we are attempting to produce lysophospholipids by using the novel phospholipase A1 that is more thermostable and more active than the wild-type enzyme.

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