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An *in vitro* evaluation of a thermostable pectate lyase by using error-prone PCR

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Abstract

Thermostable pectate lyase 47 (PL 47) prepared from *Bacillus* sp. TS 47 was mutated by a random mutation with error-prone PCR. The mutated *PL 47* gene was expressed in *Escherichia coli* and the variants were screened over the PL activity using a plate assay method. Three less-thermostable mutants were isolated and their enzymes (LT-PLs) were isolated and characterized. The LT-PLs were inferior to the PL 47 in their thermostability and thermoactivity. Analyses of the amino acids substituted in the LT-PLs suggested that Ala325 in the PL 47 contributes to its thermostability.

Keywords: Pectate lyase; Thermostability; Error-prone PCR; Random mutation

1. Introduction

Pectate lyases (PLs) are produced by a number of bacteria and some pathogenic fungi. These enzymes liberate water-soluble pectic substances by restricted hydrolysis of water-insoluble protopectin in plant cell walls and were originally named protopectinase (PPase) [1]. Sakai and Okushima [2] found a PPase in a yeast strain and, thereafter, other microbial PPases also have been found [3]. PPases are classified into two types depending on their reaction mechanisms: A-type PPases react with the polygalacturonic acid region of protopectin (inner site) and the other B-type with the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents (outer site). The A-type PPases are further classified into two sub-types: A1-type PPases catalyze the hydrolytic cleavage of polygalacturonic acid (polygalacturonase activity) and A2-type PPases catalyze the split of the glycoside linkages of polygalacturonic acid in protopectin by a *trans*-elimination reaction, and are commonly called pectate lyases [4]. Sakai et al. applied these enzymes to a biochemical process for scouring cotton fabric and named it “BioScouring” [5]. To improve the BioScouring process regarding the enzyme reaction rate, Sakai et al. attempted to use a thermostable PL.

Recently, a gene that encodes a thermostable PL (PL 47) having an optimum temperature of 70 °C from a thermophilic *Bacillus* sp. TS 47 was cloned, sequenced, and expressed in mesophilic *B. subtilis* [6]. The *PL 47* gene codes for a 416 amino acid protein. Bacterial mesophilic PLs have been found in *Erwinia* [7], *Bacillus* [8], *Clostridium* [9], *Xanthomonas* [10], and *Pseudomonas* [11]. Compared with the mesophilic PLs, less knowledge has been accumulated for the thermostable PLs; Karbassi et al. found a thermostable PL produced by *B. stearothermophilus*, a thermophilic bacterium, that showed an optimal activity at 70 °C and pH 8.0 [12]. Here, we report an *in vitro* evaluation of a thermostable pectate lyase by using error-prone PCR to elucidate the essential molecular structure that makes PL thermostable.

2. Materials and methods

2.1. Bacteria and plasmids

The host strain was *Escherichia coli* JM109 (Toyobo, Osaka, Japan) and the plasmid used was pUC18 (Amersham Biosciences Corp., New Jersey, USA). The host and plasmid

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for expressing the recombinant *PL 47* gene were *B. subtilis* MI112 [13] and pUB110 [14], respectively. To produce PL from *Bacillus* sp. TS 47, the microorganism was cultured in a medium containing 0.5% polygalacturonic acid (PGA), 1% Tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0, at 60 °C for 8 h on a shaker.

Bacillus sp. TS 47 and its mutants, which will be described below, are stocked as the collection of IGA Co. Ltd.

2.2. Error-prone PCR mutagenesis of the thermostable pectate lyase

The conditions for PCR to introduce random mutations into the *PL 47* gene were selected so as to obtain mutants having less amino acid substitutions as far as possible: a 100 μl reaction mixture contained 6.7 mM $MgSO_4$, 60 μM $MnSO_4$, 200 μM each of dNTP, 50 nM each of oligonucleotide primers, 10 ng template DNA, and 2.5 units *Taq* DNA polymerase (TaKaRa, Kyoto Japan). The oligonucleotide primers were the forward, 5′-CGACGTTGTAAAACGACGGCCAGT-3′, and the reverse, 5′-GGAAACAGCTATGACCATGATTAC-3′ (TaKaRa, Kyoto, Japan). A DNA fragment, pPL47, containing the full length of the *PL 47* gene was used as a template. PCR was performed in a PCR equipment (DNA Thermal Cycler, Sanyo Medical, Osaka, Japan) for 25 cycles, consisting of a cycle of 1 min at 94 °C, 1 min at 65 °C, and 1 min at 72 °C. The PCR was monitored by determining molecular masses of PCR products using agarose-gel electrophoresis. The amplified DNA fragments were treated with *Hin* dIII and *Pst* I, and ligated with *Hin* dIII-*Pst* I-digested pUC18 to construct a plasmid-harboring *PL 47* mutant gene. The ligations were performed by incubating at 16 °C overnight, and *E. coli* JM109 was transformed with the constructed plasmids.

2.3. Screening

Less-thermostable PL 47 mutants (LT-PL) were obtained by expressing the modified genes in *E. coli* JM109. *E. coli* JM109 cells were transformed with the constructed plasmid according to Hanahan [15]. The transformants were grown on duplicated LB agar plates containing 100 μg/ml ampicillin at 37 °C for 24 h and the agar plates were overlaid with 10 ml soft agarose composed of 1% pectin, 1% Agarose H 14 (TAKARA BIO, Shiga, Japan), and 20 mM Tris–HCl buffer (pH 8.0). After an 8-h incubation at 60 °C or 70 °C, the plates were flooded with a 0.1% ruthenium red solution. Production of PLs was detected as a formation of clear zones around bacterial colonies.

2.4. Protein expression and purification

The PLs encoded on LT-mutants were purified from culture filtrates of *B. subtilis* transformants by column chromatographies on a butyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) and Superdex 75 HR 10/30 (Amersham Biosciences Corp., New Jersey, USA). The culture filtrate of the transformants was dialyzed against 20 mM Tris–HCl buffer, pH 7.5, and made up to 50%-saturation of ammonium sulfate. The precipitated fraction was dissolved in 20 mM Tris–HCl, pH 7.5, containing ammonium sulfate of a 20% saturation and put on a butyl-Toyopearl 650 M column (10 mm × 160 mm) equilibrated with the buffer. The column was washed thoroughly with the buffer, and the adsorbed proteins were eluted with 20 mM Tris–HCl, pH 7.5, containing a descending linear gradient of ammonium sulfate from 20- to 0% saturation at a flow rate of 2 ml/min, and a 2 ml fraction for each was collected. The fractions containing PL activity were pooled and dialyzed against a 20 mM Tris–HCl buffer, pH 7.5, containing 100 mM NaCl. The solution was concentrated using Centricon-10 (Millipore, Bedford, MO, USA) and put on a Superdex 75 HR 10/30 column equilibrated with a 20 mM Tris–HCl buffer, pH 7.0, containing 100 mM NaCl. The enzyme was eluted with the buffer solution at a flow rate of 250 μl/min, and a 250 μl fraction for each was collected. The active fractions were combined and dialyzed against the buffer solution.

2.5. Enzyme assay

A reaction mixture (1 ml) containing 0.1% PGA in 100 mM Tris–HCl, 0.5 mM $CaCl_2$ (pH 8.0), and an appropriate amount of enzyme solution was incubated at 70 °C for 30 min. The reaction was stopped by keeping the mixture in a boiling water bath for 5 min. The reaction was measured as increasing at 235 nm. Liberation of 1 μmole of unsaturated galacturonate in the reaction mixture in 1 min was defined as one unit of the enzyme activity. There, the molar extinction coefficient of the unsaturated galacturonate at 235 nm was regarded to be 4,600 M^{-1} [16]. The measurements for less-thermostable PLs (LT-PLs) were done at the respective optimum temperature.

2.6. PCR mutagenesis

PCR mutagenesis on a single amino acid (Leu118Ser, Leu221Phe, Asn280Lys, Pro325Ala, Met396Ile) was carried out by a two-step PCR method [17]. The mutagenic primers (forward) for the first PCR reaction were as follows: Leu118Ser, 5′-CGGGTACGTTCGCAGAAAAAAC-3′; Leu221Phe, 5′-GGCACATACTTTGGGAGACCG-3′; Asn280Lys, 5′-CCACAATTATTATAAAAATGTCACACAGCGTTTGC-3′; Pro325Ala, 5′-GTTTTTTCTCAGATTTATGCCAAAATAATTATTT-3′; Met396Ile, 5′-GTTTTATCATGTTATACACCCTACTCCAAG-3′. The mutagenic primers (reverse) were complementary to the mutagenic primers (forward). The first PCR reaction was carried out as described above, using KOD I polymerase (Toyobo, Osaka, Japan) and combinations of the forward and reverse primers for the desired mutation. The reaction mixture was heated at 98 °C for 5 min and, then, treated for 25 cycles, consisting of a cycle of 15 s at 98 °C, 10 s at 55 °C, and 30 s

at 74 °C. The PCR products were successively heated at 98 °C for 5 min, 65 °C for 5 min, and 37 °C for 5 min. The mixture was annealed at 74 °C for 5 min after addition of KOD I polymerase. The second PCR reaction was performed as described for the first step, using the forward and reverse oligonucleotide primers. The product was purified and cloned into pUC18. The desired mutation was verified by DNA sequencing.

PCR mutagenesis on two amino acids (Leu221Phe and Pro325Ala; Asn280Lys and Pro325Ala) was also carried out similarly.

3. Results and discussion

3.1. Random mutagenesis of thermostable pectate lyase

Wan et al. found that the concentration of Mn^{2+} is a definitive factor for the random mutagenesis [18]. Based on their findings, the effects of the Mn^{2+} concentration were examined for the random mutagenesis of the *PL 47* gene using error-prone PCR. In the presence of 1.0 mM Mn^{2+}, no DNA fragment was amplified by PCR (data not shown). The frequency of inducing of the null mutants (the mutants that lost the PL activity) increased with the Mn^{2+} concentration in PCR (Table 1). Even without Mn^{2+}, a null mutant was observed at about 6.7%. To give a small null mutation with the least amino acid substitution in the *PL 47* gene, we selected 60 μM as a favorable Mn^{2+} concentration. In the course of the screening of LT-PLs, the temperature dependence of the reaction rate was measured at 5 °C intervals. Consequently, we isolated 17 mutants as three groups having different optimum temperatures; the first group (11 mutants) had an optimum temperature of 65 °C, the second group (4 mutants) was of 60 °C, and the third group (2 mutants) was of 55 °C. After the analyses of their nucleotide sequences, we found that some amino acids had been substituted, i.e. one to four amino acids for the first group, three to five amino acids for the second group and five to six amino acids for the third group.

3.2. Characterization of the less-thermostable mutants (LT mutants)

From each of above-described three groups of mutants, three representative LT-PLs (47-1, 47-2, 47-3) as a less-amino acid-substituted mutant for each group were selected, isolated, and characterized. The LT-PL 47-1 of the first group had one amino acid substitution (Pro168His), the LT-PL 47-2 of the second group had three substitutions (Val254Met, Lys384Arg and Lys409Arg) and the LT-PL 47-3 of the third group had five substitution (Ser118Leu, Phe221Leu, Lys280Asn, Ala325Pro and Ile396Met) (Fig. 1). Each of these enzyme preparations migrated as a single protein band on SDS-PAGE, and their molecular masses were determined to be almost the same 50 kDa as the PL 47.

Table 1
Effect of the Mn^{2+} concentration on the frequency of null mutant induction

Concentration (μM)	Frequency of the null mutant induction[a] (%)
0	6.7 ± 0.63 (2)
60	9.4 ± 0.56 (3)
130	11.7 ± 0.54 (3)
250	14.4 ± 1.13 (2)
500	66.2 ± 1.62 (2)

[a] With the standard deviation for experimental runs indicated in the parentheses.

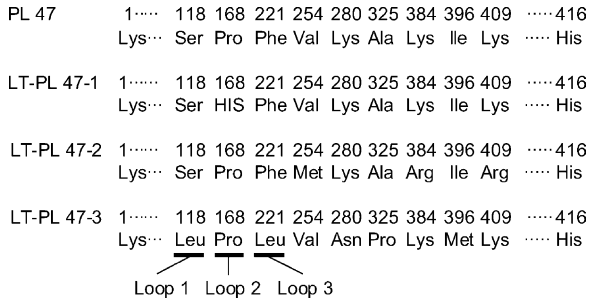

Fig. 1. Comparison of the amino acids in the PL 47 and LT-PLs. Amino acid numbers of the loops were referred to reference [19].

The thermal stabilities and activities of the LT-PL 47-1, 47-2, and 47-3 were investigated. The LT-PL 47-1 was the most thermostable of the LT-mutants and 80 and 60% of the activity remained even after 10 h incubating at 50 and 60 °C, respectively (Figs. 2a and b). Its remaining activity was 15% even after 15 min incubating at 65 °C (Fig. 2c). The LT-PL 47-2 was the second thermostable mutant and retained 20% of the activity after 4 h incubating at 60 °C. The LT-PL 47-3 was the most thermo-unstable of those and retained only 20% of the original activity even after 4 h incubating at 50 °C. All these three LT-PLs were apparently less-thermostable compared with the PL 47. The optimal temperatures for activity of the LTs were apparently lower than that of PL 47 (Fig. 3). The optimal pHs for the LT-PLs were found to be around pH 8.0 as well as for PL 47 (data not shown).

Recently, three-dimensional structures of PLs, the PelC from *E. chrysanthemi* [19] and the BsPel from *B. subtilis* [20], were reported. These enzymes have a common unique motif consisting of a parallel β-helix that is generated by coiling β-sheets into a large right-handed cylinder as exemplified for the BsPel (Fig. 4). The β-sheets domain in the enzyme has been suggested to associate with the stability of the parallel β-helix [22]. The BsPel has three long loops 1–3 that attached to the parallel β-helix domain [20]. More recently, we have preliminarily analyzed the three-dimensional structure of the PL 47 [23]. Topology of the PL 47 was close to that of the BsPel, and the PL 47 also had loops 1–3. Ser118, Pro168, and Phe221 constituting loops 1–3 in the PL 47, respectively, were conserved in the LT-PL 47-2, whereas they were substituted in the other mutants, one amino acid in the LT-PL 47-1 and two amino acids in the LT-PL 47-3 (Fig. 1). The maximum value of Q_{10} and *E*a of the LT-PL

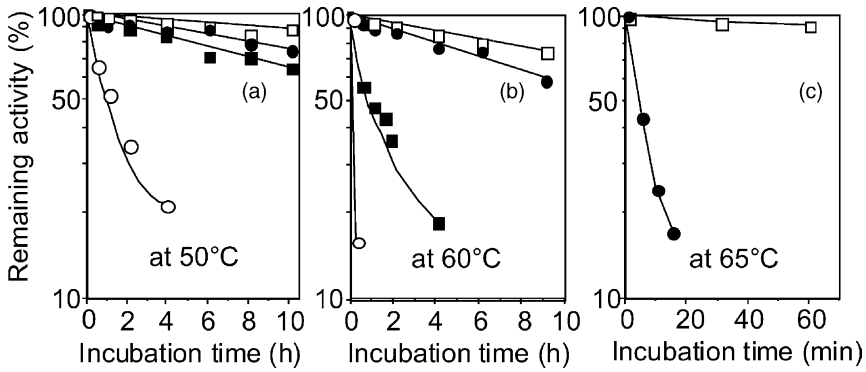

Fig. 2. Thermostability of the PL 47 and LT-PLs at three temperatures. The remaining PL activity was monitored at various times after incubation at 50 °C (a), 60 °C (b) and 65 °C (c). The PL 47 (□), the LT-PL 47-1 (●), the LT-PL 47-2 (■), and the LT-PL 47-3 (○).

Table 2
The maximum value of Q_{10} and activation energy of the PL 47 and LT-Pls[a]

	Q_{10} (temperature range)	E_a (kcal/mol)
PL 47	3.02 ± 0.09 (62.5–72.5 °C)	21.9 ± 1.03
LT-PL 47-1	1.96 ± 0.09 (55–65 °C)	14.8 ± 0.52
LT-PL 47-2	3.40 ± 0.04 (47.5–57.5 °C)	25.6 ± 0.29
LT-PL 47-3	1.99 ± 0.10 (45–55 °C)	14.2 ± 0.97

[a] With the standard deviation for 3 runs.

47-2 were close to those of the PL 47, whereas those of the two other mutants were significantly smaller than of the PL 47 (Table 2). Thus, substitutions in those mutants might be reflected on their maximum value of Q_{10} and *E*a.

As described earlier, we found that the LT-PL 47-3 has five amino acid substitutions in the PL 47 (Fig. 1). The LT-PL 47-3 was much more thermo-unstable than the PL 47 (Fig. 5). To identify the amino acid(s) to render to the thermostability of the LT-PL 47-3, these five amino acid(s) were resubstituted by each of the original amino acids or by two amino acids combinations. Among the five single-reversed

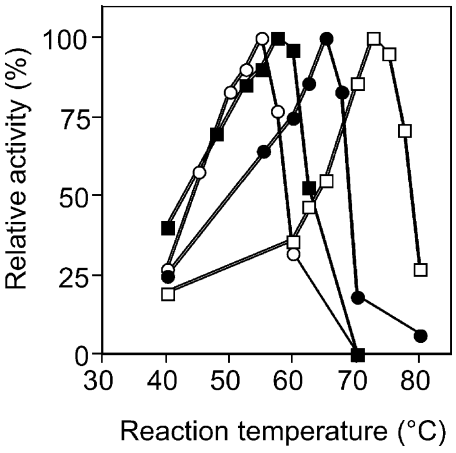

Fig. 3. Effects of temperature on the activity of the PL 47 and LT-PLs. The PL 47 (□), the LT-PL 47-1 (●), the LT-PL 47-2 (■) and the LT-PL 47-3 (○).

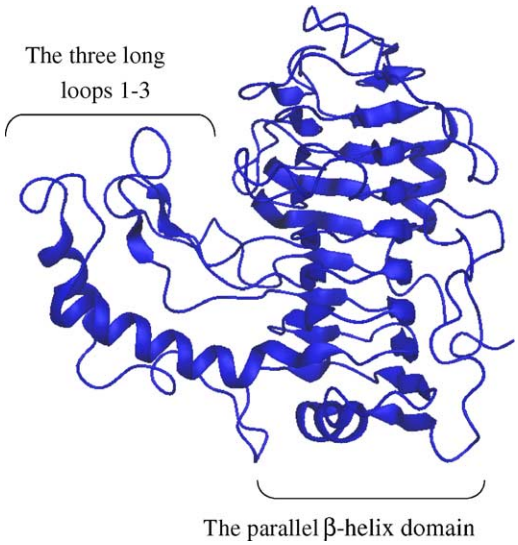

Fig. 4. Structure of BsPel [19] determined by X-ray crystallography in a representation generated by program *DINO* [21]. The parallel β-helix domain and the region formed by the association of the three long loops 1–3 are shown.

mutants (Leu118Ser, Leu221Phe, Asn280Lys, Pro325Ala, Met396Ile), the thermostability was recovered only in Pro325Ala (LT-PL mt 47-3a) up to about a half that of the PL 47 (Fig. 5). For the other four mutants, the thermostability was not regained at all (data not shown). Althogh only two mutants were isolated and analyzed as double-reversed mutants, the LT-PL mt 47-3b (Leu221Phe and Pro325Ala) and the LT-PL mt 47-3c (Asn280Lys and Pro325Ala) also somewhat enabled to regain the thermostability of the PL 47 (Fig. 5). Thus, Ala325 might have an important role for thermostability of the PL 47. To identify the amino acid(s) that have a key role for the thermostability, more structural and biochemical analyses should be done for other reverse mutants retaining Ala325. Putting together the results obtained and the three-dimensional structure of the PL 47, which is extensively analyzing in our group, important information for the thermostability of the PL 47 would be obtained. Thus, the error-prone PCR used in this study

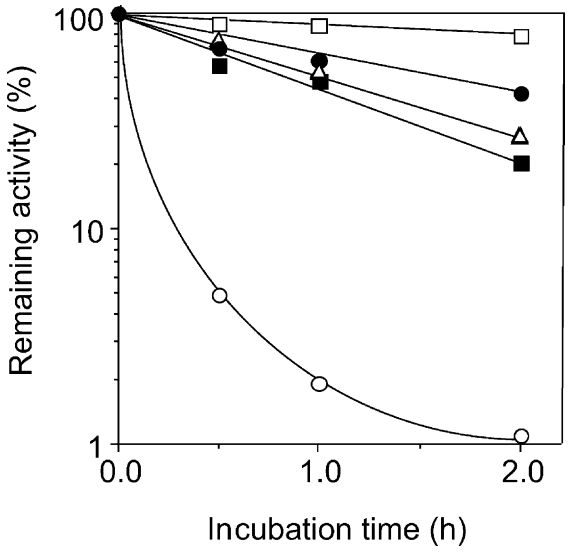

Fig. 5. Thermostability of five PLs. The LT-PL 47-3 (○) is a mutant of the PL 47 (□). The LT-PL mt 47-3a (●) is the inverse mutant, Pro325Ala, of the LT-PL 47-3, the LT-PL mt 47-3b (△) is the inverse mutant, Leu221Pro and Pro325Ala, of the LT-PL 47-3 and the LT-PL mt 47-3c (■) is the inverse mutant, Asn280Lys and Pro325Ala, of the LT-PL 47-3. Each of the mutants was incubated at 60 °C for various periods.

should be a useful technique to analyze the structure and functions of the enzymes.

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