Thermostability enhancement of the *Pseudomonas fluorescens* esterase I by in vivo folding selection in *Thermus thermophilus*

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Abstract

Prolonged stability is a desired property for the biotechnological application of enzymes since it allows its reutilization, contributing to making biocatalytic processes more economically competitive with respect to chemical synthesis. In this study, we have applied selection by folding interference at high temperature in Thermus thermophilus to obtain thermostable variants of the esterase I from Pseudomonas fluorescens (PFEI). The most thermostable variant (Q11L/A191S) showed a melting temperature (Tm) of 77.3 ± 0.1°C (4.6°C higher than the wild-type) and a half-life of over 13 hr at 65°C (7.9-fold better than the wild-type), with unchanged kinetic parameters. Stabilizing mutations Q11L and A191S were incorporated into PFEI variant L30P, previously described to be enantioselective in the hydrolysis of the (−) -enantiomer of the Vince lactam. The final variant Q11L/L30P/A191S showed a significant improvement in thermal stability (Tm of 80.8 ± 0.1°C and a half-life of 65 min at 75°C), while retaining enantioselectivity (E > 100). Structural studies revealed that A191S establishes a hydrogen bond network between a V-shaped hairpin and the α/β hydrolase domain that leads to higher rigidity and thus would contribute to explaining the increase in stability.

Keywords: directed evolution, esterase, in vivo selection, protein engineering, thermostability, Thermus thermophilus

1. Introduction

Thermal stability is an attractive property of enzymes for biocatalysis because it allows long-term storage and (re)use under harsh conditions (e.g., presence of organic solvents or detergents; Haki & Rakshit, 2003). In addition, the use of thermostable enzymes in processes at higher temperatures favors the solubility of substrates and products and makes the processes less susceptible to microbial contamination (Bommarius & Paye, 2013).

Comprehensive explorations of the natural genetic diversity, that is, metagenomic screening, are an easy and efficient way to find thermostable enzymes with a given target activity. However, the low diversification of thermophiles and their small genomes entail a limited enzymatic diversity compared to that found in mesophilic environments. Moreover, industrial reactions often require mechanisms, substrate specificit and selectivities that might not be found in nature. To overcome this limitation, directed evolution can generate thermostable variants of enzymes from mesophilic sources. Therefore, more efficient, simple and inexpensive methods are required that can be used to screen for enzymes with increased performance at high temperatures. An interesting option is the development of indirect functional selection methods in which the target property is linked to the viability of the host microorganism (e.g., by using an antibiotic reporter). There are also methods based on the connection of the target feature with the proper folding of a reporter protein. Waldo, Standish, Berendzen, and Terwilliger (1999) reported an elegant assay to increase protein solubility in which the target protein is expressed as an N-terminal fusion with a green fluorescent protein (GFP). Fisher, Kim, and DeLisa (2006) described a selection method in which a twin-arginine translocation (Tat)-based solubility reporter is used to selecting correctly folded proteins. However, these selection approaches are based on the use of a mesophilic host. Our group previously described an activity- independent selection system based on a fusion construct comprising a target protein (N-terminus) and a thermostable antibiotic resistance reporter (C-terminus) expressed in a thermophile host (Chautard et al., 2007). In this method, thermostable variants of the protein of interest would fold correctly at a high temperature allowing, in turn, the proper folding of the reporter and the growth on antibiotic-containing plates, whereas the wild-type enzyme will misfold and interfere in the folding of the reporter, yielding sensitive hosts.

The esterase I from the Gram-negative bacterium Pseudomonas fluorescens (PFEI; E.C. 3.1.1.2) is a monomeric enzyme of 31 kDa that can be overexpressed in Escherichia coli (Choi, Jeohn, Rhee, &

Yoo, 1990). Site-directed mutagenesis studies have been previously carried out on PFEI aiming to increase its activity in the hydrolysis of epoxides (Jochens et al., 2009) and lactones (Ding & Kazlauskas, 2017; D. L. Yin et al., 2010), in the perhydrolysis of carboxylic acids (D. T. Yin, Purpero, Fujii, Jing, & Kazlauskas, 2013), and to improve the enantioselectivity in the kinetic resolution of diverse chiral substrates (Schließmann, Hidalgo, Berenguer, & Bornscheuer, 2009; Torres et al., 2012). In addition, simultaneous site-saturation mutagenesis was performed on three surface positions of PFEI to enhance its thermal stability (Jochens, Aerts, & Bornscheuer, 2010).

In this study, thermal stabilization of PFEI was carried out in vivo in the extremely thermophilic bacterium Thermus thermophilus by folding interference (Chautard et al., 2007). Here, we describe how this method allowed us to find stabilizing amino acid replacements in the PFEI scaffold. In addition, the new replacements were able to overstabilize a previously reported variant that showed (–) -selectivity in the ring-opening of the Vince lactam (Torres et al., 2012), a key building block in the synthesis of nucleoside analogs used against human immunodeficiency virus (Coates et al., 1991). This final variant displayed very high stability against temperature and organic solvents, as well as the enantio-selective γ- (–) -lactamase activity, making it a very promising biocatalyst in the synthesis of antiviral drugs.

2. Experimental procedures

2.1 Reagents and enzymes

The pNCK E. coli–T. thermophilus shuttle plasmid was constructed by Chautard et al. (2007). The pGASTON-pfei expression plasmid was kindly donated by Prof Uwe Bornscheuer from Greifswald University (Germany). L-rhamnose, D-(+) glucose, SYPRO Orange Protein Gel Stain, para-nitrophenyl acetate (pNPA), N, N-dimethylformamide (DMF), 2- Azabicyclo [2.2.1] hept-5-en-3-one, (1R) -(–) -2-Azabicyclo [2.2.1] hept-5- en-3-one, (1S)-(+)-2-Azabicyclo

[2.2.1] hept-5-en-3-one, and all primers (Table S4) were purchased from Sigma-Aldrich (Madrid, Spain). Ethanol was from VWR Chemicals (Radnor, PA). Fast Red, 1-naphthyl acetate, sodium chloride, and glycerol Fluka Chemie AG (Buchs, Switzerland).

Dimethyl sulfoxide (DMSO) was from Panreac AppliChem, acetonitrile was from Scharlau and trifluoroacetic acid was from Pierce. Lysozyme was from Roche (Basel, Switzerland). Benzonase nuclease and BugBuster were purchased from Novagen (Madison, WI). PfuUltra II Fusion Hot Start DNA polymerase was from Agilent Technologies (Santa Clara, CA). NZYTaq DNA polymerase was from NZYTech (Lisboa, Portugal). Bacteriological agar, tryptone, and yeast extract were from Laboratorios Conda (Madrid, Spain). Sodium chloride and imidazole were from Merck (Merck Millipore). E. coli DH5, T. thermophilus HB27 and DNA ladder (range from 72 to 4,370 base pair [bp]) were provided by the fermentation service from the Center of Molecular Biology "Severo Ochoa" (Madrid, Spain). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards (low range) were from Bio-Rad (Hercules, CA). The NucleoSpin Gel and polymerase chain reaction (PCR) clean-up kit was from Macherey-Nagel (Düren, Germany). The GeneJET plasmid Miniprep kit and all high-fidelity restriction enzymes were from Thermo Fisher Scientific (Waltham, MA).

2.2 Cloning of PFEI into pGASTON vector

The parental pfeI gene and the 36 selected mutants were cloned into pGASTON for expression in E. coli DH5α as His-tagged products (C-terminal) under the control of the rhaPBAD promoter. A primer pair containing NdeI and BamHI sites were designed to subclone pfeI into pGASTON (Table S4).

2.3 Library construction and selection of thermostable variants

A library of the gene encoding the PFEI was obtained by epPCR using the E. coli–T. thermophilus shuttle vector pNCK as a template.

The pfeI gene was cloned using BamHI and NotI. Reaction mixture was prepared in 50 µl containing: 0.05 ng/µl DNA template, 0.5 µM direct primer, 0.5 µM reverse primer, 0.2 mM dATP and dGTP, 0.1 mM dCTP and dTTP, 0.05–0.25 mM MnCl2, and 0.05 U/µl NZYTaq DNA polymerase. Mutagenic PCR was carried out on a thermal cycler (C1000 Touch; BioRad) using the program: 95°C, 2 min (1 cycle); 95°C for 1 min, 55°C for 1 min, 74°C for 70 s (30 cycles); 74°C for 10 min (1 cycle). Primers pNCKepfw3 and pNCKeprv2 were used for sequencing (Table S4).

T. thermophilus HB27 was transformed by natural competence by adding 150 ng of plasmid DNA to 0.5 ml of cell culture at exponential phase (OD600 0.3–0.4) in Thermus broth medium (TB; 8 g/L tryptone, 4 g/L yeast extract, and 3 g/L NaCl in carbonate-rich mineral water). Cultures were incubated 4 hr after the addition of DNA and the suspension was directly spread on selection plates with antibiotic or diluted before plating for viability assays. Chemically competent E. coli DH5α cells were transformed using 45 µL of competent cells and 100–200 ng of plasmid DNA or 5 µl of ligation product.

2.4 Site-directed mutagenesis

Single mutants Q11L, L30P, and A191S were generated using the QuikChange site-directed mutagenesis kit (Agilent Genomics), following the manufacturer's protocol. The incorporation of these mutations was done on the plasmid pGASTON-pfei using the corresponding primers (Table S4).

2.5 Protein expression

T. thermophilus HB27EC (ago::ISTth7; Swarts et al., 2014) was grown in Thermus broth (TB; 8 g/L tryptone, 4 g/L yeast extract, and 3 g/L NaCl in carbonate-rich mineral water). E. coli DH5α (supE44, ΔlacU169 (Φ80 lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was used both for plasmid amplification and PFEI expression.

Expression of PFEI variants was carried out in 96-well plate format. Colonies of DH5α harboring pGASTON-pfeI and the 36 variants were picked from Luria-Bertani lysogeny broth (LB) agar plates containing 100 μg/ml ampicillin into 96 deep-well microtiter plates containing 500 μl/well of LB media with 100 μg/ml ampicillin. After cultivation for 24 hr at 30°C and 180 rpm, 100 μl of preculture was inoculated in another 96 deep-well plates with 1.8 ml autoinduction medium and incubated for 24 hr at 30°C and 180 rpm (Ecotron, Infors HT, Bottmingen, Switzerland). Cultures were harvested by centrifugation at 3,220g, 4°C for 30 min (Eppendorf centrifuge 5804 R; Eppendorf, Germany) and cell pellets were stored at −20°C.

For biocatalysis assays, PFEI variants were produced in the flask. Precultures (10 ml LB medium, 100 μg/ml ampicillin) were inoculated with single colonies from LB/ampicillin plates and incubated for 16 hr at 37°C and 180 rpm. Then, 1.5 ml of preculture were inoculated in 98.5 ml autoinduction medium (916 ml ZY solution—10 g/L tryptone and 5 g/L yeast extract; 10 ml 20% L-rhamnose; 2.5 ml 20% glucose; 20 ml 25 g/L glycerol; 50 ml phosphate buffer 20X—66 g/L ammonium sulfate 136 g/L KH2PO4 ml, and 142 g/L NaH2PO4; 1 ml 1 M MgSO4; and 500 μL 200 mg/ml ampicillin) in 500-ml flasks and cultivated at 30°C and 180 rpm for 24 hr. Cultures were harvested by centrifugation at 3,220g, 4°C for 30 min (Eppendorf centrifuge 5804 R) and cell pellets were stored at −20°C.

2.6 Protein purification

2.6.1 Purification in 96-well plate format

Cell pellets were resuspended in 300 μl/well of 50 mM sodium phosphate buffer, pH 7.5 containing BugBuster (1X in 50 mM sodium phosphate buffer, pH 7.5). After incubation at 30°C and 180 rpm for 1 hr, plates were centrifuged at 3,220g and 4°C for 30 min (Eppendorf centrifuge 5804 R). Cell lysates were added into 96-well plates containing nickel sepharose resin (HisMultiTrap FF; GE Healthcare Life Sciences, Marlborough, MA) and PFEI variants were pu-

rified following the manufacturer's protocol. The purity of wild-type PFEI and its variants was determined by 12% SDS-PAGE. The purified enzymes were dialyzed against 50 mM sodium phosphate buffer pH 7.5 and concentrated using Amicon Ultra-0.5 centrifugal filter units with 3 kDa cut-off (Merck Millipore Ltd., Burlington, MA) in a refrigerated microcentrifuge (Mikro 220 R, Hettich, Kirchlengern, Germany). Protein concentration was measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

2.6.2 Purification in column

Cell pellets (~1.5 g) were resuspended in 30 ml binding buffer (50 mM sodium phosphate buffer, 500 mM NaCl, and 20 mM imidazole, pH 7.5) and 2 µl benzonase nuclease were added. Cell disruption was carried out with a pressure homogenizer (GEA Niro homogenizer, Düsseldorf, Germany) and the lysate was centrifuged at 14,000g and 4°C for 30 min (Avanti J-25 centrifuge; Beckman Coulter). Nickel resin of 1.6 ml (Ni-NTA Superflow; QIAGEN, Hilden, Germany) were washed with 20 ml Milli-Q water and equilibrated with 20 ml binding buffer. Manufacturer's manual was followed for enzyme purification (elution buffer: 50 mM sodium phosphate buffer, 500 mM NaCl, and 20 mM imidazole, pH 7.5). Homogeneity of purified enzymes was assessed by SDS-PAGE. Then, purified samples were dialyzed against 50 mM sodium phosphate buffer pH 7.5 and concentrated Amicon ultra-15 centrifugal filter units with 3 kDa cut-off (Merck Millipore Ltd,) in a refrigerated centrifuge (Eppendorf 5804 R, Eppendorf). Protein concentration was measured with the Bio-Rad protein assay.

2.7 Biochemical characterization

2.7.1 Activity assay in the agar plate

The assay of esterase activity in plates with solid medium LB/ampicillin 100 µg/ml was used to identify E. coli clones that contain active esterase variants previously selected in T. thermophilus HB27EC. Two replicates of each clone were made with their respective controls, positive (pNCK- pfeI) and negative (pNCK), and incubated at 37°C for 16 hr. Then, one of the plates was covered with

0.5% agar solution containing 1.42 mg/ml Fast Red and 0.32 mg/ml α-naphthyl acetate.

2.7.2 Activity assay in the microtiter plate

Esterase activity was determined at room temperature in 96-well plate using 20 µl of suitable enzyme dilutions, 210 µl of Tris-HCl buffer (pH 7.5), and 20 µL of 62.5 mM pNPA in 8% (vol/vol) DMSO. The release of para-nitrophenolate (pNP) was detected at 410 nm (εpNP = 13.3 mM−1 cm−1 at pH 7.5) over time in a microplate reader (FLUOstar OPTIMA; BMG Labtech GmbH, Ortenberg, Germany).

2.7.3 Kinetic parameters

Steady-state enzyme kinetics of PFEI variants were determined at room temperature with 0–12.5 mM pNPA and 10% (vol/vol) DMSO. Initial rates (s−1) were plotted against substrate concentration (mM) and adjusted to the Michaelis–Menten model using a simple rectangular hyperbola of two parameters with function $f(x) = a \times x/ (b + x)$, being a the kcat and b the KM.

2.7.4 Stability assays

Thermodynamic stability of wild-type PFEI and its variants was assessed by differential scanning fluorimetry (Lavinder, Hari, Sullivan, & Magliery, 2009). A 20 µM solution of enzyme in 50 mM sodium phosphate buffer (pH 7.5) was supplemented with SYPRO Orange (Sigma-Aldrich) and subjected to a temperature ramp from 35°C to 95°C at 1°C/min in a real-time thermocycler (Rotor GeneTM 6000; Corbett Life Science, Sydney, Australia). Increase in fluorescence was quantified in the HRM channel (λex: 460 ± 20 nm; λem: 510 ± 5 nm). The measurement was carried out in triplicate.

Kinetic stability of PFEI variants was determined by incubating solutions of each purified enzyme (appropriate dilution to produce a linear response) and determining the remaining activity over time using the pNPA assay described above. The enzymes were incubated in a heating block (ThermoMixer; Eppendorf, Hamburg, Germany)

at 65°C and 75°C in 50 mM Tris-HCl buffer pH 7.5. The half-life was determined from this plot as the time in which the activity is 50% of the initial activity.

The tolerance to organic solvents of wild-type PFEI and its variants was evaluated by incubating solutions of each purified enzyme (appropriate dilution to produce a linear response) and determining the remaining activity using pNPA. The enzymes were incubated in a heating block at 42°C and in the presence of 40% (vol/ vol) ethanol or DMF in 50 mM Tris-HCl buffer, pH 7.5. The half-life was determined from this plot as the time in which the activity is 50% of the initial activity.

2.7.5 Determination of enantioselectivity by high-performance liquid chromatography

Enzymatic reactions were set up in 2 ml containing 4 mM of racemic Vince lactam and 10 µg of the pure enzyme in 10 mM phosphate buffer, pH 7.5. The reactions were incubated at 37°C and 500 rpm, samples were taken at different times and subjected to enzyme inactivation at 80°C for 15 min. Samples were filtered through 0.22 µm (Millex-GV; Merck Millipore Ltd,) before loading into the column.

The stereoisomers of the Vince lactam were separated on a Lux Cellulose-1 column (250 × 4.6 mm ID, cellulose tris (3,5-dimethyl-phenylcarbamate) coated on 5 µm silica-gel; Phenomenex, CA) using a HPLC system (Waters 1525 binary HPLC pump and Waters 2489 UV/visible detector; Waters Corporation, MA). The mobile phase consisted of 95% ultrapure water containing 5% acetonitrile and 0.1% trifluoroacetic acid, the temperature was set at 30°C, the flow rate was 0.4 ml/min and the UV signal was monitored at 225 nm. The retention times for the (1R) - and (1S) -Vince Lactam enantiomers were 10.8 and 13.0 min, respectively. Coefficient of variance between repeated experiments was below 10%. Enantioselectivity was calculated as the E value using the Chen equation with the conversion and the enantiomeric excess of the recovered substrate fraction (ees) as input data (Chen, Fujimoto, Girdaukas, & Sih, 1982).

2.8 Structural modeling

Homology models of the PFEI variants were created using YASARA (YASARA Biosciences GmbH, Vienna, Austria) using the default automated macro (Krieger & Vriend, 2014). Homology modeling was carried out with the template with PDB code 1VA4. Loops were modeled using a maximum of 50 conformations per loop. Quality of homology models was verified by calculating the individual Z scores with the formula Overall = 0.145 × Dihedrals + 0.390 × Packing1D + 0.465 × Packing3D. The overall score thus captures the correctness of backbone (Ramachandran plot) and side-chain dihedrals, as well as packing interactions. Images were generated with PyMOL v0.99 (Schrödinger, LLC).

2.9 Bioinformatic analysis of gene sequences

The assembly, alignment, and translation of gene sequences were carried out using the SnapGene software version 4.2.6 (GSL Biotech).

2.10 Bioinformatic analysis of enzyme stability

Calculations of ΔG for PFEI wild-type and its variants were performed using the YASARA software with the FoldX plugin (Schymkowitz et al., 2005).

3. Results and discussion

3.1 PFEI library generation and selection of thermostable variants

The incorporation of mutations into the gene encoding the PFEI was carried out by error-prone PCR (epPCR). Four MnCl2 concentrations ranging from 0.05 to 0.15 mM were tested to evaluate the mutagenic frequencies on the pfeI gene (843 bp; Figure S1). Gene sequences of randomly picked clones were analyzed, leading to nucleotide substitution frequencies from 0.7 to 1.9 per gene. We used

0.15 mM MnCl2, to introduce between 1 and 5 nucleotide substitutions (1–3 amino acid substitutions) in the pfeI gene, which is a mutational load suitable for directed evolution studies (Mate et al., 2016).

The selection of thermostable variants of PFEI was carried out using the folding interference principle at high temperature in T. thermophilus HB27EC, a hypertransformable mutant of T. thermophilus (Swarts et al., 2014). To this end, the pfeiI gene was cloned in fusion with the kanamycin nucleotidyltransferase (kat) gene into the pNCK vector (Chautard et al., 2007). The selection conditions in T. thermophilus HB27EC were fixed at the minimum temperature and kanamycin concentrations at which transformants expressing the wild-type PFEI-Kat fusion could not grow. The temperatures tested were 60°C and 65°C and the kanamycin concentrations assayed were 20, 40, 60, 80, and 100 μg/ml and the chosen selection conditions were 65°C and 60 and 80 μg/ml kanamycin (Table S1).

A library of 90,000 PFEI variants was explored under the selected conditions. Considering that PFEI has 274 amino acids and that the number of sequences containing N amino acid substitutions is given by the formula $19N \cdot (274! /(274–N)! \cdot N!)$ (Arnold, 1996), our library is expected to contain all possible single substitution variants and only 0.7% of the double substitution variants. However, these numbers are calculated for an ideal scenario and not for an enzyme library generated by epPCR, since this method is limited by the bias of DNA polymerases and not all possible substitutions can occur (Wong, Zhurina, & Schwaneberg, 2006). As a result of the selection, 234 clones were obtained at 65°C, 157 on plates with 60 μg/ml of kanamycin and 77 on plates with 80 μg/ml (Table 1). Due to the fact that T. thermophilus HB27EC possesses intrinsic esterase activity precluding a direct, on-plate functional assay, the plasmids had to be extracted from Thermus and transformed in E. coli for esterase activity at 37°C. The 55 fusion variants showing activity in E. coli were transferred back to T. thermophilus HB27EC to verify their capability to confer antibiotic resistance at 65°C using a serial dilution assay. The conferred resistance values were assigned from

+1 if only the undiluted culture grew to +4 if growth was detected at a 1/ 1,000 dilution. The 36 best variants in this assay were sequenced revealing different amino acid replacements, including mutational hot- spots (i.e., positions where different amino acids replacements took place) at positions 3, 26, 168, and 192 (Figure 1; Table S2). Specifically, Thr3 was mutated to alanine, isoleucine, and serine, Ser26 to cysteine and isoleucine, Lys168 to arginine and methionine, and Thr192 to alanine and serine (Table S2).

3.2 PFEI expression and purification in the microtiter plate

To characterize the wild-type PFEI and the 36 selected mutants at once, we set up an expression and purification protocol in microtiter plate format. The collection of variants was expressed in E. coli DH5α from plasmid pGaston in 96-well deep-well plate using autoinduction medium (Studier, 2005) modified with 0.05% glucose and 0.2% rhamnose. Cell lysis was carried out using BugBuster and the 6X His-tagged wild-type protein was purified in a 96-well plate containing nickel resin. As observed in Figure S2, purification yields were very similar across all the wells and enough for further studies.

3.3 Biochemical characterization of PFEI variants

Thermal stability was evaluated as thermodynamic stability (melting temperature; Tm) and kinetic stability (half-life; Bommarius & Paye, 2013). Unexpectedly, Tm values of selected mutants spanned 32°C (Figure 2a; Table S3), between 44.7°C (variant 31) and 77.3°C (variant 34; Q11L/A191S). Actually, only two variants showed increased Tm values respect to the wild-type: Variants 34 and 49 (S157C), with Tm values 4.6°C and 2.2°C higher than the wild-type, respectively. These two variants represent only 5.6% of all selected individuals. We hypothesize that this unexpected result is due to the fact that the selection was carried out with the fusion protein PFEI-Kat in vivo in T. thermophilus, while the Tm values were determined in vitro for the individual, purified PFEI variants. Fusions of proteins to another partner can alter properties such as

stability, selectivity, or activity. For instance, fusions to superfolder GFP or maltose-binding domains have been shown to positively influence solubility or prosequences of lipases, for example, Rhizopus oryzae, influence stability as well as substrate specificity (Beer, Wohlfahrt, Schmid, & McCarthy, 1996; Takahashi, Ueda, & Tanaka, 1999). Nevertheless, Tm values were in good agreement with the corresponding kinetic stability. Variant 34 showed a half-life of 13.1 hr at 65°C (7.9-fold longer than the wild-type), and variant 49 showed a half-life of 4.6 hr at 65°C (2.8-fold longer than the wild-type; Figure 2b; Table S3).

Table 1. *Number of T. thermophilus HB27^{EC} transformants containing pNCK-pfeI wild-type or the PFEI gene library after selection at 65°C and 60 and 80 μg/ml kanamycin*

	Kanamycin (μg/ml)	
Construct	60	80
pNCK-pfeI wild-type	0	0
pNCK-pfeI gene library	157	77

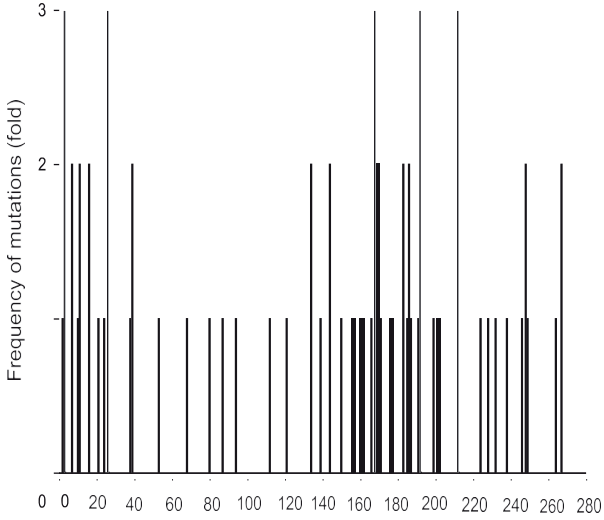
The effect of the substitutions Q11L and A191S from variant 34 on stability was studied independently. A191S had a more pronounced effect on the enhancement of thermostability than Q11L (Table S3). In addition, the triple variant E82N/K87L/E88D was incorporated into the study. This variant was previously obtained by site-saturation mutagenesis on three surface positions identified by B-fitter (Jochens et al., 2010), resulting in a Tm 9°C higher than the wild-type (6°C according to our results; Table S3). Variant 34 (Q11L/A191S) showed an increased kinetic thermostability compared with variant E82N/K87L/E88D, reflected in 6.5 hr longer half-life at 65°C. According to these results, the epPCR approach led to higher stabilization of the PFEI than structure-based studies, in good agreement the fact that location-agnostic approaches (e.g., epPCR) afford more stabilization for α/β hydrolases than structure-based methods (Jones, Lim, Huang, & Kazlauskas, 2017).

Steady-state kinetic constants of the wild-type and the 36 variants were determined with para-nitrophenyl acetate (pNPA; Figure 2b; Table S3). These values drift neutrally, as expected, since they were not involved in the selection criteria used. The most thermostable variant, PFEI 34, showed very similar KM and kcat values to those of the wild-type enzyme. In addition, we characterized the previously reported variant E82N/K87L/E88D resulting in a 2.1-fold lower KM and a 1.7-fold increased kcat compared with the wild-type (Table S3). Interestingly, variant 14 showed the mutation K87M, which increased kcat by 1.7-fold, very similar to that of the variant E82N/K87L/E88D (Table S3).

3.4 Lactamase activity of thermostable variants

The next step was to investigate whether substitutions Q11L and A191S gave rise to a stabilizing effect when incorporated into a biocatalytically relevant PFEI variant, namely L30P (L29P according to the numbering of amino acids in the crystal structure). This particular esterase shows enantioselective lactamase activity towards the (–) - enantiomer of the Vince lactam (2-azabicyclo [2.2.1] hept-5-en-3-one), the synthetic precursor in the synthesis of carbovir and abacavir, two DNA polymerase inhibitors used in the treatment of human immuno- deficiency virus and hepatitis B virus (Torres et al., 2012; Figure S3).

Figure 1. *Frequency of mutations in the 36 PFEI variants selected at 65°C*

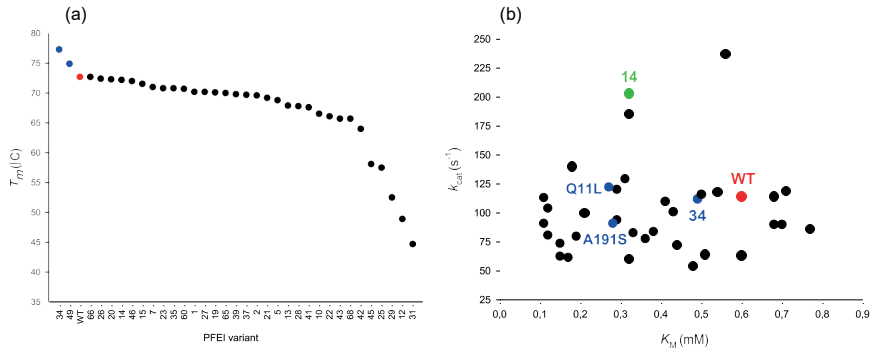


First, the single variant L30P, the double mutants Q11L/L30P and L30P/A191S, and the triple variant Q11L/L30P/A191S were cloned into the expression plasmid pGASTON, subsequently produced in E. coli and purified to homogeneity. The enantioselectivity of the promiscuous hydrolysis reaction of the Vince lactam was verified. As shown in Table 2, wild-type PFEI, the double mutant Q11L/A191S, the single variant L30P, and the triple mutant Q11L/L30P/A191S were still enantioselective with a preference towards the (−) enantiomer. Furthermore, variants containing the substitution L30P showed a much higher conversion rate of the (−) -enantiomer than the wild-type and the double mutant Q11L/A191S, as previously reported (Torres et al., 2012).

Regarding thermal stability, L30P led to a significant additional stabilizing effect, resulting in a 4.3°C increase in Tm over the wild-type and a threefold increase in half-life at 75°C, very similar to the stability of variant 34 (Q11L/A191S; Table 2). Amino acid replacements to proline have been reported to enhance stability (Huang,

Jones, & Kazlauskas, 2015; Watanabe, Masuda, Ohashi, Mihara, & Suzuki, 1994). In fact, proline limits the flexibility of the unfolded protein compared with other amino acids, causing a decrease in the configurational entropy of unfolding and shifting the equilibrium towards the folded form (Matthews, Nicholson, & Becktel, 1987).

Figure 2. *Biochemical characterization wild-type PFEI and its variants. (A) T_m values of wild-type PFEI and its variants. Measurements were done in triplicate. T_m of wild-type is colored in red and T_m of variants 34 (Q11L/A191S) and 49 (S157C) are in blue. (B) Kinetic parameters with pNPA of wild-type PFEI and its variants. k_{cat} is represented versus K_M. Wild-type is colored in red, variant 34 (Q11L/A191S) and single mutants Q11L and A191S in blue and 14 (K87M) in green. pNPA, para-nitrophenyl acetate; T_m, melting temperature; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]*



Addition of L30P into the single variants Q11L and A191S and into the double variant Q11L/A191S caused a remarkable enhancement in stability. This effect was accompanied by the apparition of lactamase activity and the reduction in the activity with the model substrate pNPA (Table 2), as previously reported (Ding & Kazlauskas, 2017). In fact, L30P seems to be a promiscuous generalist (Khersonsky & Tawfik, 2010) showing esterase, lactonase (Ding & Kazlauskas, 2017; D. L. Yin et al., 2010), perhydrolase (D. T. Yin et al., 2013), and lactamase activities (Torres et al., 2012). In addition, Tm of Q11L/L30P/A191S was 3.5°C and 8.1°C higher than those of Q11L/A191S

and the wild-type enzyme, respectively. These results prove the additive effect of combining the three stabilizing mutations Q11L, L30P, and A191S into the same protein scaffold. Moreover, half-life at 75°C was drastically increased from 10 min for variant Q11L/A191S to 65 min for Q11L/L30P/A191S (Table 2).

Table 2. *Enantioselectivity in the kinetic resolution of the racemic Vince lactam, specific activity and thermal stability (Tm and half-life) of PFEI wild-type and its variants*

PFEI	E value[a]	Conversion of (−) -Vince lactamb	Tm (°C)	Half-life at 75°C (min)	Specific activity (U/mg)c
Wild-type	>100 (39%)	8.7	72.7 ± 0.2	3.2 ± 0.2	2723 ± 80
Q11L/A191S	>100 (35%)	13.0	77.3 ± 0.2	10.0 ± 0.4	2453 ± 69
L30P	>100 (27%)	98.5	77.0 ± 0.1	9 ± 1	54 ± 1
Q11L/L30P	n.d.	n.d.	77.7 ± 0.1	16 ± 1	45 ± 2
L30P/A191S	n.d.	n.d.	79.9 ± 0.1	41 ± 4	53 ± 2
Q11L/L30P/A191S	>100 (43%)	100	80.8 ± 0.1	65 ± 5	50 ± 2

Abbreviations: n.d., not determined; pNPA, para-nitrophenyl acetate; Tm, melting temperature.

aE values were calculated at the conversion indicated in parentheses.

bConversion of (−)-Vince lactam after 2 hr at 37°C.

cSpecific activity was determined with pNPA.

The stabilizing effect of the discovered mutations was also analyzed in terms of tolerance to organic solvents. All variants containing stabilizing mutations showed improved half-lives in the presence of 40% (vol/vol) ethanol and DMF (Figure 3a,b). The most thermostable variant, the triple mutant Q11L/L30P/A191S, resulted to be also the most resistant to organic solvents, with half-lives of

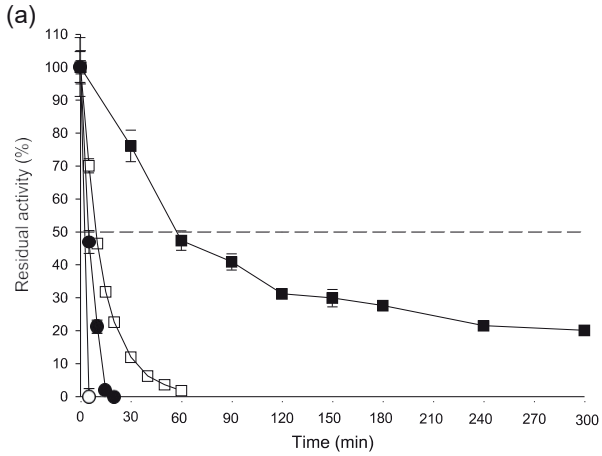
57 and 272 min with 40% ethanol and DMF, respectively, 23-fold and 60-fold higher when compared with the wild-type (2.5 min with 40% ethanol and 4.5 min with 40% DMF).

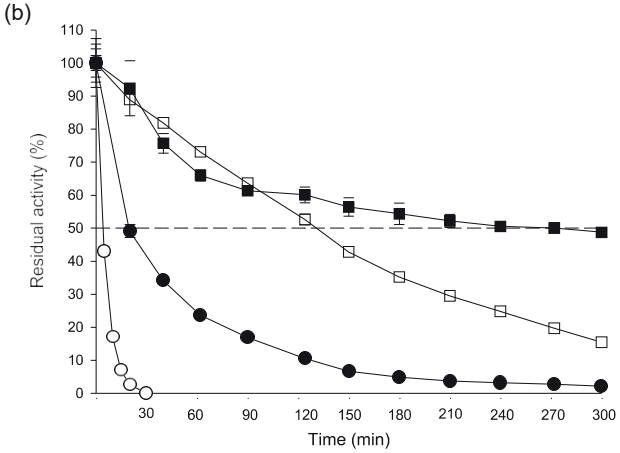
3.5 Structure–function analysis

Once activity and stability of selected variants were evaluated, we carried out a structural analysis of the found mutations. To this end, homology models of the most thermostable variant Q11L/L30P/A191S were built using the crystal structure of wild-type PFEI as a template (PDB code: 1VA4).

Gln11 is a surface-exposed residue located in the β-strand 2 (residues 9–17) that establishes two hydrogen bonds with the nearby Asp62 (Figure S4). Mutation to leucine at this position did not alter these interactions or gave rise to new ones. However, Leu11 generates a wide hydrophobic surface that could explain partial protein aggregation observed during purification of Q11L variants.

Figure 3. *Residual activity of wild-type PFEI and its variants in the presence of 40% (vol/vol) ethanol (a) and DMF (b) at 42°C. White circles, wild-type; black circles, variant L30P; white squares, variant 34 (Q11L/A191S) and black squares, variant Q11L/L30P/A191S. DMF, N, N-dimethylformamide*



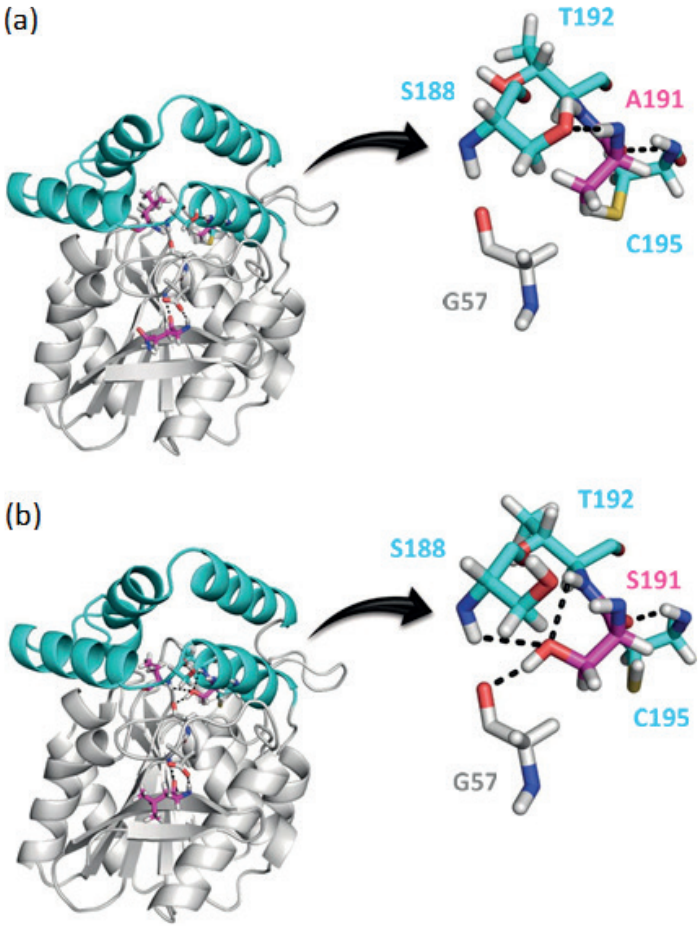


Leu30 is in the loop connecting the β-strand 3 and the α-helix 1 (residues 26–33). As previously shown using molecular dynamics, L30P leads to the pyramidal inversion of the amide nitrogen (a requisite for amide hydrolysis) 500 times faster than in the wild- type (Torres et al., 2012). Furthermore, it was hypothesized that L30P could yield different distances between the carbonyl group of the adjacent Trp29 and the scissile amide nitrogen of the substrate, broadening in that way the substrate scope of PFEI.

Ala191 is placed in the α-helix 7 (residues 189–201), which is one of the four α-helices within the V-shaped hairpin formed by the β6–β7 loop (Cheeseman, Tocilj, Park, Schrag, & Kazlauskas, 2004). This hairpin is topologically equivalent to the lid of Pseudomonas lipases (Schrag et al., 1997). In addition, Ala191 establishes hydrogen bonds with residues Ser188 and Cys195 in the wild-type PFEI. In the final variant Q11L/L30P/A191S, Ser191 keeps the interactions with Ser188 and Cys195 and forms additional hydrogen bonds with Gly57 and Thr192 (Figure 4). FoldX calculations gave rise to a ΔΔG of −16 kcal/mol for the final variant with respect to the wild-type, in line with the theoretical value for the contribution to the stability of two hydrogen bonds reported by Fleming and Rose (2005) (~12 kcal/mol). This hydrogen bond network could limit the mobility of

the β6–β7 loop, generating a more rigid and compact structure and thus explaining the increase in thermostability conferred by this mutation.

Figure 4. *Detail of the stabilizing mutation A191S in variant Q11L/L30P/A191S (b) compared with the corresponding residues in the wild- type PFEI (a). The Q11L, L30P, and A191S mutations are depicted as pink sticks. Hydrogen bonds between residues are shown as black dashes. The V-shaped hairpin (residues 138–201) is displayed as cyan cartoon and the rest of the structure as a gray cartoon [Color figure can be viewed at wileyonlinelibrary.com]*



4. Conclusions

Industrial organic syntheses require the availability of biocatalysts with high long-term stability and enantioselectivity towards the substrate of interest. The folding interference principle in T. thermophilus allowed the selection of mutations that seemingly stabilize the fusion protein PFEI-Kat, with only a small fraction of the discovered mutations able to stabilize the enzyme when it is expressed independently from the folding reporter. Even so, the method proved useful in the discovery of thermostabilizing mutations that would have been unpredictable otherwise. The best two stabilizing mutations found in the process, Q11L and A191S, conferred both improved thermal stability and higher resistance to organic solvents. Besides, when these mutations were incorporated into a highly enantioselective PFEI scaffold (L30P) the stability was increased even more, with a Tm over 80°C. Furthermore, improvement in thermal stability did not interfere with enantioselectivity towards the relevant precursor Vince lactam, since the triple variant, Q11L/L30P/A191S retained the (–) -γ-lactamase activity and selectivity.

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