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Expression, purification, refolding and characterization of a putative lysophospholipase from *Pyrococcus furiosus:* Retention of structure and lipase/esterase activity in the presence of water-miscible organic solvents at high temperatures

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ABSTRACT

A putative lysophospholipase (PF0480) encoded by the *Pyrococcus furiosus* genome has previously been cloned and expressed in *Escherichia coli*. Studies involving crude extracts established the enzyme to be an esterase; however, owing presumably to its tendency to precipitate into inclusion bodies, purification and characterization have thus far not been reported. Here, we report the overexpression and successful recovery and refolding of the enzyme from inclusion bodies. Dynamic light scattering suggests that the enzyme is a dimer, or trimer, in aqueous solution. Circular dichroism and fluorescence spectroscopy show, respectively, that it has mixed beta/alpha structure and well-buried tryptophan residues. Conformational changes are negligible over the temperature range of 30–80 °C, and over the concentration range of 0–50% (v/v) of water mixtures with organic solvents such as methanol, ethanol and acetonitrile. The enzyme is confirmed to be an esterase (hydrolyzing *p*-NP-acetate and *p*-NP-butyrate) and also shown to be a lipase (hydrolyzing *p*-NP-palmitate), with lipolytic activity being overall about 18- to 20-fold lower than esterase activity. Against *p*-NP-palmitate the enzyme displays optimally activity at pH 7.0 and 70 °C. Remarkably, over 50% activity is retained at 70 °C in the presence of 25% acetonitrile. The high organic solvent stability and thermal stability suggest that this enzyme may have useful biodiesel-related applications, or applications in the pharmaceutical industry, once yields are optimized.

Enzymes that are extremely stable to denaturation by heat are generally also stable to denaturation by water-miscible organic (nonpolar) solvents [1]. Thus, thermophilic and hyperthermophilic organisms are considered to be rich sources of enzymes that are likely to retain structure and function in mixtures of water with water-miscible organic solvents. Amongst enzymes that do retain structure and function in the presence of such solvents, several classes of hydrolases (e.g., proteases) display a reversal of the direction of the catalytic chemical reaction [2]. Lipases, in contrast, having naturally evolved to function in environments presenting a significant degree of nonpolarity, tend to act as lipases rather than as lipid synthases even in nonpolar media, under membrane-like conditions.

Notwithstanding the ability of lipases to function in mildly nonpolar environments, these enzymes, like others evolved to be water-soluble and to fold within the aqueous environment of the cytoplasm, tend to also be vulnerable to denaturation by organic solvents, albeit to a lesser degree [3,4]. In order to remain functional in nonpolar environments over extended timescales, lipases also need to be thermodynamically as well as kinetically stable to denaturation by high concentrations of water-miscible organic solvents, especially in regard to potential applications in the biodiesel and pharmaceutical industries. Since proteins show a clear correlation between stability to denaturation by organic solvents and thermal stability [1], the proteomes of thermophilic and hyperthermophilic organisms end up producing the best candidate solvent-stable lipases.

The proteome of the hyperthermophilic archaeon, *Pyrococcus furiosus* [5], contains several genes annotated as esterases or lipases on the basis of their encoded amino acid sequences. One such enzyme is a putative 'lysophospholipase', which shows limited sequence similarity with *Candida rugosa* lipase [6]. The predicted amino acid sequence of this enzyme has previously been examined and discussed, as sequence MER35299 (http://merops.sanger.ac.uk/cgi-bin/speccards?sp=SP000848&type=P) in the

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MEROPS database, with marking of the signature sequences for conserved motifs and for the catalytic triad of residues that indicate that it is a non-peptidase member of the α/β hydrolase family of enzymes. The enzyme has also been expressed as a recombinant protein in *E. coli* and tested for activity in specific chemical conversion reactions as a candidate enzyme, amongst other related enzymes from different microbial sources [6], for esterase-catalyzed hydrolysis of (*R*,*S*)-naproxen methyl ester. However, in these aforesaid studies the enzyme was found to deposit into inclusion bodies, and crude enzyme preparations made through inefficient resolubilization and extraction from pellets made up of membranes and bacterial cell debris failed to show sufficiently high levels of esterase activity in the desired chemical reactions. Thus, the enzyme was not purified further or characterized by the authors who originally produced it in recombinant form. In this paper, this is the challenge we primarily set out to address.

We describe here the cloning, overexpression, technique for solubilization, affinity purification to homogeneity through IMAC procedures, and detailed characterization of the structure, stability and function of Pfu lysophospholipase. We show that the enzyme is dimeric, that it functions as an esterase and also as a lipase, and that it is thermostable as well as stable to denaturation by water-miscible organic solvents, retaining more than half of its natural functionality as a lipase even in 25% acetonitrile.

Materials and methods

Cloning and expression

Pyrococcus furiosus genomic DNA was obtained as a gift from Drs. M.W.W. Adams, and F.E. Jenney Jr., University of Georgia, Athens, GA, USA. The gene encoding Pfu Lysophospholipase was PCR amplified from genomic DNA (database accession numbers: PF0480; NP_578209.1; GI:18976852; MER35299) using suitable forward (5′-CTGAAAATATCCCGGGACATCACCATCACCATCACCATCA CATGA CCCAGGTATACAAAGC-3′) and reverse (5′-TTTAATATGTCG ACGCGGCCGC CTAATGGTGGTGATGATGCTTCTTTATCCACTCAACTA TC-3′) primers. The forward and reverse primers were designed to incorporate N-terminal as well as C-terminal 6×His affinity tags, as well as an in-frame stop codon immediately after the C-terminal 6×His affinity tag. The PCR consisted of 30 cycles of 1 min at 95 °C (denaturation), 2 min at 55 °C (annealing), and 3 min at 72 °C (extension), with Deep Vent DNA polymerase (New England Biolabs). The reaction produced the desired product with 0, 0.5, 1.0 and 2.0 mM $MgCl_2$. The PCR product was digested with SmaI and SalI enzymes, and first cloned into the multiple cloning site of the vector pGEX-KG (Amersham Biosciences) before it was subcloned between the BamHI and XhoI sites of the multiple cloning site of the vector pET23a (Novagen Inc.) for expression. The clone was transformed into *E. coli* BL21DE3 (pLysS) and the protein was expressed through induction with IPTG (1 mM) after approximately three hours of growth of cells at 37 °C (at an A_{600} of ∼0.6–0.8). Cells were harvested after overnight growth in the presence of IPTG.

Extraction, purification and quantification

Cells were pelleted and resuspended in the presence of 6 M guanidinium hydrochloride (Gdm·HCl)[1] containing 1 M NaCl in 50 mM Tris, pH 7.0. After overnight incubation in denaturant, the resuspension was heated to 80 °C for 30 min, and centrifuged to pellet out the cell and membrane debris resulting from cell lysis. The Gdm·HCl-containing supernatant containing the lysophospholipase was loaded onto a 2.5 ml Ni-NTA affinity column (Sigma–Aldrich) using standard protocols. Following flow-through and washing steps, elution of bound lysophospholipase was carried out sequentially with 8 M urea-containing buffers of pH 5.9, and 4.5. Following elution, the urea was dialyzed out against 20 mM phosphate buffer of pH 7.0, to obtain a mixture of soluble protein and insoluble precipitated protein. The soluble protein was quantified through absorption measurements at 280 nm, using the extinction coefficient predicted for the protein, translating to a concentration of 0.97 mg/ml for an OD of 1.0 at 280 nm.

Yield estimation

For protein yield estimation, a one liter culture was grown to an OD_{600} of 0.6–0.7 and induced with IPTG to express the lysophospholipase. The culture was grown overnight before harvesting cells through low-speed centrifugation and lysis of cells in various ways, followed by IMAC purification in the presence or absence of denaturant, and refolding through dialysis where denaturant was used.

MALDI-TOF mass spectrometry

Intact protein mass was estimated using an Applied Biosystems Voyager DE STR mass spectrometer with sinapinic acid used as matrix. Peptide mass fingerprinting data after in-solution digestion of the purified Pfu lysophospholipase with mass spectrometry-grade trypsin (Sigma–Aldrich) was also collected on the same instrument, in reflector mode, using internal calibration of masses with standard ACTH fragments, using standard methods.

Circular dichroism

Far-UV CD spectra were collected on a JASCO J-810 spectropolarimeter using a cuvette path length of 1.0 mm and spectral collection in the range of 190–250 nm using a spectral bandpass of 4 nm at room temperature. Purified enzyme of 0.2 mg/ml (in 20 mM phosphate buffer, pH 7.0) was diluted to a final concentration of 0.1 mg/ml with addition of water, methanol, ethanol, or acetonitrile, as appropriate before collection of spectra. Raw ellipticity data was converted to mean residue ellipticity before plotting.

Thermal melting analyses

The mean residue ellipticity of Pfu lysophospholipase at 222 nm was monitored and plotted as a function of temperature on a JASCO J-810 spectropolarimeter, with the temperature of the protein solution being caused to rise at a rate of 3 °C/min through a Peltier block arrangement, using a cuvette of path length 1.0 mm and a 9 mm metal spacer block for heat transfer to the cuvette.

Fluorescence spectroscopy

The fluorescence emission spectrum of Pfu lysophospholipase was collected using the FMO-427 monochromator accessory of the JASCO J-810 spectropolarimeter, with excitation at 280 nm, and monitoring of emission between 300 and 400 nm using an excitation bandwidth of 8 nm and the standard emission bandwidth of the FMO-427 accessory.

Dynamic light scattering

DLS data was collected on a Wyatt, Protein Solutions DynaPro 800 instrument after filtration of the protein sample through a 0.1 μm filter, using a protein concentration of approximately

[1] *Abbreviations used:* Gdm·HCl, guanidinium hydrochloride; *p*-NP-acetate, paranitrophenyl acetate; *p*-NP-butyrate, paranitrophenyl butyrate; *p*-NP-palmitate, paranitrophenyl palmitate; PMSF, Phenyl methyl sulfonyl fluoride; MRE, mean residue ellipticity.

0.12 mg/ml, and using single-angle scatter of 824 nm laser radiation. Hydrodynamic radius distribution versus percent scattering and percent mass data were collected, together with analyses of polydispersity, using the Dynamics V6 software, and an isotropic-sphere model. Approximately twenty-five 10 second averages of scattering data were used for the analyses.

Activity assays

The activity of Pfu lysophospholipase was assayed in the following manner. Stocks (50 mM in 100% acetonitrile) were made for all three substrates tested, i.e., paranitrophenyl acetate (*p*-NP-acetate), paranitrophenyl butyrate (*p*-NP-butyrate) and paranitrophenyl palmitate (*p*-NP-palmitate). The refolded enzyme stock consisted of 0.12 mg/ml enzyme in 50 mM phosphate buffer (pH 7.0). The reaction buffer used contained 1.0% (w/v) gum arabic and 0.2% Triton X-100 in 10 mM buffer of the desired pH, i.e., citrate (pH 3–6), phosphate (pH 7–8) and carbonate (pH 9–10). The final reaction had a volume of 1 ml containing 950 μl of the reaction buffer, 40 μl of the protein stock solution and 10 μl of the substrate stock solution at the appropriate desired temperature, using the reaction buffer with the pH of choice, to assay activity at different temperatures and different pH values. Following the constitution of the reaction, incubations were made for 30 min at the desired temperature/pH for palmitate assays, and for 5 min each for butyrate and acetate assays. Development of color was monitored at 410 nm for all temperature-dependent activity assays at pH 7.0. However, for pH-dependent activity assays at 70 °C, we monitored color development at the reported isobestic point (348 nm) of the absorption spectrum of paranitrophenol [7], to avoid effects of pH-dependent changes in the molar extinction coefficient on the assay.

Activity inhibition by PMSF

Phenyl methyl sulfonyl fluoride (PMSF) was dissolved in 100% acetonitrile to generate a 50 mM stock. For inhibition studies, Pfu lysophospholipase was incubated at 37 °C for 30 min in the presence of a final concentration of 1 mM PMSF before testing for abolition of activity.

Results and discussion

The recombinant Pfu lysophospholipase construct produced and studied in this paper has the amino acid sequence shown in Fig. 1. The sequence reported in the databases is 257 amino acids long; in contrast, the construct produced by us is 286 amino acids long because of the extra N- and C-terminal affinity tags as well as residues contributed by the multiple cloning site of vectors used in the cloning and sub-cloning procedures.

Recovery using heat and denaturant

The recombinant Pfu lysophospholipase could be recovered efficiently (solubilized) from inclusion bodies through heating at 80 °C for 30 min in the presence of 6 M Gdm·HCl and 1 M NaCl (see Materials and methods). Following transfer to urea in the course of IMAC affinity purification in denatured form, the enzyme could also be easily refolded through dialysis of urea and NaCl, although partitioning of some of the population into insoluble aggregated form was also observed. However, significant concentrations of protein (0.2–0.5 mg/ml) could be retained in solution, with a net recovery of less than one milligram from a 2 L culture. The yields obtained by different methods, quantified through absorption measurements (see above section), are presented in Table 1. In work to be reported elsewhere, we have improved the solubility and yield further by using a novel expression vector (constructed by us) which produces the lysophospholipase in fusion with a different hyperthermophilic protein of high intrinsic solubility and proteolytic as well as thermal stability. Also, we note that it is possible that the yield may be improved by refolding the protein through dilution of the denaturant, instead of through dialysis performed to remove the denaturant. We hope to try this approach in combination with the fusion approach, if the fusion protein is not significantly more soluble than the Pfu lysophospholipase.

Confirmation of subunit mass

The recombinant protein produced was expected to have a length of 286 amino acids, corresponding to a calculated molecular weight of 32,037.83 Da (i.e., approx. 32 kDa). The mass of the lysophospholipase measured by SDS–PAGE analysis (Fig. 2A) is approximately 28–29 kDa. The apparently lower mass could be due to incomplete unfolding of this hyperthermophilic protein during preparation for SDS–PAGE, as it is known that hyperthermophilic proteins can display anomalous mobility [8]. The protein's mass was therefore also measured by MALDI-TOF analysis of the intact purified form (Supplementary Fig. 1) and confirmed to be 31,981 Da, with a subpopulation of 31,773 Da arising apparently from truncation of two residues at the N-terminus. The upper value (deviating from the expected mass by ~50 Da) falls within the range of error expected for mass measurements in this range of masses, using measurement with the linear mode of a mass spectrometer.

Establishment of dimeric status

A hydrodynamic radius of 4–5 nm was determined by dynamic light scattering measurements (Fig. 2B). This radius translates into a native enzyme molecular mass of approximately 82 kDa (Table 2). Given that the subunit molecular weight is 32 kDa, this indicates that the protein probably adopts a dimeric state upon refolding. Since the molecular mass calculation is based on the hydrodynamic radius, which is the actual quantity measured, mass calculations of non-spherical molecules invariably lead to overestimates, explaining how a dimer with a real quaternary structural mass of ~64 kDa could be calculated to have a mass of ~82 kDa. Gel filtration-based estimation could not be done because the enzyme interacted with Superdex (Amersham Pharmacia) and Bio-Sil (Bio-Rad) matrices.

Confirmation of protein identity

The identity of the soluble, refolded putative lysophospholipase could be clearly established through peptide mass fingerprinting, using digestion with the protease, trypsin, followed by mass spectrometric determination of tryptic fragment sizes for comparison with expected fragment sizes (Fig. 2C).

Folded tertiary structure with buried tryptophans

The fluorescence emission maximum wavelength of the lysophospholipase was established to be ~340 nm (Fig. 3A). This emission derives from the protein's four tryptophan residues (see the sequence in Fig. 1). As is well known, tryptophan emission maxima are extremely sensitive to the polarity of the environment; therefore, an unfolded protein emits at 352–353 nm whereas a folded protein with buried tryptophan(s) emits maximally at a shorter wavelength, with the blue-shifting of the emission maximum depending on the extent of burial of tryptophans (subject to a maximum blue-shift of ~35 nm). The refolded Pfu lysophospholipase

```
1   ATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCTCCCCGGGACATCACCAT
1    M  A  S  M  T  G  G  Q  Q  M  G  R  G  S  S  P  G  H  H  H
61  CACCATCACCATCACATGACCCAGGTATACAAAGCAAAATTTGGAACTCCAAACAGAGGA
21   H  H  H  H  H  M  T  Q  V  Y  K  A  K  F  G  T  P  N  R  G
121 TGGGTTATAATAGTTCACGGATTAGGAGAGCACAGTGGAAGGTACTCAAAACTGGTAAGC
41   W  V  I  I  V  H  G  L  G  E  H  S  G  R  Y  S  K  L  V  S
181 ATGCTTGTAAATGAAGGATATGCAGTCTATACATTTGACTGGCCAGGTCATGGAAAGAGC
61   M  L  V  N  E  G  Y  A  V  Y  T  F  D  W  P  G  H  G  K  S
241 CCAGGAAAAAGAGGTCATACAAGCGTTGAAGAAGCAATGGAAATAATAGACTTTATCATA
81   P  G  K  R  G  H  T  S  V  E  E  A  M  E  I  I  D  F  I  I
301 GAGGAGATAAACGATAAACCTTTTCTCTTTGGCCACAGCCTCGGAGGATTAACAGTGATA
101  E  E  I  N  D  K  P  F  L  F  G  H  S  L  G  G  L  T  V  I
361 AGATATGCAGAAACAAGGCCAGAAAAGATTAGAGGAGTTATTGCCTCCTCCCCAGCTCTG
121  R  Y  A  E  T  R  P  E  K  I  R  G  V  I  A  S  S  P  A  L
421 GCTAAGAGCCCAAAAACACCCTCTTTTATGGTTGCACTTGCAAAAATTCTTGGAGTCTTG
141  A  K  S  P  K  T  P  S  F  M  V  A  L  A  K  I  L  G  V  L
481 CTTCCAAGCTTAACACTCTCCAATGGAATAGATCCCAATCTTCTCTCAAGAAATCCAGAT
161  L  P  S  L  T  L  S  N  G  I  D  P  N  L  L  S  R  N  P  D
541 GCTGTAAAAAGATACATAGAAGATCCCCTGGTTCATGACAGAATTTCTGCCAAACTGGGA
181  A  V  K  R  Y  I  E  D  P  L  V  H  D  R  I  S  A  K  L  G
601 AGGAGCATATTCAAGAACATGGATCTTGCTCACAGAGAAGCCCACAAAATTAAAGTTCCA
201  R  S  I  F  K  N  M  D  L  A  H  R  E  A  H  K  I  K  V  P
661 GTTCTACTCTTAGTTGGGACTGGGGATGTTATAACTCCTCCAGAAGGAGCTAGAAAGTTA
221  V  L  L  L  V  G  T  G  D  V  I  T  P  P  E  G  A  R  K  L
721 TACGGGGAAATCAAAGTGGAAGATAAGGAAATTGTAGAGTTCGAAGGTGCATACCATGAG
241  Y  G  E  I  K  V  E  D  K  E  I  V  E  F  E  G  A  Y  H  E
781 ATATTTGAAGATCCAGAATGGGGAGAAGAGTTTCATAAAAAGATAGTTGAGTGGATAAAG
261  I  F  E  D  P  E  W  G  E  E  F  H  K  K  I  V  E  W  I  K
841 AAGCATCATCACCACCATTAG
281  K  H  H  H  H  H  *
```

Fig. 1. Amino acid sequence of the recombinant PF0480 (Pfu lysophospholipase) that was produced. The residues S113, E213 and H259 (in italics, brown) are predicted by the MEROPS database entry MER35299 to constitute the catalytic triad of residues for the α/β hydrolase fold predicted for this enzyme sequence which is homologous to other hydrolases of the S33 family of hydrolases. The conserved GXSXG motif characteristic of esterase enzymes containing a catalytic serine (S113) is also shown (in italics, brown). Extra residues from the expression vector and from the 6×His tags at the N- and C-termini are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Lysophospholipase yields obtained through different methods

Method of solubilization and purification	Approximate yield
Native lysis by lysozyme and sonication, followed by IMAC purification	Negligible
Lysis in 3 M Gdm·HCl and 1 M NaCl, followed by IMAC purification	Negligible
Lysis in 3 M Gdm·HCl, 1 M NaCl with heating (70 °C, 30 min) followed by IMAC purification	Negligible
Lysis in 6 M Gdm-Cl, 1 M NaCl with heating (70 °C, 30 min), followed by IMAC purification	2.3 mg/4 g cell pellet from 1 L
Refolding by dialysis of the affinity purified	~0.23 mg/4 g cell pellet from 1 L

emits maximally at 340 nm, i.e., with a blue-shift of 12–13 nm, indicating that its tryptophan residues are well-buried within the protein's hydrophobic interior, providing proof that the enzyme is folded and has tertiary structure, and is not unfolded.

Tertiary structure stable to water-miscible organic solvents

Fig. 3A also shows that the protein's fluorescence emission maximum is not red-shifted to 352–352 nm in the presence of various concentrations of water-miscible organic solvents, as would be expected if the solvents had unfolded the protein. As the data in Fig. 3A show, the protein remains folded even in the presence of 50% acetonitrile, ethanol or methanol.

Mixed alpha-beta secondary structure

The far-UV CD spectrum of the protein in water establishes that it is well folded, with formed secondary structure indicative of mixed helical and sheet content, and with beta sheets dominating the spectrum's signal intensity and helices dominating the spectrum's shape. The presence of helices is indicated by the characteristic minima observed at 208 and 222 nm, whereas the sheet content is indicated by the relatively low intensity of the spectral minimal mean residue ellipticity (MRE) value of only -4500 deg cm^2 dmol^{-1}. This MRE value would be expected to be of the order of $-35{,}000$ deg cm^2 dmol^{-1} if the protein were entirely helical, suggesting that the protein has a low helical content and high sheet content.

Stability of secondary structure to water-miscible solvents

It is seen that no changes in structure result from the presence of water-miscible organic solvents in the protein's environment, since there are only relatively minor changes in the CD spectrum of the protein in the presence of these solvents (Fig. 3B). However, the solvents do appear to increase the 222 nm signal intensity, indicating that they promote some helix formation as is common with alcohols. Thus, even though subtle changes are seen, the CD data establish that the secondary structure of the lysophospholipase is quite stable to unfolding in the presence of organic solvents, up to 50% acetonitrile, methanol or ethanol.

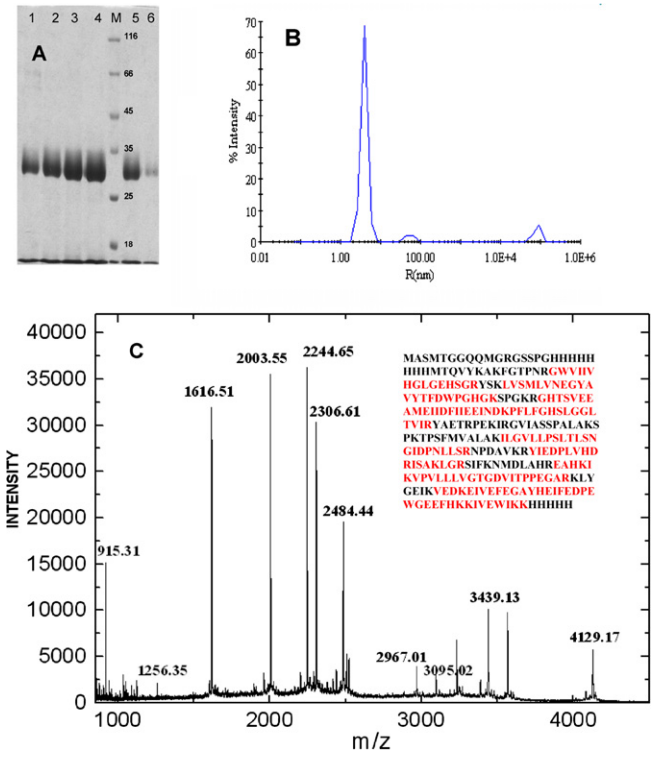

Fig. 2. Molecular weights of the subunit and native forms of Pfu lysophospholipase. (A) an SDS–PAGE of purified Pfu lysophospholipase protein by Ni-NTA affinity chromatography. Lanes marked 1–6 show 16 μl aliquots of successive fractions of 1.5 ml collected during elution from the column with urea-containing, pH 4.5, buffer. (B) The regularized size-distribution of Pfu lysophospholipase measured through dynamic light scattering analysis. (C) The MALDI-TOF peptide mass fingerprint (PMF) spectrum of fragments of Pfu lysophospholipase derived through trypsin digestion (in-solution). The expected tryptic masses which clearly matched, with 1 Da tolerance, have been labeled. The sequence coverage of these fragments is shown in red, in the inset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

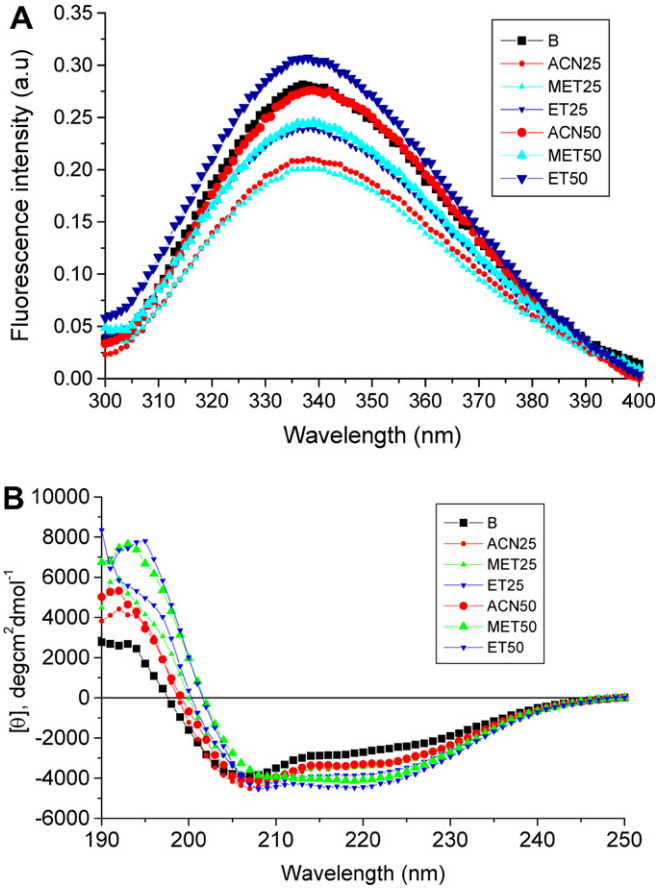

Fig. 3. Structure retention by Pfu lysophospholipase in mixtures of water and various nonpolar solvents. (A) Fluorescence emission spectra of Pfu lysophospholipase in water (marked B) and in the presence of 25% and 50% acetonitrile (ACN), methanol (MET) and ethanol (ET) as marked in the inset. (B) Far-UV CD spectra of Pfu lysophospholipase in water (marked B) and in the presence of 25% and 50% acetonitrile (ACN), methanol (MET) and ethanol (ET) as marked in the inset.

Thermal stability

From monitoring of the far-UV CD (MRE) signal at 222 nm as a function of temperature, the lysophospholipase can be seen to begin to unfold only above 85 °C, with unfolding remaining incomplete even at 95 °C (black curve; Fig. 4). This establishes that the protein is extremely thermostable in an aqueous environment. Notably, cooling does not result in the restoration of the lost CD (MRE) signal at 222 nm (red curve, Fig. 4) establishing that the thermal denaturation of the lysophospholipase is irreversible, in aqueous media, in the absence of chaotropic cosolutes. This is not entirely unexpected, since many hyperthermophilic enzymes are known to unfold irreversibly upon heating [9]. However, it may be recalled that the enzyme appears to have no problems in refolding after being extracted from inclusion bodies through the combined effect of heat (80 °C), denaturant (6 M Gdm·HCl), and additional electrolyte (1 M NaCl), during refolding through dialysis-based removal of denaturant and salt at room temperature. This observation merits further exploration.

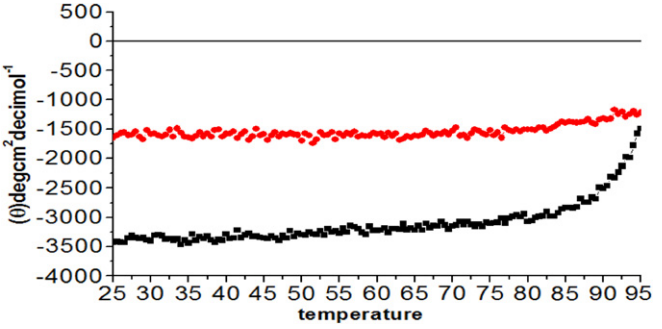

Fig. 4. Stability to thermal melting of Pfu lysophospholipase. Effects on the far-UV CD signal, measured through monitoring of mean residue ellipticity at 222 nm as a function of heating from 25 to 95 °C, shown as the black curve (thermal unfolding), and cooling from 95 to 25 °C, shown as the red curve (refolding). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 2
Molecular sizing data from dynamic light scattering studies

Radius (nm)	% Polydispersity	Molecular weight (kDa)	% Intensity	% Mass
3.9	17.62	82	88.0	100.0
54.5	20.01	38,789	4.0	0.0
82,846	17.13	1,083,790,000,000	7.9	0.0

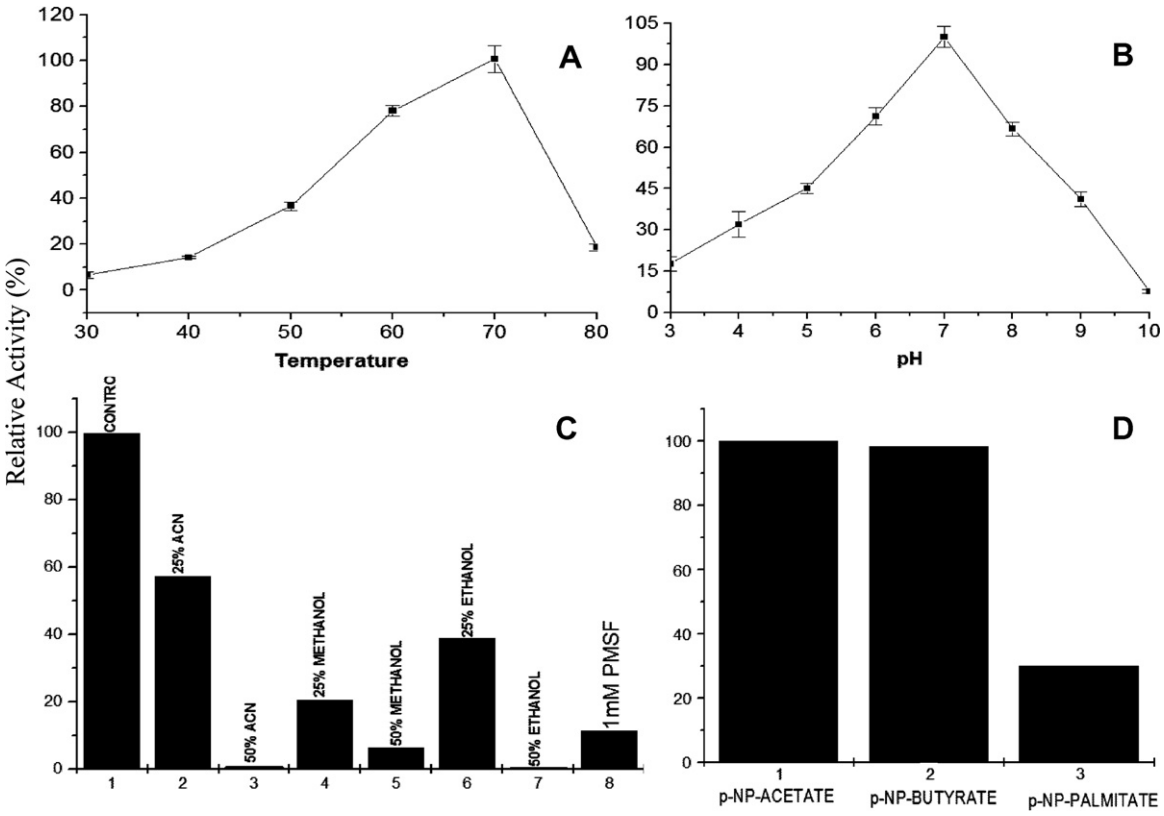

Fig. 5. Functional behavior of Pfu lysophospholipase with *p*-NP-palmitate (A–C) and also with *p*-NP-butyrate and *p*-NP-acetate (D). (A) Activity data for a fixed pH of 8.0, using the substrate *p*-NP-palmitate, at different temperatures on a relative activity scale. (B) Activity data for a fixed temperature of 70 °C, using the substrate *p*-NP-palmitate, at different pH values on a relative activity scale. (C) The effects of different cosolvents (marked in the figure) on activity at 70 °C, using 10 mM phosphate, pH 7.5, as the aqueous component. (D) The effect of changing length of the acyl chain on activity, in 10 mM phosphate, pH 7.5, at 70 °C, with bars 1 and 2 showing activity obtained through 5 min incubations against *p*-NP-acetate and *p*-NP-butyrate, and bar 3 showing the activity obtained through 30 min incubation against *p*-NP-palmitate, using identical concentrations of all substrates.

Optimum lipase activity at 70 °C and pH 7.0

With low standard error, the activity of the enzyme was found to be optimal at a temperature of 70 °C for a fixed pH of 8.0 (Fig. 5A) and at pH 7.0, for a fixed temperature of 70 °C (Fig. 5B), establishing that the protein is a neutral thermo-stable lipase that is also 'thermophilic' in regard to its functionality.

Function in organic solvents

For 25% v/v mixtures of acetonitrile, ethanol and methanol, the retention of lipase activity against the substrate p-NP-palmitate was found to be highest for 25% acetonitrile (over 50% of the control activity in aqueous buffer), followed by 25% ethanol and methanol (Fig. 5C). The retention of such high levels of activity, within the same decade of measurement, is quite remarkable. Some residual activity is still seen in 50% methanol, although only about 1/100 activity is seen in 50% acetonitrile and 50% ethanol, with the p-NP-palmitate substrate.

Confirmation of the function of the catalytic triad serine residue

Incubation in aqueous buffer of pH 8.0, with 1 mM phenylmethyl sulfonyl fluoride (PMSF), leads to a remarkable reduction in activity, establishing that the catalytic triad is inactivated through covalent modification of serine by PMSF (Fig. 5C), just as is seen to occur specifically with many serine proteases such as subtilisin and trypsin/chymotrypsin.

Relative esterase and lipase activities

The data in Fig. 5D is based on 5 min reactions for *p*-NP-acetate and *p*-NP-butyrate, but using a 30 min reaction for *p*-NP-palmitate (because the faster rate of natural hydrolysis of the control, with the smaller acyl chains, made it necessary to use shorter incubations). Given the nearly three-fold lower activity despite the use of a 6-fold higher incubation time, it can be seen that hydrolysis proceeds with nearly 18–20 times greater efficiency if the substrate has a small acyl chain (e.g., acetate or butyrate) than when the substrate has a longer acyl chain (e.g., palmitate). Clearly, therefore, the enzyme is much more efficient as an esterase than as a lipase (Fig. 5D), although, equally clearly, the enzyme is also an excellent lipase, as established by the data in Fig. 5A–C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2008.02.019.

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