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Research paper

Characterization of the first eukaryotic cold-adapted patatin-like phospholipase from the psychrophilic *Euplotes focardii*: Identification of putative determinants of thermal-adaptation by comparison with the homologous protein from the mesophilic *Euplotes crassus*

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ABSTRACT

The ciliated protozoon *Euplotes focardii*, originally isolated from the coastal seawaters of Terra Nova Bay in Antarctica, shows a strictly psychrophilic phenotype, including optimal survival and multiplication rates at 4–5 °C. This characteristic makes *E. focardii* an ideal model species for identifying the molecular bases of cold adaptation in psychrophilic organisms, as well as a suitable source of novel cold-active enzymes for industrial applications. In the current study, we characterized the patatin-like phospholipase from *E. focardii* (*Ef*PLP), and its enzymatic activity was compared to that of the homologous protein from the mesophilic congeneric species *Euplotes crassus* (*Ec*PLP). Both *Ef*PLP and *Ec*PLP have consensus motifs conserved in other patatin-like phospholipases.

By analyzing both esterase and phospholipase A_2 activity, we determined the thermostability and the optimal pH, temperature dependence and substrates of these enzymes. We demonstrated that *Ef*PLP shows the characteristics of a psychrophilic phospholipase. Furthermore, we analyzed the enzymatic activity of three engineered versions of the *Ef*PLP, in which unique residues of *Ef*PLP, Gly_{80}, Ala_{201} and Val_{204}, were substituted through site-directed mutagenesis with residues found in the *E. crassus* homolog (Glu, Pro and Ile, respectively). Additionally, three corresponding mutants of *Ec*PLP were also generated and characterized. These analyses showed that the substitution of amino acids with rigid and bulky charged/hydrophobic side chain in the psychrophilic *Ef*PLP confers enzymatic properties similar to those of the mesophilic patatin-like phospholipase, and *vice versa*.

This is the first report on the isolation and characterization of a cold-adapted patatin-like phospholipase from eukaryotes. The results reported in this paper support the idea that enzyme thermal-adaptation is based mainly on some amino acid residues that influence the structural flexibility of polypeptides and that *Ef*PLP is an attractive biocatalyst for industrial processes at low temperatures.

1. Introduction

Psychrophiles are organisms living at cold temperatures, colonizing about 70% of earth's biosphere, such as polar and alpine regions or deep-sea waters [1]. The ability of psychrophiles to survive in the cold is the result of molecular evolution and adaptations which, together, overcome the deleterious effects of low energy environments and the freezing of water [1]. One of the essential adaptive properties of psychrophiles is represented by their cold-active enzymes, which have received a great deal of attention in fundamental research for understanding the molecular basis underlying cold-adaptation [2], and for their potential utilization in certain industrial applications [3]. Cold-adapted enzymes have evolved a range of molecular features that confer a high level of structural flexibility, particularly around the active site that are translated into low activation enthalpy. This property can be useful to

Abbreviations: PLP, patatin-like phospholipase; RATE, rapid amplification of telomeric ends; *p*NP, p-nitrophenyl; *Di*C_3PC, 1,2-dipropinoyl-sn-glycero-3-phosphocholine; *Di*C_4PC, 1,2-dibutanoyl-*sn*-glycero-3-phosphocholine; *Di*C_6PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine.

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improve the efficiency of industrial processes by lowering the energetic costs [3,4].

Lipases are one of the most investigated groups of enzymes because they display both hydrolysis and synthesis activity toward many useful ester compounds. Lipases catalyze esterification, trans-esterification, interesterification, acidolysis, alcoholysis, and aminolysis, in addition to the hydrolytic activity on triglycerides. Thus, they constitute a potential source of biotechnologically interesting substances and have emerged as key enzymes for a wide variety of different industrial applications, including synthesis of biopolymer and biodiesel, production of pharmaceutical and detergents, food processing, chemical transformation, and cosmetics. In this contest, cold-adapted lipases have lately attracted attention because their high activity at low temperature is a favorable property for the production of relatively frail compounds [5].

A family of lipolytic enzymes designated as patatin-like phospholipases (PLPs, EC = 3.1.1.3, PFAM accession number: PF01734) has been previously proposed as key biocatalysts [6]. The PLPs are nonspecific lipid acyl hydrolases predominately found in potato tubers [7–9], where are essentially storage proteins with catalytic properties [10]. These proteins catalyze the nonspecific hydrolysis of phospholipids, glycolipids, sulfolipids, and mono- and diacylglycerols [11–14]. Furthermore, PLPs appear to be involved in signal transduction and to function as bioactive enzymes that protect plants from parasites [15,16]. Sequence homology studies [16,17], together with crystal structure study of a isozyme of patatin, and alanine-scanning mutagenesis study [17,18], have shown that PLPs share conserved blocks with the animal calcium-independent phospholipase A_2 ($iPLA_2$) and cytosolic phospholipase A_2 ($cPLA_2$), and possess an active dyad site instead of the Ser-His-Asp/Glu triad common of other lipolytic enzymes [19].

PLPs were intensively studied in plants, bacteria and vertebrates [20–22]. Most of these works focused mainly on subfamily classification of the *PLP* genes and on the characterization of the biological roles by informatics approaches. Relatively few PLPs have been characterized biochemically so far.

In this paper, we report the biochemical characterization of the first eukaryotic PLP from *Euplotes focardii* (*Ef*PLP), a ciliated protozoon endemic of Antarctic coastal seawaters. Ciliates provide excellent material to study adaptive evolution to cold, since they are single cells directly exposed to environmental cues. We choose *E. focardii* as model organism for this study because this eukaryotic microbe shows strictly psychrophilic phenotypes: its optimal temperature of survival and reproduction is 4–5 °C; it lacks a transcriptional response of the *Hsp70* genes to thermal shock [23,24]; it possesses cold-stable microtubules [25,26] and modified ribosomal proteins [27].

By analyzing enzymatic activities, we determined optimal pH, temperature dependence, thermostability and substrates specificity of *Ef*PLP, in comparison with the activities of a PLP from the evolutionary close mesophilic species *Euplotes crassus* (*Ec*PLP). We demonstrated that *Ef*PLP shows the characteristics of a psychrophilic phospholipase, because it is active in the cold, whereas *Ec*PLP is not. Furthermore, we analyzed the enzymatic activity of three engineered versions of the *Ef*PLP, in which unique residues of *Ef*PLP, Gly_{80}, Ala_{201} and Val_{204}, were substituted through site-directed mutagenesis by Glu, Pro and Ile, respectively (i.e. residues found in the *E. crassus* homolog). These analyses indicated that the introduction in the *Ef*PLP of amino acids with rigid and bulky charged/hydrophobic side chains confer enzymatic properties more similar to those of the mesophilic PLPs. For obtaining further support of our findings, we also carried out the reverse procedures by replacing Glu_{81}, Pro_{202} and Ile_{205} residues of *Ec*PLP with Gly, Ala and Val, respectively. Following, the effects of temperatures on enzyme properties of those variants were examined and catalytic efficiency was also investigated. These results indicate that enzyme thermal-adaptation is based on amino acid residues that influence the structural flexibility of polypeptides. Furthermore, we demonstrated that PLPs are enzymes that can display both esterase and phospholipase A_2 (PLA_2) activities, a characteristic that makes these molecules promising for industrial applications even at low temperatures.

2. Materials and methods

2.1. Materials

Restriction enzymes, recombinant *Taq* DNA polymerase and *Pfu* DNA polymerase were purchased from Fermentas (Milan, Italy). Ampicillin, Isopropyl-β-D-thio-galactoside (IPTG), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) and *p*-Nitrophenyl (*p*NP) esters were purchased from Sigma–Aldrich (USA). Arachidonoyl thio-PC was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). 1,2-dipropinoyl-sn-glycero-3-phosphocholine (*Di*C_3PC), 1,2-dibutanoyl-*sn*-glycero-3-phosphocholine (*Di*C_4PC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (*Di*C_6PC) were purchased from Avanti Polar Lipids (Alabama, USA). All chemicals were reagent grade. All oligonucleotide primers used in this study were synthesized by Sigma (Milan, Italy).

2.2. Cell strains and growth conditions

Cultures of *E. focardii* strain TN1 [28] and *E. crassus* strain G1 were used. *E. focardii* strain TN1 represents type-species material chosen from a number of wild-type strains isolated from sediment and seawater samples collected from the coastal waters of Terra Nova Bay, Antarctica (temperature −1.8 °C, salinity 35 *f* and pH 8.1–8.2). It was grown and maintained in a cold room at 4 °C. The *E. crassus* strain G1 used in this study was kindly obtained from the strain collection of Luporini's laboratory at the University of Camerino. It is a close relative species of *E. focardii* and represents the mesophilic counterpart. In a phylogenetic tree of *Euplotes* species based on 18S rDNA these two *Euplotes* belong to the same cluster (data not shown). Both cell lines were fed with the green alga *Dunaliella tertiolecta* as food.

2.3. Isolation of Euplotes PLPs nanochromosomes from macronuclear DNA via PCR and rapid amplification of telomeric ends (RATE)

E. focardii macronuclear DNA was purified as described [29]. To obtain the primary partial *E. focardii PLP* gene (*EfPLP*) sequence, we based our PCR strategy on degenerate oligonucleotide primers designed from *Euplotes EfPLP* consensus sequence (obtained by the alignment of homologous genes from *E. crassus*, which were kindly provided by the laboratory of Professor Gladyshev, Harvard Medical School, USA). Degenerate oligonucleotide primers were designed according to the improved CODEHOP primer design method [30]. The forward primer, 5′-GTKTCTGCHGGWTCCCTMAACACTTTAGGTCT-3′, covered codons 59–68 plus two nucleotides from 69, and the reverse primer, 5′- GATGTCVATSTTVCARATTACTCCACCATCAA-3′, corresponded to two nucleotides of codon 190 plus triplets 200–191. Amplification was performed using touchup PCR strategies: 95 °C for 2 min, followed by 10 cycles of 95 °C for 30 s, from 45 °C annealing temperature gradually rise 1 °C each cycle for 30 s to 54 °C, and 72 °C for 30 s, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finally a final extension at 72 °C for 5 min. The amplification products were expected to be about 1270 bp long.

Based on the organization of nanochromosomes in *Euplotes* spp. [31], RATE PCR [29] was carried out for obtaining the sequences of

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N-terminal and C-terminal coding regions and the 5′- and 3′-UTRs. The forward primer, 5′- GCAGATAACTTAGGTGACAGAACTCT-3′ and reverse primer, 5′- AGAGTTCTGTCACCTAAGTTATCTGC-3′ were individually used in combination with the telomeric oligonucleotide 5′-$(C_4A_4)_4$-3′ in RATE PCR. Amplified products were cloned into pTZ57R/T vector (Fermentas, Milan, Italy) and further transformed into *Euplotes coli* DH5α. Clones containing the *EfPLP* recombinant plasmids were sequenced in both strands (BMR Genomics, Padova, Italy).

The *E. crassus PLP* gene (*EcPLP*) was amplified from *E. crassus* genomic DNA. Oligonucleotides *Ec*NdeI (5′-CATATGAGGAAATGTAGAGCATTAGCCCT-3′) and *Ec*XhoI (5′-CTCGAGTTGAGACTCAGATTTTGGTTGTTTT-3′) were used as forward and reverse primers, respectively. The amplification primer *Ec*NdeI and *Ec*XhoI were designed to introduce an NdeI restriction site upstream from the initiation site, and an XhoI restriction site downstream from the stop codon of *EcPLP*, respectively. Amplification was performed using standard conditions: 30 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 70 s. After the amplification, the reaction was further incubated at 72 °C for 10 min and stored at 4 °C. The PCR product was ligated into pTZ57R/T vector (Fermentas, Milan, Italy) and further transformed into *E. coli* DH5α, and clones containing *EcPLP* recombinant plasmids were sequenced in both strands (BMR Genomics, Padova, Italy).

E. coli DH5α was used as host for cloning whereas *E. coli* BL21 (DE3) harbored the recombinant plasmids for gene expression. *E. coli* strains were routinely grown in LB medium at 37 °C. When required, antibiotics and chromogenic substrates were added at the following concentrations: 100 μg/mL ampicillin and 30 μg/mL 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal). The complete *Euplotes PLPs* sequences are available on GeneBank™ by accession numbers: JX257001 (*EfPLP*) and JX257002 (*EcPLP*).

2.4. Subcloning of Euplotes PLPs into the expression vector pET-22b(+)

In order to facilitate heterologous expression of *Ef*PLP protein in *E. coli*, the gene without predicted signal peptide (resulting from the deletion of the gene region encoding the first 18 amino acids) was synthesized with *E. coli* codon usage by a gene synthesis distributor (Mr. Gene, Regensburg, DE). Restriction sites NdeI and XhoI were introduced to the 5′- and 3′- respectively. The synthesized *EfPLP* gene was cloned into pET-22b(+) expression vector (Novagen, USA) previously digested by NdeI and XhoI restriction enzymes to construct an expression plasmid pET-*EfPLP*. Similarly, the *EcPLP* recombinant plasmid was digested by NdeI and XhoI restriction enzymes and *EcPLP* was cloned into pET-22b(+) expression vector previously digested by NdeI and XhoI restriction enzymes in order to obtain a pET-*EcPLP* expression vector. The expression cassette includes an in-frame C-terminal fusion purification 6× His-Tag. The DNA sequence of the resulting construct was verified by bidirectional DNA sequencing.

2.5. Plasmid constructions of mutants

The site-directed mutants were prepared by the overlap extension method [32]. It yielded six expression plasmids: pET-*EfPLP*-G80E, pET-*EfPLP*-A201P and pET-*EfPLP*-A201P/V204I for generating variants of *Ef*PLP; pET-*EcPLP*-E81G, pET-*EcPLP*-P202A and pET-*EcPLP*-P202A/I205V for obtaining mutants of *Ec*PLP. The polymerase chain reactions were carried out by using the plasmids pET-*EfPLP* and pET-*EcPLP*, respectively as templates, *Pfu* DNA polymerase, and two complementary mutagenic primers for each site-specific mutation. The sequences of the oligonucleotides used were as shown in Table 1. Each amplification reaction was performed with 30 cycles of 94 °C for 30 s, 68 °C for 1 min, and 72 °C for 10 min. The final amplified products were verified by bidirectional DNA sequencing.

2.6. Overexpression and purification of wild-type and mutant Euplotes PLPs

E. coli BL21 (DE3) cells carrying wild-types or mutants plasmids were grown overnight at 37 °C in LB medium supplemented with 100 μg/mL ampicillin. The overnight cultures were diluted to a cell density of about 0.08 OD_{600} in 600 mL of LB medium supplemented with 100 μg/mL ampicillin using a 2 L flask. Cultivation took place in a 2 L flask at 30 °C under vigorous stirring and aeration. The induction procedures were carried out when cultures reached 0.6–0.8 at OD_{600} by addition of filter-sterilized IPTG to a final concentration of 1 mM. The cultures were grown for overnight post-induction. Cells were harvested by centrifugation at 5000 *g* at 4 °C for 20 min, divided into 0.50 g aliquots and frozen at −80 °C.

2.7. Purification of PLPs from E. coli BL21 (DE3) cells

IPTG induction of recombinant *E. coli* BL21 (DE3)/PLPs cells resulted in the accumulation of recombinant wild-type and mutant proteins as inclusion bodies. To recover of PLPs from inclusion bodies, the bacterial cell pellet (0.50 g) was frozen and thawed twice, resuspended in 3 mL TE buffer (50 mM Tris–HCl, pH 8 and 40 mM EDTA). 0.75 g sucrose and 0.6 mg lysozyme were added and the solution was incubated at 37 °C for 30 min. An osmotic shock was performed by the addition of 3 mL ice-cold TE buffer. The solution was again homogenized and incubated for 30 min at 37 °C. The cells were subjected to sonication for 5 for 1 min with intervals of 2 min to prevent overheating. Subsequently, Triton-X100 was

Table 1
Nucleotide sequences of primers used for site-directed mutagenesis and generated recombinant plasmids.

Directed mutation	Primer sequence[a]	Generated plasmid
*Ef*PLP-G80E	FW: 5′-ACTGACGTTGAGGaAGCAAGCGAGTTC-3′ RV: 5′-GAACTCGCTTGCTtCCTCAACGTCAGT-3′	pET-*EfPLP*-G80E
*Ef*PLP-A201P	FW: 5′-ATCGACATTcCTTCGGCTGTTCGTCGTTGT-3′ RV: 5′-ACAACGACGAACAGCCGAAGgAATGTCGAT-3′	pET-*EfPLP*-A201P
*Ef*PLP-A201P/V204I	FW: 5′-ATCGACATTcCTTCGGCTaTTCGTCGTTGT-3′ RV: 5′-ACAACGACGAAtAGCCGAAGgAATGTCGAT-3′	pET-*EfPLP*-A201P/V204I
*Ec*PLP-E81G	FW: 5′-CAGAGGAAGTTGAAGgAGCCACTGAATTC-3′ RV: 5′-GAATTCAGTGGCTcCTTCAACTTCCTCTG-3′	pET-*EcPLP*-E81G
*Ec*PLP-P202A	FW: 5′-AACATCGACATTgCTTCAGCTATCAGAAGGT-3′ RV: 5′-ACCTTCTGATAGCTGAAGcAATGTCGATGTT-3′	pET-*EcPLP*-P202A
*Ec*PLP-P202A/I205V	FW: 5′-CATCGACATTgCTTCAGCTgTCAGAAGGTG-3′ RV: 5′-CACCTTCTGAcAGCTGAAGcAATGTCGATG-3′	pET-*EcPLP*-P202A/I205V

[a] Mismatch sites for site-directed mutagenesis are in small letters.

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added to a final concentration of 0.1% (w/v) and the solution was sonicated again for 1 min. The inclusion bodies were collected by centrifugation (20 min, 6726 *g*, 4 °C) and washed with 4 mL TE buffer. After centrifugation, the pellet was suspended in 2 mL of 10 mM Tris–HCl (pH 8). After 10 min centrifugation at 15,000 *g*, the pellet containing inclusion bodies was solubilized in 10 mL of 8 M urea and 5 mM glycine (pH 9) at room temperature. To remove the insoluble material, the solution was centrifuged 20 min at 10,000 *g*. The resulting supernatant was dialyzed in 200 mL of 20 mM glycine (pH 9), 20 mM beta-mercaptoethanol and 5 mM EDTA, at 4 °C, to allow the unfolded protein to refold. The buffer was changed three times in 24 h. Finally, the solution was dialyzed again at 4 °C against 200 mL 20 mM Tris–HCl (pH 8) to remove beta-mercaptoethanol and EDTA. Then, the refolded protein was stored at −20 °C in presence of 10% glycerol. All isolation steps were performed at 4 °C (unless otherwise specified).

2.8. SDS-polyacrylamide gel electrophoresis (PAGE) and protein concentration determination

SDS-PAGE was performed according to the method of Laemmli using 5% polyacrylamide-stacking gel and 12% polyacrylamide-resolving gel with a Bio-Rad Mini-Protean II cell unit, at room temperature. The protein concentration was determined according to the Bradford method with bovine serum albumin as the standard [33]. The protein content was measured by monitoring the optical density at 595 nm.

2.9. N-terminal sequencing of the Euplotes PLPs recombinant protein

Electroblotting was performed using PVDF membrane with 25 mM Tris Base, 192 mM glycine (pH 9) transfer buffer in the presence of 10% methanol. The membrane stained with Coomassie Brilliant Blue and destained to achieve protein band from membrane. Recombinant protein identification was carried out by N-terminal sequencing (AB Procise 49X cLC Protein Sequencing System) of the electroblotted protein band.

2.10. Enzyme assays

The esterase activities of the PLPs were measured spectrophotometrically at 405 nm by detection of *p*-nitrophenoxide released from *p*NP esters in 1-cm path-length cells with a Cary 100 spectrophotometer (Varian, Australia) equipped with a temperature controller. Assays were performed in 1 mL mixture containing purified enzyme (30 μg/mL), Teorell–Stenhagen buffer (50 mM boric acid, 10 mM phosphoric acid, 2.5 mM citric acid, adjust pH by NaOH) [34] at pH 10.5, 3% acetonitrile, and *p*NP esters at different concentrations. Stock solutions of *p*NP esters were prepared by dissolving the substrates in pure acetonitrile. Enzyme activities were determined at 25 °C for *Ef*PLP, *Ef*PLP-G80E and *Ec*PLP-P202A/I205V, at 30 °C for *Ef*PLP-A201P and *Ec*PLP-P202A, and at 35 °C for *Ec*PLP, *Ec*PLP-E81G and *Ef*PLP-A201P/V204I, respectively. Assays were carried out in duplicate or triplicate, and the results were expressed as the means of two or three independent experiments. One unit enzymatic activity was defined as the amount of protein that released 1 μmol of *p*-nitrophenoxide/min from *p*NP esters. The absorption coefficient used for *p*-nitrophenoxide was 19,000 M^{-1} cm^{-1}.

2.11. Determination of optimal pH and temperature of wild-type and mutant PLPs

The pH dependence of the esterase activity was monitored at 348 nm (the pH-independent isosbestic point of *p*-nitrophenol and the *p*-nitrophenoxide ion), with *p*NP butyrate (100 μM) as substrate. A molar absorption coefficient of 5000 M^{-1} cm^{-1} at 25 °C was introduced. The universal Teorell-Stenhagen buffer over a broad range of pH (pH 8–12) was used. Data were analyzed as described by Dixon and Webb [35]. The temperature optimum was determined by analyzing the esterase activity in the range of temperature from 0 to 50 °C in Teorell-Stenhagen buffer (pH 10.5), containing 3% acetonitrile, using *p*NP butyrate (100 μM) as substrate.

2.12. Determination of the thermostability

Half-lives of thermal inactivation were determined for purified wild-types and variants PLPs by incubating the enzymes (30 μg/mL) at 40, 55 and 70 °C for 30–180 min with regular time intervals. Initial and residual activities were measured under the standard assay conditions previously described. The first-order rate constant, k_d, of irreversible thermal denaturation was obtained from the slope of the plots of ln (initial activity/residual activity) versus time, and the half-lives ($t_{1/2}$) were calculated as $\ln 2/k_d$.

2.13. Measurement of PLA_2 activity of wild-types and mutants

PLA_2 activities toward the substrate arachidonoyl thio-PC were estimated colorimetrically by determining the release of thiol groups. Substrate in ethanol was bubbled under a gentle stream of nitrogen and then solubilized in assay buffer (80 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 4 mM Triton X-100, 30% v/v glycerol, and 1 mg/mL bovine serum albumin) and vortexed until completely dissolved. A solution in 6 mL of assay buffer contained arachidonoyl thio-PC at a final concentration of 1.5 mM. The PLA_2 assay was conducted using a 96 well microplate with a total assay volume of 225 μL. The reaction mixture contained assay buffer, arachidonoyl thio-PC substrate and purified enzyme (30 μg/mL). Reactions were initiated by the addition of purified enzyme to the wells followed by incubation for 1 h at 23 °C. Assays were stopped by addition of a mixture of 25 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 475 mM EGTA. Color was developed for 5 min and then absorbance was measured at 414 nm in a Multiskan Spectrum microplate reader (Thermo Labsystems). The determination of PLA_2 activity was calculated by following substrate manufacturer's instructions.

The PLA_2 activities toward substrates 1,2-dipropanoyl-*sn*-glycero-3-phosphocholine (or 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine or 1,2-dibutanoyl-*sn*-glycero-3-phosphocholine) were determined titrimetrically at pH 10.5 and at 25 °C with a pH-stat T50 (Mettler Toledo). Substrates in chloroform were bubbled under a gentle stream of nitrogen and then solubilized in distilled water with sonication. The standard condition contained distilled water and 10 mM substrate in a final volume of 4 mL. Substrate mix was pre-equilibrated with NaOH (0.1 mM) to pH 10.5 and pre-incubated at 25 °C for 10 min. Assays were initiated by adding enzymes to the final concentration of 30 μg/mL into substrate mix, the enzyme activities were measured by continuously monitoring the temperature amount of acid liberated from hydrolysis using the pH stat. One unit of PLA_2 activity was defined as 1 μM of fatty acid liberated under standard condition.

2.14. Kinetic measurements

The kinetic parameters K_M and k_{cat} of enzymes were measured using *p*NP esters as substrates at the condition described in the *Enzyme Assays* section. The initial velocities of substrate hydrolysis were monitored for a minimum of six substrate concentrations. The following substrate concentration ranges were used: *p*NP acetate (C_2, 100–1500 μM), *p*NP propionate (C_3, 100–1500 μM), *p*NP butyrate (C_4, 100–1700 μM), *p*NP valerate (C_5, 100–1500 μM), *p*NP

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caprylate (C_8, 50–700 μM), *p*NP caprate (C_{10}, 50–700 μM). All kinetic data were analyzed by nonlinear regression using Origin 8.0. The Standard Error (S.E.) value of each parameter was estimated from the curve fitting. Assays were done in duplicate or triplicate, and results for kinetic data were mean of two independent experiments.

2.15. Sequence analysis and comparative modeling

Sequence alignment between the putative amino acid sequences of *EfPLP* and *EcPLP* was initiated by the EMBOSS Needle Pairwise Sequence Alignment. Sequence similarity and analysis for conserved blocks were performed using BLAST programs on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Sequence alignments were performed using the tools of the T-Coffee server (http://www.ebi.ac.uk/Tools/msa/tcoffee/) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The N-terminal signal peptide was predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Molecular weights were determined using Protein Calculator V3.3 (http://www.scripps.edu/~cdputnam/protcalc.html). Comparative homology model of the *E. focardii* PLP was obtained by the program SWISS-MODEL [36,37], using the patatin from *Solanum cardiophyllum* (PDB: 1OXW) as a specific template, and the sequence identity between template and target is 19%.

3. Results

3.1. Subcloning the Euplotes PLP genes

Euplotes macronuclear DNA consists of short linear molecules (nanochromosomes) ranging in size from 0.3 to 40 kbp, each of which is present in multiple copies and generally encodes a single genetic function [38]. These nanochromosomes have telomeres at their ends that consist of repeats of the C_4A_4 motif (schematically represented in Fig. 1A). By our RATE-PCR-based cloning strategy (described under "Materials and Methods"), we obtained two entire PLP nanochromosomes from *E. focardii* and *E. crassus*, that we named *EfPLP* and *EcPLP*. These nanochromosomes were 1156 bp and 1229 bp long respectively, without introns. The coding regions were 1071 bp and 1074 bp long, respectively, and the predicted proteins of 357 and 358 amino acids, respectively. The *EfPLP* coding region was flanked by 56 and 29 bp untranslated regions at the 5′ and 3′ sides respectively. The *EcPLP* coding region was flanked by 59 and 37 bp untranslated regions at the 5′ and 3′ sides respectively.

Blastp searches of the *EfPLP* predicted amino acid sequence revealed similarity with PLPs from other protists, including three PLPs from *Tetrahymena thermophila* (XP_001014319.1, XP_001014317.1, XP_001011026.1) with 57% positives (E-value 6e-59), 51% positives (E-value 1e-51), 50% positives (E-value 9e-39), respectively, and a hypothetical patatin from the Ichthyosporea fish parasite *Capsaspora owczarzaki* ATCC 30864 (EFW47637.1) with 51% positives (E-value 1e-33). Each of the bacterial patatin-like conserved blocks [39] was identified in both *Ef*PLP and *Ec*PLP (indicated as blocks I, II, III and IV in Fig. 1B). Block I consists of a Gly-rich region, which probably serves as an oxyanion hole. Block II represents a typical lipase nucleophilic elbow Gly-X-Ser-X-Gly containing the putative active-site Ser (Gly_{59}–Val_{60}–Ser_{61}–Ala_{62}–Gly_{63} in *Ef*PLP and Gly_{60}–Val_{61}–Ser_{62}–Ala_{63}–Gly_{64} in *Ec*PLP), which is located 10–20 AA downstream the Block I. Block III contains a conserved ASXXXP motif of unknown function. Block IV contains the active-site Asp (Asp_{191} in *Ef*PLP and Asp_{192} in *Ec*PLP) that, together with the active-site Ser, forms the catalytic Ser–Asp dyad.

Further sequence analysis performed using SignalP 3.0 server indicated the presence of a signal peptide typical of secreted proteins with a putative cleavage site located between Ser_{18} and Lys_{19} in *Ef*PLP and Ala_{19} and Arg_{20} in *Ec*PLP. Molecular weight of the cleaved mature *Ef*PLP and *Ec*PLP proteins were calculated as 37.6 kDa and 37.9 kDa, respectively. The *Ef*PLP and *Ec*PLP amino acid sequences were 80.2% identical.

3.2. Sequence analysis

Cold active enzymes have been reported to be structurally modified by an increasing flexibility of the polypeptide chain enabling an easier accommodation of substrates at low temperature [3,40]. Therefore, to characterize unique characteristics of *Ef*PLP that may be responsible for cold-adaptation, we chose to focus our attention to residue substitutions which were found in the *Ef*PLP with respect to *Ec*PLP from sequence comparison, and would be expected to increase intramolecular flexibility: 1) Pro to Ala in position 201; 2) charged residue to Val/Gly/Asn/Ala in positions 49/80/96/347, respectively; 3) Ile to Val/Val/Gly in positions 204/229/317, respectively. The substitutions of bulky/rigid amino acid with small ones have been observed for cold-adapted variants of several proteins and are expected to increase intramolecular flexibility [25,27,41]. In addition, these substitutions are similar to those that transform mesophilic subtilisin-like proteases into cold-active variants [42]. Fig. 2A′ and A″ show the low-resolution homology model of *Ef*PLP (see Materials and Methods) that give an approximation of the three-dimensional organization of the conserved residues that compose the catalytic dyad, the conserved block domains, and the localization of the mutated residues. We observed, that three of the unique residues of *Ef*PLP, Gly_{80}, Ala_{201} and Val_{204} (underlined in Fig. 1B), that surround the catalytic sites, may be potential candidates for cold adaptation of *Ef*PLP. To test this hypothesis, we decided to compare the enzymatic activity of *Ef*PLP and *Ec*PLP, and to investigate the activity of three engineered versions of *Ef*PLP and *Ec*PLP, respectively. In the former three mutants, *E. focardii* residues were substituted as they were in the *E. crassus* sequence: 1- *Ef*PLP-G80E, in which Gly_{80} was substituted by Glu; 2- *Ef*PLP-A201P, in which the Ala_{201} was substituted by Pro; 3- *Ef*PLP-A201P/V204I, in which the Ala_{201} and Val_{204} were substituted by Pro and Ile, respectively. In the latter three mutants, the Gly, Ala and Val together of *Ef*PLP were introduced into *Ec*PLP. Therefore, these mutants were named as *Ec*PLP-E81G, *Ec*PLP-P202A and *Ec*PLP-P202A/I205V.

Wild-types and six mutants were subcloned in the expression vectors pET-22b(+), as described under "Materials and Methods". After confirming the correct ORF and mutations by DNA sequencing, the plasmids were transformed into *E. coli* BL21 (DE3) cells for protein expression and purification. As estimated from SDS-PAGE (Fig. 2B) the purified proteins were about 95% pure.

3.3. Esterase activity of the PLP wild-types and mutants: pH dependence, thermophilicity and thermostability

As a first step, we determined the optimal pH for the esterase activity of the *Euplotes* PLPs using *p*NP butyrate as a substrate. Kinetics was followed spectrophotometrically at 348 nm in the pH range of 8.0–12.0, which is the pH independent isosbestic wavelength between the *p*-nitrophenol and *p*-nitrophenolate ion produced by the reaction. As shown in Fig. 3, the maximum activities for both wild-type *Ef*PLP and *Ec*PLP were recorded at pH 10.5 in Teorell-Stenhagen buffer. Similar results were obtained using the mutated versions of the *Ef*PLP and *Ec*PLP (data not shown).

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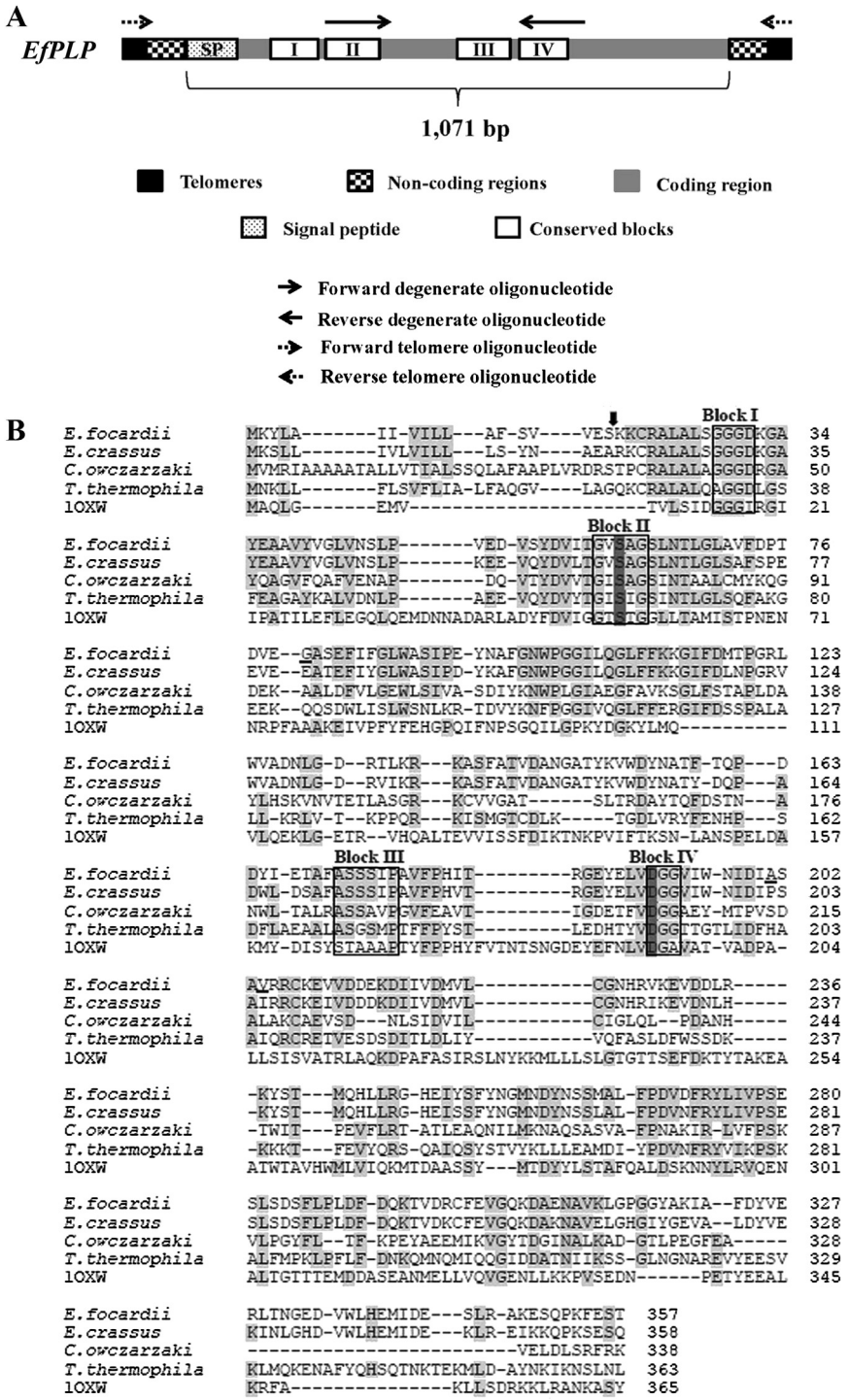

Fig. 1. *EfPLP* sequence analysis. (A) Schematic representation of the *EfPLP* macronuclear gene. The coding regions (gray squares) are flanked by non-coding regions (chequered squares) of differing lengths that terminate in telomeres composed of four repetition of the C_4A_4/G_4T_4 motif (black squares). The telomeric sequence can be used as template of oligonucleotides for PCR amplification in combination with oligonucleotides that bind DNA sequence of the coding region, indicated by horizontal arrows (see text). Signal peptide (SP) and conserved blocks (I, II, III, IV) are also represented; (B) Alignment of the predicted amino-acid sequences of *EfPLP* (JX257001) and *EcPLP* (JX257002) with the homologs from *T. thermophila* (XP_001014319.1), *C. owczarzaki* (EFW47637.1) and *Solanum cardiophyllum* (PDB accession code: 1OXW). Conserved residues are light shaded. Gray shaded Ser and Asp constitute the PLPs catalytic dyad. The conserved patatin block domains (Block I, Block II, Block III and Block IV) are boxed. The putative cleavage site of signal peptide between Ser_{18} and Lys_{19} are labeled with an arrow. The residues that are substituted by site-directed mutagenesis are underlined.

The effect of temperatures on esterase activity for both wild-types and mutants was determined using *p*NP butyrate as substrate in the temperature range of 0–50 °C. As shown in Fig. 4A, about 50% of the hydrolytic activity of *Ef*PLP was found at 0 °C. The highest percentage activity was observed at 25 °C. No activity was detected at 50 °C. In contrast, *Ec*PLP showed no activity at 0 °C, the highest activity was found at 35 °C, and the enzyme was still active at 50 °C (Fig. 4A).

The analysis of the esterase activity of the *Ef*PLP-G80E mutant showed that it behaved similarly to the wild-type, i.e. about 50% of

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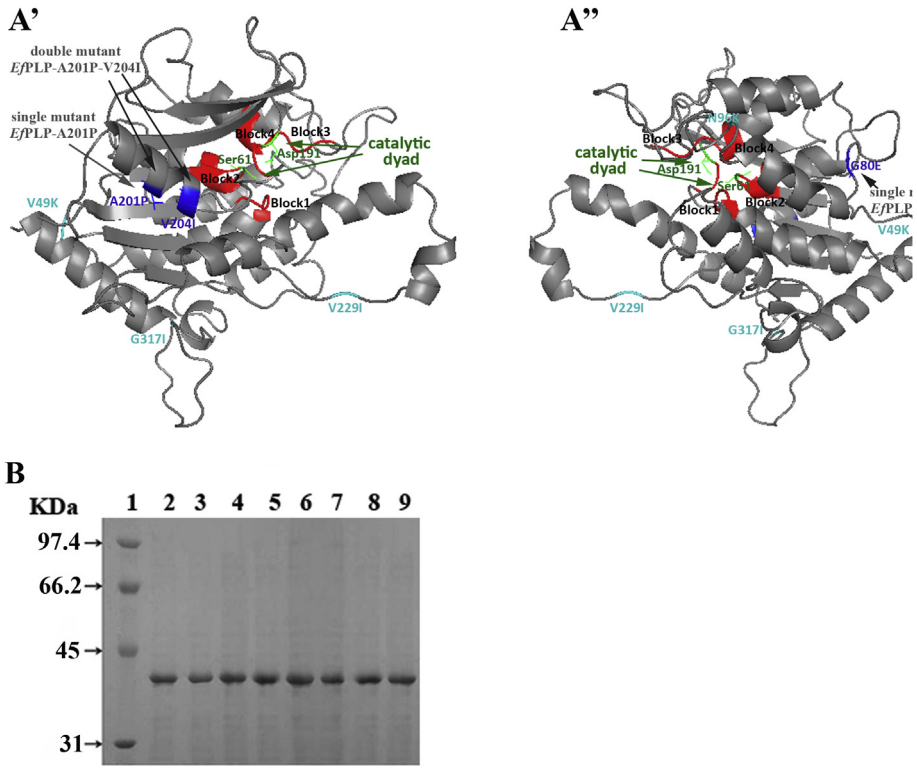

Fig. 2. (A′) Low-resolution homology model of *Ef*PLP. Side chains of the catalytic dyad aspartate and serine are shown in green; the conserved patatin block domains (Block I, Block II, Block III and Block IV) are shown in red; the mutated residues are shown in blue; other substitutions of amino acids that may influence the intramolecular flexibility are shown in light blue; (A″) Reverse side of Fig. A′; (B) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of recombinant proteins *Ef*PLP (lane 2), *Ec*PLP (lane 3), *Ef*PLP-G80E (lane 4), *Ef*PLP-A201P (lane 5), *Ef*PLP-A201P/V204I (lane 6), *Ec*PLP-E81G (lane 7), *Ec*PLP-P202A (lane 8), *Ec*PLP-P202A/I205V (lane 9) at the end of purification procedure. Five-microliter aliquots were withdrawn from each samples and loaded on a 12% SDS-PAGE gel in a Mini Protean III cell gel electrophoresis unit (Bio-Rad). The bands in the gel were revealed by Coomassie blue staining. Molecular mass markers (in kDa) are shown on the left (lane 1).

the activity at 0 °C, and the maximum activity at 25 °C, as shown in Fig. 4B. However, about 35% of the *Ef*PLP-G80E activity was maintained at 50 °C, whereas the wild-type showed no activity at this temperature. Also the *Ef*PLP-A201P mutant displayed activity at 0 °C similar to *Ef*PLP, but the maximum activity was recorded at 30 °C (Fig. 4B). The double mutant *Ef*PLP-A201P/V204I showed about 50% of maximum activity at 0 °C (Fig. 4B). However, the optimal temperature was 35 °C, and it maintained about 60% of activity at 50 °C as for *Ec*PLP, suggesting that the insertion of

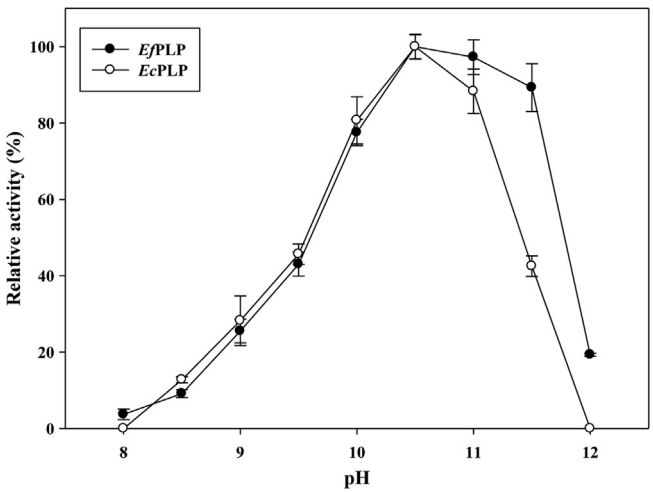

Fig. 3. Effect of pH on the esterase activity of wild-types.

E. crassus residues into *Ef*PLP conferred the capacity to efficiently exercise his enzymatic function at higher temperature than the cold-adapted variant of the PLPs.

When compared to the wild-type, the *Ec*PLP-E81G mutant showed an increased activity of about 20% at lower temperatures (15–25 °C) and decreased activity of about 10% at 40–50 °C (Fig. 4C). In contrast, the *Ec*PLP-P202A mutant displayed a dramatically increased activity at low temperature, in particular at 20–25 °C, when compared to the wild-type, and reached the highest activity at 30 °C (Fig. 4C). The double site mutant *Ec*PLP-P202A/I205V showed a 20% of activity at 0 °C, and its maximum activity was recorded at 25 °C (Fig. 4C). The double site mutant displayed a 30% loss of activity at 50 °C with respect to the wild-type. These results indicate that the residues chosen for mutations have a role in the temperature dependence of the enzyme activity.

To analyze the thermostability of the wild-type PLPs and their variants, we incubated the enzymes at 40, 55 and 70 °C for 30–180 min before measuring the residual esterase activities at their optimal temperatures, using *p*NP butyrate as substrate (Fig. 5A, B and C). As shown in Table 2, compared to *Ec*PLP which showed 3.38 and 3.11 h half-lives at 40 and 55 °C respectively, the psychrophilic counterpart *Ef*PLP displayed higher stability at the same temperatures with 3.72 and 3.65 h as half-lives. However, when the incubation temperature increased to 70 °C, *Ef*PLP showed a lower stability with 2.07 h half-life compared to *Ec*PLP which recorded 2.57 h half-life at the same temperature. All variants of *Ef*PLP with replacement of *Ec*PLP residues exhibited increased stability at 70 °C, with 2.21, 2.19 and 2.31 h half-lives for *Ef*PLP-G80E, *Ef*PLP-A201P and *Ef*PLP-A201P/V204I respectively. In contrast,

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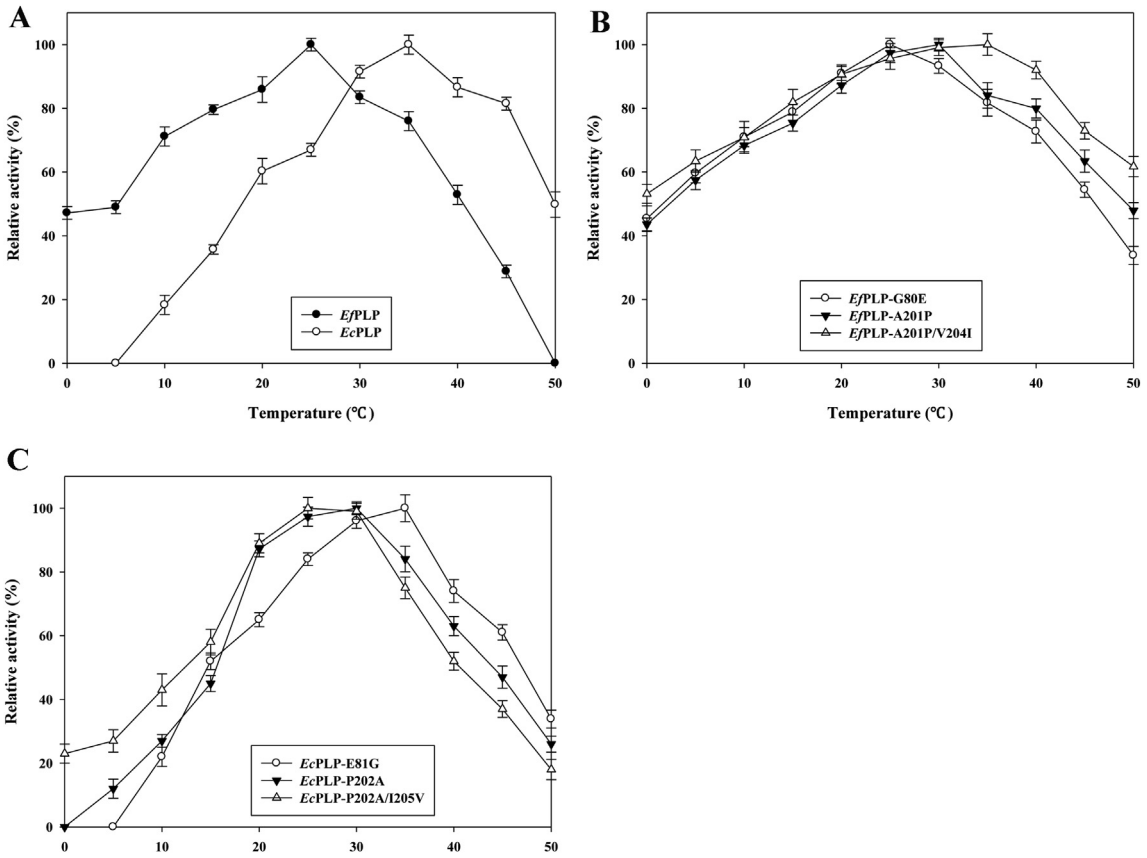

Fig. 4. Effect of temperature on the esterase activity of wild-types and mutants. (A) Temperature dependence of *Ef*PLP and *Ec*PLP; (B) temperature dependence of *Ef*PLP-G80E, *Ef*PLP-A201P and *Ef*PLP-A201P/V204I; (C) temperature dependence of *Ec*PLP-E81G, *Ec*PLP-P202A and *Ec*PLP-P202A/I205V.

mutants *Ec*PLP-E81G, *Ec*PLP-P202A and *Ec*PLP-P202A/I205V showed decreased stability at 70 °C with 2.45, 2.48 and 2.28 h half-lives respectively, when compared to their wild-type parent. To conclude, the introduction of *Ec*PLP residues into *Ef*PLP resulted to an increase of the thermostability of this protein, and *vice versa*.

3.4. PLA_2 activity of wild-types and mutants

To investigate the PLA_2 activity of *Ef*PLP, *Ec*PLP and mutants, we used the synthetic substrates *Di*C_3PC, *Di*C_4PC, *Di*C_6PC and arachidonoyl thio-PC in our assays (described under "Materials and Methods"). As shown in Table 3, *Ef*PLP, *Ec*PLP-E81G and *Ec*PLP-P202A/I205V showed increased activities with increase of substrate acyl chain length. In contrast, the *Ec*PLP and *Ef*PLP-A201P/V204I displayed higher activities toward substrates with shorter acyl chain length, and showed slightly decrease of activities with the increasing of the acyl chain length of substrates. When the substrate arachidonoyl thio-PC was used in the assay, *Ef*PLP displayed a distinct low activity compared to *Ec*PLP (Fig. 6). The double site mutant *Ef*PLP-A201P/V204I showed an increased activity compared to the *Ef*PLP. In contrast, all mutants of *Ec*PLP exhibited decreased activity compared to the wild-type; however, the double site mutant *Ec*PLP-P202A/I205V showed similar activity with *Ef*PLP.

3.5. Kinetic analysis of wild-types and mutants

Table 4 reports the kinetic constants of wild-types and mutants measured against six substrates of *p*NP esters with different acyl chain length. In general, all enzymes recorded the highest catalytic efficiency (k_{cat}/K_M) toward *p*NP butyrate as substrate. Three variants of *Ef*PLP exhibited slightly increase in esterase activity with all substrates and the most considerable increase of catalytic efficiency was observed with the double site mutants *Ef*PLP-A201P/V204I (1.9-fold increase in catalytic efficiency toward *p*NP caprate, compared to the wild type). Whereas variants of *Ec*PLP showed a minor decrease in catalytic efficiency with most of the substrates and the most marked decrease was recorded with the double site mutant *Ec*PLP-P202A/I205V (0.8-fold decrease toward *p*NP caprate, compared to the wild type). In spite of the fact that compared to the wild-type enzymes, the changes of catalytic efficiency of mutants were not substantially different. It is still remarkable that all these changes showed the trend to converting the thermal-adapted enzymes to their opposite counterparts. In general the k_{cat} of the cold adapted enzyme *Ef*PLP is lower than that of the mesophilic homolog (we should remind that the values reported in Table 4 are relative to the optimal temperature for any enzyme which is 25 °C for *Ef*PLP and 35 °C for *Ec*PLP). On the other hand from Table 4 it can be observed that *Ef*PLP shows higher substrate specificity, in comparison with *Ec*PLP, toward *p*NP butyrate (C_4 in the Table 4) among all the other tested substrates.

4. Discussion

In this study, we report the biochemical characterization of the PLP from the Antarctic ciliated protozoa *E. focardii* and that from *E. crassus*, its mesophilic counterpart. To our knowledge, this is the first biochemical characterization of an eukaryotic cold-active PLP.

The PLP from *E. focardii* shows most of the characteristics of a psychrophilic enzyme. It is 50% active at 4 °C, and it displays the

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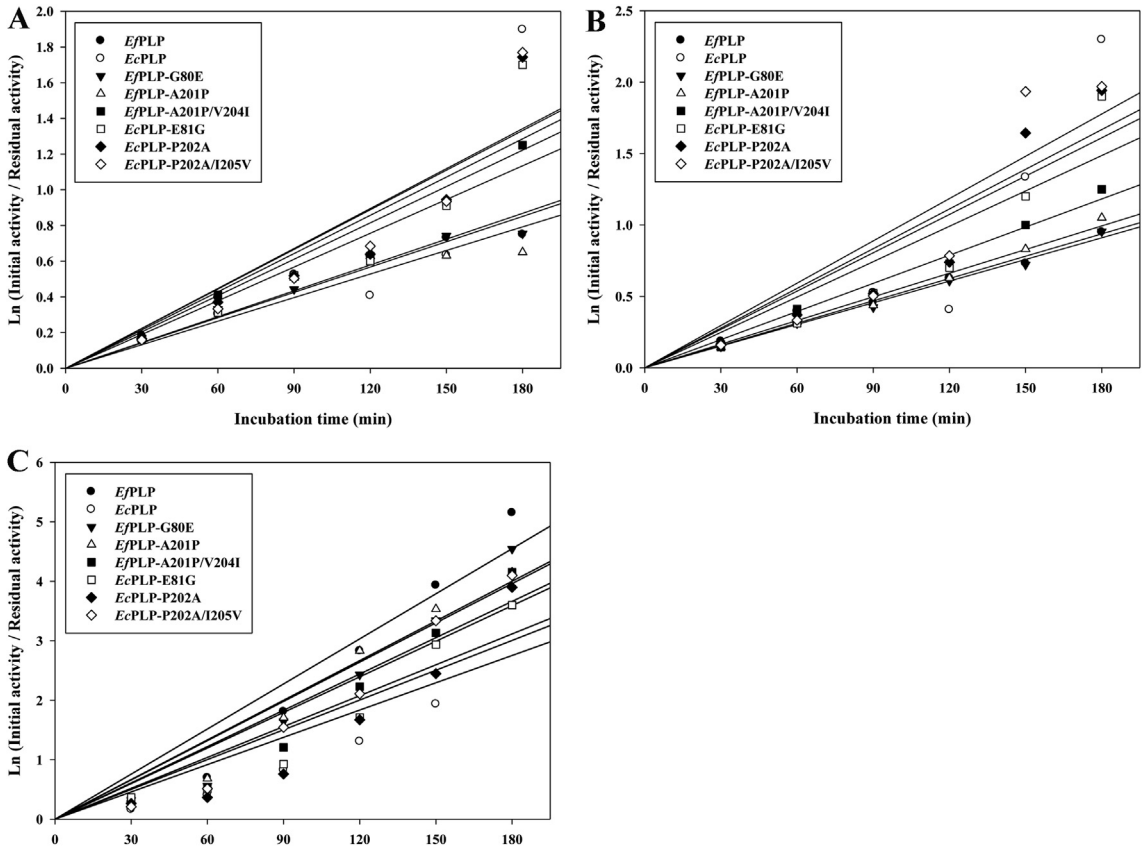

Fig. 5. Thermostability of wild-types and mutants. All enzymes were incubated at 40 °C (A), 55 °C (B) and 70 °C (C), samples were removed and assayed with *p*NP butyrate (100 μM) as substrate at regular time intervals at their optimal temperatures.

highest esterase activity at 25 °C. No activity was measured at 50 °C. In contrast, the PLP from the mesophilic *E. crassus* is not active at 4 °C, the highest activity is displayed at 35 °C and 50% of activity is maintained at 50 °C. We hypothesize that these features of cold-adaptation are consequence of adaptive substitutions of *Ef*PLP that confer structural flexibility to the polypeptide chain. This belief is first supported by the evidence that three mutants of *Ef*PLP (*Ef*PLP-G80E, *Ef*PLP-A201P, and *Ef*PLP-A201P/V204I) in which small amino acids are substituted by rigid and bulky amino acids, display esterase activities similar to the *E. crassus* phospholipase. In particular, *Ef*PLP-A201P/V204I shows its maximum activity at 35 °C, and 60% of its activity is maintained at 50 °C, whereas the wild-type showed no activity at 50 °C. These results suggest that the rigid side chain of Pro and the larger bulky hydrophobic side chain of Ile introduced by mutagenesis affected the structural flexibility of the *E. focardii* phospholipase, requiring an increase of the optimal temperature for the enzymatic activity. Furthermore, the single mutant *Ef*PLP-G80E displays the highest esterase activity at 25 °C as the wild type, but it shows 40% of activity at 50 °C, whereas the wild-type does not, suggesting that the single substitution of the *E. focardii* Gly_{80} with Glu confers enzymatic properties similar to the mesophilic *Ec*PLP without affecting its ability to function in the cold. It is noteworthy that the double site mutant *Ef*PLP-A201P/V204I displays esterase activity in a wide range of temperature, from 0 to 50 °C, a characteristic that makes it an attractive enzyme for industrial applications. In contrast, the substitution of Pro and Ile into Ala and Val in the *Ec*PLP clearly confer characteristics of a cold adapted enzyme, since the optimal temperature of *Ec*PLP-P202A/I205V was at 25 °C, but not 35 °C as for the wild-type. This evidence confirms that small residues confer an increased structural molecular flexibility to enzymes allowing to better function at low temperatures.

Table 2
Stability properties (half-lives) of wild-type PLPs and mutants.

Enzyme	$t_{1/2}$ (40 °C, h)	$t_{1/2}$ (55 °C, h)	$t_{1/2}$ (70 °C, h)	T_{opt} (°C)
*Ef*PLP	3.72 ± 0.06	3.65 ± 0.03	2.07 ± 0.07	25
*Ec*PLP	3.38 ± 0.13	3.11 ± 0.17	2.57 ± 0.15	35
*Ef*PLP-G80E	3.75 ± 0.04	3.68 ± 0.02	2.21 ± 0.07	25
*Ef*PLP-A201P	3.82 ± 0.07	3.59 ± 0.02	2.19 ± 0.06	30
*Ef*PLP-A201P/V204I	3.46 ± 0.04	3.42 ± 0.03	2.31 ± 0.08	35
*Ec*PLP-E81G	3.33 ± 0.11	3.19 ± 0.11	2.45 ± 0.09	35
*Ec*PLP-P202A	3.31 ± 0.11	3.07 ± 0.11	2.48 ± 0.13	30
*Ec*PLP-P202A/I205V	3.29 ± 0.11	3.01 ± 0.13	2.28 ± 0.08	25

Table 3
PLA_2 activity of wild-types and mutants toward different PC substrates.

Enzyme	Activity (μmol/min/ml)		
	DiC_3PC	*Di*C_4PC	*Di*C_6PC
*Ef*PLP	930 ± 32	1217 ± 32	1348 ± 22
*Ec*PLP	1329 ± 53	1084 ± 31	1078 ± 57
*Ef*PLP-G80E	1183 ± 23	985 ± 59	1024 ± 38
*Ef*PLP-A201P	1156 ± 49	977 ± 36	1038 ± 47
*Ef*PLP-A201P/V204I	1209 ± 43	1059 ± 35	1003 ± 38
*Ec*PLP-E81G	1076 ± 39	1123 ± 52	1181 ± 40
*Ec*PLP-P202A	1236 ± 54	1028 ± 47	1095 ± 29
*Ec*PLP-P202A/I205V	1103 ± 37	1144 ± 33	1230 ± 59

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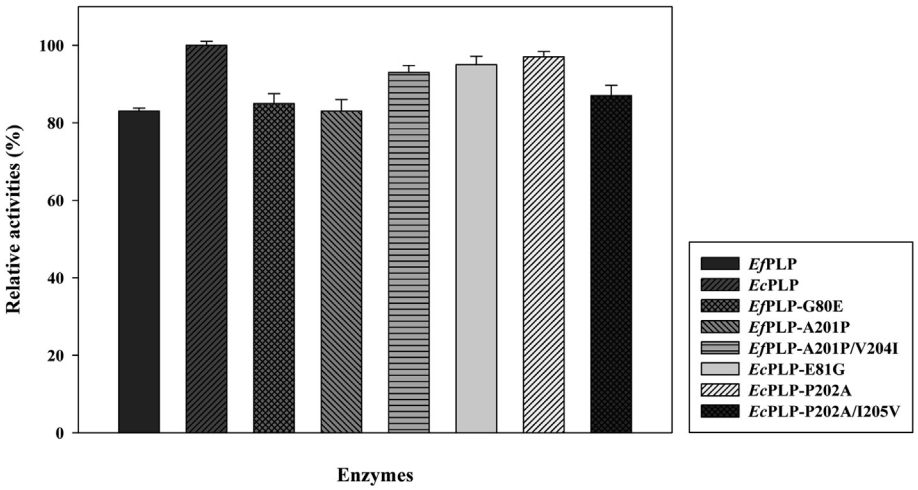

Fig. 6. Phospholipase A_2 activity of wild-types and mutants toward synthetic substrate arachidonoyl thio-PC.

The observation that mutants of *Ef*PLP behave more similarly to *Ec*PLP than the wild-type is confirmed by the kinetic analysis of these mutated enzymes, which showed a slightly increased catalytic efficiency (k_{cat}/K_M) compared to the wild-type *Ef*PLP (Table 4). However, the changes on catalytic efficiency of all mutants are relatively low to the previous reported researches [43–45], thus suggesting that the mutation sites may not directly be involved in the conformation of the catalytic core of the enzymes, which is also confirmed by the modeling analysis of *Ef*PLP (Fig. 2A′ and A″). The optimal pH for both *Ef*PLP and *Ec*PLP esterase activity is about pH 10.5, which indicates that these two proteins may be considered as alkaline phospholipases. However, *Ef*PLP displays a wider range of optimal pH with respect to *Ec*PLP, as demonstrated in Fig. 3, it maintained about 90% maximal activity at pH 11.5, a property that makes *Ef*PLP even more attractive for industrial applications.

Another remarkable property of *Ef*PLP is its higher thermostability after incubation at 40 °C and 55 °C when compared with *Ec*PLP. Furthermore, *Ef*PLP possesses relatively high thermostability showing 2.07 h half-life even after incubation at 70 °C. This is in contrast to many cold-active lipases from *Moraxella TA144* [46], *Acinetobacter baumannii* [47], *Candida albicans* [48], *Aspergillus nidulans* [49], and *Pseudomonas* spp. [50] which show decreased activity only after 20 min incubation at 40–50 °C. In other words, *Ef*PLP shows higher stability at moderate temperatures compared to its mesophilic counterpart, and also to most of the reported cold active enzymes [3]. This result is noteworthy considering the fact that cold-active enzymes usually possess a high level of structural flexibility, generally accompanied by a trade-off heat-lability [4]. However, the behavior of *Ef*PLP is not contradictory: it has been previously proposed that structural flexibility and rigidity of psychrophilic enzymes may co-exist in the same molecule, as only

Table 4
Kinetic constants of wild-types and mutants of *Euplotes* PLPs against six substrates of *p*NP esters with different acyl chain length.

Enzyme	Constant[a]	Substrate					
		C_2	C_3	C_4	C_5	C_8	C_{10}
***Ef*PLP**	k_{cat} (s^{-1})	**7.3**	**6.0**	**14.5**	**5.9**	**0.7**	**0.4**
	K_M (μM)	**373**	**405**	**566**	**366**	**194**	**183**
	k_{cat}/K_M (s^{-1} mM^{-1})	**19.6**	**14.8**	**25.6**	**16.1**	**3.6**	**2.2**
***Ec*PLP**	k_{cat} (s^{-1})	**11.5**	**7.0**	**19.2**	**7.7**	**0.8**	**0.7**
	K_M (μM)	**377**	**325**	**594**	**434**	**135**	**255**
	k_{cat}/K_M (s^{-1} mM^{-1})	**30.5**	**21.5**	**32.3**	**17.7**	**5.9**	**2.7**
***Ef*PLP-G80E**	k_{cat} (s^{-1})	**6.9**	**7.2**	**15.5**	**5.9**	**0.8**	**0.6**
	K_M (μM)	**311**	**420**	**555**	**357**	**129**	**164**
	k_{cat}/K_M (s^{-1} mM^{-1})	**22.2**	**17.1**	**27.9**	**16.5**	**6.2**	**3.7**
***Ef*PLP-A201P**	k_{cat} (s^{-1})	**12.8**	**8.0**	**22.1**	**6.9**	**1.5**	**0.7**
	K_M (μM)	**405**	**531**	**684**	**401**	**244**	**164**
	k_{cat}/K_M (s^{-1} mM^{-1})	**31.6**	**15.1**	**32.3**	**17.2**	**6.1**	**4.3**
***Ef*PLP-A201P/V204I**	k_{cat} (s^{-1})	**13.3**	**9.3**	**23.9**	**7.6**	**1.7**	**0.7**
	K_M (μM)	**410**	**541**	**695**	**408**	**260**	**169**
	k_{cat}/K_M (s^{-1} mM^{-1})	**32.4**	**17.2**	**34.4**	**18.6**	**6.5**	**4.1**
***Ec*PLP-E81G**	k_{cat} (s^{-1})	**10.4**	**6.2**	**17.9**	**7.2**	**0.7**	**0.5**
	K_M (μM)	**334**	**301**	**548**	**418**	**125**	**204**
	k_{cat}/K_M (s^{-1} mM^{-1})	**31.1**	**20.6**	**32.7**	**17.2**	**5.6**	**2.4**
***Ec*PLP-P202A**	k_{cat} (s^{-1})	**10.2**	**5.9**	**16.3**	**6.5**	**0.7**	**0.5**
	K_M (μM)	**348**	**275**	**530**	**401**	**118**	**197**
	k_{cat}/K_M (s^{-1} mM^{-1})	**29.3**	**21.4**	**30.7**	**16.2**	**5.9**	**2.5**
***Ec*PLP-P202A/I205V**	k_{cat} (s^{-1})	**9.1**	**5.8**	**15.2**	**6.3**	**0.6**	**0.4**
	K_M (μM)	**336**	**259**	**519**	**389**	**108**	**173**
	k_{cat}/K_M (s^{-1} mM^{-1})	**27.1**	**22.4**	**29.3**	**16.2**	**5.5**	**2.3**

[a] The standard error was not higher than 5%.

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protein domains involved in the conformational changes during catalysis need to be flexible [51]. This hypothesis has been supported by several researches on characterization of cold-active enzymes [52–55].

Both wild-types and mutants show broad substrate specificity toward short and long acyl chain-length (C_2–C_{10}) of *p*NP esters, and higher activities were showed on short acyl chain-length (C_2–C_5). These results contrast sharply with those obtained for other patatins [17] which showed maximum relative activity on decanoate (C_{10}, 100%) and moderate activity on palmitate (C_{16}, 40%), and very low activities toward butyrate (C_4, 4%) and hexanoate (C_6, 25%) esters. In particular, for what concerns the K_M and k_{cat} values (Table 4), the affinity constants decreased on both short (C_2) and long (C_8, C_{10}) acyl chain length of substrates for both wild-type enzymes. In parallel the k_{cat} values become much lower for the same substrates, resulting in a decrease of the catalytic efficiency. Consequently, k_{cat}/K_M values displayed a trend, leading to *p*NP butyrate (C_4) as the most preferable substrate for both enzymes. The increased substrate specificity toward *p*NP butyrate (C_4) is thus manifested more by an increase in k_{cat} than by a decrease in K_M, that is, by an increase in maximum rate rather than by an increase in substrate binding. This behavior is an example of the so called "induced fit" phenomenon, in which the enzyme works better when is able to bind more tightly the transition-state than the ground-state of the substrate. Furthermore, comparing the substrate specificity between two wild-types, we can observe that the *Ec*PLP showed a higher activity toward all *p*NP esters used for test. This result can be explained by the previous finding that mesophilic enzymes have a higher kinetic efficiency compared with psychrophilic ones at their respective optimal conditions [4].

Besides esterase activities of *Ef*PLP and *Ec*PLP, the enzymes were also found to be active toward diacylphospholipids (*Di*C_3PC, *Di*C_4PC and *Di*C_6PC) and arachidonoyl thio-PC. The results showed an increased activity for *Ef*PLP according to the increase of substrates chain length, in contrast to *Ec*PLP which displayed a decreased activity when the substrates chain length increased (Table 3). By comparing the activities on arachidonoyl thio-PC, the double site mutant *Ef*PLP-A201P/V204I and the mesophilic enzyme *Ec*PLP showed higher efficiency than the psychrophilic counterpart *Ef*PLP (Fig. 6), probably because the mutant was constructed by replacing the specific residues of *Ef*PLP with those of *Ec*PLP. Additionally, the decreased activity of three variants of *Ec*PLP again confirmed that the trend of the shifted catalytic behavior of mutants toward the wild type enzyme from which they obtained the substituted residues.

Patatins are known as the major storage protein in potato tubers [18]. Plant PLPs were originally defined as vacuolar enzymes [9], but several of these molecules were found in the cytoplasm or partially bound to chloroplasts and other endomembranes, suggesting a potential role in signal transduction [16]. However, the hydrolase and in particular the galactolipase activity of plant patatins have been implicated in the mechanisms of defenses against parasites [56]. Actually, PLPs in *Arabidopsis* have been characterized as pathogen-inducible enzymes and were identified to be active in a context of pathogen defense [57]. Relatively few indications are available about the localization and the physiological role of PLPs in protozoans. For example, it has been reported that a patatin-like from *Toxoplasma* is localized in the cytosol and it may function as storage protein, like patatin in potato tuber, to aid parasite survival during physiological stress conditions [58]. However, this patatin-like lacks phospholipase activity [58]. Research on phospholipases isolated from the extracellular medium of the ciliate *T. thermophila* demonstrated that these enzymes possess hemolytic activity suggesting that they may serve to destroy competitors and predators [59]. The phospholipase activity and the putative signal peptide typical of secreted proteins of both *Ef*PLP and *Ec*PLP, suggest that they may represent extracellular enzymes, which are translocated to the extracellular environment in response to predators' attack as defense or to facilitate ciliate's feeding. However, the evidence that *Euplotes* PLPs possess both phospholipase and esterase activity suggest that they may be involved in key metabolic events such as membrane turnover and signal transduction, as well diverse functions in the degradation of food and fat [60]. Further study will help to unravel the physiological role of *Euplotes* PLPs.

PLPs have been used in industrial applications especially in food production, such as emulsification for mayonnaise, sauces, and salad dressings production [61,62]. These processes are based on enzymes expressed in microorganisms and produced in large quantities, making them cost competitive. Furthermore, with the development of protein engineering, it is possible to produce tailor-made phospholipases of characteristics and specificity for many applications [61]. The PLP from *E. focardii* possesses both phospholipase and esterase activity, and can work in the cold and wide range of pH conditions making it a good candidate for the implementation of new and harsh industrial applications.

To conclude, the PLP from the Antarctic ciliate *E. focardii* shows the characteristics of a psychrophilic enzyme. These characteristics appear related to amino acid residues that confer structural flexibility to the polypeptides; mutations at some of these residues are able to influence the thermal-adaptation of the enzyme. Furthermore, *Ef*PLP shows high thermostability, a characteristic that distinguishes this enzyme from other cold-adapted lipases, and make it an interesting biocatalyst for industrial applications.

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