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Novel Cold-Adapted Esterase MHLip from an Antarctic Soil Metagenome

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Received: 3 December 2012; in revised form: 4 January 2013 / Accepted: 11 January 2013 /

Published: 25 January 2013

Abstract: An Antarctic soil metagenomic library was screened for lipolytic enzymes and allowed for the isolation of a new cytosolic esterase from the α/β hydrolase family 6, named MHLip. This enzyme is related to hypothetical genes coding esterases, aryl-esterases and peroxydases, among others. MHLip was produced, purified and its activity was determined. The substrate profile of MHLip reveals a high specificity for short *p*-nitrophenyl-esters. The apparent optimal activity of MHLip was measured for *p*-nitrophenyl-acetate, at 33 °C, in the pH range of 6–9. The MHLip thermal unfolding was investigated by spectrophotometric methods, highlighting a transition (T_m) at 50 °C. The biochemical characterization of this enzyme showed its adaptation to cold temperatures, even when it did not present evident signatures associated with cold-adapted proteins. Thus, MHLip adaptation to cold probably results from many discrete structural

modifications, allowing the protein to remain active at low temperatures. Functional metagenomics is a powerful approach to isolate new enzymes with tailored biophysical properties (e.g., cold adaptation). In addition, beside the ever growing amount of sequenced DNA, the functional characterization of new catalysts derived from environment is still required, especially for poorly characterized protein families like α/β hydrolases.

Keywords: α/β hydrolase; lipolytic enzymes; metagenomics; *p*-nitrophenyl-ester; cold-adaptation

1. Introduction

Lipases and esterases are enzymes active towards different substrates. Some of these enzymes are used to access carbon sources (e.g., cinnamoyl esterase) [1], some of them are regarded as pathogenic factors [2], others are involved in biocide degradation [3], but many of them have as yet uncharacterized physiological functions. Because of the increasing amount of available sequenced genomes and the possibility to construct DNA libraries from uncultured microbial consortia, many new hypothetical enzymes, including lipases and esterases, are now accessible. This finding provides the possibility to isolate new biotechnologically relevant catalysts from extreme environments [4] and to test the robustness of hypotheses derived from cultivable microorganisms [5].

Cold-adapted enzymes from psychrophilic organisms are supposed to display higher activity at low and moderate temperature when compared to their mesophilic homologs. Such a high activity at low temperature is associated with higher thermal instability [6]. In cold-adapted enzymes the active site is regarded as the most heat labile structural element whereas some other parts of the proteins can remain correctly folded over a wider range of temperatures [7]. Often, when increasing the temperature, the temperature of inactivation does not correspond to the apparent melting temperature of the protein [6,8].

Many cold-adapted enzymes have been characterized; xylanase [9–11], cellulase [12], amylase [13–15] and lipase-esterase [16,17], among others. Their analysis reveals the molecular basis of protein adaptation to cold. The adaptation to low temperatures is thought to proceed by minor structural changes of the protein through modification of non-covalent interactions leading to an increase of the flexibility of crucial parts of the protein rather than to a global increase of the flexibility [8]. A decrease of the amount of proline and arginine residues and an increase of the amount of glycine are also assumed to contribute to the increase of the flexibility, in some cases [12]. In addition, a more accessible active site is considered as an adaptation to low temperature [18].

Interestingly, the metagenomic approach offers a unique opportunity for isolating new enzymes from uncultivated psychrophilic microorganisms [19,20]. Although few cold-adapted enzymes have been isolated using the metagenomic approach [21–23], little is known about their thermal stability.

In this work a metagenomic library, constructed from an Antarctic soil sample, was partially screened for lipolytic activity. One clone displaying esterase activity was isolated. After production and purification, its adaptation to cold temperatures was analyzed regarding both the activity and the stability.

2. Results and Discussion

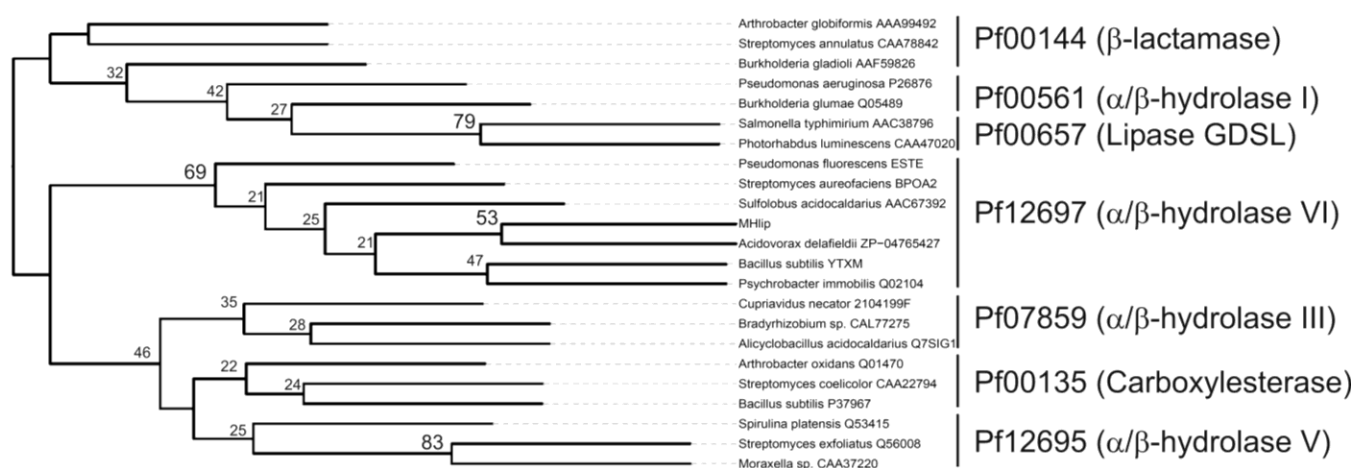
2.1. MHlip Isolation and Sequence Characterization

From the Antarctic soil metagenomic library [24], one clone producing a blue halo after one week incubation at 18 °C on SBA-tributyryn media was selected as a potential esterase/lipase producer.

Complete DNA sequencing of the insert (3 kb) revealed a unique putative gene, named *mhlip* (Genbank access. number. GU550075). The gene encodes a 262 amino acids hypothetical protein with a theoretical molecular mass of 28,075 Da and a pI of 5.2. The encoded protein, MHlip, displayed 83% amino acid identity to a putative cytosolic α/β hydrolase derived from *Acidovorax delafieldii* 2AN (Acc. Numb. ZP-04765427). The sequence also showed significant identity with many other mesophilic proteins including the arylesterase from *Pseudomonas fluorescens* (PFE-ESTE 26% id., acc. no. P22862.4), the non-heme bromoperoxidase (BPOA2, 30% id., acc. no. P29715.3) and the non-heme chloroperoxidase (PRXC-STRAU, 29% id., acc. no. O31168.1) from *Streptomyces aureofaciens*, and with the crystallized esterase YTXM from *Bacillus subtilis* (id. 26%, acc. no. P23974).

We constructed a phylogenetic tree using the MHlip sequence and some sequences derived from various esterase-related families [25]. Next, Pfam numbers were assigned to each sequence using Pfam_scan (<http://pfam.sanger.ac.uk/>) [26] (Figure 1). MHlip was associated with the α/β hydrolase family 6 (Pfl2697). This fold is observed in many enzymes having multiple functions (*i.e.*, transacetylase, lipase, hydroxynitrile lyase, methylesterase, peptidase, haloperoxidase, carboxylesterase) [27].

Figure 1. Unrooted neighbour-joining tree built with enzymes belonging to different families of esterases. The Jones-Taylor-Thornton method was used to compute distances [28]. Higher bootstrap values are displayed at the nodes and expressed as percentages of 1000 replicates. The Pfam numbers (names) are displayed on the right side of the tree.



Sequences alignment highlighted all the residues considered as involved in substrate binding and catalysis (Figure 2) [29]. In addition, residues W₂₉ and M₉₅ assumed to limit the active site of PFE-ESTE are displayed. These two residues limit the diffusion of large substrates in the catalytic cleft, independently of any lid structure. Thus resulting in an enzyme having high affinity for short substrates (*i.e.*, pNPA) [29].

Figure 2. Sequences alignment of the MHLip enzyme and some related proteins. PFE-ESTE (P22862.4), aryl esterase from *P. fluorescens*; ZP-04765427, putative α/β hydrolase from *Acidovorax delafieldii*; BPO-A2 (P29715.3), bromoperoxidase from *Streptomyces aureofaciens* and YTXM (P23974.2), lipase from *Bacillus subtilis*. ★ residues forming the catalytic triad (Ser₉₆, D₂₁₃ and H₂₄₁), ▲ residues forming the oxyanion hole involved in hydrogen bonding interactions, located upstream the active site (HG), ○ conserved residues involved in the formation of the GX SXG conserved pentapeptide containing the active-site serine, ● residues involved in the active site occlusion in PFE-ESTE [29].

MHLip	25	FSHG	LLMD	DSSM	85	LG	VQKAV	LV	GM	SQGGY	200	AI	TV	PA	LV	VHG	AL	DHA	239	GG	HASNL	TH	PE	P	
PFE	25	FSHG	WLLD	DADM	84	LD	LKEVT	LV	FS	SMGG	GDVARY	210	KI	DV	PT	LV	IHG	GD	DQI	250	AP	HG	FAV	TH	AQ	Q
ZP-04765427	25	FSHG	LLMD	DGSM	85	LG	VKTAV	LV	GM	SQGGY	200	AI	TV	PA	LV	VHG	TL	DHA	239	AG	HAANL	TH	PE	P	
BPO-A2	29	LI	HGFPL	SGHS	88	LD	LQDAV	LV	GF	SMGT	GEVARY	216	RI	DV	PA	LV	IHG	TG	DRT	256	AP	HG	LLW	TH	AEE	
YTXM	29	CL	HGFTG	SKQS	88	LK	LHKVK	LI	GY	SMGG	.RLAYS	213	EI	DV	PA	LV	LICG	EW	DEK	251	AG	H	TVHV	EQ	PR	L
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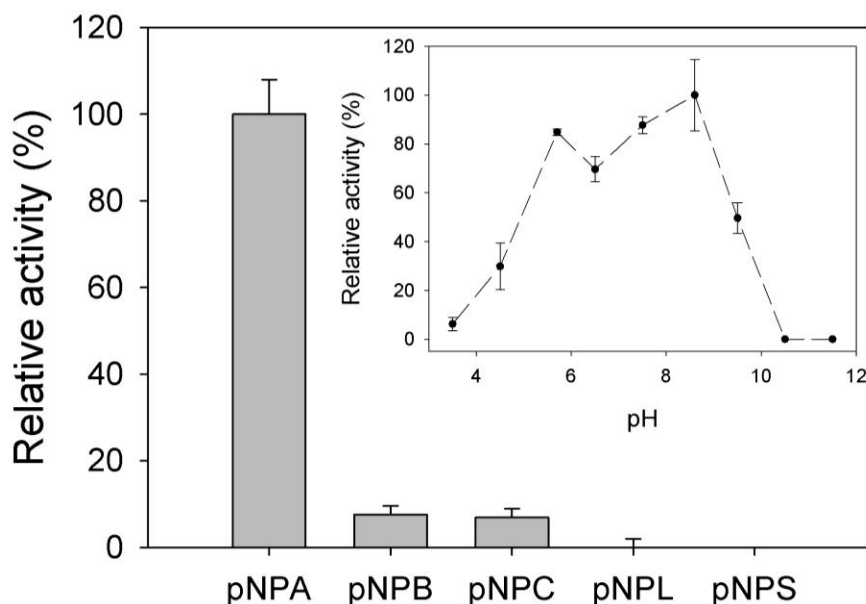
2.2. Biochemical Characterization

The MHLip-encoding gene was PCR-amplified and introduced in the pET22b vector in order to express a C-terminal His-tagged MHLip. The recombinant protein was purified from the cytoplasmic fraction of transformed *E. coli* BL21(DE3) using Ni-NTA columns.

Since MHLip was first isolated on tributyrin containing media, its activity was investigated on various *p*-nitrophenyl (pNP)-esters at pH 8.0 (Figure 3). MHLip is only active on small substrates such as pNPA; activity on pNPB is less than 5% of the pNPA hydrolysis and no activity was detected on substrates with longer acyl-chain lengths.

The pH-dependence of the MHLip activity on pNPA was investigated by monitoring the absorbance at 348 nm between pH 3.5 and 11.5, in a 20 mM pH-adjusted poly-buffer. MHLip was significantly active over a wide range of pH, from 4.5 to 9 (Figure 3).

Figure 3. MHLip substrate specificity measured at pH 8 and influence of the pH on the MHLip activity (the inset).



The thermal sensitivity of the MHLip activity towards pNPA was recorded at pH 8.0 in a thermal gradient ranging from 4 to 55 °C. The rate of pNPA hydrolysis rapidly increased from 4 to 35 °C and then quickly decreased to few percents of residual activity at 45 °C. Interestingly, MHLip remained significantly active when temperature decreased (20% at 4 °C) (Figure 4).

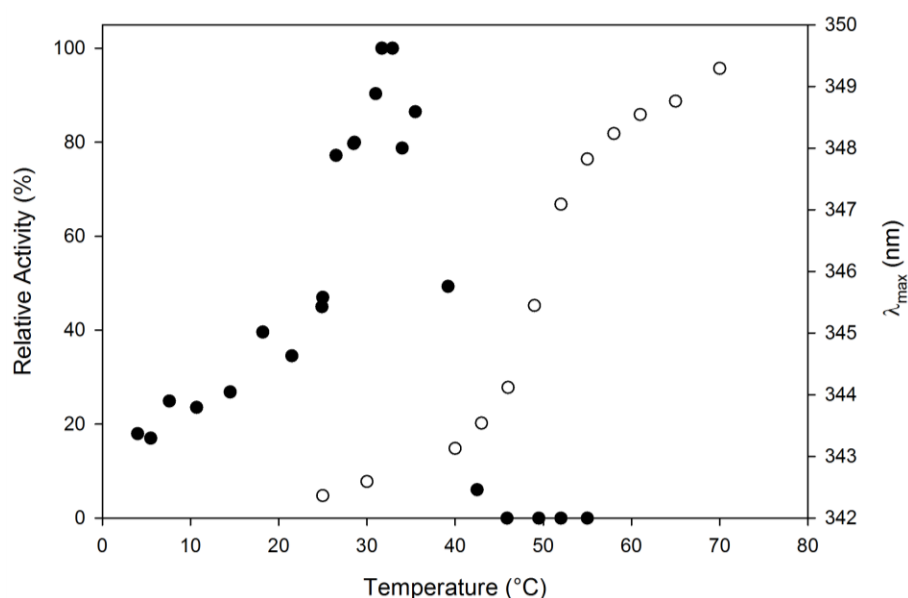
Kinetic parameters for pNPA hydrolysis were determined under initial rate conditions using a nonlinear regression analysis of the Michaelis–Menten equation. Hydrolysis was measured at 30 °C using pNPA as substrate at final concentrations ranging from 0 to 10 mM in 20 mM Tris-HCl (pH 8). For MHLip, the kinetic constants were: $K_m = 1.2 \sim 0.1$ mM, $k_{cat} = 3.13 \cdot 10^{-2} \sim 0.2 \cdot 10^{-2}$ sec⁻¹ and $k_{cat}/K_m = 0.025$ mM⁻¹ sec⁻¹.

2.3. Thermal Stability

MHLip fluorescence spectra recorded at 20 °C and 90 °C were compared. When MHLip was incubated at 80 °C its fluorescence emission spectrum, compared to the spectrum obtained at 20 °C, revealed its unfolding. A bathochromic effect was observed when the temperature increased as the maximum fluorescence was shifted to higher wavelengths (from 338 to 350 nm). In order to determine the MHLip melting temperature, the fluorescence emission at 338 nm was recorded in a thermal gradient, from 20 to 90 °C (data not shown).

This approach appeared to be inappropriate since the increase of temperature caused a decrease of the fluorescence emission at 338 nm, with no clear transition. The determination of the apparent melting temperature (T_m) was eventually achieved by recording the wavelength of the maximum fluorescence emission (λ_{max}) as a function of the temperature. At an excitation wavelength of 280 nm, a clear transition from a native to an unfolded state was observed at ~50 °C (Figure 4).

Figure 4. Influence of temperature on the MHLip activity (●). The enzyme was pre-incubated for 5 min at temperature ranging from 4 to 55 °C. Enzyme activity was determined under standard conditions on pNPA, at pH 8.0. The activity measured at 30 °C was taken as 100%. MHLip thermal denaturation following the maximum of fluorescence emission (λ_{max}) in a thermal gradient ranging from 20 to 80 °C (○).



3. Experimental Section

3.1. Metagenomic Library Construction and Screening

A previously described short-insert metagenomic library (average insert size of 5.1 kb) was used for the present work [19]. Screening for lipolytic activity was carried out by plating 3,000 recombinant *E. coli* clones on Spirit-Blue Agar (SBA) Media (BD-Difco, MD) containing chloramphenicol (12.5 µg/mL) and 1% emulsified Tributyrin (Sigma-Aldrich). The plates were incubated at 37 °C for 24 hours and kept at 18 °C for one week.

3.2. Expression and Purification

Forward primer (5'-GAGCACATATGCCTTTCGCGCA-3') and the reverse primer (5'-GCTTCAGAGGCGCTCTCGAGCTTGTTCGAGA-3'), containing *NdeI* and *XhoI* restriction sites, respectively (underlined), were used for PCR amplification of the full length MHLip-encoding gene. The obtained amplicon was ligated in an *NdeI/XhoI* double digested pET22b plasmid (Novagen, NJ) that allowed the production of a C_{term}-6×HisTag fused protein. After confirmation of the sequence, the pET22b:MHLip was introduced in competent *E. coli* BL21(DE3) cells (Novagen). Transformed cells were cultivated at 18 °C in LB media containing ampicillin (100 µg/mL). Heterologous protein expression was carried out for four hours by adding 0.4 mM isopropyl-D-1-thiogalactopyranoside (IPTG) when the OD_{600nm} reached ~0.5. After centrifugation, the cell pellet was resuspended in 20 mM Tris-HCl (pH 8.0) and the cells were disrupted by sonication (3 cycles of 30 sec at amplitude of 10–12 µm). Proteins from the cytoplasmic fractions were recovered by centrifugation at 20,000 × g for 40 min, and MHLip was purified by affinity chromatography on a 5 mL Ni-NTA columns (GE Healthcare) in a linear gradient of imidazole (from 0 to 250 mM). Protein concentration was determined by the Bicinchoninic Acid (BCA)-protein quantitation assay (Pierce) using serum albumin as standard. Finally, 30 mg of pure protein was obtained per litre of culture. The purified protein was subjected to automatic Edman degradation for the determination of the N-terminal amino acid sequence on an Applied Biosystems 492 Protein Sequencer (Perkin Elmer, Waltham, MA, USA). The resulting sequence (MPFAH) was found to match the predicted one. The purified protein was used for further characterization.

3.3. Activity Assays

Lipase/esterase activity assays were carried out on *p*-nitrophenyl-esters (pNP-Acetate, pNPA; pNP-Butyrate, pNPB; pNP-Caprilate, pNPC; and pNP-Laurate, pNPL; all purchased from Sigma) by spectrophotometric methods. The assay mixture was prepared as follow: 940 µL of 20 mM Tris-HCl (pH 8.0), 10 µL of 10 mM *p*-nitrophenyl-ester in acetonitrile, and 50 µL of enzyme solution. The release of *p*-nitrophenol was followed taking into account a molar extinction coefficient at 405 nm (ϵ_{405nm}) of 16,500 M⁻¹.cm⁻¹.

The pH-dependence of the enzyme's activity on pNPA, between pH 3.5 and 11.5, was investigated by monitoring the absorbance at 348 nm and replacing the Tris-HCl buffer by a 20 mM pH-adjusted polybuffer consisting of Tris, KCl, Na₂HPO₄, CH₃COO.Na and dihydrogen citrate [30].

The thermal sensitivity of the MHlip activity toward pNPA was recorded as described above, over a temperature range between 4 and 55 °C.

3.4. Thermal Stability and Unfolding

In order to investigate the thermal stability of MHlip, the purified protein was submitted to increasing temperatures and the process of denaturation was followed by intrinsic fluorescence spectra. The spectrum of the native MHlip at 20 °C was recorded on a Perkin Elmer LS50B spectrofluorometer using a 1 cm cell path length. Maximum fluorescence emission was observed at a wavelength of 338 nm using an excitation wavelength of 280 nm. The T_m of the enzyme was determined by plotting the wavelength of the maximum emission (λ_{max}), measured at temperatures ranging from 20 to 80 °C.

4. Conclusions

The functional metagenomic is a powerful methodology to access the genetic material of environmental microorganisms. By the past, this approach has been used to isolated new enzymes from various environments [22–24,31–33]. Here, a new esterase form Antarctica is described.

MHlip belongs to the α/β -hydrolase family 6 and is part of a large group of uncharacterized proteins. The physiological function of these enzymes has not been clearly elucidated [34]. In addition, MHlip displays significant identity (~30%) to various haloperoxidases and proteases. Although similarity between some esterases, oxidases and proteases was reported [29,35,36], MHlip does not show any detectable activity when tested for the conversion of the substrate phenol red to bromophenol blue [37] or azo-casein hydrolysis [38] (data not shown).

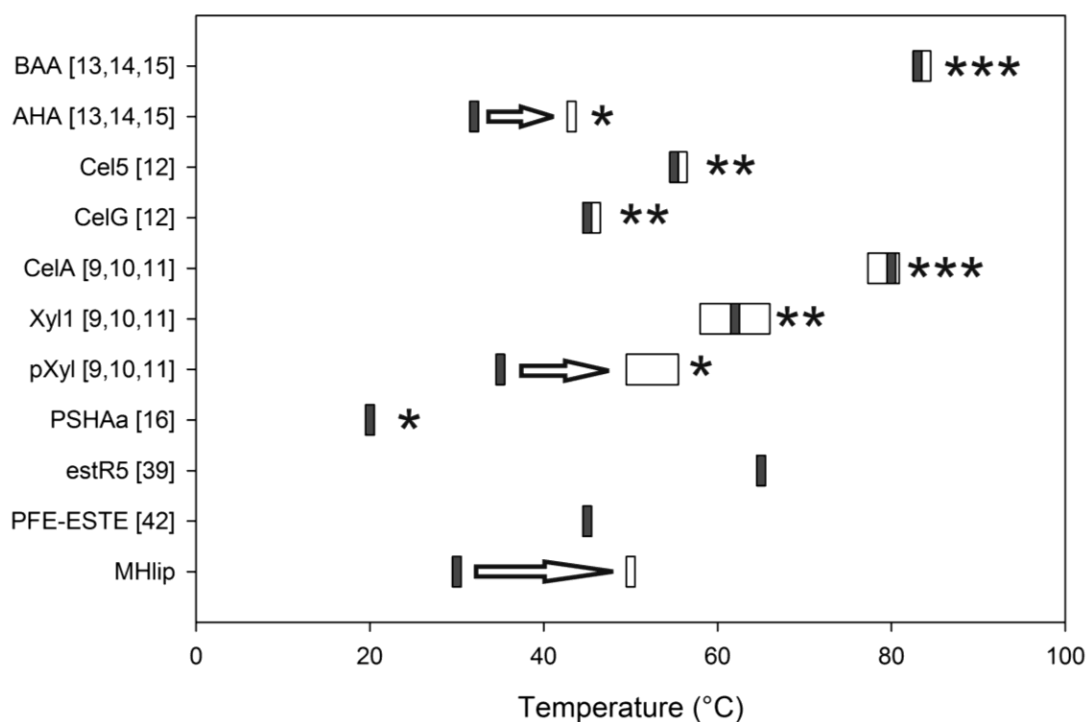
Among the tested substrates, the MHlip activity is highly specific for short esters such as *p*-nitrophenyl-acetate. Similar specificity for short substrates (e.g., pNPA) has been observed for the aryl-esterase derived from the goat rumen metagenome [39] and for the aryl-esterase from *Pseudomonas fluorescens* (PFE) [29]. Concerning PFE, such a high specificity is the consequence of a sterically hidden active site. Indeed, the catalytic cleft of PFE is delimited by W28 and M96 [29,40] whereas cumbersome residues, L29 and Q97, are observed in the corresponding positions of MHlip, and could thus have a similar effect. The kinetic parameters for the pNPA hydrolysis suggest that the MHlip primary function is not yet well understood. However, the measured K_m for the pNPA hydrolysis (1.2 mM) is similar to some values obtained for previously characterized aryl-esterases, including the enzyme from *Lactobacillus casei* [41] and an aryl-esterase derived from the goat rumen metagenome [39].

The characterization of purified MHlip reveals its adaptation to cold temperatures. Indeed, MHlip retains 20% activity at 4 °C. This is consistent with the temperature of the Antarctic soil sample used for the metagenomic library construction [24]. The MHlip activity increases from 4 to ~30 °C and is lost at higher temperatures. This suggests that our screening method, including a long incubation at cold temperature, is suitable for allowing the isolation of this highly thermo-sensitive protein. The aryl-esterases derived from the goat rumen metagenome (estR5) [39] and from *Pseudomonas fluorescens* (PFE) [42] are regarded as mesophilic homologs of MHlip since their optimal activity is observed at 60 and 45 °C, respectively (Figure 5).

The MHlip unfolding begins at 30 °C. At this temperature the MHlip activity is optimal. When increasing the temperature above 30 °C, the protein undergoes a partial denaturation and quickly loses

its activity. Complete denaturation is apparently achieved at a temperature higher than 65 °C. Based on the fluorescence transition curve, the apparent T_m is determined to be 50 °C.

Figure 5. Shift in the observed optimal temperature of activity (gray rectangle) and the melting temperature (T_m , white rectangle) of some characterized proteins. *, ** and *** stand for protein considered psychrophilic, mesophilic and thermophilic in the corresponding references, respectively.



The thorough characterization of cold active proteins unravels different ways for a protein to remain active at low temperatures [5–8,43]. Among these paths, increasing the length of the unstructured loop has been described in several cases [44,45]. However, MHlip sequence analysis does not reveal any extra stretches of amino acid.. Increasing the accessibility of the active site, as described for a cold adapted protease [18], was reported to increase the activity at low temperature. This is unlikely to occur here since MHlip may present cumbersome residues surrounding its active site. Moreover, the overall amino acid composition of MHlip is not significantly different from that of related proteins from mesophilic organisms. Nevertheless, this new enzyme, like most of the previously characterized cold adapted proteins, is an extremely heat labile enzyme.

Indeed, the MHlip's loss of activity corresponds to the early stages of its unfolding. This suggests that the catalytic site is extremely thermo-sensitive compared to the overall protein structure. Shifts between the temperature at which proteins lose their activity and the unfolding temperature (T_m) have been recorded for many cold-adapted enzymes (Figure 5) [6,8,11–14]. Thus, even if no signature associated with cold-adapted protein can be observed, at the sequence level, we assume the MHlip adaptation to cold results from many discrete structural modifications that allow the protein to remain active when temperature is low.

Acknowledgments

This project was supported by the First-Postdoc Program, Grant no. 916984 from the Région Wallonne (Belgium) and by a grant from FNRS (FRFC, 2.4561.07) to MG.

P. Power is a researcher from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and was a recipient of a Postdoctoral fellowship from the Belgian Science Policy Office (BELSPO).

Authors thank Charles Gerday and Georges Feller for their skilful technical assistance and their discussion of the manuscript.

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