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Purification, characterisation and expression in *Saccharomyces cerevisiae* of LipG7 an enantioselective, cold-adapted lipase from the Antarctic filamentous fungus *Geomyces* sp. *P7* with unusual thermostability characteristics

Tomasz Florczak[a,b,1], Maurycy Daroch[b,d,1], Mark Charles Wilkinson[b], Aneta Białkowska[a], Andrew Derek Bates[b], Marianna Turkiewicz[a], Lesley Ann Iwanejko[b,c,*]

[a] *Faculty of Biotechnology and Food Sciences, Institute of Technical Biochemistry, Technical University of Lodz, ul. B. Stefanowskiego 4/10, 90-924 Lodz, Poland*
[b] *Institute of Integrative Biology, University of Liverpool, Biosciences Building, Crown Street, Liverpool L69 7ZB, United Kingdom*
[c] *Institute of Ageing and Chronic Disease, Faculty of Health & Life Sciences Department of Musculoskeletal Biology, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, United Kingdom*
[d] *School of Environment and Energy, Peking University Shenzhen Graduate School, Shenzhen 518055, China*

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ABSTRACT

A lipase, LipG7, has been purified from the Antarctic filamentous fungus *Geomyces* sp. *P7* which was found to be cold-adapted and able to retain/regain its activity after heat denaturation. The LipG7 exhibits 100% residual activity following 1 h incubation at 100 °C whilst simultaneously showing kinetic adaptations to cold temperatures. LipG7 was also found to have industrial potential as an enantioselective biocatalyst as it is able to effectively catalyse the enantioselective transesterification of a secondary alcohol. The *LipG7* coding sequence has been identified and cloned using 454 pyrosequencing of the transcriptome and inverse PCR. The LipG7 protein has been heterologously expressed in *Saccharomyces cerevisiae* BJ5465 and shown to exhibit the same characteristics as the native protein.

1. Introduction

Lipases (triacylglycerol hydrolases E.C.3.1.1.3) are co-factor independent enzymes of outstanding industrial importance, widely employed in the food, laundry, textile, pulp and paper industries, production of biodiesel and in the synthesis of fine chemicals [1–4]. Although hundreds of different lipases from bacteria, plants fungi and animals have been identified to date, most industrially-relevant lipases originate from relatively few evolutionary groups: *Burkholderia*, *Pseudomonas*, *Candida*, *Rhizomucor* [5].

Despite the large number of lipases available they tend to exhibit very similar substrate specificities, perhaps as a consequence of the relatively short evolutionary distances between the host organisms. In spite of these limitations, lipases are often the biocatalyst of choice because of their co-factor independence, stereospecificity and solvent stability [3]. As the ability to catalyse lipase-like reactions is an essential requirement of most organisms, it is proposed that by expanding the search list of host organisms it should be possible to identify new lipase-like enzymes and potentially enzymes that combine the desirable characteristics of traditional lipase catalysts with improved, industrially relevant, properties.

The cold-adapted extremophiles are an underexploited source of enzymes. Although extremozymes have been used as a source of industrially relevant enzymes for some time [6], most of the spotlight to date has been on enzymes from thermophilic organisms, due to their high robustness in process conditions [7]. There is now an increasing focus on cold-adapted enzymes from psychrophilic organisms, since they tend to have increased molecular flexibility, which makes them superior in stereoselective reactions in organic media, compared to enzymes from meso- and thermophilic organisms [8]. Additionally, cold-adapted enzymes are capable of conducting transformations at lower temperatures; this is particularly advantageous in kinetic resolution reactions as it

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; CalB, *Candida antarctica* lipase B.

* Corresponding author at: Institute of Integrative Biology, University of Liverpool, Biosciences Building, Crown Street, Liverpool L69 7ZB, United Kingdom. Tel.: +44 1517064663.
E-mail address: iwanejko@liverpool.ac.uk (L.A. Iwanejko).

[1] Both authors contributed equally to this publication.

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slows down the transformation of the isomer with higher activation energy [9].

A major advantage of cold-adapted enzymes is that large scale processes do not necessarily have to be maintained at higher temperatures to ensure functionality of the enzyme, thereby potentially reducing both the carbon-footprint and energy costs associated with biocatalysis. However, one disadvantage of psychrophilic proteins is that they are frequently temperature-labile and this can limit their applicability in industrial catalysis. Currently the only cold-adapted lipases applied in industry are *Candida antarctica* lipase B (CalB), immobilised on an acrylic resin known under the brand name of Novozyme 435 [10,11] and *Yarrowia lipolytica* lipases [12]. Enzyme immobilization is a well established and widely employed method to increase the stability of lipases [13,14] but the requirement for some enzymes to be immobilised may limit their application, a feature which potentially could be overcome using enzymes which are both cold-adapted and able to withstand high temperatures.

The main objective of this study was to purify and characterise an enantioselective cold-adapted lipase isolated from the Antarctic fungus *Geomyces* sp. *P7* that shows unusual characteristics in thermostability assays. The second objective was to utilise inexpensive low coverage, high throughput 454 transcriptome sequencing to identify and facilitate cloning, via inverse-PCR, of the coding sequence for the lipase. The final objective was to express the protein in the *Saccharomyces cerevisiae* in order to investigate whether the heterologously expressed lipase would retain its cold-adaption and unusual thermostability characteristics.

2. Materials and methods

2.1. Geomyces sp. P7 strain origin and genus confirmation

The filamentous fungus strain *P7* used in this study was obtained from the Institute of Technical Biochemistry (Technical University of Lodz, Poland) collection of Antarctic microorganisms isolated from the soil samples in the neighbourhood of Henryk Arctowski Polish Antarctic Station at King George Island (Southern Shetlands, 62°10′S, 58°28′W). 18S ribosomal DNA sequencing was performed to identify the genus of the *P7* strain. Genomic DNA was isolated and 18S rRNA genes amplified, using primers NS1-EUK C_Fwd and UNIV1390_Rev, as described by Lockhart [15] and sequenced.

2.2. Protein purification

2.2.1. Growth conditions

For protein purification the fungus was pre-cultivated at its optimal temperature of 10 °C for 1 week on agar medium containing 0.3% (w/v) $NaNO_3$, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4$, 0.001% (w/v) $FeSO_4$, 3% (w/v) sucrose, 3% (w/v) agar (prep. grade). Mycelium was washed out into 200 ml liquid medium in a 1 l flask (the same as above but agar was replaced by 0.3% (w/v) solid corn steep liquor and 0.5% (v/v) olive oil) and the fungus was cultivated for a further 3 weeks at 10 °C, with periodical shaking. The resulting mycelium was isolated by vacuum filtration and dried by washing with acetone (3 times).

2.2.2. Protein purification steps

Cell-free extracts from the mycelium of strain *P7* were obtained by homogenization in 20 mM Tris–HCl pH 8.0 with glass beads ($\varphi \approx 0.4$ mm) followed by freezing–thawing (twice at −75 °C/room temperature) and centrifugation (8000 × *g*, 4 °C, 20 min). The cell debris was discarded. The supernatant obtained from 5 g of fresh dried biomass was filtered (45 μm), applied onto a HiTrapQ 5 ml column (Amersham) and eluted with 0.5 M NaCl in 20 mM Tris–HCl pH 8.0. Fractions exhibiting activity towards *p*-nitrophenyl acetate (section 2.3.3) were pooled, concentrated using ultrafiltration (Amicon Ultra-15 3 kDa MWCO, Millipore) and purified by gel filtration chromatography on a HiLoad Superdex 200 16/60 column (Amersham), previously equilibrated with 20 mM Tris–HCl buffer pH 8.0. Next, the active fractions were pooled and injected on a HiTrapQ column (Amersham). Proteins were eluted with a linear NaCl gradient (0–0.5 M) in the starting buffer at a flow rate of 1 ml min^{-1} and NaCl was then removed using HiTrap Desalting column (Amersham) and at the same time buffer was exhcanged into 20 mM Tris-HCl pH 8.0.

2.3. Protein characterisation

Unless stated otherwise all reagents were of analytical grade.

2.3.1. Initial activity screening

Lipolytic activity of *Geomyces* sp. *P7* was detected in toluene extracted mycelium obtained according to Vakhlu et al. [16] using the enzymatic activity detection protocols described below.

2.3.2. Assay for lipolytic activity

Lipolytic activity of both native and heterologously expressed proteins was determined as a mean of three independent technical replicates. The titrimetric assay has been performed in emulsion containing synthetic triacylglycerols (triacetin, tributyrate, tricaprylate, tripalmitate or tristearin) each at a final concentration of 500 mM in 20 mM Tris–HCl pH 8.0 as described by Szczesna-Antczak et al. [17]. After the reaction was completed the enzyme was inactivated with ethanol and released free fatty acids were titrated to pH 10 with base to determine their concentration and the resultant enzymatic activity.

2.3.3. Carboxylesterase assay

Carboxylesterase activity was determined as a mean of three independent technical replicates by spectrophotometric measurement (at 420 nm) of *p*-nitrophenol released from *p*-nitrophenyl ester (*p*-nitrophenyl acetate) (Sigma). Reaction mixtures containing 0.6 ml of 5 mM *p*-nitrophenyl acetate were dissolved in acetonitrile: 20 mM Tris–HCl (1:10 v/v) and 0.15 ml of enzyme solution (buffered in 20 mM Tris–HCl pH 8.0) (1:10 v/v). The reaction mixture was incubated at 35 °C for 10 min. One unit (U) of carboxylesterase activity was defined as the activity that releases 1 μmol of *p*-nitrophenol per minute under these standard reaction conditions.

2.3.4. Temperature profiles

The carboxylesterase assay was used to determine the temperature activity profiles over the temperature range 0–55 °C.

2.3.5. Thermostability

The thermostability of both the recombinant and native lipases was determined using the carboxylesterase assay (as a mean of three independent experiments). Enzymes were pre-incubated for 1 h at temperatures ranging between 10 °C and 100 °C, then allowed to cool to room temperature before assaying for residual activity.

2.3.6. Effects of pH

A variation of the carboxylesterase assay was employed to determine the effect of pH on the activity and stability of the LipG7 proteins. The 20 mM Tris–HCl pH 8.0 buffer was replaced with 20 mM Britton Robinson buffer in the pH range 3–9. The enzyme was then incubated for 24 h at 4 °C prior to determination of enzymatic activity.

2.3.7. Effects of salts, detergents, thiol, PMSF

The carboxylesterase assay was also employed to determine the inhibitory effects of salts, detergents, thiol compounds and phenylmethanesulfonylfluoride (PMSF). The effect of salts ($CaCl_2$, $HgCl_2$, $ZnSO_4$, $MgSO_4$, $MnSO_4$) on enzymatic activity was performed by adding the salt at the desired concentration (1 mM, 5 mM or 10 mM) to the 20 mM Tris–HCl pH 8.0 buffer and incubating the sample for 10 min prior to activity determination. The effect of detergents was tested by adding 62.5 ppm-0.5% of Tween20, Tween80, Triton X-100, sodium choleate, Brij 35, SDS to the reaction mixture prior to activity determination. The effect of 2-mercaptoethanol at 2% (v/v) final concentration was tested on samples incubated at 100 °C and cooled to room temperature before residual enzymatic activity was tested. The effect of PMSF (dissolved in 50% (v/v) water/isopropanol) on activity of both the native and the recombinant enzymes was determined under standard assay conditions. The enzymes were incubated with 2 mM PMSF for 120 min at 35 °C, and enzymatic activity assayed against control samples containing the enzyme in a solution of water/isopropanol.

2.3.8. Molecular mass and pI

SDS-PAGE was used to determine the molecular mass of both native and recombinant LipG7 essentially as described by Laemmli [18]. Protein samples (20 μg) were loaded onto slabs (10 × 5.5 cm) of 6% stacking and 8% resolving polyacrylamide gel. Page Ruler Plus (Thermo Scientific) was used as molecular weight marker. Protein concentration was determined according to Bradford [19], using BSA as a standard. The isoelectric points of the native and recombinant LipG7 were determined by binding to ion exchangers during purification and precipitation in a pH gradient using buffers in the pH range 6–8, using the following columns: Mono Q 5/50 GL, Mono S 5/50 GL, HiTrap Q FF, HiTrap SP FF, DEAE-Sepharose 5/50, POROS 50. The pI for the LipG7 was also deduced from translated protein sequence using the ExPASy server [20].

2.4. Transesterification reaction and gas chromatography

The transesterification reaction was performed with either dried mycelium powder or lyophilized protein powder (protein content corresponding to 0.035 U of *p*-nitrophenyl acetate hydrolysis activity) added to 50 μl of (R,S)-1-phenylethanol, 200 μl of vinyl acetate, 0.5 g of molecular sieve 4 Å and 750 μl of cyclohexane. Solvents for the reaction experiments were dehydrated with molecular sieves 4 Å for