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BIOCATALYSIS

Characterization of three new carboxylic ester hydrolases isolated by functional screening of a forest soil metagenomic library

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Abstract Three new lipolytic genes were isolated from a forest soil metagenomic library by functional screening on tributyrin agar plates. The genes *SBLip1*, *SBLip2* and *SBLip5.1* respectively encode polypeptides of 445, 346 and 316 amino acids. Phylogenetic analyses revealed that SBLip2 and SBLip5.1 belong to bacterial esterase/lipase family IV, whereas SBLip1 shows similarity to class C β-lactamases and is thus related to esterase family VIII. The corresponding genes were overexpressed and their products purified by affinity chromatography for characterization. Analyses of substrate specificity with different *p*-nitrophenyl esters showed that all three enzymes have a preference for short-acyl-chain *p*-nitrophenyl esters, a feature of carboxylesterases as opposed to lipases. The β-lactamase activity of SBLip1, measured with the chromogenic substrate nitrocefin, was very low. The three esterases have the same optimal pH (pH 10) and remain active across a relatively broad pH range, displaying more than 60 % activity between pH 6 and 10. The temperature optima determined were 35 °C for SBLip1, 45 °C for SBLip2 and 50 °C for SBLip5.1. The three esterases displayed different levels of tolerance to salts, solvents and detergents, SBLip2 being overall more tolerant to high concentrations of solvent and SBLip5.1 less affected by detergents.

Keywords Soil DNA extraction · Library construction · Functional metagenomics · Carboxylic ester hydrolases · Lipolytic enzymes

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Introduction

Biocatalysts are increasingly used in industrial processes. As compared to chemical catalysis, enzyme-catalysed reactions are generally more selective (high chemo-, regio- and stereoselectivity of enzymes). This results in higher product purity and reduces costs linked to elimination of unwanted by-products. Processes based on enzymatic catalysis require milder conditions than conventional chemical methods and thus consume less energy. Moreover, enzymes are biodegradable and their use helps reduce the environmental impact of the chemical industry. Carboxylic ester hydrolases (EC 3.1.1.x) are among the biocatalysts most exploited industrially [6, 8, 12, 18]. In an aqueous environment, these enzymes catalyse cleavage of carboxylic ester bonds, with formation of an alcohol and a carboxylic acid. Many of them can also catalyse esterification and transesterification reactions in the presence of an organic solvent. Of particular interest are lipolytic enzymes acting on fatty acid esters. These notably include triacylglycerol lipases (EC 3.1.1.3), able to hydrolyse water-insoluble triglycerides with long acyl chains, and carboxylesterases (EC 3.1.1.1), preferring esters with shorter acyl chains at least partly soluble in water. These enzymes find applications in many sectors such as the food, detergent, cosmetic, leather, textile, paper and pharmaceutical industries. They are also promising biocatalysts for biodiesel production [8, 25].

Lipolytic enzymes are widely distributed in nature. Enzymes of microbial origin are the most used in biotechnological applications, mainly because of the ease with which they can be produced and genetically manipulated as compared to plant or animal enzymes. They are also often more stable and offer a wider diversity of catalytic activities [4, 8, 25, 28]. So far, only enzymes from culturable

microorganisms have been used, but today, with the development of metagenomics, new enzymes with potentially interesting properties can be isolated from uncultured organisms [5, 31]. For this purpose, we have constructed a metagenomic library from a forest soil sample and screened colonies for lipolytic activity on tributyrin agar plates. We have thus isolated, purified and biochemically characterized three new bacterial enzymes.

Materials and methods

Sample collection and DNA extraction

A forest soil sample was collected from the upper 5-cm layer below the litter in Groenendaal (Belgium) at the end of October 2010. The method used to extract the metagenomic DNA was based on the protocols of Zhou et al. [39] and Pang et al. [19], with several modifications. The soil sample (5 g) was suspended in 13.5 ml DNA extraction buffer (100 mM Tris–HCl pH 8, 100 mM sodium phosphate pH 8, 100 mM sodium EDTA pH 8, 1.5 M NaCl and 5 mg/ml lysozyme) and incubated at 37 °C for 1 h. It was next incubated at 65 °C for 30 min after adding 100 µl proteinase K 10 mg/ml and 350 µl SDS 20 %. The incubation was then continued for 75 min with additional SDS (1.15 ml) and for another 15 min with 1.5 ml cetyltrimethylammonium bromide 10 %. The supernatant was collected after centrifugation at 6,000*g* for 10 min and mixed with an equal volume of chloroform–isoamyl alcohol (24:1). To remove humic acids, the aqueous phase was recovered by centrifugation (10 min, 3,850*g*) and purified twice by precipitation with polyethylene glycol 6000 (PEG)/NaCl. One volume of 20 % PEG was first added to the extract. After a 2-h incubation at room temperature, the DNA was pelleted (14,000*g*, 15 min) and dissolved in 5 ml TE (10 mM Tris–HCl, 1 mM sodium EDTA, pH 8). The second precipitation was performed with 5 ml of 10 % PEG/1.4 M NaCl. The DNA was further purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction. The aqueous phase was precipitated with 0.1 volume NaCl (5 M) and 2 volumes ethanol. The DNA pellet was washed with 70 % ethanol, air-dried and dissolved in 500 µl of 10 mM Tris–HCl pH 8.

Construction of metagenomic libraries

Metagenomic DNA was partially restricted with *Sau*3AI, and DNA fragments below 3 kb were removed by size-selective precipitation with PEG (5 %)/NaCl (0.6 M) before separation in a 1 % low melting point agarose gel. DNA fragments 4–9 and 9–20 kb in size were recovered from the agarose with AgarACE enzyme (Promega) according to the manufacturer's instructions. The digested DNA fragments (50 ng) were incubated overnight at 16 °C with the *Bam*HI-linearized, dephosphorylated vector pHT01 (MoBiTec, 50 ng) and 1 U T4 DNA ligase (Roche). The ligation products were introduced into electrocompetent *E. coli* (ElectroMAX DH10B, Invitrogen). The average insert size in each library was determined by analysing 20 randomly selected clones. Their plasmids were extracted and digested with *Bam*HI and *Hin*dIII.

Screening for lipolytic activity

Libraries were screened for lipolytic activity by plating the transformants onto 2× YT medium containing 1.5 % agar, 50 µg/ml ampicillin and 3 % Lipase Reagent (Difco), a mixture of tributyrin and polysorbate 80. After a 2-day incubation at 37 °C, positive clones were identified by formation of a clear halo around the colonies.

Subcloning, DNA sequencing and sequence analysis

The relatively short inserts encoding SBLip1 (7.7 kb) and SBLip2 (4.9 kb) were completely sequenced at GATC Biotech (Germany). The 18-kb plasmid expressing SBLip5.1 was partially digested with *Sau*3AI and fragments 1–4 kb long were subcloned in the pHT01 vector. Four independent subclones were selected on tributyrin and their plasmids sequenced. The four sequences were assembled into a contig totalling 3,166 bp. Sequence similarity searches were carried out with the BLASTX and BLASTP programs. Multiple sequence alignments were performed with the ClustalW program [33] and phylogenetic analysis was done with MEGA5 [32]. Signal peptides were predicted with the SignalP 3.0 server [3].

Overexpression and purification of recombinant esterases

The coding sequences of the three esterase genes were amplified with Platinum *Pfx* DNA Polymerase and 1x PCRx Enhancer Solution (Invitrogen). The following pairs of primers were used for SBLip1: 5′-gggaattcCATATGGCT TCGGTCTCCAAAGCCA-3′ and 5′-ttcccAAGCTTT CATCA**GTGGTGGTGGTGGTGGTG**ATCGATAATC GCCTGAGCCA-3′, for SBLip2: 5′-gggaattcCATATGAG CGAGCGAGCCACACAT-3′ and 5′-cgcGGATCCTCAT CA**GTGGTGGTGGTGGTGGTG**GCCCAGCCGCTGG CGCA-3′, for SBLip5.1: 5′-gggaattcCATATGTCCCTACA TCCACAATGC-3′ and 5′-cgcGGATCCTCATCA**GTGGT GGTGGTGGTGGTG**ACCGGCCACGGCCGTCGGC-3′. The restriction sites (*Nde*I, *Bam*HI, *Hin*dIII) are underlined and the histidine tag sequences are written in bold. The PCR products were cloned into the pET-30b(+) vector (Novagen)

and the resulting plasmids introduced into *Escherichia coli* Rosetta 2(DE3) (Novagen) after checking the inserts by DNA sequencing. To induce protein expression, overnight cultures of transformed Rosetta cells were diluted to an OD_{600nm} of 0.005 in 1-l shake flasks containing 200 ml 2xYT medium and antibiotics (30 μg/ml kanamycin and 30 μg/ml chloramphenicol). Cultures were grown at 37 °C with an agitation rate of 160 rpm until the OD_{600nm} reached 0.4. IPTG was then added to a concentration of 1 μM (SBLip1 and SBLip2) or 10 μM (SBLip5.1). Overexpression was induced for 16 h at 25 °C (SBLip1 and SBLip2) or for 4 h at 37 °C (SBLip5.1). The recombinant esterases were purified by Ni-NTA affinity chromatography (Qiagen) as previously described [16]. The purified proteins were dialysed at 4 °C against 20 mM Tris–HCl pH 7.5 and analysed by SDS-PAGE.

Lipolytic activity assays

The lipolytic activities of the purified enzymes were determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl (pNP) esters at 348 nm, the pH-independent isosbestic wavelength of *p*-nitrophenoxide and *p*-nitrophenol. After pre-incubation of the reaction mix (390 μl), the reaction was started by adding 10 μl enzyme and stopped after 5 min with 400 μl of 0.066 N ice-cold HCl. To check the linearity of the reaction, two different concentrations of enzyme were tested for each condition. One unit of esterase activity was defined as the amount of enzyme needed to release 1 μmol *p*-nitrophenol in 1 min. All experiments were performed in triplicate. Results are expressed as mean values ±SE of the mean. Comparisons with the control condition or between enzymes were performed by Student's *t* tests.

Substrate specificity was estimated with pNP-acetate (C2:0), pNP-butyrate (C4:0), pNP-octanoate (C8:0), pNP-laurate (C12:0) and pNP-palmitate (C16:0) (Sigma-Aldrich). The reactions were carried out at 35 °C in 100 mM sodium phosphate buffer pH 8 with 1 mM substrate, 0.4 % Triton X-100, 0.1 % arabic gum and 10 % acetonitrile. Isopropanol was used instead of acetonitrile for SBLip1. To determine the optimal pH of the enzymes, esterase activities were measured over a pH range of 4–10 in a mix containing 5 mM pNP-butyrate, 0.3 % Triton X-100 and 2 % acetonitrile. The buffers tested were 100 mM sodium acetate pH 4–6; 100 mM sodium phosphate buffer pH 6–8; 100 mM Tris–HCl pH 7.5–10. To minimize substrate autohydrolysis (which increases with the pH), the reaction temperature was decreased to 22 °C (room temperature). The temperature optimum was determined by incubating the reaction mixture (100 mM sodium phosphate buffer pH 7, 3 mM pNP-acetate, 3 % acetonitrile) at various temperatures from 10 to 60 °C. Activities were measured at neutral pH to limit autohydrolysis at high temperature and pNP-acetate was used instead of pNP-butyrate to minimize variation of substrate solubility with the reaction temperature. The effects of different additives (phenylmethanesulfonyl fluoride (PMSF), EDTA, ions, detergents, organic solvents) were evaluated at 35 °C in 100 mM Tris–HCl buffer pH 8, 3 mM pNP-butyrate, 0.3 % Triton X-100 and 2 % acetonitrile. To determine the kinetic parameters of SBLip1, SBLip2 and SBLip5.1, each enzyme was incubated for 5 min at its optimal temperature in 100 mM Tris–HCl pH 10, 0.3 % Triton X-100, 2 % acetonitrile (or isopropanol for SBLip1) and various concentrations of pNP-butyrate or pNP-octanoate (0.5–4 mM).

The activity of the purified enzymes was also tested on agar plates containing different emulsions of triglycerides and a pH indicator [29]. The wells of a 96-well microtitre plate were filled with 50 μl of a 1.5 % agar solution containing 1 % emulsified substrate [tributyrin, trioctanoin or olive oil (Sigma-Aldrich)], 10 mM $CaCl_2$ and 0.003 % phenol red. The pH of the solution was adjusted to 7.3–7.5 with NaOH. The substrate emulsions were prepared by mixing 1 ml substrate with 4 ml of a 5 % solution of arabic gum followed by 3 min of sonication on ice [30]. Two microlitres of pure enzyme (200 ng) was added to each well and incubated at 29 °C.

β-Lactamase assay

The β-lactamase activity of SBLip1 was determined spectrophotometrically with nitrocefin (Calbiochem) according to the manufacturer's instructions. The enzyme was incubated with 1 mM nitrocefin (in 100 mM sodium phosphate buffer pH 8) for 1 h at 35 °C.

Nucleotide sequence accession numbers

The nucleotide sequences of the inserts containing the *SBLip1*, *SBLip2* and *SBLip5.1* genes were deposited in the GenBank database under accession numbers JQ780827, JQ780828 and JQ780829.

Results

Construction and screening of metagenomic libraries

Two plasmid libraries were constructed with metagenomic DNA isolated from a soil sample taken from the A horizon of a Belgian deciduous forest. Environmental DNA was recovered according to a modified protocol based on the direct extraction method of Zhou et al. [39]. To eliminate the humic acids coextracted with the genomic DNA, the latter was precipitated twice with PEG/NaCl [1]. Pure

DNA with a final yield of 15 μg/g soil was thus obtained. After partial digestion with *Sau*3AI and subsequent elimination of small fragments by size-selective precipitation, the DNA was resolved by electrophoresis. Fragments 4–9 and 9–20 kb long were purified from the gel and inserted into the pHT01 vector (MoBiTec, 8 kb), yielding two libraries with respective average insert sizes of 7 and 12 kb. The two libraries were obtained with similar transformation efficiencies (6 and 4×10^6 transformants/μg respectively) and contained about 99 % insert-bearing plasmids. Approximately 35,000 *E. coli* colonies from each library were screened for lipolytic activity on tributyrin agar plates. Three positive clones were found, out of 665 Mb DNA analysed. To identify the genes encoding lipolytic enzymes, the 7.7- and 4.9-kb inserts isolated from the small-insert library were entirely sequenced, whereas subcloning was performed on the 18-kb insert from the medium-insert library.

Sequence analysis

Sequence similarity searches identified three open reading frames of 1,335, 1,038 and 948 bp respectively, showing homology to known lipolytic enzymes. The new genes, named *SBLip1*, *SBLip2* and *SBLip5.1*, have respective GC contents of 61.1, 71.4 and 63.1 %. The highest similarity was found for SBLip2, which shares 77 % amino acid identity (over 329 aa) with a lipolytic enzyme (AEM45126) identified by functional metagenomics in the B horizon of a German forest soil [17]. SBLip5.1 was also most similar (56 % identity over 312 aa) to a metagenome-derived enzyme: a cold-adapted esterase (ADR31550) from a mountain soil in Korea [15]. Both SBLip2 and SBLip5.1 are predicted to display the typical α/β hydrolase fold common to many different hydrolases and most lipolytic enzymes. In contrast, SBLip1 contains a structural domain conserved in β-lactamases, D-Ala carboxypeptidases/transpeptidases, penicillin-binding proteins and a few esterases and is most closely related (42 % identity over 417 aa) to a β-lactamase from *Caulobacter* sp. K31 (YP_001682220). This protein is also distinguishable from SBLip2 and SBLip5.1 by the presence of a putative 24-aa signal sequence (signal peptide probability of 0.999).

Bacterial lipolytic enzymes have been classified into eight families based on amino acid sequence similarity and physiological properties [2]. In phylogenetic analyses, SBLip1 clustered with family VIII esterases, whereas SBLip2 and SBLip5.1 could be assigned to family IV of bacterial lipolytic enzymes (Fig. 1). Multiple alignments with representative members of family IV (Fig. 2a) showed that SBLip2 and SBLip5.1 contain the catalytic triad residues serine, aspartate and histidine typical of serine hydrolases. As in many other lipolytic enzymes, the nucleophilic serine is located within the highly conserved pentapeptide motif GxSxG. Family IV comprises bacterial enzymes showing significant similarity to mammalian hormone-sensitive lipases (HSL) [10]. These enzymes have in common a conserved HGGG motif, also present in SBLip2 and SBLip5.1. This motif is involved in hydrogen-bonding interactions stabilizing the oxyanion hole and plays a role in catalysis [37]. In SBLip1 (Fig. 2b), the catalytic serine is not located in a GxSxG motif but rather in the SxxK motif characteristic of class C β-lactamases, penicillin-binding proteins and family VIII carboxylesterases [36]. The protein also contains the conserved catalytic tyrosine [14, 24] and an LLxHxxG motif found in several family VIII esterases [22].

Purification of the recombinant esterases

The lipolytic enzymes were overproduced as C-terminal His-tag fusion proteins by means of the pET-30b(+) expression system in the *E. coli* Rosetta strain. SBLip1 was produced without its putative signal peptide. As all three recombinant proteins were at least partly soluble in the cell extract and not detected in the culture supernatants, they were all purified from the soluble fraction under non-denaturing conditions (Fig. 3).

Substrate specificities of the recombinant esterases

The substrate preferences of the purified enzymes were estimated with *p*-nitrophenyl esters of varying acyl chain length (Table 1). All three enzymes were significantly more active towards short- to medium-length acyl chains (C2 to C8) than long acyl chains (C12, C16), with which less than 2 % activity was detected. SBLip1 and SBLip2 showed the highest activity towards pNP-butyrate (C4), whereas SBLip5.1 was slightly more active towards pNP-octanoate (C8). The ability of the recombinant enzymes to hydrolyse triglycerides was next investigated on agar plates containing different lipid emulsions and a pH indicator [29]. Lipolytic activity was detected by the red-to-yellow colour change of the phenol red indicator due to fatty acid release. Figure 4 shows the results after a 16-h incubation at 29 °C. As expected, the tributyrin (C4) emulsion became yellow almost instantly after addition of any one of the purified enzymes. Slight hydrolytic activity towards trioctanoin (C8) was also detectable for the two family IV esterases, but only after a few hours at 29 °C, and no enzyme was able to hydrolyse significantly the long acyl chains (∼C18) of olive oil triglycerides. This preference for short- to medium-length acyl chains suggests that the new lipolytic enzymes are carboxylesterases and not lipases.

Some family VIII esterases display β-lactamase activity in addition to their ability to hydrolyse esters [13, 23, 38]. The β-lactamase activity of SBLip1 was evaluated with the

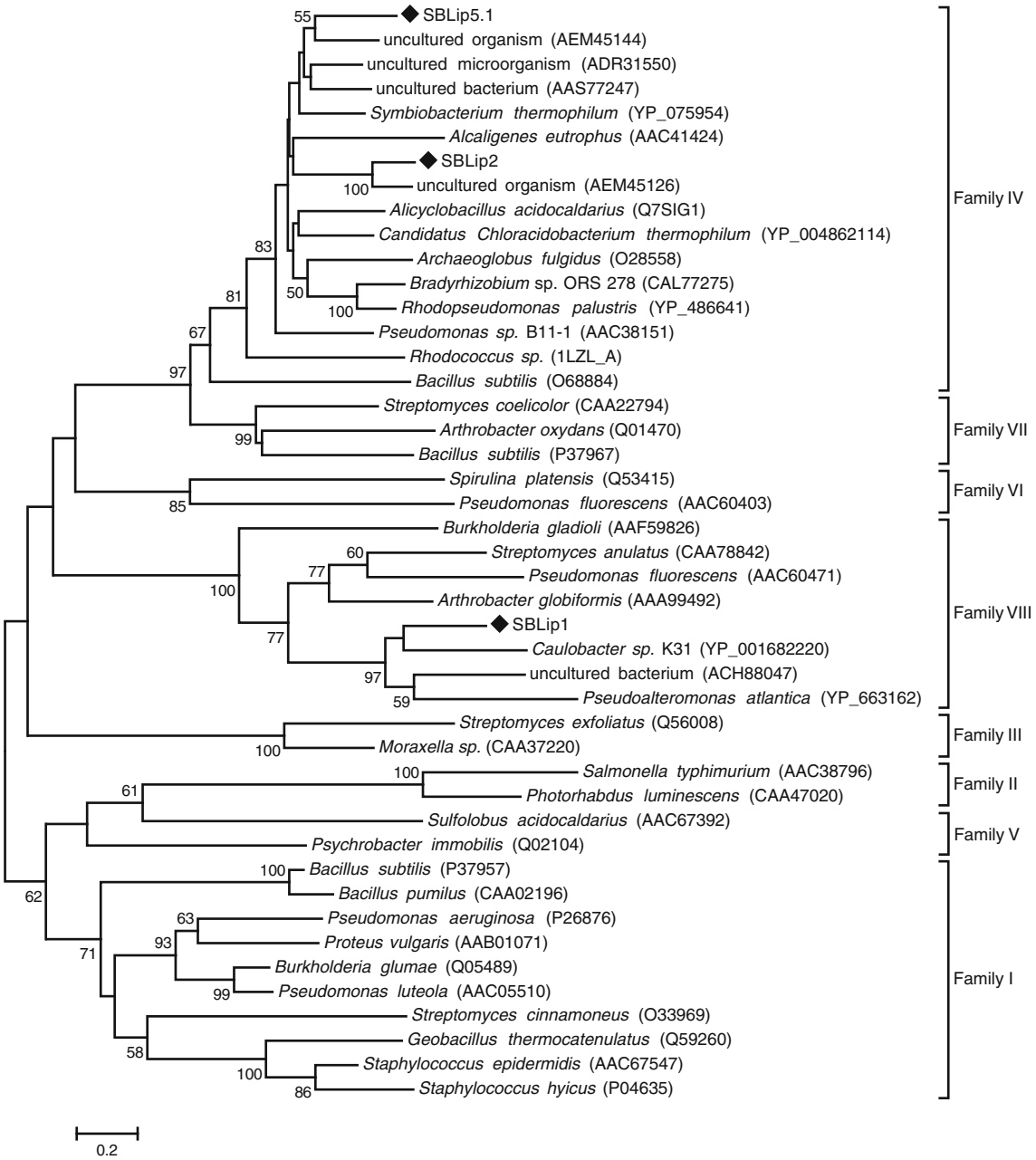

Fig. 1 Neighbour-joining tree built with lipolytic enzymes belonging to different families of esterases. The Poisson correction method was used to compute evolutionary distances. The *scale bar* indicates the number of amino acid substitutions per site. Bootstrap values are expressed as percentages of 1,000 replications and are shown at the *nodes*. Only bootstrap values higher than 50 % are indicated

chromogenic substrate nitrocefin. Only a very low activity (9×10^{-4} U/mg) was measured at pH 8, corresponding to about 0.005 % of the lipolytic activity towards pNP-butyrate at the same pH.

Temperature and pH optima of the esterases

The activity ranges of the three esterases were then evaluated between pH 4 and 10 and between 10 and 60 °C (Fig. 5). Optimal temperatures of 50 and 45 °C were observed for SBLip5.1 and SBLip2 respectively, whereas SBLip1 appeared more mesophilic, with maximal activity at 35 °C. The enzymes proved to be active across a relatively broad pH range, with an optimum at pH 10. Enzymatic activities were not evaluated at higher pH because of substrate instability under alkaline conditions. All esterases displayed more than 60 % activity between pH 6 and 10 but little to no activity below pH 4. The new family IV esterases appeared a little less sensitive to low pH than SBLip1 and retained about 40 % activity at pH 5.

a

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                                          *                                  *                                  *
SBLip2    116 AYFHGGGWVQGD   188 AVAGDSAGGNLSAVVSQ    283 VTAGFDPLRDEGRAYADRLRGAGIDVVYREYPGQIHA
SBLip5.1   79 VYFHGGGWVIGG   151 AVGGDSSGGNLAAVVTL    247 ITAECDLLRDQGELYAQKLREAGVPVSVKRYDGMIHP
AEM45126   99 VYLHGGGWVQGD   171 AVAGDSAGGNLSAVVSQ    266 ITAGFDPLRDEGRAYADRLSAAGVEVVHREYPGQIHA
ADR31550   80 VFFHGGGWVICD   152 AVGGDSAGGNLTAVVAQ    247 ITAEYDPLRDEGESYGKQLQEAGVPVTISRYDGMIHG
1LZL_A     83 LWIHGGGFAIGT   155 AVGGQSAGGGLAAGTVL    255 STMELDPLRDEGIEYALRLLQAGVSVELHSFPGTFHG
O68884    122 VYTHGGGMTILT   197 VVQGESGGGNLAIATTL    303 AVNELDPLRDEGIAFARRLARAGVDVAARVNIGLVHG
Q7SIG1     78 VYYHGGGWVVGD   150 AVGGDSAGGNLAAVTSI    247 ATAQYDPLRDVGKLYAEALNKAGVKVEIENFEDLIHG
AAC41424  127 VYFHGGGFTVGS   199 AVGGDSAGGTLAAACAV    297 AVAGYDPLHDEGVAYAEKLRAAGVAATLADYPGMIHD
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b

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                          *   *                                                                *
SBLip1       91 ATMFRVASMTKPVTSVALMMM   180 TVAAAREVTIKDLLTHVSGLASG   228 RLGSTALEFQPGSRWAYSAQAGHD
YP_0016822  103 DDIFRMMSSTKPTIGVAVLMM   173 LVPAERDITIRDLMTHTSGLSSG   218 RLGAAALDFQPGSRWSYSASDGID
AAA99492     52 DSVTGVFSCSKGVSGLVIALL    94 --EGKATITVAQLLSHQAGLLG-   132 KLAQMRPLWKPGTAFGYHALTIGV
AAC60471     60 DTIVNLFSCTKTFTAVTALQL   102 --AGKEAITLRQLLCHQAGLP--   140 TLAAEAPWWTPGQGHGYEAITYGW
LAC1         73 QTLFELGSVSKTFTGVLGGDA   114 -AKQWNGITLLHLATYTAGG---   150 FYQNWQPAWAPGTQRLYANSS-IG
LAC2         77 QTLFELGSISKTFTGVLGGDA   118 -GKQWQGIRMLDLATYTAGG---   154 FYQNWQPQWKPGTTRLYANAS-IG
PBP1         97 DFRFRIGSVTKTFTATVVLQL   140 NGYDGNKITIQEILNHTSGIAEY   185 MGISFPPDFAPGKGWSYSNTG-YV
PBP2         86 TDRFRVGSVTKSFSAVVLLQL   128 ----DDRITVRQVMSHRSGLYDY   174 LSLKHGVTNAPGAAYSYSNTN-FV
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Fig. 2 Multiple amino acid sequence alignments of SBLip1, SBLip2, SBLip5.1 and related esterases. Conserved residues are shaded in *grey*. *Asterisks* conserved catalytic residues. **a** Conserved sequence blocks from multiple alignments of SBLip2, SBLip5.1 and other family IV carboxylesterases. **b** Conserved sequence blocks from multiple alignments of SBLip1, other family VIII carboxylesterases, class C β-lactamases (LAC) and penicillin-binding proteins (PBP). *LAC1* AAA23441, *LAC2* P05364, *PBP1* CAA09676, *PBP2* P15555

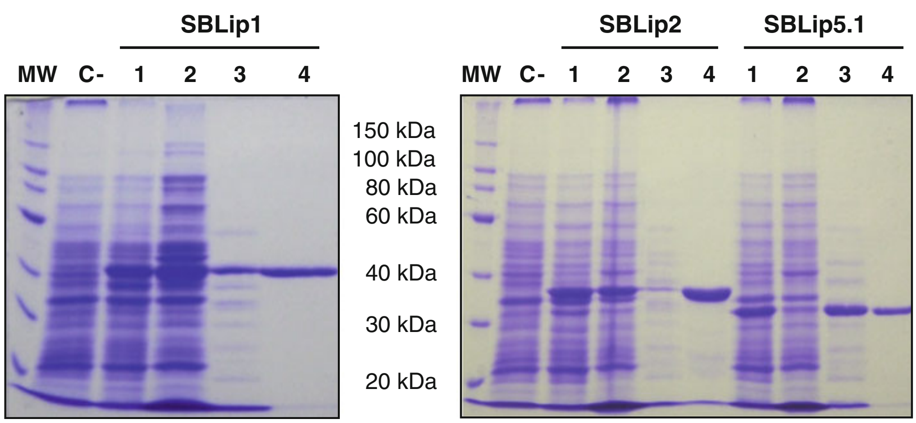

Fig. 3 Expression and purification of recombinant esterases. Proteins recovered during different purification steps were separated by SDS-10 % polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. *MW* protein molecular weight marker; *C−* total extract of induced *E. coli* Rosetta 2(DE3) cells transformed with the empty vector pET-30b(+); *lane 1* total cell extract of induced bacteria overexpressing SBLip1, SBLip2 or SBLip5.1; insoluble (*lane 2*) and soluble (*lane 3*) fractions of the cell extract; *lane 4* purified protein

Table 1 Substrate specificity towards *p*-nitrophenyl esters (1 mM)

p-Nitrophenyl esters	Specific activity (U/mg purified protein)		
	SBLip1	SBLip2	SBLip5.1
pNP-acetate (C2)	8.80 ± 0.47	756 ± 40	508 ± 9
pNP-butyrate (C4)	**29.0 ± 0.4**	**4,006 ± 79**	822 ± 21
pNP-octanoate (C8)	3.80 ± 0.20	1,904 ± 78	**891 ± 17**
pNP-laurate (C12)	0.14 ± 0.01	70.4 ± 1.9	2.65 ± 1.74
pNP-palmitate (C16)	ND	4.54 ± 1.29	13.0 ± 5.6

The highest activity of each enzyme is indicated in bold

ND not detected

Kinetic parameters

Although alkaline conditions and high temperature are not suitable for accurate determination of kinetic parameters with short-acyl-chain *p*-nitrophenyl esters, we tried to estimate the maximal velocity (V_{max}) and the Michaelis affinity constant (K_m) of each enzyme using a Lineweaver–Burk plot. For this, the initial velocity of each enzyme was measured at optimal pH and temperature with a variable concentration of its preferred pNP-ester. The activity of SBLip1 and SBLip2 did not increase with substrate concentration but remained constant over the entire range of substrate concentrations tested (0.5–4 mM). This suggests that both enzymes were saturated with substrate and thus had already reached their maximal velocity. The highest V_{max} was obtained for SBLip2, with a mean of 3,192 ± 63 U/mg protein. This is about 100 times the V_{max} of SBLip1 (29.6 ± 0.7 U/mg). To evaluate their K_m values, which corresponds to the substrate concentration at which the reaction rate is half of V_{max}, we tried to measure activities in

the presence of limiting concentrations of substrate. But it was not possible to measure reliable activities below 0.5 mM because of the high rate of non-enzymatic substrate hydrolysis occurring at alkaline pH. At pH 10, the substrate concentration dropped rapidly during the reaction and the total amount of product formed by chemical and enzymatic hydrolysis was not negligible, sometimes even exceeding that of the substrate. We can thus only conclude that the K_m of SBLip1 and SBLip2 was inferior to 0.5 mM.

As for SBLip5.1, its higher temperature optimum (50 °C) did not allow us to measure sufficiently reliable activities, even for substrate concentrations above 0.5 mM. We thus were unable to estimate its kinetic parameters. The highest specific activity measured for SBLip5.1 under these conditions was about 350 U/mg.

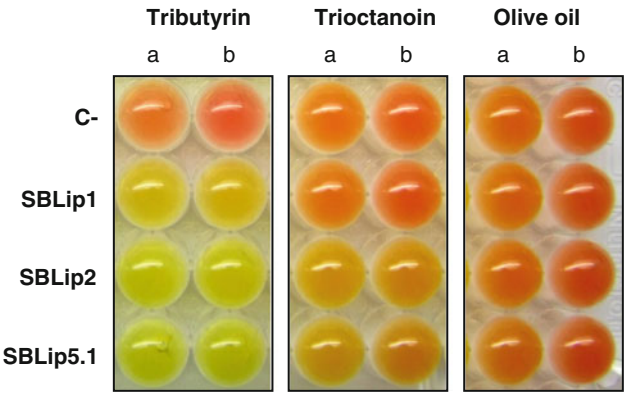

Fig. 4 Activity of recombinant esterases on triglyceride substrates. Two microliters of purified enzyme (200 ng) was added to 50 µl tributyrin, trioctanoin or olive oil emulsion and incubated for 16 h at 29 °C. Lipid hydrolysis was followed over time by monitoring the colour change (from *red* to *yellow*) of a pH indicator (phenol red) due to release of fatty acids from lipids. The emulsion pH was initially adjusted to 7.3 (**a**) or 7.5 (**b**). Tris–HCl (20 mM, pH 7.5) was used as a negative control (*C*−) (colour figure online)

Effect of additives on esterase activity

Enzymatic activity was next assayed in the presence of various additives (Fig. 6). We first investigated the effect of the modifying agent PMSF, which covalently binds to the catalytic serine of many carboxylesterases and some lipases. All esterases were inhibited by PMSF, suggesting that their catalytic site is not covered by a hydrophobic domain (lid) and thus does not require interfacial activation to be fully active. A metal cofactor also appeared unnecessary, as deduced from the absence of inhibition by the chelating agent EDTA. When added at 5 mM concentration, Na^+, K^+, Co^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} had no or very little effect on the activity of the recombinant enzymes. In contrast, all three enzymes were severely inhibited by 5 mM Zn^{2+} and to a lesser extent by Cu^{2+}, whereas Fe^{2+} and Fe^{3+} had variable effects according to the enzyme. Both of these cations slightly increased the activity of SBLip5.1 but impaired that of SBLip2. SBLip1 was affected only by Fe^{3+}. The three enzymes were not inhibited by 5 mM $MnCl_2$ but the activity of SBLip5.1 was impaired by 5 mM $MnSO_4$. This suggests that this esterase was affected by sulphate. A high salt concentration (1 M NaCl) only moderately affected the activity of the two family IV esterases, but reduced SBLip1 activity by about 50 %.

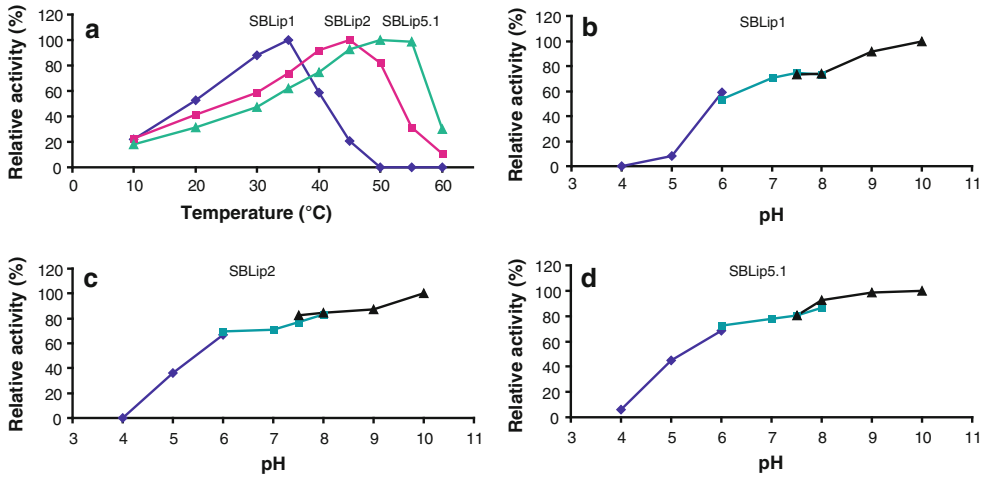

Fig. 5 Effect of temperature and pH on lipolytic activity. **a** Temperature-dependent activity of recombinant esterases, measured with pNP-acetate as substrate. The specific activities of SBLip1 (*filled diamond*), SBLip2 (*filled square*) and SBLip5.1 (*filled triangle*) at optimal temperature (100 % activity) were respectively 10.2, 1,085 and 221 U/mg. **b–d** Effect of pH on the relative activity of recombinant esterases. The ability to hydrolyse pNP-butyrate was assayed at 35 °C in different buffers at a pH ranging from 4 to 10. *Filled diamond* 0.1 M Na-acetate buffer pH 4–6, *filled square* 0.1 M Na-phosphate buffer pH 6–8, *filled triangle* 0.1 M Tris–HCl buffer pH 7.5–10. The specific activities corresponding to a relative activity of 100 % (pH 10) were 10.1, 428 and 132 U/mg for SBLip1, SBLip2 and SBLip5.1, respectively

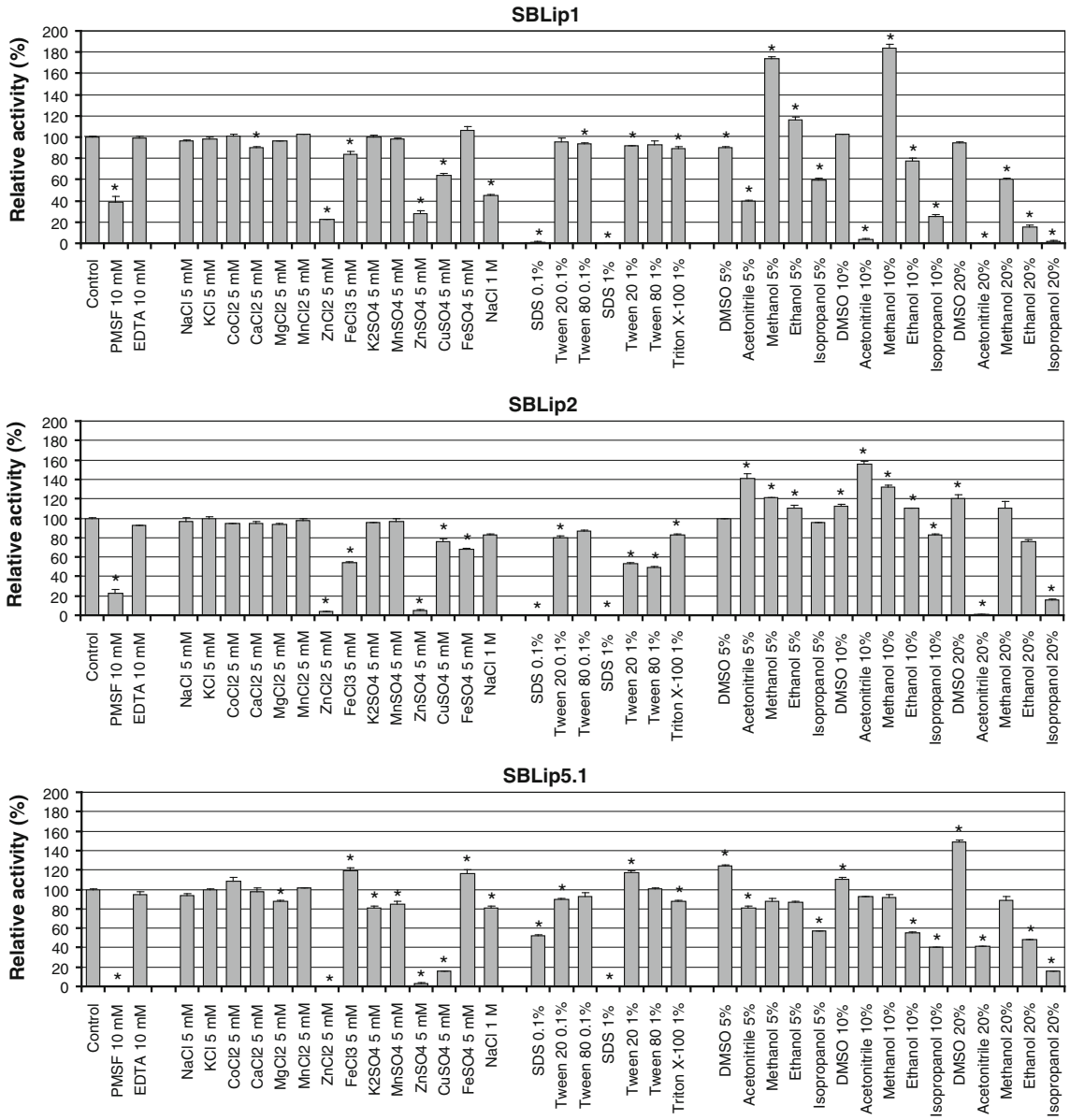

Fig. 6 Effects of various additives on the lipolytic activity of SBLip1, SBLip2 and SBLip5.1. The hydrolysis rate of pNP-butyrate was measured at 35 °C and pH 8 with 0.3 % Triton X-100, 2 % acetonitrile and variable additives. Specific activities of 28.0, 1,774 and 224 U/mg were measured for SBLip1, SBLip2 and SBLip5.1 under control conditions. *$p < 0.05$ (vs. control condition)

Enzymatic activities were also measured in reaction mixtures containing different concentrations of detergent (0.1 or 1 %) or organic solvent (5, 10 or 20 %). The enzymes were only slightly affected by 1 % Triton X-100 and by 0.1 % Tween 20 or 80 but all were completely inhibited by 1 % SDS. They were variably affected by 0.1 % SDS and 1 % Tween 20 or 80. SBLip5.1 showed the highest tolerance to 0.1 % SDS and 1 % Tween 20. Unlike SBLip1 and SBLip2, it still retained about 50 % activity in 0.1 % SDS ($p < 0.001$ vs. SBLip1 or SBLip2) and was even slightly activated by 1 % Tween 20 ($p < 0.05$ vs. SBLip1 or SBLip2). SBLip2 was the most affected by high concentrations of both Tween 20 and 80 ($p < 0.05$ vs. SBLip1 or SBLip5.1) but was highly tolerant to the organic solvents tested. Its activity was stimulated by addition of 10 % DMSO, acetonitrile, methanol or ethanol and only slightly reduced by 10 % isopropanol. Increased activity was still measurable with 20 % DMSO and methanol.

Discussion

To find new lipolytic enzymes, we have constructed metagenomic libraries with total DNA extracted from a forest

soil sample. DNA isolated from this kind of soil is generally contaminated by humic acids, which prevent further enzymatic processing. Several methods have been used to purify crude extracts, the most common being gel filtration with various resins or agarose gel electrophoresis [21, 39]. DNA precipitation with PEG/NaCl has also been successfully employed in a few studies, but always in combination with other methods [1, 18]. Here, humic acids were removed with a double PEG/NaCl precipitation and no additional purification step. PEG/NaCl was again used after partial digestion to eliminate small DNA fragments that would otherwise have integrated preferentially into the cloning vector. This enabled us to efficiently clone large fragments up to 18 kb long and to construct a plasmid library with an average insert size of 12 kb, which is higher than usually obtained [17, 34]. Hence PEG/NaCl precipitation, in addition to being a good means of purifying soil DNA, can be used advantageously in constructing any DNA library, including a BAC or fosmid library.

Screening of the libraries identified three carboxylic ester hydrolases with only moderate similarity to known proteins (42–77 % identity). As often occurs in metagenomic studies, it was not possible to determine the phylogenetic origin of the newly isolated DNA fragments. Sequence analysis suggests that the esterase genes are not part of an operon, and revealed conserved protein motifs enabling us to classify the enzymes as family IV and family VIII esterases. Like the other known members of their families, the new hydrolases act preferentially on short-chain *p*-nitrophenyl esters and triglycerides. SBLip1 can also hydrolyse the amide bond of the β-lactam ring of nitrocefin, a chromogenic substrate used to detect β-lactamase activity. Only a few family VIII esterases have been shown to retain both activities. The β-lactamase activity of SBLip1 (9×10^{-4} U/mg) was negligible, however, as compared to other reported β-lactamase activities (>10 U/mg) [23, 38] and to its ability to hydrolyse esters ($\sim$30 U/mg).

Several esterases are reported to have a temperature optimum higher than the temperatures encountered by the source organisms in their environment [7, 11, 20, 27]. This is also true of the new enzymes, which display maximal activity at 35, 45 and 50 °C. Surprisingly, all three esterases also appear to be most active at a pH significantly higher than the soil pH (acid mull). This is most probably due to a bias in the library screening procedure as a result of alkalinization of the 2xYT medium during *E. coli* growth [26, 35]. After 2 days, the pH is usually near 9, favouring the activity of alkaliphilic enzymes. The use of a well-buffered medium could likely overcome this bias and allow the discovery of enzymes with lower pH optima.

The preference of these enzymes for alkaline conditions and their resistance to chelating agents make them potentially useful in the detergent industry. Lipolytic enzymes are mainly added in detergents used in household and industrial laundry and in household dishwashers. They improve their efficiency and also help reduce the environmental impact of the detergents (e.g. by reduction of the amount of non-biodegradable products used and lower wash temperature) [9]. SBLip5.1 seems to be most adapted because it is more resistant to detergents.

SBLip2 seems particularly attractive for the chemical industry because of its high solvent tolerance, a condition notably required for many esterification and transesterification reactions. The enzyme also displayed a very high specific activity (3,000–4,000 U/mg) enabling it to retain sufficient activity even under suboptimal conditions. It could therefore be used, for example, as a cold-active enzyme as it is still highly active at 10 °C, even if it retains only 20 % residual activity. SBLip2 might thus find many biotechnological applications.

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