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ORIGINAL PAPER

Thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* SOL1 and *Caldanaerobacter subterraneus* subsp. *tengcongensis*

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Abstract Two novel genes encoding for heat and solvent stable lipases from strictly anaerobic extreme thermophilic bacteria *Thermoanaerobacter thermohydrosulfuricus* (LipTth) and *Caldanaerobacter subterraneus* subsp. *tengcongensis* (LipCst) were successfully cloned and expressed in *E. coli*. Recombinant proteins were purified to homogeneity by heat precipitation, hydrophobic interaction, and gel filtration chromatography. Unlike the enzymes from mesophile counterparts, enzymatic activity was measured at a broad temperature and pH range, between 40 and 90°C and between pH 6.5 and 10; the half-life of the enzymes at 75°C and pH 8.0 was 48 h. Inhibition was observed with 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and phenylmethylsulfonylfluorid indicating that serine and thiol groups play a role in the active site of the enzymes. Gene sequence comparisons indicated very low identity to already described lipases from mesophilic and psychrophilic microorganisms. By optimal cultivation of *E. coli* Tuner (DE3) cells in 2-l bioreactors, a massive production of the recombinant lipases was achieved (53–2200 U/l) Unlike known lipases, the purified robust proteins are resistant against a large number of organic solvents (up to 99%) and detergents, and show activity toward a broad range of substrates, including triacylglycerols, monoacylglycerols, esters of secondary alcohols, and *p*-nitrophenyl esters. Furthermore, the enzyme from *T. thermohydrosulfuricus* is suitable for the production of optically pure compounds since it is highly *S*-stereoselective toward esters of secondary alcohols. The observed *E* values for but-3-yn-2-ol butyrate and but-3-yn-2-ol acetate of 21 and 16, respectively, make these enzymes ideal candidates for kinetic resolution of synthetically useful compounds.

Keywords Thermophiles and thermophilic enzymes · Anaerobic bacteria · Genecloning and expression

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Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are best defined as carboxylesterases that catalyze both the hydrolysis and synthesis of long-chain acylglycerols (Jaeger et al. 1999). True lipases can be defined as carboxylesterases that catalyze the hydrolysis and synthesis of relatively long-chain acylglycerols with acyl chain lengths of >10 carbon atoms. Lipases share a similar active site consisting of three residues: a nucleophilic serine residue in a Gly-X-Ser-X-Gly motif, an acidic residue (aspartic acid or glutamic acid), and a histidine. These residues act cooperatively in the catalytic mechanism of ester hydrolysis. The enzymes also display a common α/β hydrolase fold (Ollis et al. 1992) which is also found in other hydrolases, such as haloalkane dehalogenase, acetylcholinesterase, dienelactone hydrolase, and serine

carboxypeptidase. Based on comparisons of amino acid sequences and biological properties, prokaryote-derived lipases have been classified into eight different families (I–VIII) (Arpigny and Jaeger 1999).

Lipases are an important group of biotechnologically relevant enzymes, and they find applications in food, diary, detergent, and pharmaceutical industries. Most lipases, that are derived from mesophilic microorganisms, can act in a wide range of pH but are mostly unstable at temperatures above 70°C. Bacterial lipases generally have temperature optima in the range of 30–65°C. Recently, reports on diverse microbial, moderate thermoactive lipases from mesophiles have been published (Chung et al. 1991; Gilbert et al. 1991; Sugihara et al. 1991, 1992; Shabtai and Daya-Mishne 1992). Several lipases have been purified and characterized from moderate thermophilic isolates, mainly representatives of the genus *Bacillus* (Sugihara et al. 1991; Fakhreddine et al. 1998), such as the lipases from *Bacillus thermoleovorans* ID-1 (Lee et al. 1999), *Bacillus thermocatenulatus* (Schmidt-Dannert et al. 1994, 1996, 1997), *Bacillus stearothermophilus* (Kim et al. 1998, 2000; Sinchaikul et al. 2002), *Bacillus* sp. J33 (Nawani and Kaur 2000) or the lipase from *Bacillus* strain A30-1 (Wang et al. 1995). Several *Pseudomonas* (Sugihara et al. 1992; Lee and Rhee 1993; Ahn et al. 1999) and *Lactobacillus* (Lopes Mde et al. 2002) species have also been reported to produce moderate thermoactive lipases.

Microorganisms living at temperatures above 70°C (extreme thermophiles), however, are an interesting source of stable enzymes (extremozymes). They are in general superior to the traditional biocatalysts, because they produce proteins with unique properties and show reasonable activity even at 100°C and in the presence of organic solvents and detergents (Antranikian 2008). Little, however, is known on the lipolytic enzyme systems of extreme thermophiles, especially from strictly anaerobic bacteria. Their enzymes are expected to be a powerful tool in industrial biotransformation processes (Coolbear et al. 1992; Lasa and Berenguer 1993; Haki and Rakshit 2003).

The ability of *Thermosyntropha lipolytica* gen. nov., sp. nov., to utilize short- and long-chain fatty acids was described (Svetlitshnyi et al. 1996) and the cloning, purification, and characterization of a thermostable esterase from *Thermoanaerobacter tengcongensis* was reported (Zhang et al. 2003). To our knowledge, however, there are no reports on detailed characteristics of lipases from extreme thermophilic anaerobic bacteria.

In this work, we report on the properties of novel recombinant thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* and *Caldanaerobacter subterraneus* subsp. *tengcongensis* (Royter 2006).

Materials and methods

Bacterial strains and plasmids

The strain DSM 7021 *Thermoanaerobacter thermohydrosulfuricus* (basonym: *Clostridium thermohydrosulfuricum*) SOL1 was isolated from a Solar Lake and belongs to the genus *Thermoanaerobacter* (Klingeberg et al. 1990; Lee et al. 1993).

Caldanaerobacter subterraneus subsp. *tengcongensis* (basonym: *Thermoanaerobacter tengcongensis*) was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ) number DSM 15242, Braunschweig, Germany (Fardeau et al. 2004). The genome of *C. subterraneus* subsp. *tengcongensis* has been sequenced (Bao et al. 2002) (GenBank AE008691). The extremely thermophilic anaerobic bacterium, designated strain MB4T, was isolated from a Chinese hot spring in Tengcong (Xue et al. 2001).

The *Escherichia coli* strains used in DNA manipulations were: TOP-10 [F- *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) *φ*80*lac*ZΔM15 Δ*lacX*74 *rec*A1 *deo*R *ara*D139 Δ (*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G] (Invitrogen), NovaBlue [*endA1 hsdR17(rK12 – mK12 +) supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA + B + lacIqZ ΔM15* ::Tn*10*(TcR)] (Novagen) and Tuner^{TM}(DE3)pLacI [F– *ompT hsdS*B(rB – mB –) *gal dcm lacY1* (DE3) pLacI (CamR)] (Novagen). *E. coli* TOP-10 was used in combination with the cloning vector pCR 2.1-TOPO (Invitrogen) suitable for blue/white assays. *E. coli* Tuner^{TM}(DE3)pLacI and NovaBlue were used in combination with vector pETBlue-1 (Novagen) containing the T7 promoter to clone and express the lipase gene.

Media and culture conditions

The basal medium for cultivation of *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* was prepared by the modified Hungate technique (Balch and Wolfe 1976). Medium for cultivation of the thermophilic anaerobic strains contained (per liter): NaCl 3.0 g; KH_2PO_4 2.5 g; NaH_2PO_4 0.8 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; $CaCl_2{\cdot}2H_2O$ 0.05; $FeCl_3{\cdot}6H_2O$ 0.01 g; $(NH_4)_2SO_4$ 1.5 g; $SrCl_2{\cdot}6H_2O$ 0.03 g; H_3BO_3 0.03 g; Na_2WO_4 0.03 g; yeast extract 1.5 g; peptone 1.5 g; trace element solution (×10) [according to medium 141 (DSMZ 1998)] 1 ml; vitamin solution (×10) (according to medium 141) 1 ml; resazurin 0.001 g; $NaHCO_3$ 1.0 g; cysteine 0.3 g; pH 7.2.

Prior to inoculation, 1 mg $Na_2S{\cdot}9H_2O$ was added to 20 ml of medium and followed by the addition of 0.05 g $Na_2S_2O_3$.

For lipase production, the thermophilic anaerobic strains were cultivated on a rotary shaker (160 rpm) for 32 h at

65°C in 50-ml bottles under anaerobic conditions containing 20 ml of the corresponding liquid medium. All *E. coli* strains were cultivated in Luria–Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl per liter of deionized water, pH 7.0] at 37°C. Carbenicillin, when added, was used at a final concentration of 50 µg/ml.

Recombinant DNA techniques

DNA manipulations were performed as described by Sambrook et al. (2001), unless otherwise stated. Restriction enzymes and other DNA-modifying enzymes were used according to the manufacturer's (Fermentas International Inc, Canada) recommendations. Genomic DNA was extracted from *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* using Qiagen column technology (Ausubel et al. 1987). *E coli* cells were transformed by "heat shock" method. Small-scale purification of plasmid DNA was performed using Qiagen column technology (Birnboim and Doly 1979; Birnboim 1983; Kraft et al. 1988). DNA fragments were isolated from agarose gels using a QIAquick gel extraction kit (Qiagen, USA), according to the manufacturer's instructions.

Sequencing and analysis of the genes

To determine the sequences of lipase gene of *T. thermohydrosulfuricus*, the PCR products and vectors with inserts were sequenced by SeqLab (Sequences Laboratories), Göttingen, Germany. The DNA and deduced amino acid sequences and chromatogram readouts were analyzed using the sequence analysis software VectorNTI and Chromas 1.45 (shareware).

Sequence and database similarity searches were done using the server at the National Center for Biotechnology Information, Bethesda, MD (http://www.ncbi.nlm.nih.gov). Analysis of the lipase genes was done with the Expasy Molecular Biology Server (http://www.expasy.org/tools). Sequence alignments were generated using ClustalW and Pairwise BLAST. Open reading frame analysis, translations, restriction mapping, polarity, hydrophobicity, and isoelectric point prediction were analyzed with the VectorNTI software (Infor-Max Inc., Oxford, UK).

Cloning and expression of the *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* lipase genes

The N-terminal sequence from *T. thermohydrosulfuricus* was compared with those of various known lipases from bacteria deposited in EMBL 29.0 and Swissprot 20.0 databases. On the basis of the best hits of the BLAST analysis, twelve various oligonucleotide primers were synthesized (Table 1a). Using these primers in all possible combinations, different internal fragments of the *T. thermohydrosulfuricus* DNA were amplified by PCR.

The PCR-products were sequenced and compared with other known hydrolase sequences. The DNA fragments identified as parts of hydrolase gene were completed using inverse-PCR techniques, in order to be able to express it actively in *E. coli*. The genomic DNA (~1.4 µg) was digested into small fragments with restriction enzymes BamHI and HindIII. The DNA-fragments were ligated and

Table 1 Oligonucleotides used for PCR-screening and inverse PCR

Number	Primer	Sequence (5′–3′)	Function
(a) PCR-screening			
1F	LF/NT/CTT	CTTAAGGGGGATGTTGCATCTTC	Forward
2F	LF/NT/ATT	ATTAAGGGGGGTACTGCATCTG	Forward
3F	LF/OAH/CAT	CATGGGTTTACCGGAAATAAAGTGG	Forward
4F	F/CRI/TTC	TTCAGGCGAAAGCGACGGAG	Forward
5F	F/CRI/GGA	GGAACAGGTGAAAGTGATGGAGAATT	Forward
6F	F/CRI/GCG	GCGGTGAAAGTGATGGAGACTTT	Forward
7R	R/CRI/TCC	TCCGTCGCTTTCGCCTGAAC	Reverse
8R	R/CRI/AAA	AAATTCTCCATCACTTTCACCTGTTCC	Reverse
9R	R/CRI/TCT	TCTCCATCACTTTCACCGCTG	Reverse
10R	R/CRII/CAA	TCCTCCCATGCTGAGTCCCAA	Reverse
11R	R/CRII/AAG	TCCTCCCATGCTGAAGCCAAG	Reverse
12R	R/CTI/TTT	TTTTGTATGGTCCGCTCCTTCTAT	Reverse
(b) inverse PCR			
	1F_Inv2Tth	GACATTTAGCAGTGAATTGGAAGATGC	Forward
	2F_Inv2Tth	TTTGTGAAAGAGCCTACGACTGACC	Forward
	3R_Inv2Tth	GCACTTTACCCTTAACATCATCAGGC	Reverse
	4R_Inv2Tth	GACTCTACTTTATTGCCTGTAAAACCG	Reverse

the circular DNA-fragments were used as templates for amplification of lipase fragments using specific primers (Table 1b).

The program ContigExpress™ (Vector NTI®, software package for Mac OS users developed by InforMax, Inc., North Bethesda, MD, USA) was used for analysis of the sequences and to complete the lipase gene.

AccepTor Vector Kit (Novagen) was used for IPTG-inducible expression of lipase genes in *E. coli* under the control of the T7lac promoter in pETBlue-1 vector. Purified genomic DNAs from *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* were used as templates for amplification of complete lipase genes. The primers used for amplification of the *T. thermohadrosulfuricus* lipase gene were LipTth-for (5′-ATGCAAAAGG CT-GTTGAAATTAC-3′) and LipTth-rev (5′-TTATCCC TTTAACAATTCCTTTTTG-3′). The *C. subterraneus* subsp. *tengcongensis* lipase gene was amplified using constructed primers LipCs-for (5′-ATGCAGAAGGCTG TAGAGTTTAC-3′) and LipCs-rev (5′-TTATCCCTT-T AATTCTCTTTCAAAG-3′).

In the expression phase of the experiment, the clone containing lipase gene in reverse orientation was used as a negative control. 5 ml of starter culture of the pETBlue-1 recombinant in an *E. coli* (DE3) pLacI expression host strain was grown in LB medium with 50 μg ml^{-1} of carbenicillin, 34 μg ml^{-1} of chloramphenicol and 1% glucose. 200 ml medium inoculated with starter culture was incubated to an OD_{595} of 0.9. The cells were induced with 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG), and cultures were incubated with shaking at 37°C for 4 h for full induction.

E. coli clone containing *T. thermohydrosulfuricus* lipase gene was cultivated in a 2-l fermentor (Bioengineering, Switzerland) with a working volume of 1.5 l for 6 h at 37°C, agitation at 1000 rpm and aeration (30 l h^{-1}).

Lipase assay

Positive recombinant clones isolated from triolein plates were cultivated in Luria–Bertani medium and assayed for lipase activity after 24 h incubation.

Spectrophotometric assay with p-nitrophenyl palmitate as substrate

Cleavage of *p*-nitrophenyl palmitate (Sigma, USA) was determined at 70°C in 0.025 M Tris–HCl buffer, pH 8.0, according to Winkler and Stuckmann (1979). A buffered *p*-nitrophenyl palmitate emulsion was sonicated for 2 min at room temperature before the kinetic measurement was started by the addition of the enzyme. A blank absorption at 410 nm was measured immediately after enzyme addition. All values were determined in triplicates and corrected for autohydrolysis using an extinction coefficient of $\varepsilon = 12750\ M^{-1}\ cm^{-1}$. The activity toward other *p*-nitrophenyl esters was measured in the same manner, by using 1 mM of each substrate. One unit (1 U) of lipase activity is defined as the amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol per minute at the conditions described above.

Spectrophotometric assay with olive oil as substrate

In order to determine pH optimum of the enzymes a modified assay was used (Schmidt-Dannert et al. 1994). The hydrolytic activity of the lipase was measured by the spectrophotometric assay at 430 nm using the formation of copper soaps for the detection of free fatty acids. Enzyme reaction was carried out under shaking for 90 min at 70°C. One unit (1 U) of lipase activity is defined as the amount of enzyme needed to liberate 1 μmol of free fatty acids per minute at the conditions described above.

Purification of recombinant lipases

After induction *E. coli* cells were harvested by centrifugation at 10000*g* at 4°C for 30 min. All purification procedures were performed at room temperature. After harvesting, the cells were washed twice with 50 mM Tris–HCl buffer pH 8.0, resuspended in 10 ml of this buffer and stored at −20°C for 1 h. After thawing, lysozyme was added at a concentration of 100 mg/ml and Triton-X 100–0.1%. The cells were incubated in the lysing reagents for 1 h on ice. DNAse was added to 100 mg/ml to one set and incubated at 37°C for 30 min. After centrifugation the cell free extract was heated at 60°C for 30 min. Proteins were precipitated and separated by centrifugation for 20 min at 10000*g*. NaCl was added to a concentration of 1 M, and 20 ml of this supernatant were loaded on a 35-ml phenyl sepharose high performance hydrophobic column (20.0 × 2.6 cm, packed with 60 ml 6F high substituted resin) (Pharmacia, Sweden) preequilibrated with 50 mM Tris–HCl buffer pH 8.0 containing 1 M NaCl. The protein was eluted with a linear reverse gradient from 1 to 0 M NaCl at a flow rate of 1 ml min^{-1}. Lipase containing fractions were pooled and dialyzed against 3 l of standard buffer (pH 8.0). The protein solutions were concentrated by ultrafiltration through a 10-kDa cut-off membrane (Amicon). The samples were then fractionated on a HiLoad 16/60 Superdex 200 preparative grade column (160 × 16 cm) (Pharmacia, Sweden) preequilibrated with standard buffer (pH 8.0) containing 1% NaCl and 1% DMSO. The flow rate was adjusted to 1 ml/min. Protein fractions with lipase activity were collected and dialyzed against standard buffer (pH 8.0). The purified enzyme was stored at +4°C. Protein concentration was measured according the method of

Bradford (1976). Bovine serum albumin was used as standard.

Gel electrophoresis

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel according to the method of Laemmli (1970) after the samples had been heated at 95°C for 5 min. The low molecular markers (Amersham, UK) used were: phophorylase b (97 kDa), albumin (66 kDa), ovalbumin 45 kDa), carbonic anhydrase (830 kDa), trypsin inhibitor 20 kDa), a-lactalbumin (14.4 kDa). Proteins were visualized in the gels by staining with Coomassie brilliant blue R-250 (Sigma, USA). The zymogram staining with α-naphthyl acetate for lipolytic activity was performed according to the method of Khalameyzer et al. (1999). When activity staining was performed, the sample applied to the gel was not boiled, and after electrophoresis SDS was removed by washing the gel with 0.5% Triton X-100 for 20 min and with distilled water for 10 min.

Influence of pH and temperature

The lipase activity was measured at a pH range from 4.0 to 12.0 using various buffers (100 mM). All activity assays were performed at 70°C. To study the pH stability of the enzyme, the lipase was incubated for 1 and 2 h at 30°C in the following buffers (100 mM): acetic acid/sodium acetate (pH 4.0–5.5), potassium phosphate (pH 5.0–8.0), Tris/HCl (pH 7.5–9.0) and glycine/NaCl/NaOH (pH 9.0–13.0) without substrate. To determine the influence of the temperature on the enzymatic activity, samples were incubated at temperatures from 30 to 95°C for 15 min. Thermostability was investigated after incubation of the samples at different temperatures from 70 to 90°C and pH 8.0 at various time intervals.

Effect of various compounds on lipase activity

The effects of various substances (metal ions, inhibitors, organic solvents and surfactants) on lipase activity were examined using the spectrophotometric assay with *p*-nitrophenyl palmitate as substrate. The purified lipase was preincubated with various reagents in different concentrations at 30°C for 90 min without substrate, and residual lipase activity was measured using *p*-nitrophenyl palmitate.

Substrate range

The enzyme specificity was studied with *p*-nitrophenyl alkanoate esters of varying alkyl chain lengths from C2 to C18 compared with *p*-nitrophenyl palmitate as substrate. The reaction was carried out at 70°C for 15 min. The lipase specificity was tested toward the following triglycerides: triacetin (C2:0), tributyrin (C4:0), tricaproin (C6:0), tricaprylin (C8:0), tricaprin (C10:0), trilaurin (C12:0), trimyristin (C14:0), tripalmitin (C16:0), tristearin (C18:0), triolein (C18:1), compared with olive oil as substrate. Incubation was performed at 70°C for 24 h.

Further substrates tested were: *p*-nitrophenyl esters of the following: benzoate, 2-(4-isobutylphenyl) propanoate (ibuprofen), 2-phenylpropanoate, 3-phenylbutanoate, cyclohexanoate, 2-(3-benzoylphenyl) propanoate (ketoprofen), 2-naphthoate, 1-naphthoate, adamantanoate and 2-(6-methoxynaphthalene-2-yl) propanoate (naproxen). The reaction was carried out at 70°C for 40 min. The reaction mixture contained 950 µl of buffer (100 mM Tris–HCl, pH 7.5), 50 µl substrate (5 mg/ml in DMSO) and 0.3 µg of lipase. Activity was determined at 410 nm after 40 min of incubation at 75°C.

Determination of catalytic activity and enantioselectivity by GC analysis

The enantioselectivity of the lipase from *T. thermohydrosulfuricus* toward racemic compounds was studied with 8 substrates. 6 of them were acetates, one a butyrate of a secondary alcohol and one an acetate of a tertiary (3-phenyl-but-1-in-3-yl-acetate) alcohol. The general procedure for the preparation of the acetates **1–3** and **5–6** was already described (Musidlowska-Persson and Bornscheuer 2002; Schmidt et al. 2005). (*R*,*S*)-racemic substrates were dissolved in sodium phosphate buffer (50 mM, pH 7.5) giving 1 ml of a 10 mM solution. The solution was mixed by vortex for 2 min. The hydrolysis was carried out in 1.5-ml reaction vials in a thermomixer (Eppendorf) at 70°C. 0.1 U of the *T. thermohydrosulfuricus* lipase (based on the spectrophotometric *p*-NPP assay) was used for each reaction. The samples were taken after 2, 4, 16, 24, and 40 h. Reactions were terminated by extraction with methylene chloride and the organic phases were dried over anhydrous sodium sulfate. For the detection of enantiomeric purity and conversion, gas chromatography was used (gas chromatograph, GC-14A, Shimadzu). The following conditions were used for the GC analyses: injection temperature 200°C, detection temperature 200°C, column (Heptakis-(2,6-*O*-methyl-3-*O*-pentyl)-*β*-cyclodextrin, 25 m × 0.25 mm), carrier gas: H_2, flame ionization detector (FID), temperature 110°C (isothermal) for 1-phenyl-1-ethyl acetate (**1**), 1-phenyl-2-pentyl acetate (**2**), 1-phenyl-2-butyl acetate (**3**), and 2-phenyl-but-1-in-3-yl acetate (**4**), column temperature 120°C (isothermal) for 1-phenyl-2-butyl acetate (**5**) and 1-phenyl-2-propyl acetate (**6**), column temperature 40°C (isothermal) for but-3-yn-2-ol acetate (**7**) and but-3-yn-2-ol butyrate (**8**) (Schmidt et al. 2006). The volume of the injected sample was 0.1 µl. With

the help of the chromatogram the enantioselectivity (E) and conversion (c) were calculated according to Chen et al. (Schmidt et al. 2006).

Other esters (listed in Table 6) were incubated while the lipase from *T. thermohydrosulfuricus* in a mixture of 400 µl buffer (125 mM Tris–HCl, pH 7.5), 50 µl substrate (10 mg/ml in Acetonitrile) and 3 µg enzyme at 30°C. Sample were taken after 1 h, 24 h and 2 days (500 µl each), extracted with 300 µl dichloromethane and analyzed by GC. The regioisomers of pentan-1,4-diyl diacetate-hydrolysis were resolved using a DB-Wax column [Hewlett-Packard, Palo Alto, CA, USA, 30 m (length), 0.32 mm (diameter), 0.25 µm (film thickness)]. The relative activity was calculated on the basis of initial rates.

Results

Sequence analysis of lipases from *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis*

Sequences of the N-terminal amino acid (17 amino acids) of the lipase from *T. thermohydrosulfuricus* indicated high identity to the sequences of the hydrolase from *C. subterraneus* subsp. *tengcongensis* (NP 623397.1). Based on these identities, the hydrolase sequence *C. subterraneus* subsp. *tengcongensis* was compared to in NCBI BLAST database. Highest identity was obtained to hydrolases from *Clostridium acetobutylicum* and *Deinococcus radiodurans*. 12 different oligonucleotide primers were synthesized and used to amplify the gene of *T. thermohydrosulfuricus* by PCR (Table 1a). The 142-bp fragment was amplified and had 84% identity to the nucleotide sequence of the hydrolase α/β superfamily from *C. subterraneus* subsp. *tengcongensis* (NP 623397.1). On the basis of this sequence, specific oligonucleotide primers were synthesized (Table 1b) to obtain the complete gene sequences of the lipase from *T. thermohydrosulfuricus* shown in Fig. 1. Nucleotide sequencing of this fragment revealed the presence of a unique open reading frame (ORF), starting at a putative ATG start codon and being 780-bp long. The lipase from *T. thermohydrosulfuricus* exhibits a low $G + C$ content (36.15%). Preceeding the ATG start codon (6 bp upstream), a potential ribosome-binding site Shine-Dalgarno sequence (5′-GGAGG-3′) was found. The deduced protein encoded by the lipase from *T. thermohydrosulfuricus* is composed of 259 residues, with a predicted molecular mass of 29161 Da and a pI of 5.41. 35% of the amino acids are hydrophobic, 18% polar, 16% are acidic, and 13% are basic. The N-terminus of this protein contains no signal peptide (SignalP V3 program).

Cloning and expression of the *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* lipase genes

A 780-bp fragment containing the *T. thermohydrosulfuricus* lipase encoding gene (Fig. 1) and a 777-bp ORF

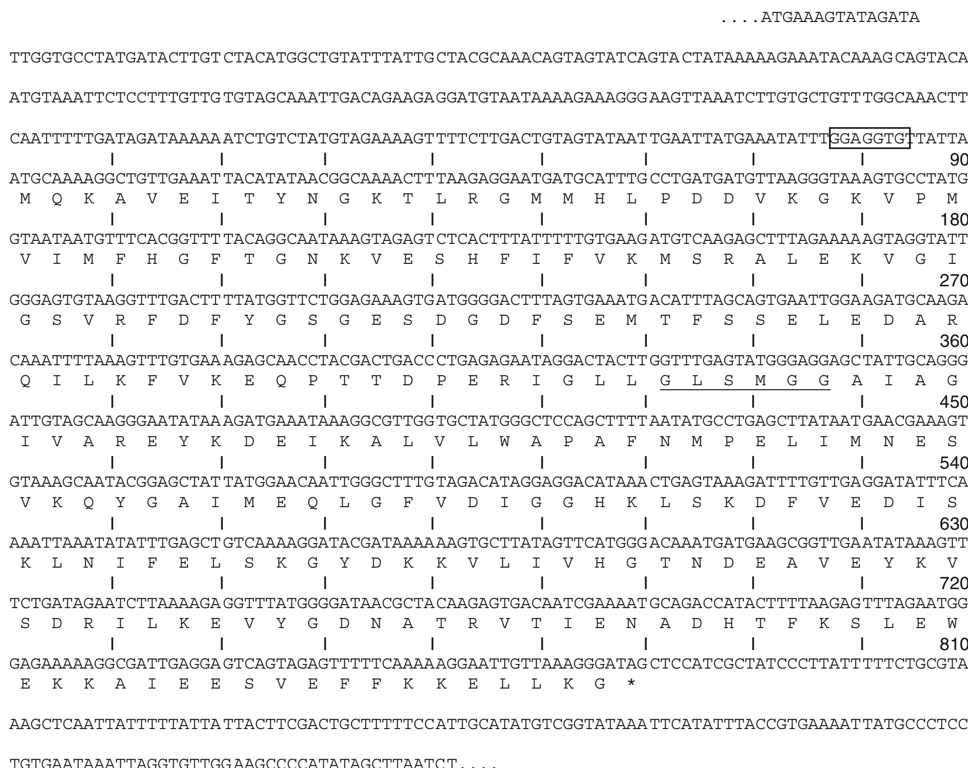

Fig. 1 Nucleotide sequence of the lipase from *T. thermohydrosulfuricus* and open reading frame translation. Only the coding strand for Lipase1 is shown. A putative ribosome-binding (Shine-Dalgarno) site is *boxed*. An active center containing the putative active-site serine is *underlined*

(AE013133) encoding the *C. subterraneus* subsp. *tengcongensis* hydrolase (NP 623397.1) were amplified by PCR with specific primers (LipTth-for and LipTth-rev) and (LipCst-for and LipCst-rev), respectively (Royter 2006). Purified DNAs from *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis* were used as template. *T. thermohydrosulfuricus* lipase encoding gene containing the ATG start codon was amplified without modification. A GTG start codon in the *C. subterraneus* subsp. *tengcongensis* hydrolase encoding gene, however, was replaced with the *E. coli*-specific ATG start codon at the 5′ end using special constructed primers. The purified PCR products with single 3′-dA overhangs were ligated into containing T7lac promoter linearized expression vector pETBlue-1 (Novagen, USA) designed for IPTG-inducible expression of target genes.

For initial expression studies in *E. coli*, plasmids pETBlue-1 containing complete lipase genes were constructed and transformed into the expression host strains Tuner™(DE3)pLacI. Expression was induced by the addition of Isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture had attained late log phase ($OD_{600} = 0.9$). The expression was optimized by testing various IPTG concentrations (0.4, 0.6, 0.8, 1.0, 1.5, and 2 mM). A halo around the colonies with lipase activity was observed on tributyrin plates. Expression levels were measured by SDS-PAGE (data not shown). Lipase activities were tested in cell-free supernatant and in cell lysate with *p*-nitrophenyl palmitate as substrate. The best expression (53–59 U/l) was achieved with 1 mM IPTG for 4 h. The enzyme level increased dramatically when cultivation was performed in a 2-l fermentor at the optimal process parameters. The production of the recombinant lipase of *T. thermohydrosulfuricus* by *E. coli* Tuner (DE3) was increased 42-fold (from 53 to 2200 U/l).

Purification of recombinant lipases

The recombinant lipases were purified by employing a three-step procedure: heat precipitation of cell free extract, hydrophobic interaction chromatography and gel filtration (Table 2). On phenyl sepharose column the enzymes were eluted at NaCl concentration between 0.90 and 0.95 M. The final gel filtration resulted in one peak of the active protein and in electrophoretically homogeneous preparations. The *T. thermohydrosulfuricus* lipase was purified 108.7-fold with 13% recovery and *C. subterraneus* subsp. *tengcongensis* lipase 93.6-fold with 8.1% recovery. The specific activity of the recombinant enzymes ranged from 10.90 to 12.15 U/mg. The purified enzymes showed on SDS-PAGE single bands with molecular weight of 34.2 kDa for *T. thermohydrosulfuricus* and 32.1 kDa for *C. subterraneus* subsp. *tengcongensis* (Fig. 2).

Physicochemical properties

The purified recombinant lipases exhibited maximum activity at a temperature of 75°C (Fig. 3) at pH 8.0. Both lipases were stable without significant loss of activity for 24-h incubation at temperatures up to 70°C (Fig. 4). After incubation at 85°C for 50 min, 90% of the lipase activity of *T. thermohydrosulfuricus* was measured.

The enzyme from *C. subterraneus* subsp. *tengcongensis* is less thermostable so that after 10-min incubation at 85°C, around 20% of residual activity was detected. The half-lives of the *T. thermohydrosulfuricus* lipase at 90°C is 50 min and of the *C. subterraneus* subsp. *tengcongensis* is 6 min. The lipases were stable to freezing (−20°C) and thawing. After first cycle of freezing and thawing, the enzymes showed 90% of residual activity and after second cycle the remaining activity was 85%.

Table 2 Purification of the recombinant lipases from *T. thermohydrosulfuricus* and *C. subterraneus* subsp. *tengcongensis*

	Total protein (mg)	Total activity[a] (U)	Specific activity[a] (U/mg)	Yield (%)	Purification (-fold)
(a) *Thermoanaerobacter thermohydrosulfuricus*					
Crude extract[b]	233.60	26.1	0.11	100.0	1.0
Heat precipitation	18.20	25.3	1.39	96.9	12.4
Phenyl sepharose	2.30	12.6	5.48	48.3	49.0
Superdex 200	0.28	3.4	12.14	13.0	108.7
(b) *Caldanaerobacter subterraneus*					
Crude extract[b]	254.70	29.7	0.12	100.0	1.0
Heat precipitation	15.40	19.2	1.25	64.6	10.7
Phenyl sepharose	2.10	8.9	4.24	30.0	36.3
Superdex 200	0.22	2.4	10.91	8.1	93.6

[a] Activity was determined photometrically with *p*-nitrophenyl palmitate (0.7 mM, pH 8.0) as substrate. Reactions were carried out at 70°C for 10 min

[b] 5 g of cells (*E. coli*) were resuspended in 10 ml 50 mM Tris–HCl buffer pH 8.0

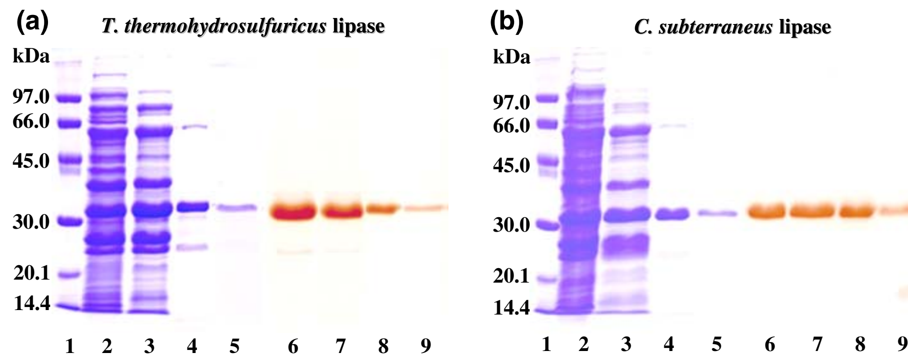

Fig. 2 SDS-PAGE analysis and zymogram of samples from purification steps of the recombinant lipases: **a** LipTth (*T. thermohydrosulfuricus)* and **b** LipCst (*C. subterraneus* subsp. *tengcongensis*). On the *left panel*, proteins were detected with coomassie blue (0.1%). The *right panel* shows a zymogram with α-naphthyl acetate. *Lane 1* molecular markers (2 μl); *lanes 2* and *6* cell-free extract (12 μg); *lanes 3* and *7* heat precipitation pool (10 μg); *lanes 4* and *8* phenyl sepharose pool (8 μg); *lanes 5* and *9* superdex pool (5 μg)

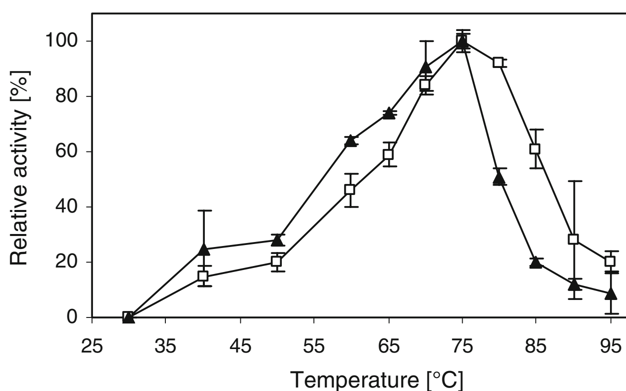

Fig. 3 Temperature profile of the recombinant lipases from *T. thermohydrosulfuricus* (*filled triangle*) and *C. subterraneus* subsp. *tengcongensis* (*opened square*). Enzyme activity of the recombinant lipases was determined over a temperature range from 30 to 95°C. The substrate mixture [0.7 mM *p*-nitrophenyl palmitate, 50 mM Tris–HCl pH 8.0, 0.1% (w/v) gum Arabic] was prewarmed prior to addition of 6 mU of the enzyme. Reactions were carried out for 10 min. Reaction was terminated by placing the samples on ice and by addition of Na_2CO_3 to a final concentration of 10 mM. Samples were centrifuged for 2 min at 9400*g*. Photometrical measurements at wavelength 410 nm were done in triplicates and corrected for autohydrolysis of the substrate

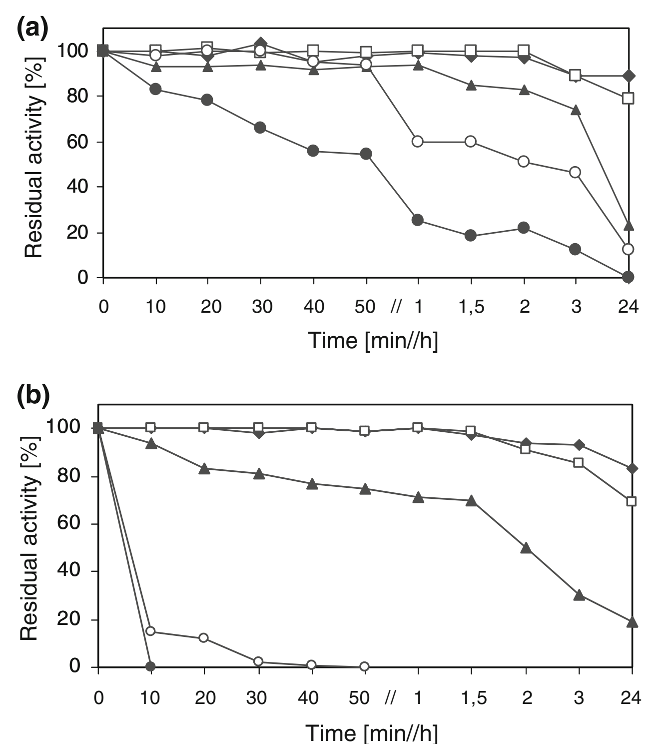

Fig. 4 Effect of temperature on stability of the recombinant lipases from *T. thermohydrosulfuricus* (**a**) and *C. subterraneus* subsp. *tengcongensis* (**b**). Thermostability of the recombinant lipases was determined by preincubation of the lipases at pH 8 and different temperatures 70°C (*filled diamond*), 75°C (*opened square*), 80°C (*filled triangle*), 85°C (*opened circle*) and 90°C (*filled circle*) for various time intervals up to 24 h. Standard assays with 0.7 mM *p*-nitrophenyl palmitate were conducted at pH 8 for 10 min at 70°C to determine the residual enzyme activity. Reaction was terminated by placing the samples on ice and by addition of Na_2CO_3 to a final concentration of 10 mM. Photometrical measurements at 410 nm were done in triplicates and corrected for autohydrolysis of the substrate

The purified *T. thermohydrosulfuricus* lipase is active over a broad range of pH and showed maximum activity at pH 8.0 and above 80% of activity at pH of 6.5 and 9.0 (Fig. 5). The *C. subterraneus* subsp. *tengcongensis* lipase showed an optimum activity at pH 7.0 and above 60% of activity at pH 6.5 and 9.0 (Fig. 5). The enzymes were completely stable in a pH range between 7.5 and 12 for 2 h.

Effects of different reagents

The effect of various compounds is shown in Table 3. The following metal ions up to 10 mM did not have any influence on the enzymes: Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Ag^{+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Rb^{+}, Co^{2+}, Ni^{2+}, and Al^{3+}. In contrast, Zn^{2+}, Fe^{3+}, and Cr^{3+} ions were inhibitory. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) has a slight activating effect on both enzymes. A decrease in

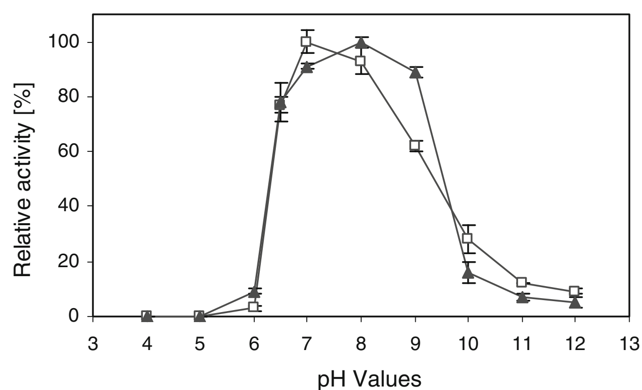

Fig. 5 Effect of pH on activity of the recombinant lipases from *T. thermohydrosulfuricus* (*filled triangle*) and *C. subterraneus* subsp. *tengcongensis* (*opened square*) was determined over a pH from 4 to 12 using 40 mM universal buffer. The substrate mixture [10 mM tripalmitin, 50 mM Tris–HCl pH 8.0, 0.1% (w/v) gum Arabic] was prewarmed prior to addition of 120 mU of the enzyme. Reactions were carried out at 70°C for 1 h. Measurements were done in triplicates

enzyme activity was observed after incubation with Tween-20, Tween-80, Triton X-100 or SDS. The two lipases showed high activity in the presence of various solvents such as *tert*-butanol, acetonitrile, isopropanol, pyridine, and acetone up to a concentration of 99% (v/v). In contrast, DMSO (dimethyl sulfoxide), benzene, toluol, amylalcohol, and methanol at a concentration of 99% (v/v) caused a significant reduction in enzyme activity (Table 3).

β-Mercaptoethanol, DTT (dithiotreitol), guanidine hydrochloride, Urea, pHMB (*p*-hydroxymercuribenzoate), and iodo-acetate showed no effect. From the deduced amino acid sequences, it was suggested that these lipases harbor a catalytic triad consisting of Ser, His, and Asp, and accordingly they were inhibited by PMSF (phenylmethylsulfonyl fluoride) and pefablock (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) (Table 3).

Substrate specificity

The recombinant lipases were active on a wide range of substrates. First, the specificity of the enzymes toward the length of different acyl chains of *p*-nitrophenyl esters was investigated (Table 4a). The enzymes were active on *p*-nitrophenyl esters of carboxylic acids of medium chain length (C6–C14). For both enzymes, *p*-nitrophenyl caprate (C10) was the most suitable substrate among the *p*-nitrophenyl esters examined. Both lipases exhibited very low levels of activity (<10%) toward the short- (C2) and long-chain (C18) substrates. The lipases from *T. thermohydrosulfuricus* and from *C. subterraneus* subsp. *tengcongensis* were able to hydrolyze further *p*-nitrophenyl ester substrates (Table 4b). Neither enzyme was able to hydrolyze *p*-nitrophenyl 2-naphthoate. Unlike the enzyme from *C. subterraneus* subsp. *tengcongensis* the lipase from *T. thermohydrosulfuricus* was active toward 4-nitrophenol cyclohexanoate but not with *p*-nitrophenyl-1-naphthoate or *p*-nitrophenyl adamantanoate. Accordingly, the described lipases displayed different substrate spectra (Table 4b).

Both lipases showed high activities in the presence of the triacylglycerols with chain lengths of C6 and C8 (80 and 100%) and lower activity with C16 (30%) (Table 4c).

Enantioselectivity

In order to determine the stereoselectivity of the thermoactive lipase from *T. thermohydrosulfuricus*, the substrates shown in Table 5 were investigated. With (*R*,*S*)-but-3-yn-2-ol butyrate and (*R*,*S*)-but-3-yn-2-ol acetate, the lipase showd (*S*)-preference and catalyzed the synthesis of the building block (–)-but-3-yn-2-ol. After 2-h incubation, the enzyme exhibited the highest enantioselectivity and an *E* value of 21 toward the butyrate was determined.

As shown in Table 5 the increase in conversion rate was accompanied with a decrease in the *E* value. The *T. thermohydrosulfuricus* lipase showed higher preference to (*S*)-enantiomers; but over time, their ability to distinguish between the two enantiomers decreased. For two other substrates, 1-phenyl-2-propyl-acetate and 1-phenyl-1-ethyl-acetate, the enantioselectivities of the enzyme where very low ($E \geq 1$) but constant over time.

Furthermore, a broad range of esters was tested, and it was found that the lipase from *T. thermohydrosulfuricus* converted preferentially esters of secondary alcohols rather than esters of primary alcohols (Table 6).

Discussion

Microorganisms that thrive in extreme habitats especially at elevated temperatures (70–100°C) are able to produce thermoactive enzymes that in general show high catalytic activity at the optimal growth conditions. Few moderate thermophilic strains (50–60°C), especially representatives of the genus *Bacillus,* are able to produce extracellular enzymes, e.g., proteases that are even active at temperatures above their growth optimum (Schmidt-Dannert et al. 1994, 1997; Kambourova et al. 1996; Lee et al. 1999; Markossian et al. 2000; Nawani and Kaur 2000). Regarding lipases, however, there are few reports on the production of such enzymes by extreme thermophilic microorganisms (70–80°C), especially strict anaerobes. The thermophilic bacterium *Thermosyntropha lipolytica* gen. nov., sp. nov., is able to utilize short- and long-chain fatty acids indicating its ability to produce lipase (Svetlitshnyi et al. 1996). The thermophilic bacterium *T. tengcongensis* produces an esterase, which is inactive toward olive oil and shows very low activity toward long chain *p*-NP esters (C16) (Zhang

Table 3 Effects of various compounds on the lipase activity

Compounds	Concentration	Relative activity (%) on lipases from	
		T. thermohydrosulfuricus	*C. subterraneus* subsp. *tengcongensis*
None	–	100	100
NaCl	10 mM	102	105
KCl	10 mM	101	95
$CaCl_2$	10 mM	95	94
$CuCl_2$	10 mM	102	100
$AgNO_3$	10 mM	98	99
$MgCl_2$	10 mM	100	102
$MnCl_2$	10 mM	88	103
$SrCl_2$	10 mM	91	90
$RbCl_2$	10 mM	101	102
$CoCl_2$	10 mM	91	94
$NiCl_2$	10 mM	84	96
$AlCl_3$	10 mM	97	61
$ZnCl_2$	10 mM	47	20
$FeCl_3$	10 mM	50	71
$CrCl_3$	10 mM	18	16
CHAPS	10% (w/v)	129	111
Polyvinyl alcohol	10% (w/v)	94	114
Tween-80	10% (v/v)	22	8
Tween-20	10% (v/v)	11	21
Triton X-100	10% (v/v)	11	12
SDS	10% (w/v)	0	0
Tert-butanol	99% (v/v)	113	118
Ethanol	99% (v/v)	76	127
Acetonitrile	99% (v/v)	110	100
Isopropanol	99% (v/v)	96	111
Pyridine	99% (v/v)	93	97
DMSO	50% (v/v)	106	96
	99% (v/v)	0	1
Acetone	99% (v/v)	105	104
Dimethylformamide	99% (v/v)	80	49
Methanol	99% (v/v)	56	50
Hexadecane	99% (v/v)	79	63
Heptane	99% (v/v)	76	71
n-Hexane	99% (v/v)	77	72
Isooctane	99% (v/v)	68	54
Amylalcohol	99% (v/v)	39	51
n-Decyl alcohol	99% (v/v)	34	26
Toluol	99% (v/v)	18	13
Benzene	99% (v/v)	0	4
β-Mercaptoethanol	10.0 mM	106	107
DTT	10.0 mM	103	107
pHMB	1.0 mM	104	106
Guanidine-HCl	10.0 mM	105	100
Urea	100.0 mM	102	116
2-Iodoacetate	10.0 mM	99	97
EDTA	10.0% (w/v)	76	66
PMSF	0.1 mM	21	6
	1.0 mM	2	0
Pefablock	0.1 mM	2	3
	1.0 mM	0	0

Prior to the standard activity assay, the enzymes (6 mU) were preincubated with various reagents at 30°C for 90 min. Activity was determined with *p*-nitrophenyl palmitate (0.7 mM, pH 8.0) as substrate. Reactions were carried out at 70°C for 10 min. Enzyme activity determined in the absence of metal ions, detergents, inhibitors and denaturing agents was defined as 100% activity

Table 4 Substrate specificity of the recombinant lipases

Substrate	Relative activity (%) on lipases from	
	T. thermohydrosulfuricus	*C. subterraneus* subsp. *tengcongensis*
(a)[a] *p*-Nitrophenyl esters		
p-Nitrophenyl acetate (C2:0)	9	8
p-Nitrophenyl butyrate (C4:0)	57	60
p-Nitrophenyl caproate (C6:0)	81	84
p-Nitrophenyl caprylate (C8:0)	90	90
p-Nitrophenyl caprate (C10:0)	100	100
p-Nitrophenyl laurate (C12:0)	84	82
p-Nitrophenyl myristate (C14:0)	68	39
p-Nitrophenyl palmitate (C16:0)	32	30
p-Nitrophenyl stearate (C18:0)	8	9
(b)[b] *p*-Nitrophenyl ester substrates		
p-Nitrophenyl benzoate	10	31
p-Nitrophenyl 2-(4-isobutylphenyl) propanoate	3	0
p-Nitrophenyl 2-phenylpropanoate	28	100
p-Nitrophenyl 3-phenylbutanoate	31	99
p-Nitrophenyl cyclohexanoate	100	0
p-Nitrophenyl 2-(3-benzoylphenyl) propanoate	9	20
p-Nitrophenyl-2-naphthoate	0	0
p-Nitrophenyl-1-naphthoate	0	22
p-Nitrophenyl adamantanoate	5	40
p-Nitrophenyl 2-(6-methoxynaphthalen-2-yl) propanoate	16	0
(c)[c] Triacylglycerols		
Triacetin (C2:0)	5	2
Tributyrin (C4:0)	10	7
Tricaproin (C6:0)	74	34
Tricaprylin (C8:0)	100	100
Tricaprin (C10:0)	15	15
Trilaurin (C12:0)	11	12
Trimyristin (C14:0)	8	10
Tripalmitin (C16:0)	22	29
Tristearin (C18:0)	9	14
Triolein (C18:1)	12	12
Olive oil	10	12

[a] Reactions were initiated by addition of 6 mU lipase and performed with 1 mM *p*-nitrophenyl esters (pH 8.0) as substrates for 10 min at 70°C. The highest activity observed with *p*-nitrophenyl caprate was defined as 100%

[b] Reactions were initiated by addition of 6 mU lipase and carried out with 0.25 mg/ml *p*-nitrophenyl esters (pH 7.5) as substrates for 40 min at 70°C. The highest activity observed with *p*-nitrophenyl cyclohexanoate was set as 100%

[c] Reactions were initiated by addition of 120 mU lipase and performed with 10 mM triacylglycerols (pH 8.0) as substrates for 24 h at 70°C The highest activity observed with tricaprylin was defined as 100%

et al. 2003). The analysis of the robust lipolytic enzyme systems from extreme thermophiles allows a comparative analysis of these enzymes with enzymes from their mesophilic and phsychrophilic counterparts. Comparison of the gene sequences of the heat stable lipases indicate very low identity to already known sequences. The lipase gene from *Thermoanaerobacter thermohydrosulfuricus* has 19% identity with the lipase 3 from the human pathogen *Mycoplasma pneumoniae* (PIR accession number S73694), 17% with the lipase 3 from the psychotropic strain *Moraxella* sp. TA144 (PIR accession number S14276) and 16% with the lipase 1 from the psychrophilic strain *Psychrobacter immobilis* (PIR accession number S57275). Therefore, it is speculative to make any predictions regarding structure–function relationships of enzymes from various groups. However, it was interesting to note that both the studied lipases lack signal peptides that are usually present in enzymes that are secreted by bacteria. It can be speculated that probably another mechanism for enzyme secretion is present in these extremophiles or the signal peptide could not be identified by SignalP V3 program.

Analysis of the deduced amino acid sequence of newly described lipases indicated that these enzymes belong to family V of lipolytic enzymes, described by Agripny and

Table 5 Determination of the stereoselectivity of the lipase from *T. thermohydrosulfuricus* toward secondary and tertiary alcohols

Substrate	Incubation time			
	2 h *C* (%)[a]/*E*[b]	4 h *C* (%)/*E*	16 h *C* (%)/*E*	24 h *C* (%)/*E*
Butinolbutyrate	41/21	53/18	67/16	68/15
Butinolacetate	27/10	39/13	50/16	50/14
1-Phenyl-2-propyl-acetate	5/1	9/1	27/1	30/1
1-Phenyl-1-ethyl-acetate	9/1	12/1	47/1	52/2
1-Phenyl-3-butyl-acetate	–[c]/–	–/–	–/–	–/–
1-Phenyl-2-pentyl-acetate	–/–	–/–	–/–	–/–
1-Phenyl-2-butyl-acetate	–/–	–/–	–/–	–/–
2-Phenyl-but-1-in-3-yl-acetate	–/–	–/–	–/–	–/–

[a] *C* conversion (%), [b] *E* enantiomeric ratio, [c] No product

Table 6 Conversion of esters with different alcohol moieties by the lipase from *T. thermohydrosulfuricus*

Substrate	Relative activity (%)	*E* value
Phenyl acetate	100	
Octyl acetate[a]	1.157	
(*R*,*S*)-2,3-glycerindibutylether acetate[a]	2.179	2.7
(*R*,*S*)-IPG acetate[a]	1.134	1.1
(*R*,*S*)-2-Phenyl-1-propyl acetate[a]	2.761	1.25
(*R*,*S*)-β-Citronellyl acetate[a]	0.627	1.6
(*R*,*S*)-Pentane-1,4-diyl diacetate[abd]	0.716	
(*R*,*S*)-Octane-2-yl acetate[b]	0.455	>200
(*R*,*S*)-Octane-3-yl acetate[b]	0.657	>200
(*R*,*S*)-1-Phenylethyl acetate[b]	0.873	35 (*R*-Enantiomer)
(*R*,*S*)-1-Phenylpropyl acetate[b]	0.313	23
(*R*,*S*)-1-Indanol acetate[b]	3.731	5
(*R*,*S*)-1-Cyclohexylethyl acetate[b]	0.224	110
(*R*,*S*)-Cyclohexyl acetate[b]	0.590	>200
(*R*,*S*)-1-(2-Naphthyl)-ethyl acetate[b]	0.276	5
(*R*,*S*)-3,3-Dimethyl-2-butan-2-yl acetate[b]	0.015	
tert-Butyl octanoate[c]	0	
(*R*,*S*)-3-methyl-3-Nonanol octanoate[c]	0	
(*R*,*S*)-3-methyl-3-Nonanol acetate[c]	0	
(*R*,*S*)-Linalyl acetate[c]	0	
(*R*,*S*)-2-Phenyl-2-butanol octanoate[c]	0	

Reactions were initiated by addition of 120 mU lipase and performed with 1 mg/ml substrate at 30°C. Analysis of the reaction product was done by GC. The highest activity observed with phenyl acetate was set as 100%

[a] primary ester, [b] secondary ester, [c] tertiary ester, [d] pentane-1,4-diyl diacetate is bifunctional

Jaeger (1999). In spite of the low identity, the four conserved regions shown in Fig. 6 are present: the HGF block containing the hydrophobic stretch in front of the lid putative oxyanion hole, the SMGG block containing the putative active-site serine, the G(DK)D block containing the putative active-site aspartic acid, and the GH block with the putative active-site histidine. The enzymes grouped in family V so far originate from mesophilic bacteria (*Pseudomonas oleovorans*, *Haemophilus influenzae*, *Acetobacter pasteurianus*) as well as from cold-adapted bacteria (*Moraxella* sp., *Psy. immobilis*) and from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* (Arpigny and Jaeger 1999). Interestingly, the lid region of the thermostable described lipases contains no tryptophane (W) residue, which is usually located in close contact with the active-site serine (Woolley and Petersen 1994).

Comparison of the 3D structure of lipases from psychrophiles, mesophiles and thermophiles indicates that these enzymes have the same catalytic mechanism with a similar 3D architecture (Vieille and Zeikus 2001). Due to the high sensitivity of cysteine to oxidation at elevated temperatures, its content seems to be reduced in thermoactive enzymes. Both lipases have no cysteines.

The lipase from *T. thermohydrosulfuricus* shows also enantioselectivity in the kinetic resolution of various racemic esters of secondary and tertiary alcohols (Table 5). Several biotransformations using lipases are already performed on an industrial scale (Bornscheuer and Kazlauskas 1999; Bornscheuer et al. 2000), but novel enzymes with high selectivity in combination with high stability under process condition are still needed. The majority of compounds investigated so far are secondary alcohols, because most hydrolases show sufficient enantioselectivity toward these compounds (Theil 1997) and they are important chiral modules for organic synthesis (Bornscheuer and Kazlauskas 1999). Lipase LipTth shows moderate *S*-enantioselectivity toward (*R*,*S*)-but-3-yn-2-ol, which is an important building block (Lesot et al. 1997; Graner et al. 2001; Cappelli et al. 2002; Schmidt et al. 2006). Furthermore, the lipase from *T. thermohydrosulfuricus* is able to convert other industrially relevant substrates. Interestingly, the enzyme shows a high preference for esters of secondary alcohols and a high selectivity for (R) enantiomers of pharmaceutically important substrates as well as *C. antarctica* lipase B (CALB) (Orrenius et al. 1995), Alcaligenes QL (Naemura et al. 1996), *C. rugosa* and *P. cepacia* lipases (PCLs) (Kazlauskas et al. 1991). It is highly enantioselective for (*R*,*S*)-octan-2-yl acetate, (*R*,*S*)-octan-3-yl acetate and (*R*,*S*)-1-(2-naphthyl)-ethyl acetate with *E* value >200. The *E* value for (*R*,*S*)-1-cyclohexylethyl acetate was also synthetically useful with $E = 110$. The enzyme prefers (*R*)-enantiomers of chiral alcohols according to the Kazlauskas-rule (Lesot et al. 1997; Graner et al. 2001; Cappelli et al. 2002; Schmidt et al. 2006).

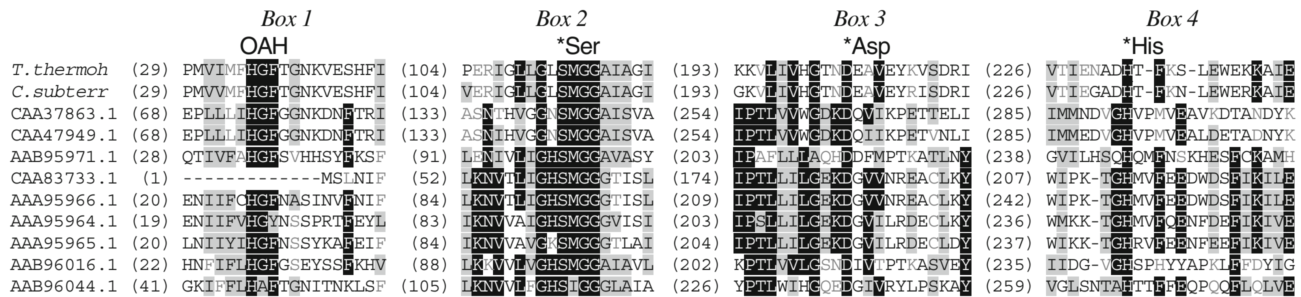

Fig. 6 Amino acid sequence blocks conserved in the deduced amino acid sequence of the *T. thermohydrosulfuricus* lipase and homologous lipases. Multiple amino acid sequence alignments of the *T. thermohydrosulfuricus* lipase, the *C. subterraneus* subsp. *tengcongensis* lipase and their homologs. The accession numbers of the aligned sequences are as follows: CAA37863.1, triacylglycerol lipase from *Moraxella* sp. (S14276, X53869); CAA47949.1, triacylglycerol lipase from *Psychrobacter immobilis* (S57275, X67712); AAB95971.1, triacylglycerol lipase 3 from *Mycoplasma pneumoniae* (strain ATCC 29342) (S73649); CAA83733.1, triacylglycerol lipase T from *Mycoplasma capricolum* (S77776, Z33059); AAA95966.1, triacylglycerol lipase 1 from *Mycoplasma mycoides* subsp. *mycoides* (JC4111); AAA95964.1, triacylglycerol lipase 1 from *Mycoplasma mycoides* subsp. *mycoides* (JC4109); AAA95965.1, triacylglycerol lipase 1 from *Mycoplasma mycoides* subsp. *mycoides* (JC4110); AAB96016.1, triacylglycerol lipase 2 from *Mycoplasma pneumoniae* (strain ATCC 29342) (S73694, AE000035); AAB96044.1, triacylglycerol lipase 3 from *Mycoplasma pneumoniae* (strain ATCC 29342) (S73722, AE000038). The accession numbers are indicated to the *left* of the amino acid sequences. Identical residues have a *black background* and similar residues have a *gray background*. *Amino acids forming a catalytic triad

The finding that the investigated lipases are active at elevated temperatures and high pH (90°C, pH 11), in addition to their resistance against organic solvents (up to 99%) makes these enzymes very attractive for biotransformation processes in water-free media (Jaeger et al. 1994; Kirk et al. 2002; Bommarius and Riebel 2004).

In summary, we were able to identify, clone and functionally express two novel lipases showing very high thermoactivity and stability. Moreover, both enzymes accept a very broad range of esters in hydrolysis and show good to excellent stereoselectivities in the kinetic resolution of a broad set of synthetically useful compounds important in organic synthesis.

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