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Highly enantioselective recombinant thermoalkalophilic lipases from *Geobacillus* and *Bacillus* sp

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Abstract—Thermoalkalophilic lipases from *Geobacillus* and *Bacillus* strains have been cloned and characterised, several of which show excellent activity and enantioselectivity towards various substrates. The hydrolysis of methyl 2-methyldecanoate proceeds to give (*R*)-2-methyldecanoic acid in good yield and >99% enantiomeric excess (*E* >200).

1. Introduction

Enzymes such as hydrolases play an important role in biotechnology because of their extreme versatility with respect to substrate specificity and stereoselectivity.[1,2] The use of lipases as catalysts for enantioselective organic reactions is often limited by unacceptably low selectivities. Lipases are versatile enzymes that can provide an environmentally friendly means of breaking down fats for environmental clean-up and the production of value-added products. They can also be used as biocatalysts in sophisticated reactions in chemistry for the pharmaceutical industry.[3] Lipases catalyse three types of reactions: hydrolysis, esterification and acyl transfer. The hydrolytic reaction mode is frequently used for the preparation of both carboxylic acids and alcohols in, for example, the lipase-catalysed hydrolysis of a racemic carboxylic ester. One significant application of lipases in organic chemistry is the production of optically active compounds that may be used in sophisticated procedures for the synthesis of pharmaceuticals. Lipases are useful catalysts for the preparation of a broad range of optically active compounds and labile organic compounds. As well as regiospecific and stereoselective hydrolysis, they can be used for esterification or transesterification reactions in organic solvents, such as *tert*-butylmethylether, toluene and heptane. Other potential industrial applications of lipases include the production of fatty acids and glycerol via hydrolysis of oils and fats, and there are opportunities for both environmental enhancement and adding value to beef tallow by its hydrolysis to fatty acids. Beef tallow and some other fats are not in a liquid form at mesophilic enzyme temperatures and clearly, efficient enzymatic processes for handling these and related by-products require suitable thermostable lipases capable of hydrolysing long-chain fatty acids. Most lipases on the market are impure and poorly characterized and importantly, operate under relatively mild conditions at low temperatures. We have described consensus primers for the isolation of lipases by Genomic Walking PCR[4] and from expression libraries.[5] The genes for thermophilic lipases from several *Geobacillus* strains and from biomass from microbial tallow hydrolysis at high temperature have been isolated and sequenced. The enzymes have had a His-tag added and they have been isolated and purified after expression in *Escherichia coli*. We have previously described the cloning and expression of a lipase from a thermophilic *Geobacillus* strain, Tp10A.1, and demonstrated its high enantioselectivity on selected racemic ester substrates.[6]

Here, we present biochemical data on three other cloned thermophilic lipases in hydrolytic reactions and describe

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their outstanding enantioselectivity towards the hydrolysis of (*RS*)-1-phenylethyl acetate and methyl (*RS*)-2-methyldecanoate.

2. Results and discussion

2.1. Characterisation of lipases

The purified recombinant lipases were assayed for optimal temperature and pH and chain-length specificity towards fatty acid *p*-nitrophenyl esters (PNP esters). The results are shown in Table 1.

Table 1. Optimal pH, temperature and substrate selectivity (fatty acid PNP esters) of the lipases

Lipase	Optimal pH	Optimal temperature (°C)	Substrate selectivity
B. therm.	9	50	C:8, 10
Mk1 A.1	9	50	C:10
OK4 A.2	9	50	C:10
Tp10 A.1	8	60	C:8, 10, 12

The optimal conditions of the enzymes correlate with their sequence similarities, shown in Table 2. The B. therm., Mk1 A.1 and OK4 A.2 lipases exhibit similar pH and temperature optima, and fatty ester selectivities. These enzymes are remarkably similar in terms of their amino acids sequences, differing by at most three residues in 389. The TP10 A.1 enzyme, which exhibits slightly different pH and temperature optima and substrate selectivity has significant primary sequence differences, including a 29-residue N-terminal extension and several residue substitutions.

Table 2. Sequence similarities of the lipases (%)

	B. therm.	Mk1 A.1	OK4 A.2	Tp10 A.1
B. therm.	100	99.2	99.7	93.4
Mk1 A.1	99.2	100	99.5	93.2
OK4 A.2	99.7	99.5	100	93.7
Tp10 A.1	93.4	93.2	93.7	100

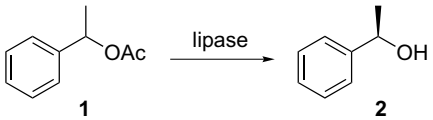
2.2. Enantioselectivities of lipases

The enantioselectivities of the three new lipases and Tp10 A.1 lipase were investigated for a number of substrates. Tp10 A.1 lipase was previously used in assays of the hydrolysis of (*RS*)-1-phenylethyl acetate **1** and methyl (*RS*)-2-methyldecanoate **3** and showed excellent enantioselectivity (E >200) for both substrates at pH 7.5, 50 °C. (In general, synthetically useful enzyme-catalysed resolutions require an E value of 50 or greater, with values higher than 100 considered excellent.)[7]

In the current work, the Tp10 A.1 lipase and the three new lipases were assayed for the hydrolysis of (*RS*)-1-phenylethyl acetate **1** at 20 and 50 °C, and at pH 7.5 and 9.0 (results shown in Table 3). Tp10 A.1 lipase again showed excellent selectivity at 20 and 50 °C at pH 7.5, with only a slight drop in E value at pH 9.0, presumably due to an increase in the background rate of hydrolysis. Of the new enzymes, both OK4 A.2 and B. therm lipases are slightly more selective than Tp10 A.1, and also more active, particularly under thermoalkalophilic conditions (pH 9, 50 °C). The Mk1 A.1 lipase, however, is considerably less selective, and does not exhibit thermoalkalophilic properties, with no increase in activity occurring at high temperature and pH. Indeed, a dramatic decrease in selectivity is observed under thermoalkalophilic conditions. All lipases were selective for hydrolysis of the (*R*)-enantiomer of **1**.

Assays of the hydrolysis of methyl (*RS*)-2-methyldecanoate **3** (Table 4) indicated that the Tp10 A.1, OK4 A.2 and B. therm lipases all exhibit excellent enantioselectiv-

Table 3. Hydrolysis of (*RS*)-1-phenylethyl acetate



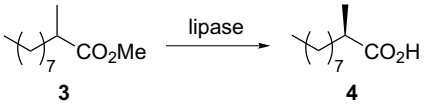
1 **2**

Lipase	Temp (°C)	pH	Time (h)	% Conv.	% Ee (P)	E
B. therm	RT	7.5	187	22.0	99.0	>200
B. therm	50	7.5	23	37.2	98.9	>200
B. therm	RT	9	69	47.1	98.6	>200
B. therm	50	9	19	49.7	96.1	188
Mk1 A.1	RT	7.5	147	41.9	97.1	142
Mk1 A.1	50	7.5	47	9.3	95.5	47
Mk1 A.1	RT	9	121	30.9	97.0	99
Mk1 A.1	50	9	70	17.2	71.3	6.9
OK4 A.2	RT	7.5	190	24.9	98.9	>200
OK4 A.2	50	7.5	71	37.2	98.4	>200
OK4 A.2	RT	9	193	48.4	94.4	103
OK4 A.2	50	9	23	49.6	97.1	>200
Tp10 A.1	RT	7.5	168	17.0	98.8	198
Tp10 A.1	50	7.5	46	20.7	98.5	174
Tp10 A.1	RT	9	170	44.4	97.3	174
Tp10 A.1	50	9	42	43.4	96.5	124

ity for this substrate. These three lipases also display distinct thermoalkalophilic properties, with high activity at pH 9 and 50 °C. The Mk1 A.1 lipase does show excellent selectivity at pH 7.5, but again appears to be unstable under thermoalkalophilic conditions. All the lipases show the same sense of enantioselectivity, being selective for hydrolysis of the (*R*)-enantiomer of methyl 2-methyldecanoate. Confirmation of the absolute configuration of the products was obtained by isolating the hydrolysed product acid and determination of its specific rotations. This enantioselectivity is the opposite sense to nearly all other lipases towards this substrate.[8,9]

Given the high selectivity of three of the four lipases to these two substrates, **1** and **3**, further assays were conducted. The hydrolysis of methyl 2-phenylpropionate **5** was tested, but all lipases showed virtually no enantioselectivity for this substrate (Table 5). The lipase-catalysed acetylation of 1-phenylethanol **2** in organic solvent was also investigated (Table 6). While the Tp10 A.1 and B. therm lipases showed very poor activity in this transformation, Mk1 A.1 and OK4 A.2 lipases were reasonable catalysts. The OK4 A.2 lipase was the most selective enzyme, giving *E* values of up to 68, though its activity was still only moderate.

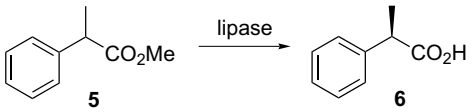
Table 4. Hydrolysis of methyl (*RS*)-2-methyldecanoate



3 **4**

Lipase	Temp (°C)	pH	Time (h)	% Conv.	% Ee (P)	*E*
B. therm	RT	7.5	261	34.1	99.5	>200
B. therm	50	7.5	42	20.3	99.3	>200
B. therm	RT	9	67	45.7	99.7	>200
B. therm	50	9	20	37.8	99.4	>200
Mk1 A.1	RT	7.5	234	17.8	99.7	>200
Mk1 A.1	50	7.5	141	42.3	99.1	>200
Mk1 A.1	RT	9	187	25.4	99.2	>200
Mk1 A.1	50	9	48	0.77	99.1	>200
OK4 A.2	RT	7.5	145	27.5	99.1	>200
OK4 A.2	50	7.5	95	23.2	99.4	>200
OK4 A.2	RT	9	45	49.1	99.3	>200
OK4 A.2	50	9	17	45.9	99.6	>200
Tp10 A.1	RT	7.5	264	45.0	99.6	>200
Tp10 A.1	50	7.5	69	32.6	99.6	>200
Tp10 A.1	RT	9	97	36.7	99.7	>200
Tp10 A.1	50	9	18	48.7	99.7	>200

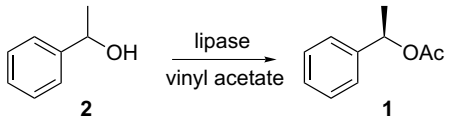
Table 5. Hydrolysis of (*RS*)-2-phenylpropionate



5 **6**

Lipase	Temp (°C)	pH	Time (h)	% Conv.	% Ee (P)	*E*
B. therm	RT	7.5	238	0.17	38.5	2.3
B. therm	50	7.5	142	0.71	9.3	1.2
B. therm	RT	9	241	1.9	2.0	1.0
B. therm	50	9	143	6.2	1.7	1.0
Mk1 A.1	RT	7.5	239	0.12	8.0	1.2
Mk1 A.1	50	7.5	142	0.34	8.2	1.2
Mk1 A.1	RT	9	244	2.1	2.7	1.1
Mk1 A.1	50	9	122	9.0	2.5	1.1
OK4 A.2	RT	7.5	19	0.65	28.6	1.8
OK4 A.2	50	7.5	119	1.5	10.4	1.2
OK4 A.2	RT	9	166	1.2	2.8	1.1
OK4 A.2	50	9	118	10.1	2.5	1.1
Tp10 A.1	RT	7.5	139	0.05	6.3	1.1
Tp10 A.1	50	7.5	143	0.21	13.3	1.3
Tp10 A.1	RT	9	142	0.96	3.0	1.1
Tp10 A.1	50	9	147	3.5	0.56	1.0

Table 6. Acylation of (*RS*)-1-phenylethanol



Lipase	Temp (°C)	Time (h)	% Conv.	% Ee (P)	*E*
B. therm	RT	148	4.6	92.6	27
B. therm	50	44	3.2	90.6	21
Mk1 A.1	RT	146	15.5	93.3	34
Mk1 A.1	50	44	6.0	89.0	18
OK4 A.2	RT	142	19.0	96.4	68
OK4 A.2	50	43	17.1	93.2	34
Tp10 A.1	RT	195	0.7	71.5	6.1
Tp10 A.1	50	44	0.2	43.7	2.6

Given that the sequence similarities of these enzymes are very high the differences in their selectivities at high temperature and pH is surprising. The Mk1 A.1 lipase exhibits a dramatic decrease in selectivity under these conditions, particularly for the hydrolysis of (*RS*)-1-phenylethyl acetate **1** and methyl (*RS*)-2-methyldecanoate **3** (Tables 3 and 4). The Mk1 A.1 lipase differs from the OK4 A.2 lipase by just two amino acid substitutions—P228R and G393S—and the B. therm. lipase differs by just an additional V156M substitution. In order to define the role of these substitutions in modifying the Mk1 A.1 activity at high temperature and pH, the thermostabilities of the enzymes were calculated (Table 7).

Table 7. Lipase thermostabilities

Lipase	Temp. (°C)	$t_{1/2}$ (min)
B. therm.	67	21
	51	87
Mk1 A.1	67	50
	51	101
OK4 A.2	67	17
	51	26
Tp10 A.1	67	15

These results show that all the lipases show significant thermophilicity (mesophilic lipases typically have a half-life <10 min at 67 °C). Interestingly, the Mk1 A.1 lipase is the most thermostable of the lipases, suggesting that its different reactivity under thermoalkalophilic conditions is due to high pH rather than high temperature. This is consistent with the single substitution of a proline residue for an arginine in Mk1 A.1 lipase. It is feasible that at high pH the arginine is no longer in the protonated form, thereby dramatically changing the electrostatic environment around this residue. Further investigations of this effect are currently underway.

In conclusion, several new thermoalkalophilic lipases have been cloned and characterised, which show excellent activity and enantioselectivity towards various substrates, particularly methyl 2-methyldecanoate. The Mk1 A.1 lipase shows a substantial loss of activity and selectivity at high pH, attributed to a single Pro-Arg substitution. The OK4 A.2 lipase is the most active and selective of those isolated, considerably more so than the previously isolated Tp10 A.1 lipase, retaining high activity and enantioselectivity towards various substrates at high pH and temperature.

3. Experimental

3.1. Bacteria and genes

All genes were expressed in *Escherichia coli* BL21AP (Invitrogen, Carlsbad, CA). This strain allows a tight regulation of lipase expression, which is induced on induction with arabinose and IPTG. The lipase from *Geobacillus* sp. Tp10A.1 was prepared as previously described.[6] The genes for lipases from *Geobacillus* strains OK4 A.2 and Mk1 A.1 and *Bacillus thermoleovorans* were cloned by PCR amplification from genomic DNA. The following primers were used: Mk1 A.1 and *B. thermoleovorans*, BthFpet. AAAAAACGGGATCCAGCGGC AGTTTCACGCGCCA; and BthRpet, GATAATCCCTAAGCTTAAGGCCGCAAACTCGCC. The OK4 A.2 lipase gene was amplified by nested PCR, with the following degenerate primers: BLPF2, GAG/TAA/GC/GATGATGAAAT/GGCTG and BLPR2, CC/TC/T G/TC/TA/TTTAAGGCCGCAAACTC. The PCR product from this amplification was used as template with the BthFpet and BthRpet primers above for the second amplification. The PCR products were ligated into pET26B(+) and induced as previously described.[6] In addition, the pET-26B(+) vector carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus a C-terminal His-Tag sequence for easy purification.

3.2. Enzyme purification

A small-scale (50 mL) culture was performed to check expression levels and to establish the optimal induction period. The lipase was purified from the cell fraction using Ni-NTA Magnetic Agarose Beads and samples were analysed by SDS PAGE. Then a larger scale protein expression (700 mL) was performed to produce

enough enzyme for biochemical characterisation. Cells were induced with arabinose (final concentration 2%) and IPTG (final concentration 1 mM) and lysed by passage through a French pressure cell. After centrifugation, the resulting cleared cell lysate was used for further purification. The recombinant His-tagged lipase was purified from the cell fraction using a Ni-column on a BIOLOGIC LP chromatographic system (Amersham/Pharmacia). The lipases appeared as a single band when analysed by SDS-PAGE and had a molecular weight of approximately 45 kDa.

3.3. Substrate preparation

Fatty acid *p*-nitrophenyl esters were purchased from Sigma. Lipase substrates (*RS*)-1-phenylethyl acetate **1** and methyl (*RS*)-2-phenylpropionate **5** were prepared from *sec*-phenethyl alcohol **2** and 2-phenylpropionic acid **6**, respectively, according to standard procedures. 2-Methyldecanoic acid **4** was prepared according to the procedure of Berglund et al.[10] Methyl (*RS*)-2-methyldecanoate **3** was prepared from **4** as described below.

3.4. Methyl (*RS*)-2-methyldecanoate 3

Thionyl chloride (2.04 mL, 28.0 mmol) was added to ice cold methanol (60 mL) and the solution treated with methyl (*RS*)-2-methyldecanoic acid[10] **4** (2.78 mL, 21.0 mmol). The solution was stirred at room temperature under nitrogen for 1 h. The reaction mixture was concentrated in vacuo and the crude product purified by flash chromatography, eluting with 5% ethyl acetate/hexane, yielding a colourless oil (3.64 g, 85%). ^{1}H NMR (300 MHz) δ 3.66 (s, 3H), 2.43 (m, 1H), 1.63 (m, 1H), 1.40 (m, 1H), 1.26 (s, 12H), 1.13 (d, $J = 6.9$ Hz, 3H), 0.87 (t, $J = 6.4$ Hz, 3H); data in accordance with literature values.[11]

3.5. Enantioselectivity assays

Assays were analysed by gas chromatography using a Hewlett Packard 5890 Series II chromatograph with a Supelco Beta Dex 120 fused silica capillary column (length 30 m, diameter 0.25 mm, film thickness 0.25 μm, carrier gas He at 110 kPa, Injector temperature 270 °C, flame ionisation detector temperature 270 °C). The extent of conversion and the enantiomeric excess (e.e.) of the products were calculated from the relative integrals of the peaks, which were corrected by experimentally determined molar response factors. *E* values (defined as the ratio of the specificity constants of the enzyme for the fast-reacting and slow-reacting substrate enantiomers, respectively) were calculated according to the method of Sih and co-workers[12] using the equation

$$E = \frac{\ln[1 - c(1 + \mathrm{ee(P)})]}{\ln[1 - c(1 - \mathrm{ee(P)})]}$$

where '*c*' denotes the extent of conversion and 'ee(P)' denotes the enantiomeric excess of the reaction product.

3.6. Assay 1: Hydrolysis of (*RS*)-1-phenylethyl acetate 1

To a stirred suspension of (*RS*)-1-phenylethyl acetate **1** (115 μL, 0.72 mmol) and Triton X-100 (1 drop, ~16 mg) in aqueous Tris buffer (100 mM, pH 7.5 or 9, 2.5 mL) at room temperature was added purified lipase solution (50 μL). A constant pH was maintained by dropwise addition of 1 M NaOH. The progress of the reaction was monitored periodically by withdrawing an aliquot (200 μL) of the reaction mixture. The aliquot was extracted with ethyl acetate (200 μL), the organic layer dried (Na_2SO_4) and subsequently analysed by gas chromatography, using a temperature profile of 80 °C for 1 min, then increasing by 2 °C/min to 130 °C for 5 min. The observed retention times were as follows: (*S*)-**1**, 23.9 min; (*R*)-**1**, 24.8 min; (*R*)-**2**, 25.3 min; (*S*)-**2**, 26 min. The extent of conversion and the enantiomeric excess of **2** were calculated from the relative integrals of the peaks, which were corrected using experimentally determined molar response factors: (*RS*)-**1**, 1×10^4 counts nmol^{-1}; (*RS*)-**2**, 8.18×10^3 counts nmol^{-1}. Results are displayed in Table 3.

3.7. Assay 2: Hydrolysis of methyl (*RS*)-2-methyldecanoate 3

To a stirred suspension of methyl (*RS*)-2-methyldecanoate **3** (80 μL, 0.40 mmol) and Triton X-100 (1 drop, ~16 mg) in aqueous Tris buffer (100 mM, pH 7.5 or 9, 1.4 mL) at room temperature was added purified lipase solution (50 μL). A constant pH was maintained by dropwise addition of 1 M NaOH. The reaction was periodically monitored by withdrawing an aliquot (200 μL) of the reaction mixture. The aliquot was extracted with ethyl acetate (200 μL), the organic layer dried (Na_2SO_4) and subsequently analysed by gas chromatography, using a temperature profile of 80 °C for 1 min, then increasing by 15 °C/min to 170 °C for 20 min, the observed retention times were as follows: (*RS*)-**3**, 10.02 min; (*S*)-**4**, 18.22 min and (*R*)-**4**, 18.75 min. The extent of conversion and the enantiomeric excess of **4** were calculated from the relative integrals of the peaks, which were corrected by the following experimentally determined molar response factors: (*RS*)-**3**, 1.57×10^4 counts nmol^{-1}; (*RS*)-**4**, 1.29×10^4 counts nmol^{-1}. Results are displayed in Table 4.

3.8. Isolation of (*R*)-2-methyldecanoic acid (*R*)-4 and methyl (*S*)-2-methyldecanoate (*S*)-3

Methyl (*RS*)-2-methyldecanoate **3** (50 μL, 0.25 mmol) was treated with Tp10 A.1 lipase for 69 h as described above. The assay mixture was extracted with ethyl acetate (3×10 mL) and the combined extracts were dried ($MgSO_4$), concentrated and the residue was purified by chromatography on silica, eluting with ethyl acetate/hexanes (1:9). The ester (***S***)-**3** was isolated as a clear oil (11.3 mg, 23%). The acid (***R***)-**4** was also isolated as a clear oil (17.4 mg, 40%), $[\alpha]_D = -15.4$ (*c* 0.84, MeOH) {lit.[13] $[\alpha]_D = -15.6$ (neat)}.

3.9. Assay 3: Hydrolysis of methyl (*RS*)-2-phenylpropionate 5

To a stirred suspension of methyl (*RS*)-2-phenylpropionate **5** (115 μL, 0.72 mmol) and Triton X-100 (1 drop, ~16 mg) in aqueous Tris buffer (100 mM, pH 7.5 or 9, 2.5 mL) at room temperature was added purified lipase solution (50 μL). A constant pH was maintained by dropwise addition of 1 M NaOH. The progress of the reaction was monitored periodically by withdrawing an aliquot (200 μL) of the reaction mixture. The aliquot was acidified with 3 M HCl (1 drop), extracted with dichloromethane (200 μL), the organic layer dried (Na_2SO_4) and subsequently analysed by gas chromatography, using the temperature profile of 85 °C for 65 min, then increasing by 10 °C/min to 200 °C for 10 min, the observed retention times were as follows: (*S*)-**5**, 67.7 min; (*R*)-**5**, 68.0 min; (*S*)-**6**, 79.0 min; (*R*)-**6**, 79.1 min. The extent of conversion and the enantiomeric excess of **6** were calculated from the relative integrals of the peaks, which were corrected using the experimentally determined molar response factors: (*RS*)-**5**, 9.36×10^3 counts $nmol^{-1}$; (*RS*)-**6**, 7.49×10^3 counts $nmol^{-1}$. Results are displayed in Table 5.

3.10. Assay 4: Acylation of (*RS*)-1-phenylethanol 2

An aliquot of purified lipase (50 μL) was evaporated under reduced pressure. To the dry residue was added vinyl acetate (2.5 mL) and (*RS*)-1-phenylethanol **2** (87 μL, 0.72 mmol) and the resulting mixture was stirred at room temperature. The reaction was monitored periodically by withdrawing an aliquot (200 μL) of the reaction mixture. The aliquot was filtered and the filtrate analysed by gas chromatography, as described for assay 1. Results are displayed in Table 6.

Acknowledgements

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