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Mapping the substrate selectivity of new hydrolases using colorimetric screening: lipases from *Bacillus thermocatenulatus* and *Ophiostoma piliferum*, esterases from *Pseudomonas fluorescens* and *Streptomyces diastatochromogenes*

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Abstract—Recent advances in biochemistry and molecular biology have simplified the discovery and preparation of new hydrolases. Although these hydrolases might solve problems in organic synthesis, measuring their selectivity, especially enantioselectivity, remains tedious and time consuming. Recently, we developed a colorimetric screening method to measure the enantioselectivity of hydrolases. Here we apply this rapid screening method to map the substrate selectivity of four new hydrolases: lipases from the thermophilic *Bacillus thermocatenulatus* (DSM 730, BTL2) and a filamentous fungus *Ophiostoma piliferum* (NRRL 18917, OPL) and esterases from two bacteria, *Pseudomonas fluorescens* (SIK-W1, esterase I, PFE) and *Streptomyces diastatochromogenes* (Tü 20, SDE). We screened a general library of 29 substrates and a chiral library of 23 pairs of enantiomers. All four hydrolases catalysed the hydrolysis of unnatural substrates, but the two lipases accepted a broader range of substrates than the two esterases. As expected, the two lipases favoured more hydrophobic substrates, while the two esterases showed a preference for smaller substrates. Several moderately enantioselective reactions were identified for the solketal esters: BTL2, butyrate, $E=7.9$ (R); octanoate, $E=4.9$ (R) and 3-bromo-2-methyl propionate methyl esters, PFE, $E=12$ (S); SDE, $E=5.6$ (S). OPL showed low enantioselectivity toward all substrates tested. The current colorimetric screen could not measure the selectivity for several slow-reacting substrates. Traditional screening identified high enantioselectivity of BTL2 and PFE toward one of these slow substrates, 1-phenylethyl acetate ($E>50$).

1. Introduction

Recently, we developed a rapid method for measuring the stereoselectivity of hydrolases, called Quick E.[1] In this paper we apply this method to map the substrate selectivity, especially the enantioselectivity, of four new hydrolase enzymes: two lipases and two esterases. Their biochemical properties suggest that they may be useful for organic synthesis (Table 1). They tolerate wide pH ranges and elevated temperatures. Three of the four hydrolases have already been cloned and overexpressed in *E. coli*. However, they have not yet been applied to problems in organic synthesis because little is known about their substrate selectivity.

Lipase from a thermophilic *Bacillus*, *B. thermocatenulatus* (DSM 730), has been cloned and overexpressed in

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Table 1. Biochemical properties of esterases and lipases studied in this paper

Hydrolase	BTL2	OPL	PFE	SDE
Host microorganism	*Bacillus thermocatenulanatus*, DSM 730	*Ophiostoma piliferum*, NRRL 18917	*Pseudomonas fluorescens*, SIK W1	*Streptomyces diastatochromogenes*, Tü 20
Mol. wt. (kDa)	43	60	30	30.9
Aa[a]	417	Unknown	272	290
Sequence[b]	Q59260	Unknown	P22862	Q59837
Expression host	*E. coli*	[c]	*E. coli*	*E. coli*
Optimal temp.	60°C	35°C[d]	45°C	44°C
Stable at pH	9–10	3–8	5–10	4.5–9.5
Optimal pH	8–9	7	7.5–8	7.5

[a] Number of amino acids in the mature protein.
[b] TrEMBL or Swiss-Prot accession numbers for nucleic acid or amino acid sequences.
[c] Not genetically engineered. Isolated from natural source.
[d] Denatures starting above 35°C.

E. coli.[2] This lipase tolerates high temperatures, some detergents and organic co-solvents. These properties suggest that it may be a rugged catalyst for organic synthesis. However, no information is available about the stereoselectivity of this lipase. Researchers have measured its apparent acyl chain length selectivity and found that it has typical lipase behaviour. For 4-nitrophenyl esters, the C10 ester reacted fastest, but C12, C14, and C16 also reacted at rates similar to C10. For triglycerides, tributyrin reacted fastest (C4 acyl chain), but triglycerides with C6, C8, C10, and C12 acyl chains also reacted at similar rates to C4.

The second lipase comes from the filamentous fungus *Ophiostoma piliferum* (NRRL 18917). The forestry industry uses an albino strain of this fungus for two reasons.[3] First, it secretes a lipase that breaks down pitch in wood, which minimizes fouling of equipment during paper manufacture. Second, colonization by *O. piliferum* excludes other fungi, including those that stain wood dark blue, thereby reducing its value. Brush et al. recently isolated a lipase from this strain of *O. piliferum*.[4] It has molecular weight of approximately 60 kDa, a pI of 3.9, a pH optimum near 7, and is stable in solution up to 35°C. Surprisingly for a lipase, butanoate esters of 4-nitrophenol reacted faster than esters of longer chain fatty acids. (C4 esters reacted approximately 20 times faster than C18 esters.) This lipase may also be useful for organic synthesis. Although Gao and Breuil isolated a lipase from another *Ophiostoma* species, *O. piceae*,[5] it differs significantly from the *O. piliferum* lipase. The *O. piceae* lipase has a molecular weight of only 35 kDa, a pH optimum near 5, and temperature optimum near 30°C.

Researchers have isolated a number of different hydrolases from *Pseudomonas fluorescens* (SIK-W1). This work focuses on an 'arylesterase' first isolated and cloned by Choi et al.[6] and later resequenced to correct errors.[7] The amino acid sequence of this esterase is similar to a non-heme haloperoxidase and indeed this esterase shows low haloperoxidase activity.[7] Previous substrate mapping of this esterase[8] showed that it accepts a range of unnatural substrates and, as expected for an esterase, favours esters of shorter chain carboxylic acids (propanoates, butyrates) over the longer chain caprylates (C8) or decanoates (C10). Aryl acetates were good substrates, but not acetates of benzyl alcohol or 2-phenylethanol. Esters of aromatic acids (methyl benzoate, methyl salicylate) were also found to be unreactive.

Previous work also identified several stereoselective reactions catalysed by PFE.[8] PFE showed high enantioselectivity in the hydrolysis of 1-phenylethyl acetate ($E>50$), but the closely related 1-phenylpropyl acetate reacted slowly and with low enantioselectivity ($E=2$). PFE catalysed the acylation of the primary alcohol solketal (2,2-dimethyl-1,3-dioxolane-4-methanol) and five closely related derivatives, but showed low enantioselectivity ($E=1.1$–4.9). PFE also showed low enantioselectivity in the hydrolysis of methyl 3-phenylbutyrate ($E=3.5$). The positional isomer ethyl 2-phenyl butyrate did not react.

Streptomyces are Gram-positive soil bacteria, many of which produce useful antibiotics. A number of strains, especially plant pathogens, secrete esterases that degrade the protective waxy coat of leaves. Wei et al. solved the X-ray structure of an esterase from the plant pathogen *Streptomyces scabies*.[9] This structure showed that the active site does not consist of the usual Ser-His-Asp triad, but a Ser-His diad. In addition, the protein fold differed slightly from the α,β-hydrolase fold. It is not clear how these unusual features might change the stereoselectivity. This paper examines the stereoselectivity of a closely related esterase. Tesch et al. isolated and cloned an esterase from *Streptomyces diastatochromogenes* (Tü 20, SDE).[10] Khalameyzer and Bornscheuer have overexpressed this esterase in *E. coli*.[11] It shows a similar amino acid sequence (31% identity in a region of 156 amino acids) to the *S. scabies* esterase, including the active site residues. Consistent with its designation as an esterase, it favoured esters of shorter chain fatty acids over longer chains: 4-nitrophenyl octanoate (C8, rate = 100), 4-nitrophenyl laurate (C12, rate = 10), 4-nitrophenyl palmitate (C16, rate = 2).[10] Although SDE catalysed the hydrolysis of unnatu-

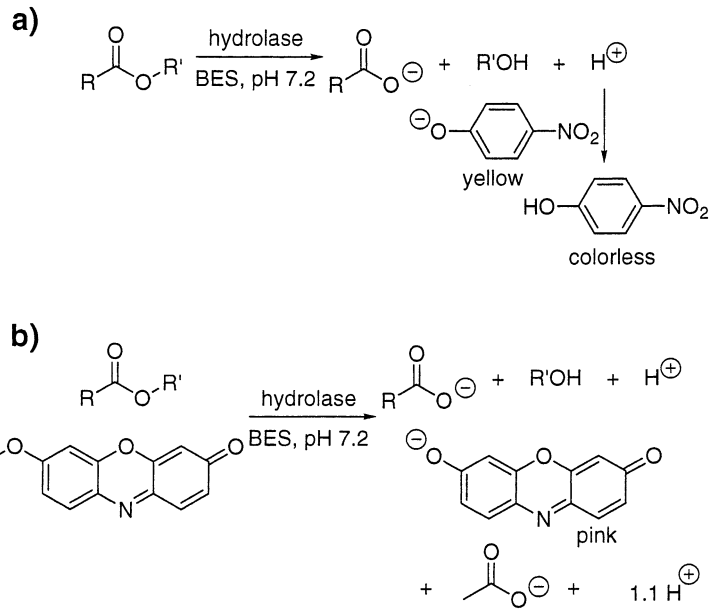


Figure 1. Measuring selectivity of hydrolases. (a) For estimated selectivities, the initial rate of hydrolysis of different esters is measured colorimetrically using 4-nitrophenol as a pH indicator. The intensity of the yellow colour (monitored at 404 nm) diminishes as hydrolysis proceeds. (b) Quantitative measure of selectivity requires a competitive experiment. We used resorufin acetate or another resorufin ester as the competitive substrate because its hydrolysis generates the easily measured resorufin anion (monitored at 574 nm). The total amount of hydrolysis is measured using 4-nitrophenol as the pH indicator as in part a. Only 10% of resorufin is deprotonated to the anion at pH 7.2.

ral substrates, it showed an unusually low enantioselectivity toward both secondary alcohols tested: $E=3.3$ for 1-phenylethyl acetate, $E=1$ for 1-phenylpropyl acetate. SDE is available commercially from Jülich Fine Chemicals.[12]

Our rapid screening method uses pH indicators to monitor ester hydrolysis colorimetrically[1] (Fig. 1). We carry out the screening in 96-well plates where each well contains a different substrate. Comparing the rates of reaction of different substrates gives an estimated or apparent selectivity, but not the true selectivity (Fig. 1a).[‡‡] Indeed, we previously showed that such comparisons can be in error by more than a factor of 20 from the true selectivity.[1] To measure the true selectivity we added a resorufin ester as a reference compound to reintroduce competition (Fig. 1b). This competition between the substrate and the resorufin ester accounts for differences in K_M and yields the correct selectivity.

[‡‡] The substrate selectivity of an enzyme is the ratio of the specificity constants (k_{cat}/K_M) for each substrate (Fersht, A. *Structure and Mechanism in Protein Science*; Freeman: New York, 1999, pp. 116–117). By measuring initial rates of the substrates separately, we eliminate competitive binding between the two substrates and therefore do NOT measure the true selectivity. For example, at saturating substrate concentrations, where $[S]>K_M$, the relative initial rates equal the relative k_{cat} values; at partially saturating conditions, where $[S]<K_M$, the initial rates also depend on the K_M values. Thus, the ratio of separately measured initial rates ignores some or all of the effect of K_M on selectivity.

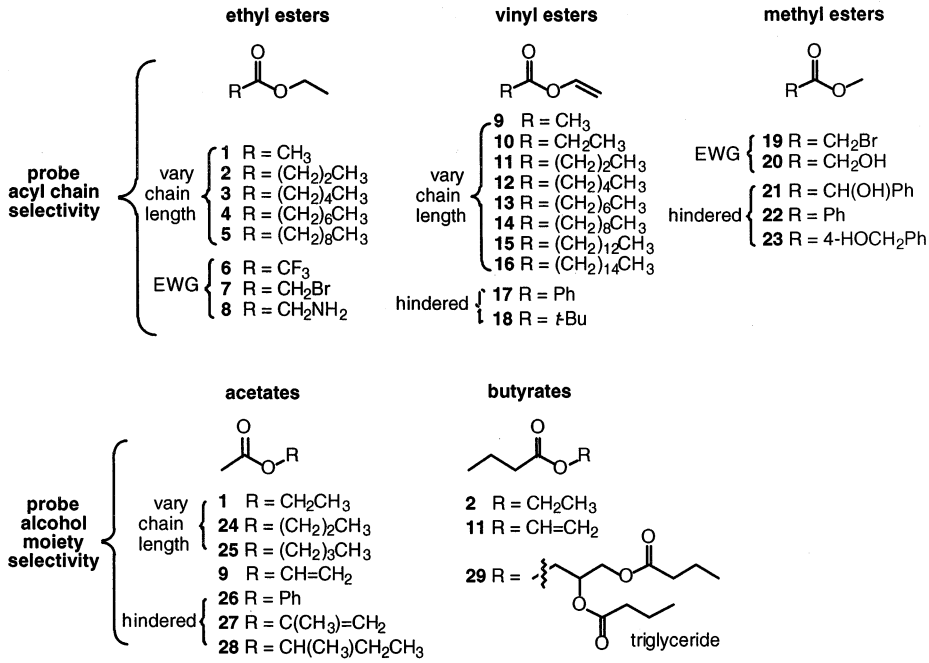
2. Results

2.1. General ester library

The general ester library consisted of 29 commercially available esters (Scheme 1). The esters were chosen to identify acyl chain length preferences of the hydrolases and also their ability to hydrolyse hindered or charged substrates. Simple alkyl esters as well as activated esters (vinyl and phenyl esters, esters with electron-withdraw ing substituents in the acyl portion) were included to test whether activated esters would react faster.

The four hydrolases accepted most of the substrates in the general ester library (Table 2), but the most polar molecules ethyl glycine, **8**, and methyl hydroxyacetate, **20**, were poor substrates. The hydrolase with the lowest overall activity was BTL2, which came from a thermophilic microorganism. At room temperature, only 12 of the 29 esters in the general library and only four of 23 pairs in the chiral library (see below) reacted. Upon increasing the temperature to 40°C, almost all of the esters reacted: 26 of the 29 esters in the general library and at least one ester in all of the 22 pairs in the chiral library. For this reason, all data on BTL2 in this paper refer to a reaction temperature of 40°C. Even at this increased temperature, BTL2 was less active than the other three hydrolases were at 25°C.

Surprisingly, the two lipases, BTL2 and OPL, catalysed the hydrolysis of small activated and small unactivated



Scheme 1. Esters used to survey the substrate selectivity of hydrolyses. Substrates that fit into two categories are listed twice. EWG = electron-withdrawing group.

Table 2. Hydrolytic activity of the four hydrolases towards the general ester library[a]

Substrate	BTL2	OPL	PFE	SDE
1, Ethyl acetate	2.9	23	Nd	Nd
2, Ethyl butanoate	3.9	38	3.2	0.75
3, Ethyl hexanoate	1.1	**53**	Nd	Nd
4, Ethyl octanoate	1.3	**1000**	13	3.5
5, Ethyl decanoate	0.72	**110**	Nd	Nd
6, Ethyl trifluoroacetate	2.9	37	**2140**	**1300**
7, Ethyl bromoacetate	0.60	**110**	**240**	**120**
8, Ethyl glycine	<0.20	2.3	<0.20	0.66
9, Vinyl acetate	0.84	9.8	**2900**	**2100**
10, Vinyl propanoate	1.3	**110**	**4600**	**1600**
11, Vinyl butanoate	0.83	**250**	**1900**	**650**
12, Vinyl hexanoate	17	**910**	**670**	**340**
13, Vinyl octanoate	28	**1700**	**1000**	**270**
14, Vinyl decanoate	29	**1300**	**91**	18
15, Vinyl tetradecanoate	1.4	27	8.1	5.1
16, Vinyl hexadecanoate	<0.20	9.6	0.69	<0.20
17, Vinyl benzoate	2.1	**250**	7.4	2.4
18, Vinyl trimethylacetate	<0.20	14	12	4.8
19, Methyl bromoacetate	0.81	35	**220**	**90**
20, Methyl hydroxyacetate	0.85	1.0	0.47	<0.20
21, Methyl mandelate	0.62	<0.20	<0.20	<0.20
22, Methyl benzoate	1.2	<0.20	<0.20	<0.20
23, Methyl 4-(hydroxy-methyl)benzoate	0.71	33	<0.20	<0.20
24, Propyl acetate	0.45	12	1.0	<0.20
25, Butyl acetate	0.85	3.0	0.52	<0.20
26, Phenyl acetate	2.8	**130**	**290**	**74**
27, Isopropenyl acetate	0.58	6.6	**170**	46
28, Isobutyl acetate	0.80	1.2	<0.20	<0.20
29, Tributyrin	7.8	**640**	6.5	1.9

[a] Activities are in units/mg of protein where a unit is 1 μmol of ester hydrolysed/minute. The detection limit was 0.20 unit/mg. Screening was done at 25°C except for BTL2, which was screened at 40°C. Nd = not determined. To highlight the fast-reacting examples, all activities over 50 U/mg are shown in bold.

esters with similar rates. For example, ethyl acetate, **1**, ethyl trifluoroacetate, **6**, and vinyl acetate, **9**, reacted at similar rates. On the other hand, larger activated esters reacted significantly faster than larger unactivated esters, especially in the OPL-mediated reactions. For example, vinyl decanoate, **14**, reacted 12–40 times faster than the ethyl decanoate, **5**. Similarly, with OPL, vinyl benzoate, **17**, reacted more than 1000 times faster than methyl benzoate, **22**. However, for BTL2, vinyl benzoate, **17**, reacted at a similar rate as methyl benzoate, **22**. The similar reaction rate for activated and non-activated esters suggests that a non-chemical step (possibly lid opening) limits the rate of reaction for BTL2.[13] This lid opening may be more difficult for small substrates with the thermophile-derived BTL2.

For esterases PFE and SDE, activated esters reacted faster than unactivated esters regardless of their size. For example, ethyl trifluoroacetate, **6**, and vinyl acetate, **9**, reacted more than 600 times faster than similar unactivated esters like ethyl butanoate, **2**, or propyl acetate, **24**. Similarly, vinyl octanoate, **13**, reacted approximately 70 times faster than the unactivated ethyl octanoate, **4**.

The apparent acyl chain length selectivity of the hydrolases varied dramatically with the nature of the leaving group.[§§] For the vinyl esters (Fig. 2) the lipases favoured C8 or C10 acyl chains, while the esterases favoured C2 or C3 chains. However, the preferred acyl group for the esterases differed upon changing from vinyl to ethyl esters. The esterases now preferred not the butanoate chain, but the longer octanoate analogues.

Previous work on *p*-nitrophenyl esters also showed a different selectivity: the ideal for BTL2 was a C10–C16 chain, OPL favoured the butanoate analogues, whilst SDE favoured the octanoate esters. For BTL2 and triglycerides, the chain length preference shifted yet again and tributyrin was the best substrate. Although short length acyl chains were usually the ideal for esterases, the lipases usually favoured longer chain lengths; the true acyl chain selectivity cannot be measured by this non-competitive approach. The apparent differences in chain length selectivity upon changing the alcohol fragment of the ester probably reflect changes in K_M upon changing the alcohol portion of the ester.

[§§] The apparent chain length selectivity is the relative rate of reaction for different esters in *separately measured* reactions. It is not the true selectivity for the reasons detailed in the previous footnote.

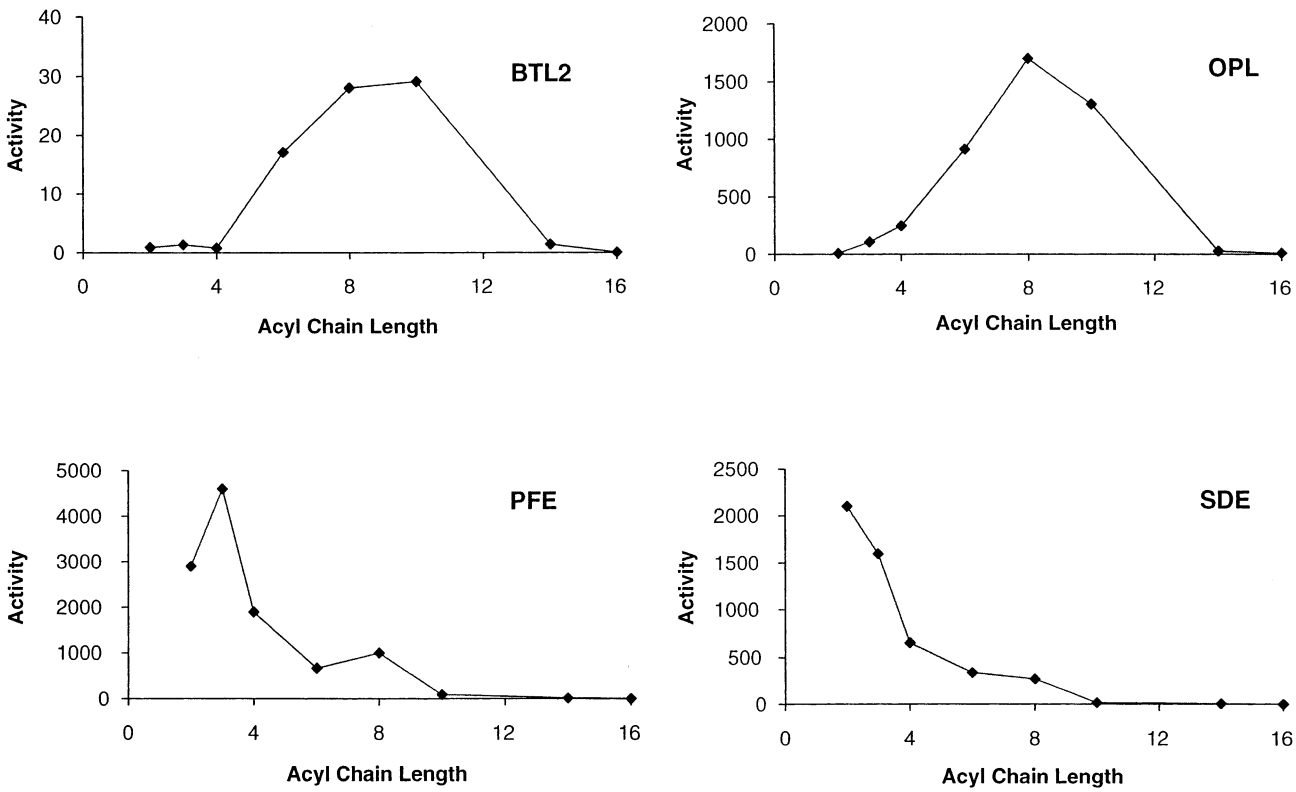
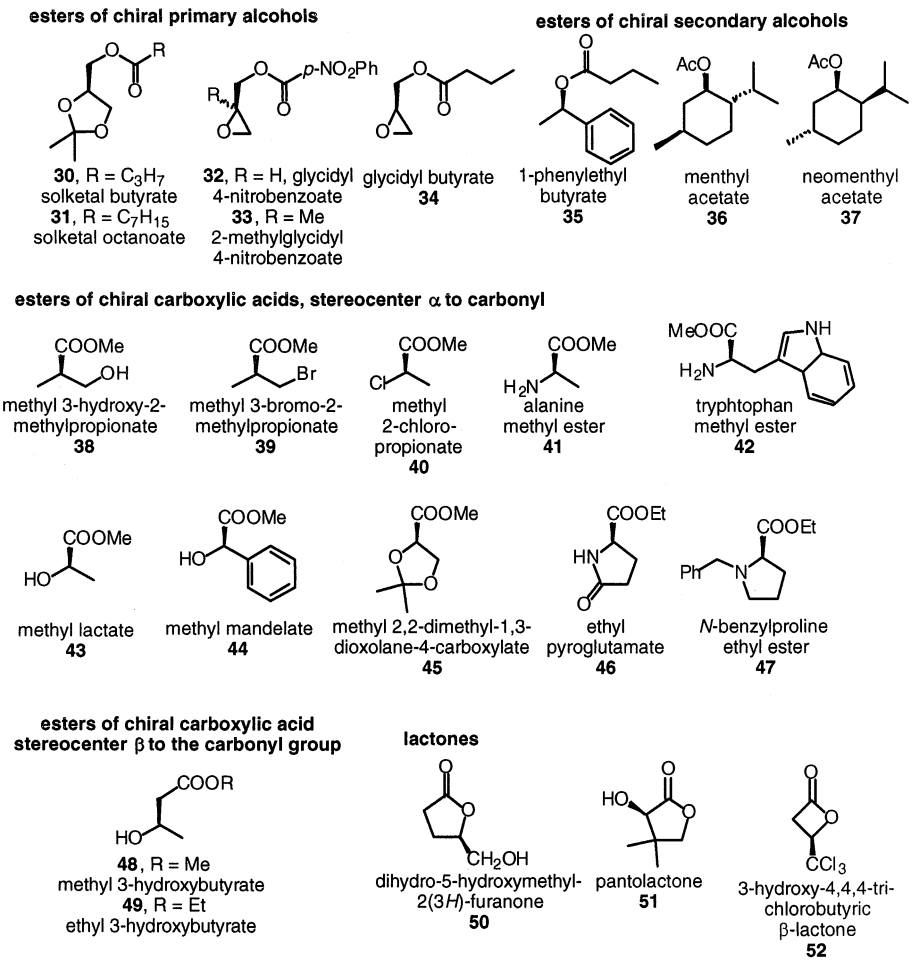


Figure 2. Initial rates of reaction of vinyl esters with the four hydrolases. The two lipases (BTL2 and OPL) both favour medium chain length (C6 to C10), while the esterases (PFE and SDE) show preference for short chain acyl groups (C2 to C4). The initial rates of hydrolysis for each ester were measured separately. For this reason the variations do not give the true selectivity, but only the apparent selectivity.



Scheme 2. Pure enantiomers used to survey the enantioselectivity of hydrolyses. For simplicity only the (*R*)-enantiomers are shown, but both were screened.

2.2. Chiral ester library

The chiral ester library contained 23 pairs of enantiomers, as shown in Scheme 2. We grouped the substrates according to the location of the stereocentre: esters of chiral primary alcohols (five pairs), esters of secondary alcohols (three pairs), esters of chiral carboxylic acids with a stereocentre at the α-position (ten pairs), esters of chiral carboxylic acids with a stereocentre at the β-position (two pairs), and lactones (three pairs).

The first stage of screening measured the initial rate of hydrolysis of each enantiomer separately to identify which esters reacted. In addition, for those cases where both enantiomers reacted, we also calculated the ratio of initial rates as an estimated enantioselectivity. Note that this ratio is NOT the true enantioselectivity, or enantiomeric ratio, *E*, because we measured the reaction rates of the pure enantiomers separately.

In the second stage of screening, we measured the true enantioselectivity using the colorimetric Quick E method.[1] This method adds a reference compound, a resorufin ester, to reintroduce competition (Fig. 1b). This competition between the substrate and the resorufin ester accounts for differences in K_M and yields the correct enantioselectivity. For slow-reacting substrates, we used slower-reacting reference compounds such as resorufin pivalate or isobutyrate instead of the faster-reacting acetate, which permits more accurate measurement of the relative rate of hydrolysis of the substrate and reference compound. However, for the slowest reacting substrates, even these resorufin esters reacted too rapidly. During the competitive experiment, we observed only hydrolysis of the reference compound and no detectable reaction of the substrate. In these cases, we could not measure the enantioselectivity using Quick E, but could only set a lower limit.

2.3. Enantioselectivity of BTL2

The chiral esters reacted more slowly than the esters in the general library with all hydrolases (Table 3). For BTL2 (40°C) at least one enantiomer reacted for all but one of the enantiomer pairs, but the rates were much slower. The fastest rates for BTL2 in the general library were 17–29 units/mg protein, while the fastest rates in the chiral library were 2.6–3.2 units/mg protein. BTL2 also catalysed the hydrolysis of chiral lactones, which are often poor substrates for hydrolases.

Table 3. Hydrolytic activity[a] and estimated enantioselectivity[b] of the four hydrolases towards the chiral ester library

Substrate	BTL2		OPL		PFE		SDE	
	Activity	Est. *E*	Activity	Est. *E*	Activity	Est. *E*	Activity	Est. *E*
30*R*	0.18	>4.0 *R*	**150**	2.9 *R*	0.50	1.0	0.25	1.0
30*S*	<0.045	>4.0 *R*	**52**	2.9 *R*	0.48	1.0	0.24	1.0
31*R*	2.6	3.2 *R*	**8400**	1.4 *R*	1.6	1.1 *S*	<0.20	Nr
31*S*	0.8	3.2 *R*	**6000**	1.4 *R*	1.8	1.1 *S*	<0.20	Nr
32*R*	0.23	5.2 *R*	0.30	>1.0 *R*	<0.40	Nr	0.21	1.3 *S*
32*S*	0.045	5.2 *R*	<0.30	>1.0 *R*	<0.40	Nr	0.27	1.3 *S*
33*R*	0.14	1.2 *R*	0.31	1.3 *S*	<0.40	Nr	<0.20	>1.1 *S*
33*S*	0.11	1.2 *R*	0.40	1.3 *S*	<0.40	Nr	0.23	>1.1 *S*
34*R*	<0.045	Nr	16	1.3 *S*	Nt	–	Nt	–
34*S*	<0.045	Nr	21	1.3 *S*	Nt	–	Nt	–
35*R*	<0.045	Nr	**73**	6.9 *R*	0.52	>1.3 *R*	0.22	>1.1 *R*
35*S*	<0.045	Nr	11	6.9 *R*	<0.40	>1.3 *R*	<0.20	>1.1 *R*
36*R*	0.099	2.4 *S*	6.4	2.3 *S*	0.94	1.1 *S*	0.64	1.1 *R*
36*S*	0.23	2.4 *S*	15	2.3 *S*	0.99	1.1 *S*	0.56	1.1 *R*
37*R*	0.18	1.4 *S*	<0.30	Nr	0.93	1.1 *S*	0.57	1.0
37*S*	0.25	1.4 *S*	<0.30	Nr	1.0	1.1 *S*	0.57	1.0
38*R*	0.73	1.7 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
38*S*	0.43	1.7 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
39*R*	1.7	1.0	<0.30	Nr	2.3	8.3 *S*	0.37	13 *S*
39*S*	1.7	1.0	<0.30	Nr	19	8.3 *S*	4.9	13 *S*
40*R*	3.0	1.1 *S*	28	1.0 *S*	**59**	2.2 *R*	<0.20	Nr
40*S*	3.2	1.1 *S*	28	1.0 *S*	27	2.2 *R*	<0.20	Nr
41*R*	1.1	1.3 *S*	28	1.0 *R*	<0.40	Nr	<0.20	Nr
41*S*	1.5	1.3 *S*	25	1.0 *R*	<0.40	Nr	<0.20	Nr
42*R*	0.87	2.0 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
42*S*	0.44	2.0 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
43*R*	0.90	1.4 *R*	2.7	10 *S*	0.50	1.7 *S*	0.21	1.4 *S*
43*S*	0.65	1.4 *R*	27	10 *S*	0.86	1.7 *S*	0.29	1.4 *S*
44*R*	2.5	2.8 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
44*S*	0.89	2.8 *R*	<0.30	Nr	<0.40	Nr	<0.20	Nr
45*R*	0.65	1.1 *R*	0.63	1.6 *R*	0.44	1.1 *R*	<0.20	Nr
45*S*	0.57	1.1 *R*	0.39	1.6 *R*	0.40	1.1 *R*	<0.20	Nr
46*R*	0.31	2.1 *S*	<0.30	Nr	<0.40	Nr	<0.20	Nr
46*S*	0.63	2.1 *S*	<0.30	Nr	<0.40	Nr	<0.20	Nr
47*R*	0.20	2.6 *S*	<0.30	Nr	<0.40	Nr	<0.20	Nr
47*S*	0.51	2.6 *S*	<0.30	Nr	<0.40	Nr	<0.20	Nr
48*R*	1.1	1.6 *R*	34	1.1 *R*	<0.40	Nr	<0.20	Nr
48*S*	0.69	1.6 *R*	32	1.1 *R*	<0.40	Nr	<0.20	Nr
49*R*	0.48	1.4 *R*	36	1.6 *R*	<0.40	Nr	<0.20	Nr
49*S*	0.35	1.4 *R*	**58**	1.4 *R*	<0.40[c]	Nr	<0.20[c]	Nr
50*R*	1.0	1.4 *R*	<0.30	Nr	<0.40[c]	Nr	<0.20[c]	Nr
50*S*	0.72	1.4 *R*	<0.30	Nr	<0.40[c]	Nr	<0.20[c]	Nr
51*R*	0.93	1.0	<0.30	Nr	<0.40[c]	Nr	<0.20[c]	Nr
51*S*	0.97	1.0	<0.30	Nr	<0.40[c]	Nr	<0.20[c]	Nr
52*R*	0.51	1.0	1.8	1.6 *R*	Nt	–	Nt	–
52*S*	0.50	1.0	1.1	1.6 *R*	Nt	–	Nt	–

[a] Activities are in units/mg of protein where a unit is 1 μmol of ester hydrolysed/min. To highlight the fast-reacting examples, all activities over 50 U/mg are shown in bold. Reaction conditions: 1 mM substrate, 7 vol% acetonitrile, 0.45 mM 4-nitrophenol, 1.0 mM BES at pH 7.20, 25°C except 40°C for BTL2. Nt = not tested, Nr = substrate did not react so no ratio can be calculated.

[b] Estimated *E* is the rate of the faster enantiomer over the slower one. The configuration of the fast-reacting enantiomer is shown in italics following the value.

The first stage of screening suggested that BTL2 may be enantioselective toward solketal butyrate, **30** (estimated $E>4.0$ favouring the (*R*)-enantiomer), and glycidyl 4-nitrobenzoate, **32** (estimated $E=5.2$ favouring the (*R*)-enantiomer). Glycidyl 2-methyl 4-nitrobenzoate, **33**, an ester similar to **32**, showed a low estimated *E*. Several other esters showed low estimated enantioselectivity (~ 2.5): menthyl acetate, **36**, methyl mandelate, **44**, and *N*-benzyl-proline ethyl ester, **47**.

Quick E measurements on BTL2 showed moderate enantioselectivity toward solketal butanoate ($E=7.9$) (Table 4). The true enantioselectivity of BTL2 toward the other substrates could not be measured with Quick E because they reacted too slowly compared to resorufin esters. We used three different reference compounds: resorufin acetate, resorufin isobutyrate, and resorufin pivalate. The increasing steric bulk of the acyl group slowed down the relative rate of reaction, allow-

Table 4. Enantioselectivity of the hydrolases towards selected chiral substrates measured by Quick E

Hydrolase	Substrate	Est. E[a]	Rate subs.[b]		Rate ref.[c]		Ref.[d]	Quick E
			R	*S*	*R*	*S*		
BTL2	**30**	3.3 (*R*)	12	4.0	1.7	2.3	*i*-But	7.9 (*R*)[e]
BTL2	**31**	3.2 (*R*)	78	26	26	42	Ac	4.9 (*R*)
OPL	**31**	1.4 (*R*)	15	12	140	130	Ac	1.3 (*R*)
OPL	**34**	1.3 (*S*)	15	21	0.37	0.39	Pv	1.5 (*S*)
OPL	**35**	6.9 (*R*)	44	30	0.19	0.11	Pv	1.5 (*R*)
OPL	**36**	2.3 (*S*)	0.064	0.087	17	18	*i*-But	1.4 (*S*)
OPL	**40**	1.0 (*S*)	5.7	8.4	0.23	0.39	Pv	1.5 (*S*)
PFE	**39**	8.3 (*S*)	16	19	4.3	4.3	Ac	12 (*S*)[f]
PFE	**40**	2.2 (*R*)	57	27	4.0	3.3	Pv	1.8 (*R*)
SDE	**39**	13 (*S*)	0.9	8.2	1.5	2.4	Pv	5.6 (*S*)

[a] Estimated enantioselectivity from Table 3. This is the ratio of the initial rates of hydrolysis of the two enantiomers measured without competition.
[b] Initial rate of hydrolysis of the substrate in units/mg protein in the presence of resorufin ester.
[c] Initial rate of hydrolysis of the resorufin ester in units/mg protein in the presence of substrate.
[d] Different reference compounds were used: Ac = resorufin acetate, *i*-But = resorufin isobutyrate, Pv = resorufin pivalate.
[e] The concentration of the slow-reacting (*S*)-enantiomer was 2 mM in the assay to improve accuracy.
[f] The concentration of the slow-reacting (*R*)-enantiomer was 10 mM to improve the accuracy. Also, the BES buffer concentration was 5 mM, also to increase accuracy. A check using the endpoint method yielded an enantioselectivity of 13±2 (mean and standard deviation for four measurements). Representative data: 35.5% conversion, 43.6% *e.e.* for remaining starting material, 79.3% *e.e.* for product; these data correspond to $E = 13.2$.

ing us to measure the enantioselectivity of slower substrates. For BTL2, the pivalate did not react at all, so the slowest available reference compound was resorufin isobutyrate.

Since the earlier general ester screening showed that BTL2 prefers longer acyl chains, we prepared solketal octanoate, **31**, and added it to the chiral library. As expected, the rate of reaction increased more than 10-fold: 2.6 units/mg protein for the octanoate as compared to 0.18 for the butyrate. The enantioselectivity decreased slightly with $E = 4.9$ for the octanoate as compared to 7.9 for the butyrate. We confirmed the accuracy of this result using the endpoint method, detailed in Table 5. A small-scale resolution of racemic solketal octanoate yielded the remaining starting material with 40.1% *e.e.* and product with 53% *e.e.* at 43.1% conversion corresponding to $E = 4.5$. The mean of five measurements was 4.4 with a standard deviation of 0.4, in good agreement with the Quick E measurement.

Changing the organic co-solvent increased the rate of BTL2-catalysed hydrolysis, but to a lesser extent. The screening solution contained 7 vol% acetonitrile to dissolve the substrate and, when needed, the reference compound. Replacing acetonitrile with *iso*-propanol increased the rate of the BTL2-catalysed hydrolysis of tributyrin 1.6-fold. Several other solvents showed more modest changes; with acetone a 1.1-fold rate increase was seen, whilst with tetrahydrofuran led to a 1.3-fold increase in the rate.

Although the first stage screening showed no reaction for 1-phenylethyl butyrate, **35**, many other lipases showed high enantioselectivity toward 1-phenylethyl esters. For this reason, we measured the enantioselectivity of BTL2 toward 1-phenylethyl esters using traditional methods. Although the reactions were very slow ($\sim 10^5$–10^6 times slower than tributyrin), BTL2 did catalyse both hydrolysis of the 1-phenylethyl acetate and acylation of 1-phenylethanol with vinyl acetate. In

Table 5. Enantioselectivity of BTL2 toward solketal, 1-phenylethanol, and 1-phenylpropanol

Reactants	Conv. (%)	% *e.e.* S	% *e.e.* P	E	Time (h)
Solketal octanoate/H_2O	43	40	53	4.4 ± 0.4[a]	1[b]
1-Phenylethanol/vinyl acetate	43	74	100	>100	92
1-Phenylethanol/vinyl butyrate	43	75	100	>100	91
1-Phenylpropanol/vinyl acetate	21	26	100	>100	91
1-Phenylethyl acetate/H_2O	41	69	100	>100	93

[a] Mean and standard deviation for five measurements. Data are for one of these measurements.
[b] Different amounts of enzyme were used so the times cannot be directly compared.

both cases the enantioselectivity was excellent, $E>100$ (Table 5). BTL2 catalysed the acylation of 1-phenylpropanol with similar rates and with excellent enantioselectivity.

BTL2 catalysed hydrolysis of one β-lactone and two δ-lactones, but with low estimated enantioselectivity (est. $E=1.1$–1.4). We checked the enantioselectivity of BTL2 toward several γ- and δ-lactones using traditional screening, but the enantioselectivity was also low. For the γ-lactones, the enantiomeric purity of the unreacted lactone was as follows (conversion not determined): γ-valerolactone no reaction, γ-octalactone 2% *e.e.*, γ-nonalactone 2% *e.e.*, γ-decalactone 4% *e.e.*, γ-undecalactone 7% *e.e.*, γ-dodecalactone 12% *e.e.* For the δ-lactones the enantiomeric purity of the unreacted lactone was as follows (50% conversion): δ-octalactone 0% *e.e.*, δ-nonalactone 0% *e.e.*, δ-decalactone 5% *e.e.*, δ-undecalactone 5% *e.e.*, δ-dodecalactone 10% *e.e.* Enantioselectivity in the BTL2-catalysed hydrolysis of lactones was found to be so low as to be of no practical use.

2.4. Enantioselectivity of OPL

In the OPL-catalysed hydrolysis of a more limited range of chiral esters, at least one enantiomer reacted for only 14 of the 23 pairs. By far the fastest substrate was solketal octanoate, **31**. Most of the esters in the chiral library were acetates and butyrates. Screening with the general ester library showed these are poor substrates for this lipase.

OPL showed the highest estimated enantioselectivities toward 1-phenylethyl butyrate, **35** (estimated $E=6.9$ favouring the (*R*)-enantiomer), and methyl lactate, **43** (estimated $E=10$ favouring the (*S*)-enantiomer). OPL showed low estimated *E*s toward solketal butyrate, **30** (estimated $E=2.9$), and menthyl acetate, **36** (estimated $E=2.3$). True enantioselectivities measured by Quick E were low, all <2: solketal octanoate, **31**, glycidyl butyrate, **34**, 1-phenylethyl butyrate, **35**, menthyl acetate, **36**, and methyl 2-chloropropionate, **40**. The true enantioselectivities for solketal butyrate, **30**, and methyl lactate, **43**, could not be measured because they reacted too slowly compared to the reference compounds. Thus, OPL showed low enantioselectivity toward all the substrates that we tested.

2.5. Enantioselectivity of PFE and SDE

PFE and SDE catalysed the hydrolysis of a limited number of esters in the chiral ester library. For PFE at least one enantiomer reacted for only nine of the 21 pairs and for SDE only eight of the 21 pairs. (Two esters were not tested with the esterases.) Neither PFE nor SDE catalysed hydrolysis of chiral lactones or most of the chiral carboxylic derivatives. PFE did not catalyse hydrolysis of the two 3-hydroxy butyrate esters **48** and **49**. Bornscheuer et al. also found that PFE did not catalyse the hydrolysis of a related 3-hydroxy ester, but used directed evolution to find a PFE mutant that did.[14]

Methyl 3-bromo-2-methyl propionate, **39**, showed the highest estimated enantioselectivity: 8.3 for PFE and 13 for SDE, both favouring the (*S*)-enantiomer. The true enantioselectivity was a moderate 12 for PFE and 5.6 for SDE. Small-scale resolutions of racemic **39** with PFE, where the enantiomeric purity of the remaining starting material and product were measured by gas chromatography, showed an enantioselectivity of 13±2, in good agreement with the value measured by Quick E.

In addition, the estimated enantioselectivity was >1.1 for 2-methylglycidyl 4-nitrobenzoate, **33**, with SDE, >1.1 for 1-phenylethyl butyrate, **35**, with both PFE and SDE, and 2.2 for methyl 2-chloropropionate, **40**, with PFE. The true enantioselectivity of PFE toward **40** was only 1.8. The true enantioselectivities for 2-methylglycidyl 4-nitrobenzoate, **33**, and 1-phenylethyl butyrate, **35**, could not be measured because they reacted too slowly compared to the reference compounds. However, previous work using traditional methods measured an enantioselectivity of these esterases toward a related compound, 1-phenylethyl butyrate. PFE showed an enantioselectivity >100,[8] while SDE showed an enantioselectivity of only 3.3.[11]

3. Discussion

Mapping the substrate selectivity of hydrolases using the Quick E screening was dramatically faster than traditional methods. Once the library was assembled, both the general and chiral library could be screened for all four hydrolases in one or two afternoons. Using traditional methods, such a mapping would require at least one month. There are at least four reasons for this dramatic increase in throughput. Most importantly, the Quick E screening eliminates the need to measure enantiomeric purity. Measuring the enantiomeric purity for 23 different substrates and products would require at the very least several different expensive chiral HPLC and GC columns and each measurement would require at least one hour; secondly, Quick E screening measures only initial rates (>5% conversion) so there is no need to wait for the reaction to reach 30 or 40% conversion; thirdly, the colorimetric method allows 96 reactions to be followed simultaneously using a microplate reader. Finally, the small scale of the reaction (typically 0.1 μmol) allows the use of more enzyme per mole of substrate and thus the reaction is faster.

The only limitation of substrate mapping with Quick E is that the true enantioselectivity for very slowly reacting substrates cannot be measured. This arises from the relative reactivity of the substrate and the resorufin ester used as a reference compound. For poorly reactive substrates, only the resorufin ester reacted; thus, we could not measure the relative rate. Utilizing several different resorufin esters—acetate, isobutyrate, and pivalate—minimized this problem. The increasing steric bulk of the acyl group slowed down the reaction rate,

Table 6. Summary of substrate selectivity

Enzyme	Information
BTL2	Low activity at room temperature For small esters, both activated and unactivated esters react at similar rates Favours longer acyl chains, e.g. *n*-octanoate and *n*-decanoate vinyl esters High enantioselectivity toward 1-phenylethanol and 1-phenylpropanol, but slow reaction Moderate enantioselectivity toward primary alcohols, e.g. solketal butanoate and octanoate Catalyses hydrolysis of lactones, but with low enantioselectivity
OPL	High activity toward hydrophobic substrates Strongly favours long acyl chains, e.g. *n*-octanoate and *n*-decanoate vinyl esters Low enantioselectivity May show enantioselectivity toward methyl lactate
PFE	Favours vinyl esters and medium chains (some preference to C4) and activated esters (vinyl, α-halo) High enantioselectivity toward 1-phenylethanol Moderate enantioselectivity toward methyl (*S*)-3-bromo-2-methylpropionate and methyl (*R*)-2-chloropropionate
SDE	Favours vinyl esters and long chains (some preference to C6) and activated esters (vinyl, α-halo) Moderate enantioselectivity toward methyl (*S*)-3-bromo-2-methylpropionate

so pivalate often served as a reference compound for substrates with low reactivity. To further extend the range of Quick E, a wider range of reference compounds is required, especially deactivated ester substrates. Several new reference compounds are currently being evaluated.[15] Otherwise, the mapping gave a good overview of the selectivity of each hydrolase and identified several interesting, though not yet useful, reactions. An alternative is to identify which substrates react using pH indicators coupled with standard GC methods to measure the actual enantioselectivity.[16]

A summary of the substrate mapping (Table 6) provides useful guidelines for applications of these hydrolases. As expected, the lipases usually favoured compounds carrying longer chain acyl groups, while the esterases preferred esters with short chain acyl groups. It may be useful to use longer chain acyl donors, e.g. vinyl octanoate, instead of shorter chain acyl donors such as vinyl acetate. The apparent acyl chain length selectivity changed dramatically with the nature of the leaving group. This inconsistency is most likely due to the fact that we measure not the true selectivity with a competitive experiment, but an apparent (or estimated) selectivity using separately measured rates. Establishing the true acyl chain selectivity of these lipases could be accomplished using an appropriate reference compound, but was not a goal of this research.

BTL2 catalysed the slow hydrolysis of solketal butyrate with moderate enantioselectivity, $E=7.9$, favouring the (*R*)-enantiomer. By increasing the length of the acyl chain to octanoate, the rate increased 10-fold with similar enantioselectivity, $E=4.9$. Other ways to increase the reaction rate were to increase the temperature or to change the co-solvent from acetonitrile to *iso*-propanol. Although this enantioselectivity is too low for preparative use, it represents a good starting point for directed evolution. Indeed, directed evolution has yielded several more enantioselective mutants.[17]

OPL showed good activity toward a wide range of unnatural substrates, but low enantioselectivity. The use of acetonitrile co-solvent in our assay may have reduced the enantioselectivity of OPL, since OPL often denatures in the presence of organic co-solvents. One possible application of this lipase could be the deprotection of synthetic intermediates under very mild conditions.

Both PFE and SDE showed moderate enantioselectivity toward methyl 3-bromo-2-methylpropanoate. Synthetic chemists have used this important chiral synthon to prepare retroviral protease inhibitors,[18] unnatural amino acids,[19] cyclooxygenase inhibitors,[20] captopril (a treatment for high blood pressure)[21] and alkaloids.[22] Although this moderate enantioselectivity is insufficient for preparative use, we are currently increasing the enantioselectivity using directed evolution. Other hydrolases also show moderate enantioselectivity toward this acid.[21] Henke and Bornscheuer increased the enantioselectivity of PFE toward chiral 3-phenylbutanoic acid using directed evolution.[23]

In spite of the unusual structure of the active site of SDE, its stereoselectivity was similar to that of PFE. The only major difference between PFE and SDE was in the enantioselectivity toward 1-phenylethyl acetate, where the PFE mediated reaction was highly enantioselective, whilst SDE showed low enantioselectivity for this substrate.

4. Experimental

4.1. General

Unless otherwise noted BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer (1.0 mM, pH 7.20) was used for screening.

4.2. Enzyme solutions

Lipase from *O. piliferum* (NRRL 18917) was prepared as previously described[4] and diluted 10-fold with BES buffer for screening. SDE[11] and PFE[8] were prepared as previously described and lyophilized. The lyophilized powder was dissolved in BES buffer (7 mg powder/mL, 1 mM BES) and the pH was readjusted to 7.2. Protein assay (BioRad dye binding assay calibrated with bovine serum albumin) showed a protein concentration of 5 mg protein/mL. A 1:10 dilution of this solution was used for both Quick E measurement and the scale-up reactions. BTL2 was expressed in *E. coli* JM105 as described previously.[2] After growing at 30°C to an

OD_{600} of 1.0, the culture was heated to 42°C for 3 h to induce lipase expression. (A heat shock promoter controlled the lipase expression.) The culture was centrifuged (4°C, 6000×*g*, 15 min) and the cells were washed twice with BES buffer (1 mM, pH 7.20). The bacterial pellet was resuspended in BES buffer (5 mL), sonicated (2 min on ice), and centrifuged to remove cell debris (4°C, 6000×*g*, 10 min). The supernatant was adjusted to pH 7.20 and used in screening.

4.3. Ester library

Unless otherwise noted esters for the substrate library were purchased from Aldrich (Oakville, ON) or Fluka (Oakville, ON). Resorufin esters, solketal butyrates **30** and **31** and 1-phenylethyl butyrate **45** were available from previous work.[1] Stock solutions of ester (100 mM in acetonitrile) were stored at −20°C. These stock solutions were diluted to 14.3 mM for preparing the screening solution.

4.4. Screening

Rates of ester hydrolysis both with and without a reference compound were measured colorimetrically as described previously.[1] The reaction mixtures contained 1.0 mM substrate, but the buffer concentration was reduced from the recommended 5.0 mM BES to 1.0 mM BES to increase the sensitivity of the assay, while compromising some accuracy.

4.5. (*R*)- and (*S*)-solketal octanoate

Octanoyl chloride (13 mmol, 2.1 mL) was added dropwise to a cold (ice bath), stirred solution of (*R*)- or (*S*)-solketal (5.0 mmol, 660 mg) and pyridine (9.0 mmol, 750 μL) in anhydrous diethyl ether (20 mL). After stirring for an additional 2 h, TLC (silica gel, 8:2 hexane/ethyl acetate) showed no remaining solketal. The mixture was washed with water, saturated sodium bicarbonate, and brine and dried over magnesium sulfate. Flash chromatography (silica gel, 8:2 hexane/ethyl acetate) yielded a clear oil: 63–72% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, 3H, octanoyl CH_3), 1.28 (m, 8H, octanoyl CH_2), 1.36 (s, 3H, solketal CH_3), 1.42 (s, 3H, solketal CH_3), 1.61 (m, 2H, octanoyl CH_2), 2.33 (t, 2H, octanoyl CH_2), 3.72 (m, 1H, 1H of CH_2), 4.0–4.27 (m, 3H, 1H of CH_2, CH_2), 4.30 (m, 1H, CH); MS (CI, NH_3) *m/z:* 259 ($M+H^+$, 5); 243 (81); 201 (50); 127 (47); 101 (100); 82.9 (36); 72 (42). The enantiomeric purities were ≥98% *e.e.* as determined by GC (Chiralsil-DEX CB, 25 m×0.25 mm, Chrompack, Raritan, NJ) 120°C, $k_R = 36.2$, $k_S = 36.5$ and $\alpha = 1.01$.

4.6. Enantioselectivity of BTL2 toward solketal octanoate 31

A BTL2 solution was added to a suspension of (±)-solketal *n*-octanoate (13.5 mg, 0.0522 mmol) in BES buffer (50 mL, 10.0 mM, pH 7.2) containing 7.0 vol% acetonitrile. The mixture was stirred rapidly and the pH was maintained at 7.2 by the addition of NaOH (0.10 M) controlled by a pHstat. When the consumption of base indicated 25–75% conversion, the mixture was extracted with ethyl ether. The extracts were dried, concentrated and analysed by gas chromatography on the column noted above. Enantiomers of both solketal ($\alpha = 1.03$, $k_R = 6.47$, $k_S = 6.64$) and solketal octanoate ($\alpha = 1.03$, $k_S = 24.2$, $k_R = 24.8$) were separated using the following temperature program: initially 20 min at 100°C, then increased to 150°C over 10 min, then held at 150°C for 30 min. Data are shown in Table 5.

4.7. Enantioselectivity of PFE toward methyl 3-bromo-2-methylpropionate 39

The enzyme solution (100 μL in 1 mM BES, pH 7.2) was added to a solution of (±)-methyl 3-bromo-2-methylpropionate (5.0 mM) in BES buffer (1.0 mM, pH 7.2, 10 mL) containing 7 vol% acetonitrile and Triton X-100 (0.3 mM). The mixture was stirred rapidly and the pH was maintained at 7.2 by the addition of NaOH (0.10 M) controlled by a pHstat. The reaction vessel was sealed with parafilm to minimize evaporation of acetonitrile. When the consumption of base indicated 40% conversion, the mixture was extracted with ethyl acetate to remove the unreacted ester. The extracts were dried, concentrated, dissolved in acetonitrile and analysed by gas chromatography on the column noted above: isothermal at 95°C, $k_S = 11.8$, $k_R = 12.3$. The aqueous phase containing the product acid was acidified to pH 1 or 2 with conc. HCl and extracted with ethyl acetate. The extracts were dried and concentrated by rotary evaporation. The residue was dissolved in methanol (1–2 mL containing 5–6 drops of conc. H_2SO_4) and refluxed under nitrogen for 1–2 h. The reaction was then neutralized with NaOH (1N) to pH 6–7 and ethyl actetate (7 mL) and water (5 mL) were added, and the phases separated. The organic layer was washed with water (5 mL), which was then added to the aqueous fraction. The aqueous fraction was washed with ethyl acetate (3×3 mL) and the washes were added to the organic fraction, which was then dried over $MgSO_4$, and concentrated. The resulting methyl ester was dissolved in acetonitrile and analysed by gas chromatography as above. Data are shown in the footnotes of Table 4.

4.8. Enantioselectivity of BTL2 toward 1-phenylethanol and 1-phenylpropanol

The hydrolysis of racemic 1-phenylethyl acetate (0.3 mmol) was performed in sodium phosphate buffer (1 mL, 50 mM, pH 7.5) and toluene (5 mL). Acylation of 1-phenylethanol and 1-phenylpropanol (0.3 mmol) was performed using vinyl acetate or vinyl butyrate (1 mmol) in toluene (5 mL). Both reactions were accomplished at 40°C using 1000 U (based on pHstat assay using tributyrin) crude extract of BTL. Samples from the reaction mixture were centrifuged and, for hydrolysis experiments, extracted with methylene chloride (300 μL). The organic phase was analysed by gas chromatography (130°C isothermal, 60 kPa pressure, FS-Cyclodex β-I/P CS-Fused silica capillary column, 50 m×0.25 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) to give the enantiomeric excess

of both remaining starting material and product. The enantioselectivity was calculated as described by Chen et al.[24] Absolute configurations were assigned by comparison with pure enantiomers.

4.9. Enantioselectivity of BTL2 toward γ- and δ-lactones

The hydrolysis was carried out in phosphate buffer (0.1 M, pH 7) for 48 h and the enantiomeric excess of the remaining substrate was measured by gas chromatography on a chiral stationary phase as described previously.[25]

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