Quantitative Screening of Hydrolase Libraries Using pH Indicators: Identifying Active and Enantioselective Hydrolases

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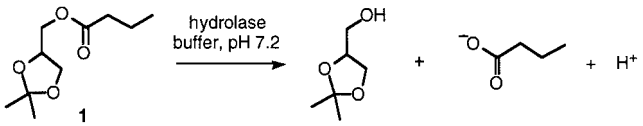
Abstract: The slowest step in finding a selective hydrolase for synthesis is often the screening step. Researchers must run small test reactions and measure the amounts of stereoisomers formed by HPLC, GC, or NMR. We have developed a colorimetric method to speed up this screening. We quantitatively detect ester hydrolysis using a pH indicator, 4-nitrophenol. We estimate the selectivity by measuring the initial rates of hydrolysis for pure stereoisomers separately. To demonstrate the utility of this method, we screened seventy-two commercial enzymes for enantioselective hydrolysis of racemic solketal butyrate, an important chiral building block. First, we eliminated the twenty hydrolases that did not catalyze hydrolysis of either enantiomer. Next, we measured initial rates of hydrolysis of the pure enantiomers of solketal butyrate. For horse-liver esterase, these initial rates differed by a factor of twelve. Subsequent GC experiments confirmed an enantiomeric ratio of fifteen for this hydrolase. Although this enantioselectivity is moderate, it is the highest enantioselectivity reported for a hydrolysis of solketal esters.

Keywords: enantiomeric resolution · enzyme catalysis · lipases · screening · synthetic methods

Introduction

Chemists often exploit the high stereoselectivity and regioselectivity of hydrolytic enzymes to solve synthetic problems.[1] For example, researchers use selective reactions catalyzed by lipases to prepare enantiomerically pure pharmaceutical intermediates and to selectively deprotect sensitive synthetic intermediates. One limitation to the wider use of hydrolases is the difficulty in finding the best hydrolase for a given reaction. Although several empirical rules[2] can assist in the selection of likely candidates from the fifty to one hundred hydrolases commercially available, most researchers also use screening. To screen for selective hydrolases today, researchers run a small reaction for each hydrolase, work up the reaction, and determine the ratio of stereoisomers by HPLC, GC, or NMR.[3] At best, one determination takes four hours. To save time researchers rarely screen all commercial hydrolases and likely miss good hydrolases.

To speed up this screening, we have developed a quantitative, colorimetric assay for hydrolysis of esters using pH indicators. Hydrolysis of an ester at neutral pH, for example solketal butyrate (**1,** butyryl ester of 2,2-dimethyl-1,3-dioxolane-4-methanol), releases a proton (Scheme 1). We measure the rate of proton release using a pH indicator.



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Scheme 1. Hydroylsis of solketal butyrate at neutral pH.

Researchers have used pH indicators to monitor the progress of enzyme-catalyzed reactions that release or consume protons since the 1940's.[4, 5] For example, researchers have monitored reactions catalyzed by amino acid decarboxylase,[6] carbonic anhydrase,[7] cholinesterase,[8] hexokinase,[9, 10] and proteases.[11] Many researchers either used a pH indicator assay qualitatively or calibrated the color change with additional experiments.

However, by choosing the reaction conditions carefully, one can ensure that the color change is proportional to the number of protons. In particular, both the buffer and the indicator must have the same affinity for protons (pK_as within 0.1 unit of each other) so that the relative amount of protonated buffer to protonated indicator stays constant as the pH shifts during the reaction. Researchers defined the proportionality between the rate of indicator absorbance change and reaction rate as the buffer factor, Q.[6, 7, 12] When the pK_as of the indicator and buffer are the same, Q is given by Equation (1), where C represents the total molar concentration (sum of acid

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0947-6539/98/0411-2324 $ 17.50+.50/0

and base forms) of buffer (B) or indicator (In), $\Delta\varepsilon$ represents the difference in extinction coefficient between the protonated and deprotonated forms of the indicator, and l represents the path length. The true reaction rate is given by Equation (2), where dA/dt is the rate of indicator absorbance change. The highest sensitivity (largest dA/dt) occurs when Q is small. Thus, lowering the buffer concentration or increasing the indicator concentration increases the sensitivity of the assay.

$$Q = \frac{C_B}{C_{In}} \times \frac{1}{\Delta\varepsilon_{404\,nm} l} \quad (1)$$

$$\text{rate } (\mu\text{mol min}^{-1}) = \frac{dA}{dt} \times Q \times \text{reaction volume} \times 10^6 \quad (2)$$

We used this assay to screen for enantioselective hydrolases in 96-well microplates (Figure 1). Using pure enantiomers, we separately measured the initial rates of hydrolysis of each enantiomer. Hydrolases that showed large differences in the initial rates of hydrolysis of the two enantiomers were further analyzed by traditional methods to determine enantioselectivities.[3] Note that the ratio of separately measured initial rates of hydrolysis of the enantiomers is not the true enantioselectivity, so this screening provides only an estimated enantioselectivity.[13] To minimize the amount of pure enantiomers needed, we first screened using racemic substrate to eliminate hydrolases that did not catalyze hydrolysis of the racemic substrate. Whittaker et al. measured the esterase activity of proteases in 96-well microplates using a similar assay.[11] However, their assay required additional calibration experiments because it did not use an indicator–buffer pair with the same pK_a values.

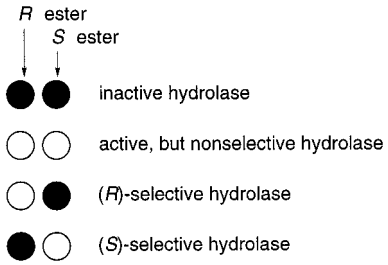

Figure 1. Schematic diagram of the colorimetric screen for enantioselectivity. The circles represent wells in a microplate containing either the *R* or the *S* enantiomer. Hydrolysis of an ester releases acid that decolorizes the pH indicator. Dark circles represent no reaction, while the white circles represent wells in which hydrolysis occurred. This diagram exaggerates the color change; in practice the color change is not visible to the eye. The hydrolase is rejected if neither enantiomer reacts or if both enantiomers react at similar rates. If one enantiomer reacts significantly faster than the other, then the hydrolase is tested further. Note that measuring the rates of hydrolysis of pure enantiomers separately gives only an estimated enantioselectivity, not the true enantioselectivity (see ref. [13]).

To demonstrate this method, we screened a library of seventy-two commercial hydrolases (lipases, esterases, and proteases) for enantioselective hydrolysis of solketal butyrate, an important chiral building block in the synthesis of pharmaceuticals and biologically active compounds.[14] Many researchers have searched, without success, for a highly enantioselective hydrolase that could resolve this substrate.[15] For hydrolysis in water, the highest enantiomeric ratio was 9 for a proteinase from *Aspergillus oryzae*,[16] while for acylation of solketal in organic solvent, the highest enantiomeric ratio was 20–25 for a lipase from *Pseudomonas* species (lipase AK).[15] Our screening, which is easily completed in one afternoon, has identified a new hydrolase, horse-liver esterase, with an enantiomeric ratio of 15 for the hydrolysis of solketal butyrate. This is the highest enantioselectivity yet reported for hydrolysis of a solketal ester.

Results

Optimizing sensitivity of the assay: Since most hydrolases have maximal activity near neutral pH, we developed the assay for pH 7.2. As a pH indicator, we chose 4-nitrophenol. The similarity of its pK_a (7.15[17]) to the pH of the reaction mixture ensures that changes in pH give a large and linear color change.[6] The large difference in the extinction coefficients of the protonated and deprotonated forms (200 versus $18000\,M^{-1}cm^{-1}$ at 404 nm) gives good sensitivity.[18] Finally, nitrophenols bind less strongly to proteins than some polyaromatic indicators.[19] The concentration of the pH indicator should be as high as possible to maximize sensitivity [Eq. (1)]. In our assay, the high initial absorbance of 4-nitrophenoxide/4-nitrophenol limited the concentration to 0.45 mM.[20] This concentration gave a starting absorbance of ≈ 1.2, where the accuracy is still not compromised by low light levels.

As a buffer, we chose BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] because its pK_a (7.15[21]) is identical to that of 4-nitrophenol. This ensures that changes in proton concentration during the reaction give linear changes in absorbance.[22] We empirically determined an optimum buffer concentration of approximately 5 mM (see below). This value is a compromise between low buffer concentrations to maximize sensitivity [Eq. (1)], and high buffer concentrations to ensure accurate measurements and small pH changes throughout the assay (< 0.05 pH units for 10% hydrolysis under our conditions). The small pH changes are important because kinetic constants can change with changing pH.

Suitable substrate concentrations ranged from 0.5 to 2 mM, typically 1 mM. At substrate concentration below 0.5 mM, the absorbance changes are too small to be detected accurately under our standard conditions. For example, hydrolysis of 5% of a 0.25 mM substrate concentration under our standard conditions (pH 7.2, 0.45 mM 4-nitrophenol, 5 mM BES), changes the absorbance by only 0.005 absorbance units. Solubility in water sets the upper limit of substrate concentration because spectrophotometric measurements require clear solutions. Typical organic substrates dissolve poorly in water, so we added organic cosolvent—7 vol% acetonitrile. For very insoluble substrates, we used previously prepared clear emulsions with detergents.[23]

Quantitative validation of the assay: To confirm that the color changes accurately measured the release of protons, we experimentally determined the factor Q and compared it with the theoretical Q, calculated using Equation (1). First, we mimicked the proton release upon hydrolysis of the substrate

by addition of HCl, Figure 2. The absorbance decreased linearly owing to protonation of the 4-nitrophenoxide. The reciprocal of the slopes corresponds to the buffer factor, Q,

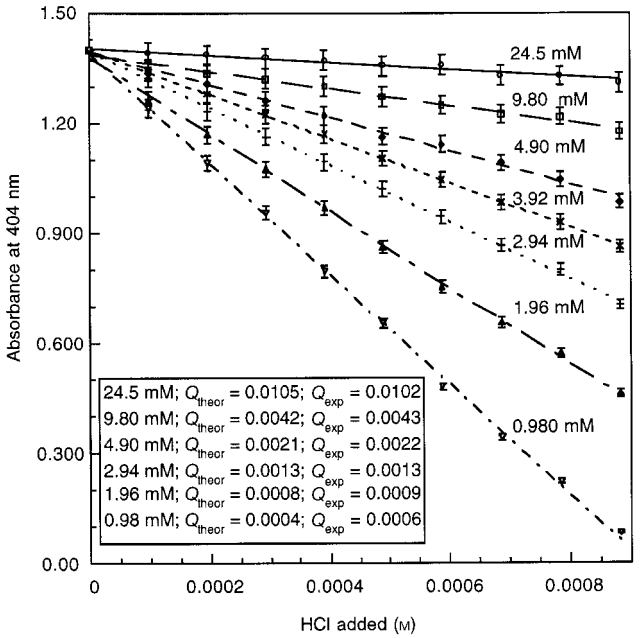

Figure 2. Sensitivity of the assay solution to added acid. The yellow color of the assay solution decreased linearly (regression factor = 0.97 for 24.5 mM; > 0.99 for all other concentrations) in each solution. For a given amount of acid, solutions with lower concentrations of buffer showed larger color changes. For buffer concentration above 2 mM, the experimentally measured slopes were within 5% of the theoretical slopes calculated by means of Equation (1) (where $\Delta\varepsilon = 17800\,\text{M}^{-1}\text{cm}^{-1}$, $l = 0.292$ cm, $C_{In} = 0.447$ mM). For buffer concentrations below 2 mM, the experimental and theoretical slopes disagreed by > 10%. We chose a buffer concentration of 5 mM to maximize sensitivity without compromising accuracy. Each point is an average of four measurements, with variation < 3% between each measurement. All lines are normalized to a starting absorbance of 1.4.

calculated by means of Equation (1). As expected, the decreases were all linear and the slopes increased with decreasing buffer concentration. However, below 2 mM buffer, the experimentally measured slopes disagreed with the theoretical slopes by more than 10%. We chose 5 mM as the buffer concentration for our assay as a compromise between accuracy and sensitivity.

Small changes in reaction conditions did not compromise the sensitivity or accuracy of this assay. The measured value of Q did not change by more than the experimental error ($\approx$5%) upon addition of 7% of acetonitrile or dimethyl sulfoxide. The measured value of Q also was not changed by unknown buffer salts in the commercial hydrolases or by added $CaCl_2$ (2 mM) in the stock solutions of proteases.

As a test reaction, we monitored the hydrolysis of racemic solketal butyrate catalyzed by horse-liver esterase. The decrease of the indicator absorbance was linear (Figure 3a), and corresponded to a specific activity of 1.85 μmol min^{-1} mg^{-1} protein.[24] Control experiments with no substrate or with no esterase showed no change in absorbance over one hour. When we scaled up the reaction a hundredfold and monitored the reaction with a pHstat, we

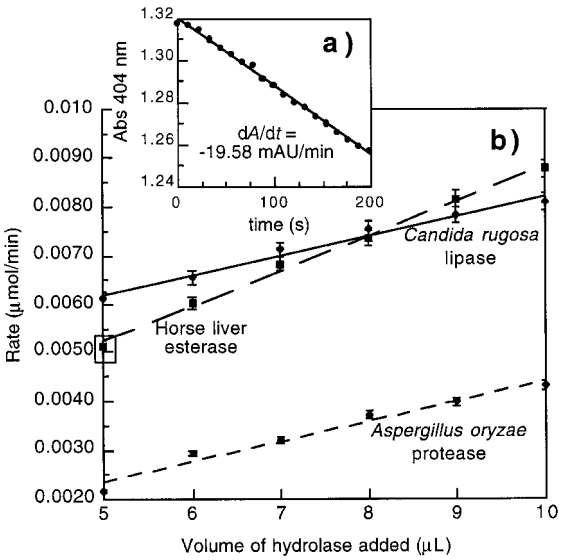

Figure 3. a) Initial rate measurement for boxed point showing the measured absorbance change in the horse-liver esterase-catalyzed hydrolysis of (±)-solketal butyrate with 5 μL hydrolase solution. The calculated rate [Eq. (2)] equals 0.0050 μmol min^{-1} and the specific activity equals 1.85 μmol min^{-1} mg^{-1} protein. The measured points fit a straight line with regression factor of 0.997. b) Increased rates of hydrolysis of 1 mM (±)-solketal butyrate with increased amount of hydrolase. Rates are calculated over 200 seconds by means of Equation (2), where $\Delta\varepsilon = 17300\,\text{M}^{-1}\text{cm}^{-1}$, $l = 0.365$ cm (final volume was 125 μL), $C_B = 4.70$ mM, $C_{In} = 0.365$ mM, 5.9% acetonitrile. The measured points fit a straight line with regression factor of 0.97 for each hydrolase. Each point is an average of four initial rate measurements, which differed by < 2%.

measured a higher specific activity, 4.99 μmol min^{-1} mg^{-1} protein. We attribute the difference to activation by the rapid mechanical stirring in the pHstat experiment.

Reaction rates increased linearly with the amount of enzyme added for three typical hydrolases, one from each class of hydrolases in our library, indicating that the enzyme-catalyzed reaction rates determined with this assay are proportional to the total enzyme concentration; see Figure 3b.

Screening for hydrolases enantioselective for solketal butyrate: To demonstrate the utility of our method, we screened a library of seventy-two commercial hydrolases for enantioselective hydrolysis of (±)-solketal butyrate. All hydrolases were dissolved in the assay buffer, 5 mM BES buffer at pH 7.2. Since some solid hydrolase preparations contain buffer salts and extenders, the pH of each solution was checked and readjusted to pH 7.2 when necessary. First, we screened with racemic solketal butyrate to eliminate hydrolases that did not catalyze hydrolysis of either enantiomer. This screen eliminated the twenty hydrolases listed in note [a] of Table 1.

Next, we estimated the enantioselectivity of the remaining fifty-two hydrolases for solketal butyrate by separately measuring the initial rates of hydrolysis of the pure enantiomers, Table 1. We used the ratio of these rates as an estimated enantioselectivity.[25] Note that the ratio of these rates is not the true enantioselectivity, or enantiomeric ratio E, because we measured the rates of hydrolysis of the enantiomers separately. Nine hydrolases showed estimated enantioselectivity $\geq$4. The seven lipases and proteases

0947-6539/98/0411-2326 $ 17.50+.50/0 Chem. Eur. J. 1998, 4, No. 11

Table 1. Activity of commercial hydrolases towards (±)-solketal butyrate and its enantiomers.

Source of active hydrolase[a]	Wt.[b]	Prot.[c]	Supplier	Activity (±)[d]	Activity (*R*)[d]	Activity (*S*)[d]	Estimated *E*[e]
Lipases							
Aspergillus niger	30	0.61	[f]	0.029	1.72	2.01	1.17 (*S*)
Aspergillus oryzae	**7.1**	**4.5**	**[g]**	**0.039**	**0.157**	**0.0317**	**4.95 (*R*)**
Candida antarctica lipase A	34	4.9	[h]	0.035	0.066	0.036	1.83 (*R*)
Candida antarctica lipase B	29	3.9	[i]	1.15	0.667	0.556	1.20 (*R*)
Candida lipolytica	35	0.23	[f]	0.114	0.381	0.340	1.12 (*R*)
Candida rugosa	31	0.35	[j]	2.76	4.25	1.85	2.30 (*R*)
Candida rugosa (cylindracea)	37	0.71	[k]	2.03	3.06	1.77	1.73 (*R*)
Humicola sp.	13	4.0	[h]	0.027	0.372	0.147	2.53 (*R*)
Penicillin camembertii	86	0.92	[f]	0.060	0.189	0.498	2.63 (*S*)
Penicillin roquefortii	57	0.74	[f]	0.151	0.844	1.23	1.46 (*S*)
Pseudomonas cepacia	**31**	**3.1**	**[f]**	**0.038**	**0.085**	**0.021**	**4.05 (*R*)**
Pseudomonas fluorescens	5.2	0.73	[g]	0.091	0.572	0.565	1.01 (*R*)
Rhizopus javanicus	**44**	**2.7**	**[f]**	**0.063**	**0.286**	**0.066**	**4.33 (*R*)**
Rhizopus oryzae	**53**	**4.1**	**[f]**	**0.040**	**0.124**	**0.011**	**11.3 (*R*)**
Thermus aquaticus	1.1	0.29	[g]	0.148	0.665	0.606	1.10 (*R*)
Esterases							
Acetylcholine esterase	0.26	0.18	[j]	0.745	7.29	5.42	1.35 (*R*)
Bacillus sp.	1.1	0.58	[g]	0.112	0.532	0.180	2.96 (*R*)
Bacillus stearothermophilus	1.1	0.57	[g]	0.066	2.77	1.65	1.68 (*R*)
Bacillus thermoglycosidasius	0.82	0.76	[g]	0.065	1.22	0.470	2.59 (*R*)
Bovine cholesterol esterase	9.8	0.99	[l]	0.402	1.43	1.73	1.21 (*S*)
Candida lipolytica	3.5	1.4	[g]	0.056	0.244	0.306	1.25 (*S*)
Cutinase	2.1	1.1	[m]	2.38	10.0	4.40	2.28 (*R*)
E001	0.40	0.14	[n]	2.08	11.4	7.65	1.49 (*R*)
E002	0.38	0.16	[n]	1.27	1.39	0.721	1.93 (*R*)
E003	1.0	0.23	[n]	2.91	4.81	3.64	1.32 (*R*)
E004	1.0	0.29	[n]	2.21	3.24	3.40	1.05 (*S*)
E005	1.0	0.27	[n]	1.88	1.38	1.10	1.25 (*R*)
E006	0.64	0.13	[n]	3.59	12.3	8.80	1.40 (*R*)
E007	2.1	0.97	[n]	1.26	1.22	1.35	1.11 (*S*)
E009	1.0	0.37	[n]	2.65	4.66	4.01	1.16 (*R*)
E010	1.0	0.26	[n]	1.56	4.19	3.01	1.39 (*R*)
E011	0.80	0.22	[n]	3.44	4.21	7.30	1.73 (*S*)
E013	1.0	0.24	[n]	0.869	0.860	0.843	1.02 (*R*)
E014	1.0	0.31	[n]	0.136	0.875	0.498	1.76 (*R*)
E016	1.0	0.26	[n]	1.22	1.76	0.960	1.83 (*R*)
E017b	1.0	0.33	[n]	1.12	0.694	0.620	1.12 (*R*)
E018	2.0	0.76	[n]	0.135	0.279	0.548	1.96 (*S*)
E019	0.60	0.20	[n]	3.70	6.45	7.79	1.21 (*S*)
E020	0.44	0.16	[n]	4.97	8.35	9.21	1.10 (*S*)
Pig-liver esterase	**–**	**0.09**	**[j]**	**71.9**	**3.03**	**14.9**	**4.91 (*S*)**
Pig-liver esterase	0.36	0.49	[g]	5.79	2.26	6.17	2.73 (*S*)
Horse-liver esterase	**1.7**	**0.59**	**[g]**	**0.975**	**0.168**	**2.05**	**12.2 (*S*)**
Saccharomyces cerevisiae	1.2	0.26	[g]	0.085	0.219	0.074	2.96 (*R*)
Proteases							
Aspergillus oryzae	**29**	**7.0**	**[j]**	**0.097**	**0.157**	**0.032**	**4.91 (*R*)**
Aspergillus satoi	32	0.40	[j]	0.748	3.17	3.13	1.01 (*R*)
Bacillus licheniformis	5.2	2.1	[g]	0.335	0.261	0.083	3.14 (*R*)
Bac. subtilis var. Biotecus A	**4.2**	**1.7**	**[g]**	**0.351**	**0.221**	**0.0303**	**7.29 (*R*)**
Subtilisin Carlsberg	9.7	4.4	[j]	0.083	0.213	0.082	2.60 (*R*)
Streptomyces griseus	20.1	7.2	[o]	0.012	0.032	0.0135	2.37 (*R*)
Thermolysin, Type X	2.4	0.15	[j]	0.095	0.255	0.738	2.89 (*S*)
Proteinase, bacterial	**4.7**	**2.1**	**[g]**	**0.186**	**0.154**	**0.028**	**5.50 (*R*)**
Proteinase K	0.42	0.07	[g]	2.14	3.08	2.29	1.34 (*R*)

[a] The following hydrolases showed no detectable activity (<0.01 μmol min^{-1} mg^{-1} protein) towards racemic solketal butyrate: Lipases: Amano lipases from *Rhizopus stolonifer*, *Mucor javanicus* and *Mucor miehei*, wheatgerm lipase (Sigma), porcine pancreatic lipase (Biocatalysts), lipase from *Rhizopus niveus* (Boehringer Mannheim); Esterases: ThermoGen esterases E008, E012 and E015, Fluka esterases from *Thermoanaerobium brockii* and *Mucor miehei*; Proteases: α-chymotrypsin (Sigma), pepsin from porcine stomach (Fluka), subtilisin from *Bacillus licheniformis* (Fluka), thrombin from human plasma (Fluka), trypsin (Worthington, Freehold, NJ), Sigma proteases from *Bacillus polymyxa*, bovine pancreas type 1, papaya, *Streptomyces caespitosus*. [b] Amount (mg) of solid enzyme per mL of buffer in the stock solutions. [c] Protein concentration of stock solutions in mg protein mL^{-1} determined by the Bio-Rad assay using BSA as a standard. [d] Observed rate of hydrolysis in μmol min^{-1} mg^{-1} protein. Rates calculated by Equation (2) were divided by the protein content in the well. The values are an average of four measurements, which typically varied by less than 2%. [e] Ratio of the separately measured initial rates for the enantiomers. This ratio is *not* the true enantioselectivity, but is a useful estimate of the enantioselectivity. The absolute configuration of the faster reacting ester is in parentheses. Note that hydrolysis of the *R* ester yields the *S* alcohol due to a change in the priority of the substituents. [f] Amano Enzyme USA (Troy, VA). [g] Fluka Chemie (Oakville, ON). [h] Boehringer-Mannheim (Mannheim, Germany). [i] Novo-Nordisk (Baagsverd, DK). [j] Sigma-Aldrich (Oakville, ON). [k] Biocatalysts (Pontypridd, Mid-Glamorgan, Wales, UK). [l] Genzyme (Cambridge, MA). [m] Unilever Research Labs (Vlardingen, the Netherlands). [n] ThermoGen (Chicago, IL). [o] Calbiochem/Behrig Diagnostics (La Jolla, CA).

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favored the *R* ester, while the two esterases favored the *S* ester. The highest estimated enantioselectivities were found with horse-liver esterase (HLE, estimated enantioselectivity = 12), *Rhizopus oryzae* lipase (ROL, estimated enantioselectivity = 11), and protease from *Bacillus subtilis*, variation *Biotecus A* (BSP, estimated enantioselectivity = 7). Previous workers identified *Aspergillus oryzae* protease (AOP) as an enantioselective hydrolase.[16] This hydrolase was also among the nine enantioselective hydrolases (estimated enantioselectivity = 5). The identification of HLE, ROL, and BSP as enantioselective hydrolases for solketal butyrate are new results from this screening.

Although we used only the first 3–4 minutes of data in the calculations, we monitored the reactions for one hour to ensure that we did not miss slow hydrolases or hydrolases that show a lag time. All substrate/hydrolase solutions were prepared and measured in quadruplicate to ensure accuracy. The total screening time for seventy-two hydrolases in quadruplicate was 180 minutes plus several minutes between each plate to fill the 96-well plates. This time could be easily reduced to less than an hour with a shorter screening time. Complete screening of the library towards the racemate and its enantiomers is easily completed in an afternoon.

We also changed reaction conditions in the assay in an attempt to increase the estimated enantioselectivity for solketal butyrate. For example, the activity of hydrolases, especially lipases, often increases in the presence of an interface. We reasoned that this interfacial activation may also change the enantioselectivity. We screened the hydrolase library with Triton X-100 (a nonionic detergent) added to create micelles. The reaction rates increased for eight hydrolases (three lipases and five esterases), decreased for thirty-two hydrolases, and stayed constant for twelve hydrolases. Unfortunately, the estimated enantioselectivities remained unchanged or decreased slightly for the best hydrolases: AOP (decrease from 4.9 to 4.2), ROL (decrease from 11.3 to 9.6), HLE (decrease from 12.2 to 7.6), and BSP (decrease from 7.3 to 5.4). With nonselective hydrolases, the estimated enantioselectivities showed small increases or decreases. For example, subtilisin Carlsberg increased from 2.6 to 2.8, esterase from *Bacillus stearothermophilus* increased from 1.7 to 2.4, and *Aspergillus oryzae* lipase decreased from 4.95 to 1.8. Overall, the selectivities for solketal butyrate did not significantly change upon addition of Triton X-100.

To confirm these screening results, we measured the enantioselectivity of three selective hydrolases and three poorly selective hydrolases using the conventional endpoint method[3] (Table 2). Under conditions similar to those in the screening solutions (1 mM substrate, 7% acetonitrile as cosolvent), the true enantioselectivity and the estimated enantioselectivity agreed to within a factor of 2.3. Since 1 mM solketal butyrate is too dilute for practical preparative reactions, we also measured the enantioselectivity of these hydrolases at 50 mM solketal butyrate, where the reaction mixture contained insoluble droplets of substrate. The enantioselectivity under these conditions also agreed with the enantioselectivity estimate from screening to within a factor of 2.6.

The most enantioselective hydrolase was HLE, $E = 14.8$ and 9.7 at substrate concentrations of 1 mM and 50 mM, respectively. At 50 mM substrate without acetonitrile, the enantioselectivity of HLE declined slightly again to $E = 8.7$ ($c = 17.1\%$ after 2.5 h). These values agree well with the estimated enantioselectivities of 12.1 (without Triton X-100) and 7.6 (with Triton X-100). Although Partali et al. reported an enantiomeric ratio of 9 for AOP,[16] we measured an estimated enantioselectivity of 4.9 and a true enantioselectivity of 4.8 under our conditions. Although the estimated enantioselectivity for ROL was also high (11.3), the true enantioselectivity was lower, $E = 4.8–4.9$. Hydrolases with low estimated enantioselectivities (CRL, Esterase E013, cutinase) also showed low true enantioselectivities. Thus, hydrolases identified as enantioselective were indeed enantioselective and hydrolases identified as nonselective were not enantioselective.

Our screening procedure quickly identified HLE as a new hydrolase for the resolution of solketal butyrate with modest enantioselectivity. It is the most selective hydrolase reported in the literature to date for the hydrolysis of an ester of solketal.

Table 2. True enantioselectivities of hydrolases towards solketal butyrate measured by the endpoint method.

Hydrolase	S[a] [mM]	Time[b] [h]	ee_s[c] [%]	ee_p[c] [%]	C[d] [%]	True E[e]	Estimated E[f]
Rhizopus oryzae lipase	1	16.5	95.4	23.6	80.2	5.0 ± 0.1 (*R*)	11.3 (*R*)
Rhizopus oryzae lipase	50	1.25	37.8	51.2	42.5	4.4 ± 0.1 (*R*)	
Horse-liver esterase	1	4.0	40.3	81.8	33.0	14.8 ± 0.7 (*S*)	12.2 (*S*)
Horse-liver esterase	50	2.5	22.1	77.3	22.2	9.7 ± 0.1 (*S*)	
Cutinase	1	2.0	92.8	27.4	77.2	5.0 ± 0.02 (*R*)	2.28 (*R*)
Cutinase	50	0.42	70.3	41.8	62.7	4.8 ± 0.04 (*R*)	
Aspergillus oryzae protease	1	4.0	10.5	65.8	13.8	5.4 ± 0.1 (*R*)	4.91 (*R*)
Aspergillus oryzae protease	50	14	11.1	62.0	15.2	4.8 ± 0.1 (*R*)	
Candida rugosa lipase	1	2.5	86.3	14.4	85.7	3.0 ± 0.1 (*R*)	1.73 (*R*)
Candida rugosa lipase	50	0.1	15.7	40.5	27.8	2.7 ± 0.02 (*R*)	
Esterase E013	1	9.5	0	0	20	1.0	1.02
Esterase E013	50	2.0	0	nr[g]	nr	nr	

[a] Substrate concentration in the reaction mixture. [b] Reaction time. [c] Measured enantiomeric purity of the starting material (ee_s) or product (ee_p). [d] Degree of conversion calculated by $ee_s/(ee_s + ee_p)$. [e] The true enantioselectivity was calculated from ee_s and ee_p according to ref. [3]. The absolute configuration of the fast-reacting ester is in parentheses. The error limits were estimated from enantioselectivities measured from three separate GC injections. [f] Values from Table 1. [g] nr = no reaction detected by GC.

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Discussion

The most important part of the assay design was to ensure that it accurately measured the rates of hydrolysis. The first requirement is that the buffer and indicator have pK_as within 0.1 units. A difference in pK_a of 0.3 units causes an 8% error when the pH changes by 0.1 unit.[12] In a typical assay, the pH changed by 0.05 units (10% hydrolysis of the substrate); thus, differences in pK_as can lead to nonlinear and inaccurate rates. If different pK_as cannot be avoided, one can still get accurate results by using calibration experiments[10] or a more complex equation.[12] The linear relationship between the amount of acid added and the color change, as shown in Figure 2, confirms that the pK_as lie within the acceptable range in our experiment. Further, the agreement of the theoretical and experimental slopes to within 5% establishes that the assay is quantitative.

The assay tolerates small changes in reaction conditions, such as the addition of 7% acetonitrile. Indeed, the pK_a of 4-nitrophenol changes only slightly from 7.15 to 7.17 upon addition of 10% ethanol.[26] This result suggests that cosolvent concentrations below 10% do not compromise the accuracy of the assay. Also, small amounts of salts present in the hydrolase solutions (buffer salts in commercial hydrolase preparations, 2 mM $CaCl_2$ in the protease solutions) did not affect the accuracy.

This assay is approximately seven times less sensitive than the one using hydrolysis of 4-nitrophenyl esters. For example, if the rate of hydrolysis for a nonchromogenic ester and a 4-nitrophenyl ester were identical, then our assay would require seven times more hydrolase to observe the same change in absorbance. The assay with 4-nitrophenyl esters releases one molecule of 4-nitrophenol (53% of these will be deprotonated at pH 7.2), while our assay protonates one 4-nitrophenoxide for every twelve protons released.

There are several advantages to this screening method. First, it is hundreds of times faster than conventional screening. The 96-well format allows the analysis of large numbers of samples simultaneously. However, speed is not gained at the expense of accuracy; variation between quadruplicate measurements for a reaction is typically $<2\%$. Moreover, our method is quantitative, unlike screening for hydrolytic activity by TLC. Second, since all the reactions and analyses take place in the microplate wells, workup and analysis by GC, HPLC, or NMR is avoided. Third, it requires hundreds to thousands of times less substrate (typically 20 μg/well) and hydrolase (we used between 0.6–35 μg protein/well). For this reason, it may be useful in the screening of mutant hydrolases in directed evolution experiments. Fourth, this assay measures the hydrolysis of any ester, not just chromogenic esters. The most important rule of screening is "You get what you screen for", so the ability to screen the target compound, not an analogue of the target compound, is an important advantage. For speed, we screened in microplates, but one could also adjust the concentrations to use cuvettes and a conventional UV-vis spectrophotometer.

There are a few disadvantages with our screening method. First, it requires pure enantiomers, albeit in small amounts. We screened the hydrolase library in quadruplicate with only six milligrams of each enantiomer of solketal butyrate. Second, it provides only an estimated enantioselectivity. This method ignores some or all of the differences in K_M of the enantiomers. Third, it requires clear solutions. To obtain clear solutions with water-insoluble substrates, experimentation is sometimes required to find the best cosolvent or emulsion conditions.

In this paper we assayed for hydrolase activity at pH 7.2, but other buffer indicator pairs may be suitable for screening at other pH values. For example, at pH 6 chlorophenol red (p$K_a = 6.0$) and MES [2-(*N*-morpholino)ethanesulfonic acid, p$K_a = 6.1$] may be suitable; at pH 8 phenol red (p$K_a = 8.0$) and EPPS [*N*-(2-hydroxylethyl)piperazine-*N*′-(3-propanesulfonic acid), p$K_a = 8.0$] may be suitable; at pH 9 thymol blue (p$K_a =$ 9.2) and CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid, p$K_a = 9.3$] may be suitable.

We are currently adapting this acid–base indicator assay to measure true enantioselectivity by extending our Quick E method for measuring true enantioselectivity.[23]

Experimental Section

General: Chemicals were purchased from Sigma-Aldrich (Oakville, ON) and were used without further purification unless otherwise stated. Triton X-100 was purchased from ESA (Chelmesford, MA). Standardized acid was purchased from A&C American Chemicals (Montréal, QC). Enzyme suppliers are noted in the footnotes of Table 1. All microplate assays were performed on a Spectramax 340 microplate reader with SOFTmax PRO version 1.2.0 software (Molecular Devices, Sunnyvale, CA). Polystyrene 96-well flat-bottomed microplates (maximum volume 360 μL/well, Corning Costar, Acton, MA) were filled using Eppendorff 8-channel pipettes (5–100 μL, 50–1200 μL) and solution basins for multichannel pipettes (Fisher Scientific, Nepean, ON). The initial rate of the small-scale horse-liver esterase-catalyzed ester hydrolysis reaction was measured with a Radiometer RTS822 pHstat.

(±)-Solketal butyrate (1): Butyric anhydride (2.87 mL, 17.5 mmol, 1.5 equiv), 4-dimethylaminopyridine (0.071 g, 0.58 mmol, 0.05 equiv), and anhydrous sodium carbonate (1.86 g, 17.6 mmol, 1.5 equiv) were added to a solution of (±)-solketal (1.54 g, 11.7 mmol, 1.0 equiv) in ethyl acetate (40 mL) and stirred overnight. The reaction mixture was washed several times with water, then with brine, and the organic extract was dried with magnesium sulfate. Flash chromatography (3:1 hexanes/ethyl acetate) afforded the pure butyryl ester as a yellow oil in 91% yield. $R_f = 0.56$ (3:1 hexanes/ethyl acetate); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.98$ (t, ^3J(H,H) = 7.4 Hz, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.67 (sextet, ^3J(H,H) = 7.4 Hz, 2H, CH_2), 2.33 (t, ^3J(H,H) = 7.3 Hz, 2H, CH_2), 3.7 (m, 1H, 1H of CH_2), 4.05–4.16 (m, 3H, 1H of CH_2, CH_2), 4.27–4.32 (m, 1H, CH); ^{13}C NMR (200 MHz, $CDCl_3$): $\delta = 15.3$ (CH_3), 19.9 (CH_2), 26.9 (CH_3), 28.1 (CH_3), 37.3 (CH_2), 65.5 (CH_2), 67.3 (CH_2), 74.6 (CH), 110.3 (C), 173.1 (C=O).

(*S*)-Solketal butyrate and (*R*)-solketal butyrate: Samples were prepared from enantiomerically pure solketal as outlined above for the racemate. The enantiomeric purities of the butyrates measured by GC (see below) were 99.2% and 99.8%, respectively. No contaminating butyric acid or solketal were detected by GC or ^{1}H NMR.

Hydrolase library: The hydrolases were dissolved in BES buffer (5.0 mM, pH 7.2 containing 0.02% NaN_3 as preservative) at the concentrations listed in Table 1 (0.5–40 mg solid/mL solution). $CaCl_2$ (2 mM) was added to the protease solutions since some proteases require calcium ions to maintain their structure. For hydrolase samples with low protein content, we used saturated solutions (up to 40 mg solid/mL), and for hydrolase samples with high protein content, we chose lower concentrations (typically, 1 mg solid/mL). Each solution was centrifuged to remove insoluble material (5 min, 2000 rpm) and titrated to a final pH of 7.2. The protein concentrations were determined using a dye-binding assay from Bio-Rad (Mississauga, ON)

with bovine serum albumin (BSA) as the standard. Solutions were stored in a 96-well assay block mother plate equipped with aluminum sealing tape (2 mL maximum volume in each well, Corning Costar, Acton, MA) at −20 °C. This mother plate speeds up repeated screens that use the same hydrolases, and is a convenient way to store large libraries of hydrolases. Hydrolytic activity of the libraries is maintained over several months.

Screening of commercial hydrolases with pH indicators: The assay solutions were prepared by mixing solketal butyrate (420 μL of a 30.0 mM solution in acetonitrile), acetonitrile (470 μL), 4-nitrophenol (6000 μL of a 0.9115 mM solution in 5.0 mM BES, pH 7.2), and BES buffer (5110 μL of a 5.0 mM solution, pH 7.2). Hydrolase solutions (5 μL/well) were transferred from the mother plate to a 96-well microtiter plate using an 8-channel pipette. Assay solution (100 μL/well) was quickly added to each well using a 1200 μL 8-channel pipette. The final concentrations in each well were 1.0 mM substrate, 4.65 mM BES, 0.434 mM 4-nitrophenol, 7.1 % acetonitrile. The plate was quickly placed in the microplate reader and shaken for 10 s to ensure complete mixing, and the decrease in absorbance at 404 nm was monitored at 25 °C as often as permitted by the microplate software, typically every 11 seconds. The starting absorbance was typically 1.2. Data were collected for one hour to ensure we detected slow reactions and reactions with a lag time. Each hydrolysis was carried out in quadruplicate and was averaged. The first 10 s of data were sometimes erratic, possibly due to dissipation of bubbles created during shaking. For this reason, we typically excluded the first 10 s of data from the calculation of the initial rate. Activities were calculated from slopes in the linear portion of the curve, usually over the first two hundred seconds. The initial rates were calculated from the average dA/dt by means of Equation (2), where $\Delta\varepsilon = 17300\,\mathrm{M^{-1}cm^{-1}}$ (experimentally determined for our conditions) and $l = 0.306$ cm. To calculate specific activity ($\mu\mathrm{mol\,min^{-1}mg^{-1}}$ protein), we took into account the total amount of protein in each well.

Screening of commercial hydrolases with pH indicators under interfacial activation conditions: The procedure was the same as outlined above except that the BES buffer (5 mM, pH 7.2) contained Triton X-100 (8.45 mM). The final concentration of Triton X-100 in the wells was 2.8 mM.

Small-scale reactions with 1 mM (±)-solketal butyrate: These small-scale reactions mimic the conditions in the microplate during pH indicator activity screening except that no indicator is present. Hydrolase solutions (50 μL) were added to solutions of (±)-solketal butyrate (3.50 mL of a 14.4 mM solution in acetonitrile) and BES buffer (46.45 mL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 50 mL (1.0 mM substrate, 4.65 mM BES, 7 % acetonitrile). After stirring at room temperature for a time estimated from the pH indicator screening, the mixture was extracted with diethyl ether (3 × 20 mL). The extracts, which contained both the ester substrate and the alcohol product, were combined, washed with water and dried with magnesium sulfate, filtered, and evaporated to dryness.

Small-scale reactions with 50 mM (±)-solketal butyrate: Hydrolase solutions (250 μL for CRL, ROL, HLE, AOP, E013; 50 μL for cutinase) were added to solutions of (±)-solketal butyrate (352 μL of a 0.715 M solution in acetonitrile) and BES buffer (4,398 μL of a 5.0 mM solution, pH 7.2) for a final reaction volume of 5.0 mL (50 mM substrate, 4.65 mM BES, 7 % acetonitrile). Reactions were worked up as outlined above.

Determination of enantiomeric purity by GC: Gas chromatography analysis was performed on a Hewlett Packard 5890 Series II Gas Chromatograph equipped with a Chirasil-DEX CB chiral stationary phase (25 m × 0.25 mm × 0.25 μm Chrompack, Raritan, NJ). For analysis, solketal was converted to the acetate by dissolving the mixture of solketal and solketal butyrate in ethyl acetate (5 mL) containing acetic anhydride, 4-pyrrolidinopyridine, and anhydrous potassium carbonate. The solution was stirred for one hour at room temperature, then filtered, washed with brine, then water, dried with magnesium sulfate and evaporated to dryness. Both the starting material, solketal butyrate, and the acetate of the product were simultaneously separated with baseline resolution by means of a temperature gradient (100 °C to 130 °C, 2 °C min^{-1}). Solketal butyrate: $k'_1 = 8.11$ (*S*), $\alpha = 1.04$; solketal acetate: $k'_1 = 4.21$ (*S*), $\alpha = 1.10$. The *ee* values reported in the tables are the mean of three injections. We did not observe any racemization of solketal or its esters during derivatization.

Acknowledgments: We thank NSERC (Canada) for financial support, Alexandra N. Weissfloch for the gift of the pure enantiomers of solketal butyrate, Fluka (Buchs, Switzerland), Thermogen (Chicago, IL), Dr. Maarten Egmond (Unilever Research), and Biocatalysts (Pontypridd, UK) for gifts of hydrolases, and the Swedish Chemical Society and the Bengt Lundqvist Commemorative Foundation for fellowships to ACL.

Received: April 22, 1998 [F1114]

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