Quick *E*. A Fast Spectrophotometric Method To Measure the Enantioselectivity of Hydrolases

Lana E. Janes and Romas J. Kazlauskas*

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec H3A 2K6, Canada

Received May 27, 1997

Hydrolase-catalyzed resolutions of racemates are often the best route to enantiomerically pure compounds.[1] These reactions are often more selective and cheaper than chemical methods. To find an enantioselective hydrolase for a target compound, researchers first screen commercial enzymes and cultures of microorganisms and then optimize the reaction conditions. Both screening and optimization require measuring the enantioselectivity of the reaction. The enantioselectivity of an enzyme is the ratio of the specificity constants, k_{cat}/K_M, for the enantiomers, eq 1.[2,3]

$$\text{enantiomeric ratio} = E = \frac{(k_{cat}/K_M)_{\text{fast enant}}}{(k_{cat}/K_M)_{\text{slow enant}}} \quad (1)$$

Currently, the best method to measure *E* is the endpoint method developed by Sih's group,[3] but screening hundreds of commercial enzymes or cultures of microorganisms by this method is time-consuming. To measure *E*, researchers run a test resolution, work up the reaction, and measure conversion and enantiomeric purity of the starting material or product. A typical example, measuring the enantioselectivity of a hydrolase toward **1**, required approximately 4.5 h.

Recognizing this difficulty, researchers have reported alternative methods to measure *E* by measuring initial rates of samples with varying ratios of enantiomers[4] or by analyzing reaction progression curves.[5] Unfortunately, they are not significantly faster and can be less accurate than the endpoint method. In this paper, we report a method to measure *E* in 1 min from relative initial rates of hydrolysis of pure enantiomers and a reference compound. Researchers previously used mixtures of substrates to measure enzyme selectivity.[6] We extend these techniques to enantioselectivity and to rapid spectrophotometric measurements.

Hydrolyses of pure enantiomers of 4-nitrophenyl 2-phenylpropanoate, (*S*)-**1** and (*R*)-**1**, and 4-nitrophenyl 2-(4-isobutylphenyl)propanoate (ibuprofen 4-nitrophenyl ester), (*S*)-**2** and (*R*)-**2**, liberates the yellow *p*-nitrophenoxide ion.[7] The increase in absorbance at 404 nm revealed the initial rates of hydrolysis of each enantiomer, but the ratio of these rates did not give the enantiomeric ratio, Table 1. The ratio of rates over- and underestimated *E* by as much as 70% because it ignored competitive binding of the two enantiomers to the enzyme. In other cases, researchers found that differences in K_M for the enantiomers contributed a factor of 3−4 to the enantioselectivity.[8]

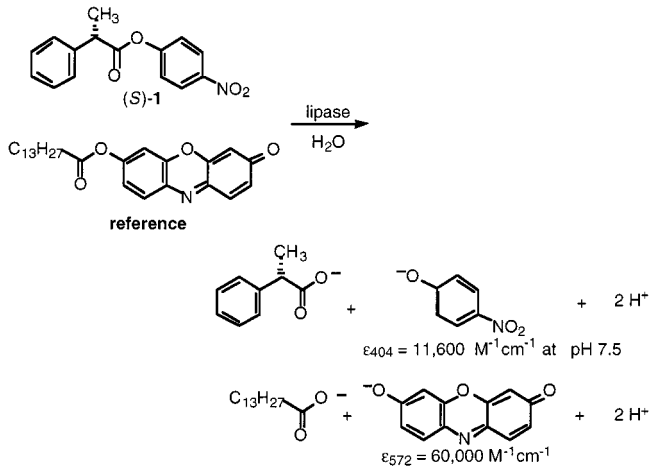

Figure 1. First step of the quick *E* measure of enantioselectivity of lipases toward 4-nitrophenyl 2-phenylpropanoate, **1**. Lipase-catalyzed hydrolysis of (*S*)-**1** and the reference compound, resorufin tetradecanoate, yields yellow and pink chromophores, respectively. The solution turns deep orange if both substrates are hydrolyzed, pink if only the reference compound is hydrolyzed. The second step of the quick *E* is the same, except that it uses the (*R*)-enantiomer of the chiral ester. Equations 2 and 3 yield the enantioselectivity.

To reintroduce competition, we added resorufin tetradecanoate as a reference compound.[9] We monitored the initial rates of hydrolysis of (*S*)-**1** at 404 nm and the reference compound at 572 nm in the same solution, Figure 1. After taking into account the initial concentrations of both substrates, the ratio of these rates yielded the selectivity of the hydrolase for (*S*)-**1** over the reference compound, eq 2.

$$\frac{(S)\text{-}\mathbf{1}}{\text{reference}}\text{selectivity} = \frac{(k_{cat.}/K_M)_{(S)\text{-}\mathbf{1}}}{(k_{cat.}/K_M)_{\text{reference}}} = \frac{v_{(S)\text{-}\mathbf{1}}}{v_{\text{reference}}}\frac{[\text{reference}]}{[(S)\text{-}\mathbf{1}]} \quad (2)$$

A second experiment using (*R*)-**1** and the reference compound yielded the selectivity of (*R*)-**1** over the refer-

(1) Roberts, S. M., Ed. *Preparative Biotransformations*; Wiley: New York, 1992−1997. Faber, K. *Biotransformations in Organic Chemistry*, 2nd ed.; Springer: Berlin, 1995.

(2) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985; pp 103−106.

(3) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.

(4) (a) Jongejan, J. A.; van Tol, J. B. A.; Geerlof, A.; Duine, J. A. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 247−254. (b) van Tol, J. B. A.; Jongejan, J. A.; Geerlof, A.; Duine, J. A. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 255−262.

(5) (a) Lu, Y.; Zhao, X.; Chen, Z. N. *Tetrahedron: Asymmetry* **1995**, *6*, 1093−1096. (b) Rakels, J. L. L.; Romein, B.; Straathof, A. J. A.; Heijnen, J. J. *Biotechnol. Bioeng.* **1993**, *43*, 411−422. (c) Fourneron, J. D.; Combemel, A.; Buc, J.; Piéroni, G. *Tetrahedron Lett.* **1992**, *33*, 2469−2472.

(6) (a) Berman, J.; Green, M.; Sugg, E.; Anderegg, R.; Millington, D. S.; Norwood, D. L.; McGeehan, J.; Wiseman, J. *J. Biol. Chem.* **1992**, *267*, 1434−1437. (b) Petithorny, J. R.; Masiarz, F. R.; Kirsch, J. F.; Santi, D. V. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11510−11514. (c) Schellenberger, V.; Siegel, R. A.; Rutter, W. J. *Biochemistry* **1993**, *32*, 4344−4348.

(7) Enantiomerically pure acid and 4-nitrophenol were coupled using 1 equiv of *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide and 1 equiv of 1-hydroxybenzotriazole in anhydrous dichloromethane at 0 °C for 15 min and then stirred at room temperature for 48 h. Esters were purified by column chromatography on silica gel eluted with ethyl acetate and recrystallized from hexanes/ethyl acetate, 44−60% yield. (*R*)-**1**, 99.7% ee; (*S*)-**1**, 99.4% ee; (*R*)-**3**, 98.2% ee; (*S*)-**3**, 99.6% ee. Starting acids showed the same enantiomeric purity. All enantiomeric purities were measured by HPLC using a Chiracel OD-H column (Daicel) at 25 °C, eluted at 1 mL/min. 2-Phenylpropanoic acid (98/2/1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 3.35$; $k_S' = 4.05$; $\alpha = 1.2$; $R_s = 2.13$); 2-(4-isobutylphenyl)propanoic acid (100/1/0.1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 3.31$; $k_S' = 4.27$; $\alpha = 1.29$; $R_s = 2.43$); **3** (100/1/0.1 hexanes/2-propanol/trifluoroacetic acid, $k_R' = 2.41$; $k_S' = 2.93$; $\alpha = 1.21$; $R_s = 1.66$). For analysis, samples of **1** were hydrolyzed to the acid in aqueous NaOH.

S0022-3263(97)00780-9 CCC: $14.00

Table 1. Enantiomeric Ratios of Hydrolases toward (±)-1 and (±)-3 Measured Using the Endpoint Method[a] and the Ratio of Separately-Measured Initial Rates of Hydrolysis of the Enantiomers

substrate	lipase[b]	ee_s[c] (%)	ee_p[c] (%)	c[d] (%)	E[e] endpoint	initial rate[f] (S)	initial rate[f] (R)	rate ratio S/R[g]
1	PCL	nd	67	60	29 ± 3	15.32	0.771	20 ± 1
1	CRL	17	50	25	3.5 ± 0.2	2.81	2.38	1 ± 0.2
1	IPA−CRL	43	98	30	>100	3.56	0.092	40 ± 2
1	PPL	nd	2	4	1.1 ± 0.1	1.11	0.757	1.4 ± 0.1
1	CAL−A	20	23	37	1.9 ± 0.1	1.40	0.355	4 ± 0.2
3	PCL	28	43.2	39	3.3 ± 0.1 (R)	0.535	0.579	1.1 ± 0.1 (R)
3	CRL	5.3	14.5	27	1.4 ± 0.1	0.330	0.274	1.2 ± 0.1
3	IPA−CRL	40.1	>99	29	>100	0.702	0.0128	55 ± 5

[a] Reaction conditions: room temperature, 10 mM Tris buffer, pH 8, 100 mg of crude lipase or 0.7 mg of protein for IPA−CRL, 1 mmol of substrate dissolved in 1 mL of acetonitrile. [b] For spectrophotometric measurements, the lipases were dissolved in Tris buffer (25 mM, pH 7.5) and centrifuged to remove insoluble material. PCL (lipase from *Pseudomonas cepacia*, Amano lipase PS, 29.3 mg solid/mL), CRL (lipase from *Candida rugosa*, Sigma, 36.9 mg of solid/mL; 0.5 mg protein/mL by the Bio-Rad protein assay), IPA−CRL (CRL treated with 2-propanol as in ref 12a; 0.7 mg/mL of protein by the Bio-Rad protein assay), PPL (porcine pancreatic lipase, Sigma, 42.3 mg of solid/mL), CAL-A (lipase A from *Candida antarctica*, Boehringer Mannheim, 18.2 mg of solid/mL). [c] ee_s = enantiomeric excess of remaining substrate, ee_p = enantiomeric excess of product. Determined by HPLC as described in ref 7. [d] Extent of conversion. [e] Enantiomeric ratio calculated using eq 2. Error limits for E were estimated assuming an error of ±1% for enantiomeric purity. All hydrolyses favored the (S)-enantiomer except the PCL-catalyzed hydrolysis of **3**, which favored the (R)-enantiomer. [f] Substrates were emulsified in aqueous solution according to: Vorderwulbecke, T.; Kieslich, K.; Erdmann, H. *Enzyme Microb. Technol.* **1992**, *14*, 631−639. An acetonitrile solution of **1** (7.8 mM, 0.5 mL) or **3** (9.04 mM, 0.5 mL) was added dropwise to Tris buffer (9 mL, 50 mM, pH 8.0) containing 0.45−0.90 w/v % Triton X-100 (Pierce Surfact-Amps) and vortexed until clear. This emulsion remained clear for at least 3 h. To measure initial rates of hydrolysis, the lipase solution (100 μL) was added to the substrate emulsion (900 μL) at 25 °C, and the increase in absorbance at 404 nm was monitored for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance per second × 10^3. [g] Average and standard deviation for three experiments.

Table 2. Enantiomeric Ratios of Hydrolases toward (±)-1 and (±)-3 Using the Quick *E* Method

		(S)-enantiomer + ref[b]		(R)-enantiomer + ref[b]		
substrate	lipase[a]	404 nm	572 nm	404 nm	572 nm	E[c] quick E
1	PCL	13.2	9.79	0.379	7.50	29 ± 3
1	CRL	1.02	9.48	0.28	9.02	3.5 ± 0.3
1	IPA−CRL	1.89	7.58	0.004	3.68	210 ± 20
1	PPL	0.199	0.186	0.125	0.164	1.4 ± 0.2
1	CAL−A	2.15	1.53	0.824	1.47	2.3 ± 0.2
3	PCL	0.3341	6.78	0.5750	4.709	2.5 ± 0.3 (R)
3	CRL[d]	3.408	49.3	1.342	52.18	3 ± 2
3	IPA−CRL	2.154	8.78	<0.01	5.767	>140

[a] See Table 1. [b] Substrate solutions as for Table 1, but an acetonitrile solution of resorufin tetradecanoate (0.5 mL, 1.6 mM) was also added. The liberated 4-nitrophenoxide and resorufin were measured in two separate measurements at 404 and 572 nm, respectively, at 25 °C for 15 s. No spontaneous chemical hydrolysis was detected. Values are in absorbance per second × 10^3. [c] Enantiomeric ratio calculated using eq 3. Average and standard deviation for three measurements. [d] CRL (lipase from *Candida rugosa*, Sigma, 214 mg solid/mL; 1.9 mg protein/mL by the Bio-Rad protein assay).

ence compound. The ratio of these two selectivities yields the enantiomeric ratio, eq 3.

$$E = \frac{(S)\text{-}\mathbf{1}}{(R)\text{-}\mathbf{1}}\text{selectivity} = \frac{\dfrac{(S)\text{-}\mathbf{1}}{\text{reference}}\text{selectivity}}{\dfrac{(R)\text{-}\mathbf{1}}{\text{reference}}\text{selectivity}} \quad (3)$$

The quick E method agreed with the endpoint method for both **1** and **2** using five different lipases, Table 2. We measured low (E = 1.4), average (E = 27), and excellent (E = 210) enantiomeric ratios correctly by this technique. Each hydrolysis experiment requires 30 s; thus, the measurement time for E was only one minute.

There are three advantages to the quick E method. First, it is many times faster than a typical endpoint measurement, yet has equivalent or better accuracy. Accuracy may be particularly important for screening in directed evolution where the improvements of each generation are small.[10] Note that quick E is based on the same equations as the endpoint methods, so inaccuracies of the endpoint method also apply quick E.[11] Second, it requires much smaller amounts of hydrolase because the entire reaction occurs in the spectrophotometer. This advantage may be especially useful for screening biocatalyst libraries. Third, the quick E method can measure high enantioselectivities more easily than the endpoint method.

There are also several disadvantages to this screen. First, it requires pure enantiomers, albeit in small amounts (~20 μg per measurement in a 200 μL cuvette). Second, the current version of quick E measures the enantioselectivity only for chromogenic substrates. Future versions of quick E will extend the range to all esters. Third, spectrophotometric measurements require clear solutions. Dissolving hydrophobic substrates in aqueous solution requires addition of surfactants.

Acknowledgment. Financial support of this work by NSERC (Canada) gratefully acknowledged. Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. We thank Ms. Cecile Vaugelaude, from INSA Rouen, France, for the synthesis of (±)-**1** and Dr. Michael Trani of the NRC Biotechnology Research Institute, Montréal, for the gift of (R)- and (S)-ibuprofen.

JO9707803

(8) Wu, S. H.; Guo, Z. W.; Sih, C. J. *J. Am. Chem. Soc.* **1990**, *112*, 1990−1995; van der Lugt, J. P.; Elfrink, H.; Evenaar, J.; Doddema, H. J. In *Microbial Reagents in Organic Synthesis*; Servi, S., Ed.; Kluwer Academic: Dordrecht, 1992; pp 261−272.

(9) Resorufin acetate can also serve as a reference compound. Previous use of resorufin acetate as a chromogenic lipase substrate: Kramer, D. N.; Guilbault, G. G. *Anal. Lett.* **1964**, *36*, 1662−1663. Herrmann, R. *Chimia* **1991**, *45*, 317−318.

(10) Moore, J. C.; Arnold, F. H. *Nature Biotechnol.* **1996**, *14*, 458−468.

(11) Due to assumptions made in deriving eq 1, both the endpoint method and quick E will give inaccurate enantioselectivities in two common situations—impure biocatalyst and reactions inhibited by product.