Biocatalysis

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Rapid Determination of Both the Activity and Enantioselectivity of Ketoreductases**

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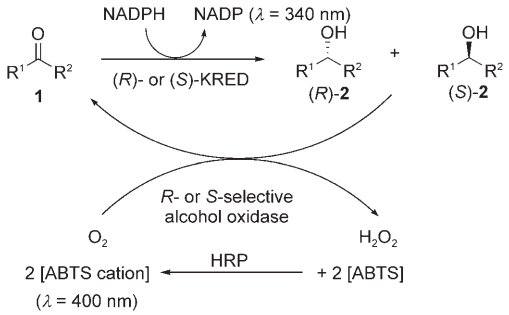
The application of biocatalysis for the production of key chiral intermediates in the pharmaceutical and fine chemicals industries is rapidly growing.[1,2] As the number of biocatalysts increases, screening these enzymes for activity and enantioselectivity against target molecules becomes a major bottleneck in the process-development timeline.[3] The current state of the art for resolving enantiomers of chiral products involves the use of chiral stationary phases in HPLC or GC format.[4,5] While these methods are very precise, assays generally require sample preparation and run times of approximately 20 minutes per sample.

Efforts have been made to devise rapid assays with a variety of technologies, including fluorescence in reaction microarrays,[6] infrared thermography,[7] mass spectrometry,[8] capillary electrophoresis,[9] circular dichroism,[10] and pH indicators.[11] One drawback of many of these techniques is that they require reference reactions involving pure enantiomers of the compound of interest to calibrate the system. Additionally, it would be beneficial to obtain not only selectivity information from the screen, but activity information as well.

Herein we report two new high-throughput, 96-well microtiterplate-format screening methods that are based on employing an enantioselective oxidase enzyme for determining both the rate and the enantioselectivity of asymmetric ketone reduction. Ketoreductases (KREDs) were selected as ideal targets for initial development in view of their increasing importance and application in the asymmetric reduction of ketones.[12] Although a large number of KREDs (more than 100) are available, difficulty remains in predicting which particular enzyme will provide the best enantioselectivity simply based on substrate structure. Minor substituent changes can lead to different "best hits", thus highlighting the need for extensive screening.[13]

Scheme 1 outlines the basis of an assay for detecting the activity and enantioselectivity of KREDs. Reduction of ketone **1** by the NADPH-dependent KRED generates alcohols (*R*)- and (*S*)-**2**, the ratio of which can be determined by addition of an alcohol oxidase of known enantioselectivity. The alcohol oxidase catalyzes oxidation of (*R*/*S*)-**2** back to ketone **1** with concomitant production of H_2O_2, which can be detected spectrophotometrically. The alcohol oxidase employed herein is derived from galactose oxidase and has been evolved to have high activity and *R* enantioselectivity towards substituted 1-phenylethanol analogues.[14]



Scheme 1. Screening for KREDs using an alcohol oxidase.

Using acetophenone (R^1 = Ph, R^2 = Me) as substrate, the rate of ketone reduction by a KRED was initially determined by monitoring the consumption of NADPH through the decrease in absorbance at 340 nm. A limiting quantity of NADPH (0.15 mM) was present in the assay mixture along with an excess (2 mM) of ketone **1** substrate to ensure rapid reduction and hence a short assay time. Upon complete consumption of NADPH, the *R*-selective alcohol oxidase was added, resulting in oxidation of any (*R*)-**2** present in the reaction mixture back to the ketone **1** with concomitant production of one mole of H_2O_2 for every mole of alcohol molecule oxidized. The precise amount of hydrogen peroxide, and hence (*R*)-alcohol, produced in the reaction was monitored at 400 nm through the colorimetric HRP/ABTS (HRP = horse radish peroxidase, ABTS = 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) assay.[15] Ensuring that NADPH is the limiting reagent is essential, as the cofactor is known to be oxidized by HRP/H_2O_2.[16]

Monitoring this assay at two different wavelengths (340 and 400 nm) generates all of the required data, namely 1) the rate of reduction of the ketone, 2) the total amount ($R+S$) of alcohol produced [Eq. (1)], 3) the amount of (*R*)- and (*S*)-alcohol produced [Eqs. (2) and (3)], and 4) the enantioselectivity of the KRED, expressed in terms of the enantiomeric excess (*ee*) of alcohol produced [Eq. (4)].

$$[(R)\text{-}\mathbf{2}] + [(S)\text{-}\mathbf{2}] = [\mathrm{NADPH}]_o - [\mathrm{NADPH}]_f \text{ (at 340 nm)} \quad (1)$$

$$[(R)\text{-}\mathbf{2}] = [\mathrm{ABTS\ cation}](2)^{-1} \text{ (at 400 nm)} \quad (2)$$

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$$[(S)\text{-}\mathbf{2}] = [\mathrm{NADPH}]_o - [\mathrm{NADPH}]_f - [\mathrm{ABTS\ cation}](2)^{-1} \quad (3)$$

$$ee = \frac{[(R)\text{-}\mathbf{2}] - [(S)\text{-}\mathbf{2}]}{[(R)\text{-}\mathbf{2}] + [(S)\text{-}\mathbf{2}]} \quad (4)$$

A panel of 17 different KREDs[17] was then screened for the enantioselective reduction of acetophenone to 1-phenylethanol. Screening was carried out both by conventional means (1-mL-scale reactions with HPLC analysis) and by the oxidase assay (100-μL-scale reactions in a 96-well plate). Table 1 reveals an extremely good correlation between the new assay and the conventional HPLC analysis for both biocatalyst activity and enantioselectivity. However, the oxidase-based assay results in substantial time savings, which are necessary for any high-throughput assay. Obtaining rate data using conventional HPLC analysis required sampling multiple time points for each reaction over the course of 4 h, dilution of each reaction sample in the mobile phase, and filtration of each sample. The run time for reverse-phase HPLC analysis was ten minutes per sample. Obtaining *ee* data using conventional normal-phase chiral HPLC analysis required product extraction from the aqueous reaction system, solvent evaporation, and resuspension in the mobile phase. The run time for normal-phase chiral HPLC analysis was 20 minutes per sample. Therefore, the total time to run the reactions, prepare samples, and analyze data by conventional HPLC means was approximately 20 h. By comparison, the entire run time for the dual-wavelength oxidase assay, including reaction time and data collection for all reactions, was ten minutes.

Table 1: Comparison of relative rate and enantiomeric excess data for the reduction of acetophenone by various KREDs as determined by the dual-wavelength oxidase assay and HPLC assay.[a]

Catalyst	Rate (oxidase)	Rate (HPLC)	*ee* (oxidase)	*ee* (HPLC)
KRED1	100	100	19	17
KRED2	0	0	–	–
KRED3	0	0	–	–
KRED4	0	0	–	–
KRED5	8	2	−100	−100
KRED6	0	0	–	–
KRED8	16	8	−100	−100
KRED9	0	0	–	–
KRED10	0	0	–	–
KRED11	136	115	−76	−82
KRED15	126	131	−84	−85
KRED16	8	7	−100	−100
KRED17	4	6	−100	−100
KRED20	4	12	−100	−100
KRED21	55	58	−97	−97
KRED23	44	52	−83	−88
KRED24	12	15	−100	−100

[a] Relative rate is the activity relative to KRED1. *R* selectivity is positive (*ee* values shown in %).

An alternative, simpler, single-wavelength method was also devised, which relies upon monitoring the reaction activity trajectory for a rapid understanding of enantiomeric excess (ATRUee). This method requires only the substrate ketone (0.15 mM) together with excess NADPH (1.5 mM) and the (*R*)-oxidase. By monitoring the trajectory of NADPH consumption, both the activity and enantioselectivity of the KRED can be determined by comparing the progress of the reaction to a kinetic model (Figure 1), which is based on the regeneration of ketone substrate from the *R*-selective oxidase. For example, a perfectly *S*-selective KRED would require only 0.15 mM NADPH conversion to NADP. The more *R*-selective the KRED, the greater the conversion of NADPH, as (*R*)-alcohol is recycled by the oxidase to ketone and reduced again by the KRED. The ATRUee method required approximately 2 h to collect all of the data. Table 2 shows that the ATRUee method gives a reliable rapid assessment of the KRED activity, but the data on enantioselectivity is not as accurate as for the dual-wavelength method.

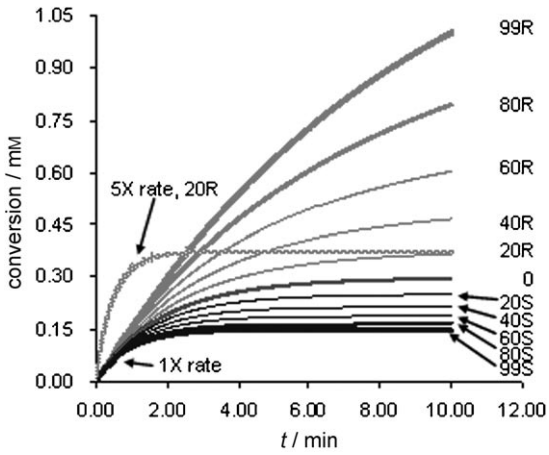

Figure 1. Kinetic model reaction profile versus KRED enantioselectivity for single-wavelength ATRUee assay. Demonstration of reaction trajectories for different activities and enantioselectivities. The numbers on the right refer to enantioselectivity. For example, 99R corresponds to 99% *R* selectivity.

Table 2: Enantiomeric excess and rate data for the reduction of acetophenone by various ketoreductases as determined by the single-wavelength ($\lambda = 340$ nm) ATRUee method.[a]

Catalyst	Rate	*ee*
KRED1	100	25
KRED11	136	−68
KRED15	140	−63
KRED23	48	−60

[a] Relative rate is the activity relative to KRED1. *R* selectivity is positive (*ee* values shown in %).

A key advantage of both methods is the small quantity of substrate needed for the screen. The sensitivity of the detection system is such that 100-μL-scale reactions were run with 0.024 mg (2 mM) or 0.0018 mg (0.15 mM) substrate acetophenone per well for the dual- and single-wavelength assays, respectively. The entire screen of 17 ketoreductases required only 0.41 mg (dual wavelength) or 0.03 mg (single wavelength) substrate.

In summary, we have developed two new high-throughput methods for the determination of KRED activity and enantioselectivity. The dual-wavelength spectrophotometric tracking of two chromogens (NADPH and ABTS cation at 340 and 400 nm, respectively), enabled the rapid determination of both the rate of KRED-catalyzed ketone reduction and the enantioselectivity of the process. Although the single-wavelength ATRUee assay is not as accurate as the dual-wavelength assay, it provides a rapid and sensitive way of determining enantioselectivity and could prove useful in situations where a suitable peroxidase detection system cannot be implemented, or where a large number of different enzymes needs to be screened (e.g. arising from a directed-evolution program). Both assays involve the use of an *R*-selective alcohol oxidase whose substrate specificity has been broadened to encompass a wide range of substituted phenylethanol substrates.[14] Further directed evolution of this enzyme, or alternatively the use of other alcohol oxidases, will enable a greater range of substrates to be screened using the methods described herein.

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[1] D. J. Pollard, J. M. Woodley, *Trends Biotechnol.* **2007**, *25*, 66.
[2] K. M. Koeller, C.-H. Wong, *Nature* **2001**, *409*, 232.
[3] M. G. Finn, *Chirality* **2002**, *14*, 534.
[4] W. H. Pirkle, J. M. Finn, *J. Org. Chem.* **1981**, *46*, 2935.
[5] Y. Okamoto, E. Yashima, *Angew. Chem.* **1998**, *110*, 1072; *Angew. Chem. Int. Ed.* **1998**, *37*, 1020.
[6] G. A. Korbel, G. Lalic, M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123*, 361.
[7] N. Millot, P. Borman, M. S. Anson, I. B. Campbell, S. J. F. Macdonald, M. Mahmoudian, *Org. Process Res. Dev.* **2002**, *6*, 463.
[8] M. T. Reetz, M. H. Becker, H.-W. Klein, D. Stöckigt, *Angew. Chem.* **1999**, *111*, 1872; *Angew. Chem. Int. Ed.* **1999**, *38*, 1758.
[9] M. T. Reetz, K. M. Kühling, A. Deege, H. Hinrichs, D. Belder, *Angew. Chem.* **2000**, *112*, 4049; *Angew. Chem. Int. Ed.* **2000**, *39*, 3891.
[10] K. Ding, A. Ishii, K. Mikami, *Angew. Chem.* **1999**, *111*, 519; *Angew. Chem. Int. Ed.* **1999**, *38*, 497.
[11] F. Morís-Varas, A. Shah, J. Aikens, N. P. Nadkarni, J. D. Rozzell, D. C. Demirjian, *Bioorg. Med. Chem.* **1999**, *7*, 2183.
[12] J. D. Stewart, *Curr. Opin. Chem. Biol.* **2001**, *5*, 120.
[13] M. D. Truppo, D. J. Pollard, P. N. Devine, *Org. Lett.* **2007**, *9*, 335.
[14] F. Escalettes, N. J. Turner, *ChemBioChem* **2008**, DOI: 10.1002/cbic.200700689.
[15] H. E. Müller, *J. Microbiol. Methods* **1984**, *2*, 101.
[16] V. De Sandro, C. Dupuy, J. Kaniewski, R. Ohayon, D. Deme, A. Virion, J. Pommier, *Eur. J. Biochem.* **1991**, *201*, 507.
[17] KREDs were obtained from Biocatalytics Inc., Pasadena, USA.

