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ORIGINAL RESEARCH PAPER

A simple method to determine concentration of enantiomers in enzyme-catalyzed kinetic resolution

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Abstract Kinetic resolutions play important roles in industrial biotransformations for production of optical pure compounds from racemic substrates. A simple method, based on enantiomeric excess of both substrate (ee_S) and the corresponding product (ee_P), was developed for determination of concentration of enantiomers in kinetic resolution. Since only relative quantity (ee) was required in the proposed method, calibration and cumbersome quantitative sample handling can be avoided and analytical accuracy can be greatly improved.

Keywords Amidase · Chiral balance · Concentration of enantiomers · Enantiomeric excess · Kinetic resolution

Introduction

With the steady growth of the importance of enantiomerically pure or enriched compounds in pharmaceuticals, agrochemicals and food additives, the so-called ''chiral market'' has become an expanding area of the fine chemicals industry (Reetz 2001). Kinetic resolution plays key roles in industrial biotransformations for production of enantiomerically pure compounds from racemic precursors (Straathof et al. 2002). In order to characterize enantioselectivity of enzymes and monitor the kinetic resolution progress, definitions, determination methods and relationships between vital parameters such as enantiomeric ratio (E), enantiomeric excess (ee) and the extent of conversion (c) have been established (Chen et al. 1982; Rakels et al. 1993; Straathof 1997). Besides these parameters, the concentration of enantiomers of both substrate and product is also of great importance to evaluate the kinetic resolution efficiency. However, direct determination of enantiomers usually requires quantitative treatment of samples such as solvent extraction, evaporation and derivatization steps, which might decrease the analytical precision.

With the development of chiral analysis technology (Ward 2002), enantioselective separations can be achieved for a wider variety of chiral compounds. Both enantiomeric excess of substrate (ee_S) and the corresponding product (ee_P) can be accurately measured in enzyme-catalyzed kinetic resolutions (Xu et al. 2004; Wielechowska and Plenkiewicz 2005; Zimmermann et al. 2006; Kotik et al. 2005; Romano et al. 2006; Berti et al. 2006), which make the determination of the concentration of enantiomers much simpler.

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In this paper, we propose a simple method to determine concentration of enantiomers in kinetic resolution progress based on ee_S, ee_P and chiral balance (Straathof et al. 1995), which might be a useful alternative to the traditional method.

Theory

Suppose that enzyme involved in kinetic resolution is *S*-stereospecific, ee_S and ee_P can be expressed as Eqs 1 and 2.

$$ee_s = \frac{S_R - S_S}{S_R + S_S} \quad (1)$$

$$ee_p = \frac{P_S - P_R}{P_S + P_R} \quad (2)$$

Where S_R, S_S, P_R and P_S are molar concentrations of enantiomers of both substrate and product.

For clarity, it is assumed that (*R*)/(*S*) configuration does not change during the reaction, which means that (*R*)-substrate is transformed into (*R*)-product and (*S*)-substrate into (*S*)-product. Using chiral balance, Eqs 3 and 4 are afforded as follows:

$$S_S + P_S = S_{S0} \quad (3)$$

$$S_R + P_R = S_{R0} \quad (4)$$

Here, S_{S0} and S_{R0} are the initial concentrations of the two enantiomers of substrate. If the substrate is racemic, $S_{S0} = S_{R0}$.

Using this set of 4 equations, 4 variables, S_R, S_S, P_R and P_S, can be calculated. For example, P_R is calculated by substitution of the other three variables.

$$P_R = \frac{(1 - ee_P)[ee_S(S_{R0} + S_{S0}) - (S_{R0} - S_{S0})]}{2(ee_S + ee_P)} \quad (5)$$

Materials and methods

Materials

Racemic 2,2-dimethylcyclopropanecarboxamide and racemic 2,2-dimethylcyclopropanecarboxylic acid were from Huakang Chemicals Ltd. (Zhejiang, China). All the other chemicals were obtained from commercial sources and were of analytical reagent grade purity.

Microorganisms and cultivation conditions

Two strains capable of producing enantioselective amidase, *Delftia tsuruhatensis* CCTCC M 205114 and *Rhodococcus* sp. N595, were isolated through a colorimetric high-throughput screening system (Zheng et al. 2007) in our laboratory. *D. tsuruhatensis* was grown at 30°C for 24 h (150 rpm) in a medium of the following composition: 10 g glucose l^{-1}, 3.56 g acetamide l^{-1}, 7.0 g yeast extraction l^{-1}, 0.7 g peptone l^{-1}, 1 g NaCl l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g K_2HPO_4 l^{-1} (pH 7.5). *Rhodococcus* sp. was grown at 30°C for 72 h (150 rpm) in a medium consisted of 10 g glucose l^{-1}, 5 g yeast extract l^{-1}, 2 g peptone l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g K_2HPO_4 l^{-1}, 1 g NaCl l^{-1}, 0.2 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.03 g $FeSO_4{\cdot}7H_2O$ l^{-1}, and 1 g ε-caprolactam l^{-1} (pH 7.0).

Cells were harvested by centrifugation and the cell pellets were then suspended in 50 mM Tris–HCl buffer (pH 8.0) to give a resting cell concentration of 11.5 g l^{-1} (cell dry).

Biotransformations

Racemic amide, 0.3 mmol dissolved in 1 ml methanol and added to 9 ml of the above mentioned cell suspensions. The reaction mixtures were incubated at 35°C with stirring (120 rpm). Samples (500 µl each) were withdrawn at intervals and the reaction was quenched by addition of 30 µl HCl (5 M). After centrifugation, 200 µl supernatant was extracted with 800 µl ethyl acetate and vigorously mixed. The ethyl acetate layer was collected and dried over anhydrous Na_2SO_4.

Analytical methods

Enantiomeric compositions of residual substrate and the corresponding product were determined by GC on an enantioselective capillary column BGB-175. GC analysis was performed under the following conditions: carrier gas, helium; flow rate, 1.6 ml min^{-1}; inlet temperature, 220°C;

detector temperature, 220°C. For enantioselective separation of the acid, the initial column temperature was 90°C, with a 5°C min^{-1} gradient to 170°C, final 6 min at 170°C. For the amide, the column temperature was isothermal at 170°C.

Enantiomeric excess (*ee*) was defined as $(A_1 - A_2)/(A_1 + A_2) \times 100$, where A_1 and A_2 were peak areas of the enantiomers, and A_1 was the larger peak area.

Results

The theory outlined above was used experimentally to determine the enantiomers of both substrate and product during the reaction progress. The kinetic resolution involved was amidase-catalyzed enantioselective hydrolysis of racemic 2,2-dimethylcyclopropanecarboxamide, which have been developed into industrial operation (Shaw et al. 2003) for the production of *S*-(+)-2,2-dimethylcyclopropanecarboxamide, a key chiral intermediate of Cilastatin (Birnbaum et al. 1985). *Delftia tsuruhatensis* produces an *R*-stereospecific amidase while *Rhodococcus* sp. contained nitrile hydratase associated.

S-stereospecific amidase

Changes of both ee_S and ee_P of the two reactions are listed in Table 1. The ee_P of the reaction catalyzed by *D. tsuruhatensis* was higher than that catalyzed by *Rhodococcus* sp., which indicated that the amidase from *D. tsuruhatensis* had greater stereospecificity. Concentrations of the four enantiomers of substrates and products were sequentially calculated according to the equations. Fig. 1 shows changes in concentration of the four enantiomers during the reaction progress. As depicted in Fig.1B, *R*-amide was rapidly converted to *R*-acid while only 5.9% of *S*-amide was degraded when ee_S reached 100%. In the *Rhodococcus* sp. catalyzed resolution, although the *S*-amide was degraded faster than the *R*-amide, the *R*-acid steadily accumulated. The residual concentration of the *R*-amide was 11.6 mM after 4 h when the ee_S was only 92% (see Fig.1A).

Discussion

This method was successfully used to determine concentration of enantiomers in kinetic resolution. Enantiomeric excess, determined from the ratio of peak areas of (*R*) and (*S*)-enantiomers, is a *relative quantity*. When it is applied to determine concentration of enantiomers (*absolute quantity*), calibration and quantitative treatment of samples can be circumvented and analytical accuracy is ensured. For example, since chiral gas capillary columns coated with cyclodexin as chiral stationary phases are usually sensitive to water, solvent extraction should be performed prior to analysis. Quantitative extraction becomes a key step in the direct determination of enantiomers.

Table 1 Changes of enantiomeric excess of substrate (ee_S) and enantiomeric excess of product (ee_P) in enantioselective amidase-catalyzed kinetic resolution progress

Rhodococcus sp. N595			*Delftia tsuruhatensis* CCTCC M 205114		
Time (min)	ee_S(%)	ee_P(%)	Time (min)	ee_S(%)	ee_P(%)
30	16.9 ± 0.1	84.4 ± 0.8	15	17.1 ± 0.1	97.4 ± 0.1
60	28.6 ± 0.1	80.0 ± 0.6	30	36.9 ± 0.1	97.1 ± 0.1
90	49.2 ± 0.3	78.3 ± 0.1	45	60.1 ± 0.2	95.8 ± 0.2
120	59.4 ± 0.3	76.4 ± 1.9	60	82.9 ± 0.8	95.2 ± 0.3
150	76.1 ± 0.1	75.6 ± 0.4	75	94.7 ± 0.7	92.3 ± 0.1
180	87.8 ± 0.8	72.7 ± 0.1	90	100	88.8 ± 0.7
210	90.2 ± 0.3	67.8 ± 0.7	105	100	87.5 ± 0.1
240	92.2 ± 0.3	62.0 ± 0.1	120	100	83.9 ± 0.4

ee values were determined in triples from ratio of peak areas of the enantiomers

Kinetic resolution means the achievement of partial or complete resolution by virtue of unequal of reaction of the enantiomers in a racemate with a chiral agent

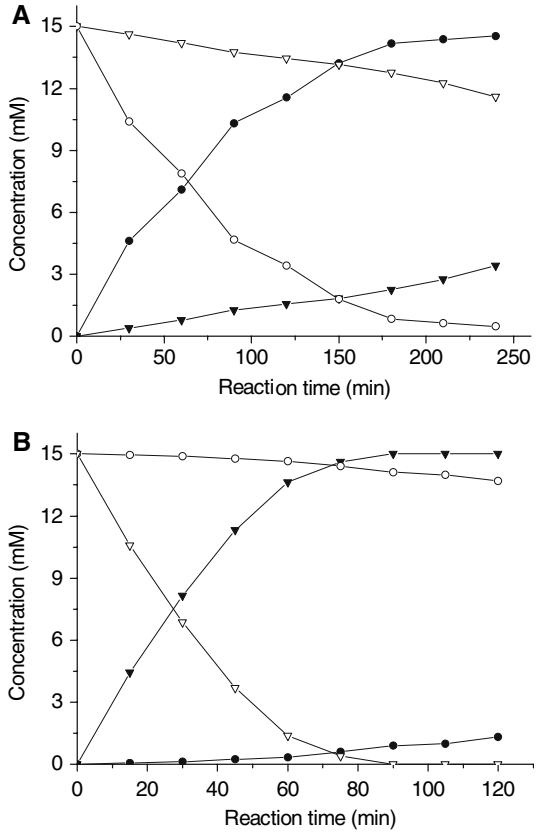

Fig. 1 Enantioselective amidase-catalyzed kinetic resolution of racemic-**1** by (**A**) *Rhodococcus* sp. N595 and (**B**) *Delftia tsuruhatensis* CCTCC M 205114. Concentrations of enantiomers were calculated from corresponding ee_S and ee_P listed in Table 1: *S*-amide (○), *R*-amide (∇), *S*-acid (●), *R*-acid (▼)

With this method, extraction efficiency is no longer as important as it was because only the ratio of peak areas of the two enantiomers is required.

Determination of enantiomeric ratio (*E*) derived from enantiomeric excess of substrate and product has been reported (Rakels et al. 1993). Here we established a relationship between ee_S, ee_P and the concentration of enantiomers, allowing for the determination of *absolute quantity* from the *relative quantity*. As shown in Eq. 5, accumulation of errors and the sensitivity of the calculations for inaccuracies from the input data are not high. No model assumptions were involved in the establishment of the relationship and it was not necessary for the substrate to be racemic. The only limitation is that it is not valid when racemization occurs.

In summary, the method proposed here may be a useful alternative to determine concentration of enantiomers in kinetic resolution if ee_S and ee_P are available. Simple, rapid and accurate determination of enantiomers in reaction mixture will greatly facilitate investigations into enzyme-catalyzed kinetic resolutions.

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