Quick Analysis of Enantioselectivity by a Chromatographic Reactor

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The transient behavior of enzyme-catalyzed reaction in a proposed chromatographic reactor was investigated, and application of the technique to evaluate the capability of the enzyme in the process of kinetic resolution was developed. The theoretical result reveals that the enantioselectivity of the enzyme can be obtained directly from the amount of substrates eluted without any further treatment if a large amount of enzyme is provided. Because of its simplicity in operation and data analysis yet without any adverse consequences induced by enzyme deactivation, the technique presented can facilitate the development of an optimal condition for enzymatic kinetic resolution.

Introduction

Enzymatic chiral resolution of racemates is a useful method for the production of enantiomerically pure compounds. An extensive research has been conducted on this application (Capewell et al., 1996; Mustranta, 1992; Tsai et al., 1996). The enantioselectivity or enantiomeric ratio, E, of the enzyme is the most important parameter in the quantitative description of kinetic resolution processes. It is defined as the ratio of the specificity constants (k_2/K_m) of the enzyme for the R- and S-enantiomer. For example, the enantiomeric ratio for an R-enantiomer preferentially selective process can be obtained from

$$E = \frac{k_2^{\mathrm{R}}/K_m^{\mathrm{R}}}{k_2^{\mathrm{S}}/K_m^{\mathrm{S}}}. \tag{1}$$

A better performance of the resolution can be expected if the enantiomeric ratio increases. Since many factors, such as the type of solvent, cosubstrate, reaction temperature, and even the water content, can affect the enatioselectivity and reaction rate, an optimal resolution condition can be only achieved by trying out different experiments. Therefore, an efficient implementation of the enantioselective analysis is the key factor to expedite the establishment of resolution condition.

Chen et al. (1982) modeled kinetic resolutions for uni-uni reactions and derived a relationship among the enantiomeric ratio (E), the degree of conversion of racemate (ξ), and enantiomeric excess of the remaining substrate (ee_s):

$$E = \frac{\ln[(1-\xi)(1-ee_s)]}{\ln[(1-\xi)(1+ee_s)]}, \tag{2}$$

in which

$$\xi = 1 - \frac{C^{\mathrm{S}} + C^{\mathrm{R}}}{C_0^{\mathrm{S}} + C_0^{\mathrm{R}}} \tag{3}$$

$$ee_S = \frac{C^{\mathrm{S}} - C^{\mathrm{R}}}{C^{\mathrm{S}} + C^{\mathrm{R}}}. \tag{4}$$

This equation is valid for homogeneous reactions in batch reactors, barring the reactions with multiple substrates for which this equation might lead to an erroneous apparent E-value. However, it is widely known that many commercial enzymes, such as esterases, proteases, and lipases, can be described kinetically by a ping-pong bi-bi or substituted enzyme mechanism. The mechanisms for multisubstrate enzymatic reactions, on the other hand, lead to more complicated rate expressions. Therefore, the enantiomeric ratio may not be evaluated properly by this method.

In order to resolve the drawback stated earlier, a progress-curve analysis was proposed to further the E-value estimation (Rakels et al., 1994). The progress-curve analysis is complicated and might predict inaccurate results despite the fact that it is applicable to a larger number of kinetic mechanisms, and the kinetic parameters associated with the enzyme-catalyzed reaction can also be determined. Because

the development of the progress curve is a tedious process, enzyme deactivation is often considered to be the major bottleneck in this analysis. Therefore, an enzyme deactivation model must be incorporated to overcome this problem (Rakels et al., 1994). As the optimal condition of resolution system is screened, the analysis becomes an even more intricate and time-consuming task.

In order to simplify the operation and reduce the possibilities of causing errors, a new method for the study of enzymatic resolution systems is proposed in the current work. A chromatographic reactor was proposed here for carrying out the transient-response experiments. The plug-flow model was employed to describe the behavior of the transient system. A simple relationship between the exit concentrations of enantiomers and the enantiomeric ratio was thus established.

Methods and Theoretical Model

Most enzymatic reactions involve at least two substrates. Ping-pong bi-bi is one of the most common mechanisms for describing two-substrate, two-product enzymatic reactions. For an irreversible reaction between the mixture of chiral substrates (S-enantiomer and R-enantiomer) and a cosubstrate B, the ping-pong bi-bi mechanism can be represented as:

S-enantiomer

$$S + EY \underset{k^S_{-1}}{\overset{k^S_1}{\rightleftharpoons}} SEY_S \xrightarrow{k^S_2} P_S + EX_S \tag{5}$$

$$EX_S + B \underset{k^S_{-3}}{\overset{k^S_3}{\rightleftharpoons}} BEX_S \xrightarrow{k^S_4} Q_S + EY. \tag{6}$$

R-enantiomer

$$R + EY \underset{k^R_{-1}}{\overset{k^R_1}{\rightleftharpoons}} SEY_R \xrightarrow{k^R_2} P_R + EX_R \tag{5'}$$

$$EX_R + B \underset{k^R_{-3}}{\overset{k^R_3}{\rightleftharpoons}} BEX_R \xrightarrow{k^R_4} Q_R + EY. \tag{6'}$$

First of all, S binds to the enzyme EY in the ping-pong mechanism for the S-enantiomer. Then P_S, the first product, forms and leaves before the cosubstrate B arrives. B cannot bind to the enzyme EY except to attach to the modified enzyme EX_S. The second product, Q_S, is then formed and liberated, leaving the enzyme in its original form. Since only one substrate is present in enzyme at any time, it is possible to treat the enzyme in EY form. As shown in Eqs. 5 and 5', when the enzyme molecules are pretreated in EY form, a perturbation of a mixture of enantiomers—S- and R-enantiomer—will transfer the enzyme into EX_S and EX_R form and generate P_S and P_R, respectively. The enzymatic resolution takes place during these two steps.

A fixed-bed reactor with packed enzyme is proposed to analyze the enantioselectivity. The proposed setup of the apparatus is shown in Figure 1. The enzyme molecules in the reactor are first pretreated with sufficient cosubstrate B-containing solution. This pretreatment will transform the enzyme molecules into EY form entirely (as shown in Eqs. 6 and 6').

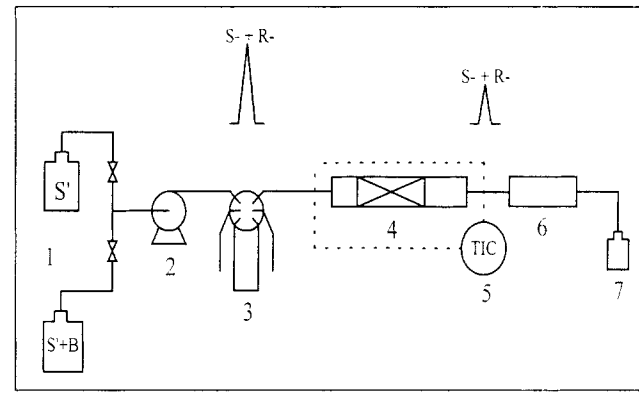

Figure 1. Suggested setup of experimental apparatus.

(1) Reservoirs, *S'*: solvent; (2) pump; (3) injection port; (4) fixed-bed reactor; (5) temperature controller; (6) detector; (7) elution collector.

After the residual cosubstrate B has been removed by infusing the packed-bed reactor with substrate-free solvent, a pulse of enatiomers in then introduced. The S-enantiomer input will combine with enzyme EY to form complex SEY_S, and enzyme EX_S and product P_S will finally be generated (as shown in Eq. 5). For an uncompleted reaction, S-enantiomer and the product P_S can be monitored at the exit of the chromatographic reactor. When both enantiomers are introduced, the eluted amount of each enantiomer can be determined simultaneously.

The pulse input of enantiomers stimulates the system to an unsteady state. Under a condition without mass-transfer limitation, the plug-flow model for the S-enantiomer in the system could be formulated as

$$-v\frac{\partial C^S}{\partial z} + \frac{1-\epsilon}{\epsilon} r_S = \frac{\partial C^S}{\partial t} \tag{7}$$

$$r_S = -k^S_1 C_e C^S + k^S_{-1} C^S_X \tag{8}$$

$$\frac{\partial C^S_X}{\partial t} = k^S_1 C_e C^S - (k^S_{-1} + k^S_2) C^S_X \tag{9}$$

$$t = 0 \qquad C^S = C^S_X = 0 \tag{10}$$

$$z = 0 \qquad C^S = C^S_{in}(t). \tag{11}$$

For the R-enantiomer, the superscript S is changed to R in Eqs. 7–11.

If the total amount of the enantiomers (S- and R-) input to the reactor is far less than that of the enzyme molecules in the reactor, which is easily achievable, the concentration of C_e can be assumed to be a constant C_{eo} during the operation. After taking the Laplace transform with respect to time, t, the transfer function of S-enantiomer at the exit can be obtained to be

$$F^S(S) = \left.\frac{\overline{C^S}}{\overline{C^S_{in}}}\right|_{z=L} = \exp(-\Phi^S \tau), \tag{12}$$

where

$$\Phi^S = s + \frac{1-\epsilon}{\epsilon} \frac{C_{eo}(S + k_2^S)}{\underset{k_1^S}{S + K_m^S}} \tag{13}$$

$$\tau = \frac{L}{v} \tag{14}$$

$$K_m^S = \frac{k_{-1}^S + k_2^S}{k_1^S}. \tag{15}$$

From the definitions of the Laplace transform, the following relationship can be obtained

$$\lim_{s \to 0} F^S(s) = \frac{\int_0^\infty C^S(t)_{z=L}\, dt}{\int_0^\infty C_{in}^S(t)\, dt}. \tag{16}$$

Since

$$\frac{\int_0^\infty C^S(t)|_{z=L}\, dt}{\int_0^\infty C_{in}^S(t)\, dt} = 1 - x^S, \tag{17}$$

Eq. 16 can be expanded in the form of

$$1 - x^S = \exp\left(- \frac{1-\epsilon}{\epsilon} \frac{k_2^S C_{eo}}{K_m^S} \tau \right). \tag{18}$$

A similar result can be obtained for the R-enantiomer

$$1 - x^R = \exp\left(- \frac{1-\epsilon}{\epsilon} \frac{k_2^R C_{eo}}{K_m^R} \tau \right), \tag{19}$$

in which

$$K_m^R = \frac{k_{-1}^R + k_2^R}{k_1^R}. \tag{20}$$

It is very surprising that the individual conversion of each enantiomer (x^S and x^R) is directly related to the specificity constant of the enzyme.

Conclusion and Discussion

From Eqs. 18 and 19, it is obvious that the ratio of the remaining fraction in logarithmic scale of the two enantiomers equals the ratio of the specificity constants of both enantiomers:

$$\frac{\ln(1-x^R)}{\ln(1-x^S)} = \frac{k_2^R/K_m^R}{k_2^R/K_m^S} \tag{21}$$

or

$$E = \frac{\ln(1-x^R)}{\ln(1-x^S)}. \tag{22}$$

A very significant conclusion is obtained from Eq. 22. That is, the only thing that needs to be done is to analyze the remaining fraction of each enantiomer at the exit in order to determine the enantioselectivity. Moreover, it must be emphasized that this method is applicable to systems with enantiomers introduced in any proportion. Due to this distinctive character, it is even more reliable when studying the highly enantioselective enzymatic reaction since the instrumentation error caused by the large concentration difference between the two enantiomers can thus be reduced.

The unsteady-state method introduced in this work can be utilized to analyze the enantioselectivity of an enzyme through only one experiment, provided that a large amount of enzyme is used. The experimental setup is very simple and requires no special apparatus. The operation is very easy and time saving, and therefore, it rules out the problem of the stability of the enzyme. The data analysis does not require a complicated treatment. Furthermore, the availability of chiral substrates becomes more flexible, since the enantiomers in any proportion can be used to start the test. Therefore, it is believed that the proposed method is destined to be a very promising technique for developing an optimal condition for enzymatic chiral resolution system.

Notation

C = concentration of the chiral substrate, mol/cm^3
C_0 = initial concentration of the chiral substrate in a batch system, mol/cm^3
C_{in} = inlet concentration of substrate A, mol/cm^3
C_e = concentration of enzyme in EY form, mol/cm^3
C_{eo} = total concentration of enzyme, mol/cm^3
C_X^S = concentration of complex SEY, mol/cm^3
k_1, k_3 = second-order rate constant, cm^3/mol/min
k_{-1}, k_2, k_{-3}, k_4 = first-order rate constant, min^{-1}
K_m = Michaelis constant for substrate A
L = length of the packed bed, cm
s = Laplace transform variable, min^{-1}
v = interstitial velocity, cm/min
x = individual conversion
z = axial distance, cm
ϵ = void fraction of bed
τ = space time, min

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