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On-Line Determination of Lipase Activity and Enantioselectivity Using An Immobilized Enzyme Reactor Coupled To A Chiral Stationary Phase

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Abstract: Lipase has been immobilized on an artificial immobilized membrane HPLC phase and the enzyme reactor coupled to a HPLC chiral stationary phase. The resulting system was used for on-line determination of the enantioselectivity of the immobilized enzyme.

We have recently reported the development of a coupled chromatographic system in which an immobilized enzyme reactor (IMER) was coupled to a column containing a chiral stationary phase (CSP).[1] In this work, the IMER consisted of α-chymotrypsin (ACHT) immobilized on a silica-based HPLC support, the ACHT-IMER, and the CSP was based on a chiral crown ether (CR-CSP). The ACHT-IMER and the CR-CSP were connected through a switching valve.

The ACHT-IMER was enzymatically active and was able to hydrolytically cleave esters of L-tryptophan (L-TRP) as well as the corresponding esters of D-TRP. The extent of the hydrolysis of the D-TRP esters could be regulated by altering the time that the compound was in contact with the immobilized ACHT. The enantioselectivity of the enzymatic conversion on the ACHT-IMER could be determined by directing the eluent containing the hydrolyzed D- and L-TRP from the ACHT-IMER to the CR-CSP where the enantiomers were stereochemically resolved and quantitiated.

This communication reports the development of an IMER-CSP system containing an IMER based upon lipase from *Candida cylindracea* (Sigma Type VII-S) and a CSP utilizing immobilized human serum albumin, HSA-CSP. In these experiments, the enzyme was immobilized by entrapment in the interstitial spaces of an artificial immobilized membrane (IAM)[2] stationary phase using previously described procedures.[3] Booth loose stationary phase and stationary phase packed into a 250 mm x 4.1 mm I.D. steel HPLC column were used in the study.

When lipase was immobilization on loose IAM stationary phase, 50 mg of the phase was washed 3 times with 1 ml portions of sodium phosphate buffer [0.1 M, pH 7] (BUFFER) and crude *Candida cylindracea* lipase [10 mg in 2 mL BUFFER] was added. The mixture was stirred for 2 h, centrifuged and the supernatant decanted. The resulting lipase containing IAM stationary phase (L-IAM-SP) was washed with 4 x 1 mL

BUFFER and the washing collected. The uv absorbancies of the supernatant and the washings were measured separately at λ = 241.4 nm and compared to the absorbency of the initial enzyme/BUFFER solution. In this manner it was determined that 3.61 mg of lipase had been immobilized on the support.

The enzymatic activity of the L-IAM-SP was determined by measuring the hydrolysis of racemic-ibuprofen methyl ester[4] (rac-IBU-ME). rac-IBU-ME [5 mg in 0.2 mL of ACN] was added to 50 mg of the L-IAM-SP (representing 3.61 mg lipase) in 2 mL BUFFER. The resulting mixture was stirred at room temperature for 6 h and the reaction quenched by centrifugation and decanting of the supernatant. The L-IAM-SP was washed with 2 ml BUFFER, the washing and supernatant combined and extracted with CH_2Cl_2, the organic solvent evaporated, the residue dissolved in 0.5 mL ACN/BUFFER [1:1, v/v] and analyzed by HPLC.[5] The residue contained 0.34 mg of IBU with an *ee* (S-IBU) of 50.5%.

The degree of enzymatic activity retained by the immobilized lipase was determined through a comparison with the activity of an equivalent amount of the nonimmobilized enzyme. In this experiment 3.61 mg of the free enzyme and 5 mg of rac-IBU were dissolved in 0.2 mL ACN and added to 2 mL BUFFER. The mixture was stirred at room temperature for 6 h, the reaction quenched by extraction with CH_2Cl_2, the organic solvent evaporated, the residue dissolved in 0.5 mL ACN/BUFFER [1:1, v/v] and analyzed by HPLC.[5] The residue contained 0.38 mg of IBU with an *ee* (S-IBU) of 36.2% indicating that the enzymatic activity of the L-IAM-SP was 90% of the equivalent amount of non-immobilized lipase and that immobilization had produced a slight increase in enantioselectivity.

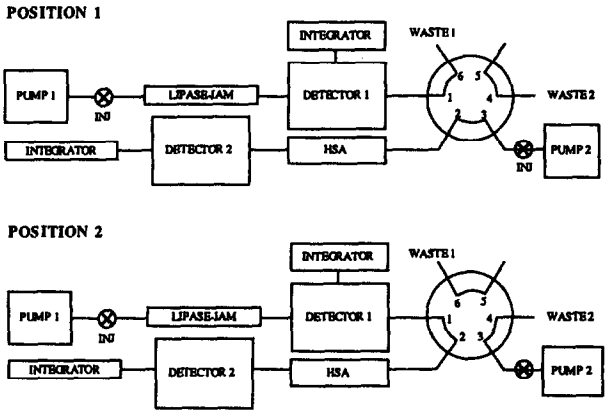

Figure 1. IMER-CSP system where: the substrate is loaded onto the IMER using Position 1; the products are switched to the CSP using Position 2; the enantiomeric composition of the product is measured on the CSP using Position 1.

The synthesis of the lipase-immobilized enzyme reactor (L-IAM-IMER) was accomplished by circulating a solution of lipase [50 mg in 50 mL BUFFER] through a 250 mm x 4.1 mm I.D. HPLC column containing IAM stationary phase. The uv absorbency of the mobile phase was monitored at $\lambda = 237$ until a stable base line was established. The decrease in the absorbency of the mobile phase in the reservoir indicated that 32 mg of the enzyme had been immobilized on the column.

The HPLC columns containing the L-IAM-IMER and HSA-CSP were connected through a 6-port switching valve, Figure 1, and the activity and enantioselectivity of the L-IMER investigated using the hydrolysis of (R,S)-naproxen chloroethyl ester[4] (R,S-NCE) as a probe. In these experiments, (R,S)-NCE [20 µl of 0.165 µg/mL BUFFER] was injected onto the L-IMER and allowed to remain in contact with the enzyme for from 2 to 14 h, Table 1. The substrate and product were then eluted from the L-IMER using a mobile phase containing ACN:BUFFER (30:70, v/v). A representative chromatogram is presented in Figure 2A. The eluent containing the product, (R,S)-naproxen, was selectively transferred to the HSA-CSP and the enantiomers were stereoselectively resolved and individually eluted using a mobile phase composed of 4 mM octanoic acid in sodium phosphate [0.05 M, pH 6.9] modified with ACN (15% v/v). A representative chromatogram is presented in Figure 2B.

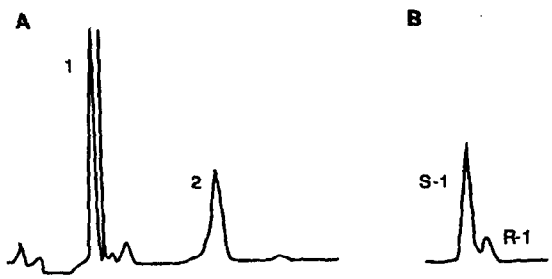

Figure 2. The lipase catalyzed hydrolysis of (R,S)-naproxen chloroethyl ester (NCE) on an IMER-CSP system. A. The elution of (R,S)-naproxen (1) and (R,S)-NCE (2) from the L-IMER. B. The elution of (S)-naproxen (S-1) and (R)-naproxen (R-1) from the HSA-CSP.

The percent conversion of the substrate on the L-IMER and the enantiomeric content of the product, as a function of the length of contact between the enzyme and substrate are presented in Table 1. After elution of the substrate and product from the L-IMER, the column was washed with 30 ml BUFFER. Under the experimental conditions employed in this study, the L-IAM was active for four months.

As illustrated in Table 1, the conversion ratio increases as a function of time while the *ee* decreases. This is agreement with previous observations.[6] However, since the enantioselectivity of the crude enzymatic preparation was low and the reaction slow, the observed differences are not very great. This can be changed

by using purified or modified enzymes.[7]

The system described in this report can be used to rapidly screen the enantioselectivity and activity of a lipase preparation towards a series of substrates as well as to screen a series of lipase preparations for a particular synthetic application. The system can also be used to optimize reaction conditions, including temperature and ionic strength, although the ACN content of the mobile phase is limited to 30%. In addition, if the solute capacity of the column containing the CSP is great enough, the coupled IMER-CSP system can be used to prepare significant quantities of the desired enantiomers.

Table 1. Degree of enzymatic hydrolysis of (R,S)-naproxen chloroethyl ester on the lipase immobilized enzyme reactor and the enantioselectivity of the reaction as a function of time.

Time (h)	Percent Conversion	Enantioselectivity (*ee*)
2	18.8	59.2
3	17.7	58.2
4	19.1	54.0
6	33.2	51.9
7	31.1	55.7
10	30.6	56.7
14	46.1	52.4

References and Notes

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4. Ibuprofen methyl ester and naproxen chloroethyl ester were synthesized using the following procedure: To 50 mmol MeOH or 30 mmol of chloroethanol in a 5 mL flask equipped with a condenser were added IBU (5.0 mmol) or naproxen (0.9 mmol) and 1 drop H_2SO_4. The mixture was heated at 80 °C for 10 h, poured into 10 mL of saturated $NaHCO_3$ and the resulting mixture was extracted with AcOEt (3 x 3 mL). The combined organic washings were washed with $NaHCO_3$ and water, dried over $MgSO_4$ and gave IBU methyl ester (90% yield) or naproxen chloroethyl ester (86% yield).
5. R- and S-IBU were enantioselectively resolved and quantified using a HPLC column (250 mm x 4.1 mm ID) packed with human serum albumin-based chiral stationary phase and a mobile phase composed of 4 mM octanoic acid in sodium phosphate buffer [0.05 M, pH 6.90]:ACN (85:15, v/v). Under these conditions, the chromatographic retention, expressed as capacity factors, k', for S- and R-IBU were 7.5 and 10.0, respectively and the observed enantioselectivity factor (α) was 1.33.
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