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Enantioselective Adsorption of (+)-(*S*)-Naproxen in a Crosslinked Lipase in Organic Solvents

CHUN-SHENG CHANG and SHAU-WEI TSAI*
DEPARTMENT OF CHEMICAL ENGINEERING
NATIONAL CHENG KUNG UNIVERSITY
TAINAN, TAIWAN 70101, REPUBLIC OF CHINA

ABSTRACT

Enantioselective adsorption of (+)-(*S*)-naproxen from the racemate in cyclohexane, *n*-hexane, *n*-heptane, or isooctane, but not in a less hydrophobic solvent such as toluene, was obtained by using a crosslinked lipase (ChiroCLEC-CR) as the adsorbent. However, this behavior was not observed in cyclohexane when a crude lipase, a purified lipase, or the crosslinked lipase preexposed to the organic solvent for 24 hours was employed. Effects of racemic naproxen concentration and solvent hydrophobicity on the amount of enantiomer adsorbed to the adsorbent, the partition coefficient (i.e., the ratio of enantiomer concentration in the adsorbent to that in the organic solvent), and the selectivity factor [i.e., the ratio of partition coefficients for (+)-(*S*)- and (−)-(*R*)-naproxen] were determined. Moreover, desorption of the enantiomer from the absorbent was compared when a fresh organic solvent was substituted or a displacer such as steric acid was added to the solution after the adsorption operation.

Key Words. Enantioselective adsorption; Crosslinked lipase; Naproxen; Selectivity factor

INTRODUCTION

Chiral separations have become increasingly important over the past decade. The advent of a variety of chiral stationary phases (CSP) in high perfor-

* To whom correspondence should be addressed. FAX: 886-6-2344496, E-mail: t62647@mail.ncku.edu.tw

mance liquid chromatography (HPLC) has widened the range of enantiomers that can be analyzed (1, 2). An important recent innovation has been the development of chiral HPLC columns using immobilized proteins as the stationary phase for chiral discrimination in pharmaceutical and agricultural chemical research. These proteins include albumins such as bovine serum albumin (3, 4) and human serum albumin (5), glucoproteins such as orosomucoid (6) and ovomucoid (7), and enzymes such as α-chymotrypsin (8) and fungal cellulase (9). Many advantages of using protein-based columns have been reported, such as direct resolution of drug enantiomers without derivatization and unusual retention characteristics by adjusting column temperature, mobile phase pH, or organic modifiers as the additive. However, to the best of our knowledge, all such stationary phases are associated with reversed phase LC separations. This is primarily due to the fact that such proteins are denatured when subjected to the presence of modest organic modifier concentrations.

Enzymes have been established as catalysts in carbohydrate, antibiotic, and aminoacid industries (10). The enzyme's ability to distinguish enantiotopic moieties of chiral and prochiral molecules, combined with the regioselectivity and chemoselectivity, has led an explosion in biocatalysis research in organic synthesis in the past 20 years (11–13). Many works have focused on the synthesis or resolution of optically active drugs or their intermediates by suspending powdered enzymes in microaqueous organic solvents (14–16). However, there is still no report on the effect of enzyme preparations and solvent hydrophobicity on the enantioselective adsorption of the racemate on the enzyme. When this effect is not negligible, an error arises in determining the enzyme enantioselectivity from the equation (17) relating the enantiomeric ratio to the conversion and the enantiomeric excess for the substrate (or the product).

The motivation of the present research is to investigate the adsorption and desorption behavior of optical isomers of naproxen on *Candida rugosa* lipase from different preparations in organic solvents. Since the lipase in crosslinked form was found to enantioselectively adsorb (+)-(*S*)-naproxen, it may have the potential as a CSP for HPLC columns operated in the normal phase mode or as an adsorbent in chiral separations in organic solvents. The present results may also impact kinetic data in which the crosslinked lipase is the biocatalyst in the kinetic resolution of racemates in microaqueous organic solvents.

EXPERIMENTAL

Materials

Pure (+)-(*S*)-naproxen [2-(6-methoxy-2-naphthyl) propionic acid] and α-lactose were purchased from Sigma (St. Louis, MO). The enantiomer was

racemized at 140°C in ethylene glycol with sodium hydroxide for 4 hours to obtain the desired racemate (18). Isooctane and cyclohexane of HPLC grade from Tedia (Fairfield, OH), *n*-heptane, *n*-hexane, and toluene of ACS grade from Merck (Darmastadt, Germany) were used without further purification. A crosslinked lipase (triacylglycerol ester hydrolases, EC 3.1.1.3) of Chiro-CLEC-CR was purchased from Altus Biologics Inc. (Cambridge, MA). A crude lipase of Lipase MY (30 units/mg solid) from *Candida rugosa* was provided by Meito Sangyo (Nagoya, Japan). A purified lipase from the same microorganism was kindly given by Professor J. S. Dordick of the University of Iowa. It was originally donated by Altus Biologics Inc., dialyzed against phosphate buffer (50 mM, pH 7.0) overnight, and lyophilized for 24 hours.

Analytical Conditions

HPLC was utilized to determine the concentration of (−)-(*R*)- and (+)-(*S*)-naproxen with 2-phenylalcohol as an internal standard (capacity factors *k* as 4.5, 5.0 and 3.5, respectively). A Chiralcel OD column (Daicel Chemical Industries Ltd., Japan) was used with the mobile phase consisting of a mixture of *n*-hexane:isopropanol:acetic acid (v/v, 97:3:1), at a flow rate of 1.0 mL/min. UV detection at 270 nm was employed for quantification at 25 C. Since the crosslinked lipase is very expensive, only a single measurement was made for each experiment.

Adsorption of Enantiomers

To the desired concentration of racemic naproxen in 1.5 mL of various organic solvents was added 1 mg/mL of ChiroCLEC-CR. The resultant mixture was stirred with a magnetic stirrer at 37°C. Samples were removed for an HPLC analysis at different time intervals. Similar experiments were carried out in cyclohexane except that 5 mg/mL of α-lactose, 5 mg/mL of Lipase MY, or 1 mg/mL of the purified lipase was substituted.

Desorption of Enantiomers

After 12 hours of the adsorption experiment in cyclohexane, the crosslinked lipase was filtered off and quickly washed out three times with each of 1.5 mL of fresh cyclohexane. The filtrate and the washing solvent were collected and analyzed to determine the enantiomer concentration and the fraction of adsorption for (−)-(*R*)- and (+)-(*S*)-naproxen (A_R and A_S, respectively). Then the lipase was poured into 1.5 cm^3 of fresh cyclohexane or methanol for 24 hours. The resultant solution was analyzed to determine the remaining A_R and A_S. Another desorption experiment was also carried out by adding 4

mM of steric acid to the resultant solution after 12 hours of the adsorption in cyclohexane.

RESULTS AND DISCUSSION

Figure 1 illustrates the variation of (−)-(*R*)- or (+)-(*S*)-naproxen concentration in the organic solvent with the enantiomer concentration in the cross-linked lipase at 37°C at the sampling time of 4.5 hours. An enantioselective adsorption of the (+)-(*S*)-enantiomer in cyclohexane was found, which indicated a stronger binding of (+)-(*S*)-naproxen to this enzyme preparation. A linear relationship between the concentrations in the adsorbent and the organic solvent for each enantiomer is also illustrated in the figure, where the highest (+)-(*S*)-naproxen concentration of 1.8×10^{-4} mmol/mg in the former was obtained. This indicates that each lipase molecule has adsorbed 10.8 molecules of (+)-(*S*)-naproxen if the molecule weight of the lipase is taken as 60,000 (19).

When a less hydrophobic solvent, toluene, was used, not only did the adsorption of each enantiomer on the enzyme greatly decrease but also the

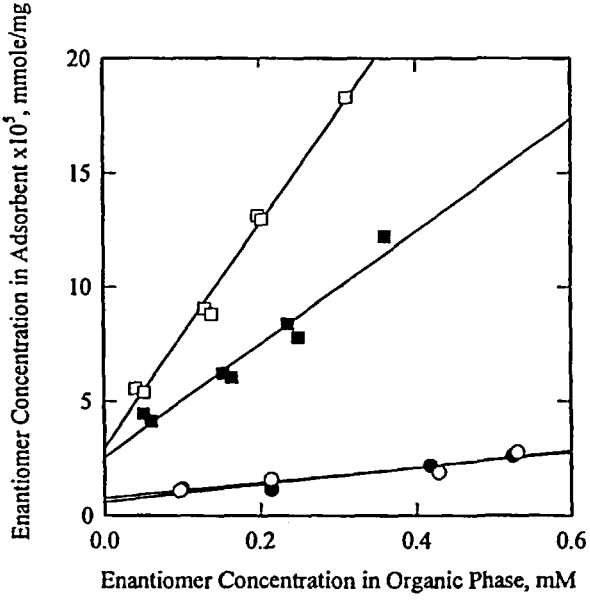

FIG. 1 Effects of enantiomer concentration in organic solvents on that in the adsorbent at 4.5 hours of the adsorption experiment: 1.0 mg/mL ChiroCLEC-CR. Filled symbols for (−)-(*R*)-naproxen and empty symbols for (+)-(*S*)-naproxen. (■, □) in cyclohexane; (●, ○) in toluene.

TABLE 1
Effects of Solvent Hydrophobicity (in terms of log P) on the Partition Coefficients of K_R and K_S (mmol/mg mM) and the Selectivity Factor of α (i.e., K_S/K_R) at 4.5 hours: 1 mg/mL ChiroCLEC-CR; 0.17 mM Racemic Naproxen in Isooctane and 0.20 mM in Other Solvents

	Toluene	Cyclohexane	*n*-Hexane	*n*-Heptane	Isooctane
Log P	2.50	3.20	3.50	4.00	4.50
$K_R \times 10^3$	0.13	0.87	1.83	1.43	1.12
$K_S \times 10^3$	0.13	1.43	2.24	2.09	1.83
α	1.00	1.64	1.22	1.46	1.63

enzyme lost its enantioselectivity. This suggests that both enantiomers may compete for the same binding site(s) of ChiroCLEC-CR in cyclohexane, whereas the presence of toluene will change the microenvironment of the binding sites, and hence eliminate the chiral discrimination. Another possibility is that there may exist multibinding sites, enantioselective and nonselective, for the adsorption of the racemate. Then, changing the solvent from cyclohexane to toluene may affect enzyme conformation, especially around the enantioselective sites, and destroy the enantioselectivity of the lipase to (+)-(*S*)-naproxen.

When solvents of greater hydrophobicity, such as *n*-hexane, *n*-heptane, and isooctane, were used, higher partition coefficients for both enantiomers (K_R and K_S) were obtained (see Table 1) compared to those in cyclohexane and toluene. Thus far, we have no explanations as to why *n*-hexane produces the maximum partition coefficient and the maximum selectivity factor ($\alpha = K_S/K_R$) was found in cyclohexane (Table 1). In general, the selectivity factor increases with the log *P* of the solvent (where *P* is the partition coefficient of solvent between *n*-octanol and water) except for cyclohexane (20). Therefore, one may conclude that solvent hydrophobicity has marked effects on the affinity and selectivity of (−)-(*R*)- and (+)-(*S*)-naproxen to ChiroCLEC-CR.

The time courses of the fraction of adsorption for each enantiomer on ChiroCLEC-CR in cyclohexane and isooctane are presented in Fig. 2. In the initial stage (within 1 hour) both enantiomers rapidly adsorb to the enzyme in isooctane without enantioselectivity, and then gradually desorbs [mainly the (−)-(*R*)-naproxen] to give the selectivity factor of 1.75 at 24.6 hours. A similar result of 1.85 at 24 hours in cyclohexane was also determined (Fig. 2), where a slight increase of the fraction of adsorption for (+)-(*S*)-naproxen was obtained. These results imply that the organic solvent may gradually change the enzyme conformation, which may reduce the binding force, and hence the adsorption, between the lipase molecule and (−)-(*R*)-naproxen.

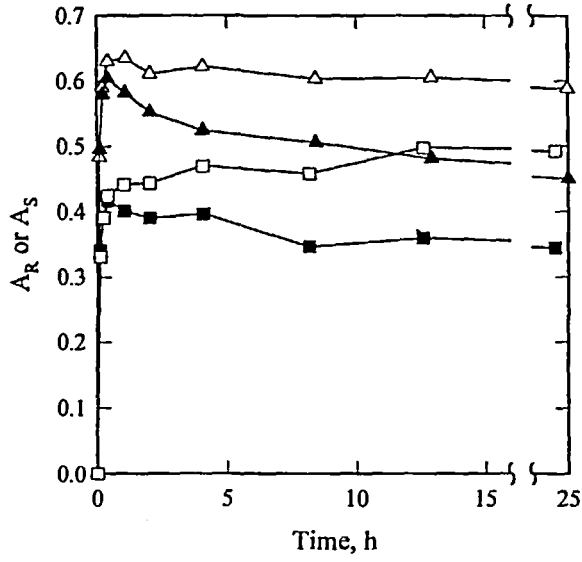

FIG. 2 Time courses of the fraction of adsorption for each enantiomer in organic solvents: 1 mg/mL ChiroCLEC-CR. Filled symbols for (−)-(*R*)-naproxen and empty symbols for (+)-(*S*)-naproxen. (▲, □) *in isooctane with 0.17 mM racemic naproxen;* (■, □) *in cyclohexane* with 0.22 mM racemic naproxen.

Similar behaviors for the enantioselective adsorption of (+)-(*S*)-ibuprofen and (+)-(*S*)-surprofen in cyclohexane were found (data not shown). More studies on the mechanism of enantioselectivity of (+)-(*S*)-profen to the crosslinked enzyme in hydrophobic solvents are needed.

By employing the crosslinked lipase preexposed in cyclohexane for 24 hours as the adsorbent, not only did the fraction of adsorption for each enantiomer decrease but also nearly no enantioselectivity at 25 hours was observed (see Fig. 3). This implies that the pre-exposure time of 24 hours is long enough to alter the binding sites to a stable conformation, which does not discriminate between enantiomers. Moreover, the adsorption of the enantiomers to the binding sites might enhance the resistance of the enzyme from conformation alternation, and then the desorption of the racemate. Otherwise, one should obtain the same final A_R and A_S in cyclohexane which is not seen from the data in Fig. 3.

When a purified lipase was used as the adsorbent in cyclohexane (see Fig. 3), a similar time-course A_R and A_S was obtained. This indicates that the enzyme conformation of the purified lipase is less suitable for the binding of the racemate and the chiral discrimination between enantiomers. Therefore,

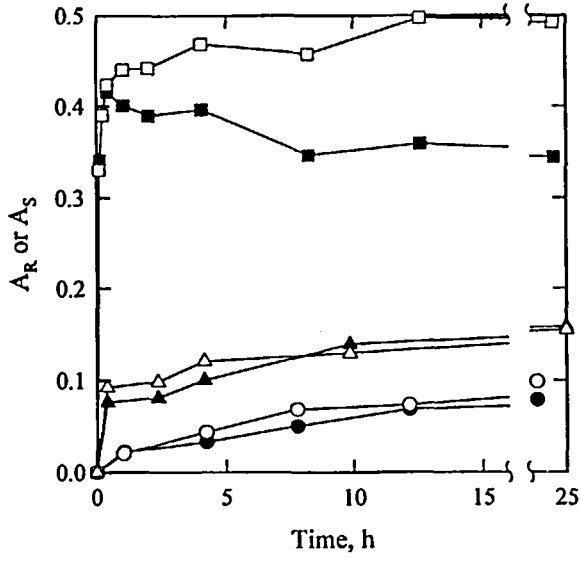

FIG. 3 Time courses of the fraction of adsorption for each enantiomer in cyclohexane: 1 mg/mL lipase, 0.22 mM racemic naproxen. Filled symbols for (−)-(*R*)-naproxen and empty symbols for (+)-(*S*)-naproxen. (■, □) for ChiroCLEC-CR; (●, ○) for ChiroCLEC-CR preexposed to cyclohexane for 24 hours; (▲, △) for the purified lipase.

the crystallization and covalent crosslinking between the molecules of the purified lipase might change the enzyme conformation so that the obtained ChiroCLEC-CR possesses the enantioselective property.

When using Lipase MY as the adsorbent in isooctane, nearly the same A_R and A_S was found (see Table 2). A similar behavior is also shown in the table when using α-lactose, a main additive of Lipase MY, as the adsorbent. Therefore, from the difference of A_R (and A_S) in both cases, it is concluded that

TABLE 2
Fractions of Adsorption of A_R and A_S in α-Lactose, Lipases from Different Preparations in Isooctane at 24 hours: 0.17 mM Racemic Naproxen

	Enzyme concentration (mg/mL)	A_R (%)	A_S (%)
ChiroCLEC-CR	1.0	47.13	63.09
α-Lactose	5.0	23.40	24.00
Lipase MY	5.0	39.40	38.71

TABLE 3
Desorption of (−)-(*R*)- and (+)-(*S*)-Naproxen at 24 hours after 12 hours Adsorption in Cyclohexane: 1 mg/mL ChiroCLEC-CR; 0.22 mM Racemic Naproxen; Unit of K_R and K_S as mmol/mg mM

		Adsorption	Desorption
With fresh cyclohexane	A_R (%)	33.43	16.53
	A_S (%)	52.92	42.04
	$K_R \times 10^3$	0.50	0.98
	$K_S \times 10^3$	1.12	3.87
	α	2.24	3.95
With fresh methanol	A_R (%)	30.25	12.00
	A_S (%)	47.34	32.98
	$K_R \times 10^3$	0.43	0.65
	$K_S \times 10^3$	0.90	2.29
	α	2.09	3.52
Adding 4 mM steric acid to the solution	A_R (%)	33.89	18.97
	A_S (%)	48.04	31.16
	$K_R \times 10^3$	0.51	0.23
	$K_S \times 10^3$	0.92	0.45
	α	1.80	1.97

the enzyme molecules in Lipase MY in isooctane have no enantioselectivity. Although a preliminary comparison of the enzyme composition, molecule structure, and stability against temperature and solvent polarity for the crude, the purified, and the crosslinked lipases was reported (21), we have no explanations as to why the purified lipase after crystallization and covalent crosslinking can enantioselectively adsorb (+)-(*S*)-naproxen.

The desorption of the adsorbed enantiomers on ChiroCLEC-CR was investigated by adding a fresh solvent to the lipase which was filtered off after the adsorption experiment in cyclohexane. As shown in Table 3, when fresh cyclohexane was utilized, the fractions of adsorption for (−)-(*R*)- and (+)-(*S*)-naproxen reduced from 33.43 to 16.53% and from 52.92 to 42.04%, respectively. This indicates that the (−)-(*R*)-enantiomer is more easily desorbed from the binding sites, as indicated in Fig. 2. Moreover, the partition coefficient for each enantiomer increases, and about 1.8-fold enhancement of the selectivity factor is obtained after the desorption experiment. When fresh methanol was applied, a similar behavior for the change of the selectivity factor was found. However, by comparing the variation of K_R or K_S before and after the desorption experiment in both systems, methanol is more effective for stripping the adsorbed enantiomers off the adsorbent.

Nature has designed lipases to hydrolyze oils and fats to give monoglyceride, diglyceride, fatty acids, and glycerol. Past research has also indicated

that these enzymes have a high affinity to fatty acids during ester synthesis. Therefore, by adding 4 mM of steric acid to the solution at 12 hours of the adsorption experiment in cyclohexane, about the same amounts of (−)-(*R*)- and (+)-(*S*)-naproxen are displaced after comparing the changes of A_R and A_S before and after the desorption (see Table 3). This implies that the slightly different binding force between the lipase and each enantiomer has no effect on the enantioselective replacement of the enantiomer by the additive. Moreover, adding steric acid is more effective for desorbing (+)-(*S*)-naproxen, as shown by the changes of A_S for all systems in Table 3. A further study on employing ChiroCLEC-CR as a CSP of a HPLC column operated in the normal phase mode will be an interesting subject for the future. The present crosslinked lipase might also be tested as an adsorbent or a CSP of a HPLC column operated in the reversed phase mode, which might shed light on the mechanism for the enantioselective adsorption of (+)-(*S*)-profen.

CONCLUSION

Preliminary experiments on using *Candida rugosa* lipases from different preparations as adsorbents for racemic naproxen have been carried out in organic solvents. Enantioselective adsorption for (+)-(*S*)-naproxen was found in *n*-hexane, *n*-heptane, cyclohexane, or isooctane, but not in a less hydrophobic solvent such as toluene, when a crosslinked lipase (ChiroCLEC-CR) was employed. However, this stereospecificity was lost when a crude lipase, a purified lipase, or the crosslinked lipase, preexposed to the solvent, was utilized in cyclohexane.

Solvent hydrophobicity has a strong influence on the adsorption performance of crosslinked lipase. However, no correlation was found for the variation of the enantiomer concentration in the adsorbent, the partition coefficient, and the selectivity factor with the log *P* of the solvent. Compared with cyclohexane, methanol was more effective for stripping the adsorbed naproxen off the adsorbent. Desorption of the adsorbed enantiomer was also observed when a displacer such as a steric acid was added in cyclohexane.

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REFERENCES

1. D. W. Armstrong and S. M. Han, *CRC Crit. Rev. Anal. Chem.*, *19*, 175–224 (1988).
2. W. H. Pirkle and T. C. Pochapsky, *Chem. Rev.*, *89*, 347–362 (1989).

3. S. Allenmark and S. Andersson, *Chirality, 1*, 154–160 (1989).
4. R. C. Williams, J. F. Edwards, and M. J. Potter, *J. Liq. Chromatogr., 16*, 171–196 (1993).
5. T. A. G. Noctor, G. Felix, and I. W. Wainer, *Chromatographia, 31*, 55–59 (1991).
6. J. Hermansson and M. Eriksson, *J. Lip. Chromatogr., 9*, 621–639 (1986).
7. H. Fujima, H. Wada, T. Miwa, and J. Haginaka, *Ibid., 16*, 879–891 (1993).
8. I. Marle, A. Karlsson, and C. Petterson, *J. Chromatogr., 604*, 185–196 (1992).
9. P. Erlandsson, I. Marle, L. Hamsson, R. Isaksson, C. Pettersson, and G. Pettersson, *J. Am. Chem. Soc., 112*, 4573–4574 (1990).
10. W. Gerhartz, *Enzymes in Industry*, VCH, Weiheim, Germany, 1990.
11. K. Drauz and H. Waldmann, *Enzyme Catalysis in Organic Synthesis—A Comprehensive Handbook*, VCH, Weiheim, Germany, 1995.
12. K. Faber, *Biotransformations in Organic Chemistry—A Textbook*, 2nd ed., Springer-Verlag, New York, NY, 1995.
13. C-H. Wong and G. M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon, Tarrytown, NY, 1994.
14. A. M. Klibanov, *Acc. Chem. Res., 23*, 114–120 (1990).
15. E. Santaniello, P. Ferraboschi, P. Grisenti, and A. Manzocchi, *Chem. Rev., 92*, 1071–1140 (1992).
16. E. Schoffers, A. Golebiowski, and C. R. Johnson, *Tetrahedron, 52*, 3769–3826 (1996).
17. C. S. Chen, Y. Fujimoto, G. Girdaukas, and C. Sih, *J. Am. Chem. Soc., 104*, 7294–7299 (1982).
18. S. W. Tsai and W. J. Wei, *Enzyme Microb. Technol., 16*, 328–333 (1994).
19. *ChiroCLEC-CR Information Booklet*, Altus Biologics, Inc., Cambridge, MA, 1995.
20. C. S. Laane, K. V. Boeren, and C. Veeger, *Biotechnol. Bioeng., 30*, 81–87 (1987).
21. J. J. Lalonde, C. Govardhan, N. Khalaf, A. G. Martinez, K. Visuri, and A. L. Margolin, *J. Am. Chem. Soc., 26*, 6845–6852 (1995).

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