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Candida Rugosa Lipase: Enantioselectivity Enhancements in Organic Solvents

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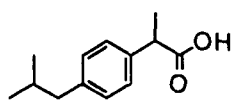
Abstract: Chiral resolutions of carboxylic acids (**1-3**) and alcohol (**4**) were carried out through esterification or transesterification in organic solvents using cross-linked enzyme crystals (CLEC®) of *Candida rugosa* lipase (CRL). Comparison of these results with those of crude CRL reveal significant differences. As was seen in resolution through hydrolysis,[1] a marked improvement in enantioselectivity is realized with the CLEC. Additionally, the stability afforded the enzyme in CLEC form leads to a higher activity in organic solvent.

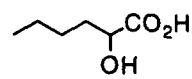
Recent studies have shown that the enantioselectivity of enzyme-catalyzed resolutions can be enhanced significantly by employing purified lipase preparations.[2] These enhancements, however, have been achieved only in hydrolytic reactions due to the lower activity and stability of purified lipase in organic solvents.[3,4] Yet lipase-catalyzed acylations in organic solvents[5] have lead to a variety of valuable optically active compounds, including many potential pharmaceuticals.[6] In order to fully explore the synthetic potential of lipases, it is imperative that a form of catalyst be developed which combines high purity (and thus maximal enantioselectivity) with activity and stability in organic solvents.

We have recently reported the use of Cross-Linked Enzyme Crystals (CLECs)[7] of different hydrolases in the synthesis of peptides[8] and in chiral resolutions.[1] This highly pure form has exhibited much greater stability than the commercially available soluble protein. CLECs of *Candida rugosa* lipase (CLECs-CRL) are not only much more stable in different reaction media than the crude CRL, but are significantly more enantioselective in hydrolytic resolutions. Here we report examples of resolutions with dry CLECs-CRL[9] in organic solvents exhibiting high enantioselectivity in esterification and transesterification. The optical resolution of three racemic acids, (*R,S*)-Ibuprofen (**1**), (*R,S*)-2-hydroxyhexanoic acid (**2**) and (*R,S*)-(4-chloro)-2-phenoxypropionic acid (**3**) were carried out by esterification in nonpolar organic solvents (Table 1). The resolution of the secondary alcohol (+/-) menthol (**4**) was effected through esterification (Table 2) and transesterification (Table 3).

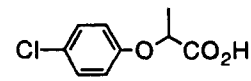
FIGURES 1-4: CRL SUBSTRATES



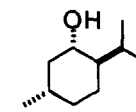
1
(*R,S*)-Ibuprofen



2
(*R,S*)-2-Hydroxyhexanoic Acid



3
(*R,S*)-(4-Chloro)-
2-Phenoxypropionic Acid



4
(+/-) Menthol

TABLE 1 Enantioselectivity of CRL-CLEC and Crude CRL in the Resolution of Acids.[a]

Acid	Catalyst	ee,[e] % ester	ee,[e] % acid	c, %	time, hrs	E
1[b]	CLEC	>99.5 (*S*)	37.9 (*R*)	27.7	24	300
	Crude	68.8 (*S*)	29.6 (*R*)	30	18	7.2
2[c]	CLEC	94.5 *S*		46.0	1	88
	Crude	33.8 *S*		32.8	40	2.0
3[d]	CLEC	99.5 *S*	89.4 *R*	47.5	1.5	>1000
	Crude	37.0 *S*	18.8 *R*	30.1	0.3	2.5

a) E equals the ratio of k_{cat}/K_m for the two enantiomers and is a constant specific for a first order, irreversible resolution reaction. Due to a dependence on the presence of effectors[10] and to product inhibition, E becomes an apparent value (E_{app}). For mixtures of enzymes, E_{app} depends on the amount of each enzyme, their enantioselectivities, substrate concentrations etc.[11] This may explain the variations for published E values. b) 100 mg (0.49 mmol) acid (**1**); 250 µl n-amyl alcohol; 5 mg CRL-CLEC or 75 mg commercial CRL in 5 ml isooctane. c) 26.4 mg (0.2 mmol) acid (**2**); 36.6 µl (0.4 mmol) n-butanol; 10 mg CRL-CLEC or 50 mg commercial CRL in 1 ml toluene. d) 20.0 mg (0.1 mmol) acid (**3**); 36.6 µl (0.4 mmol) n-butanol; 10 mg CRL-CLEC or 25 mg commercial CRL in 1 ml n-heptane. e) Values of ee were determined by chiral HPLC (**1**-Whelk-01 column, **3**-Chiralcel OJ column) or by chiral GC (**2**- Cyclodex B column).

The increase in enantioselectivity seen for these CLEC-CRL-catalyzed acid resolutions is dramatic. Enhancements in enantioselectivity (measured by the ratio of E values for CLEC and crude catalysts (Table 1)), of ~40-fold for (**1**) and (**2**) and more than 400-fold for (**3**) were achieved using this pure form of the lipase. The difference among the acids may be attributable to the variation in activity of the contaminating enzymes found in the commercial preparation of CRL[1] for these substrates. Interestingly, these enhancements in E are greater than those observed in the corresponding ester hydrolyses.[1]

Having studied CLEC-catalyzed resolutions of chiral acids in organic solvent, we were interested in testing CLEC-CRL in acylation of a chiral secondary alcohol (**4**) with different carboxylic acids (Table 2) and with activated esters, such as vinyl acetate (VA) and trichloroethyl- and vinyl butyrates (TCEB and VB) (Table 3). The results shown in Table 2 reveal similar activities for the CLEC and crude CRL, but again a greater enantioselectivity (~2-3-fold) for the CLEC-CRL catalyzed esterifications. The greater enantioselectivity with the pure and stable form of CRL is even more pronounced in the transesterification of (**4**) where E enhancements range from 30-70 fold (Table 3).

As in hydrolytic reactions, the significant increase in enantioselectivity can, at least in part, be attributed to the purity of the CLEC-CRL. However, the comparison between CLEC-CRL and pure lyophilized CRL in organic solvents is more complicated than that in water. Indeed, when pure lyophilized CRL was used in the resolution of menthol with vinyl butyrate in toluene the enantioselectivity increased to E=74 at 97%ee and 10.9% conversion (conditions: menthol (0.2 mmol), vinyl butyrate (0.2 mmol) in 1ml toluene, 1 µl water, 40mg pure CRL-49.5% protein; 42 h reaction time). Since the activity of pure CRL (0.45 nmol/min mg protein) was six orders of magnitude lower than that catalyzed by CLEC-CRL (1220 µmol/min mg protein), the higher conversion could not be achieved. The combination of several additional effects, such as the presence of surfactants[9] and maintaining the optimal water activity balance,[12] and thus enzyme flexibility[13] in the dry CLEC-CRL formulations, may account for further increase in enantioselectivity of CLEC-CRL, compared with pure lyophilized CRL. These same factors, as well as a general instability of pure lipases in organic solvents,[3,4] may account for the dramatic increase in activity of pure lipases in CLEC form.

TABLE 2 Enantioselectivity[a] of CRL-CLEC and Crude CRL in the Esterification of Menthol[b]

Acid	Rate of esterification for (+) and (-) menthol, nmol/min mg solid		Ratio of rates, $V_{(+)}/V_{(-)}$	
	CLEC	Crude	CLEC	Crude
Butyric	6.01 (+) 0.035 (-)	5.94 (+) 0.11 (-)	173	54
Pentanoic	2.62 (+) 0.025 (-)	1.67 (+) 0.043 (-)	103	39
Heptanoic	3.16 (+) 0.025 (-)	1.03 (+) 0.044 (-)	128	23
Lauric	6.50 (+) 0.035 (-)	7.67 (+) 0.194 (-)	188	40
Myristic	0.72 (+) 0.028 (-)	1.56 (+) 0.113 (-)	26	14

a) Enantioselectivity was determined as the ratio of initial rates (V) for (+) and (-) isomers of menthol, respectively. b) 62.4 mg (0.4 mmol) of (+) or (-) menthol (**4**); acid (0.4 mmol): 36.6 µl (0.4 mmol) butyric; 43.5 µl (0.4 mmol) pentanoic; 56.7 µl (0.4 mmol) heptanoic; 80.1 mg (0.4 mmol) lauric; 91.4 mg (0.4 mmol) myristic; 5 mg CRL-CLEC or 50 mg commercial CRL in 2 ml toluene.

TABLE 3 CRL-CLEC and Crude CRL in the Transesterification of Menthol

Acylating Agent	Catalyst	ee,[d] % ester	c, %	time, hr	E
TCEB[a]	CLEC	>99.5 (-)	49	1	>1000
	Crude	95.4 (+)	49	144	44
VA[b]	CLEC	99.9 (-)	29	0.5	900
	Crude	88.0 (-)	8	38	16
VB[c]	CLEC	>99.5 (-)	47	0.5	>1000
	Crude	86.6	20	24	17

a) 156.3 mg (1.0 mmol) (+/-) menthol (**4**); 170 µl (1.0 mmol) TCEB; 25 mg CRL-CLEC or 250 mg commercial CRL in 5 ml toluene. b) 15.6 mg (0.1 mmol) (+/-) menthol (**4**); 18.4 µl (2.0 mmol) VA; 25 mg CRL-CLEC or 100 mg commercial CRL in 1 ml isooctane. c) 156.3 mg (1.0 mmol) (+/-) menthol (**4**); 135 µl (1.0 mmol) VB; 50 mg CRL-CLEC or 250 mg commercial CRL in 5 ml toluene. d) Values of ee from Chiral GC (Cyclodex B column).

Much has been published on controlling, enhancing and, ultimately, predicting enzyme selectivity in organic solvents by manipulating solvent parameters,[14,15] but the conclusions vary with the enzyme, the substrate, and the physical parameters measured. It is also worth mentioning that until recently the researchers had to use crude lipase preparations even for mechanistic studies,[16] due to the low activity and stability of pure lipases in organic solvents. It is clear from the results presented here that purity of the enzyme can play a crucial role in determining its true enantioselectivity in non-aqueous transformations. The availability of pure crystalline CRL as well as *Pseudomonas cepacia* lipase[9], that are highly active in organic solvents, will facilitate our understanding of the parameters controlling enantioselectivity of these synthetically important enzymes.

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