Cross-Linked Crystals of *Candida rugosa* Lipase: Highly Efficient Catalysts for the Resolution of Chiral Esters

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Abstract: To date, most enzyme-based organic syntheses have employed enzymes in the form of a crude protein extract. The instability and expense of highly purified proteins has all but obviated their use as catalysts for enantioselective hydrolyses. Herein, we describe the use of the major hydrolase from commercial *Candida rugosa* lipase (CRL) in the form of a cross-linked enzyme crystal (CLEC) for the enantioselective hydrolysis of chiral racemic esters. The enantioselectivity of CRL-CLECs in the hydrolysis of many important chiral esters is vastly superior to that of the crude CRL extract. Since the CRL-CLEC is insoluble, recoverable, and 2−3 orders of magnitude more stable than the soluble protein, the CRL-CLEC is an attractive replacement for the crude enzyme preparation. The use of this catalyst in the resolution of chiral esters **1**−**11** and in the preparative scale (**1a**) and multicycle resolution (**2a**) of important anti-inflammatory drugs is described.

Introduction

The ability of enzymes to discriminate between the enantiomers of racemic substrates or enantiotopic groups in prochiral compounds makes them valuable tools in the preparation of optically pure compounds.[1] Although the high stereoselectivity of enzymes in the resolution of various esters, acids, alcohols, and amines is well documented,[2] their stereoselectivity in the resolution of many unnatural synthetic substrates, such as pharmaceuticals, is far from adequate.[3] The lower stereoselectivity of commercial enzymes often results from the use of crude enzyme preparations.[4] Crude enzyme preparations are commonly chosen as biocatalysts over highly purified enzymes because of their availability, low cost, and higher operational stability. A serious disadvantage of crude enzyme preparations, however, is the presence of several competing enzymes. These contaminating enzymes may have opposing stereo- and regioselectivities and in this way reduce both the chemical and optical purities of the final product.[5] Furthermore, crude enzyme preparations have lower acidity on a weight basis and normally contain cell debris, nucleic acids, inactive proteins, and pigments. Such components contaminate the final product and make the workup difficult and expensive.

A good illustration of a typical commercial enzyme preparation is *Candida rugosa* lipase (CRL). CRL[6] is one of the most versatile and widely used enzymes in the resolution of esters, acids,[5,7] and alcohols[8] in both aqueous and organic media. In addition to stereoselective ester conversions, CRL can be used to perform regioselective[9] and chemoselective[10] acylations[11] and deacylations and simple hydrolysis of esters under mild reaction conditions.[12] Yet in its most widely used form, this preparation is a mixture of several hydrolases including at least one protease (see below). It is not surprising, therefore, that in some notable resolutions of carboxylic acids the enantioselectivity of CRL is not sufficient.[13] Recent results from several groups indicate that purified CRL and the lipase from *Aspergillus niger* may be much more enantioselective than the crude mixtures of hydrolases,[14] although alternative explanations for this boost in enantioselectivity have been offered.[5,13]

From a purely scientific perspective, the presence of contaminants in commercial enzyme preparations has led to confusing and contradictory conclusions in the field of biocatalysis. Widely disparate enantioselectivity in the use of CRL, and many other biocatalysts, has been observed for identical substrates by seemingly small changes in reaction procedures (e.g., pH, concentration, catalyst supplier, and enzyme history). From a practical view, such irreproducibility has caused many organic chemists to exclude biocatalysts from their synthetic repertoire.

Problems resulting from catalyst purity can, in principle, be addressed by first purifying enzymes to homogeneity followed

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Table 1. Purification of Commercial CRL on Q-Sepharose

fraction	lipase activity[a,b]	esterase activity[c]	lipase:esterase activity ratio	E^d for the resolution of **2b**
CRL_1	1875	13	144	66
fraction II	1082	38	28	3.8

[a] Activity in μmol/(min mg) protein. [b] Olive oil assay (see Experimental Section). [c] *p*-Nitrophenyl acetate (*p*-NPA) assay (see Experimental Section). [d] Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.

by immobilization onto solid supports.[15] Enzyme immobilization is useful in many processes, but by itself does not guarantee enzyme stability[16] and, moreover, creates a new problem—low specific activity of the catalyst. Indeed, the amount of an enzyme in an immobilized preparation is typically less than 5% by weight. Consequently, high reaction rates with synthetic substrates can rarely be achieved using immobilized enzymes. Other approaches to increase the enantioselectivity of enzyme preparations (i.e., solvents, reaction conditions, and additives) have recently been reviewed by Kazlauskas.[13]

Many problems of enzyme-catalyzed resolutions, including synthesis of several optically pure drugs, can be solved by designing a catalyst which is well defined in composition and performance, combines high stereoselectivity and high operational stability and activity, and can be recycled and reused many times, thus reducing its cost. It has been demonstrated that cross-linked enzyme crystals (CLECs), microcrystals grown from aqueous solution and cross-linked with a bifunctional agent such as glutaraldehyde, retain high specific activity for extended periods at elevated temperatures, in near-anhydrous organic solvents and aqueous−organic solvent mixtures, and in the presence of proteases.[17,18] Here we describe properties of CRL-CLECs and the use of this catalyst in the synthesis of optically pure compounds.

Results and Discussion

The major lipase in commercial CRL (designated CRL_1) was selected as the protein to be converted to a CLEC because of its high enantioselectivity and lipolytic activity (Table 1). CRL_1 was separated from the mixture by ion-exchange chromatography as previously described[19] and then crystallized using 2-methyl-2,4-pentanediol[20] to give a highly purified (more than 98%) protein catalyst. The mixture remaining after purification, referred to in this paper as fraction II, contains at least three major protein bands (Figure 1) in addition to unrecovered CRL_1.

Preparation of cross-linked enzyme crystals of CRL from the purified protein consists of crystallization, followed by chemical cross-linking of the enzyme crystal with glutaraldehyde (see Experimental Section). The CRL_1 crystals are small uniform plates 30 μm in length and no more than 2 μm thick (Figure 2).[21] After cross-linking, the CRL-CLECs are insoluble in aqueous buffer[22] and organic solvents, so they can easily be used in a variety of reaction media and then recovered and reused. CRL-CLECs, like other protein crystals,[23] are macroporous, containing about 50% solvent by volume. Channels traverse the body of the crystal, permitting diffusion of solvents, substrates, and products. The large pore diameter of CLECs has been shown to allow rapid diffusion of relatively large molecules into the protein crystal.[18]

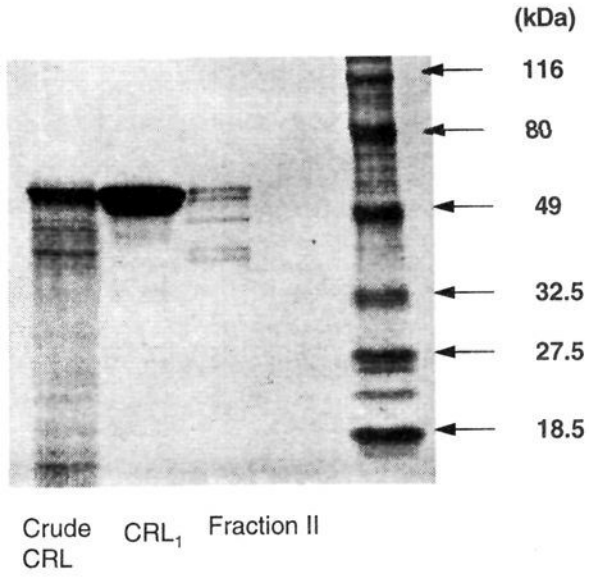

Figure 1. SDS polyacrylamide gradient gel electrophoresis (4−20%) of crude CRL (lane 1), dissolved crystals of CRL_1 (lane 2), and fraction II (lane 3) (see the Experimental Section for details on fraction II). Molecular weight standards are in lane 4.

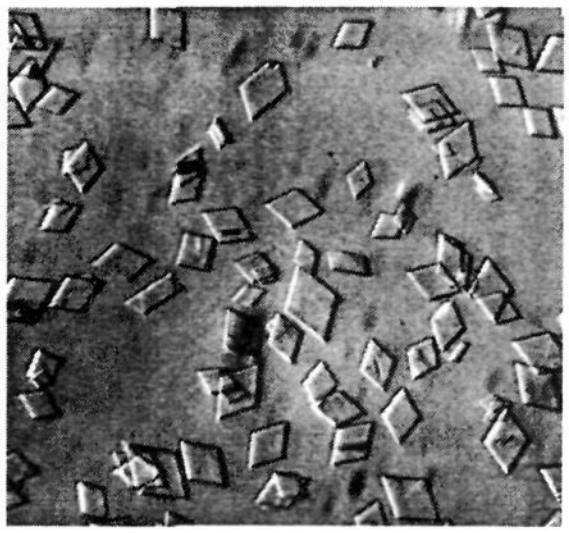

Figure 2. Optical microscope photograph of cross-linked *C. rugosa* lipase crystals (250× magnification (reproduced at 50% of original size)).

Enantioselectivity. CRL-CLECs consist of only one enzyme, and this homogeneity has profound stereochemical consequences. Several α-substituted carboxylic acids and secondary alcohols have been resolved via ester hydrolysis by both CRL-CLECs and commercial CRL (Table 2). The enantioselectivity of CRL-CLECs in the hydrolysis of the esters of (*R,S*)-2-arylpropionic acids **1a**−**5a** (Chart 1) is 3−50 times higher than that of a crude enzyme preparation. Significant enhancement in enantioselectivity of CRL-CLECs in the resolution of acids is especially important in the preparation of optically pure drugs. Indeed, compounds **1a** (Ibuprofen), **2a** (Ketoprofen), and **3a** (Flurbiprofen) are major nonsteroidal anti-inflammatory drugs that are currently marketed as racemates, despite the fact that their anti-inflammatory activity predominantly resides in their respective (*S*)-enantiomers.[3] Moreover, the (*R*)-enantiomers of Ketoprofen[24] and Flurbiprofen[25] have

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(21) The size and shape of CRL crystals do not change after cross-linking.

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Table 2. Enantioselectivity of Chiro-CLEC-CR and Crude Commercial CR Lipase

	% ee		% conv (time, h)		E_{app}[a]	
compd	CLEC	crude	CLEC	crude	CLEC	crude
1	94.6, (*S*)-**1a**	81.9, (*S*)-**1a**	22 (5)[c]	39.3 (34)[c]	47	17
1	93.0, (*S*)-**1a**		38 (20)[c]		43	
2	88.6, (*R*)-**2b**	54.7, (*R*)-**2b**	49.3 (40)[b]	36.7 (40)[b]	64	5.2
	91.1, (*S*)-**2a**	33.3, (*S*)-**2a**				
3	49.5, (*R*)-**3b**	31.2, (*R*)-**3b**	34.4 (48)[d]	34.0 (16)[d]	55	5.6
	94.3, (*S*)-**3a**	61.1, (*S*)-**3a**				
4	97.3, (*S*)-**4a**	76.2, (*S*)-**4a**	39.0 (20)[e]	46.8 (20)[e]	>100	12
	62.8, (*R*)-**4b**	66.9, (*R*)-**4b**				(>100)[h]
5	88.7, (*R*)-**5b**	73.2, (*R*)-**5b**	48 (12)[d]	70.4 (12)[d]	>100	3.7
	96.9, (*S*)-**5a**	88.7, (*S*)-**5a**				
6	30.0, (*R*)-**6b**	85.3, (*R*)-**6b**	24 (4.5)[d]	51 (24)[d]	64	26
	96.0, (*S*)-**6a**	80.9, (*S*)-**6a**				
7	94.5, **7b**	92.5, **7b**	60.0 (9)[d]	63.2 (13)[d]	15.2	10.4
	63.8, **7a**	53.9, **7a**				
8	99.5, (+)-(1*S*)-**8b**	73.7, (+)-(1*S*)-**8b**	51.3 (72)[d,f]	43.0 (72)[d,f]	>100	>100
	95.2, (−)-(1*R*)-**8a**	98.2, (−)-(1*R*)-**8a**				(8.6)[k]
9	0, **9b**	10, **9b**	54.8 (4)[d]	74.3 (4)[d]	1	1.4
	0, **9a**	8, **9a**				
10	26.0, (1*S*,4*R*)-**10a**	27.0, (1*S*,4*R*)-**10a**	90.5 (0.5)[d]	84.3 (0.5)[d]	1	1
	10a	**10a**				
11[g]	69.8, (*S*)-**11b**	71.9, (*S*)-**11b**	48.6 (20)[d]	46.1 (20)[d]	13	24
	73.7, (*R*)-**11a**	84.2, (*R*)-**11a**				

[a] See footnote *d* in Table 1. Ideally, *E* (the ratio of k_{cat}/K_m for the two enantiomers) is a constant specific for a first-order, irreversible resolution reaction. *E* becomes an apparent value (E_{app}) dependent on the presence of effectors[48] and product inhibition. In addition, as was pointed out by Kazlauskas,[28] for mixtures of enzymes, E_{app} depends on the amount of each enzyme, their enantioselectivites, substrate concentrations, etc. This may explain the variations for published *E* values. [b] Reaction buffer was 0.1 M pH 5 sodium acetate. [c] Reaction buffer was 0.1 M pH 6 sodium acetate. [d] Reaction buffer was 0.1 M pH 7 sodium phosphate. [e] 50% PEG 1000−50% pH 5 ammonium acetate. [f] Ten-fold enzyme level. [g] Fraction II: alcohol ee = 97.5, ester ee = 35.1, *c* = 26.5, *E* = 111. [h] Gu, Q.-M.; Chen, C.-S.; Sih, C. J. *Tetrahedron Lett.* **1986**, *27*, 1763−1766. [k] Calculated from ref 44a.

commercially useful pharmacological activities of their own, distinct from anti-inflammatory applications. Clearly, the enantioselectivity of crude CRL is not sufficient to justify its use for the resolution of these compounds (Figure 3). In contrast, high enantioselectivity with CRL-CLECs makes it possible to produce both enantiomers in high optical purity and yield.

The increased enantioselectivity of CRL-CLECs over that of the crude CRL preparation is clearly due to the removal of competing hydrolases. The enantioselectivity of crude CRL in the resolution of **2b** (E_{app} = 5.2; Table 2) is much lower than the enantioselectivity of CRL-CLECs (*E* = 64; Table 2) but much higher than that of fraction II (E_{app} = 3.8; Table 1). The enantioselectivity of CRL-CLECs is due to the high purity of the enzyme rather than a result of the crystalline structure of the material. The resolution of **2b** with highly purified soluble CRL (non-cross-linked) proceeds with the same high enantioselectivity (*E* > 50) as the CRL-CLECs.

The fact that crude CRL consists of several hydrolases with distinct substrate specificities and enantioselectivities is further illustrated by the resolution of the tertiary ester **11b** (Chart 1). It has been reported that crude CRL can be used in the stereoselective hydrolysis of **11b** with fair enantioselectivity (E_{app} > 20) to produce (*R*)-tertiary alcohols.[26] Surprisingly, we found that highly purified CRL-CLECs exhibit lower enantioselectivity in this reaction than the crude enzyme. However, when fraction II was applied to the hydrolysis of **11b**, it gave much higher enantioselectivity (E_{app} > 100) than either crude CRL (E_{app} = 24) or CRL-CLECs (*E* = 13). These results indicate that one or several enzymes in the fraction II mixture have much higher enantioselectivity in the resolution of the tertiary alcohol **11a** than does CRL_1.

Another example, indicating that some of the activities attributed to CRL reside with other enzymes in the mixture, is the presence of protease activity of crude CRL. Crude CRL catalyzes the hydrolysis of L-leucine naphthylamide with a rate of 0.095 nmol/(min mg) (0.1 M Tris, pH 8, 10 mM Ca^{2+}, room temperature), while CRL-CLECs show no detectable hydrolysis of this amide substrate. In addition, purified CRL does not hydrolyze methyl esters of (*Z*)-Nor and (*Z*)-Phe, while the crude enzyme does.[14b] It is quite possible that some of the reactions reported for the preparation of chiral amides reported to be catalyzed by CRL[27] are actually catalyzed by the protease impurities present. Moreover, protease impurities are common in crude enzyme preparations and have a negative impact on the long-term stability of these proteins.

In general, this increase in enantioselectivity was more profound for the resolution of α-substituted carboxylic acids **1a**−**7a** than for secondary alcohols **8a**−**10a** (Chart 1). The difference between the increase in enantioselectivity of CRL-CLECs toward carboxylic acids and secondary alcohols is consistent with the model of the binding site of this enzyme proposed by Cygler and colleagues.[28] On the basis of several lipase X-ray crystal structures, they proposed that the alcohol binding site is formed by conserved structural elements and is similar in all lipases in the mixture. The amino acid composition of the acid binding site, however, is not conserved among different lipases. If this model is correct, one may expect lipase purity to affect the enantioselectivity of carboxylic acid resolutions more than that for alcohols.[14c]

Although a number of mechanisms, such as conformational changes during purification[5] or the removal of contaminating small molecules,[5b] may be invoked to explain the dramatic increase in enantioselectivity of lipases on purification,[14b] the

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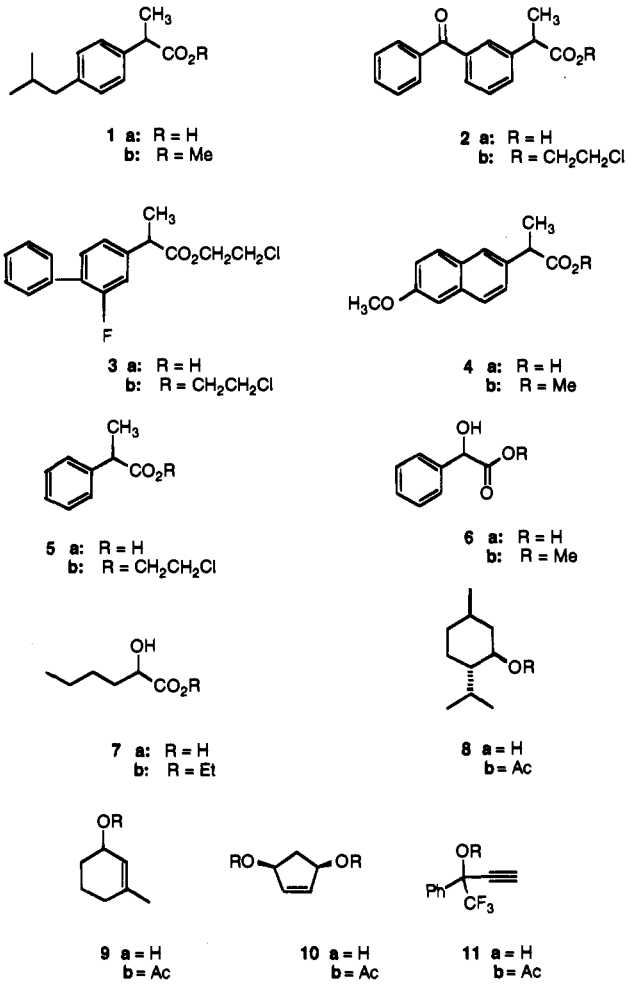
Chart 1



simplest and thus the most attractive explanation is the removal of contaminating hydrolases. Irrespective of the mechanism, it is clear that both substrate specificity and enantioselectivity of CRL-CLECs are markedly different from those of the crude enzyme mixture.[29] In our opinion it is imperative to critically reexamine important synthetic applications of crude CRL to determine which enzyme(s) catalyzes a particular transformation. Our results clearly show the benefit of using a single pure protein catalyst and suggest that crude CRL contains new hydrolases with unexpected synthetic properties.

Stability and Activity of CRL-CLECs. Although the enantioselectivity of both pure CRL and CRL-CLECs are equally high, pure CRL cannot be used in synthesis because of its poor stability against heat and organic solvents. Indeed at 40 °C and pH 7, the half-life of pure CRL is less than 5 h while that of CRL-CLEC is more than 13 days (Figure 4). The stabilization effect in the mixtures of water and water-miscible organic solvents is even more profound. While crude CRL quickly loses activity in 50% organic solvents (the half-lives are less than 1 h), CRL-CLECs remain stable for many days, demonstrating a 300−3000-fold increase in stability (Figure 5). This dramatic stabilization is extremely important when using an insoluble catalyst, since many organic compounds are poorly soluble in water. Because soluble lipases accept organic oils as substrates, solubilization of the substrate is only relevant when a heterogeneous form of the enzyme is used (such as the CLEC form or immobilized enzyme), or when the substrate itself is a water-insoluble solid (such as **4b**). The rate of hydrolysis in these triphasic systems is exceedingly slow. Attempts to increase substrate concentration by the addition of water-miscible organic solvents leads to the complete inactivation of

(29) It is also quite possible that the reported enantioselectivity increase of crude CRL after its treatment with organic solvents such as 2-propanol[13] is due to differences in stability of a lipase and other enzymes in the mixture toward organic solvents. One can envisage situations where an organic solvent treatment selectively denatures a sensitive hydrolase or reaction conditions which favor only one of the component enzymes, thus improving the resultant enantioselectivity.

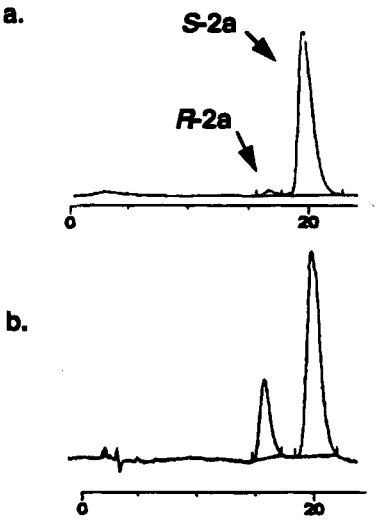


Figure 3. Chiral HPLC determination of the optical purity of product (*S*)-ketoprofen from **2b** hydrolysis: (a) CRL-CLEC, 34.7% conversion, $E = 66$; (b) commercial CRL at 36.7%, hydrolysis, $E = 5$.

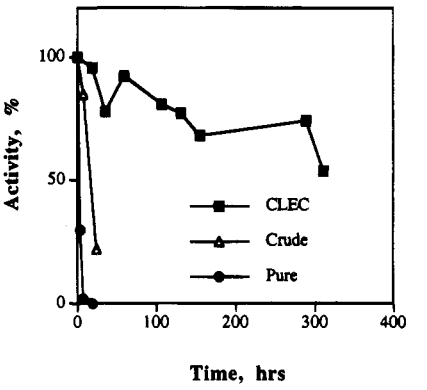


Figure 4. Thermostability of the different lipase preparations. Crude CRL (3 mg), pure soluble lipase (2.5 mg), and CRL-CLEC (7 mg) were suspended in 1 mL of 10 mM Tris, 10 mM $CaCl_2$, pH 7.0, and incubated at 40 °C. Aliquots were withdrawn at the times indicated, and the activity was measured by triacetin assay (see the Experimental Section).

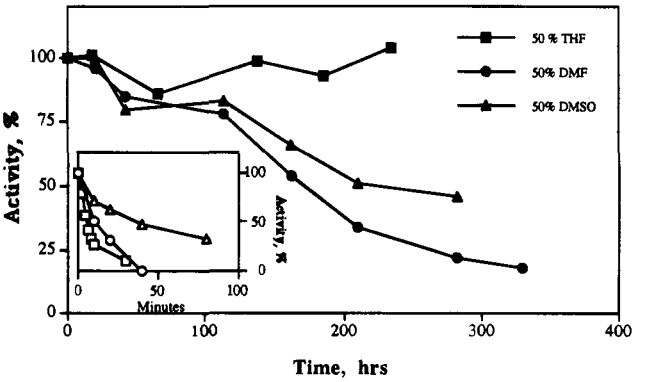


Figure 5. Stability at 25 °C of crude CRL (inset) and CRL-CLECs in water-miscible organic solvents. The aqueous component was 10 mM Tris, 10 mM $CaCl_2$, pH 7.0. The activity was measured by triacetin assay.

soluble CRL. The stability of CRL-CLECs, on the other hand, allows for the use of water-miscible organic solvents, greatly extending the range of possible substrates and reaction conditions.

As we have discussed previously,[18] we believe that the mechanism of stabilization of enzymes in CLEC form arises from the combination of the crystallinity of the material and covalent cross-linking between enzyme molecules.

In the crystal lattice, where the concentration of enzyme is close to the theoretical packing limit, many protein−protein interactions (both polar and hydrophobic) are realized.[30] These multipoint contacts among protein molecules may significantly enhance their stability against heat and denaturants[31] by preventing unfolding, aggregation, or dissociation. As in the case of thermolysin−CLEC,[18] both the crystal structure and intermolecular cross-linking are an absolute requirement for the CLEC stability. Neither cross-linked soluble CRL nor its precipitate, both lacking the crystal structure, exhibits stability beyond the level of the soluble enzyme. The importance of the chemical cross-linking, on the other hand, can be illustrated by the fact that when non-cross-linked CRL crystals are removed from the crystallization liquor, they quickly dissolve, thus losing their stability. Our results clearly show that while the high enantioselectivity of CRL-CLECs is due to the purity of the enzyme (compare Tables 1 and 2), the high stability of CRL-CLECs is a result of the crystallinity and cross-linking of the catalyst.

One of the most essential features of a synthetically useful biocatalyst is high specific activity. Clearly, purification, crystallization, and cross-linking may lead to certain losses in CRL activity. In addition, the final product CRL-CLECs are insoluble in either aqueous or organic media and the rates of CLEC-catalyzed reactions may be limited by mass transfer. We decided to compare the activity of CRL-CLECs and purified CRL in the hydrolysis of triacetin (water-soluble analog of the lipase natural substrates) and in the hydrolysis of synthetic chiral substrates **2b** and **6b**.

Negligible esterolytic activity is lost on the conversion from a soluble protein to an insoluble crystalline particle. The specific activities of both CRL-CLECs and purified CRL are very similar in monophasic solutions of triacetin (below 6%; Figure 6A). When the triacetin concentration is increased above the solubility limit, the activity of soluble CRL also increases, while the activity of CRL-CLECs levels off. For triacetin, even when a second phase is present, the difference between soluble CRL and CRL-CLEC activity is only 2−3-fold (Figure 6A). Surprisingly, the activity of CRL-CLECs in the hydrolysis of **6b** is even higher than that of soluble CRL in both monophasic and biphasic mixtures (Figure 6B). The mechanism of this activation effect is presently unknown.

Although it is well known that lipases exhibit 10^3-10^4 increases in activity when their natural substrates, such as lipids, are present as an emulsion,[32] the need for the interfacial activation in the hydrolysis of more water-soluble or small synthetic substrates is less obvious. For example, the activation effect of soluble CRL in triacetin hydrolysis is only 3-fold (Figure 6A) while the activity of soluble CRL in the hydrolysis of **6b** does not change at all before and after the separation of phases (Figure 6B).[33] In other words, in many resolution reactions, in both soluble and crystalline forms, CRL behaves as a simple esterase.

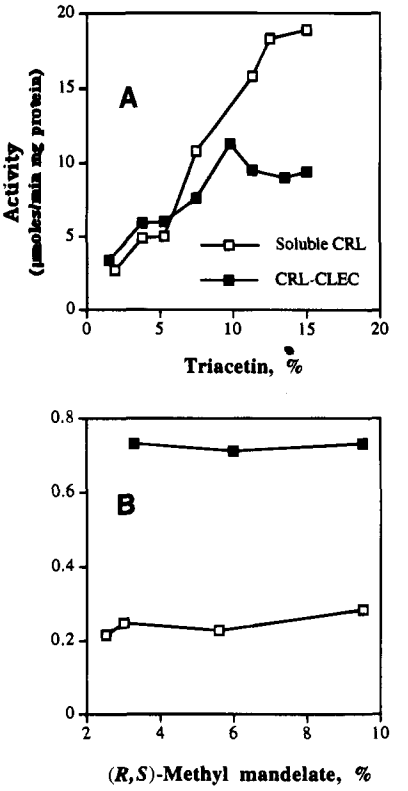


Figure 6. (A) Effect of substrate concentration on the rate of hydrolysis of triacetin at 25 °C (note: at substrate concentrations greater than 6% the system is biphasic). (B) Effect of substrate concentration on the rate of hydrolysis of a synthetic substrate (**6b**) at 43 °C. The reaction mixture is under biphasic conditions at substrate concentrations greater than 6%.

The activities of CRL-CLECs (1.4 μmol/(h·mg)) and of the soluble CRL (1.2 μmol/(h·mg)) toward a water-insoluble substrate, **2b**, are very similar.[34] The reaction system (CRL-CLECs, water-insoluble substrate, and water) is complex, and the proper contact between particles of the catalyst and the droplets of substrate is difficult to achieve. Any additive which helps to improve the contact between catalyst particles and a substrate will increase the activity of CRL-CLECs. We found that polyethylene glycol (PEG) is particularly helpful in achieving this goal. The addition of 50% PEG increased the CRL-CLECs activity 3-fold to 4.6 μmol/(h·mg)), while the activity of the pure CRL slightly dropped to 0.9 μmol/(h·mg) under the same conditions. We used this increased activity of CRL-CLECs in the resolution of water-insoluble methyl Naproxen (**4b**). This resolution using commercial CRL has been

(30) Islam, S. A.; Weaver, D. L. *Proteins: Struct., Funct., Genet.* **1990**, *8*, 1−5.
(31) Guisan, J. M. *Enzyme Microb. Technol.* **1988**, *10*, 375−382.
(32) Chapus, C.; Semeriva, M.; Bovier-Lapierre, C.; Desnuelle, P. *Biochemistry* **1976**, *15*, 4980−4987.

(33) Given the fact that, in CRL, Gly 124 and Ala 210, the residues that stabilize the transition state analog by forming a hydrogen bond, remain in the same position in the opened and closed structures (Kazlauskas, R. J. *TIBTECH* **1994**, *15*, 4980−4987), one may question whether the lid movement is at all necessary in these reactions. It is quite possible that the lid remained half-open at all times (Rubin, B. *Struct. Biol.* **1994**, *1*, 568−572). Another indication comes from the comparison of "open" and "closed" crystal forms of CRL. These different crystal forms can be prepared by using 2-methyl-2,4-pentanediol[20] and PEG 8000 (Grochulski, P.; Li, Y.; Schrag, J. D.; Cygler, M. *Protein Sci.* **1994**, *3*, 82−91) as the precipitants, respectively. Although both the activity and enantioselectivity of the closed (0.48 μmol/(min mg); $E = 25$) form were lower than those of the open one (1.4 μmol/(min mg); $E = 64$) in the resolution of **2a**, the fact that the closed form can efficiently catalyze this reaction suggests that the lid movement may not be crucial in the resolution of small chiral substrates.
(34) The activity of crude commercial preparation of CRL in this reaction is 0.4 μmol/(h·mg). Since the total lipase content (including CRL and other hydrolases with activity toward **2a**) in the commercial preparation is in the range of 5−8%, the maximum CRL or CRL-CLECs activity should be 5−8 μmol/(h·mg). It is quite possible that the lower than expected activity of the pure CRL is due to the lower stability of this material.

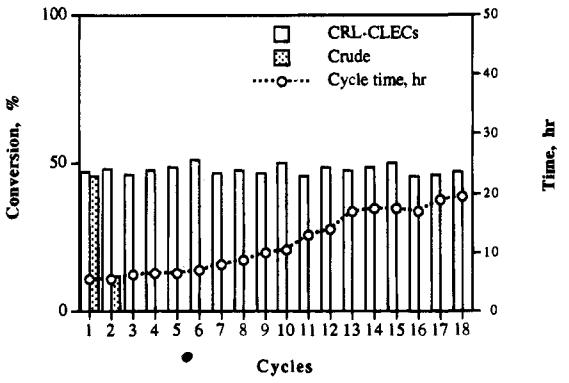


Figure 7. Multicycle resolution of (*S*)-Ketoprofen (10% chloroethyl ketoprofen in 50:50 PEG 1000–pH 5 ammonium acetate buffer, 40 °C, 5 mg/mL CRL-CLEC).

reported to proceed with excellent enantioselectivity ($E > 100$), but with an exceedingly slow rate (6 days for the substrate: enzyme ratio of 2.4:1).[35] The use of 50% PEG reduces the reaction time to 20 h for both CRL-CLECs and soluble CRL (Table 2, compound **4**). The effect of the addition of PEG on enantioselectivity, however, is drastically different. While the high enantioselectivity of CRL-CLECs ($E > 100$) and purified CRL ($E = 54$) is maintained in the organic–aqueous mixture, the enantioselectivity of crude CRL drops almost 10-fold. These results indicate that the increase in the soluble substrate concentration most probably leads to the higher activity of contaminating hydrolases with lower enantioselectivity. We believe that the high activity of CLECs in water-miscible organic cosolvents will be of general utility to organic chemists since it facilitates reactions with poorly water-soluble substrates.

The combination of high stability and activity of CRL-CLECs in mixed aqueous–organic media allowed us to conduct preparative scale resolution of methyl Ibuprofen (**1b**) on a 100 g scale (Table 2, entry 2) to give 30.6 g of (*S*)-**1a** with 93% ee (>99% ee after recrystallization; see Experimental Section for details). The high stability and activity of CRL-CLECs, along with the ability to reuse the solid catalyst, were also demonstrated in repetitive batch resolution of Ketoprofen (**2a**; Figure 7). Since the CRL-CLECs are stable, insoluble, and mechanically strong particles, they can be easily recovered by filtration or centrifugation and be reused. In contrast, crude or pure soluble CRL could not be recycled. After one cycle, followed by extraction of the product, virtually all activity is lost. The comparison with commercial immobilized CRL is quite telling. Immobilized CRL (not shown), while recoverable and reusable, has prohibitively low activity. Even with 35% of the reaction weight and 90% of the reaction volume being immobilized CRL, as compared to only 0.27% by weight of CLEC, the CLECs were cycled eight times before one immobilized enzyme cycle was completed.[36] It is worth mentioning that CRL-CLECs maintain their high enantioselectivity and activity throughout cycling. The selectivity factor E was 50 and 47 in the first and the last cycles, respectively, while the specific activity in the triacetin assay dropped only 44% from 6.66 units/mg to 3.79 units/mg after 18 reaction cycles. Since this loss of activity cannot account for the almost 5-fold increase in cycle time (Figure 7), it is quite possible that the longer cycle time is due, at least partially, to mechanical losses of the catalyst.

Conclusions. The enantioselectivity of the individual, pure hydrolases from *C. rugosa* lipase is greatly superior to the combined effects of the catalysts in this crude extract. Up until the present, the expense of purifying an enzyme to homogeneity and the low stability of purified proteins have all but excluded the use of pure biocatalysts for organic synthesis. Over the last 10–15 years there have been hundreds of studies optimizing the enantioselectivity of hydrolyses[2] by altering such parameters as pH, alkyl chain length, reactivity, organic cosolvents, activators, inhibitors, etc. It is probable that in many cases the effect of these adjustments is not in the catalytic properties of one enzyme, but a shift in relative rates of the hydrolases in these multicomponent enzyme preparations. Conditions that favor the more enantioselective enzyme in a multi-enzyme mixture will result in a higher optical selectivity in the kinetic resolution. One of the most important parameters in the optimization of biocatalyst selectivity, i.e., catalyst purity, has largely been ignored.

By chemically cross-linking enzyme crystals of the major component of CRL, the resultant crystalline protein particle is recoverable, active, and dramatically stable. On the basis of the findings reported herein, purified stabilized biocatalysts should be used to reexamine many of the important biotransformations where crude enzyme extracts have been found to be inappropriate.

Experimental Section

General Procedures. *C. rugosa* lipase (crude CRL) was obtained from Meito Sangyo (Tokyo, Japan). Methyl mandelate, (*R,S*)-Ketoprofen, (*S*)-Ketoprofen, (*S*)-Ibuprofen, (*R,S*)-Flurbiprofen, and (*S*)-Naproxen were purchased from Sigma Chemical Co. (St. Louis, MO). (*R,S*)-Methyl Ibuprofen and (*R,S*)-Ibuprofen were gifts from Sepracor, Inc. (Marlborough, MA). *cis*-3,5-Dihydroxycyclopent-1-ene and (trimethylsilyl)diazomethane were purchased from Fluka (Ronkonkoma, NY). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were of reagent grade.

Preparation of CRL-CLECs. Ion-exchange purification was used to separate the two activities as reported previously.[19] The lipase component was crystallized according to published procedures,[20] with minor modifications: the extraction of protein with 50% 2-propanol was omitted. The yield of CRL_1 from crystallization was 90%, and the purity of the crystalline lipase was determined to be 98% by densitometric scanning (Pharmascan) of a Coomassie stained gradient gel (4–20%). CRL-CLECs were obtained by cross-linking of these crystals with glutaraldehyde according to the published procedure.[17] Cross-linked enzyme crystals of *C. rugosa* lipase are sold under the trade name ChiroCLEC-CR and are a commercial product of Altus Biologics, Inc. (Cambridge, MA).

Preparation of Racemic Esters. Methyl and chloroethyl esters of the 2-arylpropionic acids **1b–5b** were prepared by treatment of the carboxylic acid with thionyl chloride in dichloromethane, followed by addition of the appropriate anhydrous alcohol.[37] (*R,S*)-Menthyl acetate (**8b**), (*R,S*)-3-methylcyclohex-2-enyl acetate (**9b**), and *cis*-3,5-diacetoxycyclopent-1-ene (**10b**) were prepared by acetylation of the appropriate alcohol with acetic anhydride and pyridine in dichloromethane.[38] 4,4,4-Trifluoro-3-phenyl-3-hydroxybutyne (**11a**) and 4,4,4-trifluoro-3-phenylbutyn-3-yl acetate (**11b**) were prepared in a manner identical to that previously described.[26] Spectral analyses of all compounds were consistent with those previously described for these compounds (see references below).

Determination of Enzymatic Activity. Lipase/Esterase Activity. The specific activity of lipase and initial rate kinetic measurements were determined either spectrophotometrically using *p*-nitrophenyl acetate (*p*-NPA) as substrate or titrimetrically using a Radiometer VIT90 autotitrator (Baegsvard, Denmark) with triacetin or olive oil as substrate. Details of each assay are outlined below.

***p*-NPA Assay.** The enzymatic release of *p*-nitrophenol from *p*-NPA (1 mM) was followed at 410 nm in 50 mM acetate buffer at pH 6.5.

(35) Sih, C. J. U.S. Patent 5,322,791, 1994.

(36) CRL immobilized on macroporous acrylic beads (Sigma) with an activity of 518 U/g (olive oil). The comparison was performed under conditions reported in footnote *b* in Table 2.

(37) Allenmark, S.; Ohlsson, A. *Chirality* **1992**, *4*, 98–102.

(38) Johnson, C. R.; Penning, T. *J. Am. Chem. Soc.* **1988**, *110*, 4726–4735.

The extinction coefficient for *p*-nitrophenol at pH 6.5 is 2500 M^{-1} cm^{-1}. The typical enzyme concentration during the assay was 1−5 μg/mL of assay solution.

Triacetin Assay. Unless specified otherwise, the triacetin concentration in the assay was 25% in 1 mM phosphate, pH 7.0, 25 °C, and the enzyme concentration was 0.5−1 mg/20 mL of assay solution.

Olive Oil Assay. The procedure employed is similar to that reported previously.[39] The temperature was maintained at 37 °C during the assay, and the set point on the titrator was 7.7 [E] = 1 μg/mL (E = enzyme) during the assay.

Amidase Assay. The amidase activity of commercial CRL and the cross-linked enzyme crystal was determined using L-leucine naphthylamide as a substrate at pH 8.[40]

Methyl Mandelate Hydrolysis. The initial rates of hydrolysis of racemic methyl mandelate by the soluble lipase and CRL-CLECs was followed titrimetrically in 1 mM phosphate, pH 6. The temperature was maintained at 43 °C during this experiment. The higher temperatures avoided precipitation of the substrate, particularly at high substrate concentrations.

Thermostability Studies. The thermostabilities of crude, pure CRL and CRL-CLECs were tested under different conditions. The activity for these studies was measured against triacetin as described earlier, and the incubation conditions are described in the figure legends.

General Procedure for Enzymatic Resolutions. An emulsion of the substrate (unless specified otherwise in the text, 100 mg) in the appropriate buffer (1 mL) and the cross-linked enzyme crystal (2 mg) or the crude CRL (25 mg) in a 1.5 mL microcentrifuge tube were shaken in a G24 incubator (New Brunswick Scientific, Edison, NJ) at 200 rpm and 40 °C. Alternatively, the suspensions could be stirred with a magnetic stirrer with equivalent results. Specific reaction details are given below the appropriate figures. The extent of hydrolysis was determined by periodically analyzing a 10 μL aliquot of the evenly suspended reaction mixture by HPLC for compounds **1**−**6** (Microsorb MV 5 cm C_{18} column (Rainin, Woburn, MA) 60:40:0.1 acetonitrile−water−trifluoroacetic acid, flow rate 1 mL/min, UV detection at 254 nm) or by capillary GC for compounds **7**−**11** (DB1701 15 m × 0.25 mm GC column, 25 μm film thickness (J&W Scientific, Folsom, CA), split ratio 1:100, helium flow at 25 cm/s).

Preparative Scale Preparation of (*S*)-Ibuprofen (1a). (*R,S*)-Ibuprofen methyl ester (**1b**) (100 g) and ChiroCLEC-CR (1.5 g) were suspended in 1.5 L of distilled water. The suspension was heated in a water bath to 40 °C and stirred vigorously with an overhead stirrer. The pH dropped to 4.5 during the reaction; no pH control was necessary. After 20 h, the hydrolysis had proceeded to 38% (by HPLC). The reaction mixture was allowed to cool to room temperature, and the CLECs were removed by filtration. The catalyst was washed with 100 mL of *tert*-amyl alcohol and then stored for reuse in pH 7 Tris, 2 mM calcium acetate. The pH of the reaction mixture was adjusted to between 2 and 3, and then extracted with diethyl ether (2 × 800 mL). The combined ether extracts were extracted with saturated pH 9.5 sodium carbonate (5 × 300 mL), and then the combined aqueous layers were back-extracted with diethyl ether (3 × 300 mL). The combined ether layers were washed with saturated sodium chloride and dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure to give (*R*)-**1b** as a colorless oil (59.1 g, 95.3% of theoretical yield, 55.8% ee by chiral HPLC). The acid (*S*)-**1a** was precipitated from the combined sodium carbonate extracts by adjusting the pH of the aqueous layer to 2 with 6 N HCl, saturating with sodium chloride, and then extracting with diethyl ether (2 × 500 mL). The ether extracts were extracted with saturated sodium chloride (100 mL) and then dried over anhydrous sodium sulfate, and the ether was evaporated under reduced pressure to give (*S*)-**1a** as a crystalline white solid (30.6 g, 87% of theoretical yield, 93% ee by chiral HPLC analysis). (*S*)-Ibuprofen of optical purity >90% can then be crystallized to an optical purity of >99% by simple *in situ* conversion to the sodium salt.[41]

Determination of Optical Purity. Assignments of absolute configuration are made from original citations (references given below) and by comparison with authentic samples.

α-Methyl-4-(2-methylpropyl)benzeneacetic Acid (Ibuprofen, 1a) and Its Methyl Ester (1b). Chiral HPLC conditions for **1b**: Chiracel OJ 25 cm column (Chiral Technologies, Exton, PA), mobile phase 99.1% hexane and 0.9% 2-propanol, flow rate 1 mL/min, UV detection at 235 nm. Retention times: (*S*)-**1b**, 8.13 min; (*R*)-**1b**, 9.57 min. Chiral HPLC conditions for **1a**: (*R,R*)-Whelk-O1 (Regis Technologies, Morton Grove, IL), 5 μm, 100 Å, 25 cm column, mobile phase 98.5% hexane, 1% 2-propanol, and 0.5% acetic acid, flow rate 1 mL/min, UV detection at 254 nm. Retention times: 8.7 (*S*)-**1a**, min; (*R*)-**1a**, 10.2 min.

2-(3-Benzoylphenyl)propionic Acid (Ketoprofen, 2a) and Its Chloroethyl Ester (2b).[5a] Chiral HPLC conditions: Chiracel OJ 25 cm column, mobile phase 90% hexane (0.05% acetic acid) and 10% 2-propanol, flow rate 1 mL/min, UV detection at 235 nm. Retention times: 16.6 (*R*)-**2a**, min; (*S*)-**2a**, 19.9 min; (*S*)-**2b**, 31.4 min; (*R*)-**2b**, 34.5 min.

2-Fluoro-α-methyl-4-biphenylacetic Acid (Flurbiprofen, 3a) and Its Chloroethyl Ester (3b).[5a] Chiral HPLC conditions: Chiracel OJ 25 cm column, mobile phase 99% hexane (0.05% acetic acid) and 1% 2-propanol, flow rate 1 mL/min, UV detection at 235 nm. Retention times: (*R*)-**3a**, 45 min; (*S*)-**3a**, 54 min; (*S*)-**3b**, 79 min; (*R*)-**3b**, 95 min.

6-Methoxy-α-methyl-4-naphthaleneacetic Acid (Naproxen, 4a) and Its Methyl Ester (4b).[5a] Chiral HPLC conditions: (*R,R*)-Whelk-O1, 5 μm, 100 Å, 25 cm column, mobile phase 90% hexane, 9.5% ethanol, and 0.5% acetic acid, flow rate 1 mL/min, UV detection at 254 nm. Retention times: (*S*)-**4b**, 16.8 min; (*S*)-**4a**, 19.2 min; (*R*)-**4b**, 21.8 min; (*R*)-**4a**, 34.3 min.

α-Methylphenylacetic Acid (5a) and Its Chloroethyl Ester (5b).[5a] Chiral HPLC conditions: Chiracel OJ 25 cm column, mobile phase 97.5% hexane (0.05% acetic acid) and 2.5% 2-propanol, flow rate 1 mL/min, UV detection at 235 nm. Retention times: (*R*)-**5a**, 20.1 min; (*S*)-**5a**, 27.9 min; (*S*)-**5b**, 31.0 min; (*R*)-**5b**, 33.6 min.

α-Hydroxyphenylacetic Acid (Mandelic Acid) (6a) and Its Methyl Ester (6b).[14b] Chiral HPLC conditions: Chiracel OJ 25 cm column, mobile phase 92% hexane (0.1% trifluoroacetic acid) and 8% 2-propanol, flow rate 0.8 mL/min, UV detection at 222 nm. Retention times: (*R*)-**6b**, 18.6 min; (*S*)-**6b**, 21.8 min; (*R*)-**6a**, 27.2 min; (*S*)-**6a**, 42.2 min.

α-Hydroxyhexanoic Acid (7a) and Its Ethyl Ester (7b).[42] Chiral GC conditions: Cyclodex B capillary GC 25 m column, 25 mm i.d. (J&W Scientific, Folsom, CA), N_2, 1 mL/min, 80 °C isothermal. Retention time: methyl ester from trimethylsilyldiazomethane treatment of acid,[43] 28.5 and 31.3 min; ethyl ester, 36.9 and 38.0 min.

Menthol (8a) and Menthyl Acetate (8b).[44] Chiral GC conditions are as in ref 44b. The enantiomeric purity was also determined by ^1H NMR via preparation of the Mosher esters with (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride:[45] δ = 3.61 ppm (minor) and 3.55 ppm (major).

3-Methylcyclohex-2-enol (9a) and 3-Methylcyclohex-2-enyl Acetate (9b).[46] Chiral GC conditions: Cyclodex B capillary GC 25 m column, 25 mm i.d., N_2, 1 mL/min, 100 °C isothermal. Retention time: **9a**, 7.5 min; **9a**, 7.7 min; **9b**, 11.1 min; **9b**, 12.2 min.

***cis*-4-Acetoxy-2-cyclopenten-1-ol (10a).**[47] Chiral GC conditions: Cyclodex B capillary GC 25 m column, 25 mm i.d., N_2, 1 mL/min, 80 °C isothermal. Racemic *cis*-4-acetoxy-2-cyclopenten-1-ol retention times: 22.5 and 24.0 min. (1*S*,4*R*)-*cis*-4-Acetoxy-2-cyclopenten-1-ol (Fluka) retention times: 22.3 min.

(39) *Pharmaceutical Enzymes-Properties and Assay Methods*; Ruyssen, R., Lauwers, A., Eds.; E. Story-Scientia PVBA, Scientific Publishing Co.: Gent/Belgium, 1978; pp 206−211.

(40) Sarath, G.; De La Motte, R. S.; Wagner, F. W. In *Proteolytic Enzymes*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: New York, 1989.

(41) Manimaran, T.; Stahly, G. P. *Tetrahedron: Asymmetry* **1993**, *4*, 1949−1954.

(42) The absolute configuration was not determined. It is most probably *S* on the basis of the preference of CRL for the analogous 2-hydroxyisocaproate ester.[14b]

(43) Aoyama, T.; Shiori, T. *Chem. Pharm. Bull.* **1981**, *29*, 1475−1478.

(44) (a) Langrand, G.; Baratti, J.; Buono, G.; Triantaphylides, C. *Tetrahedron Lett.* **1986**, *27*, 29−32. (b) Caron, G.; Tseng, W.-M.; Kazlauskas, R. J. *Tetrahedron: Asymmetry* **1994**, *1*, 83−92.

(45) Kalyanam, N.; Lightner, D. A. *Tetrahedron Lett.* **1979**, *5*, 415−418.

(46) The absolute configuration of the product alcohol was not determined. It is most likely *R* due to the preference of CRL for the (*R*)-alcohol in the transesterification with vinyl acetate.[11b]

(47) Laumen, K.; Schneider, M. *Tetrahedron Lett.* **1984**, *25*, 5875−5878.

(48) Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* **1989**, *111*, 6836−6841.

4,4,4-Trifluoro-3-phenyl-3-hydroxybutyne (11a) and 4,4,4-Trifluoro-3-phenylbutyn-3-yl Acetate (11b).[26] Chiral GC conditions: Chiraldex GTA capillary GC 20 m column, 25 mm i.d. (Astec, Whippany, NJ), N_2, 1 mL/min, 100 °C isothermal. Retention time: (*R*)-**11b**, 13.1 min; (*S*)-**11b**, 13.6 min; (*R*)-**11a**, 20.5 min; (*S*)-**11a**, 22.1 min.

Recovery and Storage of the CRL-CLECs. After each reaction, the insoluble CRL-CLECs could be recovered by filtration or centrifugation, followed by washing with a nondeactivating solvent such as water-saturated *tert*-amyl alcohol or aqueous 75% polyethylene glycol (average MW 1000) and then stored in 20 mM pH 7 Tris, 2 mM $CaCl_2$. The repetitive use of the CRL-CLECs on a small scale with minimal physical loss of catalyst is best achieved using a solid phase extraction funnel fitted with a 45 μm polymeric frit (Poly-Prep 10 mL column, BioRad, Hercules, CA).

Multiple Cycle Experiments. Ketoprofen (2a) Resolution. Ketoprofen chloroethyl ester ((*R,S*)-**2b**) (500 mg) and CRL-CLECs (25 mg) were suspended in 5 mL of 50:50 0.5 M ammonium acetate−PEG (average MW 1000) in a 10 mL solid phase extraction funnel. The tube was shaken in a 40 °C incubator at 200 rpm. Periodically 10 μL aliquots of the evenly suspended reaction mixture were removed and analyzed by HPLC. Once approximately 50% conversion had been reached, the reaction mixture was removed by filtration and the catalyst was washed once with 5 mL of water-saturated *tert*-amyl alcohol and once with 5 mL of 20 mM pH 7 Tris, 2 mM $CaCl_2$, followed by 5 mL of 50:50 0.5 M ammonium acetate−PEG 1000. The catalyst was stored overnight in the reaction buffer, and a new hydrolysis cycle was initiated the following day by the addition of racemic **2b**.

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