Cross-Linked Enzyme Crystals as Highly Active Catalysts in Organic Solvents

Nazer Khalaf, Chandrika P. Govardhan, Jim J. Lalonde, Rose A. Persichetti, Yi-Fong Wang, and Alexey L. Margolin*

Altus Biologics Inc., 40 Allston Street Cambridge, Massachusetts 02139-4211

Received January 10, 1996

The low activity of enzymes in organic solvents limits their impressive synthetic potential.[1] It is not unusual to see processes employing more enzyme than substrate by weight.[2] In the case of lipases, this low efficiency can be explained, at least in part, by the low purity of commercial preparations. For example, the commercial preparations of two of the most synthetically useful lipases—from *Candida rugosa* (CRL) and *Pseudomonas cepacia* (LPS)—contain less than 5−6% and 1% of the lipase, respectively. The large amount of impurities makes the downstream processing difficult and expensive, and complicates our understanding of the mechanistic aspects of enzymatic catalysis in organic solvents.

One would think that by using lipases in purified form much higher activity could be achieved. In addition, recent pulbications have indicated that purified lipases exhibit higher enantioselectivity in hydrolytic resolutions, simply by eliminating contaminating enzymes with opposite enantioselectivity.[3,4] Surprisingly, the potential synthetic benefits of purified lipases in organic solvents have not been realized to date.[5,6] Not only is the cost of purified lipases higher, but their stability and activity in organic solvents are lower than those of their crude counterparts.[7,8]

Recent work on cross-linked enzyme crystals (CLECs)[9] has demonstrated that enzymes in this form acquire high stability while preserving their activity in high-water mixtures.[10] By definition, CLECs are highly purified since their preparation includes crystallization of the enzymes. Subsequent chemical cross-linking locks the enzymes in the crystalline form, thus affording insolubility and stability. Here we disclose a novel form of CLEC catalysts which combines high activity and productivity in neat organic solvents.

Enzyme activity in organic solvents is intimately related to water content,[11] the size and morphology of the catalyst particles, and the enzyme microenvironment. These parameters can be adjusted by preparing lyophilized complexes of enzymes with carbohydrates,[12] organic buffers,[13] and salts.[14] However, despite the wide use of lyophilization for preparation of enzymes for catalysis in organic solvents, the process is not fully understood and may cause significant reversible denaturation of enzymes.[12]

Since CLECs are solid crystalline particles insoluble in both water and organic solvents, their drying can be achieved by washing them with solutions of different surfactants in organic solvents, thus completely avoiding lyophilization. A number of anionic,[15] cationic,[16] and nonionic[17] surfactants were used in the initial screening. The activity was measured after drying and then repeated after 12 days storage at room temperature.[18] On the basis of this screening, the best surfactants were chosen and dry CLECs were prepared on a large scale. In a typical experiment CLECs[19] of LPS (30 g protein) suspended in 10 mM Tris, 10 mM $CaCl_2$, pH 7.0, were transferred to a sintered glass funnel (porosity ∼5 μm). The buffer above the CLECs was decanted or removed by suction. The equal volume of 2-butanone containing 30 g of the detergent *N*,*N*′,*N*′-poly(oxyethylene(10))-*N*-tallow-1,3-diaminopropane was added to the CLEC cake. The solvent and surfactant were removed by gentle suction. The mixture was transferred to a fritted pressure filter funnel after breaking up any lumps and dried in a stream of nitrogen to a water content of about 2−3 % as determined by Karl Fisher titration.

The activities of the resulting CLECs[21] in the resolution of alcohols, acids, and amines are presented in Table 1. Not only are the CLECs of three different enzymes (two lipases and subtilisin) much more active than their crude counterparts on a weight basis (columns 4 and 5), but in all of the reactions shown in the table their specific activity per milligram of protein is higher as well (column 6). Thus, in order to achieve the same activity in the resolution of menthol or sulcatol, for example,

(1) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114−120. Poppe, L.; Novák, L. *Selective Biocatalysis*; VCH PUblishers: New York, 1992. Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon: New York, 1994.

(2) For some representative examples, see: Bovara, R.; Carrea, G.; Ferrara, L.; Riva, S. *Tetrahedron: Asymmetry* **1991**, *2*, 931−938. Wang, Y.-F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 7200−7205. Palomaer, A.; Cabre, M.; Ginesta, J.; Mauleon, D.; Carganico, G. *Chirality* **1993**, *5*, 320−328. Gotor, V.; Brieva, R.; Gonzales, C.; Rebolledo, F. *Tetrahedron* **1991**, *47*, 9207−9214. Therisod, M.; Klibanov, A. *J. Am. Chem. Soc.* **1987**, *109*, 3977−3981. Njar, V. C. O.; Caspi, E. *Tetrahedron Lett.* **1987**, *28*, 6549−6552. Burgess, K.; Henderson, I. *Tetrahedron Lett.* **1991**, *32*, 5701−5704. Bevinakatti, H. S.; Banerji, A. A.; Newdakar, R. V. *J. Org. Chem.* **1989**, *54*, 2453−2455. Gutman, A. L.; Shapira, M.; Boltanski, A. *J. Org. Chem.* **1992**, *57*, 1063−1065. Parida, S.; Dordick, J. *J. Am. Chem. Soc.* **1991**, *113*, 2253−2259.

(3) Wu, S.-H.; Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* **1990**, *112*, 1990−1995. Hernaiz, M. J.; Sanchez-Montero, J. M.; Sinisterra, J. V. *Tetrahedron* **1994**, *50*, 10749−10760. Ng-Youn-Chen, M. C.; Serreqi, A. N.; Huang, Q.; Kazlauskas, R. J. *J. Org. Chem.* **1994**, *59*, 2075−2081.

(4) Lalonde, J. J.; Govardhan, C. P.; Khalaf, N. K.; Martinez, O. G.; Visuri, K. J.; Margolin, A. M. *J. Am. Chem. Soc.* **1995**, *117*, 6845−6852.

(5) Nishio, T.; Kamimura, M. *Agric. Biol. Chem.* **1988**, *52*, 2631.

(6) Yamane, T.; Ichiryu, T.; Nagata, M.; Ueno, A.; Schimozu, S. *Biotechnol. Bioeng.* **1990**, *36*, 1063−1069. Tsai, S.-W.; Dordick, J. *Biotechnol. Bioeng.*, in press.

(7) Bovara, R.; Carrea, G.; Ottolina, G.; Riva, S. *Biotechnol Lett.* **1993**, *15*, 169−174. Wehtje, E.; Aldercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1993**, *41*, 171−178.

(8) Ottolina, G.; Carrea, G.; Riva, S.; Sartore, L.; Veronese, F. *Biotechnol. Lett.* **1992**, *14*, 947−952.

(9) St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314−7316.

(10) Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 2732−2737.

(11) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141−144. It is accepted that effect of water on enzyme activity in organic solvents is better described in terms of thermodynamic water activity. See: Bell, G.; Halling, P. J.; Moore, B. D.; Partridge, J.; Rees, D. G. *Trends Biotechnol.* **1995**, *13*, 468−473. Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178−206.

(12) Dabulis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **1993**, *41*, 566.

(13) Blackwood, A. D.; Curran, L. J.; Moore, B. D.; Halling, P. J. *Biochim. Biophys. Acta* **1994**, *1206*, 161−165.

(14) Khmelnitsky, Y. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647−2648.

(15) Aerosol 22, dioctyl sulfosuccinate (AOT), Niaproof Types 8 and 4, TEEPOL HB7.

(16) Methyltrioctylammonium chloride (Aliquat 336), *N*,*N*′,*N*′-poly(oxyethylene(10))-*N*-tallow-1,3-diaminopropane.

(17) Brij 30, Brij 35, Tritons X-15, X-100, and X-405, sorbitan sesquiolerate (Arlacel 83), Span 85, Tergitols NP 4 and NP 35.

(18) The activity of the dry samples was measured in transesterification of *n*-amyl alcohol with ethyl acetate in toluene for CRL and LPS and transesterification of *N*−Ac-PheOEt with *n*-propanol in isooctane for ABL. While several surfactants exhibited high activity right after drying, only a few maintained this high level after storage.

(19) The preparation of CRL−CLEC was described in ref 4. LPS was crystallized according to: Bornscheuer, U.; Reif, O-W.; Laush, R.; Fretag, R.; Scheper, T.; Kolisis, F. N.; Menge, U. *Biochim. Biophys. Acta* **1994**, *1201*, 55−60. Alcalase (Novo) from *Bacillus licheniformis* (ABL) was crystallized according to the procedure reported for subtilisin Carlsberg (Tuchsen, E.; Ottesen, M. *Carlsberg Res. Commun.* **1977**, *42*, 407−420) with slight modifications. CLECs were obtained by cross-linking with glutaraldehyde according to the published procedure.[9] Cross-linked enzyme crystals of CRL, LPS, and ABL are sold under the trade name Chiro-CLEC™-CR, ChiroCLEC-PC, and ChiroCLEC-BL, respectively, and are commercial products of Altus Biologics, Inc. (Cambridge, MA).

(20) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.

(21) A similar procedure was applied to the preparation of CRL−CLECs and ABL−CLECs. The optimal surfactants for CRL− and ABL−CLECs were Tergitol Type TMN-6 (poly(oxyethylene glycol ether)) and Tergitol Type 15-S-3 (poly(oxyethylene glycol ether)), respectively.

S0002-7863(96)00081-9 CCC: $12.00

Table 1. Enzyme-Catalyzed Resolutions in Organic Solvents (Rates (V) are in μmol/min·mg at 25 °C)

compound	actylating agent[a] and solvent	enzyme[b]	V_{CLEC}[c] $\times 10^3$	V_{crude}[d] $\times 10^3$	V_{CLEC}/V crude; equal protein[e]	E[t] CLEC	E crude
menthol (449 mM)	VA (449 mM); toluene	CRL	1220	4	62	≫100	16
Ibuprofen (97 mM)	*n*-amyl alcohol (460 mM) isooctane	CRL	57.5	0.13	90	≫100[g]	7.2[g]
hydroxyhexanoic acid (532 mM)	BuOH (1060 mM) toluene	CRL	85	2.2	7.9	55[g]	2[g]
sec-phenethyl alcohol (200 mM)	VA (200 mM) toluene	LPS	15000	90	1.7	≫100	≫100
sulcatol (80 mM)	VA (120 mM) toluene	LPS	6700	20	3.4	28	35
2-octanol (80 mM)	VA (120 mM) toluene	LPS	11700	44.5	2.6	9.8	9.9
methyltryptamine (200 mM)	TFB (400 mM) *t*-BuOH	ABL	12.5	2.9	3.6	41	46

[a] VA, vinyl acetate; TFB, trifluoroethyl butyrate. [b] The water contents of crude enzyme preparations were 9.3%, 2.5%, and 5% for CRL, LPS, and ABL, respectively. [c] The water contents for CLEC were 13.3%, 2.3%, and 2.5% for CRL, LPS, and ABL, respectively. CLEC concentrations were 4, 1, 10, 1.2, 0.4, 0.4, and 16 mg/mL. The amounts of the surfactants in the final preparations of CLECs were 16%, 40%, and 50% (w/w) for CRL, LPS, and ABL, respectively. [d] Crude enzyme concentrations were 20, 15, 50, 8.3, 40, 40, and 28 mg/mL, respectively. [e] The total protein contents in crude commercial preparations of CRL, LPS and ABL were 10%, 0.7%, and 7%, respectively. The protein content of crude lyophilized ABL was 50% (w/w). [f] Reactions rates and enantioselectivities were assayed by capillary gas chromatography on Cyclodex B, (alcohols) (R,R)Whelk-OH (Ibuprofen) GC, and Chiracel OJ HPLC (methyltryptamine) columns. E was calculated according to ref 20. For discussion on applicability of E for reactions affected by inhibition or catalyzed by crude enzymes see ref 4. [g] E_{app} was calculated as in an irreversible case on the basis of the ee of the product at low conversion.

one will need to use about 300-fold less catalyst. The striking difference in activities between crude enzyme preparations and CLECs cannot be explained by differences in water content in the case of CRL and ABL (Table 1, footnotes b and c). When crude CRL and ABL preparations were dried to the same water contents as those of CLECs (13.3% for CRL and 2.5% for ABL), the activities changed by less than 12%.

To the best of our knowledge, this is the first demonstration that pure lipases can be extremely active in organic solvents.[22] In addition, CRL−CLECs exhibit much higher enantioselectivity than crude CRL preparation (Table 1, entries 1−3). In this case the increased enantioselectivity of CRL−CLECs is clearly due to the removal of competing hydrolases.[4]

In addition to the surfactants chosen for the preparative procedures, several others (Tritons and AOT for CRL and AOT and Brij 30 for ALB) exhibited high activity after storage. The combination of several effects may account for this dramatic increase in activity of pure lipases in CLEC form. All of our data indicate that surfactants play a crucial role in the activity enhancement. Indeed, when CLECs were dried to a similar water content without surfactants, their activity was 19 (ABL), 79 (LPS), and more than 100 (CRL)-fold lower than that reported in Table 1.[23] The presence of amphiphilic surfactants may help to maintain a better water balance or the native conformation of amphiphilic lipases,[24] which in crude preparations can in part be achieved by different contaminants such as lipids, celite, and other proteins. In addition, surfactants may simply facilitate the transfer of hydrophobic substrate molecules through the layer of tightly bound water to the binding site of an enzyme. While much more work is necessary to elucidate the exact mechanism of the effects of surfactants on CLEC activity,[25] the practical consequences of this phenomenon are immediately clear: the high specific activity, purity, and stability of CLECs result in the high catalyst productivity in neat organic solvents.

In order to demonstrate the productivity of CLECs in organic solvents on a preparative scale, we chose the resolution of *sec*-phenethyl alcohol (50 mmol; 6.1 g) with vinyl acetate (50 mmol; 4.3 g) in toluene (100 mL) catalyzed by LPS−CLECs (1.3 mg solid; 1 mg protein). The reaction mixture was allowed to stir at room temperature for 16 h at which time the conversion reached 50%. The isolated yield of phenethyl acetate was 4.5 g (98.5% ee) giving the volumetric productivity of 30 g/L and a substrate to catalyst ratio of 4700.[26] The high productivity of low molecular weight synthetic catalysts is thought to be their key advantage over high molecular weight enzymes.[27] This example clearly demonstrates that despite the high molecular weight and nonaqueous reaction medium, CLECs can be highly productive and compare favorably even with the best of synthetic asymmetric catalysts.

JA960081S

(22) High activity was also demonstrated for purified subtilisin soluble in organics (Paradkar, V. M.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5009−5010). High activity of lipase complexes soluble in neat organic solvents was achieved only with crude lipases: Kamiya, N.; Goto, M.; Nakashio, F. *Biotechnol. Prog.* **1995**, *11*, 270−275. Okahata, Y.; Fujimoto, Y.; Ijiro, K. *J. Org. Chem.* **1995**, *60*, 2240−2250.

(23) The water content in these experiments was 13.3% for CRL−CLECs, 1.7−2% for LPS−CLECs, and 2.2−2.4% for ABL−CLECs.

(24) Broos, J.; Visser, J. W. G.; Engbersen, J. F. J.; Verboom, W.; van Hoek, A.; Reinhoudt, D. N. *J. Am. Chem. Soc.* **1996**, *117*, 12657−12663.

(25) Rather than accelerating the resolution reactions in water, surfactants slowed them down: Bornemann, S.; Crout, D. H. G.; Dalton, H.; Hutchinson, D. W. *Biocatalysis* **1994**, *11*, 191−221.

(26) The productivity reached 8000 after 72 h reaction when 100 mmol *sec*-phenethyl alcohol was used.

(27) Jacobsen, E. N.; Finney, N. S. *Chem. Biol.* **1994**, *1*, 85−90.