Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 4599–4605

TETRAHEDRON:
ASYMMETRY

Enantioselectivity of alcohol-treated *Candida rugosa* lipase in the kinetic resolution of racemic methyl 2-aryloxypropionates in water and aqueous organic media

Antonio Cipiciani,* Francesca Bellezza, Francesco Fringuelli and Massimiliano Stillitano

Dipartimento di Chimica, Università di Perugia, Via Elce di Sotto 8, 06100 Perugia, Italy

Received 5 November 1999; accepted 10 November 1999

Abstract

Racemic methyl 2-aryloxypropionates (±)-**1** were subjected to hydrolysis in water and in a series of two-phase aqueous organic media in the presence of *Candida rugosa* lipase (CRL). The biocatalytic material used was the enzyme of commercial CRL purified by treatment with different alcohols. The purification of CRL and the reaction medium play an important role in the enantioselection of racemates (±)-**1**. While it is not possible to use the same protocol for all substrates, by combining the different ways of purifying the enzyme with the various reaction media, it is possible to achieve high enantioselectivities of racemic esters.

1. Introduction

Lipases are enzymes widely used in organic synthesis because of their ability to regio-, diastereo- and enantioselectively catalyze hydrolysis and esterification in water as well as in aqueous organic media.[1] The enzyme is sometimes more active in aqueous organic media than in water and this can be explained by hypothesizing an oil–water interfacial activation associated with a conformational change of the protein molecule.[2] The activity of lipases is also increased by using a purified or a semipurified native material.

Several protocols have been proposed to obtain a biocatalyst with different degrees of purification, with the most common ones being a multi-step purification,[3–7] dialysis,[8] direct treatment of crude lipase with deoxycolate followed by ethanol–ethyl ether,[7] and the *iso*-propanol treatment.[9,10]

Candida rugosa lipase (CRL) is an extracellular protein. Structural analysis reveals that the catalytic triad is not exposed to the reaction medium, and the polypeptide lid, which covers the active site of the

* Corresponding author. E-mail: cipan@unipg.it

0957-4166/99/$ - see front matter
PII: S0957-4166(99)00500-5

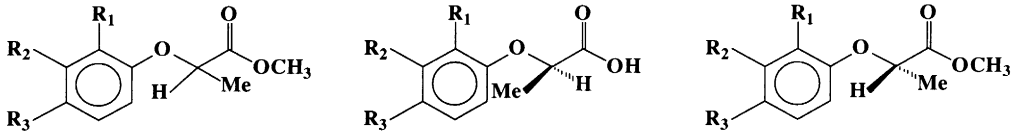
native enzyme, is displaced before the substrate approaches the active site.[11] Scattered data indicate that the method of CRL purification and the reaction medium[12] strongly influence the activity and selectivity of lipase but systematic studies in this area are rare.[10,13]

Optically active 2-aryloxypropionic acids are a family of compounds of great biological interest[14] which, in principle, should be easily obtained by lipase kinetic resolution of parent esters. The literature data show that crude CRL in water is poorly enantioselective towards this class of compounds and with the exception of our recently reported hydrolysis of racemic methyl 2-(2,4-dichlorophenoxy)propionate by treated CRL,[10] no investigation in an aqueous organic medium has been reported.

As a continuation of the research, we report a systematic study on the ability of CRL, purified by treatment with different alcohols, in the kinetic resolution of racemic methyl aryloxypropionates (±)-**1** in both water and aqueous organic media. The hydrolysis of esters was chosen rather than the transesterification reaction because the acid reaction product can be easily separated by washing with alkali and no reagent other than the substrate is required.

2. Results and discussion

Firstly we studied the hydrolyses of esters (±)-**1** (Fig. 1) in water by CRL purified by treatment with simple aliphatic alcohols (methanol, ethanol, *n*-propanol and *iso*-propanol).



(±) **1** (+)-R **2** (-)-S **3**

	a	b	c	d	e
R_1	H	Cl	H	H	Cl
R_2	H	H	Cl	H	H
R_3	H	H	H	Cl	Cl

Fig. 1.

The results of the reactions carried out at room temperature and at pH=7.2 are illustrated in Table 1. The acids (+)-(*R*)-**2** and the esters (−)-(*S*)-**3** were isolated in all the experiments. It is known that an enantiomeric ratio value of E≥20 indicates a high level of enantioselection.[15] On this basis, the MeOH- or *n*-PrOH-purified CRL in water enantioselectively hydrolyzes only the (+)-(*R*) enantiomer of (±)-**1d** yielding enantiomerically pure forms of (+)-**2d** and (−)-**3d**. Treatments with EtOH and *i*-PrOH allow CRL to also recognize the (+)-(*R*) esters of (±)-**1b** and (±)-**1e** and to obtain (+)-**2b** and (−)-**3e** with high enantiomeric excesses.

The effect of the reaction medium was evaluated by carrying out the hydrolyses at room temperature and pH=7.2 with *iso*-propanol-treated CRL in homogeneous and heterogeneous 10:1 v/v aqueous organic media. The 10:1 v/v water:organic solvent ratio was chosen based on previous experiences[10] which indicated that beyond 10% of the amount of organic fraction, the E value does not change, while the rate of hydrolysis decreases. The organic solvents (acetone, diethyl ether, benzene and cyclohexane) were chosen in order to cover a wide range of hydrophobicity values expressed by log P (from −0.23 to

Table 1
Hydrolysis of (±)-methyl 2-aryloxypropionates **1** in water at room temperature and pH 7.2 by CRL treated with the simplest aliphatic alcohols

Substrate	t (h) C (%)[a] (ee) (%) E_{app}[b]	Alcohol purified CRL			
		MeOH	EtOH	n-PrOH	i-PrOH
1a	t	1.3	1.3	0.3	1.8
	C	59	56	50	48
	2a (ee)	88	66	73	62
	3a (ee)	60	84	72	56
	E_{app}	11	13	14	8
1b	t	5.3	1.2	2.3	1.3
	C	47	43	27	48
	2b (ee)	**90**	**84**	79	62
	3b (ee)	73	65	30	58
	E_{app}	22	23	11	7
1c	t	2.3	1.0	0.6	0.6
	C	49	47	54	44
	2c (ee)	46	69	55	60
	3c (ee)	48	62	65	48
	E_{app}	4	10	7	6
1d	t	1.5	0.8	0.7	1.3
	C	48	43	50	45
	2d (ee)	**92**	**91**	**87**	**83**
	3d (ee)	**85**	70	**88**	67
	E_{app}	66	44	42	22
1e	t	4.5	12	9.3	9.3
	C	36	39	57	54
	2e (ee)	63	68	61	75
	3e (ee)	36	42	82	**88**
	E_{app}	6	8	10	20

[a] Conversion of reaction ; [b] enantioselectivity factor[16]

3.20). The results are illustrated in Table 2. The isolated compounds were again the acids (+)-(*R*)-**2** and the esters (−)-(*S*)-**3**. The aqueous–organic medium influences the enantioselectivity, as well as the rate of hydrolyses. The water–benzene medium gives the best results: the reactions are slower than in water but the acids (+)-**2b** and (+)-**2e** are isolated in optically pure form while the acids (+)-**2a** and (+)-**2d** are enantiomerically pure after only one recrystallization.

In water–acetone and water–diethyl ether media the CRL purified by *i*-PrOH only recognizes the (+)-(*R*) enantiomer of racemic **1a**, **1b** and **1d**. The water–cyclohexane medium deactivates the enzyme.

On the basis of these results, the water–benzene medium was also used to test the enantioselectivity of the methanol-, ethanol- and *n*-propanol-purified CRL. The results are illustrated in Table 3 along with those of *iso*-propanol-treated CRL. The water–benzene medium always improves the enantioselectivity of the enzyme with respect to water only (Tables 1 and 3). This is particularly important for the hydrolysis of (±)-**1c** because only by using this protocol is it possible to obtain enantiomerically pure (+)-**2c**.

The ee of products obtained in the hydrolyses with E≥20, using different protocols are summarized in Table 4. The table shows that none of the protocols is effective for all five esters (±)-**1**, rather it is

Table 2
Hydrolysis of (±)-methyl 2-aryloxypropionates **1** by *iso*-propanol purified CRL in different aqueous organic media at room temperature and pH=7.2

Substrate	t (h) C (%)[a] (ee) (%) E_{app}[b]	Reaction medium			
		H_2O/PhH[c]	H_2O/Cy[c]	H_2O/Et_2O [c]	H_2O/Me_2CO[c]
1a	t	2	2.5	7.5	1.3
	C	24	63	38	30
	2a (ee)	**88**	33	81	**92**
	3a (ee)	28	57	50	48
	E_{app}	21	3	16	37
1b	t	4.5	2	23	7
	C	22	61	25	40
	2b (ee)	**96**	53	83	**83**
	3b (ee)	27	83	27	75
	E_{app}	60	8	14	24
1c	t	6	1.3	26	3
	C	40	54	33	42
	2c (ee)	75	36	62	52
	3c (ee)	50	53	61	31
	E_{app}	12	4	8	4
1d	t	6.3	1.6	23	1
	C	38	43	50	30
	2d (ee)	**89**	55	**92**	67
	3d (ee)	55	88	**94**	45
	E_{app}	30	16	85	16
1e	t	151	9	48.5	9
	C	47	33	35	45
	2e (ee)	**95**	47	80	70
	3e (ee)	**83**	32	44	58
	E_{app}	70	4	14	10

[a] Conversion of reaction ; [b] enantioselectivity factor[16]; [c] phosphate buffer (20mM, pH 7.2) / organic solvent 10:1

necessary to combine the methods of CRL purification and the reaction medium in order to achieve the best results.

Enantiomerically pure acids (+)-**2b** and (+)-**2d** can be easily obtained by using several procedures (seven for the former and nine for the latter: entries 1, 2, 5, 8–11 and 1–5, 7, 9–11, respectively) while (+)-**2c** can only be prepared by using the protocol of entry 9. The (+)-**2** and (−)-**3** are simultaneously obtained, with a high enantiomeric excess, only from (±)-**1d** (entries 1, 3 and 7) and from (±)-**1e** (entry 5). All acids (+)-**2** and the esters (−)-**3d** and (−)-**3e** are obtained enantiomerically pure in good yield by using the suitable protocol, while no procedure yields (−)-**3a**, (−)-**3b** and (−)-**3c** with high ee value and satisfactory yield. The procedure that uses *i*-PrOH-purified CRL and water–cyclohexane as reaction medium is ineffective for all substrates.

In conclusion, the CRL purification and the reaction medium play an important role in the enantioselection of racemates (±)-**1** but it is not possible to use the same protocol for all substrates. The purification eliminates non-essential material or that which hinders the reaction, while the reaction medium influences

Table 3
Enantioselection of (±)-methyl 2-aryloxypropionates **1** in water–benzene[a] at room temperature by CRL treated with the simplest aliphatic alcohols

Substrate	t (h) C (%)[b] (ee) (%) E_{app}[c]	Alcohol purified CRL			
		MeOH	EtOH	n-PrOH	i-PrOH
1a	t	7	9	7	2
	C	53	26	47	24
	2a (ee)	67	**89**	**87**	**88**
	3a (ee)	76	32	77	28
	E_{app}	11	23	33	21
1b	t	8.5	48	3	4.5
	C	40	46	32	22
	2b (ee)	**87**	**85**	**95**	**96**
	3b (ee)	57	73	44	27
	E_{app}	23	27	61	60
1c	t	7	168	6.5	6
	C	26	20	48	40
	2c (ee)	**90**	65	78	75
	3c (ee)	32	16	73	50
	E_{app}	29	6	17	12
1d	t	6	7	2.8	6.3
	C	45	36	46	38
	2d (ee)	**90**	**90**	**92**	**89**
	3d (ee)	72	50	77	55
	E_{app}	42	30	56	30
1e	t	7	120	30	151
	C	32	42	42	47
	2e (ee)	**87**	62	**95**	**94**
	3e (ee)	41	**85**	70	**83**
	E_{app}	22	24	82	70

[a] phosphate buffer (20mM, pH 7.2) / benzene 10:1 ; [b] Conversion of reaction ; [c] enantioselectivity factor[16]

the conformational state of the enzyme (i.e., open or closed form) which then determines the geometry of the active site, allowing one compound to be recognized more easily than another.

3. Experimental

3.1. General

Lipase from *Candida rugosa* (crude CRL E.C. 3.1.1.13 type VII) was purchased from Sigma Chemical Co. Racemic 2-aryloxypropionic acids were obtained from Aldrich Chemical Co. All the organic solvents were of reagent grade and used without further purification. The ees of acids (+)-**2** were determined on corresponding methyl esters obtained by treatment with CH_2N_2. [Eu(hfc)$_3$] was used as chiral shift reagent to determine enantiomeric excess recording the ^{1}H NMR spectra in $CDCl_3$ solution on a Bruker AC 200 MHz spectrometer. The ees were determined after the usual work-up of the reaction mixture

Table 4
Enantioselection of (±)-methyl 2-aryloxypropionates **1** by CRL under different conditions

Entry	Treatment of CRL	Reaction medium	ee (%) with E_{app}[a] ≥ 20									
			2a	3a	2b	3b	2c	3c	2d	3d	2e	3e
1	MeOH	H_2O			90				92	85		
2	EtOH	H_2O			84				91			
3	n-PrOH	H_2O							87	88		
4	i-PrOH	H_2O							83			88
5	i-PrOH	H_2O/PhH	88		96				89		94	83
6	i-PrOH	H_2O/Cy										
7	i-PrOH	H_2O/Et_2O							92	94		
8	i-PrOH	H_2O/Me_2CO	92		83							
9	MeOH	H_2O/PhH			87		90		90		87	
10	EtOH	H_2O/PhH	89		85				90			85
11	n-PrOH	H_2O/PhH	87		95				92		95	

[a] enantioselectivity factor[16]

before the purification of the reaction products. Racemic esters **1** were prepared in 90% yield by treating commercial (±)-**2** with anhydrous methanol in the presence of catalytic amounts of *p*-toluenesulfonic acid at reflux for 3 h. The products were separated and purified by usual chemical work-up and spectral analyses were consistent with those previously described.[15] Purification of crude lipase from *Candida rugosa* by treatment with different alcohols was carried out according to the general procedure reported below. The specific activity of purified lipase was determined as previously described.[10] The hydrolysis of (±)-**1d** with *iso*-propanol-treated CRL in water–ethyl ether is reported as the typical procedure.

3.2. Alcohol-treated CRL

Commercial material (10 g, Sigma Chemical Co.) was mixed in phosphate buffer (50 mL, 50 mM, pH 7.2, 4°C) by stirring for 30 min. Alcohol (Table 1) (50 mL) was added dropwise over 40 min at 4°C. This heterogeneous medium was stirred at 4°C for 46 h. The mixture was centrifuged (3000 rpm, 30 min, 4°C) to remove the precipitate. The supernatant was dialyzed against phosphate buffer solution (1×2 L, 50 mM, pH 7.2) and then against deionized distilled water (1×2 L). The protein solution was stored at 4°C and used within one week. Protein concentrations were obtained by spectroscopic determination at 288 nm using bovine serum albumin (Sigma Chemical Co.) as standard (3 mg/mL).

*3.3. Hydrolysis of methyl 2-(4-chlorophenoxy)propionate (±)-**1d** with* iso*-propanol-treated CRL in water–ethyl ether*

Ethyl ether (1.2 mL) was added to a phosphate buffer (20 mM, pH 7.2) solution of *iso*-propanol-treated CRL (12 mL, 260 units, with *p*-NPA assay) and the mixture stirred for 15 min at room temperature in a closed vessel. The resulting enzymatic system was added to the substrate (214 mg) and the mixture

stirred and maintained at pH 7.2 by automatic titration with NaOH (0.2N) using a Mettler DK pH-Stat. When the hydrolysis reached 50% conversion (23 h), a saturated solution of NaCl (15 mL) was added to the reaction mixture, the pH was adjusted to 2 using 6N HCl and extracted with ether (3×30 mL). The combined ether extracts were treated with saturated aqueous $NaHCO_3$ (3×40 mL) and the layers were separated. The ether layer was dried with Na_2SO_4 and concentrated in vacuo giving the ester (−)-**3d**: 104 mg (90% yield), ee 94%, $[\alpha]_D^{25}$ −38.6, lit.[17] $[\alpha]_D^{25}$ −41.1 (c=50, EtOH). The combined aqueous layers were acidified to pH 2, extracted with ether (3×30 mL), dried with Na_2SO_4 and concentrated in vacuo giving the acid (+)-**2d**: 84 mg (85% yield), ee 92%, $[\alpha]_D^{25}$ +36.6, lit.[18] $[\alpha]_D^{25}$ +39.8 (c=30, EtOH). After one recrystallization from hexane:ethyl acetate (10:1), ee 99%.

Acknowledgements

The Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) Consiglio Nazionale delle ricerche (CNR, Rome) and Università degli Studi di Perugia are thanked for financial support.

References

1. Klibanov, A. M. *Acc. Chem. Res*. **1990**, *23*, 114. Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev*. **1992**, *92*, 1071. Wong, C. H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon: New York, 1994.
2. Holmquist, M.; Martinelle, M.; Berglund, P.; Clausen, I. G.; Patkar, S.; Svendsen, A.; Hult, K. *J. Prot. Chem*. **1993**, *12*, 749.
3. Tomizuka, N.; Ota, Y.; Yamada, K. *Biol. Chem*. **1966**, *30*, 576, 1090.
4. Veeragavan, K.; Gibbs, B. F. *Biotechnol. Lett*. **1989**, *11*, 345.
5. Shaw, J. F.; Chang, C. H.; Wang, Y. J. *Biotechnol. Lett*. **1989**, *11*, 779.
6. Brahimi-Horn, M. C.; Guglielmino, M. L.; Elling, L.; Sparrow, L. G. *Biochim. Biophys. Acta* **1990**, *1042*, 51.
7. Wu, S. H.; Guo, Z. H.; Sih, C. J. *J. Am. Chem. Soc*. **1990**, *112*, 1990.
8. Hernaiz, M. J.; Sànchez-Montero, J. M.; Sinisterra, J. V. *Tetrahedron* **1994**, *50*, 10749.
9. Colton, I. J.; Sharmin, N. A.;. Kazlauskas, R. J. *J. Org. Chem*. **1995**, *60*, 212.
10. Cipiciani, A.; Cittadini, M.; Fringuelli, F. *Tetrahedron* **1998**, *54*, 7890.
11. Grochulski, P.; Li, Y.; Schrag, J. D.; Bouthillier, F.; Smith, P.; Harrison, D.; Rubin, B.; Cygler, M. *J. Biol. Chem*. **1993**, *268*, 12843. Grochulski, P.; Li, Y.; Schrag, J. D.; Cygler, M. *Protein Sci*. **1994**, *3*, 82.
12. Rua, M. L.; Diaz-Maurino, T.; Fernandez, V. M.; Otero, C.; Ballesteros, A. *Biochim. Biophys. Acta* **1993**, *1156*, 181.
13. Cipiciani, A.; Fringuelli, F.; Mancini, V.; Piermatti, O.; Scappini, A. M.; Ruzziconi, R. *Tetrahedron* **1997**, *53*, 11853.
14. *The Pesticide Manual;* Worthing, C. R., Ed.; 6th ed.; BCPC Publication: London, 1979; p. 329. Secor, J.; Cséke, C.; Owen, W. J. In *Biocatalysis in Agricultural Biotechnology*; Whitaker, J. R.; Sonnet, P. E., Eds.; American Chemical Society: Washington, 1989; p. 265. ConteCamerino, D.; Mambrini, M.; De Luca, A.; Tricarico, D.; Bryant, S. H.; Tortorella, V.; Bettoni, G. *Pflugers Arch. Eur. J. Physiol*. **1988**, *413*, 105. Fredga, A.; Weidler, A. M.; Gronwall, C. *Ark. Kemi* **1960**, *17*, 265.
15. Chen, C. S.; Wu, S. H.; Girdauskas, G.; Sih, C. J. *J. Am. Chem. Soc*. **1987**, *109*, 2812.
16. Chen, C. S.; Fujimoto, Y.; Girdauskas, G.; Sih, C. J. *J. Am. Chem. Soc*. **1982**, *104*, 7294.
17. Kirchner, G.; Scollar, M. P.; Klibanov, A. M. *J. Am. Chem. Soc*. **1985**, *107*, 7072.
18. Witiak, D. T.; Ho, T. C.-L.; Hakney, R. E.; Connor, W. E. *J. Med. Chem*. **1968**, *11*, 1086.