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Improving the Enantioselectivity of *Candida rugosa* Lipase in the Kinetic Resolution of Racemic Methyl 2-(2,4-dichlorophenoxy)propionate

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Abstract: Racemic methyl 2-(2,4-dichlorophenoxy)propionate (±)-**1**, was 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 commercial preparation and enzyme purified by using different procedures. The (+)-R- and (−)-S-2-(2,4-dichlorophenoxy)propionic acids (**3**) were obtained in excellent yield and high enantiomeric excess when the hydrolysis of (±)-**1** was performed in water/benzene in the presence of 2-propanol treated CRL. The kinetic resolution of (±)-**1** was scaled-up.

The use of hydrolytic enzymes as synthetic chiral catalysts has received considerable attention in recent years.[1] The lipase from *Candida rugosa* (CRL) is one of the most versatile and widely used enzymes in the resolution of esters, acids and alcohols [2] in both aqueous and organic media. Researchers often use commercial product, which is a crude preparation, because of easy availability, low cost and higher stability in different reaction media. In its most widely used form, this crude preparation contains at least two hydrolases [3] that differ in their enzymatic activity. These two different enzymes may have co-operative regio- and chemo-selectivities or opposing stereochemical effects, in wich case, both the chemical and optical purities of the final product would be reduced.[4] Furthermore, using crude CRL (C-CRL) often leads to results that are irreproducible making it difficult to draw correct conclusions about the enzymatic effects and consequently making it very difficult to design a model for correctly predicting stereo preference. To overcome these difficulties, some different strategies have been adopted such as CRL having different degree of purification,[3-5] employing cross-linked enzyme crystals,[6] simple treatment with 2-propanol [7] or changing of reaction conditions by adding an organic phase,[8] inorganic salts [9] or surfactants.[10] Purification influences the stability of the resulting enzymatic system because as the purity of the enzyme increases, its stability decreases.

While comparisons of stereoselective effectiveness between crude and purified enzymes are frequently reported,[11] systematic studies concerning the solvent effect on the activity of purified enzyme have been rare. To date, the solvent effect on the enantioselectivity of 2-propanol treated CRL (PT-CRL) and on ammonium sulfate precipitate CRL (ASP-CRL) has not been previously investigated. With this in mind and on the bases of our previous experience in the field[12] we decided to investigate the hydrolysis of (±) methyl 2-(2,4-dichlorophenoxy)propionate **1** in water and in a series of aqueous-organic media. The organic solvents were chosen on the basis of their log P [13] even though no reliable relationship has been reported between the various proposed parameters that describe the solvent properties and biocatalytic effects.[14] The ester (±)-**1** was chosen

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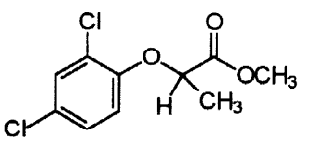
as probe because the parent enantiomer acids (+)-**2** and (−)-**2** are important members of the family of phenoxypropionic acids, which, in optically active form, are compounds of great biological interest,[15-17] and because previous attempts at enzymatic kinetic resolution of (±)-**1** using C-CRL have been unsuccessful as proven by the obtained low enantioselectivity factor values (E).[18]

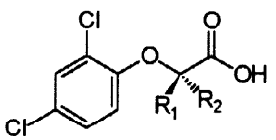
The hydrolysis of ester (±)-**1** was performed rather than the transesterification reaction because the reaction product is easily separated simply by washing with alkali and no reagent other than the substrate is required.

Results and Discussion

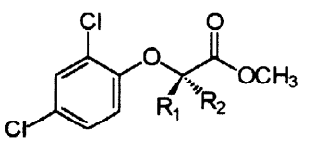
The results of the hydrolysis of (±)-**1** by C-CRL at room temperature in different aqueous-organic media by using organic solvents with different hydrophobicities (log P) are summarized in Table 1.



(±)1



(-)-S-2 $R_1 = H$, $R_2 = CH_3$
(+)-R-2 $R_1 = CH_3$, $R_2 = H$



(-)-S-3 $R_1 = H$, $R_2 = CH_3$
(+)-R-3 $R_1 = CH_3$, $R_2 = H$

Table.1 Hydrolysis of (±) methyl 2-(2,4-dichlorophenoxy)propionate by C-CRL in different aqueous organic media at room temperature.

Organic Solvent	Log P	t (h)	C[a] (%)	Products (ee %)				E_{app}[b]
none -	-	6.0	50	-		-		1.0
Acetonitrile[c]	-0.33	6.5	47	(−)-**S2**	(19)	(+)-**R3**	(17)	2.0
Acetone[c]	-0.23	6.5	47	(−)-**S2**	(17)	(+)-**R3**	(15)	1.6
THF[c]	0.49	6.0	46	(−)-**S2**	(31)	(+)-**R3**	(27)	2.5
Diethyl Ether[d]	0.85	6.0	33	(−)-**S2**	(79)	(+)-**R3**	(39)	12.0
Benzene[d]	2.00	24	32	(−)-**S2**	(28)	(+)-**R3**	(13)	2.0
Toluene[d]	2.50	23	24	(−)-**S2**	(43)	(+)-**R3**	(14)	3.0
Dibutyl ether[d]	2.90	26	28	(−)-**S2**	(60)	(+)-**R3**	(24)	5.0
Cyclohexane[d]	3.20	4.2	50	(−)-**S2**	(18)	(+)-**R3**	(18)	1.7
Hexane[d]	3.50	8.8	61	(−)-**S2**	(49)	(+)-**R3**	(77)	6.5
Heptane[d]	4.00	13.0	67	(−)-**S2**	(36)	(+)-**R3**	(72)	4.5

[a] Conversion of reaction ; [b] enantioselectivity factor[23]; [c] organic solvent/phosphate buffer (20mM, pH 7.2) 1:2; [d] organic solvent/phosphate buffer (20mM, pH 7.2) 1:9.

In all the experiments, the biocatalytic system shows the same stereopreference for ester (±)-**1** giving the (−)-S acid **2** and the unreacted (+)-R ester **3** as hydrolysis products. The reaction occured in a short time but the enantioselectivity was always low.

Subsequently, the hydrolysis was carried out using a partially purified lipase ASP-CRL, obtained by precipitating the protein with ammonium sulfate. The results are reported in Table 2. In this case, the reaction rate was faster with ASP-CRL than with C-CRL. While enantiodifferentiation depends on the reaction medium, even with treated lipase, E values were low.

Table.2 Hydrolysis of (±) methyl 2-(2,4-dichlorophenoxy)propionate by ASP-CRL in different aqueous organic media at room temperature.

Organic Solvent	Log P	t (h)	C[a] (%)	Products (ee %)				E_{app}[b]
none	-	3.0	54	(+)-**R2**	(6)	(−)-**S3**	(7)	1.2
Acetonitrile[c]	0.33	5.0	62	(+)-**R2**	(6)	(−)-**S3**	(10)	1.2
Acetone[c]	-0.23	4.5	60	(+)-**R2**	(2)	(−)-**S3**	(3)	1.1
THF[c]	0.49	5.3	52	(−)-**S2**	(13)	(+)-**R3**	(14)	1.5
Diethyl ether[d]	0.85	7.0	24	(−)-**S2**	(63)	(+)-**R3**	(20)	5.0
Benzene[d]	2.00	28.0	59	(−)-**S2**	(13)	(+)-**R3**	(19)	1.5
Toluene[d]	2.50	32.0	49	(−)-**S2**	(23)	(+)-**R3**	(22)	2.0
Dibutyl ether[d]	2.90	6.3	30	(−)-**S2**	(59)	(+)-**R3**	(26)	5.0
Cyclohexane[d]	3.20	3.0	50	(+)-**R2**	(4)	(−)-**S3**	(4)	1.1
Hexane[d]	3.50	4.5	56	(−)-**S2**	(48)	(+)-**R3**	(61)	5.0
Heptane[d]	4.00	9.0	58	(−)-**S2**	(32)	(+)-**R3**	(44)	3.0

For footnotes *a, b, c, d*, see Table 1.

Finally we used 2-propanol treated lipase (PT-CRL) purified by using a modified version of the procedure described by Kazlauskas.[7] The results in Table 3 show that the enantiopreference of the enzyme was opposite that of C-CRL and high ee were otained. The reaction times were longer in organic media than in those using C-CRL or ASP-CRL and a much higher enantiomeric ratio (E=70) was achieved with benzene/water. The acid (+)-**2** can be separated easily and is optically pure after one recrystallization. Similarly the unreacted ester (-)-**3** is isolated in high ee and optically pure acid (-)-**2** is obtained after hydrolysis and recrystallization.

It is known that the activity and enantioselectivity of CRL in the kinetic resolution of racemic esters by hydrolysis is influenced by: (i) the chemical nature of the cosolvent, (ii) the proportion between the water phase and cosolvent, and (iii) the reaction temperature.[19] To establish optimum reaction conditions we studied the hydrolysis of (±)-**1** in the presence of PT-CRL in benzene/water and water/acetone at different solvent ratios (% v/v), stopping the hydrolysis at C=30% (Fig. 1 and 2). In both cases, the rate of hydrolysis decreased as the % v/v of organic solvent increases, while the E value trends were different for the two reaction media. In benzene/water, the E value increases sharply with small amounts of benzene and then remained constant,

whereas in acetone/water, the E value decreased with small amounts of cosolvent and then levelled off. With benzene as cosolvent, the influence of temperature was then investigated. The hydrolysis of racemic **1** was carried out at 40°C in a two-phase benzene/water (1:10 v/v), in the presence of PT-CRL. Under these conditions the reaction was about four times faster than at room temperature with no decrease in yield and enantioselectivity.

Given the stability and activity of PT-CRL in mixed aqueous organic media we then conducted a preparative scale hydrolysis of (±)-**1**. Two gr of racemic methyl ester of **1** in a two-phase aqueous benzene at 40°C were converted into (+)-**2** and (−)-**3** in 70 hr with the same ee as in the micro-scale experiment. The hydrolysis was quenched when the conversion approached 46% and the optically enriched acid (+)-**2** (ee 94%, yield 93%) was separated from the ester (−)-**3** (ee 80%, yield 92%) by extraction in basic medium followed by acidification. Subsequent hydrolysis of (−)-**3** afforded (-)-**2**. One recrystallization from hexane/ethylacetate affords optically pure acids (+)-**2** and (−)-**2**.

Once again it is evident that the enzyme specificity and enantioselectivity strongly depend on the purification of the biocatalytic material and on the nature of the reaction medium. Only a qualitative suggestion can be advanced to explain these facts because no reliable relationship between the single parameters that describe the solvent properties and the biocatalytic effect has been proposed [13] nor is the crystalline structure of the acting enzyme known.[20]

Table 3. Hydrolysis of (±) methyl 2-(2,4-dichlorophenoxy)propionate by PT-CRL in different aqueous organic media at room temperature.

Organic Solvent	Log P	t (h)	C[a] (%)	Products (ee %)		E_{app}[b]
none	-	5.3	50	(+)-R-**2** (75)	(−)-S-**3** (75)	16
Acetonitrile[c]	0.33	8.5	41	(+)-R-**2** (75)	(−)-S-**3** (53)	12
Acetone[c]	-0.23	8.8	45	(+)-R-**2** (79)	(−)-S-**3** (58)	10
THF[c]	0.49	8.8	36	(+)-R-**2** (75)	(−)-S-**3** (42)	10
Diethyl ether[d]	0.85	116.0	31	(+)-R-**2** (75)	(−)-S-**3** (34)	10
Benzene[d]	2.00	151.0	47	(+)-R-**2** (95)	(−)-S-**3** (83)	70
Toluene[d]	2.50	43.0	31	(+)-R-**2** (76)	(−)-S-**3** (35)	11
Dibutyl ether[d]	2.90	130.0	41	(+)-R-**2** (75)	(−)-S-**3** (53)	12
Cyclohexane[d]	3.20	6.0	51	(+)-R-**2** (72)	(−)-S-**3** (74)	13
Hexane[d]	3.50	24.3	43	(+)-R-**2** (68)	(−)-S-**3** (51)	9
Heptane[d]	4.00	30.0	41	(+)-R-**2** (68)	(−)-S-**3** (54)	14

For footnotes *a, b, c, d*, see Table 1.

The differences observed in reactivity and enantioselectivity between hydrolysis conducted in pure water or in water/benzene might be due to a conformational change of the enzyme (among those permitted, the open and closed forms for instance) which then influences the structure of the active site. When the benzene/water ratio

increases (Fig. 1), the rate of enzymatic hydrolysis of (±)-**1** decreases because the substrate is diluted in the organic phase.

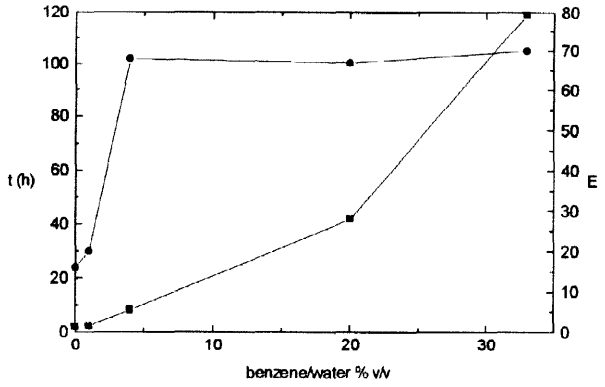


Fig.1 Plot of benzene/water (%v/v) against the reaction time t(h) (▪) and against the E (•) value at a reaction conversion of 30% at room temperature.

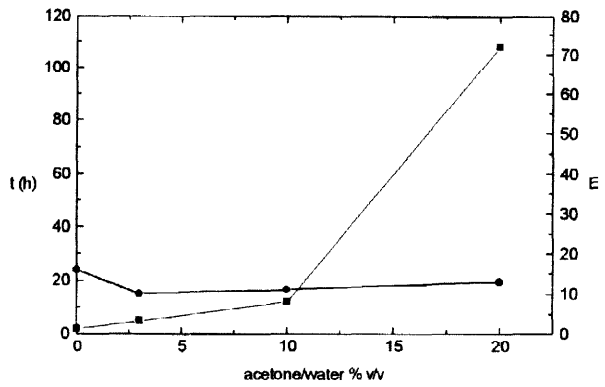


Fig.2 Plot of acetone/water (%v/v) against the reaction time t(h) (▪) and against the E (•) value at a reaction conversion of 30% at room temperature.

In summary this work shows the advantage of using the semi-purified CRL and the importance of organic cosolvent. For the first time the enzymatic kinetic resolution of methyl 2-(2,4-dichlorophenoxy)propionate (±)-**1** has been performed on a gram quantity scale; (+)- and (-)-2-(2,4-dichlorophenoxy)propionic acids **2** can now be prepared with excellent yield and in optically pure form.

Experimental Section

General. *Candida rugosa* lipase (crude CRL E.C.3.1.1.13 type VII, from Sigma Chemical CO.) was used as supplied. Racemic 2-(2,4-dichlorophenoxy)propionic acid were obtained from Aldrich Chemical Co.
All the organic solvents were of reagent grade and used without further purification.
Europium (III) tris(3-heptafluoropropyl)hydroxymethylene]-(+)camphorate [Eu(hfc)$_3$] were used as chiral shift reagent for enantiomeric excess determination recording the ^{1}H-NMR spectra in $CDCl_3$ solution on a Bruker AC 200 MHz spectrometer.
Racemic **1** were prepared in 90% yield by treating commercial (±)-**2** with anhydrous methanol in the presence of catalytic amount of p-toluenesulfonic acid at reflux for 3 hr. The product was purified and separated by usual chemical work-up and spectral analyses were consistent with those previously described.[20]

ASP-CRL Crude powder of lipase from *Candida rugosa* (10 g, Sigma Chemical CO.) was suspended in phosphate buffer (150 ml, 25 mM, pH 7.2) and the suspension was stirred at 4°C for 0.5 hr. The mixture was centrifuged at 15000 g for 30 min and the supernatant was collected. Solid ammonium sulfate (51g) was added to the supernatant in small portions over 4 hr to give 50% saturation. The suspension was centrifuged (10.000 rpm, 20 min, 4°C) and the supernatant discarded. The pellet was dissolved in 10 ml phosphate buffer (25 mM, pH 7.2) and dialyzed against phosphate buffer solution (1x1L, 25mM, pH 7.2) for one night and then against distilled water (1x1L) for 4 hr. The enzymatic activity was 9U/mg protein.

PT-CRL *Candida rugosa* lipase (CRL) was purified by a modified versionof the recently described procedure of Kazlauskas.[7] Commercial material (10 g, Sigma Chemical CO.) was dispersed in phosphate buffer (50 ml, 50 mM, pH 7.2, 4°C) by stirring for 30 min. 2-Propanol (50 ml) was added dropwise over 40 min at 4°C. This heterogeneous medium was stirred at 4°C for 46 hrs. The mixture was centrifuged (3000 rpm, 30 min, 4°C) to remove the precipitate. The supernatant was dialized against phosphate buffer solution (1×2L, 50 mM, pH 7.2) and then against deionized distilled water (1×2L). The solution of protein 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).

Determination of Enzymatic Activity. The specific activity of purified lipase was determined spectrophotometrically using p-nitrophenylacetate (p-NPA) as substrate. Activity is given in units/wt and 1unit (1U) equals 1 μmol of ester hydrolyzed per minute. In a typical experiment, an aliquot (2-5μl) of enzyme solution was added to phosphate buffer (3 ml, 10mM, pH 7.2), equilibrated at room temperature and then an aliquot of p-NPA (50μl, 100 mM solution in acetonitrile) was added. The formation of p-nitrophenolate was monitored at 346 nm for 1min and the activity of lipase was calculated using an extinction coefficient[21] of 4800 M^{-1} cm^{-1}. This value accounts for the incomplete ionization of p-nitrophenolate at pH 7.2. A typical activity of C-CRL was 36 U/g solid (1U/mg prot) measured by pH-Stat at pH 7.2 with p-nitrophenylacetate as substrate.

Determination of enantiomeric excesses. The enantiomeric purity (ee_s) of methyl ester of 2-(2,4-dichlorophenoxy)propionic acid was determined by ^{1}H-NMR in the presence of chiral shift reagent $Eu(hfc)_3$. The acid product in the enzymatic reaction was first converted to the corresponding methyl ester by treatment with diazomethane and the enantiomeric excess (ee_p) was then determined by ^{1}H-NMR analysis. For each analysis the sample contained 5 mg of the ester, 60 mg of the chiral shift and 0.6 ml of $CDCl_3$.

Hydrolysis of (±)-1 in presence of C-CRL. In a standard experiment, C-CRL (120 mg, Aldrich Chemical CO.) was suspended in phosphate buffer (NaH_2PO_4/ Na_2PO_4 12 ml, 0.1 M, pH 7.2), stirred for 15 min. at room temperature and the pH adjusted at 7.2. The organic solvent (6 ml for immiscible organic solvent and 0.4 ml for miscible organic solvent) was added and the mixture stirred at room temperature for 10 min. The obtained enzymatic mixture was added to the racemic ester **1** (1.0 mmol) and the mixture maintained at pH 7.2 under stirring at room temperature by automatic titration with NaOH 0.2 M using a Mettler DK pH-Stat. When the hydrolysis reached the conversion indicated in the tables the reaction mixture was worked up as described below.

Hydrolysis of (±)-1 in presence of ASP-CRL and PT-CRL. In a standard experiment, the organic solvent (6 ml for immiscible organic solvent and 0.4 ml for miscible organic solvent) was added to a 12 ml solution of treated *Candida rugosa* lipase (260 units, with p-NPA assay) and phosphate buffer (20mM, pH 7.2) and the mixture stirred for 15 min at room temperature. The resulting enzymatic system was added to substrate (261 mg) and the mixture maintained at pH 7.2 by automatic titration with NaOH 0.2 N using a Mettler DK pH-Stat. When the hydrolysis reached 30-50% conversion, 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 $NaHCO_3$ (3×25 ml) and the layers were separated. The ether layer was dried with Na_2SO_4 and concentrated *in vacuo* giving the unreacted ester **3**. 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 **2**. The apparent enantiomeric ratio (E_{app}) was calculated from the measured ee of the starting material (ee_s) and of the product (ee_p) using $C = ee_s/ee_s+ee_p$ and $E_{app} = \ln (1-C)(1-ee_s) / \ln (1-C)(1+ee_s)$.[23]

Hydrolysis of (±)-1 on preparative scale and preparation of (+)-2 and (-)-2. The reaction conditions of enzymatic kinetic resolution of racemic **1** were optimized so that: two ml of benzene were added to phosphate buffer (20mM, pH 7.2) solution of PT-CRL (12 ml, 500 units, with p-NPA assay) and the mixture stirred for 15 min at 40°C temperature. The resulting enzymatic system was added to substrate (2g) and the mixture stirred at 40°C and maintained at pH 7.2 by automatic titration with NaOH 0.2 N using a Mettler DK pH-Stat. When the hydrolysis reached 46% conversion (70 hr) a saturated solution of NaCl (25 ml) was added to the reaction mixture, the pH was adjusted to 2 using 6N HCl and extracted with ether (3×40 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. The combined aqueous layers were acidified to pH 2, extracted with ether (3×40 ml), dried with Na_2SO_4 and concentrated *in vacuo* giving the acid.(+)-**2** : 0.81g (94% yield) ee 93%. After recrystallization from hexane-ethyl acetate (10:1) $[\alpha]^{20}_D$ +28.7° (c 1.6 EtOH) [Lit.[24] $[\alpha]^{25}_D$ +28.9°]. The ester (−)-**3**: 0.99g (92% yield) ee 80%, $[\alpha]^{20}_D$ −30.2° Lit.[22] $[\alpha]^{25}_D$ −37.5° (c 2.5, EtOH). The ester was hydrolyzed in a solution consisting of 7 mL of aqueous 2N NaOH and 7mL of methanol at 40°C. The resulting acid was recrystallized twice giving pure (−)-**2** $[\alpha]^{20}_D$ −28.6° (c 1.6, EtOH). Methylation with diazomethane gives (−)-**3** $[\alpha]^{20}_D$ −37.1°

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