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Influence of pH and temperature on the enantioselectivity of propan-2-ol-treated *Candida rugosa* lipase in the kinetic resolution of (±)-4-acetoxy-[2,2]-paracyclophane

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Abstract—The reaction temperature, pH of the aqueous medium and use of an aqueous-*n*-hexane reaction medium markedly influenced the hydrolysis rate and enantioselectivity of (±)-4-acetoxy-[2,2]-paracyclophane with propan-2-ol-treated *Candida rugosa* lipase. The results have been justified on the basis of a possible conformational change in the enzyme as a consequence of the displacement of its polypeptide lid.

1. Introduction

The use of enzymes as chiral catalysts to prepare optically active compounds from either racemic or prochiral substrates has been widely studied. Hydrolases and in particular lipases, have been used most often, in both aqueous and organic media.[1–3] The lipase from *Candida rugosa* is one of the most versatile and widely used enzymes due to its great activity and ability to accept a large number and variety of substrates.[1–3]

The commercial *Candida rugosa* lipase is a crude form (C-CRL) that contains non-essential materials and several other hydrolases, including a protease, which sometimes hinders the desired reaction.[4,5] Several purification procedures have been developed to obtain a biomaterial with increased hydrolytic and stereospecific activity.[6–8]

Structural analysis of native CRL reveals that the active site of the enzyme is protected by a polypeptide segment which blocks the approach of very large molecules such as lipids.[9,10] External conditions can displace the polypeptide lid effecting a conformational change of the protein, and hence, of the active site. Different reactivity and selectivity are then observed with respect to the native form. Experimental data supports the idea that the conformational change occurs when an organic solvent is used to purify the C-CRL or when the enzyme is used in organic or organic-aqueous medium. It can also be inferred that temperature and pH influence the 'flipping of the lid' and, if this is the case, it should be possible to evidence the conformational changes in the enzyme by comparing the enzyme activity against these chemical–physical parameters.

2. Results and discussion

As part of our studies on the synthetic applications of C-CRL and purified CRL,[11–13] we recently reported[13] that the lipase prepared by treating C-CRL with simple aliphatic alcohols (methanol, ethanol, *n*-propanol and propan-2-ol) and the reaction medium (water and aqueous organic solvent) greatly influence lipase reactivity and its enantioselectivity in the hydrolysis of 2-aryloxypropanoates. We explained these results on the basis that the enzyme present in these lipolytic preparations assumes an open-form by changing its native conformation by moving the flap at the binding site.

Herein, we report evidence that changing the reaction pH and temperature can also move the polypeptide lid of propan-2-ol-treated CRL (PT-CRL). To investigate this we used the kinetic resolution of (±)-4-acetoxy-[2,2]-paracyclophane **1** as a probe; this compound has planar chirality[14] and is well accepted by CRL. We have shown that C-CRL selectively hydrolyzes (±)-4-acetoxy-[2,2]-paracyclophane **1** in aqueous medium at

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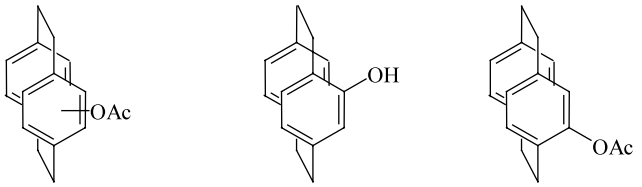
pH 7.2 and 40°C affording high enantiomeric excesses of (+)-(*R*)-4-hydroxy-[2,2]-paracyclophane **2** and (+)-(*S*)-4-acetoxy-[2,2]-paracyclophane **3**.[11]



1 (±) **2** (+)-(*R*) **3** (+)-(*S*)

The enantiopreference of an enzyme in the kinetic resolution of a racemate, as evaluated by ln*E*,[15] varies linearly with the inverse of reaction temperature according to the equation $\ln E = \Delta\Delta S^{\neq}/R - \Delta\Delta H^{\neq}/RT$ [16,17] if there are no marked differences in the conformational structure of the active site as a result of different temperatures.

The hydrolysis of (±)-**1** carried out with PT-CRL in aqueous medium at pH 7.2 is slow and occurs with low enantioselectivity in the 22–30°C range. In contrast, at higher temperatures (50–60°C), the reaction is rapid and occurs with high enantiopreference. Practically no change in the enantioselectivity occurs in the 40–60°C range. These results listed in Table 1 and plotted in Fig. 1 suggest that a marked conformational change of PT-CRL such as that caused by the flipping of the polypeptide segment, probably occurs and this results in a marked structural change at the active site.

Analogous behavior was observed by studying the effect of pH on the enantioselectivity of PT-CRL. Actually this subject has been scarcely investigated. Schneider et al.[18] found that the *E* value of the kinetic resolution of a prochiral meso-diester into a chiral monoester by porcine liver esterase, was higher at pH 7 than at 8 and Xu et al.[19] reported that the highest enantioselectivity in the hydrolysis of 2-chloroethyl ester of ketoprofen by *Candida rugosa* lipase was obtained at pH 2.2, whereas the highest activity of this enzyme is usually observed in neutral or slightly acidic medium. No data are known about the effect of a strong alkaline medium on the enantioselectivity of the enzyme.

Table 2 and Fig. 2 illustrate the effect of pH on the enantioselectivity of PT-CRL in the hydrolysis in water of **1** at 22°C in the pH 5.2–8.2 range. While the reactivity of the enzyme was practically constant over the entire pH range explored, the *E* value decreased in the Ph range 5.2–7.7 and then suddenly increased as the pH was increased by only 0.5 units. When the reaction was carried out in water/*n*-hexane the enantioselectivity was very high at pH 6.2 and collapses (from $E=100$ to $E=15$) in the pH 6.2–7.2 range (Table 3 and Fig. 3). A minor change was observed in the pH 7.2–11.0 range showing that when the PT-CRL works in aqueous organic medium, at neutral or basic pH, it probably assumes a less flexible conformation. Once again these results can be justified on the basis of a conformational change of the enzyme as a consequence of the displacement of its polypeptide lid.

We checked if the spontaneous hydrolysis of ester **1** occurred at alkaline pH in the absence of CRL and found that hydrolysis does not occur at pH 10, while at pH 11 a very small degree of spontaneous hydrolysis of **1** is seen. The *E* value was corrected for this effect.

The reaction conditions described in Table 3, entry 1, allowed excellent kinetic resolution of **1** on a multigram scale; 1.5 g of racemic **1** in aqueous *n*-hexane 6:1 v/v at 22°C and pH 6.2 were quantitatively converted into **2** (e.e. 97%) and **3** (e.e. 96%) in 7 h by 450 U of PT-CRL.

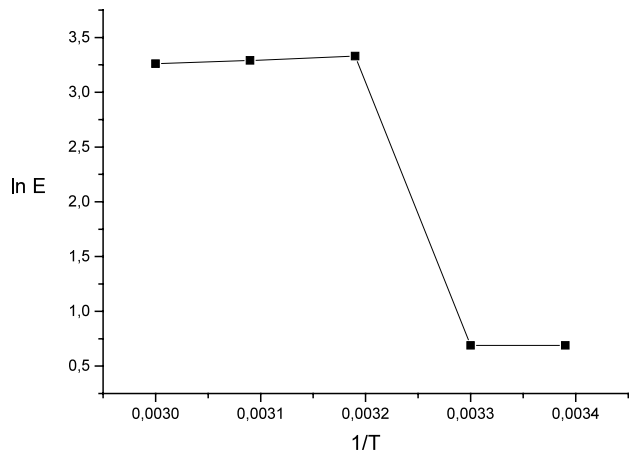


Figure 1. Effect of temperature on the enantioselectivity of PT-CRL in the kinetic resolution of **1** in water at pH 7.2.

Table 1. Effect of temperature on the reactivity and enantioselectivity of PT-CRL in the hydrolysis of **1** in water at pH 7.2

Entry	*T* (°C)	Conv. (%)[a]	*t* (h)	(% e.e.)[b]		*E*[c]
				(+)-(*R*)-**2**	(+)-(*S*)-**3**	
1	22	34	65	23	12	2
2	30	48	23	25	23	2
3	40	50	23	82	83	28
4	50	51	8	82	85	27
5	60	52	8	78	87	26

[a] % Conversion of reaction determined by GC after the required amount of NaOH 0.2N was consumed.
[b] Isolated products.
[c] Enantioselectivity factor.

Table 2. Effect of pH on the reactivity and enantioselectivity of PT-CRL in the hydrolysis of **1** in water at 22°C

Entry	pH	Conv. (%)[a]	*t* (h)	(% e.e.)[b]		*E*[c]
				(+)-(*R*)-**2**	(+)-(*S*)-**3**	
1	5.2	42	55	67	48	8
2	5.7	43	69	60	45	6
3	6.2	32	31	50	23	4
4	6.7	29	49	44	18	3
5	7.2	34	65	23	12	2
6	7.7	59	65	20	28	2
7	8.2	61	63	56	87	10

[a] % Conversion of reaction determined by GC after the required amount of NaOH 0.2N was consumed.
[b] Isolated products.
[c] Enantioselectivity factor.

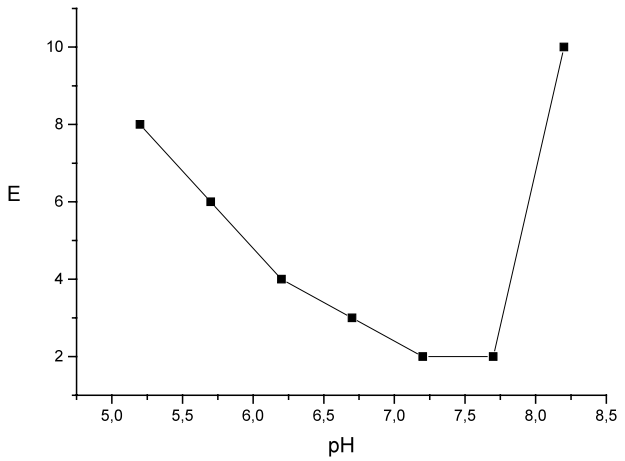


Figure 2. Effect of pH on the enantioselectivity of PT-CRL in the kinetic resolution of **1** in water at 22°C.

3. Conclusion

In closing, the present study shows that temperature, pH and reaction medium determine the conformation of PT-CRL, which in turn markedly influences the structure of the active site, and consequently the enantiopreference of the enzyme. CRL is usually used in neutral or acidic medium, so it is of interest to know that PT-CRL is active in water at pH 8.2 and in aqueous *n*-hexane up to pH 11.0.

4. Experimental

4.1. General

Compound **1** was synthesized as previously described.[11] *Candida rugosa* lipase (crude CRL E.C.3.1.1.13 type VII, LOT. 107H1024) was purchased from Sigma Chemicals Co. All the organic solvents were of reagent grade and used without further purification. All other chemicals were obtained from commercial sources. GC analyses were performed on a Hewlett–Packard 5890 chromatograph with HP-5-fused silica capillary column (30 m, 0.25 internal diameter, 0.25 μm film thickness), an 'on column' injector system, and a FID detector with hydrogen as gas carrier.

The e.e. values were calculated by measuring the specific optical rotation in $CHCl_3$ solution on a JASCO-DIP polarimeter and by ^1H NMR chiral shift experiments with $Eu(hfc)_3$ in $CDCl_3$ solution on a Brucker AC 200 MHz spectrometer. The error in the measure of specific optical rotation (±1.5%) makes significantly different also small *E* values as 2 and 4 for example.

Crude *Candida rugosa* lipase (C-CRL) was purified as previously described.[12] The specific activity of purified lipase was determined as previously described.[12]

4.2. Hydrolysis of 1 with PT-CRL in aqueous medium

In a standard experiment (Table 1, entry 3), a solution of propan-2-ol-treated *Candida rugosa* lipase (250 units, with *p*-NPA assay) in aqueous phosphate buffer (20 mM, pH 7.2, 13 mL) were stirred at 40°C for 15 min and the pH was adjusted to 7.2 with aqueous NaOH (0.2N) or aqueous HCl (0.1N). The resulting enzymatic solution was added to substrate **1** (0.75 mmol, 200 mg). The mixture was maintained at the pH of the reaction under stirring by automatic titration with aqueous NaOH (0.2 M) using a Mettler DK pH-Stat. When the hydrolysis reached 50% conversion, a saturated solution of NaCl (15 mL) was added to the reaction mixture. The mixture was then extracted with diethyl ether (3×30 mL). The organic phases were dried (Na_2SO_4), evaporated under reduced pressure and the residue was chromatographed on silica gel eluting with petroleum ether/diethyl ether/$CHCl_3$, 75/15/10 to afford (+)-(*R*)-4-hydroxy-[2,2]-paracyclophane **2** (71 mg, 85%), $[\alpha]_D^{20}$ +6.9 (*c* 1.0 $CHCl_3$) e.e. = 82% and (+)-(*S*)-4-acetoxy-[2,2]-paracyclophane **3** (100 mg, 92%), $[\alpha]_D^{20}$ +34.2 (*c* 1.0 $CHCl_3$) e.e. = 83%.

4.3. Hydrolysis of 1 with PT-CRL in water-*n*-hexane

In a standard experiment (Table 3, entry 1), a solution of PT-CRL (250 units, with *p*-NPA assay) in aqueous phosphate buffer (20 mM, pH 6.2, 13 mL) was stirred at 22°C for 15 min and the pH was adjusted to 6.2 with NaOH 0.2N or HCl 0.1N. The resulting enzymatic solution was added to a solution of **1** (0.75 mmol, 200

Table 3. Influence of pH on the reactivity and enantioselectivity of PT-CRL in the hydrolysis of **1** in water/*n*-hexane[a] at 22°C

Entry	pH	Conv. (%)[b]	*t* (h)	(% e.e.)[c]		*E*[d]
				(+)-(*R*)-**2**	(+)-(*S*)-**3**	
1	6.2	49	8	98	96	100
2	7.2	38	7	81	49	15
3	8.2	38	9	69	43	9
4	9.2	37	9	54	53	7
5	10.2	25	9	43	30	4
6	11.0	25	15	78	25	11

[a] Phosphate buffer/*n*-hexane: 6/1.
[b] % Conversion of reaction determined by GC after that the required amount of NaOH 0.2N was consumed.
[c] Isolated products.
[d] Enantioselectivity factor.

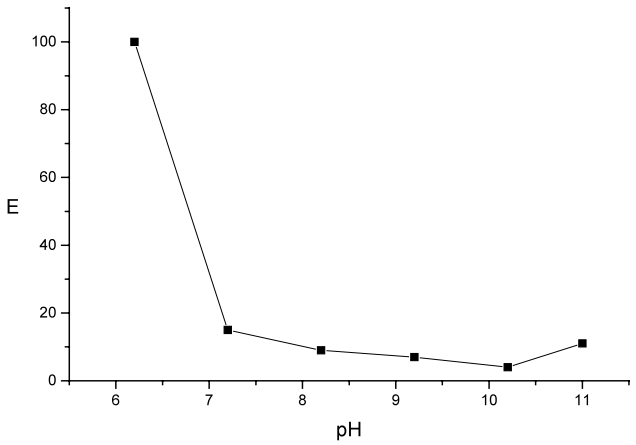


Figure 3. Effect of pH on the enantioselectivity of PT-CRL in the kinetic resolution of **1** in water-*n*-hexane 6:1 at 22°C.

mg) in *n*-hexane (2 mL). The mixture was maintained at the pH of the reaction under stirring by automatic titration with NaOH 0.2 M using a Mettler DK pH-Stat. When the hydrolysis reached 49% conversion, a saturated solution of NaCl (15 mL) was added to the reaction mixture. The mixture, worked up as described above afforded (+)-(*R*)-4-hydroxy-[2,2]-paracyclophane **2** (72 mg, 87%), $[\alpha]_D^{20}$ +8.3 (*c* 1.0 $CHCl_3$) e.e. = 98% and (+)-(*S*)-4-acetoxy-[2,2]-paracyclophane **3** (97 mg, 97%), $[\alpha]_D^{20}$ +39.6 (*c* 1.0 $CHCl_3$) e.e. = 96%.

4.4. Hydrolysis of 1 with PT-CRL on a preparative scale.

A solution of PT-CRL (450 units, with *p*-NPA assay) in aqueous phosphate buffer (20 mM, pH 6.2, 20 mL) was stirred at room temperature for 15 min in a closed vessel. The resulting enzymatic preparation was added to a solution of **1** (1.5 g) in *n*-hexane (7 mL). The pH of the mixture was maintained at pH 6.2 under stirring by automatic titration with aqueous NaOH (0.2 M) using a Mettler DK pH-Stat. When the hydrolysis reached 50% conversion, a saturated solution of NaCl (15 mL) was added to the reaction mixture. The mixture, worked up as described above, afforded (+)-(*R*)-4-hydroxy-[2,2]-paracyclophane **2** (0.67 g, 90%), e.e. = 97% and (+)-(*S*)-4-acetoxy-[2,2]-paracyclophane **3** (0.71 g, 95%), e.e. = 96%.

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