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# Small water amounts increase the catalytic behaviour of polar organic solvents pre-treated *Candida rugosa* lipase

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**Abstract**

Following a simple and quick treatment based on dissolving the crude lipase of *Candida rugosa* in different percentages (v/v) of several polar organic solvents (methanol, ethanol, acetone, 1-propanol, 2-propanol, 1-butanol and 2-butanol), and a further dialysis, different preparations with enhanced activity and thermal stability were obtained. This improved catalytic behaviour was observed in aqueous media (more efficient triglyceride hydrolysis) as well as in anhydrous organic solvents (better enantioselectivity and/or yield in the esterification of *R*- and *S*-ketoprofen).

Combination of this treatment with organic solvents with a correct control of water activity in the reaction media allowed a much better catalytic behaviour, dramatically increasing the reaction yield and the enantiodiscrimination ability in the esterification of *S*-ketoprofen vs. the *R* counterpart. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* *Candida rugosa* lipase; Esterification; Ketoprofen; Organic solvents; Ammonium sulphate; Water activity

## 1. Introduction

Crude enzyme preparations are used more often than pure enzymes because of their easier availability, lower cost and higher operational stability. Nevertheless, the use of these impure enzymes shows some disadvantages, mainly due to the presence of other protein contaminants which could display different (or even reversal) activities [1]. *Candida rugosa*lipase (CRL) has been widely used in the resolution of racemic acids [2,3] due to its high enantioselectivity. However, crude commercial extracts of CRL may be highly heterogeneous, showing different enzymatic activity and selectivity depending on the commercial lot [4]. Thus, it is difficult to systematise the synthetic properties of these mixtures in biotransformations [5], so that the use of pure enzymes would be required for this purpose. In 1993, Rúa et al. [6] developed a procedure for the purification of CRL obtaining two isoenzymes. On the other hand, some different treatments on crude CRL, such as bioimprinting with amphiphilic molecules [7,8], or organic solvents treatment [9–12], have been described with the aim of increasing the enzymatic catalytic performance in a sort of semi-purification strategy. It is also known that the enzymatic enantioselectivity can be enhanced or even

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reversed through the rational choice of organic solvent [13,14] as reaction media, although in these cases, the adjustment of the hydration degree of the enzyme molecules is crucial for the control of the catalytic activity [15]. In a previous paper [16], we have analysed the water sorption isotherms of CRL in organic media, and this strategy proved to be a good qualitative method to estimate the pre-equilibrium $a_w$ value of the system.

In the current paper, we describe how this control of the water activity on an esterification reaction catalysed by organic solvent-treated CRL leads to a much better activity and enantioselectivity in the esterification of both enantiomers of ketoprofen.

## 2. Experimental

### 2.1. Materials

Lipase (EC 3.1.1.3) Type VII from *C. rugosa* from different commercial lots (54HO260, and 85HO629), MES and tributyrin were obtained from Sigma. *S*(+)- and *R*(−)-2(3-Benzoylphenyl)propionic acid [*S*(+)- or *R*(−)-ketoprofen] were kindly supplied by Menarini Laboratories (Spain), and ammonium sulphate was purchased from Aldrich. Standard solutions for calibration of water activity were supplied from Rotronic (Switzerland). Organic solvents as well as all other chemicals were all of analytical grade.

### 2.2. Precipitation of CRL using ammonium sulphate

Crude lipase (1 g) was dissolved in phosphate buffer (50 mM, 25 ml, pH 6.8, 4°C) by stirring for 30 min. Optimum saturation percentage of ammonium sulphate (60%, 9.15 g of solid; SA-CRL) was very slowly added at 4°C and stirred for 1 h at 4°C. The precipitate was collected by centrifuging at 5000 rpm for 40 min at 4°C, dissolved in Tris/HCl buffer (10 ml, 50 mM, pH 8.0, 4°C) and dialysed against deionised distilled water (4 × 5 l). This solution contained 783 U/mg of specific activity as previously described [12,17] using tributyrin as substrate.

### 2.3. Partial purification of CRL using organic solvents

Crude lipase (2 g) was dissolved in MES buffer (50 mM, pH 6.0, 4°C) and stirred for 30 min. Different volume percentages of each organic solvent [40% 2-propanol (2P-CRL), 30% 1-propanol (1P-CRL), 40% ethanol (E-CRL), 30% acetone (A-CRL), 30% methanol (M-CRL), 30% 2-butanol (2B-CRL) and 60% 1-butanol (1B-CRL)] were added dropwise at 4°C up to a final volume of 50 ml. This translucent solution became cloudy after stirring at 4°C for 24 h. The precipitates were removed by centrifuging at 5000 rpm for 30 min at 4°C. The supernatants were dialysed against deionised distilled water (4 × 5 l) and concentrated to 25 ml by ultrafiltration (Amicon PM-30 filter). Thus, different solutions with specific activities ranging from 188 to 337 U/mg (tributyrin assay) were obtained as mentioned [12].

### 2.4. Assays for lipase activity

The hydrolysis of tributyrin was monitored in a pH stat at 25°C. The assay mixture (10 ml) consisted of 4 ml of 68 mM emulsified substrate (Novo Industry Analytical Method AF 95/5-6B), 5 ml of buffer solution (MES 50 mM; pH 6.0) and different amounts of lipase solutions to give the final protein concentration of 40 mg/ml. One unit of lipase activity is the amount of enzyme needed to produce 1 μmol of fatty acid per minute under the mentioned conditions.

### 2.5. Thermal stability assays

A study of thermal stability at 50°C was carried out on both the treated fractions and native enzymes. Native enzymes and the organic solvent-treated preparations (5 mg, 1 mg/ml) were dissolved in MES buffer (50 mM; 5 ml, pH 6.0). SA-CRL preparation (5 mg, 1 mg/ml) was dissolved in phosphate buffer (50 mM, 5 ml, pH 6.8) by stirring at 4°C. The assay mixture (10 ml) consisted of 10 ml of 68 mM emulsified tributyrin and 500 μl of enzyme and water, respectively. The activity values (using tribu-

tyrin as substrate) were fitted, using the program EXFIT from the package SIMFIT v. 4.0, to a simple or double exponential decay, depending on the sample.

### 2.6. Water sorption isotherms

The water sorption isotherms were registered by measuring the water activity values ($a_w$) of the samples after equilibration with different amounts of water using a Rotronic-Hygroscop model BT-RS1 at 30°C, which was previously calibrated using standard fixed water activity values (0, 0.35, 0.5 and 0.85).

One-hundred milligrams of dry commercial lipase, 42 mg of lipase treated with acetone (A-CRL) or 48 mg of lipase treated with ammonium sulphate (SA-CRL) were extensively dehydrated at vacuum in the presence of anhydrous $P_2O_5$ at room temperature, and added to 1 ml of dry isooctane. Once the sample has been added, the first $a_w$ value was registered and small amounts of water (2–5 μl) were subsequently added, measuring the $a_w$ values after reaching the equilibrium (1–2 h). Water sorption isotherms were obtained by plotting the value of milligram of water per milligram of sample vs. $a_w$.

### 2.7. Esterification of S(+)- or R(−)-ketoprofen

The standard reaction mixture in the absence of water was composed of isooctane (10 ml), *R*(−)- or *S*(+)-ketoprofen (66 mM), and 1-propanol (264 mM); in the presence of water, the reaction mixture was the same as described above with the addition of 5 μl of water. The reaction was carried out at 30°C by magnetically stirring in 25 ml-flasks, and started in all the cases by adding the same lipase units of treated and non-treated lipases. Then, aliquots of 0.1 ml were taken from the solution and added to 1.4 ml of isooctane; subsequently, the ester conversion was analysed by HPLC using a Chiracel-OD column (Daicel Chemical Ind., Japan). The mobile phase used was hexane/2-propanol/acetic acid (90/10/1, v/v/v), flow rate = 0.5 ml/min, and the compounds were detected at 254 nm, at retention times of 20 min (for *R*- or *S*-ketoprofen) and 13 min for the esters.

## 3. Results and discussion

### 3.1. Hydrolytic activities of native and treated lipases

Hydrolysis of tributyrin was the reaction used to characterise different commercial enzymatic powders in aqueous medium. As can be see in Table 1, lots 54 and 85 (entries 1 and 6) displayed similar specific activities in tributyrin hydrolysis. Following the procedure described in Section 2, upon ammonium sulphate precipitation [17], we obtained a preparation, named SA-CRL, about six times more active (entry 2) in hydrolysis than the crude enzyme, as can be seen from the data shown in Table 1.

Upon organic solvent treatment [12], we also obtained different enzymatic preparations more active in hydrolysis of tributyrin than the crude enzyme (Table 1), ranging from a 1.5-fold increase using 1B-CRL to 2.7-fold employing A-CRL. Thus, we are reporting an increase in tributyrin hydrolysis with both methodologies, which can be related to classical

Table 1
Partial purification of CAL according to hydrolytic activity

| Entry | Fraction | Specific activity (U/mg) | Fold |
|---|---|---|---|
| 1 | CRL (L 54)[a,b] | 133 | 1 |
| 2 | SA-CRL[c,b] | 783 | 5.7 |
| 3 | E-CRL[d,b] | 317 | 2.4 |
| 4 | 1P-CRL[e,b] | 223 | 1.7 |
| 5 | 2P-CRL[f,g] | 319 | 2.4 |
| 6 | CRL (L 85)[g] | 124 | 1 |
| 7 | A-CRL[h,i] | 337 | 2.7 |
| 8 | M-CRL[j,i] | 286 | 2.3 |
| 9 | 2B-CRL[k,i] | 279 | 2.2 |
| 10 | 1B-CRL[l,i] | 188 | 1.5 |

[a] Crude lipase from *C. rugosa*, lot 54HO260 from Sigma.
[b] Obtained by treating Lot 54.
[c] 60% ammonium sulphate on CRL.
[d] 40% (v/v) ethanol on CRL.
[e] 30% (v/v) 1-propanol on CRL.
[f] 40% (v/v) 2-propanol on CRL.
[g] Crude lipase from *C. rugosa*, lot 85HO629 from Sigma.
[h] 30% (v/v) acetone on CRL.
[i] Obtained treating Lot 85.
[j] 30% (v/v) methanol on CRL.
[k] 30% (v/v) 2-butanol on CRL.
[l] 60% (v/v) 1-butanol on CRL.

procedures for the purification of proteins, as it has been previously described [9–12,18]

### 3.2. Thermal stability of native and partially purified enzymes

A study of thermal stability at 50°C on both the treated fractions and the native enzyme was carried out. The tributyrin hydrolytic activities were fitted to single or double exponential decay equations using the deactivation model proposed by Henley and Sadana [19] (Table 2).

As can be seen, the half-life time of the preparations (except for SA-CRL and 1B-CRL) is higher than those obtained using the crude enzymes. In fact, for those preparations obtained from CRL (L 54): (E-CRL), (1P-CRL) and (2P-CRL), the stabilisation factor and the half-life time were enhanced. This effect was higher for those preparations obtained from CRL (L 85), acetone (A-CRL), methanol (M-CRL) and 2-butanol (2B-CRL). This fact may be due the higher half-life time of CRL (lot 54) vs. CRL (lot 85). Thus, we are somehow stabilising the biocatalysts by applying the organic solvent treatment. This fact is really remarkable because it is generally observed that any purification step on crude lipases results in a decrease in thermal stability [20].

Table 2
Thermal stability of native CRL and treated enzymes at 50°C

| Fraction | $t_{1/2}$(h)[a] | $F$[b] |
|---|---|---|
| CRL (L 54)[c,d] | 4.5 | 1 |
| SA-CRL[e,d] | 0.6 | 0.1 |
| 2P-CRL[f,d] | 5.9 | 1.3 |
| 1P-CRL[g,d] | 10.6 | 2.3 |
| E-CRL[h,d] | 8.2 | 1.8 |
| CRL[g] (L 85)[i,j] | 3.9 | 1 |
| A-CRL[k,j] | 10.8 | 2.8 |
| M-CRL[l,j] | 47.2 | 12.1 |
| 2B-CRL[m,j] | 7.9 | 2.0 |
| 1B-CRL[n,j] | 3.1 | 0.8 |

[a] Half-life time.
[b] Stabilisation factor related to the crude enzyme.
[c] Crude lipase from *C. rugosa*, lot 54HO260 from Sigma.
[d] Obtained by treating Lot 54.
[e] 60% ammonium sulphate on CRL 40% (v/v).
[f] 40% (v/v) 2-propanol on CRL.
[g] 30% (v/v) 1-propanol on CRL.
[h] 40% (v/v) ethanol on CRL.
[i] Crude lipase from *C. rugosa*, lot 85HO629 from Sigma.
[j] Obtained by treating Lot 85.
[k] 30% (v/v) acetone on CRL.
[l] 30% (v/v) methanol on CRL.
[m] 30% (v/v) 2-butanol on CRL.
[n] 60% (v/v) 1-butanol on CRL.

### 3.3. Esterification of S(+)- or R(−)-ketoprofen by crude and treated lipases in the absence of water

It has been recently shown [4] that the hydrolysis of tributyrin, commonly used to quantify lipase activity, is not useful for predicting the catalytic activity in lipase-catalysed reactions carried out in slightly hydrated media. Thus, different causes for explaining the irreproducibility of this type of reactions have been proposed:

(i) Relative proportion of isoenzymes, which seems to depend on the fermenter conditions;
(ii) Amount of water in the lyophilised crude enzyme; and
(iii) Protein content of the commercial powders.

The lack of repetitive results is a problem that becomes even more serious when dealing with chiral substrates, because not only the enzymatic activity but also the stereoselectivity (and even the stereopreference) may be different.

Thus, the esterification of *R*(−)- and *S*(+)-ketoprofen with *n*-propanol using anhydrous isooctane as organic solvent was catalysed by the crude enzyme as well as by the treated preparations (see Section 2). The results are shown in Table 3. The enzymatic activity in the esterification of both enantiomers has been quantified by means of the specific activity, obtained through the initial reaction rate calculated by fitting the progress curve [ester concentration (mM) vs. time (h)] to first-order kinetics, and dividing by the enzyme amount (expressed in hydrolytic units). On the other hand, the ratio of the specific activities for both enantiomers ($S/R$) was used to quantify the enzymatic enantioselectivity.

As can be seen from Table 3, the specific activity of CRL (L85) is slightly higher than that obtained with CRL (L54) (entry 2 vs. 1), although the $S/R$ ratio remained unaltered. The *R*-stereopreference observed does not agree with published data [4,21].

Table 3
Esterification of $S(+)$ or $R(-)$-ketoprofen by CRL and treated lipases in the absence of water

| Entry | Lipase | $S(+)$-Ketoprofen | | $R(-)$-Ketoprofen | | $S/R$[a] |
|---|---|---|---|---|---|---|
| | | Specific activity[b] | $\chi$[c] | Specific activity[b] | $\chi$[c] | |
| 1 | CRL (L 54)[d,e] | 6.9 | 5 | 11.5 | 9 | 0.6 |
| 2 | CRL (L85)[f,g] | 17.1 | 8 | 28.9 | 17 | 0.6 |
| 3 | SA-CRL[h,e] | 15.3 | 7 | 15.2 | 5 | 1.0 |
| 4 | E-CRL[i,e] | 15.9 | 2 | 28.1 | 7 | 0.6 |
| 5 | 1P-CRL[j,e] | 27.6 | 6 | 30.7 | 8 | 0.9 |
| 6 | 2P-CRL[k,e] | 30.2 | 8.5 | 26.1 | 7.5 | 1.2 |
| 7 | A-CRL[l,g] | 20.4 | 5 | 18.2 | 5 | 1.1 |
| 8 | M-CRL[m,g] | 22.5 | 5 | 20.7 | 5 | 1.1 |
| 9 | 2B-CRL[n,g] | 41.3 | 7.5 | 14.2 | 3 | 2.9 |
| 10 | 1B-CRL[o,g] | 18.1 | 3 | 22.1 | 3 | 0.8 |

[a] Ratio between specific synthetic activities obtained with each enantiomer.
[b] Specific ester synthetic activity (mM $U^{-1}$ $h^{-1}$) $\times 10^7$.
[c] Reaction yield (%) at 200 h.
[d] Crude lipase from *C. rugosa*, lot 54HO260 from Sigma.
[e] Obtained by treating Lot 54.
[f] Crude lipase from *C. rugosa*, lot 85HO629 from Sigma.
[g] Obtained by treating Lot 85.
[h] 60% ammonium sulphate on CRL.
[i] 40% (v/v) ethanol on CRL.
[j] 30% (v/v) 1-propanol on CRL.
[k] 40% (v/v) 2-propanol on CRL.
[l] 30% (v/v) acetone on CRL.
[m] 30% (v/v) methanol on CRL.
[n] 30% (v/v) 2-butanol on CRL.
[o] 60% (v/v) 1-butanol on CRL.

This lack of reproducibility in the stereopreference cannot be related either to the relative proportion of isoenzymes (tested to be around 80% Lipase A and 20% Lipase B in the commercial preparations used [4,22]) or to the different protein content (similar in all cases, according to the suppliers) of the commercial preparations, but rather is caused by the different hydration degree of the enzymatic preparations, as would be later confirmed. Nevertheless, it is clear from Table 3 that the $S/R$ ratio is increased when the esterification is catalysed by treated CRL (with ammonium sulphate or organic solvents) because the specific activity in the esterification of $S(+)$-ketoprofen is increased in a higher extent than for the $R(-)$ counterpart. It is known that crude CRL contains several protein contaminants, some of them being hydrolases [23,24]. These enzymes may be present at different proportions in the commercial preparations, but the treatment with organic solvents does not remove these enzymes, as it has been demonstrated [12] by SDS-PAGE electrophoresis.

Thus, in order to correlate this reversal in the stereopreference observed upon the treatments on crude CRL with the hydration degree of the samples, the esterification of $R(-)$- and $S(+)$-ketoprofen was carried out by increasing the water content of the reaction medium. This strategy was based on some previous results published in which we had stated the increase in the yield and enantioselectivity of crude CRL upon the addition of small water amounts [25].

### 3.4. Water sorption isotherm

In fact, it is widely known that the presence of water in the reaction medium affects the enzymatic activity [26]. In our reaction system, the water activity will depend on the nature of the biocatalyst

(heterogeneity of the commercial preparations), the nature of the treatment employed (solvent or ammonium sulphate), and the nature of the solvent used (because of their different affinity for the water). In a previous study, we have shown the usefulness of water sorption isotherms as a tool for measuring and controlling the reaction performance [27].

In Fig. 1, we show the water sorption isotherm of several enzymatic preparations: commercial enzyme (CRL), acetone-treated CRL (A-CRL) and CRL treated with ammonium sulphate (SA-CRL). We have used these treated samples to compare the different shapes of the water sorption isotherms. The water sorption isotherms were performed with the system (biocatalyst + isooctane) because we have previously shown [16,28] that the isotherms of pure components cannot be used to describe the hydration level of the microenvironment of the solid biocatalyst. We can see that the profiles of all the isotherms are similar for low water contents. The addition of 5 μl of water to the reaction system leads to an $a_w$ value around 0.5. We have chosen this initial $a_w$ value in order to start all the esterifications from the same hydration state for all the systems; this similar starting point is absolutely necessary for comparison due to the fact that the enzymatic preparations possess different initial $a_w$ values; therefore, the initial rate would be affected. For $a_w$ values higher than 0.5, the curve profiles are different because of the hydrophobicity of the preparations. Similar qualitative results were obtained with the other preparations of CRL (data not show).

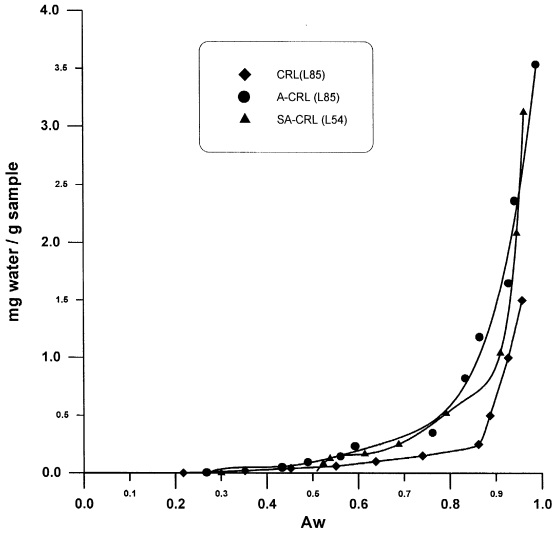


Fig. 1. Water sorption isotherms of dry commercial lipase lot 85 CRL (L85), lipase-treated with acetone (A-CRL) and lipase-treated with ammonium sulphate (SA-CRL).

### 3.5. Esterification of S(+)- or R(−)-ketoprofen by crude and treated lipase in presence of water

In Table 4, we show the results obtained in the esterification of both ketoprofen enantiomers with crude and treated CRL, adding 5 μl of water to the medium. This addition increases the initial water activity up to a value of around 0.5 for all the preparations. As can be seen, crude CRL (entries 1 and 2) displays now an *S*-enantiopreference, and this stereobias is always maintained in all cases with treated CRL. In the literature, as we mentioned before, the *S*-stereopreference of CRL in the recognition of the enantiomers of ketoprofen has been always reported [4,21,25]. We have shown here that the presence of a small water amount in the reaction system is necessary for displaying this behaviour clearly. The increase of the $(S/R)$ ratio is caused by the remarkable increase in the specific activity in the esterification of $S(+)$-ketoprofen, while the tendency for the $R(-)$ antipode is not that clear. Furthermore, the yield is dramatically increased for the esterification of $S(+)$-ketoprofen as can be seen in Table 4, so that the enhancement observed in the catalytic behaviour of CRL upon the treatment is reflected both in the reaction yield as well as in the enantioselectivity.

The control of the water activity of the system is allowing to show a similar enantiobias for all the treated preparations, as can be seen in Fig. 2, so we can conclude that the change in the enantioselectivity observed upon changing to slightly hydrated isooctane from a dry system in the esterification catalysed by the treated enzymes is pointing towards a 'water-mimicking' effect, because we are obtaining similar $S/R$ values both with the (crude enzyme + water) catalyst and with the treated preparations without adding any water. Nevertheless, when we combine the effect of the semipurification with the addition of a small water amount, a synergetic effect is obtained, increasing the enzymatic activity and stereoselectiv-

Table 4
Esterification of *S*(+) or *R*(−)-ketoprofen by CRL and treated lipases in presence of water (5 μl)

| Entry | Lipase | *S*(+)-Ketoprofen | | *R*(−)-Ketoprofen | | *S*/*R*[a] |
|---|---|---|---|---|---|---|
| | | Specific activity[b] | $\chi$[c] | Specific activity[b] | $\chi$[c] | |
| 1 | CRL (L 54)[d,e] | 47.7 | 27.5 | 12.2 | 9.0 | 3.9 |
| 2 | CRL (L 85)[f,g] | 21.3 | 15 | 19.2 | 11.5 | 1.2 |
| 3 | SA-CRL[h,e] | 83.8 | 45 | 10.6 | 8.5 | 7.9 |
| 4 | E-CRL[i,e] | 79.3 | 41 | 12.4 | 10 | 6.4 |
| 5 | 1P-CRL[j,e] | 96.2 | 45 | 11.1 | 9 | 8.7 |
| 6 | 2P-CRL[k,e] | 109 | 49 | 15.8 | 12 | 6.9 |
| 7 | A-CRL[l,g] | 183 | 51 | 15.1 | 7 | 12.1 |
| 8 | M-CRL[m,g] | 92.3 | 39 | 37.2 | 15 | 2.5 |
| 9 | 2B-CRL[n,g] | 172 | 47 | 29.4 | 11.5 | 5.8 |
| 10 | 1B-CRL[o,g] | 117 | 40.5 | 28.9 | 15 | 4.1 |

[a] Ratio between specific synthetic activities obtained with each enantiomer.
[b] Specific ester synthetic activity (mM $U^{-1}$ $h^{-1}$) $\times 10^7$.
[c] Reaction yield (%) at 200 h.
[d] Crude lipase from *C. rugosa*, lot 54HO260 from SIGMA.
[e] Obtained by treating Lot 54.
[f] Crude lipase from *C. rugosa*, lot 85HO629 from Sigma.
[g] Obtained by treating Lot 85.
[h] 60% ammonium sulphate on CRL.
[i] 40% (v/v) ethanol on CRL.
[j] 30% (v/v) 1-propanol on CRL.
[k] 40% (v/v) 2-propanol on CRL.
[l] 30% (v/v) acetone on CRL.
[m] 30% (v/v) methanol on CRL.
[n] 30% (v/v) 2-butanol on CRL.
[o] 60% (v/v) 1-butanol on CRL.

ity. Furthermore, with this last methodology, we do find clear differences in the behaviour of the treated enzymes, showing a clear maximum (shown in Fig. 2) when the semipurification is carried out using solvents with log *P* values ranging between 0.1 and 0.6. As we stated before, the treatment with organic solvents does not seem to be removing low-weight protein contaminants which are described to be hydrolases [23,24], so the enantioselectivity increase cannot be attributed to the removal of esterases possessing opposite enantioselectivity. What is more, because the activities are measured considering specific activities (i.e., we are expressing the results dividing by the enzymatic units used in each case), this enhancement in the esterification cannot be related either to a single semipurification process, so we must conclude that there must be another cause to explain the better enzymatic performance.

In the literature, it has been described [9,11] that organic solvents may convert the closed form of CRL (whose lid is covering the active site) into the open one (whose active site is exposed to the media, as the lid is open). The opening of the lid requires a *cis* to *trans* isomerisation of a prolyl amide at residues Ser91–Pro92 [9], and organic solvents have been described to accelerate the *cis–trans* isomerisation of prolyl-amides residues [29]. Therefore, the higher specific activity and the increased reaction yield obtained with the organic solvent-treated preparations are consistent with a more accessible active site in the open form.

Another possible explanation for the enhanced enzymatic activity can be found in literature. In fact, it has been reported that water plays the role of a 'molecular lubricant' in enzymes [30], and it has been also proposed that water mimics acts by a similar mechanism, i.e., increasing protein flexibility by the creation of multiple hydrogen bonds with the enzyme in anhydrous organic solvents [31]. Therefore, the increased activity observed with our treat-

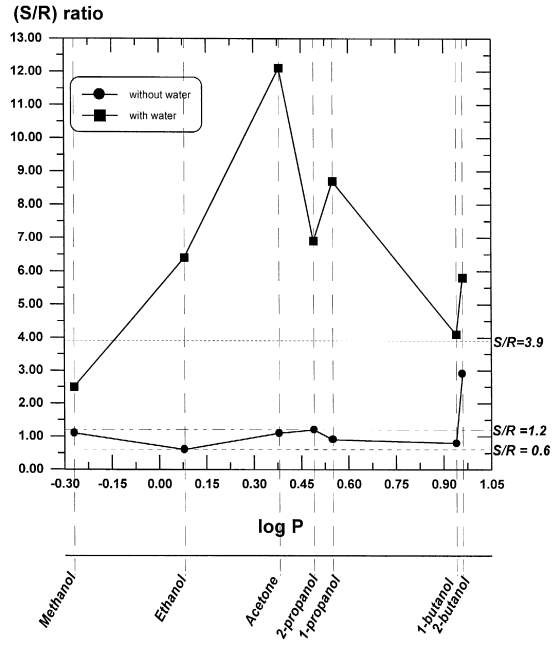


Fig. 2. Variation of $(S/R)$ ratio with log $P$ values of different solvents in crude and treated lipases with and without addition of water.

ment could be related to similar effects by the retention of some molecules of the solvent used for purification in the enzyme microenvironment after the lipophilisation, which are acting as water mimics when the treated lipase is placed in isooctane, as it has been described for subtilisin, α-chymotrypsin and *Rhizomucor miehei* lipase [32]. What is more, the increase not only in activity but also in enantioselectivity for subtilisin upon acetone and isopropanol treatment in transesterification in dioxane has also been reported [33], although this increase is smaller than that presented (Fig. 2) in this work for CRL.

## 4. Conclusions

Crude enzymatic preparations marketed by different commercial origin show an irreproducible behaviour due to multiple reasons. Following a simple and quick treatment on crude lipase of *C. rugosa* with different organic solvents, we can obtain enzymatic preparations with higher and reproducible activity and stereoselectivity. As a second step, the control of the water activity of the reaction media allows an even superior enhancement of the catalytic performance of CRL upon a substrate (ketoprofen) that is poorly esterified by the crude enzyme.

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