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Esterase activity of biocomposites constituted by lipases adsorbed on layered zirconium phosphate and phosphonates: selective adsorption of different enzyme isoforms

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Abstract

Aqueous solutions of crude extract of lipase from *Candida rugosa* (C-CRL) and of a semipurified material obtained by treating C-CRL with propan-2-ol (PT-CRL) were used as a source of biomaterial for its adsorption onto the surface of layered micro-crystals of α-zirconium phosphate $Zr(HPO_4)_2$ and phosphonates such as $Zr(C_6H_5PO_3)_2$, $Zr(HPO_4)(C_6H_5PO_3)$ and $Zr(HOOCCH_2PO_3)(HPO_4)$, which possess groups with different hydrophobic character anchored to the inorganic matrix. Several biocomposites have been obtained by changing the temperature and the time of equilibration of the various supports with the C-CRL and PT-CRL solutions. The biocomposites have shown different esterase activities and enantio-selectivities in the hydrolysis of *p*-nitrophenylacetate (*p*-NPA), ethyl butyrate and (±)-methyl-2-(4-chlorophenoxy) propionate as a function of the nature of the support and of the time and temperature of equilibration. These results have been interpreted on the basis of a selective adsorption of different isoforms of the enzyme. The biocomposites can be stored for more than 1 month at 4 °C and can be used for several cycles without a significant decrease in catalytic activity.

Keywords: Lipase; Enzyme immobilization; Zirconium phosphates; Zirconium phosphonates; Adsorption

1. Introduction

The immobilization of enzymes on insoluble supports is an interesting topic of research in enzyme technology, especially for industrial applications [1,2]. The supports employed and the methods of immobilization used are chosen so as to ensure the highest retention of enzyme activity, stability and durability. Among several immobilization techniques that can be used, adsorption seems to be the most suitable because it is simple, inexpensive and induces fewer modifications in the active conformation of the enzyme [3,4].

Lipases are interesting enzymes because of their versatility in catalyzing hydrolysis and esterification reactions with extremely simple processes, the superior quality and high yields of the final products [5–7]. Therefore, research on lipase immobilization has increased [8].

In a recent paper, we showed that layered zirconium phosphates and phosphonates are effective supports of *Candida rugosa* lipase (CRL) and, in some instances, the obtained biocomposites contained large amounts of protein adsorbed onto the surface of

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the micro-crystals which showed good catalytic activity towards the hydrolysis of *p*-nitrophenylacetate (*p*-NPA) [9]. It was also observed that the uptake capacity of the supports, as well as the catalytic activity of the biocomposites, were strongly affected by the chemical characteristics of the surfaces of the micro-crystals. Based on those results, it seemed of interest to explore the possibility of selectively immobilizing different isoforms of lipase by exploiting the different surface characteristics of layered zirconium phosphates and phosphonates.

Several lipase isoforms have been described in many organisms. These isoenzymes may behave differently when catalyzing organic synthesis. In fact, very slight differences in conformation (physical–chemical properties) can cause significant differences in the activity and stereospecificity towards different substrates [10,11]. However, it may be extremely difficult to separate these isoforms by using conventional chromatographic techniques due to their structural similarities. The possibility of separating, or at least enriching, different isoforms by selective adsorption and immobilization on supports can be achieved by taking into account the nature of the enzyme–support interactions (ionic, hydrophobic, Van der Waals, etc.) and the different rates and intensities of adsorption obtained by varying the experimental conditions of the uptake process such as equilibration time, pH, ionic strength and temperature of the equilibrating solutions [12–14].

Layered zirconium phosphates and phosphonates are very insoluble, have good chemical, thermal and mechanical resistance and are easy to handle when designing and preparing solids with various functionalities. Different types of layered phosphates and phosphonates are known and differ according to the structure of the layers [15,16]. The most investigated ones are indicated with the Greek prefix alpha and have the general formula $Zr(RPO_3)_2$, R being H, OH or a functional organic group. The layered structure arises by the packing of lamellae, each constituted by a plane of Zr(IV) atoms sandwiched between tetrahedral O_3P–R groups placed alternately above and below the metal plane. In many cases, compounds can be obtained which have two different functional groups pointing towards the interlayer region and a general formula $Zr(RPO_3)_{2-x}(R'PO_3)_x \cdot nS$. Therefore, these materials provide many advantages if used as supports for lipases. One advantage is the possibility of functionalizing the layers with a large variety of organic groups (alkyl, aryl, carboxilic, aminoacidic, sulphophenyl, ...) which changes the hydrophobic/hydrophilic character of the surface [16]. This allows different interactions with amino-acid residues of proteins, which may lead to the binding of isoenzymes with different surface characteristics.

The present paper describes the selective immobilization of isoforms of *C. rugosa* lipase via adsorption onto layered zirconium phosphate and onto various zirconium phosphonates focusing on the experimental parameters, like temperature and time of adsorption.

The lipases used for the immobilization were obtained from the crude extract (C-CRL) and by treating C-CRL with propan-2-ol (PT-CRL).

The catalytic activity of the biocomposites obtained was assayed in the hydrolysis reactions of *p*-nitrophenylacetate and ethyl butyrate.

The different biocomposites were also used to catalyze the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate in order to examine the effects of immobilization on the enantioselective ability of CRL.

2. Experimental

2.1. General

Lipase from *C. rugosa* (crude CRL E.C.1.1.13, type VII) was purchased from Sigma Chemical Co. Coomassie Brilliant Blue G-250 was purchased from Bio-Rad. Ethyl butyrate, *p*-nitrophenol and raceme 2-(4-chlorophenoxy) propionic acid were obtained from Aldrich Chemical Co. Racemic methyl 2-(4-chlorophenoxy) propionate was prepared as described in [17]. All the organic solvents were of reagent grade and used without further purification.

^{1}H NMR spectra were recorded in $CDCl_3$ solution on a Bruker AC 200 MHz spectrometer. The enantiomeric excesses (e.e.) of methyl esters (−)-*S* **3** were determined by ^{1}H NMR spectroscopy by using Europium tris[3-(heptafluoropropylhydroxymethylene)-(+)camphorate] as chiral shift reagent. The e.e. of acids (+)-*R* **2** were determined on corresponding methyl esters obtained by treatment with CH_2N_2.

2.2. Preparation of the supports

Layered zirconium phosphates and phosphonates were prepared according to the method of the slow decomposition of zirconium fluoro-complexes in the presence of phosphoric and/or phosphonic acids [18]. Details of the synthesis, as well as the physical–chemical properties, are reported in [9].

2.3. Preparation of C-CRL and PT-CRL solutions

C-CRL solution was prepared as described in [9]. PT-CRL was prepared by treating C-CRL with propan-2-ol according to the procedure reported in [17].

2.4. Protein assay of CRL solutions

The protein concentrations of the C-CRL and PT-CRL solutions were measured using the Bradford method [19].

2.5. Free CRL activity assay

In a typical experiment, one ml of *p*-nitrophenylacetate solution (0.1 M in CH_3CN) or one ml of ethyl butyrate ($d = 0.8$ g/ml) was added to 10 ml of phosphate buffer (10 mM, pH 7.2). An aliquot of C-CRL or PT-CRL solution was added to the mixture that was maintained under vigorous stirring. As the hydrolysis proceeded, acetic or butyric acid formed in solution were continuously titrated with a 0.02 M NaOH solution by using the Autotitrator Mettler Toledo DL21.

One lipase unit (U) has been defined as the amount of enzyme required to liberate 1 μmol of acetic or butyric acid per minute, under the assay conditions.

2.6. CRL immobilization

Sixty milligrams of the chosen micro-crystals were placed in contact with 4 ml of C-CRL solution ($c =$ 0.5 mg/ml) or PT-CRL solution (0.1 mg/ml) in a sealed vessel and stirred for 1.5 h (or 16 h) at 4 °C (or 22 °C). The suspension was then centrifuged at 3000 rpm for 10 min and the supernatant was separated from the solid. The solid with the immobilized enzyme was washed with distilled water (2×2 ml), the suspension centrifuged at 3000 rpm for 10 min and the supernatant separated from the solid.

The supernatants were collected and the protein concentration determined according to Bradford's method. The amount of bound protein was determined indirectly by calculating the difference between the amount of protein introduced into the reaction mixture and the amount of protein that remained in the supernatants.

Residual free activity of the supernatant was measured as described above.

Activity loading (U/g support) was determined by subtracting the residual free activity (after immobilization) from the original free lipase activity (before immobilization).

2.7. Hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**) in water in the presence of free C-CRL

In a typical experiment (Table 5, entry 1), 12 ml of C-CRL solution (55 U with *p*-NPA assay) in phosphate buffer (20 mM, pH 7.2) were stirred for 15 min at room temperature and then added to substrate **1** (214 mg, 1 mmol). The mixture was stirred and the pH was maintained at 7.2 by automatic titration with NaOH 0.2 M. When the hydrolysis reached 67% conversion, a saturated solution of NaCl (15 ml) was added to the reaction mixture. The pH was adjusted to 2 using HCl 6N and then the mixture was extracted with ethyl 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 ester (−)-*S* **3**: 63 mg (90% yield), e.e. 34%.

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 (+)-*R* **2**: 114 mg (85% yield), e.e. 17%.

2.8. Hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**) in water in the presence of free PT-CRL

In a typical experiment (Table 6, entry 1), 12 ml of PT-CRL solution (250 U with *p*-NPA assay) in phosphate buffer (20 mM, pH 7.2) were stirred for 15 min

at room temperature and then added to substrate **1** (214 mg, 1 mmol). The mixture was stirred and the pH was maintained at 7.2 by automatic titration with NaOH 0.2 M. When the hydrolysis reached 45% conversion, the reaction mixture was worked up as described above and afforded (−)-*S* **3**: 106 mg (90% yield), e.e. 67% and (+)-*R* **2**: 76 mg (85% yield), e.e. 83%.

2.9. Hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (*1*) in water in the presence of immobilized C-CRL or PT-CRL

In a typical experiment (Table 5, entry 2), 180 mg of bio-composite was added to 12 ml of phosphate buffer (20 mM, pH 7.2) and stirred for 15 min at room temperature. The mixture was then added to substrate **1** (214 mg, 1 mmol), stirred and the pH was maintained at 7.2 by automatic titration with NaOH 0.2 M. When the hydrolysis reached 33% conversion, the reaction mixture was filtered. The biocatalyst was washed with distilled water (2 × 2 ml) and ethyl ether(1 × 1 ml). The reaction mixture was worked up as described above and afforded (−)-*S* **3**: 128 mg (90% yield), e.e. 24% and (+)-*R* **2**: 56 mg (85% yield), e.e. 48%.

2.10. Repeated hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (*1*) in water in the presence of PT-CRL immobilized on α-ZrP-BP

The operational stability of the immobilized PT-CRL was assayed using 240 mg of biocatalyst dispersed in 10 ml of phosphate buffer (10 mM, pH 7.2). The suspension was added to substrate **1** (214 mg, 1 mmol), stirred at room temperature and the pH was maintained at 7.2 by automatic titration with NaOH 0.2 M. When the hydrolysis reached the conversion shown in Table 7, the reaction mixture was centrifuged at 3000 rpm for 10 min and the supernatant separated from the solid. The immobilized enzyme was then washed with distilled water (2 × 2 ml) and ethyl ether (1 × 1 ml), the suspension centrifuged at 3000 rpm for 10 min and the supernatant separated from the solid which was reused for the successive experiment. The supernatants containing the reaction mixture were collected and worked up as described above.

In a separated experiment, we tested the supernatants for the presence of protein leaching from the support. The absence of the activity in the supernatant and the negative Bradford's test indicate that the enzyme is not leached from the support.

3. Results and discussion

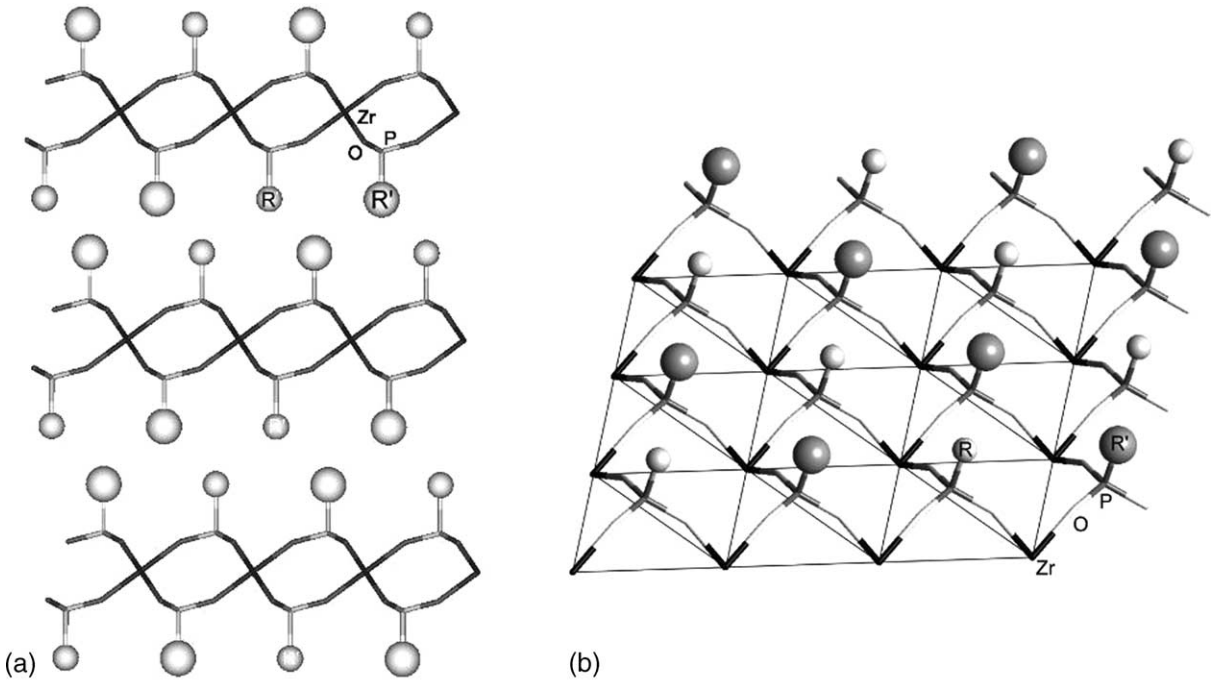
3.1. Immobilization of C-CRL on different zirconium phosphates and phosphonates: effect of the hydrophobic/hydrophilic character of the support

We selected zirconium phosphates and phosphonates that had different functional groups on the surface in order to evince their ability to recognize CRL isoenzymes and to preferentially take up the isoenzymes that interact better with the surface groups of a given support. The chemical name and acronym of the supports chosen, as well as their structural characteristics, are illustrated in Fig. 1.

According to the surface properties of the various supports, different protein-support interactions can be hypothesized. For example, when considering hydrophilic materials like α-ZrP and α-ZrP-CEP that can transfer protons to basic centers, ionic interactions may be established between the protein and the support. Hydrophobic interactions are also possible when using α-ZrP-BP but only hydrophobic interactions are envisaged for α-ZrBP.

We decided to use two different substrates to measure the immobilized catalytic activity in order to exploit the possible selectivity of the supports towards CRL isoforms. The selected test reactions were the hydrolysis of carboxylic acid esters, such as *p*-NPA and ethyl butyrate, which have different chain lengths and different sizes [20].

Table 1 reports the amount of protein immobilized on the different supports and the catalytic activity of the biocomposites towards the two test reactions. It can be seen that lipases, that are highly hydrophobic compared to of conventional proteins, show the maximum affinity for the supports that have the most hydrophobic groups (Table 1, entries 3 and 4). With regard to the catalytic activity, 48% of the esterase activity (with *p*-NPA as substrate) was immobilized on α-ZrBP, while 28% was adsorbed onto α-ZrP-CEP



R	R'	X	Support	Acronym
OH	OH	0	α-Zirconium phosphate	α-ZrP
Ph	OH	0.38	α-Zirconium phosphate benzenphosphonate	α-ZrP-BP
CH_2CH_2OH	OH	0.30	α-Zirconium phosphate carboxyethanphosphonate	α-ZrP-CEP
Ph	Ph	0	α-Zirconium benzenphosphonate	α-ZrBP

Fig. 1. Schematic representation of the layered structure of $Zr(RPO_3)_{2-x}(R'PO_3)_x$ micro-crystals. (a) Sequence of the layers of the structure. (b) View of the surface of one side of the layer. The table reports the chemical composition and the acronym of the investigated supports.

Table 1
Immobilization of C-CRL on different zirconium phosphates and phosphonates[a]

Entry	Support	Immobilized protein (%)[b]	Immobilized activity (%)[c]	
			p-Nitrophenylacetate	Ethyl butyrate
1	α-ZrP	42	14	42
2	α-ZrP-CEP	42	28	42
3	α-ZrP-BP	74	41	52
4	α-ZrBP	78	48	46

[a] Protein concentration 0.5 mg/ml, 4 °C, 16 h, pH 4.0.
[b] Calculated from the protein remaining in solution after immobilization (determined by Bradford method).
[c] Calculated from the activity remaining in solution after immobilization.

and only 14% onto α-ZrP. These different percentages of immobilized activity indicate that, among the CRL isoforms, those that recognize *p*-NPA as substrate are preferentially adsorbed onto α-ZrBP. In contrast, the immobilized activity determined with ethyl butyrate as substrate was very similar in all cases. We may conclude that the different isoforms have the same enzymatic activity in the hydrolysis of ethyl butyrate and therefore this reaction is not suitable for demonstrating selective adsorption. However, the overall results indicate that this kind of immobilization allows biocomposites to be obtained that have different catalytic properties depending on the support used.

3.2. Immobilization of C-CRL on α-ZrP-BP at different equilibration times

Among the various supports, we selected α-ZrP-BP, characterized by the presence of hydrophilic and hydrophobic groups on the surface. The hydrolysis of *p*-NPA was selected as the test reaction to study the effect of the experimental conditions on the uptake of protein and on the activity of the biocomposites. When the immobilization of C-CRL on α-ZrP-BP was obtained after equilibration for 1.5 h at 4 °C, the bound protein was 40% of the total amount offered to the support, while the immobilized activity was 32% (see Table 2, entry 1).

By increasing the contact time between the protein solution and the support to 16 h, the bound protein reached 74% of total amount, while the immobilized activity was similar to the previous value. The isoforms that recognized *p*-NPA as substrate were immobilized very rapidly on α-ZrP-BP probably due to their great affinity for the hydrophobic support [12].

Table 2
Effect of the immobilization time on C-CRL adsorption on α-ZrP-BP[a]

Entry	*t* (h)	*T* (°C)	Immobilized protein (%)[b]	Immobilized activity (%)[c]
1	1.5	4	40	32
2	16	4	74	41

[a] Protein concentration 0.5 mg/ml, 4 °C, pH 4.0.
[b] Calculated from the protein remaining in solution after immobilization (determined by Bradford method).
[c] Calculated from the activity remaining in solution after immobilization (determined by hydrolysis of *p*-NPA).

Table 3
Effect of temperature on C-CRL adsorption onto α-ZrP-BP[a]

Entry	*t* (h)	*T* (°C)	Immobilized protein (%)[b]	Immobilized activity (%)[c]
1	1.5	4	40	32
2	1.5	22	70	28

[a] Protein concentration 0.5 mg/ml, 1.5 h, pH 4.0.
[b] Calculated from the protein remaining in solution after immobilization (determined by Bradford method).
[c] Calculated from the activity remaining in solution after immobilization (determined by hydrolysis of *p*-NPA).

Successively, proteins which lacked *p*-NPA activity or that had minor affinity for the α-ZrP-BP surface also bonded to the support.

3.3. Immobilization of C-CRL on α-ZrP-BP at different temperatures

Temperature is another parameter that must be controlled during immobilization because it can influence the stability and conformational structure of proteins. The temperature may influence adsorption driven by hydrophobic interactions. At higher temperatures proteins tend to expose their hydrophobic amino-acid residues on the surface. The hydrophobic interactions with the support should increase resulting in a greater adsorption capacity of the support [21].

As can be seen in Table 3, the protein loading on α-ZrP-BP at 22 °C was higher than that observed at 4 °C. In other respects, the esterase activity immobilized at these different temperatures was about the same. The affinity of isoforms with esterase activity for the α-ZrP-BP surface seems to be independent of the temperature at which the immobilization process was carried out.

3.4. Effect of CRL purification on the immobilization onto different zirconium phosphates and phosphonates

Recently, we reported that C-CRL treated with simple aliphatic alcohols, such as propan-2-ol, produces a biomaterial (PT-CRL) that has greatly modified catalytic properties towards the hydrolysis of methyl esters of 2-phenoxy-propionic acids [7]. The explanation given was that the enzyme present in these lipolytic preparations changes its native conformation

Table 4
Immobilization of PT-CRL on different zirconium phosphates and phosphonates[a]

Entry	Support	Immobilized protein (%)[b]	Immobilized activity (%)[c]	
			p-Nitrophenylacetate	Ethyl butyrate
1	α-ZrP	40	35	20
2	α-ZrP-CEP	30	26	7
3	α-ZrP-BP	46	17	17
4	α-ZrBP	49	11	25

[a] Protein concentration 0.1 mg/ml, 4 °C, 16 h, pH 4.0.
[b] Calculated from the protein remaining in solution after immobilization (determined by Bradford method).
[c] Calculated from the activity remaining in solution after immobilization.

and assumes an open-form by moving the flap at the binding site. In particular, the flap at the binding site moves and arranges itself in an open-form which determines the geometry of the active site, allowing one substrate to be recognized more easily than another. We therefore, decided to use PT-CRL biomaterial as the source of protein to be immobilized on different zirconium phosphates and phosphonates and to study the effect of the treatment of C-CRL on the affinity of the different isoforms for the supports and on the catalytic properties of the biocomposites obtained.

PT-CRL has a lower affinity for supports like α-ZrBP and α-ZrP-BP, when compared to C-CRL (compare Table 4 with Table 1, entries 3 and 4). Furthermore, the loading of esterase activity (with *p*-NPA as substrate) decreased as the hydrophobic character of the support increased. In fact, 35% of the activity was adsorbed onto α-ZrP, while only 11% were immobilized on α-ZrBP.

On the basis of these results, we assume that the treatment of the crude extract with propan-2-ol affects the protein conformation, leading to a general decrease in the affinity of the isoforms for a given support. The PT-CRL immobilization on zirconium phosphates and phosphonates leads to biocomposites with catalytic properties that are different from those obtained with C-CRL.

3.5. Effect of selective adsorption on different zirconium phosphates and phosphonates on the stereoselective properties of CRL

The use of differently immobilized derivatives has been shown to be a valid method for modulating the activity and selectivity of lipases [22,23].

The catalytic behavior of CRL immobilized on different zirconium phosphates and phosphonates was compared with that of a free enzyme in the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (±)-**1** (see Fig. 2). This substrate was chosen because the acids obtained, (+)-*R* **2** and (−)-*S* **2,** are of great biological interest [24].

The hydrolysis of (±)-**1,** catalyzed by free C-CRL, was fast but the enantioselectivity was disappointing (Table 5, entry 1). The immobilized C-CRL showed similar reactivity and enantioselectivity towards (±)-**1** (Table 5, entries 2, 4, 6, and 8) and no significant differences in the enantioselectivity were observed when the supernatant solution, recovered from the immobilization procedure, was used to catalyze the hydrolysis (Table 5, entries 3, 5, 7, and 9).

These results suggest that all C-CRL isoforms are able to catalyze the hydrolysis of (±)-**1** with low enantioselectivity, hence the selective adsorption onto zirconium phosphates and phosphonates does not change the enantioselective properties.

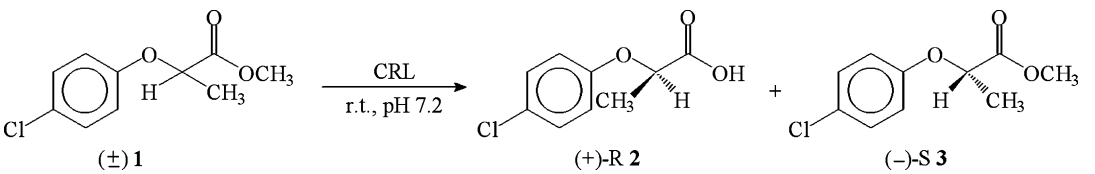


Fig. 2. Catalytic behavior of CRL.

Table 5
Enantioselectivity of C-CRL immobilized on different zirconium phosphates and phosphonates in the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**) in water

Entry	Catalyst	*t* (h)	*C* (%)[a]	e.e. (**2**)	e.e. (**3**)	*E*[b]
1	C-CRL free	0.7	67	17	34	2
2	C-CRL/α-ZrP	1.5	33	48	24	4
3	Supernatant	0.5	54	31	36	3
4	C-CRL/α-ZrP-CEP	1.0	55	48	58	5
5	Supernatant	1.5	51	32	33	3
6	C-CRL/α-ZrP-BP	2.0	53	46	52	4
7	Supernatant	1.5	52	38	41	3
8	C-CRL/α-ZrBP	5.0	44	32	25	2
9	Supernatant	3.0	59	27	39	3

[a] Conversion of reaction.
[b] Enantioselectivity factor [25].

On the other hand, PT-CRL is an efficient catalyst in the hydrolysis of (±)-**1**, which has been resolved with good eantioselectivity (Table 6, entry 1). It has been found that the enantioselectivity of PT-CRL can be modulated by immobilization on zirconium phosphates and phosphonates. The α-ZrP-CEP derivative showed an enantioselectivity that was slightly higher than that of free PT-CRL (Table 6, Entry 4), while a slight decrease in the *E*-value was observed when the supernatant was used (Table 6, entry 5).

With α-ZrP and α-ZrBP derivatives the enantioselectivity decreased when either the biocomposites or the supernatant solutions were used (Table 6, entries 2, 3, and 8, 9).

The immobilized derivative prepared on α-ZrP-BP showed the most significant results compared to the free PT-CRL (Table 6, entries 6 and 7). The enantioselectivity of the immobilized lipase increased from $E = 22$ to $E = 42$, while the *E*-value of free lipase present in the supernatant decreased to 9. This result could be explained considering the specific groups (hydrophilic/hydrophobic) present on the surface of α-ZrP-BP which selectively immobilize the CRL isoforms that have the best enantioselectivity in the hydrolysis of (±)-**1**, while leaving the isoforms that have the worst enantioselectivity in the supernatant.

Furthermore, the binding of protein and support seems to be strong because the enantioselectivity of

Table 6
Enantioselectivity of PT-CRL immobilized on different zirconium phosphates and phosphonates in the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**) in water

Entry	Catalyst	*t* (h)	*C* (%)[a]	e.e. (**2**)	e.e. (**3**)	*E*[b]
1	PT-CRL free	1.3	45	83	67	22
2	PT-CRL/α-ZrP	1.0	27	87	31	15
3	Supernatant	1.0	52	63	79	9
4	PT-CRL/α-ZrP-CEP	1.0	48	82	76	24
5	Supernatant	1.5	54	64	76	10
6	PT-CRL/α-ZrP-BP	2.0	51	85	90	42
7	Supernatant	0.6	55	62	75	9
8	PT-CRL/α-ZrBP	3.0	27	80	29	11
9	Supernatant	1.5	51	71	74	13

[a] Conversion of reaction.
[b] Enantioselectivity factor [25].

Table 7
Enantioselectivity of PT-CRL immobilized on α-ZrP-BP in the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**) in successive cycles

No. of cycle	*t* (h)	*C* (%)[a]	e.e. (**2**)	e.e. (**3**)	*E*[b]
1	2.0	51	85	90	42
2	2.5	50	84	86	37
3	3.0	51	84	88	35
4	4.0	52	82	89	30
5	4.5	47	85	75	27

[a] Conversion of reaction.
[b] Enantioselectivity factor [25].

immobilized PT-CRL is similar even after repeated assays (Table 7).

Finally, Fig. 3 reports the results of a set of experiments designed to study the effect of treating CRL with propan-2-ol during and after the immobilization protocol.

In the first experiment (a), the immobilization of C-CRL on α-ZrP-BP was carried out in water/propan-2-ol medium for 16 h at 4 °C. The resulting biocomposite was used in the hydrolysis of (±)-**1**; an *E*-value of 9 was obtained.

In the second experiment (b), the immobilization of C-CRL was carried out as previously described (water, 16 h, 4 °C). The immobilized derivative was treated with water/propan-2-ol at 4 °C for 6 h and then used in the hydrolysis of (±)-**1**. The enantioselectivity increased from 4 to 9.

In the third experiment (c), after the adsorption of C-CRL onto α-ZrP-BP for 16 h at 4 °C, the derivative was used in the hydrolysis of (±)-**1** in water/propan-2-ol medium. The reaction was very slow but the enantioselectivity increased to $E = 9$.

In all the experiments carried out, the *E*-value surpassed that obtained with the C-CRL derivative immobilized on α-ZrP-BP ($E = 4$), but the value never reached that obtained with the immobilized PT-CRL

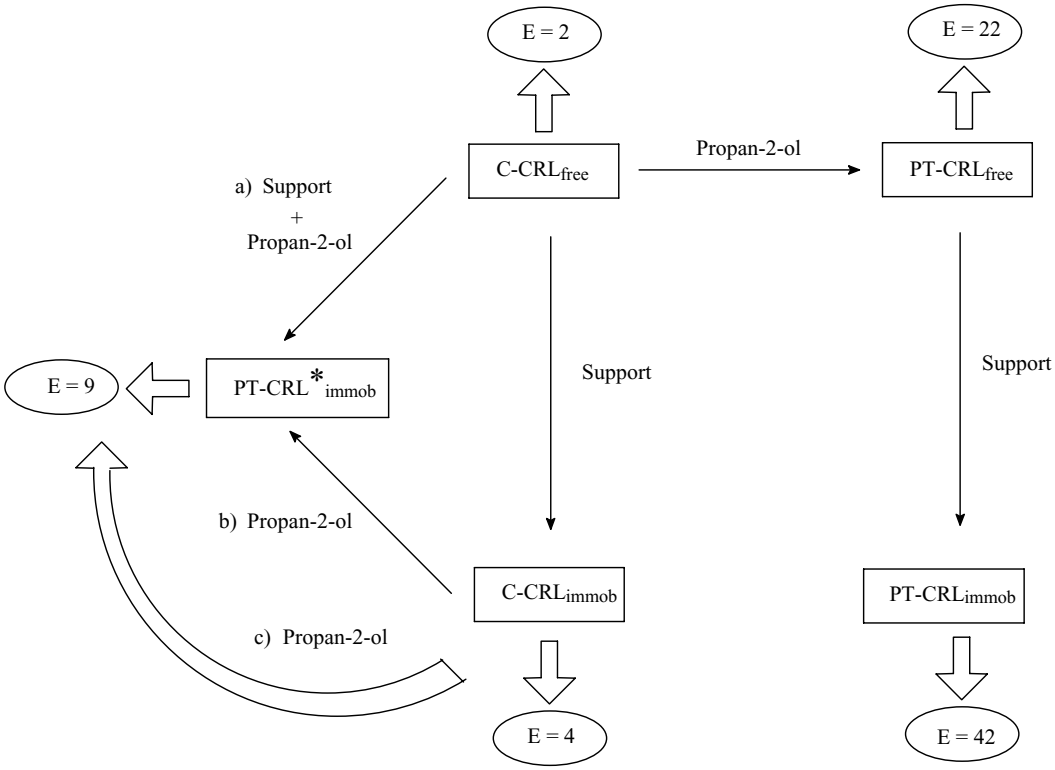


Fig. 3. The effect of propan-2-ol on the enantioselective properties of free and immobilized enzymes on α-ZrP-BP CRL in the hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate (**1**).

($E = 42$). These results suggest that the active protein is quickly immobilized on the support and then undergoes a structural change due to the effect of propan-2-ol with a consequent increase of enantioselectivity.

One possible explanation for this behavior is that the mechanism of immobilization involves the interaction between functional groups present on the surface of the support and functional groups from amino-acid residues on the surface of the enzyme far from the active site. In this way, the immobilized enzyme probably undergoes changes at the active site such as the free enzyme.

4. Conclusions

Layered zirconium phosphates and phosphonates have been shown to be an interesting new class of supports for CRL due to their versatility. Exploiting the surface characteristics of the supports by changing the nature of the functional groups and their distribution on the surface and by varying the experimental parameters such as temperature and time of contact, it has been possible to immobilize isoenzymes of CRL which have different catalytic properties.

In a simple and rapid way, we have prepared a new type of lipase derivatives that are very useful for catalyzing interesting reactions such as the enantioselective hydrolysis of chlorophenoxy propionates, compounds that are of great biological interest [24].

The immobilization of PT-CRL on α-ZrP-BP shows that the enzyme maintains a certain flexibility (less than that of free enzyme) even after it has been bound to the support.

The immobilized derivatives can be stored for more than 1 month at 4 °C without loss of enzymatic activity and can be used for several cycles without a significant decrease in the enantioselectivity of the biocatalyst.

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