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Journal of Molecular Catalysis B: Enzymatic 32 (2005) 67–76
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Acetylenic polymers as new immobilization matrices for lipolytic enzymes

F. Panzavolta, S. Soro*, R. D'Amato, C. Palocci, E. Cernia, M.V. Russo

Department of Chemistry, University of Rome "La Sapienza", P.le A. Moro, 5-00185 Rome, Italy

Received 21 November 2003; received in revised form 17 September 2004; accepted 29 September 2004
Available online 21 November 2004

Abstract

Polyphenylacetylene (PPA) was studied and characterized as a new matrix for *Candida rugosa* lipase (CRL) immobilization, establishing π interactions with the enzyme. Performances of the new biocatalytic system were compared to different hydrophobic matrices (cross-linked polyvinyl alcohol esterified with lauric acid—CL-PVA-C_{12}, commercial polypropylene—EP-100, commercial Sepabeads functionalized with C_{18} chains, treated and untreated with polyethyleneimine—C_{18}-SEPA-PEI and C_{18}-SEPA). Hydrophobic interactions between carriers and enzyme are the main forces involved. The immobilized enzyme showed a higher stability to *T* and pH changes and to organic solvents as media than the free one. Moreover, for transesterification reactions carried out in organic solvents, increased initial rate and enantioselectivity were observed for the immobilized enzyme compared to those of the free one. PPA, when compared to the other supports, showed a good performance in terms of activity and enantioselectivity suggesting a possible use as a new lipase carrier.

Keywords: *Candida rugosa* lipase; Immobilization; Hydrophobic; Polyphenylacetylene; Carrier

1. Introduction

Immobilization is very important in the industrial application of enzymes, as immobilized biocatalysts offer unique advantages in terms of better process control, enhanced stability, enzyme-free products, predictable decay rates, and improved economics. The methods used for immobilizing enzymes are varied in complexity and efficiency [1]. Mainly, they are based on physical adsorption of the enzyme on a carrier material, on its entrapment or microencapsulation in a solid support or on its covalent binding to a solid matrix.

The selection of an immobilization strategy is based on process specifications for the biocatalyst, which include parameters such as overall enzymatic activity, effectiveness of enzyme utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized biocatalyst. The procedures involved in physical adsorption are quite simple, making it one of the most widely used methods of enzyme immobilization. A wide number of carrier materials exist, fulfilling a wide range of requirements related to industrial applications. Mechanical strength, chemical, and physical stability, hydrophobic/hydrophilic character, enzyme loading capacity, recycling capacity and low cost are the most important characteristics for carrier classification.

Among the most used enzymes for industrial applications lipases are one of the most suitable and studied for immobilization. Initially, mineral supports such as porous glass beads [2,3], diatomaceous earth [4], silica [5,6], and alumina [7,8] were used. Today, the most used supports are ion exchange resins [9,10], organic and inorganic supports [11–14], and biopolymers [15–17]. In the most recent years, adsorption on carriers having the dimension of micro and nanoparticles has been carried out [18]. Since in protein adsorption matri-

* Corresponding author. Present Address: Department of Biochemical Science "A. Rossi Fanelli", University of Rome "La Sapienza", P.le A. Moro, 5-00185 Rome, Italy. Tel.: +39 0649910556; fax: +39 0649913340.
E-mail address: simonetta.soro@uniromal.it (S. Soro).

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doi:10.1016/j.molcatb.2004.09.011

ces interact with the biomolecule through low energy binding forces (Van der Waals interactions, hydrogen bonds, hydrophobic interaction, etc.), immobilization can be enhanced by using the suitable carrier for the surface chemical characteristics of the enzyme.

The success and efficiency of physical adsorption on the enzyme on a solid support depends on several parameters: the size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface, in terms of functional groups, porosity, and pore size are all crucial. The amount of adsorbed enzyme per amount of support increases with the enzyme concentration, reaching a plateau at the saturation of the carrier: different adsorption isotherms are obtained for different enzyme–carrier systems [19]. Another important parameter is the pH at which the adsorption is conducted. Moreover, addition of proper water-miscible solvents during the immobilization process could reduce the solubility of the enzyme in the aqueous phase, favoring adsorption.

The study of immobilized lipases can be of great importance also in basic research, as for a better understanding of the immobilization mechanisms, as well as for its effects on the structure and the behavior of these enzymes. By choosing different carriers, it could be possible to obtain different effects on the "open-lid" conformation of the immobilized lipase; discovering the connection between structure of the carrier and achieved effect is essential to understand the lipase activation mechanism in depth.

Our work was focused on the physical adsorption of *Candida rugosa* lipase (CRL), a fungal lipase, on different polymeric matrices (polyphenylacetylene—PPA-, polypropargyl alcohol—PPOH-, commercial pellets of polypropylene—EP-100-, cross-linked polyvinyl alcohol—CL-PVA-, cross-linked polyvinyl alcohol functionalized by C_{12} chains—CL-PVA-C_{12}, polyacrylic resins covered by C_{18} chains in the presence of polyethyleneimine-C_{18}-SEPA-PEI-); some of them are commercial, while the others (PPA, PPOH, CL-PVA-C_{12}) were synthesized in our laboratories with the aim of improving enzyme performance, characterizing new immobilization systems and finding the relationship between lipolytic activity and nature of CRL–stationary phase interaction. The used lipase is well known, widely employed, easy to handle, stable, and accepts a broad range of substrates (for its capability of working as an esterase).

2. Experimental

2.1. Reagents

Lipase Type VII from *Candida rugosa* was obtained from Sigma (St. Louis, MO, USA); the preparation contained 7.7% of proteins. All solvents were purchased from Carlo Erba Analytical (Milano, Italy). Vinyl acetate (99%) was obtained from Merck (Darmstadt, Germany). Tributyrin, (±)-l-phenyl ethanol, oleic acid, 1-heptanol were purchased from Sigma (St. Louis, MO, USA). CRL (Type VII from Sigma) immobilized on EP-100 (polypropylene in pellets-Accurel from Akzo Nobel) was kindly provided by Prof. Patrick Adlercreutz (Dept. of Biotechnology, Lund University, Lund, Sweden) [14].

CRL (Type VII from Sigma) immobilized on C_{18}-Sepabeads and then treated with PEI was kindly provided by Prof. Jose M. Guisan (CSIC, Universidad Autonoma de Madrid, Madrid, Spain) [20].

2.2. Synthesis of the polymer matrices

The synthesis of PPOH was carried out starting from propargyl alcohol and using a Pd complex as a catalyst, according to the procedure reported in the literature [21]. The polymer was characterized using IR, UV, and elemental analysis, data obtained were in agreement with literature data.

The synthesis of PPA was carried out starting from the monomer of phenylacetylene in a mixture of NaOH and CH_3OH and using a Rh complex as a catalyst [22], and characterized using IR, UV, and elemental analysis.

The synthesis of CL-PVA-C_{12} was carried out starting from PVA in NaOH and using epichlorohydrin as a cross-linker [23]. CL-PVA was then functionalized with C_{12} chains (from lauroyl chloride in pyridine) [24]. The obtained polymer was characterized using IR, UV, and elemental analysis.

2.3. Lipase immobilization

In a typical reaction, 100 mg of PPA or PPOH were suspended in 2 ml of enzyme solution (50 mg/ml in K/Na phosphate buffer 0.1 M, pH 7.6) and incubated at room temperature under magnetic stirring (300 rpm) for 4.5 h. The mixture was then filtered and the solid fraction was washed four times with 5 ml of buffer. Filtered and washing solutions were tested using the standard assay for enzymatic activity (see Section 2.4). The found International Unit (μmoles of acid released per minute—IU) of lipase was then subtracted from the total IU employed for the immobilization: the resulting IU were equal to the total quantity of adsorbed enzyme.

One gram of CL-PVA-C_{12} was suspended in 15 ml of HEPES 20 mM/EDTA 2 mM buffer (pH 7.6) at room temperature and under magnetic stirring (300 rpm) for 1 h. Ten milliliters of enzyme solution (20 mg/ml in HEPES 20 mM/EDTA 2 mM buffer, pH 7.6) were then added to the swelled polymer and incubated at room temperature under magnetic stirring (300 rpm) for 3 h. The mixture was then filtered, the solid fraction was washed four times with 10 ml of buffer and the solution tested as described above for PPA and PPOH. No lipolytic activity was found in the second and in the further washing solutions.

2.4. Enzyme assay

Lipolytic activity was assayed by alkalimetric final titration. The assay mixture, containing 2.5 ml of K/Na phos-

phate buffer (0.1 M, pH 7.6), 0.5 ml of tributyrin and 100 μl of the enzyme solution (0.5–50 mg/ml in K/Na phosphate buffer 0.1 M, pH 7.6), was shaken for 30 s and incubated at 37 °C under magnetic stirring (600 rpm) for 30 min. An immobilized enzyme assay was performed by suspending a quantity of solid carrying an amount of immobilized enzyme corresponding to 5 mg of crude powder of CRL (Type VII from Sigma, protein content: 36% in weight), previously freeze-dried, in 2.5 ml of buffer solution (K/Na phosphate buffer 0.1 M, pH 7.6) and 0.5 ml of tributyrin as substrate were added. Then the reaction was stopped with 2.5 ml of ethanol/acetone 1:1 (v/v). The reaction mixture was titrated with NaOH 0.1 M in the presence of phenolphthalein using an automatic burette (Metrohm 775 Dosimat). A measurement of lipase activity by continuous automatic titration was performed with a pH-STAT system (Compact Titrator from CRISON). One hundred microliters of enzyme solution (50 mg/ml of crude powder of CRL Type VII from Sigma) and 10 ml of water were added into a thermostatically controlled reactor vessel. The initial pH of the reaction mixture was adjusted to 8.0 with NaOH. Then, 2 ml of the substrate (tributyrin) were placed in the reaction mixture and titration at constant stirring (300 rpm) was carried out. The relationship between time and hydrolysis rate was linear for the first 30 min of reaction time.

2.5. Calculation of Langmuir equation parameters

Data obtained from adsorption isotherms were fitted to the Langmuir isotherm Eq. (1) using the SigmaPlot program package.

$$[A] = \frac{[A]_{\max}[E]}{[E] + K_{\mathrm{L}}} \quad (1)$$

where [*A*] is the amount of protein adsorbed per weight unit or area unit of the carrier, $[A]_{\max}$ the maximum amount of protein adsorbed per unit weight or unit area of the carrier, [*E*] is the concentration of enzyme in the liquid phase at the adsorption equilibrium, and K_{L} is the Langmuir constant.

2.6. Lipase catalyzed reactions in organic solvent

The transesterification reactions were carried out in duplicate in screw capped vessels in which all reagents were added in appropriate ratios: racemic 1-phenyl ethanol (final concentration = 250 mM) was added to 2 ml of organic solvent, followed by vinyl acetate (final concentration = 1.25 M) and then by the immobilized enzyme (corresponding to 5 mg of crude powder of CRL Type VII from Sigma). The content of water in the used organic solvents was 0.02%. Samples were incubated in a thermostatic bath at 40 °C under magnetic stirring at 600 rpm, along with its respective control (sample with no enzyme). No reaction took place in the absence of enzyme.

2.7. Analytical determination of the reaction products

The transesterification reactions were followed by chiral GC. A Carlo Erba Model SFC 3000 gas chromatograph, equipped with a FID, was used for all GC evaluations and separations. The injection port and detector temperature were set at 250 °C. Nitrogen was used as the carrier gas (pressure: 120 kPa; flow rate: 4 s/m). Separations were performed on a 25 m × 0.25 mm i.d. chiral capillary column coated with Megadex 5 (30% 2,3-(9-dimethyl-6-0-pentyl-*P*-cyclodextrin in OV 1701). In order to achieve the necessary overall selectivity, for both alcohol and ester, tandem arrangements of chiral (enantio- and diastereoselective) and achiral (diastereoselective) columns were performed, connecting in series the chiral column above and a 5 m × 0.20 mm i.d. fused-silica achiral capillary column, coated with cross-linked methyl silicone HP1. A temperature gradient was set starting from 70 °C for 2 min and then rising to 130 °C with a gradient of 40 °C per minute. The injection volume was 0.5 μl.

Synthetic yield of the transesterification reaction was determined on the basis of the amount of unreacted substrate and formed product by the percent area method, using peak area integration by on-line software. Mixtures of alcohol and ester enantiomers were baseline resolved with the aid of a multi-column approach in four peaks, with the following retention times (t_{R}, min):

R-1-phenylethanol	18.10
S-1-phenylethanol	18.42
S-1-phenylethyl acetate	21.38
R-l-phenylethyl acetate	21.70

2.8. Analytical determination of specific area and porosity of the polymer matrices

Specific area and porosity of the carriers were determined with the BET (after Brunauer, Emmett and Teller) method, based on adsorption and desorption of an inert gas (we used N_2) on the surface to be measured. A Micromeritics (Norcross, GA, USA) instrument (model ASAP 2000) was used for the measurements.

3. Results

3.1. Characterization of polymers

Synthesized polymers were characterized by IR, UV, NMR, and elemental analysis, according to literature data [25,26]. A further characterization, consisting in the determination of specific area and porosity with the BET method, was carried out in order to obtain information about the in-

Table 1
Surface characteristics of the carriers

Carrier	Specific area (m^2/g)	Average pore radius (Å)
PPA	44	100–200
CL-PVA-C_{12}	0.86	≅1000
EP-100[a]	90	≅250
C_{18}-Sepabeads[b]	900	50–60

[a] Data given by the manufacturer.
[b] Data given by the manufacturer and referred to the non-functionalized carrier (Sepabeads).

Table 2
Loading capacity of the carriers toward commercial CRL

Carrier	Loading capacity[a] (mg of crude enzyme/g of polymer)	Specific loading capacity (mg of crude enzyme/m^2 carrier surface)
C_{18}-Sepa-PEI	180	0.2
EP-100	1000	11
CL-PVA-C_{12}	150	174
PPA	160	4

[a] Error is within ±5% for loading capacity.

teraction between enzyme and carrier. Results are reported in Table 1.

Determination of surface characteristics was performed on polymers synthesized in our laboratories, while for EP-100 and C_{18}-Sepabeads we relied on the data provided by the manufacturer.

3.2. Lipase adsorption

Loading capacity of all the used matrices, measured after performing the immobilization according to the standard procedure (final titration assay), is reported in Table 2. Each sample was treated with an enzymatic solution at a concentration of 50 mg/ml. The adsorbed enzyme is not removed by repeated washings. The specific activity of the enzyme solution, of the recovered enzyme solution after immobilization procedure on PPA and the first and second washing solutions were respectively, 100, 40, 38, and 35% of the starting enzyme solution value. Data for PVA-CL-C_{12} were respectively, 100, 28, 25, and 20% of the starting enzyme solution value. The values of specific loading capacity are especially related to the amount of available surface for lipase adsorption (the inner surface of pores being too small to let lipase molecules enter is not available for adsorption) and to the affinity of CRL for the carrier.

The grade of affinity of the enzyme molecules for each carrier was evaluated by adsorption kinetics and adsorption isotherms of CRL on PPA and CL-PVA-C_{12}. The assays for adsorption kinetics were carried out at room temperature and for both polymers the maximum adsorption was reached after 1 h.

The assays for adsorption isotherms were carried out at 25 °C only for matrices synthesized in our laboratories.

Table 3
Residual activity of the immobilized lipase

Sample	Relative residual activity (%)
Free enzyme	100
CL-PVA-C_{12}	67 ± 2
C_{18}-Sepa-PEI	63 ± 2
C_{18}-Sepa	54 ± 3
EP-100	64 ± 2
PPA	30 ± 3

The related plot is reported in Fig. 1: PPA plot has a sigmoidal shape (Freundlich equation trend), while CL-PVA-C_{12} plot shows a hyperbola-shaped growth (Langmuir equation trend). The shape difference is due to the different behavior of lipase toward the carrier. The calculated constants of the Langmuir equation are: K_L (mg/ml) = 3.07, $[A]_{max}$ (mg/mg) = 0.04, $[A]_{max}$ (mg/m^2) = 42.7 for CL-PVA-C_{12}. Values of K_L and $[A]_{max}$ for the Langmuir equation of CRL adsorption on EP-100 (K_L (mg/ml) = 0.33, $[A]_{max}$ (mg/mg) = 0.21, $[A]_{max}$ (mg/m^2) = 2.3) were drawn from literature [14].

To evaluate if electrostatic forces play a role in adsorption, immobilization experiments on PPA and CL-PVA-C_{12} were performed with lipase preparation dissolved in Na acetate buffer of pH 3.6, 4.6, and 5.6 having the same ionic strength of the phosphate buffer used in the previous experiments. These pH values correspond to positive, electroneutral, and negative net charge of the protein. No change in the loading capacity of both matrices was observed as a function of pH. Similar results were obtained by Adlercreutz and coworkers for EP-100 [14].

3.3. Enzymatic reactions in an aqueous environment

It is known that the catalytic activity of a lipase could decrease as it is immobilized [27]. Residual activity, determined by the final titration assay, of immobilized CRL in the hydrolysis of the tributyrin reaction related to the activity of the free enzyme is reported in Table 3. Samples of CRL adsorbed on polymers through long-chain hydrophobic interactions show similar activities, while CRL adsorbed on PPA through π interactions is less active.

Figs. 2 and 3 show the activity, determined by the continuous titration assay, of immobilized CRL in the reaction of hydrolysis of tributyrin versus temperature and pH. If compared to the free enzyme, immobilized CRL is more stable at temperatures higher than 50 °C. Maximum activity is reached between 40 and 50 °C for each sample.

pH changes affect the lipolytic activity of immobilized CRL as well, but less than they do for the free enzyme. The highest activity occurs when pH is around 7.

3.4. Lipase catalyzed reactions in organic solvents

Before testing the activity of immobilized CRL in organic solvents, enzyme stability in these media was evaluated.

Residual hydrolytic activity (final titration assay) of CRL immobilized on different matrices was tested after treatment at 40 °C with organic solvent and then eliminating the solvent under vacuum. Results are reported in Table 4. In most cases, treated samples show increased activity compared to untreated ones.

We studied lipase catalyzed transesterification reaction between 1-phenylethanol and vinyl acetate as a model reaction

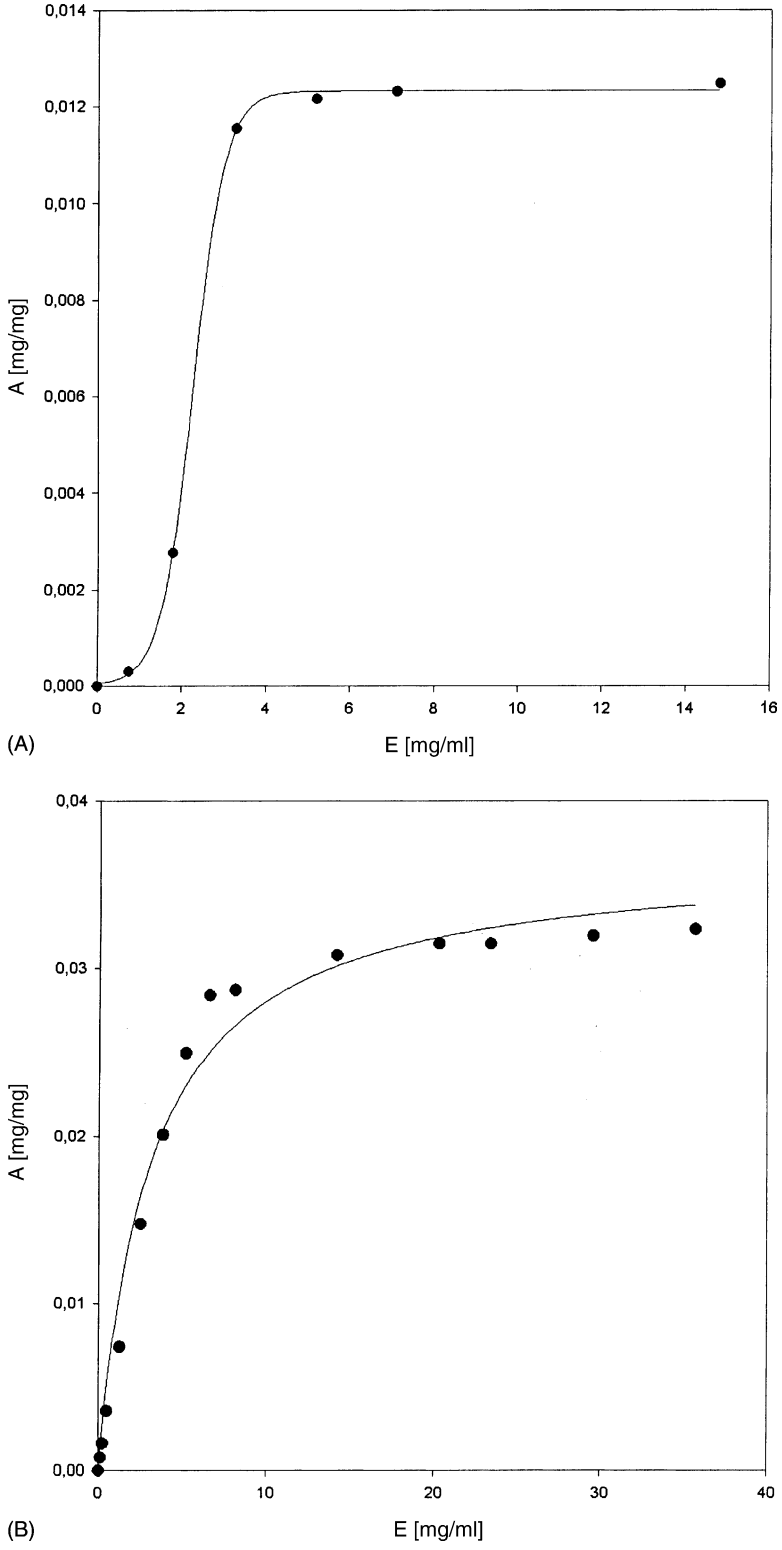

Fig. 1. Adsorption isotherm at 25 °C of CRL on PPA (A) and CL-PVA-C_{12} (B).

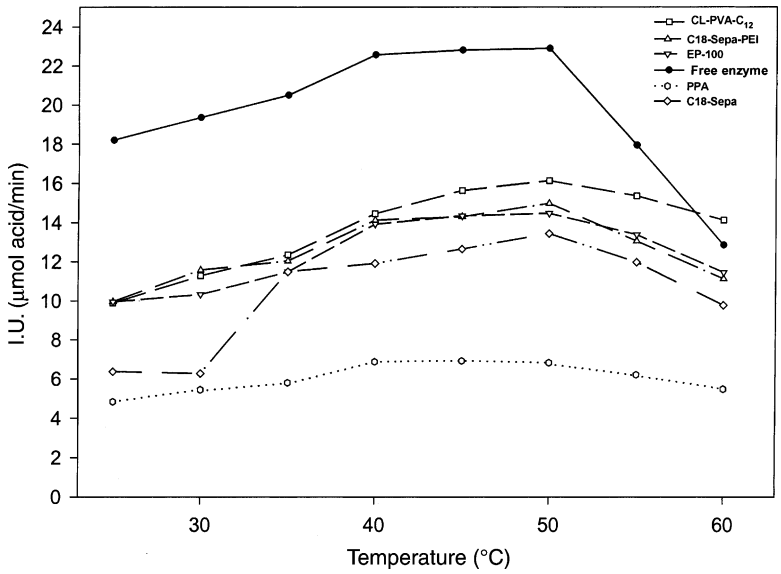

Fig. 2. Effect of temperature on the activity of the immobilized enzyme.

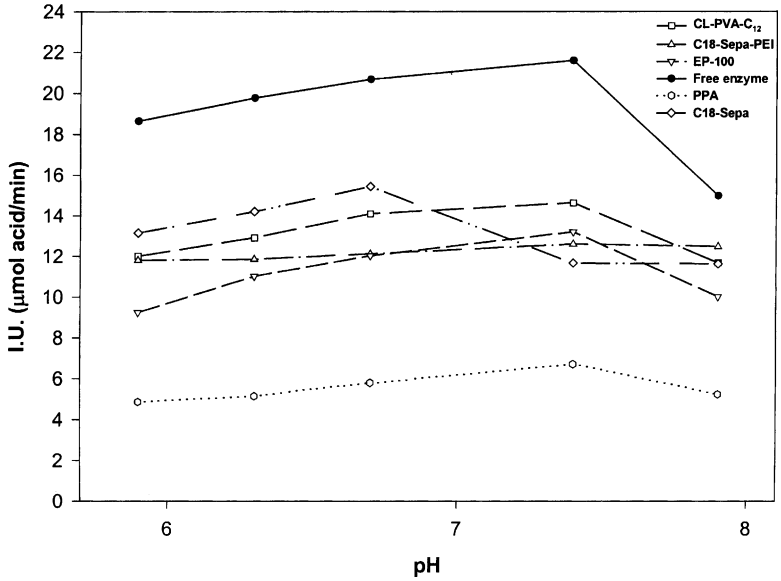

Fig. 3. Effect of pH on the activity of the immobilized enzyme.

[28]. The values of initial rate, yield after 24 h, enantiomeric excess (ee_p%) and enantiomeric ratio (*E*) in hexane, toluene and chloroform are reported in Table 5 (PPA was studied only in hexane, being soluble in the other solvents). Enantiopreference was toward *R* enantiomer. In many cases, we observed an increased initial rate and enantiomeric excess in comparison with the reaction carried out with free CRL.

Table 4
Residual activity[a] of free and immobilized CRL in the hydrolysis of tributyrin before and after treatment with organic solvents

Sample	Untreated	Hexane	Toluene	Chloroform
Free enzyme	100	62 ± 2	68 ± 3	59 ± 2
CL-PVA-C_{12}	67 ± 2	83 ± 2	88 ± 2	78 ± 2
C_{18}-Sepa-PEI	63 ± 2	73 ± 2	67 ± 2	68 ± 2
C_{18}-Sepa	54 ± 3	20 ± 3	33 ± 3	31 ± 3
EP-100	64 ± 2	54 ± 3	62 ± 2	57 ± 3
PPA	30 ± 3	47 ± 3	–	–

[a] In % of the activity of the free untreated enzyme.

4. Discussion

Carriers with different physico-chemical properties were employed in order to obtain physical adsorption of CRL; in all cases the adsorption was due to hydrophobic interactions between the enzyme and polymer matrices; in fact no adsorption based on H bond was obtained using cross-linked polyvinyl alcohol (CL-PVA) and polypropargyl alcohol (PPOH) as matrices carrying OH as functional groups. Moreover, in our experiment lipase immobilization was not

Table 5
Initial rate and enantiomeric excess of lipase catalyzed transesterification reaction between 1-phenylethanol and vinyl acetate in organic solvent

Sample	V_0 (mmol product/h) in hexane	Yield after 24 h in hexane[a]	ee_p (%)	E	V_0 (mmol product/h) in toluene	Yield after 24 h in toluene[a]	ee_p (%)	E	V_0 (mmol product/h) chloroform	Yield after 24 h in chloroform[a]	ee_p (%)	E
Free enzyme	4.5	8.1	55	2	1.5	6.7	50	3	0	0	n.d.	–
CL-PVA-C_{12}	28.8	8.2	71	6	16.3	7.8	60	4	4.0	5.3	38	2
C_{18}-Sepa-PEI	9.3	31.8	62	6	6.3	27.3	58	5	1.0	6.1	46	3
C_{18}-Sepa	2.5	11.1	63	5	1.8	9.6	54	4	0	0.2	n.d.	–
EP-100	11.8	8.8	60	4	7.5	8.7	57	4	2.5	4.9	34	2
PPA	19.5	2.5	60	4	–	–	–	–	–	–	–	–

[a] Error is ±0.5 for the yield.

affected by pH changes in the adsorption solution, suggesting that hydrophobic interactions are the main forces involved.

All the matrices are provided with long hydrocarbon chains or, as EP-100, are cross-linked hydrocarbon chains themselves: previous investigations showed that CRL affinity for alkyl chains in CL-PVA reaches maximum when their length is between 8 and 14 carbon atoms [24]. The matrix called C_{18}-Sepa-PEI was treated (by Guisan et al. [20]), after CRL immobilization, with polyethylenimine (PEI), a polar substance that seems to help keeping the enzyme in the active conformation, especially when it is employed in organic solvent, playing the role usually played by the layer of water surrounding the enzyme molecule [20].

The only exception is PPA, which carries phenyl rings as hydrophobic groups and may establish π interactions with the lipase. Our hypothesis about the adsorption mechanism suggests that CRL interacts with PPA through two areas on its surface which are particularly rich in aromatic amino-acidic groups. One of these two areas surrounds the opening of the catalytic tunnel of the lipase (Fig. 4A), while the other one is placed at the "antipodes" (Fig. 4B). When the former area interacts with the carrier, the access of substrates to the catalytic site can be hindered. This would explain the high loss of catalytic activity as CRL is adsorbed on PPA. Moreover, after purification of PPA through precipitation from chloroform [29], we observed that its loading capacity decreases by 70%. This behavior could be explained considering the polymer structure. Recent studies with X-ray crystallography have shown that in pristine PPA two different structures are mainly present: (a) *cis-transoidal* head–tail–head–tail (prevalent) and (b) *trans-transoidal* head–tail–tail–head (Fig. 5) [30]. PPA, when solubilized in chloroform, modifies its chain conformation [29] and is believed to transform itself from (a) to (c) *trans-transoidal* head–tail–head–tail structure, which lets the polymer form more ordered structures, in which most of the aromatic rings are packed together more closely and are not free to interact with CRL molecules to generate adsorption.

The adsorption isotherm of CRL on PPA has a sigmoidal shape (Fig. 1). This trend indicates that the enzyme–enzyme forces at the carrier surface are significant relative to the enzyme–carrier ones, so the energy for removing a molecule of adsorbate is clearly raised by the presence of its neighbors, and "cooperative adsorption" occurs [31]. At this point, the adsorption phenomenon is irreversible (no release of enzyme under washing was observed) and highly favorable and the protein forms a monolayer surrounding the polymer. CRL adsorption on CL-PVA-C_{12} isotherm shows a typical Langmuir trend, indicating that polymer–protein interactions are stronger than protein–protein ones. Adsorption of secondary protein layers can be observed as a kink in the adsorption isotherm after an apparent saturation of the carrier. The same trend was obtained by Adlercreutz and coworkers for CRL adsorption on EP-100 [14].

The specific activity of the lipase in solution during the adsorption process on PPA and CL-PVA-C_{12} showed a selective

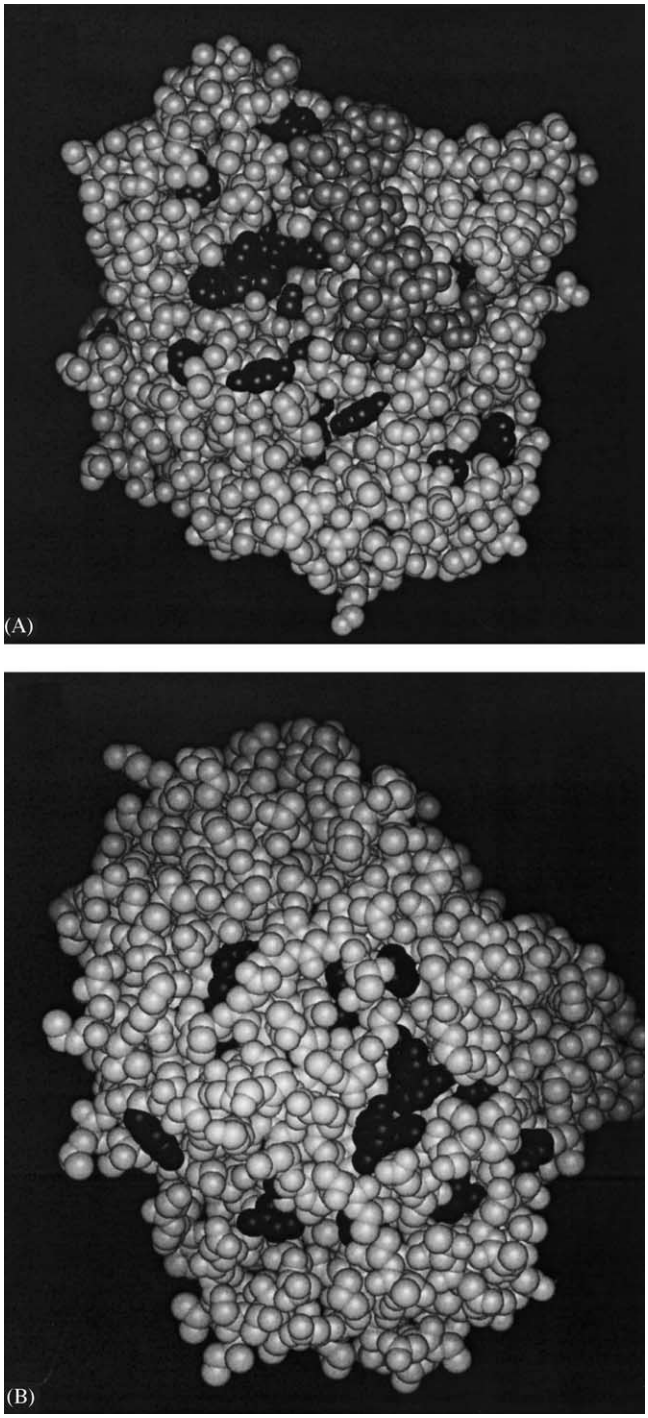

Fig. 4. Lipase model. Side chain of aromatic residues in black (in dark grey those of active site); lid in light grey. (A) Front side (lid side); (B) back side.

adsorption for the lipase protein compared to the other proteins. The reason of this high selectivity is a stronger affinity of the lipase for the polymer surface compared to that of the other proteins. Moreover, negligible CRL desorption from both polymers was detected, while other proteins were released during the washing procedures. These data support the hypothesis that the CRL molecule is fixed to the surface by multiple attachments. All attachments must break simultaneously to have lipase desorption, but, of course, this is not very likely [32].

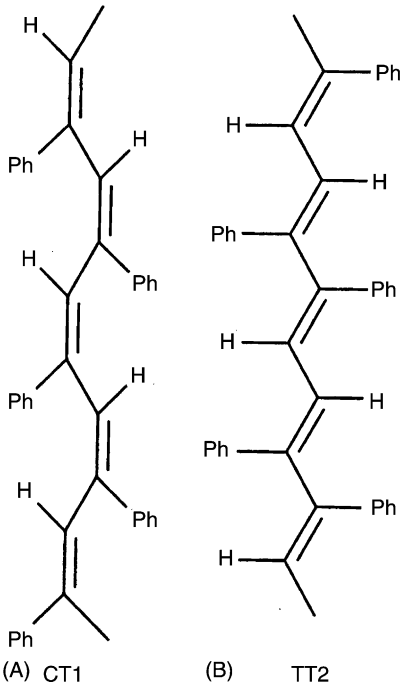

Fig. 5. PPA different structures: (A) *cis-transoidal* head–tail–head–tail; (B) *trans-transoidal* head–tail–tail–head.

According to the values of K_L and specific loading capacity we can affirm that EP-100, if compared to PPA and CL-PVA-C_{12}, seems to be the matrix showing the best characteristics for CRL immobilization. Specific loading capacity parameter is a combination between area of the surface and number of sites useful for the immobilization; it can be considered as equivalent to the calculated value of $[A]_{max}$ of the Langmuir equation. Analyzing the values of K_L, loading capacity and specific loading capacity, it becomes evident that the morphological characteristics of the carrier are often the main factor influencing its "quality". As a matter of fact, the dimensions of pores on CL-PVA-C_{12} surface are more suitable for substrates and products diffusion than those on EP-100, as known in literature for reactions catalyzed by immobilized enzymes [33].

As well known, immobilized enzymes often acquire a more constrained conformation, giving them a lower catalytic activity and a higher stability: as a matter of fact, immobilization systems are often described as a way to produce enzymes with altered properties [34]. In particular, residual activity for all the hydrophobic immobilization systems is about 60%, while for PPA, a matrix that can establish π interactions with the lipase, the residual activity is 30%. This further loss of activity can be explained by steric hindrance, which indicates that only half of the immobilized protein is able to act as a catalyst. From this point of view, the residual activity of CRL immobilized on PPA can be compared to that obtained to the other matrices, suggesting that π and hydrophobic interactions are equivalent toward lipase catalytic performance.

Temperature and pH variations show a minor negative effect on immobilized CRL compared to free enzyme. At 60 °C the immobilized enzyme keeps up to 90% of its activity in the standard reaction of hydrolysis of tributyrin, while this value is 60% for the free enzyme (Fig. 2). This gain in thermal stability can be ascribed to the higher conformational rigidity of the immobilized lipase, related to a lower number of degrees of freedom. Similar considerations can be made by varying bulk pH (Fig. 3).

Another effect of stabilization of CRL by physical adsorption is evident by observing residual activity in the standard reaction of hydrolysis of tributyrin after treatment with dehydrated organic solvents.

For free enzymes, the capability of solvents to subtract water from their surface (being related to water solubility in the solvent) is a crucial point for enzyme performance: the enzyme loses activity as it loses the layer of water covering it [35]. CRL immobilized on CL-PVA-C_{12} and PPA shows an increased activity after treatment, reaching values even higher than those of free treated enzyme (Table 4). Data obtained for C_{18}-Sepa and C_{18}-Sepa-PEI samples confirm the importance of the effect due to the presence of PEI in substituting the layer of water surrounding the enzyme molecule [20] as the polymer alone is not able to stabilize the enzyme.

The use of chiral substrates in the transesterification reaction points to an increased enantioselectivity when lipase is immobilized on polymer matrices (Table 5). Enantiopreference was toward *R* enantiomer, as usually happens when using CRL as a catalyst. The value of *E* is also a function of the organic solvent used as the reaction medium. The best results in terms of *E* were obtained for CL-PVA-C_{12} and C_{18}-Sepa-PEI in hexane; these result could be ascribed to a higher enzyme rigidity in the carrier–solvent system. The highest yield was obtained with C_{18}-Sepa-PEI matrix, probably because of better protection from the long-term denaturing effect of the organic solvent given by this carrier. The different kinetic behavior of the biocatalytic systems can be directly ascribed to matrix morphology and diffusion phenomena, instead of to the denaturing effects of the organic solvents. Moreover, for immobilized enzymes diffusion of substrates and products may be hampered by partitioning of the enzyme in the carrier layer: a hydrophobic matrix will attract hydrophobic substrates (i.e. triglycerides in the hydrolysis reaction in aqueous medium), while the concentration of charged species (acids as reaction products and water as reaction substrate) will be lower in its surroundings due to disruption of the water structure around the hydrophobic surface. The more favorable diffusion and repartition of substrates and products due to the employment of immobilized enzymes can be related to the better performance of these systems when compared to that of the free enzyme. Among the studied systems, PPA, which is a π-conjugated polymer never investigated in the context of enzyme carriers (to the best of our knowledge), shows a good performance in terms of initial rate and *E* similar to those obtained with the other carriers. The above results suggest that PPA could act as a useful carrier for enzyme immobilization.

In conclusion, the behavior of the immobilizing systems, in terms of stability, activity, and selectivity, may be explained as a compromise between strength of hydrophobic interaction, possible hindrance effects of the stationary phase, diffusion phenomena and rigidity of the adsorbed biocatalyst. On this basis, the efficiency of a biocatalyst reflects the complexity of the catalytic system, composed not only of the enzyme, but also of the immobilizing matrix and the reaction medium. The use of different polymeric matrices allows a possible modulation of the catalytic behavior of lipases, with the aim of fulfilling the different needs of biocatalytic processes.

Acknowledgements

This research was partially supported by Italian MIUR (40% National Co-Financed Projects). The authors are indebted to Prof. P. Adlercreutz and Prof. J.M. Guisan for providing lipase immobilized, respectively on EP-100 and C_{18}-Sepabeads.

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