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Bio-imprinting of lipases with fatty acids

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Abstract

Bio-imprinting of lipases with fatty acids was shown to be a feasible, effective method for obtaining highly active enzymes in organic solvents. The increase in activity was dependent on the enzyme type, the solvent type and the imprint molecule itself. A correlation between the initial activity of caprylic acid-imprinted *Candida rugosa* lipase (CRL), and solvent hydrophobicity was observed. In addition, the combination of bio-imprinting with adsorption onto an inert support such as celite, proved to be a powerful technique for obtaining an even more active and stable enzyme preparations. In the case of lipase from *Pseudomonas* sp., the increase in activity resulting from bio-imprinting with caprylic acid and immobilization onto celite, was 20-fold. Porcine pancreatic lipase (PPL), treated in the same manner, retained 70% of its initial activity at the end of 20 consecutive reaction cycles, compared to only 20% residual activity for the non-treated control.

Keywords: Lipases; Organic solvents; Bio-imprinting; Biocatalysis; Immobilization

1. Introduction

In recent years, the employment of biocatalysis for organic synthesis has become an increasingly effective alternative to conventional chemical methods [1–3]. The use of organic solvents as reaction media for biocatalytic reactions has proven to be an extremely useful approach for expanding the range and efficiency of practical applications of biocatalysis. The improvement of enzymatic activity and stability in non-aqueous media, still remains a major challenge. One strategy for attaining this goal is bio-imprinting [4,5].

The concept of imprinting of organic polymers was first introduced by Wulff in 1972 [6]. In this method, a cross-linked polymer is formed around a molecule that acts as a template. After removal of the template, an imprint containing functional groups capable of chemical interaction remains in the polymer. Imprinting of biopolymers (bio-imprinting) is based on the same idea [7,8]. In an aqueous solution, the enzyme's active site is loaded with a substrate analogue by forming a complex which is similar to an enzyme–substrate complex. This process is believed to be accompanied by small conformational changes—the induced fit. When the complex is placed in an essentially anhydrous organic solvent, the ligand is washed away, but the enzyme is unable to adopt its former conformation due to its rigid structure which results from strong electrostatic interactions in the media possessing low dielectric constants. As a consequence, the three-dimensional structure of the enzyme remains 'frozen' in a modified form, as if 'remembering' the structure of the ligand. Bio-imprinting is restricted to

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anhydrous or microaqueous organic solvents, since the memory is lost in aqueous systems, unless additional stabilization measures are used.

Bio-imprinting of enzymes was first described by Russell and Klibanov in their work on subtilisin [9]. Following lyophilization in the presence of a competitive inhibitor, they obtained an enzyme with 100-fold rate enhancement. Mosbach and co-workers [10] demonstrated that imprinting of chymotrypsin with D-amino acids enabled the enzyme to accept and esterify the unnatural enantiomer. Rich and Dordick [11] obtained a 50-fold activation in the subtilisin-catalyzed acylation of nucleosides in organic solvents, following lyophilization of the enzyme from an aqueous solution containing the substrate. Imprinting of nine lipases with *n*-octyl-β-D-glucopyranoside as an imprint amphiphile, resulted in a rate acceleration and an increase in the reaction yield, although the response was markedly lipase type dependent [12,13]. The method is not restricted to hydrolases. Myoglobin imprinted in aqueous solution with ligands bound to its heme iron, followed by lyophilization, catalyzed the epoxidation of styrene with H_2O_2 or *tert*-butyl hydroperoxide in organic solvents much faster than the non-imprinted protein [14].

The combination of bio-imprinting and surfactant coating, was described by Okahata et al. [15]. *Candida rugosa* lipase (CRL) was imprinted with various molecules and then coated with an amphiphilic molecule. The lipid coating was important both to maintain the imprinted structure, and to solubilize the enzyme. When (*R*)-1-phenylethanol was the imprint molecule, the enantioselectivity of the enzyme in the esterification of the same alcohol, increased by a factor of 14.

In an effort to maintain the imprinted properties of proteins in an aqueous environment, an enzyme was first vinylated and then cross-linked with ethylene glycol dimethylacrylate to form a rigid, stable conformation [16]. Thus, the ability of the enzyme to hydrolyze *N*-acetyl-D-tryptophan ethyl ester in phosphate buffer was possible using a cross-linked enzyme initially imprinted with *N*-acetyl-D-tryptophan.

In a previous study, we described the use of fatty acids for coating of enzymes, thereby improving their activity in organic solvents by rendering them more hydrophobic [17]. In this paper, we describe the use of fatty acids for bio-imprinting of lipases. The demonstrated synergistic effect of imprinting and immobilization is yet another important finding of this study.

2. Experimental

2.1. Materials

Candida rugosa lipase type VII and porcine pancreatic lipase (PPL) type II were purchased from Sigma (Rehovot, Israel). Lipase from *Pseudomonas* sp. (PSL) was purchased from Amano Co. (Tokyo, Japan). Fatty acids were purchased from Sigma. Methyl caproate, caproic acid and Tween 20 were from Aldrich (Rehovot, Israel). (*R*)-1-Phenyl ethanol and (*S*)-1-phenyl ethanol, racemic 1-phenylethanol and vinyl acetate were from Merck (Germany). Celite was purchased from Fluka (Switzerland) and cellulose beads were from Sigma.

All other solvents and reagents were obtained commercially and were of analytical grade.

2.2. Modification of lipases by coating with stearic acid

An amount of 500 mg of PPL was dissolved in 200 ml phosphate buffer (pH 6, 0.05 M), and mixed at 4 °C. Stearic acid (125 mg) was dissolved in 5 ml warm ethanol and was added dropwise to the enzyme solution. Following 30 min mixing, the solution was sonicated for 9 min (Sonicor SC-52H, Sonicor Instrument Corp., Capiague, NY), and then mixed again for 1 h. The mixture was kept overnight under refrigeration and the precipitate separated by centrifugation (23,500 × *g*, 4 °C for 20 min) and lyophilized. Protein content of the powder was calculated from elemental analyses measurements of the percentage nitrogen.

2.3. Bio-imprinting procedure

In a glass vial, PPL (300 mg) was dissolved in phosphate buffer (9 ml, pH 6.5, 10 mM). In the initial experiment described in Table 1, caprylic acid (50 mg, 0.35 mmol) was added to the enzyme and the solution was mixed for 20 min. In all other experiments, caprylic acid and Tween 20 (100 mg) were dissolved in ethanol (1 ml). The ethanol solution was added to the enzyme solution followed by 20 min mixing at

Table 1
Influence of coating with stearic acid and bio-imprinting with caprylic acid on the transesterification of 1-phenylethanol with PPL

Enzyme	Treatment of PPL	Initial specific activity (μmol/h mg protein)	Yield of process (wt.% protein)
A	Coating with C_{18}-acid	3.12	31
B	Imprinting with C_8-acid and then coating with C_{18}-acid	5.32	43
C	Imprinting with C_8-acid	1.52	88
D	PPL in buffer, lyophilization	0.02	85
E	None	0.99	100

PPL (300 mg) was dissolved in 30 ml phosphate buffer (pH 6.5, 10 mM). Fifty milligram of C_8-acid were added to the enzyme solution and mixing was carried out at RT for 20 min (B and C). Fifty milligram stearic acid were dissolved in 3 ml warm ethanol and added dropwise to the enzyme solution (A and E). The mixtures were stored at 4 °C overnight and the modified enzymes only were separated by centrifugation (A and B). All the enzymes were lyophilized and washed with 5 ml hexane. Mixtures of C and D were lyophilized as a whole. Enzymatic reaction conditions were 1 mmol 1-phenylethanol, 3 mmol vinyl acetate, 10 ml diisopropyl ether and 25 mg protein with continuous shaking at 35°.

room temperature. The mixture was then frozen and lyophilized. The enzyme powder was washed with 5 ml hexane to remove the imprint molecule, and vacuum dried for 2 h to remove the solvent. The dry enzyme powder was kept under refrigeration.

2.3.1. Variations

- CRL or PSL were used instead of PPL (300 mg).
- Celite or cellulose (200 mg) were added to the enzyme mixture following the addition of the imprint containing ethanol solution, then mixing was carried out for 20 min as described.
- Different fatty acids were used (0.35 mmol).

2.4. Enzymatic hydrolysis of methyl caproate in mixed water–ether medium

The following reagents were added into a 20 ml vial: PPL (300 mg) or imprinted PPL (400 mg originating from 300 mg crude enzyme), phosphate buffer pH 7.5, 0.05 M (6 ml), and diisopropyl ether (4 ml). A control without imprint molecule, but with Tween 20 and ethanol was also checked. The bi-phasic mixture was magnetically stirred until a uniform emulsion was formed (5 min). Methyl caproate (100 mg) dissolved in diisopropyl ether (1 ml) was added to the enzyme mixture to begin the hydrolysis reaction. Samples (1 μl) were withdrawn in 10 min intervals and analyzed using gas chromatography (Hewlett-Packard 5890 Series II equipped with flame ionization detector). GC analyses were conducted using a 15 m capillary column (i.d. = 0.25 mm) packed with Rtx-1 (Restek Corp.). The temperature profile was constant at 75 °C. Under these conditions, the retention times were $R_f = 1.9$ min for methyl caproate and $R_f = 2.9$ min for caproic acid. A calibration curve for caproic acid was prepared separately.

2.5. Enzymatic transesterification of 1-phenylethanol

The following reagents were added into a 25 ml flask: 1-phenylethanol (120 μl, 1 mmol), vinyl acetate (275 μl, 3 mmol) and diisopropyl ether (10 ml). CRL (300 mg) or imprinted CRL (∼400 mg originating from 300 mg crude enzyme) was added to start the reaction. The reaction was performed at 35 °C and at a shaker speed of 140 rpm in a water-shaking bath (Haake SWB 20 shaker), unless stated otherwise. Samples were taken out periodically and analyzed by GC with 1-heptanol as an internal standard. Sample preparation: 200 μl reaction mixture, 800 μl solvent and 5 μl 1-heptanol (100 mg/ml); injection of 2 μl to the GC.

When (*R*)- or (S)-1-phenylethanol was used as substrate, all of the quantities were reduced by a factor of 2. All experiments were performed in duplicates unless stated otherwise. In cases where the difference in the initial reaction rate between the two experiments was greater than 5%, the experiment was repeated again.

GC analyses were conducted using a 15 m capillary column (i.d. = 0.25 mm) packed with Rtx-1 (Restek Corp.). The temperature was programmed: $T_1 = 80$ °C, 2 min; $dT/dt = 10$ °C/min; $T_2 = 150$ °C;

$dT/dt = 20\,^{\circ}C/min$; $T_3 = 250\,^{\circ}C$. Under these conditions, the retention times were $R_f = 2.3$ min for 1-heptanol (internal standard), $R_f = 3.3$ min for PHE and $R_f = 4.9$ min for 1-phenyl ethyl acetate.

2.6. Successive batch reactions

The acetylation reaction was performed in successive batches using the same enzyme. PPL (300 mg) was imprinted according to the above-described procedure. The transesterification reaction was performed as described, using racemic 1-phenylethanol. After 5 h of reaction, the solvent was removed, the enzyme washed with 10 ml of diisopropyl ether, and kept refrigerated in fresh diisopropyl ether until the next day. Substrates were added and the reaction was started again for another 5 h. The conversion was determined by GC analysis at the end of each cycle.

3. Results and discussion

3.1. Combination of coating and imprinting

Whereas coating of lipases has been commonly done with amphiphilic molecules, bio-imprinting can be done with a substrate analogue, provided it has some solubility in water (where the formation of the enzyme–imprint complex occurs). However, one cannot rule out the possibility that during the coating process, some surfactant molecules could also bind to the active site and subsequently detach when the enzyme is placed in the organic solvent. It thus seems logical that the coating process may also encompass bio-imprinting. We therefore turned to explore the possibility of bio-imprinting lipases with fatty acids as imprint molecules.

The modification procedure, involving the coating of PPL with stearic acid, was combined with bio-imprinting (with caprylic acid), in order to determine the mutual effect on the enzymatic activity of PPL. The results are shown in Table 1. The combination of imprinting and coating afforded the most active enzyme (B). Imprinting improved the activity of the coated enzyme by 60% (B versus A). The bio-imprinting technique resulted in higher protein yields compared with the coating technique, since the entire enzyme preparation is freeze dried (C versus A). Coating with stearic acid resulted in a more active enzyme compared to the imprinted preparation (A versus C). The results imply that imprinting contributes to the fixation of the active site in its catalytically active form during the coating process. Klibanov has shown that lipases undergo interfacial activation in water in the presence of triolein but are not activated in organic solvents, presumably due to lack of hydrophobic interactions [18]. This interfacial activation is accompanied, for most lipases, with the opening of an α-helical "lid" covering the active site in aqueous surroundings. Employment of caprylic acid enabled to demonstrate the entrapment of the activated, "open" conformation, through bio-imprinting. Stearic acid, a similar molecule although of by far lower water solubility, is more hydrophobic and surely able to form the required interface for activation of lipases. Even in a state of aggregation, single molecules are in equilibrium with the micelles, and consequently are likely to penetrate the active site. It is therefore postulated that activation of the coated enzyme is primarily due to better compatibility with the organic solvent, but also partly due to changes in the conformation of the active site caused by imprinting.

Bio-imprinting was further studied and the procedure was improved by adding ethanol and Tween 20 to facilitate the solubility of C_8-acid in the aqueous solution. The inclusion of Tween 20 significantly improved the reproducibility of the results, and consequently Tween 20 was used in all subsequent bio-imprinting experiments. We examined the influence of Tween 20 on the activity (without the C_8 imprint acid) and found that it enhanced the activity by 12 and 15%, above that of the non-treated CRL and PPL, respectively. A reaction profile of imprinted PPL is shown in Fig. 1. The initial specific activity of the non-treated PPL and of the C_8-acid-imprinted enzyme in this reaction was 48 and 178 μmol/h mg protein enzyme, respectively (3.7-fold increase in initial activity). This major increase in activity was definitely attributed to the fatty acid and not to Tween 20, and we therefore continued the study of imprinted enzymes in comparison with the non-treated enzyme as the control. The activation brought about by imprinting diminished when as little as 0.04% water was added to the solvent. The addition of an equal amount of water to the non-imprinted control had no effect on its activity. This phenomenon is a known characteristic of bio-imprinting [11]. The

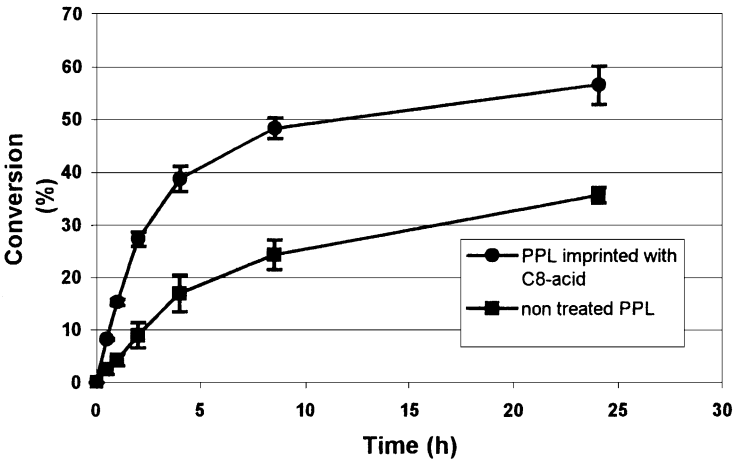

Fig. 1. A reaction profile for PPL and C_8-acid-imprinted PPL. Bio-imprinted PPL was prepared by dissolving 300 mg PPL in 9 ml phosphate buffer (pH 6.5, 0.1 M) and mixing the enzyme with 50 mg caprylic acid, initially dissolved in 1 ml ethanol and 100 mg Tween 20. Following 20 min of stirring, the enzyme was lyophilized and the resulting powder washed twice with 10 ml of hexane. Reaction conditions were 1 mmol 1-phenylethanol, 3 mmol vinyl acetate, 10 ml diisopropyl ether and 300 mg PPL (crude or imprinted) with continuous shaking at 35 °C. Data points represent an average of three determinations ± S.D.

water molecules which evidently interact preferentially with the hydrophilic enzyme, introduce flexibility into the molecule, which enables the enzyme to revert to its native structure, thus canceling out the structural changes induced by the imprint molecule. It was of further interest to evaluate the amount of active enzyme molecules following the imprinting process. For this purpose, we examined the activity of imprinted PPL in an aqueous medium using the hydrolysis of methyl caproate as a model reaction. The enzyme exhibited 90% of the activity presented by the non-imprinted (but treated with Tween 20 and ethanol), lyophilized enzyme (0.04 ± 0.001 and 0.045 ± 0.003 μmol product/min mg protein, respectively). Crude PPL, not imprinted and not lyophilized, showed a slightly higher activity of 0.048 ± 0.003. It can therefore be concluded that most of the enzyme molecules retain their viability during the imprinting and the lyophilization processes, and whereas this activity is similar to a non-imprinted enzyme in water, it is markedly increased in a non-aqueous medium.

3.2. Influence of fatty acid carbon chain length on the activity and enantioselectivity of imprinted CRL

PPL is a very selective enzyme towards (*R*)-1-phenylethanol in the acetylation reaction, and therefore, in order to study the effect of bio-imprinting on enzyme selectivity, we used CRL which is less selective in the reaction. The acetylation reaction was carried out separately with (*S*)- and (*R*)-phenylethanols and the selectivity was determined from the ratio $V_{[R]}/V_{[S]}$ [19]. CRL was imprinted with various fatty acids to determine whether the imprinting effect is a general feature of fatty acids, and what is the influence of the carbon chain length (Table 2). The chain length was varied from C_6-acid to C_{12}-acid because longer fatty acids are only sparingly soluble in water and would therefore be poor imprint molecules. The results indicated that all fatty acids tested were useful imprint molecules for enhancement of the enzymatic activity in organic solvents. The extent of activation was dependent on the carbon chain length, with an apparent optimum at C_{10}-acid. The selectivity was essentially unaffected by the imprinting.

The main requirements from an imprint molecule are solubility in the aqueous phase and resemblance to the natural substrate of the enzyme. Short-chain fatty acids satisfy both conditions. However, hexanoic acid (C_6) had a very slight activation effect (Table 2), whereas decanoic acid (C_{10}) was a much better imprint molecule. It seems that if the imprint molecule is too soluble in water, the partitioning into the active sites or the binding affinity to the enzyme active

Table 2
Influence of fatty acid carbon chain length on the initial activity and selectivity of imprinted CRL

Specific activity with 1-phenylethanol	Chain length of imprinting fatty acid				Non-treated CRL
	C_6	C_8	C_{10}	C_{12}	
$V_{[R]}$ (μmol/h mg protein)	4.00	5.40	7.00	6.40	3.10
$V_{[S]}$ (μmol/h mg protein)	1.02	1.50	2.07	1.96	0.97
Enantioselectivity	3.90	3.60	3.50	3.30	3.50

CRL was imprinted with 0.35 mmol of various fatty acids according to the legend in Fig. 1. The initial specific reaction rate was determined from the acetylation reaction containing: (*R*)- or (*S*)-1-phenylethanol (0.5 mmol), vinyl acetate (1.5 mmol), diisopropyl ether (5 ml) and CRL (150 mg).

site is low, and therefore activation is marginal. Other results (not presented in this work) indicated that decanoic acid was a better substrate than hexanoic acid in the esterification reaction of butanol in diisopropyl ether. This implies that there could be a correlation between the activity of CRL on fatty acids of different chain lengths, and their ability to serve as imprint molecules. This issue needs further investigation. The idea is supported by a recent work on CRL immobilized in a sol–gel matrix following pretreatment of the enzyme with substrate analogues such as long-chain alcohols [20]. The researchers found that the activity of the pretreated immobilized lipase increased with the increase in chain length of the alcohol used as an imprinting template. Due to its relatively good solubility in water, caprylic acid was used in further studies.

3.3. Influence of solvent type on the activity and enantioselectivity of C_8-acid-imprinted CRL

The nature of the solvent is an important factor in enzymatic catalysis. The solvent can influence the rate of the reaction, as well as the enantioselectivity [21]. The acetylation reaction was therefore performed in various solvents, to examine the influence of this parameter on the imprinted enzyme. All of the solvents were dried over molecular sieves for three days prior to use.

Table 3 summarizes the results obtained with various solvents as a function of log *P* (the solvent hydrophobicity index). The initial specific activity of both CRL and C_8-acid-imprinted CRL generally increased with the log *P* value (results not shown) in accordance with [22]. This gradual increase in activity is less orderly for the imprinted enzyme. It is also evident that the increase in activity brought upon by imprinting is solvent dependent (Table 3) and varies from 5 to 230% activation. In highly hydrophilic solvents, CRL was completely inactive. Such solvents with low log *P* values, have a strong tendency to remove the essential water from the vicinity of the enzyme, leading to inactivation [23]. It is noteworthy, that the process of imprinting with caprylic acid did not turn an inactive enzyme into an active one (e.g. THF, acetone).

Imprinting did not affect the enantioselectivity, with the exception of DCM and chloroform (decrease of

Table 3
Influence of solvent type on the activity and selectivity of C_8-acid-imprinted CRL

Solvent	log *P*[a]	Activation ratio[b]	Selectivity ratio[c]
DMF	−1	No activity	
Acetonitrile	−0.33	No activity	
Acetone	−0.23	No activity	
THF	0.49	No activity	
Pyridine	0.71	No activity	
Ether	0.85	3.0	1 ± 0.1
Methyl isobutyl ketone	1.3	2.0	1 ± 0.1
DCM	1.5	2.0	0.7 ± 0.1
Diisopropyl ether	1.9	2.8	1 ± 0.1
Chloroform	2.0	2.5	0.8 ± 0.1
Toluene	2.5	3.3	1 ± 0.1
Tetrachloromethane	3.0	1.9	1 ± 0.1
Hexane	3.5	1.1	1 ± 0.1
Isooctane	4.5	1.2	1 ± 0.1

[a] Values taken from [24].

[b] *V* (μmol/h mg protein) was calculated from the acetylation of the fast reacting enantiomer (*R*)-1-phenylethanol with vinyl acetate by both control and imprinted enzymes using.

[c] $E = V_{[R]}/V_{[S]}$. All solvents were dried on molecular sieves for 48 h prior to their use. Modified CRL was prepared according to description in legend of Fig. 1. Reaction conditions were: (*R*) or (*S*)-1-phenylethanol (0.5 mmol), vinyl acetate (1.5 mmol), solvent (5 ml) and CRL (140 mg) with shaking at 35 °C.

20–30% in the selectivity ratio). In these solvents, the activity of CRL was rather low, especially for the 5-enantiomer (0.16, 0.18 μmol/h mg protein for CRL in DCM and chloroform, respectively). This could have resulted in higher inaccuracies in the calculation of the selectivity.

Previous work on bio-imprinted hydrolases has been carried out in different solvents as isooctane [15], cyclohexane [10], or in solvent-free systems [13]. Nonetheless, the influence of solvent type on the activity of imprinted enzymes has not been described in the literature. We have shown a correlation between the enzymatic activity and the hydrophobicity (described by the log *P* value) of the solvent. The results indicated that the imprinted enzyme operated well in the more hydrophobic solvents in which its structure remained rigid. In highly hydrophobic solvents, like hexane and isooctane, the native enzyme was very active and imprinting with caprylic acid had only a negligible effect (5–15% increase in the activation). In solvents having moderate hydrophobicity (log *P*: 1.0–2.5), the activation following imprinting was most pronounced (200–300%). Imprinted CRL showed no activity in hydrophilic solvents (DMF, acetonitrile, etc.), as did the native enzyme. Hydrophilic solvents are known to strip water from the hydration shell of the enzyme thereby leading to inactivation [25]. In such solvents, with high dielectric constants, enzymes are more flexible and are liable to denaturation [26]. Imprinted enzymes operating in these solvents have no advantage over native enzymes, because the entire essence of imprinting stems from the rigidity of enzymes in organic solvents, in contrast to water. Therefore, in hydrophilic solvents, as in water, imprinting had no positive influence.

3.4. Bio-imprinting of soluble CRL with caprylic acid

The commercial CRL preparation is not completely soluble in water. In the bio-imprinting process, the entire enzyme mixture is lyophilized with the imprint molecule. We wanted to ascertain that the activation induced by caprylic acid is in fact connected to the enzyme and is not an artifact caused by the presence of insoluble compounds. The imprinting process was therefore performed with the soluble enzyme fraction obtained after centrifugation and removal of the precipitate. The results are described in Table 4.

It was discovered that imprinting of the soluble fraction of CRL with caprylic acid resulted in a 1.6-fold increase in activity compared to the non-treated control (for example, from 1.50 to 2.45 μmol/h mg protein for the (*R*)-1-phenylethanol). This implies that the activation induced as a result of imprinting is associated with the enzyme molecules.

The soluble fraction by itself was nearly inactive after lyophilization, and the addition of trehalose protected the enzyme to some extent during lyophilization. The soluble-imprinted enzyme was almost 20 times more active than the non-imprinted trehalose-protected CRL (for example, 2.45 versus 0.13 μmol/h mg protein for (*R*)-1-phenylethanol). This fact, among others, indicates that imprinting does not enhance the activity of the enzyme merely by protection during lyophilization.

Table 4
Transesterification of 1-phenylethanol with different kinds of CRL preparations

Enzyme preparation	$V_{[R]}$ (μmol/h mg protein)	$V_{[S]}$ (μmol/h mg protein)	Selectivity $V_{[R]}/V_{[S]}$
Soluble[a] and C_8-acid imprinted	2.45	1.16	2.1
Soluble	0.00	0.00	
Soluble and lyophilized with trehalose[b]	0.13	0.06	2.3
C_8-acid imprinted	5.30	2.30	2.3
Non-treated CRL	1.50	0.70	2.1

[a] CRL was dissolved in buffer and centrifuged the precipitate removed and imprinting performed on the clear aqueous phase according to the regular procedure.

[b] Trehalose (1:1 w/w with CRL) was added to the enzyme solution in order to protect it during lyophilization. Modified CRL was prepared according to description in the legend of Fig. 1. The protein content in each preparation was determined from elemental analysis and 15.5 mg protein were used in each experiment. The results represent the average of three different runs. Reaction conditions were the same as in Table 3 using diisopropyl ether as a solvent.

Another important observation was that the presence of insoluble material in the commercial enzyme preparation seemed to further improve the effect of bio-imprinting (the crude-imprinted enzyme was more active than the soluble-imprinted enzyme). The nature of the insoluble material is not specified by the supplier but could be stabilizing aids.

3.5. Combination of imprinting with immobilization on an inert support

The results obtained with soluble CRL, in comparison with the crude enzyme, led us to investigate the possibility of adding a support to the imprinted enzyme. Three lipases were imprinted with caprylic acid and celite, and evaluated in the model reaction. The results are shown in Table 5.

The enantioselectivity of the enzymes was not influenced by the addition of celite (results not shown). Celite by itself did not have a positive effect on the activity of PPL and CRL under the conditions used despite the common use of celite as an immobilization support. This could be the result of the relatively small ratio of celite/enzyme used. The addition of celite to lipase PSL did have a positive effect on its activity (nearly 10-fold). Imprinting with caprylic acid brought about an increase in activity as described previously. The addition of celite however, further enhanced the activity of the already activated enzyme by another two-fold factor.

The results presented in Table 5 indicate that combining imprinting with immobilization is a synergistic process that leads to further enhancement of the activity of lipases in organic solvents. It is likely that celite increases the effective surface area available to the enzyme, thus facilitating mass transfer of substrates and products. Another possible explanation is that the support provides a favorable micro-environment for the enzyme, thereby enhancing the catalytic activity [27]. Addition of cellulose had similar effects as celite (results not shown).

Table 5
Activity of lipases (μmol/h mg protein) imprinted with caprylic acid and immobilized onto celite in the transesterification of 1-phenylethanol

Enzyme	C_8 imprinted	C_8 + celite imprinted	Celite	Non-treated control
PPL	2.3	4.2	0.48	0.61
CRL	3.3	6.6	0.30	0.52
PSL	1400	2600	1200	138

Protein content of enzymes according to elemental analysis: CRL, 8%; PSL, 4.8%; PPL, 27%. Experiments were performed in duplicates. Modified lipases were prepared according to the description in legend of Fig. 1. Amount of celite incorporated: for CRL and PPL 200 mg celite/300 mg crude enzyme, PSL 25 mg/30 mg crude enzyme. Reaction conditions were the same as described in Table 4.

3.6. Successive recycling of bio-imprinted PPL

It was by this time established, that imprinting with fatty acids (with or without a support), activates lipases in organic solvents. Nonetheless, activation alone is usually not sufficient for achieving economic feasibility of a process. High enzymatic stability under reaction conditions is an essential prerequisite. It was therefore important to evaluate the stability of the imprinted enzymes using successive transesterification reactions.

Each reaction cycle was conducted for 5 h after which the enzyme was washed with solvent and reused in the transesterification of 1-phenyl ethanol. The conversion in the first cycle was considered as 100%. Fig. 2 describes the performance of C_8-acid-imprinted PPL, with and without the addition of celite.

It is evident from the results that the combination of imprinting with immobilization contributes to the stability of the enzyme as well as to the activity. The non-treated control lost 80% of its initial activity by the end of 20 cycles, whereas the C_8-acid-imprinted PPL adsorbed on celite, lost only 30% of its activity. This is a major improvement in enzyme performance.

The immobilized-imprinted enzyme exhibited up to 20-times higher activity, as well as, greater operational stability compared with that of the non-treated, crude enzyme. It has been proven lately, that good enzyme dispersion, achieved by immobilization or by modification with PEG, was a crucial parameter for the expression of high lipase activity in organic solvents [28]. Adsorption of the enzyme onto microporous matrices not only improves enzyme dispersion, but also reduces diffusional limitations and favors substrate access to individual enzyme molecules [29]. CRL imprinted with menthol, followed by entrapment in sol–gel [20] is the only other example in the literature in which the activity of an imprinted lipase

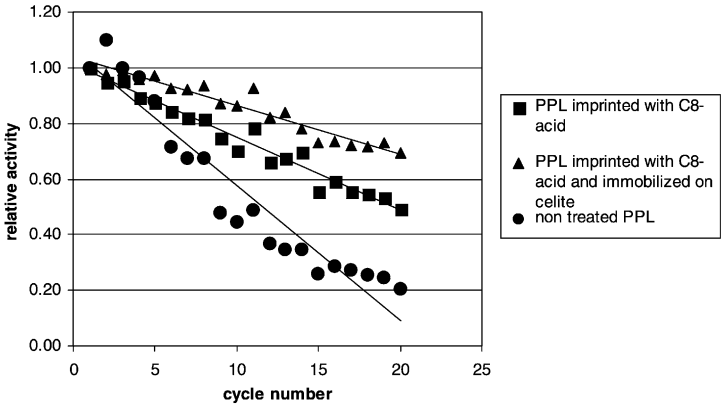

Fig. 2. Operational stability of PPL preparations catalyzing a transesterification reaction. Imprinting of PPL was performed as described in Fig. 1. Reaction conditions were 1 mmol 1-phenylethanol, 3 mmol vinyl acetate, 10 ml diisopropyl ether and 300 mg PPL, with shaking at 35 °C. Each cycle lasted 5 h.

was further enhanced by immobilization. In two other cases [16,30], the immobilization was performed by cross-linking of the pre-derivatized enzyme, resulting in enhanced stability but with a decrease in activity. Furthermore, these complex enzyme preparations were used in aqueous media, in contrast to the current imprinted and adsorbed lipase which has been utilized in organic solvents. High operational stability is of great importance for commercial use of enzymes. We have shown high stability of imprinted-immobilized PPL in 20 consecutive cycles of operation. Thus, despite the sensitivity of the system, proper use of dry solvents and maintenance of reaction conditions, can result in high activity for long-term, repetitive use.

4. Conclusions

Bio-imprinting of lipases with fatty acids was shown to be a simple, effective method for obtaining highly active enzymes in organic solvents. The imprinted lipases exhibited increased 3–10-fold activity in the acetylation of 1-phenylethanol with vinyl acetate. An increase in the fatty acid chain length from C_6-acid to C_{12}-acid had a positive influence on the activity, but no influence on the enantioselectivity compared to the crude enzyme. The extent of activation induced by imprinting CRL with caprylic acid was dependent on the solvent type, ranging from 1.05- to 2.3-fold for a wide range of solvent polarity spectrum. Addition of celite during the imprinting process further enhanced the activity of the imprinted enzyme by an additional two-fold factor, whereas celite by itself had a negligible influence on the activity. PPL, imprinted with caprylic acid and celite, retained 70% of its initial activity at the end of 20 consecutive reaction cycles, compared to only 20% residual activity for the non-treated control. It can therefore be concluded that the combination of imprinting with fatty acids, and a simple immobilization technique such as adsorption, can lead to highly active and stable enzymes in organic solvents.

References

[1] S.C. Stinson, Chem. Eng. News 79 (2001) 45.
[2] A. Zaks, Curr. Opin. Chem. Biol. 5 (2001) 130.
[3] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Nature 409 (2001) 258.
[4] A. Klibanov, Nature 409 (2001) 241.
[5] J.O. Rich, V.V. Mozhaev, J.S. Dordick, D.S. Clark, Y.L. Khmelnitsky, J. Am. Chem. Soc. 124 (2002) 5254.
[6] G. Wulff, Angew. Chem. Int. Ed. Engl. 34 (1995) 1812.
[7] C.J. Slade, E.N. Vulfson, Biotechnol. Bioeng. 57 (1998) 211.
[8] K. Faber, Biotransformations in Organic Chemistry, fourth ed., Springer-Verlag, Berlin, 2000, pp. 400–401.
[9] A.J. Russell, A.M. Klibanov, J. Biol. Chem. 263 (1988) 11626.
[10] M. Stahl, U. Wistrand, M. Mansson, K. Mosbach, J. Am. Chem. Soc. 113 (1991) 9366.
[11] J. Rich, J.S. Dordick, J. Am. Chem. Soc. 119 (1997) 3245.
[12] I. Mingarro, C. Abad, L. Braco, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 3308.

[13] H. Gonzalez-Navarro, L. Braco, J. Mol. Catal. B: Enzymatic 3 (1997) 111.
[14] S. Ozawa, A.M. Klibanov, Biotechnol. Lett. 22 (2000) 1269.
[15] Y. Okahata, A. Hatano, K. Ijiro, Tetrahedron: Asymmetry 6 (1995) 1311.
[16] F. Peisker, L. Fischer, Bioorg. Med. Chem. 7 (1999) 2231.
[17] A. Fishman, S. Basheer, S. Shatzmiller, U. Cogan, Biotechnol. Lett. 20 (1998) 535.
[18] A. Louwner, G.J. Drtina, A.M. Klibanov, Biotechnol. Bioeng. 50 (1995) 1.
[19] H. Noritomi, O. Almarsson, G.L. Barletta, A.M. Klibanov, Biotechnol. Bioeng. 51 (1996) 95.
[20] S. Furukawa, T. Ono, H. Ijima, K. Kawakami, J. Mol. Catal. B: Enzymatic 15 (2001) 65.
[21] A. Zaks, in: A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Blackie, London, 1996, pp. 70–94.
[22] Z. Yang, A.J. Russell, in: A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Blackie, London, 1996, pp. 43–69.
[23] Y.L. Khmelnitsky, A.B. Belova, A.V. Levashov, V.V. Mozhaev, FEBS Lett. 284 (1991) 267.
[24] C. Laane, S. Boeren, K. Vos, C. Verger, Biotechnol. Bioeng. 30 (1987) 81.
[25] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 3194.
[26] R. Affleck, C.A. Haynes, D.S. Clark, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5167.
[27] P. Aldercreutz, in: A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Blackie, London, 1996, pp. 9–42.
[28] F. Secundo, S. Spadaro, G. Carrea, P.L.A. Overbeeke, Biotechnol. Bioeng. 62 (1999) 554.
[29] O. Carrea, S. Riva, Angew Chem. Int. Ed. Engl. 39 (2000) 2226.
[30] N.A.E. Kronenburg, J.A.M. de Bont, L. Fischer, J. Mol. Catal. B: Enzymatic 16 (2001) 121.