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Pegylated enzyme entrapped in poly(vinyl alcohol) hydrogel for biocatalytic application

Francesco Maria Veronese [a,*], Cristina Mammucari [a], Franco Schiavon [a], Odolone Schiavon [a], Silvano Lora [a], Francesco Secundo [b], Adriana Chilin [b], Adriano Guiotto [b]

[a] *Department of Pharmaceutical Sciences, University of Padua, Center of Chemical Investigation of Drugs CNR, Padova Via Marzolo n. 5, 35131 Padua, Italy*

[b] *Institute of Biocatalysis and Molecular Recognition CNR, Via Mario Bianco n. 9, 20131 Milan, Italy*

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Abstract

A procedure for enzyme entrapment into matrices suitable for biocatalytic applications is reported. The method, which takes advantage of the stable formation of polyvinyl alcohol (PVA) hydrogels by freezing and thawing PVA aqueous solutions, was assayed using lipase as model enzyme. The leakage of lipase was minimised by using high molecular weight PVA and by previous conjugation of the enzyme to PEG.

The immobilised PEG enzyme maintained its catalytic activity in organic solvents also, thus allowing enzymatic activity towards water insoluble substrates. The activity was largely increased reducing the diffusional constrain by cutting the matrices into slices of micron size. Matrix-entrapped lipase-PEG, when used in the hydrolysis of acetoxycoumarins, showed a conversion rate of about 10 times lower than the enzyme-PEG in the free form, and maintained regioselectivity when a diacetylated product was used as substrate.

Keywords: PEG; PVA; Poly(ethylene glycol); Poly(vinyl alcohol); Enzyme immobilisation; PVA hydrogels; Lipase; Biocatalysis; Coumarins; Regioselectivity

1. Introduction

Organic synthesis received an unexpected acceleration from the use of enzymes as catalysts, due to their high rate of conversion, specificity, regio- and, frequently, stereo-selectivity [1]. Moreover, the use of enzymes in solid state finely dispersed in organic solvents, as first described by Klibanov [2,3], widened its application to the conversion of water insoluble compounds. More recently, solubility and activity of enzymes in organic solvents was also achieved by covalent linking to amphiphilic polymers, poly(ethylene glycol) (PEG) in particular [4–6]. The extensive use of enzymes as catalysts was related to the possibility of their easy recovery from the reaction mixture at the end of the reaction, thus allowing a further catalytic step; this possibility is achieved by linking the enzymes to an insoluble matrix. In the majority of cases, the enzyme is covalently linked to an organic polymer, either natural or synthetic, but important examples of physical entrapment have also been reported [7]. The entrapment into polysaccharides, proposed by Chribata [8], is an example of successful industrial application of the latter technology. Unfortunately, the immobilisation by covalent binding is generally accompanied by a loss of enzyme activity, and it is always a delicate and tedious process. On the other hand, physical entrapment is limited to aqueous operating conditions and is often accompanied by enzyme leakage.

Poly(vinyl alcohol) (PVA) presents the unusual property of affording stable hydrogels, through extremely mild freezing and thawing conditions of its aqueous solutions, a property that was exploited for the preparation of new biomaterials and for drug release [9–14].

* Corresponding author. F.M. Veronese, Dipartimento di Scienze Farmaceutiche via Marzolo, 5, 35131 Padova.

E-mail address: Francesco.Veronese@unipd.it (F.M. Veronese).

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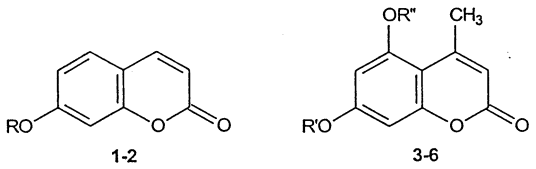
We recently demonstrated that the release of biomolecules from PVA hydrogels can be modulated by the polymer molecular weight, and/or, most importantly, by linking hindered PEG chains to the entrapped products [15]. This method allowed the preparation of new biosensors based on PVA with entrapped glucose oxidase, choline oxidase, acethylcoline esterase, enzymes that by themselves would escape from the gels [16,17].

On the basis of these prerequisites, we are now reporting the preparation of PVA hydrogels with entrapped lipase, previously conjugated to PEG, for the bioconversion of a monoacetoxycoumarin and a diacetoxycoumarin to the corresponding hydroxycoumarins, which are important synthons in the coumarin syntheses [18,19]. A diacetylated coumarin was chosen as substrate, in which two esterified functions with different steric surroundings are present. Organic solvents were used to solubilise the substrates, thanks to the possibility to equilibrate the PVA hydrogels with *n*-hexane without losing their physical properties and by conveying lipophilicity to lipase by PEG conjugation. This study further demonstrated the great potential of PEG to overcome physical and chemical problems in enzyme technology [20–22].

2. Experimental

2.1. Reagents

Lipase B from *Candida Antarctica* (MW 35 000) [23] was kindly supplied by Dr. G. Carrea, CNR, Milan. Poly(vinyl alcohol) (PVA) 31–50 kDa, 124–186 kDa (99% hydrolyzed) were purchased from Aldrich Chemie (Stenheim, Germany). Monomethoxypoly(ethylene glycol)-norleucine (PEG-Nle) 5 kDa hydroxysuccinimide ester was obtained from Shearwater Polymers (Huntsville, USA). *p*-Nitrophenylbutyrate was obtained from Sigma (St. Louis, USA). *n*-Hexane was obtained from Lab-Scan (Dublin, Ireland). 7-Hydroxy-cumarin (**1**), 7-acetoxy-coumarin (**2**), 5,7-dihydroxy-4-methylcoumarin (**3**), 5,7-diacetoxy-4-methylcoumarin (**4**), 5-acetoxy-7-hydroxy-4-methylcoumarin (**5**) and 7-acetoxy-5-hydroxy-4-methylcoumarin (**6**) (see Scheme 1) were synthesised in our laboratory and their synthesis will be reported elsewhere (Guiotto et al., personal communication). SDS-page reagents were purchased from Biorad (Richmond, CA, USA), solvents and salts were Merck products (Darmstadt, Germany).



	R	R'	R"
1	H	-	-
2	Ac	-	-
3	-	H	H
4	-	Ac	Ac
5	-	H	Ac
6	-	Ac	H

Scheme 1.

2.2. Equipment

Spectrophotometric measurements were carried out with a Perkin–Elmer (Norwalk, CT, USA) LAMBDA 5 Spectrophotometer. The ultrafiltration were performed by an ultrafiltration apparatus, Amicon (Passirana di Rho, Milan, Italy), equipped with a 30 kDa cut-off membrane. A gel filtration column operated with a Pharmacia FPLC system was used for the conjugates purification. For the activity measurements of immobilised lipase, the matrices were maintained under stirring at room temperature, using a floating stirrer of Bicasa (Milano, Italy). HPLC analyses were performed on a Shimadzu LC-10 AT instrument, equipped with SPD-10 A UV–Vis detector. A Lichrocart RP18 column was used, eluting with H_2O/CH_3CN, 60:40; flow rate 0.3 ml min^{-1}.

2.3. Lipase modification by PEG

Lipase was dissolved in 0.2 M borate buffer at pH 8.0 and PEG-Nle 5 kDa activated as hydroxysuccinimidylester was added as dry powder in two portions. The amount of PEG was in a tenfold molar excess over lipase amino groups. After 2 h the reaction mixture was washed with water and reduced to 5 ml by ultrafiltration (30 kDa membrane). This procedure was repeated five times. Alternatively, the reaction mixture was fractionated by GPC and ultrafiltered. The activity of lipase-PEG was determined as follows: 100 μl of *p*-nitrophenylbutyrate solution in isopropanol was diluted to 1 ml with buffer (0.02 M NaH_2PO_4, 0.9% NaCl, pH 7.0) and the reaction was initiated by addition of an aliquot of the lipase-PEG solution. The increase of absorbance at 410 nm against buffer for a period of about 5 min was recorded. The lipase-PEG activity was compared to the native lipase activity evaluated under the same experimental conditions. The unreacted amino groups following the PEG conjugation were evaluated by colorimetric assay [24].

2.4. Lipase immobilisation and hydrogel preparation

An aqueous solution of 20% PVA was heated at 105°C in a closed vial for 6 h under gentle stirring and

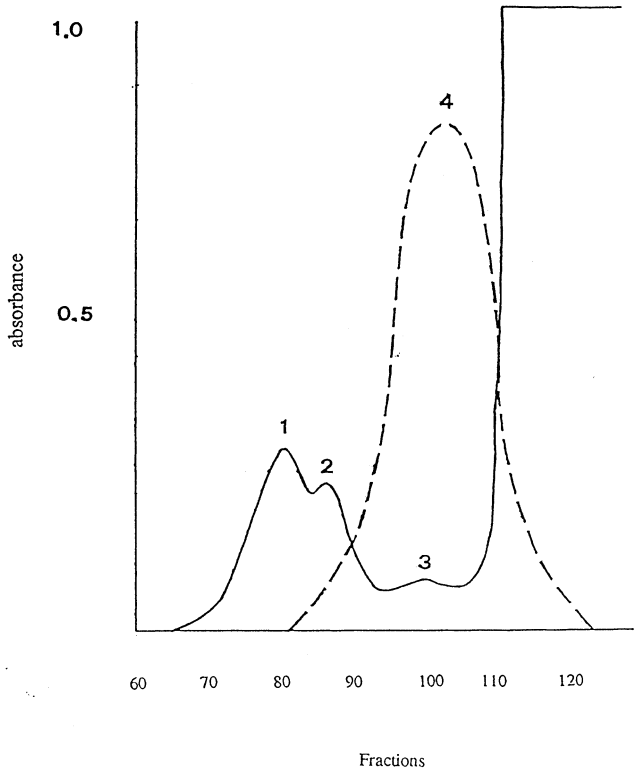


Fig. 1. FPLC gel filtration pattern of the lipase PEGylation mixture as revealed by 280 nm absorption for lipase and lipase-PEG detection, and iodine reaction for PEG: (1) PEGylated lipase first peak; (2) PEGylated lipase second peak; (3) unreacted lipase; (4) unreacted PEG.

then cooled at room temperature. Lipase-PEG (2 mg, expressed as enzyme content), was added under stirring and the mixture was poured into a mould of the desired shape. The mixture was frozen at −25°C for 4 h. The mould was warmed up to room temperature for 30 min to thaw the PVA-lipase solid. The process of freezing and thawing was repeated five times. Hydrogel cylinders of 5 mm diameter and 2.5 mm thickness were obtained. The hydrogels were soaked many times in the solvent used for the measurements of the enzyme activity in order to reach equilibrium with the external solvent. For the experiments of acetoxycoumarin bioconversion, cylinders of 1.5 cm diameters and 0.3 cm thicknesses, containing 0.5 mg of lipase-PEG (expressed as enzyme content) were used.

2.5. Activity of PVA entrapped lipase-PEG

The activity of lipase-PEG entrapped in PVA hydrogel cylinders was determined spectrophotometrically. In this way, *p*-nitrophenylbutyrate was dissolved in isopropanol, the reaction was started by dipping the lipase-PEG containing hydrogel into the substrate solution and the incubation mixture was maintained under stirring. At different time intervals, 50 μl of the reaction mixture were removed, diluted with the buffer (0.02 M NaH_2PO_4, 0.9% NaCl, pH 7.0) and the activity measured by monitoring the absorbance increase at 410 nm.

The experiment was repeated by dipping the carefully washed matrix into a fresh reaction mixture. To reduce the diffusional constraints, the hydrogels were lyophilised and cut with a microtome maintained at −20°C, into 8 μm thick slices. The slices were maintained in *n*-hexane/isopropanol (10:1), under delicate stirring. At different time intervals, portions (50 μl) of the supernatant were diluted in the phosphate buffer and the absorbance was measured at 410 nm as above reported.

2.6. Matrices dimension in different solvents

Cylindrical matrices of 1.5 cm diameter and 0.3 cm thickness, prepared as reported above, were dipped into various solvents and incubated for 48 h, exchanging the external liquid every 8 h. The solvent was then removed, the matrices were gently wiped with a paper towel, and their dimension recorded.

2.7. Evaluation of acetoxycoumarin hydrolysis

7-Acetoxycoumarin (**2**) and 5,7-diacetoxy-4-methylcoumarin (**4**) were dissolved in *n*-hexane/isopropanol, 7:1, at a 10^{-5} M concentration. To 5 ml of the coumarin solution a matrix with entrapped lipase-PEG (1.5 cm diameter × 0.3 cm thickness) was added and the mixture was shaken at 36°C. Alternatively, free lipase-PEG was also added. From the incubating mixture, 250 μl samples were drowned at scheduled times and assayed by HPLC or UV absorption. In particular, **2** was evaluated by UV absorption only, while for **4** evaluation samples were withdrawn, concentrated to dryness, dissolved in H_2O/CH_3CN and analysed by HPLC under the conditions reported above.

3. Results and discussion

3.1. PEG conjugation of lipase

According to colorimetric assay, 60% of lipase amino groups appeared to be modified by PEG conjugation. The conjugation mixture, fractionated by gel filtration, showed the presence of two peaks, centred at fractions 80 and 87, respectively, while free lipase was eluted within a third peak centred at fraction 102, as shown in Fig. 1. The fractions of the two peaks, pooled separately and examined by SDS electrophoresis, showed the pattern reported in Fig. 2. From the migration, by comparison with proteins standard, an apparent molecular weight of 65 and 50 kDa, respectively, was calculated for the two samples of lipase-PEG. This

corresponds to a linking of approximately six and three PEG chains. The amount of bound PEG in the first peak fraction could also be evaluated by its norleucine content, obtained by amino acid analysis after acid hydrolysis [25]. A number of 7.3 polymer chains were found, in agreement with the value obtained by colorimetric and electrophoretic analysis. Unfortunately, the second peak could not be examined by amino acid analysis, since it contained an excess of unbound PEG due to the partial overlapping of the lipase-PEG and free PEG in the chromatogram.

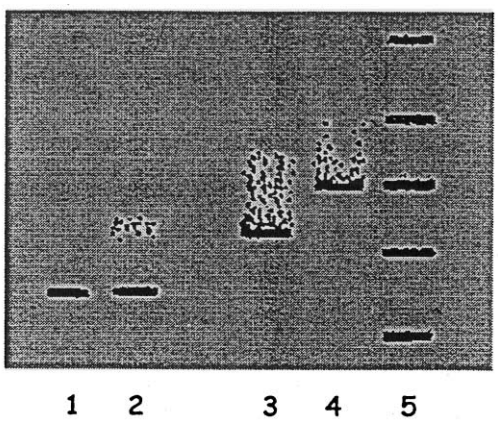


Fig. 2. SDS electrophoresis of the two lipase-PEG fractions and of standard proteins: (1) lipase reference standard; (2) unreacted lipase, third peak of Fig. 1; (3) lipase-PEG, second fraction of Fig. 1; (4) lipase-PEG first fraction; and (5) reference standard proteins of 175, 93, 67, 47.5, 32.5 kDa.

Table 1
PVA-hydrogel shrinking (%) following soaking in different solvents

Solvent	Shrinking (%)	
	20 (h)	130 (h)
Phosphate buffer	0	4.2
n-Hexane/isopropanol 10:1	0	5
Phosphate buffer/isopropanol 1:9	29.2	33.4
n-Butanol	31.7	33.4
Benzene	4.2	12.5
Dichloromethane	12.5	40.8
Ethyl acetate	33.4	38.3

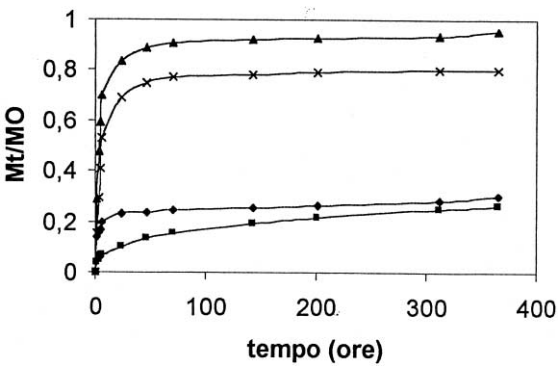


Fig. 3. Leakage from PVA hydrogel of (▲) lipase, (×) lipase-PEG from 31–50 kDa PVA, and (◆) lipase and (■) lipase-PEG from 124–186 kDa PVA. M_t: total amount of released enzyme at time t; M_o: total amount of enzyme in the hydrogel.

Table 2
Activity of PEG-lipase immobilised in PVA hydrogel matrix (1.5 cm diameter × 0.3 cm thickness), in different solvents

Reaction solvent	Activity [a] (%)
Buffer pH 7.00	100
Buffer pH 7.00/isopropanol 1:9	5.66
n-Hexane/isopropanol 10:1	7.66

[a] The activity of immobilised lipase-PEG in solvent was calculated after six washings and is relative to that found in phosphate buffer. The substrate was *p*-nitrophenylbutyrate.

For the experiments reported below, the pooled lipase-PEG mixture of the two peaks was employed. It was found to possess an activity, evaluated towards the substrate *p*-nitrophenylbutyrate, superior to the native one (about 120%). This behaviour, already observed in conjugates of the enzymes with PEG and with low molecular weight reagents, was generally ascribed to increase in substrate affinity or subtle positive modifications at the active site geometry [26].

3.2. Lipase-PEG entrapment in PVA hydrogels and activity evaluation

Lipase-PEG entrapment into PVA was carried out following a procedure that, with other enzymes, was found suitable for the maintenance of the native conformation and activity. As a preliminary investigation, several solvents were evaluated as potential organic medium for PVA gels incubation. The shrinking of the gel, after 20 and 130 h of soaking in different solvents, was evaluated and referred to the dimension of the gel in water (see Table 1). In *n*-hexane/isopropanol the dimensions remained practically the same as in the phosphate buffer, minor shrinking occurred in benzene, while it was high in all the other tested solvents.

The effect of the molecular weight of PVA and of PEG conjugated to lipase in the release of the enzyme from the matrix was evaluated. The large influence of both parameters in preventing lipase leakage is shown in Fig. 3 and it was found in agreement with what has been already reported for other proteins [15].

The activity of the entrapped lipase-PEG carried out in the phosphate buffer, buffer/isopropanol, and *n*-hexane/isopropanol as solvents is reported in Table 2. It appears that the solvent used is critical for the enzyme activity. Furthermore, the loss of entrapped lipase-PEG took place at the first change of the incubation solvent, to reach an amount of lipase-PEG permanently entrapped that corresponds to 37% of the starting one (see Table 3a).

Since diffusion could be the major reason for the decreased activity of entrapped lipase, an experiment was carried out after reducing the thickness of the hydrogel to 8 μm size. As in the experiment reported in Table 3b, *n*-hexane/isopropanol, 10:1, was used as solvent. At intervals the hydrogel was removed from the substrate solution, and soaked into a new one, before the activity was measured again. This process was repeated seven times. After three changes of the substrate solution the activity remained constant to about one half of the starting value (see Table 3b). The increase in activity following matrix slicing is impressive, about 100 times greater than the original. This result demonstrates the great influence of diffusional constraints that can be successfully minimised by reducing the thickness of the gel slices.

The use of organic solvents in these experiments demonstrated also the suitability of the immobilisation procedure to carry out biocatalysis in organic environment.

3.3. Acetoxycoumarin hydrolysis

(a) Comparisons between free and gel entrapped lipase-PEG activity. The hydrolysis rate of 7-acetoxycoumarin (**2**) in *n*-hexane/isopropanol could be evaluated by UV absorption since a decrease of absorption at 280 nm accompanied by an increase of absorption at 310 nm was observed (see Fig. 4). A comparison between the rate of the free and immobilised lipase-PEG under the same experimental conditions demonstrated that the free enzyme is approximately 10 times more active than the gel-entrapped one. Taking into account the loss of enzyme that occurs during the washings needed to reach the permanently entrapped enzyme into the matrix we could in fact calculate that the specific activity of free lipase-PEG towards 7-acetoxycoumarin (**2**) is 0.43×10^{-3} μM h^{-1} while that of the entrapped enzyme is 0.072×10^{-3} μM h^{-1}. According to the experiments reported above, such reduction of activity may be mainly related to the diffusion limitations.

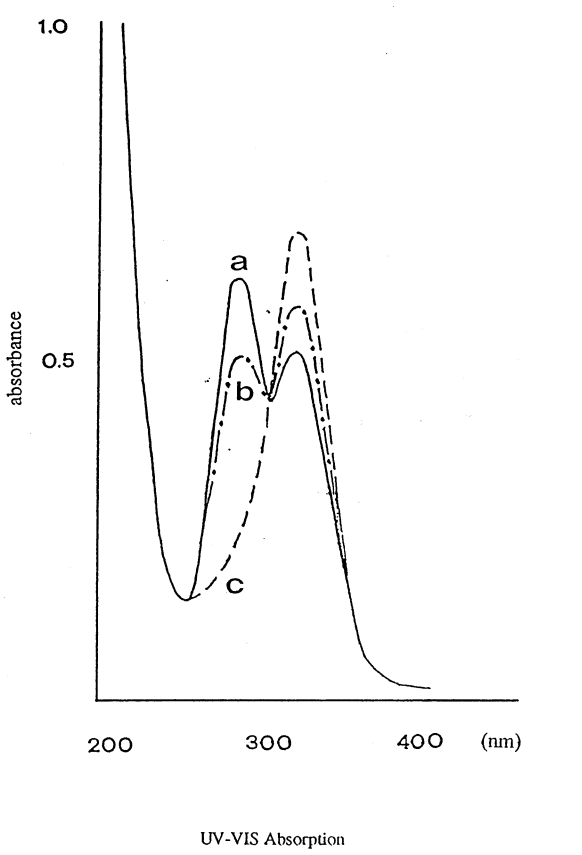


Fig. 4. Time course of 7-acetoxycoumarin (**2**) hydrolysis operated by lipase-PEG hydrogels and monitored by UV absorption. 7-Acetoxycoumarin after (a) 45 min incubation; (b) 4 h incubation; and (c) at the end of hydrolysis (21 h).

Table 3
Activity of PEG-lipase immobilised into PVA hydrogels (1.5 cm diameter × 0.3 cm thickness) in *n*-hexane/isopropanol, 10:1, following repeated incubation before (a), and after cutting the same matrix in micron size slices (b)

	Number of incubations	Activity (%)
(a) Before cutting [a]	First	100
	Second	76.78
	Third	30.63
	Fourth	35.57
	Fifth	36.56
	Sixth	37.05
(b) After cutting [b]	First	9061
	Second	9680
	Third	6272
	Fourth	4330
	Fifth	4128
	Sixth	4422
	Seventh	4294

[a] The activity following incubations is referred to the freshly prepared matrix. After each incubation the matrix is gently washed with fresh incubation mixture. The substrate was *p*-nitrophenylbutyrate and the solvent was *n*-hexane/isopropanol, 10:1.

[b] The matrices were cut with a microtome at −20°C to obtain 8 μm thick slices. The percentage of activity of the sliced matrix is calculated assuming 100 as the activity of the unsliced one from the sixth incubation, when no more enzyme leakage occurs. At the seventh washing an increase in activity of 100 fold, due to the slicing, could be calculated.

*(b) Regioselectivity in the 5,7-diacetoxy-4-methylcoumarin (**4**) hydrolysis reaction*. 5,7-Diacetoxy-4-methylcoumarin (**4**) was incubated with the immobilised enzyme and the reaction mixture was examined by HPLC, since the multiplicity of the products did not allow direct monitoring of the reaction by UV absorption. Four products were revealed by HPLC during the time course of the catalysis, corresponding to the starting product, eluted at 14.97 min, the fully deacetylated 5,7-dihydroxy-4-methylcoumarin (**3**), eluted at 5.75 min

and the two monoacetylated coumarins 7-acetoxy-5-hydroxy-4-methylcoumarin (**6**) and 5-acetoxy-7-hydroxy-4-methylcoumarin (**5**), eluted at 7.92 and 9.36 min, respectively (see Fig. 5).

After 150 h the reaction slows down and three products are present, the two monoacetoxy-monohydroxy-coumarines and the fully deacetylated dihydroxy-coumarin. In Fig. 6 the time course of the enzymatic reaction, as obtained from the HPLC analysis, is shown. It appears that a preferential regiospecificity was achieved in favour of the formation of the product deacetylated in position 5. This seems to indicate that the hydrolysis of the second acetyl group is a slow process, the monoacetylated compounds being worse enzyme substrates than the diacetylated one.

4. Conclusions

The most striking information coming out from this study was the demonstration that PEGylated lipase, entrapped in poly(vinyl alcohol) hydrogel by freezing and thawing, can be conveniently used in organic solvent biocatalysis. This enzyme immobilisation method is much more convenient in comparison to other type of immobilisation, that take advantage of enzyme covalent linkage to insoluble matrix, since the chemical step which is time consuming and harmful to enzyme activity, is avoided.

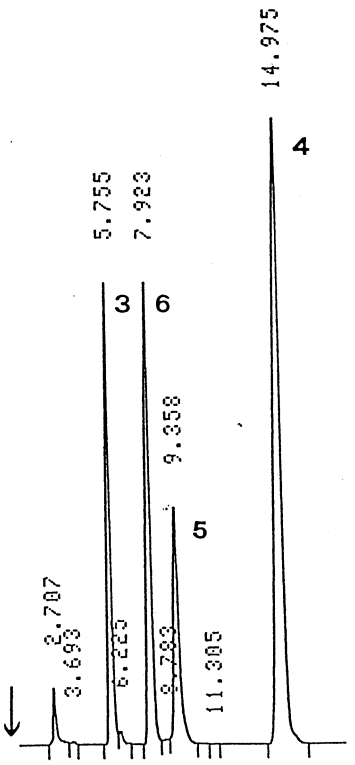


Fig. 5. HPLC analysis of the hydrolysis mixture of 5,7-diacetoxy-4-methylcoumarin: 5,7-dihydroxy-4-methylcoumarin (**3**), 7-acetoxy-5-hydroxy-4-methylcoumarin (**6**), 5-acetoxy-7 hydroxy-4methyl-coumarin (**5**) and starting 5,7-diacetoxy-4-methylcoumarin (**4**).

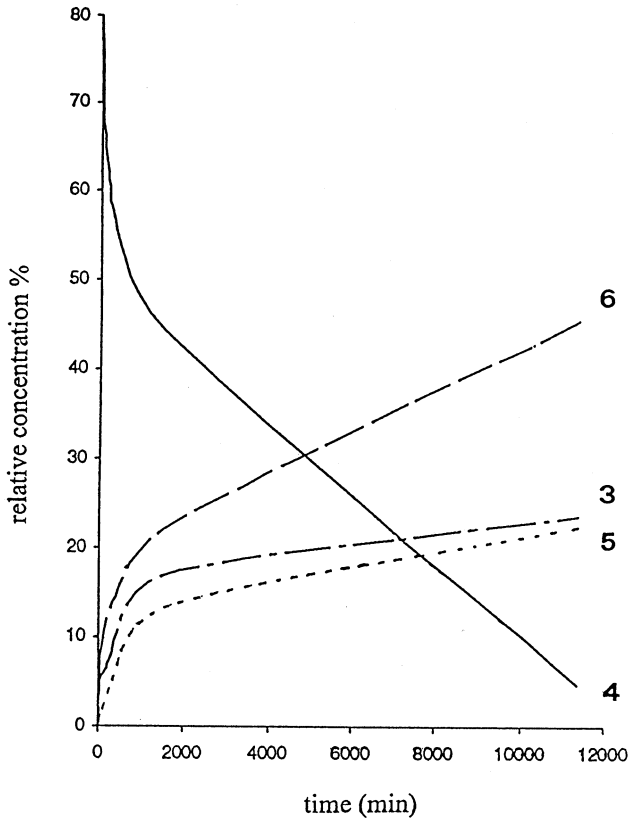


Fig. 6. Time course of disappearance of 5,7-diacetoxy-4-methylcoumarin (**4**) and formation of the completely deacetylated product (**3**), and of the products of **6** and **5**.

Furthermore, it was found that non-aqueous solvents are compatible with gel stability and enzyme activity, thus allowing a choice for easy dissolution of the products of interest.

The PEGylation step is a straightforward procedure known for many enzymes, which is characterised by the high maintenance of activity [14–16]. This modification is the critical step to increase the enzyme size needed to improve retention inside the gel and to convey at the same time the solubility of the enzyme inside the matrix.

As expected, the entrapment inside the gel reduces the catalytic rate of lipase to about one order of magnitude, due to diffusional constraints. However, these limitations can be minimised by a proper slicing of the gel matrix.

Finally, the application of the lipase bioconversion to diacetoxycoumarin derivatives demonstrated the feasibility of the proposed method in the hydrolysis of products of pharmaceutical interest and to obtain regioselective enrichment of one of the two monodeacetylated derivatives.

Application of the PEGylated gel entrapped enzymes with different specificity is presently under investigation and will be reported separately.

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