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Different phyllosilicates as supports for lipase immobilisation

Isidoro Emilio de Fuentes [a], Cesar Antonio Viseras [b], Daniela Ubiali [c], Marco Terreni [c], Andrés Rafael Alcántara [a,*]

[a] *Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain*
[b] *Departamento de Farmacia y Technología Farmacéutica, Facultad de Farmacia, Universidad de Granada, Granada, Spain*
[c] *Pharmaceutical Biocatalysis Laboratories, Department of Pharmaceutical Chemistry, University of Pavia, Pavia, Italy*

Abstract

The aim of this work was to determine the enzymatic activities resulting from the adsorption of *Rhizomucor miehei* lipase (RML) and *Candida cylindracea* lipase (CCL) onto three different phyllosilicates (sepiolite, palygorskite and montmorillonite), comparing the resultant activities with those obtained following similar immobilisation technique on a widely used resin (Duolite A-568). Due to the different adsorption mechanisms produced, different derivatives with higher hydrolytic activities can be obtained. Comparing the clays tested, the results showed that, in comparison with the laminar silicate (montmorillonite sample) and Duolite A-568 (spherical particles), fibrous materials (palygorskite and sepiolite) resulted in derivatives with higher hydrolytic activities in the hydrolysis of different ethyl esters. Moreover, according to the data obtained with the electrophoresis, the selectivity of immobilisation for RML in the case of fibrous silicates was optimal. As a conclusion, and according to the activities and selectivities measured, at least two out of the four studied materials (sepiolite and palygorskite) would be useful as supports for immobilisation for proteins of relatively low molecular weight (such as RML) for further use in biotransformations, while for *C. cylindracea* the immobilisation onto duolite rendered a derivative specially active in the hydrolysis of ethyl formiate (esterasic activity).

Keywords: *Rhizomucor miehei* lipase; *Candida cylindracea* lipase; Phyllosilicates; Immobilisation

1. Introduction

Extensive studies have been made in recent years in order to obtain immobilised lipase derivatives suitable for different reactions [1–12]. Among the several methods of immobilisation, adsorption (resulting from both hydrophobic [2,8] or hydrophilic [3–6,13] interactions) is the method which induces less modifications on the active conformation of the enzymes [1,4]. Consequently, adsorption is one of the preferred methods for enzyme immobilisation.

The use of phyllosilicates, mainly sepiolite, to immobilise enzymes was described some years ago [13,14]; nevertheless, silicate-type materials have been usually used in a two-step covalent immobilisation method, which involves the previous activation of the clay and a posterior covalent binding of the enzyme [3,7,14].

The employ of silicates as lipase carriers presents numerous advantages, such as the high specific sur-

* Corresponding author. Tel.: +34-91-3941820; fax: +34-91-3941822.
E-mail address: andresr@eucmax.sim.ucm.es (A.R. Alcántara).

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face available (between 200 and 800 m^2/g), the facility of water dispersion/recuperation, the high water uptake capacity, and the excellent mechanical resistance of these materials [15]. Last, but not least, their natural origin and their low cost make them even more attractive from an applied point of view.

Due to all these reasons, the aim of this work was to determine the activities resulting from the adsorption of *Rhizomucor miehei* lipase (RML) and *Candida cylindracea* lipase (CCL) onto different phyllosilicates (sepiolite, palygorskite and montmorillonite), comparing the resultant activities with those obtained through the immobilisation onto a widely used anionic exchange resin (Duolite A-568).

A scanning electron microscopy (SEM) analysis of all supports used was carried out. The selected test reaction was the hydrolysis of ethyl esters of linear carboxylic acids with different chain length. These substrates were chosen because of its relevance in the food industry [3].

2. Materials and methods

2.1. Enzymes and chemicals

Crude RML (Lipozyme 10 000L) was kindly donated by Novo-Nordisk Industries (Bagsvaerd, Denmark); *C. cylindracea* was purchased from Sigma (Alcobendas, Madrid, Spain); ethyl formiate, ethyl butyrate, ethyl capriate and tributyrin were purchased from Fluka Chemic (Buchs, Switzerland); the rest of the chemicals were used without previous purification.

2.2. Supports

Three natural phyllosilicates were studied: sepiolite from Vicalvaro (Madrid, Spain), palygorskite from Turón (Ciudad Real, Spain) and montmorillonite (bentonite) from Cabo de Gata (Almeria, Spain). All samples were kindly supplied by Tolsa (Madrid, Spain). For each sample, three dry sieve fractions between 75 and 125 μm were selected and then stored at 40% relative humidity at room temperature (22–25°C) for at least 48 h before any study was performed. Finally, Duolite A-568 [16] from Rohm and Haas (Paris Cedex) was included for comparative purposes, as a typical anionic exchange resin, which have been used for obtaining a widely used RML-based immobilised catalyst (Lipozyme IM [17]).

2.3. Enzyme immobilisation

The adsorption of the enzymes (RML and CCL) on the different supports was carried out as follows: Fixed amounts of enzyme and previously washed support were added to a flask containing 20 ml of the washing buffer (phosphate buffer 0.1 M pH = 6 for RML or pH = 7 for CCL), and stirred at room temperature for 24 h. After this time, samples were filtered and stored at −15°C before use.

The protein content of the initial enzymatic solution and filtrate solutions were determined (to calculate the amount of bounded protein) according to Bradford's method, using bovine serum albumin (Alcobendas, Madrid, Spain) as the standard. Thus, different derivatives possessing different enzymatic loading were obtained, these derivatives were named as SEP-RML, PAL-RML, MON-RML and DUO-RML for those obtained from the immobilisation of RML on sepiolite, palygorskite, montmorillonite and duolite, respectively, and similar names (SEP-CCL, PAL-CCL, MON-CCL, DUO-CCL) for those obtained using CCL (see Table 1 for loading).

2.4. Electrophoresis assays

An electrophoresis in SDS-PAGE, using Laemli's method [18], was performed in a Miniprotean II apparatus, (Bio-Rad Laboratories Hercules, California, USA), using Kaleidoscope Prestained standards, also purchased from Bio-Rad.

2.5. Stability assays

In order to test the hypothetical desorption of lipases from the derivatives, these ones were stirred in the reaction medium for 30 min; after filtration, the protein concentration in the elute was measured by Bradford's method, observing in all cases no traces of any protein amount.

Table 1
Immobilisation loading of lipases in the different supports

Derivative	mg of added protein/g derivative	Loading (mg protein fixed/g derivative)	% Immobilisation	Tributyrin activity (mM min^{-1} mg $protein^{-1}$)
SEP-RML	42	**30.2**	72	0.090
	63	40.8	65	0.092
	105	44	42	0.138
PAL-RML	42	**28.1**	67	0.090
	63	47.9	76	0.027
	105	88.2	84	0.005
MON-RML	42	**30.7**	73	0.090
	63	55.4	88	0.016
	105	78.7	74	0.008
DUO-RML	42	**36.5**	87	0.033
SEP-CCL	28	**23.5**	84	0.020
	45	36	80	0.055
	90	48.6	54	0.025
PAL-CCL	28	**22.7**	81	0.050
	45	32.8	73	0.023
	90	50.4	56	0.008
MON-CCL	28	**24.4**	87	0.071
	45	44.1	98	0.038
	90	43.2	48	0.005
DUO-CCL	28	**26.9**	96	0.016
	45	35	78	0.008

Experimental error ± 5% in all cases.

2.6. Determination of lipase hydrolytic activity

The hydrolytic activity of immobilised lipases was assayed by monitoring the release of the acid moiety of the different esters by titration with NaOH (50 mM) using a pH-stat (Metrohm, Switzerland). The reaction mixture consisted in 4 ml of the ester of the corresponding acid, and 36 ml of phosphate buffer 0.1 M, pH 7. The reaction was carried out with continuous magnetic stirring at 25°C, and it was initiated by the addition of determined amount of the immobilised lipase.

2.7. Scanning electron microscopy (SEM)

Finally, particle morphology (shown in Fig. 1) was studied using a Zeiss® DSM 950 scanning electron microscope.

3. Results and discussion

Table 1 shows the results obtained in the immobilisation of RML and CCL on the different silicates. As can be seen, the derivatives were obtained with different immobilisation percentages depending on the initial protein amount. Generally speaking, this immobilisation percentage is slightly higher for the same carrier for CCL compared to RML, although the initial amount of protein used was different due to the different physical state (liquid for RML, solid for CCL) of the enzymes. We can also note that we have not reached the maximum loading capacity of the supports, because in all cases the higher the initial enzyme amount, the higher the loading obtained. Nevertheless, if we consider the activity for all the derivatives (quantified by means of tributyrin hydrolysis, see Table 1), it is clear that the immobilisation on palygorskite (PAL-RML, PAL-CCL) and montmorillonite (MON-RML, MON-CCL) using increasing amounts of initial enzyme is leading to derivatives with smaller activities; this fact is indicating a possible adsorption of the new enzymes molecules onto the previously adsorbed ones rather than onto the support surface, therefore leading to lower activities because of diffusional problems. If

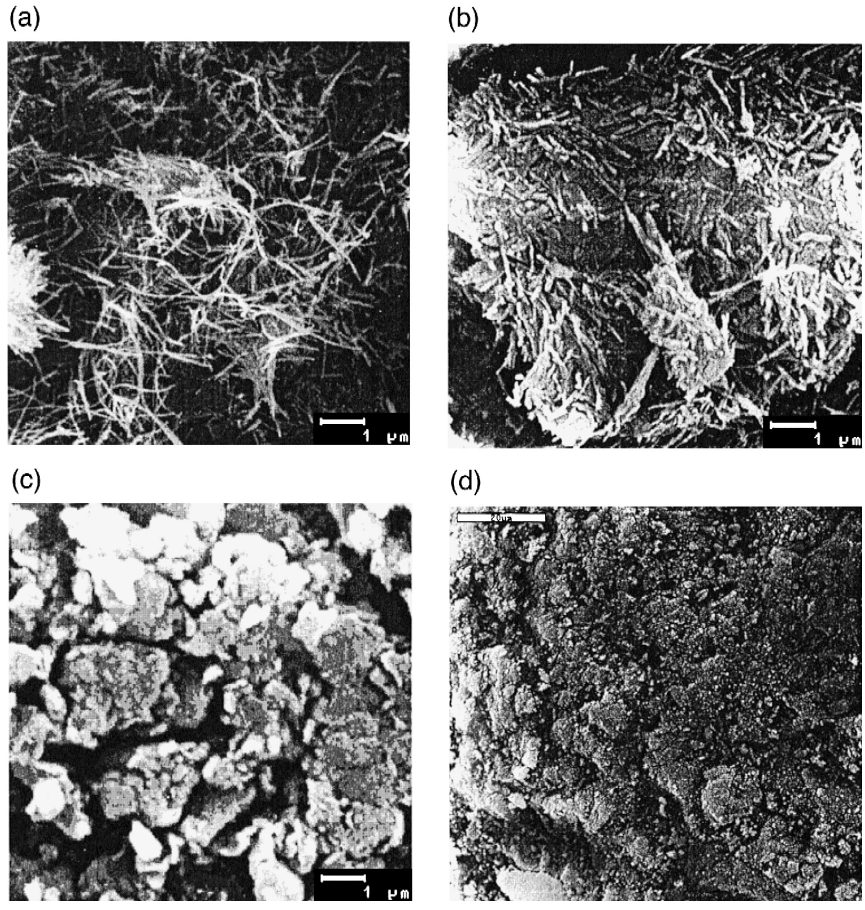

Fig. 1. SEM photographs of the different supports used by lipase adsorption. (a) Sepiolite, (b) palygorskite, (c) montmorillonite and (d) duolite.

we look at the derivatives adsorbed on sepiolite, the activity obtained for SEP-RML increases with the increasing loading, while for SEP-CCL the maximum specific activity is obtained with the intermediate loading; thus, this support seems to be more adequate as a enzymatic carrier.

For DUO-RML, it has been reported previously [19] that the maximum specific activity is obtained for those derivatives possessing the loading shown in Table 1; rather than, the immobilisation of CCL on duolite (Table 1) also renders derivatives possessing smaller specific activity when the loading is increased.

Those derivatives marked in bold in Table 1 were used for the further study of the hydrolysis of ethyl esters of different carboxylic acids, because of their similar loading.

In all cases, the adsorption of the enzymes onto different supports was demonstrated through the concentration of protein solutions before and after immobilisation. Due to the different nature of the supports used for immobilisation, different mechanisms can be expected: while for duolite (anionic exchange resin) an interaction between the enzymatic carboxylic groups and positive charges on the carrier surface must be the responsible for the adsorption, we can suppose that for silicates, which are able to promote cationic exchange through silanol residues, the adsorption mechanism must be produced through positive charges (protonated lysines) of the enzymes.

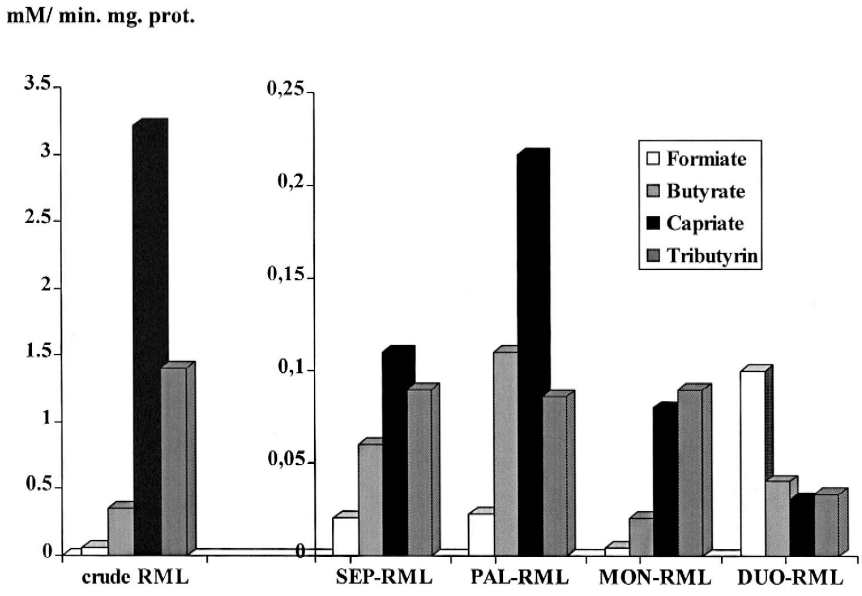

Fig. 2. Initial rate (mM min^{-1} mg $protein^{-1}$) obtained in the hydrolysis of the substrates with different derivatives of *R. miehei*.

Furthermore, the absence of any measurable desorption upon the test (see Materials and methods) indicates that some hydrophobic interaction between the support and the enzymes also does exist.

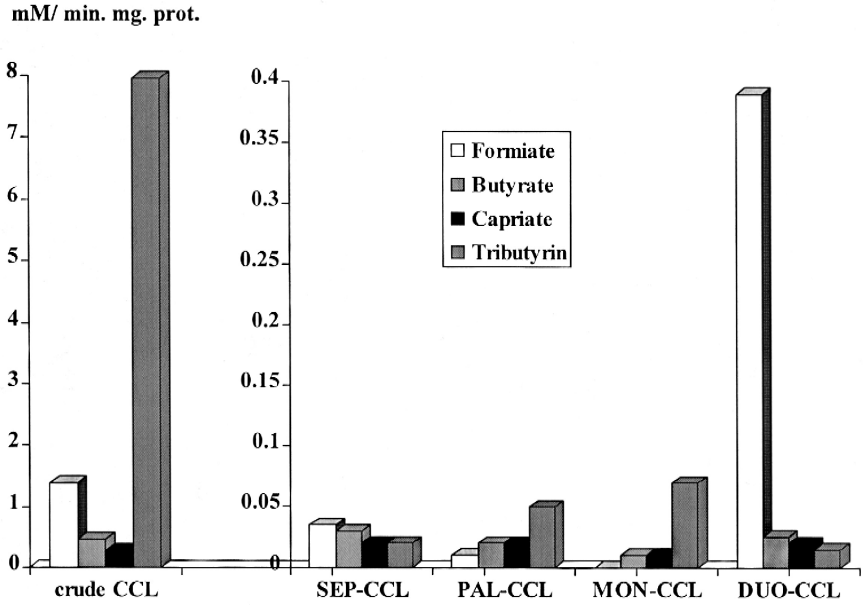

Fig. 3. Initial rate (mM min^{-1} mg $protein^{-1}$) obtained in the hydrolysis of the substrates with different derivatives of *C. cylindracea*.

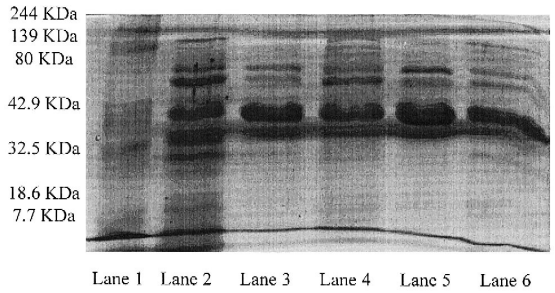

Fig. 4. Electrophoretical analysis under denaturing conditions (SDS-PAGE) of the different immobilisation of RML. Lane 1, protein standards; Lane 2, crude RML; Lane 3, filtration liquids after immobilisation on sepiolite; Lane 4, filtration liquids after immobilisation on palygorskite; Lane 5, filtration liquids after immobilisation on montmorillonite; Lane 6, filtration liquids after immobilisation on duolite.

For measuring the hydrolytic activity of the different derivatives in the hydrolysis of ethyl carboxylates of different chain length, as it is described that the non-derivatised support can independently catalyse the hydrolysis of ethyl acetate [20], thus a blank assay for each support was measured and the activity assays were corrected considering the blank activity, which was negligible for duolite, while for silicates supports using only ethyl formiate we obtained some significant activity. In Fig. 2, we present the corrected initial rates in the hydrolysis of the substrates. We can observe that in the hydrolysis of ethyl butyrate, capriate and tributyrin the higher initial rate was obtained with the SEP-RML and PAL-RML derivatives, while MON-RML generally renders lower activities. For ethyl formiate, DUO-RML showed a much better hydrolytic activity versus the others derivatives. On the other hand, SEP-RML, PAL-RML and MON-RML present similar activity in tributyrin hydrolysis, higher than that obtained with DUO-RML.

In general, the rates obtained with fibrous materials (sepiolite and palygorskite, see Fig. 1) were higher than those obtained with the laminar silicate (montmorillonite) or duolite (spherical particles). This pattern is more pronounced for the hydrolysis of butyrate and capriate.

The results obtained with CCL are presented in Fig. 3. It must be noticed that the hydrolytic activities obtained with the derivatives on clay (SEP-CCL, PAL-CCL and MON-CCL) were lower than those obtained with RML derivatives, independently of the substrate. The extremely higher initial rate obtained with DUO-CCL upon the hydrolysis of ethyl formiate deserves to be pointed out, specially considering that for the other substrates, DUO-CCL and DUO-RML presented similar activity values.

In Fig. 4 we present the electrophoretical determination of the protein content of the medium after immobilisation for RML. We can say that the selectivity in the adsorption was very similar for all the silicates; in fact, the low molecular weight bands (including 30 kDa, approximate molecular weight of RML [21]) have almost disappeared in the electrophoresis after immobilisation for the three supports; nevertheless for CCL (data not shown) the band at 60 kDa (approximate molecular weight of CCL [22,23]) is present with high intensity after immobilisation, both in the silicates and duolite, so we can conclude that CCL is not completely immobilised in any support under these conditions.

As a consequence, we can state that the tested clays are more adequate for the adsorption of low molecular weight proteins, such as RML, versus larger enzymes (CCL) and, specially, the fibrous nature of the support (SEP, PAL) induces gaining of even better results, maybe because of a better geometrical congruence with the enzyme structure. The high activity obtained with DUO-CCL and DUO-

Table 2
Consecutive immobilisation on silicate and duolite

Derivative	mg protein offered/g support	mg protein fixed/g support	% Immobilisation	Specific activity (mM min^{-1} mg $protein^{-1}$)			
				Ethyl formiate	Ethyl butyrate	Ethyl capriate	Tributyrin
PAL-RML	42	22	52	0.029	0.140	0.277	0.110
DUO-RML	20	14.5	72.5	0.131	0.041	0.028	0.014

Experimental error $\pm$ 5% in all cases.

RML on the hydrolysis of ethyl formiate (the more esterasic substrate) could be caused by the presence of proteinaceous low-weight impurities (esterases) in the crude materials [24], which could be preferentially immobilised on the anionic resin. Therefore, we would obtain a derivative with a higher esterasic activity. This fact obviously would lead to an enhanced reaction rate upon the hydrolysis of the smaller and more polar substrate. To check this hypothesis, the immobilisation of RML on palygorskite was repeated, and the supernatant (not adsorbed) was offered to duolite (obtaining a new derivative named DUO′-RML). These results are shown in Table 2. As can be seen, it is clear that our hypothesis was correct, because DUO′-RML shows an enhanced activity upon the hydrolysis of ethyl formiate.

4. Conclusions

Immobilisation of CCL and RML on silicates is obtained by ionic adsorption through the positively charged groups of the protein and the silanol residues of the supports. However, a steric factor correlated with the textural characteristics of the materials must be taken into account in order to explain the differences in the hydrolytic behaviour of the derivatives.

For RML, which in general was immobilised in higher loading than CCL, the employ of fibrous silicates (sepiolite, palygorskite) resulted in an enhanced behaviour compared to the laminar silicate (montmorillonite) and duolite. This was especially clear for the hydrolysis of butyrate and capriate esters, and tributyrin.

Duolite derivatives of RML and CCL presented very high hydrolysis on ethyl formiate, which indicates the possible preferential immobilisation of the esterases that accompany the lipase in the commercial preparations.

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