J Am Oil Chem Soc (2011) 88:801–807
DOI 10.1007/s11746-010-1727-2

ORIGINAL PAPER

# Cross-Linking of Lipases Adsorbed on Hydrophobic Supports: Highly Selective Hydrolysis of Fish Oil Catalyzed by RML

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Received: 18 February 2010 / Revised: 14 July 2010 / Accepted: 17 November 2010 / Published online: 5 December 2010


**Abstract** Organic cosolvents may improve the properties of lipases (e.g., selectivity); however, organic cosolvents also promote the desorption of the enzyme from its hydrophobic supports. In this study, adsorbed lipase molecules were cross-linked with polyfunctional polymers, such as aldehyde-dextrans, to prevent this desorption. The desorption of adsorbed lipases was greatly reduced by optimizing the polymer size, polymer/lipase ratio, and cross-linking time. More than 95% of cross-linked, immobilized *Rhizomucor miehei* lipase (RML) remained adsorbed on the support after washing with cosolvents or detergents. This new, immobilized RML preparation mediated the hydrolysis of sardine oil in the presence of organic cosolvents. The presence of cosolvents promoted small losses of hydrolytic activity. Interestingly, however, 50% 2-propanol also promoted increased selectivity in the release of eicosapentaenoic acid (EPA) in relation to docosahexaenoic acid (DHA). An EPA/DHA ratio of 4:1 in the absence of 2-propanol was increased to a ratio of 22:1 in the presence of 2-propanol. The new RML derivatives were relatively stable under the selected reaction conditions. Their overall half-life was 100 h, but, in a second inactivation phase (below 60% of remaining activity), it took 600 h to reach 30% of their remaining activity.

**Keywords** *Rhizomucor miehei* lipase · Enzyme immobilization · Polyfunctional polymers · Selective release of eicosapentenoic acid · Hydrolysis of sardine oil · Omega-3 fatty acids

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## Introduction

Lipases are some of the most widely used enzymes in biocatalysis [1]. Lipases recognize a wide variety of substrates, as they can catalyze many different reactions under diverse experimental conditions. Thus, lipases may be used in a broad range of applications: food technology (e.g., hydrolysis of fish oils, synthesis of structured lipids) [2], energy (e.g., synthesis of biodiesel) [3] and fine chemistry [4].

The hydrolysis of fish oils by lipases immobilized on porous supports has been recently reported [2]. In this case, immobilized lipases cannot undergo their typical interfacial activation by interaction with oil interfaces, as oils cannot penetrate the porous structure of the catalyst. This lack of activation on oils can be compensated for by promoting interfacial activation of lipases on the surface of the porous hydrophobic support. In a homogeneous aqueous medium, lipases present the following two structural forms: the closed form, where a polypeptide chain (lid or flat) isolates the active center from the medium; and the open form, where the lid moves and the active center is exposed to the

medium [5–7]. This equilibrium is shifted toward the open form in the presence of hydrophobic surfaces, such as drops of oil, as well as in the presence of hydrophobic supports [8] and other hydrophobic structures [9].

The release of omega-3 fatty acids [e.g., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] from fish oil could represent a first step in the preparation of highly enriched triglycerides (70–100% purity of one or both polyunsaturated fatty acids, PUFAs). These triglycerides have been described as excellent functional ingredients [10]. Recently, there has been a dramatic surge in interest among health professionals in the beneficial effects of the omega-3 fatty acids derived from fish oils, mainly consisting of DHA and EPA. DHA is required at high levels in the brain and retina as a physiologically essential nutrient to enable optimal neuronal functioning (learning ability, mental development) and visual acuity in the early stages of life [11]. EPA is considered to have beneficial effects in the prevention of cardiovascular diseases in adults [12]. Therefore, the preparation of triglycerides enriched in only one omega-3 fatty acid (DHA or EPA) could be an interesting method to promote different health effects at different ages and in specific circumstances. For this reason, the enzymatically-induced, selective release of either EPA or DHA may be of paramount biotechnological interest.

*Rhizomucor miehei* lipase (RML), immobilized on octyl-Sepharose exhibits high catalytic properties in the hydrolysis of sardine oil. In addition to a high hydrolytic activity, RML exhibits high specificity in producing EPA compared to DHA—an EPA purity of 80% was observed during the initial stages of release of both omega-3 fatty acids. Further improvements of this selectivity could be quite interesting.

The functional properties of lipases (e.g., activity toward different substrates, enantioselectivity, stability, etc.) can be modulated by preparing different immobilized derivatives with organic cosolvents and by working at different pH levels and temperatures [13, 14]. Unfortunately, RML adsorbed on octyl-Sepharose cannot be modulated in the presence of cosolvents because cosolvents and detergents promote the desorption of the lipase away from the hydrophobic supports.

To avoid this problem, a novel protocol to reinforce the adsorption of the open structure of lipases is proposed in this paper. Intermolecular cross-linking of adsorbed lipase molecules with polyfunctional polymers (e.g., aldehyde–dextran) was tested. The rationale behind our idea was that intermolecular-cross-linked lipases would only desorb if all of the cross-linked lipase molecules were simultaneously released from the support. Therefore, the higher the number of lipase molecules cross-linked with a polymeric structure, the stronger the adsorption achieved for the aggregate.

Accordingly, the cross-linked and hyperactivated RML derivatives were tested for the hydrolysis of sardine oil in the presence of different organic cosolvents to find conditions with highly efficient selectivity of EPA over DHA.

## Materials and Methods

### Materials

Triton® X-100, glutaraldehyde, 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), dextrans of different sizes and diglyme (diethylene glycol dimethyl ether) were from Sigma Chemical Co. (St. Louis, MO, USA). 1,4-Dioxane, *p*-nitrophenyl butyrate (*p*-NPB) and 1,2-ethylenediamine (EDA) were from Fluka Chemical Co. Octyl agarose gels (Octyl-Sepharose™ CL-4B) were from GE Healthcare (Uppsala, Sweden). Sucrose laurate was from Mitsubishi-Kagaku-Food Corporation (Tokyo, Japan). *Rhizomucor miehei* lipase, *Thermomyces lanuginosa* lipase and *Candida Antarctica* lipase B were from Novozymes (Denmark). Sardine oil was a kind gift from BTSA (Madrid, Spain). The fatty-acid composition of sardine oil is given in Table 1. Other reagents and solvents used were of analytical or HPLC grade.

### Methods

#### *Determination of the Activities of Various Soluble and Immobilized Lipases*

To assay the immobilization process, the activities of the soluble lipases and their immobilized derivatives were analyzed spectrophotometrically, measuring the increase in absorbance at 348 nm ($\epsilon = 5.150\ M^{-1}\ cm^{-1}$) produced by the release of *p*-nitrophenol (*p*-NP) by the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate buffer at pH 7 and 25 °C. Spectrophotometric cells provided with a stirring device were used. To initialize the reaction, 0.05–0.2 ml of

**Table 1** Fatty acids composition of sardine oil

| Fatty acid | Percentage |
|---|---|
| C:20:5n-3 (EPA) | 18.6 |
| C:22:6n-3 (DHA) | 12.7 |
| SAFA | 27.2 |
| MUFA | 24.5 |
| PUFA | 48.3 |
| Omega-3 | 40.1 |
| Omega-6 | 2.6 |

*EPA* Eicosapentaenoic acid, *DHA* Docosahexaenoic acid, *SAFA* Saturated fatty acids (mainly palmitic acid), MUFA Monounsaturated fatty acids (mainly oleic acid), *PUFA* Polyunsaturated fatty acids

lipase solution (blank or supernatant) or suspension was added to 2.5 ml of substrate solution. Enzymatic activity was calculated as micromoles of hydrolyzed *p*-NPB per minute per milligram of enzyme (International unit) under the conditions described above.

### *Purification of Lipases*

The purification of *Bacillus thermocatenolatus* lipase (BTL) prior to the immobilization was carried out as previously described [15]. All commercial lipases (CALB, TLL, RML) were semi-purified extracts that were completely purified after immobilization by interfacial activation on octyl-Sepharose [16].

### *Immobilization of Lipases on Octyl-Sepharose*

The purified or semi-purified extracts used for immobilization contained 7, 15, 12 and 5 mg protein/ml for lipases from RML, TLL, CALB and BTL, respectively, as specified by Bradford′s method [17]. To improve enzyme cross-linking, highly loaded derivatives were prepared. Forty milligrams of each enzyme were mixed with 100 ml of 10 mM sodium phosphate buffer at pH 7.0, and 10 g of octyl-Sepharose support was added at 4 °C. The supernatant activity was periodically assayed by the method described above. A blank with soluble enzyme and no support was also assayed to verify that the decrease of activity in the supernatant was due to immobilization of lipase on the support. Once the enzyme was adsorbed, the immobilized preparations were washed thoroughly, filtered and stored at 4 °C.

### *Determination of the Activities of Highly Loaded Lipase Derivatives*

Because of diffusional problems, the activity of the highly loaded immobilized enzyme derivatives was less than 10% of the activity of the soluble enzyme that was immobilized. Thus, after complete immobilization of lipases, highly loaded derivatives were broken down to small particle sizes by strong magnetic stirring at 4 °C. The activity of broken derivatives increased during stirring until reaching a maximum value after one or two hours of stirring. When the highest activity was reached, the particle size was lower than 1 μm (observed by optical microscopy). In this way, the problem of diffusional limitations was reduced.

At this maximum value, BTL, TLL and RML became 2.5, 20 and 10 times more active, respectively, when adsorbed to octyl-Sepharose, compared with soluble enzymes; whereas, CALB retained 100% of the activity of the soluble enzyme. These recoveries of activity were similar to those obtained with lightly loaded lipase derivatives (e.g., 0.2 mg of lipase/g of support).

### *Cross-Linking of Octyl-Sepharose Lipase Derivatives*

*Preparation of Aldehyde-Dextrans* Dextrans of different sizes (1.5–70 kDa) were fully oxidized with sodium periodate as previously described [18]. In this way, polyfunctional polymers containing two aldehyde groups per glucose unit could be synthesized (Dx-CHO).

*Chemical Amination of Lipases* To increase the number of exposed amino groups for a more efficient reaction with the dextran aldehyde, the immobilized lipases were aminated prior to the cross-linking. A total of 1 g of immobilized lipase was added to 10 ml of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension for a final concentration of 10 mM, which ensures the full modification of all exposed carboxylic groups of the protein (aspartic and glutamic acids) [19]. After 90 min of gentle stirring at 25 °C, the immobilized and modified preparations were vacuum-filtered and washed thoroughly with distilled water.

*Cross-Linking of Immobilized Lipase Molecules* The aminated lipase derivatives were incubated with a 33 mg/ml solution of DX-CHO in 100 mM sodium phosphate buffer at pH 7 for 1, 24 or 48 h at 25 °C. The amount of Dx-CHO per milligram of enzyme in the cross-linking treatment was 1, 5 or 10 mg in the case of RML and 1 mg for the rest of the enzymes. The molecular weights of different aldehyde-dextrans tested were 1,500, 6,000 and 70,000 Da. After reaction with DX-CHO, the treated derivatives were incubated in 1 mg/ml of $NaBH_4$ in 200 mM sodium bicarbonate, at pH 10 and 25 °C under gentle stirring. After 1 h, the derivative was washed thoroughly with distilled water and stored at 4 °C.

*Desorption of Lipases from Octyl-Sepharose* To desorb the lipases from the octyl-Sepharose, 1 g of the derivative, prepared as previously described, was suspended in 30 ml of a 1% Triton X-100 (v/v) solution, 1% sucrose laurate solution, or 50% propanol in 10 mM sodium phosphate buffer at pH 7.0. Sucrose laurate was used in place of Triton X-100 to desorb TLL derivatives because Triton caused a strong inactivation of the enzyme. The desorption in the presence of detergent was carried out at 25 °C; whereas, when using 50% propanol, the derivatives were incubated at different temperatures (4, 25 or 37 °C), as stated in the text. The desorbed activity was measured as the difference between the initial activity and the remaining activity. After desorption, the remaining activities of these highly loaded derivatives were analyzed after breaking down the derivatives, as described above.

*Hydrolysis of Fish Oil by Immobilized Lipases*

Hydrolysis of fish oil was performed in a water–organic solvent two-phase system [2]. The procedure was as follows: 4.5 ml of cyclohexane, 5 ml of Tris buffer (0.1 M) pH 6, and 0.5 ml of fish oil were placed in a reactor and pre-incubated under the conditions tested for 30 min. The reaction then was initiated by adding 0.3 g of highly loaded lipase derivatives, and the reaction mixture was mechanically stirred at 600 rpm. A pH-stat Mettler Toledo DL50 graphic was used to maintain a constant pH value during the reactions. The concentration of polyunsaturated free fatty acids was determined at various time points by the high performance liquid chromatography with an ultraviolet detection (HPLC–UV) assay.

*Analysis of PUFAS by HPLC*

After a given time, aliquots of 0.1 ml of organic phase were withdrawn from the reaction and dissolved in 0.8 ml of acetonitrile. The unsaturated fatty acids produced by the reaction were analyzed by RP-HPLC (Spectra Physic SP 100 coupled with a Spectra Physics SP 8450 UV detector) using a Kromasil C8 (15 × 0.4 cm) column. Products were eluted at flow rate of 1.0 ml/min using acetonitrile–10 mM ammonium Tris buffer at pH 8 (70:30, v/v). UV detection was performed at 215 nm. The retention times for the unsaturated fatty acids were 9.4 min for EPA and 13.5 min for DHA [2].

*Composition of Fatty Acids in Sardine Oil*

Analysis was performed by the BTSA Company. Hydrolysis of oil was performed according to protocol 994.10 from AOAC (Association of Official Analytical Chemists). Percentages are obtained from the areas of different peaks obtained by gas chromatography. The values corresponding to EPA and DHA were very similar to those obtained by HPLC–UV in our laboratory—17.9 for EPA and 12.5 for DHA. The EPA/DHA ratio in sardine oil was 1.5:1.

*Inactivation of Immobilized Derivatives in the Presence of Organic Cosolvents*

Enzyme derivatives were washed with water–cosolvent mixtures containing 50% cosolvent in 25 mM sodium phosphate solution at pH 7 and 4 °C. The cosolvents tested were acetone, dioxane and 2-propanol. Subsequently, the enzyme derivatives were resuspended in those solutions and incubated at 25 °C. Samples were withdrawn periodically, and their activity was measured by the assay described above. Experiments were carried out in triplicate, and the standard error was under 5%.

## Results

Desorption of Lipases Adsorbed on Octyl-Sepharose Under Various Experimental Conditions

*Effect of Temperature*

Immobilization of lipases was performed at 4 °C and was quantitative. Thus, no enzyme desorption should be expected at low temperatures. In many instances, it may be necessary to use the immobilized enzymes at high temperatures (e.g., in the hydrolysis of hydrophobic substrates). The adsorbed lipases were incubated for at least 30 min at the highest temperatures at which they were fully active. Desorption was not found, even at temperatures as high as 70 °C (results not shown). Thus, in fully aqueous medium, even using low ionic strength, the adsorption of all studied lipases was strong enough to permit their use under any temperature, with the only limitation being the intrinsic stability of the lipase, not the desorption of the lipase from the support. The interaction of proteins and hydrophobic supports is a complex phenomenon [20]. While elevated temperatures may reinforce hydrophobic interactions, they may also increase the solubility of the protein in the aqueous medium.

RML adsorbed on octyl-Sepharose retained 65% of its initial activity after cross-linking with aldehyde-dextran under optimal conditions. After this cross-linking, only 5% of its initial activity was released upon incubation with Triton X-100.

*Effect of Organic Solvents*

Incubation of lipase derivatives in solutions composed of up to 20% of various organic solvents (e.g., dioxane, diglyme and 2-propanol) did not cause enzyme desorption from the support. However, using 50% solutions of 2-propanol, it was possible to desorb 72% of enzyme from the TLL derivative, 42% from the CALB, 39% from BTL and 30% from the RML derivatives. The effect of the cosolvent was temperature-dependent. For example, 14% of adsorbed RML was desorbed at 4 °C; whereas at 25 and 37 °C desorptions reached 30 and 40%, respectively, in the presence of 50% 2-propanol. Therefore, the practical application of these derivatives would be hindered by the continuous desorption of enzyme from the immobilized derivative, which in these conditions would not resist a single reaction cycle. This result further supports the need for an additional post-immobilization strategy that permits the retention of the activated lipase molecules on the hydrophobic support.

*Effect of Detergents*

Incubation of the BTL, CALB and RML octyl derivatives with 1% Triton for 30 min at 25 °C resulted in full desorption of the enzymes. Octyl derivatives of TLL were incubated with sucrose laurate under the same conditions, as Triton proved to inhibit the enzyme activity. This enzyme was also fully desorbed when incubated with 1% sucrose laurate. As mentioned above, a reversible adsorption of lipases on octyl-Sepharose might be advantageous, since it provides the possibility of reusing the support through successive cycles of enzyme desorption upon inactivation and further immobilization. However, desorption of the enzyme by detergents or cosolvents limits their use, considering that detergents or cosolvents may be necessary to improve the properties of lipases.

## Aldehyde-Dextran Cross-Linking of RML Adsorbed on Octyl-Sepharose

The cross-linking of several immobilized lipases was studied, under the hypothesis that the treatment would increase the adsorption strength of the enzymes on the support.

As a proof of this concept, the octyl-Sepharose RML derivative was selected. The effects of the concentration and MW of aldehyde-dextran (Dx-CHO) on the intensity of cross-linking were evaluated. The desorption of non-cross-linked lipases after the cross-linking of immobilized enzymes was evaluated by incubation of cross-linked derivatives in the presence of 1% Triton. The effect of aldehyde-dextran concentration was not dramatic, but cross-linking seems to be more effective when using lower concentrations of aldehyde-dextran—desorption was only at 4% (the better cross-linking) when a low concentration of aldehyde-dextran (1 mg of activated polymer per mg of immobilized lipase) was used. On the other hand, 14% of adsorbed lipase was desorbed after cross-linking of derivatives with 10 mg of aldehyde-dextran per mg of immobilized lipase. An increase in the Dx-CHO MW significantly reduced the desorption of non-cross-linked RML from the cross-linked derivative after incubation with detergent. For example, only 5% of lipase was desorbed after cross-linking with 70 kDa aldehyde-dextran; whereas, 30% was desorbed after cross-linking with 1.5 kDa aldehyde-dextran. This result implies a better ability of large aldehyde-dextran molecules to cross-link a higher number of adsorbed enzyme molecules, preventing their release by competition with the detergent. Moreover, longer dextran–enzyme reactions were associated with lower 24-h enzyme desorption (Fig. 1).

RML adsorbed on octyl-Sepharose retained 65% of its initial activity after cross-linking with aldehyde-dextran under optimal conditions. After this cross-linking, only 5% of RML's initial activity was released upon incubation with Triton X-100.

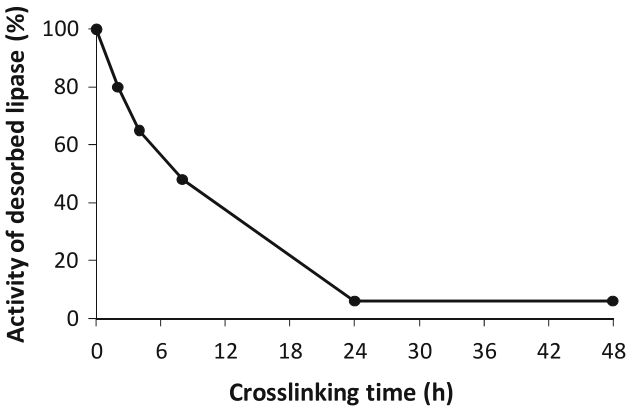


**Fig. 1** Cross-linking of RML adsorbed on octyl-Sepharose with aldehyde-dextrans: effect of the cross-linking time. Immobilized lipase derivatives were incubated in a solution of aldehyde-dextran (1 mg of aldehyde-dextran per mg of protein) and then incubated for different times. At each incubation time, cross-linking was stopped by borohydride reduction of the derivatives. The non-cross-linked lipases present in each cross-linked derivative were desorbed in the presence of 0.5% of Triton X-100

## Cross-Linking of Different Lipases Immobilized on Octyl-Sepharose with Aldehyde-Dextran

The strategy optimized for RML was also used to cross-link TLL, CALB and BTL. Each enzyme preserved more than 50% of its initial activity after treatment with 1 mg of Dx-CHO (70,000 Da) per mg protein during 24 h—CALB, TLL, BTL and RML retained 100, 87, 66 and 65% of their activities, respectively.

The RML derivatives were incubated with 1% Triton X-100 to desorb all non-cross-linked lipase molecules. After 1 h of incubation, derivatives were filtered, washed with water to eliminate the detergent and the desorbed enzyme and incubated again in aqueous media. Table 2 shows the percentage of enzyme activity desorbed away in the presence of 0.5% of detergent. Although, in all cases, some activity was desorbed, a large percentage (85–95%) of each lipase activity remained adsorbed after washing with detergent or in the presence of 50% propanol.

## Hydrolysis of Fish Oil by Immobilized RML in the Presence of Organic Cosolvents

Various cosolvents exerted different effects on the activity and selectivity of RML adsorbed on hydrophobic supports and stabilized by cross-linking with aldehyde-dextrans (Table 3). The most relevant result was the high increase in EPA/DHA selectivity promoted by the presence of 50% 2-

**Table 2** Desorption of different crosslinked octyl-lipase derivatives using 0.5% of detergents

| Enzyme | Detergent | Desorbed activity (%) |
|---|---|---|
| TLL | Sucrose laurate | 15 |
| RML | Triton X-100 | 5 |
| CALB | Triton X-100 | 20 |
| BTL | Triton X-100 | 7 |

**Table 3** Hydrolysis of sardine oil catalyzed by crosslinked derivatives of RML in the presence of 50% of different cosolvents

| Cosolvents | Activity[a] | Selectivity[b] |
|---|---|---|
| Tris buffer | 0.034 | 3.45 |
| PEG | 0.043 | 3.68 |
| 2-propanol | 0.015 | 22.4 |
| Dioxane | 0.009 | 2.89 |
| Acetone | 0.012 | 3.23 |

[a] Activity is expressed as μmol of PUFAS (EPA + DHA) released per minute and per gram of immobilized enzyme

[b] Selectivity is expressed as the ratio between released EPA and DHA. Selectivity was measured at the first stages of hydrolysis (conversions lower than 2%)

propanol, which produced a selectivity ratio above 20:1. Similar modulation and improvement of catalytic properties for the same derivative of the same lipase working under different experimental conditions (pH, temperature and presence of cosolvents) has been found for several lipases in selective processes for fine chemistry. Alterations of the shape and size of the open active center of the lipases has been proposed as a possible cause of the modulation of catalytic properties. The improved selectivity reported here for EPA enables the production, at the first stages of hydrolysis, of mixtures of omega-3 fatty acids with 95% EPA purity. After the complete release of EPA as free omega-3 fatty acid, a subsequent hydrolysis of glycerides enriched in DHA, with a very active lipase derivative (even a non-selective one), could also permit the further release of DHA as free omega-3 fatty acid. In contrast, aside from polyethylene glycol (PEG), all other cosolvents promoted a significant loss of catalytic activity compared to enzymatic hydrolysis in fully aqueous media [21]. Still, this activity regarding oils was much higher than that obtained with small substrates; at 30% 2-propanol, the RML derivatives exhibited no *p*-NPB hydrolysis activity. However, at 50% 2-propanol, 40% activity was exhibited for oil hydrolysis (Fig. 2). The presence of cosolvents improved the sequestration of the oil into the aqueous phase (from 0.2 to 3%). In addition, the possible inhibitory effects of cosolvents, preventing the adsorption of lipase substrates on the

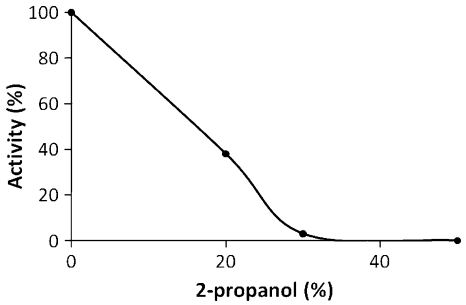


**Fig. 2** Inhibition of the esterase activity of immobilized lipases by 2-propanol. The effect of different concentrations of 2-propanol on the rate of hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) catalyzed by cross-linked RML-octyl-Sepharose. *p*-NPB hydrolysis was performed as described in the "Methods"

hydrophobic enzyme active center, should be less intense for large oil molecules than for small substrates.

## Stability of Cross-Linked Derivatives in the Presence of Organic Cosolvents

The novel RML derivatives reported in this paper exhibited suitable stability in the presence of 50% solutions of different cosolvents (Fig. 3). For example, the half-life of RML derivatives in the presence of 50% propanol was around 60 h (similar to the half-lives of TLL, CALB and BTL derivatives). Moreover, time courses of inactivation were clearly biphasic, with a second phase (starting around at 60% of remaining activity) exhibiting a half-life of 600 h. However, biocatalysts used in food and oil technology have to be cost-effective and extremely robust. Thus, new strategies to improve the stability of these selective and hyperactivated RML derivatives in the

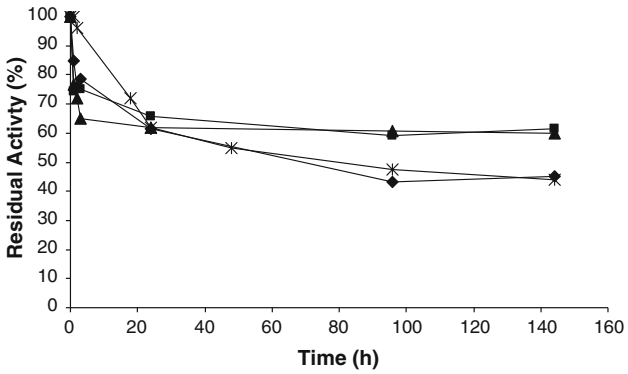


**Fig. 3** Time-courses of inactivation in the presence of 2-propanol of cross-linked derivatives of different lipases. 1 gram of cross-linked lipase derivative was suspended in 10 ml of 50% of 2-propanol at pH 7.0 and 25 °C. At different times, aliquots of the suspensions were withdrawn and assayed (*p*-NPB hydrolysis) in the absence of cosolvent. Octyl-Sepharose derivatives are RML (*asterisks*), BTL (*diamonds*), CALB (*triangles*) and TLL (*squares*)

presence of organic cosolvents will be the subject of a forthcoming paper.

## Conclusions

Polyfunctional aldehyde-dextrans are useful for reinforcing the adsorption of four lipases (RML, TLL, CALB and BTL) on hydrophobic supports by intermolecular cross-linking of adsorbed lipases. The degree of cross-linking strongly depends on the size of the polymer, the ratio of polymer/enzyme and the cross-linking time. The optimal cross-linking of adsorbed lipase molecules promotes a slight reduction of their catalytic activity but prevents almost completely their desorption in the presence of detergents or cosolvents; furthermore, more than 85% of the total enzyme may remain adsorbed on the supports.

With these novel derivatives of RML, the hydrolysis of sardine oil in the presence of 50% cosolvents in the aqueous phase (where the immobilized lipase is acting) could also be studied. In spite of the strong inhibitory effect of cosolvents, the hydrolytic rate was slightly lower than that obtained in a fully aqueous medium. More interestingly, the selectivity of EPA over DHA was greatly increased from a 3 to 4:1 ratio in aqueous media to a 22:1 ratio in the presence of 50% 2-propanol. Under these conditions, RML derivatives were fairly stable, and a major fraction (with 60% remaining activity) showed a half-life longer than 500 h.

**Acknowledgments** This work was sponsored by the Spanish Ministry of Science and Innovation (project AGL-2009-07526) and the Comunidad Autonoma de Madrid (Project S0505/PPQ/03449). We gratefully recognize the Spanish Ministry of Science and Innovation for the "Ramón y Cajal" contract for Dr. Fernandez-Lorente and Dr. Betancor.

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