J Am Oil Chem Soc (2011) 88:819–826
DOI 10.1007/s11746-010-1728-1

ORIGINAL PAPER

Hydrolysis of Fish Oil by Lipases Immobilized Inside Porous Supports

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Received: 11 December 2009 / Revised: 16 July 2010 / Accepted: 17 November 2010 / Published online: 14 December 2010

Abstract A new assay was designed to measure the release of omega-3 acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] from the hydrolysis of sardine oil by lipases immobilized inside porous supports. A biphasic system was used containing the fish oil dissolved in the organic phase and the immobilized lipase suspended in the aqueous phase. The assay was optimized by using a very active derivative of *Rhizomucor miehei* lipase (RML) adsorbed onto octyl-Sepharose. Standard reaction conditions were: (a) an organic phase composed by 30/70 (v:v) of oil in cyclohexane, (b) an aqueous phase containing 50 mM methyl-cyclodextrin in 10 mM Tris buffer at pH 7.0. The whole reaction system was incubated at 25 °C. Under these conditions, up to 2% of the oil is partitioned into the aqueous phase and most of the 95% of released acids were partitioned into the organic phase. The organic phase was analyzed by RP-HPLC (UV detection at 215 nm) and even very low concentrations (e.g., 0.05 mM) of released omega-3 fatty acid could be detected with a precision higher than 99%. Three different lipases adsorbed on octyl-Sepharose were compared: *Candida antarctica* lipase-fraction B (CALB), *Thermomyces lanuginosa* lipase (TLL) and RML. The three enzyme derivatives were very active. However, most active and selective towards polyunsaturated fatty acids (PUFA) versus oleic plus palmitic acids (a fourfold factor) was CALB. On the other hand, the most selective derivatives towards EPA versus DHA (a 4.5-fold factor) were TLL and RML derivatives.

Keywords Enzymatic release of omega-3 acids · Selective enzymatic release of EPA · Solubilization of oil by randomly methylated β-cyclodextrins

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Introduction

The release of omega-3 fatty acids (e.g., EPA and DHA) from fish oil may mean an interesting first step in the preparation of highly enriched triglycerides (70–90% of content in one or both polyunsaturated fatty acids, PUFA). These triglycerides have been described as excellent functional ingredients [1]. Recently, PUFA have meant a breakthrough amongst health care professionals of the beneficial effects of omega-3 fatty acids derived from fish oils—mainly consisting of docosahexaenoic acid (DHA) plus eicosapentaenoic acid (EPA) mixtures. DHA is required in high levels in the brain and retina as a physiologically essential nutrient to provide for optimal neuronal functioning (learning ability, mental development) and visual acuity, in the early stages of the life [2]. On the other hand, EPA is considered to have beneficial effects in the prevention of cardiovascular diseases in adults [3, 4]. In general, both PUFA have been reported useful in preventing a significant number of health disorders [5–10].

The enzymatic release of PUFA may have important advantages over the chemical release: mild reaction conditions, absence of undesirable byproducts, etc. Furthermore,

the use of immobilized lipases may have additional technological and economical advantages for the hydrolysis.

Immobilization of enzymes inside porous supports is an interesting challenge in enzyme biotechnology. Commercially available supports with excellent mechanical properties and high enzyme loading capacity can be selected. In this way, a very active enzyme catalyst can be used in any type of industrial reactor (stirred tank, packed bed, etc.). In addition, since the easy workability of porous supports, some immobilization protocols have been used to improve enzyme properties (e.g., stabilization by a very intense multipoint covalent immobilization [11], hyperactivation of lipases by immobilization and post-immobilization techniques [12, 13], reactivation of partially inactivated lipases [14], etc.)

Hydrolysis of fish oils by lipases immobilized inside porous supports is an outstanding example of lipase application in biotechnology. These oils from marine origin are the most important source in omega-3-PUFA in nature, hence their importance in food technology to make new food additives. In nature, soluble lipases suffer interfacial activation by oil drops. In contrast, using immobilized lipases on porous materials, the enzyme can only hydrolyze the oil molecules partitioned into the aqueous phase (Fig. 1). However, this limitation is compensated for by the plethora of advantages that immobilization gives to enzymes in order to be used in industrial biotransformations. From a practical point of view, immobilized lipases can be re-used for a number of reaction cycles and lipases (inside a porous structure) cannot be inactivated by hydrophobic interfaces present in strongly stirred reactors (e.g., oil interfaces, solvent interfaces, small bubbles of oxygen, etc.).

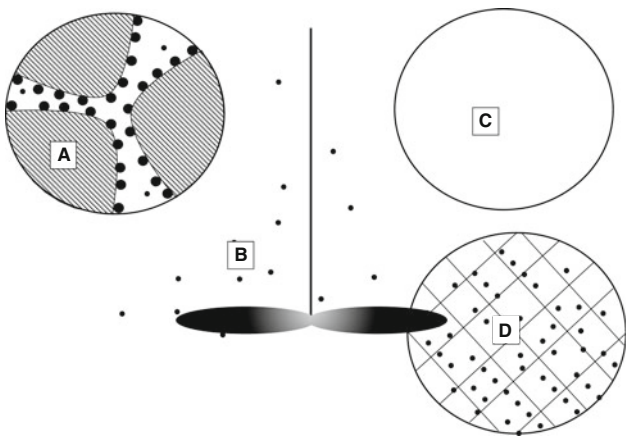

Fig. 1 Some special features of the hydrolysis of oils are strongly stirred biphasic systems catalyzed by lipases immobilized on porous supports. **a** Immobilized lipase is only acting on fully water soluble oil molecules. **b** There is a low partition between oil in the organic phase and oil in the aqueous phase. **c** Gas bubbles promoted by strong stirring are not able to penetrate the porous structure of the catalyst. **d** Drops of the organic phase are not able to penetrate inside the porous structure of the catalyst

On the other hand, a rapid evaluation of the intrinsic properties of new lipases on these oily substrates (activity, stability, selectivity) may be directly tested using immobilized derivatives even before any previous purification process. Developing this partial but very rapid and highly sensitive assay for such purposes may become of great interest.

Using biphasic systems (water/immiscible solvents) may be interesting for the above mentioned assays. Oil will be partitioned into the organic phase and the enzymatic catalyst will be in the aqueous phase only acting on isolated oil molecules that are partitioned into the aqueous phase. Pure oils are very viscous and difficult to work with, but mixtures oil/organic solvents (e.g., cyclohexane) could simplify the reaction design. In the case of fish oil, their high content in PUFA (as acyl chains forming part of triacylglycerol in the oil) could facilitate the aqueous/organic partition of oil molecules. Additionally, released PUFA could be easily detected by RP-HPLC with UV detector. The use of cyclodextrins to increase the solubility of apolar compounds in water has been widely utilized [15, 16]. Randomly methylated β-cyclodextrins (RMBCD) seem to be much more effective than unmodified β-cyclodextrins in order to improve the solubility of hydrophobic compounds in the aqueous phase [17, 18]. A degree of substitution (DS) of 1.8 has been reported as the optimal one. [19].

Materials and Methods

Materials

Lipases from CAL-B (Novozym 525L), *Rhizomucor miehei* (Palatase) and *Thermomyces lanuginosa* (Lypozyme TL) were generously donated by Novozymes (Bagsvaerd, Denmark). Randomly methylated BCD (RMBCD, methylcyclodextrin) was purchase from Cyclodextrin Resource, CTD Inc. (High Springs, FL, USA). Triton X-100, *p*-nitrophenyl butyrate (*p*NPB), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Octyl Sepharose^{TM} CL-4B was purchased from GE Healthcare (Uppsala, Sweden). Sardine Oil was generously donated by BTSA, Biotecnologías Aplicadas, S.L. (Madrid, Spain). Other reagents and solvents were of analytical or HPLC grade.

Enzyme Activity Assay

Enzyme activity was measured by monitoring the increase in absorbance at 348 nm produced by the release of

p-nitrophenol in the hydrolysis of 0.4 mM *p*-nitrophenyl butyrate (*p*NPB) in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.1 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. An International Unit of *p*NPB activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB/min (IU) under the above described conditions.

Immobilization of Lipases on Octyl-Sepharose Supports

Ten grams of octyl-Sepharose were added to 900 mL of 5 mM sodium phosphate buffer at pH 7 containing 250 mg of commercial extracts of lipase (containing a 35% of RML, a 65% of TLL and a 95% of CALB) and the suspension was incubated at 25 °C. A blank of each soluble enzyme with the same concentration was incubated under the same experiment conditions. The activity of the blanks was fully preserved for 4 h. In contrast, the activity of the supernatant of the immobilization suspension decreased down to less than 5%. That is, in all cases, more than 95% of the lipase enzyme was immobilized. After 4 h, the immobilized preparation was filtered and washed with distilled water. The percentage of recovered activity of immobilized derivatives was very low because of mass transfer limitations. Hence, the derivatives were broken (to reduce particle size down to 1 μm) by vigorous magnetic stirring. After this treatment the recovered activities (towards *p*NPB) were 120% for CALB derivative, 1,000% for RML derivative and 2,000% for TLL derivative. The same hyperactivation of lipases adsorbed on octyl-Sepharose was also observed for very low loaded derivatives (e.g., 0.1 mg of lipase per gram of support) and this hyperactivation is due to interfacial activation of lipases on hydrophobic supports [20–22]. The activity of low loaded derivatives in the absence of diffusional problems were 2.4 IU/wet gram of CALB, 39 IU/wet gram of TLL and 9 IU/wet gram of RML. Lipase derivatives were stored in wet form after a final filtration with 10 mM of Tris–Cl pH 7.0 containing 0.02% of sodium azide in order to prevent microbial contamination.

All experiments describe here and in the next sections were carried out in triplicate with an error lower than 5%. The values used in tables and figures were the average value of the three determinations.

Hydrolysis of Sardine Oil

The hydrolysis of sardine oil was performed in an organic/aqueous two-phase system. The procedure was as follows: 5 mL of cyclohexane, 5 mL of Tris–Cl buffer (0.1 M) pH 7, and 0.5 mL of sardine oil were placed in a reactor and pre-incubated at 25 °C for 30 min. The reaction then was initiated by adding different amounts (from 0.1 up to 1 g) of lipase derivative and the reaction suspension was stirred at 150 rpm. A pH–stat Mettler Toledo DL50 graphic was used to maintain the pH at a constant value during the reactions. The concentration of free fatty acids was determined at various times by HPLC–ELSD and HPLC–UV methods.

Analysis of Polyunsaturated Free Fatty Acids (PUFA) by HPLC–UV

After a given time, aliquots of 0.1 mL of organic phase were withdrawn and dissolved in 0.8 mL of acetonitrile. The organic phase was easily separated (in less than 5 min) from the aqueous phase when stirring of the biphasic system was stopped. The unsaturated fatty acids produced were analyzed by RP-HPLC [Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450 (Spectra Physics, Santa Clara, CA. USA)] using a Kromasil C8 (15 cm × 0.4 cm) column. Products were eluted at a flow rate of 1.0 mL/min using acetonitrile-10 mM Tris–OH buffer at pH 3 (70:30, v/v) and UV detection performed at 215 nm. The retention times for the unsaturated fatty acids were: 9.4 min for EPA and 13.5 min for DHA. These enzymatically produced PUFA were compared to their corresponding pure commercial standards.

Analysis of Free Fatty Acids by HPLC–ELSD

After a given time, aliquots of 0.1 mL of the organic phase were withdrawn and dissolved in 0.8 mL of propanol. The fatty acids produced were analyzed by RP-HPLC (Spectra Physic SP 100 coupled to a ELSD 2000 evaporative laser light scattering detector). N_2 was used as the nebulizing gas at a flow of 3 mL/min, and a nebulizing temperature of 30 °C. A Kromasil C18 (25 cm × 0.4 cm) column was used and a stepwise elution was performed using solvent A (acetonitrile/water, 98:2, v/v), solvent B (acetonitrile/propanol, 95:5, v/v) and solvent C (pure acetonitrile). The stepwise elution was performed as follows: 0–6.5 min: 100% A; 6.5–7.5 min: 50% A and 50% B; 7.6–15 min: 100% B; 15.1–18 min: 50% B and 50% C. 18.1–22 min: 100% C. The flow rate was set to 1 mL/min. The retention times for the 4 main fatty acids [EPA, DHA, oleic acid (OA) and palmitic acid (PA)] were between 5 and 13.5 min. The column was thermostated at 40 °C.

Partition of Sardine Oil into the Aqueous Phase

Sardine oil exhibit a high absorbance in UV detection because of the presence of around 30% PUFA in its structure. In this way, by using a very hydrophobic mobile phase (98% acetonitrile/2% water) and a short and lowly hydrophobic

column (Kromasil C4-15 cm) a number of peaks were obtained from RP-HPLC with RT comprised between 15 and 25 min. The total area of these peaks was proportional to the amount of injected oil. For example, an injected sample of 20 μL of 0.5 mM of sardine oil (at 1 ml/min) gave a total area of triglycerides of 20,000,000. On the other hand, these peaks completely disappeared after total saponification of the oil.

A biphasic system was generated by mixing 20 ml of aqueous phase (different buffer and methyl-cyclodextrin concentration) and 20 ml of organic phase (1:1 oil/cyclohexane, 0.54 M of oil). The biphasic mixture was vigorously stirred for 1 h and then stirring was stopped. Both phases were rapidly separated (in less than 5 min). The organic phase was diluted 1,000-fold with mobile phase and 20 μL of the new solution were analyzed by HPLC. The aqueous was diluted 5-fold with mobile phase and 20 μL of the new solution were analyzed by HPLC. The ratio between the concentration of sardine oil in the aqueous phase and the concentration of sardine oil in the organic phase could be easily calculated.

Results and Discussion

Detection of Traces of PUFA by Using Isocratic RP-HPLC with UV Detection

Polyunsaturated fatty acids (PUFA) exhibited a high absorbance at low wavelengths in the UV region. EPA exhibited a very high absorbance at 210, 215 and 220 nm (Table 1). A wavelength of 215 was selected in order to minimize interference by impurities present in sardine oil samples. By using pure commercial EPA and DHA different standard solutions with different concentration of each omega-3 fatty acids in cyclohexane were prepared. Then, 20-μL samples of each standard solution were analyzed by RP-HPLC with UV detection at a constant 1 ml/min flow rate. Integration areas were perfectly proportional to PUFA concentrations (Fig. 2). The absorbance of DHA was a 50% higher than such one from EPA. UV–HPLC chromatograms of aliquots of the organic phase of the reaction mixture were compared to the corresponding calibration curves and hence the rates of hydrolysis (EPA + DHA) and the EPA/DHA ratios could be easily calculated.

Table 1 UV detection of eicosapentanoic acid (EPA) eluted from a RP-HPLC system

λ (nm)	Peak area (EPA)
210	8,130,482 (100%)
215	3,224,882 (40%)
220	128,049 (15%)

Mobile phase: (70/30), acetonitrile: 10 mM ammonium phosphate pH 3.0, flow rate: 1 ml/min, injected sample: 20 μL of 0.1 mM EPA

DHA exhibited an absorbance 50% higher than EPA at 215 nm (Table 2). In contrast, a similar concentration of oleic acid absorbed around 1% in comparison to PUFA. Besides, the polyunsaturated structure of PUFA makes them less apolar and hence the retention times in RP-HPLC under isocratic conditions are relatively low (Table 2).

Therefore, using an isocratic HPLC under the previously mentioned conditions, a rapid, easy and highly precise analysis of omega-3 fatty acids released by enzymatic hydrolysis of fish oil could be designed. For instance, when analyzing a sample of 0.1 mM DHA utilizing a 1 ml/min flow the peak area was very high (over 4,800,000 ± 10,000 units with a very low baseline noise) (Table 2). On the other hand, sardine oil was not spontaneously hydrolyzed at pH 6.0 for 24 h. Thus, EPA and DHA conversions lower than 0.2% in 24 h (concentration of 0.05 mM of PUFA in the organic phase) were easily detected. A rapid preliminary evaluation of very lowly active immobilized lipases was performed by this protocol. In addition to that, the enzyme selectivity (EPA/DHA ratio) can also be measured using this protocol because of the high resolution to separate both EPA and DHA using HPLC.

Use of HPLC with a Light Scattering Detector for the Detection of Traces of Fatty Acids

A "light scattering" detector (ELSD) was used to analyze the ratios between polyunsaturated fatty acids and the abundant oleic and palmitic acids also present in fish oil

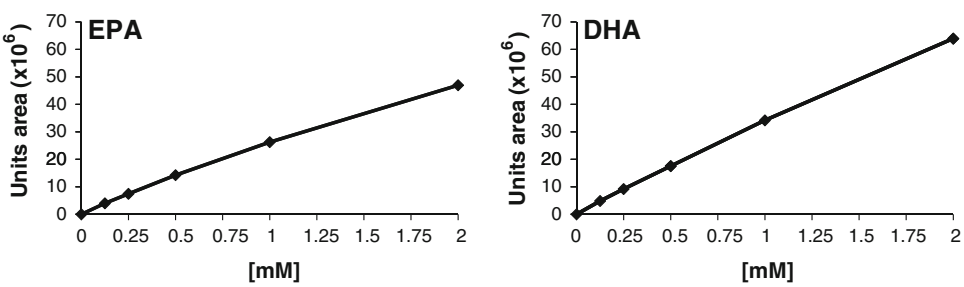

Fig. 2 UV response of EPA and DHA at different concentrations. The response is expressed as absorbance units observed for the corresponding peaks in the RP-HPLC chromatograms. Injected samples: 20 μL of solutions with different concentrations (from 0.125 to 2 mM) of PUFA

Table 2 UV detection of different fatty acids separated in a RP-HPLC system

Fatty acid	Peak area, units	Retention time (min)
Oleic acid	35,899	21.5
EPA	3,224,882	9.4
DHA	4,807,103	13.5

Mobil phase: (70/30), acetonitrile: 10 mM ammonium phosphate pH 3.0, flow rate: 1 ml/min, injected sample: 20 μL of 0.1 mM fatty acid, wavelength: 215 nm

Table 3 Effect of buffer concentration on the rate of hydrolysis of sardine oil catalyzed by immobilized RML

Aqueous phase	Activity	Selectivity
10 mM Tris–Cl buffer	8.91	4.85
100 mM Tris–Cl buffer	3.67	3.03

A water/cyclohexane biphasic reactor at pH 7.0 and 25 °C was used

Activity is expressed as micromoles of PUFA (EPA + DHA) released per minute and per gram of immobilized enzyme

Selectivity is expressed as the ratio between EPA and DHA

[23]. The detection of oleic and palmitic acids (at high concentrations, e.g., 0.5–1 mM) was much more sensitive using this detector than the UV one and hence they were easily compared to the PUFA. Now, a much more apolar mobile phase was used in order to decrease the retention time of oleic and palmitic acids (RT of 9 min approximately). Anyway, representation of the absorbance versus concentration follows a complex sigmoidal trend and hence calibration curves have to be used to calculate the exact concentration of each fatty acid (Fig. 3) [24].

Design of a Biphasic Reactor Water-Cosolvent for the Hydrolysis of Fish Oil Using Lipases Immobilized on Porous Supports

Oils are very viscous and difficult to handle (they become adsorbed on the walls of the containers, on moderately hydrophobic supports or on the stirring devices). Thus, in order to make oil manipulation feasible (especially at low oil concentrations) the biphasic systems water-immiscible cosolvents (e.g., cyclohexane) was approached. Firstly, it was proposed 1:1 ratio aqueous/organic phase where the aqueous phase contained the lipase immobilized inside porous structures (e.g., agarose gels). In this case, the enzyme inside the porous could only hydrolyze the oil molecules present in the aqueous phase of the system.

The biphasic system and the reaction conditions were optimized by using a very active RML derivative containing 5 mg of RML adsorbed per ml of octyl-Sepharose 4BCL and showing 10-fold hyperactivation due to stabilization of the active (open) form of the lipase [25].

Partitioning of the Released Fatty Acids

The partition of a DHA standard in a biphasic system 1:1 at different pH values was studied. Over a wide range of pH (pH 5–8) the non ionized acid was mainly found in the organic phase. Similar values were obtained with EPA. Since, it has been reported for PUFA a p*K* around 9–10 [26], pH 7 seems to be suitable for performing the hydrolysis of fish oils with a number of lipases independently of their corresponding optimal pH. In this sense, the product to analyze (PUFA) can be directly withdrawn from the organic phase. Another advantage of this system is the low concentration of released acids in the aqueous phase which would avoid product inhibitions issues and/or saponification processes even at high hydrolytic conversions.

Optimization of the Reaction Conditions

The composition of the aqueous phase was also optimized (Tables 3, 4) observing two highlightable effects; low buffer concentration contributed to increase the reaction rate and added methyl-cyclodextrin to the aqueous phase had a positive effect in the hydrolytic activity. Considering those two beneficial effects induced by both low pH and methyl-cyclodextrin, lipase activity increased from 3.67 U at high buffer concentrations to 20.14 U at low ones. This activity improvement seems to be related to a better

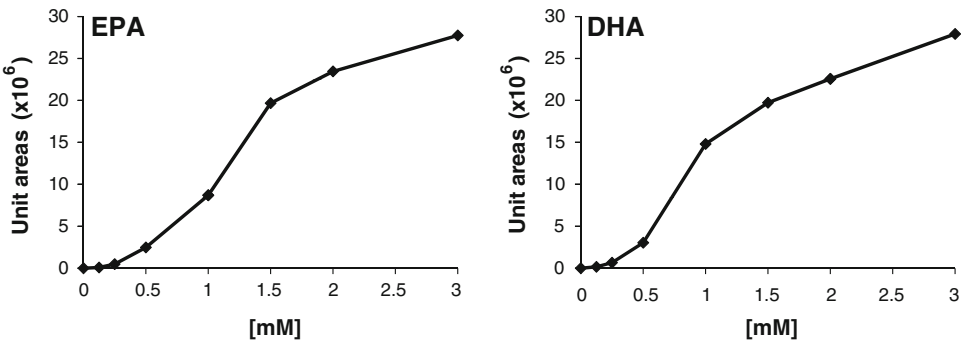

Fig. 3 "Light-scattering" response of EPA and DHA at different concentrations. Response is expressed as absorbance units observed for the corresponding peaks in the RP-HPLC chromatograms. Injected samples: 20 μL of solutions with different concentrations of PUFA (from 0.125 to 3 mM)

Table 4 Effect of methyl-cyclodextrin concentration on the rate of hydrolysis of sardine oil catalyzed by immobilized RML

Methyl-cyclodextrin (mM)	Activity[a]	EPA/DHA ratio[b]
0	8.91	4.95
10	10.53	6.34
25	14.63	5.43
50	20.14	5.31

A water/cyclohexane biphasic reactor at pH 7.0 and 25 °C was used. The presence of different concentrations of methyl-cyclodextrin in the aqueous phase was analyzed

[a] Activity is expressed as micromoles of PUFA (EPA plus DHA) released per minute and per gram of immobilized enzyme

[b] Selectivity is expressed as the ratio between EPA and DHA

partition of the oil in the aqueous phase from 0.15% in 100 mM Tris–Cl up to 2% in 20 mM Tris–Cl and 50 mM methyl-cyclodextrin.

On the other hand, the rate of hydrolysis improved when increasing the pH and curiously it also improved the selectivity EPA/DHA (Table 5). This result might be related to the intrinsic properties of RML. This result is also quite interesting for showing a possible modulation of activity-selectivity of lipases by using different experimental conditions. However, pH 7 is here proposed as the standard pH for a hydrolytic assay useful for most of lipases because of activity-stability binomials.

The effect of oil concentration in the organic phase on the hydrolytic activity was also tested. From this study it can be observed that concentrations of up to 50% of fish oil enhances the lipase activity, probably because of the higher concentration of oil in the aqueous phase, increasing the substrate availability to be used by the lipase in that phase (Table 6). Optimal reaction conditions were reached at 30% of fish oil, keeping low viscosity, making oils easier to work with at the same time the lipase showing a high hydrolytic activity. Under these suitable reaction conditions the rate of reaction was proportional to the amount of catalyst used (Fig. 4).

Table 5 Effect of pH on the hydrolysis of sardine oil catalyzed by immobilized RML

pH	Activity	Selectivity
5	7.84	2.46
6	20.14	4.95
7	21.58	5.52
8	28.35	8.01

A water/cyclohexane biphasic reactor at 25 °C was used. The effect of PH in the aqueous phase was analyzed. Acetate buffer was used at pH 5.0 and 6.0, Tris–Cl at pH 7.0 and borate at pH 8.0. In all cases, buffer concentration was 10 mM

Activity is expressed as micromoles of PUFA (EPA plus DHA) released per minute and per gram of immobilized enzyme

Selectivity is expressed as the ratio between EPA and DHA

Table 6 Effect of the concentration of sardine oil in the organic phase on the hydrolysis catalyzed by RML derivatives

Sardine oil (M)	Activity	Selectivity
0.11	20.14	4.95
0.32	68.67	4.47
0.54	109.8	5.1

A biphasic water/cyclohexane reactor at pH 6.0 and 25 °C was used

Activity is expressed as micromoles of PUFA (EPA plus DHA) released per minute and per gram of immobilized enzyme. Selectivity is expressed as the ratio between EPA and DHA

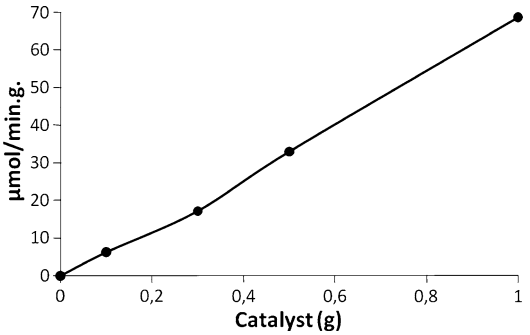

Fig. 4 Effect of the amount of immobilized lipase on hydrolytic rates. Activity is expressed as micromoles of PUFA (EPA plus DHA) released per minute. The amount of immobilized lipase is expressed as the weight of catalyst added to 10 mL of the biphasic reactor (from 0.1 to 1 g of biocatalyst). RML derivatives were evaluated

Hydrolysis of Sardine Oil Using Three Different Immobilized Lipases

Using octyl-Sepharose derivatives of three different lipases the hydrolysis of sardine oil under standard reaction conditions was studied. In addition to the HPLC–UV analysis to follow the rate of PUFA release and the selectivity EPA/DHA, HPLC-light scattering analysis was also carried out to determine lipase selectivity towards polyunsaturated fatty acids and towards oleic acid (OA) (monounsaturated fatty acid d) and palmitic acid (PA) (a saturated fatty acid). The comparison between PUFA and OA and PA was made because OA and PA are very abundant in many oils. In this way, the validity of the release of PUFA for the accurate measurement of lipolytic activity of lipases could be checked. RML and TLL were the most selective enzymes towards EPA with regard to DHA (EPA/DHA ratio of 4.5) and CALB exhibited a low EPA/DHA ratio but a high EPA + DHA/oleic (OA) + palmitic acids (PA) ratio (Table 7). Anyway, the rate of the release of PUFA and the rates of release of oleic or palmitic acids were of the same order of magnitude for these three lipases.

In this paper the interfacial activation is directly promoted on the support [21] and the possible inactivation by hydrophobic interfaces was avoided because of the

Table 7 Hydrolysis of sardine oil catalyzed by three commercial lipases immobilized by adsorption on octyl-Sepharose

Lipase	Activity	Selectivity 1	Selectivity 2
RML	69	4.47	1.22
TLL	53	4.45	0.95
CALB	105	1.5	4.2

A biphasic system 1:1 cyclohexane water was used. Organic phase: 30/70 of oil/cyclohexane. Aqueous phase: 10 mM Tris–Cl buffer, 50 mM methyl-cyclodextrin at pH 7.0. Reaction temperature: 25 °C

Activity is expressed as micromoles of PUFA (EPA plus DHA) released per minute and per gram of immobilized enzyme

Selectivity 1 is expressed as the EPA/DHA ratio

Selectivity 2 is expressed as the EPA + DHA/OA + PA

protection of the immobilized enzymes inside the porous structures [27, 28]. In addition to this hyperactivation on hydrophobic supports, other hyperactivation protocols during and after immobilization have been reported [29].

Catalytic Activity of High Loaded Lipase Derivatives

By using the synthetic substrate (*p*NPB) the intrinsic activity of the highly loaded derivatives was very high 6,330 IU/ml of immobilized CALB, 6,500 IU/ml of immobilized RML and 15,000 IU/ml of immobilized TLL. Intrinsic activity was measured after breaking the derivatives under a very vigorous magnetic stirring for 2 h. In this way particle sizes were reduced down to less than 1 μm and the broken derivatives do not exhibit diffusional limitations. The amount of commercial lipase preparations was the same for the three derivatives (25 mg of protein per ml of support). However, the purity of the commercial lipase samples was different (qualitatively shown by SDS-PAGE). CALB had a purity higher than 95%, TLL had a purity of 65% and RML had a purity of 35%. In this way, the specific activity was even much higher for immobilized TLL.

Highly loaded lipase derivatives also exhibited interesting activities for the release of PUFA from sardine oil: 69 (RML), 50 (TLL) and 104 (CALB) IU (micromole of released PUFA per minute) per ml of immobilized derivative. These good results were obtained in spite of using impure commercial samples of lipases and commercial hydrophobic supports with very large pore size octyl-Sepharose 4BCL. If the purity of commercial preparations was taken into consideration, the specific activity of the different immobilized lipases were: 11.5 IU/mg of immobilized RML, 4.2 IU/mg of immobilized TLL and 5.2 IU/mg of immobilized CALB.

Interestingly, lipases are small proteins so that they could be immobilized on supports having a much smaller pore size (e.g., 10–15% agarose gels) and consequently a much higher enzyme loading capacity. In this way, lipase loadings of 100–200 mg of pure enzyme per ml of support would be obtained. Activities of these derivatives would be more than tenfold higher than those reported here (e.g., intrinsic activities around 2,300 IU of released PUFA/ml of immobilized RML).

Conclusions

The standard conditions for an easy assay of sardine oil hydrolysis by lipases immobilized on porous supports were the following: a biphasic system at 25 °C, where the organic phase was composed of a 30/70 mixture of oil and cyclohexane and an aqueous phase containing 50 mM methyl-cyclodextrin in 10 mM TRIS buffer at pH 7.0. Under these conditions any derivative of any lipase (even immobilized from crude extracts) can be easily assayed: conversions as low as 0.2% of hydrolysis in 24 h can be easily detected. In addition, the initial rate of PUFA hydrolysis (release of DHA and EPA) and the selectivity of the hydrolysis (EPA/DHA ratio) can be easily calculated.

The use of fish oil as a substrate has a great practical interest because of the PUFA released, being the first step in synthesizing new key functional ingredients (triglycerides highly enriched with PUFA). From a more basic point of view, hydrolysis of fish oil (e.g., from sardines) may also be a very good test of lipolytic activities of a number of lipases: (a) sardine oil has a high percentage of the main types of fatty acids (PUFA, monounsaturated and saturated); (b) TLL, CALB and RML (and probably a number of other lipases) hydrolyze all kinds of fatty acids within the same order of magnitude and thus the release of PUFA can provide a fair semi-quantitative measurement of the whole lipolytic activity of different enzymes; (c) PUFA can be easily detected by UV–HPLC (at 215 nm) with a very high sensitivity and this fact would allow a simple test of even poorly active derivatives (e.g., new crude extracts) at very low conversion values (e.g., 0.2%) over a long time (e.g., 24 h), (d) at higher conversions, and using a slightly more complex system (light scattering-HPLC) the release of saturated and monounsaturated fatty acids could also be easily detected.

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. We thank the Spanish Ministry of Science and Innovation (MICINN) grant Consolider INGENIO 2010 CSD2007-00063 FUN-C-FOOD and the Comunidad de Madrid (CAM) ALIBIRD-S2009/AGR-1469 for financial support.

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