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Release of Omega-3 Fatty Acids by the Hydrolysis of Fish Oil Catalyzed by Lipases Immobilized on Hydrophobic Supports

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Abstract The release of omega-3 fatty acids by the mild enzymatic hydrolysis of sardine oil was studied. The derivatives of different lipases physically adsorbed on hydrophobic porous supports Hydrophobic Lipase Derivatives (HLD) were tested. These immobilized lipases can only hydrolyze oil molecules partitioned into the aqueous phase of a biphasic reaction system. HLD biocatalysts were compared to other enzyme derivatives that were obtained by very mild covalent immobilization on CNBr-activated Sepharose Cyanogen bromide Lipase Derivatives (CNLD) that behave almost identically to soluble enzymes (CNLD). In general, HLD biocatalysts were found to be more active and more selective for the release of eicosapentaenoic acid (EPA) than CNLD. The most interesting biocatalyst was the HLD derivative of *Yarrowia lipolytica* lipase, which was found to be sevenfold more active and tenfold more selective than CNLD. On the other hand, the most active (but non-selective) derivative was the HLD of *Pseudomonas fluorescens* lipase (PFL). The activity of this derivative was 0.6 International Units under non-optimal reaction conditions. High-loaded PFL derivatives could be very interesting for the release of mixtures of EPA and docosahexaenoic acid. Hydrophobic supports promote the interfacial activation of lipases, similar to the interaction promoted by oil drops on soluble enzymes. The most effective overactivation obtained in this work ranged from 6- to 20-fold. The hydrolytic process was carried out under very mild conditions (pH 7.0 and 25 °C), and all lipase derivatives remained fully active for at least 15 days under these conditions.

Keywords Eicosapentaenoic acid · Sardine oil · Overactivation of lipases

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Introduction

The release of omega-3 fatty acids [e.g., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] from fish oil represents the first step in the synthesis of valued functional ingredients such us triglycerides that are highly enriched in omega-3 fatty acids [containing 70 to 100% of one or both polyunsaturated fatty acids (PUFA)] [1]. Recently, the demonstrated beneficial effects of omega-3 fatty acids derived from fish oils (mainly consisting of DHA and EPA) have prompted increasing interest amongst health professionals. DHA is required at high levels by the brain and retina as a physiologically essential nutrient to not only optimize neuronal functioning, but also improve learning ability, mental development and visual acuity in the early stages of life [2]. In addition, EPA is considered to be beneficial in the prevention of cardiovascular diseases in adults [3, 4]. Therefore, the preparation of triglycerides enriched in either or both DHA and EPA could be very interesting when used to promote different health effects depending on the age, geographic location or health conditions of the end consumer.

The enzymatic release of omega-3 fatty acids from fish oil represents an attractive alternative to conventional

chemical approaches. For instance, enzymatic processes can be carried out under very mild conditions without the formation of undesirable byproducts. In addition, the selective enzymatic approach provides the advantage of preparing pure samples of each omega-3 fatty acid, avoiding the hurdle of separating EPA from DHA, two compounds with very similar physical properties.

The use of immobilized lipases may have additional technological and economical advantages for the hydrolysis of fish oils. Enzyme immobilization represents an interesting tool in enzyme biotechnology that overcomes many of the drawbacks of using soluble enzymes (e.g., limited stability, difficulty of separation, product contamination, limited reuse, etc.). Currently, immobilized derivatives with excellent mechanical properties and high enzyme-loading capacity are commercially available; therefore, very active enzyme catalysts can be potentially used in any type of industrial reactor (stirred tank, packed bed, etc.).

The utilization of porous supports confers supplementary advantages upon enzyme immobilization. Due to their ease of manipulation, they facilitate the design of protocols to improve enzyme properties during immobilization (e.g., stabilization by a very intense multipoint covalent immobilization [5], the reactivation of partially inactivated lipases [6], etc.).

The hydrolysis of oils by immobilized lipases inside porous supports may be quite useful. Soluble lipases undergo interfacial activation against drops of oils [7, 8]. When using a porous support-immobilized biocatalyst, the enzymes can only act on oil molecules partitioned into the aqueous phase. However, this limitation can be overcome by immobilizing lipases through adsorption on hydrophobic supports. These supports resemble oil drops, and they promote interesting overactivation of immobilized lipases toward small substrates (e.g., *p*-nitrophenyl butyrate, pNPB) [9]. On the other hand, enzymes immobilized inside porous supports are protected from inactivation by hydrophobic interfaces caused by strongly stirred reactors (e.g., solvent interfaces, small bubbles of oxygen) [10].

In this study, we used a number of immobilized derivatives for the release of omega-3 fatty acids from fish oil. Our approach included the utilization of two different immobilization strategies and seven relevant lipases. On one hand, lipases were mildly immobilized on CNBr-activated Sepharose very likely via a one point covalent attachment through the amino terminal group. We are here proposing the use of immobilized lipase derivatives in order to re-use lipases for a number of reaction cycles. In this way, lipase activity of these mildly covalent immobilized derivatives is taken as the intrinsic lipase activity of the native enzyme after the mildest immobilization protocol. More than 50 enzymes immobilized for short times at low temperature CNBr-Sepharose exhibit identical activity-stability properties than diluted and pure soluble enzymes in aqueous media. On the other hand, lipases were adsorbed, at low ionic strength, on hydrophobic supports (octyl-Sepharose). This adsorption involves the large and hydrophobic active center of the lipase and it mimics the interaction of soluble lipases enzymes undergoing interfacial activation on oil drops (Fig. 1). In fact, several previous papers clearly demonstrate this hypothesis: lipases selectively adsorb on hydrophobic supports at low ionic strength, immobilized lipases became overactivated (from 2- to 20-fold) after adsorption [9]. On the other hand, covalent modification with inhibitors (by modification of the catalytic Ser) is much faster after adsorption on hydrophobic adsorption than after covalent immobilization [11].

Materials and Methods

Materials

Buffering salts, Triton® X-100 (TX), pNPB, DHA and EPA standards were from Sigma Chemical Co. (St. Louis, USA). Octyl Sepharose™ CL-4B and CNBr-Sepharose were purchased from GE Healthcare (Uppsala, Sweden). Lauryl sucrose was from Mitsubishi-Kagaku-Food Corporation (Tokyo, Japan). Lipases from *Rhizomucor miehei* (RML), *Thermomyces lanuginose* (TLL), *Candida antarctica* lipase B (CALB) were generously donated by Novo Nordisk (Bagsvaerd, Denmark). Lipases from *Candida rugose* (CRL) and *Rhizopus oryzae* (ROL) were from

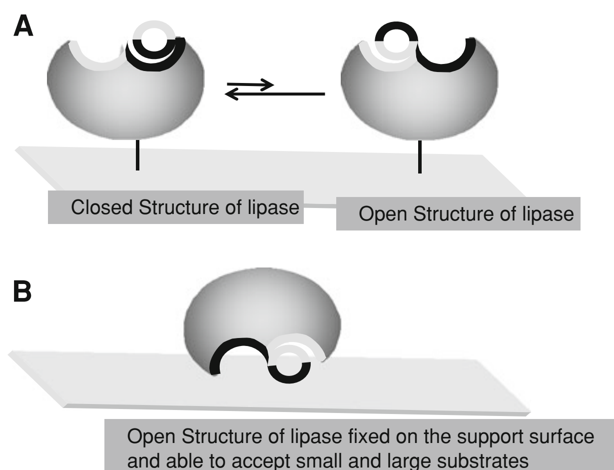

Fig. 1 The two different lipase derivatives used in this work. **a** Lipases mildly immobilized on CNBr-activated Sepharose (CNLD). Closed inactive molecules are in equilibrium with open active ones. The equilibrium is shifted toward the closed form that protects the hydrophobic active site. **b** Lipases adsorbed on hydrophobic supports (HLD). The open active form is stabilized on the support surface

Sigma Chemical Co. (St. Louis, USA). Lipase from *P. fluorescens* was purchased from Amano Enzyme Inc. (Nagoya, Japan). *Bacillus thermocatenolatus* lipase (BTL) was expressed in *E. coli* and produced as previously described [12]. Sardine oil was donated by BTSA, Biotecnologías Aplicadas, S.L (Madrid, Spain). Other reagents and solvents used were of analytical or HPLC grade.

Methods

Determination of the Activity of Different Soluble and Immobilized Lipases

In order to evaluate the immobilization process, the activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically by measuring the increase of absorbance at 348 nm ($\epsilon = 5.150\ M^{-1}\ cm^{-1}$), which is produced by the release of *p*-nitrophenol (pNP) in the hydrolysis of pNPB [9]. Enzymatic activity was calculated as micromoles of hydrolyzed pNPB per minute per milligram of enzyme (International Units) under the conditions described above. Hydrolysis of pNPB is an esterase assay but it is very simple and all lipases exhibit esterase activity in aqueous media. This assay was performed in magnetically stirred cells and it could be used both for soluble and immobilized lipases.

Purification of Lipases

Commercial samples of lipases were slightly impure. Full purification was achieved via selective adsorption, at low ionic strength (e.g., 10 mM phosphate buffer pH 7.0 at 25 °C, on octyl-Sepharose as previously described [9]. After selective adsorption, adsorbed lipases were washed and then desorbed away from the support by using detergents (TRITON X-100 or sucrose laurate). Detergents are able to stabilize the open form of lipases in aqueous medium and also they are able to cover the surface of hydrophobic supports.

Immobilization of Lipases on CNBr-Activated Sepharose Supports

Dry commercial CNBr-activated Sepharose was swollen by suspension in an acidic aqueous solution 0.1 M of HCl (pH 2) for 1 h. Then, the swollen support was washed with the same acid solution in order to remove additives and then filtered by vacuum filtration. One gram of wet CNBr-Sepharose was added to 15 mL (0.3 mg mL^{-1}) of the purified lipase solution. The mixture was then stirred at 4 °C and 250 rpm for 20 min, and the solution was then removed by filtration. Following filtration, the supported lipase was twice washed with 100 mM $NaHCO_3$ at pH 8 and then resuspended in 15 mL of 1 M ethanolamine at pH 8 for 90 min to block the unreacted imidocarbonate reactive groups [13]. Subsequently, the reaction mixture was filtered and washed thoroughly with distilled water. The immobilization yield was between 20 and 40%. The immobilization was followed by the enzymatic activity of the supernatant by using the assay described above. A blank of soluble enzyme under the same experimental conditions preserved 100% of catalytic activity.

Immobilization of Lipases on Octyl-Sepharose

Seven microbial lipases were immobilized on octyl-Sepharose by adsorption, at low ionic strength (5 mM) in sodium phosphate buffer at 25 °C and pH 7 [9]. 100 mL of 0.1 mg of pure lipase were added to 10 mL of gravity settled support (7 g of filtered wet support). The suspension was gently stirred for 2 h. Periodically, the activity of the suspension and supernatant phases were analyzed by the pNPB assay as described above. In all cases, the enzyme loading of the immobilized preparations was higher than 0.95 mg of purified lipase per milliliter of gravity settled support. In all cases the yield higher than 95%, which was calculated based on the remaining activity assayed in the supernatant after immobilization. A blank sample containing soluble lipase under the same experimental conditions preserved 100% activity during the immobilization time.

Hydrolysis of Sardine Oil

The fatty acid composition of sardine oil has been previously reported [14]. The main fatty acids are EPA (18.6%), DHA (12.7%), palmitic acid (16.1%) and oleic acid (11.7%). The hydrolysis of sardine oil was performed in an organic and aqueous biphasic system. The procedure was as follows. First, 5 mL of cyclohexane, 5 mL of TRIS–HCL 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 was then initiated by adding 0.3 g of different lipase derivatives, and the reaction suspension was stirred at 150 rpm. The concentration of free fatty acids was determined at various times by the HPLC–UV method [14]. The UV–HPLC chromatograms of the aliquots from the organic phase of the reaction mixture were compared to the corresponding calibration curves to facilitate the calculation of the rates of hydrolysis (EPA + DHA) and the EPA/DHA ratios. The initial reaction rates were measured withdrawing and analyzing the release of omega 3 in the first stages of the reaction (between 15 and 120 min). Release of omega-3 (EPA + DHA) was always lower than 5% of the total content in the amount of oil used as substrate.

All experiments were carried out in triplicate and experimental errors were always lower than 5%.

Results

Release of Omega-3 Fatty Acids from Sardine Oil Hydrolysis Catalyzed by Immobilized Derivatives of Different Lipases

The release rate obtained when adsorbed lipases on octyl-Sepharose were tested as biocatalysts was generally higher than the release rate obtained when lipases were mildly attached to CNBr-activated Sepharose (Table 1). In both derivatives, lipases cannot undergo interfacial activation by oil drops when they are immobilized on porous supports; therefore, they can only hydrolyze oil molecules partitioned into the aqueous phase of the biphasic reaction system. Notably, lipases adsorbed on hydrophobic supports can be overactivated because of the fixation of the open structure of the enzyme on the hydrophobic surface of the support. On the other hand, lipases immobilized on CNBr-activated Sepharose behave as they do in solution for soluble substrates—in an equilibrium between a higher percentage of closed and inactive molecules and in a lower percentage of open and active molecules (Fig. 1). For several lipase derivatives (e.g., RML, TLL, CALB and PFL), the specific activities were fairly high quite interesting in spite of working under non optimal conditions. From an applied point of view, specific activities could be increased by improving the solubilization of triglycerides into the aqueous phase. For instance, the activity of RML derivatives could be increased by sixfold by lowering the TRIS–HCL buffer concentration to 10 mM and by using 50 mM of methyl cyclodextrin [14]. On the other hand, Commercial octyl-Sepharose 4B supports have a very large pore diameter (2,000 Å) in order to be useful to adsorb any kind of protein even those having a very large MW (up to 400,000). In this way, these supports have a low loading enzyme capacity (around 25 mg of protein per milliliter of support). However, lipases have a low molecular weight (around 30–40 kDa) and they could be immobilized on hydrophobic supports having a much lower pore diameter (e.g., octyl-agarose 10% with 200-Å diameter) and then having a tenfold higher enzyme loading capacity. This optimal supports for lipases would provide up to 200 $m^2\ g^{-1}$ of support and be able to immobilize up to 100–150 mg of pure lipase. Combining both improvements, activities around 100–200 International Units for the release of omega-3 fatty acids could be obtained.

Table 1 Hydrolysis of sardine oil catalyzed by different lipases adsorbed on octyl-Sepharose and covalently attached to CNBr-activated Sepharose

Lipase	Activity[a]	
	CNBr-derivative (CNLD)	Octyl-derivative (HLD)
RML	0.085	0.235
CALB	0.080	0.260
TLL	0.015	0.150
CRL	0.010	0.035
PFL	0.30	0.35
ROL	0.065	0.060
YLL	0.005	0.035

[a] Activity is expressed as micromoles of PUFA (EPA + DHA) released per minute and per milligram of immobilized lipase. Hydrolysis was carried out in a biphasic system at 25 °C, and the aqueous phase contained 100 mM TRIS–HCl buffer at pH 7.0

Overactivations of Lipases toward Different Substrates

The ratio between activities of different lipases (HLD and CNLD) is shown in Table 2. The overactivation phenomenon was studied for two different substrates: high-molecular weight sardine oil was chosen as the "large" substrate and the pNPB was chosen as the "small" substrate. The overactivation values were high; however, a general trend was not observed because some lipases showed a higher overactivation toward the large substrate and others toward the small one. The highest overactivation activities consisted of a 10-fold increase for both TLL and YLL towards sardine oil and a 20-fold increase for TLL towards pNPB.

EPA/DHA Selectivity of Different Lipase Derivatives During the First Stages of Sardine Oil Hydrolysis

In general, the release of EPA was faster than the release of DHA (Table 3). The selectivity is different for the different

Table 2 Overactivation of lipase derivatives toward two different substrates

Lipase	Relative activity (%)[a]	
	Sardine oil	pNPB
RML	280	950
CALB	320	110
TLL	1,000	2,000
CRL	350	200
PFL	115	350
ROL	92	700
YLL	700	100

[a] Relative activity is expressed as the ratio between the activities of lipase adsorbed on octyl Sepharose versus lipase mildly immobilized on CNBr-activated Sepharose. Experiments on pNPB were previously described [10]

Table 3 Ratio between the release of EPA versus the release of DHA during the first stages of the hydrolysis of sardine oil catalyzed by immobilized derivatives (CNLD and HLD) of different lipases

Lipase	Selectivity[a]	
	CNBr-derivative (CNLD)	Octyl-derivative (HLD)
RML	3.0	3.4
CALB	3.2	2.8
TLL	3.2	6
CRL	2.5	4.8
PFL	1.5	1.1
ROL	6.9	9.8
YLL	1.1	10.5

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

Table 4 Rate of initial release of DHA catalyzed by both derivatives of different lipases

Lipase	Rate of release of DHA[a]	
	CNBr-derivative (CNLD)	Octyl-derivative (HLD)
RML	0.021	0.053
CALB	0.019	0.068
TLL	0.003	0.021
CRL	0.003	0.006
PFL	0.12	0.166
ROL	0.008	0.005
YLL	0.002	0.003

[a] Micromoles of DHA released per minute and per milligram of immobilized lipase

lipase derivatives. Some biocatalysts are poorly selective, and they exhibit the same rate of release of both omega-3 fatty acids (e.g., CNLD of YLL and both derivatives of PFL). In contrast, high selectivities toward EPA (approximately tenfold higher) were found for the HDL derivatives of ROL and YLL. In general, lipases adsorbed on octyl-Sepharose were more active and more selective than lipases immobilized on CNBr-activated Sepharose. The most dramatic change was observed for YLL, in which activity was increased by sevenfold and selectivity was increased more than tenfold. In addition, adsorption on octyl-Sepharose proved advantageous because it is more simple and reversible, as supports can be reused after inactivation of the enzyme. The inactivated enzyme can be desorbed with detergents, and the cleaned supports can be reused for the immobilization of fresh enzyme.

The stability of the derivatives proved suitable for the process because they all remained fully active after incubation for 15 days under the reaction conditions (pH 7.0 and 25 °C).

Enzymatic Release of DHA

The different activities and selectivities of the biocatalysts promote very different rates of release of DHA (Table 4). In CNLD derivatives, the differences of activity of different lipases were very high—reaching the highest ratio of 60-fold for PFL compared to YLL. Among HLD derivatives, the differences in activity reached 50-fold. PFL derivatives were the most active and least selective for the omega-3 acids release. They exhibit the highest rate of release of DHA: 0.166 International Units per milligram of immobilized enzyme.

This PFL derivative could be very useful in obtaining mixtures of EPA and DHA from sardine oil hydrolysis. On the other hand, the production of near pure EPA and DHA is also quite relevant, as mentioned above. In this case, EPA could be released by a highly selective biocatalyst (e.g., HDL in ROL). Then, EPA could be purified from mono-, di- and tri-glycerides containing DHA. Finally, DHA could be released by HDL in PFL.

Conclusions

In general, octyl-Sepharose derivatives of several lipases were more active and more selective than covalently immobilized derivatives. By using different lipases, very different activities and selectivities were obtained.

Interestingly, the overactivation of lipases on hydrophobic supports was different for the large and small substrates. For example, PFL was overactivated almost fourfold for pNPB, and it was not overactivated for oils. In contrast, YLL lipase was overactivated sevenfold for oils, and it was not overactivated toward the smaller substrates.

All lipases released EPA faster than DHA (e.g., selectivity was around tenfold for YLL and for ROL). The most active, but least selective, derivative was PFL adsorbed on octyl-Sepharose. The derivatives prepared here are useful for both the release of a mixture containing omega-3 fatty acids or the release of pure DHA (from mono-, di- and triglycerides) after a first selective release and separation of free EPA.

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