HYDROLYSIS OF FISH OIL BY HYPERACTIVATED *Rhizomucor miehei* IMMOBILIZED BY MULTIPOINT ANION EXCHANGE

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ABSTRACT
Rhizomucor miehei lipase (RML) is greatly hyperactivated (around 20-25 fold towards small substrates) in the presence of sucrose laurate. Hyperactivation seems to be an intramolecular process because it is very similar for soluble enzymes and covalently immobilized derivatives. The hyperactivated enzyme was immobilized (in the presence of sucrose laurate) on CNBr activated agarose (very mild covalent immobilization through the amino terminal residue), on glyoxyl agarose (intense multipoint covalent immobilization through the region with the highest amount of Lys residues) and on different anion exchangers (by multipoint anionic exchange through the region with the highest density of negative charges). Covalent immobilization does not promote the fixation of the hyperactivated enzyme, but immobilization on Sepharose Q retains the hyper-activated enzyme, even in the absence of detergent. Hydrolysis of fish oils by these hyperactivated enzyme derivatives was 7 fold faster than by covalently immobilized derivatives and 3.5 fold faster than by enzyme hyperactivated on octyl-agarose. The open structure of hyperactivated lipase is fairly exposed to the medium and no steric hindrance should interfere with the hydrolysis of large substrates. These new hyperactivated derivatives seem to be more suitable for hydrolysis of oils by RML immobilized inside porous supports. In addition, the hyperactivated derivatives are fairly stable against heat and organic cosolvents.

Keywords: lipase hyperactivation, fixation of hyperactivated lipases, multipoint interaction with activated supports

INTRODUCTION
Recently, the hydrolysis of fish oils by lipases immobilized on porous supports has been reported (1). Immobilized lipases cannot undergo interfacial activation by interaction with oil or solvent interfaces because oil drops are not able to penetrate inside the porous structure of the catalyst. This lack of interfacial activation can be compensated for by promoting the activation of lipase during its immobilization. Hyperactivation of lipases by the adsorption of lipases on the surface of porous hydrophobic supports already been reported (19 y 21). In this case, the hyperactivation towards small substrates is usually higher than hyperactivation towards large substrates (e.g., oils). The access of large substrates to the enzyme active center can be hindered by the close contact between the open active center and the support surface. 

On the other hand, several lipases are also hyperactivated with the presence of low concentrations of detergents, even when the active center is fairly well exposed to the reaction medium. In homogeneous aqueous medium, lipases exist in two structural forms, the closed one where a polypeptide chain (lid or flat) isolates the active center from the medium, and the open form where this lid moves and the hydrophobic active center is exposed to the medium (2, 3, 4). This equilibrium may be shifted towards the open form in the presence of detergents that partially cover the hydrophobic active center and make it much more hydrophilic (5). The presence of detergents during the catalytic reaction could interfere with the adsorption of oils on the active center of the immobilized lipase. If the open and active form of lipase promoted by detergents could be fixed after detergent removal, the hyperactivated form of immobilized lipase could be used for the hydrolysis of fish oils. Fixation of these open forms of lipases can be developed on the basis of the distribution of the surface groups
of lipases and differences between the open and closed states. In this case, the open
structure of the lipase hyperactivated in the presence of an optimal concentration of
an optimal detergent could have a very different distribution of negative charges,
lysine groups, etc. than the distribution corresponding to the closed structure. This
open structure generated in the presence of detergents could be fixed via multipoint
physical or chemical immobilization. The immobilized fixed open structure of lipase
could then be used in the absence of detergents.

The release of omega 3 (e.g., eicosapentaenoic acid (EPA) and
docosahexaenoic acid (DHA)) from fish oil represents the first key step in the
preparation of highly enriched triglycerides (70 to 90% of content in one or both
polyunsaturated fatty acids, PUFAs). These triglycerides have been described as
excellent functional ingredients (6). Recently, there has been a dramatic surge in
interest among health professionals in the beneficial effects of omega-3 fatty acids
derived from fish oils, mainly consisting of EPA and DHA. 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 life (7). On the other hand, EPA is considered to have beneficial effects
in the prevention of cardiovascular diseases in adults (8,9). In this way, the
preparation of triglycerides enriched in both of the omega 3 acids (DHA and EPA) or in
only one of them could be very interesting. The first key step for the production of
triglycerides of omega 3 is the rapid and selective release of polyunsaturated fatty
acids from fish oils.
Rhizomucor miehei lipase exhibits interesting catalytic properties for the hydrolysis of sardine oil. In addition to a good hydrolytic activity, RML exhibits a good specificity towards EPA (eicosapentaenoic acid) compared with DHA (docosahexaenoic acid), with an 80% purity in EPA during the initial stages of release of both omega-3 acids. Improvement in the activity and/or selectivity could be quite interesting in order to produce very pure omega-3 acids (1).

This work studies the hyperactivation of RML by different concentrations of different detergents. Then, the fixation of the most active open form was attempted via intense multipoint anion exchange or intense multipoint covalent attachment. The activity and selectivity of the immobilized and hyperactivated RML were finally evaluated for the hydrolysis of sardine oil.
MATERIALS AND METHODS

Materials

Sucrose laurate was a generous gift from the Mitsubishi-Kagaku Food Corporation (cat. No. C-1216). Triton® X-100 (TX) was obtained from Sigma. 1,4-Dioxane and p-nitrophenyl butyrate (p-NPB) were purchased from Fluka. Octyl sepharose™, Q-sepharose, CNBr activated Sepharose 4B (CNBr-agarose) and inert agarose (Sepharose 4B) were purchased from GE Healthcare. The enzyme from *Rhizomucor miehei* was kindly donated by Novozymes (Palatase 2000L). Sardine oil was a kind gift from BTSA (Madrid, Spain). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and sodium borohydride (NaBH4) were obtained from Sigma Chemical Co. (St. Louis, USA). Other reagents and solvents used were of analytical or HPLC grade.

Methods

Enzymatic activity assay

The activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically by measuring the increase in absorbance at 348 nm (ε = 5150 M⁻¹cm⁻¹) produced by the release of p-nitrophenol (pNP) in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate buffer at pH 7 and 25°C. To initialize the reaction, 0.05-0.2 ml of lipase solution (blank or supernatant) or suspension was added to 2.5 ml of substrate solution. Enzymatic activity is given as μmol of pNP produced per minute per mg of enzyme (IU) under the conditions described above.
Hyperactivation of RML performed by different detergents

To a solution of crude Palatase, (1 ml of crude extract - 1.6 mg of RML - diluted with 19 ml of 25 mM NaH$_2$PO$_4$ buffer solution at pH 7.0) various concentrations of different detergents (Triton X-100, Sucrose laurate and CTAB) were added and the activity was periodically checked by the enzymatic activity assay described above.

Immobilization of RML

Usually, the enzyme was incubated with the desired support, and a similar suspension was prepared under identical conditions but using inert agarose. Samples of the suspensions were withdrawn using cut pipette tips, and samples of the supernatants were taken using a filter pipette tip.

To perform immobilization and stability studies, each preparation was loaded with 2 mg/g of pure RML in order to prevent diffusion problems. In the enzymatic hydrolysis, highly loaded derivatives (up to 10 mg/g) were prepared.
Immobilization of RML on octyl-Sepharose

A volume of 1.5 ml of Palatase commercial solution (6,5 mg total protein/ml, about 1,6 mg RML as determined by Bradford’s assay) (10) were mixed with 30 ml of 10 mM sodium phosphate at pH 7.0 and 4ºC, and then 1 g of octyl-Sepharose previously equilibrated with the immobilization buffer was added. The supernatant and suspension activities were periodically checked by the method above described. After immobilization, the enzyme derivative was recovered by filtration under vacuum. These adsorbed lipases were used as biocatalysts or to purify RML.

Desorption of the RML from the octyl-Sepharose with sucrose laurate

To obtain a pure lipase, RML was desorbed from the RML-octyl-Sepharose; 1 g of this immobilized preparation was suspended in 10 ml of 0.5% (v/v) sucrose laurate in 25 mM NaH$_2$PO$_4$ buffer solution at pH 7.0 for 1 h. The progress of the desorption was monitored by periodically checking the enzymatic activity of the supernatant and suspension until they became equal. The final concentration of the purified lipase solution was 0.2 mg lipase/ml. The enzymatic solution obtained was then used for immobilization of the different supports, when a pure enzyme was required.

Immobilization of lipases on different ionic supports

Standard studies were performed as previously described. Then, 1 g of different ionic exchanger supports (MANAE (11), Q-Sepharose or PEI(12)) equilibrated with the
immobilization buffer were added to the pure RML solution obtained as described above (about 2 mg in 10 ml), and the pH was adjusted to 7. The mixture was then stirred at 25ºC. To finalize the immobilization, the supernatant was removed by filtration and the supported lipase was washed several times with distilled water.

Immobilization of lipase on a CNBr-activated support

These support and immobilization conditions were used as reference conditions for lipase behavior. In general, immobilization of enzymes under these conditions will occur by only a few bonds (very likely even just one bond), and these immobilized preparations result in a good model of the enzyme properties in absence of intermolecular phenomena (13-16). Lipases have been shown to exhibit a strong tendency to form lipase-lipase aggregates, making it very complex to study isolated lipase molecules in a soluble form (13-16).

Commercial CNBr activated Sepharose support was activated prior to use by its suspension in an acidic aqueous solution (pH 2-3) for one hour. Then, the support was dried by filtration under vacuum. Once activated, 1 g of CNBr-Sepharose was added to 15 ml of the purified lipase solution (0,2 mg/ml). The mixture was then stirred at 4ºC and 250 rpm for 20 minutes. After that, the solution was removed by filtration, the supported lipase was washed twice with 100 mM NaHCO₃ at pH 8 and then re-suspended in 15 ml of 1 M ethanolamine at pH 8 for 90 minutes to block the unreacted imido carbonate reactive groups (17). Subsequently, the reaction mixture was filtered
and washed with abundant distilled water. The immobilization yield was 60% with a protein loading amount of about 2 mg/g support. Following immobilization, the enzymatic activity assay described above was performed.

**Immobilization of RML on glyoxyl-agarose**

Glyoxyl agarose support (1 g) (18) was added to 13 ml of RML solution (0.2 mg of enzyme/ml) purified as described above. Immobilization was carried out at pH 10.2 over 24 h at 25 ºC under very gentle stirring. To finalize the immobilization reaction, solid NaBH₄ was added to the suspension at 1 mg/ml and the suspension was gentle stirred for 30 minutes. Borohydride reduces the Schiff’s bases formed between the enzyme and the support and the unreacted aldehyde groups remaining in de support. Subsequently, the suspension was filtered and the glyoxyl derivative was washed with abundant distilled water. The percentage of immobilization was 60% with about 2 mg/g support. The immobilization was followed by the enzymatic assay described above.

**Thermal inactivation of different RML immobilized derivatives.**

The different RML derivatives loaded at low levels (0.5 mg of enzyme/g of support) were incubated in 25 mM sodium phosphate at pH 7.0 and 50ºC. Samples were withdrawn periodically and their activities were measured using the pNPB assay. The thermal stability of glyoxyl derivatives was studied with an enzymatic hydrolytic assay modified by adding 0.3% of Triton X-100 in the cuvette. The experiments were carried out in triplicate and error was never more than 5%.
Stability of different RML immobilized preparations in organic solvents

The different RML preparations were incubated in 25 mM sodium phosphate at pH 7.0 and 50% of diglyme or isopropanol at 25 ºC. Samples were periodically withdrawn and their activities were checked with the enzymatic activity assay described above. The stability of glyoxyl derivatives was studied with an enzymatic hydrolytic assay modified by adding 0.5% of sucrose laurate in the cuvette. The experiments were carried out in triplicate and the error was never more than 5%.

Hydrolysis of Fish Oil by immobilized lipases

Hydrolysis of fish oil was performed in a water-organic two-phase system. The procedure was as follows: 4.5 ml of cyclohexane, 5 ml of phosphate buffer (0.1 M) at pH 6, and 0.5 ml of sardine oil were placed in a reactor and preincubated for 30 min. The reaction was then initiated by adding 0.3 g of lipase derivative and shaking at 150 rpm. A Mettler Toledo DL50 Graphit titrator was used to maintain a constant pH value during the reactions. The concentration of polyunsaturated free fatty acids was determined at various times by HPLC assay.

Analysis of Polyunsaturated free fatty acids (PUFAS) by HPLC

After 10 minutes, 0.1 ml aliquots of the organic phase were withdrawn and dissolved in 0.8 ml of acetonitrile. The unsaturated fatty acids produced were analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450)
using a Kromasil C8 column (15 cm × 0.4 cm). Products were eluted at a flow rate of 1.0 ml/min using an acetonitrile-10 mM ammonium Tris buffer at pH 8 (70:30, v/v), and 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.

Determination of Hydrolysis Conversion

The percent hydrolysis was computed as the amount of EPA and DHA (PUFAS) released as a percentage of their original content in the oil, considering that the PUFAS content in the fish oil was 30%. The percent of peak area in an experiment was assumed to indicate the percent content of the corresponding compound. PUFAS productivity (%) was calculated according to the following equation:

\[
PUFAS (g/min) = PUFAS (%) \times \text{fish oil (g/ml)},
\]

In which fish oil content was the weight of the fish present in the substrate mixture.
RESULTS

1. Hydrolysis of a small substrate (pNPB) by soluble RML in the presence of detergents.

The presence of several detergents during the hydrolytic assay exerts very different effects on the catalytic activity of soluble RML (Figure 1). Some detergents (e.g., CTAB) promote a strong inhibition even at very low concentrations (0.001%). The presence of TRITON X-100 promotes a small hyperactivation at very low concentrations (0.01%) but exerts inhibitory effects at higher concentrations (0.1% or higher). Sucrose laurate exerts a remarkable hyperactivating effect: the enzyme is 10-fold more active in the presence of 0.4% of this detergent than in the reference assay in the absence of detergents. This hyperactivation of RML is similar to that obtained for the same enzyme adsorbed on octyl agarose (8-10 fold more active than soluble RML) (19).

2. Long-term Incubation of soluble RML in the presence of detergents.

In order to study the kinetics of hyperactivation as well as to avoid possible inhibiting effects of the presence of high concentrations of detergents in the enzymatic assay, the hyperactivation of RML was also studied by incubating the soluble enzyme in the presence of detergents outside the reaction cell. The catalytic activity of soluble RML increased over 1 hour (figure 2), at which point the concentration of detergent in the assay mixture was almost negligible (lower than 0.01%). The observed activities were constant for 5-10 minutes. Hyperactivation was faster and more intense when using high concentrations of detergent. RML incubated in the presence of 0.5% sucrose
laurate for 30 minutes exhibits a 25-fold increase in activity. This hyperactivation was much greater than that observed in the reaction cell. The enzyme was then hyperactivated for longer incubation times and was assayed in the absence of detergent. The hyperactivation was moderately slow, stable and very high. Similar experiments were conducted with TRITON X-100, in which a 7-fold hyperactivation was observed, also much greater than the hyperactivation previously observed when detergent was added to the reaction cell.

3. Hyperactivation of RML immobilized on CNBr activated agarose.

The mild immobilization of enzyme on CNBr activated agarose conducted here (for 15 minutes at 4 °C) strongly reduces any possibility of multipoint covalent attachment. In fact, these immobilized derivatives of a number of enzymes have activity-stability properties that are very similar to those of the corresponding pure and diluted soluble enzymes in the absence of stirring (17). However, one-point covalently immobilized derivatives fully dispersed on the internal support surface cannot undergo interactions with hydrophobic interfaces (air, oil, solvents) and cannot undergo any kind of intermolecular process (aggregation, proteolysis, etc.) (20). These RML derivatives were hyperactivated similarly to soluble RML (Figure 3). These derivatives were hyperactivated 20-fold in the presence of 0.5 % sucrose laurate. The subsequent washing of the derivatives, done in order to remove the detergent, promoted a slow and complete loss of hyperactivation. This slow and high level of hyperactivation seems to be an intramolecular process that is completely reversible.
4. Sucrose laurate hyperactivation of RML adsorbed on octyl-agarose.

RML was selectively adsorbed and hyperactivated by adsorption on octyl-agarose (an 8-10 fold hyperactivation). The adsorbed enzyme could be desorbed away from the support by washing the derivatives with 0.5 % sucrose laurate. The desorbed enzyme was immediately hyperactivated by up to a factor of 20 (very similar to the slow hyperactivation of the soluble enzyme). Hyperactivation by detergent is higher but compatible with the hyperactivation on hydrophobic supports, but full hyperactivation is much more rapid.

5. Immobilization of hyperactivated RML on different activated supports.

Hyperactivated soluble RML (purified on octyl-agarose and desorbed away with 0.5 % sucrose laurate) was immobilized using 3 different immobilization protocols (TABLE 1). In protocol a, mild immobilization was performed on CNBr-activated agarose (very likely a one-point immobilization through the most reactive amino group, e.g., the terminal amino group). The enzyme preserved its hyperactivation following immobilization, but this hyperactivation was completely lost when the derivative was washed and the detergent was removed at the end of immobilization. In protocol b, immobilization was performed on highly activated glyoxyl agarose at pH 10 (multipoint covalent attachment through the enzyme region with the highest amount of Lys residues (18). The enzyme lost 50% of its hyperactivation during immobilization and lost 100% after the washing away of derivatives and removal of the detergent at the
end of immobilization. In protocol c, the enzyme was physically adsorbed on Sepharose Q (multipoint anion exchange via the enzyme region having the highest density of net negative charge). The enzyme preserved more than 90% of its hyperactivation, and this level of hyperactivation was maintained even after the complete removal of the detergent and after incubation of the derivative for several hours in the absence of detergent. This multipoint anionic exchange allows the fixation, in the absence of detergent, of the hyperactivated form of the enzyme that was previously activated in the presence of 0.5 % sucrose laurate.

6. Immobilization of hyperactivated RML on different anion exchangers.

A similar fixation of the hyperactivated structure of the enzyme was also attempted using different anion exchangers: ionic exchangers containing a very high concentration of ionized primary and secondary amino groups (MANAE-agarose) (11) and very highly activated polymeric exchangers obtained by chemical modification of aldehyde-agarose with polyethyleneimine (PEI-agarose) (12). In both cases, the hyperactivation was partially maintained (at 50%) and the final results of hyperactivation, evaluated in the absence of detergent, were similar to those obtained by adsorption on hydrophobic octyl-agarose (Table 2).

7. Stability of derivatives of hyperactivated RML.

The three derivatives where the hyperactivated form of RML was fixed were very stable under mild experimental conditions. For example, they maintained 100% of their activity after incubation for 3 weeks at 25 °C and pH 7.0. The stability of the derivatives was different under more severe conditions. At 37 °C, the most stable derivative was RML-Sepharose Q (Figure 4), which preserved 80 % of its activity (20-
fold enhanced compared with soluble enzyme) after a 24 hr incubation. On the contrary, in the presence of cosolvents (e.g., 30 % of diglyme), RML-Sepharose Q was the least stable.

8. Hydrolysis of fish oil by different derivatives of RML.

The hydrolysis of sardine oil was performed using RML-CNBr-Sepharose, RML-octyl Sepharose and hyperactivated RML-Sepharose Q derivatives (TABLE 3). RML hyperactivated on octyl-Sepharose was 2-fold more active than non-hyperactivated RML immobilized on CNBr Sepharose. This increase was 9.5-fold for the hydrolysis of small substrates (e.g., pNPB). The hyperactivated RML-Sepharose Q was 7-fold more active for oil hydrolysis than non hyperactivated RML-CNBr-agarose and 3.5-fold more active than RML hyperactivated on hydrophobic supports. In this case, the difference was also lower than that obtained using small substrates (an 18-20-fold increase for pNPB when using CNBr agarose derivatives). The EPA/DHA selectivity of these new hyperactivated derivatives could make them attractive for industrial application, for example, allowing the production of 3:1 EPA:DHA mixtures from sardine oil hydrolysis. This hyperactivation of RML is especially important given that once it is immobilized inside porous supports, RML cannot undergo interfacial activation towards drops of oil or drops of solvents containing oil because these drops are unable to penetrate inside the porous structure of the catalyst.
DISCUSSION

The 20-25-fold hyperactivation obtained for soluble or immobilized RML in the presence of high concentrations of sucrose laurate is one of the highest hyperactivations obtained for lipases via non-natural methods (different from interfacial activation on drops of oil): TLL was hyperactivated 20-fold by adsorption on hydrophobic supports (21), BTL was hyperactivated 2-fold in the presence of detergents (22), BTL was hyperactivated 3-fold in the presence of cosolvents (22), and BTL was hyperactivated 3-fold via site-directed chemical modification (23). Furthermore, BTL was hyperactivated 18-fold by the additive effects of chemical modification, detergents and cosolvents (22), etc. However, the experiments reported in this paper represent the first trial in which hyperactivation by detergents was induced outside of the reaction cell and was studied over a long period of time. Similar experiments could likely give similar interesting results with other lipases. However, at the moment, an additional hyperactivation during the desorption by detergents of lipases adsorbed and hyperactivated on hydrophobic supports had never been observed. Hyperactivations on both hydrophobic supports and in the presence of detergents seem to be related to the opening of the lipase active center. This large hydrophobic pocket of the open form of lipases may be stabilized by adsorption on hydrophobic supports and by interaction with the hydrophobic moiety of the detergent. However, in the case of RML, the detergent seems to be more effective than hydrophobic surfaces, and the new active structure of RML could be slightly different from the structure formed on hydrophobic interfaces.
The fixation of the hyperactivated form of RML by multipoint anion exchange of Sepharose Q is quite interesting. The immobilized enzyme remains hyperactivated after removing the hyperactivating agent (the sucrose laurate). A similar fixation is not possible by very mild (possibly one-point) covalent immobilization on CNBr-activated agarose. Furthermore, the hyperactivated form of the enzyme is not fixed by covalent immobilization on glyoxyl-agarose (multipoint attachment through the enzyme region with the highest amount of Lys). This region seems to be unaltered between the closes and hyperactivated forms of the enzyme. On the contrary, the region with the highest density of net negative charge (involved in the adsorption on Sepharose Q and other anionic exchangers) seems to be dramatically changed when the closed form of the lipase in transformed into the hyperactivated open form. Logically, this region with the highest negative charge is different from the enzyme active center, which is in a large hydrophobic pocket. One important finding from this work is that a complex change in the active center may be associated with a dramatic change in other regions of the enzyme, and the fixation of the associated change preserves the first and most important change in the active center. This type of long-distance stabilization of conformational changes in active centers of enzymes (namely lipases) has not been previously reported in the literature.

These results also showed a 7-fold improvement of the hydrolytic activity of RML towards fish oil with a significant selectivity towards EPA versus DHA. This allows for the rapid production of mixtures of omega-3 acids fairly enriched in EPA via enzymatic methods. This system would allow for the exploitation of all of the advantages of lipases immobilized on porous supports: re-use of the biocatalyst, resistance to strongly stirred reactors because of the lack of interaction of hydrophobic
interfaces with air, solvents, oils, etc., and resistance to intermolecular phenomena such as aggregation and proteolysis. Additionally, the main drawback of these reaction systems, the absence of interfacial activation on oil drops, is compensated for by the hyperactivation of immobilized derivatives.

The hyperactivated RML derivatives are very stable under the mild experimental conditions (e.g., pH 7.0 and 5 °C) required to hydrolyze fish oil in the absence of undesirable oxidations of omega 3. Therefore, these hyperactivated derivatives could be used for fish oil hydrolysis for a number of reaction cycles. Under more harsh experimental conditions, the derivatives are less stable. The presence of a high concentration of quaternary amino groups on the supports may fix distorted enzyme structures formed under distorting conditions (high temperatures, cosolvents, etc.). The distorted enzyme could interact more with the highly activated support.

CONCLUSIONS

RML was 20-fold hyperactivated (towards small substrates) by incubation in the presence of high concentrations (0.4%) of sucrose laurate. Hyperactivation was intermolecular, slow, moderately stable and completely reversible. Hyperactivation is likely related to the stabilization of the large hydrophobic pocket exposed to the medium in the hyperactivated form of RML.

The hyperactivated form of RML was successfully fixed by multipoint adsorption on anionic exchangers and is stabilized even after removing hyperactivating agents (the detergent). The fixed region is not the hydrophobic active center. A clear correlation
between the hydrophobic active center and the region with the highest density in net
negative charge may be responsible for the stabilization of the hyperactivated form of
RML.

RML immobilized inside porous supports becomes very active, very stable and fairly
selective for the mild hydrolysis of fish oils (25 ºC and pH 7.0) in the absence of
undesirable phenomena such as interaction with interfaces and aggregation
phenomena. Now, interfacial activation of soluble lipases on drops of oil is not
necessary since a similar activation was achieved via a careful non-natural
hyperactivation of the immobilized enzyme.

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FIGURE LEGENDS

Figure 1. Hydrolysis of p-nitrophenyl butyrate in the presence of different concentrations of detergents by soluble RML. Experiments were carried out as described in the Methods section. The activity of the enzyme assayed in the absence of detergents is assigned as 100%. Squares: Hexadecyltrimethylammonium bromide (CTAB); triangles: TRITON X-100; circles: sucrose laurate.

Figure 2. Time-courses of the hyperactivation of soluble RML incubated in the presence of different concentrations of sucrose laurate. Experiments were carried out as described in the Methods section. The enzyme was incubated in the presence of detergents and aliquots of the incubation mixtures were assayed in the presence of minimal traces of detergent (less than 0.01%). The activity of the enzyme incubated in the absence of detergent was assigned as 100%. Triangles: RML incubated in 0.1 % sucrose laurate; squares: RML incubated in 0.2 % sucrose laurate; circles: RML incubated in 0.5 % sucrose laurate.

Figure 3. Reversible hyperactivation of immobilized RML incubated in the presence of 0.5% sucrose laurate. RML was very mildly immobilized on CNBr-activated agarose gels. Column 1: activity of immobilized RML before incubation in the presence of detergent; Column 2: activity of immobilized RML after incubation for 1 hour in the presence of 0.5 % sucrose laurate; Column 3: activity of immobilized RML after a final buffer wash that was performed in order to remove the detergent.
Figure 4. Time-courses of the thermal inactivation of different RML derivatives (hyperactivated enzyme adsorbed on different anion exchangers). Derivatives were incubated at 37 ºC and pH 7.0. Squares: hyperactivated RML adsorbed on MANAE-agarose; triangles: hyperactivated RML adsorbed on PEI-agarose; circles: hyperactivated RML adsorbed on Sepharose Q.

Figure 5. Time-course of cosolvent inactivation of different RML derivatives (hyperactivated enzyme adsorbed on different anion exchangers). Derivatives were incubated in the presence of 30% of diglyme at pH 7.0 and 25 ºC. Squares: hyperactivated RML adsorbed on MANA-agarose; triangles: hyperactivated RML adsorbed on PEI-agarose; circles: hyperactivated RML adsorbed on Sepharose Q.
REFERENCES


Table 1.- Immobilization of hiperactivated RML on different activated supports. Experiments are described in Methods. Relative activities are reported using 100% as the activity of the soluble enzyme before hyperactivation and immobilization.

<table>
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<th>Steps</th>
<th>Relative activity (%)</th>
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<tr>
<td>Soluble enzyme</td>
<td>100</td>
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<tr>
<td>Adsorption to Octyl-sepharose</td>
<td>950</td>
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<tr>
<td>Desorption with 0.5% lauryl sucrose</td>
<td>2000</td>
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<td>Q-Sepharose 1880</td>
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<td>Glyoxyl-agarose 1200</td>
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<td>Immobilized derivatives after washing and removing the detergent</td>
<td>100</td>
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<td>1800</td>
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Table 2.- Immobilization of hiperactivated RML on different anion-exchange supports. Experiments are described in Methods. Relative activities are reported using 100% as the activity of the soluble enzyme before hyperactivation and immobilization.

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<td>Adsorption on Anion Exchange Resins</td>
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<td>PEI</td>
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<td>Immobilized derivatives after washing and removing the detergent</td>
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</table>
Table 3. Hydrolysis of sardine oil catalyzed by different derivatives of RML. Experiments were carried out as described in Methods.

Activity\(^a\) represents the rate of hydrolysis of a small synthetic substrate (p-nitrophenyl butyrate). Activity\(^b\) represents the rate of hydrolysis of sardine oil. In both cases, 100% is the activity of derivative obtained by very mildly immobilization on CNBr-activated Sepharose. EPA/DHA is the ration between both omega 3 released in the first stages of hydrolysis of sardine oil (up to a 10% of conversion). \(\omega-3/\text{oleic plus palmitic acid}\) is the ration between both omega3 and saturated and monounsaturated acids obtained in the first stages of hydrolysis of sardine (up to a 10% of conversion).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Activity(^a) (%)</th>
<th>Activity(^b)(%) (10^{-3})</th>
<th>EPA/DHA</th>
<th>(\omega-3/\text{Oleic plus palmitic acids})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNBr-</td>
<td>100</td>
<td>100</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>OCTIL</td>
<td>950</td>
<td>200</td>
<td>3.45</td>
<td>0.89</td>
</tr>
<tr>
<td>O</td>
<td>2000</td>
<td>700</td>
<td>4.2</td>
<td>1.06</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 5