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1 **IMPROVED PERFORMANCE OF LIPASES IMMOBILIZED ON**
2 **HETEROFUNCTIONAL OCTYL-GLYOXYL AGAROSE BEADS.**

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27 **Abstract**

28 A new heterofunctional support, octyl-glyoxyl agarose, is proposed in this study. The
29 supports were prepared by simple periodate oxidation of the commercial octyl-agarose,
30 introducing 25 μmol of glyoxyl groups per wet gram of support. This support was assayed with
31 three different lipases (those from *Candida antarctica* (form B), *Thermomyces lanuginosus*
32 (TLL) or *Rhizomucor miehei*) and the artificial phospholipase Lecitase Ultra. Used at pH 7, the
33 new support maintained as first immobilization step the lipase interfacial activation. Thus, it
34 was possible to have the purification and immobilization of the enzyme in one step. Moreover,
35 stabilization of the open form of the lipase was achieved. The covalent enzyme/support bonds
36 cannot be obtained if the immobilized enzyme was not incubated at alkaline pH value. This
37 incubation at pH 10 of the previously immobilized enzymes produced a smaller decrease in
38 enzyme activity when compared to the direct immobilization of the enzymes on glyoxyl-agarose
39 at pH 10, because the immobilization via interfacial activation promoted a stabilization of the
40 lipases. Except in the case of TLL (covalent attachment involved 70% of the enzyme
41 molecules), covalent immobilization yield was over 80%. The non-covalent attached enzyme
42 molecules were discarded by washings with detergent solutions and the new biocatalysts were
43 compared to the octyl-agarose immobilized enzymes. While the stability in thermal and organic
44 solvents inactivations was increased for Lecitase Ultra, CALB and RML, TLL improved its
45 stability in organic media but its thermal stability decreased after covalent attachment of the
46 interfacially activated enzyme. This stabilization resulted in octyl-glyoxyl-lipase preparations
47 which presented higher activity in the presence of organic solvents. Finally, while octyl-agarose
48 released enzyme molecules after incubation at high temperatures or in the presence of organic
49 solvents and detergents, the covalently immobilized enzyme remained attached to the support
50 even after boiling the enzyme in SDS, eliminating the risks of product contamination.

51 **Key words:** Heterofunctional supports, interfacial activation, lipase, phospholipase, covalent
52 immobilization, glyoxyl supports, octyl supports.

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53 **1.- Introduction**

54 Lipases are among the most used enzymes in biocatalysis, due to their broad
55 specificity[1], stability in different reaction media [2] and versatility [1, 3]. Generally, lipases, as
56 most enzymes, require the previous immobilization to facilitate their recovery and the reactor
57 control for their use as industrial biocatalysts [2c, 4]. Moreover,, immobilization has been
58 developed as a tool that, if properly used, may allow the improvement of other enzyme
59 properties, such as stability, activity, selectivity, specificity or resistance to inhibition. [5]

60

61 Lipases have a peculiar mechanism of action, called interfacial activation [6]. Most
62 lipases have two different conformations, open and closed conformation, where the active center
63 may be secluded by a polypeptide chain from the medium [6-8], [8]. This open form is unstable in
64 aqueous medium, but becomes stabilized by adsorption on hydrophobic interfaces, such as drops
65 of oils [6c, 9]. This adsorption of the open form of the lipase also occurred on any hydrophobic
66 surface, like a hydrophobic protein, another lipase in its open form, or a support bearing
67 hydrophobic surfaces. [10]

68 In this regard, octyl-agarose has been proposed for a long time as a simple method to
69 reach a one-step immobilization, stabilization, purification and hyperactivation protocol for
70 many lipases [10c, 11]. This support has been used to immobilize many enzymes that have been
71 employed in many different reactions [12]. Although the adsorption of lipases on octyl-agarose
72 beads is quite strong, the enzyme may become released from the support if the derivative is
73 incubated in the presence of high concentrations of organic cosolvents (used to solubilize some
74 substrates or products), [13] or in presence of detergents (used to modulate enzyme properties)
75 [11a, 14]. Moreover, lipase desorption may occur after enzyme inactivation, contaminating the
76 medium and losing one of the advantages of employing immobilized enzymes.

77 Crosslinking of the immobilized enzymes on octyl-agarose beads with aldehyde-dextran
78 has been proposed as a way to prevent the enzyme release of the enzyme from the support [13, 15]

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79 . In fact, crosslinking with glutaraldehyde may give a similar effect, because lipases immobilize
80 very rapidly on these supports and the enzyme molecules are packed together.[16]
81 In this paper, a new strategy to use octyl-agarose beads to immobilize, purify, stabilize
82 and hyperactivate lipases (and useful to be used in the presence of organic solvents or
83 detergents) is proposed. It is based on the idea of heterofunctional supports, bearing different
84 groups on the support surface with different functions that may permit to control immobilization
85 [17]. In this case, it is based on the conversion of octyl-agarose into a heterofunctional octyl-
86 glyoxyl support. Crosslinked octyl agarose beads have some diols (resulting from the opening of
87 the epoxy moieties during the support preparation) that may be easily oxidized to glyoxyl
88 groups by oxidation with periodate. This makes immobilization of the enzyme possible via a
89 first interfacial activation (with the advantages that this approach has) followed by covalent
90 attachment(s) to avoid undesired enzyme release. Using hierarchical meso-macroporous silica,
91 there is a recent report on the construction of octyl/glyoxyl heterofunctional supports, with good
92 results in stabilization, but without a discussion on the advantages and drawbacks of the
93 different preparations (was used in organic media) nor a clear demonstration of the
94 establishment of covalent attachment between the enzyme and the support [18].
95 Glyoxyl activated supports have been proposed as very suitable candidates to stabilize
96 enzyme by multipoint covalent attachment via reaction with the primary amino groups of the
97 enzyme [19]. For developing this new strategy, the glyoxyl residues have a further advantage;
98 they cannot immobilize soluble enzymes at pH 7, because protein immobilization on glyoxyl
99 supports requires the simultaneous production of, at least, two imino attachments [20]. This
100 permits that, at non alkaline pH value, the immobilization of the enzyme on the glyoxyl-octyl
101 heterofunctional supports may be expected to proceed via interfacial activation [19, 20b]. Later, the
102 proximity of the enzyme to the support, the addition of a thiol compound [21] or the increase of
103 the pH [21a], could permit to increase the reactivity of the protein residues to have a covalent
104 linkage between enzyme and support (after reduction a very stable secondary amino bond) [17].

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105 Thus, this immobilized enzyme cannot be released to the medium under any circumstance.
106 Obviously, this strategy requires at least one primary amino group of the enzyme to be located
107 in a position where it can react with the support after adsorption on the enzyme.

108 The strategy has been assayed using 4 different enzymes. Lipase B from *Candida*
109 *antarctica* (CALB) is one of the most used lipases in biocatalysis [22]. The 3D-protein structure
110 of this lipase has been resolved [23]. Although it has a very small lid and does not suffer from an
111 increase in activity by interfacial activation, it may still become adsorbed on hydrophobic
112 surfaces. Lipases from *Thermomyces lanuginosus* (TLL) [24] and from *Rhizomucor miehie*
113 (RML) [25], have been also utilized, they have a proper lid and are probably among the most
114 popular lipases after CALB. We have also included Lecitase Ultra in these studies, a
115 commercial chimeric phospholipase built from the gen of the lipase from *Thermomyces*
116 *lanuginosus* (to obtain good stability) and that of the phospholipase from *Fusarium oxysporum*
117 (to get the phospholipase activity) [26]. The activity will be assayed with different substrates to
118 check if the enzyme specificity is changed by the likely distortions on its structure produced by
119 the covalent attachment, as it has been reported in many papers dealing with the immobilization
120 of lipases [27].

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123 **2. Materials and methods**

124 **2.1. Materials**

125 Solutions of lipase B from *C. antarctica (CALB)* (6.9 mg of protein /mL), lipase from
126 *Thermomyces lanuginosus (TLL)* (36 mg of protein /mL), lipase from *Rhizomucor miehie* (*RML*)
127 (13.7 mg of protein/ mL) and the phospholipase Lecitase Ultra (16 mg of protein /mL) were a
128 kind gift from Novozymes (Spain). Octyl-agarose beads were from GE Healthcare. Methyl
129 mandelate, ethyl hexanoate, p-nitrophenyl butyrate (p-NPB) were from Sigma Chemical Co.
130 (St. Louis, MO, USA). All reagents and solvents were of analytical grade.

131

132 **2.2. Standard determination of enzyme activity**

133 This assay was performed by measuring the increase in absorbance at 348 nm
134 produced by the released p-nitrophenol in the hydrolysis of 0.4 mM p-NPB in 100 mM sodium
135 phosphate at pH 7.0 and 25 °C (ε under these conditions is 5150 M^{-1} cm^{-1}). To start the
136 reaction, 50–100 μL of lipase solution or suspension was added to 2.5 mL of substrate solution.
137 One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1
138 μmol of p-NPB per minute under the conditions described previously. Protein concentration was
139 determined using Bradford's method [28] and bovine serum albumin was used as the reference.

140

141 **2.3. Preparation of glyoxyl supports**

142 The preparation of both glyoxyl supports (directly agarose 4BCL or using octyl agarose
143 4BCL) respectively activated with 30 or 25 μmol of aldehyde groups/g of wet support was
144 carried out by directly oxidizing the diols of the support using sodium periodate (an
145 equimolecular ratio was used in the reaction) following the standard protocol described in [19,20].
146 A wet support is defined as the agarose beads with the pores full of aqueous medium, but
147 without interparticle water (dried using vacuum filter). The suspensions containing the supports
148 and the sodium periodate were gently stirred for 3 h at 25°C, and then the supports were filtered

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149 and washed with distilled water. The non-consumed periodate was measured by titration of the
150 filtrate with KI in saturated bicarbonate[29].
151
152 **2.4. Immobilization of enzymes**
153
154 **2.4.1 Immobilization of enzymes on octyl (OC) and octyl-glyoxyl (OCGLX) supports**

155 The immobilization was performed using 1 or 5 mg of protein per g of wet support,
156 except in maximum loading determination where the volume of enzyme was increased to reach
157 the 60 mg of enzyme/g of support. The commercial samples of the enzymes were diluted in the
158 corresponding volume of 5 mM sodium phosphate at pH 7. Then, the supports were added. The
159 activity of both supernatant and suspension was followed using p-NPB. After immobilization
160 the suspension was filtered and the supported enzyme was washed several times with distilled
161 water.

162 In the case of OCGLX, the washed immobilized enzyme was re-suspended in certain
163 instances at pH 10 for different times, to favor the enzyme-support covalent reaction [20a].
164
165 **2.4.2 Immobilization of enzymes on glyoxyl (GLX) support**

166 The immobilization was performed using 1 or 5 mg of protein per g of wet support.
167 The enzymes were diluted in a 50 mM sodium bicarbonate buffer at pH 10. Then, the support
168 was suspended in the enzyme solution under gentle stirring. Periodically, samples of the
169 supernatant and suspension were withdrawn, and the enzyme activity was measured as described
170 above.
171
172 **2.4.3 Reduction with sodium borohydride**

173 To end the enzyme-support covalent reaction, solid sodium borohydride was added to
174 a concentration of 1 mg/mL to the OCGLX and GLX suspensions (at pH 10) and were
175 submitted to gentle stirring for 30 min. This treatment reduces reversible Schiff´s bases to very

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176 stable secondary amino bonds and unreacted aldehydes groups to fully inert hydroxy groups [19-
177 21]. Finally the reduced derivatives were filtered, washed with abundant distilled water and
178 stored at 4°C.
179

180 **2.5. Desorption of the enzyme from the supports**

181 To analyze if the enzymes were really covalently attached to the support, and to keep
182 only the covalently attached enzyme molecules for further studies, the reduced OCGLX
183 derivatives were incubated with a growing concentration of the appropriate detergent, using OC
184 derivatives as reference. This treatment only releases the enzyme molecules adsorbed by
185 interfacial activation. Thus, samples of 1 g of different biocatalysts were suspended at 25 °C in
186 10 mL of 10 mM sodium phosphate at pH 7. Then, Triton X-100 (for CALB, RML and
187 Lecitase) or CTAB (for TLL) were progressively added to a final concentration of 1.5% and 2%
188 (v/v) respectively. Intervals of 30 min were allowed before taking a sample of the supernatant to
189 determine the released enzyme and performing a new detergent addition. A reference
190 suspension, having inert support and the same amount of lipase was submitted exactly to the
191 same treatment, to detect the effects of the detergent on enzyme activity or stability.
192

193 **2.6. Study of the stability of the different lipase biocatalyst**

194

195 **2.6.1. Thermal inactivation of different enzyme immobilized preparations**

196 1g of immobilized enzyme was suspended in 5 mL of 50 mM of sodium acetate at pH
197 5, sodium phosphate at pH 7 or sodium bicarbonate at pH 9 at different temperatures.
198 Periodically, samples were withdrawn and the activity was measured using p-NPB. Half-lives
199 were calculated from the observed inactivation courses.
200

201 **2.6.2. Inactivation of different preparations in the presence of organic co-solvents**

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202 Enzyme preparations were incubated in mixtures of acetonitrile or 1,4-dioxane / 100
203 mM Tris–HCl pH 7 at different temperatures. Periodically, samples were withdrawn and the
204 activity was measured using p-NPB as described above. Half-lives were calculated from the
205 observed inactivation courses. The organic co-solvents presented in the samples did not have a
206 significant effect on enzyme activity (results not shown).

207 **2.7. Determination of the hydrolytic activity of the biocatalyst versus different substrates**

208 **2.7.1. Hydrolysis of ethyl hexanoate**

209 Enzyme activity was determined by using ethyl hexanoate; 200 mg of the immobilized
210 preparations were added to 1 mL of 25 mM substrate in 50 mM sodium phosphate at pH 7, in
211 some instances containing CH_3CN to have a homogenous system instead of a biphasic system.
212 All experiments were carried out at 25 °C under continuous stirring. The conversion degree was
213 analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP
214 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 µL) were injected and
215 eluted at a flow rate of 1.0 mL/min using acetonitrile/10 mM ammonium acetate aqueous
216 solution (50:50, v/v) and pH 3.2 as mobile phase and UV detection was performed at 208 nm.
217 When a substrate/enzyme suspension biphasic system existed, a sample of 100 µL was
218 withdrawn under very vigorous stirring, mixed with a volume of acetonitrile and filtered before
219 injection in the HPLC. Hexanoic acid had a retention time of 3.4 minutes while the ester
220 presented a retention time of 14.2 minutes. One unit of enzyme activity was defined as the
221 amount of enzyme necessary to produce 1 µmol of hexanoic acid per minute under the
222 conditions described above. Activity was determined by triplicate with a maximum conversion
223 of 20–30%, and data are given as average values.

224

225 **2.7.2. Hydrolysis of methyl mandelate**

226 Enzyme activity was also determined using methyl mandelate. 200 mg of the
227 immobilized preparations were added to 1 mL of 50 mM substrate in 50 mM sodium phosphate

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228 at pH 7 and 25 °C under continuous stirring. In some instances, organic solvents were added.
229 The conversion degree was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV
230 detector Spectra Physic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20
231 μL) were injected and eluted at a flow rate of 1.0 mL/min using acetonitrile/10 mM ammonium
232 acetate (35:65, v/v) at pH 2.8 as mobile phase and UV detection was performed at 230 nm. The
233 acid presented a retention time of 2.4 minutes while the ester had a retention time of 4.2
234 minutes. One unit of enzyme activity was defined as the amount of enzyme necessary to
235 produce 1 μmol of hexanoicacid per minute under the conditions described above. Activity was
236 determined by triplicate with a maximum conversion of 20–30%, and data are given as average
237 values.
238

239 **2.8. SDS-PAGE experiments**

240 SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [30]
241 using a Miniprotean tetra-cell (Biorad), 12% running gel in a separation zone of 9 cm × 6 cm,
242 and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized
243 enzyme samples was re-suspended in 1 mL of rupture buffer (2% SDS and 10%
244 mercaptoethanol), boiled for 5 min and a 20 μL aliquot of the supernatant was used in the
245 experiments. This treatment released all enzyme just interfacially activated on the support [11a].
246 Gels were stained with Coomassie brilliant blue. Low molecular weight markers from
247 Fermentas were used (10–200 kDa).
248

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249 **3.- Results**
250

251 **3.1.Preparation of OCGLX agarose**

252 OC-agarose was submitted to oxidation with sodium periodate. The results point out
253 that 25 μmol aldehyde groups per g of wet support could be introduced (see Figure 1). The
254 support was incubated in the presence of Schiff's reactive, confirming the existence of aldehyde
255 moieties on the support. After reduction with sodium borohydride, this reactivity disappeared.
256 The support modification with ethylenediamine permitted to introduce one primary and one
257 secondary amino groups [31], their titration in a pHstat confirmed the values obtained using
258 determination of remaining periodate.

259 Thus, a support bearing octyl moieties plus 25 μmol aldehyde groups per g of wet
260 support have been easily prepared from the commercial sample of OC-agarose. Using naked
261 4BCL agarose beads activated with glycidol, around 70 μmol glyoxyl groups/g could be
262 introduced [32], thus the octyl-glyoxyl support has a reasonable amount of aldehyde groups for
263 our purposes. The direct oxidation of non-activated 4BCL agarose with periodate produced 30
264 μmol aldehyde groups per g of wet support. This was the support used as reference of covalently
265 bonded-only biocatalyst even though it presented some more glyoxyl groups than the OCGLX.

266

267 **3.2. Immobilization of lipases on octyl, glyoxyl and octyl glyoxyl**

268 Figures 2-3 (and Figure 1 in supporting information) show the immobilization courses
269 of the 4 lipases on the different supports. All lipases immobilized slower on GLX agarose than
270 on any of the octyl supports. Moreover, in three of the four cases the enzymes became almost
271 fully inactivated when immobilizing on glyoxyl support (Figure 2). The enzyme stability at pH
272 10 was low in certain cases [33]. CALB immobilized around 30% of the offered activity after 48
273 h, the activity of the suspension remained almost unaltered. The use of mercaptoethanol
274 improved the immobilization yields in all cases, but only in the case of CALB this treatment

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275 permitted to have biocatalysts with higher activities, as in the other cases the enzymes were
276 inactivated during immobilization. Thus, only GLX-CALB could be prepared for further
277 comparisons with OCGLX biocatalysts..

278 At pH 7, the GLX supports were unable to immobilize any of the lipases (results not
279 shown), this experiment is necessary to confirm that using OCGLX, the first immobilization on
280 it is via interfacial activation. This is expected, as protein immobilization via glyoxyl groups
281 requires the involvement of several amino groups of the protein[19, 20], and at pH 7 the ε-amino
282 groups of the Lys residues will be in an ionized form and, therefore, unreactive.

283 Using OC-support (Figure 1S), immobilization rates are very high, and enzyme activity
284 significantly increased upon immobilization, as previously reported in many instances[10c, 11a].
285 Lecitase reached an activity of 270% compared to the initial one, RML and TLL activity
286 increase to more than a 300%, CALB is the only enzyme whose activity remained almost
287 unaltered after immobilization on octyl, very likely due to the very small lid that not fully
288 secludes the active center from the reaction media[23].

289 The use of OCGLX supports (Figure 3) produced a slightly higher immobilization rate
290 when compared to OC supports (see Figure 1S) in all cases (perhaps because the support is now
291 slightly more hydrophobic, see Scheme 1), and the effects on the enzyme activity are similar to
292 those observed using octyl agarose.

293 To study if the enzyme molecules had been covalently attached to the support, the
294 immobilized preparations were reduced using borohydride and submitted to analysis via SDS-
295 PAGE (this prevented the release of the enzyme molecules covalently attached to the reaction
296 medium by this treatment). It was observed that most of the immobilized enzyme molecules
297 could be released to the media (results not shown) after boiling the biocatalysts in the presence
298 of SDS and mercaptoethanol, suggesting that at pH 7 the reactivity of the amino groups of the
299 enzymes was not high enough to produce a covalent attachment with the glyoxyl groups, even

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300 though the enzyme is very near to the support and the reaction is an "intramolecular reaction"
301 [34].

302 Thus, we decided to increase the pH value of the medium after enzyme adsorption on
303 octyl-glyoxyl agarose to favor the enzyme-support reactivity. At alkaline pH the ε amino group
304 of the Lys will greatly increase its reactivity with glyoxyl groups [20a]. Moreover,
305 mercaptoethanol has been reported to be able to stabilize the imino bonds. tThus we studied the
306 effect of mercaptoethanol to covalently immobilize the adsorbed enzyme molecules [35]. Figure
307 2S in supplementary information shows the effect of this incubation at pH 10 on the activities of
308 the four enzymes.

309 After 24 h of incubation at pH 10, octyl-glyoxyl–CALB retained the enzyme activity
310 almost unaltered (98%). In the case of Lecitase, the preparation decreased its activity by around
311 17% in 4 h. In the presence of mercaptoethanol, the decrease in activity was slightly higher.
312 Using RML, after 4 h of incubation enzyme activity decreased to 41% in the absence of
313 mercaptoethanol and to 32% in the presence of mercaptoethanol. TLL decreased the activity
314 after incubation at pH 10 after 4 h by only 20%, becoming 28% in the presence of
315 mercaptoethanol. This result greatly contrasted with the results obtained when TLL was
316 immobilized directly at pH 10 on glyoxyl agarose, where the enzyme was almost fully
317 inactivated [33]. This could be explained by the stabilization toward high pH caused by the
318 interfacial activation of the enzyme on the octyl support.[24].

319 The SDS-PAGE analysis (Figure 4) of the enzyme desorbed from the supports after
320 boiling in the presence of SDS showed that for CALB, RML and Lecitase, only around 15% of
321 the enzyme could be released, that is, more than 85% of the enzyme molecules were covalently
322 attached to the support in the worst case scenario (CALB). Using TLL the percentage of enzyme
323 that did not become covalently immobilized on the OCGLX support was higher than in the other
324 casesover 30% of the enzyme was released by this treatment. The presence of mercaptoethanol
325 has no relevance in altering these results, suggesting that at pH 10 the stabilization of the imino

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326 bonds by the thiol compound is not necessary. As expected due to the reversibility of the imino
327 bonds, non-reduced OCGLX preparations released a quantity of protein that produced a band in
328 SDS-PAGE with an intensity similar to that obtained by analyzing the octyl preparation (Figure
329 4).

330 To compare enzyme molecules just adsorbed on OC supports versus the enzyme
331 molecules that were moreover covalently immobilized on the OCGLX matrices, it seemed
332 convenient to eliminate all non-covalently immobilized enzymes from the supports. To reach
333 this goal, the enzyme preparations were washed with the corresponding detergent concentration
334 able to release all the enzyme molecules from the octyl support (optimization of the washing
335 conditions is not shown). SDS-PAGE analysis of the 4 detergent washed octyl-glyoxyl
336 preparations (Figure 4) revealed that most enzyme molecules remaining in the support were
337 covalently attached to the support.

338 The loading capacities of the octyl and octyl-glyoxyl were identical as the
339 immobilization cause was the same for all biocatalyst (e.g., 17-20 mg/wet gram using CALB).
340 However, we used moderate loadings to prevent diffusion artifacts in the further analysis (see
341 methods).

342 The properties of these preparations were evaluated, compared to octyl and, in the case
343 of CALB, to glyoxyl derivatives.

344

345 **3.3.- Thermal stability**

346 Table 1 shows the half-lives of the different enzyme preparations at pH 5, 7 and 9.
347 Studying CALB, OCGLX preparations were more stable than OC-CALB preparations, except at
348 pH 5. The stabilization factor was over 10 at pH 9 and 4.5 at pH 7. The incubation of OCGLX in
349 the presence of mercaptoethanol to stabilize the enzyme-support imino bonds during biocatalyst
350 preparation reduced the stability of the enzyme, although it remained more stable than the OC
351 preparation. GLX-CALB was by far the least stable preparation in all studied pH values. Using

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352 Lecitase, at pH 5, the stabilization of the OCGLX compared to the OC preparations reached a
353 value of 12, at pH 7 was 7.7 folds, and at pH 9 was 4.9, mercaptoethanol presence during
354 alkaline incubation to get covalent bonds did not alter enzyme stability. When RML was
355 studied, stabilization values were 12 at pH 5, 4 at pH 7 and 7.8 at pH 9. The presence of
356 mercaptoethanol during alkaline incubation produced a light increase of enzyme stability at pH
357 7, had not effect at pH 9 and was even negative at pH 5.

358 OCGLX-TLL was the only exception, this preparation being less stable than the enzyme
359 which was only adsorbed, OC-TLL even by a 5 fold factor at pH 7. This decrease in stability
360 due to the covalent immobilization was complex to understand. To analyze likely causes of this
361 destabilization after immobilization on OCGLX compared to OC, we prepared different
362 biocatalysts with the different treatments that suffer the OCGLX-TLL except the possibility of
363 covalent reaction between enzyme and support. The enzyme was immobilized on reduced
364 OCGLX (to check if the chemical changes in the support can be responsible for the lower
365 stability), a support that is identical to the final OCGLX-TLL, but that cannot covalently react
366 with the enzyme. An amount of this immobilized enzyme was incubated at pH 10, to check if
367 the incubation produced any conformational or chemical change in the enzyme or on the support
368 that could generate some decrease on enzyme stability. Finally, a portion of this alkaline
369 incubated preparation was reduced with sodium borohydride, to check if the reduction step was
370 responsible for the decrease on enzyme stability (TLL has several disulfide bonds). The enzyme
371 stability of these three preparations remained similar to that of the OC-TLL. Thus, the reaction
372 between enzyme and support is the likeliest explanation for this decrease in stability; perhaps
373 some distortion caused during alkaline incubation can produce a unstable nzyme structure that
374 the low number of covalent attachments between enzyme and support cannot stabilize (the fact
375 that to have just one covalent bond with this enzyme is difficult suggested that achieving an
376 intense multipoint attachment is not very likely).

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378 **3.4.- Stability in organic solvents**

379 The different enzyme derivatives were incubated in different organic solvents and
380 concentrations, looking for conditions where the different OC-enzymes preparations became
381 significantly inactivated in a reasonable time (Table 1).

382 In opposition to thermal inactivations, OC-CALB preparation, inactivated at 30ºC in
383 80% dioxane at pH 7, was the least stable CALB preparation, including the GLX-CALB that
384 was now 33% more stable. The most stable preparation was OCGLX-CALB, with a
385 stabilization factor of 1.67, and results when the preparation of OCGLX-CALB was performed
386 in the presence of mercaptoethanol were worse, with no significant difference with the OC-
387 CALB stability. The comparatively low stability of the non-covalent preparation compared to
388 the covalent one could be related to the weakening of the enzyme/support interactions caused by
389 the cosolvent.

390 OCGLX-Lecitase in 45% acetonitrile at pH 7 and 30ºC was over 3 folds more stable than
391 OC-Lecitase. OCGLX-RML was 6 folds more stable than OC-RML in 30% acetonitrile at 30ºC.
392 The TLL half live of OCGLX in 60% dioxane at pH 7 and 30ºC was 11.9 times higher than that
393 of OC-TLL. In these three cases, the incubation in the presence of mercaptoethanol during the
394 preparation of the OCGLX biocatalysts did not alter the final results. Except in the case of
395 CALB, the stabilizations observed in the presence of organic solvents were quite significant by
396 using OCGLX instead of OC supports, suggesting that the covalent immobilization may play an
397 important role in enzyme stability in this medium.

398

399 **3.5.-Desorption of enzyme molecules during inactivation from octyl supports.**

400 As previously visualized (see figure 4), the enzyme cannot be desorbed during
401 inactivation from OCGLX supports, even in a SDS-PAGE treatment the enzyme remains
402 attached to the support, because of the high stability of the secondary amino bonds formed
403 between enzyme and support after reduction. To check if OC-lipase preparations released

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404 enzyme molecules during the thermal or organic solvents inactivations, the amount of protein
405 adsorbed to the support before and after inactivations was compared using SDS-PAGE analysis.
406 As stated before, the boiling of the OC-lipases in the presence of SDS released all protein from
407 the OC support to the medium. Thus, the OC preparations of the 4 different enzymes before and
408 after enzyme inactivation were submitted to this study. Figure 5 shows that the amount of
409 enzyme in octyl supports after enzyme inactivation in organic solvent significantly decreased
410 during enzyme inactivation. The release of the enzyme covalently attached to the support via
411 secondary amino bonds is no longer possible, increasing the interest of this new methodology.
412 Thus, enzyme leakage from the octyl support could explain why the OCGLX preparations were
413 much more stable than the OC biocatalysts in the presence of organic solvents. CALB was an
414 exception, the enzyme did not seem to be released from the support during inactivation in
415 organic media, and perhaps this explains why the stabilization observed with this enzyme in the
416 presence of organic cosolvent was relatively low.
417 Furthermore, we performed a similar analysis on OC-enzyme preparations thermally
418 inactivated in aqueous medium at different pH values. Figure 6 shows that there was a massive
419 release of the immobilized enzyme from the octyl support to the medium at high temperatures.
420 The release of the enzyme molecules may be before or after enzyme inactivation, and, in all
421 cases, the enzyme can be finally incorporated to the reaction media and contaminate the product.
422 This can explain the positive effects of the covalent attachment in thermal inactivations.
423 Lecitase did not release from the octyl support during thermal inactivation at any of the analyzed
424 pH and T values (the amount of enzyme that remained adsorbed on the support is very similar),
425 but this did not prevent the clear stabilization of the enzyme using OCGLX support compared to
426 the OC ones.
427 Thus, the prevention of the release of enzyme molecules from the support may be a
428 reason for enzyme stabilization when using OCGLX, both in thermal and organic solvent

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429 inactivation. This is also important because if the enzyme is released from the support, it may
430 contaminate the reaction media, an important point if the enzyme is used in food modification.
431
432 **3.6.- Activity of the different enzyme preparations**
433 Table 2 shows the activity of the different enzyme preparations versus two different
434 substrates, methyl mandelate and ethyl hexanoate.
435 In the hydrolysis of methyl mandelate in aqueous media, OC and OCGLX-CALB
436 presented very similar activity per mg of immobilized enzyme, almost triplicating the activity of
437 the GLX preparation. The activity versus ethyl hexanoate was determined in growing
438 concentrations of acetonitrile (from 50 to 90%). The activity decreased in the presence of
439 solvent, but this decrease on enzyme activity was sharper when using the OC-CALB than when
440 using OCGLX (in 90% acetonitrile this preparation presented 30% more activity than OC-
441 CALB). GLX-CALB was 9
442 fold less active than OCGLX in 50% acetonitrile and 4 times less active in 90%.
443 Thus, the specificity of the GLX-CALB was different than that of OC preparations
444 (comparing the results in water with both substrates), while the organic solvents have a lower
445 impact on the enzyme activity of the covalent preparation. OC-and OCGLX preparations
446 presented a similar specificity but different resistance to organic solvents.
447 Analyzing Lecitase, OC- and OCGLX-Lecitase presented a similar activity in the
448 hydrolysis of methyl mandelate in aqueous media. However, in the hydrolysis of ethyl
449 hexanoate in aqueous media, OCGLX-Lecitase is around 3 fold more active than OC-Lecitase.
450 In the presence of 50% acetonitrile, this difference becomes almost 64 fold factor. OCGLX-
451 RML (remember that incubation at alkaline pH value decreased the activity by almost a 3 fold
452 factor using pNPB) is 4 fold less active in the hydrolysis of methyl mandelate than OC-RML. In
453 the hydrolysis of ethyl hexanoate, OCGLX becomes slightly more active than OC-RML in fully
454 aqueous media, and the differences become a factor of 2 in the presence of 50% acetonitrile. In

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455 the case of TLL, in aqueous media OC-TLL is almost 2 fold more active than the OCGLX using
456 methyl mandelate, while it is slightly less active in the hydrolysis of ethyl hexanoate in aqueous
457 media, but in 50% acetonitrile the OCGLX become almost 100 fold more active than OC-TLL.
458 The four OCGLY biocatalysts can be reused for 5 cycles in hydrolysis in aqueous media
459 of methyl mandelate or ethyl hexanoate without any appreciable decrease in enzyme activity
460 (Figure 3 S).
461 The results show that the covalent immobilization after interfacial activation on octyl
462 agarose of lipases produces some changes in enzyme specificity, perhaps not very significant
463 compared to other changes reported in literature after using different immobilization
464 protocols[27], but the most relevant effect is the retention of the activity in the presence of
465 organic solvents, as previously shown in the enzyme which is just interfacially activated versus
466 octyl agarose which may become desorbed in the presence of high concentrations of organic
467 solvents.
468
469 **4.-Conclusions**
470 The new octyl-glyoxyl supports, prepared by periodate oxidation of commercially
471 available octyl-agarose, have shown a great potential to be used in the immobilization of lipases.
472 The first immobilization is via interfacial activation, as showed by the release of most enzyme
473 molecules after immobilization if boiled in SDS. That way, the advantages of the use of octyl-
474 agarose remained: one step immobilization/purification, stabilization of the open form of the
475 lipases. The use of an alkaline pH value after enzyme adsorption is required to achieve some
476 covalent enzyme-support attachments. The enzyme molecules covalently attached usually
477 presented a higher thermal stability, a higher stability and activity in presence of organic
478 solvents, and the enzyme cannot contaminate the reaction media, because the covalent bonds are
479 irreversible. This last advantage may be also considered a drawback, as this strategy converted a
480 reversible immobilization method in an irreversible one. This avoids the reuse of the support

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481 after enzyme inactivation; but may also open the possibility to analyze the reactivation of the
482 immobilized enzyme by unfolding/refolding strategies [36] of the OCGLX immobilized lipases
483 (studies in course in our research group).

484 In general, a high percentage of the lipase molecules interfacially adsorbed resulted
485 covalently attached (with exception of TLL). This resulted in good yields in covalent enzyme
486 immobilization being obtained after the adsorption of the enzyme on the support that facilitates
487 the enzyme/support reaction, on a relative dense layer of aldehydes groups (considering the
488 activation achieved and the specific area of agarose 4B, around 5-6 aldehyde groups/1000A^2).
489 However, the aldehyde residues will be under a layer of longer octyl groups, and lipases are not
490 generally very rich in Lys residues. The previous interfacial activation of the lipases on the octyl
491 support improves the enzyme stability, producing a lower impact of the incubation of enzymes
492 at pH 10. In fact, three of the used enzymes cannot be immobilized on glyoxyl agarose, with
493 even higher activation than the OCGLX, because of the slow immobilization rate and low
494 stability under these conditions.

495 After confirming the advantages of a mixed interfacial activation/covalent
496 immobilization, it should be convenient to elaborate strategies where 100% of the enzyme
497 molecules interfacially activated versus the octyl support could become covalently attached. In
498 this sense, an enrichment of the enzyme surface in new amino groups (chemically or
499 genetically) seems a convenient strategy, and it has been assayed with success to improve the
500 covalent attachment of some enzymes on glyoxyl supports [33, 37]. This way, this new support
501 opens new research lines to improve the results and explore the advantages that offer. Other
502 heterofunctional supports, combining other chemical reactive moieties on the support and other
503 causes for enzyme immobilization (e.g., ion exchange, interaction with immobilized metal
504 chelate , thiol) are very useful for other enzymes, but they cannot guarantee the immobilization-
505 stabilization of the open form of lipases as the strategy proposed in this paper [17].

506

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514

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661 **Figure legends**

662

663 **Scheme 1. Preparation of OCGLX supports**

664

665 **Figure 1. Oxidation of OC support to obtain OCGLX agarose support.** Experiments
666 have been performed as described in Section 2.

667

668 **Figure 2. Immobilization courses of different lipases on glyoxyl agarose support.**
669 Experiments have been performed as described in Section 2. Immobilization on GLX;
670 Panel A: (CALB), Panel B: (Lecitase), Panel C: (RML) and Panel D: (TLL). Rhombus
671 (suspension), Square (Supernatant), Triangle (Soluble enzyme), Solid black line (pH 10
672 without mercaptoethanol), dash line (pH 10 with mercaptoethanol).

673

674

675

676 **Figure 3. Immobilization courses of different lipases on octyl-glyoxyl agarose**
677 **supports.** Experiments have been performed as described in Section 2. Panel A (CALB):
678 Triangles, solid black line: soluble enzyme; square, dash line: supernatant. Panel B
679 (Lecitase): Rhombus, solid black line: suspension; Squares, solid black line: supernatant;
680 Triangles, solid black line: soluble enzyme. Panel C (RML): Rhombus, solid black line:
681 suspension; Squares, solid black line: supernatant; Triangles, solid black line: soluble enzyme.
682 Panel D (TLL): Rhombus, solid black line: suspension; Squares, solid black line: supernatant;
683 Triangles, solid black line: soluble enzyme.

684

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686 **Figure 4. SDS-PAGE analysis of different biocatalysts preparations.** The
687 immobilized enzymes were submitted to the processes described in Section 2. Panel A:
688 CALB, Panel B: Lecitase, Panel C: RML and Panel D: TLL. Lane 1: Molecular weight
689 marker, Lane 2: OC, Lane 3: OCGLX, Lane 4: OCGLX incubated to pH10, Lane 5:
690 OCGLX incubated to pH10 and reduced with NaBH4, Lane 6: OCGLX incubated to
691 pH10 with mercaptoethanol, Lane 7: OCGLX incubated to pH10 with mercaptoethanol
692 and reduced with NaBH4, Lane 8: OCGLX incubated to pH 10, reduced with NaBH4
693 and washed with detergent, Lane 9: OCGLXCALB incubated to pH10 with
694 mercaptoethanol, reduced with NaBH4 and washed with detergent.
695
696 **Figure 5. SDS-PAGE analysis of different octyl-biocatalysts preparations after**
697 **inactivation in the presence of organic solvents at 30ºC for 8 h**. Experiments have
698 been performed as described in Section 2. The gel shows the enzyme that remains bound
699 to the support after inactivation. Panel A: OCCALB, Lanes 1 and 7: Molecular weight
700 marker, Lane 2: OCCALB, Lane 3: OCCALB incubated in 90% of Dioxane and Lane 5:
701 OCCALB incubated in 90% of ACN. Panel B: TLL, RML and Lecitase. Lanes 1, 7 and
702 10: Molecular weight marker, Lane 2: OCTLL, Lane 3: OCTLL incubated in 60%
703 Dioxane, Lane 5: OCRML incubated in 30% ACN, Lane 6: OCRML, Lane 8:
704 OCLecitase and Lane 9: OCLU incubated in 45% ACN.
705
706 **Figure 6. SDS-PAGE analysis of different octyl biocatalysts preparations after**
707 **thermal inactivation at different pH values for 8 h**. Experiments have been performed
708 as described in Section 2. . The gel shows the enzyme that remains bound to the support
709 after inactivation. Panel A: Lane 1: Molecular weight marker, Lane 2: OCTLL, Lane 3:
710 OCTLL incubated at pH 5 and 70°C, Lane 4: OCTLL incubated at pH 7 and 70°C, Lane
711 5: OCTLL incubated at pH 9 and 60°C, Lane 6: Molecular weight marker, Lane 7:

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712 OCCALB, Lane 8: OCCALB incubated at pH 5 and 70°C, Lane 9: OCCALB incubated
713 at pH 7 and 70°C, Lane 10: OCCALB incubated at pH 9 and 60°C. Panel B: Lane 1:
714 Molecular weight marker, Lane 2: OCRML, Lane 3: OCRML incubated at pH 5 and
715 60°C, Lane 4: OCRML incubated at pH 7 and 50°C, Lane 5: OCRML incubated at pH 9
716 and 45°C, Lane 6: Molecular weight marker, Lane 7: OCLU, Lane 8: OCLU incubated
717 at pH 5 and 60°C, Lane 9: OCLU incubated at pH 7 and 50°C, Lane 10: OCLU
718 incubated at pH 9 and 45°C.

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	Experimental conditions						
BIOCATALYST	pH 5	pH 7	pH 9	Dioxane 80%	ACN 45%	ACN 30%	Dioxane 60%
OCCALB	150 ± 7.5	24 ± 1.2	10 ± 0.5	144 ± 7.2	-	-	-
OCGLXCALB pH 10	120 ± 6.0	108 ± 5.4	100 ± 5.0	240 ± 12.0	-	-	-
OCGLXCALB pH 10 – Mercaptoethanol	108 ± 5.4	88 ± 4.4	80 ± 4.0	150 ± 7.5	-	-	-
GLXCALB	5 ± 0.3	5 ± 0.3	5 ± 0.3	192 ± 9.6	-	-	-
OCLU	5 ± 0.3	110 ± 5.5	105 ± 5.3	-	5 ± 0.3	-	-
OCGLXLU pH 10	60 ± 3.0	850 ± 42.5	515 ± 25.8	-	15 ± 0.8	-	-
OCGLXLU pH 10 – Mercaptoethanol	60 ± 3.0	850 ± 42.5	515 ± 25.8	-	15 ± 0.8	-	-
OCRML	10 ± 0.5	42 ± 2.1	5 ± 0.3	-	-	5 ± 0.3	-
OCGLXRML pH 10	120 ± 6.0	168 ± 0.9	39 ± 2.0	-	-	30 ± 1.5	-
OCGLXRML pH 10 – Mercaptoethanol	100 ± 5.0	180 ± 9.0	42 ± 2.1	-	-	30 ± 1.5	-

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OCTLL	240 ± 12.0	150 ± 7.5	492 ± 24.6	-	-	-	72 ± 3.6
OCGLXTLL pH 10	180 ± 9.0	30 ± 1.5	150 ± 7.5	-	-	-	860 ± 43.0
OCGLXTLL pH 10 – Mercaptoethanol	210 ± 10.5	30 ± 1.5	150 ± 7.5	-	-	-	860 ± 43.0

720
721
722 **Table 1**. Half-lives of the different biocatalyst under different conditions (in minutes).. CALB (pH 5 - 80°C, pH 7 - 70°C, pH 9 - 60°C), LU and RML (pH 5 - 60°C, pH 7 -
723 50°C, pH 9 - 45°C) and TLL (70°C at pH 5 and pH 7, 60°C at pH 9). All enzymes derivatives were incubated at 30°C in organic solvents.
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726

	Substrate and experimental conditions			
BIOCATALYST	Methyl Mandelate, aqueous media	Ethyl Hexanoate, aqueous media	Ethyl Hexanoate, 50% ACN	Ethyl Hexanoate, 90% ACN
OCCALB	61.8 ± 3.1	-	708.0 ± 35.4	37.2 ± 1.9
OCGLXCALB pH 10	68.0 ± 3.4	-	902.2 ± 45.1	50.9 ± 2.5
OCGLXCALB pH 10 – Mercaptoethanol	65.8 ± 3.3	-	626.7 ± 31.3	46.9 ± 2.3
GLXCALB	21.0 ± 1.1	-	100.0 ± 5.0	12.5 ± 0,6
OCLU	23.7 ± 1.2*	4.9 ± 0,2	1.4 ± 0.1*	-
OCGLXLU pH 10	23.4 ± 1.2*	13.2 ± 0.7	89.8 ± 4.5*	-
OCGLXLU pH 10 – Mercaptoethanol	20.8 ± 1.0*	12.5 ± 0.6	79.0 ± 4.0*	-
OCRML	22.5 ± 1.1*	6.7 ± 0,3	26.2 ± 1.3**	-
OCGLXRML pH 10	5.9 ± 0.3*	7.5 ± 0,4	55.0 ± 2.8**	-
OCGLXRML pH 10 – Mercaptoethanol	4.9 ± 0.2*	7.3 ± 0,4	43.0 ± 2.2**	-
OCTLL	8.6 ± 0.4*	8.6 ± 0.4	0.2 ± 0,02**	-
OCGLXTLL pH 10	4.4 ± 0.2*	10.7 ± 0.5	19.4 ± 1.0**	-
OCGLXTLL pH 10 – Mercaptoethanol	5.4 ± 0.3*	11.0 ± 0.6	23.3 ± 1.2**	-

727
728 Table 2. Activity of the different biocatalyst versus methyl mandelate (50 mM) and ethyl hexanoate(25 mM) at pH 7 and 25ºC. Experiments were performed as described in
729 Section 2.7. The activity is given in µmoles of substrate hydrolyzed per minute and mg of immobilized enzyme, .*Activity (x10^3), **Activity (x10^2).

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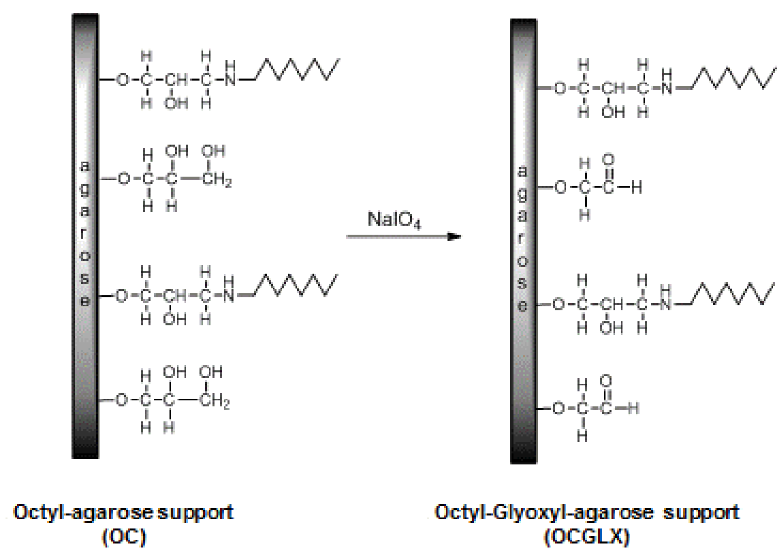
Scheme 1



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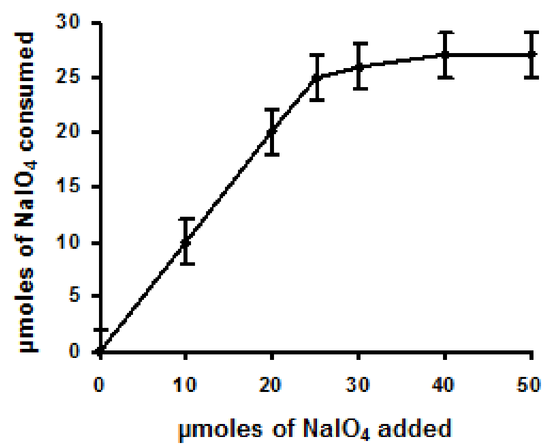

731 **Figure 1**

732

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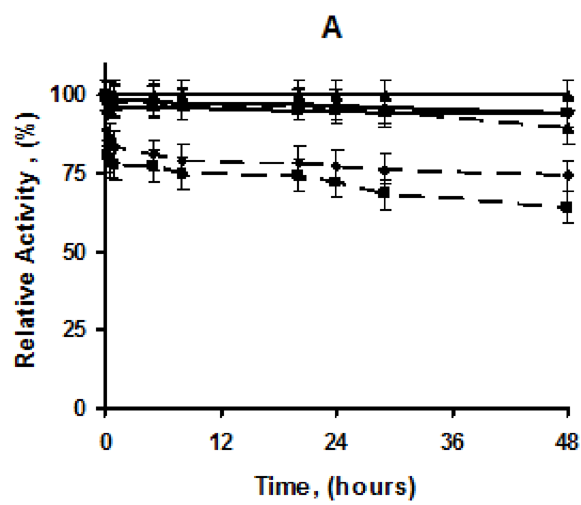

733 **Figure 2**

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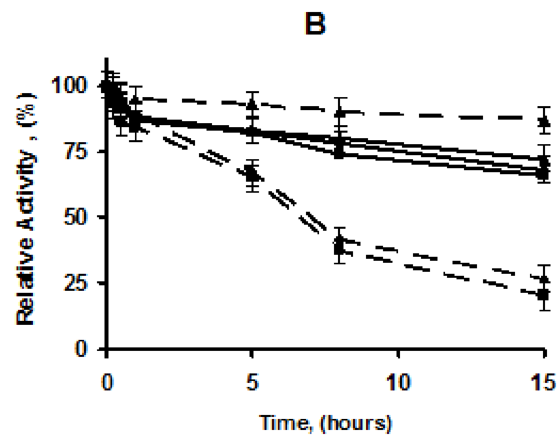

734 **Figure 2**

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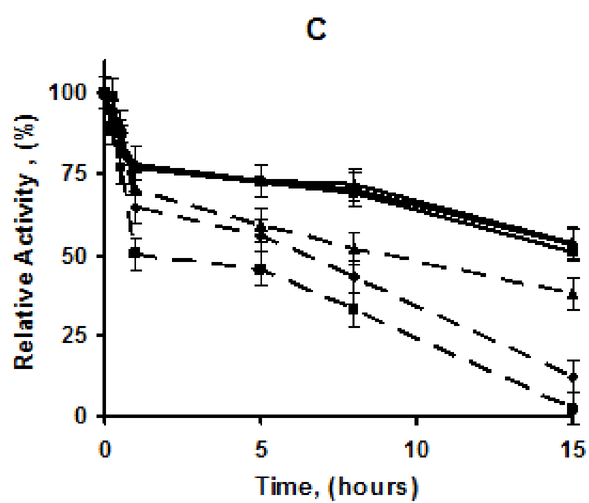

735 **Figure 2**

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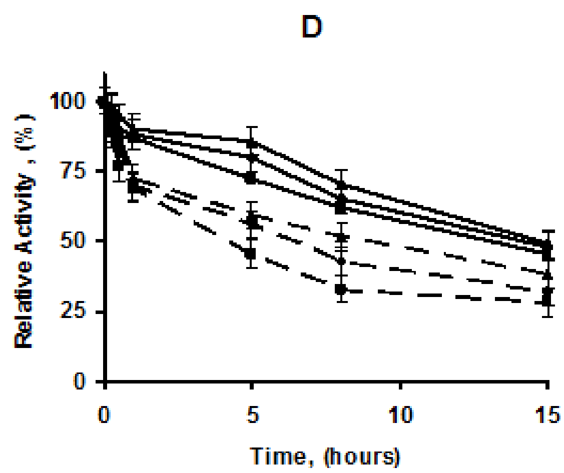

736 **Figure 2**

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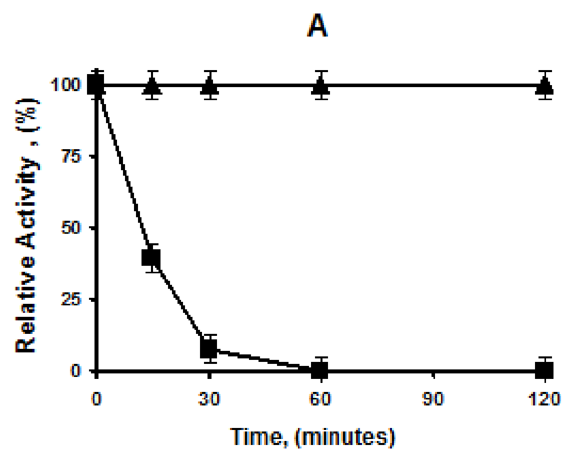

737 **Figure 3**

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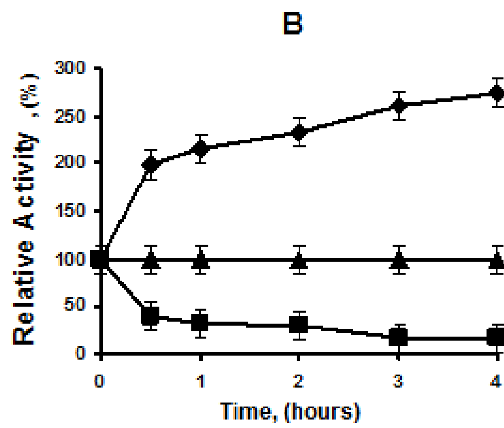

738 Figure 3

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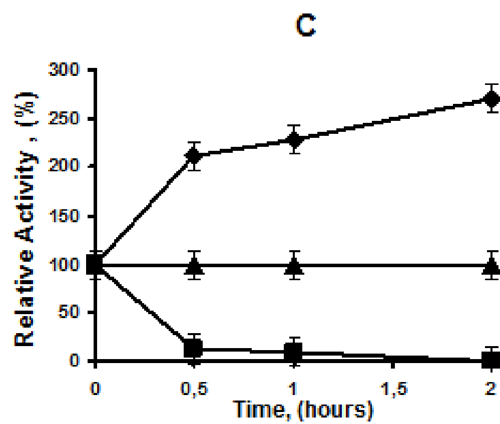

739 Figure 3

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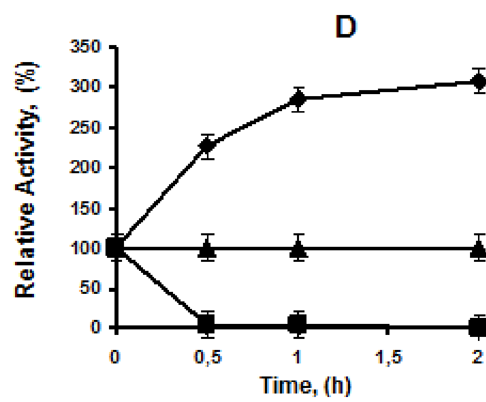

740 Figure 3

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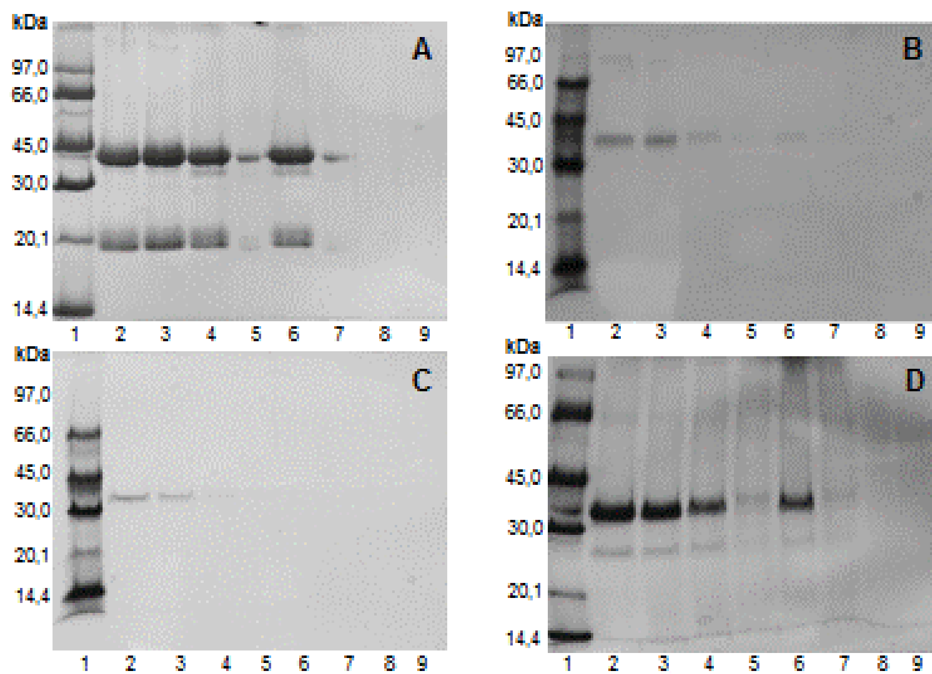

FIGURE 4

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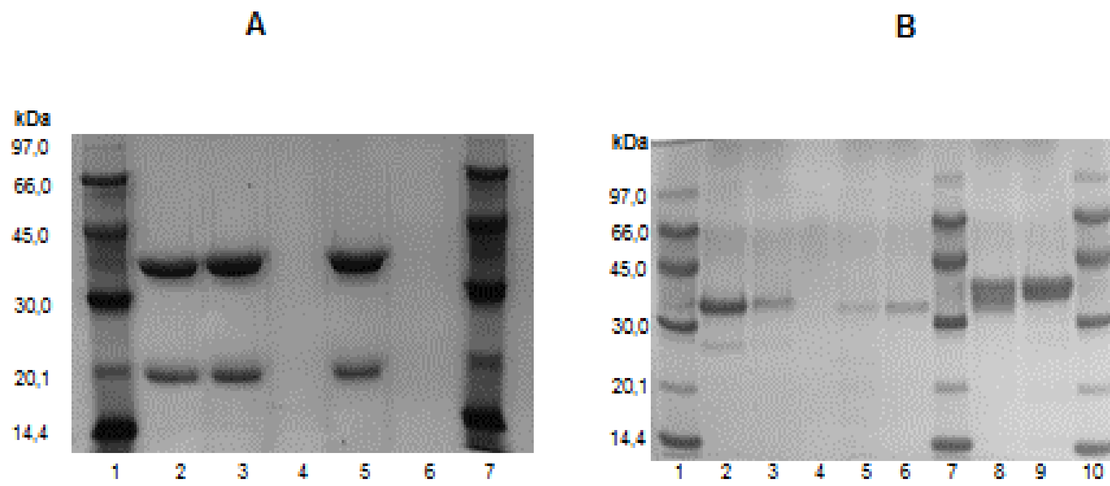

FIGURE 5

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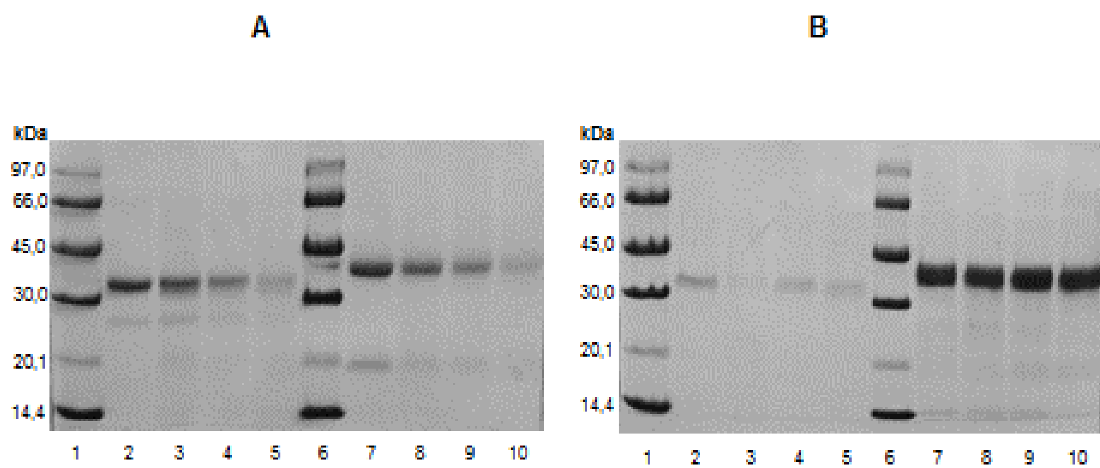

FIGURE 6

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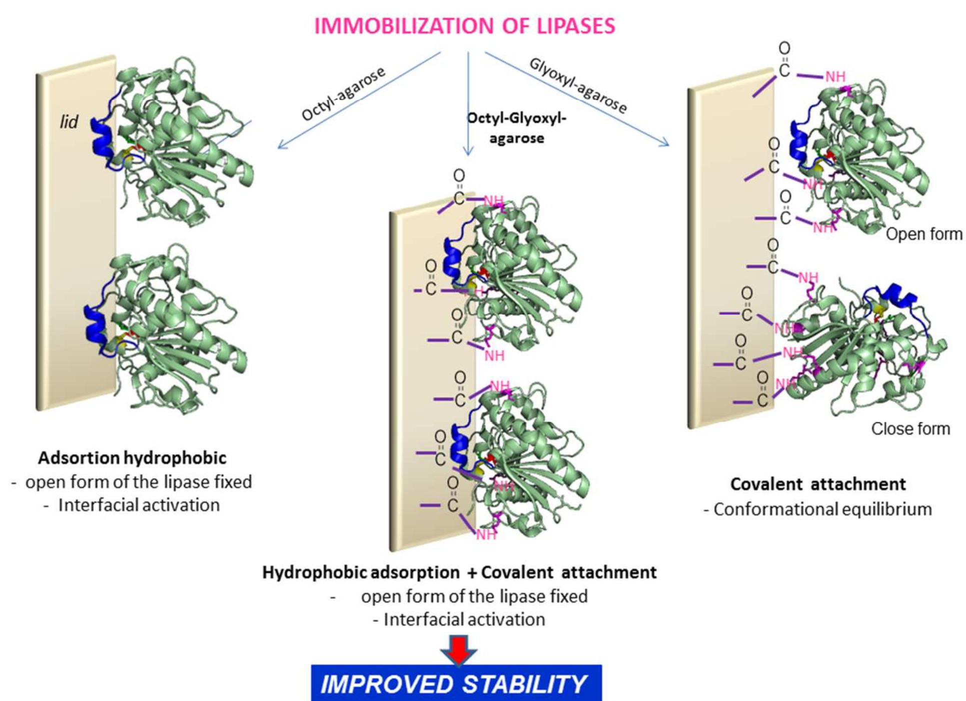

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