Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 51 (2008) 110–117
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Lecitase® ultra as regioselective biocatalyst in the hydrolysis of fully protected carbohydrates
Strong modulation by using different immobilization protocols

Gloria Fernandez-Lorente [a], Marco Filice [b], Marco Terreni [b], Jose M. Guisan [a], Roberto Fernandez-Lafuente [a,*], Jose M. Palomo [a,*]

[a] *Departamento de Biocatálisis, Instituto de Catálisis (CSIC), Campus UAM, Cantoblanco, 28049 Madrid, Spain*
[b] *Dipartimento di Chimica Farmaceutica, Università di Pavia, via Taramelli 12, 27100 Pavia, Italy*

Received 6 August 2007; received in revised form 14 November 2007; accepted 20 November 2007
Available online 26 November 2007

Abstract

This paper shows that Lecitase Ultra is an enzyme preparation with a great interest as regioselective biocatalyst in the deprotection of 4 different peracetylated sugars: 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**4**), 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**7**) and 2,3,4,6-tetra-*O*-acetyl-β-D-galacto pyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**). The enzyme properties (specificity, preference for the per-acetylated sugar and regio-selectivity) were strongly modulated by the immobilization conditions, for example the octyl-LECI preparation was 10 fold more active than the PEI-LECI preparation, while it was more than 40 fold less active against some other substrates. Very interestingly, these changes also affected the regioselectivity, depending on the preparation used it was possible to get free OH groups in anomeric position, position 6 or the mixture of both. Finally, the octyl-LECI preparation did not recognize the α-sugars, favouring the β-isomers (in opposition to most commercial lipases or the other LECI preparations). This is potentially useful to obtain pure α-peracetylated monosaccharides from a mixture of anomers.

Keywords: Regioselective reaction; Enzyme modulation; Enzyme specificity; Phospholipase; Peracetylated carbohydrates

1. Introduction

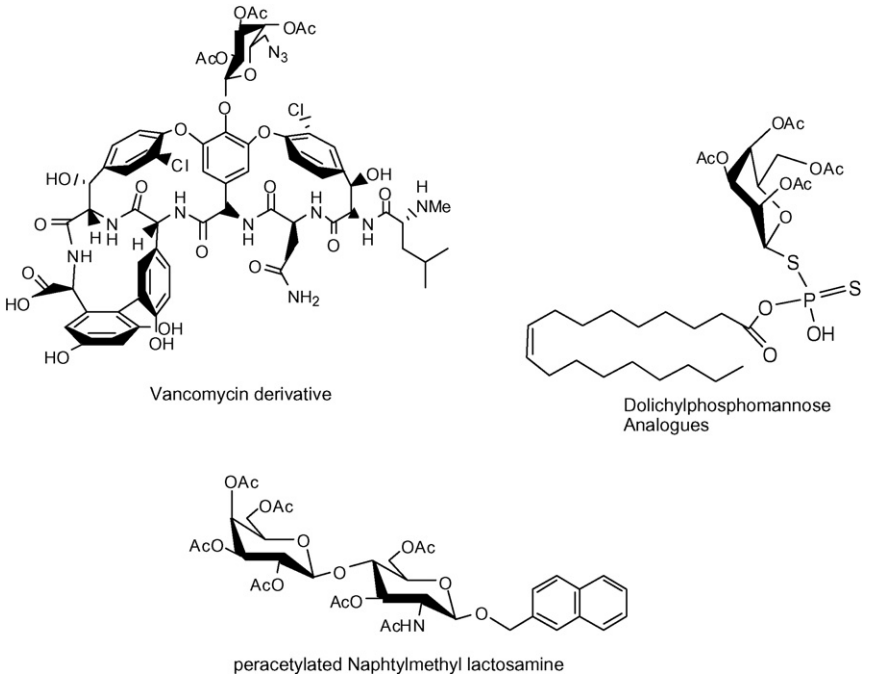
Phospholipases A_1 are of particular interest for industrial applications, to produce 2-acyl-lysophospholipids (good emulsifiers) with interesting fatty acid composition (eicosapentaenoic acid, conjugated linoleic acid and docosahexaenoic acid) and degumming process of oils [1]. The interest on finding new phospholipases is shown by the high number of sources for this enzyme discovered in the recent years and the trials to improve their performance by diverse techniques [2]. Recently, a new enzyme preparation with phospholipase A_1 activity, namely Lecitase® Ultra (LECI), was patented [3] and made commercially available. Following the supplier information, this is a preparation obtained from the fusion of the genes of the lipase from *Thermomyces lanuginosa* and the phospholipase from *Fusarium oxysporum* developed mainly for degumming processes [3]. This new enzyme presented the stability of the lipase from *T. lanuginosa* and the activity of the enzyme from *F. oxysporum* [3]. However, it also has biocatalytic properties that are useful in the synthesis of tailored phospholipids, although due to the relative novelty of this product only few manuscripts may be found in literature on its use [4].

Phospholipases have been described to suffer interfacial activation [5], similarly to lipases [6]. Lipases present a broad substrate specificity combined with a high regio and enantioselectivity, and may be used in many different reaction media [7]. However, to our knowledge, LECI as biocatalyst for fine chemistry – using substrates far from phospholipids – has been only recently utilized to resolve racemic mixtures of chiral compounds [8].

Here, we present a first study on the use of this new commercial enzyme in the regioselective hydrolysis of peracetylated carbohydrates. Pure regioisomers of *O*-acetyl-glycopyranosides

* Corresponding authors. Tel.: +34 91 585 48 09; fax: +34 91 585 47 60.
E-mail addresses: rfl@icp.csic.es (R. Fernandez-Lafuente), josempalomo@icp.csic.es (J.M. Palomo).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.11.017



Scheme 1. Several biological glycoderivatives.

presenting only one free hydroxyl group may be employed as key intermediates in the preparation of different glycoderivatives, such as oligosaccharides, glycolipids or glycopeptides [9] (Scheme 1).

Per-*O*-acetyl-glycopyranoses could be used as raw material to obtain these monohydroxy peracetylated carbohydrates, although the reported procedures of chemical hydrolysis usually afford the deacetylation at the anomeric position [10]. The synthesis of the monohydroxy derivatives in primary or secondary positions is very difficult by classical chemical approaches, making it necessary to use many chemically selective protection/deprotection steps [11,26].

Consequently, the use of enzymatic catalysts, especially lipases, because of their high versatility recognizing a broad range of substrates with high regio and enantioselectivity [12] could be an attractive alternative. Previous studies have reported the enzyme-catalyzed hydrolysis of different peracetylated carbohydrates although mainly in the anomeric position [13].

To perform the enzymatic synthesis of mono-deacetylated carbohydrates following this strategy, it is necessary to find biocatalysts exhibiting two different properties:

1. *A high specificity*—the enzyme must recognize the peracetylated sugar as substrate much better than it hydrolyzes the mono deacetylated sugar, allowing the accumulation of monohydroxy product.
2. *A high regioselectivity*—the enzyme must produce only one of the different possible regioisomers.

The catalytic mechanism of lipases, and phospholipases, implies dramatic conformational changes of the enzyme molecule between a 'closed' and an 'open' form [14]. This mechanism of action – in the case of lipases – has permitted the use of different immobilization protocols involving different areas of the enzyme – the so-called "conformational engineering of lipases" – causing a strong modulation of the lipase properties in kinetic resolution of racemic mixtures [15,16], regioselective deprotection of peracetylated sugars [17] and asymmetric hydrolysis [18]. Here, we hypothesize that, in a similar way, the use of different immobilization protocols may be used to modulate the regioselectivity of LECI in the hydrolysis of peracetylated carbohydrates in aqueous media.

Four very different immobilization protocols have been applied: (i) immobilization on hydrophobic supports at low ionic strength by interfacial activation of the lipase on the hydrophobic surface of the support, involving the hydrophobic area surrounding the active site of the lipase [19], generating a highly hydrophobic environment around the enzyme active site; (ii) immobilization on agarose activated with CNBr *via* covalent attachment throughout the most reactive amino group (very likely the terminal NH_2) on the enzyme surface [20]; (iii) immobilization on glyoxyl-agarose *via* multipoint covalent attachment throughout the richest area/s in Lysines on the enzyme surface [20]; (iv) immobilization on agarose beads coated with PEI *via* anionic exchange through the areas with the highest negative net charge of the enzyme [21].

2. Experimental section

2.1 Materials

Lecitase® Ultra (LECI) was obtained from Novozymes (Denmark). Agarose 6BCL, octyl-agarose 4BCL and cyanogen bromide agarose (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), Triton X-100, Triton

X-45, Tween-20, diethylamine (DEA), hexadecyltrimethylammonium bromide (CTAB), *p*-nitrophenyl butyrate (pNPB), 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**4**), 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-1,2,-3,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**) were from Sigma. 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**7**) was from Extrasynthese. Glyoxyl-agarose [22] and PEI-agarose [21] were prepared as previously described. Dimethylaminopyridine (DMAP) was from Fluka. HPLC analyses were performed using HPLC spectra P100 (Thermo Separation products). The column was a Kromasil-C_8 (250 mm × 4.6 mm and 5 μm) from Analisis Vinicos (Tomelloso, Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 215 nm. The eluent was an isocratic mixture of 30% acetonitrile in phosphate buffer (10 mM) at pH 4; flow rate 1.0 mL/min. TLC Columns for flash chromatography were made up with Silica Gel 60 (Merck) 60–200 or 40–63 μm. ^{1}H NMR were recorded in $CDCl_3$ (δ = ppm) on a Bruker AMX 400 instrument.

2.2 Standard enzymatic activity assay determination

This assay was performed by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of *p*NPB activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of *p*NPB per minute (IU) under the conditions described above.

2.3 Immobilization of lipases on different supports

Different immobilized preparations were prepared following the procedures previously described: (i) ionically adsorbed lipase on solid supports coated with PEI [21] at pH 7 and 25 °C, (ii) covalent immobilization on CNBr-activated support at pH 7 and 25 °C [20], (iii) multipoint covalent attachment on glyoxyl-agarose at pH 10 and 25 °C [22] and (iv) interfacial adsorption on a hydrophobic support, octyl-agarose at 5 mM sodium phosphate buffer at pH 7 [19]. Enzyme loading was 12 mg/g of support. Protein concentration was determined by the Bradford method [23].

2.4 Enzymatic hydrolysis of peracetylated sugars

1,**4** or **9** (0.02 mmol, 8 mg) and **7** (0.02 mmol, 16 mg) was added to 10 mL solution of phosphate buffer 25 mM with 10% acetonitrile for **1**,**4** and **9**, or 20% acetonitrile for **7** at pH 5, 25 °C and the reaction was initialized by adding 0.8 g (**1**) and 1 g (**4**, **7** or **9**) of biocatalyst, The reaction was performed at pH 5 in order to avoid the chemical acyl-migration in the monohydroxy per-*O*-acetylated carbohydrates [24,26]. The hydrolytic reaction was carried out under mechanical stirring, and pH was controlled by automatic titration. Hydrolysis reactions were followed by HPLC. Finally, after filtration of the immobilized enzyme, the products were isolated and identified by ^{1}H NMR, and 2D-COSY NMR.

2.4.1. *2, 3, 4, 6-Tetra-O-acetyl- α/β-D-galactopyranose (2)*

1 (390 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g glyoxyl-LECI preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2 mL × 10 mL), separated and dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum to afford **2** as a white solid (215 mg, 55%). HPLC analysis: t_R = 8.3 (β anomer), 9.8 min (α-anomer). ^{1}H NMR (400 MHz, $CDCl_3$), δ = ppm: 5.52 (bd, 1H, *J* = 3.4 Hz; H-1), 5.48 (dd, 1H; *J* = 1.25 Hz; H-4), 5.41 (dd, 1H; *J* = 3.4 Hz; H-3), 5.19 (dd, 1H; *J* = 3.4 Hz, H-2), 4.72 (dt, 1H; *J* = 6.5 Hz; H-5), 4.12–4.08 (dd, 2H; *J* = 11.5 Hz, H-6a,b), 2.15–1.99 (s, 12H, 4 × CH_3).

2.4.2. *1, 2, 3, 4-Tetra-O-acetyl-β-D-galactopyranose (3)*

1 (390 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g octyl-LECI preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum to afford **3** as a white solid (370 mg, 95%). HPLC analysis: t_R = 10.6 min. ^{1}H NMR (400 MHz, $CDCl_3$). δ: 5.73 (d, 1H, *J* = 8.26 Hz, H-1), 5.44 (d, 1H, *J* = 3.39 Hz, H-4), 5.32 (t, 1H, *J* = 8.37 Hz, H-3), 5.13 (dd, *J* = 3.42 Hz, *J* = 10.4 Hz, H-2), 3.91 (dt, *J* = 6.45 Hz, H-5), 3.8–3.51 (m, 2H, ABX system, 2 × H-6), 2.14–1.97 (s, 12H, 4 × CH_3).

2.4.3. *2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-α/β-D-glucopyranose (5)*

4 (390 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 8 g PEI-LECI preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2 mL × 10 mL), separated and dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum. After that, purification by the flash chromatography column was performed with 95:5 dichloromethane:methanol (71 mg, 18%). HPLC analysis: t_R = 6.5 min. ^{1}H NMR (500 MHz, $CDCl_3$). δ: 6.04 (d, 1H, *J* = 9.5 Hz, NH), 5.25 (dd, 1H, $J_{3,2}$ = 10.0, $J_{3,4}$ = 9.5 Hz, H-3), 5.15 (d, 1H, *J* = 3.5 Hz, H-1), 5.07 (t, 1H, *J* = 9.5 Hz, H-4), 4.73 (d, 1H, *J* = 8.5 Hz, H-1 β-anomer), 4.22 (m, 1H, H-5), 4.16 (m, 2H, H-2, H-6), 4.02 (m, 1H, H-6), 2.03 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.90 (s, 3H, N-2 CH_3).

*2.4.4. **2**-Acetamido-**2**-deoxy-**1**,**3**,**4**-tri-O-acetyl-β-D-glucopyranose (**6**)*

4 (390 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g PEI-LECI preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2 mL × 10 mL), separated and dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum. After that, purification by the flash chromatography column was performed with 95:5 dichloromethane:methanol (253 mg, 65%). HPLC analysis: t_R = 8.4 min. ^{1}H NMR (400 MHz, $CDCl_3$). δ: 5.80 (d, J = 3.51 Hz, H-1), 5.50 (d, J = 9 Hz, 1H-NH), 5.30 (t, J = 9.9 Hz, H-3), 5.10 (t, J = 9.6 Hz, H-4), 4.35 (dd, J = 9.8 Hz, J = 6.70 Hz, H-2), 4.28–4.20 (m, 2H, H-6), 4.19–4.10 (m, H-5), 2.21 (s, 9H, 3 × CH_3), 1.96 (s, 3H, CH_3).

*2.4.5. **1**,**2**,**3**,**4**,**6**-Tetra-O-acetyl- α/β -D-mannopyranose (**8**)*

7 (390 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g PEI-LECI preparation at pH 5. When the substrate disappeared (check by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum to afford **8** as a white solid (370 mg, 95%). HPLC analysis: t_R = 9.49 (β anomer), 13.60 min (α-anomer). (α:β = 4:1). ^{1}H NMR (400 MHz, $CDCl_3$).δ: 5.43 (dd, 1H, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 10.0 Hz, H-3), 5.31 (t, 1H, J = 9.5 Hz, H-4), 5.28 (dd, 1H, $J_{1,2}$ = 1.8 Hz, H-2), 5.25 (dd, 1H, J = 4.0 Hz, H-1), 4.24 (m, 2H, $J_{5,6}$ = 2.5 Hz, $J_{5,6'}$ = 5.0 Hz, H-5 and H-6), 4.13 (dd, 1H, $J_{6,6'}$ = 8.0 Hz, H-6′), 3.16 (d, 1H, OH), 2.17 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 2.00 (s, 3H, CH_3).

*2.4.6. **2**,**3**,**4**,**6**-Tetra-O-acetyl-β-D-galactopyranosyl-(1 → 4)-**2**,**3**,**6**-tri-O-acetyl-β-D-gluco pyranose (**10**)*

9 (780 mg, 20 mM) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-LECI preparation at pH 5. When the substrate disappeared (check by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5 mL × 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, then filtered and reduced under vacuum to afford **10** as a white solid (702 mg, 90%). HPLC analysis: t_R = 8.82 min (α-anomer), 9.33 (β anomer). (α:β = 1:1.1). ^{1}H NMR (400 MHz, $CDCl_3$).δ: 5.51 (t, 1H, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H_α-3), 5.36 (d, 1H, $J_{1,2}$ =3.4 Hz, H_α-1), 5.34 (dd, 1 H, $J_{4',5'}$ = 0.5, H-4′), 5.22 (t, 1H, $J_{2,3}$ = 9.3 Hz, $J_{3,4}$ = 9.3 Hz, H_β-3), 5.11 (dd, $J_{2',3'}$ = 10.5 Hz, H_α-2′), 5.09 (dd, $J_{2',3'}$ = 10.6 Hz, H_β-2′), 4.94 (1H, dd, $J_{3',4'}$ = 3.2 Hz, H-3′), 4.81 (dd,1H, H_α-2), 4.76 (m, 2H, H_β -1, H_β-2), 4.49 (d, $J_{1',2'}$ = 7.9 Hz, H_α-1′), 4.48 (1H, dd, $J_{5,6b}$ = 3.4 Hz, $J_{6a,6b}$ = 11.2 Hz, H_b-6),4.47 (d, $J_{1',2'}$ = 7.7 Hz, H_β-1′), 4.22–4.00 (m,4H, H-4, H-5, H_a-6, H_b-6′), 3.86 (1H, dt, $J_{5',6'}$ 6.3, 5′-H), 3.75 (dd, $J_{4,5}$ = 9.3 Hz, H_α-4), 2.15–1.96 (s × 7, 21H, 7 × CH_3).

Table 1
Specificity and regioselectivity of different immobilized preparation of LECI in the hydrolysis of **1**

Support	Specific activity[a]	c[b] [%]	Yield[c] [%]	2[d] [%]	3 [%]
Octyl	0.56	100	96		96
BrCN	0.05	84	79	71	7
PEI	0.17	100	90	80	10
Glyoxyl	0.05	100	59	59	

[a] Specific activity was defined as μmol × $mg_{protein}^{-1}$ × h^{-1}. It was calculated at 10–15% conversion.
[b] c = conversion.
[c] Yield of the monohydroxy peracetylated product.
[d] The proportion of the anomers was α/β (1.3:1).

Table 2
Effect of the additive addition over the specific activity and the specificity of the CNBr-LECI immobilized preparation in the hydrolysis of **1**

Additive[a]	Specific activity[b]	c [%]	Yield[c] [%]
–	0.05	84	79
CTAB	0.05	85	81
tritonX100	0.083	100	79
triton X45	0.083	100	79
DMAP	0.07	86	86
tween 20	0.073	90	79
DEA	0.073	91	81

[a] The final concentration of the additive was 0.05 mM.
[b] Specific activity was defined as μmol × $mg_{protein}^{-1}$ × h^{-1}. It was calculated at 10–15% conversion (c).
[c] Yield of the monohydroxy peracetylated product.

3. Results and discussion

3.1 Activity of different immobilized preparations of LECI in the hydrolysis of acetylated-β-glycopyranosides

The initial activities displayed by different immobilized preparations from LECI in the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**4**), 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**7**) and 2,3,4,6-tetra-*O*-acetyl-β-D-galacto pyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**) are shown in Tables 1–5.

In the hydrolysis of **1**, LECI immobilized on octyl-agarose displayed the highest specific activity, even up to more than 10 times higher than the CNBr-LECI or glyoxyl-LECI prepara-

Table 3
Specificity and regioselectivity of different immobilized preparation of LECI in the hydrolysis of **4**

Support	Specific activity[a]	Reaction time [h]	c [%]	Yield[b] [%]	5 [%]	6 [%]
Octyl	0.006	144	34	19	6	13
BrCN	0.027	144	100	89	27	62
PEI	0.283	48	100	99	24	75
Glyoxyl	0.004	144	25	25	10	15

[a] Specific activity was defined as μmol × $mg_{protein}^{-1}$ × h^{-1}. It was calculated at 10–15% conversion (c).
[b] Yield of the monohydroxy peracetylated product.

Table 4
Specificity and regioselectivity of different immobilized preparation of LECI in the hydrolysis of **7**

Support	Specific activity[a]	Reaction time [h]	*c* [%]	Yield[b] [8[c],%]
Octyl	0	300	0	0
BrCN	0.038	80	100	86
PEI	0.026	100	100	100
Glyoxyl	0.030	96	100	91

[a] Specific activity was defined as μmol × $mg_{protein}^{-1}$ × h^{-1}. It was calculated at 10–15% conversion (c).
[b] Yield of the monohydroxy peracetylated product.
[c] Relation anomers α:β (4:1).

Table 5
Specificity and regioselectivity of different immobilized preparation of LECI in the hydrolysis of **9**

Support	Specific activity[a]	Reaction time [h]	*c* [%]	Yield[b] [10[c],%]
Octyl	0	205	0	0
BrCN	0.064	28	100	96
PEI	0.9	3	100	77
Glyoxyl	0.001	205	25	20

[a] Specific activity was defined as μmol × $mg_{protein}^{-1}$ × h^{-1}. It was calculated at 10–15% conversion (c).
[b] Yield of the monohydroxy peracetylated product.
[c] Relation anomers α:β (1:1.1).

tions and 3 times higher than using the PEI-LECI immobilized preparation (Table 1).

Furthermore, the effect of the presence of several additives (e.g., detergents, bases) [25] on the specific activity of the CNBr-LECI preparation was evaluated (Table 2). Using Triton X-100 or Triton X-45, the enzyme activity increased by a 1.66 fold factor. However, the use of that concentration of CTAB did not alter the activity value.

When the hydrolysis of **4** was performed, the PEI-LECI preparation was the most active immobilized preparation, presenting a specific activity 10 times higher than the CNBr-LECI preparation and 47 times higher than the octyl-LECI immobilized preparation (Table 3).

In the hydrolysis of **7**, the CNBr-LECI preparation was the most active catalyst. In this case the glyoxyl-LECI preparation displayed a specific activity slightly higher than the PEI-LECI. Surprisingly, LECI immobilized on octyl-agarose did not hydrolyze the substrate (Table 4).

This is an interesting characteristic observed by this enzyme immobilized on octyl-agarose – not observed with other lipases –, because also with other peracetylated monosaccharides (glucose, glucosamine, galactose, galactosamine) in α-configuration

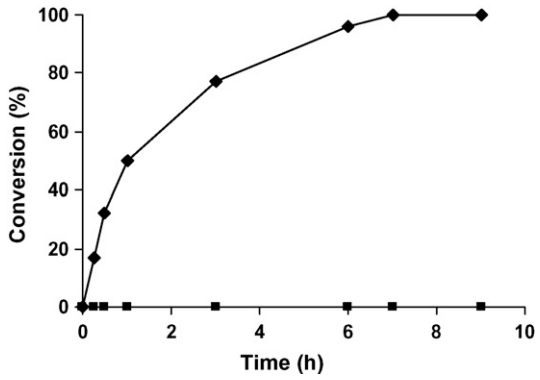


Fig. 1. Hydrolytic reaction course of different 1,2,3,4,6-penta-*O*-acetyl-D-galactopyranose anomers catalyzed by the octyl-LECI preparation. β-anomer (rhombus),α-anomer (squares).

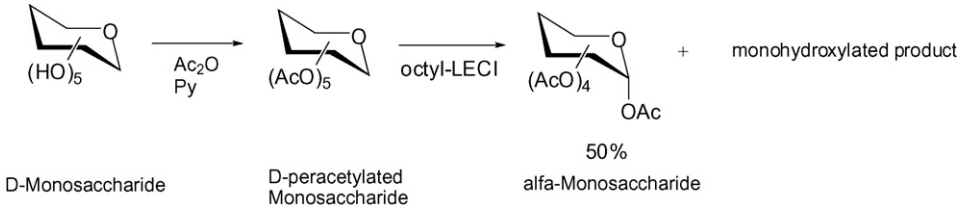
the enzyme was not active at all. This is the unique case where an enzyme recognized only the β-monosaccharides. Therefore the use of this biocatalyst is an easy and rapid strategy to obtain regio pure-α-peracetylated monosaccharides starting from the cheap D-sugars (Scheme 2 and Fig. 1).

In the hydrolysis of the disaccharide **9** (Table 5), LECI immobilized on octyl-agarose was not active whilst the immobilization on PEI-agarose permitted to hydrolyze the substrate at 0.9 U/h. This biocatalyst was over 10 times more active than the CNBr-LECI preparation and more than 900 times more active that the glyoxyl-LECI immobilized preparation.

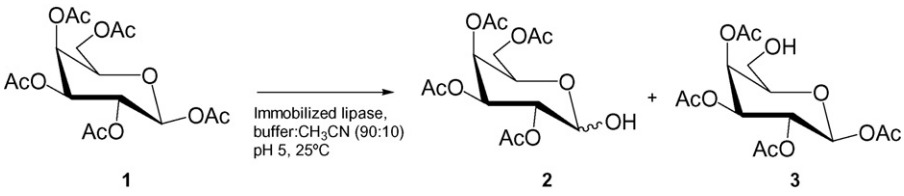
From the analysis of the above results, they clearly show that the use of different immobilization protocols greatly alters the specificity of the enzyme for different per-*O*-acetylated carbohydrates. Thus, the most active immobilized preparation was different when the substrate was different, although all preparations presented the same lipase.

3.2 Specificity and regioselectivity of the different immobilized preparations of LECI catalyzing the hydrolysis of different carbohydrates

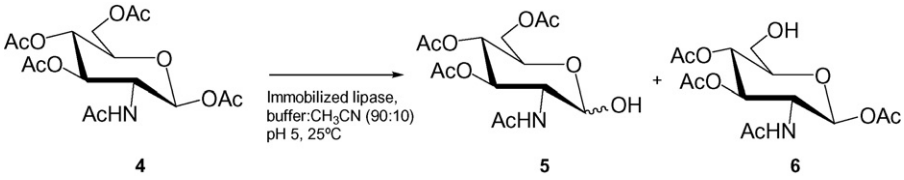
In the hydrolysis of **1** (Table 1, Scheme 3), the octyl-LECI preparation was very specific towards the peracetylated monosaccharide (that is, there is almost no hydrolysis of monodeacetyleated products) and very regioselective towards the *C*-6 position (Scheme 3), permitting to obtain > 95% 6-OH free hydroxytetraacetylated product **3** (Table 1). However, when the enzyme was immobilized on PEI-agarose or CNBr-agarose, it hydrolyzed **1** in the anomeric (**2**) and in *C*-6 (**3**) positions, although with a different yield (Table 1). When the glyoxyl-



Scheme 2. Preparation of pure α-peracetylated monosaccharides from D-monosaccharides by enzymatic hydrolysis using the octyl-LECI preparation.



Scheme 3. Specific and regioselective hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**).



Scheme 4. Specific and regioselective hydrolysis of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**4**).

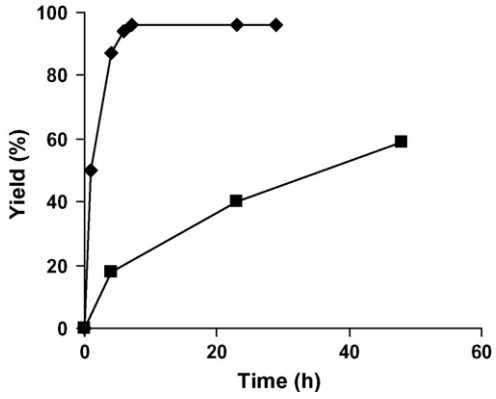
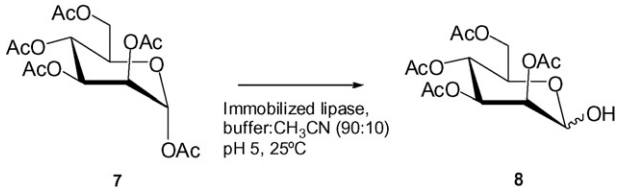


Fig. 2. Specificity profile of two different immobilized preparations of LECI in the hydrolysis of **1.** octyl-LECI (rhombus), glyoxyl-LECI (squares).



Scheme 5. Specific and regioselective hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**7**).

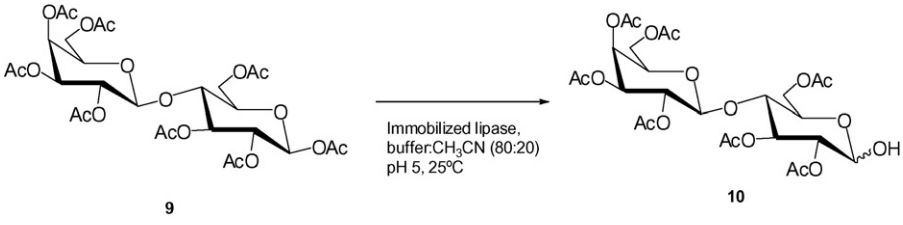
LECI preparation was used, only monohydroxy product in the anomeric position was produced with a 59% yield. The reaction courses of the reaction catalyzed by the octyl-LECI (the most specific) and the glyoxyl-LECI (the less specific) preparations are compared in Fig. 2, where it is possible to visualize the very different behaviour of LECI immobilized on different supports.

The specificity of the CNBr-LECI preparation also was influenced by the presence of additives (Table 2), although the regioselectivity seems to be less sensitive. Detergents in general decreased the amount of monohydroxy products, except using CTAB, which produced a certain increment. The best result was obtained in the presence of DMAP, because all the hydrolyzed substrate was transformed into monohydroxy products (Table 2). Thus, the use of these conditions permitted to get a high yield of **2**.

When the hydrolysis of **4** was performed (Table 3), the immobilization of LECI on PEI-agarose permitted to obtain a yield of monohydroxy products **5** and **6** (Scheme 4) near to 100%. The octyl-LECI preparation was the worst catalyst. That was the opposite that hydrolyzing **1**, with very poor activity and specificity (only 19% of monohydroxy products at 34% conversion). In this way, selecting PEI-LECI as biocatalyst, it was possible to get 75% of monohydroxy regioisomer in *C*-6 position (**6**).

In the hydrolysis of **7** (Scheme 5), the PEI-LECI immobilized preparation was the most specific, hydrolyzing the substrate in the anomeric position in 100% yield with a proportion of 80:20 between α and β anomers (**8a**:**8b**). The glyoxyl-LECI and CNBr-PECI preparations displayed the same regioselectivity with a yield in monohydroxy product around 90% (Table 4).

In the hydrolysis of **9** (Table 5), a disaccharide, LECI immobilized on octyl-agarose was not able to hydrolyze the substrate. However, the CNBr-LECI immobilized preparation presented a good activity and it was the most specific one with 95% of yield of **10** (Scheme 6), whilst the PEI-LECI preparation only produced 77% of **10**. Reaction courses of the reactions catalyzed



Scheme 6. Specific and regioselective hydrolysis of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**).

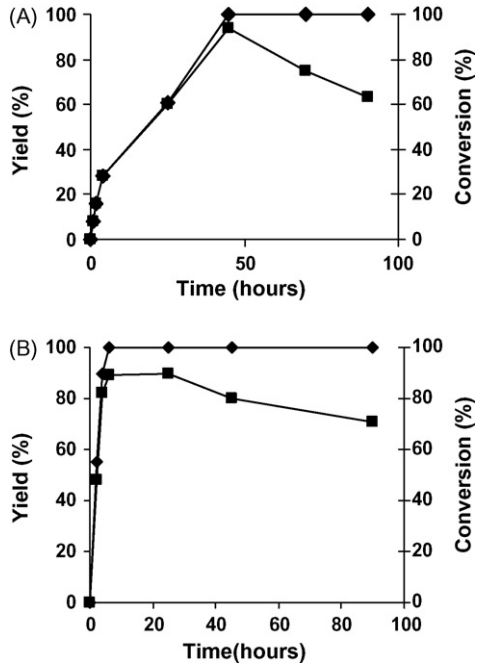


Fig. 3. Reaction course of different immobilized preparations of LECI in the hydrolysis of **9**. (A) octyl-agarose preparation and (B) CNBr-agarose preparation (rhombus = conversion, squares = yield) of monoprotected product.

by these biocatalysts are shown in Fig. 3. Here, the glyoxyl-LECI preparation produced only 20% of **10** after more than 200 h.

4. Conclusion

In this paper, we presented the first report on the use of the commercial phospholipase, Lecitase-Ultra, in the regioselective hydrolysis of peracetylated mono and disaccharides. The properties of the enzyme were greatly modulated by the immobilization protocol employed and the different preparations presented very different specificity, and regioselectivity.

For example, the octyl-LECI immobilized preparation was the most active one catalyzing the hydrolysis of **1** while the PEI-LECI preparation was the most active biocatalyst in the hydrolysis of **4**, **7** or **9.** Furthermore, the octyl-LECI was the most specific and regioselective producing exclusively 1,2,3,4-tetra-*O*-acetyl-β-D-galactopyranose whereas up to 80% of 2,3,4,6-tetra-*O*-acetyl- α/β-D-galactopyranose was obtained using the PEI-LECI preparation.

Moreover, whilst LECI immobilized on PEI-agarose was highly specific and regioselective in the hydrolysis of **4**, producing 75% of 2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-β-D-glucopyranose (**6**)**,** other preparations such as octyl-agarose or glyoxyl-agarose were not specific and very slowly.

In the hydrolysis of **7** and **9**, the octyl-LECI preparation was not active. When **7** was selected as substrate, the most active catalyst was the CNBr-LECI preparation although the most specific one was the PEI-LECI preparation, hydrolyzing with a 100% yield the anomeric position. Nevertheless, in the hydrolysis of **9**, the PEI-LECI immobilized preparation was the most active catalyst although the most specific in the production of monohydroxy product was the CNBr-LECI preparation (>95% yield of **10**).

Therefore, the immobilized preparations of LECI presented a great potential in these regioselective reactions. In this way, we have been able to find a pair immobilized LECI /peracetylated sugar that permitted to have good activity, specificity and regioselectivity. In fact, this modulation was even clearer than observed using "conventional" lipases [17].

Moreover, an interesting characteristic is that the octyl-LECI preparation did not hydrolyze the α-monosacharides, being specific towards the β-anomer. This did not occur with the other enzyme preparations nor with other lipases used in literature, where it has been described a higher activity towards the α-anomer [25]. This could permit to use this preparation to eliminate traces of β-anomers in α-monosaccharide preparations, being possible to get regiopure the latter ones.

Acknowledgements

This work has been sponsored by the Spanish CICYT (project BIO-2005-8576). We gratefully recognize CSIC by I3P contracts for Dr. Palomo and Dr Fernandez-Lorente (FEDER founds). The authors would like to thank Dr. Angel Berenguer (Universidad de Alicante) for his help during the writing of this paper and Dr. R. Martinez (Novo-Nordisk) for the gift of the enzyme.

References

[1] (a) J.G. Yang, Y.H. Wang, B. Yang, G. Mainda, Y. Guo, Food Technol. Biotechnol. 44 (2006) 101–104;
(b) A.C. Na, S.G. Eriksson, E. Erikson, E. Osterberg, K. Holmberg, J. Am. Oil Chem. Soc. 67 (1990) 766–770;
(c) M. Devos, L. Poisson, F. Ergan, G. Pencreac'h, Enzyme Microb. Technol. 39 (2006) 548–554;
(d) Y. Yamamoto, M. Hosokawa, K. Miyashita, J. Mol. Catal. B: Enzym. 41 (2006) 92–96.

[2] (a) M. Hartmann, A. Guberman, M. Florin-Christensen, A. Tiedtke, Appl. Microbiol. Biotechnol. 54 (2000) 390–396;
(b) H.N. Higgs, M.H. Han, G.E. Johnson, J.A. Glomset, J. Biol. Chem. 273 (1998) 5468–5477;
(c) S. Merino, A. Aguilar, M.M. Nogueras, M. Regue, S. Swift, J.M. Tomas, Infect. Immun. 67 (1999) 4008–4013;
(d) T. Okazaki, J.F. Strauss, G.L. Flickinger 3rd, Biochim Biophys. Acta 487 (1977) 343–353;
(e) J.K. Song, J.S. Rhee, Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1547 (2001) 370–378.

[3] K. Bojsen, A, Svendsen, C.C. Fuglsang, S. Patear, K. Borch, J. Vind, A.G. Petri, S.S. Gladd, G. Budolfsen, G.S.O. Schroder. Novozymes A/S, Denmark. PCT Internacional application WO2000/32758, 2000.

[4] (a) J. Yang, B. Yang, J. Meng, China Oils and Fats 28 (2003) 10–13;
(b) J. Yang, B. Yang, Q. Li, China Oils and Fats 28 (2003) 31–34.

[5] (a) R. Verger, M.C.E. Mieras, G.H. De Haas, J. Biol. Chem. 248 (1973) 4023–4034;
(b) Y. Matoba, M. Sugiyama, Proteins: Struct. Funct. Gen. 51 (2003) 453–469;
(c) A.G. Singer, F. Ghomashchi, C. Le Calvez, J. Bollinger, S. Bezzine, M. Rouault, M. Sadilek, E. Nguyen, M. Lazdunski, G. Lambeau, M.H. Gelb, J. Biol. Chem. 277 (2002) 48535–48549.

[6] (a) K.-E. Jaeger, M.T. Reetz, Trends Biotechnol. 16 (1998) 396–403;
(b) P. Dominguez de Maria, C. Carboni-Oerlemans, B. Tuin, G. Bargeman, A. Van Der Meer, R. Van Gemert, J. Mol. Catal. B Enzym. 37 (2005) 36–46;

(c) A. Ghanem, H.Y. Aboul-Enein, Chirality 17 (2005) 1–15;
(d) D. Lambusta, G. Nicolosi, A. Patti, C. Sanfilippo, J. Mol. Catal. B Enzym. 22 (2003) 271–277.

[7] (a) A. Ghanem, H.Y. Aboul-Enein, Chirality 17 (2005) 44–50;
(b) O. Pamies, J.-E. Backvall, Adv. Synth. Catal. 344 (2002) 947–952;
(c) S. Akai, K. Tanimoto, Y. Kanao, M. Egi, T. Yamamoto, Y. Kita, Angew. Chem. Int. Ed. 45 (2006) 2592–2595.

[8] G. Fernandez-Lorente, J.M. Palomo, J.M. Guisan, R. Fernandez-Lafuente, J. Mol. Cat B Enzym. 47 (2007) 99–104.

[9] (a) K.J. Doores, D.P. Gamblin, B.G. Davis, Chem. Eur. J. 12 (2006) 656–665;
(b) T.K.-K. Mong, L.V. Lee, J.R. Brown, J.D. Esko, C.-H. Wong, Chem. Biochem. 4 (2003) 835;
(c) M. Filice, D. Ubiali, R. Fernandez-Lafuente, G. Fernandez-Lorente, J.M. Guisan, J.M. Palomo, M. Terreni, J. Mol. Cat B:Enzym. doi:10.1016/j.molcatb.2007.10.013.

[10] R. Khan, P.A. Konowicz, L. Gardossi, M. Matulova, S. de Genaro, Aust. J. Chem. 49 (1996) 293–298.

[11] C.-H. Wong, G.M. Whitesides, Enzymes in Synthetic Organic Chemistry, Pergamon Press, Oxford, 1994.

[12] U.T. Bornscheuer, Curr. Opin. Biotechnol. 13 (2002) 543–547.

[13] (a) H.M. Sweers, C.-H. Wong, J. Am. Chem. Soc. 108 (1986) 6421–6422;
(b) J.-F. Shaw, A.M. Klibanov, Biotechnol. Bioeng. 29 (1987) 648–651;
(c) G.T. Ong, K.Y. Chang, S.H. Wu, K.T. Wang, Carbohydr. Res. 265 (1994) 311–318;
(d) O. Kirk, M.W. Christensen, F. Beck, T. Damhus, Biocatal. Biotrans. 12 (1995) 91–97.

[14] (a) L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, U. Menge, Nature 43 (1990) 767–770;
(b) A. Aloulou, J.A. Rodriguez, S. Fernandez, D. van Oosterhout, D. Puccinelli, F. Carrieère, Biochim. Biophys. Acta 1761 (2006), 995-1013.

[15] (a) J.M. Palomo, G. Fernández-Lorente, C. Mateo, M. Fuentes, R. Fernández-Lafuente, J.M. Guisán, Tetrahedron Asymmetry 13 (2002) 2653–2659;
(b) J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, M. Fuentes, J.M. Guisán, R. Fernández-Lafuente, J. Mol. Cat B Enzym. 21 (2003) 201–210;
(c) A. Chaubey, R. Parshad, S. Koul, S.C. Taneja, G.N. Qazi, J. Mol. Catal. B Enzym. 42 (2006) 39–44;
(d) J.M. Palomo, R.L. Segura, C. Mateo, M. Terreni, J.M. Guisán, R. Fernández-Lafuente, Tetrahedron Asymmetry 16 (2005) 869–874.

[16] (a) H. Yu, J. Wu, B.C. Chi, Biotechnol. Lett. 26 (2004) 629–633;
(b) J.M. Palomo, G. Fernández-Lorente, C. Mateo, C. Ortiz, R. Fernández-Lafuente, J.M. Guisán, Enzyme Microb. Technol. 31 (2002) 775–783;
(c) G. Fernandez-Lorente, J.M. Palomo, C. Mateo, R. Munilla, C. Ortiz, Z. Cabrera, J.M. Guisan, R. Fernandez-Lafuente, Biomacromolecules 7 (2006) 2610–2615.

[17] (a) J.M. Palomo, M. Filice, R. Fernandez-Lafuente, M. Terreni, J.M. Guisan, Adv. Synth. Catal. 349 (2007) 1969–1976;
(b) M. Filice, R. Fernandez-Lafuente, M. Terreni, J.M. Guisan, J.M. Palomo, J. Mol. Cat B Enzym. 49 (2007) 12–17.

[18] (a) Z. Cabrera, J.M. Palomo, G. Fernández-Lorente, R. Fernández-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 40 (2007) 1280–1285;
(b) Z. Cabrera, F. Lopez-Gallego, G. Fernández-Lorente, J.M. Palomo, T. Montes, V. Grazu, J.M. Guisan, R. Fernández-Lafuente, Enzyme Microb. Technol. 40 (2007) 997–1000.

[19] (a) R. Fernández-Lafuente, P. Armisen, P. Sabuquillo, G. Fernández-Lorente, J.M. Guisán, Chem. Phys. Lipids 93 (1998) 185–197;
(b) A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, J.M. Guisán, Biotechnol. Bioeng. 58 (1998) 486–493;
(c) G. Fernández-Lorente, C. Ortiz, R.L. Segura, R. Fernández-Lafuente, J.M. Guisán, J.M. Palomo, Biotechnol. Bioeng. 92 (2005) 773–779;
(d) J.M. Palomo, R.L. Segura, G. Fernandez-Lorente, M. Pernas, M.L. Rua, J.M. Guisan, R. Fernandez-Lafuente, Biotechnol. Prog. 20 (2004) 630–635.

[20] C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernández-Lorente, J.M. Palomo, V. Grazu, B.C.C. Pessela, C. Giacomini, G. Irazoqui, A. Villarino, K. Ovsejevi, F. Batista, R. Viera, J.M. Fernández-Lafuente, Guisán Enzyme Microb. Technol. 37 (2005) 456–462.

[21] C. Mateo, O. Abian, R. Fernández-Lafuente, J.M. Guisán, Biotechnol. Bioeng. 68 (2000) 98–105.

[22] C. Mateo, J.M. Palomo, M. Fuentes, L. Betancor, V. Grazú, F. Lopez-Gallego, B.C.C. Pessela, A. Hidalgo, G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 39 (2006) 274–280.

[23] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.

[24] T. Horrobin, Ch.H. Tran, D. Crout, J. Chem. Soc. Perkin Trans. 1 (1998) 1069–1080.

[25] (a) J.E. Mogensen, P. Sehgal, D.E. Otzen, Biochemistry 44 (2005) 1719–1730;
(b) P. Helistö, T. Korpela, Enzyme Microb. Technol. 23 (1998) 113–117;
(c) G. Fernandez-Lorente, J.M. Palomo, Z. Cabrera, R. Fernandez-Lafuente, J.M. Guisan, Biotechnol. Bioeng. 97 (2007) 242–250.

[26] B. La Ferla, Monatshefte fur chemie. 133 (2002) 1–18.