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Different derivatives of a lipase display different regioselectivity in the monohydrolysis of per-*O*-acetylated 1-O-substituted-β-galactopyranosides

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ABSTRACT

Different immobilized preparations of three different lipases – those from *Aspergillus niger* (ANL), *Candida rugose* (*CRL*) and *Candida antarctica* B (CAL-B) – have been used in the regioselective monohydrolysis of different peracetylated-β-galactopyranosides. Three very different immobilization strategies – covalent attachment, anionic exchange and interfacial activation on a hydrophobic support – were employed for each lipase. The role of the immobilization strategy on the hydrolytic activities, specificities and regioselectivities of the lipases were investigated. Moreover, the effect the biocatalysts performance of the presence of different moieties in the anomeric position of the substrate was analyzed. The PEI-ANL immobilized preparation was six times more active than the CNBr-ANL in the hydrolysis of 1-thioisopropyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside whereas the CNBr-ANL showed 2 times more activity than the PEI-ANL in the hydrolysis of galactal. Using CRL, the octyl-CRL was completely specific and regioselective in the hydrolysis of galactal, producing the *C*-6 monohydroxylated product in 99% yield. The PEI-CRL showed low specificity and poor regioselectivity, hydrolyzing in *C*-6 but also in *C*-3 positions whereas the PEI-CRL preparation showed good specificity although low regioselectivity, hydrolyzing in *C*-6, *C*-4 and *C*-3 positions.

1. Introduction

Lipases are among the most used enzymes in biotransformations because they can recognize many different substrates, in many instances, with high regio and enantioselectivity [1–4]. The mechanism of catalysis of lipases implies dramatic conformational changes of the enzyme molecule between a 'closed' and an 'open' form [5,6]. This mechanism of action produces that the use of different immobilization protocols (involving different areas of the lipase, rigidity, micro-environments, etc.) [7] causes a strong modulation of the lipase catalytic features. In fact, this strategy has been used to improve the performance of some lipases in kinetic resolutions of different esters and alcohols and in asymmetric reactions [8–10].

Fully acetylated alkyl glycosides are commonly used for the chemical synthesis of *O*-glycosides in view of their ready availability, low cost and easy preparation [11]. These per-*O*-acetyl-glycosides could be used as raw materials to obtain regioisomers of *O*-acetyl-anomeric substituted glycopyranosides presenting only one free hydroxyl group. These compounds could be key intermediates in the preparation of different neo-glycoderivatives (oligosaccharides, glycolipids, glycopeptides, glycolipopeptides, etc.) [12–15].

The application of lipases to catalyze these processes has represented an interesting alternative to the more complex chemical strategies to remove only one acetyl group in a regioselective way [16,17]. However in most cases the enzymatic deacylation of fully acylated pyranoses is very slow or proceeds with poor selectivity and yield [18]. Thus, it would be necessary to find biocatalysts exhibiting good catalytic activity, high specificity for the peracetylated substrate to improve the yield of mono-deacetylated compound and a high regioselectivity. In this way, different immobilization protocols may be used to modulate the lipase regioselectivity [16,17].

Moreover, the use of different substrates, e.g. with the anomeric position blocked with different chains, may further modify the lipase performance, perhaps permitting to obtain new monodeacetylated sugars.

Three very different immobilization protocols have been applied: (i) immobilization on hydrophobic supports at low ionic strength by interfacial activation of the lipases, involving the hydrophobic area surrounding the active site of the lipase [19]; (ii) immobilization on agarose activated with CNBr *via* covalent attachment at neutral pH value throughout the most reactive amino group (usually the terminal NH_2) on the enzyme surface [20]; (iii)

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immobilization via anionic exchange through the areas with highest negative net charge of the lipase on agarose beads coated with PEI [21].

These different immobilization strategies were used with three very employed lipases those from *Candida antarctica* (isoform B) (CAL-B), from *Aspergillus niger* (ANL) and from *Candida rugosa* (CRL). The immobilized enzymes were applied as biocatalysts in the hydrolysis of different 1-substituted per-*O*-acetylated β-galactopyranosides in aqueous media to study if the immobilization protocol may alter the lipase properties and if the substituent in the anomeric position may also influence the lipase performance.

2. Experimental

2.1. Material

Lipase from *Aspergillus niger* (ANL) was purchased from Fluka (Neu Ulm, Germany). Lipase from *C. antarctica* B (CAL-B) was kindly supplied by Novo Nordisk (Denmark). Octyl-agarose (4BCL) and cyanogen bromide (CNBr-activated Sepharose 4BCL) beads were purchased from GE-Healthcare (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), Triton X-100, *p*-nitrophenyl butyrate (*p*NPB), lipase from *Candida rugosa* (CRL), 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 1-thioisopropyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**4**) and 3,4,6-tri-*O*-acetyl-galactal (**7**) were from Sigma Chem. Co. Agarose beads coated with PEI were prepared as previously described [21]. Columns for flash chromatography were made up with Silica Gel 60 (Merck) 60–200 or 40–63 μm. The elution was performed with 40:60 hexane–ethyl acetate. ^{1}H NMR were recorded in $CDCl_3$ (δ = ppm) on a Bruker AMX 400 instrument.

2.2. Standard enzymatic activity assay determination

In order to follow the immobilization process, the activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) (ϵ = 5.150 $M^{-1}cm^{-1}$) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. Enzymatic activity is giving as μmol of hydrolyzed *p*NPB per minute per mg of enzyme (IU) under the conditions described above.

2.3. Purification of lipases

The purification of CAL-B and CRL was performed using a previously described protocol, based on the adsorption of lipases – by interfacial activation – on hydrophobic supports at low ionic strength [19]. 0.16 g of CRL commercial solid powder (30 mg protein) or 2.5 mL CAL-B commercial solution (12 mg protein/mL) [22] were offered to 95 mL of 10 mM sodium phosphate at pH 7.0. In each case, 5 g of octyl-agarose support were added. After 4 h, the enzyme derivative was filtrated under vacuum using a sintered glass funnel and abundantly washed with distilled water. In all cases, more than 95% of lipase was adsorbed on the support. ANL was purified from the commercial extract crude as previously described [23]. 6 mg lipase per gram of support was prepared. The SDS-PAGE analysis of the adsorbed proteins (after boiling in the presence of SDS) showed only a single band with a molecular weight corresponding to that of the different native lipases. These adsorbed lipases were used in some instances directly as biocatalysts in some of the studies. In some other cases, the purified lipases were finally immobilized in other supports (see below). To have a pure lipase from the octyl derivatives, the adsorbed enzymes were re-suspended in a solution of 10 mM sodium phosphate containing 1% triton (v/v) at pH 7.0 and 4 °C for 1 h, obtaining a purified lipase solution with a final concentration of 0.6 mg lipase/mL. Then, the enzymatic solution was used for immobilization in the other supports.

2.4. Immobilization of lipases on other supports

Lipases previously purified containing 1% triton – enough detergent to prevent any lipase–lipase interaction that could produce the immobilization of lipase dimers with altered properties [24] – were immobilized on agarose support activated with CNBr [20] or agarose beads coated with polyethyleneimine (PEI) [21]. No functionalized agarose and reduced glyoxyl-agarose were used as control experiment as references, non-specific binding during the immobilization processes could be discarded. 10 mL of lipase solution (0.6 mg lipase/mL) was added to 30 mL of 10 mM sodium phosphate solution at pH 7. After that, 1 gram of the CNBr-agarose or PEI-agarose support was added. The mixture was then stirred at 25 °C and 250 rpm for 1 or 4 h, respectively. In the immobilization on CNBr-agarose support, the supported lipase – after the supernatant was removed by filtration – was added to 40 mL of 3 M glycine at pH 8 for 1 h to block the possible remaining electrophilic groups on the support. The further exhaustive washing with distilled water of the immobilized lipases permitted to fully eliminate the detergent. The immobilization yields were in both methods and with all lipases more than 95%. The recovered activity in all cases was more of 95% of the initial enzyme activity value follow by the assay described above.

2.5. Enzymatic hydrolysis of peracetylated β-galactopyranosides

2 mM of **1**,**4**,**7** in 50 mM sodium acetate with 20% acetonitrile at pH 5 and 25 °C were prepared. 0.5 g of biocatalyst was added to 3 mL (**1**,**4**) or 10 mL (**7**) of the previous solution to initialize the reaction. The pH value was selected to avoid the chemical acyl-migration in the per-*O*-acetylated carbohydrates hydrolysis. The hydrolytic reaction was carried out under mechanical stirring, and the pH value was kept constant using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland). Reactions were followed by HPLC using a HPLC spectrum P100 (Thermo Separation products). The column was a Kromasil-C_{18} (250 × 4.6 and 5 μm) from Analisis Vinicos (Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 215 nm. The mobile phase was an isocratic mixture of 40% acetonitrile/60% 10 mM sodium phosphate at pH 4; flow rate 1.0 mL/min. Finally, the products were isolated and identified by ^{1}H NMR.

2.5.1. 2,3,4,6-Tetra-O-acetyl-α/β-D-galactopyranose (**2**)

1 (390 mg, 1 mmol) was hydrolyzed in 45 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g CNBr-ANL preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL).

The collected organic layers were dried over anhydrous Na_2SO_4 – which was then removed by filtration – and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 40:60 hexane–ethyl acetate as eluent to afford **2** as a white solid (129 mg, 33%). ^{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). The NMR-data are in agreement with the reported value [24].

2.5.2. 1,2,3,4-Tetra-O-acetyl-β-D-galactopyranose (**3**)

1 (390 mg, 1 mmol) was hydrolyzed in 45 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g CNBr-ANL

preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL).

The collected organic layers were dried over anhydrous Na_2SO_4 – which was then removed by filtration – and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 40:60 hexane–ethyl acetate as eluent to afford **3** as a white solid (156 mg, 40%).

^{1}H NMR (500 MHz, $CDCl_3$). δ: 5.73 (d, J = 8.26 Hz; H-1), 5.44 (d, J = 3.39 Hz; H-4), 5.32 (t, 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, ABX system, 2H; H-6), 2.14–1.97 (s, 12H, 4×CH_3).

2.5.3. 1-Thioisopropyl-2,3,4-tri-O-acetyl-α/β-D-galactopyranose (**5**)

4 (117 mg, 0.3 mmol) was hydrolyzed in 10 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g octyl-CRL preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **5** as a white solid (111 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$), δ = ppm: 5.40 (d, 1H, J = 2.93 Hz; H-3), 5.23 (t, 1H; J = 8.07 Hz; H-4), 5.08 (dd, 1H; J = 3.423 Hz; H-2), 4.58 (d, 1H; J = 10.03 Hz; H-1), 3.78–3.68 (m, 2H; H-5, H-6), 3.52–3.46 (m, 1H; H-6), 3.23–3.14 (septete, 1H, J = 6.6 Hz; 1H, CH), 2.16 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 1.30 (m, 6H, 2 × CH_3).

2.5.4. 1-Thioisopropyl-2,3,6-tri-O-acetyl-α/β-D-galactopyranose (**6**)

4 (117 mg, 0.3 mmol) was hydrolyzed in 10 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g PEI-ANL preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL).

The collected organic layers were dried over anhydrous Na_2SO_4 – which was then removed by filtration – and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 50:50 hexane–ethyl acetate as eluent to afford **6** as a white solid (4.68 mg, 4%). ^{1}H NMR (400 MHz, $CDCl_3$), δ = ppm: 5.36 (d, 1H, J = 3.00 Hz; H-3), 5.03 (dd, 1H; J = 3.423 Hz; H-2), 4.4 (d, 1H, J = 9.50 Hz; H-1), 4.33–4.10 (m, 2H, 2 × H-6), 3.91 (t, 1H; J = 8.2 Hz; H-4), 3.66–3.56 (m, 1H; H-5), 3.20–3.12 (septete, 1H; J = 6.8 Hz; 1H, CH), 2.15 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 1.29 (m, 6H, 2 × CH_3).

2.5.5. 3,4-Di-O-acetyl-D-galactal (**8**)

7 (117 mg, 0.3 mmol) was hydrolyzed in 10 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g octyl-CRL preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **8** as a white solid (111 mg, 95%). ^{1}H NMR (500 MHz, $CDCl_3$), δ = ppm: 6.50 (dd, 1H; J = 6.27, 1.8 Hz; H-1), 5.60–5.55 (m, 1H, H-3), 5.50–5.44 (dt, 1H; J = 7.4, 3.6 Hz; H-4), 4.73 (dt, 1H; J = 5.9, 2.8 Hz; H-2), 4.22–4.16 (m, 1H; J = 7 Hz; H-5), 3.83–3.61 (dd, 2H, J = 11.6, 5.8 Hz; H-6a,b), 2.31 (bs, 1H, OH), 2.16 (s, 3H, CH_3), 2.05 (s, 3H, CH_3).

2.5.6. 3,6-Di-O-acetyl-D-galactal (**9**)

7 (117 mg, 0.3 mmol) was hydrolyzed in 10 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g CNBr-CAL-B preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL).

The collected organic layers were dried over anhydrous Na_2SO_4 – which was then removed by filtration – and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 40:60 hexane–ethyl acetate as eluent to afford 9 as a white solid (56 mg, 48%). ^{1}H NMR (500 MHz, $CDCl_3$), δ = ppm: 6.43 (dd, 1H; J = 6.3, 1.8 Hz; H-1), 5.37 (dt, 1H; J = 4.7, 3.8 Hz; H-3), 4,76 (dd, 1H; J = 3.8, 1.7 Hz; H-2), 4.38–4.57 (dd, 2H; J = 12.2, 7.7 Hz; H-6a,b), 4.01 (m, 1H; J = 7.7, 1.6 Hz; H-5), 3.84 (bt, 1H, H-4), 3.54 (bs, 1H, OH), 2.14–2.16 (s, 6H, 2 × CH_3).

2.5.7. 4,6-Di-O-acetyl-D-galactal (**10**)

7 (117 mg, 0.3 mmol) was hydrolyzed in 10 mL solution of 50 mM phosphate buffer (80%) and acetonitrile (20%) using 5 g octyl-ANL preparation at pH 5. When the substrate disappeared (checked by HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL).

The collected organic layers were dried over anhydrous Na_2SO_4 – which was then removed by filtration – and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 40:60 hexane–ethyl acetate as eluent to afford 10 as a white solid (7 mg, 6%). ^{1}H NMR (500 MHz, $CDCl_3$), δ = ppm: 6.40 (dd, 1H; J = 6.2, 1.6 Hz; H-1), 5.48 (m, 1H, H-4), 4.75 (m, 1H, H-2), 4.30–4.15 (m, 1H, H-6a,b), 4.14–4.10 (m, 1H, H-3), 2.11 (s, 3H, CH_3), 2.03 (s, 3H, CH_3).

3. Results and discussion

3.1. Effect of the immobilization protocol on the lipase activity in the hydrolysis of different acetylated-β-1-O-substituted-galactopyranosides

The specific activity displayed by different immobilized preparations from CAL-B, ANL and CRL in the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 1-thioisopropyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**4**) and 3,4,6-tri-*O*-acetyl-galactal (**7**) are shown in Table 1.

The presence of different groups in the anomeric position of peracetylated galactose affected in a different way to the enzyme activity.

When using CAL-B, the highest activity was achieved in the hydrolysis of **1** for all the immobilized preparations. With respect to the preparation showing the highest activity, the CNBr-CAL-B was the most active biocatalyst in the hydrolysis of **1** and **7**, ranging from 25 times using peracetylated galactopyranose 1–33 times using galactal **7** when compared to the PEI-CAL-B. The CNBr-CAL-B was almost 3 times more active than the octyl-CAL-B preparation in the hydrolysis of **1** and more than four times in the hydrolysis of **7**. The activity of all CAL-B immobilized preparations was not significant in the hydrolysis of **4** (Table 1).

Table 1
Specific activity different immobilized preparations of lipases in the hydrolysis of per-*O*-acetylated 1-*O*-substituted-β-galactopyranosides.

Enzyme	Support	**1**[a]	**4**[a]	**7**[a]
CAL-B	Octyl	9	<0.002	2.55
	CNBr	25	<0.002	11
	PEI	1	<0.002	0.31
ANL	Octyl	52	3.9	360
	CNBr	162	74	3056
	PEI	600	49	3770
CRL	Octyl	1.5	10	22
	CNBr	0.35	0.5	3.5
	PEI	0.28	1	6

[a] The initial rate in nmol × mg_{prot}^{-1} × min^{-1}. It was calculated at 10–15% conversion.

Table 2
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **1**.

Entry	Enzyme	Support	Reaction time [h]	c[a] [%]	**2**[b] [%]	**3**[b] [%]
1		Octyl	120	100	41	49
2	CAL-B	CNBr	120	100	39	47
3		PEI	168	32	12	14
4		Octyl	168	100	37	47
5	ANL	CNBr	75	100	43	51
6		PEI	75	100	40	47
7		Octyl	168	100	7	39
8	CRL	CNBr	168	36	5	21
9		PEI	168	32	11	19

[a] Conversion.
[b] Yield of the monohydroxy peracetylated product.

Table 3
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **4**.

Entry	Enzyme	Support	Reaction time [h]	c[a] [%]	**5**[b] [%]	**6**[b] [%]
1		Octyl	168	17	17	
2	ANL	CNBr	168	43	32	
3		PEI	168	39	13	9
4		Octyl	96	100	99	
5	CRL	CNBr	168	29	26	3
6		PEI	168	55	40	5

[a] Conversion.
[b] Yield of the monohydroxy peracetylated product.

Using ANL as biocatalyst, all immobilized preparations exhibited the highest activity in the hydrolysis of galactal **7**. The PEI-ANL immobilized preparation was more than 10 times more active than the octyl-ANL in the hydrolysis of **1**, **4** and **7**, although the CNBr-ANL was almost two-folds more active than PEI-ANL and 19 times more active than the octyl-ANL preparation in the hydrolysis of thioisopropyl derivative **4**.

The octyl-ANL showed almost 100 times more activity towards **7** than **4** whereas the PEI-ANL exhibited 73 times more activity towards **7** than **4**.

When using CRL, the octyl-CRL preparation was the most active catalyst in the hydrolysis of three substrates used.

The octyl-CRL was five times more active than the PEI-CRL preparation in the hydrolysis of **1** and 10 times in the hydrolysis of **4**. When the octyl-CRL was compared with the CNBr-CRL, the former showed 20 times more activity in the hydrolysis of **5** than the latter. In the hydrolysis of galactal **7**, the octyl-CRL was six times more active than the CNBr-CRL.

The octyl-CRL showed the highest activity in the hydrolysis of **7**, being almost 20 times more active than in the hydrolysis of **1**, whereas the CNBr-CRL or PEI-CRL preparations exhibited seven times more activity compared with that in the hydrolysis of **1**.

3.2. Specificity and regioselectivity of the different immobilized lipases preparations in the hydrolysis of different acetylated-β-1-O-substituted-galactopyranosides

In the hydrolysis of peracetylated galactose **1**, all the immobilized preparations of CAL-B or ANL exhibited high specificity; although a low regioselectivity was found, producing a mixture of monohydrolyzed products in *C*-1 and *C*-6 (Scheme 1 and Table 2).

When CRL was employed, the octyl-CRL preparation showed low specificity and accumulating the 1-OH and 6-OH products, although producing more than five times **3** compared with **2**. The CNBr-CRL displayed also low specificity and regioselectivity, in this case producing **2** and **3** in a relation 1:4. The PEI-CRL was specific, accumulating monodeacetylated product, although produced **2** and **3** in similar amount (Table 2).

Next, the hydrolysis of substrates with the anomeric position blocked was performed in order to study the effect of this modification on the behavior of the different lipases biocatalysts.

First the peracetylated galactoderivative **4** with a thioisopropyl moiety in the anomeric position was used. This blocking is a reactive group for glycosylation and therefore this methodology would permit an easy way to prepare different neo-glycoderivatives [25,26]. Furthermore, it has been reported that the thio-sugars displayed specific pharmacological activity and may have therapeutic use [25,26].

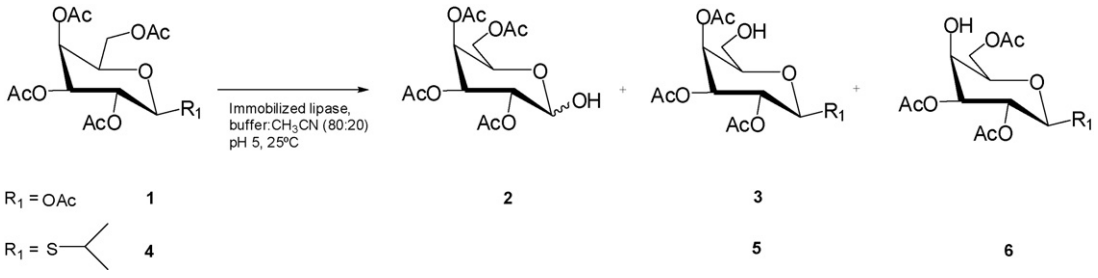
Using ANL, the octyl-ANL and CNBr-ANL exhibited high specificity and regioselectivity, producing only the monohydrolylated product in *C*-6 **5**. However, the PEI-ANL preparation showed lower specificity and low regioselectivity, accumulating the 6-OH **5** and also the interesting 4-OH product **6**, which it was not detect when using the peracetylated molecule **1** as substrate (Scheme 1, Table 3). The use of this substrate with the anomeric position blocked, avoided the mixture of products 1-OH/6-OH, permitting to improve the yield in 6-OH or the hydrolysis in new positions.

When CRL was used, the octyl-CRL was highly specific and regioselective, producing exclusively the 6-OH product (**5**) in 99% yield. The CNBr-CRL or PEI-CRL exhibited high specificity towards **4**, although a partial regioselectivity, producing 6-OH, but also hydrolysis in *C*-4 was observed (Table 3). Again, the blocking in the anomeric position permitted to improve the yield in 6-OH or the hydrolysis in new positions.

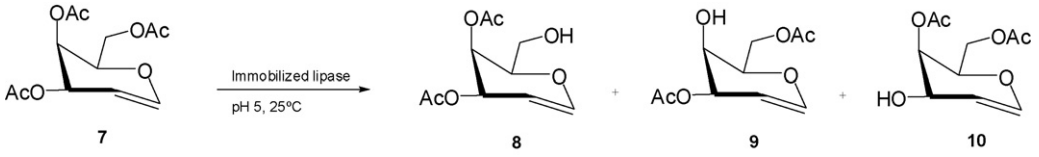
Very interesting results were found when galactal **7** – 1,2-anhydrosugar with great synthetic potential as building blocks in oligosaccharide synthesis – was used as substrate (Table 4).

Using CAL-B, the three immobilized preparations displayed low specificity, with a moderate yield of monodeacetylated sugar, but only 3-OH derivative **10** (58% yield using the CNBr-CAL-B) could be found (Scheme 2 and Table 4).

When ANL was employed, the PEI-ANL exhibited the highest specificity among all biocatalysts studied (around 50% yield in monodeacetylated products at 100% conversion). The regioselectivity of this catalyst was low, which permitted to produce a mixture of monohydrozylated products in *C*-6 (40%) and *C*-3 (10%) but also a small amount of *C*-4 (3%) (Scheme 2 and Table 4). Using the other



Scheme 1. Specific and regioselective hydrolysis of different 1-substituted 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosides by different immobilized lipases.



Scheme 2. Regioselective hydrolysis of **7** by different immobilized preparations of lipases.

Table 4
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **7**.

Entry	Enzyme	Support	Reaction time [h]	c[a] [%]	**8**[b] [%]	**9**[b] [%]	**10**[b] [%]
1		Octyl	168	70			43
2	CAL-B	BrCN	168	100			58
3		PEI	168	12			6
4		Octyl	168	96	25	10	13
5	ANL	BrCN	41	100	38	4	12
6		PEI	10	100	40	3	10
7		Octyl	17	100	99		
8	CRL	BrCN	168	100	64	8	7
9		PEI	168	90	52		7

[a] Conversion.
[b] Yield of the monohydroxy peracetylated product.

preparations the results were similar, although 10% of **9** using the octyl-ANL – a very interesting product and none described by enzymatic methods as far we know – was synthesized.

Using CRL in the hydrolysis of **7**, the octyl-CRL was completely specific and regioselective, producing only the monohydroxylated **8** in 99% yield. The PEI-CRL showed low specificity and poor regioselectivity, hydrolyzing in *C*-6 but also in *C*-3 positions (Table 4, Scheme 2). However, the PEI-CRL preparation showed good specificity although low regioselectivity, because together **8**, the enzyme hydrolyzed also in *C*-4 (**9**) and *C*-3 (**10**) positions (Table 4).

The double bond in 1,2 positions and the lack of this acetyl groups permitted to hydrolyze in other positions different of anomeric or *C*-6 with high yields, such as *C*-3, quite difficult to obtain using **1**.

4. Conclusion

In this paper, the enzymatic mono-deacetylation – using three different lipases – of different 1-O-substituted-peracetylated galactopyranosides has been presented. The immobilization strategy was a key point to find the optimal catalysts in each case, defining the activity, specificity and regioselectivity of the final biocatalyst. The presence of different moieties in the anomeric position of the substrate also altered greatly the catalytic properties of these immobilized lipases. For example, the PEI-ANL immobilized preparation was six times more active than the CNBr-ANL in the hydrolysis of thioisopropyl derivative **4** whereas the CNBr-ANL showed two times more activity than PEI-ANL in the hydrolysis of galactal **7**.

Other feature that was altered by the immobilization was the enzyme specificity by the different substrates. For example, the octyl-CRL showed the highest activity in the hydrolysis of **7**, being almost 20 times more active than in the hydrolysis of **1**, whereas the CNBr-CRL or PEI-CRL preparations exhibited seven times more activity compared with that in the hydrolysis of **1**.

About the modulation of the regioselectivity by the immobilization strategy, for example, the octyl-CRL was highly specific and regioselective in the hydrolysis of **4**, producing exclusively the 6-OH product (**5**) in 99% yield. The CNBr-CRL or PEI-CRL exhibited high specificity towards **4**, although a partial regioselectivity, producing 6-OH, and also the 4-OH product.

In the hydrolysis of galactal **7**, the CNBr-CAL-B displayed low specificity, with a moderate yield of monodeacetylated sugar, but accumulating only 3-OH derivative **10** (58%). Using CRL, the octyl-CRL was completely specific and regioselective, producing only the monohydroxylated **8** in 99% yield. The PEI-CRL showed low specificity and poor regioselectivity, hydrolyzing in *C*-6 but also in *C*-3 positions whereas the PEI-CRL preparation showed good specificity although low regioselectivity, because together **8**, the enzyme hydrolyzed also in *C*-4 (**9**) and *C*-3 (**10**) positions.

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