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Regioselective Hydrolysis of Different Peracetylated β-Monosaccharides by Immobilized Lipases from Different Sources. Key Role of The Immobilization

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Abstract: The effect of the immobilization strategy on the activity, specificity and regioselectivity of three different lipases [those from *Thermomyces lanuginose* (TLL), *Aspergillus niger* (ANL) and *Candida antarctica* B (CAL-B)] in the hydrolysis of peracetylated β-monosaccharides has been evaluated. Three very different immobilization strategies were utilized, covalent attachment, anionic exchange and interfacial activation on a hydrophobic support. The octyl-TLL immobilized preparation was the most efficient biocatalyst in the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose, producing specifically 6-hydroxy-1,2,3,4-tetra-*O*-acetyl-β-D-galactopyranose in 95% overall yield, whereas the CNBr-TLL preparation was 48 times slower and regioselective towards the anomeric position, producing the 1-hydroxy derivative in 70% yield. The PEI-TLL immobilized preparation was the most efficient catalyzing the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, permitting us to obtain up to 70% of the 6-hydroxy product. In the hydrolysis of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose, the octyl-CALB preparation was not selective at all for the production of monohydroxy products whereas when CAL-B was immobilized on PEI-agarose, the enzyme was highly specific and regioselective producing the 6-hydroxy-2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-β-D-glucopyranose in 70% yield.

Keywords: immobilization; lipase; lipase modulation; peracetylated monosaccharides; regioselectivity

Introduction

Carbohydrates exist in very different forms in nature and are playing a very important role in many biological processes.[1]. Furthermore, many compounds with medical relevance are glycosylated.[2]

Pure regioisomers of *O*-acetyl-glycopyranoses presenting only one free hydroxy group may be employed as key intermediates in the preparation of different glyco derivatives, such as oligosaccharides, glycolipids or glycopeptides.[3–7]

Per-*O*-acetyl-glycopyranoses could be used as raw material to obtain monohydroxy peracetylated carbohydrates, although the reported procedures of chemical hydrolysis usually afford the deacetylation at the anomeric position.[8] 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,[9] – with a poor final overall yield – because of the low regioselectivity to remove only one acetyl group among different esters with similar reactivity.

Consequently, the use of enzymatic catalysts could be an attractive and important alternative. However, in most cases the enzymatic deacylation of fully acylated pyranoses is very slow or proceeds with poor selectivity and yield. In addition, these reactions often afford complex mixtures of tetra-, tri-, di-, and monoacetylated derivatives, along with free monosaccharides.[10]

To perform the enzymatic synthesis of mono-deacetylated carbohydrates following this strategy, it is necessary to find biocatalysts exhibiting good catalytic activity and a high regioselectivity. Moreover, in case that the enzyme is able to hydrolyze in several positions – producing a mixture of monodeacetylated products – it would be convenient that the enzyme preparation prefers the per-acetylated monosaccharide instead of the mono-deacetylated one, in order to

accumulate the latter (that is, to exhibit a high specificity for the per-acetylated sugar).

Lipases recognize a broad range of substrates with high regio- and enantioselectivity in different processes such as racemic mixture resolutions and asymmetric reactions.[9b,11–13]. Indeed, lipase-catalyzed hydrolyses of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose have been reported to yield deacetylation at the primary and the anomeric positions.[14]

The mechanism of catalysis of lipases implies dramatic conformational changes of the enzyme molecule between a 'closed' and an 'open' form.[15,16] This mechanism of action has permitted the use of different immobilization protocols (involving different areas of the lipase, rigidity, micro-environments, etc.)[17] causing a strong alteration of the lipase properties in the kinetic resolution of racemic mixtures[17–19] and asymmetric hydrolysis.[20] Here, we hypothesize that, in a similar way, the use of different immobilization protocols may be used to modulate the regioselectivity of lipases in the hydrolysis of per-acetylated carbohydrates in aqueous media.

Herein, in order to demonstrate this hypothesis, three very different immobilization protocols have been applied to the immobilization of different lipases: (i) Immobilization on supports coated with a dense layer of hydrophobic groups at low ionic strength by interfacial activation of the lipases, involving the hydrophobic area surrounding the active site of the lipase,[21] generating a highly hydrophobic environment around the enzyme active site. (ii) Immobilization on agarose activated with CNBr *via* covalent attachment throughout the most active amino group (usually the terminal NH_2) on the enzyme surface.[22] (iii) Immobilization on agarose beads coated with PEI *via* anionic exchange through the areas with highest negative net charge of the lipase.[23]

These different immobilization strategies were used with lipases from *Candida antarctica* (isoform B) (CAL-B),[24] *Thermomyces lanuginose* (TLL)[25] and *Aspergillus niger* (ANL),[26] applying them as biocatalysts in the hydrolysis of several per-*O*-acetylated monosaccharides in aqueous media.

Results and Discussion

Effect and Key Role of the Immobilization Protocol on the Lipase Activity in the Hydrolysis of Acetylated β-Glycopyranoses

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

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

Entry	Enzyme	Support	Initial rate[a]	Reaction time [h]	Yield[b] [%]	**2**[c] [%]	**3** [%]	Others
1	TLL	Octyl	0.48	23	99		99	
2		BrCN	0.01	168	96	77		19
3		PEI	0.02	84	25	14.5	7.5	3
4	ANL	Octyl	0.77	29	75	75		
5		BrCN	45	1.5	100	100		
6		PEI	31	1	100	97	3	
7	CAL-B	Octyl	0.01	96	70	58		12
8		BrCN	0.06	15	100	85		15
9		PEI	0.08	49	100	89	12	

[a] The initial rate in μmol × mg_{prot}^{-1} × h^{-1}. It was calculated at 10–15% conversion.
[b] Yield of the monohydroxy peracetylated product at 100% conversion.
[c] The proportion of the anomers was α/β (1.3:1).

Table 2. Specificity and regioselectivity of different immobilized preparation of lipases in the hydrolysis of **4**.

Entry	Enzyme	Support	Initial Rate[a]	Reaction time [h]	Yield[b] [%]	**5**[c] [%]	**6** [%]
1	TLL	Octil	0.0022	193	33	9	24
2		BrCN	0.0022	193	100	28	72
3		PEI	0.0165	25	43	15	28

[a] The initial rate in μmol × mg_{prot}^{-1} × h^{-1}. It was calculated at 10–15% conversion.
[b] Yield of the monohydroxy peracetylated product at 100% conversion
[c] The proportion of the anomers was α/β (1.1:1)

Table 3. Specificity and regioselectivity of different immobilized preparation of lipases in the hydrolysis of **7**.

Entry	Enzyme	Support	Initial Rate[a]	Reaction time [h]	Yield[b] [%]	**8**[c] [%]	**9** [%]	Other[d]
1	TLL	Octyl	0.004	96	95	26	69	
2		BrCN	0.002	194	90	20	70	
3		PEI	0.006	68	100	33	67	
4	ANL	Octyl	0.10	84	96	33	63	
5		BrCN	29	0.5	100	30	70	
6		PEI	32	0.42	100	33	67	
7	CAL-B	Octyl	0.003	312	0			80
8		BrCN	0.095	102	35	15	20	65
9		PEI	0.074	122	100	30	70	

[a] The initial rate in μmol × mg_{prot}^{-1} × h^{-1}. It was calculated at 10-15% conversion.
[b] Yield of the monohydroxy peracetylated product at 100% conversion.
[c] The proportion of the anomers was α/β (1.1:1).
[d] Di-deacetylated product.

First, we will comment the results obtained with TLL: In the hydrolysis of **1**, TLL immobilized on octyl-agarose presented the highest activity value compared with the other immobilized preparations from this lipase, even up to 48 times higher compared with that one of the CNBr-TLL preparation or 24 times higher in relation of the activity of PEI-TLL preparation (Table 1, entries 1–3). However, the PEI-TLL immobilized preparation was the most active catalyst in the hydrolysis of **4** (Table 2), with a specific activity seven times higher with respect to the other TLL preparations. In the hydrolysis of **7** (Table 3), the PEI-TLL preparation was again the most active biocatalyst (more than three times compared with the other preparations). Thus, the presence of an acetamido group in position 2 (**7**) (compared with **4**) produces a very different effect on the activity of the different TLL preparations. The activity of the PEI-TLL preparation decreased by a factor of almost three times whereas the activity of the octyl-TLL immobilized preparation increased by a factor of almost two times while the activity of the CNBr-TLL preparation remained very similar.

Using ANL as biocatalyst, the CNBr-immobilized preparation presented the highest activity in the hydrolysis of **1**, being more than 50 times more active than the octyl-ANL immobilized preparation. The PEI-ANL preparation also showed a very high activity, although lower than that of the CNBr-ANL preparation (Table 1). In the hydrolysis of **7**, the immobilization of ANL on PEI-agarose permitted us to obtain the most active preparation (around 300 times more active compared to the enzyme immobilized on octyl-agarose) (Table 3).

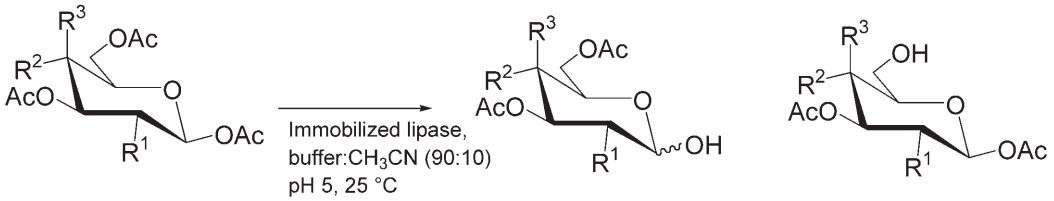
In the case of CAL-B, the lipase showed the highest activity after immobilization on PEI-agarose in the hydrolysis of **1**, being eight times more active than the octyl-CAL-B immobilized preparation (Table 1). However, in the hydrolysis of **7**, CAL-B immobilized on agarose activated with CNBr presented the highest activity value, around 30-fold higher than using the octyl-CAL-B preparation (Table 3).

Taken together, the above results clearly show that the use of different immobilization protocols greatly alters the catalytic activity of the enzyme for different per-*O*-acetylated carbohydrates. This may be clearly appreciated because, for each particular lipase, the most active immobilized preparation was different when the substrate was different, although all preparations presented the same lipase.

Specificity and Regioselectivity of the Different Immobilized Lipase Preparations Catalyzing the Hydrolysis of 1

The different immobilized preparations of the lipases presented a different specificity and regioselectivity in the hydrolysis of **1** (Table 1, Scheme 1).

When TLL preparations were used, the octyl-agarose preparation was very specific towards the peracetylated monosaccharide and very regioselective towards the 6 position (Scheme 1), permitting us to obtain 99% of the 6-OH free monohydroxyperacetylated sugar **3** (entry 1, Table 1). However, this enzyme immobilized on CNBr-agarose produced 96% of monohydroxy products of which 77% was in the anomeric position (α/β, 56%/44%) (**2**) (entry 2,Table 1). On the other hand, the PEI-TLL immobilized preparation was neither specific towards peracetylated **1** – with only a 25% yield in monohydroxy product – nor totally regioselective, giving a mixture of monohydroxy products deprotected in the anomeric and 6-OH positions (entry 3, Table 1). Thus, Figure 1 shows how, depending of the immobilization strategy, the profiles of the course in the hydrolysis of **1** catalyzed by the TLL were quite different.



R^1 = OAc, R^2 = H, R^3 = OAc **1**
R^1 = OAc, R^2 = OAc, R^3 = H **4**
R^1 = NHAc, R^2 = OAc, R^3 = H **7**

R^1 = OAc, R^2 = H, R^3 = OAc **2**
R^1 = OAc, R^2 = OAc, R^3 = H **5**
R^1 = NHAc, R^2 = OAc, R^3 = H **8**

R^1 = OAc, R^2 = H, R^3 = OAc **3**
R^1 = OAc, R^2 = OAc, R^3 = H **6**
R^1 = NHAc, R^2 = OAc, R^3 = H **9**

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

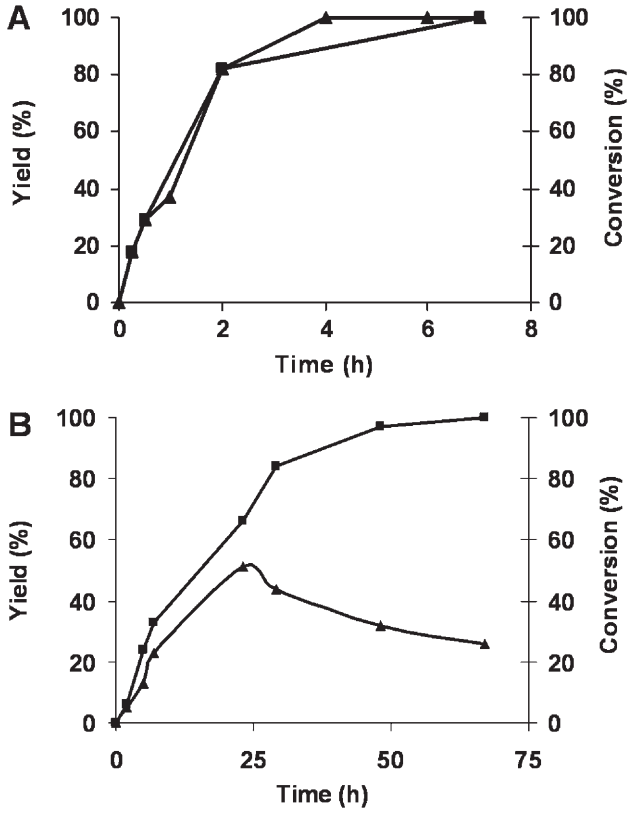


Figure 1. Reaction course of different immobilized preparations of TLL in the hydrolysis of **1**: **A**) octyl-agarose preparation; **B**) PEI-agarose preparation (squares = conversion, triangles = yield of monoprotected product).

In the case of using ANL, the different immobilized preparations preferentially hydrolyzed in the anomeric position producing the tetra-acetylated product **2** with a different specificity (Scheme 1); 100% yield using the CNBr-ANL preparation and 75% yield using the octyl-ANL preparation. (Table 1). However, when ANL was immobilized on PEI-agarose, the enzyme was completely specific towards the peracetylated sugar although not totally regioselective, giving a mixture of monohydroxy products, 97% of **2** and 3% of **3** (entry 6 , Table 1).

When CAL-B was studied, the octyl-CAL-B and the CNBr-CAL-B immobilized preparations were regioselective, preferentially hydrolyzing the anomeric position. The octyl-CAL-B preparation hydrolyzed **1** producing 70% of monohydroxy compound of which 58% was monohydroxy **2** whereas this enzyme immobilized on CNBr-agarose was specific for the mono-deacetylation of **1** with a 100% yield of which 85% was monohydroxy **2** (entries 7 and 8, Table 1). The immobilization of CAL-B on PEI-agarose permitted us to get a high specific biocatalyst towards the peracetylated monosaccharide, but the enzyme was not totally regioselective, giving 89% of **2** and 12% of **3** (entry 9, Table 1).

For a more high scale production, the hydrolysis was performed using 8 g/L substrate concentration using octyl-TLL immobilized preparation as catalyst. **3** was isolated in 95% overall yield. A similar result was obtained in the production of **2** using the CNBr-ANL immobilized preparation as catalyst.

Specificity and Regioselectivity of the Different Immobilized TLL Preparations Catalyzing the Hydrolysis of 4

The different preparations of TLL presented a different specificity and regioselectivity in the hydrolysis of **4** (Table 2). TLL immobilized on CNBr-agarose was quite specific, producing only monohydroxy product, and yielding 24% of **5** and 72% of **6** (Scheme 1). However, when the enzyme was immobilized on octyl-agarose or on PEI-agarose, the production of mono-deacetylated product decreased with yields lower than 50% (entry 1, 3, Table 2), suggesting a lower preference by the peracetylated substrate. The regioselectivity was similar to that observed with the CNBr-TLL preparation.

Using 8 g/L substrate, the hydrolysis of **4** was performed using the CNBr-TLL immobilized preparation, and **6** was isolated in 63% overall yield.

Specificity and Regioselectivity of the Different Immobilized Lipase Preparations Catalyzing the Hydrolysis of 7

Table 3 summarizes the results observed using this substrate. When TLL was used, the immobilization on octyl-agarose or PEI-agarose permitted us to obtain a highly specific catalyst with a yield of monohydroxy products **8** and **9** (Scheme 1) near to 100% whereas the enzyme immobilized on CNBr-agarose gave 70% yield (Table 3). It is interesting to note that the most specific preparations in the hydrolysis of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**7**) (Table 3) presented a poor specificity towards 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**4**) (Table 2).

When using ANL, the three immobilized preparations were quite specific in the hydrolysis of the peracetylated compound, with a regioselectivity towards anomeric (30%, **8**) and 6-OH (70%, **9**) positions.

The most interesting results were found when we used the different preparations of CAL-B as biocatalysts (entries 7–9, Table 3). Here, the immobilization of CAL-B on octyl-agarose produced a biocatalyst with no specificity towards **7**, and in fact the main products were di-deacetylated compounds (80%) (entry 7, Table 3). When the enzyme was immobilized on CNBr-agarose, it presented a certain specificity towards the production of mono-deacetylated product (35%) with a regioselectivity towards **8** and **9**, (entry 8, Table 3) although still 65% were di-deacetylated products. However, the immobilization of CAL-B on PEI-agarose permitted us to obtain a highly specific biocatalyst, with 100% yield of a mixture of two monohydroxy derivatives: **8** (30%) and **9** (70%) (entry 9, Table 3). When the hydrolysis was performed using 8 g/L substrate and using the CNBr-ANL immobilized preparation, **9** was isolated in 65% overall yield.

Conclusions

In this paper, we have described a simple approach for preparing monohydroxy peracetylated monosaccharides, very interesting and useful building blocks for carbohydrate chemistry, by specific and regioselective hydrolysis catalyzed by immobilized lipases. Here, we presented the key role of the immobilization strategy in the hydrolytic activity, specificity and regioselectivity of three different lipases in the hydrolysis of peracetylated sugars. Thus, different immobilized preparations of different lipases presented quite different behaviour in the hydrolysis of several fully protected monosaccharides. For example, with respect to the reaction rate, the octyl-TLL preparation was the most active biocatalyst in the hydrolysis of **1** while the PEI-TLL immobilized preparation was the most active one catalyzing the hydrolysis of **4**. Furthermore, the octyl-TLL was the most specific and regioselective producing exclusively the 6-OH monoxydroxy derivative **3** in 95% overall yield, whereas the CNBr-TLL preparation was regioselective towards the anomeric position, producing **2** from the hydrolysis of **1**. The octyl-CAL-B immobilized preparation was not selective at all for the production of monohydroxy derivatives in the hydrolysis of **7**, whereas when CAL-B was immobilized on PEI-agarose, the enzyme was highly specific and regioselective, producing 70% of **9**. Therefore, the use of this lipase engineering strategy *via* immobilization have permitted to obtain an optimal biocatalyst for the regioselective hydrolysis of different peracetylated β-monosaccharides producing only one free hydroxy group in different positions in high overall yields and suitable reaction times.

Experimental Section

General Remarks

Lipase from *Aspergillus niger* (ANL) was purchased from Fluka (Neu Ulm, Germany). The lipases from *T. lanuginose* (TLL) and *C. antarctica* B (Novozym 525 L) (CAL-B) were kindly supplied by Novo Nordisk company. PEI-agarose[23] were prepared as previously described. Octyl-agarose (4BCL) and cyanogen bromide (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), Triton X-100, *p*-nitrophenyl propionate (pNPP), 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1**), 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**4**) and 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**7**) were from Sigma Chem. Co. The pH of the solutions during the enzymatic hydrolysis was kept constant using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland). HPLC analyses were performed using a HPLC spectra P100 (Thermo Separation products). The column was a Kromasil-C_{18} (250×4.6 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 for **1** and **4**, and 20% acetonitrile in phosphate buffer for **7**; flow rate 1.0 mL min^{-1}. The retention times of the substrates in these conditions were; **1**: 30 min, **4**: 25 min, **7**: 15.5 min. 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.

Standard Enzymatic Activity Assay

In order to follow the immobilization process, the activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically by measuring the increment in absorbance at 348 nm ($\in = 5.150\,M^{-1}cm^{-1}$) produced by the release of *p*-nitrophenol (pNP) in the hydroly-

sis of 0.4 mM *p*NPP in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution (blank or supernatant) or suspension was added to 2.5 mL of substrate solution. Enzymatic activity was determined as μmol of hydrolyzed pNPP per minute per mg of enzyme (IU) under the conditions described above.

Purification of Lipases

The purification of the lipases was performed using a previously described protocol, based on the interfacial activation of lipases on hydrophobic supports at low ionic strength.[21] 0.5 g of ANL commercial solid powder (22 mg protein)[27] and 5 mL TLL or CAL-B commercial solution (12 mg protein/mL)[27] were dissolved in 95 mL of 10 mM sodium phosphate buffer at pH 7.0. In each case 5 g of octyl-agarose support were added. The supernatant and suspension activities were periodically checked by the method described above and the immobilization was terminated after 5 h by filtration. In all cases, more than 90 % of lipase was immobilized. Following this protocol, the SDS-PAGE analysis of the protein adsorbed to the octyl-Sepharose[21] only showed a single band with a molecular weight corresponding to that of the different native lipases. These adsorbed lipases were used as the biocatalyst in some studies. The percentage of immobilization and the enzyme loading in each case are shown in Table 4.

If the lipases need to be released from the octyl-agarose, the preparation was added to a solution of 1 % Triton (v/v) in 10 mM sodium phosphate buffer at pH 7.0 and 4 °C for 1 h obtaining a purified lipase solution with a final concentration of 1.2 mg lipase/mL. Then the enzymatic solution was used for immobilization on the different supports.

Table 4. Immobilization yield of lipases on different supports.

Lipase	Support[a]	Immobilization yield [%][b]	Protein loading[c]	Recovered activity [%]
TLL	soluble	-	-	100
	octyl	100	12	1800
	CNBr	100	12	40
	PEI	100	12	60
ANL	soluble	-	-	100
	octyl	100	4.5	115
	CNBr	48	2.4	100
	PEI	48	2.4	95
CAL-B	soluble	-	-	100
	octyl	100	12	100
	CNBr	16	2	90
	PEI	42	5	95

[a] Soluble is referred to the purified lipase by the methodology described in Experimental Section.
[b] The percentage of immobilized enzyme was determined comparing the enzymatic activity left in the supernatant with the activity of free enzyme – which is 100 % – using the pNPP assay.
[c] As mg of purified lipase/g of support.

Immobilization of Lipases on CNBr-Activated Support

Commercial agarose support activated with CNBr was suspended in an acidic aqueous solution (pH 2–3) during one hour. After that the solid support was dried by filtration under vacuum.

10 mL (for TLL or CAL-B) and 4 mL (for ANL) of the purified lipase solution (1.2 mg mL^{-1}) were added to 8 mL of 10 mM sodium phosphate buffer solution at pH 7. After that, 1 g of the CNBr-agarose support was added. The mixture was then shaken at 25 °C and 250 rpm for 18 h. After that, the solution was removed by filtration and the supported lipase was washed several times with distilled water. The percentage of immobilization and the amount of immobilized lipase in each case are shown in Table 4. The immobilization was followed by the assay described above.

Immobilization of Lipases on PEI-Agarose Support

20 mL (for TLL or CAL-B) and 8 mL (for ANL) of the purified lipase solution (1.2 mg mL^{-1}) were added to 16 mL of 10 mM sodium phosphate buffer solution at pH 7 for TLL, ANL and pH 9 for CAL-B. After that, 2 g of the glyoxyl-agarose beads coated with polyethyleneimine (PEI) were added. The mixture was then shaken at 25 °C and 250 rpm for 4 h. After that, the supernatant was removed by filtration and the supported lipase washed several times with distilled water. The percentage of immobilization and the enzyme loading in each case are shown in Table 4.

Enzymatic Hydrolysis of Peracetylated Monosaccharides

Standard assay was performed as follows: **1**, **4** or **7** (0.02 mmol, 8 mg) was added to 10 mL solution of phosphate buffer 25 mM with 10 % acetonitrile at pH 5, 25 °C and the reaction was initialized by adding 0.8 g (**1**) or 1 g (**4**, **7**) of the biocatalyst. The reaction was performed at pH 5 in order to avoid the chemical acyl-migration in the per-*O*-acetylated carbohydrates hydrolysis.[28] The hydrolytic reaction was carried out under mechanical stirring, and the pH value was controlled by automatic titration. Hydrolysis reactions were followed by HPLC. Finally, the optimization of the reaction in each case was performed using a 8 g/L substrate and the products were isolated and identified by ^{1}H NMR.

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

Substrate **1** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80 %) and acetonitrile (20 %) using 5 g CNBr-ANL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered and saturated with NaCl and then extracted with ethyl acetate (5 × 50 mL). The collected organic layers were washed with a 5 % $NaHCO_3$ solution (2 × 10 mL), separated and dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **2** as a white solid; yield: 370 mg (95 %). HPLC analysis: $t_R = 8.3$ (β anomer), 9.8 min (α-anomer). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.52$ (bd, 1H, $J = 3$, 4 Hz; H-1), 5.48 (dd, 1 H, $J = 1.25$ Hz, H-4), 5.41 (dd, 1 H, $J = 3.4$ Hz, H-3), 5.19 (dd, 1 H, $J = 3$, 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 ,4CH_3). The NMR data are in agreement with the reported values.[29]

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

Substrate **1** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g octyl-TLL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered 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 **3** as a white solid; yield: 370 mg (95%). HPLC analysis: $t_R=10.6$ min. ^{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, 4CH_3).

2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranose (5)

Substrate **4** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-TLL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered 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;yield: 78 mg (20%). HPLC analysis: $t_R=$ 8.2 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ=5.47 (t, $J=9.7$ Hz, H-3, α anomer), 5.38 (d, $J=3.5$ Hz, H-1, α-anomer), 5.18 (t, $J=9.6$ Hz, H-3, β-anomer), 5.03 (t, $J=9.7$ Hz, H-4, α/β anomers), 4.86 (dd, $J=8.1$, 9.7 Hz, H-2, β-anomer), 4.83 (dd, $J=3.6$, 10.1 Hz, H-2, α-anomer), 4.70 (d, $J=8.0$ Hz, H-1, β-anomer), 3.95–4.25 (m, 3H, H-5, α anomer, H-6, α/β anomers), 3.71 (m, ABX system, H-5, β-anomer), 1.90–2.20 (s, 12H, 4CH_3, α/β anomers). The NMR data are in agreement with the reported values .[29]

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose (6)

Substrate **4** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-TLL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered 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; yield: 246 mg (63%). HPLC analysis: $t_R=$ 10 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ=5.70 (d, 1H, $J=$ 8.4 Hz, H-1), 5.27 (t, $J=9.7$ Hz, H-3), 5.07 (m, $J=8.4$ and 9.7 Hz, 2H, H-2, H-4), 3.73 (dd, $J=2.3$ and 12.5 Hz, H-6), 3.62 (ddd, $J=2.3$, 4.2 and 9.7 Hz, H-5), 3.55 (dd, $J=4.2$ and 12.5 Hz, H-6), 2.08 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.99 (s, 3H, CH_3). The NMR data are in agreement with the reported values.[30]

2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α/β-D-glucopyranose (8)

Substrate **7** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-ANL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered and saturated with NaCl and then extracted with ethyl acetate (5×50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2×10 mL), separated and 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 95:5 dichloromethane:methanol as eluent; yield: 97.5 mg (25%). HPLC analysis: $t_R=6.5$ min. ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.04 (d, $J=9.5$ Hz, NH), 5.25 (dd, $J_{3,2}=10.0$, $J_{3,4}=9.5$ Hz, H-3), 5.15 (d, $J=3.5$ Hz, H-1), 5.07 (t, $J=9.5$ Hz, H-4), 4.73 (d, $J=$ 8.5 Hz, H-1, β-anomer), 4.22 (m, H-5), 4.16 (m, 2H, H-2, H-6), 4.02 (m, 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). The NMR data are in agreement with the reported values .[29]

2-Acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-β-D-glucopyranose (9)

Substrate **7** (390 mg, 1 mmol) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-ANL preparation at pH 5. When the substrate had disappeared (checked by TLC and HPLC), the aqueous solution was filtered and saturated with NaCl and then extracted with ethyl acetate (5×50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2×10 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; yield: 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, 3CH_3), 1.96 (s, 3H, CH_3).

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