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Regioselective monohydrolysis of per-*O*-acetylated-1-substituted-β-glucopyranosides catalyzed by immobilized lipases

Adriano A. Mendes [†,‡], Dasciana S. Rodrigues [†,‡], Marco Filice, Roberto Fernandez-Lafuente, Jose M. Guisan [*], Jose M. Palomo [*]

Departamento de Biocatálisis, Instituto de Catálisis (CSIC), Campus UAM Cantoblanco, 28049 Madrid, Spain

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ABSTRACT

The regioselective monohydrolysis of different peracetylated-β-glucopyranosides in aqueous media using immobilized preparations of three different lipases—those from *Aspergillus niger* (ANL), *Candida rugose* (CRL) and *Candida antarctica* B (CAL-B)—has been studied. 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 and the effect of the presence of different moieties in the anomeric position of the substrate on the hydrolytic activities, specificities and regioselectivities of the lipases were investigated. For example, the PEI-ANL preparation exhibited 800 times higher activity than the octyl-ANL in the hydrolysis of 2-acetamido-2-deoxy-1-(4-nitrophenyl)-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside—producing 4-OH derivative in 18% yield—whereas the octyl-ANL was five times more active than the PEI-ANL in the hydrolysis of 1-(4-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-xylopyranoside, producing 4-OH monohydroxy product in >99% yield.
The octyl-CRL preparation hydrolyzed regioselectively 3,4,6-tri-*O*-acetyl-glucal in position 6 in 68% yield while the PEI-CRL produced the 3-OH product in 11% yield, although with moderate specificity. The CNBr-CAL-B hydrolyzed specifically and regioselectively the glucal producing the 3-OH product in >99% yield.

1. Introduction

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

Specially, alkyl and aryl glycosides have attracted tremendous interest in recent years in view of their diverse applications as food emulsifiers, antimicrobial agents, drug carriers or inhibitors of carbohydrate–lectin interactions.[3–8]

Fully acetylated alkyl/aryl glycosides are commonly used for the chemical synthesis of *O*-glycosides in view of their ready availability, low cost and ease preparation.[9] 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, key intermediates in the preparation of different neo-glycoderivatives (oligosaccharides, glycolipids, glycopeptides, glycolipopeptides, etc.).[10–12]

However, the synthesis of monohydroxy derivatives is very difficult by classical chemical approaches, being necessary to use many chemically selective protection/deprotection steps—with a poor final overall yield—because of the low regioselectivity to remove only one acetyl group.[13] Consequently, the use of enzymes in aqueous media could be an attractive alternative. However, in most cases the enzymatic deacylation of fully acylated pyranoses is very slow or proceeds with poor selectivity and yield.[14]

To perform the enzymatic synthesis of monodeacetylated carbohydrates following this strategy, it is necessary to find biocatalysts, specially in an immobilized form,[15] exhibiting good catalytic activity and a high regioselectivity. Moreover, the yield of monodeacetylated product will be defined by the specificity of the enzyme: the biocatalyst should prefer to hydrolyze the peracetylated substrate instead of hydrolyzing the monodeacetylated product to accumulate the last.

Lipases may be a good option to catalyze these processes, because they recognize a broad range of substrates with high regio and enantioselectivity in many instances.[13b,16,17]

The mechanism of catalysis of lipases implies dramatic conformational changes of the enzyme molecule between a 'closed' and

* Corresponding authors. Tel.: +34 91 585 54 78; fax: +34 91 585 47 60.
E-mail addresses: jmguisan@icp.csic.es (J.M. Guisan), josempalomo@icp.csic.es (J.M. Palomo).
† Present address: Departamento de Engenharia Química, Universidade Federal de São Carlos Rodovia Washington Luis, Km 235-676, 13565-905 São Carlos, Brazil.
‡ Both authors contributed equally to this work.

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an 'open' form.[18,19] This mechanism of action produces that the use of different immobilization protocols (involving different areas of the lipase, rigidity, micro-environments, etc.)[20] causes a strong modulation of the lipase properties. In fact, this strategy has been used to modulate the lipase properties.[21–23] In a similar way, the use of different immobilization protocols may be used to modulate the lipase regioselectivity. For example, the hydrolysis of several per-*O*-acetylated monosaccharides by different immobilized preparations of lipases—to deacetylate them at the primary and the anomeric positions—has been recently studied.[24] Now, differently immobilized lipases have been used in the hydrolysis of alkyl or aryl anomeric substituted peracetylated-β-glucopyranosides 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, producing new monodecatylated regioisomers.

Three 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 lipases;[25] (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;[26] (iii) immobilization via anionic exchange through the areas with the highest negative charge of the lipase on agarose beads coated with PEI.[27]

These different immobilization strategies were applied to three very used lipases: those from *Candida antarctica* (isoform B) (CAL-B), from *Candida rugosa* (CRL) and from *Aspergillus niger* (ANL). The immobilized enzymes were utilized as biocatalysts in the hydrolysis of different 1-substituted per-*O*-acetylated glucopyranosides in aqueous media.

2. Results and discussion

2.1. Effect of the immobilization protocol on the lipase activity in the hydrolysis of different peracetylated-β-1-substituted-glucopyranosides

The specific activity displayed by different immobilized preparations from CAL-B, ANL and CRL in the hydrolysis of 1-butyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**1**),1-phenyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**5**), 1-(4-nitrophenyl)-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**), 2-acetamido-2-deoxy-1-(4-nitrophenyl)-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (**13**), 1-(4-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (**17**) and 3,4,6-tri-*O*-acetyl-glucal (**19**) are shown in Table 1. The activities were compared with that obtained in the hydrolysis of the peracetylated glucopyranose (**22**).

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

Table 1
Specific activity of different immobilized preparations of lipases in the hydrolysis of per-*O*-acetylated 1-substituted-β-glucopyranosides[a]

Enzyme	Support	1	5	9	13	17	19	22
CAL-B	Octyl	0.9	8	Nd	0.003	25	486	124
	CNBr	12	6	1.20	0.075	70	1940	132
	PEI	Nd	0.024	Nd	0.004	Nd	23	29
ANL	Octyl	15	48	390	0.45	53,340	60	660
	CNBr	630	5370	25	7	27,150	2790	374,400
	PEI	540	8640	21	15	13,710	1410	99,930
CRL	Octyl	0.75	1.80	0.28	0.002	8	52	187
	CNBr	0.04	0.21	0.06	0.006	23	5	0.2
	PEI	Nd	Nd	0.06	0.004	Nd	4	1

Nd: activity not detected.
[a] The initial rate in $nmol \times mg^{-1}_{prot} \times min^{-1}$. It was calculated at 10–15% conversion.

Using CAL-B, the highest activity for the octyl-CAL-B and CNBr-CAL-B immobilized preparations was achieved in the hydrolysis of **19**, more than 100 times higher, in the case of using the CNBr-CAL-B, than the activity of this biocatalyst in the hydrolysis of peracetylated glucose **22**. In contrast, CAL-B immobilized on PEI-agarose displayed its maximal activity in the hydrolysis of **22**.

The CNBr-CAL-B was the most active biocatalyst in the hydrolysis of **1**, **9**, **13**, **17** and **19**, 13 times more active—using peracetylated butyl-glucopyranoside **1**—and 16 times—using 4-nitrophenyl glucosamine derivative **13**—when it was compared to the octyl-CAL-B preparation. However, the octyl-CAL-B preparation exhibited the highest activity when a phenyl moiety was linked in the anomeric position (**5**), up to 300 times more active compared to the PEI-CAL-B preparation. The PEI-CAL-B was always the less active preparation, and product was not detected in the hydrolysis of **1**, **9** and **17**.

Using ANL as biocatalyst, the CNBr-ANL and PEI-ANL preparations exhibited the highest activity in the hydrolysis of the peracetylated product **22**, while the octyl-ANL exhibited the highest activity towards xylopyranoside **17**, 80 times higher compared to the activity displayed for it in the hydrolysis of **22**.

The CNBr-ANL immobilized preparation was the most active preparation in the hydrolysis of **1**, **19** and **22**, 40 times—using peracetylated butyl-glucopyranoside **1**—and 45 times—using glucal **19**—when it was compared to the octyl-ANL. However, the octyl-ANL preparation showed the highest activity in the hydrolysis of **9** and **17**, e.g., more than 15 times highest compared to the activity displayed by other preparations hydrolyzing **9**.

The PEI-ANL preparation displayed the highest activity in the hydrolysis of **5** and **13**, around 180 times more active towards **5** when it was compared to the enzyme immobilized on octyl-agarose. Interestingly, the activity of the octyl-ANL decreased more than 800 times in the hydrolysis of glucosamine **13** whereas the PEI-ANL activity slightly decreased.

When using CRL, the highest activity for the octyl-CRL was achieved in the hydrolysis of **22**. The CNBr-CRL preparation exhibited the highest activity using xylopyranoside **17** while the PEI-CRL was the most active catalyst towards glucal **19**.

The octyl-CRL preparation was the most active catalyst in the hydrolysis of **1**, **5**, **9** and **19**, 18 times—using peracetylated butyl-glucopyranoside **1**—and 10 times—using glucal **19**—when it was compared to the CNBr-CRL.

However, the CNBr-CRL was the most active catalyst in the hydrolysis of **13** and **19**, with three fold highest activity than the octyl-CRL preparation.

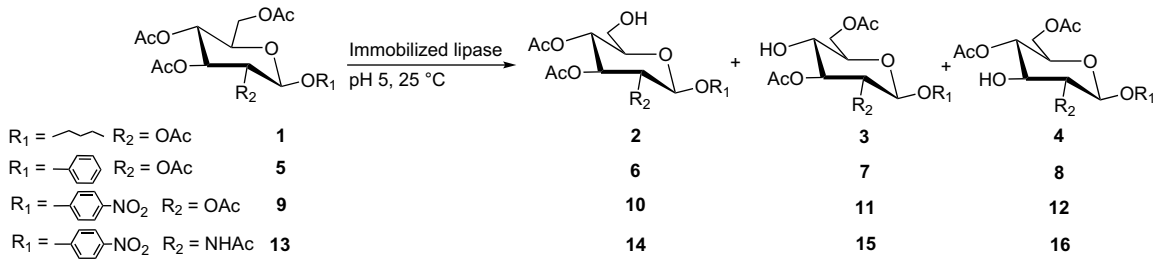
The activity of the CRL immobilized on octyl-agarose decreased more than 100 times when the anomeric position was blocked in substrates **1**, **5**, **9** and **13**. However, the CNBr-CRL showed the same activity using **22** and **5** and more than 100 times higher activity when the xylopyranoside was used.

The PEI-CRL was always the less active preparation, and any products were not detected in the hydrolysis of **1**, **5** and **17**. However, this lipase immobilized preparation showed better activity (four fold) towards glucal **19** than using **22**.

In this way, the modification in the anomeric position of different peracetylated-β-1-substituted-glucopyranosides presented a very different effect on the activity exhibited by lipases immobilized following different protocols. That way, lipases specificity was strongly affected by the immobilization protocol.

2.2. Specificity and regioselectivity of the different immobilized lipases preparations

Recently we found that different immobilized biocatalysts of these three lipases exhibited a regioselectivity towards anomeric or 6-OH position in the hydrolysis of the peracetylated **22**.[24] In this



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

study, the anomeric position was blocked expecting that to alter these previous results.

2.2.1. Hydrolysis of *1*

When CAL-B was studied, the octyl-CAL-B preparation was not specific towards the peracetylated substrate, giving the non-desired multi-hydrolyzed products (data not shown). The CNBr-CAL-B preparation was also not specific, giving 11% of monodeacetylated product, although accumulating the 6-OH product **2** (Scheme 1, Table 2).

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	CAL-B	Octyl	168	51	0	0
2		CNBr	90	100	11	
3		PEI	168	—	—	—
4	ANL	Octyl	168	43	10	5
5		CNBr	6	96	77	
6		PEI	24	96	73	5
7	CRL	Octyl	168	90	47	8
8		CNBr	168	7	4	3
9		PEI	168	0	—	—

[a] Conversion.
[b] Yield of the monodeacetylated product.

Using ANL, the CNBr-ANL immobilized preparation was quite specific (accumulating the monodeacetylated products) and regioselective (hydrolyzing at position 6), producing the tri-acetylated product **2** in 77% yield (Scheme 1, Table 2). ANL immobilized on PEI-agarose was also quite specific but not totally regioselective, producing 73% of **2** but also 5% of 4-OH product **3** was achieved (entry 6, Table 2). The hydrolysis at position 4, although still minority, may be interesting. However, the octyl-ANL preparation showed low specificity towards the peracetylated **1**, giving only 15% yield of monodeacetylated products **2** and **3** (2:1) (entry 4, Table 2).

When CRL immobilized preparations were used, the octyl-CRL was not very specific with 55% yield in monohydroxy product at 100% conversion, neither it was regioselective, because both monohydroxy 6-OH (**2**) and 4-OH (**3**) products were obtained (47% **2**, 8% **3**). The hydrolysis at position 4—although minority—may be interesting. The CNBr-CRL preparation was not regioselective, with the production of both **2** and **3** in similar amounts (Table 2).

2.2.2. Hydrolysis of *5*

When CAL-B was used, the octyl-CAL-B or CNBr-CAL-B preparations were not specific, giving 17% or 3% of monodeacetylated product, although accumulating the product hydrolyzed at position 6 (**6**). The PEI-CAL-B only gave 5% conversion after 168 h producing **6** (Scheme 1, Table 3).

Using ANL, the immobilization on CNBr-agarose or PEI-agarose was not very specific, producing 61% and 49% of 6-OH product **6**, respectively. The octyl-ANL was specific, although hydrolyzing at position 6 (**6**, 38%) and also, interestingly, at position 4, but with a low yield (**7**, 6%) (Table 3).

In the case of CRL, the immobilization on octyl-agarose permitted to obtain a not very specific catalyst to produce **6** in 35% yield. The other CRL preparations hydrolyzed at positions 6 and 4 gave a mixture of **6** and **7** (Table 3, entries 8 and 9). The production of **7**—even although at low yields—opens the possibility of obtaining this interesting product.

2.2.3. Hydrolysis of *9*

Using CAL-B, the CNBr-CAL-B preparation was the unique preparation active of this lipase towards **9**. It was non-specific, producing 14% of 6-OH monodeacetylated product **10** (Scheme 1, Table 4).

Table 3
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **5**

Entry	Enzyme	Support	Reaction time [h]	*c*[a] [%]	**6**[b] [%]	**7**[b] [%]
1	CAL-B	Octyl	168	100	17	
2		CNBr	168	100	3	
3		PEI	168	5	5	
4	ANL	Octyl	168	44	38	6
5		CNBr	5	100	61	
6		PEI	5	100	49	
7	CRL	Octyl	160	100	35	
8		CNBr	168	36	19	6
9		PEI	168	3.3	1.8	1.5

[a] Conversion.
[b] Yield of the monodeacetylated product.

Table 4
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **9**

Entry	Enzyme	Support	Reaction time [h]	*c*[a] [%]	**10**[b] [%]	**11**[b] [%]
1	CAL-B	Octyl	168	0		
2		CNBr	168	96	14	
3		PEI	168	0	—	—
4	ANL	Octyl	54	100	42	5
5		CNBr	168	100	50	8
6		PEI	168	73	13	6
7	CRL	Octyl	168	90	6	5
8		CNBr	168	33	10	12
9		PEI	168	89	7	5

[a] Conversion.
[b] Yield of the monodeacetylated product.

Table 5
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **13**

Entry	Enzyme	Support	*c*[a] [%]	**14**[b] [%]	**15**[b] [%]	**16**[b] [%]
1	CAL-B	Octyl	5			1
2		CNBr	24			12
3		PEI	3	3		
4	ANL	Octyl	6	5		1
5		CNBr	84	51	11	7
6		PEI	92	37	18	3
7	CRL	Octyl	2	2		
8		CNBr	9	3		
9		PEI	5	5		

[a] Conversion at 168 h.
[b] Yield of the monodeacetylated product.

Table 6
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of 17

Entry	Enzyme	Support	Reaction time [h]	*c* [%]	**18**[a] [%]
1	CAL-B	Octyl	168	14	14
2		CNBr	168	41	41
3		PEI	168	0	—
4	ANL	Octyl	25	100	>99
5		CNBr	35	100	>99
6		PEI	74	100	>99

[a] Yield of the monodeacetylated product.

When ANL was employed, all the preparations showed different specificity (Table 4) producing an interesting mixture of monodeacetylated 6-OH (**10**) and 4-OH (**11**) products. Using the CNBr-ANL immobilized preparation was possible to achieve a yield of 50% of **10** and 8% of **11** (Table 4, entry 5).

In the case of CRL, the octyl-CRL or PEI-CRL preparation showed very low specificity. The CNBr-CRL immobilized preparation presented better specificity producing in the same amounts **10** and the interesting **11** (Table 4).

2.2.4. Hydrolysis of *13*

In the hydrolysis of 4-nitrophenyl glucosamine derivative (**13**), the CAL-B immobilized on octyl-agarose or CNBr-agarose yielded less than 25% of monodeacetylated sugar but only producing the very interesting product **16**. However, the PEI-CAL-B preparation hydrolyzed the acetyl group producing only the 6-OH **14** although with low yield (Table 5).

Employing ANL, the CNBr-ANL preparation was highly specific in the hydrolysis of peracetylated **13**, producing 6-OH product **14** in 51% yield, and also the interesting 4-OH **15** in 11% and 3-OH **16** in 7% yield. Using the PEI-ANL, the results were quite similar (entry 6, Table 5). The octyl-ANL hydrolyzed specifically at positions 6 and 3 in a relation 5:1.

Using CRL, all immobilized preparations exhibited very low activity and the regioselectivity was not analyzed.

2.2.5. Hydrolysis of *17*

In the regioselective hydrolysis of the xylopyranoside **17** (Table 6, Scheme 2), all ANL and the octyl- and CNBr-CAL-B preparations displayed a very high specificity and regioselectivity towards the production of the interesting product 4-OH **18**. Using the octyl-ANL preparation—such as optimal catalyst—was possible to obtain **18** in >99% yield in 25 h (Table 6).

Any of the CRL immobilized preparations did not hydrolyze this substrate.

2.2.6. Hydrolysis of *19*

Using CAL-B, all the three immobilized preparations hydrolyzed specifically and regioselectively the glucal **19**—1,2-anhydrosugar with great synthetic potential as building blocks in oligosaccharide synthesis—at position 3 (**21**) in >99% yield (Table 7, Scheme 3).

The ANL preparations were also regioselective producing the monohydrolyzed 3-OH derivative **21** although with different yields for the three ANL immobilized preparations. Using the PEI-ANL preparation, 75% of **21** was obtained whereas only 34% of **21** was achieved employing the CNBr-ANL preparation (Table 7, entries 5 and 6).

Table 7
Specificity and regioselectivity of different immobilized preparations of lipases in the hydrolysis of **19**

Entry	Enzyme	Support	Reaction time [h]	*c*[a] [%]	**20**[b] [%]	**21**[b] [%]
1	CAL-B	Octyl	17	100		>99
2		CNBr	5	100		>99
3		PEI	120	100		>99
4	ANL	Octyl	168	27		10
5		CNBr	24	100		34
6		PEI	168	100		75
7	CRL	Octyl	24	100	68	
8		CNBr	168	100		9
9		PEI	168	90		11

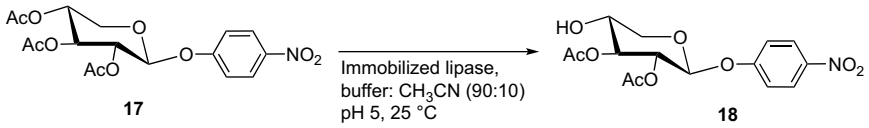
[a] Conversion.
[b] Yield of the monodeacetylated product.

When CRL was used, the octyl-CRL preparation showed good specificity and regioselectivity, hydrolyzing exclusively at position 6 of **19**, producing the monodeacetylated product **20** in 68% yield. The other CRL biocatalysts gave low yield in the monodeacetylation of **19**, accumulating the 3-OH product **21** in 10% yield (Table 7, entries 7–9).

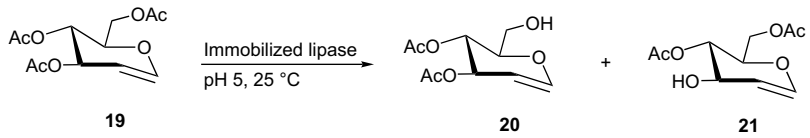
3. Conclusion

In this manuscript, we have presented the enzymatic deacetylation—using three different lipases—of different 1-substituted-peracetylated glucopyranosides by hydrolysis in aqueous media. The immobilization strategy was a key point to determine the optimal catalysts in each case. The immobilization strategy defines the activity, specificity and regioselectivity of the final biocatalyst. The presence of different moieties in the anomeric position of the substrate altered the catalytic properties of these immobilized lipases compared to the use of sugars with an acetyl group in the anomeric position.

The variation of the specific activity caused by the immobilization strategy may be exemplified by the ANL. The PEI-ANL immobilized preparation exhibited 800 times higher activity in the hydrolysis of **13** compared with the activity displayed by the octyl-



Scheme 2. Regioselective hydrolysis of **17** by different immobilized preparations of lipases.



19 20 21

Scheme 3. Regioselective hydrolysis of **19** by different immobilized preparations of lipases.

ANL whereas the octyl-ANL preparation was five times more active than the PEI-ANL in the hydrolysis of **17**. About the modulation of the regioselectivity by the immobilization strategy, for example, the octyl-CRL hydrolyzed regioselectively the position 6 of **19**, producing the monodeacetylated product **20** in 68% yield while the PEI-CRL produced the 3-OH product **21** in 11% yield, although with moderate specificity.

Using this biocatalytic approach, the 4-OH monohydroxy derivative **18** was produced in >99% yield using the octyl-ANL. Moreover, the monodeacetylated 3-OH **16** and **21** was also obtained in 12% and >99% yields using the CNBr-CAL-B preparation. These monodeacetylated glucosides are very interesting and useful building blocks for carbohydrate chemistry and are not produced using the unmodified peracetylated sugars. Thus, the combination of combinatorial biocatalysis (use of different enzymes immobilized following different strategies) and a small collection of modified substrates may permit to produce monodeacetylated sugars in no standard positions, with a high interest in the production of neo-glycoderivatives or glycoconjugates for biological studies.

4. Experimental

4.1. General

Lipase from *A. 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) (M_r 25,000), Triton X-100, *p*-nitrophenyl butyrate (*p*-NPB), lipase from *C. rugosa* (CRL), 1-butyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**1**), 1-phenyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**5**), 1-(4-nitrophenyl)-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**9**), 2-acetamido-2-deoxy-1-(4-nitrophenyl)-3,4,6-tri-*O*-acetyl-β-D-glucopyranose (**13**), 1-(4-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (**17**), 3,4,6-tri-*O*-acetyl-glucal (**19**) and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranoside (**22**) were from Sigma Chem. Co. Agarose beads coated with PEI were prepared as previously described.[27] 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. The different products obtained by enzymatic hydrolysis were characterized by COSY 2D NMR homocorrelation studies in order to assign the exact position of the hydrolysis.

4.2. 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 given as μmol of hydrolyzed *p*-NPB per minute per mg of enzyme (IU) under the conditions described above.

4.3. Purification of lipases

The purification of CAL-B and CRL was performed using a previously described protocol, based on the selective adsorption of lipases on hydrophobic supports at low ionic strength.[25] CRL commercial solid powder (0.16 g, 30 mg protein) or 2.5 mL CAL-B commercial solution (12 mg protein/mL)[28] was offered to 95 mL of 10 mM sodium phosphate at pH 7.0. In each case, 5 g of octyl-agarose support was added. After 4 h, the enzyme immobilized preparation was filtered 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.[29] Six milligrams lipase per gram of support was prepared. The SDS–PAGE analysis of the proteins after the purification treatment 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 immobilized preparations, the adsorbed enzyme was added to 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.

4.4. Immobilization of lipases on other supports

Lipases were immobilized in the presence of 1% triton, as obtained after the enzyme purification, to prevent any lipase–lipase interaction that could produce the immobilization of lipase dimers with altered properties.[30] The further exhaustive washing with distilled water of the immobilized lipases permitted to fully eliminate the detergent.

4.4.1. Immobilization of lipases on CNBr-activated support

Commercial agarose support activated with CNBr was suspended in an acidic aqueous solution (pH 2–3) for 1 h. After that, the solid support was dried by filtration under vacuum using a sintered glass funnel. Lipase solution (10 mL, 0.6 mg lipase/mL) was added to 30 mL of 10 mM sodium phosphate solution at pH 7. After that, 1 g of the CNBr-agarose support was added. The mixture was then stirred at 25 °C and 250 rpm for 2 h. After, the supernatant was removed by filtration using a sintered glass funnel and the supported lipase added to 40 mL of 3 M glycine at pH 8 for 1 h. Finally, the immobilized lipases were filtered using a sintered glass funnel and washed several times with distilled water. The immobilization yield was—in all cases—more than 95%.

4.4.2. Immobilization of lipases on PEI-agarose support

Lipase solution (10 mL, 0.6 mg lipase/mL) was added to 30 mL of 10 mM sodium phosphate solution at pH 7. After that, 1 g of the glyoxyl-agarose beads coated with polyethyleneimine (PEI) was added. The mixture was then stirred at 25 °C and 250 rpm for 4 h.

After, the supernatant was removed by filtration and the supported lipase was washed off several times with distilled water. The immobilization was—in all cases—more than 95%.

4.5. Enzymatic hydrolysis of peracetylated-β-glucopyranosides

Compounds **1**, **5**, **19** and **22** (2 mM) or 0.2 mM of **9**, **13** and **17** in 50 mM sodium acetate with 20% acetonitrile at pH 5 and 25 °C were prepared. Biocatalyst (0.5 g) was added to 3 mL (**1**, **5**, **9**, **13**, **17**) or 10 mL (**19**, **22**) 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.[31] 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). At least triplicates of each assay were made. 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.

4.6. 1-Butyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (2)

^{1}H NMR ($CDCl_3$): δ (ppm): 5.25 (t, 1H, *J*=9.5 Hz, H-3), 5.00 (t, 1H, *J*=9.8 Hz, H-4), 4.98 (dd, 1H, *J*=7.9, 9.6 Hz, H-2), 4.43 (d, 1H, *J*=7.8 Hz, 1H-1), 3.90–3.79 (m, 2H, 2×H-6), 3.64 (m, 1H, *J*=9.5 Hz, H-5), 3.46–3.41 (m, 2H, $CH_{2\alpha}$), 2.01–1.93 (s, 12H, 4×CH_3), 1.55–1.46 (m, 2H, CH_2), 1.32–1.22 (m, 2H, CH_2), 0.90 (t, 3H, *J*=7.4 Hz CH_3). The NMR data are in agreement with the reported values.[24c]

4.7. 1-Butyl-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (3)

^{1}H NMR ($CDCl_3$): δ (ppm): 5.23 (t, 1H, *J*=9.5 Hz, H-3), 4.97 (dd, 1H, *J*=7.9, 9.6 Hz, H-2), 4.42 (d, 1H, *J*=7.8 Hz, 1H-1), 4.34–4.09 (m, 2H, 2×H-6), 3.98 (t, 1H, *J*=9.8 Hz, H-4), 3.64 (m, 1H, *J*=9.5 Hz, H-5), 3.45–3.40 (m, 2H, $CH_{2\alpha}$), 2.10–2.03 (s, 12H, 4×CH_3), 1.46–1.45 (m, 2H, CH_2), 1.35–1.27 (m, 2H, CH_2), 0.94 (t, 3H, *J*=7.4 Hz CH_3).

4.8. 1-Phenyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (6)

^{1}H NMR ($CDCl_3$): δ (ppm): 7.35–7.11 (m, 5H, Ar–H), 5.26 (t, 1H, *J*=9.5 Hz, H-3), 5.16 (dd, 1H, *J*=7.9 Hz, *J*=9.6 Hz, H-2), 4.80 (d, 1H, *J*=7.8 Hz, H-1), 4.65 (t, 1H, *J*=9.8 Hz, H-4), 3.90–3.82 (m, 2H, 2×H-6), 3.64 (m, 1H, *J*=9.5 Hz, H-5), 2.11–1.98 (s, 9H, 3×CH_3).

4.9. 1-Phenyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (7)

^{1}H NMR ($CDCl_3$): δ (ppm): 7.34–7.12 (m, 5H, Ar–H), 4.79 (t, 1H, *J*=9.4 Hz, H-3), 5.14 (dd, 1H, *J*=7.8, 9.5 Hz, H-2), 4.75 (d, 1H, *J*=7.8 Hz, H-1), 4.34–4.09 (m, 2H, 2×H-6), 3.91 (t, 1H, *J*=9.5 Hz, H-4), 3.65 (m, 1H, *J*=9.5 Hz, H-5), 2.09–1.96 (s, 9H, 3×CH_3).

4.10. 1-(4-Nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (10)

^{1}H NMR ($CDCl_3$): δ (ppm): 8.10 (d, 2H, *J*=8.5 Hz, H-3′, H-5′), 6.88 (d, 2H, *J*=8.3 Hz, H-2′, H-6′), 5.25 (t, 1H, *J*=9.4 Hz, H-3), 5.14 (dd, 1H, *J*=7.8, 9.5 Hz, H-2), 4.81 (d, 1H, *J*=7.7 Hz, H-1), 4.66 (t, 1H, *J*=9.7 Hz, H-4), 3.92–3.84 (m, 2H, 2×H-6), 3.65 (m, 1H, *J*=9.4 Hz, H-5), 2.19 (s, 3H, CH_3), 2.11–2.09 (s, 6H, 2×CH_3). The NMR data are in agreement with the reported values.[32]

4.11. 1-(4-Nitrophenyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (11)

^{1}H NMR ($CDCl_3$): δ (ppm): 8.14 (d, 2H, *J*=8.6 Hz, H-3′, H-5′), 6.98 (d, 2H, *J*=8.5 Hz, H-2′, H-6′), 5.18 (t, 1H, *J*=9.6 Hz, H-3), 5.06 (m, 1H, H-2), 4.77 (d, 1H, *J*=7.8 Hz, H-1), 4.34–4.12 (m, 2H, 2×H-6), 3.91 (t, 1H, *J*=9.5 Hz, H-4), 3.69 (m, 1H, *J*=9.5 Hz, H-5), 2.09–1.96 (s, 9H, 3×CH_3). The NMR data are in agreement with the reported values.[32]

4.12. 2-Acetamido-2-deoxy-1-(4-nitrophenyl)-3,4-di-*O*-acetyl-β-D-glucopyranoside (14)

^{1}H NMR ($CDCl_3$): δ (ppm): 8.14 (d, 2H, *J*=8.6 Hz, H-3′, H-5′), 7.1 (d, 2H, *J*=8.5 Hz, H-2′, H-6′), 5.28 (t, 1H, *J*=9.5 Hz, H-3), 4.85 (d, 1H, *J*=7.7 Hz, H-1), 4.79 (m, 1H, H-2), 4.66 (t, 1H, *J*=9.5 Hz, H-4), 3.85–3.70 (m, 2H, 2×H-6), 3.65 (m, 1H, H-5), 2.20 (s, 6H, 2×CH_3), 1.84 (s, 3H, CH_3).

4.13. 2-Acetamido-2-deoxy-1-(4-nitrophenyl)-3,6-di-*O*-acetyl-β-D-glucopyranoside (15)

^{1}H NMR ($CDCl_3$): δ (ppm): 8.15 (d, 2H, *J*=8.4 Hz, H-3′, H-5′), 6.9 (d, 2H, *J*=8.6 Hz, H-2′, H-6′), 4.85 (d, 1H, *J*=8.0 Hz, H-1), 4.80 (t, 1H, *J*=9.3 Hz, H-3), 4.65 (m, 1H, H-2), 4.20–4.08 (m, 2H, 2×H-6), 3.90 (m, 1H, H-4), 3.75 (m, 1H, H-5), 2.17 (s, 6H, 2×CH_3), 1.85 (s, 3H, CH_3).

4.14. 2-Acetamido-2-deoxy-1-(4-nitrophenyl)-4,6-di-*O*-acetyl-β-D-glucopyranoside (16)

^{1}H NMR ($CDCl_3$): δ (ppm): 8.12 (d, 2H, *J*=8.4 Hz, H-3′, H-5′), 7.0 (d, 2H, *J*=8.5 Hz, H-2′, H-6′), 4.85 (d, 1H, *J*=7.8 Hz, H-1), 4.66 (m, 1H, H-2), 4.4 (t, 1H, *J*=9.5 Hz, H-4), 4.30–4.09 (m, 2H, 2×H-6), 3.99 (t, 1H, *J*=9.0 Hz, H-3), 3.80 (m, 1H, H-5), 2.16–2.00 (s, 6H, 2×CH_3), 1.89 (s, 3H, CH_3).

4.15. 1-(4-Nitrophenyl)-2,3-di-*O*-acetyl-β-D-xylopyranoside (18)

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 8.10 (d, 2H, *J*=8.4 Hz, H-3′, H-5′), 6.88 (d, 2H, *J*=8.31 Hz, H-2′, H-6′), 5.21 (d, 1H, *J*=5.3 Hz, H-1), 5.16 (m, 1H, H-2), 4.78 (m, 1H, H-3), 4.12 (m, 1H, H-5), 3.92 (m, 1H, H-4), 3.55 (m, 1H, H-5), 2.10 (s, 3H, CH_3), 2.05 (s, 3H, CH_3). The NMR data are in agreement with the reported values.[33]

4.16. 3,4-Di-*O*-acetyl-glucal (20)

^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.49 (dd, 1H, *J*=6.1 Hz, H-1), 5.41–5.50 (m, 1H, H-3), 5.22 (dd, 1H, *J*=9.0, 6.5 Hz, H-4), 4.81 (dd, 1H, *J*=5.9, 2.8 Hz, H-2), 3.98–4.09 (m, 1H, H-5), 3.66–3.86 (m, 2H, H-6A,B), 2.07–2.13 (2s, 6H, 2×CH_3). The NMR data are in agreement with the reported values.[34]

4.17. 4,6-Di-*O*-acetyl-glucal (21)

^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.35 (dd, 1H, *J*=6.2 Hz, H-1), 4.95 (dd, 1H, *J*=8.4, 6.2 Hz, H-4), 5.41–5.50 (m, 1H, H-3), 4.84 (dd, 1H, *J*=6.2, 5.2, 3, H-2), 4.27 (dd, *J*=12.9, 6.2 Hz, 1H-3), 4.11 (ddd, 1H, *J*=3.1 Hz, H-5), 4.20–4.37 (m, 2H, H-6A,B), 2.55 (br s, 1H, OH), 2.16 (s, 3H, CH_3), 2.11 (s, 3H, CH_3). The NMR data are in agreement with the reported values.[34]

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