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Regio-selective deprotection of peracetylated sugars via lipase hydrolysis

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Abstract—Purified lipases (via interfacial activation on hydrophobic supports) from different microbial extracts have been evaluated in the regio-selective hydrolysis of peracetylated sugars (peracetylated glucose, ribose and sucrose). Among the enzymes tested, lipases from *Candida rugosa* (CRL) and from *Pseudomonas fluorescens* (PFL) exhibited the best properties in these reactions.Then, we have prepared two different immobilized lipase preparations obtained by interfacial activation on hydrophobic supports or by covalent attachment on glutaraldehyde agarose. Interfacially activated lipases exhibited a higher activity than covalently attached enzymes (even by a 100-fold factor), giving the higher yields of mono deacetylated sugars (in some instances by more than a threefold factor) in short reaction times. In the hydrolysis of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose catalyzed by PFL adsorbed on octyl agarosa, hydrolyzed mainly the 3 position (30% of yield) while the CRL gave the hydrolysis only in position 5 (about 50% of yield). Depending on the enzyme immobilized preparation, we have been able also to obtain selective hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α/β-D-glucopyranose obtaining a free hydroxyl group in position 1, 4 or 6. Moreover, selective hydrolysis in the 4′ position of peracetylated sucrose was achieved when the hydrolysis is performed with CRL immobilized on octyl-agarose (yield was 77%).
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1. Introduction

Pure regioisomers of *O*-acetyl-pyranosides presenting only one free hydroxyl group (AP) may be used as key intermediates for the synthesis of a large number of glycoderivatives (oligosacharides, sugar esters, glycopeptides, etc.).[1,2] AP intermediates can be readily and selectively modified at this free hydroxyl group and they are soluble in most organic solvents. Moreover, the protected final products can be easily deacetylated by very mild chemical or enzymatic processes. However, the preparation of APs with only one free hydroxyl group by classical chemical synthesis requires multi-step reactions that may cause environmental problems for large-scale production, due to the use of organic solvents and toxic reagents.[3–9] These problems make the use of enzymatic catalysts such as lipases or esterases an attractive alternative route for AP preparation,[10,11] although in many instances, the reactions are very slow or proceed with poor selectivity and yield. In addition, these reactions often afford complex mixtures of tetra-, tri-, di-, and monoacetates, along with free monosaccharide.[12–14]

In the design of this kind of reactions, we have considered two different aspects:

First, the enzyme must mainly recognize the peracetylated sugar as substrate, permitting the accumulation of mono deacetylated sugar. This is related to the specificity of the enzyme-immobilized preparation by the different available substrates. Second, it is convenient that the enzyme produces only one of the different possible regioisomers. This is related to the regioselectivity of the enzyme.

When using lipases as biocatalyst of organic reactions, it is necessary to bear in mind that their mechanism of catalysis implies dramatic conformational changes of the enzyme molecule between a 'closed' and an 'open' form.[15–21] This equilibrium between two very disimilar molecular forms also exists in most immobilized lipases.

Our hypothesis is that by using immobilization techniques that involve different areas of the enzyme, that confer different rigidity to the enzyme structure, it is possible to restringe the mobility of the enzyme and, in this way, altering their catalytic properties (Fig. 1). Thus, the same lipase molecule can present very different catalytic properties after immobilization on different supports. This has shown in resolution of racemic mixtures with lipases,[22–25] to permit a strong modulation of the lipase properties. In this

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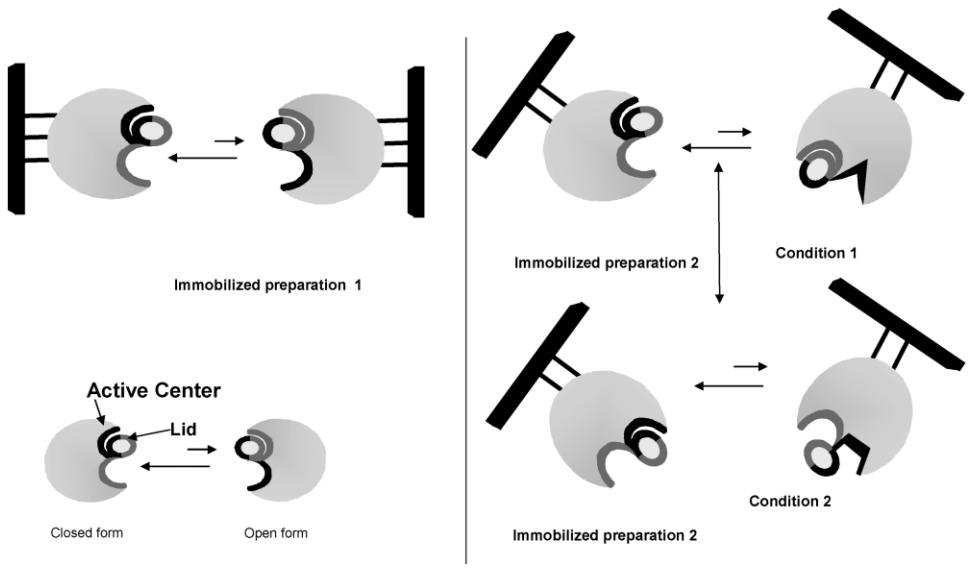


Figure 1. Conformational engineering of lipases.

way, it is necessary not only to state the source, but also the utilized form. Even the use of different enzyme concentrations may alter the behavior of the enzyme when used in soluble form.[26,27]

In this work, we have studied if the immobilization of lipases on different supports may also alter their behavior when employed them as catalyst in the regio-selective deprotection of peracetylated sugars. Therefore, two different immobilized lipase preparations have been produced: one by interfacial activation on hydrophobic supports[28] and one by covalent attachement on glutaraldehyde agarosa.[29] These immobilized preparations have been compared in the hydrolysis of peracetylated ribose, glucose and sucrose.

2. Results and discussion

Table 1 shows the different activities and yields obtained in the hydrolysis of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose **1** when using lipases adsorbed on octyl agarose[30] from seven different sources: *Pseudomonas fluorescens* (PFL), *Mucor miehei* (MML), *Humicola lanuginosa* (HLL), *Aspergillus oryzae* (AOL), *Candida rugosa* (CRL), *Candida antarctica* A (CAL-A) and B (CAL-B).

Table 1. Regio-selective hydrolysis of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose catalyzed by lipases immobilized on octyl-agarose supports in aqueous media

Lipase	Reaction rate[a]	Time (h)	Yield[b] (%)
CRL	3.07	3	47
PFL	0.1	31	40
MML	0.0075	140	33
HLL	0.0073	140	25
AOL	0.008	140	20
CAL-A	0.012	92	5
CAL-B	0.03	25	2

Experiments were carried out as described in Section 4.
[a] Reaction rate was expressed in IU/mL (μmol of substrate hydrolyzed per minute per mL of immobilized protein).
[b] Accumulated percentage of isolated tris-*O*-acetyl-β-D-ribofuranoside for 100% hydrolysis of substrate.

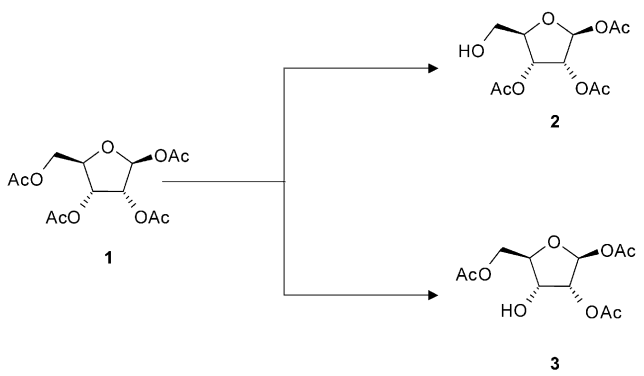
From the data obtained, only CRL and PFL seemed offer good activity values: 3.07 and 0.1 IU/mL of biocatalyst. These enzymes also gave the highest yields on triacetate compound, showing an affinity for the peracetylated sugars much higher than that towards the first product of hydrolysis. MML, HLL and AOL showed slightly lower yields than those, but much lower activities (around 0.0075 IU/mL). The yields achieved with *C. antarctica* lipases were far lower than these values. Therefore, CRL and PFL were chosen to carry out further studies.

Table 2 shows the results achieved when using differently immobilized preparation of these enzymes. CRL adsorbed on octyl-agarose catalyzed hydrolysis of **1** in sodium phosphate buffer yielding a single triacetate in 47% yield (the rest of the substrate was hydrolyzed to lower acetylated products). This product was isolated and identified by ^{1}H NMR spectroscopy as 1,2,3-tri-*O*-acetyl-β-D-ribofuranoside **2** (Scheme 1). Using the covalently immobilized preparation, activity decreased by a 100-fold factor respect to the octyl-agarose preparation, while the yield of triacetatate was 33%, being only isolated the compound **2**.

Table 2. Enzymatic hydrolysis of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose catalyzed by CRL and PFL preparations

Lipase	Immobilized preparation	Reaction rate[a]	Yield[b] (%)	Deacetylated sugar
CRL	Octyl	3.07	47	**2**
	Glutaraldehyde	0.03	33	**2**
PFL	Octyl	0.1	40	**2**, **3** (1:3)
	Glutaraldehyde	0.007	30	**3**

Experiments were carried out as described in Section 4.
[a] Reaction rate was expressed in IU/mL ?μmol of substrate hydrolyzed per minute per mL of immobilized protein).
[b] Accumulated percentage of isolated tris-*O*-acetyl-β-D-ribofuranoside for 100% hydrolysis of substrate.



Scheme 1.

α/β **5** to be obtained in quantitative yields at all pH values tested (results not shown).

The activity of CRL towards substrate **4** was higher than towards compound **1**, for both covalently (glutaraldehyde derivative) and interfacially (octyl derivative) immobilized preparations. The activity of the interfacially activated enzyme was again around 100 times higher than that obtained for the covalent preparation, when the reaction was performed to acid pH. The yields of tetraacetate using the different immobilized preparations at pH 5 were 75% for the interfacially adsorbed lipase and 52% for the covalently immobilized one. In both cases the unique tetraacetate produced by hydrolysis resulted the 1,2,3,4,-tetra-*O*-acetyl-α-D-glucopyranose **6** (Scheme 2).

Table 3. Enzymatic hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose catalyzed by different CRL and PFL preparations

Lipase	Immobilized preparation	pH	Reaction rate[a]	Yield[b] (%)	Deacetylated sugar
PFL	Octyl	7	1.7	>98	**5** ?α/β?
	Glutaraldehyde	7	0.07	56	**5** (α/β)
CRL	Octyl	5	7	75	**6**
	Glutaraldehyde	5	0.006	52	**6**
CRL	Octyl	7	1	57	**6**, **7** (3:7)
	Glutaraldehyde	7	0.008	34	**6**, **7** (3:7)

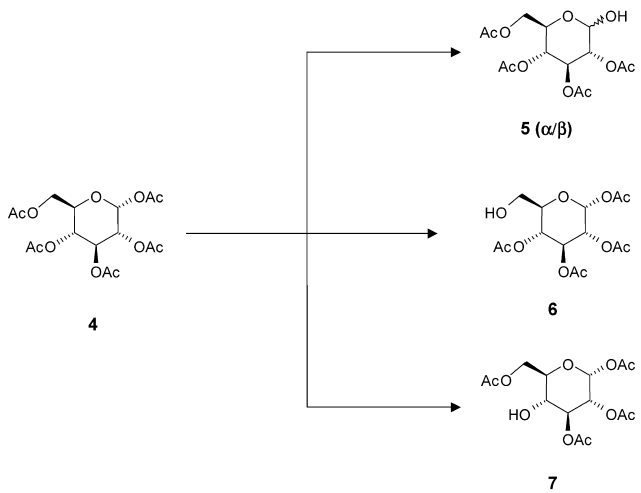
Experiments were carried out as described in Section 4.
[a] Reaction rate was expressed in IU/mL (μmol of substrate hydrolyzed per minute per mL of immobilized protein).
[b] Accumulated porcentage of isolated tetra-*O*-acetyl-α-D-glucopyranoside for 100% hydrolysis of substrate.

When the hydrolysis was catalyzed by octyl-PFL preparation, two compounds, 1,2,3-tri-*O*-acetyl-β-D-ribofuranoside **2** and 1,2,5-tri-*O*-acetyl-β-D-ribofuranoside **3**, were isolated after 30 h of reaction in 10 and 30% yield, respectively. Covalently immobilized preparation exhibited lower enzyme activity (by a 14-fold factor), but in this case an improvement on the selectivity was achieved: only product **3** in 30% yield was in fact obtained.

The influence of the immobilization strategy on the properties of CRL and PFL in the hydrolysis of the 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose **4** (Table 3) has been also investigated. In this case, the influence of acyl group migration occurring at neutral or basic pH[11] should be carefully considered. For that reason the different immobilized preparations have been studied considering the effect pH value on the regioselectivity and yields achieved. PFL and CRL allow, at acidic pH, the regio-selective hydrolysis only in position 1 and 6, respectively (Scheme 2) to obtain the corresponding APs: 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose **5** and 1,2,3,4-tetra-*O*-acetyl-α-D-glucopyranose **6**.

Using PFL lipase (Table 3), the change of substrate **1** by substrate **4** promoted an increment of the enzyme activity by a 17-fold factor using the interfacially activated lipase, and a 10-fold factor using the covalently immobilized preparation. Thus, interfacially activated lipase was almost 24 times more active than the covalently immobilized preparation. Yields of tetraacetate using the interfacially adsorbed preparation was next to 100%, while the covalently preparation gave only a 56%. In both cases the 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose **5** was the only reaction product (mixture of α and β anomers). PFL allowed

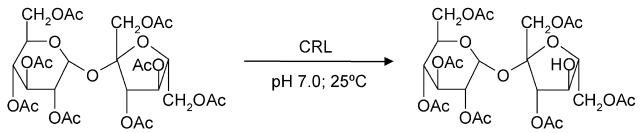
Important changes, in yield and selectivity, were observed using CRL at neutral pH. The yields of AP and the regioselectivity of the reaction decreased increasing the pH. At neutral pH, 34 and 57% yields were obtained, respectively, with the covalently attached and hydrophobically adsorbed preparations (Table 3). In these reactions the formation of a mixture (30:70) of compound **6** and 1,2,3,6-tetra-*O*-acetyl-α/β-D-glucopyranose **7** (Scheme 2) were observed. For example, in the reaction performed with CRL adsorbed on octyl agarose, compound **7** was isolated as major product in 40% yield. The decrease in yield and the change in regioselectivity observed with CRL at neutral pH are consequences of an acetyl group migration from position 4 to 6 occurring in neutral or basic medium.[11] Under these conditions, the enzyme only hydrolyzed the position 6 of the



Scheme 2.

substrate, but because of the acyl group migration, the product obtained **7** can be a new substrate to the lipase being hydrolyzed to 1,2,3-tri-*O*-acetyl-α/β-D-glucopyranose, with a consequent reduction of the overall yield.

We studied also the performance of the two immobilized preparation of CRL in the hydrolysis of 1′,3′,4′,6′,2,3,4,6-octa-*O*-acetyl sucrose **8** (Scheme 3). This substrate gave the lowest activity compared with the other two substrates considered in this work. For instance, the activity of octyl-CRL was 13-fold lower when this substrate that using compound **1**, while decreased by a 33-fold factor using the covalent preparation (Table 4). Thus, the interfacially activated preparation was more than 75-fold more active than the covalently immobilized preparation, permitting to reach a 77% of 1′,3′,6′,2,3,4,6-hepta-*O*-acetyl sucrose **9** (Scheme 3) versus the 22% obtained by using the other enzyme preparation. Both immobilized preparations gave the same product as consequence of the regio-selective hydrolysis in position 4′ (compound **9**).



8 9

Scheme 3.

Table 4. Regio-selective hydrolysis of 1′,3′,4′,6′,2,3,4,6-octa-*O*-acetyl sucrose catalyzed by different immobilized lipases

Immobilized preparation	Reaction rate[a]	Yield[b] (%)	Deacetylated sugar
Octyl	0.23	77	**9**
Glutaraldehyde	0.003	22	**9**

Experiments were carried out as described in Section 4.

[a] Reaction rate was expressed in IU/mL (μmol of substrate hydrolyzed per minute per mL of immobilized protein).

[b] Accumulated percentage of isolated product of the deacetylated sugar for 100% hydrolysis of substrate.

3. Conclusions

In this paper we have described some simple approach for preparing useful building blocks for sugar chemistry, by regio-selective hydrolysis peracetylated ribofuranoses, glucopyranoses and sucrose. Depending from the enzyme immobilized preparation and the reaction condition used, protected sugars bearing only one free hydroxy group in different position have been obtained with good yields and suitable reaction times.

These results have been achieved by an integrated approach that involves the study of lipases from different organisms with different properties (selection of the enzyme with the best properties) and following different protocols immobilization of the enzyme. In fact, the activity, selectivity and regioselectivity of lipases has been modulated by the use of different immobilization techniques. Interfacially activated lipases adsorbed on hydrophobic supports gave a very high activity with the peracetylated sugar, while activity against the product bearing only one free hydroxyl group (AP) was greatly reduced, facilitating the accumulation of the mono deacetylated sugar AP. The difference in hydrophobicity between the substrate and the product may be a key factor in the higher selectivity exhibited by the interfacially activated lipases compared to the covalent one. The very hydrophobic environment formed by the hydrophobic active center adsorbed near the surface of the hydrophobic support, could favor the entry of the most hydrophobic substrate (peracetylated sugars) compared with the partially hydrolyzed sugars, that result more hydrophilic. This yields an apparent increment in selectivity.

4. Experimental

4.1. General

The lipase from *P. fluorescens* (Lipase PS 'Amano') (PFL) was purchased from Amano Pharmaceutical Co., Ltd; (Nagoya, Japan). *C. rugosa* lipase Type VII (lot. 78H1147) (CRL) was from Sigma and the lipases from *H. lanuginosa* (Novozym 871) (HLL), *M. miehei* (Novozym 388) (MML) and *C. antarctica* A (Novozym 868) (CAL-A) and B (Novozym 525L) (CAL-B) were kindly supplied by Novo Nordisk company. *A. oryzae* lipase (No. 2326691) (AOL) was obtained from Fluka. Manae-agarose 6BCL was kindly donated by Hispanagar and prepared as previously described[31] (Burgos, Spain). The glutaraldehyde support was prepared as previously described.[29] Octyl agarose 4BCL was purchased from Pharmacia Biotech (Uppsala, Sweden). *p*-Nitrophenyl propionate (*p*NPP) and 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose and per-acetate sucrose were from Sigma Chem. Co. Other reagents and solvents used were of analytical degree.

4.2. Enzyme activity assay

The activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically measuring the increment in the absorption at 348 nm promoted by the hydrolysis of *p*NPP. The reaction mixture consisted of 0.4 mM *p*NPP in 25 mM sodium phosphate buffer at pH 7.00 and 25°C.

An activity unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPP per minute under the described conditions.

4.3. Preparation of lipase solutions

50 mg of commercial preparations were dissolved in 100 mL of 10 mM sodium phosphate buffer at pH 7.0. These solutions were centrifuged and dialyzed overnight against 50 volumes of 10 mM sodium phosphate buffer at pH 7.0 and 4°C. The lipase solutions were stored at 4°C by a maximum of 24 h. The protein concentration was measured using Bradford method.[32] The protein calibration curve was obtained using BSA.

4.4. Immobilization and purification of lipases by interfacial adsorption on octyl agarose

10 mL of octyl-agarose was added to 100 mL of the standard solution of lipase (20 mg/mL). Periodically, samples of supernatant, suspension and reference was withdrawn and analyzed by *p*NPP assay. When the adsorption was finalized, the suspension was filtered and the immobilized preparation was washed abundantly with distilled water. Under these conditions, the immobilization was very selective for lipases.[33–36]

This preparation was used as the interfacially adsorbed preparation.[28] To obtain soluble pure lipase, the enzyme was desorbed from the octyl agarose using a gradient of Triton X-100 (from 0 to 3%), achieving a yield over 80% in all cases.

4.5. Covalent immobilization of lipases on glutaraldehyde activated agarose

10 g of glutaraldehyde agarose 6BCL was suspended in 100 mL of the purified lipase. The suspensions were maintained under constant stirring during 3 h at pH 7 and 4°C. Periodically, samples of suspension and supernatant were withdrawn and analyzed using the *p*NPP assay. After immobilization, 100 mL of 0.1 M of sodium bicarbonate buffer at pH 8.5 and 4°C containing 200 mg of $NaBH_4$ was added. After 30 min, the immobilized preparation was filtered and washed with abundant distilled water.

4.6. Enzymatic hydrolysis of peracetylated sugars

4.6.1. 1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (1) or 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose (4). The title compounds were dissolved at 25°C in a 10 mL solution of 25 mM sodium phosphate buffer (85%, v/v) and acetonitrile (15%, v/v) at pH 7.0 to a substrate concentration of 10 mM and 1 g of biocatalyst were added. Using octaacetate of Sucrose (**8**), the acetonitrile concentration was increased to 20%.

The pH was maintained constant at pH 7.0 by using a pH-stat. Reactions were followed by HPLC. Finally, after filtration of the immobilized preparation, the products were isolated by flash chromatography, and the products were identified by ^{1}H NMR.

The hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose catalyzed by CRL was studied also at pH 5 in order to avoid possible influence arising from the acyl migration occurring at neutral pH.[11]

For the HPLC analysis, it was used an 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 a L-7300 column oven and UV detector SP 8450 at 215 nm. Columns for flash chromatography were made up with Silica Gel 60 (Mecrk) 60–200 or 40–63 μm. The elution was performed with 40:60 hexane–ethyl acetate. ^{1}H NMR was 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. For sucrose heptacetate identification TOXY 2D NMR homocorrelation studies were also performed.

4.6.2. 1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (1). HPLC analysis: 30% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.5 mL/min. Retention time (t_R)=13 min. ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.01–2.11 (4s, CH_3, 12H), 4.09 and 4.28 (AB part of ABX system, $J^{1,3}$=5.23, 5.22 Hz, $J^{1,2}$=11.82 Hz, 2H-5), 4.31–4.33 (m, 1H-4), 5.27–5.32 (m, 1H-3, 1H-2), 6.10 (s, 1H-1).

4.6.3. 1,2,3-Tri-*O*-acetyl-β-D-ribofuranose (2). HPLC analysis: 18% acetonitrile in ammonium phosphate buffer (10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=11 min, ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.00–2.20 (3s, CH_3, 9H), 3.63 and 3.83 (AB part of ABX system, $J^{1,3}$=4.1, 3.2 Hz, $J^{1,2}$=12.5 Hz, 2H-5), 4.15–4.30 (m, 1H-4), 5.38 (dd, *J*=4.9, 20.5 Hz, 1H-3), 5.36 (d, *J*=4.9 Hz, 1H-2), 6.13 (s, 1H-1).

4.6.4. 1,2,5-Tri-*O*-acetyl-β-D-ribofuranose (3). HPLC analysis: 18% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=15 min. ^{1}H NMR in $CDCl_3$ (δ, ppm):2.00–2.10 (3s, CH_3, 9H), 4.08 and 4.35 (AB part of ABX system, $J^{1,3}$=5.6, 3.6 Hz, $J^{1,2}$=12.2 Hz, 2H-5), 4.35–4.40 (m, 1H-4), 5.03 (dd, *J*=3.8, 16.3 Hz, 1H-3), 5.30 (d, *J*=3.8 Hz, 1H-2), 6.03 (s, 1H-1).

4.6.5. 1,2,3,4,6-Penta-*O*-acetyl-α-D-glucopyranose (4). HPLC analysis: 30% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.5 mL/min. t_R=16 min. ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.00–2.25 (5s, CH_3, 15H), 4.10 (m, ABX system, 1H-5), 4.10 and 4.26 (AB part of ABX system, $J^{1,3}$=6.0, 3.0 Hz, $J^{1,2}$=12.5 Hz, 2H-6), 5.10 (dd, *J*=9.4, 4.0 Hz, 1H-2), 5.15 (t, *J*=9.5 Hz, 1H-4), 5.45 (t, *J*=9.4 Hz, 1H-3), 6.32 (d, *J*=4.0 Hz, 1H-1).

4.6.6. 2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranose (5). HPLC analysis: 20% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=18 min. (α-isomer 70%) and t_R=19.5 min (β-isomer 30%). ^{1}H NMR (α/β anomers 3:1) in $CDCl_3$ (δ, ppm): 1.90–2.20 (4s, CH_3 α/β anomers, 12H) 3.71 (m, ABX system, 1H-5 β anomer), 3.95–4.25 (m, 1H-5 α anomer, 2H-6 α?β anomers) 4.70 (d, *J*=8.0 Hz, 1H-1 β anomer), 4.83 (dd, *J*=3.6, 10.1 Hz, 1H-2 α anomers), 4.86 (dd, *J*=8.1, 9.7 Hz, 1H-2 β anomers), 5.03 (t, *J*=9.7 Hz, 1H-4 α/β anomers), 5.18 (t, *J*=9.6 Hz, 1H-3 β anomer), 5.38 (d, *J*=3.5 Hz, 1H-1 α-anomer), 5.47 (t, *J*=9.7 Hz, 1H-3 α anomer).

4.6.7. 1,2,3,4-Tetra-*O*-acetyl-α-D-glucopiyranose (6). HPLC analysis: 20% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=8.9 min. Identification by ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.00–2.25 (4s, CH_3, 12H), 3.61–3.75 (AB part of ABX system, $J^{1,3}$=4.2, 2.4 Hz, $J^{1,2}$=12.9 Hz, 2H-6), 3.96 (m, ABX system, 1H-5), 5.10 (dd, *J*=3.7, 9.9 Hz, 1H-2), 5.14 (t, *J*=9.9 Hz, 1H-4) 5.56 (t, *J*=9.9 Hz, 1H-3), 6.38 (d, *J*=3.7 Hz, 1H-1).

4.6.8. 1,2,3,6-Tetra-*O*-acetyl-α-D-glucopiyranose (7). HPLC analysis: 20% acetonitrile in phosphate buffer

(10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=8.5 min. ^{1}H NMR in $CDCl_3$ (δ, ppm): 1.92–2.14 (4s, CH_3, 12H), 3.58 (t, J=9.3 Hz, 1H-4), 3.91 (m, ABX system, 1H-5), 4.21 and 4.41 (AB part of ABX system, $J^{1,3}$=4.0, 2.1 Hz, $J^{1,2}$=12.6 Hz, 2H-6), 4.96 (dd, J=10.2, 3.7 Hz, 1H-2), 5.28 (t, J=9.8 Hz, 1H-3) 6.23 (d, J=3.5 Hz, 1H-1).

4.6.9. 1′,3′,4′,6′,2,3,4,6-Octa-*O*-acetyl sucrose (8). HPLC analysis: 50% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.0 mL/min. t_R=11 min. ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.00–2.30 (8s, CH_3, 21H), 4.15–4.45 (m, 2H-1′AB, 1H-5′ ABX, 2H-6′AB, 1H-5 ABX, 2H-6AB), 4.92 (dd, J=10.3, 3.7 Hz, 1H-2), 5.11 (t, J=10.0 Hz, 1H-4), 5.41 (t, J=5.8 Hz, 1H-4′), 5.47 (t, J=9.8 Hz, 1H-3), 5.49 (d, J=5.8 Hz, 1H-3′), 5.70 (d, J=3.9 Hz, 1H-1).

4.6.10. 1′,3′,6′,2,3,4,6-Hepta-*O*-acetyl sucrose (9). HPLC analysis: 30% acetonitrile in phosphate buffer (10 mM) pH 3.0, flow rate 1.5 mL/min. t_R=19 min. ^{1}H NMR in $CDCl_3$ (δ, ppm): 2.00–2.30 (7s, CH_3, 21H), 3.25 (s, OH, 1H), 4.00–4.34 (m, 2H-1′AB, 1H-5′ABX, 2H-6′AB, 1H-5 ABX, 2H-6AB), 4.35 (t, J=7.8 Hz, 1H-4′), 4.92 (dd, J=10.4, 3.8 Hz, 1H-2), 5.06 (t, J=10.1 Hz, 1H-4), 5.26 (d, J=7.8 Hz, 1H-3′), 5.45 (t, J=9.8 Hz, 1H-3), 5.70 (d, J=3.7 Hz, 1H-1).

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References

1. (a) Koto, S.; Morishima, N.; Kihara, Y.; Suziki, H.; Kosugi, S.; Zen, S. *Bull. Chem. Soc. Jpn* **1979**, *56*, 188–191. (b) Koto, S.; Morishima, N.; Zen, S. *Bull. Chem. Soc. Jpn* **1979**, *52*, 784–787. (c) Leroux, F.; Perlin, A. S. *Carbohydr. Res.* **1978**, *67*, 163–167.
2. Kirk, O.; Christensen, M. W.; Beck, F.; Damhus, T. *Biocatal. Biotransform.* **1995**, *12*, 91–97.
3. Allen, P. Z. *Meth. Carbohydr. Chem.* **1962**, *1*, 372.
4. Rowell, R. M.; Feather, M. S. *Carbohydr. Res.* **1967**, *4*, 486.
5. Excoffier, G.; Gagnaire, D.; Utille, J.-P. *Carbohydr. Res.* **1975**, *39*, 368.
6. Watanabe, K.; Ithoh, K.; Araki, K.; Ishido, Y. *Carbohydr. Res.* **1986**, *154*, 165.
7. Khan, R.; Konowicz, P. A.; Gardossi, L.; Matulova, M.; de Genaro, S. *Aust. J. Chem.* **1996**, *49*, 393.
8. Hall, D. M.; Lawler, T. B.; Childress, B. C. *Carbohydr. Res.* **1974**, *38*, 359–363.
9. Koppen, B. H. *Carbohydr. Res.* **1972**, *24*, 154–158.
10. Bastida, A.; Fernández-Lafuente, R.; Fernández-Lorente, G.; Guisan, J. M.; Pagani, G.; Terreni, M. *Biorg. Med. Lett.* **1999**, *9*, 633–636.
11. Terreni, M.; Salvatti, R.; Linati, L.; Fernández-Lafuente, R.; Fernández-Lorente, G.; Bastida, A.; Guisan, J. M. *Carbohydr. Res.* **2002**, *337*, 1615–1621.
12. Shaw, J. F.; Klibanov, A. M. *Biotechnol. Bioengng* **1987**, *29*, 648–652.
13. Hennen, W. J.; Sweers, H. M.; Wang, Y. F.; Wong, C. H. *J. Org. Chem.* **1988**, *53*, 4939–4945.
14. Khan, R.; Groppen, L.; Konpwicz, P. A.; Matulová, M.; Paoletti, S. *Tetrahedron Lett.* **1993**, *34*, 7767.
15. Sarda, L.; Desnuelle, P. *Biochim. Biophys. Acta* **1958**, *30*, 513–521.
16. Brzozowski, A. M.; Derewenda, U.; Derewenda, Z. S.; Dodson, G. G.; Lawson, D. M.; Turkemburg, J. P.; Bjorkling, F.; Huge-Jensen, B.; Patkar, S. S.; Thim, L. *Nature* **1991**, *351*, 491–494.
17. Brady, L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J. P.; Christiansen, L.; Huge-Jensen, B.; Norskov, L.; Thim, L.; Menge, U. *Nature* **1990**, *343*, 767–770.
18. Bornscheuer, U.; Reif, O.-W.; Lausch, R.; Freitag, R.; Scheper, T.; Kolisis, F. N.; Menge, U. *Biochim. Biophys. Acta* **1994**, *1201*, 55–60.
19. Derewenda, Z. S. *Struct. Biol.* **1995**, *2*, 347–349.
20. Noble, M. E. M.; Cleasby, A.; Johnson, L. N.; Egmond, M. R.; Frenken, L. G. J. *FEBS Lett.* **1993**, *331*, 1265–1269.
21. Winkler, F. K.; D'Arcy, A.; Hunziker, W. *Nature* **1990**, *343*, 771–774.
22. Palomo, J. M.; Fernandez-Lorente, G.; Mateo, C.; Ortiz, C.; Fernandez-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2002**, *31*, 775–783.
23. Fernández-Lorente, G.; Terreni, M.; Mateo, C.; Bastida, A.; Fernández-Lafuente, R.; Dalmases, P.; Huguet, J.; Guisán, J. M. *Enzyme Microb. Technol.* **2001**, *28*, 389–396.
24. Palomo, J. M.; Fernández-Lorente, G.; Mateo, C.; Fuentes, M.; Guisan, J. M.; Fernández-Lafuente, R. *Tetrahedron: Asymmetry* **2002**, *13*, 2653–2659.
25. Palomo, J. M.; Muñoz, G.; Fernandez-Lorente, G.; Mateo, C.; Fuentes, M.; Guisan, J. M.; Fernandez-Lafuente, R. *J. Mol. Catal. B: Enzym.* **2003**, *21*, 201–210.
26. Palomo, J. M.; Fuentes, M.; Fernández-Lorente, G.; Mateo, C.; Guisan, J. M.; Fernández-Lafuente, R. *Biomacromolecules* **2003**, *4*, 1–6.
27. Fernández-Lorente, G.; Palomo, J. M.; Fuentes, M.; Mateo, C.; Guisan, J. M.; Fernández-Lafuente, R. *Biotechnol. Biengng* **2003**, *82*, 232–237.
28. Bastida, A.; Sabuquillo, P.; Armisen, P.; Fernández-Lafuente, R.; Huguet, J.; Guisan, J. M. *Biotechnol. Bioengng* **1998**, *58*, 486–493.
29. Fernández-Lafuente, R.; Rodríguez, V.; Santana, C.; Soler, G.; Bastida, A.; Guisán, J. M. *Enzyme Microb. Technol.* **1998**, *10*, 375–382.
30. Fernández-Lafuente, R.; Armisen, P.; Sabuquillo, P.; Fernández-Lorente, G.; Guisán, J. M. *Chem. Phys. Lipids* **1998**, *93*, 185–197.
31. Fernández-Lafuente, R.; Rosell, C. M.; Rodríguez, V.; Guisán, J. M. *Enzyme Microb. Technol.* **1993**, *15*, 550–556.
32. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
33. Sabuquillo, P.; Reina, J.; Fernández-Lorente, G.; Guisán, J. M. Fernández-Lafuente. *Biochem. Biophys. Acta* **1988**, 337–348.

34. Palomo, J. M.; Muñoz, G.; Fernández-Lorente, G.; Mateo, C.; Fuentes, M.; Fernández-Lafuente, R.; Guisán, J. M. *J. Mol. Catal. B: Enzym.* **2002**, *19–20*, 279–286.
35. Palomo, J. M.; Fernandez-Lorente, G.; Mateo, C.; Fuentes, M.; Fernandez-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2002**, *31*, 775–783.
36. Sugiura, M.; Isobe, M. *Chem. Pharm. Bull.* **1976**, *24*, 72–78.