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Enzymatic synthesis of *N*-acetylglucosaminobioses by reverse hydrolysis: characterisation and application of the library of fungal β-*N*-acetylhexosaminidases☆

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Abstract

The regioselectivity of 20 extracellular β-*N*-acetylhexosaminidases of fungal origin was screened in the reverse hydrolysis with 2-acetamido-2-deoxy-D-glucopyranose. Most of the enzymes used yielded 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose (**3**) and 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-glucopyranose (**4**). So far unknown product of enzymatic condensation, 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 3)-2-acetamido-2-deoxy-D-glucopyranose (**2**) was synthesised using the β-*N*-acetylhexosaminidases from *Penicillium funiculosum* CCF 1994, *P. funiculosum* CCF 2325 and *Aspergillus tamarii* CCF 1665. Addition of salts ($(NH_4)_2SO_4$ or $MgSO_4$ (0.1–1.0 M)) to the reaction increased the yields and also enhanced the β-*N*-acetylhexosaminidase regioselectivity.

Keywords: Reverse hydrolysis; Enzymatic synthesis; β-*N*-Acetylhexosaminidase; *N*,*N*′-Diacetylchitobiose; Filamentous fungi; 2-Acetamido-2-deoxy-D-glucopyranose

1. Introduction

Glycosidases, cheap, robust and relatively well-available enzymes, are often used for glycoside synthesis. They are characteristic by strict stereoselectivity but often poor regioselectivity that is a serious drawback hampering their practical application. Regioselectivity can be achieved either by a rational choice of enzymes, e.g. from enzyme libraries composed of fully characterised enzymes [1–9] or by various tricks, including the use of selectively protected acceptors [10,11] or modification of the reaction conditions [12–17]. Thus, the decisive parameter of glycosidase regioselectivity is its source [18–21]. Therefore, detailed knowledge of regioselectivity and other parameters (yields, etc.) of a large number of enzymes is of utmost importance for the choice of effective synthetic tools in carbohydrate chemistry.

The possibility of replacement of rather expensive *p*-nitrophenyl glycosides (e.g. *p*NP-β-GlcNAc) by cheaper substrates such as 2-acetamido-2-deoxy-D-glucopyranose brings numerous advantages, namely lower price and less complex reaction mixtures free from *p*-nitrophenol and *p*NP-β-GlcNAc autocondensation products (*p*-nitrophenyl oligoglycosides). Reverse hydrolysis catalysed by β-*N*-acetylhexosaminidase (EC 3.2.1.52) was first demonstrated by our group [22]. Here, we demonstrate the enzymatic synthesis of all possible regioisomers of *N*,*N*′-diacetylchitobiose and the synthetic characterisation of a large library of extracellular fungal β-*N*-acetylhexosaminidases.

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2. Experimental

2.1. Materials

2-Acetamido-2-deoxy-D-glucopyranose and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside were purchased from Senn Corp., Switzerland, Toyopearl HW-40F (100 Da–10 kDa) was obtained from Tosoh Corp., Japan.

All β-*N*-acetylhexosaminidases used in this work originated from the Library of glycosidases of the Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic in Prague, and were prepared by cultivation of the respective fungi as described previously [23–25]. Briefly, respective microoganisms were grown in shaken liquid cultures in the media with chitin hydrolysates as inductors (2 g/l), typically for 10 days at 28 °C. The enzymes were partially purified from the culture supernatant by fractional precipitation with $(NH_4)_2SO_4$ (80% saturation). β-*N*-Acetylglucosaminidase activity was assayed using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. One unit of β-*N*-acetylhexosaminidase activity was defined as the amount releasing 1 μmol of *p*-nitrophenol per minute at pH 5.0 and 35 °C [24]. The producing strains are deposited with the Czech Collection of Fungi (CCF) at the Department of Botany, Charles University in Prague, Czech Republic.

2.2. Analytical HPLC

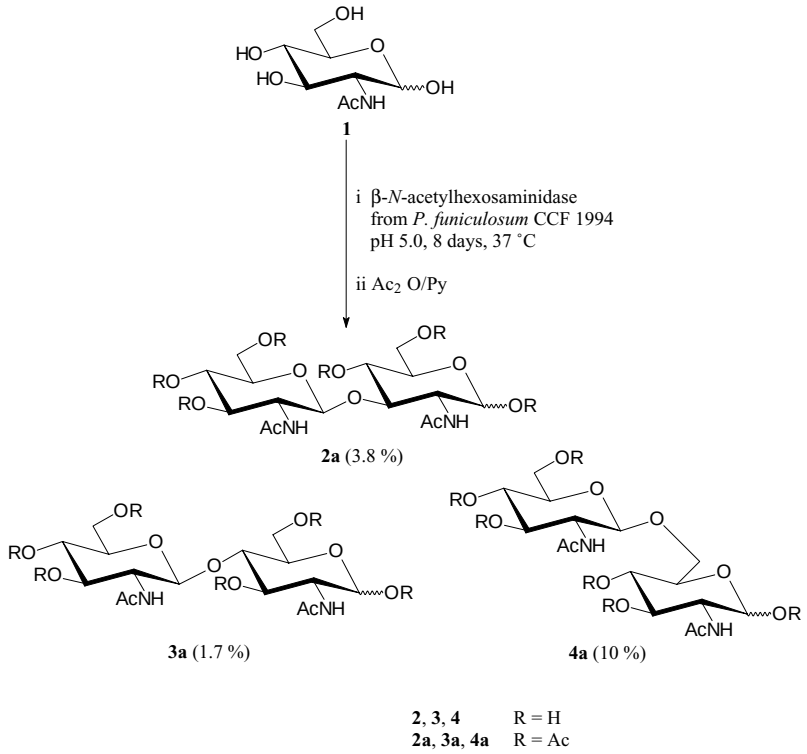
Analytical HPLC was carried out on a modular system (Spectra Physics, USA) consisting of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV-Vis detector. The analyses were performed on a Lichrospher 100-5 NH_2 column (250 mm × 4 mm, Watrex, Czech Republic); mobile phase acetonitrile–water 79:21, flow rate 0.6 ml/min at 24 °C, UV detection at 200 nm; retention times: β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**, 19.26 min), β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc (**3**, 16.40 min), β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4**, 19.76 min) and for GlcNAc (**1**, 9.56 min). Compounds **2**, **3** and **4** were obtained after Zemplén deacetylation of the respective acetates **2a**, **3a** and **4a** (Scheme 1). For the identification of these isomers, the authentic standards **2** [26], **3** [27] and **4** [22] were used (co-chromatography).

2.3. TLC

TLC was carried out on silica gel plates (Kieselgel 60 F_{254}, Merck) with mobile phase A (propan-2-ol/water/ammonia = 7/2/1, v/v/v), twice developed. Compounds were visualised by charring with 5% H_2SO_4 in ethanol. The condensation products **2** ($R_f = 0.58$), **3** ($R_f = 0.67$), **4** ($R_f = 0.54$) were compared (co-chromatography) with authentic standards **2** [26], **3** [27], **4** [22] and starting GlcNAc (**1**, $R_f = 0.79$).

2.4. NMR and mass spectroscopy

NMR spectra of **2a** were measured on a Varian ^{UNITY}*Inova*-400 spectrometer (399.88 and 100.55 MHz, respectively) in $CDCl_3$ at 303 K. Residual signal of $CDCl_3$



Scheme 1.

was used as an internal standard ($\delta_H = 7.265$, $\delta_C = 77.00$). ^{1}H NMR, COSY, TOCSY, and HMQC spectra were measured using standard manufacturers' software (Varian Inc., Palo Alto, USA). Selective 1D-TOCSY was measured with sequence published by Uhrín and Barlow [28]. Protons were assigned by COSY and TOCSY and the assignment was transferred to carbons by HMQC. Chemical shifts are given in δ-scale (ppm), and coupling constants in Hz. Digital resolution allowed us to report chemical shifts of protons to three and coupling constants to one decimal place. Carbon chemical shifts were read out from HMQC (protonated carbons) and are reported to one decimal place.

2.5. Condensation reactions on an analytical scale

To test the synthetic ability of β-*N*-acetylhexosaminidases for β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**) formation by reverse hydrolysis, condensation reactions with 2-acetamido-2-deoxy-D-glucopyranose were screened. Screening system composed of 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc, **1**, 2–16 mg, 0.2–1.6 M) in citrate–phosphate buffer (0.1 M, 45 μl; pH 5.0) using respective β-*N*-acetylhexosaminidase (2–4 U) incubated for 8 days at 37 °C. Reactions were monitored by TLC, the formation of β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**) (depending on the enzyme source) was observed only at a substrate (**1**) concentration of 0.6–1.2 M. Optimum GlcNAc concentration (1.0 M) was employed for preparative procedures. Respective blank reactions void of enzyme were run to exclude the possibility of a non-enzymatic GlcNAc condensation—no spontaneous autocondensation was observed.

2.6. Condensation reactions on a preparative scale

Analogously to Section 2.5, 2-acetamido-2-deoxy-D-glucopyranose (**1**, 1.0 M) was treated with a series of 20 fungal β-*N*-acetylhexosaminidases (4–10 U) from the genera of *Acremonium*, *Aspergillus*, *Penicillium*, *Talaromyces* and *Trichoderma* (Table 1). All reactions were carried out using the same procedures as described in Section 2.7.

2.7. *2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 3)-2-acetamido-2-deoxy-D-glucopyranose [β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc, **2**], 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose [β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc, **3**] and 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-glucopyranose [β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc, **4**]*

Substrate (**1**, 114 mg, 0.516 mmol) was dissolved in citrate–phosphate buffer (0.1 M, 500 μl; pH 5.0) and the β-*N*-acetylhexosaminidase from *Penicillium funiculosum* CCF 1994 (3 U) was added. After 2 days another 5 U of the enzyme were added. The reaction mixture, monitored by TLC, was incubated for 8 days at 37 °C. Then the enzyme was deactivated by heating (10 min, 100 °C). The mixture was centrifuged and the supernatant was loaded on a Toyopearl HW-40F column (900 mm × 26 mm, flow rate 17 ml/h) and eluted with water. Fractions (ca. 2.5 ml) containing disaccharides were collected and lyophilised. This fraction (21.5 mg) was peracetylated (Ac_2O/Py, 30 h, 24 °C) and separated by silica gel flash chromatography ($CHCl_3$:MeOH = 97:3) yielding **2a** (6.6 mg, 3.8%), **3a** (3.0 mg, 1.7%) and **4a** (17.5 mg, 10%) (Scheme 1). The 1 → 3 linkage of peracetate **2a** was inferred from chemical shifts of H-3 showing that this position is not acetylated and downfield resonating C-3 (with respect to parent GlcNAc), experiencing a glycosylation shift. NMR and MS data were identical with the data published previously **3a** [27] and **4a** [22].

Table 1
Regioselectivity of fungal β-*N*-acetylhexosaminidases-production of *N*-acetylglucosaminobioses

Source of enzyme	Condensation products[a] (isolated yields %)		
	β(1 → 3)	β(1 → 4)	β(1 → 6)
Acremonium persicinum CCF 1850	–	–	7.4
Aspergillus flavipes CCF 2026	–	–	3.2
A. flavofurcatis CCF 3061	–	8.4	–
A. flavus CCF 1129	–	–	4.0
A. fumigatus CCF 1059	–	3.2	5.1
A. niveus CCF 3057	–	–	–
A. oryzae CCF 147	–	–	2.3
A. oryzae CCF 1066	–	–	2.1
A. sojae CCF 3060	–	2.2	1.2
A. tamarii CCF 1665	1.6	–	5.2
A. terreus CCF 2539	–	–	2.2
Penicillium brasilianum CCF 2155	–	–	–
***P. funiculosum* CCF 1994**[b]	**3.8**	1.7	10.0
P. funiculosum CCF 2325	2.0	1.5	7.3
P. chrysogenum CCF 1269	<1.5	1.6	3.1
P. multicolor CCF 2244	–	–	<1.5
P. oxalicum CCF 2315	–	–	2.4
P. pittii CCF 2277	–	2.0	2.8
Talaromyces flavus CCF 2686	–	–	–
Trichoderma harzianum CCF 2687	<1.5	3.3	4.6

[a] Yields (%) of condensation reactions with GlcNAc (**1**), the respective β-*N*-acetylhexosaminidase (8 U), GlcNAc (1.0 M), 0.1 M citrate–phosphate buffer (pH 5.0), 8 days, 37 °C—the yields of the respective peracetates **2a**, **3a**, **4a** after reaction mixture peracetylation and separation.

[b] The maximum yield of product β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2a**).

2.7.1. ***2a*** *(α:β = 64:36)*

2a *α-anomer*: ^{1}H NMR (399.88 MHz, $CDCl_3$, 303 K) 1.937, 2.008, 2.018, 2.050, 2.065, 2.071, 2.079, 2.200 (24H, s, Ac), 3.165 (1H, ddd, J = 7.2, 7.9, 10.4 Hz, H-2′), 3.774 (1H, ddd, J = 2.5, 4.1, 10.0 Hz, H-5′), 3.953 (1H, dd, J = 9.1, 10.5 Hz, H-3), 3.996 (1H, ddd, J = 2.4, 4.3, 10.2 Hz, H-5), 4.053 (1H, dd, J = 2.5, 12.4 Hz, H-6′a), 4.077 (1H, dd, J = 2.4, 12.4 Hz, H-6a), 4.247 (1H, dd, J = 4.3, 12.4 Hz,

H-6b), 4.445 (1H, dd, $J = 4.1$, 12.4 Hz, H-6′b), 4.480 (1H, ddd, $J = 3.7$, 9.2, 10.5 Hz, H-2), 5.003 (1H, dd, $J = 9.2$, 10.0 Hz, H-4′), 5.026 (1H, dd, $J = 9.1$, 10.2 Hz, H-4), 5.201 (1H, d, $J = 7.9$ Hz, H-1′), 5.586 (1H, d, $J = 9.2$ Hz, 2-NH), 5.649 (1H, dd, $J = 9.2$, 10.4 Hz, H-3′), 5.792 (1H, d, $J = 7.2$ Hz, 2′-NH), 6.108 (1H, d, $J = 3.7$ Hz, H-1); ^{13}C NMR (100.55 MHz, $CDCl_3$, 303 K) 51.4 (C-2), 56.7 (C-2′), 61.6 (C-6), 61.8 (C-6′), 67.8, 68.7 (C-4, C-4′), 69.6 (C-5), 70.7 (C-3′), 71.4 (C-5′), 75.5 (C-3), 91.0 (C-1), 98.7 (C-1′).

2a *β-anomer*: ^{1}H NMR (399.88 MHz, $CDCl_3$, 303 K) 1.946, 2.012, 2.018, 2.029, 2.061, 2.086, 2.116 (24H, s, Ac), 3.394 (1H, ddd, $J = 7.6$, 8.1, 10.5 Hz, H-2′), 3.710 (1H, ddd, $J = 2.6$, 4.5, 9.9 Hz, H-5′), 3.839 (1H, ddd, $J = 2.3$, 4.9, 9.8 Hz, H-5), 3.840 (1H, ddd, $J = 8.3$, 8.4, 9.7 Hz, H-2), 4.118 (1H, dd, $J = 2.6$, 12.5 Hz, H-6′a), 4.127 (1H, dd, $J = 2.3$, 12.4 Hz, H-6a), 4.237 (1H, dd, $J = 8.8$, 9.7 Hz, H-3), 4.284 (1H, dd, $J = 4.9$, 12.4 Hz, H-6b), 4.334 (1H, dd, $J = 4.5$, 12.5 Hz, H-6′b), 5.001 (1H, dd, $J = 8.8$, 9.8 Hz, H-4), 5.008 (1H, dd, $J = 9.2$, 9.9 Hz, H-4′), 5.091 (1H, d, $J = 8.1$ Hz, H-1′), 5.470 (1H, dd, $J = 9.2$, 10.5 Hz, H-3′), 5.792 (1H, d, $J = 7.6$ Hz, 2′-NH), 5.835 (1H, d, $J = 8.4$ Hz, 2-NH), 5.958 (1H, d, $J = 8.3$ Hz, H-1); ^{13}C NMR (100.55 MHz, $CDCl_3$, 303 K) 55.3 (C-2), 55.9 (C-2′), 61.7 (C-6, C-6′), 67.8, 68.7 (C-4, C-4′), 71.5 (C-3′), 71.7 (C-5′), 72.4 (C-5), 76.8 (C-3), 91.8 (C-1), 98.7 (C-1′); MALDI-TOF MS: *m/z* $[M + Na]^+$ $C_{28}H_{40}N_2O_{17}$ calculated 676.23; measured 699.40.

3. Results and discussion

β-*N*-Acetylhexosaminidases cleave (and also transglycosylate) both β-GlcNAc and β-GalNAc moieties [23]. However, reports on the reverse hydrolysis with this type of enzymes are rather scarce [10,22] especially because of low yields (<1%) and due to a strong feedback inhibition of β-*N*-acetylhexosaminidase by 2-acetamido-2-deoxy-D-glucopyranose (**1**) (the β-*N*-acetylhexosaminidase from *A. oryzae*, $K_i = 1.6$ mM).

Twenty extracellular β-*N*-acetylhexosaminidases of fungal origin were prepared [24,25] and screened for the regioselectivity by the reverse hydrolysis with 2-acetamido-2-deoxy-D-glucopyranose (**1**, maximum solubility ca 1.7 M) [22] as a substrate. Almost all enzymes exhibited a rather high selectivity for β(1 → 6) bond formation (Table 1, Scheme 1) at a GlcNAc concentration of 0.3–1.4 M.

The three β-*N*-acetylhexosaminidases from *Aspergillus tamarii* CCF 1665, *P. funiculosum* CCF 2325 and *P. funiculosum* CCF 1994 produced relatively a high proportion of a rare chitobiose isomer β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**, yields approx. 5%). To our knowledge, the formation of β(1 → 3) linkage by β-*N*-acetylhexosaminidases has not been described yet. Recently, the compound **2** has been prepared by chemical synthesis [26].

The β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994 as the enzyme with the most pronounced formation of

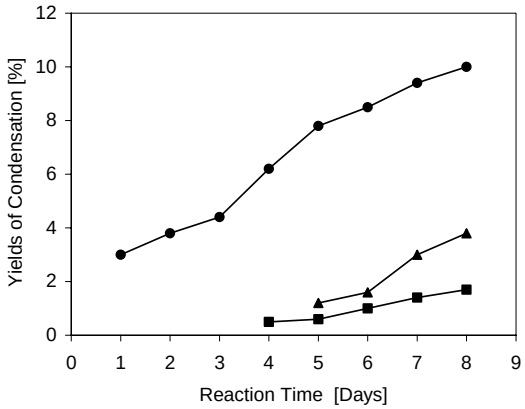


Fig. 1. Time profile of the formation of chitobiose isomers in the reaction catalysed by the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994, (▲) β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**), (■) β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc (**3**), (●) β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4**).

2 was further examined regarding the following criteria: the influence of reaction time and GlcNAc concentration on the respective isomer (**2**, **3**, **4**) production.

In the course of the first 3 days of the reaction (from the total reaction time 8 days-maximum yield of isomer **2**), exclusively β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4**) was formed. During the next 5 days another isomers β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc (**3**) and β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**) (from the fourth day for **3** and the fifth day for **2**) were produced (Fig. 1).

Fig. 2 shows the effect of substrate (**1**) concentration on the preference of the isomer formation. Doubling GlcNAc concentration (0.6–1.0 M), the formation of **2** was tripled (1.3–4.0%). A further increase of the substrate concentration caused the decrease of the product formation, probably due to the enzyme inhibition by substrate.

We have previously observed that the addition of some inorganic salts or organic solvents could stimulate the

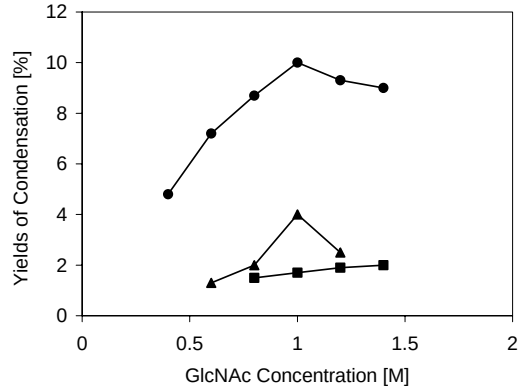


Fig. 2. Influence of GlcNAc concentration on the isomer yields in reaction catalysed by the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994, (▲) β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**), (■) β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc (**3**), (●) β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4**).

Table 2
Influence of salts addition on the isomer production in reaction catalysed using the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994

Salt added	Salt concentration (M)	Isolated yields[a] (%)	
		β(1 → 3)[b]	β(1 → 6)[c]
None (control)	–	3.8	10.0
$(NH_4)_2SO_4$	0.1	4.3	11.2
$(NH_4)_2SO_4$	0.5	–	12.5
$(NH_4)_2SO_4$	1.0	–	14.0
$MgSO_4$	0.1	–	10.5
$MgSO_4$	0.5	–	11.7
$MgSO_4$	1.0	–	13.2

[a] Conversion of GlcNAc (**1**, 1.0 M) into *N*-acetylglucosaminobioses (two isomers—**2**, **4**), the respective salt ($(NH_4)_2SO_4$, $MgSO_4$) concentration, the β-*N*-acetylhexosaminidase (8 U), 0.1 M citrate–phosphate buffer (pH 5.0), 37 °C, 8 days. The yields of the respective peracetates **2a** and **4a** after reaction mixture peracetylation and separation.
[b] β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2a**).
[c] β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4a**).

β-*N*-acetylhexosaminidase activity and shift the equilibrium in favour of product formation [20,22,23]. Therefore, the influence of inorganic salts ($(NH_4)_2SO_4$ and $MgSO_4$) on the product formation was studied as well (Table 2).

The above inorganic salts stimulated strongly the β(1 → 6) isomer production. The isomer **2** was formed only at low $(NH_4)_2SO_4$ concentration (0.1–0.2 M). The formation of product **3** was totally suppressed by the inorganic salt addition.

Although products **2**, **3** and **4** were enzymatically synthesised in a single step and the starting material GlcNAc (**1**) can easily be recovered from the reaction mixture after gel filtration, the separation of respective isomers was not trivial. The course of the GlcNAc condensation could not be monitored by HPLC due to the similar retention time values of isomers (19.26 min for **2** and 19.76 min for **4**). Therefore, the formation of β(1 → 3), β(1 → 4), β(1 → 6) linkages (depending on the enzyme source) could be monitored easily by TLC that gave a clear resolution ($R_f = 0.58$ for **2**, $R_f = 0.67$ for **3** and $R_f = 0.54$ for **4** with mobile phase A). The compounds can be separated by silica gel chromatography in their peracetylated forms.

The β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 displayed a selective preference for the β(1 → 4) product formation (**3**) out of β-*N*-acetylhexosaminidases tested (Table 1).

4. Conclusions

The regioselectivity of a series of fungal β-*N*-acetylhexosaminidases in the reverse hydrolysis mode was investigated. The required regioselectivity can be achieved by a rational choice of a specific enzyme from the library of glycosidases possessing a large variability.

Disaccharides β-D-Glc*p*NAc-(1 → 3)-D-Glc*p*NAc (**2**), β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*NAc (**3**) and β-D-Glc*p*NAc-(1 → 6)-D-Glc*p*NAc (**4**) were synthesised by condensation of 2-acetamido-2-deoxy-D-glucopyranose (**1**) using respective β-*N*-acetylhexosaminidases.

It was also demonstrated that addition of salts $(NH_4)_2SO_4$ or $MgSO_4$, could affect the β-*N*-acetylhexosaminidase regioselectivity and enhanced or modulated the yields of the respective isomers.

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