Glycosidase-catalysed oligosaccharide synthesis of di-, tri- and tetra-saccharides using the *N*-acetylhexosaminidase from *Aspergillus oryzae* and the β-galactosidase from *Bacillus circulans*

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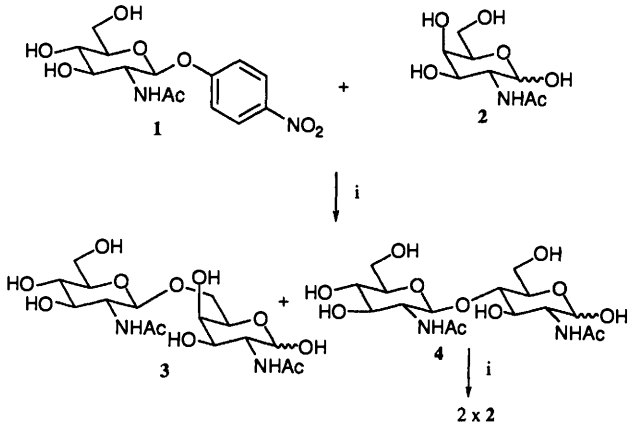
The *N*-acetylhexosaminidase from *Aspergillus oryzae* catalyse transfer of *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl residues selectively on to the 6-OH group of *N*-acetylgalactosamine to give the corresponding (1→6)-linked disaccharides 3 and 6 in 26 and 38% yield, respectively. The disaccharide β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc 3 thus synthesized acts as acceptor for transfer of a β-D-galactosyl residue from the corresponding *p*-nitrophenyl glycoside on to the 4-OH group of the non-reducing unit to give the ovarian cyst fluid mucin fragment β-D-Gal*p*-(1→4)-β-Glc*p*NAc-(1→6)-D-Gal*p*NAc 8 in 48% yield together with the tetrasaccharide β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal*p*NAc 9 in 7% yield. With lactose as acceptor, the trisaccharide β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p* 12, a growth factor for human intestinal bifidobacteria, is produced in 52% yield.

Introduction

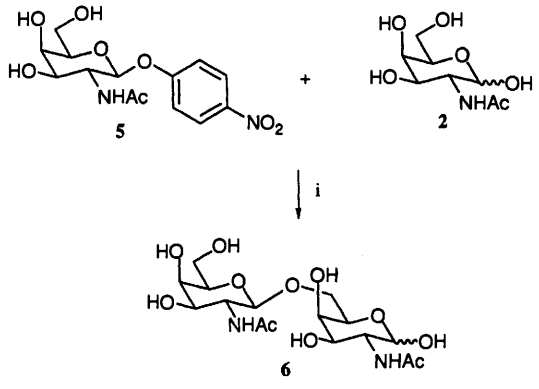
The complexities of oligosaccharide synthesis by conventional procedures have stimulated much interest in enzymic methods. The use of glycosidases provides an attractive route, since protection–deprotection sequences are avoided and complete control is exerted over newly generated anomeric centres by the specificity of the particular glycosidase used. In addition, the use of *exo*-glycosidases guarantees that transfer from suitable glycosidic donors is directed exclusively to the non-reducing terminal monosaccharide component of the acceptor. Although transfer can occur to more than one hydroxy group of this terminal unit, in practice, transfer proves to be highly selective; rarely are more than two products formed in significant amounts. In addition, experience to date indicates that as the acceptor gets bigger (monosaccharide ⟶ disaccharide ⟶ trisaccharide, *etc.*), it is bound at the active site in a conformation that increases selectivity of transfer with the result that a single product greatly predominates, to the extent that frequently one transfer product only can be detected.

In our hands, the *N*-acetylhexosaminidase from *Aspergillus oryzae* has proved to be an effective biocatalyst for transfer of *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl residues onto D-glucose and *N*-acetyl-D-glucosamine, using readily synthesized *p*-nitrophenyl glycosides as donors.[1] We also observed that D-galactose can act as acceptor[2] and now report similar findings with respect to *N*-acetyl-D-galactosamine as acceptor. Thus with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **1** (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide) as donor and 2-acetamido-2-deoxy-D-galactopyranose (*N*-acetyl-D-galactosamine) **2** as acceptor, the disaccharide β-D-Glc*p*NAc-(1→6)-D-Gal*p*NAc **3** was produced (Scheme 1). The product was contaminated by 4% of di-*N*-acetylchitobiose **4** formed by *N*-acetyl-β-D-glucosaminyl transfer on to the hydrolysis product of the donor. This impurity was removed by making use of the selectivity of the enzyme towards hydrolysis of (1→4) links in this disaccharide. For this, the total disaccharide product was first isolated by charcoal–Celite chromatography. It was then incubated with the enzyme, which selectively hydrolysed the disaccharide **4** (Scheme 1). The product mixture then contained a single disaccharide **3**. The mixture was applied to a charcoal–Celite column and monosaccharides were eluted with 5% ethanol in water. The single disaccharide product **3** was then eluted in 26% yield with 10% ethanol in water as eluent.



Scheme 1 *Reagent:* i, β-*N*-acetylhexosaminidase from *A. oryzae*

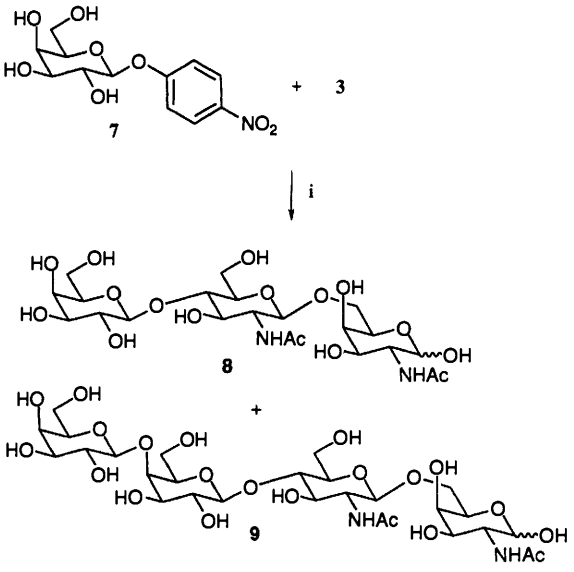
When the same procedure was applied with *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide **5** as donor, only a single disaccharide, β-D-Gal*p*NAc-(1→6)-D-Gal*p*NAc **6**, was formed in 38% yield (Scheme 2). This was readily isolated by charcoal–Celite chromatography as before.



Scheme 2 *Reagent:* i, β-*N*-acetylhexosaminidase from *A. oryzae*

To date, investigations with glycosidases as biocatalysts for oligosaccharide synthesis have been largely exploratory. However, the selectivities of a number of *exo*-glycosidases are now sufficiently well understood that they can be used in the rational design of target oligosaccharides. Thus it was decided

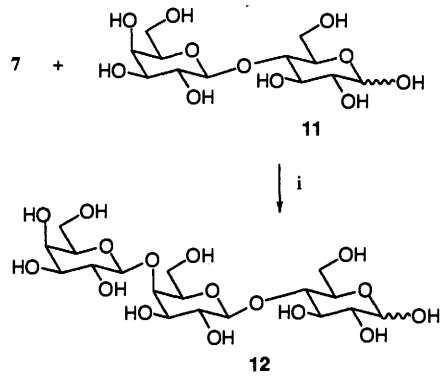
to employ the β-(1→4) selectivity of the β-galactosidase from *Bacillus circulans* ('Biolacta')[3] in an attempt to synthesize the core trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal*p*NAc **8** of ovarian cyst fluid mucin **10**.[4] Thus when *p*-nitrophenyl β-D-galactopyranoside **7** was incubated with disaccharide **3** as acceptor, the target trisaccharide **8** was formed in 48% yield, together with 7% of the tetrasaccharide **9** (Scheme 3). These products were readily isolated and separated



Scheme 3 *Reagent:* i, β-galactosidase from *B. circulans*

β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-β-D-GalNAc-Ser/Thr
3
↑
1
β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-β-D-Gal
3
↑
1
α-L-Fuc-(1→4)-β-D-GlcNAc
3
↑
1
β-D-Gal **10**

by preparative HPLC. The formation of the tetrasaccharide **9** illustrates the remarkable selectivity of the β-galactosidase from *B. circulans*. Although the acceptor trisaccharide **8** has available a sterically unencumbered 6-OH group, the specificity exerted by the enzyme directs glycosyl transfer to the 4-OH group, notoriously one of the least reactive positions in glycosyl acceptors. To demonstrate the generality of the procedure, a similar transfer was carried out with lactose as acceptor. A single trissaccharide, β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p* **12**, was produced in 52% yield (Scheme 4). Again, the



Scheme 4 *Reagent:* i, β-galactosidase from *B. circulans*

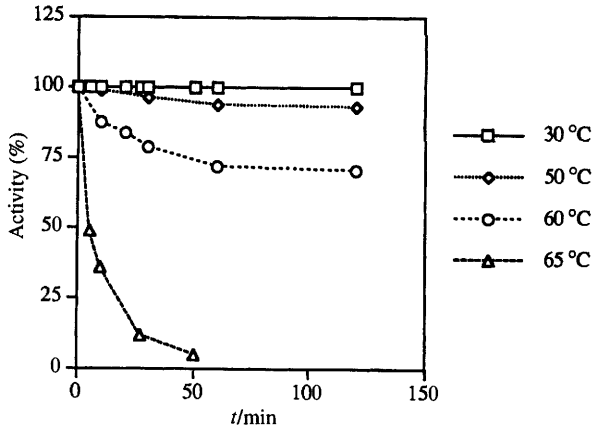


Fig. 1 Temperature stability of *N*-acetylglucosaminidase activity

remarkable selectivity of the β-galactosidase from *B. circulans* was demonstrated. The trisaccharide **12** has been reported as a growth factor for human intestinal bifidobacteria[5] and it is the β-analogue of globotriose [α-D-Gal*p*-(1→4)-β-D-Gal*p*-D-(1→4)-Glc*p*], the human p^k antigen in the P blood group system.[6] A mixture of trisaccharide **12** and the corresponding tetrasaccharide with an additional β-(1→4)-galactosyl residue has been reported as a fermentation product.[7]

The structures of the disaccharides, trisaccharides and tetrasaccharide reported here, particularly with respect to the linkage positions, were confirmed by NMR spectroscopy (DEPT, ^1H–^1H, ^{13}C–^1H COSY).† Overall compositions were confirmed by mass spectrometry.

The β-*N*-acetylhexosaminidase from *A. oryzae* was purified by ammonium sulfate precipitation, hydrophobic interaction chromatography, hydroxyapatite chromatography and gel chromatography (Table 1). The final product appeared by electrophoresis to be nearly homogeneous. Only a trace of contaminating protein was observed. The K_m and V_{max} values for the purified enzyme (Table 3) were in reasonable agreement with the values reported in the literature.[8] The temperature stability was reasonable up to 60 °C but fell rapidly at 65 °C (Fig. 1).

Because the β-*N*-acetylhexosaminidase appeared to transfer *N*-acetylhexosaminyl residues with nearly equal facility on to *N*-acetylglucosamine and *N*-acetylgalactosamine, it was important to obtain evidence that a single enzyme was involved and not a mixture. Accordingly the enzyme was purified to near homogeneity by fast-protein liquid chromatography (FPLC) using hydrophobic interaction chromatography followed by chromatography on hydroxyapatite and Sephacryl S-200-HR. The quotient V_{max}/K_m for the two donors was 4.3. During purification, the relative specific activities of the enzyme fractions towards *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide remained constant at 1.8. It was concluded that a single enzyme activity was involved. To answer the same question with respect to the acceptors, the experimental procedure was more difficult as, under the conditions of the preparative experiments, the acceptor was initially present in quantities greater than could be dissolved, in order to maintain maximum concentrations. During the course of the incubation, the acceptor gradually dissolved until a homogeneous solution was obtained. Accordingly, the acceptors were incubated separately and in combination with the enzyme for a fixed time period. The reaction was then stopped and the ratio of transfer products was determined by HPLC. It would be expected that if separate activities were responsible for transfer to the two acceptors, the ratio of

† DEPT: distortionless enhancement by polarization transfer; COSY: 2D homonuclear chemical-shift correlation.

Table 1 Purification of *N*-acetylhexosaminidase

Purification step	Protein/ mg	Units	Activity/ μmol min^{-1} mg^{-1}	Purification (fold)	Recovery (%)
Ammonium sulfate precipitation	833	174	0.12		100
Hydrophobic interaction	6	126	20.8	173	73
Hydroxyapatite	0.4	48	132	1100	28
Gel chromatography	0.05	15	370	3080	9

Table 2 Relative activities as acceptors of *N*-acetylglucosamine and *N*-acetylgalactosamine, singly and in combination

	Quotient of transfer to *N*-acetylglucosamine/*N*-acetylgalactosamine		
Acceptor	Fraction 1	Fraction 2	Fraction 3
Individual acceptors	4.15	2.27	2.1
Combined acceptors	16.0	4.5	4.9

products would not change when the acceptors were incubated together, whereas if a single enzyme were involved, transfer to one of the substrates would be favoured at the expense of transfer to the other. This experiment was repeated with three cuts from the β-*N*-acetylhexosaminidase fraction from the final (Sephacryl) FPLC column to see if there was any variation in behaviour across this peak. The results are given in Table 2, which shows that when the acceptors were incubated together, the ratio of transfer products increased significantly in favour of transfer to *N*-acetylglucosamine. The same relative effect was found for all three peak fractions. It was concluded that a single enzyme was responsible for transfer of *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl residues on to both *N*-acetylglucosamine and *N*-acetylgalactosamine. No particular significance can be attached to the difference between the absolute values for the three peak fractions as incubation times were different in order to permit sampling of homogeneous solutions for the final product analyses.

The present results demonstrate the versatility of the *N*-acetylhexosaminidase from *A. oryzae*, in that in addition to D-glucose, *N*-acetyl-D-glucosamine[1] and D-galactose,[2] *N*-acetylgalactosamine is now shown also to act as an acceptor of glycosyl transfer, exclusively to the 6-OH group. The yields of disaccharide obtained compare favourably with those obtained by multi-step non-enzymic procedures. The substitution of D-gluco to D-galacto monosaccharides as acceptors causes a switch in transfer from predominantly the 4-OH group to the 6-OH group. By contrast, the β-galactosidase of *B. circulans* retains a strong selectivity for transfer to the 4-OH group of acceptors with either the D-gluco or the D-galacto configuration. It has been observed previously that this selectivity is maintained with *N*-acetylglucosamine,[3] *N*-acetylgalactosamine,[9] *p*-nitrophenyl β-D-galactosaminide[9] and thioglycosides of D-glucose, D-galactose, *N*-acetyl-D-galactosamine and D-xylose[10] as acceptors, although the regiochemistry of transfer to *p*-nitrophenyl *N*-acetylglucosaminide was reported to be modulated by organic solvent (acetonitrile), high concentrations favouring 1→4 transfer and low concentrations 1→6 transfer.[9]

We have previously observed that the regiochemistry of glycosyl transfer becomes more specific as the substrate becomes bigger. Transfer to disaccharides and higher oligosaccharides becomes more selective than transfer to monosaccharides or monosaccharide glycosides.[1] This is borne out by the present results in which no significant amounts of regioisomers were detected during formation of trisaccharides **8** (Scheme 3) and **12** (Scheme 4). This no doubt reflects the restriction of the conformations of bound disaccharides to a single form and indicates that molecular recognition of the substrates extends over at least two monosaccharide units.

Experimental

NMR spectra were obtained using Bruker 250 MHz ACF250 and Bruker 400 MHz WH400 spectrometers. NMR coupling constants (*J*) are quoted in Hz. MS spectra were determined using a VG Analytical Quattra 2 spectrometer and high-resolution mass spectra on a VG Analytical ZAB-E mass spectrometer. Optical rotations were determined using an AA-1000 polarimeter (Optical Activity Ltd.) with a 2 dm cell, and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Protein analyses were carried out using the PhastSystem from Pharmacia LKB Biotechnology. Quantitative protein purification was carried out using a Waters 650E Advanced Protein Purification System (Millipore). Analytical and preparative high-performance liquid chromatography (HPLC) separations were carried out using Gilson 302 and 305/306 solvent delivery systems, respectively. Spectrophotometric enzyme and protein assays were monitored using a Pye Unicam SP1800 spectrophotometer and scanning measurements were obtained using a Philips PU8720 spectrophotometer.

2-Acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-galactopyranose 3

A mixture of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **1** (0.22 g, 0.64 mmol) and *N*-acetyl-D-galactosamine **2** (1 g, 4.52 mmol) in 0.05 M sodium phosphate buffer (pH 6.5; 4.4 ml) was incubated with β-*N*-acetylhexosaminidase from *A. oryzae* (0.175 ml; 149 mg protein ml^{-1}; 0.094 U mg^{-1} protein) at 30 °C for 30 h. By HPLC it was determined that the ratio of disaccharides **3** and **4** was 96:4. The reaction was stopped by heating the mixture in a boiling water-bath for 5 min. The mixture was applied to a charcoal–Celite column chromatography. The column was first eluted with 5% ethanol–water to remove the monosaccharides and then with 10% ethanol–water to elute the disaccharide mixture. The disaccharide fraction was evaporated under reduced pressure and then redissolved in 0.04 M sodium phosphate buffer (pH 6.5; 2 cm^3) and incubated with β-*N*-acetylhexosaminidase from *A. oryzae* (20 μl, as above) for 4 h at 30 °C to hydrolyse the *N*,*N*′-diacetylchitobiose **4**. The mixture was again purified by charcoal–Celite chromatography (as above) to give *disaccharide* **3** (71 mg, 26%), $[\alpha]_D^{26}$ +19.2 (*c* 0.85, water); δ_H(400 MHz; D_2O) 1.98 (6 H, s, 2 × CH_3), 3.37–3.50 (3 H, m, HC-O), 3.62–3.96 (8 H, m, HC-O), 4.05 (0.44 H, dd, *J* 11 and 3.64, H-2α), 4.10–4.13 (0.56 H, m, H-5α), 4.501 (0.56 H, d, *J* 8.44, H-1′), 4.504 (0.44 H, d, *J* 8.48, H-1′), 4.55 (0.44 H, d, *J* 8.40, H-1β) and 5.14 (0.56 H, d, *J* 3.64, H-1α); δ_C(100.6 MHz; D_2O) 22.60, 22.80, 22.85, 50.89, 54.35, 56.12, 61.33, 67.86, 68.43, 69.12, 69.77 (C-6), 69.81, 69.85 (C-6), 70.51, 71.60, 74.35, 74.48, 76.50, 91.67 (C-1α), 96.05 (C-1β), 102.24 (C-1′), 102.37 (C-1′), 175.36, 175.40 and 175.64 [Found: M^+ (FAB), 425.1758. $C_{16}H_{28}N_2O_{11}$ requires *M*, 425.1771].

2-Acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-deoxy-D-galactopyranose 6

A mixture of *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide **5** (0.250 g, 0.73 mmol) and *N*-acetyl-D-galactosamine **2** (1.125 g, 5.09 mmol) in citrate–phosphate buffer (0.04 M; pH 4.5; 5 cm^3) was incubated with the β-*N*-acetylhexosaminidase from *A. oryzae* (0.175 cm^3; 149 mg protein cm^{-3}; 0.048 U mg^{-1} protein) at 30 °C for 47 h. The reaction was stopped by heating the mixture in a boiling water-bath for 5 min. HPLC showed only the formation of disaccharide **6**. The product was purified by charcoal–Celite column chromatography as above to give disaccharide **6** (119 mg, 38%); $[\alpha]_D^{26}$ +47.63 (*c* 0.74, water) {lit.,[11] $[\alpha]_D^{25}$ +46.0 (*c* 2.05, water)}; δ_H(400 MHz; D_2O) 1.98 (6 H, s, 2 × CH_3), 3.61–3.98 (11 H, m, HC-O), 4.05 (0.55 H, dd, *J* 11.00 and 3.68, H-2α), 4.11–4.14 (0.55 H, m, H-5α), 4.436 (0.55 H, d, *J* 8.44, H-1′), 4.439 (0.45 H, d, *J* 8.44, H-1′), 4.55 (0.45 H, d, *J* 8.40, H-1β) and 5.14 (0.55 H, d, *J* 3.68, H-1α); δ_C(100.6 MHz; D_2O) 22.61, 22.86, 50.91, 53.01, 54.36, 61.65, 67.87, 68.41, 69.09, 69.66 (C-6), 69.74 (C-6), 69.82, 71.57, 74.50, 75.79, 91.68 (C-1α), 96.05 (C-1β), 102.74 (C-1′), 102.85 (C-1′), 175.35, 175.59 and 175.63 [Found: M^+ (FAB), 425.1749. Calc. for $C_{16}H_{28}N_2O_{11}$: *M*, 425.1771].

2-Acetamido-6-*O*-[2-acetamido-4-*O*-(β-D-galactopyranosyl)-2-deoxy-β-D-glucopyranosyl]-2-deoxy-D-galactopyranose 8 and 2-acetamido-6-*O*-{2-acetamido-4-*O*-[β-D-galactopyranosyl-4-*O*-(β-D-galactopyranosyl)]-2-deoxy-β-D-glucopyranosyl}-2-deoxy-D-galactopyranose 9

A solution of *p*-nitrophenyl β-D-galactopyranoside **7** (45 mg, 0.15 mmol) and disaccharide **3** (190 mg, 0.45 mmol) in sodium phosphate buffer (0.04 M; pH 6.5; 0.75 cm^3) was incubated with the β-galactosidase from *B. circulans* (20 μl; 9 mg cm^{-3}; 5000 U g^{-1} protein) at 30 °C for 32 h. The reaction was stopped by heating the mixture in a boiling water-bath for 5 min. Products were separated by preparative HPLC [Hypersil 5APS column (25 cm × 20 mm), UV detection at 210 nm, with MeCN–water (75:25) as eluent, flow rate 10 cm^3 min^{-1}] to give the trisaccharide **8** (42 mg, 48%) and the tetrasaccharide **9** (8 mg, 7%).

Trisaccharide 8. $[\alpha]_D^{26}$ +13.56 (*c* 0.52, water); δ_H(400 MHz; D_2O) 1.96 (6 H, s, 2 × CH_3), 3.43–3.94 (17 H, m, HC-O), 4.03 (0.52 H, dd, *J* 3.68 and 10.96, H-2α), 4.08–4.11 (0.52 H, m, H-5α), 4.39 (1 H, d, *J* 7.76, H-1″), 4.51 (1 H, d, *J* 8.56, H-1′), 4.53 (0.48 H, d, *J* 8.52, H-1β) and 5.12 (0.52 H, d, *J* 3.65, H-1α); δ_C(100.6 MHz; D_2O) 22.59, 22.81, 50.88, 54.34, 55.64, 60.64, 61.68, 67.86, 68.43, 69.12, 69.19, 69.37, 69.81, 69.89, 71.13, 71.60, 72.97, 73.13, 74.47, 75.39, 76.00, 79.00 (C-4′), 91.67 (C-1α), 96.04 (C-1β), 102.15 (C-1′), 102.27 (C-1′), 103.52 (C-1″), 175.36 and 175.64; *m/z* (FAB) 609 $(M + Na)^+$, 587 $(M + H)^+$.

Tetrasaccharide 9. $[\alpha]_D^{26}$ +7.16 (*c* 0.293, water); δ_H(400 MHz; D_2O) 1.95 (3 H, s, CH_3), 1.96 (3 H, s, CH_3), 3.45–3.94 (22 H, m, HC-O), 4.08–4.11 (1.57 H, m, HC-O and H-5α), 4.03 (0.57 H, dd, *J* 11.04, 3.72, H-2α), 4.42 (1 H, d, *J* 7.84, H-1′), 4.50 (1 H, d, *J* 7.68, H-1‴), 4.52 (1 H, d, *J* 7.76, H-1″), 4.53 (0.43 H, d, *J* 8.44, H-1β) and 5.12 (0.57 H, d, *J* 3.72, H-1α); δ_C(100.6 MHz; D_2O) 22.60, 22.83, 50.89, 54.35, 55.63, 60.61, 61.37, 61.63, 67.87, 68.43, 69.13, 69.25, 69.81, 69.88, 70.65, 71.61, 72.00, 72.09, 72.96, 73.44, 73.57, 74.47, 75.15, 75.39, 75.78, 77.79 (C-4″), 79.16 (C-4′), 91.67 (C-1α), 96.05 (C-1β), 102.16 (C-1′), 102.29 (C-1′), 103.54 (C-1″), 104.85 (C-1‴), 175.37 (CO) and 175.66 (CO); *m/z* (FAB) 771 $(M + Na)^+$, 749 $(M + H)^+$.

4-*O*-[4-*O*-(β-D-Galactopyranosyl)-β-D-galactopyranosyl]-D-glucopyranose 12

A solution of lactose monohydrate **11** (0.43 g, 1.19 mmol) and *p*-nitrophenyl β-D-galactopyranoside **7** (62.3 mg, 0.20 mmol) in sodium acetate buffer (50 mM; pH 5.0; 4 cm^3) was added β-D-galactosidase from *B. circulans* (1.39 mg). After 3.5 h, all of the donor **7** had been consumed as determined by HPLC. The reaction was stopped by heating the mixture at 100 °C for 5 min. The mixture was filtered and to the filtrate was added water (15 cm^3). The solution was extracted with diethyl ether (4 × 20 cm^3). The aqueous residue was concentrated under reduced pressure to 3 cm^3 and applied to a column of Biogel P2 (100 × 2 cm). The column was eluted with water at a flow rate of 0.38 cm^3 min^{-1} to give the trisaccharide **12** (53 mg, 52%) (Found: C, 42.7; H, 6.3. $C_{18}H_{32}O_{16}$ requires C, 42.89; H, 6.34%); R_f 0.41 [silica gel G; 2-propan-2-ol–acetone–0.1 M lactic acid (4:4:2)]; $[\alpha]_D^{25}$ +37.9 {lit.,[7] $[\alpha]_D$ +45 (*c* 1.0, water)}; δ_H(400 MHz; D_2O) 3.23 (1 H, dd, *J* 8.0 and 9.2, H-2 [β-anomer]), 3.50–3.83 (15 H, m, 15 × HC-O), 3.86 (1 H, d, *J* 3.3, H-4″), 3.90 (1 H, dd, *J* 2.0 and 12.2, H-6[a] [α-anomer]), 4.15 (1 H, d, *J* 3.1, H-4′), 4.44 (1 H, d, *J* 7.8, H-1′), 4.56 (1 H, d, *J* 7.8, H-1″), 4.62 (0.53 H, d, *J* 7.9, H-1β) and 5.18 (0.47 H, d, *J* 3.7, H-1α); δ_C(100.6 MHz; D_2O), 60.5, 60.7, 61.4, 61.6, 69.2 (C-4″), 70.7, 71.7, 72.0, 72.1, 73.4, 73.6, 74.4, 75.0, 75.1, 75.4, 75.7, 77.8 (C-4′), 78.9 (C-4β), 79.1 (C-4α), 92.4 (C-1α), 96.4 (C-1β), 103.5 (C-1′) and 104.8 (C-1″); *m/z* (FAB) 527 $(M + Na)^+$.

Purification of the β-*N*-acetylhexosaminidase from *A. oryzae*

The source of the enzyme was a commercial preparation of β-galactosidase (grade XI, Sigma) containing various contaminating glycosidase activities (*e.g.*, α-galactosidase, β-glucosidase, β-mannosidase, β-*N*-acetylhexosaminidase).

Assay method

A sample of enzyme solution (10 μl) was added to 90 μl of 0.05 M McIlvain buffer (pH 5.0) containing 5 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **1**. The reaction mixture was incubated for 10 min at 30 °C. The absorbance was read at 420 nm upon addition of 3.9 cm^3 of 0.1 M aq. Na_2CO_3. Activities of other glycosidases were assayed in the same way using the corresponding *p*-nitrophenyl glycosides as substrates. Protein concentration was determined with a commercial implementation of the Bradford method (Bio-Rad) using bovine serum albumin as a standard.

One unit of enzyme activity (U) is defined as that quantity of enzyme hydrolysing 1 μmol of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **1** per min under the conditions stated above. The specific activity is expressed as units per mg of protein.

Purification procedure

All unit operations (extraction, ammonium sulfate precipitation, dialysis, ultrafiltration) were carried out at 4 °C. Chromatography columns (XK series, Pharmacia) were packed with commercial chromatography media according to the manufacturer's instructions. A Waters 650E Advanced Protein Purification System (Millipore, USA) was employed for column development at 20 °C. Samples were centrifuged on a Sorvall RC-5B refrigerated superspeed centrifuge (8500 g; 10 °C; 40 min). Proteins were concentrated by membrane ultrafiltration (Centriprep, Amicon, 30 kDa relative molecular mass cut-off).

Extraction and ammonium sulfate precipitation

A sample of 10 g of commercially available β-galactosidase from *A. oryzae* (Grade XI, Sigma) was extracted with 100 cm^3 of stirred sodium phosphate buffer (50 mM; pH 6; 0.5% EDTA) for 2 h, and the extract was centrifuged. The supernatant was slowly saturated with ammonium sulfate (100% saturation) and allowed to mature for several hours. The precipitate collected by centrifugation was re-suspended in sodium phosphate buffer (20 mM; pH 6.5) and dialysed against the same buffer overnight. A minor precipitate was removed by filtration, and the enzyme solution was concentrated by membrane ultrafiltration. This crude preparation was used for further purification.

Hydrophobic interaction chromatography

A portion of the crude enzyme preparation (300 mg protein) was loaded onto a column (*l* = 25 cm, *id* = 2.6 cm) packed

with 'high substituted' Phenyl Sepharose (Pharmacia) equilibrated with sodium phosphate buffer (20 mM; pH 7) containing 0.5 M ammonium sulfate. Proteins were eluted with a linearly decreasing salt concentration (starting with sodium phosphate buffer [20 mM; pH 7], ammonium sulfate [0.5 M]; final concentrations sodium phosphate buffer [10 mM; pH 7], ammonium sulfate [0.0 M]). β-*N*-Acetylhexosaminidase emerged towards the end of the gradient. Active fractions were pooled, and concentrated by membrane ultrafiltration as above.

Hydroxyapatite chromatography

Concentrated active fractions from hydrophobic interaction chromatography (6 mg protein) were applied to a column (l = 50 cm, id = 1 cm) packed with hydroxyapatite (Bio-Gel HTP, Bio-Rad) and equilibrated with sodium phosphate buffer (10 mM; pH 6). Enzyme was eluted with a linearly increasing phosphate buffer concentration (starting conditions: 10 mM buffer; pH 6.0; final conditions: 0.3 M buffer; pH 6.0). Active fractions were pooled, and concentrated by membrane ultrafiltration. This preparation was free of α- and β-galactosidase, β-glucosidase and β-mannosidase activities.

Gel chromatography

To eliminate minor impurities of lower molecular mass the concentrated sample from hydroxyapatite chromatography (0.4 mg protein) was loaded onto a Sephacryl S-200-HR column (Pharmacia, l = 100 cm, id = 1.6 cm) equilibrated and eluted with McIlvain buffer (50 mM; pH 5). Active fractions were pooled, and concentrated by membrane ultrafiltration. β-*N*-Acetylhexosaminidase was purified more than 3000-fold (Table 1). The ratio of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities did not change throughout the purification procedure. The specific activity of the preparation was 370 and 205 μmol min^{-1} mg^{-1} protein for β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activity, respectively. According to polyacrylamide gel electrophoresis (PAGE) the protein was found to be accompanied by a minor impurity of higher relative molecular mass.

Characterization of the β-*N*-acetylhexosaminidase

The isoelectric point was determined using the PhastGel isoelectrofocussing kit (Pharmacia) with precast IEF 3-9 gel slabs. Gels were calibrated with an appropriate mixture of standard proteins supplied by the manufacturer. The pI of the enzyme is 4.1. The relative molecular mass of the enzyme was determined by native and sodium dodecyl sulfate (SDS)/PAGE performed with PhastGel kit (Pharmacia) on precast Gradient 8-25 gel slabs. The protein calibration kit (HMW electrophoresis calibration kit, Pharmacia) was used to determine the relative molecular mass of the enzyme and its subunits. The enzyme has a relative molecular mass of ~117 kDa, and consists of two identical subunits (~60 kDa).

Influence of pH, temperature and substrate concentration on β-*N*-acetylglucosaminidase activity

pH optimum. The enzyme was added to a substrate solution (5 mM) in McIlvain buffer (50 mM) of different pHs. Enzyme activity was assayed as above. The pH optimum is 5 and 4.5 for β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activity, respectively.

pH stability. The enzyme was incubated in 50 mM McIlvain buffer of the respective pH at 30 °C. After 2 h the residual activity of the enzyme was determined as stated above. The enzyme is stable within the range pH 4–6. Inactivation (> 20%) was detected at pH 7.

Temperature optimum and stability. The enzyme was pre-incubated in McIlvain buffer (50 mM; pH 5) at different temperatures to determine its temperature stability. Residual activity was assayed at various time intervals and compared with that of a reference sample kept at 30 °C (Fig. 1). The enzyme shows an increase in activity with increasing temperature (up to 60 °C). The enzyme is remarkably stable at temperatures below 60 °C. At 60 °C a 30% decrease in activity was observed after 2 h. More than 50% loss of activity within 5 min was observed at 65 °C. No activity was detected at 70 °C.

Table 3 K_m and V_{max} values

Substrate	Concn. (mM)	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
p-Nitrophenyl β-GlcNAc	0.3–1.6	0.79	3.0×10^2
p-Nitrophenyl β-GalNAc	0.6–4.4	2.80	2.5×10^2

Kinetic constants. Adequately diluted enzyme was incubated at 30 °C for 2, 4 and 8 min with various concentrations of the substrate in 50 mM McIlvain buffer of pH 5. Initial rates of substrate hydrolysis were obtained for each substrate concentration, and K_m and V_{max} were calculated from Lineweaver–Burk plot (Table 3).

Determination of the relative activities of *N*-acetylglucosamine and *N*-acetylgalactosamine as acceptors, singly and in combination

The peak corresponding to the β-*N*-acetylhexosaminidase activity from the Sephacryl S-200-HR column was split into three components, fraction 1 from the leading edge, fraction 2 from the middle of the peak and fraction 3 from the trailing edge. For each fraction, the activities of the two acceptors were determined separately and together as follows. To a mixture of *p*-nitrophenyl glycoside **1** (7 mg, 0.0204 mmol; final concentration 0.51 M) and *N*-acetylglucosamine (45 mg, 0.203 mmol; final concentration 0.51 mM) was added the enzyme (fraction 1, 50 mm^3; fraction 2, 20 mm^3; fraction 3, 15 mm^3). The mixtures were incubated at 30 °C respectively for 338, 308 and 248 min. The reactions were stopped as above and the product mixture was analysed by HPLC. Exactly the same procedure was followed with *N*-acetylgalactosamine **2** as acceptor. For incubation with the combined acceptors, the concentration of donor was kept as in the experiments with the individual substrates and the amounts and concentrations of acceptors were as for the individual experiments (*i.e.* 45 mg each; final concentration of each 0.51 M). The volumes of enzyme fractions 1–3 were as for the individual experiments. Analysis was carried out by HPLC as before. The results are shown in Table 2.

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