Purification of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* and the β-mannosidases from *Helix pomatia* and *A. oryzae* and their application to the enzymic synthesis of the core trisaccharide of the *N*-linked glycoproteins

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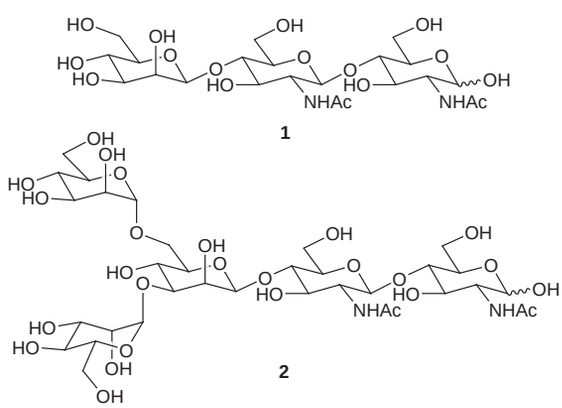
The β-*N*-acetylhexosaminidase from *Aspergillus oryzae* and the β-mannosidases from *Helix pomatia* and *A. oryzae* were purified and used to synthesise the core trisaccharide of *N*-linked glycoproteins, Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc.

Introduction

The trisaccharide β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc **1** comprises the proximal trisaccharide of the pentasaccharide **2**, which is the universal core pentasaccharide of *N*-linked glycoproteins. Elaboration of this core pentasaccharide gives rise to the great diversity of oligosaccharide structures observed in this class of protein. Synthetic routes to the pentasaccharide **2** are therefore of great interest. However, a considerable obstacle to be overcome in any synthesis of the core pentasaccharide is construction of the β-mannosidic bond to the internal *N*,*N*′-diacetylchitobiose unit D-Glc*p*NAc-(1→4)-D-Glc*p*NAc. Whereas α-mannosidic bonds are amongst the easiest glycosidic bonds to generate, the β-mannosidic bond is the most difficult. Both steric and polar effects conspire with the anomeric effect to favour strongly formation of the α-mannosidic linkage. Consequently, great ingenuity has been demonstrated in formulating solutions to the problem. Although a number of methods give mixtures of α- and β-mannopyranosides, relatively few are highly stereoselective. The first clean solution was provided by Barresi and Hindsgaul[1,2] who linked a protected mannopyranosyl donor as the ethyl thioglycoside to an acceptor *via* an isopropylidene bridge. Activation of the ethylthio group with *N*-iodosuccinimide (NIS) gave the corresponding β-mannopyranoside accompanied by no detectable α-isomer (Scheme 1a). A variation of this method has been described in which a bridging *p*-methoxybenzylidene group was used.[3] Stork *et al.* described a closely analogous method in which a temporary silicon bridge was used (Scheme 1b).[4,5] Kunz and Günther inverted the configuration at C-2 through triflate displacement by intramolecular attack of an adjacent phenylurethane group (Scheme 1c).[6] Lichtenthaler and Schneider-Adams coupled 2-oxohexosyl bromides to selectively protected acceptors and reduced the disaccharide products with borohydride. High β/α anomeric ratios were obtained by appropriate choice of solvent, temperature, and protecting group in the donor 2-oxohexosyl bromide (Scheme 1d).[7] Earlier, the group of Jeanloz had followed a similar reductive strategy, but with generation of the appropriate axial 2-OH group in selectively protected β-D-Glc*p*-(1→4)-α-D-GlcNAc[8] or β-D-Glc*p*-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc[9] glycosides.

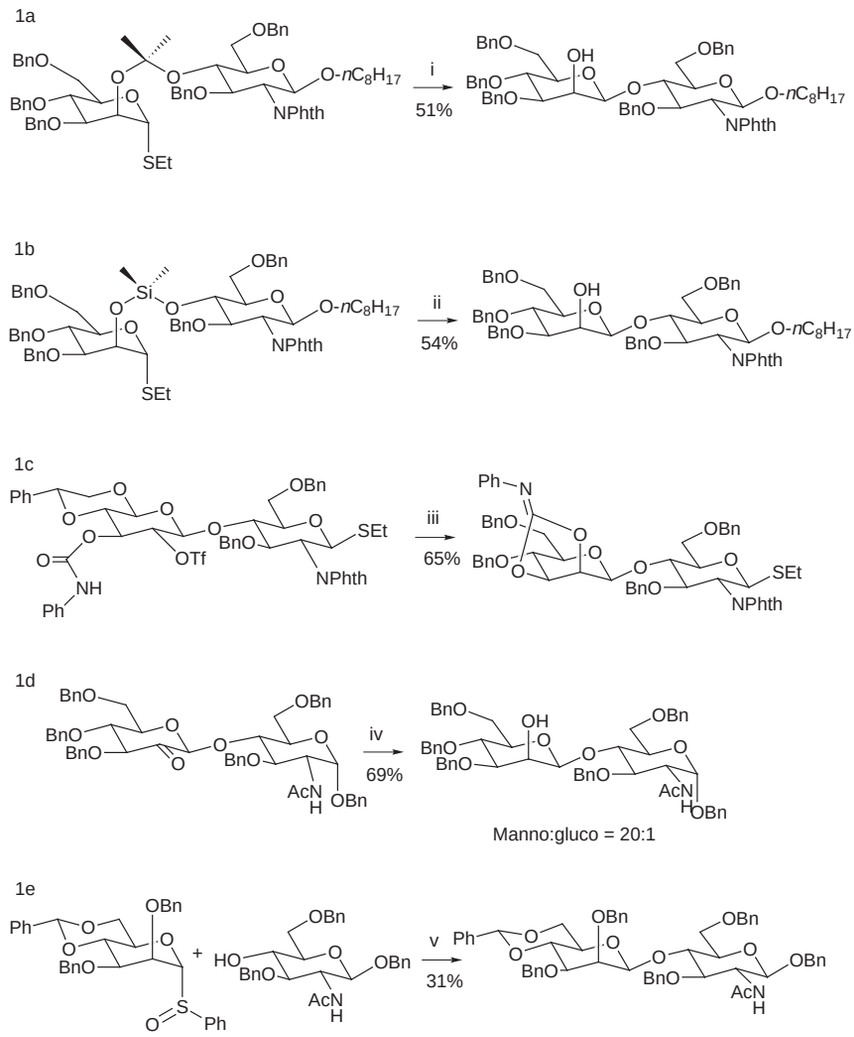
Very recently, Crich and Sun have described a variation of Kahne's sulfoxide glycosylation method[10,11] in which the sulfoxide is activated by triflic anhydride. Trapping of the intermediate oxycarbenium ion by axial attack of triflate, followed by an S_N2-like attack of the selectively protected acceptor, gives predominantly the β-mannoside (Scheme 1e).[12] Although the reaction shown (chosen for its relevance to the present paper) was not exceptionally selective, β:α anomer ratios of >25:1 are achievable using this method.



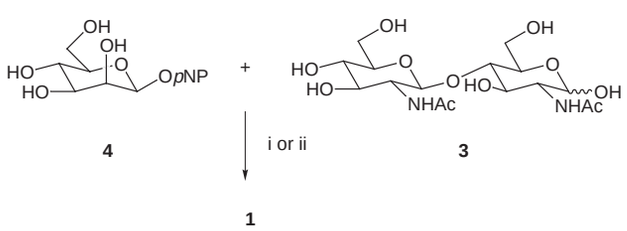
Results

Although the above methods are selective or highly selective, considerable synthetic effort is required to prepare glycosyl donor and acceptor for coupling or to prepare the appropriate keto-oligosaccharide for stereoselective reduction. The products are obtained in variously protected forms. Although several of the procedures described (Scheme 1b–e) give β-D-Man-D-GlcNAc derivatives, only one of them[9] appears to have been applied to the synthesis of the core trisaccharide **1** of the pentasaccharide itself.

In the face of such synthetic problems, enzymic methods offer an attractive alternative. In particular, β-mannosidases, which have been used for the synthesis of simple β-mannose glycosides, offer an efficient and selective solution of this problem.[13–15] Incubation of a suitable glycoside donor with an acceptor, both completely unprotected, with an appropriate β-mannosidase gives the disaccharide product. Since we had access to considerable quantities of *N*,*N*′-diacetylchitobiose **3**,[16] this was used in our first experiments with the β-mannosidase from the edible snail *Helix pomatia* (Scheme 2). *p*-Nitrophenyl β-D-mannopyranoside **4**, readily available in two steps from mannose,[17] was incubated with disaccharide **3** in the presence of the enzyme. The required trisaccharide **1** was isolated by carbon-Celite chromatography but in only 3% yield. The donor was largely hydrolysed to mannose and *p*-nitrophenol. Clearly, the disaccharide **3** was only weakly recognised. How-



Scheme 1 *Reagents and conditions*: i, NIS, 2,6-di-*tert*-butyl-4-methylpyridine, −5 °C; ii, Tf_2O, 2,6-di-*tert*-butyl-4-methylpyridine, −100 °C; iii, pyridine, DMF, 75 °C; iv, $NaBH_4$, MeOH–CH_2Cl_2 (1 : 1); v, Tf_2O, 2,6-di-*tert*-butyl-4-methylpyridine.



Scheme 2 *Reagents*: β-mannosidase from i, *H. pomatia*; ii, *A. oryzae*.

ever, encouraged by this positive result, we examined a number of glycosidase sources and identified significant activity in the crude β-galactosidase from *Aspergillus oryzae*. When *p*-nitrophenyl β-D-mannopyranoside **4** was incubated with this enzyme in the presence of a 6.7-fold molar excess of the acceptor **3**, the core trisaccharide **1** was obtained in 26% yield (unoptimised).† On a larger scale, using 0.5 g of the donor **4**, the trisaccharide **1** was obtained in 16% yield, but approximately 25% of the donor remained unchanged. This suggests that the enzyme is inhibited either by the *p*-nitrophenol or by the trisaccharide product **1**. Since the unchanged acceptor **3** can be recovered essentially quantitatively from the carbon-Celite column [elution with ethanol–water (90 : 10)], this procedure provides a rapid and effective route to the completely unprotected core trisaccharide **1**. It was noteworthy that no other trisaccharide products could be detected, indicating high selectivity of the β-mannosidase for 1→4 glycosyl transfer.

When using glycosidases for the synthesis of oligosaccharides larger than disaccharides, it is essential that the enzyme preparation used should be free of activity that might catalyse hydrolysis of the acceptor. In the present case it was essential that β-*N*-acetylglucosaminidase activity should be absent. The enzyme from *H. pomatia* was prepared essentially free from β-*N*-acetylglucosaminidase activity by removing unwanted glycosidase activities by adsorption on DEAE- and CM-Sepharose. The enzyme from *A. oryzae* was obtained in homogeneous form by chromatography on Phenyl-Sepharose and anion-exchange resins. The β-*N*-acetylhexosaminidase, which was used for the production of the *N*,*N*′-diacetylchitobiose used in these experiments, was also purified from the same source. The procedure was identical up to the separation on Phenyl-Sepharose. The β-*N*-acetylglucosaminidase was then purified to homogeneity by successive passage through hydroxyapatite and Sephacryl columns. The purification data for the two enzymes from *A. oryzae* are given in Table 1 and the SDS-PAGE analysis is shown in Fig. 1.

The relative molecular masses as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were 72 kDa for the β-mannosidase and 60 kDa for the β-*N*-acetylhexosaminidase. However, the latter enzyme on a native gel had an apparent relative molecular mass of 117 kDa. Under non-denaturing conditions it therefore appears to exist as an α_2 dimer.

Kinetic constants and pI-values for the two enzymes are

† A preliminary communication describing this result has been published.[18]

Table 1 Purification of the β-mannosidase and the β-*N*-acetylhexosaminidase from *A. oryzae*

	Protein/ mg	Total activity[a] (units)	Specific activity (units/mg protein)	Yield (%)	Purification factor
β-Mannosidase					
Crude extract	970	40	0.04	100	1.0
Phenyl-Sepharose	285	28	0.10	72	2.5
DEAE-Sepharose	64	21	0.33	53	8.0
Mono-Q	12.1	12	0.99	30	25.0
β-*N*-Acetylhexosaminidase					
Crude extract	970	202	0.21	100	1
Phenyl-Sepharose	7	146	21	73	100
Hydroxyapatite	0.42	56	132	28	630
Sephacryl G-200-HR	0.045	17	370	9	1760

[a] Activity is defined for hydrolysis of *p*-nitrophenyl β-D-mannopyranoside and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide respectively. One unit is the amount of enzyme required to hydrolyse the respective substrate at the rate of 1 μmol min^{-1} at 30 °C.

Table 2 Kinetic constants and pI-values for the β-mannosidase and the β-*N*-acetylhexosaminidase from *A. oryzae*

	(μmol/min/mg protein)		
	K_m/mM	V_{max}	pI
β-Mannosidase	0.853	1.71	4.2
β-*N*-Acetylglucosaminidase[a]	0.54	269	4.3
β-*N*-Acetylgalactosaminidase[a]	1.71	211	(4.3)

[a] Activity was assayed with *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide respectively.

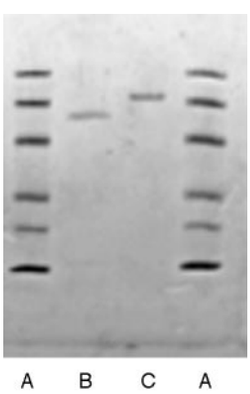


Fig. 1 Polyacrylamide gel electrophoresis of the β-*N*-acetylhexosaminidase and the β-mannosidase of *Aspergillus oryzae.* Lane A, relative molecular mass markers; lane B, β-*N*-acetylhexosaminidase; lane C, β-mannosidase. The standards had relative molecular masses (top to bottom) of 94, 67, 43, 30, 20.1 and 14.4 kDa, respectively.

given in Table 2. A β-*N*-acetylhexosaminidase has been purified from a different crude β-galactosidase (Takadiastase) from *A. oryzae*. The specific activity (242 U mg^{-1}), K_m (0.85 mM)[19] and pI (4.0)[20] values are similar to those for the enzyme described here (Tables 1 and 2). The relative molecular mass was estimated to be 72–74 kDa by SDS-PAGE and 146 kDa on a native gel.[20] As with our enzyme, the Takadiastase enzyme appears to exist as a homodimer. The pH optima for the enzymes were 2.6 (β-mannosidase), 5.0 (*N*-acetylglucosaminidase) and 4.5 (*N*-acetylgalactosaminidase activity). The corresponding values for the Takadiastase enzyme were 4.5 and 3.7 for Glc*p*NAc and Gal*p*NAc respectively.[20]

For practical applications of glycosidases in oligosaccharide synthesis, stability at elevated temperatures and over the useful pH range are desirable properties. Accordingly the temperature stabilities of the β-mannosidase and the β-*N*-acetylhexosaminidase were investigated. The results are shown in Fig. 2. Both enzymes showed high stability at temperatures up to

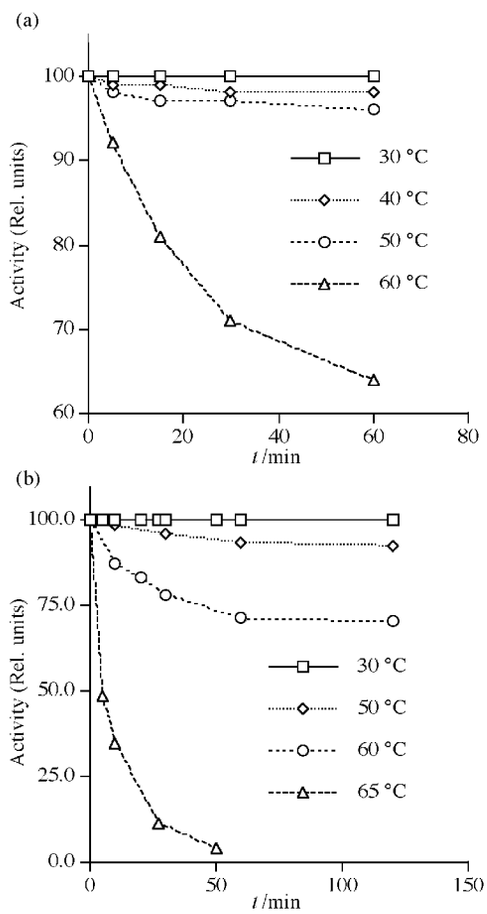


Fig. 2 Temperature stabilities of (a) the β-mannosidase; (b) the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*.

50 °C. At higher temperatures there was a significant decrease in activity with time. Finally, the pH stabilities of the enzymes were studied. The enzymes were maintained in McIlvain (citrate–phosphate) buffer at the appropriate pH for 16 h. The remaining activity was then determined. The results are shown in Fig. 3. The β-mannosidase showed maximum stability in the pH range 3.5–4.5. The β-*N*-acetylhexosaminidase showed maximum stability in the pH range 4.0–6.0.

The present paper describes a synthesis of the core trisaccharide of *N*-linked glycoproteins in acceptable yields using two glycosidase-catalysed steps. A related approach has been reported using the β-mannanase from *Aspergillus niger*.[21] This method required the use of the trisaccharide β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man*p* as donor. This was prepared by enzymic hydrolysis of copra mannan followed by fractionation

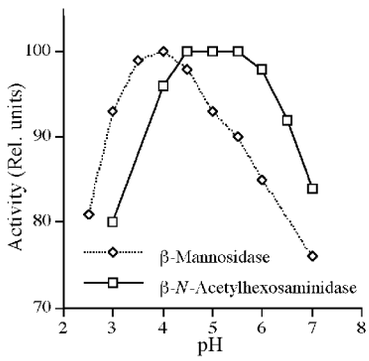


Fig. 3 pH Stabilities of the β-*N*-acetylhexosaminidase and the β-mannosidase of *Aspergillus oryzae.*

of the resulting oligosaccharide mixture. The enzyme used was a crude mannanase from a *Streptomyces* strain. The core trisaccharide **1** was obtained in 3.7% yield. Very recently, the alternative enzymic approach to oligosaccharide synthesis using glycosyl transferases has been applied to this problem.[22] A recombinant β-mannosyl transferase was incubated with phytanylpyrophosphoryl α-*N,N′*-diacetylchitobioside and guanosine 5′-diphosphomannose (GDP-mannose). The product was hydrolysed with alkali to give the core trisaccharide (1.5 mg). Although the reaction proceeded in high (80%) yield, the complex nature of both mannosyl donor and acceptor would be a consideration if this method were to be applied to larger scale synthesis of the trisaccharide **1**. On the other hand, the glycosidase-catalysed method is capable of producing readily the trisaccharide **1** on a >100 mg scale even under non-optimised conditions. Immobilisation of the enzyme will greatly increase the efficiency with which it can be used and is likely to lead to even higher yields. Recently[23] a new method for the synthesis of β-mannosides has been described based on activation of stannylene acetals with caesium fluoride. The caesium alkoxides produced were used to displace triflates in acceptors. Although this method gave high stereocontrol with respect to formation of β-mannoside, it has yet to be applied to the creation of the β-D-Man*p*-(1→4)-β-D-GlcNAc linkage.

Experimental

NMR spectra were recorded on a Bruker WH400 (400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane. NMR coupling constants (J) are quoted in Hz. Mass spectra were determined on a Bruker BioApex 9.4 T FTICR mass spectrometer. Optical rotations were determined at 598 nm with an Optical Activity LTD AA-1000 polarimeter with a 2 dm cell. $[\alpha]_D$-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). HPLC analyses were carried out using a Gilson HPLC instrument with a Hypersil 5APS (aminopropylsilica) column (20 cm × 4.6 mm), UV detection at 210 nm and with acetonitrile–water (83 : 17) as eluent at a flow rate of 1.75 cm^3 min^{-1}. Preparative HPLC was carried out using a Hypersil 5APS column (25 cm × 2 cm) with UV detection at 210 nm and with acetonitrile–water (76 : 24) as eluent at a flow rate of 10 cm^3 min^{-1}. Spectrophotometric enzyme and protein assays were monitored using a Pye Unicam SP1800 spectrophotometer and scanning measurements were obtained using a Philips PU8720 spectrophotometer.

Enzyme extraction and purification

General procedures. All operations were carried out at 4 °C. Extraction and elution buffers prepared by mixing corresponding stock solutions were supplemented with 2-mercaptoethanol (final concentration 5 mM). Samples were centrifuged on a Sorvall RC-5B refrigerated superspeed centrifuge (8 500 ***g***; 20 min; 4 °C). Proteins were concentrated by membrane ultra filtration (Centriprep, Amicon, 30 kDa cut off) on a Mistral 2000R refrigerated centrifuge (4 000 ***g***; repeated 15 min runs at 4 °C). Samples of small volume were concentrated in Minicon concentration cells (Amicon, 15 kDa cut off). 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.

Assay methods. The enzyme solution (0.01 cm^3) was added to McIlvain (citrate–phosphate) buffer (0.09 cm^3; pH 5.0) containing *p*-nitrophenyl glycoside (5 mM). The mixture was incubated for 10 min at 30 °C. The assay solution was diluted with aq. sodium carbonate (0.1 M; 3.9 cm^3) and the absorbance at 400 nm was determined. The *p*-nitrophenolate concentration was determined from a standard curve. One unit of enzyme activity is defined as the quantity of enzyme that hydrolyses 1 μmol of *p*-nitrophenyl glycoside under the conditions given above. The specific activity is expressed as units per mg protein. Protein concentration was determined according to Bradford[24] with bovine serum albumin as standard.

Materials

A crude preparation of β-galactosidase from *A. oryzae* (grade XI) was kindly donated by the Sigma Chemical Company. *p*-Nitrophenyl glycosides of various monosaccharides used as enzyme substrates were obtained from Sigma. The following chromatography media were used: high substituted Phenyl Sepharose, Sephacryl S-200-HR (both from Pharmacia), DEAE-Sepharose (Fluka), Macro-Prep ceramic hydroxyapatite (Bio-Rad). Prepacked Mono Q column (Pharmacia) was employed in final purification steps. For the estimation of relative molecular mass and pI of the enzymes, precast gel slabs, buffer strips and protein standards were used (Pharmacia). All other chemicals were purchased from commercial sources as reagent grade.

The β-mannosidase from *Helix pomatia*

Freeze-dried digestive juice of *H. pomatia* [kindly supplied by Dr V. Kren (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague), 1 g] was dissolved in sodium phosphate buffer [50 mM; pH 6.0; 10 cm^3, containing EDTA (0.5%)]. The solution was filtered from insoluble debris. The filtrate (352 mg protein, 134 U [see above for the assay method]) was added to DEAE-Sepharose (30 cm^3 of gel slurry) equilibrated with sodium phosphate buffer (50 mM; pH 6.0) and the mixture was gently swirled in an ice-bath for 30 min. The β-mannosidase and other unbound proteins were washed from the gel using the same buffer solution (3 washings, each of 20 cm^3). The combined washings were cooled in ice–water and proteins were precipitated by ammonium sulfate (100% saturation). The resulting precipitate was collected by centrifugation as described in the "general procedures", above. The protein pellet was exhaustively dialysed against McIlvain (citrate–phosphate) buffer (20 mM; pH 4.3) and added to CM-Sepharose (20 cm^3 of gel slurry equilibrated with the same buffer). The mixture was gently swirled in an ice-bath for 30 min. The β-mannosidase and other unbound proteins were washed from the gel with the same buffer solution (2 washings, each of 15 cm^3). Proteins in the combined washings were precipitated by ammonium sulfate (100% saturation) and collected by centrifugation. The final β-mannosidase preparation (1.9 U/mg protein) was purified five-fold with a recovery of 52%. Only traces of β-*N*-acetylglucosaminidase, β-galactosidase and β-glucosidase activities were detectable.

The β-mannosidase from *A. oryzae*

Extraction and ammonium sulfate precipitation. A sample of commercially available β-galactosidase from *A. oryzae* (Grade XI, Sigma, 50 g) was extracted with sodium phosphate buffer [50 mM; pH 6.8 containing EDTA (0.5%); 600 cm^3] on ice for 2 h with stirring. The extract was centrifuged. The supernatant was slowly saturated with ammonium sulfate (100% saturation) and allowed to mature overnight. The precipitate was collected by centrifugation and resuspended in sodium phosphate buffer (20 mM; pH 6.5).

Hydrophobic interaction chromatography.—A portion of the crude sample (300 mg protein, approximately) was loaded on to a Phenyl-Sepharose column (Pharmacia, 5 × 20 cm) in sodium phosphate buffer (20 mM; pH 6.5; containing ammonium sulfate, 0.5 M). The column was developed with a linear decrease of the salt concentration in the same buffer (0.5–0.0 M ammonium sulfate; flow rate 1.5 cm^3 min^{-1}). Approximately 100 fractions (15 cm^3) were collected. β-Mannosidase activity was found at the beginning of the gradient (fractions 16–28) together with most of the contaminating proteins. The β-*N*-acetylhexosaminidase activity was tightly bound to the column and emerged at zero salt concentration (fractions 79–85). Fractions containing the various glycosidase activities were pooled and the proteins were precipitated with ammonium sulfate (100% saturation). The precipitates were collected by centrifugation and dialysed against sodium phosphate buffer (10 mM; pH 6.5). β-*N*-Acetylhexosaminidase activity is completely removed from the β-mannosidase fraction in this step.

DEAE-Sepharose chromatography.—The β-mannosidase-rich fraction (approximately 100 mg protein) was applied to a DEAE-Sepharose column (Fluka; 2.6 × 20 cm; 20 mM sodium phosphate buffer; pH 6.5) and eluted with an increasing salt concentration (0.0–0.25 M NaCl, linear gradient; flow rate 1.5 cm^3 min^{-1}). Active fractions were pooled, concentrated, and dialysed against sodium phosphate buffer (5 mM; pH 6.5) overnight. As the β-mannosidase preparation obtained still contained some β-galactosidase activity, the sample was further purified on a Mono Q column (0.02 M sodium phosphate buffer; pH 6.5, with elution with a salt gradient 0.0–0.1 M NaCl) to obtain an electrophoretically homogeneous preparation for biochemical studies. However, for synthetic experiments a partially purified preparation obtained from the DEAE-Sepharose column was used.

The β-*N*-acetylhexosaminidase from *A. oryzae*

Purification procedure. The concentrated and dialysed fractions containing the β-*N*-acetylhexosaminidase activity from the Phenyl-Sepharose chromatography, above, was concentrated by ultrafiltration (approximately 7 mg protein) and loaded on to a Macro-Prep ceramic hydroxyapatite column (1.6 × 33 cm) equilibrated with potassium phosphate buffer (5 mM; pH 6.8). The column was washed with the starting buffer, and the enzyme was eluted with increasing buffer concentration (linear gradient of potassium phosphate buffer 5–500 mM; pH 6.8; flow rate 1.5 cm^3 per min). Fractions containing β-*N*-acetylhexosaminidase activity were pooled and concentrated by ultrafiltration. A homogeneous preparation of the β-*N*-acetylhexosaminidase free of other glycosidase activities was obtained after further chromatography on a Sephacryl S-200-HR column (1.6 × 100 cm) equilibrated with potassium phosphate buffer (0.02 M; pH 6.5; containing 0.1 M NaCl). The column was eluted with the same buffer (flow rate 0.5 cm^3 per min; 1.5 cm^3 per fraction), and active fractions were pooled, and concentrated by membrane ultrafiltration.

Relative molecular mass and isoelectric point

Relative molecular mass of the purified proteins was determined by native and SDS-PAGE on PhastSystem (Pharmacia) using precast gel slabs PhastGel Gradient 8-25 (continuous 8 to 25% gradient gel zone, 2% crosslinking) calibrated with a protein standard mixture. The relative molecular masses found were: β-mannosidase, 72 kDa (SDS), 72 kDa (native gel); *N*-acetylhexosaminidase, 60 kDa (SDS), 117 kDa (native gel).

Isoelectric focusing was carried out with PhastSystem using precast PhastGel IEF media with a linear pH gradient covering the range pH 3–9, calibrated with a protein standard mixture. The proteins in the gel were visualised with Coomassie blue staining in PhastSystem apparatus.

pH Dependence

The pH influence on the activity was studied using the pure enzymes. To 90 mm^3 of 5 mM substrate solution in buffer [50 mM; McIlvain (citrate–phosphate) buffer for the β-*N*-acetylhexosaminidase; 50 mM sodium acetate buffer for the β-mannosidase] was added 10 mm^3 of enzyme solution. The enzyme-activity assay was performed as described above. Stability at various pHs was investigated by incubation of the enzyme solution with McIlvain (citrate–phosphate) buffer (100 mM; range pH 2.5–7.0) at room temp. overnight. The enzyme activity assay was performed with enzyme solution aliquots under standard conditions.

Temperature stability

The enzymes were incubated at various temperatures in McIlvain (citrate–phosphate) buffer (50 mM; pH 5.0). At preset time intervals, aliquots were taken and assayed for the activity. A sample kept at 30 °C was assumed to maintain a 100% activity. These studies were carried out using pure enzymes.

Enzyme kinetics

The influence of the substrate concentration on the initial velocity was measured using pure enzymes under standard assay conditions. Initial rates of substrate hydrolysis were obtained for each substrate concentration, and apparent Michaelis constants (K_m) were calculated from direct linear plots.

β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc 1

a. Using the β-D-mannosidase from *H. pomatia*. *p*-Nitrophenyl β-D-mannopyranoside **4** (0.2 g, 0.66 mmol) and *N*,*N*′-diacetylchitobiose **3** (1.75 g, 4.13 mmol) in McIlvain (citrate–phosphate) buffer (0.05 M; pH 4.5; 4 cm^3) were incubated with the β-D-mannosidase (25 × 10^{-3} cm^3; 1.9 U/mg protein, 35 U cm^{-3}) at 30 °C for 30 h. The mixture was heated in a boiling water-bath for 5 min. HPLC analysis indicated the formation of a single transfer product. This was purified by carbon-Celite chromatography. The column (45 × 3 cm) was eluted first with 5% aq. ethanol to remove monosaccharide, 10% aq. ethanol to recover disaccharide and 15% aq. ethanol to give the trisaccharide **1** (12 mg, 3% yield). The spectroscopic data were as for the product prepared using the enzyme from *A. oryzae*, below.

b. Using the β-D-mannosidase from *A. oryzae*. *p*-Nitrophenyl β-D-mannopyranoside **4** (75 mg, 0.25 mmol) and *N*,*N*′-diacetylchitobiose **3** (690 mg, 1.63 mmol) in McIlvain (citrate–phosphate) buffer (0.05 M; pH 4.5; 1 cm^3) were incubated with the β-D-mannosidase (0.1 cm^3; 0.24 U/mg protein; 46 mg protein cm^{-3}) at 30 °C for 5 h. The mixture was heated in a boiling water-bath for 5 min. The product was purified by preparative HPLC to give the trisaccharide **1** (38 mg, 26%), $[\alpha]_D^{21}$ −12.2 (*c* 1.0, water) {lit.,[8] $[\alpha]_D^{20}$ +0.2 (*c* 1.5, water); lit.,[9] $[\alpha]_D^{20}$ 0.5 (*c* 0.44, water); lit.,[21] $[\alpha]_D^{25}$ +0.2 (*c* 1.0, water)}; δ_H (400 MHz; D_2O) 2.01 (s, 3 H, Me), 2.03 (s, 3 H, Me), 3.41–3.39 (m, 1 H), 3.91–3.50 (m, 16 H), 4.03 (d, 1 H, *J* 3.2, H-2″), 4.57 (0.45 H, d, *J* 7.70, H-1′), 4.58 (0.55 H, d, *J* 7.72, H-1′), 4.67 (0.45 H, d, *J* 8.16, H-1),

4.73 (1 H, s, H-1″) and 5.16 (0.55 H, d, *J* 2.76, H-1); δ_C (100 MHz; D_2O) [α and β refer to the configuration at the reducing-end anomeric centre] 22.55 (α-Me), 22.79 (Me), 22.84 (β-Me), 54.32 (α-C-2), 55.72 (C-2′), 56.78 (β-C-2), 60.67 (β-C-6), 60.74 (α-C-6 and C-6′), 61.60 (C-6″), 67.29 (C-4″), 69.92 (C-3′), 70.67 (α-C-3), 71.18 (α-C-5), 72.66 (C-2″), 73.15 (β-C-3), 73.43 (C-3″), 75.26 (C-5′ and β-C-5), 77.10 (C-5″), 79.31 (C-4′), 79.91 (β-C-4), 80.36 (α-C-4), 91.12 (α-C-1), 95.50 (β-C-1), 100.75 (C-1″), 102.05 (C-1′), 175.15 (α-CO, reducing end), 175.26 (CO) and 175.42 (β-CO, reducing end); *m/z* (M^+) (Found: M^+, 586.2317. $C_{22}H_{38}N_2NaO_{16}$ requires M, 586.2216).

Large-scale reaction

p-Nitrophenyl β-D-mannopyranoside **4** (0.5 g, 1.66 mmol) and *N,N′*-diacetylchitobiose **3** (5 g, 16.6 mmol) in McIlvain (citrate–phosphate) buffer (0.05 M; pH 4.5; 5 cm^3) were incubated with the β-D-mannosidase (0.5 cm^3; 0.49 U/mg protein, 108 mg protein cm^{-3}) at 30 °C for 4 h. The mixture was heated in a boiling water-bath for 5 min. The product was purified by carbon-Celite chromatography as for the product obtained using the enzyme from *H. pomatia*, above, to give the trisaccharide **1**, 157 mg, 16%.

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