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Glycosidases—a great synthetic tool

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Abstract

Glycosidases were used to prepare oligosaccharide structures of physiological and medicinal relevance. The study included an extensive screening of crude enzymatic preparations for α- and β-galactosidase, α- and β-mannosidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase and α-L-fucosidase activities. The enzymes were assessed with respect to regioselectivity of glycosyl transfer on to carbohydrate acceptors. The purification procedures for individual biocatalysts are described in detail.

Keywords: Glycosidases; Screening; Enzymatic glycosylation; Oligosaccharide synthesis

1. Introduction

A number of carbohydrate ligands play a crucial role in the most fundamental processes in living organisms [1]. The elucidation of their function greatly depends on the availability of the corresponding oligosaccharide structures. Even though the carbohydrate ligands of glycoproteins and glycolipids contain only a limited number of monosaccharide building blocks, they are linked in a specific manner presenting considerable difficulties with respect to synthesis by conventional methods [2].

Notwithstanding the impressive development of new and efficient methods for the synthesis of complex carbohydrates, the real limitation still remains—the need to employ extensive protection–deprotection sequences. In recent years, this problem has been addressed by the application of enzymes [3–5]. Enzyme-catalysed glycosidic bond formation takes place in a single step, avoiding protection–deprotection sequences, and with complete control of the configuration at the anomeric centre. Accordingly, the time and effort required for the synthesis of oligosaccharides is drastically reduced.

Of the two enzymatic approaches, one using glycosyl transferases and the other glycosidases, the former offers regioselective transfer and high glycosylation yields. In some well-tuned systems yields are almost quantitative, at the expense of nucleotide donors that are costly or have to be generated in situ using additional complex enzymatic machinery. Moreover, glycosyl transferases are also highly selective with respect to the glycosyl acceptors, and the glyco-

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syl transfer rarely occurs on to a modified or exogeneous substrate. Glycosidases accommodate much simpler and readily available substrates. However, in the presence of water as a competing nucleophile the transglycosylation yields are less impressive. On the other hand, glycosidases are quite robust and can be handled easily in an organic chemistry laboratory. Their occurrence in animal and plant tissues is widespread, and they are abundantly produced by a host of micro-organisms.

To establish a range of glycosidases that can be used to generate interglycosidic linkages of the kind found in glycoproteins and glycolipids (Fig. 1), an extensive screening and assessment of the regioselectivity of glycosyl transfer was needed. The glycosidase activities present in several dozen enzymatic preparations are reviewed. The isolation and purification of some enzymes applicable to the synthesis of oligosaccharides are presented in detail together with the assessment of the regioselectivity of the glycosyl transfer on to certain carbohydrate acceptors.

2. Materials and methods

2.1. Sources of glycosidases

Most enzyme preparations were obtained from commercial sources, i.e., Amano (Japan), Biocatalysts (UK), Daiwa Kasei (Japan), Genencor (USA), Interspex (USA), K.I. Chemicals (Japan), Kikkoman (Japan), Megazyme (Ireland), Rohm & Haas (USA), Sankyo (Japan), Sigma (USA).

Strains of *Aspergillus niger* var. Tieghem were obtained from IMI culture collection (Cambridge, UK), and the gram-negative soil *Coccobacillus* 13949 was from ATCC (MD,

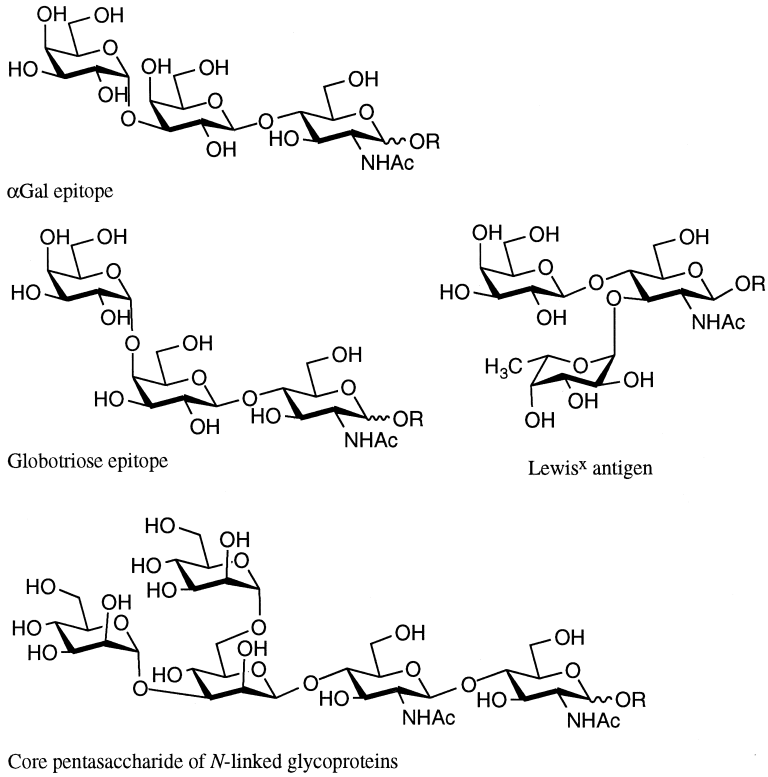


Fig. 1. Target molecules for a glycosidase-catalysed synthesis: complex carbohydrate structures.

USA). A crude β-*N*-acetylhexosaminidase from *A. terreus* was obtained by ammonium sulfate fractionation of spent cultivation media (Dr. V. Kren, Institute of Microbiology, Prague, Czech Republic). A sample of Helikase, a crude enzyme mixture from the digestive juice of the edible snail *Helix pomatia*, was also provided by Dr. V. Kren.

Fresh white edible mushrooms, alfalfa seeds, and 'Golden Delicious' apples were purchased in a local supermarket. Liver and epididymis from freshly sacrificed pigs were either immediately processed or quick-frozen and stored at −70°C.

2.2. Screening

An assay based on the ability of some glycosidases to release *p*-nitrophenol from corresponding *p*-nitrophenyl glycosides was designed to identify the sources of α-galactosidase (EC 3.2.1.22), β-*N*-acetylhexosaminidase (EC 3.2.1.52), β-mannosidase (EC 3.2.1.25), α-mannosidase (EC 3.2.1.24), and α-L-fucosidase (3.2.1.51). The *p*-nitrophenyl moiety not only provides a good leaving group in transglycosylation reactions, but these derivatives are readily available at low cost.

2.3. Assay methods

A sample of enzyme solution (10 μl) was added to McIlvain buffer (50 mM, pH 5.0, 90 μl) containing the corresponding *p*-nitrophenyl glycoside as substrate (5 mM), and incubated for 10 min at 30°C. The reaction was stopped by addition of Na_2CO_3 (0.1 M, 3.9 ml) and the released *p*-nitrophenol was determined spectrophotometrically at 400 nm.

Protein concentration was assayed according to Bradford [6] with bovine serum albumin as a standard (Bio-Rad).

One unit of activity was defined as the amount of the enzyme releasing 1 μmol of *p*-nitrophenol/min. The specific activity was expressed as units per milligram of protein.

2.4. General procedures

All enzyme preparations were handled at 4°C, unless otherwise stated in the text. The protein extraction from crude sources was performed with ice-bath stirring of the powder with extraction buffer for 1 h. A Waters 650 E Advanced Protein Purification System (Millipore, USA) was employed for chromatography of proteins on columns equipped with a cooling jacket (Pharmacia XK series). CM-Sepharose, DEAE-Sepharose, high substituted Phenyl-Sepharose and Q-Sepharose were from Pharmacia. Macro-Prep ceramic hydroxyapatite was from Bio-Rad. Elution buffers were prepared with ultra-pure water (Elga); other chemicals were at least of an analytical grade. A Sorvall RC-5B refrigerated superspeed centrifuge with a GSA or SS-34 rotor was used for centrifugation. Large volumes of diluted protein samples were concentrated by ammonium sulfate precipitation (100% saturation) while membrane ultra-filtration (30 kDa molecular weight cut-off) was used with smaller sample volumes. The enzyme preparations were stored either in ammonium sulfate (approximately 2.5 M) or in the presence of 0.02% sodium azide.

2.5. Glycosidase preparations

A. niger var. Tieghem was maintained on potato–dextrose agar (old potatoes 100 g, D-glucose 7.5 g, agar 10 g, water to 0.5 l). The production liquid medium (yeast extract 0.4 g, bacto-peptone 4 g, dihydrogen potassium phosphate 2.4 g, di-potassium hydrogen phosphate 4 g, water to 0.8 l) was supplemented with hog gastric mucin (4 g) and cultures were grown for 6 days (25°C, 120 rpm). The mycelium was filtered off, spent medium was centrifuged and the proteins precipitated between 50–100% ammonium sulfate saturation were harvested by centrifugation.

The *Coccobacillus* 13949 was maintained on minimal medium (ammonium sulfate 1 g, di-potassium hydrogen phosphate 2 g, hog gastric

mucin 10 g, agar 10 g, water to 1 l, pH 7.1) [7]. The bacterium was grown in the production medium (ammonium sulfate 1 g, di-potassium hydrogen phosphate 2 g, hog gastric mucin 1 g, water to 1 l, pH 7.1) for 3 days (35°C, 150 rpm). The bacteria were pelleted by centrifugation, resuspended in potassium phosphate buffer (10 mM, pH 7.1, 0.1 mM PMSF) and disrupted by sonication. After centrifugation (10,000 × *g*, 10 min) the supernatant was treated with streptomycin sulfate (20 ml of 10% w/v solution/100 ml of cell-free extract, 30 min), clarified by centrifugation (10,000 × *g*, 10 min), and fractionated by ammonium sulfate (30–100% saturation). This treatment afforded 0.5 units of α-fucosidase activity from 6 l of processed medium.

White closed cup mushrooms (*Agaricus* sp.) were washed with distilled water, diced and homogenised in potassium phosphate buffer (50 mM, pH 6.5, 0.1 mM PMSF). The suspension was stirred on ice for 30 min and centrifuged (8500 × *g*, 20 min). The proteins in the supernatant precipitated by ammonium sulfate (100% saturation) were collected by centrifugation (8500 × *g*, 20 min).

'Golden Delicious' apples (*Malus domestica* Borhk.) were rinsed with distilled water, peeled, and the diced cortex (620 g) was homogenised in 1 l of sodium acetate buffer (25 mM, pH 4.0, 0.1 mM EDTA). The homogenate was filtered through a fine cloth and centrifuged (8500 × *g*, 20 min). The proteins in the supernatant were precipitated by ammonium sulfate (100% saturation) and harvested by centrifugation (8500 × *g*, 20 min).

Alfalfa seeds (*Medicago sativa*, 140 g) were surface sterilised (0.5% sodium hypochlorite solution, 10 min), thoroughly rinsed with distilled water, and kept in distilled water for 6 h with several changes of water. Soaked seeds were spread on filter papers kept moist, and allowed to germinate for three days at room temperature. The germinated seeds were homogenised in a food processor with 1 l of potassium phosphate buffer (50 mM, pH 6.5). The mash was incubated at 35°C for 1 h and was then re-processed. After centrifugation (8500 × *g*, 30 min) the proteins from the supernatant were precipitated by ammonium sulfate (65% saturation) and dialysed against potassium phosphate buffer (20 mM, pH 6.5). Chromatography on DEAE-Sepharose (20 mM potassium phosphate buffer pH 6.5, elution gradient 0–0.25 M NaCl) and subsequently on hydroxyapatite (5 mM potassium phosphate buffer pH 6.5, elution gradient 0.005–0.5 M potassium phosphate buffer pH 6.5) yielded fractions enriched in β-*N*-acetylgalactosaminidase, β-*N*-acetylglucosaminidase, α-galactosidase and α-mannosidase activities, respectively. α-L-fucosidase activity detected in the starting material was not recovered in the final step. All buffers for the handling of α-mannosidase were supplemented with 0.1 mM zinc sulfate.

Defatted almond meal (20 g, Sigma) was extracted with 200 ml of sodium acetate buffer (10 mM, pH 4.5, 5 mM 2-mercaptoethanol, 0.1 mM zinc sulfate) and filtered through a fine cloth. The precipitate collected between 30–75% ammonium sulfate saturation was dialysed against the above buffer, clarified by centrifugation (8500 × *g*, 20 min), and chromatographed on CM-Sepharose (20 mM sodium acetate buffer pH 4.6, 0.1 mM zinc sulfate, 5 mM 2-mercaptoethanol, elution with linear 0–0.3 M NaCl gradient). α-Mannosidase was collected by ammonium sulfate precipitation (100% saturation).

Fresh pig epididymis (60 g) was cut into small pieces and homogenised with 150 ml of McIlvain buffer (50 mM, pH 4.5). The homogenate was filtered, centrifuged, and the concentrated sample was fractionated by ammonium sulfate (20–70% saturation). The collected precipitate was dialysed against McIlvain buffer (50 mM, pH 5.0), clarified by centrifugation and stored at 4°C.

Pieces of pig liver tissue from deep-frozen stock were homogenised in volume of potassium phosphate buffer (25 mM, pH 7.6) containing potassium chloride (50 mM), EDTA (1 mM), 2-mercaptoethanol (5 mM), pepstatin

(0.1 mM), and PMSF (0.1 mM). The homogenate was centrifuged, filtered through a fine cloth, and the protein precipitate collected between 40–70% ammonium sulfate was dialysed against potassium phosphate buffer (2 mM, pH 7.6, 5 mM 2-mercaptoethanol, 10% glycerol). The dialysate was treated with polyvinylpyrroline (2% w/v, 30 min stirring on ice), the precipitate was removed by centrifugation and the protein sample was chromatographed on a Q-Sepharose column (same buffer, elution with linear 0–1 M NaCl gradient). The proteins in active fractions were collected by ammonium sulfate precipitation (100% saturation).

Amylase from barley malt (type VIII, Sigma) was extracted with McIlvain buffer (50 mM, pH 5.0). The extract was clarified by centrifugation and proteins were fractionated by ammonium sulfate. Most glycosidases were recovered between 40–60% ammonium sulfate saturation.

Cellulase *A. niger* (Sigma) was extracted with McIlvain buffer (50 mM, pH 5.3, EDTA), clarified by centrifugation, and proteins were

Table 1
Substrates used in transglycosylation reactions

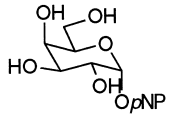
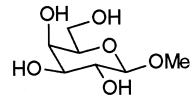
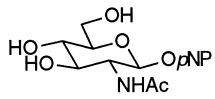
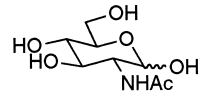
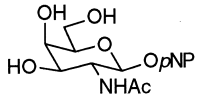
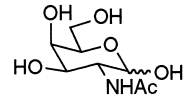
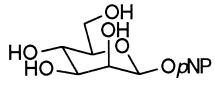
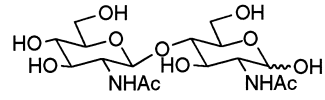
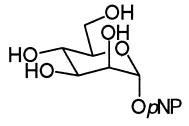
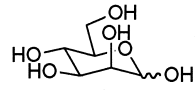
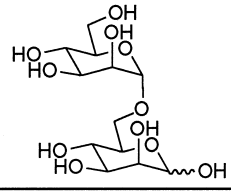
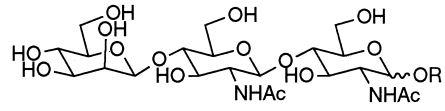
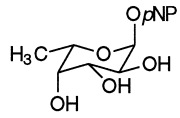
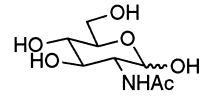
Enzyme	Glycosyl donor	Carbohydrate acceptor
α-Galactosidase		
β-*N*-Acetyl-glucosaminidase		
β-*N*-Acetyl-galactosaminidase		
β-Mannosidase		
α-Mannosidase		
α-Mannosidase		
α-L-Fucosidase		

Table 2
Glycosidase activities detected in enzymatic preparations

Preparation (origin)	Glycosidase activity[a]						
	α-Gal	β-Gal	α-M	β-M	β-GlcN	β-GalN	α-Fuc
Agaricus sp. (homogenate)	+	0	0	0	+	+	0
Alfalfa (seed homogenate)	+	+	+	+	+	+	0
Almond meal (Sigma)	+	+	+	0	+	+	0
Amylase *A. oryzae* X-A (Sigma)	+	+	0	0	+	+	0
Amylase *Bacillus* sp. (Sigma)	0	0	0	0	0	0	0
Amylase barley malt VIII-A (Sigma)	+	+	+	+	+	+	0
Amylase porcine pancreas VI-B (Sigma)	0		0	0	0		0
Amyloglucosidase *Rhizopus* (Sigma)	+	+	0	0	+	+	0
Apple (homogenate)	0	+	0	+	+	+	0
Aspergillus terreus (cultured)	0	+	0	0	+	+	0
A. niger IBM 60286 (cultured)	+	+	0	0	+		0
A. niger IBM 50565 (cultured)	+	+	0	0	+		0
A. niger IBM 329399 (cultured)	+	+	0	0	+		0
A. niger IBM 358033 (cultured)	+	0	0	0	+		0
Biolacta *B. circulans* (Daiwa Kasei)	0	+	0	0	+	+	0
Cellulase A *A. niger* (Amano)	+	+	0	+	+	+	0
Cellulase *A. niger* (Sigma)	+	+	+	+	+	+	0
Cellulase *P. funiculosum* (Sigma)	+	+	0	+	+	+	0
Cellulase *T. reesei* ATCC 26921 (Sigma)	+	+	0	0	+	+	0
Cellulase T *T. viride* (Amano)	+	+	0	+	+	+	0
Cellulase *T. viride* (Sigma)	+	+	0	0	+	+	0
Cellulase C013L (Biocatalysts)	+	+	0	0	+	+	0
Cellulase 5A (Lyven)	+	+	0	0	+	+	0
Coccobacillus ATCC 13949 (cultured)	+	+	0	0	+	+	+
Cyclodextrin glucanotransferase *B. macerans* (Amano)	0	0	0	0	+	0	0
Driselase (Interspex)	+	+			+	+	
Ficin (Sigma)	+	0	0	0	0	0	0
α-Fucosidase bovine epidydimis (Sigma)	0	0	0	0	0	0	+
α-Fucosidase bovine kidney (Sigma)	0	0	0	0	0	0	+
α-Fucosidase human placenta (Sigma)	0	0	0	0	0	0	+
α-Galactosidase *A. niger* (Sigma)	+	+	0	0	0	0	0
α-Galactosidase coffee beans (Sigma)	+	0	0	0	0	0	0
α-Galactosidase guar (Megazyme)	+	0	0	0	0	0	0
β-Galactosidase *A. oryzae* XI (Sigma)	+	+	0	+	+	+	0
β-Glucuronidase bovine liver B-1 (Sigma)	+	+	+	+	+	+	+
β-Glucuronidase *E. coli* IX-A (Sigma)	0	0	0	0	0	0	0
β-Glucuronidase limpets L-II (Sigma)	+	+	+	+	+	+	+
β-Glucuronidase mollusc (Sigma)	+	+	+	+	+	+	+
β-Glucuronidase scallops S-1 (Sigma)	0	0	0	0	+	+	+
β-Glucuronidase snail H-1 (Sigma)	+	+	0	+	+	+	
β-Glucuronidase snail H-5 (Sigma)	+	+	0	+	+	+	
Helikase (V. Kren)	+	+	0	+	+	+	+
β-*N*-acetylhexosaminidase *A. niger* (Sigma)					+		
β-*N*-acetylhexosaminidase jack beans (Sigma)	0	0	0	0	+	+	0
Hemicellulase *A. niger* (Amano)	+	+	0	+	+	+	+
Inolage 44L (Genencor)	+	+	0	0	+	+	0
Irgazyme (Genencor)	+	+	0	0	+	+	0
Lactase F *A. oryzae* (Amano)	+	+	0	+	+	+	0
Lactase P *P. multicolor* (K.I. Chemicals)	+	+	0	0	+	+	+
α-Mannosidase jack beans (Sigma)	0	0	+	0	0	0	0
Molsin (Kikkoman)	+	+	+	+	+	+	0
Pig epididymis (homogenate)		0			+		

Table 2 (continued)

Preparation (origin)	Glycosidase activity[a]						
	α-Gal	β-Gal	α-M	β-M	β-GlcN	β-GalN	α-Fuc
Pig liver (homogenate)					+		
Primafast 100 (Genencor)	+	+	0	0	+	+	0
Rhozyme HP 150 *A. niger* (Rohm & Haas)	+	+	0	+	+	+	0
Takadiastase *A. oryzae* (Sankyo)	+	+	0	0	+	+	0
Transglucosidase L *A. niger* (Amano)	+	+	0	+	+	+	0
Viscozyme (Interspex)	+	+	0	+	+	+	+

[a] α-Gal = α-galactosidase; β-Gal = β-galactosidase; α-M = α-mannosidase; β-M = β-mannosidase; β-GlcN = β-*N*-acetylglucosaminidase; β-GalN = β-*N*-acetylgalactosaminidase; α-Fuc = α-L-fucosidase.

precipitated by ammonium sulfate (100% saturation). The glycosidases were separated by chromatography on Phenyl-Sepharose potassium phosphate buffer pH 6.5, 1 M ammonium sulfate; eluting with descending gradient of salt concentration 1–0 M ammonium sulfate) followed by separation on DEAE-Sepharose (20 mM potassium phosphate buffer pH 6.5, elution gradient 0–0.5 M NaCl). α-Galactosidase free of β-galactosidase activity was obtained by further chromatography on CM-Sepharose (20 mM sodium acetate buffer, pH 4.4, elution gradient 0–0.5 M NaCl).

β-Galactosidase from *A. oryzae* (type XI, Sigma) was a source of β-*N*-acetylhexosaminidase, β-mannosidase and two α-galactosidases [8]. The purification procedures for β-*N*-acetylhexosaminidase and β-mannosidase have been described in detail [9]. After extraction with potassium phosphate buffer (50 mM, pH 6.5, 1 mM EDTA) and ammonium sulfate precipitation (100% saturation), chromatography on Phenyl-Sepharose (20 mM potassium phosphate buffer pH 6.5, 0.5 M ammonium sulfate, eluted with decreasing salt concentration 0.5–0 M ammonium sulfate gradient) followed by separation on DEAE-Sepharose (20 mM potassium phosphate buffer pH 6.5, elution with linear 0–1 M NaCl gradient) yielded an α-galactosidase preparation free from of β-*N*-acetylhexosaminidase and β-mannosidase activities. Chromatography on ceramic hydroxyapatite (0.005 M potassium phosphate buffer pH 6.5, elution with increasing buffer concentration 0.005–0.15 M gradient) separated two forms of α-galactosidase with remarkably different regioselectivities of galactosyl transfer [10].

Hemicellulase from *A. niger* (10 g; Amano) was extracted with 50 ml potassium phosphate buffer (10 mM, pH 6.5, 5 mM 2-mercaptoethanol, 1 mM EDTA) and fractionated by ammonium sulfate. The proteins collected between 40–80% saturation were dialysed against potassium phosphate buffer (20 mM, pH 6.8). Protein fractions enriched in α-galactosidase, β-mannosidase, β-*N*-acetylhexosaminidase, and α-L-fucosidase activities, respectively, were obtained after chromatography on DEAE-Sepharose (same buffer, elution with linear 0–0.4 M NaCl gradient).

Lactase P from *Penicillium multicolor* (3 g; K.I. Chemicals) was extracted with 40 ml of distilled water. Proteins precipitated by dropwise addition of cold ethanol (50% v/v) were collected by centrifugation, dialysed against potassium phosphate buffer (10 mM, pH 6.5), and chromatographed on DEAE-Sepharose (same buffer, elution with linear 0–0.8 M NaCl gradient) to obtain α-L-fucosidase and α-galactosidase rich fractions. Further purification of α-galactosidase on ceramic hydroxyapatite (5 mM potassium phosphate buffer pH 6.5, elution with linear 0.005–0.25 M buffer concentration gradient) removed all but traces of β-galactosidase activity so abundant in the original crude sample [11].

Transglucosidase L from *A. niger* (Amano) was diluted with distilled water (two-fold) and

proteins obtained between 40–70% ammonium sulfate saturation were collected and dialysed against potassium phosphate buffer (20 mM, pH 6.5). Chromatography on Q-Sepharose (same buffer, elution with linear 0–0.27 M NaCl gradient) separated two forms of α-galactosidase with strikingly different transglycosylation regioselectivities [12]. An enriched α-mannosidase fraction was also obtained in this step. Further purification of the α-galactosidase on ceramic hydroxyapatite (0.005 M potassium phosphate buffer pH 6.5, elution with linear 0.005–0.25 M buffer concentration gradient) yielded a preparation free from β-galactosidase.

2.6. Transglycosylation reactions

The acceptor saccharide was used in five-fold molar excess except in the case of the mannose core trisaccharide which was used in equimolar ratio based on the activated *p*-nitrophenyl glycoside donor (Table 1). The transglycosylations were carried out in McIlvain buffer (50 mM, pH range 4.0–6.5 depending on the pH optimum of the biocatalyst) at 30°C with gentle stirring. Aliquots (30 μl) were diluted with distilled water (50 μl), boiled for 2 min and assayed by HPLC.

2.7. Product identification

HPLC analyses were carried out using a Gilson HPLC instrument with a Hypersil 5AP (aminopropylsilica) column (20 cm × 4.6 mm), either UV (210 nm) or light scattering detector, and with acetonitrile–water (approximate ratio 80:20; varying according to the saccharide assayed) as eluent at a flow rate of 1.75 cm^3/min. The reaction products were compared to standards prepared as described elsewhere [13–16].

3. Results

3.1. Screening

The screening included a number of enzyme preparations, i.e., commercially available crude mixtures, products of microbial cultivations, or plant and animal tissue homogenates that can be easily prepared in any laboratory without the need for sophisticated equipment (Table 2). It has provided us with a valuable information regarding the choice of a starting material for subsequent glycosidase purification. It also alerted us of the presence of other glycosidases that had to be removed from the preparation prior to its use with more complex carbohydrate acceptors, e.g., β-galactosidase contamination in preparations to be used with lactose or lactosamine as acceptors.

A few facts became obvious from studying the glycosidase profile of individual enzyme sources. First, most of the preparations considered as sources of α-galactosidase had also an abundant β-galactosidase activity. This was quite unfortunate, as it precluded the use of a crude enzyme with some acceptors (e.g., lactose, lactosamine), and a further purification had to be carried out.

β-*N*-Acetylhexosaminidase was yet another glycosidase widely distributed in the samples tested. Both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities were often present, and the ratio between these two activities remained constant throughout the purification. The homogenate from alfalfa seeds was exceptional in this respect as further purification separated the two activities.

The almost ubiquitous occurrence of the above glycosidases contrasted strongly with the far less abundant β-mannosidase. We identified only a few microbial preparations containing this enzyme, mainly from *Aspergillus* sp., and some tissue homogenates (alfalfa, barley, bovine liver, molluscs). In all instances a further purification was necessary to eliminate β-*N*-acetylhexosaminidase activity if *N*,*N*′-diacetylchitobiose was to be used as an acceptor to obtain the core trisaccharide structure of the *N*-linked glycoproteins.

α-Mannosidase activity was detected in plant seeds (alfalfa, almonds, barley), some tissue homogenates (bovine liver, salt-water molluscs)

Table 3
Transglycosylation products obtained with α-galactosidases

Enzyme preparation	Major product	Other products
Agaricus sp. (homogenate)	α(1 → 6)	
Almond meal (Sigma)	α(1 → 6)	
Amylase barley malt VIII-A (Sigma)	α(1 → 6)	
A. niger IBM 60286	α(1 → 6)	
A. niger IBM 50565	α(1 → 6)	
A. niger IBM 329399	α(1 → 6)	
A. niger IBM 358033	α(1 → 6)	
Cellulase A *A. niger* (Amano)	α(1 → 6)	
Cellulase C013L (Biocatalysts)	α(1 → 6)	
α-Galactosidase *A. niger* (Sigma)	α(1 → 6)	α(1 → 3)
α-Galactosidase coffee beans (Sigma)	α(1 → 6)	α(1 → 3)
α-Galactosidase guar (Megazyme)	α(1 → 6)	α(1 → 3)
β-Galactosidase[a] *A. oryzae* XI (Sigma)	α(1 → 6)	α(1 → 3)
β-Galactosidase[a] *A. oryzae* XI (Sigma)	α(1 → 6)	α(1 → 3)
β-Glucuronidase bovine liver B-1 (Sigma)	α(1 → 6)	
β-Glucuronidase limpets L-II (Sigma)	α(1 → 6)	
β-Glucuronidase snail H-1 (Sigma)	α(1 → 6)	
Hemicellulase *A. niger* (Amano)	α(1 → 6)	
Irgazyme (Genencor)	α(1 → 6)	
Lactase P *P. multicolor* (K.I. Chemicals)	α(1 → 3)	
Molsin (Kikkoman)	α(1 → 6)	
Rhozyme HP 150 *A. niger* (Rohm & Haas)	α(1 → 6)	
Transglucosidase L[a] *A. niger* (Amano)	α(1 → 3)	α(1 → 6)

[a]Two different α-galactosidases have been purified from this preparation [8,12].

Table 4
Transglycosylation products obtained with β-*N*-acetylhexosaminidases

Enzyme preparation	Major product	Other products
Agaricus sp. (homogenate)	β(1 → 4)	
Alfalfa[a] (seed homogenate)	β(1 → 6)	β(1 → 4)
Alfalfa[b] (seed homogenate)	β(1 → 4)	β(1 → 6)
Almond meal (Sigma)	β(1 → 6)	β-(1 → 4)
Amylase barley malt VIII-A (Sigma)	β(1 → 4), β(1 → 6)	
Apple (homogenate)	β(1 → 6)	
A. terreus (cultured)	β(1 → 4)	β(1 → 6)
Cellulase A *A. niger* (Amano)	β(1 → 4)	β(1 → 6)
Cellulase *A. niger* (Sigma)	β(1 → 4)	
Cellulase *P. funiculosum* (Sigma)	β(1 → 4), β(1 → 6)	
β-Galactosidase *A. oryzae* XI (Sigma)	β(1 → 4), β(1 → 6)	
β-Glucuronidase limpets L-II (Sigma)	no transfer product	
β-Glucoronidase mollusc (Sigma)	no transfer product	
Hemicellulase *A. niger* (Amano)	β(1 → 4)	β(1 → 6)
β-*N*-acetylhexosaminidase *A. niger* (Sigma)	no transfer product[c]	
β-*N*-acetylhexosaminidase jack beans (Sigma)	β(1 → 6)	
Irgazyme (Genencor)	β(1 → 4)	β(1 → 6)
Pig epididymis (homogenate)	β(1 → 6)	
Pig liver (homogenate)	no transfer product	

[a]β-*N*-Acetylglucosaminidase fraction with GlcNAc/GalNAc activities ratio 6.5.
[b]β-*N*-Acetylgalactosaminidase fraction with GlcNAc/GalNAc activities ratio 0.12, *p*-nitrophenyl *N*-acetyl-β-D-galactopyranoside used as glycosyl donor.
[c]Hydrolyses *N,N′*-diacetylchitobiose.

Table 5
Transglycosylation products obtained with β-mannosidases

Enzyme preparation	Product
Amylase barley malt VIII-A (Sigma)	no transfer product
β-Galactosidase *A. oryzae* XI (Sigma)	β(1 → 4)
Helikase	β(1 → 4)

and two microbial preparations. Even though there is evidence for the widespread occurrence of microbial α-mannosidases [17], many of these enzymes were not detected by our screening protocol as they do not utilise *p*-nitrophenyl α-D-mannopyranoside as a substrate. Nevertheless, we included the α-mannosidase from *A. niger* (Transglucosidase L Amano) which utilised Manα(1 → 6)Man as substrate [16].

α-L-Fucosidase activity was detected in tissue homogenates (bovine liver, molluscs), almond meal and some microbial preparations. Attempts to elicit α-L-fucosidase production by supplementing the microbial cultivation media with hog gastric mucin were successful only with the *Coccobacillus* culture, as reported earlier [7].

3.2. Regioselectivity of glycosyl transfer

With *p*-nitrophenyl α-D-galactopyranoside as an activated donor and methyl β-D-galactopyranoside as an acceptor, the majority of α-galactosidases produced the α(1 → 6)-linked disaccharide, in some cases accompanied with traces of α(1 → 3)-linked product (Table 3).

A remarkable result was obtained with the α-galactosidase isolated from *P. multicolor* (Lactase P) which yielded exclusively the α(1 → 3) disaccharide [11].

β-*N*-Acetylhexosaminidases were assayed with *p*-nitrophenyl *N*-acetyl-β-D-glucopyranoside as donor and *N*-acetylglucosamine as acceptor. β-*N*-acetylhexosaminidases of plant and microbial origin proved to be efficient catalysts yielding a mixture of β(1 → 4)- and β(1 → 6)-linked disaccharides. Preparations from *Agaricus* and *A. niger* (Cellulase, Sigma) gave exclusively *N,N′*-diacetylchitobiose, while the apple homogenate produced selectively the β(1 → 6)-linked disaccharide. No glycosyl transfer was observed with the enzymes from molluscs and pig epididymis and, interestingly, neither with the commercially available pure β-*N*-acetylhexosaminidase from *A. niger*, even though this enzyme readily hydrolysed *N,N′*-diacetylchitobiose (Table 4).

The purification of an alfalfa seed homogenate yielded a protein fraction rich in β-*N*-acetylgalactosaminidase activity (the activity towards *p*-nitrophenyl *N*-acetyl-β-D-galactopyranoside exceeded more than eight times the activity towards *p*-nitrophenyl *N*-acetyl-β-D-glucopyranoside) which gave predominantly GalNAcβ(1 → 4)GalNAc in the reaction with *p*-nitrophenyl *N*-acetyl-β-D-galactopyranoside and *N*-acetylgalactosamine as substrates (Table 4).

Table 6
Transglycosylation products obtained with α-mannosidases

Enzyme preparation	Product	
	With mannose[a]	Core trisaccharide[a]
Alfalfa (seed homogenate)	α(1 → 2), α(1 → 3)	–
Almond meal (Sigma)	α(1 → 2), α(1 → 3)	no transfer product[b]
β-Glucuronidase limpets L-II (Sigma)	α(1 → 2), α(1 → 3)	–
α-Mannosidase jack beans (Sigma)	no transfer product	–
Transglucosidase L *A. niger* (Amano)	α(1 → 6)	no transfer product[c]

[a] Used as a glycosyl acceptor.
[b] Mixture of Manα(1 → 2)Man and Manα(1 → 3)Man used as glycosyl donor.
[c] Manα(1 → 6)Man used as glycosyl donor.

Table 7
Transglycosylation products obtained with α-L-fucosidases

Enzyme preparation	Product
Driselase (Interspex)	no transfer product
α-L-Fucosidase bovine epididymis (Sigma)	traces
α-L-Fucosidase bovine kidney (Sigma)	no transfer product
α-L-Fucosidase human placenta (Sigma)	no transfer product
β-Glucuronidase limpets L-II (Sigma)	no transfer product
Lactase P *P. multicolor* (K.I. Chemicals)	α(1 → 3)
Viscozyme (Interspex)	no transfer product

Only a few enzymatic preparations were tested for their ability to transfer the β-mannosyl residue from *p*-nitrophenyl β-D-mannopyranoside on to *N,N′*-diacetylchitobiose. That was because β-mannosidase preparations had to be extensively purified to remove any β-*N*-acetylhexosaminidase activity present to prevent the hydrolysis of the disaccharide acceptor. β-Mannosidases from *A. oryzae* (crude β-galactosidase) and snail digestive juice (Helikase) both gave a single product, Manβ(1 → 4)GlcNAcβ(1 → 4)GlcNAc [9], while the enzyme from barley did not yield any trisaccharide (Table 5).

The transfer activity of α-mannosidase was first assayed with *p*-nitrophenyl α-D-mannopyranoside as donor and mannose as acceptor. The α-mannosidase from alfalfa, almonds and limpets produced a mixture of α(1 → 2)- and α(1 → 3)-linked disaccharides. When the core trisaccharide structure was used as an acceptor with these enzymes, no transfer product was detected (Table 6). Regrettably, that was also true for the α-mannosidase from *A. niger* (Transglucosidase L), in this case using Manα(1 → 6)Man as sugar donor.

We intended to use lactosamine as a substrate for α-L-fucosidases. However, it proved to be extremely difficult at this stage to separate the enzyme from a crude mixture. Accordingly, a monosaccharide acceptor, *N*-acetylglucosamine, was employed with *p*-nitrophenyl α-L-fucoside as glycosyl donor. Glycosyl transfer occurred with the α-L-fucosidase from *P. multicolor* (Lactase P) to give L-Fucα(1 → 3)GlcNAc, as reported earlier [18]. Only traces of disaccharide product were observed with a commercial preparation from bovine epididymis (Table 7).

4. Conclusion

For the preparation of complex oligosaccharides glycosyl transferases are often preferred to much cheaper glycosidases, not so much for their higher glycosyl transfer yields but rather owing to the strict regioselectivity of these biocatalysts. It becomes clear, however, that some of the glycosidases are also capable of regioselective transfer [11,19,20]. The inspection of glycosidases undertaken in this project supports this view. Such enzymes when discovered and sufficiently characterised can provide an efficient and cost effective tool for the preparation of complex oligosaccharides so much in demand for advancing biological and medicinal research.

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