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A HIGHLY REGIOSELECTIVE SYNTHESIS OF MANNOBIOSE AND MANNOTRIOSE BY REVERSE HYDROLYSIS USING SPECIFIC 1,2-α-MANNOSIDASE FROM *ASPERGILLUS PHOENICIS*

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SUMMARY

A specific 1,2-α-mannosidase was isolated from *A. phoenicis* and used in the equilibrium-controlled synthesis of α1→2-linked mannobiose and mannotriose. Yields of 22.33 % disaccharide, 8.21 % trisaccharide and 2.74 % tetrasaccharide were obtained. Regioselectivity was absolute for the 1→2 linkage.

INTRODUCTION

Glycosidases are potentially useful catalysts for the synthesis of small bioactive and novel oligosaccharides (Rastall and Bucke, 1992). The most significant problem with these enzymes is a tendency toward low regioselectivity, many glycosidic linkages being formed in most reported syntheses. Most of these studies, however, have used non linkage-specific enzymes. It is not, perhaps, surprising that they catalyse the formation of several linkages. In this paper we report the synthesis regioselective of α1→2 linked mannobiose and mannotriose, common components of "high mannose" oligosaccharides, using α1,2-mannosidase from *Aspergillus phoenicis*. The equilibrium reactions used gave good yields and allowed starting material to be recycled with minimal losses.

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MATERIALS AND METHODS

Materials.

All materials were obtained from Sigma (Poole, Dorset) with the exception of Sephadex G-100 and DEAE-Sephadex A-50 (Pharmacia-LKB, Milton Keynes, UK), a Spherisorb-NH_2 column (PhaseSep, Clwyd, UK), HPLC grade acetonitrile (Rathburn, Walkerburn, UK), BioGel-P2 (Bio-Rad, Watford, Herts)

Extraction and partial purification of 1,2-α-mannosidase

Aspergillus phoenicis (ATCC -14332) was grown on wheat bran, and the crude enzyme was extracted and concentrated in a 80% saturated ammonium-sulphate solution and 65% ethanol according to Ichishima *et al* (1981). Partial purification of 1,2-α-mannosidase was carried out by gel-filtration chromatography on Sephadex G-100, eluted with 0.1 M sodium acetate buffer pH 5.0/0.2 M NaCl. All fractions exhibiting 1,2-α-mannosidase were collected and dialysed against 0.01 M sodium acetate buffer pH 5.0. The enzyme solution was then applied to a column of DEAE Sephadex A-50 and eluted with 0.01 M sodium acetate buffer pH 5.0. Fractions containing 1,2-α-mannosidase activity were concentrated by ultrafiltration. The activity of this enzyme was determined using baker's yeast mannan (Ichishima *et al*, 1981) and the released mannose was measured as reducing sugar by the Nelson-Somogyi method (Somogyi, 1952). The protein content of the partially-purified enzyme was determined by the Lowry method (Lowry *et al*, 1951).

Equilibrium synthesis reactions

Synthesis reactions were performed at different concentrations of total mannose (30%-80%, w/w) in 100 mM sodium acetate buffer pH 5.0. To each reaction mixture, 1,2-α-mannosidase (0.011 units per ml reaction mixture) was added and the reaction mixture incubated at 55°C for 7 days. After incubation, the sugar solution was heated at 100°C for 1-2 minutes to inactivate the enzyme. Products were analysed by HPLC.

Analysis of products by HPLC

Mannose, mannobiose and mannotriose were quantified by HPLC on a 3 μm Spherisorb amino column (4.6 x 150 mm) in 80% acetonitrile in water (v/v). The column was eluted at 1.5 ml/min and carbohydrate detected by refractive index.

Purification of products

Products were purified by gel filtration on a column (2.5 cm x 120 cm) of Bio-Gel P-2 (fine grade) eluted with degassed deionised water at 0.5 ml/min. Carbohydrate was detected by refractive index. All fractions containing mannobiose and mannotriose were collected and lyophilised prior to structural analysis.

Methylation analysis

Product structures were analysed by methylation analysis. The methylation protocol of Ciucanu and Kerek (1984) and the hydrolysis and reduction protocol of Harris *et al* (1984) were followed. Partially methylated alditol acetates (PMAA) were analysed

on a BPX-5 capillary column (50.0 m x 0.32 mm) at 100-250^{0}C (3^{0}C/min temperature program) with flame ionisation detection.
Mass spectra were recorded with an HP-5972 mass selective detector calibrated from 33 to 400 amu with a scan rate of 1.9 scan/s . The electron impact ionisation current was set at 50.0 mA.

RESULTS

The 1,2-α-mannosidase used in this experiment has 99.5% specific activity relative to non-specific (*p*-nitrophenyl α-mannoside hydrolysis) activity. Various concentrations of mannose (30% to 80%, w/w) were incubated at 55^{0}C for 7 days in the presence of 0.011 units of *Aspergillus phoenicis* 1,2-α-mannosidase per ml reaction misxture. The reverse hydrolysis reaction resulted in a mixture of substrate and products (Figure 1).

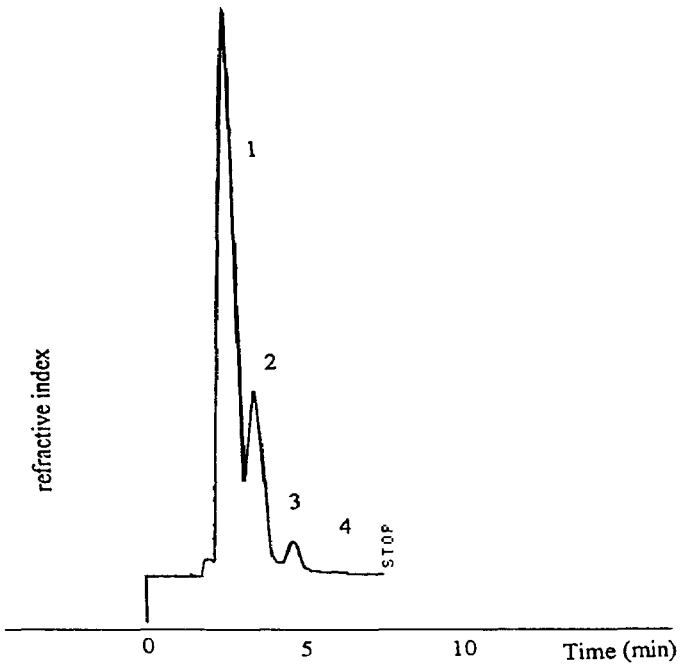

Figure 1. Products of the reverse reaction catalysed by *Aspergillus phoenicis* 1,2-α-mannosidase in 70% (w/w) D-mannose. Products were analysed on a Spherisorb-NH_2 column eluted with 80% acetonitrile in water (v/v) at a flow rate of 1.5 ml/min with refractive index detection. Peaks are assigned as: (1) mannose, (2) mannobiose, (3) mannotriose, and (4) tetrasaccharide.

By gel filtration chromatography (data not shown) and methylation analysis, Peak 1 was found to be mannose, while peaks 2 and 3 were identified as α-D-man1→2-D-man and α-D-man1→2-α-D-man1→2-D-man, respectively (Table 1). A small trace of tetrasaccharide (Peak 4) was also seen. The structure of this was not analysed since the yield obtained was very low.

Table 1. Methylation analysis of peaks 2 and 3.

Derivative	Primary ions (m/z)	Area ratio (Peak 2)	Area ratio (Peak 3)
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	45, 117, 161, 205	1.00	1.00
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol	45,161, 189	0.97	2.39

The 1,2-mannosidic linkage was found exclusively, with no other linkages detected. The yield of products at various mannose concentrations was also investigated (Figure 2).

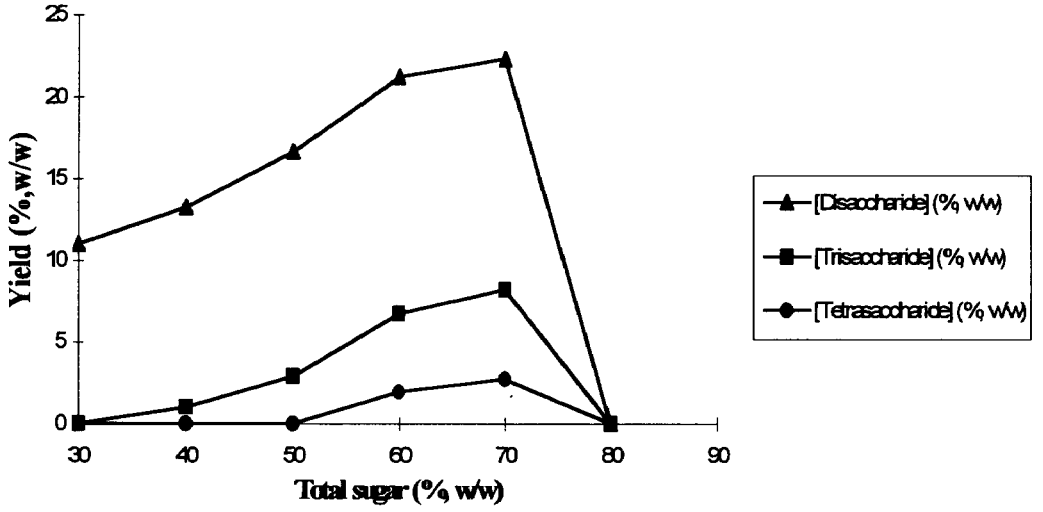

Figure 2. Products of the synthesis reaction at various concentrations of α-D-mannose in the presence of 0.055 U/ml of *Aspergillus phoenicis* 1,2-α-mannosidase. Reaction mixtures were incubated at 55°C for 7 days. Yields are expressed as percentage of the total sugar by HPLC.

The yield of mannobiose and mannotriose increase with increasing concentration of D-mannose (Figure 2). Tetrasaccharide is also seen at 60-70% (w/w) mannose. It is noteworthy that there is no product at all at 80% mannose. This behaviour has been reported by Rastall *et al* (1992) for the α-mannosidase from Jack bean.

DISCUSSION

Although glycosidases display lower regioselectivity than glycosyltransferases, these enzymes are still of interest for the synthesis of small, novel, oligosaccharides. Jack Bean α-mannosidase, for instance, has been exploited for the synthesis of mannobiose, mannotriose, and mannotetraose (Johansson *et al*, 1989) as well as for the synthesis of novel hetero-oligosaccharides (Rastall *et al*, 1992). With this enzyme, however, a mixture of α-1,2, α-1,3, α-1,4, and α-1,6 linked isomers are obtained. This is a recurring observation with such systems (Rastall and Bucke, 1992). To date, glycosidases with absolute linkage specificity have not been evaluated for their synthetic potential in equilibrium synthesis reactions. *Aspergillus phoenicis* is the synonym for *Aspergillus saitoi* (Raper and Fennell, 1965), from which a specific 1,2-α-mannosidase has been isolated and purified (Ichishima *et al*, 1981). This enzyme cleaves single mannose residues from the nonreducing terminal of the α-1,2-D-linked side chains from baker's yeast mannan and has no activity towards *p*-nitrophenyl-α-D-mannopyranoside. When various glycoproteins were treated with this enzyme, only manα1→2man linkages were hydrolysed (Yamashita and Ichishima, 1980).
Most high-mannose type sugar chains in glycoprotein are characterised by the presence of α-D-man1→2-D-man and α-D-man1→2-α-D-man1→2-D-man sequences. These two compounds are commercially available but are very expensive. In this paper we have demonstrated that this enzyme can be used to synthesise α1→2 linked di- and trisaccharide with absolute regioselectivity. Optimisation of the reaction and investigation of the potential of the enzyme for hetero-oligosaccharide synthesis are currently under way.

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