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Regioselective synthesis of mannobiose and mannotriose by reverse hydrolysis using a novel 1,6-α-D-mannosidase from *Aspergillus phoenicis*

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Abstract

A novel 1,6-α-D-mannosidase was produced by *Aspergillus phoenicis* grown on a commercial manno-oligosaccharide preparation in liquid culture. The enzyme hydrolysed only α-D-Man*p*-(1 → 6)-D-Man*p* and did not act on α-D-Man*p*-(1 → 2)-D-Man*p*, or α-D-Man*p*-(1 → 3)-D-Man*p*. The 1,6-α-D-mannosidase was used for synthesis of manno-oligosaccharides by reverse hydrolysis reaction. The highest yields, expressed as percentages (w/w) of total sugar, were ∼21% mannobiose and ∼5% mannotriose, and they were obtained with 45% (w/w) initial mannose concentration at pH 4.5 after 12 days incubation at 55 °C. The disaccharide and trisaccharide products were separated and their structures determined by methylation analysis. Only 1–6 linkages were found in both of them.

Keywords: Mannobiose; Mannotriose; Oligosaccharide synthesis; 1,6-α-D-Mannosidase; *Aspergillus phoenicis*

1. Introduction

Glycosidases are useful catalysts for the synthesis of small bioactive and novel oligosaccharides [1]. The most significant problem with this approach is that most of these enzymes have low regioselectivity. This can be overcome by the use of linkage specific enzymes. Mannosidases have been exploited for the synthesis of manno-oligosaccharides but the products are usually a mixture of isomers [2,3]. Previous research has established facile enzymatic synthesis of α-D-Man*p*-(1 → 2)-D-Man*p* [4]. The 1–6 linkage is thermodynamically favoured, but although it is the predominant product of most enzymes [2,5], it has not been synthesised with absolute regioselectivity. The current work has extended this approach to the manufacture of α, 1–6 linked di and trisaccharide with absolute regioselectivity.

2. Materials and methods

2.1. Materials

Mannose was purchased from Sigma (Poole, Dorset, UK). Mannobioses linked α-D-Man*p*-(1 → 2)-D-Man*p*, α-D-Man*p*-(1 → 3)-D-Man*p* and α-D-Man*p*-(1 → 6)-D-Man*p* were purchased from Dextra Laboratories Ltd., Reading, UK. Bio-Mos® was given by Nick Adams, Alltech (UK) Ltd., Stamford, UK. All other chemicals used were purchased from BDH-Merck. The TLC plates used were 10 cm Silica Gel 60 by Merck, Darmstadt, Germany. Bio-Gel P-2 fine grade with bead size 45–90 μm, MW fractionation range 100–1800 was purchased from Bio-Rad Laboratories Ltd., Hertfordshire, UK.

2.2. Fermentation, extraction and concentration of mannosidase

Aspergillus phoenicis ATCC 14332 (syn. *Aspergillus saitoi*) [6] was obtained from the American Type Culture Collection, Rockville, USA. It was grown in submerged culture in 500 ml Erlenmeyer flasks, each containing 200 ml of mineral solution and 1% (w/v) Bio-Mos®. The composition of the mineral solution was: $(NH_4)_2SO_4$ 0.14%, KH_2PO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 0.03%, $CaCl_2$ 0.03%, $FeSO_4$ 0.001%, $ZnCl_2$ 0.0005%, $MnSO_4{\cdot}H_2O$ 0.0005%, $CoCl_2{\cdot}5H_2O$ 0.0005%, $CuSO_4{\cdot}5H_2O$ 0.0005%, (w/v), pH 5.0. The flasks were autoclaved at 110 °C for 10 min. This sterilised liquid broth was inoculated with a fresh spore suspension and then incubated at 30 °C for 3 days on a shaker adjusted to 130–140 rpm.

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The submerged culture filtrates were initially collected using Miracloth (Calbiochem), cooled at 4 °C and then centrifuged at 16,000 × *g* for 30 min to remove large particles. The clear supernatant was sterilised by filtration through a 0.2 μm filter using vacuum and the pH was adjusted to 5.0 before being concentrated.

The crude extracts were concentrated ten times in a Gyrosep 300 (Techmate Ltd., Milton Keynes, UK) stirred ultrafiltration unit using 40 cm^2 flat sheet 10,000 MWCO membranes under nitrogen pressurisation to 2.5–4 bar. Extracts were cleaned further by two-fold diafiltration using 0.01 M sodium acetate buffer, pH 5.0.

2.3. Enzyme assays

A qualitative enzyme detection was performed as follows. The concentrated enzyme extract was assayed for enzymatic activity by incubating 5 μl, with 5 μl of 2% (w/w) α-D-Man*p*-(1 → 2)-D-Man*p*, α-D-Man*p*-(1 → 3)-D-Man*p* and α-D-Man*p*-(1 → 6)-D-Man*p*, at pH 5.0, 30 °C for up to 24 h. Samples (1 μl) were removed at regular time intervals and applied to TLC plates to monitor the hydrolysis of the differently linked disaccharides. The mobile phase used was butanol:ethanol:acetic acid:water 9:6:3:1 by volume, with a double ascent. Plates were developed with 5 ml of 0.1 M $Ce(SO_4)_2$ diluted in 100 ml of 15% (v/v) H_2SO_4. The plates were dipped in the developer and the sugar spots visualised at 100 °C for 15 min.

Enzyme activity was quantified as follows. An appropriate enzyme dilution was incubated at 1% (w/w) α-D-Man*p*-(1 → 6)-D-Man*p*, total volume 240 μl, at pH 4.5 and 30 °C for 20 min. The reaction was stopped by immersion in boiling water for 2 min followed by cooling in ice bath. The mannose released was determined by HPLC. The extent of the reaction was always less than 15% substrate conversion. One unit is defined as the amount of enzyme that releases 2 μmol of mannose per min at 30 °C.

2.4. Synthesis reaction

The synthesis reaction was performed at different concentrations of total mannose (30–60% w/w) dissolved in 0.1 M sodium acetate buffer, pH 5.0, with ∼0.4 units of mannosidase/g of reaction mixture. To observe the effect of pH, similar reaction mixtures were set up at different pH values (3.5–6.0). The synthesis yield was monitored over 12 days at various temperatures. After incubation, the sugar solution was heated at 100 °C in a water-bath for 2 min to inactivate the enzyme.

2.5. HPLC

Manno-oligosaccharides were quantified by HPLC. The column used at ambient temperature was an Apex Carbohydrate amino column (250 mm × 4.6 mm i.d., Jones Chromatography, Hengoed, UK) and carbohydrates were detected using a refractive index detector. The eluent was acetonitrile/water:75/25 (v/v), the injection volume 20 μl and the column was eluted at a flow rate of 1 ml/min. Standards of mannose, lactose and raffinose were used to calibrate the analysis.

2.6. Separation of oligosaccharides

The manno-oligosaccharides were separated by gel filtration on a Bio-Gel P-2 column (6.0 cm × 100 cm) with degassed deionised water as an eluent at 2 ml/min and carbohydrate elution was monitored using a refractive index detector. Fractions were collected and freeze-dried prior to structural analysis.

2.7. Analysis of sugar structure

Product structures were determined by methylation analysis performed by methylation [7], followed by hydrolysis and reduction [8]. Partially methylated alditol acetates (PMAA) were analysed by GCMS. The gas chromatographic analysis was accomplished with a HP 5890 Series II gas chromatograph equipped with a split/splitless injector. Separations were performed on a Low Bleed/MS WCOT fused Silica column, 60 m × 0.25 mm i.d., with CP-SIL 8 CB coating, by Chrompack. Mass spectra were recorded with an MS detector, scanned from 33 to 400 amu with a scan rate of 3.92 scans/s, at an electron multiplier voltage of 1894 V with an ionisation current of 35 μA and an ion-source temperature of 172 °C.

3. Results and discussion

3.1. Production of 1,6-α-D-mannosidase

A. phoenicis grew in the form of many separate pellets in the shake flasks. Bio-Mos® was the sole carbon source and the only α-D-mannosidase activity detected was that on α-D-Man*p*-(1 → 6)-D-Man*p* (Fig. 1). This was the only α-D-mannosidase produced on this substrate and it is induced by it. Indeed, when grown on different substrates, *A. phoenicis* produces a 1,2-α-D-mannosidase and a non-linkage specific enzyme [9,4]. The specificity of the hydrolytic reaction towards the 1–6 linkage and the absence of any other mannosidase activity justified the use of concentrated extracts for synthesis without any further purification. A 1,6-α-D-mannosidase has been previously reported in the literature [10] but it has not been available in sufficient quantities to allow its use for oligosaccharide synthesis.

3.2. Synthesis of manno-oligosaccharides

The reverse hydrolysis reaction resulted in a mixture of mannose substrate and manno-oligosaccharide products. A

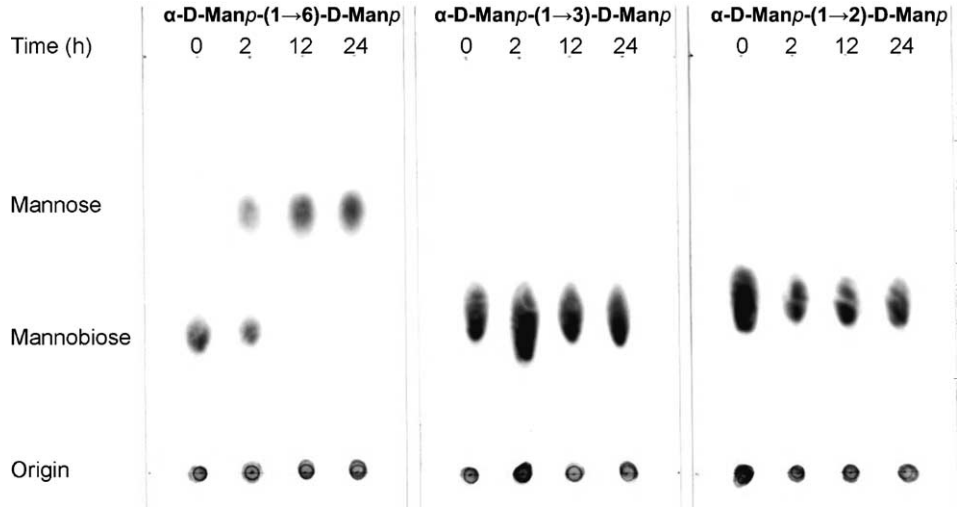

Fig. 1. TLC plates showing the hydrolytic activity of the enzyme extract towards differently linked disaccharides, monitored over 24 h incubation time at 30 °C, pH 5.0, using two ascents of butanol:ethanol:acetic acid:water 9:6:3:1 by volume. The plates were dipped in 5 ml of 0.1 M $Ce(SO_4)_2$ diluted in 100 ml of 15% (v/v) H_2SO_4 and the sugar spots visualised at 100 °C for 15 min.

typical HPLC chromatogram of a reverse hydrolysis reaction mixture is presented in Fig. 2.

3.3. Optimisation of the parameters of the synthesis reaction

The effect of initial mannose concentration on synthesis yield is shown in Fig. 3. Maximum yields are observed at 40–45% (w/w) mannose. This result is in contrast with previous studies on mannosidases that have reported maximum yields at 70–85% (w/w) mannose [4,2]. These yields are probably not the equilibrium yields since a time course was not constructed.

The synthesis reaction was performed at different pH values and the results are presented in Fig. 4. The buffers used were: sodium acetate/HCl for pH 3.5, phosphate for pH 6.0 and sodium acetate/acetic acid for all the rest. Maximum yields are observed at pH 4.0–5.0 with the peak at pH 4.5. These yields, like those in Fig. 3, are probably not the equilibrium yields since a time course was not constructed.

The mannobiose and mannotriose synthesis time courses at various temperatures are presented in Figs. 5 and 6,

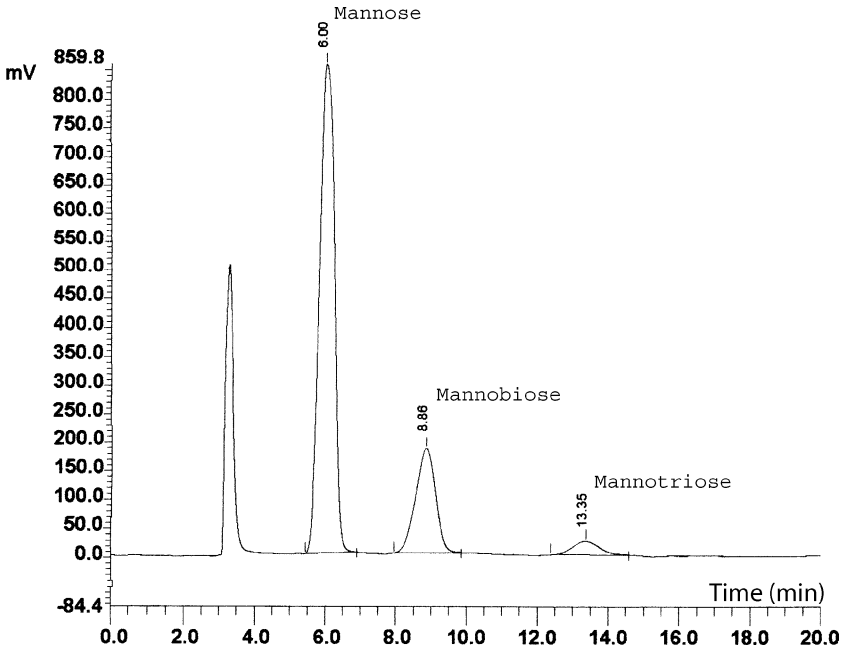

Fig. 2. A typical HPLC chromatogram of a reverse hydrolysis reaction mixture catalysed by *A. phoenicis* 1,6-α-D-mannosidase (~0.4 units/g of reaction mixture), at 45% (w/w) initial mannose concentration after 7 days incubation at 55 °C, pH 5.0.

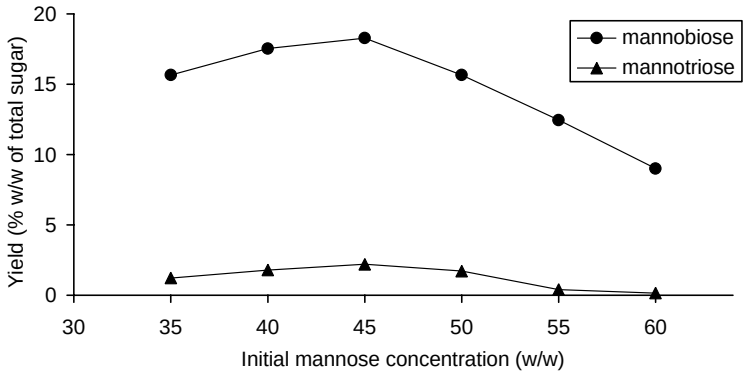

Fig. 3. Effect of initial mannose concentration on disaccharide and trisaccharide synthesis yield after 7 days incubation with ∼0.4 units of mannosidase/g of reaction mixture at 55 °C and pH 5.0. Values are means of duplicate determinations of two separate reactions of 0.5 g each.

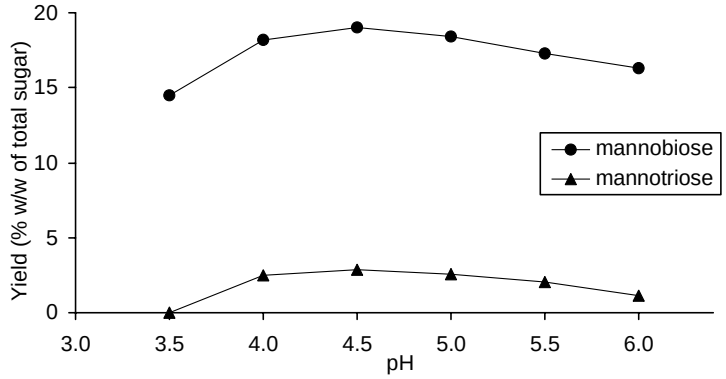

Fig. 4. Effect of pH on disaccharide and trisaccharide synthesis yield after 7 days incubation with ∼0.4 units of mannosidase/g of reaction mixture, at 55 °C with 45% (w/w) initial mannose concentration. Values are means of triplicate determinations of three separate reactions of 0.5 g each, with R.S.D. < 2.4 for mannobiose and R.S.D. < 9.2 for mannotriose.

respectively. From Fig. 5 is apparent that the initial rate of synthesis is higher at higher temperatures, although the final yield is lower (16% at 70 °C) than at lower temperatures (21% at 45 °C). The results can be explained by the enzyme activity/inactivation phenomena at high temperatures. The higher the temperature the faster the enzyme acts, but the faster it becomes inactivated. Therefore, at lower temperature yields will exceed those at higher temperatures at some point in time. From Fig. 6 similar observations can be made, but there is a time lag as trisaccharide synthesis can only start when disaccharides have accumulated in the reaction.

The optimum conditions for synthesis are: an initial mannose concentration of 45% (w/w), pH 4.5 and 10 days incubation at 55 °C. Two large scale reactions, (20 g each, containing 9 g mannose) were set up at the optimum conditions, the products were separated by Bio-Gel P-2 and

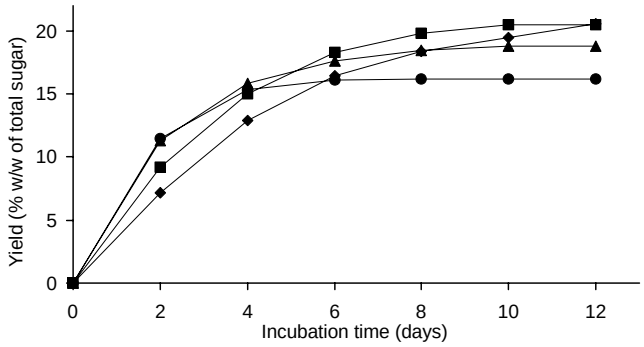

Fig. 5. Mannobiose synthesis time courses at various temperatures (45 °C, ◆; 55 °C, ■; 65 °C, ▲ and 70 °C, ●), using ∼0.4 units of mannosidase/g of reaction mixture, at pH 4.5 and 45% (w/w) initial mannose concentration. Values are means of triplicate determinations of three separate reactions of 0.5 g each, with R.S.D. < 2.0.

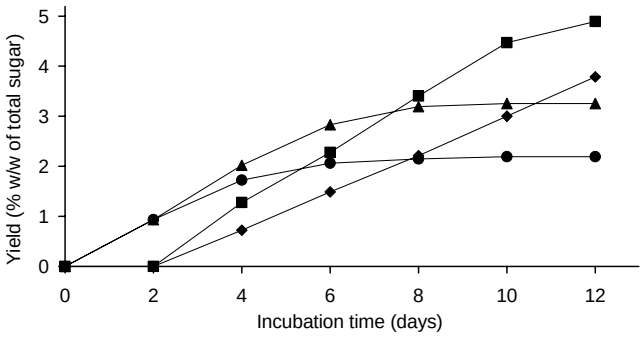

Fig. 6. Mannotriose synthesis time courses at various temperatures (45 °C, ◆; 55 °C, ■; 65 °C, ▲ and 70 °C, ●) using ∼0.4 units of mannosidase/g of reaction mixture, at pH 4.5 and 45% (w/w) initial mannose concentration. Values are means of triplicate determinations of three separate reactions of 0.5 g each, with R.S.D. < 4.5.

a small amount of tetrasaccharide was detected (less than 1% (w/w) of the total sugar, not previously detected in the HPLC). From the large scale reactions a total of ∼3.7 g mannobiose, ∼0.8 g mannotriose and ∼100 mg mannotetraose were separated.

3.4. Structure analysis

The pattern of mass spectra were compared to those found in the literature [11]. The primary fragments suggested that the disaccharide was α-D-Man*p*-(1 → 6)-D-Man*p* and the trisaccharide α-D-Man*p*-(1 → 6)-α-D-Man*p*-(1 → 6)-D-Man*p*. Methylation analysis of the chemically synthesised α-D-Man*p*-(1 → 6)-D-Man*p* gave identical result to the mannobiose synthesised by the enzyme. The tetrasaccharide structure was not determined.

4. Conclusions

A. phoenicis can grow in liquid culture using a mineral medium and Bio-Mos® as the sole carbon source and produces an extra-cellular 1,6-α-D-mannosidase. This is the only α-D-mannosidase produced on this substrate and it is induced by it. The linkage specificity was proven in the hydrolytic reaction. The enzyme cleaved only α-D-Man*p*-(1 → 6)-D-Man*p*. It did not hydrolyse other chemically synthesised mannobioses. A 1,6-α-D-mannosidase has been previously reported in the literature but it has not been available in sufficient quantities to allow its use for oligosaccharide synthesis. In this study we have shown an economic way to produce a 1–6 linkage specific microbial mannosidase. The enzyme can be easily produced by liquid fermentation and can be used for synthesis after concentration without the need for lengthy purification procedures. The parameters of the reverse hydrolysis reaction have been studied and optimised. The enzyme exhibits maximum synthesis yield at low initial mannose concentrations making the recycling of mannose easier. The yields of mannobiose and mannotriose, 21 and 5%, respectively, as percentage (w/w) of total sugar, are sufficiently high.

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