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Glycosidase-catalysed synthesis of mannobioses by the reverse hydrolysis activity of α-mannosidase: partial purification of α-mannosidases from almond meal, limpets and *Aspergillus niger*

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Abstract

Two disaccharides, α-D-Man*p*-(1→2)-D-Man*p* **6** and α-D-Man*p*-(1→3)-D-Man*p* **7**, were synthesised from mannose using the reverse hydrolysis activity of the partially purified α-mannosidases from almond (*Prunus amygdalus*) meal and limpets (*Patella vulgata*). Both disaccharides were isolated by carbon–Celite chromatography. Attempts were also made towards the synthesis of core pentasaccharide using the purified α-mannosidase from *Aspergillus niger*.

1. Introduction

α-D-Man*p*-(1→2)-D-Man*p* **6** and α-D-Man*p*-(1→3)-D-Man*p* **7** (see Scheme 1) units are found in the high mannose type sugar chains of glycoproteins. α-D-Man*p*-(1→2)-D-Man*p* is a repeating unit of the *O*-antigen polysaccharide of *Salmonella thompson*[1] and is also found in a mannohexaose isolated from cells of *Candida albicans* NIH A-207 serotype strain.[2] α-D-Man*p*-(1→3)-D-Man*p* is a core disaccharide of N-linked glycoproteins.

The prevalence of α-D-Man*p*-(1→2,3,6) linkages in nature has stimulated a number of investigations into the formation of these linkages using glycosidases. The principle of this approach is illustrated in Fig. 1.

A glycosyl donor **1** is incubated with the glycosidase E to give a glycosyl-enzyme intermediate **2** with inversion of configuration at the anomeric centre, according to the normal mechanism of retaining glycosidases.[3] In hydrolysis reactions, this intermediate is intercepted by water to give the hydrolysis product **3**. Alternatively, it may be intercepted by a different acceptor, ROH, such as another carbohydrate molecule, to give a new glycosidic product **4** with inversion of configuration and overall retention of

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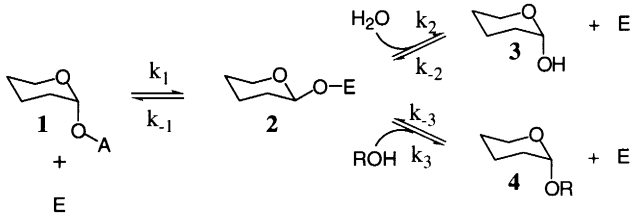
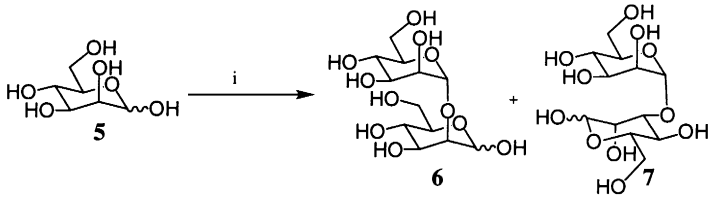


Fig. 1. Kinetics of glycosidase-catalysed glycoside hydrolysis and synthesis

configuration with respect to the substrate **1**. The reaction leading to product formation can be carried out under either kinetic or thermodynamic control. With an activated donor such as a nitrophenyl glycoside (**1**, AOH=nitrophenol), $k_{-1} \approx 0$ and the efficiency of formation of the product **4** is determined by the competition between water and the acceptor ROH for the glycosyl-enzyme intermediate. The reaction is under kinetic control. However, when the donor is not activated, as in the simplest case where A=H in substrate **1**, the product evolves towards an equilibrium mixture of donor **1**, hydrolysis product **3** and glycosyl transfer product (or products) **4**. The reaction is under thermodynamic control and this mode of operation is frequently referred to as 'reverse hydrolysis'. Although this latter method can lead to mixtures of regioisomeric di-, tri- and higher oligosaccharides, its simplicity has much to commend it where problems of product isolation and purification can be overcome effectively.

Early studies showed that the α-mannosidase from Jack bean (*Canavalia ensiformis*) could be used to catalyse reverse hydrolysis with D-mannose as substrate.[4,5] Johansson et al. obtained a product mixture in an overall yield of 37%.[4] It consisted largely of α-D-Man*p*-(1→6)-D-Man*p* with smaller and comparable amounts of the corresponding (1→2) and (1→3) disaccharides. Ajisaka et al.[6] used an α-mannosidase from *Aspergillus niger* to convert mannose into α-D-Man*p*-(1→6)-D-Man*p* (23%), together with the corresponding 1→2 (3%) and 1→3 (0.4%) isomers and trace amounts of trisaccharides. Suwasono and Rastall[7] showed that by using an α-mannosidase preparation from *Aspergillus phoenicis* (=*A. saitoi*) a single disaccharide product α-D-Man*p*-(1→2)-D-Man*p* (22%) was obtained together with minor amounts of α-D-Man*p*-(1→2)-α-D-Man*p* (1→2)-D-Man*p* and traces of tetrasaccharide.[7] Surprisingly, when the enzyme preparation was immobilised on Biofix-E-2 (a china clay product) and cellulose DE-52, the α1→6 disaccharide was the major product, whereas when alginate-immobilised enzyme was used, the expected α1→2 dimannoside was the major product, as expected.[8] However, it is known that *A. phoenicis* produces two α-mannosidases, one with 1→2 selectivity, the other with selectivity in the order 1→3>1→6≈1→2.[9,10] The result observed may therefore be attributable to a differential effect of immobilisation on the activities of these enzymes.

In this paper we report the synthesis from D-mannose **1** (Scheme 1) of α-D-Man*p*-(1→2)-D-Man*p* and α-D-Man*p*-(1→3)-D-Man*p* using α-mannosidases from almond (*Prunus amygdalus*) meal and limpet (*Patella vulgata*). Emphasis was placed on developing simple methods for the isolation of these products in pure form. We also report on efforts to synthesise the core pentasaccharide of N-linked glycoproteins using these enzymes.

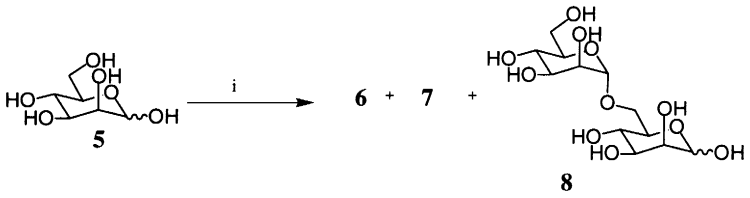


Scheme 1. (i) α-Mannosidase from almond meal and limpets

2. Results and discussion

α-Mannosidases were partially purified from defatted almond meal and the visceral humps of limpets (*Patella vulgata*) to remove most of the other glycosidase activities present in these crude preparations. Incubation of mannose **5** at high concentration with the α-mannosidase from almond meal at 45°C for 13 days gave a mixture α-D-Man*p*-(1→2)-D-Man*p* **6** and α-D-Man*p*-(1→3)-D-Man*p* **7** in a 65:35 ratio. The disaccharides were readily separated using carbon–Celite chromatography. Similarly, incubation of mannose **5** with the α-mannosidase from limpet gave a mixture of disaccharides **6** and **7** in a 57:43 ratio.

Disaccharides **6** and **7** were obtained repeatedly throughout our experiments when different sources of almond meal were used. However, the α-mannosidase isolated from almond meal made from pre-soaked almonds gave a mixture of three disaccharides. The main component was α-D-Man*p*-(1→6)-D-Man*p* **8** (Scheme 2) (~50%) and the remainder consisted of a mixture of the 1→2 **6** and 1→3 **7** (Scheme 1) isomers. Upon closer examination of the pre-soaked almonds a substantial fungal growth with apparent black conidiophores was observed. It has been reported in the literature[6] that the α-mannosidase from *Aspergillus niger* produced the disaccharide α-D-Man*p*-(1→6)-D-Man*p* **8** from D-mannose. We repeated the synthesis of disaccharide **8** using the α-mannosidase from *A. niger* according to the published procedure (Scheme 2).[6]

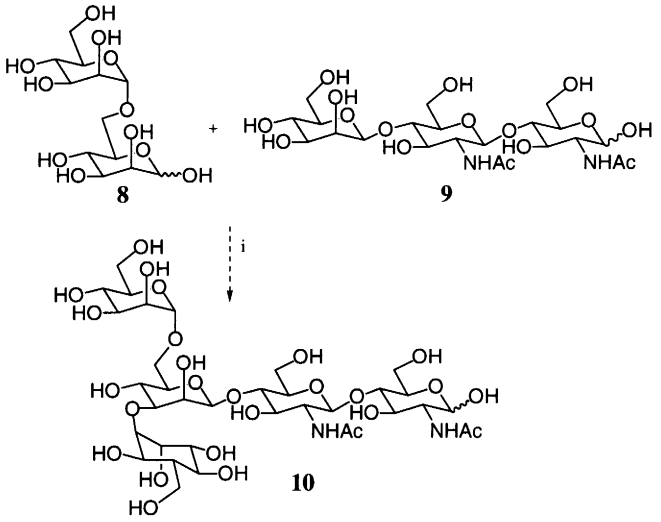


Scheme 2. (i) α-Mannosidase from *Aspergillus niger*

These findings supported our view that the enzyme preparation from pre-soaked almonds was contaminated with *A. niger* which was then responsible for the formation of the disaccharide α-D-Man*p*-(1→6)-D-Man*p* **8**.

We then moved our attention towards the synthesis of the core pentasaccharide **10** of N-linked glycoproteins (Scheme 3). We had previously synthesised the trisaccharide, β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-D-GlcNAc*p* **9**, of this core pentasaccharide using glycosidases.[11] Transglucosidase L from *A. niger* contained, in addition to a host of other glycosidases, a considerable amount of α-mannosidase activity. Unwanted glycosidases were efficiently removed in our procedure after two purification steps. The purified preparation of α-mannosidase was then used in an attempted synthesis of core pentasaccharide **10** starting from the disaccharide **8** as glycosyl donor and the trisaccharide **9** as acceptor. However, in this reaction no transfer product was observed by HPLC. The only reaction observed was hydrolysis of the donor **8**.

With the reverse hydrolysis procedure for the synthesis of mannobiosides described above, all three common linkage types (1→2, 1→3, 1→6) can be synthesised in a single step. The success of the method depends on the selectivities of the enzymes employed and on the availability of straightforward methods for product purification. Although the isolated yields of products from the reactions with almond and limpet enzymes are nominally low, the disaccharides are readily obtained in hundred milligram amounts and the unused mannose can be recovered quantitatively, giving a high overall efficiency for the syntheses in terms of substrate consumed.



Scheme 3. (i) α-Mannosidase from *Aspergillus niger*

3. Experimental

^{1}H NMR and ^{13}C NMR spectra were determined at 250 or 400 MHz and 62.89 or 100.62 MHz, respectively, using either Bruker AC250 or WH 400 spectrometers. Mass spectra were determined with a Bruker 9.4 T BioApex FTICR mass spectrometer. Optical rotations were determined using an AA-10000 polarimeter (Optical Activity Ltd.) with a 2 dm cell. Optical rotations are given in units of 10^{-1} deg cm^2 g^{-1}. Celite 535 was obtained from Fluka and activated carbon (Darco G-60, 100 mesh) was obtained from Aldrich. Thin layer chromatography was carried out using Silica Gel 60 GF254 (Merck) with the solvent system propan-1-ol:nitromethane:water (10:9:2). Oligosaccharides were visualised by spraying with 10% H_2SO_4 and charring.

*3.1. α-D-Manp-(1→2)-D-Manp **6** and α-D-Manp-(1→3)-D-Manp **7***

3.1.1. Using the α-mannosidasae from almond meal

Mannose **5** (4 g) in sodium acetate buffer (0.1 M, 1.25 ml; pH 4.8) was incubated with α-mannosidase from almond meal (1 ml; 6.3 U ml^{-1}) at 45°C for 13 days. The reaction was stopped by heating in the boiling water bath for 5 min. The mixture was purified by carbon–Celite (290 g, 1:1) column chromatography. The column was eluted first with water (150 ml) followed by 2% ethanol (4 l), 3% ethanol (1 l) and 4% ethanol (1 l) to give starting mannose **5**, α-D-Man*p*-(1→2)-D-Man*p* **6** (190 mg, 5%) and α-D-Man*p*-(1→3)-D-Man*p* **7** (113 mg, 3%).

For disaccharide **6** (mixture of alpha and beta anomers, α:β=88:12): $[\alpha]_D^{22}$=+38.7 (*c* 1.19, H_2O), lit.[12] $[\alpha]_D$=+60 (*c* 0.1, H_2O), lit.[13] $[\alpha]_D$=+51.8 (*c* 0.52, 1:1 H_2O:MeOH); ^{1}H NMR (400 MHz, D_2O): 5.34 (d, 0.88H, *J*=1.32 Hz, H-1α), 5.10 (d, 0.12H, *J*=1.64 Hz, H-1′β), 4.99 (d, 0.88H, *J*=1.64 Hz, H-1′α), 4.87 (d, 0.12H, *J*=1.32 Hz, H1β) 4.07–4.05 (m, 0.12H, H-2′), 4.03 (dd, 1H, *J*=3.22 and 1.64 Hz, H-2′), 3.91–3.56 (m, 10.88H), 3.33–3.39 (m, 0.12H); ^{13}C NMR (400 MHz, D_2O): 102.82, 102.07, 94.07, 93.16, 79.75, 78.81, 77.34, 74.05, 73.86, 73.48, 73.09, 70.94, 70.75, 70.60, 67.67, 67.49, 67.38, 67.27, 61.69, 61.58, 62.51; *m/z* (M^+) found: 365.106. $C_{12}H_{22}NaO_{11}$ requires: 365.1054.

For disaccharide **3** (mixture of alpha and beta anomers, α:β=70:30): $[\alpha]_D^{22}$=+48.11 (*c* 1.09, H_2O), lit.[14] $[\alpha]_D^{27}$=+48.4 (*c* 0.72, H_2O); ^{1}H NMR (400 MHz, D_2O): 5.11 (d, 0.70H, *J*=1.96 Hz, H-1α), 5.10 (d,

0.70H, *J*=2 Hz, H-1′α), 5.09 (d, 0.30H, *J*=2.32 Hz, H-1′β) 4.87 (d, 0.30H, *J*=1.00 Hz, H-1β), 4.04–4.02 (m, 2H, H-2 and H-2′), 3.92–3.79 (m, 4.70H), 3.75–3.58 (m, 6H), 3.40–3.35 (m, 0.30H); ^{13}C NMR (400 MHz, D_2O): 102.99, 102.85, 94.66, 94.16, 80.99, 78.55, 76.60, 73.95, 73.20, 71.58, 71.00, 70.70, 67.47, 67.41, 66.88, 66.69, 61.65, 61.50; *m/z* (M^+) found: 365.105. $C_{12}H_{22}NaO_{11}$ requires: 365.1054.

3.1.2. α-Mannosidase from limpet

Mannose (**5**, 4 g) in sodium acetate buffer (0.1 M, 1.25 ml; pH 4.8) was incubated with α-mannosidase from limpet (0.1 ml; 14 U ml^{-1}) at 35°C for 12 days. The reaction was stopped by heating in the boiling water bath for 5 min. The mixture was purified as above to give α-D-Man*p*-(1→2)-D-Man*p* (**6**, 181 mg) and α-D-Man*p*-(1→3)-D-Man*p* (**7**, 137 mg).

3.2. Preparation of α-D-*Man*p-*(1→6)*-D-*Man*p ***8*** *using the α-mannosidase from* Aspergillus niger

Mannose (**5**, 8 g) in sodium acetate buffer (0.1 M, 2.5 ml, pH 4.8) was incubated with transglucosidase L (1.7 ml) from Amano at 50°C for 17 days. The reaction was stopped by heating in the boiling water bath for 5 min. The mixture was purified as above to give disaccharide **8** (1.76 g).

3.3. Purification of α-mannosidases

3.3.1. Materials and general procedures

Sweet almonds (Tesco Stores; defatted almond meal from Sigma), visceral hump from limpets (*Patella vulgata*) and transglucosidase L (Amano) from *Aspergillus niger* were identified as sources of α-mannosidase. *p*-Nitrophenyl derivatives of various monosaccharides used as enzyme substrates were obtained from Sigma. Buffers were prepared with ultra-pure water (Elga). Other chemicals were of analytical grade from commercial sources. All operations were carried out at 4°C. Chromatography columns (XK series, Pharmacia) with a water cooling jacket were used for protein purification. The following chromatography media were used: Macro-Prep ceramic hydroxyapatite (Bio-Rad), Q-Sepharose and CM-Sepharose (both from Pharmacia). A Waters 650E Advanced Protein Purification System (Millipore, USA) was employed for column development. Extraction and elution buffers were supplemented with 2-mercaptoethanol and zinc sulphate (5 mM and 0.1 mM, respectively). Samples were centrifuged on a Sorvall RC-5B refrigerated superspeed centrifuge (8500 g, 20 min, 4°C). Proteins were concentrated by membrane ultra filtration (Centriprep, Amicon, 30 kDa cut-off) on a Mistral 2000R refrigerated centrifuge (3500 g; repeated 15-min runs at 4°C). Samples of a small volume were concentrated in Minicon concentration cells (Amicon, 15 kDa cut-off).

3.4. Enzyme activity assay

Activities of glycosidases were detected and quantified using appropriate *p*-nitrophenyl glycosides as substrates. The assay mixture contained a corresponding 5 mM *p*-nitrophenyl glycoside and the enzyme in McIlvain (citrate–phosphate) buffer (50 mM, pH 5.0). The mixture was incubated at 30°C for 10 min. The reaction was terminated by the addition of Na_2CO_3 (0.1 M, 3.9 ml) and absorbance was read at 400 nm. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. The specific activity was expressed as units per mg of protein. Protein content was estimated by Bio-Rad protein assay[15] using bovine serum albumin as standard. As the α-mannosidase from *A. niger* does not accept *p*-nitrophenyl α-mannopyranoside as a substrate the activity was assayed only qualitatively (TLC) using α-D-Man*p*-(1→6)-D-Man*p* **8** as an alternative substrate.

3.5. Enzyme extraction and purification

3.5.1. α-Mannosidase from almonds (Prunus amygdalus)

Almonds (95 g dry weight) were extensively washed in distilled water, rinsed with acetone and blotted dry. The material was homogenised in a stainless steel blender and chilled acetone was added (200 ml, kept on dry ice). The homogenation was carried out in four 30 s bursts (high power). The finely ground material was filtered through a fine cloth, and washed with chilled acetone followed by hexane. Residual solvent was removed under reduced pressure at 30°C for several hours and the dry almond meal (39 g) was kept at 4°C. Almond meal (10 g) was extracted with sodium acetate buffer (20 mM, pH 4.5) for 30 min, filtered and centrifuged. The proteins were fractionated by ammonium sulphate precipitation (35–75% saturation), the precipitate was collected by centrifugation and extensively dialysed against the same buffer. Dialysed proteins were applied to a column of CM-Sepharose (13×2.6 cm i.d.) equilibrated with sodium acetate buffer (20 mM, pH 4.5). The column was eluted with an increasing salt concentration (0–0.4 M NaCl) in the same buffer. α-Mannosidase emerged in a broad elution profile together with β-galactosidase and β-glucosidase (Table 1).

Table 1
Purification of α-mannosidase from almond meal

Sample	Prot [mg]	Units	α-Man[a]	α-Gal[a]	β-Gal[a]	β-Glc[a]	Hex[a]
crude	31	16	0.524	0.142	3.788	13.708	0.513
CM-Sepharose	4	11.5	2.792	0	2.930	11.387	0

[a] Specific activity (units per mg protein) of α-mannosidase (α-Man), α-galactosidase (α-Gal), β-galactosidase (β-Gal), β-glucosidase (β-Glc) and β-*N*-acetylglucosaminidase (Hex).

3.5.2. α-Mannosidase from limpets (Patella vulgata)

Limpets (*Patella vulgata*) were collected in the Plymouth (UK) area. The visceral humps removed from the organisms (each batch approx. 100 g) were homogenised with 300 ml of ice-cold acetone for 1 min at maximum speed. The solvent was filtered off and the tissue debris was extracted once more with ice-cold acetone in the blender. The acetone was removed, the material was resuspended in ice-cold diethyl ether and filtered. The residual solvent was removed under reduced pressure and the desiccated powder was kept at 4°C. The glycosidases were extracted from the crude powder (112 g) with 500 ml of McIlvain buffer (50 mM, pH 5.5) at 4°C overnight. The debris was removed by centrifugation and the proteins in the extract were precipitated by ammonium sulphate. A fraction collected between 40–60% ammonium sulphate saturation was enriched in α-mannosidase activity (specific activity 0.260 unit per mg protein, 745 units in total).

3.5.3. α-Mannosidase from Aspergillus niger

Transglucosidase L from *A. niger* (Amano; 50 ml) was diluted with 50 ml of distilled water, 2-mercaptoethanol was added to a final concentration of 5 mM and the proteins were fractionated by ammonium sulphate precipitation (40–70% saturation). The collected precipitate was dialysed overnight against potassium phosphate buffer (20 mM, pH 6.5) and loaded onto a Q-Sepharose column (30×2.6 cm i.d.) equilibrated with the same buffer. Unbound proteins were eluted with the same buffer and the column was developed with an increasing salt concentration (0.0–0.27 M NaCl linear gradient; 1.5 ml per min). α-Mannosidase activity (detected with α-D-Man*p*-(1→6)-D-Man*p* **8** as a substrate) was eluted at high salt concentration and the proteins in active fractions were collected by ammonium sulphate precipitation (100% saturation). No β-mannosidase activity was detected in this preparation. The protein

precipitate was exhaustively dialysed against potassium phosphate buffer (2 mM, pH 6.5) and loaded onto a hydroxyapatite column (33×1.6 cm) equilibrated with the same buffer. The column was eluted with increasing phosphate buffer concentration (0.002–0.2 M linear gradient; 1.5 ml per min). α-Mannosidase activity was detected in the first major peak after the onset of the gradient. The active fractions were pooled, concentrated by ultra filtration and stored at 4°C in the presence of sodium azide (0.02%). This purification step completely removed those contaminating glycosidase activities still present after the chromatography on Q-Sepharose.

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