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Enzymatic synthesis of mannobioses and mannotrioses by reverse hydrolysis using α-mannosidase from *Aspergillus niger*

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Abstract

Various manno-oligosaccharides including α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man were formed when a highly concentrated mannose solution was incubated in the presence of α-mannosidase from *Aspergillus niger*. α-D-Man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man were isolated by activated carbon chromatography followed by high performance liquid chromatography using an amino-silica column. In addition to the above oligosaccharides, α-D-man-(1 → 3)-D-man, α-D-man-(1 → 6)-D-man, and α-D-man-(1 → 2)-α-D-man-(1 → 6)-D-man were also isolated.

Keywords: Mannobioses; Mannotrioses; α-Mannosidase; *Aspergillus niger*

1. Introduction

Most high-mannose type sugar chains in glycoproteins contain α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man units. Although chemical synthesis of these chains has been achieved by many researchers, the process is complicated by the use of numerous protection and deprotection reactions [1,2]. Recently, α-1,2-mannosyltransferase was used for the synthesis of α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man-serine [3]. This enzymic reaction produces only one product in high yield. In

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contrast, the reaction using hydrolases displays comparatively low regioselectivity, but several different oligosaccharides can be obtained simultaneously. We consider this to be an advantage, especially if the separation process can be optimized. The enzymatic synthesis of manno-oligosaccharides using α-mannosidase has been investigated by Johansson et al. [4,5] and by Rastall et al. [6], but these reports did not describe the details of the separation procedure or the structure of the products. Another disadvantage of these previous methods is the use of α-mannosidase from jack bean, since enzymes of plant origin are difficult to obtain in amounts necessary for large scale preparation.

In the present study, we report a reaction using an enzyme of microbial origin and a new separation procedure for large scale preparation.

2. Results and discussion

For the enzymatic synthesis of oligosaccharides, two different classes of reactions are known: transglycosylation reactions and reverse hydrolysis reactions. In transglycosylation, an activated donor substrate such as a 4-nitrophenyl glycoside or a disaccharide is employed. In general, the transglycosylation reaction proceeds regioselectively if the enzyme is properly chosen [7,8]. In contrast, the free sugar can be used in the reverse hydrolysis reaction, which is regulated by the equilibrium between the monosaccharide, resulting oligosaccharides, and water [9,10].

α-Mannosidase from *Aspergillus niger* is known to hydrolyze the α-D-man-(1 → 2)-D-man linkage but not 4-nitrophenyl-α-D-mannopyranoside [11]. This means that 4-nitrophenyl-α-D-mannopyranoside cannot be used as a donor in a transglycosylation

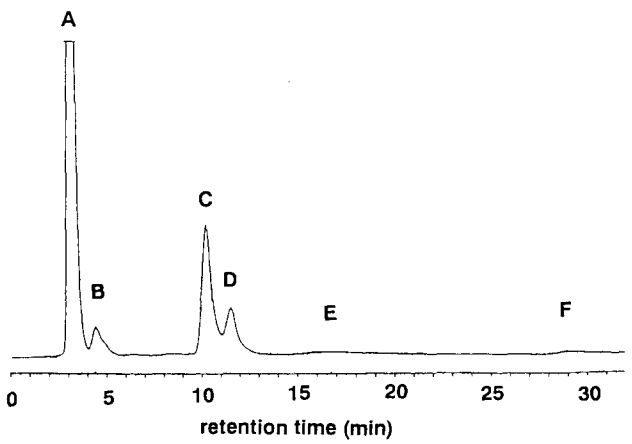

Fig. 1. HPLC of reaction using semi-purified α-mannosidase from *A. niger*. Column: Carbon-500; eluent: water; flow rate, 0.5mL/min; detection, pulsed amperometric detector (Dionex). Peaks A – F were assigned as: (A) mannose; (B) α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man; (C) α-D-man-(1 → 6)-D-man; (D) α-D-man-(1 → 3)-D-man; (E) and (F) trisaccharides.

reaction. Since the transglycosylation reaction was not an option for our purposes, we concentrated our efforts on the condensation (hydrolase) reaction.

A highly concentrated solution of mannose was incubated at 37°C or 50°C in the presence of α-mannosidase from *A. niger*. This equilibrium driven reaction resulted in a mixture of products as determined by HPLC (Fig. 1). The products corresponding to each peak were isolated and their structures determined by comparing ^{13}C NMR chemical shifts with those found in the literature [12] or with those of the corresponding methyl glycosides [13–15]. Peak A was found to be mannose, while peaks C and D were identified as α-D-man-(1 → 6)-D-man and α-D-man-(1 → 3)-D-man, respectively. Peak B consisted of α-D-man-(1 → 2)-D-man, but also contained a minor amount of α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man. Peaks E and F were found to be trisaccharides, but the structures were not determined.

The α-mannosidase used in the above reaction was semi-purified from a commercial preparation of Transglucosidase Amano™, which is a culture broth of *A. niger* containing various glycosidase activities. For purification, the crude enzyme mixture was applied to a DEAE-MemSep™ column and fractions exhibiting activity to hydrolyze the α-D-man-(1 → 2)-D-man linkage were collected and concentrated for use in the

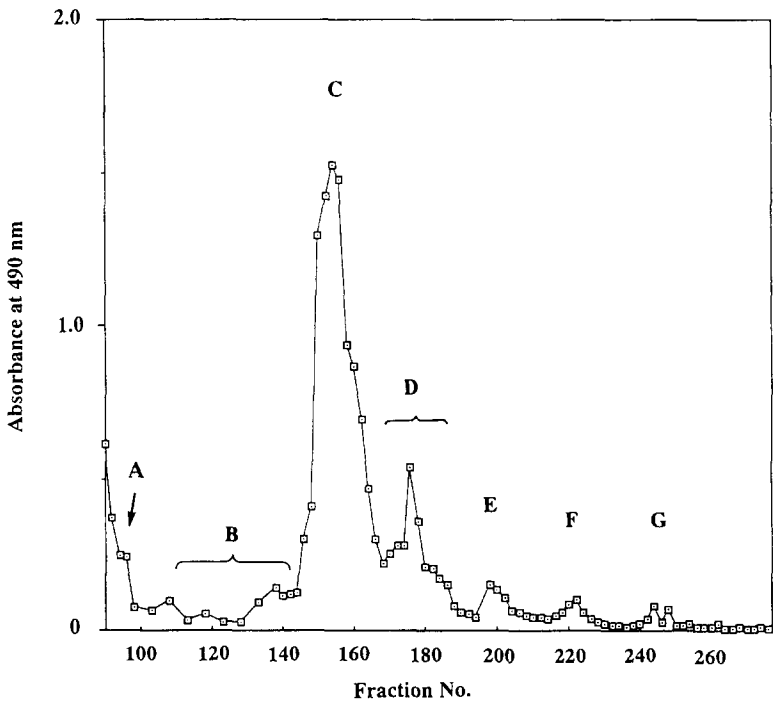

Fig. 2. Activated carbon column chromatogram of the large scale reaction using crude enzyme of Transglucosidase Amano™. Column was eluted with a gradient of 0 to 30% aqueous ethanol. Peaks were assigned as: (A) mannose; (B) α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man; (C) α-D-man-(1 → 6)-D-man; (D) α-D-man-(1 → 3)-D-man and two trisaccharides (one trisaccharide was assigned as α-D-man-(1 → 2)-α-D-man-(1 → 6)-D-man.); and (E) – (G) trisaccharides of unknown structure.

condensation reaction. We also found that the crude enzyme preparation could also be used for the synthesis, since enzyme activities other than α-mannosidase do not significantly affect the reaction.

A large scale synthetic reaction was performed using the crude enzyme mixture. The manno-oligosaccharides were first separated by activated carbon column chromatography. The column was washed with water to remove mannose and then eluted with a gradient of 0 to 30% aqueous ethanol. The elution pattern measured by the phenol-sulfuric acid method [16] is shown in Fig. 2. Near the end of the water elution, α-D-man-(1 → 2)-D-man appeared gradually in region B together with the tail of the monosaccharide elution (region A). As can be seen in Fig. 2, the absorbance of α-D-man-(1 → 2)-D-man was so weak that the presence of the disaccharide in each fraction had to be confirmed by HPLC using a CarboPac PA-1 column (Dionex) and pulsed amperometric detector. The reason for the weak absorbance of α-D-man-(1 → 2)-D-man by the phenol–sulfuric acid method may be due to the linkage at the 2-position at the reducing end of α-D-man-(1 → 2)-D-man. The fractions in region C contained α-D-man-(1 → 6)-D-man in essentially pure form. In contrast, the fractions of region D contained small amounts of α-D-man-(1 → 6)-D-man, α-D-man-(1 → 3)-D-man, and two unidentified trisaccharides.

It should be noted that α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man was eluted in region B together with α-D-man-(1 → 2)-D-man. Normally the order of elution in activated carbon column chromatography is monosaccharides, disaccharides, and trisaccharides, however in this case, α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man eluted before α-D-man-(1 → 6)-D-man and α-D-man-(1 → 3)-D-man. Apparently the hydrophobicity of α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man is less than that of α-D-man-(1 → 6)-D-man and α-D-man-(1 → 3)-D-man.

α-D-Man-(1 → 3)-D-man and the trisaccharides in region D were separated by HPLC using a Carbon-500 column (Toso Co.), and the structure of one trisaccharide was identified as α-D-man-(1 → 2)-α-D-man-(1 → 6)-D-man by ^{13}CNMR spectroscopy. Johansson et al. [4,5] reported the presence of α-D-man-(1 → 1)-D-man in the reaction mixture using α-mannosidase from jack bean, but we could not detect this disaccharide. Similarly, α-D-man-(1 → 4)-D-man was not detected in any HPLC fraction or by activated carbon column chromatography.

Although α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man co-eluted with α-D-man-(1 → 2)-D-man, these two oligosaccharides could be easily separated by HPLC using an amino-silica column or gel-permeation chromatography. The HPLC pattern of the separation for these oligosaccharides is shown in Fig. 3. In addition, TLC of the peracetylated derivatives of mannose, α-D-man-(1 → 2)-D-man, and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man showed spots at R_f values of 0.52, 0.24, and 0.11, respectively, when the solvent system of *n*-hexane-ethyl acetate (4:5) was used. A preliminary separation of these sugars was performed by silica-gel column chromatography using an *n*-hexane-ethyl acetate solvent system. This approach proved very convenient for our purposes, as we desired the peracetylated form for use in the chemical synthesis of a high-mannose type oligosaccharide.

Representative results for the synthesis and isolation of manno-oligosaccharides are summarized in Fig. 4. Although the yield of α-D-man-(1 → 2)-D-man is only about 2%,

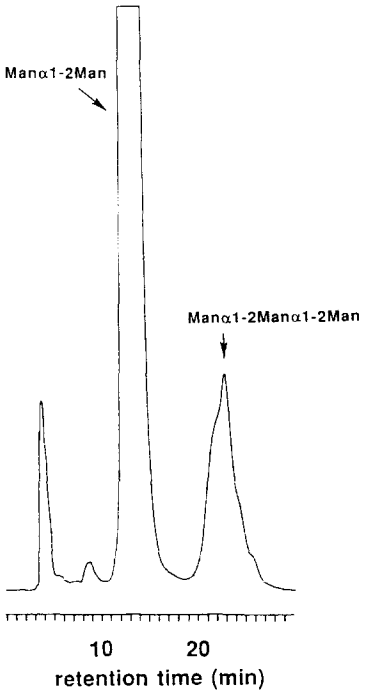

Fig. 3. Preparative HPLC of the mixture of α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man. Column: preparative LiChrosorb–NH_2; eluent, 75% acetonitrile; flow rate: 3mL/min; detection, refractive index monitor.

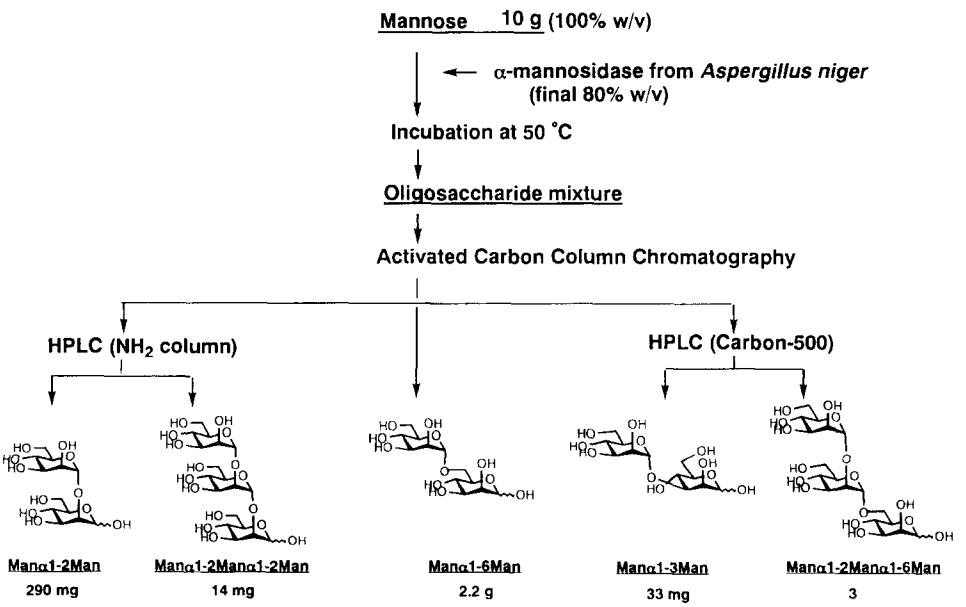

Fig. 4. Summary of the reaction and isolation of manno-oligosaccharides.

this procedure has several advantages: (1) the starting material is mannose which is inexpensive; (2) and the enzyme mixture does not require purification; and (3) α-D-man-(1 → 2)-D-man, α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man, α-D-man-(1 → 6)-D-man, and α-D-man-(1 → 3)-D-man can be obtained from a single reaction.

3. Experimental

Materials.—Transglucosidase Amano™ was purchased from Amano Phamaceuticals Co., (Osaka, Japan). LiChrosorb–NH_2, Carbon-500, and CarboPac PA-1 are products of Merck (Darmstadt, Germany), Toso Co., (Osaka, Japan), and Dionex (CA, USA), respectively. DEAE-MemSep™ is a product of Millipore (MA, USA).

Methods.—^{13}CNMR spectra were measured with a Varian Unity-500 NMR spectrometer for samples dissolved in D_2O containing a small amount of acetonitrile for chemical shift reference (1.27 ppm). The α-mannosidase activity was measured using α-(1 → 2)-linked mannan as a substrate [17], and the liberated mannose measured colorimetrically using 4-hydroxybenzoic acid hydrazide [18]. Protein concentration of the semi-purified α-mannosidase was measured using a protein assay reagent kit (Micro BCA™, Pierce, Ill, USA).

Semi-purification of α-mannosidase from A. niger *using a DEAE-MemSep™.*—Transglucosidase Amano™ (0.24 U/mg protein, 3mL) was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and the solution applied to DEAE-MemSep™. After washing with water for 40 min at a flow rate of 5 mL/min, the DEAE-MemSep™ was eluted using a 0 to 0.2 M NaCl gradient in 10 mM Tris—HCl buffer (pH 7.5) over 50 min. The α-mannosidase activity of each 10-mL fraction was measured and the fractions exhibiting α-mannosidase activity collected and concentrated to ~ 1 mL. The concentrated solution showed an α-mannosidase activity of 3.40 U/mL (3.86 U/mg protein)

Synthesis of manno-oligosaccharides via condensation reaction in the presence of semi-purified α-mannosidase from A. niger.—Mannose (100 mg) was dissolved in 0.1 M acetate buffer (pH 5.0) to a final volume of 100 μL, and to this, 20 μL of the semi-purified α-mannosidase solution was added. After incubation of the reaction mixture for 72 h at 50°C, the solution was heated in boiling water for 5 min to inactivate the enzyme. The composition of the products was analyzed by HPLC using a Carbon-500 column.

Preparative-scale synthesis of manno-oligosaccharides using crude enzyme.—Mannose (10 g) was dissolved in 0.1 M acetate buffer (pH 5.0) to a final volume of 10 mL. Transglucosidase Amano™ (2.5 mL) was added to this solution and the mixture incubated at 50°C for 20 days. After the inactivation of the enzyme by heating in boiling water for 5 min, the solution was applied to an activated carbon column (10 cm∅ × 50 cm). After washing the column with 2 L of water at a flow rate of 10 mL/min, the column was eluted with a 0 (2 L) to 30% (2 L) aqueous ethanol gradient. Fractions (20 mL) were collected and the sugar concentration in each was measured by the phenol–sulfuric acid method [16]. After concentration of the fractions of the main peak, 2.2 g of α-D-man-(1 → 6)-D-man was obtained exclusively.

Each fraction between monosaccharide and α-D-man-(1 → 6)-D-man was examined by HPLC using a CarboPac PA-1 column, and the fractions containing α-D-man-(1 → 2)-D-man and α-D-man-(1 → 2)-α-D-man-(1 → 2)-D-man were collected and concentrated to give ~ 320 mg of syrup. The syrup was dissolved in 1 mL of water and 100 μL portions of this solution were applied to a preparative HPLC LiChrosorb–NH_2 column (10 mm∅ × 20 cm). Elution of the column with 70% aqueous acetonitrile, yielded two compounds. Following 10 such separations, 290 mg of α-D-man-(1 → 2)-D-

By concentration of the shoulder fractions of the α-D-man-(1 → 6)-D-man peak, ~ 150 mg of syrup was obtained. To isolate the oligosaccharides in this fraction, two methods were attempted. The syrup was dissolved in 600 μL of water, and the resulting 700 μL solution was divided into 600 μL and 100 μL portions. The 600 μL portion was subdivided into three 200 μL portions which were applied to a preparative HPLC Carbon-500 column, (7.5 cm∅ × 15cm). The combined product of these three separations was 33 mg of α-D-man-(1 → 3)-D-man. The 100 μL portion was subdivided into four 25 μL portions which were applied to a LiChrosorb–NH_2 HPLC column and the column was eluted with 75% aqueous acetonitrile. The combined product of the separations was ~ 3 mg of α-D-man-(1 → 2)-α-D-man-(1 → 6)-D-man. ^{13}C NMR (125 MHz, D_2O): 102.9 (C-1), 100.2 (C-1′), 93.2 (C-1″), 80.0 (C-2′), 73.9, 73.4 (C-5, C-5′), 71.6 (C-5″), 71.3, 71.1 (C-2, C-2″), 70.9, 70.7, 70.6 (C-3, C-3′, C-3″), 66.0 (C-6″), 61.6 (C-6, C-6′).

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