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Enzymic synthesis of the trisaccharide core region of the carbohydrate chain of *N*-glycoprotein

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Transmannosylation from mannotriose (Manβ1-4Manβ1-4Man) to the 4-position at the nonreducing end N-acetylglucosaminyl residue of *N*,*N*′-diacetylchitobiose was regioselectively induced through the use of β-D-mannanase from *Aspergillus niger*. The enzyme formed the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc (3.7% of the enzyme-catalysed net decrease of *N*,*N*′-diacetylchitobiose) from mannotriose as a donor and *N*,*N*′-diacetylchitobiose as an acceptor. Mannobiose (Manβ1-4Man) was also shown to be useful as a donor substrate for the desired trisaccharide synthesis.

Keywords: Manβ1-4GlcNAcβ1-4GlcNAc, β-D-mannanase in carbohydrate synthesis, trisaccharide core of *N*-glycoprotein

Abbreviations: Man, D-mannose; (M_n) (n = 1–5), β-linked n-mer of mannose; $GlcNAc_2$; 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-2-deoxy-D-glucose.

Introduction

There is at present great interest in developing synthetic routes to oligosaccharide involved in glycoconjugates. From a practical viewpoint, the use of glycosidases is very attractive in oligosaccharides synthesis, utilizing the transglycosylation reaction. Our interest was directed to an enzymic approach to the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc (**1**), which is the common structural feature at the core region of most of the *N*-glycoproteins. This compound will be useful as an exogenous substrate for β-D-mannosidase [1, 2], a probe for lectin [3], and a common synthetic intermediate of *N*-linked glycan. Organic chemical methods for obtaining **1** have been developed [5–8], but are characterized by variously elaborated protection, glycosylation, and deprotection. Especially, site-selective glycosylation for the introduction of the β-D-Man residue is known to be a key reaction of synthesizing compound **1** [7]. From a practical viewpoint, the use of glycosidases, which are commercially available, is very attractive in the synthesis of an oligosaccharide in glycoconjugates, utilizing transglycosylation. However, use of glycosidases in synthesis has been limited due to the preponderant formation of the (1-6) linkage over of the (1-2), (1-3), and (1-4) linkages [9, 10]. This paper reports that β-D-mannanase (1,4-β-D-mannan mannohydrolase; EC 3.2.1.78) from *A. niger* regioselectively forms **1** (Manβ1-4GlcNAcβ1-4GlcNAc) through a transglycosylation reaction from M_3 (Manβ1-4Manβ1-4Man) and $GlcNAc_2$ (GlcNAcβ1-4GlcNAc).

Materials and methods

Materials

Commercially available hemicellulase (AC, Shinnihon Kagaku Co., Ltd, Japan) prepared from the culture filtrates of *A. niger* was used as an enzyme source. Mannooligosaccharides (d.p. 2–5) were prepared by the method of Kusakabe [11]. The charcoal-Celite column for the separation of transglycosylation products was prepared as follows: equal parts by weight of dry charcoal and Celite were slurried in water and packed into a glass column. All other chemicals were obtained from commercial sources.

Enzyme assays

β-D-Mannanase activity was assayed as follows. The mixture containing 0.5 mg of Konjac mannan in 0.1 ml, 20 mM sodium acetate buffer (pH 5.0) and enzyme solution in a total volume of 0.2 ml was incubated at 40 °C for 10 min. The amount of reducing activity produced was determined by the Somogyi-Nelson method [12, 13]. One unit of activity was defined as the amount of enzyme

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liberating 1 μmol of reducing sugar equivalent as mannose, per min under the assay conditions. *N*-Acetyl-γ-D-hexosaminidase (NAHase, EC 3.2.1.52) activity was assayed with *p*-nitrophenyl β-*N*-acetylglucosaminide as the substrate. One unit of activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

Preparation of affinity adsorbent

Copra mannan was prepared according to the method of Takahashi *et al.* [14]. Soluble short-chain glucomannan prepared by formic acid treatment of glucomannan from Konjac [15] was used as affinity ligands. Short-chain glucomannan-AH Sepharose CL 6B was prepared as follows. Three grams of short-chain glucomannan were reacted with 3 g of cyanogen bromide in acetonitrile (4 ml) with vigorous stirring for 5 min at room temperature, and then AH-Sepharose (30 ml gel packed vol.) prepared by the procedure of Kaufman [16] was added. The reaction was carried out overnight at 5 °C with shaking. The resulting short-chain glucomannan-AH-Sepharose CL 6B was sequentially washed with 1 M $NaHCO_3$ (300 ml), 2 M KCl (300 ml), 2 M sodium acetate (300 ml) and distilled water (1.5 l), and finally suspended in 20 mM sodium acetate buffer at pH 5.0 (buffer A).

Partially purified β-D-mannanase from A. niger

β-D-Mannanase completely devoid of NAHase activity was prepared as follows. Hemicellulase AC (2 g) was suspended in 10 ml of buffer A and stirred for 1 h, and insoluble material was removed by centrifugation. To the solution, solid ammonium sulfate was added to give 75% saturation. After centrifugation of the precipitate formed, it was dissolved in 10 ml of the same buffer, and then dialysed using a Micro Acilyzer (Asahikasei Kogyo Co. Ltd).

DEAE-Sepharose fast flow ion-exchange chromatography (a)

One fifth volume of the desalted enzyme solution obtained by solid ammonium sulfate precipitation was directly applied to a column (3.0 × 16 cm) of DEAE-Sepharose Fast Flow equilibrated with 0.1 M sodium acetate buffer at pH 5.0 (buffer B). The column was washed with buffer B (tubes 0–34) and then eluted with a linear gradient of 0 (2 l)–0.3 M (2 l) NaCl in buffer B. The eluate was collected in 18 ml fractions. Most of the β-D-mannanase activity (tubes 98–110) eluted with 0.1 M NaCl. Eluates of this fraction were combined and concentrated to low volume (2 ml) using the Amico Diaflo unit, then lyophilized (6.1 mg: 16.7 U mg^{-1}).

Glucomannan-AH-sepharose CL 6B affinity chromatography (b)

The enzyme from step (a) was dissolved in 2 ml of 20 mM sodium acetate buffer at pH 5.0 (buffer C). The enzyme solution was loaded on a glucomannan-AH-Sepharose CL 6B column (1.2 × 26 cm) equilibrated with buffer C in 0.5 M NaCl. When the column was changed to buffer C in 1.0 M NaCl, most of the mannanase activity (tubes 35–45) associated with the protein peak emerged from the column a little behind a NAHase peak. The eluate was collected in 2 ml fractions. The combined solutions were desalted using the Amicon Diaflo unit. A peak showing the mannanase activity (tubes 38–49) was completely separated from the NAHase contaminant peak. The combined solutions were concentrated to 2 ml using the Amicon Diaflo unit, lyophilized (10.5 mg:17.7 U mg^{-1}), and then stored over $CaSO_4$ at 4 °C. The partially purified β-D-mannanase was used for the enzymic synthesis.

Analytical method

HPLC was performed with an Asahipak packed column NH2P-50 (4.6 × 250 mm) in a Hitachi L-4000 ultraviolet detector. Elution was effected with water:acetonitrile (1:3, by vol). The flow rate was 1.0 ml min^{-1} at a pressure of 60 kg cm^{-2}. ^{13}C- and ^{1}H-NMR spectra were determined with a Varian XL-500 spectrometer operating at 125.7 MHz in the pulsed Fourier-transform mode with complete proton decoupling and 499.84 MHz, respectively. Chemical shifts are expressed in ppm relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an internal standard. Mass spectrometric analysis of the sugar chain was carried out as follows. After reduction of oligosaccharide, the oligosaccharidiol was permethylated according to the method of Hakomori [17]. The permethylated product was purified through a Wakogel S-1 column (0.3 × 3 cm, Wako Pure Chemical Ltd., Japan) with methanol as eluent at 37 °C. The mass spectrum of the purified permethylated oligosaccharidiol was obtained by a direct inlet system and recorded using a Jeol JNS-DX302 spectrometer equipped with a Jeol DA-500 mass-data system at an ionizing voltage of 70 eV. FABMS/MS spectrum of oligosaccharides was recorded with a Jeol JMS HX/HX 110A mass spectrometer, operating at the full accelerating potential (10 kV) and coupled to a Jeol DA-800 mass data system. The sample (3 μl) in distilled water was added to the magic bullet. The molecular weight of the sample was estimated from *m/z* value of quasi molecular-ion $[M + H]^+$ peak. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd).

Results and discussion

Enzymic synthesis

A β-D-mannanase from *A. niger* was partially purified by successive chromatographies on DEAE-Sepharose Fast Flow and short-chain glucomannan-AH-Sepharose CL 6B in order to separate from contaminant NAHase activity. The β-D-mannanase completely devoid of NAHase activity was used for the enzymic synthesis, because the latter enzyme acts on $GlcNAc_2$ as an acceptor substrate as

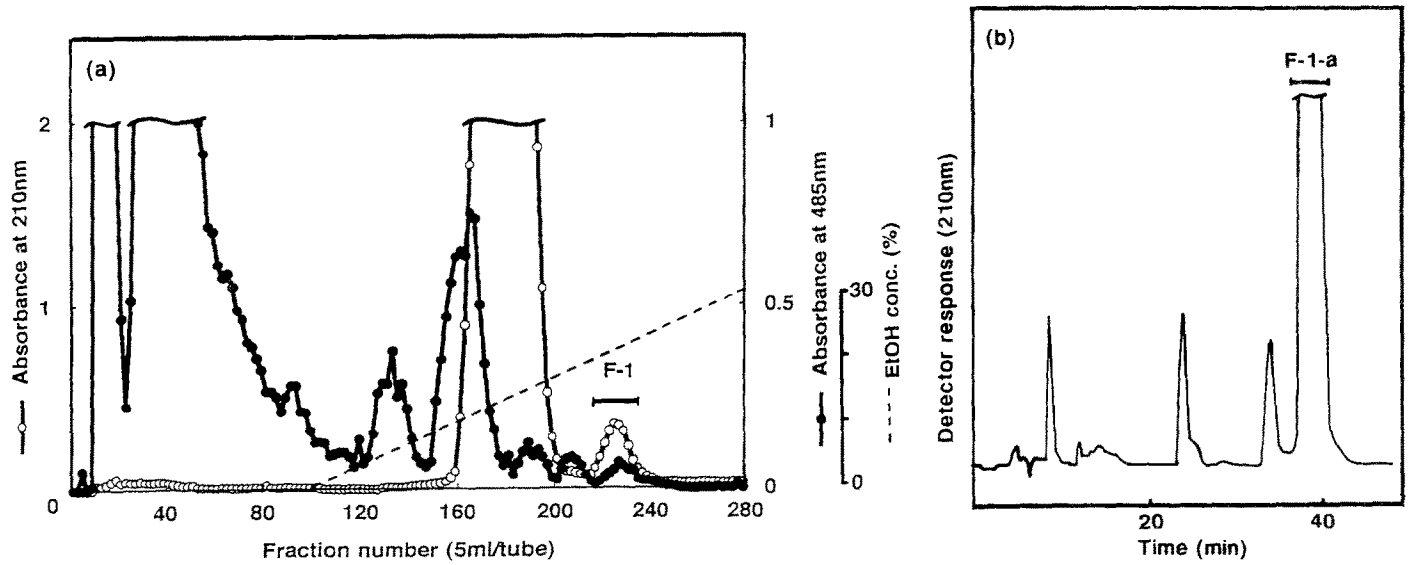
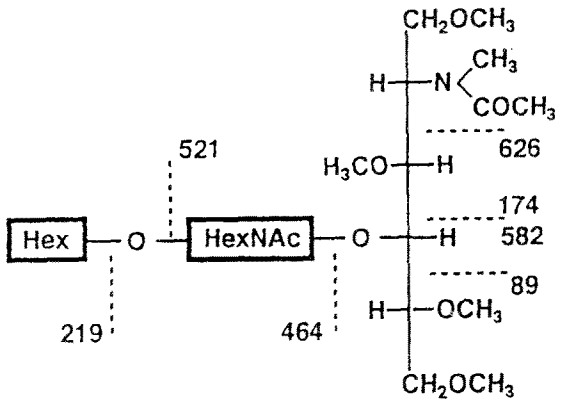


Figure 1.

mentioned below. To a solution of M_3 (357 mg) and $GlcNAc_2$ (150 mg) in 50 mM sodium acetate buffer (3 ml, pH 4.0) was added the partially purified β-D-mannanase (50 U). The mixture was incubated at 40 °C for 10 h and terminated by heating at 95 °C for 10 min. The molar ratio of the former and latter compounds was 2:1 and the total substrate concentration was 14.5%. The resulting insoluble material was centrifuged off. The supernatant was directly applied to a charcoal-Celite column as in Fig. 1(a). The column was first eluted with water (500 ml) and then with a linear gradient of 0 (450 ml)–30% (450 ml) ethanol. The elution was monitored by measuring the absorbance at 210 nm (characteristic absorption of the *N*-acetyl group) and at 485 nm (carbohydrate content, determined by the phenol-sulfuric acid method). The chromatogram shows one small peak for which the absorption at 210 nm coincides with that at 485 nm. The F-1 peak (tubes 216–235) was combined, concentrated, and lyophilized. The fraction was dissolved in 1 ml of distilled water and 1/10 vol of the solution was applied to HPLC. The F-1 fraction was separated into one main peak (F-1a) as shown in Fig. 1(b). The eluates corresponding to the F-1a peak were combined, concentrated, and lyophilized. The remaining aliquots were similarly worked as above. As a result, F-1a gave a yield of 5.5 mg.

Characterization

The FAB-MS indicated that F-1a is a trisaccharide consisting of Hex-HexNAc-HexNAc. Thus, the sugar sequence was confirmed by the presence of *m/z* 204 (fragment from the reducing end of HexNAc), 407 (fragment from the reducing end of Hex-HexNAc) and 587 ($[M + H]^+$). F-1a was also identified by its EI mass spectrum as the permethylated alditol of a Hex-HexNAc-1,4-HexNAcitol. Fragment ions at *m/z* 174 and 582 indicate the linkage of the disaccharide unit (Hex-HexNAc) to HexNAcitol at the C-4 position. Other fragment ions at *m/z* 89, 219, 464, 521, and 626 are characteristic for the fragmentation indicated below.



^1H- and ^{13}C-NMR were used for further elucidation of the structure of the product involved in F-1(a). Figure 2 shows the HMQC spectral map of F-1a. ^1H-NMR (^2H_2O, 50 °C): δ5.208/4.718 (α/β, d, *J* 2.93/8.06 Hz); 4.770 (s, *J* < 1 Hz, H-1″, due to the mannosyl residue; 4.626/4.621 (d, *J* 7.70/7.69 Hz, H-1′, due to α,β-anomerization of the *N*-acetylglucosamine); 2.085 (s, 3H, CH_3CON–) and 2.059 (s, 3H, CH_3CON–). The carbon resonances were assigned by the ^{13}C-^1H shift-correlation map and by comparing the spectrum with the data for the corresponding model $GlcNAc_2$ [18] and mannooligosaccharides [19] as in Table 1. These results indicated clearly that F-1a is a trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc: $[\alpha]_D^{25}$ + 0.2 (*c* 1.0, water).

Time course of reaction for the production of **1**

When $GlcNAc_2$ was used as the acceptor, D-mannosyl transfer occurred regioselectively at 0-4 of the nonreducing end *N*-acetylglucosamine residue of the oligomer. Compound

Table 1. Carbon-13 chemical shifts of fraction F-1a in ^2H_2O solution.

	C-1	C-2	C-3	C-4	C-5	C-6	CH_3 ($NHCOCH_3$)	C=O ($NHCOCH_3$)
Iα	93.279	56.481	72.097	82.586	72.844	62.863	24.742	177.256
Iβ	97.649	58.972	77.429	82.160	77.429	62.989	25.023	177.537
II	104.204	57.923	74.836	81.534	77.429	62.949	24.983	177.356
III	102.915	73.347	75.317	69.472	79.250	63.769		

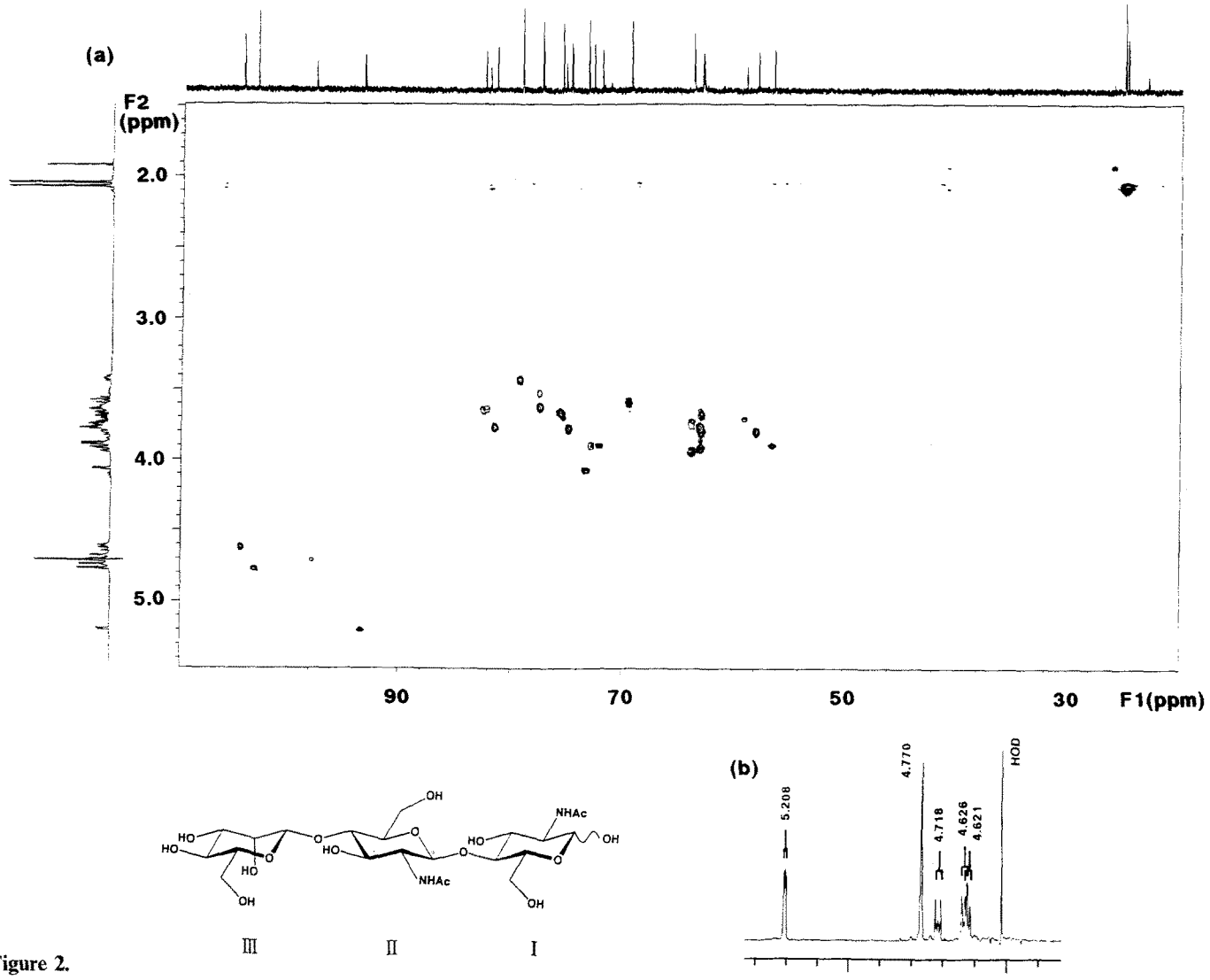


Figure 2.

1 was obtained in a yield of 3.7% based on the $GlcNAc_2$ added. Figure 3 is a transglycosylation profile of the reaction of β-D-mannanase with M_3 and $GlcNAc_2$. The amount of compound **1** produced as a function of time was examined on the 0.5 ml scale according to the method mentioned above. The samples (20 µl) were taken at intervals during the incubation, inactivated by heating at 95 °C for 10 min, and then diluted with 8 vol of water for analysis by HPLC. The time at which maximum production of **1** was reached was 8 h. Once formation of **1** reached its maximum, the amount remained almost unaltered during the subsequent reaction. Furthermore, when M_2 (Manβ1-4Man) was the donor instead of M_3, the enzymic reaction also gave exclusively **1**. However, the maximum rate for the production of **1** decreased markedly and its yield dropped to 2.5% (based on the $GlcNAc_2$ added).

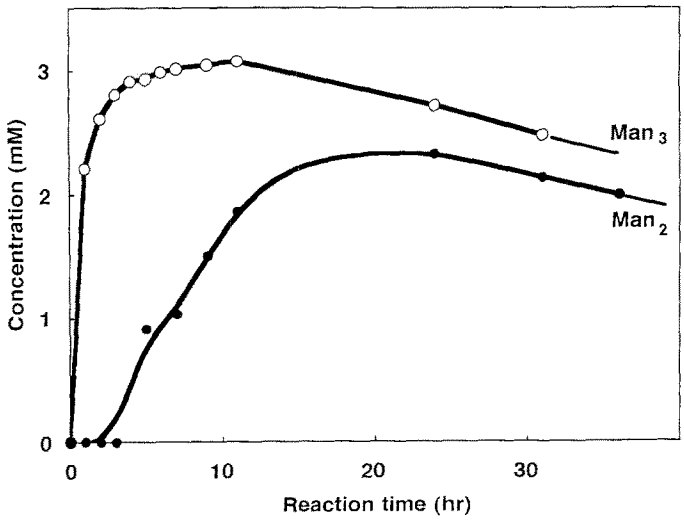


Figure 3.

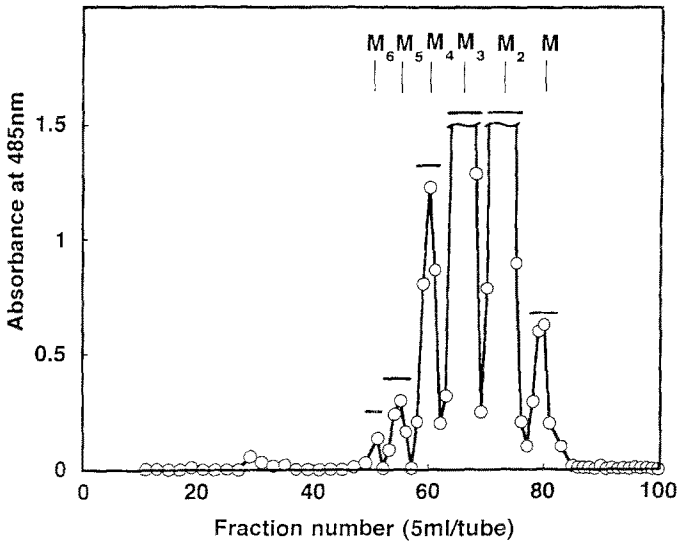


Figure 4.

Transglycosylation mode of the β-D-mannanase

A disproportionation of M_3 as the donor substrate suggested that the β-D-mannanase shows site-selective glycosylation of $GlcNAc_2$. Thus, M_3 and $GlcNAc_2$ were reacted by the following major reaction processes:

$$MMM^* + E \rightleftarrows M \cdot E + MM^* \quad (1)$$

$$M \cdot E + GG^* \rightleftarrows MGG^* + E \quad (2)$$

(E = β-mannanase; M = mannose; G = *N*-acetylglucosamine, * = reducing-end group).

In equations (1) and (2), M_3 serves as the donor and $GlcNAc_2$ as the acceptor, and **1** is regioselectively synthesized by transferring mannosyl residues to the $GlcNAc_2$ acceptor molecule by use of excess of substrate. We further investigated a transglycosylation reaction by M_3 to elucidate mannosyl transfer from the substrate to the acceptor. When a 20% M_3 solution was reacted with the enzyme for 5 h at 40 °C, the chain-elongation reaction could be observed with the substrate as shown in Fig. 4. The molar ratio of M_1 (Man), M_2, M_3, M_4, (Manβ1-4Manβ1-4Manβ1-4Man), and M_5 (Manβ1-4Manβ1-4Manβ1-4Manβ1-4Man) obtained by transglycosylation reaction was 1.0:9.0:5.1:0.5:0.1. This indicates that M_3 serves in both as the donor and the acceptor. Thus, a mannosyl residue split from the donor is able to be predominantly transferred to another M_3 acceptor to form M_4. M_4, once accumulated. is quickly hydrolysed to M_2, which is mainly produced as the final reaction product. In this case, accumulation of M_1 is to a lesser extent observed during the entire course of reaction. No chain-elongation reaction occurred with M_4 as an initial substrate. M_3 might be a good substrate for the transglycosylation reaction of this enzyme. Polysaccharide hydrolyases such as amylase [20], amylomaltase [21], cellulase [22], and lysozyme [23, 24], exhibit high regioselectivity for the hydroxyl group of the acceptor and this selectivity is much more predictable than that of glycosidases. An interesting result from these studies is the capacity of the β-D-mannanase to catalyse regiospecific formation of the β-(1-4)-linked D-mannosyl trisaccharide, which is important as a common core unit of *N*-glycoproteins. With this reaction it was also possible to perform a one pot preparation of **1**. We are presently engaged in expanding the method to other enzymes and oligosaccharides.

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