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Transfer of $Man_9GlcNAc$ to L-fucose by endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*

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We have reported that transglycosylation activity of endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (endo-A) can be enhanced to near completion using GlcNAc as an acceptor in a medium containing 30% acetone (Fan J-Q, Takegawa K, Iwahara S, Kondo A, Kato I, Abeygunawardana C, Lee YC (1995) *J Biol Chem* **270**: 17723–29). In this paper, we found that the endo-A can also transfer an oligosaccharide, $Man_9GlcNAc$, to L-Fuc using $Man_9GlcNAc_2Asn$ as donor substrate in a medium containing 35% acetone. The transglycosylation yield was greater than 25% when 0.2 M L-Fuc was used as acceptor. The transglycosylation product was purified by high performance liquid chromatography on a graphitized carbon column and the presence of L-Fuc was confirmed by sugar composition analysis and electrospray mass spectrometry. Sequential exo-glycosidase digestion of pyridyl-2-aminated transglycosylation product, $Man_9GlcNAc$-L-Fuc-PA, revealed that a β-anomeric configuration linkage was formed between GlcNAc and L-Fuc. The GlcNAc was found to be 1,2-linked to L-Fuc by two methods: i) collision-induced decomposition on electrospray mass spectrometry after periodate oxidation, reduction and permethylation of $Man_9GlcNAc$-L-Fuc; and ii) preparation of $Man_9GlcNAc$-L-Fuc-PA, its periodate oxidation and reduction, followed by hydrolysis and HPLC analysis. Thus, the structure of the oligosaccharide synthesized by endo-A transglycosylation was determined to be $Man_9GlcNAc\beta(1,2)$-L-Fuc. Methyl β-L-fucopyranoside, L-Gal are also acceptors for the enzymic transglycosylation. However, transglycosylation failed when methyl α-L-fucopyranoside, D-Fuc and D-Gal were used. These results indicate that the endo-A requires not only 3-OH and 4-OH to be equatorial but also a $^4C_1$-conformation or equivalent conformation of the acceptor to perform transglycosylation.

Keywords: transglycosylation to fucose, endo-β-*N*-acetylglucosaminidase, periodate oxidation, pyridylamination, electrospray mass spectrometry

Abbreviations: endo-A, endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*; PA, pyridyl-2-amino-; AP, aminopyridine; GlcNAc, *N*-acetyl-D-glucosamine; Man, mannose; Gal, galactose; Fuc, fucose; Glc, glucose; PA-C_2, PA-glycolaldehyde; PA-C_3, PA-L-glyceraldehyde; PA-C_4, PA-D-threose; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detector; HPLC, high performance liquid chromatography; ODS, octadecylsilyl; ES-MS, electrospray mass spectrometry; CID, collision-induced decomposition.

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Introduction

The enzymatic method for synthesis and reconstruction of glycoproteins is becoming increasingly popular, because it is more stereo- and regio-selective than chemical methods [1, 2]. Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (endo-A) was found to perform transglycosylation [3, 4], and is a potential tool for reconstruction and remodelling of glycopeptides and glycoproteins [5]. In fact, we have reported [6] that the transglycosylation activity by endo-A could be enhanced by inclusion of 30% acetone in the reaction medium, and some useful neoglycoconjugates have been synthesized [7].

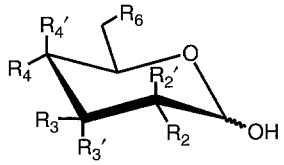
The study on the acceptor specificity of endo-A transglycosylation toward various monosaccharides[‡] revealed that the 3- and 4-OHs of an acceptor sugar residue must be equatorial, in a normal conformation, but 1-, 2-, and 6-OHs are relatively unimportant [6]. For instance, endo-A could transfer oligosaccharide in about 90% yield to GlcNAc, Glc, Man, and 2-deoxy-Glc, all possessing equatorial 3-OH and 4-OH (Scheme 1), in contrast with Gal, allose, and 3-deoxy-Glc which are not acceptors. When methyl α-GlcNAc is used as acceptor, the newly formed linkage by the endo-A transglycosylation was determined to be GlcNAcβ1,4GlcNAc by ^{1}H-NMR [6]. During the course of studying the acceptor specificities, we found that the enzyme could also transfer the oligosaccharide to L-Fuc to a significant level. This result appears to contradict our previous conclusion, because the 4-OH of L-Fuc is axial.

L-Fuc is a component sugar found in some of the *N*-linked glycoproteins. It often links to the Asn-bound GlcNAc through an α-1,3- or α-1,6 linkage [8, 9], or occupies non-reducing terminal positions of outer branches. Recently, existence of difucosylated GlcNAc-Asn was found in an insect glycoprotein [10]. Since the GlcNAc is the target acceptor of endo-A transglycosylation, any possible acceptance of oligosaccharide by L-Fuc would be of great concern, because it could lead to undesirable products. Therefore, it is necessary and important to elucidate the mechanism of transglycosylation to L-Fuc by endo-A. In this paper, we report the determination of the GlcNAc-L-Fuc linkage synthesized by the endo-A tranglycosylation and the further information on the acceptor specificity of endo-A transglycosylation.

Methods and materials

Materials

Endo-A was purified by the published method [11]. $Man_9GlcNAc_2Asn$ and $Man_9GlcNAc_2AsnPhe$ were prepared from soybean agglutinin by exhaustive Pronase digestion, followed by gel filtration on Sephadex G-50 and further purification with HPLC on a graphitized carbon column [12]. 2-Aminopyridine (re-crystallized from 1-hexane before use) and Dimethylamine-borane complex were obtained from Aldrich Chemical Co. (Milwaukee, WI). Alpha-mannosidase from jack bean meal was purchased from V-labs, Inc. (Covington, LA). Beta-mannosidase from *Helix pomatia* and β-*N*-acetylhexosaminidase from jack bean meal were obtained from Oxford GlycoSystems (Rosedale, NY). GlcNAc, L-Fuc, D-Fuc, Gal, and Man were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL) and L-Gal was obtained from Sigma Chemical Co. (St Louis, MO). Methyl α- and β-L-Fuc were synthesized by the conventional method. PA-glycolaldehyde, PA-L-glyceraldehyde and PA-D-threose were prepared according to the previous report [13].



Sugar	Transglycosylation (%)	Substituent
GlcNAc	97	R_3=R_4=R_6=OH, R_2'=R_3'=R_4'=H, R_2=NHAc;
Glc	89	R_2=R_3=R_4=R_6=OH, R_2'=R_3'=R_4'=H;
Man	87	R_2'=R_3=R_4=R_6=OH, R_2=R_3'=R_4'=H;
Gal	0	R_2=R_3=R_4'=R_6=OH, R_2'=R_3'=R_4=H;
All	0	R_2=R_3'=R_4=R_6=OH, R_2'=R_3=R_4'=H;
Xyl	24	R_2=R_3=R_4=OH, R_2'=R_3'=R_4'=H;
2-deoxy-Glc	91	R_3=R_4=R_6=OH, R_2=R_2'=R_3'=R_4'=H;
3-deoxy-Glc	0	R_2=R_4=R_6=OH, R_2'=R_3=R_3'=R_4'=H;
6-deoxy-Glc	77	R_2=R_3=R_4=OH, R_2'=R_3'=R_4'=R_6=H;
3-OMe-Glc	36	R_2=R_4=R_6=OH, R_2'=R_3'=R_4'=H, R_3=OMe.

Scheme 1.

[‡]All monosaccharides used are of D-configuration, unless otherwise noted.

High performance anion exchange chromatography

An HPAEC system that consisted of a Bio-LC (Dionex Corp., Sunnyvale, CA) equipped with a pulsed amperometric detector (PAD-II) and a Dionex CarboPac PA-1 column (4 × 250 mm) was used for analysis of the enzyme reaction. The separation of endo-A transglycosylation products was accomplished by elution with 100 mM sodium hydroxide with a linear gradient of sodium acetate (0–80 mM) developed in 24 min at a rate of 1.0 ml min^{-1}. A cycle of a 5 min washing with 100 mM sodium hydroxide-200 mM sodium acetate and a 15 min equilibration with the starting buffer was inserted between runs. The quantification of transglycosylation product was by substraction of the hydrolysis product ($Man_9GlcNAc$) and remaining donor substrate ($Man_9GlcNAc_2Asn$) from the starting donor substrate. Quantification is based on the assumption that the PAD response is approximately equal on molar basis.

High performance liquid chromatographies

HPLC were performed with a Gilson HPLC system equipped with a Rheodyne 7125 injector, a Fiatron CH-30 column heater, and an uv detector (ISCO, Lincoln, NE) for unmodified oligosaccharide or a fluorescence detector (LS-40, Perkin Elmer) for PA-oligosaccharide. The flow rate used was 1.0 ml min^{-1}. To purify $Man_9GlcNAc$-L-Fuc from the reaction mixture, a Shandon Hypercarb graphitized carbon column (4.6 × 100 mm, Shandon Scientific, UK) with a Direct-Connect guard cartridge column (Alltech Associates Inc., Deerfield, IL) was used. The elution was at 70 °C with 10 mM NH_4OH and a linear gradient of CH_3CN (6–11%) developed in 40 min, and the effluent was monitored at 215 nm. PA-oligosaccharide was analysed with a TSK-GEL Amido-80 column (4.6 × 250 mm, Tosoh, Tokyo) and an Ultrasphere ODS column (4.6 × 250 mm, Beckman, CA). The conditions for elution of the Amido-80 column were 3% acetic acid-triethylamine buffer (pH 7.3) with: A) a linear gradient of CH_3CN (90–65%) developed in 30 min; B) a gradient of 65–50% CH_3CN in 50 min. The elution of the ODS column was 50 mM citrate buffer (pH 4.0) with 1% CH_3CN. The effluent was monitored by measuring fluorescence (λ_{ex} 320 nm; λ_{em} 390 nm).

Transglycosylation with endo-A

A transglycosylation reaction at an analytical level was performed with a mixture of 3 nmol of $Man_9GlcNAc_2Asn$, 1 mU of enzyme and 2 μmol of acceptor, in a total volume of 10 μl of 25 mM ammonium acetate buffer (pH 6.0) containing 35% acetone (by vol). After incubation at 37 °C for 15 min, the enzymic reaction was terminated by boiling for 3 min in a water bath, and the mixture was evaporated with a Speedvac using a vacuum pump. An aliquot of the reconstituted sample was analysed by HPAEC-PAD.

Preparation of $Man_9GlcNAc$-L-Fuc by endo-A transglycosylation

In order to prepare a larger quantity of $Man_9GlcNAc$-L-Fuc for detailed determination of the linkage structure, 200 nmol of $Man_9GlcNAc_2Asn$ was incubated with 90 μmol L-Fuc and 47 mU endo-A in 180 μl of 0.1 M NH_4OAc buffer (pH 6.0) containing 35% acetone at 37 °C for 25 min. After terminating the reaction by boiling, the product was purified by HPLC using a graphitized carbon column.

Pyridylamination of $Man_9GlcNAc$-L-Fuc

Pyridylamination was carried out according to the published method [14]. To a thoroughly dried oligosaccharide sample (*ca.* 70 nmol), 10 μl of a 2-aminopyridine solution (2.76 g ml^{-1} gl. acetic acid) was added and heated in a heating block (Lab-line, Melrose Park, IL) at 90 °C for 60 min, followed by addition of a 10 μl of 20% dimethylamine-borane complex in gl. acetic acid and heating at 80 °C for 50 min. After the reaction, the sample was diluted with 0.3 ml H_2O and purified on a Sephadex G-10 column (1 × 57 cm) eluting with 50 mM NH_4HCO_3.

Sequential exo-glycosidase digestion of PA-oligosaccharide

i) α-Mannosidase digestion. A sample of pyridylaminated oligosaccharide ($Man_9GlcNAc$-L-Fuc-PA, *c.* 30 nmol) was incubated with 1 U of α-mannosidase in 50 μl of 20 mM ammonium acetate buffer (pH 5.0) at 37 °C for 48 h. The reaction was monitored and the product was purified by HPLC on an Amido-80 column using the condition B.

ii) β-Mannosidase digestion. The α-mannosidase digested sample was incubated with 0.1 U of enzyme in 75 μl of 25 mM citrate-phosphate buffer (pH 4.0) at 37 °C for 18 h. The completion of the reaction was determined by HPLC on the Amido-80 column using the elution condition A.

iii) β-N-Acetylhexosaminidase digestion. The PA-disaccharide (500 pmol) after β-mannosidase digestion, presumably GlcNAc-L-Fuc-PA, was treated with 250 mU of the enzyme in 55 μl of 20 mM citrate-phosphate buffer (pH 6.0) at 37 °C for 18 h. An additional 250 mU of the enzyme was added to the reaction mixture and incubated for another 18 h. Completion of the reaction was confirmed by two HPLC analyses using the Amido-80 and ODS columns.

Determination of the linkage position between GlcNAc and Fuc in $Man_9GlcNAc$-L-Fuc by HPLC

The method used is based on Omichi and Hase [15, 16]. A PA-oligosaccharide ($Man_9GlcNAc$-L-Fuc-PA, 5 nmol) was dissolved into 15 μl of 0.1 M sodium acetate buffer

(pH 4.0) and oxidized with 15 μl of 0.1 M sodium metaperiodate for 40 h in a dark cold room. The reaction was terminated by adding 40 μl of 0.26 M sodium borohydride at room temperature for 3 h to destroy the excess reagent ($NaIO_4$) and reduce the newly formed aldehyde groups. Acid hydrolysis of the reaction mixture was accomplished by addition of 2.4 μl of 3 M H_2SO_4 and heating at 80 °C for 30 min, followed by adjusting the pH to *ca.* 8.5 by 1 M NaOH. A small portion of the 2-aminopyridine formed during the reaction was removed by addition of dry Dowex 50X8 (NH_4^+-form) beads (a few mg). The final fluorescent derivative was analysed by comparing with the elution time of the standards on HPLC of ODS column.

Methylation of oligosaccharide

The $Man_9GlcNAc$-L-Fuc was directly methylated by adding 100 μl of a NaOH/DMSO suspension, prepared by vortexing DMSO and powdered sodium hydroxide [17]. After 1 h at room temperature, 35 μl of methyl iodide was added and the suspension set for 1 h at room temperature with occasional vortexing [18]. The methylated product was extracted into chloroform and back washed with water until neutral.

Periodate oxidation and derivatization

Periodate oxidation [19, 20] of $Man_9GlcNAc$-L-Fuc was performed using a 9 mM solution of $NaIO_4$ buffered with 0.1 M sodium acetate at pH 5.5 in a dark cold room (4 °C) for 48–72 h. The reaction was quenched with ethylene glycol and the mixture was kept for an additional 24 h under the same conditions. The sample was neutralized with 0.1 M NaOH to which was added 5 mg of solid $NaBH_4$, and the reaction mixture was kept at room temperature for an additional 16 h. Excess reducing reagent was destroyed by the addition of acetic acid and the solution dried under vacuum. Borate was removed as its methyl ester by repeated addition of methanol followed by evaporation. The sample is vacuum-desiccated prior to permethylation.

Electrospray ionization mass spectrometry [21]

The instrument used in this study was a Finnigan-MAT TSQ-700 (Finnigan-MAT Corp., San Jose, CA) equipped with an electrospray ion source. Methylated samples were dissolved in methanol:water (6:4, by vol) containing 0.25 mM sodium hydroxide and analysed by syringe pump flow-injected at a rate of 0.75 μl min^{-1} directly into the electrospray chamber through a stainless steel hypodermic needle. The voltage difference between the needle tip and the source electrode was −3.5 kV. For collision-induced decomposition (CID) studies, multiply charged precursor ions were selectively transmitted by the first mass analyser and directed into the collision cell containing argon at roughly 0.27 Pa (2 mtorr) with acceleration voltages of 30–40 V.

Results and discussion

We have demonstrated that endo-A requires both 3-OH and 4-OH of the acceptor (e.g. GlcNAc) in the normal conformation to be equatorial for efficient transglycosylation and the transglycosylation occurs at 4-position when GlcNAc is used as an acceptor [6, 7]. However, when L-Fuc was used as acceptor, an unidentified peak eluting at 17 min was found on the chromatogram of HPAEC-PAD (Fig. 1). This peak represented more than 25% of the starting substrate. If this unidentified product represents a transglycosylation product to L-Fuc, it is a deviation from our previous conclusion, because the 4-OH in L-Fuc is axial. Therefore, the nature of this product was carefully investigated.

To obtain a sufficient amount of the putative transglycosylation product for structure determination, a larger scale reaction was carried out with 200 nmol donor substrate. The reaction products were well separated by HPLC using a graphitized carbon column (Fig. 2), and each of the peaks showing absorbance at 215 nm was collected and reinjected into the HPAEC-PAD for identification. The peaks *a* and *b* corresponded to the hydrolytic product, $Man_9GlcNAc$, and the remaining substrate, $Man_9GlcNAc_2Asn$, respectively. Peak *c*, eluted at the same position as the unidentified peak in Fig. 1, was presumed to be the transglycosylation product. Indeed, when a portion of the peak *c* (*c.* 1 nmol) was hydrolysed with 2 M TFA at 100 °C for 4 h, the sugar

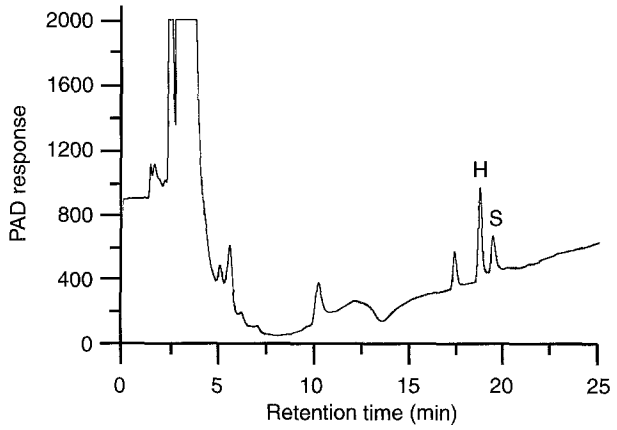


Figure 1. Endo-A transglycosylation with L-Fuc as acceptor. The reaction mixture containing 3 nmol of $Man_9GlcNAc_2Asn$, 2 μmol of L-Fuc and approximate 1 mU of the enzyme in 10 μl of 25 mM ammonium acetate buffer (pH 6.0) containing 35% acetone was incubated at 37 °C for 15 min. After lyophilization, one fourth of the sample (50 μl) was injected into the HPAEC-PAD system for analysis. The elution was performed with 100 mM NaOH and a linear gradient of NaOAc: 0–8% in 24 min. S, remaining substrate, $Man_9GlcNAc_2Asn$; H, hydrolytic product, $Man_9GlcNAc$.

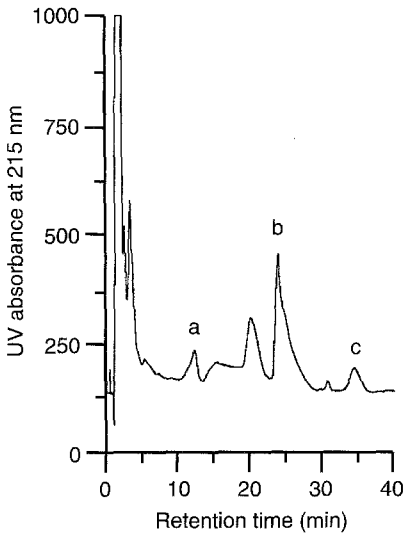


Figure 2. HPLC purification of endo-A transglycosylation product by graphitized carbon column. The enzymatic reaction was carried out as described in Materials and methods. The sample was purified by HPLC on a graphitized carbon column. The elution was with 10 mM NH_4OH and a linear gradient of CH_3CN (6–11%) in 40 min at 70 °C. The effluent was monitored at 215 nm, and each of the peaks was collected, evaporated, and re-analysed by HPAEC-PAD system. *a*, the hydrolytic product, $Man_9GlcNAc$; *b*, the remaining substrate, $Man_9GlcNAc_2Asn$; *c*, the transglycosylation product.

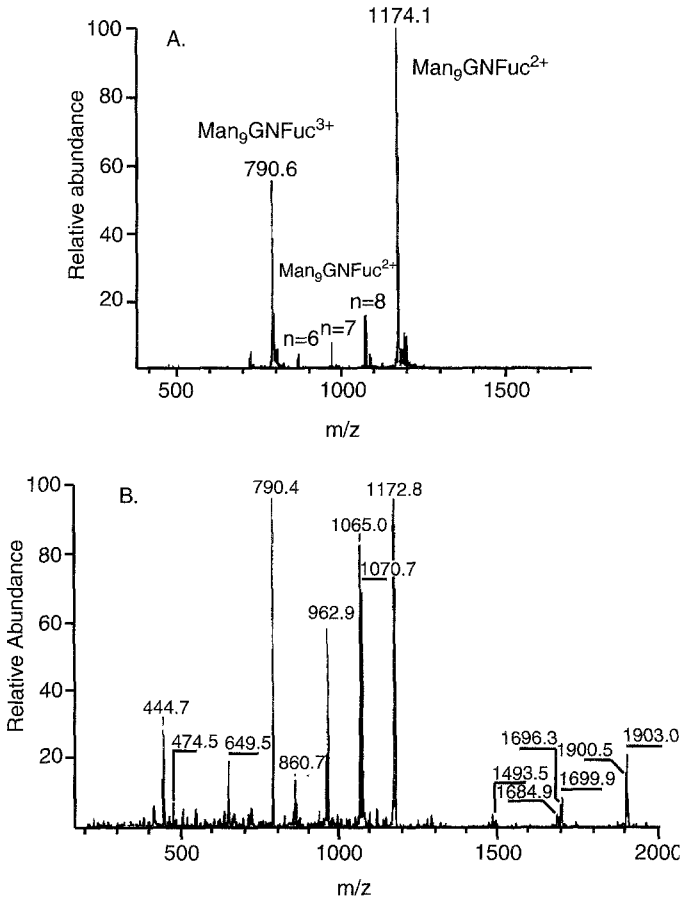


Figure 3. Electrospray mass spectra of transglycosylation product of endo-A. A. ES-MS profile analysis of the sample showing three and two charged states. Small ions represent $Man_nGlcNAc^{2+}$, where $n = 6$, 7 and 8. B. ES-MS-CID-MS analysis of the peak of *m/z* 790.6^{3+}.

composition was found to be Man:GlcNAc:Fuc = 9:1:1 by HPAEC-PAD [22].

The newly formed linkage between GlcNAc and L-Fuc in the transglycosylation product, $Man_9GlcNAc$-L-Fuc was then investigated. ^{1}H-Nuclear magnetic resonance spectroscopy could be employed for this purpose [23], but it requires *c.* 1 μmol of the purified material. We chose to use a combination of sequential exo-glycosidase digestion and ES-MS and also periodate oxidation of the PA-derivative, because these methods require only several nmols.

For structural investigation by ES-MS, the Man_9-GlcNAc-L-Fuc was first permethylated. The MS analysis of methylated oligosaccharide indicated two major ions representing the two (*m/z* 1174.1^{2+}), and three (*m/z* 790.6^{3+}) charge states where charging arises by the addition of sodium cations and a series of lower high mannose type homologues, ($Man_nGlcNAc$-L-Fuc, $n = 6$, 7, 8, Fig. 3A). The triple charged ion was selected and analyzed by CID which provided the spectrum in Fig. 3B. The five major ions above the parent, *m/z* 1172.8^{2+}, 1070.7^{2+}, 1065.0^{2+}, 962.9^{2+} and 860.7^{2+}, can be explained by a loss of sodium, along with four terminal losses: methylated Fuc from the reducing terminal, and one, two, and three methylated mannosyl residues from the non-reducing end, respectively. The latter three ions were also observed in the single charge state *m/z* 1903.0^+, 1699.9^+ and 1493.5^+. The satellite peaks adjacent to these ions represent a combined loss from different termini, e.g. adjacent to *m/z* 1699.9^+ are two lower mass ions *m/z* 1696.3^+ and 1684.9^+ which can be accounted for as the loss of methylated Man-Man and Fuc, and methylated Man-Man and Man, respectively. Two ions, *m/z* 649.5^+ and 444.7^+ represent the sodiated tri-, and di-mannosyl fragments [(methylated Man-Man-Man)Na]$^+$ and [(methylated Man-Man)Na]$^+$. An additional fragment has a mass that can only be methylated GlcNAc-Fuc, *m/z* 474.5^+, suggested that the compound found in peak *c* is the product of endo-A transglycosylation.

In another approach to elucidation of the linkage between GlcNAc and L-Fuc, the fucosyl oligosaccharide ($Man_9GlcNAc$-L-Fuc, *c.* 70 nmol) was first derivatized with 2-aminopyridine. PA-oligosaccharide ($Man_9GlcNAc$-L-Fuc-PA) was purified by a Sephadex G-10 column and was shown to be homogeneous by HPLC with an Amido-80 column (Fig. 4A1). The PA-oligosaccharide was then subjected to sequential exo-glycosidase digestion. After

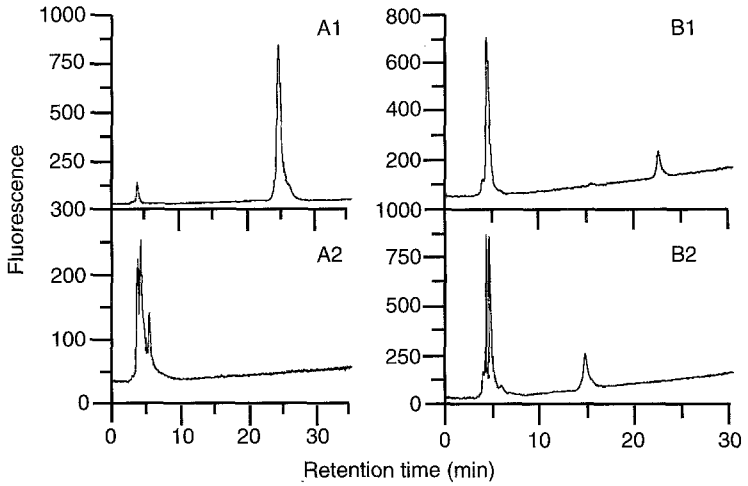


Figure 4. HPLC analysis of PA-oligosaccharide digested with α-mannosidase (A) and then with β-mannosidase (B). The samples were analysed by an Amido-80 column eluted with 3% acetic acid-triethylamine buffer (pH 7.3) using a linear gradient of 65–50% CH_3CN in 50 min (A) or 90–65% CH_3CN in 30 min (B). $Man_9GlcNAc$-L-Fuc-PA (30 nmol) was digested with 1 U of α-mannosidase (A), followed by digestion with 0.1 U of β-mannosidase (B). A1, before α-mannosidase digestion; A2, after α-mannosidase digestion; B1, before β-mannosidase digestion; B2, after β-mannosidase digestion.

the exhaustive digestion with α-mannosidase, the elution time of PA-oligosaccharide on the Amido-80 column was shifted from 25 min (Fig. 4A1) to 5.7 min (Fig. 4A2) under the condition B. This material was collected, concentrated and further digested with 0.1 U β-mannosidase. After the digestion, the peak eluted (under the condition A) at 22 min attributable to ManGlcNAc-L-Fuc-PA (Fig. 4B1), disappeared and a new peak at 15 min was found (Fig. 4B2), suggesting that the β-linked Man residue was completely removed. This also proves that the α-mannosidase completed its task and the β-Man was exposed. To determine the anomeric configuration of GlcNAc linked to L-Fuc, the PA-disaccharide (GlcNAc-L-Fuc-PA) was treated with β-*N*-acetylhexosaminidase. As shown in Fig. 5, the peak size of PA-disaccharide eluted (condition A) at 15 min decreased (Fig. 5A1), and a new peak at 7.3 min, cochromatographing as PA-L-Fuc, emerged (Fig. 5A2, A3). The newly formed peak was also confirmed to be PA-L-Fuc on the ODS column (Fig. 5B1 and B2) by its co-elution with a synthetic standard. Thus, the GlcNAc is β-linked to L-Fuc.

The linkage position between GlcNAc and L-Fuc was solved by ES-MS following periodate oxidation, reduction, and methylation as outlined in Scheme 2. After these chemical modifications, increments to the molecular weight would vary with each linkage position of L-Fuc: 4-*O*-, 3-*O*-, or 2-*O*-linkage providing +133 u, +177 u and +119 u, respectively. The $Man_9GlcNAc \rightarrow$ moiety would also be modified, but to a constant value of 1980.0 Da. Two major ions, *m/z* 1036.1^{2+} and 1072.1^{2+}, were detected (Fig. 6A), and the latter ion can be accounted for as a 119 u increment to the modified $Man_9GlcNAc \rightarrow$ moiety adducted two sodium ions, (i.e., 1980 + 119 + 46 = 2145; 2145/2 = 1072.5). To confirm the structure of the 1072.1^{2+}, it was analysed by CID which provided the spectrum in Fig. 6B. The interpretation of these fragments in terms of the periodate

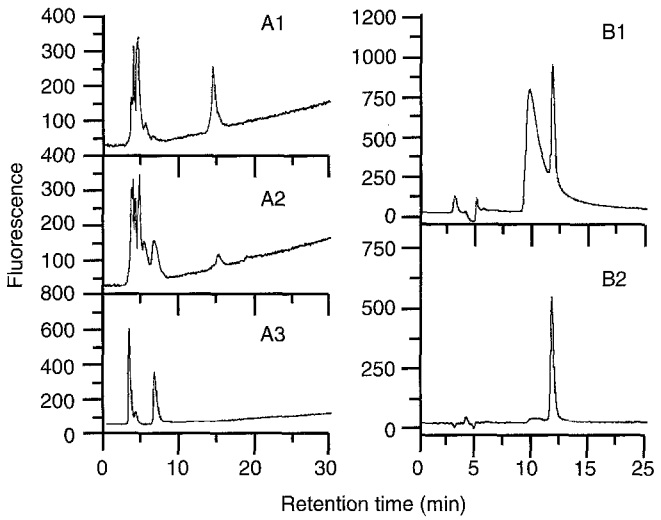
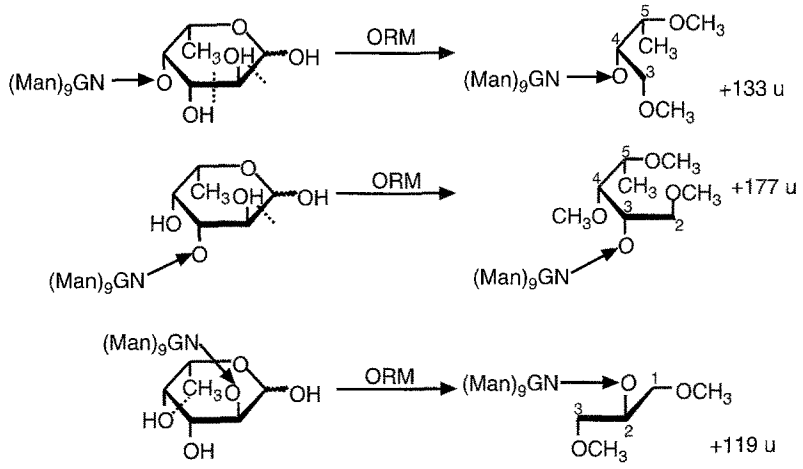


Figure 5. HPLC analysis of the β-acetylhexosaminidase-treated GlcNAc-L-Fuc-PA by an Amido-80 (A) and an ODS column (B). GlcNAc-L-Fuc-PA generated by α- and β-mannosidase digestions was incubated with 250 mU of β-acetylhexosaminidase in 55 μl of 20 mM citrate-phosphate buffere (pH 6.0) at 37 °C for 18 h, followed by an addition of 250 mU of the enzyme and further incubation for 18 h. The eluent for the Amido-80 column (A) was 3% acetic acid-triethylamine buffer (pH 6.0) with a gradient of 90–65% CH_3CN developed in 30 min, and for the ODS column (B) was 50 mM citrate buffer (pH 4.0) with 1% CH_3CN. A1, before the β-hexosaminidase digestion; A2, after the enzyme digestion; A3, standard PA-L-Fuc; B1, after the enzyme digestion; B2, standard PA-L-Fuc.



Scheme 2. Outline of determination of GlcNAc-L-Fuc linkage. ORM, oxidation, reduction and methylation; GN, GlcNAc; Man, mannose.

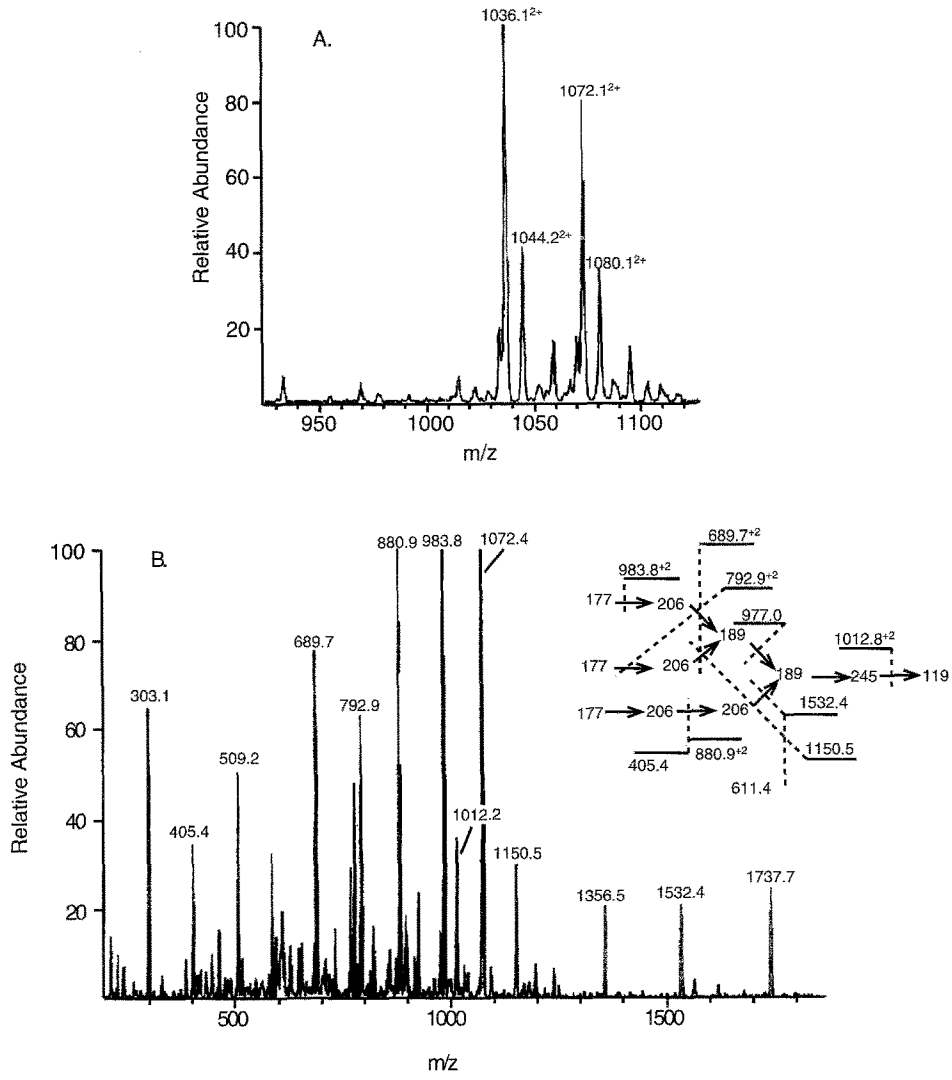


Figure 6. Electrospray mass spectra of $Man_9GlcNAc$-L-Fuc following oxidation, reduction and permethylation. (A) ES-MS profile analysis of the sample following periodate oxidation, reduction and permethylation. Major ion and satellite peaks are identified in the text. (B) ES-MS-CID-MS analysis of *m/z* 1072.4^{2+}. Fragments are a mixture of ions in single or double charged state.

oxidation residues is given in the insert. The former ion, *m/z* 1036.1^{2+} was determined by CID to be a hydrolysis product that occurred during the chemical reactions: the methylated alditol of the modified $Man_9GlcNAc$ (data not shown). Thus, the MS data strongly suggests a GlcNAc(1,2)-L-Fuc linkage.

The linkage between GlcNAc and L-Fuc, was also confirmed by a recently developed method based on separation of PA-derivatized C_2-C_5 sugars [15]. Briefly, the PA-oligosaccharide was oxidized with periodate to cleave *vic.* glycols, followed by reduction of the aldehydes generated by the oxidation. After acid hydrolysis of the glycosidic bonds, the fluorogenic compound derived from L-Fuc is analysed by HPLC using an ODS column. The different fluorogenic compounds will result from different linkage positions, e.g. PA-L-glyceraldehyde (PA-C_3) would be formed from GlcNAc(1,2)-L-Fuc-PA, whereas PA-D-threose (PA-C_4) or PA-glycolaldehyde (PA-C_2) would be formed, if the linkage is 1,3 or 1,4, respectively.

After periodate oxidation, reduction and acid hydrolysis

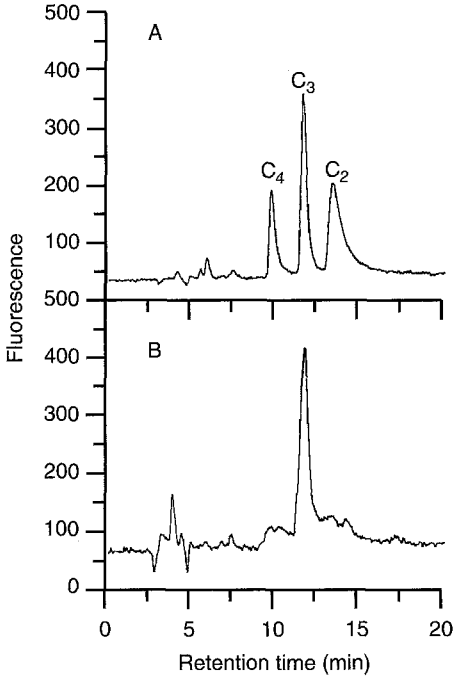
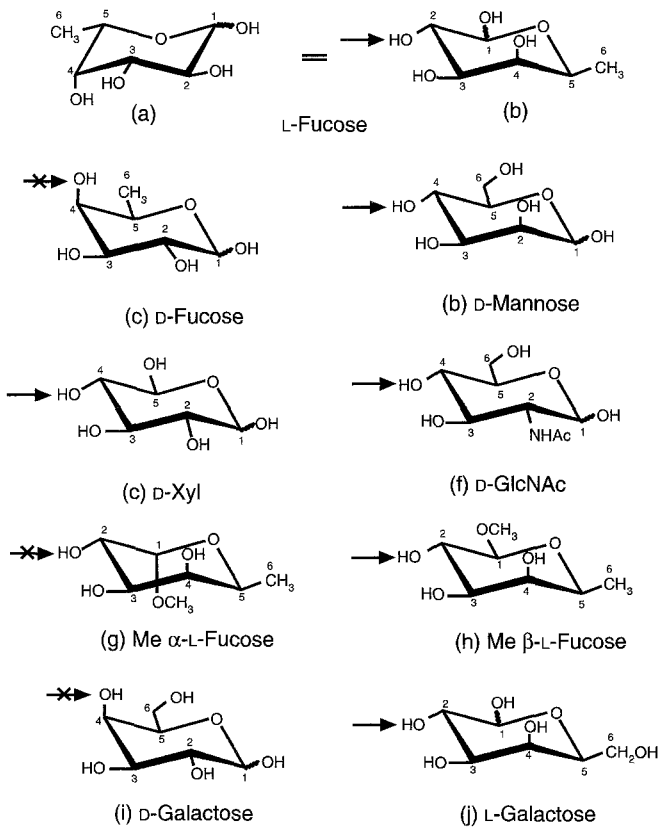


Figure 7. Determination of periodate oxidation product of Man_9-GlcNAc-L-Fuc-PA by HPLC. The sample ($Man_9GlcNAc$-L-Fuc-PA, 5 nmol) was periodate oxidized, reduced, and hydrolyzed as described in Materials and methods. The product was injected into an ODS column and eluted with 50 mM citrate buffer (pH 6.0) with 1% CH_3CN. The elution rate was 1 ml min^{-1} and the effluent was monitored by a fluorescence detector (λ_{ex} = 320 nm, λ_{em} = 390 nm). The chromatograms of three standards (100 pmol each) and the reaction mixture (about 20 pmol) were shown in A and B, respectively. C_2, PA-glycolaldehyde; C_3, PA-L-glyceraldehyde; C_4, PA-D-threose.

Table 1. Transglycosylation to monosaccharides and their derivatives. The enzyme reaction mixture contained 3 nmol $Man_9GlcNAc_2Asn$, 2 μmol acceptor and 1 mU enzyme in 10 μl of 25 mM ammonium acetate buffer (pH 6.0) containing 35% (vol.) acetone. The incubation was performed at 37 °C for 15 min, and more than 90% of the starting substrate was consumed during the reaction. The product was analyzed with HPAEC-PAD using the elution condition A.

Acceptors (0.2 M)	*Transglycosylation*[a] (%)
GlcNAc	90.5
Man[b]	82.6
D-Fuc	0.0
L-Fuc	25.8
Me-α-L-Fuc	0.0
Me-β-L-Fuc	33.5
D-Gal	0.0
L-Gal	19.4

[a] Transglycosylation was defined as the ratio of the transglycosylation product to the consumed substrate, which is the sum of transglycosylation and hydrolysis products.
[b] $Man_9GlcNAc_2AsnPhe$ was used as substrate.



Scheme 3. Illustration of sugar conformation. Arrow indicates where transglycosylation occurs; arrow with an X means transglycosylation does not occur.

of $Man_9GlcNAc$-L-Fuc-PA, the major fluorescent product was eluted at the same retention time as standard PA-C_3 from an ODS column of HPLC (Fig. 7). Thus, the inevitable conclusion is that the GlcNAc is 1,2-linked to L-Fuc in the transglycosylation product.

To study the acceptor specificity further, several fucosyl glycosides were tested as acceptors (Table 1). Both L-Fuc and L-Gal could serve as acceptors for endo-A transglycosylation, although the transglycosylation efficiencies (L-Fuc, 25.8%; L-Gal, 19.4%) were not as good as GlcNAc (90.5%) and Man (82.6%). On the other hand, D-Fuc and D-Gal could not serve as acceptors at all. Methyl β-L-Fuc_p could also act as an acceptor, and the transglycosylation ratio (33.5%) was even better than L-Fuc. However, methyl α-L-Fuc failed to accept the oligosaccharide by the same enzyme under the same conditions.

The basis of rationalization for the results obtained in Table 1 was summarized in Scheme 3. Based on our previous knowledge (Scheme 1), equatorial 3-OH and 4-OH of the acceptor are important for the endo-A transglycosylation [6]. In addition, a $^4C_1$-conformation or equivalent conformation of the acceptor is probably also required for the transglycosylation, suggesting the ring oxygen maybe involved. L-Fuc is known to exist in the $^1C_4$-conformation (Scheme 3a), which can also be viewed in the '$^2C_5$-conformation' (Scheme 3b). The arrangement of the configurations for C2-C4 in L-Fuc in the '$^2C_5$-conformation' is similar to that of Man in $^4C_1$-conformation, in which 2-OH and 3-OH of L-Fuc, corresponded to 4-OH and 3-OH of Man, are all equatorial. If the 4-OH of Man can accommodate oligosaccharide by the endo-A transglycosylation, the 2-OH of L-Fuc could also accept oligosaccharide. On the other hand, the $^4C_1$-conformation is a more stable form for D-Fuc, in which the 4-OH is axial, and thus could not serve as acceptor for the endo-A transglycosylation. When both methyl α-L-Fuc and methyl β-L-Fuc are viewed in the '$^2C_5$-conformation', ^{1}OMe in methyl α-L-Fuc is axial, and it is equatorial in methyl β-L-Fuc. The reactivity of endo-A to methyl β-L-Fuc, but not to methyl α-L-Fuc, indicates that the bulky axial substituent on the C1 position of L-Fuc may interfere with the transglycosylation. Since L-Fuc is often linked to other sugars through an α-linkage in glycoproteins, the transglycosylation to fucosyl residue in glycoprotein is not likely, because methyl α-L-Fuc failed to serve as acceptor. The conformation of L-Gal is similar to L-Fuc which is 6-deoxy-L-Gal. Therefore, L-Gal also serves as acceptor and the transglycosylation is most likely to be at the 2-OH of L-Gal. However, the 4-OH of D-Gal is axial which could not accept oligosaccharide by the endo-A transglycosylation.

Transglycosylation is a competition against hydrolysis, where the acceptor competes for water at the enzyme active centre. Therefore, it is possible to enhance the transglycosylation activity by re-designing the active centre of enzyme to gain stronger affinity for the acceptor. In fact, Matsui *et al.* [24] have reported that transglycosylation activity of α-amylase from *Saccharomycopsis* increased after changing an aromatic residue in the active centre by site-directed mutagenesis. Our results provide useful information on the interaction between the acceptor and the active centre of the endo-A, and contribute to a better understanding for engineering of active centre of endo-A to gain a high transglycosylation activity.

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