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Transglycosylation of intact sialo complex-type oligosaccharides to the *N*-acetylglucosamine moieties of glycopeptides by *Mucor hiemalis* endo-β-*N*-acetylglucosaminidase

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Abstract

The endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) of *Mucor hiemalis*, endo-M, was found to transfer the sialo complex-type oligosaccharides from transferrin glycopeptide to the *N*-acetylglucosamine (GlcNAc) moieties of peptidyl-GlcNAc. Disialo complex-type oligosaccharide of transferrin glycopeptide was transferred to 9-fluorenylmethyloxycarbonyl (Fmoc)–asparaginyl-*N*-acetylglucosaminide (Fmoc–Asn–GlcNAc) by endo-M in a high yield. The structure of the reaction product was confirmed to be Fmoc–Asn–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal–NeuAc)_2$ by mass spectrometry. Endo-M also transferred disialo complex-type oligosaccharide to the GlcNAc residue of chemically synthesized H–Ile–Asn(GlcNAc)–Ala–Thr–Leu–OH. Asn-linked asialo complex-type oligosaccharide and Asn-linked high-mannose type oligosaccharide were also effective as oligosaccharide donors. Transfer of disialo complex-type oligosaccharide to the GlcNAc-peptide was the most effective among the three types of oligosaccharides, although the disialo complex-type oligosaccharide attached to the peptide was the poorest substrate for the hydrolytic activity of endo-M. © 1996 Elsevier Science Ltd.

Abbreviations: Fmoc, 9-fluorenylmethyloxycarbonyl; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid; Gal, D-galactose; Man, D-mannose

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1. Introduction

Many exoglycosidases have been shown to have transglycosylation activities in addition to hydrolytic activities. Such activities are useful for the enzymatic synthesis of various oligosaccharides, since chemical synthesis of oligosaccharide is complicated and is usually accompanied by the formation of unwanted anomeric isomers [1]. Transglycosylation activities of endoglycosidases are considered to be very useful for obtaining artificial glycoconjugates, because well-characterized oligosaccharides can be transferred to peptides, proteins, or lipids. Only a limited number of reports, however, are available on the transglycosylation activities of endoglycosidases [2–7]. Among the known endo-β-*N*-acetylglucosaminidases (endo-β-GlcNAc-ases), endo-F from *Flavobacterium meningosepticum* [2], endo-A from *Arthrobacter protophormiae* [3], and endo-M from *Mucor hiemalis* [4], have been found to have transglycosylation activities for the transfer of oligosaccharides to acceptors.

Transglycosylation activities of endo-β-GlcNAc-ases catalyze the following reaction:

$$\underset{\text{(Glycoside donor)}}{\text{R}-(\text{GlcNAc})_2-\text{Asn}} + \underset{\text{(Acceptor)}}{\text{GlcNAc}-\text{Asn}-\text{R}'} \rightarrow \underset{\text{(Transglycosylation product)}}{\text{R}-(\text{GlcNAc})_2-\text{Asn}-\text{R}'} + \text{GlcNAc}-\text{Asn}$$

(R: oligosaccharide, R′: peptide or peptide derivatives).

Endo-F was the first enzyme found to transfer the oligosaccharide of a glycopeptide to glycerol [2]. Next, endo-A was found to transfer high-mannose type oligosaccharides to the *N*-acetylglucosamine moiety of glycoproteins [3,8]. However, endo-A hydrolyzes only the high-mannose type sugar chains of glycoproteins, and can transfer only these oligosaccharide groups. On the other hand, endo-M was found to hydrolyze all three types of N-linked sugar chains, namely high-mannose, hybrid, and complex-type oligosaccharides [9,10], and it is also able to transfer high-mannose type and asialo complex-type oligosaccharides from glycopeptides to suitable acceptors [4].

Although endo-M is the only endo-β-GlcNAc-ase known to hydrolyze sialo complex-type oligosaccharides, the hydrolytic velocity is much lower than that for asialo complex-type oligosaccharides. Despite this unfavourable situation, we recently found that endo-M can effectively transfer intact sialo complex-type oligosaccharides from transferrin glycopeptide to the *N*-acetylglucosamine moiety of *N*-acetylglucosaminyl peptides. Moreover, it was found that the transfer of sialo complex-type oligosaccharide was more effective than that of the non-sialylated one.

In this paper, the transglycosylation of sialo complex-type oligosaccharides to synthetic peptides containing a GlcNAc moiety is reported. Since the sialic acid moiety of sialo complex-type oligosaccharides is physiologically important, the technique enabling transfer of the sialo complex-type oligosaccharide to protein and peptide is promising from the glycotechnological point of view. The transglycosylation activity of endo-M can be effectively used to synthesize potentially useful neo-glycoproteins and neo-glycopeptides containing sialo complex-type oligosaccharides. This is the first

report on the transfer of intact sialo complex-type oligosaccharides to glycoside acceptors.

2. Materials and methods

Preparation of the enzyme.—Endo-M was partially purified from the culture medium of *Mucor hiemalis*, as described by Kadowaki et al. [9]. The enzyme preparation was free from other glycosidase activities but contained a very little protease activity.

Preparation of substrates.—Disialotransferrin glycopeptide (STF-GP): Asn–$(GlcNAc)_2$–Man–(Man–GlcNAc–Gal–NeuAc)$_2$, was obtained by exhaustive pronase digestion of human transferrin, followed by purification on a Sephadex G-25 gel column as described by Kadowaki et al. [11]. Asialotransferrin glycopeptide (ASTF-GP): Asn–$(GlcNAc)_2$–Man–(Man–GlcNAc–Gal)$_2$, was prepared by sialidase digestion of STF-GP [11]. The high-mannose type glycopeptide (M_6-GP): Asn–$(GlcNAc)_2$–$(Man)_6$, was prepared from ovalbumin by the procedure of Tai et al. [12].

Fmoc–Asn–GlcNAc was synthesized by the method of Inazu and Kobayashi [13]. β-*N*-Acetylglucosaminyl–hCG(β12–16) peptide [hCG(β12–16)–GlcNAc; H–Ile–Asn(GlcNAc)–Ala–Thr–Leu–OH], was synthesized by the method described by Inazu et al. [14].

Transglycosylation reaction.—Transglycosylation was carried out in a reaction mixture composed of 250 nmol (final 25 mM) of a glycoside donor (STF-GP, ASTF-GP or M_6-GP), 100 nmol (final 10 mM) of an acceptor [Fmoc–Asn–GlcNAc sodium salt or hCG(β12–16)–GlcNAc], 40 μunit (final 4 mU/mL) of endo-M and 60 mM potassium phosphate buffer (pH 6.25) in a total volume of 10 μL. EDTA (final concentration of 50 mM) was added to the reaction mixture containing hCG(β12–16) in order to protect it from the action of the trace amount of peptidase contaminating the endo-M preparation. After incubation for several h at 37 °C, the mixture was heated for 3 min in boiling water to terminate the reaction, diluted to 250 μL with cold distilled water, and analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis.—HPLC of Fmoc derivatives was performed by using a reverse-phase (ODS) column (4.6 × 150 mm, Inertsil ODS-2, G-L Science, Japan). The reaction mixture was analyzed by an Hitachi L-6200 chromatograph equipped with an L-4250 ultraviolet spectrophotometer. Elution was carried out with a linear gradient of acetonitrile (20–26%) containing 0.1% trifluoroacetic acid in 30 min at a flow rate of 1 mL/min. The reaction products were monitored by absorption at 254 nm.

HPLC of peptide derivatives was performed by using an ODS column. Elution was carried out with 12% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min. Peptide derivatives were detected by absorption at 214 nm.

Yield (mol%) of the transglycosylation product was calculated by the following equation, based on the assumption that the molar absorption coefficient (ε) of the transglycosylation product was the same as that of the acceptor.

$$\text{Yield of product (mol\%)} = \frac{\text{Area of the peak of the product}}{\text{Initial area of the peak of the acceptor added}} \times 100.$$

Mass spectrometry.—Electrospray (ESI) mass spectrometry was performed in the negative-ion mode on an Analytica of Branford/JEOL JMS-SX102A mass spectrometer (JEOL). Matrix-associated laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the negative-ion mode by using α-cyano-4-hydroxycinnamic acid as a matrix on a Finnigan Lasermat mass spectrometer (Finnigan Mat, UK).

Materials.—Human serum transferrin was obtained from Seikagaku Kogyo, Japan. Sialidase from *Arthrobacter ureafaciens* was obtained from Nacalai Tesque, Japan. All other chemicals were obtained from commercial sources.

3. Results

*Transglycosylation of sialo complex-type oligosaccharides from STF-GP to asparaginyl-*N*-acetylglucosaminide.*—Endo-M was previously shown to transfer high-mannose type oligosaccharide to asparaginyl-*N*-acetylglucosaminide and asialo complex-type oligosaccharide from ASTF-GP to peptidyl-GlcNAc prepared from bovine pancreatic ribonuclease B [4].

We examined whether endo-M could transfer sialo complex-type oligosaccharide from STF-GP to the *N*-acetylglucosamine moiety of Fmoc–Asn–GlcNAc, which was chosen as a model compound of a synthetic peptide containing a GlcNAc moiety.

HPLC profiles of the reaction products after incubation for 18 h are shown in Fig. 1. A new peak was observed in front of the peak of remaining Fmoc–Asn–GlcNAc, and was considered to correspond to the transglycosylation product (Fig. 1a). The yield of

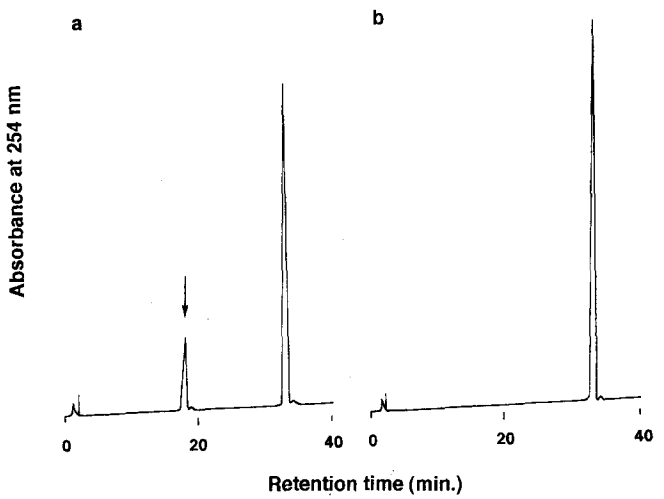

Fig. 1. HPLC profiles of the reaction mixture incubated with Fmoc–Asn–GlcNAc and STF-GP in the presence or absence of endo-M. A sample of each reaction mixture with (a) or without endo-M (b) was analyzed by using an ODS column and monitoring the UV absorption at 254 nm. The arrow in (a) indicates the transglycosylation product. A large peak at a retention time of 32–34 min corresponds to the remaining Fmoc–Asn–GlcNAc.

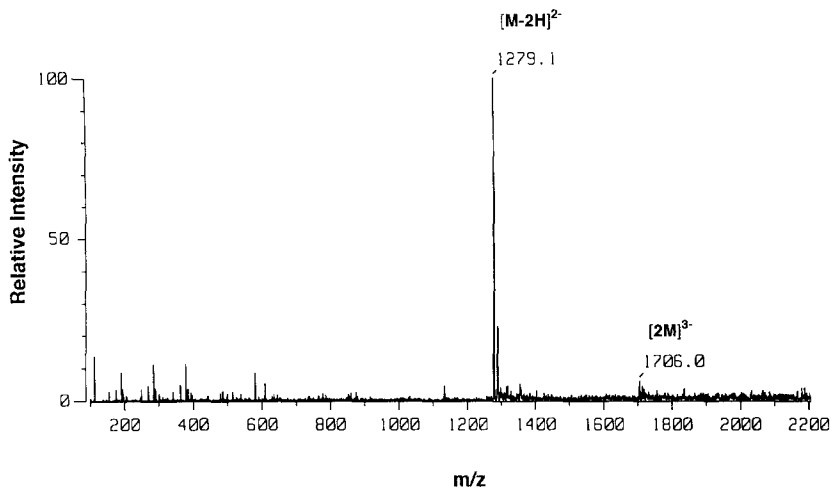

Fig. 2. Mass spectrum of the reaction product with Fmoc–Asn–GlcNAc and STF-GP. The peak indicated by an arrow in Fig. 1a was isolated and analyzed by ESI mass spectrometry.

product was estimated to be 20.0% (mol/mol) of the original amount of acceptor added. The new peak was not detected when any one of STF-GP, Fmoc–Asn–GlcNAc, or endo-M (shown in Fig. 1b) was omitted from the reaction mixtures.

The fraction containing the new peak was collected, freeze-dried, and subjected to ESI mass spectrometry. A doubly-charged mass ion $[M - 2H]^{2-}$ with m/z of 1279.1 was detected (Fig. 2). The molecular mass of 2560.1 calculated from this value accorded with the theoretical value of Fmoc–Asn–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal–NeuAc)_2$ (MW 2560.4). After treatment of the isolated product with sialidase, the molecular ion $[M - 2H]^{2-}$ shifted to the value corresponding to the theoretical value of Fmoc–Asn–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal)_2$ (MW 1977.9). Based on these data, the product was identified as the transglycosylation product of disialo biantennary complex-type oligosaccharide to Fmoc–Asn–GlcNAc. This result indicates that endo-M can transglycosylate the sialo biantennary complex-type oligosaccharides of transferrin to the GlcNAc moiety of Fmoc–Asn–GlcNAc.

When ASTF-GP or M_6-GP was used as an oligosaccharide donor, a single transglycosylation product for Fmoc–Asn–GlcNAc was detected in each reaction mixture. Both transglycosylation products were isolated and identified as Fmoc–Asn–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal)_2$ (MW 1977.9) and Fmoc–Asn–$(GlcNAc)_2$–$(Man)_6$ (MW 1747.6) by MALDI-TOF mass spectrometry (data not shown).

Transglycosylation of sialo complex-type oligosaccharide from transferrin glycopeptide to synthetic human chorionic gonadotropin peptide containing an N-acetylglucosamine moiety.—We found that endo-M could transfer sialo complex-type oligosaccharide from transferrin glycopeptide to hCG(β12–16)–GlcNAc (MW 733.8).

The reaction mixture after incubation for 1 h was analyzed by HPLC. As shown in Fig. 3a by an arrow, a new peak was detected in the enzyme reaction with an earlier retention time than that of the remaining hCG(β12–16)–GlcNAc. The yield of the new

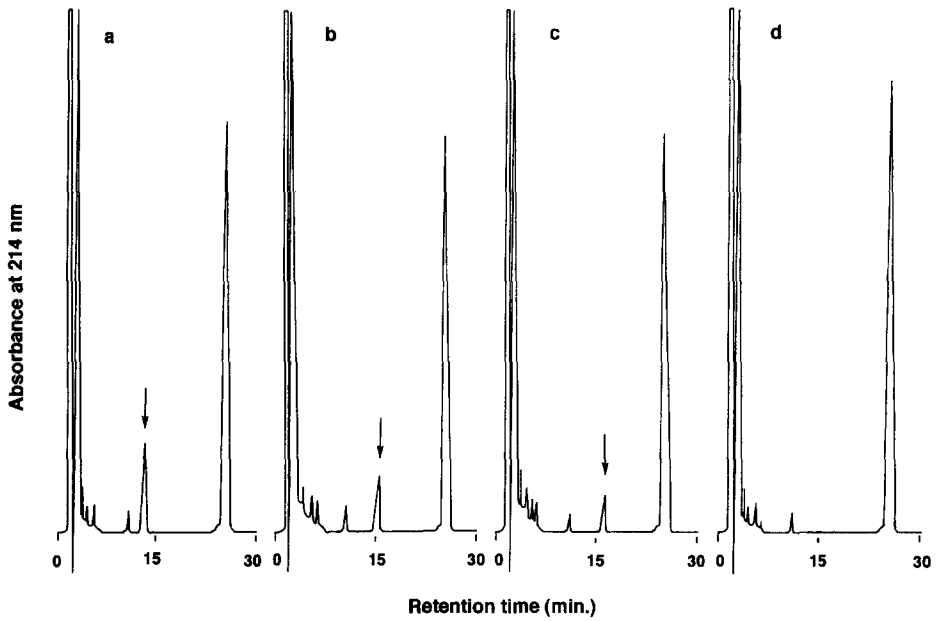

Fig. 3. HPLC profiles of the products formed by incubation of hCG(β12–16)–GlcNAc and various glycoside donors. A sample of each reaction mixture containing STF-GP (a), ASTF-GP (b), or M_6-GP (c) as a donor was analyzed by using an ODS column and monitoring the UV absorption at 214 nm. The reaction mixture without a donor was also analyzed (d). The arrows indicate the transglycosylation products. A large peak at retention time of 25–27 min corresponds to the remaining hCG(β12–16)–GlcNAc.

peak was 10.1% (mol/mol) of the original acceptor added. The new peak was not detected when STF-GP, hCG(β12–16)–GlcNAc or endo-M was omitted from the reaction mixture (Fig. 3d).

The fraction corresponding to the new peak was collected, freeze-dried, and analyzed by ESI mass spectrometry. A doubly-charged mass ion $[M - 2H]^{2-}$ with m/z of 1367.2 was detected (Fig. 4). The molecular mass of 2736.4 calculated from this value coincided with the theoretical value for hCG(β12–16)–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal–NeuAc)_2$ (MW 2736.6).

When ASTF-GP or M_6-GP was used as a glycoside donor, a single transglycosylation product, as shown by an arrow in Fig. 3b or c, was detected in each reaction mixture. Both products were isolated and identified as hCG(β12–16)–$(GlcNAc)_2$–Man–$(Man–GlcNAc–Gal)_2$ (MW 2154.1) and hCG(β12–16)–$(GlcNAc)_2$–$(Man)_6$ (MW 1909.9) by MALDI-TOF mass spectrometry.

Comparison of yields of transglycosylation products.—By using Fmoc–Asn–GlcNAc as the acceptor, we examined the yield of each transglycosylation product from STF-GP, ASTF-GP, or M_6-GP by changing the incubation time. The reaction mixtures were the same as those described in 'Materials and methods'. After incubation at 37 °C for the designated period, the reaction mixture was heated and analyzed by HPLC. As shown in Fig. 5, the yield of the transglycosylation product from STF-GP was the highest and

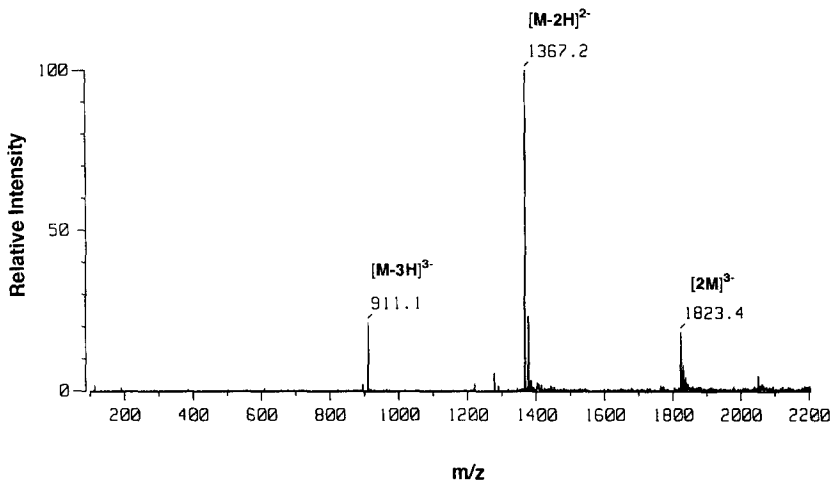

Fig. 4. Mass spectrum of the reaction product with hCG(β12–16)–GlcNAc and STF-GP. The peak indicated by an arrow in Fig. 3a was isolated by HPLC, and analyzed by ESI mass spectrometry.

20% of the acceptor was converted into the product after 18 h of incubation. The yield of product from ASTF-GP was lower than that from STF-GP, reaching 15% after 18 h. On the other hand, the yield of the product from M_6-GP was low, and at the maximum only one-third ($\sim$ 8%) of that from STF-GP after 18 h of incubation. Similar results

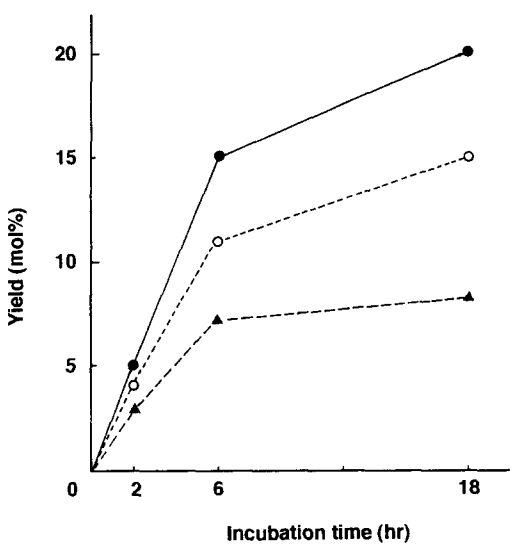

Fig. 5. Time course study of the transglycosylation from various oligosaccharide donors to Fmoc–Asn–GlcNAc by the action of endo-M. After incubation for designated periods, each transglycosylation product to Fmoc–Asn–GlcNAc from STF-GP (●————●), ASTF-GP (○----○) or M_6-GP (▲- - - -▲) was analyzed by HPLC to determine the yield.

were obtained when hCG(β12–16)–GlcNAc was used as an acceptor. The yields of the transglycosylation products from STF-GP, ASTF-GP, and M_6-GP after 1 h of incubation were about 10, 7, and 4%, respectively (Fig. 3).

4. Discussion

Recently, Yamamoto et al. reported that endo-M could transfer asialo complex-type oligosaccharide of human serum transferrin to suitable acceptors such as GlcNAc or peptidyl-GlcNAc [4]. Takegawa et al. also reported that endo-A could transfer high-mannose type oligosaccharide to ovalbumin peptide or ribonuclease B, which were deglycosylated by incubation with *Flavobacterium* sp. endo-β-GlcNAc-ase [8].

Transglycosylation catalyzed by endoglycosidase is an attractive method for remodelling of glycoproteins or synthesis of neoglycoproteins, because a preformed oligosaccharide can be transferred to a glycoside acceptor in a one-step reaction. However, several drawbacks were noted for the practical application of this method. Many endoglycosidases show rather high specificity toward donors and acceptors. Furthermore, yields of transglycosylation products were very low.

We tried to resolve these problems by using endo-M under the conditions described in this paper, and a substantial amount of sialo complex-type oligosaccharide was successfully transferred to such acceptors as peptidyl-GlcNAc by action of the enzyme. It is important to note that the enzyme transfers the complex-type oligosaccharides to appropriate acceptors more effectively than the high-mannose type ones. Furthermore, it is of interest that the effectiveness of transfer reactions from various donor glycopeptides is the reverse of their hydrolysis reactions [10]. M_6-GP was the most readily hydrolyzed by endo-M, but it was the least effective in the transglycosylation reaction. This evidence indicates that elucidation of the difference in the mechanisms of hydrolysis and transglycosylation is essential for increasing the yield of transglycosylation products.

We also found that the yield of transglycosylation product became higher by increasing the concentrations of both glycoside donor and glycoside acceptor to extremely high levels, and by limiting the amount of enzyme (the details of the reaction conditions will be reported in another paper). Thus, the yield of transglycosylation product of sialo complex-type oligosaccharide attained almost 20% (mol/mol).

It is well known that a sialic acid moiety in an oligosaccharide is physiologically important for cell–cell or effector–receptor interactions. Therefore, development of a technique for transglycosylation of the sialo complex-type oligosaccharide to peptides, as reported in this paper, is important for glycobiology.

Our strategy for the synthesis of neoglycopeptides is briefly summarized in Fig. 6. The first step is the chemical synthesis of the peptide. The peptide having a GlcNAc moiety can be readily prepared by solid-phase synthesis based on the Fmoc strategy in which Fmoc–Asn–GlcNAc is used instead of Fmoc–Asn [12,13]. The second step is the transglycosylation process catalyzed by endo-M. endo-M transfers both complex-type and high-mannose type oligosaccharides to the GlcNAc moiety of the synthetic peptide.

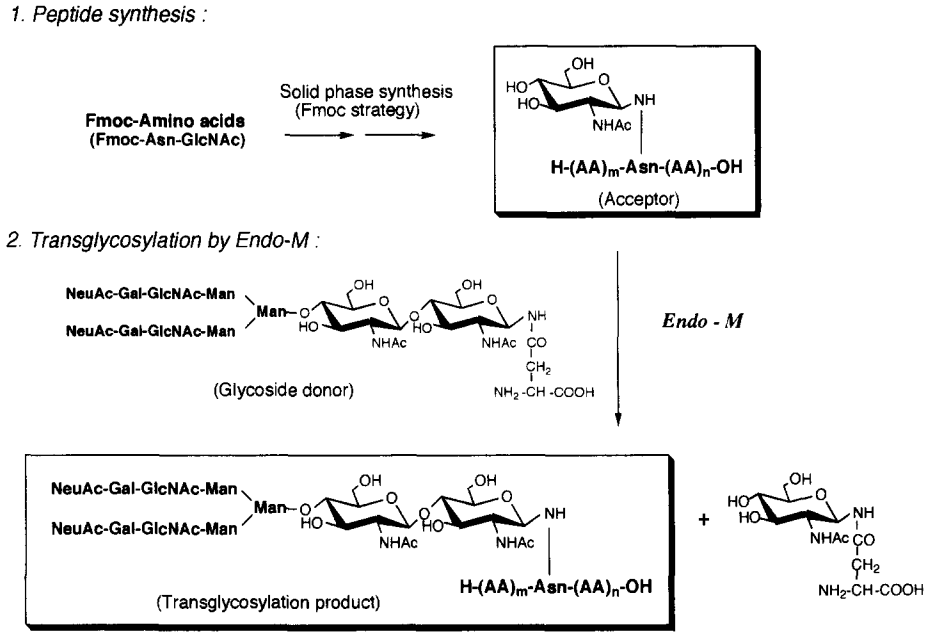

Fig. 6. A strategy of neo-glycopeptide synthesis by using endo-M. Abbreviations: NeuAc, *N*-acetylneuraminic acid; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; AA, amino acid.

The transglycosylation reaction catalyzed by endo-M will, therefore, be useful for the synthesis of neoglycopeptides and should become a practical technique in protein engineering.

References

[1] G.L. Cote and B.Y. Tao, *Glycoconjugate J.*, 7 (1990) 145–162.
[2] R.B. Trimble, P.H. Atkinson, A.L. Tarentino, T.H. Plummer, F. Maley, and K.B. Tomer, *J. Biol. Chem.*, 261 (1986) 12000–12005.
[3] K. Takegawa, S. Yamaguchi, A. Kondo, I. Kato, and S. Iwahara, *Biochem. Int.*, 25 (1991) 829–835.
[4] K. Yamamoto, S. Kadowaki, J. Watanabe, and H. Kumagai, *Biochem. Biophys. Res. Commun.*, 203 (1994) 244–252.
[5] R.M. Bardales and V.P. Bhavanandan, *J. Biol. Chem.*, 264 (1989) 19893–19897.
[6] Y.-T. Li, B.Z. Carte, B.N.N. Pao, H. Schweingruber, and S.-C. Li, *J. Biol. Chem.*, 266 (1991) 10723–10726.
[7] H. Ashida, Y. Tsuji, K. Yamamoto, H. Kumagai, and T. Tochikura, *Arch. Biochem. Biophys.*, 305 (1993) 559–562.
[8] K. Takegawa, M. Tabuchi, S. Yamaguchi, A. Kondo, I. Kato, and S. Iwahara, *J. Biol. Chem.*, 270 (1995) 3094–3099.
[9] S. Kadowaki, K. Yamamoto, M. Fujisaki, and T. Tochikura, *J. Biochem.*, 110 (1991) 17–21.
[10] K. Yamamoto, S. Kadowaki, M. Fujisaki, H. Kumagai, and T. Tochikura, *Biosci. Biotech. Biochem.*, 58 (1994) 72–77.
[11] S. Kadowaki, K. Yamamoto, M. Fujisaki, K. Izumi, T. Tochikura, and T. Yokoyama, *Agric. Biol. Chem.*, 54 (1990) 97–106.

[12] T. Tai, K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, and A. Kobata, *J. Biol. Chem.*, 250 (1975) 8569–8575.
[13] T. Inazu and K. Kobayashi, *Synlett.*, (1993) 869–870.
[14] T. Inazu, K. Kobayashi, and H. Yaginuma, in Okada, Y. (Ed.), *Peptide Chemistry* 1993, Protein Research Foundation, Osaka, Japan, 1994, pp 101–104.