ELSEVIER

Carbohydrate Research 305 (1998) 415–422

CARBOHYDRATE RESEARCH

Chemoenzymatic synthesis of a novel glycopeptide using a microbial endoglycosidase

Kenji Yamamoto [a,*], Kenya Fujimori [a], Katsuji Haneda [b], Mamoru Mizuno [b], Toshiyuki Inazu [b], Hidehiko Kumagai [a]

[a] *Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*

[b] *The Noguchi Institute, 1-8-1 Kaga, Itabashi, Tokyo 173, Japan*

Received 1 July 1997; accepted 5 September 1997; accepted in revised form 5 September 1997

Abstract

The chemoenzymatic synthesis of a glycopeptide by chemical synthesis of *N*-acetylglucosaminyl peptide and enzymatic transfer of an oligosaccharide is described. We synthesized glycosylated Peptide T which blocks infection of human T cells by human immunodeficiency virus. The first step of the chemoenzymatic method is the solid-phase chemical synthesis of *N*-acetylglucosaminyl Peptide T (Ala-Ser-Thr-Thr-Thr-Asn(GlcNAc)-Tyr-Thr) with an *N*-acetylglucosamine moiety bound to the asparaginyl residue by a solid-phase method. This product was prepared in high yield by the dimethylphosphinothioic mixed anhydride method without protecting the hydroxyl functions of the sugar moiety using Fmoc-*N*-acetylglucosaminyl asparagine instead of Fmoc-asparagine. The second step was transglycosylation of complex type oligosaccharide to *N*-acetylglucosaminyl Peptide T by a microbial endoglycosidase. The endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* transfer the oligosaccharide of human transferrin glycopeptide to *N*-acetylglucosaminyl Peptide T. The transglycosylation product was confirmed to be the glycosylated Peptide T with a sialo biantennary complex type oligosaccharide by mass spectrometry. The glycosylated Peptide T was highly stable against proteolysis in comparison to native Peptide T and *N*-acetylglucosaminyl Peptide T.

Keywords: Peptide T; Endoglycosidase; Transglycosylation; Glycopeptide; Endo-β-*N*-acetylglucosaminidase

1. Introduction

During the past two decades, the oligosaccharide moieties of some glycoconjugates have been shown to play important roles in biological phenomena such as cellular recognition, lectin binding and viral infec-

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; Dns, dansyl (5-dimethylaminonaphthalene-1-sulfonyl); Fmoc, 9-fluorenyl-methyloxycarbonyl; Boc, butyloxycarbonyl; Bu^t, *t*-butyl; EDTA, ethylenediaminetetraacetic acid

* Corresponding author. Fax: +81-75-753-6275; e-mail: yamamotk@kais.kyoto_u.ac.jp

0008-6215/98/$19.00
PII S0008-6215(97)10018-0

tion. Progress in the field of glycobiology requires the synthesis and construction of glycoconjugates to elucidate the significance and functions of the oligosaccharides. Although methods for the chemical synthesis of oligosaccharides in glycoconjugates have been developed, they are labor-intensive and involve complicated steps of protection and deprotection steps. On the other hand, enzymatic methods have the advantages of high stereo- and regio-selectivities. However, many kinds of glycosyltransferases are required to synthesize oligosaccharides with complex structures. We examined the use of a chemoenzymatic method for synthesis of glycoconjugates, and synthesized a bioactive glycopeptide by this method.

Several strategies for glycopeptide synthesis have been reported. These include the chemical addition of oligosaccharide to peptide by reductive amination [1], solid-phase synthesis using glyco-amino acids as building blocks [2] and enzymatic synthesis using peptidases and glycosyltransferases [3]. Recently, using the transglycosylation activity of microbial endoglycosidases, Yamamoto et al. [4] and Takegawa et al. [5] reported the addition of oligosaccharide to *N*-acetylglucosaminyl peptide obtained as a building material for glycopeptide synthesis from natural sources by enzymatic degradation of glycoprotein. Wang et al. [6] described the synthesis of a glycopeptide analog with a C-glycosidic linkage using the transglycosylation activity of endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) from *Arthrobacter protophormiae* (Endo-A). We also described the synthesis of a glycopeptide fragment using the transglycosylation activity of *Mucor hiemalis* endo-β-GlcNAc-ase (Endo-M) [7].

Endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) is a unique endoglycosidase that hydrolyzes *N*,*N*′-diacetylchitobiosyl linkages in oligosaccharides bound to asparaginyl residues of various glycoproteins and glycopeptides, and leaves one *N*-acetylglucosamine (GlcNAc) residue on the protein and peptide moi-

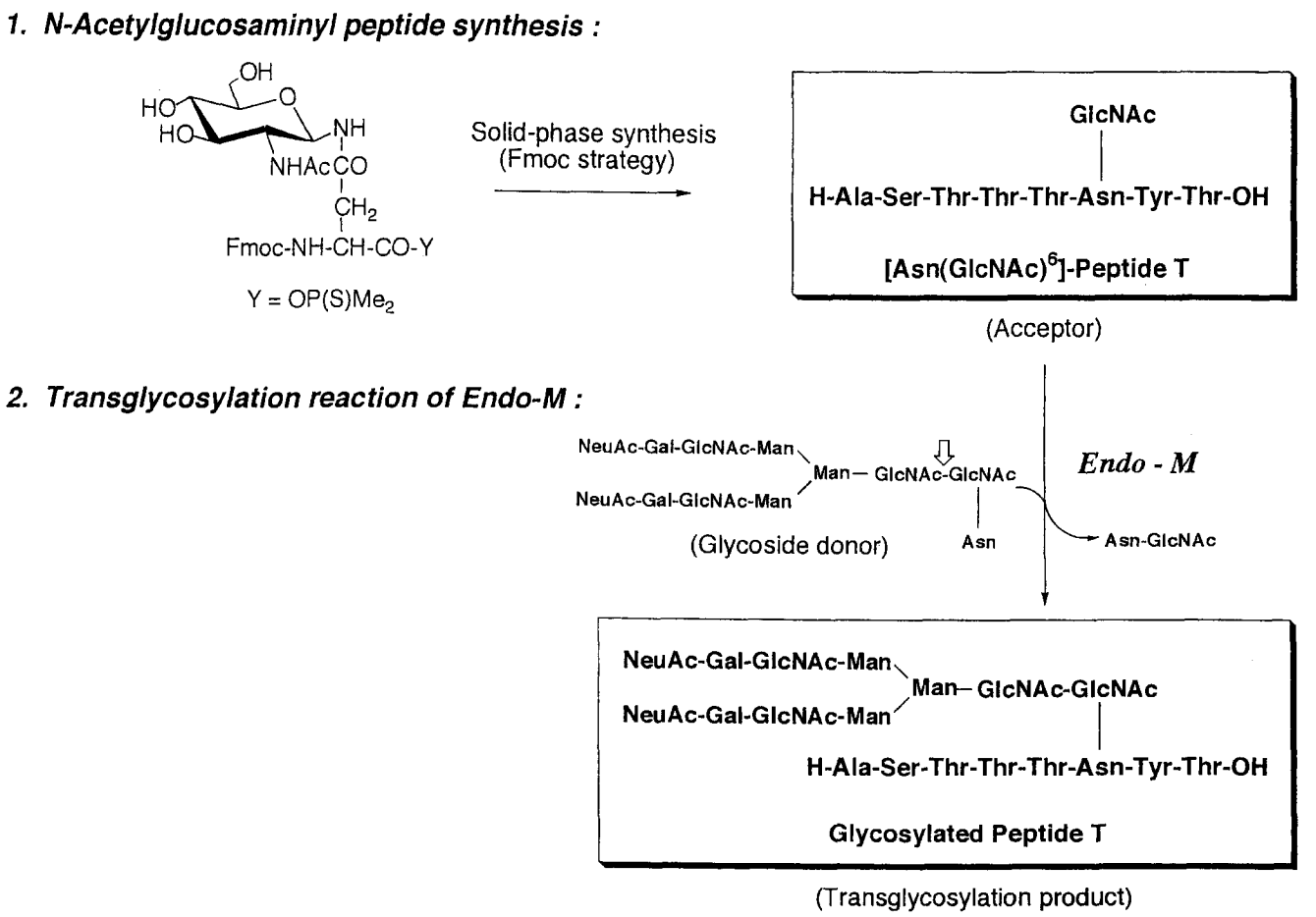

Fig. 1. The strategy of chemoenzymatic synthesis of glycosylated Peptide T containing *N*-linked oligosaccharide. Abbreviations: NeuAc, *N*-acetylneuraminic acid (sialic acid); Gal, D-galactose; GlcNAc, *N*-acetylglucosamine; Man, D-mannose.

eties. We found a novel endo-β-GlcNAc-ase in the culture medium of *M. hiemalis* isolated from soil, which could cleave not only the high-mannose type and hybrid type of asparagine-linked oligosaccharides but also the complex type of oligosaccharide [8], unlike other endo-β-GlcNAc-ases which can act on only high-mannose and hybrid type oligosaccharides. As mentioned above, this enzyme showed transglycosylation activity and could transfer the oligosaccharides from glycopeptides to suitable acceptors with a GlcNAc residue during hydrolysis of the glycopeptide [4]. The transglycosylation activity of Endo-M catalyzes the following reaction, R-GlcNAc-GlcNAc-Asn (glycoside donor) + GlcNAc-Asn-R′ (acceptor) → R-GlcNAc-GlcNAc-Asn-R′ (transglycosylation product) + GlcNAc-Asn where R = any type of oligosaccharide and R′ = peptide or peptide derivatives. These observations suggested that the transglycosylation of Endo-M might be useful for synthesis of glycopeptides.

On the other hand, Inazu et al. [9] recently developed a convenient synthetic method to prepare *N*-acetylglucosaminyl peptides by solid-phase synthesis based on the Fmoc strategy in which Fmoc (9-fluorenylmethyloxycarbonyl)-Asn-(GlcNAc)–OH is used instead of Fmoc-Asn–OH. We developed a procedure for the synthesis of glycopeptides by combining chemical and enzymatic methods. Here, we describe the chemoenzymatic synthesis of glycosylated Peptide T. Peptide T is a bioactive peptide composed of eight amino acids of the sequence Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr, and can block infection of human T cells by human immunodeficiency virus (HIV) [10]. Our synthetic strategy for glycosylated Peptide T is shown in Fig. 1. This is the first report of the synthesis of a bioactive glycopeptide by a chemoenzymatic method.

2. Experimental

Preparation of endo-β-N-acetylglucosaminidase from Mucor hiemalis.—The endo-β-GlcNAc-ase of *M. hiemalis* was partially purified from the culture medium of the fungus as described previously [11]. The enzyme preparation was almost free from other glycosidase and protease activities.

Preparation of substrates.—Sialotransferrin glycopeptide was prepared by repeated exhaustive Pronase digestion of human transferrin followed by Sephadex G-25 gel filtration as described previously [8]. Asialotransferrin glycopeptide was prepared from sialotransferrin glycopeptide by reaction with sialidase before the last Pronase digestion. Both glycopeptides were further purified by high performance liquid chromatography (HPLC) using a reverse-phase column before use.

Dansyl derivatives of glycopeptides were prepared according to the method of Gray [12].

Chemical synthesis of N-*acetylglucosaminyl peptide.*—The building block N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc)-N^{β}-(*N*-acetylglucosaminyl)-asparagine [Fmoc-Asn(GlcNAc)–OH], was synthesized by reaction of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranosyl azide and Fmoc-aspartic acid α-*t*-butyl ester in the presence of triethylphosphine [13]. Using Fmoc-Asn(GlcNAc)–OH instead of Fmoc-Asn–OH, the solid-phase synthesis of Peptide T containing GlcNAc, that is [Asn(GlcNAc)^6] Peptide T(Ala-Ser-Thr-Thr-Thr-Asn(GlcNAc)-Tyr-Thr), was performed by the dimethylphosphinothioic mixed anhydride (Mpt-MA) method without protecting the hydroxyl groups of the sugar moiety [9].

N-Acetylglucosaminyl Peptide T([Asn(GlcNAc)^6] Peptide T) was synthesized via the Fmoc strategy except for coupling reactions, starting from Fmoc-Thr(Bu′)–OH coupled to Wang resin (*p*-benzyloxybenzyl alcohol resin, Thr; 0.45 meq/g) in the vessels of a Multi-Peptide Solid-Phase Synthesizer (Kokusan Chemical Works, Japan). For coupling of the Asn(GlcNAc) residue, Fmoc-Asn(GlcNAc)–OH was used without protecting the hydroxyl groups. Coupling reactions were carried out with a two-fold excess of protected amino acid Mpt-MAs in the presence of *N*,*N*-diisopropyl ethylamine (DIEA) for 60 min in *N*,*N*-dimethylformamide (DMF). For the coupling reaction for Asn(GlcNAc), double coupling was necessary. Amino acid Mpt-MAs were prepared from the Fmoc-amino acid and dimethylphosphinothioic chloride (Mpt-Cl) [14] in the presence of DIEA in DMF. Threonine^8 and tyrosine^7 residues were used as the *O*-Bu^t derivatives to prevent diketopiperazine formation. The alanine^1 residue was used as N^{α}-*tert*-butyloxycarbonyl (Boc)-Ala-OMpt. Deprotection of the Fmoc group was performed with 20% piperidine/DMF solution in the usual manner. Cleavage of the glycopeptide from the resin was carried out with TFA (trifluoroacetic acid)–phenol–water–thioanisol–ethanedithiol (82.5:5:5:5:2.5; TFA-cocktail) for 30 min at room temperature, which also removed the side chain protections of Tyr, Ser and N^{α}-Boc groups of Ala. The crude glycopeptide was isolated as a precipitate after evaporation and washing with diethyl ether. After purification by preparative HPLC using

an Inertsil C_{18} column (20 × 250 mm, G-L Science, Japan), the desired peptide, [Asn(GlcNAc)^6] Peptide T, was obtained.

For preparation of N^{α}-dansyl (Dns)-[Asn(GlcNAc)^6] Peptide T, Dns-Ala–OH was used instead of Boc-Ala–OH.

Enzyme reaction.—The transglycosylation reaction of Endo-M was carried out in 10 μL of reaction mixture including 25 mM glycoside donor (sialo- or asialotransferrin glycopeptide), 12.5 mM acceptor (*N*-acetylglucosaminyl peptide or its derivative), 60 mM potassium phosphate buffer (pH 6.0) and 40 mU of Endo-M (final 4 mU/mL). EDTA-3Na was added to the reaction mixture to a final concentration of 50 mM to protect the enzyme from the action of trace amounts of peptidase in the enzyme preparation. The reaction mixture was incubated at 37 °C for a suitable period. To terminate the reaction, the mixture was diluted 25-fold with cold distilled water and immediately frozen. Aliquots of the reaction mixture were analyzed by HPLC after filtration. Yield of the transglycosylation product was calculated by the following equation [7]: Yield (%) = (Area of the peak of the product/Initial area of the peak of the acceptor) × 100.

The enzyme activity of Endo-M was measured as hydrolysis activity using dansyl asialotransferrin glycopeptides, as described previously [8].

HPLC analyses.—HPLC analyses were carried out with a Hitachi L-6200 chromatograph with an F-1050 fluorescence spectrophotometer and L-4200 UV–VIS detector. HPLC analyses of dansyl derivatives were performed with a reverse-phase column (J'sphere ODS-M80, 4.6 × 250 mm: YMC, Japan) at room temperature. Elution was carried out with a linear gradient of acetonitrile (15–25%) containing 0.05% TFA in 30 min at a flow rate of 0.8 mL/min. Dansyl derivatives were detected by fluorescence with excitation and emission wavelengths of 320 and 540 nm, respectively. HPLC analyses of peptides and glycopeptides were performed with a reverse-phase column (J'sphere ODS-M80, 4.6 × 250 mm: YMC, Japan). Elution was carried out with a linear acetonitrile gradient (0–10%) in 30 min at room temperature at a flow rate of 0.8 mL/min. Peptides and glycopeptides were detected by UV absorbance at 214 nm.

Mass spectrometry.—Electrospray ionization (ESI) mass spectrometry was performed in the negative-ion mode on an Analytica of Branford/JEOL JMS-SX 102 A mass spectrometer (JEOL, Japan). Matrix-associated laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the negative-ion mode using α-cyano-4-hydroxycinnamic acid as a matrix on a Finnigan Lasermat mass spectrometer (Finnigan Mat, UK).

Materials.—Human serum transferrin was purchased from Seikagaku Kogyo, Japan. Peptide T was obtained from Peptide Institute, Japan. Pronase (Actinase E) was purchased from Kakenkagaku, Japan. Fmoc-amino acids were purchased from Kokusan Chemical Works, Japan. All other chemicals used were obtained from commercial sources.

3. Results

Solid-phase synthesis of N-acetylglucosaminyl Peptide T.—The first step of our strategy of chemoenzymatic synthesis of glycosylated Peptide T containing *N*-linked oligosaccharides was the solid-phase synthesis of *N*-acetylglucosaminyl Peptide T which had GlcNAc attached to the Asn residue of the peptide. The scheme of the solid-phase synthesis of [Asn(GlcNAc)^6] Peptide T, is shown in Fig. 2. The yield was about 39%. The structure of [Asn(GlcNAc)^6] Peptide T obtained was identified by ^{1}H NMR spectroscopy, MALDI-TOF mass spectrometry and amino acid analysis.

Transglycosylation of oligosaccharides to N-acetylglucosaminyl Peptide T derivative.—The second step of our strategy was the addition of oligosaccharide from glycopeptide to *N*-acetylglucosaminyl Peptide T using the transglycosylation activity of Endo-M. Firstly, dansyl [Asn(GlcNAc)^6] Peptide T was chosen as the acceptor of the oligosaccharide in the transglycosylation reaction by Endo-M because this compound is protected from the trace amounts of peptidase contamination in the Endo-M preparation by a dansyl group on its N-terminal and it can be sensitively detected by fluorescence of the dansyl group on HPLC. Transglycosylation of Endo-M with dansyl [Asn(GlcNAc)^6] Peptide T was carried out using asialo- or sialotransferrin glycopeptide as a glycoside donor. The reaction mixture was incubated at 37 °C for 6 h. After terminating the reaction, the mixture was filtered and analyzed by HPLC with a reverse-phase column as shown in Fig. 3. In the profiles of reaction mixtures containing glycoside donors (Fig. 3b and c), a new peak was found in front of the peak of each acceptor and was considered to be the transglycosylation product. The new peak from the reaction mixture with sialotransferrin glycopeptide as a donor (indicated by an arrowhead in Fig. 3c)

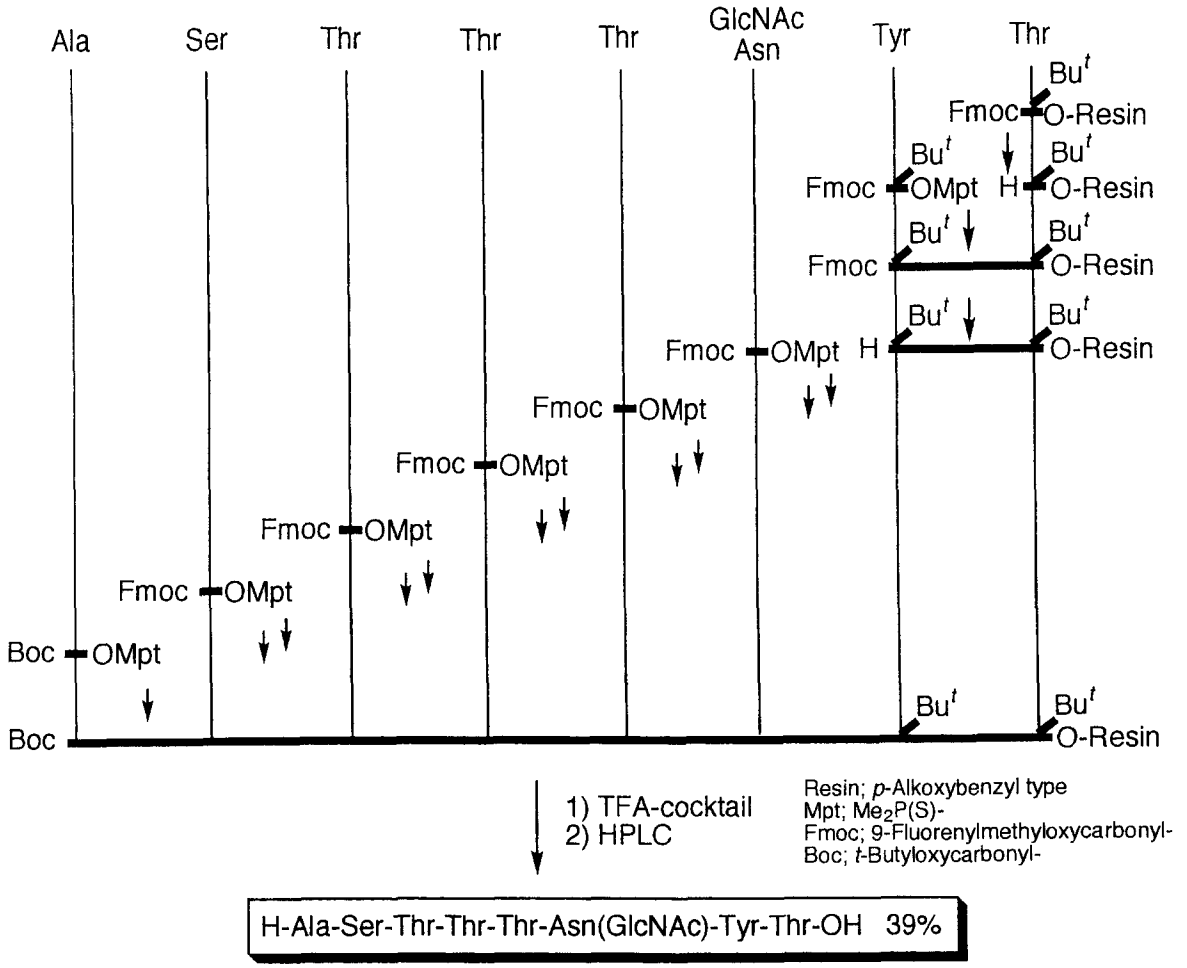

Fig. 2. Solid-phase synthesis of *N*-acetylglucosaminyl Peptide T containing GlcNAc attached to the Asn residue.

was collected and identified by MALDI-TOF mass spectrometry as the transglycosylation product in which a sialo biantennary complex type oligosaccharide was transferred to the GlcNAc moiety of dansyl [Asn(GlcNAc)^6] Peptide T (MW 3297.2) (data not shown). Yields of transglycosylation products obtained using asialo- and sialotransferrin glycopeptides as donors were 15.3 and 16.3%, respectively.

Transglycosylation of oligosaccharides to N *-acetylglucosaminyl Peptide T.*—Unprotected *N*-acetylglucosaminyl Peptide T ([Asn(GlcNAc)^6] Peptide T) was used as the acceptor in the transglycosylation of Endo-M. The reaction was carried out in the same manner using 25 mM sialotransferrin glycopeptide as the glycoside donor and 10 mM [Asn(GlcNAc)^6] Peptide T as the acceptor. After incubation for 4.5 h at 37 °C, the reaction mixture was subjected to HPLC. The profiles are shown in Fig. 4. Several peaks were observed in the HPLC profile of the reaction mixture containing the donor (Fig. 4c). Among these, two peaks (peaks 3 and 4 in Fig. 4c) corresponded to those in the profile of the reaction mixture without donor. They were considered to be degradation products of [Asn(GlcNAc)^6] Peptide T by peptidase contamination in the Endo-M preparation. They were confirmed by MALDI-TOF mass spectrometry to be fragments of [Asn(GlcNAc)^6] Peptide T which had lost Ala-Ser-Thr (peak 3) and Ala (peak 4), respectively. The new peaks 1 and 2 were collected and analyzed by ESI mass spectrometry. In the spectrum of peak 1, a mass ion with a charge of -3, $[M-3H]^{3-}$ with m/z of 1019.4, was detected (Fig. 5). The molecular mass of 3061.2 calculated from this value agreed with the theoretical value of a transglycosylation product in which sialo biantennary complex type oligosaccharide was transferred to [Asn(GlcNAc)^6] Peptide T (MW 3063.9). The yield of the product was about 9%. The spectrum of peak 2 revealed a transglycosylation product in which a monosialo biantennary complex type oligosaccharide was transferred to [Asn(GlcNAc)^6] Peptide T (MW 2772.6).

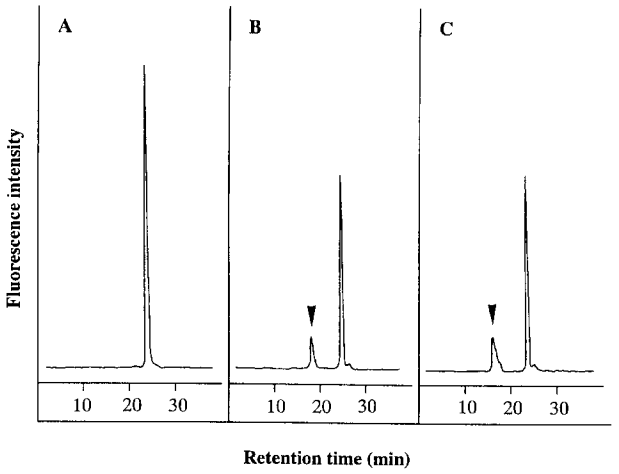

Fig. 3. HPLC profiles of the reaction mixtures incubated with DNS-[Asn(GlcNAc)^6] Peptide T and sialo- and asialotransferrin glycopeptides in the presence or absence of Endo-M. (A) Reaction mixture without Endo-M. (B) Reaction mixture with asialotransferrin glycopetide and Endo-M. (C) Reaction mixture with sialotransferrin glycopeptide and Endo-M. The arrowheads indicate the transglycosylation products. A large peak at a retention time of 22–24 min corresponds to the remaining DNS-[Asn(GlcNAc)^6] Peptide T.

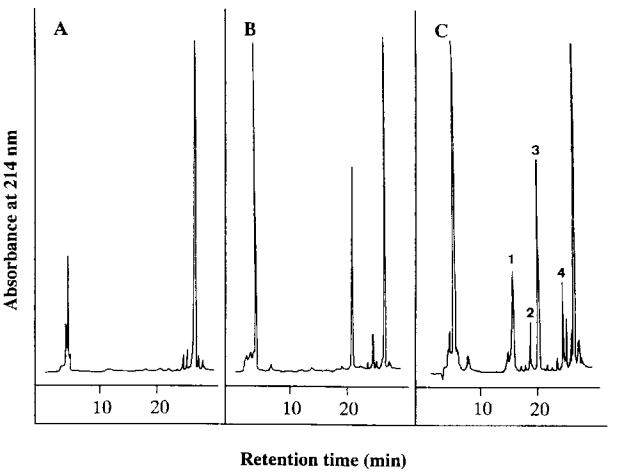

Fig. 4. HPLC profiles of the reaction mixtures incubated with [Asn(GlcNAc)^6] Peptide T and sialotransferrin glycopeptide in the presence or absence of Endo-M. (A) Reaction mixture without Endo-M. (B) Reaction mixture without sialotransferrin glycopetide in the presence of Endo-M. (C) Reaction mixture with sialotransferrin glycopeptide in the presence of Endo-M. The large peak at a retention time of 24–25 min corresponds to the remaining [Asn(GlcNAc)^6] Peptide T.

Stability of native and glycosylated Peptide T against protease digestion.—Generally, glycosylated peptides or proteins are more stable against heat denaturation, freezing and protease digestion than the original molecule. We investigated the resistance of glycosylated Peptide T against protease digestion. The transglycosylation product of Endo-M obtained using sialotransferrin glycopeptide as a glycoside donor, which is Peptide T containing sialo bianten-nary complex type oligosaccharide, was used as the glycosylated Peptide T. Native Peptide T, [Asn(GlcNAc)^6] Peptide T and glycosylated Peptide T (0.7 mM each) were each incubated with 0.8 mg Pronase (Actinase E) in 83 mM Tris–HCl buffer (pH 7.5) containing 17 mM $CaCl_2$ at 37 °C in a total volume of 0.1 mL. An aliquot of each reaction mixture was withdrawn at intervals and incubated in a boiling water bath for 5 min to terminate the

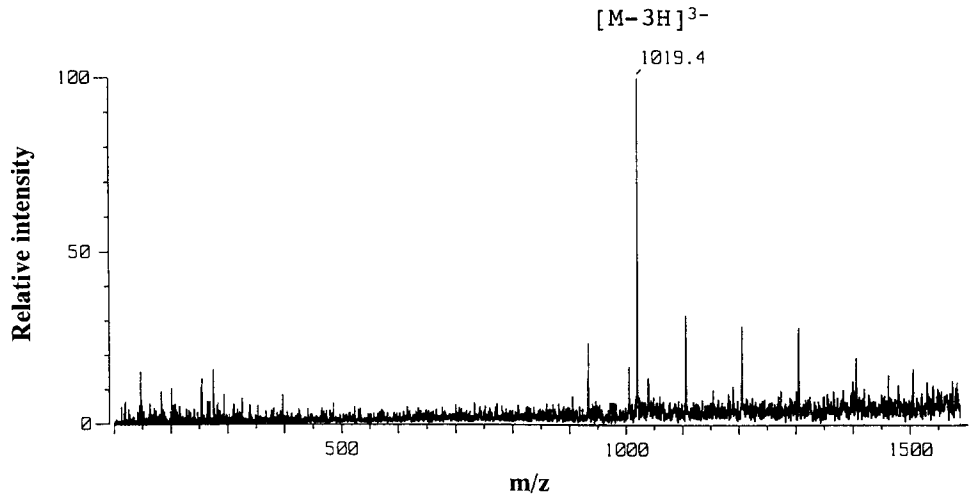

Fig. 5. ESI mass spectrum of the transglycosylation product of Endo-M with [Asn(GlcNAc)^6] Peptide T and sialotransferrin glycopeptide.

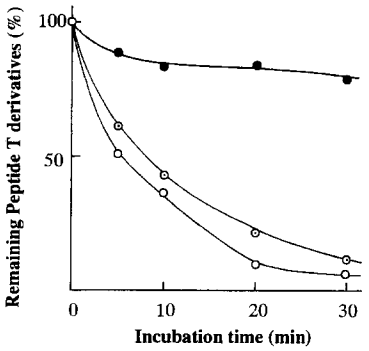

Fig. 6. Effects of protease digestion on Peptide T and Peptide T derivatives. Each peptide was incubated with pronase for the designated periods and the ratio of the remaining amount of each peptide was analyzed by HPLC. ○: Peptide T, ⊙: [Asn(GlcNAc)^6] Peptide T, ●: glycosylated Peptide T.

reaction. Each reaction mixture was analyzed by HPLC, and the ratio of remaining Peptide T derivatives was calculated (Fig. 6). In contrast to the observation that almost all Peptide T was degraded within 30 min, about 80% of the original amount of glycosylated Peptide T remained at this time point. [Asn(GlcNAc)^6] Peptide T showed only slight resistance against pronase digestion.

4. Discussion

Peptide T is a partial sequence of the HIV envelope glycoprotein gp120, which has been reported to block infection of human T cells by HIV [10]. Peptide T and a number of its analogs proved quite potent in triggering human monocyte chemotaxis through the CD4 receptor [15–17]. A few attempts to add saccharides to Peptide T have been performed to improve the conformational stability and resistance against protease digestion. Marastoni et al. [18] prepared some Peptide T analogs in which D-glucopyranose was *O*-glycosidically linked to Thr^4 and/or Thr^5 of Peptide T. They reported that a glycosyl unit at Thr^5 increased CD4 receptor interaction. Kosch et al. [19] synthesized lactosamine-binding Peptide T with an *N*-glycosidic bond between lactosamine and Asn^6 of Peptide T. Urge et al. [20] also synthesized various Peptide T analogs in which monosaccharide was incorporated into the middle, or C- or N-termini of the peptide. They reported that these modified peptides were conformationally more stable than native Peptide T. However, monosaccharide-bound Peptide T derivatives such as *N*-acetylglucosaminyl Peptide T in which GlcNAc is attached to an Asn residue did not have higher resistance against proteolysis in the present study. Peptide T contains the consensus sequence of amino acids for *N*-linked oligosaccharide binding (Asn-X-Thr or Ser). We have, thus, attempted to add oligosaccharide, to the GlcNAc residue in Asn^6 of Peptide T by transglycosylation with Endo-M. We reported previously that sialooligosaccharide was a better glycoside donor for the transglycosylation reaction of Endo-M than asialooligosaccharide [7]. The same result was obtained in the case of Dns-[Asn(GlcNAc)^6] Peptide T which was used as an acceptor. Finally, we successfully synthesized glycosylated Peptide T with sialooligosaccharide, and this glycopeptide showed high resistance against protease digestion.

Peptide T analogs such as [D-Ala^1] Peptide T-amide and Peptide T_{4-8} (the core pentapeptide of Peptide T) were also reported to be potent inhibitors of the binding of HIV to CD4 [15,17]. We synthesized *N*-acetylglucosaminyl Peptide T analogs such as [D-Ala^1] Peptide T-amide including GlcNAc at Asn^6 and Peptide T_{4-8} including GlcNAc at Asn^6, and confirmed that Endo-M could also transfer the oligosaccharide of transferrin glycopeptide to these *N*-acetylglucosaminyl Peptide T analogs. Investigations of the bioactivities of glycosylated Peptide T and such Peptide T analogs are currently underway in our laboratory. It will be interesting to determine whether glycosylated Peptide T and its analogs may be better inhibitors for binding of HIV. The chemoenzymatic method combined with chemical synthesis of *N*-acetylglucosaminyl peptide and transglycosylation of endoglycosidase should be highly useful for the synthesis of many glycopeptides.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 06660102) from the Ministry of Education, Science, and Culture of Japan, by the Grant-in Aid for Scientific Research on Priority Areas (No. 05274102) from the Ministry of Education, Science, and Culture of Japan, and by The Inamori Foundation. This work was partially supported by Special Coordination Funds of the Science and Technology Agency of the Japanese Government.

References

[1] Y.C. Lee and R.T. Lee, *Glycoconjugates*, in H.J. Allen and E.C. Kisailus (Eds.), Marcel Dekker, NY, 1992, pp. 121–165.

[2] M. Meldal, *Glycoconj. J.*, 11 (1994) 59–63.

[3] M. Schuster, P. Wang, J.C. Paulson, and C.H. Wong, *J. Am. Chem. Soc.*, 116 (1994) 1135–1136.

[4] K. Yamamoto, S. Kadowaki, J. Watanabe, and H. Kumagai, *Biochem. Biophys. Res. Commun.*, 203 (1994) 244–252.

[5] K. Takegawa, M. Tabuchi, S. Yamaguchi, A. Kondo, I. Kato, and S. Iwahara, *J. Biol. Chem.*, 270 (1995) 3094–3099.

[6] L.-X. Wang, J.-Q. Fan, and Y.C. Lee, *Tetrahedron Lett.*, 37 (1996) 1975–1978.

[7] K. Haneda, T. Inazu, K. Yamamoto, H. Kumagai, Y. Nakahara, and A. Kobata, *Carbohydr. Res.*, 292 (1996) 61–70.

[8] S. Kadowaki, K. Yamamoto, M. Fujisaki, K. Izumi, T. Tochikura, and T. Yokoyama, *Agric. Biol. Chem.*, 54 (1988) 97–106.

[9] T. Inazu, K. Kobayashi, and H. Yaginuma, *Peptide Chemistry 1993*, in Y. Okada (Ed.), Protein Research Foundation, Osaka, Japan, 1994, pp. 101–104.

[10] C.B. Pert, J.M. Hill, R.M. Berman, W.G. Robey, L.O. Arthur, F.W. Ruscetti, and W.L. Farrar, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 9254–9258.

[11] S. Kadowaki, K. Yamamoto, M. Fujisaki, and T. Tochikura, *J. Biochem.*, 110 (1991) 17–21.

[12] W.R. Gray, *Methods Enzymol.*, 11 (1967) 139–151.

[13] T. Inazu and K. Kobayashi, *Synlett.*, (1993) 869–870.

[14] M. Ueki, T. Inazu, and S. Ikeda, *Bull. Chem. Soc. Jpn.*, 52 (1979) 2424–2427.

[15] M.R. Ruff, B.M. Marin, E.I. Ginns, W.L. Farrar, and C.B. Pert, *FEBS Lett.*, 211 (1987) 17–22.

[16] M. Marastoni, S. Salvadori, G. Balboni, S. Spisani, R. Gavioli, S. Traniello, and R. Tomatis, *Int. J. Peptide Protein Res.*, 35 (1990) 81–88.

[17] M. Marastoni, S. Salvadori, G. Balboni, V. Scaranari, S. Spisani, E. Reali, S. Traniello, and R. Tomatis, *Int. J. Peptide Protein Res.*, 41 (1993) 447–454.

[18] M. Marastoni, S. Spisani, and R. Tomatis, *Arzneim. Forsch. / Drug Res.*, 44 (1994) 984–987.

[19] W. Kosch, J. Marz, and H. Kunz, *Reactive Polymer*, 22 (1994) 181–194.

[20] L. Urge, L. Gorbics, and L. Otvos, *Biochem. Biophys. Res. Commun.*, 184 (1992) 1125–1132.