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Linkage of sugar chains to a fragment peptide of FGF-5S by a chemoenzymatic strategy and changes in the rate of proteolytic hydrolysis

Katsumi Ajisaka[1]*, Mariko Miyasato[1], Chikako Ito[2], Yasuko Fujita[2], Yoshimitu Yamazaki[2] and Syuichi Oka[2]

[1]*Nutrition Science Institute, Meiji Milk Products Co., Ltd., 540 Naruda, Odawara, Kanagawa 250-0862, Japan,* [2]*National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1 Higashi, Tsukuba-shi, Ibaraki 305-8566, Japan*

Various *O*-linked and *N*-linked sugar chains were linked enzymatically to a fragment peptide (Leu-Ser-Gln(or Asn)-Val-His-Arg) of FGF-5S. First, galactose was linked with β-(1 → 3)-linkage to GalNAc-linked peptide by a transglycosylation using β-galactosidase from *Bacillus circulans* (recombinant). Then sialic acid was linked with the aid of sialyltransferase from rat liver (recombinant) to give NeuAcα-(2 → 3)-Galβ-(1 → 3)-GalNAc-linked hexapeptide. Further, a sialylated 2-chain biantennary sugar chain was linked by a transglycosylation using endo *N*-acetyl-β-D-glucosaminidase from *Mucor hiemalis* (endo M, recombinant). The activity of DNA synthesis in a fibroblast cell line was increased by this glycosylation. The resistance of the obtained glycopeptides towards proteolytic hydrolysis by rat serum and by five proteases was compared with that of original peptide. The resistance was remarkably enhanced by the glycosylation.

Keywords: glycopeptide, enzymatic synthesis, FGF-5, proteolytic hydrolysis

Abbreviations: FGH, fibroblast growth factor; Cbz, benzyloxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; Gal-β-pNP, *para*-nitrophenyl-β-D-galactopyranoside.

Introduction

One of the roles of sugar chains in glycoproteins is to protect the peptide bond from proteolytic degradation [1–3]. However the roles and functions of sugar chains in glycopeptides have not been studied as extensively as those in glycoproteins [4]. One reason may be that the synthesis of glycopeptides is not so easy [5]. To our knowledge, only one paper has reported about the effect of sugar chains on the hydrolysis of peptide bond by proteases [6]. In that paper, the linkage of disaccharide (Galβ-(1 → 4)-Glc) was demonstrated to protect the peptide from the hydrolysis by proteases. However, the model peptide used in the above report was not a natural peptide with a biological function.

We have previously reported an efficient chemoenzymatic method to synthesize mucin-type glycopeptide [7], but in that study we did not examine the changes of the activity and functions induced by the glycosylation. In the present study we planned to synthesize glycopeptides and to examine the changes of activity and functions compared with the original peptides. As a model peptide, we selected a fragment peptide of FGF which has the biological function to stimulate DNA synthesis.

From chemical syntheses and activity measurements of various fragment peptides of human FGF-5 [8], Leu-Ser-Gln-Val-His-Arg **2** was found to have a stimulating effect on DNA syntheses in a fibroblast cell line, BALB/3T3 A31, similar to FGF-5 itself. The peptide **2** is a C-terminal sequence of FGF-5S, which is a short form of the FGF-5 and is generated from alternative splicing of FGF-5 mRNAs. FGF-5 mRNAs are generated by splicing out the second exons of FGF-5 genes encoding 123-amino acid polypeptides. The alternative splicing results in frame shifts and the introduction of immediate stop codons in the third exons [9,10]. We used the peptide **2** as a model peptide to which sugar chains were linked and to examine the changes in activity and functions. The structures of peptides and glycopeptides used in this study are summarized in Table 1.

In the present study, we linked various sugar chains to the peptide **2** and examined the relation between the structure of sugar chains and the degree of resistance toward the proteolytic

*To whom correspondence should be addressed: Dr. Katsumi Ajisaka. Tel. : +81-465-37-3661, Fax: +81-465-36-2776, E-mail: kajisaka@mbc.sphere.ne.jp

Table 1. Structure of peptides and glycopeptides used in the present study

Peptide or glycopeptide	*Structure*
1	Cbz-Leu-Ser-Gln-Val-His-Arg
2	Leu-Ser-Gln-Val-His-Arg
3	Cbz-Leu-[GalNAc-]Scr-Gln-Val-His-Arg
4	Leu-[GalNAc-]Ser-Gln-Val-His-Arg
5	Cbz-Leu-[Galβ-(1 $\rightarrow$ 3)-GalNAc-]Ser-Gln-Val-His-Arg
6	Leu-[Galβ-(1 $\rightarrow$ 3)-GalNAc-]Ser-Gln-Val-His-Arg
7	Cbz-Leu-[NeuAcα-(2 $\rightarrow$ 3)-Galβ-(1 $\rightarrow$ 3)-GalNAc-]Ser-Gln-Val-His-Arg
8	Leu-[NeuAcα(2 $\rightarrow$ 3)-Galβ-(1 $\rightarrow$ 3)-GalNAc-]Ser-Gln-Val-His-Arg
9	Cbz-Leu-Ser-Asn-Val-His-Arg
10	Leu-Ser-Asn-Val-His-Arg
11	Cbz-Leu-Ser-[GlcNAc-]Asn-Val-His-Arg
12	Leu-Ser-[GlcNAc-]Asn-Val-His-Arg
13	Cbz-Leu-Ser-[Complex type sugar chain-]Asn-Val-His-Arg
14	Leu-Ser-[Complex type sugar chain-]Asn-Val-His-Arg
15	Lys-Val-Ala-[Complex type sugar chain-]Asn-Lys-Thr
16	Lys-Val-Ala-[GlcNAc-]Asn-Lys-Thr

hydrolysis. We also examined the changes in function due to the linkage of sugar chains, because there would be no merit in linking sugar chains if function is thereby lost, even though the resistance to proteolytic hydrolysis can be enhanced.

Materials and methods

Materials

HMP-resin of *N*-Fmoc-Arg and cartridges of *N*-Fmoc-Val, *N*-Fmoc-His, and *N*-Fmoc-Asn were purchased from Applied Biosystems Japan Ltd. (Tokyo, Japan) and *N*-Cbz-Leu from Sigma. *N*-Fmoc-*O*-[(2-acetamido-3, 4, 6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)]-Ser and *N*-Fmoc-*O*-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)]-Asn were the products of Nissinbo Co. (Tokyo, Japan). All the proteases used in the present study were purchased from Sigma. Sialylated biantennary sugar chain (SGP) was the product of Taiyo Kagaku Co., Ltd. (Yokkaichi, Japan). Sialyltransferase from rat liver was purchased from Calbiochem. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and [^{3}H]-thymidine were purchased from Sigma, Sanko Jyunyaku Co. Ltd. (Tokyo, Japan), and Moravek Biochemicals (Brea, CA), respectively.

Analytical method

HPLC analysis was conducted on a Pharmacia Akta Purifier system with UV monitor (215 nm and 256 nm). Glycopeptides were separated on a Mightysil RP-18 column (Kanto Chemical Co., Tokyo, Japan) by eluting with a gradient (45 min) of 0–20% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min at room temperature. ^{1}H and ^{13}C NMR spectra were obtained by a Varian Inova 500 spectrometer. TOF-MS was measured in the positive and negative mode using Lasermat 2000 spectrometer (Finnigan mat, CA) using 2,5-dihydroxybenzoic acid as a matrix.

Synthesis of GalNAc-linked hexapeptide **3**

A preloaded HMP-resin of *N*-Fmoc-Arg (0.25 mmol) and cartridges of *N*-Fmoc-Val, *N*-Fmoc-His, and *N*-Fmoc-Gln (1 mmol each), *N*-Fmoc-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-Ser (0.25 mmol), and *N*-Cbz-Leu (1 mmol) were applied to the peptide synthesizer (model 431A, Perkin-Elmer Japan Co., Chiba, Japan). The peptide synthesis was performed with DCC-HOBt-activation method. The obtained resin-linked glycopeptide was suspended in 10 ml of 95% TFA solution and the mixture was stirred for 1.5 h. After filtration and concentration, the obtained syrup was dissolved in 10 ml of water. The solution was then stirred for 1.5 h in an ice-cold water bath by maintaining pH 10 with a 0.1 N NaOH solution. The glycopeptide was isolated by a preparative Mightysil RP-18 column (3.5 cm $\times$ 25 cm) eluted with a gradient (160 min) of 0–20% aqueous acetonitrile containing 0.1% TFA. After freeze-drying, 122 mg of Cbz-Leu-[GalNAc-]Ser-Glc-Val-His-Arg **3** was obtained (45% yield). Selected ^{1}H-NMR data (500 MHz, D_2O)-δ: 1.892 (s, 3H, *N*-Ac(GalNAc)); 3.073(t, ^3J (H,H) = 6.51 Hz, 2H, Hβ(His)); 3.452(broad s, 1H, H-5(GalNAc)); 3.760(broad s, 1H, H-4(GalNAc)); 3.875(broad d, ^3J (H,H) = 10.40 Hz, 1H, H-3(GalNAc)); 3.945(d, ^3J (H,H) = 7.40 Hz, 1H, Hα(Val)); 4.320(dd, ^3J (H,H) = 3.96 Hz, 10.32 Hz, 1H, H-2(GalNAc)); 4.241(dd, ^3J (H,H) = 5.52 Hz, 8.12 Hz, 1H, Hα(Arg)); 4.791 (d, ^3J (H,H) = 3.96 Hz, 1H, H-1(GalNAc)); 7.156(s, 1H); 8.487(s, 1H). TOF-MS: calcd. for [M + H], 1091.5; found, 1090.2; calcd. for [M + Na], 1113.5; found, 1111.4.

Synthesis of Galβ-(1 → 3)-GalNAc-linked hexapeptide **5**

GalNAc-linked hexapeptide **3** (50 mg, 46 μmol) and Gal-β-pNP (70 mg, 230 μmol) were dissolved in 430 μl of 0.1 M phosphate buffer (pH 6.0) containing 10% DMF. To this solution, 70 μl of β-galactosidase from *Bacillus circulans* ([EC 3.2.1.23], recombinant, 25 units/ml) [11] was added. The solution was incubated at 37°C, and HPLC was carried out at appropriate time intervals. After 6.5 h, the solution was heated at 100°C for 3 min to deactivate the enzyme. Isolation of the products by HPLC with preparative Mightysil RP-18 column afforded 7.4 mg (13% yield) of Cbz-Leu-[Galβ-(1 → 3)-GalNAc]Ser-Gln-Val-His-Arg **5**. Selected ^{1}H-NMR data (500 MHz, D_2O)-δ: 1.898(s, 3H, *N*-Ac(GalNAc)); 3.073(t, ^{3}J (H,H) = 7.14 Hz, 2H, Hβ(His)); 3.439(broad s, 1H, H-5(GalNAc)); 3.735(broad s, 1H, H-4(GalNAc)); 3.796(broad s, 1H, H-5(Gal)); 3.880(broad d, ^3J (H,H) = 10.51 Hz, 1H, H-3(GalNAc)); 3.951(d, ^3J (H,H) = 7.36 Hz, 1H, Hα(Val)); 4.210(dd, ^3J (H,H) = 4.09 Hz, 10.51 Hz, 1H, H-2(GalNAc)); 4.251(dd, ^3J (H,H) = 5.57 Hz, 8.08 Hz, 1H, Hα(Arg)); 4.299(d, ^3J (H,H) = 7.25 Hz, 1H, H-1(Gal)); 4.791(d, ^3J (H,H) = 4.09 Hz, 1H, H-1(GalNAc)); 7.214(s, 1H); 8.494(s, 1H). TOF-MS: calcd. for [M + H], 1238.6; found, 1239.3; calcd. for [M + Na], 1260.6; found, 1261.5.

Synthesis of NeuAcα-(2 → 3)-Galβ-(1 → 3)-GalNAc-linked hexapeptide **7**

Glycopeptide **5** (8.0 mg, 7.4 μmol) and CMP-NeuAc (15.4 mg, 22 μmol) were dissolved in 450 μl of a 50 mM cacodylate buffer (pH 6.25) containing 0.1% triton X and 1 mg/ml of BSA. To this solution, 300 μl (100 mU) of sialyltransferase from rat liver was added. After incubation at 37°C for 1.75 h, the reaction was stopped. Isolation with a preparative Mightysil RP-18 column afforded 4.8 mg of Cbz-Leu-[NeuAcα-(2 → 3)-Galβ-(1 → 3)-GalNAc]Ser-Gln-Val-His-Arg **7** (42% yield) and 2.5 mg of glycopeptide **5**. Selected ^{1}H-NMR data (500 MHz, D_2O)-δ:1.796 (t, ^3J (H,H) = 12.41 Hz, 1H, 1-H_{ax}(NeuAc)); 2.021(s, 3H, *N*-Ac(GalNAc)); 2.030(s, 3H, *N*-Ac(NeuAc)); 2.752(dd, *J* (H,H) = 4.51 Hz, 12.41 Hz, 1H, H-3$_{eq}$(NeuAc)); 4.318(dd, ^3J (H,H) = 3.66 Hz, 11.02 Hz, 1H, H-2(GalNAc)); 4.358(broad dd, 1H, Hα(Gln)); 4.551(d, ^3J (H,H) = 7.80 Hz, 1H, H-1(Gal)); 4.664(t, ^3J (H,H) = 5.26 Hz, 1H, Hα(Ser)); 4.720(dd, ^3J (H,H) = 1.85 Hz, 7.14 Hz, 1H, Hα(His)); 4.775(d, ^3J (H,H) = 3.66 Hz, 1H, H-1(GalNAc)); 7.344(broad s, 1H), 8.634(d, ^3J (H,H) = 1.33 Hz, 1H). TOF-MS: calcd. for [M + H], 1395.6; found, 1397.2; calcd. for [M + Na], 1417.6; found, 1418.8.

Synthesis of GlcNAc-linked hexapeptide **11**

The synthesis of this glycopeptide was performed by a similar procedure as that of GalNAc-linked hexapeptide **3** by using *N*-Fmoc-*O*- (2-acetamido-3, 4, 6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-Asn instead of the corresponding GalNAc-Ser derivatives. By purification with preparative HPLC column, 70.8 mg of Cbz-Leu-Ser-[GlcNAc]Asn-Val-His-Arg **11** was obtained (26% yield). Selected ^{1}H-NMR data (500 MHz, D_2O)-δ: 1.902(s, 3H, *N*-Ac(GalNAc)); 3.025(t, ^3J (H,H) = 7.01 Hz, 2H, Hβ(His)); 3.496(t, ^3J (H,H) = 8.96 Hz, 1H, H-3(GlcNAc)); 4.350(t, ^3J (H,H) = 5.90 Hz, 1H, Hα(Ser)); 4.954(d, ^3J (H,H) = 9.81 Hz, 1H, H-1(GlcNAc)); 7.192(s, 1H); 8.513(s, 1H). TOF-MS: calcd. for [M + H], 1091.5; found, 1090.2; calcd. for [M + Na], 1113.5; found, 1112.1.

Synthesis of sialylated biantennary sugar chain linked peptide **13**

GlcNAc-linked peptide **11** (10 mg, 9.3 μmol) and SGP **15** (120 mg, 51 μmol) were dissolved in 0.06 M phosphate buffer (pH 6.5) containing 0.05 M EDTA and 0.01% triton X. Then 26.6 μl (13.3 mU) of endo-M solution was added, and the mixture was incubated for 1.5 h at 37°C. By purification through HPLC, 2 mg of glycopeptide **13** was obtained (7.4% yield). Selected ^{1}H-NMR data (500 MHz, D_2O)-δ:1.607 (t, ^3J (H,H) = 11.75 Hz, 1H, 1-H_{ax}(NeuAc)); 1.913(s, 9H, *N*-Ac(GlcNAc)); 1.947, 1.954(s, 3H × 2, *N*-Ac(NeuAc and GlcNAc)); 2.556(dd, ^3J (H,H) = 3.90 Hz, 12.14 Hz, 1H, H-3$_{eq}$(NeuAc)); 7.183(s, 1H), 8.486(s, 1H).

General procedures for the removal of Cbz-group

Cbz-linked glycopeptide (10 mg) was dissolved in 10 ml water and 1 mg of palladium hydroxide was added. The aqueous solution was stirred in a hydrogen gas atmosphere for 4 h. After filtration of the catalyst, the filtrate was freeze-dried to give Cbz-free glycopeptide quantitatively.

Measurement of stimulatory activity of DNA synthesis

BALB/3T3 fibroblasts (4×10^4 cells/500 μl) were grown in DMEM containing 10% heat-treated FBS for 24 h in 48-well plates. The medium was then changed to 0.3% charcoal-absorbed heat-treated FBS/DMEM and incubation was continued for another 48–56 h. After the addition of 5 μg/ml heparin and 10^{-3}–10^{-5} M test peptides, incubation was continued for another 16 h. To this medium, [^{3}H]-thymidine was added and incubation proceeded for another 4 h. At the end, cells were solubilized with 200 μl of 0.025% trypsin-0.02% of EDTA2Na-PBS and the radioactivity was measured by a liquid scintillation counter [13].

Measurement of proteolytic hydrolysis of the hexapeptide and sugar linked hexapeptides

Protease solution containing 0.02 U/ml of each protease was prepared using 0.1 M phosphate buffer (pH 7.5). Hexapeptide or glycopeptide was dissolved in the same buffer with a concentration of 1 μmol/ml. Then 50 μl of enzyme solution or 100 μl of rat serum was added to the 100 μl of substrate solution, and the mixture was incubated at 37°C. An aliquot of solution was withdrawn at 0, 1.0, 3.0, 6.0, 18 h, and HPLC

was carried out. The internal standard was not used, and the absolute peak area was obtained by the Hitachi 2500 Integrater by injecting exactly 20 µl of sample solution. The extent of hydrolysis was estimated from the area of the peak produced by the remaining (glyco)peptide. In Table 2, the peak area of the (glyco)peptide was normalized with that of the time zero of the reaction set to 100.

Results and discussion

As shown in Figure 1, 10^{-4} and 10^{-3} M of original hexapeptide **2** increased the DNA synthesis rate in BALB/3T3 fibroblasts to 155% and 295%, respectively, of that in the control medium. Similarly, 10^{-4} and 10^{-3} M of the Asn derivative **10** also increased the DNA synthesis rate to 130% and 249%, respectively, of control. In addition, around the sequence of the peptide **2**, we synthesized various peptides by a chemical method, of which Pro(105)-Asp-Gly-Lys-Val-Asn-Gly-Ser-His-Glu-Ala-Asn-Met-Leu-Ser (119)Gln-Val-Gis-Arg increased DNA synthesis in BALB/3T3 A31 (data not shown). Moreover, His(113)-Asp-Gly-Lys-Val-Asn-Gly-Ser-His-Glu-Ala-Asn-Met (117) showed megakaryopoietic activity *in vitro* and platelet increasing activity *in vivo* [12,13]. In the present study, we used peptide **2** as a model peptide to see the effect of sugar chains on the activity and functions.

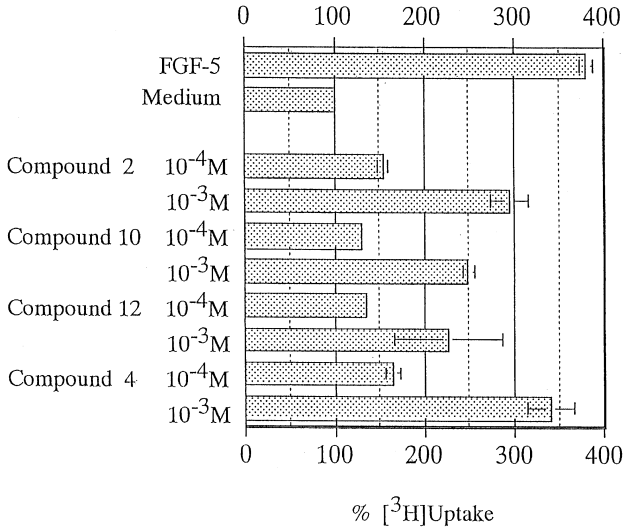


Figure 1. Measurement of stimulation of DNA synthesis by free and sugar-linked peptides. Compound **2**; Leu-Ser-Gln-Val-His-Arg, Compound **10**; Leu-Ser-Asn-Val-His-Arg, Compound **12**; Leu-Ser-[GlcNAc-]Asn-Val-His-Arg, Compound **4**; Leu-[GalNAc-]Ser-Gln-Val-His-Arg. Serum-starved BALB/3T3 A31 cells were stimulated with each peptide at the indicated concentration, or stimulated with 2×10^{-10} M of FGF-5. Each point represents mean +/− standard deviations determined from triplicate samples.

Synthesis of sugar chain linked FGF fragment peptides

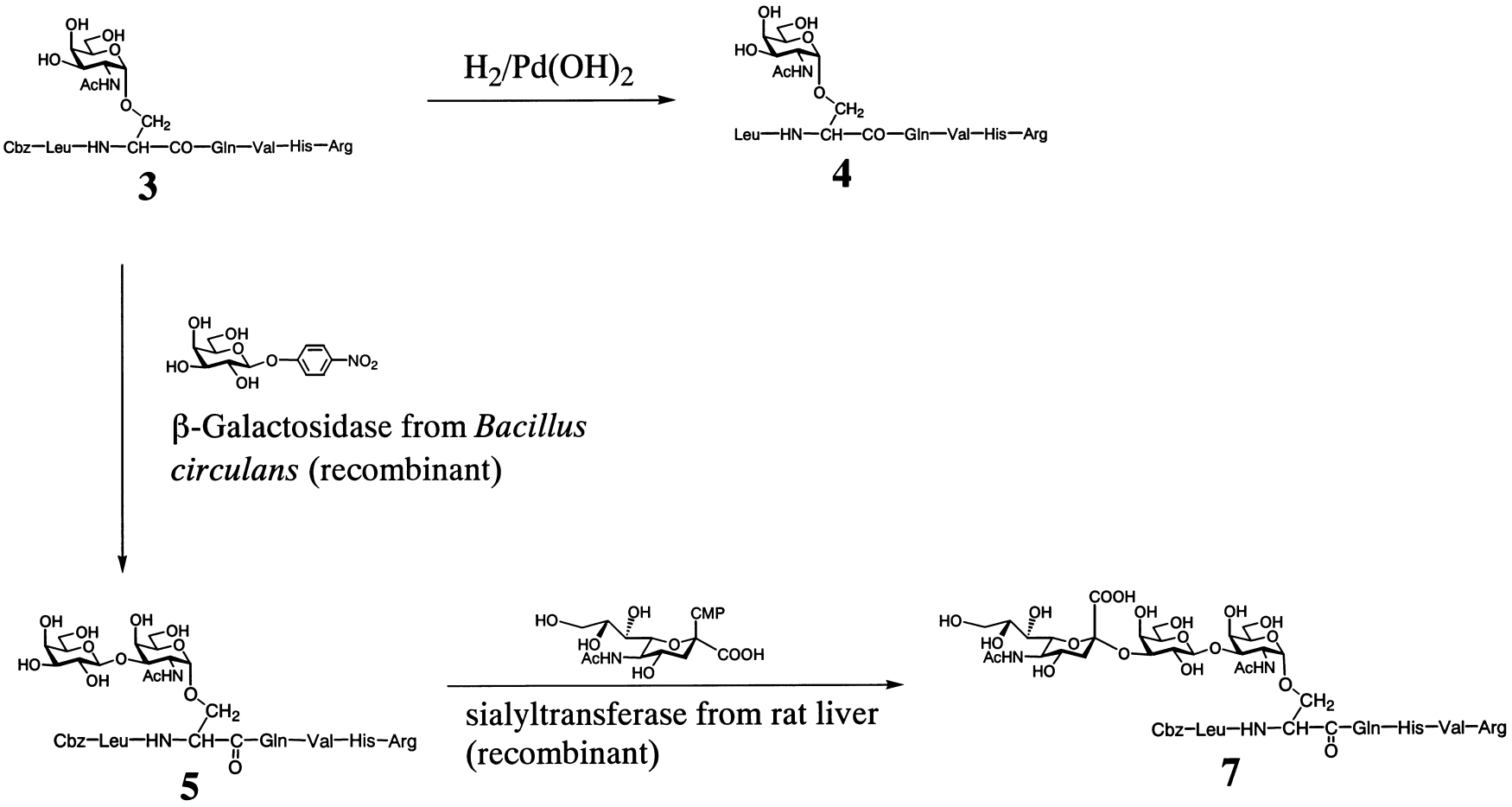
We have previously reported the chemoenzymatic synthesis of mucin-type glycopeptide [7]. Our strategy was to synthesize a GalNAc-linked peptide first by use of peptide synthesizer and subsequently link galactose and sialic acid enzymatically to form sialyl T-antigen linked peptide. In the present study, we applied this technology to extend sugar chains from the FGF fragment peptides.

To make the separation of the products by a reversed phase HPLC easier, we used Cbz-protected amino acid for the *N*-terminal of the peptide. The synthesis path is shown in Scheme 1. GalNAc-linked hexapeptide **3** was prepared by a peptide synthesizer using peracetylated GalNAc-linked *N*-Fmoc-Ser. Then, galactose was linked to the GalNAc residue of the GalNAc-linked hexapeptide **3** by a transglycosylation using Gal-β-pNP as a donor with the aid of β-galactosidase from *Bacillus circulans* (recombinant) [14,15], with a 20% yield. The Gal-β-(1 → 3)-GalNAc-linkage in glycopeptide **5** was confirmed by the lower field shift of C-3 signal of GalNAc residue in ^{13}C NMR spectrum. The relatively low yield of this reaction was due to the low solubility of the GalNAc-linked hexapeptide. Then, sialic acid was linked with α-(2 → 3)-linkage by use of a commercial sialyltransferase from rat liver to obtain NeuAc-α-(2 → 3)-Gal-β-(1 → 3)-GalNAc-linked peptide **7**.

Table 2. Degree of hydrolysis of peptide or glycopeptides by six kinds of proteases (A ~ F)

	Proteases					
	A	*B*	*C*	*D*	*E*	*F*
Cbz-LSQLVHR **1**	0	0	0	0	9	4
Cbz-L[GalNAc-]SQVHR **3**	100	3	93	63	15	30
Cbz-L[Galβ-(1 → 3)-GalNAc-]SQVHR **5**	100	26	100	96	30	31
Cbz-L[NeuAcα-(2 → 3)-Galβ-(1 → 3)-GalNAc-]SQVHR **7**	100	40	100	100	41	36
Cbz-LS[GlcNAc-]NVHR **11**	98	56	47	96	17	7
Cbz-LS[Complex type sugar chain-]NVHR **13**	100	100	51	96	26	33

(A): Leucine amino peptidase, (B): Elastase, (C): Proteinase K, (D): Protease type VIII, (E): Carboxy peptidase, (F) rat serum. Degree of hydrolysis was expressed by the percentages of the remaining peptide or glycopeptides after the hydrolysis in the presence of protease at 37°C for 1 h; 100 means that nothing was hydrolyzed, and zero means that the peptide was completely hydrolyzed.

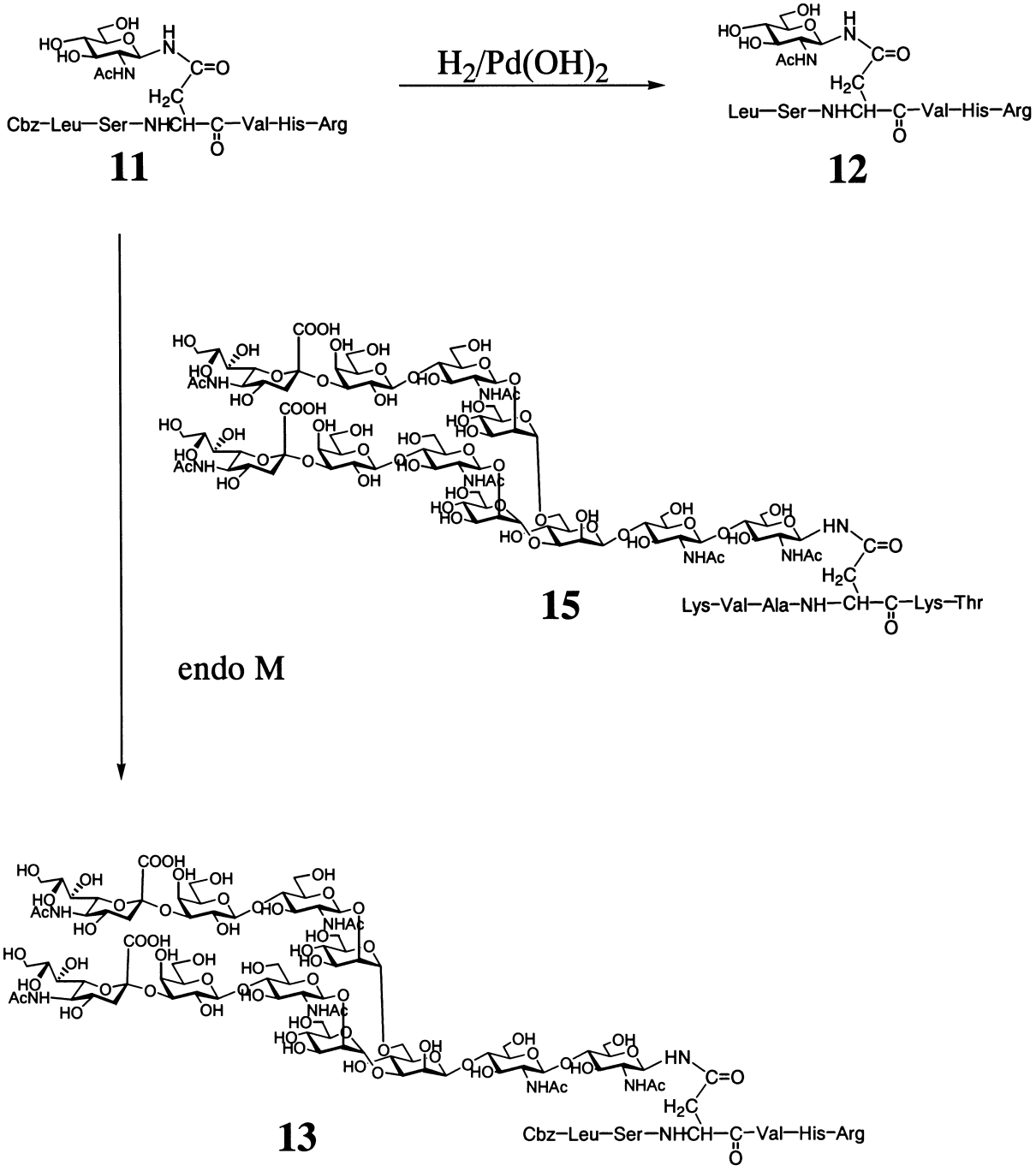


Scheme 1. Synthesis path for the linkage of *O*-linked sugar chain to the FGF fragment peptides.

For the synthesis of *N*-linked glycopeptide, we used sialylated biantennary sugar chain-linked hexapeptide **15** from egg yolk as a donor, and endo-*β*-*N*-acetylglucosaminidase from *Mucor hiemalis* (endo M) as an enzyme for the transglycosylation (Scheme 2)[16]. As GlcNAc-linked *N*-Fmoc-Gln was not commercially available, we synthesized GlcNAc-linked peptide **11** by changing Gln to Asn in the peptide **3**. In the transglycosylation reaction using endo M, the yield of the reaction is generally in the range of 40–60%. The yield of the present transglycosylation reaction was very low because of the low solubility of GlcNAc-linked hexapeptide **11**.

Changes of activity by glycosylation

In Figure 1 is shown the changes in rate of DNA synthesis in BALB/3T3 fibroblasts induced by the peptide linked with GalNAc (glycopeptide **4**) or GlcNAc (glycopeptide **12**). The



Scheme 2. Synthesis path for the linkage of complex type sugar chain to the FGF fragment peptides.

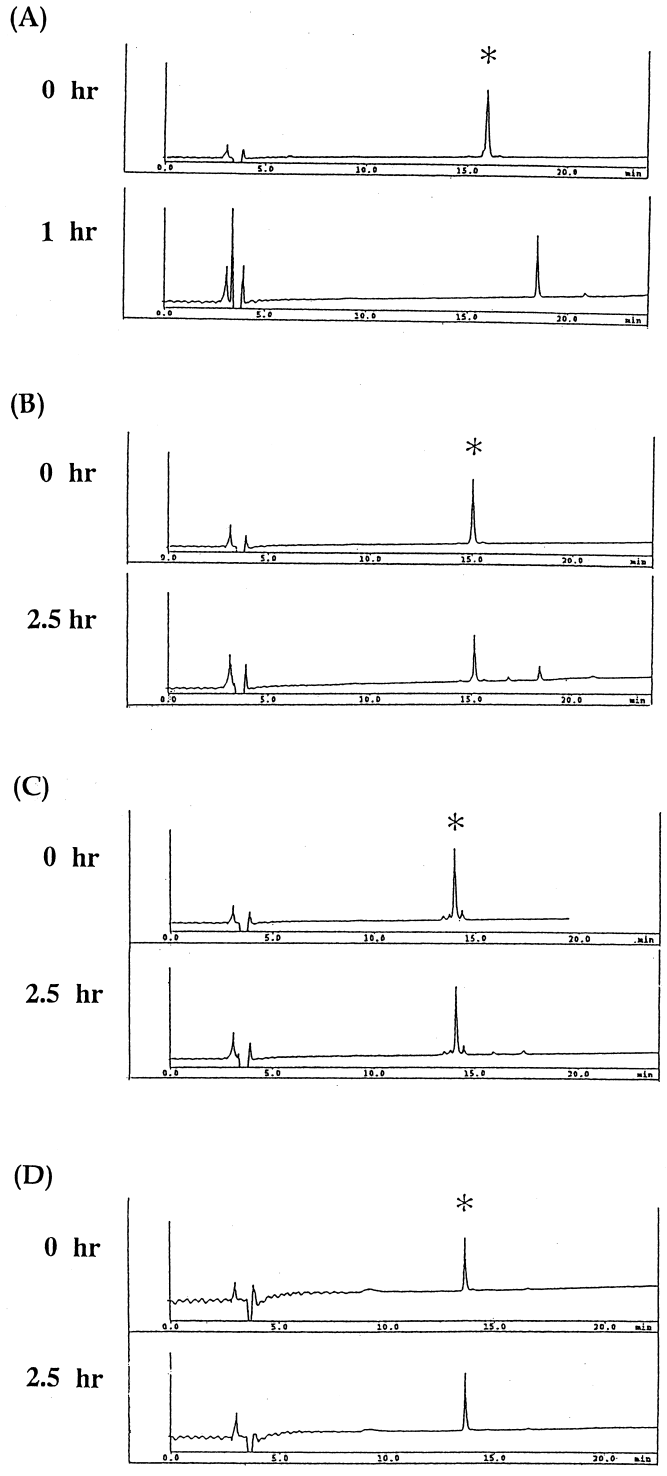


Figure 2. HPLC time charts showing the course of hydrolysis of original peptide and *O*-linked glycopeptides in the presence of proteinase K. (A)–(D) in the chart shows the results for Cbz-Leu-Ser-Gln-Val-His-Arg **1**, Cbz-Leu-[GalNAc-]Ser-Gln-Val-His-Arg **3**, Cbz-Leu[Galβ-(1 → 3)-GalNAc-]Ser-Gln-Val-His-Arg **5**, and Cbz-Leu[NeuAcα-(2 → 3)-Galβ-(1 → 3)-GalNAc-]Ser-Gln-Val-His-Arg **7**, respectively. The peak marked with * on each chart at 0 h shows the area of peak of intact peptide or glycopeptide.

DNA synthesis rate induced by GlcNAc-linked glycopeptide **12** was the same as the rate induced by non-glycosylated peptide **10**, but this rate was increased slightly more by GalNAc-linked glycopeptide **4** than by non-glycosylated peptide **2**. This difference can be explained by the difference of the glycosylation site in the peptide. Although the mechanism of the enhancement of the stimulating activity of DNA synthesis by this peptide is not clear at present, it is obvious that the glycosylation does not interfere with the binding of this peptide to the acceptor in the fibroblast cells.

Changes in the resistance to proteolytic hydrolysis

In the proteolytic hydrolysis measurements, we used five kinds of proteases of different origins or rat serum. The HPLC of the peptide solution in the presence of protease was carried out at appropriate time intervals and the degree of hydrolysis of a (glyco)peptide sample was estimated from the area of the peak in HPLC due to that (glyco)peptide. A time chart of HPLC for proteinase K is demonstrated in Figure 2. As can be seen in Figure 2-(A), the peak of free peptide **1**, marked with asterisk, disappeared within 1 h completely. In contrast, peaks due to glycosylated peptides, **3**, **5**, and **7**, marked with asterisk in Figure 2-(B), (C), and (D), respectively, remained after 2.5 hr. For the other proteases, the results are summarized in Table 2. We did not use an internal standard for the estimation of the remaining (glyco)peptide, because we could not find a material which elutes at a particular retention time without overlapping with any other peaks of the decomposed fragment peptides. However the present procedure is enough for the purpose of comparing the profiles of the proteolytic hydrolysis of differently glycosylated peptides.

Even with the linking of monosaccharide, **3** or **11**, the resistance toward the hydrolysis was enhanced for all the proteases tested. Especially in the case of proteinase K and protease type VIII, this tendency was prominent, because these enzymes cleave the peptide bond between Ser and Gln residue. Among *O*-linked glycopeptides, the rate of hydrolysis was slower as the length of sugar chain became longer. But the difference in degree of resistance was not remarkable between GalNAc-linked peptide **3**, Gal-β-(1 → 3)-GalNAc-linked peptide **5**, and NeuAc-α-(2 → 3)-Gal-β-(1 → 3)-GalNAc-linked peptide **7**. Among *N*-linked glycopeptides, GlcNAc-linked peptide **11** and sialylated biantennary sugar chain-linked peptide **13** also exhibited resistance, and the difference was small between these glycopeptides. The same tendency was observed in the hydrolysis using rat serum too. It may be said that the structure of sugar chain is not important in the resistance to proteolytic hydrolysis, but the position where the sugar chain is linked is important.

Conclusion

We linked sugar chains to FGF fragment peptide by the method we developed previously. In the present study, the

approach was confirmed to be applicable to any peptides. The stimulatory activity on DNA synthesis of the fragment peptide of FGF-5S was not decreased but rather increased by the linkage of GalNAc. The resistance toward the proteolytic hydrolysis was enhanced remarkably by the glycosylation. However the difference in resistance due to the linkage of monosaccharide and larger sugar chains was not as large as expected.

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