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Identification of the oligosaccharide structures of human coagulation factor X activation peptide at each glycosylation site

HIROAKI NAKAGAWA[1]*, NORIKO TAKAHASHI[1], KAZUHISA FUJIKAWA[1], YOSHIYA KAWAMURA[1], MASAKI IINO[2], HIROYUKI TAKEYA[2], HIROYUKI OGAWA[2] and KOJI SUZUKI[2]

[1]*GlycoLab, Nakano Vinegar Co. Ltd, Nakamura-cho 2-6, Handa-city, Aichi 475, Japan*

[2]*Department of Molecular Biology on Genetic Disease, Mie University School of Medicine, Edobashi 2-174, Tsu-city, Mie 514, Japan.*

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Human blood coagulation factor X has two N-linked oligosaccharides at Asn^{39} and Asn^{49} residues and two O-linked oligosaccharides at Thr^{17} and Thr^{29} residues in the region of the factor X activation peptide (XAP) which is cleaved off during its activation by factor IXa. We determined the structure of oligosaccharides in the XAP region of human factor X. Four glycopeptides each containing a glycosylation site were isolated by digestion of XAP with endoproteinase Asp-N followed by reversed-phase HPLC. N-linked oligosaccharides released from the glycopeptides by glycoamidase A digestion were derivatized with 2-aminopyridine. Pyridylamino(PA)-oligosaccharides were separated by HPLC into neutral and sialyl oligosaccharides using an anion-exchange column. Structures of oligosaccharides and their contents at each glycosylation site were determined by a two-dimensional sugar mapping method. The contents of the neutral oligosaccharides at Asn^{39} and Asn^{49} residues were 32.5% and 30.0%, respectively. Six neutral and twelve monosialyl oligosaccharides isolated from both N-linked glycosylation sites showed similar elution profiles composed of bi-, tri- and tetra-antennary complex type oligosaccharides. The predominant component in neutral oligosaccharides was biantennary without a fucose residue. Two major monosialyl oligosaccharides were also biantennary without fucose and with a Neu5Acα2→6 residue. In addition, the structures of O-linked oligosaccharides at Thr^{17} and Thr^{29} residues were suggested to be disialylated Galβ3GalNAc sequences by their component analyses.

Keywords: oligosaccharide structure, coagulation factor X activation peptide

Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; HPLC, high-performance liquid chromatography; NDV, Newcastle disease virus; Neu5Ac, 5-*N*-acetylneuraminic acid; ODS, octadecylsilyl; PA, pyridylamino; RVV-X, Russell's viper venom factor X activator; TBS, Tris-buffered saline; XAP, factor X activation peptide.

Introduction

Human blood coagulation factor X, a vitamin K-dependent plasma glycoprotein with molecular mass 59 000 Da, is composed of a light chain with molecular mass 17 000 Da and a heavy chain with molecular mass 42 000 Da. They are linked by a disulfide bond [1, 2]. In the presence of Ca^{2+}, factor X is proteolytically activated by factor IXa complexed with factor VIIIa, in the intrinsic coagulation pathway, and also by factor VIIa complexed with tissue factor, in the extrinsic coagulation pathway, on the surface of cellular phospholipid membrane [2]. Activated factor X (factor Xa) physiologically and specifically activates prothrombin into α-thrombin which acts as a key enzyme in thrombosis and haemostasis [3, 4], and also evokes various biological responses from a variety of cells including platelets [5]. Thus the activation of factor X is a critical event in the course of thrombin formation. In the activation of factor X, both factor IXa and factor VIIa cleaved a peptide bond between Arg^{52} and Ile^{53} residues of the heavy chain of factor X and the factor X activation peptide (XAP) is released from the resultant factor Xa [1]. Our previous study [6] suggested that the XAP region in human factor X directly interacts with factor IXa, and the factor Xa region other than XAP interacts with factor VIIIa, and also indicated that the sialic acids in the oligosaccharides in the XAP region contribute in the part of the interaction with factor IXa. The amount of oligosaccharides in the XAP region accounts for

*To whom correspondence should be addressed.

67.1% of the total amount of the oligosaccharides present in factor X [1]. All N-linked oligosaccharides in human factor X are present in the XAP region at Asn^{39} and Asn^{49} residues [7]. The XAP region has also O-linked oligosaccharides at Thr^{17} and Thr^{29} residues [8]. Recently Sinha and Wolf [9] showed that *Sambucus nigra* agglutinin, a lectin recognizing Siaα2,6Gal or Siaα2,6GalNAc, inhibited the factor X activation by factor IXa-factor VIIIa complex and by factor VIIa-tissue factor complex. They also showed that sialidase-digested factor X was only slightly activated. Their results in combination with our previous findings suggest that the oligosaccharide moieties in the XAP region are most crucial for factor X activation.

In the present study, we have determined the structures of N-linked oligosaccharides at each glycosylation site in the XAP region of factor X by the use of a two-dimensional sugar mapping technique with HPLC, which is a powerful method for structural analysis of minute quantities of N-linked oligosaccharides [10].

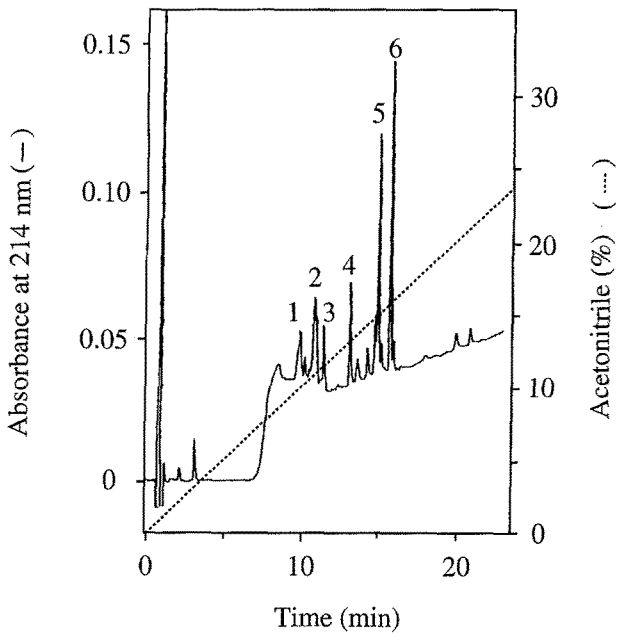

Figure 1. Reversed-phase HPLC of XAP fragments derived from XAP digested with endoproteinase Asp-N. Following the digestion of XAP, a part of the digest was applied to a column of COSMOSIL 5C18-AR (4.6 × 100 mm). Chromatographic conditions are described in the text. The peaks 1 to 6 correspond to the following sequence in XAP region : peak 1, Asp^{47}-Arg^{52}; peak 2, Asp^{37}-Gly^{46}; peak 3, Ser^{1}-Pro^{13}; peak 4, Asp^{27}-Phe^{33}; peak 5, Asp^{22}-Leu^{26}; peak 6, Asp^{14}-Tyr^{21}. These peaks cover all the regions of XAP excepted for Asp^{34}-Leu^{36}.

Experimental procedures

Materials

Glycoamidase A (Glycopeptidase A; EC3.5.1.52) from almond, β-galactosidase and β-*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo (Tokyo, Japan). α-L-Fucosidase from bovine kidney and endoproteinase Asp-N were from Boehringer Mannheim Biochemicals (Mannheim, Germany). Sialidase from *Arthrobacter ureafaciens* was obtained from Nacalai Tesque (Kyoto, Japan). Sialidase from Newcastle disease virus was a gift from Dr Akira Takatsuki (The Institute of Physical and Chemical Research, Wako, Japan). The PA-derivatives of isomaltooligosaccharides, (4–20 glucose oligomers) and those of N-linked oligosaccharides, corresponding to code numbers 200.4, 210.4, 300.8, 300.31 and 400.16 [10, 11], were purchased from Nakano Vinegar (Handa, Japan). PA-oligosaccharides, corresponding to code numbers 300.18 and 310.18, were prepared as described previously [12]. The structures of these standard PA-oligosaccharides are illustrated in Table 1.

DEAE-Sephacel, sulfated Sephadex and Sephadex G-15 were obtained from Pharmacia-LKB (Uppsala, Sweden). Sodium cyanoborohydride was from Aldrich Chemicals (Milwaukee, WI, USA). 2-Aminopyridine was from Wako Pure Chemicals (Osaka, Japan). Russell's viper venom factor X activator (RVV-X) was purified from crude snake venom *Vipera Russelli* obtained from Sigma (St Louis, MO, USA) by the method previously described [13].

Methods

Purification of factor X and factor X activation peptide (XAP) Factor X was purified from human plasma by DEAE-Sephacel and sulfated Sephadex ion exchange chromatography [14]. It was activated overnight at 37°C by RVV-X-coupled to Cellulofine (Seikagaku Kogyo) in Tris-buffered saline (TBS) consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 10 mM $CaCl_2$, and 10 mM benzamidine chloride, as described previously [6, 15].

XAP was isolated from human factor X as follows [6]. After activation of factor X by RVV-X-coupled Cellulofine, the solution containing activated factor X was dialysed overnight against 0.1 M potassium phosphate, pH 7.0, and was applied to a column of hydroxyapatite (1.5 × 15 cm) (Seikagaku Kogyo) equilibrated with the same buffer to separate XAP from factor Xa, XAP eluted in the passed-through fractions from the column was further purified by reversed-phase HPLC using a column of COSMOSIL 5C18-AR (4.6 × 100 mm) (Nacalai Tesque) at a flow rate of 1 ml min^{-1}. XAP was eluted from the column at room temperature by a linear gradient of acetonitrile in 13 mM (maximum concentration) of trifluoroacetic acid as a single peak. The isolated XAP was lyophilized and frozen at –30°C.

Preparation of glycopeptides from XAP Four glycopeptides from XAP, each containing a sugar chain linked to Asn^{39}, Asn^{49}, Thr^{17} or Thr^{29}, were prepared as follows. XAP (200 nmol) in 25 mM potassium phosphate, pH 8.0, containing 1 M urea was digested by endoproteinase Asp-N at an enzyme: substrate molar ratio of 1:200 at 37°C overnight. The digested fragments were separated by reversed-phase HPLC using a column of COSMOSIL 5C18-AR (4.6 × 100 mm) under the

Table 1. Elution positions on HPLC and the proposed structures of PA-oligosaccharides derived from Asn^{39} and Asn^{49} of coagulation factor X.

Peak name (Code No.)	Structure	*Elution position on* ODS (Glucose unit)	Amide (Glucose unit)	Relative quantity (%) Asn^{39}	Asn^{49}
Neutral oligosaccharides		observed (reported)[a]			
B (200.4)	Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	10.3 (10.2	6.8 7.0)	17.3	10.3
F (210.4)	Galβ4GlcNAcβ2Manα6 Fucα6 \ \ Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	13.9 (13.8	7.4 7.4)	3.6	4.5
E (300.8)	Galβ4GlcNAcβ2Manα6 \ Galβ4GlcNAcβ4 Manβ4GlcNAcβ4GlcNAc-PA \ / Manα3 / Galβ4GlcNAcβ2	12.8 (12.7	8.2 8.3)	3.5	7.1
A (300.18)	Galβ4GlcNAcβ6 \ Manα6 / \ Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	8.2 (8.1	8.4 8.6)	4.2	2.6
D (310.18)	Galβ4GlcNAcβ6 \ Manα6 Fucα6 / \ \ Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	(11.2	11.3 9.0)	8.8 2.2	1.9
C (400.16)	Galβ4GlcNAcβ6 \ Manα6 / \ Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc-PA Galβ4GlcNAcβ4 / \ / Manα3 / Galβ4GlcNAcβ2	10.6 (10.6	9.6 9.9)	1.8	3.5

Peak name	Structure	*Elution position on* ODS (Glucose unit) observed (reported)	*Elution position on* Amide (Glucose unit) observed (reported)	Relative quantity (%) Asn[39]	Relative quantity (%) Asn[49]
Monosialyl oligosaccharides					
I	Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc-PA / Neu5Acα2,6Galβ4GlcNAcβ2Manα3	9.9	7.0	16.5	12.8
L	Neu5Acα2,6Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	12.0	7.0	17.4	13.4
N, P Neu5Acl	Galβ4GlcNAcβ2Manα6 Fucα6 \ \ Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	13.9 15.4	7.3 (N) 7.2 (P)	3.1	7.5
K, O, Q Neu5Acl	Galβ4GlcNAcβ2Manα6 \ Galβ4GlcNAcβ4 Manβ4GlcNAcβ4GlcNAc-PA \ / Manα3 / Galβ4GlcNAcβ2	11.7 14.8 15.7	– (K) – (O) – (Q)	9.6	15.4
G, H Neu5Acl	Galβ4GlcNAcβ6 \ Manα6 / \ Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	8.2 9.5	9.1 (G) – (H)	9.5	6.9
M a Neu5Acl	Galβ4GlcNAcβ6 \ Manα6 Fucα6 / \ \ Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc-PA / Galβ4GlcNAcβ2Manα3	12.7	–	2.9	5.6
J, Mb Neu5Acl	Galβ4GlcNAcβ6 \ Manα6 / \ Galβ4GlcNAcβ2 \ Manβ4GlcNAcβ4GlcNAc-PA Galβ4GlcNAcβ4 / \ / Manα3 / Galβ4GlcNAcβ2	10.1 12.7	– (J) – (Mb)	8.5	8.3

[a]. reference [11]

same conditions used for purification of XAP is shown in Fig. 1. The peptides containing the respective glycosylation sites were identified by amino acid sequence analysis using a protein sequencer (PSQ-1, Shimadzu, Kyoto, Japan).

Preparation and derivatization of N-linked oligosaccharide moiety Oligosaccharides were released from 50 nmol of each glycopeptide with 0.2 mU glycoamidase A in 30 μl of 0.1 M citrate-phosphate buffer, pH 4.5, at 37°C for 16 h [16]. The reducing ends of the released N-linked oligosaccharides were reductively aminated with 2-aminopyridine by the use of sodium cyanoborohydride. The PA-derivatives of oligosaccharides were purified by gel filtration on a Sephadex G-15 column eluted with 10 mM ammonium bicarbonate.

Isolation of PA-oligosaccharides by three successive HPLC steps PA-oligosaccharides were separated by HPLC with an anion exchange column, TSKgel DEAE-5PW (7.5 × 75 mm) (TOSOH, Tokyo, Japan) according to the acidity of their sialic acid residues. Elution was performed using solvent A (10% acetonitrile in water adjusted to pH 9.5 with triethylamine) and solvent B (a mixture of 90% of 0.53 M acetic acid-triethylamine buffer, pH 7.3, and 10% acetonitrile) at a flow rate of 1.0 ml min^{-1} at 35°C. The column was equilibrated with solvent A. After injection of a sample containing 1–10 pmol of PA-oligosaccharides for the purpose of structural analysis, the column was eluted with solvent A for 5 min. For another 40 min the ratio of solvent B was increased to 20% on a linear gradient.

Neutral PA-oligosaccharides were separated and identified by two different modes of HPLC, using the two-dimensional sugar mapping technique [10]. First, PA-oligosaccharides were separated on a reversed-phase column (Nakanopak ODS-A, 6 × 150 mm) (Nakano Vinegar). Elution was performed at a flow rate of 1.0 ml min^{-1} at 55°C using solvent C (10 mM phosphate buffer, pH 3.8) and solvent D (0.5% 1-butanol in solvent C). The column was equilibrated with a buffer composed of 80% solvent C and 20% solvent D. After injection of a sample containing 1–10 pmol PA-oligosaccharides, the ratio of solvent D in the buffer was increased with a linear gradient to 50% in 60 min. Each oligosaccharide fraction separated on the ODS column was collected separately and then applied to a size fractionation column (Nakanopak Amide-A, 4.6 × 250mm, Nakano Vinegar). Elution was performed using solvent E (a mixture of 35% of 0.53 M acetic acid-triethylamine buffer, pH 7.3, and 65% acetonitrile) and solvent F (a mixture of 50% of 0.53 M acetic acid-triethylamine buffer pH 7.3, and 50% acetonitrile) at a flow rate of 1.0 ml min^{-1} at 40°C. The column was equilibrated with solvent E. After injection of the sample, the concentration of solvent F was increased to 100% with a linear gradient in 50 min.

In the above three modes of HPLC using the Shimadzu LC-10A HPLC system, PA-oligosaccharides were detected by fluorescence using excitation and emission at 320 and 400 nm, respectively. The elution positions of the PA-oligosaccharides on the HPLC columns were expressed as glucose units defined as the elution times relative to reference PA-glucose oligomers. The glucose units of each sample oligosaccharide on the ODS and amide columns were plotted on the two-dimensional sugar map and compared with the coordinates for the standard N-linked oligosaccharides which include 220 different compounds [11, 19].

Exoglycosidase digestion procedure Each PA-oligosaccharide (50 pmol) isolated on the ODS and/or the amide columns was digested by exoglycosidases (β-galactosidase, β-*N*-acetylhexosaminidase and α-L-fucosidase) under the conditions described previously [11]. Sialidase from *Arthrobactor ureafaciens* (20 mU) or that from Newcastle disease virus (sialyl α2 → 3 specific [21], 800 μU) was incubated with 50 pmol of substrate in 15 μl of 0.1 M citrate-phosphate buffer, pH 5.0 at 37°C for 16 h. The necessary amounts of both sialidases for hydrolysis of Neu5Acα2 → 3 and Neu5Acα2 → 6 linkages were freshly determined in each case using Neu5Acα2 → 3 lactose and Neu5Acα2 → 6 lactose as substrates. Sialic acids in PA-oligosaccharides were also released by a weak acid hydrolysis at pH 2.0 at 90°C for 60 min.

Desialylation of oligosaccharides during analytical procedures We estimated that no desialylation was caused under the conditions used for fractionation of glycopeptides (incubation of 13 mM trifluoroacetic acid at room temperature for 60 min, then freeze dried), by determining sialic acid liberated from disialyl biantennary PA-oligosaccharide and human IgG as model compounds. By the use of disialyl biantennary oligosaccharide of human fibrinogen as a model compound, we confirmed that the procedure of derivatization with 2-aminopyridine did not affect the content of sialic acid of sialyl oligosaccharides. Furthermore, we showed that desialylation did not occur during β-galactosidase or β-*N*-acetylhexosaminidase digestion of PA-oligosaccharides unless a large excess of enzyme was added to the reaction mixture. We took great care to keep sialic acids intact. It is possible, however, that during the entire process of the experiments including dilution, concentration or storage of the sample, some desialylation might have occured.

Monosaccharide analysis The carbohydrate composition of O-linked oligosaccharides was determined after hydrolysis of 2 nmol of each glycopeptide obtained from XAP with 2.5 M trifluoroacetic acid at 100°C for 7 h. The reaction mixture was analysed by high-performance anion exchange chromatography (Dionex BioLC system) on a column of CarboPac PA1 (Dionex, Sunnyvale, CA, USA) and carbohydrate was detected by a pulsed amperometric detector as described previously [22, 23].

Results

Separation of N-linked neutral and monosialyl oligosaccharides N-linked oligosaccharides at Asn^{39} or Asn^{49} were

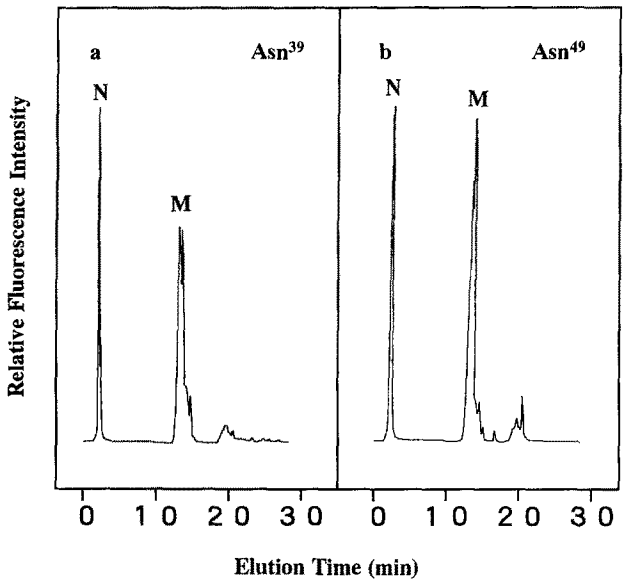

Figure 2. Separation of N-linked neutral and monosialyl oligosaccharides on a DEAE column. a) oligosaccharides linked to Asn^{39}; b) oligosaccharides linked to Asn^{49}; N, neutral oligosaccharides; M, monosialyl oligosaccharides.

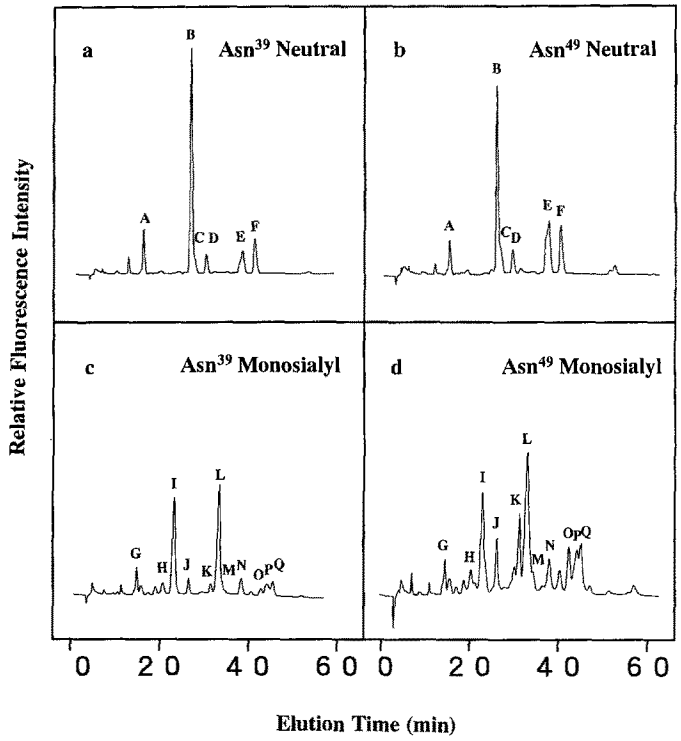

Figure 3. Comparison of HPLC profiles on an ODS column for PA-oligosaccharides. a) Neutral oligosaccharides linked to Asn^{39}; b) neutral oligosaccharides linked to Asn^{49}; c) monosialyl oligosaccharides linked to Asn^{39}; d) monosialyl oligosaccharides linked to Asn^{49}.

released from 50 nmol of the respective glycopeptide. The PA-oligosaccharides were separated by the first HPLC using a DEAE column into two fractions, N (neutral oligosaccharides) and M (monosialyl oligosaccharides) (Fig. 2-a and -b) Hase [24] reported that several different neutral PA-oligosaccharides mainly have the same molar fluorescence intensity in spite of their different structures. By the use of disialyl biantennary oligosaccharide and its desialylated form as model compounds, we determined that both of these oligosaccharides have the same fluorescence intensity. This result indicated that attachment of sialic acid residues does not influence fluorescence intensity. Therefore, peak response proportion corresponds to the molar ratio. The molar ratio of fractions N:M was 32.5:67.5 for oligosaccharides from Asn^{39} residue, and 30.0:70.0 for oligosaccharides from Asn^{49} residue.

Identification of neutral oligosaccharides Neutral PA-oligosaccharide fractions released from both Asn^{39} and Asn^{49} were separated into six fractions (termed A–F) on the ODS column chromatogram (Fig. 3-a and -b). Oligosaccharides A–F were further examined for homogeneity by the use of amide column. The elution positions of the oligosaccharides on the amide column reflect primarily the molecular sizes of the oligosaccharides. It was found that oligosaccharide fraction B+C was separated into distinct peaks B and C on the amide column (data not shown). Other peaks were all homogeneous not only on the ODS column chromatogram but also on the amide column. The types and the molar ratios of the oligosaccharides were essentially the same for the oligosaccharides from the Asn^{39} residue (Fig. 3-a) and those from the Asn^{49} residue (Fig. 3-b).

The process to identify each sample PA-oligosaccharide by the two-dimensional mapping technique is as follows:

a) First, the coordinates of the elution positions of a sample PA-oligosaccharide (expressed as glucose units on an ODS and an amide column) are determined experimentally.

b) Then the coordinates of the sample are compared with those of known standard PA-oligosaccharides on the two-dimensional map as reported previously. By computer searching, two candidate PA-oligosaccharides are chosen with coordinates which coincided with those of the sample PA-oligosaccharide within allowable error (±5%).

c) The sample PA-oligosaccharide and one of the candidate standard PA-oligosaccharides (the structure of which was previously established) are coinjected into two different HPLC columns to determine whether they give a single peak.

d) The sample PA-oligosaccharide and the candidate standard PA-oligosaccharides are digested with several glycosidases, always at the same time and in the same manner. After a glycosidase digestion, the elution position of the sample PA-oligosaccharide is again compared with that of the candidate standard PA-oligosaccharide.

e) The comparison is continued until both PA-oligosaccharides yield the common trimannosyl core Manα6(Manα3) Manβ4GlcNAcβ4GlcNAc.

The elution positions of oligosaccharides A, B, C, D, E, and F respectively coincided with the reported values for the compounds 300.18, 200.4, 400.16, 310.18, 300.8 and 210.4 within allowable error (Table 1). For example, oligosaccharide F was identified as follows (Fig. 4). Oligosaccharide F was eluted on the ODS column at glucose unit 13.9 and on the amide column at glucose unit 7.4. This coordinate 13.9 and 7.4 (13.9 on the X axis and 7.4 on the Y axis) coincided within allowable error with that of standard oligosaccharide code number 210.4 Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4(Fucα6)GlcNAc(14.1, 7.4 [11]). After α-fucosidase treatment, the elution position of oligosaccharide F moved to position 10.1 and 6.7 on the two-dimensional sugar map. This coordinate was identical to that of the isolated oligosaccharide B and standard oligosaccharide code number 200.4, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (10.2, 7.0 [11]). Furthermore, α-fucosidase digested oligosaccharide F, untreated oligosaccharide B and standard oligosaccharide 200.4 were then treated with β-galactosidase. After the enzyme digestion, the coordinate of each oligo-saccharide was reduced by 2 glucose units on the amide column (corresponding to the loss of two galactose residues) yielding the elution position 8.8 and 4.8. This coordinate was identical to that of standard oligosaccharide code number 200.1, GlcNAcβ2Manα6(GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (8.9, 5.1 [11]). After subsequent β-*N*-acetylhexosaminidase treatment of the resultant oligosaccharide, the product showed glucose units of 7.3 and 4.1. The data coincided with that of oligosaccharide 000.1 (trimannosyl core) 7.4, 4.3 [11]) on the two-dimensional sugar map. In the same manner, the structures of neutral oligosaccharides A through E were identified as shown in Table 1.

Structures of monosialyl oligosaccharides Monosialyl oligosaccharide fractions derived from Asn^{39} or Asn^{49} were separated into 11 fractions (termed G to Q) on the ODS column chromatogram (Fig. 3-c and -d). Oligosaccharide fraction L+M was separated into three different peaks, L, Ma and Mb, respectively, on the amide column (data not shown). The structure of oligosaccharide I was determined as follows. After treatment with a weak acid (pH 2, 90°C, 60 min) or by *Arthrobacter* sialidase digestion, oligosaccharide I changed to a neutral oligosaccharide. However, the sialic acid residue was not released from the oligosaccharide I by treatment with Newcastle disease virus sialidase (data not shown). Desialylated I was eluted on the ODS column at glucose unit 10.4 and on the amide column at glucose unit 6.8 (Fig. 5). This coordinate (10.4 on the X axis and 6.8 on the Y axis) coincided within the allowable error with that of the standard oligosaccharide code number 200.4, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (10.2, 7.0 [11]). Furthermore, by direct β-galactosidase digestion of oligosaccharide I, the elution position of the oligosaccharide moved to position 9.3 and 6.2 on the two-dimensional sugar

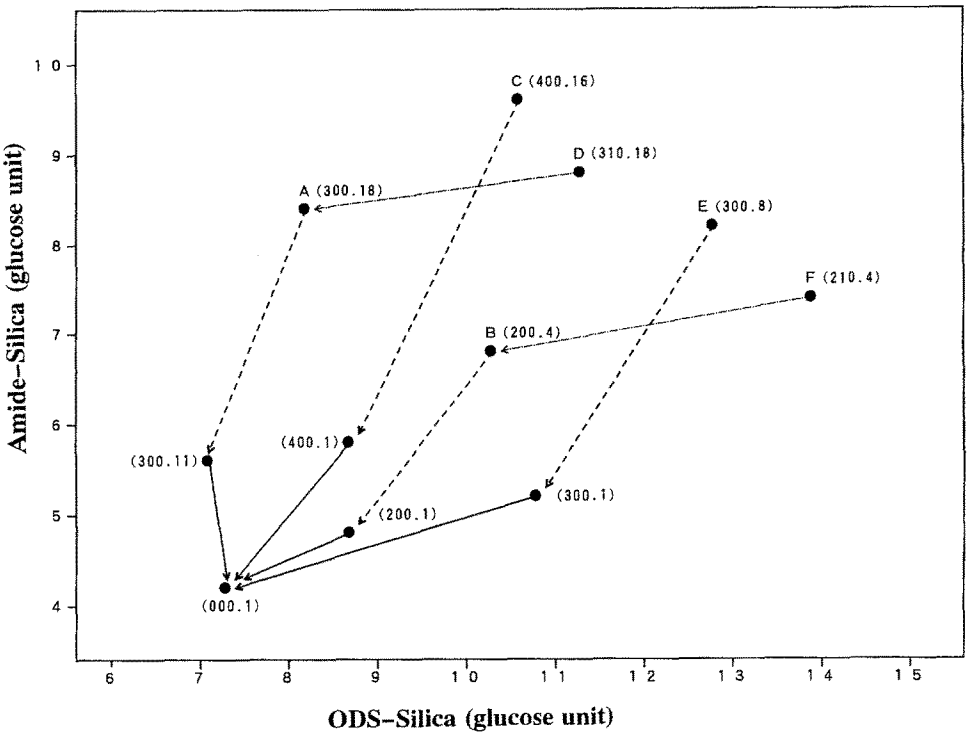

Figure 4. Characterization of structures of oligosaccharides A–F obtained from a neutral oligosaccharide fraction of XAP using the two-dimensional mapping technique. Arrows indicate the direction of changes in the coordinates of oligosaccharides after digestion with exoglycosidases: -·-·-·-·-, α-fucosidase; ————, β-galactosidase; ————, β-N-acetylhexosaminidase.

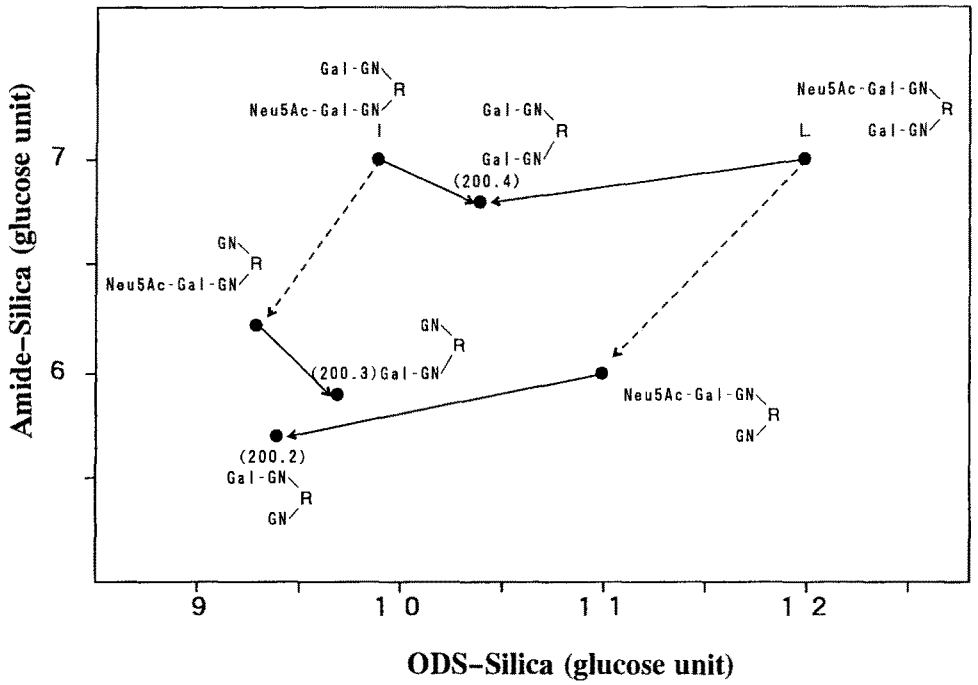

Figure 5. Characterization of structures of monosialyl oligosaccharides I and L derived from XAP using the two-dimensional mapping technique. Arrows indicate the direction of changes in the coordinates of oligosaccharides after digestion with exoglycosidases: ————, sialidase from *Arthrobactor ureafaciens*; – – – – – – –, β-galactosidase; R, trimannosyl core.

map; then after *Arthrobactor* sialidase digestion, the resultant oligosaccharide moved to elution position 9.7 and 5.9. This coordinate was identical to that of the standard oligosaccharide code number 200.3 GlcNAcβ2Manα6 (Galβ4GlcNAc β2Manα3)Manβ4GlcNAcβ4GlcNAc(9.6, 6.1 [11]). From these results, the structure of oligosaccharide I was determined as Galβ4GlcNAcβ2Manα6(Neu5Acα6Galβ4GlcNAcβ2Man α3)anβ4GlcNAcβ4GlcNAc. In an analogous way, the structure of oligosaccharide L was determined as Neu5Acα6 Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4Glc NAcβ4GlcNAc (Fig. 5). A minor fraction of oligosaccharide J was desialylated and eluted on the ODS column at glucose unit 10.2 and on the amide column at glucose unit 9.6. This coordinate coincided within allowable error with that of the standard oligosaccharide code number 400.16, Galβ4Glc NAcβ6(Galβ4GlcNAcβ2)Manα6{Galβ4GlcNAcβ4(Galβ4Gl cNAcβ2)Manα3}Manβ4GlcNAcβ4GlcNAc(10.6, 9.9 [11]). The structure of J was thus determined as monosialylated 400.16. In the same manner, the structures of all other monosialylated oligosaccharides were determined as shown in Table 1. It was found that monosialyl oligosaccharide fractions N and P; K, O and Q; G and H; J and Mb were separated into distinct peaks on the ODS column, although each fraction contained the same neutral oligosaccharide portion along with one sialic acid residue. They were different due to the differences in linkage position of the sialic acid. N-linked oligosaccharides obtained from XAP were all complex type and were fully galactosylated, but not fully sialylated. In spite of the presence of bi-, tri- and tetraantennary oligosaccharides, only monosialyl oligosaccharides were found.

Analysis of O-linked oligosaccharides The monosaccharide component analysis of the separate glycopeptides containing Thr^{17} or Thr^{29} showed that the molar ratio of Neu5Ac : Gal : GalNAc for Thr^{17} was 1.8 : 1.0 : 0.7, while the ratio for Thr^{29} was 2.0 : 1.0 : 0.7, respectively. These findings suggest that the oligosaccharide structures linked to both Thr^{17} and Thr^{29} are $(Neu5Ac)_2(Gal)_1(GalNAc)_1$.

Discussion

In the present study, we determined the structures of N-linked oligosaccharide moieties at Asn^{39} and Asn^{49} in the XAP region. As illustrated in Table 1, at least six neutral and twelve monosialyl oligosaccharides were identified at both N-linked glycosylation sites, and the distribution of the respective oligosaccharides was similar. It is of interest that although these oligosaccharides are bi-, tri- and tetraantennary, they are mostly monosialylated. The sialic acid linkage in the N-linked oligosaccharides of XAP was found to be Neu5Acα6Gal by use of sialylα2→3 specific sialidase digestion. This finding is in agreement with that of the report by Sinha and Wolf [9] in which factor X bound specifically to *Sambucus nigra* agglutinin that was specific for the sialylα2→6 linkage. On the other hand, bovine factor X is known to have a single N-linked oligosaccharide at the Asn^{36} residue of the heavy chain, which may cor-

respond to the Asn^{39} residue of the human factor X. Two types of bovine factor X oligosaccharides which are completely different from those in the human factor X molecule have been determined to be linked to the Asn^{36} residue [25]. They have three or four sialic acid residues in the biantennary oligosaccharides, and the sialic acids are bonded to galactose or *N*-acetylglucosamine residues through $\alpha 2 \rightarrow 3$ or $\alpha 2 \rightarrow 6$ linkages.

In addition, we suggest the structure of the two O-linked oligosaccharides linked to Thr^{17} and Thr^{29} in the XAP region to be $(Neu5Ac)_2(Gal)_1(GalNAc)_1$. Bovine factor X [25] has been determined to have at least two O-linked oligosaccharides at the Thr^{26} and Thr^{300} residues of the heavy chain. The former may correspond to the Thr^{29} residue of the human molecule. The structure of O-linked oligosaccharides of bovine factor X has been identified to be NeuAcα3Galβ3(NeuAcα6) GalNAc [25], which appears also to be present in human plasma glycoproteins involving factor X.

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