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Structural Analysis of *N*-Linked Sugar Chains of Human Blood Clotting Factor IX[1]

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The structures of *N*-glycans of human blood clotting factor IX were studied. *N*-Glycans liberated by hydrazinolysis were *N*-acetylated and the reducing-end sugar residues were tagged with 2-aminopyridine. The pyridylamino (PA-) sugar chains thus obtained were purified by HPLC. Each PA-sugar chain was analyzed by two-dimensional sugar mapping combined with glycosidase digestion. The major structures of the *N*-linked sugar chains of human factor IX were found to be sialotetraantennary and sialotriantennary chains with or without fucose residues. These highly sialylated sugar chains are located on the activation peptide of the protein.

Key words: human factor IX, *N*-linked sugar chains, pyridylamination, two-dimensional sugar mapping.

Human blood clotting factor IX (Factor IX, Christmas factor) is a vitamin K–dependent plasma glycoprotein that plays an essential role in blood coagulation (*1*). A deficiency of Factor IX results in hemophilia B. This disease is currently treated with Factor IX derived mainly from human plasma. Recently, Schnieke *et al.* reported the use of a transgene designed to express Factor IX in sheep milk (*2*). Recombinant Factor IX produced in milk would be of lower cost and free of the potential infectious risk associated with products derived from human blood. However, the clinical use of recombinant Factor IX necessitates detailed knowledge of the sugar chain structures of the native Factor IX because they can influence biological properties of the glycoprotein such as its pharmacokinetics, stability, and antigenicity.

Factor IX undergoes several post-translational modifications, including γ-carboxylation of 12 glutamic acid residues near the N-terminal, β-hydroxylation of Asp-64, and incorporation of both *N*-linked and *O*-linked oligosaccharides. Four sites have been identified in Factor IX where *O*-linked oligosaccharides can be attached, including Ser-53 (*3*) and Ser-61 (*4*, *5*) within the first epidermal growth factor (EGF)–like domain. There are also two sites for the attachment of *N*-linked oligosaccharides in the activation peptide at Asn-157 and Asn-167 (*6*). The detailed structures of these *N*-linked oligosaccharides have not yet been reported except for those of bovine blood clotting factor IX (*7*). In the present study, the structures of *N*-linked sugar chains of Factor IX were analyzed.

MATERIALS AND METHODS

Materials—Factor IX purified from human plasma by immunoaffinity chromatography (*5*) was provided by the Chemo-Serotherapeutic Research Institute (Kumamoto). The purified preparation gave a single band on SDS-PAGE (data not shown). PA-isomaltooligosaccharides were purchased from Takara Biomedical (Kyoto). A Shodex Asahipak NH2P-50 column (4.6 × 50 mm) was obtained from Showa Denko (Tokyo), Cosmosil 5C18-P columns (1.5 × 250 and 10 × 250 mm) from Nacalai Tesque (Kyoto), a Mono Q HR 5/5 (5.0 × 50 mm) from Pharmacia (Uppsala, Sweden), Dowex 50W-X2 (200–400 mesh) from Dow Chemicals (Richmond, VA), and a TSK-gel Sugar AXI column (4.6 × 150 mm) and TSK-gel HW-40F from Tosoh (Tokyo). *Arthrobactor ureafaciens* sialidase was purchased from Nacalai Tesque, Newcastle disease virus (NDV) sialidase and *Streptococcus pneumoniae* β-galactosidase from Boehringer Mannheim (Mannheim, Germany), bovine epididymis α-fucosidase and jack bean β-*N*-acetylhexosaminidase from Sigma (St. Louis, MO), *Aspergillus* sp. β-galactosidase from Toyobo (Tokyo), *Xanthomonas manihotis* β-galactosidase from New England BioLabs (Beverly, MA), and *Escherichia freundii* endo-β-galactosidase from Seikagaku (Tokyo). PA-Fuc, PA-GalNAc, PA-Glc, PA-GlcNAc, and PA-Gal were prepared as described previously (*8*). The PA-sugar chains listed in Table I were prepared as reported (*9*). A mixture of PA-sialo sugar chains was prepared as reported previously (*10*) from α_1-acid glycoprotein obtained from Sigma.

Preparation of PA-Sugar Chains from Factor IX—Sugar

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Abbreviations: NDV, Newcastle disease virus; PA-, pyridylamino; EGF, epidermal growth factor; Factor IX, human blood clotting factor IX. The structures and abbreviations of the sugar chains used are listed in Table I.

TABLE I. **Structures and designations of pyridylaminated sugar chains used in the study.**

Structure	Sugar Chain	HPLC: Reversed-phase[a]	HPLC: Size-fractionation[b]
Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	1	43.0	4.5
Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	2	40.5	5.2
GlcNAcβ1-6Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	3	44.7	5.2
Manα1-6 GlcNAcβ1-4Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	4	47.8	5.2
GlcNAcβ1-2Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	5	53.4	5.2
Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	6	44.6	6.1
Galβ1-4GlcNAcβ1-6Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	7	45.7	6.1
Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	8	49.1	6.1
Galβ1-4GlcNAcβ1-2Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	9	56.0	6.1
GlcNAcβ1-2Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	10	49.4	6.3
GlcNAcβ1-6 GlcNAcβ1-2Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	11	40.0	6.8
GlcNAcβ1-6 GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	12	46.9	6.8
GlcNAcβ1-6Manα1-6 GlcNAcβ1-4Manα1-3 GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	13	52.2	6.8
GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manα1-3 GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	14	58.5	6.8
GlcNAcβ1-6 GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manα1-3 GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	15	50.1	7.5
Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	16	54.5	7.8
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	17	44.8	9.2
Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	18	65.0	9.2
Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Fucα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	19	64.0	9.9
Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	20	73.5	9.7
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	21	51.9	9.9
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	22	54.0	9.9
Galβ1-4GlcNAcβ1-6Manα1-6 GlcNAcβ1-2 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	23	54.3	9.9
GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	24	57.2	9.9
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	25	55.0	10.6
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Fucα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	26	53.2	11.4
Galβ1-6Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4Manα1-3 Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA	27	63.4	11.1

[a]The reversed-phase scale is taken from Ref. 9. [b]Glucose units.

chains were released from 20 nmol of Factor IX by hydrazinolysis (40°C for 350 h) followed by *N*-acetylation (*11*). The sugar chains were pyridylaminated as described previously (*11*). After excess reagents had been removed by evaporation (*11*), the residue was dissolved in a small amount of water, and the resulting solution was placed on an HW-40F column (1.5 × 67 cm) equilibrated with 10 mM ammonium acetate buffer, pH 6.0. PA-sugar chains were eluted with the same buffer.

Glycosidase Digestion of PA-Sugar Chains—PA-sugar chains (20 pmol) were digested with 10 milliunits of α-L-fucosidase in 50 μl of 40 mM citrate–phosphate buffer, pH 6.0; with 1 milliunit of *Streptococcus* β-galactosidase in 10 μl of 50 mM sodium acetate buffer, pH 6.0; with 6 units of *Xanthomonas* β-galactosidase in 10 μl of 50 mM sodium acetate buffer, pH 4.5; with 13 units of *Aspergillus* β-galactosidase in 10 μl of 50 mM ammonium acetate buffer, pH 5.0; with 10 milliunits of endo-β-galactosidase in 10 μl of 50 mM ammonium acetate buffer, pH 5.0; with 25 milliunits of β-*N*-acetylhexosaminidase in 10 μl of 50 mM citrate–phosphate buffer, pH 5.0; with 2 milliunits of NDV sialidase in 100 μl of 50 mM ammonium acetate buffer, pH 6.5; or with 2.5 milliunits of *Arthrobacter* sialidase in 50 μl of 100 mM ammonium acetate buffer, pH 5.0. The digestions were carried out at 37°C for 16 h except for the NDV sialidase digestion, which was performed at 37°C for 2 h. Each enzymatic reaction was terminated by heating the solution at 100°C for 3 min.

High-Performance Liquid Chromatography (HPLC)—Size-fractionation HPLC was carried out on a Shodex Asahipak NH2P-50 column at 25°C at the flow rate of 0.6 ml/min. Two eluents, A and B, were used. Eluent A was acetonitrile:water:acetic acid (200:800:3, v/v/v) titrated to pH 7.0 with aqueous ammonia, and Eluent B was acetonitrile:water:acetic acid (930:70:3, v/v/v) titrated to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent B:Eluent A (32:1, v/v). After injecting a sample, linear gradient elution was performed to Eluent B:Eluent A (2:1, v/v) in 1 min and then to Eluent B:Eluent A (3:7, v/v) in 34 min. PA-sugar chains were detected by measuring the fluorescence during the elution (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Reversed-phase HPLC was performed on a Cosmosil 5C18-P column (1.5 × 250 mm) at the flow rate of 150 μl/

min at 25°C. The column was equilibrated with 20 mM ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. After injecting a sample, the concentration of 1-butanol was raised linearly to 0.4% in 90 min. PA-sugar chains were detected by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

Reversed-phase HPLC was also performed on another Cosmosil 5C18-P column (10 × 250 mm) at the flow rate of 3.0 ml/min at 25°C. Two eluents, C and D, were used. Eluent C was water:acetic acid (1,000:6, v/v) titrated to pH 4.0 with aqueous ammonia, and Eluent D was water:acetic acid:1-butanol (1,000:6:10, v/v/v) titrated to pH 4.0 with aqueous ammonia. The column was equilibrated with Eluent C:Eluent D (32:1, v/v). After injecting a sample, linear gradient elution was performed to Eluent C:Eluent D (1:3, v/v) in 125 min. PA-sugar chains were detected as described above for reversed-phase HPLC.

Anion-exchange HPLC was carried out on a Mono Q HR 5/5 column at 25°C at the flow rate of 1.0 ml/min. Two eluents, E and F, were used. Eluent E was aqueous ammonia, pH 9.0, and Eluent F was 500 mM ammonium acetate buffer, pH 9.0. The column was equilibrated with Eluent E. After injecting a sample, linear gradient elution was performed to Eluent E:Eluent F (22:3, v/v) in 3 min, to Eluent E:Eluent F (3:2, v/v) in 14 min, and then to Eluent F in 5 min. PA-sugar chains were detected as described above for size-fractionation HPLC.

Anion-exchange HPLC for determining PA-monosaccharides was performed on a TSK-gel Sugar AXI column as reported (*12*).

Reducing-End Analysis of PA-Sugar Chains—The reducing-end residues of PA-sugar chains (each 20 pmol) were quantified by HPLC on a TSK-gel Sugar AXI column after hydrolysis with 4 M hydrochloric acid at 100°C for 8 h in evacuated sealed tubes, followed by *N*-acetylation (*8*).

RESULTS AND DISCUSSION

Preparation of PA-N-Linked Sugar Chains from Factor IX—PA-sugar chains obtained from 20 nmol of Factor IX were gel-filtered on an HW-40F column (1.5 × 67 cm). Fractions of 8.6 ml were collected. To determine the elution positions of the *N*-linked sugar chains, reducing-end PA-monosaccharides were analyzed as described under "MATERIALS AND METHODS." PA-sugar chains with the reducing-end PA-GlcNAc were only eluted in Fraction 5, indicating that all PA-*N*-linked sugar chains were eluted in this fraction (data not shown). *O*-Linked sugar chains with reducing-end PA-GalNAc, PA-Fuc, or PA-Glc were detected in Fractions 6–17 (data not shown).

Preparation and Separation of Desialylated PA-Sugar Chains—PA-*N*-linked sugar chains were digested with *Arthrobactor* sialidase, the digests were separated on a Mono Q column, and the neutral fraction corresponding to 85% of the PA-sugar chains was collected as Fraction X (data not shown). Fraction X thus obtained was separated into Fractions A–H by reversed-phase HPLC (Fig. 1-1). Fraction C was further separated by size-fractionation HPLC and a major fraction, C1, was collected (data not shown). All the fractions obtained gave a single peak on size-fractionation HPLC (data not shown). The eight main fractions (A, B, C1, D, E, F, G, and H) comprised 85% of the amount of Fraction X.

Structural Analysis of Fractions A–H—To determine the structures of the PA-*N*-linked sugar chains, reversed-phase and size-fractionation HPLC (two-dimensional sugar map) were performed. The elution positions of 93 standard PA-*N*-linked sugar chains have already been reported, and the introduction of a reversed-phase scale made it possible to predict the elution positions even if standard PA-*N*-linked sugar chains were not available (*9*).

Fractions A–H except for Fraction D were eluted at the positions of standard PA-sugar chains, which could be well distinguished from other PA-sugar chains. Their structures were further analyzed by exoglycosidase digestion combined with two-dimensional HPLC mapping (Table II). Fractions A and F were susceptible to digestion with α-L-fucosidase, and the difference in their elution positions on reversed-phase HPLC is characteristic of a Fucα1-3 residue. Fractions E and H were also susceptible to digestion with α-L-fucosidase, and the difference in their elution positions is characteristic of a Fucα1-6 residue. Fractions B, C, D, and G, and α-L-fucosidase digests of four fractions (A, E, F, H) were susceptible to digestion with *Streptococcus* β-galactosidase, which specifically hydrolyzes Galβ1-4GlcNAc (*13*), but were resistant to digestion with *Xanthomonus* β-galactosidase, which hydrolyzes Galβ1-3GlcNAc (*14*), indicating that all the Gal residues were linked through the β1-4 linkage (data not shown). The results are summarized in Table III.

Fraction D was not identified as one of the standard PA-

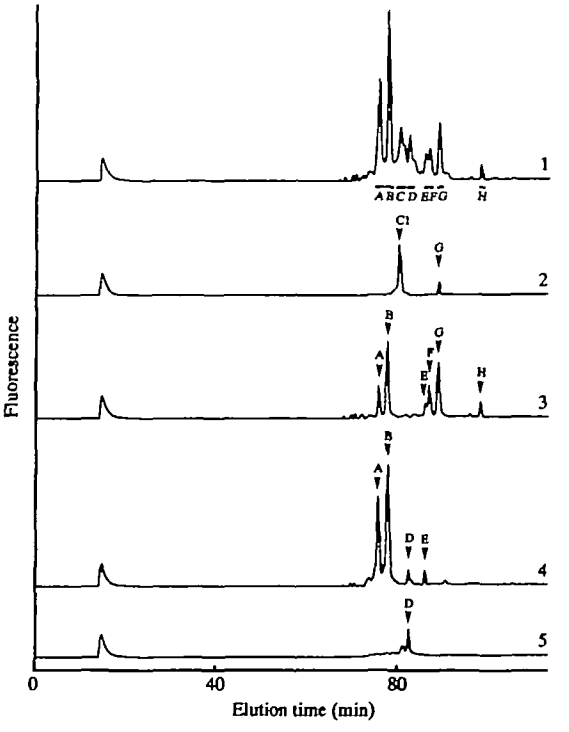

Fig. 1. **Reversed-phase HPLC of Fractions X, S2N, S3N, S4N, and S5N.** Elution profiles: 1, Fraction X; 2, Fraction S2N; 3, Fraction S3N; 4, Fraction S4N; 5, Fraction S5N. HPLC was performed on a Cosmosil 5C18-P column (10.0 × 250 mm). Fractions were pooled as indicated by bars A–H. Arrowheads A, B, C1, D, E, F, G, and H indicate the elution positions of PA-sugar chains A, B, C1, D, E, F, G, and H, respectively. The peaks appearing at around 15 min are due to contaminating material.

TABLE II. **HPLC analysis of sequential glycosidase digests of Fractions A–H, and their reducing ends.**

Fraction	Successive treatment with glycosidases	HPLC		Sugar chain identified[c]
		Reversed-phase[a]	Size-fractionation[b]	
A	No treatment	53.9	11.4	26
	α-L-Fucosidase	55.4	10.6	25
	Streptococcus β-galactosidase	50.7	7.5	15
	β-*N*-Acetylhexosaminidase	43.9	4.5	1
B	No treatment	54.6	10.6	25
	Streptococcus β-galactosidase	50.3	7.5	15
	β-*N*-Acetylhexosaminidase	42.1	4.5	1
C1	No treatment	55.0	7.8	16
	Streptococcus β-galactosidase	50.1	6.2	10
	β-*N*-Acetylhexosaminidase	43.1	4.5	1
D	No treatment	60.5	12.2	—
	Endo-β-galactosidase	57.0	9.9	24
	β-*N*-Acetylhexosaminidase	65.6	9.2	18
	Streptococcus β-galactosidase	59.5	6.8	14
	β-*N*-Acetylhexosaminidase	42.7	4.5	1

	No treatment	60.5	12.2	—
	Streptococcus β-galactosidase	55.0	8.9	—
	β-*N*-Acetylhexosaminidase	45.2	6.1	7
	Streptococcus β-galactosidase	45.0	5.2	3
E	No treatment	63.3	11.2	27
	α-L-Fucosidase	54.0	10.6	25
	Streptococcus β-galactosidase	50.7	7.5	15
	β-*N*-Acetylhexosaminidase	43.8	4.5	1
F	No treatment	64.1	9.9	19
	α-L-Fucosidase	65.0	9.2	18
	Streptococcus β-galactosidase	57.4	6.8	14
	β-*N*-Acetylhexosaminidase	42.1	4.5	1
G	No treatment	66.1	9.2	18
	Streptococcus β-galactosidase	59.0	6.8	14
	β-*N*-Acetylhexosaminidase	42.9	4.5	1
H	No treatment	73.0	9.7	20
	α-L-Fucosidase	63.8	9.2	18
	Streptococcus β-galactosidase	58.0	6.8	14
	β-*N*-Acetylhexosaminidase	43.0	4.5	1

[a]Reversed-phase scale (*9*). [b]Glucose units. [c]The numbers refer to the sugar chain structures shown in Table I.

TABLE III. **Proposed structures of PA-sugar chains from fuman factor IX.**

Sialic acid	Proposed structure	Molar ratio (%)	Fraction
Sia_3- 20% Sia_4- 80%	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 2 Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA Fucα1-3 Galβ1-4GlcNAcβ1	22	A
Sia_3- 40% Sia_4- 60%	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	35	B
Sia_2- 100%	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	10	C1
Sia_4- 40% Sia_5- 60%	Galβ1-4GlcNAcβ1-Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	8	D
Sia_3- 10% Sia_4- 90%	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	5	E
Sia_3- 100%	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Fucα1-3 2 Manα1-3 Galβ1-4GlcNAcβ1	5	F
Sia_2- 20% Sia_3- 80%	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	12	G
Sia_3- 100%	Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3	3	H

sugar chains; however, the digest with endo-β-galactosidase was eluted at the position of Sugar Chain 24, which was separated from the positions of Sugar Chains 21–23 on the two-dimensional sugar map (Table II). When the product was further digested with β-*N*-acetylhexosaminidase, a new peak appeared at the position of Sugar Chain 18. On further digestion with *Streptococcus* β-galactosidase, the product was eluted at the position of Sugar Chain 14, which could be well distinguished from Sugar Chains 11–13. In addition, the digestion of Fraction D with *Streptococcus* β-galactosidase reduced the molecular size by four galactose residues. On further digestion with β-*N*-acetylhexosaminidase, the product was eluted at the position of Sugar Chain 7. When the product was further digested with *Streptococus* β-galactosidase, a new peak appeared at the position of Sugar Chain 3, which could be well separated from Sugar Chains 2, 4, and 5 on the two-dimensional sugar map (Table II). These results indicated that Fraction D was a PA-tetraantennary sugar chain with a lactosamine residue on the GlcNAcβ1-6Manα1-6Man branch, and all the Gal residues of Fraction D were linked through the β1-4 linkage (Table III).

Analysis of the Sialic Acid Residues—PA-*N*–linked sugar chains were separated by Mono Q HPLC (Fig. 2). Fractions S2–S5 were collected, and a part of Fraction S5 was partially desialylated with *Arthrobactor* sialidase. Five peaks appeared at the elution positions of authentic asialo, monosialo, disialo, trisialo, tetrasialo PA-sugar chains, indicating that Fraction S5 contained pentasialo PA-sugar chains (data not shown). The molar ratio of Fractions S2:S3:S4:S5 was 10:32:46:12.

Fraction S2 was completely digested with *Arthrobactor* sialidase, which hydrolyzes Siaα2-3/6Gal. The products were purified by Mono Q HPLC, and the neutral fraction (S2N) was collected and analyzed by reversed-phase HPLC (Fig. 1-2). Peaks were observed at the elution positions of Sugar Chains C1 and G, indicating that 100% of Sugar Chain C1 and 30% of Sugar Chain G are disialylated in human Factor IX (Table III).

Fractions S3–S5 were analyzed in the same way. When these fractions were desialylated with *Arthrobactor* sialidase, about 15% of them was not hydrolyzed, but a peak appeared at the monosialo sugar chain position. This fraction was resistant to re-digestion with *Arthrobactor* sialidase. This fraction was not studied further because it was found to give many small peaks on reversed-phase HPLC. Neutral fractions (S3N–S5N) were analyzed in the same manner as S2N (Fig. 1 and Table III). The NDV sialidase digests of Fractions S2–S5 revealed that 20% of the sialic acid residues were linked through an α2-3 linkage (data not shown). The *N*-linked sugar chains of human factor IX thus consist of complex-type chains with 2–5 acidic residues. The existence of five sialic acid residues in tetraantennary sugar chains (Fraction D) may indicate the existence of Siaα2-6GlcNAc. This structure predominantly exists in *N*-linked sugar chains of bovine factor IX, but all of them are linked to Galβ1-3GlcNAc (*7*). In comparison with bovine factor IX, human factor IX contains fucosylated *N*-linked sugar chains (35%), some of which may have a sialyl Le^x structure.

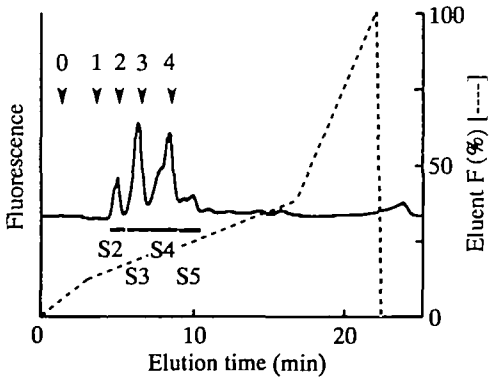

Fig. 2. **Separation by Mono Q anion-exchange HPLC of PA-*N*–linked sugar chains.** Arrowheads 0–4 indicate the elution positions of authentic asialo, monosialo, disialo, trisialo, and tetrasialo PA-*N*–linked oligosaccharides, respectively. Fractions were pooled as indicated by bars S2, S3, S4, and S5. The peaks appearing at around 24 min are due to contaminating material.

Bovine factor IX contains three *N*-linked sugar chains in the activation peptide (residues 147–182) at Asn-158, -168, and -173. These sugar chains were heavily sialylated, bi- and tri-antennary sugar chains with 2–5 sialic acid residues (*7*). Human factor IX is composed of a single polypeptide chain of 416 amino acid residues. Four sites for attachment of *O*-linked sugar chains are present in human factor IX, including Thr-159 and -169 in the activation peptide (residues 146–180). There are also two sites for the attachment of *N*-linked oligosaccharides in the activation peptide at Asn-157 and -167. These oligosaccharides on the activation peptide have a total of 7.9 acidic residues (*15*). The abundance of sialic acid residues in a small region of only 35 amino acid residues may contribute to maintenance of the conformation of factor IX, and may prevent an undesirable proteolytic attack until its activation by factor XIa takes place in the coagulation process (*7*).

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