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Analysis of Fluorogenic Smith Degradation Products of 7-(1,3-Disulfonaphtyl)amino-Disaccharides for Linkage Position Analysis of Carbohydrates

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The linkage position of a glycosidic bond to the reducing-end residue of a pyridylamino (PA-) sugar can be determined sensitively by Smith degradation and HPLC [K. Omichi and S. Hase, (1994) *J. Biochem.* 115, 429–434]. With the aim of enhancing the sensitivity of this method of linkage position analysis to the fmol-level, use of the 7-(1,3-disulfonaphtyl)amino (DSNA-) group instead of the PA-group as a fluorescent tag was examined. Smith degradation of DSNA-disaccharides with a DSNA-hexose, DSNA-*N*-acetylglucosamine, or DSNA-*N*-acetylgalactosamine reducing-end residue was carried out. HPLC and FAB-MS of the fluorogenic Smith degradation products showed that the DSNA-group was stable under the Smith degradation reaction conditions, and that the reaction proceeded in a manner similar to that using PA-disaccharides to give the predicted products. Fluorogenic Smith degradation products specific to the glycosidic linkage position were well separated by reversed-phase HPLC, and were easily assignable by comparing the HPLC elution positions with those of standard compounds. The method was successfully applied to analyzing the structure of an *N*-linked sugar chain.

Key words: DSNA-disaccharide, linkage position analysis, Smith degradation.

The pyridylamination of sugars is an effective means of analyzing their structures because (i) the pyridylamino (PA-) group is stable to reagents employed in sugar chemistry, such as periodate, borohydride, acids, and alkalis, and (ii) PA-sugars can be sensitively analyzed by HPLC under various separation modes (*1, 2*).

The linkage position of the glycosidic bond of an oligosaccharide is usually determined by methylation analysis or NMR spectrometry. However, relatively large amounts of sample are required for these methods. As an alternative, periodate oxidation also provides structural information about the linkage position (*3–5*). Previously, we proposed a method for determining the glycosidic linkage position involving Smith degradation of PA-disaccharides and HPLC analysis of the fluorogenic products (*6, 7*). The fluorescence intensity of PA-derivatives, however, is rather weak. 7-(1,3-Disulfonaphtyl)amino (DSNA-) sugars have been reported to possess strong fluorescence and to be amenable to highly sensitive analysis (*8*). If the DSNA-group is sufficiently stable under the Smith degradation reaction conditions and fluorogenic Smith degradation products of DSNA-sugars are separatable from each other, very sensitive linkage position analysis will be possible.

This paper describes the separation of the Smith degradation products of DSNA-disaccharides by HPLC and subsequent fmol-level linkage position analysis.

MATERIALS AND METHODS

Materials—Glyceraldehyde, L-threose, D-erythrose, maltose (Glcα1-4Glc), isomaltose (Glcα1-6Glc) and Wakosil-II 5C18 HG columns (10 × 250 mm, 4.6 × 250 mm) were purchased from Wako Pure Chemicals (Osaka); Glcα1-2Glc, Glcα1-3Glc, Manα1-3Man, Galβ1-3GlcNAc, Galβ1-4GlcNAc, and Galβ1-6GlcNAc were from Sigma (St. Louis, MO, USA); Galβ1-3GalNAc was from Funakoshi (Tokyo); TSK-gel HW-40F from Tosoh (Tokyo); a Shodex Asahipak NH2P-50 column (4.6 × 150 mm) was from Showa Denko (Tokyo); Jack bean α-mannosidase and *Achatina fulica* β-mannosidase were from Seikagaku Kogyo (Tokyo). Monopotassium 7-amino-1,3-naphtalenedisulfonate from Aldrich (Milwaukee, WI, USA) was twice crystallized from water.

Tagging Sugars with DSNA—Glyceraldehyde, threose, erythrose, and disaccharides were converted to their DSNA-derivatives, basically according to the method of Lee *et al.* (*8*). A lyophilized sample (0.5 mg) was mixed with 50 μl of 50% (w/v) monopotassium 7-amino-1,3-naphtalenedisulfonate solution, the pH of which was adjusted to 6.2 with 5 M sodium hydroxide, and the mixture was heated at 80°C for 1 h. To the reaction mixture was added 10 μl of 1.6 M sodium cyanoborohydride, and the mixture was heated at 70°C for 6 h. The reaction mixture was applied onto a TSK-gel HW-40F column (1 × 80 cm) equilibrated with 20 mM ammonium acetate buffer, pH 6.0. The elution was

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Abbreviations: DSNA-, 7-(1,3-disulfonaphtyl)amino; FC2, DSNA-glycolaldehyde; FC3, DSNA-glyceraldehyde; FC4e, DSNA-erythrose; FC4t, DSNA-threose; FNC3, DSNA-2-acetamido-2-deoxy-glyceraldehyde; FNC4t, DSNA-2-acetamido-2-deoxy-threose; FNC5a, DSNA-2-acetamido-2-deoxy-arabinose; FNC5x, DSNA-2-acetamido-2-deoxy-xylose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; Man, mannose; PA-, pyridylamino.

monitored by measuring the absorbance at 314 nm.

Standard Linkage Position Analysis—DSNA-disaccharides were subjected to Smith degradation in a manner similar to that used for PA-disaccharides (*4*). A DSNA-disaccharide (50–200 fmol) in 25 μl of 0.1 M sodium acetate buffer, pH 4.0, was mixed with 25 μl of 0.1 M sodium metaperiodate solution at 0°C and kept at 0°C for 1 min in the dark. Then, 10 μl of the reaction mixture was added to 40 μl of 0.26 M sodium borohydride solution. After letting the mixture stand for 1 h at 25°C, 10 μl acetic acid was added to decompose the residual borohydride. The pH of the reaction mixture was adjusted to 6.0 by adding 30 μl of 2.2 M sodium hydroxide solution. Finally, the mixture was frozen and stored at –60°C until required for HPLC analysis.

Oxidation of the remaining solution was continued for 24 h at 4°C. After treatment with sodium borohydride (60 μl) followed by the addition of acetic acid (15 μl) and pH adjustment to 6.0 with 50 μl of 2.2 M sodium hydroxide, the reaction mixture was applied onto a Wakosil-II 5C18 HG column (10 × 250 mm) to isolate the fluorogenic oxidation–reduction product. The elution buffer was 0.1 M ammonium acetate, pH 6.0, containing 0.1% 1-butanol, and the flow rate was 3.0 ml/min at 25°C. The elution was monitored by measuring the fluorescence at 452 nm (excitation at 314 nm).

A part (1 ml) of the oxidation–reduction product collected was added to 100 μl of 1.1 M sulfuric acid and the mixture was heated at 80°C for 10 min. After adjusting the pH to 6.0 with 100 μl of 2.2 M sodium hydroxide, a part (300 μl) of the hydrolysate (1.2 ml) was injected into a Wakosil-II 5C18 HG column (10 × 250 mm) as described above.

HPLC of the stored 1-min reaction mixture was carried out under the same conditions.

Fast-Atom-Bombardment Mass Spectrometry (FAB-MS)—Molecular masses were measured by FAB-MS using a JEOL JMS-HX100 mass spectrometer equipped with an FAB ion source and a DA-5000 data processor. A mixture of a sample and glycerol (0.5 μl) was bombarded with a neutral atom beam accelerated at a potential of 10 keV.

Preparation of PA-N-Glycans from Japanese Medaka (Oryzias latipes)—*N*-Glycans were released from finely powdered freeze-dried liver (2.2 mg) by hydrazinolysis (0.5 ml, 100°C for 10 h) followed by *N*-acetylation, and the reducing ends of the *N*-glycans liberated were pyridylaminated as described previously (*9*). The pH of the reaction mixture was brought to 10 with 6 M aqueous ammonia, and then excess reagents were extracted five times with an equal volume of chloroform. After pH adjustment to 6.0 with acetic acid, the water phase was concentrated to a small volume, and then the solution was chromatographed on an HW-40F column (1.2 × 50 cm) using 0.01 M ammonium acetate buffer, pH 6.0. The fraction eluted between the void volume and 50 ml was collected as a PA-*N*-glycan fraction.

Preparation of DSNA-Disaccharides from PA-N-Glycan—To 100 pmol of PA-*N*-glycan, 100 μl of 1 M trifluoroacetic acid was added, and the mixture was hydrolyzed at 100°C for 10 min. The hydrolysates were then freeze-dried and *N*-acetylated (*9*). The sample was then disulfonaftylaminated, and the reaction mixture was subjected to gel filtration on a TSK-gel HW-40F column (1.0 × 110 cm). DSNA-disaccharide fractions were combined and lyophilized.

High-Performance Liquid Chromatography (HPLC)—Reversed-phase HPLC of PA-*N*-glycans was performed on a Wakosil-II 5C18 HG column (4.6 × 250 mm) at the flow rate of 0.9 ml/min at 25°C. The column was equilibrated with 50 mM ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. Afetr injecting a sample, the concentration of 1-butanol was raised linearly to 1.125% in 40 min. PA-sugar chains were detected by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

Size Fractionation HPLC of PA-*N*-glycans was carried out on a Shodex Asahipak NH2P-50 column at 25°C at the flow rate of 0.9 ml/min. Two eluents, A and B, were used. Eluent A was acetonitrile:water:acetic acid (100:900:3, v/v/v) titrated to pH 7.0 with aqueous ammonia, and Eluent B was acetonitrile:water:acetic acid (900:100:3, v/v/v) titrated to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent B:Eluent A (95:5, v/v). After injecting a sample, linear gradient elution was performed to Eluent B:Eluent A (60:40, v/v) in 10 min, to Eluent B:Eluent A (40:60, v/v) in 40 min, and then to Eluent B:Eluent A (20:80, v/v) in 10 min. PA-sugar chains were detected by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Reversed-phase HPLC of DSNA-disaccharides was performed on a Wakosil-II 5C18 HG column (10 × 250 mm) at the flow rate of 3.0 ml/min at 25°C. The column was equilibrated with 100 mM ammonium acetate buffer, pH 6.0, containing 0.01% 1-butanol. Afetr injecting a sample, the concentration of 1-butanol was increased linearly to 0.15% in 40 min. DSNA-disaccharides were detected by measuring the fluorescence (excitation wavelength, 314 nm; emission wavelength, 452 nm).

RESULTS AND DISCUSSION

Possible Smith Degradation Products from DSNA-Disaccharides—DSNA-derivatives theoretically produced by Smith degradation of DSNA-disaccharides are summarized in Fig. 1. DSNA-glyceraldehyde (FC3) should be formed from 2-substituted disaccharides with DSNA-hexose as the reducing-end residue, and DSNA-threose (FC4t) or DSNA-erythrose (FC4e) from 3-substituted disaccharides. DSNA-glycolaldehyde (FC2) should be formed from 4- or 6-substituted disaccharides. However, as is the case with PA-disaccharides, at an early stage of periodate oxidation the products should be different (*4*): FC3 or FC4t (or FC4e) should be characteristic of 6-substituted disaccharides and

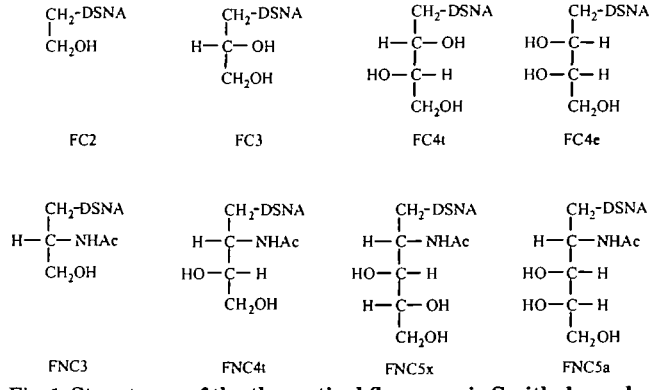

Fig. 1. Structures of the theoretical fluorogenic Smith degradation products examined.

never be produced from 4-substituted ones (Fig. 2). Hence, the identification of either of these products implies a 6-substituted disaccharide.

In the case of DSNA-disaccharides with DSNA-*N*-acetylglucosamine or DSNA-*N*-acetylgalactosamine as the reducing-end residue, the linkage positions should be more simply determined by the identification of three different fluorogenic products: DSNA-2-acetamido-2-deoxy-threose (FNC4t) should be formed from 3-substituted disaccharides, DSNA-2-acetamido-2-deoxy-xylose (FNC5x) or DSNA-2-acetamido-2-deoxy-arabinose (FNC5a) from 4-substituted disaccharides, and DSNA-2-acetamido-2-deoxy-glyceraldehyde (FNC3) from 6-substituted disaccharides.

Stability of DSNA-Residues to Smith Degradation—To ascertain whether the DSNA-group is stable enough for the reaction conditions of the Smith degradation, eight model DSNA-disaccharides were oxidized with sodium metaperiodate at pH 4.0 and 4°C for 24 h. After reduction with sodium borohydride, the reaction mixtures were analyzed by reversed-phase HPLC as described in "MATERIALS AND METHODS" (Fig. 3). The fluorogenic products indicated by bars in Fig. 3 were collected and hydrolyzed with 0.1 M sulfuric acid at 80°C for 10 min and the hydrolysates were

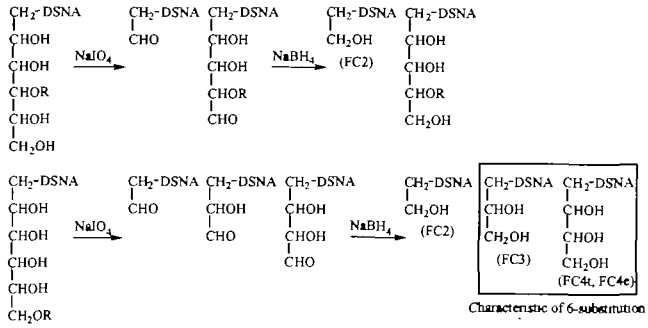

Fig. 2. **Possible fluorogenic Smith degradation products derived from 4- or 6-substituted DSNA-disaccharides at an early stage of periodate oxidation.**

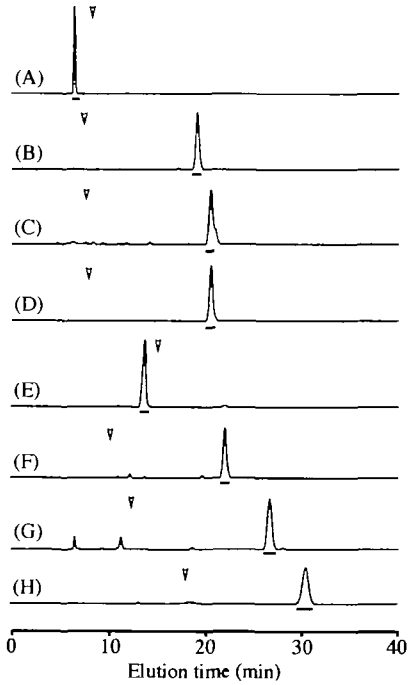

Fig. 3. **HPLC of fluorogenic periodate oxidation–reduction products from DSNA-disaccharides.** Elution profile of periodate oxidation–reduction product from A, Glcα1-6Glc-DSNA; B, Glcα1-2Glc-DSNA; C, Glcα1-3Glc-DSNA; D, Manα1-3Man-DSNA; E, Galβ1-6GlcNAc-DSNA; F, Galβ1-3GlcNAc-DSNA; G, Galβ1-4GlcNAc-DSNA; H, Galβ1-4GalNAc-DSNA. Open arrowheads indicate the elution positions of intact DSNA-disaccharides. The fractions indicated by bars were collected for further analysis.

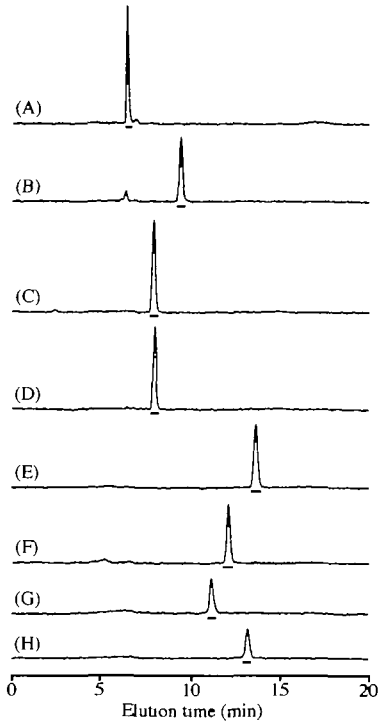

Fig. 4. **HPLC of acid hydrolysates of fluorogenic oxidation–reduction products obtained in Fig. 3.** Elution profile of acid hydrolysate obtained from A, Glcα1-6Glc-DSNA; B, Glcα1-2Glc-DSNA; C, Glcα1-3Glc-DSNA; D, Manα1-3Man-DSNA; E, Galβ1-6GlcNAc-DSNA; F, Galβ1-3GlcNAc-DSNA; G, Galβ1-4GlcNAc-DSNA; H, Galβ1-4GalNAc-DSNA. For FAB-MS, the eight fractions indicated by bars were collected as Fractions A–H, respectively.

TABLE I. **Molecular ions of fluorogenic Smith degradation products.**

Fraction (structure)	Molecular ion [M + 1] observed (calc. M)	(calc. 1Na)	(calc. 2Na)
A (FC2)	(347.0)	(369.0)	391.5 (391.0)
B (FC3)	377.7 (377.0)	(399.0)	(421.0)
C (FC4t)	408.2 (407.0)	430.2 (429.0)	(451.0)
D (FC4e)	(407.0)	430.2 (429.0)	452.2 (451.0)
E (FNC3)	419.2 (418.1)	441.3 (440.0)	463.3 (462.0)
F (FNC4t)	449.2 (448.1)	(470.0)	(492.0)
G (FNC5x)	(478.1)	501.5 (500.1)	523.4 (522.0)
H (FNC5a)	479.1 (478.1)	501.1 (500.1)	523.2 (522.0)

then subjected to HPLC (Fig. 4). Large-scale preparation of the fluorogenic Smith degradation products (Fractions A–H, indicated by bars in Fig. 4) was carried out to determine their molecular weights by FAB-MS (Table I), which revealed them to be FC2, FC3, FC4t, FC4e, FNC3, FNC4t, FNC5x, and FNC5a, respectively. FC2 was ob-tained from Glcα1-6Glc-DSNA (200 fmol) with a yield of 60%. This total yield included the loss of FC2 during purification by reversed-phase HPLC. The loss was measured by the use of authentic FC2. Taking the loss into consideration, about 80% of FC2 was liberated from Glcα1-6Glc-DSNA under the Smith degradation conditions established. These results show that the DSNA-group is stable enough for the reaction conditions used and that the Smith degradation proceeds in the expected manner to give the predicted products.

As shown in Fig. 5, the fluorogenic Smith degradation products obtained from DSNA-disaccharides with DSNA-hexose, DSNA-*N*-acetylglucosamine, or DSNA-*N*-acetylgalactosamine reducing-end residues, were separated by reversed-phase HPLC. The chromatogram in Fig. 5 shows that the epimers FC4t and FC4e were not actually separated from one another under the chromatographic conditions employed, but this did not affect the linkage position analysis because they both indicate the 3-substitution of DSNA-hexose.

Early Stage Periodate Oxidation of 4- or 6-Substituted Disaccharides—To determine the periodate oxidation reaction conditions that would allow detection of the characteristic intermediates of 6-substituted disaccharides with DSNA-hexose as the reducing-end residue, DSNA-isomaltose and DSNA-maltose were oxidized at 0°C for an appropriate periods and reduced with sodium borohydride as described in "MATERIALS AND METHODS." The first glycol cleavage should occur at the flexible glycols of the DSNA-reducing-end residue rather than at the glycols of the rigid non-reducing-end pyranose ring residue (*6*). HPLC of the reaction mixtures revealed that FC3 was rapidly liberated from DSNA-isomaltose and had almost disappeared within 5 min (Fig. 6I). The peaks appearing at 8, 12, and 14 min are thought to be intermediates of the oxidation reaction, such as an adduct with a periodic acid (Fig. 6I, ii). FC4t was not detected throughout the oxidation, indicating that the first glycol cleavage might occur between C-2 and C-3 or between C-3 and C-4, but not between C-4 and C-5. This inference is supported by a previous finding that PA-threose and PA-erythrose were also undetected from several 6-substituted PA-disaccharides (*6*). On the other hand, in the case of DSNA-maltose, the characteristic product was not

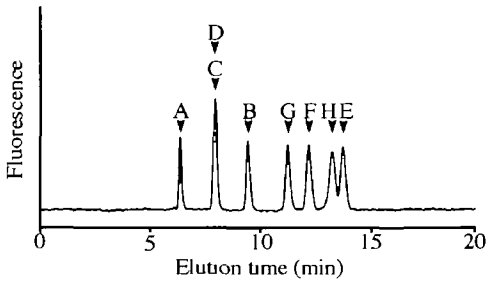

Fig. 5. **HPLC of fluorogenic Smith degradation products from DSNA-disaccharides.** A, FC2; B, FC3; C, FC4t; D, FC4e; E, FNC3; F, FNC4t; G, FNC5x; H, FNC5a.

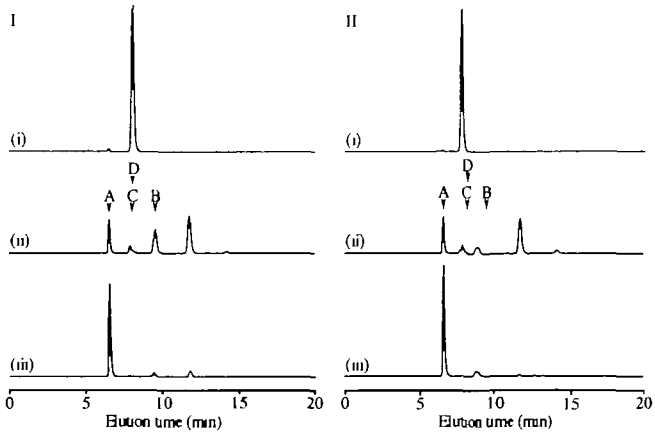

Fig. 6. **Time courses of periodate oxidation of DSNA-isomaltose (I) and DSNA-maltose (II) at an early stage.** Each DSNA-disaccharide (50 fmol) was oxidized with sodium metaperiodate for 0 min (i), 1 min (ii), or 5 min (iii). After reduction with sodium borohydride, the reaction mixtures were analyzed by HPLC as described in "MATERIALS AND METHODS." Black arrowheads indicate the elution positions of the standard compounds: A, FC2; B, FC3; C, FC4t; D, FC4e.

TABLE II. **Fluorogenic Smith degradation products detected by HPLC and their linkage positions.** This method can be applied to disaccharides with DSNA-reducing-end residues derived from pyranose without any alteration.

Fluorogenic Smith degradation product	Fluorogenic product of 1-min reaction	Linkage position
FC2	FC3	-6Hex
FC2	noFC3	-4Hex
FC3	—	-2Hex
FC4t	—	-3Hex
FC4e	—	-3Hex(Man)
FNC3	—	-6HexNAc
FNC4t	—	-3HexNAc
FNC5x	—	-4GlcNAc
FNC5a	—	-4GalNAc

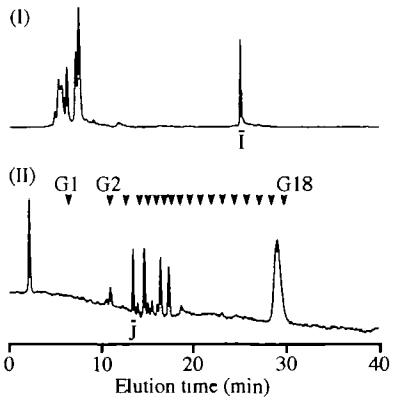

Fig. 7. **HPLC of PA-*N*-glycans from the liver of a Japanese medaka.** (I) Reversed-phase HPLC of PA-*N*-glycans from the liver of a Japanese medaka (*Oryzias latipes*). HPLC was performed on a Wakosil-II 5C18 HG column (4.6 × 250 mm). Fraction I was collected as indicated by the bar. (II) Size-fractionation HPLC of Fraction I. Arrowheads G1–G18 indicate the elution positions of PA-glucose–PA-isomaltooctadecaose. Fraction J was collected, as indicated by the bar.

detected throughout the oxidation (Fig. 6II). A 1-min reaction at 0°C was chosen as the periodate oxidation condition for differentiating 4- and 6-substitution of DSNA-hexose.

Linkage Position Analysis—The linkage position could be determined by combining Smith degradation and HPLC as described in "MATERIALS AND METHODS." The relation between the linkage position and the fluorogenic Smith degradation product detected by HPLC is summarized in Table II. This method can be applied to disaccharides with DSNA-reducing-end residues derived from pyranose without any alteration.

It was found that when a column was used for HPLC analysis of the enzymatic reaction products, proteins naturally accumulated at the head of the column. Such a column should not be used for the sensitive analysis of DSNA-derivatives since the proteins may adsorb fmol-level DSNA-derivatives, leading to a failure of linkage position analysis.

Application of Linkage Position Analysis to an N-Glycan in Japanese Medaka—Two-dimensional sugar mapping of PA-sugar chains, combining reversed-phase and size-fractionation HPLC, is a convenient and useful method to estimate the structure, when the amount of sample is very small and usual methods of structural analysis are not usable (*10, 11*). The present method for linkage position analysis was expected to offer more information about the structure, and was applied to confirm the estimated structure of PA-*N*-glycan from the liver of Japanese medaka (*Oryzias latipes*). PA-*N*-glycans were prepared from the liver of a mature female medaka as described in "MATERIALS AND METHODS." The PA-*N*-glycan fraction obtained was separated by reversed-phase HPLC (Fig. 7I). Fraction I was collected and further separated by size-fractionation HPLC (Fig. 7II). Fraction J, which eluted at the positions of Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA on the two-dimensional sugar map, was collected (*12*). The yield of Fraction J was 120 pmol.

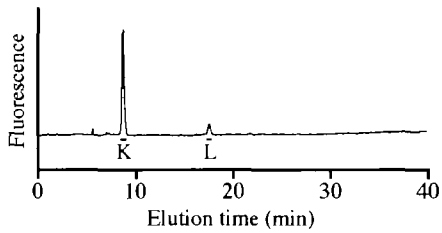

Fig. 8. **Reversed-phase HPLC of DSNA-disaccharides from Fraction J.** HPLC was performed on a Wakosil-II 5C18 HG column (10 × 250 mm). Fractions K and L were collected as indicated by the bars.

Part (100 pmol) of Fraction J was partially hydrolyzed with acid, and the hydrolysate was converted to DSNA-derivatives as described in "MATERIALS AND METHODS." The DSNA-disaccharides obtained were separated on a Wakosil-II 5C18 HG column (Fig. 8). Fractions K and L were collected and confirmed to be susceptible to digestion with α-mannosidase and β-mannosidase, respectively (data not shown). Part (100 fmol) of Fraction K was treated as described in "MATERIALS AND METHODS." HPLC chromatograms of the oxidation–reduction mixture and the hydrolysate of the oxidation product are shown in Fig 9. FC2 was detected as the Smith degradation product, indicating 4- or 6-substitution (Fig. 9II). HPLC of the 1-min oxidation–reduction mixture resulted in the detection of FC3 (Fig. 9III). This clearly shows that α-Man is linked to the 6-position of the DSNA-Hex residue. FNC5x was detected as the Smith degradation product of Fraction L (Fig. 10). This result indicates that the structure of Fraction L is Manβ1-4GlcNAc-DSNA. The detection of Manα1-6Hex and Manβ1-4GlcNAc in the partial acid hydrolysates of Fraction J is consistent with the structure of Fraction J estimated by two-dimensional HPLC mapping (Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA).

Replacement of the PA-group with the DSNA-group enhances the sensitivity of the linkage position analysis by about 100-fold, and FC2, FC3, and FNC5x at the fmol-level could easily be detected by HPLC.

Partial acid hydrolysis of an oligosaccharide must give various disaccharides. Determining the linkage positions of all the disaccharides produced will allow all the linkage

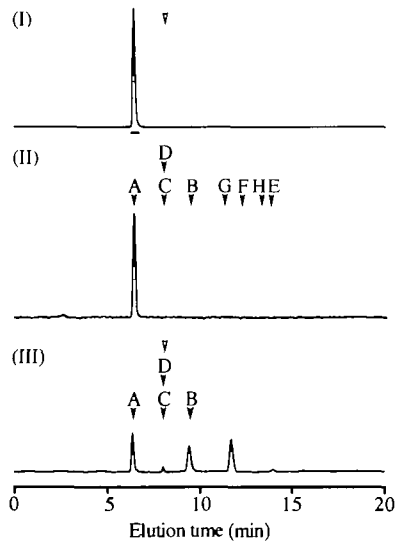

Fig. 9. **HPLC analysis of the Smith degradation product of Fraction K.** HPLC of I, the 24-h oxidation–reduction mixture; II, the acid hydrolysate of the 24-h oxidation–reduction product; III, the 1-min oxidation–reduction mixture. The fraction indicated by the bar was collected for further analysis. Open and closed arrowheads, respectively, indicate the elution positions of intact Fraction K and the standard compounds: A, FC2; B, FC3; C, FC4t; D, FC4e.

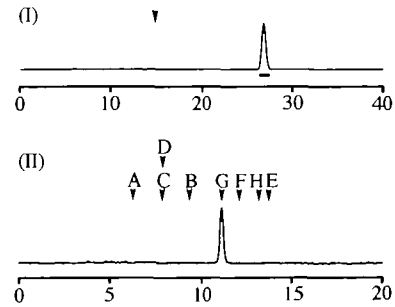

Fig. 10. **HPLC analysis of the Smith degradation product of Fraction L.** HPLC of I, the 24-h oxidation–reduction mixture; II, the acid hydrolysate of the 24-h oxidation–reduction product. The fraction indicated by the bar was collected for further analysis. Open and closed arrowheads, respectively, indicate the elution positions of intact Fraction L and the standard compounds: A, FC2; B, FC3; C, FC4t; D, FC4e; E, FNC3; F, FNC4t; G, FNC5x; H, ENC5a.

positions in the oligosaccharide to be determined.

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