The Structure of Nephritogenoside

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Nephritogenoside is a minor component of the basement membrane of normal animals (including humans). It is a glycopeptide with the ability to induce chronic progressive glomerulonephritis (end stage kidney) when administered as a single footpad injection, and contains a novel carbohydrate–peptide linkage. The total chemical structure was investigated. It was revealed that nephritogenoside is a simple glycopeptide composed of three glucose residues [α-Glc-(1→6)-β-Glc-(1→6)-Glc] and twenty-one amino acids [^{1}Asn–Pro–Leu–Phe–^{5}Gly–Ile–Ala–Gly–Glu–^{10}Asp–Gly–Pro–Thr–Gly–^{15}Pro–Ser–Gly–Ile–Val–^{20}Gly–^{21}Gln], and that the glucose residues are linked α-*N*-glycosidically to the *N*-terminal amino acid.

Keywords nephritogenoside; ion-exchange resin; amido linkage; amino acid sequencer; HPLC; dansylation; carboxypeptidase

We have isolated a glycopeptide named nephritogenoside, which is a minor component of the glomerular basement membrane of normal animals (2—3 μg/kidney).[1,2] Shibata and Nagasawa[3] have established a new experimental animal model for adult human progressive glomerulonephritis, which was induced in homologous animals by a single footpad injection of nephritogenoside and Freund's incomplete adjuvant. Six to eight months later the injected animals became afflicted with chronic glomerulonephritis (contracted kidney). Typical histological changes of contracted kidney were observed in 96—98% of the injected animals.[4] This finding has been confirmed by Nishii *et al.*[5]

From methylation analysis,[6] concanavalin A testing,[7] and carbon-13 nuclear magnetic resonance (^{13}C-NMR) data compared with those of related synthetic glycosylamine derivatives,[8] Shibata and Nakanishi[9] proposed, in 1980, the following structure for nephritogenoside: α-Glc(1→6)-β-Glc-(1→6)-α-Glc(1→N)-peptide. The number of amino acids (various kinds of common amino acids) is 21, and the amino acid profile is characterized by high contents of glycine and proline. There have been no examples reported of an α-*N*-glycosyl linkage in D-glucose glyco-peptides among natural compounds. In the field of nucleoside-type antibiotics, many *N*-glycosides have been reported.[10] However, among glycoproteins, almost all of the compounds reported up to now have an *O*-glycosidic linkage between the sugar portion and the amino acids. As a compound with an *N*-glycosidic linkage, only *N*-acetylglucosaminyl-asparagine is known. In 1983, Wieland *et al.* found an asparaginylglucose having an *N*-glycosidic linkage between glucose and an asparagine from *Halobacterium* strain M_1,[11] but this linkage was found to be β-*N*-glycosidic.

In our previous paper,[12] we reported a tentative structure of the peptide portion of nephritogenoside. However, the exact sequence at positions 4, 7, 13, 16 and 19 was not suggested at that time. In this paper, we describe the total structure of nephritogenoside.

We wished to isolate the peptide moiety from nephritogenoside to analyze its structure, because it has been difficult to determine the amino acid sequence of nephritogenoside and the site of carbohydrate attachment in the glycopeptide. We reported the cleavage of the amido linkage of various glycosylamine derivatives,[13] such as *N*-(L-γ-glutamyl)-α-D-glucopyranosylamine and *N*-(L-β-aspartyl)-α-D-glucopyranosylamine, by Amberlite IRA-410 (OH^-), as evidenced by the appearance of a glycosyl amine and related amino acid and the disappearance of the starting amide. This resin treatment was successfully applied to nephritogenoside. After the cleavage with the resin of the amido linkage between carbohydrate and peptide of nephritogenoside, the part of the aqueous eluate which showed a color reaction with anthrone–sulfuric acid reagent was discarded. Then, the 1 N HCl eluate, which did not show a color reaction with anthrone–sulfuric acid reagent, was lyophilized and chromatographed on a column of Sephadex G-100. The effluent was collected in 10 ml fractions. The

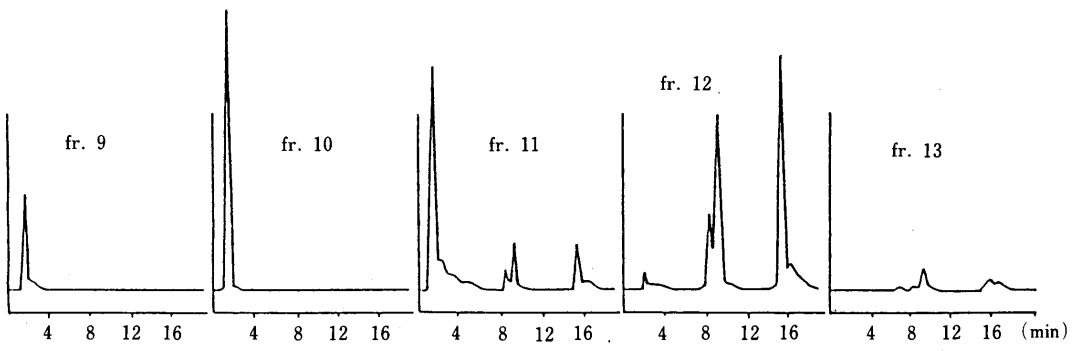

Fig. 1. Chromatogram of OPA-Derivatized Peptide

column effluent was monitored by measuring the absorbance at 210 nm with a UV detector. From fractions 9 to 13, a broad single peak was observed. Each fraction of this peak was analyzed by high-performance liquid chromatography (HPLC) using a column of ISC-07/S-1504. As shown in Fig. 1, fractions 9 and 10 (fr. 9, fr. 10) were shown by HPLC (with a fluorescence detector) to be homogeneous. The method, which involves derivatization of the amino acid with *O*-phthalaldehyde (OPA), is extremely sensitive.[14] The recovery of fr. 10 was 32% starting from nephritogenoside. Fraction 10, which was obtained by the cleavage utilizing ion-exchange resin treatment, was concluded to contain only the peptide portion of nephritogenoside. Fraction 10 was hydrolyzed with 6 N HCl for 24 h at 110 °C and the acid hydrolysate gave Asp_2, Thr_1, Ser_1, Glu_2, Pro_3, Gly_6, Leu_1, Ile_2, Ala_1, Val_1 and Phe_1. There were 21 amino acid residues in all and the composition coincided with that of nephritogenoside. The *N*-terminal sequence analysis of fr. 10 was performed by sequential Edman degradation in the presence of polybrene using an Applied Biosystems sequencer, model 470A.[15] The sequence determined is as follows[16]: ^{1}Asp–Pro–Leu–Phe–^{5}Gly–Ile–Ala–Gly–Glu–^{10}Asp–Gly–Pro–Thr–Gly–^{15}Pro–Ser–Gly–Ile–Val–^{20}Gly–^{21}Gln. The N- and C-terminal amino acids of the peptide portion have been confirmed to be aspartic acid and glutamine, respectively. Dansylated Asp was clearly detected as the N-terminal amino acid of the peptide portion by reverse-phase HPLC on Develosil C 18, using a linear gradient formed from Tris–HCl buffer (pH 7.75) and methanol.[17] The C-terminal amino acid of the peptide portion has been confirmed by carboxypeptidase Y[18] digestion to be glutamine, because the release of one mol of glutamine and very small amounts of glycine, valine and isoleucine was observed. Since we have reported that the peptide portion of nephritogenoside is directly bonded to the glucose chain, the following four binding types can be considered: 1) binding at the N-terminal Asp, 2) binding at the 9th Glu, 3) binding at the 10th Asp, 4) binding at the C-terminal Gln. To further elucidate this problem, nephritogenoside was treated with carboxypeptidase Y at pH 6.5 (37 °C) for 2 d, and then lyophilized. The sample thus obtained was applied to a column of Sephadex G-100. As shown in Fig. 2, five peaks of sugar and peptide (fractions 4, 7, 9, 11 and 16) were observed. The amino acid content in a 6 N HCl hydrolysate of each fraction was then examined. The amino acid content of fraction 4 (C-fr. 4) was the same as that of the starting material. In fraction 7 (C-fr. 7), Gly, Ile, Val, Gly and Gln residues were reduced in comparison to the starting material. In fraction 9 (C-fr. 9), Thr, Gly, Pro and Ser residues were reduced in comparison to C-fr. 7. In fraction 11 (C-fr. 11), Asp, Gly and Pro residues were less than in C-fr. 9. In fraction 16 (C-fr. 16), Leu, Phe, Gly, Ile, Ala, Gly, and Glu residues were less than in C-fr. 11. In this fraction we could find only Asp and Pro as amino acids. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of C-fr. 16 (δ, D_2O) exhibited three anomeric protons at 5.58 (d, J=4.02 Hz, H-1), 4.92 (d, J=4.02 Hz, H-1″), and 4.52 (d, J=7.96 Hz, H-1′) due to carbohydrate. The structure of C-fr. 16 was confirmed by the ^{13}C-NMR data. ^{1}H- and ^{13}C-NMR spectra were in accordance with those of synthetic α-Glc-(1→6)-β-Glc-(1→6)-α-Glc-(1→Asn–Pro).

Based on these results, nephritogenoside is now established as the glycopeptide in which the trisaccharide chain is α-*N*-glycosidically linked to the peptide portion through the amido nitrogen of the N-terminal asparagine residue (Fig. 3).

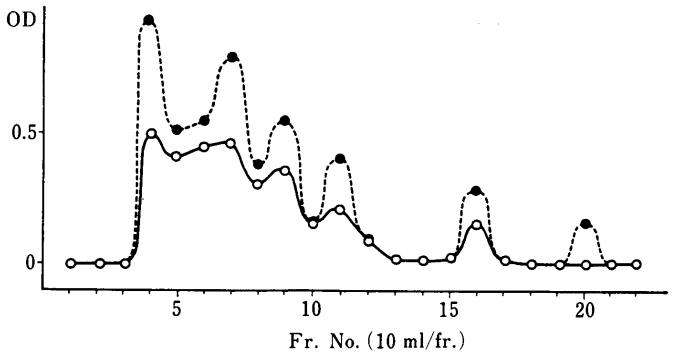

Fig. 2. Gel Filtration after Carboxypeptidase Y Treatment
—— 490 nm; ------ 210 nm.

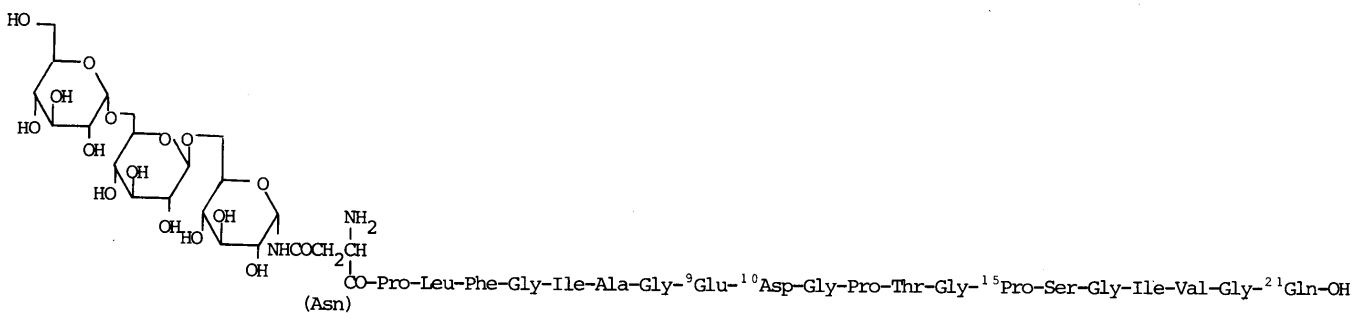

Fig. 3. Proposed Chemical Structure of Nephritogenoside

Experimental

General Methods The amino acid composition was determined by HPLC, on a Shimadzu LC-3A chromatograph, with a Shimadzu RF-530 fluorescence spectromonitor, after hydrolyzing the samples in 6 N HCl for 24 h at 110 °C in an evacuated sealed tube. A stainless-steel ISC-07/S1504 column (15 cm × 4 mm i.d.) was used. The detector settings were excitation 350 nm, emission 450 nm.

Ion-Exchange Resin Treatment Nephritogenoside (1.0 mg) was dissolved in 1 ml of water containing 2 g of Amberlite IRA-410 (OH^-), and stirred for 10 h. The 1 N HCl eluate was lyophilized to give a white powder, which was chromatographed on Sephadex G-100 (25 × 2.4 cm). The column effluent was monitored by measuring the absorbance at 210 nm with a ultraviolet (UV) detector, and the carbohydrate fraction was analyzed by the anthrone method at 490 nm.

Dansylation of Fr. 10 Dansyl amino acids and Dns–Cl were purchased from Seikagaku Kogyo Co., Tokyo. The HPLC apparatus consisted of a Shimadzu LC-6A liquid chromatograph with a linear gradient marker. A stainless steel column (250 × 4.0 mm i.d.) was packed with Develosil C 18

(Nomura Chemical Co., Seto, Japan). Fluorescence was monitored with a Shimadzu RF-530 spectrofluorophotometer. Excitation and emission wavelengths were 340 and 530 nm, respectively. The solvent consisted of 0.01 M Tris–HCl buffer (pH 7.75) and methanol (10:3 by volume). The flow rate of the mobile phase was 1.0 ml/min, which produced a column pressure of about 150 kg/cm^2. Column temperature was set at 50 °C. Dansylation for the NH_2-terminal analysis of fr. 10 was done according to the method of Gray[19] for proteins. Dansylated peptide was hydrolyzed with 6 N HCl in a sealed ampoule at 110 °C for 18 h. After the removal of the acid *in vacuo*, the residue was dissolved in 50 μl of methanol. The retention time of Dns–Asp was 11.1 min. In the case of Dns–OH and Dns–NH_2, which are the most abundant by-products of the dansylation reaction, the retention times are 25.0 and 60.0 min, respectively. Dns–Asp was clearly detected as the NH_2-terminal amino acid.

N-Terminal Sequence Analysis of Fr. 10 Fr. 10 was submitted to NH_2-terminal amino acid sequence analysis in the Applied Biosystems model 470A sequenator, and the following result was obtained: Asp–Pro–Leu–Phe–Gly–Ile–Ala–Gly–Glu–Asp–Gly–Pro–Thr–Gly–Pro–Ser–Gly–Ile–Val–Gly–Gln.

Carboxypeptidase Y Treatment Carboxypeptidase Y was purchased from Peptide Institute, Inc., Osaka, Japan. Nephritogenoside (1.0 mg) was dissolved in pH 6.5, 0.05 M *N*-ethylmorpholine at a concentration of 1 mg/50 ml and the solution was re-adjusted to pH 6.5. An *N*-ethylmorpholine solution (1 ml) of carboxypeptidase Y (0.1 mg) was added and the mixture was agitated gently at 37 °C for 48 h. At the end of the digestion period, the reaction mixture was taken to pH 6.0 and centrifuged to remove carboxypeptidase Y. The clear supernatant was lyophilized. The lyophilized product was chromatographed on a column of Sephadex G-100 (25 × 2.4 cm i.d.). The elution profile is shown in Fig. 2. The column effluent was monitored by the same methods as described above. The yields and the component ratios of each fraction are as follows: C-fr. 4; 0.32 mg, C-fr. 7; 0.18 mg, C-fr. 9: 0.14 mg, C-fr. 11; 0.09 mg, C-fr. 16; 0.07 mg. Amino acid ratios in a hydrolysate: C-fr. 4; Asp 2.08, Thr 1.03, Ser 1.02, Glu 2.07, Gly 6.00, Ala 1.02, Val 1.04, Ile 2.04, Leu 1.02, Phe 1.01, Pro 3.06 (recovery of Gly 94%), C-fr. 7; Asp 2.06, Thr 1.02, Ser 0.84, Glu 1.03, Gly 4.00, Ala 1.02, Ile 1.02, Leu 1.03, Phe 1.01, Pro 3.06 (recovery of Gly 93%), C-fr. 9; Asp 2.06, Glu 1.02, Gly 3.00, Ala 1.02, Ile 1.02, Leu 1.03, Phe 1.01, Pro 2.02 (recovery of Gly 92%), C-fr. 11; Asp 1.30, Glu 1.02, Gly 2.00, Ala 1.02, Ile 1.02, Leu 1.02, Phe 1.02, Pro 1.01 (recovery of Gly 96%), C-fr. 16; Asp 1.03, Pro 1.02.

^{1}H- and ^{13}C-NMR Spectrometry NMR spectroscopy of C-fr. 16 was performed on a JEOL GSX-400 MHz spectrometer. The sample was dissolved in 0.4 ml of D_2O. Chemical shifts in both the proton and carbon spectra were obtained by reference to internal 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt in D_2O ($\delta = 0.00$). The ^{1}H spectrum was taken with a pulse width of 8.2 μs and a computer memory of 16K. For the ^{13}C spectrum, a 1.5 s relaxation delay was employed.

Characterization of C-fr. 16 $[\alpha]_D^{23}$ +33.4° ($c = 0.014$, H_2O). ^{1}H-NMR (D_2O) δ: 5.58 (d, $J = 4.02$ Hz, H-1), 4.92 (d, $J = 4.02$ Hz, H-1″), 4.52 (d, $J = 7.96$ Hz, H-1′). ^{13}C-NMR (D_2O) δ: 77.6 (C-1), 70.4 (C-2), 72.6 (C-3), 70.4 (C-4), 73.0 (C-5), 69.5 (C-6), 103.8 (C-1′), 74.1 (C-2′), 77.0 (C-3′), 70.6 (C-4′), 75.4 (C-5′), 66.6 (C-6′), 99.0 (C-1″), 73.0 (C-2″), 74.2 (C-3″), 70.6 (C-4″), 74.2 (C-5″), 61.6 (C-6″), 49.7 (Asn-α), 35.5 (Asn-β), 59.5 (Pro-α), 29.0 (Pro-β), 24.4 (Pro-γ), 46.6 (Pro-δ).

Acknowledgements We wish to thank Mr. Y. Sengoku and Mr. S. Fukushima of Japan Scientific Instrument Co., Ltd. for determination of the amino acid sequence with the Applied Biosystems sequenator, model 470A. We also thank S. Kato for the ^{1}H- and ^{13}C-NMR measurements.

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