The Structure of Nephrigenoside

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Nephrigenoside 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 nephrigenoside is a simple glycopeptide composed of three glucose residues [α-Glc-(1→6)-β-Glc-(1→6)-Glc] and twenty-one amino acids [Asn-Pro-Leu-Phe-3-Gly-Ile-Ala-Gly-Glu-10-Asp-Gly-Pro-Thr-Gly-15-Pro-Ser-Gly-Ile-Val-20-Gly-23-Gln], and that the glucose residues are linked α-N-glycosidically to the N-terminal amino acid.

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

We have isolated a glycopeptide named nephrigenoside, which is a minor component of the glomerular basement membrane of normal animals (2–3 μg/kidney).1,2 Shibata and Nagasawa11 have established a new experimental animal model for adult human progressive glomerulonephritis, which was induced in homologous animals by a single footpad injection of nephrigenoside and Freund’s incomplete adjuvant. Six to eight months later the injected animals became afflicted with chronic glomerulonephritis (nephrinosis). Typical histological changes of contracted kidney were observed in 96–98% of the injected animals.41 This finding has been confirmed by Nishii et al.42

From methylation analysis,61 concanavalin A testing,71 and carbon-13 nuclear magnetic resonance (13C-NMR) data compared with those of related synthetic glycosylamine derivatives,81 Shibata and Nakanishi99 proposed, in 1980, the following structure for nephrigenoside: 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.100 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-acetylglicosaminyl-asparagine is known. In 1983, Wieland et al. found an asparagusinylglucose having an N-glycosidic linkage between glucose and an asparagine from Halobacterium strain M1,111 but this linkage was found to be β-N-glycosidic.

In our previous paper,121 we reported a tentative structure of the peptide portion of nephrigenoside. 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 nephrigenoside.

We wished to isolate the peptide moiety from nephrigenoside to analyze its structure, because it has been difficult to determine the amino acid sequence of nephrigenoside 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-(1-γ-glutamyl)-α-D-glucopyranosylamine and N-(1-β-asparagyl)-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 nephrigenoside. After the cleavage with the resin of the amido linkage between carbohydrate and peptide of nephrigenoside, 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

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Fig. 1. Chromatogram of OPA-Derivatized Peptide

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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. 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<sub>2</sub>, Thr<sub>1</sub>, Ser<sub>1</sub>, Glu<sub>2</sub>, Pro<sub>3</sub>, Gly<sub>6</sub>, Leu<sub>1</sub>, Ile<sub>2</sub>, Ala<sub>1</sub>, Val<sub>1</sub>, and Phe<sub>1</sub>. 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. The sequence determined is as follows:<sup>15</sup> Asp-Pro-Leu-Phe<sup>5</sup>Gly-Ile-Ala-Gly-Glu<sup>10</sup>Asp-Gly-Pro-Thr-Gly<sup>14</sup>Pro-Ser-Gly-Ile-Val<sup>20</sup>Gly<sup>21</sup>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 Devosol C 18, using a linear gradient formed from Tris-HCl buffer (pH 7.75) and methanol.<sup>17</sup> The C-terminal amino acid of the peptide portion has been confirmed by carboxypeptidase Y 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 (1H-NMR) spectrum of C-fr. 16 (δ, D₂O) 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 13C-NMR data. 1H- and 13C-NMR spectra were in accordance with those of synthetic α-Glc-(1→6)-β-Glc-(1→6)-α-Glc-(1→4)-Asn-Asp-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).

**Experimental**

**General Methods** The amino acid composition was determined by HPLC, on a Shimadzu LC-3A chromatograph, with a Shimadzu RF-530 fluorescence spectrometer, 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 x 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 x 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 x 4.0 mm i.d.) was packed with Devosol C 18.
Characterization of C-fr. 16  \[ \text{[\text{gly}]}^2 + 33.4' \ (c = 0.014, \text{H}_{2}\text{O}), \ \text{H-NMR} (\text{D}_{2}\text{O}) \delta : 5.58 (d, J = 4.02 \text{Hz}, \text{H}-1), 4.92 (d, J = 4.02 \text{Hz}, \text{H}-1'), 5.42 (d, J = 7.96 \text{Hz}, \text{H}-1), \ 13\text{C-NMR} (\text{D}_{2}\text{O}) \delta : 77.6 (\text{C}-1), 70.4 (\text{C}-2), 72.6 (\text{C}-3), 70.4 (\text{C}-4), 73.0 (\text{C}-5), 69.3 (\text{C}-6), 103.8 (\text{C}-7), 74.1 (\text{C}-2'), 77.0 (\text{C}-3'), 70.6 (\text{C}-4'), 75.4 (\text{C}-5'), 66.6 (\text{C}-6'), 99.0 (\text{C}-1'), 73.0 (\text{C}-2'), 74.2 (\text{C}-3'), 70.6 (\text{C}-4'), 74.2 (\text{C}-5'), 61.6 (\text{C}-6'), 49.7 (\text{Asn}-\alpha), 35.5 (\text{Asn}-\beta), 59.5 (\text{Pro}-\alpha), 29.0 (\text{Pro}-\beta), 24.4 (\text{Pro}-\gamma), 46.6 (\text{Pro}-\delta).

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