[Chem. Pharm. Bull. 32(9)3698—3701(1984)]

Selective Cleavage of the Amido Linkage of Glucopyranosylamine Derivatives by Ion-Exchange Resin Treatment[1)]

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(Received January 18, 1984)

The amido linkage of various glycosylamine derivatives, *N*-(L-γ-glutamyl)-α-, and -β-D-glucopyranosylamine, *N*-(L-β-aspartyl)-α- and -β-D-glucopyranosylamine and *N*-glycyl-α-D-glucopyranosylamine, can be cleaved by Amberlite IRA-410 (OH^-), as evidenced by the appearance of the aglycone amino acids and the disappearance of the starting amide.

Keywords——nephritogenoside; glucopyranoslylamine; amido linkage; ion-exchange resin; HPLC; fluorescence detection; ^{13}C-NMR

In a previous paper, we reported the synthesis of *N*-(L-β-aspartyl)-α-D-glucopyranosylamine,[2)] *N*-(L-γ-glutamyl)-α-D-glucopyranosylamine[3)] and two trisaccharide glucosylamine derivatives, *O*-α-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→6)-*N*-(L-β-aspartyl)-[4)] and -(L-γ-glutamyl)-α-D-glucopyranosylamine[3)] as models of nephritogenoside,[5)] a compound that has activity for the induction of glomerulonephritis in homologous animals, and which may be present in the glomerular basement membrane of rats.

The isolation of peptide-free oligosaccharides from glycoproteins and glycopeptides is desirable for the structural analysis of carbohydrate moieties. There have been several reports[6-12)] on the complete release of the asparagine-bound oligosaccharides in glycoproteins. Hydrazinolysis was originally described by Matsushima and Fujii,[6)] and later developed by Bayard and Montreuil[7)] and also Kobata,[8)] Osawa[9)] and others to cleave specifically the GlcNAc→Asn linkage. Lee *et al.*[10)] have described the cleavage by alkaline borohydride treatment. Enzymic methods liberate asparagine-linked carbohydrate chains as oligosaccharides. For example *endo*-β-*N*-acetylglucosaminidases[11)] and almond glycopeptidase[12)] have been used to determine the structures of hen egg albumin and stem bromelain glycopeptide.

We wished to isolate peptide(s) from nephritogenoside, which has a new type of carbohydrate-protein linkage among natural compounds (a direct *N*-glycosyl linkage between a glycosyl and an amino acid residue) for structural analysis of the peptide moiety. In preliminary experiments, we studied several synthetic model compounds. We now report the cleavage of amido linkage between glycosylamine and aspartic acid, glutamic acid and/or glycine by ion-exchange resin treatment. *N*-(L-γ-Glutamyl)-α-D-glucopyranosylamine was passed through a column of Amberlite IRA-410 (OH^-) and the column was washed with distilled water. α-D-Glucopyranosylamine ($[\alpha]_D$ +112.5°) was obtained quantitatively; the proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a signal due to the anomeric proton at 5.60 ppm (d, $J=4$ Hz), and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum showed an anomeric carbon atom at δ 77.3 ppm. Elution with 2 N HCl gave L-

glutamic acid, which was identified by high-performance liquid chromatography (HPLC) with a fluorescence detector. The method, which involves derivatization of the amino acid with *O*-phthalaldehyde (OPA), is extremely sensitive.[13] A reaction time of only 5 h is sufficient to effect the complete release of the glutamine-bound glycosylamine. In the case of *N*-(L-γ-glutamyl)-β-D-glucopyranosylamine, L-glutamic acid was released more slowly than in the case of the α-isomer. After resin treatment for approximately 96 h, β-D-glucopyranosylamine, ($[\alpha]_D$ +13.8°) was obtained quantitatively; the ^{1}H-NMR spectrum showed a signal due to the anomeric proton at 5.06 ppm (d, J=8 Hz), and the ^{13}C-NMR spectrum showed an anomeric carbon at δ 80.4 ppm.

In order to determine the reactivity for the release of amino acid, we treated *N*-(L-β-aspartyl)-α- and -β-D-glucopyranosylamine with ion-exchange resin in a batchwise operation. Both the above compounds and aspartic acid were determined by HPLC. A typical chromatogram showing the separation of *N*-(L-β-aspartyl)-α-D-glucopyranosylamine is shown in Fig. 1(a). The result with *N*-(L-β-aspartyl)-β-D-glucopyranosylamine is shown in Fig. 1(b). The amounts of aspartic acid released from glucosylamine derivatives at various times during ion-exchange resin treatment are shown in Figs. 2 and 3. The amount of aspartic acid released from the α-anomer as determined by HPLC after resin treatment reached a

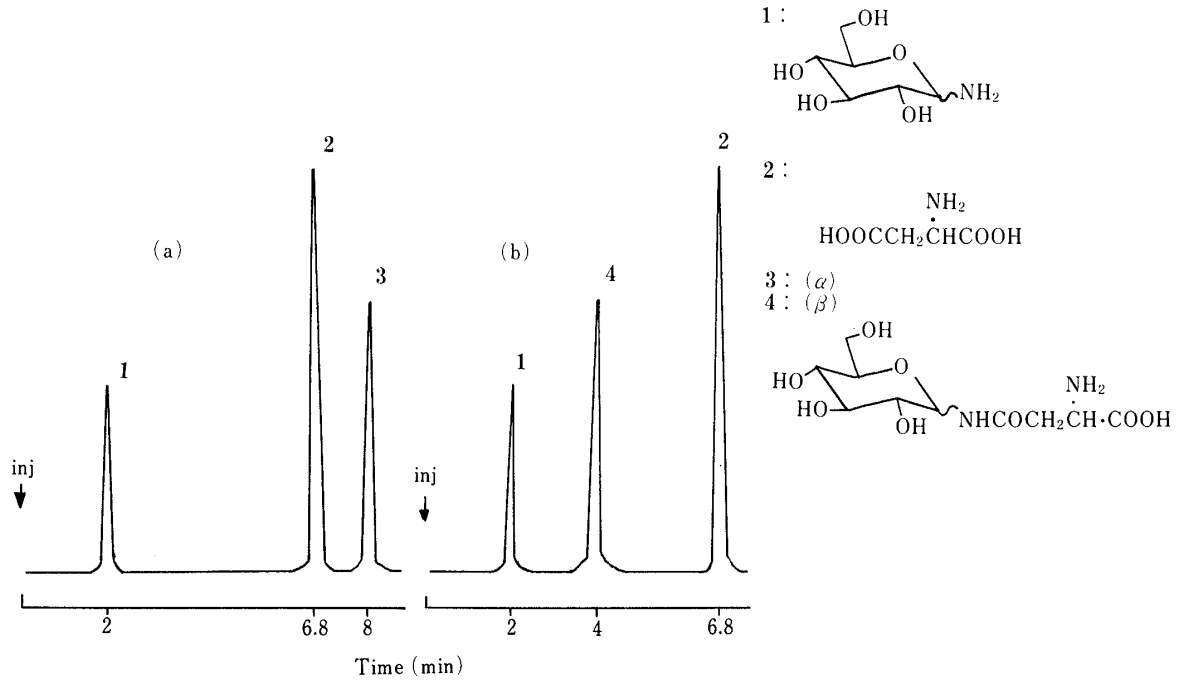

Fig. 1. Chromatogram of OPA-Derivatized Amino Acid and Related Compounds

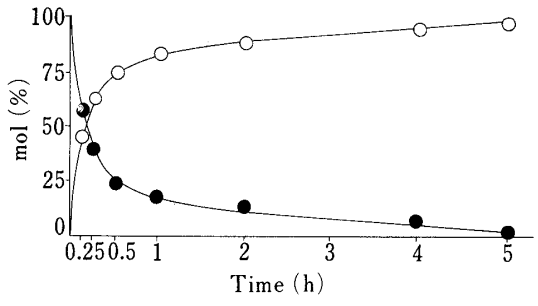

Fig. 2. Release of L-Aspartic Acid Obtained by Ion-Exchange Resin Treatment of *N*-(L-β-Aspartyl)-α-D-glucopyranosylamine

○—○, L-aspartic acid; ●—●, starting material.

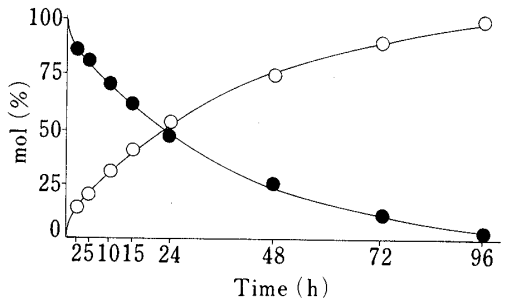

Fig. 3. Release of L-Aspartic Acid Obtained by Ion-Exchange Resin Treatment of *N*-(L-β-Aspartyl)-β-D-glucopyranosylamine

○—○, L-aspartic acid; ●—●, starting material.

maximum at approximately 5 h (Fig. 2). The starting material was rapidly converted into α-D-glucopyranosylamine and aspartic acid, and the starting material had almost disappeared at 5 h. On the other hand, as shown in Fig. 3, the reaction of the β-anomer is very slow. After 5 h of resin treatment, about 20% of the asparagine-linked glucopyranosylamine derivative was cleaved. After 96 h the starting material had almost disappeared. Similarly, *N*-glycyl-α-D-glucopyranosylamine, which was prepared as described later, was passed through a column of Amberlite IRA-410 (OH^-). α-D-Glucopyranosylamine and glycine were obtained quantitatively. After 12 h *N*-glycyl-α-D-glucopyranosylamine had disappeared.

N-Glycyl-α-D-glucopyranosylamine (**3**) was synthesized as follows. Condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosylamine with *N*-benzyloxycarbonyl-glycine in the presence of diethylphosphorocyanidate gave 2,3,4,6-tetra-*O*-acetyl-*N*-benzyloxycarbonyl-glycyl-α-D-glucopyranosylamine (**1**) in 69.7% yield. The ^{13}C-NMR spectrum showed the anomeric carbon atom at 73.9, and the carbon of the glycine residue at 42.6 ppm. Removal of the benzyloxycarbonyl group of **1** by catalytic hydrogenation afforded 2,3,4,6-tetra-*O*-acetyl-*N*-glycyl-α-D-glucopyranosylamine (**2**). Subsequently, **2** was de-*O*-acetylated with triethylamine in 50% methanolic solution at room temperature to give *N*-glycyl-α-D-glucopyranosylamine (**3**) ($[\alpha]_D$ +93.9°). The ^{1}H-NMR spectrum showed anomeric proton at δ 5.57, and the ^{13}C-NMR data showed the anomeric carbon atom at δ 77.8 ppm.

Anion-exchange resin Amberlite IRA-410 does not split of the peptide bond of glycyl-glycine or glycyl-glycyl-glycine under the same conditions. Whitaker and Deatherage have detected little or no hydrolysis of insulin on the anion-exchange resins Amberlite IH-4B, Amberlite XE-67, and Dowex 2, even after heating under reflux for 150 h.[14]

This resin treatment procedure was applied to the *N*-acetylglucosaminylasparagine and -glutamine linkages, but these linkages were not cleaved upon gentle shaking with Amberlite IRA-410 (OH^-). This method does not have wide applicability at the present stage, but it might be applicable to nephritogenoside.

Experimental

General Methods——For HPLC a Shimadzu LC-3A chromatograph with a Shimadzu RF-530 fluorescence spectromonitor was employed. The column was housed in a Shimadzu CTO-2A column oven at 60 °C. The flow of the mobile phase was controlled by means of a Shimadzu SGR-1A step gradient elution unit. Mobile phases: A, 6% ethanol in 0.2 N sodium citrate (pH 3.2); B, 0.2 N sodium citrate (pH 4.25); C, 0.6 N sodium citrate (pH 6.0); D, 0.6 N sodium citrate (pH 10.5); E, 0.2 N NaOH Stepwise gradient program of mobile phase: A, 24 min; B, 11 min; C, 13 min; D, 11 min; E, 5 min. A stainless-steel ISC-07/S1504 column (15 cm × 4 mm i.d.) was used. The flow rate was 0.5 ml/min. The detector settings were excitation 350 nm, emission 450 nm. Reagent A was 0.1% NaClO solution in 0.3 M borate buffer (pH 10.5). Reagent B was OPA reagent (400 mg of OPA and 1 ml of 2-mercaptoethanol in 500 ml of 0.3 M borate buffer (pH 10.5). ^{1}H-NMR spectra were recorded with a JNM MH-100 spectrometer, and ^{13}C-NMR spectra with an FX-100 instrument, tetramethylsilane being used as an external standard in both cases. Optical rotations were measured with a JASCO DIP-4 digital polarimeter.

Materials——*N*-(L-β-Aspartyl)-α- and -β-D-glucopyranosylamine and *N*-(L-γ-glutamyl)-α- and -β-D-glucopyranosylamine were obtained by the procedures described in the previous paper.[2,3] *N*-(L-β-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine and *N*-(L-γ-glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine were obtained by the procedure of Tsukamoto *et al.*[15] The basic physical and chemical properties were coincident with those reported earlier. Glycyl-glycine and glycyl-glycyl-glycine were purchased from the Protein Research Foundation, Osaka, Japan.

α-D-Glucopyranosylamine——A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosylamine (40 mg) in 50% methanolic solution (4 ml) was treated with 0.7 ml of triethylamine and the mixture was kept overnight. The solution was concentrated *in vacuo* to provide a syrup (19 mg, 92%), $[\alpha]_D^{22}$ +112.5° (c=0.5, H_2O), ^{1}H-NMR (D_2O) δ: 5.60 (d, J=4 Hz, H-1), ^{13}C-NMR (D_2O) δ: 77.3 (C-1), 70.2 (C-2), 73.4 (C-3), 70.2 (C-4), 73.9 (C-5), 61.4 (C-6), *Anal.* Calcd for $C_6H_{13}NO_5$: C, 40.22; H, 7.31; N, 7.82. Found: C, 40.35; H, 7.42; N, 7.89.

β-D-Glucopyranosylamine——β-D-Glucopyranosylamine was obtained from 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine in the same manner described above, $[\alpha]_D^{25}$ +13.8° (c=0.5, H_2O), ^{1}H-NMR (D_2O) δ: 5.06 (d, J=8 Hz, H-1), ^{13}C-NMR (D_2O) δ: 80.4 (C-1), 72.7 (C-2), 77.5 (C-3), 70.4 (C-4), 77.7 (C-5), 61.5 (C-6), *Anal.*

Calcd for $C_6H_{13}NO_5$: C, 40.22; H, 7.31; N, 7.82. Found: C, 40.28; H, 7.45; N, 7.90.

2,3,4,6-Tetra-*O*-acetyl-*N*-(*N*-benzyloxycarbonyl-glycyl)-α-D-glucopyranosylamine (1)——*N*-Benzyloxycarbonyl-glycine (150 mg), DEPC (170 mg), and 0.2 ml of triethylamine were added to a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosylamine (200 mg) in THF (18 ml). The mixture was stirred for 12 h at 0 °C. The reaction mixture was diluted with AcOEt (30 ml), and successively washed with 5% HCl, water, saturated $NaHCO_3$, and saturated NaCl. Drying followed by evaporation gave a syrup (**1**) (216 mg, 69.7%); $[\alpha]_D^{25}$ +78.1° ($c=0.5$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 7.32 (br s, 5H, Arom. H), 5.10 (s, 2H, $-CH_2-$ ), 2.00 (s, 6H, 2×OAc), 2.04 (s, 6H, 2×OAc). ^{13}C-NMR ($CDCl_3$) δ: 73.9 (C-1), 68.3 (C-2), 69.5 (C-3), 67.3 (C-4), 70.0 (C-5), 61.8 (C-6), 42.6 (Gly C_α).

2,3,4,6-Tetra-*O*-acetyl-*N*-glycyl-α-D-glucopyranosylamine (2)——A solution of **1** (100 mg) in EtOH (8 ml) was hydrogenated in the presence of 10% Pd/C (30 mg) for 12 h at room temperature. The catalyst was filtered off, and the filtrate evaporated to dryness to give **2** (70 mg, 93%); $[\alpha]_D^{24}$ +85.7° ($c=0.8$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 2.05 (s, 6H, 2×OAc), 2.08 (s, 3H, OAc), 2.10 (s, 3H, OAc). ^{13}C-NMR ($CDCl_3$) δ: 73.2 (C-1), 68.4 (C-2), 70.0 (C-3), 67.4 (C-4), 70.6 (C-5), 62.0 (C-6), 46.4 (Gly C_α).

***N*-Glycyl-α-D-glucopyranosylamine (3)**——Triethylamine (0.1 ml) was added to a solution of 2 (70 mg) in 50% methanol (20 ml) and the mixture was kept overnight at room temperature. The solution was concentrated *in vacuo* to provide a syrup, yield 35 mg (85.4%); $[\alpha]_D^{22}$ +93.9° ($c=0.9$, H_2O); ^{1}H-NMR (D_2O) δ: 5.57 (d, $J=4$ Hz, H-1). ^{13}C-NMR (D_2O) δ: 77.8 (C-1), 70.5 (C-2), 73.7 (C-3), 70.5 (C-4), 74.2 (C-5), 61.7 (C-6), 47.9 (Gly C_α). *Anal.* Calcd for $C_8H_{16}N_2O_6$: C, 40.68; H, 6.83; N, 11.86. Found: C, 40.56; H, 6.78; N, 11.99.

Ion-Exchange Resin Treatment——The five kinds of glucosylamine derivatives and two kinds of 2-acetamido-2-deoxy-glucosylamine derivatives and two kinds of peptide (each 1.8 mg) were each dissolved in 1 ml of water containing 2 g of Amberlite IRA-410 (OH^-), and stirred for various periods. Then 50 ml of 2 N HCl was added and the resin was filtered off. The filtrate was freeze-dried. The residue was dissolved in 100 μl of water and 2 μl aliquots were injected into the HPLC column. The peak area was measured using a Shimadzu Chromatopac CE-1B.

Release of L-Aspartic Acid Obtained by Ion-Exchange Resin Treatment of *N*-(L-β-Aspartyl)-α-D-glucopyranosylamine——7.5 min; 44%, 15 min; 62%, 30 min; 73%, 1 h, 84%, 2 h; 86%, 4 h; 92%, 5 h; 100%.

***N*-(L-β-Aspartyl)-β-D-glucopyranosylamine**——2 h; 14%, 5 h; 23%, 10 h; 30%, 15 h; 38%, 24 h; 51%, 48 h; 74%, 72 h; 90%, 96 h; 100%.

***N*-Glycyl-α-D-glucopyranosylamine**——2 h; 3%, 4 h; 9%, 6 h; 68%, 8 h; 85%, 10 h; 92%, 12 h; 100%. The glycosylamine, glycine and the starting material were identified by HPLC comparison with authentic samples (HPLC retention times, 2 min, 13.3, 13.3 min, respectively). In order to determine the reactivity for the release of glycine, the starting material was eluted with water and glycine was eluted with 2 N HCl from column.

***N*-(L-β-Aspartyl)-, *N*-(L-γ-Glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine**——The retention times of *N*-(L-β-aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine, *N*-(L-γ-glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine and 2-acetamido-2-deoxy-β-D-glucosylamine under the present HPLC conditions were 6.8 min, 8.1 min and 27.6 min, respectively.

Glycyl-glycine, Glycyl-glycyl-glycine——The retention times of glycyl-glycine and glycyl-glycyl-glycine under the present HPLC conditions were both 20.0 min.

Acknowledgement A part of this study was supported by Suzuken Memorial Foundation. We thank S. Kato for the ^{1}H-NMR and ^{13}C-NMR spectral measurements.

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