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Mutual separation of hinge-glycopeptide isomers bearing five *N*-acetylgalactosamine residues from normal human serum immunoglobulin A1 by capillary electrophoresis

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Abstract

Immunoglobulin A1 (IgA1) from normal human serum is known to have *O*-linked sugar chains, sialylated Galβ1,3GalNAc, in the hinge portion. In order to reduce the microheterogenity of the sugar chain, the hinge glycopeptide prepared from IgA1 was sequentially treated with neuraminidase and β-galactosidase. The asialo-, agalacto-hinge glycopeptide (HGP-SG) composed of a 33-mer peptide (HP33) and *N*-acetylgalactosamine (GalNAc) residues was obtained. The HGP-SG was separated into three major peaks, A, B and C, by high-performance liquid chromatography (HPLC). Each glycopeptide fraction was further separated by capillary electrophoresis (CE). Peaks A, B and C with HPLC abundantly contained HP33 bearing five and six *N*-acetylgalactosamine residues (HGP33-5,6GN), HGP33-4,5GN and HGP33-3,4GN, respectively. Among these glycopeptide peaks, only the HGP33-5GN peak was partly split into two peaks based on the CE analysis – HGP33-5GN-α and -β. The glycopeptide, HGP25-5GN shortened by the thermolysin digest of HGP33-SG was also well separated into the α and β forms by CE analysis. No differences in their mass and peptide portion were observed between HGP25-5GN-α and -β. Therefore, the obtained result might indicate that HGP25-5GN-α was an isomer of HGP25-5GN-β differing in its stereospecific structure of the peptide portion and/or the attachment site of the GalNAc residue. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Immunoglobulin A1; Hinge glycopeptides; Oligosaccharides

1. Introduction

Human serum immunoglobulin A1 (IgA1) is one of the most exceptional glycoproteins among the serum glycoproteins because it has *O*-linked oligosaccharides in its hinge portion in addition to the *N*-linked carbohydrate chains in its structure [1–3]. In our previous report, the glycoform of the *O*-linked oligosaccharide of the IgA1 subclass from a healthy control and from myeloma patients was analyzed. Three glycoforms for IgA1 from myeloma patients and only one glycoform from healthy individuals were found [4]. However, IgA1 from a healthy individual could be further fractionated dependent on

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its different affinity toward jacalin and its different heat stability. These subfractions had mutually different glycoforms of the *O*-linked oligosaccharide. Aggregated IgA1 was abundant in asialo-Gal-β1,3GalNAc, and the heat-stable IgA1 subfraction was abundant in a sialylated disaccharide [5]. These results suggested the possibility that the incomplete structure of the hinge *O*-linked oligosaccharide is related to the stability of IgA1 molecule and is one of the causes of the production of such an aggregated IgA1. Additionally, we found a phenomenon in which the enzymatic removal of neuraminic acid from normal human serum IgA1 induced the self-aggregation of part of the IgA1 [6,7].

IgA nephropathy is a common disease characterized by predominant IgA deposits in the renal mesangium. It is well-known that IgA1 among two subclasses, IgA1 and IgA2, is a dominant deposit in the glomeruli during IgA nephropathy [8,9]. The most prominent structural difference between the IgA1 and IgA2 subclasses was the duplicated proline-rich hinge portion and the characteristic *O*-linked oligosaccharide chains on the IgA1 hinge portion. There are many reports on the presence of an incompletely glycosylated *O*-linked oligosaccharide(s) on the IgA1 hinge region in some of the IgA nephropathy patients [10–20].

In this report, capillary electrophoresis (CE) of the asialo-, agalacto-hinge glycopeptide was carried out. Hinge glycopeptides (HGPs) having three to six GalNAc residues were clearly separated from each other. Among them, only an HGP having five GalNAc residues was further separated into two components having a common mass number and a common peptide portion. Thus, the presence of isomeric structures of a major hinge glycopeptide was first described for normal human serum IgA1.

2. Materials and methods

2.1. Materials

The following compounds and materials were commercially obtained: normal human serum was from China Newtech Development and Trade and was stored at −20°C. PD-10 and Sephacryl S-300 were from Pharmacia Biotech (Uppsala, Sweden); Jacalin-agarose was from Vector Labs. (Burlingame, USA); 4-vinyl pyridine was from Aldrich (Milwaukee, WI, USA); α-*N*-acetylgalactosaminidase (EC 3.2.1.49) from *Acremonium* sp. was from Seikagaku (Tokyo, Japan); neuraminidase (EC 3.2.1.18) from *Arthrobacter ureafaciens* was from Boehringer Mannheim (Germany); β-galactosidase (EC 3.2.1.23) from bovine testes and trypsin (EC 3.4.21.4) were from Sigma (St. Louis, MO, USA); and thermolysin (EC 3.4.21.14) was from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of IgA1 by jacalin-agarose affinity chromatography

Unless otherwise stated, the jacalin-agarose affinity chromatography was carried out at room temperature [5]. Ten ml of serum was applied to the jacalin column (6×1.5 cm I.D.) and washed with 0.1 *M* Tris–HCl buffer, pH 7.6, containing 0.02% sodium azide at a flow-rate of 2 ml/5 min. The thoroughly washed column was first eluted with 0.8 *M* glucose (50 ml) and then with 0.8 *M* galactose (50 ml) in the above buffer. The IgA1 fraction obtained on stepwise elution with galactose was dialyzed against distilled water and then lyophilized.

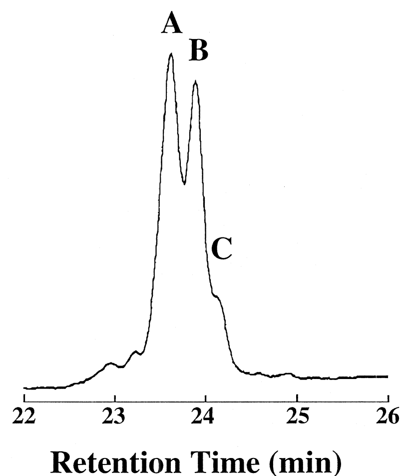

Fig. 1. HPLC analysis of asialo-, agalacto-hinge glycopeptide (HGP33-SG). Asialo-, agalacto-hinge glycopeptide (HGP33-SG) was separated into three peaks, A, B and C, by reversed-phase HPLC. Purified subfractions were subjected to further analysis.

2.3. Preparation of S-pyridylethylated α1 chain from IgA1 [20]

About 2 mg of IgA1 was first desalted using a PD-10 column. The desalted sample was dissolved in 1 ml of 0.4 *M* Tris–HCl buffer, pH 8.6, containing 6 *M* guanidine–HCl and 0.2 *M* ethylenediaminetetra-acetic acid (EDTA). To dissociate the disulfide linkage, 10.8 μl of dithiothreitol solution (200 mg/ml) was added, and stirred. After heating at

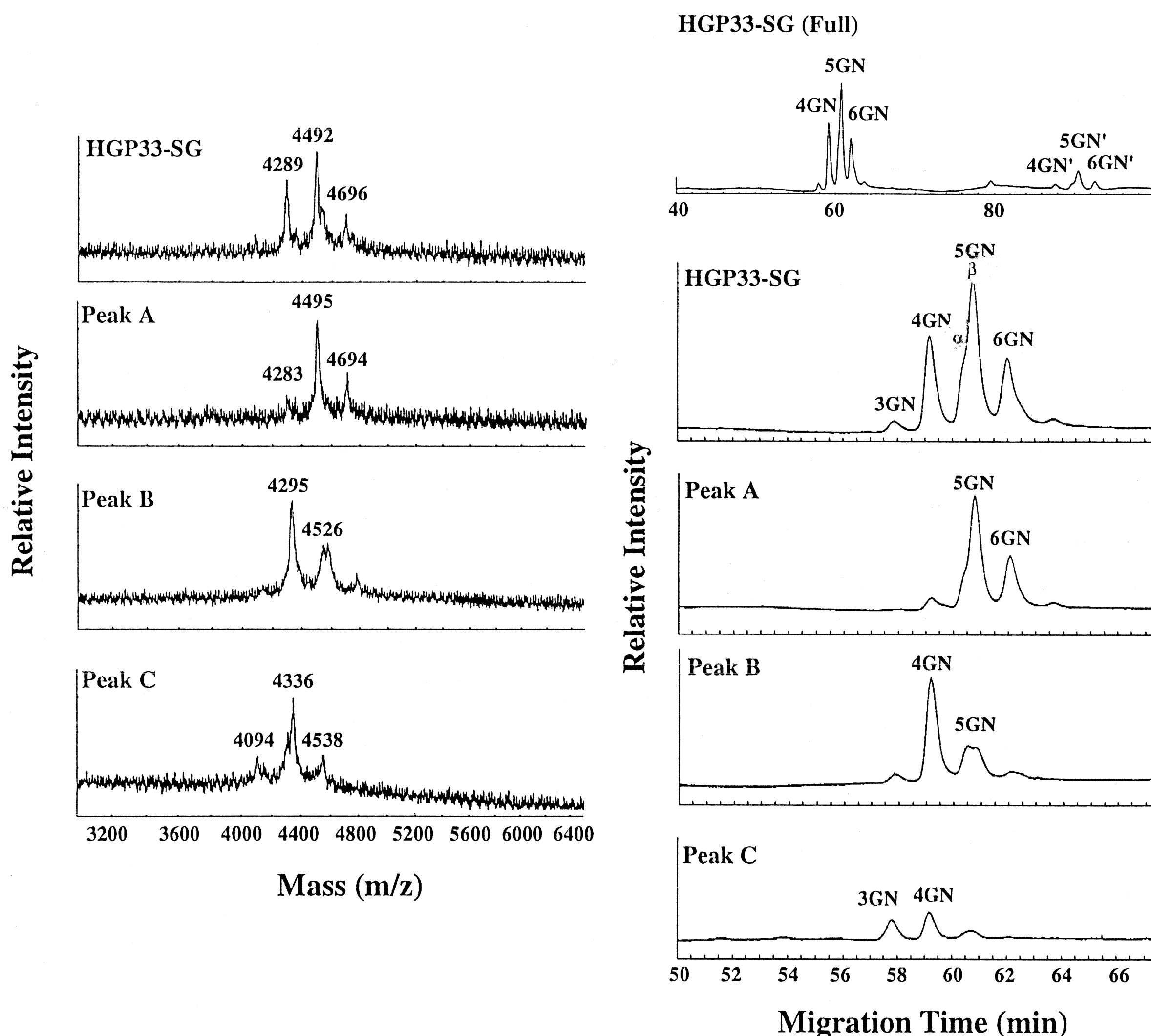

Fig. 2. MALDI–TOF-MS analysis and CE of HGP33-SG and three peaks A, B and C from HGP33-SG with HPLC. Left: HGP33-SG and the peaks A, B and C with HPLC (Fig. 1) were analyzed by MALDI–TOF-MS analysis. The observed mass number for the each peak corresponds to the calculated mass numbers, 4690.8, 4490.8, 4287.6 and 4084.4, for HP33 (HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPS) having six, five, four and three GalNAc residues, respectively. Right: HGP33-SG (Full); CE of HGP33-SG indicated the presence of the major peaks, assigned as 3GN, 4GN, 5GN and 6GN, and additional minor peaks, 4GN′, 5GN′ and 6GN′. These additional peaks correspond to the formylated by-products produced during preparation of HGP33. Indications of α and β on the 5GN peak are explained in the text. HGP33-SG, peaks A, B and C; only major peaks on CE are displayed for the original HGP33-SG and peaks A, B and C with HPLC.

50°C for 4 h, 3.2 μl of 4-vinyl pyridine was added and the reaction mixture was allowed to stand for 90 min at room temperature. The reaction was terminated by the addition of 100 μl of 2.0 *M* formic acid.

The α-chain dissociated as described above was fractionated by high-performance liquid chromatography (HPLC) using a Cosmosil 5C4-300 column (Nacalai Tesque, 150×4.6 mm) equipped with Shimadzu LC-4A. Elution was carried out by a linear gradient for 60 min from 10% to 90% acetonitrile in 0.1% trifluoroacetic acid (TFA). The material eluted at the peak position around 30 min. was collected and concentrated by a SpeedVac concentrator.

2.4. Preparation of hinge glycopeptide from a trypsin digest of the heavy chain [20]

About 0.5 mg of heavy chain was dissolved in 160 μl of 50 m*M* Tris–HCl buffer, pH 8.0, containing 2.0 *M* urea. Twenty μl of trypsin solution (10 μg trypsin/20 μl of the above buffer) and 20 μl of 0.1 *M* $CaCl_2$ were added, and the reaction mixture was incubated overnight at 37°C. The trypsin digest was made up to 1ml by adding 0.8 ml of 0.175 *M* Tris–HCl buffer, pH 7.6. The sample was applied to a jacalin agarose column (2 ml), and the passed fraction was eluted with 6 ml of the above buffer. After further washing the column with 6 ml of the buffer, the hinge glycopeptide fraction was eluted with 6 ml of 0.1 *M* melibiose in the buffer. Purification of the glycopeptide by HPLC was carried out using a Cosmosil 5C18-300 column (150×4.6 mm). Elution was carried out using a linear gradient for 60 min. from 0% to 90% acetonitrile in 0.1% TFA. Detection was performed by monitoring UV absorption at 220 nm. The material eluted at the peak position of the hinge glycopeptide (HGP33) around 23 min. was collected and concentrated as above.

2.5. Treatment of glycopeptide (HGP33) with exoglycosidases [20,21]

Purified HGP33 was dissolved in 95 μl of 0.2 *M* acetate buffer, pH 5.0. Five microliters of neuraminidase from *A. ureafaciens* (1 U/100 μl) was added to the solution followed by incubation at 37°C overnight. The asialo-glycopeptide (HGP33-S) was then treated with β-galactosidase. The sample was dissolved in 30 μl of 0.2 *M* sodium acetate buffer, pH 5.0, and then 20 μl of β-galactosidase from bovine testes (1.0 U/ml) was added to the sample. The reaction mixture was incubated overnight at room temperature. The asialo-glycopeptide (HGP33-S) and asialo-, agalacto-glycopeptide (HGP33-SG) were purified by HPLC using a Cosmosil 5C18-300 column (150×4.6 mm I.D.) under the same conditions described above.

2.6. Treatment of the asialo-, agalacto-glycopeptide (HGP33-SG) with thermolysin

Purified HGP33-SG was dissolved in 10 μl of thermolysin solution (0.1 mg/ml – 100 m*M* ammonium bicarbonate). The reaction mixture was incubated 2 h at room temperature. HPLC purifica-

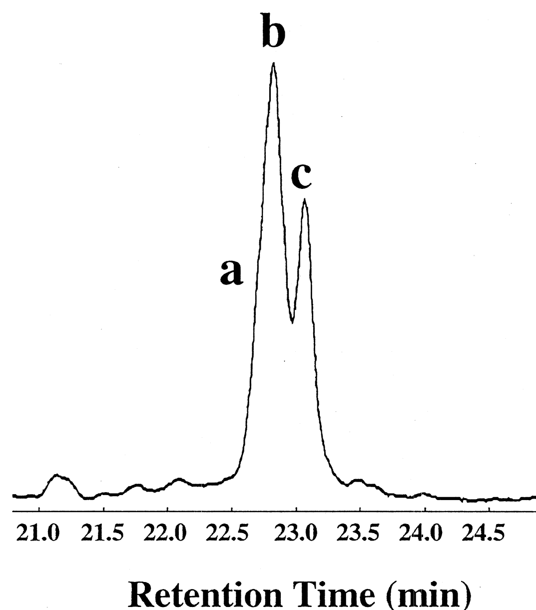

Fig. 3. HPLC analysis of HGP25-SG prepared by the digestion of HGP33-SG with thermolysin. The peptide portion of HGP33-SG was shortened to HGP25-SG by thermolysin digestion. Peaks a, b and c were prepared from the HGP25-SG composed of HP25 (VTVPCPVPSTPPTPSPSTPPTPSPS) and GalNAc residues.

tion of HGP25-SG was carried out as described above.

2.7. Treatment of HGP25-SG with α-N-acetyl-galactosaminidase

Purified HGP25-SG was dissolved in 83 μl of 50 m*M* sodium citrate buffer, pH 4.5, and then 17 μl of α-*N*-acetylgalactosaminidase from *Acromonium* sp. was added to the solution. The incubation was performed overnight at room temperature. HPLC purification of the peptides was carried out as above.

2.8. CE of hinge glycopeptide

CE experiments were performed on a Hewlett-Packard (Waldbronn, Germany) Model ^{3D}CE capillary electrophoresis instrument that was equipped with a capillary cartridge containing a 80.5 cm (effective length 72 cm)×75 μm I.D. Hewlett-Packard CEP coated capillary. The detection wavelength

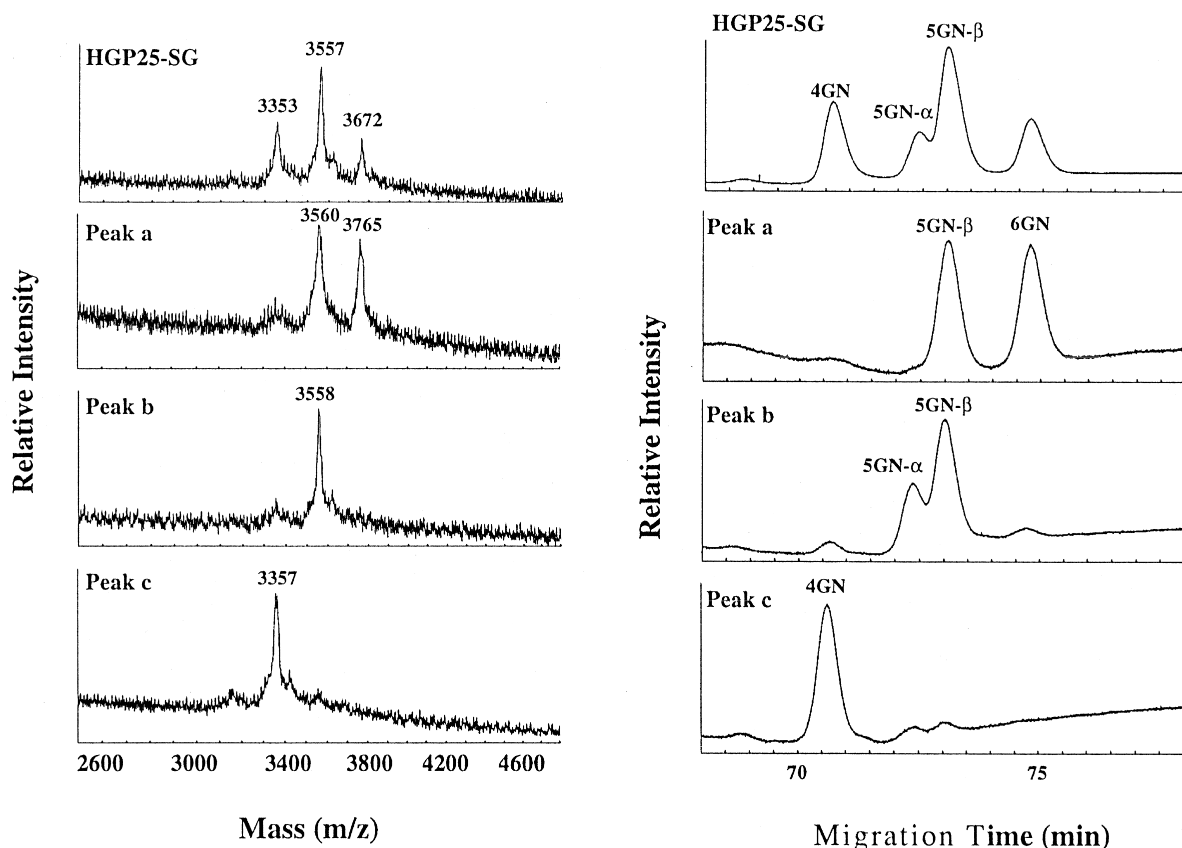

Fig. 4. MALDI–TOF-MS analysis and CE of HGP25-SG, and three peaks, a, b and c from HGP25-SG with HPLC. Left: HGP25-SG and the Peaks a, b and c with HPLC (Fig. 3) were analyzed by MALDI–TOF-MS analysis. The observed mass number for the each peak corresponds to the calculated mass numbers, 3751.1, 3547.9 and 3344.7, for HP25 (VTVPCPVPSTPPTPSPSTPPTPSPS) having six, five and four GalNAc residues, respectively. The deviation in the mass number will be due to the glycopeptide mixture exhibiting a different relative content of the glycopeptide and its formyl derivative (+60 form). Right: HGP25-SG, peaks a, b and c; only the major peaks on CE are displayed for the original HGP25-SG, and peaks a, b and c with HPLC. 4GN, 5GN and 6GN correspond to the HP25 having four, five and six GalNAc residues, respectively. 5GN was split into two peaks, named HGP25-5GN-α and -β.

was set at 214 nm and the operating temperature controlled at 40°C. Samples were loaded onto the capillary by pressure injection (200 mbar s). Electrophoresis was performed at a constant voltage of 20 kV using 100 m*M* sodium phosphate buffer (pH 2.5) as the running buffer.

2.9. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF-MS) analysis of glycosidase-treated hinge glycopeptide

The hinge glycopeptide was analyzed by MALDI–TOF-MS in the negative ion mode to suppress the production of the adduct ion. The mass spectrometer used in this work was a Finnigan Lasermat (Finnigan MAT, Hemel Hempstead, UK). The sample solution (0.5 μl) was mixed with 0.5 μl α-cyano-4-hydroxy cinnamic acid (10 mg/ml in acetonitrile–water, 70:30). The mixture (1 μl) was applied to the sample target and allowed to air-dry prior to MALDI–TOF-MS analysis. The mass spectra involved less than a 0.3% error.

3. Results and discussion

As previously reported [20], the number of *O*-linked oligosaccharides per heavy chain of normal human serum IgA1 was determined by MALDI–TOF-MS. Removal of sialic acid and galactose by exoglycosidase treatment from hinge glycopeptide (HGP33) resulted in the production of a 33-mer peptide, HYTNPSQDVTVPCPVPSTPPTPSPSTPP-TPSPS having three to five GalNAc residues (7, 20, 21). The same sample was analyzed with a combination of HPLC, MALDI–TOF-MS and CE (Figs. 1 and 2). In addition to the previously detected components, the presence of HGP33 having six GalNAc residues was also detected as another component. CE was also useful to remove the contaminated glycopeptide having an N-terminal histidine residue blocked by a formyl residue. It was found that the formyl glycopeptides (4GN′, 5GN′ and 6GN′) were accidentally produced by heating the glycopeptide during the sample preparation (Fig. 2). Interestingly, the component having five GalNAc residues was further separated into two components named HGP33-5GN-α and -β. In order to analyze this component further, the 33-mer peptide was shortened to a 25-mer peptide, VTVPCPVPSTPPTPSPSTPPTPSPS, by thermolysin digestion (Figs. 3 and 4). The two components composing the 25-mer peptide and five GalNAc residues were well separated from each other by CE, although components having six and four GalNAc exhibited symmetrical peaks (Fig. 4). Because peak b with HPLC in Fig. 3 was mainly composed of HGP25-5GN, this peak was divided into three portions, b-1, b-2 and b-3 as indicated in Fig. 5. As shown in Fig. 6, faster eluted peaks b-1 and b-2 were enriched by HGP25-5GN-β. MALDI–TOF-MS analysis of these three portions indicated that no mass difference was observed among these portions (Fig. 6). Removal of GalNAc residues from HGP25 produced three peaks on HPLC (Fig. 7). Among

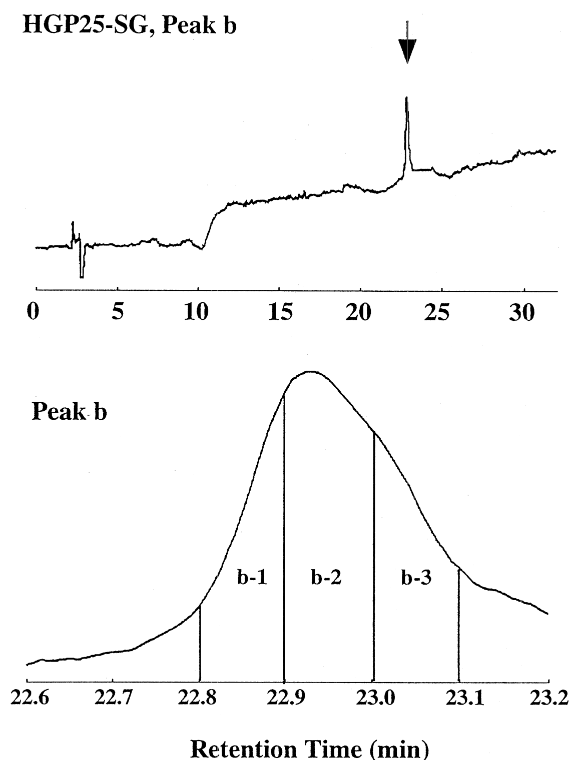

Fig. 5. Dividing peak b into three portions based on the elution order with HPLC. HGP25-SG, peak b; purified peak b in Fig. 4 was rechromatographed with HPLC. Arrowhead indicates purified peak b. Peak b; the purified peak b was divided into three portions, b-1, b-2 and b-3.

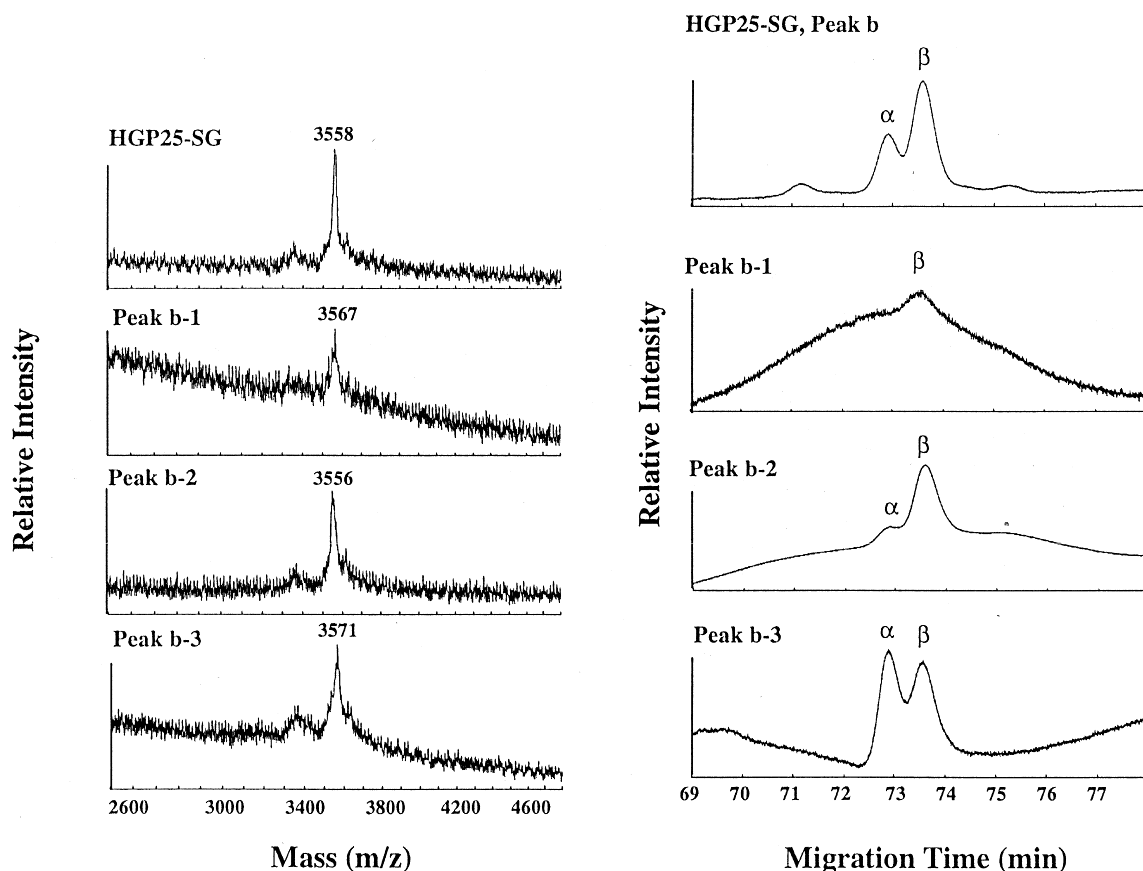

Fig. 6. MALDI–TOF-MS analysis and CE of purified HGP25-SG, peak b and its subfractions. Left: HGP25-SG, peak b; mass chromatogram of purified peak b. Peaks b-1, b-2 and b-3; mass chromatograms of peaks b-1, b-2 and b-3 prepared from purified peak b (Fig. 5). Mass numbers indicated in the figure were close to the calculated mass number 3547.9 for HP25 having five GalNAc residues. Right: HGP25-SG, peak b; profile of CE of purified peak b. Peaks b-1, b-2 and b-3; profiles of CE of three portions prepared from purified peak b. The α and β on the peak indicate HGP25-5GN-α and -β peaks, respectively (Fig. 4).

them were two peptides corresponding to a 25-mer peptide and a 19-mer peptide which would be produced from the 25-mer peptide by a contaminated protease in α-*N*-acetylgalactosaminidase preparation (Figs. 7 and 8). Thus, the mass number and peptide portions of HGP25-5GN-α and -β were not different. Therefore, HGP25-5GN-α should be a structural isomer of HGP25-5GN-β.

Recently, analysis of the attachment site of the sugar chains on the hinge portion of recombinant IgA1s indicated that some of them was attached to a threonine residue but not a serine residue [22]. This was different from the report for myeloma IgA1 by Baenziger and Kornfeld [2]. The attachment site of *O*-linked oligosaccharide for normal human serum IgA1 has not been determined.

As we reported before, it was found that the unsubstituted GalNAc residue was present only on about half of the α1 chain [20], and the number of attached sugar chains varied from three to six as indicated in this report. Thus, transfer of the GalNAc residue toward serine or threonine, the first step in constructing the *O*-linked oligosaccharide on the hinge portion, does not seem to be a well-controlled process and is determined by the environment around the hydroxyamino acid. Such microheterogeneity of a glycoprotein is a well-known phenomenon. However, the importance of the structure of the hinge

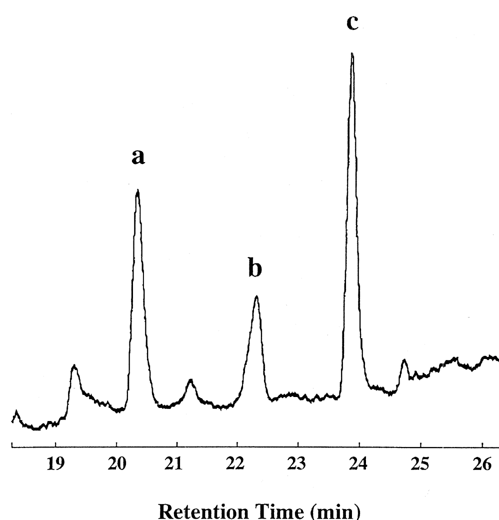

Fig. 7. HPLC analysis of α-*N*-acetylgalactosaminidase-treated HGP25-SG. HGP25-SG was treated with α-*N*-acetylgalactosaminidase, and the HGP25-SGG was fractionated with HPLC as described in the text. Among the three peaks a, b and c, peak b did not contain the peptide component.

portion of IgA1 should not be ignored because of the relationship with IgA nephropathy, because incompleteness of a sugar chain on the hinge portion would be related to the stability of the IgA1 molecule and the self aggregation of IgA1 [6,7,16–19].

In this report, analysis of the asialo-,agalacto-hinge glycopeptide was carried out by CE. A new hinge glycopeptide having six GalNAc residues was found. Among these hinge glycopeptides bearing different numbers of GalNAc residues, only an HGP25-5GN component was separated into structural isomers. The structural differences between HGP25-5GN-α and -β might be due to the attachment of a specific GalNAc residue, because HGP25-4GN and HGP25-6GN did not show the presence of such isomeric structures. That is, an addition of a fifth GalNAc residue to either of two possible attachment sites on the hinge peptide may produce two possible structures of the hinge peptide.

Therefore, further analysis of the attachment site of each GalNAc residue on these isomers will be

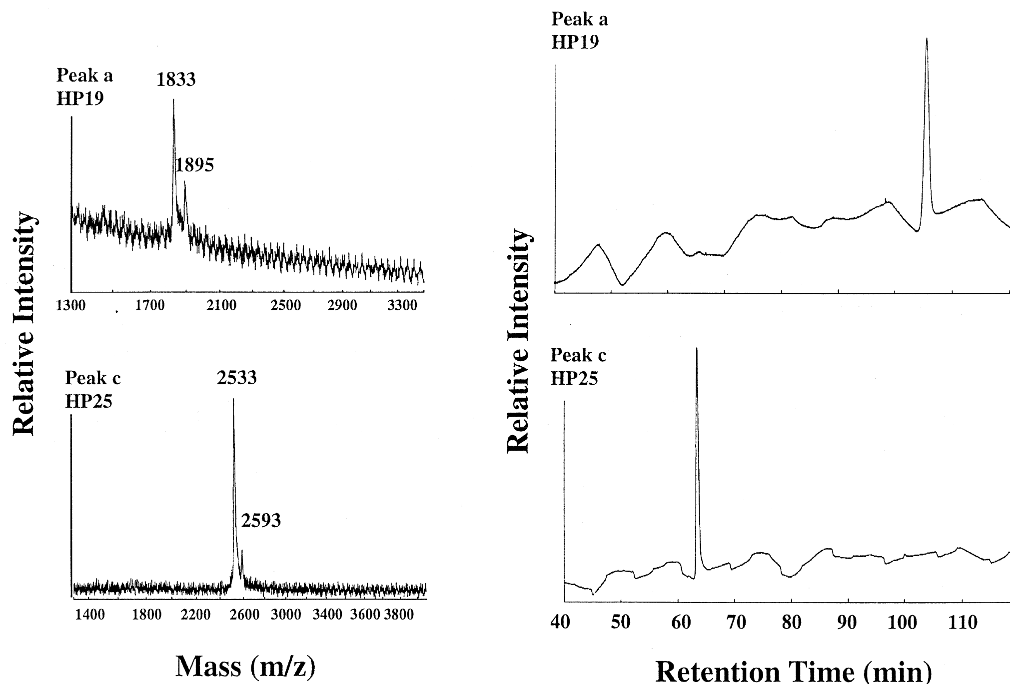

Fig. 8. MALDI–TOF-MS analysis of HGP25-SGG. Left: Peaks a and c in Fig. 7 were analyzed by MALDI–TOF-MS. Mass numbers for peaks a and c coincided with the calculated mass numbers, 1832.0 for HP19 (VPSTPPTPSPSTPPTPSPS) and 2533.9 for HP25 (VTVPCPVPSTPPTPSPSTPPTPSPS), respectively. Right: peaks a and c in Fig. 7 were analyzed by CE. The retention time of HP19 was prominently different from that of HP25.

needed to understand the structural differences in the hinge portion from normal and patient human serum IgA1.

Acknowledgements

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References

[1] J. Baenziger, S. Kornfeld, J. Biol. Chem. 249 (1974) 7260.
[2] J. Baenziger, S. Kornfeld, J. Biol. Chem. 249 (1974) 7270.
[3] A. Torano, Y. Tsuzukida, Y.-S. Victor Liu, F.W. Putnum, Proc. Natl. Acad. Sci. 74 (1977) 2301.
[4] H. Iwase, I. Ishii-Karakasa, E. Fujii, K. Hotta, Y. Hiki, Y. Kobayashi, Anal. Biochem. 206 (1992) 202.
[5] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, I. Ishii-Karakasa, Y. Kobayashi, K. Hotta, J. Biochem. 120 (1996) 92.
[6] T. Kokubo, Y. Hiki, H. Iwase, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 9 (1998) 2048.
[7] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, T. Sano, I. Ishii-Karakasa, K. Toma, Y. Kobayashi, K. Hotta, J. Chromatogr. B, in press.
[8] M.E. Conley, M.D. Cooper, A.F. Michael, J. Clin. Invest. 66 (1980) 1432.
[9] R.J. Glassock, Am. J. Kidney Dis. 12 (1988) 449.
[10] P.M. Andre, P. Le Pogamp, D. Chevet, J. Clin. Lab. Anal. 4 (1990) 115.
[11] A.C. Allen, S.S. Harper, Clin. Exp. Immunol. 100 (1995) 470.
[12] A.C. Allen, P.S. Topham, S.J. Harper, J. Feehally, Nephrol. Dial. Transplant. 12 (1997) 701.
[13] J. Mestecky, M. Tomana, P.A. Crowley-Nowick, Z. Modoneanu, B.A. Julian, S. Jackson, Contrib. Nephrol. 104 (1993) 172.
[14] M. Tomana, K. Matousovic, B.A. Julian, J. Radl, K. Konecny, J. Mestecky, Kidney Int. 52 (1997) 509.
[15] Y. Hiki, A. Horii, H. Iwase, A. Tanaka, Y. Toda, K. Hotta, Y. Kobayashi, Contrib. Nephrol. 111 (1995) 73.
[16] Y. Hiki, H. Iwase, M. Saitoh, Y. Saitoh, A. Horii, K. Hotta, Y. Kobayashi, Nephron 74 (1996) 429.
[17] T. Kokubo, Y. Hiki, H. Iwase, A. Horii, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 8 (1997) 915.
[18] H. Iwase, Y. Hiki, K. Hotta, Trend in Glycoscience and Glycotechnology 10 (1998) 13.
[19] Y. Hiki, A. Tanaka, T. Kokubo, H. Iwase, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 9 (1998) 577.
[20] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, I. Ishii-Karakasa, Y. Kobayashi, K. Hotta, J. Biochem. 120 (1996) 393.
[21] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, I. Ishii-Karakasa, J. Nishikido, Y. Kobayashi, K. Hotta, J. Chromatogr. B 709 (1998) 145.
[22] T.S. Mattu, R.J. Pleass, A.C. Willis, M. Lilian, M.R. Wormald, A.C. Lellouch, P.N. Rudd, J.M. Woof, R.A. Dwek, J. Biol. Chem. 273 (1998) 2260.