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Short communication

Application of matrix-assisted laser desorption ionization time-of-flight mass spectrometry to the analysis of glycopeptide-containing multiple *O*-linked oligosaccharides

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Abstract

In our previous report [Iwase et al., J. Biochem., 120 (1996) 393], the number of *O*-linked oligosaccharide chains on the hinge region of IgA1 was estimated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOFMS). In this experiment, the number of non-substituted *N*-acetylgalactosamines and Galβ1,3GalNAc residues, as the core *O*-linked oligosaccharide structure per heavy chain of normal human serum IgA1, was estimated by digestion of the asialo-hinge glycopeptide with α-*N*-acetylgalactosaminidase (GalNAc-ase) or endo-α-*N*-acetylgalactosaminidase (endo-GalNAc-ase). GalNAc-ase treatment of the asialoglycopeptide produced two major peaks, one being a glycopeptide containing four GalNAc and four Gal residues, and the other contained three GalNAc and three Gal residues. Treatment with endo-GalNAc-ase also produced a nearly equal amount of the two peaks, with the naked hinge peptide and the peptide having one GalNAc residue. From those results, we concluded that the asialo-hinge glycopeptide was composed of three components bearing four Galβ1,3GalNAc and one GalNAc, only four Galβ1,3GalNAc, and three Galβ1,3GalNAc and one GalNAc, respectively. This method was useful for determining the glycoforms on the IgA1 molecule with respect to the core *O*-linked oligosaccharide structure. © 1998 Elsevier Science B.V.

Keywords: Glycopeptides; Oligosaccharides

1. Introduction

Human serum IgA1 is one of the most exceptional of the human serum glycoproteins because it has five *O*-linked oligosaccharides in its hinge portion, in addition to the two *N*-linked carbohydrate chains in its structure [1–3]. In our previous report, the glycoform of the *O*-linked oligosaccharide of the IgA1 from a healthy control, IgA1 myeloma patients [4] and IgA nephropathy patients [5] was analyzed using gas-phase hydrazinolysis with respect to their sialic acid content of Galβ1,3GalNAc. Three glycoforms for IgA1 from myeloma patients, only one glycoform from healthy individuals, and an aberrant glycoform from IgA nephropathy patients were found, although it was later revealed that the IgA1 from the healthy control was composed of heterogeneous components that had mutually different glyco-

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forms [6]. All of the results were described qualitatively as the relative content of each sialylated Galβ1,3GalNAc. Aberrant glycosylation of the hinge portion of IgA1 from an IgA nephropathy patient was also reported from other research groups [7–11].

Subsequently, the number of *O*-linked sugar chains per heavy chain of IgA1 could be estimated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOFMS) using the hinge glycopeptide [12]. Sequential removal of peripheral sialic acid and galactose residues resulted in simple spectra that exhibited the number of attached sugar chains by counting the remaining *N*-acetylgalactosamine residues.

With respect to the glycoform of the *O*-linked oligosaccharide of IgA1, Baenziger and Kornfeld [2] reported that one out of five possible glycosylation sites of the α1 chain from a myeloma patient had only a GalNAc residue, thus differing from the other sites. They suggested that there might be one specific site that was difficult to glycosylate.

In this experiment, we developed a method for counting the number of non-substituted *N*-acetylgalactosamine and Galβ1,3GalNAc residues per asialo hinge glycopeptide of normal human serum IgA1 by the combination of exo- or endo-glycosidase treatment and MALDI–TOFMS analysis.

2. Experimental

2.1. Reagents and chemicals

The following materials were purchased from the sources indicated: Normal human serum was from China Newtech Development and Trade; PD-10 from Pharmacia Biotech (Uppsala, Sweden); 4-vinyl pyridine from Aldrich (Milwaukee, WI, USA); jacalin–agarose from Vector Laboratories (Burlingame, CA, USA); neuraminidase from *Arthrobacter ureafaciens* from Boehringer Mannheim (Mannheim, Germany); β-galactosidase, from bovine testes, and trypsin from Sigma (St Louis, MO, USA); α-*N*-acetylgalactosaminidase from *Acremonium* sp. and *O*-glycanase were from Seikagaku (Tokyo, Japan).

2.2. Preparation of IgA1 by jacalin–agarose affinity chromatography

If not stated, the jacalin–agarose affinity chromatography was carried out at room temperature as reported before [12]. A 10-ml volume of serum was applied to the jacalin column (10 ml) and thoroughly washed with 0.1 *M* Tris–HCl buffer, pH 7.6, containing 0.02% sodium azide. 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 absorbance of the eluate at 280 nm was used to detect the protein. Nonspecifically bound materials were eluted with glucose. The IgA1 fraction, obtained by stepwise elution with galactose, was dialyzed against distilled water and then lyophilized.

2.3. Preparation of hinge glycopeptide from human serum IgA1

About 1 mg of IgA1 was first desalted using a PD-10 column. The desalted sample was dissolved in 500 μl of 0.4 *M* Tris–HCl buffer, pH 8.6, containing 6 *M* guanidine–HCl and 0.2 *M* EDTA. To dissociate the disulfide linkage, 5.4 μl of dithiothreitol solution (200 mg/ml) was added and the mixture was stirred. After heating at 50°C for 4 h, 1.6 μl of 4-vinyl pyridine were added, and the reaction mixture was allowed to stand for 90 min at room temperature. The reaction was terminated by the addition of 50 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 (150×4.6 mm; Nacalai Tesque, Kyoto, Japan) 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). Elution was monitored by UV absorption at 280 nm. The material eluted at the peak position, around 30 min, was collected and concentrated.

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. A 20-μl volume 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 1 ml 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 above buffer. Purification of the glycopeptide by HPLC was carried out using a Cosmosil 5C18-300 column (Nacalai Tesque, 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 the UV absorbance at 220 nm. The material eluted at the peak position of the hinge glycopeptide, at around 23 min, was collected and concentrated, as shown in Fig. 1.

2.4. Treatment of glycopeptide with exo- and endo-glycosidases

Enzyme treatment of the glycopeptide was usually performed overnight, with the exceptions of endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) using 50 mU of neuraminidase from *Arthrobacter ureafaciens*, 20 mU of β-galactosidase from bovine testes, 0.2 U of α-*N*-acetylgalactosaminidase from *Acromonium* sp. and/or 5 mU of *O*-glycanase in 20 m*M* sodium acetate buffer, pH 5.0, where enzyme treatment was for three days. The incubation mixture was directly analyzed by MALDI–TOFMS without the further purification of the glycopeptide.

2.5. MALDI–TOFMS analysis of glycosidase-treated hinge glycopeptide

The hinge glycopeptide was analyzed by MALDI–TOFMS 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 of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 70% acetonitrile–30% water). The mixture (1 μl) was applied to the sample target and allowed to air-dry prior to MALDI–TOFMS analysis. The error involved in the mass determination was less than 0.3%. TOFMS analysis of the sequentially treated glycopeptide with exo- and endo-glycosidase was also carried out under the above conditions.

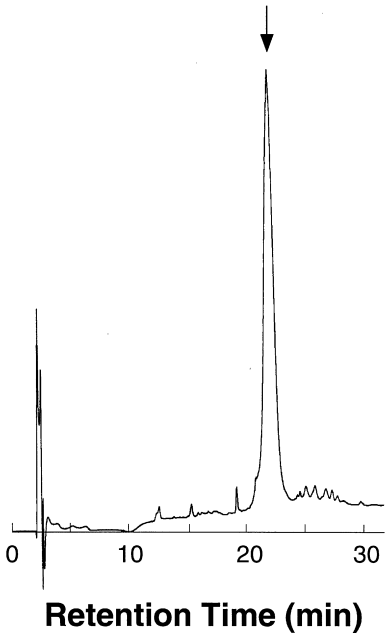
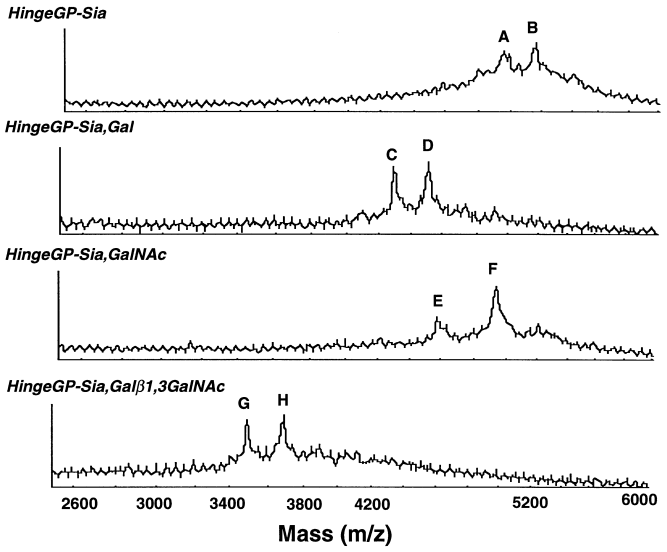


Fig. 1. HPLC profile of hinge glycopeptide purified from a trypsin digest of an α1 chain of IgA1 using a jacalin–agarose column. The hinge glycopeptide purified on the jacalin–agarose column was further purified by HPLC under the conditions described in Section 2. The arrow indicates the elution position of the hinge glycopeptide.

3. Results and discussion

Counting of number of non-substituted *N*-acetylgalactosamine and Galβ1,3GalNAc residues per asialo-hinge glycopeptide from normal human serum IgA1 was carried out by the combination of exo- and endo-α-*N*-acetylgalactosaminidase treatment and MALDI–TOFMS analysis. Neuraminidase-treated hinge glycopeptide was used as the starting material, since the glycopeptide bearing sialic acid did not produce clear peaks. The profile of the MALDI–TOFMS analysis and the mass number of each glycopeptide treated with enzyme are summarized in Fig. 2 [12].

The following three results with respect to the



Detected Peak	Composition	Mass Number Calculated [M-H]⁻	Observed (m/z)
hingeGP-Sia :			
A	Peptide + 4GalNAc + 4Gal	4935	4943
B	Peptide + 5GalNAc + 4Gal	5138	5150
hingeGP-Sia,Gal :			
C	Peptide + 4GalNAc	4287	4288
D	Peptide + 5GalNAc	4490	4495
hingeGP-Sia,GalNAc :			
E	Peptide + 3GalNAc + 3Gal	4570	4575
F	Peptide + 4GalNAc + 4Gal	4935	4942
hingeGP-Sia,Galβ1,3GalNAc :			
G	Peptide	3475	3479
H	Peptide + 1GalNAc	3678	3682

Fig. 2. Profiles of MALDI–TOFMS analysis and mass numbers of asialo-hinge glycopeptide treated with various glycosidases. Peaks are sequentially designated as A to H, and the estimated composition of each peak is also indicated in the table. The structure of the peptide portion was HYTNPSQDVTVP-CPVPSTPPTPSPSTPPTPSPS, as reported previously [12]. Cysteine (C) in the sequence indicated an *S*-pyridylethylated residue.

structure of the asialo-hinge glycopeptide of IgA1 from normal human serum were obtained:

(1) The major glycopeptide had four or five sugar chains. The previously reported minor component, having three sugar chains, was not detected as a clear peak in this experiment.

(2) Asialo-hinge glycopeptides with three or four Galβ1,3GalNAc residues were the major components.

(3) Approximately half of the hinge glycopeptide had non-substituted GalNAc residues, but the other half had none.

The proposed structure of the three components for the asialo-hinge glycopeptide is shown in Table 1. Among them, 1 and 2 were major components and 3 was a minor one, which could not be detected as a peak in neuraminidase-treated hinge glycopeptide. Thus, the presence of three glycoforms was found to be the core structure of the *O*-linked oligosaccharide on the hinge portion of the α1 chain from normal human serum. On comparing the results with those of our previous study, the myeloma IgA1 differed from that of a healthy control in that the molecule had a core structure whose major component had

Table 1
Three glycoforms estimated for the core structure of *O*-linked oligosaccharide on hinge glycopeptide

Component	Estimated glycoform
1	Peptide+4×(Galβ1,3GalNAc)+1×(GalNAc)
2	Peptide+4×(Galβ1,3GalNAc)
3	Peptide+3×(Galβ1,3GalNAc)+1×(GalNAc)

The numbers of non-substituted *N*-acetylgalactosamine and Galβ1,3GalNAc residues for each glycoform are deduced from the results in Fig. 2.

only four GalNAc residues. Also, most of the sugar chain was fully galactosylated in an identical manner to that of component 2 in Table 1 [12].

In this experiment, about half of the heavy chain had non-substituted *N*-acetylgalactosamines and the other half had none. This result was partly consistent with the research by Baenziger and Kornfeld [2], who reported that one of five possible glycosylation sites on the hinge portion of the α1 chain has GalNAc alone. Our results support the conclusion that there is likely to be one specific site where it is difficult for transference by galactose and *N*-acetylgalactosamine. Jentoft [13] reported that the presence of a GalNAc residue directly attached to the peptide portion in a mucin-type glycoprotein was most important for the stabilization of peptide conformation. The importance of this residue will be the same for the IgA1 molecule.

Recently, aberrant glycosylation of an IgA1 molecule from IgA nephropathy patients was reported by some research groups [7–11]. It is difficult to present a view consistent with their results, due to the differences in the techniques used. Since the structural difference in the hinge portion of IgA1 between a healthy control and an IgA nephropathy patient seems to be reliable, application of this method to the analysis of the glycoform of the *O*-linked oligosaccharide core on IgA1 from a healthy control and that from an IgA nephropathy patient might be useful for investigating the role of the *O*-linked oligosaccharide in the etiology of IgA nephropathy.

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