

ラット脳の Thy-1 の N-結合型糖鎖については、これまでに抗体カラム等を用いて精製された Thy-1 をトリプシン消化後、糖ペプチドごとに糖鎖を切り出し、ゲルろ過や、エキソグリコシダーゼ消化等を用いて分析した例が報告されている。Asn23 には、高マンノース型 (M5, 6) が、Asn74 には、コアがフコシル化された複合型糖鎖と、マイナー糖鎖としてハイブリッド型糖鎖及び高マンノース型糖鎖が結合していることが明らかにされているが^{16, 17)}、電気泳動と LC/MS/MS を組み合わせることによって、微量の膜タンパク質を、抗体を使用せずに簡便且つより詳細に解析できることがわかる。尚、Asn98 に結合する糖鎖及び GPI アンカーについては、トリプシン消化によって得られた糖ペプチドの親水性が高く、LC カラムに保持されなかったため、他の酵素消化によって得られた糖ペプチドを解析することによって明らかにしている (データ省略)。

以上のように、LC/MS/MS によるペプチド・糖ペプチドマッピングにおいて、糖鎖に特徴的なオキシニウムイオンを用いて糖ペプチドのプロダクトイオンスペクトルを取り出す方法は、タンパク質を同定すると同時に、糖鎖結合部位を決定し、結合部位ごとの糖鎖構造を推定することを可能にする。2次元 HPLC との組み合わせや、2次元電気泳動で分離された糖タンパク質の解析も可能であることから、今後、グライコプロテオミクスの分野で役立つことが期待される。

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要 約

LC/MS/MS は、アミノ酸配列情報に加え、糖鎖構造に関する情報についても得ることができ、糖ペプチドの解析にも有用である。QqTOF-MS を用いた LC/MS/MS は、糖ペプチドのピークを特定し、ペプチドを同定し、結合糖鎖構造に関する情報も得ることができる。本稿では、糖ペプチドの解析例として、APF 及び電気泳動で分離された GPI アンカー型タンパク質の解析例を示す。

Kinetic Analysis of Pepsin Digestion of Chicken Egg White Ovomuroid and Allergenic Potential of Pepsin Fragments

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Key Words

Ovomuroid · Allergen · Digestion · Simulated gastric fluid · Fragment, pepsin-digested · Human serum IgE

Abstract

Background: The allergenic potential of chicken egg white ovomucoid (OVM) is thought to depend on its stability to heat treatment and digestion. Pepsin-digested fragments have been speculated to continue to exert an allergenic potential. OVM was digested in simulated gastric fluid (SGF) to examine the reactivity of the resulting fragments to IgE in sera from allergic patients. **Methods:** OVM was digested in SGF and subjected to SDS-PAGE. The detected fragments were then subjected to N-terminal sequencing and liquid chromatography/mass spectrometry/mass spectrometry analysis to confirm the cleavage sites and partial amino acid sequences. The reactivity of the fragments to IgE antibodies in serum samples from patients allergic to egg white was then determined using Western blotting (n = 24). **Results:** The rate of OVM digestion depended on the pepsin/OVM ratio in the SGF. OVM was first cleaved near the end of the first domain, and the resulting fragments were then further digested into smaller fragments. In the Western blot analysis, 93% of the OVM-reactive sera also bound to the 23.5- to 28.5-kDa fragments, and 21% reacted with

the smaller 7- and 4.5-kDa fragments. **Conclusion:** When the digestion of OVM in SGF was kinetically analyzed, 21% of the examined patients retained their IgE-binding capacity to the small 4.5-kDa fragment. Patients with a positive reaction to this small peptide fragment were thought to be unlikely to outgrow their egg white allergy. The combination of SGF-digestibility studies and human IgE-binding experiments seems to be useful for the elucidation and diagnosis of the allergenic potential of OVM.

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Introduction

Chicken egg white is one of the strongest and most frequent causes of food allergies among young children [1–5]. Egg white contains several allergens, including ovalbumin, ovotransferrin, lysozyme and ovomucoid (Gal d 1, OVM). OVM accounts for about 11% of all egg white proteins [6] and has a molecular weight of 28 kDa, containing a carbohydrate content of 20–25% [7]. OVM is known to be stable to digestion and heat, and cooked eggs can cause allergic reactions in OVM-specific allergic patients [8–11]. One possible reason for this is that OVM contains linear epitopes that are only slightly affected by conformational changes induced by heat denaturation.

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OVM consists of 186 amino acids divided into three domains of about 60 amino acids each; the third domain has been reported to be the most important domain with regard to allergenicity [12]. In a previous report, N-glycans in the third domain were suggested to be essential for allergenicity [13]; however, a recent report found that the deletion of the N-glycans did not affect the allergic reactivity.

We previously reported the digestibility of 10 kinds of food proteins in simulated gastric fluid (SGF) [8, 14]. OVM was digested relatively rapidly, but several fragments were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue (CBB) staining. The reactivity of these fragments with IgE antibodies from the sera of patients with egg white allergy is very important to understanding the mechanism of OVM allergy.

A few previous reports have described the reactivity of IgE in sera from patients with egg white allergies with OVM-derived fragments. Kovacs-Nolan et al. [15] separated pepsin-digested fragments of OVM using high-performance liquid chromatography (HPLC) and examined the IgE-binding activities of each fragment using an enzyme-linked immunosorbent assay (ELISA). Besler et al. [16] investigated the reactivity of pepsin-digested fragments with patient IgE using Western blotting and showed that the fragments retain their binding capacity to human IgE in some serum samples from OVM-allergic patients. However, little attention has been paid to the digestive conditions, and the number of serum samples has been somewhat small in these studies. Urisu et al. [17] reported that the sera of subjects that tested positive or negative during an oral egg white challenge exhibited a significant difference in their reactivity with pepsin fragments.

In the present report, kinetic data for different generations of SGF-stable OVM fragments were obtained, and the reactivity of the fragments with serum IgE from patients with egg white allergies was investigated using Western blotting.

Materials and Methods

Pepsin (catalog number P6887) and chicken egg white OVM (T2011, Trypsin Inhibitor, Type III-O) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). The concentration of the OVM test solution was 5 mg/ml of water. The gels and reagents used for the SDS-PAGE analysis were purchased from Invitrogen (Carlsbad, Calif., USA).

Serum Specimens

Sera from 24 patients with egg white allergies and a healthy volunteer were used after obtaining informed consent from the patients and ethical approval by the Institutional Review Board of the National Institute of Health Sciences. Twenty-two of the patients had been diagnosed as having an egg white allergy at hospitals in Japan, based on their clinical histories and positive IgE responses to egg white proteins by radioallergosorbent test (RAST), while the remaining 2 allergen-specific sera were purchased from Plasma Lab International (Everett, Wash., USA); the commercial sera originated from adult Caucasians who had been diagnosed as having several food allergies, including egg white, based on their clinical history and skin tests. The commercial sera also showed positive IgE responses to egg white proteins when examined using RAST.

Preparation of SGF

Pepsin (3.8 mg; approximately 13,148 units of activity) was dissolved in 5 ml of gastric control solution (G-con; 2 mg/ml NaCl, pH adjusted to 2.0 with distilled HCl), and the activity of each newly prepared SGF solution was defined as the production of a ΔA_{280} of 0.001/min at pH 2.0 and 37°C, measured as the production of trichloroacetic acid-soluble products using hemoglobin as a substrate. The original SGF was prepared at a pepsin/OVM concentration of 10 unit/ μ g, and this solution was diluted with G-con for the experiments performed at pepsin/OVM concentrations of 1 and 0.1 unit/ μ g. The SGF solutions were used within the same day.

Digestion in SGF

SGF (1,520 μ l) was incubated at 37°C for 2 min before the addition of 80 μ l of OVM solution (5 mg/ml). The digestion was started by the addition of OVM. At each scheduled time point (0.5, 2, 5, 10, 20, 30, and 60 min), 200 μ l of the reaction mixture was transferred to a sampling tube containing 70 μ l of 5 \times Laemmli buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8) and 70 μ l of 200 mM Na₂CO₃. For the zero-point samples, the OVM solution (10 μ l) was added to neutralized SGF (190 μ l of SGF, 70 μ l of 5 \times Laemmli buffer, and 70 μ l of 200 mM Na₂CO₃). All neutralized samples were then boiled at 100°C for 3 min and subjected to SDS-PAGE.

SDS-PAGE Analysis and Staining Procedure

Samples (15 μ l/lane) were loaded onto a 10–20% polyacrylamide Tris/Tricine gel (Invitrogen, Carlsbad, Calif., USA) and separated electrophoretically. The gels were fixed for 5 min in 5% trichloroacetic acid, washed for 2 h with SDS Wash (45.5% methanol, 9% acetic acid), stained for 10 min with CBB solution (0.1% Coomassie Brilliant blue R, 15% methanol, 10% acetic acid), and destained with 25% methanol and 7.5% acetic acid. The stained gel images were then analyzed using Image Gauge V3.1 (Fuji Film, Tokyo, Japan), and the density of each band was quantified. Periodic acid-Schiff (PAS) staining [18] was used to detect the glycosylated fragments.

N-Terminal Sequence Analysis

OVM (1.5 mg) was digested in SGF containing 1 unit/ml pepsin, concentrated by centrifugation using Centriprep YM-3 (Millipore Corporation, Bedford, Mass., USA) and subjected to SDS-PAGE followed by electrical transblotting to a 0.2- μ m polyvinylidene difluoride membrane (Bio-Rad, Richmond, Calif., USA) and CBB staining. The detected fragment bands were then cut out and sequenced using a Procise 494HT Protein Sequencing System (Applied Biosys-

tems, Foster City, Calif., USA) or an HP G1005A Protein Sequencing System (Hewlett-Packard, Palo Alto, Calif., USA); each fragment was analyzed for 5 cycles.

Carboxymethylation and Peptide Mapping Using Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS)

The digested OVM sample was separated electrophoretically as described above, stained with CBB, and the stained bands were cut out. The gel pieces were homogenized in 20 mM Tris-HCl (pH 8.0) containing 0.1% SDS and the proteins were extracted. The extracts were concentrated and purified by acetone precipitation. The acetone precipitates were incubated with 2-mercaptoethanol (92.5 mM) in 72 µl of 0.5 M Tris-HCl buffer (pH 8.6) containing 8 M guanidine hydrochloride and 5 mM EDTA at room temperature for 2 h. To this solution, 1.5 mg of monoiodoacetic acid was added, and the mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was desalted using a MicroSpin G-25 column (Amersham Bioscience, Uppsala, Sweden) and lyophilized. Reduced and carboxymethylated proteins were digested with trypsin (50 ng/µl in 50 mM NH₄HCO₃).

Tandem electrospray mass spectra were recorded using a hybrid quadrupole/time-of-flight spectrometer (Qstar Pulsar i; Applied Biosystems, Foster City, Calif., USA) interfaced to a CapLC (Magic 2002; Michrom BioResources, Auburn, Calif., USA). Samples were dissolved in water and injected into a C18 column (0.2 × 50 mm, 3 µm, Magic C18, Michrom BioResources). Peptides were eluted with a 5–36% acetonitrile gradient in 0.1% aqueous formic acid over 60 min at a flow rate of 1 µl/min after elution with 5% acetonitrile for 10 min. The capillary voltage was set to 2,600 V, and data-dependent MS/MS acquisitions were performed using precursors with charge states of 2 and 3 over a mass range of 400–2,000.

Western Blotting of Digested Fragments with Human Serum IgE

The digested OVM samples were applied to a 10–20% polyacrylamide Tris/Tricine 2D gel, followed by electrical transfer to a nitrocellulose membrane. The membrane was then blocked with 0.5% casein-PBS (pH 7.0) and cut into 4-mm strips. The strips were incubated with diluted human serum (1/4 to 1/5) in 0.2% casein-PBS (pH 7.0) at room temperature for 1 h and then at 4°C for 18 h. After washing with 0.05% Tween 20-PBS, the strips were incubated with rabbit anti-human IgE (Fc) antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) at room temperature for 1 h, and then with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibodies (Amersham Biosciences, Little Chalfont, UK) at room temperature for 1 h. Finally, the strips were reacted with Konica ImmunoStain HRP-1000 (Konica, Tokyo, Japan), according to the manufacturer's protocol.

Results

Kinetics of OVM Digestion by Pepsin

OVM was digested in SGF containing various concentrations of pepsin, and the fragments were separated by SDS-PAGE and stained with CBB (fig. 1). The molecular weight of OVM, based on its amino acid sequence, is about 20 kDa, but a broad band representing intact OVM

appeared at about 34–49 kDa in the SDS-PAGE gel because of the presence of five N-linked sugar chains. The pepsin band was detected at 39 kDa, overlapping with the intact OVM band, and lysozyme (14 kDa) contamination was detected in the OVM sample that was used. Intact OVM rapidly disappeared within 0.5 min in SGF (pepsin/OVM = 10 unit/µg), and a fragment band was detected at 7 kDa. When the pepsin content in SGF was reduced to 1 and 0.1 unit/µg, the digestion rate markedly decreased. Intact OVM was still detected after 30 min when the pepsin/OVM ratio was 0.1 unit/µg. The fragment bands were clearer (fig. 2) when a concentrated SGF-digested OVM solution (pepsin/OVM = 1 unit/µg, digestion times 5 and 30 min) was used, followed by SDS-PAGE. As shown in figure 2, a strong 23.5- to 28.5-kDa band (FR 1) was detected at 5 min, while 10- (FR 2), 7- (FR 3) and 4.5- to 6-kDa (FR 4) bands were detected after 30 min. FR 1 and FR 2 were both positively stained by PAS, suggesting that the FR 1 and FR 2 fragments have high carbohydrate contents. The time courses for the amounts of intact OVM and the four fractions are plotted in figure 3, where the pepsin/OVM ratio is 1 unit/µg. FR 1 rapidly increased but slowly disappeared after 2 min. FR 2 and FR 3 also rapidly reached maximum values at 5 min and then slowly disappeared. On the other hand, FR 4 gradually increased throughout the entire period of the experiment.

Preheating (at 100°C for 5 or 30 min) of the OVM solution (5 mg/ml in water) did not influence the digestion pattern (fig. 1).

Table 1. N-Terminal sequences of pepsin fragments

Digestion period	Fraction	Fragment Residues	Sequence	Ratio % ^a	
5 min	FR 1	1-1	50-54	FGTNI	73.1
		1-2	51-55	GTNIS	11.6
		1-3	1-5	AEVDC	6.9
5 min	FR 2	2-1	1-5	AEVDC	68.8
		2-2	134-138	VSVDC	28.2
5 min	FR 3	3-1	1-5	AEVDC	48.4
		3-2	134-138	VSVDC	24.3
		3-3	104-108	NECLL	9.6
		3-4	85-89	VLCNR	6.5
30 min	FR 4	4-1	134-138	VSVDC	30.6
		4-2	104-108	NECLL	24.0
		4-3	19-23	VLVCN	20.6

^a Molar ratios of the fragments to the total amount in each fraction.

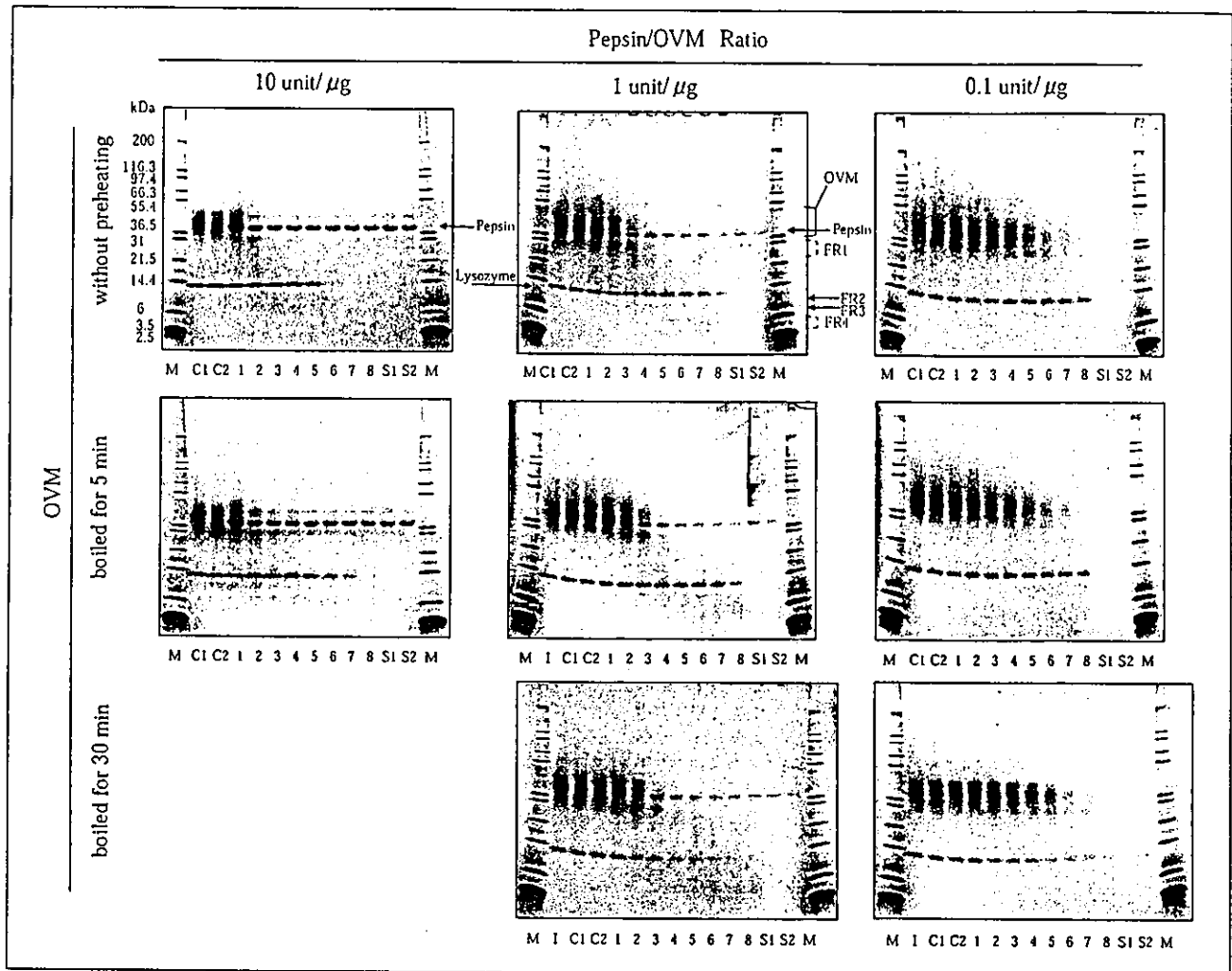


Fig. 1. Kinetic patterns of OVM digestion in SGF-containing pepsin. Digested samples were analyzed by SDS-PAGE followed by CBB staining. The digestion patterns of OVM without preheating (upper panels), preheated at 100°C for 5 min (middle panels), and preheated at 100°C for 30 min (lower panels) are shown. The ratio of pepsin to OVM was 10 unit/1 μg (left), 1 unit/1 μg (middle), and 0.1 unit/1 μg (right). Lane M = Molecular weight markers; lanes C1 and

C2 = OVM without pepsin at 0 (C1) and 60 (C2) min; lanes 1–8 = SGF-digested OVM at 0, 0.5, 2, 5, 10, 20, 30 and 60 min, respectively; lanes S1 and S2 = SGF alone at 0 (S1) and 60 (S2) min; lanes I = OVM without preheating; FR 1 = fraction 1 containing a fragment at 23.5–28.5 kDa; FR 2 = fraction 2 containing a 10-kDa fragment; FR 3 = fraction 3 containing a 7-kDa fragment; FR 4 = fraction 4 containing 4.5- to 6-kDa fragments.

Sequence Analysis of OVM Fragments

The sequences of the five N-terminal residues in each fragment were analyzed, and the data are summarized in table 1. Figure 4 schematically depicts the identified fragments; the arrows in the upper panel indicate the sites of pepsin cleavage.

The internal sequences of the FR 1, FR 3, and FR 4 fragments were also identified by LC/MS/MS and are shown in table 2 and in the upper panel of figure 4.

Reactivity of the Fragments with Serum IgE from Patients with Egg White Allergy

Western blot analysis using patient sera as the source of the primary antibodies was performed to identify sera that reacted with intact OVM and the SGF fragments. Representative blotting data are shown in figure 5, and all the results are listed in table 3. Ninety-two percent of the serum samples from allergic patients reacted with OVM, and 93% of the OVM-positive sera reacted with FR 1

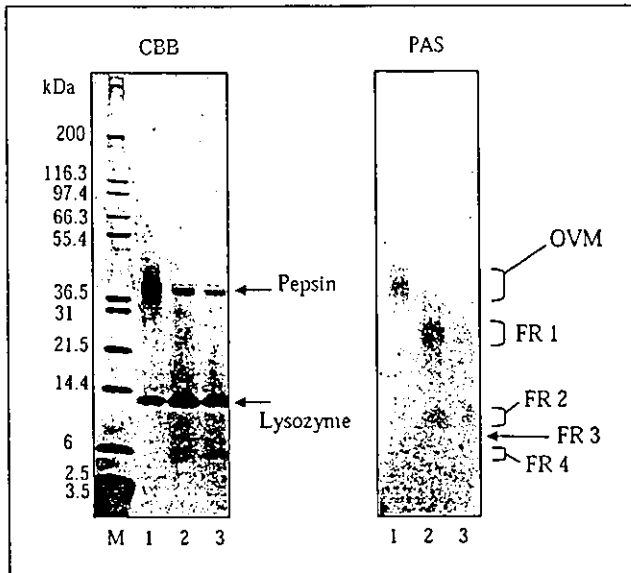


Fig. 2. CBB and PAS staining of OVM fragments following digestion in SGF (pepsin/OVM = 1 unit/ μ g) for 5 and 30 min. Lane M = Molecular weight markers; lane 1 = original OVM (2.5 μ g/lane); lanes 2 and 3 = OVM digested for 5 and 30 min, respectively, and concentrated (12 μ g, equivalent to the original OVM/lane). Samples were applied to two SDS-PAGE gels and electrophoresed. One plate (left panel) was stained with CBB reagent, and the other (right panel) was stained with PAS reagent.

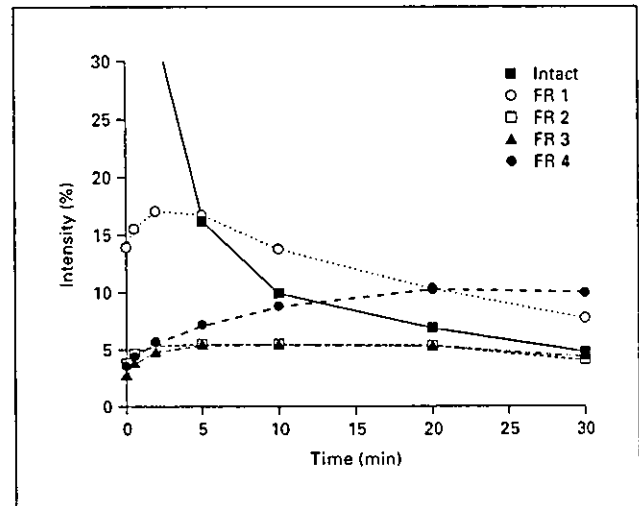
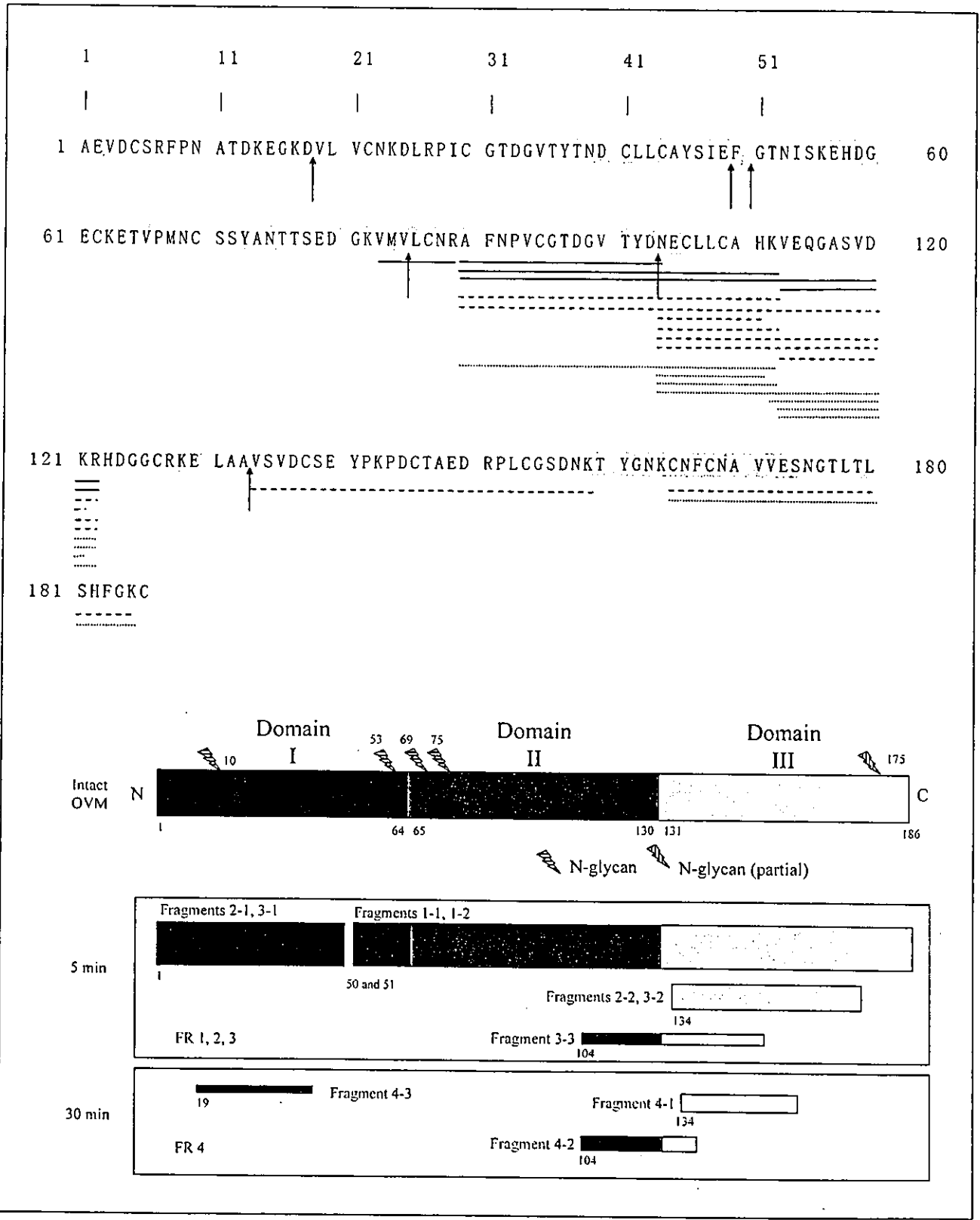


Fig. 3. Quantification of the SGF-digestion pattern of intact OVM and the digestion fragments at a pepsin/OVM ratio of 1 unit/ μ g. The intensity of each band was calculated using the ratio of the band's density to the total density of the originally detected band at $t = 0$. Values are the mean of duplicate analyses. Similar results were observed in another set of experiments.

Table 2. Identified inside sequences in pepsin- and trypsin-digested OVM

Pepsin digestion	Fraction	Residues	Sequence
5 min	FR 1	83-89	VMVLCNR
		90-103	AFNPVCGTDGVTYD
		90-112	AFNPVCGTDGVTYDNECLLCAHK
		90-122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		113-122	VEQGASVDKR
5 min	FR 3	90-112	AFNPVCGTDGVTYDNECLLCAHK
		90-122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		104-111	NECLLCAH
		104-112	NECLLCAHK
		104-121	NECLLCAHKVEQGASVDK
		104-122	NECLLCAHKVEQGASVDKR
		113-122	VEQGASVDKR
		134-159	VSVDCSEYKPDCTAEDRPLCGSDNK
165-185	CNFCNAVVESNGTLTSLHFGK		
30 min	FR 4	90-112	AFNPVCGTDGVTYDNECLLCAHK
		104-111	NECLLCAH
		104-112	NECLLCAHK
		104-122	NECLLCAHKVEQGASVDKR
		112-122	KVEQGASVDKR
		113-121	VEQGASVDK
		113-122	VEQGASVDKR
		165-185	CNFCNAVVESNGTLTSLHFGK



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after 5 min of digestion. Three of the serum samples also reacted with FR 2, FR 3, and FR 4 after 30 min of digestion.

The three samples that react with FR 2, FR 3, and FR 4 were obtained from patients who exhibited persistent allergies to egg white. One of these serum samples, No. 4, was obtained from a 3-year-old girl who is presently 6 years old; her total IgE level has decreased slightly to 4,450 IU/ml, but the specific IgE level for egg white remains at more than 100 IU/ml, and the patient has not outgrown her hypersensitivity to eggs. Another patient, No. 13, was a 1-year-old boy; 7 years later, his total and egg white-specific IgE levels had been reduced to 947 and 6.85 IU/ml, respectively, but eating raw eggs still caused allergic symptoms. The third FR 4-positive patient, No. 19, was an 11-year-old boy whose total IgE level decreased to 3,940 IU/ml and whose egg white-specific IgE decreased to 13.5 IU/ml after a period of about 2 years; however, this patient has also not outgrown his allergies. These cases and our previously reported data [17] indi-

cate that the induction of egg white tolerance may be difficult in patients whose serum IgE exhibits binding activity to digested small fragments of OVM.

Discussion

In the SGF-digestion system, preheating the OVM (100°C for 5 or 30 min) did not affect the OVM digestion pattern (fig. 1), consistent with the results of previous reports [9] in which heat treatment did not markedly decrease the allergenicity of OVM. On the other hand, a decrease in the pepsin/OVM ratio dramatically reduced the digestion rate, suggesting that digestibility may vary depending on the amount of OVM intake and the conditions of the individual's digestion system. In its native state, OVM possesses serine protease inhibitor activity. Fu et al. [11] and our group [10] previously reported that intact OVM was stable for 60 min in simulated intestinal fluid. Kovacs-Nolan et al. [15] also reported that pepsin-

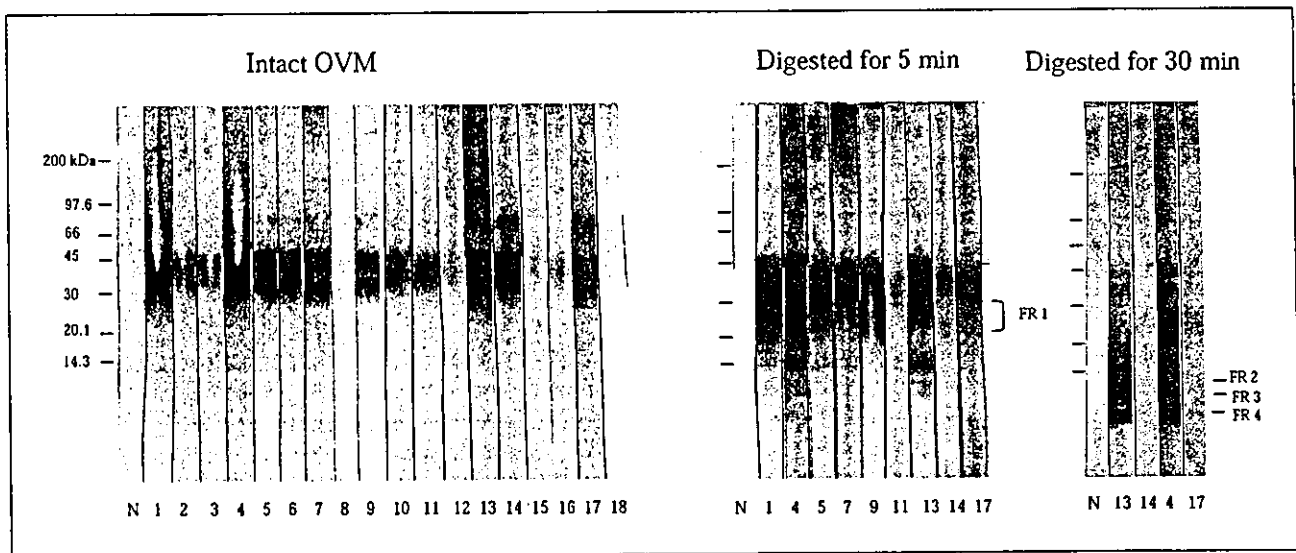


Fig. 4. Amino acid sequence and schematic representation of the SGF-digestion pattern of OVM. The amino acid sequence of OVM is shown in the upper panel. The arrows indicate the SGF-digested points according to the results of an N-terminal analysis of the OVM fragments (table 1), and the underlined regions indicate sequences identified by LC/MS/MS. Solid line = FR 1; dotted line = FR 3; dashed line = FR 4. Shaded areas represent reported human IgE epitopes [16]. The lower panel is a summary of the OVM digestion pattern according to N-terminal analysis.

Fig. 5. Western blot analysis of intact OVM and the fragments with serum IgE from egg white allergic patients and a normal volunteer. The fragments were prepared as described in the legend of figure 2. The number of each strip corresponds to the sample numbers in table 2.

Table 3. Reactivity of OVM and pepsin fragments with patient serum IgE

No.	IgE content, IU/ml		Reactivity with patient IgE ¹				
	total	egg white-specific	intact OVM	FR 1	FR 2	FR 3	FR 4
1	3,700	>100	+++	++	-	-	-
2	402	3.74	+	n.d.	n.d.	n.d.	n.d.
3	251	6.85	+	n.d.	n.d.	n.d.	n.d.
4	6,510	>100	+++	+++	+	+	++
5	2,060	>100	++	++	-	-	-
6	1,240	12.4	++	n.d.	n.d.	n.d.	n.d.
7	4,180	31.3	++	++	-	-	-
8	56	20.1	±	n.d.	n.d.	n.d.	n.d.
9	1,355	50.7	++	++	-	-	-
10	22,810	2.11	+	n.d.	n.d.	n.d.	n.d.
11	1,463	4.65	+	-	-	-	-
12	14,230	0.70-3.49	±	n.d.	n.d.	n.d.	n.d.
13	8,000	>100	+++	+++	+	+	++
14	22,490	1.05	+++	±	-	-	-
15	934	66.3	+	n.d.	n.d.	n.d.	n.d.
16	345	20.1	+	n.d.	n.d.	n.d.	n.d.
17	1,500	80	++	+	-	-	-
18	3,300	>10	-	n.d.	n.d.	n.d.	n.d.
19	20,500	26.8	+++	++	±	±	±
20	138	45.4	++	+	-	-	-
21	940	2.44	+	+	-	-	-
22	91	0.70-3.49	+	±	-	-	-
23	828	0.9	++	+	-	-	-
24	21	3.50-17.4	-	n.d.	n.d.	n.d.	n.d.
	positive/tested		22/24 (92%) ²	13/14 (93%) ³	3/14 (21%) ³	3/14 (21%) ³	3/14 (21%) ³

n.d. = Not done.

¹ Intensity of the reactivity of each band was evaluated by the ratio to normal serum: - = <1; ± = 1-2; + = 2-5; ++ = 5-10; +++ = >10.² Percent of egg white-positive samples.³ Percent of intact OVM-positive samples.

digested OVM retains its trypsin inhibitor activity. Therefore, OVM and its pepsin-digested fragments were thought to be stable in the small intestine.

At a pepsin/OVM ratio of 1 unit/μg, FR 1 reached a maximum level after 2 min of digestion, while both FR 2 and FR 3 reached maximum levels after 5 min of digestion; thereafter, FR 1, FR 2, and FR 3 gradually decreased. However, FR 4 increased continuously throughout the 30-min period of digestion and the major fragments were seen after 30 min of digestion (fig. 3). FR 4 was mainly composed of three fragments whose N-terminals were 134V, 104N and 19V (table 1). A C-terminal sequence, 165N-185C, was also identified in FR 4 (table 2). These fragments contain known IgE epitopes [19] and therefore may cause allergic responses. Three of the

OVM-positive sera from patients with egg white allergy reacted positively with the FR 4 fragments (table 3).

The present results are consistent with the previous finding that pediatric subjects with a higher IgE-binding activity to pepsin-treated OVM were unlikely to outgrow their egg allergy [17]. For peanut allergies, differences in IgE-binding epitopes have been reported between the patients with clinically active peanut allergies and those who developed a tolerance, regardless of the presence of high or low peanut-specific IgE levels [20].

The N-terminal residue of the major fragment (4-1) of FR 4 was Val-134 (30%; table 1). This fragment retains most of domain III, which has been reported to have significantly higher human IgG- and IgE-binding activities than those of domains I and II [12]. A domain-III OVM

variant has also been reported to cause a reduction in immunogenicity and allergenicity [21].

Domains I, II, and III contain one, three, and one N-glycosylation sites, respectively [7]. The possible relation between the carbohydrate chain in domain III and allergenicity is interesting. One report suggested that this carbohydrate chain may play an important role in allergenic determinants against human IgE antibody [13], and another report suggested that the carbohydrate chains of OVM may protect against peptic hydrolysis [22]. However, the carbohydrate moieties have been shown to have only a minor effect on allergenicity [23]. As shown in figure 2, intact OVM, FR 1, and FR 2 fragments were detected using PAS staining, suggesting the presence of carbohydrate chains, but FR 4 was not stained with the PAS reagent, despite being clearly detected with CBB. Therefore, FR 4 might contain little or no carbohydrate chains. Since FR 4 seems to maintain its allergenic potential, as described above, the absence of the carbohydrate chains in FR 4 suggests that they are not necessary for OVM allergenicity. Since the minimum peptide size capable of eliciting significant clinical symptoms of allergic reactions is thought to be 3.1 kDa [24], FR 4 may be able to trigger mast cell activation and elicit clinical symptoms.

In this report, the SGF-digestion kinetic pattern of OVM was investigated in detail, and the partial sequences

of the fragments in the 4 fractions separated by SDS-PAGE were determined. Furthermore, the reactivity of the fragments with a number of serum samples from patients with egg white allergies was detected using Western blotting. The four fractions were separated according to their molecular weight and consisted of more than one fragment, as determined by N-terminal analysis. The identified sequences that started at Asn-104 and Val-134 in FR 3, as determined using LC/MS/MS (table 2), coincided with the 3-2 and 3-3 fragments in the N-terminal analysis (table 1), and the sequence that started at Asn-104 in FR 4 coincided with fragment 4-2. Moreover, the LC/MS/MS analysis indicated that FR 3 and FR 4 contained other parts of domain II and the C-terminal sequence N165-C185, which are thought to be minor components of these fractions. The combination of SGF digestion and patient IgE may provide useful information for the diagnosis and prediction of potential OVM allergenicity.

Acknowledgement

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Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography–mass spectrometry

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Abstract

We have previously demonstrated that liquid chromatography/mass spectrometry equipped with a graphitized carbon column (GCC-LC/MS) is useful for the structural analysis of carbohydrates in a glycoprotein. Here, we studied the monosaccharide composition analysis and quantitative oligosaccharide profiling by GCC-LC/MS. Monosaccharides were labeled with 2-aminopyridine and then separated and monitored by GCC-LC/MS in the selective ion mode. The use of tetradeuterium-labeled pyridylamino (d_4 -PA) monosaccharides as internal standards, which were prepared by the tagging of standard monosaccharides with hexadeuterium-labeled 2-aminopyridine (d_6 -AP), afforded a good linearity and reproducibility in ESIMS analysis. This method was successfully applied to the monosaccharide composition analysis of model glycoproteins, fetuin, and erythropoietin. For quantitative oligosaccharide profiling, oligosaccharides released from an analyte and a standard glycoprotein were tagged with d_0 - and d_6 -AP, respectively, and an equal amount of d_0 - and d_4 -PA oligosaccharides were coinjected into GCC-LC/MS. In this procedure, the oligosaccharides that existed in either analyte or a standard glycoprotein appeared as single ions, and the oligosaccharides that existed in both analyte and a standard glycoprotein were detected as paired ions. The relative amount of analyte oligosaccharides could be determined on the basis of the analyte/internal standard ion-pair intensity ratio. The quantitative oligosaccharide profiling enabled us to make a quantitative and qualitative comparison of glycosylation between the analyte and standard glycoproteins. The isotope tag method can be applicable for quality control and comparability assessment of glycoprotein products as well as the analysis of glycan alteration in some diseases.

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Keywords: Monosaccharides; Oligosaccharides; Pyridylation; Isotope tag

1. Introduction

A variety of recombinant glycoproteins and modified glycoproteins are developed as medical agents, and most of them exist in heterogeneous forms because of the various combinations of oligosaccharides. Alteration of glycosylation is

known to affect the biological activity, mobilization, and biophysical properties of glycoproteins [1], so assessments of their carbohydrate structure and heterogeneity are essential in many stages of development and quality control of glycoprotein products. Since glycosylation varies in response to changes in the manufacturing condition, monosaccharide composition analysis and/or oligosaccharide profiling are needed for the characterization and as a test for constancy and comparability assessments of glycosylation [2]. Several analytical procedures using HPLC have been reported for oligosaccharide profiling and structural analysis of carbohydrates [3–5]. The oligosaccharide profiling using liquid chromatography/mass spectrometry (LC/MS) is especially known to provide structural information from their chromatographic behavior and molecular mass [6–8]. We have developed mass spectrometric oligosaccharide profiling using a graphitized carbon column (GCC), which can separate

Abbreviations: AP, 2-aminopyridine; d_0 , non-deuterium-labeled; d_4 , tetradeuterium-labeled; d_6 , hexadeuterium-labeled; Fuc, fucose; Gal, galactose; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; GCC, graphitized carbon column; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; PA, pyridylamino; R.S.D., relative standard deviation; SIM, selected ion mode; TFA, trifluoroacetic acid; TIC, total ion chromatogram

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oligosaccharides based on subtle differences in branch, position, and linkage with volatile solution [9,10]. This method enables us to distinguish the glycosylation among some glycoprotein products produced in different cells [11].

A use of internal standards is known to improve the precision and linearity in quantitative analyses. Isotopic analogs of the analytes are currently the preferred internal standards for quantification by mass spectrometry (MS) procedures. For instance, Gygi et al. [12] demonstrated the approach for the accurate quantification of the proteins within complex mixture using isotope-coded affinity tags (ICATs). The use of the isotope-labeled carbohydrates as internal standards can make it possible to quantify the carbohydrates by LC/MS. Reductive pyridylation is frequently used for the tagging of carbohydrates in HPLC analysis [13,14]. This derivatization is known to afford higher sensitivity in MS analysis [15], and PA oligosaccharides were reported to be separated by GCC [16]. Here, we study quantitative analysis of carbohydrates using tetradeuterium-labeled pyridylamino (d_4 -PA) carbohydrates as internal standards. First, we study the monosaccharide composition analysis by using d_4 -PA monosaccharides as internal standards. Next, the isotope tag method is used for the quantitative oligosaccharide profiling using recombinant human chorionic gonadotropin (rhCG) and human chorionic gonadotropin (hCG) as an analyte and standard glycoproteins, respectively.

2. Materials and methods

2.1. Materials

All monosaccharide standards were purchased from Seikagaku-kogyo (Tokyo, Japan). The pyridylation apparatus (PALSTATION), reagents for the pyridylation reaction, and PA monosaccharide standards were available from TaKaRa Biomedicals (Otsu, Japan). The hexadeuterium-labeled 2-aminopyridine (d_6 -AP) was purchased from Wako (Osaka, Japan). Human chorionic gonadotropin (hCG) and recombinant hCG (rhCG) were bought from Sigma (St. Louis, MO, USA). *N*-glycosidase F was purchased from Roche Diagnostics. All other chemicals and reagents were of analytical grade and were commercially available.

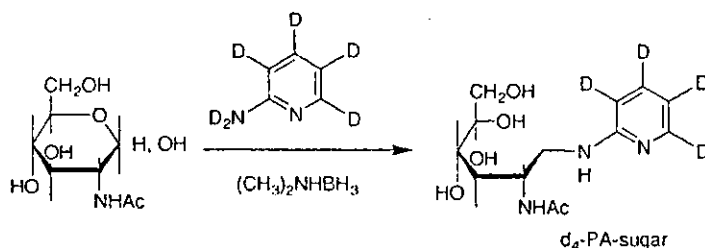


Fig. 1. Synthesis of d_4 -PA monosaccharide internal standard.

2.2. Pyridylation of monosaccharides

For the pyridylation of amino sugars, free amino groups of monosaccharides (GlcN, GalN, 1–1000 pmol) were acetylated by incubation in 50 μ l of methanol/pyridine/distilled water (30/15/10, v/v/v) with 2 μ l of acetic anhydride for 30 min at room temperature. The mixture was dried using a vacuum centrifuge evaporator without heating. Acetic acid (50 μ l), methanol (60 μ l), and 10 μ l of coupling reagent prepared by mixing 100 mg of AP was added to monosaccharides (Fuc, Gal, Glc, Man, GlcNAc, GalNAc, 1–1000 pmol). The mixture was heated at 90 $^{\circ}$ C for 20 min by PALSTATION, and the excess reagents were removed by evaporation under a stream of nitrogen gas at 60 $^{\circ}$ C for 20 min. Then 10 μ l of a reducing reagent, prepared just before use by mixing 6 mg of borane–dimethylamine complex and 100 μ l of acetic acid, was added, and the mixture was heated at 90 $^{\circ}$ C for 35 min. The reaction mixture was dried three times under a stream of nitrogen gas at 50 $^{\circ}$ C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of isotope analogs, the tetradeuterium-labeled PA (d_4 -PA) monosaccharide, d_0 -AP was just replaced by d_6 -AP (Fig. 1).

2.3. Monosaccharide composition analysis of a glycoprotein

A glycoprotein (25 pmol) was placed in a hydrolysis tube fitted with a Teflon-lined screw cap. Fifty microliters of 2M HCl–2M trifluoroacetic acid (TFA) was added to the sample, which was then heated at 100 $^{\circ}$ C for 6 h. Simultaneously, a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analytes. The solution obtained was freeze-dried. The monosaccharides obtained from the analyte glycoproteins and standard monosaccharides were tagged with non-deuterium-labeled 2-aminopyridine (d_0 -AP) and d_6 -AP, respectively. Each tagged oligosaccharide mixture was dissolved into purified water, and a mixture of d_0 - and d_4 -PA monosaccharides was injected into the GCC-LC/MS.

2.4. Preparation of N-linked oligosaccharides

N-linked oligosaccharides were released from hCG as described previously [17]. Briefly, hCG and rhCG (100 μ g)

were dissolved in 360 μ l of 0.5 M Tris–HCl buffer (pH 8.6), containing 8 M guanidine hydrochloride and 5 mM ethylenediaminetetra-acetic acid (EDTA). After an addition of 2.6 μ l of 2-mercaptoethanol, the mixture was allowed to stand at room temperature for 2 h. To this solution, 7.56 mg of monoiodoacetic acid was added, and the resulting mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was applied to a PD-10 column (Amershambioscience, Uppsala, Sweden) to remove the reagents, and the eluate was lyophilized.

Carboxymethylated hCG and rhCG were dissolved in 100 μ l of 0.1 M sodium phosphate buffer, pH 7.2, and incubated with 5 units of PNGase F at 37 °C for 2 days. Protein was precipitated with 340 μ l of cold ethanol, and the supernatant was dried.

2.5. Pyridylation of oligosaccharides from hCG

To the lyophilized oligosaccharides released from rhCG we added 10 μ l of coupling reagent prepared by mixing 300 mg of d_0 -AP, and 100 μ l of acetic acid, and the reaction mixture was heated at 90 °C for 60 min. Then, 10 μ l of a reducing reagent, prepared just before use by mixing 20 mg of borane–dimethylamine complex and 100 μ l of acetic acid, was added, and the mixture was heated at 80 °C for 60 min. The reaction mixture was dried three times under a stream of nitrogen gas at 60 °C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of the tetradeuterium-labeled (d_4)-PA oligosac-

charide isotope analogs, d_0 -AP was just replaced by d_6 -2-aminopyridine.

2.6. LC/MS analysis

LC was carried out using a Magic 2002 HPLC system (Michrom BioResources Inc., Auburn, CA, USA) using a Hypercarb column (0.2 mm \times 150 mm, Thermoelectron, San Jose, CA, USA). The flow rate was set at 2–3 μ l/min through a splitter system. The mobile phases were 5 mM ammonium acetate (pH 8.5) with 2% of acetonitrile (pump A) and 80% of acetonitrile (pump B). A gradient of 10–35% of B in 60 min was used for the monosaccharide analysis. For oligosaccharide profiling, we used a gradient of 5–20% of B in 20 min, 20–70% of B in 15 min, and 70–95% of B in 5 min. The mass spectrometer used was a TSQ 7000 (Thermoelectron) equipped with a nanoelectrospray ion source (AMR Inc., Tokyo, Japan). The ESI voltage was set to 2000 V (positive ion mode) or 1500 V (negative ion mode), and the capillary temperature was 175 °C.

3. Results

3.1. Monosaccharide composition analysis using the isotope tag method

First, we examined the possibility of the isotope-tag method for the monosaccharide composition analysis of gly-

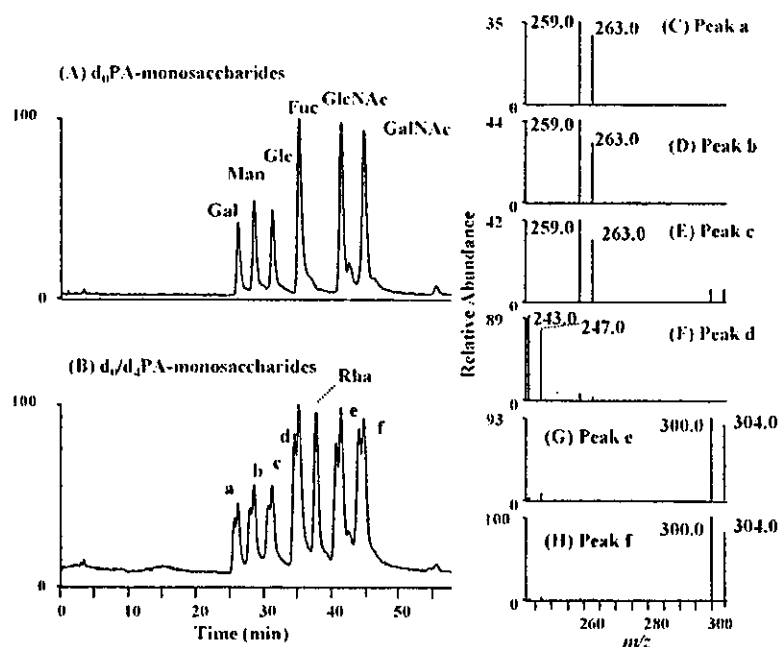


Fig. 2. (A) Extracted ion chromatogram (set m/z values, 243, 259, and 300) of d_0 -PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, GlcNAc, and GalNAc). (B) Extracted ion chromatogram (set m/z values, 243, 247, 259, 263, 300, and 304) of a mixture of d_0 - and d_4 -PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, Rham, GlcNAc and GalNAc). (C) Mass spectra of peaks a (C), b (D), c (E), d (F), e (G), and f (H).

coproteins. An equal molar of each d_0 -PA monosaccharide (Gal, Man, Glc, Fuc, GlcNAc, and GalNAc, 1 pmol each) was analyzed by GCC-LC/MS in the positive ion mode. The ions monitored were m/z 259 (for d_0 -PA-Gal, d_0 -PA-Man, and d_0 -PA-Glc), m/z 243 (d_0 -PA-Fuc), and m/z 300 (d_0 -PA-GlcNAc and d_0 -PA-GalNAc). Fig. 2A shows the mass chromatogram of the d_0 -PA monosaccharides. All six d_0 -PA monosaccharides were retained and separated by GCC. The detection limit at a signal-to-noise ratio of 3 was 45 fmol.

The d_4 -PA monosaccharides were prepared as internal standards by tagging of standard monosaccharides with d_6 -AP and combined with d_0 -PA monosaccharides. Fig. 2B shows the chromatogram of a mixture of d_0 -, d_4 -PA monosaccharides and PA-labeled Rhamnose, which is frequently used as an internal standard in the monosaccharide composition analysis. Paired ions with a difference of m/z 4 were detected in the mass spectra of peaks a–f (Fig. 2C–H). When 0.5 pmol d_0 -PA monosaccharides were determined in the presence of d_4 -PA monosaccharides or Rhamnose by GCC-LC/MS, the relative standard deviation ($n=5$) was 1.8–4.8% or 5.6–8.3%, respectively.

To assess the linearity and reproducibility of the whole procedure, including reacetylation, pyridylation, the removal of excess derivatization reagents, and GCC-LC/MS, we tagged different amounts of monosaccharides (Gal, Man, Glc, Fuc, GlcN, and GalN, 1–1000 pmol) with d_0 -AP, and d_4 -PA monosaccharides (4 or 20 pmol) were added to the d_0 -PA monosaccharides (1–10 pmol or 10–1000 pmol, respectively). The whole process of the isotope tag method was found to be linear for all six monosaccharides over the tested range of 1–1000 pmol (Fig. 3). The accuracy of this method was approximately 80–100% (Fig. 3), and the relative standard deviations (%R.S.D.) were less than 7.2% for all monosaccharides (based on the peak area ratio of monosaccharides from five samples).

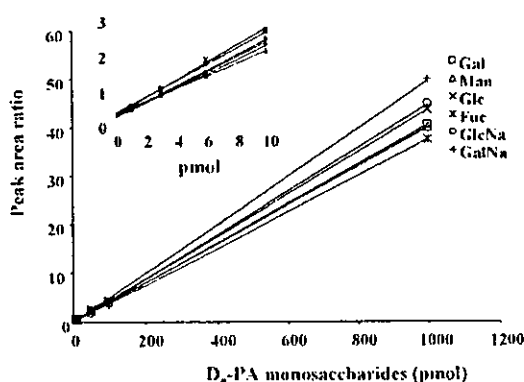


Fig. 3. Linearity on the isotope-tag method for monosaccharide quantification. For the internal standards, 1000 pmol monosaccharides were derivatized to d_4 -PA monosaccharides. Different amounts of monosaccharides were derivatized to d_0 -PA monosaccharides and co-injected with 4 pmol (A) or 20 pmol (B) internal standards into GCC-LC/MS.

We used this method for the monosaccharide composition analysis of fetuin and erythropoietin. Accuracy in the monosaccharide composition analysis of a glycoprotein relies on the condition of hydrolysis. Fan et al. [18] studied the hydrolysis of N-linked oligosaccharides and recommended 4 h with 2 M TFA at 100 °C for neutral sugars, and 6 h with 4 M HCl at 100 °C for amino sugars. While these hydrolysis conditions result in the complete release of neutral and amino sugars with no degradation, it takes two hydrolyses for a single sample. To quantify both neutral and amino sugars in glycoproteins in the same run, fetuin and erythropoietin (25 pmol) were heated in 2 M HCl-2M TFA at 100 °C for 6 h [19], and a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analyte glycoproteins. After hydrolysis, the analyte and standard monosaccharides were tagged with d_0 - and d_6 -AP, respectively. Fig. 4A and E show the mass chromatogram of monosaccharides prepared from fetuin and erythropoietin in the presence of d_4 -PA monosaccharides, respectively.

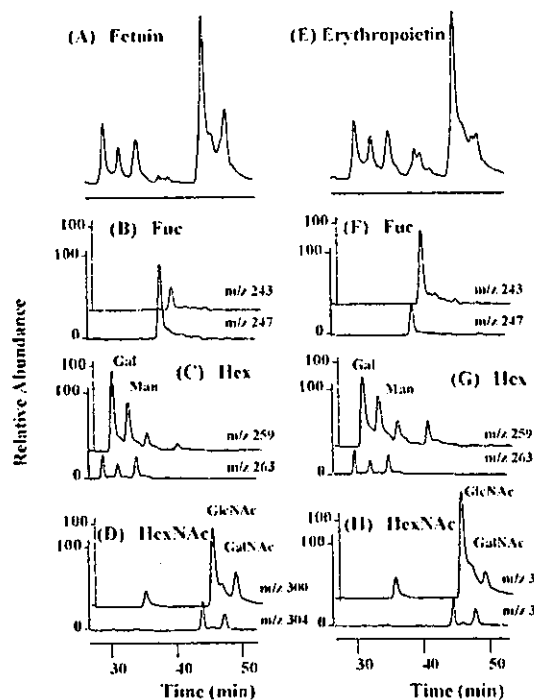


Fig. 4. Monosaccharide composition analysis of glycoproteins. Extracted ion chromatograms of d_0 -PA monosaccharides from fetuin and d_4 -PA standard monosaccharides (set m/z values, 243, 247, 259, 263, 300, and 304) (A), d_0 -PA Fuc from fetuin and d_4 -PA standard Fuc (set m/z values, 243 and 247) (B), d_0 -PA Hex from fetuin and d_4 -PA standard Hex (set m/z values, 259 and 263) (C), and d_0 -PA HexNAc from fetuin and d_4 -PA standard HexNAc and (set m/z values, 300 and 304) (D). Extracted ion chromatograms of d_0 -PA monosaccharides from erythropoietin and d_4 -PA standard monosaccharides and (set m/z values, 243, 247, 259, 263, 300, and 304) (E), d_0 -PA Fuc from erythropoietin and d_4 -PA standard Fuc (set m/z values, 243 and 247) (F), d_0 -PA Hex from erythropoietin and d_4 -PA standard Hex (set m/z values, 259 and 263) (G), and d_0 -PA HexNAc from erythropoietin and d_4 -PA standard HexNAc (set m/z values, 300 and 304) (H).

Table 1
Monosaccharide composition analysis by isotope-tag method

Glycoprotein	Monosaccharide	mol/mol ^a	mol/mol
Fetuin	Fuc	0.3	0 [20]
	Gal	10.4	12
	Man	7.6	9
	GlcNAc	14.7	15
	GalNAc	3.4	3
Erythropoietin	Fuc	3.4	4.1 [21]
	Gal	12.8	13.8
	Man	8.1	8.7
	GlcNAc	15.6	17.2
	GalNAc	1.5	0.9

^a Values were expressed as mol detected in 1 mol glycoprotein.

Fig. 4B, and F show the mass chromatograms of d₀- and d₄-PA fucose, Fig. 4C and G indicate those of d₀- and d₄-PA hexose, and Fig. 4D and H show those of d₀- and d₄-PA HexNAc. The monosaccharide compositions of fetuin and erythropoietin calculated from the peak area ratios (d₀-PA/d₄-PA monosaccharides) were in good agreement with the reported values (Table 1) [20,21]. By heating the standard monosaccharides simultaneously the decomposition of monosaccharides during hydrolysis can be corrected, and a use of isotope analogs as the internal standards can reduce deviation in ESIMS analysis.

3.2. Quantitative oligosaccharide profiling using the isotope tag method

Next, we explored the capability of the isotope-tag method for the quantitative oligosaccharide profiling. When d₀-PA oligosaccharides prepared from an analyte glycoprotein are analyzed with an equal part of d₄-PA oligosaccharides prepared from a standard glycoprotein, oligosaccharides which link to both the analyte and the standard glycoproteins are expected to appear as paired ions with a difference of 4 Da, and the individual oligosaccharides in the analyte glycoprotein can be quantified based on the analyte/internal standard ion-pair intensity ratio. On the other hand, any oligosaccharides that link to either the analyte or the standard glycoprotein ought to be detected as single ions. Oligosaccharides released from rhCG and hCG were tagged with d₀- and d₆-AP, respectively, and the tagged oligosaccharides were analyzed by GCC-LC/MS in both positive and negative ion modes.

Fig. 5A and B show the mass spectra of the peak which was detected at 21.5 min in the positive and the negative ion mode, respectively. In the positive ion mode, ions at *m/z* 863.0, 1359.4 and 1197.2 were detected (Fig. 5A), and they can be assigned to d₄-PA [Hex]₅[HexNAc]₄²⁺ (an asialobiantennary oligosaccharide), d₄-PA[Hex]₃[HexNAc]₄⁺ (a fragment of the asialobiantennary form) and d₄-PA[Hex]₄[HexNAc]₄⁺ (a fragment of the asialobiantennary form), respectively. In contrast, only an ion at *m/z* 860.9 (d₄-PA[Hex]₅[HexNAc]₄²⁻, asialobiantennary oligosaccharide) was detected in the negative ion mode (Fig. 5B). This result suggests that mass spectra

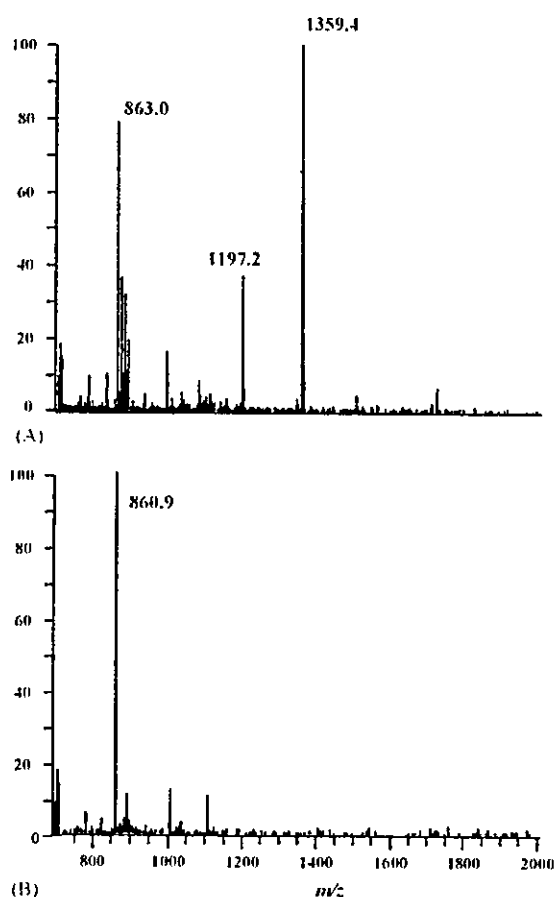


Fig. 5. Mass spectra of d₄-PA oligosaccharide. D₄-PA oligosaccharide eluted at 21.5 min from GCC was analyzed by ESIMS in the positive ion mode (A) and negative ion mode (B).

of PA oligosaccharides become complicated due to fragmentation in the positive ion mode, while only molecular ions can be detected in the negative ion mode. Therefore, ESI analysis in the negative ion mode was chosen for the PA oligosaccharide profiling.

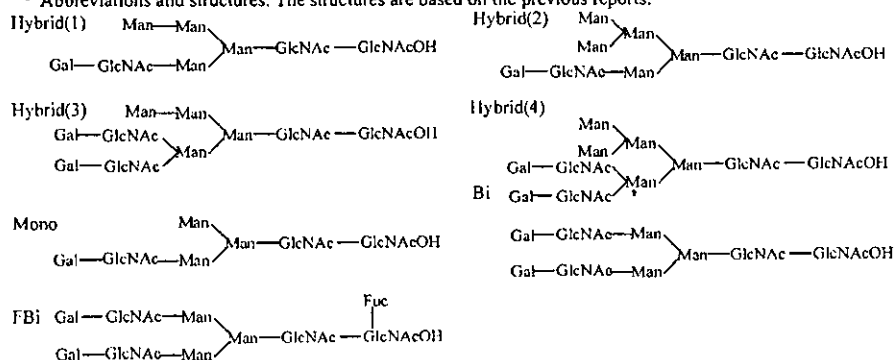
Fig. 6A and B show the TIC of a mixture of equal parts of d₀-PA oligosaccharides prepared from rhCG and d₄-PA oligosaccharides from hCG, and its two-dimensional display (retention time versus *m/z*), respectively. The carbohydrate structures, which can be deduced from *m/z* values, are indicated in Table 2. Paired ions at *m/z* 757.5, 759.5 were observed in the mass spectrum of peak a1. Based on carbohydrate composition [Hex]₅[HexNAc]₃, it can be assigned to a hybrid type oligosaccharide. Likewise, peak 11, 12, 14, 15, p1, p2, and p4 consisted of paired ions and can be assigned to monosialylated (11, 12, 14, 15) and disialylated (p1, p2) biantennary oligosaccharide without Fuc. Fig. 7 shows TIC of d₀- and d₄-PA oligosaccharides (A), extracted ion chromatograms of d₀-PA (B), d₄-PA (C), and d₀- and d₄-PA monosialylated biantennary form (D). The mass spectra of peaks 11–15 are shown in Fig. 7E–I. Peak 13 was not observed in Fig. 7D and only

Table 2
Structural assignment of peaks in Fig. 6B

Peak nos.	Carbohydrate composition ^a	Deduced structure ^b	Theoretical mass (d ₀ -PA-sugar)	Observed <i>m/z</i>			Ion-pair intensity ratio d ₀ /d ₄
				d ₀ -PA-rhCG		d ₄ -PA-hCG	
				M ²⁻	M ³⁻	M ²⁻	
a1	[Hex] ₅ [HexNAc] ₃	Hybrid (1)	1517.5	757.5		759.5	0.27
b1	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	Bi + NA ₂	2303.1		768.2		
c1	[Fuc] ₁ [Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	FBi + NA ₂	2449.3		816.7		
d1	[Hex] ₄ [HexNAc] ₃ [NeuNAc] ₁	Mono + NA	1646.6			824.3	
d2	[Hex] ₄ [HexNAc] ₃ [NeuNAc] ₁	Mono + NA	1646.6			824.0	
e1	[Hex] ₆ [HexNAc] ₃	Hybrid (2)	1679.6	838.6			
e2	[Hex] ₆ [HexNAc] ₃	Hybrid (2)	1679.6			840.6	
f1	[Hex] ₅ [HexNAc] ₄	Bi	1720.7	858.9			
f2	[Hex] ₅ [HexNAc] ₄	Bi	1720.7			861.2	
g1	[Hex] ₅ [HexNAc] ₃ [NeuNAc] ₁	Hybrid (1) + NA	1807.7	902.9			
g2	[Hex] ₅ [HexNAc] ₃ [NeuNAc] ₁	Hybrid (1) + NA	1808.7			905.0	
h1	[Fuc] ₁ [Hex] ₅ [HexNAc] ₄	FBi	1866.8			934.0	
i1	[Hex] ₆ [HexNAc] ₄	Hybrid (3)	1882.8	940.2			
j1	[Hex] ₅ [HexNAc] ₅	Bi + GN	1924.9			962.7	
k1	[Hex] ₆ [HexNAc] ₃ [NeuNAc] ₁	Hybrid (2) + NA	1970.8			986.8	
k2	[Hex] ₆ [HexNAc] ₃ [NeuNAc] ₁	Hybrid (2) + NA	1970.8			986.2	
l1	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	Bi + NA	2011.9	1004.7		1006.7	0.77
l2	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	Bi + NA	2011.9	1004.6		1007.3	0.56
l3	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	Bi + NA	2011.9	1004.6			
l4	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	Bi + NA	2011.9	1004.6		1006.5	0.67
l5	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	Bi + NA	2011.9	1004.6		1006.4	0.49
m1	[Hex] ₇ [HexNAc] ₄	Hybrid (4)	2044.9	1021.4			
n1	[Fuc] ₁ [Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	FBi + NA	2158.0			1079.8	
n2	[Fuc] ₁ [Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	FBi + NA	2158.0			1079.8	
n3	[Fuc] ₁ [Hex] ₅ [HexNAc] ₄ [NeuNAc] ₁	FBi + NA	2158.0			1079.8	
o1	[Hex] ₆ [HexNAc] ₄ [NeuNAc] ₁	Hybrid (3) + NA	2174.0	1085.6			
o2	[Hex] ₆ [HexNAc] ₄ [NeuNAc] ₁	Hybrid (3) + NA	2174.0	1085.7			
p1	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	Bi + NA ₂	2303.1	1150.3		1152.1	5.76
p2	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	Bi + NA ₂	2303.1	1150.2		1152.2	5.92
p3	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	Bi + NA ₂	2303.1	1150.1			
p4	[Hex] ₅ [HexNAc] ₄ [NeuNAc] ₂	Bi + NA ₂	2303.1	1150.3		1152.4	0.45

^a Hex, hexose; HexNAc, *N*-acetyl hexosamine; NeuNAc, *N*-acetyl neuraminic acid; Fuc, fucose.

^b Abbreviations and structures. The structures are based on the previous reports.



single ion was detected in Fig. 7G. These results suggest that one of monosialylated binantennary oligosaccharides isomers links to only rhCG.

We determined relative amounts of some oligosaccharides in rhCG on the basis of ion-pair intensity ratios (Table 2). The amount of monosialylated biantennary forms (l1, l2, l4, and l5) linked to rhCG were 50–70% of those to hCG. The amount of disialylated biantennary forms (p1 and p2) linked to rhCG

was five-fold of those to hCG, and the linkage of p4 to rhCG was one-half of that of hCG. The isotope tag method clearly shows the difference in distribution of isomers between rhCG and hCG.

In this procedure, oligosaccharides linked to either rhCG or hCG were detected as single ions. As shown in Table 2, nine oligosaccharides were detected as single ions in rhCG, and they are reduced to hybrid type and complex type.

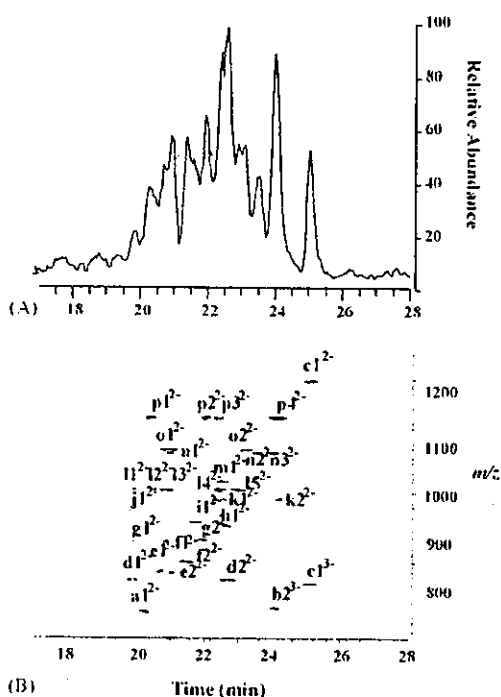


Fig. 6. TIC of a mixture of equal amount of d_0 -PA N-linked oligosaccharides from rhCG and d_4 -PA N-linked oligosaccharides from hCG (A), and its 2D display (B). Oligosaccharides (from 2 μ g rhCG and hCG) were analyzed by GCC-LC/MS in the negative ion mode.

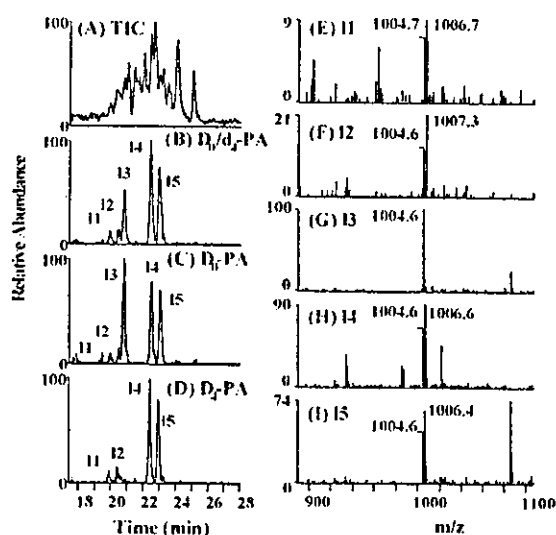


Fig. 7. TIC of a mixture of equal amount of d_0 -PA N-linked oligosaccharides from rhCG and d_4 -PA N-linked oligosaccharides from hCG (A). Extracted ion chromatograms of d_0 - and d_4 -PA monosialylated biantennary (set m/z values, 1004–1007) (B), d_0 -PA monosialylated biantennary (set m/z values, 1004–1005) (C), and d_4 -PA monosialylated biantennary oligosaccharides (set m/z values, 1006–1007) (D). Mass spectra of peak 11–15 (E–I).

Fourteen oligosaccharides were detected only in hCG, and most of them were fucosylated complex type. These results show the differences in glycosylation between rhCG and hCG and suggest that many hybrid type oligosaccharides linked to rhCG, while fucosylated oligosaccharides attach to hCG.

4. Discussion

Alteration of glycosylation is known to cause many changes in the biological activity as well as the physical properties of proteins. Several procedures of oligosaccharide profiling have been reported for the assessment of alteration of glycosylation, however, most of them can be used for only either qualitative or quantitative analysis. Although mass spectrometric oligosaccharide profiling is useful for the qualitative analysis, it has a problem on precision, and some isomers are still indistinguishable if their retention times are closed to others. In this study, we demonstrated that the use of isotope-tagged internal standards and GCC-LC/MS made it possible to do both quantitative and qualitative carbohydrate analysis.

First, we demonstrated the monosaccharide composition analysis using the isotope tag method. The use of internal standards that were heated under the same hydrolysis condition as an analyte glycoprotein resulted in good precision and accuracy in the monosaccharide composition analysis. Several HPLC methods for determination of monosaccharides have been reported. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been widely used for monosaccharide composition analysis [20,22–25]. Although HPAEC-PAD gives high resolution of all common monosaccharides and has the advantage of not requiring derivatization, this method is also known to have a disadvantage of limited selectivity [26]. The isotope tag method with SIM mode is equal to the HPAEC-PAD in sensitivity and is better than it in selectivity.

Next, we demonstrated the potentiality of the isotope tag method for quantitative oligosaccharide profiling using rhCG and hCG as model glycoproteins. hCG consists of an α subunit (MW 14.7 kDa) and a β subunit (MW 23.0 kDa), and oligosaccharides link to Asn52, and 78 in the α subunit and Asn13 and 30 in the β subunit. It has been reported that the majority of N-linked oligosaccharides in rhCG and hCG are fucosylated or non-fucosylated di-, tri-, and tetra-antennary forms with a various level of sialylation [27–30]. We prepared d_0 -PA oligosaccharides and d_4 -PA oligosaccharides from rhCG and hCG, respectively, and an equal part of d_0 -PA and d_4 -PA oligosaccharides was injected into LC/MS. We demonstrated that the oligosaccharides existing in one side protein were detected as single ions, whereas common oligosaccharides were detected as paired ions. We could easily realize that monosialo-, and disialobiantennary oligosaccharides linked to both hCG and rhCG, while fucosylated oligosaccharides and some hybrid type oligosaccharides linked to only hCG and rhCG, respectively. In addition, we demonstrated the pos-

sibility of the quantitative comparison the oligosaccharides between two quite similar glycoproteins. This quantitative oligosaccharide profiling is expected to be a powerful tool in various stages, including quality control and comparability assessment of glycoprotein products, and elucidation of glycan alteration in some diseases.

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Site-specific glycosylation analysis of human apoB100

Title: Site-specific glycosylation analysis of human apolipoprotein B100 using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

Human apolipoprotein B100 (apoB100) has 19 potential *N*-glycosylation sites, and 16 asparagine residues were reported to be occupied by high-mannose type, hybrid type, and monoantennary and biantennary complex type oligosaccharides. In the present study, a site-specific glycosylation analysis of apoB100 was carried out using reversed-phase high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). ApoB100 was reduced and carboxymethylated, and then digested by trypsin or chymotrypsin. The complex mixture of peptides and glycopeptides was subjected to LC/ESI/MS/MS, where product ion spectra of the molecular ions were acquired data-dependently. The glycopeptide ions were extracted and confirmed by the presence of carbohydrate-specific fragment ions such as *m/z* 204 (HexNAc) and 366 (HexHexNAc) in the product ion spectra. The peptide moiety of glycopeptide was determined by the presence of the b- and y-series ions derived from its amino acid sequence in the product ion spectrum, and the oligosaccharide moiety was deduced from the calculated molecular mass of the oligosaccharide. The heterogeneity of carbohydrate structures at 17 glycosylation sites was determined using this methodology. Our data showed that Asn2212, not previously identified as a site of glycosylation, could be glycosylated. It was also revealed that Asn158, 1341, 1350, 3309, and 3331 were occupied by high-mannose type oligosaccharides while Asn 956, 1496, 2212, 2752, 2955, 3074, 3197, 3438, 3868, 4210 and 4404 were predominantly occupied by mono- or disialylated oligosaccharides. Asn 3384, the nearest *N*-glycosylation site to the LDL-receptor binding site (amino acids 3359-3369), was occupied by a variety of oligosaccharides including high-mannose, hybrid and complex types. These results are useful for understanding the structure of LDL particles and oligosaccharide function in LDL-receptor ligand binding.