



Purification and molecular characterization of a truncated-type Ara h 1, a major peanut allergen: oligomer structure, antigenicity, and glycoform

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Abstract

Peanut allergies are among the most severe food allergies, and several allergenic proteins referred to as Ara h 1–Ara h 17 have been identified from peanut seeds. The molecular characterization of Ara h 1 (63 kDa), a glycosylated allergen, has almost been completed, and the occurrence of two homologous genes (clone 41B and clone P17) has been identified. In this study, we found a new variant of Ara h 1 *i.e.* 54 kDa, in which the N-terminal amino acid sequence was EGREGGEQ-, indicating that the N-terminal domain of 63 kDa Ara h 1 had been removed. This new isoform was obtained from the run-through fraction of hydrophobic interaction chromatography while 63 kDa Ara h 1 was tightly bound to the hydrophobic resins, suggesting that the removal of the N-terminal domain resulted in extreme hydrophilic properties. We found that 63 kDa Ara h 1 occurs as higher order homooligomeric conformations such as decamer or nonamer, while 54 kDa Ara h 1 occurs exclusively as a homotrimer, indicating that the N-terminal domain of the 63 kDa molecule may be involved in higher order oligomerization. When antisera from peanut-allergic patients were treated with both the Ara h 1 molecules, the immunoglobulin E (IgE) antibodies in these sera reacted with each Ara h 1 molecule, suggesting that the C-terminal as well as the N-terminal domains of Ara h 1 contribute significantly to the epitope formations of this peanut glycoallergen. Furthermore, the glycoform analyses of *N*-glycans linked to 63 kDa and 54 kDa Ara h 1 subunits revealed that both typical high-mannose type and β -xylosylated type *N*-glycans are linked to the molecules. The cross-reactivity of IgE against Ara h 1 in the serum of one peanut allergy patient was completely lost by de-*N*-glycosylation, indicating the *N*-glycan of Ara h 1 was the sole epitope for the Ara h 1-specific IgE in the patient.

Keywords Peanut allergy · Ara h 1 · Glycoallergen · Immunogenic *N*-glycan

Abbreviations

RP-HPLC	reversed-phase high performance liquid chromatography
NP-HPLC	normal-phase HPLC
PA-	pyridylamino
Hex	hexose
HexNAc	<i>N</i> -acetylhexosamine
Pen	pentose
Deoxyhex	deoxyhexose

Man	D-mannose
GlcNAc	<i>N</i> -acetyl-D-glucosamine
Xyl	D-xylose
Fuc	L-fucose
MX	Xyl β 1–2Man β 1–4GlcNAc β 1–4GlcNAc-PA
M3X	Man α 1–6(Man α 1–3)(Xyl β 1–2)Man β 1–4GlcNAc β 1–4GlcNAc-PA
M4X	Man α 1–3Man α 1–6(Man α 1–3)(Xyl β 1–2)Man β 1–4GlcNAc β 1–4GlcNAc-PA
Mn	(Man) <i>n</i> GlcNAc2-PA

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Introduction

Peanut allergies are one of the most common food allergies, and many allergenic proteins referred to as Ara h 1–Ara h 17 have been identified from peanut seeds [1]. The most predominant allergens are Ara h 1 and Ara h 2 to which the sera of 70–

90% of the patients with peanut allergy respond [1]. Ara h 1 consists of three identical N-glycosylated subunits (63 kDa) and accounts for 12–16% of the total peanut proteins [2, 3]. The molecular characterization of Ara h 1 has nearly been completed, and two isoforms (clone 41B and clone P17) have been identified and characterized [4]. Ara h 1 belongs to the vicilin family of legume seed storage proteins (7S globulin) and is a bicupin seed storage glycoprotein, which has been identified as a homotrimer consisting of a 63–66 kDa subunit [5, 6]. The stable trimeric structure contributes to its resistance to protease degradation in the human gastrointestinal tract. Hydrophobic amino acids play a critical role in trimer formation, and these important amino acids are located at the distal ends, where monomer-monomer interactions occur. It is believed that the majority of immunoglobulin E (IgE)-binding epitopes are located at these association sites of the Ara h 1 subunit [7]. Wichers *et al.* [8] reported that Ara h 1 occurs as a protein (64 kDa) in which the first 84 (clone 41B) and 78 (clone P17) amino acids are cleaved off from the nascent protein or preprotein in peanut seeds during the maturation process. The cleaved-off N-terminal peptides carry the epitope(s) against IgEs in patients with peanut allergy.

Burks *et al.* and Shin *et al.* [5, 6] reported that the N-terminal domain and hydrophobic region on the trimer of Ara h 1 are involved in its allergenicity; therefore, if a variant molecule of Ara h 1 lacking the N-terminal domain of the mature molecule (63–66 kDa) is available, it must provide important molecular information regarding allergenicity. In previous reports, Ara h 1 and other peanut allergens have been purified by a combination of ion exchange chromatography, gel filtration, and affinity chromatography after ammonium sulfate fractionation (ASF) [6, 9–11]. Furthermore, Masuyama *et al.* [12] have developed a simple method to purify Ara h 1, Ara h2, and Ara h3 using only the ASF method. However, a truncated Ara h 1 molecule lacking the N-terminal domain, which contains the putative pathological epitope, has not been prepared or purified thus far. In this study, during the purification of Ara h 1 using conventional procedures, we succeeded to purify a new variant molecule of Ara h 1 lacking the N-terminal domain (~7 kDa) of the mature Ara h 1 molecule (63 kDa in this study), although at this moment it has been obscure whether this truncated type Ara h 1 was produced by a natural processing or artificial cleavage by endogenous protease(s). Interestingly, we found that, in the native state, the 63 kDa subunit occurs as higher order oligomeric structures such as homodecameric conformation, while the 54 kDa subunit occurs exclusively as a homotrimer, indicating that the N-terminal domain may be involved in the formation of higher order oligomeric structures. We evaluated the immunoactivities of the two isomers of Ara h 1 against IgEs obtained from patients with peanut allergy. Immunoblotting analysis showed that both Ara h 1 subunits could be recognized by IgEs in patient antisera, suggesting

that the C-terminal domain carries other epitope(s) in addition to the N-terminal region of the 63 kDa subunit.

Furthermore, it has been reported that Ara h 1 has a single N-glycosylation site to which both antigenic β 1–2 xylosylated N-glycans and non-antigenic high-mannose type N-glycans are linked [13, 14]. In this study, we also analyzed the structural features of N-glycans in both the 63 kDa and 54 kDa Ara h 1 molecules and demonstrated that the cleavage of the N-terminal domain had an insignificant effect on N-glycan processing in the Golgi apparatus.

Materials and methods

Materials

Peanut seeds (*Arachis hypogaea* L.) were harvested in Chiba, Japan. Diethylaminoethyl (DEAE)-cellulose, Sephadex G75, and Sephacryl S-200 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). The Toyopearl Butyl column was produced by Tosoh (Tokyo, Japan). The Sephadex® G-25 Superfine column was manufactured by GE Healthcare Japan (Tokyo, Japan). The Shodex Asahipak NH2P-50 4E column (0.46 × 25 cm) was developed by Showa Denko (Tokyo, Japan). The Cosmosil 5C18-AR-II column (0.6 × 25 cm) was produced by Nacalai Tesque (Kyoto, Japan). Authentic PA-sugar chains (M1, MX, M3X, M4, M4X, M5, and M6) were prepared as described in our previous papers [15–17]. PNGase F PRIME™ Glycosidase was purchased from N-Zyme Scientifics, LLC (Doylestown, PA, USA). Ginkgo β 1,2-xylosidase was prepared from *Ginkgo biloba* seeds as described previously [18].

Purification of Ara h 1

All protein purification steps were carried out at 4 °C to control the endogenous protease(s) activity. The protein concentration was determined by measuring the absorbance at 280 nm with bovine serum albumin (BSA) as the standard.

Step 1. Preparation of crude extract Step 1. Preparation of crude extract

Peanut seeds (970.0 g) were homogenized in acetone. The resulting defatted powder (548.7 g) was suspended in 5 L of 50 mM Tris-HCl buffer (pH 8.3) containing 0.2 M NaCl and stirred at 4 °C for several hours. The mixture was then filtered through two layers of gauze and centrifuged at 17,390×g for 20 min. The supernatant was saturated with 100% ammonium sulfate. Half of the precipitated protein was dissolved in a small amount of 25 mM Tris-HCl buffer (pH 7.8) containing 50 mM NaCl and dialyzed against the same buffer.

Step 2. *DEAE-cellulose column chromatography* Step2.
DEAE-cellulose column chromatography

The crude extract (465 mL) was loaded onto a DEAE cellulose column (4 × 39 cm) previously equilibrated with 25 mM Tris-HCl buffer. The run-through fraction containing the major peanut allergen Ara h 1 with a molecular mass of approximately 63 kDa was pooled as shown in Fig. 1a and saturated with 100% ammonium sulfate.

Step 3. *Gel filtration through the Sephadex G75 column* Step 3. *Gel filtration through the Sephadex G75 column*

The precipitate obtained in Step 2 was dissolved in 25 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl and dialyzed against the same buffer. The resulting protein solution (75 mL) was loaded onto the Sephadex G75 column (6.2 × 115 cm) previously equilibrated with the same buffer. A fraction containing Ara h 1 (the horizontal bar in Fig. 1b) was collected.

Step 4. *Butyl-Toyopearl column chromatography* Step 4.
Butyl-Toyopearl column chromatography

Solid ammonium sulfate (1.5 M final concentration) was added to the protein solution (200 mL). The resulting protein solution was loaded onto the *Butyl-Toyopearl* column (4 × 39 cm) previously equilibrated with 25 mM Tris-HCl buffer (pH 7.8) containing 1.5 M ammonium sulfate. After washing the column with the same buffer, the adsorbed proteins were eluted with a linear gradient (1.5–0 M) of ammonium sulfate in the same buffer. Five fractions (as shown in Fig. 2) were collected and saturated with 100% ammonium sulfate. After centrifugation, each fraction was dialyzed against deionized water and lyophilized.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method described by Laemmli *et al.* [19] on 15% acrylamide gel in 0.1 M Tris-glycine buffer using a slab gel apparatus under reducing conditions with 5% 2-methanol. Precision Plus Protein™ Standards (Bio-Rad) were used as the marker proteins for molecular mass determination (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, and 15 kDa). The proteins on the gel were stained with Coomassie Brilliant Blue R-250.

N-terminal amino acid sequence

The proteins on the gel were transferred to Amersham Hybond-P polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA, USA) for 1 h at 100 mA on a blotting system (Nihon Eido Co. Ltd., Japan) using blotting buffer (0.1 M Tris, 0.192 M glycine, and 5% methanol). The membrane was stained with Coomassie Brilliant Blue R-250 and used for analysis of the N-terminal amino acid sequence with a PPSQ-31A protein sequencer (Shimadzu, Kyoto, Japan).

Analysis of the native molecular weights of Ara h 1 oligomers by gel filtration

The lyophilized proteins (F-1 and F-3) obtained in Step 4 were dissolved in 25 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl. Each fraction was loaded onto a Sephacryl S-200 column (2 × 105 cm) previously equilibrated with the same buffer at a flow rate of 0.7 mL/min. Standard proteins, including thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa), and ovalbumin (44 kDa), were used to estimate the native molecular masses of the Ara h 1 oligomers.

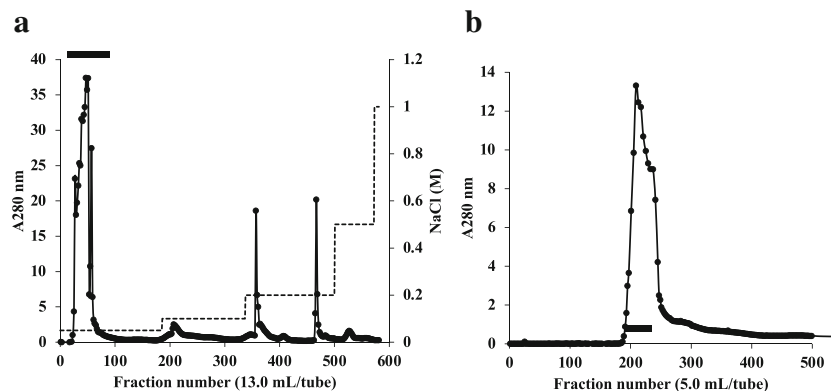


Fig. 1 Partial purification profile of peanut allergen Ara h 1, using DEAE-cellulose chromatography and gel-filtration. **a**, DEAE cellulose chromatography. Proteins were eluted as described in the Materials and Methods section. The protein fractions indicated using the horizontal bar

were pooled for the further steps. **b**, Sephadex G75 chromatography. The column was developed using 25 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl. The protein fractions indicated using the horizontal bar were pooled for final purification

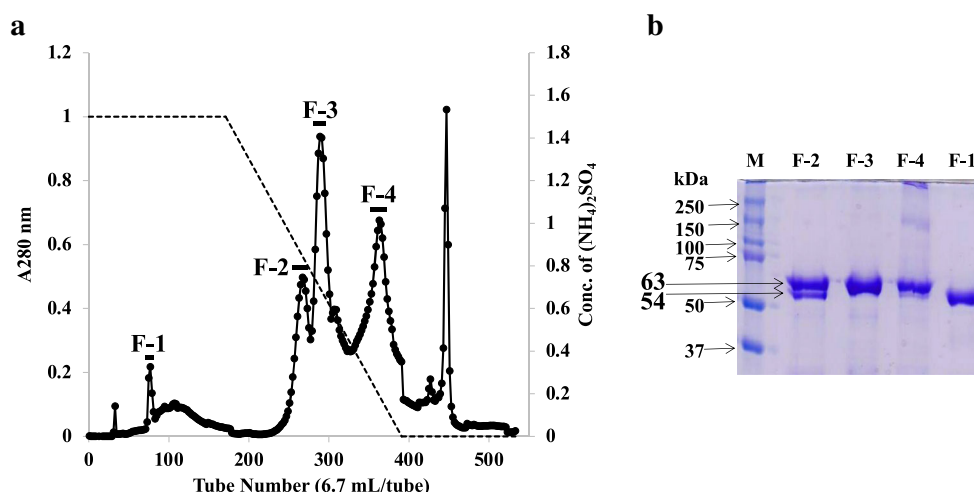


Fig. 2 Purification of two kinds of subunits of Ara h 1: 54 kDa and 63 kDa subunits. **a**, Butyl-toyopearl chromatography. Proteins were eluted using a reverse linear gradient of ammonium sulfate from 1.5 M to 0 M in the same buffer. Five protein fractions indicated using the horizontal bar were pooled separately. **b**, SDS-PAGE of the purified Ara h 1 subunit.

Proteins in four fractions (F-1, -2, -3, -4) were analyzed using SDS-PAGE with 15% polyacrylamide gel and were stained with CBB R-250. M, Marker proteins (Precision Plus Protein™ Standards, Bio-Rad, 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, and 15 kDa)

Sera from patients with peanut allergy

Nine patients and 12 healthy individuals were selected on the basis of a convincing clinical history of peanut allergy at the Aichi Children's Health and Medical Center. This study was approved by the institutional ethics committees at the Aichi Children's Health and Medical Center (approval number: 2019100) and the Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (approval number: KEN1912–021).

Immunoblotting

After SDS-PAGE (12% gel) of two species of Ara h 1 (63 kDa and 54 kDa; approximately 2 µg), the proteins in the gels were transferred onto a PVDF membrane as described in section 2.4. The membrane was blocked with 1% BSA in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.05% Tween-20 (TTBS) at 4 °C for 24 h. After washing with TTBS, the membrane was probed with sera from patients with peanut allergies at a 1:20 dilution in TTBS at 4 °C for 24 h. After washing with TTBS, the membrane was probed with a KPL peroxidase-labeled antibody to human IgE (ε) (SeraCare, Milford, MA, USA) at a 1:10000 dilution in TTBS for 1 h at room temperature. The IgE-Ara h 1 complex was detected with Amersham ECL SELECT™ detection reagent (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's protocol.

Multiple sequence alignment

A multiple sequence alignment was generated using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>).

PNGase F digestion

According to the instruction manual, freeze-dried proteins (100 µg each) were dissolved in 22 µL of 1X phosphate-buffered saline (PBS). The proteins were denatured by boiling for 10 min in the presence of 5% SDS (2 µL) under reduced conditions with 1 M DTT (2 µL). After the addition of 10% NP-40 (4 µL), half of the samples (15 µL) were digested with 1000 units of recombinant PNGase F PRIME Glycosidase at 37 °C for 1 h.

Preparation of pyridylaminated N-glycans

The N-glycans of glycoproteins were released by hydrazinolysis at 100 °C for 10 h. The resulting oligosaccharides were N-acetylated and labeled with 2-aminopyridine (PA) according to the method of Natsuka and Hase [20].

Fractionation and separation of pyridylaminated N-glycans

The PA-labeled oligosaccharides were separated by RP- and NP-HPLC on a Jasco 2080-PU HPLC instrument equipped with a Jasco 920-FP Intelligent Spectrofluorometer (excitation 310 nm, emission 380 nm). For RP-HPLC using the Cosmosil 5C18-AR-II column (0.6 × 25 cm), the PA-labeled oligosaccharides were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 7% over 40 min at a flow rate of 1.2 mL/min. For NP-HPLC using the Shodex Asahipak NH2P-50 column (0.46 × 25 cm), the PA-labeled oligosaccharides were eluted by increasing the water content in the water-acetonitrile mixture from 26% to 50% linearly over 40 min at a flow rate of 0.7 mL/min.

Exoglycosidase digestions

Digestion of Jack bean α -mannosidase (13.2 U) was performed in 130 μ L of 0.1 M sodium acetate buffer (pH 4.5) containing 10 mM ZnSO_4 at 37 °C for 48 h. Digestion with partially purified Ginkgo β -1,2-xylosidase (1.0 mg) [18] was performed in 280 μ L of 0.1 M sodium acetate buffer (pH 4.5) at 37 °C for 24 h. The reaction was stopped by heating in a boiling water bath for 3 min, and the digestion contents were analyzed by NP-HPLC and ESI-MS.

Electrospray ionization (ESI) mass spectrometry (MS)

LC/MS and MS/MS analyses of PA-labeled oligosaccharides were performed using the Agilent 6500 series HPLC-Chip/QTOF-MS system (Santa Clara, CA, USA) equipped with a microwell-plate auto sampler (maintained at 10 °C), a capillary sample loading pump, a nanopump, an HPLC-Chip interface, and the Agilent 6520 Q-TOF LC/MS system as described previously [21, 22]. The Agilent porous graphitized carbon (PGC) chip was used to separate the PA-labeled sugar chains.

Results and discussion

Identification of 54 kDa Ara h 1, a new subunit of the common peanut allergen

The variants of Ara h 1 were purified from peanut seeds using a combination of ion-exchange chromatography, gel filtration, and hydrophobic interaction chromatography (Fig. 2a). As shown in Fig. 2a, four fractions (F-1, -2, -3, and -4) were obtained by hydrophobic interaction chromatography. Since F-4 contained unknown brown-colored materials and some proteins other than 63 kDa Ara h 1 with very low yields, we used F-1, F-2, and F-3 in the following experiments (approximately 10–40 mg yield). As shown in Fig. 2b, SDS-PAGE showed that F-1 contained the 54 kDa protein, F-2 contained both the 54 kDa and 63 kDa (Ara h 1) proteins, and F-3 contained the 63 kDa (Ara h 1) protein. The N-terminal amino acid sequences of each protein band are shown in Supplementary Table 1. The N-terminal sequence of the 63 kDa protein in F-1 and F-2 was R-S/H-P-P-G-E-R-, suggesting that two Ara h 1 molecules encoded by two clone P41B and clone P17 genes are present in F-1 [4]. In contrast, the N-terminal sequence of the 54 kDa protein in F-1 and F-2 was E-G-R-E-G-E-Q-, suggesting that this 54 kDa protein resulted from proteolysis of the 63 kDa Ara h 1 (Fig. 3) and that it is a novel subunit of Ara h 1 with hydrophilic properties that has not been reported to date. These results suggest that the removal of the N-terminal domain (~7 kDa) contributes significant hydrophobic properties to the 63 kDa molecule. To

our knowledge, the 54 kDa Ara h 1 has been found and purified in this study for the first time, since these other studies have not used the run-through fraction of the hydrophobic chromatography for the purification of Ara h 1 or they focused on the 63 kDa Ara h 1 (homo-oligomer) molecule in the gel-filtration step. At this moment, it has been obscure whether the 54 kDa Ara h 1 molecule was a natural-processed product like 63 kDa Ara h 1 by an endogenous protease(s) or an artificial product occurred during the purification process by other protease(s). However, the 54 kDa Ara h 1 molecule have been reproducibly prepared or purified in relatively large amounts by the purification procedure described above without protease inhibitor(s), and this variant Ara h 1 must be a valuable molecule to see biochemical function(s) of the N-terminal domain of 63 kDa Ara h 1. At this moment, however, a possibility that the 54 kDa molecule was artificially generated in large amount by the action of endogenous peptidase(s) during the purification process cannot be excluded.

Oligomerization of 54 kDa Ara h 1 and 63 kDa Ara h 1 subunits

Since Ara h 1 has been reported as a homotrimer [6, 8], we analyzed the native and oligomeric structures of the 54 kDa Ara h 1 subunit to determine whether the N-terminal domain of 63 kDa Ara h 1 plays a critical role in trimer formation. The oligomeric structures of the two Ara h 1 subunits were analyzed by gel filtration using a Sephacryl S-200 column. As shown in Fig. 4, the elution profile of 54 kDa Ara h 1 showed a single peak (peak-1) with a molecular mass of approximately 170 kDa, suggesting that the 54 kDa subunit occurs as a trimeric conformation in a native state. In contrast, the elution profile of 63 kDa Ara h 1 was observed as a broad peak (peak-2). Although the molecular mass of the peak top was estimated to be approximately 640 kDa, which corresponds to a decameric conformation, the broad peak may consist of some other different oligomeric conformations, such as a undecamer or nonamer. These results demonstrate that the 63 kDa subunit occurs as higher-order oligomeric conformations in addition to the trimeric structure (found as a small shoulder in Fig. 4-B) in its native state, corroborating previous reports [2, 10, 11] and suggesting that the N terminal domain of the 63 kDa Ara h 1 subunit may enhance multi-oligomerization while the hydrophilic 54 kDa Ara h 1 subunit mainly occurs as a trimeric oligomer in its native state due to the missing N-terminal domain from the 63 kDa subunit.

As shown in Fig. 3, the 54 kDa subunit was found in F-2 along with the 63 kDa subunit, and F-2 appears to be hetero-oligomeric Ara h 1 consisting of the hydrophilic 54 kDa and hydrophobic 63 kDa subunits. Such hetero-oligomeric Ara h 1 molecules might become more hydrophobic than the homo-oligomers of the 54 kDa subunits and less hydrophobic than the homo-oligomers of 63 kDa subunits depending on the

Fig. 3 Sequence alignment of Ara h 1 encoded by two homologous genes: clone P17 and P41B. Sequence alignment was performed using the ClustalW program. The N-terminal sequence of 63 kDa Ara h 1 was R-H/S-P-P-G-E-R-. 54 kDa Ara h 1 was E-G-R-E-G-E-Q-. The consensus sequence of N-glycosylation, -NAS-, was indicated using the horizontal double bar

clone P17	MRGRVSPLMLLLGILVLASVSATQAKS-PY-RKTENPCAQRCLQSCQQEPDDLKQKACES	58
clone P41B	MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEPDDLKQKACES	60
clone P17	RCTKLEYDPRCVYD----TGATNQRHPPGERTGRGQPGDYDDRRQPRREEGGRWGPAEP	114
clone P41B	RCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTGRGQPGDYDDRRQPRREEGGRWGPAEP	120
63 kDa Subunit		
clone P17	REREREEDWRQPREDWRRPSHQQPRKIRPEGREGEQEWGTPGSEVREETSRRNNPFYFSPR	174
clone P41B	REREREEDWRQPREDWRRPSHQQPRKIRPEGREGEQEWGTPGSHVREETSRRNNPFYFSPR	180
54 kDa Subunit		
clone P17	RFSTRYGNQNGRIRVLQRFDQRSKQFQNLQNHRIVQIEARPNTLVLPKHADADNILVIQQ	234
clone P41B	RFSTRYGNQNGRIRVLQRFDQRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQ	240
clone P17	GQATVTVANGNNRKSFNLDGHALRIPSGFISYILNRHDNQLRVAKISMPVNTPGQFED	294
clone P41B	GQATVTVANGNNRKSFNLDGHALRIPSGFISYILNRHDNQLRVAKISMPVNTPGQFED	300
clone P17	FFPASSRDQSSYLQGFSRNTLEAAFNAAFNEIRRVLLLENAGGEQEERGQRRRSTRSS-D	353
clone P41B	FFPASSRDQSSYLQGFSRNTLEAAFNAAFNEIRRVLLLENAGGEQEERGQRRWSTRSS-EN	360
clone P17	NEGVIVKVSKEHVQELTKHAKSVSKKGSEE-EDITNPINLRDGEPLDSNNFGRLFEVKPD	412
clone P41B	NEGVIVKVSKEHVEELTKHAKSVSKKGSEEEDITNPINLRDGEPLDSNNFGKLFVKPD	420
clone P17	KKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVKGTGNLELVAVRKEQQQRGR	472
clone P41B	KKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVKGTGNLELVAVRKEQQQRGR	480
clone P17	EQEWEDEDEDEEGSNREVRRYTARLKEGDFVIMPAAHVPVAINASSELHLLGFGINAEN	532
clone P41B	EEE---EDEDEDEEGSNREVRRYTARLKEGDFVIMPAAHVPVAINASSELHLLGFGINAEN	537
clone P17	NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLKQKESHFVSARPSQSQSP-----	587
clone P41B	NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLKQKESHFVSARPSQSQSQSPSSP	597
clone P17	--SSPEKEDQEEENQGGKGPLLSILKAFN	614
clone P41B	EKESPEKEDQEEENQGGKGPLLSILKAFN	626

abundance ratio of each subunit. In this study, we have not performed the further analysis on the quaternary structure of F-2, and it is obscure whether F-2 contains higher-order

multimeric (such as decamer or undecamer) structures, but we believe that Ara h 1 forms a hetero-oligomeric structure, which maybe more than trimer, composed of the 54 kDa and 63 kDa molecules.

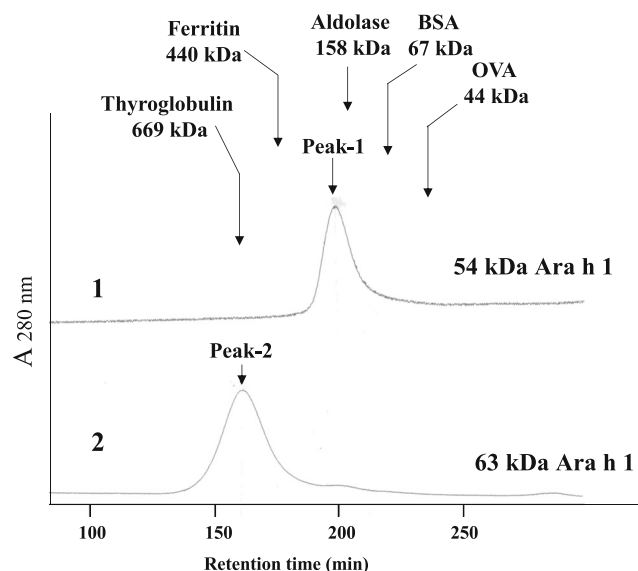
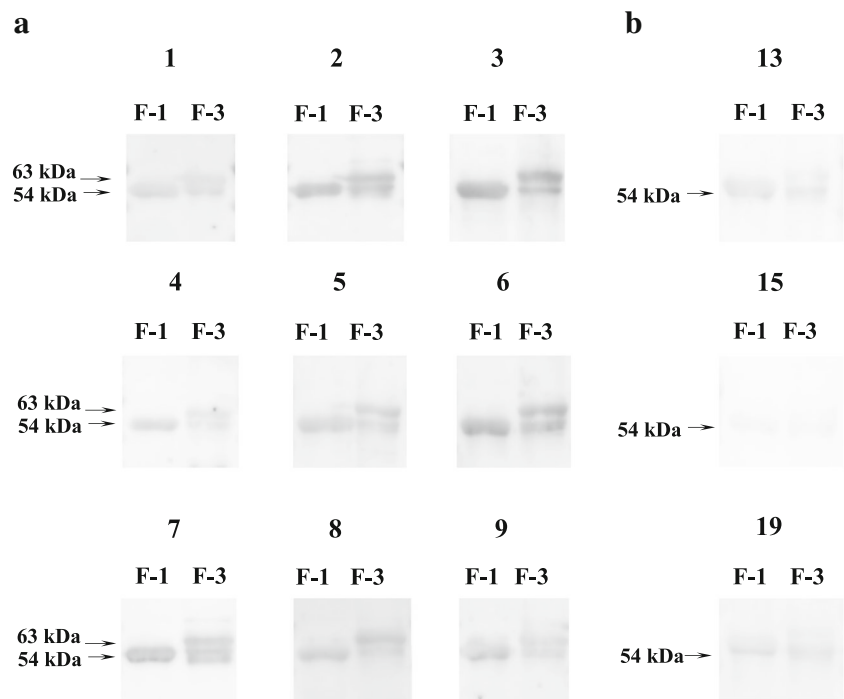


Fig. 4 Gel-filtration analysis of native conformation of the 54 kDa (F-1) and 63 kDa (F-3) subunits. **a**, an elution profile for the 54 kDa Ara h 1 purified in Fig. 2. **b**, an elution profile for the 63 kDa Ara h 1. Gel-filtration profile on Sephacryl S-200 column (2×105 cm). The column was developed with 25 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl at a flow rate of 0.7 mL/min. The marker proteins used were as follows: **(a)** thyroglobulin (669 kDa); **(b)** ferritin (440 kDa); **(c)** aldolase (158 kDa); **(d)** BSA (67 kDa); and **(e)** ovalbumin (44 kDa)

Immunoblot analysis of the 54 kDa and 63 kDa subunits of Ara h 1 with antisera from patients with peanut allergy

To determine the immunological significance of the N-terminal domain in the 63 kDa Ara h 1 subunit, we compared the allergenicities of the 54 kDa and 63 kDa Ara h 1 subunits by immunoblotting analysis using antisera from nine patients with peanut allergy after SDS-PAGE. The results are shown in Fig. 5; A (1, 2, 3, 4, 5, 6, 7, 8, and 9) for peanut allergy patients, B (10, 11, 12, and 13) for healthy individuals. The analysis showed that the IgEs in sera of all nine patients strongly reacted to both subunits of Ara h 1 (Fig. 5a), indicating that the deletion of the 63 kDa molecule N-terminal domain exerts an insignificant effect on allergenicity. Although the SDS-PAGE analysis indicated that F-3 contained the 63 kDa Ara h 1 only as shown in Fig. 2b, both sera from peanut allergy patients and healthy individuals recognized the 54 kDa Ara h 1 in F-3, indicating the trace amount of a contamination of the 54 kDa molecule that could not be detected by the Coomassie brilliant blue staining. On the other hand, interestingly, some sera from healthy individuals slightly reacted with the 54 kDa Ara h 1 (Fig. 5b) but not 63 kDa

Fig. 5 Immunoblot analysis of the 54 kDa subunit (F-1) and 63 kDa subunit (F-3). Immunoblotting using sera from nine patients with peanut allergy was performed as described in the Materials and Methods section. **a**, results with sera from nine patients with severe peanut allergy. **b**, three representative results with sera from 12 individual with no peanut allergy



Ara h 1, suggesting that they carried the IgE(s) reacting with epitope(s) on the 54 kDa molecule. These observations might suggest that the epitope(s) on the N-terminal domain of the 63 kDa Ara h 1 might be relevant to severe peanut allergy symptoms. To verify this hypothesis, further detail analyses using many more sera from peanut allergy patients and healthy individuals must be prerequisite. At least, however, these results indicated that the epitopes for IgEs against the Ara h 1 molecules occur on several portions of both subunits in addition to the N-terminal domain of the 63 kDa subunit, although it is obscure whether all IgEs recognizing the Ara h 1 molecules in sera are significantly involved in the development of peanut allergy.

Burks *et al.* [5] identified 23 IgE-binding epitopes on the full-length Ara h 1 subunit (clone P418). Nine out of twenty-three epitopes were located in the N-terminal region on the full-length subunit, A (25)-R (152), containing the N-terminal domain of the 63 kDa subunit, R (85)-S-P-P-G-E-R—I-R-P (149) as shown in Fig. 3. Since Gibbs *et al.* [23] reported that the amino acid sequence of the 63 kDa subunit N-terminal region is unique to this peanut vicilin and does not share significant similarity with vicilins from other legumes, it appears that the N-terminal domain may play a significant role in the high allergenicity of 63 kDa Ara h 1. However, Burks *et al.* [5] also found some epitopes in the internal portion, T(294)-V(470), and the C-terminal portion, R(498)-D(606), of the 63 kDa subunit. It is thus reasonable to assume that recognition of the 54 kDa subunit by Ig Es in the sera of peanut-allergic individuals was due to the presence of certain epitopes in the internal or C-terminal portions, although the

respective roles of these epitopes in peanut allergy symptoms remain unclear. Furthermore, Eiwegger *et al.* [24] reported that the gastro-duodenal digestion products (6–19 kDa fragments) of Ara h 1 retain allergenic potential, suggesting that relevant epitopes occur widely on the Ara h 1 molecule.

Structural analysis of N-glycans linked to the 54 kDa Ara h 1 subunit

Although it has already been reported that the both β -xylosylated immunogenic type and typical high-mannose type *N*-glycans are linked to Ara h 1 (63 kDa subunit in this study) [12, 13], we analyzed those of the 54 kDa subunit to determine whether the deletion of the *N*-terminal domain affects *N*-glycan processing. When both Ara h 1 subunits were treated with PNGase F, the apparent molecular weights of both subunits decreased slightly, indicating the presence of *N*-glycans (Supplementary Fig. 1). Thus, we released these *N*-glycans from both Ara h 1 subunits by hydrazinolysis, and the resulting free *N*-glycans were *N*-acetylated and pyridylaminated. The PA-labeled *N*-glycans were fractionated by RP-HPLC and pooled as shown in Fig. 6a. The structures of these *N*-glycans were analyzed by NP-HPLC, exoglycosidase digestion, and MS and MS/MS analysis. Since the structural analyses of *N*-glycans from both subunits presented roughly the same results, the data for the 54 kDa subunit are described below in detail. As shown in Fig. 6B-1, five peaks (peak-a, -b, -c, -d, and -e) were detected by NP-HPLC. The elution position of peak-a coincided with that of Man₃Xyl₁GlcNAc₂-PA (M3X), peak-b with that of Man₄GlcNAc₂-PA (M4), peak-c with that of

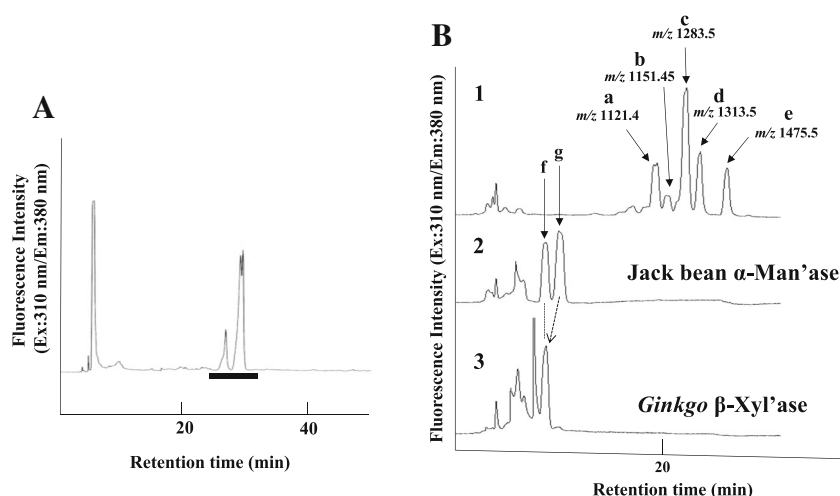


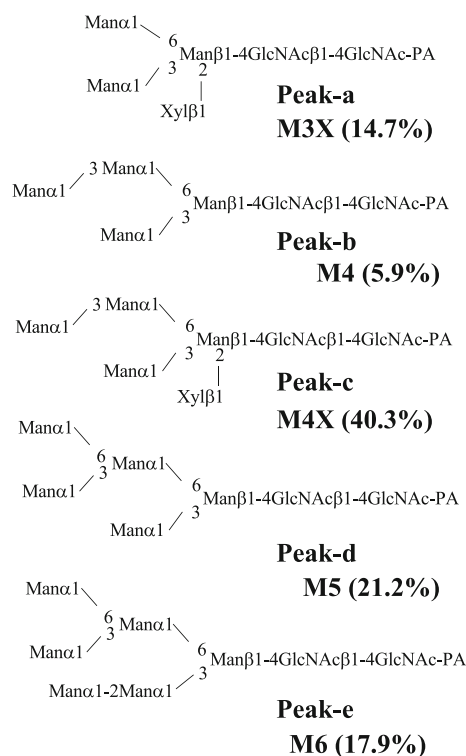
Fig. 6 Purification and structural analysis of pyridylaminated N-glycans prepared from 54 kDa and 63 kDa Ara h 1. **a**, RP-HPLC profile of the pyridylaminated (PA-) sugar chains. The N-glycan-fraction was pooled, as indicated using the horizontal bars, and was applied to NP-HPLC. **b**, NP-HPLC analysis of the exoglycosidase-digests of the pyridylaminated

N-glycans obtained in A. 1, PA-sugar chains prepared from 54 kDa Ara h 1. 2, Jack bean α -mannosidase digest of 1. 3, Ginkgo β -xylosidase digest of 2. The pyridyl aminated N-glycans obtained in A were successively digested with Jack bean α -mannosidase and Ginkgo β -xylosidase [18]. The results obtained from 54 kDa Ara h 1 are shown as a typical example

Man₄Xyl₁GlcNAc₂-PA (M4X), peak-d with that of Man₅GlcNAc₂-PA (M5), and peak-e with that of Man₆GlcNAc₂-PA (M6). When these PA-glycans were treated with Jack bean α -mannosidase, they were converted to two peaks (f and g); peak-g had the same elution position as Man₁Xyl₁GlcNAc₂-PA (MX), and peak-f corresponded to Man₁GlcNAc₂-PA (M1) (Fig. 6B-2). Peak-g was converted to peak-f by Ginkgo β -1,2-xylosidase [18] (Fig. 6B-3), indicating that the N-glycans linked to the 54 kDa Ara h 1 subunit are typical high-mannose type and β -xylosylated type N-glycans.

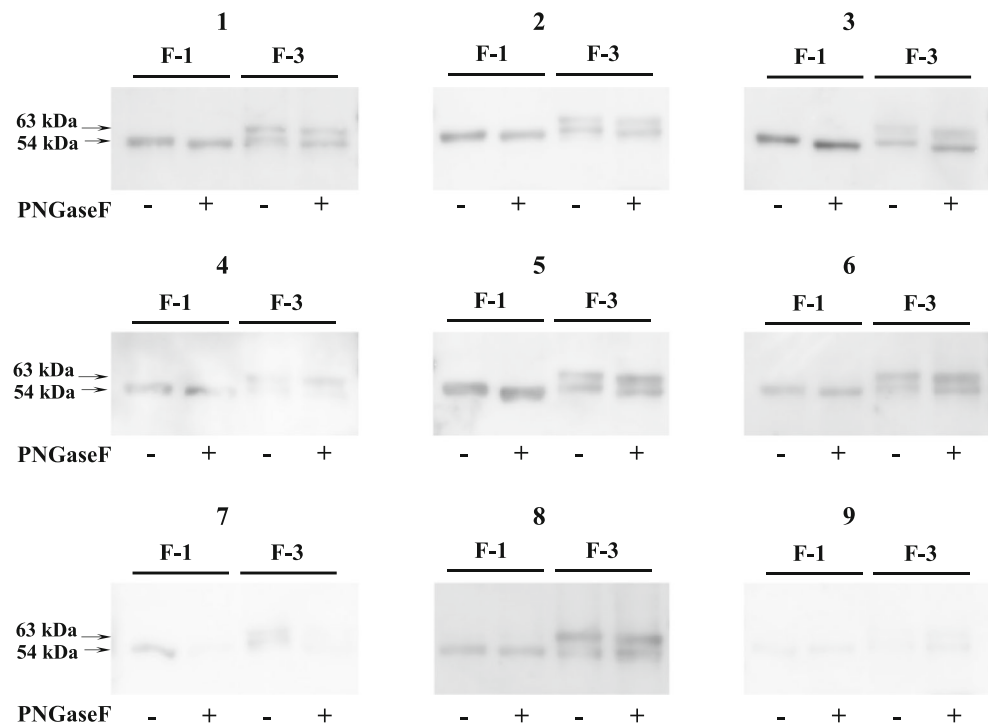
The deduced structures of the five PA-glycans (peak-a, -b, -c, -d, and -e) were further analyzed by LC/MS and MS/MS using the Agilent 6500 Series HPLC-Chip/QTOF-MS system. As shown in Supplementary Fig. 2, ESI-MS analysis of peak-a exhibited an N-glycan ion at m/z 1121.4 $[M + H]^+$ corresponding to $[(\text{Hex})_3(\text{Pen})_1(\text{HexNAc})_1(\text{HexNAc-PA}) + H]^+$ or M3X; peak-b, m/z 1151.4 corresponding to $[(\text{Hex})_4(\text{HexNAc})_1(\text{HexNAc-PA}) + H]^+$ or M4; peak-c, m/z 1283.5 $[M + H]^+$ corresponding to $[(\text{Hex})_4(\text{Pen})_1(\text{HexNAc})_1(\text{HexNAc-PA}) + H]^+$ or M4X; peak-d, m/z 1313.5 $[M + H]^+$ corresponding to $[(\text{Hex})_5(\text{HexNAc})_1(\text{HexNAc-PA}) + H]^+$ or M5; and peak-e, m/z 1475.5 $[M + H]^+$ corresponding to $[(\text{Hex})_6(\text{HexNAc})_1(\text{HexNAc-PA}) + H]^+$ or M6. In MS/MS analysis of these PA-glycans (peak-a, -b, -c, -d, and -e), all produced fragment ions were assigned as product ions from each putative N-glycan structure as shown in Supplementary Fig. 3. Although all fragment ions from peak-b, -d, and -e were assigned as typical fragment ions from the high-mannose type N-glycans, a fragment ion at m/z 959.4 was observed from peak-a and -c, indicating the occurrence of a pentose (xylose) residue. The structural features of N-glycans from the 54 kDa and 63 kDa Ara h 1 subunits are nearly

the same. As shown in Scheme 1, the N-glycan structures of both subunits purified in this study were in agreement with those reported in previous studies [12, 13], although a small amount of the Man₄GlcNAc₂ structure was newly discovered in this study. These results suggest that removal of the N-terminal domain has an insignificant effect on N-glycan processing machinery.



Scheme 1 Structural features of N-glycans linked to the 54 kDa and 63 kDa Ara h 1 subunits

Fig. 7 Immunoblot analysis of the de-*N*-glycosylated 54 kDa subunit (F-1) and 63 kDa subunit (F-3) with sera from peanut-allergy patients. The 54 kDa and 63 kDa Ara h 1 were deglycosylated with PNGase-F. Immunoblotting using sera from nine patients with peanut allergy was performed as described in the Materials and Methods section



In terms of the immunogenic activity of *N*-glycans, the plant specific *N*-glycans bearing α 1–3fucosyl and β 1–2xylosyl residues are highly immunogenic against mammals and known as cross-reactive carbohydrate determinants (CCDs). Since the β 1–2xylosylated *N*-glycans are linked to both 54 kDa and 63 kDa Ara h 1 subunits, we analyzed whether the peanut-allergy patients carry IgE(s) recognizing the plant *N*-glycans. After the treatment of the 54 kDa and 63 kDa Ara h 1 with PNGase F, the cross-reactivity of IgE(s) of peanut-allergy patients against the *N*-glycosylated and de-*N*-glycosylated Ara h 1 subunits was analyzed by the immunoblot analysis. As shown in Fig. 7, the cross-reactivity of IgE(s) in the serum of one patient (#7) was completely lost by de-*N*-glycosylation, suggesting the *N*-glycan, probably β 1–2xylosylated *N*-glycans (Man₄.₃GlcNAc₂), of the Ara h 1 subunits was the sole epitope for the IgE(s) against the peanut allergen. To our knowledge, this is the first report indicated clearly that the β 1–2xylosylated but not α 1–3fucosylated *N*-glycans of Ara h 1 was the epitope for IgE(s) [25], although it is still obscure whether the β 1–2xylosylated *N*-glycans are directly involved in the onset of peanut allergy. In the previous report [26], we also reported that about 20% Japanese cedar pollen allergy-patients carry IgE(s) against the plant antigenic *N*-glycans linked to Cry j1, a major allergen of the pollen allergens.

Conclusion

In this study, we purified a new isoform of Ara h 1 with a molecular weight of 54 kDa by hydrophobic interaction

chromatography. The N-terminal amino acid sequence analysis suggested that this 54 kDa Ara h 1 subunit was derived from 63 kDa Ara h 1 by proteolysis with the N-terminal domain removed. Although at this moment it is obscure whether the truncated type Ara h 1 was artificial product during the purification procedure, this 54 kDa molecule was reproducibly obtained. Since the 54 kDa subunit was purified in the run-through fraction of hydrophobic interaction chromatography, it appears that the N-terminal domain of the 63 kDa subunit provided hydrophobic properties to Ara h 1. Gel filtration analysis demonstrated that the 54 kDa Ara h 1 subunit exclusively occurs in a trimeric conformation while 63 kDa occurs in a decameric conformation as an average oligomeric structure. However, IgE(s) in sera of the patients with peanut allergy recognized the 54 kDa and 63 kDa subunits at the same level, suggesting that several epitopes are distributed on the Ara h 1 molecule in addition to the hydrophobic N-terminal domain of 63 kDa Ara h 1. Interestingly we found that some sera from healthy individuals slightly reacted with the 54 kDa Ara h 1 but not the 63 kDa molecule. We found that a serum obtained from one peanut-allergy patient carried IgE(s) against the *N*-glycans, probably β 1–2xylosylated ones, of the 54 kDa and 63 kDa Ara h 1 subunits. The structural features of *N*-glycans linked to the 54 kDa and 63 kDa subunits and presence of both β -xylosylated and high-mannose types were nearly the same, indicating that the N-terminal domain deletion had an insignificant effect on the processing or maturation of *N*-glycans.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10719-020-09969-1>.

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Author's contributions Y.K. shared responsibility for writing the manuscript with A.M., M.M., T.M., Y.T., and K.I. All authors were responsible for the study concept and design. A.M., M.M., T.M., and Y.T. carried out the study. All authors contributed to the critical revision of the manuscript.

Compliance with ethical standards

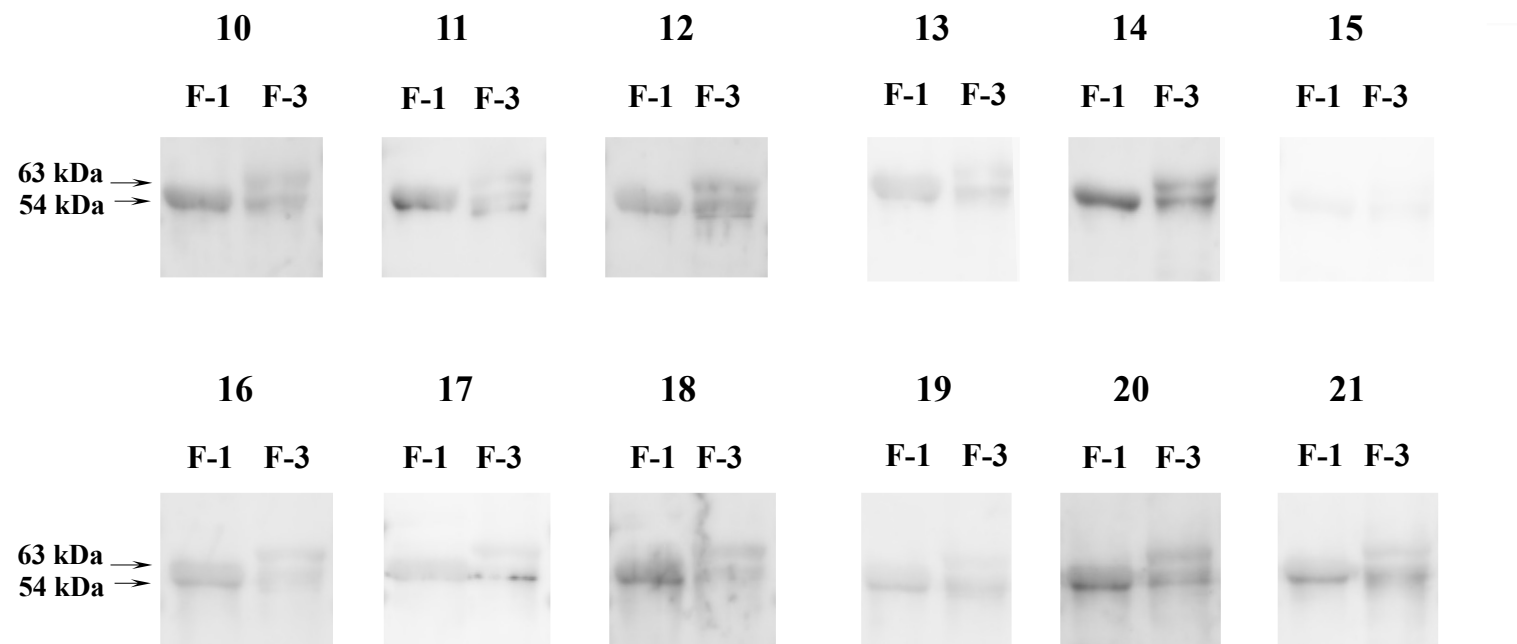
Conflict of interest No potential conflicts of interest have been reported by the authors.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

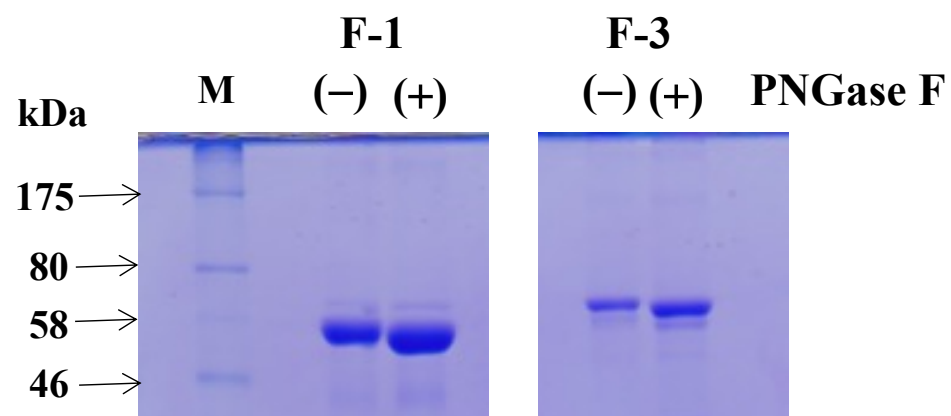
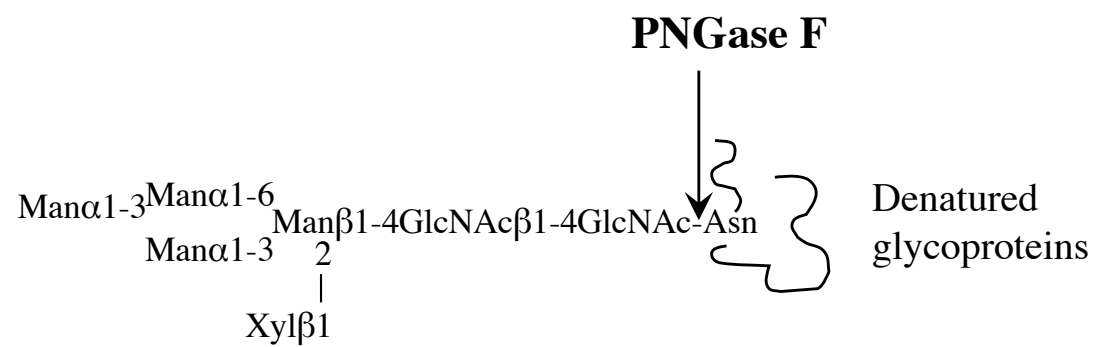
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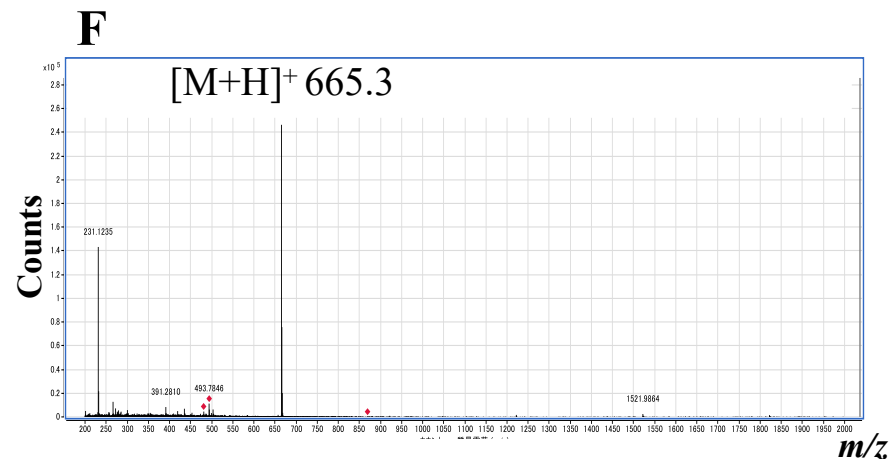
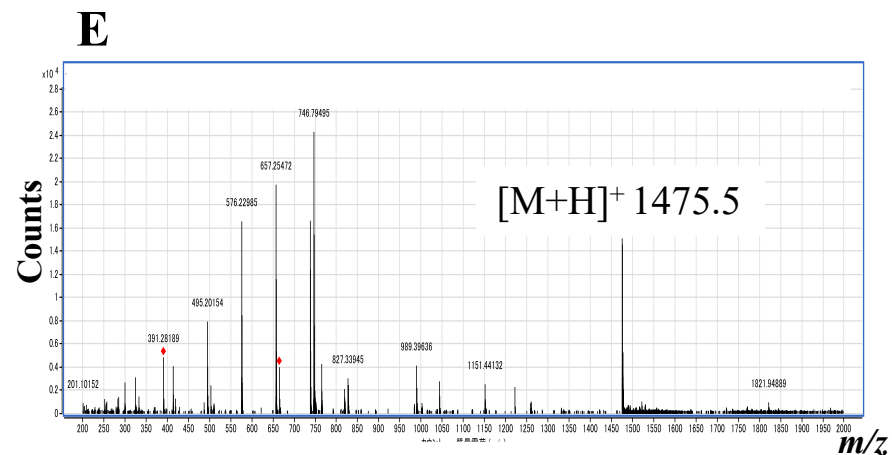
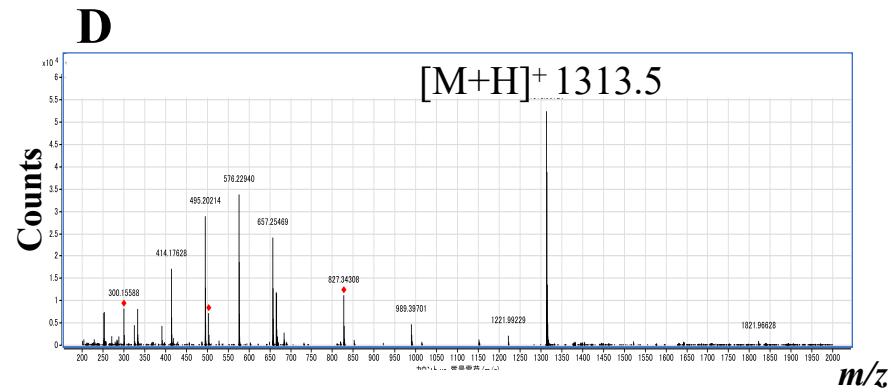
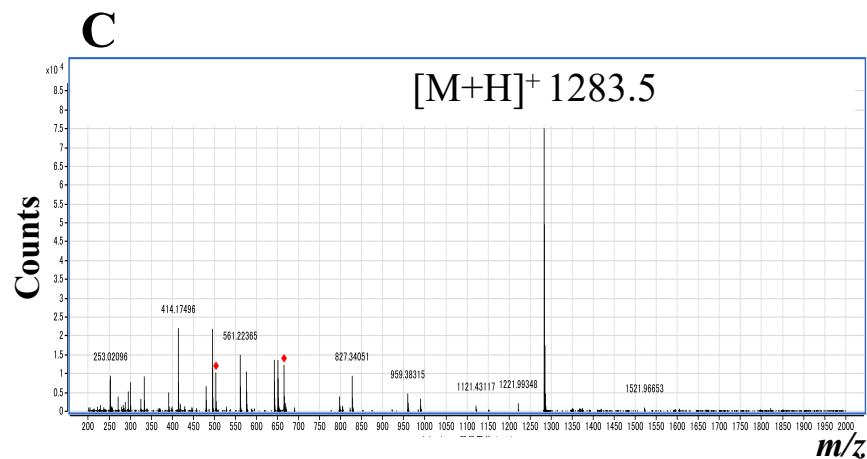
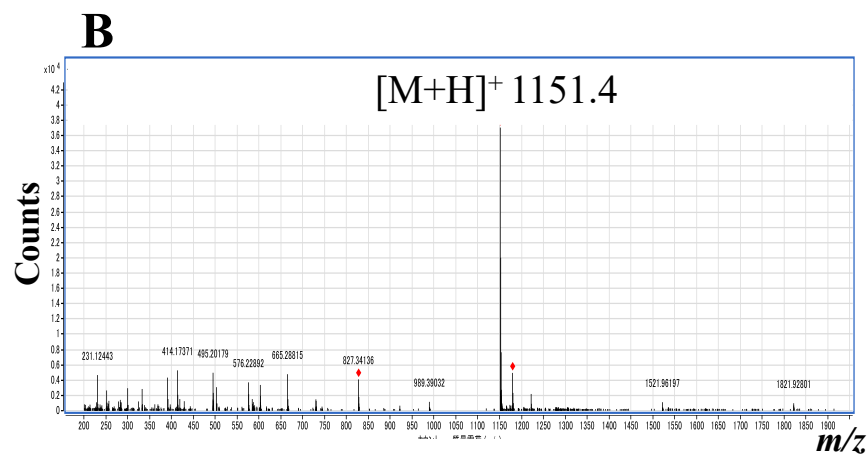
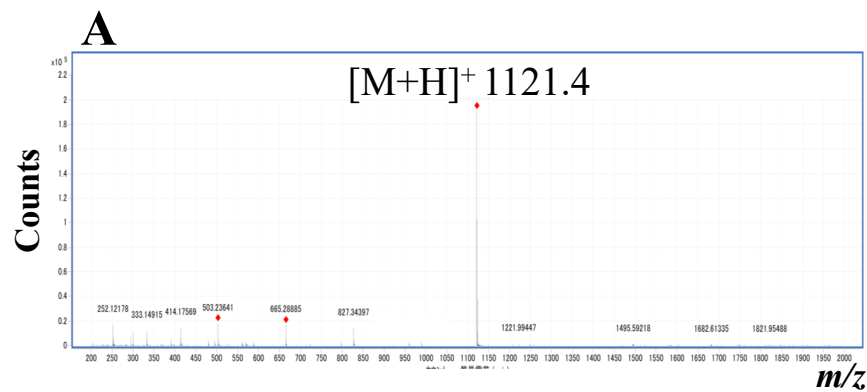
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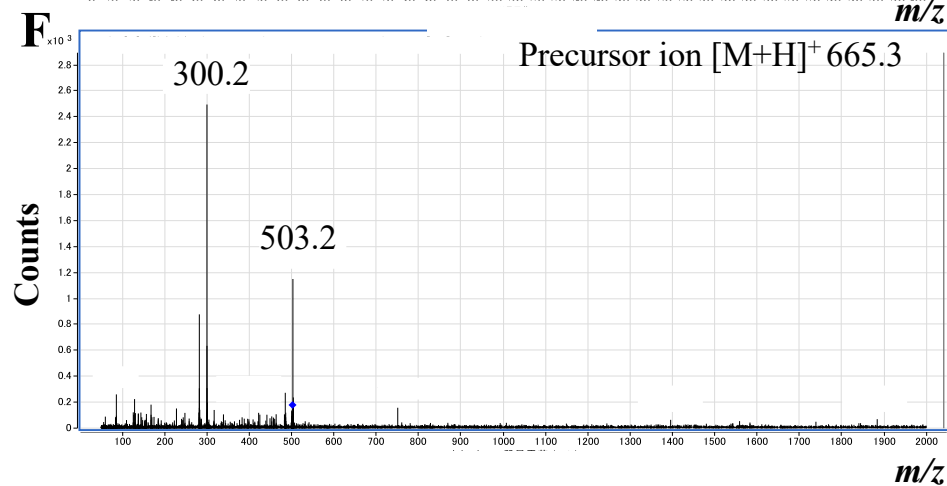
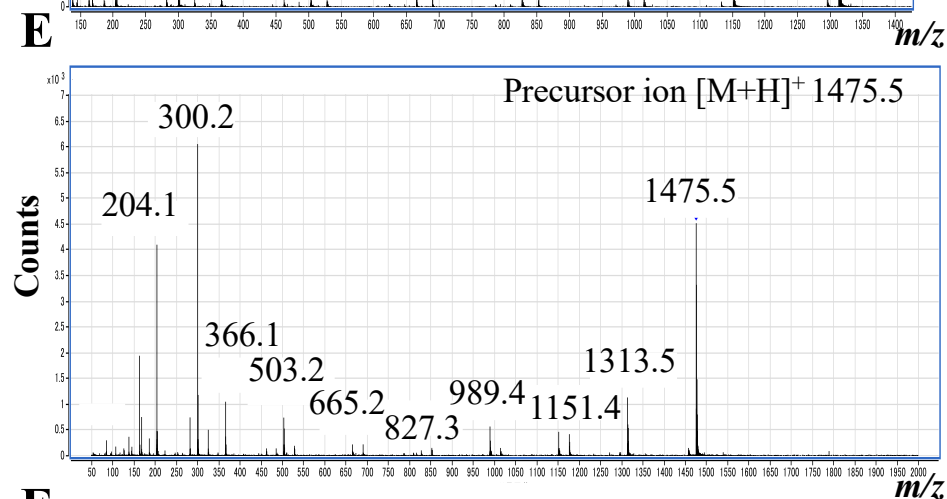
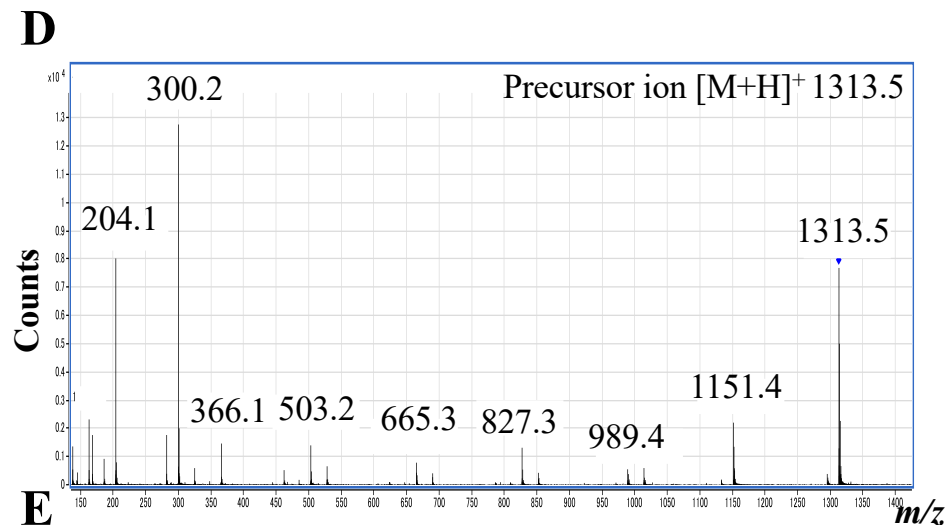
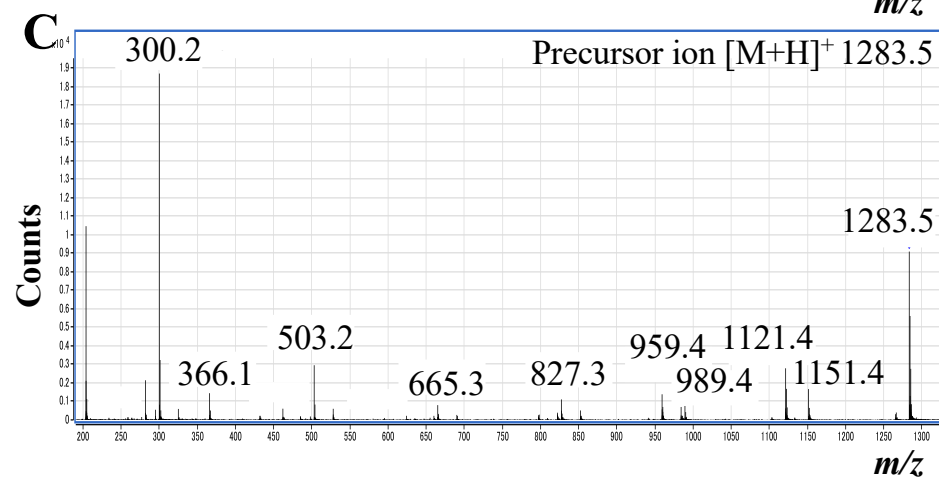
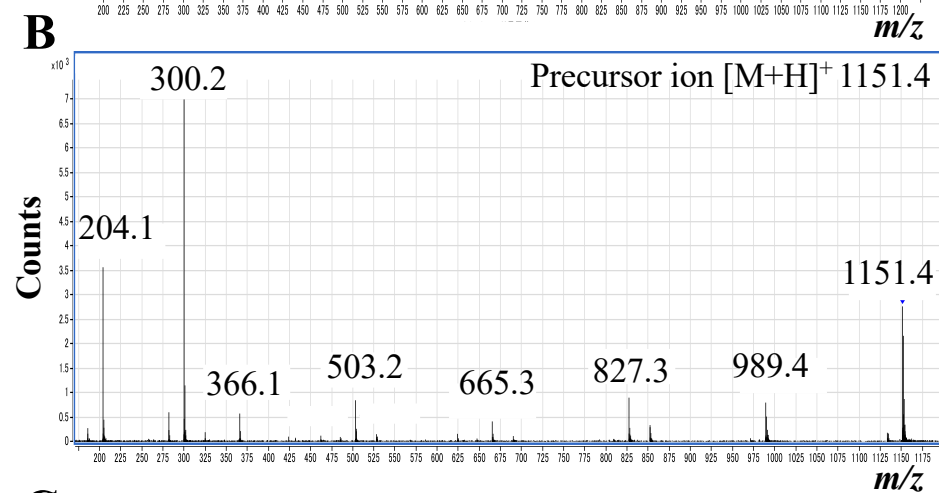
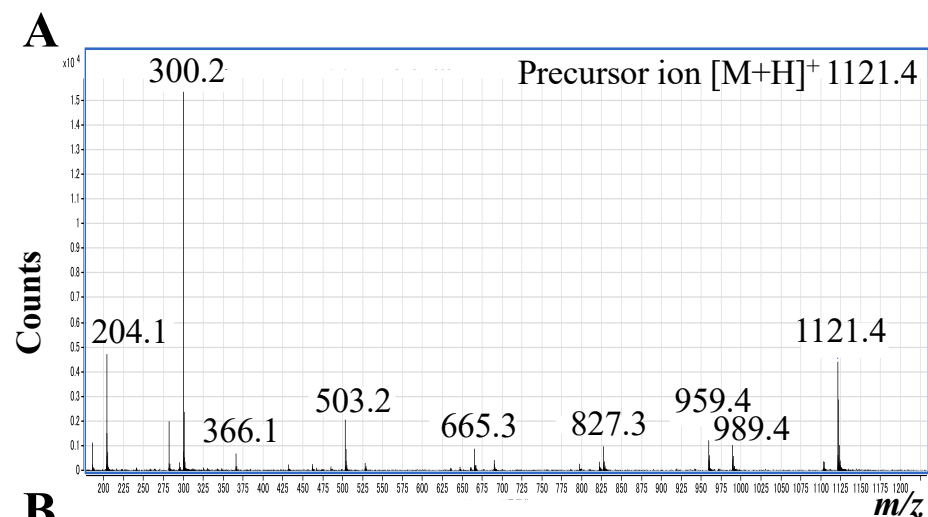
Supplemental Fig. 1 Asaduzzaman MD *et al.*



Supplemental Fig. 2 Asaduzzaman MD *et al.*



Supplemental Fig. 3 Asaduzzaman MD *et al.*



Supplemental Fig. 4 Asaduzzaman MD *et al.*

Supplementary Table 1. N-Terminal amino acid sequences of 63 kDa and 54 kDa Ara h 1 subunits found in each fraction obtained by the hydrophobic interaction chromatography

Protein bands	N-terminal sequences
F-1 (54 kDa)	E-G-R-E-G-E-Q-
F-2 (63 kDa)	R-S/H-P-P-G-E-R-
F-2 (54 kDa)	E-G-R-E-G-E-Q-
F-3 (63 kDa)	R-S/H-P-P-G-E-R-
F-4 (63 kDa)	R-X-X-X-G-E-X-

Supplementary Table 2. MS/MS analysis of five *N*-glycans from 63 kDa and 54 kDa Ara h 1 subunits

Peak a (Man3Xyl1GlcANc2-PA)	<i>m/z</i>
[HexNAc-PA+H] ⁺	300.2
[(HexNAc2-PA)+H] ⁺	503.2
[(Hex)1(HexNAc2-PA)+H] ⁺	665.3
[(Hex)2(HexNAc2-PA)+H] ⁺	827.3
[(Pen)1(Hex)2(HexNAc2-PA)+H] ⁺	959.4
[(Hex)3(HexNAc2-PA)+H] ⁺	989.4
[(Pen)1(Hex)3(HexNAc2-PA)+H] ⁺	1121.4

Peak b Man4GlcNAc2-PA	<i>m/z</i>
[HexNAc-PA+H] ⁺	300.2
[(HexNAc2-PA)+H] ⁺	503.2
[(Hex)1(HexNAc2-PA)+H] ⁺	665.3
[(Hex)2(HexNAc2-PA)+H] ⁺	827.3
[(Hex)3(HexNAc2-PA)+H] ⁺	989.4
[(Hex)4(HexNAc2-PA)+H] ⁺	1151.4

Peak c (Man4Xyl1GlcNAc2-PA)	<i>m/z</i>
[HexNAc-PA+H] ⁺	300.2
[(HexNAc2-PA)+H] ⁺	503.2
[(Hex)1(HexNAc2-PA)+H] ⁺	665.3
[(Hex)2(HexNAc2-PA)+H] ⁺	827.3
[(Pen)1(Hex)2(HexNAc2-PA)+H] ⁺	959.4
[(Hex)3(HexNAc2-PA)+H] ⁺	989.4
[(Pen)1(Hex)3(HexNAc2-PA)+H] ⁺	1121.4
[(Hex)4(HexNAc2-PA)+H] ⁺	1151.4
[(Pen)1(Hex)4(HexNAc2-PA)+H] ⁺	1283.5

Peak d (Man5GlcNAc2-PA)	<i>m/z</i>
[HexNAc-PA+H] ⁺	300.2
[(HexNAc2-PA)+H] ⁺	503.2
[(Hex)1(HexNAc2-PA)+H] ⁺	665.3
[(Hex)2(HexNAc2-PA)+H] ⁺	827.3
[(Hex)3(HexNAc2-PA)+H] ⁺	989.4
[(Hex)4(HexNAc2-PA)+H] ⁺	1151.4
[(Hex)5(HexNAc2-PA)+H] ⁺	1313.5

Peak e (Man6GlcNAc2-PA)	<i>m/z</i>
[HexNAc-PA+H] ⁺	300.2
[(HexNAc2-PA)+H] ⁺	503.2
[(Hex)1(HexNAc2-PA)+H] ⁺	665.3
[(Hex)2(HexNAc2-PA)+H] ⁺	827.3
[(Hex)3(HexNAc2-PA)+H] ⁺	989.4
[(Hex)4(HexNAc2-PA)+H] ⁺	1151.4
[(Hex)5(HexNAc2-PA)+H] ⁺	1313.5
[(Hex)6(HexNAc2-PA)+H] ⁺	1475.5