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ARTICLE *in* CARBOHYDRATE RESEARCH · SEPTEMBER 2009

Impact Factor: 1.93 · DOI: 10.1016/j.carres.2009.08.001 · Source: PubMed

Carbohydrate Research 344 (2009) 2182–2187

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structural analysis of O-glycans of mucin from jellyfish (*Aurelia aurita*) containing 2-aminoethylphosphonate

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ARTICLE INFO

Article history:
Received 11 April 2009
Received in revised form 30 June 2009
Accepted 1 August 2009
Available online 7 August 2009

Keywords:
Jellyfish
Glycoprotein
Mucin
Glycan
Structure
2-Aminoethylphosphonate

ABSTRACT

The structure of O-glycan in qniumucin (Q-mucin), which is a novel mucin extracted from jellyfish, was analyzed by a combination of NMR and ESI-MS/MS. A previously unidentified monosaccharide involved in the glycan chains was determined to be *N*-acetylgalactosamine (GalNAc) substituted by 2-aminoethylphosphonate (AEP) at the C-6. The O-glycans in Q-mucin from *Aurelia aurita* were proved to be mainly composed of three monosaccharides: GalNAc, AEP-($O\rightarrow$6)-GalNAc, and P-6-GalNAc. To the best of our knowledge, this is the first example of an O-glycan structure of glycoproteins containing AEP. This exceptionally simple structure of Q-mucin and its potential use in material science and technology are revealed.

1. Introduction

Since jellyfish populations have been increasing worldwide, they have been accumulating as huge amounts of marine waste at coastal industries, such as power plants and fishing sites.[1] Recently, during the screening of jellyfish for the valuable materials to compensate for the economic damage caused by their mass occurrence, a novel glycoprotein (qniumucin: Q-mucin), a member of the mucin family, has been extracted from several species of jellyfish.[2] In that report, the peptide main chain of Q-mucin was shown to consist of a tandem repeat unit of eight amino acids, -Val-Val-Glu-Thr-Thr-Ala-Ala-Pro-, with a small amount of -Val-Ile-Glu-Thr-Thr-Ala-Ala-Pro-.[2] The sequence of amino acids was also confirmed by NMR experiments on native Q-mucin, and no other protein sequences were recognized other than this tandem repeat unit.[3] NMR experiments on native Q-mucin also demonstrate that the two threonine residues in the tandem repeat unit are mostly O-glycosylated by α-*N*-acetylgalactosamine (GalNAc), and this GalNAc residue is partially substituted by unidentified substances at the C-6.

In contrast to other conventional mucins or glycoproteins with mucin domains, such as gastric and submaxillary mucins from domestic animals,[4] human mucins, and intra-articular glycoproteins,[5,6] Q-mucin has been proved to have exceptionally simple chemical structure that is suitable for applications material science and technology. However, in the previous monosaccharide analysis, an unidentified sugar component was detected in Q-mucin from several species of jellyfish.[2]

In this study, we thoroughly analyzed the chemical structure of O-glycan of Q-mucin and identified the unknown monosaccharide as GalNAc substituted by 2-aminoethylphosphonate (AEP) using NMR and ESI-MS/MS.

2. Results

2.1. Amino acid analysis of Q-mucin

Q-mucin extracted from *Aurelia aurita* after purification by ion-exchange chromatography was hydrolyzed by 5.7 M HCl and analyzed using an amino acid analyzer. Consequently, Thr, Glu,

Abbreviations: ABEE, 4-aminobenzoic acid ethyl ester; AEP, 2-aminoethylphosphonate; DPFGSE, double pulsed field gradient spin echo; DQF-COSY, double-quantum-filtered correlation spectroscopy; ESI FT-ICR MS, electrospray ionization ion-cyclotron resonance Fourier transform mass spectrometry; HMBC, heteronuclear multiple-bond coherence; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; MS, mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; Q-mucin, qniumucin; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

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doi:10.1016/j.carres.2009.08.001

Pro, Ala, Val(+Ile), and GalN were detected as major components at a molar ratio of approximately 2:1:1:2:2:2. Although this result is consistent with that in our previous publication,[2,3] where we were not aware of the existence of AEP, we additionally recorded the amount of AEP as a major component in the present study using commercially available AEP as the standard. The AEP content in samples was found to vary from batch to batch and the molar fraction of AEP was observed to be 0.5–1.0 of GalN or Thr. This fluctuation in AEP content can be attributed to the condition of the jellyfish used as the source and/or reactions occurring during the extraction and purification.

2.2. Monosaccharide and O-glycan analysis

The monosaccharide composition of Q-mucin was reinvestigated in the present study. Purified Q-mucin was hydrolyzed by 4 M TFA and the released monosaccharides were N-acetylated, ABEE-derivatized, and then analyzed by HPLC on a C_{18} column based on a method described elsewhere.[7,8] GalNAc and unidentified saccharide 1, which was also observed in our previous study, always existed as two major components. Galactose and arabinose were also commonly found as minor components whereas sialic acids were not detected. These results are essentially the same as those in our previous study.[2] An example of a HPLC chart after hydrolysis is shown in Figure 1A, where the obtained molar ratio of GalNAc/saccharide 1 was 1.0:0.4.

Next, purified Q-mucin was hydrazinolyzed for the examination of the O-glycan structure. Released O-glycans were analyzed by HPLC using the same method as that used for examining the monosaccharide. GalNAc and saccharide 1 were again detected as major O-glycans. These results indicate that the majority of O-glycans in Q-mucin from *A. aurita* are monosaccharides, that is, GalNAc and saccharide 1. The HPLC result for the same sample as that shown in Figure 1A is indicated in Figure 1B with a molar ratio of GalNAc/saccharide 1 of 1.0:0.8.

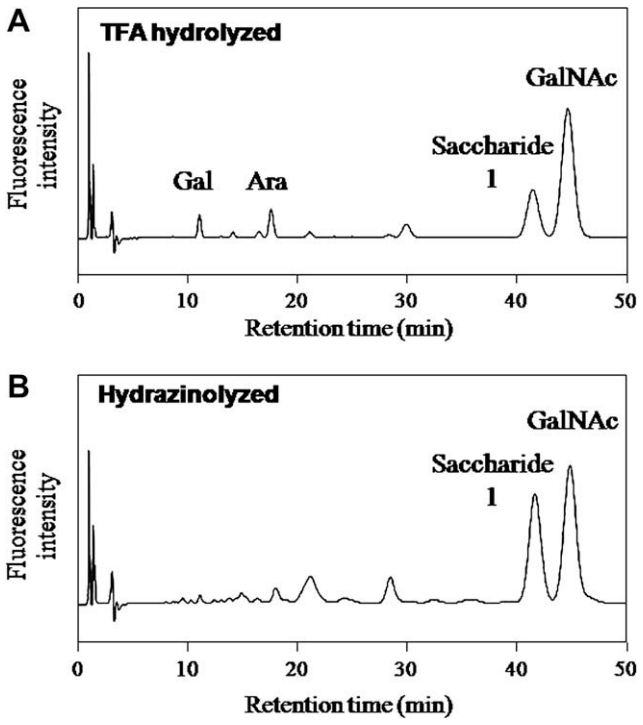


Figure 1. HPLC of N-acetylated and ABEE-derivatized saccharides released from Q-mucin. (A) TFA hydrolysate, (B) hydrazinolysate.

2.3. Structural analysis of saccharide 1

To perform further structural analysis, saccharide 1 was isolated from the N-acetylated hydrazinolysate of Q-mucin by anion-exchange column chromatography using Dowex 1-X8. The adsorbed fraction was passed through a Sep-Pak Plus C18 cartridge and then ABEE-derivatized. This fraction exhibited a single peak in HPLC analysis in a C_{18} column (data not shown). Using NMR and MS analyses of this purified, N-acetylated, and ABEE-derivatized sample (hereafter referred to as 'adduct 1', see Formula 1), saccharide 1 was finally determined to be GalNAc substituted by AEP in the manner described below.

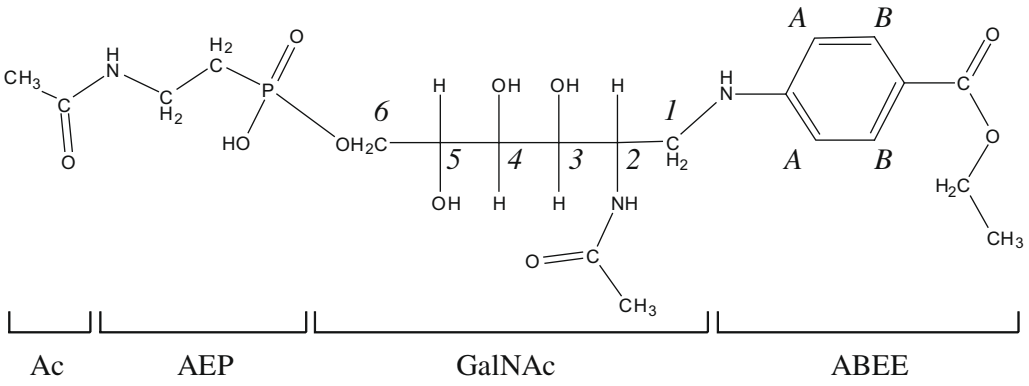
Figure 2A shows the ^{1}H–^{13}C HSQC spectrum of adduct 1. By comparison with the NMR data of ABEE-derivatized GalNAc, two distinctive proton signals were revealed at 1.87 and 3.36 ppm that correlated with carbon signals at 28.5 and 37.5 ppm, respectively. The combination of DPFGSE–TOCSY, DPFGSE–NOESY, DQF-COSY, HSQC, and HMBC experiments allowed us to assign most of the ^{1}H and ^{13}C signals to those of adduct 1, as summarized in Table 1. A ^{31}P NMR experiment produced a peak in the downfield region at 25.2 ppm suggesting the existence of a C–P bond in saccharide 1, indicating the presence of phosphonates, not phosphates. A ^{1}H–^{31}P HMBC experiment indicated cross peaks from this ^{31}P signal at 25.2 ppm to three proton signals at 1.87, 3.36, and 3.91 ppm (Fig. 2B). The proton signals at 1.87 and 3.36 ppm were assigned to CH_2P (2J) and CH_2N (3J) of the AEP group, respectively, with reference to the AEP molecule used as the standard ($\delta_H(CH_2P)$ = 1.81 ppm and $\delta_H(CH_2N)$ = 3.04 ppm, δ_P = 19.5 ppm). Since the proton signal at 3.91 ppm is assigned to H-6 of GalNAc (3J), the AEP moiety is found to connect with GalNAc at the C-6. These NMR data clearly demonstrated the structure of saccharide 1 to be AEP-(*O*→6)-GalNAc. In other words, a ^{31}P signal correlating with three protons at CH_2P, CH_2N, and H-6 can be used as a marker of the AEP-(*O*→6)-GalNAc moiety.

To confirm the present assignment, high-resolution ESI FT–ICR MS analysis of adduct 1 in the positive mode was performed, which indicated a protonated precursor ion ($[M+H]^+$) at *m/z* = 520.2056 as calculated to be $C_{21}H_{35}N_3O_{10}P$. ESI-MS/MS analysis of the same sample in the negative mode also indicated a precursor ion ($[M-H]^-$) at *m/z* = 518.2 and its fragment ion at *m/z* = 166.0 suggesting the presence of N-acetylated AEP (Fig. 3). The MS results are in good agreement with those of the NMR analyses.

2.4. NMR analyses of native Q-mucin

To confirm the existence of the AEP moiety in O-glycans of native Q-mucin, ^{31}P NMR experiments were performed on a native sample extracted from *A. aurita*. All the ^{1}H and ^{13}C signals in main chain and branched GalNAc were assigned in a separate paper.[3] To clarify the anomeric configuration of GalNAc in native Q-mucin, the coupling constant between the anomeric proton and carbon was examined by non-decoupling HMQC and obtained a large coupling constant of 176 Hz indicating that the α configuration is favorable.[9,10]

Since ^{31}P NMR measurement of native Q-mucin produced three signals at 0.86, 21.7, and 22.1 ppm with an integral intensity ratio of (<0.05):0.33:1, a ^{1}H–^{31}P HMBC experiment was then performed, the results of which are depicted in Figure 4. Similarly to the case of adduct 1, two of the three ^{31}P signals independently exhibited cross peaks with three protons: the ^{31}P signal at 22.1 ppm with proton signals at 1.95, 3.14, and 3.95 ppm, and the ^{31}P signal at 21.7 ppm with proton signals at 1.84, 3.08, and 4.37 ppm. These two sets of cross peaks are compatible with the existence of AEP-(*O*→6)-GalNAc. The proton signals at 1.95 and 3.14 ppm, which correlate with the ^{31}P signal at 22.1 ppm were assigned to CH_2P and CH_2N of the AEP moiety, respectively, and the signal at 3.95 ppm was confirmed to H-6 of GalNAc by a ^{1}H–^{31}P HMBC–



Formula 1. Structure of N-acetylated, ABEE-derivatized saccharide 1 (adduct 1).

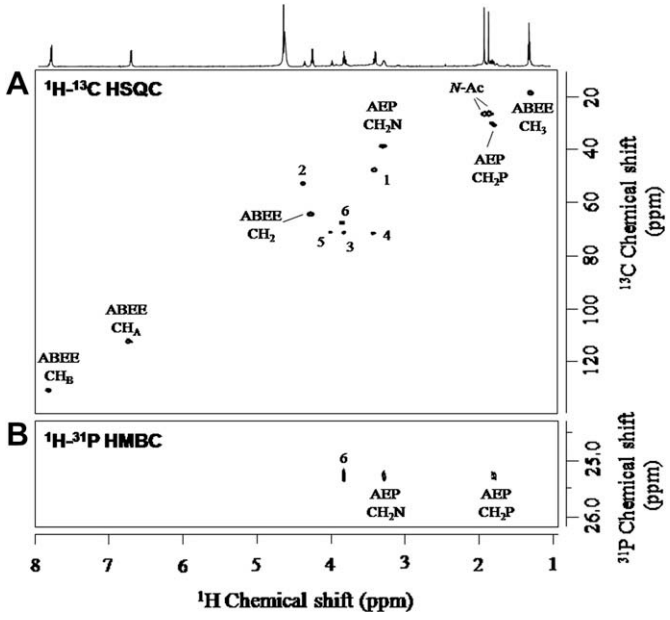


Figure 2. NMR spectra of adduct 1 in D_2O recorded at 30 °C. (A) ^1H–^{13}C HSQC spectrum, (B) ^1H–^{31}P HMBC spectrum.

Table 1
^1H and ^{13}C NMR chemical shifts (δ) of adduct 1 recorded in D_2O at 30 °C

Residues		δ (^1H)	δ (^{13}C)
GalNAc	1	3.48	47.0
	2	4.44	52.2
	3	3.89	71.9
	4	3.49	72.2
	5	4.07	71.5
	6	3.91	67.9
	CH_3[a]	1.92	24.6
N-Acetylated AEP	CH_2P	1.87	28.5
	CH_2N	3.36	37.5
	CH_3[a]	1.98	24.8
ABEE	CH_3	1.37	16.3
	CH_2	4.34	64.3
	CH_A	6.80	115.0
	CH_B	7.88	134.4

[a] These chemical shifts were interchangeable.

TOCSY experiment where the interaction reached to H-5. Therefore this group of signals shows the existence of AEP-(*O*→6)-GalNAc which is similar to adduct 1. On the other hand, however, the other group of proton signals at 1.84, 3.08, and 4.37 ppm, which correlate with ^{31}P at 21.7 ppm, cannot be straightforwardly assigned to the same species. The proton at 4.37 ppm showing a large low field shift exhibited no HSQC correlation with ^{13}C involved in any methylene groups. We speculate that this species may be an isomer of AEP-(*O*→6)-GalNAc with AEP connecting at other positions (C-3 or C-4). We tried a ^1H–^{31}P HMBC–TOCSY measurement from the proton at 4.37 ppm to other protons which resulted in unsuccessful. Consequently, the origin of ^{31}P at 21.7 ppm is still uncertain. Although the most probable candidate is an isomer of AEP-(*O*→6)-GalNAc, the possibility of other AEP compounds than glycans could not be excluded.

Additionally, a single cross-peak was found between the ^{31}P signal at 0.86 ppm and the proton signal at 4.04 ppm without coupling to any other protons. This proton signal was also assigned to H-6 of GalNAc and differs from the above AEP-(*O*→6)-GalNAc. The smaller chemical shift (0.86 ppm) of ^{31}P implies that the Q-mucin from *A. aurita* contains phosphates and a small amount of GalNAc residues that were phosphorylated at the C-6 (P-6-GalNAc). A standard molecule, P-6-Gal-1-Ph-NO_2, was used to confirm this assignment of 4.08 ppm for H-6 and 2.33 ppm for ^{31}P.

Present NMR experiments on the native Q-mucin and the previous study on another sample[3] produced the conclusion that the major O-glycans of native Q-mucin from *A. aurita* were GalNAc, AEP-(*O*→6)-GalNAc, and P-6-GalNAc.

2.5. Proteinase digestion of Q-mucin

Another possible approach to investigating the O-glycan structure is through the digestion of native Q-mucin. The main part of the peptide main chain of Q-mucin is composed of a tandem repeat unit of eight amino acid residues.[2,3] Since each repeat unit has one glutamic acid, Q-mucin is assumed to be digested selectively by endoproteinase Glu-C, which cleaves on the C-terminal of glutamic acid residues. Since the total AEP content fluctuates from batch to batch as observed in amino acid analysis, we selected a sample in which the AEP content was almost equal to that of Thr and GalN.

The digestion product was analyzed by LC–MS/MS with a C18 column, and a single peak appeared indicating one of major products after digestion. ESI-MS/MS analysis of this peak component in the positive mode indicated a precursor ion ($[M+2H]^{2+}$) at m/z = 704.3 as calculated to be the repeat unit of eight amino acids containing two AEP-GalNAc residues (Fig. 5). The loss of one and two AEP-GalNAc residues from the precursor ion generated fragment ions at m/z = 1097.7 (formal neutral loss of $C_{10}H_{19}N_2O_7P$ from $[M+H]^+$) and 787.5 (loss of $2 \times [C_{10}H_{19}N_2O_7P]$ from $[M+H]^+$), respectively. Fragment ions at m/z = 311.1 and 443.2 were also detected, which were AEP-GalNAc ($[C_{10}H_{19}N_2O_7P]^+$) and y_4 (Pro-Val-

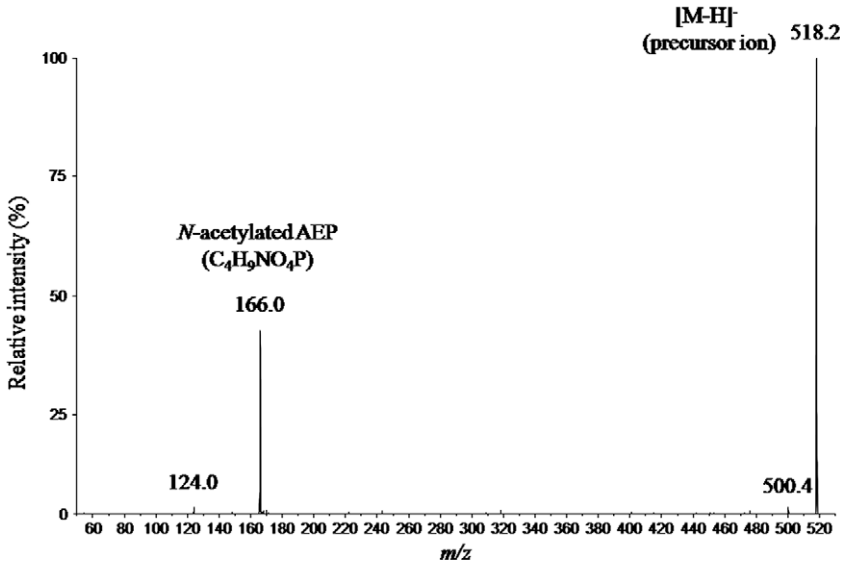


Figure 3. ESI-MS/MS fragmentation pattern of adduct 1 in negative mode.

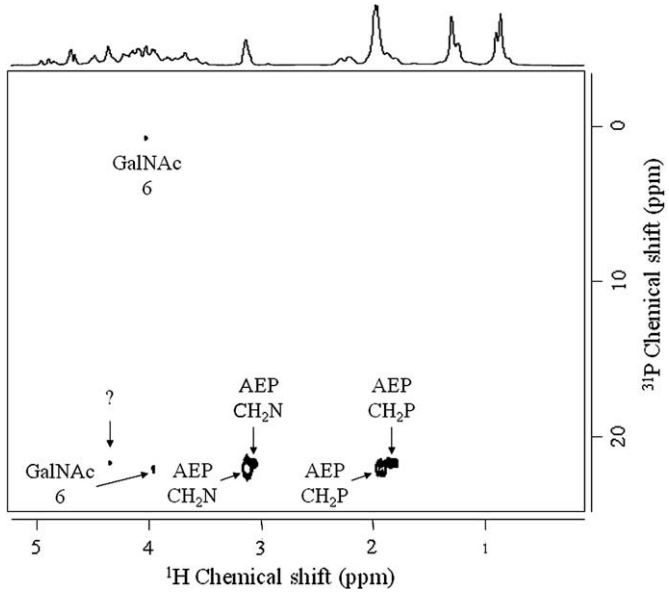


Figure 4. ^{1}H–^{31}P HMBC spectrum of native Q-mucin in D_2O recorded at 30 °C.

Val-Glu) (a product of 'proline effect';[11] $[Pro\text{-}Val\text{-}Val\text{-}Glu+H]^+$), respectively. These results support the above determination of O-glycans by the NMR study of native Q-mucin.

3. Discussion

The results obtained here indicate that the identified O-glycans of Q-mucin from *A. aurita* were GalNAc, AEP-(*O*→6)-GalNAc, and P-6-GalNAc. These three forms may mutually convert by hydrolysis, although the conversion from AEP-(*O*→6)-GalNAc to P-6-GalNAc is not considered to occur because C–P bond cleavage is thought to be difficult. It should be noted that the ester bond in AEP-(*O*→6)-GalNAc, different from that in P-6-GalNAc, is resistant to hydrolysis or hydrazinolysis before the monosaccharide or O-glycan analysis. Although the fractions of these three components vary from batch to batch as found in the amino acid analysis, no evidence of dynamic interconversion was recognized in the sample solutions prepared for NMR analysis. Assuming that our extraction method is satisfactorily established, we speculate that the fractions of these three forms are affected by some of the biological activities of jellyfish itself.

To the best of our knowledge, this is the first report of an O-glycan structure of a glycoprotein containing AEP. Moreover, the AEP-(*O*→6)-GalNAc group has only been previously reported as a component of triphosphonoglycosphingolipid in the nervous system of the sea hare, *Aplysia kurodai*.[12] Historically, AEP was the first compound containing C–P bonds to be found in a biological material.[13] Since its discovery, it has been found in lipids[14–16] and polysaccharides[17,18] in a wide variety of organisms, and in N-glycans of glycoprotein from the insect *Locusta migratoria*.[19] AEP was reported as a component of sphingophosphonolipids in *A. aurita*.[20] In our previous report, AEP-(*O*→6)-GalNAc (referred to as the unknown component in the previous paper) was detected as a common component of the monosaccharide in Q-mucin from several species of jellyfish including *Nemopilema nomurai* and *Chrysaora melanaster*.[2] Indeed, AEP-(*O*→6)-GalNAc was detected in the present study as a major O-glycan of Q-mucin from *N. nomurai* by hydrazynolysis (data not shown).

The function of AEP has been predicted by a number of researchers which provides a high resistance to enzymatic hydrolysis because of the existence of the C–P bond.[21,22] On the other hand, it has been reported that phospho-ethanolamine, an analog of AEP, contained in O-glycans of the glycoprotein from the wasp, *Vespula germanica,* may act as an alternative compound to sialic acid for conferring charges to the glycoprotein.[23] Since no sialic acids have been previously detected in jellyfish,[24] it is probable that the AEP in Q-mucin may also act as a substitute for sialic acid.

This study and previous ones prove that both the peptide and glycan structures of Q-mucin are remarkably simple without sialic acids or extra peptide sequences. This mucin is different from those conventionally found probably because it originates from a more primitive species. In mucins found in mammals, for example, the wide variety of glycan chains (glycoforms) realizes flexible and complex molecular recognition such as glycan–lectin interactions although they are difficult to treat as a single material owing to their inhomogeneous structure. In sharp contrast, Q-mucin is as simple and homogeneous as artificial polymers although its complete synthesis is still impossible using currently available techniques. Similarly to chitins and chitosans, Q-mucin can be treated as a single material. For example, one can use it as a starting material for generating designer mucins that are as complicated as those of higher animals.

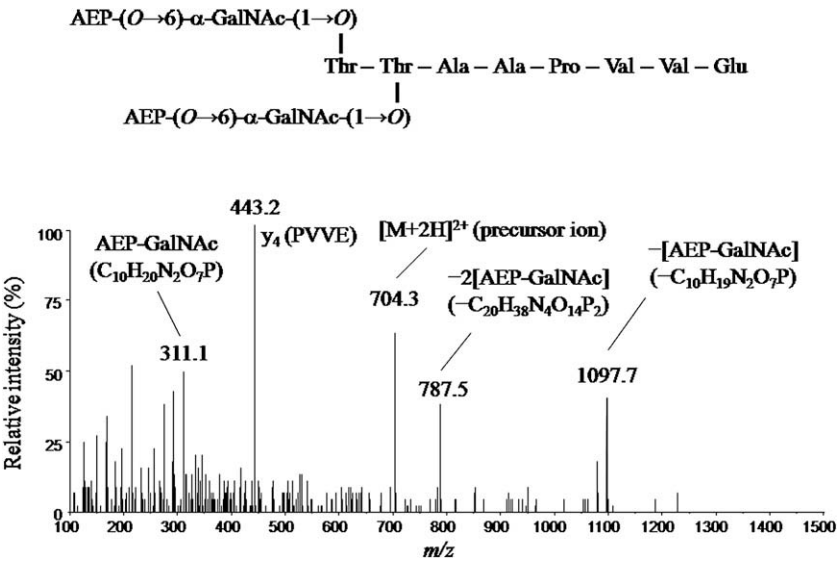


Figure 5. ESI-MS/MS fragmentation pattern of Glu-C digestion products of Q-mucin in positive mode.

In conclusion, the O-glycan structures of Q-mucin from *A. aurita* are clarified in the present study. The majority of the structures are monosaccharide GalNAc taking three forms: GalNAc, AEP-(*O*→6)-GalNAc, and P-6-GalNAc. Because of its exceptional simplicity and homogeneity, Q-mucin has the potential to be used in material science and technology.

4. Experimental

4.1. Materials

Q-mucin was extracted from *A. aurita* collected in Hakata Bay, Fukuoka, Japan and was purified by anion-exchange chromatography as described previously.[2] AEP was obtained from Wako Chemical Co., Ltd., and was used as received as the standard for amino acid analysis and NMR measurement.

4.2. Amino acid analysis

The amino acid composition was analyzed using an L-8500A automatic amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 5.7 M HCl for 20 h at 110 °C. The analysis of AEP content was also performed synchronously by the same procedure using the additional standard in HPLC.

4.3. Monosaccharide analysis

Q-mucin was hydrolyzed by 4 M TFA at 100 °C for 3 h, and the monosaccharides thus obtained were N-acetylated, labeled with ABEE, and analyzed by HPLC with a Honenpak C18 column (J-Oil Mills Inc., Tokyo, Japan) in accordance with the supplier's instructions.[7,8] A mixture of L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-ribose, D-xylose, D-galacturonic acid, D-glucuronic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine was treated as described above and each of which was then employed as a standard substance.

4.4. O-Glycan analysis

Q-mucin was hydrazinolyzed by Hydraclub Y2100 (J-Oil Mills Inc., Tokyo, Japan) in accordance with the supplier's instructions. Briefly, Q-mucin was hydrazinolyzed at 80 °C for 8 h, and the thus obtained hydrazinolysate was N-acetylated (the reduced end of the saccharide modified with hydrazide was simultaneously converted to an aldehyde group), labeled with ABEE, and analyzed by HPLC under the same conditions as those of the monosaccharide analysis.

4.5. Isolation of saccharide 1

Saccharide 1 was isolated from the N-acetylated hydrazinolysate of Q-mucin as an acidic saccharide, as described previously.[25] The N-acetylated hydrazinolysate mixture was placed to a Dowex 1-X8 (The Dow Chemical Company, Midland, MI, USA) anion-exchange chromatography column, washed with 0.2 M HOAc, eluted with 2 M HOAc, and lyophilized. The resultant solid was dissolved in MilliQ water, passed through a Sep-Pak Plus C18 cartridge (Waters Co., Milford, MA, USA), and derivatized with ABEE. Excess ABEE reagent was removed by Et_2O, and the water phase was lyophilized. The residual solid was redissolved in MilliQ water and applied to a Sep-Pak Plus C18 cartridge, washed with MilliQ water, and eluted with MeOH. The obtained fraction exhibited only a single peak of saccharide 1 in HPLC analysis.

4.6. NMR experiments

All NMR spectra were recorded at 600 MHz (^{1}H), 150 MHz (^{13}C), and 243 MHz (^{31}P) using an ECA 600 instrument (JEOL Ltd, Tokyo, Japan). Chemical shifts were given in δ (ppm), using sodium 3-trimethylsilylpropionate-2, 2, 3, 3-d_4 (TSP), whose ^{1}H and ^{13}C resonate at 0 ppm, or H_3PO_4 for ^{31}P as an external reference for samples measured in D_2O at 30 °C. Assignments of signals were made using a combination of various experimental techniques such as DPFGSE–TOCSY, DPFGSE–NOESY, DQF-COSY, HSQC, ^{1}H-^{31}P HMBC and ^{1}H-^{31}P HMBC–TOCSY.

4.7. Mass spectrometry

N-Acetylated, ABEE-derivatized saccharide 1 was separated in an ODS column (L-column ODS-L, 100 mm × 1.50 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan) by HPLC (1100 series; Agilent Technologies Inc., Santa Clara, CA, USA) using a linear gradient of MeCN (10–50%) with 0.1% formic acid and flow rate of 100 μL/min and detected by a 4000Q TRAP (Applied Biosystems Inc., Carlsbad, CA, USA).

The N-acetylated, ABEE-derivatized saccharide 1 was also analyzed by ESI FT-ICR Mass Spectrometer (BIOAPEX-II; Bruker Daltonics Inc., Billerica, MA, USA).

4.8. Proteinase digestion of Q-mucin

The Q-mucin was digested by endoproteinase Glu-C from *Staphylococcus aureus* V8 (Sigma–Aldrich Co., St. Louis, MO, USA) for 16 h at 37 °C in a sodium phosphate–EDTA buffer (pH 7.4), as described previously.[26]

The digestion product was analyzed by LC–MS/MS with an ODS column (Inertsil ODS-3, 200 mm × 1.5 mm, GL Sciences Inc., Tokyo, Japan) using a gradient of MeCN (2–70%) with 0.1% formic acid and a flow rate of 100 μL/min.

Acknowledgments

We are grateful to H. Koshino, RIKEN for his valuable discussion. We acknowledge H. Morishita at the Research Resources Center, Brain Science Institute, RIKEN for amino acid analysis, and R. Sato and T. Momma for their help in extracting mucin, as well as K. Kihira for his encouragement and suggestions. This research is partly supported by Grants-In-Aid for Scientific Research (Kakenhi) No. 17034067 in Priority Area 'Molecular Nano Dynamics', No. 17651051, No. 17300166, and No. 19590002 from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. This study was partly supported by the project to develop innovative seeds (Creation and Support Program for Start-ups from Universities) of the Japanese Science and Technology Agency.

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