Anal. Chem. **2004,** *76,* 5990−6001

Articles

Strategy for Profiling and Structure Elucidation of Mucin-Type Oligosaccharides by Mass Spectrometry

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A strategy combining accurate mass determination, tandem mass spectrometry, structure homology, and exoglycosidases is described that allows the structural characterization of mucin-type O-linked oligosaccharides. The method is used to profile with quantitation the O-linked oligosaccharide (both neutral and anionic) components of the only diploid *Xenopus* frog, *Xenopus tropicalis*. Collision-induced dissociation was used to determine connectivity, to identify previously characterized oligosaccharides, and to determine the presence of structural motifs in unknown oligosaccharides. Exoglycosidase digestion was used to identify the individual residues along with the linkages. The enzymes were also used to cleave larger oligosaccharides to smaller units that are similar to previously elucidated components. By using CID, isomeric structures were compared to determine whether they were identical. In this way, the exoglycosidases were more effectively used, and their use was minimized. A total of 35 oligosaccharides including neutral, sialylated, and sulfated were characterized in this way. The relative abundances of all components were also determined based on HPLC.

More than half of all proteins in biological systems are glycosylated.[1] Carbohydrate moieties in glycoprotein mediate a number of essential functions such as cell−cell and cell−matrix recognition, cellular adhesion, inter- and intracelluar interaction, and protection. Unlike DNA and proteins, carbohydrates can be highly branched molecules with monomeric units connected by linkages differing in position and stereochemistry. This high degree of complexity made structural determination a difficult task. To date, no single technique has shown the capability of providing complete structures with the necessary sensitivity to be applicable to biological samples. Traditionally, analysis of glycoprotein oligosaccharides involves a combination of techniques such as mass spectrometry (MS), NMR, monosaccharide composition analysis, and chemical derivatization.[2−6] These methods are time-consuming and require milligram quantities of glycoproteins. The lack of a rapid method for the complete structural elucidation of oligosaccharides remains a major barrier in understanding structure−function relationships of this important class of compounds.

MS has emerged as the premier technique for characterizing glycan moieties in various biologically important molecules. Mass spectrometry offers distinct advantages over conventional approaches because of its inherent sensitivity and its capability to obtain structure information through tandem mass spectrometry (MS^n) techniques. However, the identification of individual monomeric units, linkages, and anomericity have posed big challenges to mass spectrometry. This is especially true for O-linked oligosaccharides where the structure diversity is great. A few years ago, this laboratory developed a method called the "catalog library approach (CLA)",[7] in which groups of fragment ions, present in collision-induced dissociation (CID) spectra, were assigned to specific structural motifs. These fragment ions were then used to identify the specific motifs in the CID spectra of unknown oligosaccharides. Using this approach, Tseng et al.[8] successfully elucidated the structures of O-linked oligosaccharides in the egg jelly coat of *Xenopus laevis* with great speed and sensitivity. However, only five motifs were identified based upon fragmentation patterns of known structures determined by NMR.[9]

Exoglycosidase digestion, either sequentially or in arrays, has been used extensively for the structural analysis of carbohydrates

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10.1021/ac049666s CCC: $27.50 © 2004 American Chemical Society
Published on Web 09/09/2004

and glycoconjugates.[10−15] The coupling of exoglycosidase digestion with matrix-assisted laser desorption/ionization (MALDI)-MS combines the high specificity of enzymes with the high resolution, speed, and sensitivity of MS. This combination has been successfully used for N-linked oligosaccharides;[15−18] however, the large structural variations of O-linked oligosaccharides prohibit the use of only exoglycosidase arrays for complete structural elucidation. A few reports mentioned the use of exoglycosidase digestion on very simple structures.[19,20] Rudd et al.[21,22] determined the structures of 2-aminobenzamide-labeled O-linked oligosaccharides using the combination of HPLC, mass spectrometric composition and mass fragmentation data, and exoglycosidase digestions. In a previous publication,[23] exoglycosidase digestion was used to confirm and refine the O-linked oligosaccharide alditol structures obtained from the CLA. Here, the first extensive use of exoglycosidase for the complete structural elucidation of O-linked oligosaccharides is described.

O-Linked oligosaccharides in the jelly coat of amphibian eggs are known to be essential in fertilization events. *X. laevis*, the South African frog, has been an invaluable model in studies of fertilization and developmental mechanisms. As the only diploid species in the *Xenopus* genus, *Xenopus tropicalis*, a small, fast-breeding, close relative of *X. laevis*, has become "the prince of new model organism"[24,25] to the developmental biologist. It shares all advantages of *X. laevis*, the well-studied tetraploid model system. Recently Lindsay et al.[26] have demonstrated that cross-fertilization between *X. laevis* and *tropicalis* was much lower in both directions when compared to the homologous crosses. The carbohydrate chains present in the egg jelly coats can be involved in species-specific gamete interactions.[27] Oligosaccharides have been postulated to provide definite structural recognition sites.[28] Therefore, the structural analysis of carbohydrate components is critical for understanding the structure−function relationships of oligosaccharides in the fertilization process.

In the present study, we develop a strategy to determine the structures of O-linked oligosaccharides in the egg jelly coat of *X. tropicalis*. CID was used to elucidate the sequences of the unknown oligosaccharides. Based on the preliminary sequence information, a series of specific exoglycosidases were chosen to sequentially digest the target oligosaccharide with the resulting enzymatic products monitored by MALDI Fourier transform ion cyclotron resonance (FTICR) MS. Tandem MS analysis was performed on the digested products whose fragmentation patterns were compared with previously determined structures. In this way, the use of the expensive and time-consuming exoglycosidase digestion was minimized.

EXPERIMENTAL SECTION

Materials. The exoglycosidases, α1−2 fucosidase, β1−3 galactosidase, β-*N*-acetylhexosaminidase, α-*N*-acetylgalactosaminidase, α2−6 neuraminidase, and α2−3 neuraminidase were purchased from New England Biolabs (Beverly, MA). The α-fucosidase and α1−6 fucosidase was purchased from Sigma-Aldrich (St. Louis, MO). The α1−3,4-fucosidase were purchased from ProZyme (San Leandro, CA). The α-galactosidase, β-galactosidase, and β-*N*-acetylglucosaminidase were purchased from Boerhinger Mannheim (Indianapolis, IN). Original enzyme suspensions from the company were stored at −20 or 4 °C according to the instruction. Just before use, 1 μL of enzyme suspension was dialyzed against deionized water for 30 min. All the enzymes used in this study and their biological sources, EC number, and specificity are listed in Table 1.

Release, Isolation Oligosaccharides from Frog Egg Jelly. *X. tropicalis* eggs were collected and washed with De Boers buffer. The jelly coat was solubilized by 0.3% (v/v) β-mercaptoehanol. The solubilized jelly coat was lyophilized. O-Linked oligosaccharides were released from egg jelly glycoproteins in the alditol form by treating dried jelly coat with 1.0 M $NaBH_4$ and 0.1 M NaOH for 24 h at 42°C. Then, 1.0 M HCl was added to stop the reaction and destroy the excess $NaBH_4$ in an ice bath.

The resulting mixture of oligosaccharides was purified and fractionated on a porous graphitized carbon solid-phase extraction cartridge (PGC-SPE; Alltech Associate inc., Deerfield, IL). The PGC-SPE cartridge was washed with 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) followed by water prior to use. The sample was applied to the column and allowed to run into the absorbents at a flow rate of ∼0.5 mL/min. Salts were washed off with three column volumes of water. The glycans were sequentially eluted with three solvents (one column volume each): (1) 10% (v/v) acetonitrile/water solution, (2) 20% (v/v) acetonitrile/water solution, and (3) 40% (v/v) acetonitrile in 0.05% (v/v) TFA−water solution. Each effluent was collected into 4−6 fractions. After checking for the presence of glycans by MALDI-FTICR MS analysis, the fractions that contained neutral and anionic oligosaccharides were pooled separately and concentrated by evaporating the solvents in a vacuum evaporation system. The

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Table 1. Specificity and Reaction Conditions of Exoglycosidases

glycosidase	source	EC no.	specific for	pH	temp (°C)	time (h)
α-L fucosidase	bovine kidney	3.2.1.51	α-L-fucose	5.5	25	~20
α1−2 fucosidase	*Xanthomonas manihotis*	3.2.1.63	α (1−2)-fucose	6.0	37	~20
α1−6 fucosidase	recombinant, expressed in *Escherichia coli*	3.2.1.127	α (1−6)-fucose	5.0	37	~20
α1−3,4 fucosidase	almond meal		α (1−3,4)-fucose	5.0	37	~20
α-galactosidase	green coffee beans	3.2.1.22	α (1−3,4,6)-galactose	7.0	25	<10
β-galactosidase	bovine testes	3.2.1.23	β-galactose	4.3	37	<10
β1−3 galactosidase	cloned from *X. manihotis* and expressed in *E. coli*		β (1−3)-galactose	4.5	37	<10
β-*N*-acetylhexosaminidase	cloned from *Streptomyces plicatus* and overexpressed in *E. coli*	3.2.1.52	β-*N*-acetylhexosamine	4.5	37	<5
α-*N*-acetylgalactosaminidase	cloned from a proprietary strain and expressed in *E. coli*	3.2.1.49	α-*N*-acetylgalactosamine	7.0	37	<5
β-*N*-acetylglucosaminidase	bovine kidney		β-*N*-acetylglucosamine	5.0	40	<5
α 2−6 neuraminidase	cloned from *Clostrdium perfringens* and overexpressed in *E. coli* at NEB	3.2.1.18	α(2−6)-sialic acid	6.0	37	<5
α2−3 neuraminidase	cloned from *Salmonella typhimurium* and overexpressed in *E. coli*	3.2.1.18	α(2−3)-sialic acid	6.0	37	<5

resulting samples were submitted for further MS or HPLC analysis.

HPLC Separation of Oligosaccharide Mixtures. Glycan mixtures purified from *X. tropicalis* egg jelly by the above procedures were separated by HPLC on a Hypercarb porous graphitized carbon column (2.1 × 100 mm, 5 μm, ThermQ Hypersil Division, Bellfonte, PA). The flow rate was 0.25 mL/min. For the separation of neutral oligosaccharide mixture, solvent A was H_2O and solvent B was acetonitrile. The gradient elution was 5−32% B during 0−85 min. For the separation of anionic oligosaccharide mixture, solvent A was 0.05% TFA−H_2O (v/v) and solvent B was 0.05% TFA−40% acetonitrile−H_2O (v/v). The gradient elution was 12.5−75% B during 0−85 min. The effluents were monitored at 206 nm by a diode-array detector. HPLC effluents were collected as 1-min fractions and analyzed by MALDI-FTICR MS.

MALDI-FTICR MS Analyses. MALDI-FTICR MS was performed on a commercial instrument (Ion Spec, Irvine, CA) with a 4.7-T superconducting magnet and an Nd:YAG laser operating at 355 nm. The MALDI sample was prepared by concentrating 1−20 μL of the HPLC fraction directly on the probe. One microliter of 0.4 M 2,5-dihydroxybenzoic acid in ethanol was added as the matrix. For the positive mode, 1 μL of 0.1 M NaCl in ethanol−water solution (50:50) was added to the probe tip to enrich the Na^+ concentration and produce primarily sodiated species. Warm air was used to quickly crystallize the sample on the probe.

MALDI-CID-FTICR MS Analyses. Sustained off-resonance irradiation (SORI)-CID experiments were performed to obtain structural information. The desired ion was isolated in the ion cyclotron resonance (ICR) cell with the use of an arbitrary waveform generation and synthesizer excitation. The ions were excited +1000 Hz of their cyclotron frequency for 1000 ms at 2−8 V (base to peak), depending on the desired level of fragmentation and the size of the oligosaccharides. Two argon pulses were used during the CID event to maintain a pressure of 10^{-5} Torr.

Methylation of Sialylated Oligosaccharides. The sialylated oligosaccharide from HPLC fractions was first dried by speed-vacuum centrifuge and was dissolved with 10 μL of methyl iodide and 50 μL of anhydrous dimethyl sulfoxide (DMSO). The suspension was thoroughly mixed by the vortex mixer and allowed to react for 2 h at room temperature. After another 100 μL of DMSO was added into the mixture, DMSO solvent was removed by lyophilization. The methylated glycan was reconstituted in water prior to MALDI-FTICR MS analysis.

Exoglycosidase Digestion. Ammonium acetate buffer solution (0.1 M) for enzyme digestions was prepared. The desired pH was adjusted by the addition of acetic acid. The digestion temperature, the pH value, and the time used for each specific exoglycosidase digestion are presented in Table 1. This table is an extension of one presented earlier by Xie et al.[23] To perform sequential exoglycosidase digestion of oligosaccharides released from *X. tropicalis* egg jelly, an amount of the HPLC fraction that contains the target oligosaccharide(s) was dried by speed-vacuum centrifuge. It was redissolved in 4 μL of deionized water or 0.1 M buffer solution, depending on the requirement of the exoglycosidase. The sample was then added with dialyzed enzyme solution and incubated. A 1-μL aliquot of the resulting enzymatic products was analyzed by MALDI-FTICR MS. Since the enzymes retain their activity and specificity in the presence of other exoglycosidases used in previous digestion steps, a second and third exoglycosidase can be sequentially added into the reaction mixture, allowing further MALDI-FTICR MS analyses of the digested product without any sample purification.

RESULTS

Profile Oligosaccharide Components. The neutral and anionic glycan components released from the glycoproteins were separated by employing the proper eluting solvent on a PGC-SPE cartridge.[29] The neutral components were eluted out first by 10% (v/v) acetonitrile−water solution and after with 20% (v/v) acetonitrile−water solution. The anionic glycans were eluted out by 40% (v/v) acetonitrile in 0.05% (v/v) TFA−water solution. The fractions containing neutral and anionic oligosaccharides were separately pooled, concentrated, and analyzed by MALDI-FTICR mass spectrometry. The resulting positive and negative mode MALDI-FTICR mass spectra are shown in Figure 1a and b, respectively. Nearly all of observable peaks correspond to neutral, sulfated, and sialylated oligosaccharides as determined by the

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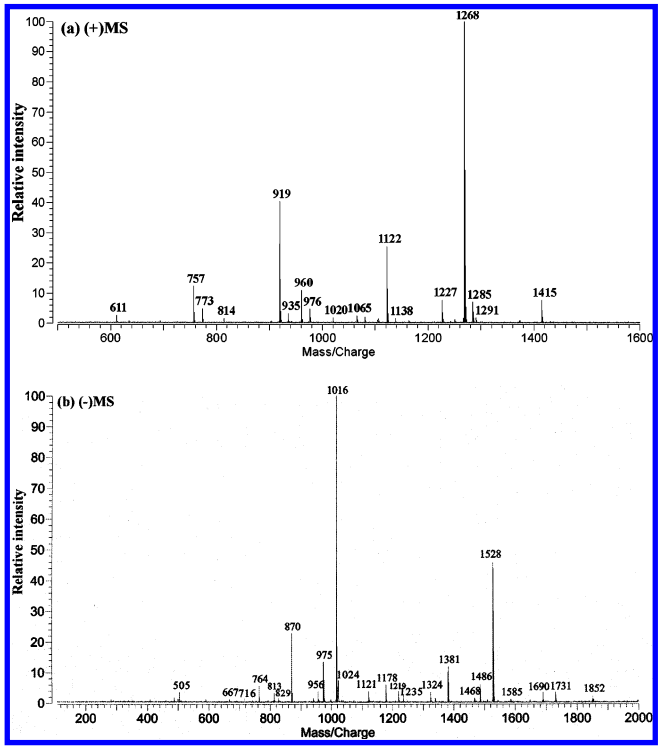

Figure 1. MALDI-FTICR mass spectra of (a) neutral oligosaccharide components and (b) anionic oligosaccharide components after PGC-SPE purification.

Table 2. Masses and Composition of Neutral Oligosaccharides

no.	expt (m/z)	theor (m/z)	Hex	Fuc	HexNAc
1	611.223	611.227	1	0	2
2	757.278	757.285	1	1	2
3	773.273	773.280	2	0	2
4	814.298	814.306	1	0	3
5	919.329	919.337	2	1	2
6	960.354	960.364	1	1	3
7	976.352	976.359	2	0	3
8	1065.385	1065.396	2	2	2
9	1106.410	1106.422	1	2	3
10	1122.401	1122.417	2	1	3
11	1163.427	1163.444	1	1	4
12	1227.431	1227.449	3	2	2
13	1268.463	1268.475	2	2	3
14	1414.512	1414.533	2	3	3

accurate mass of each peak, and are summarized in Table 2 and Table 3, respectively. It should be pointed out that all neutral oligosaccharides produced sodiated peaks in the positive mode while sulfated and sialylated oligosaccharides produced strong $[M - H]^-$ peaks in the negative mode.

Porous graphitized carbon had been extensively explored for HPLC separation of O-linked oligosaccharides.[30] PGC-HPLC has a unique ability to resolve isomeric oligosaccharides.[31,32] In Figure 2a and b, the PGC-HPLC chromatograms are shown for neutral and anionic oligosaccharides, respectively. For the neutral oligosaccharide separation, a gradient of acetonitrile and water was

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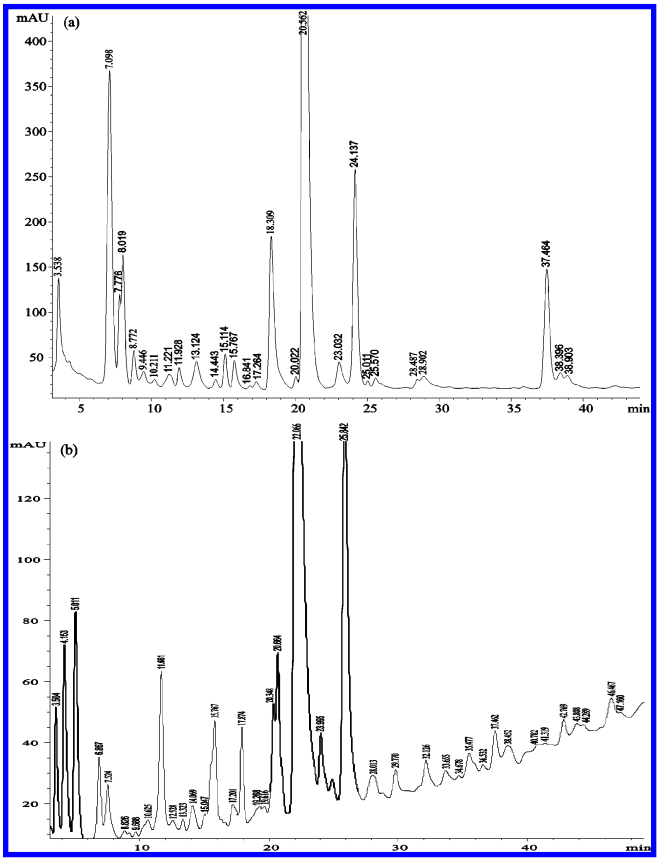

Figure 2. PGC-HPLC chromatograms of (a) neutral and (b) anionic oligosaccharides.

used as described in the Experimental Section. For the anionic oligosaccharide separation, TFA was added into both the acetonitrile and water solvents to elute out the acidic carbohydrate component. The neutral components were eluted out between 3 and 40 min. At 20.6 min, the most dominant compound was eluted out, which produced $[M + Na]^+$ at m/z 1268.46 (2 Fuc, 2 Hex, and 3 HexNAc) on the MALDI-FTICR mass spectrum. The anionic components were eluted out between 3 and 48 min. At 22.1 min, the most dominant compound was eluted out, which produced $[M - H]^-$ at m/z 1016.32 on the MALDI-FTICR mass spectrum corresponding to a singly sulfated oligosaccharide (1 Fuc, 1 Hex, 3 HexNAc, and 1 sulfate).

MS and MS/MS analysis were conducted on each HPLC fraction in order to identify and elucidate each oligosaccharide component. The majority of the HPLC fractions contained a single component. The isomers were completely resolved in the HPLC. For instance, three isomers of m/z 960 were identified with significantly different HPLC retention times (11.9, 18.3, and 38.9 min). Two isomers of m/z 1268 were found at retention times of 15.1 and 20.1 min. A total of 16 neutral, 13 sulfated, and 6 sialylated oligosaccharides were identified. The diversity in terms of total number of both neutral and anionic oligosaccharides in the egg jelly of *X. tropicalis* is significantly less than that of *X. laevis*[8] in which over 30 neutral and over 50 anionic oligosaccharides were found.

Sequence Determination of Unknown Oligosaccharide by MALDI-CID-FTICR MS. Tandem MS (MS^n) experiments (more specifically CID) were performed on both the neutral and anionic components to obtain the connectivity and to identify previously

Table 3. Masses and Composition of Anionic Oligosaccharides

no.	expt (*m*/*z*)	theor (*m*/*z*)	Hex	Fuc	HexNAc	Na	sulfate	Neu5Ac
1	505.138	505.135	0	0	2		1	
2	667.189	667.187	1	0	2		1	
3	716.268	716.273	0	0	2			1
4	764.286	764.283	1	2	0			1
5	813.242	813.245	1	1	2		1	
6	829.244	829.240	2	0	2		1	
7	870.265	870.267	1	0	3		1	
8	956.216	956.212	0	1	3	1	2	
9	975.297	975.298	2	1	2		1	
10	1016.324	1016.324	1	1	3		1	
11	1024.386	1024.384	1	1	2			1
12	1121.364	1121.356	2	2	2		1	
13	1178.387	1178.377	2	1	3		1	
14	1219.414	1219.404	1	1	4		1	
15	1324.443	1324.436	2	2	3		1	
16	1381.477	1381.456	2	1	4		1	
17	1467.411	1467.401	1	2	4	1	2	
18	1486.452	1486.448	3	2	3		1	
19	1527.517	1527.514	2	2	4		1	
20	1585.545	1584.536	2	1	5		1	
21	1689.575	1689.568	3	2	4		1	
22	1730.601	1730.594	2	2	5		1	
23	1851.629	1851.621	4	2	4		1	

determined structural motifs. Based on the product ions, a rudimentary structure containing the connectivity of unidentified residues can be produced.

The elucidation of an unknown oligosaccharide XT-1268-2 is illustrated. Based on the accurate mass analyses, the quasi-molecular ion $[M + Na]^+$ *m*/*z* 1268.46 is composed of two Fuc, two Hex, and three HexNAc (theoretical mass *m*/*z* 1268.48). CID fragmentation of neutral oligosaccharides often occurs from the nonreducing end toward the reducing end. Therefore, the losses of Fuc (*m*/*z* 1122) and Hex (*m*/*z* 1106) from the quasimolecular ion, shown in Figure 3a, indicate that both fucose and Hex are present as nonreducing ends. In Figure 3b, the presence of *m*/*z* 1065 in the MS^3 (*m*/*z* 1268/1122) spectrum indicates a loss of HexNAc from the quasimolecular ion, another nonreducing terminus. The MS^4 experiment yielded the core structure. In Figure 3c, the group of fragment ions *m*/*z* 611, 449, 431, 413, 408, and 228 are identical to the fragment ions of a known trisaccharide core structure, Gal(β1−3)[GlcNAc(β1−6)]GalNAc-ol, which is also found in the egg jelly of *X. laevis*.[7] The presence of *m*/*z* 388, which corresponds to a Hex-HexNAc combination, further suggests that the terminal Hex is bound to the GlcNAc leaving the other terminal HexNAc bound to the Gal of the core, as shown in the inset structure. The exoglycosidase experiments described below confirm the proposed structure and yield the linkages.

All 16 neutral oligosaccharides were sequenced in this way. Their preliminary structures and their corresponding retention times in HPLC separation are listed in Chart 1. There are at least eight known core structures for mucin-type O-linked oligosaccharides.[33] Most of the compounds contained the trisaccharide core structure Gal(β1−3)[GlcNAc(β1−6)]GalNAc-ol, also known as core 2.[33] Another core structure is represented in XT-757-2. Accordingly, there are two possibilities for the Hex-HexNAc-ol core of XT-757-2, Gal(β1−3)GalNAc-ol (core 1), or Gal(α1−3)-

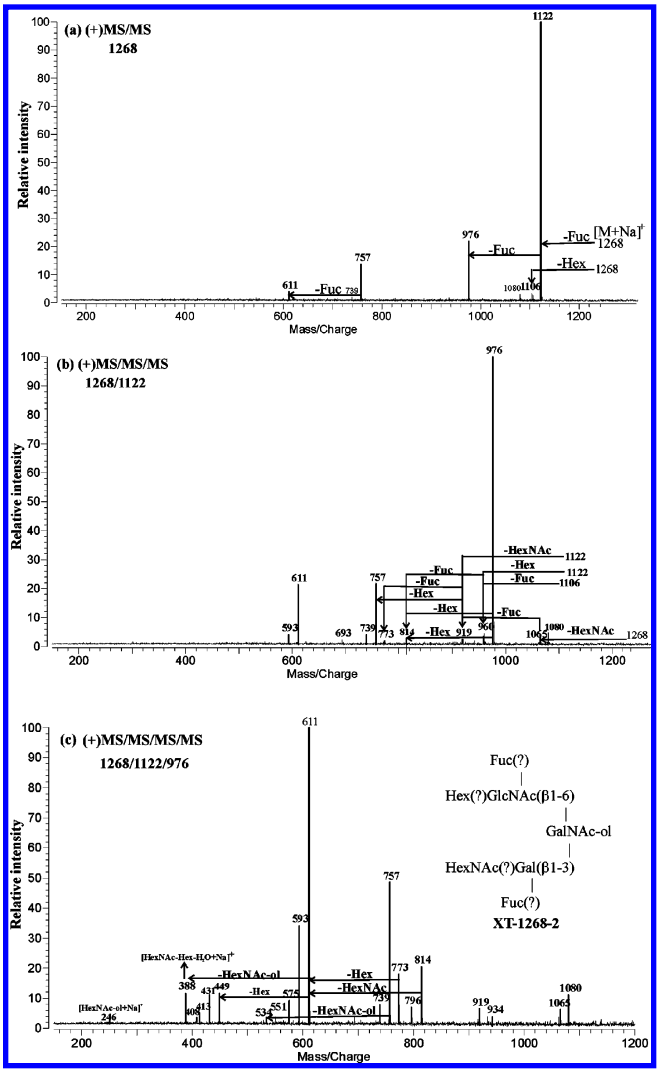

Figure 3. CID spectra of XT-1268-2. (a) MS/MS spectrum. (b) MS^3 spectrum (*m*/*z* 1268 → 1122). (c) MS^4 spectrum (*m*/*z* 1268 → 1122 → 976). The proposed primary structure is inset.

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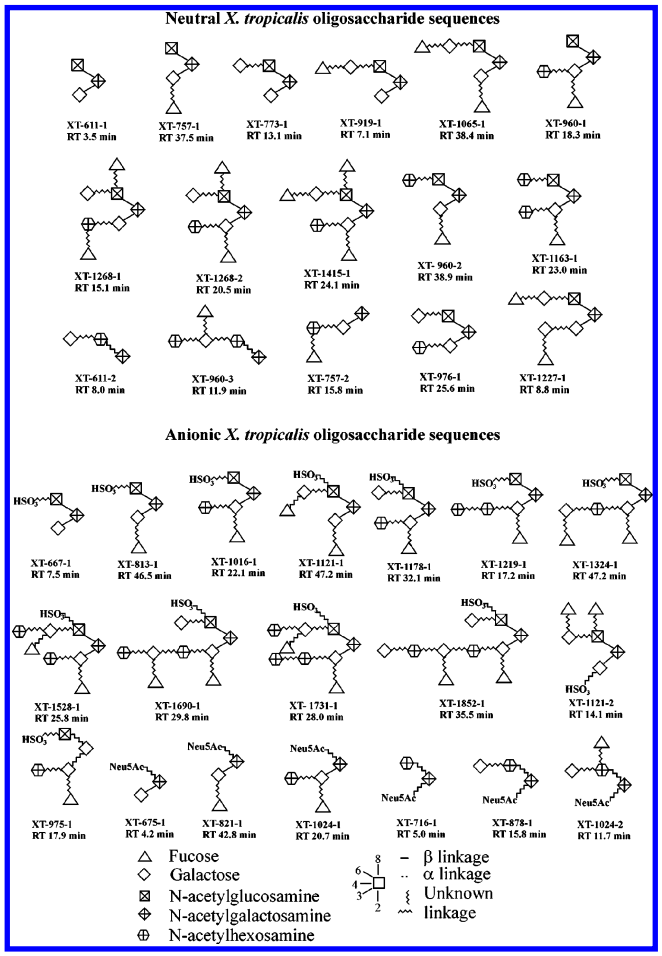
Chart 1



GalNAc-ol (core 8). NMR analyses have revealed that the Hex-HexNAc-ol disaccharide core in *X. laevis*[34] and all other amphibian species[35−47] is exclusively Gal(β1−3)GalNAc-ol**.** We believe, therefore, that the Hex-HexNAc-ol core shown in *X. tropicalis* is also Gal(β1−3)GalNAc-ol. However, because of its size and the limitations in the low-mass range of the instrument, we could not unambiguously assign this structure at this time.

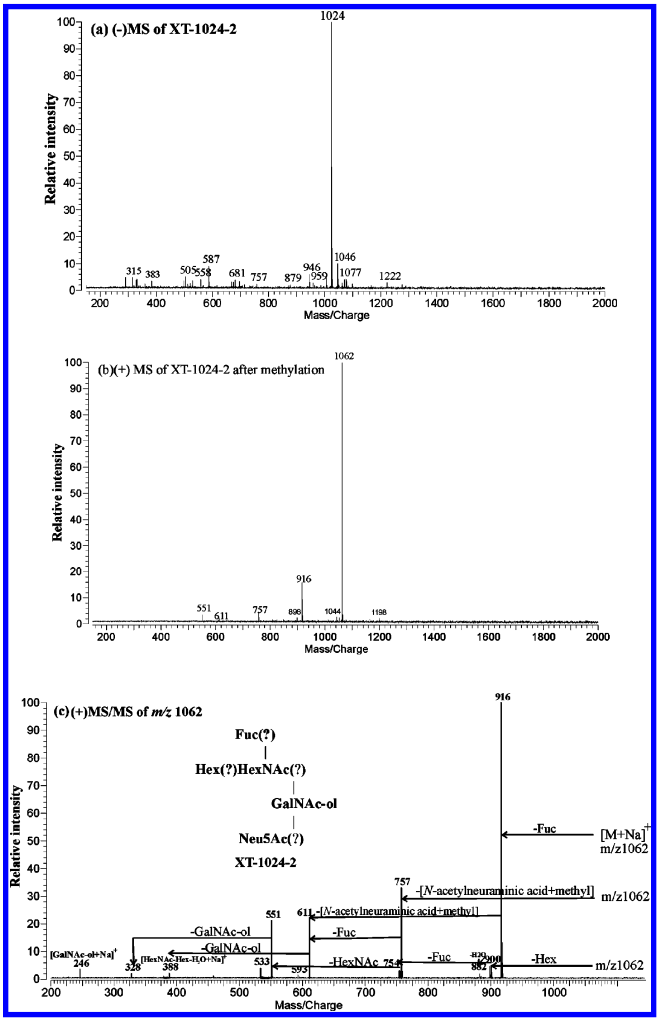


Figure 4. MALDI-FTICR mass spectra of sialylated oligosaccharide XT-1024-2. (a) MS of XT-1024-2 in the negative mode. (b) MS of methylated compound in the positive mode. (c) CID spectra of methylated compound and the proposed primary structure is inset.

The core structure of XT-611-2 and XT-960-3, HexNAc-HexNAc-ol, has four possibilities based on the putative cores, which are GlcNAc(β1−3)GalNAc-ol, GlcNAc(β1−6)GalNAc-ol, GalNAc(α1−3)GalNAc-ol, and GalNAc(α1−6)GalNAc-ol. Interestingly, this core is not found in other amphibian egg jelly.

The anionic oligosaccharides in the egg jelly of *X. tropicalis* are either sulfated or sialylated. Figure 4a shows the negative mode MALDI-FTICR mass spectrum of XT-1024-2 (m/z 1024.38, 1 Fuc, 1 Hex, 2 HexNAc, and 1 Neu5Ac). The signals, except m/z 1024, shown in Figure 4a are due to either impurities or matrix peaks. The sialic acid is generally labile in MALDI-MS and is readily cleaved in the CID process. In the negative mode, tandem MS yields a single product (m/z 290) corresponding to the deprotonated sialic acid residue. To get the structural information, methylation of the sialic acid group is needed to minimize the loss of the sialic acid residue. Figure 4b shows the positive mode MALDI-FTICR mass spectrum of the methylated product; the peak at m/z 1062 corresponds to the methyl-derivatized quasimolecular ion. The peaks at m/z 916 and 757 correspond to the loss of terminal fucose and methylated sialic acid from the quasimolecular ions, respectively, due to the metastable decay. For carbohydrates analyzed by MALDI-FTMS, with the relatively long time between

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ionization and detection, the unimolecular metastable fragmentations are sometimes observed leading to substantial loss of fucose and sialic acid residues. Extensive work has been conducted in the past several years on the development of MALDI methods to reduce the problem of metastable decay such as using large alkali cations,[48] collisional cooling with high pressure,[49−54] atmospheric pressure sources,[55−60] and infrared MALDI.[61] The CID spectrum of m/z 1062 in the positive mode is shown in Figure 4c. The losses of Fuc (m/z 916), Hex (m/z 900), and methylated sialic acid (m/z 757) from the quasimolecular ion indicate that these groups are present at the nonreducing ends. The smallest fragment at m/z 246, corresponds to $[GalNAc\text{-}ol + Na]^+$ the reducing terminus in its alditol form. Again, the presence of m/z 388 yields the [HexNAc-Hex] combination. Based on the fragment ions, the relative connectivity of the monosaccharide units and the position of sialic acid are shown in the inset structure. Because all Hex residues found in egg jelly of amphibian species are exclusively galactose,[34−47] the Hex in Chart 1 is assumed to be galactose. This assumption is verified by the exoglycosidase reactions described below. Tandem MS of sulfated oligosaccharides was performed in the anionic mode without derivatization. The structures of the sialylated and sulfated oligosaccharides deduced from tandem MS and their corresponding retention time are summarized in Chart 1.

Determination of the Complete Oligosaccharide Structure Employing Exoglycosidase, CID, and Structure Homology. The ability to elucidate oligosaccharide structures with exoglycosidases depends on the ability of enzymes to specifically remove the terminal monosaccharide from the nonreducing end. The glycosidase reactions are sensitive to the monosaccharide, the linkage, and the anomeric configuration. The most commonly used procedure employs glycosidase digestion either in arrays[10] or sequentially[11] with the products monitored by MALDI-MS. However, these methods require splitting the samples for the array, which either decreases the overall sensitivity or increases significantly the cost. More critically, the large diversity of O-linked structures as compared to N-linked makes these approaches prohibitively time-consuming and expensive. In this study, small oligosaccharides were elucidated first and their CID spectra were used as references to compare with exoglycosidase products of large oligosaccharides. Specific exoglycosidase were chosen based on the connectivity shown in Chart 1. MALDI-FTICR MS analyses were used to monitor the resulting enzymatic products. Tandem MS experiments were performed on the exoglycosidase products, which were isomeric to those previously elucidated structures, allowing direct comparison of the CID spectra. Thus, a series of reference compounds and their corresponding CID spectra can be built up and used to elucidate the complete structures of unknown oligosaccharides.

This strategy is first illustrated with XT-611-1 (Chart 1), which is identical in mass to a known trisaccharide core from *X. laevis*.[7,8] The CID of XT-611-1 can be compared directly to the known *X. laevis* compound.[7,8] Because the CID mass spectrum of this compound (data not shown) is identical to that of known oligosaccharide XL-611-1 (please refer to Figure 2a in ref 7), the structure is therefore Gal(β1−3)[GlcNAc(β1−6)]GalNAc-ol. This structure is also the most common core structure for mucin-type *O*-glycans from the jelly coat of amphibians.[34]

The second structure XT-757-1 is a tetrasaccharide composed of a fucose connected to the β-Gal of the above core. This compound has a fragmentation pattern identical to that of XL-757-1 found in *X. laevis*, which consists of a fucose α1−2 linked to β-Gal (please refer to Figure 8b in ref 7). This compound was further digested with a α1−2 fucosidase, resulting in a loss of Fuc (146 amu), confirming its structure (data not shown).

The third structure XT-773-1 is also a tetrasaccharide, a Hex connected to the β-GlcNAc of the trisaccharide core. To assign the terminal Hex, galactosidases (α and β) were chosen to cleave the hexose residue. The mass spectra yielded (data not shown) the loss of Hex (162 amu) from the quasimolecular ions after treatment with a nonspecific β-galactosidase. The exact linkage was determined after the compound was reacted with the β1−3 galactosidase (shown in Figure 5 a). The ion at m/z 682.3 is an impurity from the enzyme but does not correspond to an oligosaccharide. Figure 5b is the CID spectrum of XT-773-1. As this structure is incorporated in large oligosaccharides, this spectrum will be used later for comparison.

The compound XT-919-1 was found via CID to be composed of a Fuc-Hex combination attached to the core structure through the β-GlcNAc residue (inset in Figure 6a). To assign the linkage of the terminal Fuc, three α-fucosidases including α1−2, α1−6, and α1−3,4 were added sequentially to the HPLC fraction. There was no reaction when the first two were added. When the α1−3,4 fucosidase was added, the resulting MALDI-FTICR mass spectrum (Figure 6a) indicated a loss of the fucose residue. The subsequent product of the fucosidase reaction was identical in mass to XT-773-1. To determine whether the product was structurally identical to XT-773-1, CID of the two were compared (Figure 6b and Figure 5b). The CID spectra yielded identical products in nearly approximately the same abundances, indicating that the two species are identical. To verify the structure, the glycosidase reaction product was further reacted with β1−3 galactosidase to yield the spectrum in Figure 6c. The loss of Hex confirmed the structure inset in Figure 6c. The structure XT-773-1 is a common motif for the oligosaccharides in *X. tropicalis*. In subsequent

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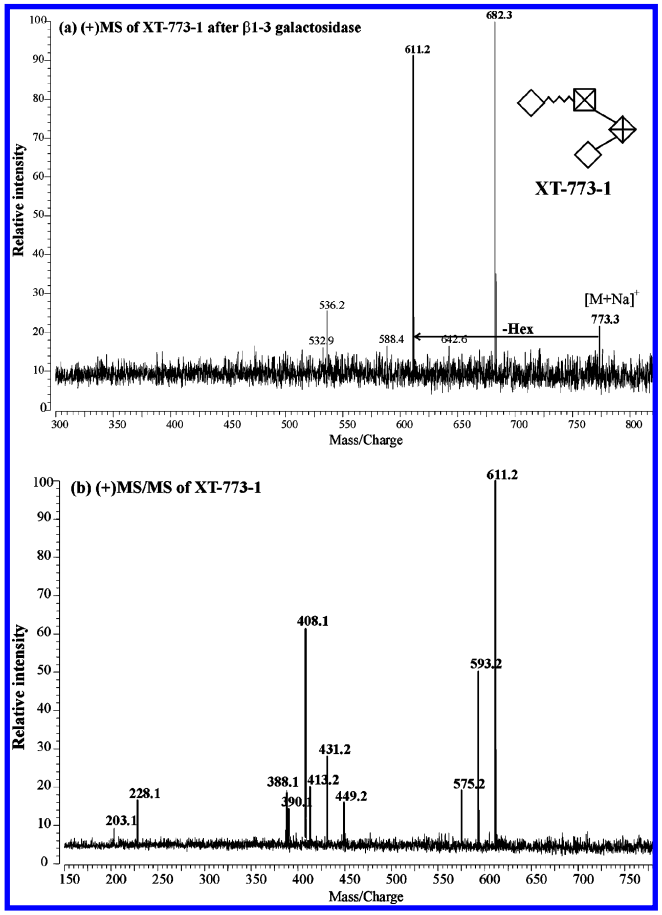


Figure 5. (a) MALDI-FTICR mass spectrum of XT-773-1 after treatment with β1−3 galactosidase. A loss of a galactose is observed. (b) CID spectrum of XT-773-1.

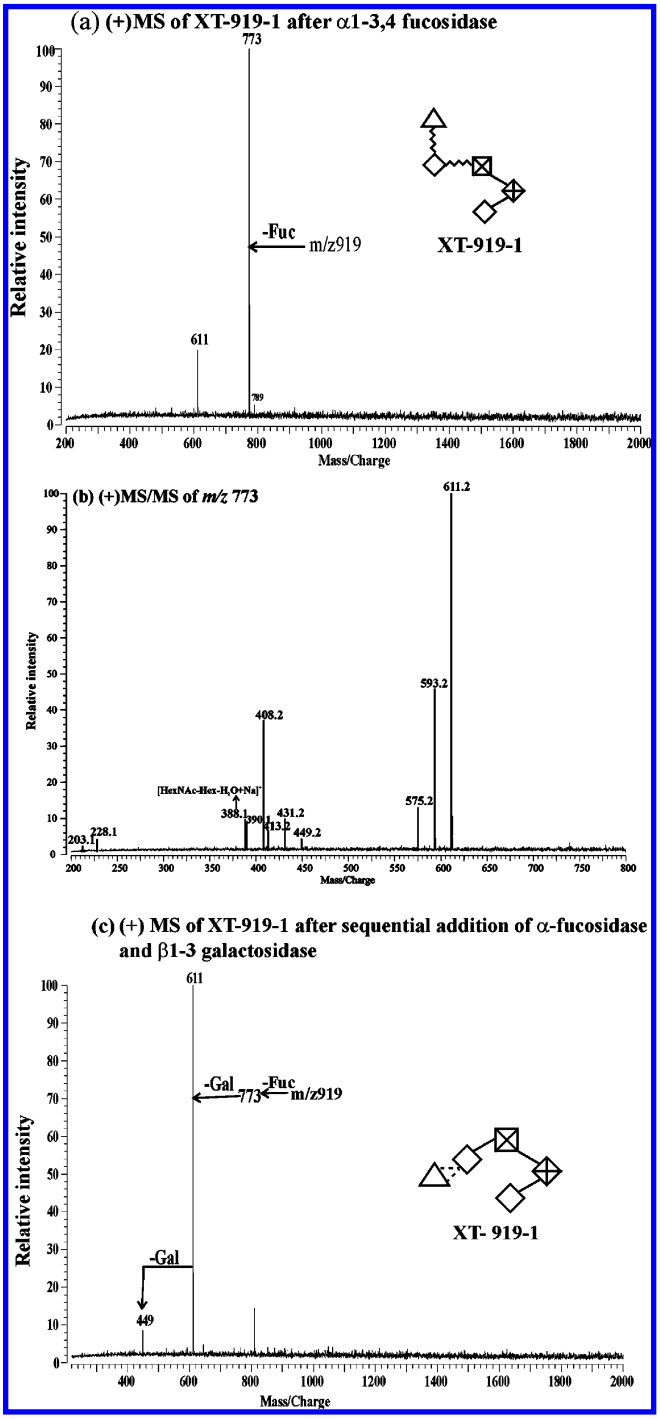


Figure 6. (a) MALDI-FTICR mass spectrum of XT-919-1 (sequence inset) after treatment with α1−3,4 fucosidase. A loss of fucose is observed. (b) CID spectrum of resulting *m*/*z* 773 product. The fragmentation pattern is identical to that of XT-773-1. (c) β1−3 galactosidase after α-fucosidase. The loss of galactose from *m*/*z* 773 confirms the structure (inset).

compounds, its presence was identified based solely on the CID spectrum.

The compound elucidated in Figure 7 illustrates potential pitfalls of employing only glycosidase reactions. The compound, XT-960-1 contains a HexNAc and a Fuc attached to the Gal(β1−3) of the core as shown (inset) in Figure 7a. Reaction with a nonspecific α-*N*-acetylgalactosaminidase yielded the loss of the HexNAc residue (Figure 7a), identifying the residue. However, there are no linkage-specific α-*N*-acetylgalactosaminidases commercially available so that the linkage of this residue could not be determined. The resulting product, however, was similar to XT-757-1. CID of the product (Figure 7b) yielded a spectrum that was identical to that of XT-757-1. Interestingly, the reaction of XT-960-1 with fucosidase did not yield the expected product. However, when the terminal GalNAc was removed, the reaction with the α1−2 fucosidase yielded the appropriate product (Figure 7c). Apparently, the presence of the neighboring GalNAc prevented the reaction of the fucose residue connected to an internal residue. It is therefore advantageous to employ CID with the glycosidase reaction.

XT-1268-2 is the most abundant species with two fucoses, one connected to the Hex and another to the HexNAc of the core structure (inset in Figure 8a). This compound reacted with α-*N*-acetylgalactosaminidase to yield the reaction product shown in Figure 8a. Removal of the GalNAc and reaction with α-fucosidase yielded the product shown in Figure 8b. This product has the same mass as XT-773-1. CID of this product yielded a spectrum identical to that of XT-773-1 (Figure 5b). The structure based on these results is inset in Figure 8c. As an additional note, one of the α-fucose moieties was determined by exoglycosidase reaction as being α1−2 (data not shown) and the other as either α1−6 or α1−3,4. The ambiguity stems from fact that a limited number of specific α-fucosidases are commercially available.

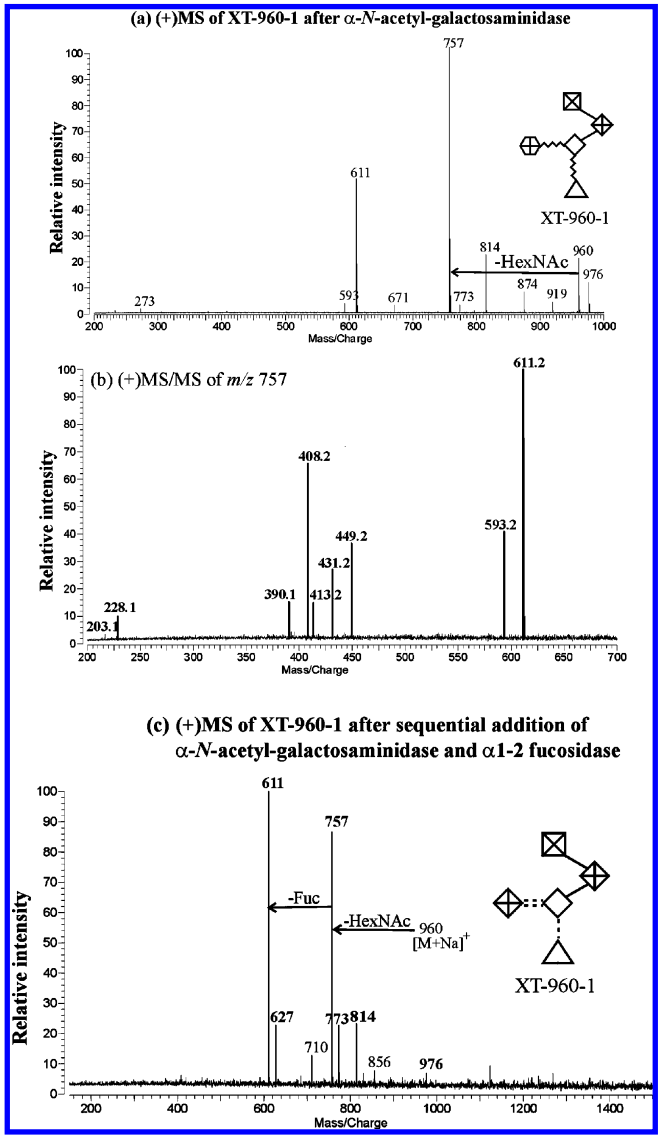


Figure 7. (a) MALDI-FTICR mass spectrum of XT-960-1 (sequence inset) after treatment with α-*N*-acetylgalactosaminidase. A loss of HexNAc is observed. (b) CID spectrum of resulting *m*/*z* 757 product. The fragmentation pattern is identical to that of XT-757-1. (c) A loss of fucose from *m*/*z* 757 after α1−2 fucosidase digestion confirms the inset structure.

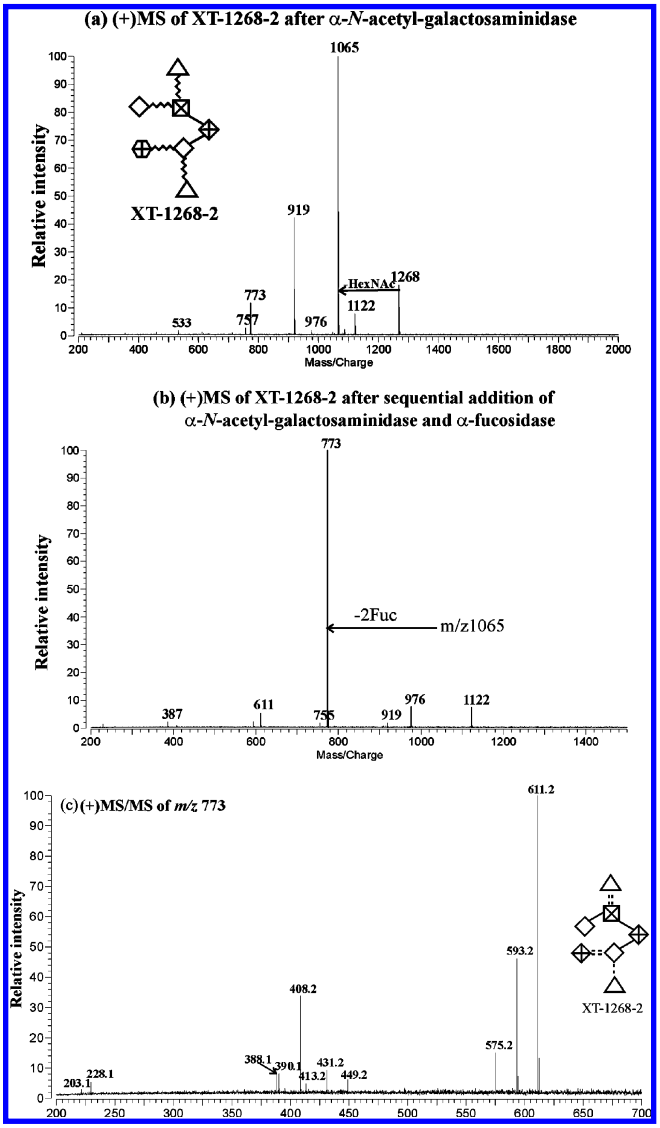


Figure 8. (a) MALDI-FTICR mass spectrum of XT-1268-2 (sequence inset) after treatment with α-*N*-acetylgalactosaminidase. A loss of HexNAc is observed. (b) After addition of α-fucosidase to the mixture, two fucoses are lost. (c) CID spectrum of resulting *m*/*z* 773 product. The fragmentation pattern is identical to that of XT-773-1. The proposed structure is shown.

A total of 16 neutral oligosaccharide structures were determined in the above manner. There were some structural ambiguities in the assignments due to the absence of specific exoglycosidases. The structures and relative abundances based on HPLC responses are listed in Chart 2. The relative abundances were measured with an array detector set to 206 nm. This wavelength corresponds to the absorption of the *N*-acetyl group. There is a general relationship, though not linear, between the absorptivity and the number of HexNAc residues. The values were corrected based on standard solutions of model compounds.[62]

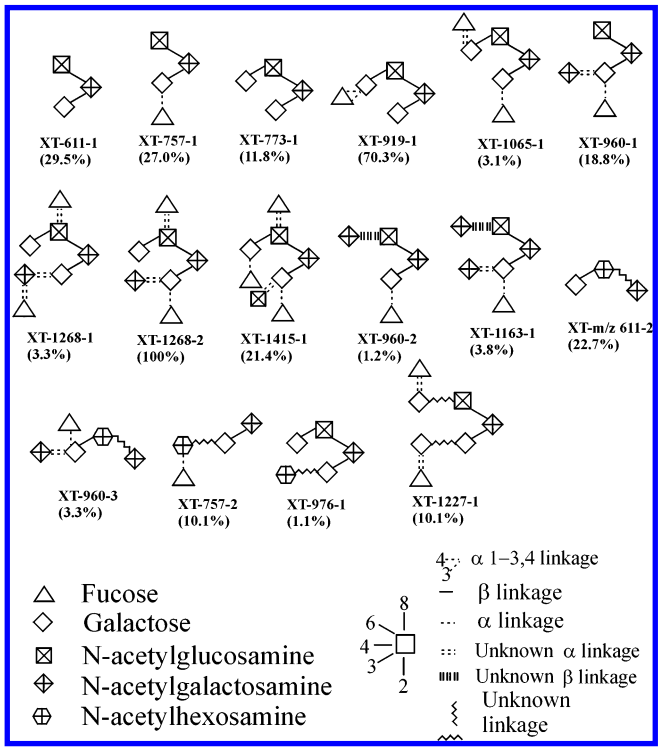
At least 13 distinct sulfated oligosaccharides were obtained in the egg jelly of *X. tropicalis*. Compared to *X. tropicalis*, the diversity of sulfated oligosaccharides from *X. laevis* was found to be much greater.[8] Complete structures of six sulfated oligosaccharides from *X. laevis* were determined previously by NMR,[34] all with a common trisaccharide core corresponding to Gal(β1−3)[(SO_3-6)GlcNAc-(β1−6)]GalNAc-ol (the designation indicates a sulfate ester group located at the C-6 position of the GlcNAc residue). Tandem MS analyses employing CID was performed on all six oligosaccharides, and a characteristic fragmentation of the core was obtained consisting of the fragment ions at *m*/*z* 667, 505 ([SO_3-HexNAc-HexNAc-ol]$^-$), and 282 ([HexNAc − SO_3]$^-$). CID analyses of oligosaccharides in *X. tropicalis* revealed that all but two sulfated species contained this core. An example demonstrating the characteristic signals of the sulfated core is shown in the CID spectrum of XT-1016-1 in Figure 9a (sequence inset).

The structure of XT-1219-1 was determined based on the comparison of the CID data with a known structure from *X. laevis* previously determined by NMR.[34] The two compounds had identical CID spectra. The structure of XT-1219-1 is provided in Chart 3.

The structure of XT-667-1 corresponds to the basic core Gal-(β1−3)[(SO_3-6)GlcNAc(β1−6)]GalNAc-ol with a sulfate group

(62) Zhang, J.; Xie, Y.; Hedrick, J. L.; Lebrilla C. B., 2004.

Chart 2. Neutral Oligosaccharide Structures[a]



[a] Relative abundances (in parentheses) are based on the HPLC peak area ratio relative to the most abundant compound XT-1268-2.

attached to the GlcNAc residue. The exact mass and the presence of the diagnostic peaks in CID spectra all point to this structure. The compound XT-813-1 has an additional fucose attached to the hexose (Gal) that was assigned as α1−2 after fucosidase digestion (data not shown). XT-1016-1 was a compound one HexNAc larger than XT-813-1. Exoglycosidase reaction determined this structure to be an α-GalNAc (Figure 9b). The CID spectrum of the product *m*/*z* 813 is shown in Figure 9c. This spectrum is identical to that of XT-813-1 (data not shown). Unfortunately, due to the lack of linkage-specific α-GalNAc exoglycosidases, the exact linkage could not be determined.

Other sulfated compounds including XT-1121-1, XT-1178-1, and XT-1528-1 were determined in this manner. Glycosidase was used to determine a new connection, and CID was used on the glycosidase product to determine whether the rest of the structure corresponds to a known smaller structure. It should be noted that the presence of the terminal GalNAc in XT-1528-1 hindered the enzymatic cleavage of the two fucoses. Glycosidase cleavage could be obtained when the GalNAc terminus was removed. There were larger compounds whose core structures were identified, but the complete structures could not be due to either limitation on amounts of compounds, lack of exoglycosidase, or both. These compounds are shown with their rudimentary structure in Chart 1.

The two compounds with atypical core structures were XT-1121-2 and XT-975-1 (Chart 1). With XT-1121-2, the sulfate was bound to the *β*-Gal residue of the trisaccharide core. Because there are two common sulfotransferases acting on mucin-type oligosaccharides,[63] which transfer sulfate from 3′-phosphoadenosine 5′-

(63) Brockhausen, I. *Biochem. Soc. Trans.* **2003**, *31* (2), 318−325.

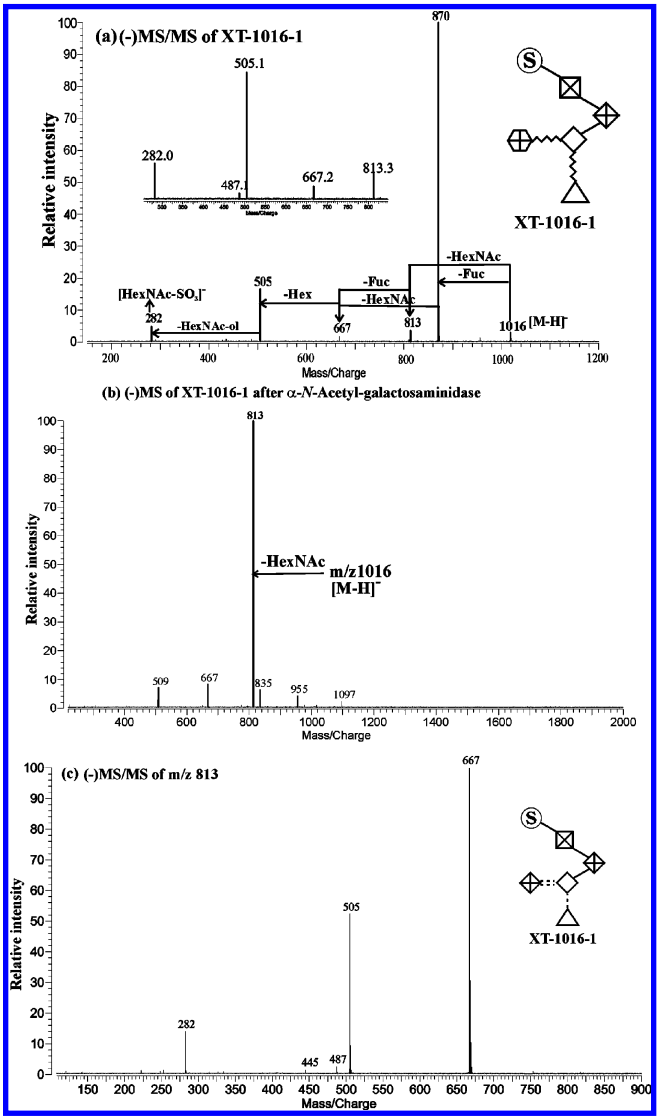
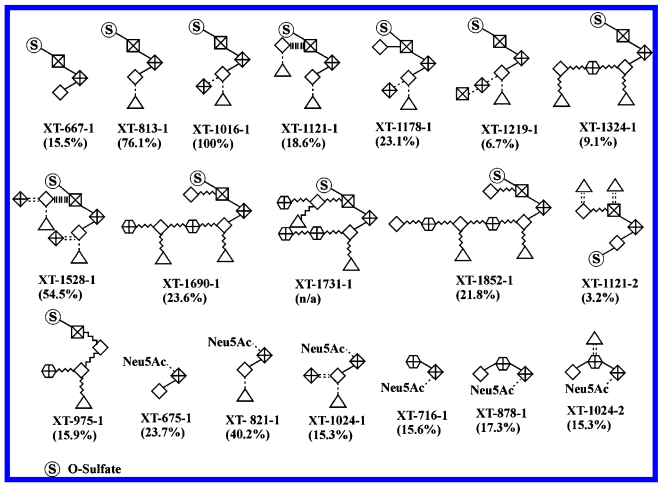


Figure 9. (a) CID spectrum of sulfated oligosaccharide XT-1016-1 (sequence inset). (b) MALDI-FTICR mass spectrum of compound after treatment with α-*N*-acetylgalactosaminidase. A loss of HexNAc is observed. (c) MS/MS of resulting *m*/*z* 813 product. The CID data are identical to that of XT-813-1. The proposed structure is shown.

phosphosulfate to either the 3-position of Gal (Gal3ST) or the 6-position of GlcNAc(GlcNAc6ST), the sulfate can be putatively assigned as being on the 3-position of *β*-Gal. All of anionic oligosaccharide structures and the relative abundance based on their HPLC peak area are listed in Chart 3. The structure of XT-975 has not yet been assigned. It contains a very unusual Gal core.

Only one sialylated oligosaccharide has been elucidated from egg jelly of *X. laevis*.[34] This oligosaccharide has a Neu5Ac connected to the GalNAc reducing end through an α2−6 linkage. The sialylated oligosaccharides found in egg jelly of *X. tropicalis* were more diverse and abundant. The Neu5Ac residues of all six sialylated oligosaccharides found in *X. tropicalis* were cleaved after digestion with either α2−6 or α2−3 neuraminidase. An example is shown in Figure 10a with XT-1024-1 reacted with α2−6 neuraminidase. The ion at *m*/*z* 757 is the sodiated product after loss of Neu5Ac. The six sialylated oligosaccharides could be classified into two groups. One included three oligosaccharide structures (XT-716-1, XT-878-1, XT-1024-2), where a HexNAc and

Chart 3. Anionic Oligosaccharide Structures[a]



[a] Relative abundances (parentheses) based on the HPLC peak area ratio relative to the most abundant compound XT-1016-1.

a neuraminic acid residue were attached directly to the GalNAc core residue. α2−3 neuraminidase cleaved the respective residue, which meant the unidentified HexNAc residue was attached to the 6-position, the only other site available on the GalNAc core. After removal of sialic acid of XT-878-1 by α2−3 neuraminidase, the positive mode MALDI-FTICR MS yielded the sodiated peak at *m*/*z* 611 (not shown). Tandem MS of *m*/*z* 611 (data not shown) yielded the same fragmentation pattern as the neutral oligosaccharide XT-611-2, where a *β*-Gal is connected to the HexNAc-GalNAc-ol core through the 1−3 site. Therefore, the structure of XT-878-1 was assigned as shown in Chart 3. The same motif is also present in compound XT-1024-2. This novel motif Gal(*β*1−3)HexNAc(α, *β*1−6)[Neu5Ac(α2−3)]GalNAc-ol appears specific to *X. tropicalis*.

The other group of sialylated structures included three structures (XT-675-1, XT-821-1, XT-1024-1), where a Neu5Ac and a Hex were attached directly to the GalNAc core. Again, glycosidases were useful for determining fine structure. For example, XT-1024-1, after treatment with α2−6 neuraminidase (vide supra, *m*/*z* 757 Figure 10a), was treated with α-*N*-acetylgalactosaminidase digestion to yield *m*/*z* 554 (Figure 10b), indicating that the terminal residue was an α-GalNAc. Subsequent treatment of the product with α1−2 fucosidase yielded the product *m*/*z* 408 (Figure 10c). As discussed above, the Hex-GalNAc-ol core is exclusively Gal(*β*1−3)GalNAc-ol in oligosaccharides of amphibian eggs. It was found that the sialic acid creates steric hindrance to both GalNAc and fucose exoglycosidases. The reaction of both α-*N*-acetylgalactosaminidase and α1−2 fucosidase were only observed after the removal of sialic acid.

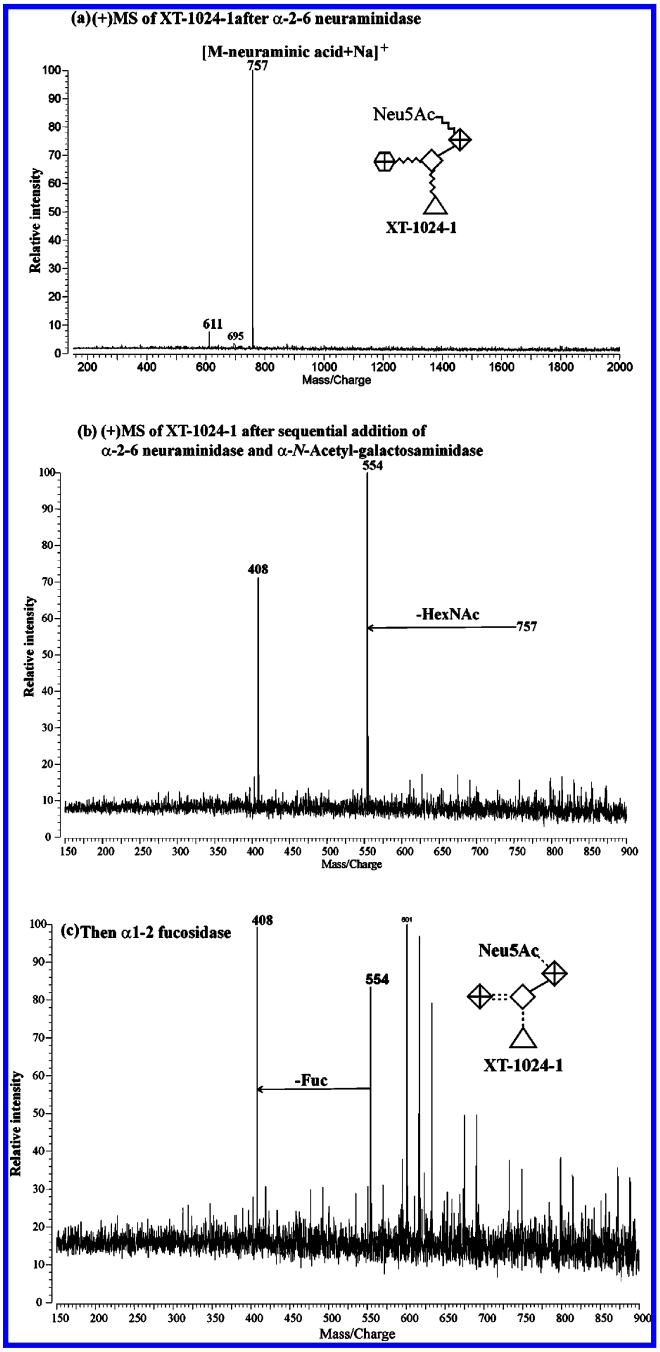


Figure 10. (a) Positive mode MALDI-FTICR mass spectrum of sialylated oligosaccharide XT-1024-1 (sequence inset) after treatment with α-2−6 neuraminidase. The peak at *m*/*z* 757 corresponds to the sodiated product after loss of sialic acid. (b) α-*N*-Acetylgalactosaminidase is added to the reaction mixture yielding the loss of a HexNAc. (c) The reaction mixture is treated with additional α1−2 fucosidase yielding a loss of fucose. The proposed structure is inset.

DISCUSSION

The comparison of *X. laevis* and *tropicalis* is informative. We find significantly fewer oligosaccharides with the diploid *X. tropicalis* compared to *laevis*. For example, we found 16 neutral structures for *tropicalis*, while we found over 30 for *laevis*. We found over 60 sulfated structures in *laevis* compared to 13 for *tropicalis*. *Tropicalis* is also dominated by a small number of highly abundant species. The neutral oligosaccharide XT-1268-2 is the most abundant (100%) followed by XT-919-1 with 70.3%. All other structures have abundances of 30% or less of the most abundant species (100%). Similarly for the anionic oligosaccharides, one compound, XT-1016-1, is the most abundant followed by XT-813-1 with 76% and XT-1528-1 with 54.5%. All others have abundances less than 40% of the most abundant species.

The core structure as defined by XL-611-1 is common to most of the oligosaccharides found in *laevis* and *tropicalis*.[8,34] However, the oligosaccharides are distinct. Among the neutral oligosaccharides, only two structures are common. While among the anionic

compounds, four structures are common. The variation in oligosaccharide structures may explain the low rate of cross-fertilization. Strecker et al.[9,34−47] using NMR analyses have determined the structures of mucin-type O-linked oligosaccharides released from 15 different amphibian egg jelly coats. Their studies also show that the ligosaccharides were highly specific to each amphibian species.

When this paper was in preparation, a report on the oligosaccharide determination from *X. tropicalis* egg jelly by NMR was published by Strecker et al.[64] In that study, eight neutral, one sulfated, and three sialylated oligosaccharides were identified. In this study, 16 neutral, 13 sulfated, and 6 sialylated oligosaccharides were found. The common structures found in both studies include XT-611-1, XT-757-1, XT-960-1, XT-919-1, XT-1016-1, and XT-675-1.

CONCLUSIONS

A rapid and sensitive structural elucidation approach for O-linked oligosaccharides is illustrated. Unlike N-linked, O-linked oligosaccharides possess a high level of structural diversity in core structure, monosaccharide composition, and connectivity. Consequently, enzyme sequencing for O-linked oligosaccharide is significantly more complicated than for the N-linked glycans. In this report, targeted exoglycosidase reactions were applied sequentially based on the preliminary structures obtained by tandem mass spectrometry. CID fragmentation patterns of the isomeric structures were then compared to previously determined structures. Because all of the enzyme reactions were performed in a single vial and the analyte consumption for MALDI-MS analysis was minimal, sensitivity was highly improved. Most importantly, only a small number of enzymes were needed to obtain complete structure information in a short time. This strategy is applicable to most O-linked oligosaccharides, particularly those found in mucins.

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ACKNOWLEDGMENT

Funding provided by the National Institute of Health is greatly acknowledged.

Received for review March 2, 2004. Accepted July 21, 2004.

AC049666S