Targeted Use of Exoglycosidase Digestion for the Structural Elucidation of Neutral O-Linked Oligosaccharides

Yongming Xie, Ken Tseng, Carlito B. Lebrilla
Department of Chemistry, University of California, Davis, California, USA

Jerry L. Hedrick
Section of Molecular and Cellular Biology, University of California, Davis, California, USA

Exoglycosidase digestion in combination with the catalog-library approach (CLA) is used with matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) to obtain the complete structure of oligosaccharides. The CLA is a collision-induced dissociation (CID)-based method used to determine the structure of O-linked neutral oligosaccharides. It provides both linkage and stereochemical information. Exoglycosidases are used to confirm independently the validity of the CLA. In some cases, the CLA provides structural information on all but a single residue. Exoglycosidase is used to refine these structures. In this way, exoglycosidase use is targeted employing only a small number of enzymes. Exoglycosidase arrays, which have been used with N-linked oligosaccharides, is avoided despite the larger variations in structures of O-linked species. (J Am Soc Mass Spectrom 2001, 12, 877–884) © 2001 American Society for Mass Spectrometry

Oligosaccharides, as glycoconjugates, are widely distributed in nature and play key roles in several fundamental cellular processes [1]. Their relative importance in cellular recognition has spurred the development of a large number of methods for structural elucidation. Despite the significant efforts in their developments however, no single method has emerged. The problem lies in the complexity of oligosaccharides. The variations in connectivity, the number of different linkage combinations, the possibility of branching, and the stereochemistry of each monosaccharide unit all conspire to produce major challenges in structural elucidation. Moreover, the large number of oligosaccharides and the variations in their structures from even a single source produce an often intractable problem that has hindered the study of structure–function relationships in oligosaccharides.

Mass spectrometry offers an attractive method for structural elucidation as it is unsurpassed in both speed and sensitivity. Mass spectrometry has long been used to obtain the composition and the connectivity of unknown oligosaccharides. Several studies have been published where mass spectrometry, and specifically CID was employed to obtain linkage information [2–7]. There have even been efforts towards obtaining stereochemistry with CID [8]. These latter studies have focused on monosaccharides but they may be extended to larger oligomers.

Until recently, the use of mass spectrometry to determine the complete structure was still not possible. In a preceding publication, we described a mass spectrometry (MS) based method to obtain the structure of oligosaccharides complete with linkage, stereochemistry, and anomericity [9]. The method was used to profile most of the O-linked neutral oligosaccharide components in a library released from the jelly coat of the *Xenopus laevis* egg [10]. This frog is a common biological model for the study of reproduction [11–13]. Oligosaccharides released from this source were found to have several substructural motifs in common (Chart **1**). Even the small number of motifs combine to produce large variation in structures. The motifs also yield distinct fragmentation patterns under CID that are preserved from one compound to the next. These patterns are sensitive to the linkage and even the stereochemistry of the substructural unit. The determination of new structures requires the identification of various patterns corresponding to substructures in the spectra. Unknown structures can be deduced by recombining various motifs found in the CID spectra (Scheme **1**). The key to this method is to identify the substructural motifs and their corresponding CID spectra to develop a catalog for the library [9]. For this reason the method was named the catalog-library approach (CLA). CLA

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Address reprint requests to Dr. C. B. Lebrilla, Department of Chemistry, University of California, Davis, CA 95616. E-mail: cblebrilla@ucdavis.edu

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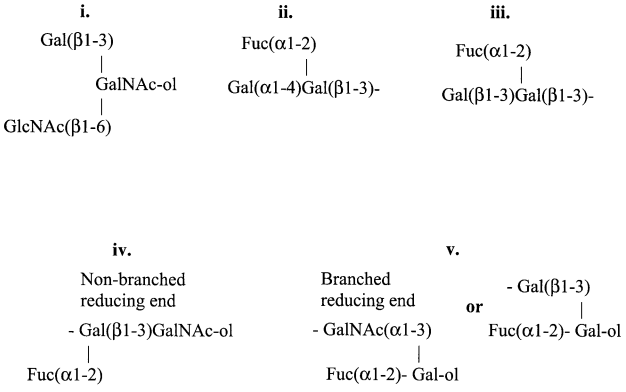


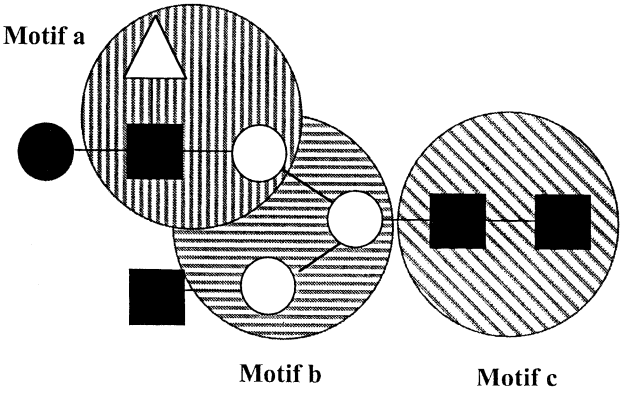
Chart 1

was used to determine the structures of twelve new oligosaccharides in *Xenopus laevis* [9].

Although the CLA was tested with several known compounds, the structures of the unknowns could not be independently verified. Additionally, limitations in material or the lack of fragments in the CID spectra yielded only partial structures with the linkage or the identity of residue not properly identified.

In this investigation, enzymatic degradation was used to verify and refine the structures obtained by CLA. The combination of exoglycosidases and CLA produces a method capable of obtaining the complete structure of oligosaccharides in a relatively short period (hours) with small amounts of material (picomoles). Exoglycosidases produce highly specific glycosidic bond cleavages that are sensitive to the linkage, the stereochemistry, and even the configuration of the anomeric carbon. This method involves digestion of the oligosaccharide with defined exoglycosidases, followed by analysis of the digestion products by MALDI-FTMS analysis. The sequence of the oligosaccharide and the specific linkages between the individual sugar moieties are deduced from the pattern of observed carbohydrate fragments and the specificity of the enzymes.

It is feasible in some cases to use only exoglycosidase to sequence the entire oligosaccharide. This is particularly true for N-linked oligosaccharides where the number of residues and the variations in structures are relatively small. Indeed, specific procedures have been devised based on the enzymatic degradative method and mass spectrometry of N-linked oligosaccharides [14–18]. More recently, Mechref et al. [19], Colangelo et al. [20], and Geyer et al. [21] have illustrated exoglycosidase digestions followed directly on the MALDI-MS analysis. Orlando and co-workers optimized reaction conditions to allow incubation times of only a few mintes [18]. Zhao et al. reported the exoglycosidase digestion of synthetic glycopeptides [22].



Scheme 1

O-linked oligosaccharides have far more complex structures and exhibit far greater structural diversity than N-linked oligosaccharides. The large structural variations make it difficult to use only exoglycosidase arrays for the complete structural elucidation. There are also fewer specific exoglycosidases for O-linked oligosaccharides and inherent complications such as steric effects that prohibit the action of specific glycosidase (vide infra). Perhaps for these reasons, the use of exoglycosidase in conjunction with MS on O-linked oligosaccharide has not been well studied. To our best knowledge, there is only one other report that mentions the use of exoglycosidase for O-linked oligosaccharides [21].

Experimental

Materials

The exoglycosidases were purchased from Boerhinger Mannheim (Indianapolis, IN). Original enzyme suspensions from the company were stored at 4 °C. Just before use, 1μL of enzyme suspension was dialyzed against deionized water for 30 min. All the enzymes used in the study and their biological sources are listed in Table 1. The oligosaccharide standards were purchased from Oxford GlycoSciences (UK).

Oligosaccharide Release

The oligosaccharides were released and purified as described in detail in earlier publications [9, 23]. In brief, alkaline borohydride reduction followed by methanol distillation and Bio-Gel P2 column desalting procedure was utilized to release and purify the oligosaccharides from the *Xenopus laevis* egg jelly coats. The partially purified mixture of the oligosaccharides was then separated by high-performance liquid chromatography (HPLC) on a primary amine-bonded silica column (4.6 × 25.0 cm; Supelco, Bellefonte, PA) using aqueous acetonitrile gradient. The HPLC fractions were collected and analyzed by MALDI-FTMS.

Exoglycosidase Digestion

Buffer solutions for enzyme digestions were prepared by dissolving ammonium acetate in deionized water and adjusting to the desired pH by addition of glacial

Table 1. Specificity and optimal conditions of exoglycosidases

Glycosidase	Source	Specific for	pH*	Temperature
α-L-Fucosidase	bovine kidney	α(1-2,6)-fucose	7.0 (5.0)	40°C
α-Galactosidase	green coffee beans	α(1-3,4,6)-galactose	7.0 (7.2)	25°C
β-Galactosidase	bovine Testes	β-galactose	4.3	37°C
Sialidase (Neuraminidase)	*Clostridum perfringens*	α(2-3,6,8)-sialic Acid	5.0	37°C
N-Acetyl-β-D-glucosaminidase	*Diplococcus pneumoniae*	N-acetyl-β-D-glucosamine	5.0	40°C
N-Acetyl-β-D-hexosaminidase	bovine kidney	N-acetyl-β-D-hexosamine	4.5	37°C

* The pH values listed were found optimal in this study. The manufacturer prescribed pH is in parenthesis when different from the optimal conditions. pH 7.0 was achieved in deionized water while all the other pH values were achieved with 0.1M ammonium acetate buffer.

acetic acid. The final concentration of the buffer solution was 0.1 M. To perform exoglycosidase digestion of oligosaccharide standards, a 4 μL aliquot (1mg/mL) oligosaccharide solution was added to 4 μL deionized water or 0.1-M ammonium acetate buffer solution. The dialyzed enzyme solution was added into the oliogsaccharide solution and the mixture incubated at the specified temperature (Table 1). To monitor the enzymatic digestion, 1 μL aliquot of sample solution was removed at various reaction times and subjected to MALDI-FTMS analysis.

To perform exoglycosidase digestion of oligosaccharides released from egg jelly coats, an amount (microliters to hundreds of microliters depending on the concentration of the oligosaccharide) of the HPLC fraction that contained the target oligosaccharide(s) was dried by speed-vacuum centrifuge. It was then redissolved in 4 μL deionized water or 0.1 M ammonium acetate buffer, depending on the requirement of the exoglycosidase. The buffer solution was then combined with the dialyzed enzyme solution and incubated.

MALDI-FTMS Analysis

The MALDI-FTMS (IonSpec Corp., Irvine, CA) used in this study is equipped with an external MALDI source, a 4.7 tesla superconducting magnet, and a 337-nm nitrogen laser. The detailed description of this instrument is found in previous publications [24, 25]. To analyze the digestion products, 1 μL aliquot of sample solution was placed on the MALDI probe, and 1 μL of 0.01M NaCl was added to increase the Na^+ concentration and produce primarily sodiated species. One μL of 0.4 M 2,5-dihydroxybenzoic acid (DHB) was added as matrix before the analysis.

For neutral oligosaccharides, the MALDI-FTMS analyses were performed in the positive mode, as the compounds form strong sodium adducts during the MALDI process. Anionic oligosaccharides were analyzed in the negative mode as the deprotonated species produced abundant anionic signals. However, for the digestion of oligosaccharides containing sialic acid with sialidase, it was difficult to detect the anionic oligosaccharides and the neutral products simultaneously. Anionic oligosaccharides produce strong signals in the negative mode, while neutral oligosaccharides produce strong signals in the positive mode. Anionic dopants, such as sulfuric acid and sulfanilic acid, were therefore utilized to observe the neutral products in the negative mode [26, 27].

Results and Discussion

Optimization of Exoglycosidase Digestion Conditions

To determine the optimal conditions, the exoglycosidases were used on oligosaccharide standards first. For example, lacto-N-fucopentaose I (LNFP-I) was utilized to optimize the digestion conditions for α-fucosidase (bovine kidney), and lacto-N-fucopentaose V (LNFP-V) for β-galactosidase (bovine testes).

For compatibility with MALDI-FTMS, ammonium acetate buffers (0.1 M) were used instead of the recommended one. For example, the recommended pH 5.0 buffer for α-fucosidase (bovine kidney) is achieved in a sodium citrate buffer. However, this buffer produced severe chemical interferences in the MALDI-FTMS spectrum probably because of the clustering of sodium citrate molecules. Ammonium acetate produced favorable results because it produced volatile products during MALDI that did not interfere with the mass spectrometry. Activity as discussed in this report is based on the ability of the enzyme to produce the expected cleavage products (as monitored by mass spectrometry) in a reasonable amount of time. All the enzymes used in this study retained the desired activity. A pH 4.3 ammonium acetate buffer solution was found optimal for β-galactosidase. After only one hour incubation, LNFP-V (*m/z* 876, Figure 1a) was completely converted to the product, *m/z* 714, by losing a β-galactose group (162 mass units) (Figure 1b). The optimal digestion conditions of all the exoglycosidases used in this study are listed in Table 1. In some cases, deionized water was sufficient for the enzymatic digestion. Both α-fucosidase and α-galactosidase produced the desired cleavages in solutions with only deionized water.

Incubation temperature was found to be another important parameter for enzymatic digestion. Three

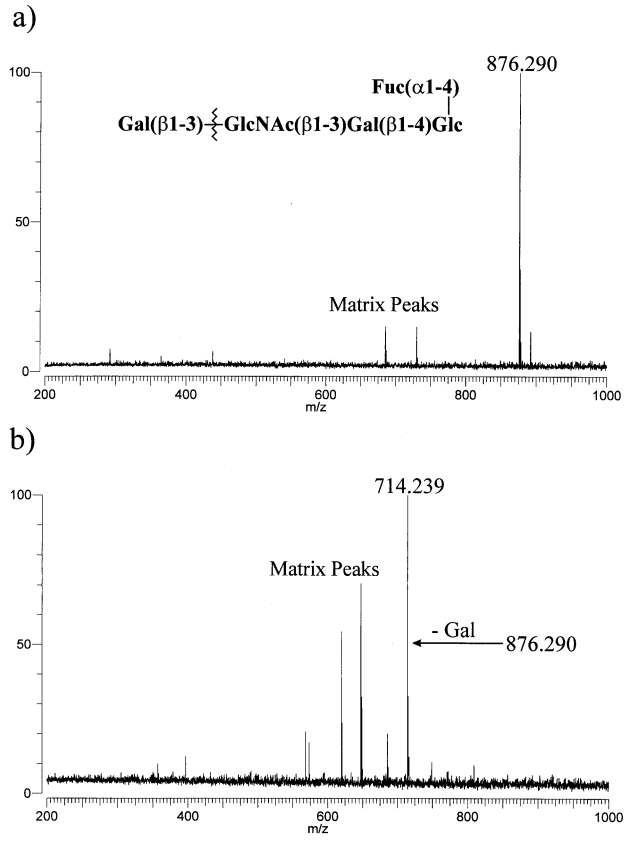


Figure 1. MALDI-FTMS spectrum of LNFP-V after addition of β-galactosidase (**a**) immediately and (**b**) after one hour. The loss of a galactose during digestion is consistent with the structure of LNFP-V.

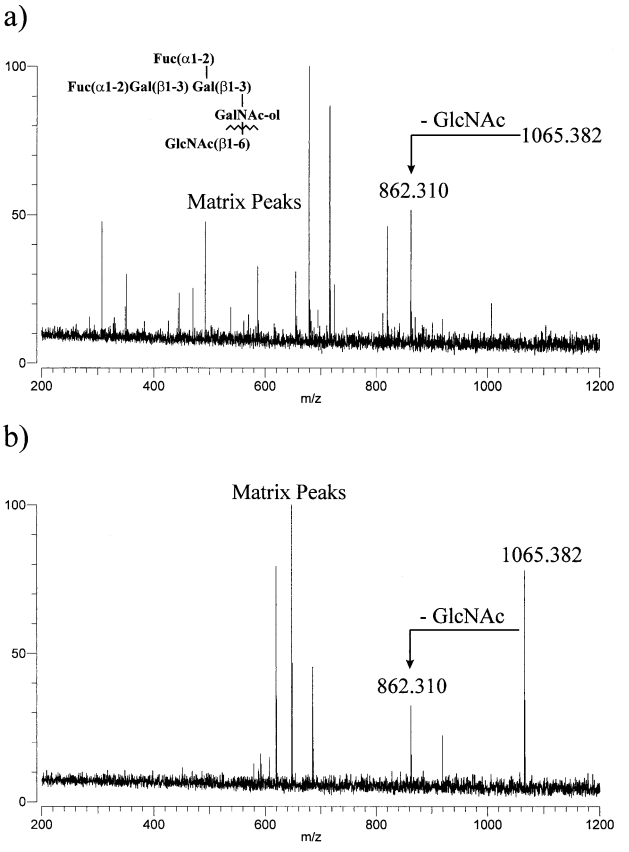


Figure 2. MALDI-FTMS spectrum of compound **XI** in digestion mixture with N-acetyl-β-D-glucosaminidase for four hours at (**a**) 40 °C and (**b**) 37 °C.

different incubation temperatures, room temperature (25–27 °C), 37 °C, and 40 °C were examined. In general, the prescribed temperature of 37 °C provided the suitable condition. For example, α-fucosidase was found to completely defucosylate LNFP-I at 37 °C after an incubation period of 20 hours in a water-only solution (data not shown). In some instances, other temperatures produced more satisfactory results. For example, α-galactosidase produced the best result at ambient temperature (25 °C). When the sample was incubated at 37 °C, the sample produced a mass spectrum with significant chemical interference and poor signal-to-noise. In other cases, increasing the temperature helped speed up the digestion without affecting the analysis. For example, N-acetyl-β-D-glucosaminidase fully converted compound **XI** (*m/z* 1065) to its product (*m/z* 862) at 40 °C within four hours (Figure 2a, *m/z* 1065→*m/z* 862), while the reaction at 37 °C of the same compound produced only partial digestion (Figure 2b). For further confirmation N-Acetyl-β-D-hexosaminidase was used. This enzyme provided the desired result but the reaction was significantly slower than the glucosaminidase.

LS tetrasaccharide a (LSTa), which has a sialic acid unit and appears as $[M-H^-]$ (*m/z* 997) in negative mode (Figure 3a), was digested with sialidase. After adding the sialidase a 1 μL aliquot of a sulfuric acid solution (0.01M) was added to the digest and the mixture analyzed by MALDI-FTMS in the negative mode. Both the deprotonated species (*m/z* 997) and the digestion product (*m/z* 804 $[M-NeuNAc+SO_3-H]^-$) were detected (Figure 3a). The *m/z* 804 corresponds to the LSTa minus the sialic acid residue plus $[HSO_3]^-$ as the anionic dopant. After one hour, the LSTa was completely converted into the trisaccharide compound that lacked the sialic acid residue (Figure 3b).

We did not observe any cleavage of β-galactose after incubating lacto-N-fucopentaose II (μLNFP-II) with β-galactosidase in pH 4.3 buffer solution for 24 hours (Figure 4a). It appeared that steric hindrance by an adjacent α-fucose prevented the β-galactosidase digestion. Indeed, if the α-fucose is first cleaved by α-fucosidase, the β-galactosidase was then able to cleave the β-galactose (Figure 4b). The same phenomenon was observed with other oligosaccharides containing similar structural arrangements. However, α-galactose is readily cleaved by α-galactosidase, whether or not an adjacent α-fucose is present. Since α-fucoses are often found on residues adjacent to β-galactoses in the egg jelly of *Xenopus laevis*, this reaction may provide a good diagnostic test for the presence of this structural motif.

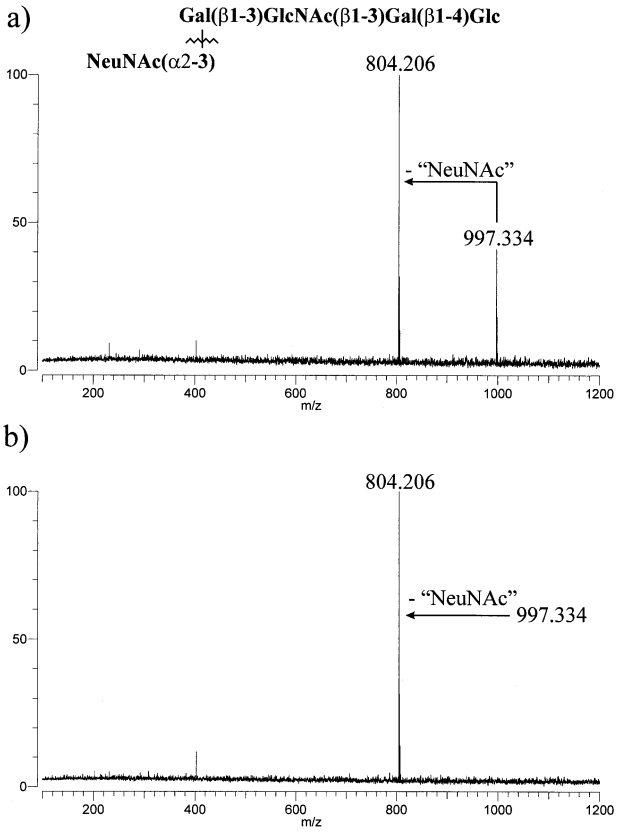


Figure 3. MALDI-FTMS spectrum in the negative mode of LSTa (a sialylated oligosaccharide) in the negative mode after digestion for (**a**) several min and (**b**) one hour. The quasimolecular ion is the deprotonated species while the product is obtained with a sulfate dopant to produce the sulfated complex.

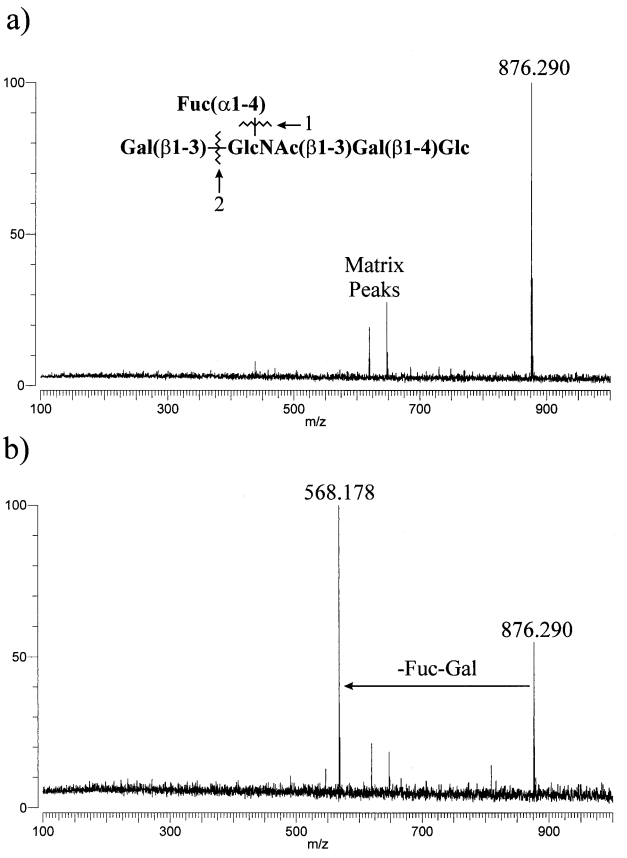


Figure 4. (**a**) MALDI-FTMS spectrum of LNFP-II in the presence of β-galactosidase after a 24-hour reaction period. No product is observed. (**b**) Same reaction mixture with α-fucosidase added. Losses of a fucose and a galactose are observed.

Confirmation of Oligosaccharide Structures Revealed by NMR

Twelve of the most abundant oligosaccharides were isolated and characterized using NMR by Strecker et al. [28]. The structures of a selected number of the twelve compounds characterized by Strecker were verified by exoglycosidase digestion (Chart **2**). Compound **VII** (*m/z* 919, Chart **2**) has a terminal α-galactose with a terminal α-fucose on the adjacent residue. (The compound designation in previous papers will be used in this report for the compound name [9, 28]. Treatment of **VII** with α-galactosidase produced the mass spectrum shown in Figure 5a. The most abundant fragment (*m/z* 757) corresponded to the loss of a galactose, presumably the terminal α-galactose. Subsequent addition of α-fucosidase to the same reaction mixture produced the loss of fucose (*m/z* 611, Figure 5b).

Compound **X** (*m/z* 919, Chart **2**), an isomer of compound **VII**, has a terminal β-galactose, but both have an α-fucose on an adjacent residue. Based on an earlier result, this compound should not yield products with β-galactosidase. Indeed, digestion of the compound with β-galactosidase for 48 hours did not produce digestion products (spectrum not shown). For completeness, the compound was also treated with α-galactosidase which produced no digestion products. Interestingly, treatment of the compound with α-fucosidase produced dissociation on only a small fraction of the compound. Only about 10% of the compound exhibited loss of fucose even after 48 hours (Figure 6a). Treatment of the compound with α-fucosidase and β-galactosidase produced the loss of two β-galactoses (Figure 6b). Even

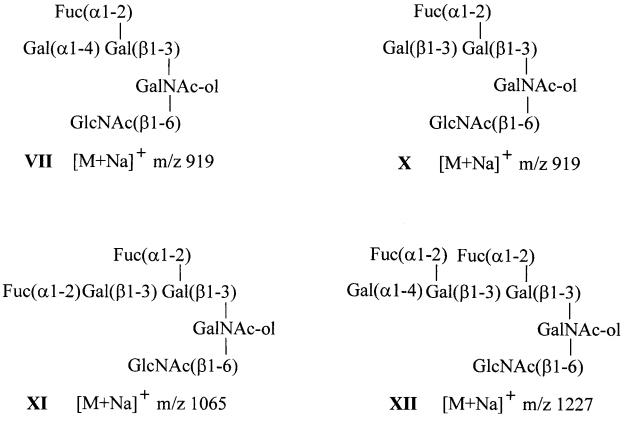


Chart 2

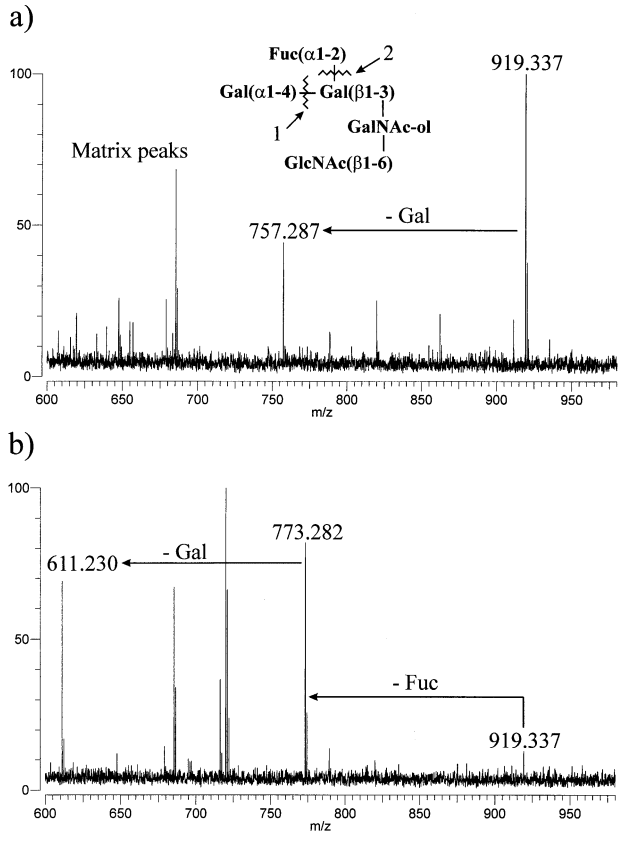


Figure 5. (**a**) MALDI-FTMS spectrum of compound **VII** treated with α-galactosidase. A loss of a galactose is observed. (**b**) α-Fucosidase added to the reaction mixture.

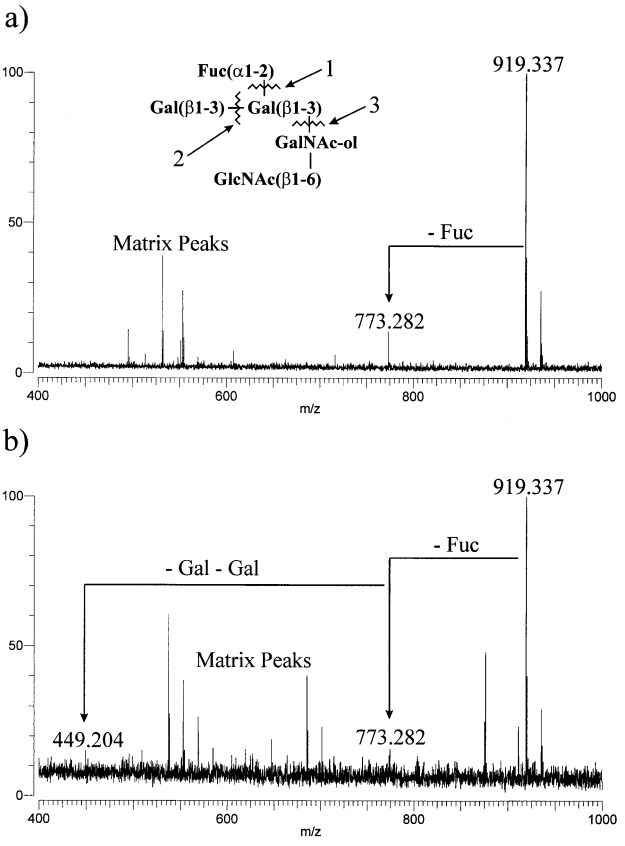


Figure 6. (**a**) MALDI-FTMS spectrum of compound **X** treated with β-galactosidase after 48 hours of reaction time. (**b**) The same reaction mixture treated with an additional α-fucosidase.

with the poor signal-to-noise ratio in Figure 6b, we are confident of the mass assignment. This reaction was repeated two additional times and the presence of *m/z* 449 is observed only after the β-galactosidase was added.

That α-fucosidase does not react effectively when the α-fucose is attached to a residue adjacent to the branched point was observed with other oligosaccharides. When **XI** (*m/z* 1065, Chart **2**) was digested with α-fucosidase at 40 °C for four hours, we observed the loss of one fucose group (Figure 7a) while only about 30% of the second fucose is cleaved. Even after incubation for an additional 16 hours, the relative abundance of the signal at *m/z* 773 was littled changed (Figure 7b). The digestion of compound **XII** (*m/z* 1227, Chart **2**) with α-fucosidase produced similar results (data not shown).

These results and those described earlier confirm the structures derived from NMR and also validate the use of glycosidases for O-linked oligosaccharides.

Validation and Refinement of Structures Obtained from CLA

Twelve new oligosaccharides from *Xenopus laevis* were structurally elucidated in our laboratory by the CLA [9]. In five of those structures, one residue could not be precisely located. Two of them (compound **1** and **2**) were found in the same HPLC fractions, (Chart **3**–initial structure). The corresponding quasimolecular ions had *m/z* 1373 and *m/z* 1389, respectively (Figure 8a). From the MS data, compound **1** was known to have three fucose units with the linkages and positions of two assigned by CLA. The position and the linkage of the third fucose could not be determined. Compound **2** was known to contain two fucose units with the position and linkage of only one determined by CLA. The identity and linkage of a hexose unit was also not determined, although it was determined that the hexose was attached to a GlcNAc group. For these two compounds, exoglycosidase digestion was used to validate the known parts of the structure and to refine the remainder.

Glycosidase digestions were performed directly on the mixture. Digestion with α-fucosidase for three hours showed that three and two fucose units were cleaved from **1** and **2**, respectively (Figure 8b). Therefore, all fucose units of both compounds were α-linked. Digestion of the mixture with β-galactosidase alone did not produce any digestion product even after a 24 hour reaction period. The incubation of the mixture with both with α-fucosidase and β-galactosidase simultaneously produced the same product as with the α-fu-

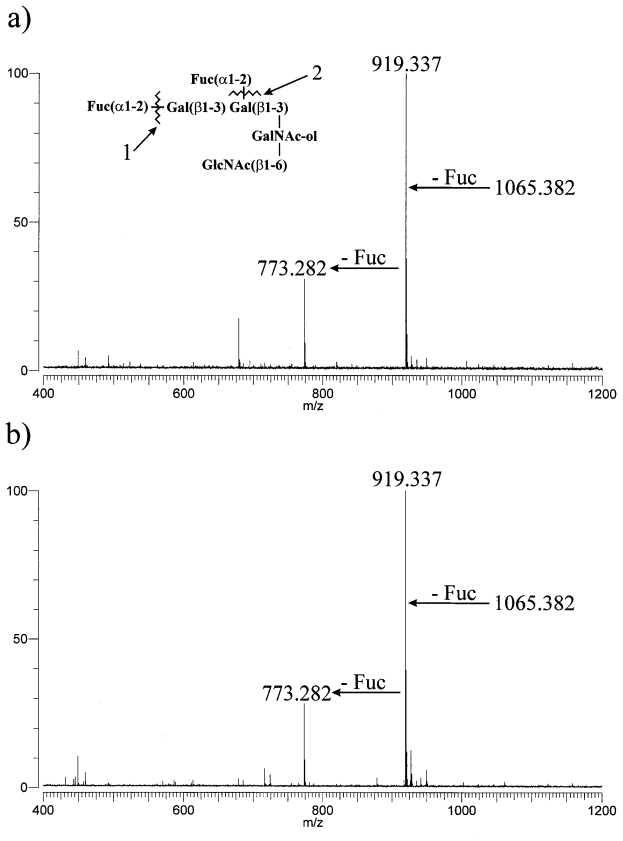


Figure 7. (**a**) MALDI-FTMS spectrum of compound **XI** treated with α-fucosidase at 40 °C for four hours. (**b**) After 16 hours of reaction time.

cosidase alone with no fragmentation due to losses of hexoses. We therefore concluded that both 1 and 2 did not contain terminal β-galactose.

Digestion of the mixture with α-galactose produced reaction products instantly (Figure 8c). Compound **1** yielded the loss of a single hexose unit (*m/z* 1211)

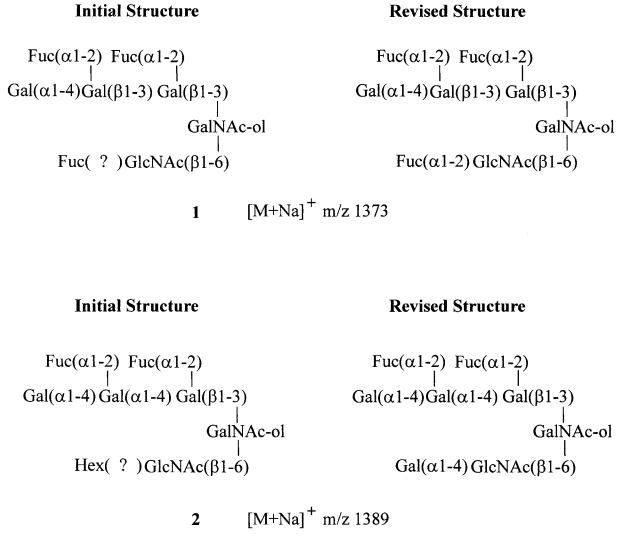


Chart 3

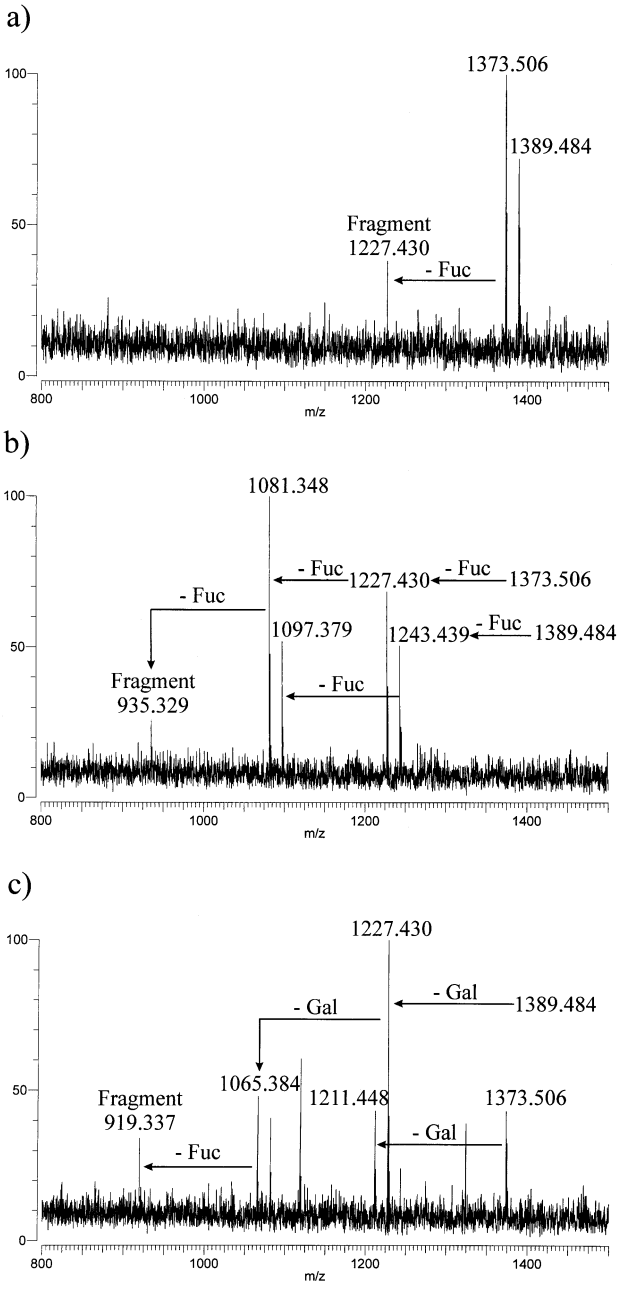


Figure 8. (**a**) MALDI-FTMS of HPLC fraction containing mixture of compounds **1** and **2** (*m/z* 1373 and 1389, respectively). Structures are presented in Chart **3**. (**b**) After treatment with α-fucosidase for three hours. (**c**) After addition of α-galactosidase to the mixture.

suggesting the presence of at least one α-galactose. This confirmed the linkage and the identity of the residue obtained via CLA. The reaction product also yielded the position of the third fucose group. The loss of a hexose would not be observed if the fucose were linked to the hexose. Therefore, the α-fucose was linked to the β-GlcNAc. In the same reaction mixture it was observed that the digestion of compound **2** yielded the loss of two hexose units. At least one originated from the terminal end of the long antenna indicating that the unknown hexose is an α-galactose. The cleavage of the α-galacto-

ses also confirms the position of the fucose residues. The glycosidase products are consistent with the assigned positions of the fucoses. They are not on the terminal α-galactoses as these would not have cleaved if the fucose residues where directly linked to them. Similarly, the presence of a fucose residue on the internal α-galactose prevented the loss of a third α-galactose from compound **2**. The revised structures are presented in **Chart 3**.

Conclusion

Exoglycosidase digestion was used to confirm the oligosaccharide structures obtained from the CLA. It was also used to refine the structure and remove structural ambiguities from the original CLA assignments. The use of glycosidase arrays was avoided. Although these arrays are sometimes used in conjuction with mass spectrometry to fully sequence N-linked oligosaccharides, they are not feasible with O-linked species. N-linked species have a small number of distinct monosaccharide units that can be readily examined with a correspondingly small number of glycosidases. O-linked oligosaccharides have no common core and may be composed of more different monosaccharide units. They do not exhibit the same type of structural similarities found in most N-linked species. For this reason, O-linked oligosaccharide libraries have greater heterogeneity and their structures have significantly greater diversity than the N-linked species.

The combination of exoglycosidases, CLA, and FTMS provides a powerful tool for the structural elucidation of oligosaccharides. In most cases, it yields the complete structure of the oligosaccharide. The sensitivity of this method has been evaluated based on known standards; compounds as little as a few picomoles may be analyzed by this method. At the moment the limiting step is the speed of the glycosidase reaction, however these procedures are still being optimized. Nonetheless, it is now possible to fully elucidate the structure of oligosaccharides in a matter of minutes or hours with as little as a few picomoles of material.

Acknowledgment

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