

Fig. 7. Product ion spectra of the doubly charged GPI-peptide at m/z 1051 (A), and at m/z 1151 (B) in peak A1-2. The inset is the deduced structure of the GPI-peptide, and the core structure of GPI is the inside dashed line. Man, mannose; HexNAc, *N*-acetylhexosamine; GlcNH₂, glucosamine; EtNH₂-P, phosphorylethanolamine; Ino-P, inositol-phosphate.

3.5. Analysis of Asp-N digested Thy-1

Glycopeptides obtained by Asp-N digestion were also localized by in-source CID using marker ions at m/z 204 and 292 (Fig. 6(D and E)), and neutral loss of 81 u by data-dependent CID-MS/MS (Fig. 6(F)). Product ion spectra of glycopeptides were sorted by using B series ions as marker ions from those acquired around localized elution positions. Consequently, peaks A2-7 were identified as those of glycopeptides (Fig. 6(A)). The oligosaccharide structures in the glycopeptides were then characterized based on their product ion spectra (Table 1). In addition to the high-mannose-type oligosaccharides, M5, 6, and 7 deduced by LC/MSⁿ of tryptic digests, the oligosaccharide at Asn23 was characterized

as dHex₀₋₁Hex_{3,5,6}HexNAc₃₋₅NeuAc_{0,1}, complex-type and hybrid-type oligosaccharides containing Lewis a/x or bisecting GlcNAc as a partial structure. Asn74 is attached by *N*-glycans with dHex₀₋₂Hex₃₋₆HexNAc_{2,4,5}NeuAc_{0,1}. They were high-mannose-type oligosaccharide, M5, complex-type oligosaccharides containing core Fuc and Lewis a/x as a partial structure, and hybrid-type oligosaccharides with core Fuc. Asn98 is occupied by high-mannose-type oligosaccharides, M5, and *N*-glycans with dHex₀₋₂Hex_{3,5,6}HexNAc₂₋₅NeuAc_{0,1}, hybrid-type oligosaccharides containing Lewis a/x or blood group H-determinant as a partial structure, which were found to be of greater diversity than those deduced by analysis of tryptic digests.

4. Discussion

In the present study, we have developed an efficient and convenient strategy for characterization, including protein identification and glycosylation analysis, of a small amount of unknown protein. We used gel electrophoresis, which is a powerful tool for separation of a small amount of protein from complex proteins mixture, especially from insoluble membrane fractions. For the complete glycosylation analysis, we examined the extraction of a whole glycoprotein from the gel, followed by trypsin digestion. Additionally, for the effective glycopeptide analysis, we studied mass spectrometric peptide/glycopeptide mapping by LC/MSⁿ with in-source CID and data-dependent MSⁿ. The glycopeptides were localized in the peptide/glycopeptide map by using oxonium ions as marker ions such as HexNAc⁺ and NeuAc⁺, which were generated by in-source CID, and neutral loss by data-dependent CID-MS/MS. For simultaneous identification of both peptides and glycopeptides, we conducted the database search analysis using search parameters containing a possible glycosylation at Asn with GlcNAc (203 Da). We successfully determined the sequences of peptides and some of the glycopeptides, which were localized by in-source CID and data-dependent CID-MSⁿ. The database search analysis using these search parameters was useful for identifying the glycopeptides resulting from predictable proteinase digestion. Glycopeptides caused by irregular digestion could be identified by assignment of peptide b and y series ions, which arose from further MSⁿ. The oligosaccharide structures of the identified glycopeptides were characterized on the basis of their product ion spectra. In this way, we were able to isolate rat brain Thy-1 and to elucidate *N*-glycosylation at Asn23, 74, and 98 as well as the structure of the GPIs at Cys111.

Post-translationally modified peptides could not be identified by the database search analysis. It has been particularly difficult to identify glycopeptides by database search analysis due to their complicated product ions resulting from the cleavage of glycosidic bonds. It has recently been reported that peptide + GlcNAc ion generated from a glycopeptide by CID-MS/MS yields b and y series ions by further MSⁿ, and that these ions can be utilized for identification of the peptide backbone and its glycosylation site [15,16,18]. Additionally, search analysis using the database including the possibility of glycosylation at Asn with all possible cleavage products of the known glycopeptides can be utilized for identification of glycopeptides in the peptide/glycopeptide map [19]. This ability would be helpful in the identification of glycoproteins whose glycosylation are already known. In the present study, we carried out a database search analysis using search parameters containing a possible glycosylation at Asn with only GlcNAc (203 Da), and successfully identified an unknown glycoprotein and *N*-glycosylated sites. This search analysis can be used for the identification of *O*-glycosylation, which has no consensus amino acid sequence, by using search parameters containing

possible glycosylations at Ser/Thr with Hex, HexNAc, and dHex.

Precursor ion scans have been used for the localization the glycopeptides in peptide/glycopeptide mapping [10,11,13]. Although this method can be used for monitoring the peptides with predictable modification by setting mass of fragment ions prior to scanning, peptides with unpredictable modification cannot be detected. In contrast, in-source CID and CID-MS/MS are capable of localizing of the modified peptides after just one data acquisition using objective oxonium ions and neutral losses. In the present study, we were able to localize GPI-peptides in the peptide/glycopeptide map using EtN-PO₄-Man⁺ and GlcN-Inositol-PO₄⁺ generated by in-source CID [25] and to elucidate the GPI structures. We also could localize the glycopeptides with dHex, HexNAc, and NeuAc at the non-reducing ends as well as Hex using neutral loss by CID-MS/MS.

Site-specific glycosylation analysis of rat brain Thy-1 was performed after purification with monoclonal antibody affinity chromatography. Released oligosaccharides from fractionated trypsin-digested glycopeptides were analyzed by conventional analytical methods, including exoglycosidase digestion and methylation analysis [26]. In the present study, we separated PIPLC-treated GPI-anchored proteins of rat brain by SDS-PAGE, and conducted site-specific glycosylation analysis by LC/MSⁿ. Using a simpler step, we could elucidate the glycosylation at each glycosylation site with a greater variety of oligosaccharides than that reported previously and four GPI structures, including two novel attached structures.

Our strategy presented herein can relatively simply facilitate complete site-specific glycosylation analysis that used to require a series of complicated steps and is applicable to characterization of unknown proteins on 2-DE gel in proteomic study. Even in a mixture of multiple unknown glycoproteins, glycosylation of each glycoprotein can be determined based on the product ion spectra. Our method would be helpful for study of the alternation of glycosylation with growth, aging, and disease [27,28].

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Core Research for the Evolutional Science and Technology Program (CREST) of the Japan Science and Technology Agency (JST), and Research on Health Science focusing on Drug Innovation from The Japan Health Science Foundation (N.K.).

We appreciate Dr. A. Hachisuka of the National Institute of Health Science for her technical advice.

We would also like to thank Dr. M. Kubota and Mr. M. Yoshida of Thermo Electron K.K. (Japan), for their technical support.

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Mass Spectrometry of Glycoproteins

糖タンパク質の質量分析

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Key Words: MS, MS/MS, MSⁿ, glycoprotein, oligosaccharide, glycopeptide

Abstract

This review presents mass spectrometric methods for glycoprotein identification, determination of glycosylation sites, structural elucidation of carbohydrates, and their applications to glycomics and proteomics.

要 約

本レビューでは、質量分析を用いた糖タンパク質同定、糖鎖結合位置の決定、糖鎖構造解析、及びグライコミクス・プロテオミクスへの応用について紹介する。

A. Introduction

Mass spectrometry (MS) has become a powerful tool for glycoprotein identification, and the determination of glycosylation sites, and structural features of carbohydrates, such as sequence, linkage and branching at each glycosylation site. Generally, the mass spectrometric characterization of glycoprotein involves the following steps: 1) fractionation of enzymatically or chemically liberated glycans followed by MS, and 2) fractionation of glycopeptides from proteolytic digests followed by MS. Here we present the MS of glycan and glycopeptides using the latest applications.

B. MS of Liberated Glycans

Matrix-assisted laser desorption/ionization (MALDI) (1), and electrospray ionization (ESI) (2), which are soft ionization techniques, are often used for glycan molecular mass determination. MALDI has been used by preference for rapid microanalyses, however, it generates metastable ions and the consequent various fragmentations, including the depletion of terminal sialic acids (known as post-source decay, PSD) (3). Although ESI used to have a problem with sensitivity, the introduction of nanospray technology allows us to use ESI to analyze femtomole levels of glycans (4). To measure all types of glycans, including neutral glycans and sulfated or sialylated acidic glycans, we suggest mass spectrometric glycan analysis in both positive and negative ion modes.

MALDI and ESI are combined with several types of

A. 緒 言

質量分析 (MS) は、糖タンパク質の同定、糖鎖結合位置の決定、並びに各結合位置における糖鎖の配列、結合位置、及び分岐等を含む構造特性解析の有用な手段として利用されている。現在、MSを用いた糖鎖解析のアプローチとしては、1) 酵素的または化学的に切り出された糖鎖の分画と MS、及び 2) 糖タンパク質酵素消化物からの糖ペプチドの分画と MS、が一般的である。そこで、ここでは、遊離糖鎖と糖ペプチドの MS について、最近の分析例を取り上げながら解説する。

B. 遊離糖鎖の MS

糖鎖分子の分析に適したソフトイオン化法として、マトリクス支援レーザー脱離イオン化法 (MALDI) (1)、及びエレクトロスプレーイオン化法 (ESI) (2) がよく利用されている。MALDI は迅速分析及び微量分析に適しているが、準安定イオンが生成し、MSⁿ スペクトル上にはシアル酸が脱離したイオンをはじめとする様々なフラグメントが検出される (ポストソース分解、PSD) (3)。ESI は感度上の問題が指摘されてきたが、現在ではナノスプレーの開発によって、フェムトモルレベルの糖鎖分析が可能となっている (4)。糖鎖には中性糖鎖だけでなく、シアル酸や硫酸基などが結合した酸性糖鎖が存在するので、未知試料を分析する際には、ポジティブ及びネガティブ両イオンモードで測定するのが望ましい。

MALDI や ESI は様々なアナライザー (分析計) と組み合

analyzer, such as quadrupole (Q) (5), quadrupole ion trap (IT) (6), time-of-flight (TOF) (7), and Fourier transform ion cyclotron resonance (FTICR) (8). Tandem mass spectrometers with various combinations of these analyzers have recently become available. Tandem mass spectrometry (MS/MS) is widely recognized as an effective means of structural elucidation, including molecular mass measurement by MS¹ and oligosaccharide sequencing by collision-induced dissociation (CID)-MS/MS (9–11). In particular, ITMS instruments are becoming popular for multistage tandem mass spectrometry (MSⁿ), which offers multiple precursor selections and CID experiments (12, 13).

Fig. 1A shows types of carbohydrate fragmentation by CID-MS/MS (14). In the positive ion mode the most common fragmentation involves cleavage of the glycosidic bond with retention of the glycosidic oxygen atom by species formed from the reducing end (Fig. 1B). Fragment ions generated by this cleavage are represented as B-ion (non-reducing end) and Y-ion (reducing end). The cleavage of carbon-carbon bonds of the sugar ring yields A-ion and X-ion. These cross ring fragments are often used as decisive ions in linkage determination (15, 16).

Fig. 2 shows the positive ion ESI-MS/MS and MS³ spectra of pyridylaminated agalacto-triantennary oligosaccharide (I) (Figs. 2A, A') and bisected agalacto-biantennary oligosaccharide (II) (Figs. 2B, B'). These are positional isomers whose one GlcNAc is attached to either α 1-3/6 Man or β 1-4Man in the trimannosyl core and cannot

わせて利用されている。アナライザーには四重極型 (Q) (5)、イオントラップ型 (IT) (6)、飛行時間型 (TOF) (7)、及びフーリエ変換イオンサイクロトロン共鳴型 (FTICR) (8) MS 装置などが用いられている。現在では、これらの分析装置を様々に組み合わせたタンデム質量分析装置の利用が可能である。タンデム質量分析 (MS/MS) は、MS¹ による糖鎖の質量測定と、衝突誘起解離 (CID)-MS/MS による糖鎖の配列解析を同時に行うことができる (9–11)。特に ITMS 装置は、前駆イオンの選択と CID-MS/MS を繰り返す多段階 MS/MS (MSⁿ) が可能であることから、近年、糖鎖解析用装置としての人気が高い (12, 13)。

図 1A は、CID-MS/MS における糖鎖の開裂を示したものである (14)。ポジティブイオンモードで測定した糖鎖の CID-MS/MS では主に、グリコシド結合の酸素原子を還元末端側糖鎖に残した開裂が生じる (図 1B)。そのとき生じた非還元末端側イオンは B イオン、還元末端側イオンは Y イオンと呼ばれ、糖鎖の配列解析に利用される。ピラノース環の炭素-炭素間の結合が開裂して生じたイオンは A、及び X イオンと呼ばれ、糖鎖結合位置の決め手となることがある (15, 16)。

図 2 は、ピリジルアミノ化されたアガラクト 3 本鎖糖鎖 (I) 及びアガラクトバイセクト 2 本鎖糖鎖 (II) のポジティブイオン MS/MS (図 2A, B)、及び MS³ (図 2A', B') スペクトルである。これらの糖鎖は GlcNAc 1 分子がトリマンノシルコアの α 1-3/6Man または β 1-4Man に結合した位置異性体で、MS¹ による分子量測定では区別することはできない。しかし、MSⁿ によっ

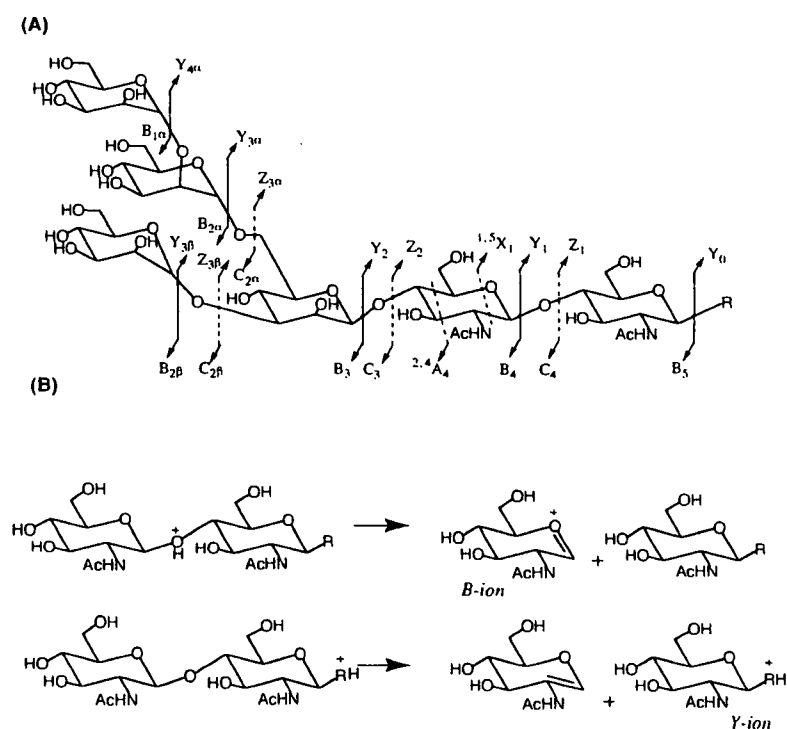


Fig. 1. (A) Types of carbohydrate fragmentation. (B) Production of B- and Y-ions in the positive ion mode.

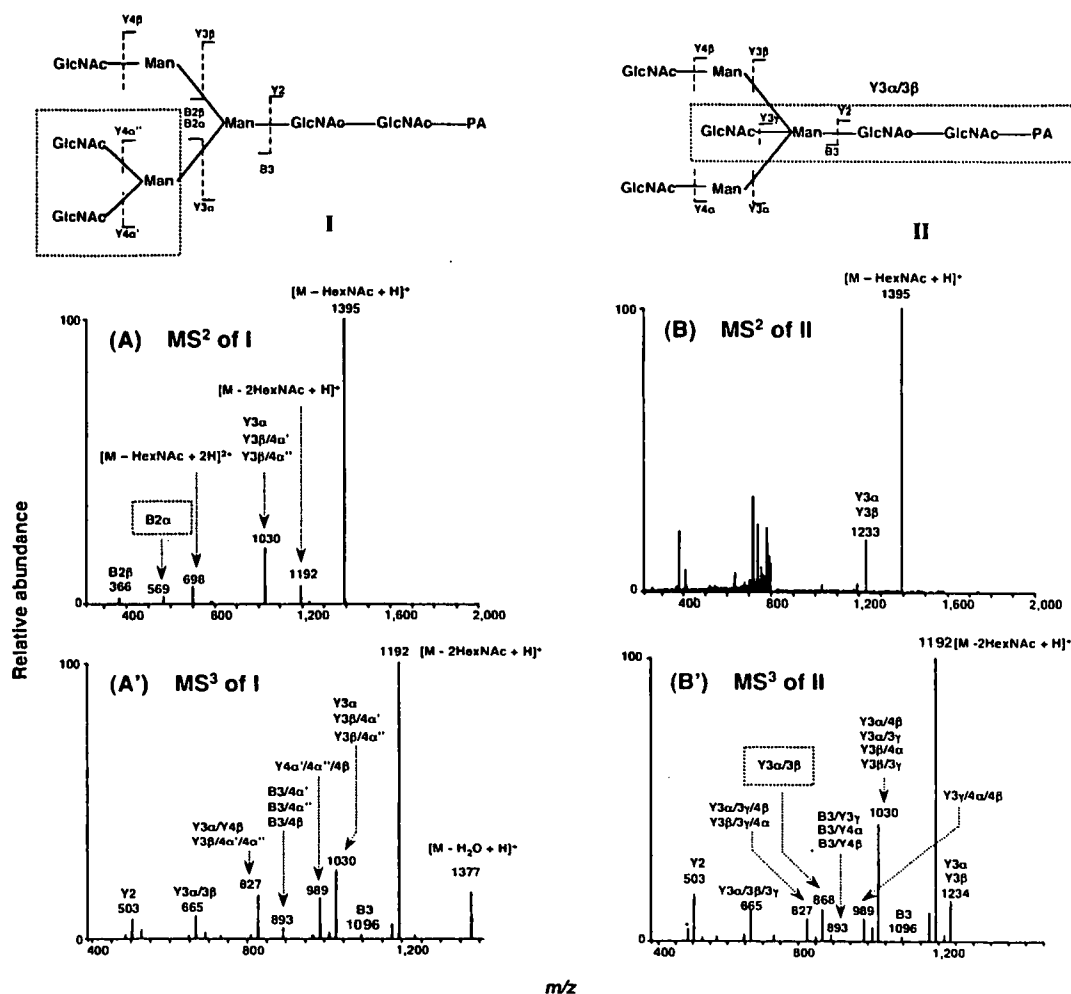


Fig. 2. MSⁿ spectra of oligosaccharides. (A) MS/MS spectrum of oligosaccharide I (precursor ion: *m/z* 800.8). (A') MS³ spectrum of oligosaccharide I (precursor ion: *m/z* 1,395.4). (B) MS/MS spectrum of oligosaccharide II (precursor ion: *m/z* 800.5). (B') MS³ spectrum of oligosaccharide II (precursor ion: *m/z* 1,395.4). MS: LTQ (Thermo Electron).

be discriminated by MS¹ (molecular mass determination). In contrast, MSⁿ spectra clearly show structural differences between two glycans. B-ion corresponding to GlcNAc₂Man⁺ is observed at *m/z* 569 in the MS/MS spectrum of glycan I (Fig. 2A), while Y-ion at *m/z* 868 corresponding to [GlcNAc-Man-GlcNAc-GlcNAc-PA + H]⁺ is detected in MS³ spectrum of glycan II (Fig. 2B'). In particular, glycan II can be determined as bisected oligosaccharides on the basis of a bisected N-glycan diagnostic ion at *m/z* 868.

The MSⁿ spectra of some glycans exhibit characteristic fragment patterns. Hence, even if no diagnostic ion, such as a bisected glycan-specific fragment, is detected, the glycan structure can be sometimes deduced from ion intensity ratios obtained by MSⁿ (17,18). For instance, structures of branched arms were deduced from mass spectrometric patterns obtained by MS/MS which causes a cleavage of α1-3 linkage more than α1-6 linkage (19–21). However, complete structural

て両者の構造の違いを明確にすることができる。例えば、糖鎖 I の MS/MS スペクトルには *m/z* 569 に GlcNAc₂Man⁺ に相当する B イオンが検出され (図 2A)、糖鎖 II の MS¹ スペクトル (前駆イオン: *m/z* 1395 [M-HexNAc + H]⁺) には [GlcNAc-Man-GlcNAc-GlcNAc-PA + H]⁺ に相当するイオンが *m/z* 868 に検出されている (図 2B')。特に、[GlcNAc-Man-GlcNAc-GlcNAc-PA + H]⁺ (*m/z* 868) はバイセクト糖鎖に特異的なフラグメントであり、糖鎖 II がバイセクト糖鎖であることを決定づけている。

糖鎖の MSⁿ は特徴的なスペクトルパターンを示すことが多いので、構造特異的イオンが検出されない場合でも、MSⁿ スペクトルのパターンより糖鎖構造を推定できる場合がある (17,18)。例えば、トリマンノシルコアの α1-3 結合が α1-6 結合よりも開裂しやすいことを利用して、分岐構造を解析した例が報告されている (19–21)。しかし、MS 単独で糖鎖構造を完

elucidation by MS alone is still a great challenge, and additional experiments are required, such as exoglycosidase digestion (22), lectin affinity chromatography (23), and sugar mapping (17,24,25).

C. Glycan Profiling by LC/MS

Many different oligosaccharides are attached to a glycoprotein. The development of derivatization and separation techniques for glycans has been an important part of structural glycobiology (26-34). The derivatization of glycan with a hydrophobic molecule improves the ionization efficiency of hydrophilic glycans and offers higher sensitivity (31,35). A combination of various HPLC techniques with off-line MALDI-MS or on-line ESI-MS has been successful in oligosaccharide profiling as well as the elucidation of structural details (36-39). As an example, we present the mass spectrometric *N*-glycan profiling of CHO cells and cells transfected with *N*-acetylglucosaminyltransferase III, which catalyzes the addition of GlcNAc to β 1-4Man in the trimannosyl core (Fig. 3) (40). Mass spectrometric analysis reveals the difference in glycosylation between two samples and the appearance of peaks with additional HexNAc (203 Da) in the transfected cells.

We recently demonstrated quantitative oligosaccharide

全に同定することが困難である場合が多く、MSはエキソグリコシダーゼによる段階的消化法(22)、レクチンアフィニティークロマトグラフィー(23)、及び糖鎖マッピング(17,24,25)などと組み合わせて用いられることが多い。

C. LC/MSを用いた糖鎖プロファイリング

糖タンパク質には様々な糖鎖が結合しているので、糖鎖の標識法と分離技術の開発は、構造糖鎖生物学において重要な位置を占めている(26-34)。糖鎖を分離・検出するために開発された疎水性物質による誘導体化は、親水性の高い糖鎖のイオン化効率を向上させるので、MSにおける高感度化においても有用である(31,35)。さらに、様々なHPLCによる分離法とオフラインMALDI-MS、あるいはオンラインESI-MSを組み合わせた分析方法は、糖鎖プロファイリングと糖鎖構造解析を兼ね備えた方法として利用され、多くの成果を上げている(36-39)。一例として図3にCHO細胞、及びトリマンノシルコアの β 1-4ManにGlcNAcを付加させる*N*-アセチルグルコサミン転移酵素III(GnT-III)遺伝子を導入したCHO細胞のMSを用いた糖鎖プロファイリングの結果を示す(40)。MSを利用することによって、GnT-III導入細胞で新たに出現した糖鎖は、HexNAc1分子(203Da)増加した糖鎖であることが確認できる。

筆者らは最近、安定同位体標識化2-アミノピリジン(AP)を用いた標識法とLC/MSを組み合わせた定量的糖鎖プロファ

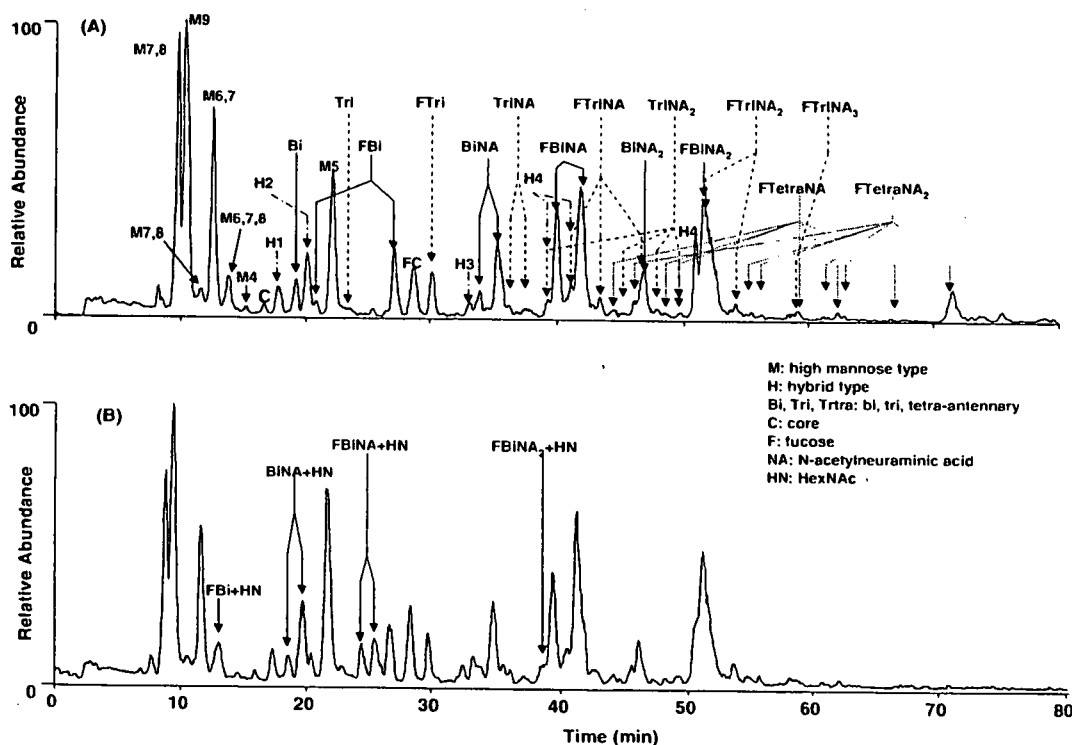


Fig. 3. TICs of *N*-linked oligosaccharides released from the insoluble fractions. (A) CHO cells. (B) *N*-acetylglucosaminyltransferase III-transfected CHO cells. Column: Hypercarb (0.2 × 150 mm, Thermo Electron), LC: Magic 2002 (Michrome BioResources), MS: TSQ-7000 (Thermo Electron), Eluent: 5 mM ammonium acetate containing acetonitrile.

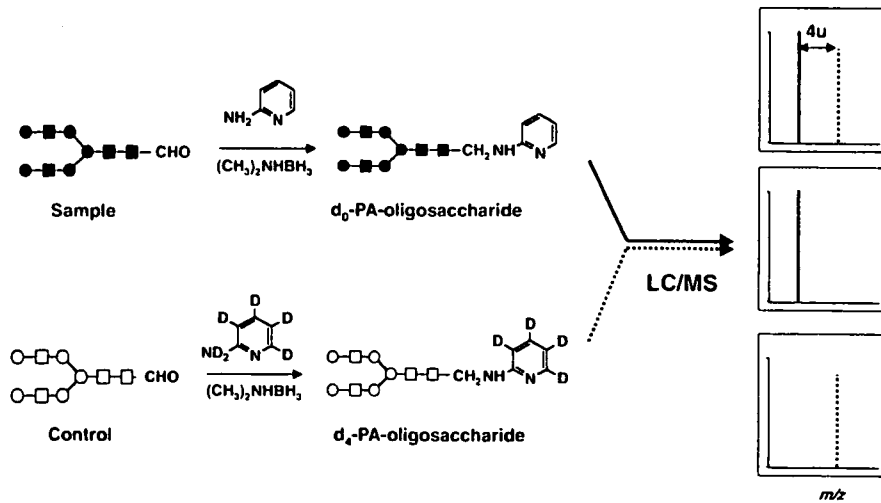


Fig. 4. Quantitative oligosaccharide profiling using LC/MS and an isotope tagging method.

profiling using isotope-labeled 2-aminopyridine (AP) and LC/MS (Fig. 4) (41). In this procedure, oligosaccharides released from an analyte and standard glycoprotein are tagged with d₀-AP and d₄-AP, respectively, and an equal amount of d₀-PA and d₄-PA oligosaccharides is analyzed by LC/MS. Oligosaccharides existing in either the analyte or standard glycoprotein appear as single ions, and oligosaccharides that exist in both analyte and standard glycoprotein are detected as paired ions with a difference of 4 u. The relative amount of analyte oligosaccharides can be determined on the basis of the analyte/ internal standard ion pair intensity ratio. This method can improve the precision of the mass spectrometric quantification and be used for glycan differential analysis among multiple samples.

イリングを開発した(図4)(41)。この方法は、標準糖タンパク質及び検体糖タンパク質から切り出した糖鎖をそれぞれ6重水素置換 AP(d₆-AP)、及び未置換 AP (d₀-AP)で標識し、得られた d₄-PA 糖鎖及び d₀-PA 糖鎖を1対1の混合物として LC/MS で分析するものである。標準糖タンパク質または検体糖タンパク質のどちらか一方にしか結合していない糖鎖は、d₄-PA 糖鎖または d₀-PA 糖鎖どちらかの単独イオンとして検出される。標準糖タンパク質及び検体糖タンパク質に共通して存在する糖鎖は 4u 異なる1対のイオンとして検出され、d₄-PA 糖鎖及び d₀-PA 糖鎖のイオン強度比から相対糖鎖結合量を求めることができる。同位体標識糖鎖を内部標準物質として用いることによって、MSを用いた定量解析における再現性が改良されるので、定量的糖鎖プロファイリングは、複数のサンプル間の糖鎖の差異を質的量的に比較する場合に有用である

D. MS of Glycopeptides

When glycans are released from protein, all information about their attachment to the protein is lost. Protein identification, determination of glycosylation sites and site-specific glycosylation analyses are generally achieved by the mass spectrometric analysis of glycopeptides. ESI and MALDI coupled with several analyzers are employed for glycopeptide analysis. ESI allows the accurate mass measurement of relatively large glycopeptide/protein because of the generation of multiple charged ions, whereas ions of large glycopeptide molecule ions are sometimes missed by MALDI-MS due to PSD and their poor ionization efficiency (42).

Fig. 5 illustrates three possible cleavages of the peptide backbone. The most common C-terminal and N-terminal fragment ions are b-ion and y-ion, respectively (37). In many cases, CID induces glycosidic bond cleavages rather than peptide backbones. Electron capture dissociation with FT-ICRMS is reported as a means for preferential cleavage of the

D. 糖ペプチドの MS

タンパク質から糖鎖を切り離すと、糖鎖とタンパク質間の結合に関する情報が失われてしまうので、糖鎖含有タンパク質の同定、糖鎖結合位置の決定、及び部位特異的糖鎖不均一性の解析などには糖ペプチドの MS が適している。糖ペプチドの分析においても、ESI あるいは MALDI に様々なアナライザーを組み合わせた装置が利用されている。MALDI-MS では、ほぼ一価イオンが生成するが、ESI-MS では多価イオンが生成するため比較的高分子量の糖ペプチドの質量を正確に測定することが可能である。また、MALDI-MS を用いて糖鎖の割合が高い糖ペプチドを分析する場合、イオン化の抑制や PSD によって、分子関連イオンが測定されにくいとする報告がある(42)。

図5はペプチド骨格の開裂を示している。ペプチド結合の開裂によって生じた N 末端側は b イオン、C 末端側は y イオンと呼ばれる(43)。通常、糖ペプチドの CID-MS/MS では、ペプチドよりも糖鎖の開裂が優先される。ペプチド部分が優先的に開裂する方法として FT-ICRMS 装置を用いた電子捕獲解

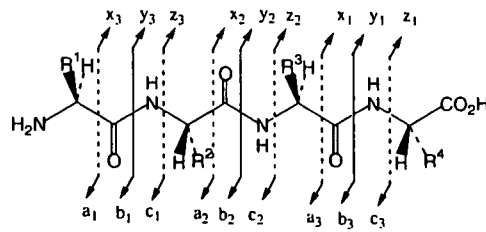


Fig. 5. Types of peptide fragmentation.

peptide backbone (44,45).

For the cleavages of both glycosidic and peptide bonds CID-MS/MS is carried out with relatively high energy (approx. 50V/1000 u) (46-51). Hence, it allows both peptide sequencing and estimation of the carbohydrate structure on the basis of b/y-ions and B/Y-ions. Fig. 6A presents the CID-MS/MS spectrum of a glycopeptide derived from human α -fetoprotein using the ESI-QqTOFMS instrument. Carbohydrate-specific B-ions, such as HexNAc⁺ and NeuAc⁺ are observed at m/z 204, 292 as well as y-series ions. Based on the y-series ions and peptide ion, this peptide is identified

離法が報告されている (44,45)。

糖鎖とペプチド部分を同時に開裂させるためには比較的高いエネルギー(約50V/1000 u)を与えてCID-MS/MSを行う(46-51)。生成したb、yイオン、及びB、Yイオンから、ペプチド部分の氨基酸配列や、糖鎖構造を解析することができる。図6Aは、ESI-四重極飛行時間型MSを用いたCID-MS/MSによって得られたヒトアルファフェトプロテイン由来糖ペプチドのMS/MSスペクトルである。糖鎖Bイオンである m/z 204 (HexNAc⁺)、及び292 (NeuAc⁺)等と一緒に、ペプチドに由来する一連のyイオンが検出されていることがわかる。これら一

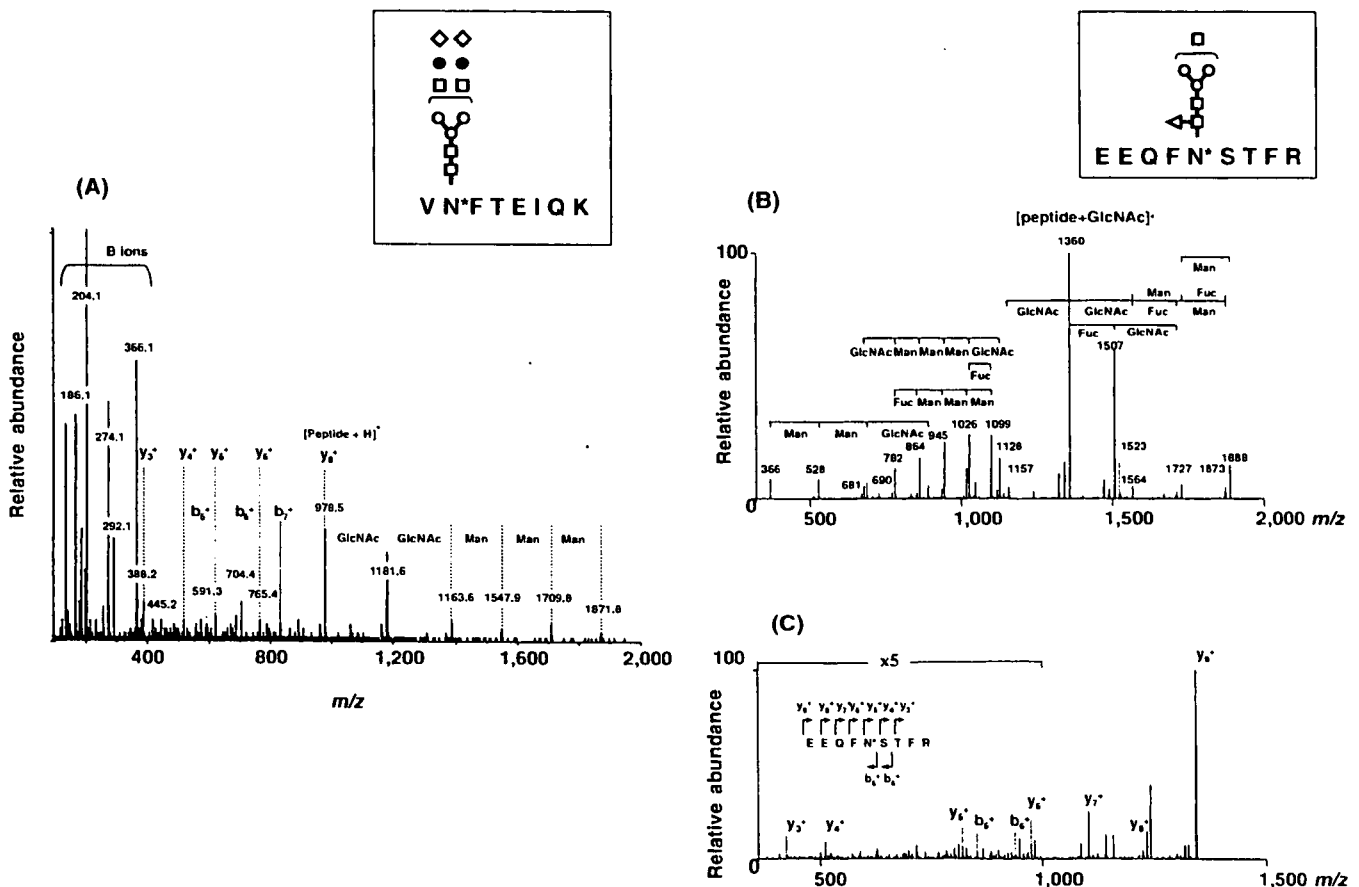


Fig. 6. CID-MSⁿ spectra of glycopeptides. (A) CID-MS/MS with relatively high energy (precursor ion, m/z 1,061.8). Sample: glycopeptide from human α -fetoprotein, MS: QSTAR (Applied Biosystems). (B) Low-energy CID-MS/MS (precursor ion, m/z 1,200). (C) Low-energy CID-MS³ (precursor ion, m/z 1,360), Sample: glycopeptide from mouse IgG1, MS: LTQ (Thermo Electron).

as VNFTEIQK. The oligosaccharide structure can be deduced as disialylated biantennary from the molecular mass of the carbohydrate moiety together with B-ions in the MS/MS spectrum.

B- and Y-series ions are produced by the low-energy CID-MS/MS of glycopeptides (12, 52–55). Fig. 6B shows the low energy (approx. 5–20V/1000 u) CID-MS/MS spectrum of glycopeptide derived from mouse IgG1. Based on the carbohydrate related-ions, the carbohydrate structure could be determined as fucosylated biantennary. When using an ITMS instrument, MS³ is automatically carried out for intense ions. In this experiment, [peptide +HexNAc +H]⁺, which is generally detected as intense ion, was subjected to further product ion scan, and b- and y-series ions appeared in the MS³ spectrum (Fig. 6C). Peptides can be identified by comparing experimental fragment ions with predictable fragment ions derived from proteins in a database. Moreover, the database analysis with the possibility of glycosylation at Asn and Ser/Thr with HexNAc, Hex, and dHex allows the identification of glycopeptides and glycosylation sites (56). For instance, this peptide was identified as EEQFN*STFR glycosylated with HexNAc at N*. This method would enable the glycosylation analysis of unknown glycoproteins and a mixture of glycoproteins.

E. Site-Specific Glycosylation Analysis of Glycoproteins

Fig. 7 illustrates the strategy for the site-specific glycosylation analysis of glycoproteins. First, a glycoprotein is digested with an appropriate proteinase, which provides

連の y イオン、及びペプチドイオンの質量から、このペプチド部分は VNFTEIQK と同定することができる。また、糖鎖構造は、糖鎖部分の分子量と B イオンからジシアロ 2 本鎖糖鎖と推定される。

低エネルギー CID-MS/MS では、グリコシド結合が優先して開裂し、B、及び Y イオンが検出される (12, 52–55)。図 6B はマウス IgG1 から得られた糖ペプチドの低エネルギー CID-MS/MS (約 5–20V/1000 u) スペクトルで、糖鎖関連イオンから、糖鎖構造を推定することができる。糖ペプチドの MS/MS では一般に、[ペプチド + HexNAc + H]⁺ が比較的強く検出される。そこで、ITMS 装置を用いて [ペプチド + HexNAc + H]⁺ を前駆イオンとして選択し、さらにプロダクトイオンスキャンを行うと、MS³ スペクトル上に b 及び y イオンが検出される (図 6C)。これらの b 及び y イオンの実測値をタンパク質データベースに登録されているタンパク質の予測プロダクトイオンの理論質量と比較することによって、ペプチドを同定することができる。さらに、Asn や Ser/Thr に HexNAc、Hex、あるいは dHex 等による糖鎖修飾の可能性を追加してデータベース検索をすることによって、糖鎖結合位置を決定できる場合がある (56)。例えば、ここで分析された糖ペプチドはデータベース検索エンジンを用いて、EEQFN*STFR (N* は HexNAc 修飾 Asn) と同定された。この方法を利用することによって、混合物中の糖タンパク質や、未知の糖タンパク質の部位特異的な糖鎖解析が可能となるものと期待される。

E. 糖タンパク質の部位特異的な糖鎖解析

糖タンパク質の部位特異的な糖鎖解析の流れを図 7 にまと

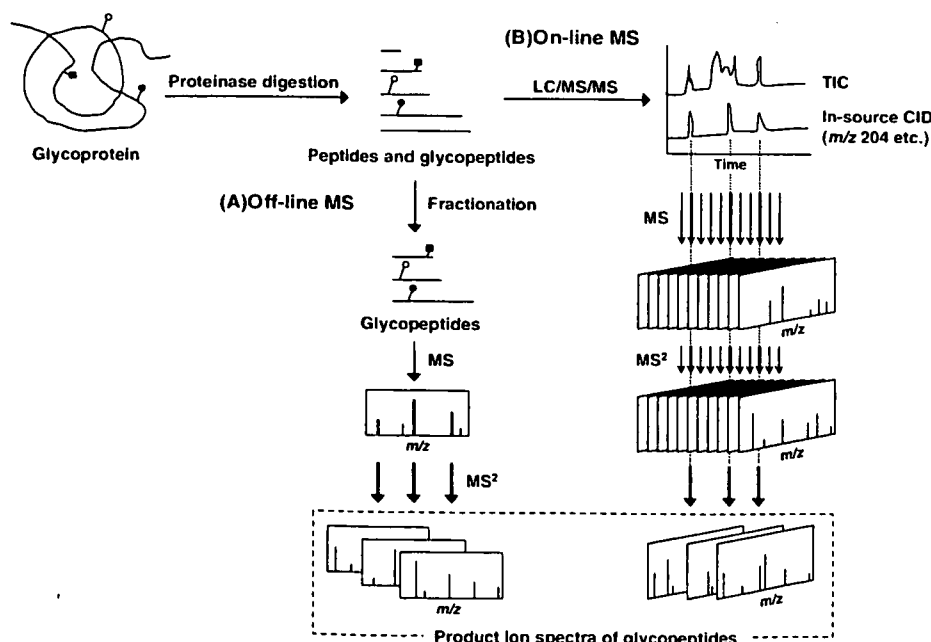


Fig. 7. Strategy for site-specific glycosylation analysis by LC off-line MS (A) and on-line LC/MS (B).

glycopeptides, including a single glycosylation site. Trypsin, Lys-C, Glu-C and Asp-N are commonly used for glycosylation analysis. Since glycopeptide ions are sometimes missed by MS in the presence of excess peptides due to their lower ionization efficiency, several methods have been proposed for the fractionation of glycopeptides, such as HPLC and affinity chromatography (57,58), followed by off-line MALDI-MS. LC on-line ESI/MS is an efficient method for the direct elucidation of glycopeptides in a complex mixture. Although LC/MS provide a complicated chromatogram, glycopeptides in a chromatographic position can be localized by the appearance of marker ions, such as HexNAc⁺ and HexHexNAc⁺, resulting from precursor ion scanning and in-source CID (59-66). However, for peptide identification these two means require additional CID-MS/MS scan for some intense ions (data-dependent CID-MS/MS scan). Recently we presented an alternative method, with which product ion spectra of glycopeptides can be selected directly using marker ions arising from glycopeptides by data-dependent CID-MS/MS scan with relatively high energy (49).

める。まず、糖タンパク質を適切な酵素で糖ペプチドに断片化する。この際、同一ペプチドに複数の糖鎖結合部位が含まれないように酵素を選択する。基質特異性の高いトリプシン、Lys-C、Glu-C、及び Asp-N 等がよく用いられている。糖ペプチドはペプチドに比べてイオン化効率が悪く、ペプチドが混在するとマススペクトルが得られにくい。そのため、アフィニティークロマトグラフィーや、HPLC 等で予め糖ペプチドを分画してから (57,58)、マススペクトルを測定するオフライン法 (図 7A) や、C18 カラム等を用いた LC/MS^{*} でペプチド・糖ペプチドを分離しながら直接マススペクトルを測定するオンライン法がよく用いられている (図 7B)。オンライン LC/MS では複雑なクロマトグラムが得られるが、プリカーサーイオンスキャンやインソース CID によって生じた糖鎖に特徴的な B イオン (例えば、HexNAc⁺、*m/z* 204 や HexHexNAc⁺、*m/z* 366 など) を利用することによって、糖ペプチドの溶出位置を推定することができる (59-66)。ただしこれらの方法は、ペプチドを同定するために、別途、強度の高いイオンに対する自動的 CID-MS/MS スキャン (データ依存的 CID-MS/MS スキャン) を行う必要がある。そこで、我々は別法として、データ依存的 CID-MS/MS によって生成した B イオンを利用して糖ペ

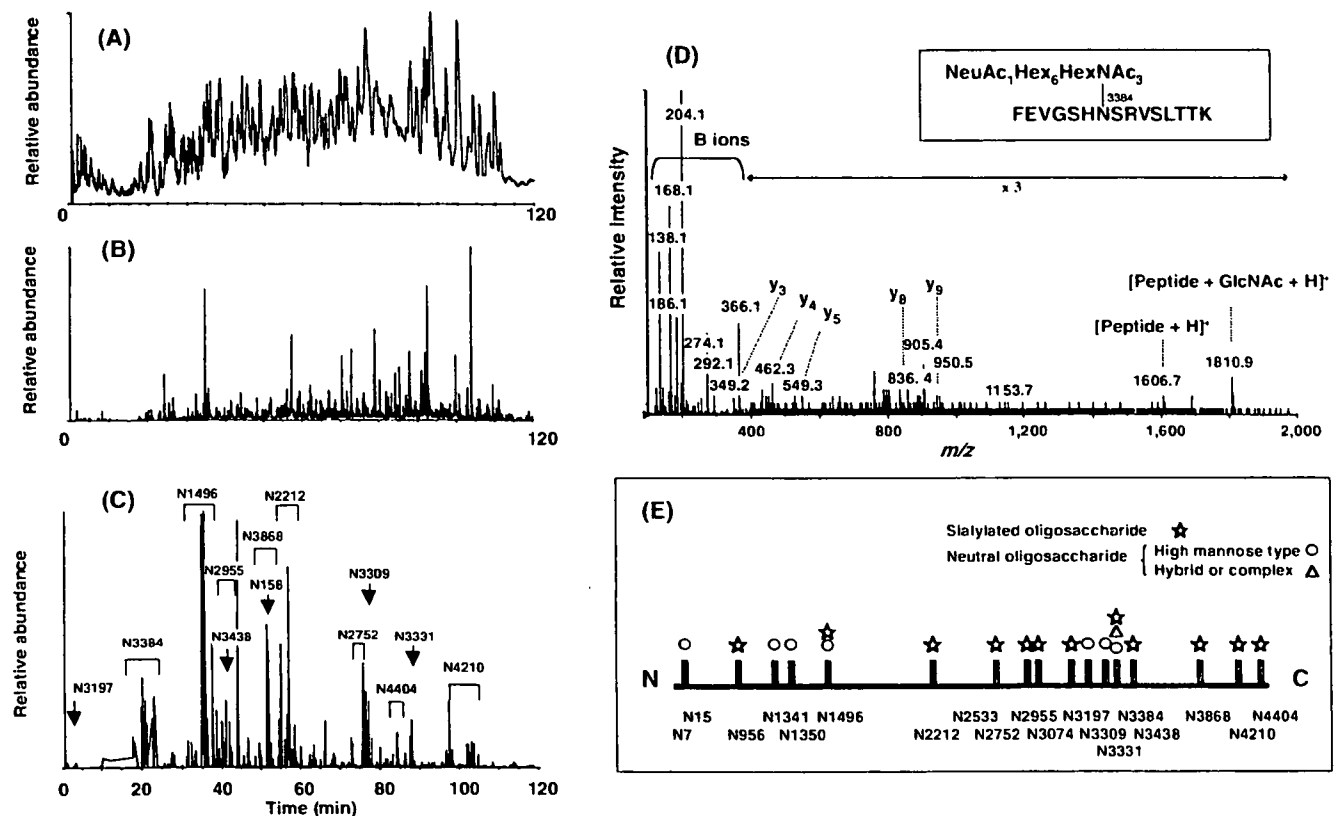


Fig. 8. Site-specific glycosylation analysis of apoB100 by LC/ESI-MS/MS. (A) TIC of full MS scan (*m/z*: 400-2,000). (B) TIC of data-dependent CID-MS/MS scan (*m/z* 100-2,000). (C) Mass chromatogram at *m/z* 204 in data-dependent CID-MS/MS scan. (D) MS/MS spectrum of glycopeptide (precursor ion: *m/z* 1,160.4). (E) *N*-glycosylation of apoB100. Sample: tryptic digest of apoB100 (4 µg), LC: Paradigm (Michrome BioResources), Column: Magic C18 (0.2 × 50 mm, Michrome BioResources), MS: QSTAR (Applied Biosystems).

Here we demonstrate the site-specific glycosylation analysis of human apolipoprotein B100 (apoB100). Tryptic digest of apoB (4 μ g) was injected into an LC/ESI-QqTOFMS instrument equipped with a C18 column. Figs. 8A and B are total ion chromatograms (TIC) obtained by full MS¹ scan and data-dependent CID-MS/MS scan, respectively (49). These chromatograms appear complicated due to a number of peptide-related ions derived from a large glycoprotein molecule of 500,000 Da. In order to localize glycopeptides in the peptide map, carbohydrate marker ion, HexNAc⁺ at *m/z* 204, was extracted from the TIC of data-dependent CID-MS/MS scan (Fig. 8C). The MS/MS spectra of glycopeptides were then sorted from the peaks appearing in the mass chromatogram (Fig. 8D). We successfully identified 17 *N*-glycosylation sites among 19 potential *N*-glycosylation sites and deduced glycosylation at each glycosylation site from the mass of carbohydrate moieties (Fig. 8E).

F. Application in Proteomics and Glycomics

MS enables us to elucidate a small number of glycoproteins isolated by electrophoresis (67, 68) and micro HPLC (69). This method could be applied in comprehensive or carbohydrate structure-specific glycoprotein analysis by a combination with proteomic approaches such as 2-dimensional (2D) gel electrophoresis (GE) and 2D-LC followed by MS. Some applications have been already demonstrated, for instance, glycoprotein expression analysis using 2D-GE coupled with carbohydrate-specific dyeing or immunoblotting (70), and LC/MS combined with lectin affinity chromatography (71,72). These glycomic/proteomic technologies are expected to be a powerful tool for the functional study of glycoproteins, finding disease-related glycoproteins and identifying proteins attached to some glyco-epitopes.

プチドの MS/MS スペクトルを選び出す方法を見出した (49)。

図 8 に、我々が最近行ったオンライン法によるヒトアポリポプロテイン B100 (apoB100) の部位特異的な糖鎖解析例を示す (49)。apoB100 のトリプシン消化物 (4 μ g) を C18 カラムを用いた LC/ESI-QqTOFMS 装置で分析した。図 8A 及び B はフル MS¹ スキャン (*m/z* 1,000-2,000) 及びデータ依存的 CID-MS/MS スキャンによって得られたトータルイオンクロマトグラム (TIC) である。apoB100 は分子量約 500,000 Da の大きな糖タンパク質なので、非常に多くのペプチドイオンが検出されている。そこで、糖ペプチドの溶出位置を推定するために、データ依存的 CID-MS/MS スキャンによって生じた *m/z* 204 イオンのみを抜き出した (図 8C)。出現したピーク周辺から B イオンを指標に糖ペプチドの MS/MS スペクトルを探し出し、それらのスペクトル上のプロダクトイオンを帰属した (図 8D)。その結果、19 カ所の推定 *N* 結合型糖鎖結合部位のうち 17 カ所に糖鎖が結合していることを明らかにするとともに、それぞれの部位に結合している糖鎖を推定することができた (図 8E)。

F. グライコミクス・プロテオミクスへの応用

現在では、電気泳動 (67,68) やマイクロ液体クロマトグラフィー (69) で分離された僅かな糖タンパク質からでも、MS によって、多くの糖鎖構造情報を得ることができるようになった。これらの糖鎖解析技術とプロテオミクスのアプローチ、すなわち、2次元電気泳動や2次元クロマトグラフィーによるタンパク質発現解析と MS を組み合わせることによって、糖タンパク質の網羅的解析や、任意の糖鎖構造を持つタンパク質の解析が可能になるものと期待されている。すでに、2次元電気泳動と糖タンパク質特異的染色法や免疫プロットを組み合わせた糖タンパク質発現解析や (70)、レクチンアフィニティクロマトグラフィーと各種 LC/MS を組み合わせた糖タンパク質の網羅的解析の例が報告されている (71,72)。今後、これらのグライコミクス・プロテオミクス解析技術が、糖鎖の機能研究や、疾患等に関与する糖鎖・糖タンパク質の探索、並びに様々な糖鎖エピトープ結合タンパク質の特定に貢献できるものと期待される。

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Received on June 3, 2005, accepted on July 19, 2005

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N-linked oligosaccharide analysis of rat brain Thy-1 by liquid chromatography with graphitized carbon column/ion trap-Fourier transform ion cyclotron resonance mass spectrometry in positive and negative ion modes

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Received 25 July 2005; received in revised form 10 November 2005; accepted 14 November 2005

Available online 20 December 2005

Abstract

We have previously described the site-specific glycosylation analysis of rat brain Thy-1 by LC/multistage tandem mass spectrometry (MSⁿ) using proteinase-digested Thy-1. In the present study, detailed structures of oligosaccharides released from Thy-1 were elucidated by mass spectrometric oligosaccharide profiling using LC/MS with a graphitized carbon column (GCC-LC/MS). First, using model oligosaccharides, we improved the oligosaccharide profiling by ion trap mass spectrometry (IT-MS) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). Sequential scanning of a full MS¹ scan with FT-ICR-MS followed by data-dependent MSⁿ with IT-MS in positive ion mode, and a subsequent full MS¹ scan with FT-ICR-MS followed by data-dependent MSⁿ with IT-MS in negative ion mode enabled the monosaccharide composition analysis as well as profiling and sequencing of both neutral and acidic oligosaccharides in a single analysis. The improved oligosaccharide profiling was applied to elucidation of N-linked oligosaccharides from Thy-1 isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was demonstrated that Thy-1 possesses a significant variety of N-linked oligosaccharides, including Lewis a/x, Lewis b/y, and disialylated structure as a partial structure. Our method could be applicable to analysis of a small abundance of glycoproteins, and could become a powerful tool for glycoproteomics.

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Keywords: Mass spectrometric oligosaccharide profiling; Graphitized carbon column; Ion trap mass spectrometry; Fourier transform ion cyclotron resonance mass spectrometry; Data-dependent MSⁿ; Thy-1

1. Introduction

Glycosylation is one of the most abundant post-translational modifications of proteins [1]. It is already known that glycosylation influences the biological functions as well as the physicochemical properties of proteins, i.e., folding, solubility, aggregation, and stability. A number of reports have noted a positive relationship between a change in glycosylation and

development, aging, and certain diseases [2–4]. Elucidation of structural detail in oligosaccharides is necessary to clarify the biological properties of glycoproteins.

MS is now a powerful tool for structural analysis of glycoproteins. There are two major mass spectrometric approaches to the structural analysis of glycoproteins, i.e., MS of glycopeptides [5–7] and of oligosaccharides [8–13]. For oligosaccharide sequencing, tandem mass spectrometry as well as exoglycosidase digestions in conjunction with MS is recognized as an effective means of oligosaccharide sequencing [14–16]. Mass spectrometric peptide/glycopeptide mapping by LC coupled with tandem mass spectrometry (LC/MS/MS) is effective for the determination of glycosylation sites and the analysis of site-specific heterogeneity [17–22]. However, structural detail in

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oligosaccharides is not always available by product ion spectra of glycopeptides, as many of the precursor ions consist of uniform peptides carrying different oligosaccharides with identical m/z values. LC/MS/MS of glycopeptides has limitations for the structural analysis of carbohydrates due to the difficulty of isolating of glycopeptide isomers. Mass spectrometric oligosaccharide profiling through the separation of isomers by LC can supply the structural detail of each oligosaccharide although it cannot provide information regarding glycosylation sites and site-specific glycosylation [23–29]. MS of both glycopeptides and oligosaccharides is needed for glycosylation analysis of a glycoprotein [30].

Thy-1 is a cell adhesion molecule that belongs to the immunoglobulin superfamily and is attached to the cell membrane via a glycosylphosphatidylinositol (GPI)-anchor. We recently, studied the glycosylation of Thy-1 in rat brain by mass spectrometric peptide/glycopeptide mapping, and demonstrated that Thy-1 possesses various *N*-glycans at Asn23, 74, and 98 [31]. The monosaccharide composition of *N*-glycan at each glycosylation site was estimated by masses of molecular ions; however, structural detail regarding some of the oligosaccharides could not be elucidated by MSⁿ since many glycopeptides with identical m/z values contained several oligosaccharide isomers and yielded product ions from a mixture of these glycopeptide isomers. Mass spectrometric oligosaccharide profiling is necessary for detailed structural analysis of oligosaccharides.

We have previously demonstrated a simple means of oligosaccharide profiling using liquid chromatography/electrospray ionization mass spectrometry with a graphitized carbon column (GCC–LC/MS) [32–34], in which oligosaccharides can be separated on the basis of their branching, sequence, and linkage, and can be characterized based on their monosaccharide compositions estimated from their calculated molecular masses. Here, we study the glycosylation of Thy-1 by oligosaccharide profiling with GCC–LC/MS. First, we improved our oligosaccharide profiling by ion trap mass spectrometry (IT–MS) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR–MS). This instrument is capable of both monosaccharide composition analysis by acquisition of accurate masses and data-dependent multistage tandem MS (MSⁿ) for sequencing with fast switching between positive and negative ion modes. Using a mixture of typical oligosaccharides, including high-mannose-type, and asialo-, trisialylated, and tetrasialylated complex-types, we confirmed that the improved method can be used for monosaccharide composition analysis and detailed structural analysis of both neutral and acidic oligosaccharides. The method was then applied to *N*-linked oligosaccharide analysis of rat brain Thy-1.

2. Experimental

2.1. Materials

Man7/D1, Man7/D3, and asialo-triantennary (Tri) were obtained from Oxford Glycosystems (Abingdon, UK). Trisialylated triantennary (TriNA₃) and tetrasialylated tetraantennary (TetraNA₄) were purchased from Dionex (Sunnyvale, CA,

USA). Rat brain was purchased from Nippon SLC (Hamamatsu, Japan). Phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus cereus* was purchased from Molecular Probes (Eugene, OR, USA). Peptide-*N*-glycosidase F (PNGase F) was purchased from Roche Diagnostics (Mannheim, Germany). SimplyBlue SafeStain was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Release of *N*-linked oligosaccharides from rat brain Thy-1 by in-gel PNGase F digestion

PIPLC-treated GPI-anchored proteins were prepared from rat brain as reported previously [31]. Briefly, the homogenate of rat brain was defatted and solubilized with 2% Triton X-114 at 4 °C overnight [35,36]. After centrifugation, the supernatant was subjected to Triton X-114 phase-partitioning at 37 °C. Solubilized membrane proteins in the detergent phase were precipitated with cold acetone, and the precipitates were digested with PIPLC. After resubjecting the digest mixture to Triton X-114 phase-partitioning, PIPLC-treated soluble GPI-anchored proteins in aqueous phase were precipitated by adding cold acetone. These proteins were carboxyamidomethylated [30], and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5%) followed by staining with SimplyBlue SafeStain.

In-gel PNGase F digestion of Thy-1 and extraction of *N*-linked oligosaccharides were performed as previously described

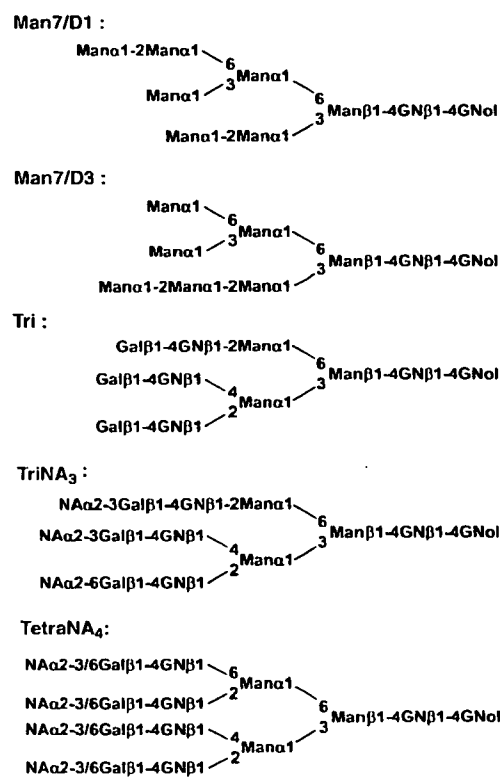


Fig. 1. Structures of major oligosaccharides and their abbreviations. Man: mannose, Gal: galactose, GN: *N*-acetylglucosamine, GNol: *N*-acetylglucosaminitol, NA: *N*-acetylneuramic acid.

[15]. The protein band of Thy-1 (20–25 kDa) was excised, cut into pieces, and destained. The gel pieces were dehydrated with 50% acetonitrile. The dried gels were then equilibrated with 50 mM sodium phosphate buffer (pH 7.2) and incubated at 37 °C with 3 units of PNGase F. The released *N*-glycans were extracted three times from gel pieces by intermittent sonication for 30 min in water. All extracts were combined and lyophilized. The released *N*-linked oligosaccharides were reduced with NaBH₄, as previously reported [33], and subjected to GCC-LC/IT-MS-FT-ICR-MS.

2.3. *N*-linked oligosaccharide analysis by GCC-LC/IT-MS-FT-ICR-MS

GCC-LC/MS was carried out using a MAGIC 2002 system (Michrom BioResource, Auburn, CA, USA) connected to IT-MS instrument coupled with FT-ICR-MS instrument

(Finnigan LTQ FT, Thermo Electron Corp., San Jose, CA, USA). The eluents consisted of 5 mM ammonium acetate, pH 9.6, containing 2% CH₃CN (pump A), and 5 mM ammonium acetate, pH 9.6, containing 80% CH₃CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on Hypercarb (150 mm × 0.2 mm, 5 μm, Thermo Electron Corp.) as GCC with a linear gradient of 5–30% for pump B over a period of 60 min at a flow rate of 2 μl/min.

The MS^{*n*} experiment includes sequential scans, as follows: a full MS¹ scan (*m/z* 700–2000) by FT-ICR-MS in positive ion mode, data-dependent MS^{*n*} scans by IT-MS for most abundant ions regardless of their charge state, a full MS¹ scan (*m/z* 700–2000) by FT-ICR-MS in negative ion mode, and data-dependent MS^{*n*} scans by IT-MS for the most intense ions regardless of their charge state. For the data-dependent MS^{*n*}, the following settings were used: the isolation window for precursor masses, ±2.5 Da; collision energy, 35%; dynamic exclusion

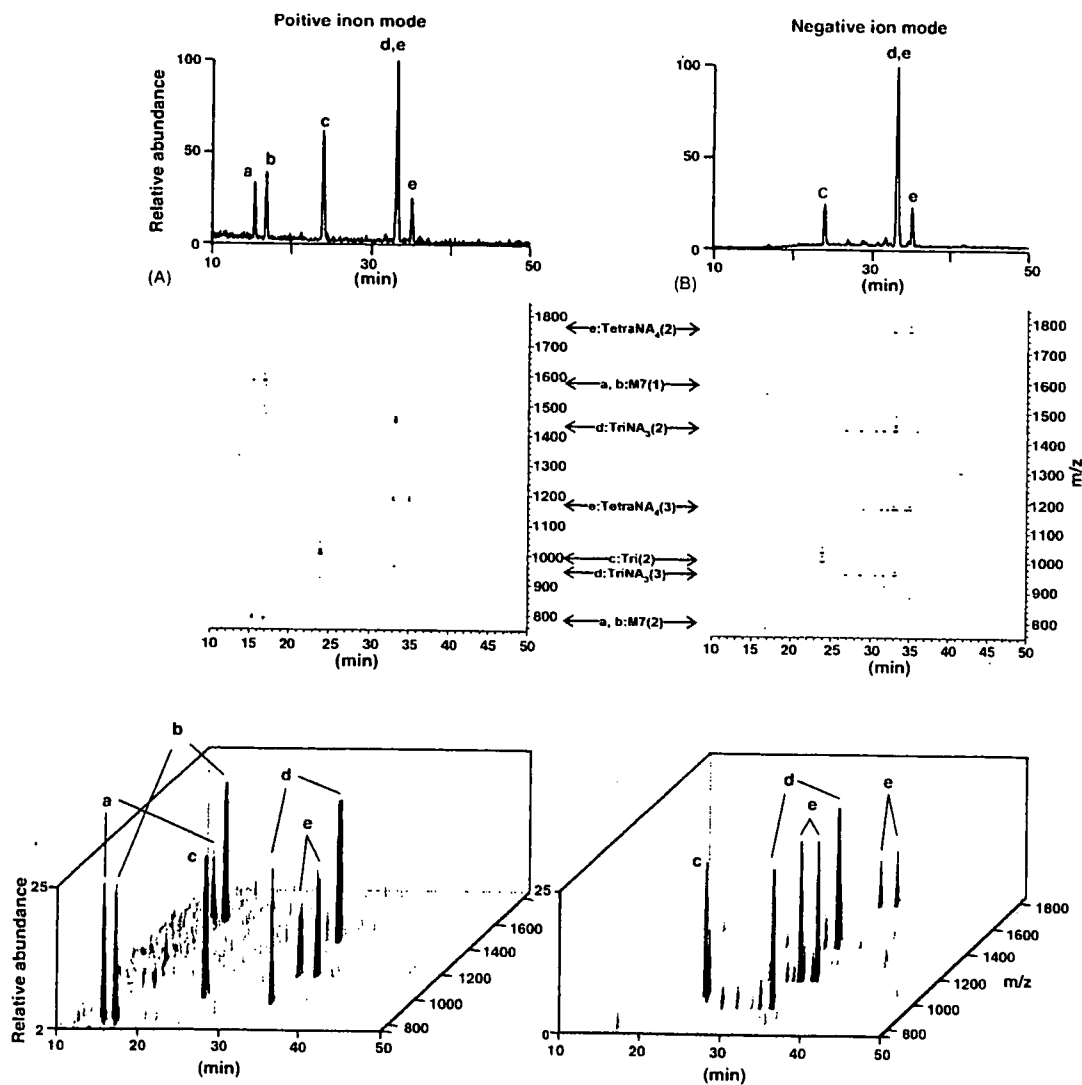


Fig. 2. Typical oligosaccharide profiles obtained by full MS¹ scans with FT-ICR-MS. (A) total ion chromatogram (TIC) (upper), two-dimensional (2D) display (retention time vs. *m/z*) (middle), and three-dimensional (3D) display (lower) in positive ion mode. (B) TIC (upper), 2D (middle) and 3D display (lower) in negative ion mode. Numbers in parentheses after the abbreviation of the model oligosaccharides refer to the charge state.

time, 15 s. The operating conditions employed for LC/MSⁿ were as follows: tube lens offset, 120 V; capillary voltage, 2.0 kV; and capillary temperature, 200 °C.

3. Results

3.1. GCC-LC/IT-MS-FT-ICR-MS of model oligosaccharides

By using the IT-MS-FT-ICR-MS instrument, the oligosaccharide profiling was shown to be more rapid, accurate, and informative. Man7/D1, Man7/D3, Tri, TriNA₃, and TetraNA₄, which were chosen as model neutral and acidic oligosaccharides (Fig. 1), were analyzed by alternative scans in positive and negative ion modes, which are consisting of a full MS¹ scan by FT-ICR-MS followed by data-dependent MSⁿ scans by IT-MS. Fig. 2A and B show the oligosaccharide profiles obtained by a full MS¹ scan with FT-ICR-MS (*m/z* 700–2000) in positive and negative ion modes, respectively. The monosaccharide compositions of individual oligosaccharides could be easily determined by accurate *m/z* values, and the major peaks of a, b, c, d, and e were assigned to Man7/D1 or D3, Man7/D3 or D1, Tri, TriNA₃ and TetraNA₄, respectively. Oligosaccharides detected at the same *m/z* values are positional isomers. Man7/D1 and D3 were detected in positive ion mode, but were only slightly detectable in negative ion mode. The major isomers of TriNA₃ and TetraNA₄ were detected in both ion modes, whereas their minor isomers were detected only in negative ion mode. These results demonstrate the advantage of alternative scans in both ion modes.

We confirmed the possibility of data-dependent MSⁿ scans for sequencing neutral and sialylated oligosaccharides. Man 7/D1 and D3 could be distinguished from each other by data-dependent MSⁿ (Fig. 3). Oligosaccharide eluted at 15 min could be assigned to Man7/D1 by the relatively intense ions at *m/z*

913 (Y_{3a}⁺) and 1237 (Y_{3b}⁺), which would be predominantly produced from Man7/D1 by the cleavage of the α1–6-linked or α1–3-linked branch arm of the core mannose (Fig. 3A) (nomenclature proposed by Domon and Costello [37]). Likewise, the oligosaccharide at 17 min could be Man7/D3 based on the intensity of Y_{3x}⁺ at *m/z* 1075 generated from Man7/D3 by the cleavages of both the α1–6-linked and α1–3-linked branch arms (Fig. 3B).

Fig. 4A and B show the product ion spectra of TetraNA₄ in positive and negative ion modes, respectively. In positive ion mode, the characteristic B ions such as *m/z* 454 (B_{2x}⁺), 657 (B_{3x}⁺), 1475 (B_{4x}⁺), and 1658 (B₆²⁺), and a ladder of several Y ions with intervals corresponding to Hex, HexNAc, and NeuAc were detected. B/Y ions were also detected at *m/z* 366, 527, 819 (B₅/Y_{3x}²⁺), and 1330 (B₆/Y_{4x}²⁺). In negative ion mode, only sialic acids were predominantly eliminated by MS² and MS³. The structural information was provided by MS⁴, whereby both B and Y ions were originated from TetraNA₂, together with the internal fragmentation ions and cross ring cleaved ions (Fig. 4B). In addition to the B and Y ions, which were predominantly produced in positive ion mode, fragment ions at *m/z* 470 (C_{2x}⁻), 1322 (Z_{6x}²⁻, [Y_{6x}-H₂O]²⁻), 1241 (Z_{5x}²⁻, [Y_{5x}-H₂O]²⁻), and 1057 (Y_{5x}⁻/Z_{4x}²⁻, Y_{4x}⁻/Z_{5x}²⁻, Y_{4x}⁻/Z_{5x}²⁻, Y_{5x}⁻/Z_{4x}²⁻, [Y_{4x}/Y_{5x}-H₂O]²⁻, [Y_{4x}/Y_{5x}-H₂O]²⁻) were detected in negative ion mode. These ions were also useful for the structural characterization of oligosaccharides.

3.2. Glycosylation analysis of Thy-1 by GCC-LC/IT-MS-FT-ICR-MS

The improved oligosaccharide profiling using IT-MS-FT-ICR-MS was applied to the glycosylation analysis of Thy-1. PIPLC-treated Thy-1 in rat brain was isolated by SDS-PAGE [31]. N-linked oligosaccharides were extracted from the gel after in-gel PNGase F digestion and were reduced

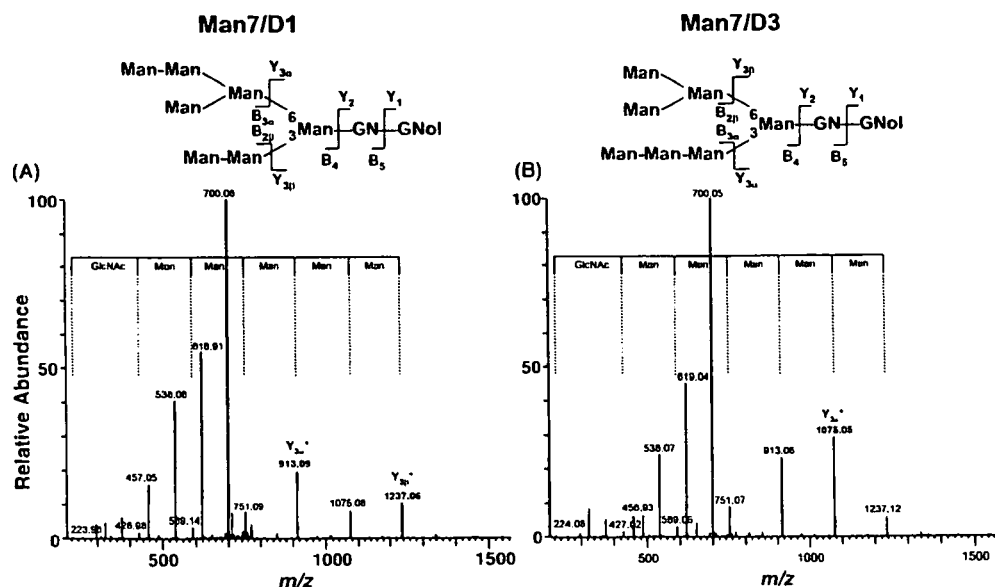


Fig. 3. Product ion spectra of oligosaccharide Man 7/D1 (A) and Man 7/D3 (B).