13

Tandem Mass Spectrometry of Tagged and Permethylated Polysaccharides

Wen-Bin Yang
Genomics Research Center, Academia Sinica
Taiwan R.O.C.

1. Introduction

Polysaccharides are the most abundant materials occurring in organism. In addition, the increasing biological importance of saccharides is difficult to detect because their lack an ionization natural and low sensitivity in mass spectrometry than other biomass (Zaia 2010; Chang et al., 2011a; Harvey, 2011; Mischnick 2011). The development of methods for mass spectrometry of isolated glycan from glycoconjugates and polysaccharides are commonly used chemical operation to form the tagged or/and permethylated glycan for mass determination and structural analysis (Fig. 1). Because of most the hydrophobic aldo-derivatives gives a higher signals than the native glycan in instrumental determination so in recent years, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS with tandem mass instrument has become a powerful tool for the structural determination of the non-derivatived/derivatived glycans.

MS is capable of providing structural information for oligosaccharides, although complete structural determination typically required several analytical technologies including tandem mass, GC/MS linkage analysis, *exo*-/*endo*-glycosidase digestions, and NMR (Yoo & Yoon 2005; Kim et al., 2006; Kukushkin et al., 2011; Yang et al., 2011). MS methods are used to have molecular weights and fragment ions information producing with tiny sample loading, on the other hand, MS provides a higher sensitivity than other glycan analytic methods. Combinations of chemical labeling, tandem mass spectrometry may be used to build signals to define the structures of glycans present from biological sources. Here we introduce four commonly used labeling methods for glycan. One is reductive amination labeling (Harvey 2011), a number of regents are commercially available for reductive amination reaction such as 2AB, 2AA and AP tags. Second is a new glycan tagging method with NAIM tag (Lin et al., 2008; Lin et al., 2010a; Chang et al., 2011; Lin et al., 2011). Aromatic *otho*-diamines are used to label reducing end of glycan presence with catalytic amount of iodine to form a serial of glycan-NAIM derivatives. These glycan-NAIMs provide superiority in enhancement ionization of saccharides base on their size, molecular weight and linkage since saccharide-NAIM derivatives exhibit a stronger mass intensity than native sugar in MALDI time-of-flight (TOF) MS analysis. The third is 1-phenyl-3-methyl-5-pyrazolone (PMP) derivation (Taga et al., 2001; Kodama et al., 2006), which condensd PMP with some monosaccharides to form aldo-(PMP)2 derivatives, and these derivatives were resolved by micellar electrokinetic chromatography (MEKC) using (*S*)- or (*R*)-dodecoxycarbonylvaline as the chiral selector for enantiomeric analysis. The last is permethylation of glycan

(Hakamori 1964) results in the conversion of all hydrogen atoms that are bound to oxygen and nitrogen atoms to methyl groups and serves to render glycans hydrophobic. Permethylated carbohydrates are considerably more stable than native glycans and produce more information on tandem mass spectra. Tandem MS with MALDI and ESI applied permethylated polysaccharides in glycan mixtures is powerful tool for saccharides' linkage analysis. In this context we described mass based approaches for chemical derivatized glycans such as tagged and permethylated oligo-/poly-saccharides. And also these linkages information of permethylated glycans can be elucidated by tandem mass for structural determination.

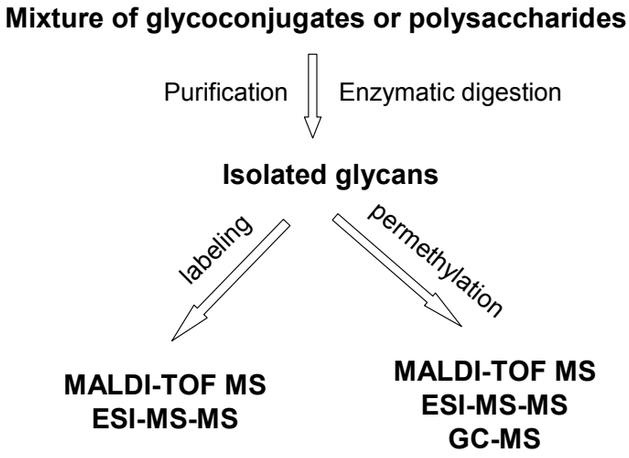


Fig. 1. The strategy for the mass determination of the glycans.

2. Mass ion production and ionization methods

Mass spectrometric ionization of carbohydrates has been reviewed recently (Zaia, 2008; Havery, 2011). Therefore, this section will provide a brief description of two main ionization methods, MALDI and ESI, and highlight some specific requirements for glycan sample preparation that improve overall efficiency of the MS ionization process. The analysis of complex polysaccharides by mass spectrometry is limited by the phenomenon of ion suppression. MALDI entails mixing analyte molecules with an organic matrix, such as 2,5-dihydroxybenzoic acid (2,5-DHB) and 2',4',6'-trihydroxyacetophenone (THAP).

Singly charged ions predominate in MALDI-TOF determination is common. It is typically used for analysis of neutral or charged saccharides. MALDI MS has the advantage of sample preparation and a relatively high tolerance to salts and other contaminants. However, acidic carbohydrates (such as phosphorylated, sulfated, or sialylated glycans) are quite different during MALDI ionization and may be in various ionization models and mechanism.

ESI entails spraying a solution containing the analyte appropriately charged droplets move toward the mass spectrometer orifice while undergoing solvent evaporation. This process results in the formation of multiple charged gas phase ions that are analyzed in the mass spectrometer. ESI is used with many types of mass spectrometers and is well suited to the analysis of methylated saccharides. ESI produces inherently better resolved peaks for

glycans due to the absence of matrix adduct peaks and provides better sugar-ring fragmentation ability than MALDI (Harvey 2000). In addition, permethylation protects all the hydroxyl groups on glycans to provide superior ionization than native glucans due to the low volatility of hydroxyl groups for ionization during the MALDI and ESI process.

2.1 MALDI mass spectrometry of polysaccharides

The MALDI-TOF ionization efficiency for neutral carbohydrates oligomers has been reported (Harvey et al., 1996; Zaia, 2004), where the ionization efficiency decreases with an increasing molecular weight. Therefore, chemical or enzymatic degradation and purification are essential prior to MALDI-TOF MS analysis for subsequent identification and structure elucidation. Analysis of *N*- and *O*-linked glycans from glycoproteins using MALDI-TOF mass spectrometry is common manipulation. The clean-up profiles of native and permethylated oligosaccharides for an efficient MALDI-TOF MS analysis have been described (Morelle et al., 2007; Zaia, 2010).

There are less of native polysaccharides giving MALDI-TOF MS analysis without degradation and derivation. For examples, Sturiale1 et al. (2005) resolved and identified of Gram-negative bacteria through MALDI-MS of native R-type LPSs. The simples can be successfully and systematically adopted for the analysis of these complex biomolecules without prior chemical degradation. This is quite important since a bacterial R-type LPS is actually a mixture of similar molecules and a MALDI mass spectrum provides the relative intensities among the different species. Structure determination of β-glucans from *Ganoderma lucidum* with MALDI mass spectrometry also reported (Hung et al., 2008). The mass range of detectable polysaccharides is 2000 Da in average.

Pullulans are polysaccharides produced from different strains of the fungus *Aureobasidium pullulans*. Pullulans play an important role in analytical chemistry since they are commonly used as calibration standards in aqueous SEC. Masses below 5 kDa are detectable by mass spectrometry as an alternative method providing direct data on the molar masses. NanoESI-MS analysis of pullulans was successfully carried out with a sample of weights average molar mass of approximately 5900 Da (Bahr et al., 1997). It was possible to obtain a more or less uniform charge state by addition of three sodium ions per molecule. For samples with higher masses it is increasingly difficult to get such simple spectra. However, the MALDI process is known to produce predominately singly charged ions. Different matrices have been used for the analysis of pullulans, like 2,5-dihydroxybenzoic acid (Stahl et al., 1991; Garozzo et al., 2000), 2,4,6-trihydroxyacetophenone (Hsu et al., 2007) and nor-harmane (Fukuyama et al., 2005). The use of the ionic liquid matrix like 2,5-dihydroxybenzoic acid butylamine (DHBB) turned out to be well suited for the analysis of pullulan samples in terms of signal intensities of very high mass polymers (Chang et al., 2011b).

In recent years, MALDI MS has become a powerful tool for the determination of the characteristic molecular weights of polymers. Other techniques like electrospray ionization are known to generate multiple charged ions and provide complex spectra due to a superposition of mass and charge distribution. Due to the huge mass range of polymers with broad distributions the commonly used combination of MALDI ion source with TOF that is well suited for the determination of molar mass distributions. Moreover, TOF instruments have a nearly unlimited mass range if the polysaccharides can be departed with matrix by laser beams. There are some further effects that influence mass like the voltage parameters of the MALDI-TOF instrument, the laser power, the choice of the matrix and ionizing agent and the nature of the analytes are also involved.

Some polysaccharides containing charges are easier to determine than neutral glycans. For examples, the characterization of polysialic acids by a high diversity mass based method allows a rapid, highly sensitive, and unambiguous identification of native polysialic acid as well as fluorescently labeled sialic acid polymers without the need of standard substances due to exact mass determination (Galuska et al., 2007). In addition, some tandem mass spectrometry for structural determination of permethylated sialic acid oligosaccharides are also reported (Wheeler & Harvey, 2000; Yoo & Yoon, 2005; Pabst & Altmann, 2008) in recent years.

2.2 Effect of matrix in MALDI MS for polysaccharides determination

The first investigation of non-derivatized oligosaccharides by MALDI with 3-amino-4-hydroxybenzoic acid as matrix was reported by Mock et al. (1991). Stahl et al. (1991) subsequently discovered that 2,5-DHB yielded better reproducibility and higher signal-to-noise ratio than 3-amino-4-hydroxybenzoic acid. Since then, DHB has become the primary choice of matrix for oligosaccharides. Improvements in sensitivity with a concomitant improvement in resolution were achieved with the addition of 10% 2-hydroxy-5-methoxybenzoic acids to DHB (Wang et al., 1993) and these co-matrices were referred as 'super-DHB'. 1-Hydroxyisoquinoline (Mohr et al., 1995) was also found to be an effective additive to DHB to produce more homogeneous samples for MALDI detection. DHB with or without additives has been broadly used as matrix on MALDI for neutral saccharides. Several other matrices have also been reported (Nonami et al., 1998; Harvey, 1999; Mirza et al., 2004). Recently, Hsu et al. (2007) reported THAP as matrix for MALDI of neutral polysaccharides with molecular weight up to approximately 5,000 Da. For example, a linear neutral polysaccharide with m/z higher than 47,000 was readily detected by MALDI using THAP. Use of THAP as matrix always yielded high quality spectra with good reproducibility in their study.

In addition, positive-/negative-ion MALDI MS of saccharides such as dextran 8,000 Da with 2,5-DHB as matrix (Hao, et al., 1998). The matrix-to-analyte mole ratio was about 10,000. The matrix plays a more important role in the ionization process for oligosaccharides, while in the desorption process for polysaccharides (Chang et al., 2007). There have been only a few matrices reported for detection of polysaccharides with molecular weight higher than 3,000 daltons by MALDI mass spectrometry (Hao, et al., 1998; Hsu et al., 2007). Large polysaccharides, dextrans, glycoproteins and polysialic acids are still under challenge to detect by MALDI MS with various matrices.

2.3 Ionic liquid-assisted electrospray ionization of polysaccharides

Ionic liquids are organic or semiorganic salts with a low vapor pressure. Due to their ability to dissolve a wide range of analytes they have been used in a number of analytical techniques (Schnöll-Bitai, et al., 2008). In 2001, ionic liquids were introduced as matrices in MALDI MS for the analysis of biomolecules and synthetic polymers (Tholey & Heinzle, 2006). The first ionic liquid matrices (ILMs) were synthesized of the commonly used matrix substances sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) combined with a variety of cations based on amine structures (Armstrong et al., 2001). An example of spectroscopic application is the determination of pullulans. The combination of 2,5-DHB with butylamine (DHBB) turned out to be well suited for the analysis of oligosaccharides and glycolipids (Mank et al., 2004; Laremore et al., 2006, 2007). The same ILM was used for

the determination of the molecular mass distribution of polysaccharides (pullulans) which are used as calibration standards in aqueous size-exclusion chromatography (Schnöll-Bitai et al., 2008). In most cases the ionization process in ILMs led to protonated species of the analytes, very similar to solid matrices. However, the analysis of large glycans by ILMs seems to be impeded by the fact that these molecules tend to form complexes with the cations or anions of the matrix.

Recently, ionic liquid-assisted ESI (ILA-ESI) mass spectrometry has been improved for detection of large neutral polysaccharides (Chang et al., 2011). Detection sensitivity of polysaccharides by adding various ionic liquid compounds into samples was improved by ESI or MALDI-TOF mass spectra. Mass spectra obtained were greatly simplified and appeared to be similar to spectra from MALDI due to the narrow charge number distribution.

2.4 ESI mass spectrometry of saccharides

Electrospray ionization techniques for saccharides determination are used in the large amount of published work from 1980 era (Whitehouse et al., 1985; Meng et al., 1988; Zaia, 2007). Conventional ESI-MS (Meng et al., 1988; Fenn et al., 1990) involves the pumping of a solution into the ion source, and has been observed to produce relatively weak ion signals for native oligosaccharides compared to those for peptides and proteins (Burlingame et al., 1994; Reinhold et al., 1994; 1995). ESI produces ion signals that are comparable between the peptide and carbohydrate compound classes. Therefore, it appears that the hydrophilicity of oligosaccharides limits the surface activity in ESI droplets and their sensitivity is significantly enhanced. The sensitivity increase observed when oligosaccharides are derivatized cause by reducing their hydrophilicity that increased their volatility in the surface (Karas et al., 2000). In fact the ESI of carbohydrates appears to be less effective upon the nano grams than MALDI-TOF for glycans. Interfaces for on-line ESI LC/MS typically produce droplet sizes to those produced by the use of MALDI for neutral oligosaccharide analysis, particularly for applications that involve the profiling of mixtures released from glycoproteins. Although fragmentation allows the analysis of carbohydrate ions, which is caused by the higher internal energies imparted to the ions for structural analysis using ESI method, however, ESI is low efficiency in its ionization process in native oligo-/polysaccharides.

2.5 LC-MS/MS spectrometry of glycoproteins

Mass spectrometry evolved as a key technique in the analysis of proteins and their post translational modifications. *N*-linked oligosaccharide provides a relative chromatographic quantification via HPLC and subsequent identification via MS. The procedure demonstrates that the glycan hydrolysis, derivitization, and chromatographic separation. Subsequent analysis of the chromatographic peaks via LC/MS or LC/MS/MS will yield additional data to confirm the identity of the glycan, and allow deconstruction of the glycan for additional information on its branch and sub-unit structure. Some of peptide mapping methods will give glycosylation sites, identification of the glycan and its structures. The information may provide insights into the heterogeneity of the glycosylation. Sequence-based peptide analysis by LC-ESI-MS/MS is most often applied for identification or quantification of proteins in typical "proteomics" projects. Careful evaluation of the peptide-mass fingerprinting data allows determining the glycan composition at individual glycosylation

sites. A precious side product is the possibility of confirming the protein termini and of eventually revealing other post-translational modifications. Both MALDI-TOF MS and LC-ESI-MS can generate such data but LC-ESI generally provides higher sequence coverage. The multiple charged ions formed by ESI also facilitate MS/MS experiments, which substantiate any conclusions on the nature of presumed glycopeptides.

Recently, Wang et al. (2011) described consists of 2D HPLC fractionation of intact proteins and liquid chromatography multistage tandem mass spectrometry (LC-MS/MS^n) analysis of digested protein fractions. A digital ion trap mass spectrometer with a wide mass range is then used for LC-MS/MS^n analysis of intact glycopeptides from the 2D HPLC fractions. The standard approach for peptide-based glycoprotein analysis starts with bands of Coomassie-stained polyacrylamide gel. After S-alkylation and digestion with trypsin the resulting mixture of peptides and glycopeptides is separated by capillary reversed-phase HPLC and analyzed by ESI-MS and/or ESI-MS/MS. The free glycan analysis from isolated cells or from whole tissues are preferred by MALDI-TOF MS especially in the case of neutral glycans, e.g. from plant, fungi or bacteria cell wall polysaccharides. LC-ESI-MS is chose for sialylated oligomers or mixtures of sialylated and neutral glycans especially when structural information is desirable.

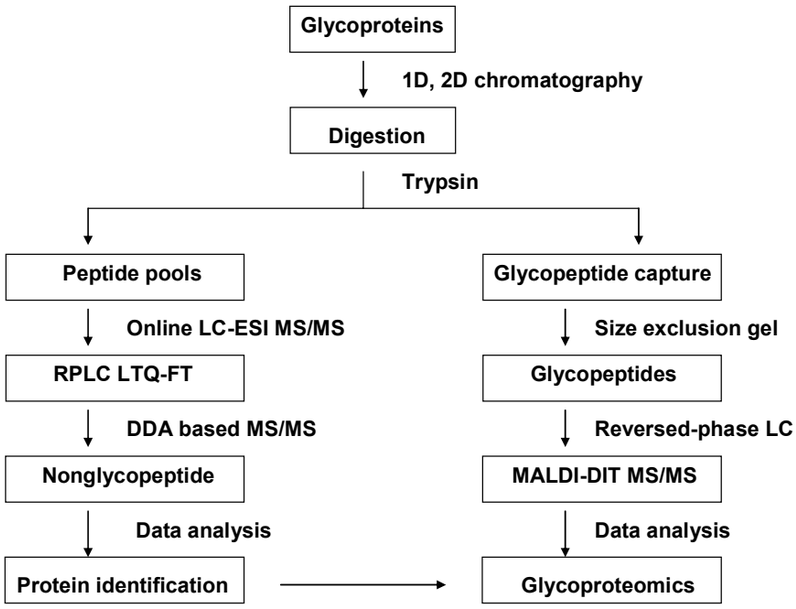


Fig. 2. A general overview of the procedure for profiling glycoproteins. Sample is subjected to an immuno-depletion chromatography followed by 2D HPLC fractionation. The digested 2D HPLC fractions are then analyzed by LC-MS/MS (Wang, et al., 2011). Abbreviation: DDA represents data dependent acquisition, DIT represents digital ion trap, LTQ-FT represents linear trap quadrupole Fourier transformation, and RPLC represents reversed phase liquid chromatography.

3. Strategy for the mass determination of glycans

Tandem MS of native and tagged glycans may be acquired using either positive or negative ions. Commonly, positive ion model of tandem MS is abundant glycosidic bond product ions that occur adjacent to HexNAc, sialic acid, and Fuc particularly abundant (Zaia, 2010; Harvey 2011). In summary, tandem MS produces the greatest structural detail on permethylated glycans. Negative ion tandem MS is effective for producing useful structural information on native and tagged glycans, such as sialic acid containing, sulfated and phosphorylated glycan and those classes are commonly not compatible with permethylated glycans (Wheeler & Harvey, 2000; Larsen et al., 2006; Miller et al., 2006; Mechref et al., 2006; Lei et al., 2009; Yu et al., 2009; Barnes et al., 2011). For tagged glycans the mass shift varies according to the glycan's derivatives. The differentially labeled samples are combined prior to MALDI MS analysis to minimize sample-to-sample variation in peak abundances and maximize the ability to perform the comparison of two samples. Thus, it is possible to analyze the mixtures directly in the MS mode, or to select the nominal masses for subsequent tandem mass spectrometric analysis of glycoform mixtures.
Stable labels are used commonly in saccharide analysis to improve determination of glycans in the sample. Chemical derivatized saccharides increase volatility and stability for MS analysis and helpful to purify them in chromatography when they were labeled. Depending on the sample and extent of the information needed, several different labels can be used as described in subsections below.

3.1 Tagged polysaccharide with reductive amination labeling

Reductive amination derivatization is very useful tool for mass spectral analysis of glycans. A number of reductive amination reagents are commercially available (Yuan et al., 2005; Hitchcock et al., 2006; Zaia, 2008). During the derivatization, an aromatic amine forms a Schiff base at the acyclic reducing sugar residue. The resulting Schiff bass is then chemically reduced by sodium cyanoborohydride ($NaBH_3CN$) to form a stable labeled glycan. Both steps of the derivatization can be performed in a single reaction (Klapoetke et al., 2010). Nevertheless, glycan tagging has higher ionization efficiency than native glucans. A recent review for derivatization of carbohydrates for analysis by chromatography and mass spectrometry was published by Harvey (2011). The most approach is labeling the reducing end of the glycans with reductive amination to generate fluorescence for glycan profile by LC-fluorescence and by MS for glycan identification. In this approach, glycan profiles were readily obtained due to high fluorescent sensitivity imparted by the labeling agent.

3.2 Tagged polysaccharide with aldo-NAIM labeling

An alternative method for the conversion of native aldose to aldo-naphthimidazole (aldo-NAIM) has been developed (Lin et al., 2008; Lin et al., 2010a; Lin et al., 2010b; Lin et al., 2011). Using iodine as a catalyst in acetic acid solution, a series of mono-, oligo-, and polysaccharides, including those containing carboxyl and acetamido groups, progresses an oxidative condensation reaction with aromatic vicinal diamines at room temperature to give the corresponding aldo-NAIM products in high yields (Fig. 3). In addition, a series of aldo-NAIMs was determined by MALDI–TOF MS to analyze molecular weight and ion intensity. For instance, 2,3-naphthalene diamine-labeled Ling-zhi polysaccharides showed enhanced signals in MALDI–TOF MS (Fig. 4; Lin et al., 2010b).

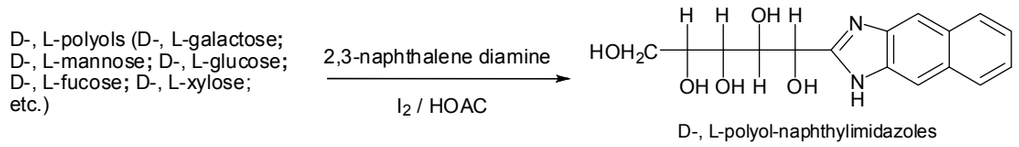
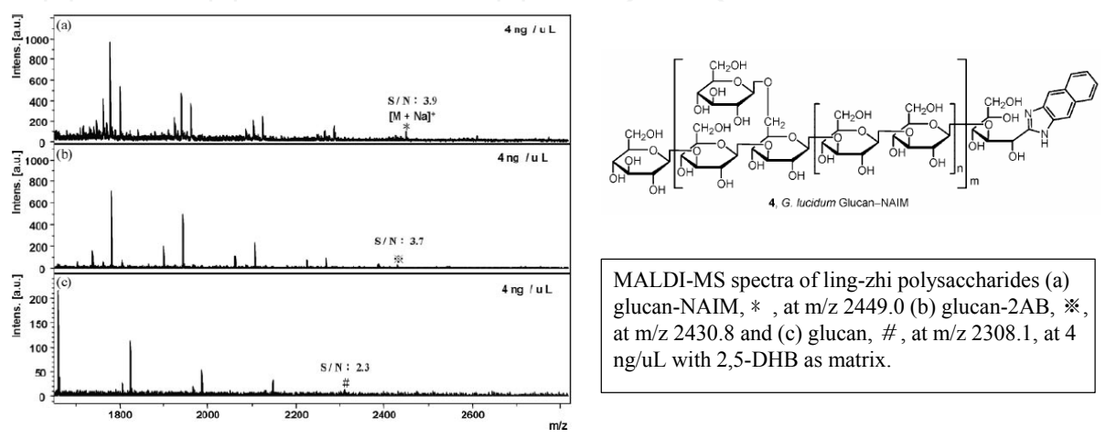


Fig. 3. The preparative method for the conversion of native aldose to aldo-NAIM.



MALDI-MS spectra of ling-zhi polysaccharides (a) glucan-NAIM, ＊ , at m/z 2449.0 (b) glucan-2AB, ※, at m/z 2430.8 and (c) glucan, ＃, at m/z 2308.1, at 4 ng/uL with 2,5-DHB as matrix.

Fig. 4. The corresponding naphthimidazole derivative of Ling-zhi polysaccharides showed the enhanced signals property in the MALDI–MS spectrum.

These aldo-tagged derivatives give a higher sensitivity than the native glycan in common. Not only mono-/disaccharides but also oligo-/polysaccharides were labelled in straightway method. For example, pullulan (molecular mass distribution 2,500~6,000) was tagged with 2,3-naphthalene diamine to obtain pullulan-NAIM derivatives for MALDI-TOF mass spectrometry analysis (Fig. 5). Because this pullulan sample is a polydispersable natural polysaccharide with 15–40 DP (degree of polymerization), pullulan-NAIM displayed their mass signals with a difference of 162.1 Da between neighboring peaks. For instance, the characteristic signals [pullulan-NAIM (DP = 18 + Na]$^+$ at *m/z* 3096 and [pullulan (DP = 36) + Na] $^+$ at *m/z* 6012 are shown in Fig. 5, respectively. Even with as little amount of analyte the signal is still measurable.

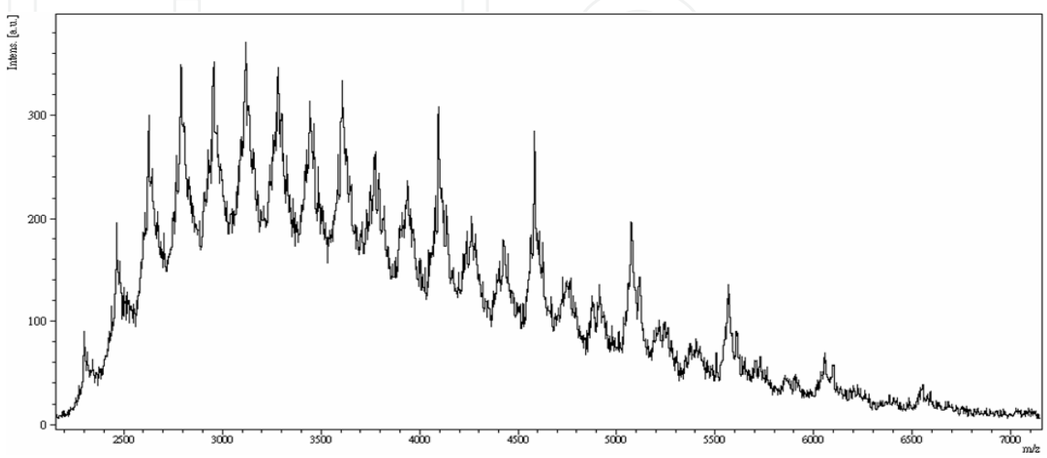


Fig. 5. The corresponding NAIM derivative of pullulan polysaccharides in the MALDI–MS.

3.3 Tagged saccharide with PMP and indole

Glycan labeling strategies are useful in identification and quantification of saccharides. (Ruhaak et al., 2010). Honda have reported the condensation derivatives of some monosaccharides with 1-phenyl-3-methyl-5-pyrazolone (PMP) in Fig. 6, which were resolved by micellar electrokinetic chromatography (MEKC) using (*S*)- or (*R*)-dodecoxycarbonylvaline as the chiral selector (Honda et al., 1997; Taga et al., 2006). The racemic monosaccharides such as PMP-D-/L-Man, PMP-D-/L-Gal, and PMP-D-/L-Fuc were enantioseparated by ligand-exchange capillary electrophoresis (Kodama et al., 2001). Kuo et al. (2011) reported a series of aldo-bis-indole (aldo-BIN) derivatives (Fig. 6) was prepared by aromatic *C*-alkylation reaction to condense aldose with two molecular indoles in water/acetic acid solution for enantioseparation of racemic monosaccharides. Common monosaccharides were derivatized smoothly to form the UV absorbable aldo-BINs. However, both tagging reagents failed in polysaccharide labeling due to the low reactivity.

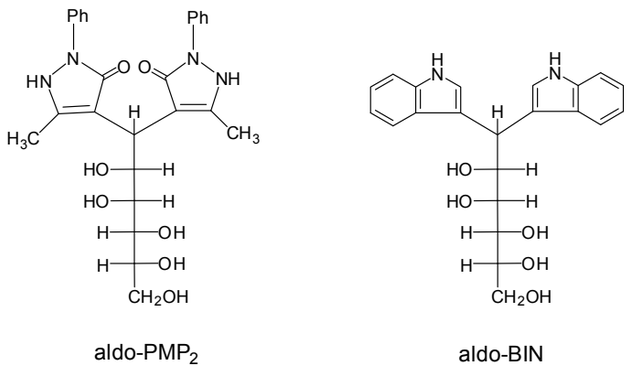


Fig. 6. The chemical structures of aldo-$(PMP)_2$ and aldo-BIN.

3.4 Permethylation labeling of polysaccharides

Permethylation and reductive amination derivatization is most common tool for mass spectral analysis of glycans. Permethylation approach required methylation of all hydroxyl groups on saccharides. Permethylation improves MS sensitivity and stabilizes saccharides as well as glycuronic acids and sialic acids by converting the carboxylic groups into methyl esters. However, permethylation involves complicated sample preparation and clean up with liquid-liquid extraction. In brief, the desiccated samples were dissolved in dimethyl sulfoxide (DMSO) suspension, which was prepared by vortexing DMSO and powdered sodium hydroxide (NaOH) or sodium hydride (NaH) at room temperature, excess of methyl iodide (MeI) was added, and the solution was kept for hours at room temperature with occasional vortexing (Ciucanu & Kerek, 1984). After finished reaction, the sample was partitioned by adding chloroform, the suspension was extracted times with diluted acetic acid aqueous solution, and the chloroform layer was concentrated. The sample was stored at -20 °C prior to analysis. An example of this labeling approach is demonstrated using a comparative glycoform mapping method (C-GlycoMAP), developed based on differential stable isotope labeling (Kang et al., 2007). The differentially isotope labeled samples are combined prior to MALDI-TOF MS analysis to minimize sample-to-sample variation in

peak abundances and maximize the ability to perform the comparison of samples. However, to purify permethylated glycans (no chromophore) is quite difficult, so for smaller amounts of samples, they were analyzed directly after extraction and washed with aqueous dilute bicarbonate solutions.

Permethylation is also reported to be particularly useful for in-depth analysis of glycans as it provides information on linkage and branching. So, methylation is a traditional method that provides GC/MS and tandem mass for structural determination of glycans. Strategies for acquisition and interpretation of multistage MS have been most fully developed for permethylated glycans (Ashline et al., 2005). For example, Hung et al. (2008) measured permethylated *G. lucidum* glucans using 2,5-DHB as a matrix. The *G. lucidum* glucans were observed as sodium attached ions and molecular masses were calculated as 219.13 (a terminal sugar) + (204.13)n + 22.99 Da and 219.13 + (204.13)n + 31.02 (mass of reducing end residue) + 22.99 Da, respectively (Fig. 7).

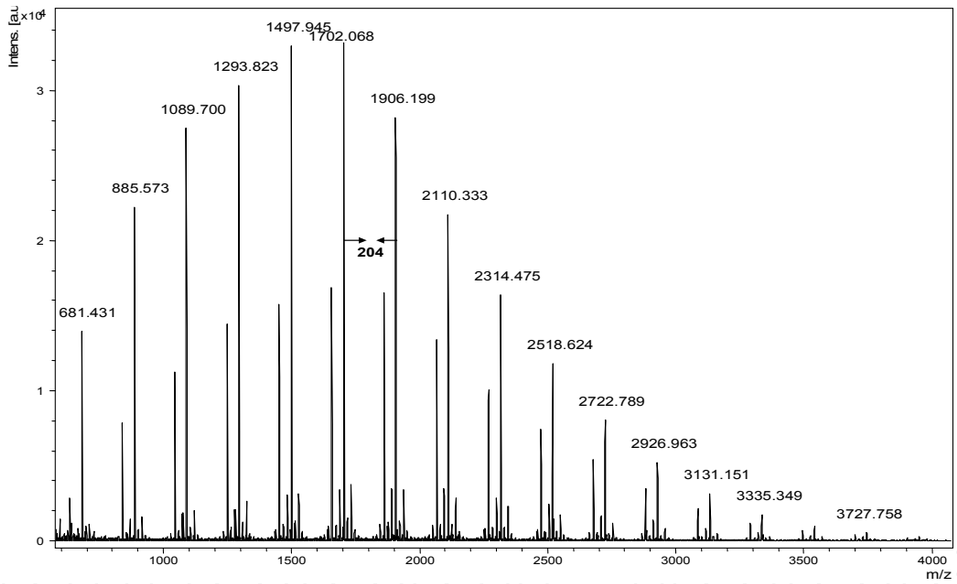


Fig. 7. MALDI-TOF mass spectrum of permethylated *G. lucidum* glucans with 2,5-DHB as matrix. A peak-to-peak mass difference of 204.1 Da is observed. The molecular masses were measured as sodium attached ions at 681.4; 855.6; 1089.7; 1293.8; 1497.9; 1702.1; 1906.2; 2110.3; 2314.5; 2518.6; 2722.8; 2927.0; 3131.2; 3335.4; 3539.6 and 3727.8 (DP = 3~18), respectively.

In addition, permethylated xylans were observed as sodium attached ions with peak-to-peak mass difference of 160.1 Da (Fig. 8). One of polysaccharides from alginic acid, which is a kind of polysaccharide mixture of hexose and aldouronic acid was derivatived by NaH/MeI in DMSO to get permethylated alginic acids and following determined by MALDI-TOF MS. The peak-to-peak mass difference was observed in two series 204.1 Da (Hex) and 218.2 Da (HexA), respectively (Fig. 9).

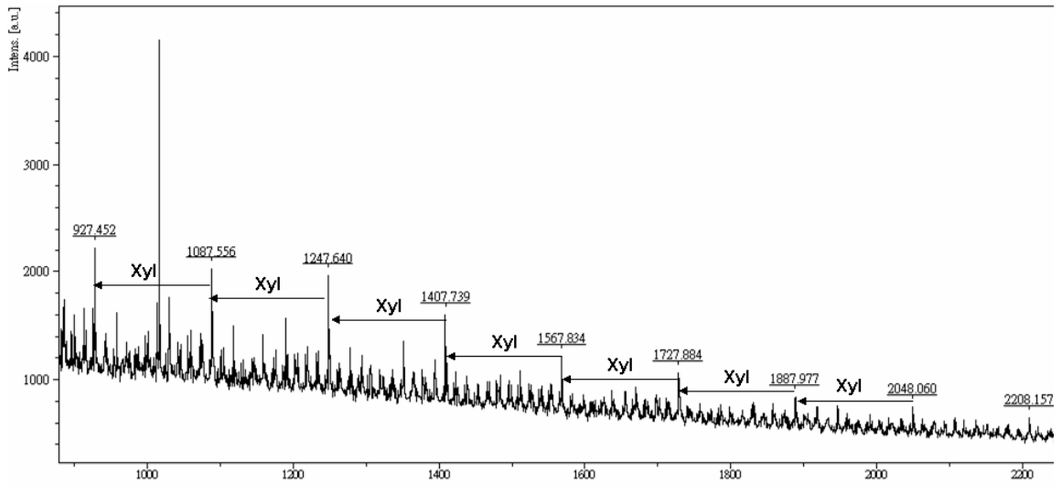


Fig. 8. MALDI-TOF mass spectrum of permethylated xylans with 2,5-DHB as matrix. A peak-to-peak mass difference of 160.1 Da is observed.

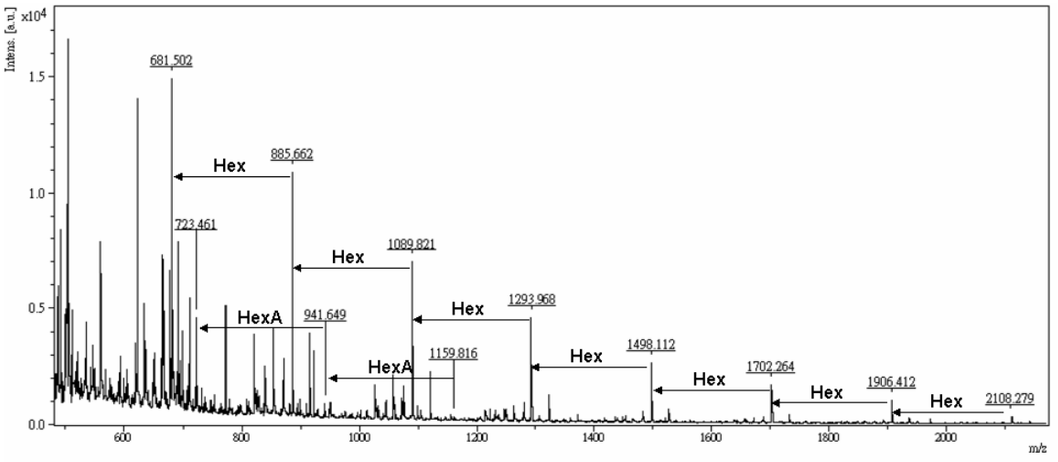


Fig. 9. MALDI-TOF mass spectrum of permethylated alginic acids with 2,5-DHB as matrix. The peak-to-peak mass difference of two series is 204.1 Da and 218.2 Da, respectively.

The advantage to this approach is that tandem mass spectrometric dissociation of a glycosidic bond leaves a site that lacks a methyl group that is clearly indicated by mass. Thus, the linkage position is indicated by the mass of crossring cleavage ions (A- or X-types). It is possible to differentiate some types of positional isomers based on the formation of specific product ion types. Tandem MS of permethylated glycans produces more structural detail than does that of native and reductively aminated glycans.

4. Tandem mass of methylated polysaccharides for structural determination

The challenges to polysaccharides determination are that the glycan-moiety has different chemical properties than proteins or nucleic acids (Forsberg et al., 2000; Faber et al., 2001; Lattová et al., 2005; Mischnick et al., 2005; Nielsen et al., 2010) and most of them are

insoluble that makes problems in chemical manipulation such as permethylation and reducing end labeling. Since tandem MS for polysaccharide hasn't been reviewed in detailed, only a few of methodology and reports have been described. However the key aspects of fragmentation procedures are same with those glycoproteomics studies on oligosaccharides and may applied for further study on other source of polysaccharides. Here we introduce and review some results on tagged and permethylated polysaccharides by tandem mass spectrometry.

Tandem MS with MALDI and ESI used permethylated polysaccharides is powerful tool for saccharides' linkage analysis. The use of tandem MS is driven by the need to produce structural information on glycans (Harvey, 1999; Zaia, 2004). The tandem MS experiment is to analyze a mixture of positional isomers directly. Sequential stages of tandem MS are performed in series and the stages of MS are abbreviated MS^n. The masses of the product ions of glycan substructures may be selected for dissociation in further stages. In addition, tandem mass analysis of permethylated glucan can be refered to GC/MS on their methylated acetyled alditols and in comparison with the spectra of synthetic standards for structural analysis of polysaccharides. Most glycan tandem mass spectra are produced by collisional induced dissociation (CAD), a technique in which selected precursor ions are dissociated by collision with gas atoms in a collision cell. Typically, the weakest bonds rupture to produce the most abundant product ions. It is possible to dissociate permethylated glycans using high energy CAD that uses a MALDI TOF/TOF or ESI MS instrument, under which conditions bond rupture is kinetically controlled and cross-ring cleavage ions are more abundant for structural analysis.

4.1 Nomenclature for tandem mass spectrometric ions of glycans

The nomenclature for glycan and glycoconjugate product ions (Domon & Costello, 1988) is shown in Fig. 10 and will be used throughout this section. Product ions containing a non-reducing terminus are labeled as A, B, C, and those containing the reducing end are labeled X, Y, Z. Cleavages across residue rings (A-and X-type ions) are particularly useful for determining linkages.

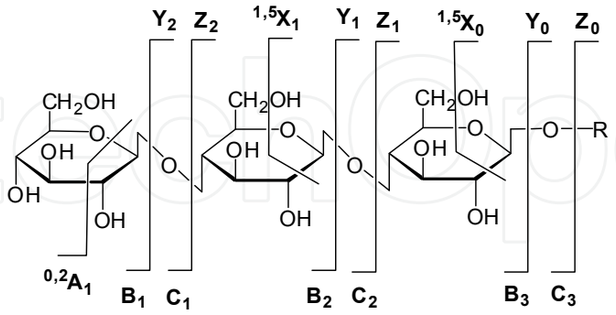


Fig. 10. Nomenclature of tandem mass spectromic ions of glycans and glycoconjugates (Domon & Costello, 1988).

4.2 Characterization of *N*-, *O*-linked glycans from glycoproteins

Recently, Zaia (2010) has reported tandem MS of isomeric glycan mixtures. For glycoconjugates tandem mass spectrometric ions are cleavaged glycan occurs by rupture of glycosidic bonds (B, C, X, Y types) or across rings (A and X types) based on defined by

Domon & Costello (1988). Product ions containing the original nonreducing oligosaccharide end are A, B, and C types, and those containing the original reducing end or aglycon are X, Y, and Z types. Permethylation of glycans has particular advantages for tandem mass spectrometric structural analysis because all glycan OH and NH groups are derivatized. As a result, glycan bond scission occurring during tandem MS creates unmodified sites, which indicate which bond has been cleaved. The linkage position for a given monosaccharide is therefore indicated by the masses of the crossring cleavage ions (A or X types). The crossring cleavage ion masses are useful for determining the linkages and masses of substituents. These principles have been developed into a strategy for determination of glycan linkage and branching structure (Ashline et al., 2005). Tandem mass spectra of native and reductively aminated glycans produce less definitive structural information because the glycosidic bonds cleaved during dissociation do not leave a mass of ring fragments. Thus, it is only possible to determine residue linkage sites when a crossring cleavage is observed to that residue in which the substituents remain intact.

For example, in positive ion mode, native and reductively aminated glycans form abundant product ions from cleavages adjacent to labile NeuAc, HexNAc, and Fuc residues. Crossring cleavages to branching residues are typically low in abundances. Such linkages may also be differentiated in the negative mode using MS^2 of deprotonated ions in which an ion corresponding to A ions correlates with an α-2,6 isomer (Wheeler & Harvey, 2000). In addition, an MS^n series may be used to differentiate glycan linkages by dissociation of C- or D-ions in sialic acid linkage isomers (Deguchi et al., 2007; Ito et al., 2007). For permethylated glycans, the masses of specific A-type ions occurring to the saccharide residues. These fragment ions have been used to differentiate sialic acid linkages using modern MALDI-TOF instruments (Mechref et al., 2006). The determination of the glycan branching structures using tandem MS entails the observation of crossring cleavage ions occurring about the branching residues. Native or reductively aminated glycans observed as sodiated precursor ions dissociate to form A-type ions to the core branching mannose residue of *N*-glycans that may be used to determine the compositions of the three and six branches (Harvey, 2000). Such ions are often observed in increasing abundances for deprotonated precursor ions in the negative mode, as has been observed for branched milk oligosaccharides (Chai et al., 2002) and *N*-glycans (Harvey, 2005).

The formation of the D-ion is particularly useful since it occurs only for three-linked residues. Electron detachment dissociation (EDD) of native glycans has been shown to produce tandem mass spectrometric patterns that are particularly useful for deriving structural information (Adamson & Hakansson, 2007). On the other hand, a single stage of dissociation gives rise to B- and Y- type ions. Single-stage MS of permethylated glycans is a well-established approach for determining molecular weights. A-type crossring cleavages are often abundant, and serve to define the compositions of antenna when they occur to branching residues. Tandem MS of permethylated glycans carries the advantage that ions formed from cleavage of glycosidic bonds have a unique mass value that distinguishes an internal fragment from a single bond cleavage. Multistage dissociation of permethylated glycans has been used to determine detailed linkage information for saccharide units formed by gas phase disassembly of the glycans (Zhang et al., 2005; Prien et al., 2008, 2009; Jiao et al., 2011). The key to multistage dissociation of glycans is the selection of a series of precursor ions that isolate structural branches for subsequent

stages. Subsequent dissociation of B-type ions yields crossring cleavages that are useful for determining linkage.

4.3 Characterization of plant glycans

Naturally occurring plant saccharides are huge and comprise mixtures of various conjugated polymers. More recently ESI and MALDI combined with tandem mass spectrometry has been shown to provide valuable structural information for plant polysaccharides. For instance, three D-xylan type per-*O*-methylated saccharides with various types of linkages between the D-xylopyranose units were examined by mass spectrometry (Bagag et al., 2008). In addition, polysaccharides are the principal components of the plant cell wall from its main structural framework. In high plants the three main polysaccharides of the cell wall are cellulose, pectin and hemicellulose. Fernández et al. (2003) has reported the structural characterization of arabinoxylans from wheat by MALDI-TOF and ESI-Q-TOF MS. An arabinoxylan sample digested with endoxylanase was analyzed, the resulting in identification of molecular ions for saccharide residues with up to 22 DP. The permethylated arabinoxylan was also performed to obtain structural information regarding arabinose branching and xylose backbone.

4.4 Characterization of fungi glycans from *Ganoderma lucidum*

Ganoderma lucidum (a medicinal fungi in Asia) has been used for a long time to prevent and treat various human diseases (Lin et al., 2006; Cheng et al., 2007; Hua et al., 2007; Ji et al., 2007; Zhu et al., 2007; Chuang et al., 2009; Chen et al., 2010; Lai et al., 2010). The mainly components of *G. lucidum* polysaccharides are (1→3) and (1→6)-β-D-glucan. Beta-D-glucan is a carbohydrate polymer with chains of glucose molecules linked together by β-glycosidic linkages (Sone et al., 1985; Usui et al., 1983; Wang et al., 2002). Beta-glucan isolated from *G. lucidum* having quite huge of molecular weights and is challenge for studies using the MALDI-MS method. Hung et al. (2008) have analyzed non-derivatized and through permethylated derivatized *G. lucidum* polysaccharides. The permethylated *G. lucidum* glucan was measured, which is derived from acidic degradation (Fig. 11 up). And its tandem mass MS^2 at *m/z* = 1293.7 of this permethylated *G. lucidum* hexasaccharide was dominated by peaks resulting from cleavage at glycosidic bonds, giving the C/Y ion series and a less intense series of B/Z ions (Fig. 11 middle), which are same as the observation in the curdlan (one of fungi polysaccharide with β-1→3-D-glucan). The MS^2 spectrum of permethylated *G. lucidum* glucan B5 ion at *m/z* 1058.8 is shown in Fig. 11 bottom. The major fragment ions are the Y and C ions (1277.1, 1075.5, 871.3, 667.2, 463.1 *m/z*), respectively. However, the B5 ions differ between the permethylated *G. lucidum* hexasaccharide and other linkaged permethylated hexasaccharide such as malto- or dextro-hexaose. The fragmented ions from *m/z* at 1058.8 ~ 871.3 are 940.7 ($^{0,3}A_5$), 928.7 ($^{1,3}A_5/^{2,4}A_5$), 912.3 ($^{0,4}A_5$), 898.7 ($^{1,5}X_5$) and 883.1 ($^{2,3}A_5$), respectively. For the ions with m/z at 912.3 ($^{0,4}A_5$) and 898.7 ($^{4,5}A_5$), it indicates that *G. lucidum* glucan has 1→6 linkage and 883.1 ($^{2,3}A_5$) indicates out the presence of 1→3 linkage. Based on A and X ions in Fig. 11 bottom, we confirm that this methylated glucan has 1→3 and 1→6 linkage between glycosidic bonds.

The aforementioned example of the utility of multistage fragmentation of B-ions generated from permethylated *G. lucidum* glycan. A retro-Diels-Alder reaction in a 1,3-linked B5-type pentaose shows formation of fragment ions. The generic cross ring cleavages that may be formed from B-type ions of various linkages.

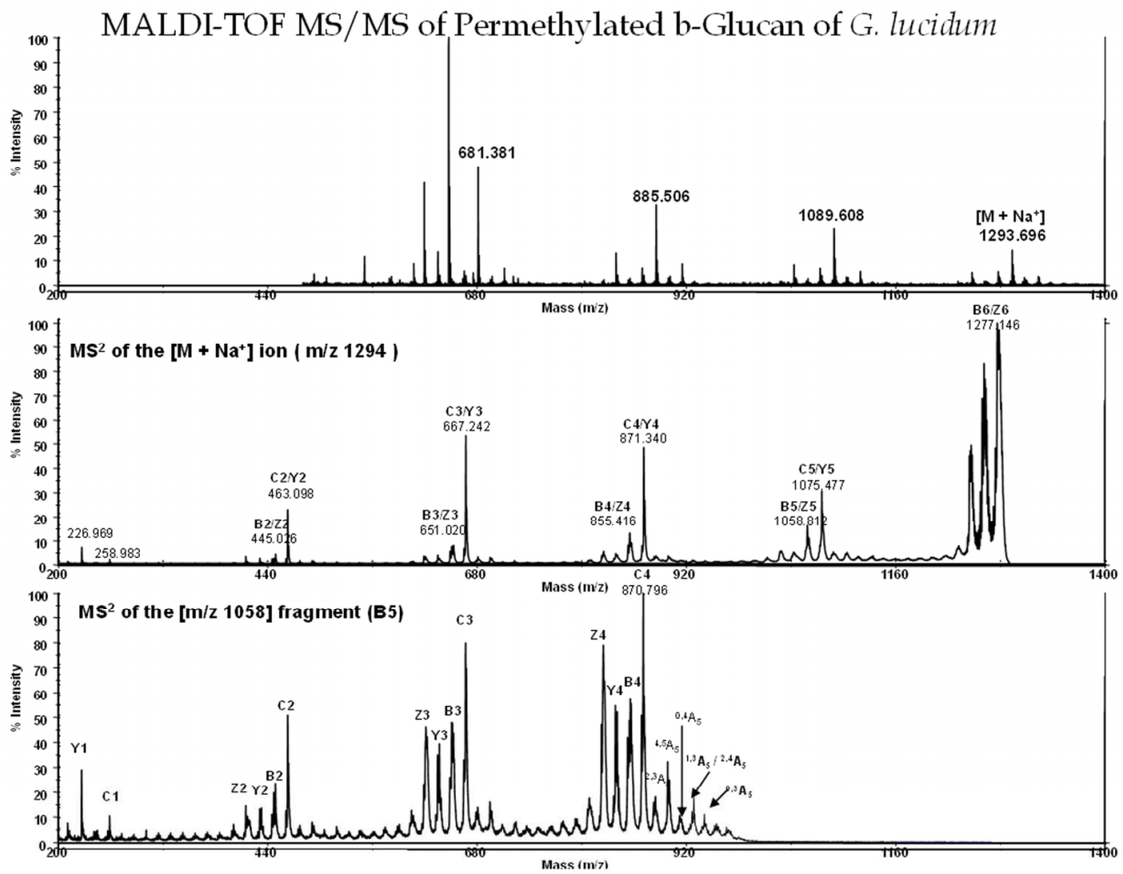


Fig. 11. MALDI-TOF MS/MS of permethylated *G. lucidum* glucans. The ion of permethylated *G. lucidum* hexasaccharide (*m/z* = 1293.7, $[M + Na]^+$) shown in up panel. MS^2 at the hexasaccharide ion ($[M + Na]^+$, *m/z* = 1293.8) shown in middle panel and MS^2 at the B5 sodiated fragment ion (*m/z* = 1058.8) shown in bottom panel. The major cross-ring fragment ions at B5~C4 region are 940.7 ($^{0,3}A_5$), 928.7 ($^{1,3}A_5/^{2,4}A_5$), 912.3 ($^{0,4}A_5$), 898.7 ($^{1,5}X_5/^{4,5}A_5$) and 883.1 ($^{2,3}A_5$), respectively.

5. Conclusion

This review introduced mass spectrometry of tagged glycans and the uses tandem mass spectrometry for permethylated glycans. These chemical derivatives are useful for the structural analysis of glycans and have been used to study the glycosylation of isolated complex glycoconjugates or polysaccharides in medicinal herbs or fungi. The structural information obtained from tandem mass studies is complicate but useful for glycan linkage information. The glycan in tandem mass also compatible with the derivatization conditions, permethylation remains the alternative choice. Using permethylation, ionization responses are increased over those of underivatized glycans, and the chemical stability improved. Multistage tandem mass spectrometric dissociation of permethylated glycans produces the greatest level of detail possible when using mass spectral techniques. Glycan classes modified with sulfate or other fragile substituents are not compatible with permethylation, but may use reductive amination method to label glycan in tandem mass determination. A

number of LC/MS and CE/MS approaches have been incorporated into comprehensive analysis for tagged and permethylated glycans. Biological important biomass glycans and glycoconjugates may be analyzed using tandem mass a combination of various electron dissociation methods.

6. Acknowledgment

The author appreciated a Grant-in-Aid for scientific research from the Genomics Research Center, Academia Sinica, Taiwan R.O.C.

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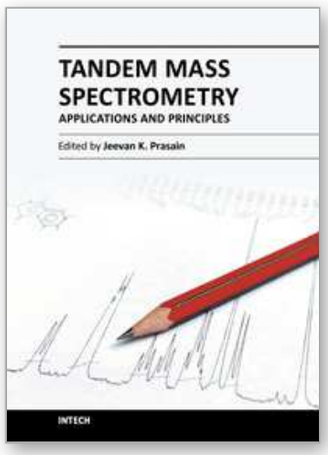

Tandem Mass Spectrometry - Applications and Principles

Edited by Dr Jeevan Prasain

ISBN 978-953-51-0141-3
Hard cover, 794 pages
Publisher InTech
Published online 29, February, 2012
Published in print edition February, 2012

Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Wen-Bin Yang (2012). Tandem Mass Spectrometry of Tagged and Permethylated Polysaccharides, Tandem Mass Spectrometry - Applications and Principles, Dr Jeevan Prasain (Ed.), ISBN: 978-953-51-0141-3, InTech, Available from: http://www.intechopen.com/books/tandem-mass-spectrometry-applications-and-principles/tandem-mass-spectrometry-of-tagged-and-permethylated-polysaccharides

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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