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Structural characterization of glycoprotein digests by microcolumn liquid chromatography-ionspray tandem mass spectrometry

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ABSTRACT

An in-house modified microcolumn liquid chromatography (LC) system has been coupled to a PE-SCIEX API III triple-quadrupole mass spectrometer through an ionspray interface for the structural characterization of model glycoproteins, bovine ribonuclease B and human α_1-acid glycoprotein. In conjunction with enzymatic digestion approaches using trypsin and peptide-N-glycosidase F, the feasibility of packed-capillary (250 μm I.D.) LC columns, coupled with ionspray mass spectrometry (MS) in a tandem format, have been assessed for glycopeptide mapping and structural determination. This configuration demonstrates a highly promising approach for the determination of glycosylation sites and the corresponding sequence structures of related tryptic fragments. A glycosylated tetrapeptide, Asn-Leu-Thr-Lys with carbohydrate moieties on Asn-34, was readily located for bovine ribonuclease B. Preliminary results using micro-LC-MS also show the identification of a class A carbohydrate attachment on a tryptic fragment of human cc,-acid glycoprotein. The microheterogeneity of carbohydrate moieties can be quickly screened using this approach for either tryptic digests or the intact glycoprotein. These methods demonstrate potential applications for structural characterization of recombinant glycoproteins of pharmaceutical interest.

INTRODUCTION

Rapid advances in recombinant DNA techniques have stimulated great interest in proteins for novel therapeutic uses in the pharmaceutical industry. Glycoproteins are among the most attractive subjects because of their structural specificity and biological roles of oligosaccharides. During recent years, the fundamental understanding of biological functions of glycosylation of proteins has been explored. These include intracellular transport, the influences on the activity, stability, and solubility of the protein, antigenicity, molecular recognition, thermal stability, and the rate of proteolysis [1–5]. Structural characterization of glycoproteins, including protein sequence information, carbohydrate compositions and the determination of glycosylation sites, presents a significant analytical challenge.

The detection and identification of glycoproteins have been traditionally accomplished using polyacrylamide gel electrophoresis followed by direct gel staining [6], membrane staining techniques [7–9] or lectin affinity chromatography [10–12]. Further structural characterization is often performed by chromatographic separation in conjunction with appropriate enzymatic methods. In recent years, anion-exchange chromatography with pulsed amperometric detection has been widely utilized for analysis of complex mixtures of oligosaccharides [13,141 and structural classification and microheterogeneity of carbohydrate moieties at specific attachment sites in glycoproteins while combined with fast atom bombardment mass spectrometry

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(FAB-MS) [15,161. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been well practiced for peptide mapping of protein digests and can often be adapted to map individual glycoforms of glycoproteins [16–18]. On-line LC–MS, especially with FAB-MS and electrospray MS, has received considerable attention in peptide mapping and subsequent structural characterization [15,19–22].

In any case, it is clear that the chemical or enzymatic cleavage of glycoproteins and subsequent separation and identification of the glycosylated peptide fragments is an important approach for the structural characterization of glycoproteins. Enzymatic and chemical digestion of glycoproteins usually generate complex mixtures of peptides, glycopeptides, and even oligosaccharides after the cleavage of carbohydrate side chains. Thus, a preliminary separation step with high resolution and high detection sensitivity and the feasibility of easy interfacing with a mass spectrometer is essential. Microcolumn liquid chromatography (micro-LC), especially using packed fused-silica capillaries of 50–320 μm I.D. has been a very sucessfull technique for biological applications [23–25]. This technique uses the same separation method as conventional HPLC but with a miniaturized format and optimized instrumentation, providing very high mass sensitivity and excellent analytical resolution. The introduction of electrospray or ionspray (pneumatically assisted electrospray) interfaces for MS and the increased use of continuous flow (CF) FAB-MS have further stimulated the use of micro-LC to facilitate the on-line LC-MS configuration. The very low volumetric flow-rates (2-5 μl/min) attained with micro-LC techniques are well suited for either an electrospray or ionspray MS interface, or CF-FAB-MS for the conditions of potential high-sensitivity performance. There have been several reports which demonstrate the utility of these techniques for peptide mapping and protein characterization [26–29], however, techniques for the characterization of glycoproteins utilizing microcolumn separation techniques, especially coupled with MS, have not been well explored. This is partially due to the lack of instrumentation for performing micro-LC and also the structural complexity of glycoproteins.

In this paper, a micro-LC system in combination with tandem mass spectrometry (micro-LC–MS–MS) is used for comparative glycopeptide mapping of model glycoprotein digests prior to. and after. proteolytic cleavages. This method has been applied to the determination of a glycosylation site and associated peptide structure of bovine ribonuclease B. Preliminary results for the partial determination of oligosaccharide attachment sites of a complex glycoprotein, human α_1-acid glycoprotein, are also discussed.

EXPERIMENTAL

Materials

Protein sequencing-grade trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA), UV-grade acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) and HPLC-grade water (Fisher, Fair Lawn, NJ, USA) were used. Ribonuclease B (Type III-B, from bovine pancrease), α_1-acid glycoprotein (human), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, dithiothreitol (DTT), iodoacetamide, and ammonium bicarbonate were all purchased from Sigma. Peptide-N-glycosidase F (PNGase F) was obtained from Boehringer Mannheim (Indianapolis, IN, USA).

Reduction and S-carboxymethylation

The reduction and S-carboxymethylation of selected glycoproteins were performed prior to digestion. In this study, 500 μl of ribonuclease B (5 mg) dissolved in water were mixed with 500 μl of 0.5 *M* Tris–HCl buffer (pH 8.25) containing 2 m*M* EDTA and 6.0 *M* guanidine–HCl, and 100 μl of 0.4 *M* DTT. The mixed solution was incubated at 37°C for 3 h. A volume of 200 μl of 0.8 A4 iodoacetamide was then added and incubated for 1 h. A 3.5-mg sample of human α_1-acid glycoprotein was treated in an identical manner. The samples were desalted overnight using Spectra/Por 6 membranes from Fisher and dried for further treatment.

Enzymatic digestion

The reduced and S-carboxymethylated ribonuclease B (RCM-ribonuclease B) (*ca.*1 mg) and human α_1-acid glycoprotein *(ca. 0.8* mg) were digested with TPCK-trypsin in 0.1 A4 ammonium bicarbonate buffer (pH 8.00) with a substrate-enzyme ratio of 50: 1 (w/w). The mixtures were incubated at 37°C for 24 h. After digestion. 100 μl of the tryptic digest

(approximate 10 nmol) was incubated with two units of peptide-N-glycosidase F (PNGase F) in 0.1 *M* ammonium bicarbonate (pH 8.30) containing 2 m*M* EDTA at 37°C for 24 h. The reaction mixtures were either directly used for chromatographic analysis or dried and stored in a freezer and redissolved in 0.1% TFA prior to use.

Microcolumn liquid chromatography

A Beckman System Gold conventional HPLC system was modified for performing micro-LC at low flow-rates. Solvent gradients were directly delivered into a micromixer obtained from Upchurch Scientific (Oak Harbor, WA, USA), at flow-rates of 0.2–0.4 ml/min. A precolumn splitting device was used to obtain appropriate output flow-rates (approximately 3 μl/min) for packed capillary columns. The split ratio was easily regulated by adjusting the length of the restriction line (fused-silica capillary with 50 μm I.D. and 361 μm O.D.). The capillary columns with 250 μm I.D. and 348 μm O.D. were packed in-house with C_{18}, 5-μm particles of 300 Å pore size, from Vydac (Hesperia, CA, USA) using an ISCO μLC-500 pump. The columns were directly connected into a Valco micro-injector with 100 nl or 500 nl internal loops. The transfer line from column outlet consisted of fused-silica capillary with 50 μm I.D. and 190 μm O.D. on-line connected to UV detector and mass spectrometer. An ABI Model 785A UV detector (Applied Biosystem, Foster City, CA, USA) equipped with an Z-shape capillary flow cell obtained from LC Packings (San Francisco, CA, USA) was used in this study. The mobile phase used consisted of 0.1% TFA in water (solvent A) and solvent A–acetonitrile (20:80) with 0.1% TFA (solvent B). Solvent gradients (0%–60% solvent B over 120 min) were used for all separations.

Ionspray muss spectrometry

A PE-SCIEX (Thornhill, Ontario, Canada) API III triple-quadrupole mass spectrometer equipped with an ionspray interface was used on-line with the micro-LC system and UV detector described above. Micro-LC effluent was introduced directly into the ionspray source. Micro-LC-MS experiments were performed while scanning from ***m/z*** 300 to 1800 at a scan-rate of 4 s/scan. For the daughter MS-MS operation. the parent ions were selected in the first quadrupole mass analyzer and transmitted into the second quadrupole (collision cell) with collision energy of 50 eV and argon collision gas thickness of $400 \cdot 10^{12}$ molecules/cm^2.

RESULTS AND DISCUSSION

Electrospray (or ionspray) MS has been increasingly used for determining molecular mass and structural analysis of peptides and proteins. Generally, multiple-charge states with a Gaussian distribution of relative intensities can be observed for the intact protein from which a single peak indicative of the molecular weight can be converted by deconvolution [30]. However, glycoprotein analysis appears to be more complicated. In the present study, we have selected bovine ribonuclease B, a glycoprotein with a single glycosylation site and high mannose content, as a model compound. Initial screening was performed directly by ionspray MS and the results are shown in Fig. 1. A deconvoluted mass spectrum was obtained for the intact glycoprotein without pretreatment as shown in Fig. 1A, exhibiting a pattern of microheterogeneity of glycosylation. It is known that structural heterogeneity of carbohydrate moieties attached on the peptide backbone of the protein frequently occurs in glycoproteins, especially for asparagine-linked glycoproteins [3 1,321. This phenomenon was further noticed after the treatment with PNGase F, an enzyme which specifically cleaves N-linked carbohydrates between the di-N-acetylchitobiose unit and the asparagine residue of the polypeptide backbone. As shown in Fig. 1B, most glycosylated peaks disappear. The molecular mass of deglycosylated protein was found to be 13 692 which is consistent with the calculated value of 13 69 1 based on its known amino acid sequence. Actually, this peak was also observed in native glycoprotein, but can only be confirmed to be a deglycoform after the release of oligosaccharides. It could be due to the presence of ribonuclease A, a non-glycoprotein that has the same polypeptide backbone as ribonuclease B. Also noted was a component with an M_r of 13 895 corresponding to protein-GlcNAc. A possible explanation for this phenomenon is due to the enzymatic cleavage of carbohydrate moieties by Endo-F (Endo-b-N-acetylglucosaminidase F), an enzyme often co-existing with PNGase F, releasing a peptide at-

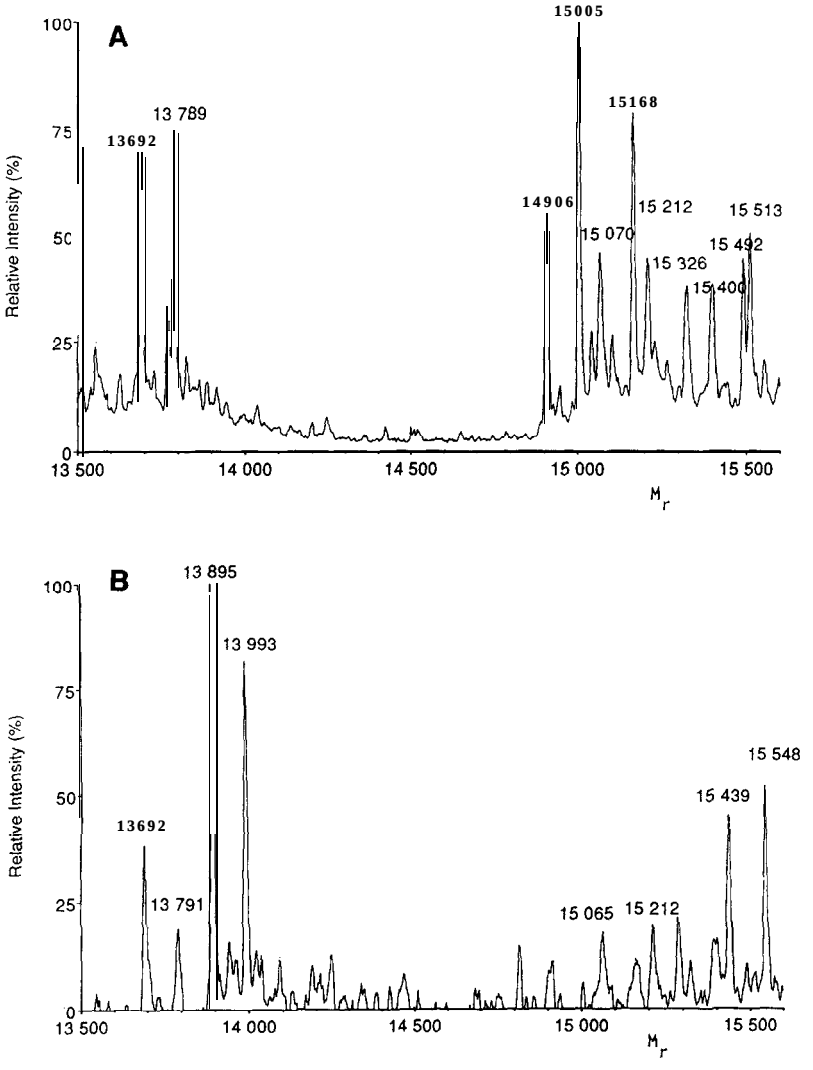

Fig. 1. &convoluted mass spectra of bovine ribonuclease B by ionspray-MS analysis. (A) Native protein; (B) PNGase F treated protein.

tached with one N-acetylglucosamine residue, peptide-GlcNAc, which is not itself a substrate for PNGase F [33]. Based on these studies, the carbohydrate content constitutes about 12% of the total molecular mass. This approach provides a method for quickly screening the microheterogeneity of oligosaccharide substructures of a glycoprotein and the determination of carbohydrate content. Table I lists possible glycoforms observed from these experiments in terms of diagnostic increments of M_r 162 for a hexose residue and increments of M_r 203 for N-acetylhexosamine residue.

Further structural analysis of glycoproteins generally requires fragmentation, either by chemical or enzymatic cleavage, and subsequent separation or isolation. Chromatographic isolation has been a traditional method for glycoprotein analysis, such as the use of lectin affinity chromatography. Peptide mapping is an attractive approach while utilized in conjunction with the appropriate enzymatic methods. By comparing peptide maps prior to, and after enzymatic cleavage, peptide fragments attached to oligosaccharides may be located and distinguished from non-glycosylated peptides. Mass spectrome-

TABLE I

HETEROGENEITY OF RIBONUCLEASE B GLYCOFORMS OBSERVED BY IONSPRAY-MS ANALYSIS*

Native protein	M_r	PNGase F-treated protein	M_r
Polypeptide chain	13 692	Polypeptide chain	13 692
Polypeptide adduct (phosphate)	13 789	Polypeptide adduct (phosphate)	13 791
Hex_5-$(HexNAc)_2$-polypeptide	14908	HexNAc-polypeptide	13 895
Hex_5-$(HexNAc)_2$-(polypeptide + phosphate adduct)	15 005	HexNAc-(polypeptide + phosphate adduct)	13 993
Hex_6-$(HexNAc)_2$-polypeptide	15 070		
Hex_6-$(HexNAc)_2$-(polypeptide + phosphate adduct)	15 168		
Hex_7-$(HexNAc)_2$-(polypeptide + phosphate adduct)	15 326		
Hex_8-$(HexNAc)_2$-(polypeptide + phosphate adduct)	15492		

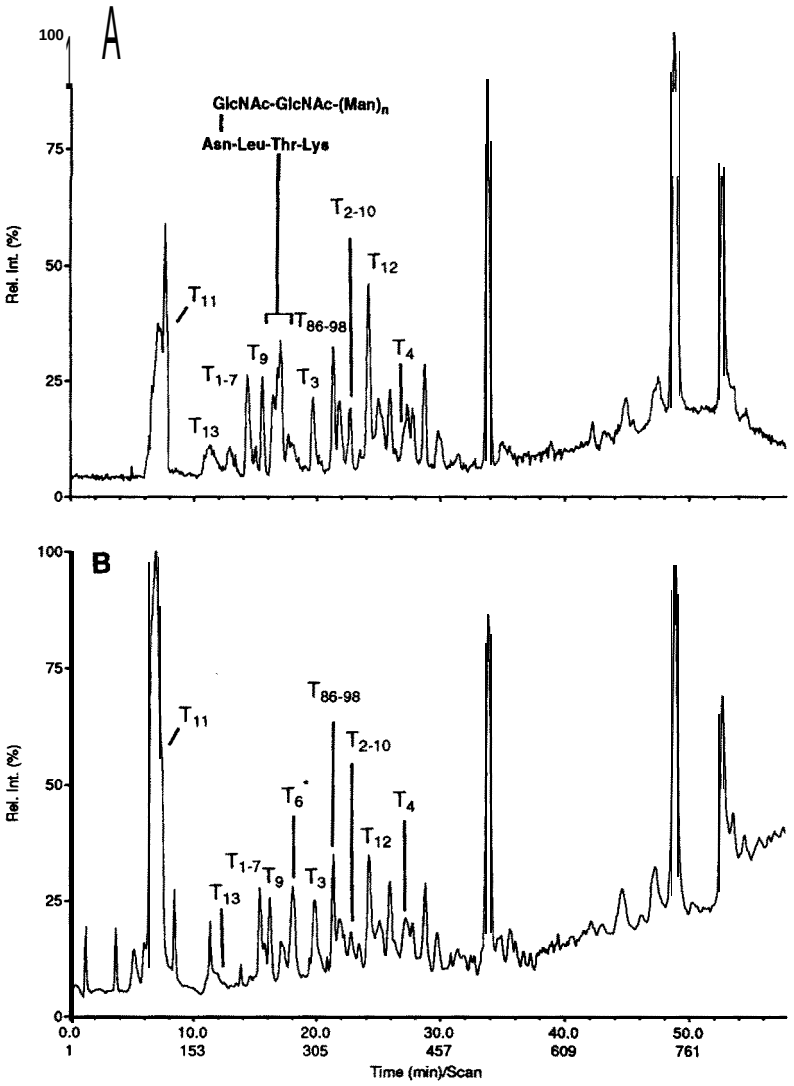

Fig. 2. Comparative peptide mapping of enzymatic digests of bovine ribonuclease B by micro-LC-lonspray MS. (A) TIC trace of digested RCM-glycoprotein with trypsin; (B) TIC trace of digested RCM-glycoprotein with trypsin and PNGase F. Capillary column: 33 cm × 250 μm I.D. (348 μm O.D.) packed with C_8. Gradient conditions described in Experimental section. Approximate 52 picomol of digests were injected onto the column.

try, especially FAB-MS, has been utilized to determine the structure of oligosaccharides and the classification of glycosylation. However. an ionspray interface combined with micro-LC separation techniques is particularly attractive for mapping protein digests [34,35]. Fig. 2 shows the total ion current (TIC) MS chromatograms of enzymatic digests of bovine ribonuclease B. This glycoprotein was denatured, reduced, and *S*-carboxymethylated prior to enzymatic treatment. Trypsin was first used to digest the RCM-ribonuclease B, generating a mixture of peptides and glycopeptides. The fragments were separated and detected by micro-LC on-line with a UV detector and ionspray mass spectrometer. The tryptic map shown in Fig. 2A as the TIC profile, which correlates well with the UV absorbance trace, exhibits abundant signals that could be assigned to most of the expected normal peptide fragments (Fig. 2). Of particular interest is a unique broad peak between retention time 16 and 17.5 min which was determined to be related to the heterogeneity of glycoforms attached to a specific tryptic peptide. To establish the identity and the attachment of the carbohydrate substructure, tryptic fragments of ribonuclease B were further reacted with PNGase F. After treatment with PNGase F, this particular broad peak disappeared and a new peak with a retention time of 18 min (T_6^*), which was absent in original tryptic map, was observed in the enzymatic digest (Fig. 2B). This new component corresponds to a peptide fragment with a molecular mass of 474.

A full scan mass spectrum (Fig. 3) averaging all the glycoforms of the glycosylated peptide, corresponding to the broad peak from 16 to 17.5 min (Fig. 2A), provides further detailed information for the confirmation of the presence of glycoforms attached on a single peptide. As shown in Fig. 3, a series of singly and doubly charged ions represent diagnostic increments of M_r 162 and 203 corresponding to hexose and GlcNAc substructures, respectively. The table inserted in Fig. 3 suggests the structures of the proposed glycopeptide with homogeneous components differing in the number of hexose units. For example, the arithmetic difference between the component of M_r 1691 (MH^+ at m/z 1692. MH_2^{2+} at m/z 846) and peptide substructure (M_r 474) is M_r 12 17 which corresponds uniquely to a carbohydrate composition of five mannose and

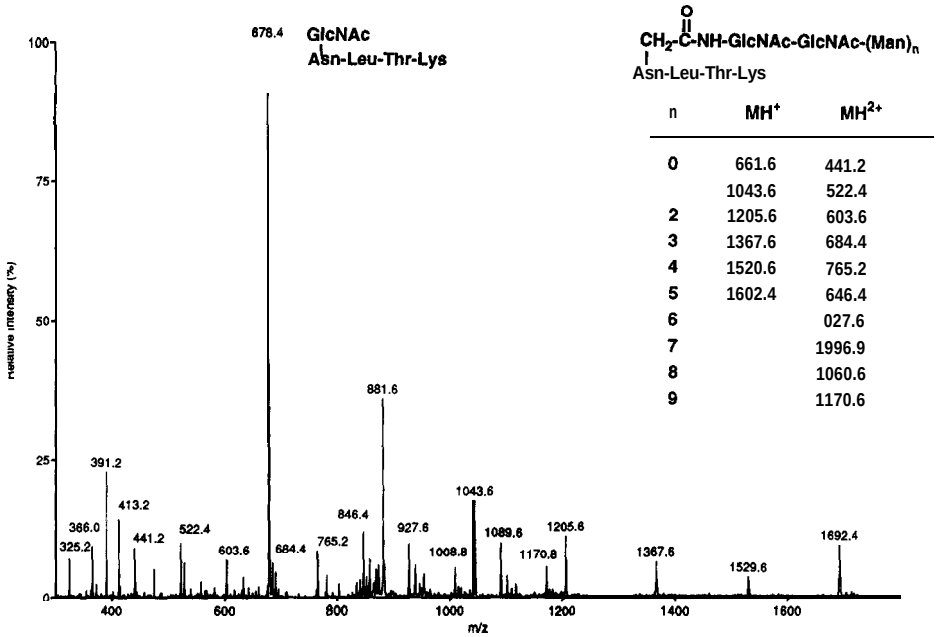

Fig. 3. Averaged full scan mass spectrum of glycoforms corresponding to the peaks indicated in Fig. 2A

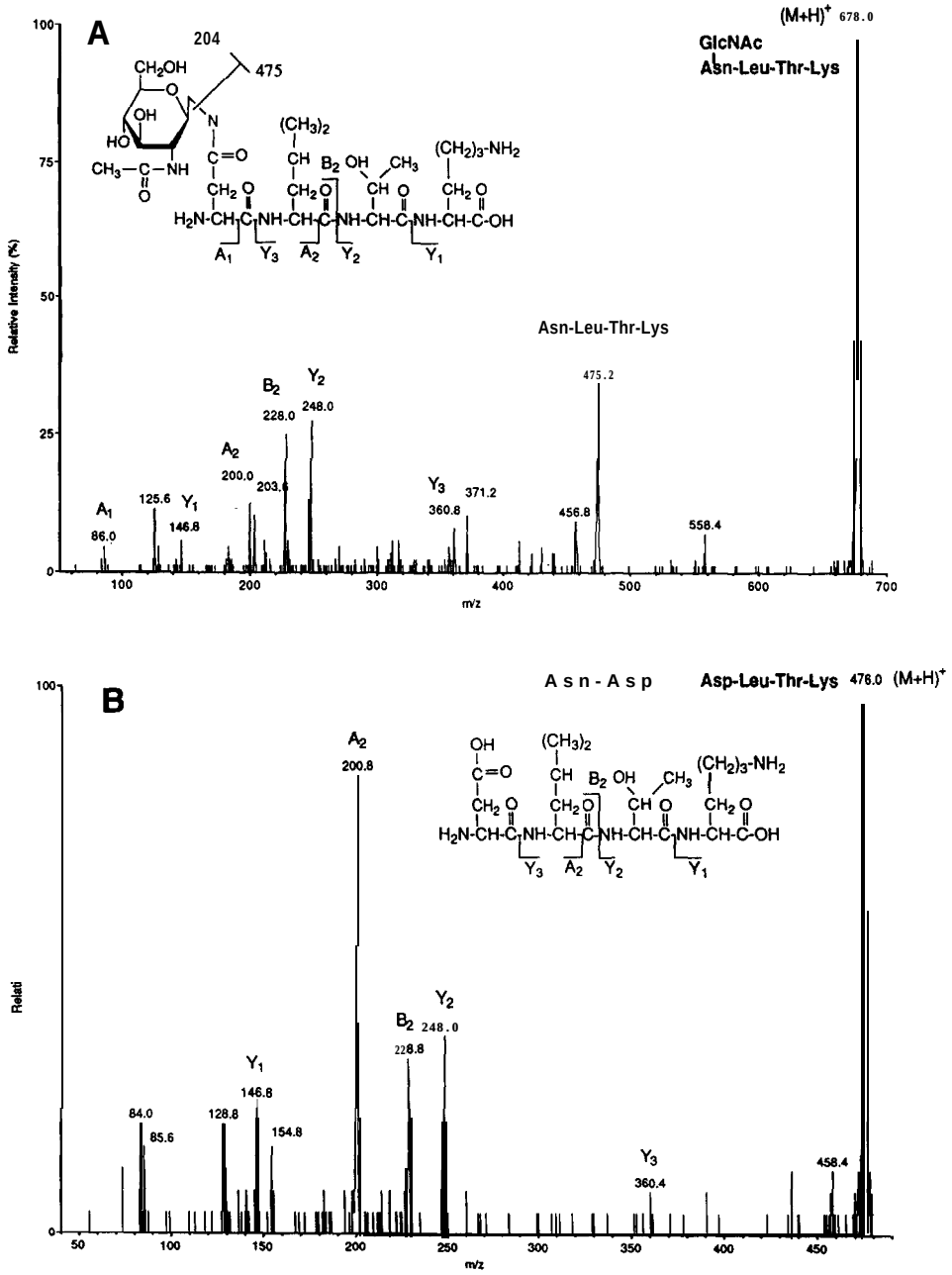

Fig. 4. Structural determination of a glycopeptide fragment from enzymatic digests of bovine ribonuclease B by micro-LC-MS-MS. (A) Daughter MS-MS spectrum of glycosylated peptide ion at m/z 678. (B) Daughter MS-MS spectrum of deglycosylated peptide ion at m/z 476.

two N-acetylglucosamine residues. Similarly, a mass difference of 1054 between M_r 1528 (MH^+ at m/z 1529, MH_2^{2+} at ***m/z*** 765) and the peptide confirms a sugar unit of four mannoses linked to two N-acetylglucosamine residues. Additional carbohydrate units can be derived in a similar manner. The presence of an intense ion at ***m/z*** 678 in the full scan mass spectrum should be noted. As will be dis-

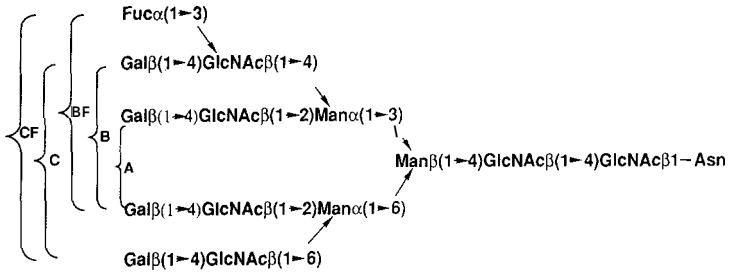

Fig. 5. Classes of carbohydrate moieties for human a,-acid glycoproteins [37].

cussed later, MS–MS experiments indicated that this ion corresponded to the tetrapeptide attached to a single N-acetylglucosamine residue. The presence of this glycoform is somewhat unusual since it was not observed in the original glycoprotein. It is most likely a mass spectrometric fragment ion formed during the ionization process. In conjunction with MS-MS studies, such fragmentation in the ion source facilities the identification of the glycosylation site.

To confirm this structure and the carbohydrate attachment site, micro-LC–MS–MS experiments were performed prior to, and after the treatment of tryptic digest with PNGase F (Fig. 4). Fig. 4A shows a daughter MS-MS spectrum of the glycosylated fragment from the tryptic digest at m/z 678. The ion at m/z 475 represents the tetrapeptide substructure (Fig. 4A) due to the facile cleavage of N-linked sugar. A weak ion at m/z 204 corresponding to the N-acetylglucosamine residue fragment was also observed. The confirmation of this peptide sequence was based on typical Y-series fragment ions due to the fragmentation between peptide bonds of each amino acid as well as the A- and B-series ions. For example, Y_1 represents Lys at the C-terminus, Y_2 corresponds to the dipeptide fragment, Thr-Lys. and Y_3 corresponds to the tripeptide fragment, Leu–Thr–Lys. Amino acid residues from N-terminus were confirmed by A_1, A_2, and B_2 ions, indicating the presence of Asn, Asn-Leu, and Asn–Leu-Thr fragments, respectively.

After enzymatic cleavage of the sugar substructure by PNGase F, a new tryptic fragment, T_6^*, was observed. A daughter MS-MS spectrum of the deglycosylated fragment T_6^* (m/z 476) was obtained and is shown in Fig. 4B. The M_r of 475 and structural information obtained from the MS-MS spectrum are consistent with the conversion of asparagine into aspartic acid in the tetrapeptide after treatment by PNGase F [36]. This phenomenon was readily detected by ionspray MS and is an important piece of evidence for determining the site of carbohydrate attachment. From Fig. 4B, it is clear that the Y-series ions from the C-terminus of the deglycosylated peptide are the same as those found in glycosylated peptide. The observed M_r l increase for the A_2 and B_2 fragments from the N-terminus provides further evidence supporting the conversion of asparagine to aspartic acid. This information confirms that the carbohydrate is attached on the tryptic tetrapeptide, Asn–Leu–Thr–Lys, at the asparagine of position 34 in the polypeptide backbone of the glycoprotein.

We have applied this approach to a more complicated glycoprotein, human α_1-acid glycoprotein, a protein consisting of a single polypeptide chain but with a relatively high carbohydrate content. Furthermore, these carbohydrates are N-linked oligosaccharides with complex degrees of branching and structural variability due to the microheterogeneity and they account for 4.5% of the total molecular mass of the protein. There are five classes of carbohydrate moieties reported [37] for this glycoprotein as shown in Fig. 5.

The major core is class A. Obviously, these branched oligosaccharides complicate the chromatographic separation as well as spectral interpretation. The preliminary results of this mapping strategy are shown in Fig. 6 which were obtained by treating the glycoprotein sequentially with trypsin and PNGase F. By comparing these two maps, it is apparent that fragment T_9 from the deglycosylated

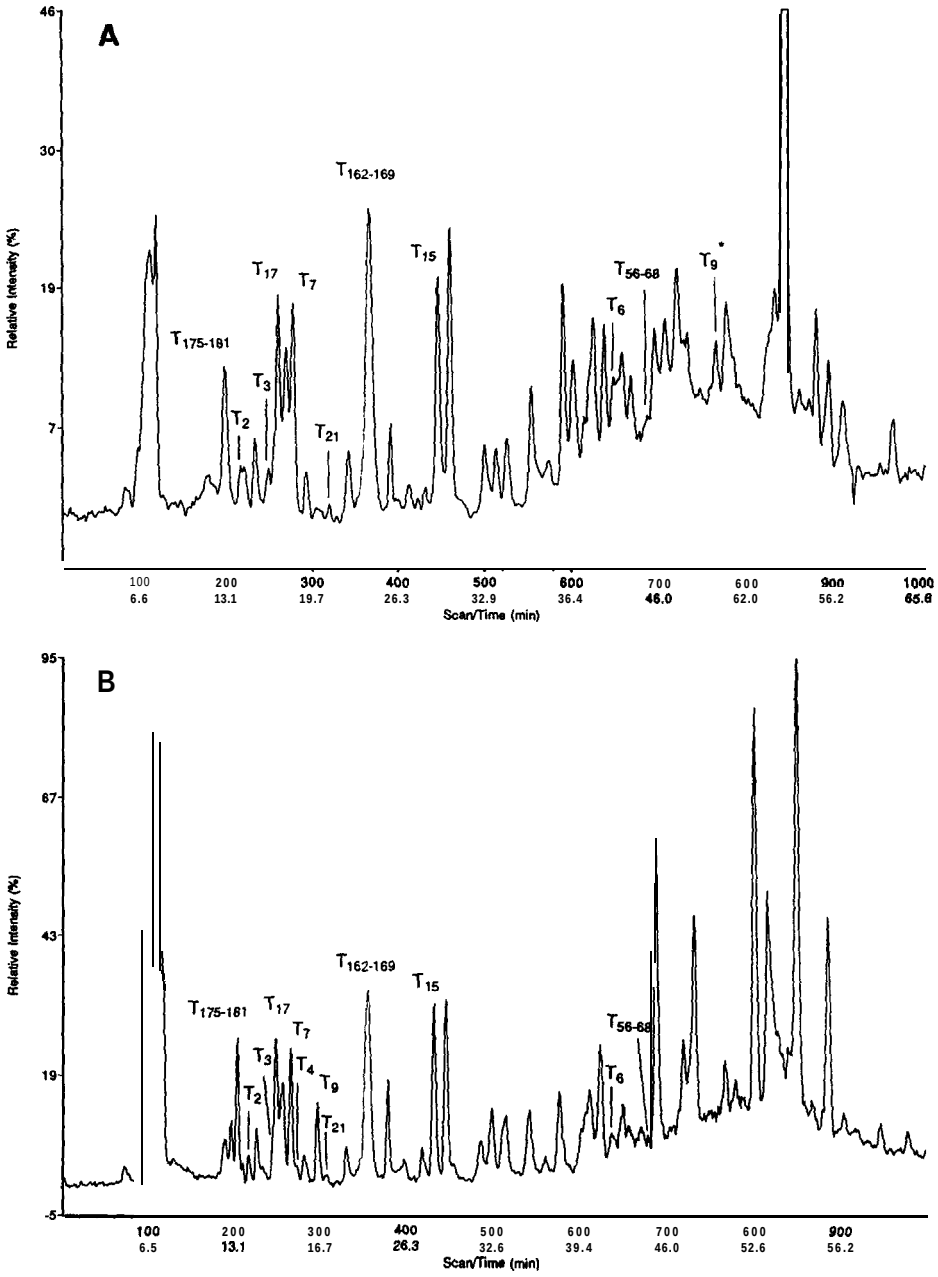

Fig. 6. Comparative peptide mapping of enzymatic digests of human α_1-acid glycoprotein by micro-LC-ionspray-MS. (A) TIC trace of digested RCM-glycoprotein with trypsin; (B) TIC trace of digested RCM-glycoprotein with trypsin and PNGase F. Experimental conditions are the same as in Fig. 3.

digest (Fig. 6B) is unique since it was not observed in the glycosylated digest (Fig. 6A). In addition, a glycosylated peptide was located as T* shown in Fig. 6A, which possibly corresponds to the proposed carbohydrate structure based on the classification of the oligosaccharides demonstrated above. A full scan mass spectrum of the glycosylated tryptic fragment T* was obtained and is shown in Fig.

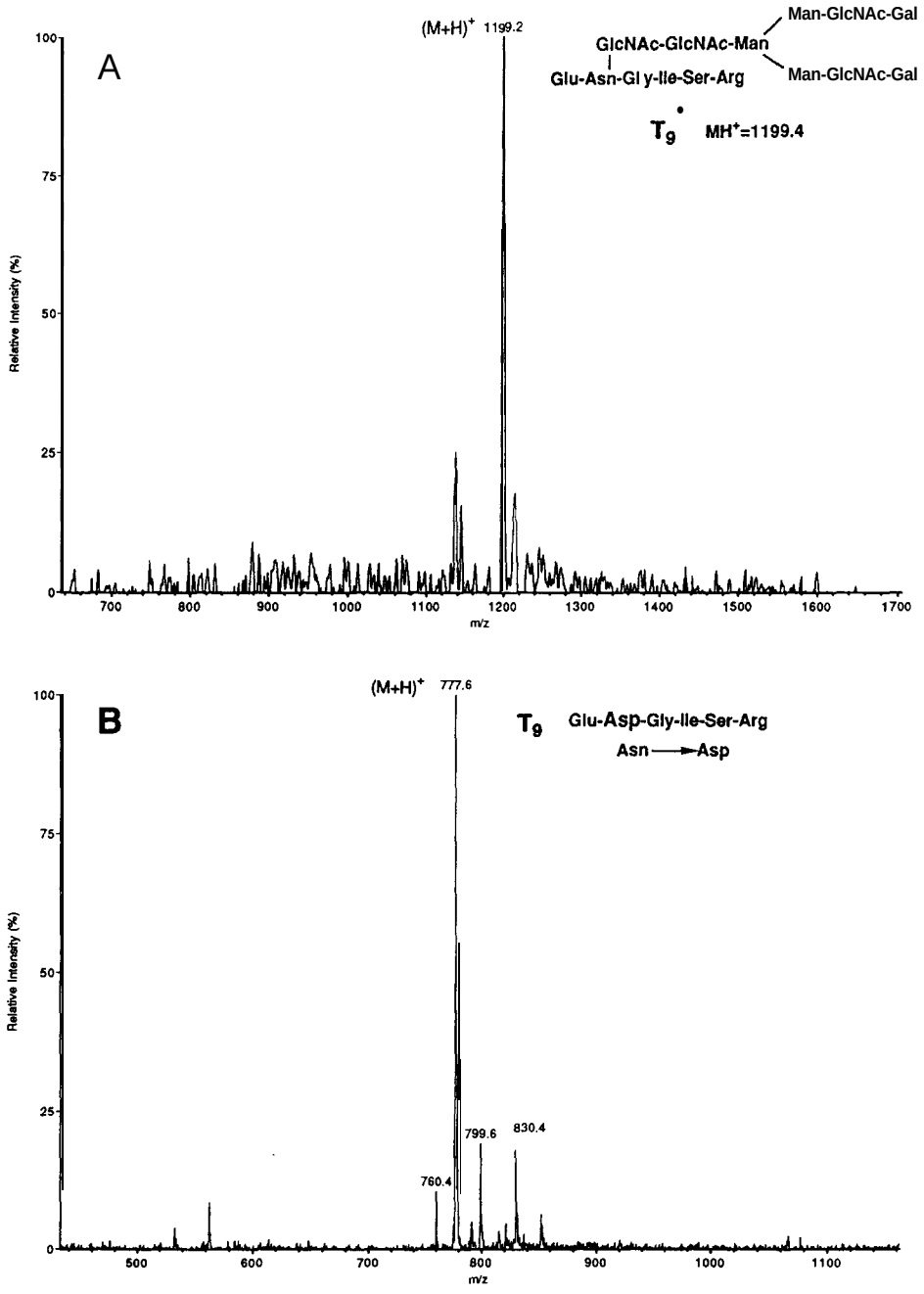

Fig. 7. Full scan mass spectra of a glycopeptide fragment from enzymatic digests of human a,-acid glycoprotein. (A) Glycosylated peptide; (B) deglycosylated peptide.

7A. A doubly charged ion (MH_2^{2+} 1199.2) was found to be related to the proposed glycopeptide that has molecular mass of 2396.8 (giving MH_2^{2+} 1199.4). Based on the known peptide sequence of human α_1-acid glycoprotein, the corresponding deglycosylated tryptic fragment could have a molecular mass of 775. The mass difference of M_r 1622 between the total molecular mass of glycosylated

peptide and the deglycosylated peptide agrees well with a class A carbohydrate chain. The full scan mass spectrum of deglycosylated fragment T_9 shown in Fig. 7B provides further evidence for this glycosylation. A singly charged ion (MH^+) at *m/z* 777 was observed, M_r 1 more than the predicted peptide $(MH^+$ at *m/z* 776) described above. This is consistent with studies on bovine ribonuclease B since enzymatic cleavage of the N-linked sugar unit by PNGase F results in conversion of asparagine to aspartic acid. Since this heavily glycosylated glycoprotein is highly branched, it appears that further studies utilizing highly efficient separations and the utilization of alternate enzyme combinations should be pursued in conjunction with micro-LC-MS-MS experiments for substructure determination and confirmation.

CONCLUSIONS

A strategy involving the use of micro-LC-MS-MS techniques has been shown to be capable of providing a highly sensitive method for peptide mapping of glycoproteins. In conjunction with appropriate enzymatic methods such as trypsin and PNGase F, high-resolution chromatographic separations of complex mixtures of peptides and glycopeptides provide valuable comparative maps for rapidly locating glycosylated peptide fragments.

Electrospray (or ionspray) ionization MS is an extremely attractive and novel technique for structual characterization of glycoproteins. Quick screening of the intact native glycoprotein by ionspray-MS provides information of the microheterogeneity profile as well as the carbohydrate content. On-line coupling of micro-LC to MS is a very powerful configuration which permits rapid identification of enzymatically generated peptide and glycopeptide fragments present in the maps. Micro-LC–MS-MS techniques provide an extra dimension of information allowing for the characterization of peptide and glycopeptide substructures and the determination of carbohydrate attachment sites. The MS-MS fragmentation patterns of the tryptic fragments permits confirmation of carbohydrate attachments.

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