Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 1859–1865
Carbohydrate RESEARCH

Oligosaccharide analyses of glycopeptides of horseradish peroxidase by thermal-assisted partial acid hydrolysis and mass spectrometry

Bao-Shiang Lee,* Sangeeth Krisnanchettiar, Syed Salman Lateef, Nabila Salman Lateef and Shalini Gupta

Protein Research Laboratory, Research Resources Center, 835 S. Wolcott Avenue, University of Illinois, Chicago, IL 60612, USA

Received 15 February 2005; accepted 23 April 2005
Available online 16 June 2005

Abstract—Thermal-assisted partial acid hydrolysis of the carbohydrate moieties of N-glycosylated peptides of horseradish peroxidase (HRP) is used to generate oligosaccharide cleavage ladders. These ladders allow direct reading of components of the oligosaccharides by mass spectrometry. Acid hydrolysis performed with 1.4, 3.1, 4.5, or 6.7 M trifluoroacetic acid at 37, 65, or 95 °C for 30 min to 24 h hydrolyzed mainly the oligosaccharide units of glycopeptides with least peptide bond or amino acid side chain hydrolysis. Tryptic N-glycosylated peptides from HRP with molecular weights of 2533, 2612, 3355, 3673, and 5647 Da were used as test systems in these experiments. Data showed that the most labile group of oligosaccharides is the fucose (Fuc) and the majority of the end cleavage products are peptides with one or no *N*-acetylglucosamine (GlcNAc) residue linked to Asparagine (Asn). Additionally, the data agree with previous reports that glycopeptides 3355 and 3673 Da carry an oligosaccharide (Xyl)Man_3(Fuc)$GlcNAc_2$, glycopeptide 5647 Da carries two oligosaccharides (Xyl)Man_3(Fuc)$GlcNAc_2$, and glycopeptides 2612 and 2533 Da carry (Xyl)$Man_3GlcNAc_2$ and (Fuc)GlcNAc, respectively. However, the glycosylation site of the 2612 Da peptide at Asn_{286} is partially occupied. This method is particularly useful in identifying glycopeptides and obtaining monosaccharide compositions of glycopeptides.

Keywords: Horseradish peroxidase; N-glycosylated peptides; MALDI-TOFMS; Acid hydrolysis; Trifluoroacetic acid; Proteomics

1. Introduction

Addition of oligosaccharides is one of the most important steps in the process of post-translational modification (PTM) of proteins. Majority of proteins found on the mammalian cell surface and on internal endoplasmic reticulum are glycoproteins.[1] Characterization of the carbohydrate moiety of glycoproteins is essential to understand the function of a protein.[2,3] Typically, oligosaccharides in a glycoprotein are characterized by various methods that begin with chemical or enzymatic removal of the oligosaccharides and subsequently derivatization and hydrolysis of the released glycan. The hydrolyzed products are analyzed by various methods[4–7] such as HPLC, capillary electrophoresis, NMR, and mass spectrometry. However, these processes are labor-intensive, and may yield incomplete cleavage of the glycan thereby providing unrepresentative oligosaccharide structures. Although recently mass spectrometry with MS/MS mode has been used effectively to study oligosaccharide structures in horseradish peroxidase (HRP) by direct analysis of the glycopeptide,[8] data interpretation is a challenge.

In our previous study,[9] trifluoroacetic acid (TFA) and microwaves were used for hydrolysis of glycopeptides to obtain information on oligosaccharide structures of HRP. In this study, conventional oven heating along with trifluoroacetic acid is used for the hydrolysis of

Abbreviations: PTM, post-translational modification; HPLC, high-performance liquid chromatography; RP, reversed phase; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CHCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; Xyl, xylose.

* Corresponding author. Fax: +1 312 996 1898; e-mail: boblee@uic.edu

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.04.018

```
  1  QLTPTFYDNS  CPNVSNIVRD  TIVNELRSDP  30
             *
 31  RIAASILRLH  FHDCFVNGCD  ASILLDNTTS  60
                                    *
 61  FRTEKDAFGN  ANSARGFPVI  DRMKAAVESA  90
 91  CPRTVSCADL  LTIAAQQSVT  LAGGPSWRVP  120
121  LGRRDSLQAF  LDLANANLPA  PFFTLPQLKD  150
151  SFRNVGLNRS  SDLVALSGGH  TFGKNQCRFI  180
            *
181  MDRLYNFSNT  GLPDPTLNTT  YLQTLRGLCP  210
       *            *
211  LNGNLSALVD  FDLRTPTIFD  NKYYVNLEEQ  240
      *
241  KGLIQSDQEL  FSSPNATDTI  PLVRSFANST  270
                     *             *
271  QTFFNAFVEA  MDRMGNITPL  TGTQGQIRLN  300
                     *
301  CRVVNSNS
```

Figure 1. Amino acid sequence of horseradish peroxidase. Potential N-glycosylation sites are marked with asterisks.

the N-glycosylated peptide of HRP.[10,11] Tryptic N-glycosylated peptides of horseradish peroxidase are used as test cases. C_{18} reversed-phase HPLC following tryptic digestion of HRP shows several glycopeptides,[8,12–15] of which the glycopeptides with molecular weights of 2533, 2612, 3355, 3673, and 5647 Da are used in this study. Compared to acid hydrolysis of glycopeptide using HCl,[16] TFA produces less extraneous hydrolysis products from the peptide.

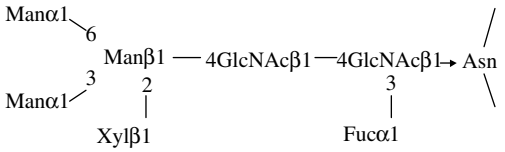

Figure 2. Structure of the major carbohydrate moiety of HRP.

2. Experimental

2.1. Materials

All chemical and biochemicals were obtained from Sigma Chemical Co. (S. Louis, MO) and used without further purification.

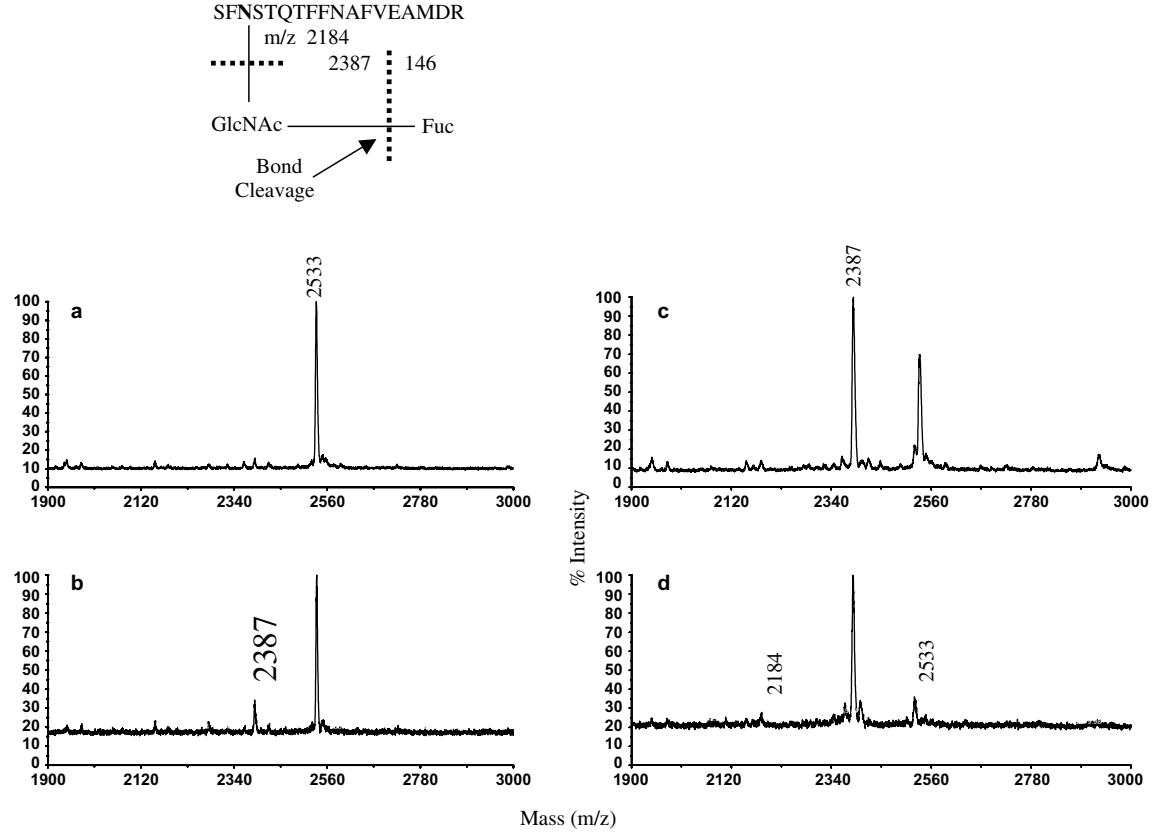

Figure 3. MALDI-TOF mass spectra of thermal-assisted acid hydrolysis product ions from the glycopeptide 2533 Da of HRP corresponding to peptide S_{265}-R_{283} under different conditions of hydrolysis. (a) 14 mM TFA and no thermal heating; (b) 4.5 M TFA and 3.5 h thermal heating at 37 °C; (c) 1.4 M TFA and 1 h thermal heating at 65 °C; (d) 4.5 M TFA and 30 min thermal heating at 95 °C.

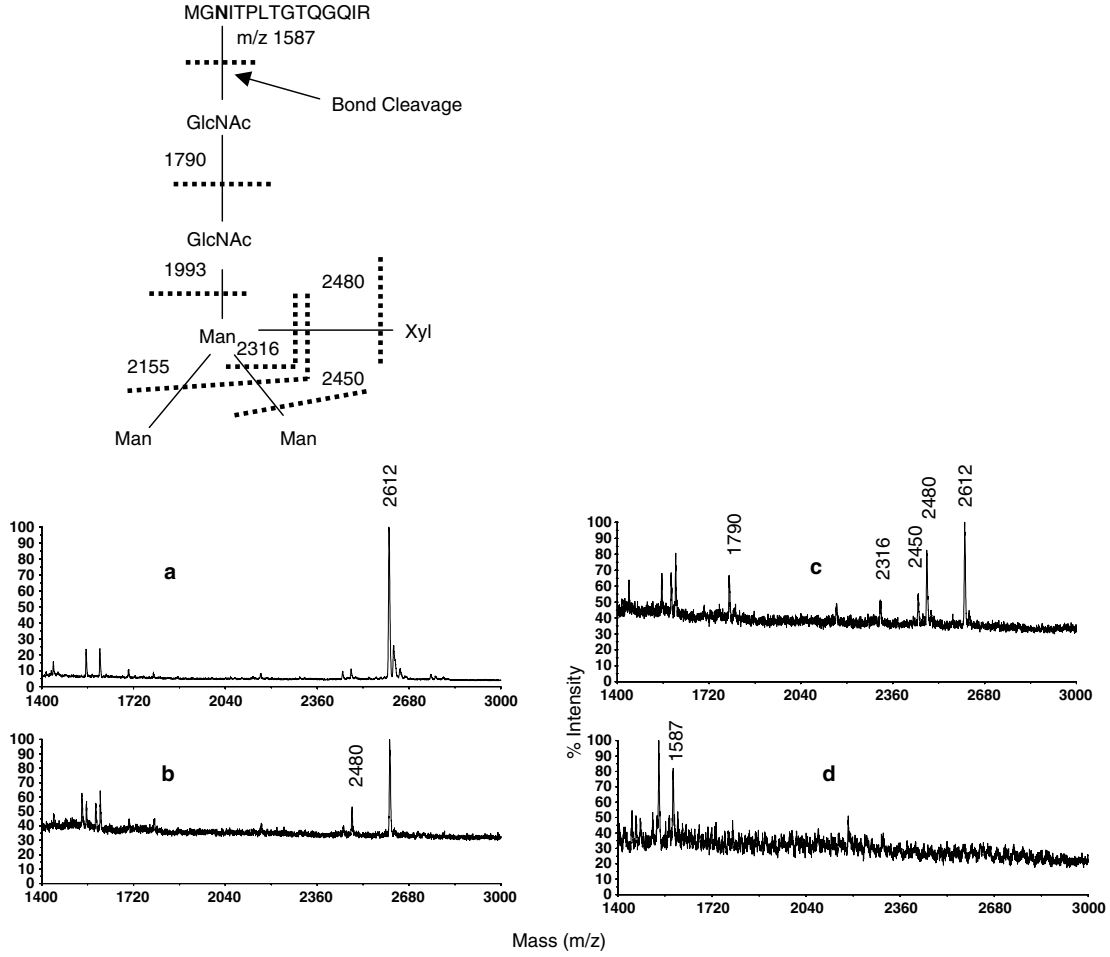

Figure 4. MALDI-TOF mass spectra of thermal-assisted acid hydrolysis product ions from the glycopeptide 2612 Da of HRP corresponding to peptide M_{284}-R_{298} under different hydrolysis conditions. (a) 14 mM TFA and no thermal heating; (b) 3.1 M TFA and 1 h thermal heating at 65 °C; (c) 1.4 M TFA and 30 min thermal heating at 95 °C; (d) 1.4 M TFA and 2.5 h thermal heating at 95 °C.

2.2. Methods

2.2.1. Preparation of the N-glycosylated peptides. HRP (5 mg) was dissolved in 2 mL of 50 mM tris buffer (pH 7.0) solution containing 0.1% SDS. To the HRP solution 40 μL of 50 mM tris(2-carboxyethyl)phosphine solution was added and heated at 95 °C for 10 min. After the solution was cooled to room temperature, 500 μL of acetonitrile and 200 μg of trypsin in 2.5 mL of double-distilled water (DD H_2O) were added, and the solution was incubated overnight at 37 °C. HRP trypsin digest was fractionated using a reversed-phase C_{18} Vydac column (218TP1010) on an Agilent model HP1100 HPLC system. Elution was conducted at a flow rate of 5 mL/min using solvent A (0.1% TFA in DD H_2O) and solvent B (0.1% TFA in acetonitrile). The column was equilibrated with 5% solvent B. After sample injection, the column was eluted by a linear gradient from 5% solvent B to 100% solvent B in 60 min. The fractions corresponding to glycopeptides with MW of 2533, 2612, 3355, 3673, and 5647 Da, detected by mass spectrometry, were collected at 64% B, 27%B, 61%B, 44%B, and 35%B, respectively. These glycopeptide solutions (~0.1 μg/μL) were used directly in the experiment.

2.2.2. Thermal-assisted acid hydrolysis. To the glycopeptide solution (10 μL) in a 1.5 mL Eppendorf tube, 1, 3, 5, or 10 μL TFA was added, and the solutions were heated at 37, 65, or 95 °C for 30 min, 1, 2.5, 3.5, 5, or 20 h in a traditional heating oven. Increasing the volume of each reaction mixture three times did not produce any significantly different results. The products were centrifuged and then cooled to room temperature prior to mass analysis.

2.2.3. MALDI-TOF mass spectrometric analyses. Cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix for mass spectrometric analysis of the glycopeptide solutions. Compared to 2,5-dihydroxybenzoic acid, higher intensity signals for larger mass glycopeptides were obtained with the use of CHCA. Samples were spotted onto a matrix-assisted laser desorption/ionization

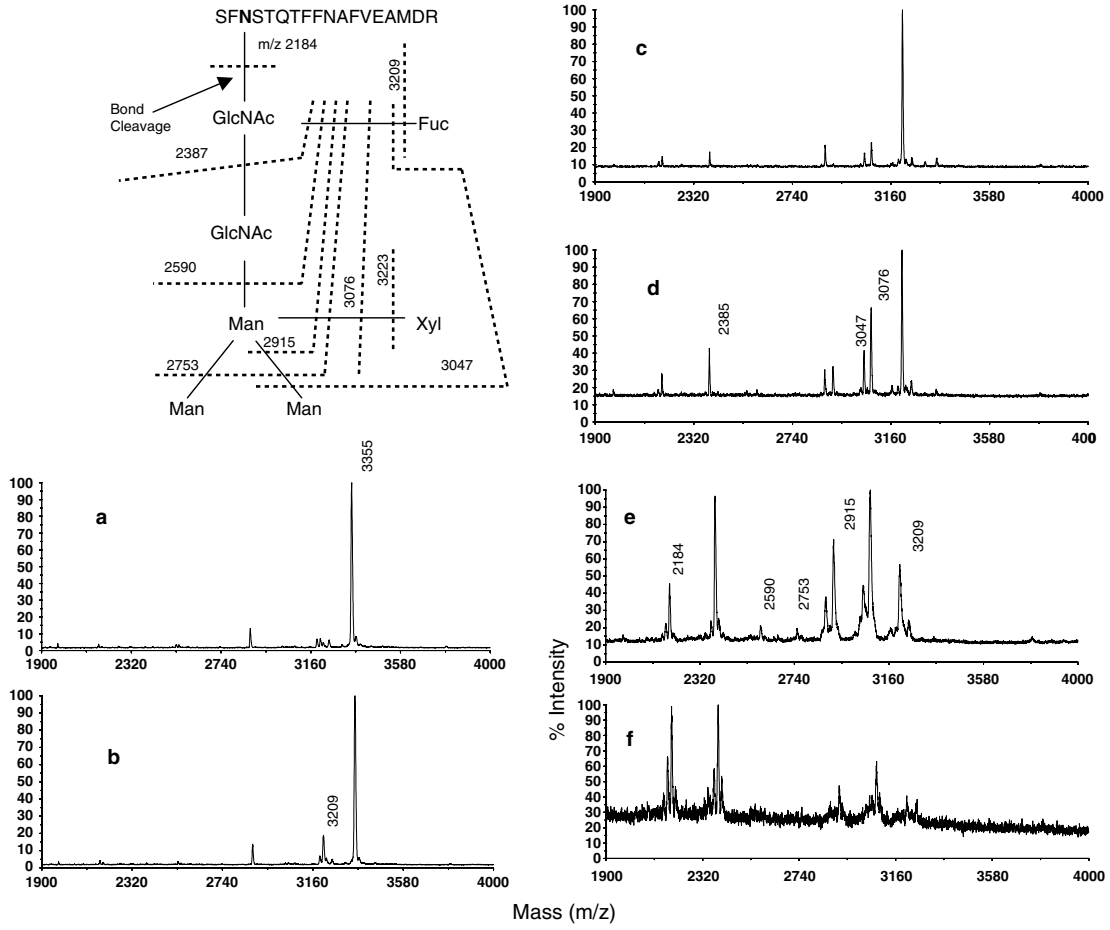

Figure 5. MALDI-TOF mass spectra of thermal-assisted acid hydrolysis product ions from the glycopeptide 3355 Da of HRP corresponding to peptide S_{265}-R_{283} under different hydrolysis conditions. (a) 14 mM TFA and no thermal heating; (b) 1.4 M TFA and 3.5 h thermal heating at 37 °C; (c) 3.1 M TFA and 1 h thermal heating at 65 °C; (d) 1.4 M TFA and 30 min thermal heating at 95 °C; (e) 3.1 M TFA and 30 min thermal heating at 95 °C; (f) 3.1 M TFA and 5 h thermal heating at 65 °C.

time-of-flight (MALDI-TOF) target and analyzed by a Voyager- DE PRO Mass Spectrometer (Applied Biosystems) equipped with a 337 nm pulsed nitrogen laser. Peptide mass was measured using a positive-ion linear mode over the range of *m*/*z* 1000–6500. External mass calibration was performed using peaks corresponding to a mixture of bradykinin fragments 1–7 at *m*/*z* 757, angiotensin II (human) at *m*/*z* 1046, $P_{14}R$ (synthetic peptide) at *m*/*z* 1533, adrenocorticotropic hormone fragments 18–39 (human) at *m*/*z* 2465, insulin oxidized B (bovine) at *m*/*z* 3494, and insulin (bovine) at *m*/*z* 5735.

3. Results and discussion

The amino acid sequence of horseradish peroxidase (HRP) is shown in Figure 1, and the nine N-glycosylation sites of Asn are indicated by asterisks. The structure of the major oligosaccharide of the HRP is shown in Figure 2. Acid hydrolysis of N-glycosylated peptides of horseradish peroxidase (HRP) is used to generate oligosaccharide ladders with MWs of 2533, 2612, 3355, 3673, and 5647 Da. Sequencing information for the carbohydrate moieties of glycoproteins is collected using these ladders. Generally,[4,15] linkages involving *N*-acetyl hexosamines are the most difficult to hydrolyze and require 4 M HCl with heating at 100 °C for 6 h. Linkages between hexoses, deoxyhexoses, and pentoses are easier to hydrolyze requiring 2 M trifluoroacetic acid (TFA) and heating at 100 °C for 6 h. Sialic acid family is even more acid labile requiring only 50–100 mM TFA and heating at 37 °C for 1 h. Heating for 2 h at 121 °C in 4 M TFA is also used to release *N*-acetyl hexosamines, hexoses, deoxyhexoses, pentoses, and the sialic acid family successfully.[11] However, some degradation products of peptide and sugars have been observed in all acid hydrolysis procedures. It is noted that acid cleavage of Asn-linked carbohydrate can N-deacetylate all amine-based sugar residues.

Figures 3–7 show the MALDI-TOF mass spectra of the glycopeptide 2533, 2612, 3355, 3672, and 5647 Da associated with peptides S_{265}-R_{283}, M_{284}-R_{298}, S_{265}-

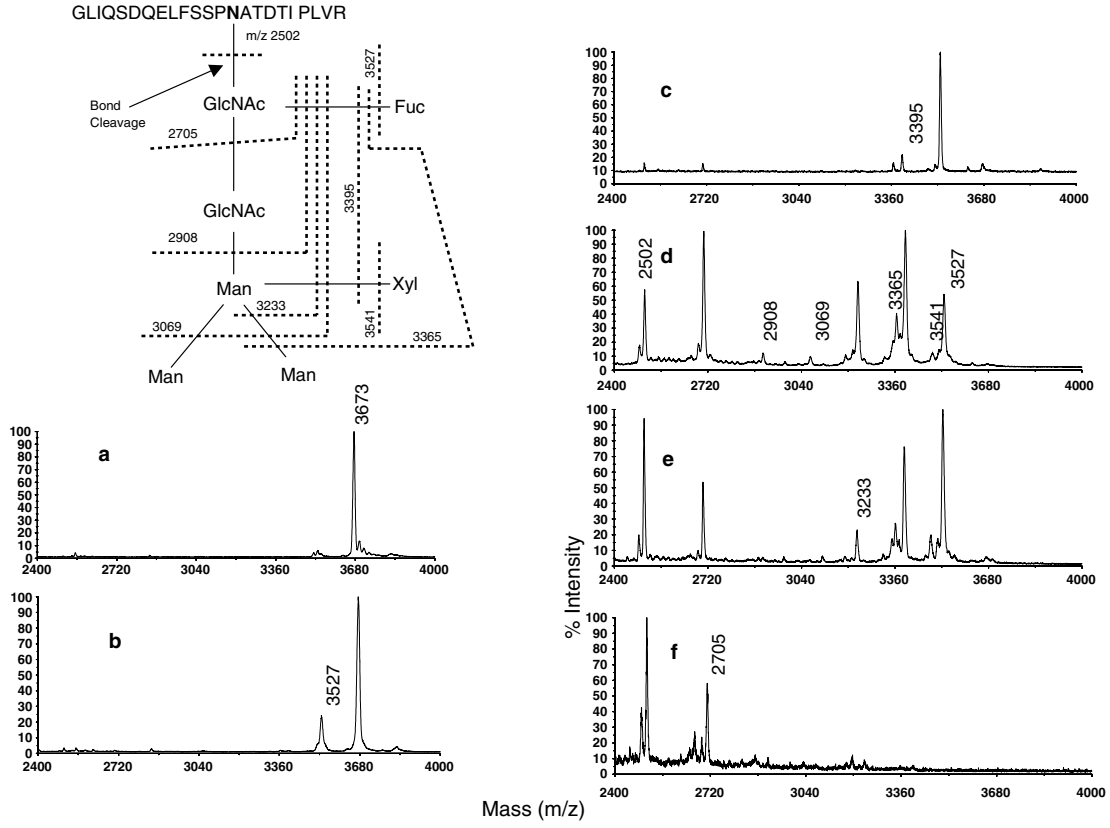

Figure 6. MALDI-TOF mass spectra of thermal-assisted acid hydrolysis product ions from the glycopeptide 3673 Da of HRP corresponding to peptide G_{242}-R_{264} under different hydrolysis conditions. (a) 14 mM TFA and no thermal heating; (b) 3.1 M TFA and 20 h thermal heating at 37 °C; (c) 3.1 M TFA and 1 h thermal heating at 65 °C; (d) 3.1 M TFA and 30 min thermal heating at 95 °C; (e) 1.4 M TFA and 5 h thermal heating at 65 °C; (f) 1.4 M TFA and 2.5 h thermal heating at 95 °C.

R_{283}, G_{242}-R_{264}, and F_{179}-R_{206}, respectively, following thermal-assisted acid hydrolysis in 1.4, 3.1, 4.5, or 6.7 M TFA for 30 min, 1, 2.5, 3.5, 5, or 20 h. Glycopeptides, except 2612 Da, show a loss of an *m/z* 146 species with 2.5 h of heating at 37 °C in 1.4 M TFA solution. The cleavage product ions are observed at *m/z* 2387, 3209, 3527, and 5355 and 5501 for 2533, 3355, 3672, and 5647 Da glycopeptides, respectively. These data indicate that all glycopeptides studied here except 2612 Da contain deoxyhexose (Fuc) at the terminals. Some minor product ions from N-deacetylation indicate that amine-based sugar residues (GlcNAc) are present in all the glycopeptides tested. Upon increasing the TFA concentration (to 3.1 M) and duration of thermal heating, glycopeptides except 2533 Da show losses of *m/z* 132 and 162 species, indicating the presence of pentose and hexose (Xyl and Man). The cleavage product ions are observed at *m/z* 2316, 2450, and 2480 for the 2612 Da glycopeptide; at *m/z* 3076 and 3047 for the 3355 Da glycopeptide; at *m/z* 3395 and 3365 for the 3673 Da glycopeptide; and at *m/z* 5223, 5195, 5091, 5061, 5031, 4929, and 4899 for the 5647 Da glycopeptide. Further increasing the TFA concentration to 4.5 or 6.7 M with thermal heating at 65 °C for 5 h, glycopeptides lose more *m/z* 162 species of Man and *m/z* 203 species of *N*-acetyl-α-D-glucosamine (GlcNAc). Majority of the final cleavage products are peptides with no sugar residues and/or one GlcNAc residue linked to the Asn. The final cleavage product ions are observed at *m/z* 2184, 1587, 2184, 2502, and 3305 for the 2533, 2611, 3355, 3672, and 5647 Da glycopeptides, respectively. The pattern of acid hydrolysis and observance of the final cleavage product ion of 5647 Da at *m/z* 3305 (5647–2 × 1171) suggests that this glycopeptide contains two glycosylation sites with identical oligosaccharide structure $(Xyl)Man_3(Fuc)GlcNAc_2$. Minor cleavage product ions from loss of acetyl groups of GlcNAc (e.g., *m/z* 3191 of *m/z* 3233 in Fig. 6e) or water (e.g., *m/z* 2484 of *m/z* 2502 in Fig. 6g) have also been detected. Some other cleavage product ions, for example, *m/z* 1786, 2104, and 2363 of 3673 Da glycopeptide, resulting from the hydrolysis of the peptide bond have also been detected (data not shown).

The MALDI-TOF mass spectra for the thermal-assisted acid hydrolysis products from non-glycopeptide 1587 Da (Met_{284}-Arg_{298}) in varied amounts of TFA for varied temperature and duration show no change in MW as predicted (data not shown). An unchanging intense signal was seen for the 1587 Da peptide, which suggested that the N-glycosylation site at Asn_{286} is not occupied.[8,12–14] However, a weak glycopeptide peak at

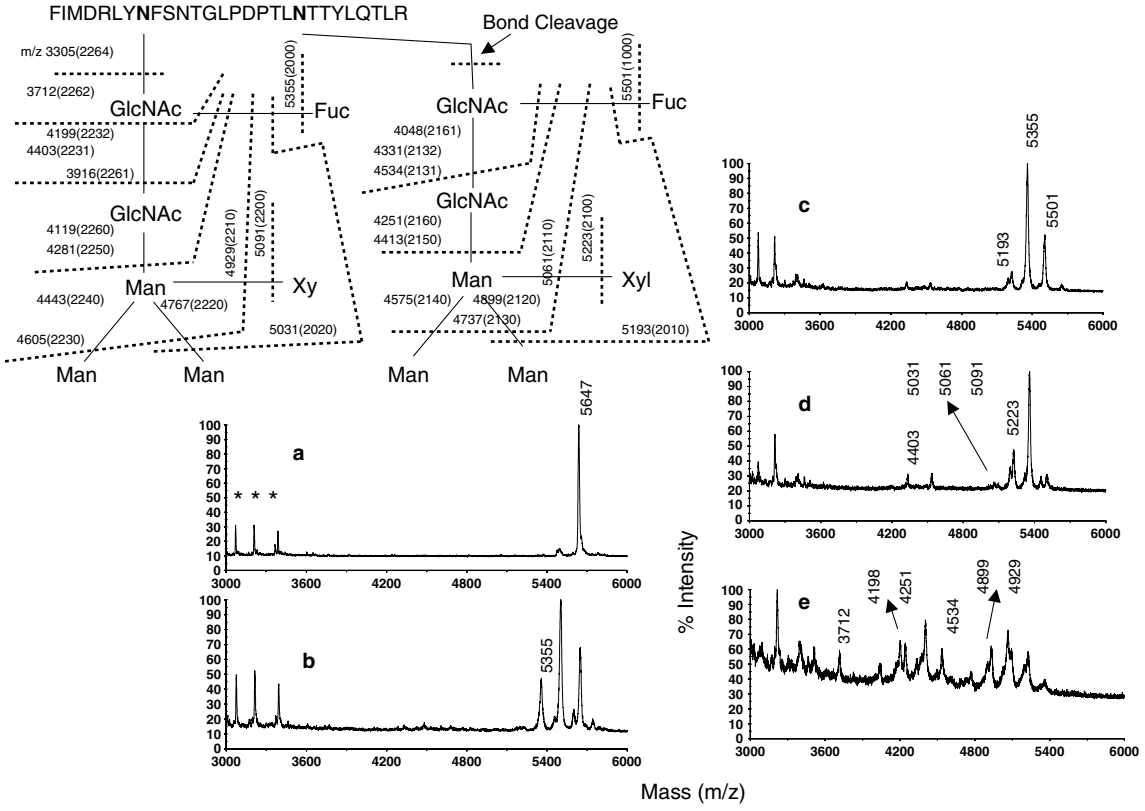

Figure 7. MALDI-TOF mass spectra of thermal-assisted acid hydrolysis product ions from the glycopeptide 5647 Da of HRP corresponding to peptide F_{179}-R_{206} under different hydrolysis conditions. (a) 14 mM TFA and no thermal heating; (b) 4.5 M TFA and 3.5 h thermal heating at 37 °C; (c) 1.4 M TFA and 1 h thermal heating at 65 °C; (d) 3.1 M TFA and 1 h thermal heating at 65 °C; (e) 3.1 M TFA and 30 min thermal heating at 95 °C. Asterisks indicate minor impurities. The first, second, third, and fourth number in the parentheses represent number of lost Fuc, Xyl, Man, and GlcNAc, respectively.

2612 Da that also corresponds to peptide M_{284}-R_{298}, as discussed above, provides an evidence for the occupancy of the Asn_{286}.[8,9,15] It is therefore likely that Asn_{286} is partially occupied. New cleavages of the HRP by trypsin observed here, which were absent in previous reported HRP data,[8] provided more information about the structures of the oligosaccharides.

4. Conclusions

Thermal-assisted heating and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were successfully used to study the glycan structures of glycopeptides. Tryptic N-glycosylated peptides of horseradish peroxidase with MWs of 2533, 2612, 3355, 3673, and 5647 Da were used as model systems. By thermal heating the glycopeptides at 37, 65, or 95 °C in 1.4, 3.1, 4.5, or 6.7 M aqueous trifluoroacetic acid solution, partial and complete cleavages of the glycopeptide glycan were observed. The hydrolysis ladders suggested that N-glycosylated peptides 3355, 3673, and 5647 Da have a glycan structure of $(Xyl)Man_3(Fuc)GlcNAc_2$. There were two glycosylation sites for the 5647 Da peptide. Glycopeptide 2612 Da had a glycan structure of $(Xyl)Man_3GlcNAc_2$. However, majority of the glycosylation sites of the 2612 Da peptide at Asn_{286} were not occupied. Glycopeptide 2533 Da had a core glycan structure of (Fuc)GlcNAc. The most labile group was the Fuc, and the majority of the final cleavage products were the peptides with no sugar residues linked to the Asn. The data also showed species that have lost one water molecule and acetylation, and some hydrolyzed products of the carbohydrate moiety of the glycopeptide. Further work on glycopeptides with more complex glycan structures is underway.

Acknowledgments

We thank the Research Resources Center at the University of Illinois at Chicago for support.

References

1. Gahmberg, C.; Tolvanen, M. *Trends Biochem. Sci.* **1996**, *21*, 308–311.
2. Varki, A. *Glycobiology* **1993**, *3*, 97–130.

3. Freeze, H. *Glycobiology* **2001**, *11*, 129–143.
4. Michalski, J.; Capon, C. *Methods Mol. Biol.* **1990**, *125*, 159–180.
5. Chiesa, C.; O'Neill, R.; Horváth, C.; Oefner, P. In *Capillary Electrophoresis in Analytical Biotechnology*; Righetti, P., Ed.; CRC Press: Boca Raton, FL, 1996, Chapter 9, pp 277–430.
6. Duus, J.; Gotfredsen, C.; Bock, K. *Chem. Rev.* **2000**, *100*, 4589–4614.
7. Morelle, W.; Michalski, J. *Curr. Anal. Chem.* **2005**, *1*, 29–57.
8. Wuhrer, M.; Hokke, C.; Deelder, A. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1741–1748.
9. Lee, B.; Krishnanchettiar, S.; Lateef, S.; Gupta, S. *Rapid Commun. Mass Spectrom.* 2005, *19*, 1545–1550.
10. Fan, J.; Namiki, Y.; Matsuoka, K.; Lee, Y. *Anal. Biochem.* **1994**, *219*, 375–378.
11. Fu, D.; O'Neill, R. *Anal. Biochem.* **1995**, *227*, 377–384.
12. Gray, J.; Yang, B.; Montgomery, R. *Carbohydr. Res.* **1998**, *311*, 61–69.
13. Welinder, K. *Eur. J. Biochem.* **1979**, *96*, 483–502.
14. Veith, N. *Phytochemistry* **2004**, *65*, 249–259.
15. Kelly, J.; Locke, S.; Ramaley, L.; Thibault, P. *J. Chromatogr. A* **1996**, *720*, 409–427.
16. Zhong, H.; Marcus, S. L.; Li, L. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 471–481.