Extension of microwave-accelerated residue-specific acid cleavage to proteins with carbohydrate side chains and disulfide linkages

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ABSTRACT

This laboratory has introduced a chemical method for residue-specific protein cleavage and has provided a preliminary assessment of the suitability of microwave-accelerated acid cleavage as a proteomic tool. This report is a continuing assessment of the fate of common protein modifications in microwave-accelerated acid cleavage. We have examined the cleavage of ribonuclease A and the related N-linked glycoprotein ribonuclease B, and the O-linked glycoprotein alpha crystallin A chain, using MALDI-TOF and LC–ESI-MS to identify the peptide products. RNase A and B each contains four disulfide bonds, and the addition of a reducing reagent, such as dithiothreitol, was found to be required to achieve efficient acidic proteolysis. The linkage of the glycosidic group to the asparagine side chain in ribonuclease B was found not to be cleaved by brief microwave treatment in 12.5% acetic acid. The distribution of the heterogeneous carbohydrate side chain in the glycopeptide products of acid cleavage was compared to that of the glycopeptide products of tryptic digestion. Hydrolysis within the carbohydrate chain itself is minimal under the conditions used. The O-linked side chain on alpha crystalline A was found to be cleaved during acid cleavage of the protein.

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1. Introduction

Recently the Fenselau group has reported that microwave-assisted acid incubation cleaves proteins at aspartic acid residues, with high specificity in seconds or minutes [1] and that tandem mass spectra of the peptide products of this proteolytic reaction are suitable for bioinformatics searching in high throughput proteomic strategies [2,3].

Acetic (or other) acid serves as a chemical enzyme that can cleave at both sides of aspartic acids, via a mechanism that has been considered previously [3,4]. This method was demonstrated for rapid identification of viruses [5] and *Bacillus* spores [1,6]. More recently, the effect of hot acid has been evaluated on acetylation, oxidation and phosphorylation of proteins [3]. In the present study, we evaluate the suitability of this chemical proteolysis for analysis of glycoproteins and proteins with disulfide bonds.

Ribonuclease A and B were used to validate the method for analysis of N-linked glycoproteins and proteins with internal disulfide bonds. Ribonuclease A is a non-glycosylated protein, which serves as a control for ribonuclease B, as they have the same pri-

mary structure. Ribonuclease B carries an N-linked glycan at Asp 34, whose structure has been characterized as a high-mannose type with a *N*-acetylglycosamine core (GlcNAc) and 4–9 mannose residues (Man) attached to the core [7,8]. Additionally, both of these relatively small proteins have four internal disulfide bonds, which make their structures stable and relatively difficult to digest. In proteomic strategies, an incubation time of an hour or longer is usually required to reduce disulfide bonds with relatively large amounts of reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT). We demonstrate in this report that we were able to cleave the disulfide bonds in RNase A and B and digest these model proteins concurrently within 5 min, by adding dithiothreitol to the acidic microwave reaction. The acetic acid cleaves the protein at one or both sides of aspartic acid when the disulfide bridges are reduced by DTT.

It was of primary interest to determine if carbohydrate–protein linkages would be affected by the microwave-accelerated acid digestion and if the oligosaccharide chain would be cleaved. Carbohydrate heterogeneity in the acid cleavage products of RNase B was characterized by LC–ESI-MS mass spectrometry and compared to that of the products of a parallel tryptic digestion. Alpha crystallin A chain carries a single O-linked GlcNac at S162 [9], which allowed evaluation of the stability of O-linkages during acid catalyzed proteolysis.

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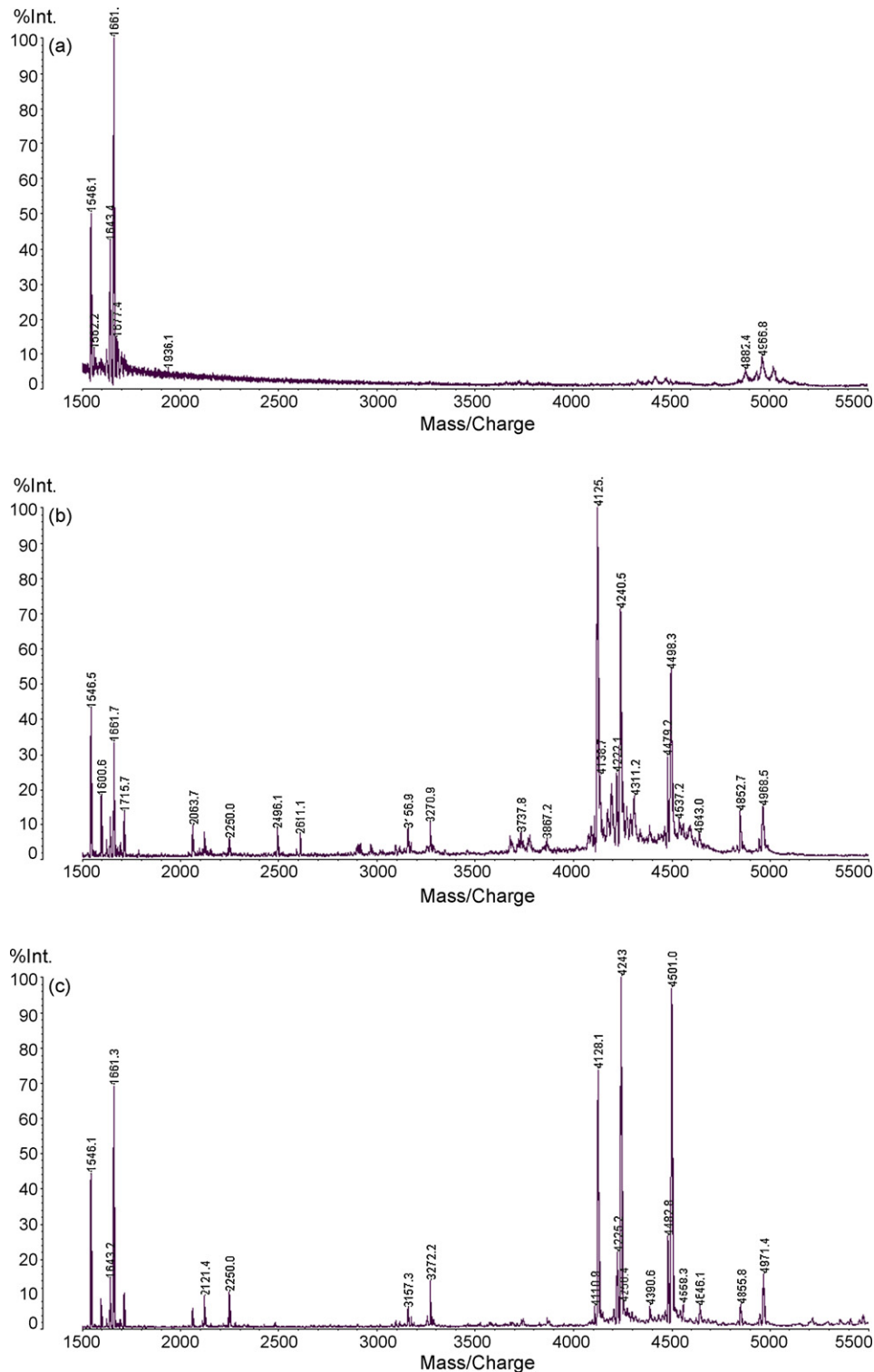
Fig. 1. MALDI-MS spectra of peptide products of the acetic acid digestion (a) of RNase A without the DTT (b) of RNase A with 5 mM DTT, and (c) ribonuclease B with 5 mM DTT.

2. Experimental section

2.1. Materials

HPLC gradients (acetonitrile, water, formic acid) were purchased from Burdick & Jackson (Morristown, NJ), glacial acetic acid was purchased from Fisher (Fair lawn, NJ), α-cyanohydroxycinnamic acid (CHCA) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO), protein calibration standard kit, ribonuclease A, and ribonuclease B were purchased from Sigma. Alpha crystallin A chain was purchased from Stressgen Bioreagents (Ann Arbor, MI). Trypsin was purchased from Promega (Madison, WI). All chemicals and proteins were used without further purification.

Table 1
Peptides from the acid digestion of ribonuclease A identified by MASCOT

Position	Obs.	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
1–13	1546.5000	1545.4926	1546.7496	−1.2570	0	-.KETAAAKFERQHM.D
1–14	1660.6000	1659.5926	1661.8371	−2.2444	1	-.KETAAAKFERQHMD.S
1–14	1661.7000	1660.6926	1661.8371	−1.1444	1	-.KETAAAKFERQHMD.S
15–37	2496.1000	2495.0926	2496.7533	−1.6606	0	D.SSTSAASSSNYCNQMMKSRNLTK.D
15–38	2611.1000	2610.0926	2611.8407	−1.7480	1	D.SSTSAASSSNYCNQMMKSRNLTKD.R
39–53	1715.7000	1714.6926	1715.9275	−1.2349	1	D.RCKPVNTFVHESLAD.V
39–82	4852.7000	4851.6926	4855.4469	−3.7543	2	D.RCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSIT.D
39–83	4968.5000	4967.4926	4970.5343	−3.0417	3	D.RCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITD.C
54–82	3156.9000	3155.8926	3157.5347	−1.6421	0	D.VQAVCSQKNVACKNGQTNCYQSYSTMSIT.D
54–83	3270.9000	3269.8926	3272.6221	−2.7295	1	D.VQAVCSQKNVACKNGQTNCYQSYSTMSITD.C
84–120	4125.0000	4123.9926	4127.6406	−3.6480	0	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHF.D
84–121	4240.5000	4239.4926	4242.7280	−3.2354	1	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFD.A
84–124	4498.3000	4497.2926	4500.0143	−2.7217	2	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV.-

2.2. Microwave-assisted acetic acid digestion

A Discover Benchmate microwave system (CEM Corp, Matthews, NC) was utilized to perform the protein digestions which allowed control of the temperature, pressure, power, and time. Ribonuclease A was dissolved in Milli-Q water to make a 2 mg/ml solution. Two and a half microliters of this protein solution was mixed with 6.25 μL of acetic acid, 25 μL of 10 mM DTT, and 16.25 μL of Milli-Q water in a 300 μL micro-glass vial and exposed to microwave irradiation in the open vessel mode. The incubation time was 5 min with a fixed microwave irradiation power at 300 W and a maximum temperature of 140°. The same procedure was used for ribonuclease B.

Alpha crystallin A chain came in phosphate buffer solution at 1.2 mg/mL (0.15 M NaCl, 0.05 M phosphate, pH 7.2). Two and one-tenth microliters of this protein solution was mixed 6.25 μL acetic acid and 25 μL of 10 mM DTT, and water was added to reach a final volume of 50 μL.

2.3. Tryptic digestion

Ammonium bicarbonate (79 mg) was mixed with 10 mL of Milli-Q water to reach a final concentration of 100 mM. Ammonium bicarbonate solution (1.6 ml) was added directly to a vial containing 20 μg immobilized trypsin. Ribonuclease B solution (2.5 μL) was incubated with 30 μL 100 mM DTT for 3 h at 57 °C and allowed to cool to room temperature. Fifty microliters trypsin was added to the protein solution, followed by incubation overnight at 37 °C. For the alpha crystallin, 5 μL solution was mixed with 45 μL 10 mM DTT, and processed as described above.

2.4. MALDI-TOF-MS analysis

The samples from the acetic acid digestion were analyzed using an Axima-CFR Plus MALDI-TOF mass spectrometer (Shimadzu Biotech, Columbia, MD). The matrix solution was prepared by dis-

solving 10 mg CHCA in 1 mL water containing 70% acetonitrile and 0.1% TFA. The sample was applied to the MALDI plate via a sandwich method. A layer of 0.5 μL matrix was pipetted onto the plate first, followed by 0.5 μL sample solution and another layer of 0.5 μL matrix. Each layer was allowed to air dry before the next layer was applied. The spectra were obtained in the linear positive ion mode, with a 337 nm N₂ laser, by collecting and summing 200 profiles from 1500 to 5500 Da.

## 2.5. HPLC–ESI-MS analysis

The peptide mixtures of RNase B from both the tryptic digestion and the acetic acid digestion were each separated on a nanoACQUITY UPLC System (Waters, Milford, MA). Ten microliters of sample solution was introduced onto a reverse phase C-18 column (1.0 mm i.d. ×100 mm) with 1.7 μm bridged ethyl hybrid (BEH) particle packing (Waters, Milford, MA). The peptides were eluted via a binary solvent system (solvent A: 0.1% TFA and 1% water in acetonitrile; solvent B: 0.1% TFA and 1% acetonitrile in water) using a
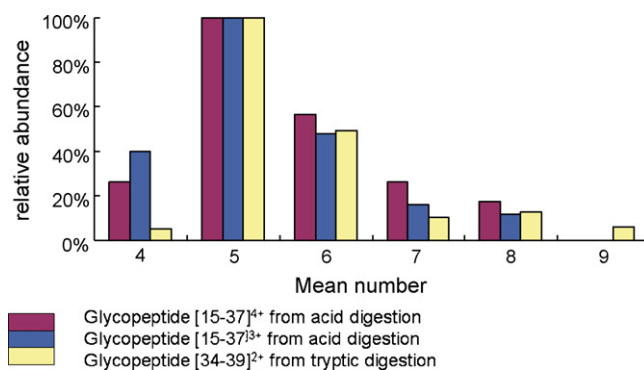
Fig. 2. Relative abundances of glycopeptides produced by acetic acid and tryptic digestions of ribonuclease B.

Table 2
Peptides from the acid digestion of ribonuclease B identified by MASCOT

Position	Obs.	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
1–13	1546.1000	1545.0926	1546.7496	−1.6570	0	-.KETAAAKFERQHM.D
1–14	1660.2000	1659.1926	1661.8371	−2.6444	1	-.KETAAAKFERQHMD.S
1–14	1661.3000	1660.2926	1661.8371	−1.5444	1	-.KETAAAKFERQHMD.S
39–53	1715.3000	1714.2926	1715.9275	−1.6349	1	D.RCKPVNTFVHESLAD.V
39–82	4855.8000	4854.7926	4855.4469	−0.6543	2	D.RCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSIT.D
39–83	4971.4000	4970.3926	4970.5343	−0.1417	3	D.RCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITD.C
54–82	3157.3000	3156.2926	3157.5347	−1.2421	0	D.VQAVCSQKNVACKNGQTNCYQSYSTMSIT.D
54–83	3272.2000	3271.1926	3272.6221	−1.4295	1	D.VQAVCSQKNVACKNGQTNCYQSYSTMSITD.C
84–120	4128.1000	4127.0926	4127.6406	−0.5430	0	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHF.D
84–121	4243.0000	4241.9926	4242.7280	−0.7354	1	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFD.A
84–124	4501.0000	4499.9926	4500.0143	−0.0217	2	D.CRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV.-
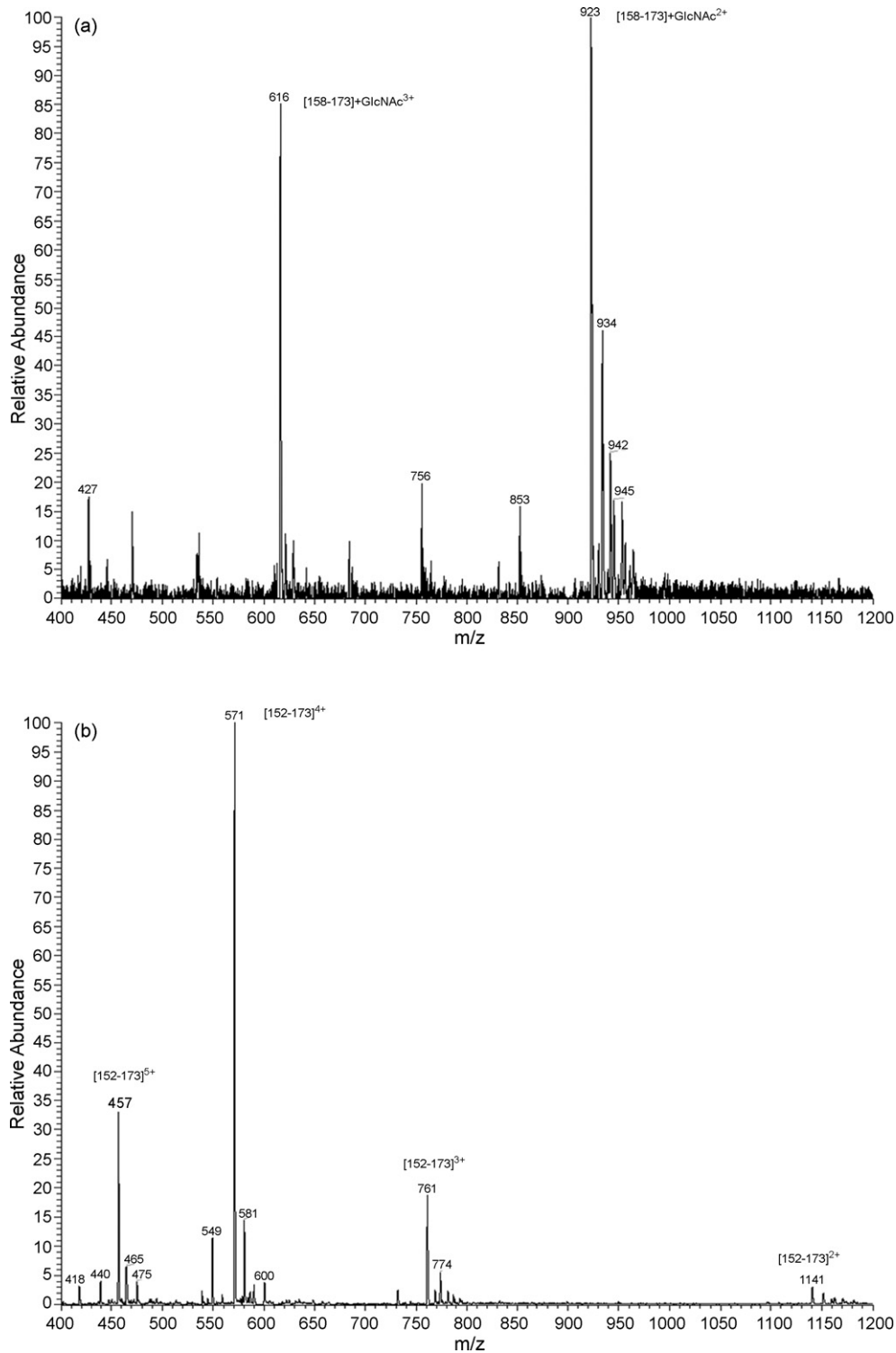
Fig. 3. (a) MS spectrum of the glycopeptide [158–173] + GlcNAc from tryptic digestion and (b) deglycosylated peptide [152–173] from acid digestion of alpha crystallin A chain.

linear gradient from 0% B to 40% B in 40 min. The flow rate was set at 50 μL/min. The HPLC system was directly coupled to the ESI-MS. All mass spectra were acquired between 400 and 2000 Da on an LTQ XL ion trap (Thermo Electron, San Jose, CA). The electrospray voltage was 4 kV, and the ESI nebulizing gas was nitrogen.

The peptide mixture obtained from alpha crystallin A chain by acetic acid digestion was separated and analyzed using the same system. A linear 60 min gradient from 0% to 50% B was applied.

2.6. Mascot database search

A Mascot search (Matrix Science Ltd., London, UK) was carried out using the peptide mass fingerprint to analyze the MS spectra from acetic acid digestion. All the *m*/*z* values in the spectrum in Fig. 1 were entered except for the doubly charged ions. The search was performed against SwissProt database and all species using "formic acid" as the enzyme with a peptide mass tolerance of 4 Da.

Four missed cleavages were allowed. The average mass of $[M+H]^+$ was selected, while no modification was entered.

3. Results and discussion

Ribonuclease A and B each contains four disulfide bonds. For acid cleavage (as well as tryptic cleavage) good digestion could not be realized without reduction of the disulfide bonds. This is illustrated for RNase A in Fig. 1a, where it can be seen that only the N-terminal peptide [1–13] and [1–14] is released by acid treatment of the native protein. When DTT was added directly to the protein solution with acetic acid before microwave radiation, the reduction of disulfide bonds and cleavage of the peptide backbone were performed concurrently in 5 min, as analyzed in Fig. 1b. No further cleanup was needed before the MS analysis.

4. N-linked glycoprotein—RNase B

The MALDI spectra of peptides recovered from reduced ribonuclease A and B are shown in Fig. 1b and c. Pairs of peaks separated by 115 Da can be observed in the spectra, because acetic acid can cleave the polyamide chain on both sides of aspartic acid. These pairs help to recognize and identify peptide products. Peptides were identified that provide 100% sequence coverage of RNase A, as listed in Table 1. For RNase B, the peptides identified covered 80% of the amino acid sequence (Fig. 1c and Table 2). The missing sequence was associated with the glycopeptide [15–38], which carries carbohydrate attached to Asn34. The glycopeptides were subsequently analyzed using ESI, following fractionation by HPLC.

Relative abundances of the glycoforms produced from the same sample by acid cleavage and trypsin, were compared in order to evaluate the stability of bonds within the carbohydrate chain during the short treatment with hot acid. Four small tryptic glycopeptides were detected, [34–49], [32–49], [34–37], [32–37], while the acetic acid digestion produced two glycopeptides [15–37], [15–38] with molecular masses around 4000 Da. Analysis of the former was optimized for [34–39] and the latter for [15–37]. The latter glycopeptides weighed around 4 kDa, including the carbohydrate, and were ionized mainly in two charge states. The relative abundances of glycoforms of $[34–39]^{2+}$, $[15–37]^{3+}$ and $[15–37]^{4+}$ are plotted in Fig. 2. The relative abundances of glycoforms containing Man-5, 6, 7 and 8 Man residues are comparable. However, a glycopeptide with Man-9 is observed only among the tryptic products, and the abun-dance of the Man-4 glycoforms is higher in the acid products. These differences may reflect the preferential analysis and detection of the smaller ions in the series of heavy glycopeptides, however the possibility of a small amount of glycolysis cannot be ruled out.

5. O-linked glycoprotein—alpha crystallin A chain

The electrospray spectrum of the O-linked glycopeptide produced by tryptic digestion of alpha crystallin A is shown in Fig. 3a. The intact glycopeptide [158–173] is characterized in two charge states, +2 and +3. Fig. 3b presents the spectrum of peptide [152–173], recovered from the acid digestion of alpha crystallin A chain. The spectrum supports the conclusion that the O-linked GlcNAc attached to Ser162 was cleaved during the microwave acid digestion process.

6. Conclusion

Studies of the model proteins ribonuclease A and B lead to the conclusions that reduction of disulfide bonds is required to allow rapid and comprehensive residue-specific acid cleavage; that such reduction is effectively carried out concurrently with proteolysis; and that the classic N-linkage of carbohydrate chains to asparagine residues is not cleaved by acid under microwave irradiation. Analysis of glycoforms indicates that degradation of the high mannose side chain occurs slowly if at all, relative to proteolysis, and that glycopeptide products will allow an estimation of glycoforms heterogeneity. However, the O-linked glycan was cleaved completely by the acid treatment of a model O-linked glycoprotein.

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