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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.

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 primary 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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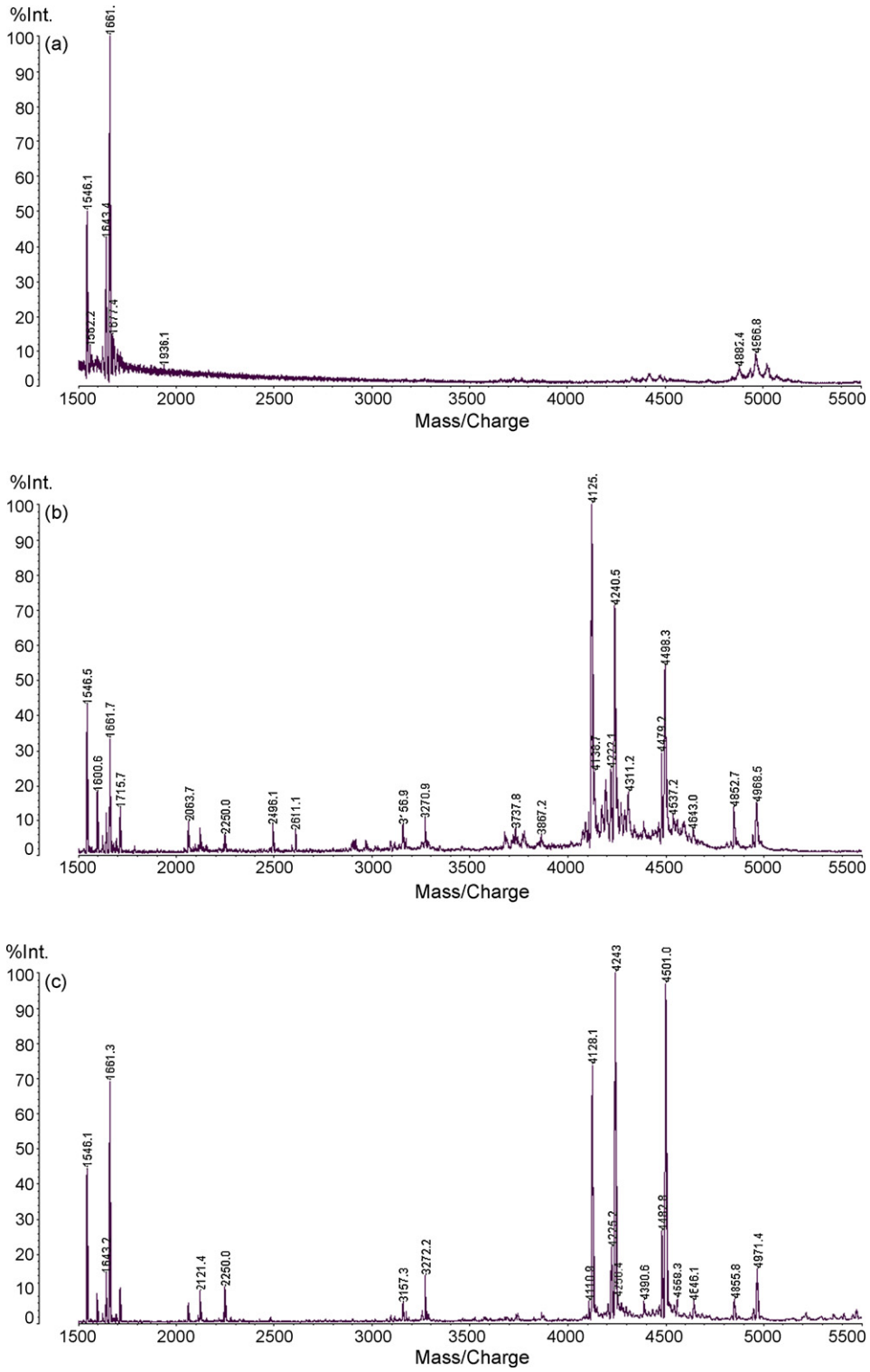

Fig. 1. MALDI-MS spectra of peptide products of the acetic acid digestion (a) of RNase A without the DTT (b) of RNase A with 5 mM DTT, and (c) ribonuclease B with 5 mM DTT.

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.