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Online Microwave D-Cleavage LC-ESI-MS/MS of Intact Proteins: Site-Specific Cleavages at Aspartic Acid Residues and Disulfide Bonds

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An online nonenzymatic digestion method utilizing a microwave-heated flow cell and mild acid hydrolysis at aspartic acid (D) for rapid protein identification is described. This methodology, here termed microwave D-cleavage, was tested with proteins ranging in size from 5 kDa (insulin) to 67 kDa (bovine serum albumin) and a bacterial cell lysate (*Escherichia coli*). A microwave flow cell consisting of a 5 μL total volume reaction loop connected to a sealed reaction vessel was introduced into a research grade microwave oven. With this dynamic arrangement, the injected sample was subjected to microwave radiation as it flowed through the reaction loop and was digested in less than 5 min. Different digestion times can be achieved by varying the sample flow rate and/or length of the loop inside the microwave flow cell. The microwave flow cell can be operated individually with the output being collected for matrix assisted laser ionization/desorption (MALDI) mass spectrometry (MS) or connected online for liquid chromatography (LC) electrospray ionization (ESI)-MS. In the latter configuration, the microwave flow cell eluates containing digestion products were transferred online to a reversed phase liquid chromatography column for direct ESI-MS and ESI-MS/MS analyses (specifically, Collision Induced Dissociation, CID). Concurrently with the microwave D-cleavage step, disulfide bond reduction/cleavage was achieved by the coinjection of dithiothreitol (DTT) with the sample prior to online microwave heating and online LC-MS analysis and so eliminating the need for alkylation of the reduced protein. All protein standards, protein mixtures, and proteins in a bacterial cell lysate analyzed by this new online methodology were successfully identified via a SEQUEST database search of fragment ion mass spectra. Overall, online protein digestion and identification was achieved in less than 40 min total analysis time, including the chromatographic step.

Introduction

The advent of new ionization techniques including electrospray ionization (ESI)[1–3] and matrix assisted laser desorption/ionization (MALDI)[4,5] have allowed mass spectrometry to become a powerful tool in protein identification. Proteins can be identified by performing a database search on tandem mass spectrometry (MS/MS) data generated from a peptide derived from the intact protein.[6–9] In this bottom-up proteomic approach, proteins are cleaved at a specific amino acid residue creating a mixture of peptides, which contains all of the sequence information for the protein. Further refinement of this approach to incorporate 2-dimensional chromatography has allowed these proteomic methods to be applied to mixtures of proteins.[10–12]

The enzymatic digestion of proteins has proven to be quite effective as a preliminary step in the routine identification of proteins. Trypsin is the most commonly used enzyme because of its high specificity in cleaving at the C-terminus of the amino acids arginine (R) and lysine (K). Because of the high frequency of these amino acids in protein sequences, peptides produced are in the mass range of 500–2000 u, making them amenable to mass analysis with most quadrupole-based mass analyzers and collision induced dissociation (CID). The digestion of proteins with trypsin (or any other enzyme) is usually carried out off-line and requires controlled conditions of pH, concentration, and temperature. Moreover, digestion times range from 30 min to overnight, depending on conditions (i.e., enzyme immobilization) and sample concentration and volume. As a result of these conditions, proteomic analyses may not be suitable for field applications, or where a rapid turnaround time is required, as in forensic investigations or for high throughput proteomic measurements.

As part of the protein enzymatic digestion protocol for bottom-up protein identification, disulfide bonds are often cleaved to increase protein sequence coverage and to facilitate the formation of small peptides and their detection by tandem MS.[13,14] Cleavage of these bonds is commonly accomplished by chemical reduction of disulfide bonds with the addition of dithiothreitol (DTT), 2-mercaptoethanol, or tris(2-carboxyethyl)-phosphine (TCEP).[15–18] Conditions for disulfide bond reduction range in time from 30 min to 24 h while maintaining the solution temperature in the range from 25 to 60 °C,

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depending on the protocol used.[19–21] Moreover, alkylation of the reduced cysteines (C) is required in order to prevent the residues from reforming their bonds through oxidation. This alkylation step requires incubation of the reduced protein with iodoacetamide for 30 min in the dark and at room temperature, further adding to the total analysis time required for protein identification. Obviously, the incorporation of a disulfide bond cleavage step considerably increases the analysis time for bottom-up proteomic assays. In fact, the enzymatic digestion of proteins remains the rate limiting step in bottom-up proteomic analysis as the speeds of the MS measurement and the chromatographic step are faster (assuming a 30 min gradient).

Several approaches have been taken to decrease the time for protein digestion prior to MS analysis. In one approach, online digestion was performed by coating a fused silica capillary with a pepsin-containing gel.[22] Other methods of enzyme immobilization on a capillary have been implemented including physical adsorption,[23] covalent bonding,[24] and other sol-gel techniques.[25,26] These approaches were successful in eliminating the sample handling step prior to mass analysis and reducing the digestion time to minutes; however, the construction of these enzyme immobilized columns was time-consuming (days), requiring extensive preparation of the inner wall of the capillary and using conditions that allowed the enzymes to retain their biological activities. Hence, a protein digestion protocol incorporating a nonenzymatic site-specific cleavage into the bottom-up proteomic measurement would decrease the analysis time and allow for simplification and complete automation of the sample preparation and analytical measurement.

One such nonenzymatic protein digestion method is the chemical cleavage of proteins by acid hydrolysis at aspartic acid (D).[27,28] Aspartyl bonds are cleaved optimally at pH 2.0 while heating above 108 °C for 2 h, leading the β-carboxyl group to act as a proton donor and its hydroxyl oxygen as a nucleophile toward the adjacent carbonyl carbons in the peptide bond.[29] Cleavage at the C-terminus of D is the result of the formation of a five-membered ring and its subsequent hydrolysis, releasing the C-terminus peptide and reforming the aspartic acid group.[28] N-Terminus hydrolysis is also possible but not observed as frequently. Formic acid,[30] dilute hydrochloric acid,[31,32] and trifluoroacetic acid (TFA)[33] have all been used to keep the pH sufficiently low for the hydrolysis reaction to take place. Sand baths or hot plates traditionally have been used to keep the temperature at an elevated level.[30–32] More recently, however, microwave radiation has been used to quickly raise the temperature of the reaction vessel to speed the digestion at D.[34–36] Instead of a 2 h incubation time, it has been shown that digestion at D by microwave heating can take place in 1−10 min.[36,37] Microwave ovens are now extensively used to increase the speed of both enzymatic[38,39] and acid hydrolysis digestion of proteins,[36,40] and their importance in the field of proteomics is illustrated in a recent review article.[41] Recently, the analysis of *bacillus* spores and viruses was carried out by adopting this technique and digesting soluble proteins with 90 s of microwave heating.[40,42] However, in all these studies, the microwave digestion was performed off-line; that is, samples inside the microwave oven were placed in either a 1.5 mL eppendorf capped tube,[35] a 5 mL loosely capped vial,[42] or a septum-sealed 10 mL glass test tube,[40] requiring the handling of relatively large sample volumes and manual sample transfer for MALDI-MS analysis.

In this study, we describe an online microwave digestion technique coupled to ESI-MS and LC-ESI-MS which takes advantage of the speed, simplicity, and specificity of the acid hydrolysis at D. The technique described here, termed microwave D-cleavage, incorporates a novel microwave radiation flow cell design that couples directly to ESI-MS or LC-ESI-MS for fast online digestion and analysis of proteins in small sample volumes without transfer losses. In addition, this technique also incorporates a rapid online microwave disulfide bond reduction step carried out simultaneously with the microwave D-cleavage step, and as a result, the alkylation step is not required. With this combined approach and coupled online with LC-MS/MS, we demonstrate protein identification using either standard database searches (SEQUEST) or manual mass assignments. Overall, the methodology described herein was successful in decreasing the protein digestion time, increasing the protein sequence coverage detected (when compared to acid hydrolysis at D only), eliminating the need for an alkylation step, and reducing potential sample losses during off-line microwave heating and sample handling.

Experimental Section

Chemicals. Insulin (bovine pancreas), α-lactalbumin (bovine milk), lysozyme (chicken egg white), myoglobin (horse skeletal muscle), carbonic anhydrase (bovine erythrocytes), albumin (bovine serum), dithiothreitol (DTT), and sinapic acid were all purchased from Sigma (St. Louis, MO). HPLC grade deionized water and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI). ACS reagent grade formic acid was obtained from Sigma-Aldrich (St. Louis, MO).

Bacteria. *Escherichia coli* (*E. coli*; ATCC 15597) was purchased from American type Culture Collection (ATCC, Manassas, VA). Bacteria were grown under optimum laboratory conditions using tryptic soy agar (TSA; BD Science, Sparks, MD) at 37 °C for 12–15 h. Bacterial cells were transferred to 1 mL of water using a sterile tungsten loop inoculator. The cells were vortexed for 10 s and centrifuged for 5 min at 12 000 rpm to remove the media. The supernatant was removed; the pellet was resuspended in 1 mL of water; and the washing step was repeated. After three washing steps the cells were lysed by ultrasonication on ice. The cells were subjected to a sonication pulse duration of 10 s, followed by an idle 10 s, and repeated 10 times. The sample was then centrifuged at 5000 rpm for 15 min to remove any cell material. A 100 μL aliquot of the supernatant was diluted to 1 mL in 12.5% FA and 100 mM DTT just before being analyzed.

Protein Standards Preparation. All proteins were prepared at a concentration of 5−20 μg/mL in 12.5% FA and 100 mM DTT from a 1 mg/mL in 100% deionized water stock protein solution.

Instrumentation. Microwave heating was achieved using a CEM Focused Microwave Synthesis System (model: Discovery; CEM, Matthews, NC). Figure 1 shows the diagram of the online microwave cell used in all of the experiments described in this work, including photographs of the modified reaction vessel with the capillary flow cell.

The standard CEM reaction vessel (P/N 302780) was modified by drilling two threaded holes into the top cap and fitting it with two 5/16-24 to 10-32 adapters (Rheodyne P/N 6000-076, Rohnert Park, CA). A 1/16″ tubing was inserted by using a RheFlex nut/ferrule (P/N 6000-076) connecting directly into the port adapter. A 5 μL microwave reaction loop was made with 108.7 cm of fused silica capillary (Polymicro, Phoenix, AZ)

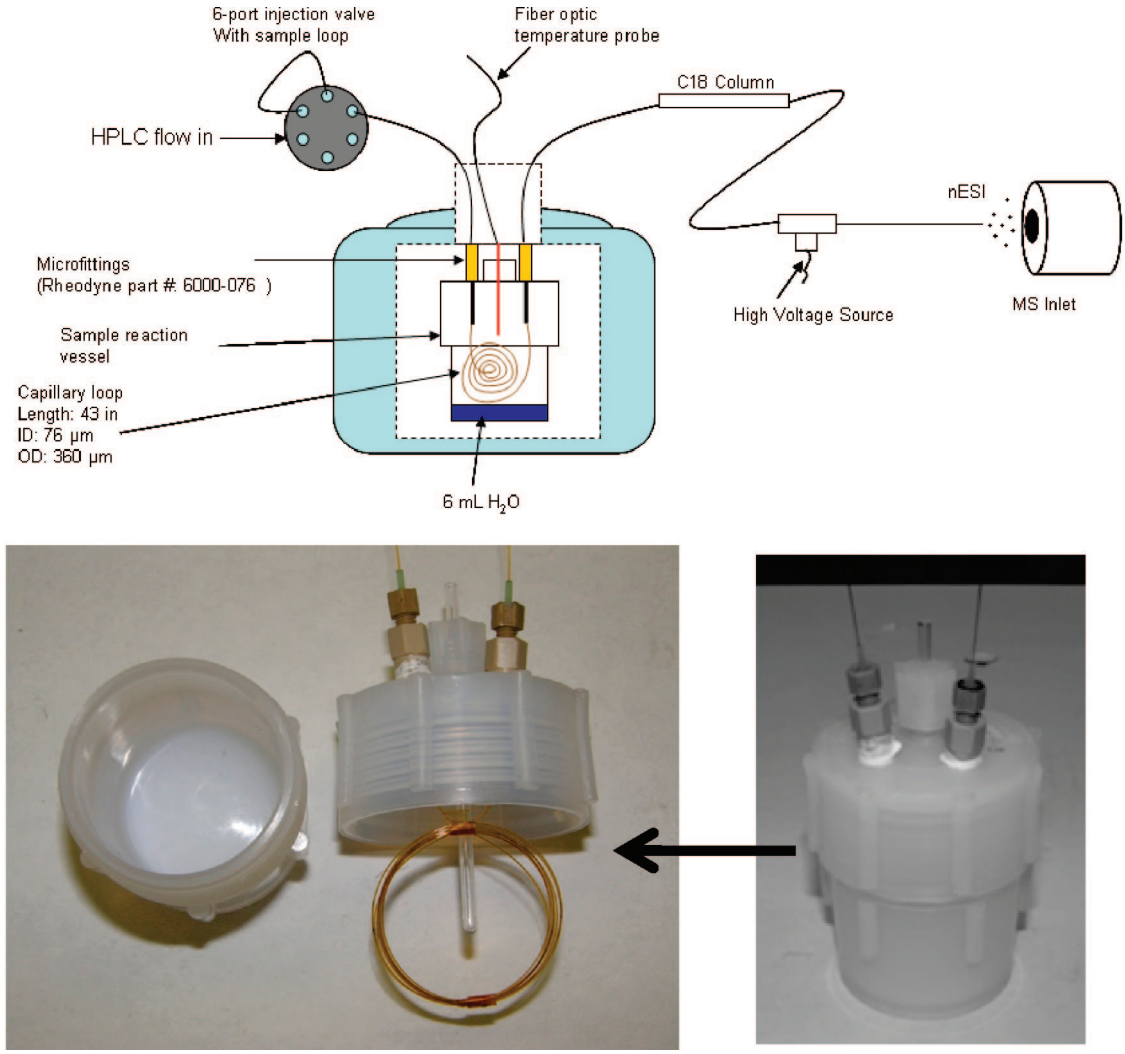


Figure 1. Top figure: schematic of the microwave digestion flow cell for online microwave D-cleavage. Pictures: the microwave flow cell is based on modification of the sample reaction vessel of a commercially available research-grade microwave oven. The reaction loop was 5 μL in total internal volume and was operated at a flow rate of 1 μL/min for a total digestion time of 5 min.

with an outer diameter of 360 μm and an inner diameter of 76 μm. Each end of the reaction loop was inserted into the underside of the adapters and tightened into place using 360 μm ID sleeves (Silica Sealtight, Upchurch Scientific, Oak Harbor, WA). About 6 mL of water was added to the bottom of the reaction vessel as suggested by the manufacturer. The top of the cell was tightened onto the bottom of the reaction vessel so that the microwave reaction loop was completely inside the vessel. The use of these microfittings allowed for the reaction loop to be inserted into the standard reaction vessel of the CEM microwave apparatus while maintaining pressure within the vessel during heating. The fused silica capillaries connected to the modified microwave vessel fit through the existing microwave oven opening at the top of the CEM unit. An advantage of this simple microwave flow cell is that the actual microwave heating unit is not modified, and as a result the operational safety of the microwave oven is not compromised. Moreover, the current design allows for multiple reaction loops to be added in order to perform parallel protein digestions. All the components used to fit the online microwave cell described herein have not shown any deterioration and/or heat damage under the microwave heating conditions used in this work.

All chromatographic experiments were performed with a dual gradient HPLC system (Ultimate, LC Packings, Sunnyvale, CA) capable of delivering a flow of 0.25–1 μL/min through the microwave flow cell. Samples were manually injected using a 2 μL loop (Cheminert injector, Valco Instruments Co. Inc., Houston, TX) fitted to a 6-port injection valve.

Online microwave digestion parameters were as follows: The temperature of the system was brought up to 130 °C over a 2 min interval with the microwave oven using a power between 50 and 250 W and then held at 130 °C for 20 min, for a total of 22 min of microwave heating. Note that the flow rate used determined how long the sample was subjected to the microwave heating. In this study, the flow was set at 1 μL/min for a total digestion time of 5 min (using a 5 μL internal volume microwave loop). The temperature was measured using a noncontact infrared sensor (CEM, Matthews, NC).

For online microwave digestion-LC-MS/MS, the flow output of the microwave flow cell was directly connected to a home-built packed column (Reversed phase POROS R2, Applied Biosystems, Framingham, MA) with dimensions of 100 mm length, 360 μm OD, and 76 μm ID. Using 100% solvent A (12.5% FA in water) during the microwave digestion step, product peptides were held on the column as they eluted from the microwave digestion process. At the conclusion of the microwave digestion cycle, these peptides were then separated using a gradient of increasing solvent B (solvent B: 0.1% FA in

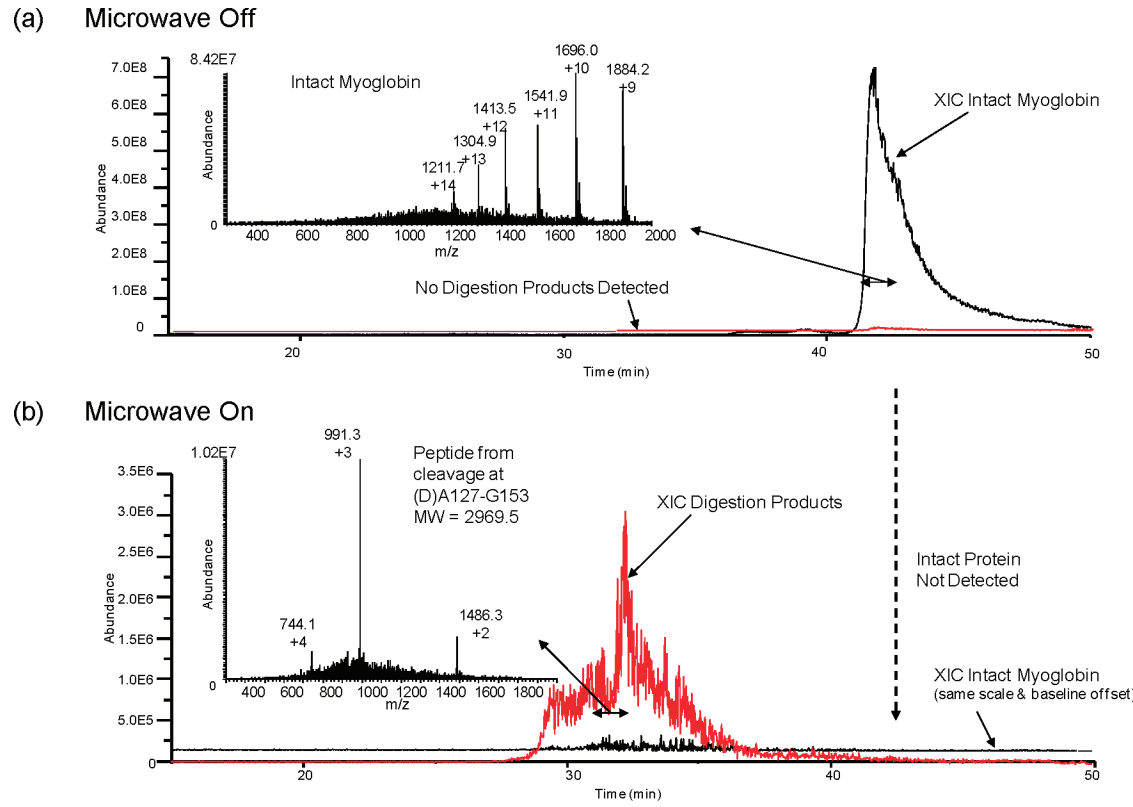


Figure 2. Microwave heating flow cell online with LC-MS: (a) extracted ion chromatogram (XIC) from the analysis of myoglobin using the microwave flow cell online with LC-MS without microwave heating of myoglobin, and (b) XIC for the same analysis but with the microwave heating turned on. Microwave digestion occurs during the first 10 min of the analysis (MS detector not on during this time; *m*/*z* values of digestion products used in XIC are listed in Table 1).

acetonitrile) at a flow rate of 1 μL/min. Overall, the solvent B gradient was set at 0% for 0–23 min to allow for the 5 min digestion time and sufficient time to remove/flush the DTT reagent, followed by a step-jump to 25%, and increased from 25 to 95% in 27 min, followed by a 20 min flush with 100% solvent B.

The HPLC column eluate was connected to the nanoelectrospray ionization (nESI) source of a quadrupole ion trap MS (LCQ Classic, ThermoFinnigan, San Jose, CA). Tandem MS experiments with the ion trap MS were performed by acquiring a full-scan mass spectrum between *m*/*z* 300 and 2000 followed by three data-dependent product ion mass spectral scans of the most intense precursor ions (a.k.a., "big-three" scan). The collision energy for dissociation was set at 35% with a 50 ms activation time. The dynamic exclusion feature of the Xcalibur software was enabled with a repeat count of 3, a repeat duration of 0.5 min, and exclusion duration of 3 min.

MALDI-time-of-flight-MS (Voyager DE-Pro, Applied Biosystems, Foster City, CA) analyses were conducted in the linear mode using sinapic acid as the matrix. In these experiments, proteins were digested in the microwave flow cell and fractions were collected prior to MALDI-MS analyses.

Database Search. The SEQUEST software (ThermoFinnigan, San Jose, CA) was used to carry out all database searches. An in silico "enzyme" was created within the BioworksBrowser to perform the digestion at the C-terminus of D residues. Even though cleavage can occur N-terminus to D residues, the favored reaction is for the cleavage C-terminus.[30] The parameters used in the search included up to 10 missed cleavage sites, group scan of 50, minimum group count of 1, and minimum ion count of 12, and the charge state option was set to auto.

Results and Discussion

Online Microwave D-Cleavage of Proteins. A digestion time of 5 min (1μL/min flow, 5 μL total volume reaction loop) was used for all experiments since it yielded peptide products above our instrument detection limit with the shortest digestion time. Myoglobin was chosen as a test protein for online microwave D-cleavage due to its moderate size (16.9 kDa) and its known sequence. Figure 2 shows the extracted ion chromatogram from the analysis of myoglobin using the microwave flow cell online with LC-MS without microwave heating (Figure 2a) and with microwave heating (microwave D-cleavage, Figure 2b). In Figure 2a, only the intact protein was detected and eluted at approximately 42 min, corresponding to 65–70% solvent B. No detectable peptides were observed between retention times of 25 and 35 min (30–50% solvent B composition). Figure 2b shows the extracted ion chromatogram for the online microwave D-cleavage and LC-MS analysis of myoglobin with the microwave heating on. By contrast, the chromatogram shows many new peaks of compounds eluting between retention times 28 and 35 min, corresponding to a 40–58% solvent B composition in the LC gradient. An average mass spectrum from a region of this retention time range shows signals corresponding to some of the predicted masses of peptide products due to microwave D-cleavage digestion (see Table 1). No detectable intact protein signal is observed in Figure 2b (XIC for intact protein is baseline offset for clarity). The experiment illustrated in Figure 2 clearly demonstrates the feasibility of carrying out the microwave D-cleavage digestion in an online configuration with MS. This was achieved using the simple microwave flow cell described in Figure 1. The

Table 1. Expected and Observed Peptides from Online Microwave D-Cleavage of Myoglobin[a]

expected peptides from myoglobin	peptides observed	MW (Da)	*m*/*z* observed
GLSD*GEWQQV LNVWGKVEAD*	(D)I142-G153	1325.5	663.3
IAGHGQEVLI RLFTGHPETL	(D)K45-D60	1893.2	946.2
EKFD*KFKHLK TEAEMKASED*	(D)A127-D141	1665.9	833.5
LKKHGTVVLT ALGGILKKKG	(D)A127-G153	2972.4	991.2, 744.5
HHEAELKPLA QSHATKHKIP	(D)I21-D60*	4582.25	917.7, 764.1
IKYLEFISDA IIHVLHSKHP	(D)L61-D109*	5422.1	1086.7, 905.4, 776.2
GD*FGAD***AQGA MTKALELFRND***IAAKYKEL FQG*	(D)A110-G153*	4773.4	796.4, 682.9

[a] Peptides were matched via SEQUEST database search (*manual interpretation).

amount of protein injected in this analysis was 0.01 μg (5 μg/mL, 2 μL injected). Injections of higher concentrations of protein sample resulted in the presence of undigested protein, indicating the need to optimize digestion time for different sample loads (data not shown).

The analysis illustrated in Figure 2b was repeated, however, this time performing LC-nESI-MS/MS online with the microwave D-cleavage digestion followed by database protein identification. A confident sequence match was obtained for three out of the four peptides detected by SEQUEST as their Xcorr values were above 1, delta correlation values above 0.1, preliminary scores (Sp) above 400, and ranks (RSp) of 1. It is well-known that under acidic conditions deamidation reactions at asparagines (Asn) and/or glutamine (Gln) can take place with an overall mass change of +1. The SEQUEST database search was also performed taking into consideration this modification; however, it did not result in any new peptides matched by the database search, and as a result, all subsequent searches were performed without using this modification parameter. Figure 3 shows a full scan mass spectrum and the subsequent product ion mass spectrum of the most intense peak in the full scan mass spectrum. Based on the ion at *m*/*z* 833.5, this signal

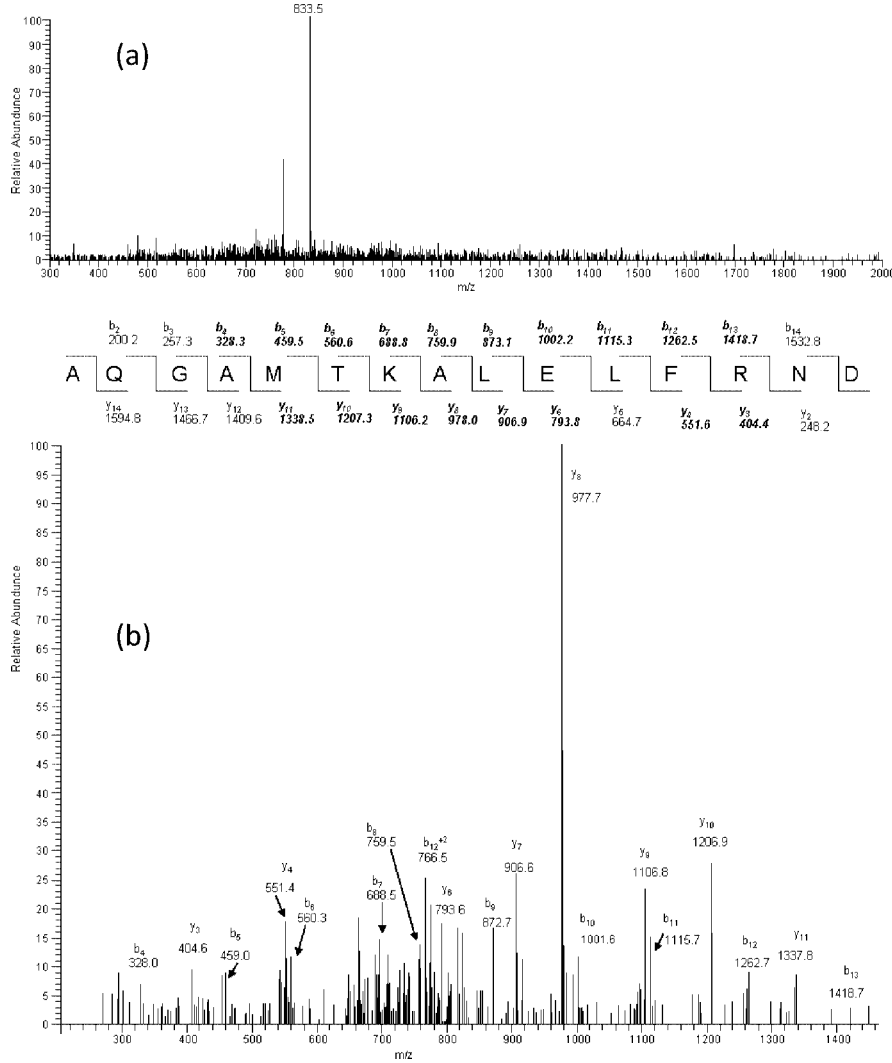


Figure 3. (a) Full scan ESI-mass spectrum (single scan, t_r 29.64 min) of a mixture of peptides created from specific cleavage at the aspartyl residues of myoglobin. (b) Tandem mass spectrum of the ion at *m*/*z* = 833.5. The peptide sequence and expected ions are shown. Observed ions are in bold.

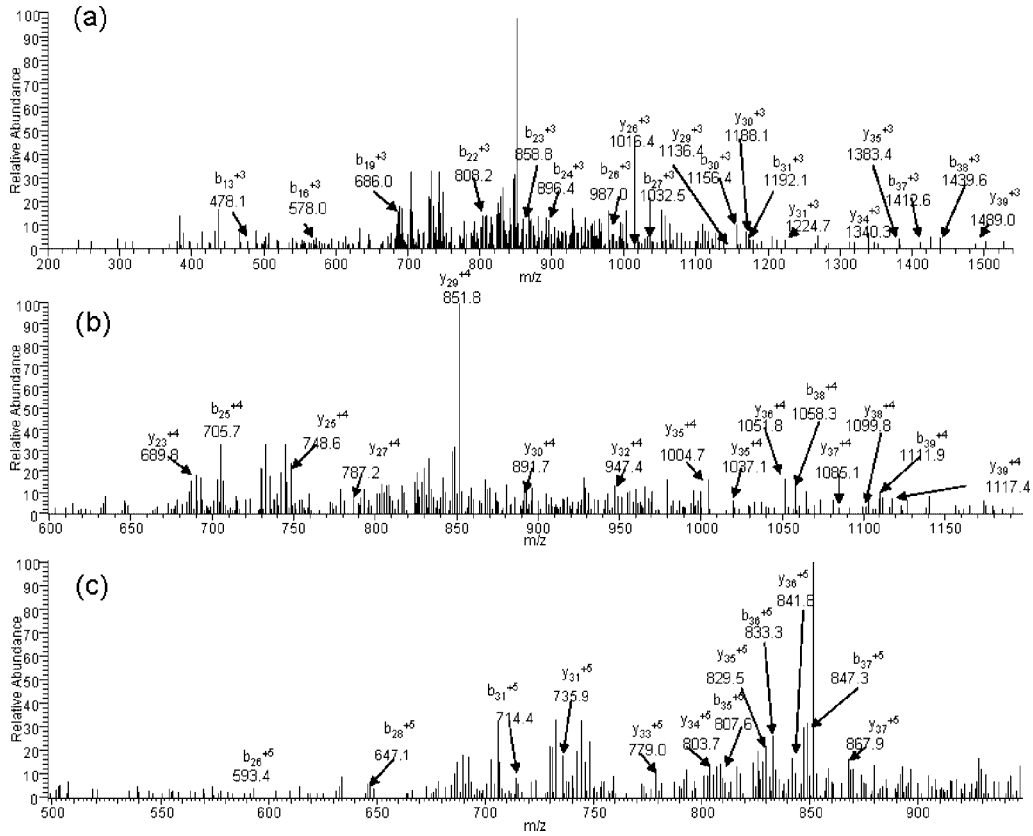


Figure 4. Product ion mass spectrum of $m/z = 764.1$ which is the $(M + 6H)^{6+}$ ion from the peptide formed from microwave D-cleavage of myoglobin at D20-D60, sequence: *IAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASED*. Each spectrum is a zoomed section of the same tandem mass spectrum labeled to show only the (a) 3+ ions, (b) 4+ ions, and (c) 5+ ions (the peptide expected ions are listed in Table 1S in the Supporting Information).

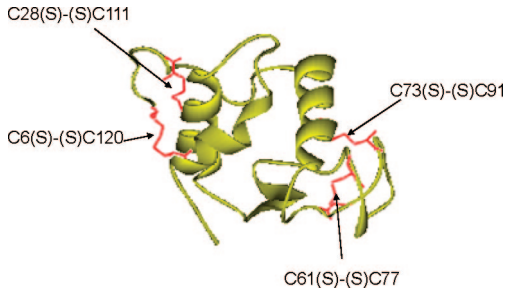


Figure 5. Structure of α-lactalbumin showing the intrachain disulfide bond sites.

should correspond to the $(M + 2H)^{2+}$ of the peptide formed from cleavage at D127 and D141 in myoglobin. Fragment ions in the product ion mass spectrum corroborate the expected sequence for the peptide at m/z 833.5, that is, of sequence (D)AQGAMTKALELFRND.

These results show that online microwave D-cleavage occurs within five minutes of heating and that protein identification can be achieved with this methodology. In addition, the resulting protein sequence coverage obtained via SEQUEST data analysis was 28%; however, further inspection of the data revealed the presence of more peptides than those detected by the database search. These peptides are large, about 20–40 amino acids in length, due to the relative low abundance of aspartyl residues and/or from missed D-cleavages. These peptides are detected in the full scan spectrum with a charge state of +4 or higher (data not shown), making their tandem mass spectra complex and difficult to match by SEQUEST search alone. A manual assignment of the fragment ions to their respective amino acid sequences is difficult to achieve due to the fact that many of the fragment ion masses may overlap. However, because the sequence of myoglobin is known, these higher charge state ions found within the spectra can be assigned to specific peptides by their mass and by manual sequencing of the fragment ions. For example, Figures 4(a−c) show the product ion mass spectra of the ions fragmented from a precursor ion at m/z 764.30 which is the $(M + 6H)^{6+}$ of the peptide formed from cleavage at D21-D60, MW 4582.2 (see Supporting Information, Table 1S for sequence information of this peptide and all of the expected CID ions from the +6 charge state). As a result, by including assigned fragment ions from these large peptides, the protein sequence coverage for myoglobin is increased to 88%. The expected peptides formed from microwave D-cleavage along with the observed peptides after a 5 min digestion at 130 °C are shown in Table 1.

The protein α-lactalbumin, containing 13 D amino acids and 4 intrachain disulfide bonds, was also tested with the online microwave D-cleavage method. Figure 5 illustrates the secondary structure of α-lactalbumin, including disulfide bonds. The four disulfide bonds occur between C6-C122, C28-C111, C61-C77, and C73-C91.

Unexpectedly, microwave D-cleavage online LC-nESI-MS/MS analysis of the protein α-lactalbumin produced no detectable peptides for successful protein identification (data not shown). Further digestion product analysis by MALDI-MS also showed that no detectable peptides were formed for this protein (shown in Figure 6). Similar analysis of the protein

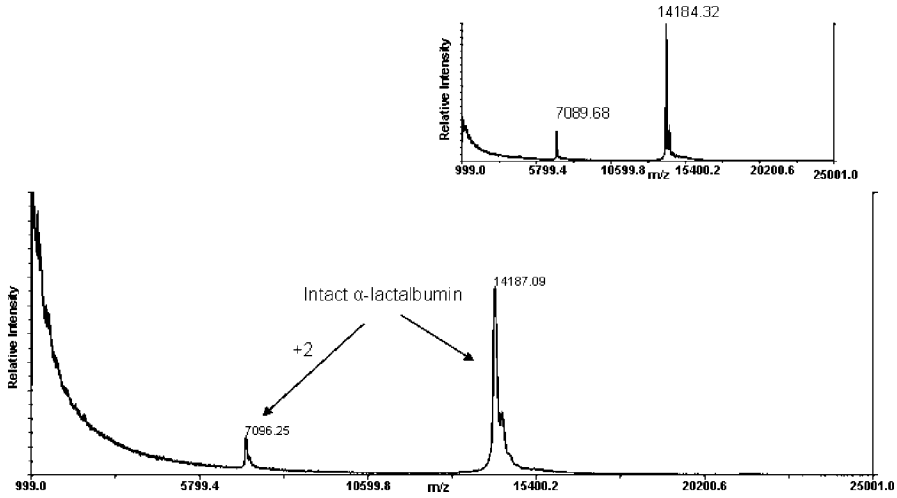


Figure 6. Effect of disulfide bond presence in proteins digested with the microwave D-cleavage method. MALDI-mass spectra of α-lactalbumin before (inset) and after microwave D-cleavage. No peptides are observed after microwave treatment, most likely due to the presence of intrachain disulfide bonds. MALDI matrix: sinapic acid.

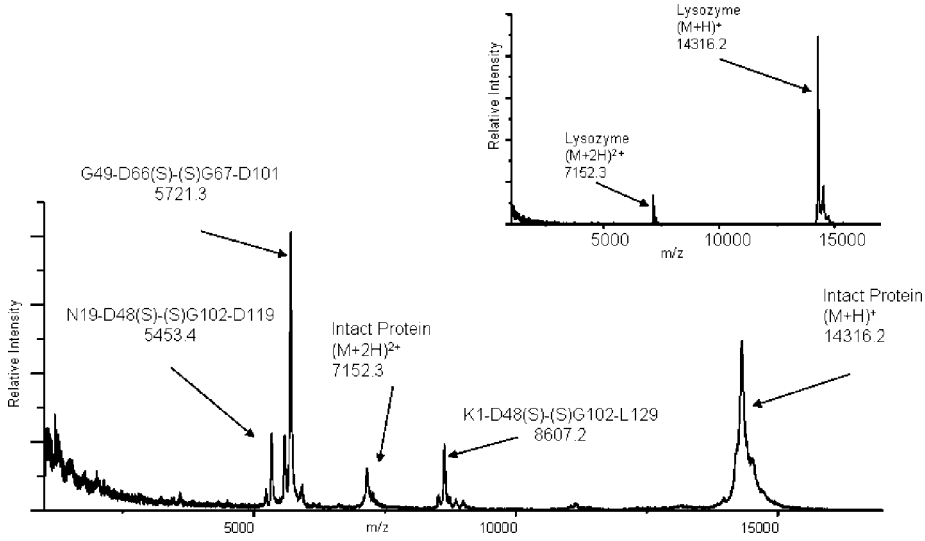


Figure 7. MALDI-mass spectra of lysozyme before (inset) and after microwave D-cleavage. Peptides that are detected are attributed to peptides formed from cleavage at D but with intrachain disulfide bonds still intact. MALDI matrix: sinapic acid.

lysozyme, which contains 7 D amino acids and 4 disulfide bonds, produced three detectable peptides resulting from site specific cleavage at D (shown in Figure 7). However, these peptides were still linked by interchain disulfide bonds. These outcomes indicated that disulfide bonds either hinder microwave D-cleavage, as it may be the case for α-lactalbumin, or lead to the production of large peptide chains cross-linked by disulfide bonds, as in the case of lysozyme. The latter situation is known to reduce the amount of protein sequence coverage that can be obtained. These facts, coupled with the prevalence of disulfide bonds in proteins, led us to implement a microwave-disulfide bond cleavage method that is carried out concurrently with the microwave D-cleavage step. Data illustrating this method are presented next, followed by examples of online microwave digestion of larger proteins.

Online Microwave Disulfide Bond Cleavage. The breaking of the disulfide bonds prior to or during the microwave D-cleavage process is expected to make the aspartyl residues more accessible and/or susceptible to hydrolysis and form small peptides, the latter being more conducive to collision-induced dissociation (CID). One of the most common methods of reducing disulfide bonds (R-S−S-R′) to thiol groups (R-SH + HS-R′) is by incubating the protein sample with dithiothreitol (DTT) at 37 °C for 30 min.[43] The reaction is driven entropically,[44] and if excess DTT is removed, the thiol groups tend to oxidize spontaneously to reform the disulfide bridges. To prevent this from taking place, thiol group alkylation is often carried out by reaction of the reduced protein with iodoacetamide in the dark and at room temperature for 30 min. Combined, these steps add an additional one hour of sample preparation time to the protein digestion procedure. In order to circumvent this problem, we developed a microwave-based method to cleave disulfide bonds that is performed at the same time as the microwave D-cleavage step. The new methodology is based on the addition of DTT as well as formic acid to the protein solution prior to injection and microwave heating through the flow cell. The rapid increase in temperature during the microwave heating process increases the rate of reduction of the disulfide bonds as well as cleaving the protein at the D sites.

To illustrate the microwave disulfide cleavage process, the model protein insulin was tested since it is composed of two

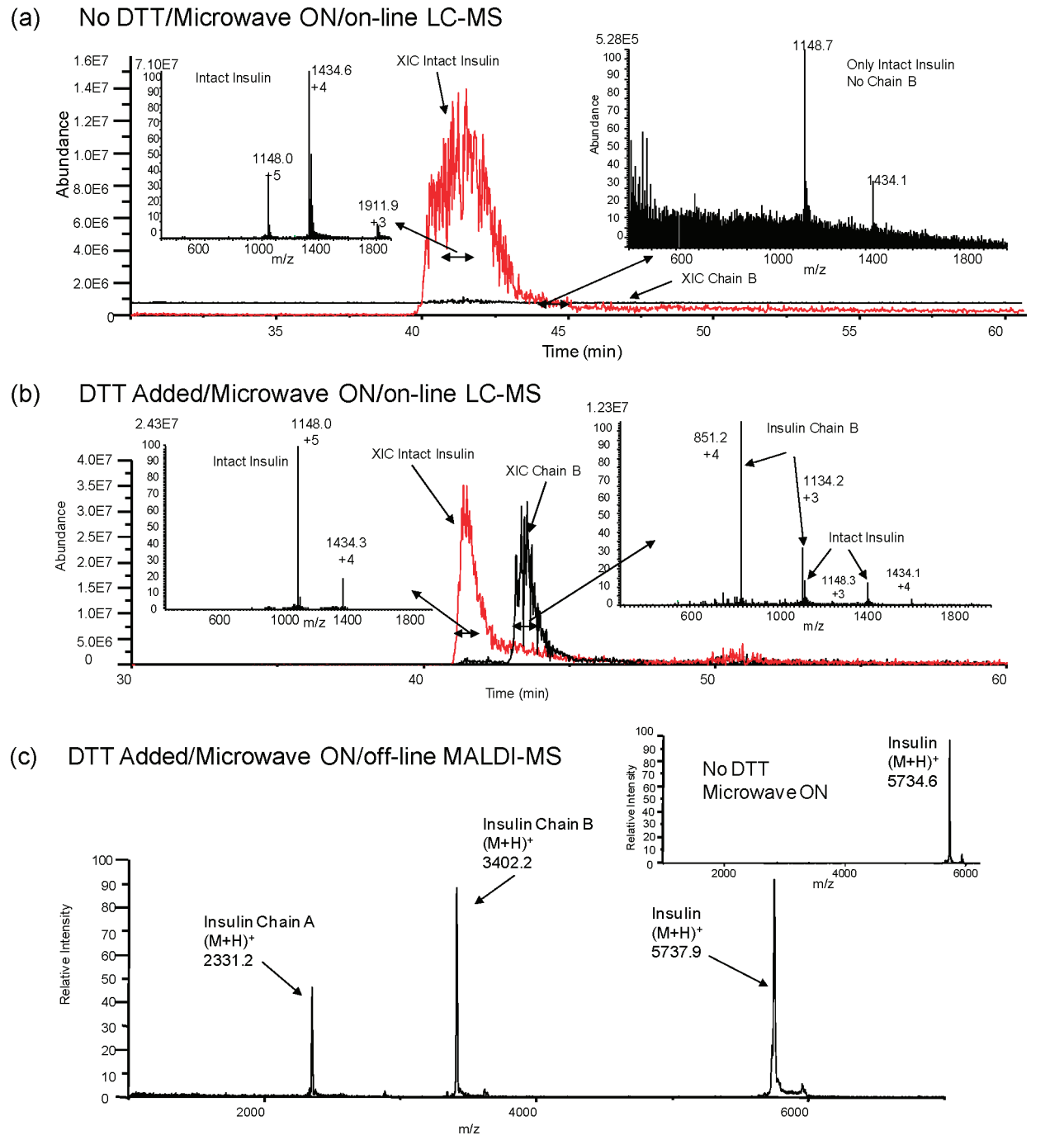


Figure 8. Online microwave disulfide bond cleavage LC-nESI-mass spectrum of insulin: (a) nESI-mass spectrum of insulin after microwave D-cleavage treatment with no DTT added. No nonspecific amino acid cleavages as well as disulfide bond cleavages are observed. (b) nESI-mass spectrum of insulin after microwave D-cleavage with the addition of DTT (microwave disulfide bond cleavage). (c) MALDI mass spectrum of insulin after microwave D-cleavage with DTT. Both chain A and B are observed. No nonspecific cleavages are observed in insulin (see inset in Figure 8c).

peptide chains, chain A and chain B, linked by two interchain disulfide bonds (plus one intrachain disulfide bond) and lacks the amino acid D (hence, this measurement is also a negative control test for the specificity of the microwave D-cleavage step). Successful implementation of the microwave disulfide bond reduction/cleavage using the microwave flow cell would produce the two separate peptide chains.

Using the microwave flow cell and loading a solution of insulin with DTT and formic acid, disulfide bond reduction in insulin was carried out, and results are illustrated in Figure 8. The addition of DTT to the solution followed with a 5 min online microwave heating treatment (and LC-ESI-MS) was sufficient to reduce the disulfide bonds and break apart the two peptide chains of insulin. When insulin is subjected to online microwave cleavage with no DTT added, the two chains remain linked (Figure 8a). However, when the sample was subjected to microwave D-cleavage in the presence of DTT, the disulfide bonds were cleaved, evident by the detection of the free insulin chain B. Figure 8b shows the extracted ion chromatograms for intact insulin and the chain B product.

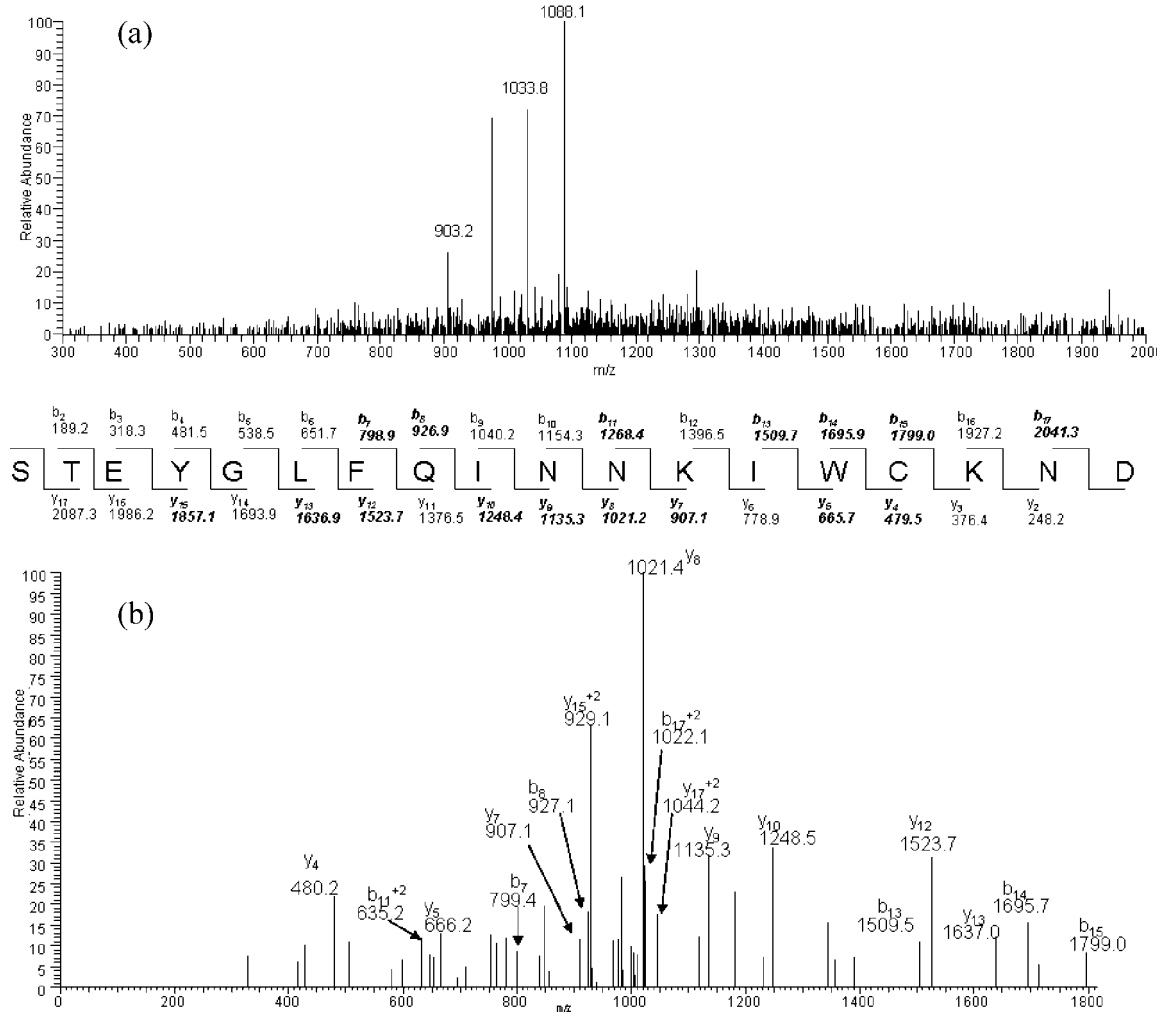


Figure 9. Full scan ESI-mass spectrum (single scan, retention time 33.12 min) of peptides resulting from online microwave D-cleavage and disulfide bond cleavage of the protein α-lactalbumin. (b) The corresponding CID mass spectrum of the ion at $m/z = 1088.1$ and its sequence.

Because no signal corresponding to the chain A product was observed in the LC-ESI-MS measurement, the digestion products were also analyzed by MALDI-MS. Figure 8c shows the MALDI-mass spectrum of insulin treated with microwave D-cleavage and DTT. Both signals for chain A and B are observed with no additional signals detected due to nonspecific hydrolysis of any other bonds. Finally, it is worth noting that the addition of DTT reagent does not interfere with the ESI process since it is eliminated or flushed during the first 10 min of the LC gradient, while digestion products are still retained in the LC column. Furthermore, in experiments using insulin as a test protein, the low pH used for the microwave D-cleavage (pH 1–2) was found to lower the efficiency of the microwave disulfide bond cleavage, most likely due to thiol group protonation (data shown in Figure 1S in the Supporting Information). However, the DTT reagent was still effective in reducing the disulfide bond at the low pH used and allowed for the microwave D-cleavage step to take place (vide infra). The fact that cleavage at D is carried out simultaneously and online with the disulfide cleavage step prevents the thiol groups from oxidizing and reforming disulfide bonds, precluding the implementation of an alkylation step.

Table 2. Observed Cleavage Peptides of α-Lactalbumin from Online Microwave D-Cleavage in the Presence of DTT[a]

MH^+	charge	sequence	Xcorr	δ Cn	Sp	RSp
2174.4	2	(D)S47-D64	2.26	0.745	549.9	1
2518.8	3	(D)L15-D37	3.087	0.62	381.2	1
3090.7	2	(D)K98-L123	3.717	0.563	560.8	1
2708.5*[b]	3	(D)Q65-D88	×	×	×	×
3503.8*[b]	4	(D)L15-D46	×	×	×	×
3689.1*[b]	4	(D)S47-D78	×	×	×	×
4131.1*[b]	5	(D)I89-L123	×	×	×	×

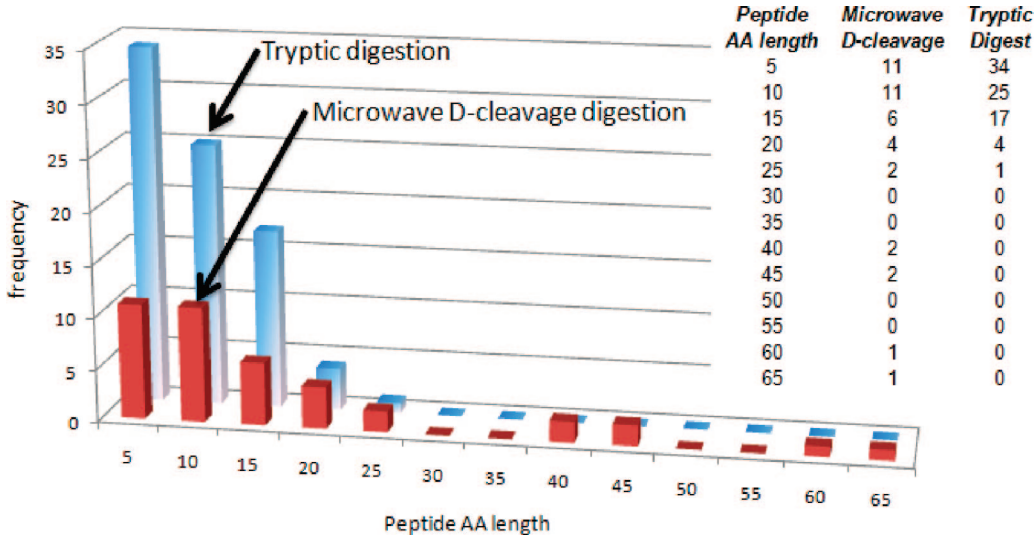
[a] Asterisk denotes sequence confirmed manually. [b] Peptides were not matched by SEQUEST and have no SEQUEST statistics.

To illustrate this combined advantage, the protein α-lactalbumin was again digested with the online microwave D-cleavage process, however this time with the addition of DTT. Recall from Figure 6 that digestion of this protein with the microwave D-cleavage method did not produce any detectable peptide products. Figure 9 shows the full scan mass spectrum (single scan at t_r 33.12 min) of the peptides detected from this digestion, along with the tandem mass spectrum of the $(M + 2H)^{2+}$ ion at m/z 1088, matched to the peptide sequence STEYGLFQINNKIWCKND. Overall in this analysis, three peptides were matched with high confidence using SEQUEST and with protein sequence coverage of 53%. The addition of DTT during the microwave heating process enabled the cleavage of

Table 3. Proteins and the Peptides Identified by MS/MS database Search and by Manual Assignment[a]

protein	PubMed accesion #	peptide sequence from D-cleavage	peptide MW (Da)	Xcorr	δ Cn	Sp	RSp
α-Lactalbumin (14.2 kDa)	1F6S_F	LKGYGGVSLP EWVCTTFHTS GYD	2517.8	3.087	0.62	381.2	1
80.5% Sequence Coverage		LKGYGGVSLP EWVCTTFHTS GYDTQAIVQN ND∗	3501.8	×	×	×	×
		STEYGLFQIN NKIWCKDD	2174.4	2.26	0.745	549.9	1
		QNPHSSNICN ISCDKFLDDD LTDD∗	2708.5	×	×	×	×
		STEYGLFQIN NKIWCKDDQN PHSSNICNIS CD∗	3688.1	×	×	×	×
		IMCVKKILDK VGINYWLAHK ALCSEKLDQW LCEKL∗	4130.1	×	×	×	×
		KVGINYWLAH KALCSEKLDQ WLCEKL	3089.6	3.717	0.563	560.8	1
Lysozyme (14.3 kDa)	5LYM_B	KVFGRCELAA AMKRHGLD	2002.4	3.908	0.773	470.5	1
71.3% Sequence Coverage		KVFGRCELAA AMKRHFLDNY RGYSLGNWVC AAKFESNFNT QATNRNTD∗	5427.1	×	×	×	×
		GSTDYGILQI NSRWWCND	2128.3	2.916	691	346.2	1
		GNGMNAWVAW RCKGTDVQAW IRGCRL∗	3219.7	×	×	×	×
		VQAWIRGCRL	1201.5	1.887	0.514	365.3	1
Myoglobin (16.9 kDa)	P68082	IAGHGQEVLI RLFTGHPETL EKFDKFKHLK TEAEMKASED∗	4582.2	×	×	×	×
86.9% Sequence Coverage		KFKHLKTEAE MKASED	1892.2	1.579	0.345	415.9	1
		LKKHGTVVLT ALGGILKKKG HHEAELKPLA QSHATKHKIP IKYLEFISD∗	5425.4	×	×	×	×
		AIIHVLHSKH PGDFGADAQG AMTKALELFR NDIAAKYKEL GFQG∗	4767.4	×	×	×	×
		AQGAMTKALE LFRNDIAAKY KELGFQG	2971.4	4.571	0.758	1239.9	1
		IAAKYKELGF QG	1324.5	1.234	0.236	312.4	1
Carbonic Anhydrase (28.9 kDa)	1V9E_A	FPIANGERQS PVNIDTKAVV QD∗	2398.7	×	×	×	×
33.2% Sequence Coverage		TKAVVQD	759.6	1.409	0.227	488.9	1
		SQDKAVLKD	1003.1	1.701	0.324	443.6	1
		FGTAAQQPD	933.4	1.44	0.331	307.4	1
		GLAVVGVFLK VGDANPALQK VLDALD∗	2623.1	×	×	×	×
		ANPALQKVLD	1068.2	1.772	0.435	413.7	1
		ALDSIKTKGK STDFPNFD∗	1984.2	×	×	×	×
Bovine Serum Albumin (66.5 kDa)	CAA76847	RADLAKYCID NQDTISSKLK ECCD∗	2733.1	×	×	×	×
19.0% Sequence Coverage		AIPENLPPLT ADFAED	1712.9	2.515	0.574	446.5	1
		PHACYSTVFD KLKHLVD	1973.4	3.507	0.679	897.9	1
		PHACYSTVFD KLKHLVDEPQ NLIKQNCD	3256.7	2.394	0.521	297.3	1
		ETYVPKAFDE KLFTFHAD∗	2158.4	×	×	×	×
		KCCAADDKEA CFAVEGPKLV VSTQTALA	2869.3	2.781	0.655	410.3	1

[a] Asterick denotes sequence assigned manually; "x" denotes no SEQUEST score obtained.



Peptide AA length	Microwave D-cleavage	Tryptic Digest
5	11	34
10	11	25
15	6	17
20	4	4
25	2	1
30	0	0
35	0	0
40	2	0
45	2	0
50	0	0
55	0	0
60	1	0
65	1	0

Figure 10. Comparison of the expected distributions of peptide amino acid length derived from the microwave D-cleavage and tryptic digestions of the protein BSA. Only no missed cleavages were considered in this calculation.

the disulfide bonds while the hydrolysis of the aspartyl residues was taking place (also demonstrating that the addition of DTT does not interfere with the D-cleavage process). This combined process resulted in the production of smaller peptides that were

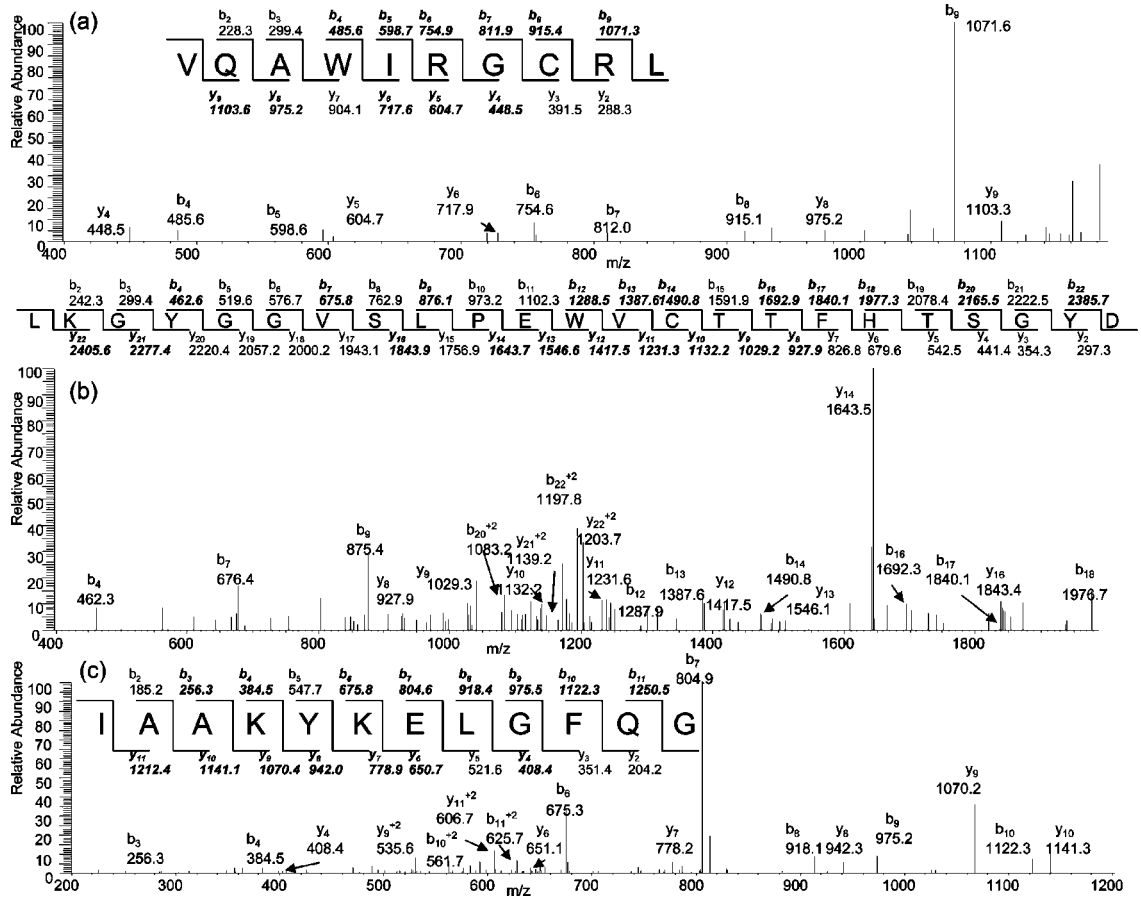


Figure 11. ESI-MS/MS spectra of peptides formed from a mixture of lysozyme, α-lactalbumin, and myoglobin. (a) CID spectrum of the peptide formed from cleavage at D119 of lysozyme, (b) CID spectrum of the peptide formed from cleavage at D14 and D37 of α-lactalbumin, (c) CID spectrum of the peptide formed from cleavage at D141 of myoglobin.

amenable to ESI-MS and CID analyses. Table 2 shows the peptides that were observed after online microwave D-cleavage and disulfide cleavage (compare with Figure 6, where no digestion products were detected). Once again, several large peptides were not matched by SEQUEST due to their size and highly charged state fragment ions and as a result were manually interpreted. After accounting for these peptides (total of six peptides detected), the protein sequence coverage approaches 80%.

Several test proteins were identified by performing a database search of the MS/MS data resulting from online microwave D-cleavage/disulfide bond cleavage, and results are listed in Table 3. The test protein sequence is shown along with the peptides that were detected after online microwave digestion, including the peptides that were matched to a database and those interpreted manually. The proteins tested ranged in size from 5 kDa (insulin) to 67 kDa (bovine serum albumin), and all were successfully identified with a high degree of certainty.

However, the sequence coverage derived from the analysis of BSA is particularly low. A closer inspection and comparison of the peptide length distribution derived from the microwave D-cleavage and tryptic digestions may help explain this outcome. Figure 10 shows a histogram and its data on the frequency distribution of peptide amino acid length for both of these digestion methods for the protein BSA (only no missed cleavages considered). Several observations can be made from these data (for BSA only): (i) the average length of the microwave D-cleavage peptide is 15 amino acids, about twice as large as the average length of the tryptic peptides (7.4 amino acids), (ii) the largest peptide generated by microwave D-cleavage contains 64 amino acids, while the largest by tryptic digest is 21, (iii) about 74% of the BSA sequence coverage is contained in 42 tryptic peptides with 5–15 amino acids in length, and (iv) about 48% of the BSA sequence coverage is contained in six microwave D-cleavage peptides with 35 or higher amino acids in length. The disproportionate percentage of sequence information included in large peptides that result from microwave D-cleavage and the implementation of CID may explain in part the low sequence coverage observed for BSA in this work. Furthermore, when considering about 250 different proteins in the *E. coli* proteome, the average length for a D-cleavage peptide is 16 amino acids, while the average tryptic peptide is 9.5 amino acids in length. Current investigations in our laboratory are aimed at the implementation of newly developed Electron Transfer Dissociation[45] (ETD) techniques for the tandem MS analysis of these large and high charge state (>+3) peptide ions, which are known to be better suited to this type of fragmentation.[46]

A mixture of lysozyme, α-lactalbumin, and myoglobin was also digested with the microwave D-cleavage/disulfide cleavage technique and analyzed online by LC-ESI-MS/MS. Figure 11 shows the tandem mass spectra of peptides from each of the three protein components in the mixture that were successfully identified using SEQUEST. The protein lysozyme was identified with a 38% sequence coverage, including the peptide formed from cleavage at D119 (Figure 11a). Figure 11b shows the

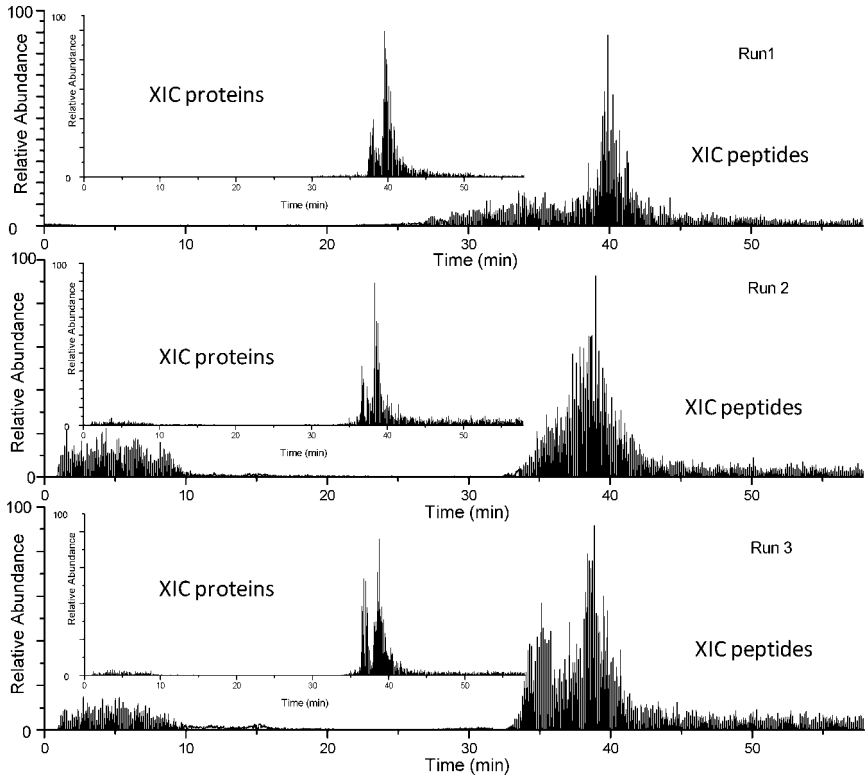


Figure 12. Replicate data-dependent LC-ESI-MS/MS analyses of the same protein standard mixture subjected to online microwave D-cleavage (data-dependent analysis: full scan followed by three data-dependent product ion mass spectral scans, a.k.a., "big three" scan).

Table 4. Top Ten Proteins Identified in *E. coli* Using the Online Microwave D-Cleavage Digestion and LC-ESI-MS/MS Analysis[a]

protein	PubMed accession #	number of peptides detected (Xcorr > 1)	Xcorr (highest)
endonuclease R [*Escherichia coli* K12]	16132171	5	1.908
rhsD protein in rhs element [*Escherichia coli* K12]	16128481	4	1.913
phosphoanhydride phosphorylase; pH 2.5 acid phosphatase	16128946	3	1.189
β-D-galactosidase [*Escherichia coli* K12]	16128329	3	1.292
BCSC_ECOLI Cellulose synthase operon protein C c-type	2851645	3	1.294
PNP_ECOLI Polyribonucleotide nucleotidyltransferase	1172545	3	1.458
putative ligase [*Escherichia coli* K12]	16132055	2	1.256
diaminopimelate decarboxylase [*Escherichia coli* K12]	16130742	2	1.674
4-hydroxy-2-ketovalerate aldolase [*Escherichia coli* K12]	16128337	2	1.892
SSNA_ECOLI SsnA protein protein [Plasmid R100]	6685984	2	1.546

[a] Protein identification via SEQUEST database search.

tandem mass spectrum of the peptide formed from cleavage at D14-D37 in the protein α-lactalbumin, with a 33% sequence coverage. The CID mass spectrum of the peptide formed from cleavage at D141 in myoglobin, detected with a 27% sequence coverage, is shown in Figure 11c. These results were obtained by including both SEQUEST and manually sequenced peptides.

The reproducibility of the microwave D-cleavage digestion online with LC-MS/MS detection was also investigated. Figure 12 shows three replicate measurements of the standard protein mixture containing lysozyme, α-lactalbumin, and myoglobin (chromatogram: peptide XIC; inset: proteins XIC). In all chromatograms, elution of the online microwave D-cleavage digestion products occurs between 30 and 45 min. Even though the same peptides were detected in all analyses, database searches identified different numbers of peptides, specifically those peptides above MW 2500. The fact that all proteins were consistently identified in all replicate measurements points to the robustness and reproducibility of the methodology here presented. It is worth noting that the poor chromatographic efficiency observed in these and other analyses in this work may be the result of the introduction of a (large) dead volume by the microwave heating flow cell (see Figure 1). In the future, this may be avoided by implementing switching valves to divert the gradient elution flow from the microwave flow cell (after the 5 min heating period) and direct it into the LC column. This system configuration, along with some other modifications, is currently being developed and tested in our laboratory to allow for the direct analysis of whole untreated cells with the microwave flow cell (i.e., online cell lysis and protein digestion LC-ESI-MS/MS).

Online Microwave D-Cleavage LC-MS/MS of *E. coli* Proteins. To demonstrate the ability of the online microwave D-cleavage and microwave disulfide bond cleavage to identify proteins in a complex mixture, a bacterial (*E. coli*) cell lysate was analyzed. Table 4 lists the top 10 proteins identified in *E. coli* along with the corresponding number of peptides derived

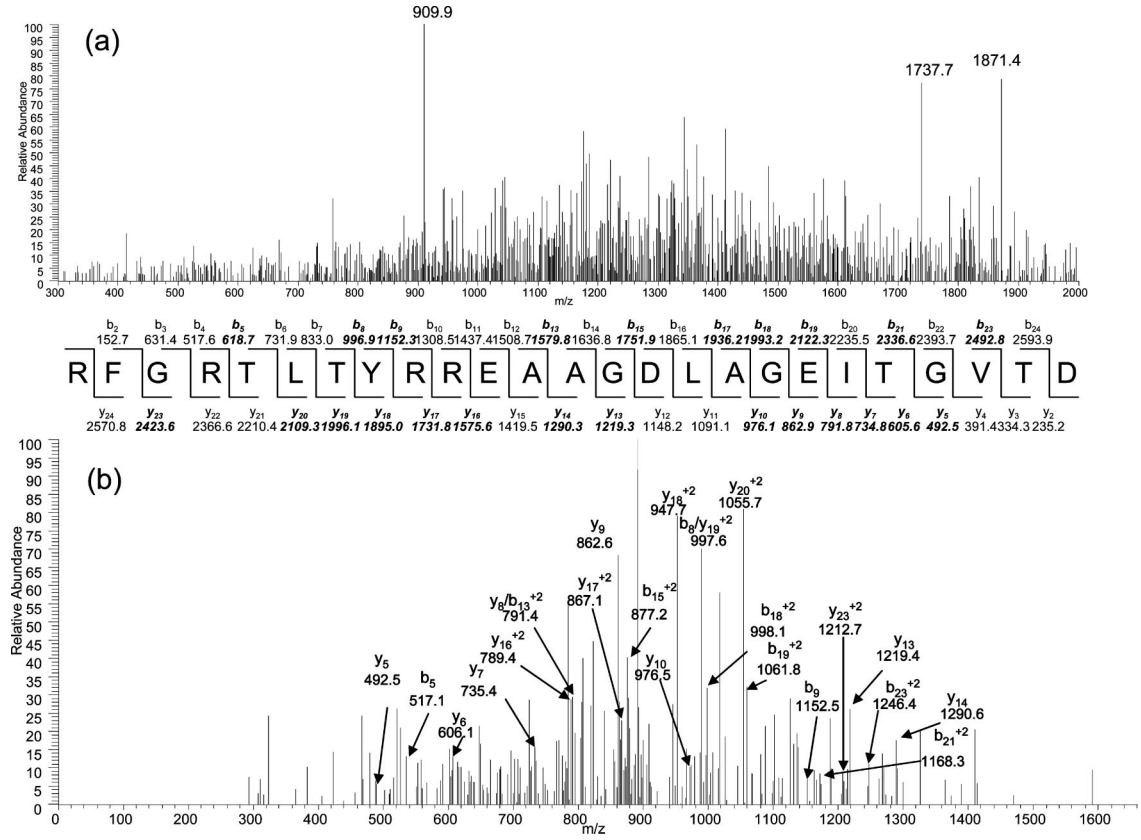


Figure 13. (a) Full scan ESI-mass spectrum (single scan, t_r = 32.32 min) of eluting peptides resulting from the microwave D-cleavage and microwave disulfide bond cleavage of an *E. coli* cell extract, (b) corresponding CID mass spectrum of the ion at *m*/*z* 909.9 and its sequence. This peptide was matched to the rhsD protein from *E. coli* by a SEQUEST search (Xcorr score of 1.302 and Sp of 239.4).

from that protein with Xcorr-scores above 1. An extended listing of all proteins identified in *E. coli* along with all peptide sequences and SEQUEST scores is available in the Supporting Information (Table 2S).

These proteins were identified with a high degree of confidence and yielded CID tandem mass spectra useful for the SEQUEST database search (Figure 13). No manual interpretation of several tandem mass spectra of highly charged peptides was attempted. However, from our analysis of protein standards above, it is expected that if these data are included in the analysis the number of proteins identified with a high degree of confidence would increase. Clearly, the methodology here presented is capable of analyzing complex proteomes in biological samples.

Conclusions

The online nonenzymatic microwave D-cleavage method is a tool that can be implemented for the rapid digestion and identification of proteins. The C-terminus cleavage at aspartic acid occurs readily to give peptides that can be analyzed by tandem MS to identify the protein. One important aspect of this method is the relatively simple sample preparation that is required to perform this bottom-up proteomic approach. The digestion, chromatographic separation of peptide products, and subsequent mass spectral analysis all occur in an online configuration, therefore there is no need to remove any of the cleavage or reduction reagents and/or sample handling prior to MS analysis. Adding DTT prior to the injection of the sample into the system allows for disulfide bond reduction/cleavage at the same time as the digestion at D, creating more peptides that are conducive for tandem MS. The need for alkylation to prevent reformation of disulfide bonds is abrogated. The implementation of the microwave D-cleavage digestion technique with protein quantitation labeling reagents like iTRAQ is expected to be compatible in an off-line mode as the labeling is performed after the digestion step. However, quantitation reagents like the ICAT may not to be compatible with this technique as the labeling is performed on the intact protein and the reagents have an acid cleavable site, which may be affected by the microwave D-cleavage step. Although not yet tested, the effects of the microwave D-cleavage digestion on PTMs like phosphorylation and glycosylation are expected to be limited due to the digestion in mild acidic conditions (pH = 2). The microwave D-cleavage data obtained to date from protein digestions, albeit limited, indicate that the cleavage mechanism is highly dependent on the disulfide bonds present and independent of the adjacent C-terminus amino acid (method cleaves the Asp-Pro bond), as no obvious correlation has been observed between cleavage frequency and neighboring amino acid residue. The efficiency of the microwave D-cleavage was found to be kinetically limited; that is, complete digestion of proteins was not always achieved. For example, for protein levels at or below 5 μg/mL (0.01 μg injected), complete digestion of the protein was observed (e.g., see Figure 2). Detailed kinetic studies[29] on the hydrolysis of proteins and peptides at Asp have determined that the rate limiting step below pH 4 is the leaving-group expulsion (rather than water attack). This may help explain the prevalence of C-terminus peptides for certain proteins undergoing microwave D-cleavage in solution and under pyrolytic conditions.[47] Peptides created

by microwave D-cleavage are inherently longer than tryptic peptides; however, many of the microwave D-cleavage peptides yield CID fragments that can be matched by a database search to correctly identify the protein from which they came, although in certain cases at the expense of a low protein sequence coverage. The implementation of newly developed Electron Capture Dissociation[48] (ECD) and Electron Transfer Dissociation[45,49,50] (ETD) techniques for tandem MS should alleviate this low sequence coverage shortcoming as the large and high charge state peptides produced by the microwave D-cleavage method may be more suitable for these fragmentation techniques. Our laboratory, in collaboration with others, is currently investigating the analysis of microwave D-cleavage peptides with ETD and already found that the ETD-MS analysis of microwave D-cleavage peptides yields a higher sequence coverage when compared to CID of these peptides, results of which will be presented in detail in a forthcoming manuscript in this Journal.

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Supporting Information Available: Tables 1S and 2S and Figure 1S. This material is available free of charge via the Internet at http://pubs.acs.org.

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