Optimization of Nonenzymatic Microwave D-Cleavage and Disulfide Bond Cleavage

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ABSTRACT: A nonenzymatic digestion technique allows proteins to be well suited for collision-induced dissociation tandem mass spectrometry. This method utilizes microwaves and Dithiothreitol (DTT) to selectively cleave aspartic acid (D) and disulfide bonds. This microwave digestion technique is reproducible and produces peptides with a shortened sequence length. Insulin I is used in this study to optimize the disulfide bond cleavage, and α-lactalbumin is used as a test-piece for the combined D-cleavage and disulfide bond cleavage technique. The digested samples in this study are analyzed using Matrix Assisted Laser Desorption (MALDI). Through optimization with pH, time, temperature and DTT concentration, a spectrum of ideal parameters for these techniques was found.

1. Introduction

Enzymes such as Trypsin are used extensively in proteomic studies due to the simplicity of use, and the enzymes ability to cleave proteins specifically at Arginine (R) and Lysine (K), which, due to their size and inherent 2+ charge when ionized via electrospray ionization, allows the residue to be easily analyzed via collision-induced dissociation (CID) fragmentation in tandem MS (MS/MS) measurements (1). The use of nonenzymatic techniques in conjunction with enzymatic digestion can give an advantage over enzymatic techniques alone, due to the speed, simplicity, robustness and automation of the technique. The nonenzymatic digestion of proteins at aspartic acid (D) and disulfide bonds has been explored and developed but not optimized (1). The cleavage of disulfide bonds is carried out using the common reducing agent dithiotreitol (DTT), and the aspartic acid cleavage is a selective, thermodynamically aided hydrolysis process, which only requires acid to occur. By optimizing these techniques based on pH, DTT concentration, time and temperature, the versatility and efficacy of the procedures increase as the reagent (DTT) and time required to carry out the operation can be reduced, thus saving researchers valuable resources. These disulfide bond and aspartic acid digestion techniques, while useful by themselves, allow for a more thorough and widely applicable digestion when used in combination with enzymatic digestion or electrochemical oxidation at tyrosine (Y) and tryptophan (W) (1).

2. Experimental

2.1 Chemicals

Insulin I (bovine pancreas), α-lactalbumin (bovine milk), dithiotreitol (DTT), and α-Cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile (ACN), sodium acetate and deionized water were purchased from Burdick and Jackson (Muskegon, MI). Reagent grade acetic acid was obtained from Sigma-Aldrich (St. Louis, MO).

2.2 Sample Preparation

All proteins were prepared at a concentration of 1 mg/mL in buffered acetic acid solutions (pH 2.0 or 5.0). Acetic acid was used to increase hydrolysis efficiency (2).

For use with Matrix Assisted Laser Desorption Mass Spectrometry (MALDI-MS) analysis, the samples were diluted with the matrix to a concentration of 50/50 (v/v) sample/α-cyanohydroxycinnamic acid (CHCA), whereas the manufacturer instructions for the preparation of CHCA were followed (0.1 % trifluoroacetate/50% ACN).

2.3 Instrumentation

Microwave heating was achieved using a CEM Focused Microwave Synthesis System (model:Discovery; CEM, Matthews, NC). Approximately 5 mL of the buffer solution was added to the bottom of the reaction vessel, as suggested by the manufacturer. Microwave digestion parameters varied according to the optimization parameters matrix.

2.4 Mass Spectrometry

The samples were analyzed using MALDI-MS (Voyager DE-Pro, Applied Biosystems, Foster City, CA). The analyses for insulin I were conducted in the reflector/positive mode using CHCA as the matrix. The accelerating voltages were set at 20,000 V, the grid at 70%, with a delay time of 600 nano-Seconds, shots/spectrum set at 50, with a mass range of 500 to 2,000 Da. The analyses for α-lactalbumin were conducted in the linear/positive mode using CHCA as the matrix. In these experiments, peptides were digested in the microwave and fractions were collected for off-line MALDI-MS analyses.
3. Results and Discussion

Disulfide Bond Cleavage Optimization

As discovered in prior research (1), in order to observe peptides from aspartic acid digestion, disulfide bonds occasionally must be cleaved, as often these disulfide bonds preserve the intact protein, thus rendering the site-specific cleavage effectively useless for the application of shotgun proteomics. Therefore, the optimization of disulfide bond cleavage using DTT is extremely useful.

Based on prior research (1), insulin I (chains A & B) was chosen to test the disulfide bond cleavage technique. Insulin I is an ideal protein for this experiment due to the relative structural simplicity, small size (approx. 5.7 kDa), lack of aspartic acid residues (so as not to see any hydrolysis cleavage) and, most importantly, the ability to clearly see when disulfide bonds are broken, since chains A and B are connected only by disulfide bonds. Insulin I was digested in a sampling matrix with the following parameters: time (1, 5, 10 minutes); temperature (0, 50, 80, 95, 105, 110, 115, 125, 135, 145 °Celsius); pH (2.0, 5.0); DTT concentration (10, 50, 60, 70, 80, 90, 100 mM).

The data from the sampling matrix was analyzed and a range of full digestion parameters was discovered (figure 3). As expected, the efficiency of the microwave disulfide bond cleavage was significantly decreased at pH 2 as compared to pH 5 (extremely small amounts of DTT was required for full cleavage at this pH), as the percentage of thiolate anions is reduced at that low pH (1). However, since microwave aspartic acid cleavage occurs optimally at pH 2, the disulfide bond cleavage technique was optimized at this pH. Figure 1 shows the effects of heat and DTT on the test protein insulin I. Figure 2a shows MALDI-mass spectra of insulin I before and after digestion (figure 2b). The digested spectrum is considered 50% cleavage, according to the formula used (figure 2c).

![Figure 1](image1)

**Figure 1.** Intact insulin I (Left) with chains A & B connected by two interchain disulfide bonds is digested using DTT and heat. The resulting product is a mixture of chains A & B (right).

![Figure 2a](image2)

**Figure 2a.** Intact insulin I (inset) with chains A & B connected by two interchain disulfide bonds is digested using DTT and heat. **Figure 2b** The resulting product is a mixture of chains A & B. **Figure 2c** the formula for calculating %cleavage is shown.

\[
\text{% Cleavage} = \frac{I_{3400 \text{ m/z}}}{\Sigma \text{Total Ions}}
\]
The factors tested (time, temperature, DTT concentration) scaled linearly, with the increase of one of the parameters resulting in smaller required parameters to achieve full cleavage. Digestion time had the largest effect on percent cleavage. Figure 3 shows contour maps that illustrate the interaction of DTT concentration (mM) and temperature at 1-minute, 5-minute and 10-minute digestion times. A regression equation, which has a fairly reliable $R^2$ value (67.9%) for the cleavage of disulfide bonds, was obtained, and can be seen in figure 3. The ideal parameters for disulfide bond cleavage are dependent on whether or not aspartic acid cleavage is simultaneously applied, however, with as little as 60 mM DTT concentration at 105°C, full disulfide bond cleavage can be achieved in just 10 minutes (as seen on figure 3.c).

Microwave Aspartic Acid Cleavage Optimization

The site-specific cleavage of aspartic acid (D) in proteins is highly useful for non-enzymatic digestion techniques, as it is a reagent-less reaction that occurs in minutes. While microwave D-cleavage can be useful for protein identification by itself, it is often necessary to couple it with disulfide bond cleavage, as proteins with disulfide bonds generally retain their intact structure regardless of these site-specific digestions (1). The coupling of D-cleavage with disulfide bond cleavage is multiply advantageous, as it prevents a further need for alkylation and reduces potential sample losses during off-line microwave heating and sample handling (1).

The protein α-lactalbumin (figure 4), which contains 13 D amino acids and 4 intrachain disulfide bonds, was used as a test-piece for the dual optimization of these techniques. This protein served as an effective test-piece, as it shows no detectable peptides for successful protein identification when subjected solely to D-cleavage without disulfide bond cleavage (figure 5b). As used in the disulfide bond optimization, a sampling matrix that tested the interaction of time, temperature and DTT concentration was utilized. As expected, a similar set of effective parameters to the disulfide bond digestion technique was observed, with higher temperatures required in order to be able to see digested peptides. The pH for this optimization was held constant at 2.0, as this was found in previous research to be ideal for this reaction (1).

Figure 3a (top left) shows a contour map of percent cleavage of insulin I (1 minute digestion time) with DTT concentration in the x-axis and temperature in the y-axis, with orange corresponding to full cleavage. Figure 3b (top right) is 5-minute digestion time. Figure 3c (bottom) shows 10-minute digestion time.

% Cleavage = -165 + 0.510 DTT Conc. + 4.59 Time + 1.56 Temperature

$R^2 = 67.9\%$

Figure 4. The structure of α-lactalbumin is shown. Disulfide bonds are highlighted in yellow, and aspartic acid residues are in red.

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1 130°C and 5 minutes digestion time were found to be effective for this technique in prior research (1).
Due to the large size of intact α-lactalbumin (~14 kDa) and the relatively small size of its cleavage products (1-10 kDa), it was difficult to obtain a full spectrum that contains both the intact and the cleavage products. Due to this difficulty, CHCA was used in linear mode, and therefore, the spectra contain average peaks, as opposed to exact peaks as would be the case in reflectron mode. For this reason, the exact cleavage products were not identified exactly, but rather reasonable guesses were made using the expected cleavage masses as predicted by PAWS protein software. Despite these shortcomings, the data obtained from these experiments is still useful.

A similar formula to that which was used in the disulfide bond-only experiment was used to calculate %cleavage for these experiments, and can be seen in figure 5. Figure 6 shows the effects of DTT concentration and temperature on %cleavage. Notice that ~50mM DTT, cleavage products begin to appear, thus demonstrating a low threshold for these parameters. As was the case with insulin, time affected the minimum DTT concentration and temperature required to achieve full cleavage. As seen in figures 6a & 6b, 5 minutes digestion time reveals nearly identical data. Due to the potential of non-site specific amino acid cleavage (denaturation) at high temperatures, it seems that the ideal parameters for aspartic acid + disulfide bond cleavage are: five-minute digestion time with 200 mM DTT held at 120°C. At these parameters, 97% cleavage was attained. However, if higher temperatures are not an issue, but DTT concentration resources are limited, near-full cleavage (91%) can be achieved with little a digestion time of 1 minute, with 100 mM DTT, held at 125°C.

![Figure 5a](image5a.png) MALDI-mass spectrum of digested α-lactalbumin, with reasonable guesses of peaks annotated. **Figure 5b.** (inset) Undigested α-lactalbumin is shown.

![Figure 6a](image6a.png) Contour map of α-lactalbumin aspartic acid + disulfide bond % cleavage as a function of temperature and DTT concentration, 1 minute digestion time. **Figure 6b** (right) 5-minute digestion time.
Conclusions

The nonenzymatic microwave D-cleavage and disulfide-bond cleavage method is a tool that can be implemented for the rapid digestion and identification of proteins. While the technique was only used and optimized on two different proteins in these experiments, it is likely that it can be applied to other proteins with similar results. The disulfide bond-only digestion data obtained is extremely reliable, as reflectron mode was used in MALDI-mass spectrometry, thus giving a high confidence level.

Due to the inability to conduct MALDI-mass spectrometry in reflectron mode for the analysis of α-lactalbumin and its cleaved products, the data is not as reliable, and should only be used as a rough guide for the technique. However, it is reasonable to use the data from these experiments, as they display a similar pattern to that found in the disulfide bond-only data. A more accurate optimization of this dual-cleavage technique could possibly be performed by using the matrix sinapinic acid for analysis of the intact protein, and CHCA for the cleavage products (both in reflectron mode, with an internal standard).

Author Contributions

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References
