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Thermal Denaturation: A Useful Technique in Peptide Mass Mapping

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The use of thermal denaturation of proteins prior to in-solution digestion and mass spectral peptide mass mapping is reported. Thermal denaturation is preferred over chemical denaturation because it does not require purification/concentration prior to mass spectral analysis. Enzymatic digestions of proteins that are resistant to proteolysis are significantly enhanced by thermal denaturation. Native proteins that are sensitive to proteolysis show similar or slightly lower digestion yields following thermal denaturation. Proteins that are resistant to digestion become more susceptible to digestion, independent of protein size, following thermal denaturation. For example, amino acid sequence coverage from digest fragments increases from 15 to 86% in myoglobin and from 0 to 43% in ovalbumin. This leads to more rapid and reliable protein identification by MALDI peptide mass mapping. Although some proteins aggregate upon thermal denaturation, the protein aggregates are easily digested by trypsin and generate sufficient numbers of digest fragments for protein identification.

Peptide mass mapping by MALDI TOF mass spectrometry has become a routine method for protein identification.[1−4] Many laboratories now use 2-D gel electrophoresis for protein separation−isolation and "in-gel" digestion and peptide mass mapping for identification.[5−11] The protein separation capabilities of 2D PAGE has made in-gel digestion suitable for analysis of very complicated protein mixtures; however, in-gel digestion requires a series of chemical reactions and extraction/purification steps to recover digest fragments from the gel. Furthermore, the extensive sample preparation procedure of 2D PAGE/in-gel digestion increases the possibility of protein contamination.

The most commonly used sample preparation method of MALDI peptide mass mapping involves dissolving the protein and digesting the protein sample with proteolytic enzymes (so-called "in-solution" digestion). Generally speaking, in-solution digestion requires considerably less time and sample preparation efforts, and it appears to be a reasonable choice for simple protein mixtures or for identification of single protein samples. However, the use of in-solution digestion has often been disfavored because some proteins are not easily digested and additional chemical denaturation/purification steps can become more cumbersome than the in-gel digestion method. Hence, a nondigestion approach, protein database (PDB) searching with partial N-terminal sequence information (10−15 residues) using HPLC/micro Edman sequencing, was recently introduced; however, this alternative is not economical for the rapid identification of single-protein or simple protein mixtures.[12]

In this paper we describe a thermal denaturation method that is highly compatible with MALDI-MS. This method is preferred over chemical denaturation because sample preparation steps are minimal and, unlike chemical denaturation, purification and concentration steps prior to MALDI mass analysis are not required. The effect of thermal denaturation prior to enzymatic digestion of dietary proteins has been previously reported.[13−15] Varying degrees of digestion rate enhancement for different proteins were observed with SDS PAGE and the amount of newly exposed N-terminal amino acids after digestion was also increased in thermally denatured proteins. Nonetheless, thermal denaturation alone has never been recognized as a substitute for chemical denaturation of in-solution digestion. One possible reason thermal denaturation is not widely used would be formation of protein aggregates during thermal denaturation. The mechanisms of protein aggregation and structural details of protein aggregates have not been fully revealed yet. Insolubility of protein aggregates has hindered more frequent use of thermal denaturation in enzymatic digestion.

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In this study, we show that thermal denaturation (50−90 °C) of a protein can be accomplished in 20 min without the requirement of adding other denaturants and the effects of thermal denaturation on in-solution protein digestion are investigated using MALDI TOF mass spectrometry. Also, protein aggregation, which is a common phenomenon of thermal denaturation, is discussed. This study aims at developing a more efficient in-solution digestion methodology, which could ultimately lead to more accurate, reliable, and rapid protein identification in MALDI peptide mass mapping.

EXPERIMENTAL SECTION

All peptides and proteins (bradykinin, horse cytochrome *c*, horse myoglobin, chicken egg ovalbumin, bovine hemoglobin, and rabbit carbonic anhydrase) were purchased from Sigma (St. Louis, MO), and sequencing grade modified trypsin was purchased from Promega (Madison, WI).

Proteins were dissolved in aqueous 50 mM ammonium bicarbonate (Sigma) solution to make a concentration of 1−5 μM. Aliquots from protein solutions were thermally denatured by incubating at 90 °C for 20 min in airtight micro-centrifuge tubes. Following incubation, the proteins were transferred to an ice−water bath to quench the denaturation process. The thermally denatured and nondenatured proteins were enzymatically digested with sequencing grade modified trypsin at 37 °C for 3 h. The concentration of trypsin was maintained at 40:1 (wt of substrate/wt of trypsin) for all experiments.

The protein samples were analyzed by MALDI using the overlayer method.[16,17] Briefly, 1−2 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich) in methanol (HPLC grade, Fisher) was deposited on the MALDI sample and the solvent evaporated to form a thin matrix base layer. Approximately 1−2 pmol of digested proteins was diluted with deionized water (Milli-Q Water System, Millipore) and mixed with 15 mM α-cyano-4-hydroxycinnamic acid in methanol. A small amount (0.1−0.2 μL) of protein/matrix solution was placed onto the previously prepared matrix base layer and allowed to air-dry.

All MALDI-TOF mass spectra were acquired using a PerSeptive Biosystems Voyager Elite XL TOF equipped with a pulse nitrogen laser (337 nm). MALDI mass spectra were obtained by using delayed extraction (DE) in the reflected mode.[16] Total ion acceleration voltage was 25 kV, and the grid voltage was 17.5 kV. The delay time between laser pulse and voltage pulsing in the extraction plate was 200 ns.[18] Signals from 100 laser shots were averaged to increase the S/N ratio of each mass spectrum. All mass spectra were internally calibrated using the matrix dimer signal (m/z = 379.0930) and bradykinin $[M + H]^+$ ion signal (m/z = 1060.5692). Internal calibration with bradykinin $[M + H]^+$ ion was used throughout the analysis to provide reliable mass accuracy for protein database searches (average of less than 5 ppm).

MS−FIT from University of California (San Francisco, CA) was used to identify digest fragments and to estimate extent of digestion. The protein database, Swiss Prot, which is known to be the most annotated, was used. No restriction was placed on species, molecular weight range, and isoelectric point of target proteins. Trypsin was chosen as a digestion enzyme. All other possible modifications, such as an alkylation of the cysteine residue, were not considered. Mass tolerance or mass error range was kept below 20 ppm throughout the entire protein search.

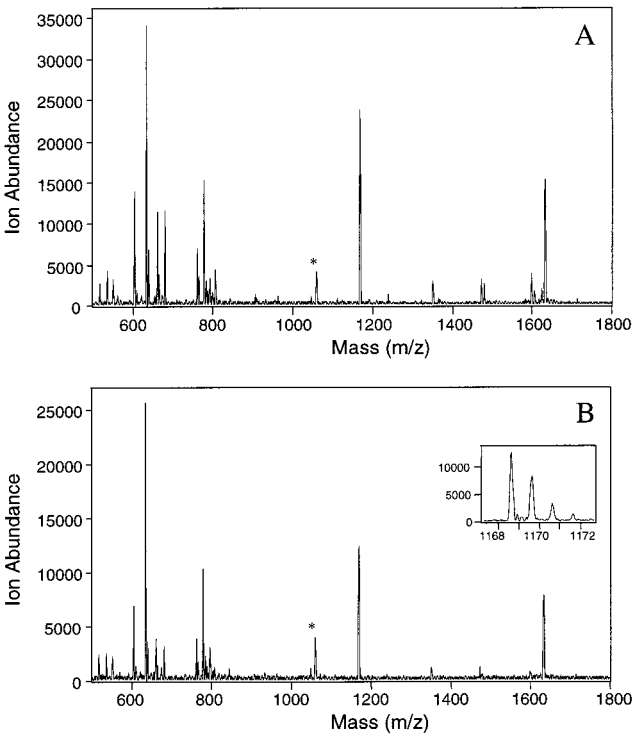

Figure 1. MALDI TOF mass spectra of horse cytochrome *c* digested by porcine trypsin (40:1 = weight of substrate/weight of trypsin) for 3 h at 37 °C (A) Nondenatured and (B) Thermally denatured cytochrome *c*. * denotes internal calibrant, bradykinin ($[M + H]^+$ = 1060.5692). Inset shows a well-resolved monoisotopic distribution of a tryptic digest fragment.

RESULTS AND DISCUSSION

Cytochrome *c* and myoglobin demonstrate significantly different responses to trypsin digestion. For example, the abundance of peptide fragments in the MALDI mass spectrum of digested cytochrome *c* is much greater than that for myoglobin under otherwise similar digestion conditions. Thus, it appears that cytochrome *c* is much easier to digest than is myoglobin. To increase the digestion efficiency, chemical denaturants such as 6−8 M guanidine HCl or urea, can be added to denature the protein; however, high concentrations of chemical denaturants suppress both MALDI and electrospray ion yields, thereby reducing the sensitivity of both methods.

Nondenatured and thermally denatured cytochrome *c* were digested for 3 h at 37 °C by trypsin and the digest fragments analyzed by high resolution MALDI TOF mass spectrometry (See Figure 1A and B). Trypsin digestion patterns of the two cytochrome *c* samples appear quite similar; however, a slightly lower abundance of peptide fragment is observed for thermally denatured cytochrome *c* as compared with the nondenatured sample (See Table 1.). The two missing digest fragments in the case of thermally denatured cytochrome *c* (m/z = 907.5439 and 1623.7929) do show up in the mass spectra, but the intensities of these two

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Table 1. Comparison of the Number of Digest Fragments and Amino Acid Sequence Coverages from Digest Fragments between Nondenatured and Thermally Denatured Proteins[a]

	Cytochrome *c* (Horse)	
	nondenatured	thermally denatured
experimental/theoretical digest fragments	14/22	12/22
amino acid sequence coverage of the protein	73% ± 0.0	64% ± 0.0

	Myoglobin (Horse)	
	nondenatured	thermally denatured
experimental/theoretical digest fragments	2/21	14/21
amino acid sequence coverage of the protein	15% ± 4.0	86% ± 1.2

	Hemoglobin (Bovine)			
	nondenatured		thermally denatured	
	α	*β*	α	*β*
experimental/theoretical digest fragments	8/14	10/18	9/14	10/18
amino acid sequence coverage of the protein	84 ± 2.9	75 ± 4.0	69 ± 4.9	73 ± 0.0

	Ovalbumin (Chicken Egg)	
	nondenatured	thermally denatured
experimental/theoretical digest fragments	0/34	13/34
amino acid sequence coverage of the protein	0%	43% ± 0.6

[a] Four separate digestions and mass analyses were performed for individual protein samples.

ions are not high enough to find a centroid for accurate mass. On the other hand, myoglobin produces far more tryptic digest fragments for the thermally denatured sample (Figure 2A,B). Thermal denaturation increases the rate of trypsin digestion for myoglobin as well as the number of digest fragments. Both the rate of enzymatic digestion and the diversity of digest fragments are strongly dependent upon the conformation of the substrate; thus, it appears that myoglobin undergoes significant conformational changes as a result of thermal denaturation.

The digest fragments present in the MALDI TOF mass spectra are identified using MS−FIT, a peptide mass mapping program. Table 1 lists the results of protein database searches from the above four MALDI mass spectra. Cytochrome *c* does not show significant differences between nondenatured and thermally denatured samples. However, the number of digest fragments identified for myoglobin increases from 2 to 14 peptide fragments (15−85% amino acid sequence coverage) which corresponds to a 6-fold increase.

To further evaluate thermal denaturation/trypsin digestion, we analyzed samples of bovine hemoglobin (MW = 60 kDa) and chicken egg ovalbumin (MW = 42 kDa) (See Table 1). Bovine hemoglobin is easily digested under the same trypsin digestion condition (40:1 = wt of substrate/wt of trypsin), but ovalbumin is hardly digested after 3 h of trypsin incubation. Two hemoglobin components, hemoglobin α and hemoglobin *β*, are individually identified by protein database searching at more than 70% amino acid sequence coverages for each component in nondenatured and thermally denatured samples. Nondenatured hemoglobin digestion demonstrates slightly greater amino acid sequence coverage than thermally denatured hemoglobin. Conversely, ovalbumin exhibits a very similar digestion pattern to myoglobin, that is, nondenatured ovalbumin does not produce any detectable tryptic digest fragments on the MALDI mass spectrum after 3 h of digestion whereas 13 tryptic digest fragments are detected for thermally denatured ovalbumin. This corresponds to 43% coverage of the amino acid sequence.

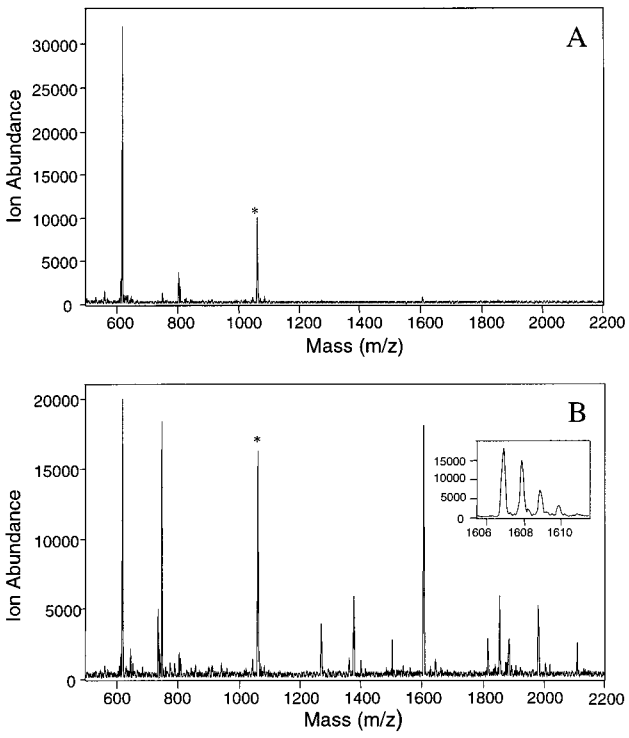

Figure 2. MALDI TOF mass spectra of horse myoglobin digested by porcine trypsin (40:1 = weight of substrate/weight of trypsin) for 3 h at 37 °C (A) Nondenatured and (B) Thermally denatured myoglobin. * denotes internal calibrant, bradykinin ($[M + H]^+ = 1060.5692$).

Rabbit carbonic anhydrase (MW = 29 kDa) was also investigated for the impact of thermal denaturation on trypsin digestion (data not shown here). Significant enhancement of digestion after thermal denaturation is also observed in carbonic anhydrase. Only the proteins that show resistance to enzymatic digestion are greatly affected by thermal denaturation in the peptide mass mapping. Proteins that are sensitive to digestion are hardly affected by thermal denaturation. All the proteins tested so far can be divided into these two classes: sensitive or insensitive to enzymatic digestion after thermal denaturation. Not a single protein has shown a drastic decrease of enzymatic digestion after thermal denaturation. A drastic increase of enzymatic digestion of proteins after heat treatment or thermal denaturation has been reported, especially in the nutritional chemistry area.[13−15] When heat-treated proteins were examined, again, none of those proteins tested showed a drastic decrease of digestion after heat treatment. This was speculated to be a result of conformational changes of proteins during heat treatment. The conformational changes in most proteins appear to be irreversible since thermal denaturation and digestion steps are separated by time and temperature.

Protein aggregation, which is one of the outcomes of thermal denaturation, is easily observed after thermal denaturation. The protein aggregation may be the reason thermal denaturation facilitates digestion in most cases. Protein aggregates are believed to be the oligomerization products of the denatured form of protein.[19] In general, hydrophobic parts of the protein are located inside and relatively less hydrophobic parts of the protein are exposed to the aqueous environment. During the thermal denaturation, intact proteins are gradually unfolded into a denatured conformation and sufficient energy is provided to prevent a fold back to its native conformation. The probability for interactions with other denatured proteins is increased, thus allowing hydrophobic interactions between exposed hydrophobic parts of the proteins. Preliminary data suggests that some protein aggregates show decreased sensitivity toward proteases recognizing hydrophobic residues. For example, chymotrypsin was tested on thermally denatured (90 °C, 20 min) and nondenatured horse cytochrome *c*. Thermally denatured cytochrome *c*, which shows definite formation of aggregates, produces some chymotryptic digest fragments with far lower ion abundance than nondenatured cytochrome *c*. However, this decreased sensitivity to chymotrypsin does not affect the results from the protein database search, because sufficient numbers of digest fragments are formed and detected using thermal denaturation/enzymatic digestion. In addition, protein aggregates of the denatured protein can have a more protease-labile structure than nondenatured proteins because more cleavage sites are exposed to the environment. Protein aggregates are easily digested, so that protein aggregates are not observed at the end of 3 h of trypsin digestion. Moreover, trypsin digestion of protein aggregates generates more specific cleavage products. Protein aggregation is closely related to the thermal denaturation condition as well as the content of disulfide bonding. The effects of disulfide bonding appear to be negligible in this study because the contents of disulfide bonding in ovalbumin and carbonic anhydrase are relatively low and they also show enhancement of digestion after thermal denaturation. Thermal denaturation in a very low pH condition or in the presence of surfactants or denaturants rarely forms protein aggregates.[20,21] This was explained by electrostatic repulsion by the charge built up on the surface of the denatured form of the proteins under the above conditions. Hence, the protein aggregation will be most pronounced if there is high protein concentration, low ionic salt concentration, and near p*I* conditions are achieved.

The relation between protein conformation and protease susceptibility has not been fully disclosed yet. α-Lytic protease, hemagglutinin, luciferase, and the prion protein PrP have shown unusually high resistance to enzymatic digestion.[22−25] This was explained by the high kinetic barrier to the unfolding process. The presence of a dynamic disordered loop structure that does not have atomic coordinates in the X-ray structure is also proposed to affect enzymatic digestion because it could be the primary target for enzymatic digestion.[26,27] Regardless of the causes of protein stability, thermal denaturation renders stable protein conformations vulnerable to proteolysis. Enzymatic digestions of protease-insensitive proteins appear to be enhanced via protein aggregation during thermal denaturation.

CONCLUSIONS

Thermal denaturation can be used in place of chemical denaturation to achieve uniform digestion of proteins which show different reactivities to in-solution enzymatic digestion. The higher yields of peptide fragments obtained by using thermal denaturation improve digestion efficiency, thereby enhancing protein identification by peptide mass mapping. Thermal denaturation is suitable for small-/large-scale protein identification and ultimately for automated peptide mass mapping because of its simple sample preparation. We have observed similar enhancements of peptide ion yields for digestion/peptide mass mapping of protein mixtures and even whole cell lysates.[28,29]

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