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Digestion of Native Proteins for Proteomics Using a Thermocycler

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Abstract

Efficient protein digestion is a critical step for successful mass spectrometry analysis. Here we describe simultaneous tryptic digestion and gradual unfolding of native proteins by application of a temperature gradient using a single cycle of 5 min or less in a PCR thermocycler. Chemicals typically used for chromatographic techniques did not affect the digestion efficiency. Tryptic digestion was performed in a small volume (3 μ L) with 1.5 μ g of trypsin without denaturing agents. This rapid procedure yielded more peptides than conventional methods utilizing chemical denaturation for 18 proteins out of 20. Samples were directly spotted on the MALDI-TOF target plate, without additional purification, thus reducing losses on reversed phase resins.

Mass spectrometry (MS) has become an indispensable technique in protein research.¹⁻⁴ Usually, denatured proteins are digested with a proteinase and the peptides can then be analyzed by MS. The mass spectrum is compared with the peptide mass fingerprints in databases for protein identification.¹⁻⁴ Such mass mapping requires purified proteins. Therefore, this technique is usually used in conjunction with protein fractionation methods such as liquid chromatography (LC) or one- and two-dimensional gel electrophoresis (1DE/2DE). Today multidimensional chromatography and analysis techniques based on computer-controlled HPLC systems are being adopted by industry and academia.^{1,5-8} MDCA generally processes proteins in their native conformations rather than denatured as in SDS gels. Native proteins are also isolated by bimolecular interaction analysis instruments, such as BIAcore, which utilize the biological specificity of native proteins for other proteins or DNA for isolation of proteins from complex mixtures.⁹⁻¹² Subsequent chemical denaturation protocols^{12,13} have been applied to the output of these separation techniques followed by conventional overnight trypsin digestion, with subsequent solid-phase capture and elution of samples for MS analysis.

The use of direct protease digestion of native proteins from liquid chromatography or BIAcore would be a great advantage for further analysis by MS. Automation of the integrative LC-MS analysis is currently difficult mainly due to the complicated process of protein digestion. Some proteins are susceptible to proteinases and can be digested under standard conditions (37 °C, neutral pH, etc.), whereas most proteins are not digested effectively. To achieve efficient digestion, those proteins require a pretreatment step that denatures the protein prior to proteinase digestion. Proteins are usually unfolded using chemical denaturants.¹³⁻¹⁵ Denaturation at 90 °C has been reported as an alternative to chemical denaturation of proteins.¹⁵ Such denatured proteins are generally aggregated, forming large clumps of protein(s). The process of digestion of such aggregates is very slow (typically overnight digestion is required).

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Moreover, the digested samples require purification and concentration due to the presence of urea or salts that are incompatible with MS. This purification is usually achieved with columns packed with hydrophobic media such as C18.^{16,17} It is well-known that not all proteins and peptides bound to such resins can be eluted. Significant amounts of material are lost reducing protein identification.

Efforts have been made to find an alternative procedure of the digestion. Thus, on-particle enzymatic digestion reduced the digestion time;^{16,18,19} on-particle digestion at elevated temperatures further improved digestion efficiency.^{20,21} Yu et al. used a novel acid-labile anionic surfactant to solubilize proteins and improve digestion rate.²² A focused ultrasound technique also greatly reduced the time of protein digestion.²³ Another alternative to the conventional method of protein digestion is microwave irradiation technology. This technology has been extensively used in organic chemistry.²⁴⁻³⁰ Pramanik et al. reported the use of microwave technology for protein digestion in 2002.³¹ Since then several groups have shown the efficiency of the microwave assisted protein digestion method using different systems³²⁻³⁴ and on-particle digestion (for review, see ref³⁹).³⁵⁻³⁸ Although there are experimental data pointing to nonthermal microwave effects,⁴⁰ some researchers thought that the thermal effect is the only basis for the microwave-assisted reaction.⁴¹ It is in the bounds of possibility that in both high-intensity ultrasound and microwave irradiation procedures the temperature is the key factor causing acceleration of the enzymatic reaction. In this report, we describe a method using a widely available instrument, the PCR-type thermocycler, to gradually expose cleavage sites of proteins by thermal melting with simultaneous digestion by ordinary proteomics grade trypsin. We show that digestion of native proteins in-solution was complete in less than 5 min at optimal temperatures. All purified proteins were digested in 3 μ L of buffers typically used for purification, without dialysis or other techniques for buffer replacement. Digestion was performed without using any denaturing agents, so the peptides obtained could be analyzed directly by MALDI-TOF.

EXPERIMENTAL SECTION

Materials and Reagents

Thin walled TempAssure PCR tubes were from USA Scientific (Ocala, FL). Centricon centrifugal filter devices with YM-10 membranes were obtained from Millipore Corp. HPLC grade water and acetonitrile (ACN) were purchased from Fisher Scientific. Modified proteomics grade trypsin, matrix (α -cyano-4-hydroxycinnamic acid), peptide standards (human angiotensin II, synthetic peptide P₁₄R, human ACHT fragment 18-39) and all other analytical grade reagents were purchased from Sigma-Aldrich (Sigma Chemicals, St. Louis, MO). Bovine serum albumin (BSA), human hemoglobin, human albumin, human transferrin and lysozyme were purchased from Sigma-Aldrich. All other proteins were obtained from Complement Technology, Inc. (Tyler, TX). The iCycler thermal cycler (Bio-Rad) with a temperature ramping speed of 3.3 °C/s and a cooling speed of 2.2 °C/s was used for these studies.

Protein Preparation

Lysozyme, BSA, human hemoglobin, human albumin and human transferrin were dissolved in water to final concentrations of 10 μ g/ μ L, aliquoted and stored at -75 °C. Prior to experiments, protein solutions were diluted in water (or PBS) to a final concentration of 1 μ g/ μ L. All other proteins from Complement Technology, Inc. were in PBS buffer in concentrations of 1 μ g/ μ L. If necessary, proteins were concentrated on Centricon centrifugal filter devices with YM-10 membranes to a final concentration of 10 μ g/ μ L.

Preparation of Trypsin

Two hundred microliters of HPLC grade water were injected into the manufacturer's vial containing 20 µg of lyophilized, proteomics grade, modified trypsin using a 1-mL tuberculin syringe. The solution was gently mixed in a laboratory rotator at room temperature for 10 min, aliquoted and dried on a SpeedVac, and then stored at -75 °C. Prior to use, trypsin pellets were dissolved in water to the desired concentration.

Conventional Digestion Protocol

In-solution digestion was performed in accordance with the recommendation of Shimadzu Biotech (Columbia, MD). Briefly, 60 µg of protein in 60 µL of PBS buffer was evaporated and resuspended in 6 µL of 8 M urea in water containing 10 mM DTT. This solution was incubated for 1 h at 37 °C. Iodoacetamide was added to a final concentration of 55 mM, the mixture was incubated for 45 min in the dark, DTT was then added to final concentration of 65 mM, and this mixture was incubated for 1 h. The solution was then diluted with 20 mM NH₄HCO₃ pH 8.0 to a final concentration of 1 M urea. Trypsin (2 µg) was added to 60 µL of diluted denatured protein, and this was incubated at 37 °C overnight. Digested samples were used without further purification by mixing with matrix and spotting on MALDI plates.

Protein Digestion at Different Temperatures

In-solution digestion at elevated temperatures was done without denaturation or alkylation of proteins. In each experiment 1 µL (1 µg) of protein in water or PBS buffer, 1 µL (140 ng) of trypsin in water and 1 µL of DTT (1.5 mM) in 100 mM NH₄HCO₃ pH 8.0 were loaded into TempAssure PCR tubes, mixed, placed in the thermocycler, and incubated for 20 s at the desired temperature.

Protein Digestion: Dependence on pH, DTT, NaCl

For the experiments with DTT, PCR tubes were prepared with 1 µL (1 µg) of protein in water or PBS buffer, 1 µL (140 ng) of trypsin in water, and 1 µL of DTT in 100 mM NH₄HCO₃, pH 8.0. Different concentrations of DTT were added to make final concentrations of 0, 0.25, 0.5, 1.0, 2.5, 5.0 or 10 mM. For the experiments with salt, PCR tubes contained 1 µL (1 µg) of protein in water, 1 µL (140 ng) of trypsin in water, 1 µL of solution containing DTT (1.5 mM) in 100 mM NH₄HCO₃ and different amounts of 4 M NaCl was added to get final concentrations of NaCl of 100 or 1000 mM. Samples were prepared and kept on ice until spotted. At least 3 replicas were used for each experiment.

Digestion of a Small Amount of Protein

Trypsin was dissolved in water, and aliquoted to have 140 ng of enzyme per tube, and dried on a SpeedVac. Protein sample (substrate) was prepared in 20 mM NH₄HCO₃ containing 0.5 mM DTT to have a final concentration of 50 ng/µL. A small amount (3 µL) of this protein solution was placed into the tube containing dried trypsin. Samples were digested in the thermocycler for 20 s at 52 °C.

SDS-PAGE Analysis of Protein Digested for Different Periods of Time and Concentrations of Trypsin

A solution (20 µL) containing 0.5 mM DTT, 4 µg of BSA, and 1.2 µg of trypsin in 10 mM NH₄CO₃ was incubated at 52 °C for desired time. The digestion reaction was stopped by adding 2 µL of 5 % TFA. For the experiments with different concentration of trypsin, 15 µL of solution containing 0.7 mM DTT and 4 µg of BSA in 15 mM NH₄CO₃ was prepared. Different amounts of trypsin in 5 µL of water were added into this solution. Samples were then incubated at 52 °

C for 5 min. Digestion was stopped by adding 2 μL of 5 % TFA. These protein digests were used for SDS-PAGE analysis.

Time Curve of Protein Digestion

A solution (20 μL) containing 0.5 mM DTT, 4 μg of BSA, and 4 μg of trypsin in 10 mM NH_4CO_3 was placed into the thermocycler preheated to 52 $^\circ\text{C}$. At particular time points (0.33, 0.8, 1.75, 5, 15 and 60 min) 1 μL of the reaction mixture was sampled and added to 2 μL of 0.5% trifluoroacetic acid. Each sample was then diluted 1:50 with 0.1% formic acid, and 6 μL of the resulting solution (containing 1.2×10^{-13} mol of BSA digest) was used for each mass spectrometric analysis.

LC-MS/MS was carried out on the $t = 60$ min fraction using a 4000 Q-Trap mass spectrometer (Applied Biosystems, Warrington, UK). Briefly, peptides were loaded at high flow rate onto a reversed-phase trapping column (0.3 mm i.d. $\times$ 1 mm), containing 5- μm C18 300 $\text{\AA}$ Acclaim PepMap media (Dionex), and eluted through a reversed-phase capillary column (75 μm i.d. $\times$ 150 mm) containing Jupiter Proteo 4- μm , 90- $\text{\AA}$ media (Phenomenex) that was self-packed using a high pressure packing device (Proxeon Biosystems, Odense, Denmark). The output from the column was sprayed directly into the nanospray ion source of the 4000 Q-Trap mass spectrometer.

Fragment ion spectra generated from the LC-MS/MS were searched using the MASCOT search tool (Matrix Science Ltd., London, UK) against a weekly updated copy of the SwissProt protein database using appropriate parameters. The criteria for protein identification were based on the manufacturer's definitions (Matrix Science Ltd.). Basically candidate peptides with probability-based MOWSE scores exceeding threshold ($p < 0.05$), and thus indicating a significant or extensive homology were referred to as "hits". Protein scores were derived from peptide-ion scores as a nonprobabilistic basis for ranking proteins.

Based upon the LC-MS/MS results of the $t = 60$ min sample, five peptides were selected to obtain relative quantitation using a multiple reaction monitoring (MRM) experiment⁴². For each peptide, a pair of diagnostic masses were chosen; these consisted of the $[\text{M} + 2\text{H}]^{2+}$ precursor ion mass, and a strong yion fragment mass

	$[\text{M} + 2\text{H}]^{2+}$	fragment	collision energy
LVNELTEFAK	582.32	951.48 (y_8)	26.38
LGEYGFQNALIVR	740.40	1017.58 (y_9)	31.91
YICDNQDTISSK	693.81	1110.47 (y_{10})	30.28
DAFLGSFLYEYSR	784.38	1121.53 (y_9)	33.45
SLHTLFGDELCK	681.84	811.37 (y_7)	29.86

Each precursor/fragment ion pair was scanned in turn for a 50-ms time period and its intensity recorded. Chromatograms were plotted for each ion pair, and peak areas were calculated using integration software (Analyst v 1.4.2, Applied Biosystems).

For each time point, MRM measurements were repeated two to five times. A blank run was recorded between each replicate and validated to ensure that there was no run-to-run carryover from the column.

Thermocycler Digestion Method

Proteins were digested in solution with trypsin, without chemical denaturation. In each experiment, 1 μL (1 μg) of protein in water or PBS buffer, 1 μL of 1.5 mM DTT in 100 mM NH_4HCO_3 and 1 μL of trypsin in water (1.5 μg , final concentration of 21 μM) were loaded into TempAssure PCR tubes and mixed. The digestion reaction was performed in the thermocycler, using a program with 20 s at the optimal digestion temperature for the protein,

or for unknowns and difficult to digest proteins, with 20 s at each of the following temperatures: 49, 50, 51, 52, 53, 54, and 55 °C.

Protein Digestion at 37 °C Without Denaturation

Proteins were digested in solution with trypsin, without chemical denaturation. In each experiment, 1 µL (1 µg) of protein in water or PBS buffer, 1 µL of 1.5 mM DTT in 100 mM NH₄HCO₃ and 1 µL of trypsin in water (1.5 µg, final concentration of 21 µM) were loaded into TempAssure PCR tubes and mixed. The digestion reaction was performed in the thermocycler at 37 °C for 5 h.

MALDI-ToF Analysis

The digested protein samples were analyzed on an Axima-CFR (Manchester, UK) mass spectrometer. The system uses a pulsed nitrogen laser, emitting at 337 nm. The ions were accelerated to kinetic energy of 20 000 V. All measurements were done under pulsed extraction mode optimised for 2500 Da.

Digested proteins (1 µL) were mixed with 5 µL matrix (α -cyano-4-hydroxycinnamic acid in 0.1% TFA/50 % ACN). A small amount of this protein/matrix solution (0.3-1.0 µL) was placed on the MALDI target and allowed to air-dry. Signals from at least 100 laser shots were averaged to increase the signal-to-noise ratio of each mass spectrum. For external calibration angiotensin II (m/z 1046.5), P₁₄R (m/z 1533.9) and ACHT fragment 18-39 (m/z 2465.2) were used and spotted in close proximity to each sample spot.

Database Searches

Protein identification was conducted using MASCOT database search software, available on the Internet (<http://www.matrixscience.com/>), against the MSDB Database. The query was made for the all entries. One missed cleavage for tryptic peptides was allowed.

RESULTS AND DISCUSSION

Simultaneous Unfolding and Digestion of Proteins

Digestion of proteins at increased temperatures was performed using proteomics grade modified trypsin on a common PCR thermocycler. Commercially available trypsin, modified by reductive methylation was used for protein mass fingerprinting. This enzyme has been shown to retain activity and specificity at higher temperatures.^{43; 44} Three model proteins were used: BSA, complement factor H and lysozyme. Digestion of proteins was performed over a range of temperatures. Because the ultimate goal of the research was protein identification, MASCOT scores (<http://www.matrixscience.com/>) were used as an indicator of the efficiency of the method.

The MASCOT score data indicated that BSA could be digested in a single 20-s cycle at temperatures between 37 and 48 °C (Figure 1A). However, the efficiency of digestion was significantly higher at temperatures between 50 and 55 °C. Above 55 °C the digestion efficiency gradually decreased and above 60 °C the protein MASCOT scores were below the significance level (dashed line on Figure 1A). Another protein, lysozyme is known to be highly susceptible to trypsin. Using the thermocycler this protein was digested with trypsin at 37 °C and identified by MASCOT software. However, the most effective digestion of lysozyme was observed at temperatures between 50 and 55 °C (Figure 1B). A third protein, complement factor H, was not digested below 48 °C (Figure 1C). The most effective digestion occurred at 50 °C, but unlike BSA the MASCOT score did not fall below the significant level even up to 72 °C, the highest temperature used.

Susceptibility of proteins to proteinase digestion depends on structure, stability, size and post-translational modifications. The digestion of some proteins is efficient, whereas under similar conditions other proteins are not digested.¹⁵ Efficient digestion of most proteins requires denaturation and disruption of disulfide bonds. In the most widely used procedure, dithiothreitol in a denaturing buffer (urea or guanidine hydrochloride) is used to reduce disulfide bonds that are subsequently alkylated. Unfortunately, proteins typically aggregate after removal or dilution of the denaturing agent. Digestion of such aggregates can be very slow process (16 h or more). The fact that conventional methods require many hours or even days to complete digestion indicates that most cleavage sites are not freely accessible in these preparations. We suggest that cleavage of the most easily accessible tryptic sites begins during the ramp up time from 21 °C to the final incubation temperature. For a final temperature of 50 °C, the ramp time would be 9 s at 3.3 °C/s. These initial cleavages destabilize the protein accelerating exposure of less susceptible cleavage sites and promoting more complete digestion by trypsin. Because cleavage and release of peptides occurs rapidly and simultaneously with denaturation, digestion occurs before large-scale aggregates can form if an adequate amount of trypsin is present. This prevents sequestration of Lys and Arg sites in large aggregates. The resulting high MASCOT scores indicate that even after only an ~ 42 s reaction (9 s ramp up, 20-s incubation at 50 °C and 13-s ramp down to 21 °C) most tryptic sites were cleaved.

Digestion of Small Amount of Protein

In the liquid chromatography experiments often a small amount of protein is purified. In this experiment protein samples were added directly to the tubes containing dried trypsin. This method makes material sampling very convenient and does not dilute the protein sample during the digestion procedure. The results indicate that small amounts of protein (50 ng/μL) can be efficiently digested and identified (data not shown).

PAGE Analysis of Protein Digestion at Different Concentrations of Trypsin

In the thermocycler digestion method, the concentration of proteinase is important. Thus, large undigested fragments were observed when BSA was digested at 52 °C for 5 min with 0.5 μM of trypsin (Figure 2A). Notably this concentration of trypsin did not completely digest protein even in 4 h (Figure 2B). At the same time, BSA was completely digested within 5 min at concentration of trypsin 21 μM (Figure 2A). Experiments with lysozyme and factor H shown similar results (not shown).

Modified trypsin used in this experiment is resistant to autolytic digestion.⁴⁵ Havlis et al. reported that modification of trypsin by reductive methylation reduced the autolysis and shifted its working optimum to a higher temperature.⁴⁴ Thus, mass spectrometric analysis revealed that the samples were not contaminated with trypsin autolysis products, despite the high concentration of this enzyme, whereas the substrates were digested effectively (data not shown).

Peptide Yield and Digestion Time

In the time course experiments accumulation of 5 peptides was analyzed: LVNELTEFAK; LGEYGFQNALIVR; YICDNQDTISSK; DAFLGSFLYEYSR; SLHTLFGDELCK (Figure 4). Maximum peptide yield was reached in 15 min. However, when the protein was digested for 1 h at elevated temperature, the loss of considerable amount of peptides was observed. The reason for this is unknown. The peak areas observed for peptides obtained using the conventional method of digestion were higher than those of obtained using the thermocycler digestion method at longer times. These results indicate that effective digestion of proteins in small volumes at elevated temperatures has to be done for short time periods.

Effect of Reducing Agent

BSA, factor H and lysozyme were digested in the presence of different concentrations of DTT. BSA and lysozyme were adequately digested without DTT, but DTT concentrations between 0.5 and 10 mM were found to give optimum digestion. Many proteins were not digested well by trypsin without reduction of disulphate bonds, but were digested in concentrations of DTT between 0.5 and 10 mM. For example, factor H is a unique protein in that it contains 20 homologous domains with two disulphide bonds in each of them. Almost every tryptic peptide of this protein is bound to another peptide through a disulfide bond. Furthermore, this protein is highly resistant to trypsin at physiological temperatures even with DTT present (Figure 1C). However, it was digested very efficiently in 20 s in the presence of 0.5 mM DTT at temperatures above 50 °C. Because factor H contained the highest proportion of disulfide bonds of the proteins tested, 0.5 mM DTT was used for digesting all proteins at elevated temperatures.

Application of the Thermocycler Digestion Method for Proteins Purified by HPLC

HPLC systems are widely used for rapid purification of proteins from complex mixtures. Typically, the buffers used in these procedures contain high concentration of salts or organic solvents. Most proteins are eluted from ion exchange columns at concentrations of salts below 600 mM. The results of digesting proteins in solution using the thermocycler digestion method in the presence of high concentrations of salt show that proteins are digested effectively. Even 1 M of NaCl did not decrease the efficiency of the thermocycler digestion method, with 26, 87 and 31% of sequence coverage and MASCOT scores of 77, 179 and 142 for BSA, lysozyme and factor H, respectively (when proteins were digested without salt sequence coverages were of 61, 46 and 92 %, with MASCOT scores 215, 260 and 201 for BSA, factor H and lysozyme, respectively). The typical MALDI mass spectra of all three proteins digested in presence of 1 M of NaCl given in Figure 3.

The results indicate that sample desalting is unnecessary and that it should be possible to analyze effluents from ion exchange columns directly. This would eliminate peptide losses and save time compared to current protocols.

Comparison of Conventional and Thermocycler Digestion Methods

We compared the digestion performance of the rapid thermocycler digestion method with that of the conventional method, which uses urea denaturation and overnight digestion. This comparison was made by examining 20 different proteins (Table 1). We digested these proteins at 37 °C for 5 h without denaturation, to find out which proteins were resistant to tryptic digestion at physiological temperature. MASCOT database-searching software was used to evaluate the quality of peptide mass fingerprints and the certainty of identification of proteins treated using three digestion methods. Protein digestion at 37 °C without denaturation failed to identify 7 proteins (marked NI in Table 1). Seven other proteins were identified but with scores below the significance level. Only 6 proteins out of 20 were effectively digested and identified with a statistically significant score (the numbers are underlined in the Table 1). Conventional overnight digestion after denaturation with urea/DTT resulted in the identification 11 proteins out of 20 with high certainty and 7 additional proteins were identified but with MASCOT scores that were below the significance level. This method failed to identify two proteins (NI in the Table 1).

For the thermocycler digestion method, the digestion reaction was performed as described in the Experimental Section. Because of the diversity of proteins being examined, we utilized a program that gradually increased the temperature by one degree between 49 and 55 °C every 20 s (total reaction time less than 3 min). All 20 proteins were efficiently digested and identified by MASCOT software with 18 out of 20 scores above the significance level. The number of identified peptides, the MASCOT scores and the sequence coverage were all higher than the

conventional method for 18 out of 20 proteins. In fact, the thermal digestion method resulted in MASCOT scores that were, on average, more than twice the scores obtained with the conventional urea/DTT-digestion method.

CONCLUSION AND PERSPECTIVES

In the conventional method of protein digestion used for mass fingerprint analysis, proteins are chemically denatured to make more cleavage sites available. But, due to aggregation, digestion of such denatured proteins is slow and sometimes not effective. We have demonstrated here that simultaneous digestion and melting at increased temperatures yields MASCOT scores twice as high as the conventional method, on average. It is very fast, easy to perform, and the equipment needed is widely available and relatively inexpensive. The results were superior to the conventional method for all 20 proteins tested. Furthermore, the thermocycler digestion method is ideally suited for automated 2D proteomic chromatographic systems now being produced for high-throughput, high-resolution separation of non-denatured biological samples. We have demonstrated that the presence of the typical solvents used in these systems does not interfere with peptide generation and MASCOT identification.

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Figure 1.

Effect of temperature on protein digestion. Proteins were digested 20 s at the indicated temperatures in a thermocycler. The reaction was performed in 3 μL of 33 mM NH_4HCO_3 solution containing 1 μg of protein, 140 ng of trypsin, and 0.5 mM DTT, pH 8.0, without denaturing agents. The quality of the mass fingerprint was evaluated using MASCOT software. Protein scores greater than 78 were considered significant (dashed lines). Panel A shows results for BSA, Panel B for lysozyme, and Panel C for human complement factor H.

Figure 2.

Trypsin digestion of BSA. (A) Different amounts of trypsin (5 μ L) were added to 15 μ L of solution containing 0.7 mM DTT and 4 μ g of BSA in 10 mM NH_4CO_3 : (1) 0.2, (2) 0.3, (3) 0.5, (4) 2, (5) 6, (6) 10 (7) 21 μ M trypsin; (8) BSA was digested using conventional method of digestion; (9) no trypsin. Samples were incubated for 5 min at 52 $^\circ\text{C}$. (B) Solution (20 μ L) containing 0.5 mM DTT, 4 μ g of BSA, and 0.5 μ M trypsin in 10 mM NH_4CO_3 was incubated at 52 $^\circ\text{C}$ for different times: (1) 0, (2) 10, (3) 30, (4) 50 s, (5) 2, (6) 10, (7) 40 min; (8) 2, (9) 4 h. $^\circ\text{C}$.

Figure 3.

Effect of high concentration of salt on protein digestion. Proteins were digested using the thermocycler digestion method with 20 s at each temperature between 49 and 55 °C. The reaction was performed in 3 μ L of 33 mM NH_4HCO_3 solution containing 1 μ g of protein, 140 ng of trypsin, 0.5 mM DTT and 1 M NaCl, pH 8.0 without denaturing agents. (A) BSA; (B) factor H; (C) lysozyme.

Figure 4.

Time curve quantitation analysis of protein digestion. Solution (20 μ L) containing 0.5 mM DTT, 4 μ g of BSA and 4 μ g of trypsin in 10 mM NH_4CO_3 was incubated at 52 $^\circ\text{C}$ for 20 s, 45 s, 1.5 min, 5 min, 15 min, and 1 h. Indicated five peptides were used for quantitation. The peak areas of peptides obtained using the conventional protein digestion method were between 2×10^{-7} and 5×10^{-6} .

Table 1
Comparison of Conventional and Thermocycler Digestion Methods

Proteins	Thermocycler Digestion Method ^a				Conventional Protein Digestion Method ^b			
	MS	NM	SC%	E-value	MS	NM	SC%	E-value
Complement C1r	151	24	35	2.6×10^{-9}	85	10	21	1.2×10^{-7}
Complement C1s	112	20	38	3.0×10^{-7}	97	20	38	4.6×10^{-6}
Complement C1q*	100	9	51	3.3×10^{-3}	NI	-	-	-
Complement C2	209	26	45	4.1×10^{-15}	72	9	14	9.7×10^{-4}
Complement C3*	162	32	32	2.0×10^{-9}	103	28	28	1.5×10^{-6}
Complement C4	203	51	35	1.6×10^{-14}	87	20	18	3.1×10^{-5}
Complement C5	165	34	28	1.0×10^{-10}	66	9	7	8.6×10^{-3}
Complement C6	171	38	43	2.6×10^{-11}	80	13	19	1.2×10^{-6}
Complement C7	192	28	39	2.0×10^{-10}	85	17	25	2.5×10^{-6}
Complement C8*	66	6	43	0.2	NI	-	-	-
Complement C9*	190	24	55	3.2×10^{-13}	72	9	20	2.0×10^{-4}
Cobra Venom Factor	170	30	28	3.2×10^{-11}	87	20	18	3.2×10^{-6}
Factor H*	260	46	46	1.2×10^{-10}	153	23	28	2.5×10^{-7}
Factor B	215	40	47	5.1×10^{-14}	87	11	24	9.0×10^{-8}
Factor D	149	11	65	4.1×10^{-9}	70	7	52	6.0×10^{-4}
Factor I	118	21	42	5.1×10^{-6}	74	10	24	2.4×10^{-5}
Factor P*	112	12	30	2.0×10^{-5}	60	6	20	4.6×10^{-3}
Haemoglobin, human*	120	10	84	3.2×10^{-6}	66	5	40	2.7×10^{-4}
Albumin, human serum	174	19	44	1.3×10^{-11}	100	20	35	1.2×10^{-6}
Transferrin, human	284	37	50	1.3×10^{-22}	113	17	40	6.4×10^{-10}

^a Proteins were digested using the thermocycler digestion method with 20 s at each temperature between 49 and 55 °C. The reactions were performed in 3 µL of 33 mM NH₄HCO₃ containing 1 µg of the indicated protein, 1.5 µg of trypsin, and 0.5 mM DTT, at pH 8.0 without denaturing agents. The quality of the mass fingerprint was evaluated using MASCOT software. MS, MASCOT scores. NM, number of matched mass values, sequence coverage. NI, proteins were not identified. Statistically significant scores are underlined.

^b Proteins were digested using the conventional overnight digestion method as described in the Experimental Section.

^c Proteins not identified by MASCOT while digested at 37 °C without denaturation were digested 5 h at 37 °C. The reaction was performed in 3 µL of 33 mM NH₄HCO₃ containing 1 µg of the indicated protein, 1.5 µg of trypsin, 0.5 mM DTT, at pH 8.0, without denaturing agents.