Microwave-assisted Protein Preparation and Enzymatic Digestion in Proteomics*

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The combinations of gel electrophoresis or LC and mass spectrometry are two popular approaches for large scale protein identification. However, the throughput of both approaches is limited by the speed of the protein digestion process. Present research into fast protein enzymatic digestion has been focused mainly on known proteins, and it is unclear whether these results can be extrapolated to complex protein mixtures. In this study microwave technology was used to develop a fast protein preparation and enzymatic digestion method for protein mixtures. The protein mixtures in solution or in gel were prepared and digested by microwave-assisted protein enzymatic digestion, which rapidly produces peptide fragments. The peptide fragments were further analyzed by capillary LC and ESI-ion trap-MS or MALDI-TOF-MS. The technique was optimized using bovine serum albumin and then applied to human urinary proteins and yeast lysate. The method enabled preparation and digestion of protein mixtures in solution (human urinary proteins) or in gel (yeast lysate) in 6 or 25 min, respectively. Equivalent (in-solution) or better (in-gel) digestion efficiency was obtained using microwave-assisted protein enzymatic digestion compared with the standard overnight digestion method. This new application of microwave technology to protein mixture preparation and enzymatic digestion will hasten the application of proteomic techniques to biological and clinical research. Molecular & Cellular Proteomics 5:769–776, 2006.

Proteomics aims to characterize a large number of proteins extracted from a cell, tissue, or organism so that a global perspective of changes in protein expression can be obtained in a rapid fashion (1, 2). Proteomic analysis of complex mixtures of proteins usually proceeds along either a bottom-up or top-down approach. In the bottom-up approach, the entire proteome is digested into a pool of possibly thousands of peptides (3–5). Two-dimensional (2D)

1 LC and MS are used to resolve and identify peptide components in the mixture. This approach, referred to as shotgun proteomics, affords the advantages of automation and sensitivity but at the loss of information regarding the intact protein. In the top-down approach, the characteristics of a protein, such as molecular weight, isoelectric point, and hydrophobicity, are used to isolate the intact protein (6, 7). Gel electrophoresis is the most commonly used top-down approach (8). Each slice or spot in the gels is excised, digested, and identified by MALDI-MS or ESI-ion trap-MS. Another top-down approach uses chromatofocusing and reverse phase chromatography in an HPLC format to separate proteins (9–12). Each fraction from LC is also digested and identified by MS. LC offers an advantage over gel electrophoresis in terms of ease of automation and protein recovery.

Although protein and peptide separation and identification can be made highly automated and rapid, sample preparation and digestion in contrast are considerably slower (more than 16 h) and limit the speed of large scale protein identification. Recently several approaches have been developed for fast protein digestion. One approach is the use of modified trypsin for in-gel digestion of proteins instead of native trypsin (13). Another approach uses on-line protein digestion during LC using a proteolytic reactor (14, 15) or an immobilized enzyme column (16, 17). Other promising approaches include microwave-assisted protein enzymatic digestion (MAPED) or acid hydrolysis (18–22).

Several recent reports have highlighted the speed and convenience of MAPED. Juan et al. (18) used microwave technology to digest several known proteins in gel with trypsin in 5 min. Pramanik et al. (19) also applied microwave technique to digest known proteins in solution or in gel with trypsin in 10 min including a protein that was tightly folded and extremely resistant to denaturation (bovine ubiquitin). Lin et al. (20) investigated microwave-assisted enzyme-catalyzed reactions in various solvent systems. They found that digestion efficiency and sequence coverage were increased when the trypsin digestion occurred in ACN−, methanol−, and chloroform-containing solutions under microwave irradiation. These authors found that ACN did not deactivate the proteolytic enzyme during the irradiation period, whereas methanol did.

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1 The abbreviations used are: 2D, two-dimensional; 1D, one-dimen-
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There are also some reports on microwave-assisted protein acid hydrolysis. Chen et al. (21) and Zhong et al. (22) used microwave irradiation to accelerate the hydrolysis of peptides and proteins with 6 m HCl. Zhong et al. (23) also described a microwave-assisted acid hydrolysis method for rapid protein degradation with trifluoroacetic acid. They applied the method to analyze a membrane protein-enriched fraction of cell lysates and identified 41 membrane proteins.

Published studies using MAPED have mainly focused on one or several known proteins, and the microwave technique is only used for protein enzymatic digestion (18–20). Whether the microwave technique can be applied to protein mixture preparation and digestion, especially for protein recovery from the gel, has still not been well investigated. For microwave-assisted protein acid hydrolysis, Zhong et al. (22, 23) have developed the method for known proteins as well as protein mixtures. But protein acid hydrolysis is known to lack cleavage specificity (21, 22) and is generally not used in proteomic studies. Therefore it is necessary to develop the proper MAPED method for protein mixtures.

In this study, we utilized a known protein (bovine serum albumin) as well as complex mixtures of proteins derived from human urine and yeast lysate to address the problems noted above. In our experiments MAPED could prepare and digest protein mixtures in solution or in gel in 6 or 25 min, respectively, and its peptide yield efficiency was the same (in solution) or better (in gel) than the present standard method (16 h or overnight). This new application of microwave technology speeds up protein sample preparation and enzymatic digestion in proteomic studies of biological and clinical samples.

MATERIALS AND METHODS

Apparatus

An LCQ Deca XP mass spectrometer was purchased from ThermoFinnigan (San Jose, CA). A Voyager-DE Pro MALDI-TOF mass spectrometer was purchased from Applied Biosystems (Foster City, CA). Accessories for capillary liquid chromatography were from Upchurch Scientific Inc. (Oak Harbor, WA). C18 reverse phase (RP) and strong cation exchange (SCX) resins (5 μm, 300 Å) were from Merck and PolyLC Inc. (Columbia, MD), respectively. A vertical mini-gel system (Mini-Protean II) was purchased from Bio-Rad. The microwave oven used in this study was solid-state Whirlpool model VIP271 (Shanghai, China), and the maximum output power was 850 W.

Reagents

Deionized water from a MilliQ RG ultrapure water system (Millipore, Bedford, MA) was used at all times. HPLC grade ACN and formic acid, trifluoroacetic acid, ammonium bicarbonate, iodoacetamide, and DTT were purchased from Merck. Sequencing grade modified trypsin, protease inhibitor PMSF, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma.

Protein Sample

BSA used in this study was purchased from LianXing (Beijing, China). Human urinary proteins were obtained from five healthy male mixed morning urine samples by acetone precipitation as described previously (24). The five urinary samples were centrifuged at 5000 × g for 30 min, and the precipitates were removed. The supernatants from five donors were mixed with the same volume, and the mixed urinary samples were precipitated by 50% acetone for 10 min following centrifugation at 12,000 × g for 30 min. The pellets were resuspended in 25 μM ammonium bicarbonate. Yeast lysate was obtained with the method described previously (25). Strain CG1945 was grown to log phase in yeast, peptone, dextrose medium. The cells were collected by centrifugation and lysed with glass beads in lysis buffer (25 mM ammonium bicarbonate, pH 8.0, 0.5 mM EDTA, 1 mM PMSF). The urine and yeast protein mixtures described above were quantitated by Bradford method.

One-dimensional Electrophoresis

BSA (10 μg) and yeast lysate (20 μg) were mixed with the same volume of glycine loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol), heated at 100 °C for 3 min, cooled to room temperature, and loaded onto the gel. Electrophoresis was performed on 12% polyacrylamide, 100 × 80-mm slab gels with 0.75-mm spacers using Mini-Protean II. Human urinary proteins (10 μg) and two tryptic digested urinary peptide mixtures from microwave irradiation (Table I, Column 6) and standard (Table I, Column 3) digestion methods were mixed with Tricine loading buffer (125 mM Tris-HCl, pH 6.8, 100 mM DTT, 5% SDS, 0.1% bromphenol blue, 10% glycerol) and run on a 15% Tricine gel as described above.

A microwave-assisted Coomassie Blue staining protocol was followed (26). The gel was incubated in the microwave oven at 850 W for 1 min in the methanol/acetic acid fixative followed by 1-min staining in the staining solution (0.1% Coomassie Blue R-250 (w/v), 40% methanol, 5% acetic acid) and 3-min destaining in the destaining solution (40% methanol, 5% acetic acid).

Protein Digestion

In-solution Digestion—BSA was used as a model protein to study in-solution digestion. 1 mg of BSA was dissolved in 1 ml of ammo-
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| Protocol | Solution | Standard, sample 6 | MAPED, sample 7 |
|----------|----------|-------------------|-----------------|
| Reduce   | 10 mM DTT| h                 | 1               |
| Alkylate | 55 mM iodoacetamide | 0.5          | 0               |
| Dehydrate| 100% ACN | 0.5               | 0               |
| Rehydrate| 0.5 μM trypsin, 25 mM ammonium bicarbonate | 0.5         | 0               |
| Digest   | 0.5 μM trypsin, 25 mM ammonium bicarbonate | 16          | 5               |
| Extract  | 5% formic acid, 50% ACN | 0.5 × 2     | 5 × 2           |
| Total time |          | 19.5             | 25              |

All lyophilized samples were redissolved in 0.1% formic acid (buffer A) before ESI-MS analysis. For BSA samples, 100 fmol each of samples 1–5 and 300 fmol of samples 6 and 7 were loaded onto self-packed C18 RP capillary columns (100 mm × 0.17-mm inner diameter) with buffer A (0.1% formic acid, 99.9% H2O). The BSA peptides were eluted with 5–30% buffer B (0.1% formic acid, 99.9% ACN; flow rate, 2 μl/min) for 60 min. Each BSA sample was run three times. For urinary proteins, 100 μg of digested protein mixture was resolved using an on-line SCX capillary column (150 mm × 0.32-mm inner diameter) and RP capillary column (150 mm × 0.17-mm inner diameter). The ammonia acetate concentration of elution steps for the SCX capillary column were 0 mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, and 1 M. The elution gradient for the RP column was from 5 to 30% buffer B for 3 h. Yeast lysate peptides extracted from gels were separated by a self-packed C18 capillary column (150 mm × 0.17-mm inner diameter) with 5–30% elution gradient (buffer B) for 3 h.

Eluted peptides were detected in a survey scan from 400 to 1500 amu (three microscans) followed by five data-dependent MS/MS scans (five microscans each; isolation width, 3 amu; 35% normalized collision energy; dynamic exclusion for 3 min) in a completely automated fashion on an LCQ-DECA XP plus ESI mass spectrometer. All MS/MS spectra were respectively searched using SEQUEST algorithm-based Bioworks 3.1 SR1 (Thermo Finnigan) against a bovine database created from a non-redundant database, a yeast database from the National Center for Biotechnology Information website (ftp.ncbi.nih.gov/), and the International Protein Index human database (version 3.07) from the European Bioinformatics Institute website (www.ebi.ac.uk/IPI/). The search criteria used were those reported in Ref. 3.

According to previous reports (31–33), the number of identified MS/MS spectra for a protein can be used as the measure of that protein’s abundance. In this study the MS/MS spectra number from protein samples was used to evaluate the efficiency of each digestion protocol. For the same sample amount, the more MS/MS spectra identified, the higher the digestion efficiency of the protocol was. Paired t test was utilized to define the statistical significance between the identified MS/MS spectral numbers from the two protocols for the same sample amount. A level of p < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

In-solution Digestion

BSA In-solution Digestion—Fig. 1 (A–E) shows the MALDI-MS results of five BSA samples (samples 1–5). The mass spectrum of sample 1 was almost the same as that of sample 2, indicating that similar peptides were formed despite the approximately 100× difference in processing time. The results from sample 3 and sample 4 showed the same trend.
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A

B

C

D

E

F

G

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different from those of samples 1 and 2, there was no statistical significance in the MS/MS spectrum number for the three protocols. So the MAPED protocol could get the same digestion efficiency as the standard protocol without alkylation. Recently it was reported that proteins could be reduced in boiled water with DTT in 5 min (34), so we tried this method and digested BSA using the MAPED protocol for sample 4. The MALDI and ESI results of sample 4 were similar to those of sample 3, which provided an even more convenient sample preparation protocol.

Sample 5 was digested using the MAPED protocol without reduction and alkylation. The MALDI and ESI results showed that although BSA could be digested into peptide fragments the number of MS/MS spectra obtained by LC-ESI-MS was significantly lower than for the other four samples. One possible reason was protein partial enzymatic digestion. Without disulfide bond reduction, specific Lys or Arg residues might not be exposed and cleaved by trypsin, so the number of tryptic peptides decreased, and correspondently the number of identified MS/MS spectra was decreased. Another possible reason for the decrease might be the database-searching algorithm. SEQUEST identifies linear peptides from MS/MS spectra but not peptides linked together with a disulfide bond. So with the SEQUEST algorithm only a part of the digested peptide pool could be identified. Regardless even without reduction and alkylation a protein could at least be partially digested by trypsin with the MAPED protocol.

**Human Urinary Proteins In-solution Digestion**—Based on the BSA results the most convenient and fastest protocol was the fourth one (Table I, Column 6), which was to reduce proteins in boiled water and to digest using the MAPED protocol. As a protein mixture, human urinary proteins were used to retest the enzymatic digestion efficiency of the MAPED protocol described above.

Fig. 3 shows the one-dimensional Tricine gel results of human urinary proteins before and after trypsin digestion. With the MAPED protocol most of the urinary proteins higher than 14 kDa disappeared, and some bands appeared below 14 kDa. But around 31 kDa there were still a few bands, indicating that part of the protein pool had not been fully digested. With the standard protocol some bands appeared below 14 kDa, but there were much more intense bands higher than 14 kDa. So for protein mixtures the MAPED protocol seemed to obtain higher digestion efficiency than the standard protocol.

To further define the digestion efficiency, the two urine...
samples digested by the standard and MAPED protocols were analyzed by 2D LC-ESI-MS. The number of proteins identified using two or more tandem mass spectra were 153 for the standard protocol and 139 for the MAPED protocol, and the total numbers of MS/MS spectra were 2,333 and 2,541, respectively (detailed data are shown in the supplemental table). Although there was about an 8.5% difference in the identified MS/MS spectrum number for the two samples, there was not a statistical significance between them. So we conclude that the ESI-MS identification efficiency from the two protocols was similar. We repeated the above experiments, including sample digestion and one-dimensional Tricine gel and 2D LC-ESI-MS analysis but obtained similar results (data not shown). From the above results we concluded that the MAPED protocol could obtain digestion efficiency similar to that of the standard protocol.

In-gel Digestion

BSA In-gel Digestion—Previously a microwave technique was applied to trypsin in-gel digestion and showed high efficiency (18, 19). Whether it could be used for protein mixture in-gel preparation and digestion, especially for in-gel peptide extraction, was still unknown. In this study BSA was used to address this question. Fig. 1 (F and G) shows the MALDI results for BSA samples 6 and 7. The peptide peak patterns were almost the same, indicating that the digested peptide fragments from the two protocols were similar.

To evaluate the in-gel protein digestion and extraction efficiency for the two protocols, the two samples (samples 6 and 7) were analyzed by 1D LC-ESI-MS, and the results are shown in Fig. 2. It has been reported that with the standard in-gel digestion protocol only about 50% of total peptide could be recovered from the gel (35). According to previous analysis (31), the identified MS/MS spectrum number from 300 fmol of BSA by 1D LC-ESI-MS should be about 20. In this study, with the standard protocol an average 11 spectra (55%) were extracted from the gel, similar to the previous report (35).
the MAPED protocol about 14 spectra (70%) were recovered from the gel. There was statistical significance between the results from the two samples \( (p < 0.05) \), so we concluded that the MAPED protocol peptide extraction efficiency from the gel was higher than that of the standard protocol.

**Yeast Lysate In-gel Digestion**—Further experiments with yeast lysate were used to prove the in-gel peptide extraction and digestion efficiency of MAPED protocol for protein mixtures. Five continuous bands of yeast lysate (Fig. 4) were cut, in-gel prepared, digested, and resolved by 1D LC-ESI-MS. The identified proteins with more than three MS/MS spectra are shown in Table III. The number of proteins identified with MAPED versus the standard protocol was approximately the same for bands 1–3 and higher in the case of bands 4 and 5. The number of identified MS/MS spectra obtained with the MAPED protocol was also more than that with the standard protocol (about 96, 200, 29, 188, and 29% for the five bands, respectively). These results indicate that the MAPED in-gel protocol could significantly improve peptide recovery efficiency from gel for protein mixtures compared with standard in-gel digestion protocols.

### Conclusion

In this study we developed a MAPED protocol for protein preparation and digestion useful for both in-solution and in-gel digestion of protein. The in-solution MAPED protocol enabled protein mixtures to be prepared and digested in 6 min and showed protein digestion efficacy similar to that using standard protocols. Using the in-gel MAPED protocol, the total time for protein mixture preparation, digestion, and peptide extraction could be shortened to 25 min, and higher peptide recovery efficiency could be achieved than with the standard protocol.

For large scale clinical proteomic research one of the important tasks is to prepare and digest tens or hundreds of protein samples. Present standard sample preparation and digestion protocols are labor-intensive and time-consuming. The MAPED protocol we present here simplifies the sample preparation and digestion procedure and will be helpful for the application of proteomics to biological clinical research.

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**TABLE III**

The identified proteins from five yeast lysate bands by 1D LC-ESI-MS with standard and MAPED in-gel preparation, digestion, and peptide extraction protocols

| GI no. | Protein name                      | Molecular mass kDa | pl   | Number of MS/MS spectra |
|--------|-----------------------------------|--------------------|------|-------------------------|
| Band 1 |                                   |                    |      |                         |
| 6319279| Pyruvate kinase                   | 54.9               | 7.57 | 22                      |
| 6332073| Pyruvate decarboxylase            | 61.7               | 5.76 | 4                       |
| 6321631| Glyceraldehyde-3-phosphate dehydrogenase 3 | 35.9 | 6.51 | 2                        |
| Band 2 |                                   |                    |      |                         |
| 6321693| Enolase I                         | 46.9               | 6.16 | 3                       |
| 6321631| Glyceraldehyde-3-phosphate dehydrogenase 3 | 35.9 | 6.51 | 1                        |
| Band 3 |                                   |                    |      |                         |
| 6321693| Enolase I                         | 46.9               | 6.16 | 17                      |
| 6321968| Enolase                           | 47.0               | 5.61 | 9                       |
| 6323333| O-Acetylimoseringer-O-acetylseryl sulfhydrolase | 48.7 | 5.97 | 9                        |
| Band 4 |                                   |                    |      |                         |
| 6324486| Alcohol dehydrogenase             | 37.3               | 6.22 | 3                       |
| 6321693| Enolase I                         | 46.9               | 6.16 | 2                       |
| 6323073| Pyruvate decarboxylase            | 61.7               | 5.76 | 2                       |
| 6321631| Glyceraldehyde-3-phosphate dehydrogenase 3 | 35.9 | 6.51 | 1                        |
| 6319279| Pyruvate kinase                   | 54.9               | 7.57 | 0                       |
| 6319314| Heat shock protein of HSP70 family, cytoplasmic | 69.9 | 4.84 | 0                        |
| Band 5 |                                   |                    |      |                         |
| 6324486| Alcohol dehydrogenase             | 37.3               | 6.22 | 12                      |
| 6323961| Alcohol dehydrogenase II          | 37.2               | 6.28 | 6                       |
| 6321693| Enolase I                         | 46.9               | 6.16 | 3                       |
| 6321631| Glyceraldehyde-3-phosphate dehydrogenase 3 | 35.9 | 6.51 | 3                        |
| 6321968| Enolase                           | 47.0               | 5.61 | 0                       |

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