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# A rapid protein sample preparation method based on organic-aqueous microwave irradiation technique

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Fast and efficient sample preparation methods are a prerequisite for protein identification in bottom-up proteomics. Here, an innovative microwave irradiation sample preparation method was developed based on an optimized organic-aqueous solvent system for protein identification. Specifically, protein solutions containing high-concentration acetonitrile were subjected to 5 min microwave irradiation. After cooling down, trypsin was added and the digestion was performed with 30 s microwave irradiation, and the resulting peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). A shortened processing time of only 5.5 min is needed with this method (more than 12 h is necessary in the traditional overnight protein sample preparation). Moreover, due to the absence of urea and other chaotropic reagents, the digests can be readily identified by MALDI-TOF MS. When an assessment of this method was performed by digesting a model protein BSA, 69% ± 3% sequence coverage corresponding to 47 ± 3 peptides was obtained, which shows better protein identification than that from the standard overnight protein sample preparation method (51% ± 2% sequence coverage and 23 ± 1 peptides). Another model protein α-casein was used for the analysis of protein phosphorylation with the newly developed method that yielded 4 phosphopeptides with 8 phosphorylation sites, whereas 3 phosphopeptides with 2 phosphorylation sites were obtained from the traditional overnight approach. Moreover, the organic-aqueous microwave irradiation method provides effective digestion for proteins down to fmol.

**MALDI-TOF MS, microwave, organic-aqueous solvent, protein identification, digestion**

## 1 Introduction

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) [1] is achieving a more significant role for protein identification due to its simplicity, excellent mass accuracy, high resolution, and sensitivity. Considering the complexity of proteins, efficient sample preparation is of fundamental importance in enabling successful MALDI-TOF MS detection [2–5]. The standard protein sample preparation method for protein identification at the peptide level in bottom-up proteomics involves protein denaturation (e.g., with 8 mol/L urea), reduction of disulfide linkages, alkylation, and protein digestion (usually overnight tryptic proteolysis). However, it suffers from several drawbacks. First, the relatively lengthy pretreatment time hampers the throughput of sample preparation [6,7]; second, potential sample loss can result from the removal of salts, that are introduced in sample preparation, which is often required prior to MALDI-TOF MS detection. Therefore, it is desirable to develop a high-throughput and clean preparation method for MALDI-TOF MS-based protein identification in bottom-up proteomics.

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To improve protein digestion throughput, we adopted a variety of methods, including microwave [8–11], infrared [12,13], laser [14], ultrasonication [15], and high pressure [16] to afford increased contact frequency between enzymes and substrates and thus to largely shorten the digestion time. Microwave-assisted digestion seems to be particularly promising. Microwaves, an electromagnetic radiation occupy the electromagnetic spectrum between infrared and radio waves. As a heating method, using convenient and cheap equipment, microwave irradiation can expedite the digestion reaction via increasing molecular movement. However, because long microwave-assisted digestion times deactivate proteases, it is important to shorten the microwave irradiation time when most highly accelerating the digestion reaction. To date, various microwave-based sample-preparation techniques have been established for the routine analysis of proteins [17–21]. For example, Sun *et al*. [17] demonstrated a 6-min microwave protein-preparation method for bovine serum by using 5-min dithiothreitol reduction in boiled water and 1-min trypsin digestion. Adequate care should be taken with this method, however, because since proteins might be irreversibly aggregated during thermal denaturation.

In addition, beneficial effects of organic solvent-assisted protein-sample preparation have been reported [21–27] based on the fact that organic solvent can be easily removed by lyophilization instead of desalting; this renders a clean sample preparation and can reduce sample loss. Moreover, an organic-aqueous solvent system often results in more-efficient trypsin digestion [26,28] than a solvent system without organic solvents. Strader *et al*. [26] evaluated different organic solvents for effective protein identification. In their study, 1 h digestion using a solvent system containing 80% acetonitrile provided the most complete protein digestion. Lin *et al*. [28] evaluated different organic solvent systems using several model proteins (myoglobin, cytochrome c, lysozyme, and ubiquitin) for trypsin digestion.

To establish a rapid protein-sample preparation method for targeted protein identification in bottom-up proteomics, we combined the organic-aqueous solvent system with the microwave irradiation technique, followed by MALDI-TOF MS analysis. In this study, we optimized the experimental conditions (e.g., the dithiothreitol reduction in organic-aqueous solvent and the microwave irradiation time) for protein sample preparation. With this approach, the complete process (i.e., denaturation, reduction, and digestion) could be completed within 5.5 min.

## 2 Experimental

### 2.1 Chemicals and reagents

Acetonitrile (ACN, HPLC-grade) was purchased from Merck (Darmstadt, Germany). AR-grade formic acid (FA) and AR-grade trifluoroacetic acid (TFA) were from Kermel (Tianjin, China). Myoglobin (horse), BSA (bovine), α-casein (bovine milk), apo-transferrin (bovine), and trypsin were all purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Acros (Morris Plains, NJ, USA). Urea was purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2 Instrumentation

A microwave oven (2.5 GHz, Midea, China) was used to perform the microwave irradiation. MALDI-TOF MS experiments were performed on an Ultraflex III MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany).

### 2.3 Standard overnight protein-sample preparation method

Proteins (1 mg) denatured by 100 μL 8 mol/L urea, and 20 μL DTT (100 mmol/L) was added for the reduction of the disulfide bonds at 60 °C for about 1 h. Afterward, 20 μL IAA (200 mmol/L) was added for alkylation, followed by dilution. Trypsin digestion was carried out by adding trypsin into the protein solution (enzyme-to-protein ratio = 1:25, *w*/*w*) at 37 °C for 12 h.

### 2.4 Aqueous microwave irradiation protein sample preparation method

Proteins (1 mg) were pretreated as described in the standard overnight digestion section before trypsin digestion. Then trypsin was mixed with the protein solution at room temperature for 30 s microwave irradiation (enzyme-to-protein ratio = 1:25, *w*/*w*).

### 2.5 Organic-aqueous microwave irradiation protein sample preparation method and MALDI-TOF analysis

Proteins (0.1 mg) were dissolved in ammonium bicarbonate solution containing 80% ACN (*v*/*v*) [26]. Next, 100 μL of this protein solution was added to 0.2 mL 100 mmol/L DTT. Afterward, 5 min microwave irradiation was performed [17] for the reduction of the disulfide bonds. Subsequently, 4 μL trypsin solution (100 ng/μL) was added into the solution followed by vortex and sonication for 10 s each. Finally, the solution was irradiated by microwave for different lengths of time (5, 10, 15, 30, 60 or 90 s), directly followed by MALDI-TOF MS identification. The standard MALDI-TOF analysis procedure was adopted according to the manufacturer's manual. Briefly, external calibration of MALDI-TOF MS was performed with 10 commercial standard peptides. Spectra were acquired from the accumulation of 1000 laser shots. The voltage was set as follows: acceleration, 21.85 kV; lens, 9.2 kV; reflector 126.39 kV; reflector 214.0 kV. A 7 mg/mL CHCA in 70% (*v*/*v*) aqueous ACN with 0.1% (*v*/*v*) TFA solution was prepared. The sample and matrix solution

were mixed with 1:1 (*v*/*v*) in a 300 μL Eppendorf tube, and 0.5 μL of the mixed solution was deposited onto a MALDI target plate.

## 3 Results and discussion

### 3.1 Organic-aqueous microwave irradiation protein-sample preparation method

Initially, myoglobin was used to evaluate the performance of the organic-aqueous microwave irradiation protein sample preparation method. Due to the absence of disulfide linkages in this protein, reduction via DTT was unnecessary. Instead, 0.1 mg/mL myoglobin solution was directly subjected to microwave irradiation and then the peptide was analyzed by MALDI-TOF MS. Figure 1 illustrates the sequence coverage changes with the increase of microwave irradiation time from 5 to 90 s.

Intriguingly, myoglobin was well digested and positively identified within a treatment of 5 s (average sequence coverage > 85%). However, we found that myoglobin protein peaks in the MALDI-TOF MS spectrum within a treatment of 5 s. An even better digestion performance, nearly 100% identification (96% ± 3% sequence coverage and 18 ± 1 unique peptides) by MALDI-TOF MS, was achieved at 30 s (Table 1).

With this method, microwave irradiation was carried out in 30 s for trypsin digestion. By contrast, myoglobin was also prepared via the standard overnight sample-preparation method and aqueous microwave irradiation protein-sample preparation method (Table 1). Results indicated that simultaneous denaturation and digestion of myoglobin could occur in the organic-aqueous microwave irradiation protein-sample preparation method and that this would lead to more-efficient digestion before apparent aggregation upon denaturation. Increased solubility of the denaturized proteins and the resulting peptides was an important factor in the improved digestion performance of the organic-aqueous microwave irradiation protein-sample preparation method.

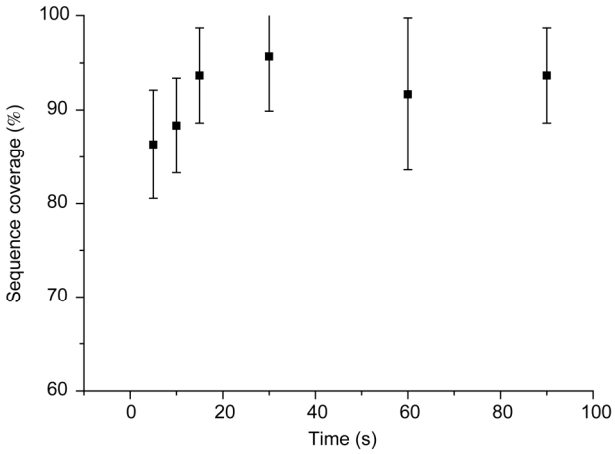


**Figure 1** Effect of microwave irradiation time (output power, 700 W) on the sequence coverage of myoglobin (300 fmol). Triplicate spots were analyzed by MALDI-TOF MS.

**Table 1** MASCOT searching results of the tryptic digests of 100 ng/μL myoglobin obtained from three protein sample preparation methods and MALDI-TOF MS identification

| | Peptide sequence | Microwave + organic-aqueous | Overnight | Microwave + aqueous |
|---|---|---|---|---|
| Position | | | | |
| 2–32 | M.GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR.L | √ | √ | √ |
| 18–32 | K.VEADIAGHGQEVLIR.L | √ | √ | √ |
| 33–43 | R.LFTGHPETLEK.F | √ | √ | |
| 33–48 | R.LFTGHPETLEKFDKFK.H | √ | √ | √ |
| 49–57 | K.HLKTEAEMK.A | √ | | |
| 58–78 | K.ASEDLKKHGTVVLTALGGILK.K | √ | √ | |
| 65–78 | K.HGTVVLTALGGILK.K | √ | √ | |
| 65–79 | K.HGTVVLTALGGILKK.K | √ | √ | |
| 65–80 | K.HGTVVLTALGGILKKK.G | √ | | |
| 79–97 | K.KKGHHEAELKPLAQSHATK.H | √ | √ | |
| 80–97 | K.KGHHEAELKPLAQSHATK.H | √ | √ | |
| 81–97 | K.GHHEAELKPLAQSHATK.H | √ | √ | |
| 98–119 | K.HKIPIKYLEFISDAIIHVLHSK.H | √ | √ | |
| 120–140 | K.HPGDFGADAQGAMTKALELFR.N | √ | | |
| 135–146 | K.ALELFRNDIAAK.Y | √ | √ | √ |
| 135–148 | K.ALELFRNDIAAKYK.E | √ | √ | |
| 141–154 | R.NDIAAKYKELGFQG.- | √ | | |
| 147–154 | K.YKELGFQG.- | √ | √ | √ |
| Digestion time | | 30 s | 12 h | 30 s |
| Peptide number | | 18 | 14 | 5 |
| Sequence coverage | | 99% | 83% | 43% |
| MASCOT score | | 170 | 131 | 51 |

In addition, due to the benefits of the absence of urea, which would interfere or suppress the ion signals of analytes in MALDI-TOF MS detection, a better crystal could be achieved with this newly developed approach. BSA containing 17 disulfide bond, was prepared as same as myoglobin, but with the inclusion of a 5-min disulfide reduction step.

As shown in Table 2, the reduction significantly improved the protein digestion by increasing the number of matched peptides 35 ± 3 to 47 ± 3 peptides), the corresponding sequence coverage (34% ± 2% to 69% ± 3%), and the MASCOT score (165 ± 8 to 311 ± 23). In the organic-aqueous microwave irradiation sample preparation method, no additional cysteine alkylation was required because the proteins were promptly digested by trypsin under microwave irradiation treatment. To further demonstrate the advantages of the organic-aqueous microwave irradiation method, other methods were also tested as described in the experimental section.

As shown in Figure 2, poor absolute-signal intensity was observed even after accumulation up to 1000 laser shots for BSA peptides obtained from the aqueous microwave irradiation method. Although BSA with the organic-aqueous microwave irradiation method gave a similar MALDI-TOF MS signal intensity as the conventional overnight sample-preparation method, a ten folds higher signal was achieved than with the aqueous microwave irradiation protein sample preparation method. Moreover, the identification result demonstrates that the organic-aqueous microwave irradiation method was superior to other methods (Table 2). More important, the time of protein sample preparation was shortened to 5.5 min, which means that the throughput of protein digestion was significantly improved over the traditional overnight protein-sample preparation method (~13.5 h) and the whole aqueous microwave method (> 1.5 h).

### 3.2 Model proteins analysis

A model protein for examining protein phosphorylation, α-casein, was digested to further evaluate the utility of the superior digestion performance of the organic-aqueous microwave irradiation method. Figure 3(a) shows the MALDI-TOF MS spectra of α-casein digests. Sequence coverage of 61% ± 0% was obtained and 17 ± 0 peptides were identified. As a control, α-casein was also prepared using the traditional overnight protein sample preparation method; only 13 ± 1 peptides were identified with 48% ± 1% sequence coverage (Figure 3(b)). Moreover, the organic-aqueous microwave irradiation method yielded 4 phosphopeptides including 8 phosphorylation sites from tryptic digest of α-casein via MALDI-TOF MS detection (the sequences were FFVAPFPEVFGKEKVNELSpK, QMEAESpISpSpSpEEIVPNSpVEQKHIQK, KYKVPQLEIVPNSpAEER, and YKVPQL-

**Table 2** MASCOT search results of the tryptic digestions of 100 ng/μL BSA obtained from three protein-sample preparation methods and MALDI-TOF MS identification

| Methods | Microwave + organic-aqueous | Overnight | Microwave + aqueous |
|---|---|---|---|
| | BSA | BSA | BSA |
| Peptide number | 47 ± 3 | 23 ± 1 | 29 ± 0 |
| Sequence coverage | 69% ± 3% | 51% ± 2% | 43% ± 0% |
| MASCOT score | 311 ± 23 | 108 ± 5 | 181 ± 6 |

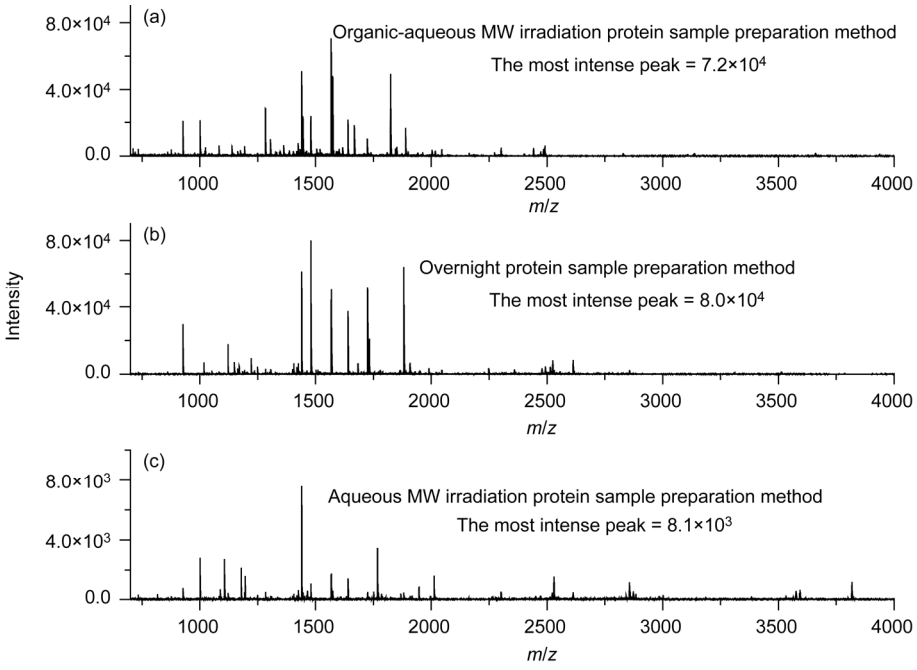


**Figure 2** MALDI-TOF MS spectra for the analysis of tryptic BSA digest via three methods. (a) Organic-aqueous microwave irradiation method; (b) overnight sample preparation method; (c) aqueous microwave irradiation sample preparation method. MW = microwave.

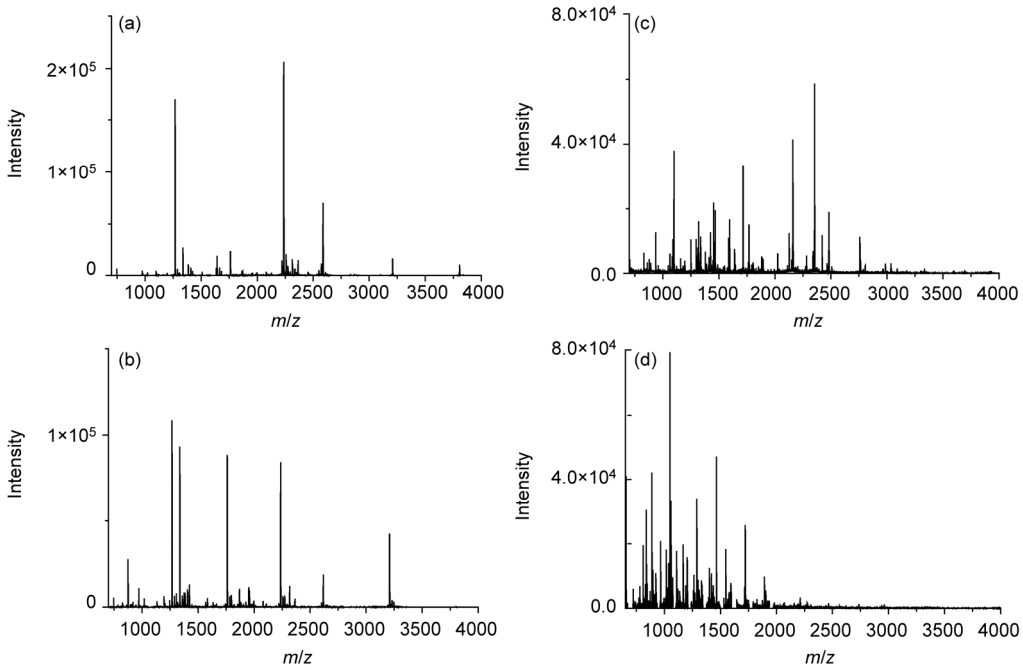


**Figure 3** MALDI-TOF MS spectra obtained from the digest of α-casein (a, b) and apo-transferrin (c, d) via the organic-aqueous microwave irradiation protein sample preparation method (a, c) and the overnight method (b, d).

EIVPNSpAEER, respectively), while only 3 phosphopeptides with 2 phosphorylation sites were obtained from the overnight protein sample preparation method. Apo-transferrin, a well-studied protein consisting of 704 amino acids with a molecular mass of 79 kDa, was also used to further evaluate our method. Figure 3(c, d) illustrates the MALDI-TOF MS spectra of apo-transferrin digests from the newly developed method and the conventional overnight protein sample preparation method. The developed method showed better digestion performance than the overnight protein sample preparation method (unique peptide number, 56 ± 3 vs. 34 ± 0 and sequence coverage, 66% ± 4% vs. 37% ± 2%).

### 3.3 Analysis of low-concentration proteins

Protein digestion is usually affected by the substrate concentration in a given sample; low abundance proteins usually cannot be completely digested. With these conditions in mind, 10 ng/μL myoglobin (300 fmol) and BSA (70 fmol) were prepared with the newly developed method, and the peptides were detected by MALDI-TOF MS (Figure 4). Positive identification was achieved even with an amount

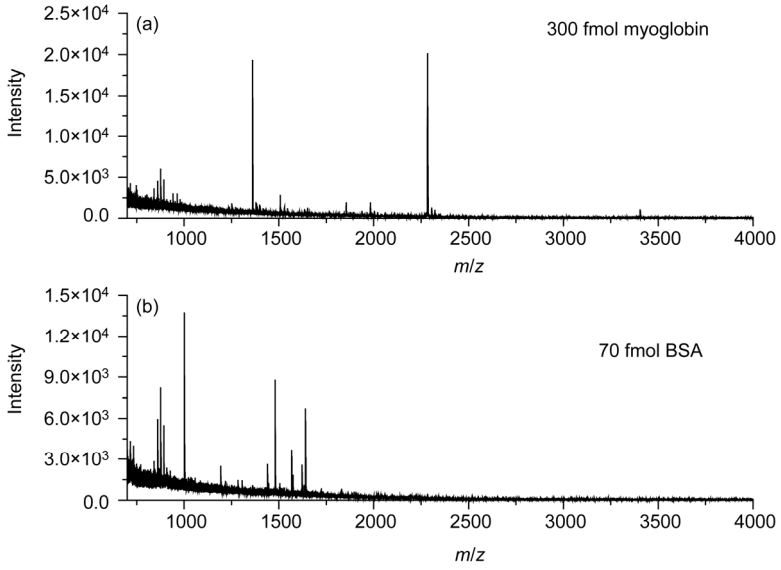


**Figure 4** MALDI-TOF MS spectra of tryptic peptides derived from the digestion of 10 ng/μL myoglobin (a) and 50 μL BSA (b) via the organic-aqueous microwave irradiation protein sample preparation method.

down to fmol, in yields of 49% ± 0% (5 ± 0 unique peptides) and 12% ± 0% (6 ± 0 unique peptides) sequence coverage for myoglobin and BSA, respectively. These results demonstrated that the organic-aqueous microwave irradiation protein sample preparation method could provide effective digestion for low-concentration proteins.

## 4 Conclusions

An organic-aqueous microwave irradiation protein sample preparation method coupled with MALDI-TOF MS analysis was developed successfully for rapid protein identification in bottom-up proteomics. The entire process, including protein denaturation, reduction of disulfide bonds, and trypsin digestion, was shortened to 5.5 min. Compared to the traditional overnight digestion and aqueous microwave irradiation, the new method provides better digestion performance in terms of the peptide number and sequence coverage. The ease, simplicity, efficiency and low cost are promising for its wider applicability even for complex samples.

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