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Alternating current-assisted on-plate proteolysis for MALDI-TOF MS peptide mapping

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In this report, alternating current-assisted on-plate proteolysis has been developed for rapid peptide mapping. Protein solutions containing trypsin were allowed to digest directly on the spots of a stainless steel MALDI plate with the assistance of low-voltage alternating current electricity. Alternating current (AC) was allowed to pass through the protein solutions *via* the MALDI plate and a platinum disc electrode. The feasibility and performance of the novel proteolysis approach were investigated by the digestion of BSA and cytochrome *c* (Cyt-*c*). It was demonstrated that AC substantially enhanced the efficiency of proteolysis and the digestion time was significantly reduced to 5 min. The digests were identified by MALDI-TOF MS with sequence coverages of 42% (BSA) and 77% (Cyt-*c*) that were comparable to those obtained by using conventional in-solution tryptic digestion. The present proteolysis strategy is simple and efficient, offering great promise for MALDI-TOF MS peptide mapping.

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Nowadays, more and more research attention is being paid to proteomics because it gives a much better understanding of an organism than genomics. Proteomics is the large-scale study of proteins, particularly their structures and functions. One of its most important tasks is to develop efficient and rapid approaches to identifying various proteins. Protein digestion is an important procedure prior to MS identification. Because the conventional in-solution digestion of proteins is time-consuming [1, 2], it is of high importance to develop novel approach to achieve a highly efficient proteolysis for MS-based peptide mapping.

MALDI-TOF MS has been widely used in protein chemistry and proteomics research for the identification of proteins. Protein samples were usually digested into peptides with proteases. Subsequently, the obtained digests were deposited on MALDI plates to perform MS measurements. To simplify the analysis process, on-plate proteolysis approaches were developed by combining digestion and spotting into one procedure [3]. Trypsin was usually immobilized on the spots of the plate by adsorption or covalent linking [4]. Protein samples were then deposited on the trypsin-immobilized spots and were allowed to digest in humidified enclosures with the aid of heat. Because a high amount of trypsin was immobilized on the plate, the typical time of the on-plate protein digestion was significantly reduced to 5−30 min. In the case of conventional in-solution tryptic digestion, the autolysis of protease would generate interfering fragments. A low weight ratio (typically 1:20−1:100) between trypsin and protein was usually employed and resulted in long digestion time (typically 12 h at 37°C). It is a challenging task to enhance the digestion efficiency of the in-solution protein digestion that is performed directly on MALDI plates.

Recently, microwaves were employed to enhance the efficiency of conventional in-solution proteolysis significantly [5, 6]. The typical digestion time of microwave-assisted proteolysis was in the range of 5 to 20 min. Moreover, it was

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Abbreviations: AC, alternating current; **Cyt-*c***, cytochrome *c*

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reported that ultrasonic waves could also accelerate the conventional in-solution digestion of proteins and the digestion time was reduced to 1 min [7]. More recently, we have employed infrared radiation as an energy source to promote tryptic proteolysis and the digestion could complete within 5−10 min [8]. Usually, alternating current (AC) refers to the form in which electricity is delivered to businesses and residences. Its amplitude and polarity vary in a succession of identical cycles to form a sine wave. The major advantage that AC electricity has over direct current is that AC voltages can be transformed to higher or lower voltages by using a transformer. As the most commonly used electricity, AC has also found a wide range of biomedical applications, such as AC conductimetric biosensors [9], electroacupuncture [10], tumor therapy [11], physiotherapy [12], *etc*. However, we are not aware of early reports on AC-assisted proteolysis. It is of high interest to demonstrate the possibility of employing alternating current to enhance the efficiency of conventional in-solution proteolysis.

In this work, protein solutions containing trypsin were allowed to digest directly on the spots of a stainless steel MALDI plate with the assistance of low-voltage AC at room temperature (~25°C). As illustrated in Fig. 1B, AC was allowed to pass through the protein solutions *via* the MALDI plate and a platinum disc electrode. The AC-assisted proteolysis approach has been coupled with MALDI-TOF MS for the digestion and peptide mapping of BSA and cytochrome *c* (Cyt-*c*) from horse heart.

The stock solutions (1 mg/mL) of BSA and Cyt-*c* were prepared in 10 mM NH_4HCO buffer solution (pH 8.1) and were denatured in a 95°C water bath for 15 min. As shown in Fig. 1, the sample solution of each protein was digested directly on the spots of the MALDI plate with the aid of alternating current. Before digestion, the stock solutions of BSA and Cyt-*c* were each diluted to 20 ng/μL with 10 mM NH_4HCO_3 buffer solution (pH 8.1) containing 0.5 ng/μL trypsin. A volume of 1 μL of each diluted protein solution was deposited on the spots of a MALDI plate (Fig. 1A). Then, the sample solution on the spots was sandwiched between the MALDI plate and the surface of a platinum disc electrode (0.5-mm diameter) with the aid of a 3-D micromanipulator (Olympus, Tokyo, Japan). The surface of the platinum electrode should touch the top of the solution and its distance to the MALDI plate was adjusted to be ~1 mm. Subsequently, an AC voltage with the peak-to-peak voltage ($V_{p\text{-}p}$) of 5 V was applied between the MALDI plate and the platinum disc electrode (Fig. 1B). The current flowed through the protein solution could be monitored with a multimeter. Its value was in the range of 1.0 to 1.2 mA. The protein samples were allowed to digest in the alternating electric field for 5 min except mentioned otherwise. The low-voltage AC employed in this work was supplied by a laboratory-made multi-output transformer (E-type, 10 W) that could convert commercial AC power (220 V, 50 Hz) into lower AC voltages (2.5, 5, 7.5, and 10 V, 50 Hz).

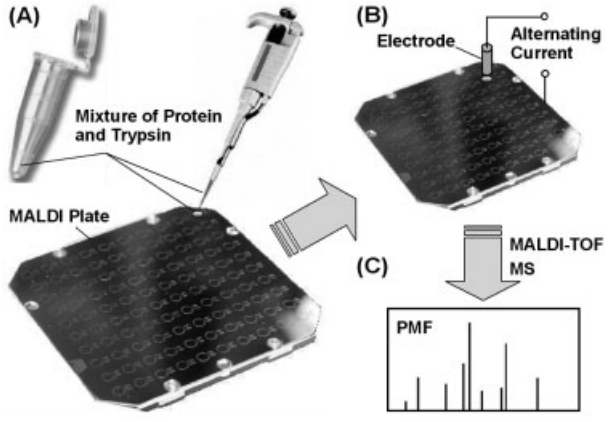

Figure 1. Schematic diagram showing the process of alternating current-assisted on-plate proteolysis. (A) Depositing the mixture of protein and trypsin on MALDI plate; (B) applying AC voltage to the drops of the sample solution on MALDI plate to accelerate the digestion; (C) MALDI-TOF MS peptide mapping.

For comparison, BSA and Cyt-*c* (20 ng/μL each) in 10 mM NH_4HCO_3 buffer (pH 8.1) were also digested by using conventional in-solution proteolysis in a 37°C water bath for 12 h. The weight ratio between trypsin and protein substrate was 1:40. The obtained digests were further identified by MALDI-TOF MS. All MS experiments were performed in positive ion mode using a 4700 proteomics analyzer (Applied Biosystems, Framingham, MA). Prior to MALDI-TOF MS analysis, a volume of 0.5 μL of each sample solution obtained by using conventional in-solution digestion was spotted on a MALDI plate. However, the present on-plate digestion obviated the spotting of the digests because digestion was directly performed on the spots of plate. After the solvent evaporated, 0.5 μL of the matrix solution (4 mg/mL CHCA dissolved in 50% aqueous ACN containing 0.1% TFA was dropped on the dried samples. The MS instrument was operated at an accelerating voltage of 20 kV. A 200-Hz pulsed Nd:YAG laser at 355 nm was used. Prior to measurements, the MS instrument was calibrated with the tryptic digest of myoglobin by using an internal calibration mode. GPS Explorer software from Applied Biosystems with MASCOT as a search engine and Swiss-Prot as a database were used to identify proteins. The search was done based on the monoisotopic MH^+ mass values of peptides. The peptide mass tolerance was set to ±100 ppm and the missed cleavages of peptides were allowed up to 1.

Figure 2 displays the PMF spectra of the tryptic digests of 20 ng/μL BSA and 20 ng/μL Cyt-*c* obtained by using AC-assisted proteolysis. The protein samples were digested and positively identified. The identified peptides in the digests of BSA and Cyt-*c* were presented in Tables 1 and 2 in Supporting Information, respectively. A total of 31 and 11 tryptic peptides were matched with the amino-acid sequence coverage of 42 and 77% for BSA and Cyt-*c*, respectively. The results indicated that 260 out of the 607 possible amino acids of BSA and 81 out of the 104 possible amino acids of Cyt-*c* were identified (Table 3 in Supporting Information). In addition,

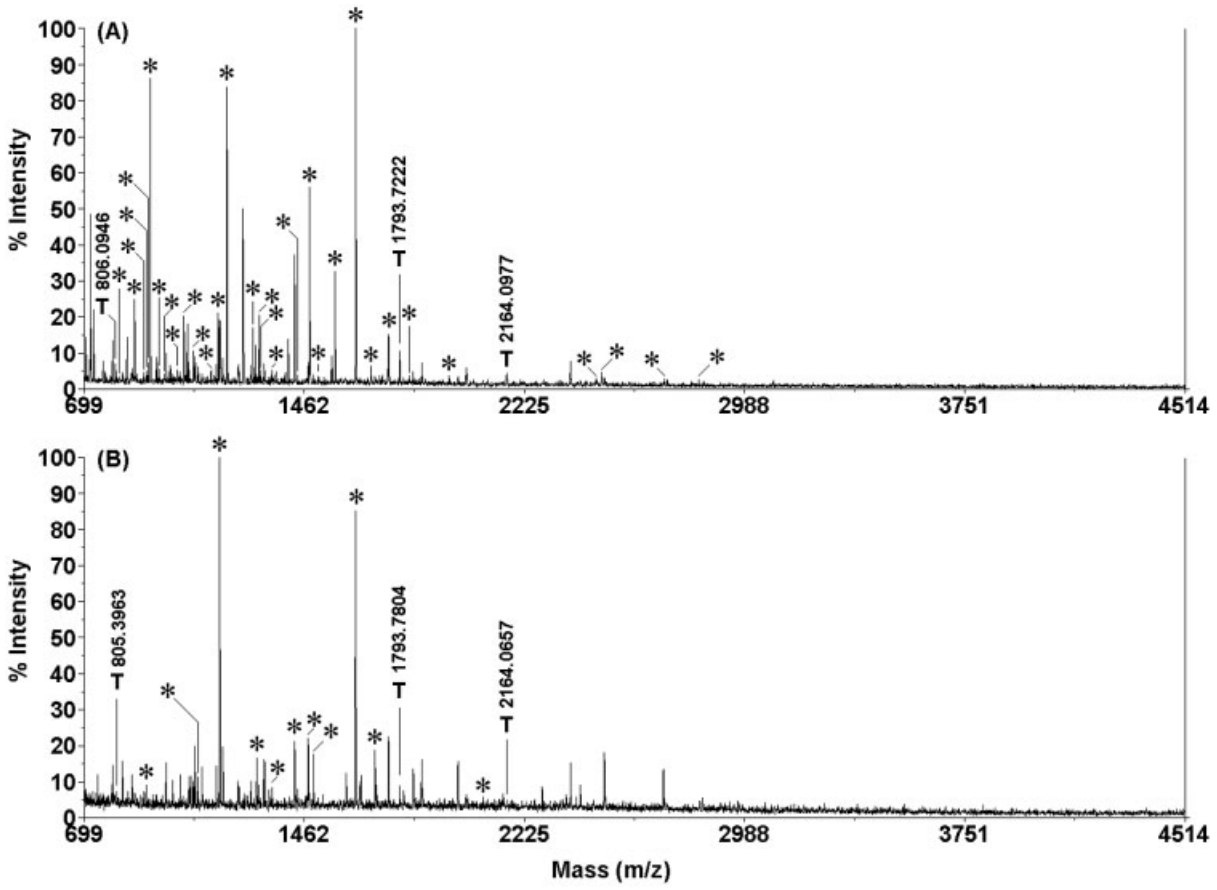

Figure 2. MALDI-TOF mass spectra of the digests of 20 ng/μL BSA (A) and 20 ng/μL Cyt-*c* (B) in 10 mmol/L NH_4HCO_3 buffer solution (pH 8.1) obtained by using AC-assisted on-plate proteolysis (trypsin/substrate ratio, 1:40; digestion time, 5 min). Matched peptides and trypsin autolysis fragments were marked with "*" and "T", respectively.

the MALDI-TOF mass spectra of both digests were also measured over an extended *m/z* range (Fig. 1 in Supporting Information). No peak of their parent proteins was found, indicating the proteins in the sample solutions were digested almost completely.

Figure 2 in Supporting Information illustrate the MALDI-TOF mass spectra of the digests of 20 ng/μL BSA and 20 ng/μL Cyt-*c* obtained by using 12-h conventional in-solution digestion. All matched peptides were presented in Tables 1 and 2 in Supporting Information. The results indicated that 21 and 10 peptides were matched with the amino-acid sequence coverage of 33% (identified amino acids, 201) and 76% (identified amino acids, 80) for BSA and Cyt-*c*, respectively. Table 3 in Supporting Information summarizes the MALDI-TOF MS results of the digests obtained by using different digestion approaches. The identification results obtained by AC-assisted proteolysis were comparable to those based on conventional in-solution digestion. More importantly, the digestion time was significantly reduced from 12 h for in-solution digestion to 5 min for the present alternating current-based digestion. In addition, the results indicated that the total numbers of miscleavages in the matched peptides increased from 10 to 17 (BSA) and from 7 to 8 (Cyt-*c*) when AC-assisted proteolysis was employed instead of conventional in-solution digestion (Table 3 in Supporting Information). It might be attributed to the fact that the digestion time of AC-assisted digestion (5 min) was very short [3].

To investigate the effect of peak-to-peak voltage on proteolysis, a sample of 20 ng/μL Cyt-*c* in 10 mM NH_4HCO_3 buffer solution (pH 8.1, containing 0.5 ng/μL trypsin) was digested on plate with the assistances of alternating currents at different peak-to-peak voltages. The tryptic digests were then analyzed with MALDI-TOF MS. At the peak-to-peak voltages of 0, 2.5, 5, and 7.5 V, the determined sequence coverage of the digests were 28, 62, 77, and 77%, respectively. Upon raising the peak-to-peak voltage from 0 to 5 V, the sequence coverage significantly increased from 28 to 77%. When $V_{p\text{-}p}$ exceeded 5 V, no increase in sequence coverage was found. In addition, a sample of 20 ng/μL Cyt-*c* in 10 mM NH_4HCO_3 buffer solution (pH 8.1, containing 0.5 ng/μL trypsin) was also digested on plate with the assistances of

alternating current at the peak-to-peak voltage of 5 V for 2.5, 5, 7.5, and 10 min. After each digestion, 0.5 μL of 0.5% TFA aqueous solution was added to the sample solution on the plate to stop the digestion process for subsequent MALDI-TOF MS measurements. Upon raising the digestion time from 2.5 to 5 min, the sequence coverage increased from 52 to 77%. However, no significant increase in the coverage was observed when the time was longer than 5 min although the absolute peak strength of PMF spectra increased to some extent.

Temperature is of crucial importance for tryptic proteolysis. In this work, a sample of 20 ng/μL Cyt-*c* in 10 mM NH_4HCO_3 buffer solution (pH 8.1, containing 0.5 ng/μL trypsin) was digested with the assistance of alternating current at 15, 25, 35, and 45°C. The temperature of the MALDI plate was controlled by a water bath. The voltage between the plate and the platinum disc electrode was 5 V. The MALDI-TOF-MS spectra of the digests were illustrated in Fig. 3 in Supporting Information. When the temperature increased from 15 to 25°C, the sequence coverage of Cyt-*c* increased rapidly from 50 to 77%. No increase in the coverage was found at higher temperatures.

The reproducibility of the present method was examined from a series of eight repetitive digestions of a 20 ng/μL Cyt-*c*. Based on the obtained eight PMF spectra (not shown), the average sequence coverage of Cyt-*c* was measured to be 75% with an RSD of 3.6%, indicating the satisfactory reproducibility of the present AC-assisted digestion approach.

The significantly enhanced digestion efficiency of the present proteolysis approach could be attributed to the assistance of alternating current. In this work, proteins were digested in 10 mM NH_4HCO_3 buffer solution (pH 8.1). Based on p*I*, trypsin (p*I* 10.1−10.5 [13]) and Cyt-*c* (p*I* 10.0–10.5 [14] carried net positive charges while BSA (p*I* 4.7 [15]) was negatively charged at pH 8.1. As illustrated in Fig. 4 in Supporting Information, both the migration directions of charged species (including proteins, trypsin, inorganic ions, etc.) and the orientations of dipoles (including proteins, trypsin, water, *etc.*) would change 100 times per second under the Coulomb force in the alternating electric field (50 Hz), resulting in the vibrations and rotations of molecules and the collisions and frictions of molecules in solutions. All these molecule movements could increase the frequency of the interaction between trypsin and protein molecules so that the digestion efficiency was enhanced. In addition, the alternating electric field also exerted alternating Coulomb force on the positively or negatively charged groups in protein molecules and led to the vibrations of peptide bonds. These vibrations could lead to more cleavage sites exposed to trypsin, resulting in easier cleavage of peptide bonds. It might be the reason why there were more matched peptides in the PMF spectra of the digests obtained by using AC-assisted digestion.

In this work, an AC voltage (typically 5 V) was applied on the metal electrodes that touched the mixture solution of protein and trypsin to accelerate tryptic proteolysis. The electrochemical reactions that possibly occurred on the electrodes included the electrolysis of water, the reduction of dissolved oxygen, and even the oxidation of some peptides in the digestion solutions. Tables 1 and 2 in Supporting Information indicated that the oxidation of peptides in the digests was not pronounced based on the fact that most matched peptides in the digests of BSA and Cyt-*c* obtained by using classical digestion were found in the products of AC-assisted proteolysis. During AC-aid digestion, small bubbles were observed to form on the electrodes slowly, indicating the main reaction occurred on the electrodes was the electrolysis of water. The gas generated on either electrode was a mixture of oxygen and hydrogen because AC voltage was applied on the electrodes.

It can be concluded that AC-assisted on-plate proteolysis coupled with MALDI-TOF MS is a promising strategy for the efficient protein digestion in peptide mapping. With the assistance of alternative current, digestion time was substantially reduced to 5 min compared to 12 h for conventional in-solution digestion. If a platinum-electrode array is used instead of the single platinum disc electrode used in this work, different protein samples on the spots of MALDI plate can be digested simultaneously by using AC-assisted on-plate digestion. Another advantage of the present approach is its minimal sample consumption. It is important for the digestion of complex protein mixtures after being separated by capillary LC or capillary array LC because the volumes of eluents are usually limited. The ease, simplicity, efficiency, and low cost of the novel proteolysis approach indicate it may find further application in automated analysis of large sets of proteins.

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The authors have declared no conflict of interest.

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