J. Am. Soc. Mass Spectrom. (2012) 23:728–735
DOI: 10.1007/s13361-011-0323-5

RESEARCH ARTICLE

Online Electrospray Ionization Mass Spectrometric Monitoring of Protease-Catalyzed Reactions in Real Time

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Abstract

Although there are a lot of well established methods for monitoring enzyme-catalyzed reactions, most of them are based on changes in spectroscopic properties during the conversion of substrates to products. However, reactions without optical changes are common, which are inapplicable to these spectroscopic methods. As an alternative technique for enzymologic research, mass spectrometry (MS) is favored due to its specificity, sensitivity, and the ability to obtain stoichiometric information. In this work, probe electrospray ionization (PESI) source coupled with a time of flight mass spectrometer was employed to monitor some typical protease-catalyzed reactions, including pepsinolysis and trypsinolysis of cytochrome *c* in real time. Due to the high electrical conductivity of each reaction system, corona discharges are likely to occur, which would decrease intensities of mass spectrometric signals. An ultra-fine sampling probe and an auxiliary vapor spray were adopted to prevent corona discharges. Experimental results from peptic and tryptic digestions of cytochrome *c* showed different and characteristic catalytic pathways. With the data presented in this study, PESI-MS can be considered as a potential tool for real-time monitoring of enzymatic reactions because of its simplicity in instrumental configuration, wide applicability under harsh conditions, and flexibility in combination with other techniques.

Key words: Online monitoring, Probe electrospray ionization, Proteolytic reactions, Electrochemical etching, Solid probe

Introduction

Proteases break proteins down to peptides and have proven to be important in many fundamental biological processes, e.g., cell differentiation and growth [1, 2], blood coagulation [3], angiogenesis [4], wound healing [5], apoptosis [6], and lipid metabolism [7], etc. Even in industries, proteases are widely utilized as effective hair removers [8] and meat tenderizers [9]. In proteomics, protease-catalyzed digestions prior to the identification of proteins are in the center of most conventional workflows and have much influence to the subsequent analysis. Incomplete digestion may mislead data

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Received: 12 August 2011
Revised: 10 December 2011
Accepted: 18 December 2011
Published online: 4 February 2012

analysis and over-digestion can also cause unexpected cleavages of the substrate, which will complicate the procedure of protein identification.

Currently, most of the established methodologies for monitoring enzyme-catalyzed reactions are based on changes of spectroscopic properties during the conversion of substrates to products. UV-Vis absorbance and fluorescence spectroscopy are well-accepted techniques [10, 11]. However, some natural substrates do not present any observable optical properties. Furthermore, in some cases, spectroscopic changes due to enzymatic catalysis are not significant enough to be detected. As an effective aid, chemical derivatization by introducing some optical active functionalities could enhance the availabilities of spectroscopic methods [12]. But these chromophoric or fluorophoric groups would change the kinetic properties of the enzymatic catalysis by altering the substrate recognition [13]. Radioactive labeling is another effective method. However, both complicated pre-/post-measurement requirements and high possibility of misjudgment of the catalysis pathway make it less favored. Additionally, radioactive wastes need special care, lacking in most laboratories [14].

In enzyme-catalyzed conversions, structural changes of substrates always lead to molecular mass changes. Moreover, the products are mostly of different molecular weights compared to their precursors. Mass spectrometry (MS) can be employed in this field due to its mass-sensitive ability. In 1997, Northrop and Simpson [15] enthusiastically suggested that mass spectrometry could be a promising technique for enzyme kinetic research for its breakthrough in detection of biological macromolecules due to the invention of soft ionization techniques such as electrospray ionization (ESI) [16, 17] and matrix-assisted laser desorption/ionization (MALDI) [18, 19]. The first approach of applying ESI-MS to real-time monitoring enzyme-catalyzed reactions was reported by Lee et al. in 1989 [20]. After this report, many notable ESI-MS based methods have been invented subsequently. Although those methods worked well in their respective reports, considering they are based on ESI, some limitations of ESI can also be identified. Because normally the transportation of sample solution in an ESI source is done by a capillary, it will cause a time delay, which is specially unwanted for real-time analysis where a quick response is favored. On the other hand, dealing with a high-salt-containing sample solution is risky to the conventional ESI source, due to the high possibility of clogging to the ESI capillary.

It was reported by Shiea et al. that the electrospray could be generated not only through capillaries but also on a solid conductor [21]. In 2007, a modified version of ESI, probe electrospray ionization (PESI), was introduced by Hiraoka et al. [22], where a solid needle with a sharp tip for both sampling and electrospraying was utilized to replace the capillary of the ESI source. PESI could bring some benefits to ESI, such as direct analysis with minimal sample pretreatments [23, 24], high tolerance to salt [25], and low sample consumption [26]. In our previous work, PESI-MS was applied to real-time monitoring of some biological and organic chemistry reactions [27]. Herein, we describe the application of PESI-MS to real-time monitoring of protease-catalyzed reactions. With the help of an auxiliary solvent vapor sprayer and an ultra-fine sampling/electrospraying probe, PESI-MS can be employed to deal with reactions occurring in highly conductive solutions. The design of the current PESI source can enhance the durability and reliability and suppress the occurrence of corona discharge for highly acidic or buffered solutions. Typical protease-catalyzed reactions were selected in this work, including peptic digestions (at pH 1 and 3) and tryptic digestion (at pH 8) of a model protein, cytochrome *c*.

Experimental

Preparation of the Ultra-Fine Sampling Probe by Electrochemical Etching

The ultra-fine PESI probe was prepared according to a well-established electrochemical etching protocol [28] with a little modification. In brief, as shown in Figure 1a, a 3.5 mm diameter platinum wire loop (Φ 0.5 mm, purity 99.98%; Nilaco, Tokyo, Japan), forming the cathode, was placed on the surface of a 30% (wt/wt) KOH solution (etchant). One piece of 7–8 mm tungsten wire (Φ 0.05 mm, purity 99.95%; Nilaco), forming the anode, was positioned centrally within the cathode and dipped into the etchant for about 1 mm. The DC voltage between the two electrodes was set at 5.0 V and the stop current was 2.2 mA. The whole etching would last for 3–5 min. After etching, this probe was rinsed with deionized water and methanol subsequently for cleaning. The scanning electron microscopic (SEM) (JSM-6500F; JOEL, Tokyo, Japan) images of an etched tungsten probe are shown in Figure 1b. The tip diameter of the probe is measured to be less than 100 nm.

Setup of the PESI Source

The configuration of the PESI source is shown in Figure 1c, which is similar to those described previously [24, 25]. An etched tungsten probe is fixed on a linear actuator system (ARIOS, Tokyo, Japan) and then aligned orthogonally to the direction of the axis of the ion sampling orifice of a mass spectrometer. This probe can be driven up and down along the vertical axis with a maximum speed of 40 mm/s. During its motion, this probe can stop at the lowest and highest positions for 40 ms and 300 ms, respectively. When the probe was at the highest position, the vertical and horizontal distances between its tip to the apex of the ion sampling orifice were both 2 mm. The motion stroke was set as 7 mm. The time for one sampling/electrospraying cycle is estimated to be 690 ms.

A home-made mini-thermostat stirring system comprised of a flexible heater (Minco Products, Minneapolis, MN, USA), a low-speed electric motor, a magnet, and two low-voltage DC power supplies (PMC-2A; Kikusui Electronics,

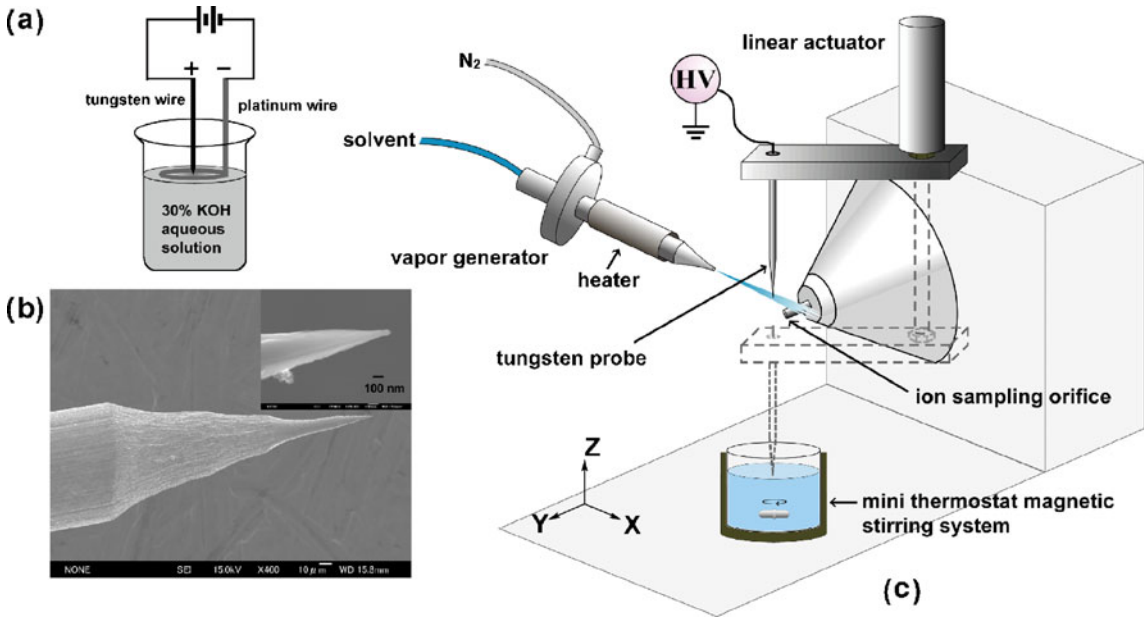

Figure 1. (**a**) Schematic depiction of the electrochemical etching of a piece of tungsten wire; (**b**) SEM images of the tip of the tungsten wire after etching at magnification of 400× and 85,000× (inset), respectively; (**c**) Schematic depiction of the setup of PESI source. The axis of the ion sampling orifice of the mass spectrometer is set as the Y axis. The probe is aligned parallel to the Z axis while the auxiliary vapor generator is aligned parallel to the X axis. For clarity, dashed lines are indicative of the movable probe mounting part when it is driven at the lowest position of its motion

Yokohama, Japan), was used as the reaction incubator in this work. All reactions were carried out with continuous stirring at 37 °C. When the sampling probe reached at the lowest position of its motion, it could load a certain amount sample solution. While, when it reached the highest position, after being applied a high voltage, typically 1.5 kV, the loaded sampled solution could be electrosprayed.

An auxiliary vapor generator was applied in this work and functioned as an online dilution means to suppress the occurrence of corona discharge. As also shown in Figure 1c, a commercially available ESI emitter (JOEL, Tokyo, Japan) combined with an automatic temperature controller forms the main body of the vapor generator. This vapor generator is placed perpendicularly to the axes of both the sampling probe and the ion sampling orifice. The heater was set at 120 °C and pure nitrogen of 0.125 MPa was used to assist the directional flow of the vapor towards the tip of the probe. When a 50:50 (vol/vol) water-acetonitrile mixture was pumped though the vapor generator at a typical flow rate of 20 μ L/min, pure solvent vapor would be generated and then condensed on the needle tip directly. The distance from the tip of the vapor generator to the tip of the probe is crucial to the whole experiment because it will affect the wetness of the needle tip. In this case, the optimized distance is 1.5 mm. No voltage was applied to the sprayer.

Mass Spectrometer

Mass spectrometric recording was carried out on an orthogonal time of flight mass spectrometer (AccuTOF; JEOL, Tokyo, Japan). The original ESI source was removed and the interlock was overridden. Typical parameters for the AccuTOF were: ion sampling orifice temperature, 120 °C; inlet orifice, 150 V; ring electrode, 10 V; first skimmer voltage, 5 V; ion guide rf amplitude, 1500 V; and the multi-channel plate detector, 2300 V. All data were acquired and recorded under positive ionization mode by using the analogue-to-digital converter (ADC)/continuous averaging ion detection system. Due to the file size limit, each continuous mass spectrometric recording was performed for 60 min.

Chemicals and Reagents

All reagents and solvents used in this work were of analytical grade or higher and were used directly without any further purification. Water was purified and deionized by a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade acetonitrile and acetic acid (HAc) was purchased from Kanto Chemicals (Tokyo, Japan). Cytochrome *c* (CytC, cat. no. C2037), pepsin (cat. # P6687), HCl, KOH, and ammonium bicarbonate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trypsin Gold (cat. # V5280) was purchased from Promega (Madison, WI, USA).

Preparation of Protein Sample Solutions and Protease Solutions

Pepsin and trypsin solution could be simply prepared by dissolving pepsin and trypsin gold in pure water and 50 mM

HAc solution, respectively, at the same final concentration of 1 μg/ μL. The protease solutions were stored in the ice-water mixture before being mixed with substrates. Three types of CytC solutions were prepared by dissolving CytC into solvents of different pHs, namely 0.1 M HCl (pH=1.13), 0.001 M HCl (pH=3.26), and 50 mM NH_4HCO_3 (pH=8.71) for peptic (pH 1), peptic (pH 3), and tryptic (pH 8) digestions, respectively. The pH value of each solution was measured by using a digital pH meter (pH Spear; Eurotech Instruments, Malaysia).

Theoretical Digestions

The sequence of bovine cytochrome *c* (accession number P62894) for the theoretical digestions was adopted from Uniprot Knowledgebase release 2011-05 [29]. The initial methionine was excluded before theoretical digestions. The tools, ExPASy Peptide Cutter [30] and MS-Digest (from Protein Prospector) [31], were used for calculation of the theoretical digestion products within a mass range of 500–5000 Da. The maximum of missed cleavages for trypsin and pepsin were set to 5 and 12, respectively.

Results and Discussions

Optimization of the PESI Source for High Conductive Solutions

Since electrospray takes place in strong electric fields, it may be accompanied by the generation of corona discharges. Corona discharges between the electrospray capillary and a counter-electrode (e.g., the ion sampling orifice) are usually considered to be a significantly unwanted side effect to electrospray [32]. When corona discharges occur, the pattern of the mass spectrum will change accordingly [33]. Ions of background gas-phase analytes produced by ion–molecule reactions induced by corona discharges would greatly suppress ions of analytes from the sample solution [34]. Both stability and reproducibility of the electrospray will get worse.

In most conditions, it is found that the onset voltage of the corona discharge is a little higher than that of the electrospray [35]. Thus, lowering the spray voltage of the ESI source will avoid the troublesome corona discharges [36]. According to Smith's theoretical work [37], the onset voltage for electrospray is proportional to $T^{1/2}r^{1/2}\ln(4h/r)$, where T is the surface tension of the solution, r is the outer radius of the ESI capillary, h is the distance from the tip of the ESI capillary to the counter electrode. Considering that the surface tension of water (T=72.75 dyn/cm, 20 °C) is much higher than that of other known solvents such as methanol (T=22.5 dyn/cm, 20 °C), the onset voltage for electrospraying of water is 1.8 times higher than that for methanol [36], which makes analysis of pure aqueous solutions by ESI-MS difficult. If the spray voltage is adjusted high enough and exceeds the corona onset voltage, corona discharges will occur.

Based on Smith's equation, one possible way to electrospray pure aqueous solutions is to decrease the outer radius of the ESI emitter. By sharpening the ESI capillary, Chait and Chowdhury achieved ESI mass spectra of proteins in highly conductive aqueous solutions without nebulization [38]. Furthermore, a finer ESI capillary will lead to charged droplets of smaller size, which will allow the ESI source to be placed closer to the mass spectrometer for minimizing sample loss.

Compared to ESI emitters, PESI probe has a much sharper tip. Dried sample residues on the probe surface will increase the irregularities of the probe surface and induce a higher likelihood of the occurrence of corona discharges. In order to enhance the performance of electrospraying pure aqueous solutions and suppress the influence from corona discharges, two major improvements to previously described PESI sources [22–25, 27, 39] were made in this work. The first concern is to reduce the size of the probe tip. As shown in Figure 1b, an electrochemically etched tungsten probe with a less than 100 nm tip was adopted as the PESI probe. The spray voltage could be reduced to about 1.5 kV, which is 1.0–2.0 kV lower than that in previous work [24, 27]. The second concern is to introduce an auxiliary vapor generator in this work. The vapor can condense on the probe's surface. Dried compounds on the probe surface can be dissolved and then electrosprayed, which will eliminate the unwanted irregularities of the probe surface. By applying thee two means described above, corona discharges could be effectively suppressed and stable ESI signals originating from pure aqueous solutions could be obtained reproducibly.

Since in PESI-MS experiments, the same probe is used for continuous sampling and electrospraying, the carryover effect, which means the current measurement is affected by the previous sampling, may exist. In our previous work [40], we have demonstrated that by providing enough solvent vapor to the probe for cleaning, the carryover effect can be avoided. In this study, because the dwell time for the probe at the highest position is longer and the flow rate of solvent is faster than before, we assume the carryover effect can be diminished to a negligible level.

Real-Time Monitoring of Pepsinolysis of CytC

As one of the major proteases in the human digestive system, pepsin is secreted by chief cells in the gastric mucosa as pepsinogen, the precursor of pepsin with 44 additional amino acids [41]. Pepsin is the enzyme whose catalytic activity is highly dependent on the environmental pH value. Porcine pepsin, the most studied pepsin, has its maximal activity at pH 2.0 and retains its activity when the pH drops to 1.0, but begins to be inactive at pH 5 [42]. Normally, pepsin is believed to have a broad but low specificity by preferring to cleave peptide bonds in which the carboxyl group is provided by amino acids with bulky

hydrophobic or aromatic side chains [43]. The pepsinolysis is susceptible to many factors such as the ratio of substrate/enzyme, temperature, and pH values of solvents [44]. Hamuro et al. have statistically analyzed large amounts of peptic digestion data, and their results showed that under low temperature (<1 °C) and low pH, the specificity of pepsin can be narrowed [45]. Furthermore, a recent UPLC-MS research suggested that the specificity of pepsin is pH-independent [46]. For the purpose of investigating the relationship between pepsin specificity and the solution pH value, peptic digestions of bovine CytC at pH 1 and 3 were performed, respectively, and monitored by PESI-MS in real time.

Time-resolved mass spectra of pepsinolysis of CytC at pH 1 are shown in Figure 2a. Some ions are identified according to their *m*/*z* values, theoretical digestion results, and literature published elsewhere [47, 48]. At the starting point (the time right after the mixing of pepsin and CytC solutions, noted as 0.0 min), ions of CytC with multiple charges (from 9+ to 21+) were detected. At 0.5 min after the starting point, the ion intensities of multi-charged CytC ions decreased sharply and several multi-charged fragment ions started to appear, especially the fragment 65–104 at *m*/*z* 583.7. After 3.0 min, the fragment 65–104 disappeared and ions of the fragment 35–101 at *m*/*z* 690.9 and the fragment 65–82 at *m*/*z* 551.7 became dominant; 60.0 min later, the most abundant peaks were ions of fragments 65–82 and 83–96. From the extracted ion chromatograms (EICs) of four typical ions shown in Figure 2b, the kinetic process of pepsinolysis of the substrate could be clearly seen. CytC was consumed very fast to generate the fragment 65–104, a short-lived intermediate. The fragment 35–101 seemed to be generated simultaneously with the fragment 65–104 but with a slower rate. The fragment of 65–82 could be viewed as one of the final digestion products during the 60 min of pepsinolysis because of its relatively stable ion intensity. During the pepsinolysis, the solution pH changed from 1.13 to 1.21.

The pepsinolysis of CytC was also examined at pH 3 to investigate how the solution pH affected the digestion process. As shown in Figure 3a, at 0.0 min, peaks of CytC with up to 15+ charges were detected. After 0.5 min, the fragment 65–82 appeared while ion intensities from CytC started to decrease. From Figure 3b, the EIC of the ion at *m*/*z* 735.2 showed a clear tendency that after the mixing of the enzyme and the substrate for 5 min, the ion intensity of the fragment 65–82 reached its maximum and then started to decrease. Ions of the fragment of 76–82 at *m*/*z* 793.6 and 67–82 at *m*/*z* 971.6 were detected uniquely compared with Figure 2. Due to our limitation of identification techniques (MS/MS), most of peaks in Figure 3 were not identified. After the reaction, the solution pH changed from the initial 3.26 to 3.50.

It has been proposed that at a certain temperature lower than the body temperature and short time contact, the catalytic mechanism of pepsin is pH-dependent [46]. On the other hand, it was hypothesized that the pH-related conformational changes of proteins might be the reason why an enzyme can catalyze the same substrate with different catalytic mechanisms at different pH values [49–51]. Apparently, our results support this speculation. Given that a typical pepsinolysis will be incubated for hours [48], experimental data in this work can only show the initial first 60 min of pepsinolysis. From the time-resolved ion intensities of a representative pepsinolysis product, the fragment 65–82, at different pH values, it can be easily summarized that pepsin has more activity at pH 3 than that at pH 1, which is in accordance with the pH effect on the activity of pepsin reported by Piper and Fenton [42]. The pH-induced conformational change of CytC and the pH-sensitive catalytic activity of pepsin make characteristic digestion pathways at different pHs.

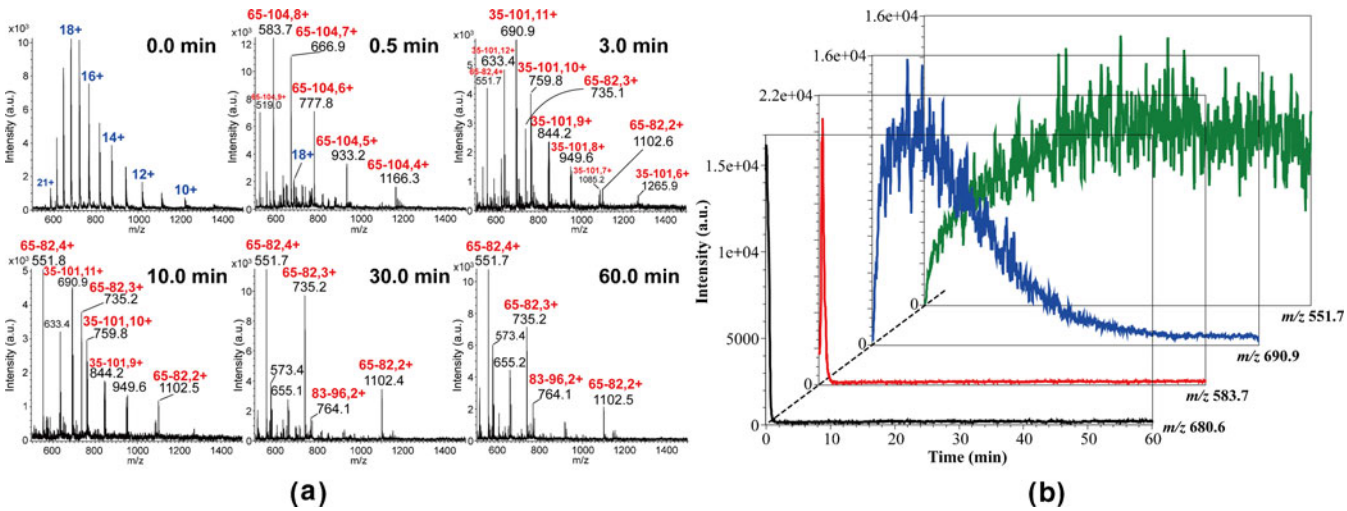

Figure 2. Online real-time monitoring of pepsinolysis of CytC at pH 1. (**a**) Time-resolved mass spectra 0.0, 0.5, 3.0, 10.0, 30.0, and 60.0 min after the mixing of 2 µL pepsin (1 µg/ µL), and 200 µL CytC (2×10^{-5} M); (**b**) EICs of ions at *m*/*z* 680.6 (CytC 18+), 583.7, 690.9, and 551.7, respectively. Charge states of CytC ions are notated in *blue*

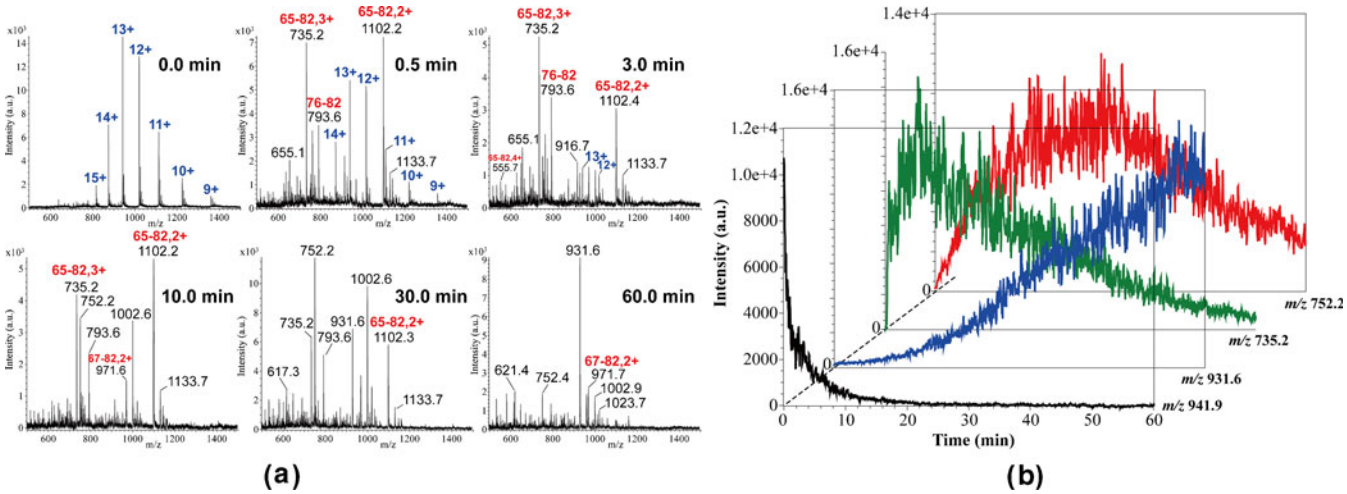

Figure 3. Online real-time monitoring of pepsinolysis of CytC at pH 3. (**a**) Time-resolved mass spectra 0.0, 0.5, 3.0, 10.0, 30.0, and 60.0 min after the mixing of 2 µL pepsin (1 µg/µL) and 200 µL CytC (2×10^{-5} M); (**b**) EICs of ions at *m/z* 941.9 (CytC 13+), 931.6, 735.2, and 752.2, respectively. Charge states of CytC ions are notated in *blue*

By comparing the peptic digestion pathways of CytC at pH 1 and 3, one significant feature of pepsinolysis is that the peptic digestion proceeds as a multi-stage process. The final digestion products may be generated from sequentially cleaved intermediates from substrates [52, 53]. As hypothesized by Belikow and Antonowa [54], pepsin may preferentially cleave peptide bonds on the surface of a substrate. When the most accessible peptide bonds on the surface have been hydrolyzed, peptide bonds on the inner parts will become accessible. To a pepsinolysis, the solution pH value is a vital factor because it can influence both the pepsin activity and the conformation of the substrate, which makes the whole reaction multi-staged.

Real-Time Monitoring of Trypsinolysis of CytC

Trypsin is another important proteolytic enzyme in the human digestive system. Trypsin is activated from the inactive trypsinogen secreted by the pancreas and functions in the lumen of the small intestine. Usually trypsin digests optimally at around pH 8 and 37 °C [55]. Trypsin breaks proteins down into peptides by following a strict rule of

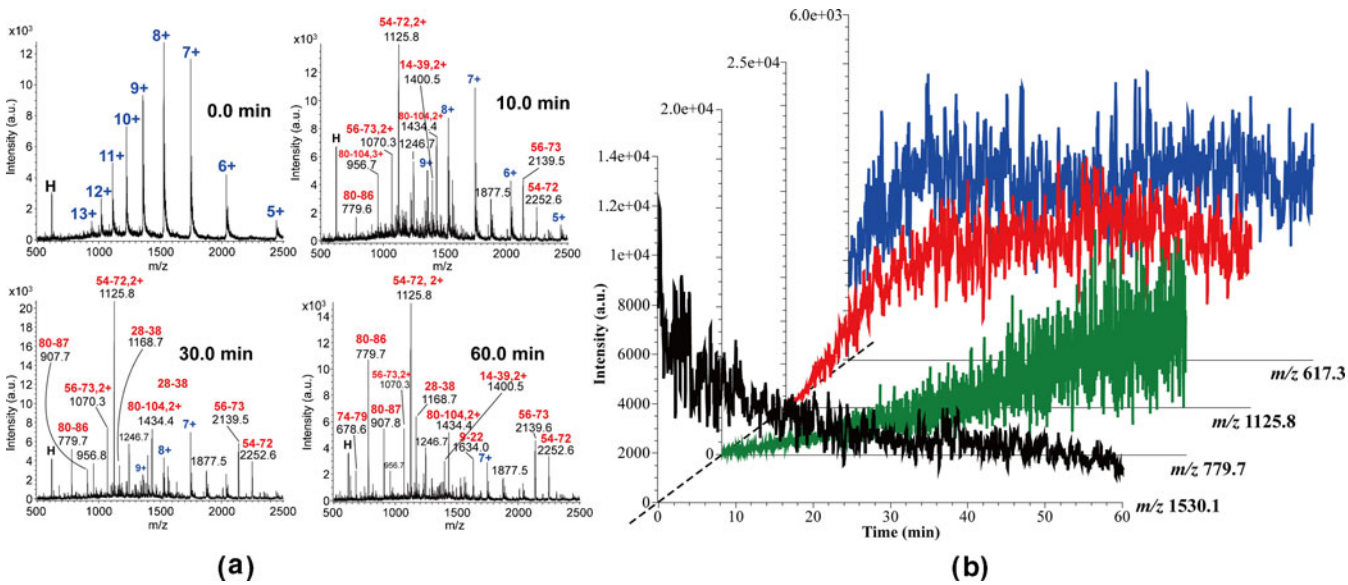

Figure 4. Online real-time monitoring of trypsinolysis of CytC at pH 8. (**a**) Time-resolved mass spectra 0.0, 10.0, 30.0, and 60.0 min after the mixing of 2 µL trypsin gold (1 µg/µL) and 200 µL CytC (5×10^{-5} M); (**b**) EICs of ions at *m/z* 1530.1 (CytC 8+), 779.7, 1125.8, and 617.3 (heme), respectively. Charge states of CytC ions are notated in *blue*. H indicates heme

cleaving on the carboxyl side of lysine and arginine except if they are followed by proline [56]. The high specificity makes trypsin the first choice protease for most MS-based proteomics [57]. Trypsin is often notated as an opposite example to pepsin due to its high but narrow specificity, requirement of an alkaline environment, and the slowness of its catalysis. In order to compare the catalytic properties of trypsin and pepsin, trypsinolysis of CytC was performed and monitored in real time by the same method as described above.

The time-resolved mass spectra of 0.0, 10.0, 30.0, and 60.0 min after the mixing of CytC and trypsin solutions are shown in Figure 4a. Due to the high specificity of trypsin, most ions of digestion products could be identified according to the theoretical digestion results and the data published previously [58–60]. Compared with the pepsinolysis results shown in Figures 2 and 3, the detection of intact ions of CytC at 60 min could be ascribed to the slowness of trypsin catalysis. In Figure 4b, the EICs of typical ions at *m/z* 1530.1, 779.7, 1125.8, and 617.3 corresponding to CytC 8+, the fragment 80–86, the fragment 54–72 and heme were shown, respectively, which also indicated that trypsin has a lower catalytic activity than pepsin.

In 1994, Noda et al. [61] reported the existence of intermediates in the trypsinolysis of a modified lysozyme, which has a similar molecular mass to CytC. In that work, the tryptic digestion was performed at insufficient time and under suboptimal conditions. In our case, however, all fragment ions seemed to be generated in parallel. There was no significant observation of intermediate species, whose ion intensities would comprise an increase followed by a decrease like the ion at *m/z* 583.7 shown in Figure 2b. As shown in Figure 4b, the intensities of three typical ions exhibited either a continuous increase (the ion at *m/z* 779.7) or a fast increase with a plateau (the ions at *m/z* 1125.8 and 617.3). There are a lot of discussions concerning intermediates of trypsinolysis, where substrates with high masses were employed [62, 63]. Additionally, a full tryptic digestion needs hours of incubation time. We thus assume the simultaneous observation of tryptic products of CytC, which could be regarded as a parallel but not consecutive process as far as the reaction time of 60 min was concerned in the current experiment. This may be ascribed to the relatively low molecular mass of CytC. However, we do not exclude the consecutive digestion process of tryptic digestions if the reaction time could be extended to that of a full digestion. The kinetic difference between peptic and tryptic digestions of CytC reflect characteristically different stages in the human digestive system, namely, a fast and extensive digestion versus a slow and intensive digestion.

Conclusions

Usually, aqueous solutions are difficult to electrospray due to the high likelihood of occurrence of corona discharges. In this work, the employment of the ultra-fine sampling probe and the auxiliary vapor spray can suppress corona discharges and reinforce stability and reproducibility of PESI-MS for analysis of reactions occurred in high conductive aqueous solutions. Two typical protease-catalyzed reactions were adopted to demonstrate the applicability of PESI-MS for real-time monitoring of enzymatic conversions. Time-resolved mass spectra and ion chromatograms indicate PESI-MS can be a suitable technique for both monitoring the change of a specific reactant/product or the whole reaction and elucidating the kinetic progress of a reaction.

The current study highlights the application of PESI-MS to real-time monitoring and direct analysis of ongoing enzymatic reactions. The flexibility in instrumental configuration and versatility in application aspects makes PESI-MS a potential tool for the research of enzymatic kinetics and related sciences. This method can also be used as a supplementary technique for proteomic research to avoid incomplete/over-digestions or to obtain digestion products at a specific time point.

Acknowledgments

The authors acknowledge financial supported for this work by the Grants-in-Aid for Scientific Research (S) and Development of System and Technology for Advanced Measurement and Analysis Program (SENTAN) from Japan Science and Technology Agency (JST). Z.Y. is grateful for the financial support (no. SY201002) from the Experimental Center of Shenyang Normal University. M.K.M. is financially supported by the Monbukagakusho Scholarship from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

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