

Selected Paper

Protein Digestion Using Immobilized Enzyme and a Microscale Vibration Unit for Structural Analysis of Phospholipase A_2 by Mass Spectrometry

Yukie Sasakura,* Rumiko Inada, Makoto Nogami, Masaki Watanabe, Tsuyoshi Ogino, and Katsuhiro Kanda

4th Department, R&D Division, Nanotechnology Product Business Group, Hitachi High-Technologies Corporation, 882 Ichige, Hitachinaka 312-8504

Received December 25, 2007; E-mail: sasakura-yukie@naka.hitachi-hitec.com

For mass spectroscopy (MS)-based protein structural analyses, effective digestion by proteases is one of the key steps. In this study, we developed a protein digestion method using immobilized enzyme and a microscale vibration unit. Phospholipase A_2 was applied as a model substrate to evaluate the new procedure due to its rigid structure and resistance to protease digestion. Digested substrates were analyzed by MS, and cleavage at all expected recognition sites was evaluated. In relation to conventional liquid-phase digestion, the number of matched peptides and sequence coverage improved significantly from 6 to 9, and 41% to 69%, respectively. These results support the efficacy of our novel method in proteomics applications, including protein structural and post-translational modification analyses.

Mass spectrometry (MS) is widely used for protein structural analysis. For these experiments, the target protein is initially digested into peptides with proteases, such as trypsin. These peptides are subjected to MS analysis, and their m/z values compared to the theoretical values. Therefore, effective protein digestion by protease is one of the key steps in MS. However, several proteins, including post-translational modification (PTM) protein and digestive enzymes, such as protease and lipase, are generally resistant to protease cleavage, and thus conventional digestion is not sufficient.[1,2] Insufficient digestion would produce peptides of different lengths, resulting in reduced individual concentrations, which may affect their detection efficiency by MS. In addition, long peptides are often generated that are usually resistant to ionization and fragmentation for MS/MS analyses. Finally, for PTM analysis, target peptides containing modification sites need to be detected by MS. Therefore, all possible peptides should be distinguished to obtain 100% sequence coverage, although a relatively limited percentage (less than 10% in some cases) is generally sufficient for protein identification. For these purposes, complete protease digestion is required, and an effective digestion method is important.

The theory of immobilized protease has been suggested to overcome the problems of low digestion efficiency.[3] In this method, increased enzyme/substrate ratio by enzyme immobilization is expected to improve the digestion. For example, Palm and Novotny immobilized trypsin to the monolith substrate, and used it to digest cytochrome *c*, BSA, and myoglobin.[4] The proteins were analyzed by MS and identified from sequence coverages of 47%, 32%, and 29%, respectively. These levels of sequence coverage are sufficient for protein identification, but insufficient for PTM analysis, since several important modifications or alterations may occur within peptides, which remain undetected. Cooper et al. reported on membrane-immobilized trypsin.[5] Cytochrome *c* was initially digested by membrane-immobilized trypsin, and dispensed to the trypsin-immobilized MALDI target plate for complete digestion. The protein was analyzed by MALDI-MS, and 83% sequence coverage was attained. The group additionally digested ovalbumin with membrane-immobilized trypsin, followed by protein analysis using MALDI-MS and ESI-MS. Sequence coverage of 97% was achieved in this case. However, there are no documented reports on the use of digestive enzymes as substrates, which are generally resistant to proteolytic cleavage.

Our group has developed a protein digestion unit involving immobilized enzyme and a vibration motor, with a view to improving the efficiency of protein digestion and accuracy of MS analyses.[6] In a previous study, we employed BSA and IgG as model substrates.[6] Here, we use phospholipase A_2 derived from the honeybee. Compared to the substrates used in other protease immobilization studies, phospholipase A_2 possesses a rigid structure and resistance to protease digestion. These characteristics possibly contribute to its role as a digestive enzyme, which would undergo auto-digestion in the absence of a rigid structure. Here, we evaluate the effectiveness of our novel digestion method on rigid proteins for the first time.

Experimental

Materials. The vibration reaction unit was manufactured by Fluidware Technologies (Tokyo, Japan). ProteoChip™ was obtained from Proteogen (Seoul, Korea). Trypsin and phospholipase A_2 were purchased from Sigma-Aldrich (St. Louis, MO), the HybriWell™ sealing system from GRACE BIO-LABS (Bend, OR), and Slide-A-Lyzer MINI Dialysis Cassette from PIERCE (Rockford, IL). Other chemicals were acquired from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich.

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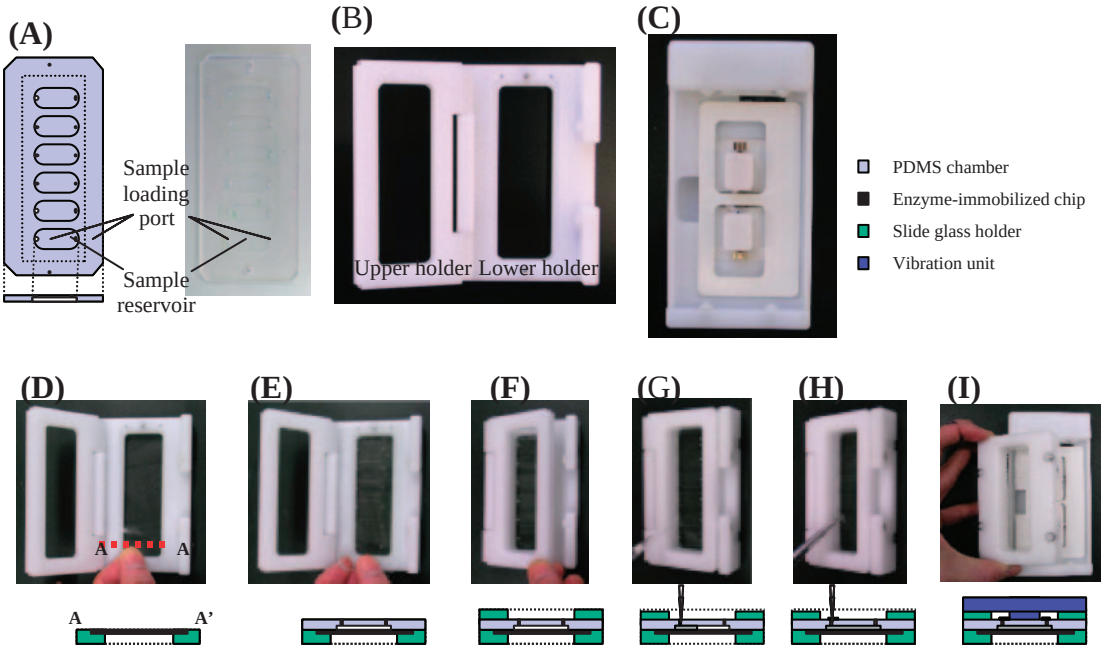

Figure 1. The vibration reaction unit. (A) The PDMS chamber contains six hollows (50 μL) that can be filled with samples and reagents on the slide glass surface. Sample loading ports were attached to each hollow. Their designs (left) and images (right) are shown. (B) The slide glass holder is composed of upper and lower holders that support the slide-affixed PDMS chamber. (C) The vibration unit generates convection within the hollows. Its operation is presented in (D)–(I). (D) The enzyme-immobilized chip is placed on the lower holder. (E) The enzyme-immobilized chip is covered by the PDMS chamber. (F) The holder is closed to clamp the PDMS chamber and the enzyme-immobilized chip. (G) The sample is loaded through the loading port. (H) Sample loading ports are sealed with the small pieces of PDMS sheet. (I) The vibration unit is attached for sample agitation.

Reduction and Alkylation of the Proteins. Phospholipase A_2 (1 mg) was diluted with 1 mL of denaturing buffer (200 mM (1 M = 1 mol dm^{-3}) Tris-HCl, pH 8.5, containing 6 M Guanidine-HCl and 2.5 mM EDTA). After the addition of 0.6 mg of DTT, the surfaces of the solutions were blown using N_2 gas, and incubated for 3 h at 37 °C. Following incubation for 5 min on ice, 1 mg of iodoacetamide was added, the surfaces of the solutions were blown with N_2 gas, and incubated for 1 h at rt under dark conditions. Guanidine-HCl was removed by dialysis against 10 mM Tris-HCl (pH 8.0) using the Slide-A-Lyzer MINI Dialysis Cassette.

Preparation of the Trypsin-Immobilized Chip. For preparation of the trypsin-immobilized chip, ProteoChip™ was used as a solid substrate. Trypsin was prepared at a concentration of 1 mg mL^{-1} with PBS (pH 7.4). The HybriWell™ sealing system was adhered to the ProteoChip™ surface beforehand. The trypsin solution was introduced into the well, and incubated overnight at 4 °C for immobilization on the surface. The chip was immersed in PBS (pH 7.4) with gentle shaking for removal of unbound trypsin, rinsed with 10 mM Tris-HCl (pH 8.0), and dried using a spin dryer, prior to use.

HPLC Analysis of Digested Phospholipase A_2. HPLC analysis was performed using the Hitachi L-2000 system (Hitachi High-technologies, Tokyo, Japan). Digested samples were applied to a CAPCELLPAK C_{18} MG column (2 mm I.D. × 75 mm, Shiseido, Tokyo, Japan). Two buffers were prepared as the mobile phase: 2% acetonitrile/98% water containing 0.1% TFA (buffer A) and 98% acetonitrile/2% water containing 0.1% TFA (buffer B). Elution was performed using a linear gradient of 100:0 to 40:60 A/B delivered at 0.2 mL min^{-1} over 60 min. Absorption at 214 nm was detected.

LC-MS Analysis of Digested Proteins. LC-MS analysis was performed using a NanoLC-Linear Ion Trap-Time of flight-Mass spectrometer NanoFrontierLD (Hitachi High-Technologies, Tokyo, Japan). Tryptic-digested protein was applied to a PicoFrit™ reverse-phase column (New Objective, Woburn, MA). Two buffers were prepared as the mobile phase: 2% acetonitrile/98% water containing 0.1% formic acid (buffer A) and 98% acetonitrile/2% water containing 0.1% formic acid (buffer B). Elution was performed using a linear gradient of 98:2 to 60:40 A/B delivered at a speed of 0.1 μL min^{-1} over 60 min. The analytical conditions of the MS detector were as follows: Ion source, electrospray; Polarity, positive ion mode; CID gain, 100%; CID time, 2 ms; Isolation window, 8 Da; Isolation time, 2 ms; Detector voltage, 2200 V; and Mass scan range, 200–2000. Proteins were identified by searching the National Center for Biotechnology Information database using MASCOT software (Matrix Science, London, UK). The following search conditions were used: Taxonomy, Other Metazoa; Enzyme, Trypsin; Missed cleavages, 0 or 1; Fixed modifications, Carbamidomethyl (C); Variable modifications, Phospho (STY); Peptide tol, ±0.3 Da; MS/MS tol, ±0.1 Da; Instrument, ESI-TRAP.

Results and Discussion

The Vibration Reaction Unit. The vibration reaction unit was originally developed for hybridization of slide glass, such as in immunostaining or microarray.[7,8] The unit, which was additionally used for microscale protein digestion with enzyme-immobilized slide glass,[6] contains PDMS chambers (Figure 1A), a slide glass holder (Figure 1B), and a vibration unit (Figure 1C). PDMS is an excellent material for use in mi-

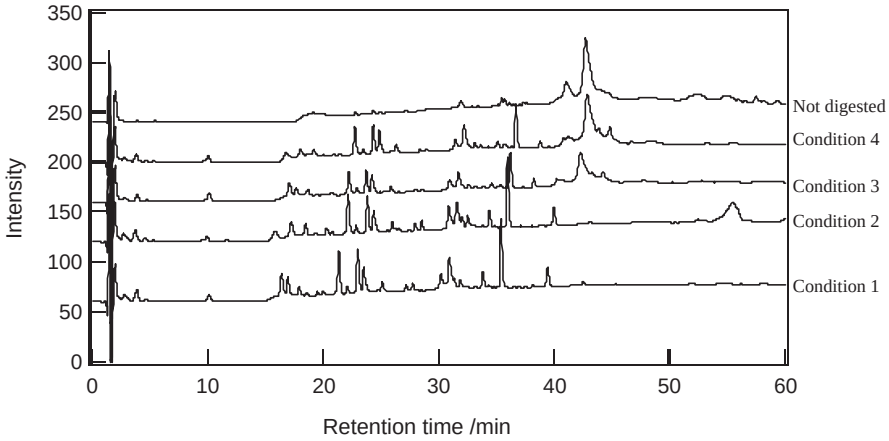

Figure 2. Phospholipase A_2 was digested with trypsin under four different conditions: 1) immobilized trypsin with vibration, 2) immobilized trypsin without vibration, 3) liquid-phase digestion (non-immobilized trypsin) with vibration, 4) liquid-phase digestion without vibration. HPLC chromatograms of the digested samples are shown.

crofluidic systems targeted towards the analysis of biological samples,[9,10] as it is inexpensive, impermeable to water, non-toxic to biomolecules, and able to attach onto glass surfaces. The PDMS chamber consists of six small hollows (50 μL volume) with sample loading ports. The enzyme-immobilized chip was initially placed on the lower holder (Figure 1D). After the PDMS chamber was attached to the chip surface to generate reaction baths in the hollows (Figure 1E), chip and chamber were clamped between the upper and lower holder (Figure 1F), samples or reagents were injected via the sample loading ports using a pipette (Figure 1G), ports were sealed (Figure 1H), and the vibration unit was attached to generate convection within the reaction baths (Figure 1I).

The convection efficiency of this reaction unit was examined using a blue-dye solution. The solution was put into the water-filled reaction bath and its diffusion was checked in the presence of vibration. It diffused across the entirety of the bath within 3 s once vibration was applied. Therefore, the vibration was suggested to be enough for the sample convection.

Digestion of Phospholipase A_2 Using the Trypsin-Immobilized Chip and Vibration Reaction Unit. The effectiveness of our novel protein digestion unit was evaluated using phospholipase A_2 as a substrate. Phospholipase A_2 has a rigid structure with seven S–S bonds, and is resistant to tryptic digestion. The enzyme is thus suitable as a model substrate to evaluate the performance of our new procedure. Two factors are expected to improve digestion efficiency, specifically, (1) increased enzyme/substrate ratio by enzyme immobilization, and (2) increased collision efficiency between enzyme and substrate molecules by vibration. To evaluate each of these effects, digestion experiments were performed under four different conditions: 1) immobilized enzyme with vibration, 2) immobilized enzyme without vibration, 3) liquid-phase digestion (non-immobilized enzyme) with vibration, 4) liquid-phase digestion without vibration.

Firstly, we prepared a trypsin-immobilized chip to digest phospholipase A_2 at 37 °C for 1–3 h with vibration. Digested samples were analyzed by SDS-PAGE (data not shown). The band corresponding to undigested phospholipase A_2 disappeared completely after 3 h reaction. Consequently, 3 h was adopted as the digestion time for subsequent experiments. We digested phosphodiesterase A_2 for 3 h under the different conditions specified above. For liquid-phase digestion without trypsin immobilization (conditions 3 and 4), a polycarbonate plate was inserted into the slide glass holder instead of the trypsin-immobilized chip. Trypsin was mixed with samples beforehand at a 1/100 substrate concentration. Uses of the high concentration of trypsin should be avoided, because it would disturb the mass spectrum of the protein. The mixture of trypsin and substrate was injected into the reaction baths, and incubated at 37 °C for 3 h with or without vibration.

All digested samples were analyzed by HPLC. Chromatograms are depicted in Figure 2. Relative digestion rates were evaluated by comparing the peak area of undigested phospholipase A_2, which displayed a retention time of 43 min. While the peak areas of all samples were decreased, differences in their chromatogram patterns were evident. To clarify these differences, sample analysis was performed using LC-MS/MS, followed by a database search with MASCOT software.

Firstly, the number of the missed cleavages was limited to zero to evaluate complete digestion. The search results are presented in Table 1A. We observed significant differences in sequence coverage between conditions 1 and 4 with sigma values of 4.8, suggesting that the combined use of enzyme immobilization and vibration is effective for phospholipase A_2 digestion.

Next, we considered the uncovered region of phospholipase A_2. The MASCOT search result under condition 1 (missed cleavages = 0) is shown in Figure 3A. Amino acid sequences in red correspond to the detected peptides. No tryptic digestion was evident at sites X and Y. Next, LC-MS/MS results were searched using different conditions, with up to one missed cleavage allowed (Figure 3B). Consequently, the peptides boxed in red were detected, suggestive of digestion at sites X and Y, albeit not with 100% efficiency. In this case, 91% sequence coverage was achieved. Other short peptides may not be identified, since they are out of the detection range of MS (underlined in green) or their ionizations are inhibited by cys-

Table 1. The MASCOT Search Results of Phospholipase A_2[a)]

Digestion conditions			Result A[b)]				Result B[c)]			
	Enzyme immobilization	Vibration	Identified peptides[d)]	Protein score	Sequence coverage /%	Identified cleavages[e)]	Identified peptides[d)]	Protein score	Sequence coverage /%	Identified cleavages[e)]
1	○	○	9.0 ± 0.0	374 ± 8.5	69 ± 0.0	14.0 ± 0.0	14.5 ± 0.7	567 ± 0.7	90 ± 2.1	17.0 ± 0.0
2	○	×	7.0 ± 1.4	313 ± 34.7	57 ± 7.1	11.5 ± 2.1	12.5 ± 2.1	473 ± 39.6	87 ± 4.2	14.5 ± 2.1
3	×	○	8.0 ± 1.0	368 ± 72.7	62 ± 8.7	13.0 ± 1.0	13.3 ± 0.6	585 ± 61.3	85 ± 8.5	14.0 ± 1.0
4	×	×	5.7 ± 0.6	231 ± 26.6	41 ± 5.8	11.3 ± 1.2	11.3 ± 2.5	424 ± 53.7	66 ± 7.0	15.7 ± 1.2

a) The MASCOT search results under four different digestion conditions were shown. The number of identified peptides, protein score, sequence coverage, and number of identified cleavage sites were compared. b) Missed cleavages were not allowed. c) Missed cleavages of up to 1 were allowed. d) Number of the identified peptides by MASCOT search with rank 1. e) Number of the identified cleavage sites. (There were 17 trypsin sites in total.)

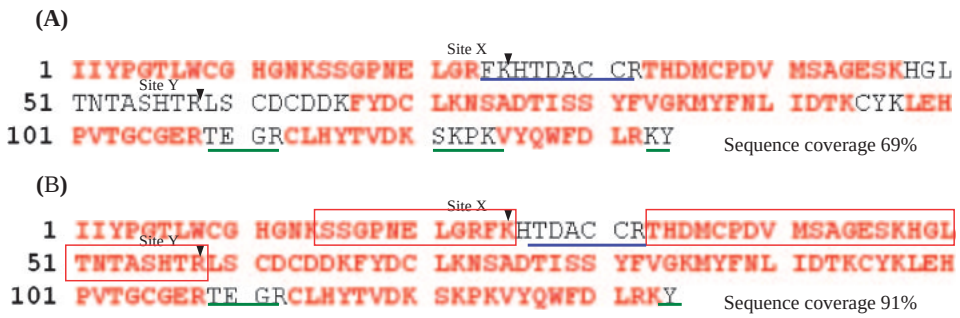

Figure 3. MASCOT search results under condition 1 (immobilized trypsin with vibration) are shown. Amino acid sequences in red correspond to the detected peptides. (A) Search result when missed cleavages were not allowed. (B) Search result when missed cleavages of up to 1 were allowed. X and Y are expected trypsin target sites, whose digestion was not confirmed under condition (A), but validated under condition (B), with the detected peptides boxed in red. The underlined peptides were not distinguished, probably since they were out of range (green line) or ionizations were inhibited by cysteine alkylation (blue line).

Figure 4. Peptides that were not detected in samples treated under condition 4 are underlined in black. Tryptic digestion efficiency is usually low around proline (P, blue), aspartic acid, and glutamic acid (D or E, red) sites (shown).

teine alkylation (underlined in blue). In view of these effects, we analyzed data obtained under four different conditions using search conditions allowing missed cleavages of up to 1 (Table 1B). We noted significant differences in sequence coverage between conditions 1 and 4. No significant differences were observed between conditions 1 and 2 (immobilized enzyme with or without vibration) or between conditions 1 and 3 (immobilized or unimmobilized enzyme without vibration). Further investigations are required to confirm the individual effects of enzyme immobilization and vibration. The amount of trypsin immobilized on the surface was 0.23 μg per well, which was calculated from the projected area of trypsin. It was about 1/50 of the substrate, suggesting that only twice amount of enzyme was used compared to the liquid-phase digestion. Therefore, enzyme stabilization by immobilization would be one of the main reasons of the improved digestion. Increasing the immobilization area to use more enzyme would be beneficial to improve the digestion efficiency.

Finally, we investigated the cause of the differences in sequence coverage. Peptides subjected to condition 4 that were not detected are underlined in black in Figure 4. The generation rates of these peptides under four different conditions were summarized in Table 2. Among these, peptide B was not detected, even under condition 1. However, A and C were detected under condition 1, suggesting that these two peptides are responsible for the differences in sequence coverage. Therefore, the digestion efficiency at the termini of these peptides was expected to be relatively low, possibly due to the amino acid sequence or high-order structure of the protein. Generally, tryptic digestion efficiency is low at the site next to proline (P, blue in Figure 4) or the neighboring areas of aspartic acid and glutamic acid (D and E, red in Figure 4). However, these amino acids were not shown around the termini of peptides A, B, and C. Therefore, higher-order protein structures would affect digestion efficiency. Peptides B and C are belonging to the rigid α-helix structure,[11] which might result

Table 2. The Generation Rates of Three Peptides under Four Different Conditions[a)]

Digestion condition	Detection rate		
	Peptide A	Peptide B	Peptide C
1	2/2	0/2	2/2
2	2/2	0/2	2/2
3	2/3	0/3	3/3
4	0/3	0/3	0/3

a) The generation rates of three different peptides were compared. The numbers of samples (detected/analyzed) are shown. Position and sequence of peptide A, B, and C are shown in Figure 4.

to the inhibition of digestion, even after reduction and alkylation treatment.

We obtained 91% sequence coverage of Phospholipase A_2, one of the substrates resistant to protease digestion, within 3 h. Compared to the conventional liquid-phase digestion, our system has several advantages; (1) it is easy to operate, (2) the time required for the digestion is short (3 h), (3) There is no contamination of the trypsin into the samples, (4) the immobilized enzyme could be stored for long time: it maintained its activity more than three months and could be reused more than two times. We also confirmed the usefulness of this method for the analysis of phosphorylated protein such as β-casein and ovalbumin, and succeeded to identify their modified amino acid (unpublished results). Although, the analysis of phosphoprotein is one of the focused areas, it is often prohibited by their poor digestion efficiency. Therefore, we think that our digestion technique is effective for the analysis of these important PTMs. Also, we are trying to adopt this method to determine the position of disulfide bonding in the protein. For this purpose, the protein digestion without reduction and alkylation would be required, and it would be our next task. Concerning about the detection limit of the substrate, we have succeeded to identify 300 fmol of BSA per one reaction bath. We are now, challenging to analyze more diluted samples. Also, BSA could be digested with the existence of detergent (4 M urea), which would be beneficial for the digestion of rigid proteins.

Our final goal is to cover 100% of the target protein sequence. To reach this objective, more suitable methods should be devised, including digestion by multiple proteases,[12] a combination of multiple ionizations[5] or several dissociation techniques.[13] For example, the combined use of different proteases, such as ArgC, AspN, GluC, LysC, or various dissociation techniques, such as CID and ECD, in MS/MS analyses would be beneficial.

Conclusion

Here, we show that the combined use of enzyme immobilization and vibration is effective for protein digestion, even for a substrate with a rigid structure such as phospholipase A_2. Sequence coverage over 90% was attained within 3 h, and digestions at all expected recognition sites were observed. Compared to the conventional digestion method (liquid-phase digestion without vibration), sequence coverage was significantly improved. Accordingly, we propose that our new digestion method is applicable in further proteomics analyses.

Abbreviations

MS, mass spectrometry; MS/MS, mass spectrometry/mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; PTM, post-translational modification; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PDMS, polydimethylsiloxane; CID, collision-induced dissociation; ECD, electron capture dissociation;

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