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Short communication

Enzymatic protein digestion using a dissolvable polyacrylamide gel and its application to mass spectrometry-based proteomics

Nobuaki Takemori[a,*], Ayako Takemori[a], Jun Ishizaki[b], Hitoshi Hasegawa[b]

[a] Proteo-Science Center, Ehime University, Shitsukawa, Toon City, Ehime, Japan
[b] Department of Hematology, Clinical Immunology and Infectious Diseases, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime, Japan

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ABSTRACT

Enzymatic protein digestion in polyacrylamide gel has been used for sample pretreatment in mass spectrometry-based proteomics due to its effectiveness in removing contaminants that interfere with sample ionization. However, the difficulty of recovering the digested peptides from the solid gel matrix has been a drawback of this method. Here we have developed a novel in-gel digestion method to enhance peptide recovery using a dissolvable, bis-acrylylcystamine (BAC)-crosslinked polyacrylamide gel. After enzymatic protein digestion in BAC gel, we completely dissolved the gel by reductive treatment with tris-(2-carboxyethyl) phosphine to release the digested peptides from the gel. Our analysis revealed that the reductive dissolution of the BAC gel enhances the peptide recovery, which has a significantly higher protein identification capability than the conventional method, using an insoluble polyacrylamide gel. In addition, protein samples trapped in dehydrated BAC gel were stable at room temperature and reproducible sample recovery was obtained after storage for one week. These results indicate that the proposed method could be an effective tool for conducting sample pretreatment for mass spectrometry-based protein analysis.

1. Introduction

A high-throughput protein characterization method using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been often utilized for shotgun proteomics studies [1,2]. In this approach, proteins are initially digested into peptide fragments with site-specific endoproteinases such as trypsin. Following LC separation, the digested peptides are subjected to MS/MS analysis for protein identification and quantitation. Therefore protein digestion is an essential process for obtaining reliable experimental results in shotgun proteomics analysis.

In-gel enzymatic digestion has been widely used for sample pretreatment in shotgun proteomics, especially for detergent-solubilized samples, due to its compatibility with various detergents [3,4]. In this method, proteins are immobilized in a polyacrylamide gel with *N*,*N*′-methylene-bis-acrylamide (Bis), and interfering contaminants are effectively removed by washing the gel. In general, in-gel digestion is performed after protein separation using electrophoresis methods such as SDS-PAGE, which require tedious operations and are not suitable for the analysis of small samples. Recently, Lu and Zhu developed a "tube-gel" digestion protocol that does not require protein electrophoresis [5]. In this protocol, the solubilized proteins are directly mixed with monomeric acrylamide prior to polymerization, which allows us to use high concentrations of detergents (e.g. 4% SDS) for the solubilization of hydrophobic proteins. Tube-gel digestion and its modified protocols are especially powerful for the analysis of membrane proteins [5–8], and have been successfully used for various proteomics studies [9–11].

Despite such significant advantages, the difficulty of recovering the peptides from the solid gel matrix often results in poor identification and low sequence coverage in proteomics analyses. Previously, Zhou et al. reported the use of bis-acrylylcystamine (BAC), a disulfide-containing analog of bis-acrylamide, to improve the peptide recovery from the polyacrylamide gel [12]. They showed that the partial degradation of BAC-crosslinked acrylamide gel by incubation with dithiothreitol (DTT) or 2-mercaptoethanol is effective for releasing the peptides from the gel. However, the weak

Abbreviations: BAC, bis-acrylylcystamine; Bis, N,N′-methylene-bis-acrylamide; DTT, dithiothreitol; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; TCEP, tris-(2-carboxyethyl)phosphine; SRM, selected reaction monitoring.

* Corresponding author. Tel.: +81 89 960 5499; fax: +81 89 960 5961.
E-mail address: takemori@m.ehime-u.ac.jp (N. Takemori).

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reductive treatment involving DTT needs long incubation times to achieve peptide extraction, and partial degradation may not be adequate for comprehensive peptide recovery. Here, we demonstrated that the complete dissolution of the BAC gel matrix using tris-(2-carboxyethyl)phosphine (TCEP), a more powerful reducing agent than DTT, is effective for shortening the preparation time and maximizing the peptide recovery in sample pretreatment for shotgun proteomics. Moreover we evaluated the reproducibility of the enzymatic digestion and peptide recovery in the proposed BAC-gel digestion method by quantitative MS analysis.

2. Materials and methods

2.1. Materials

BAC was purchased from Polysciences, Inc. (Warrington, PA, USA), and other chemicals were purchased from GE Healthcare (Pittsburgh, PA, USA). LC–MS-grade solvents and Lys-C were purchased from Wako (Osaka, Japan). Sequence-grade trypsin was purchased from Promega (Madison, WI, USA).

2.2. Sample preparation

Solubilized *Drosophila* proteins which extracted from 50 fly heads (Canton-S) in SDS lysis buffer (4% SDS in 0.15 M Tris–HCl buffer, pH 8.8) were precipitated by using the methanol/chloroform/water method [13] and stored at −80 °C until use. Human serum samples were obtained from three healthy donors after obtaining informed consent in accordance with the procedures approved by the human ethics committee of the Ehime University. After depletion of the 14 major serum proteins using MARS spin cartridges Human-14 (Agilent Technologies, Santa Clara, CA, USA), the remaining proteins were precipitated and stored at −80 °C.

2.3. Enzymatic protein digestion using BAC gel

Resolubilized protein samples (5 μg total protein) in 5 μL of SDS lysis buffer were initially reduced with 1 μL of 200 mM DTT and then alkylated with 1 μL of 1.2 M acrylamide. Reductively alkylated protein solution (total 7 μL) was mixed with 5 μL of 30%/0.5% (w/v) acrylamide/BAC solution in a 0.2 mL tube. The mixed sample was polymerized with 1 μL of 1.5% ammonium persulfate and 0.5 μL of tetramethylethylenediamine. Synthesized polyacrylamide gel was washed twice with 2 mL of wash solution A (50% [v/v] methanol, 5% [v/v] acetic acid) for 30 min and once with 2 mL of wash solution B (50 mM NH_4HCO_3, 50% [v/v] acetonitrile) for 30 min. After washing, the gel was dehydrated with 200 μL of acetonitrile for 15 min and then air-dried at room temperature for 15 min. In-gel digestion with 0.1 μg of trypsin and 0.1 μg of Lys-C was performed in 20 μL of 100 mM NH_4HCO_3 solution at 37 °C. After dual enzyme digestion, the gel was dissolved by adding 40 μL of 25 mM TCEP solution with vigorous shaking for 10 min. The sample solution was mixed with 60 μL of acetonitrile and shaken for 30 s. After centrifugation at 15,000 × *g* for 3 min, the supernatant was transferred in a fresh plastic tube and subsequently concentrated in a vacuum centrifuge. The concentrated peptides were purified by using a self-made C18 STAGE tip.

2.4. Enzymatic protein digestion using Bis-acrylamide gel

To produce the Bis-acrylamide gel, 7 μL of protein solution was mixed with 5 μL of 30%/0.8% (w/v) acrylamide/Bis solution, 1 μL of 1.5% ammonium persulfate, and 0.5 μL of tetramethylethylenediamine. After in-gel digestion with trypsin and Lys-C, the digested peptides were extracted three times with 100 μL of 50% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid for 10 min.

2.5. In-solution digestion

The proteins (5 μg total protein) solubilized in 20 μL of 8 M urea buffer (8 M Urea, 2.5 mM EDTA in 100 mM Tris–HCl buffer, pH 8.8) were digested with 0.1 μg of Lys-C at 37 °C for 4 h. After Lys-C digestion, the protein solution was diluted with 80 μL of 100 mM NH_4HCO_3 and digested with 0.1 μg of trypsin at 37 °C for 12 h. Digestion was terminated by adding 1 μL of formic acid.

2.6. LC–MS analysis

LC–MS/MS and selected reaction monitoring (SRM) analyses were carried out using a QTRAP 5500 hybrid triple quadrupole/linear ion trap MS (AB SCIEX, Framingham, USA) coupled with an Eksigent nanoLC-Ultra system via a cHiPLC-nanoflex module (AB-SCIEX). Peptide separation was performed using a nano cHiPLC C18-reversed phase column (Chrome XP C18CL, 75 μm ID × 15 cm). Database search for human serum proteome was performed with the ProteinPilot software ver.4.5 (AB-SCIEX) against the International Protein Index (IPI) human protein database using the following parameters: modifications, biological modifications; instrument type, 5500QTRAP ESI; and search type, thorough mode. Database search for *Drosophila* proteome was performed against the Universal Protein Resource (UniProt) database. Quantitative analysis of the SRM data set was performed using the Skyline software [14].

3. Results and discussion

Fig. 1 shows the workflow of the proposed method involving the use of the BAC gel and the conventional digestion using the Bis-acrylamide gel. In the conventional method, multiple extraction steps are required for recovering the peptides from the gel matrix. On the other hand, the peptide recovery in our method is performed by a single dissolution step of the BAC gel. A piece of the BAC gel (Fig. 2A) was gradually degraded during incubation with TCEP and the complete dissolution was achieved in 5 min

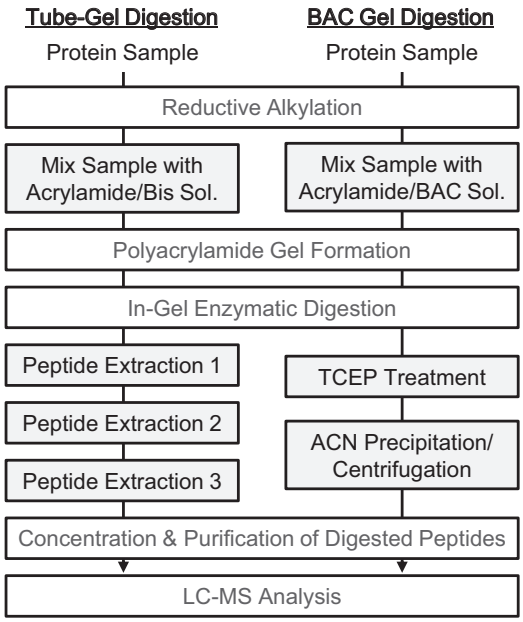

Fig. 1. Experimental workflow of the proposed digestion method using BAC-crosslinked polyacrylamide gel and conventional tube-gel digestion methods.