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Short communication

A robust new strategy for high-molecular-weight proteome research: A 2-hydroxyethyl agarose/polyacrylamide gel enhanced separation and ZnO–PMMA nanobeads assisted identification

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ABSTRACT

A new mass spectrometry based analysis strategy has been established here for high-molecular-weight (HMW) proteome research. First, a 2-hydroxyethyl agarose/polyacrylamide (HEAG/PAM) electrophoresis gel was designed for the first time to realize an easy-handling separation method with high spatial resolution for HMW proteins, good reproducibility and mass spectrometry-compatible sliver staining. Second, ZnO–polymethyl methacrylate (ZnO–PMMA) nanobeads were applied here for enriching and desalting the peptides from the HMW proteins. Third, the peptides were analyzed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) with the presence of the ZnO–PMMA nanobeads, and their MS signals were enhanced markedly. The success rate of identification for HMW proteins was significantly increased due to high enriching efficiency and salt tolerance capability as well as signal enhancing capability of the ZnO–PMMA nanobeads. We believe that this analysis strategy will inspire and accelerate the HMW proteome studies.

1. Introduction

One of the proteomics aims is to find biomarker candidates for biological or clinical research through the rapid identification of all the proteins expressed by cells or tissue. Proteins with the molecular weights above 100 kDa, which are commonly defined as high-molecular-weight (HMW) proteins, are known to be involved in a number of human diseases and some of them have been approved as cancer biomarkers, such as CA125 for monitoring ovarian cancer in serum, HMW CEA and mucin for monitoring bladder cancer in urine [1]. However, HMW proteome research is still a challenge.

One bottleneck is lack of highly efficient separation of HMW proteins from complex real samples. Polyacrylamide gel electrophoresis, which can separate proteins based on their isoelectric points (p*I*) and molecular weights, has dominated to the large-scale analysis of the proteins [2,3]. For separating HMW proteins with high resolution, the theoretic acrylamide percentage should be lower than 5% (w/v), and yet such a low-percentage polyacrylamide gel would be too fragile to handle with [4]. Besides, no polyacrylamide gel can be formed below 2.2% (w/v) [5], so the separating ability is limited. A 3–15% gradient polyacrylamide gel can be prepared to separate HMW proteins [6]; however, the other proteins are visible in the same gel, which makes the selective separation for HMW proteins inefficient. In pursuit of highly separation efficient for HMW proteins, many researchers used the agarose gel which has a larger average pore size than its polyacrylamide counterpart. For the isoelectric focusing (IEF), Oh-Ishi et al. used an immobilized pH agarose gel strip to make more HMW proteins get into the gel from the lysis buffer [7,8]. And for the vertical slab gel electrophoresis, Warren et al. reported a vertical agarose gel electrophoresis with the acrylamide plug to overcome the universal problem that the agarose gel would slide out of the plates during electrophoresis [9]. Unfortunately, the high-sensitivity staining method for the agarose gel is difficult to be compatible with mass spectrometry (MS), because it requires the complete drying of the gel to reduce excessive background staining, and dried agarose is refractory to the rehydration/dehydration cycles required for the in-gel proto-

Abbreviations: HEAG, 2-hydroxyethyl agarose gel; PAM, polyacrylamide gel; HMW, high-molecular-weight; IEF, isoelectric focusing; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; CHCA, α-cyano-4-hydroxycinnamic acid; ZnO–PMMA, ZnO–polymethyl methacrylate; APS, ammonium persulfate; SD, Sprague–Dawley; GST, glutathione-S-transferase; 1-DE, one dimensional electrophoresis; 2-DE, two dimensional electrophoresis; TOF, time-of-flight; MYO, horse heart myoglobin; BSA, bovine serum albumin; GRAVY, the grand average of hydropathy.

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cols [10,11]. Therefore, Suh and Chiari et al. introduced composite slab gels of agarose and polyacrylamide [4,12] and Roncada et al. reported a copolymer slab gel produced by acrylamide and allyl agarose [13]. Their gels can combine the advantages of the two components, and should be prepared at 50 °C by using a temperature control system to keep the agarose from gelling during the processing.

Low success rate of identification is the following problem for the HMW proteome analysis. The MS signals could be suppressed by the contaminants due to the long polypeptide chains of HMW proteins, which would be easily attacked by inorganic salts, chaotropes and detergents during separation procedure [7]. Besides, a large number of hydrophobic peptides of the HMW proteins make their ionization in matrix-assisted laser desorption/ionization (MALDI)-systems difficult by using the commercial organic matrices, such as α-cyano-4-hydroxycinnamic acid (CHCA) [14,15]. Therefore, extra two steps for HMW protein identification are needed, including desalting and signal enhancement.

Herein, we report a new analysis strategy for HMW proteome: using a new gel electrophoresis designed for HMW protein separation, ZnO–polymethyl methacrylate (ZnO–PMMA) nanobeads for enriching and desalting peptides simultaneously as well as enhancing signals in MALDI-MS.

2. Materials and methods

2.1. HEAG/PAM gel preparation

Every 800 mg 2-hydroxyethyl agarose (low-melting-point agarose, GE Healthcare) was melted in every 100 mL buffer A [375 mM Tris–HCl (Sigma) pH 8.8 (Tris–HCl 8.8), 0.1% SDS, w/v, Sigma] by a 70 °C water bath and cooled at room temperature (0.8% HEAG). 7% acrylamide (Sigma) was diluted by buffer B [375 mM Tris–HCl 8.8, 0.1% SDS, w/v, 0.05% ammonium persulfate (APS, w/v, Sigma)] from a stock solution of 30% acrylamide (w/v) and 0.08% N,N′-bis-methylene acrylamide (w/v, Sigma) at room temperature (7% PAM). Then 0.8% HEAG and 7% PAM were mixed at the volume ratio of 1:3, 1:2 or 1:1. 0.06% TEMED (v/v, Sigma) was added immediately, before transferring the mixed gel solution to the vertical gel casting mold. The three kinds of composite gels were defined as HEAG/PAM 1 (0.2% HEAG–5.3% PAM, w/v), HEAG/PAM 2 (0.3% HEAG–4.7% PAM, w/v) and HEAG/PAM 3 (0.4% HEAG–3.5% PAM, w/v), respectively. After pouring the gel, overlay the solution with water-saturated butanol to prevent exposure of the acrylamide to oxygen and create a flat gel surface.

2.2. Sample preparation

The total proteins were extracted from the colon of Sprague–Dawley (SD)-rat by a lysis buffer [8 M urea, 2 M thiourea, 4% CHAPS, 1% Nonidet P-40, 1% Triton X-100, 1 mM PMSF and Protease Inhibitor Cocktail; Sigma], purified by Clean-up Kit (GE Healthcare) before separation. Protein concentration was determined using the Bradford method. Proteins for glutathione-S-transferase (GST) pulldown experiment were extracted from the brain of SD-rat by a lysis buffer [50 mM HEPES, 25 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and Protease Inhibitor Cocktail, pH 7.4; Sigma].

For one dimensional electrophoresis (1-DE) separation, molecular mass standards from 10 to 250 kDa (Precision Plus Protein Unstained Standards, Bio-Rad) and proteins from SD-rat brain through GST pulldown experiment were electrophoresed by a discontinuous system: a 3% PAM gel as the stacking gel and the HEAG/PAM gel or a 7% PAM gel as the separating gel. For two dimensional electrophoresis (2-DE) sample preparation, every 500 μg proteins from SD-rat colon were firstly separated by 7-cm nonlinear pH 3–10 IPG strips (GE Healthcare). Then the strips were reduced, alkylated and embedded on the top of the HEAG/PAM gels. Each gel ran with Tris buffer (25 mM Tris–HCl, 192 mM glycine and 0.1% SDS) at 10 mA/gel for 2–2.5 h until the dye front ran to the end of the gel.

After the electrophoresis separation, all of the gels were visualized by silver staining method [16]. Before in-gel digestion, the excised spots were incubated with trypsin at 4 °C for 15 min and covered with 30 μL of 25 mM NH_4HCO_3. Then proteins were digested by trypsin (sequencing level, freshly diluted in 25 mM NH_4HCO_3; Roche) at 37 °C overnight. After in-gel digestion, peptides were extracted sequentially.

2.3. Enriching and desalting peptides

A 0.5-μL aliquot of ZnO–PMMA suspension (2.5 μg/μL) was used to enrich and desalt the extracted peptides of each spot from the 2-D maps according to our previous research [17]. The deposit containing the enriched sample was resuspended in 0.8 μL matrix (5 mg/mL CHCA in 50% acetonitrile/0.1% trifluoroacetic acid; Sigma) and spotted on the MALDI plate.

2.4. MALDI-MS analysis and data processing

All mass spectra were acquired by a 4700 Proteomics Analyzer MALDI-time-of-flight (TOF)/TOF-MS (Applied Biosystems). The instrument was operated at an accelerating voltage of 20 kV. A 200 Hz pulsed Nd-YAG laser (355 nm) was used for MALDI. The MS instrument was calibrated by trypsin-digested peptides of horse heart myoglobin (MYO, Sigma) with known molecular masses. Peptides were analyzed in reflector TOF detection mode. All spectra were taken from the signal average of 2000 laser shots. The laser intensity was kept constant. The five strongest peaks in each mass spectrum were automatically selected for MS/MS analysis. GPS Explorer software (version 3.6, Applied Biosystems) with Mascot (version 2.1, Matrix Science) as a search engine was used to identify proteins against the international protein index v3.25 (IPI_RAT_3.25) database. All proteins were identified using the peptide fingerprint mass spectra combined with tandem mass spectra. The searching parameters were set up as follows: the enzyme was trypsin, the number of missed cleavages was allowed up to 1, the variable modification was oxidation of methionine, the peptide mass tolerance was 100 ppm, and the tandem mass tolerance was 0.5 Da. Protein scores greater than 59 were considered significant (probability $p < 0.05$).

3. Results and discussion

3.1. Performances of the HEAG/PAM gel

To achieve highly efficient separation, the new composite slab gel of HEAG/PAM was applied here. 2-Hydroxyethyl agarose has been designed to separate HMW RNA/DNA for its melting point (m.p. 65 °C) is much lower than that of the agarose molecule (m.p. 95 °C), with the result that unacceptable thermal degradation of high-molecular-weight RNA/DNA can be prevented when recovering them from the separating gel [18]. It is also essential to use this hydroxyethylated agarose for HMW protein separation, instead of the standard agarose reported in other composite gels. The gels containing standard agarose (g.p. 45 °C) should be prepared at 50 °C to prevent them from quick gelling when being poured into the narrow gap of two assemble plates of vertical electrophoresis apparatus [4,12,13]. By contrast, the melting 2-hydroxyethyl agarose