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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

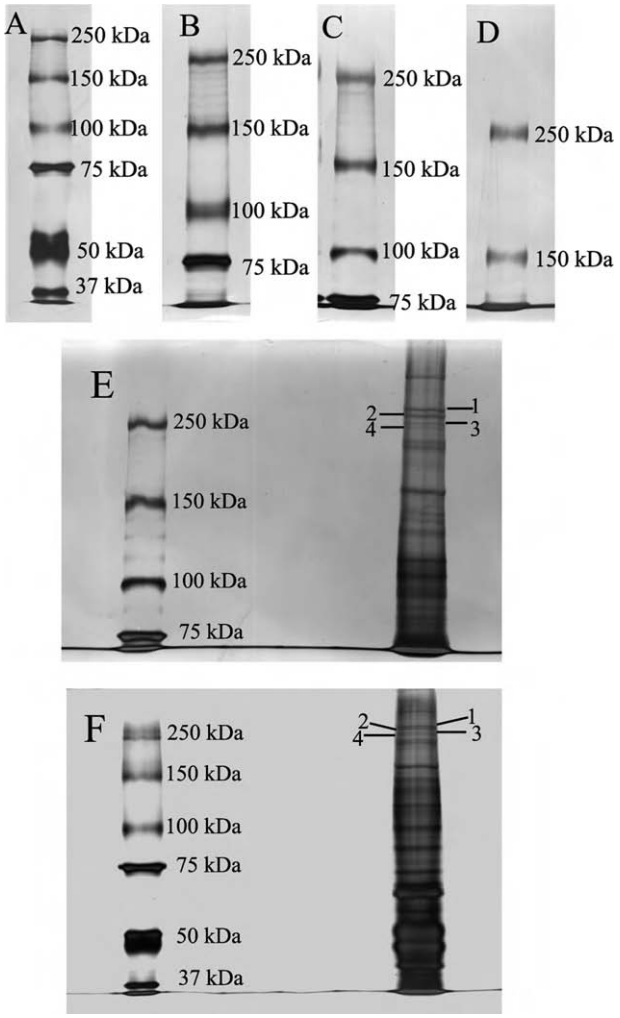


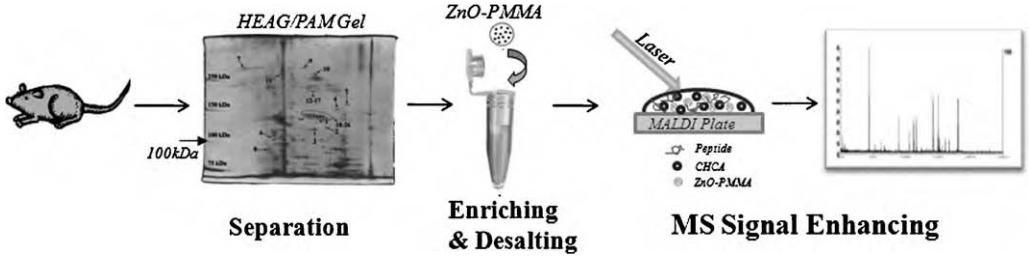
Fig. 1. 1-DE maps of the molecular mass standards (A) 7% PAM, (B) HEAG/PAM 1 (0.2% HEAG–5.3% PAM, w/v), (C) HEAG/PAM 2 (0.3% HEAG–4.7% PAM, w/v), and (D) HEAG/PAM 3 (0.4% HEAG–3.5% PAM, w/v). 1-DE maps of proteins from SD-rat brain through GST pulldown experiment with the different kinds of the second dimensional gels (E) HEAG/PAM 2, and (F) 7% PAM. After the electrophoresis separation, the gels were visualized by standard MS-compatible silver staining method. Four of the Arabic numeral mark bands assigned to the isoforms of spectrin.

can be kept in liquid phase for almost 1 h at the room temperature before it forms a gel owing to its gelling point (g.p. 26 °C). In virtue of its easy handling, the 2-hydroxyethyl agarose was utilized in gel preparation for HMW proteins separation by the vertical electrophoresis apparatus. After electrophoresis, this composite gel was visualized by standard MS-compatible silver staining method [16] with low background as a polyacrylamide gel.

The HEAG/PAM gel with high spatial resolution is displayed in Fig. 1. A 5-μL aliquot of the protein molecular mass standards from 10 to 250 kDa was electrophoresed by a discontinuous system: a 3% PAM gel as the stacking gel and a series of HEAG/PAM gel or a 7% PAM gel as the separating gel. Six of the standards of MW between 37 and 250 kDa were visualized in the 7% PAM gel (Fig. 1A). As the percentage of 2-hydroxyethyl agarose increased in the HEAG/PAM gel, the relative mobility of the same protein (the ratio of the migration distances of a protein to the dye front on the same gel) obviously increased. Fig. 1B–D shows the images of these three kinds of gels. Molecular weight curves of proteins were plotted by detecting their relative mobility (Supplementary Fig. 1). In good agreement with the Ferguson method [19], the relative mobility was linear with denary logarithm of protein MW among the four gels. Therefore, the MW range of proteins could be calculated for the 52-mm height separating gel: 35–270 kDa for a 7% PAM gel; 60–330 kDa for HEAG/PAM 1; 75–360 kDa for HEAG/PAM 2; 125–420 kDa for HEAG/PAM 3. As for the biological sample, isoforms of spectrin from SD-rat brain through GST pulldown experiment are separated with higher efficiency by the HEAG/PAM gel (HEAG/PAM 2, 0.3% HEAG–4.7% PAM, w/v) than by the 7% PAM gel, shown in Fig. 1E and F. Three spectrin isoforms are well separated in different bands by the HEAG/PAM gel, shown in Fig. 1E: spectrin alpha chain (MW: 285.3 kDa) in the band 1; non-erythroid spectrin beta (MW: 273.6 kDa), in the band 2 and 3; spectrin beta chain (MW: 271.1 kDa) in the band 4.

3.2. MS signal enhancement in the presence of ZnO–PMMA nanobeads

Interestingly, we found MS signals could be enhanced with the presence of the ZnO–PMMA nanobeads here. Every 5-ng digested bovine serum albumin (BSA, Sigma) or MYO was mixed with 4-μg CHCA as the matrix on the MALDI plate, and then 1.25-μg nanobeads were spotted on it. Fig. 2 displays the mass spectra of the tryptic digestions of standard proteins with the presence or absence of the ZnO–PMMA nanobeads. The S/N of almost all peptides increased to 2–9-fold in the presence of the ZnO–PMMA nanobeads. In MALDI-system, Kinumi and Watanabe et al. have reported to use ZnO nanoparticles as inorganic matrix for surface-assisted laser desorption/ionization of the target molecules [14,20]. Furthermore, Shastri, Kailasa and Shrivas et al. have successfully applied many other quantum dots such as functional ZnS, ZnSe and CdSe nanoparticles to assist ionization of the analytes [21–23]. It is all because that quantum dot nanoparticles have the excellent ultraviolet adsorption property and can transfer energy to the analytes [14,21–23]. In our experiment, the ZnO nanocores and ZnO–PMMA also exhibited photochemical property by ultraviolet laser, and the estimated quantum yields for each of them were 85% and 22%, respectively [24]. Influenced by PMMA-shells, our ZnO–PMMA nanobeads could not work as matrix independently for their relatively low quantum yields. However, the ZnO–PMMA nanobeads could still enhance the signal when they are dried with CHCA on the MALDI plate.



Scheme 1. A robust new analysis strategy for high-molecular-weight proteome.

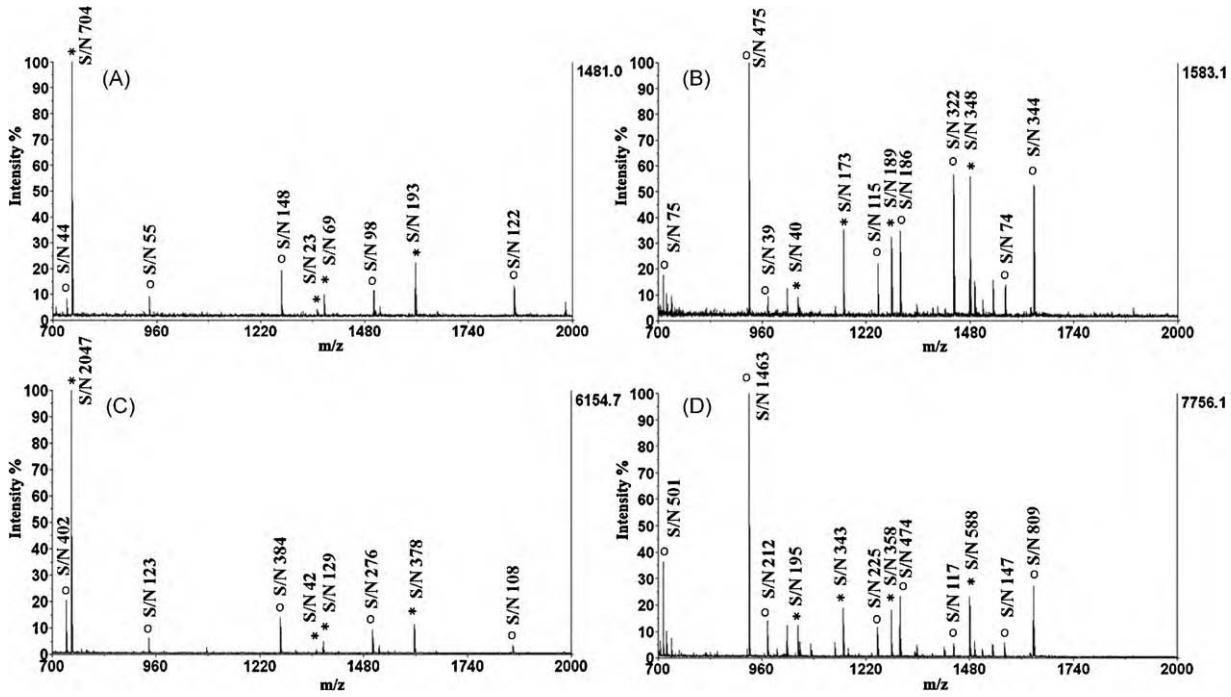


Fig. 2. Mass spectra of (A) 5-ng digested MYO, (B) 5-ng digested BSA, (C) 5-ng digested MYO and 1.25-μg ZnO-PMMA, and (D) 5-ng digested BSA and 1.25-μg ZnO-PMMA, using CHCA as the matrix. All mass spectra were acquired by a 4700 Proteomics Analyzer MALDI-TOF/TOF-MS (Applied Biosystems). Circles marked peaks assigned to hydrophilic peptides (GRAVY < 0). Asterisks marked peaks assigned to hydrophobic peptides (GRAVY < 0).

3.3. Analysis strategy for HMW proteome research

The analysis strategy for HMW proteome is shown in Scheme 1: using the HEAG/PAM gel electrophoresis for HMW protein separation, ZnO–PMMA nanobeads for enriching and desalting peptides as well as enhancing signals in MALDI-MS.

3.3.1. HEAG/PAM gel enhanced 2-DE separation

The HEAG/PAM gel was applied to separate the proteins extracted from SD-rat colon for HMW proteome research. The 65-mm height 2-DE map, of which HEAG/PAM 2 (0.2% HEAG–5.3% PAM, w/v) are used in the second dimension, is shown in Fig. 3. Good resolving ability was due to the large average pore size of the improved gel which provided large tunnels for HMW proteins to separate effectively. In addition, the HEAG/PAM gel has a good reproducibility. Three replicated 2-DE gels of this kind are shown in Supplementary Fig. 2. The average protein spots of 274 were calculated by ImageMaster 2-D (GE Healthcare) software in the range of high molecular weight (RSD: 7.6%). And all 26 protein spots which were chosen randomly on the gel in Fig. 3 could be visualized on each of the replicated gels in Supplementary Fig. 2. That is because it was easy to control the processing of gel preparation by using 2-hydroxyethyl agarose, and the components could be adequately mixed to form a homogeneous reproducible slab gel.

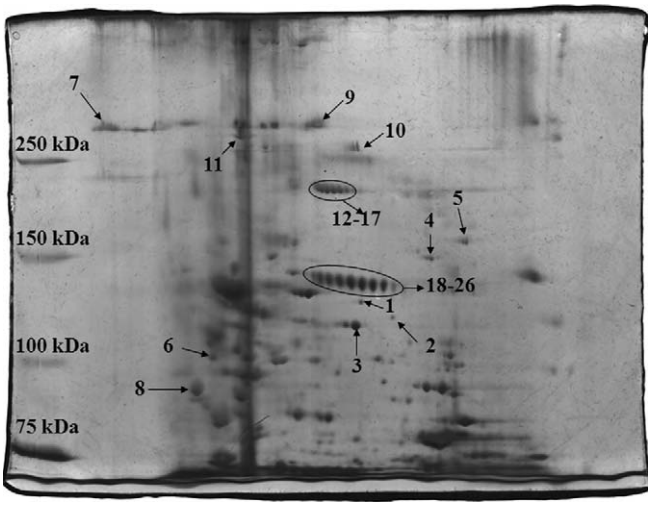


Fig. 3. 2-DE maps of proteins from SD-rat colon with HEAG/PAM 1 as the second dimensional gel. After the electrophoresis separation, the gels were visualized by standard MS-compatible silver staining method. Arabic numerals make spots assigned to the proteins shown in Table 1 and the same Arabic numeral marks the same protein in all 2-DE maps in Supplementary Fig. 2.

3.3.2. ZnO–PMMA nanobeads-assisted identification

Peptides extracted from each of the 26 HMW protein spots were identified by MALDI-MS after they were processed by the ZnO–PMMA nanobeads. After enriching/desalting steps, the ZnO–PMMA nanobeads were directly spotted on the MALDI plate without nanobeads removal and formed a homogenous layer on the MALDI plate, as we have previously described [17]. As a result, all of the 26 protein spots on the gel shown in Fig. 3 were successfully identified. The results are shown in Table 1. Spot 12–17 or spot 18–26 were identified as the same protein, respectively, which could be under the posttranslational modification condition. Without the pretreatment of ZnO–PMMA nanobeads, only 7 of these 26 protein spots could be successfully identified. The results are shown in Supplementary Table 1. High success rate of identification was ascribed to high enriching efficiency and salt tolerance capability of the ZnO–PMMA nanobeads. Moreover, in the results of the ZnO–PMMA method, 156 of 501 detected peptides (31.1%) from all the 26 identified protein spots were found with GRAVY value [25] above 0. As for the same protein spots which were lyophilized without any other processing before MS analysis, 35 of 142 detected peptides (24.6%) from 7 identified protein spots were found. Thus, it was observed that more peptides including the hydrophobic ones could be detected by the ZnO–PMMA method than those by the

Table 1
List of proteins from SD-rat colon separated by 2-DE and identified by ZnO–PMMA nanobeads method.[a].

Spot no.	Protein description	MW (Da)/p*I*	Protein score	No. of matched peptides	Sequence coverage (%)
1	Leucine rich protein 157	156554/6.20	261	20	15
2	Pc protein	129695/6.34	398	28	26
3	Similar to vinculin	116542/5.83	537	33	35
4	Similar to thyrotropin-releasing hormone degrading ectoenzyme	121900/6.85	61	17	21
5	170 kDa protein	169981/7.45	74	16	12
6	Transitional endoplasmic reticulum ATPase	89478/5.14	478	28	34
7	Similar to filamin-A	281116/5.69	65	6	3
8	Heat shock protein HSP 90-beta	83098/4.97	612	16	29
9	Similar to filamin-A	281116/5.69	58	10	5
10	Tln1 protein	270872/5.95	149	18	10
11	Spectrin alpha chain, brain	284537/5.23	344	55	25
12	Similar to alpha 3 type VI collagen isoform 1 precursor	288447/8.37	131	17	6
13			143	30	10
14			62	27	10
15			124	31	10
16			130	22	10
17			58	22	7
18	Similar to procollagen, type VI, alpha 2	109591/6.18	92	7	10
19			148	10	10
20			149	14	12
21			263	8	8
22			357	10	11
23			446	16	14
24			467	19	17
25			274	6	6
26			236	9	9

[a] The proteins were separated by 2-DE map with HEAG/PAM 1 gel (0.2% HEAG–5.3% PAM, w/v) as the second dimensional gel. Every extraction solution of protein spots was processed by the ZnO–PMMA method and then identified by a 4700 Proteomics Analyzer MALDI-TOF/TOF-MS (Applied Biosystems). Search parameters, IPI_RAT_3.25 database; digest used, trypsin; maximum of missed cleavages, 1; peptide mass tolerance, 100 ppm; tandem mass tolerance, 0.5 Da; variable modification, oxidation of methionine. GPS Explorer software from Applied Biosystems with Mascot as a search engine was used to identify proteins. All proteins were identified by using the peptide fingerprint mass spectra combined with tandem mass spectra. Protein scores greater than 59 were considered significant (probability $p < 0.05$).

traditional lyophilization method. The MS signal enhancement in the presence of ZnO–PMMA nanobeads could be another reason for high success rate of HMW protein identification.

4. Conclusion

This is the first time to use a 2-hydroxyethyl agarose/polyacrylamide gel for HMW protein separation and ZnO–PMMA nanobeads for MS signal enhancement. The strategy for HMW proteome research has been established here to provide a high efficient separation method, a useful enriching and desalting method, and a signal enhancing method. Therefore, we believe that it might be an alternative method for high-throughput proteome research of interests in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.06.034.

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