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Preparation and application of a partially degradable gel in mass spectrometry-based proteomic analysis

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ABSTRACT

In-gel digestion is an attractive route in mass spectrometry-based proteomic analysis, which, however, often suffers from a certain amount of sample loss mainly due to insufficient protein digestion and peptide extraction. To address this, herein we establish a partially degradable gel-assisted protein digestion and peptide recovery method by means of a simple replacement of bis-acrylamide (BA) with bis-acrylylcystamine (BAC). Concretely, the protein sample solubilized using high concentrations of sodium dodecyl sulfate (SDS) and urea were directly entrapped and immobilized into BAC-crosslinked gel by vacuum-dried gel absorption followed by fixation treatment. After removal of SDS and urea by repeated washing, the proteins were subjected to in-gel digestion and the gel was reductively treated. The tryptic peptides were recovered from the partial degradation of the gel and analyzed afterwards by capillary liquid chromatography coupled with tandem mass spectrometry (CapLC–MS/MS). Compared with conventional BA-crosslinked gel method, this new method increased the numbers of identified proteins and unique peptides by 20.2% and 20.4%, respectively. The further statistical analysis demonstrated that the method improved the recovery of tryptic peptides particularly larger and/or hydrophobic peptides, thereby significantly facilitating protein identification. Thus, the newly developed method is a promising alternative for BA-crosslinked gel-based shotgun workflows and has potential application in the related fields of protein chemistry and proteomics.

1. Introduction

"Bottom-up" approach has become a key technology in mass spectrometry (MS)-based proteomic analysis. When the approach is applied to large-scale analysis of highly complex protein mixtures, it is often known as "shotgun proteomics" [1,2]. In this typical analysis, protein digestion is often the most critical step as it involves reduction of intact proteins into a collection of peptides suitable size for MS analysis. In gel-based shotgun proteomics, incomplete protein digestion and insufficient peptide extraction are likely the main limitations for the identification of proteins. Effective digestion of proteins, which is one of the most important factors determining the subsequent peptide recovery, often requires good dissociation of protein complexes, denaturation and solubilization of protein individuals and protease accessibility.

Proteins exist within cells/tissues as tightly associated protein complexes or insert into lipid layers. For efficient and effective extraction and solubilization of these proteins, various detergents and chaotropes have been widely employed in biomedical studies [3–8]. In general, sodium dodecyl sulfate (SDS) is used as a powerful and universal agent for sample preparation. Unfortunately, even in small concentrations, SDS could significantly reduce the activity of proteolytic enzymes and seriously interfere with reversed-phase liquid chromatography and MS analysis [4,7]. Thus, its removal is a prerequisite for the efficient protein identification. An alternative to SDS is the use of urea for sample treatment. Urea is a chaotrope that commonly used to disrupt native hydrophobic interactions and unfold the proteins. It can readily be removed by liquid chromatography due to its not binding to ion-exchange or reversed-phase resins, thus not affecting peptide analysis by standard LC–MS/MS.

Abbreviations: BA, bis-acrylamide; BAC, bis-acrylylcystamine; CapLC–MS/MS, capillary liquid chromatography coupled with tandem mass spectrometry; GO, gene ontology; GRAVY, grand average hydrophathy value; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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However, a drawback of urea is that it causes problematic carbamylation of N termini and lysine residues by isocyanate formed in the decomposition of itself. The carbamylation could block sites of trypsin digestion and contribute to false protein identification in MS analysis due to the occurrence of artefactual modifications of peptides [6,8].

Because the use of some commonly used detergents and chaotropes in sample preparation often interferes with proteolysis and LC–MS/MS analysis, the influence must be eliminated prior to the subsequent experimental steps. To specifically target this requirement, the tube-gel digestion protocol was developed, and yet it unavoidably led to significant protein loss because proteins could not be completely incorporated into the polyacrylamide gel matrix [9–11]. For avoiding the protein loss and adverse chemical modification during the gel-embedment process [12], we established an improved sample preparation based on vacuum-dried gel absorption. Compared with the tube-gel digestion method, the improved method produced higher recovery of tryptic peptides, thereby significantly facilitating the identification of proteins especially hydrophobic proteins [13]. Nevertheless, in general, in these gel-based approaches insufficient extraction of peptides especially larger and/or hydrophobic peptides from the gel seems to be an untunably difficult problem, which can be attributed to the inherent adhesion surface provided by acrylamide network [14,15].

Aiming to further improve protein identification on the basis of the previous work, we set out to use bis-acrylylcystamine (BAC) as a reversible cross-linker in polyacrylamide-gel formation to substitute for common bis-acrylamide (BA). BAC is a disulfide-containing analogue of BA, as shown in supplementary Fig. 1. After polymerization, the BAC-crosslinked gel (BAC-gel) can be degraded in the presence of suitable reducing agents such as β-mercaptoethanol (β-ME) and dithiothreitol (DTT), which eliminate the crosslinks by reducing the disulfide bond [16–18]. This property of the gel would be in favor of the recovery of biomacromolecules from the polymerized gel [18]. In view of its particular superiority, shortly via introduction of 10% BAC-gel began to establish a new improved sample preparation method for shotgun proteomic s. Like our previous protocol, the denatured proteins were immobilized in the gel matrix via vacuum-dried gel absorption followed by fixation treatment, and the interfering substances from the protein solution were then removed by in-gel washing steps. After in-gel digestion, the tryptic peptides were released by the partial degradation of the gel by the addition of β-ME and subsequently subjected to MS analysis. Compared with the conventional BA-crosslinked gel (BA-gel) method, the newly developed method resulted in higher peptide yields, thereby significantly improving the protein identification. The valuable insights from our study demonstrate that the method holds great promise in gel-based sample preparation for shotgun proteomic analysis.

2. Materials and methods

2.1. Materials

Ammonium persulfate (AP), iodoacetamide (IAA), N,N,N′,N′-tetramethylethylenediamine (TEMED), acrylamide, BA, SDS, DTT, urea and Bradford protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). BAC was obtained from Sigma–Aldrich (St. Louis, MO, USA). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Water was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA, USA). All other reagents were domestic products of the highest grade available.

2.2. Protein sample preparation

Adult male Sprague-Dawley rats (weighting 200–250 g) were from the Chongqing Medical University (Chongqing, China). Rats were killed by decapitation, and their hippocampal tissues were quickly excised. All fresh tissues were homogenized on ice in a Teflon dounce grinder in 25 mM NH_4HCO_3 containing 2% SDS, 6 M urea, and protease inhibitors. After centrifugation, the protein mixture was reduced with 50 mM DTT at 50 °C for 1 h and alkylated with 40 mM IAA at room temperature (RT) in the dark for 45 min. The protein solution was then subjected to acetone precipitation, and the resulting pellets were resuspended in lysis buffer containing 2% SDS, 6 M urea and 25 mM NH_4HCO_3. The protein concentration was determined using Bradford protein assay kit with BSA as a standard.

2.3. Preparation of BAC- and BA-gels

An 80-μl BAC-gel (10%) was prepared in an Eppendorf tube according to the method reported by Seymour et al. with minor modifications [18]. Concretely, 40 μl of acrylamide/BAC (20% T, 3.9% C_{BAC}) solution, 20 μl of 1.5 M Tris–HCl (pH 7.0), 16.6 μl of H_2O, 0.5 μl of 1% AP and 2.9 μl of 100% TEMED were mixed in the tube, and then incubated at 40 °C for 1 h. After polymerization, the gel was thoroughly washed several times with H_2O until the pH of washing solution was near neutral. The gel was then dehydrated in 100% ACN and dried in a Speed Vac. For comparison, an 80-μl BA-gel (10%) preparation was performed as described in our previous study with slight modifications. Briefly, 26.7 μl of acrylamide/BA (30% T, 2.67% C_{BA}) solution, 48.5 μl of 25 mM NH_4HCO_3, 4 μl of 1% AP, and 0.8 μl of 10% TEMED were mixed in another tube. After complete polymerization at RT, the gel was washed several times, dehydrated in 100% ACN and dried in a Speed Vac.

2.4. Entrapment of protein sample by the vacuum-dried gels and the effect of fixation treatment on protein immobilization efficiency

For removing the SDS and urea that may interfere with the subsequent enzymolysis and MS analysis from the protein sample while retaining proteins in gel matrix, the above vacuum-dried BAC- and BA-gels were used to absorb aliquots of the protein solution (50 μg protein each). In order to improve the immobilization of the absorbed proteins in the gel matrix, the proteins-containing gels were treated with a gel fixation solution containing 40% (v/v) methanol and 10% (v/v) acetic acid at RT for 30 min. For comparison, the control gels without fixation treatment were also prepared. All the gels were separately washed four times with 50% ACN and the wash solutions of each gel were collected and pooled, and then subjected to 12% SDS-PAGE analysis, respectively, for detection of relative amounts of the protein loss.

2.5. Evaluation of protein recovery after BAC-gel degradation

As described above, the same amount of protein sample was entrapped and immobilized into 10% BAC-gel by gel absorption followed by fixation treatment. After being thoroughly washed, the gel was moderately homogenized using a pestle in 500 μl of the aforementioned sample lysis solution containing 2% β-ME (v/v) or 100 mM DTT [18], and then incubated at RT or 50 °C for 2 h, with intermittent gentle vortexing. After centrifugation at 15,000 × *g* for 10 min, the proteins in the supernatants were collected by acetone precipitation, and then used for 12% SDS-PAGE analysis.

2.6. In-gel protein digestion and peptide recovery

For digestion of the proteins embedded in gel matrix, all the gel lumps that had been treated with the fixation solution were cut into small pieces and washed four times with 50% ACN/25 mM NH_4HCO_3. The gel slices were then dehydrated in 100% ACN and dried in a Speed Vac. In-gel digestion was performed with trypsin (1:100 (w/w) enzyme-to-protein ratio) in 25 mM NH_4HCO_3 containing 10% ACN with incubation overnight at 37 °C. Peptide extraction from the BA-gel pieces was performed twice according to the conventional procedure as described previously [13], whereas the extraction from the BAC-gel was carried out mainly according to the procedure described by Seymour et al. [18]. The BAC-gel pieces were homogenized in 500 μl of 25 mM NH_4HCO_3 containing 2% β-ME, and then incubated at RT for 2 h, with intermittent gentle vortexing. The viscous partially-degraded gel was washed with 67% ACN containing 5% formic acid. The resulting sample solutions were pooled and centrifuged at 15,000 g for 10 min. The supernatants were concentrated in a Speed Vac for subsequent LC–MS/MS analysis.

2.7. CapLC–MS/MS analysis

The resultant digests were analyzed by automated capillary liquid chromatography coupled with tandem mass spectrometry (CapLC–MS/MS). LC was performed using an automated Agilent 1200 LC system equipped with an autosampler and an analytical capillary C18 PepMap column (180 μm i.d., 15 cm long, LC-Packings, Amsterdam, The Netherlands). Before separation on the reverse phase capillary column, the sample was desalted and pre-concentrated on a short C18 precolumn (Zorbax SB, 500 μm i.d., 3.5 cm long, Agilent). When the sample was separated on the C18 PepMap column, the flow rate was 3 μl/min and the column temperature was set to 25 °C. For the chromatography, the following solvents were used: solvent A (99.9% H_2O, 0.1% formic acid) and solvent B (99.9% ACN, 0.1% formic acid). The online LC separation used a gradient from 5% to 31% B in 137 min, 31% to 55% B in 10 min, 80% to 85% B in 9 min and then by 5% B for 11 min. The LC system was directly coupled to 3-D high-capacity ion trap mass spectrometer (HCTultra™, Bruker Daltonics, Bremen, Germany) with an electrospray ionization source. The system was controlled using Chemstation B01 (Agilent) and EsquireControl™ 6.0 (Bruker Daltonics) software. The nebulizer pressure was 10 psi. The flow rate of drying gas was 5 l/min. The temperature of drying gas was 300 °C. Capillary voltage was 4000 V. The full MS scan mode was standard enhanced (m/z 350–1600 Da). The five most abundant ions detected in each MS scan were selected for collision-induced dissociation (MS/MS) with 1.05 V collision energy using data-dependent MS/MS mode over the m/z range of 100–2000 Da.

2.8. Data processing

Raw spectrum data were processed and MASCOT compatible mgf files were created using Data Analysis™ 3.4 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 10,000, maximum number of compounds 100,000, retention time windows 1.0 min. Searches were performed using MASCOT™ software 2.2 (Matrixscience, London, UK), and the international protein index (IPI) rat database (vision 3.64, 39871 sequences, http://www.ebi.ac.uk/IPI) was used for peptide and protein identification. Search parameters were set as follows: enzyme, trypsin; allowance of up to one missed cleavage; peptide mass tolerance, 1.2 Da and MS/MS mass tolerance, 0.6 Da; fixed modification, carbamidomethylation (C); variable modification, oxidation (at Met). All searches were performed using MudPIT scoring model. The results reported with bold red ensured each identification protein with at least one unique peptide, and the ion score cutoff was set to 10. General protein identification was based on two or more peptides whose ions scores exceeded the threshold, $P < 0.05$, which indicated identification at the 95% confidence level [13,19]. If proteins were identified by a single peptide, the spectrum was manually inspected. For a protein to be confirmed, the assignment had to be based on four or more contiguous y- or b-series ions (e.g., y4, y5, y6, y7). Moreover, the false positive rate that calculated as described previously [20] was below 2%. Concretely, the exported mass spectra were searching using the MASCOT against a composite database that includes both regular and reverse protein sequences. Protein hits up to an accumulated false-positive rate of 2% were considered as true-positive protein identifications. This was calculated as the percentage of twice the number of hits from the decoy database to the total number of hits in both searches.

2.9. Bioinformatics analysis

The theoretical molecular weights (MW) and isoelectric points (p*I*) of the identified proteins were retrieved from MASCOT output files. The mapping of putative transmembrane domains (TMDs) for identified proteins was carried out using the TMHMM 2.0 program based on transmembrane hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM) by submitting the FASTA files [21]. The grand average hydropathy (GRAVY) values for identified proteins and peptides were calculated using the ProtParam software available at http://tw.expasy.org/tools/protparam.html. Further information on the subcellular location and function of identified proteins were predicted by gene ontology (GO) component and function terms, respectively. Text-based annotation files were available for download from GO database ftp site at ftp://ftp.geneontology.org/pub/go [22].

3. Results and discussion

3.1. Effect of gel fixation on the immobilization of proteins in gel matrix

For efficient removal of various interfering detergents and chaotropes from protein sample, various gel-based sample cleanup methods have been developed and applied [9–11,13]. In these methods, proteins were immobilized in gel matrix and the interfering substances were removed by repeated gel washing. Obviously, the effect of protein immobilization in the gel is a critical consideration. Therefore, we first evaluated the effect of gel fixation on the immobilization of proteins by determining the relative amounts of protein loss when the polyacrylamide gel (i.e., BA- or BAC-gel) was fixed or unfixed after gel absorption of the protein solutions. As shown in Fig. 1A, the wash procedure for the interference removal could cause a certain protein loss when the gels were not treated with fixation solution. In contrast, the fixation treatment after gel absorption could improve the immobilization of the proteins and decrease the protein loss during the repeated washing. The present results demonstrated that gel fixation is in favor of decreasing protein loss and is an essential experimental step in gel-based sample preparation strategies.

3.2. Protein recovery after chemical degradation of polymerized BAC-gel

Due to the strong interactions between the proteins and the commonly used BA-gel, it is usually difficult to efficiently recover intact proteins from the gel. To address this, preparation of degradable gel is one of the choices. BAC has a disulfide linkage which becomes an integral part of the crosslinkage on

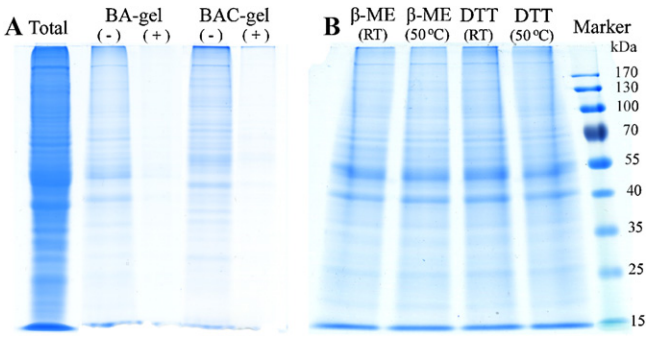

Fig. 1. SDS-PAGE analysis of proteins from the polymerized gels. (A) The effect of gel fixation on the immobilization of proteins in BA- and BAC-gels. Total, 50 μg of original total proteins; –, unfixation; +, fixation. (B) Evaluation of protein recovery after partially degradation of BAC-gel matrix. The gel was degraded by β-ME and DTT at RT or 50 °C.

copolymerization. After polymerization, the BAC-gel could be degraded by the addition of a reducing reagent such as β-ME and DTT [16–18], thus providing a possibility to extract more intact proteins from the gel for other special analyses. Likewise, an acid-degradable polyacrylamide gel had been prepared by use of N,N′-(7,7-dimethyl-3,6,8,11-tetraoxatridecane-1,13-diyl) diacrylamide as the crosslinker [23]. In the present study, protein recovery after this special degradation of BAC-gel was preliminarily assessed. From Fig. 1B, it could be found that, compared to the corresponding lane loading original total proteins in Fig. 1A, many proteins embedded in the BAC-gel matrix were recovered, and this could be explained by the great decrease in the interactions between proteins and gel that was caused by the partial degradation of the gel. In addition, there were no obvious differences between the different treatment temperatures and reducing reagents. In view of that β-ME is easier to lyophilize than DTT, we selected β-ME as the reducing reagent and a lower reaction temperature (i.e., RT) in the following experiments. The present result provides a valuable cue that BAC-gel might be applicable in gel-based protein and peptide sample preparation for proteomic analysis.

3.3. Application of BAC-gel to sample preparation for proteomic analysis

It is known that in most biological samples both soluble and insoluble proteins exist (e.g., in cell or tissue extracts). In this case, the buffer containing high concentrations of SDS and urea was commonly employed for complete extraction and solubilization of different types of proteins. Reduction/removal of detergents and chaotropes from protein sample is a prerequisite for highly efficient proteomic analysis. In this context, a partially degradable gel-assisted sample cleanup method was developed as shown in supplementary Fig. 2. Before being dried and used for adsorbing protein solution, the polymerized gel was thoroughly washed to remove residual reagents including acrylamide monomers, AP and TEMED for avoiding the possible side-reactions as mentioned in the literature [12]. After complete absorption of protein solution, the gel was treated with fixation solution and then repeatedly washed for removal of interfering components. The denatured proteins were retained in BAC-gel matrix in a protease-accessible form suitable for the subsequent digestion. The thorough gel washing provided a clean environment amenable to downstream buffer exchange and in-gel digestion to produce peptides for MS analysis. After BAC-gel degradation with the reducing reagent, the interactions between the resulting tryptic peptides and gel were greatly weakened, which in turn improved the recovery of the peptides from the gel. Therefore, the BAC-gel combined with in-gel

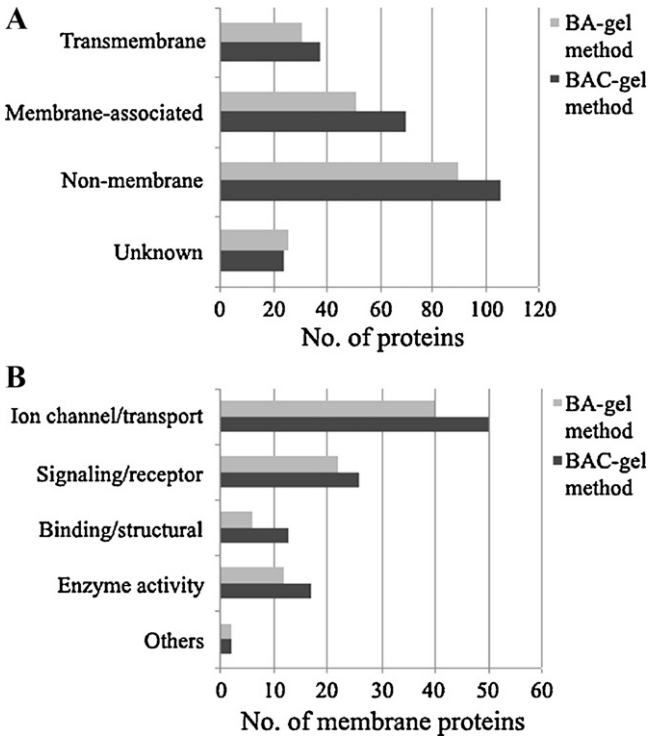

Fig. 2. Comparison and classification of the proteins and membrane proteins identified by BAC- and BA-gel methods. (A) The number of the proteins assigned based on membrane status from GO annotations. (B) The number of membrane proteins assigned based on their function classification, membrane proteins here included the transmembrane and membrane-associated proteins as described above. The consensus assignment classified each protein as being located on the plasma membrane, organism membrane or membrane proteins of uncertain localization. The merged results from triplicate analysis were used for comparison.

proteolysis and LC–MS/MS analysis would help to improve the identification of proteins in a complex biological sample.

3.4. Comparison of BAC- and BA-gels used in the analysis of rat hippocampal proteome

In the present study, the difference between new BAC-gel and conventional BA-gel methods in protein identification was probed by applying them to the shotgun proteomic analysis from a rat whole hippocampal tissue homogenate. The data of proteins identified in the two methods were compiled and compared. The identifications from triplicate experiments were merged and listed in supplementary Table S1. After de-redundance, a total of 238 and 198 non-redundant proteins were identified by BAC- and BA-gel methods, respectively. Compared with BA-gel method, BAC-gel method increased the number of identified proteins by 20.2%. As for identified membrane proteins including transmembrane and membrane-associated proteins, 108 were identified in BAC-gel method, increasing 31.7% compared with BA-gel method (Fig. 2A). In addition, BAC-gel method also led to an increase of non-membrane protein identifications. These data demonstrated that BAC-gel method could improve the identification of the embedded proteins. Moreover, it is worth noting that the increased extent of identified membrane proteins was higher than that of other proteins, suggesting that BAC-gel method may be more favorable for the identification of membrane proteins to a certain degree.

Generally, in the field of biological studies, the analysis of membrane proteins presents a special challenge due to their hydrophobic nature and low abundance, seriously complicating their solubilization and identification [24]. In this context, for further probing into the difference between the two methods in

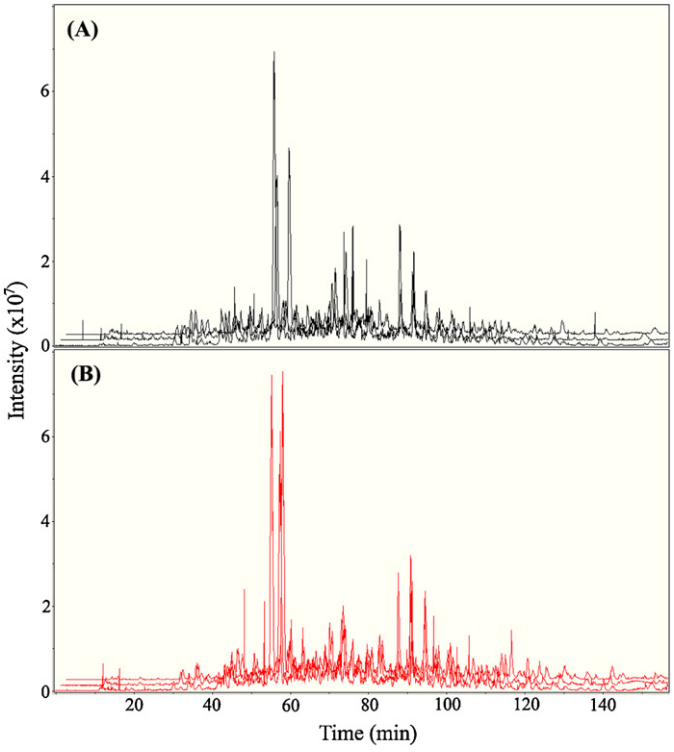

Fig. 3. LC–MS base peak chromatograms of the triplicate extracts from BA- (A) and BAC-gel (B) methods.

membrane protein identification, the identified membrane proteins were separately compiled and then categorized on the basis of their GO function annotation, although the classification was not strict due to multiple functions of a protein. As shown in Fig. 2B, of identified membrane proteins in the two methods, the proteins with ion channel/transport and signaling/receptor functions accounted for a large proportion, which were generally considered to be low abundant and hydrophobic, and the rest were binding and structural proteins, enzymes, etc. Importantly, in most bins, BAC-gel method led more proteins to be identified compared with BA-gel method, and was superior in the identification of membrane proteins.

In shotgun proteomics, protein identification is generally determined by the quality of mass spectra, which is heavily influenced by the amount of peptides introduced into mass spectrometer. After in-gel digestion, it is expected that the peptides with different physicochemical properties can be efficiently extracted from the gel by extraction buffer. However, the extraction of the peptides, especially larger and/or hydrophobic peptides, from the conventional BA-gel matrix remains unsatisfactory till the present day probably due to strong interactions between the peptides and the inherent adhesion surface provided by polyacrylamide network. In general, the peptide loss from conventional in-gel digestion protocol is 15–50% [25–27]. For improvement of the peptide recovery, we sought to take advantage of partial degradability of BAC-gel. Accordingly, BAC-gel was employed to replace BA-gel system for facilitating the extraction process of the tryptic peptides. By the way, the acid-degradable gel reported by Kim et al. [23] might also have similar applicability to our strategy. To gain insight into the efficiency of peptide extraction, the identified unique peptides in the two methods were compiled and compared. In this study, the BAC- and BA-gel methods allowed the identification of 839 and 697 peptides, respectively. It could be calculated out that BAC-gel method increased the identified peptides by 20.4%.

For further investigating the difference between BAC- and BA-gel methods in peptide recovery, the base peak chromatograms in the LC–MS analysis of the triplicate extracts were comparatively analyzed (Fig. 3). In most regions, the chromatograms of the extracts from BAC-gel (Fig. 3B) show more base peaks with higher intensity compared with those from BA-gel (Fig. 3A). It should be noted that this distribution tendency was more obvious in the time range of 80–140 min demonstrating that BAC-gel method could lead to more lager and/or hydrophobic peptides to be identified

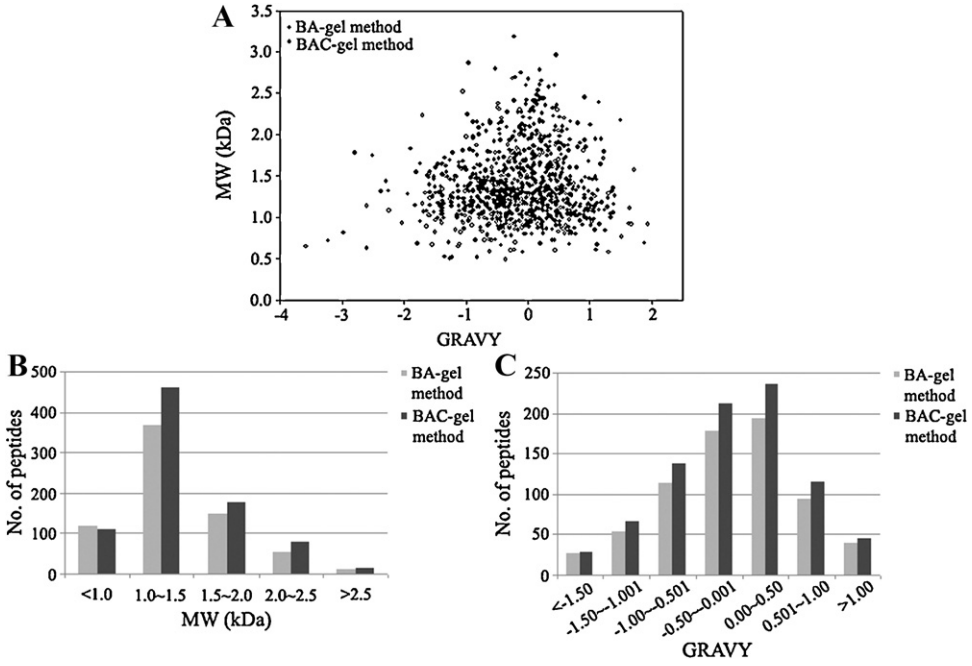

Fig. 4. Comparison of the unique peptides identified by BAC- and BA-gel methods. (A) Relationship between GRAVY value and MW of the peptides from the two methods. The white dots represent peptides from BA-gel method; the black dots represent peptides from BAC-gel method. (B) and (C) MW and hydrophobicity comparison of all peptides detected by LC–MS/MS in the two methods.

compared with BA-gel method, because these types of peptides tended to be eluted at higher organic solvent concentrations [28]. Further, the statistical analysis was carried out based on MW and GRAVY values of the unique peptides from the two methods. Fig. 4A presents the distribution ranges of MW and GRAVY value of tryptic peptides and the relationship between them. On the whole, the peptides identified by the two methods were mainly distributed in the MW range of 0.5–2.5 kDa and GRAVY range of −1.5 to +1.0. It could be observed that the larger peptides presented the hydrophobic tendency. Furthermore, Fig. 4B shows a normal distribution with the highest point at a MW value of 1.0–1.5 kDa. It could be discovered by detailed comparison that BAC-gel method led to more peptides to be identified in most subregions of MW distribution profile than BA-gel method, except for that <1.0 kDa. Regarding GRAVY value distribution, Fig. 4C shows that BAC-gel method led to identifying more peptides in every bin of the map. Taken together, these comparative results indicated that BAC-gel method produced better peptide recovery and increased the chances for the identification of larger and/or hydrophobic peptides, thus improving the identification efficiency of the proteins embedded in the gel matrix.

In conclusion, a partially degradable polyacrylamide gel was prepared and its new application in the field of MS-based proteomics was probed into. Compared with conventional BA-gel, this specific gel was more favorable for peptide recovery after in-gel digestion owing to its partial degradability, thereby giving rise to better identification efficiency of proteins particularly membrane proteins. Thus, as a robust and straightforward sample preparation matrix, it provides a particularly well-suited proteomic tool for comprehensive investigation of complex biological samples.

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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.jchromb.2011.08.017.

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