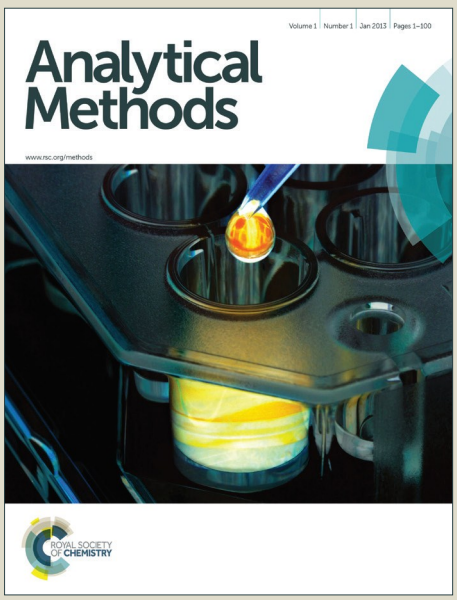
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Development and application of a wide-range gradient gel electrophoresis to proteome analysis

Hai Yu, Hui Liu, Yi-Zhong Yan, Zhi-Pan Dai, Shuai Yan, Jiang-Lin Li, Zhi-Gui Duan, Zhen Liu, Xian-Chun Wang*

Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Normal University, Changsha, 410081, P. R. China

Contact address:

Xianchun Wang
College of Life Sciences
Hunan Normal University
Changsha, Hunan 410081
P. R. China
Tel.:+86 731 8887 2556

E-mail: wang_xianchun@263.net

Footnotes:

* Corresponding author. Tel.: +86 731 8887 2556; E-mail address: wang_xianchun@263.net

ABSTRACT

SDS is widely used to treat the proteins difficult to solubilize and digest and improve protein separation in SDS-PAGE. However, SDS interferes with subsequent analyses and needs to be removed prior to digestion and LC-MS/MS analysis, whereas the conventional SDS-PAGE lacks the ability to efficiently remove SDS and retain low-molecular-weight proteins and peptides. In the present work, we developed a wide-range gradient gel electrophoresis (WGGE) system in a vertical slab gel electrophoresis cell, which was primarily composed of a 4-20% continuous gradient polyacrylamide gel separation layer and two interception layers with even higher concentrations (30% and 50%, respectively). The main advantages of the system are simultaneously cleaning up SDS-solubilized sample, separating proteins and intercepting low-molecular-weight proteins and peptides, thereby simplifying experimental operation, improving protein recovery and enhancing the total efficiency of proteome analysis. Using this system, about 87.25% of SDS in the sample and gel was electrophoretically removed and the a peptide with a molecular weight of 3.75 kDa was efficiently intercepted. Combined with CapLC-MS/MS, the WGGE system was applied to the analysis of rat liver membrane-enriched protein sample and the results indicated that the WGGE-based strategy is suitable for the identification of proteins varying in molecular weight, p*I*, hydrophobicity, etc., suggesting potential applications in global and comparative analyses of various proteomes.

Keywords: Gel electrophoresis; Wide-range gradient gel; SDS removal; Protein separation; Interception; Proteome

Introduction

In recent years, the proteomic study of soluble proteins has achieved rapid progress and, however, the analysis of the proteins difficult to solubilize such as most membrane proteins has lagged behind and needs to be strengthened. Numerous studies have shown that biological membranes play critical roles in the biological phenomena and processes, and membrane proteins perform membrane function as receptor, pore complex, ion channel, transporter and cell adhesion molecules.[1,2] Therefore, the research on the membrane proteins is greatly helpful for the elucidation of structure and functions of the biological membranes. However, up to now the membrane protein analysis is still a great challenge due to the fact that most of the membrane proteins have the nature of high hydrophobicity and low abundance.[3-7] Currently, it is considered the most effective method to use detergents such as sodium dodecyl sulfate (SDS) to improve the solubilization and extraction of insoluble membrane proteins.[8] Besides, SDS-dependent SDS-PAGE is the most classic method for the separation of a membrane protein mixture. Unfortunately, SDS of slightly higher concentrations (*e.g.* 0.1%) interferes with the activity of proteolytic enzymes, chromatographic separation of the digests, and mass spectrometric analysis.[9,10] Therefore, efficient removal of SDS from the sample and gel is crucial for the successful membrane proteome analysis. Conventionally, the SDS in the gel was removed by repeatedly washing after the completion of SDS-PAGE. Such a treatment not only complicates the experimental operation, but also causes some loss of proteins and peptides.

On the other hand, for removing SDS and other salts from a protein sample (sample cleanup), many conventional methods have been applied prior to SDS-PAGE, such as dialysis,

protein precipitation with organic solvents, gel filtration, solid-phase extraction, hydrophobic adsorption, ion-exchange chromatography, and ultrafiltration.[11-18] Although these methods can eliminate SDS and other salts from the protein samples to various degrees, they have their inherent limitations, including significant protein loss, and therefore are not suitable for the treatment of all kinds of protein mixtures particularly those in micro-amounts.

Recently, several new gel-based sample cleanup methods have been developed and applied in the field of membrane proteome research. For example, Lu *et al*.[19] have developed a gel-based sample cleanup method, tube-gel digestion (TGD), in which the SDS-containing protein sample is mixed with acrylamide monomer solution prior to gel polymerization. SDS and other salts are removed by repeatedly washing gel pieces after gel polymerization. Nevertheless, significant protein loss is still unavoidable in this method, because there is always a certain volume of sample solution excluded from the gel during the gel polymerization. Later, Liu *et al*.[20] described another form of gel-based sample cleanup method, three-layer sandwich gel electrophoresis (TSGE). The "three-layer sandwich" gel, generated in an Eluter glass tube, consists of an polyacrylamide sealing layer (bottom), a polyacrylamide concentration layer (middle) and an agarose loading layer (top). By electrophoresis, SDS is removed and the proteins are driven into the polyacrylamide concentration layer simultaneously. This method is helpful to reduce protein loss and suitable for the analysis of micro-scale protein samples. However, like the situation in TGD method, the protein mixture are is not separated during sample cleanup in TSGE method, which reduces the total efficiency of protein identification and limits the application of the method to the analysis of complex protein samples, although it can be coupled with 2D-LC-MS/MS

to reduce the complexity of the sample. In addition, the efficiencies of SDS removal and low molecular weight interception in the TSGE method have not been evaluated.

In view of the limitations of existing methods for the cleanup of detergent-solubilized protein samples particularly those in micro-amounts, our present work developed a wide-range gradient gel electrophoresis (WGGE) system, which is primarily composed of a continuous gradient (4-20%) polyacrylamide gel separation layer and two high-concentration interception layers (30% and 50%, respectively). By appropriately prolonging electrophoresis time, the WGGE system efficiently removed the SDS and at the same time separated proteins as well as intercepted the proteins and peptides with low molecular weights, thereby simplifying experimental operation and improving protein and peptide recoveries that is are helpful for the improvement of proteome analysis.

Experimental section

Materials

Proteomics sequencing-grade trypsin, ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), ammonium persulfate, phenylmethanesulfonyl fluoride (PMSF), iodoacetamide (IAA), dithiothreitol (DTT) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide, N, N´-methylenebisacrylamide, glycine, Tris and SDS were from Amresco (Solon, OH, USA). Coomassie brilliant blue G-250 and Bio-Rad RC-DC protein assay kit were from Bio-Rad (Hercules, CA, USA). Ultrapure 18.2-MΩ water obtained from a Millipore Milli-Q system (Bedford, MA, USA). All other reagents were the products of the highest grade available.

Establishment of wide-range gradient gel electrophoresis (WGGE) system

The WGGE system was established in a vertical slab gel electrophoresis cell (125 mm × 100 mm×1 mm) with a 4% polyacrylamide stacking layer, a continuous gradient (4% to 20%) polyacrylamide separation layer, and two polyacrylamide interception layers with high concentrations (30% and 50%, respectively). The concentration of crosslinker N, N′-methylene bisacrylamide was 5%. Briefly, 0.8 mL of 50% gel solution was first injected between the two glass plates, and after polymerization, 0.8 mL of 30% gel solution was overlaid on the first interception layer. Then, 10 mL of separation gel with a continuous gradient from 4% to 20% prepared with a gradient mixing device was placed over the 30% interception layer. Lastly, on the top of separation gel, 0.8 mL of 4% stacking gel solution was injected after the separation gel was polymerized.

In order to evaluate the ability of 50% polyacrylamide interception layer to intercept low-molecular-weight peptides, we designed a simple gel electrophoresis system consisting of a layer of 10% separation gel and a layer of 50% interception gel and used a 3.75-kDa peptide huwentoxin-I (HWTX-I)[21] as the model molecule. The SDS-PAGE was run at 50V for 2 h, 200 V overnight (about 11 h), and 600 V for 20 min. The bands at the interface between 10% and 50% gel layers and the 50% gel layer were excised and then subjected to in-gel digestion and mass spectrometry-based protein identification.

Rat liver membrane-enriched fraction preparation and protein extraction

Rats were purchased from Medical Academy of Central South University (Changsha, Hunan, China). Rat liver membrane-enriched sample was prepared using sucrose density gradient centrifugation and aqueous two-phase partitioning according to the methods previously described. [22-24] Briefly, the rats were sacrificed by decapitation after being starved for 18-24 h and the livers were excised. After removal of the gall bladder and blood vessels, the liver pieces were homogenized on ice in four times their weight of a cooled solution (50 mM HEPES, 1.0 mM $CaCl_2$ and 0.1 mM PMSF, pH 7.4) with a Tissue Tearor (Biospec products, CE 2000, Mexico) at 20000 rpm until completely liquefied. The mixture was transferred to 50-mL conical tubes and centrifuged at 600 g for 20 min at 4 ^{o}C. The supernatant was collected and the pellet was repeatedly treated as above. The collected supernatants were pooled and then centrifuged at 24 000 g (Ti70 rotor, Beckman, Fullerton, CA, USA) for 30 min at 4 ^{o}C. The membranes in the pellet were further enriched sequentially with a discontinuous sucrose density gradient centrifugation and an aqueous polymer two-phase system consisting of Dextran and polyethylene glycol. For density gradient centrifugation, a discontinuous sucrose density gradient (60%, 45%, 41% and 37%) was used. After centrifugation at 100 000 g (SW-28 rotor, Hitachi, Tokyo, Japan) for 2.5 h, the plasma membrane-enriched fraction at the interface between 37% and 41% sucrose solutions was collected, following followed by washing with 1.0 mM $NaHCO_3$ solution and centrifuging at 100 000 g twice. The resulting pellet was further purified on a 16 g aqueous polymer two-phase system prepared from stock solution of 20% (w/w) Dextran T500 and 40% (w/w) polyethylene glycol 3350. The two-phase system was constituted of 5.12 g of 20% Dextran, 2.56 g of 40% polyethylene glycol 3350, 0.4 ml of 0.2 M K_3PO_4 (pH 7.2) and 1.6 ml of 1.0

M sucrose, to which distilled water was added to a final weight of 14 g and then 2 g of the membrane pellet was added. After extensive mixing, phase separation was accelerated by centrifugation at 750 g for 5 min at 4 ^{o}C. The upper phase was collected and the lower phase was re-extracted with a fresh upper phase solution. The obtained two upper phase solutions were pooled and re-extracted by a fresh lower phase solution. The membrane debris in resulting upper phase was retrieved and washed. The obtained membrane-enriched fraction was stored at -80 ^{o}C until further use. All procedures conformed to the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animal.

For protein extraction, the enriched membranes were dissolved in a 4% SDS solution with sonication twice each for 30 min in a water bath. After centrifugation to remove the insoluble part, protein content of the sample was determined using a Bio-Rad RC DC protein Assay kit with BSA as a standard protein.

Electrophoretical cleanup and separation of protein sample

For electrophoretical sample cleanup and protein separation on the WGGE system, the membrane protein solution was mixed with the sample loading buffer (0.5 M Tris-HCl, 0.1 M DDT, 4% SDS, 20% glycerol and a trace of bromophenol blue, pH6.8) at a volume ratio of 1: 1, followed by boiling for 5 min and centrifugation. An aliquot of about 20 μL of the supernatant (containing 100 μg of proteins) was loaded into the sample well in the gel. The electrophoresis was run at 50 V until the bromophenol blue reached the separation gel, 100V overnight and 800 V until the tracking dye entered the last interception layer. The separated proteins in the gel were fixed in 50% methanol and 10% acetic acid (v/v) and then stained by

coomassie brilliant blue G-250. A PageRuler^{TM} Prestained Protein Ladder (Fermentas, Hanover, MD) was used as molecular weight standard.

Quantitative determination of the efficiency of SDS removal

In order to quantitatively determine the efficiency of SDS removal by WGGE system, a specially-designed electrophoresis was run under the same conditions as those described above, except that no SDS was added in the running buffer. Quantitative determination of SDS content was performed according to the method of Rusconi *et al.*[25] Briefly, the stains-all stock solution was prepared by dissolving 1 mg of stains-all dye in 1 mL of 50% isopropanol in water. Then the coloration solution was prepared by mixing the stock solution, formamide and ddH_2O at a ratio of 1:1:18. For establishment of SDS standard curve, 6 tubes were preloaded with 0, 2, 4, 6, 8 and 10 μL of 0.01% SDS stock solution, which corresponded to 0, 0.2, 0.4, 0.6, 0.8, 1.0 μg of SDS, respectively. ddH_2O was add to each tube to a final volume of 20 μL. 200 μL of the coloration solution were added into each tube, followed by vortexing. The running buffers in anode and cathode reservoirs of electrophoresis apparatus were sampled before and after electrophoresis and then treated as above. Light absorption values of all solutions at 438 nm were recorded with a spectrophotometer. For comparison, the content of SDS in a conventional SDS-PAGE with a 4.0% polyacrylamide stacking gel and an11.5% separating gel was also quantitatively determined with the same method. All the above the assays were performed in triplicate.

Protein digestion and identification

In-gel digestion of the electrophoretically-resolved proteins and capillary high performance liquid chromatography-tandem mass spectrometry (CapLC-MS/MS) analysis were carried out according to the methods reported previously in literature.[19,26,27] In brief, after the completion of WGGE, the lane gel was cut into slices of about 2 mm wide. The slices were further cut in small pieces and then the gel-bound proteins were reduced with DTT and alkylated by IAA. After the gel pieces were washed and lyophilized, trypsin dissolved in 50 mM NH_4HCO_3 was added and incubated at 37 ^oC for 16 h. The resulting peptides were extracted twice with 67% ACN containing 0.1% formic acid with sonication for 20 min. The extracts were combined and appropriately concentrated in a Speed-Vac and ready for CapLC-MS/MS analysis.

The CapLC-MS/MS analysis of the tryptic peptide samples was performed on an automated Agilent 1200 LC system (Agilent Technologies, Waldbronn, Germany) coupled with a 3D high-capacity ion trap mass spectrometer (HCTultra™, Bruker Daltonics, Bremen, Germany). In the front of the analytical capillary C18 PepMap column (180 μmi.d., 15 cm long, LC-Packings, Amsterdam, Netherlands), there was a short C_{18} precolumn Zorbax SB (500 μm i. d., 3.5 cm long, Agilent Technologies) that was used to concentrate and desalt the peptide sample. The peptides eluted from the analytical capillary column were directed into the mass spectrometer for MS/MS analysis. The instruments were controlled using Chemstation B01 (Agilent) and EsquireControl™ 6.0 (Bruker Daltonics) software. The main control parameters included: nebulizer pressure, 10 psi; flow rate of drying gas,5 L/min; capillary voltage, 4000 V; temperature of drying gas, 300 °C; full MS scan mode, standard-enhanced (m/z 350 to 1600). Peptides were analyzed in a positive mode and the five most abundant ions detected in each MS scan were selected for collision-induced dissociation (CID) using the data-dependent MS/MS mode over the m/z range of 200-2000.

The acquired raw MS and MS/MS data were processed using DataAnalysis™ 3.4 software (Bruker Daltonics, Bremen, Germany) and utilized to search against the international protein index (IPI) rat database containing 39871 protein sequences (IPI_rat_v3.64) for protein identification. The main search parameters were set as follows: enzyme, trypsin; allowance of up to one missed cleavage site; MS mass tolerance, 1.2 Da; MS/MS mass tolerance, 0.6 Da; fixed modification, carbamidomethylation (C); variable modification, oxidation (M). Proteins were identified on the basis of peptides whose ions scores exceeded the threshold, $P < 0.05$, which indicated identification at the 95% confidence level. The relevant information on the identified proteins was retrieved from the protein database.

Results and discussion

Establishment of WGGE system

The main aim of our present work is to develop an electrophoresis system that can efficiently remove SDS and separate proteins simultaneously and thus overcome the limitations of the existing sample cleanup methods. Theoretically, SDS removal can be easily achieved by prolonging the electrophoresis time, and the key issue is to develop a special gradient gel system that not only efficiently separates most of the proteins but also prevents small proteins and peptides from entering the lower reservoir as the electrophoresis time is prolonged. Thus, we developed a wide-range gradient gel electrophoresis (WGGE) system, which was primarily constituted of a 4-20% continuous gradient polyacrylamide gel layer, and two high-concentration polyacrylamide gel interception layers (30% and 50%, respectively), to meet the two criteria simultaneously. Liu *et al.*[20] used a 40% gel as the interception layer of their TSGE system and evaluated its intercepting efficiency using SDS-PAGE low-range protein standards (BSA, ovalbumin, carbonic anhydrase and lysozyme with molecular

weights of 66.43, 45, 30 and 14.4 kDa, respectively). Their results indicated that the protein recoveries approached 100%. When they applied the TSGE system in combination with 2D-LC-MS/MS to the analysis of a whole cell extract from the protozoan parasite *Toxoplasma gondii*, it was found that the smallest protein intercepted by the interception layer was a protein with a molecular weight of 7 kDa. These results suggest that there is necessity to further increase the concentration of the interception layer to intercept even smaller proteins and peptides. Therefore, in our present work, we used a 50% polyacrylamide gel as the last interception layer. In order to detect the ability of the interception layer to intercept peptides, we additionally designed a simple gel system, which was only composed of a 10% gel layer and a 50% gel layer, to electrophoretically run a 3.75-kDa peptide HWTX-I.[21]As shown in Fig.1, the peptide was electrophoretically driven to the interface between 10% and 50% gel layers and the prolonged electrophoresis time forced the peptide to extend to both sides. Using the proteomic strategy, HWTX-I was identified from the interface (Fig.2) and not from the 50% gel layer. These results indicate that 50% interception gel layer can efficiently intercept the peptide with a molecular weight as low as 3.75 kDa.

SDS removal efficiency of WGGE system

For quantitative determination of SDS, a standard curve for SDS was established according to the method of Rusconi *et al.*[25] As shown in Fig. 3, SDS standard showed strong linear correlations between concentration and absorbance value at 438 nm over the concentration range tested, with a R^2 value of 0.995, demonstrating that the standard curve could be used to reliably quantify the SDS content in the samples under the present experimental conditions.

In the gel electrophoresis specially designed for SDS determination, the loaded protein sample contained 1.8 mg SDS (a total of 60 μL of protein sample containing 3%SDS were loaded in three parallel lanes each 20 μL) and the gel slab itself contained 12.4 mg of SDS (the volumes of 50%, 30%, 4-20% and stacking gel layers were 0.8, 0.8, 10.0 and 0.8 mL, respectively, all of which were prepared with 0.1% SDS-containing buffer), with totals of 14.2 mg of SDS in the sample and the gel slab. The quantitative determination indicated that, after the completion of electrophoresis, 12.389 mg of SDS were electrophoretically driven away from the sample and the gel slab, and the SDS removal efficiency was about 87.25%.

In order to further demonstrate the superiority of the WGGE system in the removal of SDS, a conventional SDS-PAGE was run as a control to detect the changes in SDS content. Before the electrophoresis, 140 mL upper reservoir buffer and 96 mL lower reservoir buffer contained 127.157 mg and 87.193 mg SDS, respectively, the loaded protein sample contained 2.0 mg SDS, and the gel slab contained 8.088 mg SDS. After the complete completion of the electrophoresis, upper reservoir buffer and lower reservoir buffer contained 105.465 mg SDS and 90.114 mg SDS, respectively. That is to say, the SDS content in the gel slab was increased from 8.088 mg to 28.859 mg [127.157+87.193+2.0+8.088-(105.465+90.114) =28.859], indicating that the conventional gel electrophoresis not only could not remove SDS from the sample and the gel slab, but increased the SDS content in the gel slab greatly. This is attributed to the fact that electrophoretical time of the conventional SDS-PAGE is too short to drive the SDS into lower reservoir buffer.

These data demonstrate that the newly developed WGGE system can efficiently remove the SDS from the protein sample and the gel slab, and thus provide a clean environment for

the subsequent enzymatic processes. Although there were small amounts of residual SDS in the gel slab, they were speculated not to significantly interfere with the enzyme activity in the subsequent in-gel digestion due to their tightly binding to gel and/or protein molecules and their low concentration (much lower than 0.1%) in the gel.

Protein identification based on WGGE combined with CapLC-MS/MS

The newly developed WGGE system was employed to analyze the rat liver plasma membrane-enriched fraction. After the proteins in the fraction were extracted with a SDS-containing buffer, the sample was gel electrophoretically run in the WGGE system (Fig. 4). From the figure it can be seen that the 4-20% continuous gradient efficiently separated the proteins in the sample and most of the proteins in the sample had molecular weights below 170 kDa, with the highest abundant proteins being distributed in the MW range of about 40-70 kDa. There were only a few proteins distributed above 170 kDa, suggesting that for such samples the start concentration of the gradient gel can be appropriately increased so as to separate the protein mixture even better. The continuous gradient polyacrylamide gel can separate the protein mixture into sharper bands than is usually possible with a gel of uniform concentration/pore size. This is because, when the gradient gel electrophoresis is run, the moving proteins are continually entering areas of the gel with decreasing pore size, and therefore the advancing edge of the moving protein zone is retarded more than the trailing edge, thereby forming sharp protein bands. The higher resolution of our newly developed WGGE system overcomes the limitations of the existing gel-based sample pretreatment methods, including some conventional SDS-PAGE, TGD[19] and TSGE[20].

For protein identification, the lane gel was cut into slices, followed by in-gel digestion, CapLC-MS/MS analysis and database searching. As a result, a total of 537 proteins were identified based on 2838 unique peptides after data merging and strict de-redundance. On average, each protein was identified based on 5.28 unique peptides, which suggested that the proteins were identified with high reliability. GO annotation[28] showed that, of the 537 identified proteins, 512 have definite function information and 506 have definite subcellular location information (Supplementary Table 1).

According to the molecular functions, the identified 512 proteins with function annotation can be classified into several groups (Fig. 5), although this classification is not strict due to the fact that a protein often has more than one kind of biological function. From the Fig. 5, it can be seen that about 45% of the proteins with function annotation have catalytical analytic activity, indicating the rat membrane-enriched faction is rich in enzymes. The proteins involved in biosynthesis account for about 15% and are mainly ribosomal proteins (Supplementary Table 1), suggesting that the ribosomes were also enriched to a certain extent in the membrane-enriched fraction. About 15% of the proteins have binding and/or structural functions. In a cell they bind to various kinds of cellular components such as ATP, GTP carbohydrates, lipids, Ca^{2+} and other proteins to exert extensive biological functions. The proteins involved in cell signaling, metabolism regulation and substance transport also account for a relatively large proportion (23.5%), which is in agreement with the characteristics of the biological membranes.

In order to further investigate the distribution profile of the identified proteins, we analyzed the main physicochemical properties of the identified proteins (Fig.6). The

identified proteins were shown to be distributed in the MW range from 1.116 to 537.74 kDa (Supplementary Table 1, Fig. 6A), indicating that the WGGE-based strategy can identify the proteins in a wide MW range. It is worthy of noting that of the identified 537 proteins, 15 proteins (accounting for 2.79%) have molecular weights less than 10 kDa, compared to 2 proteins (accounting for 0.47%) in a conventional SDS-PAGE-based proteomic analysis.[22] Compared with the conventional SDS-PAGE, our newly developed WGGE system is helpful to identify even more proteins particularly those with low molecular weights (Table 1). In addition, the protein sample used in the present study had been undergone multiple enrichment steps, during which many small-molecular-weight non-membrane proteins and peptides were removed. It can be speculated that if this WGGE system was used to separate a whole cell extract lysate, there would be many more small-molecular-weight proteins and peptides to be identified.

When the p*I* distribution of the identified proteins was analyzed, it was found that the proteins were distributed in the p*I* range from 4.23 to 11.79. Both acidic and basic proteins including those with extreme p*I* values were efficiently identified (Fig.6B). For evaluating the efficiency of the developed WGGE system in the identification of proteins with different hydrophobicity, we analyzed the calculated grand average of hydropathy (GRAVY) values[29] and the predicted transmembrane domain (TMD) numbers[30] of the identified proteins. The proteins exhibiting negative GRAVY values are generally considered hydrophilic and those with positive GRAVY values are deemed hydrophobic.[31] Transmembrane proteins are generally more difficult to analyze than peripheral membrane proteins and soluble non-membrane proteins due to the fact that they contain one or more TMDs that are highly

hydrophobic. The statistical analysis indicated that proteins identified using the WGGE-based strategy were distributed in the GRAVY range from -2.21 to 0.833, and the proteins with positive or negative GRAVY values accounted for 20.86% (112/537) and 79.14% (425/537), respectively, demonstrating that the WGGE system is suitable for the analysis of both hydrophilic and hydrophobic proteins (Fig.6C, Supplementary Table 1). Of the identified 537 proteins, 303 proteins (56.42%) were membrane proteins whose transmembrane domain (TMD) distribution profile is shown in Fig. 6D. It was found that the proteins with one or more TMDs account for 62.38% (189/303), of which 72 proteins (38.1%) have two or more TMDs, including the protein nicotinamide nucleotide transhydrogenase (IPI00555265) with 12 TMDs, the most number of TMD in a protein identified in the present study. These results demonstrate that the WGGE-based strategy is suitable for the identification of proteins with different hydrophobicity.

Conclusions

In the present work, we have developed and applied a wide-range gradient gel electrophoresis system. The main advantages of the system are simultaneous electrophoretic removal of SDS and other salts, protein separation and low-molecular-weight protein/peptide interception. Therefore, application of the system to the analysis of SDS-solubilized complex proteomes can: (1) avoid the special sample treatment for SDS removal before gel electrophoresis; (2) avoid repeatedly washing of the gel slices for SDS removal after gel electrophoresis and thus simplify experimental operation and reduce the risk of protein loss; (3) separate proteins efficiently; and (4) overcome the limitations of conventional SDS-PAGE in the analysis of

low-molecular-weight proteins and peptides, thereby improving the protein recovery and thus the total efficiency of protein identification. The analysis of real proteome sample using WGGE combined with LC-MS/MS demonstrate that the WGGE system can be applied to the efficient separation and identification of the proteins varying in molecular weight, p*I* and hydrophobicity, *etc*.

Abbreviations

CapLC-MS/MS, capillary liquid chromatography-tandem mass spectrometry; GRAVY, grand average of hydropathy; HWTX-I, huwentoxin-I; IPI, international protein index; TGD, tube-gel digestion; TMD, transmembrane domain; TSGE, three-layer sandwich gel electrophoresis; WGGE, wide-range gradient gel electrophoresis.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (31271135,31070700), Hunan Provincial Natural Science Foundation of China (11JJ2019)and the Cooperative Innovation Center of Engineering and New Products for Developmental Biology of Hunan Province (20134486).

Supplementary data

Supplementary Table 1 associated with this article can be found, in the online version, at

References

1 J. R. 3rd.Yates, A. Gilchrist, K. E. Howell and J. J. Bergeron, *Nat. Rev. Mol. Cell Biol.*, 2005, **6**, 702.

2 J. S. Andersen and M. Mann, *EMBO. Rep*., 2006, **7**, 874.

3 V. Santoni, M. Molloy and T. Rabilloud, *Electrophoresis,* 2002, **21**, 1054.

4 T. Rabilloud, *Nat.Biotechnol.*,2003, **21**, 508.

5 A. E. Speers and C. C. Wu, *Chem. Rev.,*2007, **107**, 3687.

6 J. Wei, J. Sun, W. Yu, A. Jones, P. Oeller, M. Keller, G. Woodnutt and J. M. Short, *J. Proteome Res.,*2005,**4**, 801.

7 M. P. Washburn, D. Wolters and J. R. 3rd.Yates, *Nat.Biotechnol.,*2001, **19**, 242.

8 T. Masuda, M. Tomita and Y. Ishihama, *J. Proteome Res*., 2008, **7**, 731.

9 Y .Lin, H. Liu, Z. Liu, Y. Liu, Q. He, P. Chen, X. Wang and S. Liang, *Anal. Biochem.,*2013, **432**, 41.

10 H. Zischka, C. J. Gloeckner, C. Klein, S. Willmann, M. Swiatek-de Lange and M. Ueffing, *Proteomics,*2004, **4**, 3776.

11 N. Nagaraj, A. Lu, M. Mann and J.R. Wiśniewski, *J. Proteome Res.,*2008,**7**, 5028.

12 A.J. Furth, *Anal. Biochem.,*1980, **109**, 207.

13 F. Li, L.J. Miller, S. Naylor and M.Q. Dong, *Rapid Commun. Mass Spectrom.,*1999, **13**, 464.

14 D.Wessel and U.I.Flügge, *Anal. Biochem.,*1984, **138**, 141.

15 M. Puchades, A. Westman, K. Blennow and P.Davidsson, *Rapid Commun. Mass Spectrom.,*1999, **13**, 344.

16 G.M. Janini, T.P. Conrads, T.D. Veenstra and H.J. Issaq, *J. Chromatogr. B,*2003, **1**, 43.

17 M. Gilar, A. Belenky and B.H. Wang, *J.Chromatogr. A*, 2001,**1**, 3.

18 J.R. Wiśniewski, A. Zougman, N. Nagaraj and M.Mann, *Nat. Methods*, 2009, **5**, 359.

19 X.N. Lu and H.N. Zhu, *Mol. Cell. Proteomics,*2005, **4**, 1948.

20 T. Liu, A. M. Martin, A. P. Sinai and B.C. Lynn, *J. Proteome Res.,*2008,**7**, 4256.

21 S. P. Liang, D.Y. Zhang, X. Pan, Q. Chen and P. A. Zhou, *Toxicon*,1993,**8**, 969.

22 R. Cao, X.W. Li, Z. Liu, X. Peng, W.J. Hu, X.C. Wang, P. Chen, J.Y. Xie and S.P. Liang. *J. Proteome Res*., 2005, **5**, 634.

23 X. Xiong, S. Huang, H. Zhang, J.J. LI, J.Y. Shen, J.X. Xiong, Y. Lin, L.P. Jiang, X.C. Wang and S.P. Liang. *Proteome Sci.,* 2009,**7**, 41.

24 D.J. Morré and D.M. Morré, *Biotechniques,*1989, **7**, 946.

25 F. Rusconi, E. Valton, R. Nguyen and E. Dufourc, *Anal. Biochem.,*2001, **295**, 31.

26 Y. Lin, Y. Li, Y. Liu, W. Han, Q. He, J. Li, P. Chen, X. Wang and S. Liang, *Electrophoresis,*2009, **30**, 3626.

27 R. Cao, Q.Y. He, J. Zhou, Q. Z. He, Z. Liu, X.C. Wang and S.P. Liang, *J. Proteome Res.,* 2008, **7**, 535.

28 M. Ashburner, C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski and S.S. Dwight, *Nat. Genet.,*2000, **25**, 25.

29 J. Kyte and R.F. Doolittle, *J. Mol. Biol.,*1982, **157**, 105.

30 A. Krogh, B. Larsson, G. Heijne and E.L.Sonnhammer, *J. Mol. Biol.,*2001, **305**, 567.

31 J. Blonder, M.B. Goshe, R.J. Moore, L. Pasa-Tolic, C.D. Masselon, M.S. Lipton and R.D. Smith, *J. Proteome Res.,*2002,**1**, 351.

Table 1 Comparison of WGGE and conventional SDS-PAGE for identification of low-molecular-weight proteins and peptides

Item	WGGE	Conventional SDS-PAGE[a]
Total proteins identified	537	428
Proteins (MW ≤10 kDa)	15(2.79%)	2(0.47%)
Proteins (20 kDa≥MW≥10 kDa	77(14.34%)	27(6.31%)

a, Cao *et al*.[22]

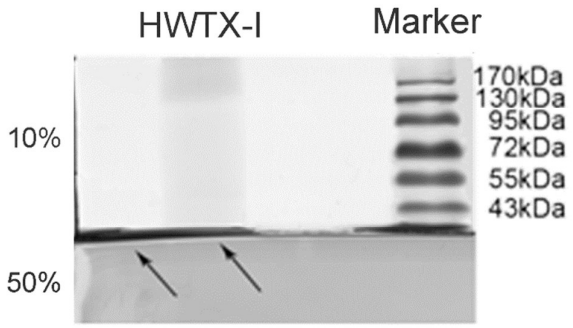


Fig. 1 Evaluation of the ability of 50% interception layer to intercept low molecular weight peptides. The arrows indicate the HWTX-I electrophoretically driven to the interface between 10% gel and the 50% interception gel layer.

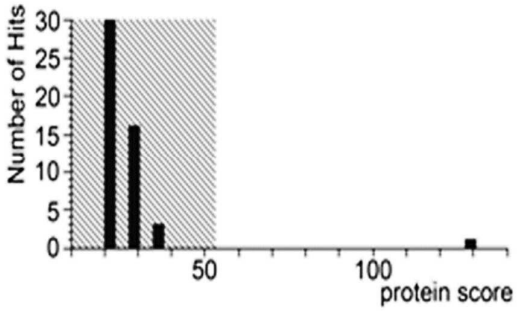


Fig. 2 Proteomic identification of HWTX-I at the interface between 10% gel and the 50% interception gel layer. By searching against protein database, the peptide was identified as huwentoxin-I with a score of 129, matches of 17(12), sequences of 2(1) and emPAI of 12.76.

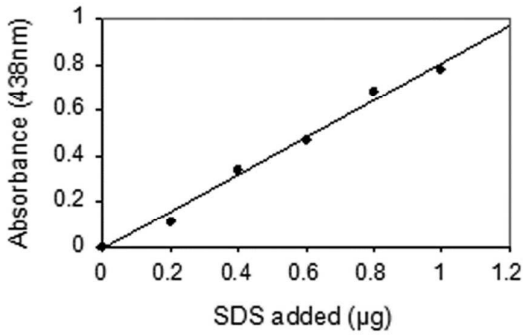


Fig. 3 SDS standard curve showing the plot of the absorbance at 438 nm versus the amounts of SDS added in the stains-all solution. $y=1.205x + 0.01813$, $R^2=0.995$.

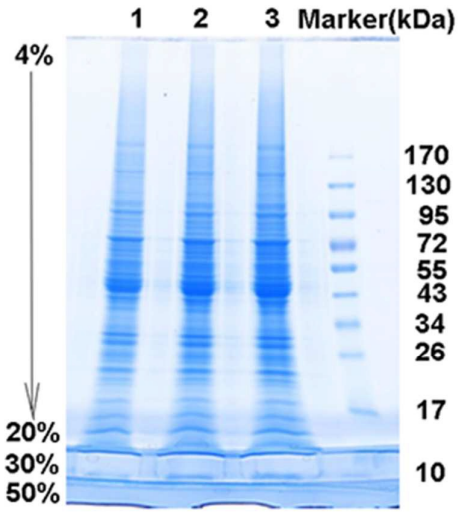


Fig. 4 WGGE image of the proteins in the rat liver membrane-enriched fraction. Lanes 1,2 and 3, protein sample.

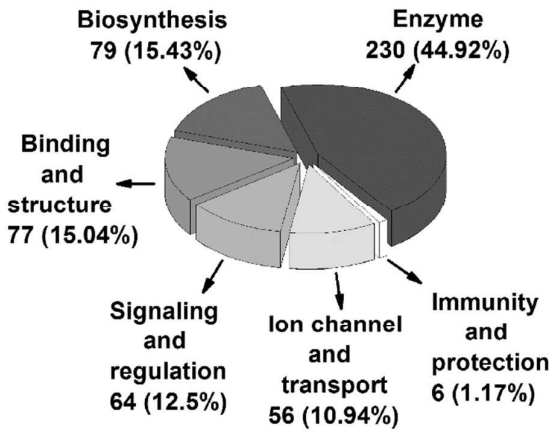


Fig. 5 Functional classification of the proteins identified from rat liver membrane-enriched fraction.

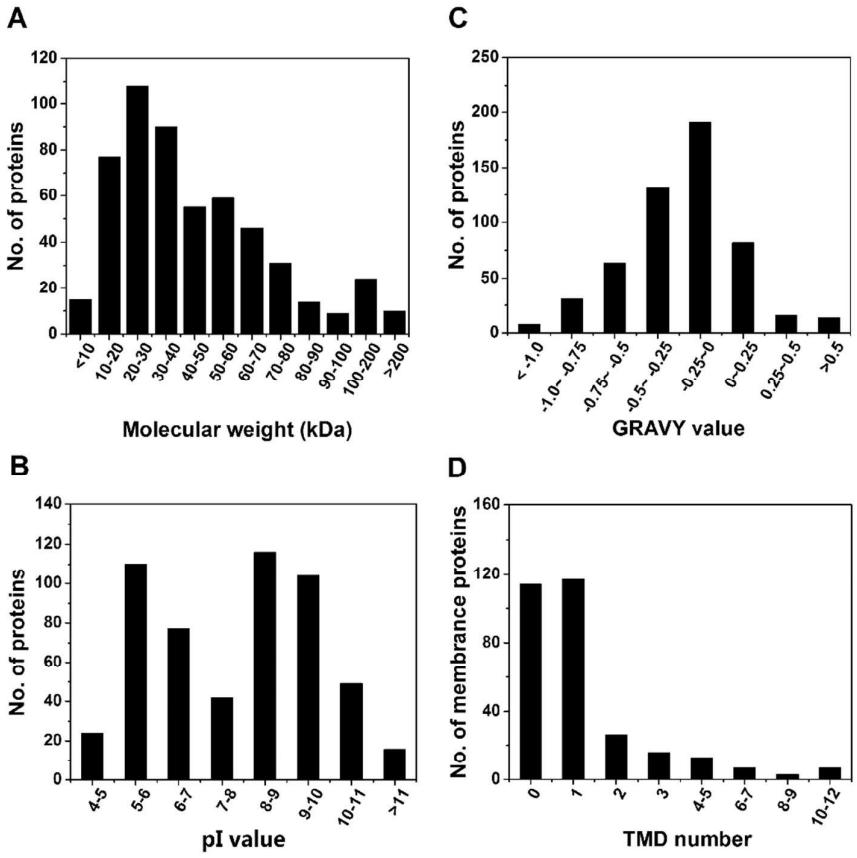


Fig. 6 Distribution profiles of the proteins identified using WGGE-based strategy

(A) Molecular weight distribution of all identified proteins; (B) p*I* distribution of all identified proteins; (C) GRAVY value distribution of all identified proteins; (D) TMD distribution of the identified membrane proteins.

Table of contents entry

A new gel electrophoresis system was developed to simultaneously remove SDS, separate proteins and intercept small proteins, thereby improving proteome analysis.