Protein fractionation for proteomics using the SAINOME-plate

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SUMMARY

The SAINOME-plate consists of a 384-well plate and a cover that contains a cutter, which can cut a polyacrylamide gel into approximately 4.5-mm square pieces following electrophoresis. In this study, we applied SDS-PAGE and the SAINOME-plate to fractionation of protein mixtures from cell extracts or serum for proteomic approaches. Compared with gel-fractionation using a cutter or a scalpel, SAINOME-plate gel-fractionation is simpler and higher-throughput. In terms of reproducibility of proteomic profiling, SAINOME-plate gel-fractionation was comparable to scalpel gel-fractionation. Additionally, human keratin contamination was lower with the SAINOME-plate than with a scalpel. In serum protein fractionation, the number of proteins identified increased approximately 2-fold and 3.7-fold relative to non-fractionation when the gel was divided into 8 and 96 fractions, respectively. The results demonstrate that the SAINOME-plate gel-fractionation will be a useful method in mass spectrometry-based proteomics.

Key words: SAINOME-plate, gel fractionation, in-gel digestion, mass spectrometry, proteomics

INTRODUCTION

Due to recent dramatic developments in mass spectrometry (MS) and peripheral technologies, ion intensity-based shotgun proteomics has become a popular approach for comprehensive analysis of proteins and their post-translational modifications (PTMs). However, due to the enormous complexity and protein dynamic range of serum samples, it remains difficult to exhaustively characterize the serum proteome. In particular, protein dynamic range is the most challenging issue facing development of serum proteomics technology.

To simplify the proteome, fractionation methods are generally used as a pretreatment for MS-based proteome analysis. In these methods, proteins are classified based on differences in their physical properties, such as electric charge, molecular weight, and isoelectric point. Among these approaches, SDS-PAGE gel fractionation is commonly used to separate proteins based on their molecular weight. Following electrophoresis, SDS-PAGE gels are diced using a scalpel, and proteins are subsequently subjected to in-gel digestion and MS analysis for protein identification.

Gel-fractionation is a powerful analytical method for MS-based proteome analysis, and is easily compared with other methods. However, gel-fractionation techniques using a scalpel are often difficult. In order to provide reproducible results while suppressing contamination by human keratin contamination, technical proficiency and scrupulous attention are required. Therefore, it is necessary to develop an easier method for gel-based fractionation.

The SAINOME-plate is a 384-well microplate produced by sainome Corporation. The plate has a cover that contains a cutter, and can cut a polyacrylamide gel into approximately 4.5-mm square pieces following electrophoresis (Fig. 1). Following dicing, the gel pieces are directly dropped into each well of the microplate, and then treated immediately. The SAINOME-plate gel-fractionation requires only a few steps and rapidly separates many gel pieces. Previously, the method was used for the DEG (Diced Electrophoresis Gel) assay, which can detect enzyme activity in a plate. In this study, we investigated the use of the SAINOME-plate for dicing of SDS-PAGE gels, followed by in-gel digestion and MS analysis. In particular, we evaluated the usefulness of the SAINOME-plate as a protein gel-fractionation tool.
acetone precipitation.

Fractionation by SDS-PAGE gel dicing
In the conventional method, unstained SDS-PAGE gels were diced into approximately 4.5-mm square pieces using a scalpel, according to the molecular weight marker. On the other hand, in the SAINOME-plate method, the unstained gels were placed on the plate according to the molecular weight marker, and subsequently diced by centrifugation at 3,000 rpm for 5 min (Fig. 1B). The plate cover plays the role of the gel cutter. We used an 18-lane XV PANTERA gel because the width of each lane is similar to those of the SAINOME-plate wells. HEK293 proteins and serum/seed proteins were separated into seven and eight fractions, respectively.

In-gel digestion
In-gel digestion procedures for the SAINOME-plate method and the conventional method were carried out in the microplate and a microtube, respectively. Gel pieces were washed with gel-washing buffer containing 60% (v/v) ACN and 50 mM NH₄HCO₃ three times, and then the liquid was evaporated. Evaporated gel pieces were rehydrated and incubated in a reducing buffer containing 10 mM DTT, 0.2 M guanidine hydrochloride, and 50 mM NH₄HCO₃, and then alkylated with 108 mM iodoacetamide in 50 mM NH₄HCO₃. Gel pieces were washed seven times with gel-washing buffer, followed by evaporation of liquid, and then subjected to in-gel digestion with trypsin. Non-fractionated proteins were subjected to in-solution digestion with trypsin after reduction with 10 mM DTT and alkylation with 25 mM iodoacetamide. The resultant peptides were desalted and concentrated by purification using a reverse-phase C18 tip column (Stage Tip). The obtained peptides were analyzed by LC-MS/
using a scalpel. Additionally, the development of a clear plate allowed us to easily observe gel pieces or solutions at each step of the SAINOME-plate gel-fractionation.

To evaluate the reproducibility of SAINOME-plate gel-fractionation, we identified the proteins derived from HEK293 cells by SAINOME-plate gel-fractionation, followed by in-gel digestion and MS analysis, in two separate experiments. A total of 4,523 (first experiment) and 4,299 (second experiment) proteins were identified by SAINOME-plate gel-fractionation, nearly equivalent to the number of proteins [4,512 (first), 4,586 (second)] identified by the conventional method using a scalpel. SDS-PAGE gel-fractionation increased the number of proteins identified by approximately 2.6-fold in comparison to non-fractionation (Fig. 2B). Furthermore, the degree of overlap between the two data sets obtained by the SAINOME-plate method was 83.9%, comparable to the overlap of the conventional method (83.8%), and was slightly higher than non-fractionation (82.2%). Accordingly, the SAINOME-plate gel-fractionation was highly reproducible, suggesting that it could be a powerful analytical tool for MS-based proteomics.

RESULTS AND DISCUSSION

Ease and reproducibility

To investigate the ease of SAINOME-plate gel-fractionation for MS-based proteomics, we fractionated protein extracts from HEK293 cells. In the conventional method using a scalpel, the time required to dice the SDS-PAGE gel into seven fractions was approximately 15 minutes. The gel pieces obtained were transferred to individual tubes, followed by in-gel digestion in each tube. Additional time was also required for opening and closing the lids of all tubes. As the number of fractions and the number of samples increased, the operation time for the conventional method also increased. By contrast, the gel-dicing time of the SAINOME-plate gel-fractionation method was very short: a maximum of 384 pieces could be fractionated from the gel at once, in approximately 5 minutes. Because the gel pieces were also dropped into the lower wells of the plate by centrifugation, in-gel digestion could be carried out in the same plate. Therefore, SAINOME-plate gel-fractionation is high-throughput and simple. Therefore, we considered that the ease was superior to that of the conventional method using a scalpel. Additionally, the development of a clear plate allowed us to easily observe gel pieces or solutions at each step of the SAINOME-plate gel-fractionation.

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Human keratin contamination

In gel-based proteomics analysis, it is important to greatly reduce human keratin contamination. To evaluate the extent of human keratin contamination during operation of the SAINOME-plate method, we analyzed the protein mixtures from cell extracts of legume seeds. After electrophoresis, proteins were fractionated using a scalpel or SAINOME-plate, and then investigated by in-gel digestion followed by MS analysis. To identify human proteins, the obtained MS and MS/MS data of each fraction were searched against human protein sequences using the MASCOT search engine. The results revealed that human keratin contamination was reduced significantly with the SAINOME-plate method (Fig. 3).

These experiments were carried out by a researcher who had little technical experience. Thus, the SAINOME-plate
method would be useful for beginners in gel-based proteomics, for two reasons: minimal contact with the gel, and short operation time.

**Application to serum protein fractionation**

In serum proteomics, pretreatment by fractionation is essential to increase the number of proteins identified by MS analysis. In this study, we investigated SDS-PAGE gel-based fractionation with the SAINOME-plate for the purpose of serum proteomics. Serum samples, from which 14 high-abundance human proteins were removed, were separated by SDS-PAGE, and then the gels were diced into eight fractions using the SAINOME-plate. The number of proteins identified by MS analysis of these eight fractions was increased approximately 2-fold vs. non-fractionation (403 vs. 202 proteins). Furthermore, the serum sample was pre-separated into 12 fractions by IEF electrophoresis using an OFFGEL Fractionator, and subsequently each fraction was separated by SDS-PAGE. After electrophoresis, the SAINOME-plate diced the SDS-PAGE gel into 96 fractions, followed by in-gel digestion and MS analysis (Fig. 4). The number of proteins identified were increased approximatel

| Protein name | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | 9th | 10th | 11th | 12th | 13th | 14th | 15th | 16th | 17th | 18th | 19th | 20th | 21st | 22nd | 23rd | 24th | 25th | 26th | 27th | 28th | 29th | 30th | 31st | 32nd | 33rd | 34th | 35th | 36th | 37th | 38th | 39th | 40th | 41st | 42nd | 43rd | 44th | 45th | 46th | 47th | 48th | 49th | 50th | 51st | 52nd | 53rd | 54th | 55th | 56th | 57th | 58th | 59th | 60th | 61st | 62nd | 63rd | 64th | 65th | 66th | 67th | 68th | 69th | 70th | 71st | 72nd | 73rd | 74th | 75th | 76th | 77th | 78th | 79th | 80th | 81st | 82nd | 83rd | 84th | 85th | 86th | 87th | 88th | 89th | 90th | 91st | 92nd | 93rd | 94th | 95th | 96th |
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approximately 3.7-fold (755 proteins) vs. non-fractionation, although MS analysis took a long time (4 days). Moreover, this analysis successfully identified proteins present at lower concentrations, such as transforming growth factor beta-1, connective tissue growth factor, and vascular endothelial growth factor C, which are difficult to detect in serum by non-fractionation or other fractionation methods. Thus, using the SAINOME-plate gel-fractionation technique, we simplified the proteome and adjusted the dynamic protein range, enabling global analysis by shotgun proteomics. Because these proteins are also involved in various diseases, including cancers\(^{11-13}\), this approach could be used for the discovery of biomarkers and drug targets. Hence, the SAINOME-plate has also the potential for wide use in proteomics.

**CONCLUSION**

In this study, we showed that the SAINOME-plate method has high ease and reproducibility for protein fractionation. Moreover, the degree of human keratin contamination was reduced significantly, and the number of proteins identified was increased, in gel-based fractionation for proteomics using the SAINOME-plate method. We expect that the SAINOME-plate will be widely adopted for gel-based proteomics in the future.

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No potential conflicts of interest were disclosed.

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