

A Multiplexed Quantitative Strategy for Membrane Proteomics – Opportunities for Mining Therapeutic Targets for Autosomal Dominant Polycystic Kidney Disease

Abstract

Towards multiplexed, comprehensive and robust quantitation of the membrane proteome, we developed a strategy combining gel-assisted digestion, iTRAQ labeling, and LC-MS/MS. The new quantitation platform not only demonstrates a high degree of accuracy (<8% error) and precision (<12% RSD) but also provides an efficient and comprehensive quantitation for membrane proteome. Under stringent identification criteria, more than 90% of quantified proteins were membrane proteins. We applied this approach to the first proteomics delineation of phenotypic expression in a mouse model of autosomal dominant polycystic kidney disease (ADPKD) and discovered 104 differentially expressed proteins among 791 quantified proteins. Some of these differentially expressed membrane proteins have been proven effective in preclinical studies of ADPKD. Our new strategy demonstrates how comparative membrane proteomics can provide insight into the molecular mechanisms underlying various diseases and discover potential biomarkers which may contribute to the diagnostics or therapeutic uses for diseases.

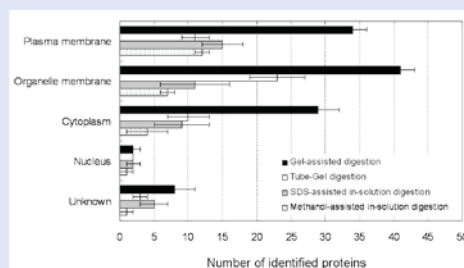
Chia-Li Han¹, Chih-Wei Chien², Wen-Cheng Chen³, Yet-Ran Chen⁴, Chien-Peng Wu⁴, Hung Li⁵, and Yu-Ju Chen⁶

¹Department of Chemistry, National Taiwan Normal University, Taipei, Taiwan ²Graduate Institute of Medical Biotechnology, Chang Gung University, Tao-Yuan, Taiwan ³Institute of Biochemistry, National Yang-Ming University, Taipei, Taiwan ⁴Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan ⁵Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan ⁶Institute of Chemistry and Genomic Research Center, Academia Sinica, Taipei, Taiwan

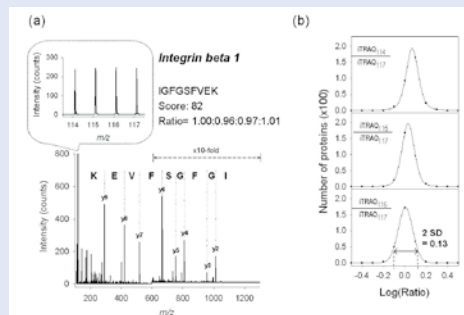
Many membrane proteins are implicated in particular diseases states and often are attractive therapeutic targets. Comprehensive and quantitative profiles of membrane proteins facilitate our understanding of their roles in regulating biological processes and in cellular signaling. However, the analysis of membrane proteins is experimentally challenging due to their hydrophobic nature and low abundance, which seriously complicates their solubilization, sample handling, preparation, separation and analysis. In this study, we report a strategy integrating enhanced gel-assisted digestion and iTRAQ labeling for multiplex quantitative profiling of the membrane proteome. Specifically, we aimed to achieve (1) higher identification/quantification numbers, (2) enhanced quantification for integral membrane proteins, and (3) broad compatibility with a variety of solubilization reagents with robust applicability to diverse biological systems.

iTRAQ uses isobaric tagging chemistry at the peptide level; thus, effective solubilization, denaturation and digestion are critical for high peptide yields and subsequent iTRAQ quantitation. To select an optimized digestion method for membrane proteins, we evaluated the digestion efficiency of our previously developed gel-assisted digestion with comparison with three recently published methods. Under the stringent thresholds in the protein identification, the comparison of three replicate experiments showed that gel-assisted digestion distinguished significantly more proteins than the Tube-Gel digestion, SDS-assisted and methanol-assisted in-solution digestion methods, identifying 75, 34, 26, and 19 non-redundant membrane proteins, respectively (Figure 1). The enhanced digestion efficiency of membrane proteins clearly demonstrated that gel-assisted digestion had the best performance among the four methods.

The reproducibility of gel-assisted digestion and its compatibility with iTRAQ labeling for multiplexed quantitation was determined by four replicate sets of membrane proteins. In the representative MS/MS spectrum of IGFGSFVEK (m/z 636.4, score 82) from integrin beta 1 shown in Figure 2(a), the cluster of peaks in the mass window of m/z 113-118 displayed the signature iTRAQ fragments in the ratio of 1.00:0.96:0.97:1.01. All the other quantified proteins also showed expected unitary ratios. The similarity of these distribution curves (Figure 2b) demonstrated the high reproducibility and precision of our quantitation strategy on the four independently prepared membrane samples. Based on a three standard deviation model (3 SD, confidence interval=99.7%), we considered a difference in abundance of 1.5-



1 Comparison of subcellular localization for identified proteins using gel-assisted digestion, Tube-Gel digestion, SDS-assisted in-solution digestion, and methanol-assisted in-solution digestion. The error bars represent \pm SD from three replicate analyses.



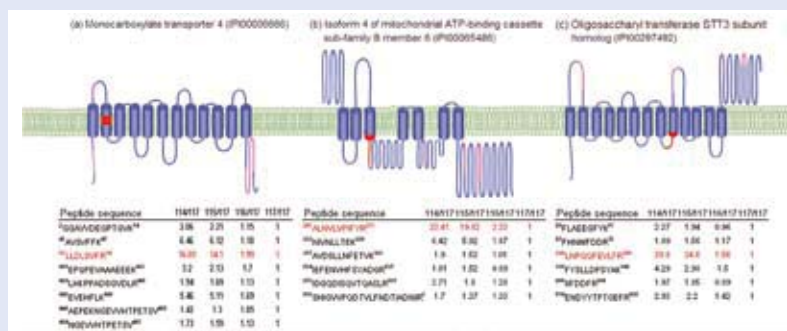
2 Proof-of-concept experiments showing the feasibility and reproducibility of the quantification strategy combining gel-assisted digestion with iTRAQ labeling. (a) A typical MS/MS spectrum for peptide IGFGSFVEK derived from integrin beta 1 prepared from four membrane fractions in a 1:1:1:1 ratio. The zoom-in figure shows the iTRAQ reporter ions (m/z 114, 115, 116, and 117). (b) Distribution of protein ratios in log scale. The variance of quantitation is shown as a dashed line, indicating the 95% confidence interval (SD = 0.065).

fold to indicate a statistically significant degree of up-regulation or down-regulation.

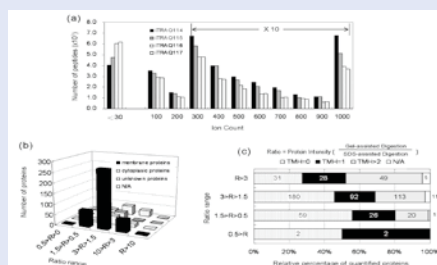
To further establish a comprehensive method for large-scale multiplexed quantitation of the membrane proteome, fractionation using a multi-dimensional peptide separation technique was incorporated into the optimized strategy. The in-depth comparison between the newly developed gel-assisted digestion and the most common used in-solution digestion as well as performance assessment were evaluated via large-scale quantitation of replicate membrane fractions from HeLa cells. A total of 696 non-redundant proteins (false discovery rate = 0%) were identified by combining LC-MS/MS data files from 42 SCX fractions. We used the TMHMM algorithm and Ingenuity Pathway Analysis to predict the number of transmembrane helices (TMH) and the subcellular location for each identified protein. Our results quantified as many as 520 membrane proteins (i.e., 91%, exclusive of 8% unknown proteins) from the crude membrane fraction. To our knowledge, no other studies have achieved a quantitative proteomic analysis with such high percentage of membrane proteins.

We constructed the topological models of quantified peptides and correlated the extracellular/intracellular domain and TMH with iTRAQ ratios. Figure 3 shows three examples of multi-pass integral membrane proteins. This strong correlation between the most dramatic changes in yield and the TMH peptides implies that the efficiency of cleavage of extremely hydrophobic peptides in proximity to the TMH was relatively enhanced by gel-assisted digestion. As shown in Figure 4(a), SDS-assisted in-solution digestion (iTRAQ₁₁₆ and iTRAQ₁₁₇) yielded more low-signal peptides (< 30 count, unquantified peptides), whereas gel-assisted digestion (iTRAQ₁₁₄ and iTRAQ₁₁₅) yielded greater numbers of quantified peptides with higher intensity. The difference is more dramatic for peptides having an ion count > 300. To address whether detection of specific categories of proteins could be preferentially improved, the ratio distribution of quantifiable proteins in various subcellular localizations was plotted (Figure 4b). The higher-yield proteins (ratio > 1.5) were mainly membrane proteins (86.1%, exclusive of unknown and unclassified proteins). We also predicted the number of TMHs for these higher-yield proteins to examine their hydrophobic characteristics (Figure 4c). Using our method, the yield of multi-pass integral membrane proteins was greater than proteins lacking a TMH. Taken together, both trends reveal that gel-assisted digestion results in greater peptide yield for a greater number of hydrophobic membrane proteins, thereby facilitating subsequent peptide-level labeling with iTRAQ and providing more complete coverage of the membrane proteome.

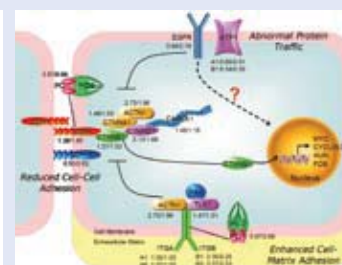
Finally, the new approach was applied to analyze the membrane proteome for the mouse model of autosomal-dominant polycystic kidney disease (ADPKD). To date, effective therapy for ADPKD is lacking, with the exception of dialysis and kidney transplantation. The development of better therapeutic treatment is founded on an understanding of the molecular pathophysiological mechanism for the development of ADPKD. Although the mechanisms underlying ADPKD pathology are not well understood, a close examination of the differentially expressed membrane proteins quantified by the current comparative approach reveals potential correlations with proteins associated with the pathogenesis of ADPKD in previous literature. Figure 5 summarizes some of these proteins that have been confirmed to be involved in the major abnormalities in epithelial cell proliferation and apoptosis, cell-cell and cell-matrix interactions, abnormal ion and fluid secretion, and alterations in membrane protein polarity. Moreover, the identification of additional potential drug target candidates will speed the discovery of an effective therapy. In this study, we discovered several potential drug target candidates for ADPKD. Among them, EGFR, COX, and Na⁺/K⁺ ATPase had already been targeted in a potential treatment for ADPKD. We expect that our new membrane proteomics platform can offer an improved quantitation platform to investigate differential expression of membrane proteins in cells, biofluids, and tissues under different environmental or pathophysiological conditions. Given the possibilities offered by our new technique for more efficient identification and quantification of large numbers of transporters/receptors, the results may systematically decipher the disease mechanism and identify drug target candidates for better diagnosis and treatment.



3 Predicted locations of identified peptides in three transmembrane proteins by TMHMM topological analysis. The identified peptides and their quantified ratios are listed. The peptides in red represent the greatest fold-change recovery using gel-assisted digestion compared with SDS-assisted in-solution digestion. Other quantified peptides are shown in pink on the topological representation.



4 Performance evaluation of quantitation strategies by gel-assisted digestion (iTRAQ₁₁₄, iTRAQ₁₁₅) and SDS-assisted in-solution digestion (iTRAQ₁₁₆, iTRAQ₁₁₇) followed by iTRAQ labeling. (a) The distribution of number of quantified peptides as a function of ion counts ranging from 30 to 1000. SDS-assisted in-solution digestion generated more quantified peptides with ion count below 30, whereas gel-assisted digestion generated relatively higher abundant peptides. (b) Differential yield of proteins sorted by subcellular localization between gel-assisted and SDS-assisted in-solution digestion (R: ratio = iTRAQ₁₁₄/iTRAQ₁₁₇). The higher-yield proteins (ratio > 1.5) were mainly membrane proteins. (c) The distribution of proteins with a different number of transmembrane helices (TMH) versus fold-change of quantified proteins. The percentage of membrane proteins with TMHs increased among proteins with higher recovery using gel-assisted digestion.



5 Partial pathways of differentially expressed proteins that have been confirmed to be involved in the major abnormalities in epithelial cell proliferation and apoptosis, cell-cell and cell-matrix interactions, abnormal ion and fluid secretion, and alterations in membrane protein polarity in polycystic kidney disease. The ratios, (2L3-1/Wt-1)/(2L3-2/Wt-1) in the figure indicate the fold change of protein expression level in PKD knockout compared to control mice.

Publication

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