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A Comparative Study of Workflows Optimized for In-gel, In-solution and On-filter Proteolysis in the Analysis of Plasma membrane Proteins

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Abstract

Proteomic studies of plasma membrane proteins are challenged by the limited solubility of these proteins and the limited activity of proteolytic enzymes in solubilizing agents such as SDS. In this work, we have evaluated three bottom-up workflows to obtain tryptic peptides from plasma membrane proteins solubilized with 2% SDS. The workflows are: in-gel digestion, in-solution digestion, and on-filter digestion. The efficiencies of these strategies, optimized to employ different matrices for trypsin cleavage, were compared using a plasma membrane sample enriched from multiple myeloma cells using a nanoparticle pellicle. Based on the number of proteins identified, number of transmembrane proteins identified, hydrophobicity, and spectral count per protein, the workflow that uses in-gel digestion is the most advantageous approach for analysis of plasma membrane proteins.

INTRODUCTION

In this technical note we report the comparison of three strategies to study proteins recovered from the eukaryotic plasma membrane (PM). These strategies have been widely used and optimized by the proteomics community, and are designed to solubilize hydrophobic proteins, provide tryptic digestion and remove SDS. They are: 1) protein digestion in polyacrylamide gel matrix [1] (in gel-digestion of proteins); 2) protein precipitation in chloroform/methanol followed by resolubilization and digestion in urea [2], (in-solution digestion); and 3) filter-aided sample preparation (on-filter digestion), in which the SDS-urea exchange and enzymatic cleavage take place in a molecular weight cut off filter. [3, 4] Each one of these strategies has its advantages and disadvantages. Briefly, the first technique, in-gel digestion, is robust, reproducible and effective; however, it is laborious and time-consuming. The second method, precipitation/in-solution digestion, requires less time for preparation, but it introduces sample losses due to a low re-solubilization of aggregated proteins. [5] The third method, on-filter digestion, has been described as universal and enhancing membrane protein identification,[3,4] but other reports dispute the universality of the method and report limited reproducibility and the loss of protein on the filter [6, 7].

Although instrumental technologies for handling complex protein mixtures are continually being improved (e.g., aspects of tandem mass spectrometry and high performance liquid

chromatography), effective strategies for preparation of hydrophobic samples are still imperfect. Ideally, bottom-up analysis of plasma membrane proteins would include enrichment of the plasma membrane followed by followed by solubilization of the hydrophobic proteins to accommodate proteolysis. Organic solvents have been successfully demonstrated for solubilization of membrane proteins, proteolysis and compatibility with mass spectrometry.[8–11] Urea and SDS solutions have classically been used to solubilize hydrophobic proteins and these have been shown to accommodate tryptic proteolysis at dilute concentrations.[7,8,12,13] Even at low concentrations these detergents must be removed after digestion to maintain optimal ionization conditions. Mass spectrometry-compatible surfactants have also been proposed to support enzymatic cleavage of membrane proteins.[9,10]

In the present study, plasma membrane proteins were enriched by cationic silica nanoparticle coating, following the method of Jacobson.[14,15] In our hands repeated incubation with 2% SDS is required to recover plasma membrane proteins from the pellicle, and this is the solution with which we have compared the three workflows. This study explores the effectiveness of each strategy in identifying plasma membrane peptides and proteins, and investigates the properties of the peptides and proteins identified, including hydrophobicity and transmembrane characteristics.

MATERIALS AND METHODS

Materials

Ludox® CL colloidal silica 30 wt.% was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were also obtained from Sigma-Aldrich unless specified otherwise. Endoproteinase Lys-C and trypsin were obtained from Promega (Madison, WI). Criterion™ Tris-HCl precast gels, Tris-glycine-SDS buffer, molecular weight standards, and protein assay kit RCDC™ (Reducing Agent and Detergent Compatible) were purchased from Bio-Rad (Hercules, CA). Amicon 3 kDa centrifugal filters were purchased from Millipore (Billerica, MA). RPMI 1640 tissue culture medium was obtained from American Type Culture Collection (Manassas, VA), and fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA).

Isolation of plasma membrane proteins using nanoparticle pellicles

Human multiple myeloma RPMI 8226 cells were grown in RPMI 1640 minimum essential medium as previously described[14]. Plasma membrane proteins were obtained from 1×10^8 RPMI 8226 cells following published methods that employ cationic silica nanoparticles[14] with slight modifications. Briefly, the cells were washed and resuspended in plasma membrane coating buffer A (PMCBA= 800 mM sorbitol, 20 mM MES, 150 mM NaCl, pH 5.3). The suspension was added in a dropwise fashion to a suspension of silica nanoparticles in PMCBA (10% weight by volume) and gently rocked at 4°C for 15 min. Silica surfaces were neutralized by placing the coated cells dropwise into a solution of 10 mg/mL polyacrylic acid in PMCBA buffer, pH 6.0–6.5, and gently rocking at 4°C for 15 min. The intact coated cells were incubated in 2.5 mM imidazole buffer with protease inhibitor cocktail (Sigma) at 4°C for 30 min. Lysis by N_2 cavitation followed at 1250 psi. The plasma membrane/nanoparticle pellicles were sedimented at 100xg for 7 min and subjected to a sucrose (2 M) cushion centrifugation. Associated cytosolic proteins were removed by washing once each with 1M KCl and 1M Na_2CO_3, pH 11.4. Protein was solubilized from the pellicles by triplicate incubations in 2% SDS, 62.5 mM Tris-HCl, and 5% β-mercaptoethanol at 100 °C for 5 min in a microwave oven (CEM Corporation, Matthews, NC). Protein concentration was determined using an RCDC™ protein assay kit (Bio-Rad).

Protein digests

In gel digestion was performed by standard procedures [1]. Proteins (80 μg) were loaded onto an 8–16% Bio-Rad Criterion precast gel and run at 200 V for 90 min. The gel was then stained with Comassie blue and 17 bands were excised. Each was reduced with 10 mM DTT at 56°C for 30 min and alkylated with 55 mM iodoacetamide for 20 min. The gel bands were destained with 50% ACN in 50 mM NH_4HCO_3 and dehydrated with 100% ACN. Trypsin digestion (13 ng/μL) was performed overnight prior to peptide extraction in 1:2 (v/v) 5% formic acid/ACN.

Precipitation and in-solution digestion were carried out using standard methods [2]. Proteins (80 μg) were precipitated by the addition of chloroform and methanol (sample:chloroform:methanol:water 1:1:4:3 by volume) and re-dissolved in 8 M urea. Reduction and alkylation were performed by incubation with 20 mM DTT at 56 °C for 30 min and 40 mM iodoacetamide in the dark for 30 min, respectively. Lys-C digestion (16 ng/μL) was performed in 8 M urea for 3 h, followed by trypsin cleavage (32 ng/μL) in 1.6 M urea for 16 h at room temperature.

Lastly, on-filter digestion was performed on a 3 kDa molecular weight cut off filter using the procedure described by Wisniewski *et.al.* [3]. Briefly, 80 μg protein was applied to the filter in Laemmli buffer containing 100 mM DTT. The solution was exchanged with 8 M urea in 0.1 M Tris HCl, pH 8.5 and the retentate was incubated for 5 minute with 50 mM iodoacetamide. The retentate was again exchanged three times with 8 M urea in 0.1 M Tris HCl, pH 8.0. Lys-C digestion (16 ng/μL) was achieved on the filter in a wet chamber at room temperature overnight. This incubation was terminated by adding 300 μL of 50 mM NH_4HCO_3 to dilute the urea to 2M and make the solution suitable for trypsin digestion. Trypsin digestion followed (32 ng/μL) at room temperature for 4 hours. Tryptic peptides prepared from the last two methods were desalted with a Thermo C18 spin column prior to HPLC separation.

HPLC-MS/MS Analysis

Analyses were carried out on a Shimadzu Prominent nanoHPLC (Shimadzu BioSciences, Columbia MD) interfaced to an LTQ-orbitrap XL (Thermo Fisher Scientific, San Jose CA). Peptide mixtures obtained from the in-solution and on-filter digestions were each injected via an autosampler in six aliquots in order to optimize precursor selection.[16] Extracts from the 17 gel slices were injected separately under the same experimental conditions. Peptides were injected into an Acclaim PepMap 300 C18 precolumn (Dionex, Sunnyvale CA) and desalted by 10% solvent A (97.5% H_2O, 2.5% ACN, 0.1% formic acid) for 10 min. The separation was performed in an t Zorbax 300SB-C18 (Agilent Technologies, Palo Alto CA) nanobore column (0.075 x 150 mm) with a linear gradient increasing from 10% to 60% solvent B (97.5% ACN, 2.5% H_2O, 0.1% formic acid) in 90 min, followed by another increase from 60% B to 85% B through 20 min. The flow rate was 10 μL/min for peptide trapping and 300 nL/min for separation. Precursor ions were scanned in the orbitrap with a resolution of 30,000 at m/z 400. In each activation cycle the nine most abundant ions were fragmented by collisional induced dissociation (CID) prior to product ion scans in the LTQ. Dynamic exclusion was enabled with 1 repeat count for 180 sec. Data were acquired using Xcalibur 2.0 software (Thermo Fisher Scientific, San Jose, CA).

Bioinformatics

Following spectral acquisition, .RAW data files from Xcalibur were submitted to the PepArML batch uploader and searched with the PepArML meta-search engine (https://edwardslab.bmcb.georgetown.edu/PepArML/) [17] against the human IPI database. PepArML combines search results from Mascot and six open source search engines. Results were

processed by an in-house tool to remove redundant peptide identifications. The spectrum level false discovery rate (FDR) filter was set lower than 10% and two or more peptides were required for protein identification. Protein localization was assigned by GO Slim annotation using the Protein Information Resource server (http://pir.georgetown.edu/). The number of transmembrane helices in proteins was predicted by TMHMM 2.0[18] Protein GRAVY scores were determined using the GRAVY Calculator (http://www.gravy-calculator.de/). A reference set of 3965 plasma membrane proteins was assembled by combining the human plasma membrane and cell surface categories in the UniProt database (http://UniProt.org) to provide theoretical values for comparison of transmembrane helices and hydrophobicity.

The general workflow is represented in Figure 1.

RESULTS and DISCUSSION

The efficiency of the three different procedures for detergent removal and digestion of PM proteins was evaluated based on the number of PM proteins identified, PM protein hydrophobicity determined by GRAVY scores, and the number of PM proteins containing transmembrane helices predicted by the TMHMM algorithm. As shown in Table 1 and Supplementary Table 1, the highest number of PM proteins identified was obtained using in-gel digestion, followed by on-filter digestion and in-solution digestion, respectively. To further test this observation the number of peptides required for protein identification was varied (Figure 2), and the relative performance of the methods was unchanged. In-gel digestion provided a significantly higher number of PM protein identifications..

While it is not unexpected that the protein-level fractionation afforded by the in-gel method might identify more low-abundance proteins than the other techniques, a comparison of spectral counts suggests that additional factors contribute to the identification of fewer plasma membrane proteins by the in-solution and on-filter methods. The Venn diagram in Figure 3 shows that 79 proteins were identified by all three strategies. (See also Table 2 in Supplementary Information.) An estimate of the relative abundances of these proteins in each of the digestion matrices was provided by spectral counting[16] with spectral counts for each of the six replicate injections of the in-solution and on-filter digestion products summed. For each of the 79 in-common proteins, the method (in-gel, in-solution, on-filter) with the largest spectral-count was identified. Of 78 proteins, 59 from the in-gel method had the largest spectral-count (p-value $< 2.3 * 10^{-15}$, χ^2-test). (See Table 3 in Supplementary Information.) One protein was discarded because the spectral-counts from the in-gel method and on-filter digestion were equivalent. This indicates that peptides from the in-common proteins provide more recorded spectra[16] when the in-gel method is used. Since all three digestions were initiated with equivalent amounts of total protein, one interpretation is that protein has been lost during precipitation/resolubilization in the solution workflow, and that on the filter incomplete resolubilization and incomplete enzyme access contribute to the reduced levels of peptide spectra recorded.

The distribution of transmembrane domains (TMD) in the PM proteins identified using the three procedures were compared to each other, and also to the distribution of transmembrane domains in a reference set of proteins listed as plasma membrane or cell surface in the UniProt database (Figure 4). The natural abundance of 7TM receptors in the plasma membrane is reflected in the distribution of the reference set, though not in the experimental samples. In-gel digestion provided 106 proteins containing one or more transmembrane helices, whereas on-filter digestion and in-solution digestion provided 37 and 33 transmembrane proteins respectively (Table 1).

The hydrophobicity[19] of the plasma membrane proteins identified in our three experiments is summarized in Figure 5, along with the reference set. Higher GRAVY scores indicate higher hydrophobicity. All sample sets are maximally represented around −2.0, however the reference set exhibits a second maximum, between 0.4 and 1.0. The in-gel values provide the best match for this second maximum. Based on the distributions and averages, PM proteins identified by in-gel digestion are more hydrophobic, compared to those identified using on-filter and in-solution digestion.

Finally, the molecular weight distributions of tryptic peptides identified from plasma membrane proteins in the three experiments are compared (Figure 6) with each other and with tryptic peptides generated *in silico* from the set of reference PM proteins. Masses are plotted beginning at 800 Da, since this is the lower limit (m/z 400 with a +2 charge state) used for precursor selection for collisional activation in the tandem mass spectrometry experiments. Figure 6 shows that in-solution digestion contributes most heavily above m/z 2000 on a percent basis. However this has to be evaluated in the context of the lower number of total identifications provided by precipitation followed by resolubilization and in-solution digestion.

CONCLUSION

This comparison of three workflows to provide tryptic digestion of plasma membrane proteins and removal of 2% SDS indicates that in-gel digestion provides advantages over workflows optimized for on-filter and in-solution digestions, based on the number of plasma membrane proteins identified, and the numbers of transmembrane proteins and hydrophobic proteins characterized, which are commonly underrepresented in proteomic analysis of the plasma membrane. Consequently, and acknowledging the additional experimental manipulation required, one dimensional gel fractionation and in-gel digestion are found to provide superior analysis of plasma membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006; 1:856–860.
2. Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 1984; 138:141–143. [PubMed: 6731838]
3. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009; 6:359–362. [PubMed: 19377485]
4. Manza LL, Stamer SL, Ham AJ, Codreanu SG, Liebler DC. Sample preparation and digestion for proteomic analyses using spin filters. Proteomics. 2005; 5:1742–1745. [PubMed: 15761957]
5. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. Electrophoresis. 2010; 31:3573–3579. [PubMed: 20967768]
6. Liebler DC, Ham AJ. Spin filter-based sample preparation for shotgun proteomics. Nat Methods. 2009; 6:785. [PubMed: 19876013]

7. Bereman MS, Egertson JD, MacCoss MJ. Comparison between procedures using SDS for shotgun proteomic analyses of complex samples. Proteomics. 2011; 11:2931–2935. [PubMed: 21656683]
8. Zhang N, Chen R, Young N, Wishart D, Winter P, Weiner JH, Li L. Comparison of SDS- and methanol-assisted protein soubilization and digestion methods for Escherichia coli membrane proteome analysis by 2-D LC-MS/MS. Proteomics. 2007; 7:484–493. [PubMed: 17309111]
9. Sun L, Tao D, Han B, Ma J, Shu G, Liang Z, Shan Y, Zhang L, Zhang Y. Ionic liquid 1-butyl-3-methyl iimidazolium tetrafluoroborate for shotgun membrane proteomics. Bioanal Chem. 2011; 399:3387–3397.
10. Chen EI, Cociorva D, Norris JL, Yates JR. Optimization of mass spectrometry-compatible surfactants for shotgun proteomics. J Proteome Res. 2007; 6:2529–2538. [PubMed: 17530876]
11. Blonder J, Chan KC, Issaq HJ, Veenstra TD. Identification of membrane proteins from mammalian cell/tissue using methanol-facilitated solubilization and tryptic digestion coupled with 2D-LC-MS/MS. Nat Protoc. 2006; 1:2784–2790. [PubMed: 17406535]
12. Yao X, Freas A, Ramirez J, Demirev P, Fenselau C. Proteolytic ^{18} O Labeling for Comparative Proteomics: Model Studies with Two Serotypes of Adenovirus. Anal Chem. 2001; 73:2836–2842. [PubMed: 11467524]
13. Sun D, Wang N, Li L. Integrated SDS removal and Peptide Separation by Strong-Cation Exchange Liquid Chromatography for SDS-Assisted Shotgun Proteome Analysis. J Proteome Res. 2012; 11:818–828. [PubMed: 22214374]
14. Rahbar AM, Fenselau C. Integration of Jacobson's pellicle method into proteomic strategies for plasma membrane proteins. J Proteome Res. 2004; 3:1267–1277. [PubMed: 15595737]
15. Chaney LK, Jacobson BS. Coating cells with colloidal silica for high yield isolation of plasma membrane sheets and identification of transmembrane proteins. J Biol Chem. 1983; 258:10062–10072. [PubMed: 6309765]
16. Liu H, Sadygov RG, Yates JR. A Model for Random Sampling and Estimationof Relative Protein Abundance in Shotgun Proteomics. Anal Chem. 2004; 76:4193–4201. [PubMed: 15253663]
17. Edwards N, Wu X, Tseng TW. An unsupervised, model-free, machine-learning combiner for peptide identifications from tandem mass spectra. Clinical Proteomics. 2009; 5:23–36.
18. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001; 305:567–580. [PubMed: 11152613]
19. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 1982; 157:105–132. [PubMed: 7108955]

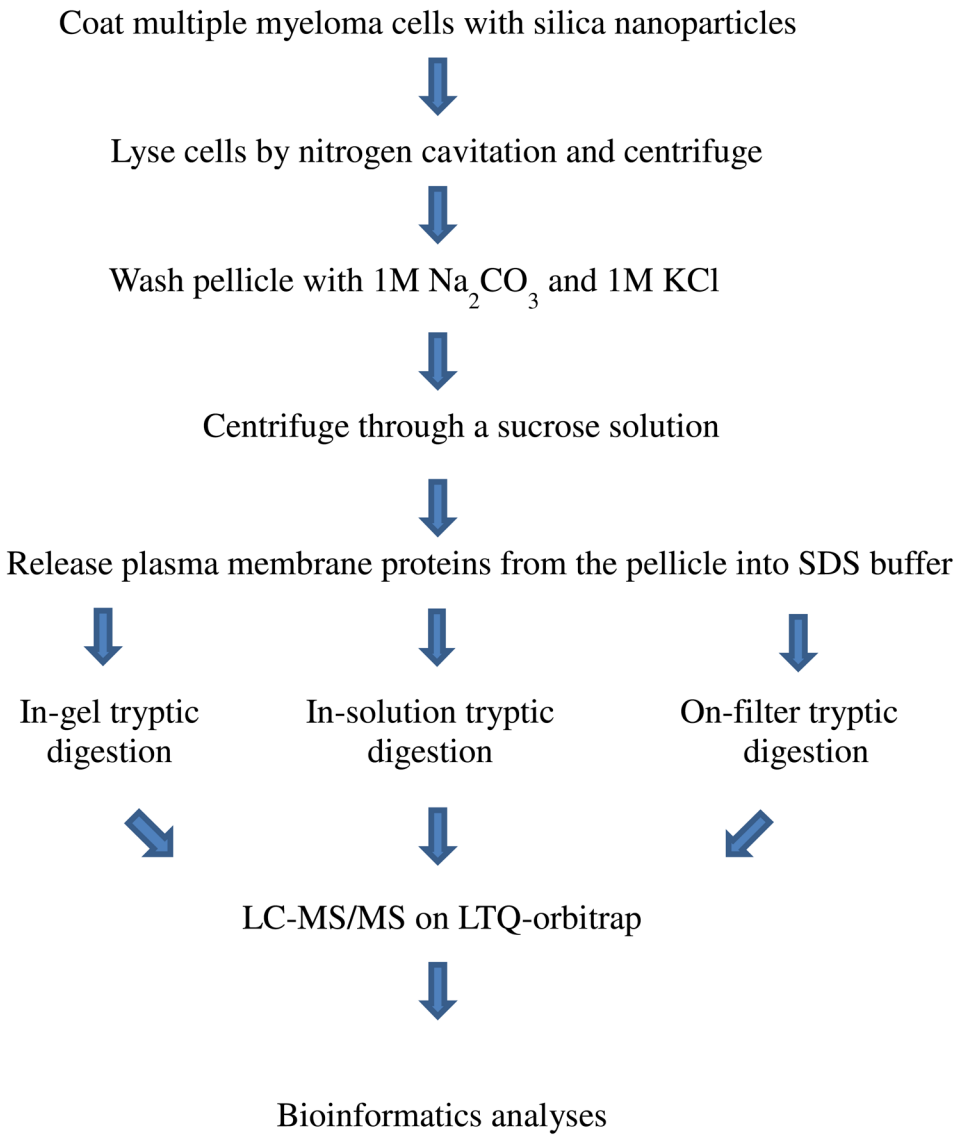

Figure 1.
The General Workflow

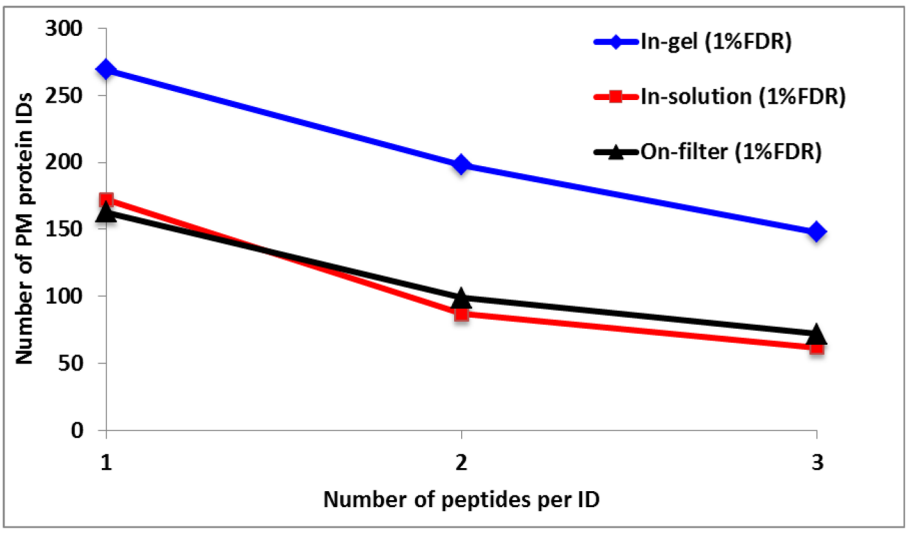

Figure 2.
Number of plasma membrane proteins identified from in-gel digestion, in-solution digestion, and on-filter digestion as a function of peptides per protein

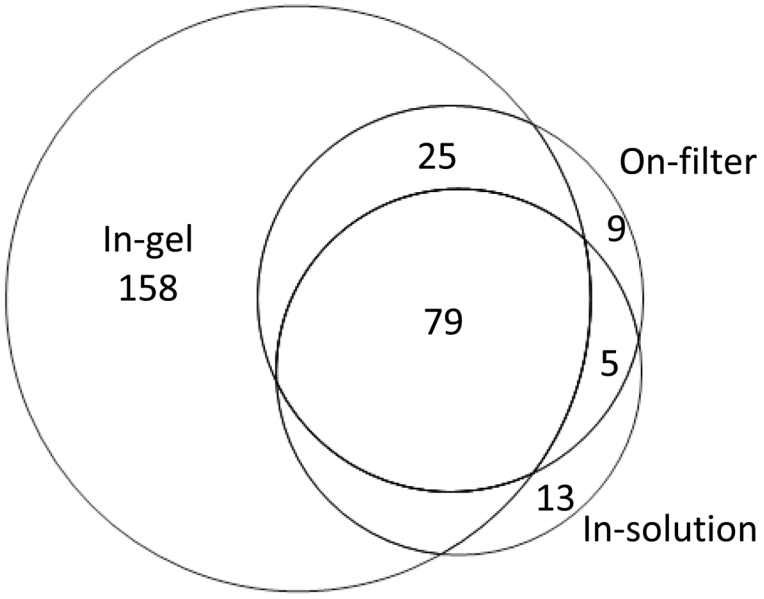

Figure 3.
Venn diagram showing overlap in proteins identified using in-gel, in-solution and on-filter digestion

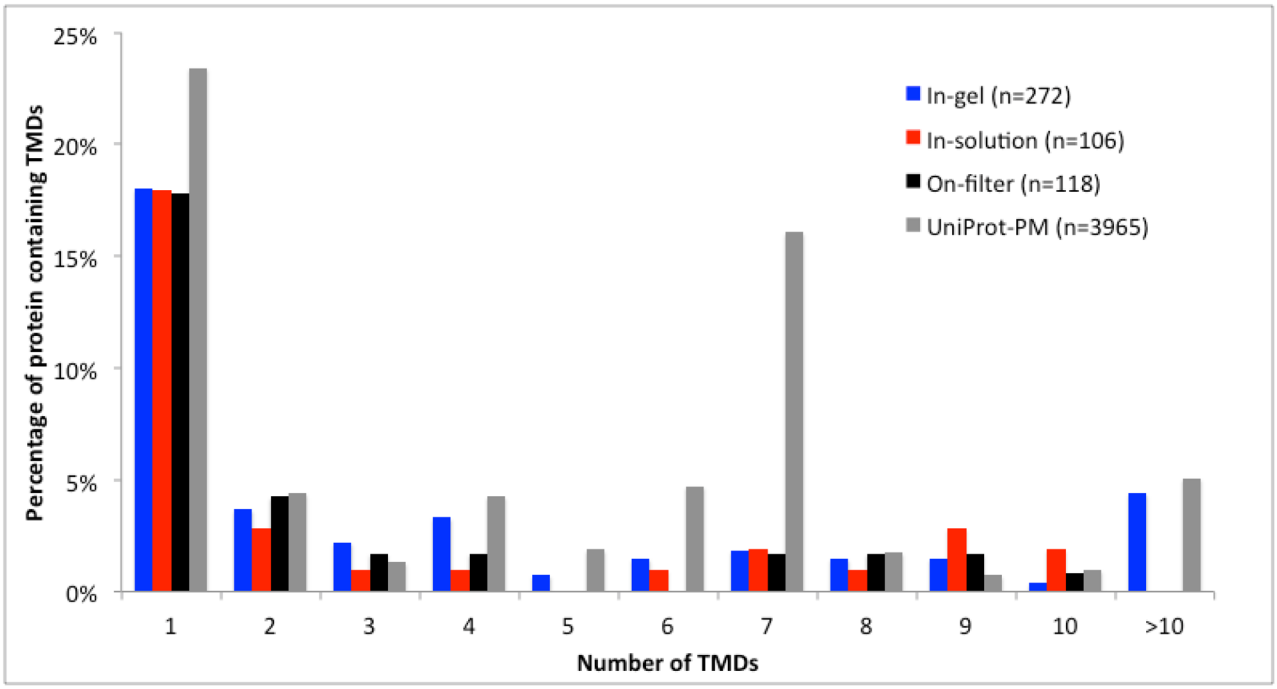

Figure 4.
Fractions of plasma membrane proteins containing transmembrane helices from in-gel, in-solution, on-filter and the 3964 protein reference set (UniProt).

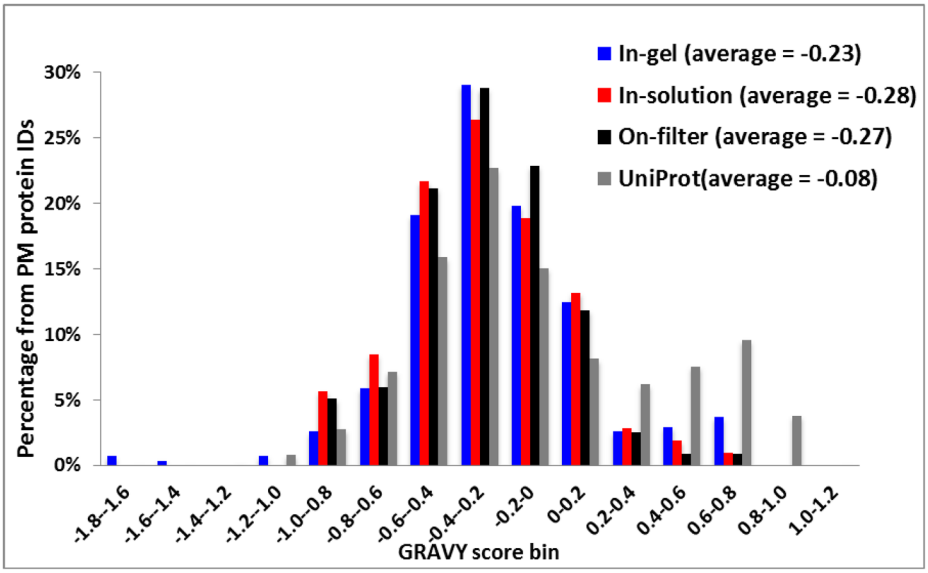

Figure 5.
Correlation between the number of plasma membrane proteins identified and their hydrophobicities

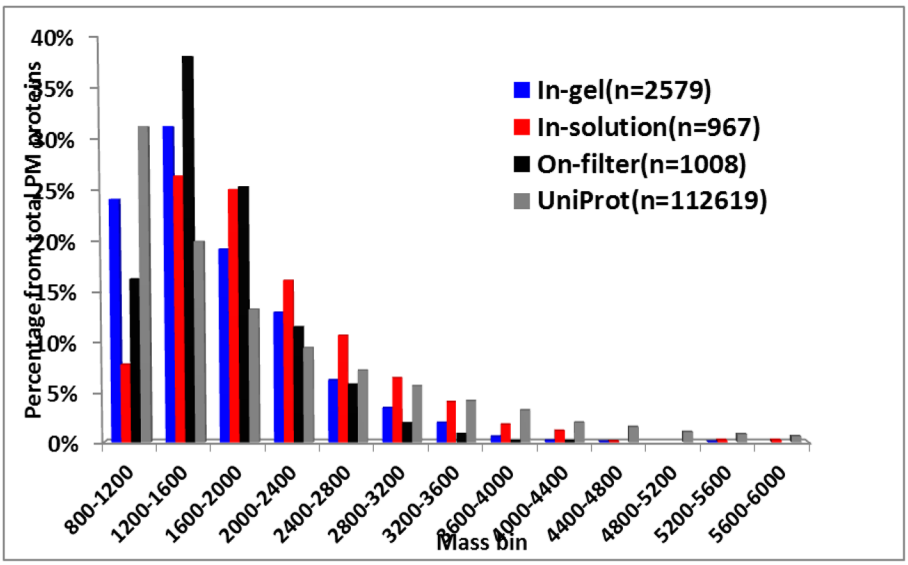

Figure 6.
Molecular weight distribution between 800 and 6000Da of PM tryptic peptides identified from in-gel, in-solution, on-filter and predicted in silico from the (UniProt) reference set

Table 1

Number of PM proteins and peptides identified using three different methods *

	In gel	In-solution	On-filter
PM protein IDs (based on 2 peptides)	272	106	118
PM proteins containing TMDs (based on 2 peptides)	106	33	37
Total PM peptide IDs	2580	967	1009

* Based on 2 peptides per protein