In-gel Digestion of Mouse Membrane Protein Extract: Increased Peptide Recovery and Identification of Very Low Abundance Hydrophobic Proteins

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Overview

- A robust, 1 hour in-gel digest protocol is presented which, in the presence of a commercially available and MS friendly surfactant, significantly increases membrane protein and peptide identification.
- The effectiveness of the new protocol is compared to that of an overnight standard in-gel digestion.
- Increased protein sequence coverage and identification of low abundance membrane proteins are investigated.
- Physiochemical differences of peptides identified in contrasting methods are evaluated.

Introduction

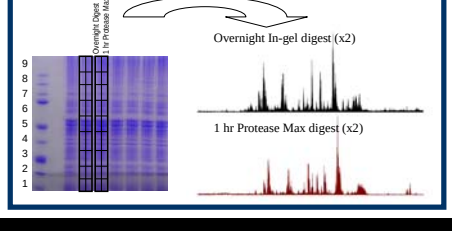
In mammalian proteomes, it is estimated that 6,000-8,000 genes encode for membrane proteins. Yet, large scale proteomic analysis of these same membrane proteins remains a challenge. The hydrophobic nature of membrane proteins most commonly results in poor protein solubility. For in-gel digestion protocols, poor protein solubility for both hydrophobic and hydrophilic peptides results in inefficient digestion and peptide extraction.

Methods

Highly purified cell membrane protein was obtained from whole tissue mouse heart homogenates. The membrane extract was run on a 1-D SDS PAGE gel. Each lane was excised into 9 regions and each region split in half. One half of the bands was digested using a standard 37°C overnight in-gel protocol as previously reported[1]. The remaining half was digested using a membrane protein-targeted protocol at 50°C for 1 hour which included 0.01% Protease Max (Promega Corp.).

NanoLC-MS was performed in identical fashion on all samples, using a self-packed 75 um ID C18 column. The mass spectrometer was a LCQ Deca XP$^+$ set in data dependent acquisition mode to perform MS/MS on the top three most intense ions with a dynamic exclusion setting of two. The .RAW files were searched using a Sage-N Sorcerer data processor which implements the SEQUEST search algorithm. All samples were run in duplicate.

SCHEME 1. Overview of general sample workup



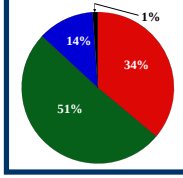
Results

TABLE 1. Protein and peptide identification numbers as a function of in-gel digestion conditions. Gel regions 1, 4, 5 & 9 contained many proteins that remained unidentified using the standard overnight digest protocol. The remaining regions reported only slight differences: 2, 3 & 8 gave a few more peptides in the overnight digest, and one additional unique protein; 6 & 7 gave a few more peptides in the 1 hr digest, and one additional unique protein.

	Gel Region	1*	2	3	4*	5*	6	7	8	9*	Total	Change
Overnight												
	Peptides	135	210	271	108	96	243	163	130	51	1407	
	Proteins	23	28	36	18	13	45	36	28	6	233	
Pmax_1hr												
	Peptides	210	198	253	253	204	266	149	147	98	1778	>371
	Proteins	31	28	36	29	24	45	37	31	12	273	>40

*These regions with distinct variations in protein and peptide identifications offer insight into the functionality of the MS friendly surfactant and were selected for further analysis as presented below.

FIGURE 1. Analysis of variation between protocols. Specifically comparing the findings within gel regions 1, 4, 5 & 9, which represent 765 peptides and 96 proteins, four scenarios prevail:

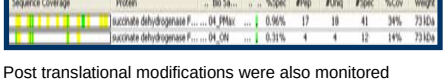


1. Protein identification under Protease Max digestion conditions only
2. Protein identification under both conditions; Sequence coverage increased by Protease Max
3. No change in identification under either digestion protocol
4. Protein identification under overnight digestion conditions only

Increased sequence coverage

Sequence coverage for previously identified proteins increased 51% of the time as a result of the use of Protease Max. In addition, 34% of the time coverage increased from zero to 2 or more peptides, enabling identification of proteins unobserved in the overnight digestion (scenario 1).

FIGURE 2. Increased sequence coverage of succinate dehydrogenase. A 20% increase in sequence coverage (13 peptides) was realized under Protease Max digestion conditions.



Post translational modifications were also monitored (phosphorylation, acetylation, oxidation): no tendencies fitting outside the general statistics were noted, other than that acetylation is preferentially observed in overnight digests. The data sets for acetylated peptides were too small for further exploration supported by statistical analysis.

Physiochemical properties

Peptides of scenario 1 (Protease Max only identified, red) and scenario 3 (no change in either digest conditions, blue) were sorted and annotated for further investigation into their physiochemical properties as demonstrated in Figures 3a and 3b below.

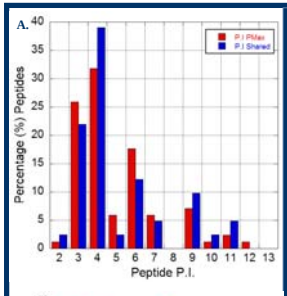


FIGURE 3a. Binned peptides by pI as calculated by GPMAW software for individual peptides which were identified only when using surfactant (red) or which were identified equally regardless of digest conditions (blue).

The deviation between sample sets is nominal. The trend toward low pI Is expected because all LC- MS/MS runs were performed using nanoESI in positive ion mode.

FIGURE 3b. Binned peptides by GRAVY value as calculated using the Expasy ProtParam function[2] for each peptide in scenario 1 (red) and 3 (blue) as detailed in Fig. 3a above.

Peptides identified only in the digests involving PMax have an average GRAVY value lower than those in the equally identified fraction, indicating a pseudo-environment soluble to very hydrophilic peptides.

In order understand the amino acid compositional trends, peptides identified in scenario 1 (red, protease max only) were plotted as % amino acid composition against the normal occurrence (blue) in proteins[3] (Figure 4, below). Significant biases toward peptides rich in alanine, and to a lesser degree aspartic acid, were identified.

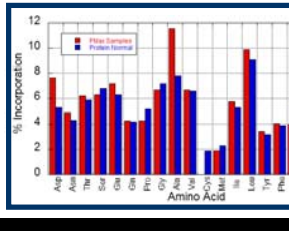


FIGURE 4. Amino acid composition of peptides preferentially detected in surfactant digest in comparison to normal protein composition.

Chromatography & carryover of surfactant

Surfactants are potentially detrimental to chromatographic methods, as they nonspecifically bind to reversed phase matrix and greatly diminish column capacity. The Protease Max surfactant used in the 1 hr digests had specific and reproducible chromatographic qualities that were unobtrusive to the eluting peptides. The amount of residual surfactant does not affect subsequent chromatographic runs (Figure 5, below).

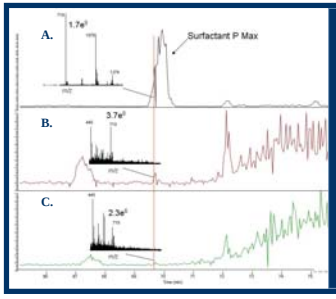


Figure 5. Chromatographic behavior: TICs of (A) Fourteenth consecutive run where Protease Max was present in the sample injection. (B) Blank run 1 and (C) Blank run 2 immediately following run A. Minimal surfactant carryover is observed, and the effect on peptide separation is negligible.

Conclusions

- The use of a commercially available MS friendly surfactant decreases digestion time from overnight (16 hrs) to 1 hr
- Peptide identification is significantly enhanced when using the surfactant:
  - Very low abundance membrane proteins that otherwise went undetected in a routine in-gel digest were identified
  - Sequence coverage was increased in over 50% of the proteins identified
- The most significant change in physiochemical properties found in the subset of peptides was a trend toward low GRAVY values (more hydrophilic peptides)
- Peptides identified with surfactant had a disproportional amount of alanine and aspartic acid
- Surfactant is visualized during a chromatographic run, but has defined unobtrusive retention characteristics and minimal carryover.

References

1. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Shevchenko, A; Tomas, H; Havlis, J; et al. NATURE PROTOCOLS Volume: 1, pp. 2856-2860. 2006.
2. http://ca.expasy.org/tools/protparam.html
3. Doolittle, R.F. (1989) Redundancies in protein sequences. In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D., ed.), pp. 599-623, Plenum Press, New York.

Acknowledgements

Special thanks to the Vincent and Stella Coates Foundation

This poster may be downloaded from the Stanford University Mass Spectrometry website at http://mass-spec.stanford.edu/Publications.html