

High pressure-assisted in-gel tryptic digestion: qualitative and quantitative aspects

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INTRODUCTION

Tryptic digestion of proteins continues to be a fundamental part of any proteomics workflow. A number of enhanced digestion protocols have been developed in recent years (1, 2). Nonetheless, a need still exists for new digestion approaches that meet the demands of qualitative and quantitative proteomics. Another integral part of proteomics is separation of proteins. All separation techniques in proteomics may be broadly classified under three groups: gel-based, liquid phase-based and hybrid liquid phase-gel-based. Recently we have developed an integrated approach combining RP-HPLC separation of proteins with direct on target tryptic digestion (3). Here we report an application of pressure cycling technology (PCT) with tryptic in-gel digestion of proteins and an evaluation of the application of this method to label-free quantitation of proteins separated by 1DE.

METHODS

SDS PAGE separation of proteins

- Individual solution of three standard proteins, i.e., glucose oxidase (GLOx), pyruvate kinase (PyKin), or catalase (Catal) and a primary liquid standard (pLS) preparation of the influenza virus A/Uruguay/716/2007 were loaded on NuPAGE 4-12% Bis-Tris gel. Pictures of the resulting Coomassie Blue stained gels are shown in Fig. 1 & 2, respectively. Individual bands were excised and proteins contained in them were digested either applying the standard approach or using Pressure Cycling Technology (PCT) as described below.

Standard Digestion

- Gel pieces were destained and proteins were reduced (using DTT) and alkylated (using iodoacetamide).
- Proteins were then digested with trypsin at enzyme:protein ratio of 1:20 for 16h at 37°C.
- Peptides were extracted by adding 0.1% TFA in CH₃CN to a final CH₃CN concentration of 50%.
- A 0.7 µL aliquot of the digest was spotted on a MALDI target in triplicate and samples were analyzed using the 4800 MALDI TOF/TOF.

PCT digestion

- Pressure-assisted digestion was performed in a Barocycler NEP3229 instrument (Pressure BioSciences, Inc., South Easton, MA)
- Gel pieces were destained and proteins were reduced and alkylated.
- Gel pieces were transferred to PCT Microtubes.
- Proteins were then digested with trypsin at enzyme: protein ratio of 1:20 using PCT for 45 cycles at 37°C, 35 kpsi, 55s up, 5 sec down.
- Peptides were extracted by adding 0.1% TFA in CH₃CN to a final CH₃CN concentration of 50%.
- A 0.7 µL aliquot of the digest was spotted on a MALDI target in triplicate and samples were analyzed using the 4800 MALDI TOF/TOF.

Relative quantitation of influenza virus proteins

1) Normalization with a co-migrating protein

- The average of the peak heights of the three most intense peptides for each influenza protein were calculated in each band. The average value of one of the proteins, e.g. NP for Bands 1,3, and 7 or HA2 for Band 11 (figure 2), was used to normalize the average value of the other co-migrating proteins. Figure 2 shows a bar graph representation of the number of folds of a co-migrating protein in relation to the protein used for normalization.

Normalization with added internal standard

- To 9 µL of the digest of each band of the pLS preparation of A/Uruguay/716/2007, 1 µL of a predigested catalase solution (concentration 200 ng/µL) was added as an internal standard.
- The average of the peak heights of the three most intense peptides of catalase were used to normalize the average peak heights of each influenza protein.

MALDI MS and Data Analysis

- Mass spectra were acquired on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA).
- The resulting data were processed and interpreted using GPS Explorer™ version 3.6
- MASCOT was used for database searching against SwissProt database, considering up to one missed cleavage, carbamidomethyl modifications of cysteine, monoisotopic peptide mass tolerance of 50 ppm, and fragment ion mass tolerance of 0.3 amu.

RESULTS

Figure 1: SDS PAGE of standard proteins. Each protein was loaded in duplicate and each band was digested either by the standard or PCT approach.

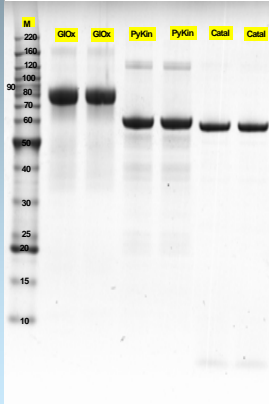
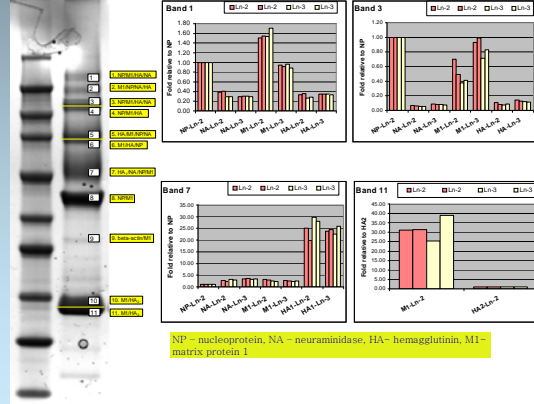


Figure 2: SDS PAGE of pLS from A/Uruguay/716/2007 depicting spot-to-spot and lane-to-lane repeatability. The average of the peak heights of one of the proteins, e.g. NP for Bands 1,3, and 7 or HA2 for Band 11, was used to normalize the average value of the other co-migrating proteins.



NP - nucleoprotein, NA - neuraminidase, HA - hemagglutinin, M1 - matrix protein 1

Figure 3: Mass spectra of glucose oxidase and pyruvate kinase comparing the standard and PCT (Barocycler) digestion approaches.

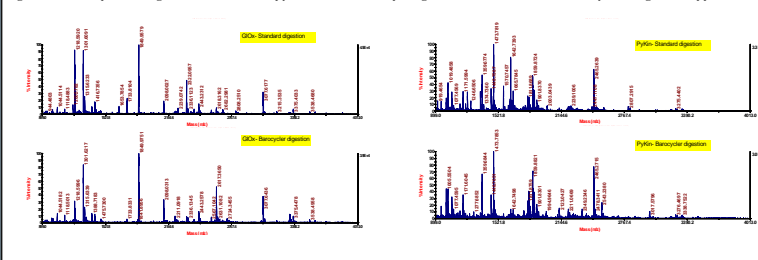
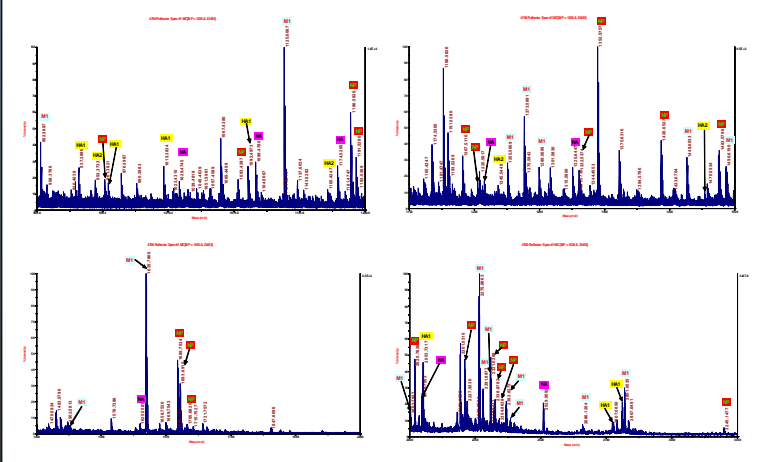


Figure 4: Mass spectra of the tryptic digest of pLS from A/Uruguay/716/2007, lane-2, band-1 (figure 2 above). The ion peaks assigned to peptides of each influenza protein are indicated with the protein name. NP - nucleoprotein, NA - neuraminidase, HA - hemagglutinin, and M1 - matrix protein 1.



RESULTS

Table 1: Summary of comparison of the standard and PCT digestions with respect to number of peaks identified, number of peptides assigned and sequence coverage.

Protein	No of peaks detected, *SNR > 20		No of peptides assigned by PMF		% Sequence Coverage		No peptides assigned by MS/MS	
	Standard	PCT	Standard	PCT	Standard	PCT	Standard	PCT
GLOx	97	92	14	17	38	49	7	9
PyKin	112	128	25	30	59	66	10	13
Catalase	86	80	18	21	45	50	11	14

Table 2: Relative quantitation of co-migrating influenza virus proteins from 1D gel. Predigested catalase was used as an internal standard. The table demonstrates spot-to-spot and lane-to-lane repeatability of the quantitation strategy.

Protein	Lane-1		Lane-2		Lane-3		Lane-1 to Lane-3	
	Average (fM)	% CV	Average (fM)	% CV	Average (fM)	% CV	Average (fM)	% CV
HA	38	3	30	5	36	10	34	12
M1-	54	1	50	11	51	3	52	4
NA	28	1	21	1	24	6	24	15
NP	92	2	67	1	79	0	79	16

CONCLUSIONS

Our results demonstrated that high-pressure assisted in-gel tryptic digestion compares with the standard in-gel tryptic digestion with respect to number of peaks detected, number of peptides identified, and sequence coverage. In all three parameters, PCT digestion performed at least as well, and sometimes better, than the standard digestion approach. For example, PCT provided sequence coverage ranging from 49% to 66% as compared to 38% to 59% obtained with standard digestion. This approach also reduced the extended time (about 16 hrs) required by the standard in-gel digestion to 45 minutes. The quantitation aspect of our study revealed good spot-to-spot and lane-to-lane reproducibility, with relative errors less than ±10 and 15%, respectively. We have shown that this high pressure assisted, label-free approach can be used for the rapid, relative quantitation of proteins after 1DE separation. We have also demonstrated that this method can be used to determine the relative quantities of co-migrating proteins using influenza virus preparations. We are currently evaluating the applicability of this approach for absolute quantitation of proteins after 1DE separation.

ACKNOWLEDGMENT

This project was supported by funding provided by CBER Pandemic Influenza Unmet Needs and by Biomedical Advanced Research & Development Authority.

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