Label-free mass spectrometry-based relative quantitation of proteins separated by 1DE

Melkamu Getie-Kebtie[1]; Maryna Eichelberger[2], Manju B. Joshi1[3], Rajesh Gupta[3], Michail A. Alterman[1]
[1]FDA/CBER/OCTGT/TVBB; [2]FDA/CBER/OVRR/LPRVD; [3]FDA/CBER/OVRR/PQLS

INTRODUCTION

The determination of differences in relative protein abundance is a vital aspect of proteomics research that is progressively used to address various biological questions. Until recently, majority of the quantitative proteomic analyses have been performed using stable isotope labeling strategies such as SILAC, ICAT, iTRAQ™, and ^{18}O labeling. In recent years, however, relative and absolute quantification of proteins by label-free mass spectrometry is gaining a significant acceptance (1, 2). Here we report a label-free approach for relative quantitation of proteins after separation by SDS PAGE. Our approach involves spiking of a known molar quantity of a pre-digested internal standard protein to the in-gel digest of the test samples. The average peak intensity of the three most abundant peptides of the internal standard will then be used to normalize that of the three most abundant peptides from each protein in the mixture. Our results demonstrated a good spot-to-spot and lane-to-lane repeatability with coefficient of variance of less than ±10 and 20%, respectively. The applicability of this method for more complex samples was further demonstrated on primary liquid standard preparation of H3N2 influenza A virus (A/Uruguay/716/2007).

METHODS

SDS PAGE separation of proteins

- A serial dilution of a mixture of five standard proteins, i.e., carbonic anhydrase (CarAn), glucose oxidase (GlOx), hemoglobin (Hem), pyruvate kinase (PyKin), and lactate dehydrogenase (LaDe) and a primary liquid standard (pLS) preparation of the influenza virus A/Uruguay/716/2007 were loaded on NuPAGE 4-12% Bis-Tris gels.
- Pictures of the resulting Coomassie Blue stained gels are shown in Figs. 1 & 3, respectively.
- Individual bands were excised and all the bands in a single lane were mixed and placed in a single tube (for standard proteins) or each band was placed in a separate tube (for pLS).
- Proteins contained in the gels were then digested using Pressure Cycling Technology (PCT) as described below.

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 CH_3CN to a final CH_3CN concentration of 50%.
- A 0.7 uL aliquot of the digest containing the internal standard was spotted on a MALDI target in triplicate and samples were analyzed using the 4800 MALDI TOF/TOF.

RESULTS

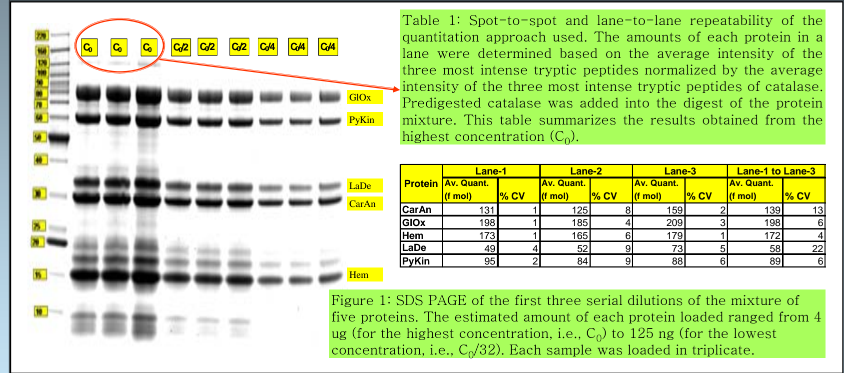

Table 1: Spot-to-spot and lane-to-lane repeatability of the quantitation approach used. The amounts of each protein in a lane were determined based on the average intensity of the three most intense tryptic peptides normalized by the average intensity of the three most intense tryptic peptides of catalase. Predigested catalase was added into the digest of the protein mixture. This table summarizes the results obtained from the highest concentration (C_0).

Protein	Lane-1		Lane-2		Lane-3		Lane-1 to Lane-3	
	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV
CarAn	131	1	125	8	159	2	139	13
GlOx	198	1	185	4	209	3	198	6
Hem	173	1	165	6	179	1	172	4
LaDe	49	4	52	9	73	5	58	22
PyKin	95	2	84	9	88	6	89	6

Figure 1: SDS PAGE of the first three serial dilutions of the mixture of five proteins. The estimated amount of each protein loaded ranged from 4 ug (for the highest concentration, i.e., C_0) to 125 ng (for the lowest concentration, i.e., $C_0/32$). Each sample was loaded in triplicate.

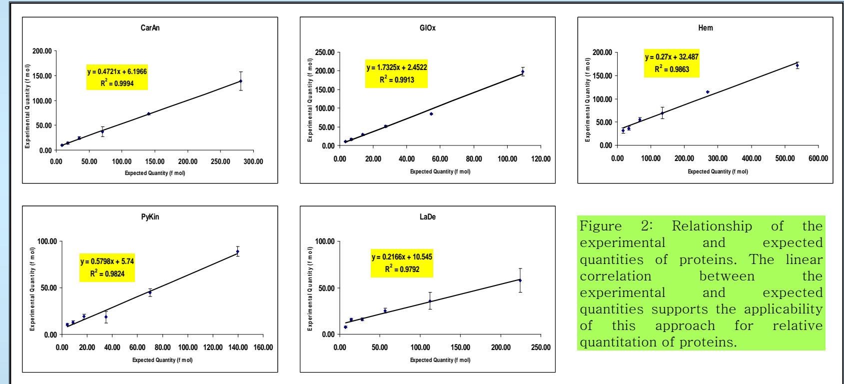

Figure 2: Relationship of the experimental and expected quantities of proteins. The linear correlation between the experimental and expected quantities supports the applicability of this approach for relative quantitation of proteins.

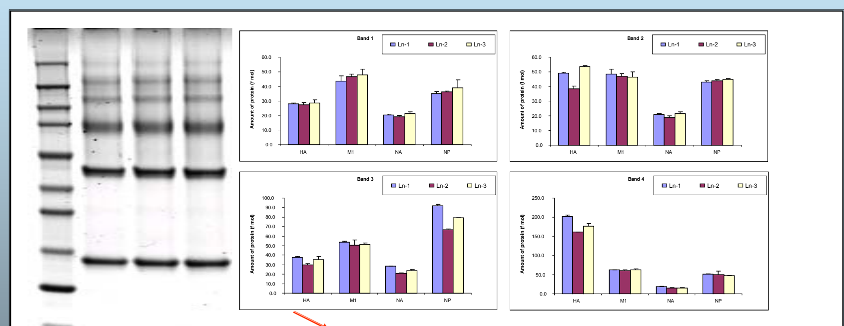

Figure 3: SDS PAGE of a primary liquid standard (pLS) preparation of the influenza virus A/Uruguay/716/2007. The bar graphs show the spot-to-spot and lane-to-lane repeatability.

Table 2: Relative quantitation of co-migrating influenza virus proteins from 1D gel (Band 3). Predigested catalase was used to normalize the average intensity of the three most intense tryptic peptides from each protein. 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	
	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% 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

METHODS

Digestion of catalase

- A stock solution of catalase was prepared in 50 mM ABC containing 0.1% RapiGest and the protein concentration was determined using a 2-D Quant Kit (GE Healthcare).
- The protein was reduced (DTT) and alkylated (iodoacetamide) before tryptic digestion at enzyme: protein ratio of 1:20 at 37 °C for 16 h.
- The catalase digest was further diluted to 50 ng/uL before addition to the test samples.

Relative quantitation of standard proteins

- Equal volumes of the internal standard tryptic digest and the in-gel digest of the protein mixtures were mixed and a 0.7 uL aliquot was spotted on a MALDI target.
- The average peak intensities of the three most intense tryptic peptides for each protein were calculated and these values were normalized by the average peak intensity of the three most intense tryptic peptides of catalase.

Relative quantitation of infulenza proteins

- To 9 uL of the digest of each band of the pLS preparation of A/Uruguay/716/2007, 1 uL of a predigested catalase solution was added as an internal standard.
- Normalization of the average peak intensities of influenza virus proteins was performed as described above for standard proteins.

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 SwisProt 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.

CONCLUSIONS

We have shown that 1DE separation of protein mixtures and tryptic digestion of excised bands followed by MALDI TOF/TOF analysis can be used as a label-free strategy for relative quantitation of proteins using purified proteins. We have also confirmed the applicability of this technique by determining the relative quantities of proteins co-migrating in SDS PAGE of an influenza virus preparation.

ACKNOWLEDGMENT

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

REFERENCES

1. Silva, J. C., Gorenstein, M. V., Li, G., Vissers, J. P. C., Geromanos, S. J., Molecular & Cellular Proteomics 2006, 5:144–156
2. Wang, W., Zhou, H., Lin, H., Roy, S., Shaler, T. A., Hill, L. R., Norton, S., Kumar, P., Anderle, M., Becker, C. H., Anal. Chem. 2003, 75, 4818–4826.

"The findings and conclusions in this presentation have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy."