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Comparison of the capabilities of liquid isoelectric focusing–one-dimensional nonporous silica reversed-phase liquid chromatography–electrospray ionization time-of-flight mass spectrometry and liquid isoelectric focusing–one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis mass mapping for the analysis of intact protein molecular masses

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Abstract

Nonporous silica reversed-phase HPLC coupled to electrospray ionization with on-line time-of-flight mass spectrometric detection (NPS-RP-HPLC–ESI-TOF-MS) is shown to be an effective liquid phase method for obtaining the molecular masses of proteins from pH fractionated cellular lysates where the method is capable of generating the same banding patterns typically observed using gel phase one-dimensional sodium dodecyl sulfatepolyacrylamide gel electrophoresis. The liquid-phase mass spectrometry-based method provides a mass accuracy of at least 150 ppm, with 4000 mass resolution and provides improved sensitivity as the protein molecular mass (MW) decreases. The liquid and gel phase methods are shown to be complementary in terms of their mass range but the liquid phase method has the advantage over the gel method in that the analysis times are 50 times shorter, the mass accuracy is 70 times better and the resolution is 130 times higher. The liquid phase method is shown to be more effective for detection of proteins below 40 kDa, while the gel phase separation can access many more proteins, including more hydrophobic proteins, at increasing MW.

Keywords: Mass mapping; Molecular mass; Proteins

1. Introduction

Recently rough drafts of the human genome were completed by both private and public organizations. The primary objective now is to understand what these sequences mean and many reviews have been written on the evolving field known as proteomics [1–3]. While some genes are already well characterized many are not and there is not yet any effective method to determine from the gene sequence alone the specific sequences that will make up expressed genes and more importantly what proteins may be expressed. The ultimate goal is to be able to determine the function of the proteins that are trans-

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lated from the genome and to thereby understand molecular mechanisms that may be related to some disease state, pharmacoproteomic research topic and/or fundamental biological problem.

Currently most proteins are identified by peptide mass mapping and tandem mass spectrometry (MS–MS) methods that typically cover from 20 to 70% of the protein sequence [4–14]. Because of the limited sequence coverage it is possible that some isoforms that share significant sequence homology may go undetected because the unique parts of the protein isoform may not be observed. For this reason it is vital that MS methods be able to cover 100% of the protein sequence. The most effective method to accomplish this is to detect the protein intact molecular mass. Other workers have attempted to obtain intact protein molecular mass (MW) values but with limited mass accuracy [15–18]. Some workers have attempted to obtain MW information from gel-separated proteins [19] however, exact MW information is difficult to obtain from proteins separated on gels. The exact MW is essential for determining the presence of posttranslational modifications and the correct sequence of the protein, which may have been modified in the cell to be different from that expected from the database. A number of methods have been used to interface matrix-assisted laser desorption ionization (MALDI) MS to analysis of intact protein MW including MALDI-MS directly from gels [19] or from proteins blotted onto membranes [18,20–26]. In either case, as the size of the protein increases, only limited resolution can be determined by MALDI-MS and a mass accuracy of only 1–5 parts-per-thousand might be expected [27]. Electrospray MS can provide very highly accurate intact MW values of larger proteins [28], but intact proteins are not readily interfaced from gels to electrospray ionization (ESI) MS.

In this work, a liquid phase separation technique is described for obtaining exact MW analysis by ESI-MS and compared to gel electrophoresis. A prefractionation based upon isoelectric point (p*I*) is used in the liquid phase as the first dimension and then a one-dimensional (1D) separation of a p*I* fraction is performed either using nonporous silica (NPS) reversed-phase high-performance liquid chromatography (HPLC) with on-line ESI time-of-flight (TOF) MS detection or alternatively a 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) separation. The resulting protein band patterns from the two separations are compared in terms of the resolution, mass accuracy and speed for determining M_r values. The accurate intact MW determination provided by the liquid phase ESI-MS method together with peptide mapping results [26] can be compared against the databases to determine the presence of posttranslational modifications and other alterations in the structure of the protein. In addition, the liquid-based method can provide detection of proteins at low MW not detected on gels or proteins that cannot be resolved on gels but can be readily resolved and detected by ESI-MS. At present though, the gels still provide a means to detect proteins at increasingly higher MW where detection by ESI-MS is still limited.

2. Experimental

2.1. HEL protein sample preparation

HEL cell-line samples were prepared in the laboratory of Dr. Michael Long at the University of Michigan in the Department of Pediatrics as in previous work [26,29]. The proteins were extracted from the cells by thawing to room temperature and adding three volumes of lysis buffer consisting of 6 *M* urea (Bio-Rad), 2 *M* thiourea (Bio-Rad), 1% *n*-octyl β-D-galactopyranoside (OG; Sigma), 6% ampholytes (Bio-Rad, 3/10), 10 m*M* Tris[2-carboxyethyl] phosphine (TCEP; Pierce), 10 m*M* dithiothreitol (DTT) (Bio-Rad) and 10 m*M* phenylmethylsulfonyl fluoride (PMSF) (Bio-Rad) [26]. The samples contained approximately $5 \cdot 10^6$ cells per mg of sample. Previous analysis of these HEL cell lines by two-dimensional (2D) SDS–PAGE [24,29] show that these samples do not contain highly over-expressed proteins such as albumin which are often found in bodily fluids and may interfere with the analyses performed in this work.

2.2. Isoelectric focusing (IEF) with the mini-Rotofor

The HEL cytosolic protein fraction was loaded to the mini-Rotofor along with a separation buffer

containing all the same ingredients as the lysis buffer with a total final separation volume of 15 ml. The minirotofor provides a much reduced volume compared to the Rotofor used in earlier work [24]. The minirotofor was run as in previous work [24,26] where the proteins were separated by isoelectric focusing over a 5-h period where the separation temperature was 10°C and the separation buffer contained 0.1% OG (Sigma), 8 *M* urea (ICN), 2% β-mercaptoethanol (Bio-Rad), and 2.5% Biolyte ampholytes, pH 3.5–10 (Bio-Rad). The 20 fractions contained in the Rotofor were collected simultaneously, into separate vials, using a vacuum source attached by plastic tubing to an array of 20 needles, which were punched through a septum. The pH of the fractions was measured using a mini-pH electrode (PH/C 900, Amersham Pharmacia Biotech). The concentration of protein in each fraction was determined using the Bradford-based assay (Bio-Rad). Protein pH fractions were stored at −80°C until loading to the nonporous reversed-phase (RP) HPLC column.

2.3. 1D SDS–PAGE

1D SDS–PAGE was performed using gradient gels in a Hoefer 14×16 cm format vertical gel apparatus using the Laemlli buffer system. The gel gradient was from 8 to 20% T with 2.6% C and was poured using a gradient maker (Hoefer Pharmacia Biotech, SG50, San Francisco, CA, USA) using gel solutions (Sigma) [T=(g acrylamide+g Bis)/100 ml solution; C=g Bis/T]. The gels were run at constant 200 V overnight. The tank buffer was 25 m*M* Tris (Sigma), 192 m*M* glycine (Sigma), 0.1% SDS (Sigma) and adjusted to pH 8.3. The gel buffer was 4× 1.5 *M* Tris–HCl, 0.4% SDS and the stacking buffer was 4× 0.5 *M* Tris–HCl, 0.4% SDS. Gels were stained using a modified silver stain procedure. Briefly the gels were fixed in 10% methanol (Sigma, HPLC grade)–12% acetic acid (Sigma, HPLC grade), washed in water (Milli-Q), sensitized in 0.02% sodium thiosulfate (Fisher, NJ, USA), washed in water, stained with 0.1% silver nitrate (Sigma, ACS reagent), washed in water and finally developed with 2% sodium hydrogencarbonate (Fisher). The development procedure was stopped using 10% acetic acid–10% methanol. Gels were imaged using the Gel-pro analyzer software (version 3.1). Calibration was performed using the unstained broad range calibrants (Bio-Rad) and Microcal Origin (version 6.0) software. The gel MW plots were converted from a log MW scale to a linear MW scale. The calibration equation {log MW= $5.57699+[-0.07940\cdot(\text{migration distance})^{1/2}]$} used related the log of the protein MW to the square root of the band/peak migration distance as is standard with gradient gels.

2.4. NPS-RP-HPLC protein separations

The separations were performed as in previous work [26]. 33×3 mm columns packed with 1.5 μm diameter nonporous silica ODS I particles (Eichrom Technologies) were used for protein separations. The use of 3 mm columns allowed for slower flow-rates than the 4.6 mm columns used in earlier work [24], which is more compatible with the on-line ESI-TOF-MS detection. The flow-rate used was 0.2 ml/min. The binary gradient was run from 0 to 20% B in 1 min, then 20 to 30% B in 2 min, then 30 to 54% B in 8 min, then 54 to 65% B in 1 min and finally up to 100% B in 1 min. Accounting for a 1-min dwell-time the actual gradient reached 54% in 12 min. The NPS allow rapid separations of large numbers of proteins with high recovery compared to porous columns [24].

2.5. ESI-TOF-MS

Electrospray ionization mass spectrometry was performed using an ESI-TOF-MS instrument (LCT, Micromass, Manchester, UK) as in previous work [26]. Typical flow-rates to the mass spectrometer were 0.2 ml/min from a total flow-rate of 0.4 ml/min that was split post-column in a 1:1 ratio. Separations were performed with a binary gradient of water [0.1% trifluoroacetic acid (TFA), 0.3% formic acid] and acetonitrile (0.1% TFA, 0.3% formic acid) ranging from 0 to 100% organic. Ions were produced from a z-spray source with the nitrogen desolvation gas at 400°C and a flow-rate of 600 l/h. The source block was held at 150°C and the nebulizer gas was held at a relatively high flow-rate. The capillary voltage was +2500 V, the sample cone was at +45 V, the extraction cone was at +3 V, the hexapole radiofrequency voltage (RF) was +1000 V with a

d.c. offset at +7 V. Data were processed using Masslynx v.3.4 software and the protein multi-charged umbrellas were deconvoluted using Maxent 1 software (Micromass). This deconvolution software is a critical element in these experiments where the complex multi-charged protein umbrellas can be deconvoluted to a unique protein MW or MW values if more than one protein is present. The mass precision was optimized by loading 0.1 μg of bovine insulin with each NPS-RP-HPLC sample and then using the 1912.197 peak as a lock mass to fine tune the external calibration for each mass spectrum. External calibration was performed prior to each separation using direct infusion of a standard NaI/CsI solution.

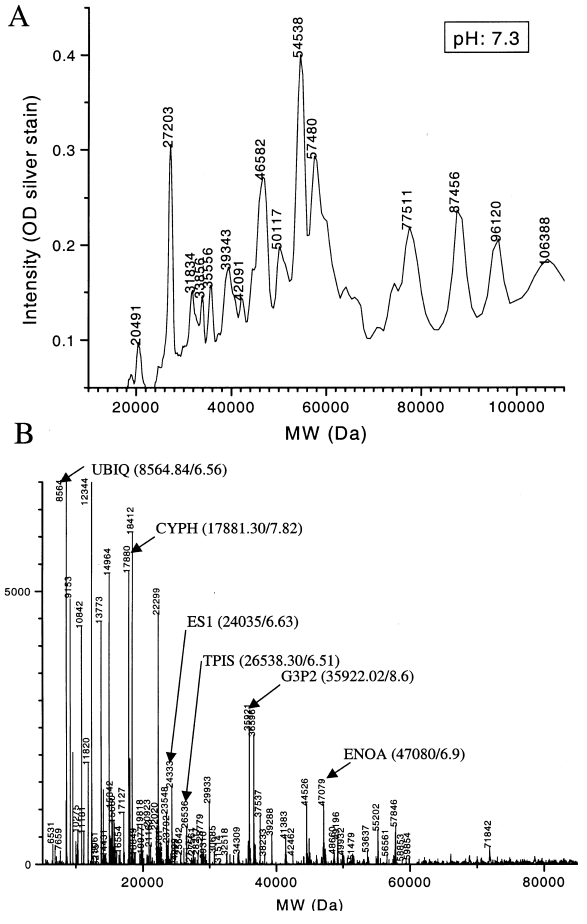

Fig. 1. Analysis of protein MW content of HEL cell sample from Rotofor pH 7.3 fraction by (a) 1D SDS–PAGE and by (b) NPS-RP-HPLC–ESI-TOF-MS. The protein loading was ~10^5 and $2{\cdot}10^5$ cells, respectively.

3. Results and discussion

Intact protein MW values are determined in this work by either 1D SDS–PAGE or NPS-RP-HPLC–ESI-TOF-MS. In the case of the liquid phase NPS-RP-HPLC–ESI-TOF-MS method the peak plots are generated by deconvolution of the protein multiply-charged umbrellas detected by the mass analyzer and then addition of all MW plots to one mass spectrum. In the case of the gel phase 1D SDS–PAGE method the peak plots are extracted from the 1D banding pattern silver stained images by use of the Gel-pro analyzer software. From this point forward these protein molecular mass plots, both liquid and gel phase, will be referred to as liquid or gel phase MW plots.

3.1. Sensitivity and mass resolution

The two IEF pH fractions analyzed in this work by these methods are pH 7.3 and pH 8.0 from the Rotofor. There are 20 pH fractions obtained from the Rotofor where these fractions have been selected for comparison. Figs. 1 and 2 show comparisons of the liquid and gel phase MW plots that result from separation of the proteins in these Rotofor fractions. There are several features that are apparent from these figures where for example the liquid MW plots show much greater sensitivity for lower MW proteins (<40 kDa). Indeed, many of the proteins below 20 kDa, for example ubiquitin (8565 Da, 6.6 p*I*), are not detected by the gel method but are detected by the liquid phase MS method. A second important feature is that the resolution of the liquid method is clearly much improved over that of the gel method due to the ability to directly measure MW from the on-line liquid separation (Fig. 3). It is possible to assign tentative protein identifications to the liquid phase MS-based MW plots as a result of the high resolution and mass accuracy detection of the intact MW at a given p*I* based upon previous such liquid maps of the HEL cell line. Verification of the protein identity was achieved in previous work by peptide mapping techniques [26]. However, the detection of accurate MW allows tentative identification in the same cell line based upon such previous measurements of exact MW, p*I*, hydrophobicity (not shown) and peptide mapping. The liquid MW plots are thus

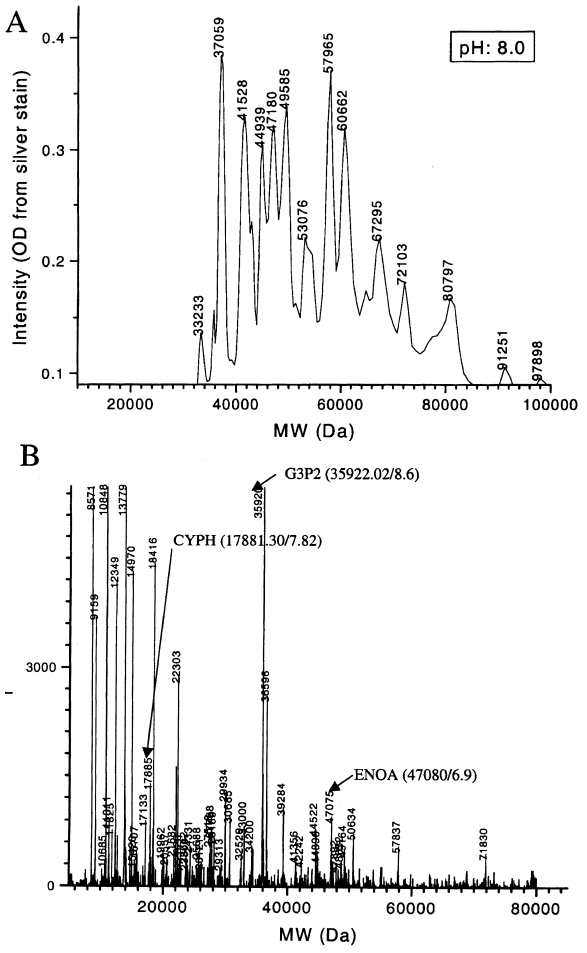

Fig. 2. Analysis of protein MW content of HEL cell sample from Rotofor pH 8.0 fraction by (a) 1D SDS–PAGE and by (b) NPS-RP-HPLC–ESI-TOF-MS. The protein loading was ~10^5 and $2{\cdot}10^5$ cells, respectively.

labeled with some possible protein identities which in turn were verified by peptide mapping results.

Table 1 shows some of the features of the MW plots. The 29 kDa average MW detected with the liquid phase MS method reflects the possible loss of larger proteins to adsorption during the IEF or RP-HPLC portions of the separation. Also with the larger proteins there are many more multiply-charged peaks thus reducing the signal-to-noise ratio. The resolution at full width half max (FWHM) was roughly 3000 to 5000 for the liquid MW plots and 30 for the gel MW plots (Table 1A and Fig. 3). This difference in resolution is demonstrated in the zoomed MW plots shown in Fig. 3. The liquid MW zoom plots of ENOA (α-enolase) and G3P2 (glyceraldehyde $3PO_4$ dehydrogenase) as detected in the pH 7.3 fraction, are shown in comparison to the zooms of the gel MW plots shown from the pH 7.3 and 8.0 plots. The resolution of proteins by gradient SDS–PAGE gel is not comparable with the liquid phase MS-based method and would not be expected to be so. More significantly though, the low resolution of the gel phase method prohibits detection of protein MW shifts of less than 1000 Da for a 27 000 Da protein thus making it impossible to detect most posttranslational modification (PTM) or sequence mass shifts. In contrast the liquid MW plots can resolve changes in MW of typically 5–7 Da for a 30 000 Da protein assuming a conservative resolution at FWHM of 3000 to 5000. Using this method such shifts are readily observed in ENOA for example. Further at low MW, below 15 kDa, the gel cannot resolve the presence of many proteins that are readily resolved and detected by ESI-TOF-MS.

3.2. Peak capacity and numbers of peaks observed: 1D SDS–PAGE vs. RP-HPLC–MS

Based on the resolution and typical peak widths it is possible to determine the maximum number of peaks, or peak capacity, that may be observed. For each liquid MW plot and separation the theoretical peak capacity was 6700 while for the gel MW plots the peak capacity was 50. The actual number of peaks observed in a given p*I* range for the liquid MW plots was 46 to 63 peaks while the gel MW plots produced 14 to 15 peaks. This is almost certainly related to sensitivity and limits of detection. The 1D SDS–PAGE gel method has peaks that are typically 1000 to 4000 Da in width and so many proteins cannot be seen with this method due to coelution to the same SDS–PAGE generated band. This becomes a major problem with 1D or 2D gels where diffusion limits resolution at low MW while the ESI-TOF-MS is most effective in this region.

3.3. Dynamic range and MW range

Proteins are expressed over at least six orders of magnitude and the liquid ESI-TOF-MS method is only capable of detecting proteins over 2–3 orders of

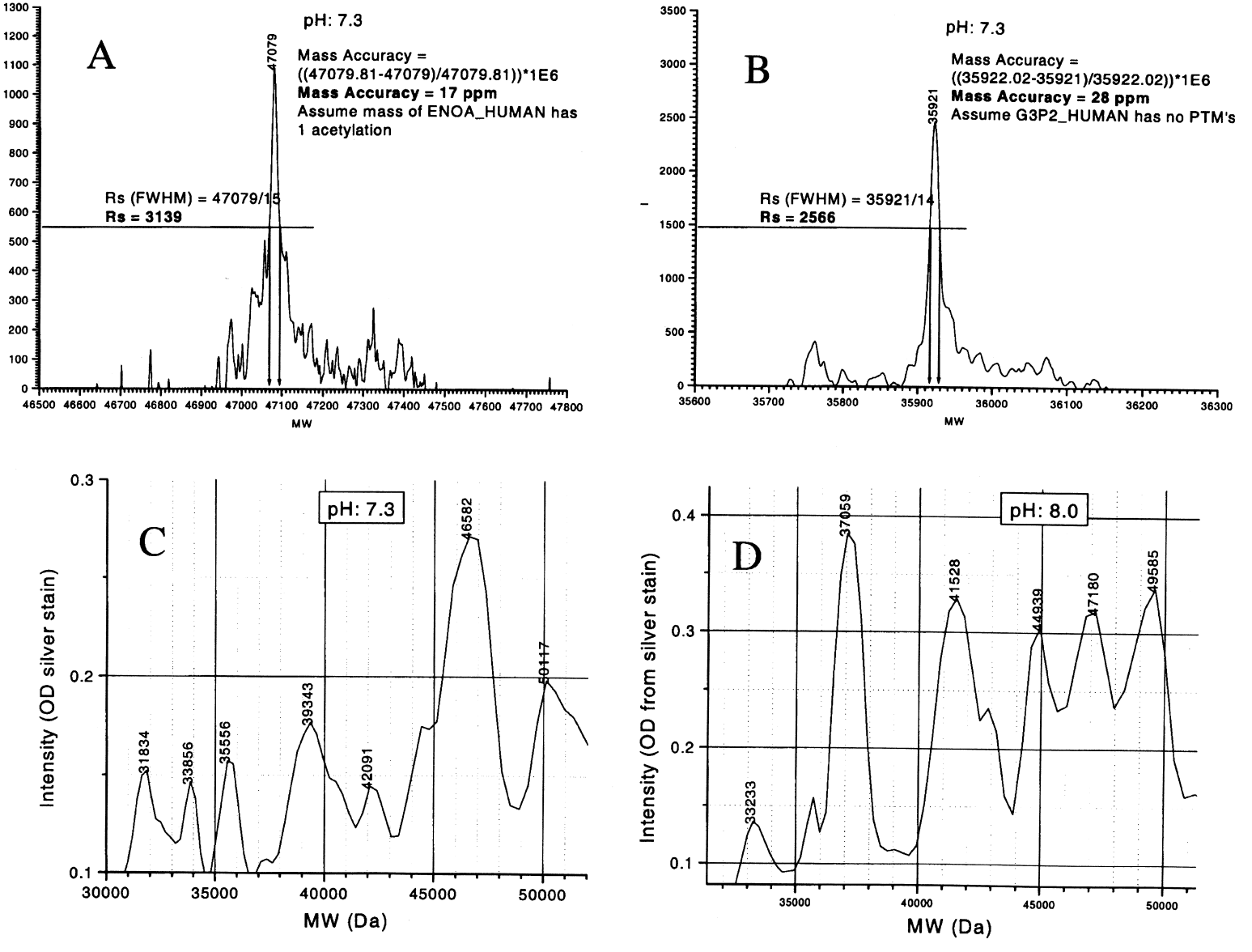

Fig. 3. Zoomed images of (a) MW peak of ENOA from pH 7.3 fraction; (b) MW peak of G3P2 from pH 7.3 fraction; and (c, d) molecular mass zoomed images of G3PD2 and ENOA from 1D SDS–PAGE gel.

Table 1
Calculations for mass analysis by (A) 1D SDS–PAGE and (B) NPS RP-HPLC–ESI-TOF-MS

		pH 7.3		pH 8.0	
		Gel	Liquid	Gel	Liquid
(A)					
Resolution	FWHM M/dM	27	3000	27	3000
Peak capacity	mass range/dM	50	6670	50	6670
Peaks observed		15	63	14	46
Mass range	kDa	20–300	5–85	20–300	5–85
Mass average		80 028	28 930	88 909	28 719
Mass range	kDa	20.5–241.3	6.5–71.8	33.2–265.4	8.6–71.8
(B)					
		G3P2 gel	G3P2 liquid	ENOA gel	ENOA liquid
	Mexp. 1, pH 7.3	35 556	35 921	46 582	47 079
	Mexp. 2, pH 7.6	36 205	35 923	46 312	47 083
	Mexp. 3, pH 8.0	37 059	35 920	47 180	47 075
	Mexp. average	36 273	35 921	46 691	47 079
	Mexp. Precision 1SD	615.50	1.25	362.69	3.27
	M database	35 922	35 922	47 080	47 080
	Mass accuracy 1	10 189	28	10 578	21
	Mass accuracy 2	7878	28	16 313	64
	Mass accuracy 3	31 652	56	2124	106
	Mass accuracy average	16 573	37	9671	64

dM=peak width at half maximum height. M=Mass from database. Mexp.=mass experimental.
Mass accuracy: $[(M-Mexp.)/M]*10^6$.

magnitude. In addition some larger more hydrophobic proteins are probably being lost to the C_{18} stationary phase of the NPS-RP-HPLC separation. Although the plots in Figs. 1 and 2 only show mass ranges below 110 kDa, the standard MW range for SDS–PAGE is from 10 to 300 kDa, while for the liquid ESI-TOF-MS method the range is from 5 to 85 kDa. The gel MW plots detected proteins from 20.5 to 265.4 kDa while the liquid MW plots detected proteins from 6.5 to 71.8 kDa.

The protein load to the gel separations in this work was 90 μl of the pH 7.3 (0.272 μg/μl) and pH 8.0 (0.307 μg/μl) IEF fractions or 24 and 28 μg of total protein, respectively. This corresponds to an estimated loading of ~10^5 cells. The protein load to the liquid separations was 200 μl of the pH 7.3 and pH 8.0 fractions or 54 μg and 61 μg of total protein, respectively. For low mass proteins (<40 kDa) the liquid phase method can detect proteins not detected by the gel with an estimated sensitivity in the <10 ng range. Under the best conditions, using either UV absorption or TOF-MS detection, we can detect down to close to 1 ng of protein which corresponds to ~1000 copy numbers per cell. For the larger proteins (>50 kDa) the sensitivity of the liquid MS-based method is significantly lower than that of the gel and is probably approaching the sensitivity levels of a Coomassie blue stained gel (100 ng +). Many of the peaks that show up in the gel above 50 kDa do not show up in the liquid phase method even though the LC–MS method was loaded with twice the amount of protein. Further improvements in detection of lower copy number proteins and higher MW proteins will require improved deconvolution software for mining greater numbers of proteins, improved MS detection of high MW proteins and improved fractionation of highly abundant from low abundance proteins.

3.4. Mass precision and mass accuracy

In Table 1B the mass precision and mass accuracy of the liquid and gel-based MW plot methods are shown. These data were taken from the results of performing the LC–MS and gel analyses on three pH

fractions from the Rotofor (7.3, 7.6, 8.0). We have used the highly expressed proteins which have been identified [26], G3P2_HUMAN and ENOA_HUMAN, which show up in multiple IEF fractions, to quantify the mass precision and mass accuracy over three different pH fractions as detected in the liquid and gel MW plots. The presence of these proteins in several p*I* fractions is due to diffusion in the liquid phase IEF fractionation. For G3P2_HUMAN the gel phase precision was 615.50 Da (1 SD) while the liquid phase precision was 1.25 Da (1 SD). For ENOA_HUMAN the gel phase precision was 362.69 Da while the liquid phase precision was 3.27 Da. The reproducibility of the liquid phase mass spectrometry-based method is far superior to that of the gel. This reproducibility will become critically important in future studies comparing protein mass maps between different samples as well as comparing mass maps between different research groups. The precision of the MW measurements may serve as a standard to compare proteins in such interlysate comparisons.

The other important characteristic of a mass map is the mass accuracy. This can be extremely important when working with proteins as the accurate mass may reflect the modified state of the protein, whereas an inaccurate mass could not discriminate between the modified or unmodified forms. Again referring to Table 1B, the mass accuracy of the liquid method for detecting G3P2 was on average 37 ppm, while the mass accuracy of the gel method was on average 16 573 ppm. For ENOA, the average mass accuracy obtained with the liquid phase method was 64 ppm, while the average mass accuracy obtained from the gel phase method was 9671 ppm. In general therefore the mass accuracy of the liquid phase mass spectrometry-based method was at least two orders of magnitude better than that of the gel phase method. This capability for high mass accuracy in measuring MW becomes significant because it leads to the conclusion that the G3P2 protein is being detected with no sequence or posttranslational modifications while the ENOA protein seems to have a PTM that increases its mass by 40 Da. From the literature it was determined that ENOA can be acetylated and so it is possible to assign a putative PTM of acetylation to ENOA [10]. The protein MW supports this result though the MALDI-MS peptide mass mapping performed on this protein (data not shown) did not find the PTM due to the fact that 100% sequence coverage was not achieved.

3.5. 1D SDS–PAGE and RP-HPLC–ESI-TOF-MS banding pattern comparisons

The band format versions of the MW plots are shown in Fig. 4 with the liquid phase bands shown in gray scale where the intensity of the peaks is reflected in the gray scale intensity of the band. For the gel phase band image the proteins are detected using silver staining and were imaged using a CCD camera. The liquid phase banding patterns are produced with an inherent linear MW dimension, while the gel phase banding patterns are produced with a logarithmic MW dimension. Calibration of a linear mass dimension is obviously easier than calibration of a logarithmic one, where this is an advantage of the liquid phase mass spectrometry-based method. For gradient gels, the log of the standard MW must be plotted vs. the square root of the migration distance to achieve calibration.

4. Conclusion

This work compares the analytical capabilities of the liquid IEF–1D SDS–PAGE and liquid IEF–NPS-RP-HPLC–ESI-TOF-MS methods in determining intact molecular mass values for proteins from whole cell lysates of HEL cell samples. The NPS-RP-HPLC–ESI-TOF-MS method exhibits 110 times greater resolution, 130 times greater peak capacity, a mass precision and mass accuracy that are two orders of magnitude improved over that of the gel. The sensitivity of the liquid phase MS-based method is better than that of the gel for proteins below 20 kDa where many more peaks are seen than in the gel method (Fig. 1 and Fig. 2). From 20 to 40 kDa the LC–MS and gel methods show more or less equal numbers of proteins and so equivalent sensitivities. Larger proteins (>40 kDa) were detected with greater sensitivity in the gel method as can be seen in Figs. 1 and 2. The average mass detected by the gel method was 80 kDa while the average mass detected by the liquid phase method was 30 kDa. Naturally

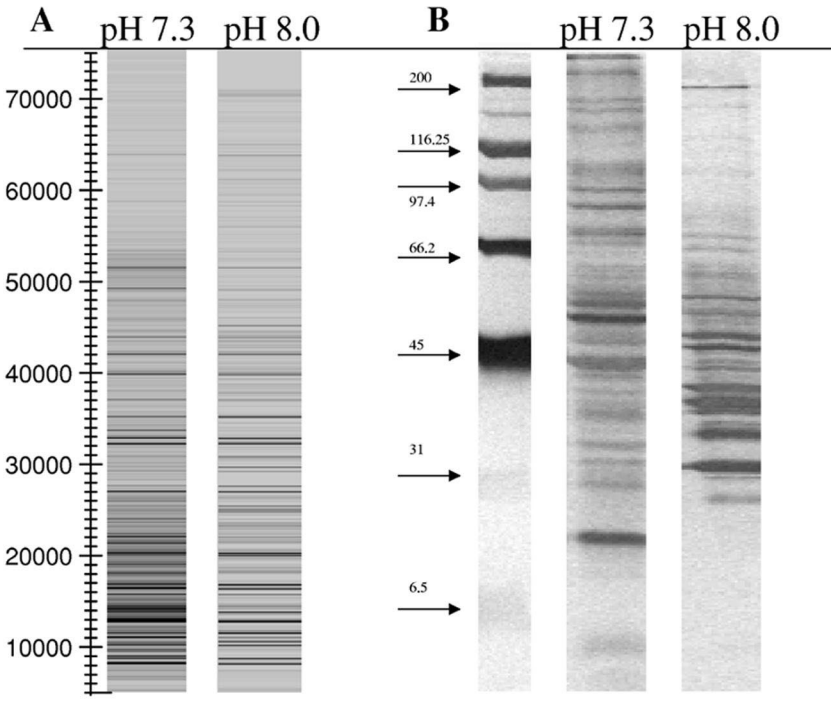

Fig. 4. A comparison of banding format images (a) of the NPS-RP-HPLC–ESI-TOF-MS of Rotofor pH fractions 7.3 and 8.0 and (b) 1D SDS–PAGE separation of the same fractions. Note gel MW markers on left hand column.

these two methods complement one another, where if one is studying low MW cytosolic proteins the method of choice would be NPS-RP-HPLC–ESI-TOF-MS while at present the 1D SDS–PAGE method can access larger more hydrophobic proteins not readily performed by the liquid method. In addition, the separation and detection by NPS-RP-HPLC–ESI-TOF-MS is much faster than by 1D SDS–PAGE where the liquid phase separation requires from 10 to 20 min to run a Rotofor pH sample depending on the gradient used and the quality of the separation. The 1D gel may require more than 10 h to run and several additional h to stain and develop. Importantly, the liquid phase MW plots are potentially capable of a high degree of reproducibility based upon an accurate MW measurement where typically <150 ppm accuracy is achieved. This may allow detection of PTM or sequence change mass shifts from one sample to the next in interlysate comparisons whereas the gel phase MW plots often do not have sufficient resolution to distinguish subtle mass shifts.

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