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Effects of chromatography conditions on intact protein separations for top-down proteomics

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Abstract

For top-down proteomics, nano-reversed phase liquid chromatography (RPLC) plays a major role in both single and multidimensional protein separations in an effort to increase the overall peak capacity for the resolution of complex protein mixtures prior to mass spectrometry analysis. Effects of various chromatography conditions, including alkyl chain length in the stationary phase, capillary column temperature, and ion-pairing agent, on the resolution of intact proteins are studied using nano-RPLC-electrospray ionization-mass spectrometry. Optimal chromatography conditions include the use of C_{18} column heated at 60 °C and the addition of trifluoroacetic acid instead of heptafluorobutyric acid as the ion-paring agent in the mobile phase. Under optimized chromatography conditions, there are no significant differences in the separation performance of yeast cell lysates present in the native versus denatured states. Denatured yeast proteins resolved and eluted from nano-RPLC can be subjected to proteolytic digestion in an on- or off-line approach to provide improved protein sequence coverage toward protein identification in a combined top-down/bottom-up proteome platform.

Keywords: ESI-MS; Intact proteins; Nano-RPLC; Top-down proteomics

1. Introduction

The vast number of proteins present in the proteome of a typical organism requires that separations be performed on the mixture prior to introduction into the mass spectrometer. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is still the method of choice for separating thousands of proteins in a single run [1–3] while offering a "differential display" of protein expression. However, the identification of gel resolved proteins and the study of protein modifications typically involve the separate excision, proteolytic digestion, peptide extraction/concentration, and mass spectrometry (MS) analysis of each protein spot [4–7]. All of these procedures, even when semi-automated using the commercially available robotic sample and liquid handling systems, are time-consuming tasks prone to significant sample loss and analyte dilution. Thus, the 2-D PAGE-MS approach remains lacking in proteome coverage, dynamic range, sensitivity, and throughput [7].

Consequently, considerable efforts have been devoted to the development of non-gel-based and bottom-up (or shotgun) proteome technologies through the combination of various chromatography methods with MS or tandem MS analysis [8–15]. These peptide separation techniques fully exploit the sensitivity achievable with conventional mass spectrometers (roughly 10^{-16} mol as opposed to 10^{-14} mol in conjunction with 2-D PAGE), allowing many additional proteins to be identified. However, the bottom-up approaches provide very limited molecular information about the intact proteins where only a fraction of the total theoretical peptide population of a given protein may be identified.

By comparing with shotgun methodologies, the top-down proteome techniques [16–23], in which intact proteins rather than peptides are measured, are advantageous for the detection of post-translational modifications (PTMs) [24]. PTMs include co- or post-translation covalent modifications to the protein structure and proteolytic processing of the translated

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protein. Furthermore, the top-down approaches to protein sequence analysis using tandem MS may allow complete protein characterization far more efficiently than shotgun proteome technologies using protein digests. In order to achieve the full potential of top-down approaches, the processing and separation of intact proteins still have to be brought to a similar level as those routinely achieved in shotgun proteomics.

A variety of separation technologies, including gel-based isoelectric focusing [25,26], capillary isoelectric focusing [27,28], capillary zone electrophoresis [16], and reversed phase liquid chromatography (RPLC) [19,29], have been utilized for resolving intact proteins prior to MS analysis. The application of only a single separation dimension, however, provides insufficient peak capacity for the resolution of complex protein mixtures. Thus, several recent attempts have involved off-line combinations of preparative scale isoelectric focusing [30–33] or acid-labile surfactant-based polyacrylamide gel electrophoresis [21,23] with RPLC in the development of multidimensional protein separation platforms. The collected protein fractions from either isoelectric focusing or gel electrophoresis were further resolved using RPLC as the second separation dimension prior to MS analysis.

It is clear that RPLC plays a major role in both single and multidimensional protein separation platforms in an effort to increase the overall peak capacity for the resolution of complex intact protein mixtures such as cell lysates. Total peak capacity improvements in protein separations contribute to increased number of proteins identified in top-down proteomics due to better use of the MS dynamic range and reduced discrimination during ionization. Thus, the main objective of the present study is to carefully examine the effects of various chromatography conditions, including alkyl chain length in the stationary phase, capillary column temperature, and ion-pairing agent, on the resolution of intact proteins. By using nano-RPLC-electrospray ionization (ESI)-MS, we further investigate potential differences in chromatography separation and ESI measurement of yeast intact proteins as the result of the protein denaturation process. Denatured proteins collected in RPLC fractions can be directly subjected to proteolytic digestion for obtaining their corresponding peptides and peptide sequences, particularly toward the MS identification of high molecular mass proteins [30–33].

2. Experimental

2.1. Materials and chemicals

Fused-silica capillaries (50 μm i.d./365 μm o.d.) were acquired from Polymicro Technologies (Phoenix, AZ). Model proteins, including bovine serum albumin (bovine, p*I* 5.60, 66,433.0 Da), cytochrome *c* (equine, p*I* 9.59, 12,362.0 Da), myoglobin (equine, p*I* 7.36, 16,951.5 Da), and ribonuclease A (bovine, p*I* 8.64, 13,688.1 Da), dithiothreitol (DTT), and iodoacetamide (IAM) were obtained from Sigma (St. Louis, MO). Acetonitrile, DNase, formic acid, glycerol, magnesium chloride, trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (Tris), and urea were purchased from Fisher Scientific (Pittsburgh, PA). Heptafluorobutyric acid (HFBA) was acquired from Pierce (Rockford, IL). All solutions were prepared using water purified by a Nanopure II system (Dubuque, IA) and further filtered with a 0.22 μm membrane (Costar, Cambridge, MA).

2.2. Soluble fraction of intact proteins from S. cerevisiae

The yeast cells (Sigma) were suspended in a buffer, which consisted of 10 mM Tris (pH 7.0), 5 mM magnesium chloride, 0.1 mM DTT, and 10% glycerol. The cells were disrupted by sonication for the release of cellular proteins [34]. After sonication, DNase was added with a final concentration of 50 μg/mL for the cleavage and removal of nucleic acids. The cellular proteins were collected in the supernatant by centrifugation at 20,000 × *g* for 10 min. The protein solution was then desalted using a regenerated cellulose membrane (Millipore, Bedford, MA) with a 5000 molecular weight cut-off.

Yeast cytosol proteins were denatured and reduced in a 20 mM Tris buffer containing 8 M urea and 0.1 M DTT for 2 h at 37 °C under a nitrogen atmosphere. Proteins were alkylated by adding excess IAM with a final concentration of 50 mM and the reaction was allowed to proceed for 30 min at room temperature in the dark. A PD-10 size exclusion column (Amersham Pharmacia Biotech, Uppsala, Sweden) was employed for buffer exchange and proteins were eluted in a solution containing 2 M urea and 10 mM Tris at pH 8.0. The total protein concentration was determined using the Bradford method (Bio-Rad, Richmond, CA) and was around 2 mg/mL.

2.3. Nano-RPLC-ESI-MS analysis of model proteins and soluble fraction of intact proteins from S. cerevisiae

The tip at the end of a 16-cm-long fused silica capillary (50 μm i.d. × 365 μm o.d.) was flame-pulled and packed with 13 cm of 5-μm C_4- or C_{18}-bonded particles (Phenomenex, Torrance, CA). The C_{18}-bonded particles in methanol were introduced into the capillary by gradually increasing the pressure from 100 to 2000 psi using an Agilent 1100 capillary LC pump (Avondale, PA). The packed capillary was left under pressure for 10 h and then depressurized overnight. A microcross (Upchurch Scientific, Oak Harbor, WA) containing a platinum electrode was employed to apply an ESI voltage of 1.8 kV and reduce the flow of Agilent 1100 capillary LC pump from 2 μL/min to an effective flow rate of 200 nL/min. A 30-min linear gradient from 10% to 65% acetonitrile (containing 0.1% TFA or 0.02% HFBA) was utilized to perform intact model protein separations. The linear gradient was further extended from 30- to 60-min for the analysis of complex yeast cell lysates.

The eluants from nano-RPLC were monitored using a Micromass Q-TOF mass spectrometer (Manchester, United

Kingdom). Mass spectra were collected from 600 to 1900 *m*/*z* using a scan time of 2.0 s. Automated analysis of the nano-RPLC-ESI-MS data files were performed using Protein Trawler software (Bioanalyte, Portland, ME) [35,36]. The program summed all data within a specified time interval, utilized Micromass MaxEnt 1 to deconvolute multiply charged ions, centered the results, carried out a threshold selection, and reported the mass, intensity, and retention time of each protein in a text file. The program repeated this process across sequential portions of the chromatogram.

3. Results and discussion

The reduction of column i.d. from few hundred μm to as small as 15 μm in capillary liquid chromatography [8,37–40] has resulted in higher peptide concentrations within smaller peak volumes, thus enabling more sensitive MS detection. Nano-RPLC equipped with a 50 μm i.d. capillary column was therefore employed in this study to evaluate the effects of various chromatography conditions on intact protein separations and allow ultrasensitive characterization of model proteins and yeast cell lysates using ESI-MS. Due to the use of ESI-MS detection, the differences in chromatography separation and protein intensity were further revealed among the samples containing the soluble fraction of yeast cell lysates in the native versus denatured/reduced/alkylated states.

3.1. Effect of alkyl chain length in the stationary phase on intact protein separations

Four model proteins, including bovine serum albumin, cytochrome *c*, myoglobin, and ribonuclease A, were prepared in 10 mM Tris at pH 7.0 with a final concentration of 0.1 μM for each model protein. The effect of alkyl chain length in the stationary phase on chromatography separation of model proteins was studied and shown in Fig. 1A and B as the base peak chromatograms acquired from capillary columns packed with 5-μm C_4- and C_{18}-bonded particles, respectively. All protein peaks were directly identified on the basis of mass spectra of protein analytes taken from the average of scans under the peaks.

As anticipated [41], the elution time of model proteins increased with increasing alkyl chain length. Furthermore, the reconstructed ion chromatograms were obtained using the most intense *m*/*z* ions from each protein envelope and displayed in Fig. 2A and B for the separations carried out by the C_4 and C_{18} columns, respectively. Both the separation efficiency and resolution of model proteins achieved in the C_{18} column were considerably better than those obtained from the C_4 column, particularly for early eluted proteins such as ribonuclease A and cytochrome *c*. Still, potential sample loss due to irreversible protein adsorption onto the C_{18} stationary phase may outweigh the benefits of better separation performance and may adversely impact the ability to perform comprehensive proteome analysis, particularly toward the identification of low abundance proteins. Thus, both C_4 and C_{18} reversed phase columns have been utilized by several research groups for the analysis complex protein mixtures such as cell lysates in top-down proteomics [21,23,29–33,35,36].

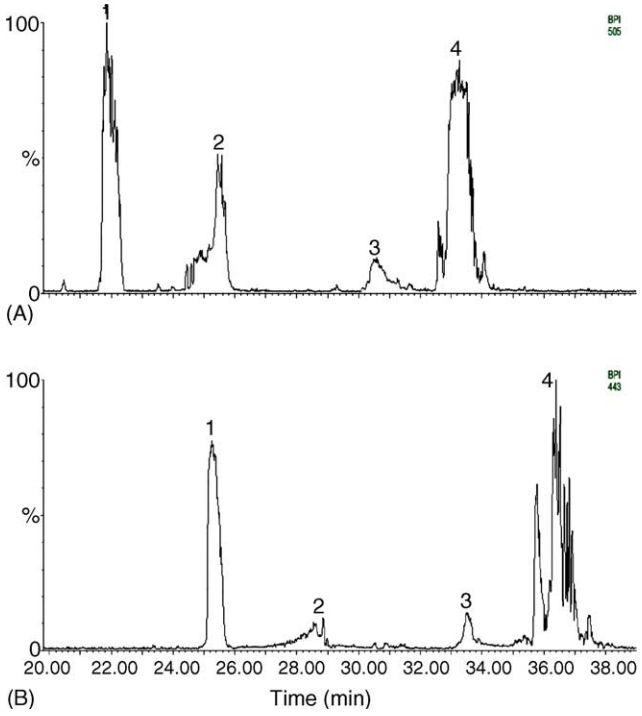

Fig. 1. Comparison of model protein separations using (A) C_4 and (B) C_{18} columns. Column temperature: 25 °C; elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) bovine serum albumin, and (4) myoglobin; mobile phase: a 30-min linear gradient from 10% to 65% acetonitrile (containing 0.1% TFA) at a flow rate of 200 nL/min; sample loading: 100 femtomole for each model protein (1 μL injection of 0.1 μM protein solution).

3.2. Effect of ion-pairing agent on intact protein separations

To improve chromatography separations, ion-pairing agents are commonly used to sharpen peak shapes [41,42]. In contrast to ion pairing agents of alkanesulfonic acids, perfluorinated carboxylic acids up to four carbon atoms are known to be volatile and therefore well suited for ESI-MS detection. The downside to the use of perfluorinated carboxylic acids is their ionization suppression effect. Some of the decrease in MS sensitivity, however, can be regained through increased analyte concentrations in sharpened peaks due to the "concentration sensitive" behavior of nano-ESI-MS [43].

In shotgun proteomics, Yates and co-workers [9,10] have substituted acetic acid with 0.02% HFBA for the analysis of a trypsin-digested sample of soluble proteins from *S. cerevisiae* using strong cation exchange chromatography coupled with RPLC. By comparing with an acetic acid buffer system, more than three times as many peptides were identified using HFBA in their work. Adding HFBA improved the dynamic range of the analysis and allowed for the acquisition of more

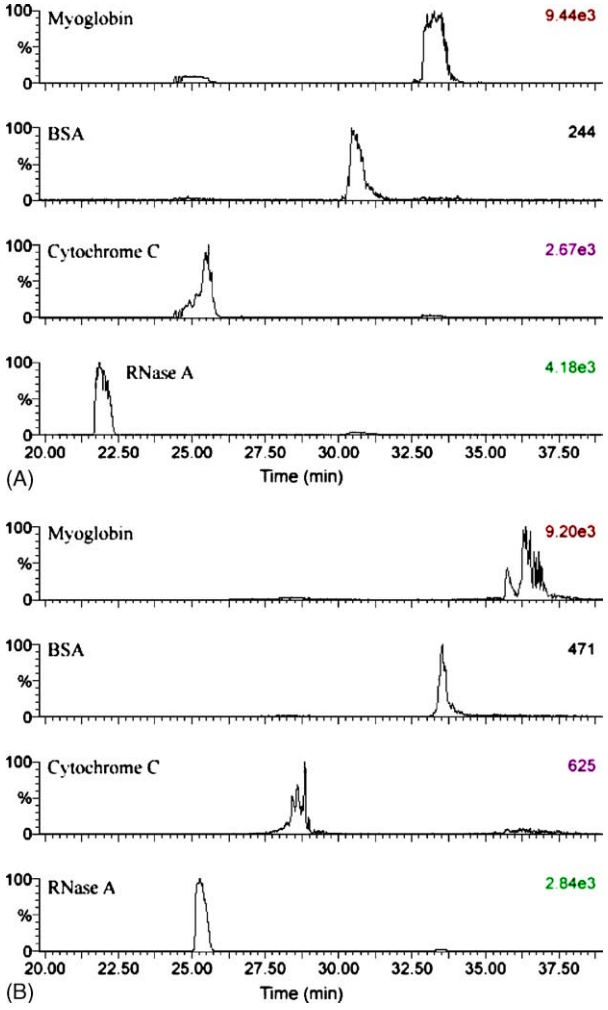

Fig. 2. Reconstructed ion chromatograms of model protein separations using (A) C_4 and (B) C_{18} columns. Each model protein is represented using its most intense *m/z* ion from the ESI protein envelope: *m/z* 893.7 for myoglobin, *m/z* 1332.9 for bovine serum albumin, *m/z* 825.4 for cytochrome *c*, and *m/z* 1245.7 for ribonuclease A.

low-abundance peptides. By using a C_{18} column, the effect of ion-paring agent on intact protein separations was investigated by adding either 0.1% TFA (Fig. 1B) or 0.02% HFBA (Fig. 3) into the mobile phase. In contrast to the conclusion obtained from shotgun proteome studies [9,10], the use of 0.02% HFBA as ion-pairing agent resulted in significant deterioration of separation resolution among model proteins, particularly between bovine serum albumin and myoglobin. Further increase in HFBA concentration contributed to dramatic reduction in protein ion intensities measured by ESI-MS (data not shown).

3.3. Effect of column temperature on intact protein separations

At high column temperatures, the mobile phase viscosity is reduced, and concomitantly, the diffusivity of the analyte is enhanced. More importantly, the sorption kinetics of

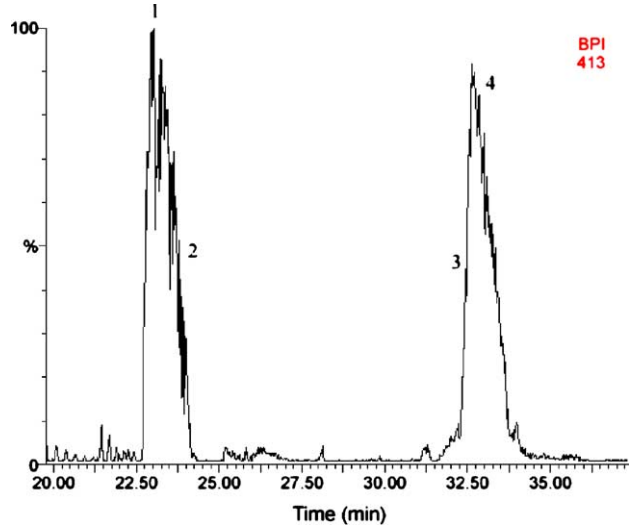

Fig. 3. Base peak chromatogram for illustrating the effect of ion-paring agent on nano-RPLC-ESI-MS separation of model proteins. C_{18} column temperature: 25 °C; elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) bovine serum albumin, and (4) myoglobin; mobile phase: a 30-min linear gradient from 10% to 65% acetonitrile (containing 0.02% HFBA) at a flow rate of 200 nL/min; sample loading: 100 femtomole for each model protein (1 μL injection of 0.1 μM protein solution).

the analyte is also accelerated with increasing temperature. Consequently, the column efficiency is expected to be higher at elevated column temperatures [44,45]. Thus, the effect of column temperature on intact protein separation performance was evaluated by raising the column temperature from 25 to 60 °C using a column heater. By comparing with the results shown in Fig. 1B, the base peak chromatogram displayed in Fig. 4 not only demonstrated improved separation resolu-

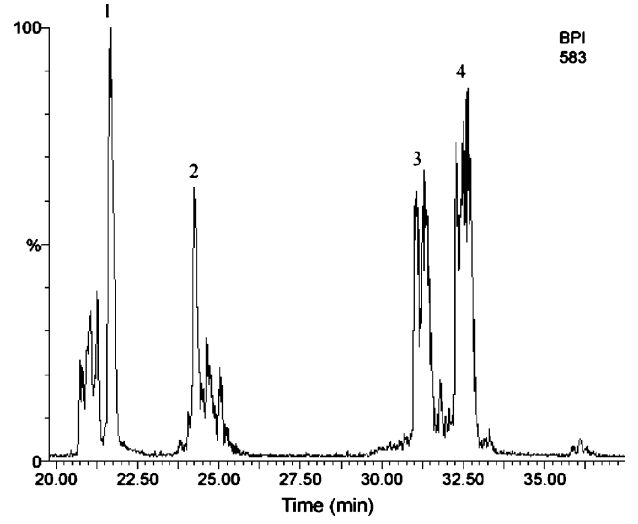

Fig. 4. Base peak chromatogram for illustrating the effect of column temperature on nano-RPLC-ESI-MS separation of model proteins. C_{18} column temperature: 60 °C; elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) bovine serum albumin, and (4) myoglobin; mobile phase: a 30-min linear gradient from 10% to 65% acetonitrile (containing 0.1% TFA) at a flow rate of 200 nL/min; sample loading: 100 femtomole for each model protein (1 μL injection of 0.1 μM protein solution).

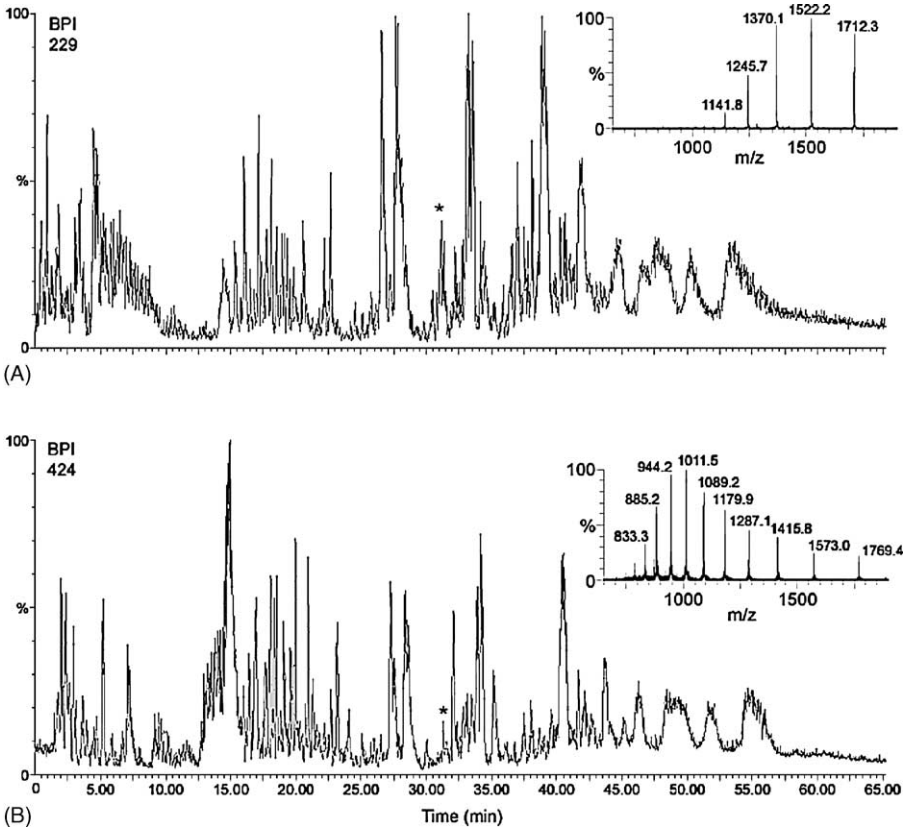

Fig. 5. Base peak chromatograms for the soluble fraction of yeast cell lysates in the (A) native and (B) denatured states using nano-RPLC-ESI-MS. C_{18} column temperature: 60 °C; mobile phase: a 60-min linear gradient from 10% to 65% acetonitrile (containing 0.1% TFA) at a flow rate of 200 nL/min; sample loading: 2 μg of total yeast protein (1 μL injection of 2 mg/mL yeast protein solution) spiked with ribonuclease A. Eluted ribonuclease A peaks are marked with * and the mass spectra taken from the average of scans under the peaks are shown as insets.

tion and efficiency among various protein conformers at high column temperature, but also presented strong protein ion intensities as the result of enhanced ESI prior to MS detection. Due to the limitation on MS scan speed, no attempts were pursued in increasing the mobile phase flow rate for further enhancing the column efficiency and achieving high speed chromatography separations.

3.4. Nano-RPLC-ESI-MS analysis of native and denatured/reduced/alkylated yeast cell lysates

In addition to the characterization of model proteins, the soluble fraction of intact proteins from *S. cerevisiae* in the native state was analyzed using nano-RPLC-ESI-MS in this study. As shown in Fig. 5A, the peak width (4σ) for a protein eluting from the column is as small as 10 s which corresponds to a peak capacity of 360 over a gradient run time of 60 min. Further enhancement in the peak capacity can be realized by slowing the solvent gradient in nano-RPLC and increasing the column length at the expense of analysis time [40]. Still, the peak capacity of any single dimension separations is insufficient for processing the number of proteins that expected from even a eukaryotic organism such as *S. cerevisiae* proteome (>6000 proteins).

It has been estimated that up to 10,000 proteins may be commonly present in human serum, most of which would be present at very low relative abundance [46,47]. The large variation of protein relative abundances having potential biological significance in mammalian systems (>6–9 orders of magnitude) also presents a major analytical challenge for proteomics. Thus, the use of multidimensional protein separations, including combinations of isoelectric focusing [30–33,48] or polyacrylamide gel electrophoresis [21,23] with RPLC, not only contributes to reduction in protein complexity prior to MS analysis, but also increases the number of proteins identified due to better use of the MS dynamic range and reduced discrimination during ionization.

Top-down proteome approaches offer excellent molecular level information for the intact proteins, but currently require the use of Fourier transform ion cyclotron resonance (FTICR)-MS/MS for obtaining protein-sequencing measurements [16–23]. Furthermore, top-down technologies are also limited by the relative scarcity of bioinformatic tools to efficiently analyze this type of data, although these are under development [49,50]. Thus, on-line proteolytic digestion of eluted proteins or off-line fraction collection of resolved proteins followed by subsequent digestion [30–33] can greatly facilitate protein identification through peptide mass mapping

or peptide sequences obtained from tandem MS using conventional mass spectrometers. Instead of spreading proteolytic peptides over the entire multidimensional separation in the bottom-up approach, the peptides and their sequences can be directly linked with the corresponding proteins in this combined top-down/bottom-up methodology, possibly resulting in increased peptide sequence coverage of identified proteins.

In order to implement the combined top-down/bottom-up proteome analysis in our future work, we therefore investigated potential differences in chromatography separation and ESI-MS measurement of yeast intact proteins present in the native versus denatured states. This is because these denatured, reduced, and alkylated proteins eluted from nano-RPLC can be directed toward to a miniaturized trypsin membrane reactor [51] for performing on-line and real time proteolytic digestion prior to MS analysis. The chromatography separation results obtained from the same yeast protein sample which was further denatured, reduced, and alkylated (Fig. 5B) were compared with the analysis using yeast proteins in the native state (Fig. 5A). In general, there were no significant differences in the separation efficiency and resolution of native versus denatured proteins using nano-RPLC. The elution time of denatured, reduced, and alkylated proteins was slightly earlier than that of corresponding native proteins.

This is evidenced by comparing the elution times of ribonuclease A (labeled by * in Fig. 5A and B) which was spiked into the yeast protein samples. By comparing with the ESI protein envelope of native ribonuclease A (inset in Fig. 5A), the mass spectrum of denatured and alkylated ribonuclease A (inset in Fig. 5B) not only confirmed complete alkylation, but also displayed an increase in its average charge state as the result of denaturation process. The deconvoluted masses of native and alkylated ribonuclease A were measured as 13,690.7 and 14,147.5 Da, respectively. The alkylation of each cysteine residue results in addition mass of 57.0 Da. Thus, the difference in protein mass between native and alkylated ribonuclease A corresponds to the alkylation of all eight cysteines present in ribonuclease A. Furthermore, the protein denaturation process often results in improved MS intensity as the denatured state of a protein exhibits an open conformation and accepts more protons than the native state.

4. Conclusion

For top-down proteomics, total peak capacity improvements in protein separations will clearly contribute to increased number of proteins identified due to better use of the MS dynamic range and reduced discrimination during ionization. Thus, the effects of various chromatography conditions, including alkyl chain length in the stationary phase, capillary column temperature, and ion-pairing agent, on the resolution of intact proteins are examined in this study using nano-RPLC-ESI-MS. Optimal chromatography conditions include the use of C_{18} column heated at 60 °C and the addition of trifluoroacetic acid instead of heptafluorobutyric acid as the ion-paring agent in the mobile phase.

We further investigate potential differences in chromatography separation and ESI measurement of yeast intact proteins as the result of the protein denaturation process. Under optimized chromatography conditions, there are no significant differences in the separation performance of yeast cell lysates present in the native versus denatured states. Denatured yeast proteins resolved and eluted from nano-RPLC can be subjected to proteolytic digestion in an on- or off-line approach to provide improved protein sequence coverage toward protein identification in a combined top-down/bottom-up proteome platform. Due to the complexity of typical cell lysates, protein digests related to individual protein peaks resolved and eluted from nano-RPLC may require further separations prior to MS analysis. In this regard, the capillary format of the nanoscale trypsin membrane reactor recently developed in our laboratory [51] lends itself to further peptide concentration and separation using high-speed capillary isotachophoresis/capillary zone electrophoresis prior to ESI-MS.

Acknowledgment

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