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Practical Considerations for Improving the Productivity of Mass Spectrometry-based Proteomics

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Abstract: Mass spectrometry (MS) is currently the most sensitive and selective analytical technique for routine peptide and protein structure analysis. Top-down proteomics is based on tandem mass spectrometry (MS/MS) of intact proteins, where multiply charged precursor ions are fragmented in the gas phase, typically by electron transfer or electron capture dissociation, to yield sequence-specific fragment ions. This approach is primarily used for the study of protein isoforms, including localization of post-translational modifications and identification of splice variants. Bottom-up proteomics is utilized for routine high-throughput protein identification and quantitation from complex biological samples. The proteins are first enzymatically digested into small (usually less than *ca*. 3 kDa) peptides, these are identified by MS or MS/MS, usually employing collisional activation techniques. To overcome the limitations of these approaches while combining their benefits, middle-down proteomics has recently emerged. Here, the proteins are digested into long (3–15 kDa) peptides *via* restricted proteolysis followed by the MS/MS analysis of the obtained digest. With advancements of high-resolution MS and allied techniques, routine implementation of the middle-down approach has been made possible. Herein, we present the liquid chromatography (LC)-MS/MS-based experimental design of our middle-down proteomic workflow coupled with post-LC supercharging.

Keywords: Electron transfer dissociation (ETD) · Higher-energy collisional dissociation (HCD) · Limited proteolysis · Middle-down proteomics · Post-column supercharging

Today, protein analysis using mass spectrometry (MS) is routine in numerous academic, commercial and clinical laboratories around the world. Depending on the goal of the study, among the most commonly employed approaches are bottom-up, top-down, and the newly emerging middle-down proteomics.[1] While bottom-up proteomics can be performed on fast, economical but low-resolution instruments, top-down and middle-down proteomics can only be performed on more expensive, high-resolution platforms, such as Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap, or state-of-the-art time-of-flight (TOF) instruments.[2]

Bottom-up proteomics is regularly used for high-throughput protein identification, quantitation, and targeted identification of post-translational modifications (PTMs). Here, proteins are cleaved into small (less than *ca.* 3 kDa) peptides with an enzyme, usually trypsin. The peptides are separated on a chromatographic column and analyzed individually, typically using data-dependent tandem mass spectrometry (MS/MS). Standardized protocols for both in-solution and in-gel digestion of proteins are well tested, separation of the small peptides is routinely achievable using both micro- and nanoflow rate liquid chromatography (LC), and high-throughput identification of thousands of proteins is possible from a single chromatographic run (*e.g.* 2,500 proteins/90 min.).[3] The MS/MS activation method can also be tailored to maximize the quality of the mass spectra. Collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD) of short tryptic peptides is usually very efficient, and the interpretation of the mass spectra is straightforward. However, these collisional activation methods lead to preferential cleavage of the weak covalent bonds, therefore localization of PTMs is cumbersome. Electron capture and electron transfer dissociation (ECD and ETD) are techniques that can be used for fragmentation of multiply charged precursor ions. The main advantage of ETD and ECD is that the labile PTMs are preserved, however, fragmentation of peptides carrying less than three charges is inefficient and the MS/MS spectra of these are often uninformative.

Although a vast number of research efforts are employing bottom-up proteomics, this conventional approach carries several limitations arising from the high sample complexity and limited instrumental performance.[4] Specifically, biological samples typically contain thousands of proteins in a wide concentration range, simultaneous digestion of these leads to tens of thousands of peptides, which greatly increases sample complexity. Due to the limited separation capabilities of liquid chromatography and the time allotted for each MS/MS scan, only the most abundant

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of the co-eluting peptides are analyzed in data-dependent MS/MS.[5] This can be partially overcome by data independent MS/MS (termed MS^E MS/MS^{ALL} or SWATH), where all peptides present in a given *m/z* window are simultaneously fragmented, however, in such approach, the fragment ions and the precursors must be precisely related on the basis of their elution profile.[6,7] An additional shortcoming of bottom-up proteomics is that many proteins are present in multiple isoforms (PTMs, splice variants, *etc*.).[8] If a protein is present in only two isoforms (for instance, single phosphorylation and the non-phosphorylated variety), the only prerequisite for identification of both isoforms is the identification of the modified and non-modified peptide. However, without prior knowledge of the number of isoforms, or if multiple isoforms exist, the relationship between the modified peptide and the originating protein sequence is lost.[9]

In contrast, top-down proteomics is the MS-based method for the analysis of intact proteins. High-throughput identification of isoforms for select proteins has been reported in recent years. On-line 2D separation of purified histones on weak cation-exchange and hydrophilic interaction chromatography (WCX-HILIC) columns followed by ETD of the intact proteins allowed for the identification of 708 isoforms present in HeLa cells.[10] High-throughput PTM localization is also possible using top-down proteomics, Tran and coworkers identified 3000 variants of 1043 human proteins.[11] However, to achieve such a result, extensive four-dimensional fractionation and multiple LC-MS/MS runs were required, which is a serious shortcoming when limited sample quantities are available. The protein fractionation is necessary due to several reasons. The efficient LC separation of proteins is technically more difficult to achieve than it is for peptides. Since proteins are present in multiple charge states in the mass spectrum, co-eluting proteins might have overlapping signals that hinder the isolation of the individual protein signals. In addition, protein fragmentation is more cumbersome than peptide MS/MS analysis, and the fragmentation mass spectra often contain intersecting multiple charge state product ion signals. Moreover, a much higher number of MS/MS scans per LC peak must be accumulated for achieving sufficient signal to noise (S/N) ratio of the fragment ions. Another consideration is the extremely diverse protein sizes present in a complex mixture. Although analysis of small proteins (<20 kDa) can be performed without major instrument modifications, MS/MS analysis of larger proteins is not trivial. Recently, up to 32% sequence coverage of ~150 kDa intact monoclonal antibodies was obtained using electron transfer dissociation (ETD) on an Orbitrap FTMS and time-of-flight MS, as well as by electron capture dissociation (ECD) on FT-ICR MS.[12] However, for these accomplishments, hundreds of ETD/ECD mass spectra had to be averaged, which is not possible for all proteins on the timescale of LC separation. In general, application of ECD/ETD to proteins yields larger sequence coverage than collisional activation-based MS/MS. Nevertheless, if the protein is highly folded or the structure protected by disulfide bonds, fragmentation of the internal backbone bonds is not efficient by either MS/MS method.

Middle-down proteomics is an approach that aims to combine the benefits of bottom-up and top-down approaches, while minimizing their above-mentioned limitations. Here, similarly to bottom-up, proteins are digested, however, a restricted (less frequent) proteolysis is employed to increase the average size of the resulting peptides (3–15 kDa), as detailed below. Due to the lower number of resulting peptides, the sample is less complex than in the bottom-up approach, but MS/MS analysis can still be performed in a high-throughput manner. Specifically, efficient separation of these long peptides can be readily performed on commercial chromatographic columns, and the elution profile and LC peak capacities are comparable to those of the bottom-up approach. Moreover, due to the decreased sample complexity, the number of co-eluting peptides is also reduced, as detailed further below. Although a longer acquisition time is necessary for recording of high resolution MS/MS spectra, this is achievable with modern instrumentation, such as the Orbitrap FTMS. In addition, the long amino acid series enhances the uniqueness of the sequence and increases the chance for identification of peptides that carry a modification.

Therefore, we consider this paradigm shift towards analysis of longer peptides to be the key for achieving increased dynamic range of protein concentrations and high-throughput identification of targeted protein isoforms. However, sample preparation, peptide separation, ionization conditions, fragmentation parameters, data acquisition and data analysis must be appropriately tailored to analysis of long peptides. Herein, we first identify a suitable protease for the middle-down approach that ensures a fast digestion into long peptides and results in high protein sequence coverage. In addition, we present post-column supercharging employed to increase the average charge state of the long peptides, and, consequently, the fragmentation efficiency; and finally consider the practical aspects of such setup for high-throughput protein analysis.

Theoretical Considerations

Although trypsin is a well-characterized protease that is routinely used in bottom-up proteomics, it is not ideal, since it produces tens of thousands of short (4–10 residue) peptides. This, in turn, is detrimental due to ineffective use of the LC column when the valuable binding sites are saturated by excessive numbers of short, uninformative peptides. In addition, an MS/MS scan is performed on all multiply charged precursor ions and, if several species elute in a narrow elution window, fragmentation of short peptides may be performed to the detriment of co-eluting longer ones. Moreover, these fragmentation mass spectra are often not useful, since the probability that the sequence is unique to a particular protein decreases with decreasing peptide length. Finally, short peptides (<1 kDa) may be removed prior to analysis using molecular weight cut-off (MWCO) filters, however, as any additional handling operation, this may also lead to sample loss. It is therefore desirable to perform the proteolysis at less frequent amino acid sites or specific amino acid patterns with a required repetition rate.

Our survey of the yeast proteome (as well as that of human and bacteria, data not shown here) suggests that cleavage at dibasic residues greatly decreases the number of peptides yielded by proteolysis. Fig. 1 shows the theoretical peptide size distribution of proteolytic peptides obtained *via in silico* digestion of the *E. coli* proteins, with zero missed cleavages allowed, with trypsin (top panel) and with a protease Sap9 (bottom panel) that cleaves after two adjacent basic amino acids, arginine and lysine, *vide infra*. Trypsin produced a total number of ~285,000 peptides with unique sequences, 280,000 of these were within the length range of 2–50 amino acid residues and had an average length of 15.2 residues. In contrast, the dibasic site cleavage yielded *ca*. 100,000 peptides, with 65,000 within the length range of 2–100 residues and had an average length of 58.2 residues. Because the peptides obtained with dibasic residue cleavage are more uniformly distributed across the 20–100 amino acid size range, one may expect that the chromatographic separation of these will be more efficient. Particularly, the elution will be spread along a wider gradient region. As a result, a better MS sampling of the eluting peptides may be obtained. This is important when considering that long, highly charged peptides yield multiply charged product ions, requiring high resolution mass analysis and, consequently, longer acquisition time per MS/MS spectrum compared to analysis of short peptides in a trap instrument.

Tailoring the proteolysis site to ensure

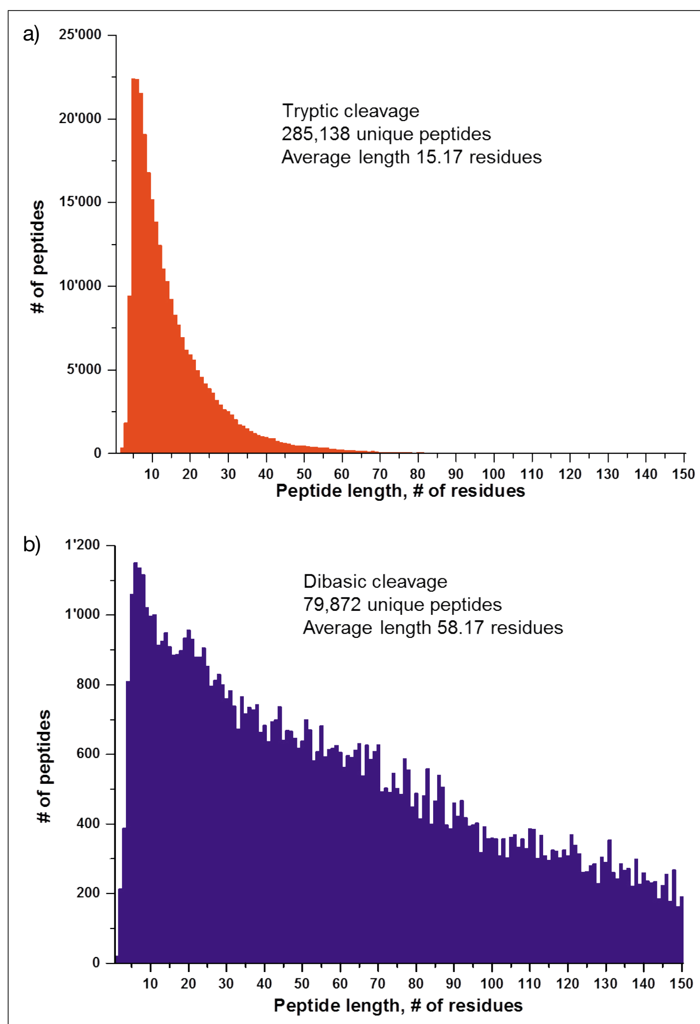

Fig. 1. Size distribution of proteolytic peptides in the 1–100 residue range obtained *via in silico* digestion of the *E. coli* proteins database with trypsin (panel a) and a dibasic protease Sap9 (panel b). Zero missed cleavages were allowed in the calculations.

longer average peptides is the driving force for the development of middle-down proteomics. Although several proteases, such as AspN, LysC and GluC, as well as microwave-assisted acid proteolysis have been utilized for obtaining long peptides,[13–17] the occurrence of these amino acid sites (data not shown) is more frequent than the occurrence of two adjacent basic residues. As a result, targeting the dibasic residues for cleavage offers more desirable peptide size range. Kex2[18] and OmpT[19] have been previously described to have dibasic site specificity. However, recombinant Kex2 is specific only to KR and RR, and not KK and RK sites therefore decreasing the cleavage possibilities and increasing the average peptide size beyond the 10 kDa mass range. The working regime for very long peptides approaches the top-down approach, where both the LC and the mass spectrometer operating parameters must be specifically tailored for efficient separation and timely fragmentation. OmpT is a protein construct which appears to be cumbersome to produce, required re-folding prior utilization, and it has been found to extensively cleave at other amino acids, as well.

Sap9, a Novel Protease for Middle-down Proteomics

In our approach, we sought to establish the enzymatic activity of the *Candida albicans* aspartic protease Sap9 overexpressed in *Pichia pastoris*. The protease production is highly efficient (in the order of g/L), it is excreted in the extracellular medium, and can be effortlessly purified from the supernatant using the His-tag approach, as described by Albrecht and coworkers.[20] For the cleavage specificity study, commercial carbonic anhydrase (29 kDa) obtained from Protea (Morgantown, WV) was digested with Sap9 at pH 4.5, enzyme:protein ratio 1:2.5, 37 °C. We performed extensive enzyme activity studies and found these conditions to be the most appropriate for efficient proteolysis. Aliquots were removed hourly for eight hours. Peptides were desalted with C18 ZipTip (Millipore, Billerica, MA) and separated on a C8 column (Thermo Scientific, 15 cm, 2 µm, 100 Å) using a 45 min. H_2O/ACN:MeOH:TFE (2:5:1) gradient. Eluted peptides were detected using LTQ Orbitrap Elite FTMS equipped with a high-field Orbitrap mass analyzer and provided with the eFT signal processing algorithm. MS scan was performed at 60,000 resolution (at 400 *m/z*) while HCD mass spectra were acquired at 15,000 resolution setting. Protein database search was performed using Sequest and Mascot against the *Bos taurus* database. We performed a fully tryptic search allowing for nine missed cleavages, as well as a no-enzyme search to avoid biasing of the results towards preferred cleavage sites.

Fig. 2 shows the sequence coverage obtained by Sequest (yellow) and Mascot after four hours of proteolysis. The regions identified by both algorithms are noted in green (tryptic peptides) and blue (nonspecific cleavage). Amino acids denoted in bold letters indicate the C terminal positions of the trypsin-like cleavages.

After four hours of digestion, using a fully tryptic database search with nine missed cleavages, Mascot identified 58.8% of the carbonic anhydrase sequence, the average peptide mass was 3.1 kDa. Sequest performed slightly better, yielding 62.3% sequence coverage, average peptide mass 2.7 kDa. The longest peptide identified was 6106.08 Da and the sequence (170–224) is unique to bovine carbonic anhydrase. When performing a no-enzyme search, we obtained 95% sequence coverage

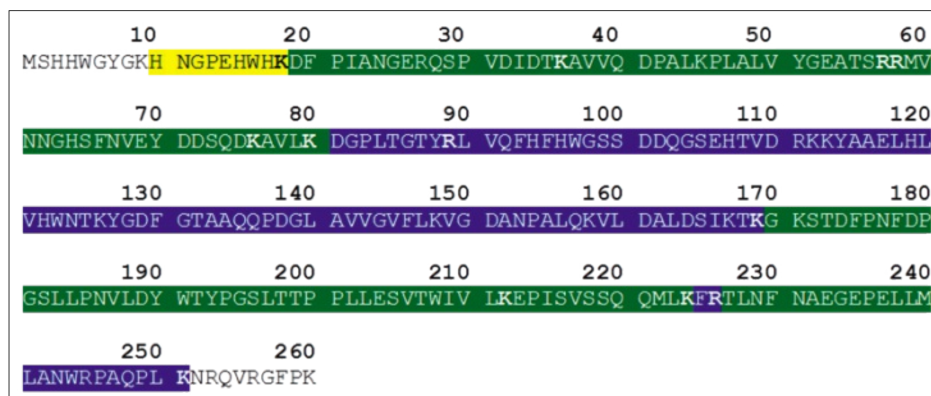

Fig. 2. Sequence coverage of bovine carbonic anhydrase digested for 4 h with Sap9 at pH 4.5, 37 °C, protein:enzyme ratio 1:2.5 (w/w). In yellow is the tryptic peptide identified by Sequest, in green are indicated the regions identified by both Sequest and Mascot. Blue indicates the protein region identified using a no enzyme search.

with both engines. Of the 81 total cleavages observed, only 6 occurred at dibasic sites, 13 were tryptic and 32 half-tryptic. In addition, we observed 26 peptides with cleavage C-terminal to hydrophobic residues. Only 5 peptides were detected with hydrophilic C terminus other than K or R. Shorter digestion times (<2 h) yielded a combination of short (<2 kDa) and long (>10 kDa) peptides, while longer interaction times (5–8 h) yielded increasingly shorter peptides.

The obtained results indicate that at the conditions where Sap9 is the most active, its site specificity is not ideal. Under the experimental conditions presented herein we observed a secondary selectivity towards hydrophobic residues in addition to the basic sites. We are currently investigating the optimal proteolysis conditions where cleavage specificity is tailored toward more predictable dibasic sites whereas the protease activity is still sufficiently high.

Peptide Fragmentation Study

We sought to establish the efficiency and quality of the mass spectra obtained using the two most commonly used fragmentation techniques, HCD and ETD, applied to the analysis of middle-down range peptides. Specifically, we investigated whether the exact location of the dibasic site cleavage, *i.e.* proteolysis after or between the two adjacent basic residues, has an effect on the quality of the MS/MS mass spectra. We performed a series of experiments where the HCD activation energy and ETD reaction time was gradually varied for all charge states of the 34 residue synthetic peptides mimicking cytochrome C sequence (Peptide Synthesis Facility, University of Lausanne, Switzerland) TGQAPGFSYTDANKNKGITWGEETLMEYLENPKK and KTGQAPGFSYTDANKNKGITWGEETLMEYLENPK. Peptides were dissolved in 50:50 ACN:H_2O solvent mixture containing 0.1% of formic acid to the final concentrations of *ca.* 10 μM. Ions were generated using a nano-electrospray ionization (nESI) ion source (Triversa Nanomate, Advion Biosciences, Ithaca, NY, USA) at a flow rate of *ca.* 300 nL/min. HCD normalized collision energy (NCE) was varied between 0 and 35 in increments of 5, while ETD interaction time was varied from 0.1 to 100 ms in increments of 5 ms. Fig. 3 shows the representative MS/MS spectra for the 5+ charge state of the two peptides obtained at NCE 20 and 25 ms ETD reaction time, respectively. The most notable difference between the HCD mass spectra of the two peptides is the absence of the b_3-b_5 ion series when both peptide termini are K. This is likely due to the sequestration of the proton by the K side-chain does not allow charge-directed fragmentation, as predicted by the mobile proton model.[21] As indicated by the absence of products from the innermost positions, the distal positioning of the two fixed charges is detrimental for the cleavage of peptide bonds in the middle of the sequence, even at the collision energy where virtually no precursor ion remains. In contrast, the c_2-c_6 ion series is completely absent from the ETD mass spectrum of the peptide with adjacent basic residues, indicating that, for these product ions, the N-terminal charge is neutralized upon ETD.[22] Nonetheless, ETD fragmentation of both peptides yielded informative mass spectra and almost complete (93%) sequence coverage. We performed similar studies on numerous peptides with varying lengths. We have found that, as expected, sequence coverage is improved for both

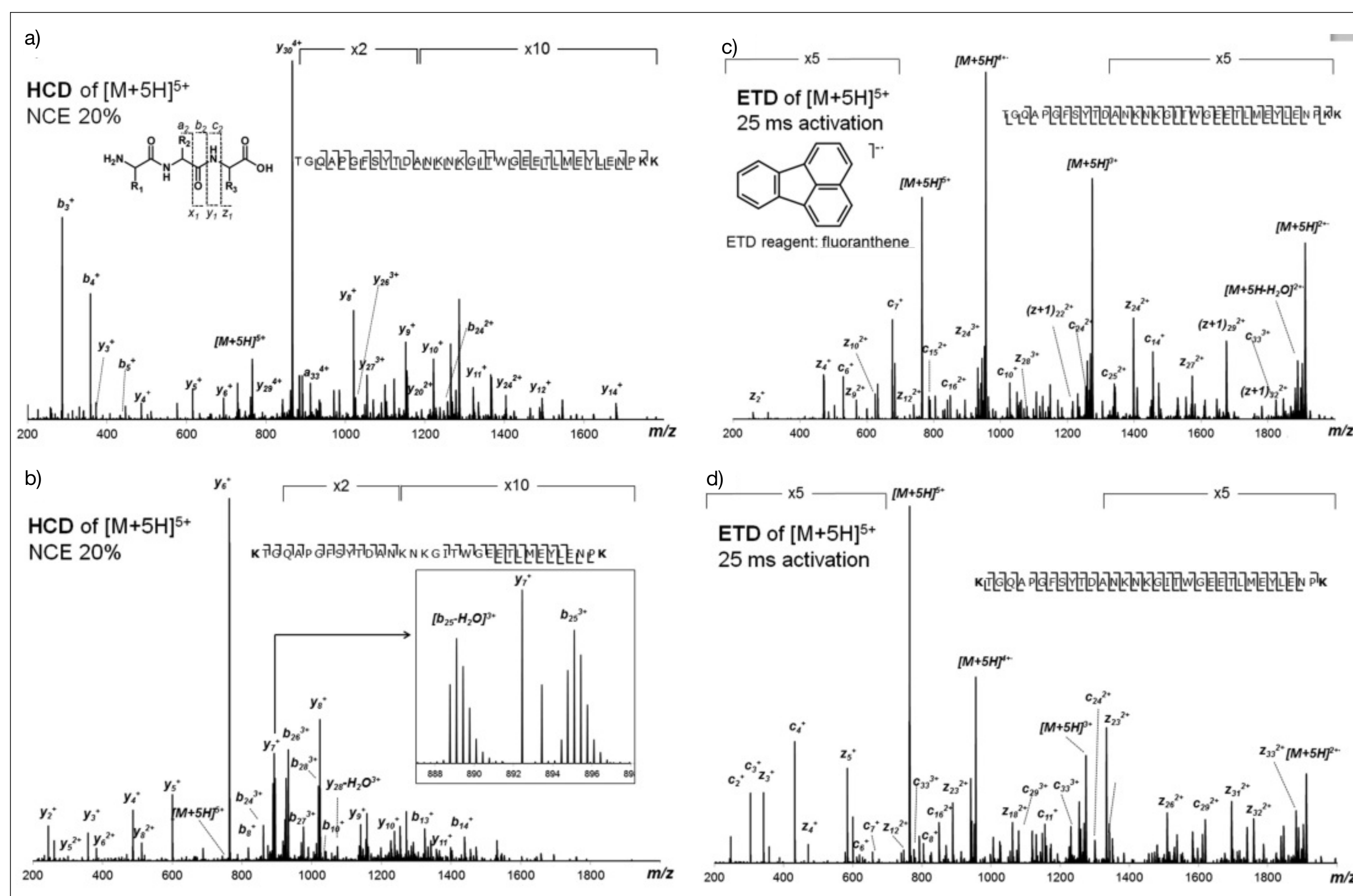

Fig. 3. Comparison of the fragmentation mass spectra of the 5+ precursor ions of the peptides TGQAPGFSYTDANKNKGITWGEETLMEYLENPKK and KTGQAPGFSYTDANKNKGITWGEETLMEYLENPK obtained with HCD (panels *a* and *b*) and ETD (panels *c* and *d*). Ion activation conditions were: NCE 20 for HCD, and 25 ms of ion activation time for ETD. The inset in panel b demonstrates the utility of high resolution in the identification and assignment of product ions.

HCD and ETD fragmentation with increasing precursor ion charge state of each peptide, regardless of the positioning of the terminal basic residues.

Post-column Supercharging

To ensure that the peptides carry the maximum number of charges and to increase the signal to noise ratio,[23] while minimizing the total number of charge states, we utilized post-column supercharging. For this, a mixture of five proteins was digested overnight with LysC, the peptides eluting from the chromatographic column were continuously reacted with 0.5% *m*-NBA (*m*-nitrobenzyl alcohol) in 50% ACN (both from Sigma Aldrich). The reagent was introduced *via* a zero-dead-volume Y junction (Idex, Oak Harbor, WA), as illustrated in Fig. 4. The supercharging reagent flow was set to match the flow from the column (0.3 µL/min) to minimize turbulence. The inset in Fig. 4 shows the minimal effect of post-column supercharging on the chromatographic peak shape. Representative mass spectra obtained with and without supercharging are shown in Fig. 5. All other experimental conditions (LC flow rate, spray voltage, number of precursor ions, maximum ion injection time, mass resolution setting) were the same for both experiments. To ensure that the change in charge state distribution is the effect of *m*-NBA, and not a solvent-effect, for the non-supercharged experiment we infused 50% ACN.

The mass spectra in Fig. 5 contain several species, two of which are highlighted in red and blue. Under normal conditions (no supercharging, top panel) the 'red' peptide was present under four different charge states (+4, +5, +6, and +7), while the 'blue' peptide was detected with +3 and +4 charges. The most abundant charge state for the 'red' and 'blue' peptides were +6, and +4, respectively. When the supercharging reagent was added, the peaks corresponding to the 'red' +4 and +5 charge states were greatly diminished, while the +7 peak now had the highest S/N. Similarly, the ratio of the +4 and +3 precursors of the 'blue' peptide increased from 2:1 to 12:1. Fig. 6 shows the total (not unique) number of precursor ions with different charge states obtained with and without supercharging. As indicated by the ratio of the red and blue columns, the number of +2 precursor ions decreased slightly, while the number of MS/MS spectra that had precursor ions of >2 charges increased. This shows that the overall S/N of high charge state species increases and more precursor ions are selected for MS/MS in the data dependent scanning event. This is an important aspect for middle-down proteomics, since higher S/N precursor ion yields better quality MS/MS spectra and requires shorter scanning times.

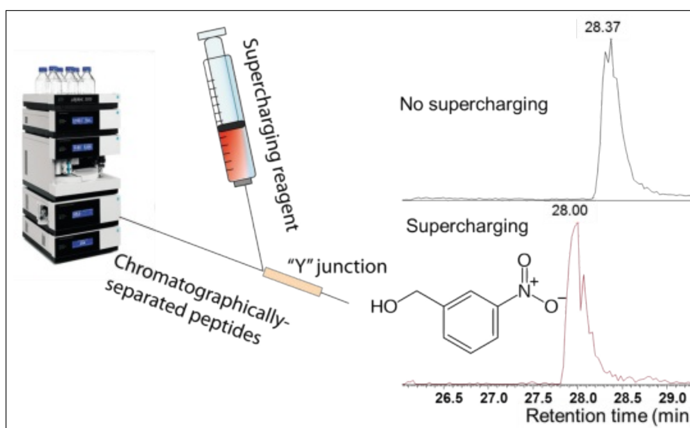

Fig. 4. Schematic representation for the introduction of the supercharging reagent after chromatographic separation, using a zero-dead-volume Y connector. The inset shows that the chromatographic peak shape is not significantly affected with introduction of the reagent.

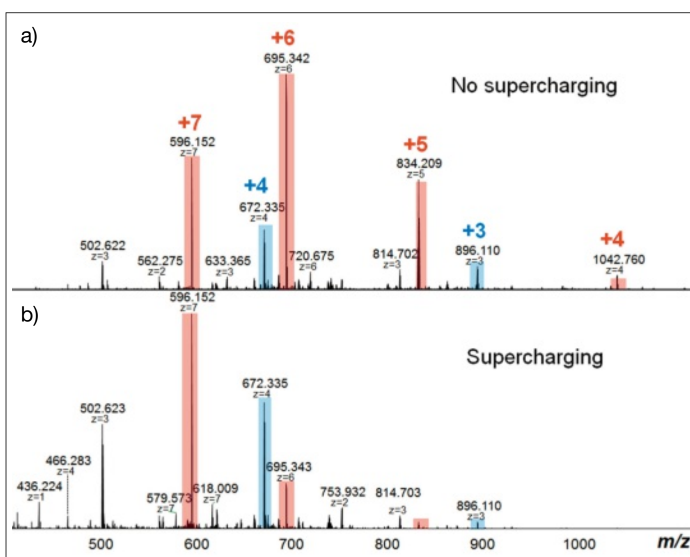

Fig. 5. Experimental mass spectra of peptides eluted from the chromatographic column a) without b) with addition of 0.5% *m*-NBA supercharging reagent. The S/N of high charge states for peptides indicated in red and blue increases by supercharging, while the signal of low charge states are significantly diminished.

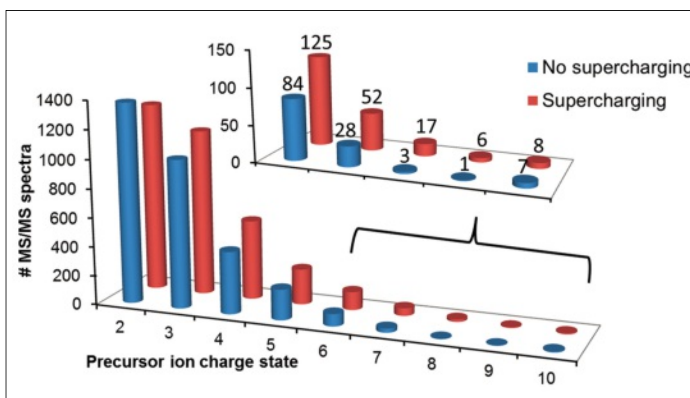

Fig. 6. Total precursor ion charge state distribution with (red) and without (blue) post-column supercharging for a mixture of five standard proteins digested overnight with LysC.

Conclusions

Sap9 is a promising protease for middle-down proteomics and yields close to complete sequence coverage in as short as four hours of digestion. This, in turn, improves the probability for detection and localization of PTMs and the identification of splice variants. It can also be important when the goal of the study is to distinguish between proteins with very similar sequences, such as in targeted species identification based on a reference protein.[24] To improve the MS and MS/MS data quality, the S/N of highly charged peptides can be increased by introduction of the supercharging reagent post column. This has the advantage of not interacting with the stationary phase, therefore eliminating contamination, and preventing changes in chromatographic separation. These improvements of the proteomic workflow can be implemented for the high throughput analysis of complex protein mixtures using state-of-the-art high resolution in-

strumentation. In addition, for the peptide size range <7 kDa, protein database search engines well established for bottom-up proteomics (Sequest and Mascot) can be successfully employed, without the need for customized in-house built algorithms.

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