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An integrated top-down and bottom-up strategy for broadly characterizing protein isoforms and modifications

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Abstract

We present an integrated top-down and bottom-up approach that is facilitated by concurrent liquid chromatography-mass spectrometry (LC-MS) analysis and fraction collection for comprehensive high-throughput intact protein profiling. The approach employs high resolution reversed phase (RP) LC separations coupled on-line with a 12T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer to profile and tentatively identify modified proteins, using detected intact protein masses in conjunction with bare protein identifications from the bottom-up analysis of the corresponding LC fractions. Selected identifications are incorporated into a target ion list for subsequent offline gas-phase fragmentation that uses an aliquot of the original fraction used for bottom-up analysis. In a proof-of-principle demonstration, this comprehensive strategy was applied to identify protein isoforms arising from various amino acid modifications (e.g. acetylation, phosphorylation) and genetic variants (e.g. single nucleotide polymorphisms, SNPs isoforms). This strategy overcomes major limitations of traditional bottom-up (e.g., inability to characterize multiple unexpected protein isoforms and genetic variants) and top-down (e.g., low throughput) approaches.

Introduction

Bottom-up and top-down strategies for mass spectrometry (MS) based protein characterization are complementary; each with its own strengths and weaknesses. In bottom-up strategies, proteomic measurements at the peptide level offer a basis for comprehensive protein identification.[1–5] However, important information may be ultimately unobtainable since only a portion of the entire protein is generally detected and different proteins can have tryptic peptides that are indistinguishable (e.g., families of highly related genes). The information on proteolytic processing is generally lost in peptide centric analyses. Similarly, information regarding the coordination of posttranslational modifications (PTMs) is generally lacking. Indeed, even for bottom-up approaches targeted at the identification of modifications, important information is inevitably lost, e.g., are two sites modified in concert or independently? From a data analysis standpoint, peptide-centric measurements significantly complicate protein quantitation due to the existence of multiple protein isoforms. For instance, PTMs can significantly affect protein activity without a change in overall protein abundance. As protein quantity is typically compiled using information obtained for multiple peptides in bottom up approaches, the assumption of “all peptides being equal” can potentially introduce significant errors since it is virtually impossible to know whether a peptide is specific to a single protein isoform or common to multiple isoforms. For these reasons, measurements at the intact protein level provide an important adjunct to peptide level measurements.

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Top-down proteomic strategies that involve gas-phase dissociation of intact proteins[6–18] have demonstrated 100% protein sequence coverage and allowed for the identification of protein isoforms, proteolytic processing events, and PTMs. [19–28] However, these strategies presently suffer from limited sensitivity and throughput.[8, 29] The most recent and significant advancements in the context of top-down proteomics are electron capture dissociation (ECD) [23, 25, 30–34] and electron transfer dissociation (ETD)[35–37]. These techniques typically provide more uniform dissociation than conventional collisionally induced dissociation (CID), while preserving the labile modifications. However, EC/TD based approaches have been demonstrated only occasionally for intact protein characterization and have not yet been effectively incorporated into a high-throughput comprehensive top-down proteomic analysis.

Top-down platforms have been applied to characterize limited subsets of proteins (~100) from microorganisms,[26, 27] human proteins,[20, 21, 24] and even proteins with molecular masses above 200 kDa.[9] However, top-down approaches face a number of challenges in addition to limited throughput. For example, on-line liquid chromatography- tandem mass spectrometry (LC-MS/MS) platforms for intact protein characterization have met with only limited success; [29] most often, MS/MS is accomplished offline (e.g., using collected LC fractions) to allow for spectral averaging and to generate sufficient information on fragment ions. Another major challenge is MS/MS data interpretation. ProSightPTM,[38–40] which uses accurate neutral masses inferred from tandem mass spectra, is virtually the only available tool for identifying intact proteins. When combined with known or predicted information, ProSightPTM facilitates PTM identification, but at present cannot address unknown or unexpected modifications and/or single nucleotide polymorphisms (SNPs). Additionally, major challenges associated with sensitivity, higher molecular mass proteins (>100 kDa), and highly hydrophobic proteins (e.g., integral membrane proteins) remain largely unsolved thus, limiting the applicability of top-down proteomics on a large scale. Similarly, effective separation of minute amounts of a complex mixture of proteins with a variety of physicochemical properties prior to MS analysis represents an overarching technical challenge.

For these reasons, various integrated approaches that combine complementary information from bottom-up and intact protein analyses have been adopted for analyzing intact proteins. [41–45] Typically, intact protein separations are employed to differentiate between protein isoforms and fractionate proteins to a level such that the reduced complexity enables the identification of protein masses from peptide level characterization of the same fraction. Based on earlier studies,[41, 46] precise intact protein mass is sufficient for identifying a limited number of proteins detected in an LC fraction. For example, application of this approach to the bacterium *Shewanella oeidensis* resulted in tentative identification of only ~10% of detected intact *S. oeidensis* proteins. However, ~60% of the tentatively identified intact proteins were modified, indicating a prevalence of modified proteins even at the microbial level.[41] The failure to identify 90% of detected intact proteins illustrates the combinatorial challenge posed by the large number of possible modifications and too many bottom-up derived candidates, which must be considered as potentially being present. This suggests that mass measurement accuracy (MMA) is still inadequate and additional data (e.g. MS/MS) is still required to increase confidence and expand the set of identified proteins. Alternatively, fractions of limited complexity (several proteins) and known composition would provide identifications that are more confident.

In response to these obstacles, we have combined intact protein separations with on-line spectral acquisition and fraction collection. A key advantage offered by this approach in comparison to conventional top-down proteomics is that it eliminates the need for MS/MS analysis of the intact proteins that often cannot be effectively performed on the separation time scale, while still providing a protein fingerprint in the form of its corresponding peptides. Importantly, unlike the earlier strategy where complex intact protein mixtures (e.g., weak anion

exchange fractions) were independently analyzed at the peptide and protein level,[41] this current approach preserves a direct link between the protein (mass) and its corresponding tryptic peptides (MS/MS data) since all tryptic peptides are confined to the chromatographic peak (i.e., on-line collected RPLC fraction) of the protein. Protein identification greatly benefits from this preserved linkage and time-consuming off-line intact protein MS/MS can be performed in a targeted fashion. For example, to confirm and/or localize specific modifications or interesting proteins (e.g., proteins showing statistically significant change in abundance between two biological states), thereby increasing overall effectiveness and/or throughput of the analysis. This combination of intact protein mass and abundance information with bottom-up derived protein identity is similar to traditional gel-based proteomics, but this integrated approach has the advantage of allowing subsequent MS/MS (top-down) or Western blot characterization of biologically relevant targets.

The platform used to demonstrate the integrated approach employs high resolution reversed phase LC (RPLC) separations coupled to on-line fraction collection and a 12 Tesla Fourier transform ion cyclotron resonance (FTICR) spectrometer. Modified proteins are tentatively identified using detected intact protein masses in conjunction with “bare” protein identifications from bottom-up analysis of the collected RPLC fraction. The bottom-up data provide a means to estimate relative abundances for each identified protein across fractions, which allows us to match the LC elution pattern generated from peptide-level data to the intact protein LC-MS profile. Here, the protein abundance was estimated in each fraction using peptide counts, i.e. counting the number of identified peptides per protein. The additional constants from the aligned top-down and bottom-up datasets limit the protein search space, thereby improving the confidence for identifications based on accurate mass measurements and facilitating the discovery of unexpected modifications and/or amino acid substitutions. Additionally, this integrated approach holds great promise for profiling protein abundances at a level that incorporates the protein modification complement and affords high-throughput selection of biologically relevant targets for subsequent offline gas-phase fragmentation using an aliquot of the original fraction used for bottom-up analysis.

We have initially evaluated this strategy by identifying modifications and isoforms from a mixture of standard proteins. Despite the simplicity of our model system, we were able to identify a surprisingly rich set of proteins that included various isoforms stemming from SNPs and different PTMs (e.g., acetylation, phosphorylation, heme-containing proteins) in addition to contaminant proteins. We further applied the integrated approach to analyze the yeast proteasome and identified several classes of PTMs, including oxidation, phosphorylation, methylation, as well as proteolytic processing activity, demonstrating the feasibility of the integrated approach and its applicability for the analysis of larger proteins.

Experimental Section

Materials

The following standard proteins were purchased from Sigma: ubiquitin (U6253), cytochrome C (C2037), β-lactoglobulin A (L7880), β-lactoglobulin B (L8005), β-casein (C6905), carbonic anhydrase II (C3934), and myoglobin (M1882). Trypsin was obtained from Promega (Madison, WI). Calmodulin [accession number MCCH (PIR database) or P02593 (SWISS-PROT database)] was cloned, expressed, and purified under standard conditions, with polyhistidine tag (i.e. GHHHHHHGGGGGIL) on the C-terminus for nickel affinity purification.[47] *S. cerevisiae* proteasome complex was purchased from BIOMOL (Plymouth Meeting, PA).

Intact protein LC-FTICR MS with on-line fractionation

LC-FTICR MS and simultaneous fractionation was accomplished using the Triversa NanoMate 100 (Advion BioSciences, Inc.; Ithaca, NY). The RPLC system used for on-line intact protein separations was similar to that previously reported.[41] Briefly, the column (70 cm × 200 μm i.d.) was packed in-house with Phenomenex Jupiter particles (C5 stationary phase, 5 μm particle diameter, 300 Å pore size). Mobile phase A was composed of 0.05% trifluoroacetic acid (TFA), 0.2% acetic acid, 5% isopropanol, 25% acetonitrile (ACN), and 69.75% water. Mobile phase B consisted of 0.1% TFA, 9.9% water, 45% isopropanol, and 45% ACN. The operating pressure was 10,000 psi, and the estimated flow rate was ~5.5 μl/min. The LC separations were carried out at constant pressure (10,000 psi) as reported earlier.[48] The RPLC system was equilibrated at 10,000 psi with 100% mobile phase A. Next, a mobile phase selection valve was switched to create a near-exponential gradient as mobile phase B displaced A in a 2.5 mL mixer. A split was used to provide an initial flow rate through the column of ~5.5 μl/min and most proteins eluted in less than 2 hours. The column eluant was split with ~300 nl/min of the flow directed to a modified Bruker 12 T APEX-Q FTICR mass spectrometer,[41] and ~5.2 μl/min collected into a 96-well Eppendorf Twin-pac plate. The standard protein mixture was prepared in mobile phase A, using the protein concentrations specified in Table 1. Total sample injection volume was 20 μl (~20 μg total protein). A pressure of 0.25–0.35 psi and a voltage of 1.45-1.7 kV was used for the nanoESI employing NanoMate 100 system. A novel compensated trapped-ion cell with improved DC potential harmonicity was employed to enhance mass measurement accuracy and sensitivity.[49] During the LC-MS analysis, a single mass spectrum was recorded using 512k data points, and the average of two mass spectra was used for data analysis.

Offline protein MS/MS analysis

One-third of the collected fraction volume (~5 μl) was used for offline MS/MS analysis. ESI-MS/MS was carried out with a NanoMate 100, a combined autosampler (a rack of 96 disposable and conductive pipette tips and a 96-well microtiter plate that contained samples to be analyzed) and a nanoESI source (a chip-based nanoESI device with 400 nozzles). CID-MS/MS analyses were accomplished by reducing the DC offset on the accumulation hexapole from 0 V to −25 V. Ten to fifty mass spectra were averaged to obtain fragment ion information of desired quality (i.e., such that the S/N ratio is larger than 3 for more than 80% fragment peaks). ECD-MS/MS analyses were performed using a heated hallow cathode located outside the ICR cell (i.e., the standard Bruker ECD arrangement). The cathode was heated with a current through the filament of 1.6–1.8 A. A 1–3 ms electron injection time was used with the potential on the solid cathode dispenser set at −7.5 V to −15 V. Again, up to 50 mass spectra were averaged to obtain fragment ion information of sufficient quality.

On-plate fraction digestion

A 50 mM ammonium bicarbonate in 30% (v/v) aqueous ACN (pH 8.2) was used as the digestion buffer. An aliquot of each fraction (~5 μl) collected during RPLC was used for top-down analysis (described above). The remaining sample was digested on-plate overnight at 37°C by adding 10 μl trypsin in digestion buffer (10 μg/ml). Samples were evaporated on the plate to remove extra organic solvent (to ~5 μl remaining volume) using SpeedVac. The final volume was adjusted to 15 μl with mobile phase A.

RPLC-Ion Trap (IT) peptide MS/MS analysis

The capillary RPLC system used for peptide separations has been previously described.[48] Briefly, the separation was performed under a constant pressure of 5000 psi, using two ISCO (Lincoln, NE) Model 100 DM high-pressure syringe pumps and a column (60 cm × 150 μm i.d.) packed in-house with Phenomenex (Torrance, CA) Jupiter particles (C18 stationary phase,

5 μm particles, 300 Å pore size). Mobile phase A consisted of 0.05% TFA, 0.2% acetic acid, and 99.75% water. Mobile phase B consisted of 0.1% TFA, 9.9% water, and 90% ACN. Approximately 5 μl of the digest from each fraction (about half of the total fraction volume) was loaded onto the column for 20 min. The gradient was produced by adding mobile phase B to a stirred mixing chamber (2.5 ml volume) that was initially filled with mobile phase A. A split was used to provide an initial flow rate through the column of ~2 μL/min. ESI using an etched fused-silica tip[50] was employed to interface the RPLC separation to a Finnigan (San Jose, CA) LTQ linear ion trap. A maximum of five MS/MS spectra were recorded for the most intense peaks in each survey mass spectrum.

Data processing

Peptide RPLC-IT MS/MS data were processed using SEQUEST[51] and a database that contained both genome derived possible *S. oneidensis* protein sequences[52] and the standard protein sequences listed in Table 1. No enzyme rules were applied, and identified peptides were filtered according to the criteria suggested by Washburn et al.[53] Provisional databases that contained proteins supported by at least two distinct peptide identifications and in the protein mass range of 5–40 kDa were created for each fraction.

Intact protein RPLC-FTICR mass spectra were processed using in-house developed software (ICR-2LS and Viper;[54] available at http://ncrr.pnl.gov/software/) as described in Sharma et al.[41] Time-domain signals were apodized (Hanning) and zero-filled (twice) prior to FT. All spectra were externally calibrated, using myoglobin and ubiquitin spectra acquired in a separate LC-MS analysis.

The resulting mono-isotopic masses were clustered into features based on neutral mass, charge state, abundance, isotopic fit, and spectrum number (relating to RPLC retention time). Spectra that corresponded to a particular feature were summed, and the resulting spectrum reprocessed as described above. Next, all charge states in the *m*/*z* range were collapsed into a zero charge state spectrum (i.e. neutral mass), which were then searched against the appropriate provisional protein database (assembled from bottom-up data) for tentative intact protein identifications. Mass measurements were manually inspected by matching the observed most abundant peak to the theoretical one. Discrepancies between the measured protein masses and the predicted masses for proteins in the provisional databases were used to search for a limited set of protein PTMs (discussed in following section).

Intact protein MS/MS spectra were analyzed using ICR-2LS and/or ProSightPTM (https://prosightptm.scs.uiuc.edu/) and protein sequences identified in bottom-up analyses.

Results and Discussion

Figure 1 depicts the integrated bottom-up and top-down approach used to analyze a mixture of standard proteins (Table 1). The total ion chromatogram reconstructed from the FTICR spectra acquired during an RPLC separation of the standard proteins is shown in Figure 2A, and the corresponding 2D display (neutral mass vs. spectrum number, i.e. retention time), in Figure 2B. Each of the 42 fractions collected over the course of the LC-MS analysis were separated into two aliquots: one for bottom-up (~2/3 of the total fraction volume), and the other for follow-up top-down analysis (~1/3 of the total fraction volume). Tentative protein and modified protein identifications were assigned by matching bottom-up results to the measured intact protein masses, while allowing for a limited set of PTMs (i.e., methylation, acetylation, oxidation, phosphorylation, heme, and loss of the N-terminal Met). (Table 2) The MMA <10 ppm used for tentative protein identification was based on the data quality estimated as described by Sharma et al.[41]

Protein LC-MS profiles were then generated, using unique peptides identified in bottom-up analyses (as illustrated in Figure 3) for the later portion of the LC-MS analysis. Proteins typically displayed distinctive elution patterns. For instance, carbonic anhydrase mainly eluted in fractions 33–36, while calmodulin eluted in fractions 31–33. As a result, the elution patterns generated using bottom-up data could be used to restrict the identity of intact protein masses (observed in LC-MS analysis) to a particular combination of PTM, SNPs, N-/C- terminal cleavage(s), etc. Tentative identifications were subsequently confirmed by MS/MS analyses of the collected intact protein fractions. In the present implementation of the integrated approach, intact protein MS/MS limits the overall throughput and is therefore performed in a targeted fashion. For instance, in a comparative proteomics experiment, only species showing statistically significant change in abundance between two states as determined by comparison of their LC-MS profiles would be selected for MS/MS identity confirmation. To increase overall sensitivity, this type of comparative analysis is currently accomplished using a 75μm i.d. column with <1 μg of total protein load compared with 200 μm i.d. column and >10 μg of total protein load typically employed for on-line fractionation.

The correlation between on-line LC-MS data and MS data obtained for a reconstructed fraction is exemplified in Figure 4. The intact protein mass spectrum acquired for reconstituted fraction 35 (Figure 4A) is virtually identical to the mass spectrum reconstructed from the LC-MS data by summing all of the spectra acquired on-line during collection of fraction 35 (Figure 4B). The shift in the charge state distribution was attributed to different solvent compositions, while the observed increase in the degree of oxidation for the reconstituted fraction likely resulted from storage and/or chip-based nanoESI.[55] The most abundant species that eluted in fraction 35 were tentatively assigned as acetylated carbonic anhydrase II by matching the intact protein mass and bottom-up data acquired for fraction 35 (MMA ~0.01 ppm). MS/MS analysis of reconstituted fraction 35 confirmed this protein to be carbonic anhydrase II acetylated at the N-terminus (Figure 4C).

Bottom-up data indicated the presence of β-lactoglobulin B in a broad range of fractions (i.e., 31 – 38). The elution profile (Figure 5A), which was generated using relative protein abundances derived from the bottom-up data, revealed two elution peaks (i.e., fractions 32–33 and fraction 36), in contrast to the majority of analyzed proteins that typically displayed a single peak. In addition, only early eluting peaks (e.g., cluster 3 in Figure 5A) could be identified using accurate intact protein masses as a β-lactoglobulin B with two disulfide bonds (MMA=0.8 ppm[56]). Hence, while the intact protein data indicated that β-lactoglobulin B protein eluted before fraction 34 was collected, the bottom-up data suggested that fraction 36 should also contain this protein. Intact protein data acquired for fractions 35–38 revealed the presence of a highly abundant protein with the molecular mass of 18363.45 Da, which was apparently incorrectly identified in the bottom-up analysis (i.e., measured intact protein mass was 86.03 Da higher than theoretical mass of β-lactoglobulin B). However, measured mass agreed well with β-lactoglobulin A theoretical mass (MMA = 0.1 ppm). A comparison of the two sequences (β-lactoglobulin A and B) revealed that they differ in only two amino acids (Figure 5B). Top-down analysis (CID), performed using reconstituted fractions confirmed the presence of β-lactoglobulin B (M_r =18277.42 Da) in fraction 32 and β-lactoglobulin A (M_r =18363.45 Da) in fraction 36 (data not shown). Thus, by combining intact protein information (mass and elution time) with bottom-up derived protein identity, this integrated approach provides information that would be challenging at present by either approach alone, particularly for a complex protein mixture.

Similarly, the bottom-up results (Figure 3) revealed a majority of β-casein eluted in fractions 26–30. However, the intact protein LC-MS data indicated that three distinct proteins with M_r =24092.34, 24023.22, and 23983.23 Da, eluted sequentially in this region (Figure 6). A protein with M_r =23983.23 Da was matched to β-casein isoform A2 with 5 phosphorylation sites

(MMA =1.8 ppm). Intact protein CID (Figure 6) and ECD (Figure 7) data, obtained using reconstituted intact protein fractions, confirmed this assignment. For example, in the ECD spectrum of isoform 1, the fragment masses that generated the sequence tag ELNVNPG that matched β-casein residues 5–11 were assigned as unmodified c ions, indicating the absence of phosphorylation in this region (Figure 7). Similarly, the sequence tag TEDELQD matched β-casein residues 41–47, but all of the associated fragments were identified as c ions bearing five phosphate groups. Finally, five phosphorylation sites were located in the region between residues 15 and 41, which contains a total of seven Thr and Ser residues. Our data indicate that the loss of phosphate moiety is much less pronounced when CID is performed at the intact protein level, as reported earlier.[8] Preferential loss of the phosphate moiety relative to the peptide backbone cleavage in the CID spectra of phosphopeptides represents a major challenge for pinpointing phosphorylation sites. Therefore, intact phosphoprotein CID appears promising for mapping phosphoproteins, determining the degree of phosphorylation, and studying phosphorylation dynamics in the context of signaling networks.

Species with molecular masses of 24092.34 Da and 24023.22 Da could not be identified as modified β-casein species using any combinations of PTMs allowed in the search, and were therefore examined by offline MS/MS. A majority of the species with M_r =24023.22 Da eluted in fraction 26, while a majority of the species with M_r =24092.34 Da were detected in the following fraction (i.e., fraction 27). As shown in Figure 6, the mass differences between the observed intact protein masses, M_r =24023.22 Da (isoform 2) and M_r =24092.34 Da (isoform 3), and the previously identified phosphorylated β-casein (isoform 1) are 39.99 Da and 109.11 Da, respectively. By comparing the CID spectra obtained for isoforms 1 and 2, we identified b62 as the last unmodified b ion, and b74 as the first modified b ion (i.e., a difference in mass of 40 Da from identified isoform 1). Therefore, the modification or amino acid substitution(s) must reside between residues 62 and 74. Considering single amino acid substitutions only, both Pro-to-His and Gly-to-Pro switches will result in a 40 Da mass shift. However, Pro-to-His substitution represents an SNP and is therefore more likely to occur. In fact, β-casein variant A1, in which Pro67 is substituted for His has been reported.[57] Similarly, MS/MS data confirmed Pro67-to-His substitution for isoform 3; however, a mass shift of 109.11 Da relative to isoform 1 (phosphorylated β-casein) suggested the presence of additional SNP(s), which resulted in a mass shift of 69.12 Da. Considering all possible amino acid substitutions, this mass shift can only be associated with a Ser-to-Arg switch (an SNP). Using combined CID and ECD data obtained for isoform 3, we were able to associate this delta mass with a substitution of one of the four Ser residues in region 74–160 to Arg. The Ser122-to-Arg mutation was confirmed by the identification of the fully tryptic peptide YPVEPFTER (XCorr =3.0539) in the same fraction (Figure 8), which is in agreement with the previously reported sequence of β-casein variant B.[57, 58] Hence, three β-casein isoforms were identified as β-casein genetic variants A2, A1, and B, respectively. The identity of all three β-casein isoforms was additionally confirmed by searching the bottom-up data against these mutant sequences. This example demonstrates the ability of the integrated approach to characterize co-existing protein isoforms that originate from SNPs. Even more importantly, this example highlights a significant pitfall of the conventional bottom-up approaches that cannot validate the presence of multiple distinctive isoforms (i.e., distinguish between one isoform with two SNPs and two isoforms each with a single SNP), even given 100% sequence coverage. As SNPs can have a major impact on how humans respond to disease, environmental insults, and therapy, a characterization strategy such as the one described here can be invaluable for biomedical research.

We further applied the integrated approach to characterize the yeast proteasome. A small sample, about 15 μg, was analyzed by LC-MS analysis with concurrent fraction collection revealing the presence of several putative proteins with mass >10 kDa. A subset of these proteins has been tentatively identified (Table 3) by matching bottom-up derived protein

identifications and elution profiles with the intact protein accurate masses and elution profiles, as illustrated in Figure 9. This hybrid analysis identified various classes of PTMs including oxidation, phosphorylation, methylation, as well as proteolytic processing events (Table 3).

Our results agreed well with previous reports in regards to the N-terminal methionine truncation of several proteasomal components. For instance, initiator Met was cleaved from 26S proteasome regulatory subunits RPN10 and RPN12, while RPN9 showed neither removal of Met residue nor N-acetylation. [59] An intact mass of 11129.34 Da was identified as 60S acidic ribosomal protein P2β with 1 phosphorylation and with no N-terminal modification, in agreement with previously reported results.[60] Similar results were found for 60S acidic ribosomal protein P2α. These acidic stalk proteins are the only ribosomal components for which there is a cytoplasmatic pool and which are present in multiple copies in each ribosome. [61] While these highly abundant cell proteins are likely to be nonspecific contaminants, a physiologically relevant interaction between proteasomes and ribosomes has been previously suggested. [62] Additionally, we were able to identify proteins with M_r >40 kDa. For instance, acetylated 26S protease regulatory subunit 6B homolog (M_r =47935.21 Da) was identified with MMA <1 ppm. These initial results support the feasibility of the integrated approach and its applicability for the characterization of larger proteins.

Conclusions

In response to the limited abilities of bottom-up approaches for identifying protein modifications and top-down approaches in terms of throughput, we developed an integrated top-down and bottom-up approach for comprehensive protein identification and profiling. This approach combines bottom-up analyses with accurate intact protein mass measurements to derive protein identity, and uses targeted top-down analyses to validate interesting protein targets. Bottom-up data are used to limit the search space, thus facilitating the discovery of unexpected modifications and/or amino acid substitutions. In an initial application with a mixture of standard proteins and yeast proteasome, the integrated approach enabled characterization of PTMs (e.g., acetylation, phosphorylation, heme-containing proteins) and genetic variants (e.g., SNP isoforms). Importantly, the unique ability of this approach for identifying (and potentially quantifying) distinctive protein isoforms (e.g., distinguish between one isoform with two SNPs and two isoforms each with a single SNP in the case of β-casein) was validated. The ability to characterize (i.e. identify and quantify) various protein variants could have a high impact in biomedical studies. For example, bioactive peptide β-casomorphin 7 (BCM-7), which is generated by successive gastrointestinal proteolytic digestion of bovine β-casein variants A1 and B, potentially plays a role in the etiology of a number of human diseases (e.g., cardiovascular diseases, type1 diabetes, sudden infant death syndrome, autism, and schizophrenia).[63] The new integrated strategy can be readily applied to measure differential protein abundances, and provides a platform for high-throughput selection of biologically relevant targets for further characterization.

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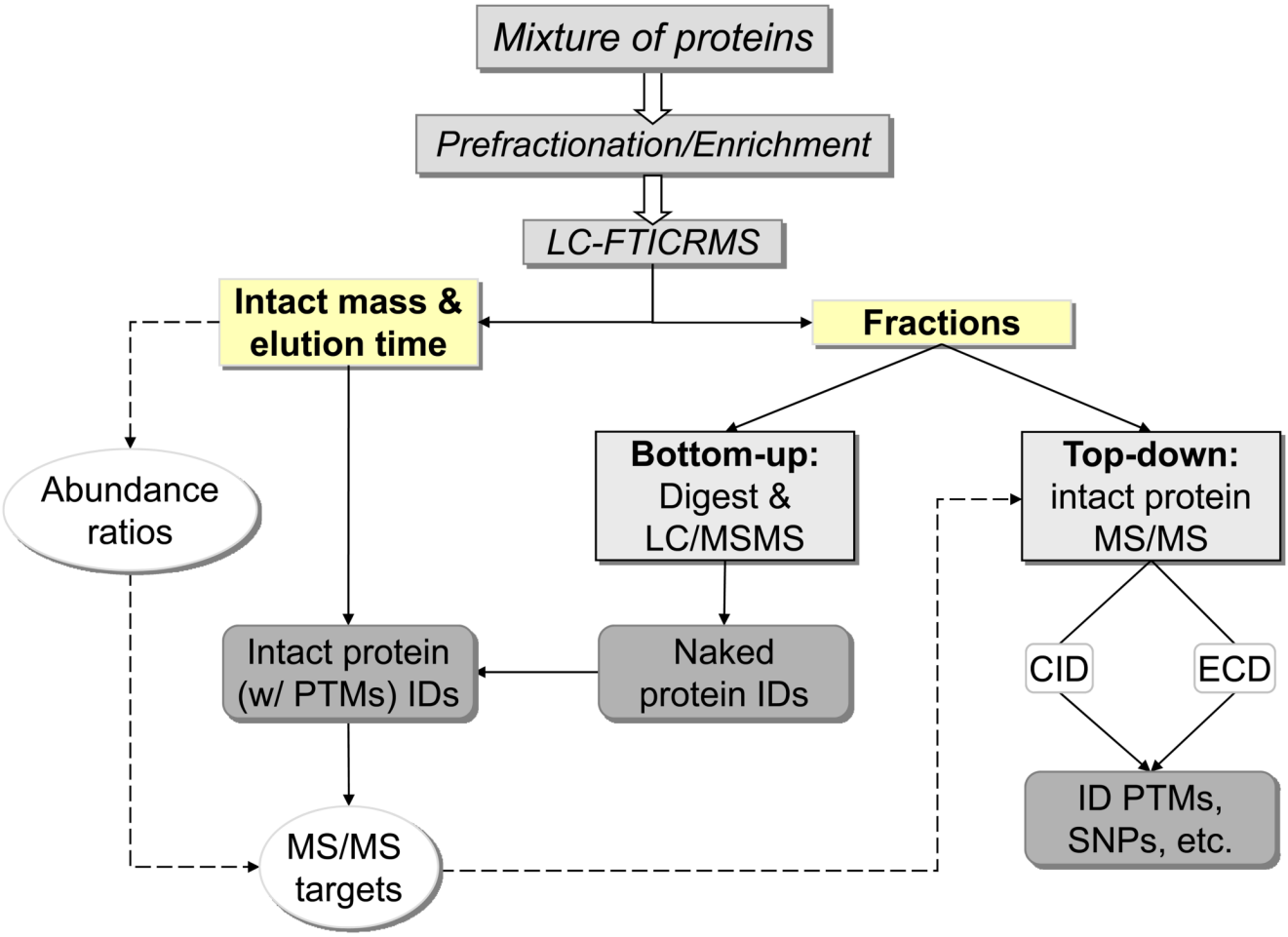
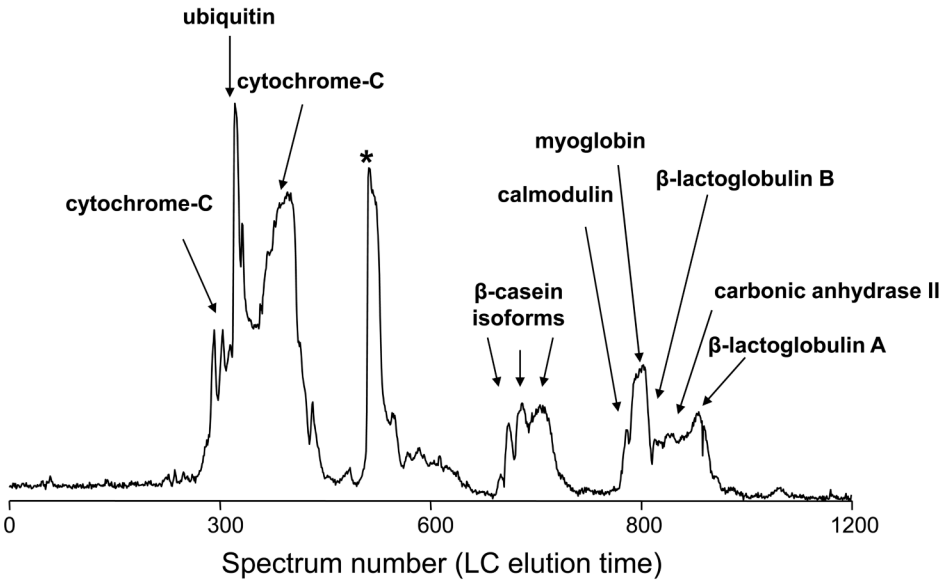


Figure 1.

Schematic of the integrated top-down and bottom-up strategy. Protein mixtures are fractionated to a level at which their reduced complexity allows the inference of proteins from peptide-level characterization of the same fraction. Accurate intact protein mass and time measurements facilitate protein abundance profiling that incorporates protein modifications, as well as high-throughput selection of biologically relevant targets for subsequent offline gas-phase fragmentation using only an aliquot of the original fraction used for bottom-up analysis.



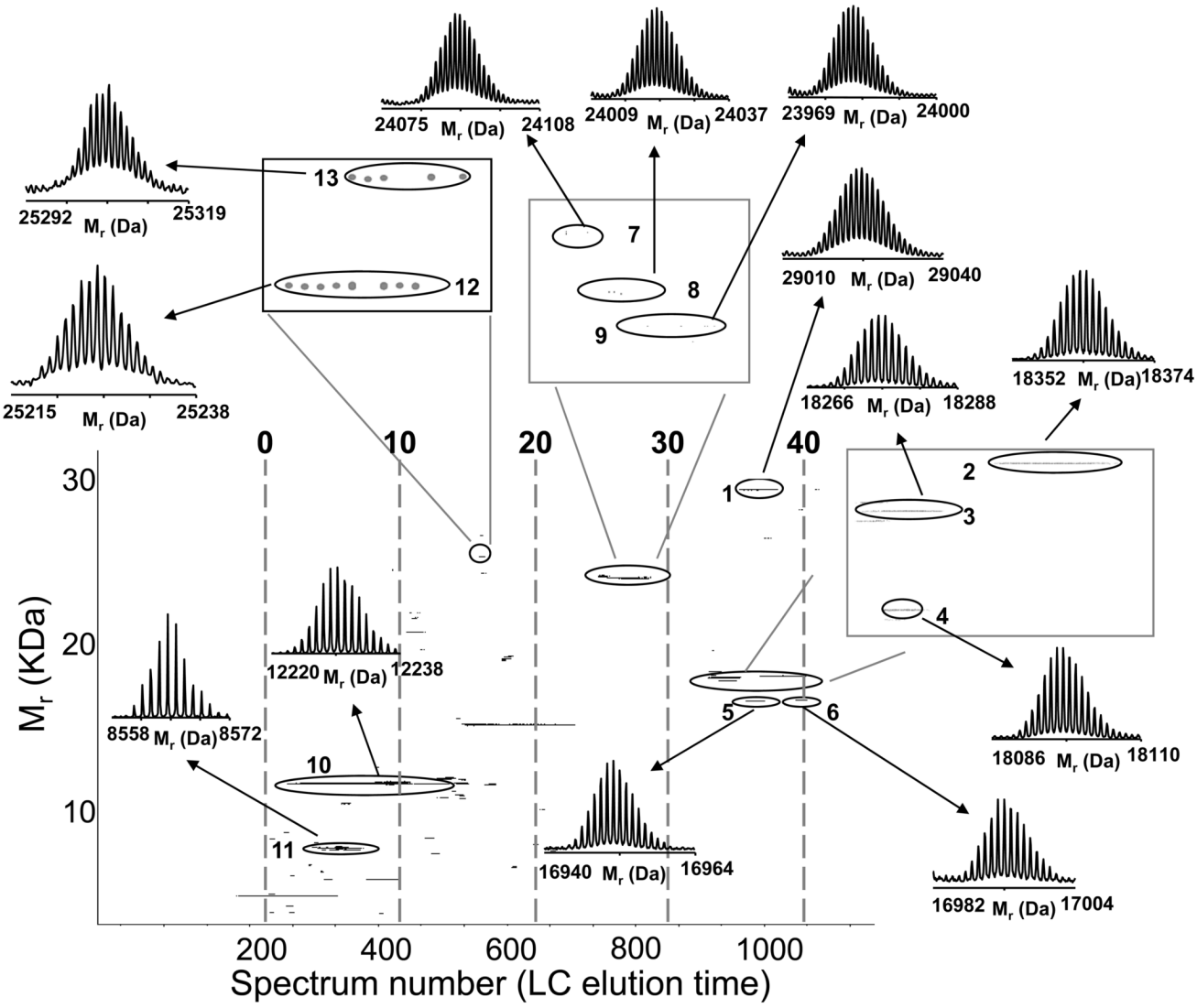


Figure 2.

Intact LC-MS analysis of a standard protein mixture: A) RPLC-FTICR MS total ion chromatogram and B) 2D display reconstructed from the LC-MS data. Proteins and modified proteins were tentatively identified (Table 2) by matching bottom-up data with the measured intact protein masses. Note that the peak labeled with * in A is assigned as a mixture of a putative myoglobin degradation fragments. The resolving power of the isotope distributions in this and Figures 5 and 9 is ~60,000. The acquired m/z data has a resolution of ~100,000. The major reduction in resolution occurs during the hyper-transform of m/z isotope isotopic distributions from multiple charge states into a single neutral charge isotope distribution.

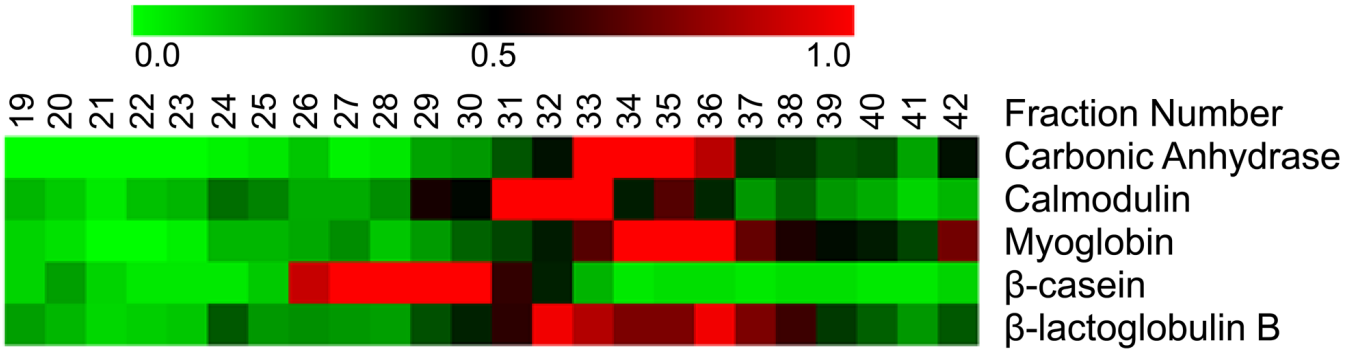
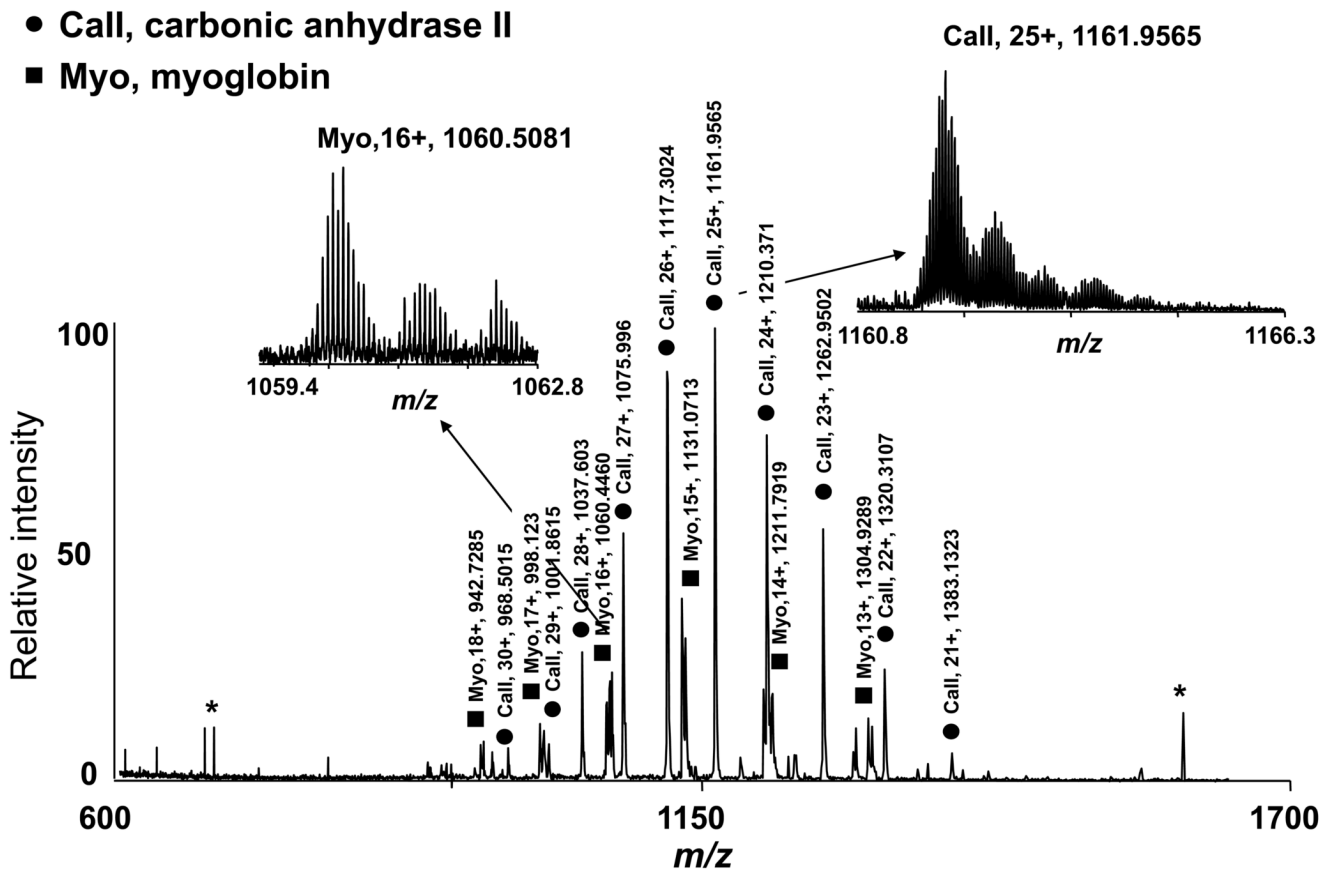


Figure 3.

Heat map representation of protein elution patterns generated for the later portion of the LC-MS analysis using tryptic peptides identified in each fraction. Observation counts of the peptides for each protein are used to derive relative protein abundances. The observation counts were normalized by dividing the value obtained for each protein with the sum of the values for the protein (row), with the scale ranging from 0 (i.e. least abundant, green) to 1 (i.e. most abundant, red). The columns in the heat map represent the RPLC fraction number.



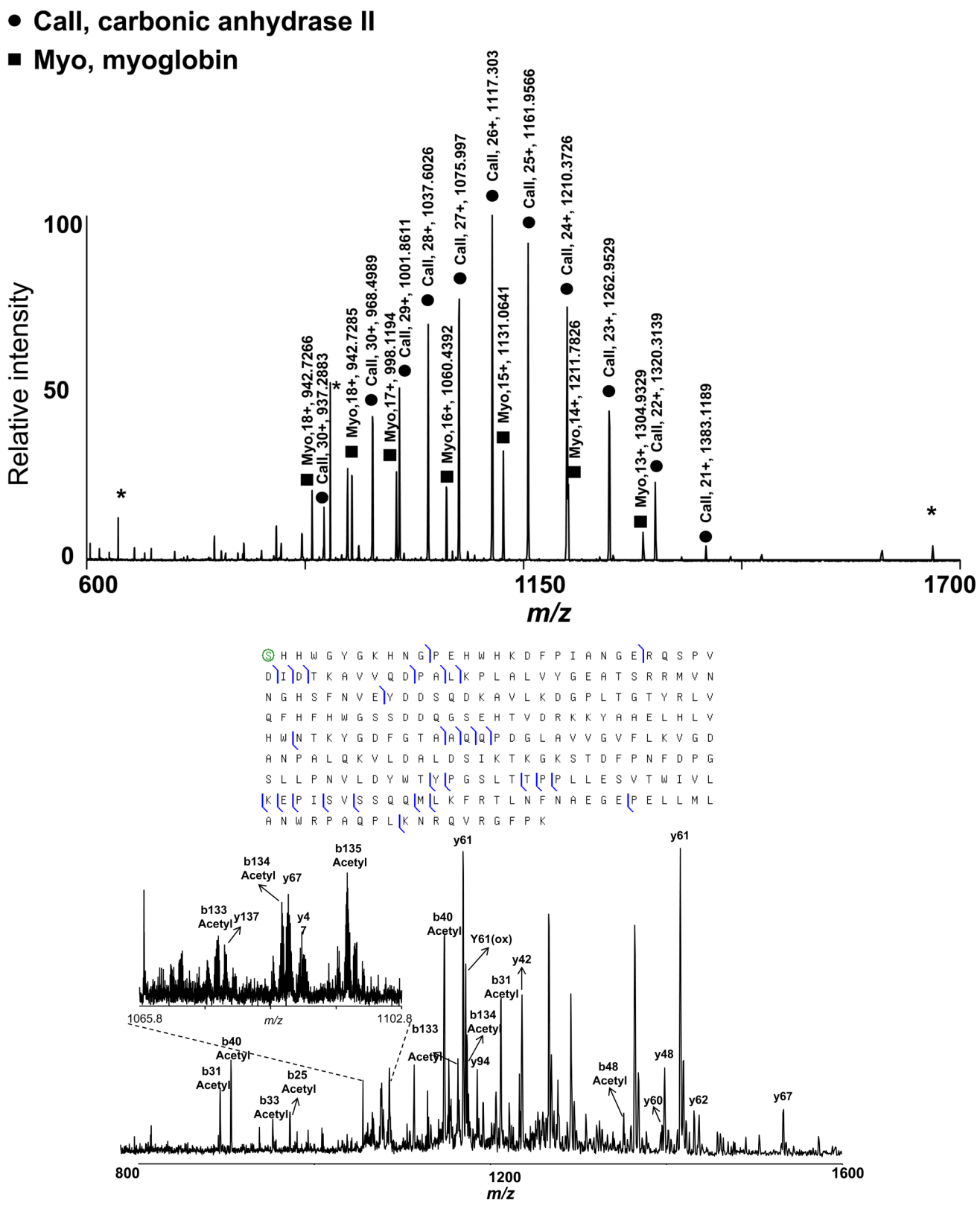
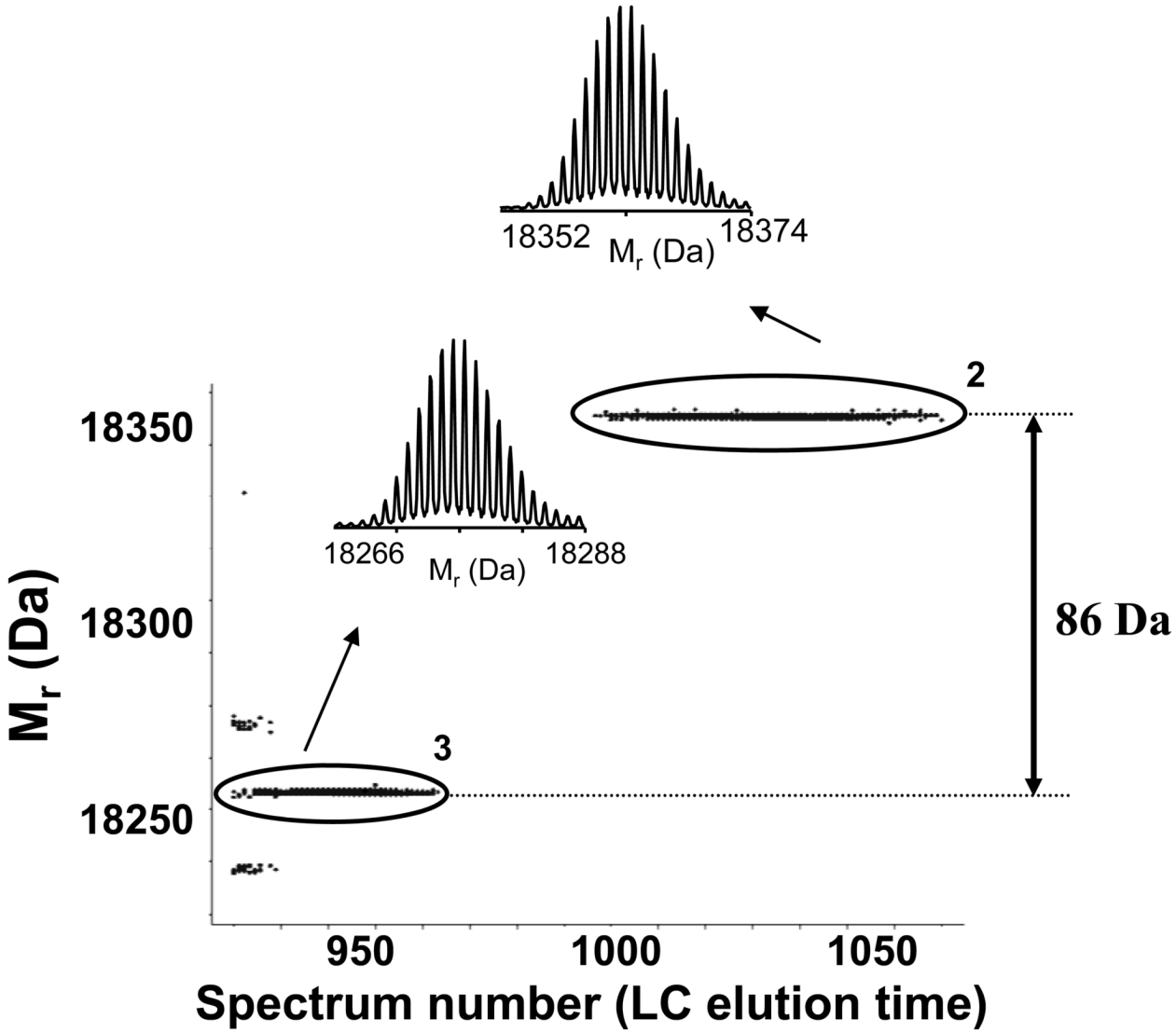


Figure 4.

Identification of carbonic anhydrase II in fraction 35. A) Mass spectrum obtained for reconstituted fraction 35; B) mass spectrum obtained by summing all of the spectra acquired online during the collection of the fraction 35; C) CID MS/MS data acquired for *m/z*=1161. (Note that the peaks labeled with * are noise peaks.)

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Lac-B    1 LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP EGDLEILLQK

Lac-A   61 WENDECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK KYLLFCMENS AEPEQSLVCQ
Lac-B   61 WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK KYLLFCMENS AEPEQSLACQ

Lac-A  121 CLVRTPEVDD EALEKFDKAL KALPMHIRLS FNPTQLEEQC HI
Lac-B  121 CLVRTPEVDD EALEKFDKAL KALPMHIRLS FNPTQLEEQC HI
```

Figure 5.

A) 2D display reconstructed from the intact LC-MS data over β-lactoglobulin B containing fractions according to its profile pattern from the bottom-up results. Insets show representative neutral mass spectra. B) Sequence alignment for β-lactoglobulin B and β–lactoglobulin A.

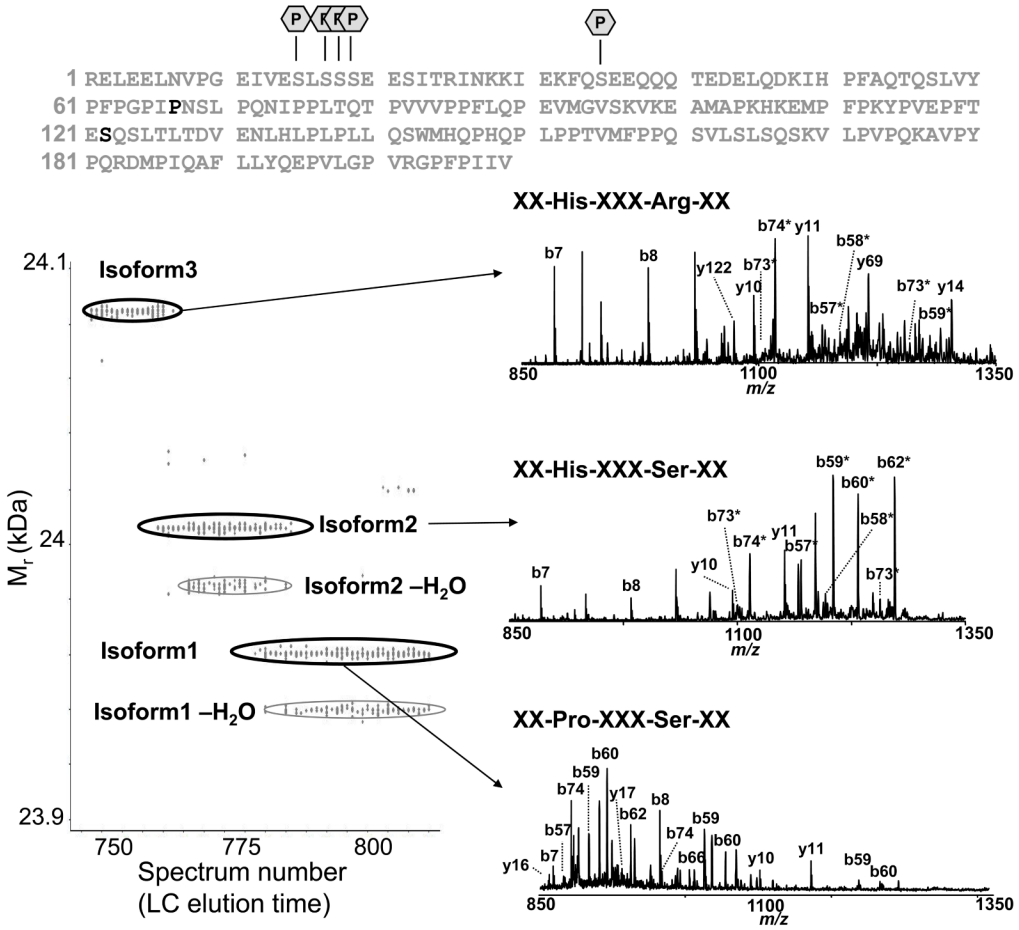

Figure 6.

Multiple SNPs for bovine β-casein were identified using the integrated approach. A) β-casein sequence with the annotations of the identified SNPs position. B) Three phosphorylated isoforms of β-casein, differing in mass by +40 Da and +109 Da, were assigned as β-casein genetic variant A2, A1, and B respectively. (Note that the annotations marked with * indicate a mass shift was observed for the identified peaks.)

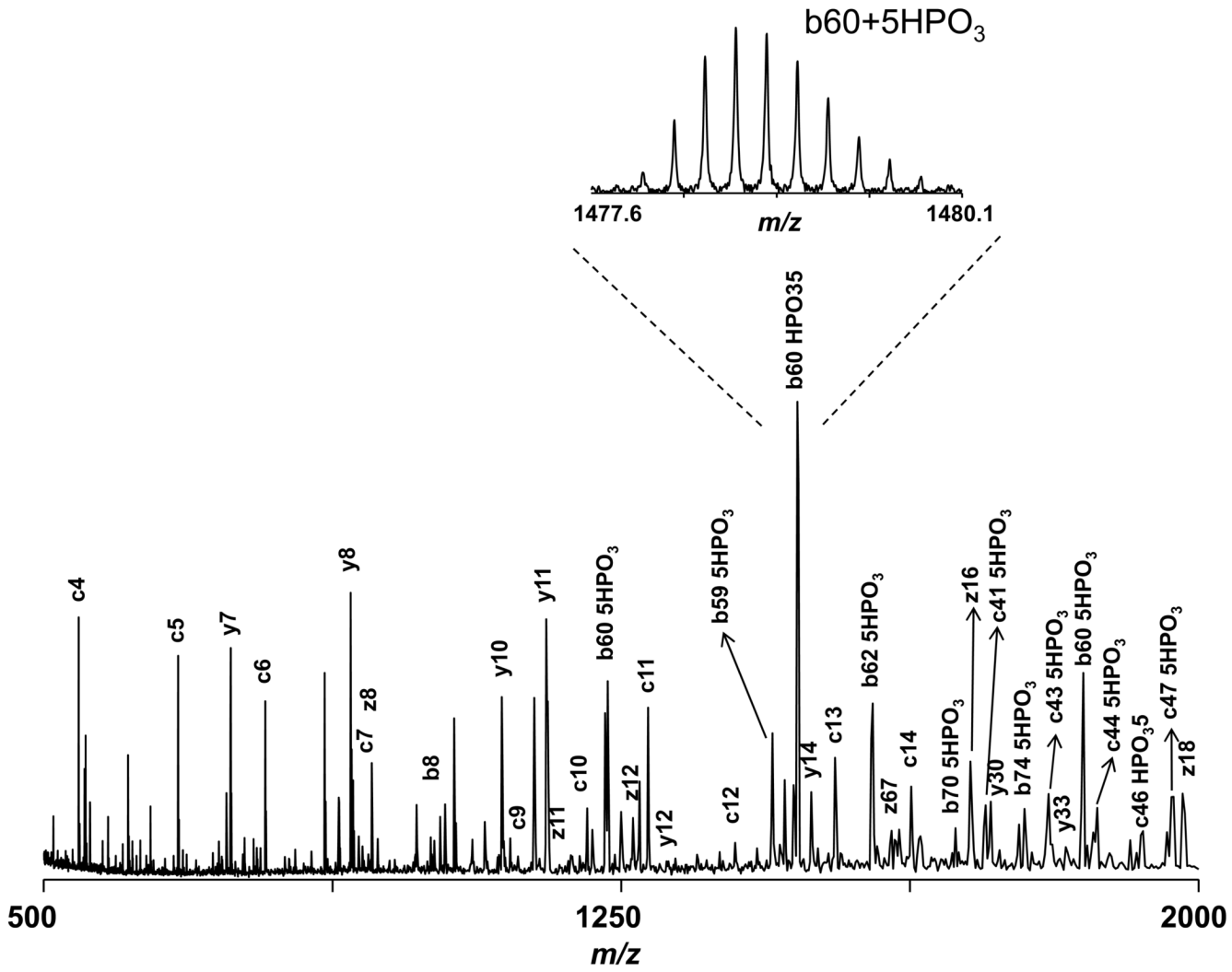

Figure 7.
ECD spectrum obtained for β-casein isoform 1 isolated in fraction 30 confirmed the identification as variant A2.

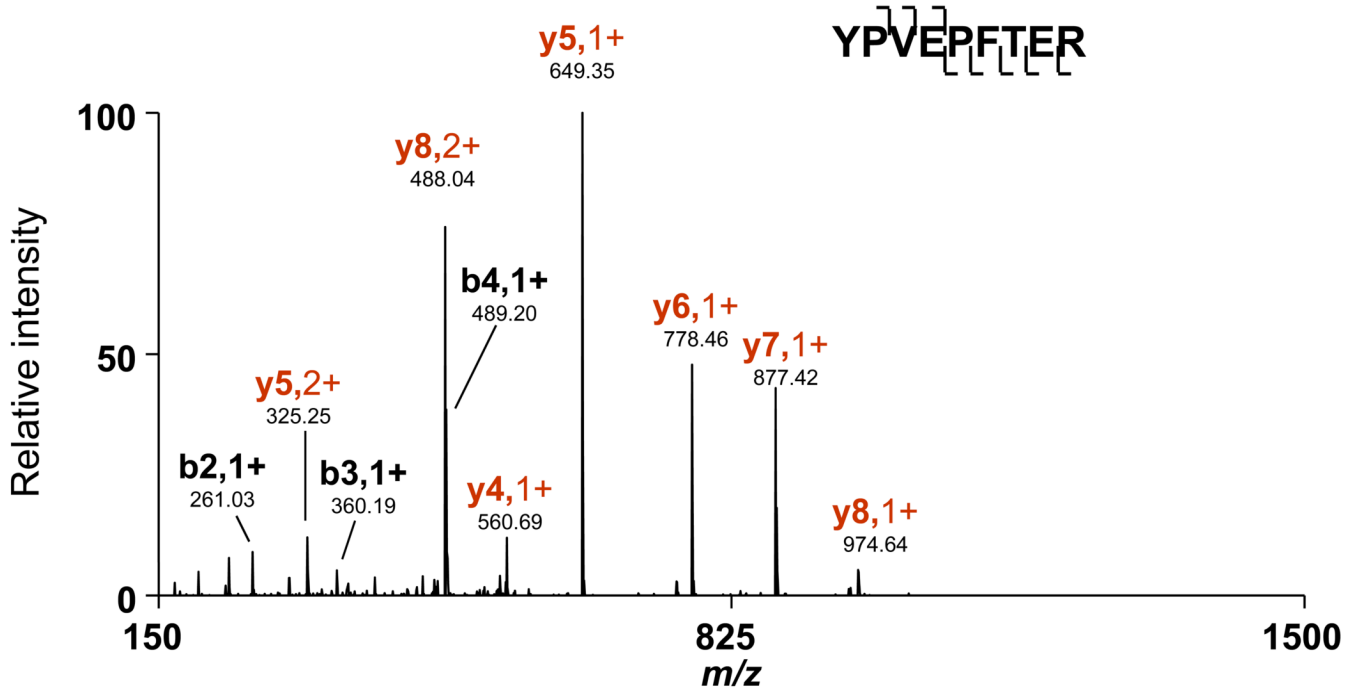

Figure 8.
Fully tryptic peptide YPVEFTER, identified in fraction 26, further confirmed the assignment of this species as β-casein variant B.

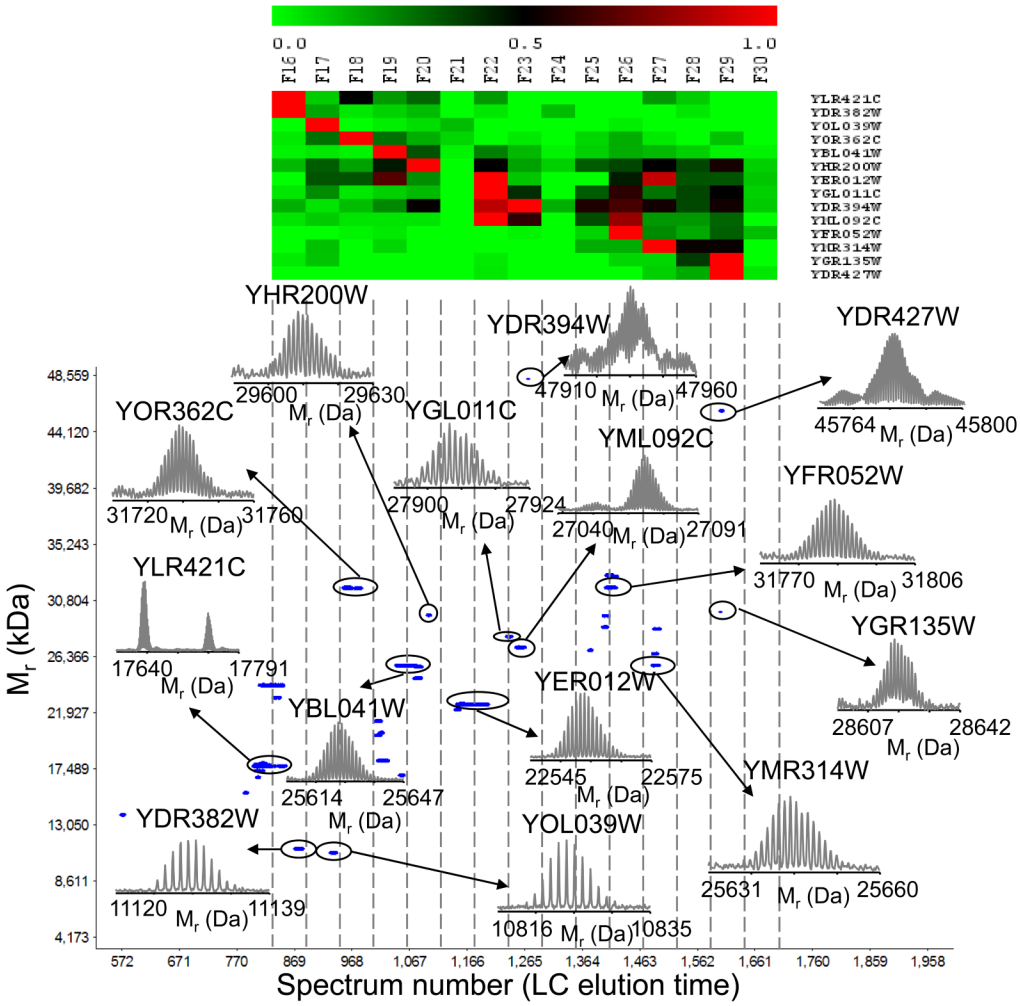

Figure 9.

Top: Heat map representation of protein elution patterns generated for the portion of the LC-MS analysis of yeast proteasome using tryptic peptides identified in each fraction. Relative protein abundances across fractions are based on normalized peptide observation counts (spectrum counting) and range from 0 (i.e. least abundant, green) to 1 (i.e. most abundant, red). The columns in the heat map represent the RPLC fraction number. Bottom: 2D display (mass vs. spectrum number or RPLC elution time) reconstructed from the intact protein LC-MS data. Insets show examples of proteins tentatively identified by matching bottom-up data with the accurate intact protein masses.

Table 1

Mixture of standard proteins used for proof-of-principle demonstration.

Protein	M_r (Da)	Conc. (pmol/μl)
ubiquitin	8564.6302	1.27
carbonic anhydrase II	29024.7312	4.51
β-lactoglobulinA	18363.4538	7.92
β-lactoglobulinB	18277.4169	7.96
calmodulin	18097.471	9.04
β-casein	23983.191	7.58
cytochrome C	12229.2193	11.89
myoglobin	16950.992	5.36

Table 2

Summary of results obtained for mixture of standard proteins using the integrated approach. Tentative identifications of proteins and modified proteins were accomplished by matching bottom-up data with the measured intact protein masses. Protein assignments (with exception of α-caseins) were subsequently confirmed by protein MS/MS analyses using collected fractions.

Spot in Fig 2B	Measured M_r (Da)	Theoretical M_r (Da)	Protein	Modifications	Eluted in Fractions	MMA (ppm)
1	29024.7313	29024.7312	carbonic anhydrase II	Removal of N-term Met, N-acetylation	[33,36]	0.00
2	18363.4561	18363.4538	β-lactoglobulin A	2 disulfide bonds	[35,37]	0.13
3	18276.4323	18276.4169	β-lactoglobulin B	2 disulfide bonds	[32,34]	0.84
4	18097.4866	18097.471	Calmodulin	with His-tag	[31.33]	0.86
5	16950.9857	16950.992	myoglobin	Intact protein	[34,36]	−0.37
6	16993.0104	16993.0025	myoglobin	Lys-acetylation	42	0.46
7	24092.3436	24092.2661	β-casein, variant B	5 phosphorylations, P67H(SNP) and S122R (SNP) to variant A2	26	3.22
8	24023.2204	24023.1971	β-casein, variant A1	5 phosphorylations, P67H(SNP) to variant A2	27	0.97
9	23983.2346	23983.191	β-casein, variant A2	5 phosphorylations	[28,30]	1.82
10	12229.2211	12229.2193	cytochrome C	Removal of N-term Met, N-acetylation, with Heme	[3,12]	0.15
11	8564.6471	8564.6302	ubiquitin	Intact protein	[4,7]	1.97
12	25226.9975	25227.0194	α-casein2	11 phosphorylations	14	0.87
13	25306.1167	25305.9875	α-casein2	12 phosphorylations	14	−5.18

Table 3

Summary of results obtained for yeast proteasome using the integrated approach. Tentative identifications of proteins and modified proteins were accomplished by matching bottom-up data with the measured intact protein masses.

ID	UniProt #	DESCRIPTION	Notes	M_{theo}	M_{exp}	MMA (ppm)
1	YLR421C	26S proteasome regulatory subunit RPN13	1 acetylation, 1 phsophorylation, loss of N-term 3 residues	17674.62	17674.58	−2.26
	YLR421C	26S proteasome regulatory subunit RPN13	1 acetylation, 2 phsophorylation, loss of N-term 3 residues	17754.58	17754.55	−1.69
2	YDR382W	60S acidic ribosomal protein P2β	1 phsophorylation	11129.36	11129.34	−1.80
3	YOL039W	60S acidic ribosomal protein P2α	1 phsophorylation	10825.22	10825.19	−2.77
4	YOR362C	Proteasome component C1	loss of N-term Met, Blocking of N-term Thr	N/A	31740.47	N/A
5	YBL041W	Proteasome component C5	loss of N-term 12 residues	25628.92	25628.91	−0.39
6	YHR200W	26S proteasome regulatory subunit RPN10	loss of N-term Met	29615.84	29615.77	−2.36
7	YER012W	Proteasome component C11	1 acetylation	22558.62	22558.60	−0.89
8	YGL011C	Proteasome component C7α	loss of N-term Met, 1 acetylation	27911.20	27911.16	−1.43
9	YDR394W	26S protease regulatory subunit 6B homolog	1 acetylation	47935.21	47935.19	−0.42
10	YML092C	Proteasome component Y7	loss of N-term Met, 1 acetylation	27072.08	27072.06	−0.74
11	YFR052W	26S proteasome regulatory subunit RPN12	loss of N-term Met	31787.19	31787.16	−0.94
12	YMR314W	Proteasome component PRE5	1 acetylation	25645.24	25645.24	0.00
13	YGR135W	Proteasome component Y13	loss of N-term Met, 1 acetylation	28624.59	28624.57	−0.70
14	YDR427W	26S proteasome regulatory subunit RPN9	No Modification	45782.85	45782.87	0.44