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Coumarin Tags for Analysis of Peptides by MALDI-TOF MS and MS/MS. 2. Alexa Fluor 350 Tag for Increased Peptide and Protein Identification by LC-MALDI-TOF/TOF MS

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The goal of this study was the development of N-terminal tags to improve peptide identification using high-throughput MALDI-TOF/TOF MS. Part 1 of the study was focused on the influence of derivatization on the intensities of MALDI-TOF MS signals of peptides. In part 2, various derivatization approaches for the improvement of peptide fragmentation efficiency in MALDI-TOF/TOF MS are explored. We demonstrate that permanent cation tags, while significantly improving signal intensity in the MS mode, lead to severe suppression of MS/MS fragmentation, making these tags unsuitable for high-throughput MALDI-TOF/TOF MS analysis. In the present work, it was found that labeling with Alexa Fluor 350, a coumarin tag containing a sulfo group, along with guanidation of ϵ-amino groups of Lys, could enhance unimolecular fragmentation of peptides with the formation of a high-intensity y-ion series, while the peptide intensities in the MS mode were not severely affected. LC-MALDI-TOF/TOF MS analysis of tryptic peptides from the SCX fractions of an *E. coli* lysate revealed improved peptide scores, a doubling of the total number of peptides, and a 30% increase in the number of proteins identified, as a result of labeling. Furthermore, by combining the data from native and labeled samples, confidence in correct identification was increased, as many proteins were identified by different peptides in the native and labeled data sets. Additionally, derivatization was found not to impair chromatographic behavior of peptides. All these factors suggest that labeling with Alexa Fluor 350 is a promising approach to the high-throughput LC-MALDI-TOF/TOF MS analysis of proteomic samples.

Various derivatization approaches to increase the information content from a sample, as well as for differential quantitation, are widely used in proteome studies.[1−3] In part 1 of the present study, the importance of derivatization agents to improve peptide signals in the MS mode of MALDI-TOF MS has been examined.[4] However, with constantly growing protein databases,[5,6] and with recent improvements in MS/MS instrumentation[7−9] and database searching algorithms,[10−12] confident peptide identification is now based on MS/MS fragmentation, in addition to exact molecular masses of peptides measured in the MS mode.[13−15] For this reason, new peptide derivatization reagents are designed, among other purposes, for improved MALDI MS/MS fragmentation.[16−20] The goal of the present study was to develop a derivatization approach that would enhance peptide identification in high-throughput LC-MALDI-TOF/TOF MS, while enhancing or maintaining signal intensity in the MS mode. In the future, isotopic labels could be incorporated in the developed tags, to allow differential quantitation of the proteins expressed in various cell states.

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Improvement in the fragmentation efficiency of peptides is of particular importance for MALDI-generated ions, because the predominant singly charged ions in MALDI generally fragment worse than doubly charged ions.[21] Compared to the MS/MS spectra of doubly charged ESI-generated ions, MS/MS spectra of singly charged MALDI ions contain ions from various fragment ion series,[22] resulting in the dissipation of MS/MS ion current and low S/N of individual fragment ion peaks.[23] This low S/N will cause poor MS/MS spectra, leading to low levels of identification and false positives.

Several derivatization approaches for improved fragmentation of singly charged ions have been presented.[2,16] The most commonly used derivatization agents have been cationic or strongly basic moieties, as they were reported to improve peptide detection sensitivity,[16,24] and to simplify high-energy collision-induced dissociation (CID) MS/MS and postsource decay (PSD)[25,26] spectra of peptides, leading to preferential formation of a- and d-ion series.[27,28]

Recently introduced MALDI-TOF/TOF mass spectrometers,[7,29] which employ both unimolecular (PSD) and high-energy CID fragmentation,[30] are well suited for high-throughput analysis of complex peptide mixtures. It has been demonstrated that MALDI-TOF/TOF CID spectra are similar to MALDI-CID and liquid secondary ion-CID spectra obtained earlier with tandem double focusing EBE-TOF mass spectrometers.[31] As a consequence, the cation tag labeling approach for the improvement of fragmentation efficiency of peptides was expected to be applicable for MALDI-TOF/TOF MS as well. On the other hand, there have been suggestions that the use of cationic tags for MALDI-TOF MS/MS may not be effective, but no detailed investigation has been published.[2,32]

Another derivatization approach that offers advantages for peptide analysis in both the MS and MS/MS modes is N-teminal labeling with weakly basic tags, which have high gas-phase proton affinity, e.g., pyridine, morpholine, and piperazine derivatives.[19,33,34] Labeling of peptides with *N*-succimidyl-3-morpholine acetate was shown to improve coverage of sequence-informative ions in MALDI-TOF/TOF MS spectra, in particular, b-ion series, which

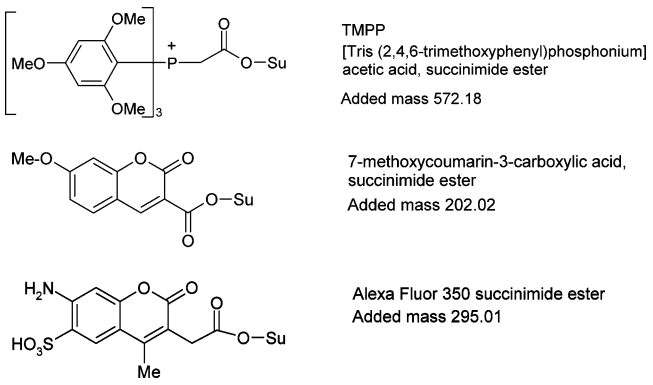

Figure 1. Structures of N-terminal peptide tags used in the study.

led to an increase in MASCOT scores and improved de novo sequencing analysis.[35,36] Recently our laboratory reported on a similar fragmentation pattern found for N-terminal coumarin tags.[37] Another advantage of the coumarin tags is that they enhance intensities of peptide signals in the MS mode via improved incorporation in hydrophobic MALDI matrixes during cocrystallization.[4]

Finally, N-terminal sulfonic acid derivatives were advanced recently for peptide sequencing by MALDI-TOF MS.[2,38−40] Despite a decrease in MALDI MS signal intensity in the positive ion mode, sulfonation was shown to be beneficial for MS/MS unimolecular fragmentation (PSD). This is due to the fact that the sulfo group facilitates MS/MS fragmentation of singly charged peptide ions by providing an additional "mobile" proton, the acidic proton from the sulfo group, which lowers amide bond strength, allowing facile unimolecular decay.[2,21] Up to now, N-terminal sulfonation has been primarily used in the analysis of in-gel digest samples and de novo sequencing. Only one attempt to use a sulfo tag, 4-sulfophenyl isothiocyanate (SPITC), with LC separation has been published, with MS/MS analysis conducted by ESI-ion trap MS.[41]

In this paper, part 2, following the previously published part 1,[4] we have utilized a combination of the coumarin structure with a sulfo group, commercially available Alexa Fluor 350 dye, as a new N-terminal derivatizing agent for LC-MALDI-TOF/TOF MS analysis (see Figure 1). Additionally, we guanidated ϵ-amino groups of lysine, to create a high proton affinity site at C-terminus, as well as protect lysines from being labeled by a second sulfo tag.[40] To demonstrate the advantages of the use of Alexa Fluor 350 labeling for the analysis of complex proteomic samples, a strong cation exchange (SCX) LC fraction of a tryptic digest of *Escherichia coli* lysate was divided into two parts; one was

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maintained native and the other modified by guanidation of lysines, followed by N-terminal labeling with Alexa Fluor 350. Both fractions were then analyzed in parallel by high-performance reversed-phase nanoLC-MALDI-TOF/TOF MS using a home-built multiplexed LC-MALDI deposition device.[42,43] All studies were performed using an AB 4700 MALDI TOF/TOF mass spectrometer in both PSD (unimolecular fragmentation) and CID modes. Significant improvements in the number and confidence of peptide and protein identifications as a result of labeling were found.

EXPERIMENTAL SECTION

Chemicals. All organic solvents, reagents, salts, standard peptides, proteins, and indicator paper strips were purchased from Sigma (St. Louis, MO). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Bruker Daltonics (Billerica, MA) and used without further purification. Coumarin dyes (*N*-hydroxysuccinimide esters), including Alexa Fluor 350, were purchased from Molecular Probes (Eugene, OR), and trypsin was from Promega (Madison, WI). An *E. coli* cell suspension was kindly provided by Prof. Kim Lewis (Department of Biology, Northeastern University). Novagen brand reagents for protein extraction were purchased from VWR Scientific (San Dimas, CA).

Synthesis of Tris(2,4,6-trimethoxyphenyl)phosphonium−acetic acid, *N*-Hydroxysuccinimide Ester (TMPP−Ac−OSu). A 25-mL dry round-bottom flask, equipped with an N_2 inlet, was filled with bromoacetic acid *N*-hydroxysuccinimde (100 mg), 4 mL of anhydrous toluene, and a 4-mL toluene solution of tris-(2,4,6-trimethoxyphenyl)phosphine (216.8 mg), and the resulting reaction mixture was stirred for 1/2 h. The solid product was collected via filtration, dried under vacuum and used without further purification.

Preparation of a Cell Lysate Digest. A protein extract of an *E. coli* cell lysate was prepared according to a protocol described by Novagen (EMD Biosciences, Madison, WI). Briefly, a wet cell pellet of *E. coli* (300 μg) was resuspended in BugBuster protein extraction reagent (5 mL of the reagent/g of wet cell paste) at room temperature. Additionally, for the same amount of wet cell paste, two enzymes were added: 1 μL (25 units) of Benzonase Nuclease (Novagen, EMD Biosciences) for DNA and RNA cleavage and 1000 units of rLysozyme (Novagen, EMD Biosciences) for efficient lysis of *E. coli* cell walls. The suspension was incubated on a shaking platform for 10−20 min at room temperature. Insoluble cell debris was removed by centrifugation at 16000*g* for 20 min at 4 °C. The protein concentration was estimated by the Bradford assay. Next, the protein extract was cleaned from the lysis detergents by acetone precipitation, specifically, treatment with 90% acetone at −80 °C for 15 min, followed by centrifugation at 14000*g* for 15 min. The precipitated protein was collected, reconstituted in 0.1 M triethylammonium bicarbonate (TEA−HCO_3) buffer, pH 8.5, denatured with 6 M guanidine hydrochloride, and reduced with 5 mM dithiothreitol (DTT). The protein extract was then diluted with TEA−HCO_3 buffer to a final guanidine concentration of less than 0.5 M, followed by digestion with trypsin at 37 °C overnight. The resulting digest was concentrated and cleaned with 1-mL Bond Elut C_{18} cartridges (Varian, Palo-Alto, CA).

Strong Cation Exchange Fractionation. SCX was conducted on a Series II 1090 liquid chromatograph (Agilent Technologies, Palo Alto, CA). *E. coli* lysate digest sample (500 μL, ∼1 mg of total protein) was injected into a polysulfoethyl A column (2.1 mm × 15 cm, PolyLC, Columbia, MD) and separated with a 1-h linear gradient at a flow rate of 200 μL/min. The composition of solvent A was 25% (v/v) ACN, 10 mM phosphate buffer at pH 3.0, and solvent B was 25% (v/v) ACN, 10 mM phosphate buffer at pH 3.0 with 350 mM KCl. The separation was monitored using a Waters 486 UV detector (Waters Corp., Milford, MA). Fractions of ion exchange eluent, collected in 6-min time intervals (1200 μL each), were concentrated by a SpeedVac (Thermo Savant, Holbrook, NY) to a final volume of 100 μL and frozen at −80 °C. Several SCX fractions were selected for labeling and analysis by reversed-phase LC-MALDI-TOF and TOF/TOF MS.

Guanidation of ε-Amino Groups of Lysine Residues. The guanidation reaction was performed with a slight modification of a protocol described elsewhere.[40] Fresh solution of *O*-methylisourea hemisulfate, 13.2 mg in 50 μL of water, was prepared immediately before the reaction. A 10-μL aliquot of this solution was added to 10−20 μL of an aqueous tryptic digest of a standard protein (10^{-4}−10^{-6} M) or a fraction of *E. coli* cell lysate digest after SCX. Roughly 5 μL of pure triethylamine (TEA) was added to raise the pH to 10.5−11.0 (as measured by indicator paper strips). After mixing, the samples were placed in a heater (Heat Block, VWR Scientific, San Dimas, CA) at 65 °C for 10 min. Typically, 100% labeling efficiency for amino groups of lysines was achieved under these conditions, provided that the reaction was maintained at pH 10.5−11.0. In some cases, especially for peptides with N-terminal glycine, N-termini partially reacted (producing an MS signal up to 30% of the intensity of the main product peak). This side reaction did not cause problems in analysis of the other portion of the peptide that was not N-terminally blocked, as it could subsequently be labeled with Alexa Fluor 350.

N-Terminal Labeling of Peptides. Labeling of peptides was performed with a minor modification of the manufacturer's protocol.[44] A 2-μL aliquot of buffer concentrate (1 M triethylammonium bicarbonate, pH 8.3) was added to 10 μL of the guanidated tryptic digest of a standard protein (10^{-4}−10^{-6} M) or cell lysate digest. The tagging solution was prepared immediately before labeling: 20 μg of the tag (*N*-hydroxysuccinimide ester) was dissolved in 100 μL of 50% (v/v) solution of ethanol in water, and 5 μL of the given solution was added to the digest. The reaction was carried out at room temperature for 30 min, and then a 5-μL aliquot of a fresh tag solution was added every 30 min, for a total of 4−6 times within 2−3 h. The reaction products were purified and analyzed by MALDI-TOF MS. The labeling was found to be complete for the majority of the peptides for the digests of standard proteins.

Sample Cleanup. Sample cleanup was required, both after the guanidation reaction for efficient N-terminal labeling and after N-terminal labeling, before nanoLC-MALDI TOF MS. In each case, the cleanup was performed with Zip-Tip C_{18} (Millipore, Bedford,

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MA) on 1−2 μg of total peptide mass, or with Ultra MicroSpin C_{18} (The Nest Group, Southborough, MA) for sample amounts up to 100 μg, according to the manufacturers' protocols.

Instrumentation and Methods. Mass spectra were acquired on an Applied Biosystems 4700 TOF/TOF Proteomics analyzer (Framingham, MA), equipped with delayed extraction and a 200-Hz repetition rate UV laser (355 nm). The instrument was controlled by the AB 4700 Data Explorer software, version 2.0. The mass resolution on the AB 4700, operating in the positive mode, was at least 10 000 (fwhm) for all peptide peaks. Typically, 600 shots/spectrum were accumulated in the MS mode and 1000 shots/spectrum in the MS/MS mode. When CID mode was used for peptide fragmentation, air was the collision gas, and the CID chamber pressure was in the range $(1-6) \times 10^{-6}$ Torr, with accelerations of 1 and 2 kV. In the PSD mode, ion acceleration of 1 kV was used. Standard 192-well stainless steel MALDI sample plates were used in this study. An internal standard was used for mass calibration, and the Plate Model calibration option, allowed by the instrument software, was used to alleviate spatial variations of the mass accuracy across the MALDI plate. For the dried droplet samples, the CHCA matrix was prepared in acetonitrile/water (60:40, v/v) containing 0.1% trifluoroacetic acid (TFA), at a concentration of 5 mg/mL. For the continuous deposition, 7 mg/mL CHCA solution in 50:50 (v/v) ACN/water, 0.1% (v/v) TFA, was used.

NanoLC-MALDI System. A multiplexed four-channel HPLC system was coupled off-line to MALDI-MS using a laboratory-built deposition interface, as described elsewhere.[43] Each separation channel consisted of an individual manual injection valve with a 2-mL sample loop and a 15 cm × 100 μm i.d. analytical column (packed with Magic C18 phase (Alltech, Nicholasville, KY), particle size 5 μm, pore size 100 Å). The mobile phases were delivered by an UltiMate system (Dionex, Sunnyvale, CA) and then split equally into four separation channels. The samples were directly injected into the analytical columns and then simultaneously separated by a 50-min linear gradient at a flow rate of 1 μL/min in each of the four channels. The composition of solvent A was 2% (v/v) ACN, 0.1% (v/v) TFA, and that of solvent B was 85% (v/v) ACN, 5% (v/v) 2-propanol, 0.1% (v/v) TFA. The eluents from individual LC columns were then subsequently mixed in Micro-Tees (Upchurch, Oak Harbor, WA) with MALDI matrix solution (7 mg/mL CHCA, 0.1% (v/v) TFA in 50% (v/v) ACN/water) at a 3:2 flow rate ratio and simultaneously deposited onto MALDI plates in the form of spots. Each spot consisted of 5 s of chromatographic time. A standard 2 in. × 2 in. MALDI plate could accommodate four 7 × 28 arrays of spots, representing 16 min of separation for each channel. During deposition, the interface was controlled by a LabVIEW program (National Instruments, Austin, TX). An internal standard, Glu-fibrinopeptide B, m/z 1570.6774, was used for mass calibration.

Data Acquisition and Processing. MASCOT server version 2.0 (Matrix Science, London, U.K.) was used for peptide and protein identifications. The data for MS/MS ion fragment and ion current distribution were extracted from MASCOT data using an in-house-written PERL program. For an SCX fraction of the *E. coli* digest, after completion of the data acquisition in the MS mode, the spectra were processed for peak picking by internally developed algorithms, MEND[45] and PRESEL,[46] to generate the list of precursor ions for MS/MS analysis. The MS peak height threshold was set at 200 counts, resulting, for each sample, in a list of ~6000 precursors submitted for MS/MS. The MS/MS data were submitted to the MASCOT server for database searching. The searches were performed against a SwissProt database. For taxonomy, "*Escherichia coli*" was specified for the β-galactosidase and *E. coli* fraction analysis, and "mammals" for the standard proteins. To analyze Alexa Fluor 350-labeled samples, N-terminal "Alexa" modification (monoisotopic mass increment 296.0228), was added to the default list of modifications, while guanidation is a standard option in MASCOT. To decrease the number of false positives, the probability of random hits (p) was set <0.01, meaning 99% confidence in the correct peptide identification. The peptide mass tolerance was set at ±50 ppm, and the fragment mass tolerance at ±0.15 Da. Trypsin was specified as the proteolytic enzyme, and the maximum of two missed cleavages was allowed. For native samples, methionine oxidation was set as a variable modification; for tagged samples, two additional modifications were added: lysine guanidation as permanent and N-terminal Alexa as variable. Under these conditions, the MASCOT significance threshold was 26 for the *E. coli* peptides. The "MUDPIT" scoring option in MASCOT was chosen,[47] ion score cutoff was set at 20, and only peptides with rank 1 were considered.

RESULTS AND DISCUSSION

The goal of the present study was to develop a derivatization approach that would enhance peptide identification in high-throughput MALDI-TOF and TOF/TOF MS. In part 1,[4] we focused on the improvement in MALDI-TOF MS signal, which was achieved through the use of commercially available N-terminal coumarin tags. In this study, several approaches for directed MS/MS fragmentation in TOF/TOF MS, with both high-energy CID and unimolecular (PSD) fragmentation, were explored. The advantage of the Alexa Fluor 350 tag for improved MS and MS/MS analysis was examined in depth in the analysis of an SCX fraction of an *E. coli* cell lysate.

Cationic Tag Derivatization. As noted in the introduction, N-terminal labeling of peptides with cationic tags leads to directed fragmentation and formation of a- and d-ion series in tandem double-sector MS/MS instruments, where CID occurs under high-energy conditions.[27,28] In directed fragmentation, peptides fragment in a predictable way, forming preferentially one (or more) nearly complete series of fragment ions. It has been observed that modern MALDI-TOF/TOF MS instruments are able to generate high-energy CID fragmentation conditions,[7,29] with ion acceleration up to 3 keV and center-of-mass energy deposition up to 255 eV,[30] comparable to tandem double-sector instruments.[48] Therefore, the use of cationic tags for MALDI-TOF/TOF MS analysis in the CID mode was examined.

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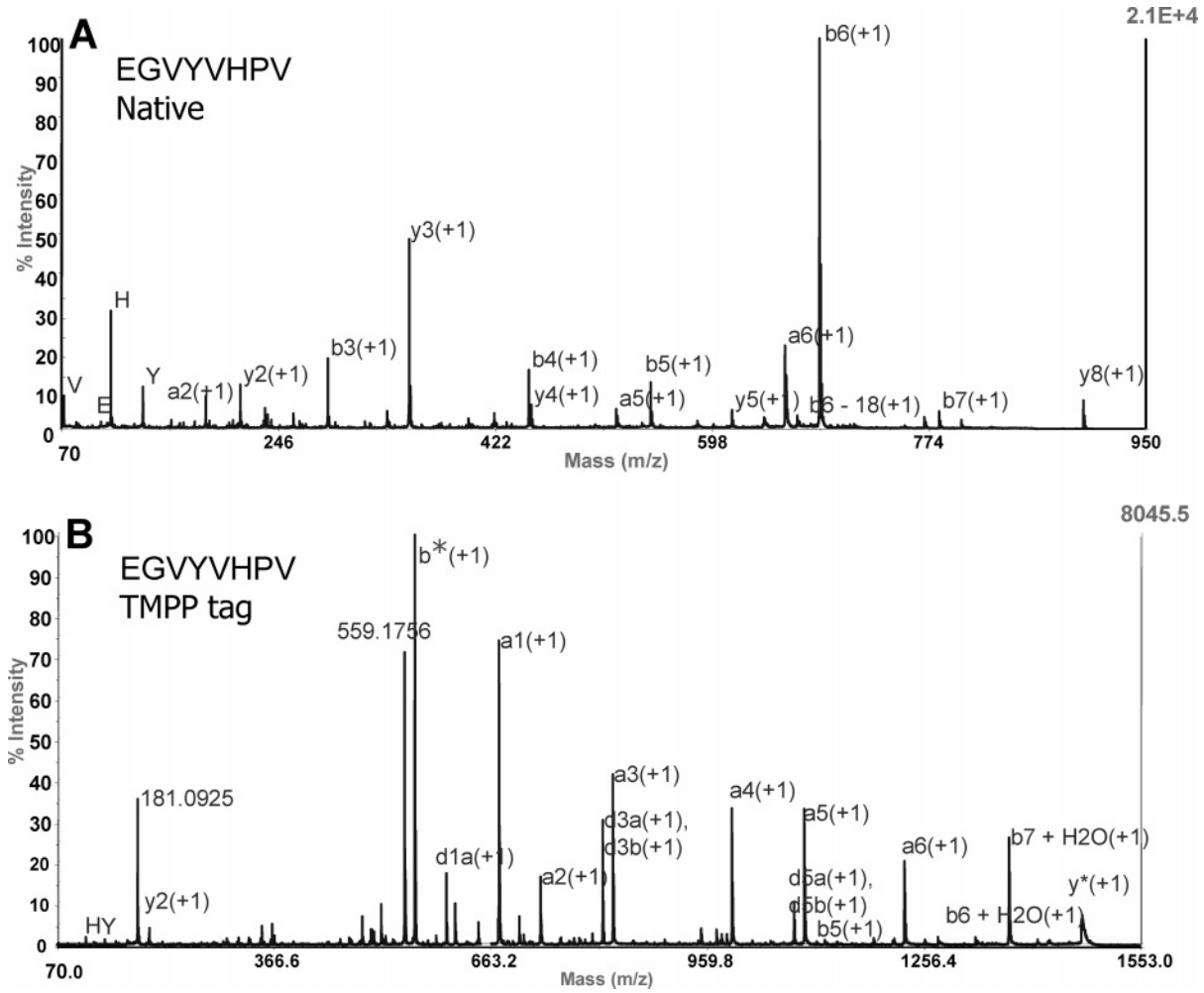

Figure 2. Comparison of N-terminal cationic derivative vs native peptide for a peptide that does not contain Arg: MALDI-TOF/TOF CID mass spectrum of a peptide EGVYVHPV, 1 pmol. MS/MS conditions are described in the Experimental Section. (A) Native peptide, *m/z* 899.4627; (B) N-terminally labeled peptide with a cationic phosphonium tag (TMPP), *m/z* 1471.6438. b* corresponds to loss of the N-terminal tag.

Standard peptides and protein digests were labeled with various ammonium and phosphonium cation tags and analyzed by MALDI-TOF/TOF MS under CID conditions. Air was the collision gas, and the CID chamber pressure was in the range $(1-6) \times 10^{-6}$ Torr, with accelerations of 1 and 2 kV. Under these conditions, the center-of-mass energy deposited in peptides was calculated to be in the range 10−40 eV.

Typical results are demonstrated in Figures 2 and 3, showing MALDI-TOF/TOF CID mass spectra of native and N-terminally labeled peptides with the tris(2,4,6-trimethoxyphenyl) phosphonium acetyl (TMPP−Ac) tag. The spectra of native peptides, shown in Figures 2 and 3A, contain incomplete series of various fragment ions, both C-terminal (e.g., *y*-ions) and N-terminal (a- and b-ions), with various intensities. When labeled with a cationic tag, directed fragmentation leading to a preferential formation of an a-series was observed, if the peptide did not contain arginine (Figure 2 B, EGVYVHP). On the other hand, if a C-terminal Arg residue was present in the sequence (Figure 3B, EGVNDNEEGFFSAR), no selectively directed fragmentation was observed, and the spectra of the labeled peptides contained both N-terminal a-, b-ions, and C-terminal y-ions. Importantly, as can be seen from Figure 3B, the MS/MS spectra typically showed considerably lower overall intensity, as well as a decreased number and intensity of structurally important fragments, compared to the spectra of native peptides (Figure 3A). Similar results were observed for several other studied cationic tags (data not shown). For all cationic tags, fragments, corresponding to the complete loss of the tag (or its fragment), were the most intense peaks in the MS/MS spectra of labeled peptides (b* ions in Figures 2 and 3 B).

For MALDI-TOF/TOF MS in the PSD mode (i.e., without a collision gas), it is known that $[M + H]^+$ ions carry less internal energy than in the CID mode,[22] and the spectra contain a reduced number of low-mass, high-energy fragment ions.[30] From our experience, for database searching, the PSD mode typically provides better MS/MS spectral quality than CID. This result is due to the fact that the fragment ion current in the PSD mode is distributed mainly over backbone b- and y-ion series, leading to a higher S/N of ions in the spectrum (see Figure 4, discussed later). Fragmentation of the cationic tag labeled peptides under MALDI-TOF/TOF PSD conditions has also been explored, and results similar to the CID mode were obtained (data not shown).

From all these results, it can be seen that labeling with cationic tags causes suppression of MS/MS fragmentation under MALDI-TOF/TOF MS conditions, both in the CID and PSD modes. A likely explanation is that the addition of a cationic moiety increases the gas-phase stability of the peptide ions, thus making such ions

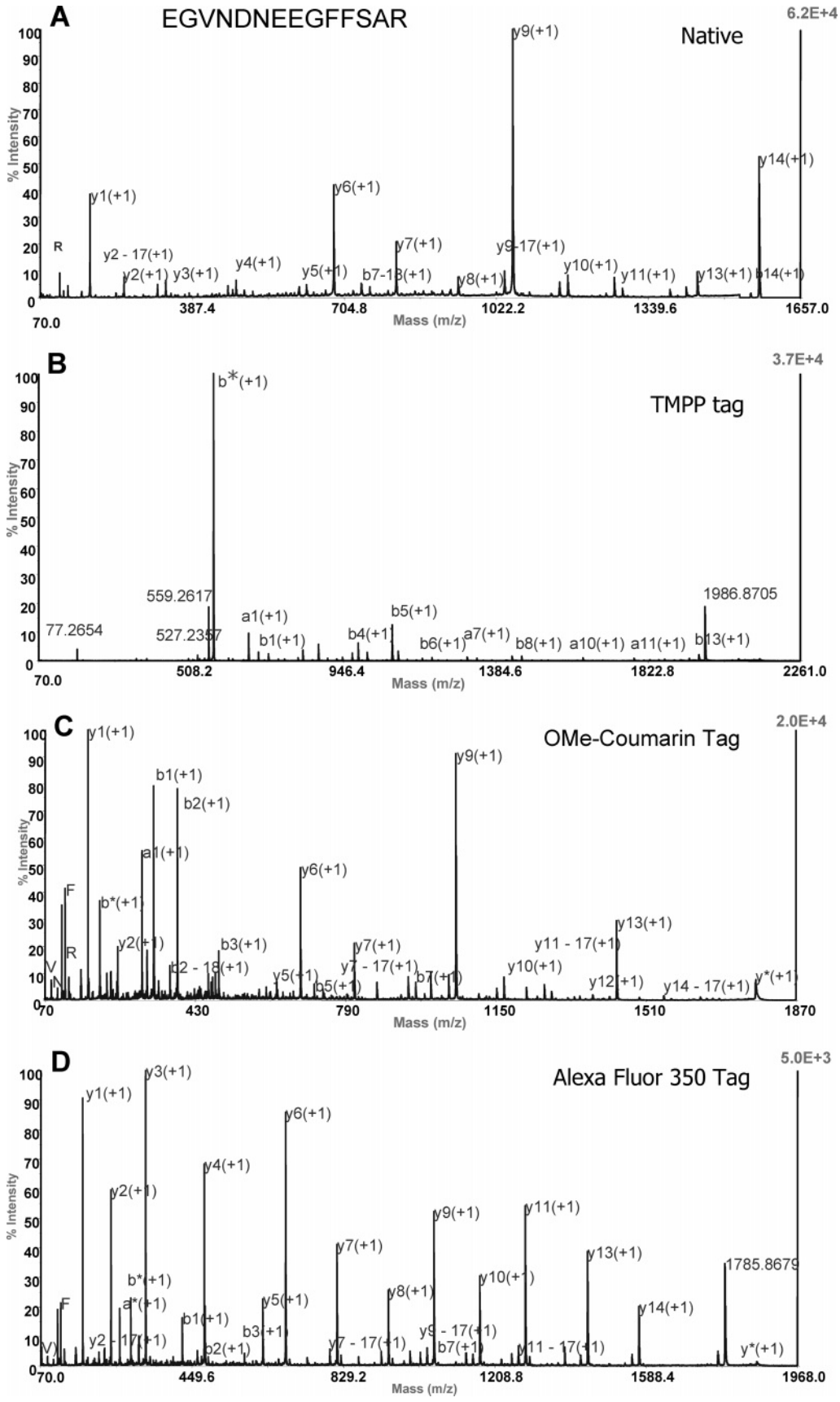

Figure 3. Comparison of N-terminal derivatives vs native peptide for a peptide that contains Arg: MALDI-TOF/TOF CID mass spectrum of a tryptic peptide EGVNDNEEGFFSAR (Glu-fibrinopeptide B), 1 pmol. MS/MS conditions are the same as in Figure 2. (A) Native peptide, *m*/*z* 1570.6774; (B) the same peptide N-terminally labeled with the cationic tag (TMPP), *m*/*z* 2142.8585; (C) N-terminally labeled with OMe-coumarin, *m*/*z* 1772.7037; D. N-terminally labeled with Alexa Fluor 350, *m*/*z* 1865.6922. Fragments labeled with an asterisk: a*, b*, and y*, correspond to loss of the N-terminal tag.

more difficult to fragment. According to the "mobile proton" theory of MS/MS fragmentation,[21] a proton, added to a peptide during ionization, plays a major role in the backbone fragmentation process. Sites with high gas-phase basicities, such as the guanidine group of Arg, retain the proton, thus limiting formation of sequence-specific product ions. In contrast to the usual $[M + H]^+$ peptide ions, the $[M]^+$ peptide ion, which is formed with cationic tag labeled peptides, does not contain a mobile proton, and fragmentation then follows an alternative pathway, i.e., a charge-remote mechanism.[48,49] It is likely that MALDI-TOF/TOF MS, in contrast to the tandem double-sector MS, does not provide sufficient energy to support this fragmentation pathway. Therefore, it can be concluded that cationic tags are not suitable as derivatizing agents for high-throughput MALDI-TOF/TOF MS analysis.

Coumarin Tags. In part 1 of this study, it was shown that N-terminal coumarin tags are beneficial for peptide analysis in the MS mode, as they enhance the intensities of signals of hydrophilic peptides.[4] Since, as noted above, moieties with high gas-phase basicity do not favor MS/MS fragmentation,[21] an alternative approach for improvement of both MALDI-TOF MS and MS/MS of peptides was explored, and small hydrophobic coumarin tags were tested in the MS/MS mode.

The same peptides and protein digests, as used above for cationic tags, were labeled with the following coumarin tags: 7-methoxycoumarin, 7-hydroxycoumarin, Marina Blue, and Pacific Blue (see part 1 of this work[4] for structures) and analyzed by MALDI-TOF/TOF MS under both CID and PSD conditions. The MALDI-TOF/TOF CID spectrum of the same peptide Glu-fibrinopeptide B (EGVNDNEEGFFSAR), labeled with the OMe-coumarin tag, is shown in Figure 3C. Compared to the MS/MS spectrum of the native peptide (Figure 3A), the spectrum of the tagged peptide reveals increased intensities of b-ions, in particular, b_1-b_3. A common feature of coumarin-tag directed fragmentation is the appearance of a_1 and b_1 ions. The intensities of these ions were enhanced in the CID mode, compared to PSD. b_1 ions are not present in the MS/MS spectra of native peptides, but can be observed in the spectra of the N-terminal derivatives formed via the amide bond, R—C(=O)—(NH-peptide).[19,35,36] The explanation for this result is that the carbonyl group of the tag participates in the formation of the five-member oxazolone ring, typical for b_n-ions ($n \geq 2$).[50] Analysis of MS/MS fragment ion distribution revealed that, despite some improvement in the intensity of the b-ion series, labeling did not result in a significant increase in the number of b-ions, and MASCOT scores of identified peptides were not improved (data not shown). Therefore, while useful for peptide mass fingerprinting (PMF), coumarin tags 7-methoxycoumarin, 7-hydroxycoumarin, Marina Blue, and Pacific Blue were not selected for high-throughput LC-MALDI-TOF/TOF MS analysis of complex proteomic samples.

Labeling with the Coumarin Tag, Alexa Fluor 350. The use of N-terminal tags containing a sulfo group for improved de novo peptide sequencing by MALDI-TOF PSD MS was pioneered by Keogh and colleagues and recently commercialized as the chemically assisted fragmentation (CAF) reagent kit.[2,17,39,40] Later, N-terminal labeling with a sulfo tag, SPITC, was suggested.[51–53] To obtain a ladder of y-ions for tryptic peptides, lysine-terminated peptides required guanidation, both to prevent double labeling and to create a high proton affinity site on the C-terminus. In part 1,[4] a sulfo group containing coumarin tag, Alexa Fluor 350, was found to provide better MALDI-TOF MS signals of peptides compared to the CAF reagent.[17] In the present work, a series of peptides, labeled with Alexa Fluor 350 were examined by MALDI-TOF/TOF MS. Figure 3D is the MALDI-TOF/TOF mass spectrum in the CID mode of the same Glu-fibrinopeptide B, N-terminally labeled with the Alexa Fluor 350 tag. It can be seen that, in the MS/MS mode, peptides labeled with Alexa Fluor 350 showed extensive y-series fragmentation, typical for peptides that have been N-terminally labeled with sulfo group-containing tags.[38–40,51–53] Moreover, the intensities of all y-ions were uniformly high, which is advantageous for the identification of the peptides over a wide concentration range (see below).

To explore in more detail the advantages of Alexa Fluor 350 labeling for peptide analysis, digests of five proteins, α-casein, *β*-casein, myoglobin, bovine serum albumin, and cytochrome *c*, were labeled and analyzed by MALDI-TOF/TOF MS in both the CID and PSD modes. The resulting MS/MS spectra were submitted to database searching and processed, as described in the Experimental Section. MALDI-TOF/TOF MS ion fragment and ion current distribution data were extracted from the MASCOT data using an in-house-written PERL program, to reveal changes in fragmentation caused by Alexa Fluor 350 labeling, compared to the native peptides, and to determine the optimum conditions for MS/MS analysis of the labeled peptides.

A summary of the distribution of ion current between the main TOF/TOF MS fragment ion series for native and labeled peptides is shown in Figure 4. Fragment ion series, typical for both the TOF/TOF CID and PSD modes and used by the MASCOT scoring algorithm, were considered, i.e., a-, b-, y-, immonium, and internal ions. Fragments corresponding to a neutral loss of water or ammonia were summed together and are represented in the figure as "% neutral". Also considered were nonidentified fragments, i.e., unassigned fragments, whose presence lower MASCOT scores. The most important result in Figure 4 is the clear shift toward preferential formation of y-ion series in the case of Alexa Fluor 350-labeled peptides, at the expense of all other fragment ion series. As can be seen, the fraction of y-series for both native and labeled peptides was significantly higher in the PSD compared to the CID mode. In contrast, CID spectra of both native and labeled peptides were richer in less informative immonium and internal ions, representing up to 50% of the total MS/MS ion current (see the bars for the native peptides). At the same time, Alexa labeling resulted in a slightly higher fraction of unidentified fragments, which could possibly be explained by the lowered fragmentation energy barrier due to the presence of the sulfo group. In the PSD mode, the fraction of the y-series was more than 70% of the total ion current, decreasing to 50% in the CID mode. This latter result confirms the unimolecular mechanism of fragmentation of sulfonated peptide ions[2] and indicates

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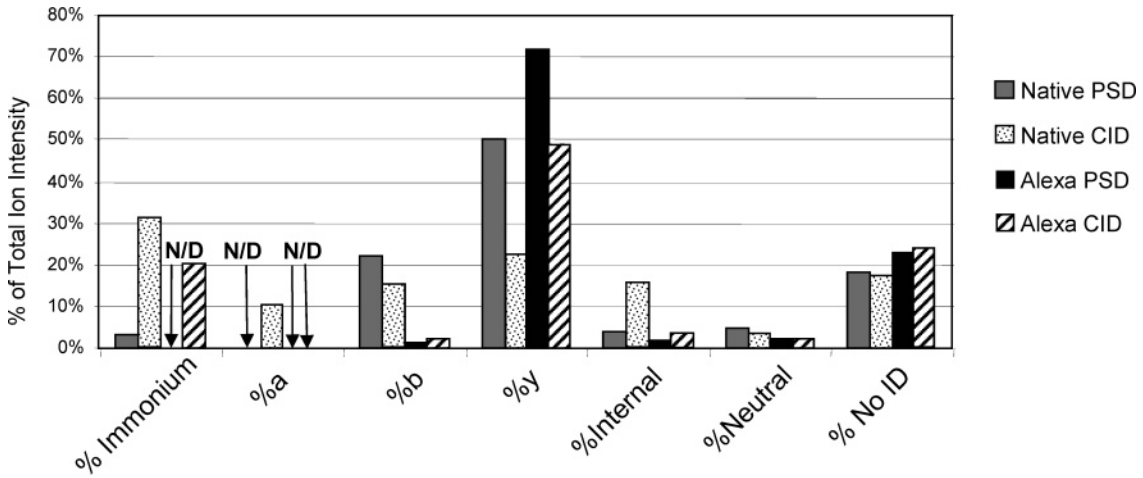

Figure 4. MALDI-TOF/TOF MS fragment ion current distribution obtained from MS/MS spectra of tryptic peptides of five proteins: α-casein, β-casein, myoglobin, bovine serum albumin, and cytochrome *c*. The percentage of the total fragment ion current for a particular fragment ion series is the ratio of the sum of the intensities of all fragment ions of this series to a sum of the intensities of all ions in the spectrum. The intensity of each fragment ion was determined from the specific cluster area, which is the sum of the peak areas in the isotopic cluster for the given ion.

that PSD is the mode of choice for the MS/MS analysis of the sulfonated peptide samples by TOF/TOF MS/MS.

Backbone ion series, such as b and y, are the most important ion series for peptide identification via database searching.[10] Typically, a combination of complementary b- and y-ions is observed in MALDI-TOF/TOF mass spectra of native tryptic peptides, with the fraction of y-ions being twice as large as that of b-ions (see Figure 4). While b- and y-ions make the largest contribution to the MASCOT scores of the peptides, the scores are improved even more if a longer sequence of these ion series is present in the spectrum. As a consequence, for the highest possible MASCOT score, it is beneficial that all fragment ion current be directed into a single fragment ion series. For MALDI-TOF/TOF MS, Lys guanidation, followed by N-terminal derivatization with agents containing a sulfo group, is an effective strategy. Moreover, isotopic labels may be introduced with guanidation,[18,54] with the N-terminal tag, or metabolic incorporation, allowing flexibility in experimental design for differential quantitation.

LC-MALDI-TOF/TOF Analysis of Complex Peptide Mixtures. Analysis of a Protein Digest. As noted earlier, in other reports, N-terminal labeling with various sulfo tags has been employed for the analysis of in-gel digests by MALDI-TOF PSD MS.[40,52,53] Since N-terminal labeling with Alexa Fluor 350 has been found to be beneficial for both MALDI-TOF MS[4] and MS/MS analysis, we next decided to explore the possibility of analyzing labeled samples by RPLC followed by MALDI-TOF/TOF MS. Approximately 100 pmol of a tryptic digest of β-galactosidase was guanidated and then N-terminally labeled with Alexa Fluor 350, and ~1/50 of this sample was analyzed by nanoLC-MALDI-TOF/TOF MS, using a home-built multiplexed LC-MALDI deposition device.[42,43] Figure 5 shows extracted ion chromatograms of representative native and derivatized peptides. Analysis of the chromatograms revealed that narrow (30−40 s) chromatographic peaks were obtained for the labeled peptides, which is comparable to the native peak widths, with an increase in the retention times of the labeled peptides, compared to the unlabeled peptides. It can be concluded that the addition of the tag to the peptide did not appear to significantly affect chromatographic performance.

Analysis of a Strong Cation Exchange Fraction of a Tryptic Digest of *E. coli* Lysate. After the successful preliminary studies, the analysis of more complex peptide mixtures was performed. An SCX LC fraction of a tryptic digest of an *E. coli* lysate was divided into two parts, one of which was maintained native and the other modified by guanidation of lysines, followed by N-terminal labeling with Alexa Fluor 350. Both fractions were then analyzed in parallel by four-channel multiplexed nanoLC-MALDI-TOF/TOF MS, each sample in duplicate, as described in the Experimental Section, and the data were submitted to MASCOT for database searching.

The results showed the expected improved fragmentation for many peptides, which led to the higher peptide scores due to labeling. As an example, Figure 6 presents the MALDI TOF/TOF MS/MS spectra of the peptide GALSAVVADSR from 50S ribosomal protein, m/z 1044.5560, which showed poor fragmentation in the native form, yielding a Mascot score of 10, with the significant threshold being 26. MS/MS of the same peptide in the labeled form resulted in the spectrum with a complete ladder of y-ions with a high MASCOT score of 95, leading to an unambiguous identification.

Another important advantage found for the Alexa Fluor 350 labeling is the ability to obtain high-quality MS/MS spectra even from very low intensity MS precursors. Figure 7 shows a typical MALDI-TOF mass spectrum from one of the deposited spots from the LC separation of the labeled sample. Peptide SVDEAANSDIVDK, from ATP synthase B chain, m/z 1698.668, produced an MS signal of low intensity (350 counts, S/N ~9). However, the inset to Figure 7 shows that the MS/MS spectrum contained the complete y-series, yielding a high MASCOT score of 119, well above the significant threshold of 26.

To demonstrate further the improvement in peptide identification due to the labeling for peptides in various MS signal intensity

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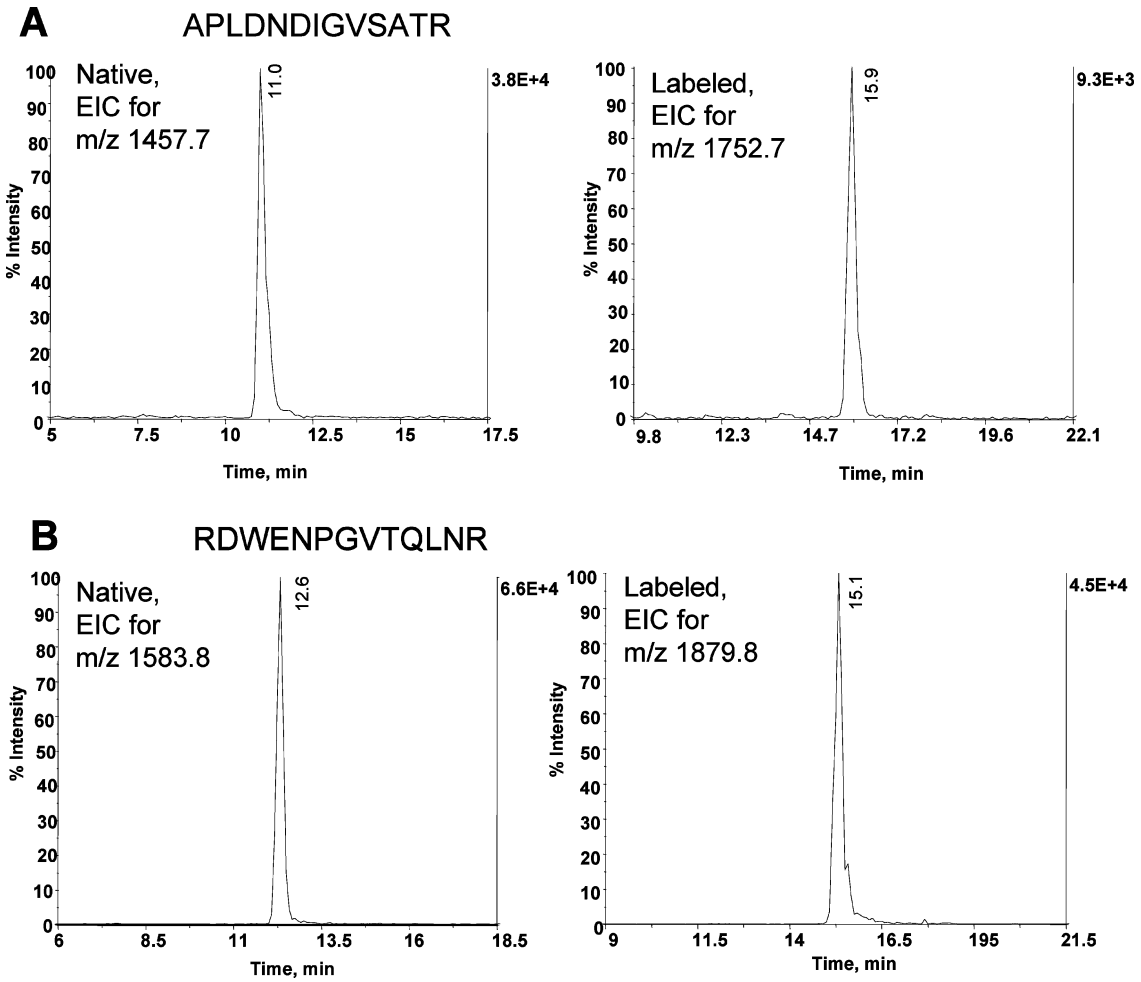

Figure 5. Extracted ion chromatograms for the native and Alexa Fluor-350-labeled β-galactosidase peptides APLDNDIGVSATR (A) and RDWENPGVTQLNR (B). In both figures, the peak widths are ~30 s. See Experimental Section for details.

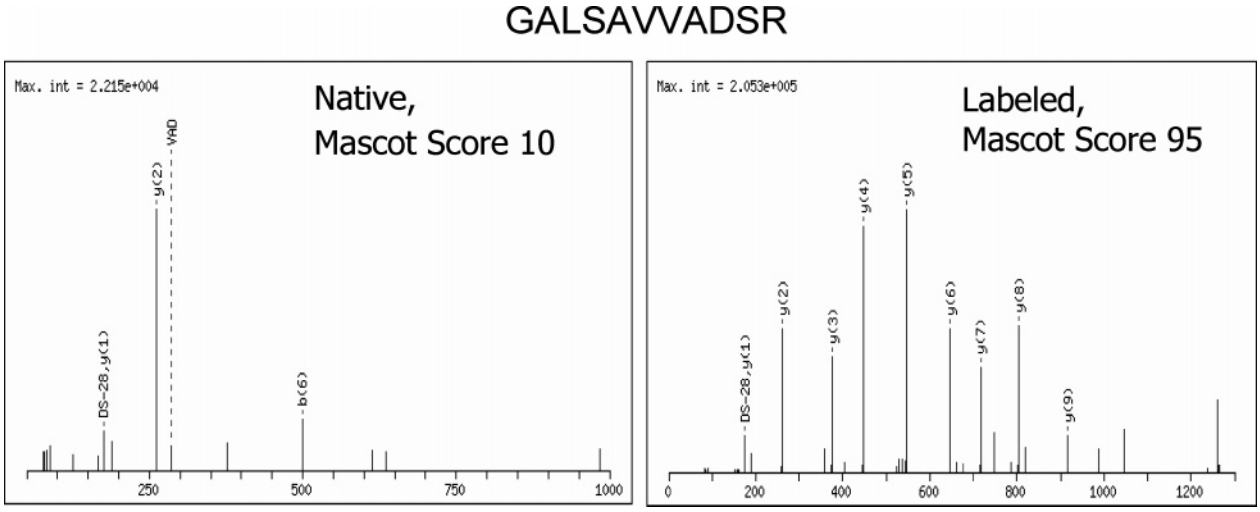

Figure 6. Example of high-confidence peptide identification in the *E. coli* fraction due to labeling. MS/MS fragmentation of GALSAVVADSR, a peptide from 50S ribosomal protein L10 (L8). Monoisotopic mass of the native peptide (neutral) *m*/*z* 1044.556, MASCOT score 10; monoisotopic mass of the labeled peptide (neutral) *m*/*z* 1339.571; fixed modifications guanidination (K); variable modifications Alexa (N-term); MASCOT score 95. The significance threshold was 26 at $p < 0.01$.

ranges, the ratios (in percentages) of identified peptides in native and tagged samples were calculated at different intensities of the MS/MS precursor signals. All MS/MS precursors were grouped by their monoisotopic MS peak height values into groups from 500 to 100 000 counts. For each group, the ratios of the number of peptides that yielded MASCOT scores above 26 (significance threshold) to the total number of submitted precursors of the same peak height range were calculated. As presented in Figure 8 A in the form of a histogram, the results indicated that the percentage of identified peptides was higher for the tagged sample, compared

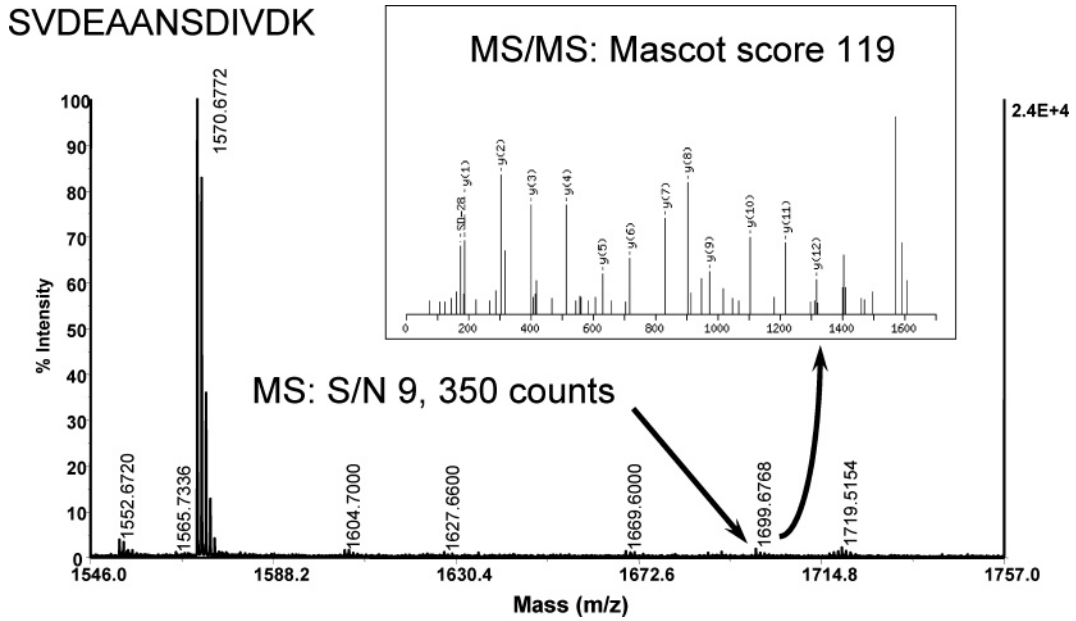

Figure 7. Example of high-confidence identification a low MS intensity peptide SVDEAANSDIVDK from ATP synthase B chain, due to labeling. Monoisotopic mass of the labeled peptide (neutral) *m*/*z* 1698.668, fixed modifications guanidination (K), variable modifications Alexa (N-term), and MASCOT score 119.

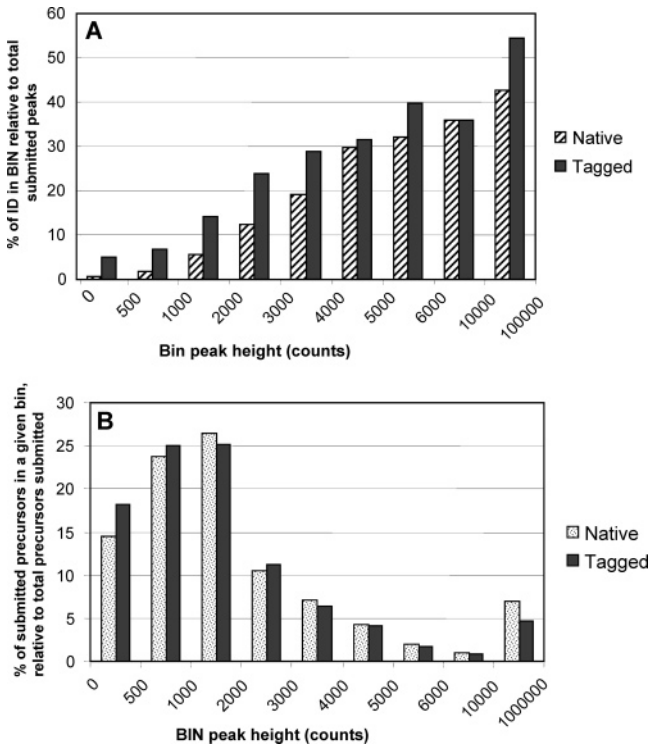

Figure 8. (A) Comparison of percentages of peptides identified in the native and Alexa Fluor 350-labeled *E. coli* fraction. Note that the peak height scale on the *X*-axis is not linear. (B) From the same experiment: a percentage of submitted MS/MS precursors of a particular peak height range, out of all submitted precursors, for native and labeled samples.

to the native. It can be further seen in Figure 8A that the percentage of identified peptides was significantly higher (on average, 2−3-fold) for the low-intensity precursors (500−1000 counts), confirming the results of Figure 7. Figure 8B shows a histogram of the distribution of the MS signal intensities of the native and labeled peptides, calculated for the same peak height ranges as in Figure 8A. From Figure 8B, it can be seen that the total number of peptides in each group was similar for native and tagged samples, and a distribution of their MS intensities was nearly identical. This important observation means that overall no significant decrease in MS ion intensity was caused by the labeling. It is important that the largest improvement was observed in the low-intensity range (below 4000 counts), as over 80% of the MS/MS precursors fell in this range (see Figure 8B).

While usually the probability of random hits (p) of less than 0.05 is typically used in MASCOT searching, we selected here the more conservative setting of less than 0.01, meaning higher than 99% confidence in the correct peptide identification, to decrease the number of false positive identifications that result from low-score hits. As seen in Figure 9A, a total of 419 peptides were identified in the native sample, while 557 peptides were identified in the labeled sample (each number consists of the number of nonredundant hits obtained using the combined data from the duplicate runs). Surprisingly, the overlap between peptides identified in both the native and labeled samples was small (14.5%, Figure 9A). At the same time, the overlap between the peptides identified in each of the duplicate samples, either native or labeled, was about 80−90% (data not shown). Figure 9B shows the identification summary on the protein level: a total of 232 proteins were identified in the native sample and 252 proteins in the labeled sample. The number of overlapped proteins was higher than was the case for peptides (43.6%, Figure 9B), indicating that many proteins were identified in both the native and labeled samples, but by different peptides. As a result, confidence in the correct identification was increased for proteins found in both samples. In particular, the fraction of protein identifications based on two or more peptides increased from 44.8% in the native sample to 64.6% in the combined native + labeled sample data sets. On the whole, 50.8% more peptides and 31.2% more proteins were identified from one SCX fraction of the *E. coli* cell lysate due to the Alexa Fluor 350 labeling.

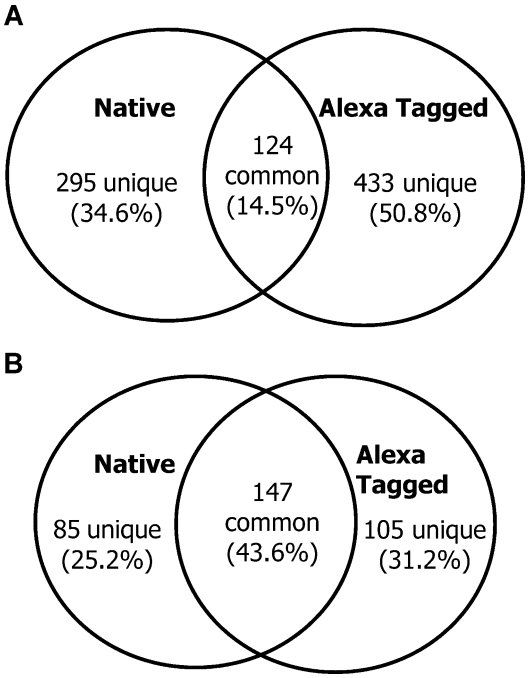

Figure 9. Summary of peptides (A) and proteins (B) identified in the native and labeled samples from the SCX fraction of an *E. coli* lysate digest. A total of 852 unique peptides were identified with 99% confidence using both the native and labeled data sets. These peptides belong to 337 proteins (MUDPIT scoring option was used; see Results and Discussion section for details).

Examination of the experimental data revealed that the nature of peptides identified in both the native and labeled data sets was different. Typically, longer peptides with C-terminal arginine prevailed among the native peptides, while shorter peptides with C-terminal lysine (guanidated) predominated in the labeled sample. There are several factors contributing to this trend. From PMF data acquired by MALDI-TOF MS, it is known that guanidation improves sequence coverage by increasing the MS intensities of lysine-terminated peptides.[55] The effect is peptide-dependent and may vary from no change to a 10-fold enhancement.[55] On the other hand, labeling with Alexa Fluor 350 also caused changes in the MS signal intensities of the labeled peptides, varying from a ~ 5-fold decrease to an enhancement (1.5-fold), depending on the hydrophobicity of the peptide.[4] The resulting effect on MALDI MS signals of peptides was that both guanidation and Alexa Fluor 350 on average counterbalanced each other, although the distribution of the MS intensities for various peptides differed. In particular, the decrease in signal due to Alexa Fluor 350 labeling was more pronounced for the Arg-terminated peptides, which were not enhanced by guanidation. The decrease in signal intensity was somewhat higher for large Arg-peptides with MW >2500, as the mass increase due to labeling (~300 Da) moved such peptides out of the optimum mass range for MALDI with the CHCA matrix. On the other hand, the bias for the detection of shorter Lys-peptides after labeling can be explained by both the enhancement caused by guanidation and the relative increase in the mass and hydrophobicity due to labeling, which facilitate the detection of such peptides by MALDI-TOF MS.[4] The results suggest that the labeled samples should be run along with the native samples, similar to the complementary use of the MALDI and ESI-MS methods.[56,57] The four-channel multiplexed continuous LC-MALDI deposition and parallel analysis technology, using a 2-kHz repetition rate laser,[43,58] should be well suited for the native and labeled sample analysis approach utilized in this work. In summary, the results demonstrate that the proposed analysis strategy leads to a significant improvement in protein identification by LC-MALDI-TOF/TOF MS.

CONCLUSIONS

This paper has shown that Lys guanidation followed by N-terminal labeling with the commercially available sulfo group coumarin tag, Alexa Fluor 350, is an effective approach for peptide analysis in both the MS and MS/MS modes in LC-MALDI-TOF/TOF MS. On average, no significant decrease in MS ion intensity was caused by the labeling. On the other hand, the percentage of identified peptides was considerably higher (on average, 2−3 times) for the tagged sample, compared to the native, due to the high-quality MS/MS spectra that could be obtained, even from very low intensity precursors.

Analysis of the MALDI-TOF/TOF MS fragment ion current before and after labeling revealed changes in the fragmentation pattern caused by the tag. In particular, labeling with Alexa Fluor 350 resulted in a dramatic increase of the y-ion fraction, while the fractions of the rest of the fragment ions, such as a-, b-, immonium, and internal ions, decreased. These trends were found to lead to significantly improved MASCOT scores for the labeled peptides. The N-terminal sulfo tag labeling approach is well suited for commercially available MALDI-TOF/TOF MS instruments, taking advantage of the high sensitivity and high mass accuracy of such instruments in both the MS and MS/MS modes, as well as the possibility of precise precursor ion selection.[7]

LC-MALDI-TOF/TOF MS analysis of the peptides labeled with a sulfo tag was successfully performed. The analysis of the data for tryptic peptides from an *E. coli* lysate revealed improved peptide scores and an increased number of peptides identified due to labeling. Moreover, derivatization did not impair chromatographic behavior of peptides, as narrow chromatographic peaks were obtained for the labeled peptides. Importantly, overlap in peptides identified in either the native or labeled samples was small (14.5%). At the same time, the overlap between the two replicate runs of the same sample, either native or labeled, was close to 90%. The number of overlapped proteins was slightly higher, suggesting that many proteins were identified in either the native or labeled samples by different peptides. As a result, confidence in the correct identification was increased for proteins found in both samples. On the whole, 50.8% more peptides and 31.2% more proteins were identified from one SCX fraction of the *E. coli* cell lysate due to the labeling.

The four-channel multiplexed continuous LC-MALDI deposition and parallel analysis technology developed in our laboratory[43,58] is well suited for the native and labeled sample analysis

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approach demonstrated in this paper. To increase the speed of analysis, TOF/TOF instruments equipped with high-repetition rate lasers (e.g., 2 kHz) can be used for data acquisition.[59] It should be further noted that fluorescent properties of the Alexa Fluor 350 tag may be beneficial for the detection of low sample amounts, as well as for quantitation.[60] Differential labeling and quantitation may be performed by metabolic labeling or by using the isotopically labeled guanidation reagents, such as ^{13}C- and $^{15}N_2$-labeled *O*-methylisourea[54] or the commercially available Lys Tag 4H reagent (Agilent Technologies).[18] A ^{13}C version of the Alexa Fluor 350 tag may be synthesized, as well. All these factors suggest that labeling with Alexa Fluor 350 has real potential for use in high-throughput multiplexed LC-MALDI-TOF MS/MS analysis of proteomic samples.

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