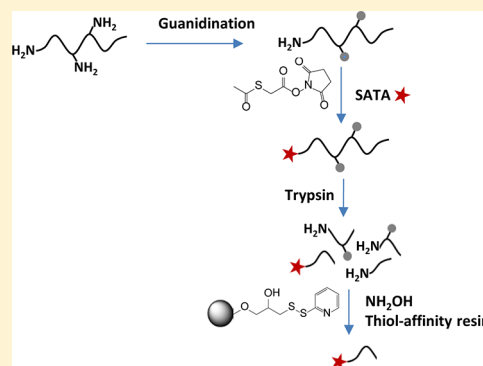


Resin-Assisted Enrichment of N-Terminal Peptides for Characterizing Proteolytic Processing

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S Supporting Information

ABSTRACT: A resin-assisted enrichment method has been developed for specific isolation of protein N-terminal peptides to facilitate LC-MS/MS characterization of proteolytic processing, a major form of posttranslational modifications. In this method, protein thiols are blocked by reduction and alkylation, and protein lysine residues are converted to homoarginines. Protein N-termini are selectively converted to reactive thiol groups, and the thiol-containing N-terminal peptides are then captured by a thiol-affinity resin with high specificity (>97%). The efficiencies of these sequential reactions were demonstrated to be nearly quantitative. The resin-assisted N-terminal peptide enrichment approach was initially applied to a cell lysate of the filamentous fungus *Aspergillus niger*. Subsequent C-MS/MS analyses resulted in the identification of 1672 unique protein N-termini or proteolytic cleavage sites from 690 unique proteins.



Proteolytic processing, the irreversible hydrolysis of peptide bonds by proteases, is an important posttranslational modification (PTM) for regulating protein functions in living organisms.¹ Because of the inherent complexity of the proteome, the identification of proteolytic cleavage sites typically requires selective enrichment of protein N-terminal peptides prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based analysis.^{2,3} Several negative and positive selection methods have been reported for enriching the N-terminal peptides. For negative selection, non-N-terminal peptides can be removed using amine-reactive scavengers^{2,4–6} or via chromatographic separations;^{3,7,8} however, drawbacks of this approach include multiple scavenging reactions^{4,6} or extensive fractionation steps.^{3,7,8} Furthermore, negative selection using scavenger materials may suffer from nonspecific binding of non-N-terminal peptides and nonspecific loss of N-terminal peptides on the resin due to the relatively large amount of amine-reactive scavenging materials required to remove all non-N-terminal peptides.

Conceptually, positive selection strategies in which N-terminal peptides are directly captured using a chemical derivatization strategy have the potential to achieve high enrichment specificity. This advantage of high specificity compensates one limitation of the positive selection strategies; that is, in vivo modified N-terminal peptides will not be enriched.⁹ In positive selection strategies, a biotin affinity tag is often introduced to the protein N-termini through either enzymatic^{10–12} or chemical labeling.^{9,13} The biotin tag enables selective capture of protein N-terminal peptides by immobilized avidin beads;^{9–13} however, a major disadvantage of avidin–

biotin enrichment is the noncovalent nature of the binding, which can lead to nonspecific binding and relatively poor enrichment specificity.^{14–16} To address this issue, a number of studies have utilized solid-phase or resin-assisted covalent enrichment approaches that offer simpler, more efficient ways of capturing cysteine-containing peptides^{16,17} and other PTMs, such as S-nitrosylation,^{14,18,19} phosphorylation,¹⁵ and tyrosine nitration.²⁰ For example, we demonstrated the utility of resin-assisted covalent enrichment for proteomics applications, using thiol-affinity resins to capture cysteine-containing peptides via disulfide-exchange.¹⁷

In this work, we introduce a chemical strategy that enables resin-assisted enrichment of N-terminal peptides from complex mixtures. The strategy involves several blocking and chemical derivatization steps that selectively convert protein N-termini into reactive thiol groups amenable to capture by thiol-affinity resins. All chemical reactions demonstrate nearly quantitative yields. Initial application of our N-terminal peptide strategy to a whole cell lysate of *Aspergillus niger* followed by LC-MS/MS revealed 1672 unique protein N-termini or proteolytic cleavage sites from 690 unique proteins, indicating a high enrichment efficiency.

■ EXPERIMENTAL SECTION

Culture Conditions. *Aspergillus niger* (NRRL3122) was grown and harvested as previously described.²¹ Briefly, *A. niger*

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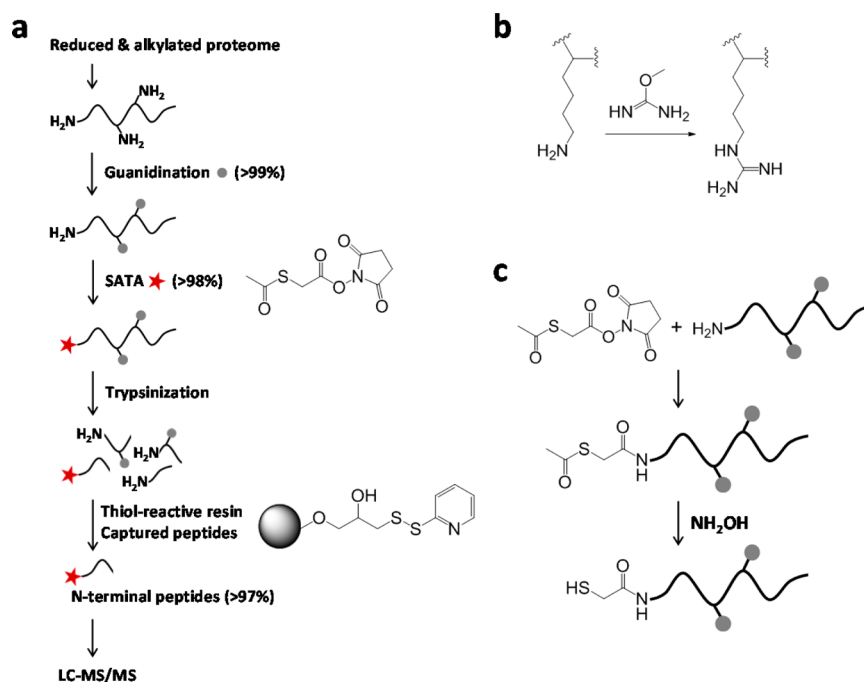


Figure 1. Schematic diagrams of thiol affinity resin-assisted enrichment of N-terminal peptides. (a) Overall enrichment strategy. The reaction or capture efficiency is indicated in parentheses. (b) Guanidination reaction. (c) SATA-labeling.

was grown on potato dextrose agar plates at 30 °C for 4 days for culture maintenance and spore production. Spores were harvested by washing with sterile 0.8% Tween 80 and counted using a hemocytometer.

Protein Extraction. To extract proteins from whole cells, the mycelia were separated from the medium by filtering through two layers of sterile miracloth (EMD Chemicals Inc., Gibbstown, NJ). Approximately 5 g of frozen mycelia were ground into fine powder in liquid nitrogen using a chilled mortar and pestle. The sample was then resuspended in 10 mL of 8 M urea buffer containing 0.5% dithiothreitol (DTT) and 0.5% protease inhibitor cocktail (for use with fungal and yeast extracts; #P8215, Sigma-Aldrich, St. Louis, MO). After incubating at room temperature for 1 h with intermittent vortexing, insoluble material was removed by centrifugation (15 000 × *g* for 15 min). The supernatant was transferred to a new tube and centrifuged again (15 000 × *g* for 15 min). The collected protein sample in the supernatant was subjected to acetone precipitation by adding four volumes of cold acetone (−20 °C) and the sample was placed at −20 °C overnight. After centrifugation, the protein pellet was redissolved in 6 M guanidine hydrochloride containing 250 mM triethylammonium bicarbonate (TEAB, Sigma-Aldrich) buffer (pH 8.2) and stored at −20 °C until further use.

Alkylation of Protein Thiols. Protein samples (100–150 μg) dissolved in 6 M guanidine hydrochloride containing 250 mM TEAB were denatured and reduced in 10 mM DTT for 1 h at 37 °C followed by alkylation of cysteine residues with 40 mM iodoacetamide (IAA) for 1 h at 37 °C in the dark. The TEAB buffer was selected for amine derivatization because it does not react with amine-reactive reagents and is easy to remove.

Chemical Derivatizations of Amino Groups. The pH of alkylated protein samples was adjusted to 10.5 by the addition of 5N NaOH solution. The samples were mixed with *o*-methylisourea solution (pH 10.5) to reach a final concentration

of 1 M *o*-methylisourea. After brief vortexing and centrifugation, the samples were incubated for >24 h at 4 °C with constant shaking. The pH of the samples was checked again after the first 12 h incubation and readjusted to 10.5 with 5N NaOH if necessary. 20% trifluoroacetic acid was added to drop the pH of the protein samples to ~8.0. The samples were then labeled using *N*-succinimidyl S-acetylthioacetate (SATA, Pierce, Rockford, IL) at a SATA/protein ratio of 50 nmol/μg without additional desalting or buffer exchange. The samples were incubated for 1 h at room temperature. The SATA-labeling step was repeated by adding a new aliquot of SATA reagent into the sample. Subsequently, a 10-fold excess of hydroxylamine to SATA was added to the sample, which was then incubated for 2 h at room temperature to quench any unreacted SATA and to release the thiol groups from the protein N-termini. Following chemical derivatization, the protein samples were desalted by buffer exchange into 50 mM ammonium bicarbonate buffer, using Amicon Ultra centrifugal filters with 10K molecular weight cut off (Millipore, Billerica, MA).

Protein Digestion. After determination of the protein concentration using a BCA protein assay (Pierce), acetonitrile was added to the desalted protein samples to reach a final concentration of 20% (v/v) in order to increase protein solubility and digestion efficiency. Samples were digested overnight at 37 °C, using sequencing-grade modified-trypsin (Promega, Madison, WI) with a 1:50 enzyme to protein ratio (w/w). Digested samples were lyophilized to remove acetonitrile and buffer solution in preparation for resin-assisted enrichment of N-terminal peptides.

Capture of N-Terminal Peptides Using Thiol-Reactive Resin. A slightly modified version of our cysteinyl peptide enrichment protocol¹⁷ was used to capture the derivatized N-terminal peptides. The lyophilized peptides were resuspended in 150 μL of 50 mM Tris-HCl/20 mM EDTA buffer (pH 7.5) and 1.0 μL of 100 mM DTT. After incubation for 1 h at 37 °C, the samples were subjected to prewashed thiol-reactive resin in

Table 1. Assessment of Guanidination Efficiency of Lysine ϵ -Amines under Different Conditions^a

samples	incubation conditions	identified peptides	lysine-containing peptides	guanidinated lysine-peptides	guanidination efficiency (%)
bovine serum albumin	4 °C, 24 h	473	262	260	99.2
bovine serum albumin	37 °C, 4 h	310	148	145	98.0
bovine serum albumin	65 °C, 20 min	839	440	207	47
<i>Aspergillus niger</i> lysate	4 °C, 24 h	2725	1300	1290	99.2

^aSpectral count of identified peptides was used for the estimation. All reactions were performed at pH 10.5. The number of identified peptides indicates all identified MS/MS spectra with FDR <0.5%.

a spin column (Pierce) that contained 35 mg of dried thiopropyl Sepharose 6B (GE Healthcare, Pittsburgh, PA) and had a final volume of ~100 μ L after hydration. The mixture was incubated for 1 h at room temperature to allow sufficient time for the resin to capture thiol-containing N-terminal peptides. Non-N-terminal peptides were removed by washing the resin five times with each of the following solutions: 2 M urea, 2 M NaCl, and 80% acetonitrile in 0.1% trifluoroacetic acid. The disulfide-based covalently captured N-terminal peptides were eluted with 2 \times 100 μ L of 20 mM DTT and then alkylated using 100 μ L of 80 mM IAA. The samples were desalted using C18 SPE prior to LC-MS/MS analysis.

LC-MS/MS Analysis. The N-terminal peptide samples were analyzed using a fully automated nano-LC system²² coupled online with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) modified with an in-house manufactured electrospray ionization interface. The reversed phase capillary column (75 μ m i.d. \times 65 cm long) column was slurry packed using 3 μ m Jupiter C18 particles (Phenomenex). The column was connected via a Valco 100 μ m i.d. stainless steel union to a chemically etched fused silica emitter (20 μ m i.d.). Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). An exponential gradient that started with 100% A and gradually increased to 60% B over the course of 100 min at a flow-rate of ~500 nL/min was utilized for the LC separation. The LTQ-Orbitrap was operated in data-dependent mode with an m/z range of 400–2000. The ten most abundant ions determined by MS were selected for MS/MS, which used a normalized collision energy setting of 35%. A dynamic exclusion of 1 min was applied to reduce repetitive analysis of the same abundant precursor ion.

Data Analysis. LC-MS/MS raw data were converted into .dta files using Extract_MSn (version 3.0) in Bioworks Cluster 3.2 (Thermo Fisher Scientific, Cambridge, MA). The SEQUEST algorithm²³ (version 27, revision 12) was used to search all MS/MS spectra against the *Aspergillus niger* JGI database (version 3.0 2006-04-04; consisting of 11200 protein entries) using a parameter file with static cysteine alkylation (+57.022 Da), static lysine guanidination (+42.022 Da), and dynamic alkylated SATA modification (+131.004 Da) on the N-terminal residue of a peptide. Peptides were filtered based on the MS-generating function (MSGF) score^{24,25} value. The final peptide identifications have <1% false discovery rate (FDR) at the unique peptide level. Only fully tryptic and semitryptic peptides were considered.

RESULTS AND DISCUSSION

Resin-Assisted Enrichment Strategy. Our strategy for enriching N-terminal peptides is depicted in Figure 1a. In this strategy, the protein cysteine residues are first blocked through reduction by DTT and alkylation by IAA. Protein lysine ϵ -amines are then converted into homoarginine by a guanidination reaction using *o*-methylisourea (Figure 1b).^{13,26} After

blocking all lysine ϵ -amines, a protected thiol-ester group is introduced to the protein N-termini by SATA, an amine reactive reagent. The protected thiol-ester group is then readily converted to free thiols by treating the samples with hydroxylamine (Figure 1c). If the reactions of these blocking and derivatization steps are achieved with high efficiencies only the protein N-termini contain free thiols. These thiol-containing N-terminal peptides can be enriched either at the protein level or at the peptide level.¹⁹ For protein-level enrichment, on-resin trypsin digestion can be performed and all non-N-terminal peptides can be removed prior to the elution of N-terminal peptides. For peptide level enrichment, the thiol-containing N-terminal peptides are captured after in-solution trypsin digestion by the thiol-affinity resin as previously described.^{17,19} In this work, the peptide-level enrichment protocol was utilized to take advantage of the better binding capacity that the resin has for peptides. All derivatization steps were performed in guanidine HCl to avoid urea-related N-terminal carbamylation, a potential artifact for N-terminal peptides.

Assessment of Derivatization Reaction Efficiencies.

Effective blocking of protein thiols is an essential first step in achieving the enrichment specificity necessary to capture N-terminal peptides using a thiol-affinity resin. Mouse plasma was used as a test sample (Supporting Information Table 1) and spectral counting as a semiquantitative measure.^{27,28} Our data showed that the conditions (10 mM DTT and 40 mM IAA) used to reduce/alkylate cysteines at the protein level resulted in nearly complete alkylation (99.6%). This finding was further confirmed by MS/MS spectra obtained for cell lysate samples of *A. niger*, that is, ~3100 spectra were identified as N-terminal peptides while only 15 spectra were identified as cysteine-containing non-N-terminal peptides (Supporting Information Table 2). These results demonstrated that the small percentage (~0.5%) of unalkylated cysteine-containing peptides had only a negligible effect on the final enrichment specificity for N-terminal peptides.

Another factor critical to the success of this approach is the efficiency and selectivity of converting lysine ϵ -amines to homoarginines by the guanidination reaction at the protein level (Figure 1b). If guanidination efficiency is low, then unblocked lysine residues compromise the enrichment efficiency of N-terminal peptides. Conversely, overguanidination of N-terminal amines can occur in some reaction conditions, for example, high temperature or poorly controlled pH.^{29,30} Overguanidination results in a loss of N-terminal peptides, as blocked N-terminal amines will not be converted to reactive thiols. The selectivity of guanidination on lysine ϵ -amines rather than N-terminal α -amines has been largely attributed to the steric protection of α -amines given by the peptide bond and the amino side chains.^{29,31} Indeed, guanidination on α -amines was often only observed on N-terminal glycine due to the reduced steric hindrance of

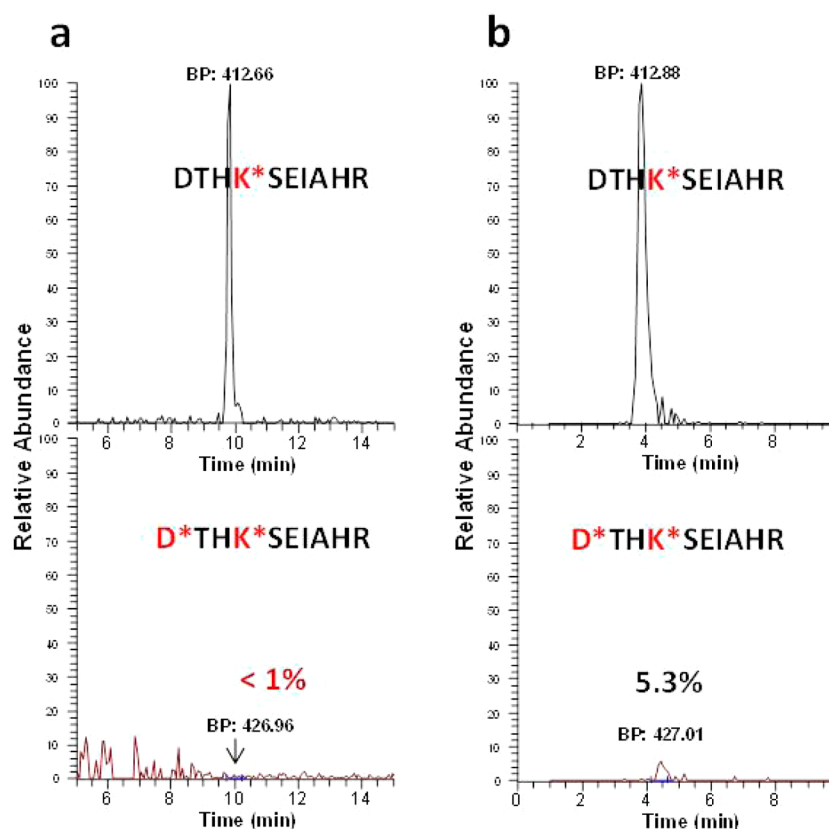


Figure 2. Specificity of guanidination on lysine amines. Extracted ion chromatographs of N-terminal peptides (DTHKSEIAHR) of bovine serum albumin from two different incubation conditions: (a) at 4 °C for 24 h and (b) at 37 °C for 4 h. The undesirably guanidinated peptide on protein N-terminal amine was displayed in the bottom. Note that there was an ~5 min shift of retention times with a 100-min separation for the observed peptides between two different runs. This was due to the relative poor reproducibility in retention times for LC separations operated under a constant pressure.

glycine.^{29,31} Another potentially important factor contributing to the selectivity is that the lysine ϵ -amines is a much better nucleophile than the N-terminal α -amine because of the electron-withdrawing nature of the adjacent carbonyl group. After exploring a range of conditions, we observed that the most effective reaction condition was incubation at 4 °C and pH 10.5 for 24 h, as shown by the spectral count data (Table 1). Figure 2a further shows the specificity of guanidination for lysine amines, which was found to be ~99% based on the extracted ion chromatogram (XIC) peak areas. In contrast, incubation at 37 °C for 4 h resulted in a ~5% increase in undesirable N-terminal overguanidination (Figure 2b). We note that in both cases the guanidination efficiency was high as only guanidinated peptides were detected.

Next, we tested the reaction efficiency of introducing a protected thiol group to primary amines. Because of the limited number of N-terminal peptides available when using a standard protein or standard protein mixture, lysine amines were utilized to examine the reaction efficiency, as N-terminal and lysine amines should exhibit similar reaction efficiencies when using amine-reactive reagents. Three reagents (dimethyl 3,3'-dithiobispropionimidate, *N*-succinimidyl 3-(2-pyridyldithio)propionate, and SATA) that contain a thiol group were initially explored using bovine serum albumin (BSA). The *N*-succinimidyl 3-(2-pyridyldithio)propionate reagent encountered a solubility issue in aqueous buffer and a substantial portion of organic solvent (~40% acetonitrile) was necessary to dissolve it in a coupling buffer, which caused protein

precipitation during the course of the reaction. The protein labeling of BSA using dimethyl 3,3'-dithiobispropionimidate reagent was observed to have a low labeling efficiency (~45%) based on MS/MS spectral count data. The SATA reagent was observed to be the best for this step based on its high labeling efficiency at the protein level, as well as its good solubility in buffer solution. On the basis of the MS/MS spectral count data, ~93% of lysine-containing peptides from BSA were labeled with SATA reagent. Moreover, ~98% reaction efficiency was achieved based on the XICs of major peaks (Supporting Information Figure 1). The observed high reaction efficiency was consistent with our previous work, which used the SATA reagent for a similar reaction and demonstrated that the release of free thiol groups from SATA labeled-peptides using hydroxylamine was close to 100%.²⁰

In our strategy, alkylation, guanidination, SATA-labeling, and thioester deprotection reactions are performed sequentially in one sample vial. This was done without additional sample cleanup in order to minimize potential protein loss (note that pH of the sample was adjusted for each reaction as necessary). We observed that this sequential reaction strategy did not compromise the efficiency of any chemical reaction based on our final enriched N-terminal peptide identification data (Supporting Information Table 2). Following buffer exchange to remove all reagents and trypsin digestion, the peptide mixture was subjected to resin-assisted enrichment of thiol-containing peptides. In general, the procedure involved a buffer

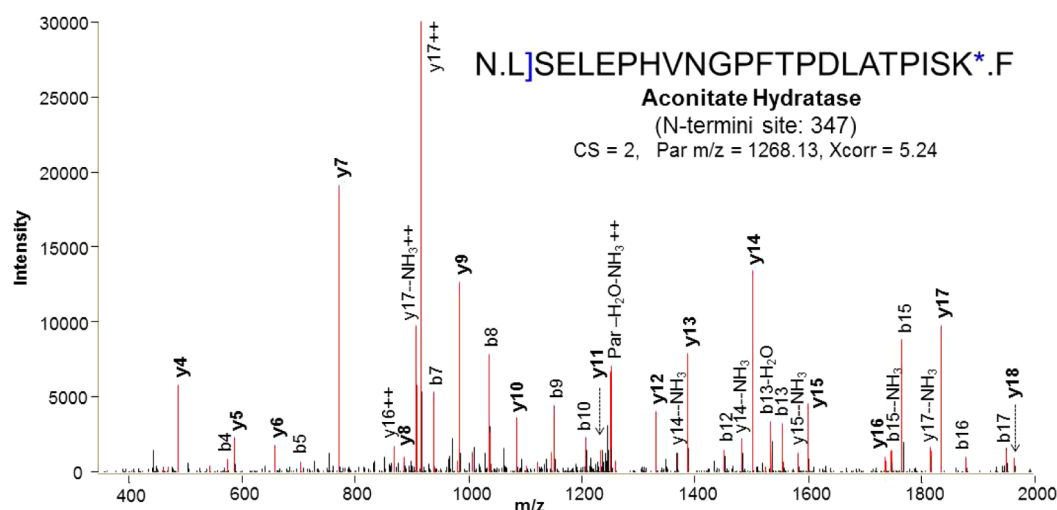


Figure 3. MS/MS spectrum of N-terminal peptide of aconitate hydratase with guanidinated lysine C-terminus. The single bracket and asterisk indicate SATA-labeled and guanidinated residues, respectively.

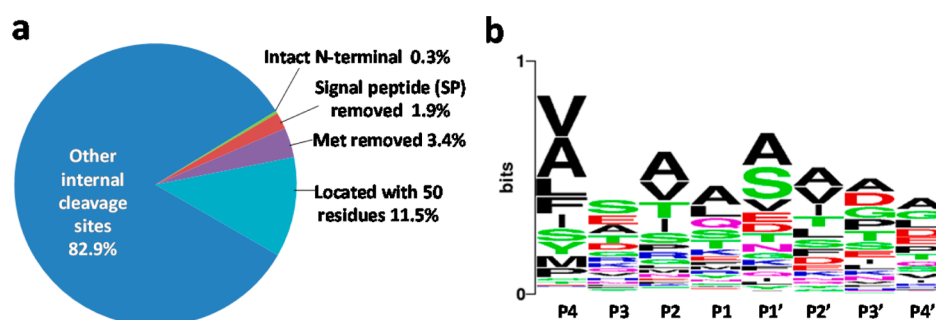


Figure 4. (a) Distribution of the identified N-terminal sites. (b) Sequence motif of N-terminal cleavage sites; potential sites of signal-peptide removal, which were located within first 50 amino acid residues.

exchange step and a single SPE cleanup step for all chemical reactions, including resin-assisted capturing.

Application to an *A. niger* Cell Lysate. Following optimization, we applied the strategy to profile proteolytic cleavage sites of proteins in *A. niger*, a filamentous fungus of interest for biomass degradation due to its ability to produce many extracellular hydrolytic enzymes.³² Based on the results from LC-MS/MS analysis of two process replicates of enriched samples, 1672 uniquely labeled N-terminal peptides from 690 proteins were identified with FDR <0.5% (Supporting Information Tables 2–5). Furthermore, ~98% of the MS/MS spectra were identified as labeled thiol-containing N-terminal peptides, demonstrating the high specificity of the enrichment strategy. Among the nonspecific peptides, 0.5% was from unblocked cysteine-containing peptides and 1.2% from non-specific bound peptides (Supporting Information Table 2).

An interesting observation related to the identified N-terminal peptide sequences is that 423 of the identified N-terminal peptides ended with a homoarginine residue (Supporting Information Table 3). This result suggests that the homoarginine residue, which is slightly longer but structurally similar to arginine (Figure 1b), remains susceptible to trypsin cleavage, although the catalytic kinetics may be slower than those of either lysine or arginine residues. Figure 3 shows a representative MS/MS spectrum of a homoarginine-ending N-terminal peptide. The ability of trypsin to cleave at the converted homoarginine residue is an advantage of our method as it allows more N-terminal peptides ending with a

lysine residue (>30%) to be identified. If the arginine residue was the only enzymatic cleavage site available after lysine residues are blocked, these peptides most likely would be too large (e.g., >5000 Da) to be identified by LC-MS/MS. For example, the long arginine-terminal peptide (54 amino acid residues and >6000 Da) shown in Figure 3 could only be identified because of cleavage on the homoarginine residue. It should be noted that the use of multiple enzymes such as trypsin and Glu-C is an alternative strategy for facilitating identification of large peptides by providing additional cleavage sites.¹³

The identified N-terminal peptides were further examined for known annotations. Figure 4a shows that only 5.6% were annotated as known or predicted sites that comprised intact N-termini, sites with methionine initiator removed, and sites with a signal peptide or a mitochondrial targeting peptide removed (Supporting Information Table 4). Our data confirmed 27 N-terminal cleavage sites predicted to remove either signal or mitochondrial targeting peptides (from SignalP^{33,34} and TargetP³³ prediction tools), as well as 62 original protein N-termini—among which are proteins with methionine initiator removed. These results lend support to the validity of our method and also the accuracy of the predictions. Six other sites that were predicted to remove mitochondrial targeting peptides were either one or two residues off the observed cleavage sites (Supporting Information Table 4), which raises the possibility that the predictions may be inaccurate.

A motif analysis was performed for the nonannotated proteolytic cleavage sites that were located within the first 50 amino acid residues (Figure 4b). The alanine-rich and relatively serine-rich sequence motifs of these peptides provide only weak evidence that some of the sites may correspond to signal peptides being removed. Overall, the majority of the identified N-terminal sites originated from internal protein cleavage sites with no apparent specific sequence motifs, which suggests a complexity of protease activities in *A. niger*. This observation is consistent with the finding that *A. niger* produces a variety of proteases.³⁵

CONCLUSIONS

Resin-assisted enrichment of N-terminal peptides coupled with LC-MS/MS provides an effective approach for proteome-wide profiling of proteolytic cleavage sites. The enrichment protocol requires relatively small amounts of starting material (100–150 μg of total proteins) to yield 5–10 μg of total N-terminal peptides for LC-MS/MS. Furthermore, resin-assisted thiol-affinity capture can be performed at either the peptide-level as in this study or at the protein-level, depending on the applications. Protein-level capture is detergent-friendly in that all reagents can be readily removed after capturing thiol-containing proteins on the resin.

The strategy also is amenable to quantitative studies (e.g., for identifying protease substrates or novel functional proteins) by using an ^{18}O -labeled “universal” reference,^{36,37} stable isotope labeling by amino acids in cell cultures,³⁸ or label-free quantitative approaches. In addition to facilitating the discovery of specific functional proteolytic isoforms in quantitative studies, the N-terminal profiling may provide a resource for other applications, such as characterization of intact protein isoforms using top-down proteomics.³⁹

ASSOCIATED CONTENT

Supporting Information

Additional tables and figure as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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