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N-Terminal Protein Characterization by Mass Spectrometry after Cyanogen Bromide Cleavage using Combined Microscale Liquid- and Solid-Phase Derivatization

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A sample preparation method for protein N-terminal peptide isolation from cyanogen bromide (CNBr) protein digests has been developed. In this strategy, the CNBr cleavage was preceded by protein α- and ε-amine acetylation and carboxyamidomethylation in a one-pot reaction scheme. The peptide mixture was adsorbed on ZipTip$_{C18}$ pipette tips for reaction of the newly generated N-termini with sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate. In the subsequent steps, the peptides were exposed in situ to hydroxylamine for reversal of potential hydroxyl group acylation, followed by reductive release of the disulfide-linked biotinamido moiety from the derivatives. The selectively thiol group-functionalized internal and C-terminal peptides were reversibly captured by covalent chromatography on activated thiol-sepharose, leaving the N-terminal fragment in the flow-through fraction. The use of the reversed-phase support as a venue for postcleavage serial modification proved instrumental to ensure throughput and completeness of derivatization. By this sequence of solid-phase reactions, the N-terminal peptide could be recognized uniquely in the MALDI-mass spectra of unfractionated digests by its unaltered mass signature. The use of the sample preparation method was demonstrated with low-picomole amounts of model protein. The N-terminal CNBr fragments were retrieved selectively from the affinity support. The sample preparation method provides for robustness and simplicity of operation using standard equipment available in most biological laboratories and is anticipated to be readily expanded to gel-separated proteins.

KEY WORDS: peptide identification, reversed-phase supports, covalent chromatography, N-terminal peptide

INTRODUCTION

Protein terminal regions are widely recognized as essential determinants of diverse cellular functions, such as protein degradation, localization, and membrane interaction.[1,2] Technologies aimed at the characterization of these positional regions involve the isolation of terminal peptides from protein digests for subsequent structural characterization by tandem mass spectrometry (MS/MS), efforts that are greatly facilitated by the high sequence specificity at the protein termini, resulting in an increased success rate of protein identification.[3]

Methods that enable N-terminal peptide enrichment from protein digests by negative or positive selection have been, thus far, of the main interest, and developments in this area, also known as N-terminomics, have been pursued for some time (reviewed by Nakazawa et al.[4]). In the former strategy, α-and ε-amines were blocked at the protein level, followed by proteolytic digestion and depletion of the internal and C-terminal peptides, via the newly generated (neo) N-termini.[5–8] A method proposed to isolate N-terminal fragments in this manner involved protein acetylation, tryptic digestion of the modified protein, followed by biotinylation of the internal and C-terminal peptides, and removal of these fragments on streptavidin sepharose.[5] In a variation of this depletion step, N-hydroxysuccinimide -activated sepharose (NHS-sepharose) was used to capture peptides with protoeolytically exposed (neo) N- termini, leaving the N-terminal peptide free in solution.[6] As a result of the lengthy individual reaction steps, nearly 2 days were needed to complete this protocol. Positive N-terminal peptide selection relied on protein ε-amine guanidination, followed by biotinylation of the N-terminus using sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate (sulfo-NHS-SS-biotin). After protein tryptic digestion, the

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doi: 10.7171/jbt.14-2501-003

N-terminal peptide was captured on avidin sepharose, from which the isolated fragment was released by oxidative cleavage of the biotinamido-linked disulfide bond.[7] In a modified version of this approach, the protein N-terminus was selectively labeled as described above, followed by tryptic digestion of the modified protein and performic oxidation of the digest. The internal and C-terminal peptides were scavenged by p-phenylenediisothiocyanate glass, leaving the unbound, oxidized N-terminal isolate in solution.[8] Although the sulfonated fragments aided MS/MS data interpretation, issues persist as to their diminished detection sensitivity in MALDI-TOF MS.[9]

In the strategy reported here, we chose cyanogen bromide (CNBr) digests as substrates for negative N-terminal peptide selection. The reagent is known to hydrolyze amide bonds C-terminal to methionine residues with exceptionally high efficiency (>90%). The chemistry has proved especially useful to prepare hydrophobic proteins for structural characterization by MS, replacing trypsination as a fragmentation method, which proved ineffective as a result of poor accessibility of proteolytic attack and difficulties associated with recovery of the high mass hydrophobic peptides.[10] Alternately, the CNBr products from hydrophilic regions often harbor multiple protonatable sites and as such, are meeting the requirements for electron transfer dissociation-based MS/MS.[11] With these benefits in mind, we developed a sample preparation method, in which protein acetylation and CNBr fragmentation were combined into a one-pot reaction scheme. The internal/C-terminal CNBr peptides were scavenged via their N-termini on NHS-activated sepharose, leaving the N-terminal fragment in the flow-through fraction. However, this depletion procedure consumed ~16 h, limiting the overall throughput of the method.[6] To remedy this situation, we incorporated a masked thiol group into the internal/C-terminal peptides, using sulfo-NHS-SS-biotin as a conjugation reagent. The disulfide-linked biotinamido moiety was then reductively released from the derivatives. The internal/C-terminal peptides thiolated in this manner were reversibly bound to activated thiol-sepharose; the N-terminal fragment remained free in solution. Less than 2 h were required to complete the affinity purification step. Moreover, the bound material may be reductively retrieved from the affinity support for further chemical manipulation, which is not possible when relying on depletion methods thus far described in the literature.

MATERIALS AND METHODS

TFA, *N*-hydroxysulfosuccinimide ester of acetic acid (sulfo-NHS acetate), sulfo-NHS-SS-biotin (EZ-Link), hydroxylamine hydrochloride, Bond-Breaker tris (2-carboxyethyl)phosphine (TCEP) solution (0.5 M), Handee spin columns (0.8 mL internal volume), and GelCode Blue Stain Reagent were obtained from Pierce (Rockford, IL, US). *N*-Octyl glucoside (OGS) was obtained from Roche Diagnostics (Indianapolis, IN, USA). Sodium phosphate dibasic dodecahydrate and CNBr were purchased from Fluka Chemical (Ronkonkoma, NY, USA). Methanol and acetonitrile were from Honeywell Burdick & Jackson (Muskegon, MI, USA). Hydrochloric acid (11.6 N), iodoacetamide, sodium phosphate monobasic monohydrate, 1 M Tris-HCl/0.1 M EDTA buffer, pH 8.0, sodium carbonate, lysozyme C from chicken egg white, RNase A from bovine pancreas, rat serum albumin (RSA), BSA, bovine carbonic anhydrase, horse heart myoglobin, human angiotensin I acetate salt hydrate [DRVYIHPFHL; mass-to-charge ratio (*m/z*) 1296.5], and human [Glu[1]] fibrinopeptide B (EGVNDNEEFFAR; *m/z* 1570.5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Activated thiol-sepharose 4B and urea (Ultra Pure grade) were from GE Healthcare (Piscataway, NJ, USA). The α-cyano-4-hyroxycinnamic acid preparation was from Agilent Technologies (Palo Alto, CA, USA). $ZipTip_{C18}$ pipette tips (0.6μL bed volume) and $ZipTip_{μ\text{-}C18}$ pipette tips (0.2 μL bed volume) were purchased from Millipore (Billerica, MA, USA). Trypsin (modified sequencing grade) was purchased from Promega (Madison, WI, USA). Polyacrylamide gels (Criterion precast gel, 1 mm, 10%) were from Bio-Rad (Hercules, CA, USA).

Protein In-Gel Proteolytic Digestion

GelCode Blue-stained bands from 5 to 50 pmoles RSA, loaded onto the gels, were destained twice with 200 μL 25 mM ammonium bicarbonate in 50% aqueous acetonitrile for 30 min at 37°C. Bands were then dried briefly in a SpeedVac and then incubated for 15 min at 37°C in 100 μL 2 mM TCEP/25 mM ammonium bicarbonate; the supernatant was removed. The gel bands were incubated in 100 μL 20 mM iodoacetamide in 25 mM ammonium bicarbonate for 30 min at 37°C; the supernatant was then discarded. The gel bands were washed three times with 200 μL 25 mM ammonium bicarbonate for 15 min at 37°C and dried in a SpeedVac. Bands were dehydrated for 10 min in 100 μL acetonitrile and then dried briefly in a SpeedVac. Dried gel bands were reswollen at room temperature in 20 μL 25 mM ammonium bicarbonate/0.01% OGS, supplemented with Promega-modified trypsin to an enzyme:substrate ratio of 1:10. After 20 min, 40 μL 25 mM ammonium bicarbonate was added, and the digestion continued for 18 h at 37°C with agitation. After incubation, the supernatant was removed. TFA (50 μL 0.1%) was added. The gel bands were incubated for 45 min at 37°C. The combined extracts were reduced in volume to 35 μL and

acidified by addition of 5 μL 10% TFA before sample immobilization was performed, according to the procedure described below. Bound peptides were desalted by passing 100 μL 0.1% TFA over the resin and typically eluted in 50% acetonitrile/0.1% TFA/0.01% OGS before MALDI-MS.

Protein In-Gel Acetylation

GelCode Blue-stained bands from 5 to 50 pmoles RSA, loaded onto the gels, were destained and carboxyamidomethylated, as described above. After alkylation, bands were washed three times with 200 μL water for 15 min and dried briefly in a SpeedVac. Bands were dehydrated for 10 min in 100 μL acetonitrile and then dried briefly in a SpeedVac. Sulfo-NHS acetate (40 μL 20 mM in 50 mM sodium phosphate buffer, pH 8.0) was then added to the dried gel bands. After incubation for 2 h at 37°C, the gel bands were washed three times with 200 μL water for 15 min at 37° and subsequently, three times with 200 μL 25 mM ammonium bicarbonate for 15 min at 37°C. Bands were taken to dryness in a SpeedVac and processed for in-gel tryptic digestion and peptide extraction, as described above.

One-Pot Protein Carboxyamidomethylation, Acetylation, and CNBr Cleavage

Model protein (25–200 pmoles) was reduced in 30 μL aqueous 10 mM sodium phosphate/4 M urea solution/0.01% OGS (pH 8.0), supplemented with 2.5 mM TCEP. After 20 min incubation at 55°C, 10 uL of a 150-mM iodoacetamide solution, prepared in 10 mM sodium phosphate/4 M urea/0.01% OGS, was added to a final concentration of 30 mM and the incubation continued for 30 min at 37°C in the dark. Sulfo-NHS acetate solution (10 μL of a 60-mM in 10 mM sodium phosphate/4 M urea/0.01% OGS urea) was added to a final concentration of 10 mM. The mixture (50 μL) was incubated for 20 min at 55°C. The CNBr stock solution was prepared by dissolving 10 mg reagent in 0.5 mL 0.6 N aqueous HCl, of which 10 μL was added to the derivatized samples. The cleavage reaction was allowed to proceed overnight at room at temperature in a mixing block with agitation and halted by immobilizing the digests on ZipTip$_{C18}$ pipette tips, according to the procedure described below. Excess of reagents was removed by passing 100 μL 0.1% TFA and 50 μL water in 10 μL aliquots over the resin.

The digest was then processed for MALDI-TOF MS or solid-phase derivatization, as described below. CAUTION: CNBr is extremely toxic and should be handled in a fume hood. All chemical waste should be disposed of properly, according to local regulations.

One-Pot Protein Carboxyamidomethylation and CNBr Cleavage

Model protein (25–200 pmoles) was carboxyamidomethylated, as described above.

The CNBr solution (10 μL; 10 mg in 0.5 N aqueous HCL) was the then added. The sample was incubated overnight at room temperature in a mixing block with agitation. The digests were processed for ZipTip$_{C18}$ pipette tip purification, according to the procedure described below.

Sample Adsorption on C18 Reversed-Phase Support

ZipTip$_{C18}$ pipette tips and ZipTip$_{\mu\text{-}C18}$ pipette tips were wetted six times with 10μL methanol, followed by six washes with 10 μL 0.1% TFA, following the manufacturer's instructions. Model peptides (10–100 pmoles), prepared in 1% TFA/0.01% OGS solutions, were transferred in 10 μL aliquots to 0.5 mL microfuge tubes and subjected to 10 sample aspiration/dispense cycles. In each cycle, the sample is withdrawn and dispensed once. The immobilized peptides were then washed three times with 10 μL 0.1% TFA. For high-volume sample enrichment (typically 40–60 μL), the CNBr digests or dilute peptide solutions were aspirated sequentially in 10-μL aliquots onto ZipTip$_{C18}$ pipette tips and dispensed into a 0.5-ml microfuge tube. The partially stripped peptide solution was then transferred back in this step-wise mode to the original sample tube. This alternating load/dispense sample enrichment was repeated five times to maximize peptide binding. Unless stated otherwise, the ZipTip pipette tips were then washed 10 times with 10 μL 0.1% TFA. To avoid resin de-wetting, ZipTips tips, loaded with test peptides or CNBr digests, were processed immediately for derivatization or kept in temporary storage. To this purpose, 10 μL 0.1% TFA was aspirated onto the supports from 60 μL solvent that had been placed into a 0.5-mL microfuge tube. The tips were left immersed in solvent prior to use.

General Procedure for Solid-Phase Peptide Derivatization

The various reagents (10 μL) were aspirated three times onto the ZipTip$_{C18}$ pipette tips or ZipTip$_{\mu\text{-}C18}$ pipette tips and dispensed to waste. The reagents (10 μL) were then loaded onto the tips from 60 μL that had been placed into 0.5 ml microfuge tubes. During incubation, the tips were left immersed in the reagents. The tips were subsequently desalted by a solvent wash passed over the resin, as specified below. Reaction products and untreated peptides (set aside in storage) were eluted in 5–10 μL 50% acetonitrile/0.1% TFA/0.01% OGS, of which 1 μL was typically used for MALDI-MS analysis. ZipTip$_{\mu\text{-}C18}$ pipette tips were eluted

in matrix, supplemented with 0/1% TFA/0.01% OGS onto the MALDI target.

On-Column Acetylation

Sulfo-NHS acetate was dissolved in 50 mM sodium phosphate buffer, pH 8.0, to a final concentration of 20 mM. ZipTip$_{C18}$ pipette tips were loaded with 10 μL reagent and incubated for 20 min at 55°C. The ZipTips were then washed 10 times with 10 μL aliquots of 0.1% TFA.

On-Column Amino Group Thiolation

A 20-mM sodium phosphate buffer solution, pH 8.0, was supplemented with 20 mM sulfo-NHS-SS-biotin. ZipTip$_{C18}$ pipette tips were incubated in 10 uL reagent for 30 min at room temperature and then washed 10 times with 10μL 0.1% TFA. Sample processing for MALDI-TOF MS was as described above.

On-Column *O*-Deacylation

Hydroxylamine hydrochloride was dissolved in 1 M sodium carbonate, pH 9.4, to a final concentration of 2%. ZipTip$_{C18}$ pipette tips were incubated in 10 μL reagent for 15 min at 37°C and then washed 10 times with 10 μL 0.1% TFA and the five times with 10 μL water. Sample processing for MALDI-TOF MS was as described above.

On-Column Disulfide Reduction

A 20-mM sodium phosphate solution, pH 8.0, was supplemented with 5 mM TCEP. Reagent (10 μL) was loaded onto the ZipTip$_{C18}$ pipette tips. After incubation for 15 min at 37°C, the tips were washed 10 times with 10 μL of a 2-mM aqueous EDTA solution and then 10 times with 10 μL 0.1% TFA. Samples were processed for MALDI-TOF MS, as described above.

Consecutive On-Column Derivatization: Sample Preparation for Thiol-Directed Affinity Depletion

N-Thiolation, *O*-deacylation, and disulfide-bond reduction were combined into the sequential reaction scheme (see Fig. 1). The intermittent desalting steps before reagent loading were as specified for the individual chemistries described above. After reaction with EZ-Link sulfo-NHS-SS-biotin and subsequent *O*-deacylation, the desalted ZipTips can be stored at least overnight at −20°C in 10 μL 0.1% TFA, while leaving the ZipTips immersed in solvent. At the conclusion of the reaction scheme, care must be taken to thoroughly remove the reductant from the support by sequentially passing 100 μL aqueous 2 mM EDTA and 100 μL 0.1% TFA in 10 μL aliquots over the resin. The final reaction products were eluted in10 μL 50% acetonitrile/0.1% TFA/0.01% OGS directly into 40 μL 50 mM sodium phosphate/2 mM EDTA/2 M urea/0.01% OGS (pH 8.0), used as coupling buffer for covalent chromatography (see section below).

Covalent Chromatography

Activated thiol-sepharose (1 g) was allowed to swell for 10 min in 10 ml water. The gel was washed by vacuum filtration with a total of 150 ml water added in 15 ml aliquots, suspended in 10 ml 10% aqueous ethanol, and stored refrigerated until use. A 75% gel slurry (100 μL) was placed into the spin column that had been washed with 200 μL acetonitrile by centrifugation at 1500 rpm for 1 min before use, a centrifugation speed and time setting that was used throughout the protocol. The affinity medium was centrifuged, resuspended in 200 μL 50 mM sodium phosphate/2 mM EDTA, pH 8.0, or in 50 mM Tris-HCl/5 mM EDTA, pH 8.0, followed by centrifugation; the flow-through was discarded. The spin column was then sealed with the plastic plugs supplied by the manufacturer. The CNBr digest conditioned for covalent chromatography was eluted from the ZipTip$_{C18}$ pipette tip with 10 μL 50% acetonitrile/0.1% TFA/0.01% OGS, directly into 40 μL 50 mM sodium phosphate/2 mM EDTA/2 M urea/0.01% OGS (pH 8.0) or in 50 mM Tris-HCl/5 mM EDTA/2 M urea/0.01% OGS (pH 8.0), used as thiol-coupling buffer. The mixture was transferred to the spin column, which was then agitated gently to resuspend the medium pellet. The spin column was capped, inserted into 1.5 mL microfuge tubes, secured with parafilm, and end-over-end-incubated for 1 h at room temperature using a rotary mixer and subsequently, centrifuged to recover unbound material, i.e., the N-terminal CNBr fragment. The affinity medium was then washed consecutively with 50 μL of the thiol-coupling buffer, 50 μL 60% aqueous acetonitrile/0.1% TFA, and 50 μL 80% aqueous acetonitrile/0.1% TFA. The combined flow-through fractions were reduced in volume by SpeedVac evaporation to 35 μL, acidified with 5 μL TFA to a final concentration of 1%. The isolates were immobilized on ZipTip$_{C18}$ pipette tips, which were then washed with 100 μL 0.1% TFA, passed over the resin in 10 μL aliquots. Peptides were eluted from the support with 10 μL 50% acetonitrile/0.1% TFA/0.01% OGS before MALDI-TOF analysis. Low-level amounts of peptide were enriched with micro ZipTips, washed 10 times with 10 μL aliquots of 0.1% TFA, and deposited in matrix containing 0.1% TFA directly onto the MALDI target. The disulfide-bound peptides, i.e., the internal and C-terminal fragments, were optionally recovered from the affinity support as follows. The affinity medium was washed with 50 μL thiol-coupling buffer to neutralize residual TFA; the flow-through was discarded. For release of bound peptides, 50 μL of the coupling buffer containing 5 mM TCEP was added to the sealed spin column, which was then incubated

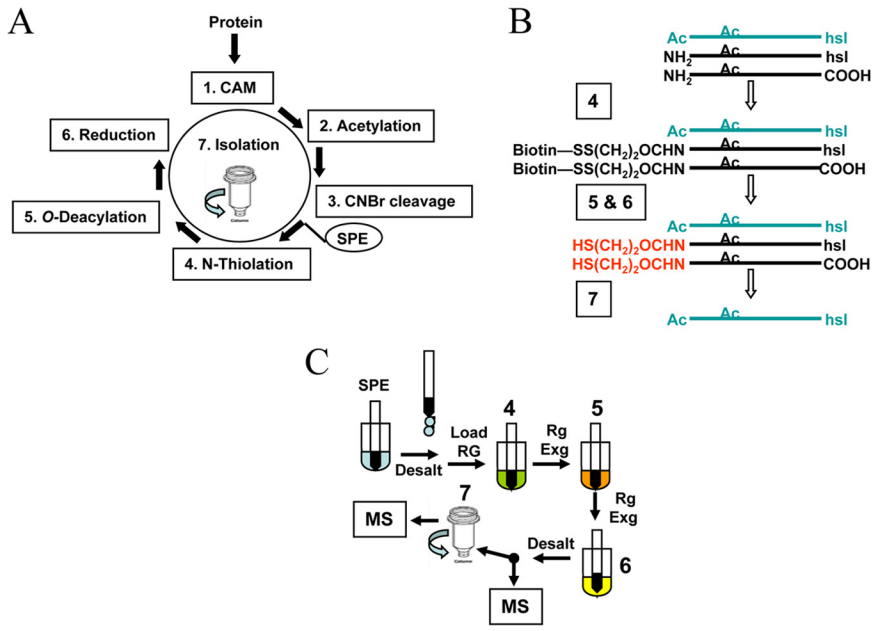

FIGURE 1

(A) Flow chart of sample preparation for N-terminal CNBr peptide isolation. The individual reaction steps are numbered and highlighted with boxes. Whole protein is reduced/carboxyamidomethylated (CAM; Step1), followed by acetylation (Step2) and CNBr cleavage (Step3) in a one-pot reaction sequence. The digest is adsorbed on ZipTip$_{C18}$ reversed-phase supports [solid-phase extraction (SLE)] for on-column reaction with sulfo-NHS-SS-biotin (Step 4). Subsequent processing on the solid-phase includes *O*-deacylation to reverse unwanted hydroxyl group transesterification (Step 5) and the reductive release of the disulfide-linked biotinamido moiety from the biotinylated derivatives (Step 6). Note that during serial incubations, the ZipTips are left immersed in the reagents. After sample transfer to activated thiol-sepharose, the thiol group-functionalized internal and C-terminal peptides are scavenged on the affinity support, leaving the N-terminal CNBr peptide in the flow-through fraction. (B) Schematic representation of the structure of the analyte and its derivatives. hsl and Ac denote homoserine lactone and acetyl group, respectively. The thiol-functionalized affinity tag is highlighted in red. (C) Schematic representation of solid-phase sequential sample handling steps. Note that reagents are sequentially exchanged on the reaction bed (Rg Exg), eliminating intermittent sample purification. The reaction cycle is concluded by a solvent wash before product elution, MALDI-TOF MS analysis, and transfer to activate thiol-sepharose.

for 30 min at room temperature with end-over-end mixing. The thiol-peptide derivatives were displaced from the affinity support by centrifugation, which was then washed with 50 µL of the reductant. The affinity resin was subsequently washed with the organic solvents, as described above. The combined fractions were reduced in volume to 35 µL, and acidified with 5 µL 10% TFA to a final concentration of 1%. The material was subsequently bound to ZipTip$_{C18}$ pipette tips, washed with 100 µL 0.1% TFA, passed over the resin in 10 µL aliquots. Prior to MALDI-TOF MS, peptides were eluted from the support with 10 µL 50% acetonitrile/0.1% TFA/0.01% OGS.

MS

A Voyager DE STR (Applied Biosystems, Foster City, CA, USA) was used and operated in the reflector mode at an accelerating voltage of 20 kV, 67% grid voltage, and 250 ns extraction delay time. Laser intensity was typically set at 1400–1600, and spectra were acquired using 100 laser shots/spectrum. When operated in the linear mode, the grid voltage was typically set at 95% and the extraction delay time at 150 ns. The data shown are based on three accumulated acquisitions averaged. Some spectra were obtained using 80 laser shots/spectrum, and spectra acquired in the linear mode from eight different positions were averaged. The analyte was prepared on the target in the dried droplet mode using α-cyano-4-hydroxycinnamic acid as matrix. For peptide fragmentation, a 4800 Proteomics Analyzer (Applied Biosystems) was used and operated in the MS and MS/MS mode; typical laser power for MS was ~3700; for MS/MS, ~4500. Usually, 1000–2000 shots were acquired for MS; 2000–20,000 for MS/MS.

RESULTS AND DISCUSSION

Reaction Scheme

As schematically illustrated in Fig. 1A, the sample preparation scheme is initiated by protein disulfide bond reduction, followed by carboxyamidomethylation of the liberated thiols (Step 1). The protein is then subjected to acetylation, to block its amino terminus, as well as all

internal lysine ε-amine side-chains (Step 2), and subsequently, to CNBr cleavage as a final step of the one-pot reaction scheme (Step 3). The reaction mixture is then adsorbed onto ZipTip$_{C18}$ pipette tips and reacted in situ with sulfo-NHS-SS-biotin targeting the chemically neo N-termini (Step 4). In the subsequent steps, the modified peptides were exposed to hydroxylamine for reversal of unwanted hydroxyl group acylation (Step 5), followed by reductive release of the disulfide-linked biotinamido moiety from the derivatives (Step 6). The serial solid-phase reaction cycle is halted by a clean-up step, followed by product elution and sample transfer to thiol-activated thiol-sepharose for subsequent depletion of the thiol-functionalized internal and C-terminal peptides, leaving the N-terminal peptide in the flow-through fraction.[7] Optionally, the depleted material can be reductively released from the affinity support for further chemical and/or enzymatic manipulation.

Sample Handling

The solid-phase sample handling format offers some notable advantages over the classical in-solution-based methods, the predominant sample preparation technique in current proteomic studies.[12] It affords easy sample cleanup before and after the derivatization. This simple desalting step replaces repeated vacuum concentration with intermittent water addition commonly practiced in proteomics studies to ensure complete removal of reagent and solvent from CNBr digests. This sample manipulation can cause adsorptive peptide loss ranging up to 50% or more of the starting solution after a single evaporation step, especially as seen with a low-level analyte.[13] The process of sample adsorption concentrates the analyte on the support, allowing the reactions to proceed in situ, in general, at higher efficiency and faster kinetics than in solution.[9,12,14,15] The solid-phase procedure should be readily amenable to automation on ZipTip$_{C18}$ pipette and tip/ZipTip$_{\mu\text{-}C18}$ pipette tip-compatible, liquid-handling stations. It is noteworthy that reaction format has evolved as the predominant sample preparation technique for trace analysis of bioorganic compounds and has found widespread application in pharmaceutical and toxicological studies.[16,17]

Serial in-solution reaction schemes, as practiced in proteomics studies, are considered as problematic because of the high potential of cumulative adsorptive sample loss incurred during intermittent sample purification.[18] In contrast, during solid-phase sequential derivatization, the various reagents are replaced directly on the support, obviating the need for sample transfer between the reaction steps.[14,19] Owing to this minimal sample handling, optimal product formation is maintained throughout the derivatization cycle, allowing the final reaction products to be recovered at minimal sample loss.

The potential for peptide adsorption to surfaces in well-recognized and strategies has been advanced to minimize losses, including use of low levels of detergents and sample processing component miniaturization. Accordingly, N-terminal peptide purification by affinity chromatography was performed on a relatively small bed of activated thiol-sepharose (>100 μL), and the sample buffer was supplemented with trace amounts of OGS (0.01%). This nonionic detergent has been shown to improve the efficiency of in-gel digestion attributed to minimized, nonspecific protein/peptide adsorption to plastics and to enhanced solubility of the digestion products.[20] To benefit from this effect, during protein derivatization, reagent solutions were also supplemented with 0.01% OGS, and test peptides were enriched from solutions in the presence of the detergent. The additive, when included in the matrix solutions, has been shown to promote peptide ionization in MALDI-TOF MS, facilitating detection of relatively large species in the mass range between 4 and 11 kDa.[21] To take advantage of this effect, peptides were eluted from the ZipTip$_{C18}$ pipette tips in the presence of the detergent. Peptides, once adsorbed onto the ZipTip$_{C18}$ pipette tips containing nanoliter sorbent beds, are rendered essentially impervious to adsorptive sample loss.

Sample Preparation at the Protein Level

As illustrated in Fig. 1A, derivatization of the protein involved a series of chemical in-solution reactions, including protein disulfide reduction, carboxyamidomethylation of the liberated thiol,s followed by acetylation of the protein's primary amino groups. As demonstrated with the test proteins, lysozyme C and RNase A, the thiol and amine-blocking step proceeded to completion. These results are discussed later in the text.

The derivatized samples were then subjected to fragmentation, allowed to proceed overnight in 0.1 N hydrochloric acid, supplemented with 33 mM CNBr. Such dilute acidic solvent conditions previously have been found advantageous to render highly hydrophobic proteins susceptible to CNBr cleavage and to minimize hydrolytic protein degradation.[10] Other side-reactions known to occur during CNBr cleavage include cysteinyl group partial conversion to cysteic acid and deamidation of asparagine and glutamine side-chains.[10] The oxidative side-reaction was prevented by the sulfhydryl group alkylation, used as an initial reaction step in the sample preparation method. As assessed by gel electrophoretic analysis of the reaction products of the well-characterized proteins lysozyme C (M_r 14.3 kDa), RNase A (M_r 13.6 kDa), BSA (M_r 66.4 kDa), carbonic anhydrase (M_r 28.9 kDa), and myoglobin (M_r

16.9 kDa), the methionyl-bond cleavage proceeded under the described reaction conditions at high efficiency (>80%), yielding fragments observed in the gel images within the predicted molecular weight range of 5–30 kDa (results not shown).

The fragmentation reaction was halted by adsorption of the digest directly from the reactant mixture on ZipTip$_{C18}$ pipette tips used as a venue for subsequent derivatization at the peptide level.

Sample Preparation at the Peptide Level

Our sample preparation strategy uses a thiolation step to introduce a disulfide moiety into internal and C-terminal CNBr fragments bearing free amino groups. Sulfo-NHS-SS-biotin, commonly used for protein and peptide biotinylation,[22] was evaluated to this purpose using angiotensin I (DRVYIHPFHL; *m/z* 1296.5) as a model peptide. In the experiments, the peptide was immobilized on ZipTip$_{C18}$ pipette tips and incubated with 20 mM sulfo-NHS-SS-biotin in buffered sodium phosphate (pH 8.0). After 30 min incubation at room temperature, the reaction was arrested by a solvent wash. The reaction product, along with untreated peptide, was eluted and submitted to MALDI-TOF MS. The MALDI-TOF MS spectra, illustrated in Fig. 2A and B, showed that the peptide's amino group reacted with the NHS-ester reagent to near completion. The mass difference of 398 Da between the starting material at *m/z* 1296.9 and its biotinylated counterpart at *m/z* 1686.3 was in accord with the expected mass addition. Furthermore, the native peptide and its derivative ionized in MALDI-TOF MS at comparable efficiency. In addition, the peptide proved susceptible to acylation of the tyrosine hydroxyl group side-chain (*O*-acylation), giving rise to the product at *m/z* 2075.5, attributable to the reactivity enhancement effect of histidine within the YIH aa triad (Fig. 2B, arrow).[22] In the subsequent reaction steps, the sample was exposed for 15 min at 37°C to alkaline 2% hydroxylamine (pH 9.4), followed by incubation in a 20-mM sodium phosphate solution, supplemented with 5 mM TCEP (pH 8.0). This treatment resulted in regeneration of tyrosine by hydrolysis of the *O*-linked ester bond, and

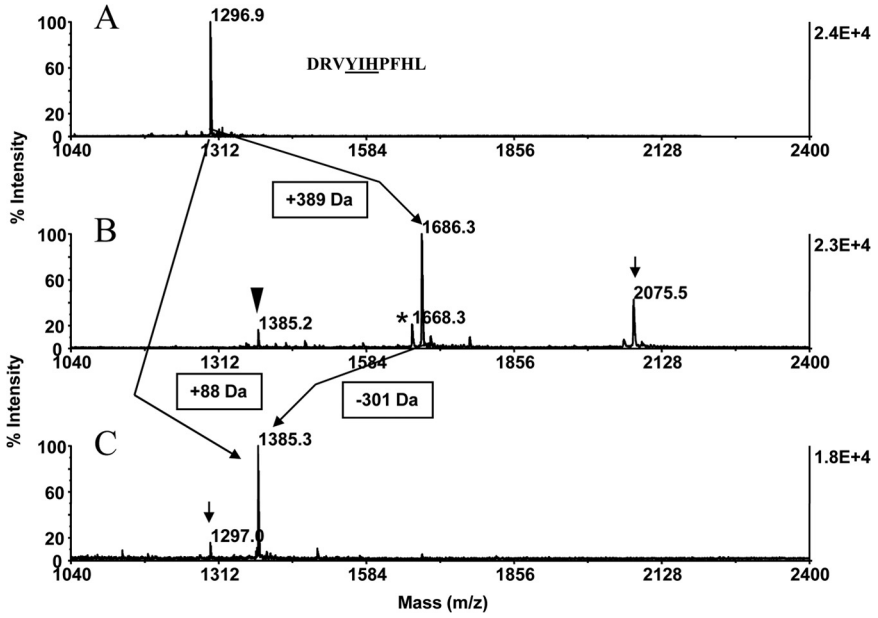

FIGURE 2

Sample preparation at the peptide level: peptide biotinylation, *O*-acylation reversal, and reductive release of thiol derivatives. Angiotensin I- DRVYIHPFHL (*m/z* 1296.9; 10 pmoles) was immobilized on ZipTip$_{C18}$ pipette tips and incubated with 20 mM sulfo-NHS-SS-biotin in sodium phosphate (pH 8.0) for 30 min at room temperature. The biotinylated peptide was then exposed for 15 min at 37°C to an alkaline 2% hydroxylamine (pH 9.4), followed by incubation in 20 mM sodium phosphate, supplemented with 5 mM TCEP (pH 8.0). MALDI-TOF MS spectra of (A) unmodified peptide; (B) after biotinylation; and (C) after *O*-deacylation and subsequent disulfide reduction. Cross-arrows indicate the native peptide at *m/z* 1269.9, its biotinylation product at *m/z* 1686.3, and its thiol-functionalized derivative at *m/z* 1385.3. The mass shifts accompanying the reactions are highlighted with boxes. (B) Arrow denotes *O*-acylated peptide. Note reversal of the transesterification at tyrosine upon the hydroxylamine treatment (C). Filled arrowhead denotes a small fraction-prereleased thiol derivative at *m/z* 1385.2. (B) Asterisk and (C) arrow designate reaction product of a peptide synthesis byproduct and partially regenerated peptide, respectively. One of 10 of the eluates corresponding to ~1 pmoles peptide was applied to the target.

subsequent formation of the thiol-functionalized derivative observed in the spectrum at *m/z* 1385.3 (Fig. 2C). Notably, sulfo-NHS acetate proved more effective to acylate hydroxyl groups in angiotensin I than the biotinylation reagent (data not illustrated). The inherently high reactivity of this NHS ester toward hydroxyl amino acids explains the finding that lysozyme C, which is devoid of the reactivity-modulating sequence motif, was prone to partial *O*-acylation, as shown later in this report. Taken together, the results highlight the use of the solid-phase platform to ensure throughput and optimal efficiency of derivatization. As a separate application, the solid-phase derivatization format may be used for small-scale preparation of biotinylated peptides and proteins.

Covalent Chromatography

The method, in general, relies on capture of proteins/peptides from a mixture on the activated thiol-sepharose by thiol-disulfide interchange, removal of the nonbound material, followed by reductive release of the disulfide-linked material.[23] In the application of this technique to the isolation of N-terminal CNBr fragments, efficient displacement of the isolate from the affinity medium was perceived as a prerequisite to ensure optimal recovery of the desired species. To test the sampling efficiency of the miniaturized retrieval system, 10 pmoles immobilized [Glu^1] fibrinopeptide B (EGVNDNEEFFAR; *m/z* 1570.5) was converted to its acetylated counterpart. The N-terminal peptide mimic was submitted to covalent chromatography. MALDI MS analysis of the samples showed that the starting material (Fig. 3A) and the peptide recovered from the flow-through fraction (Fig. 3B) displayed similar ionization yields, indicating that the acetylated peptide was excluded effectively from the affinity support. The affinity resin was then carried through a "mock" enrichment cycle, normally used to recover disulfide-linked material. A small percentage (<20%) of starting material was found in the reductively released fraction, suggesting that additional organic solvent washes would maximize the recovery of the acetylated peptide (Fig. 3C). Taken together, the data validate the use of the miniaturized covalent chromatographic system to recover amine-blocked peptides with minimal sample loss.

To assess the mass sensitivity of the peptide-displacement procedure, 0.5 pmole of the N-terminally blocked test peptide was submitted to covalent chromatography. The flow-through fraction was concentrated on a ZipTip$_{\mu\text{-C18}}$ pipette tip and eluted directly in matrix onto the MALDI plate. The high quality of the spectrum underscores the potential to extend the detection limits of the procedure (Fig. 3B, inset).

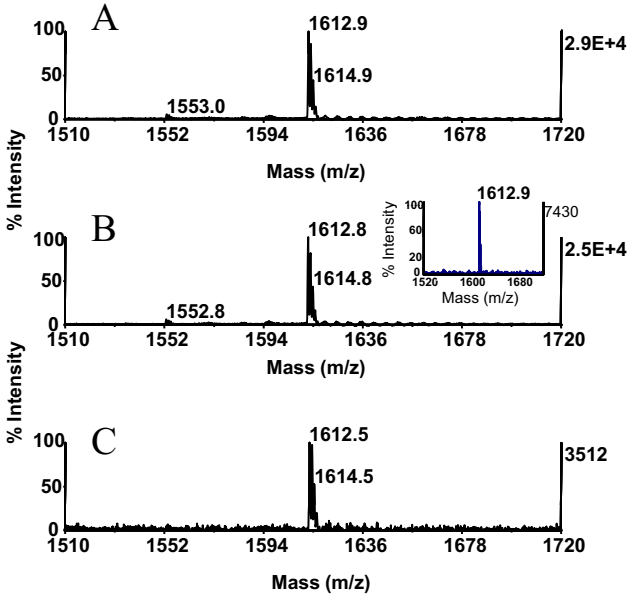

FIGURE 3

Evaluation of microscale covalent chromatography. [Glu^1] fibrinopeptide B (EGVNDNEEFFAR; *m/z* 1570.5; 10 pmoles) was adsorbed on ZipTip$_{C18}$ pipette tips exposed to a phosphate-buffered solution containing 20 mM sulfo-NSH acetate for 20 min at 55°C (pH 8.0). The acetylated peptide (serving as an N-terminally blocked peptide mimic) was subjected to covalent chromatography. The starting material, the flowthrough, and the "reductively" released fractions were analyzed by MALDI-TOF MS. Spectra were obtained using 80 laser shots/spectrum, and eight spectra acquired from spots at different positions were averaged. MALDI-TOF MS spectra of (A) starting material; (B) flow-through fraction; and (C) reductively released fraction. Note that an estimated 84.5% (average) of the starting material was displaced from the affinity support. One of 10 of the eluates corresponding to ~1 pmoles peptide was applied to the target. (B) Inset shows the spectrum of displaced peptide at a 0.5-pmole sample load. The peptide was concentrated on a ZipTip$_{\mu\text{-C18}}$ pipette tip and deposited in matrix onto the target.

Application of Sample Preparation/Affinity Isolation to Whole Protein

To test the applicability of the N-terminal isolation strategy with whole proteins, lysozyme C (25 pmoles) was carried through the series of the liquid-phase reactions, as detailed above. Eluates from the CNBr digest, generated from carboxyamidomethylated protein without and with acetylation, were analyzed by MALDI-TOF MS. As shown in Fig. 4A and B, the CNBr fragments shifted by 42 Da/modified residue, and unreacted material was not observed in the spectrum of the carbamidomethylated sample, indicating that the amine-blocking step at the protein level proceeded to completion. As shown further in Fig. 4A and B, the fragment at *m/z* 1949.3 yielded a doubly acetylated product at *m/z* 2033. 8, accompanied by the less-abundant triply acetylated counterpart at *m/z* 2075. 9, indicating that partial hydroxyl group transesterification (*O*-acylation)

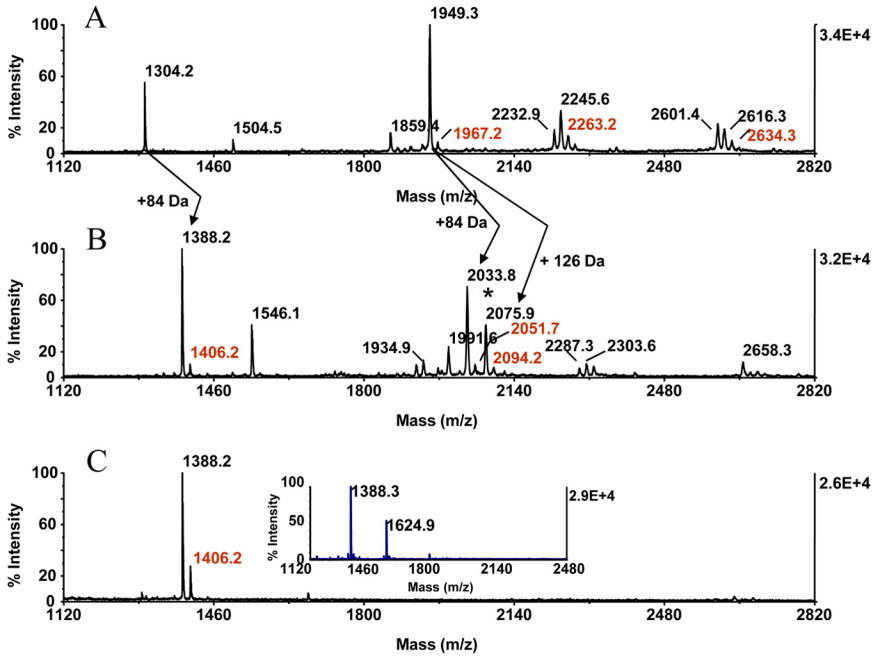

FIGURE 4

Application of method to whole protein. CNBr digests, generated from carboxyamidomethylated-acetylated, intact lysozyme C (25 pmoles), alone or from an equimolar mixture with RNase, were adsorbed on ZipTip$_{C18}$ pipette tips and carried through the sequential reaction scheme, as described in Fig. 2. Digests from the alkylated and from the alkylated and subsequently acetylated protein were submitted to MALDI-TOF MS analysis. MALDI-TOF MS spectra of (A) alkylated digests and (B) digest after subsequent acetylation. (A and B) Arrows indicate the 42-Da mass shift/amine modification. *Reaction product at *m/z* 2075.9, arising from the partial hydroxyl group acylation at the protein level within of the peptide sequence at *m/z* 1949. 3 (see text). Masses highlighted in red denote homoserine-terminated fragments. The thiol group-functionalized digests were submitted to affinity depletion on activated thiol-sepharose. The flow-through fractions were analyzed by MALDI-TOF MS (C). The peptide isolate at *m/z* 1388.2 was aligned by database search to the N-terminal sequence KVFGRCELAAAM, spanning residues 19–30. The ion at *m/z* 1624.9 was recognized by database search as the N-terminal CNBr isolate of RNase A, corresponding to the sequence KETAAAKFERQHM, spanning residues 27–39 (C, inset). One of 10 of the eluates corresponding to ~2 pmoles digest was applied to the target.

had occurred at the protein level. To test this, the resin-bound acetylated digest was exposed to alkaline 2% hydroxylamine (pH 9.4) for 15 min at 37°C and analyzed by MALDI-TOF MS. The spectra showed that the reaction product at *m/z* 2075.9 merged into the reaction product at *m/z* 2033.8, which is indicative of reversal by ester bond hydrolysis of an *O*-acylation event (results not shown).

The peptide at *m/z* 1949.3 had no counterpart among the expected three CNBr fragments. This homoserine lactone-terminated peptide was recognized by database search, as the truncation product of the large internal CNBr fragment, spanning residues 31–227 (M_r 13.4 kDa). Such C-terminal truncation events have been noted to occur with model peptides after prolonged exposure (16 h) to 2.74 N hydrochloric acid at elevated temperatures (50°C).[24] No acid-induced peptide degradation occurred when the reaction was performed in a 0.1-N hydrochloric acid solution, i.e., conditions that were relatively more stringent than used in our CNBr cleavage protocol (0.1 N HCl, 16 h, room temperature). As demonstrated by the experiments described below, the C-terminal truncation product, as well as the additional, minor, nonspecific degradation products, was, as expected, subject to affinity depletion by covalent chromatography and thus, had no impact on the selectivity of the N-terminal peptide isolation.

The lysozyme C digest derived from the modified protein was then carried through the sequential solid-phase reactions, as illustrated in Fig. 2, and analyzed by MALDI-TOF MS. The treatment imparted a mass shift of 88 Da to all peptides with the exception of the fragment at *m/z* 1388.2, which thus, could be recognized as the N-terminal CNBr peptide by its unaltered mass signature (data not illustrated). The thiol-functionalized derivatives, formed from the fragment at *m/z* 1949.3 that appeared as a doublet in the MALDI-TOF MS spectrum, collapsed, as would be expected, into a single species upon exposure to hydroxylamine (data not shown). Importantly, *O*-deacylation, used as a penultimate reaction step in the sample preparation

method, ensures that the isolates are recovered, undiluted, from the affinity support.

We note here that N-terminal peptide identification prior to enrichment is not possible when relying on depletion strategies thus far proposed in the literature. We do recognize that N-terminal peptides in more complex mixtures (i.e. produced from high molecular proteins or protein mixtures) can, owing to the signal suppression effect, be difficult to identify by differential peptide mass mapping. In this situation, N-terminal peptide identification relies solely on affinity-based depletion of the redundant material, as described below.

In the following experiments, the thiol-functionalized preparation of lysozyme C was submitted to covalent chromatography. MALDI-TOF MS of the flow-through fraction revealed the prominent ion at *m/z* 1388.2, and there was essentially no background contamination, indicating that the various internal, nonspecific fragments were effectively scavenged by the affinity support (Fig. 4C). The isolate matched within the known protein sequence to the N-terminal CNBr fragment, spanning residues 19–30, corresponding to KVFGRCELAAAM. We note here that the peptide was recovered predominantly in its homoserine lactone-terminated form. In addition, the N-terminal peptide was recognized in its fully carboxyamidomethylated form, indicating that the thiol protection step at the protein level went to completion and thereby. precluded incomplete recovery of the isolate.

The sample preparation method was then applied to a mixture of lysozyme C and RNase A containing equimolar amounts of protein (25 pmoles), followed by covalent chromatography. MALDI-TOF MS analysis of the flow-through fraction revealed two prominent ions at *m/z* 1388.3 and at *m/z* 1624.9, of which the latter isolate aligned to the known RNase A protein sequence, with the N-terminal CNBr fragment spanning residues 27–39, corresponding to KETAAAKFERQHM (Fig 4C, inset). Taken together, the data validate the use of a miniaturized sample processing/covalent chromatographic system to isolate selectively N-terminal CNBr fragments from whole protein with minimal sample loss.

In-Gel Protein Acetylation

With the use of gel-separated RSA, we next developed an in-gel protocol of the amine acetylation step of the protein sample preparation method. We reasoned that this format would be advantageous, as proteins are presented frequently in gel-separated form for chemical and enzymatic manipulation. This technique has found wide-spread use to carboxyamidomethylate gel-separated protein for subsequent in-gel proteolytic digestion and has been shown to accommodate relatively low-level amounts of protein (>2 pmoles). In the experiments, GelCode Blue-stained bands from 5 to 50 pmoles RSA were loaded onto the gels, destained, carboxyamidomethylated, and subsequently incubated in 20 mM sulfo-NSH acetate/50 mM sodium phosphate buffer (pH 8.0) with agitation. After 2 h incubation at 37°C, bands were washed briefly and subjected to in-gel tryptic digestion, along with carboxyamidomethylated controls. Gel extracts were immobilized on ZipTip pipette tips and submitted to MALDI-TOF MS. The MALDI-TOF spectra, prepared from the digests before and after in-gel acetylation, revealed that the reaction gave rise to a set of peptides generated with endoproteinase Arg-C-like specificity (Fig. 5B). For instance, the tryptic fragment observed at *m/z* 1440.9 mass matched to the sequence APQVSTPTLVEAAR (residues 439–452) and aligned with the larger fragment observed at *m/z* 2003.3, corresponding to the sequence YTQ**K**APQVSTPTLVEAAR (residues 435–452), bearing the acetylated lysine residue. Furthermore, the dominant ion at *m/z* 1456.4, shared by both spectra, matched by database search to an arginine-terminated fragment, devoid of an internal lysine. The data showed that amine acetylation could be performed effectively in the gel matrix, leaving the protein intact for in-gel CNBr cleavage or optional chemical/enzymatic treatment. To this purpose, we are

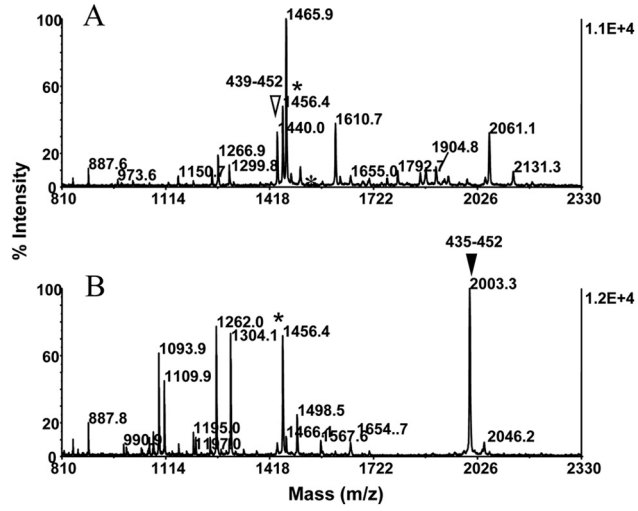

FIGURE 5

In-gel protein acetylation. RSA (20 pmoles) was subjected to in-gel alkylation or in-gel alkylation, followed by in-gel acetylation. Gel bands were then submitted to tryptic digestion. Extracts were analyzed by MALDI-TOF MS. The MALDI-TOF MS spectra of (A) digest after alkylation and (B) digest after serial alkylation and acetylation. Open and filled arrowheads denote peptides corresponding to the sequence 439APQVSTPTLVEAAR452 and its acetylated (K438) overlap peptide 435YTQ**K**APQVSTPTLVEAAR452, respectively. The corresponding masses are annotated with starting and ending amino acids. *Arginine-terminated fragment, spanning residues 361–372, devoid of internal lysine. One of 10 of the eluates corresponding to ~2 pmoles digest was applied to the target.

currently adapting our in-solution CNBr cleavage protocol to the gel-based format, with the future aim use of silver-stained protein to determine the mass detection limits of the gel-based sample preparation method.

Limitations of the Approach

N-terminal methionine excision from nascent proteins is an essential cotranslational event, targeting the majority (80%) of intracellular proteins of all organisms.[25] An estimated 14% of cytoplasmic proteins are cleaved at their N-terminal regions by signal peptidases. The relatively small subset of proteins that retains methionine is evidently not amenable to the approach described in the study, as the truncated fragment delineated by the penultimate amino acid residue would be co-depleted on activated thiol-sepharose (see Fig. 1). If the characterization of this class of unprocessed proteins is of interest, then the use of alternate cleavage methods that target rare amino residues in proteins (i.e., Trp, Cys) or that affect cleavage at specific dipeptide linkages, occurring at low frequency (i.e., Asp-Pro, Asn-Gly), deserves consideration.[26,27]

Aminopeptidase M is an exoproteinase that removes amino acid residues nonspecifically from the N-terminal domains of polypeptides in a step-wise mode, generating a sequence ladder.[28] Consequently, the incorporation of this enzymatic reaction step into our sample preparation method would result in the generation of set of N-terminal CNBr isolates, differing from each other by the mass of the hydrolyzed amino acid. MS analysis of the isolates may provide for a readout, albeit limited to a few truncation products, of the substrate's N-terminal sequence. Experiments aimed at the optimization of the digestion step subject to structure-catalytic activity modulation are currently being pursued in our laboratory.

Current and Future Experiments

MALDI-TOF/TOF and electrospray ionization tandem mass spectrometers have been designed to operate best in the intermediate mass range between 0.8 and 3 kDa, whereas high mass peptides (>3 kDa) are analyzed preferentially by Fourier transform ion cyclotron resonance MS or by linear trap quadruple MS, using higher-energy dissociation cells. However, these high-end instruments may not be available to all experimental biology laboratories. To address this situation, we adopted an earlier reported strategy, in which the CNBr reaction was exploited to render membrane proteins amenable to digestion with trypsin.[29] In the practical implementation of our expanded sample preparation workflow, the CNBr reactant mixture (60 μL) was adsorbed onto a $ZipTip_{C18}$ pipette tip, desalted, and eluted into 50 mM ammonium bicarbonate/1 M urea/0.01% OGS (pH 8.0), supplemented with trypsin to an enzyme:substrate ratio of 1:20. Alternatively, the reaction mixture may be buffer-exchanged by gel filtration, followed by the addition of the protease to the collected fraction.[14] The digestion products were extracted on a $ZipTip_{C18}$ pipette tip and desalted and carried through the series of chemical reactions, as depicted in Fig. 1B to convert the neo amine-terminated proteolytic fragments to their thiol-functionalized analogs, which are then depleted by covalent chromatography. Hence, this simple strategy results in selective sampling of the N-terminal proteolytic fragment from the corresponding CNBr counterpart. In silico analysis of well-characterized proteins (e.g., BSA, RNase A) revealed that the predicted tryptic N-terminal isolates, C-terminally delineated by arginine, were within the desirable intermediate mass range of 1.5–3.5 Da. We note here that the endoproteinases chymotrypsin and Glu-C, which remain active in up to 2 M urea solutions, provide for a means to probe, in a second pass manner, N-terminal CNBr fragments, devoid of arginine. The results from the ongoing experiments with model systems, including hydrophobic proteins and experimental samples, will be published elsewhere at a later date.

Conclusions

A sample preparation method for N-terminal peptide isolation from protein CNBr digests has been developed. The method relies on a series of chemically reactions at the protein and peptide level, which were optimized for throughput and completeness of derivatization. The use of reversed-phase supports in this process proved instrumental to recover the final reaction products at minimal sample loss. The N-terminal peptides were purified effectively by covalent chromatography. The microscale sample preparation method/covalent affinity retrieval system combines robustness with simplicity of operation, using standard equipment readily available in most biological laboratories, and is expected to be readily adaptable to gel separated protein.

ACKNOWLEDGMENTS

This work has been funded by U.S. National Institutes of Health grant #R33CA101150 to R.H.A. The authors thank the Albert Einstein College of Medicine for generous support, Mr. Edward Nieves and Dr. Richard Stanley for helpful discussions, and Ms. Junko Hihara for editorial assistance.

DISCLOSURE

The authors declare no conflict of interest associated with financial support.

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