ARTICLE

C-Terminal Protein Characterization by Mass Spectrometry using Combined Micro Scale Liquid and Solid-Phase Derivatization

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A sample preparation method for protein C-terminal peptide isolation has been developed. In this strategy, protein carboxylate glycinamidation was preceded by carboxyamidomethylation and optional α- and ϵ-amine acetylation in a one-pot reaction, followed by tryptic digestion of the modified protein. The digest was adsorbed on ZipTip$_{C18}$ pipette tips for sequential peptide α- and ϵ-amine acetylation and 1-ethyl-(3-dimethylaminopropyl) carbodiimide-mediated carboxylate condensation with ethylenediamine. Amino group-functionalized peptides were scavenged on N-hydroxysuccinimide-activated agarose, leaving the C-terminal peptide in the flow-through fraction. The use of reversed-phase supports as a venue for peptide derivatization enabled facile optimization of the individual reaction steps for throughput and completeness of reaction. Reagents were exchanged directly on the support, eliminating sample transfer between the reaction steps. By this sequence of solid-phase reactions, the C-terminal peptide could be uniquely recognized in mass spectra of unfractionated digests of moderate complexity. The use of the sample preparation method was demonstrated with low-level amounts of a model protein. The C-terminal peptides were selectively retrieved from the affinity support and proved highly suitable for structural characterization by collisionally induced dissociation. The sample preparation method provides for robustness and simplicity of operation using standard equipment readily available in most biological laboratories and is expected to be readily expanded to gel-separated proteins.

KEY WORDS: C-terminal peptide identification, multistep derivatization, reversed-phase supports, C-terminal peptide structural characterization

INTRODUCTION

Positional proteomics technologies have proven to be valuable tools to characterize protein terminal regions widely recognized as critical determinants of diverse cellular functions, such as protein degradation, complex formation, and membrane interaction.[1] These technologies involve the isolation of terminal peptides from protein digests for subsequent structural characterization by tandem mass spectrometry (MS). These efforts are greatly facilitated by the high sequence specificity at the protein termini, resulting in an increased success rate of protein identification.[2]

N-terminal proteomics projects have, so far, been of main interest, and method developments in this area have been pursued for some time.[3] In contrast, only a few C-terminal analytical techniques are currently available, including carboxypeptidase ladder sequencing and trypsin-catalyzed ^{18}O-coding, which selectively labels all peptides except the C-terminal peptide that thus can be identified by its isotopomer signature.[4,5] The efficiency of the former approach proved dependent on the substrate amino acid sequence, whereas the selectivity of ^{18}O-coding using trypsin is abrogated if the C-terminus is occupied by lysine or arginine.

Methods that enable C-terminal peptide enrichment from protein digests by negative selection include anhydrotrypsin-based peptide purification and various chemical approaches.[6–8] Anhydrotrypsin samples C-terminal peptides from tryptic digests by affinity depletion of arginine and lysine-terminating peptides.[6] However, proteins bearing these amino acid residues at their C-termini are not amenable to this approach, and nonspecific cleavage products are not retained on the support. Chemical reaction schemes have been proposed that scavenge non-C-terminal peptides from lysyl endopeptidase digests on p-phenylenediisothiocyanate (DITC) glass after α-amino group label-

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ing with succinimidyloxycarboxylmethyl tris-(2,4,6-trimethylphenyl) phosphonium bromide (TMPP-Ac-OSu) or after chemoselective α-amino group transamination.[7,8] Selective labeling with TMPP-Ac-OSu, however, could not be demonstrated generally, and several amino acid residues proved inert to transamination or afforded poor detection sensitivity by MALDI-TOF MS. Moreover, in both protocols, C-terminal peptides bearing lysine were also captured by the DITC support.

To address these shortcomings, we developed a reaction scheme that is conceptually related to an early method in which protein carboxyl groups were protected by glycinamidation followed by trypsination.[9,10] The N-terminal and internal peptides were then captured on an amino group-functionalized Sepharose support by 1-ethyl-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated carboxylate condensation. Several additional peptides, however, were co-isolated by this method, and product recoveries proved relatively low (10%). In the strategy reported here, protein carboxylate glycinamidation is preceded by optional α- and ϵ-amine acetylation in a one-pot reaction, followed by tryptic digestion of the modified proteins. The digests are then adsorbed onto C18 reversed-phase supports for subsequent, sequential in situ peptide α-amine acetylation and EDC-mediated carboxylate condensation using ethylenediamine (EDA) dihydrochloride as a nucleophile. The amino group-functionalized N-terminal and internal peptides are then depleted on N-hydroxysuccinimide (NHS)-activated Sepharose, leaving the C-terminal peptides, as rendered impervious to amination in the flow-through fractions. The final, optimized protocol affords a throughput of $<$2 days/sample.

When preparing this manuscript, we became aware of an alternative adaptation that uses protein α-amine-reductive dimethylation, followed by carboxylate EDC-catalyzed conjugation with ethanolamine.[11] Reaction at the peptide level includes α- and ϵ-amine-reductive dimethylation and subsequent depletion of the N-terminal and internal peptides on polyallylamine polymers. The spectra of fractions enriched from model digests revealed that several non-C-terminal peptides co-isolated with noticeable abundance. As a result of the lengthy individual reaction steps, nearly 1 week is needed to complete this protocol.

MATERIALS AND METHODS

TFA, formic acid, EDC, N-hydroxysulfosuccinimide (sulfo-NHS), EDA dihydrochloride, NHS ester of acetic acid, MES-buffered saline, tris-(2-carboxyethyl)phosphine solution (TCEP; Bond-Breaker, 0.5 M), urea (sequanal grade), guanidine hydrochloride (8 M), Spin Columns–Screw Caps (0.8 mL internal volume), Zeba desalt spin columns (0.5 mL resin bed), and GelCode blue stain reagent were obtained from Pierce (Rockford, IL, USA). Glycinamide hydrochloride was from Acros Organics (Morris Plains, NJ, USA). NHS-activated Sepharose 4 Fast Flow was from GE Healthcare (Piscataway, NJ, USA). Hydrochloric acid (11.6 N), iodoacetamide, sodium carbonate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic dodecahydrate, rat serum albumin, horse heart myoglobin, and bovine β-lactoglobulin were purchased from Sigma-Aldrich (St. Louis, MO, USA), as were the following synthetic peptides: bradykinin human acetate salt hydrate (RPGFSPFR, *m/z* 963.2), human fibrinopeptide B (PyrGVNDNEEGFFSAR, *m/z* 1552.5), [E^1] human fibrinopeptide B (EGVNDNEEGFFSAR, *m/z* 1570.5), human fibrinopeptide A (ADSGEGDFLAEGVR, *m/z* 1536.5), and human neurotensin (PyrLYENKPRPYIL, *m/z* 1672.9). *N*-octyl glucoside (OGS) was obtained from Roche Diagnostics (Indianapolis, IN, USA). Methanol and acetonitrile was from Burdick & Jackson (Muskegon, MI, USA). Polyacrylamide gels (Criterion precast gel, 1 mm, 10%) were from Bio-Rad Laboratories (Hercules, CA, USA). $ZipTip_{C18}$ pipette tips (0.6 μl bed volume) and $ZipTip_{μ\text{-}C18}$ pipette tips (0.2 μl bed volume) were purchased from Millipore (Billerica, MA, USA). α-Cyano-4-hydroxycinnamic acid was obtained from Agilent Technologies (Palo Alto, CA, USA). Trypsin (modified sequencing grade) was purchased from Promega (Madison, WI, USA).

Protein In-Gel Proteolytic Digestion

GelCode blue-stained bands, from 5 to 50 pmoles of rat serum albumin 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 briefly dried 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 briefly dried 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 prior to sample immobilization.

Protein In-Gel Glycinamidation

GelCode blue-stained bands, from 5 to 50 pmoles of rat serum albumin 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 briefly dried in a SpeedVac. Bands were dehydrated for 10 min in 100 μL acetonitrile and then briefly dried in a SpeedVac. Glycinamide was dissolved in aqueous 0.1 M MES/5 M guanidine hydrochloride to a final concentration of 1 M (pH 4.7). EDC and sulfo-NHS were dissolved together in aqueous 0.1 M MES/5 M guanidine hydrochloride (pH 4.7) to a final concentration of 0.5 M and 10 mM, respectively. The reagents were combined in a ratio of 1:1. The reagent mixture (40 μL) was then added to the dried gel bands. After incubation for 1.5 h at 37°C, the gel bands were washed three times with 500 μL water for 15 min at 37°C 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. Reagents were prepared fresh for daily use.

One-Pot Protein Carboxyamidomethylation and Glycinamidation

Model protein (25–200 pmoles) was reduced in 40 μL aqueous 10 mM sodium phosphate /4 M urea solution/ 0.005% OGS (pH 8.0), supplemented with 2.5 mM TCEP. After 20 min incubation at 55°C, 10 μL of a 150-mM iodoacetamide solution prepared in10 mM sodium phosphate/4 M urea/0.005% OGS was added to a final concentration of 30 mM, and the incubation continued for 30 min at 37°C in the dark. Glycinamide hydrochloride (88.43 mg) was dissolved in 0.5 mL aqueous 0.2 M MES/4 M urea /0.005% OGS (pH 4.7). EDC (38.2 mg)/2.2 mg sulfo-NHS were dissolved together in 0.5 mL aqueous 0.2 M MES/4 M urea/0.005% OGS (pH 4.7). The glycinamide solution (25 μL) was then added to the carboxyamidomethylated sample and then mixed with 25 μL of the condensation reagent to give a final concentration of 0.4 M glycinamide, 0.1 M EDC, and 5 mM sulfo-NHS, respectively. After 1.5 h incubation at 37°C, the samples were submitted to buffer exchange on Zeba desalt spin columns as described below. Reagents were prepared fresh for daily use.

One-Pot Protein Carboxyamidomethylation, Acetylation, and Glycinamidation

A model protein (50–200 pmoles) was carboxyamidomethylated as described above. A sulfo-NHS acetate solution (10 μL; 60 mM), in 10 mM sodium phosphate/4 M urea/0.005% OGS urea, was added to a final concentration of 10 mM. The mixture was incubated for 20 min at 55°C. The nucleophile solution (30 μL) and 30 μL of the condensation reagent, prepared as described above, were then added. The mixture was incubated for 1.5 h at 37°C and subjected to buffer exchange on Zeba desalt spin columns as described below. Reagents were prepared fresh for daily use.

Spin Column Gel Filtration

The derivatized protein samples were subjected to buffer exchange on Zeba desalt spin columns, according to the manufacturer's recommendations with minor modifications. A 50-mM ammonium bicarbonate/1 M urea/ 0.005% OGS solution (pH 8.0; 400 μL) was loaded on the resin bed, followed by centrifugation for 1 min at 1500 *g*. This step was repeated five times. The protein samples (100–120 μL) were applied to the spin columns and allowed to absorb on the resin. Buffer (10–20 μL) was then applied to the resin bed, followed by centrifugation for 2 min at 1500 *g*.

Trypsin Digestion

The buffer-exchanged protein samples were supplemented with 5 μL of an aqueous trypsin solution to give a substrate: enzyme ratio of 20:1. The digestion was allowed to proceed overnight at 37°C and arrested by addition of 5 μL 50% TFA. The sample volume was reduced to 40 μL by SpeedVac evaporation prior to peptide immobilization on ZipTip reversed-phase supports as described below. After sample immobilization, the $ZipTip_{C18}$ pipette tips were desalted by passing 100 μL 0.1% TFA in 10 μL aliquots over the resin.

Sample Immobilization

$ZipTip_{C18}$ pipette tips/$ZipTip_{\mu\text{-}C18}$ pipette tips were wetted six times with 10 μL methanol, followed by six washes with 0.1% TFA. Model peptides (10–50 pmoles) prepared in 0.2–1% TFA/0.01% OGS solutions were placed in 10 μL aliquots into 0.5 mL microfuge tubes and subjected to 10 sample-loading/dispense cycles. The immobilized test peptides were then washed three times with 10 μL 0.1% TFA and once with 10 μL water. For high-volume sample enrichment (<10 μL), in-gel proteolytic digests and digests prepared in solution were loaded sequentially in 10 μL aliquots onto $ZipTip_{C18}$ pipette tips and dispensed into a 0.5-ml microfuge tube. The sample was then transferred back in this step-wise mode to the original collection tube. This alternating load/dispense enrichment cycle was repeated five times. The ZipTip pipette tips were then washed five times with 10 μL 0.1% TFA. Optionally,

200 μL disposable pipette tips can be press fitted into $ZipTip_{\mu\text{-}C18}$ pipette tips or $ZipTip_{C18}$ pipette tips, as suggested by the manufacturer, and operated with a 200-μL pipettor to pass the sample slowly 10 times over the resin. Up to 200 μL sample can be processed for binding in this manner.[12]

General Procedure for Solid-Phase Peptide Derivatization

The nucleophile reagents (10 μL) were aspirated three times onto the $ZipTip_{C18}$ pipette tips or onto $ZipTip_{\mu\text{-}C18}$ 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. Unless stated otherwise, the samples were desalted by passing 100 μL 0.1% TFA over the resin in 10 μL aliquots. The products were eluted from $ZipTip_{C18}$ pipette tips in 5–10 μL 50% acetonitrile/0.1% TFA/0.01% OGS, of which 1 μL was used for MALDI-MS analysis. $ZipTip_{\mu\text{-}C18}$ pipette tips were eluted in matrix containing 0.1% TFA/0.01% OGS onto the MALDI-TOF plate. Reagents used in the solid-phase protocols described below were prepared fresh for daily use.

Solid-Phase 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. Sample processing for MALDI-TOF was as described above.

Solid-Phase Carboxylate Amination/Glycinamidation

EDA dihydrochloride or glycinamide hydrochloride was dissolved in aqueous 0.1 M MES to a final concentration of 0.8 M (pH 4.7). EDC and sulfo-NHS were dissolved together in aqueous 0.1 M MES to a final concentration of 0.2 M and 10 mM, respectively. The nucleophile solutions were combined with the condensation reagent in a ratio of 1:1. The mixtures (10 μL) were loaded on the $ZipTip_{C18}$ pipette tips. After incubation for 1.5 h at 37°C, the ZipTips were washed with 50 μL water and subsequently with 100 μL 0.1% TFA passed in 10 μL aliquots over the resin. Sample processing for MALDI-TOF was as described above.

Consecutive Solid-Phase Derivatization

Model peptides and tryptic digests derived from glycinamidated protein with and without prior acetylation were subjected to solid-phase acetylation as described above. Prior to reagent loading, the ZipTips were flushed three times to waste with 10 μL of the reagent. Sample clean-up after acetylation was omitted. Instead, the acetylation step was terminated by flushing the ZipTips three times to waste with 10 μl of the amination reagent; the reagent (10 μL) was then loaded onto the support. Reaction conditions for carboxyl group condensation were as described above. After derivatization, the ZipTips were washed with 50 μL water and subsequently with 100 μL 0.1% TFA passed in 10 μL aliquots over the resin. Products were eluted from $ZipTip_{C18}$ pipette tips in 10 μL 50% acetonitrile/0.1% TFA/0.01% OGS. During development of the protocol, 1 μL of the eluates was typically consumed for MALDI-TOF analysis.

Differential Peptide Mass Mapping

Protein digests reduced in volume to 40 μL were split in equal parts, bound to $ZipTip_{C18}$ pipette tips and subjected to acetylation. One sample was desalted and stored in 10 μL 0.1% TFA while immerged in solvent. The other sample was processed for carboxylate amination, as described above, and desalted. The tips were eluted typically in 10 μL aqueous acetonitrile, of which 1 μL was consumed for MALDI-MS. Optionally, $ZipTip_{\mu\text{-}C18}$ pipette tips were used, which were eluted directly in matrix onto the target.

NHS-Activated Sepharose Affinity Chromatography

The 50% resin slurry (100 μL), kept on ice, was placed into the spin column and centrifuged at 2000 *g* for 20 s, a centrifugation speed setting used throughout the protocol. The medium pellet was then resuspended by gentle agitation in 200 μL of an ice-cold, 1-mM hydrochloride acid solution, followed by centrifugation for 10 s. This step was repeated four times. The spin column was sealed with the plastic plugs supplied by the manufacturer, inserted into a 1.5-mL microfuge tube, and kept on ice. The derivatized protein digest was eluted from the reversed-phase support in 10 μL 50% acetonitrile/0.1% TFA/0.01% OGS directly into 50 μL 50 mM sodium phosphate/0.15 M sodium chloride/0.005% OGS (pH 8.0), used as affinity-coupling buffer. The mixture was transferred to the spin column, which was then gently agitated to resuspend the medium pellet. The spin column was inserted into 1.5 mL microfuge tubes, secured with Parafilm, incubated overnight at 4°C using a rotary mixer, and subsequently, centrifuged for 1 min to collect the unbound material. The affinity medium was then consecutively washed with 50 μL coupling buffer, 50 μL 60% aqueous acetonitrile/0.1% TFA/0.15 M sodium chloride, and 50 μL 80% aqueous acetonitrile/0.1% TFA/0.15% sodium chloride. The combined flow-through fractions were reduced in volume by SpeedVac evaporation to 60 μL and acidified with 5 μL 20% TFA. The recovered material was bound to

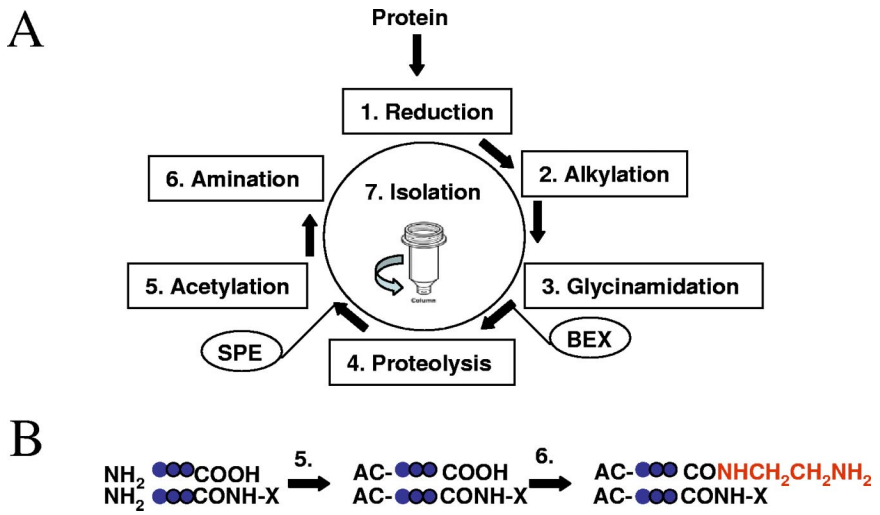

FIGURE 1

(*A*) Flow chart of sample preparation for C-terminal peptide isolation. The individual reaction steps are numbered and highlighted with boxes. Whole, intact protein is reduced (1), carboxyamidomethylated (2), followed by glycinamidation (3) in a one-pot reaction sequence. The protein preparation is then subjected to buffer exchange (BEX) by micro gel filtration and subsequently digested with trypsin (4). The digest is adsorbed on ZipTip$_{C18}$ reversed-phase supports (SPE) for on-phase sequential acetylation (5) and amination (6) of the newly exposed peptide N- and C-termini, respectively. In this procedure, the tips are loaded with the sulfo-NHS acetate solution, which after completion of the reaction, is exchanged directly on the solid phase for the EDA solution, supplemented with EDC and sulfo-NHS, abrogating the need for intermittent sample purification. After sample transfer to NHS-activated Sepharose, the amino group-functionalized N-terminal and internal peptides are covalently coupled to the affinity support. The C-terminal peptide is recovered by centrifugation in the flow-through fraction (7). (*B*) Schematic representation of peptide starting material and reaction products. X and AC denote glycinamide and acetyl group, respectively. The ethyleneamine affinity tag is highlighted in red. Note that the C-terminal peptide, since unaffected by amination, can be uniquely identified in the mass spectra of unfractionated digests by its unaltered mass signature (see Figs. 4 and 5).

ZipTip$_{C18}$ pipette tips or ZipTip$_{\mu\text{-}C18}$ pipette tips, which were then washed 10 times with 10 µL 0.1% TFA. Products were eluted with 5–10 µL 50% acetonitrile/0.1% TFA/0.01% OGS, of which 1 µL was consumed for MALDI-MS analysis. Peptides were eluted from ZipTip$_{\mu\text{-}C18}$ pipette tips in matrix directly onto the MALDI plate.

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. Laser intensity was typically set at 1690–2400, and spectra were acquired using 100–200 laser shots/spectrum. The data shown are based on three accumulated acquisitions. Some spectra were obtained in the linear mode using 80 laser shots/spectrum, and five to 10 spectra acquired from spots at different positions were averaged. For peptide fragmentation, a 4800 Proteomics Analyzer was used and operated in the MS and MS/MS modes; typical laser power for MS was ~3700. Usually, 1000–2000 shots were acquired for MS and 2000–20000 for MS/MS.

RESULTS

Reaction Scheme

As schematically illustrated in Fig. 1A, the sample preparation scheme is initiated by protein disulfide bond reduction (1), followed by carboxyamidomethylation of the liberated thiols (2). The protein is then glycinamidated using EDC to induce amide-bond formation between carboxylates and the amine nucleophile (3). The one-pot serial reaction mixture is subsequently buffer exchanged by gel filtration for tryptic digestion of the recovered fraction (4). The digest is then adsorbed onto ZipTip$_{C18}$ pipette tips for sequential solid-phase peptide amine acetylation (5) and EDC-mediated peptide carboxylate condensation with EDA (6). The conjugation reaction targeting the protoeolytically generated C-termini is halted by a clean-up step, followed by product elution and sample transfer to NHS-activated agarose for subsequent depletion of the amino group-functionalized N-terminal and internal peptides leaving the carbamidated C-terminal peptide since N-terminally blocked by acetylation in the flow-through fraction (7). The isolate is then subjected to structural characterization by collisionally induced fragmentation.

Sample Handling

To minimize adsorptive sample loss during sample handling, a miniaturized spin-column format was adopted to accommodate relatively small resin beds (50–500 μL) used for buffer exchange by gel filtration and for C-terminal peptide isolation by affinity chromatography. In addition, reagents and solvents were supplemented with trace amounts of OGS to minimize nonspecific protein/peptide adsorption. This nonionic detergent has been shown to minimize nonspecific protein/peptide adsorption and to promote peptide ionization in MALDI-MS. In addition, the additive proved fully compatible with liquid chromatography MS, owing to its strong retention on reversed-phase columns.[13,14]

As demonstrated in our study, the micro adaptation of the method proved particularly advantageous for performing serial derivatization at the peptide level using $ZipTip_{C18}$ pipette tips as highly miniaturized reaction beds (0.2–0.6 μL). Sample handling during sequential solid-phase derivatization is exceedingly simple, involving peptide adsorption on $ZipTip_{C18}$ reversed-phase pipette tips, followed by a solvent wash to remove unwanted matrix components. The tips are then loaded with reagent and left immersed in reagent during incubation. The subsequent chemical reaction is initiated by a reagent exchange in situ obviating sample transfer between the reaction steps. The reaction cycle is concluded by a clean-up step, followed by product elution.

We have used this solid-phase sample-handling format earlier for single-step phosphopeptide derivatization and showed that the chemical strategy afforded improved phosphoseryl peptide detection in unfractionated digest from model proteins and experimental samples.[15,16] We noted in this and the present study that the solid-phase derivatization format offers some notable advantages over the classical in-solution-based methods, the predominant sample preparation technique in current proteomic studies. It obviates the need for sample dry-down, commonly practiced in proteomics studies to concentrate peptide mixtures prior to derivatization in solution. This sample handling step can cause substantial adsorptive peptide loss ranging up to 50% or more of the starting solution after a single evaporation step, especially as seen with low-level samples.[17] The process of sample adsorption concentrates the analyte on the support, allowing the reaction to proceed in situ, in general, at higher efficiency and faster kinetics than in solution. Protein digests can be enriched effectively on the solid phase from dilute solutions, in which chemical reactions inherently proceed at slow reaction rates. Since the introduction of this concept to phosphoprotein characterization,[15] its apparent benefits have been exploited to prepare samples for facile de novo sequence interpretation.[18,19] As demonstrated in an earlier communication and in the current study, the solid-phase format proved particularly advantageous for performing serial chemical reactions, considered as problematic in proteomics studies because of the high-potential cumulative adsorptive sample loss incurred during intermittent sample manipulation.[17,20,21] Once the peptides are immobilized, reagents are exchanged directly on the support obviating the need for sample transfer between the reaction steps. Consequently, the analyte can be carried through the reaction scheme with minimal sample loss. It is noteworthy that derivatization on the solid phase has evolved as the predominant sample preparation technique for trace analysis of bioorganic compounds and has found wide-spread application in pharmaceutical and toxicological studies.[22]

Sample Preparation at the Protein Level

As illustrated in Fig. 1A, derivatization of the protein involved a series of chemical reactions, including protein disulfide reduction, carboxyamidomethylation of the liberated thiols, followed by glycinamidation to block the protein's C-terminal carboxyl group concomitantly with the internal asparatate and glutamate residue side-chains. To evaluate the efficiency of carboxylate condensation, a key feature of the sample preparation method, β-lactoglobulin, selected as test protein, was reduced and carboxyamidomethylated in a one-pot reaction and set aside as control. In parallel experiments, the carboxyamidomethylated sample was supplemented with glycineamide and the condensation reagent prepared in 0.1 M MES buffer/4 M urea (pH 4.7) to give a final concentration of 0.4 M nucleophile, 0.1 M EDC, and 5 mM sulfo-NHS. After 1.5 h incubation at 37°C, the sample was submitted to buffer exchange by gel filtration along with the carboxyamidomethylated control, followed by trypsination of the recovered fractions. After 18 h digestion, the samples were purified on $Zipip_{C18}$ pipette tips, eluted, and submitted to MALDI-TOF MS. Comparison of the MALDI mass spectra before and after carbamidation revealed that the reaction resulted in the expected mass addition of 56 Da/carboxyl group modification. Ions corresponding to underivatized material were not observed in the spectrum, indicating that the condensation reaction proceeded to completion (Fig. 2A and B). Comparable results were obtained when the incubation was extended for up to 8 h (results not shown). The data further showed that the modification had no significant impact on the peptides' ionization behavior, consistent with results obtained with secondary ion MS,[9] with the exception of the peptide at *m/z* 2313.7 corresponding to the tryptic peptide spanning residues 57–76 (VYVEELKPTPEGDLEILLQK), which exhibited a threefold lower signal intensity after carbamidation.

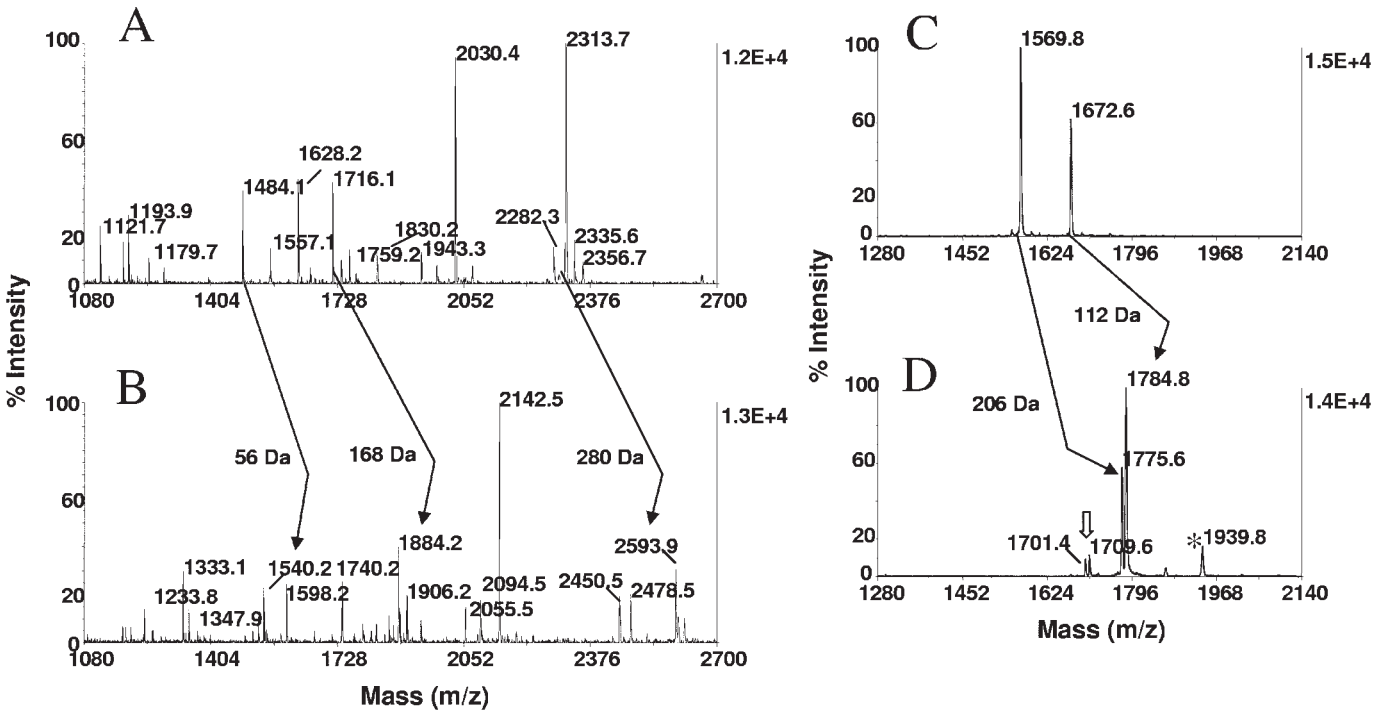

FIGURE 2

Protein/peptide glycinamidation. β-Lactoglobulin (50 pmoles) was reduced, carboxyamidomethylated, and glycinamidated in the sequential one-pot reaction sequence. Carbamidation was carried out in a 0.4-M nucleophile, 0.1 M EDC, and 5 mM sulfo-NHS solution containing 0.1 M MES buffer/4 M urea (pH 4.7). After incubation for 1.5 h at 37°C, the reaction was terminated by buffer exchange on gel filtration spin columns, followed by trypsination of the recovered fractions. After 18 h digestion, the samples were purified on $ZipTip_{C18}$ pipette tips, eluted, and submitted to MALDI-TOF MS. MALDI-TOF spectra of (*A*) digest of unmodified protein and (*B*) digest after sequential protein derivatization. Arrows indicate the 56-Da mass shifts/modified carboxylate. Note completeness of derivatization. One-tenth of the eluates was applied to the target. An equimolar mixture of [E^1] fibrinopeptide-EGVNDNEEGFFSAR and neurotensin was bound to $ZipTip_{C18}$ pipette tips and exposed on the solid phase to the conditions described above, except that urea was omitted from the reaction mixture. The glycinamidation reaction was terminated by a solvent wash, followed by product elution prior to MALDI-MS. MALDI-TOF spectra of (*C*) native peptides and (*D*) after glycinamidation. Note efficiency of derivatization. Arrows indicate derivatization mass shifts. Open arrow in *D* denotes low-abundance ions representing residual material. Asterisk denotes minor side-reaction product recognized as EDC-induced *O*-acryl isourea adduct of neurotensin (see Fig. 5). One-fifth of the eluates corresponding to ~1 pmole of peptide was applied to the target.

This result was unanticipated, as charge neutralization of acidic side-chains by carbamidation is thought to decrease the hydrophilicity of the peptide, thereby aiding its detection in positive ionization MALDI-MS. In addition, the preferred ionization of the native species itself contrasts the general perception that highly acidic peptides typically exhibit moderate signal intensities in positive ionization MALDI-MS.[23] Further studies are required to elucidate the factors involved in this ionization/desorption modulation effect.

An alternative protein carboxylate-labeling method, which we examined—methyl esterification in methanolic HCl—gave rise to partial deamidation of asparagine and glutamine, as well as oxidative side-reaction.[11,24] In this procedure, samples need to be taken to complete dryness before and after derivatization, a handling step known to promote adsorptive sample loss.[17] EDC-activated carboxyl groups reacted sluggishly with ethanolamine, an optional nucleophile, which we evaluated, requiring up to 16 h to drive the condensation reaction to completion, as reported previously.[11]

In a separate but related application of the method, a equimolar mixture of [E^1] fibrinopeptide B (EGVNDNEEGFF SAR, *m/z* 1570.5) and neurotensin (PyrLYENKPRPYIL, *m/z* 1672.9) was bound to $ZipTip_{C18}$ pipette tips and exposed on the solid phase to the conditions described in Fig. 2A and B, except that urea was omitted from the reaction mixture. The glycinamidation reaction was terminated by a solvent wash, followed by product elution prior to MALDI-MS. The MALDI-TOF spectra illustrated in Fig. 2C and D showed that the C-terminal and the internal

carboxylates of these peptides were effectively converted to their carbamidated analogs concomitantly with the formation of pyrogutamate by EDC-induced intramolecular condensation. Comparable results were obtained with fibrinopeptide A bearing four internal carboxyl groups. The nature of the side-reaction observed with neurotensin, which gave rise to the product at *m/z* 1939.8, is discussed later in the text (see Fig. 5). The protocol is anticipated to provide a tool to characterize acidic peptides by tandem MS/MS. The procedure advocated to this purpose involves carboxyl group methyl esterfication.[25] This chemistry has been shown to block in argininyl peptides the preferential fragmentation pathway C-terminally to aspartic and glutamic acid. As demonstrated with orcokinin neuropeptides, the resultant improved spectral information content facilitated localization of the substituted acidic side-chains. By analogy, glycinamidation of aspartic and glutamic acid side-chains is expected to render peptides amenable to nonselective backbone fragmentation. The chemistry proceeds under mild reaction conditions in pure aqueous solution and is devoid of side-reactions associated with methyl esterification. Further experimentation with model systems needed to establish the usefulness of this approach for acidic peptide structural characterization by tandem MS is currently being pursued in our laboratory.

Sample Preparation at the Peptide Level

In preparation for C-terminal protein characterization by MS, proteins are commonly protoeolytically digested for further experimentation. To this purpose, trypsin had been the enzyme of choice because of its high substrate specificity. In addition, protein glycinamidation has been shown to facilitate enzyme action at lysine and arginine by neutralizing proximal asparate and glutamate residues, thereby improving the quality of the peptide maps.[9] Our attempts to deplete the N-terminal and internal tryptic peptides through their newly formed carboxyl groups by EDC-mediated condensation with amino-functionalized supports proved, despite considerable effort, only partially successful. To remedy this situation, we devised a strategy to introduce an amino group-functionalized affinity tag into the N-terminal and internal peptides by EDC-catalyzed carboxylate conjugation with EDA and to remove these peptides by coupling to NHS-activated Sepharose. In this procedure, the carboxyl group labeling was preceded by amine acetylation to preclude potential EDC-induced interpeptide cross-reaction and importantly, to block this functionality in the C-terminal peptide, thereby preventing this species from coupling to the affinity support. In the practical implementation of this sequential reaction scheme, tryptic digests were bound to $ZipTip_{C18}$ pipette tips used as venue for derivatization, a sample-handling format that proved an ideal miniaturized interface between derivatization at the protein and peptide level (see Fig. 1).

To evaluate the efficiency of the serial derivatization, bradykinin at *m/z* 963.7, selected as a model peptide, was bound to $ZipTip_{C18}$ pipette tips and incubated in a phosphate-buffered 20 mM sulfo-NHS acetate solution (pH 8.0). After 20 min incubation at 55°C, the ZipTips were dispensed to waste and then loaded with a solution containing 0.4 M ethylene dihydrochloride/0.1 M EDC/5 mM sulfo-NHS in 0.1 M MES buffer (pH 4.7). In this manner, reagents were exchanged directly on the solid phase precluding sample transfer between the reaction steps. The condensation reaction was allowed to proceed for 1.5 h at 37°C. The ZipTips were desalted, eluted, and followed by MALDI-TOF MS of the recovered products. In parallel, an equimolar amount of peptide was acetylated to assess the efficiency of the initial reaction step. The MALDI-TOF spectra illustrated in Fig. 3A–C showed that the peptide's acetylation product was fully formed and that the subsequent amination reaction proceeded to completion. Minor ions observed in the spectra derived from sodium adducts and a peptide synthesis byproduct and its reaction products. Additional peptides examined with this protocol and found to react to completion included fibrinopeptide A, [E^1] fibrinopeptide B, and neurotensin (data not shown).

The protocol was then applied to tryptic digests of glycinamidated β-lactoglobulin. Comparison of the MALDI-mass maps prepared from the digest, before and after acetylation, revealed the characteristic peptide mass increment of 42 Da/modified residue (Fig. 4A and B). Subsequent amination of the acetylated peptides' free carboxyl groups imparted a mass shift of 42 Da to all peptides, with the exception of the fragment at *m/z* 1926.3, which thus, could be recognized as the C-terminal peptide by its unaltered mass signature (Fig. 4C, open arrow). The acetylation and amination steps are schematically illustrated in Fig. 1B. The C-terminal peptide identified in this simple manner can be selected from the mass map of the unfractionated digest for subsequent MS/MS structural characterization. We note here that C-terminal peptide identification prior to enrichment is not possible when relying on depletion strategies thus far proposed in the literature. For optimal use of low-level samples, $ZipTip_{\mu\text{-}C18}$ pipette tips can be used in this application. As the products can be deposited onto the target nearly undiluted, subpicomole mass detection can be readily attained.

We do recognize that C-terminal peptides in more complex mixtures can, owing to the signal suppression effect, be difficult to identify by differential peptide mass mapping. In addition, these peptides may, as a

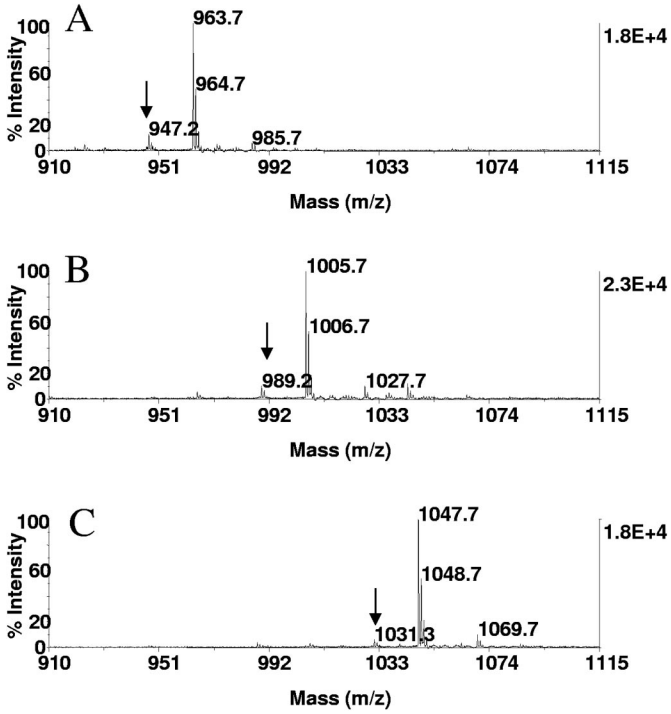

FIGURE 3

Solid-phase amino group acetylation with consecutive carboxylate amination. Bradykinin (5 pmoles) was adsorbed on ZipTip$_{C18}$ pipette tips and exposed to a PBS containing 20 mM sulfo-NSH acetate (pH 8.0). After 20 min incubation at 55°C, the reaction was terminated by a solvent wash. In parallel experiments, the acetylation reagent was replaced on the solid phase by a 0.1-M MES solution containing 0.4 M EDA dihydrochloride/0.1 M EDC and 5 mM sulfo-NSH. After 1.5 h incubation at 37°C, the condensation reaction was halted by a solvent wash. Reaction products recovered from the reversed-phase support were submitted to MALDI-MS. MALDI-TOF spectra of (*A*) native peptide, (*B*) after acetylation, and (*C*) after acetylation followed by carboxylate amination. Note completeness of sequential reactions. Arrows in *A*–*C* denote synthesis side-product and its derivatives. One-fifth of the eluates corresponding to ~1 pmole of peptide was applied to the target.

result of being too short, potentially escape identification by MS. We addressed this issue by including in the sample preparation an acetylation step preceding protein glycinamidation. As applied to β-lactoglobulin, the amine-blocking step prevents trypsin cleavage at lysines and thus, resulted in a noticeable simplification of the MALDI-TOF spectra (Fig. 5A and B). Similarly, myoglobin, processed at the protein level in this manner, yielded the C-terminal peptide at *m/z* 2002.8, generated with endoproteinase Arg-C-like specificity, and was clearly distinguishable from fragments derived from the protein's internal regions by its unaltered mass signature (Fig. 5D and E). However, digests prepared from high molecular weight proteins or protein mixtures are expected to be less amenable to this approach. In this situation, C-terminal peptide identification relies on affinity-based depletion of the redundant material, as discussed later in the text.

ZipTip$_{C18}$ Pipette Tip Chemical Compatibility Evaluation

Long-term C18 reversed-phase column testing (>1 week), with mobile phases at pH 9–12.3, has been shown to diminish to a minor extent (~2%) the retention of the silane-bonded stationary phase.[26] This effect was ascribed to mechanical attrition around the covalently attached silanes, eventually causing splitting off of the bonded organic phase, and could therefore, potentially reduce in our applications the analyte recovery from the silica-based reaction bed. In previous work, we have assessed the relative recovery of the model peptide angiotensin I (DRVYIHPFHL) from ZipTips before and after β-elimination with Michael addition (pH 12.3) using a stable isotope dilution technique.[16] The data showed that only a small fraction of the sample (~10%) could not be retrieved or was lost from the support after the alkali exposure. Accordingly, one would expect the analyte, under the considerably less-stringent reaction conditions described in Fig. 3, to remain firmly bound to the reversed-phase support.

To test the validity of this perception, fibrinopeptide B (PyrGVNDNEEGFFSAR, *m/z* 1552.5), N-terminally protected by pyroglutamate and devoid of lysine, was aminated on the solid phase, as described in Fig. 3, to fully block the internal and C-terminal carboxylates. Reaction products were eluted, briefly dried, reconstituted in 1% TFA/0.01% OGS, bound to ZipTip$_{C18}$ pipette tips in three replicates, and carried through the sequence of the chemical reactions as described in Fig. 3. Untreated samples were left aside as controls. The samples were analyzed by MALDI-TOF MS using a total of 640 laser shots/spectrum sampled from eight different spot positions. Comparison of the spectra showed that the peptide was recovered at comparable signal strength from the chemically treated and the untreated samples. Variations in signal intensity between the replicates were <15% (results not shown). The data suggest that the peptide's retention was little affected by the chemical treatment, rendering the reversed-phase support highly suitable for derivatization at minimal sample loss.

NHS-Activated Sepharose Affinity Chromatography

The method relies on capture of the amino group-functionalized N-terminal and internal peptides on the affinity support via formation of stable amide bonds between the reactants and subsequent separation of the unbound C-terminal peptides by centrifugation. Nonspecific peptide adsorption during this process is of concern, as it would reduce the recovery of the species of interest. We reasoned that micro adaptation of the method should minimize this

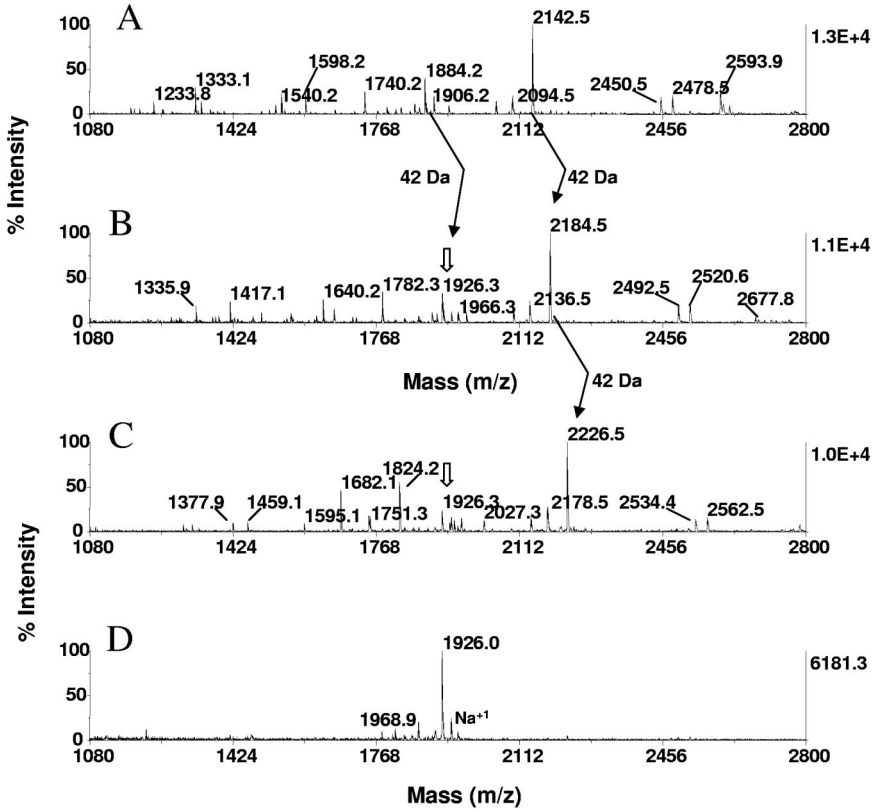

FIGURE 4

Protein digest conditioning and C-terminal peptide selection by affinity chromatography. Tryptic digests generated from glycinanmidated intact β-lactoglobulin (50 pmoles) were adsorbed on ZipTip$_{C18}$ pipette tips and carried through the sequential reaction scheme as described in Fig. 3. Eluates from the starting material, the acetylated, and sequentially derivatized samples were submitted to MALDI-TOF analysis. MALDI-TOF spectra of (*A*) unmodified digests, (*B*) digest after acetylation, and (*C*) digest after acetylation and subsequent carboxylate amination. Arrows across *A* and *B* indicate the 42-Da mass shift/amine imparted on all peptides. Arrows across *B* and *C* indicate the 42-Da mass shift/carboxylate imparted exclusively on non-C-terminal peptides. Open arrows in *B* and *C* denote the C-terminal peptide at *m/z* 1926.3, identified by its unaltered mass signature. The amino group-functionalized digest was submitted for affinity depletion on NHS-activated agarose. The flow-through fraction was concentrated on ZipTip$_{C18}$ pipette tips, eluted, and analyzed by MALDI-MS. Note that the C-terminal peptide at *m/z* 1926.0 was selectively recovered from the resin (*D*). The peptide was aligned by database search to the sequence LSFNPTQLEEQCHI, spanning residues 165–178. The ion at *m/z* 1968.9 represents residual, nonalkylated peptide.

effect, which is expected to become especially noticeable with the low-level analyte. To this purpose, a miniaturized spin column format was adopted to accommodate a relatively small reaction bed (50 μL). To assess the relative coupling/recovery efficiency of the method, equimolar amounts (1–10 pmole) of acetylated bradykinin, prepared as described in Fig. 3, and its native counterpart were eluted from ZipTip$_{C18}$ pipette tips into the affinity-coupling buffer, which was then loaded on the activated NHS support and end-over-end incubated overnight. The sample was centrifuged to recover unbound material. To collect residual material, the resin was washed with coupling buffer, 60% aqueous acetonitrile, and 80% aqueous acetonitrile containing 0.1% TFA/0.15% sodium chloride. The flow-through fractions were combined, reduced in volume, acidified, and adsorbed on ZipTip$_{C18}$ pipette tips, which were desalted and eluted. The eluates were analyzed by MALDI-TOF using a total of 640 laser shots/spectrum sampled from eight different spot positions. The MALDI-TOF spectra showed that the signal from the acetylated starting material and its recovered counterpart were of nearly equal abundance, indicating that the amino terminal-blocked peptide, mimicking a C-terminal peptide, was displaced effectively from the resin. An estimated 5–10% of the native peptide was typically found in the flow-through fraction, indicating that the coupling of the peptide to affinity support proceeded to near completion (data not illustrated). The data validate the use of the miniaturized covalent chromatographic system to selectively retrieve the analyte at minimal sample loss.

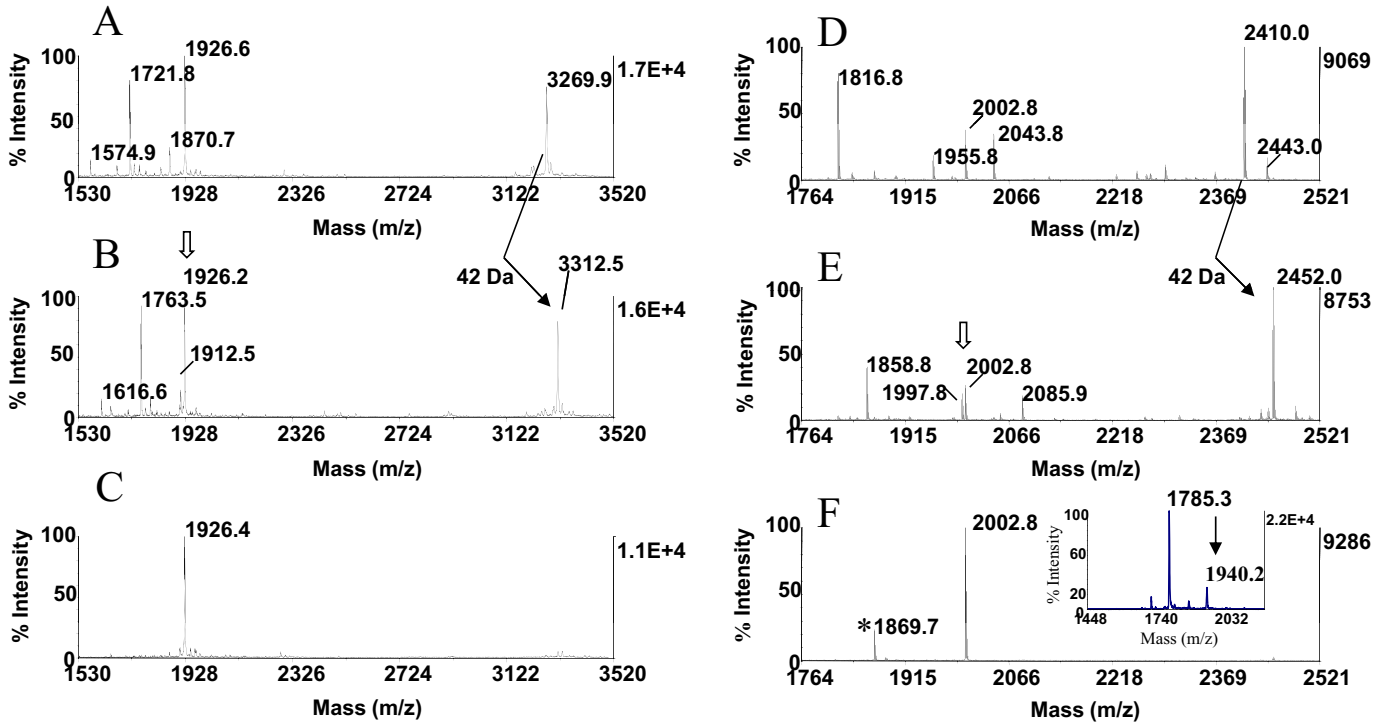

FIGURE 5

Protein-digest conditioning and C-terminal peptide selection by affinity chromatography. Whole, intact β-lactoglobulin (50 pmoles) and myoglobin (50 pmoles) were reduced, carboxyamidomethylated, acetylated, and glycinamidated in the one-pot reaction sequential scheme. The proteins were trypsinized after buffer exchange. After 18 h incubation at 37°C, the digests were purified on $ZipTip_{C18}$ pipette tips, conditioned for C-terminal peptide isolation, as described in Fig. 4, and subjected to affinity depletion on NHS-activated agarose. Expanded section of MALDI-TOF spectra of (*A* and *D*) digest of derivatized β-lactoglobulin and of derivatized myoglobin after solid-phase acetylation, respectively. (*B* and *E*) Digest of derivatized β-lactoglobulin and of derivatized myoglobin after sequential acetylation and carboxylate amination, respectively. (*C* and *F*) Isolates recovered from the flow-through fractions. Cross-arrows indicate mass shift/modified carboxylate. The C-terminal peptides recognized by the unaltered mass signatures are marked in *B* and *E* by open arrows. The C-terminal peptide at *m/z* 2002.8 matched by database search to NDIAAKYKELGFQG, spanning residues 141–154. Note that the derivative's mass is 155-Da higher than its carbamidated/acetylated counterpart attributable to EDC modification of tyrosine (see text for details). Inset in *F* shows spectrum of neurotensin (PyrLYENKPRPYIL, *m/z* 1672.2) after solid-phase glcycinamidation, exhibiting the same tyrosine modification-specific mass addition of 155 Da (arrow). Asterisk in *D* designates unidentified, low-abundance co-isolate.

The protocol was applied to the β-lactoglobulin and myoglobin digests that had been conditioned for C-terminal peptide isolation, as described in Figs. 4 and 5, respectively. The flow-through fractions were bound to $ZipTip_{C18}$ pipette tips, which were then desalted and eluted. MALDI-MS of the eluates revealed the prominent ion at *m/z* 1926.0, and there was very little background contamination (Figs. 4D and 5C). The peptide matched to the known protein sequence of the C-terminal fragment, spanning residues 156–178 corresponding to LSFNPTQLEEQCHI. The latter digest yielded the isolate at *m/z* 2002.8, which aligned to the known protein sequence, with the C-terminal fragment spanning residues 141–154 corresponding to NDIAAKYKELGFQG. The peptide sequences were confirmed by MALDI-TOF/TOF MS (Fig. 6A and B). Taken together, the data validate the use of a miniaturized sample processing/covalent chromatographic system to effectively isolate C-terminal peptides from whole, intact protein.

The nominal mass of the myoglobin isolate proved 155 Da higher than its acetylated/carbamidated counterpart, with a calculated mass of 1847.7 Da, a characteristic mass addition that is indicative for *O*-aryl isourea adduct formation between EDC and the phenolic hydroxyl group of tyrosine.[27] As shown in Fig. 5D–F, the C-terminal peptide was identified as a single product, indicating that tyrosine reacted on the whole, intact protein to completion. To gain insight in the kinetics of this modification, neurotensin (PyrLYENKPRPYL, *m/z* 1673.2) was bound to $ZipTip_{C18}$ pipette tips and exposed to the conditions described in Fig. 2D. The MALDI-TOF spectrum of the sample illustrated

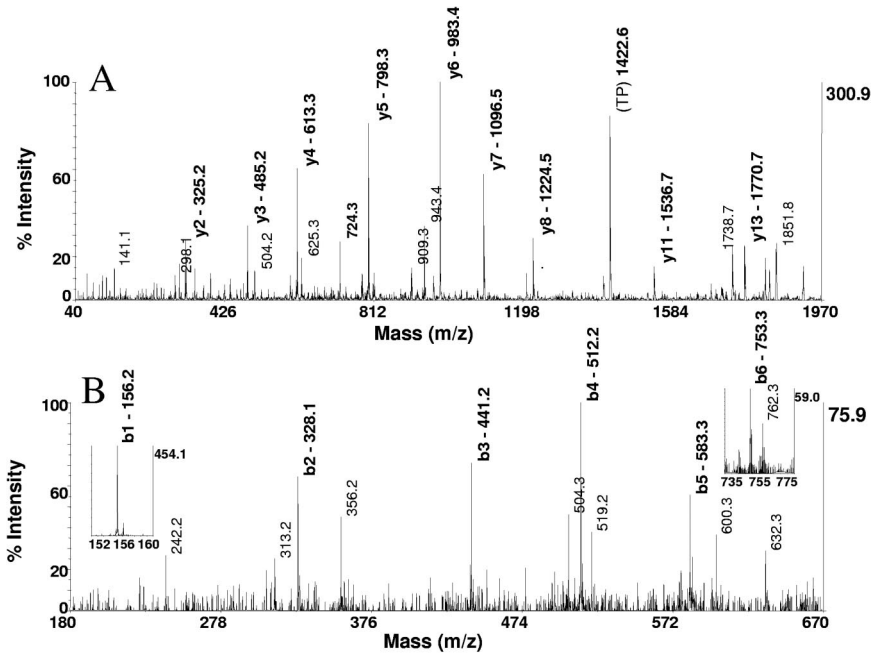

FIGURE 6

C-terminal peptide structural characterization by MALDI-TOF MS/MS. The isolates recovered from the model proteins were subjected to sequence analysis by MALDI-TOF tandem MS. MALDI-TOF/TOF spectrum of (*A*) the C-terminal peptide at *m/z* 1926.4 enriched from the tryptic digest of β-lactoglobulin as described in Fig. 5. The y ion series annotated in the spectrum produced at high abundance is nearly uninterrupted and yielded the peptide sequence ^{165}LSFNPTQLE EQCHI ^{178}. The derivatization discriminates the sites of glycinamidation as the unique residue mass of 185 Da contained in the product ions y5 and y6. The product y2 in the spectrum confirmed the presence of the label at the C-terminal residue. MALDI-TOF/TOF spectrum of (*B*) the C-terminal peptide at *m/z* 2002.8 enriched from the tryptic digest of myoglobulin as described in Fig. 5. The sequence tag (NDIAAK) deduced from the b ion series, b1–b6, yielded the sequence ^{141}NDIAAKYKELGFQG^{154} by database search. Note that the predominant b1 ion at *m/z* 156.2 results from the preferred cleavage C-terminal to the acetylated asparagine as the major pathway of fragmentation.

in Fig. 5F, inset, revealed the fully glycinamidated peptide at *m/z* 1785.3 and the low-abundance product at *m/z* 1940.2 displaying the diagnostic mass signature of 155 Da (arrow). Comparable results were obtained with angiotensin I (DRVYIHPFHL) bearing a single tyrosine residue (results not shown). The data indicate that the extent of the tyrosine modification is subject to amino acid sequence-dependent modulation. Treatment of the hydroxyl-substituted peptide with hydroxylamine, reported to reverse the tyrosine modification by ester bond hydrolysis in proteins, is expected to address this unwanted signal dilution.[27]

C-Terminal Peptide Structural Characterization

Representative MALDI-TOF/TOF data obtained from the β-lactoglobulin C-terminal isolate at *m/z* 1926.4 are illustrated in Fig. 6A. As exemplified by the resultant product ion spectrum of the derivative, a nearly uninterrupted y ion series was produced in high abundance, enabling facile MS/MS data interpretation. The glycinamide tag was readily located at Residue Positions 173, 174, and 178 in the peptide sequence ^{165}LSFNPTQLEEQCHI^{178} by the presence of the product ions y2, y4, y5, and y6. The glycinamide group proved stable under the conditions of collision-induced dissociation (CID) used. Product ion diagnostics for *O*-acylated serine and threonine were not observed in the spectrum. MS/MS analysis of the myoglobin C-terminal fragment at *m/z* 2002.8 produced the continuous b ion series b1–b6, dominated by b1 corresponding to the acetylated N-terminal fragmentation product (Fig. 6B). The preferential cleavage C-terminal to the acetylated asparagine resulted, as expected, in diminished peptide backbone fragmentation. However, the sequence tag (NDIAAK) deduced from the data enabled unambiguous identification of the C-terminal peptide ^{141}NDIAAKYKELGFQG^{154} by database search.

In-Gel Protein Glycinamidation

Using gel-separated rat serum albumin we next developed an in-gel protocol of this method. We reasoned that this format would be advantageous, as proteins are frequently presented in gel-separated form for digestion. This technique has been shown to accommodate relatively low-level amounts of protein (>2 pmoles).[17] GelCode blue-stained bands from 5 to 50 pmoles rat serum albumin were loaded

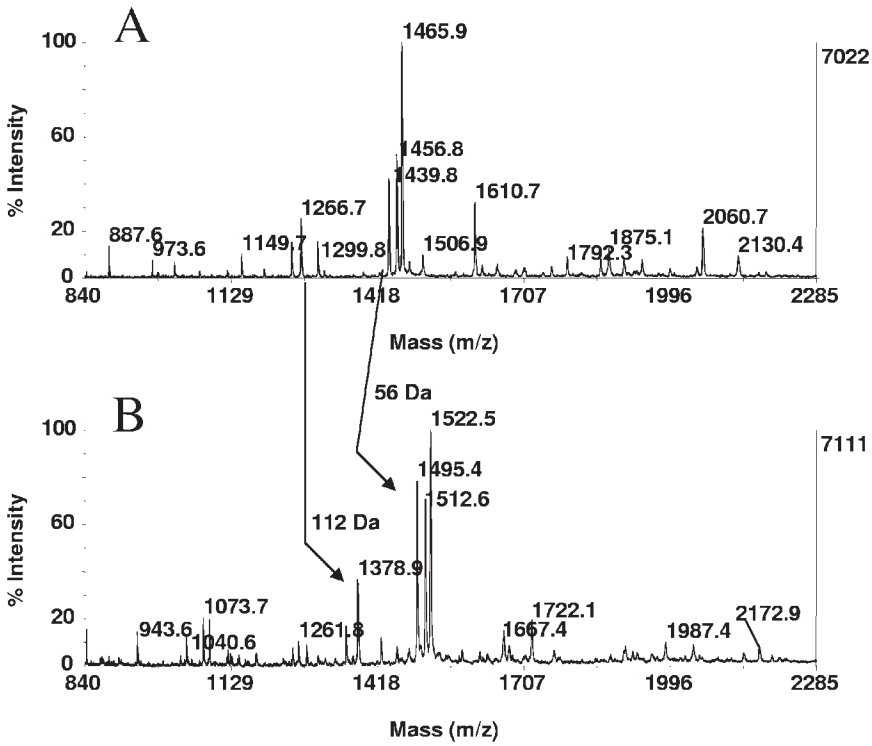

FIGURE 7

In-gel protein glycinamidation. GelCode blue-stained bands from 10 pmoles rat serum albumin, loaded onto the gels, were destained and carboxyamidomethylated and subsequently glycinamidated as described in Materials and Methods. Carbamidation was carried out in a 0.5-M nucleophile, 0.25-M EDC, and 5-mM sulfo-NHS solution containing 0.1 M MES buffer/6 M guanidine hydrochloride (pH 4.7). After incubation for 1.5 h at 37°C, gel bands were processed for in-gel tryptic digestion for 18 h at 37°C. Gel extracts from the derivatized samples and untreated controls were purified on $ZipTip_{C18}$ pipette tips. MALDI-TOF spectra of (*A*) digest of carboxyamidomethylated control and (*B*) digest after glycinamidation. Cross-arrows indicate peptide mass shifts of 56 Da/modified carboxylate. Note that peptide ionization efficiency was moderately enhanced or reduced (10–30%) as a result of the derivatization.

onto the gels, destained, carboxyamidomethylated, and subsequently incubated in a 0.1-M MES/6-M guanidine solution (pH 4.7) containing 0.5 M glycinamide hydrochloride/0.25 M EDC and 5 mM sulfo-NHS. After 1.5 h incubation at 37°C, bands were 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 glycinamidation revealed that the reaction resulted in the expected mass addition of 56 Da for each modification (Fig. 7A and B). Underivatized starting material was not observed in the spectra, indicating that the reaction proceeded to completion. The data showed that carboxylate glycinamidation could be performed effectively in the gel matrix, leaving the protein intact for further manipulation. The high quality of the spectra produced suggests that lower amounts of protein should be amenable to in-gel glycinamidation. We are currently evaluating the use of silver-stained protein to determine the mass detection limits of the gel-based sample preparation method, as well as the use of accelerated in-gel digestion, which would afford a throughput of 1 day/sample set.[28]

DISCUSSION

Future Developments: Large-Scale Proteome Profiling

We are currently exploring the use of commercially available gel filtration and reversed-phase spin columns for chemistry scale-up, needed to adapt the sample preparation method for large-scale C-terminal peptide profiling. These spin columns accommodate up to 300 μg digest, corresponding to amounts typically available for further analysis after prerequisite prefractionation of proteomics mixtures. Although selective isolation of C-terminal peptides would result in a reduction in sample complexity by one order of magnitude, orthogonal prefractionation at the protein level is required to render the isolates amenable to liquid chromatography tandem MS, currently capable of resolving ~10,000 peptides in a single operation.[29] Our preliminary experiments with model systems showed that the sample preparation method can be readily adapted to the spin-column derivatization format. In recent work, we have begun to evaluate the scaled-up sample-preparation method with HPLC fractions prepared from cell lysates, with a future view to use differential stable-isotope labeling to measure protein expression levels in complex biological systems.

Conclusions

A sample preparation method for protein C-terminal peptide isolation from protein digests has been developed. The approach uses a series of chemical reactions at the protein and peptide level. Whole, intact protein was glycinamidated after carboxyamidomethylation in a one-pot reaction to label the C-terminal carboxyl group, as well as the carboxyl groups distributed along the polypeptide chain. Optionally, the carbamidation reaction was preceded by α- and ϵ-amine acetylation. The derivatized protein was then digested with trypsin using micro gel filtration spin columns for the prerequisite buffer exchange. Subsequent sample processing by this strat-

egy involved adsorption of the digest on $ZipTip_{C18}$ reversed-phase supports for on-phase sequential acetylation and amination of the newly exposed peptide N- and C-termini, respectively. The use of reversed-phase supports as a miniaturized reaction bed promoted the efficiency of the chemical reactions and enabled analyte processing with minimal sample loss. By this sequence of solid-phase reactions, the C-terminal peptide can be recognized uniquely in mass spectra of unfractionated digests of moderate complexity, which is not possible when relying on depletion methods so far advocated in the literature. The N-terminal and internal peptide's amino group, functionalized in this manner, were readily captured on NHS supports, leaving the C-terminal peptide in the flow-through fraction. The use of the sample preparation method was demonstrated with low-level amounts of a model protein. The C-terminal peptides were retrieved selectively from the affinity resin and proved highly suitable for structural characterization by CID. An in-gel glycinamidation method was developed to extend the scope of the method to gel-separated proteins. The sample preparation method combines robustness with simplicity of operation using standard equipment readily available in most biological laboratories and is expected to be readily expanded to gel-separated proteins.

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DISCLOSURE

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

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