Chapter 3

Phosphopeptide Enrichment by Covalent Chromatography After Solid Phase Derivatization of Protein Digests on Reversed Phase Supports

Heinz Nika, Ruth Hogue Angeletti, and David H. Hawke

Abstract

The isolation of the phosphopeptide constituents from phosphoprotein digests is prerequisite to facilitate the mass spectrometric characterization of phosphorylation events. Here, we describe a chemical proteomics approach which combines solid phase derivatization of phosphoprotein digests with phosphopeptide enrichment by covalent chromatography. The use of the solid phase support for derivatization ensures for speed and completeness of reactions. The isolates proved highly suitable for mapping of the sites of phosphorylation by collisionally induced dissociation (CID). The method combines robustness with simplicity of operation using equipment available in biological laboratories, and may be readily extended to map the sites of O-glycosylation.

Key words Mass spectrometry, β-elimination/Michael addition, Reversed phase support, Multi-step solid-phase derivatization, Phosphopeptide enrichment, Covalent chromatography, Phosphorylation site determination

1 Introduction

Protein phosphorylation is recognized as a critical event in modulation of cellular processes including cellular signaling, cell cycle progression and differentiation [1].

Therefore, the determination of phosphorylation sites by mass spectrometry (MS) has become increasingly important. However, these efforts are challenged by the ionization inefficiency of the phosphopeptides, their low stoichiometry and the limited information content of the tandem MS (MS/MS) spectra due to neutral loss of the phosphate group upon collision induced dissociation (CID). Immobilized metal ion affinity chromatography (IMAC) and titanium dioxide (TiO_2) chromatography are frequently used techniques to address these problems by phosphopeptide enrichment [2, 3]. In many instances these methods had been inefficient due to co-adsorption of non-phosphorylated (acidic) peptides.

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TiO_2 chromatography has been shown to poorly enrich phosphopeptides derived from basophilic kinase substrates. Although the above strategies afford improved phosphopeptide detection, issues still persist with regard to intrinsic instability of phosphate groups often rendering site mapping subject to ambiguity under the conditions of CID.

β-elimination of phosphate from serine and threonine residues coupled with Michael addition (BEMAD) provides for a chemical strategy to enrich phosphopeptides from unfractionated protein digests. This approach has been successfully adopted by several laboratories to map phosphorylation events in isolated proteins and on the proteome-wide scale [4–9] and has also been exploited to enrich for proteolytic fragments bearing O-linked β-N-acetylglucosamine (O-GlcNAc) [10]. In this strategy, dithiothreitol (DTT) was used as nucleophile in the Michael addition reaction. The thiol adducts were then captured by thiol-disulfide interchange on activated Thiol Sepharose, and reductively released from the affinity support; a procedure based on the concept of covalent chromatography [5]. Differentiation between phosphorylation and O-glycosylation was afforded by enzymatic dephosphorylation which renders the phosphopeptide component in the mixture insensitive to enrichment [10]. The nucleophilic substitution of the phosphorylated (glycosylated) residues precluded neutral loss of phosphate (glycan); the preferential pathway of fragmentation by CID. In consequence, the isolates sequenced noticeably more informatively than their native counterparts facilitating phosphorylation site determination. However, the application of the method to the characterization of the phosphoproteome of human whole saliva revealed that the enriched fractions contained mostly singly phosphorylated peptides [9]. We encountered the same complication in attempts to isolate the DTT adduct of the tetraphosphorylated peptide of β-casein [11]. We found that cross-link formation between the adjacent phosphosites through intramolecular Michael addition rendered the peptide inert to enrichment by covalent chromatography. This observation strongly suggests that this class of hyper-phosphorylated peptides, accounting for ~37 % of phosphopeptides in eukaryotic proteins, is in general excluded from isolation by the dithiol-based affinity enrichment approach [12]. Notably, the phosphoramidate chemistry (PAC) used for large-scale phosphopeptide isolation from digests of *Drosophila melanogaster* Kc167 cells also exhibited a strong bias against the selection of multiply phosphorylated peptides [13]. In addition, this method regenerates the original phosphopeptides rendering site mapping subject to ambiguity under the conditions of CID. These shortcomings prompted us as to devise an alternative strategy and the method that evolved from these efforts is the subject of the protocol [11].

The method's work-flow and a schematic depiction of the structure of the analyte and its derivatives are illustrated in Fig. 1a, b, respectively. In this protocol the phosphoprotein digest is extracted on a ZipTip$_{C18}$ pipette tip (SPE) and submitted in situ to sequential performic acid oxidation, acetylation and BEMAD using 2-aminoethanethiol as nucleophile. The amine-protection step renders the amino groups of the Michael addition products (i.e. the *N*-acetyl *S*-2-aminoethyl-/β-methyl-*S*-2-aminoethylcysteine derivatives) as the sole targets for subsequent acylation with sulfosuccinimidyl-2-(biotinamido)-ethyl-1, 3-dithiopropionate (Sulfo-NHS-SS-Biotin). In this sequence of solid phase reactions, denoted as N-thiolation, the biotinylated digest is then exposed to hydroxylamine for reversal of hydroxyl group acylation followed by reductive release of the disulfide-linked biotinamido moiety from the conjugates. The Michael addition derivatives, selectively thiolated

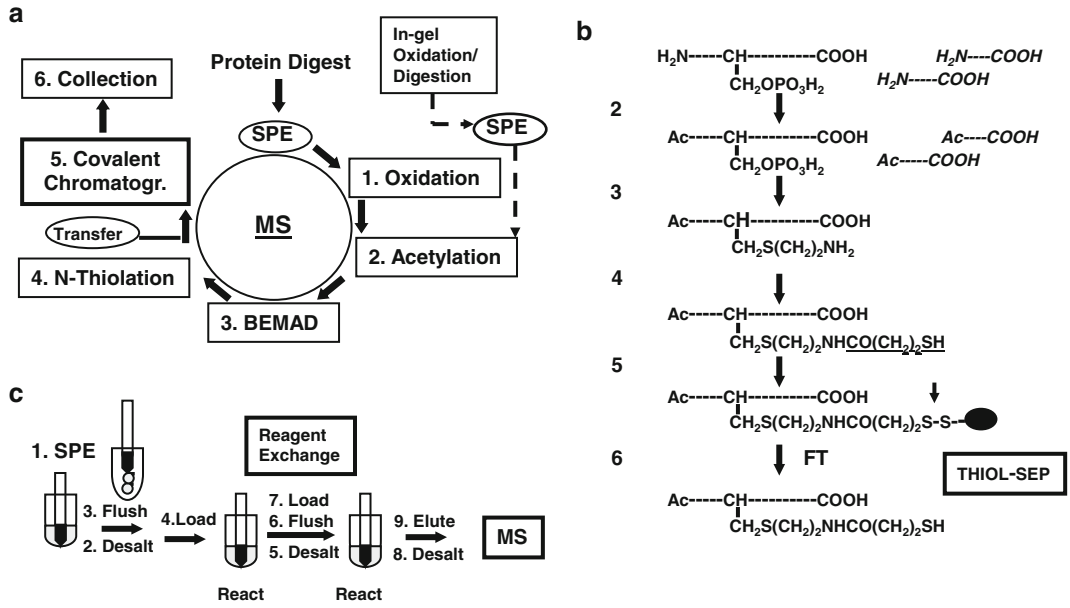

Fig. 1 (**a**) Work-flow for Phosphopeptide Enrichment. The reaction steps are numbered and highlighted with boxes. The digest is extracted on a ZipTip$_{C18}$ pipette tip (SPE), carried through the sample preparation scheme and submitted to covalent chromatography. Non-bound material is removed by a solvent wash. The disulfide-bonded phosphopeptide analogs are reductively released from the affinity support and collected in the flow-through fraction. BEMAD designates β-elimination with Michael addition. Flow path modification accommodating digests recovered from in-gel oxidized protein is indicated by *stippled arrows*. (**b**) Schematic representation of the analyte and its derivatives. The affinity tag is *underlined*. Ac and THIOL-SEP denote acetyl group and activate-Thiol Sepharose, respectively. Non-phosphorylated peptides are highlighted in *italics*. (**c**) Schematic representation of solid phase sample handling steps. After solid phase extraction (SPE) the analyte is desalted. Then the ZipTip is briefly flushed with reagent, loaded with reagent and incubated while immersed in reagent. In this manner reagents are exchanged between the reaction steps in situ eliminating intermittent sample transfer. The reaction cycle is concluded by a solvent wash prior to product elution

in this manner, are then enriched by covalent chromatography, reductively released from the affinity support and collected in the flow-through fraction. Co-isolation of cysteinyl peptides is prevented by the initial reaction step which converts cysteine to its sulfonic acid analog, thereby making cysteinyl peptides insensitive toward BEMAD. Alternatively, gel-separated protein can be oxidized and digested in the gel matrix [11]. In this way the reactivity of cysteine toward BEMAD is arrested prior to adsorption of the digest on the reversed phase support.

Figure 1c depicts the handling steps employed to carry the digest through the sample preparation method. After SPE the immobilized digest is desalted, briefly flushed with reagent which is then loaded onto the reversed phase support. In the process of adsorption the analyte is concentrated on the solid phase typically by a factor of 100 or more relative to solutions of the same original concentration [14]. As a result, chemistries proceed on the solid phase at higher efficiency and faster kinetics than in solution providing a significant advantage when dilute samples known to react poorly in solution are processed by this technique. In the process of serial derivatization reagents are exchanged in situ thereby eliminating intermittent sample transfer. In consequence, the derivatives are carried through the multi-step sample preparation scheme with minimal loss; a task that in general has met with moderate success when sequential derivatization is carried out in the solution phase; the predominant reaction format in proteomics studies [15]. It is noteworthy that modern analytical sample preparation techniques for the reasons outlined above are almost exclusively based on solid phase derivatization and have found widespread use for automated, high-throughput trace analysis of bioorganic compounds in toxicology, environmental, and pharmaceutical studies [14].

2 Materials

Prepare all solutions using ultrapure water with 18 MΩ cm resistivity at 25 °C. Unless stated otherwise prepare reagent solutions fresh for daily use. Reagents should be of analytical grade.

2.1 Gel-Electrophoresis and In-Gel Protein Digestion Reagent/Solvent Setup

1. 10 % gels of 1 mm thickness (Criterion Tris–HCl Precast gel, 133×87 mm, 18 wells, Bio-Rad, Hercules, CA) or alternatively Criterion Tris–HCl Precast 10–20 % gradient gels (same dimensions as the continuous gels).
2. GelCode Blue Stain Reagent (Pierce Corp., Rockford, IL), a Coomassie blue-based reagent.
3. Gel Destaining Solution: 25 mM ammonium bicarbonate, 50 % aqueous acetonitrile. Prepare a 0.25 M ammonium bicarbonate stock solution. Dissolve 179.6 mg of ammonium bicar-

bonate in 10 mL water. Mix 1 mL 0.25 M stock solution with 5 mL acetonitrile and 4 mL of water. Store the Destaining Solution at 4 °C.

4. Disulfide Reductant: 2 mM tris [2-carboxyethyl] phosphine hydrochloride (TCEP), 25 mM ammonium bicarbonate. Prepare a 25 mM ammonium bicarbonate solution by diluting the 0.25 M stock solution with water in a 1:10 ratio. Add 8 μL of the 0.5 M TCEP stock solution (Bond Breaker TCEP solution, Pierce Corp, Rockford, IL) to 1992 μL 25 mM ammonium bicarbonate. Store reagent at 4 °C.
5. Digestion Buffer: 25 mM ammonium bicarbonate, 0.01 % of *N*-octyl glucoside (OGS) (Roche Diagnostics, Indianapolis, IN). Dissolve 20 mg OGS in 1 mL 25 mM ammonium bicarbonate. Add 10 μL of the 2 % OGS stock solution to 1990 μL 25 mM ammonium bicarbonate. Store buffer at 4 °C.
6. Enzyme Solution: Sequencing grade modified trypsin (Promega, Madison, WI), 25 mM ammonium carbonate, 0.01 % OGS. Dissolve 25 μg of enzyme in 2 mL digestion buffer (12.5 ng/μL). Store enzyme solution in 100 μL aliquots at −20 °C. The enzyme remains active under this condition for several months.
7. Peptide Extraction Solvent: 0.1 % TFA. Prepare a 1 % aqueous TFA stock solution by mixing 10 μL of neat TFA with 990 μL water. Prepare 0.1 % TFA by diluting the stock solution with water in 1:10 ratio. Store solvent at 4 °C.

2.2 Solid Phase Derivatization Reagent/Solvent Setup

1. Performic Acid Oxidation Reagent: 0.3 % performic acid. Prepare reagent by mixing 30 % aqueous hydrogen peroxide with 96 % formic acid (5:95, v/v). Leave the reagent at room temperature for at least 30 min (*see* **Note 1**). Dilute with water at a 1:5 ratio to a final concentration of 0.3 % (pH 3.1). Store reagent at 4 °C for up to 1 h.
2. Acetylation Reagent: 20 mM of *N*-hydroxy sulfosuccinimide ester of acetic acid, 20 mM sodium phosphate (pH 8.0). Prepare a 0.25 M sodium phosphate buffer stock solution. Dissolve 3.45 g of $NaH_2PO_4 \cdot H_2O$ in 50 ml water (0.5 M Solution A). Dissolve 9.95 g of $Na_2HPO_4 \cdot 12H_2O$ in 50 ml water (0.5 M Solution B). Complete dissolution may require heating and vigorous stirring. Mix 23.7 ml Solution B with 1.3 ml Solution A and add 25 ml of water. Store the 0.25 M phosphate buffer stock solution at 4 °C. Mix 0.8 ml of the stock solution with 9.2 ml of water to give a final concentration of 20 mM sodium phosphate. Dissolve 5.2 mg Sulfo-NHS acetate (Pierce Corp., Rockford, IL) in 1 mL 20 mM sodium phosphate (*see* **Note 2**).

3. Concurrent BEMAD Reagent: 66 mM barium hydroxide, 33 mM 2-aminoethanethiol hydrochloride (pH 12.3). Prepare a 100 mM barium hydroxide stock solution by dissolving 31.54 mg of barium hydroxide octahydrate (Sigma-Aldrich, Milwaukee, WI) in 1 mL water (*see* **Note 3**). Prepare a 100 mM 2-aminoethanethiol hydrochloride stock solution by dissolving 11.36 mg of 2-aminoethanethiol hydrochloride (Sigma-Aldrich, Milwaukee, WI) in 1 mL water. Mix the barium hydroxide solution with the 2-aminoethanethiol hydrochloride solution in a ratio of 2:1. Store reagent at room temperature for daily use.
4. Consecutive BEMAD Reagents:
   (a) β-elimination Base: 50 mM barium hydroxide. Dilute 100 mM barium hydroxide with water in a 1:1 ratio. Store reagent at room temperature for daily use.
   (b) Michael Addition Reagent: 100 mM 2-aminoethanethiol hydrochloride, 75 mM barium hydroxide (pH 10.6). Mix 100 mM barium hydroxide with 400 mM 2-aminoethanethiol hydrochloride (45.44 mg/mL) in a 3:1 ratio. Store reagent at room temperature for daily use.
5. Biotinylation Reagent: 20 mM Sulfo-NHS-SS-Biotin (Pierce Corp., Rockford, IL), 20 mM sodium phosphate (pH 8.0). Dissolve 6 mg Sulfo-NHS-SS-Biotin in 1 mL 20 mM sodium phosphate (*see* **Note 4**).
6. O-deacylation Reagent: 2 % hydroxylamine hydrochloride (Pierce Corp., Rockford, IL), 1 M sodium carbonate (pH 9.4). Prepare a 1 M sodium carbonate solution by dissolving 105.9 mg sodium carbonate in 1 mL water. Dissolve 20 mg hydroxylamine hydrochloride in 1 mL 1 M sodium carbonate. Store reagent at room temperature for daily use.
7. Disulfide Reductant: 5 mM TCEP, 20 mM sodium phosphate (pH 8.0). Add 20 µL of the 0.5 M TCEP stock solution to 980 µL of 20 mM sodium phosphate (pH 8.0). Store reagent at room temperature for daily use.
8. Sample Cleanup Solution: 2 mM ethylenediaminetetraacetic acid (EDTA). Dissolve 7.3 mg EDTA in 10 mL water. Store the solution at 4 °C.
9. Sample Cleanup Solvent: 0.1 % TFA. Prepare a 1 % TFA stock solution by mixing 10 µL of neat TFA with 990 µL water. Prepare 0.1 % TFA by diluting the stock solution with water in 1:10 ratio. Store solvent at 4 °C.
10. Sample Eluate: 50 % aqueous acetonitrile, 0.1 % TFA, 0.01 % OGS. Mix 390 µL water with 500 µL acetonitrile and 100 µL 1 % TFA. Add 10 µL 2 % OGS. Store eluate at 4 °C.

2.3 Affinity Purification Reagent/ Solvent Setup

1. Activated-Thiol Sepharose 4B Gel: Weigh 1 g of activated-Thiol Sepharose 4B (GE Healthcare, Piscataway, NJ) and place the medium into a scintillation glass vial or equivalent. Add 10 ml water. Mix gently. The resin swells within 10 min and should give a ~4 ml settled medium. Wash medium in an appropriate glass filter funnel by vacuum filtration with a total of 150 ml of water added in 15 ml aliquots. Suspend medium in 10 ml of 10 % aqueous ethanol with gentle agitation, transfer medium to a 10 ml scintillation glass vial or equivalent. Store medium at 4 °C for at least 2 weeks (*see* **Note 5**).
2. Affinity Coupling Buffer: 50 mM sodium phosphate, 2 mM EDTA (pH 8.0). Add to a 15 mL Falcon tube 2 mL 0.25 M sodium phosphate stock solution and 8 mL water. Add 7.3 mg EDTA. Vortex vigorously to facilitate dissolution. Store the Coupling Buffer at 4 °C.
3. Disulfide Reductant: 50 mM sodium phosphate, 5 mM TCEP (pH 8.0). Add to a 15 mL Falcon tube 0.5 mL of the 0.25 M sodium phosphate stock solution, 1.975 mL water and 25 μL 0.5 M TCEP. Store the reductant at 4 °C.
4. Resin Wash Solvent A: 60 % acetonitrile, 0.1 %. TFA. Prepare a 10 % TFA stock solution by adding 0.1 mL neat TFA to 0.9 mL water. Mix 6 ml acetonitrile with 3.9 mL water and 0.1 ml 10 % TFA. Store solvent at 4 °C.
5. Resin Wash Solvent B: 80 % acetonitrile, 0.1 % TFA. Mix 8 mL of acetonitrile with 1.9 mL water and 0.1 mL 10 % TFA. Store solvent at 4 °C.
6. α-cyano-4-hyroxycinnamic acid (Agilent Technologies, Palo Alto, CA), 0.1 % TFA. Add 10 μL 1 % aqueous TFA to 90 μL matrix.

2.4 Test Analysis and Diagnostic Test

1. Bovine α-S1 and β-casein.
2. Test peptides: DAM1 phosphopeptide SFVLNPTNIGMp SKSSQGHVTK (AnaSpec, San Jose, CA); angiotensin I peptide DRVYIHPFHL (Sigma-Aldrich, St. Louis, MO); cysteinyl peptide Somatostatin CKNFFWKT, *m/z* 1073.2 (Sigma-Aldrich, St. Louis, MO).

2.5 Equipment

1. Gilson pipettor P20, model Pipetman classic, 2–20 μL displacement or equivalent.
2. Bench top centrifuge Eppendorf 5415 D or equivalent.
3. Rotary mixer.
4. Modular block heater.
5. Thermomixer.
6. Savant SpeedVac concentrator.

7. MALDI-TOF mass spectrometer (Voyager DE STR, Applied Biosystems, CA) or equivalent.
8. MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, CA) or equivalent.
9. Spectra were analyzed using Data Explorer (Applied Biosystems, version 4.8).

2.6 Other Materials

1. ZipTip$_{C18}$ pipette tips (0.6 µL bed volume, Millipore Corp., Billerica, MA) or with ZipTip$_{µ\text{-}C18}$ pipette tips (0.2 µL bed volume) for solid phase reactions.
2. Eppendorf LoBind microcentrifuge tubes (0.5, 1.5, and 2 mL).
3. Eppendorf pipette tips.
4. Spin Columns-Screw Caps, 0.8 mL internal volume (Pierce Corp, Rockford, IL).

3 Methods

3.1 In-Gel Digestion

After sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) and gel staining (*see* **Note 6**), carry out all manipulations in a laminar flow-vented hood or equivalent. Wear gloves at all times to prevent keratin contamination.

1. Run your protein sample on a gel.
2. Stain using Colloidal Coomassie using instructions supplied by the manufacturer (most dye-stains will work).
3. Excise protein band from the stained gel using a clean scalpel.
4. Cut band into ~1 × 1 mm wide pieces and transfer to a 0.5 mL centrifuge tube.
5. Set thermomixer to an agitation speed of 500 rpm.
6. Incubate gel pieces twice with 200 µl of the Destaining Solution for 30 min at 37 °C; discard supernatant.
7. Dry gel band briefly in a SpeedVac.
8. Add 100 µL of the reductant and incubate for 15 min at 37 °C (*see* **Note 7**).
9. Remove supernatant.
10. Dehydrate the gel pieces for 10 min in 100 µL of acetonitrile. Discard supernatant and briefly dry the gel in a SpeedVac.
11. Rehydrate gel pieces at room temperature in 20 µL Promega-modified trypsin solution (12.5 ng/µL). Discard the remainder of the enzyme solution.

12. After 30 min add 40 µL 25 mM ammonium bicarbonate and incubate for 18 h at 37 °C.
13. After incubation, add 50 µl 0.1 % TFA and collect supernatant in a 1.5 mL centrifuge tube (Extract 1). Prior to collection of the extract place 35 µL of 0.1 % TFA into the centrifuge tube. Mark the liquid level. Empty the tube.
14. Add 50 µl 0.1 % TFA to the gel pieces and incubate for 30 min at 37 °C (Extract 2).
15. Transfer Extract 2 to the collection tube.
16. Reduce the extracts in volume to 35 µL by SpeedVac evaporation (*see* **Note 8**).
17. Add 5 µL of 10 % TFA. Proceed with sample binding.

3.2 Sample Binding to the Reversed Phase Support

1. Wet the ZipTip$_{C18}$ pipette tip six times with 10 µL of methanol followed by six 10 µL washes with 0.1 % TFA according to the manufacturer's instructions. Critical! Do not allow the ZipTip to dry before sample loading. Should this inadvertently occur, condition a new ZipTip and proceed immediately with the next step. Aspirate the acidified sample in 10 µL aliquots onto the ZipTip and dispense into a 0.5 ml microfuge tube. Transfer the partially stripped peptide solution back in this manner into the original collection tube. Repeat this alternating enrichment cycle five times to maximize peptide recovery. Then wash the ZipTip with 50 µL 0.1 % TFA by passing the solvent in ten aliquots over the resin. Critical! After the desalting step do not allow the ZipTip to de-wet. Proceed immediately to the performic acid oxidation step or aspirate 10 µL 0.1 % TFA onto the ZipTip for the purpose of temporary storage (*see* **Note 9**). Prepare model peptide solutions at a concentration of 2 pmol/µL in 1 % aqueous TFA, 0.01 % OGS. Transfer the solutions in five10 µL aliquots to 0.5 mL centrifuge tubes. Bind peptides to the support using up to ten sample aspiration/dispense cycles. Wash ZipTips with 50 µL 0.1 % TFA. Proceed with the experiments described under Subheading 3.6 or store the ZipTips temporarily in 0.1 % TFA until use.

3.3 Solid Phase Derivatization

With the exceptions noted below the ZipTip$_{C18}$ pipette tip is intermittently desalted. Then the ZipTip is flushed twice with reagents. After this resin conditioning step, the reagents are loaded onto the support from the 60 µL that had been placed into 0.5 mL microcentrifuge tubes. Critical! Leave the ZipTip immersed in reagents during the incubations. Do not allow the ZipTip to dry during intermittent sample handling. If the ZipTip is inadvertently allowed to de-wet prior to reagent loading repeat the resin conditioning step. Agitation during incubations is not required. Use the modular block heater or equivalent device.

1. Flush the ZipTip to waste with 10 µL of the oxidant. Repeat this step. Load 10 µL of the oxidant onto the support. Allow reaction to proceed for 1 h at 4 °C. Halt the reaction by passing 100 µL of 0.1 % TFA in ten aliquots over the resin.
2. Condition the desalted ZipTip twice with 10 µL of the sulfo-NHS acetate reagent. Load 10 µL of reagent onto the support. Allow reaction to proceed for 20 min at 55 °C.
3. Flush the ZipTip twice with 10 µL of the BEMAD reagent mixture. Load 10 µL of the reagent mixture onto the support and allow the concurrent reaction to proceed for 1 h at 37 °C. Wash the ZipTip ten times with 10 µL 0.1 % TFA. Implement the consecutive BEMAD reaction mode as follows: (*see* **Note 10**). Condition the ZipTip twice with 10 µL β-elimination Base. Aspirate 10 µL reagent onto the ZipTip. Incubate for 30 min at 55 °C. Flush the ZipTip twice with 10 µL Michael Addition Reagent. Aspirate 10 µL of reagent onto the ZipTip and allow the addition reaction to proceed for 2 h at 55 °C. Desalt the ZipTip by passing 100 µL of 0.1 % TFA in 10 µL aliquots over the resin. *Pause point*. The ZipTip may be stored overnight in 0.1 % TFA at −21 °C.
4. Condition the desalted ZipTip twice with 10 µL of the sulfo-NHS-SS-Biotin Reagent. Load 10 µL of reagent onto the support. Incubate for 30 min at room temperature.
5. Flush the ZipTip twice with 10 µL of the hydroxylamine solution. Load 10 µL of reagent onto the ZipTip and allow the reaction to proceed for 15 min at 37 °C. Wash the resin ten times with 10 µL of 0.1 % TFA and then five times with 10 µL of water. *Pause point*. The ZipTip may be stored overnight in 0.1 % TFA at −21 °C.
6. Condition the desalted ZipTip twice with 10 µL of the reductant. Aspirate 10 µL of the reductant onto the support. Incubate for 15 min at 37 °C. Wash the ZipTip ten times with 10 µL 2 mM aqueous EDTA and then ten times with 10 µL of 0.1 % TFA. Critical! This stringent purification step is required for thorough removal the reductant. Residual TCEP would diminish the binding capacity of the affinity support. Store the ZipTip at 4 °C in 0.1 % TFA while proceeding to **step 9**, Subheading 3.4.

3.4 Covalent Chromatography

1. Place the spin column in a 2 ml centrifuge tube. Add 200 µL of acetonitrile and centrifuge at 209 × *g* for 1 min.
2. Let the activated-Thiol Sepharose gel, stored at 4 °C in 10 mL 10 % aqueous ethanol, assume room temperature.
3. Adjust the liquid level in the storage container (i.e., the scintillation tube) to one third of the height of the settled gel.

4. Swirl the container slightly to suspend the medium. Pipette 100 µl of the slurry into the spin column. To facilitate pipetting, shorten a 200 µL pipette tip by ~2 mm using a clean razor blade.
5. Centrifuge at 209 × *g* for 1 min.
6. Suspend the medium pellet in 200 µl of Coupling Buffer and centrifuge at 209 × *g* for 1 min.
7. Seal spin column with the plastic plug supplied by the manufacturer. Ensure that the plug is properly seated to avoid leaks.
8. Insert spin column into a 1.5 mL centrifuge tube.
9. Elute the digest from the ZipTip (set aside in **step 6**, Subheading 3.3) with 10 µL 50 % acetonitrile, 0.1 % TFA, 0.01 % OGS into a 0.5 mL centrifuge tube. Place 1 µL of the eluent onto the MALDI target. Immediately add 40 µl Coupling Buffer to the remainder. Mix. Checkpoint! Analyze the aliquot along with the material recovered in **steps 25** and **26**, Subheading 3.4 (*see* **Note 11**).
10. Transfer the mixture to the spin column. Swirl the spin column gently to suspend the medium pellet.
11. Attach screw cap and secure the spin column to the centrifuge tube with a piece of Parafilm.
12. End over end incubate the sample for 1 h at room temperature in the rotary mixer.
13. Remove plug from the spin column and centrifuge at 209 × *g* for 1 min. Mark the liquid level (~50 µL) on the collection tube.
14. Place 50 µl of Coupling Buffer into the spin column, suspend the pellet, and centrifuge at 209 × *g* for 1 min.
15. Place 50 µl 60 % aqueous acetonitrile, 0.1 % TFA into the spin column, suspend the pellet with agitation, and centrifuge at 209 × *g* for 1 min.
16. Place 50 µl 80 % aqueous acetonitrile, 0.1 % TFA into the spin column, suspend the pellet with agitation and centrifuge at 209 × *g* for 1 min (*see* **Note 12**).
17. Reduce the volume of the collected fractions by SpeedVac evaporation to the level indicated on the collection tube. Add 5 µL of 15 % TFA. Store sample in 0.1 % TFA at 4 °C.
18. Insert the spin column into a 1.5 mL centrifuge tube.
19. Place 50 µl Coupling Buffer into to the spin columns to neutralize residual acid. Centrifuge at 209 × *g* for 1 min; discard the flow-through.
20. Seal the spin column and suspend the medium pellet with 50 µl of the reductant. Attach screw cap. Place the spin column

into a 1.5 ml centrifuge tube and secure the spin column to the centrifuge tube with a piece of Parafilm.

21. With end-over-end mixing, incubate for 30 min at room temperature.
22. Unplug the spin column and centrifuge at 209 × *g* for 1 min. Mark the liquid level on the collection tube (~50 µL).
23. Wash the affinity resin consecutively with the organic solvents as described above.
24. Reduce volume of the collected fractions by SpeedVac evaporation to the level indicated on the collection tube (*see* **Note 13**). Add 5 µL of 15 % TFA.
25. Bind the material to a ZipTip$_{C18}$ pipette tip, pass 100 µL 0.1 % TFA in 10 µL portions over the resin and elute with 5–10 µL 50 % aqueous acetonitrile, 0.1 % TFA, 0.01 % OGS. Subject 1 µL of the eluate to MALDI MS analysis (*see* **Note 14**).
26. Concentrate the material (i.e. the non-phosphorylated peptides) kept in temporary storage (**step 17**, Subheading 3.4) on a ZipTip$_{C18}$ pipette tip. Process the ZipTip for MALDI MS analysis as described above.

3.5 Anticipated Results and Diagnostic tests

3.5.1 Application of Method to Model Protein

To exemplify the performance of the sample preparation/covalent chromatographic system an in-gel digest prepared from an equimolar mixture of bovine α-S1 and β-casein (25 pmol) was examined. The results from this experiment that demonstrate the successful implementation of the method are shown in Fig. 2a–c [11]. On the basis of the known protein sequences the ions at *m/z* 1770.3, *m/z* 2103.2, and *m/z* 3432.6 are recognized as the *N*-acetyl thiol derivatives of the α-S1 monophosphorylated fragment -^{121}VPQLEIVPNpSAEER^{134}, of the miscleavage product ^{119}YKVPQLEIVPNpSAEER^{134} and of the tetraphosphorylated fragment of β-casein-^{16}RELEELNVPGEIVEpSLpSpSpSEESITR40, respectively which proved impervious to enrichment by the dithiol-based affinity approach (*see* Fig. 2c). Ions which would indicate incomplete acetylation were not observed in the spectrum of the starting material (*see* Fig. 2a, arrow heads). The ions at *m/z* 2060.3 and at *m/z* 2390.4 matched in mass to the oxidized counterparts of fragments harboring methionine. Non-phosphorylated peptides were nearly exclusively found in the solvent wash providing evidence that these peptides were effectively depleted from the affinity support (*see* Fig. 2b). The data provide a general means for method trouble shooting. Tests proposed to survey the individual reaction steps in detail for potential complications are described below.

The results of the application of the method to a digest prepared by in solution digestion of 2 pmol α-S1 casein are shown in Fig. 2c, inset (*see* **Note 15**). The high quality of the spectrum produced from the isolate suggests that sub-picomole quantities of

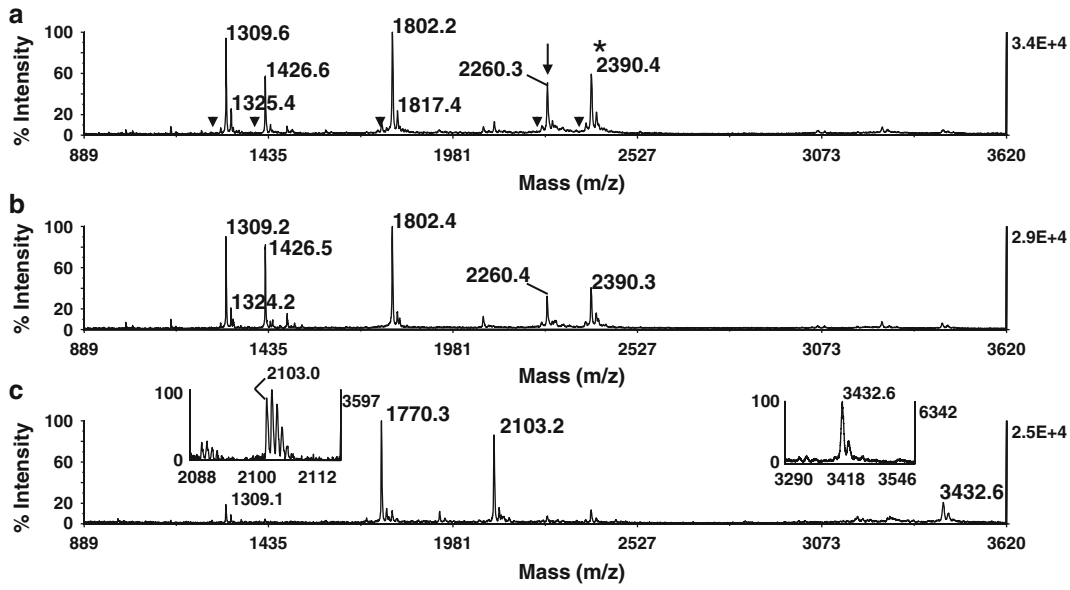

Fig. 2 Application of method to a tryptic in-gel digest prepared from equimolar mixture of 25 pmol of bovine α-S1 and β-casein. MALDI MS spectra of (**a**) digest after preconditioning reactions; (**b**) solvent wash; (**c**) reductively released fraction. The phosphopeptides are recognized at *m/z* 1770.3, *m/z* 2103.2, and *m/z* 3432.6 as the α-S1 casein fragments ^{121}VPQLEIVPpSAEER^{134} and ^{119}YKVPQLEIVPNpSAEER^{134} and the β-casein fragment ^{16}RELEELNVPGEIVEpSLpSpSpSEESITR^{40}, respectively. The fragment of β-casein and of α-S1casein targeted by oxidation is denoted by *arrow* and *asterisk*, respectively. *Inset* in (**c**), *right margin* shows expanded section of spectrum containing the tetraphosphorylated peptide. ~2 pmol of digest were applied to the target. *Inset* in (**c**), *left margin* shows expanded section of spectrum containing the α-S1 miscleavage product selected from 2 pmol of α-S1 digest. ~200 fmol of isolate were applied to the target

digest should be amenable to the chemical approach. This precept is supported by the finding that femtomole amounts of model peptides can be successfully carried through the reaction schemes [11]. See below.

3.5.2 Phosphorylation Site Determination by MALDI-TOF/ TOF MS

Phosphorylation site determination by CID in native phosphopeptides often fails due to lack of informative sequence information because gas-phase cleavage of the phosphodiester bond dominates over peptide backbone fragmentation. As previously noted the substitution of the labile phosphate group blocks this preferential dissociation event. In consequence; the sequence information content is in general improved facilitating phosphorylation site determination. Representative MALDI-TOF/TOF data obtained from the α-S1 casein tryptic isolate at *m/z* 1770.3 which reflect this benefit are illustrated in Fig. 3. The resultant product ion spectrum displayed a nearly uninterrupted y ion series produced in high abundance. The location of the modification could be readily identified by the unique residue mass of 234 Da of the fragment ion y5 and hence serine in position 130 was recognized as the site of

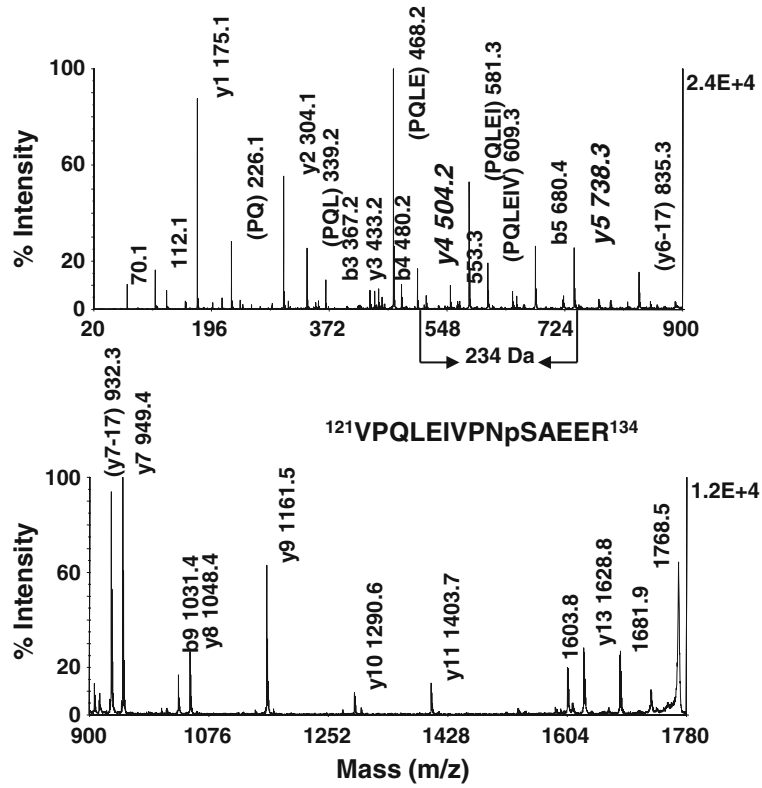

Fig. 3 Phosphorylation site determination by MALDI tandem MS. MALDI-TOF/TOF spectrum of thiol derivative at *m/z* 1770.3 enriched from α-S1/β-casein tryptic digest. The derivatization discriminates the site of phosphorylation as the unique residue mass of 234 Da contained in the product ion y5 *highlighted in italics* as is y4 which is contiguous to the modification. The presence of this ion pair enables unambiguous assignment of serine in position 130 as the site of phosphorylation. ~ 2 pmol of isolate were applied to the target

phosphorylation. Substituted phosphothreonine is recognized by its characteristic signature mass of 248 Da. The label remained stable under the condition of CID.

3.6 Diagnostic Tests

The tests are intended to monitor the efficiency of the individual reaction steps of the sample preparation method. In this way chemistry deficiency can be readily identified and remedies implemented to ensure that the final reaction products are formed to near completion.

1. Test #1. Performic acid oxidation/acetylation/BEMAD. Optimal formation of the intermediates and the final reaction product is desirable to ensure for method selectivity and to maximize the recovery of the Michael addition product. The data obtained from the application of the protocol described in **steps 1–3,** Subheading 3.3 using DAM1 phosphopeptide

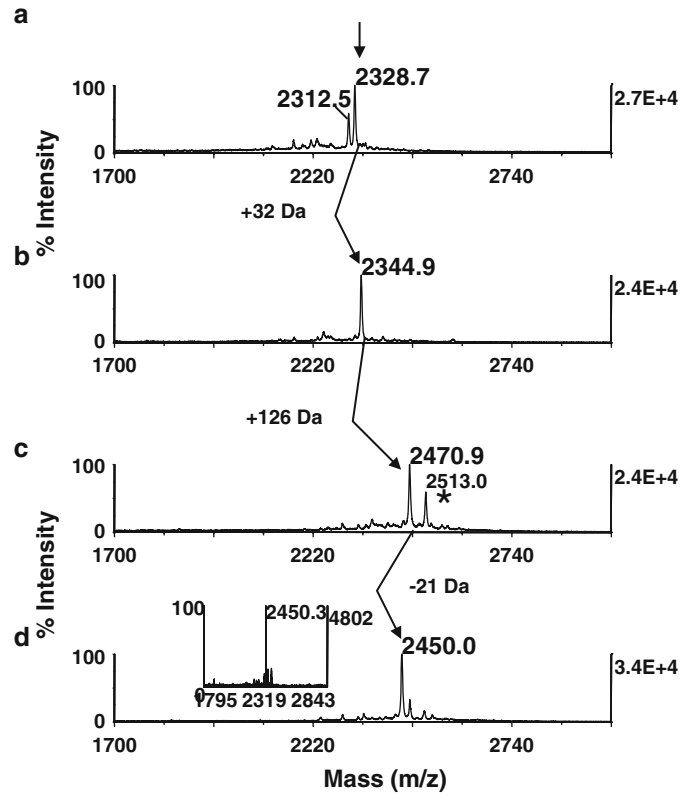

Fig. 4 Diagnostics test #1. MALDI MS spectra of (**a**) native DAM1 phosphopeptide SFVLNPTNIGMpSKSSQGHVTK, *m/z* 2312.5 (20 pm); (**b**) after oxidation; (**c**) after acetylation; (**d**) after BEMAD. *Cross arrows* denote mass shifts. *Arrow* and *asterisk* designate sulfoxide derivative and O-acylation product, respectively. ~2 pmol of peptide were applied to the target. *Inset* shows experiment at 300 fmol sample load using a μ-$_{C18}$ ZipTip from which the product was deposited in matrix onto the target

SFVLNPTNIGMpSKSSQGHVTK, *m/z* 2312.5 as test peptide are shown in Fig. 4a–d. Accordingly, the peptide was fully oxidized (*see* Fig. 4b), its primary amino groups were effectively acetylated (*see* Fig. 4c) and BEMAD resulted in nearly quantitative formation of the *N*-acetyl *S*-2-aminoethylcysteine adduct concomitant with complete O-acylation reversal (*see* Fig. 4d). Incompleteness of oxidation was observed only when the reagent was not allowed to be fully formed (*see* **Note 1**). Partial acetylation was strictly avoided by the immediate use of freshly prepared reagent (*see* **Note 2**). We have not encountered any complications during method optimization associated with BEMAD with respect to efficiency of derivatization or potential chemistry side reactions [11]. Inset shows the spectrum of the Michael addition product produced from 300 fmol of starting material. Consistent with previous results,

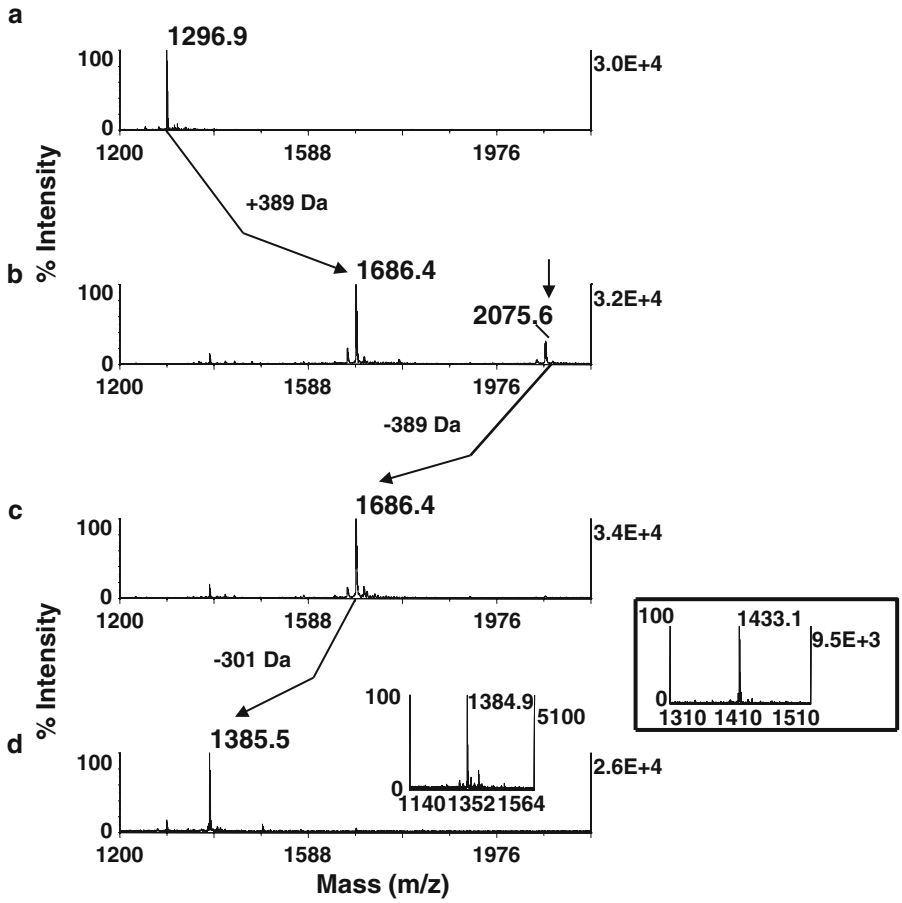

Fig. 5 Diagnostic test #2. MALDI MS spectra of (**a**) native angiotensin I DRVYIHPFHL, *m/z* 1296.9 (20 pm); (**b**) after biotinylation; (**c**) after O-deacylation; (**d**) after disulfide reduction. *Cross arrows* indicate mass shifts. *Arrow* denotes O-acylated peptide. *In-boxed area* shows sulfone derivative of thiolated angiotensin. ~2 pmol of peptide were applied to the target. *Inset* shows experiment at 500 fmol sample load using a μ-$_{C18}$ ZipTip from which the product was deposited in matrix onto the target

the data show that low-level amounts of phosphopeptide expected to occur in in vivo phosphorylated samples can be carried undiluted through the serial reaction scheme [11] (*see* **Note 16**).

2. Test #2. Another key feature of the sample preparation method is the N-thiolation sequence of serial reactions which imparts the affinity label onto the analyte. Results from the application of the N-thiolation procedure to the model peptide angiotensin I-DRVYIHPFHL, *m/z* 1296.5 are shown in Fig. 5a–d. Under the conditions described in **steps 4–6,** Subheading 3.3, the peptide was conjugated with Sulfo-NHS-SS-biotin to near completion (*see* Fig. 5b), its O-acylation product effectively hydrolyzed by hydroxylamine (*see* Fig. 5c), and the thiol-functionalized peptide analog produced in nearly quantitative

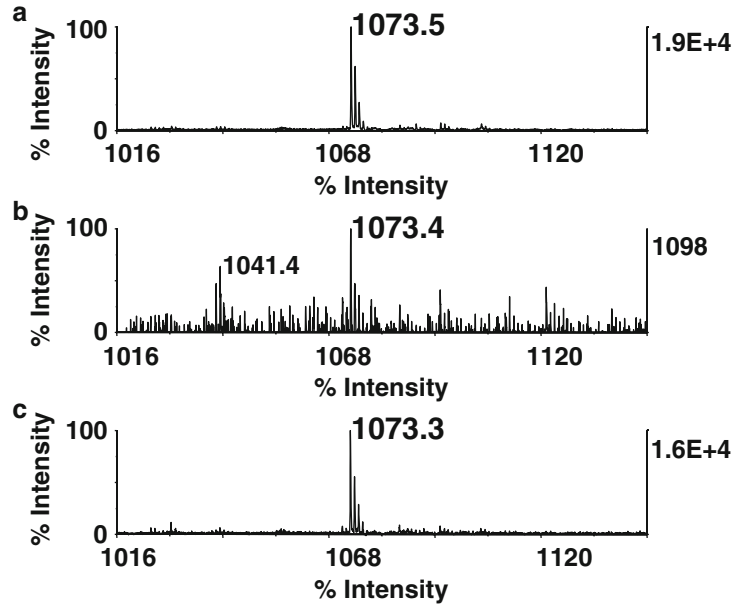

Fig. 6 Diagnostic test #3. Validation of analyte capture/release efficiency by covalent chromatography using the somatostatin fragment 3–10 (CKNFFWKT, *m/z* 1073.2) as test peptide. MALDI MS spectra of (**a**) starting material (5 pmol); (**b**) peptide recovered from the flow-through fraction; (**c**) peptide reductively released from the affinity support. ~1 pmol of peptide was analyzed. For MALDI MS, 80 laser shots acquired from eight different spot positions were summed for each spectrum

yield (*see* Fig. 5d). Inset shows the spectrum of the thiolated product produced from 500 fmol of starting material highlighting the advantage of solid phase reaction format to thiolate sub-picomole amounts of material. Failure to appreciate the recommendation with regard to the use of the NHS-ester reagent may lead to incomplete N-thiolation. As a result, the population of non-reacted Michael addition products would be subject to depletion from the affinity support resulting in diminished recovery of the isolates.

3. Test #3. The capability of the covalent chromatographic system to reversibly capture the analyte at high efficiency has been previously demonstrated with the cysteinyl peptide Somatostatin CKNFFWKT, *m/z* 1073.2 [11]. Results from such experiment are shown in Fig. 6a–c. Fractions collected before and after the reduction step were analyzed by MALDI MS along with the unprocessed sample. The MALDI MS spectra show that the signals produced from the starting material (*see* Fig. 6a) and from the reductively retrieved peptide (*see* Fig. 6c) were of comparable abundance. <10 % of the starting material was found in the initial flow-through fraction indicating

that a ~90 % coupling/release efficiency was achieved (*see* Fig. 6b). Variations between replicate experiments were <5 %. As noted, storage of the medium in the presence of bacteriostatics should be avoided as these agents impair the capture efficiency of the medium.

4 Notes

1. To avoid incomplete oxidation make sure that the mixture is left standing for at least 30 min prior to dilution to allow the reagent to be fully formed.
2. NHS-ester reagents in general have a half-life of 1 h at pH 8.0 and 25 °C. To ensure for completeness of reaction do not store the freshly prepared Sulfo-NHS acetate solution for more than 10 min prior to use. NHS-ester reagents are moisture sensitive. To prevent decomposition let the reagent equilibrate in the closed container from its low storage temperature (i.e., –20 °C) to room temperature. Obviously, do not prepare reagent in amine-containing buffers.
3. Grind barium hydroxide octahydrate crystals to a fine powder in a standard porcelain mortar. Transfer 31.54 mg of the chemical to a 1.5 mL microcentrifuge tube. Add 1 ml water and vortex vigorously for ~2 min to facilitate dissolution. Centrifuge for 1 min at 1811 × *g* to remove carbonate precipitates.
4. Considerations with regard to the proper use of Sulfo-NHS-SS-Biotin are as described in **Note 3**.
5. Do not add bacteriostatics such as sodium azide, merthiolate, or phenyl mercuric salts to the gel. These agents diminish the medium's binding capacity.
6. We used GelCode Blue Stain Reagent. We have thus far not employed other more sensitive non-covalent staining methods. We refer the reader to the report of Granvogl and coworkers in which the use of those methods is reviewed in ref. 17.
7. Standard protein in-gel digestion and in solution digestion protocols recommend alkylation after cystine reduction to promote the accessibility of the substrate to digestion. However, alkylated cysteinyl peptides are subject to BEMAD resulting in their co-isolation. This complication is addressed in our sample preparation method by post-digestion performic oxidation which renders cysteine inert toward BEMAD. Optionally, the solid phase oxidation step can be replaced by in-gel performic acid oxidation. The protein is then digested by the protease in the gel matrix. Gel extracts are bound to the reversed phase support and subsequently

subjected to acetylation (*see* Fig. 1). An effective gel-based procedure which we developed is found in the literature [11].

8. Monitor the progress of solvent evaporation. Do not dry the sample below the level indicated on the collection tube (~35 μL). Further solvent evaporation may cause substantial adsorptive peptide loss especially as seen with low-level samples up to 50 % or more of the starting solution [16].
9. The ZipTip de-wets in <2 min and should therefore be immediately processed for derivatization. Optionally, the ZipTip can be temporarily stored in 0.1 % TFA at 4 °C preferably for <60 min or at room temperature for <30 min. To this purpose place 60 μL 0.1 % TFA into 0.5 mL microcentrifuge tubes. Aspirate 10 μL of solvent onto the ZipTips. Leave the ZipTips immersed in solvent during storage.
10. Under the condition of concurrent BEMAD phosphothreonyl peptides are recalcitrant to derivatization as are phosphoseryl/threonyl peptides in which the phosphorylated residue is positioned adjacent to proline. The consecutive reaction mode has to be used to ensure efficient conversion of this class of phosphopeptides. During incubation air-induced carbonate precipitation typically occurs which has no impact on the efficiency of derivatization.
11. MALDI MS of the eluent furnishes the mass map of the starting material presented to covalent chromatography. Deviations from the anticipated result shown in Fig. 2a are typically due to incomplete digestion and/or incomplete derivatization. Do not refreeze the remainder of the enzyme solution for further use. Repeated thawing and freezing may impair enzyme activity. The presence of satellite peaks would indicate incompleteness of oxidation and/or acetylation. Remedies for these complications are discussed under Subheading 3.6.
12. Make sure that the affinity resin is properly washed to avoid cross contamination of the enriched fraction with non-phosphorylated peptides. Repeat the organic solvent wash if this problem persists.
13. To avoid adsorptive sample loss of the isolate do not dry the sample below the level marked on the collection tube (~50 μL) [16].
14. We recommend the use of ZipTipμ-$_{C18}$ pipette tips to recover phosphopeptide isolates enriched from low-level digests. With μ-tips the isolates can be deposited in 0.5 μL matrix directly on the MALDI-target. In this manner, femtomole mass detection can be readily achieved (*see* Figs. 2 and 5, insets).
15. In-solution tryptic digestion was performed in 40 μL of 25 mM ammonium bicarbonate/0.01 % OGS at an enzyme to substrate ratio of 1:100. After 18 h incubation at 37 °C, the

digest was acidified with 5 μL of 10 % TFA prior to solid phase immobilization.

16. Any other medium-size phosphoseryl peptide containing methionine may be used as test peptide.

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