

## REGULAR ARTICLE

# Quantitative analysis of both protein expression and serine/threonine post-translational modifications through stable isotope labeling with dithiothreitol

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While phosphorylation and *O*-GlcNAc (cytoplasmic and nuclear glycosylation) are linked to normal and pathological changes in cell states, these post-translational modifications have been difficult to analyze in proteomic studies. We describe advances in  $\beta$ -elimination/Michael addition-based approaches which allow for mass spectrometry-based identification and comparative quantification of *O*-phosphate or *O*-GlcNAc-modified peptides, as well as cysteine-containing peptides for expression analysis. The method (BEMAD) involves differential isotopic labeling through Michael addition with normal dithiothreitol (DTT) (d0) or deuterated DTT (d6), and enrichment of these peptides by thiol chromatography. BEMAD was comparable to isotope-coded affinity tags (ICAT; a commercially available differential isotopic quantification technique) in protein expression analysis, but also provided the identity and relative amounts of both *O*-phosphorylation and *O*-GlcNAc modification sites. Specificity of *O*-phosphate vs. *O*-GlcNAc mapping is achieved through coupling enzymatic dephosphorylation or *O*-GlcNAc hydrolysis with differential isotopic labeling. Blocking of cysteine labeling by prior oxidation of a cytosolic lysate from mouse brain allowed specific targeting of serine/threonine post-translational modifications as demonstrated through identification of 21 phosphorylation sites (5 previously reported) in a single mass spectrometry analysis. These results demonstrate BEMAD is suitable for large-scale quantitative analysis of both protein expression and serine/threonine post-translational modifications.

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## 1 Introduction

Measuring protein expression differences is a goal in proteomic studies. Additionally, post-translational modifications expand the functional diversity of proteins [1] and

represent important targets in comparative proteomics [2, 3]. Altered levels of post-translational modifications (sometimes in the absence of protein expression changes) are often linked to cellular responses and disease states. While phosphorylation is a fundamental switch mechanism [4], modifications such as *O*-GlcNAc [5, 6] also contribute to dynamic protein regulation. *O*-GlcNAc is an enzyme-mediated cytosolic and nuclear carbohydrate modification of serines and threonines by *N*-acetylglucosamine found on diverse functional classes of proteins [7, 8]. Studies which include mapping of post-translational modifications provide a basis for site-specific testing of their regulatory function.

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**Abbreviations:** BEMAD, beta-elimination/Michael addition with DTT; ICAT, isotope coded affinity tags

Seminal work on differential isotopic labeling of proteins [9] has led to the development of tools such as isotope-coded affinity tags (ICAT) [10, 11], stable isotope labeling with amino acids in culture (SILAC) [12], and others [13, 14] for use in large-scale quantitative expression comparisons through the use of mass spectrometry (MS). The common feature of these techniques is the incorporation of chemically identical but mass-differentiated constituents into a given protein or peptide from two different samples. When these two samples are mixed, measurement of the ratio of isotopic content by MS provides an accurate measure of their relative abundance. When combined with liquid chromatography and tandem MS (LC-MS/MS), relatively high-throughput analysis may be achieved that complements other approaches including 2-D gel/differential gel electrophoresis (DIGE) separation and quantitation [15]. ICAT is the most widely applied of these methods and involves labeling of cysteine residues with biotinylated “tags” of different isotopic mass. Subsequent enrichment of these cysteine-tagged peptides reduces sample complexity and is thought to increase numbers of protein identifications. This technique has been successful in comparative expression analysis of complex mixtures containing low levels of protein [11, 16, 17]. However, as the technique selectively targets cysteine residues, it is not suitable for analysis of post-translational modifications in general.

Since occupancy of post-translational modifications is often substoichiometric and chemically labile, their comparative analysis/identification is a greater challenge than expression proteomics.  $\beta$ -Elimination/Michael addition at phosphorylated serine and threonine [18, 19] with affinity tags for enrichment and site-mapping [20–23] has been employed to facilitate mapping of phosphorylation sites. Further developments include coupling of  $\beta$ -elimination/Michael addition to immobilized metal affinity chromatography (IMAC) [24] for specific enrichment of low-abundance phosphopeptides [25] and to creation of phosphospecific proteolytic sites [26].  $\beta$ -Elimination/Michael addition strategies have also been adapted for mapping of *O*-GlcNAc sites [27]. In addition to enabling enrichment of low-abundance modified peptides, the affinity tags added through Michael addition are more stable during collision-induced dissociation (CID) than the *O*-phosphate or *O*-GlcNAc they replace, facilitating mapping of specific modification sites. While there are current limitations to these chemical derivatization methods, including resistance of some modified sites to  $\beta$ -elimination [23], and potential nonspecific Michael addition to unmodified residues under relatively harsh  $\beta$ -elimination conditions [28, 29], the approach has been effective in mapping sites of both *O*-phosphate and *O*-GlcNAc modification.  $\beta$ -Elimination/Michael addition with differential isotopic tags [20, 22] has demonstrated potential for its use in quantitative proteomic comparisons of post-translational modifications. Using  $\alpha$ -casein as a model phosphoprotein, the use of a deuterated form of DTT as a Michael addition reagent has been demonstrated to be a tool for quantitative

phosphorylation site-mapping [30]. Optimization of this approach for phosphorylation site-mapping included the use of  $\text{Ba}(\text{OH})_2$  as the alkali reagent, use of solid  $\text{CO}_2$  to precipitate barium carbonate, and separation of the  $\beta$ -elimination and Michael addition steps [30]. Currently,  $\beta$ -elimination/Michael addition approaches target serine/threonine post-translational modifications and do not provide information on protein expression differences. Additionally, confident discrimination between derivatization of *O*-phosphate, *O*-GlcNAc, and nonspecific residues has been difficult to achieve. Definitive states of post-translational occupancy have not been mapped from complex mixtures of unknown proteins using  $\beta$ -elimination/Michael addition approaches.

We report an LC-MS/MS-coupled proteomic approach for measuring changes in both expression and low-abundance serine/threonine post-translational modifications using differential isotopic labeling by Michael addition with either “light” dithiothreitol (DTT) (d0) or deuterated “heavy” DTT (d6) under conditions which discriminate between derivatization of *O*-phosphate and *O*-GlcNAc, or nonspecific residues.

## 2 Materials and methods

### 2.1 Preparation of “standard mix”

A mixture was made containing 1 pmol/ $\mu\text{L}$  in 50 mM  $\text{NH}_4\text{HCO}_3$  of the following 14 proteins (Sigma, St. Louis, MO, USA, unless otherwise indicated). Transferrin (apo),  $\alpha$ -S1 casein,  $\alpha$ -lactalbumin,  $\beta$ -casein, ribonuclease B,  $\alpha$ -S2 casein,  $\beta$ -lactoglobulin, serum albumin, superoxide dismutase, and  $\alpha$ -1 acid glycoprotein were from the species bovine. Alcohol dehydrogenase was from yeast. Ig  $\gamma$  1C region was from human. Lysozyme was from chicken. The *O*-GlcNAc modified synthetic peptide PSVPV(*S*-*O*-GlcNAc)GSAPGR synthesized as described [31] was derived from the UL32 protein of human cytomegalovirus.

### 2.2 $\beta$ -Elimination/Michael addition with DDT (BEMAD) labeling of the standard mix

5  $\mu\text{L}$  of standard mix was diluted to 50  $\mu\text{L}$  in 50 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.2, reduced with 10 mM DTT at 52°C, and alkylated with 50 mM iodoacetamide (Sigma) at room temperature in darkness for 1 h. 1% w/w trypsin (Promega, Madison, WI, USA) was added and digest carried out for 4 h at 37°C. The sample was acidified to pH 4.5 with addition of trifluoroacetic acid (TFA) to 0.5% and desalted. Unless otherwise stated, desalting was by reverse phase using microspin C18 columns (Nest Group) with elution in 0.1% TFA/70% acetonitrile, followed by Speed-vac drying. One-half of sample was labeled with either “light” DTT (d0) or “heavy” deuterated DTT (d6) (C/D/N isotopes) by BEMAD as follows. The sample was suspended in 100  $\mu\text{L}$   $\beta$ -elimination/Michael addition solution (pH 12.5–13) containing 1.5% triethylamine (TEA)

(Pierce, Rockford, IL, USA), 0.15% NaOH, and 20 mM of either the d0 or d6 form of DTT and incubated for 1.5 h at 52°C, immediately acidified to below pH 5 with addition of TFA to 2%, mixed together and desalted. For thiol chromatography, Thiopropyl Sepharose 6B (Amersham Biosciences, Piscataway, NJ, USA) was swelled in degassed TBS-EDTA (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA) and 200 µL of a 50% slurry was transferred to an emptied macrospin column (Nest Group) and washed 7 times with 500 µL TBS-EDTA (centrifugation was 30 seconds at 1500 × *g*). The sample suspended in TBS-EDTA (400 µL) was then mixed with the Thiopropyl Sepharose at room temperature for 3 h, washed 7 times with 500 µL TBS-EDTA, suspended in TBS-EDTA containing 20 mM DTT, and mixed at room temperature 1 h before collecting eluent. Thiol elution was acidified to 0.5% TFA, desalted, and suspended in 0.1% formic acid for MS analysis. Where β-*N*-acetylhexosaminidase (New England Biolabs, Beverly, MA, USA) treatment is indicated, the dried trypsin digest was suspended in pH 4.7 buffer supplied by the manufacturer, split 1:1, and treated with 5 U/20 µL of either active (+Hex) or inactive enzyme which had been boiled 10 min (–Hex). The sample was then desalted and BEMAD treatment using d0 DTT for (–Hex) sample and d6 DTT for (+Hex) sample was carried out as described above.

### 2.3 Cleavable ICAT (cICAT) labeling of the standard mix

5 µL of standard mix was split in half and labeled with either “light” (<sup>13</sup>C<sub>0</sub>) or “heavy” (<sup>13</sup>C<sub>9</sub>) cICAT reagent (Applied Biosystems, Foster City, CA, USA), desalted with strong cation exchange chromatography, and the cICAT labeled peptides were enriched with avidin affinity purification followed by ICAT cleavage/elution as described [16].

### 2.4 Mouse brain lysis and BEMAD treatment

The freshly dissected brain from a male C57BL/6J 3 month old mouse was mechanically lysed with a Polytron (2 times 30 s bursts) in buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, and a cocktail of protease inhibitors. The lysate was clarified by centrifugation at 43 000 × *g* and protein concentration determined (Bio-Rad, Hercules, CA, USA). 2 mg protein was precipitated with 10% cold TCA for 20 min at 4°C and pelleted by centrifugation at 14 000 × *g* for 10 min, washed with 70% EtOH, dried and suspended in 100 mM NH<sub>4</sub>HCO<sub>3</sub> and spiked with 20 pmol of the peptide PSVPV(*S*-O-GlcNAc)GSAPGR as an internal control for monitoring BEMAD. 50 µg trypsin was added and the sample was incubated at 37°C overnight, acidified with TFA (1%), desalted with a Sep-Pak C18 cartridge (Millipore, Bedford, MA, USA) and dried in a Speedvac. The dried digest suspended in 400:1 alkaline phosphatase buffer (New England Biolabs) was split 1:1 and treated at 37°C for 3 h with 50 U active alkaline phosphatase (AP) (New England Biolabs) and 50 U active insoluble AP attached to

beaded agarose (Sigma) (+Alk sample) or 50 U inactive AP which had been boiled for 10 min (–Alk sample). The beaded AP was removed by centrifugation, samples were desalted using C18 Sep-Pak cartridges (Millipore) and dried in a Speed-Vac. For performic acid oxidation, 30% aqueous H<sub>2</sub>O<sub>2</sub> (Sigma) was mixed with 85% aqueous formic acid (Sigma) (5:95 v/v) and left at room temperature for 1 h. Dried samples were suspended in 400 µL of this solution and kept on ice for 1 h, followed by drying in a Speed-Vac. The (–Alk) and (+Alk) samples were BEMAD-labeled with DTT (d0) and DTT (d6), respectively, and prepared for MS/MS analysis as described above for the “standard mix” except that the volume of β-elimination reactions was 400 µL, desalting was with C18 Sep-Paks, and thiol chromatography was with 500 µL of swelled Thiopropyl Sepharose resin. Final clean-up steps prior to LC-MS/MS for mouse brain samples were performed with C18 ZipTips.

### 2.5 Nano-LC-ESI-Qq-TOF MS analysis

Peptides were analyzed by LC-MS/MS on a QSTAR Pulsar mass spectrometer (MDS Sciex, Concord, Ontario, Canada) operating in positive ion mode. Chromatography was by nanoflow HPLC using the 1100 series HPLC (Agilent, Technologies, Waldbronn, Germany) at flow rate 300 nL/min. Separation was achieved by a gradient of increasing ACN in water (2–34%) over 80 min using 0.1% formic acid as the ion-pairing agent on a capillary 75 µm ID column self-packed with Jupiter Proteo C12 (Phenomenex, Torrance, CA, USA) chromatographic support. The LC eluent was directed to a micro-ionspray source. Throughout the LC gradient, MS and MS/MS data were recorded continuously based on a 3 s cycle time. Within each cycle, MS data was accumulated for 1 s, followed by CID acquisition for 2 s on ions automatically selected by preset parameters. In general, ions selected for CID are the most abundant in the MS spectrum, except that singly charged ions were excluded and dynamic exclusion was employed to prevent repetitive selection of the same ions within a preset time. Collision energies were programmed to be adjusted automatically according to the charge state and mass value of the precursor ion. Peak lists for database searching were created using a script from within the Analyst software and submitted to an in-house version of the search algorithm MASCOT, Version 1.8 (Matrix Science). The code was appended to MASCOT such that the variable mass addition to unmodified serine and threonine by the d0 (136.1) or d6 (142.1), or to cysteine by the d0 (120.1) or d6 (126.1) forms of DTT, could be accounted for in automated searching. When analyzing oxidized samples, variable mass additions due to oxidation (*e.g.*, +16, +32, or +48 for cysteine and tryptophan and +16 or +32 for methionine and histidine) were also considered. The allowed mass tolerance between expected and observed masses was ± 100 ppm for MS and ± 0.2 Da for MS/MS fragment ions. Searches were performed against both the nonredundant National Center for Biotechnology Information database (NCBIInr) and Swiss-Prot.

Mascot searches of data from the “standard mix” were performed using a mammalian subdatabase. Mascot searches of data from mouse brain were performed using a rodent subdatabase. Peptides matched with a Mascot confidence score of 30 or greater were considered potentially correct and manually checked to ensure assignment of all major ions in MS/MS. Quantification of ion pair ratios was performed manually by averaging isotope envelope area over the time of elution of a given ion pair.

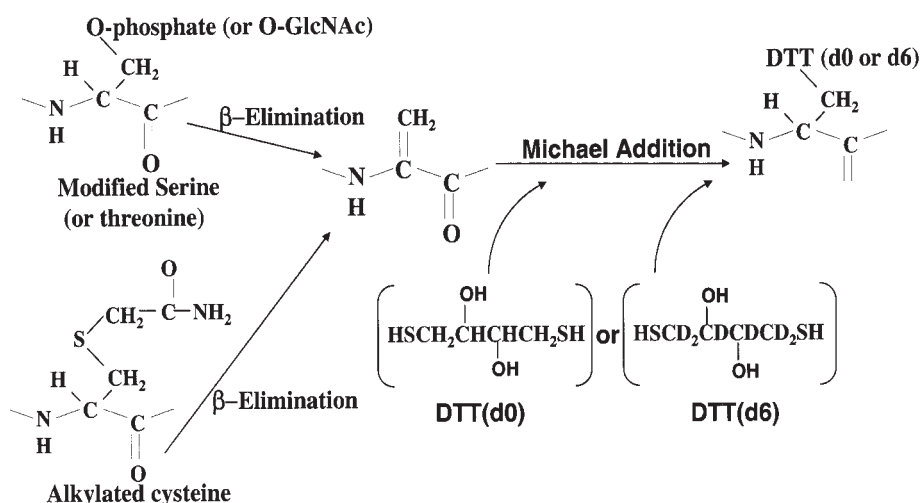
### 3 Results

#### 3.1 Isotopic tagging of cysteines and serine/threonine modifications

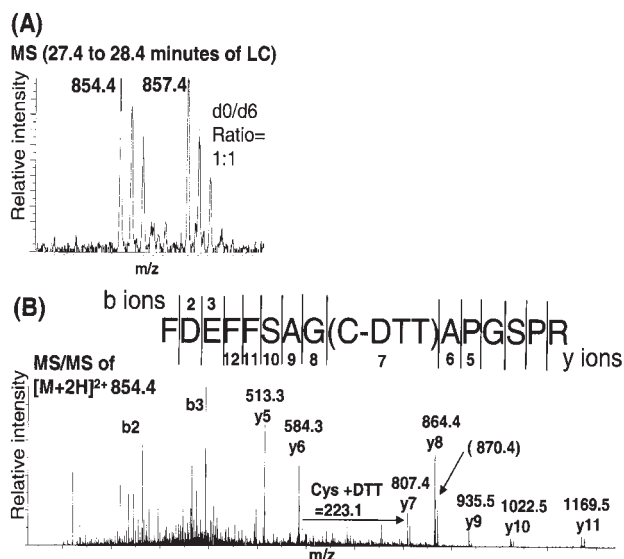
Alkylated cysteine residues have been reported to be susceptible to BEMAD [27]. Using a deuterated form of DTT (d6), differential isotopic labeling of both alkylated cysteines and serine/threonine post-translational modifications with BEMAD was evaluated as outlined in Fig. 1. A mixture of 14 cysteine-containing proteins (standard mix) defined in Section 2 which included  $\alpha$ -casein and  $\beta$ -casein (two proteins with well-characterized phosphorylation sites) and one O-GlcNAc-modified peptide derived from the human cytomegalovirus protein UL32 [31] were prepared. We then carried out analysis of the standard mix by both BEMAD and ICAT differential isotopic labeling approaches. The standard mix containing 5 pmol of each component was split in half and labeled by BEMAD with either DTT (d0) or DTT (d6), or alternatively was labeled with a cleavable form of either cICAT( $^{13}\text{C}_0$ ) or cICAT( $^{13}\text{C}_9$ ). “Light” and “heavy” isotopic labeled samples were mixed in equimolar quantities, and the labeled peptides were isolated by thiol (BEMAD) or avidin (cICAT) chromatography, respectively. An aliquot of one-fifth of each sample was analyzed in single 80 min nano-LC-MS/MS experiments using a Qq-TOF mass spectrometer. A trace of the total ion current for the BEMAD analysis is shown in

Fig. 2A. The mass spectrum of components eluting during 27.4–28.4 min of the analysis contained an ion pair at  $[\text{M}+2\text{H}]^{2+}$   $m/z$  854.4/857.4 with the expected DTT (d0) vs. DTT (d6) mass difference of 6 Da and with the expected ratio of 1:1 (Fig. 2B). The DTT (d0)-labeled peptides were observed to generally elute by reverse-phase chromatography slightly later than the d6 form, and thus accurate quantification requires averaging of the mass spectra over the full elution period of each ion pair. CID spectra derived from the “light” form of this ion pair at  $m/z$  854.4 are shown in Fig. 2C which allowed the identification of the peptide FDEFFSAG(C-DTT)APGSPR from transferrin. The 223.1 Da mass difference between the  $y_6$  and  $y_7$  ion corresponds to the BEMAD specific DTT (d0) addition to  $\beta$ -eliminated cysteine (dehydroalanine). The mass window for automated selection of precursors for CID allowed some co-eluting “heavy” ion to be selected and fragmented along with the “light” precursor. Thus, singly charged fragments differing by 6 Da corresponding to ion pair fragments retaining the DTT modification are often observed as noted for the  $y_8$  ion in Fig. 2C. A MASCOT search against the Swiss-Prot database of the CID in Fig. 2C ranked FDEFFSAG(C-DTT)APGSPR as the top match with a confidence score of 57 (data not shown).

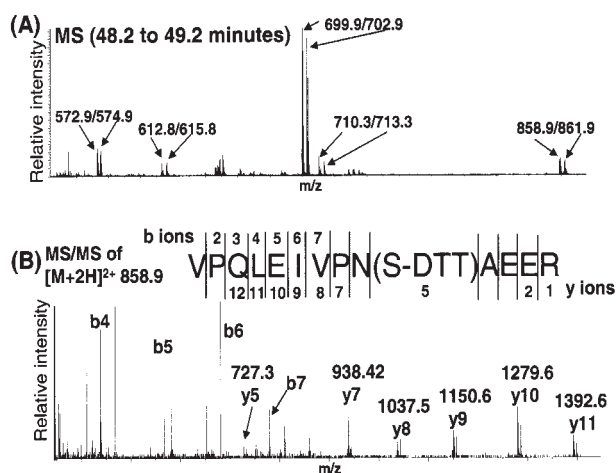
The mass spectrum from 48.2–49.2 min of the BEMAD analysis of the standard mix contained several different co-eluting ion pairs including 858.9/861.9 with a ratio of 1:1 as shown in Fig. 3A. The CID spectrum from the “light” form of this pair at  $m/z$  858.9 identified the peptide VPQLEIVPN(S-DTT)AEER (Fig. 3B) with DTT addition to a  $\beta$ -eliminated serine (dehydroalanine) corresponding to a known phosphorylation site in  $\alpha$ -casein [32] (a protein in the standard mix). A MASCOT search against the Swiss-Prot database ranked VPQLEIVPN(S-DTT)AEER as the top ranked peptide for the CID in Fig. 3B with a confidence score of 36 (data not shown). Thus, comparative quantification of cysteine-containing peptides and serine/threonine post-translationally modified peptides can be achieved using BEMAD.



**Figure 1.** Scheme for differential isotopic tagging of either cysteines or post-translationally modified serine and threonine residues using BEMAD.  $\beta$ -Elimination of alkylated cysteines and phosphorylated (or O-GlcNAc)-modified serines and threonines generates an intermediate containing an  $\alpha$ - $\beta$  unsaturated carbonyl (e.g., alkylated cysteine and modified serine residues are converted to dehydroalanine) which is susceptible to Michael type addition with either “light” DTT (d0) or “heavy” DTT (d6) containing 6 deuteriums.



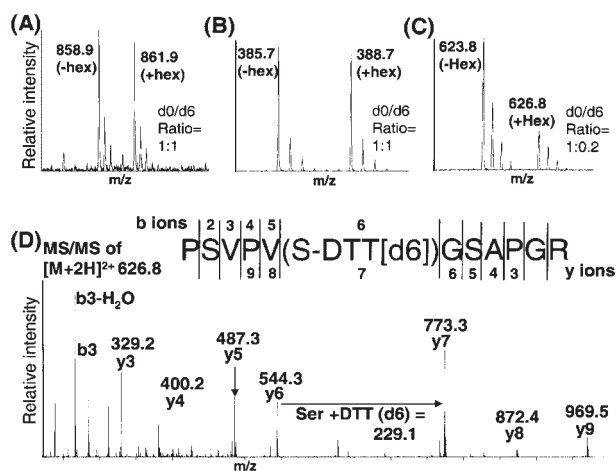
**Figure 2.** Identification and relative quantitation of a cysteine-containing peptide from the “standard” mix using BEMAD. A tryptic digest of the standard mix was derivatized by BEMAD using either light (d0) or heavy (d6) DTT and mixed 1:1. (a) MS from 27.4 to 28.4 min of the LC showing an “ion pair” at  $[M+2H]^{2+}$  854.4/857.4 with a ratio of 1:1. (b) CID spectrum of precursor  $[M+2H]^{2+}$  854.4 (d0 form of ion pair in [a]) listing y and b ions identifying FDEFFSAG(C-DTT)APGSPR and showing mass difference between the y6 and y7 ion of 223.1 Da corresponding to  $\beta$ -eliminated cysteine (dehydroalanine) modified with DTT (d0).



**Figure 3.** Identification and relative quantitation of a phosphorylation site from the “standard mix” using BEMAD. (a) MS of co-eluting “ion pairs” with characteristic mass differences for DTT (d0) vs. DTT (d6) derivatized forms from 48.2 to 49.2 min of the LC-MS/MS analysis referred to in Fig. 2b. CID spectrum of  $[M+2H]^{2+}$  858.9 from the ion pair 858.9/861.9 with ratio 1:1 is shown in (b) listing y and b ions identifying VPQLEIVPN(S-DTT)AEER, modified by DTT at a  $\beta$ -eliminated serine (dehydroalanine) which is a known  $\alpha$ -S1 casein phosphorylation site.

### 3.2 Specificity of serine/threonine isotopic tagging

$\beta$ -Elimination may occur at O-phosphate, O-GlcNAc, and in some cases at unmodified serine/threonine residues. Phosphatases and enzymes which hydrolyze O-GlcNAc (e.g., N-acetyl-hexosaminidase) have been used to help experimentally assign phosphorylation [33] and O-GlcNAc [27] modifications, respectively. To help achieve specificity in site-mapping with BEMAD, we combined the use of N-acetyl-hexosaminidase with differential isotopic labeling. 5 pmol of the standard mix was split in half and treated with equivalent amounts of inactive (–Hex) or active N-acetyl-hexosaminidase (+Hex) as described in Section 2. BEMAD labeling of the (–Hex) sample with DTT (d0) and the (+Hex) sample with DTT (d6) was then performed prior to mixing, thiol enrichment, and LC-MS/MS analysis. Thus, ion pairs of d0 vs. d6 forms corresponded to peptides derived from the (–Hex) and (+Hex) samples, respectively. As shown in Fig. 4A, a ratio of 1:1 is observed for an ion pair at  $m/z$  858.9/861.9 corresponding to the DTT derivatized  $\alpha$ -casein phosphopeptide VPQLEIVPN(S-phospho)AEER. A 1:1 ratio is observed for an ion pair at  $m/z$  385.7/388.7 corresponding to the DTT-derivatized cysteine-containing peptide AL(C-DTT)SEK from transferrin (Fig. 4B). However, N-acetyl-hexosaminidase treatment resulted in a shift of the d0/d6 ratio



**Figure 4.** Enzymatic O-GlcNAc hydrolysis coupled with differential isotopic labeling for discrimination of O-GlcNAc and O-phosphate derivatization with BEMAD. A tryptic digest of the standard mix untreated (–Hex) or treated with  $\beta$ -N-acetyl hexosaminidase (+Hex) was BEMAD-derivatized with DTT (d0) and DTT (d6) respectively. Ion pairs from a thiol-enriched 1:1 mixture analyzed by LC-MS/MS are shown for (a) the derivatized  $\alpha$ -S1 casein phospho-peptide VPQLEIVPN(S-O-phospho)AEER with a d0/d6 ratio of 1:1, (b) a cysteine-containing peptide ALCSEK from transferrin with a d0/d6 ratio 1:1, and (c) the UL32 O-GlcNAc-modified peptide PSVPV(S-O-GlcNAc)GSAPGR with a shift in the ratio of d0 vs. d6 forms to 1:0.2 in response to N-acetyl-hexosaminidase. (d) CID spectrum of  $[M+2H]^{2+}$  626.8 listing y and b ions identifying PSVPV(S-DTT[d6])GSAPGR and showing the mass difference between the y6 and y7 ions of 229.1 Da corresponding to DTT (d6) addition to a  $\beta$ -eliminated serine previously O-GlcNAc modified.

(1:0.2) for the ion pair at  $m/z$  623.8/626.8 corresponding to the DTT derivatized *O*-GlcNAc-modified peptide PSVPV(S-*O*-GlcNAc)GSAPGR (Fig. 4C). *N*-Acetyl-hexosaminidase catalyzed reduction of *O*-GlcNAc modification was readily measured by differential isotopic labeling (relative decrease in DTT [d6] form vs. DTT [d0] form) and indicates specific derivatization of *O*-GlcNAc. Analogously, specificity of *O*-phosphorylation site mapping may be achieved with BEMAD through use of phosphatases.

### 3.3 BEMAD vs. ICAT comparison

A summary of the comparison between BEMAD and ICAT analysis of the standard mix is shown in Table 1. CID spectra obtained for 40 cICAT-labeled cysteine-containing peptides allowed for identification and relative quantification of 9 proteins in the standard mix. CID spectra for 41 BEMAD-labeled cysteine-containing peptides (see Fig. 4, and data not shown) allowed for identification and relative quantification of 11 proteins in the standard mix. Nineteen of the cysteine derivatized peptides in Table 1 were identified by both ICAT and BEMAD, indicating significant overlap between the two methods. Additionally, the BEMAD analysis resulted in relative quantification and identification of two peptides (VPQLEIVPN[S-DTT]AEER and LH[S-DTT]MK) with  $\beta$ -eliminated serine residues (dehydroalanine) modified by DTT corresponding to known phosphorylation sites in the protein  $\alpha$ -S1 casein [32], one peptide (FQ[S-DTT]EEQQQTE-DELQDK) with a  $\beta$ -eliminated serine modified by DTT corresponding to a known phosphorylation site in  $\beta$ -casein [34], and one peptide (PSVPV[S-DTT]GSAPGR) with a  $\beta$ -eliminated serine modified by DTT corresponding to the *O*-GlcNAc modified site in UL32 [31]. Deviation from the expected 1:1 ion pair ratio for BEMAD identifications was at greatest 27% and averaged less than 10%, a range of error comparable to other differential isotopic methods [35, 36]. Thus, BEMAD and ICAT performed comparably in relative quantification of expression through tagging and enrichment of cysteine-containing peptides, while BEMAD additionally provided quantitative information on serine/threonine modification sites. Only 8 underivatized peptides (not modified by DTT) were identified in the analysis, indicating the effectiveness of thiol chromatography enrichment. No *N*-terminal cysteines were derivatized in the BEMAD analysis of tryptic peptides from the standard mix, while cICAT identified 5 such peptides. A synthetic peptide from laminin (CDPYIGSR) with an *N*-terminal cysteine was successfully tagged by ICAT, but was not observed to be derivatized with DTT by BEMAD (data not shown). It appears that alkylated *N*-terminal cysteine residues may be resistant to  $\beta$ -elimination under the conditions used.

### 3.4 *O*-Phosphorylation site-mapping from mouse brain

The potential use of BEMAD in relatively large-scale mapping of serine/threonine modifications from a complex mixture of unknown proteins was tested. A tryptic digest of a

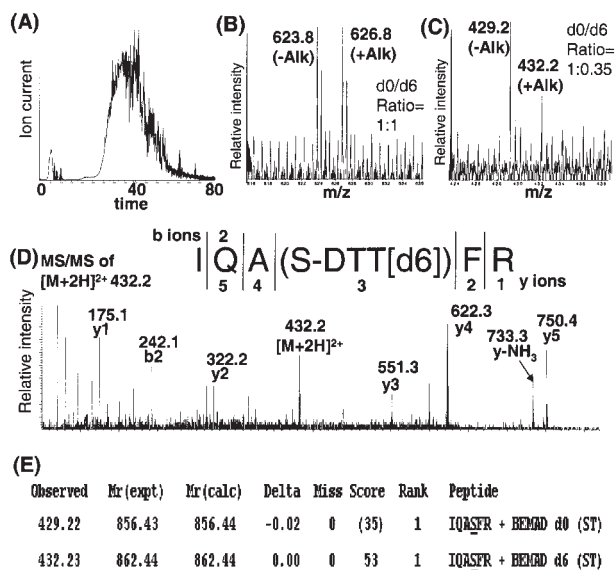
whole-brain lysate from mouse was spiked with the synthetic *O*-GlcNAc modified peptide PSVPV(S-*O*-GlcNAc)GSAPGR. Typically, post-translational modifications are substoichiometric and therefore the peptides they modify will be of low abundance compared to cysteine-containing peptides. Both alkylated cysteines (which would be derivatized by DTT) and reduced cysteines (which would be enriched during the thiol chromatography step) would thus interfere when specific targeting of serine/threonine modifications in a BEMAD analysis is desired. Therefore, we oxidized the brain tryptic digest with performic acid (converting cysteines to cysteic acid which adds 48 Da). It is important to note that performic acid oxidation not only targets cysteines, but may add 32 or 48 Da to tryptophan, and 32 Da to methionine and histidine. These potential mass additions due to oxidation must be accounted for in MS/MS interpretations. Intending to target *O*-phosphorylation, 2 mg of oxidized brain lysate was split in half and either untreated (–Alk) or treated with alkaline phosphatase (+Alk) as described in Section 2. BEMAD labeling of the (–Alk) sample with DTT (d0) and the (+Alk) sample with DTT (d6) was then performed prior to equimolar mixing, thiol enrichment, and LC-MS/MS. Total ion current for the 80 min LC-MS/MS BEMAD analysis is shown in Fig. 5A. The mass spectrum from 38.1 to 39.1 min of the analysis contained an ion pair at  $m/z$  623.8/626.8 corresponding to the *O*-GlcNAc-modified internal control peptide derivatized with DTT [PSVPV(S-DTT)GSAPGR] with a ratio of 1:1 as shown in Fig. 5B. MS for an ion pair at  $m/z$  429.2/432.2 eluting from 44.6 to 45.6 min with a ratio of 1:0.4 is shown in Fig. 5C. A CID spectrum from the DTT (d6) precursor at  $m/z$  432.2 identified the peptide IQA(S-DTT)FR derivatized with DTT at a  $\beta$ -eliminated serine (Fig. 5D). This peptide is found in mouse neurogranin and neuromodulin, and the DTT-modified residue (corresponding to serine 36 in neurogranin) is a known *in vivo* PKC substrate phosphorylation site involved in calmodulin association [37–39]. As phosphatase treatment did not affect the ion pair ratio of the *O*-GlcNAc-derivatized peptide (Fig. 5B), the phosphatase induced shift in the ion pair ratio 1:0.4 (d0/d6) corresponding to the neurogranin peptide indicates specific mapping of a phosphorylation site.

A summary of peptide identifications derived from ion pairs responsive to phosphatase treatment (d6 form having at least 40% lower intensity than the d0 form) from the BEMAD analysis of brain lysate is shown in Table 2. Several CID spectra leading to peptide identifications in Table 2 are provided in the supplemental figures.  $\beta$ -Eliminated serines modified with DTT in 5 of these 22 peptides (listed with bold font in Table 2) correspond to previously reported sites of phosphorylation (see references listed in supplementary Table 1 and Section 4 for details). In all cases, the MS/MS was of sufficient quality to unambiguously determine the site of modification. Due to sequence conservation, it was not always possible to determine which isoform(s) of a protein the DTT modified peptide came from as indicated in Table 2.

**Table 1.** Comparison of ICAT and BEMAD identification/quantitation of peptides from the standard mixture

Protein	ICAT	BEMAD
Transferrin (bov)	EC#VPNSNER (1:1) LC#QLCAGK (1:1.1) ENFEVLC#K (1:1) WC#AIGHQER (1:1) SVDDYQEC#YLAM- VPSHAVVAR (1:1) 11 peptides not shown	ATCVEK (1:1.1) SC#HTGLGR (1:1.2) TSHMDC#IK (1:0.9) ENFEVLC#K (1:1.1) EC#VPNSNER (1:1.0) WC#AIGHQER (1:1.0) 6 peptides not shown.
$\alpha$ S1 casein (bov)		LLILTC#LVAALARPK (1:1) <u>VPOLEIVPNS#AEER (1:1)</u> <u>YKVPOLEIVPNS#AEER (1:1)</u> <u>LHS#MK (1:1)</u>
Lysozyme (chick)	WWC#NDGR (1:1.1) NLC#NIPCSALLSSDITAS- VNCAK (1:1.0)	GYSLGNWVC#AAK (1:1.0)
$\alpha$ -Lactal- bumin (bov)		ALC#SEK (1:1.0) LDQWLC#EK /1:1.2) FLDDDLTDDIMC#VK (1:1.0)
$\beta$ -casein (bov) Ribonuclease (bov)	YPNC#AYK (1:1.0) C#KPVNTFVHESLADVQA- VC#SQK (1:1) HIIVAC#EGNPYPVHFD- ASV (1:1.0)	<u>FOS#EEQQTEDELQDK (1:1.1)</u> YPNC#AYK (1:0.9) STMSITDC#R (1:1.0) HIIVAC#EGNPYPVHFD- ASV (1:1.0)
Ig $\gamma$ 1C region (hum)	NQVSLTC#LVK (1:1) TPEVTC#VVVDVSHEDPE- VK (1:1.0) GPSVFPLAPC#LVK (1:1) VYAC#EVTHQGLSSPVTK (1:1)	NQVSLTC#LVK (1:1.0) VYAC#EVTHQGLSSPVTK (1:1.2) STSGGTAALGC#LVK (1:0.9) SYSC#QVTHEGSTVEK (1:1)
UL32 (EBV)		<b>PSVPV S#GSAPGR (1:1.0)</b>
$\alpha$ -S2 casein (bov)	ENLC#STFC#K (1:1.0)	
$\beta$ -Lactoglobulin (bov)	LSFNPTQLEEQC#HI (1:1.0)	LSFNPTQLEEQC#HI (1:1.1)
Alcohol de- hydrogenase	C#C#SDVFNQVVK (1:1.0)	YSGVC#HTDLHAWHGDW- PLPVK (1:1.2)
Serum albumin (bov)	QNC#DQFEK (1:1.1) C#C#TESLVNR (1:1) ETYGDMADC#C#EK (1:1) SLHTLFGDELK#K (1:1.0) 6 peptides not shown	SHCLAEVEK (1:1.0) QNC#DQFEK (1:0.9) EAC#FAVEGPK (1:1.2) SLHTLFGDELK#K (1:1.0) 7 peptides not shown
Superoxide dismutase	LAC#GVIGIAK (1:1)	AVCVLKLACGVIGIAK (1:1) LAC#GVIGIAK (1:1)
$\alpha$ -1-acid glycoprotein		EQLGEFYEALDCLR (1:1.0)

Ratios of ion pairs (light vs. heavy) are given in parentheses. For peptides not shown, ratios differed from 1:1 by less than 25%. Phosphopeptides are underlined and in italics. Q-GlcNAc peptides are in bold. # = position of ICAT or DTT tag.



**Figure 5.** Mapping phosphorylation sites from a complex mouse brain lysate with BEMAD. A performic acid oxidized tryptic digest of a cytosolic lysate derived from a whole mouse brain untreated (–Alk) or dephosphorylated with alkaline phosphatase (+Alk) was derivatized with DTT (d0) or DTT (d6), respectively, with BEMAD prior to equimolar mixing (a). Total ion current of an 80 min LC-MS/MS of the thiol-enriched mixture. Ion pairs corresponding to the derivatized forms of (b) the UL32 O-GlcNAc-modified peptide PSVPV(S-O-GlcNAc)GSAPGR (which had been spiked in to the original brain lysate) with a d0/d6 ratio of 1:1 and (c) a neurogranin peptide IQA(S-phospho)FR with a d0/d6 ratio of 1:0.4 are shown. (d) CID spectrum of  $[M+2H]^{2+}$  432.2 listing y and b ions identifying IQA(S-DTT)FR and showing a mass differential between the  $y_6$  and  $y_7$  ions of 229.1 corresponding to  $\beta$ -eliminated serine (dehydroalanine) modified with DTT (d6). (e) MAS-COT top ranked interpretations of CID corresponding to both the “light” and “heavy” form of IQA(S-DTT)FR, with the DTT modified residue corresponding to the known serine 36 phosphorylation site in neurogranin.

## 4 Discussion

Using a single approach (BEMAD), we demonstrate differential isotopic derivatization of cysteines and/or post-translationally modified serines/threonines, their enrichment from complex mixtures, and their identification/quantitation using LC-MS/MS. When applied to small subproteomes, such as protein complexes, simultaneous comparative analysis of protein composition and serine/threonine post-translational modifications should be possible. Analysis of post-translational modifications alone may not provide information on truly dynamic signaling events. For example, reduced phosphorylation of a given protein may be a consequence of reduced expression. Comparative analysis of both expression and post-translational events will help establish the truly dynamic nature of O-phosphate and/or O-GlcNAc modifications. The method is also suitable to large-scale proteomics at either the expression or post-translational level.

While certain high pH chemistries can bias  $\beta$ -elimination towards either O-phosphate [40] or O-GlcNAc [41], it has been difficult to achieve confidence in discriminating between derivatization of these alternative modifications with Michael addition. Additionally, there are recent reports of low-level nonspecific Michael addition to unmodified serines/threonines under relatively harsh  $\beta$ -elimination conditions [28, 29], adding to the potential for miss-assignment of post-translational modifications. We show that reduced levels of serine/threonine modified sites in response to enzymatic dephosphorylation or deglycosylation (as measured by differential isotopic Michael addition) aids in specifically assigning O-phosphate and O-GlcNAc sites.

Proteomic analysis of protein expression differences alone may miss important changes at the level of post-translational modifications. Techniques such as BEMAD will aid in detecting functional differences due to differential serine/threonine post-translational site occupancy. BEMAD is demonstrated to be a relatively sensitive method, through mapping of phosphorylation sites on the equivalent of 1 pmol of starting material of  $\alpha$ -casein and  $\beta$ -casein in the standard mix. As observed with CID of ICAT-labeled peptides [10], the presence of ion fragment pairs differing by 6 Da and the presence of the nonstandard masses of DTT modifying  $\beta$ -eliminated cysteine, serine, and threonine add confidence in interpretation of BEMAD-labeled peptide sequence. While fragmentation of ICAT tags may complicate MS/MS spectrums [42], no fragmentation of the smaller DTT tag in BEMAD has been observed. Compared to kits required for ICAT analysis, individual reagents required for BEMAD are commercially available and relatively inexpensive. Some sequence dependent differences in  $\beta$ -elimination efficiency may account for the unique set of “standard mix” cysteine peptides derivatized with BEMAD vs. ICAT. However, BEMAD derivatization of cysteine residues is clearly general enough to allow for large-scale expression analysis. BEMAD has recently been used to identify over 100 cysteine-derivatized peptides from liver in a single LC-MS/MS experiment (manuscript in preparation), achieving protein expression analysis at a level of complexity similar to ICAT [16].

The mapping of 21 phosphorylation sites in a single LC-MS/MS run from a mouse brain lysate demonstrates the usefulness of BEMAD in serine/threonine post-translational comparative proteomics. In addition to the PKC phosphorylation site in neurogranin at serine 36, four other mouse brain phosphorylation sites mapped by BEMAD have been previously reported. The serine 14 phosphorylation site (to which an antibody has been raised) in syntaxin 1, a protein involved in synaptic vesicle fusion, is a known *in vivo* CKII substrate site regulating association with SNAP-25 [43]. Other known phosphorylation sites mapped include the CaM kinase II substrate site serine 61 in the synaptic vesicle-associated protein VAMP [44], serine 63 in the abundant synaptic membrane protein 14-3-3 sigma [45], and at serine 178 in mouse myelin basic protein [46]. Serine 37 was iden-

**Table 2.** Serine/threonine phosphorylation sites from mouse brain determined by BEMAD

Protein(s)	Accession#(s)	Peptide	Start-end	Previously mapped site (reference)	Mascot Confidence Score
<b>VAMP 1, (2), (3)</b>	<b>Q62442 (Q64357), (Q64271)</b>	<b>LS#ELDDR</b>	<b>62-68</b>	<b>42</b>	65
<b>Neurogranin (neuromodulin)</b>	<b>gi:11528516 (P06837)</b>	<b>IQAS#FR</b>	<b>33-38</b>	<b>35</b>	53
<b>14-3-3 sigma</b>	<b>O70456</b>	<b>VIS#SIEQK</b>	<b>61-68</b>	<b>43</b>	36
<b>Myelin basic protein</b>	<b>P04370</b>	<b>FFS#GDR</b>	<b>175-181</b>	<b>44</b>	31
		DTGILDS#IGR	165-175		40
<b>Syntaxin 1 b-like</b>	<b>gi19625039</b>	<b>SAKDS#DEEEVVHVDR</b>	<b>10-25</b>	<b>41</b>	31
DPR-2(CRMP-2)	O08553	IVLEDGTLHVTEGS#GR	452-467		43
ATP synthase alpha	gi114523	VLS#IGDGIAR	64-73		36
Creatine kinase B	Q04447	LAVEALS#SLDGDLSGR	157-172		45
Rab-3A, (B), (C), (D)	P05713, (Q9CZT8), (P35276) (Q63482)	TS#FLFR	36-41		34
Na/potassium-transporting ATPase alpha	P06687	IATLAS#GLEVGK	260-271		64
Fructose-bisphosphate aldolase A	P05064	GILAADES#TGSIK	28-41		35
Cyclophilin A	gi 6679439	VS#FELFADK	20-28		32
CaMK 2 alpha, (beta), (delta), (gamma)	gi190334, (gi120177955) (gi125286), (gi118158420)	GAFS#VVR	22-18		35
ATP1A1	gi116307541	LS#LDELHR	5-12		30
14-3-3 sigma, (tau)	O70456, (P35216)	DS#TLIMQLLR	215-224		55
GTR-binding regulatory protein Gs alpha-S1	gi171887, (gi:6754006)	LLLLGAGES#GK	41-51		36
Drebin	Q9QXS6	LS#NGLAR	133-139		32
Gamma enolase	P17183	IAPALIS#SGISVVEQEK	72-88		33
Tubulin beta- (2), (3), (4) (5)	(Q7TMM9), (Q9ERD7) (Q9D6F9), (P05218)	IS#EQFTAMFR	381-390		64
Phosphoglycerate kinase	P09411	VS#HVSTGGGASLELLEG	388-405		58
Transducin beta3, (4)	Q61011, (P29387)	LLVSAS#QDQK	69-78		38

# = DTT modified. Identifications previously reported to be phosphorylated are in bold.

tified as a phosphorylation site on the small G protein Rab3A (the major Rab isoform in brain), which is found associated with synaptic vesicles and plays a role in their transport [47]. Serine 37 is adjacent to the GTP binding domain of Rab3A and phosphorylation at that site possibly modulates GTP exchange/hydrolysis. Several other phosphorylation sites were identified on proteins known to have brain specific expression patterns and functions, such as CaM-kinase II[48], cyclophilin A[49], creatine kinase B[50], DPR-2 (also known as CRMP-2) [51],  $\gamma$  enolase [52], drebrin [53], and phosphoglycerate kinase [54]. Potential regulatory roles for the newly identified amino-

terminal phosphorylation site in CaM-kinase II (Ser 25) include effects on modulating Ca<sup>++</sup>/calmodulin binding/activation of the enzyme which is critically controlled by autophosphorylation at threonine 286 [48]. Cyclophilin A is well-known for its role as an immunophilin and its binding to the immunosuppressive drug cyclosporin A. However, cyclophilin A is most highly expressed in neurons and the serine 21 phosphorylation site identified in this study may influence its known role in neuronal protein maturation and folding [49]. The creatine kinase B brain-specific isoform may be involved in ion homeostasis (K(+)-resorption) and neurotransmitter trafficking in the

brain [50], and the serine 163 phosphorylation site identified here could potentially influence these processes. There is a potential link between phosphorylation of DPR-2 (serine 465 was identified here as a site of phosphorylation) and neurodegenerative disease, as DPR-2 found associated with neurofibrillary tangles is hyperphosphorylated [51]. Potential regulatory roles of the serine 78 phosphorylation site on the neuronal and neuroendocrine specific  $\gamma$ -enolase include regulation of its dimeric glycolytic enzymatic activity [52]. Drebrin is highly phosphorylated *in vivo* with a brain-specific role in linking neurotransmitter signaling to the cytoskeleton [53], and sites of phosphorylation (serine 134 phosphorylation was identified here) may contribute to regulation of this function. Deficiency of phosphoglycerate kinase has central nervous system manifestations, such as mental retardation and epilepsy [54], and phosphorylation (serine 389 was identified here as a site of phosphorylation) may regulate its activity as a glycolytic enzyme.

It should be noted that we observed no incompletely oxidized cysteines using our performic acid conditions and thus it is likely that only +48 Da added to cysteine should have to be considered. Additionally, we did not observe halogenated tyrosine or the formation of kynurenine from tryptophan, although these have been reported as side-products of performic acid oxidation [55, 56].

In comparative proteomic experiments, we anticipate strategies in which a fraction of the original samples to be compared are labeled with DTT-d0 vs. DTT-d6 for quantitative analysis of protein levels and/or serine/threonine modification changes (with or without prior oxidation). Remaining original sample can then be subjected to treatment with  $-/+$  phosphatase and/or hexosaminidase to define specificity of DTT labeling.

Notably, all DTT-modified sites from BEMAD analysis of brain are serine residues. This is not unexpected from a single LC-MS/MS analysis of a complex mixture, as modified serines in general are more susceptible to  $\beta$ -elimination than modified threonines [40] and there is a cellular bias towards phosphorylation of serines over threonines (see phosphobase at [www.cbs.dtu.dk/databases/PhosphoBase/pb.html](http://www.cbs.dtu.dk/databases/PhosphoBase/pb.html)). Several peptide identifications containing DTT-modified serine/threonine residues were made from precursors belonging to ion pairs which did not respond to phosphatase (data not shown). These peptides likely represent mapping of either alternative serine/threonine modifications, phosphorylation sites resistant to alkaline phosphatase, or non-specific derivatization.  $\beta$ -Elimination chemistry is also used to target sites of complex O-linked glycosylation [57].

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## 5 References

- [1] Krishna, R. G., Wold, F., *Post-translational Modifications in Proteins: Analysis and Design Academic Press, San Diego, CA* 1998.
- [2] Schweppe, R. E., Haydon, C. E., Lewis, T. S., Resing, K. A., Ahn, N. G., *Acc. Chem. Res.* 2003, **36**, 453–461.
- [3] Jensen, O. N., *Curr. Opin. Chem. Biol.* 2004, **8**, 33–41.
- [4] Hunter, T., *Cell* 2000, **100**, 113–127.
- [5] Wells, L., Vosseller, K., Hart, G. W., *Science* 2001, **291**, 2376–2378.
- [6] Hanover, J. A., *Faseb J.* 2001, **15**, 1865–1876.
- [7] Hart, G. W., *Annu. Rev. Biochem.* 1997, **66**, 315–335.
- [8] Vosseller, K., Sakabe, K., Wells, L., Hart, G. W., *Curr. Opin. Chem. Biol.* 2002, **6**, 851–857.
- [9] Oda, Y., Huang, K., Cross, F. R., Cowburn, D., Chait, B. T., *Proc. Natl. Acad. Sci. USA* 1999, **96**, 6591–6596.
- [10] Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., *et al.*, *Nat Biotechnol.* 1999, **17**, 994–999.
- [11] Gygi, S. P., Rist, B., Griffin, T. J., Eng, J., Aebersold, R., *J. Proteome Res.* 2002, **1**, 47–54.
- [12] Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen *et al.*, *Mol. Cell Proteomics* 2002, **1**, 376–586.
- [13] Tao, W. A., Aebersold, R., *Curr. Opin. Biotechnol.* 2003, **14**, 110–118.
- [14] Ong, S. E., Foster, L. J., Mann, M., *Methods* 2003, **29**, 124–130.
- [15] Gharbi, S., Gaffney, P., Yang, A., Zvelebil *et al.*, *Mol. Cell Proteomics* 2002, **1**, 91–98.
- [16] Hansen, K. C., Schmitt-Ulms, G., Chalkley, R. J., Hirsch, J. *et al.*, *Mol. Cell Proteomics* 2003, **2**, 299–314.
- [17] Cutillas, P. R., Norden, A. G., Cramer, R., Burlingame, A. L., Unwin, R. J., *Contrib. Nephrol.* 2004, **141**, 155–169.
- [18] Meyer, H. E., Hoffmann-Posorske, E., Korte, H., Heilmeyer, L. M., Jr., *FEBS Lett.* 1986, **204**, 61–66.
- [19] Mega, T., Hamazume, Y., Hong, Y. M., Ikenaka, T., Nong, Y. M., *J. Biochem. (Tokyo)* 1986, **100**, 1109–1116.
- [20] Goshe, M. B., Conrads, T. P., Panisko, E. A., Angell, N. H. *et al.*, *Anal. Chem.* 2001, **73**, 2578–2586.
- [21] Oda, Y., Nagasu, T., Chait, B. T., *Nat. Biotechnol.* 2001, **19**, 379–382.
- [22] Weckwerth, W., Willmitzer, L., Fiehn, O., *Rapid Commun. Mass Spectrom.* 2000, **14**, 1677–1681.
- [23] Adamczyk, M., Gebler, J. C., Wu, J., *Rapid Commun. Mass Spectrom.* 2001, **15**, 1481–1488.
- [24] Andersson, L., Porath, J., *Anal. Biochem.* 1986, **154**, 250–254.
- [25] Thompson, A. J., Hart, S. R., Franz, C., Barnouin, K. *et al.*, *Anal. Chem.* 2003, **75**, 3232–3243.
- [26] Knight, Z. A., Schilling, B., Row, R. H., Kenski, D. M. *et al.*, *Nat. Biotechnol.* 2003, **21**, 1047–1054.
- [27] Wells, L., Vosseller, K., Cole, R. N., Cronshaw *et al.*, *Mol. Cell Proteomics* 2002, **1**, 791–804.
- [28] Li, W., Backlund, P. S., Boykins, R. A., Wang, G., Chen, H. C., *Anal. Biochem.* 2003, **323**, 94–102.
- [29] McLachlin, D. T., Chait, B. T., *Anal. Chem.* 2003, **75**, 6826–6836.

- [30] Amoresano, A., Cirulli, C., Qemeneur, E., Marino, G., *Eur. J. Mass Spectrom.* 2004, *10*, 401–412.
- [31] Greis, K. D., Gibson, W., Hart, G. W., *J. Virol.* 1994, *68*, 8339–8349.
- [32] Xhou, W., Merrick, B. A., Khaledi, M. G., Tomer, K. B., *J. Am. Soc. Mass Spectrom.* 2000, *11*, 273–282.
- [33] Larsen, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M., Roepstorff, P., *Proteomics* 2001, *1*, 223–238.
- [34] Grosclaude, F., Mahe, M. F., Voglino, G. F., *FEBS Lett.* 1974, *45*, 3–5.
- [35] Han, D. K., Eng, J., Zhou, H., Aebersold, R., *Nat. Biotechnol.* 2001, *19*, 946–951.
- [36] Washburn, M. P., Ulaszek, R., Deciu, C., Schieltz, D. M., Yates III, J. R., *Anal. Chem.* 2002, *74*, 1650–1657.
- [37] Baudier, J., Deloulme, J. C., Van Dorsselaer, A., Black, D., Matthes, H. W., *J. Biol. Chem.* 1991, *266*, 229–237.
- [38] Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., Storm, D. R., *Biochemistry* 1990, *29*, 2330–2335.
- [39] Prichard, L., Deloulme, J. C., Storm, D. R., *J. Biol. Chem.* 1999, *274*, 7689–7694.
- [40] Byford, M. F., *Biochem. J.* 1991, *280*, 261–265.
- [41] Rademaker, G. J., Pergantis, S. A., Blok-Tip, L., Langridge *et al.*, *Anal. Biochem.* 1998, *257*, 149–160.
- [42] Borisov, O. V., Goshe, M. B., Conrads, T. P., Rakov *et al.*, *Anal. Chem.* 2002, *74*, 2284–2292.
- [43] Foletti, D. L., Lin, R., Finley, M. A., Scheller, R. H., *J. Neurosci.* 2000, *20*, 4535–4544.
- [44] Hirling, H., Scheller, R. H., *Proc. Natl. Acad. Sci. USA* 1996, *93*, 11945–11949.
- [45] Aitken, A., Baxter, H., Dubois, T., Clokie, S. *et al.*, *Biochem. Soc. Trans.* 2002, *30*, 351–360.
- [46] Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y. *et al.*, *J. Biol. Chem.* 1985, *260*, 12492–12499.
- [47] Leenders, A. G., Lopes da Silva, F. H., Ghijsen, W. E., Verhage, M., *Mol. Biol. Cell.* 2001, *12*, 3095–3102.
- [48] Means, A. R., *Mol. Endocrinol.* 2000, *14*, 4–13.
- [49] Goldner, F. M., Patrick, J. W., *J. Comp. Neurol.* 1996, *372*, 283–293.
- [50] Hemmer, W., Wallimann, T., *Dev. Neurosci.* 1993, *15*, 249–260.
- [51] Charrier, E., Reibel, S., Rogemond, V., Aguera, M., Thomasset, N., Honnorat, J., *Mol. Neurobiol.* 2003, *28*, 51–64.
- [52] Deloulme, J. C., Helies, A., Ledig, M., Lucas, M., Sensenbrenner, M., *Int. J. Dev. Neurosci.* 1997, *15*, 183–194.
- [53] Shirao, T., Sekino, Y., *Neurosci. Res.* 2001, *40*, 1–7.
- [54] Sugie, H., Sugie, Y., Tsurui, S., Ito, M., *Neurology* 1994, *44*, 1364–1365.
- [55] Chowdhury, S. K., Eshraghi, J., Wolfe, H., Forde, D., Hlavac, A. G., Johnston, D., *Anal. Chem.* 1995, *67*, 390–398.
- [56] Finley, E. L., Dillon, J., Crouch, R. K., Schey, K. L., *Prot. Sci.* 1998, *7*, 2391–2397.
- [57] Huang, Y., Konse, T., Mechref, Y., Novotny, M. V., *Rapid Commun. Mass Spectrom.* 2002, *16*, 1199–1204.