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Quantification of phosphoproteins with global internal standard technology

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Abstract

Global internal standard technology (GIST) is being developed for the quantification of all primary structure and post-translational variants of proteins in a proteome. This paper is directed at an analysis of phosphorylation, primarily of serine and threonine. Quantification was achieved by acylation of primary amino groups in peptide cleavage fragments of proteins with isotopically coded derivatizing agents. Peptides from controls were globally coded with an isotopically "light" form of the reagent while those from experimental samples were coded with a "heavy" form of the reagent. The two types coding reagents used in this work were *N*-hydroxyl succinimide derivatives of acetate and 4-trimethylammoniumbutyrate. Heavy isotope forms were produced by deuteration of methyl groups. Subsequent to coding and mixing, the two samples were passed through a Ga(III) immobilized metal affinity chromatography (IMAC) column and the selected peptide fraction was further resolved by reversed-phase chromatography (RPC) and analyzed by mass spectrometry (MS). Relative differences in phosphopeptide concentration between samples were derived from isotope ratio measurements of the peptide isoforms observed in mass spectra. The method was validated with model peptides.

Keywords: Global internal standard technology; Phosphoproteins

1. Introduction

Genomic databases are being used increasingly in protein identification. A problem with this approach is that proteins also undergo a large number of post-translational modifications (PTM) that are not apparent from gene sequence data. One of the more prominent modifications is phosphorylation. Protein phosphorylation plays many roles, ranging from the promotion of protein complex formation and enhancing the biological activity of a protein to signaling. Phosphorylation is widely exploited in biological systems to cycle proteins between different activity states with minimal expenditure of energy. Determining the phosphorylation state of proteins and quantifying the extent to which phosphorylation has changed is essential in the study of many types of regulation.

Quantification by heavy isotope coding in vitro is now being utilized widely in proteomics to study protein expression [1,2]. Recent studies indicate that isotope coding is of equal utility in the study of post-translational modification [3–5]. A central element in many of these methods is proteolytic conversion of proteins to peptides that are then differentially coded according to sample origin and compared simultaneously by mass spectrometry (MS). Two issues must be confronted in applying these in vitro coding methods to PTM analysis. One is how to introduce isotope coding into peptides carrying a particular PTM. The second is how to recognize these peptides in a sea of other peptides.

Two relatively different strategies have been used in dealing with these issues. One is to isotopically code all peptides formed during proteolysis and then affinity select those with a particular structural feature for analysis, such as peptides that are glycosylated [3] or phosphorylated [4]. Coding is achieved either by derivatization of primary amines [3] or carboxyl groups [4] with isotopically distinct forms of a coding agent. The alternative route is to target the PTM directly for covalent modification and attachment of a derivatizing agent that is both isotopically coded and tagged with biotin for later selection [5]. This approach has been used successfully in the study of phosphorylation.

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This paper focuses on the first of these two strategies, using acylation of primary amines in an approach that has come to be known as the global internal standard technique (GIST) [6]. Acylation has been achieved in the GIST approach with succinic anhydride and *N*-hydroxysuccinimde activated acetate, propionate, and 4-trimethylammonium butyrate (TMAB) [7,8]. Through acylation with isotopically distinct forms of these reagents, peptides in control and experimental samples can be differentially coded and after mixing, multidimensionally fractionated and analyzed by mass spectrometry. All peptides appear in mass spectra as doublet clusters of ions separated by multiples of the mass difference in the coding agents attached to the peptide depending on the number of the labels incorporated. In the cases of $^{1}H_3/^{2}H_3$-acetate and $^{12}C_3/^{13}C_3$-propionate, the doublet mass difference is a multiple of 3 amu. Peptides labeled with $^{1}H_9/^{2}H_9$ trimethylammonium butyrate will appear in mass spectra separated by multiples of 9 amu. Relative differences in concentration among samples are quantified by isotope ratio measurements [9].

Affinity selection of serine/threonine phosphorylated peptides with either Ga(III) or Fe(III) loaded immobilized metal affinity chromatography columns is now widely used to examine this form of phosphorylation [4]. One of the concerns with the acylation of amino groups as part of stable isotope labeling for quantification is that it will alter or even preclude immobilized metal affinity chromatography (IMAC) selection by altering the charge on a peptide [6]. This paper examines the prospect of combining Ga(III) IMAC selection with the GIST approach to quantitative proteomics for quantification of serine/threonine phosphorylation. The linear dynamic range of quantification was examined with two different GIST reagents, *N*-acetoxysuccinimide and 4-trimethylammonium butyrate. Additionally, the ability to carry out Ga(III) IMAC selection on isotopically coded peptides is examined.

2. Experimental

2.1. Materials

The phosphorylated peptide P6462, trypsin, *N*-[2-hydroxyethyl] piperazine - *N*′ - [2 - ethanesulfonic acid] (HEPES), monobasic sodium phosphate, iodoacetic acid (IAA), dithiothreitol (DTT), sodium acetate trihydrate, the *N*-hydroxysuccinimide ester of acetic acid (AA), *N*-hydroxysuccinimide, D_3-acetic anhydride, hydroxylamine, glacial acetic acid, sodium chloride, gallium(III) chloride (HPLC)-grade formic acid, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and urea were purchased from Mallinckrodt (St. Louis, MO, USA). Calcium chloride ($CaCl_2$) and ammonium hydroxide (NH_4OH) were purchased from Fisher Scientific. Phosphorylated peptide standards #22914, #20291, #24504, #25060 were purchased from AnaSpec. HITrap™ Chelating HP column was obtained from Amersham Pharmacia Biotech. A 218TP54 reversed-phase C18 column was purchased from Vydac™. Bovine milk was provided by Purdue Dairy.

2.2. Instrumentation

Reduction, alkylation, digestion, and labeling of samples was done in solution while the affinity selection and reversed-phase chromatography (RPC) analyses were done in series on an Integral Micro-analytical Workstation (PE Biosystems, Framingham, MA). Analyte detection was achieved by monitoring absorbance at 215 nm. Labeled peptides were injected onto a HITrap™ Chelating HP IMAC column of 1 mL volume that had been loaded with Ga(III) for affinity selection of phosphorylated peptides. Peptides selected by the IMAC column were directly transferred to a C18 column and resolved for further analysis. Mass spectral analyses were done off-line with a MALDI-TOF Voyager-DE™ RP Biospectrometry™ (PE Biosystems) and a PE Sciex QSTAR™ Hybrid LC/MS/MS Quadrupole TOF mass spectrometer. All spectra were taken in the positive ion mode.

2.3. Proteolysis

Disulfide reduction was carried out in 8 M urea and 10 mM DTT. To facilitate reduction, the sample was heated at 37 °C for 1 h. Alkylation was then achieved through the addition of IAA to a final concentration of 10 mM and allowed to incubate at 4 °C in the dark for 30 min. The solution was diluted to 1.5 M urea with 50 mM HEPES, pH 8.0. Proteolysis with trypsin was achieved at an enzyme to protein ratio of 1/50 (w/w). The digest was allowed to incubate at 37 °C for 8 h. The resulting peptides were collected and incubated with labeling agent.

2.4. Synthesis of *N*-acetoxy-D_3-succinimide

Production of this reagent and the procedure for its use in labeling peptides has been described elsewhere [10–12]. Briefly, 1.77 g of *N*-hydroxysuccinimide were combined with 5 g of D_3-acetic anhydride under nitrogen and stirred constantly overnight at room temperature. The white crystals that formed were filtered, washed three times with hexane, left to dry under nitrogen, and stored at −20 °C until utilized.

2.5. Labeling reaction of peptides with *N*-acetoxysuccinimide

Acetic acid *N*-acetoxysuccinimide and *N*-acetoxy-D_3-succinimide were used to label the control and experimental mixtures of tryptic peptides, respectively. Tryptic digests of control and experimental samples were exposed to a 3×

molar excess (with respect to molar concentration of the peptides) of the *N*-acetoxysuccinimide coding agents and incubated at room temperature with stirring for 5 h. The pH of the solution was increased to 10 and a 3 M excess of hydroxylamine was added to the digest and incubated at room temperature for 30 min.

2.6. Synthesis and labeling of peptides with 4-trimethylammonium butyrate-H_9

4-Trimethylammonium butyric acid was prepared according to a procedure by Staros [13]. A 50 fold molar excess of the *N*-hydroxysuccinimide (NHS) ester of (H_9)4-trimethylammonium butyrate was added to the control sample and the NHS ester of (D_9)4-trimethylammonium butyrate to the experimental sample. These samples were then allowed to react overnight at room temperature.

2.7. Preparation of Ga(III)–IMAC column

The washing and loading of the IMAC column was carried out first washing with 5 CV of ddI water at 0.5 mL/min. Roughly 15 CV of mobile phase containing 100 mM Ga(III)Cl_3 and 100 mM acetic acid (pH 3.2) was then passed through column at 0.2 mL/min. A 3 CV wash of 0.1% acetic acid at 0.5 mL/min completed the preparation of the gallium loaded IMAC column.

2.8. Affinity selection

Equivalent amounts of control and experimental samples were combined and acidified with glacial acetic acid to a final pH between 2.5 and 3.0. This sample was then applied to the Ga(III)–IMAC column at 0.025 mL/min. The column was then washed with 0.1% acetic acid for an additional 3 CV, 30% ACN/0.1% AA for 3 CV, and finally with to 0.1% AA for a 3 CV wash. Next the column was washed with 3 CV of 0.5 M NaCl/0.1% AA, 3 CV of 0.1% AA. Peptides were eluted directly onto the reversed-phase column with 3 CV of 0.2 M phosphate buffer (pH 8.4).

2.9. Reversed-phase elution of the peptides

The reversed-phase column was equilibrated using 100% mobile phase A (5% ACN/95% ddI H_2O with 0.1% TFA v/v) at a flow rate of 1.00 mL/min for 2 CV. Gradient elution of the analyte was achieved over 50 min starting with 100% mobile phase A and ending with 100% mobile phase B (95% ACN/5% ddI H_2O with 0.1% TFA v/v) at a flow rate of 1 mL/min. The gradient was then held at 100% mobile phase B for an additional CV. Throughout the analysis, an on-line UV detector set at 215 nm monitored the peptide fragments. Each peak was collected manually and rotovaped to a final volume of 50 μL before analysis by MALDI-TOF mass spectrometry.

2.10. MALDI-TOF mass spectrometry

A PE Biosystems Voyager DE-RP™ was used to analyze peptide fractions. A 1 μL volume of each fraction was incubated and vortexed briefly in 20 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid solution (50% ACN/50% H_2O in 0.1% TFA v/v). A 1 μL volume of sample of this mixture was spotted onto the MALDI plate and allowed to crystallize under vacuum. Samples were analyzed in the reflector mode. Instrument parameters were an acceleration voltage of 20,000 V, a guide wire voltage at 0.05%, and delay time of 200 ns. All spectra were taken in the positive ion mode. Spectra were from an average of roughly 100 scans.

2.11. Sequencing peptides

A PE Sciex QSTAR™ Hybrid LC/MS/MS Quadrupole TOF mass spectrometer was used to examine peptides that showed a quantitative change in concentration with respect to the control sample. The original fraction was mixed with an equal volume of 100% methanol, 0.1% formic acid, and vortexed. Fractions were then electrosprayed into the mass spectrometer and masses indicating a change in intensity with respect to their respective control mass were fragmented via MS/MS.

3. Results and discussion

Primary amine derivatization and coding of all peptides in tryptic digests was achieved with either *N*-hydroxysuccinimide activated acetic or 4-trimethylammonium butyric acid (Fig. 1a and b). Peptides from experimental samples were labeled with deuterated forms of coding agents in all cases. For example, in the case of acetate coding control samples were labeled with *N*-acetoxysuccinimide and experimental samples with *N*-acetoxy-D_3-succinimide, then mixed, and subsequently fractionated as described above. Isotopically coded peptides appeared in spectra as doublet clusters separated by 3, 6, or 9 amu, depending on the number of primary amine groups in the peptide. Peptides with

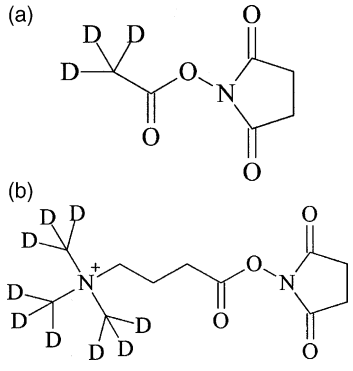

Fig. 1. *N*-Acetoxy-D_3-succinimide and trimethylammonium butyrate-D_9.

a free amino group at their N-terminus and arginine at the C-terminus with no additional amino group appeared in spectra as a double cluster of ions separated by 3 amu. Peptides with C-terminal lysine residues were separated by 6 amu. C-terminal lysine-containing peptides with an additional lysine residue from a missed-cleavage showed a 9 amu difference.

Coding with H_9/D_9 4-trimethylammonium butyrate was also examined for its use in comparative analysis of phosphopeptides. Again the number of primary amine groups in the peptide being derivatized determined the mass difference in doublet clusters appearing in spectra.

Linearity of the GIST method in quantifying relative differences in the concentration of phosphopeptides was examined with the *N*-acetoxysuccinimide coding agent by adding differing ratios of a differentially coded synthetic phosphopeptide to a tryptic digest of albumin. Concentration ratios of the coded synthetic peptide added were 0.5:1, 1:1, 1:2, 1:3, 1:5, and 1:10. Subsequent to mixing different concentration ratios of coded samples, phosphopeptides were selected with a Ga(III) loaded immobilized affinity chromatography (IMAC) column, further fractionated by reversed phase chromatography, and then analyzed by MALDI mass spectrometry. Isotope ratio versus concentration plots were linear over the entire range of concentration tested ($y = 1.094x - 0.1455$, $R^2 = 0.9976$).

The chromatographic retention times (Fig. 2) and mass of acylated and un-acylated peptides were different. This fact was exploited in evaluating the degree of acylation with the *N*-acetoxysuccinimide coding agent. Based on reversed phase chromatography and MALDI mass spectrometry, the un-acylated peptide was undetectable. It is concluded from these results that acylation is at least 99.9% complete based on the dynamic range of these two instruments.

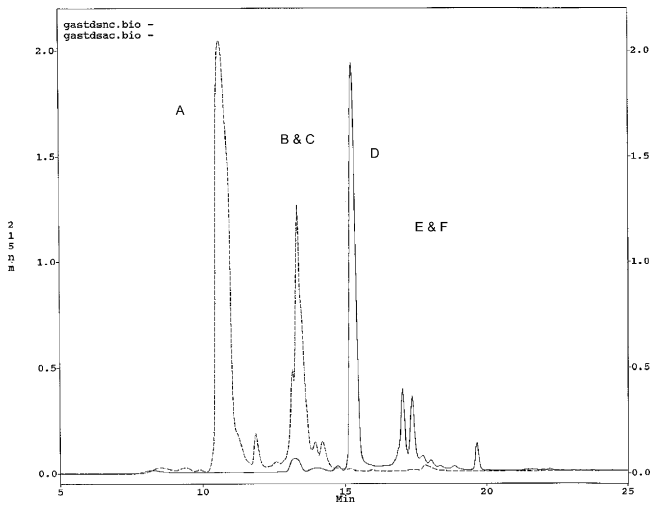

Fig. 2. Three phosphorylated peptide standards (Lys-Arg-pThr-Ile-Arg-Arg, Leu-Arg-Arg-Ala-pSer-Leu-Gly, Thr-Ser-Thr-Glu-Pro-Gln-pTyr-Gln-Pro-Gly-Glu-Asn-Leu) designated A, B, and C, respectively, selected by Ga(III) IMAC and resolved with RPC. The same peptide standards labeled with *N*-acetoxysuccinimide (D, E, and F) then selected by Ga(III) IMAC and resolved with RPC.

Subsequent to isotope coding, there was no observable loss of the coding agent during the ensuing multidimensional

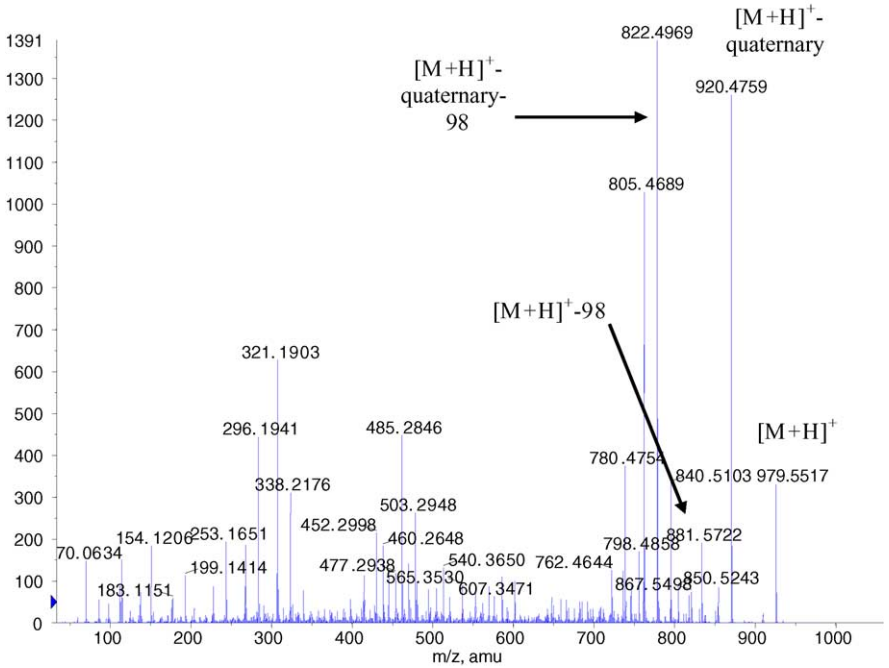

Fig. 3. MS/MS of the previous standard phosphorylated peptide (Leu-Arg-Arg-Ala-pSer-Leu-Gly) labeled with trimethylammonium butyrate-H_9 on the primary amine site within the peptide. The loss of H_2PO_3 is evident by the 881.57 ion. Fragmentation of the quaternary amine head of the labeling agent is evident by the 920.47 ion.

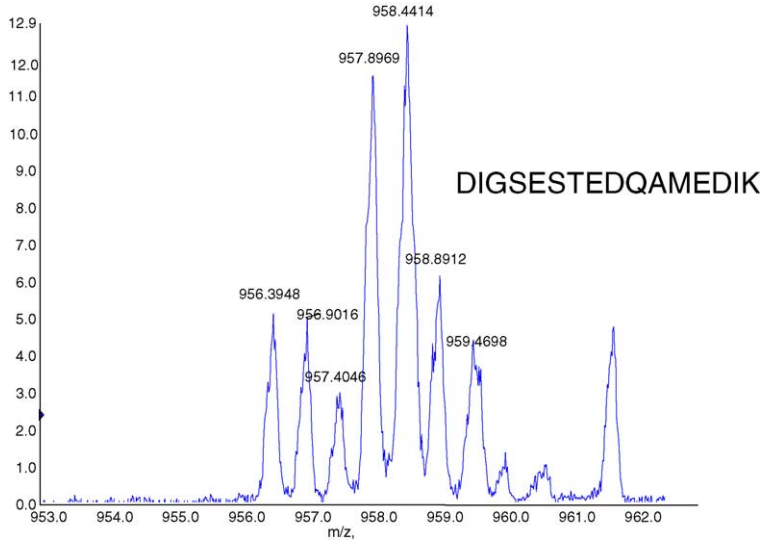

Fig. 4. Mass spectrum of $[M + 2H]^{2+}$ of 956.39 acetate-H_3 labeled peptide and its acetate-D_3 counterpart $[M + 2H]^{2+}$ of 957.89 indicating a two fold increase of the peptide originating from the cow with mastitis.

fractionation process with acidic mobile phases or during storage under acidic conditions.

Acidic peptides are often harder to detect in mass spectrometry, particularly in the positive ion mode of MALDI-MS. Because acetylation of primary amines with *N*-acetoxysuccinimide increases peptide acidity by removing positive charge, lysine-containing peptides are harder to detect. This problem can be addressed by using the quaternary amine coding agent 4-trimethylammonium butyrate. Acylation of peptides with TMAB removes a primary amine from the conjugate but in the process adds a quaternary amine. A second advantage of TMAB is that deuterium labeling can be clustered around the very polar quaternary amine to minimize chromatographic isotope effects.

Standard phosphorylated peptides were labeled with (H_9)TMAB and (D_9)TMAB on their primary amines. Acylation with a single (H_9)TMAB shifts the mass of the parent ion by 127 amu whereas addition of (D_9)TMBA causes a 136 amu shift in parent ion mass. The characteristic loss of H_2PO_3 (−98 amu) and trimethylamine (−58 amu) from the coding agent during MS/MS are unique features of TMAB coded peptides (Fig. 3).

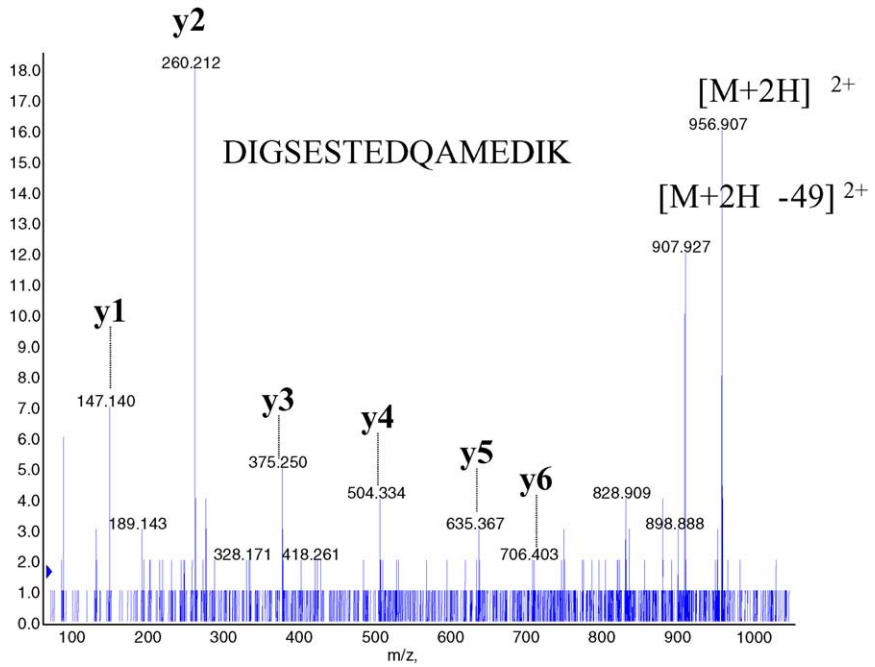

Fig. 5. MS/MS of $[M + 2H]^{2+}$ 956.9 *m/z*. Note the loss of 49 mass units due to the loss of the H_3PO_4.

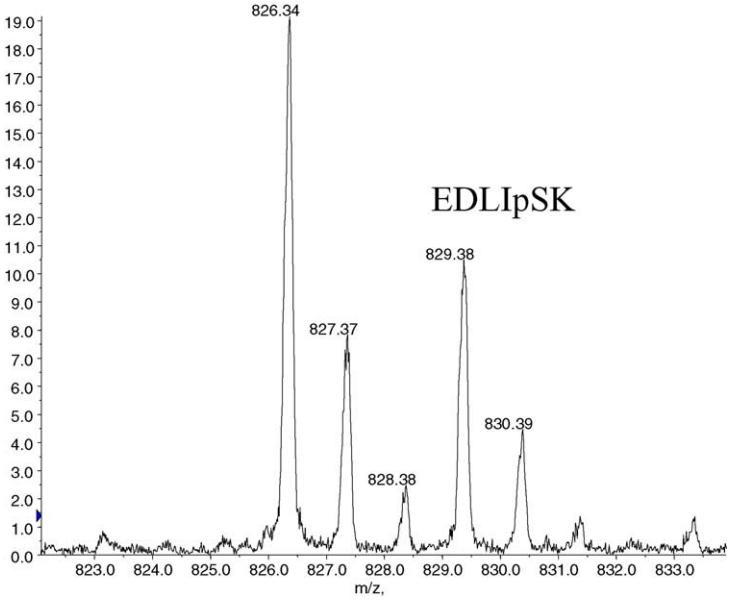

Fig. 6. The mass spectrum of the $[M+H]^+$ of the 826.34 peptide is acetate-H_3 labeled and its acetate-D_3 counterpart $[M+H]^+$ of 829.38 indicating a two fold decrease of the peptide originating from the cow with mastitis.

Table 1
Milk proteins identified by tandem mass spectrometry and Mascot

Protein	Peptide sequence	Ratio
Alpha S1 casein	AYFYPELF	+2.2
	DIGSESTEDQAMEDIK [+1 p(S or T)]	+3
	VPQLEIVPNSAEER	+1.1
	HQGLPQEVLNENLL	+2.2
	GYLEQLL	+7
Alpha S2 casein	NMAINPSK	1
Beta casein	FQSEEQQQTEDELQDK	1
	YPVEPFTER	−20
Kappa casein	FFSDK	1
T-cell receptor beta chain	QQGVLSATLLYEILLGKATLYAV [+1 pY and +1 p(S or T)]	+3.5
	LVSALVL	
T-cell receptor alpha chain	GSGTML	+2
T-cell receptor delta chain	EDSAKY	+1.2
Allograft inflammatory factor-1	DLNEDGGIDIMSLKRMMEKLGVPKTHL	+5.6
	KLIMEVSSGPGETFSY	−3
Uterine milk protein precursor	SQKMEAHPKDFAQELF	+2.5
	HHLVHLGR	
Epithelial mucin	HPMSEYSTYHTHGR [+1 p(S or T)]	+4
	QELQRSIW	
15S-Lypoxygenase type 2	SLVLREGTAKISW [+1 p(S or T)]	1
MARC 2 BOV	WLGFVF	10
MARC 3 BOV	WPLHSVSASL [+2 p(S or T)]	3.5
BARC 5 BOV	KRYTL	3
Pregnancy-associated glycoprotein 8	SCDAINSLPTL [+1 p(S or T)]	−1.7
Pregnancy-associated glycoprotein 14	FDTGSSDLW	1.3
Potassium channel protein	AEADER	−2
28 kDa Glycoprotein PP3	EDLISK	−2
RAB11 binding protein	SHCGRL	1
	STRGRNK	−2

Table 1 (*Continued*)

Protein	Peptide sequence	Ratio
Hepatoma derived growth factor	QSSSQKK	−2
	DLFPY	1
	GTHETAFL	−1.2
Inducible nitric oxide synthase 1	SQKVDL	2
β-Lactoglobulin	TPEVDDEALEK	1
Acetylcholine receptor protein, alpha chain	IHIPSEK	1.2
Alpha crystallin beta chain	ESDLFPASTSL [+1 p(S or T)]	1
Thioredoxin	HSLSEK [+1 p(S or T)]	−2
Histidine-rich glycoprotein	CPHPPFGTK	3
Immunoglobulin λ light chain	GSSSRPSGDPDR [+1 p(S or T)]	2.5
Connective tissue growth factor	KCPDGEVMK	−1.2
SMC3 protein	TSKEER [+1 p(S or T)]	8
Cytochrome P450 1A1	KGRPDL	1.1
Unc-18 protein homolog-bovine	DEDDDLW	3
Proline–arginine-rich end leucine-rich repeat protein	RLSQNQISR	2
Peroxisome proliferator activated receptor alpha	QEIYKDMYR	2
Prolactin-releasing peptide precursor	EGGAEPSRALPGRL	1.1
RAN-binding protein 2	EQFSTSHTFK	−5
Plasma membrane calcium transporting ATPase	DNENDIVTGL	−1.5
Epididymal secretory protein E1	AEPVKF	−1.4
Annexin II	DLYDAGVKRK	3
Non-muscle caldesmon	EETEIVTK [+1 p(S or T)]	6
CAAX box protein TIMAP	KVHEVPDYSMAYGNPGVADATPSWSGY [+1 pY +1 p(S or T)]	2
Myosin IB	NMVWKYCRSISPEW	1.4
Erythrodihydroneopterin triphosphate synthetase	YYFGIYKR	8
Cathepsin D	GDVFIGRYY	2
Uveal autoantigen	SEQTQK [+1 p(S or T)]	2
Apolipoprotein C-III	MTESFSSLKDYW [+3 p(S or T)]	10
G-protein-coupled receptor kinase GRK5	LDSMYFDRFLQWK	4
CAMP-dependent protein kinase, beta-1 catalytic subunit	RVKGRTWTLCGTPEY [+1 p(S or T)]	3
Cleavage and polyadenylation specificity factor protein	DDIGPSVVMASPGMMQSGL	2

The left column describes the protein from which the peptide sequence in the second column originated. The ratio column indicates the fold change in regulation of the experimental protein (mastitis stricken milk) that was labeled with acetate-D_3 with respect to the identical protein from the control sample (non-mastitis stricken milk) labeled with acetate-H_3.

TMAB labeled phosphopeptide standards were again selected with Ga(III) IMAC and resolved with RPC. TMAB derivatization had no impact on IMAC selection of phosphopeptides. Linearity of quantification was evaluated as in the case of acetylation above with a synthetic phosphopeptide using (H_9)TMAB and (D_9)TMBA coding. Differently labeled peptides were then combined in ratios of 1:1, 1:2, 1:3, 1:5, and 1:10 and added to a β-lactoglobulin tryptic digest. After Ga(III) IMAC selection the captured peptides were transferred to a reversed phase chromatography column and further fractionated before analysis by MALDI mass spectrometry. The results were linear over the entire concentration range examined ($y = 0.9105x - 0.0598$, $R^2 = 0.9983$). These findings lead to the conclusion that the use of TMAB and acetate labeling are equivalent.

Bovine milk has been widely used to evaluate methods for analysis of the phosphoproteome. Efficacy of the method described above was evaluated in a comparative analysis of milk obtained from healthy cattle and one stricken with mastitis. Fat was skimmed from each sample, the disulfide bonds in proteins were reduced, sulfhydryl groups were then alkylated, and the proteins digested with trypsin. The sample from healthy animals was coded with *N*-acetoxysuccinimide while that from the animal with mastitis was labeled with *N*-acetoxy-D_3-succinimide. These samples were combined, selected with Ga(III) IMAC, fractionated by reversed phase chromatography, and then analyzed by electrospray ionization mass spectrometry. The mass spectrum in Fig. 4 is from one of the reversed phase chromatographic peaks. Isoforms of peptides from αS1 casein differing by 3 amu are seen in the spectrum. The lysine residue in this peptide did not get labeled for an unknown reason. As indicated in the Figure, up-regulation in αS1 casein is seen in the disease state. The MS/MS spectrum of the non-deuterated peptide (Fig. 5) shows the loss of phosphate as a mass shift of 49 amu from the $[M + 2H]^{2+}$ in addition to confirming the sequence. Down-regulation of a peptide in the animal with mastitis is seen in Fig. 6. A list of peptides that varied in concentration between healthy and infected animals is seen in Table 1. Many of the peptides mentioned in this table are not phosphorylated. This is due to nonspecific binding of acidic residues which is commonly observed during IMAC selection for phosphopeptides [4].

The observed changes in expression of the proteins varied from a 20 fold decrease to up 10 fold increase based on peak intensity. It was observed that different peptides from the same protein sometimes showed different changes in relative concentration. It is possible that different phosphorylation sites are being regulated independent of each other. Further study would be necessary to determine if this is the case.

There were several proteins identified that do not seem to originate from milk. While the definite origin of these proteins is unknown, it is possible that they could result from the infection. The history of the two animals from which these samples were taken is unknown. Thus, it is impossible to draw any biological conclusions on the differences in concentration of peptides between the two animals. A more definitive study is required with samples pooled from populations of healthy and infected animals.

4. Conclusions

Utilizing *N*-acetoxysuccinimide, *N*-acetoxy-D_3-succinimide, trimethylammonium butyrate-H_9, and trimethylammonium butyrate-D_9 in the labeling of primary amines of peptides in a sample gave uniform and stable coverage. The *N*-acetoxysuccinimide and trimethylammonium butyrate labeling of peptides are linear beyond a 10 fold change in concentration of a peptide. The labeling agents do not interfere in the selection of phosphorylated peptides by Ga(III) IMAC. Ionization of the peptides in the mass spectrometer is still achieved, and post-translational modifications remain evident during MS/MS. Combining these labeling techniques with a simplification of the complex sample by Ga(III) IMAC and RPC will allow us to tackle the enormity of analyzing complex cellular samples and the ability to monitor the up and down regulation of the proteins as a result of a stress upon a system. Even though complex cellular lysates can result in upwards to a million peptides, the GIST technique allows a way to simplify and observe cellular regulation with very little sample manipulation in a matter of a few hours.

Acknowledgements

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