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Reduction of non-specific binding in Ga(III) immobilized metal affinity chromatography for phosphopeptides by using endoproteinase glu-C as the digestive enzyme

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Abstract

The selectivity of immobilized metal affinity chromatography (IMAC) systems for the purification of phosphopeptides is poor. This is particularly a problem with tryptic digests of proteins where a large number of acidic peptides are produced that also bind during IMAC. The hypothesis examined in this work was that the selectivity of IMAC columns for phosphopeptides could be increased by using endoproteinase glu-C (glu-C) for protein digestion. Glu-C cleaves proteins at acidic residues and should reduce the number of acidic residues in peptides. This method was successfully applied to a mixture of model proteins and bovine milk. The percentage of phosphorylated peptides selected from proteolytic digests of the milk sample was increased from 40% with trypsin to 70% with glu-C. Additionally, this method was coupled with stable isotope coding methods to quantitatively compare the concentration of phosphoproteins between samples.

Keywords: Phosphopeptides; Endoproteinase glu-C

1. Introduction

Phosphorylation is one of the more abundant posttranslational modifications (PTM) of proteins, being implicated in cellular functions ranging from signal transduction and gene expression to the regulation of growth and involvement in disease mechanisms. Accordingly, much effort has been expended in the study and quantification of changes in protein phosphorylation and dephosphorylation [1–14].

One of the oldest methods for measuring phosphorylation is through ^{32}P labeling with quantification by scintillation counting or audioradiography [1,2]. More recently, quantification is achieved by stable isotope labeling and mass spectrometry. Because phosphoproteins appear in low abundance, some type of enrichment is often used to obtain sufficient quantities of material for characterization. Antibodies have been relatively useful in the context of recognition and enrichment, particularly with Western blotting and immunoprecipitation methods associated with 2D gel electrophoresis [3,4]. They are fairly specific for the 0.1% of phosphoproteins that are phosphorylated on tyrosine [5] but seem to be far less useful in the enrichment of phosphoserine and phosphothreonine containing proteins [4].

At the peptide level of phosphoprotein analysis, a method for the replacement of the phosphate ester with another functional group carrying an affinity selectable tag has been described [5–8]. This approach exploits the fact that phosphate esters on serine and threonine β-eliminate with the formation of an α,β-unsaturated double bond. The β-elimination product is then alkylated at the former phosphorylation site with ethane dithiol (EDT) and subsequently labeled with a biotin tag. Following the removal of excess EDT-biotin tagging agent, biotinylated peptides are selected with an avidin column and then characterized by LC/MS. Phosphotyrosine does not β-eliminate, making this method specific for serine and threonine phosphorylated peptides. Because serine and threonine phosphorylation sites tend to cluster, it is common to encounter multiple biotin groups in the same peptide when using this process. Biotinylation adds roughly 500 amu per phosphorylation site to the mass of a peptide, causing the mass of peptides with multiple phosphorylation sites to be substantially higher after biotin tagging. It is impor-

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tant to note that *O*-glycosylated peptides also β-eliminate, producing a site indistinguishable from a phosphorylation site.

Still another method for selecting phosphopeptides is through attachment of an affinity tag to phosphate residues [9]. In this process, primary amines in peptides are first protected using *t*-butyl-dicarbonate (*t*-BOC) chemistry. An aqueous solution of the protected peptides is then treated with ethanolamine and a water-soluble carbodiimide to convert carboxyl groups to amides and phosphate groups to phosphoramidates. Phosphate groups in peptides are subsequently regenerated by treatment with weak acid and again converted to phosphoramidates with carbodiimide catalysis, but with cystamine in the second phosphoramidation. Reduction of cystamine generates a free sulfhydryl group that, following removal of non-peptide reactants through reversed phase chromatography (RPC), is covalently captured by reaction with iodoacetyl groups attached to glass beads. Phosphoramidate bonds in the captured peptides are again cleaved with trifluoroacetic acid at a concentration that cleaves the *t*-BOC protecting group as well. The original phosphopeptides are released with an overall recovery of approximately 20% [9].

Finally there are analytical methods for the characterization of phosphopeptides based on direct selection of phosphopeptides. Immobilized metal affinity chromatography (IMAC) columns loaded with Fe(III), Al(III), Zr(IV), or Ga(III) are well known to bind phosphopeptides [10,11]. Although Ga(III) has been noted to have distinct advantages in selectivity and ease of elution [11], Fe(III) loaded columns remain the most commonly used form of IMAC for phosphopeptide selection [4,12–14]. Regardless of which metal is used, IMAC is not without its problems, the largest being the binding of acidic peptides. More than half of the peptides selected by IMAC columns are generally not phosphorylated. It has been shown that this problem can be circumvented by blocking the binding of acidic peptides to IMAC columns through esterification of their carboxyl groups. The selection of phosphopeptides is not disturbed when carboxyl groups of peptides are methylated [4,14]. Methylation with either d_3- or d_0-methanol has the additional advantage that stable isotope quantification can be achieved at the same time. However, aspartate and glutamate esters readily undergo acid catalyzed hydrolysis [15]. If this happens before or during reversed phase chromatography, sample complexity will be increased and quantification will be more difficult.

This paper explores an alternative approach to phosphopeptide selection. The IMAC strategy is very simple, but suffers from lack of specificity as noted above. Recognizing that this problem could be resolved by reducing the number of carboxyl residues in peptides, V-8 protease glu-C was used for protein digestion instead of the more traditional trypsin. Glu-C cleaves proteins at aspartic and glutamic acid. In the ideal case, this enzyme would produce peptides with a single acidic amino acid per peptide at the C-terminus. The fact that this enzyme is an endopeptidase, and the presence of multiple adjacent acidic residues in proteins, makes it unlikely that acidic peptides will be completely absent in glu-C digests. The hypothesis being examined here is that glu-C proteolysis will increase the fraction of peptides bound by Ga(III) loaded IMAC columns that are phosphorylated. The objective is to enrich and select peptides from proteolytic digests that are predominantly phosphopeptides and in the course of doing so circumvent lengthy derivatization procedures.

2. Experimental

2.1. Materials

Skim milk was purchased from Wal-Mart (Lafayette, IN, USA). Trypsin, HEPES, iodoacetamide (IAM), β-casein, α-casein, guanidine hydrochloride, guanidine thiocyanate, urea, *N*-hydroxysuccinimide, d_6-acetic anhydride, *N*-acetoxysuccinimide, hydroxylamine, and gallium (III) chloride ($GaCl_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) and trifluoroacetic acid (TFA) were purchased from Pierce (Rockford, IL, USA). Acetonitrile (ACN), methanol, glacial acetic acid, sodium chloride, calcium chloride, sodium hydroxide, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Malinckrodt (Paris, KY, USA). Sodium dodecyl sulfate (SDS) was purchased from BioRad (Hercules, CA, USA). Chelating Sepharose was purchased from Amersham Pharmacia (Uppsala, Sweden). Glu-C was purchased from Roche Diagnostics (Indianapolis, IN, USA). Water was purified in house with a Milli-Q Gradient A10 water filtration system from Millipore (Bedford, MA, USA).

2.2. Instrumentation

All chromatographic separations were carried out on an Integral™ Micro-Analytical Workstation and mass spectral analyses were performed on a PE SciEx QSTAR, both from Applied Biosystems (Framingham, MA, USA).

2.3. Proteolytic digestion

The caseins (11 mg β-casein and 17 mg α-caseins) were dissolved in 4 ml of 50 mM HEPES, 10 mM $CaCl_2$ and split in half. The proteins were digested overnight with trypsin (37 °C) or glu-C (25 °C).

Milk (1 ml) was mixed with 1 ml 100 mM HEPES and a final concentration of 10 mM DTT and incubated at 80 °C to denature and reduce the proteins. IAM was added to a final concentration of 20 mM and incubated in the dark for 30 min. Cysteine was added to a final concentration of 10 mM to quench the reaction and allowed to react for 30 min at ambient temperature. Glu-C or trypsin was then added and allowed to digest overnight at 25 or 37 °C, respectively.

2.4. Acetate labeling

D_3-Acetoxysuccinimide was synthesized as previously described [16]. Briefly, 5 g of d_6-acetic anhydride was mixed with 1.77 g *N*-hydroxysuccinimide under nitrogen and stirred overnight. The resulting crystals were filtered and washed with hexane. Two different brands of skim milk were reduced, alkylated, digested, and labeled with d_0- and d_3-acetoxysuccinimide by adding them in a 30:1 ratio to protein in the sample. The mixtures were stirred for 3 h followed by the addition of hydroxylamine. The pH was raised to 12 for 10 min to eliminate tyrosine esters then reduced to three with glacial acetic acid prior to Ga(III) IMAC selection.

2.5. Ga(III) IMAC

Chelating Sepharose (about 1 ml) was centrifuged to remove the ethanol in which it was obtained. It was then washed with 15 ml of water by stirring 5 min. Centrifugation was carried out after each sequential step. The resin was charged with 15 ml of 0.1 M $GaCl_3$ in 0.1 M acetic acid for 45 min. Following this, it was washed with 15 ml of 0.1 M acetic acid for 5 min. The sample was then loaded onto the resin and incubated for 45 min to bind the phosphopeptides. The unbound peptides were decanted and saved for analysis and the resin was washed with 15 ml acetonitrile:water:acetic acid (25:74:1, v/v/v). After this, a wash of 15 ml of 0.1 M acetic acid was done and finally the bound phosphopeptides were eluted in 2 ml of 0.1 M phosphate, pH 5.

2.6. Chromatographic separation

Peptides were separated on a Vydac (Hesperia, CA, USA) C18 column. Buffer A was 0.1% TFA in water and buffer B was 80% ACN, 0.1% TFA in water. The sample was injected while running 100% buffer A and the flow through was allowed to elute. A gradient was run from 100% buffer A to 60% buffer A over 40 min. The gradient was then run from 60% buffer A to 100% buffer B over 10 min and then held at 100% buffer B for one column volume. Fractions were collected manually. Absorbance was monitored at 215 nm. The samples were then dried in a speed-vac.

2.7. Mass spectral analysis

Dried fractions were resuspended in a 50:50 mixture of methanol: water (0.1% TFA). Each fraction was analyzed on the QSTAR. Peptides from the caseins were identified by comparison to peptides predicted from a Swiss-Prot search (www.expasy.ch). Peptides from milk digests were sequenced by MS/MS and the data compared with that in databases using Mascot [17]. The NCBIr database was used most frequently. Proteolysis was achieved with either trypsin or glu-C. One missed cleavage was allowed with trypsin whereas three were allowed with the V8 protease glu-C. All cysteine residues were alkylated with iodoacetamide. Variable modifications included phosphorylation (S/T), phosphorylation (Y), sodiated (D/E), and sodiated (C-terminal). Tolerances of 0.5 Da for the peptide and 0.2 Da for the fragments were used.

3. Results and discussion

The question examined in these studies was the extent to which glu-C digestion of proteins reduced the number of acidic, non-phosphorylated peptides captured by Ga(III) loaded IMAC columns. Based on the extensive phosphorylation of milk proteins, they were selected as models for this study. Caseins are commercially available individually in approximately 90% purity with minor contamination from other caseins.

3.1. Ga(III) IMAC of caseins

A mixture of three of the caseins (αS1, αS2, and β) was digested with glu-C and the digests used to examine the selectivity of Ga(III) IMAC columns for phosphopeptides. IMAC selected peptides were transferred to a RPC column on the LC-MS instrument and analyzed for both phosphate content and amino acid sequence. Peptides were identified by mass comparison to sequences in the Swiss-Prot database. Ten phosphopeptides were identified as belonging to the three caseins (Table 1). All the captured peptides were phosphorylated, ranging up to five phosphorylation sites. But not all of the expected phosphorylation sites were observed. For example, there were two phosphopeptides from αS2 casein that were not seen in the LC-MS. The first of these, YSIG-pSpSpSEE, is rather hydrophilic and may not have been retained on the reversed phase column. The second, pSAE, is very small and again hydrophilic. Another possible factor is that these proteins may not contain all the phosphorylation sites predicted by Swiss-Prot. There may be inter-lot variations in phosphorylation.

3.2. Ga(III) IMAC of milk

Bovine milk was used to examine IMAC selectivity with a more complex sample. Skim milk was used because the lipids in whole milk interfere with the gallium selection of phosphopeptides. Heating to 80 °C for 1 h was more effective in keeping proteins in solution during denaturation of milk samples than traditional methods employing the addition of urea, guanidine hydrochloride, or guanidine thiocyanate. Although milk proteins could be kept soluble by using SDS during denaturation, SDS binds strongly to IMAC columns and interferes with the adsorption of phosphopeptides.

After denaturation, the milk samples were reduced, alkylated, and digested with either trypsin or glu-C. They were

Table 1
Phosphopeptides identified from a glu-C digest of three caseins[a]

Casein	Mass observed (charge state)	Peptide mass	Missed cleavages	Sequence	Phosphorylation sites
β	847.31 (1)	847.31	0	KFQ**S**EE	1
β	853.33 (2)	1705.66	2	KFQ**S**EEQQQTEDE	1
β	1004.84 (2)	2008.68	2	LNVPGEIVE**S**L**SSS**EE	4
αS1	1258.28 (1)	1258.28	1	AE**SI**S**SS**EE	4
αS1	997.27 (1)	997.27	1	IG**S**E**S**TED	2
αS1	938.37 (1)	938.37	0	IVPN**S**AEE	1
αS1	1038.78 (2)	2076.56	2	AE**SI**S**SS**EEIVPN**S**VE	5
αS2	953.27 (1)	953.27	0	QL**S**T**S**EE	2
αS2	1405.46 (1)	1405.46	1	SII**S**QETYKQE	1
αS2	876.23 (2)	1751.46	1	HV**SSS**EE**S**IISQE	4

[a] Bold residues indicate phosphorylation sites.

then Ga(III) IMAC selected and fractionated by RPC. Collected fractions were dried and then analyzed by MS/MS. The digestion and gallium-based selection were run in parallel for both enzymes to avoid introduction of procedural errors. The same chromatographic methods were used for digests from both enzymes.

Peptides were sequenced with the PE SciEx QSTAR and identified using Mascot. A total of 81 tryptic peptides were analyzed by tandem mass spectrometry. Of the 81, only 33 (40.7%) were phosphorylated based on either Mascot results or manual interpretation. Some peptides did not fragment well enough for definitive sequence determination, but a neutral loss of phosphate from the peptide was observed [5,18]. Fifteen (18.5%) were acidic and four (4.9%) were retained by some other mechanism. The MS/MS spectrum of an acidic peptide is shown in Fig. 1. It is concluded that this peptide is not phosphorylated based on the absence of a 98 amu loss of a phosphate group. The remaining 29 peptides also showed no apparent loss of phosphate and sequence could not be determined.

Sixty three peptides were captured and sequenced from the glu-C digest. Of these, 44 (69.8%) were confirmed to be phosphorylated by Mascot and/or manual interpretation of the spectra. Fig. 2 shows a triply phosphorylated peptide that was determined to arise from αS1 casein. Fig. 3 shows a peptide determined to be doubly phosphorylated by manual inspection, however, a sequence could not be obtained. Of the remaining selected peptides, seven (11.1%) were acidic (Fig. 4) due to missed cleavages by the enzyme and 12 (19.1%) were bound to the column through a non-specific

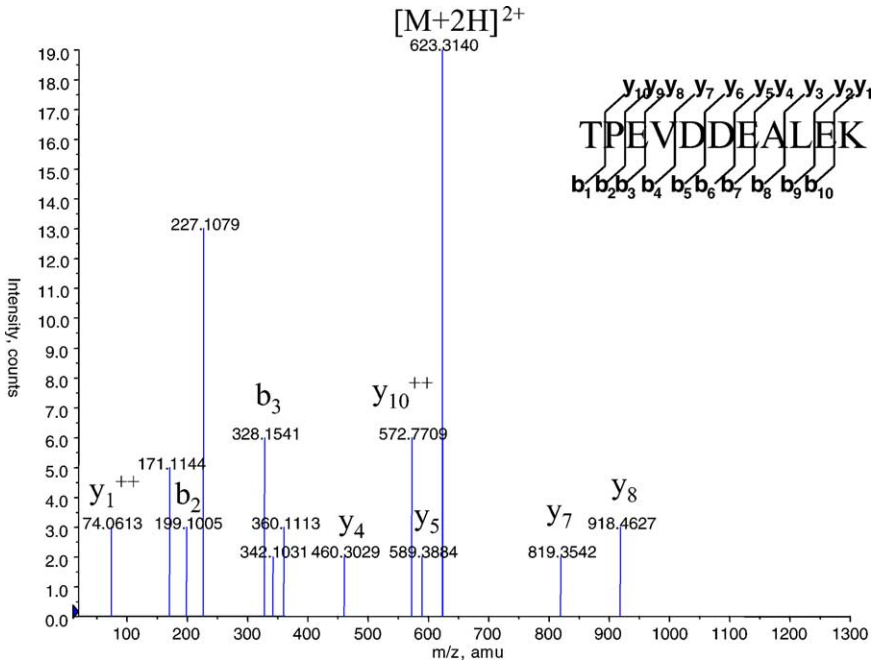

Fig. 1. An MS/MS spectrum of an acidic peptide selected by Ga(III) IMAC from the tryptic digest of milk. This peptide was concluded not to be phosphorylated based on the absence of the typical loss of a phosphate group from the parent ion in the MS/MS spectrum. This peptide was identified to be from β-lactoglobulin.

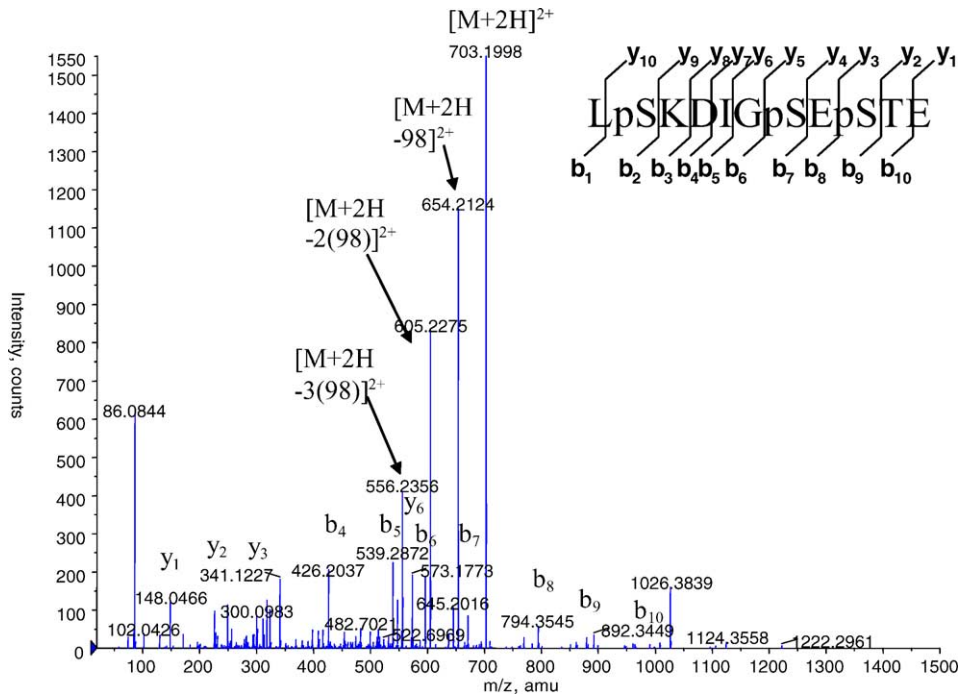

Fig. 2. An MS/MS spectrum of a phosphorylated peptide selected by Ga(III) IMAC from the glu-C digest of milk. The loss of three phosphates can be seen by the dominant peaks at multiples of 49 less than the molecular ion (charge state +2). The peptide was identified to be from αS1 casein.

mechanism (Fig. 5). The peptide in Fig. 4 from αS1 casein resulted from three missed glu-C cleavages and contained five carboxyl groups. Obviously a peptide with this degree acidity would bind to the Ga(III) IMAC column.

The four caseins account for 80% of the proteins expressed in milk [19,20]. As seen in Table 2, almost half of the peptides selected from the glu-C digest are derived from the caseins. Non-specific binding of casein peptides

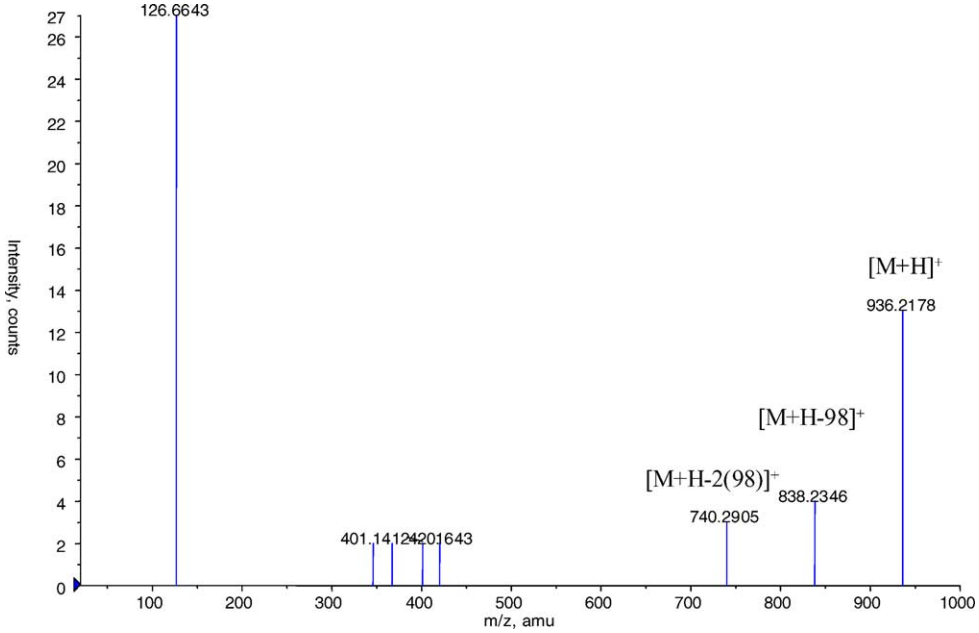

Fig. 3. The MS/MS spectrum of a phosphorylated peptide from the glu-C digest of milk. It was concluded this peptide contained at least two phosphate groups based on the peaks at *m*/*z* 838 $[M + \text{H-98}]^+$ and 740 $[M + \text{H-2(98)}]^+$. Due to poor fragmentation, it was not possible to determine the sequence of this peptide.

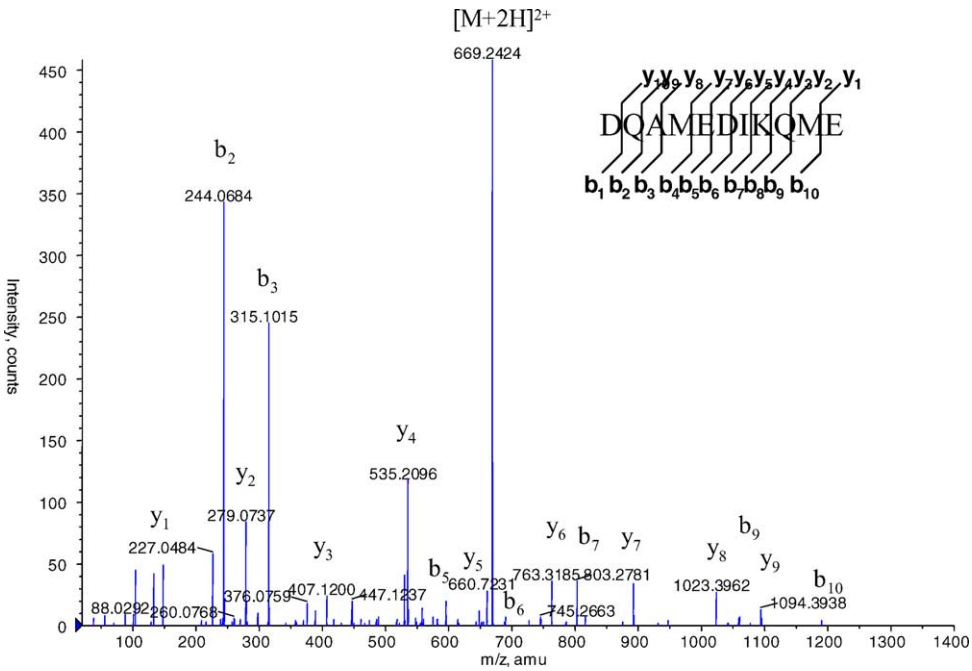

Fig. 4. An MS/MS spectrum of an acidic peptide from the glu-C digest of milk. No loss of phosphate was observed in the fragmentation. This peptide contained three missed glu-C cleavages and as a consequence had five carboxyl groups. This is the apparent reason it bound to the Ga(III) IMAC column. The peptide was determined to be from αS1 casein.

accounted for 14 out of 19 of the non-specifically selected peptides from this digest with 11 of them possibly being due to hydrophobic interactions with the sorbent. The only acidic residues present on these peptides were at the C-termini of the peptides. Additionally, there were two peptides observed from β-lactoglobulin and one from α-lactalbumin. These two proteins make up another 15% of the total protein present in milk [20]. The only non-phosphorylated peptides observed from this digest were from the six most abundant proteins in milk.

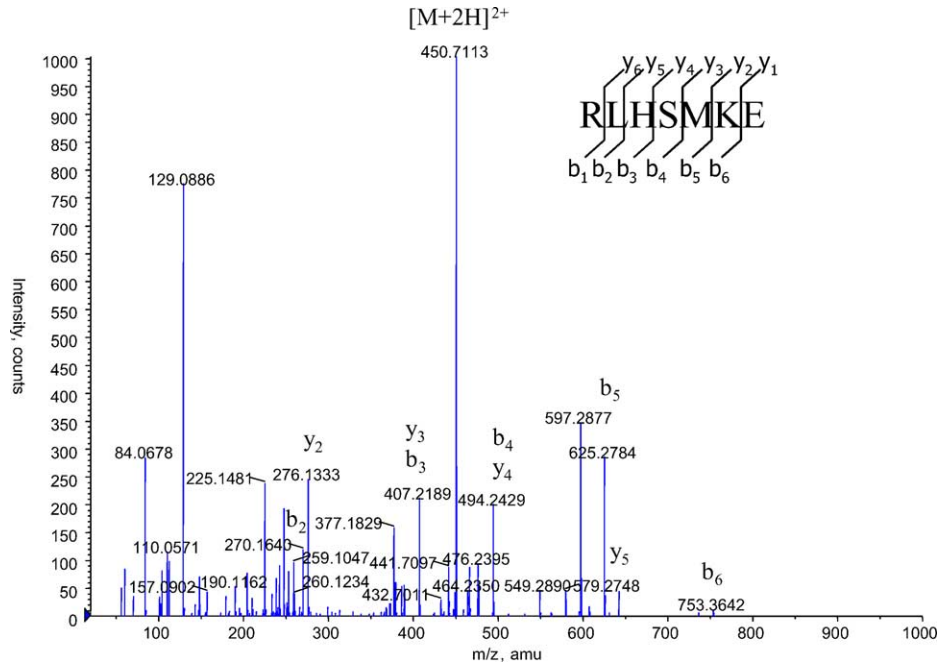

Fig. 5. An MS/MS spectrum of a peptide from the glu-C digest of milk that non-specifically bound to the Ga(III) IMAC resin. Again no loss of phosphate was observed in the fragmentation. It was also determined to be from αS1 casein.

Table 2
Proteins identified from glu-C and tryptic digests of skim milk

Glu-C	Trypsin
Caseins (16/30)	Caseins (10/19)
β-Lactoglobulin (0/2)	β-Lactoglobulin (0/4)
Lethal giant larvae homolog (1/1)	Osteopontin precursor (1/1)
Osteopontin precursor (3/3)	*N*-Acetylgalactosaminyl-transferase (1/2)
Pregnancy associated glycoprotein-14 (1/1)	Component PP3 (1/2)
Nuclear DNA helicase II (1/1)	Albumin (0/2)
Component PP3 (2/2)	Inositol 1,4,5-triphosphate receptor (0/1)
Latrophilin-2 (1/1)	Uterine milk protein (0/1)
Villin 2 (1/1)	Acidic ribosomal phosphoprotein PO (0/1)
α-Lactalbumin (0/1)	Development and differentiation enchancing factor 1 (1/1)
	PAX 2 (0/1)
	Laminin receptor 1 (1/1)
	Goodpasture antigen-binding protein D26 (1/1)
	Uveal autoantigen (0/1)
	Phosphatidylinositol 4-kinase (1/1)

The number in parentheses indicates the number of peptides that were phosphorylated out of the total number of peptides selected from that protein.

Comparing tryptic and glu-C digestion, the number of casein peptides was increased from 19 to 30 with glu-C. Moreover, only 10 of the trypsin peptides were phosphorylated whereas 16 from the glu-C digest were phosphorylated. More peptides from the tryptic digest were captured by the Ga(III) IMAC column but 29 were not phosphorylated and could not be identified. There were also several discrepancies in phosphorylated proteins selected from the trypsin digest and the glu-C digest. This may be due to peptides being too small to be retained on the RPC column or too large to be well resolved in the mass spectrometer. Suppression of ionization may also have been a factor.

3.3. Comparative study using glu-C

A glu-C digest of β-casein was split and half was labeled with d_0-acetoxysuccinimide while the other half was labeled with d_3-acetoxysuccinimide. The samples were then mixed in a 3:1 d_3-labeled to d_0-labeled ratio and selected by Ga(III) IMAC. Separation of the selected peptides was again achieved by RPC with MS based isotope ratio analysis. All selected peptides appeared in a 3:1 ratio (Fig. 6), confirming the utility of differential sample coding with stable isotope labeled derivatization agents for quantification.

Digested milk samples (1 ml) of Prairie Farms and Deans brand skim milk were labeled with d_0- and d_3-acetoxysuccinimide, respectively. The samples were then combined and gallium selected. The selected peptides were separated by RPC and the collected fractions analyzed by MS/MS. For most of the observed peptides, there was little difference in labeling ratio (Fig. 7). Although a ratio of 1.12:1 was observed for LpSKDIGpSEpSTE, this is within the limits of variation observed with the GIST method [16]. This means there was little relative difference in the amount of phosphopeptides between these two samples.

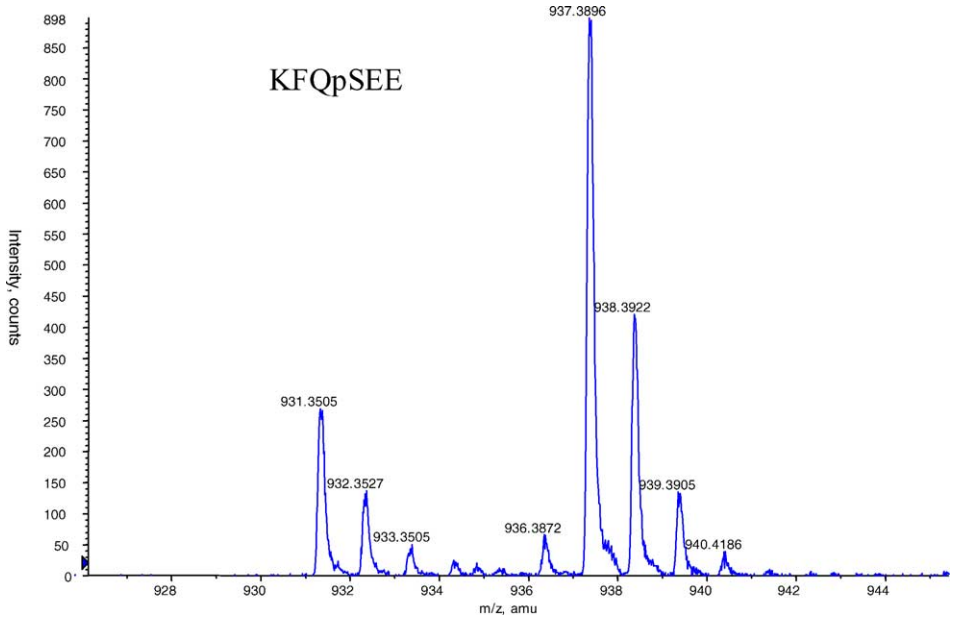

Fig. 6. The mass spectrum of differentially coded phosphopeptides from glu-C digests of β-casein. Experimental and control samples were differentially coded with d_3- and d_0-labeled acetate and mixed in a 3:1 ratio prior to selection and separation. Isotopic isoforms of the peptides were observed in roughly a 3:1 ratio in the mass spectrum based on peak height. The peptide isoforms were labeled both on their N-termini and ε-amino group of lysine, accounting for the 6 amu difference in their mass.

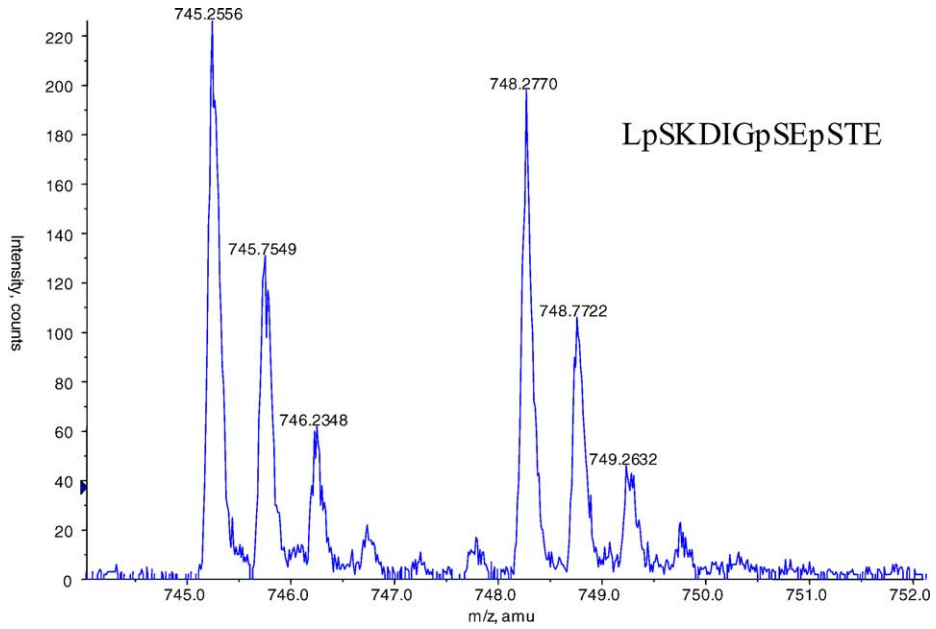

Fig. 7. A mass spectrum of a d_0/d_3-acetate coded peptide from glu-C digests of two milk samples. This peptide was present at a ratio of roughly 1.12:1 based on peak height. The 6 amu difference in the mass of the differentially coded peptides indicates that the peptide contains a lysine residue. This was later confirmed by MS/MS. The peptide was determined to be from αS1 casein.

4. Conclusions

It is concluded that substituting glu-C proteolysis for trypsin digestion of complex protein mixtures increases the fraction of phosphopeptides selected by Ga(III) loaded IMAC columns from 40 to 70%. Moreover, the use of glu-C seems not to interfere with the selection and separation of peptides. Additionally, it is concluded that acylation with stable isotope coding agents can be used to quantify the relative concentration of peptides between samples. Digestion with glu-C can be used for complex mixtures in comparative proteomics studies to substantially increase the selectivity of Ga(III) IMAC columns and in the course of doing so enhance phosphopeptide characterization by simplifying mixtures.

Acknowledgements

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