Capturing native protein states in real-time using a novel MS-compatible phosphatase and protease inhibitor formulation

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Overview

The elucidation of cellular pathways requires a thorough understanding of how proteins are processed and modified in response to external or internal stimuli. Preservation of sample integrity in the interim between sample collection and analysis is imperative. While use of stop-state inhibitor cocktails, which are designed to minimize activity from proteases and phosphatases upon sample collection, is commonplace, many formulations from commercial suppliers can lead to difficulties in fractionation of the sample or covalent modification of the sample resulting in the appearance of artifacts during mass spectrometry (MS) analyses. To minimize these effects, we have developed a new formulation of small molecules which combine phosphatase and protease inhibition in an MS-compatible format. Herein we demonstrate both the compatibility and effectiveness of that novel formulation in various workflows leading towards MS analysis.

Introduction

During a thorough evaluation of commonly used protease and phosphatase inhibitors, several components within traditional inhibitor cocktails were found to either be covalent protein modifiers or cause interferences in processing prior to MS (Table 1). Elimination of any of these problematic components within the cocktail led to significant decline in either protease or phosphatase inhibitory performance with various biological sample inputs. Through an iterative fashion we were able to formulate an optimized inhibitor cocktail which displays excellent inhibition properties while maintaining compatibility with mass spectrometry.

The resultant optimized formulation was then tested in a more stringent fashion via MS, using a mix of 10-25 synthetic phosphopeptides in both light and heavy (isotopically labeled) versions. Compatibility of IMAC enrichment was verified by quantifying enrichment of the light components with or without inhibitors and comparing recovered amounts against their isotopically labeled counterparts. The inclusion of the inhibitor mixture showed no deleterious effects on IMAC enrichment, nor did it have any effect on separation, ionization, or identification of the standardized phosphopeptides by MS.

Materials

All materials were obtained from or prepared at Sigma-Aldrich, unless noted. Product numbers are given in parentheses:
•MSSafe phosphopeptide protease inhibitor cocktail (MSSAFE). The individual inhibitors tested are given in Table 1
•PhosphoMix phosphopeptide standards (MSP1L, MSP2L, MSP3L, MSP1H, MSP2H, MSP3H)
•Phosphatases: PP2A-α (SRP-5336), PP2Ac (P1618), PP2A1 (P6993) PP1C (P1493) PTP-beta (P9864), Alkaline Phosphatase (P0114)
•Phos-Select Iron Affinity Gel (P9740)
•BSA (A7638)
•Trypsin (T6567)
•E-64 (E3132)

Methods

General phosphatase inhibition was demonstrated using a standardized assay for phosphatase activity with para-nitrophenyl phosphate (PNPP) as a phosphatase substrate. The reaction was monitored by the appearance of the hydrolysis product *p*-nitrophenol by UV-abosrbance at 405 nm.

A scheme illustrating the use of light and heavy peptides is shown in Figure 1. In general, either the heavy or light phosphopeptides were incubated with the inhibitor cocktail and subjected to either phosphatase treatment or IMAC enrichment. The heavy and light components were combined just prior to MS analysis and the relative ratios were measured to determine if any changes had occurred relative to a control sample.

E-64 adduct formation onto cysteine (Figure 2) was demonstrated on a tryptic digest of Bovine Serum Albumin in borate buffer (pH 9). Samples were analyzed via LC-MS using a Supelco Ascentis Express Peptide (ES-C18) column in line with a Waters Q-TOF.

IMAC compatibility (Table 2) was demonstrated using PHOS-Select Iron affinity gel beads incubated with phosphopeptides at room temperature for 2 hours. Following wash steps, the bound phosphopeptides were eluted using ammonium hydroxide and dried. Analysis was done by LC-MS using a Thermo Fisher LTQ-FT equipped with a Supelco Ascentis Express ES-C18-peptide column.

Phosphatase specific treatment (Figure 3, 4) was done by incubating the standard phosphopeptides with phosphatases at multiple concentrations (1x, 5x, and 25x) over a 2 hour period at 37 ºC. Analysis was done by LC-MS using a Thermo Fisher LTQ-FT equipped with Supelco Ascentis Express ES-C18-peptide column. The 1x enzyme concentration for each phosphatase was chosen based upon published activity values from the manufacturer.

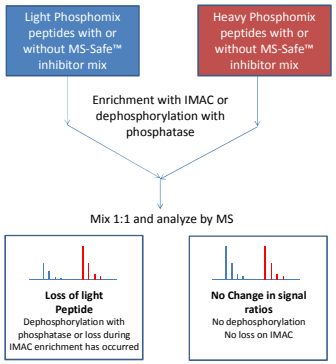

Figure 1. Workflow demonstrating the use of light and heavy isotopically labeled phosphopeptides to test inhibitor effectiveness against a wide range of phosphatases as well as IMAC enrichment prior to MS.

Results and Discussion

Through an iterative process, a suitable mix of both protease and phosphatase inhibitors was obtained to reach **>90% inhibition of phosphatase activity** in both rat liver extracts and HeLa cells and **>80% inhibition of protease activity** in pancreatin and mammalian cell extracts.

In this formulation, many of the commonly used phosphatase and protease inhibitors that have been found to be problematic in MS workflows (Table 1) have been avoided. For example, the commonly used cysteine protease inhibitor E-64 was found to give non-specific covalent modification of cysteine-containing peptides (Figure 2). Our formulation also excludes other additives found in commercial cocktails that have detrimental effects on chromatography in LC-MS applications or for IMAC enrichment when phosphopeptide enrichment was necessary (PEG, etc.).

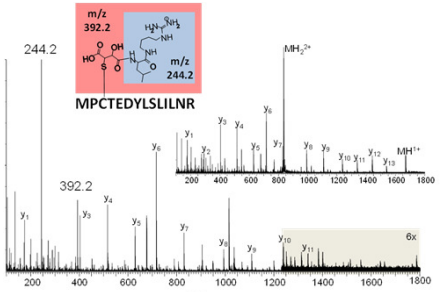

Figure 2. Incubation of E-64 with BSA prior to tryptic digestion was shown to give covalent adducts onto cysteine containing peptides. Two signature fragment ions from the E-64 adduct on a BSA peptide (structure shown) can be observed at m/z of 392.2 and 244.2. A quick comparison of the adduct fragementation as compared to the non-derivatized peptide (inset) helps to assign adduct formation to the cysteine amino acid.

Table 1 . Common inhibitors used in commercial protease and phosphatase inhibitor cocktails and their compatibility with MS analysis workflows (X - problematic, O - compatible)

	Inhibitors	Target Enzyme	Covalent Modifier	IMAC incompatible	MS-SAFE™
Protease Inhibitors	Antipain	Ser/Cys proteases			O
	Aprotinin	Ser (trypsin & elastase)			O
	Bestatin	aminopeptidases			O
	Elastatinal	elastase			O
	Leupeptin	Ser/Cys protease			O
	Nafamostat Mesylate	Ser, kallikrein			O
	Phosphoramidon	thermolysin, collagenase			O
	Pepstatin A	Aspartic proteases			O
	E-64	Cysteine proteases	X		
	AEBSF	Ser (trypsin & chymotrypsin)	X		
	PMSF	Ser/Cys proteases	X		
	TLCK	Ser proteases	X		
	EDTA	metalloproteinase		X	
Phosphatase Inhibitors	Bromotetramisole Oxalate	alkaline phosphatases			O
	Okadaic Acid	Type 1 & 2A phosphatases			O
	Sodium Fluoride	Ser/Thr, acidic phosphatase			O
	Sodium Orthovanadate	Tyr, alkaline phosphases			O
	b-glycero-phosphate	Ser/Thr phosphatases		X	
	Sodium Pyrophosphate	Ser/Thr phosphatases		X	

Table 2. Recovery of phosphopeptides from IMAC enrichment in the presence (light) of inhibitor cocktail as compared to an identical sample enriched in the absence (heavy) of inhibitor cocktail. A value of less than 1 indicates that a loss of the light peptide has occurred and the inhibitor cocktail has caused interference in the enrichment. The average for all peptides was 0.93:1 indicating little effect in the presence of the inhibitor cocktail.

Peptide	Recovery from IMAC
VLHSGpSR	0.99
RDSLGpTYSSR	0.90
pTKLtpTQLRDAK	0.76
EVQAEQPSSpSSPR	1.01
ADEPpSSEESDLEIDK	1.29
ADEPSpSEEpSDLEIDK	0.69
FEDEGAGFEESpSETGDYEEK	0.58
ELSNpSPLRENSFGpSPLEFR	1.19
SPTEYHEPVpYANPFYRPTpTPQR	0.99
Average	**0.93**

The resultant optimized formulation (Table 1) was then tested in a more stringent fashion via MS, using a mix of 10-25 synthetic phosphopeptides in both light and heavy (isotopically labeled) versions as illustrated in Figure 1.

Compatibility of IMAC enrichment was verified by quantifying enrichment of the light components with or without inhibitors and comparing recovered amounts against their isotopically labeled counterparts. The inclusion of the inhibitor mixture showed no deleterious effects on IMAC enrichment (Table 2), nor did it have any effect on separation, ionization, or identification of the standardized phosphopeptides by MS.

Following demonstration of suitability for MS we decided to further test the performance of phosphatase inhibition of the cocktail against a variety of mammalian phosphatases (and alkaline phosphatase) at multiple concentrations. The phosphorylation state of a mix of 25 light and heavy phosphopeptides was examined following incubation with 6 phosphatases at 3 concentrations either with or without inhibitor present. Dephosphorylation could be observed in the case of many of the light peptides (no inhibitor present) through a loss in signal intensity as compared to the heavy counterpart (Figure 3a). Further corroboration that dephosphorylation was occurring could be obtained by looking for the corresponding dephosphorylated version of the light and heavy peptides (Figure 3b).

A broader look at the data for a handful peptides (Figure 4) was then plotted using the ratio of Light:Heavy peptide signal in an effort to understand substrate specificity of the phosphatases against a diverse set of substrates. Work towards this area continues. Of note for this poster however, is the lack of signal for any dephosphorylated heavy peptides indicating a complete inhibition of the phosphatases at the concentrations tested.

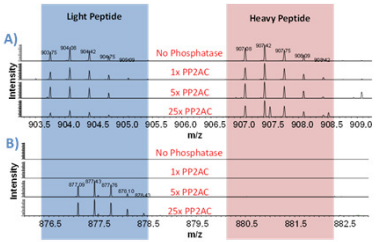

Figure 3. Loss of signal for the light phosphopeptide LGPGRPLPTFPpTSECamTSDVEPDTR is observed (A) when incubated with the phosphatase PP2AC in the absence of inhibitor (light, m/z = 903.75) as compared to incubation in the presence of inhibitor (heavy, m/z = 907.08). Likewise, appearance of the corresponding dephosphorylated version of the peptide is observed for the light version (m/z = 877.09) but not the heavy (B) indicating no dephosphorylation is occurring when the inhibitor is present.

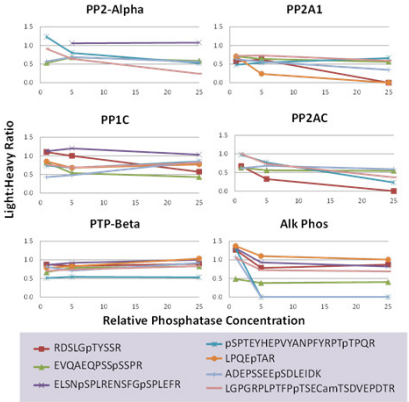

Figure 4. Demonstration of phosphatase specificity and inhibition with the Phosphomix phosphopeptide standard against different phosphatases. A decrease in the light:heavy ratio of the phosphorylated peptide at higher phosphatase concentrations indicates that the phophopeptide is a substrate for the phosphatase and additionally, that the phosphatase is being properly inhibited by the MS-SAFE cocktail.

Conclusions

•Numerous traditional phosphatase and protease inhibitor cocktails can cause issues during analysis by mass spectrometry necessitating caution with their use.
•We have derived an MS friendly cocktail which has been designed for maximal protease and phosphatase inhibition containing no irreversible covalent modifiers or substances which would interfere with enrichment or chromatographic methods.
•Enrichment of phosphopeptides with IMAC was not significantly compromised in the presence of the inhibitor formulation.
•Testing of the phosphopeptide inhibitor cocktail against a variety of phosphatases was used to demonstrate both the effectiveness of the mixture against a diverse group of enzymes as well as suitability of the components for MS analyses.