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 internal or external stimuli. Preservation of sample integrity in mass spectrometry is critical for accurate measurement and analysis of proteins. While use of step-wise inhibitor cocktails, which are designed to minimize activity from proteases and phosphatases upon sample collection, is commonplace, many workflows from commercial proteomics providers still rely on multiple-step incubation to control protease and phosphatase activity during sample processing in the appearance of artifacts during mass spectrometry (MS) analyses. To minimize these effects, we have developed a novel formulation of small molecule-selective combinable phosphatase and protease inhibition in an MS-compatible format. Herein, we demonstrate both the compatibility and effectiveness of this novel formulation in various workflows leading towards MS analysis.

Introduction
During a thorough evaluation of commonly used protease and phosphatase inhibitors, several compounds within traditional inhibitor cocktails were found to either be covalent protein modifiers or cause interference in prior MS work flow (Table 1). Elimination of any of these problematic compounds within the cocktail led to a 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 synthetic phosphopeptides in both light and heavy (isotope labeled) forms. Enzymatic reaction products were then compared to phosphopeptide standards (heavy, m/z = 907.08). Light components with or without inhibitors and comparing recovered amounts against their isotope labeled counterparts. The inclusion of the inhibitor cocktail showed no deleterious effects on IMAC enrichment, nor did it have any effect on separation, isolation, or identification of the phosphopeptides using LC-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, MSP4H, MSP5H, MSP6H)
• Phosphoramidon, bestatin, pepstatin A, aminopeptidases, Nafamostat Mesylate, ser, kallikrein, collagenase, E-64, Cysteine proteases

Methods
General phosphatase inhibition was demonstrated using a standardized assay for phosphatase inhibition (PITA assay). The reaction was monitored by the appearance of the hydroxylated product and/or UV absorbance at 405 nm. A scheme illustrating the use of light and heavy phosphopeptides 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 compared prior to MS analysis and the relative ratios were measured to determine if any changes had occurred relative to a control sample.

Results and Discussion
Through an iterative process, a suitable mix of both protease and phosphatase inhibitors was attained to reach ~95% inhibition of phosphatase activity in both crude tissue extracts and tissue homogenate and ~9o% inhibition of protease activity in parenthesis in 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 removed. For example, the common phosphatase inhibitors phosphoramidon (full-time, selective phosphatase inhibition of protein tyrosine phosphatases) and phosphoramidon were found to be incompatible with IMAC or phosphopeptide enrichment when phosphopeptides were subjected to either phosphatase treatment or IMAC enrichment. The heavy and light phosphopeptides were either not recovered or completely lost during analysis by mass spectrometry necessitating caution with their use.

Table 1. Common inhibitors used in commercial protease and phosphatase inhibitor cocktails and their compatibility with MS analysis workflow (Y = protease; O = phosphatase).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Target Enzyme</th>
<th>Covalent Modifier</th>
<th>IMAC Compatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bestatin</td>
<td>Aminopeptidases</td>
<td>No covalent modification</td>
<td>Yes</td>
</tr>
<tr>
<td>Nafamostat</td>
<td>Ser, kallikrein</td>
<td>No covalent modification</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>Protein tyrosine phosphatases</td>
<td>No covalent modification</td>
<td>No</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Aspartic proteases</td>
<td>No covalent modification</td>
<td>Yes</td>
</tr>
<tr>
<td>E-64</td>
<td>Cysteine proteases</td>
<td>No covalent modification</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The resultant optimized formulation (Table 1) was then tested in a more stringent fashion via MS using a mix of 10 synthetic phosphopeptides in both light and heavy (isotopically labeled) forms 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 respective controls. Analysis was done by LC-MS using a Supelco Ascentis Express ES-C18 peptide column. The 1x enzyme concentration for each phosphatase was chosen based upon published activity values from the manufacturer.

Conclusions

Nonspecific traditional phosphatases and protease inhibitor cocktails can cause issues during analysis by mass spectrometry necessitating cautious use with their use. We have derived an MS-friendly cocktail which has been designed for maximal protease and phosphatase inhibition without causing significant covalent modification or substrate inhibition, and further more, has been identified as being MS compatible. Enzyme inhibition of phosphapases with IMAC was not significantly compromised in the presence of this formulation.

Testing of the phosphatase 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 analysis.

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 analysis.

Figure 2. Demonstration of phosphatase specificity and inhibition with the Phospho-specific phosphatase standard against different phosphatases. A decrease in the heavy/light ratio of the phospho-specific peptide at higher phosphatase concentrations indicates that the phosphatase is selectively inhibited by the MS-SAFE cocktail.

Figure 3. Demonstration of phosphatase specificity and inhibition with the Phospho-specific phosphatase standard against different phosphatases. A decrease in the heavy/light ratio of the phospho-specific peptide at higher phosphatase concentrations indicates that the phosphatase is selectively inhibited by the MS-SAFE cocktail.