Mass Spectral Identification of Phosphopeptides Using a Selective Biotin-Tagging Method

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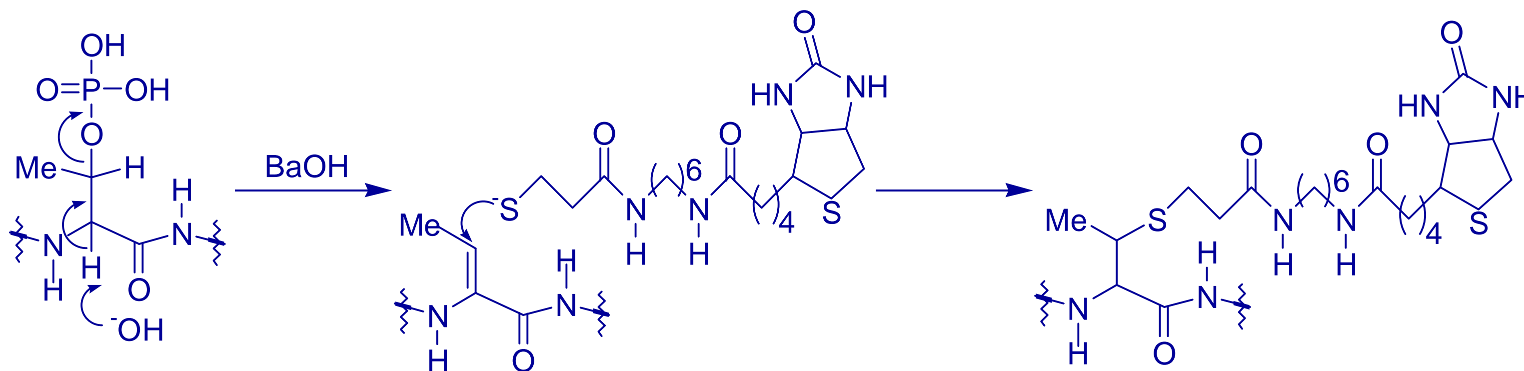
INTRODUCTION

Protein phosphorylation is of fundamental importance in a variety of cellular processes, including the biosynthesis necessary for cell proliferation.[1] Although identification of phosphorylation sites can be accomplished using mass spectral analysis of proteolytic digests,[2] improvements in sensitivity and selectivity can be achieved by separating the phosphopeptides from the digest mix.[3]

An alternative approach involves selective chemical transformations at phosphorylation sites, which provide modified residues that are more easily identified by MS. Michael addition of thiols to the β-elimination products of phosphoserine or phosphothreonine has recently been used to characterize previously unreported protein phosphorylation sites.[4]

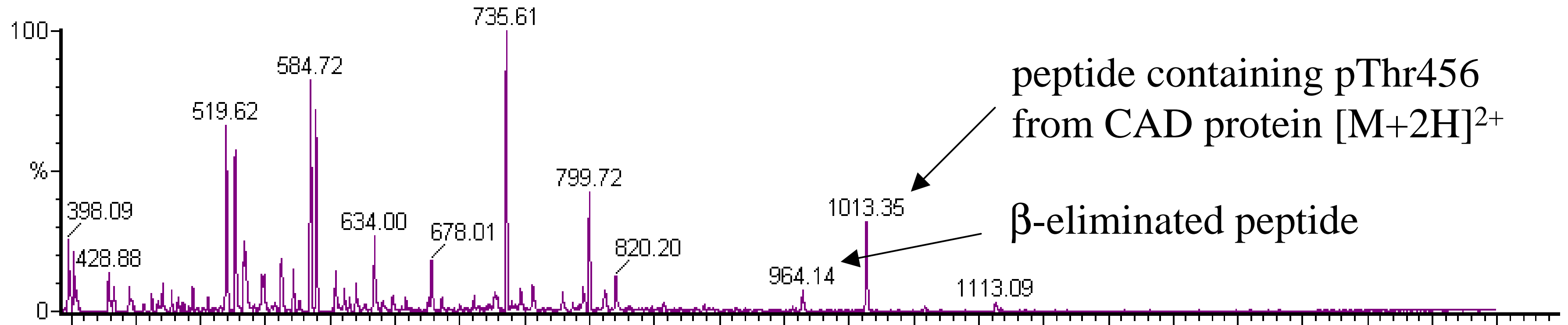
This report describes progress toward a technique that utilizes a selective chemical transformation followed by isolation of the modified phosphopeptides in order to improve MS detection. The selective phosphopeptide modification involves the covalent addition of a biotin derivative based on β-elimination / Michael addition chemistry developed over the past several years.[4,5] Our intention is to then use avidin on solid support to isolate and effectively concentrate the biotinylated peptides of interest for mass spectral characterization.[6]

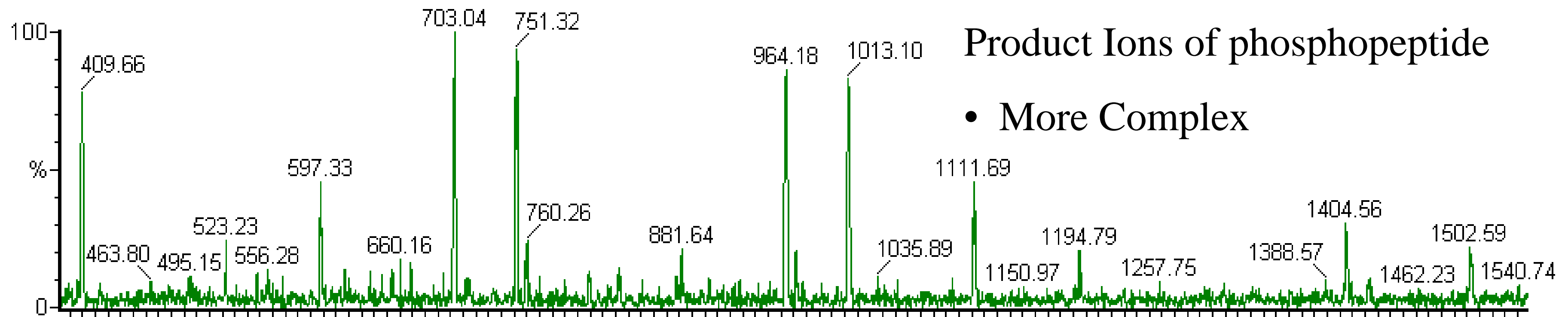
β-Elimination / Biotinylation of Phosphopeptides

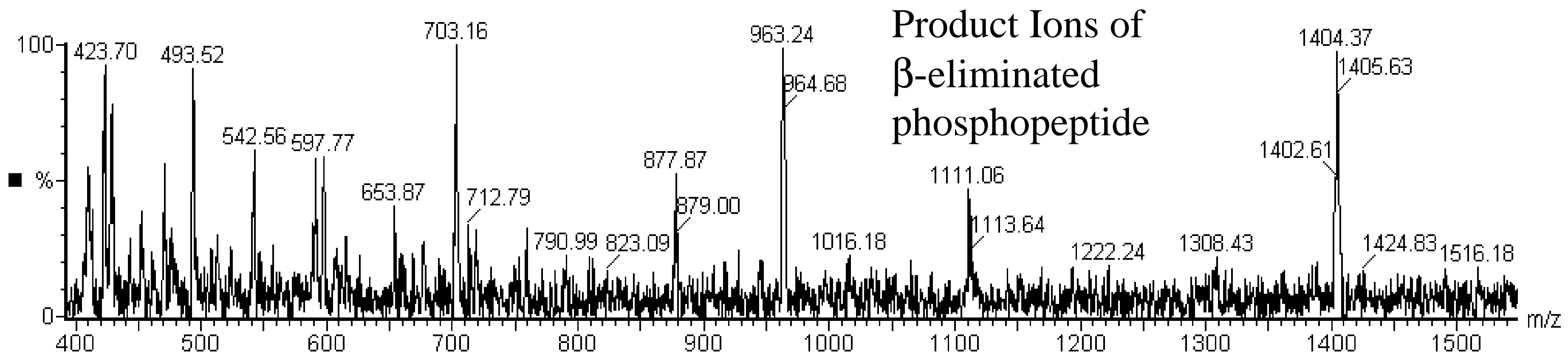


- β-elimination of a phosphothreonine residue, followed by Michael addition of reduced Biotin-HPDP to the resultant dehydroamino-2-butyric acid
- The methyl substituent on the β carbon of phosphothreonine lowers reactivity in comparison to phosphoserine.
- Phosphate undergoes β-elimination at high pH, with the reaction accelerated in the presence of Ba^{2+}.

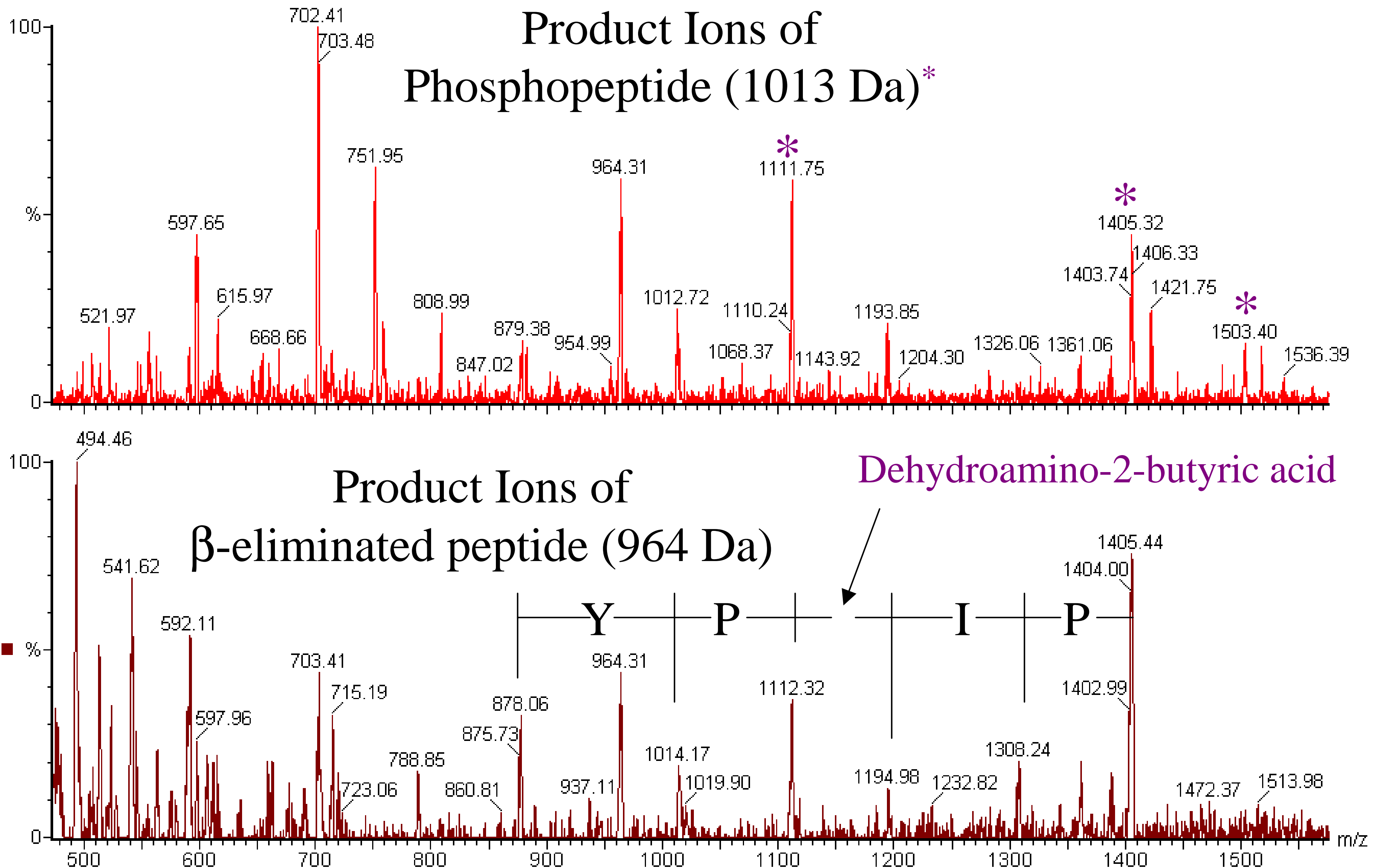
Alternatives: Direct Sequencing



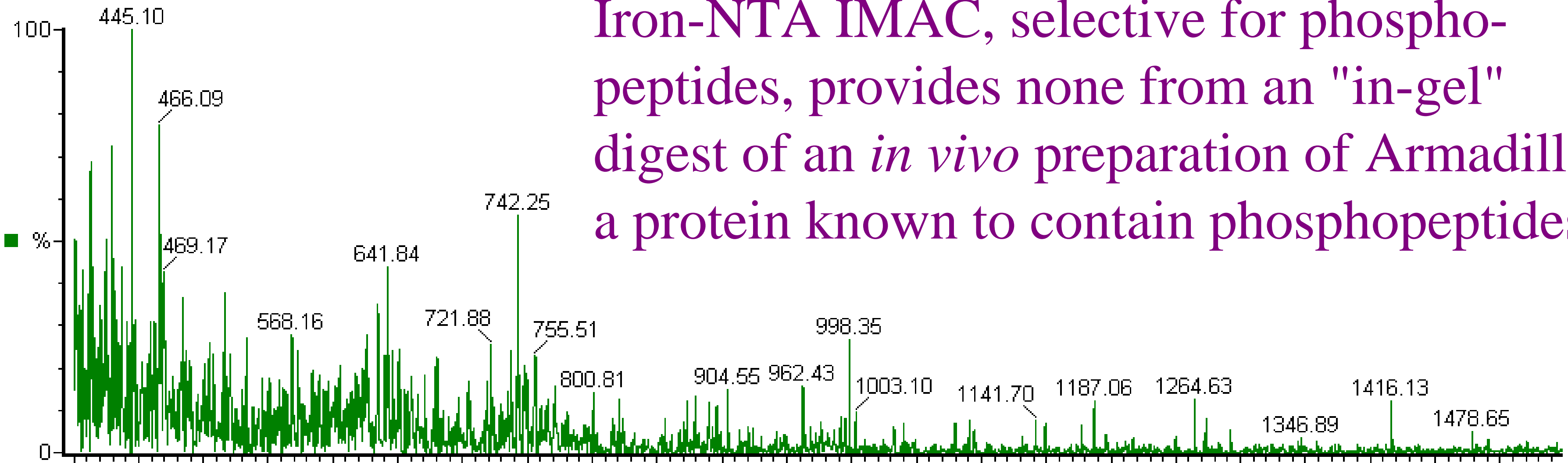




Sequencing: phospho- vs. β-elim



Alternatives: Immobilized Metal Affinity Chromatography (IMAC)



Iron-NTA IMAC, selective for phospho-peptides, provides none from an "in-gel" digest of an *in vivo* preparation of Armadillo, a protein known to contain phosphopeptides

non-phosphorylated peptides are selected from a digest of PAK (p21 activated Kinase)

MSDEEILEK

CLDMDVEK

CLDMDVEKR

T55
953.17

T31 2+
T2 2+
547.18

T28-29
T12-13
403.20

521.94

605.12

T3 3+
667.82

714.85

T7-8
T22 2+
756.38

T52 2+
986.68

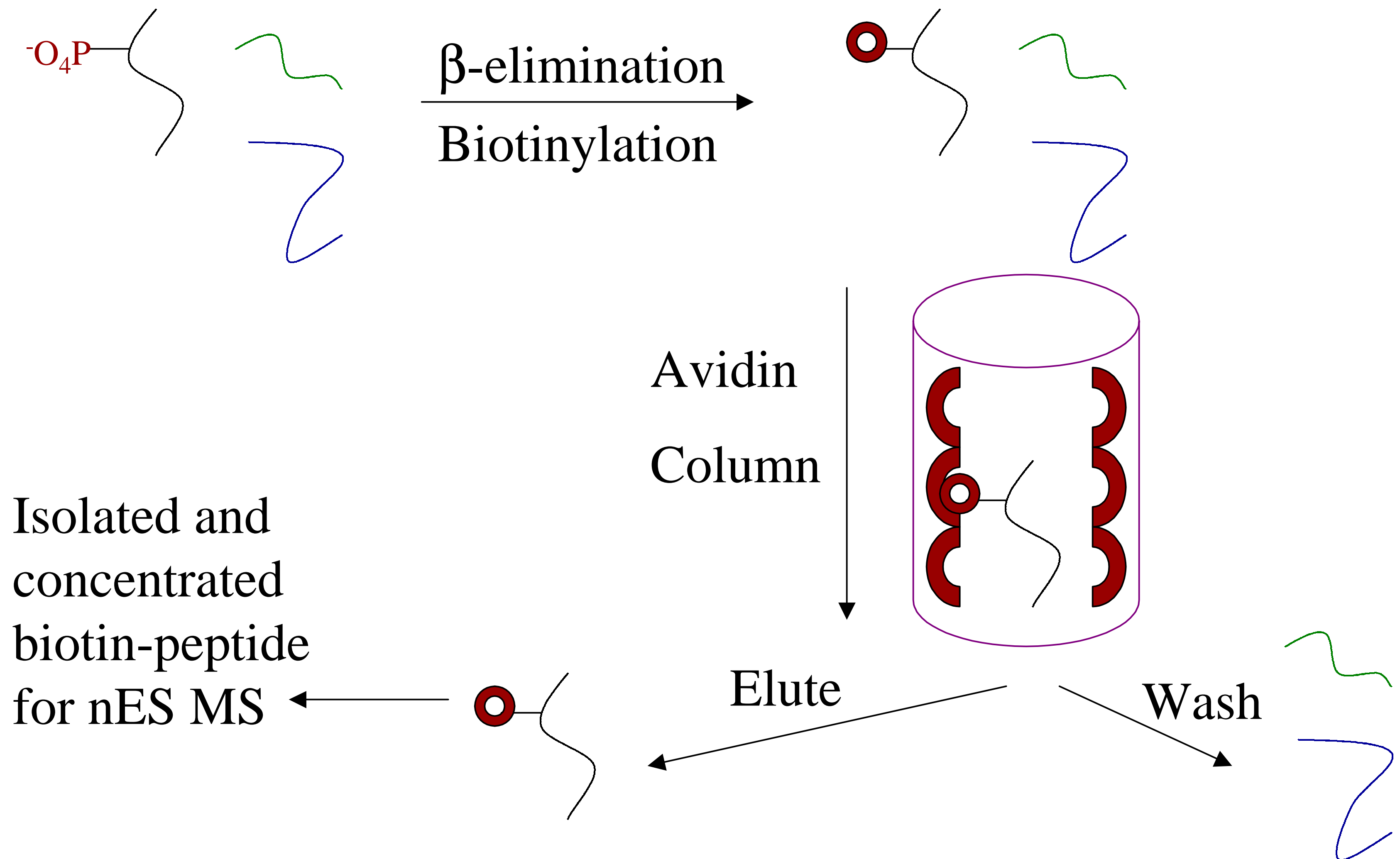
T9-10
1072.77

T23 3+
T37-38 3+
1270.31

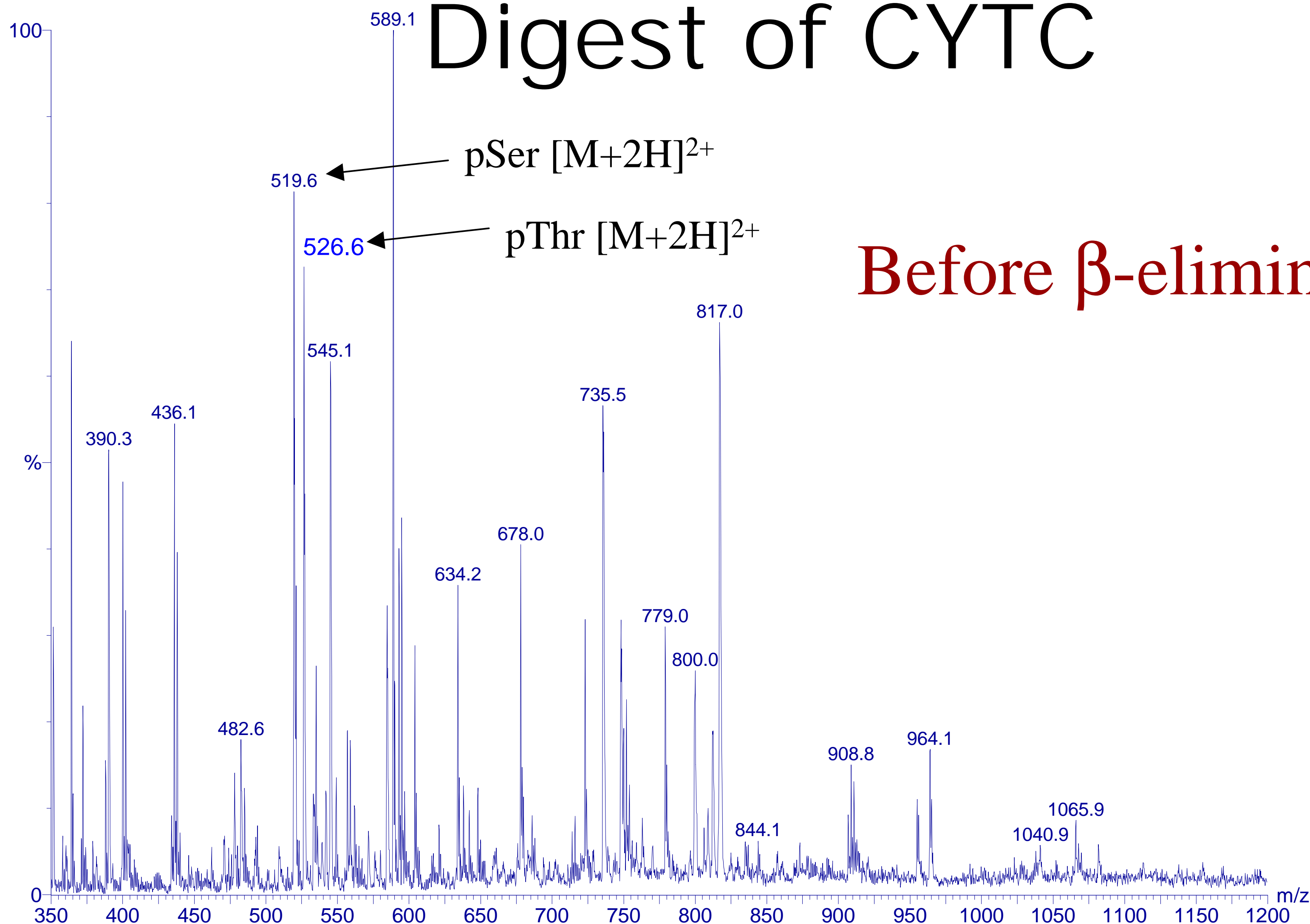
100
%
0

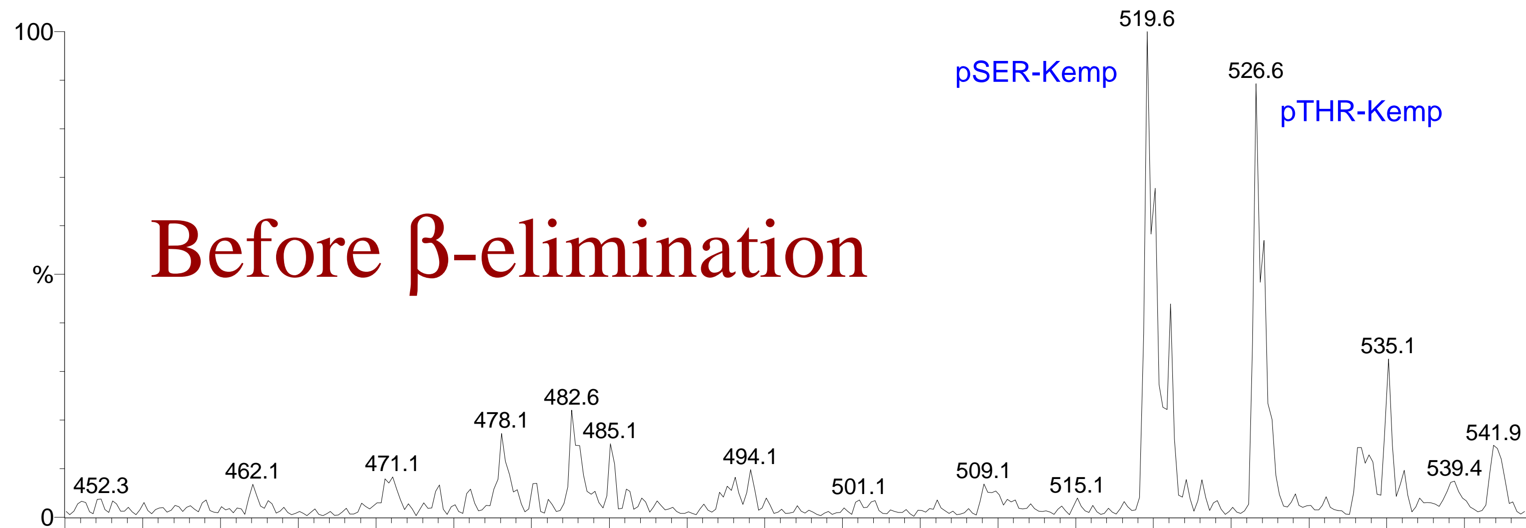
400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 m/z

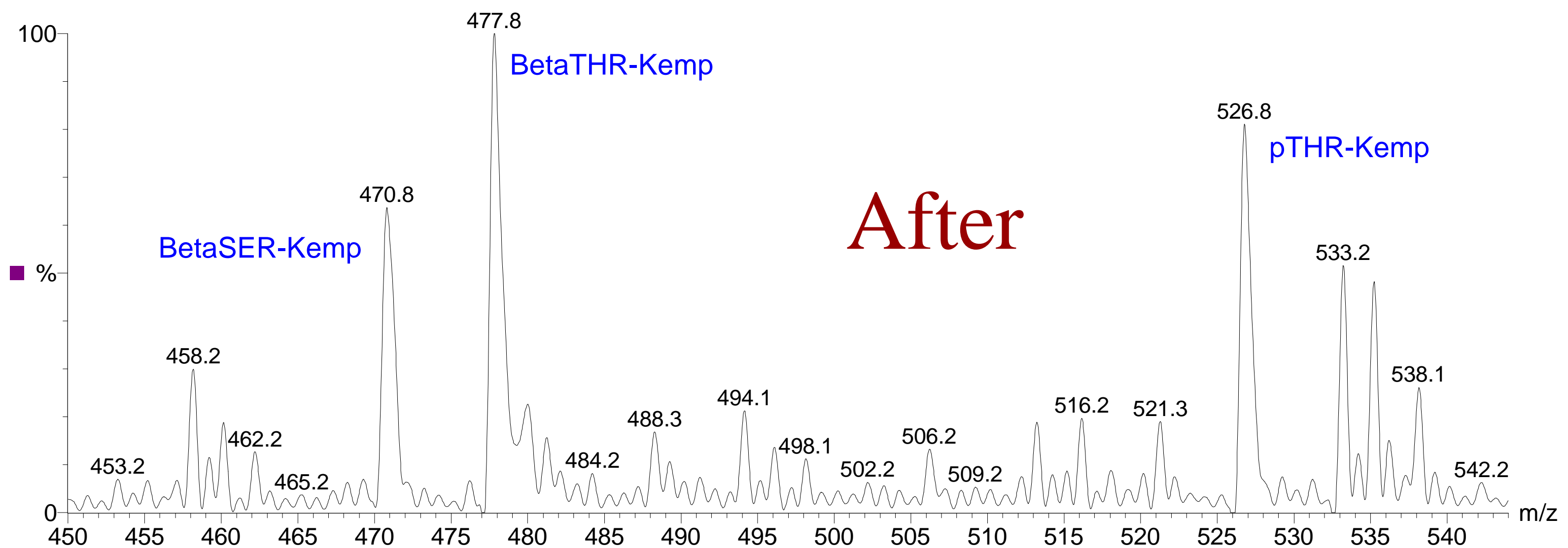
Phosphopeptide Isolation Procedure



Mass Spectra of phospho-Kemptides Spiked into a Tryptic Digest of CYTC

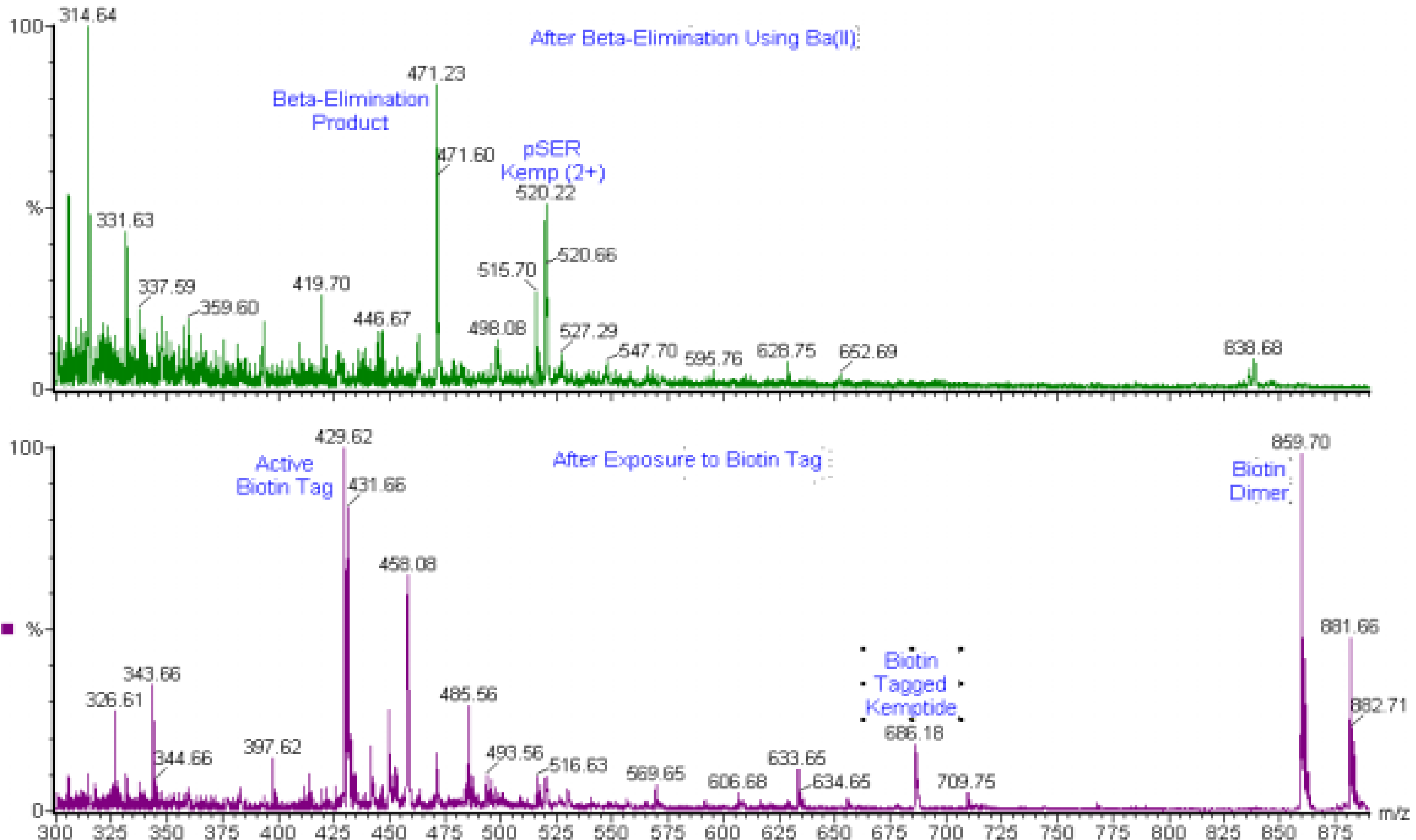






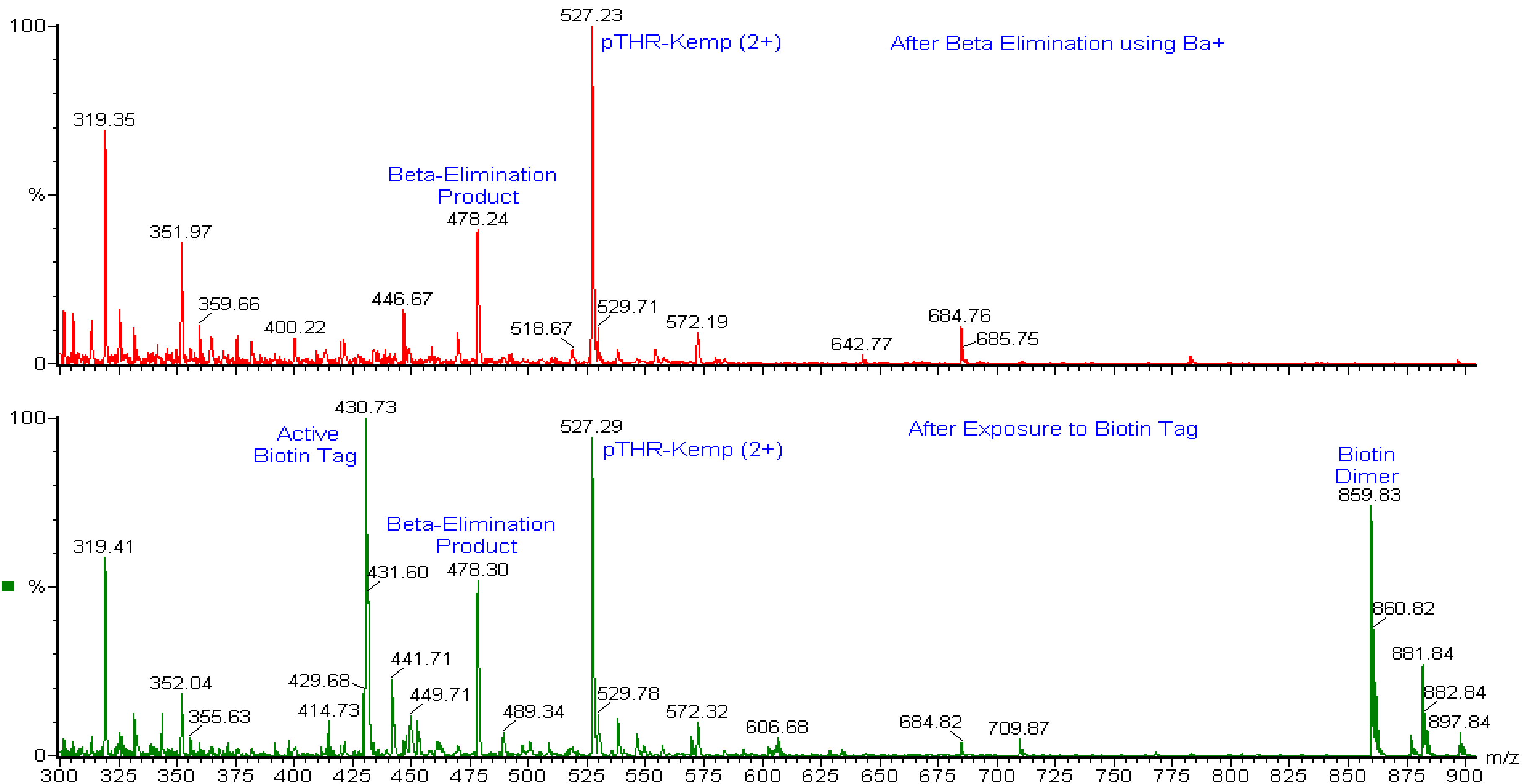
Significant β-elimination products are observed in three hours from both phospho-Kemptides, with the pSER-Kemptide reacting to completion.

β-Elimination of pSER Kemptide



Significant β-elimination / biotinylation of pSER-Kemptide was observed at room temperature after only one hour of exposure to Ba^{2+}, followed by one hour of reaction with the reduced thiolate form of Biotin-HPDP.

β-Elimination of pTHR Kemptide



The synthetic peptide pTHR-Kemp (RRLSpTLRA) undergoes β-elimination slowly and incompletely even overnight, and there is no evidence of biotinylation upon exposure to reduced Biotin-HPDP.

FUTURE DIRECTION

Two experiments must be performed before the described technique may be used to isolate unknown phospho-peptides from a complex mixture:

- It must be demonstrated that biotinylated peptides can be selectively bound to avidin on solid support and recovered in such a manner as to improve MS detection.

- Phosphopeptides must be efficiently isolated from the in-gel digest of a protein that contains native phosphorylation sites (e.g. β-casein). Phosphopeptide recovery from digests is hampered by contamination and loss.

An interesting application of this method is in determining the extent of protein phosphorylation in signaling pathway enzymes.

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