PYRIDOXAL PHOSHATE AS A TAG TO IDENTIFY ENZYMES WITHIN THE "PLP-OME"

A Thesis

by

KAYLA J. MESSER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2011

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Tadhg P. Begley
Committee Members,	Frank M. Raushel
	Coran M. Watanabe
Head of Department,	David H. Russell

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ABSTRACT

Pyridoxal Phosphate as a Tag to

Identify Enzymes Within the "PLP-ome." (May 2011)

Kayla J. Messer, B.S.,Cabrini College

Chair of Advisory Committee: Dr. Tadhg P. Begley

The main objective of this research was to develop a protocol in which pyridoxal phosphate (PLP) would act as a tag to identify PLP-dependent enzymes from complex mixtures or cell lysates. Following the purification of a PLP-dependent enzyme (CysM), a method was developed to reduce the PLP-lysine Schiff base to form a chemically stable bond between the PLP and the protein. The reduced protein was enzymatically digested resulting in multiple peptide fragments with one or more containing PLP (bound to the active site lysine). These fragments were analyzed by monitoring the absorbance or fluorescence using High Performance Liquid Chromatography. Immobilized Metal Ion Affinity Chromatography (IMAC) was then used to enrich the PLP-peptide(s) from the peptide mixture. The PLP-bound peptide(s) was then analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS).

More specifically, sodium borohydride ($NaBH_4$) was used to reduce the Lysine-PLP bond in CysM. This reaction was monitored by either UV-vis spectroscopy or mass spectrometry. Trypsin was used to enzymatically digest the reduced CysM before it was enriched with IMAC and analyzed with LC-MS. Since the objective of this project was

to develop a method which could be applied to a cell lysate, IMAC was used as an enrichment method to separate the PLP-peptide(s) from other peptides within the mixture. The PLP-peptide(s) was then located in the peptide mixture by monitoring the absorbance at 325 nm. The LC-MS results of the full reaction before IMAC treatment versus the final column, when monitoring the mass spectrum, showed that the treatment using the IMAC column separated the PLP-peptides from all other peptides within the sample. Using IMAC to enrich specifically the PLP-peptides, followed by analysis with LC-MS, may be a useful method for studying PLP-dependent enzymes within the proteome or the "PLP-ome."

DEDICATION

To my fiancé Craig for his love, understanding and constant support;

To my father and mother for their endless patience;

To my friends for pushing me to do my best;

To my Uncle John for inspiring me;

And to God

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Begley, and my committee members, Dr. Raushel and Dr. Watanabe, for their guidance and support throughout the course of this research. Thanks also to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. Finally, thanks to my mother and father for their encouragement, to my Uncle John for his inspiration and to my fiancé for his patience and love. I would also like to thank my lab mates both current and past for all their support.

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1. INTRODUCTION: THE IMPORTANCE OF PYRIDOXAL PHOSPHATE

Pyridoxal 5'phosphate (PLP) was first identified in 1951 as one of the active vitamers of vitamin B_6.[1] An extensive amount of research has been conducted since then to understand its catalytic versatility. PLP-dependent enzymes have attracted attention due to their widespread involvement in cellular processes. PLP-dependent enzymes are mainly involved in the biosynthesis of amino acids and amino acid-derived metabolites as well as in the biosynthetic pathways of amino sugars and other amine-containing compounds.[2]

PLP is a cofactor utilized by numerous biologically important enzymatic reactions including transaminations, decarboxylations and racemizations. In many of these reactions, PLP acts as an "electron sink" withdrawing electrons and forming imines with amino groups of corresponding substrates.[3] In most PLP-dependent reactions, the carbonyl group of PLP binds to the amino group of a lysine residue in the active site. This results in the formation of a Schiff base.[3] During catalysis, the amino group of the substrate replaces the enzyme lysine of the Schiff base to form an external aldimine.[4] The lysine residue within the active site plays a crucial role during catalysis. The cross-linking of the PLP to the enzyme active site, allows one to identify the essential lysine in the PLP-binding site.

Structurally characterized PLP-dependent enzymes have been found to belong to

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five distinct structural groups identified as fold-types I-V.[5] These five structural groups are presumed to correspond to five independent evolutionary lineages.[6] Fold-type I is found in a variety of amino transferases and decarboxylases including enzymes that catalyze α-, β- or γ-eliminations.[7] Fold-type I is also the most common structural group. Fold-type II is found in enzymes that catalyze β-elimination reactions.[7] Fold-type III is found in alanine racemase as well as in a subset of amino-acid decarboxylases.[7] Fold-type II is also characterized by a $(\beta/\alpha)_8$ barrel structure.[7] D-alanine aminotransferase and a few other enzymes consist of the Fold-type IV enzymes.[7] The fold-type V enzymes include glycogen and starch phosphorylases.[7] The limitations in structural diversity have been used to identify PLP-dependent enzymes from genomic sequences by using methods that identify the structural conservation of protein families.[6]

The importance of PLP-enzymes is exemplified by the number identified as drug targets. For example, the treatment of epilepsy has been improved through the inhibitors of γ-aminobutyric acid aminotransferase (GABA ATase).[8] Serine hydroxymethyl transferase (SHMT) has also been identified as a target for cancer therapy.[9] The inhibitors of ornithine decarboxylase (ODC) have even been used in the treatment of African sleeping sickness.[10] Furthermore, functional defects in PLP enzymes have also been implicated in disease pathologies including homocystinuria which is mainly linked to mutations in cystathionine β-synthase.[11] However, there are still many PLP-enzymes that have not been identified or characterized.

The number of genes that code for PLP-dependent enzymes in free-living microorganisms depends on the organism's adaptations to specific nutrient sources.[7]

Methanococcus jannaschii has the smallest number of genes (a total of 20) which is probably due to its peculiar nutrient requirements.[7] Generally, there are between 20 and 30 genes encoding PLP-dependent enzymes in archaeal organisms with slightly higher numbers found in bacteria with small genomes (ex. *Clostridium perfringens*).[7] Almost 1.5% of all prokaryotic genes code for PLP-dependent enzymes, but this number drastically decreases within higher eukaryotes.[7] This is consistent with the hypothesis that PLP-dependent enzymes are mainly involved in basic metabolism.[7] These enzymes are functionally diverse with more than 140 distinct enzymatic activities, catalogued by the Enzyme Commission, dependent upon PLP.[7] Nevertheless, detailed questions referring to the importance, distribution and diversity of PLP-dependent enzymes remain unanswered.

A two-step approach has been used to learn more about these enzymes.[7] The first step involves surveying genomic sequences to obtain an outline of the genomic distribution of PLP-dependent enzymes.[7] The second step involves investigating aspects of the results by relating them to general issues relevant to the genomic analysis of protein families.[7] This approach is not only time consuming but is problematic considering that a one-to-one correspondence between functions and genes should not be expected.[7] The assignment of function cannot rely solely on bioinformatics, but must be supplemented with emerging high-throughput technologies to supply necessary biochemical data.

Even though there is a fairly long history of research in this field, there are many unanswered questions regarding this versatile cofactor. To learn more about PLP-

dependent enzymes, a versatile method for investigating the proteome's PLP-dependent enzymes must be developed. To begin this investigation, the Schiff base could be reduced with sodium borohydride ($NaBH_4$) to form a chemically stable secondary amine. This would allow further research on the PLP-protein complex. The reduction of a Schiff base to form a covalent, chemically stable, PLP-bound protein is shown in Figure 1-1. To identify the PLP-protein from a complex mixture, an enrichment method may need to be applied in order to elucidate low-abundance proteins of interest in the presence of a large excess of relatively abundant proteins.[12] It becomes crucial to enrich the sample in order to perform effective proteome analysis. Methods for enriching specific phosphorylated proteins have been developed for the enrichment of phosphoserine/threonine-containing proteins, phosphotyrosine-containing proteins as well as for nonspecific proteins. One of the most reliable methods for enriching non-specific proteins is immobilized metal ion affinity chromatography (IMAC).[12]

Anderson and Porath first observed that phosphorylated amino acids and proteins selectively bind to Fe(III) charged iminodiacetic acid (IDA) agarose.[13] The two most commonly utilized chelating groups are iminodiacetic acid and nitrilotriacetic acid (NTA). These groups are then immobilized on chromatographic resins, membranes or magnetic beads.[13] Consequently, the number of available coordination sites depends upon the chelating group. The NTA chelating group is a quadridentate chelating absorbent.[14] The NTA derivative occupies four positions in the metal coordination sphere of nickel (Ni^{2+}) leaving two positions available for PLP to coordinate.[14] For elution, the strategy is similar to those used during the purification of his-tagged

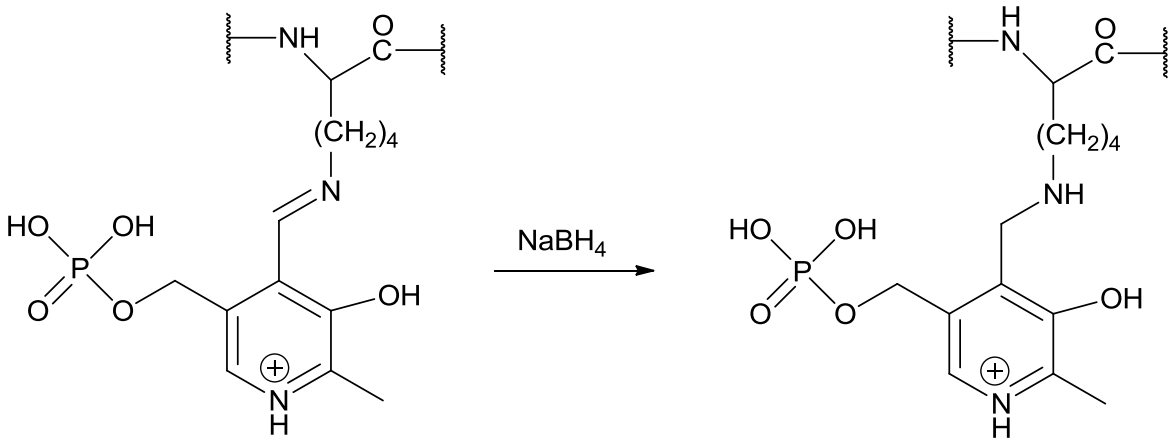

Figure 1-1. The reduction of a PLP-protein with $NaBH_4$ resulting in a chemically stable PLP-bound protein. This figure shows the results following $NaBH_4$ reduction of the Schiff base formed between the PLP and lysine residue of CysM.

proteins. Either an excess amount of free ligand (in this case being phosphates) or a shift in pH can be used to release the phosphorylated proteins from the chelating group. The selective affinity for phosphorylated proteins, as well as the reversibility of the binding, has been exploited in the enrichment of phosphorylated proteins from complex samples.[13] Overall, IMAC relies upon the affinity of the phosphate group to metal ions immobilized on a matrix via acidic compounds such as NTA.[15]

IMAC has not only been used to enrich phosphoproteins/peptides but also to separate various phosphoisoforms of the same protein. Andersson and Proath showed that they could separate phosphoisoforms of ovalbumin using a Fe(III)-IDA matrix and a pH gradient from 5.7-7.2.[13] Various phosphoisoforms of tomatosucrose synthase were also isolated by applying a basic gradient from pH 6.3-9.5.[16] IMAC has also been

applied to extract large numbers of phosphoproteins in proteomic studies. Fusion proteins have been captured by a Fe(III)-NTA matrix and then eluted with a mixture of high pH and competition of phosphate ions.[17] In a similar procedure, Jurkat T cells were analyzed by capturing the phosphoproteome on a Fe(III)-NTA matrix at acidic pH and eluted at pH 8.5.[18] Based upon these studies, IMAC seems to be a viable candidate for enriching the PLP-peptides from a peptide mixture. Nonetheless, a single PLP-dependent enzyme must first be selected as a model for method development.

2. A PLP-DEPEDENT ENZYME MODEL - CYSTEINE SYNTHASE

2.1 Introduction

Mycobacterium tuberculosis is a hazardous pathogen that wreaks havoc on the human population from both a medicinal and a financial vantage point. In the dormant phase, this organism survives in the granulomas within the lungs of the infected individual(s).[19] This environment is characterized by hypoxia, nutrient starvation as well as oxidative stress.[20] Oxidative stress results in nitrogen monoxide and spontaneously formed reactive nitrogen intermediates which are used by the host's phagocytic cells to kill the internalized bacteria. The primary targets of the nitrogen intermediates include cysteine and tyrosine side chains.[21] In *Mycobacterium tuberculosis,* the principle thiol responsible for maintaining the redox balance contains a cysteine-derived cysteamine moiety.[22] As a result, this pathogen's first line of defense against free radicals is directly dependent upon the availability of cysteine.[22] Consequently, the inhibition of cysteine biosynthesis has been studied as a potential target for antibacterial agents to combat this pathogen.[23]

In order to treat and potentially control the adverse symptoms caused by this pathogen, a full understanding of its biological machinery was first required. The *M. tuberculosis* genome affords four loci assigned as encoding cysteine synthase enzymes, one of which is notated as CysM.[24] The cysteine biosynthetic pathway of *M. tuberculosis*, which contains CysM, was previously reconstituted.[24] The chemical mechanism of CysM has been proposed by Dr. Tadhg Begley's lab. This particular

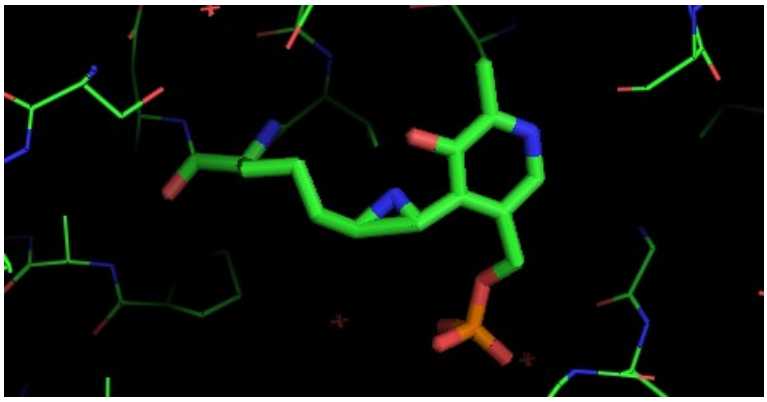

Figure 2-1. The crystal structure of CysM. This figure shows the active site of CysM with the residue Lys51, of the protein, and the PLP cofactor shown in stick format.

enzyme forms an imine between the CysM-bound pyridoxal 5'-phosphate and the amino acid substrate, followed by elimination across the α,β bond of the substrate to form a stable α-aminoacrylate intermediate.[24] A thiocarboxylate adds to this intermediate resulting in a β-substituted amino acid imine.[24] This imine then undergoes transimination with an active site lysine residue to release the product as well as regenerate the active site.[24] Using the Protein Data Bank, the active site within the crystal structure of *M. tuberculosis* CysM is shown in Figure 2-1. The PLP cofactor binds at lysine 51 making CysM a suitable candidate for PLP-peptide proteomics and method development.

2.2 Results

2.2.1 CysM Expression and Purification. The Amp-LB plate from the transformation of BL21 DE3 *E. coli* with CysM is shown in Figure 2-2. The growth

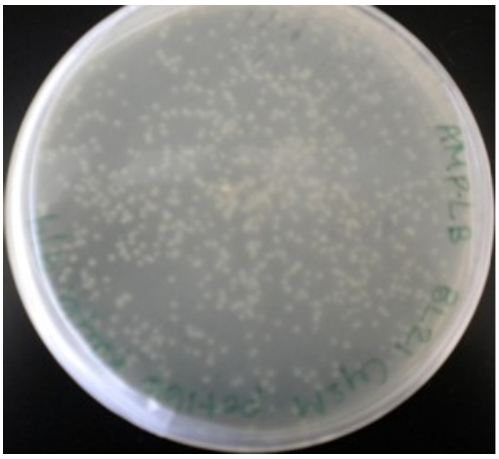

Figure 2-2. CysM-BL21 colony growth on an Amp-LB plate. This figure shows the colonies resulting from growth of the transformed *E. coli* BL12 DE3 cells.

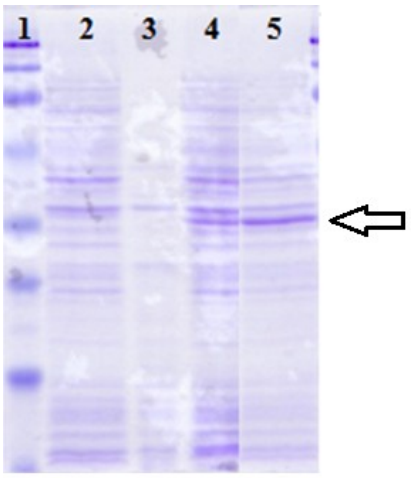

Figure 2-3. CysM expression SDS-PAGE gel results. Lane 1 – Ladder; Lane 2 – 0 hours after inducing with IPTG; Lane 3 – 3 hours after inducing with IPTG; Lane 4 – 6 hours after inducing with IPTG; Lane 5 – 20 hours after inducing with IPTG. This

Figure 2-4. CysM Ni-IMAC column during CysM purification. This image shows the yellow band (indicated with a white arrow) at the top of the nickel column which indicated that the CysM protein was bound.

resulted in many small colonies approximately 1 mM in diameter. The gel results, from the protein expression, showed the expression of a new protein at approximately 34 kDa figure shows the successful expression of the CyM protein with a band at approximately 34 kDa as indicated with an arrow which corresponds to the molecular weight of CysM (Figure 2-3). During the purification, this particular protein was bright yellow colored as shown after it was added to the Ni-IMAC column during purification as shown in Figure 2-4. CysM was concentrated at the top of the column as a bright yellow substance until an appropriate concentration of imidazole buffer was used to elute it. The protein eluted from the column between the 100-500 mM imidazole buffer washes. The gel results from the protein purification are shown in Figure 2-5. The gel results showed that most of the protein was eluted within the 500 mM imidazole buffer fraction.

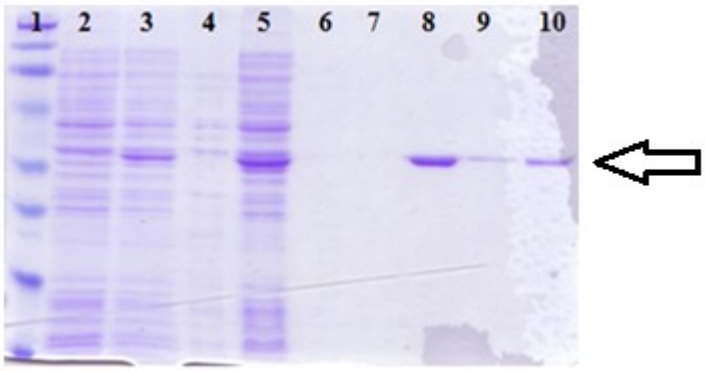

Figure 2-5. CysM purification SDS-PAGE gel results. Lane 1 – Ladder; Lane 2 – 0 hours after inducing with IPTG; Lane 3 – 20 hours after inducing with IPTG; Lane 4 – Cell pellet; Lane 5 – Cell lysate; Lane 6 – 100 mM imidazole buffer wash; Lane 7 – 200 mM imidazole buffer wash; Lane 8 – 500 mM imidazole wash (1 mL collection); Lane 9 – 50% dilution of lane 8; Lane 10 – 500 mM imidazole wash (5 mL collection). This figure shows the successful purification of the CysM protein with a band at approximately 34 kDa as indicated with a black arrow.

2.2.2 CysM Reduction Under Basic Conditions. The UV-vis spectrum of the CysM assay was taken before $NaBH_4$ reduction, as well as at 5 and 30 minutes after $NaBH_4$ addition. These results (shown in Figure 2-6) indicated that a new peak formed as a "shoulder" at approximately 320 nm over the course of the reaction. Also, the peak at 420 nm decreased over the course of the reaction. The reduction of the Schiff base occurred after only 5 minutes of reaction time. After a reaction time of 5 minutes, the assays also changed from yellow to clear as shown in Figure 2-7.

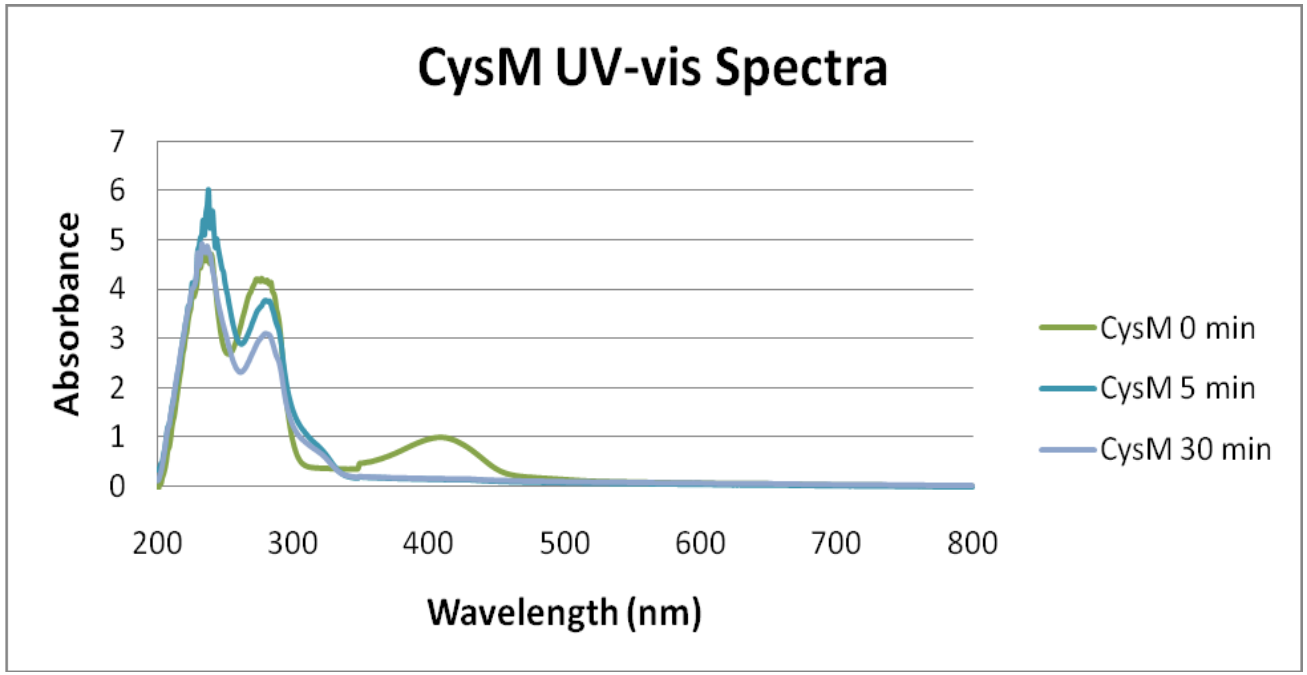

Figure 2-6. CysM UV-vis spectra following reduction with $NaBH_4$ after 0 min, 5 min and 30 min. This figure shows the successful reduction of the PLP-CysM through a decrease in the peak at 420 nm and an increase in the peak at 320 nm. The reduction occurred after only 5 minutes of reaction time and the spectrum did not change after 30 minutes of reaction time.

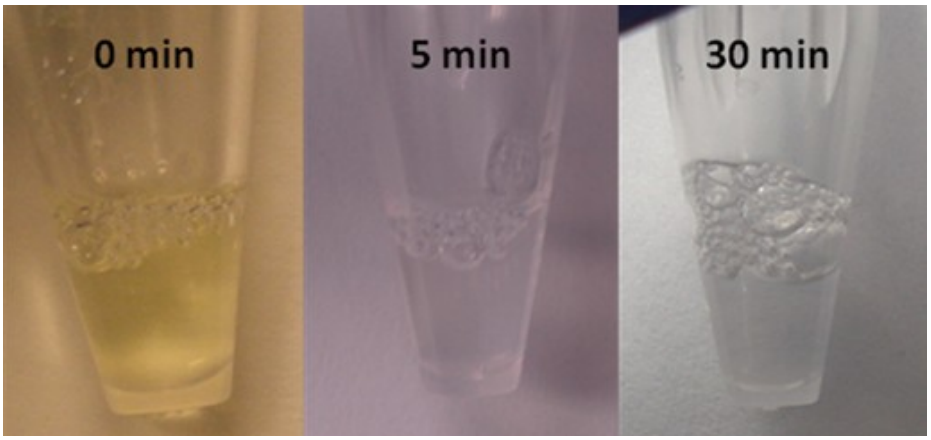

Figure 2-7. The reaction samples shown in clear 1 mL eppendorf tubes. These images show that the reduction of the PLP-CysM also resulted in a color change from yellow to clear after only a 5 minute reaction time.

```
  1 MTRYDSLLQA LGNTPLVGLQ RLSPRWDDGR DGPHVRLWAK
 41 LEDRNPTGSI KDRPAVRMIE QAEADGLLRP GATILEPTSG
 81 NTGISLAMAA RLKGYRLICV MPENTSVERR QLLELYGAQI
121 IFSAAEGGSN TAVATAKELA ATNPSWVMLY QYGNPANTDS
161 HYCGTGPELL ADLPEITHFV AGLGTTGTLM GTGRFLREHV
201 ANVKIVAAEP RYGEGVYALR NMDEGFVPEL YDPEILTARY
241 SVGAVDAVRR TRELVHTEGI FAGISTGAVL HAALGVGAGA
281 LAAGERADIA LVVADAGWKY LSTGAYAGSL DDAETALEGQ
321 LWA
```

Figure 2-8. CysM amino acid sequence. This figure shows the sequence of the CyM protein with the active site lysine residue (Lys51), with which PLP binds, shown in red.

The CysM sequence is shown in Figure 2-8. The purified protein also contained a His-tag with the following amino acid sequence: mghhhhhhhhhhssghiugrh. Using this sequence, as well as the increased mass due to the addition of PLP (321.0297 Da), the anticipated mass of the reaction product (in which the Schiff base was reduced) was determined to be 37062 Da and the mass of the control (in which the Schiff base that was not reduced) was determined to be 36829.5 Da. The results, analyzed by Pei-Jing "Peggy" Pai, Ph.D. (from Dr. Russell's group at Texas A&M University), are shown in Figure 2-9. The unmodified protein is shown in the top spectrum (the control) and the modified protein, with PLP bound, is shown in the bottom spectrum (the reaction). The reduction of the Schiff base resulted in an increase of approximately 231 Da within the reaction (37058 Da) compared to the control (36827 Da). There is also a 178 Da increase from 36827 Da to 37005 Da that corresponded to a glyconoylation. The reaction spectrum also showed the PLP modification to the gluconoylated protein as an increase in mass from 37005 Da to 37238 Da. The sample contained various salts or NaOH which complicated the sample analysis. Removal of the salts or NaOH as well as allowing the $NaBH_4$ reaction to occur for longer than 5 minutes would result in a greater concentration of PLP-modified protein and a cleaner spectrum.

2.2.3 CysM Reduction Under Neutral Conditions. The UV-vis spectrum of each sample was taken before $NaBH_4$ reduction, 5 minutes after $NaBH_4$ addition and 30 minutes after $NaBH_4$ addition. The results confirmed the $NaBH_4$ reduction of the PLP-lysine bond and are shown in Figure 2-10. A new peak formed as a "shoulder" at

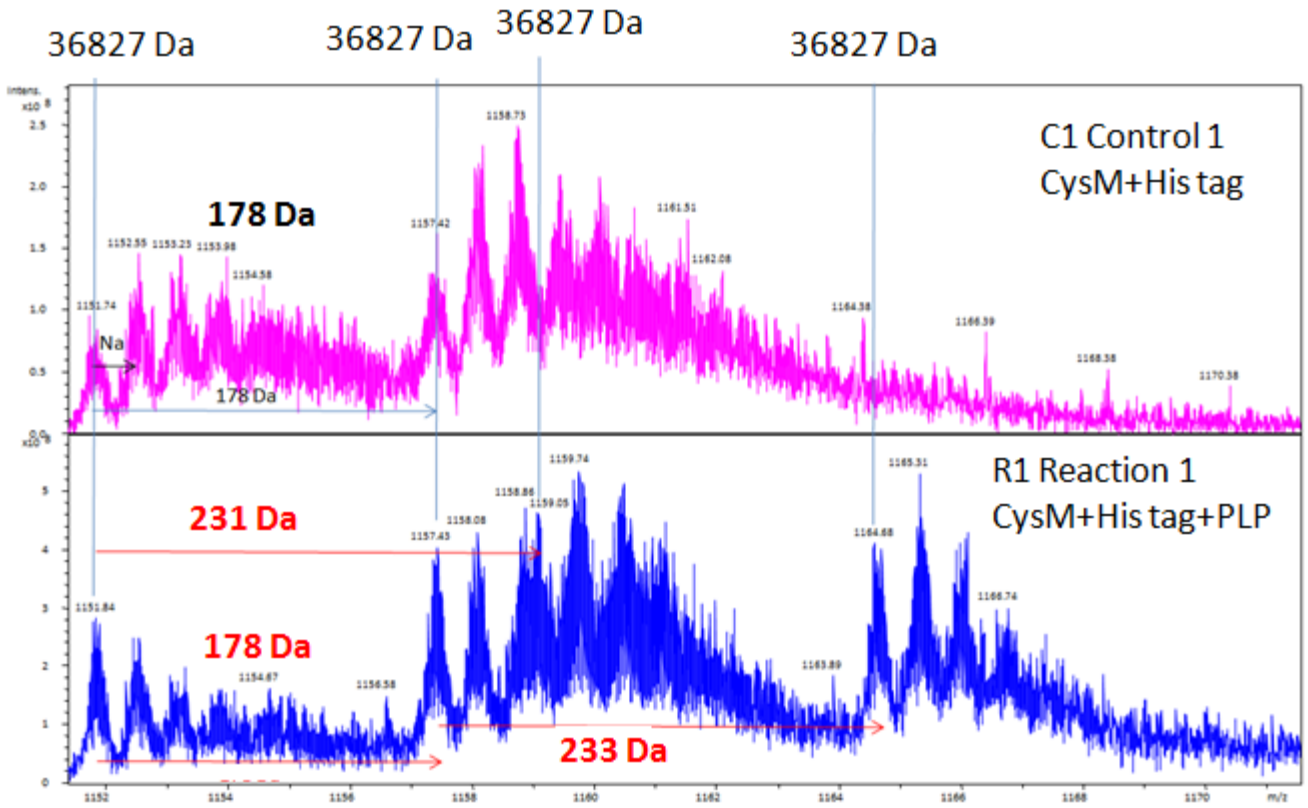

Figure 2-9. Mass spectrometry results from the $NaBH_4$ reduction of CysM under basic conditions. The top trace (pink) is the control without reduction of the Schiff base. The bottom trace (blue) is the CysM full reaction with reduction of the Schiff base. This figure shows the successful reduction of the CysM through the increase in the mass of the control (36827 Da) by 231 Da to a mass of 37058 Da in the reaction.

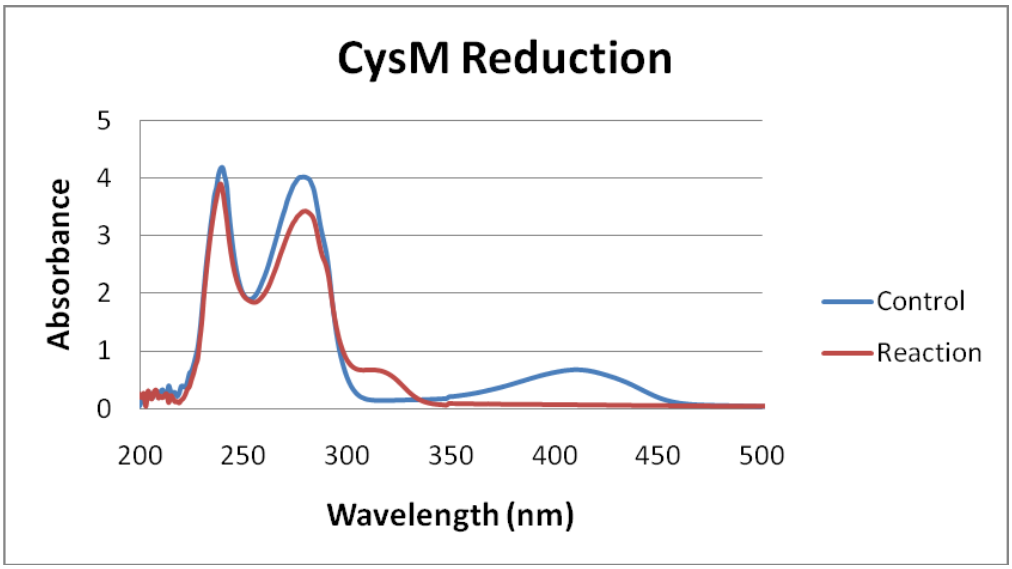

Figure 2-10. The UV-vis spectroscopy results of the $NaBH_4$ reduction. The full reaction, with the $NaBH_4$ reduction, is shown in the red trace. The control, without reduction, is shown in the blue trace. This figure shows the successful reduction of the Schiff base through a decrease in the peak at 420 nm and an increase in the peak at 320 nm.

approximately 320 nm as well as a decrease in the height of the peak at 320 nm when the Schiff base was reduced.

These samples were also submitted to Pei-Jing "Peggy" Pai, Ph.D. (from Dr. Russell's group at Texas A&M University) for analysis with mass spectroscopy. The result from the analysis of the control, without reduction of the Schiff base, is shown in Figure 2-11. The result from the analysis of the reaction, with reduction of the Schiff base, is shown Figure 2-12. These results clearly showed the addition of the PLP moiety with an increase in mass of approximately 231 Da. These results show that the current

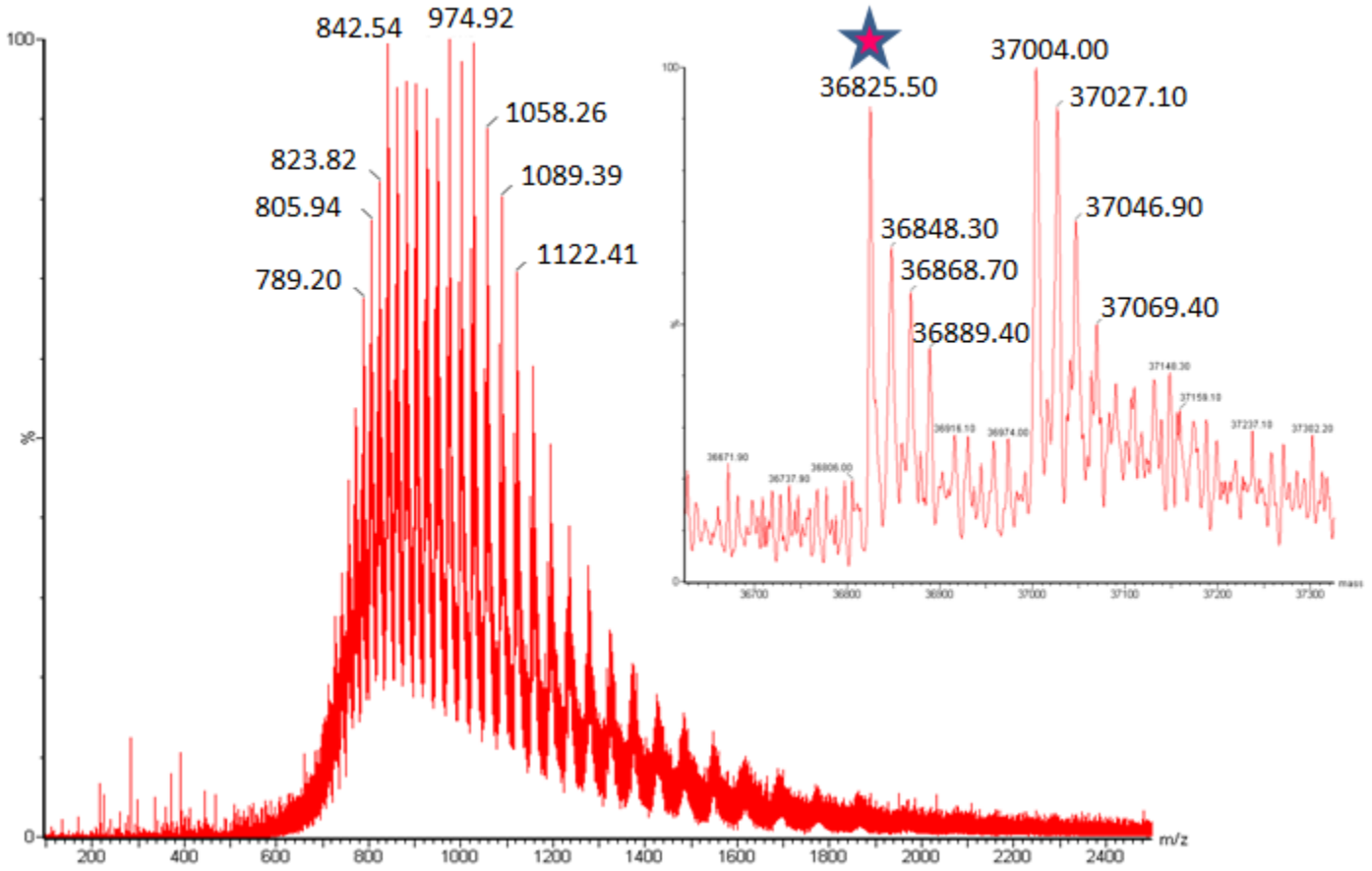

Figure 2-11. Mass spectrometry results from the analysis of the control (in which the Schiff base was not reduced). This figure shows the various ion charge states in the large spectrum with the deconvoluted spectrum shown in the inset. This figure shows the molecular weight of the control being 36825.50 Da before reduction with $NaBH_4$ (peak indicated with a star).

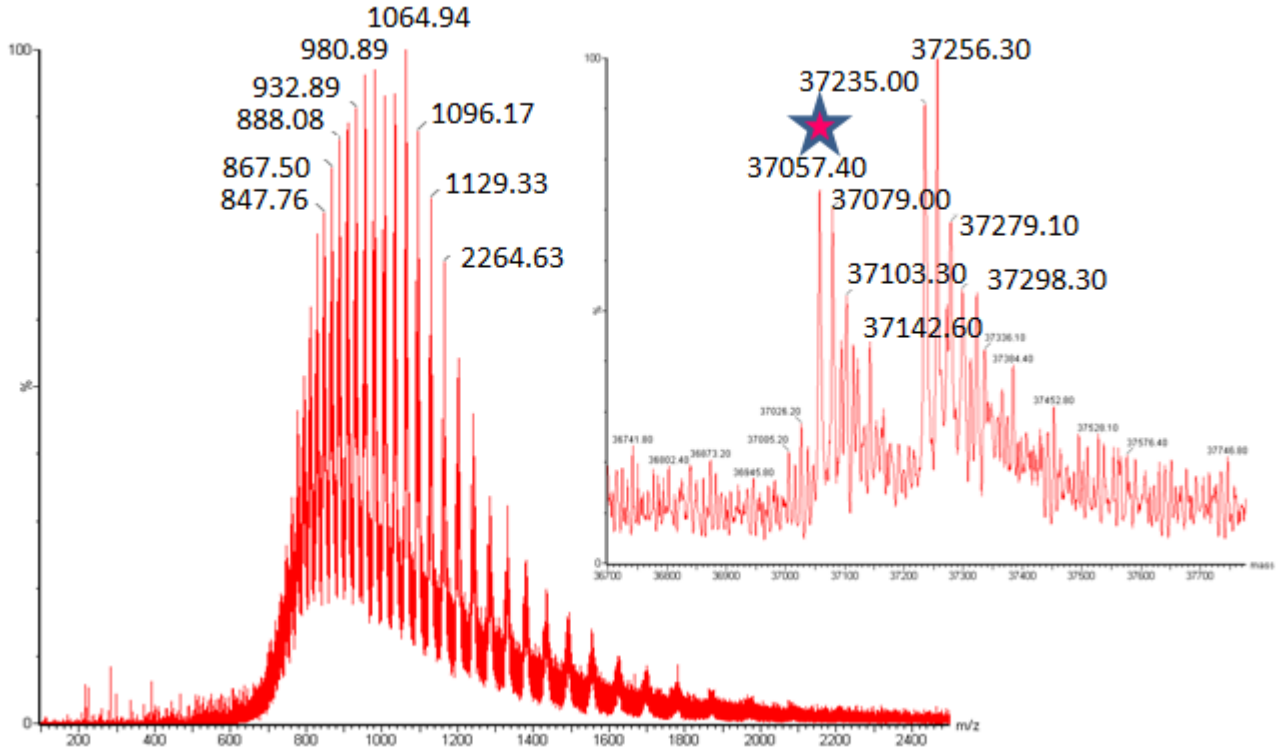

Figure 2-12. Mass spectrometry results from the analysis of the full reaction (in which the Schiff base was reduced). This figure shows the various ion charge states in the large spectrum with the deconvoluted spectrum shown in the inset. This figure shows the resulting mass of the reduced CysM as 37057.40 Da (peak indicated with a star). Reduction of the Schiff base resulted in an increase of approximately 231.03 Da which corresponds to the addition of PLP.

method for reduction worked appropriately and the reaction can be confirmed by either UV-vis spectroscopy or mass spectrometry.

2.2.4 Immobilized Ion Metal Affinity Chromatography Enrichment. MALDI was used to analyze the results after attempting to enrich the PLP-peptides from the CysM digest using the Fe-IMAC column. The samples were prepared by Phillip Lang. The peptide solutions were analyzed by Phillip in both negative and positive mode. Unfortunately, no peptides were found in the ammonium bicarbonate washings, where any bound phosphoproteins were expected to elute. Based upon the fragmentation from trypsin digestion, the following peptides, containing the active site lysine (Lys51), were expected: 1) LEDRNPTGSIK – 1229.6484 Da; w/ PLP bound – 1460.6781 Da; 2) NPTGSIK – 716.3937 Da; w/ PLP bound – 947.4234 Da; 3) NPTGSIKDRPAVR – 1410.7811 Da; w/ PLP bound – 1641.8108 Da.

2.2.5 Trypsin Digest of CysM and Analysis. The UV-vis spectra confirmed the reduction of the Schiff base through a decrease in the peak at 420 nm and an increase in the peak at 325 nm. These results are shown in Figure 2-13. During the HPLC analysis, a peak at approximately 27 minutes was apparent in the reaction sample that was not present in the control (without $NaBH_4$ reduction of the Schiff base). This peak was apparent when monitored at 214 nm or 325 nm (neither of which was present in the control). The results from monitoring the absorbance at both 214 nm and 325 nm are shown in Figure 2-14.

2.2.6 LC-MS Analysis of the Trypsin Digest of CysM. The analysis of the absorbance traces (LC-MS) resulted in a peak when monitored at 214 nm in the full

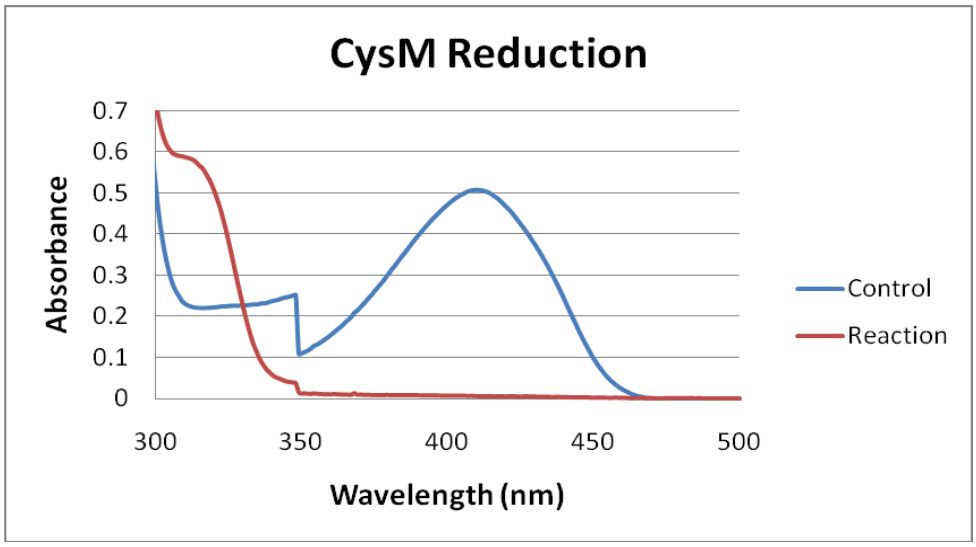

Figure 2-13. UV-vis spectra obtained from the CysM $NaBH_4$ reduction. The control (without reduction of the Schiff base) is shown in blue and the full reaction (with reduction of the Schiff base) is shown in red. This result showed the successful reduction of the PLP-CysM through a decrease in the peak at 420 nm and an increase in the peak at 320 nm.

reaction sample (with $NaBH_4$ reduction of the Schiff base) at about 27.4 minutes that was not apparent in the control (without $NaBH_4$ reduction of the Schiff base). These results are shown in Figure 2-15. Furthermore the control resulted in three peaks, when monitored at 214 nm, at about 7.4, 10.5 and 11.75 minutes that were not apparent in the reaction. When monitoring at 325 nm, a peak was apparent in the reaction at about 27.4 minutes as shown in Figure 2-16 (the same peak that showed up when monitored at 214 nm). The peak in the reaction with a retention time of about 27.4 was further analyzed using the mass spectroscopy portion of the LC-MS (see Figure 2-17). This particular

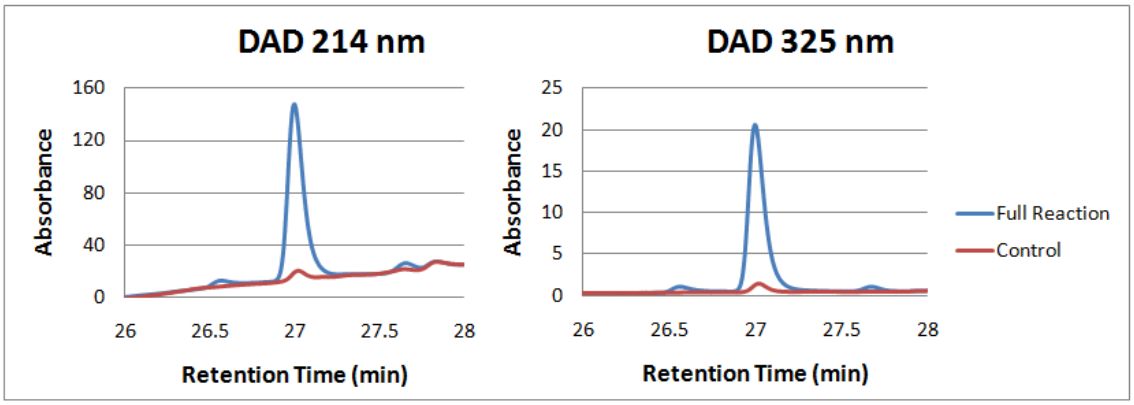

Figure 2-14. The HPLC results from analyzing the CysM trypsin digest. This figure shows the results when monitoring the absorbance at 214 nm on the left and the results when monitoring the absorbance at 325 nm on the right. The blue traces show the full reaction samples (with reduction of the Schiff base) and the red traces show the control samples (without reduction of the Schiff base).

peak corresponded to an m/z value of 821.4329. The peak was doubly charged resulting in an M+H value of 1641.8580 Da. This particular peak corresponded to the mass of a peptide with the PLP bound at lysine 51 with a skipped trypsin cleavage site after the PLP bound lysine. The expected peptide sequence was as follows: NPTGSIKDRPAVR (with a PLP bound at the center lysine). Analysis of the two of the three new peaks that were apparent in the control (without $NaBH_4$ reduction of the Schiff base) samples, when monitored at 214 nm, resulted in M+H values that corresponded to the expected peptides without PLP bound (NPTGSIK and DRPAVR).

2.2.7 Purified CysM – Fluorescence Tests. The UV-vis spectra that resulted from the reduction of CysM are shown in Figure 2-18. These results confirmed the

reduction of the PLP-peptide through the increase in the peak at 320 nm and the decrease in the peak at 420 nm when comparing the control (without $NaBH_4$ reduction of the Schiff base) and reaction (with $NaBH_4$ reduction of the Schiff base). The HPLC results when measuring fluorescence while varying the excitation wavelengths are shown in Figure 2-19. The emission wavelength was kept the same at 395 nm while the excitation wavelengths were varied as 290 nm, 280 nm, 330 nm or 355 nm. The excitation wavelengths resulting in the greatest intensity were 290 nm, 330 nm or 355 nm when the emission wavelength was 395 nm.

The HPLC results when measuring fluorescence and varying the emission wavelengths are shown in Figure 2-20. The excitation wavelength was kept the same at 290 nm while varying the emission wavelength as 395 nm, 390 nm, 400 nm or 410 nm. These results showed that the emission wavelengths resulting in the greatest intensity were 395 nm, 390 nm or 400 nm when the excitation wavelength was 290 nm.

The HPLC results when measuring fluorescence with an excitation wavelength of 290 nm and an emission wavelength of 395 nm are shown in Figure 2-21. Although the PLP-peptides at about 29.1 and 29.8 minutes were apparent when monitoring with fluorescence, there were many other peaks that were also apparent. This showed that fluorescence was not as specific as monitoring the absorbance at 325 nm even though it was slightly more sensitive.

The HPLC results when monitoring at an absorbance of 325 nm can be seen in Figure 2-22. The traces for the trypsin control (with only trypsin present), control (without $NaBH_4$ reduction of the Schiff base) and full reaction (with $NaBH_4$

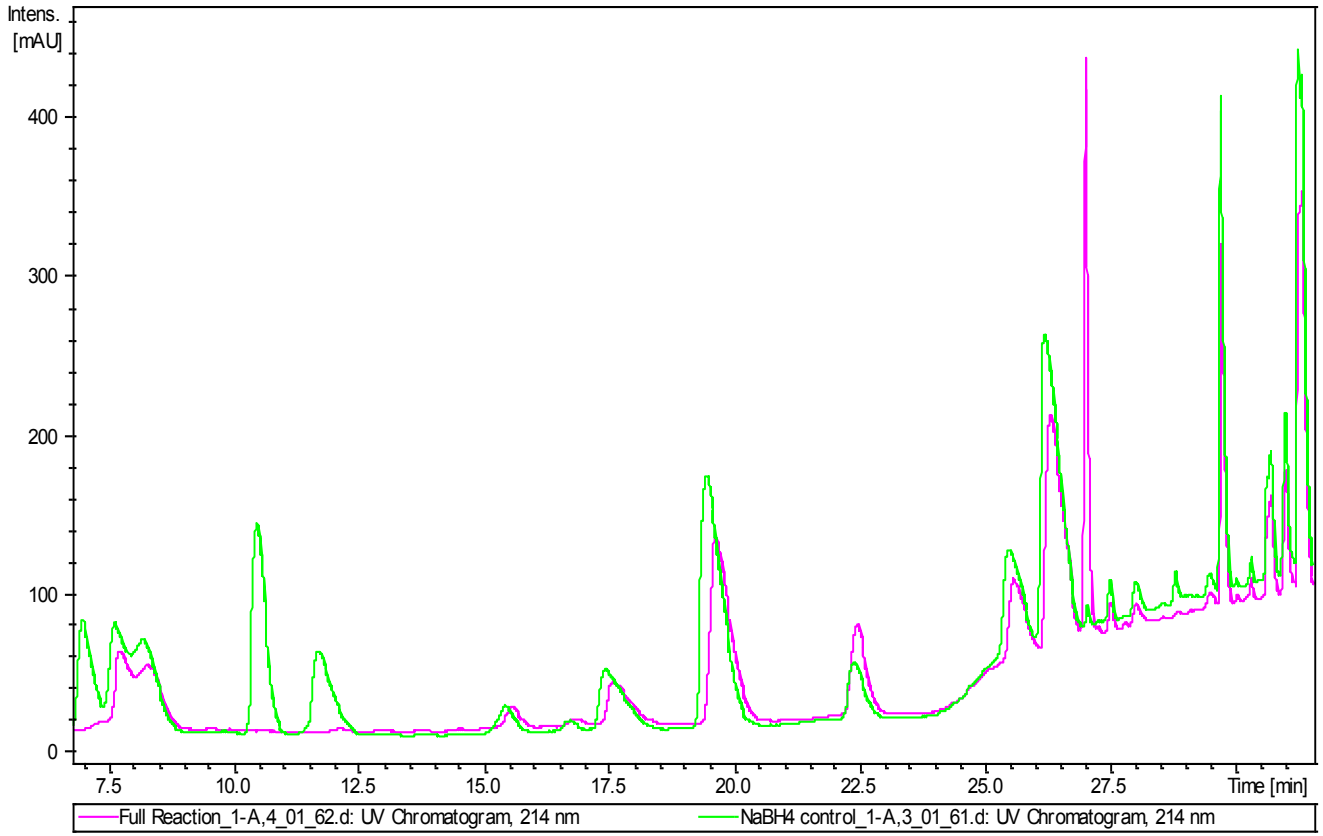

Figure 2-15. LC-MS results comparing the CysM full reaction and control when monitoring the absorbance at 214 nm. The result from the full reaction (with $NaBH_4$ reduction of the Schiff base) is shown in the purple trace and the control (without $NaBH_4$ reduction of the Schiff base) is shown in the green trace when monitoring the absorbance of both samples at 214 nm. These figure shows a peptide peak with a retention time of 27.4 minutes in the reaction that is not apparent in the control.

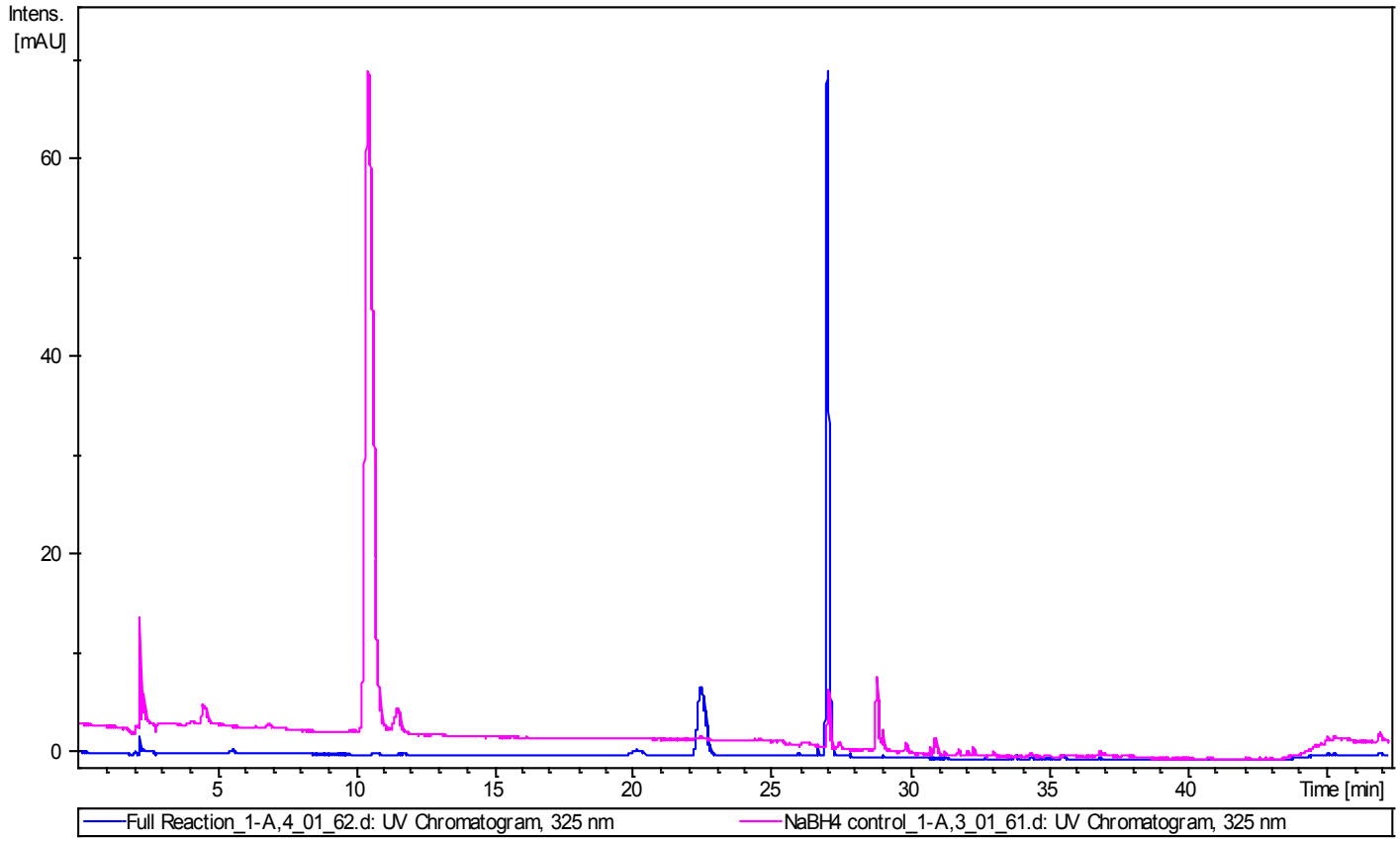

Figure 2-16. LC-MS results from the full reaction of CysM and the control monitoring absorbance at 325 nm. The full reaction is shown in the blue trace and the control (without reduction of the Schiff base) is shown in the pink trace. This figure shows that, when monitoring at 325 nm, a peak with a retention time of about 27.4 minutes was apparent in the reaction (with $NaBH_4$ reduction of the Schiff base) that was not apparent in the control (without $NaBH_4$ reduction of the Schiff base).

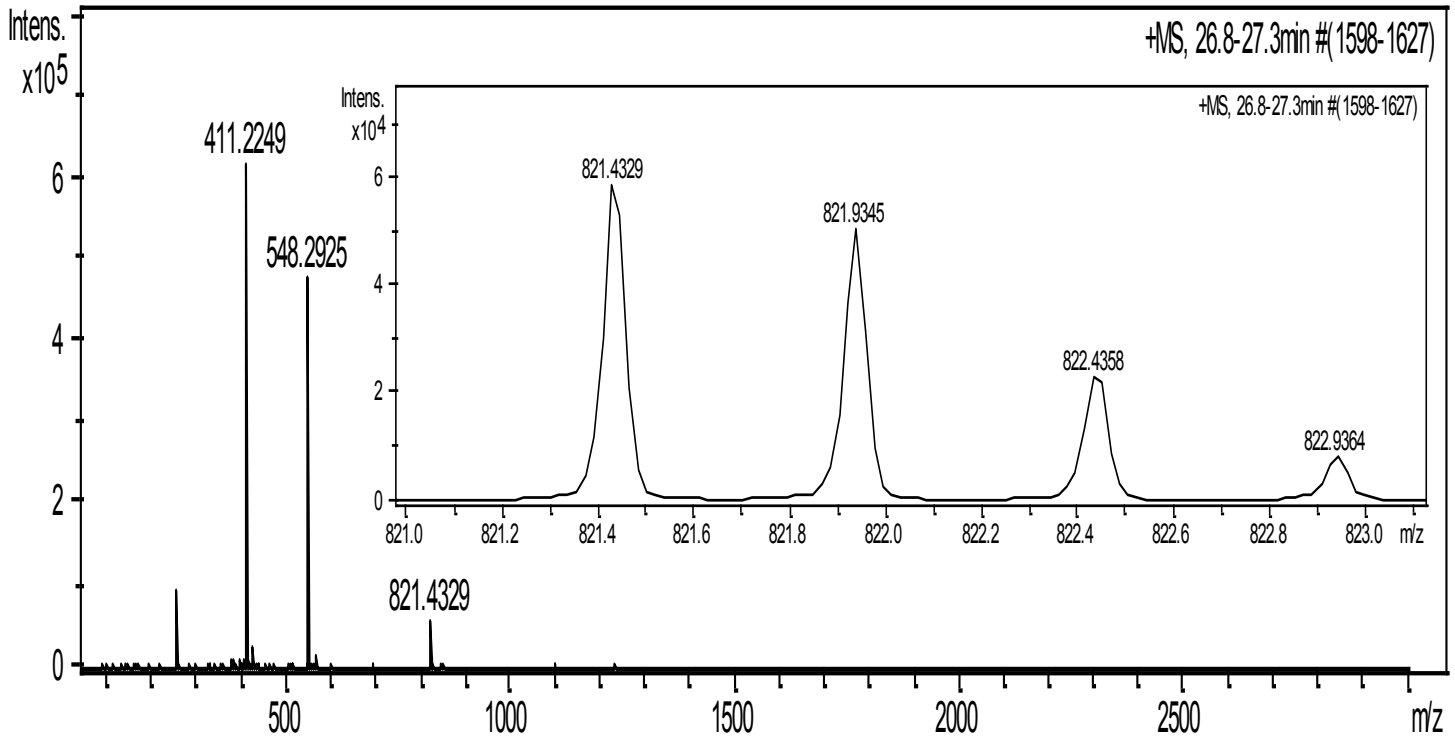

Figure 2-17. Further analysis of the peak in the reaction at 27.4 minutes (when monitoring absorbance at 325 nm) using the mass spectrum results from the LC-MS. This figure shows that the peak with a retention time of 27.1 minutes corresponded to a m/z value of 821.4329. This ion was doubly charged resulting in a "m" value or mass of about 1641.8580 Da which corresponded to the peptide: NPTGSIKDRPAVR.

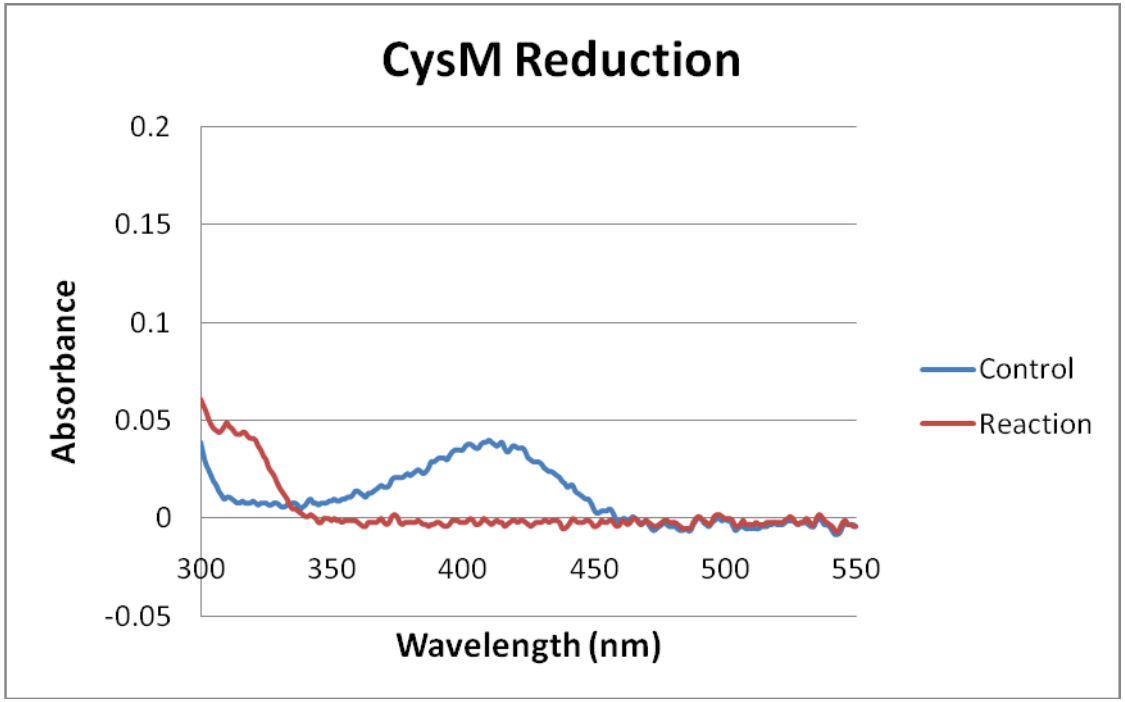

Figure 2-18. The UV-vis spectra results from the reduction of purified CysM. This figure shows the successful reduction of the Schiff base through a decrease in the peak at 420 nm and an increase in the peak at 320 nm.

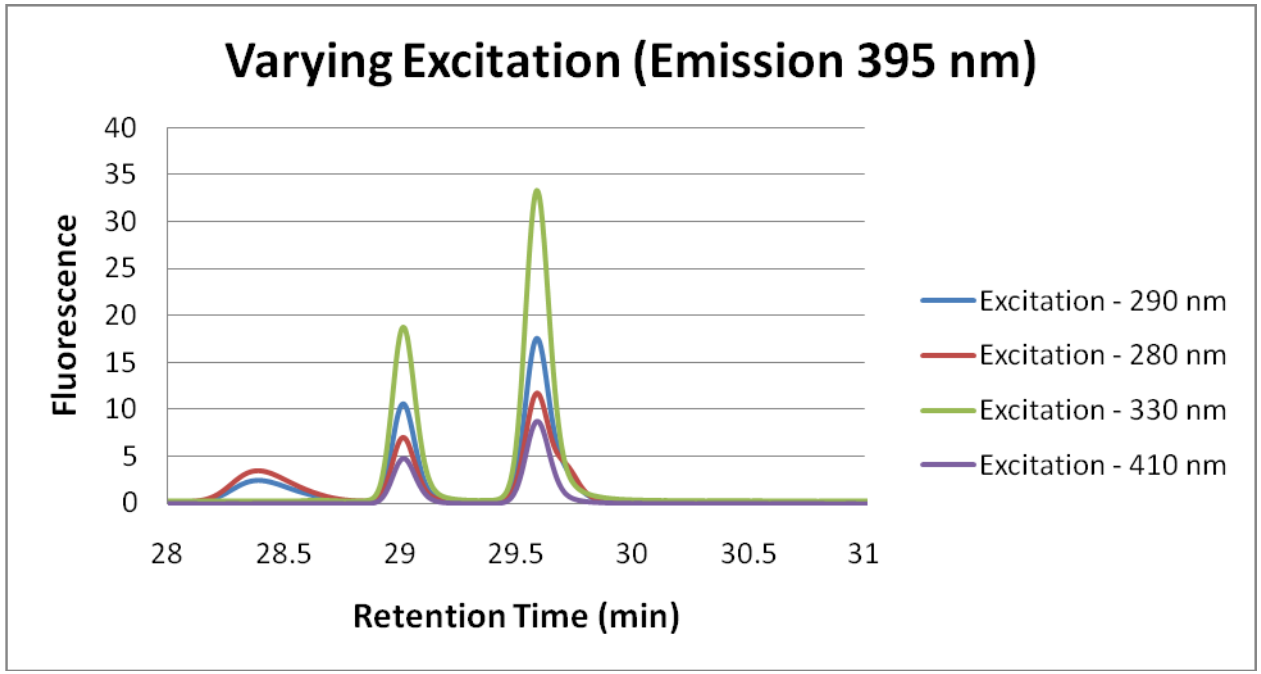

Figure 2-19. The HPLC results when measuring fluorescence and varying the excitation wavelengths using the HPLC 1200 + FC. The emission wavelength was kept at 395 nm. The excitation wavelengths were 290 nm (blue), 280 nm (red), 330 nm (green) and 355 nm (pink). This figure shows the excitation wavelengths that resulted in the greatest intensity were 290 or 330 nm when the emission wavelength was 395 nm.

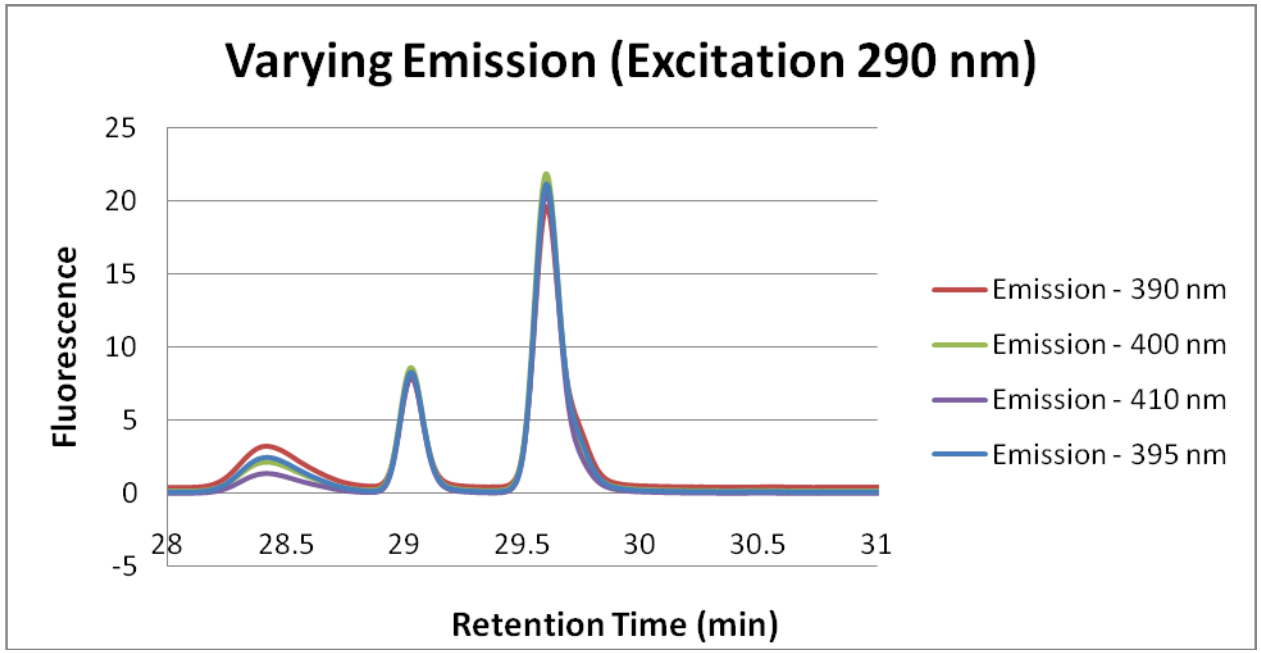

Figure 2-20. The HPLC results when measuring fluorescence and varying the emission wavelengths using the HPLC 1200 + FC. The excitation wavelength was kept at 290 nm. The emission wavelengths were 395 nm (blue), 390 nm (red), 400 nm (green) and 410 nm (pink). This figure shows the emission wavelengths that resulted in the greatest intensity were 395, 390 or 400 nm when the excitation wavelength was 290 nm.

reduction of the Schiff base) are shown. These results emphasized that monitoring the absorbance was more selective than monitoring the fluorescence. The PLP-peptides were apparent in the reaction at retention times of approximately 29.1 and 29.8 minutes.

2.2.8. Modified IMAC Enrichment. The UV-vis spectra results from the reduction of CysM are shown in Figure 2-23. The results confirmed the reduction of the PLP-peptide through an increase in the peak at 320 nm and a decrease in the peak at 420 nm when comparing the control (without $NaBH_4$ reduction of the Schiff base) and reaction (with $NaBH_4$ reduction of the Schiff base). The LC-MS results from the analysis of the full reaction versus the control when monitoring the absorbance at 325 nm showed two peaks in the full reaction that were not apparent in the control (see Figure 2-24). These peptides had retention times of 27.1 and 27.8 minutes. From previous results, these two peaks corresponded to the retention times of the expected PLP-peptides from the analysis of CysM. The LC-MS results from the analysis of the full reaction versus the flow-through after the addition of the full reaction on the IMAC column showed that the two peaks at 27.1 and 27.8 minutes were no longer apparent (see Figure 2-25). This indicated that the PLP-peptides were bound to the Fe-IMAC column.

All of the column washings were analyzed using the LC-MS, but only one of the fractions contained peptides that absorbed at 325 nm. The final column washing with ammonium bicarbonate pH 7.8 contained two peptides that absorbed at 325 nm with retention times of 27.1 and 27.8 minutes (see Figure 2-26). A comparison between the initial full reaction sample (before IMAC treatment) and this final ammonium bicarbonate washing showed that the retention times were slightly shifted.

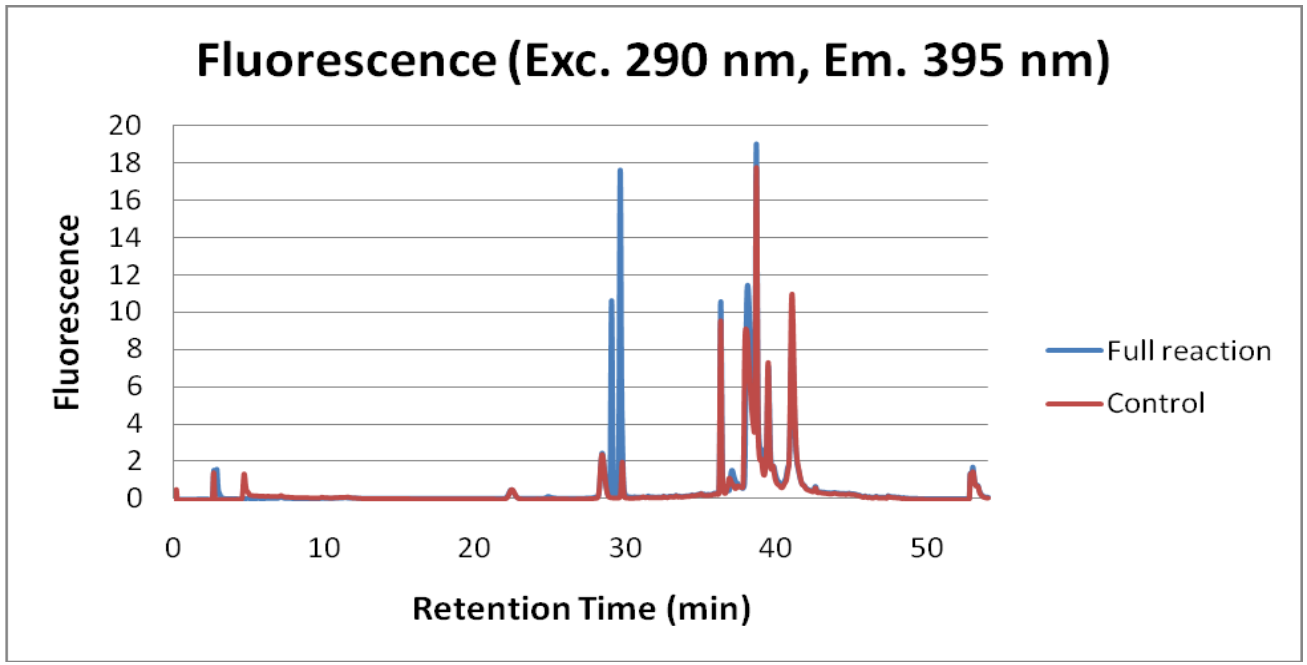

Figure 2-21. The HPLC results when measuring fluorescence with an excitation wavelength of 290 nm and an emission wavelength of 395 nm. This figure shows the PLP-peptides at about 29.1 and 29.8 minutes as well as many other irrelevant peaks. The full reaction (with $NaBH_4$ reduction of the Schiff base) is shown in the blue trace and the control (without $NaBH_4$ reduction of the Schiff base) is shown in the red trace.

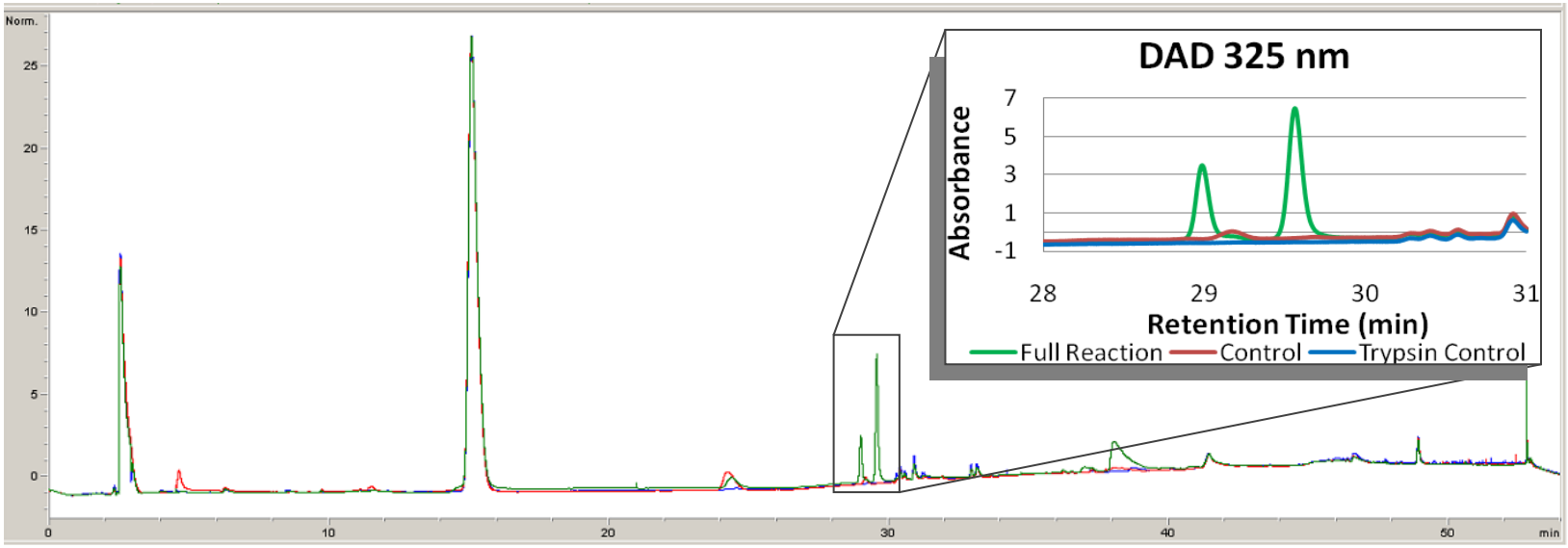

Figure 2-22. The HPLC results when monitoring the absorbance of 325 nm. The blue trace is the trypsin control (with only trypsin present), the red trace is the control (without $NaBH_4$ reduction of the Schiff base) and the green trace is the full reaction (with $NaBH_4$ reduction of the Schiff base). This figure shows that only the peaks corresponding to the PLP-peptides, with 27.1 and 27.8 minutes retention time, were apparent when monitoring the absorbance (325 nm) of the full reaction. The inset shows a zoomed in version with the full reaction (with $NaBH_4$ reduction of the Schiff base) in the blue trace, the control (without $NaBH_4$ reduction of the Schiff base) in the red trace and the trypsin control (with only trypsin present) in the green trace.

However, the peaks in both these samples corresponded to one another (see Figure 2-27). The LC-MS results of the CysM full reaction before IMAC treatment versus the final column washing with ammonium bicarbonate pH 7.8 when monitoring the mass spectrum are shown in Figure 2-28. These results showed that Fe-IMAC columns separated the PLP-peptides from other peptides within the sample.

The LC-MS results, when monitoring the mass spectrum, from analyzing the peptide in the full reaction (with $NaBH_4$ reduction of the Schiff base) with a retention time of 27.1 minutes are shown in Figure 2-29. The results showed that the m/z value was 411.2097 with a "z" value of 4. The resulting M+H was 1641.8154 Da which corresponded to the expected PLP-peptide as discussed previously (NPTGSIKDRPAVR - 1410.7811 Da; PLP – 231.0297 Da; resulting in a PLP-peptide of 1641.8108 Da). The LC-MS results, when monitoring the mass spectrum, from analyzing the peptide in the full reaction (with $NaBH_4$ reduction of the Schiff base) with a retention time of 27.8 minutes are shown in Figure 2-30. These results showed that the m/z value was 431.8200 with a "z" value of 5. The resulting M+H was 2155.0688 Da which corresponded to the expected PLP-peptide as discussed previously (LEDRNPTGSIKDRPAVR - 1924.0358 Da; PLP – 231.0297 Da; resulting in a PLP-peptide with a mass of 2155.0558 Da). The results showed that the PLP-peptides bound to the Fe-IMAC column. These peptides were eluted when the column was washed with 25 mM ammonium bicarbonate pH 7.8. The masses of the peptides with retention times of 27.1 and 27.8 minutes were 1641.8154 Da and 2155.0688 Da, respectfully (as expected and discussed earlier).

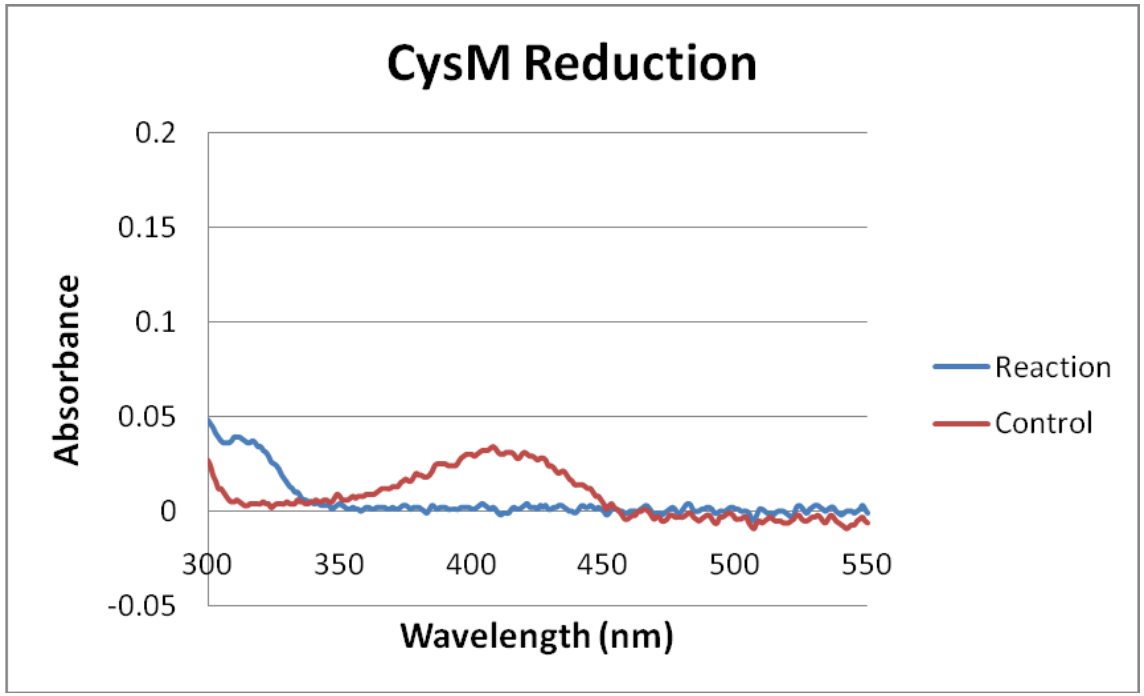

Figure 2-23. The UV-vis spectra results from the reduction of purified CysM for IMAC enrichment. This figure shows the successful reduction of the PLP-CysM through a decrease in the peak at 420 nm and an increase in the peak at 320 nm.

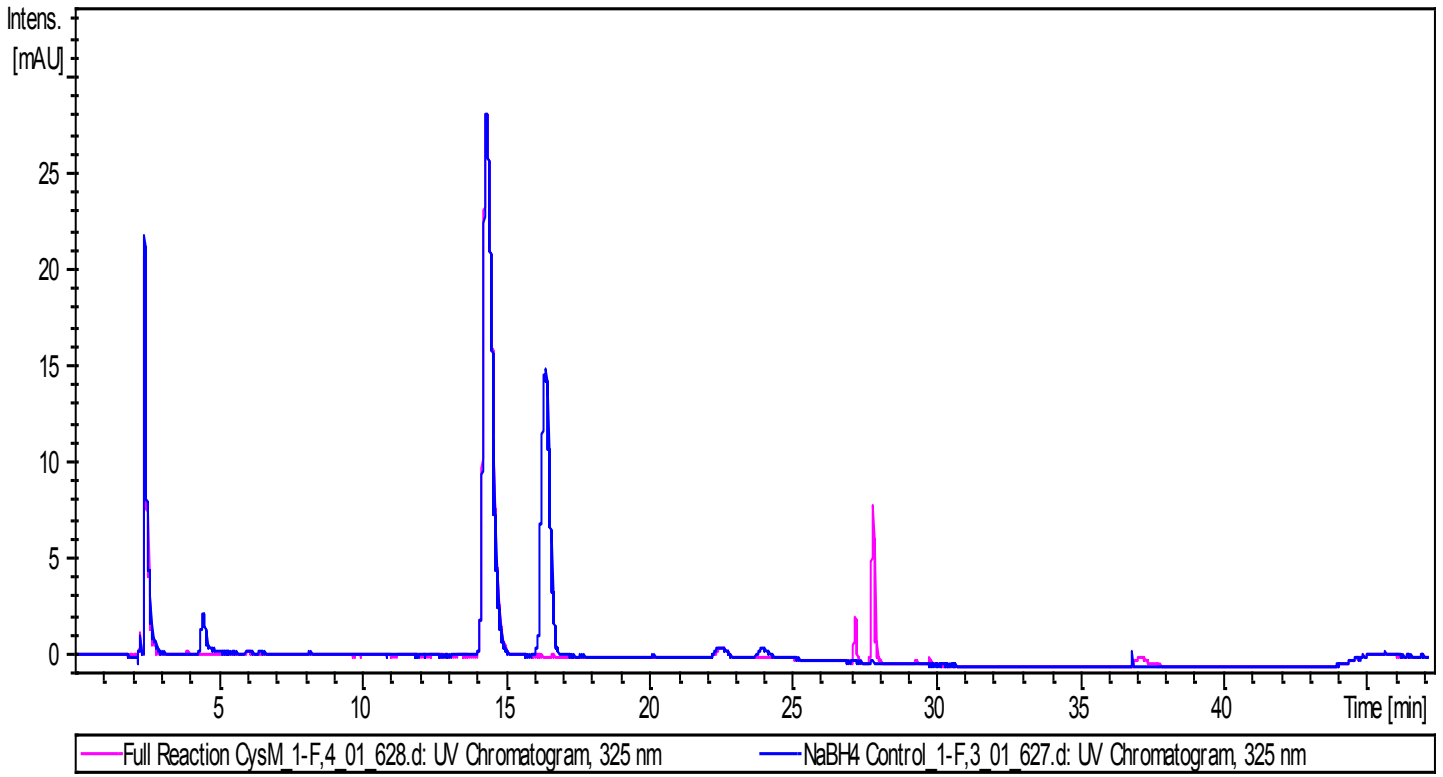

Figure 2-24. LC-MS results comparing the CysM full reaction and the control before IMAC enrichment. The result, when monitoring the absorbance of both samples at 325 nm, from the full reaction (with $NaBH_4$ reduction of the Schiff base) is shown in the pink trace and the control (without $NaBH_4$ reduction of the Schiff base) is shown in the blue trace. The figure shows the successful $NaBH_4$ reduction of the Schiff base as well as the successful trypsin digest. The PLP-peptides with retention times of 27.1 and 27.8 minutes were apparent in the reaction when monitoring at 325 nm, but not in the control.

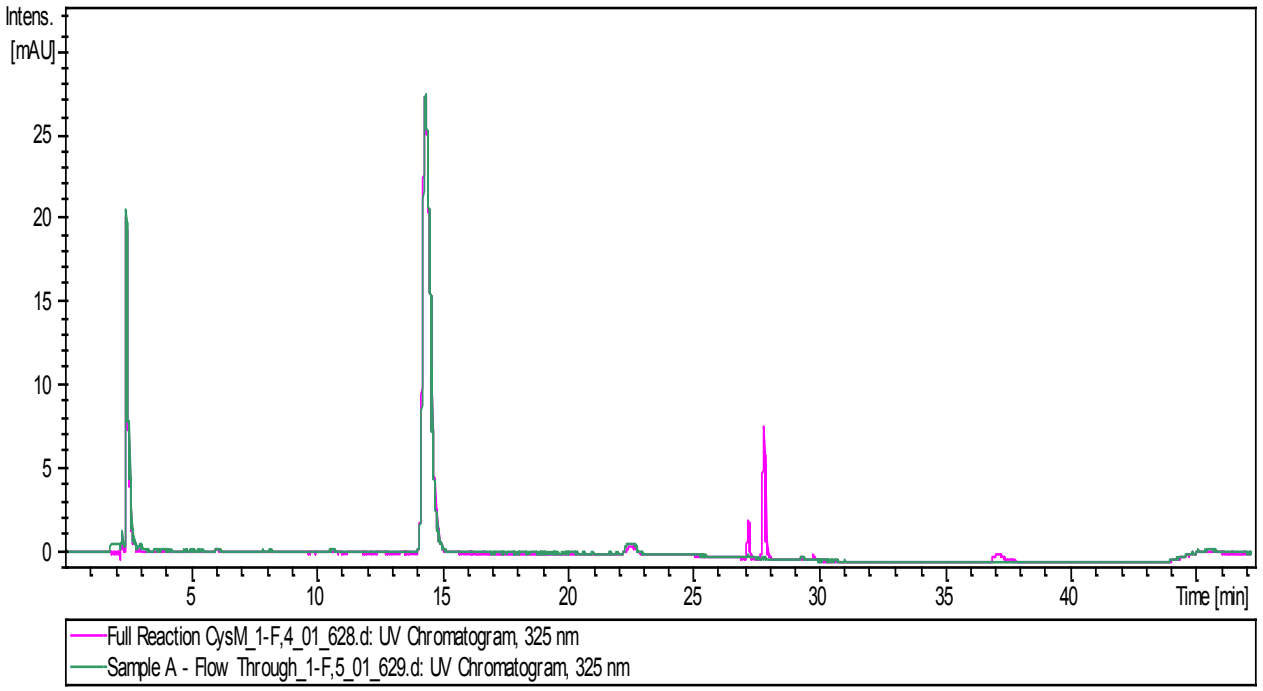

Figure 2-25. LC-MS results comparing the CysM full reaction and the flow-through after the IMAC enrichment. The full reaction (with $NaBH_4$ reduction of the Schiff base) is shown in the pink trace and the flow-through after the addition of the full reaction on the IMAC column is shown in the blue trace when monitoring the absorbance of both samples at 325 nm. This figure shows the PLP-peptides with retention times of 27.1 and 27.8 minutes were apparent in the reaction when monitoring at 325 nm. The flow-through trace shows that the PLP-peptides were no longer in the sample after enrichment with the Fe-IMAC column.

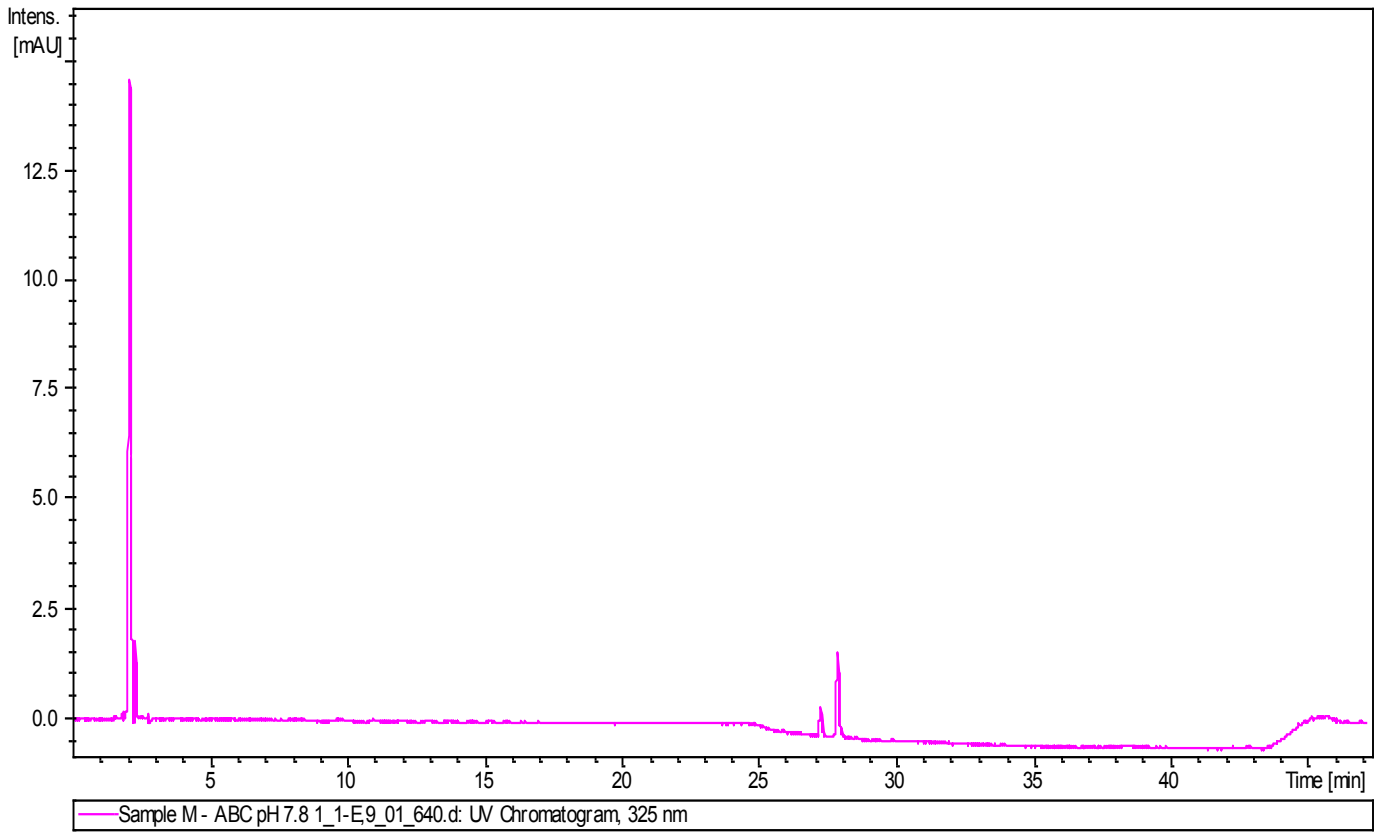

Figure 2-26. LC-MS results of the final column washing with ammonium bicarbonate pH 7.8 when monitoring the absorbance at 325 nm. The figure shows that the PLP-peptides with retention times of 27.1 and 27.8 minutes were eluted when the column was washed with 25 mM ammonium bicarbonate pH 7.8.

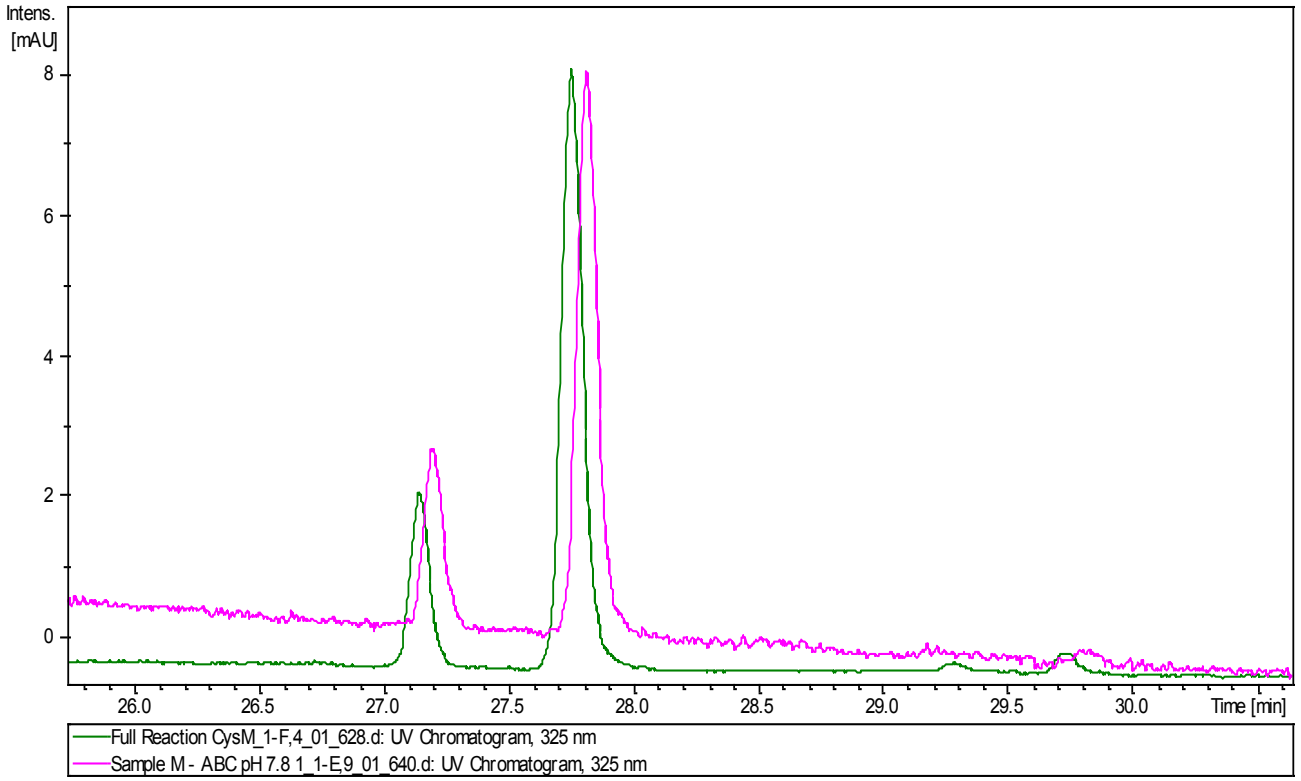

Figure 2-27. LC-MS results when comparing the full reaction and the final column washing after the IMAC enrichment. The results of the full reaction (with $NaBH_4$ reduction of the Schiff base) is shown in the green trace and the final column washing with ammonium bicarbonate pH 7.8 is shown in the pink trace, when monitoring the absorbance of both samples at 325 nm. The figure shows that the PLP-peptides had approximately the same retention times when comparing the samples before and after Fe-IMAC enrichment.

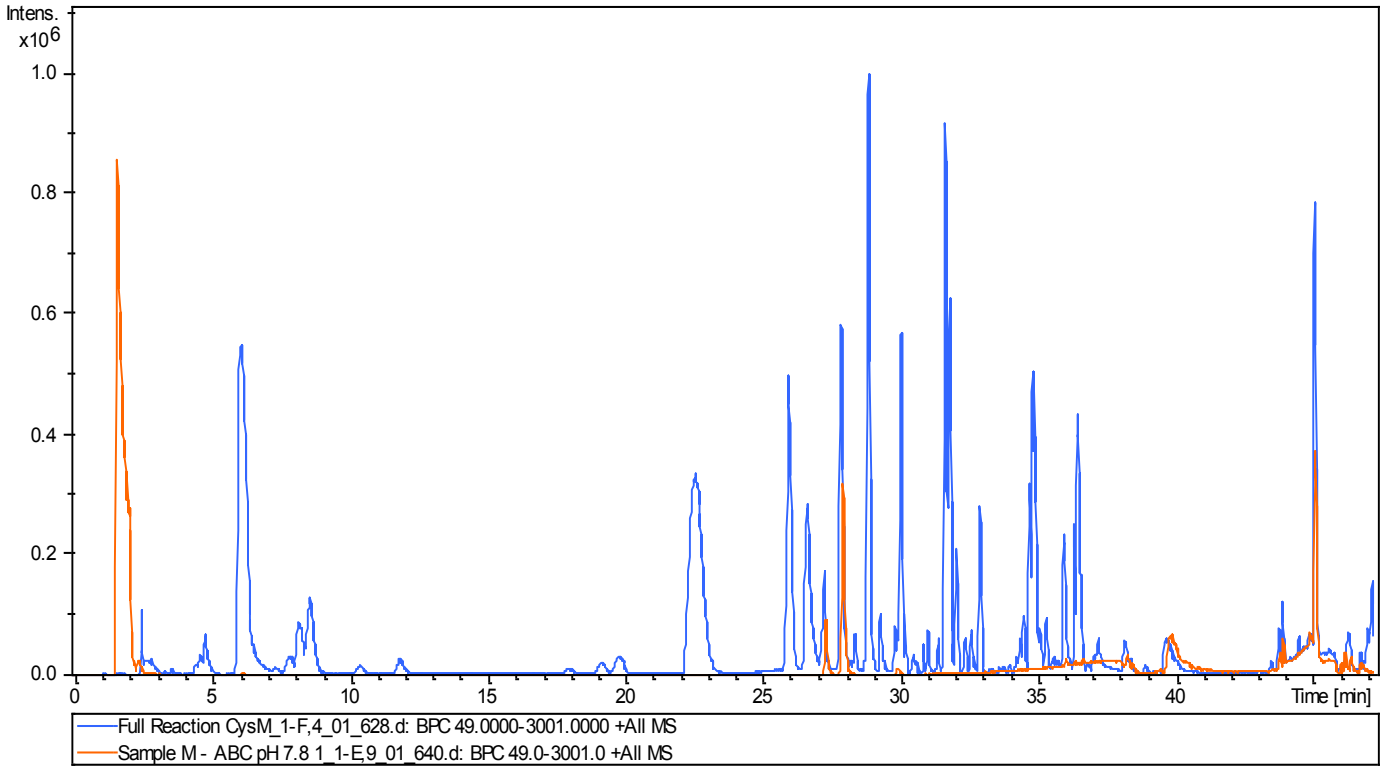

Figure 2-28. LC-MS results of the full reaction and the final column washing with ammonium bicarbonate pH 7.8 when monitoring the mass spectrum. The results of the full reaction (with $NaBH_4$ reduction of the Schiff base) before IMAC treatment is shown in the blue trace and the final column washing with ammonium bicarbonate pH 7.8 is shown in the orange trace, when monitoring the mass spectrum of both samples. This figure shows that the PLP-peptides were specifically enriched using the Fe-IMAC column.

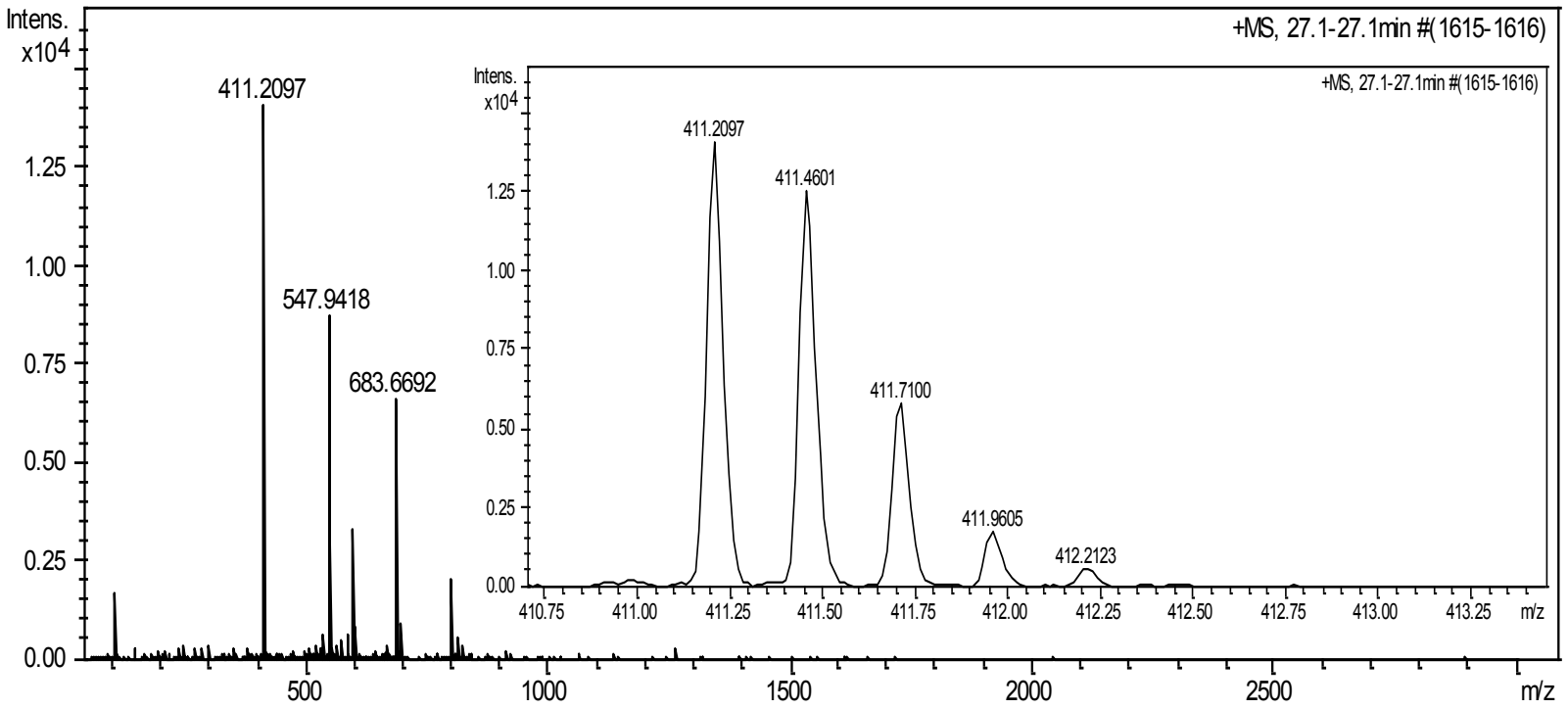

Figure 2-29. LC-MS results of the final column washing (with ammonium bicarbonate pH 7.8) by analyzing the mass spectrum of the peptide with a retention time of 27.1 minutes. The figure shows that the m/z value, of the peptide with a retention time of 27.1 minutes, was 411.2097 with a "z" value of 4. The resulting M+H was 1641.8154 Da which corresponded to the expected PLP-peptide (NPTGSIKDRPAVR - 1410.7811 Da; PLP – 231.0297 Da; resulting in a PLP-peptide of 1641.8108 Da).

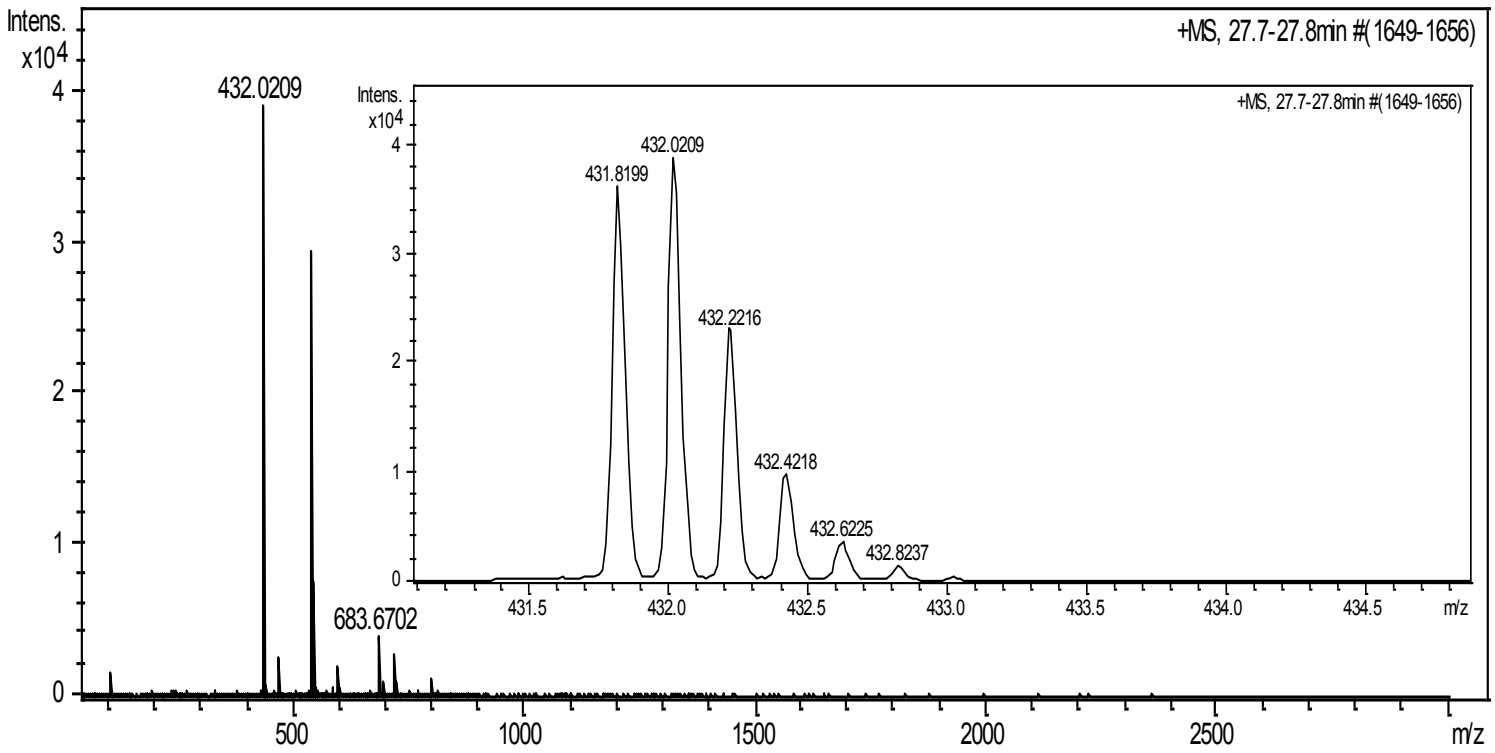

Figure 2-30. LC-MS results of the final column washing (with ammonium bicarbonate pH 7.8) by analyzing the mass spectrum of the peptide with a retention time of 27.8 minutes. This figure shows the m/z value, of the peptide with a retention time of 27.8 minutes, was 431.8200 with a "z" value of 5. The resulting M+H was 2155.0688 Da which corresponded to the expected PLP-peptide (LEDRNPTGSIKDRPAVR - 1924.0358 Da; PLP – 231.0297 Da; resulting in a PLP-peptide of 2155.0558 Da).

2.3 Discussion

The HPLC analysis of the PLP-peptides showed that fluorescence was not a very selective method for separating the PLP-peptides from the peptide mixture. When the fluorescence was monitored with an excitation wavelength of 290 nm and an emission wavelength of 395 nm, there were many irrelevant peaks present. These irrelevant peaks weren't apparent when monitoring the absorbance at 325 nm. As a result, monitoring the fluorescence was not as selective as when the absorbance was monitored at 325 nm.

The LC-MS analysis of the PLP-peptides from CysM proved to be a successful method for determining which peptide peaks corresponded to those bound to PLP. By monitoring at 325 nm, the PLP-peptide(s) were successfully located and analyzed based upon their mass spectrum. The two peaks that were consistently apparent in the reaction samples (with $NaBH_4$ reduction of the Schiff base), but not in the control (without $NaBH_4$ reduction of the Schiff base) corresponded to the correct M+H values for expected PLP-peptides. The first peptide with a retention time of 27.1 minutes had an M+H value of 1641.8154 Da. Based upon the protein sequence and trypsin digestion, the amino acid sequence of this peptide was NPTGSIKDRPAVR with an M+H of 1410.7811 Da. As expected, the covalent addition of PLP resulted in an increase of 231.0297 Da which ultimately resulted in an M+H 1641.8108 Da in the reaction sample. The second peptide with a retention time of 27.8 minutes had an M+H value of 2155.0688 Da. Based upon the protein sequence and trypsin digestion, the amino acid sequence of this peptide was LEDRNPTGSIKDRPAVR with a M+H of 1924.0358 Da.

As expected, the covalent addition of PLP resulted in an increase of 231.0297 Da which ultimately resulted in an M+H of 2155.0558 Da in the reaction sample.

These findings were important because they showed that LC-MS would be useful for determining PLP-peptides from complex peptide mixtures. Although there were many other peptide fragments that resulted from the trypsin digestion, monitoring at 325 nm made it possible to locate the PLP-peptide(s). Using the mass spectrum of the sample, it was possible to determine the mass of the resulting PLP-peptide fragment was determined. This study was relevant because it provided proof of concept that it would be possible to analyze cell lysate proteomes for PLP-peptides. Even though monitoring the absorbance at 325 nm made it possible to locate the PLP-peptides, the peptide mixture was still very complex for each sample. The mass spectrum was complex with many peptide peaks. Although the mass spectrum peak that corresponded to the absorbance peak, when monitoring the absorbance at 325 nm, could be determined from the digestion of a pure PLP-enzyme, it may be difficult to determine the mass of a PLP-peptide from a cell lysate. Therefore, it may be necessary to enrich the samples for PLP-peptides prior to analysis with LC-MS in order to decrease the number of irrelevant peptides in the sample.

Fe-IMAC was a successful method for specifically enriching the PLP-peptides. The mass spectrum of the entire CysM lysate was very complex with many peaks corresponding to various peptides. After enrichment using the Fe-IMAC column, the mass spectrum was simplified to mainly contain the PLP-peptide peaks, which had retention times of 27.1 and 27.8 minutes. Overall, the results showed that the PLP-

peptides specifically bound to the Fe-IMAC column and were eluted when the column was washed with 25 mM ammonium bicarbonate pH 7.8. Overall, the IMAC column was successful at specifically enriching PLP-peptides and may be a useful method for studying the proteomics of PLP-dependent enzymes or the "PLP-ome."

Future research should include attempting to analyze the proteome of cell lysates from various organisms. When applying this methodology to a cell lysate it may be necessary to make slight variations. For example, it may be necessary to complete the $NaBH_4$ reduction under denaturing conditions. It may also be necessary to add the cell lysate to the Fe-IMAC column multiple times in order to allow the PLP-peptides to be enriched before analysis on the LC-MS. It may also be possible to mix the cell lysate with the Fe-NTA resin in a batch reaction rather than using a column. This would allow constant mixing between the PLP-peptides and the resin and may enhance the enrichment of the PLP-peptides.

2.4 Experimental

2.4.1 CysM Expression and Purification. The gene source of CysM was *Mycobacterium tuberculosis*, the plasmid was pET16b (Ampicillin resistant) and the storage strain was DH5alpha. The storage strain was grown on an Amp-LB plate overnight for 14 hours. A single colony from this plate was used to inoculate a 15 mL starter culture consisting of ampicillin (Amp) and LB (15 μL of 100 mg/ml ampicillin). This culture was then incubated in the small shaker at 37°C for approximately 15 hours. The culture was then centrifuged for 10 minutes at 5000 RPM. The supernatant was

then discarded and the pellets were resuspended in 250 µL Buffer P1 according to the QIAprep Spin MiniPrep kit for plasmid DNA purification. The second step of the MiniPrep kit called for the addition of 250 µL of Buffer P2 and inversion of the eppendorf tube. Next, 350 µL of Buffer N3 was added and mixed immediately. The eppendorf tubes were then centrifuged for 10 minutes at 13,000 RPM. The supernatants were applied to the QIAprep spin columns by pipetting. The spin columns were centrifuged for 60 seconds and the flow-through was discarded. The QIAprep columns were washed by adding 500 µL of Buffer PB and centrifuged for 60 seconds. The columns were washed two more times with 750 µL of Buffer PE and centrifuged for an additional 60 seconds. The flow-through was discarded and centrifuged for an additional minute to remove residual wash buffer. The columns were then placed in clean 1.5 mL eppendorf tubes. The DNA was eluted by adding 50 µL of Buffer EB to the center of each spin column then they were left standing for one minute and then centrifuged for one minute. Before beginning the transformation, two electrocuvettes were placed on ice and two Amp-LB plates were placed in the 37°C incubator.

The electroporation process required the dilution of the MiniPrep product using 1 µL of MiniPrep solution and 19 µL of sterile H_2O. One µL of the above diluted MiniPrep product was added to approximately 40 µL of electrocompetent *E. Coli* BL21 (DE3) cells and then transferred to the sterile electrocuvettes that were placed on ice at the beginning of this process. All air bubbles were removed from the cuvette before being placed in the electroporator. A pulse of 2.5 kV was used to increase the permeability of the plasma membrane to allow the transformation of the cells. The cells

were then "rescued" from the cuvette by adding 1 mL of LB and transferring to sterile eppendorf tubes. These tubes were then incubated in the shaker for 45 minutes at 37°C. About 100 µL of the incubated cells were then plated on a warmed Amp-LB plate and incubated overnight at 37°C. A single colony was used to inoculate a 15 ml LB culture with 15 µL 100 mg/mL Amp. This culture was then incubated overnight for 12-15 hours at 37°C 200 RPM. This single colony culture was then used to inoculate a large 1 L AMP-LB culture and grown up to an OD_{600} of 0.6 at 37°C, 200 RPM. This culture was then placed in the cold room for approximately 20 minutes and the shaker was decreased to 15°C and 180 RPM. Protein production was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) (C_F 1 mM) and the culture was placed in the shaker. After 0 hrs, 3 hrs, 6 hrs and 20 hrs, aliquots were taken to run on a SDS-PAGE gel to monitor the expression of CysM.

A 12% SDS-PAGE gel was made and used to check the protein production after inducing with IPTG. Each aliquot of cells was mixed with 10 µL of loading dye with DTT. These samples were then incubated for 10 minutes at 100°C using the PCR machine. The samples were then centrifuged 2-3 minutes at 14,000 RPM. The samples were loaded into a 12% SDS-PAGE gel and run at 120 V for about 1.5 hours.

The following buffers were made: Start/Lysis Buffer – 50 mM KH_2PO_4, 150 mM NaCl, pH 7.8; Elution Buffer – 50 mM KH_2PO_4, 150 mM NaCl, 500 mM Imidazole, pH 7.8; 2x Final Buffer – 100 mM KH_2PO_4, 300 mM KCl, 30% glycerol, pH 7.5; IMAC Strip Buffer – 20 mM NaH_2PO_4, 500 mM NaCl, 50 mM EDTA, pH 7.4. The Elution and Start Buffers were combined to form buffers with the following concentrations of

imidazole: 10 mM, 50 mM, 100 mM, 200 mM (all buffers also contained 50 mM KH_2PO_4, 150 mM NaCl, pH 7.8).

The purification procedure required the cell pellet to be thawed and resuspended in 10 mL of 10 mM Imidazole, 50 mM KH_2PO_4, 150 mM NaCl, pH 7.8 buffer. The cells were then sonicated six times with a process time of 20 seconds, 1.5 seconds on and 1.5 seconds off at a 5.0 output. A 100 µL aliquot was then removed prior to spinning down the sonicated cells at 15,000 RPM for 35 minutes. The 100 µL aliquot was also spun down at 14,000 RPM for 5 minutes. The cell pellet and supernatants were separated for both the large sample as well as the small 100 µL sample. TCEP was then added to 5 mL aliquots of each buffer for purification containing varying concentrations of imidazole (C_F 1 mM). A Ni-IMAC column (1 mL) was prepared by running 5 cv water, 5 cv strip buffer, 5 cv water, 5 cv 0.1M $NiSO_4$, 5 cv water, and 5 cv of the 10 mM imidazole buffer (50 mM KH_2PO_4, 150 mM NaCl, pH 7.8). The cell lysate was filtered through a syringe filter and then loaded onto the column. An imidazole gradient was then applied by adding 5 cv 10 mM imidazole, 5 cv 50 mM imidazole, 5 cv 100 mM imidazole, 5 cv 200 mM imidazole, and 5 cv 500 mM imidazole (all imidazole buffers also included 50 mM KH_2PO_4 and 150 mM NaCl pH 7.8). The column was then washed with 5-10 cv water, 5 cv strip buffer, 5 cv water and 5 cv 10% ethanol.

A 12% SDS-PAGE gel was then prepared and the gel samples were prepared as follows: Expression samples - 0 hr and 20 hrs from previous gel; Supernatant/Pellet - 5 µL of the supernatant/pellet sample + 15 µL of the 10 mM imidazole + 10 µL loading dye; IMAC fractions – 20 µL sample + 10 µL dye. The samples were then boiled in the

PCR thermal cycler at 100°C for 10 minutes except for the pellet sample which was boiled for 20 minutes. The samples were then spun about 2 minutes at 14,000 RPM. The gel was loaded with 8 µL of the marker and 10 µL of each sample (resulting in 10 samples in total). The samples containing the CysM protein were then desalted and buffer exchanged into 1x final buffer consisting of 50 mM KH_2PO_4, 150 mM KCl, 15% glycerol, pH 7.5 before being concentrated to a final concentration of 130 µM.

2.4.2 CysM Reduction Under Basic Conditions. A solution of 0.24% (w/v) NaOH was made fresh before the hydrogenation reaction. Two separate assays were set up as follows: 1) 50 µL of 50 mM CysM (in 1x final buffer consisting of 50 mM KH_2PO_4, 150 mM KCl, 15% glycerol, pH 7.5) + 15 µL 10 mM $NaBH_4$ in 0.24% NaOH; 2) 50 µL of 50 mM CysM (in 1x final buffer consisting of 50 mM KH_2PO_4, 150 mM KCl, 15% glycerol, pH 7.5) + 15 µL 0.24% NaOH. The UV-vis spectrum was obtained after various reaction times. The samples that were reacted with $NaBH_4$ for 5 minutes were buffer exchanged into 20 mM ammonium acetate using the Biospin Buffer exchange columns and were then concentrated down in order to analyze the samples via mass spectroscopy. The final concentration of the reaction was 40 µM at 50 µL and the final concentration of the control (without $NaBH_4$ reduction of the Schiff base) was 50 µM at 50 µL.

2.4.3 CysM Reduction Under Neutral Conditions. Two separate assays were set up as follows: 1) 50 µL of 50 mM OAHS (in 1x final buffer consisting of 50 mM KH_2PO_4, 150 mM KCl, 15% glycerol, pH 7.5) + 15 µL 10 mM $NaBH_4$ in dH_2O; 2) 50 µL of 50 mM OAHS (in 1x final buffer consisting of 50 mM KH_2PO_4, 150 mM KCl,

15% glycerol, pH 7.5) + 15 µL in dH_2O. The UV-vis spectrum was obtained after a one hour reaction time. The samples were buffer exchanged into 20 mM ammonium acetate using the Biospin Buffer exchange columns and were then concentrated down in order to analyze the samples via mass spectroscopy. The final concentration of the reaction was 40 µM at 50 µL and the final concentration of the control (without $NaBH_4$ reduction of the Schiff base) was 50 µM at 50 µL.

2.4.4 Immobilized Ion Metal Affinity Chromatography Enrichment. Two 50 µL samples of 100 µM CysM were thawed and buffer exchanged into 100 mM HEPES pH 7.8. A 1 M $NaBH_4$ solution (in 0.24% NaOH) was made and 10 µL were added to the reaction samples while 10 µL of 0.24% NaOH were added to the control solution (without $NaBH_4$ reduction of the Schiff base). The reaction proceeded undisturbed, in the dark, for 5 minutes. The samples were then buffer exchanged into 50 mM ammonium bicarbonate (ABC) pH 7.8. The resulting concentrations of the control (without $NaBH_4$ reduction of the Schiff base) and reaction (with $NaBH_4$ reduction of the Schiff base)were 4 µM with volumes of 60 µL. The samples were thermally denatured by incubating at 90°C for 20 minutes before being quenched with ice water for 5 minutes. The thermally denatured protein was then enzymatically digested with sequencing grade trypsin for 5 hours at 37°C. The concentration of trypsin was maintained at 40:1 (weight of substrate: weight of trypsin). The trypsin digested proteins were then stored in the freezer until being enriched with Fe-IMAC.

The procedure for making the Fe-IMAC column was obtained from Phillip Lang (Dr. Russel's research group). A 200 µL pipette tip was obtained and glass wool was

placed at the bottom for packing. Then, 50 µL of NTA-agarose resin was added and a pipette was used to push through the solution and pack the beads. Next, the beads were washed by adding 50 µL of 0.1 M acetic acid three times and then a pipette was used to push through the solution after letting it flow through the column by gravity about 1 hour. The beads were then charged with $FeCl_3$ (60 mM) in 0.1 M acetic acid by adding 50 µL of 60 mM $FeCl_3$ followed by another 50 µL of 60 mM $FeCl_3$ in 0.1 M acetic acid. The column was kept at room temperature for 30 minutes. A pipette was used to push through the $FeCl_3$ eluent before a third aliquot of 50 µL $FeCl_3$ was added to the column and a pipette was used to push through the solution. Next, the column was washed with 50 µL dH_2O three times and then the column was washed with 0.1 M acetic acid an additional three times.

The peptides were added to the column through the following procedure. Approximately 10 µL of peptides were mixed with 50 µL of 0.1 M acetic acid before the entire 60 µL solution was added to the column. A pipette was used to push the solution through slightly before the tip was capped and incubated at 37˚C for 30 minutes. The following procedure was then used to collect the peptides from the column. First, the column was washed with 50 µL of dH_2O three times while the flow-through was collected. Then 50 µL of acetic acid (0.1 M) was used to wash the column three times while the flow-through was collected. The column was then washed with 50 µL of dH_2O while the flow-through was collected. Next, the peptides were eluted with 50 µL 0.1 M Ammonium bicarbonate, 20% Acetonitrile pH = 9 three times while collecting the flow-through. The peptides were then placed in the freezer for storage and the column

was covered with parafilm for storage. The resulting peptide mixtures were analyzed using MALDI with the help of Phillip Lang from Dr. Russell's lab.

2.4.5 Trypsin Digest of CysM and Analysis. Two 50 µL samples of 100 µM CysM were thawed and buffer exchanged into 100 mM HEPES pH 7.8. The resulting concentrations of the control (without $NaBH_4$ reduction of the Schiff base) and reaction (with $NaBH_4$ reduction of the Schiff base) were and 3.1 mg/ml (91 µM) and 2.9 mg/ml (83 µM) respectfully. A 1 M $NaBH_4$ solution (in 100 mM HEPES pH 7.8) was made and 10 µL were added to the reaction sample while 10 µL of 100 mM HEPES pH 7.8 were added to the control solution (without $NaBH_4$ reduction of the Schiff base). The reaction proceeded undisturbed, in the dark, for 1 hour. The samples were then buffer exchanged into 25 mM ammonium bicarbonate (ABC) with the following concentrations: control – 1.8 mg/ml (53 µM); reaction – 1.8 mg/ml (53 µM). Finally, the samples were enzymatically digested with trypsin in a 40:1 (wt of substrate: wt of trypsin) ratio overnight (about 16 hours) at 37°C.

HPLC was then used to analyze the trypsin digested samples. The method consisted of a gradient from 100% dH_2O + 0.1% formic acid to 100% acetonitrile + 0.1% formic acid. The entire method can be seen in Table 2-1. The total stop-time was 65 minutes and the total post-time was 7 minutes resulting in a 72 minute run. A total of four samples (35 µL each) were run including the 25 mM ammonium bicarbonate control (with only ammonium bicarbonate), the trypsin control (with only trypsin), the control (without $NaBH_4$ reduction of the Schiff base) and the full reaction (with $NaBH_4$

reduction of the Schiff base). The wavelengths being monitored were 210 nm, 214 nm, 254 nm, 280 nm, and 325 nm.

2.4.6 LC-MS Analysis of the Trypsin Digest of CysM. The samples resulting from the previous trypsin digest without were analyzed with the LC-MS. The modified method is shown in Table 2-2. The post-time was 47 minutes and the total runtime was 54 minutes. The following samples were analyzed: buffer control (with only ammonium bicarbonate buffer), trypsin control (with only trypsin), CysM control (without $NaBH_4$ reduction of the Schiff base) and full reaction (with $NaBH_4$ reduction of the Schiff base).

2.4.7 Purified CysM – Fluorescence Tests. A 100 µL aliquot of CysM was thawed and buffer exchanged into 100 mM HEPES pH 7.8. The resulting concentration was 2.9 mg/ml (85 µM). This aliquot was split into two CysM aliquots (50 µL each). A 1 M $NaBH_4$ solution (in 100 mM HEPES pH 7.8) was made and 10 µL were added to the reaction samples while 10 µL of 100 mM HEPES pH 7.8 were added to the control samples (without $NaBH_4$ reduction of the Schiff base). The reaction proceeded undisturbed, in the dark, for 1 hour. The samples were then buffer exchanged into 25 mM ammonium bicarbonate (ABC) with the following concentrations: control – 1.7 mg/ml (50 µM), reaction – 1.5 mg/ml (44 µM).

The samples were then denatured by adding 10 µL of 40 mM DTT in 100 mM ABC. The samples were reacted for 45 minutes at 50°C before being cooled to room temperature. To acetylate, 10 µL of 220 mM iodoacetamide (made fresh while cooling in the previous step) were added to each sample. The samples were reacted for 30 minutes at room temperature. To quench, 50 µL of 40 mM DTT were added to each

Table 2-1. The HPLC method used for the trypsin digest sample analysis. This table shows the gradient used from 100% 0.1% formic acid in water up to 100% 0.1% formic acid in acetontrile that was used during HPLC analysis.

Time (minutes)	%B (0.1% Formic Acid/Water)	%D (0.1% Formic Acid/ACN)
0	100	0
10	98	2
20	95	5
60	0	100
62	0	100
63	100	0
65	100	0

Table 2-2. The LC-MS method used for the trypsin digest samples. This table shows the gradient used from 100% 0.1% formic acid in water up to 100% 0.1% formic acid in acetontrile that was used during LC-MS analysis.

Time (minutes)	%B (0.1% Formic Acid/Water)	%D (0.1% Formic Acid/ACN)
0	100	0
10	98	2
20	95	5
40	47.5	52.5
42	0	100
47	0	100
49	100	0
54	100	0

sample. Finally, the samples were enzymatically digested by adding trypsin in a 40:1 (wt of substrate: wt of trypsin) ratio. The samples were then reacted overnight (about 16 hours) at 37°C. The samples were then quenched with 10 µL of 1% formic acid. These samples were then analyzed using the HPLC with the same method as shown in Table 2-2. The specific wavelengths analyzed included 214 nm, 254 nm, 325 nm as well as fluorescence.

2.4.8 Modified IMAC Enrichment. A 100 µL sample of 100 µM CysM was thawed and buffer exchanged into 100 mM HEPES pH 7.8. The resulting concentration was 3.5 mg/ml (100 µM). This sample was then split into two even aliquots (35 µL each). A 1 M $NaBH_4$ solution (in 100 mM HEPES pH 7.8) was made and 10 µL were added to the reaction sample while 10 µL of 100 mM HEPES pH 7.8 were added to the control sample (without $NaBH_4$ reduction of the Schiff base). The reaction proceeded undisturbed, in the dark, for 1 hour. The samples were then buffer exchanged into 25 mM ammonium bicarbonate (ABC) with the following concentrations: control – 1.7 mg/ml (50 µM); reaction – 1.5 mg/ml (50 µM). Finally, the samples were enzymatically digested by adding trypsin in a 40:1 (wt of substrate: wt of trypsin) ratio. The samples were then reacted overnight (about 16 hours) at 37°C.

Three IMAC columns were prepared using three 200 µL pipette tips with glass wool placed at the tip for packing. Next, 50 µL of NTA-agarose resin was added to each tip. A pipette was used to push through the solution and pack the beads. The beads were then washed by adding 50 µL of 0.1 M Acetic acid five times, using a pipette to push through each aliquot after letting flow by gravity for 15 minutes each. The beads

were then charged with $FeCl_3$ (60 mM) in 0.1 M acetic acid by the addition of 50 µL of 60 mM $FeCl_3$. Another 50 µL of 60 mM $FeCl_3$ in 0.1 M acetic acid was added and reacted at room temperature for 30 minutes. A pipette was used to push through the $FeCl_3$ eluent. A third aliquot of 50 µL $FeCl_3$ was added to column and pushed through with a pipette. The column was then washed with 50 µL dH_2O three times. Finally, the column was washed three times with 50 µL of 0.1 M acetic acid.

To quench the trypsin, 10 µL of 1% formic acid was added to each sample. To prepare the peptide solution, an additional 10 µL of 1% formic acid was added to each sample which decreased the pH to 3-4. The peptides were then added to the column in 50 µL increments. For each increment, a pipette was used to slightly push the solution through the column before the column was capped and incubated at 37°C for 30 minutes. The peptides were added in this manner (in 50 µL increments) until all the peptide solution has been passed over the column. The resulting flow-through was then added to the column one more time before the column was capped and incubated at 37°C for 30 minutes.

To collect the peptides, the column was first washed with 50 µL of dH_2O three times while the flow-through was collected. The column was then washed with 50 µL of acetic acid (0.1 M) three times while the flow-through was collected. The column was then washed one more time with 50 µL of dH_2O while the flow-through was collected. The column was then washed with 50 µL 0.1 M ammonium bicarbonate, 20% acetonitrile pH = 9 three times to potentially elute the PLP-peptides while the flow-through was collected. The column was then washed twice with 0.1 M phosphoric acid

while the flow-through was collected. Finally, the column was washed once with 25 mM Ammonium bicarbonate pH 7.8 while the flow-through was collected. The fractions were then analyzed using the LC-MS with the same RP C18 column and method as used previously (Table 2-2).

3. SUMMARY AND CONCLUSIONS

3.1 Summary

CysM was a suitable model for PLP-peptide proteomics and method development. The CysM was successfully purified from *E. coli* BL21 DE3 using nickel affinity chromatography. The reduction of the Schiff base with $NaBH_4$ was characterized using both UV-visible spectroscopy as well as mass spectrometry. When analyzing with UV-visible spectroscopy, a new peak formed as a "shoulder" at approximately 320 nm and the absorbance at 420 nm decreased when the Schiff base was reduced. When analyzing with mass spectrometry an increase of 231.0297 Da was expected, and seen, due to the covalent bond formed after the reduction.

For the enrichment of PLP-proteins, an initial attempt at immobilized ion metal affinity chromatography did not result in any peptides within the ammonium bicarbonate washings, where any bound phosphoproteins were expected to elute. Based upon the fragmentation from trypsin digestion, the following peptides were expected: 1) LEDRNPTGSIK – 1229.6484 Da; w/ PLP bound – 1460.6781 Da; 2) NPTGSIK – 716.3937 Da; w/ PLP bound – 947.4234 Da; 3) NPTGSIKDRPAVR – 1410.7811 Da; w/ PLP bound – 1641.8108 Da. These results indicated that either the Fe-IMAC procedure would need to be modified for PLP-peptides or another enrichment method would need to be used to enrich and/or visualize the PLP-peptides.

CysM was then analyzed using LC-MS after trypsin digestion. The method consisted of a gradient from 100% dH_2O + 0.1% formic acid to 100% acetonitrile + 0.1% formic acid. The wavelengths monitored included 214 nm, 254 nm, 280 nm and

325 nm. At least one peak, corresponding to a PLP-peptide, was expected when monitoring the full reaction at 325 nm. This particular peak was not expected to be apparent in the control (without $NaBH_4$ reduction). It was expected and found that monitoring at 325 nm would select specifically for the PLP-peptide(s). The results showed that there were two peaks in the full reaction that were not apparent in the control (without $NaBH_4$ reduction of the Schiff base) with retention times of approximately 27.1 and 27.8 minutes. Analyzing the mass spectrum at these retention times resulted in M+H values of 1641.8108 Da and 2159.1015 Da. These two M+H values corresponded to the following expected peptides with PLP-bound to the active site lysine (Lys51): 1) NPTGSIKDRPAVR; 2) LEDRNPTGSIKDRPAVR.

The enzymatically digested CysM was also analyzed by monitoring the fluorescence of the PLP-peptide. The best fluorescence wavelengths to monitor included an excitation of 290 nm and an emission of 395 nm. Although peaks corresponding to the PLP-peptides were apparent, this method for monitoring PLP-peptides was not as sensitive as monitoring by absorbance at 325 nm. There were many other peaks that were apparent at various retention times.

Due to the complexity of a cell lysate, the enrichment method Fe-IMAC was attempted once again with a modified procedure. The LC-MS results from the analysis of the full reaction versus the control (without $NaBH_4$ reduction of the Schiff base) showed two peaks, eluting at 27.1 and 27.8 minutes, in the full reaction that are not apparent in the control. The LC-MS results from the analysis of the full reaction versus the flow-through, after the addition of the full reaction on the IMAC column, showed

that the two peaks at 27.1 and 27.8 minutes were no longer apparent. This indicated that the PLP-peptides were bound to the Fe-IMAC column. The final column washing with ammonium bicarbonate pH 7.8 contained two peptides that absorbed at 325 nm with retention times of 27.1 and 27.8 minutes. The LC-MS results of the CysM full reaction before IMAC treatment versus the final column washing with ammonium bicarbonate pH 7.8, when monitoring the mass spectrum, showed that the treatment using the IMAC column separated the PLP-peptides from all other peptides within the sample. Overall, the Fe-IMAC column specifically enriched the PLP-peptides and may be a useful method for studying the proteomics of PLP-dependent enzymes.

3.2 Conclusions

In conclusion, a method was developed to analyze and identify PLP-peptides when using CysM as a model system. The Schiff base was reduced with $NaBH_4$ which was monitored by either UV-vis spectroscopy, through an increase at 320 nm and a decrease in absorbance at 420 nm or mass spectrometry, through the increase in mass of 231.0297 Da. Trypsin was used to enzymatically digest the reduced CysM before being it was analyzed using LC-MS. The PLP-peptide(s) was located in the peptide mixture by monitoring the absorbance at 325 nm. The mass spectrum was then be used to confirm the PLP-peptide(s). The PLP-peptides were also specifically enriched by using Fe-IMAC columns and eluted with an ammonium bicarbonate buffer pH 7.8. This enrichment method may provide an opportunity to identify and analyze PLP-peptides in complex peptide mixtures.

Following this method development, PLP-dependent enzymes in a cell lysate could be analyzed in a fairly quick and efficient manner. The combination of genomic and biochemical information gained from this type of study could be used to address detailed questions about PLP-dependent enzymes. For example, which PLP-dependent enzymes are minimally required for an organism to survive in a strained or favorable environment? How do these enzymes differ in quantity and function between a basic prokaryote versus a more complex higher eukaryote? Can novel, unclassified, PLP-dependent enzymes be uncovered through the use of this genomic and biochemical approach? Answering these questions could lead to substantial biochemical advancements which may further our understanding of complex biological processes.

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