CHAPTER II

Development and Evaluation of a Bacterial Model System for N-Terminal Protein Labeling with N-Myristoyl Transferase

ABSTRACT

This chapter describes the design, development, and evaluation of a model system for NMT-mediated protein labeling. First, we assembled the necessary components; their selection and design are described in the Introduction. Green fluorescent protein (GFP) was chosen to be the test protein, owing to its widespread use and its facile detection by fluorescence measurements as well as conventional protein characterization methods.

We prepared two different engineered GFP substrates, each displaying a different NMT recognition sequence, and achieved selective modification of each protein via co-expression with NMT in the presence of an azido fatty acid. The labeled GFP substrates were purified, digested with a protease, and analyzed by mass spectrometry; identification of the N-terminal peptide fragment confirmed that site-specific labeling had occurred. Whole-protein mass spectrometry was also performed to determine the extent of modification. Finally, lysate samples containing a labeled GFP substrate were treated with a reactive dye and examined by SDS-PAGE. Fluorescence detection indicated that NMT labeled only the engineered GFP substrate *in vivo*. Together, these results demonstrate that our NMT-mediated protein labeling system is site-specific, quantitative, and highly selective. These results also constitute the first example of NMT-mediated labeling of a non-natural substrate protein with a functionalized, non-natural fatty acid, paving the way for future projects with other proteins of interest and other fatty acids analogs.

INTRODUCTION

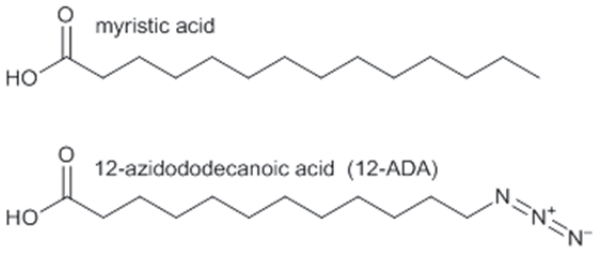
The three components comprising our NMT-mediated protein labeling model system are a reactive fatty acid, NMT, and a substrate protein. The design and development of each component are described below.

Selection of 12-Azidododecanoic Acid (12-ADA)

The natural fatty acid substrate of NMT, myristic acid (Chart II-1), is chemically inert. Fortunately, as described in Chapter I, dozens of reactive fatty acid analogs have been shown to be well-tolerated by NMT in an *in vitro* system.[1–3] Ideally, the analog employed in our protein labeling system would be readily synthesized and purified, permeable to the bacterial cell wall and membrane, bound and transferred by NMT in high yields, and able to participate in bioorthogonal chemistries. The compound 12-azidododecanoic acid (abbreviated 12-ADA henceforth; Chart II-1) was investigated with these criteria in mind.

Previous work indicated that 12-ADA was transferred to octapeptide substrates of NMT in higher yields than 9-, 11-, or 13-carbon analogs.[2] More recent work in mammalian cells yielded similar results, with 12-ADA resulting in more robust protein labeling than closely related azido fatty acids.[4] As described below, we synthesized and purified 12-ADA in high yields from adapted literature protocols. We also attempted the synthesis of an alkynyl compound, 13-tetradecynoic acid, but the reaction yields were low and the product was difficult to purify. Thus, for further work, we focused on the use of 12-ADA.

Chart II-1. Structures of myristic acid, the natural fatty acid substrate of NMT, and 12-azidodecanoic acid (12-ADA), the fatty acid analog prepared for and utilized in our studies.



The azide group can participate in three different bioorthogonal reactions: the Staudinger ligation, the copper-catalyzed azide-alkyne cycloaddition (CuAAC), and the strain-promoted azide-alkyne cycloaddition (SPAAC).[5–10] All three of these reactions may be performed in the challenging environment of cell lysate with exquisite selectivity. The Staudinger ligation and SPAAC can also be conducted in live cells and organisms. By selecting 12-ADA for our protein labeling system, we were well-positioned to explore these chemistries with N-terminally functionalized proteins.

Two-Plasmid Bacterial Co-expression System

Co-expression of NMT and a substrate protein, whether natural or engineered, requires the presence of plasmid(s) harboring genes that encode each one. In order to make our system modular, we decided to utilize two separate plasmids—one encoding NMT and the other encoding a substrate protein—rather than one plasmid encoding both. Changes to the various features of each plasmid (i.e., antibiotic resistance, origin of replication, promoter regions) could be readily made, if desired, to independently control the expression of NMT and the substrate protein. Moreover, we postulated that

replacement of our test protein, GFP, with other substrate proteins for future studies would be more straightforward if NMT were encoded on a separate plasmid.

Two GFP-based substrates, yARF-GFP and Fyn-GFP, were prepared, each displaying an NMT recognition sequence derived from a known substrate protein. The yARF (yeast ADP-ribosylation factor) protein is a GTP-binding protein that helps to control trafficking within the cell.[11] We engineered yARF-GFP to carry the first seven residues of yARF: MGLFASK.[12] The Fyn protein is a member of the Src kinase family,[13] and its first nine residues, MGCVQCKTK, are displayed by Fyn-GFP. A C-terminal 6xHis tag was also added to each protein to aid in affinity purification and detection procedures. The cloning scheme developed to construct the substrate plasmids is described in the Experimental Section of this chapter. The NMT plasmids employed in our system encode human NMT1 or NMT2, as well as methionine aminopeptidase (Met-AP). The genes encoding NMT as well as both substrate proteins were placed under the control of IPTG (isopropyl β-D-1-thiogalactopyranoside)-inducible promoters.

The co-expression protocol that we developed is shown schematically in Figure II-1. Cells were transformed with the NMT and substrate plasmids, and overnight cultures were inoculated from the resultant cell stocks. Expression cultures were then grown until cells reached an appropriate cell density, at which point 12-ADA was added and protein expression was induced. After 3-4 hours of protein expression, cells were harvested. As described in Chapter I, various applications could then be explored, utilizing intact cells, clarified lysates, or purified protein.

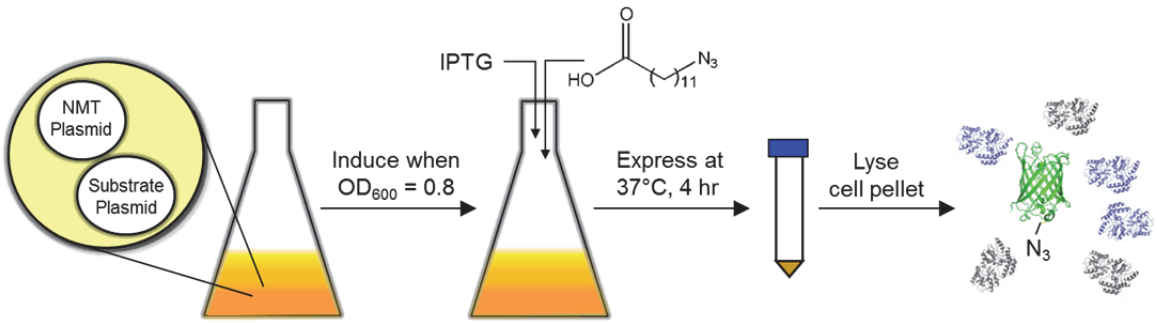


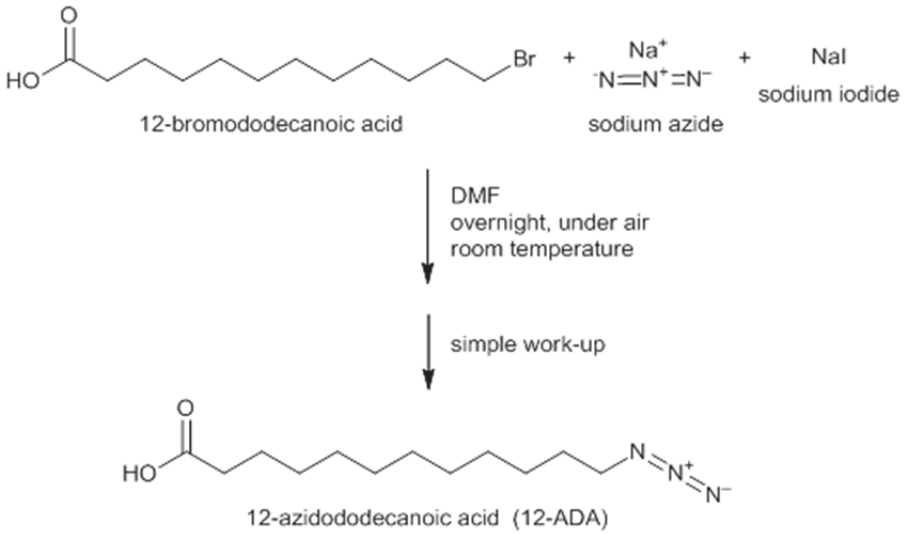
Figure II-1. Experimental overview of our bacterial co-expression system for N-terminal protein labeling with NMT.

RESULTS AND DISCUSSION

Synthesis and Characterization of 12-ADA

The compound 12-ADA was synthesized and purified as shown in Scheme II-1. The protocol we developed was adapted from literature precedent,[2] and the product identity was confirmed by comparison with published IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS data.[2,4] After optimizing the reaction, work-up, and purification steps, near-quantitative yields of 12-ADA were routinely obtained. Though literature protocols described a flash chromatography step for purification, we observed no difference in purity after a work-up only.

Scheme II-1. The azide fatty acid utilized in our studies, 12-azidododecanoic acid (12-ADA), was prepared from simple precursors.



Expression and Purification of GFP-Based Substrate Proteins

The plasmids pQE80_yARF-GFP and pQE80_Fyn-GFP were constructed using standard cloning procedures, as described in the Experimental Section of this chapter. The final plasmids were transformed into *E. coli* BL21(DE3) competent cells that had already been transformed with plasmids encoding human NMT1 or human NMT2, yielding four cell strains: BL21(DE3)/yARF-GFP/hNMT1, BL21(DE3)/yARF-GFP/hNMT2, BL21(DE3)/Fyn-GFP/hNMT1, and BL21(DE3)/Fyn-GFP/hNMT2. We moved forward with two of these cell strains: BL21(DE3)/yARF-GFP/hNMT1 and BL21(DE3)/Fyn-GFP/hNMT2. These two strains were selected due to literature reports regarding the substrate selectivity of each human NMT isoform: yARF has been shown to be myristoylated to a greater extent by hNMT1 than hNMT2,[12] while *in vitro* studies

demonstrated the preference of hNMT2 for a Src peptide substrate.[14] As noted earlier, Fyn is a member of the Src kinase family.

Co-expression of yARF-GFP or Fyn-GFP with NMT was completed as depicted in the schematic overview above (Figure II-1). Briefly, overnight cultures were diluted 1:50 into expression cultures containing the appropriate antibiotics to ensure retention of both plasmids. Protein expression was induced at a cell density of $OD_{600} \approx 0.8$ with the addition of IPTG, and at the same time, 12-ADA was added. After 4 hours of expression at 37°C, cells were harvested from each culture and lysed. Each protein, yARF-GFP or Fyn-GFP, was purified from lysate via denaturing or native Nickel-NTA chromatography. Expression and purification protocols are presented in more detail in the Experimental Section at the end of this chapter. Purification fractions were analyzed by SDS-PAGE, which confirmed the presence of each protein at the expected molecular weight of 32 kDa (Figure II-2).

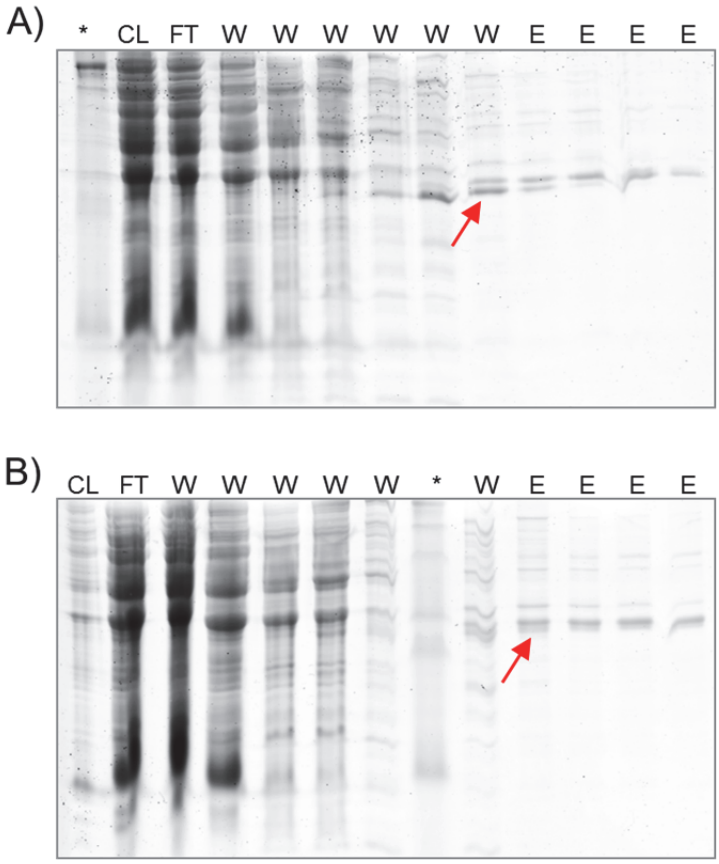


Figure II-2. SDS-PAGE analysis of native Ni-NTA purification fractions. Red arrows indicate the presence of pure yARF-GFP (A) and Fyn-GFP (B) isolated from co-expression cultures grown in the presence of 12-ADA. CL = Clarified Lysate; FT = Flow Through; W = Wash; E = Elution; * = MW marker.

MALDI-MS Analysis of Trypsinized Protein Samples

After expressing and purifying yARF-GFP and Fyn-GFP, we verified that both proteins had been labeled with 12-ADA and determined the extent of their modification. First, to confirm that labeling had occurred only at the protein N-terminus, we employed matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Purified samples of yARF-GFP and Fyn-GFP were digested with the trypsin protease, which cleaves peptides after arginine (Arg, R) and lysine (Lys, K) residues,

yielding a predictable set of fragments. We were particularly interested in detecting the N-terminal fragment of each protein. The expected masses for the N-terminal peptide of each protein—unlabeled, myristoylated, or labeled with 12-ADA—are presented in Table II-1.

Table II-1. Expected masses of the unlabeled (Mass 1), myristoylated (Mass 2), and 12-ADA-labeled (Mass 3) N-terminal peptide fragments of yARF-GFP and Fyn-GFP. Masses account for removal of the initial Met residue, trypsin digestion, reduction, and alkylation. Formation of a covalent bond between Gly and myristic acid or 12-ADA results in the loss of one water molecule. All values are in Daltons (Da).

Protein (N-terminal peptide)	Mass 1	Mass 2 (+Myr)	Mass 3 (+12-ADA)
yARF-GFP (GLFASK)	622.20	832.56	845.52
Fyn-GFP (GCVQCK)	751.22	961.58	974.54

After trypsinization, the resultant peptide fragments for each protein sample were prepared for MALDI-MS analysis using standard clean-up columns and matrix reagents. The MALDI mass spectrum for each protein is shown in Figure II-3.

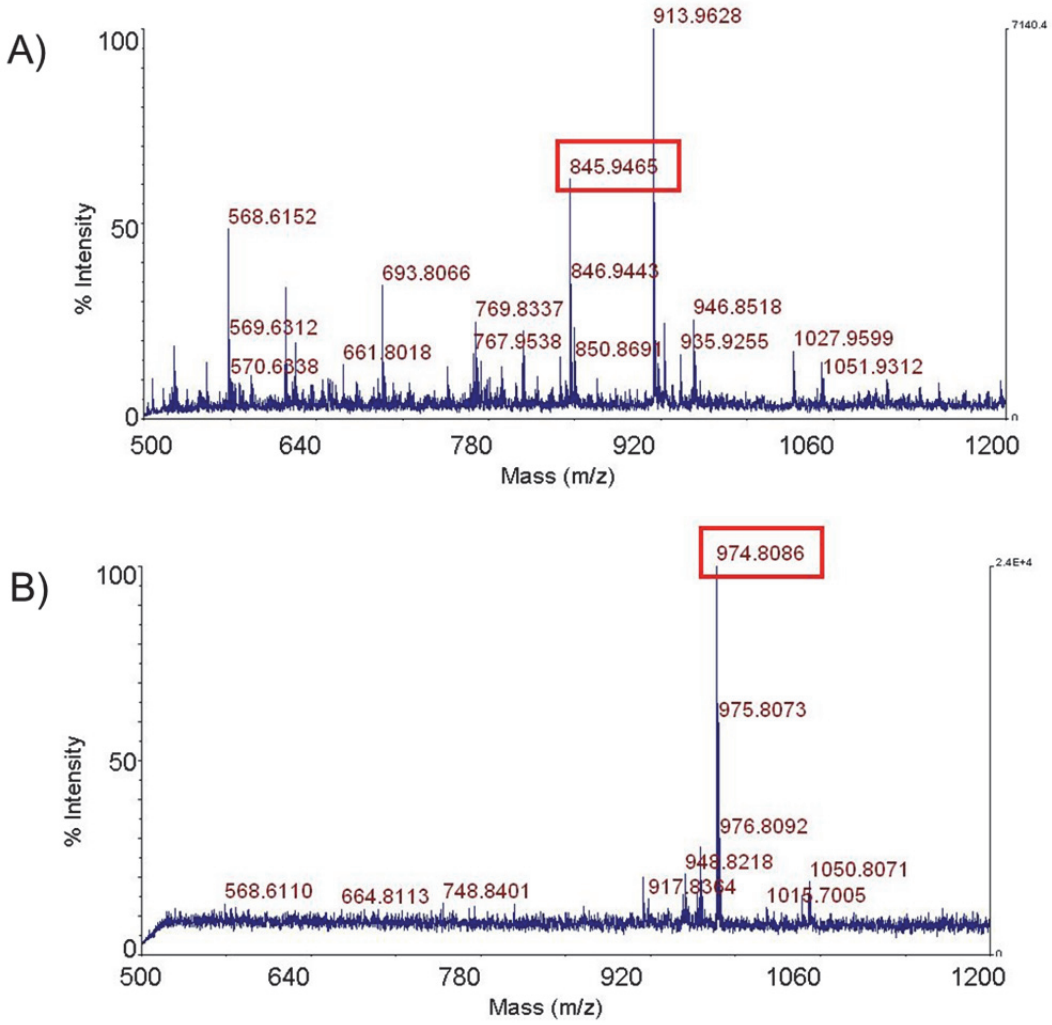


Figure II-3. MALDI mass spectra for yARF-GFP (A) and Fyn-GFP (B) indicate successful N-terminal labeling with 12-ADA. Samples were co-expressed with NMT in the presence of 12-ADA, purified, and trypsinized. The Fyn-GFP sample was also subjected to reduction and alkylation prior to trypsin digestion. No unlabeled or myristoylated N-terminal peptide fragments were observed (see Table II-1).

In analyzing each spectrum, we first looked for peaks corresponding to various expected fragments to ensure that the digestion was effective. We also searched for each N-terminal peptide. As shown in Figure II-3A, the 12-ADA-labeled N-terminal fragment for yARF-GFP was readily identified. For Fyn-GFP, initially, we found that the

12-ADA-labeled N-terminal fragment was consistently 2 Da lower than expected (data not shown), perhaps due to disulfide bonding of the two cysteine residues found in that fragment. Thus, we also examined Fyn-GFP samples that were reduced and alkylated following trypsinization. After such treatment, the 2 Da discrepancy was no longer observed (Figure II-3B). Notably, the myristoylated N-terminal fragment was not detected for either protein, indicating that competition between myristic acid and 12-ADA is not a problem in our system. In addition, negative control samples that were not exposed to 12-ADA during expression yielded spectra that lacked the peaks outlined in red in Figure II-3 (the "Mass 3" values in Table II-1), further confirming that those peaks correspond to the 12-ADA-labeled N-terminal fragments.

Intact LC-MS Analysis of Protein Samples

While MALDI-MS analysis of trypsinized yARF-GFP and Fyn-GFP samples showed that both proteins had been labeled with 12-ADA, they did not indicate what percent of the protein pool was tagged. For both proteins, we were unable to detect the unlabeled N-terminal peptide fragment (Table II-1, "Mass 1"), even for negative control samples that were not exposed to 12-ADA, likely due to the fragments' polarity, charge, and small size. The inability to detect these fragments by MALDI-MS complicated efforts to measure the extent of 12-ADA labeling of yARF-GFP and Fyn-GFP. Fortunately, intact LC-MS of purified samples proved to be a more effective approach. The expected masses of the relevant protein species, as well as those actually observed in whole-protein mass spectra of yARF-GFP and Fyn-GFP, are presented in Table II-2.

Table II-2. Expected masses of yARF-GFP and Fyn-GFP (unlabeled, myristoylated, or labeled with 12-ADA), and masses observed by intact LC-MS. Expected mass values account for removal of initial Met and loss of 20 Da upon formation of GFP chromophore. Analysis revealed quantitative labeling of both proteins with 12-ADA by NMT. All mass values are in Da. N/D = Not Detected.

Protein	Expected Mass	Observed Mass	% 12-ADA-Labeled
yARF-GFP	31,817.85	N/D	> 98 %
+Myr	32,028.20	N/D	
+12-ADA	32,041.16	32,041.31	
Fyn-GFP	32,062.17	N/D	> 98 %
+Myr	32,272.52	N/D	
+12-ADA	32,285.48	32,280.02	

For both proteins, we routinely observed a large peak corresponding to the 12-ADA labeled species, very little evidence of unlabeled protein (<5%), and no evidence of myristoylated protein. Taken together, the two sets of MS data demonstrate that yARF-GFP and Fyn-GFP are both labeled solely and quantitatively at the N-terminus with 12-ADA by NMT, with no competition presented by myristic acid.

As a step toward optimizing our co-expression system, we investigated the use of both minimal medium and rich media for our expression cultures. Certain protocols developed in the Tirrell Lab for residue-specific incorporation of ncAAs recommend the use of minimal medium in order to boost ncAA incorporation, while avoiding the use of rich media that contain a given natural amino acid. We hypothesized that myristic acid might similarly be present in rich media, which could negatively impact the extent of protein labeling with 12-ADA. Thus, we originally used M9 minimal medium for protein expression, but later tested the use of rich media (LB and 2xYT). Identical LC-MS

results were obtained for Fyn-GFP regardless of the expression medium, with no myristoylated protein species observed in any of the samples (Figure II-4).

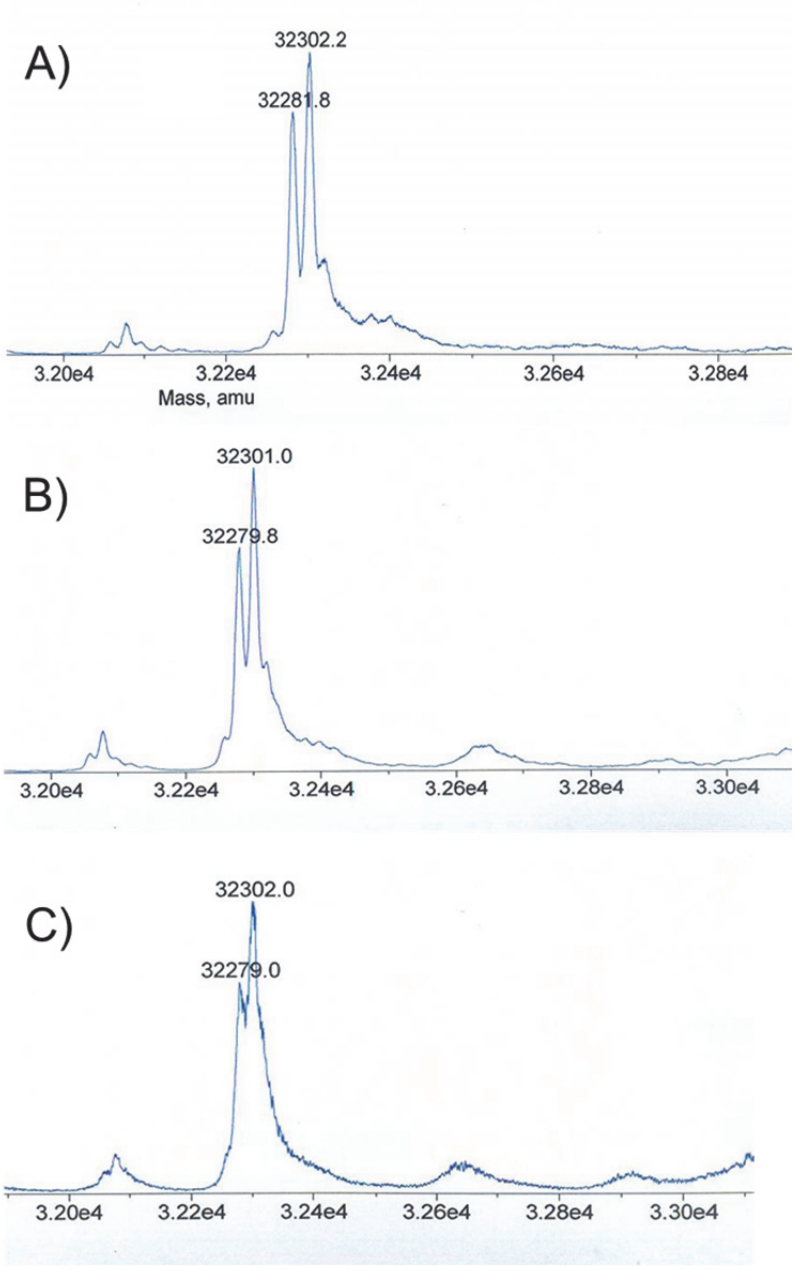


Figure II-4. Intact LC-MS data for Fyn-GFP samples purified from expression cultures grown in M9 (A), LB (B), or 2xYT (C) medium. The compound 12-ADA was added to all three cultures. No myristoylated Fyn-GFP was observed in any sample (see Table II-2).

These results are notable because the use of rich media for protein expression instead of a minimal medium often results in higher protein yields. Moreover, as with the data presented in Table II-2, very little unlabeled protein was detected.

Fluorescence Gel Analysis of Lysate Samples

As mentioned at the beginning of this chapter, one notable feature of NMT is its absence from prokaryotic systems. We were intrigued by the possibility of developing a chemoenzymatic labeling system that is not only site-specific and high-yielding, but also orthogonal to the widely used *E. coli* bacterial expression system. To investigate whether or not the yARF-GFP/hNMT1 and Fyn-GFP/hNMT2 co-expression systems fulfill this additional criterion, we took advantage of existing bioorthogonal chemistries and reagents to detect the presence of 12-ADA-labeled proteins.

For these experiments, lysates were utilized to effectively measure NMT's selectivity toward our engineered substrate proteins. Lysate samples were treated with an alkyne-TAMRA probe in a CuAAC reaction, which is known to conjugate azides and alkynes even in complex biological settings.[15,16] Proteins were subsequently precipitated out of the reaction mixture and analyzed via SDS-PAGE (Figure II-5). For each protein, the same gel was imaged for TAMRA fluorescence, then stained with Coomassie colloidal blue and imaged again. As the gel images indicate, NMT transferred 12-ADA only to yARF-GFP and Fyn-GFP and did not label natural bacterial proteins. These data demonstrated the activity and selectivity of our NMT-mediated protein labeling system, even when using a non-natural fatty acid substrate in conjunction with non-natural substrate proteins.

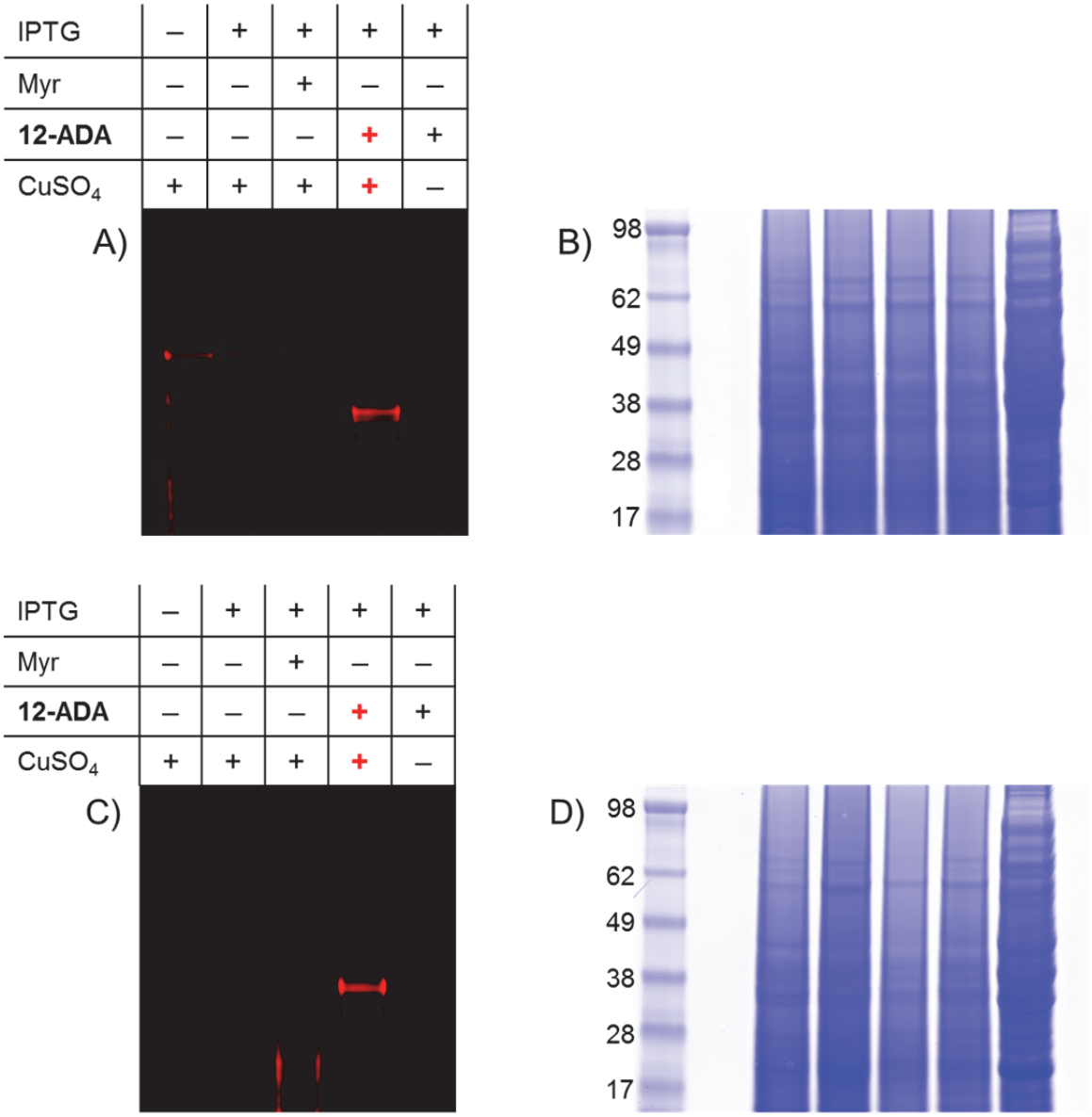


Figure II-5. SDS-PAGE analysis of lysate samples containing yARF-GFP (A, B) or Fyn-GFP (C, D) co-expressed with NMT in the presence of no fatty acid, myristic acid ("Myr"), or 12-ADA. Lysate samples were treated with alkyne-TAMRA for detection of azide-labeled protein (A, C). The same gels were stained with Coomassie colloidal blue (B, D). Comparison of each pair of gel images indicates selective 12-ADA labeling of yARF-GFP and Fyn-GFP.

CONCLUSION

In summary, we designed, constructed, and evaluated a site-specific protein labeling system centered on the eukaryotic enzyme N-myristoyl transferase (NMT). We developed two plasmids encoding GFP-based, non-natural substrates of NMT: yARF-GFP and Fyn-GFP. Both proteins display a recognition sequence derived from a known NMT substrate. We co-expressed each protein with an isoform of human NMT in the presence of the azido fatty acid, 12-ADA, which was readily synthesized. Purified protein samples were subjected to MALDI-MS analysis after trypsinization and were studied via intact LC-MS as well. The MS data sets showed that labeling by NMT was specific for the N-terminus, as expected, and essentially quantitative. Examination of lysate samples treated with an azide-reactive dye confirmed that NMT labels each engineered GFP substrate, but is inactive toward natural bacterial proteins. The site-specific, quantitative, and selective protein labeling system established here is the basis of the further research described in Chapters III, IV, and V.

EXPERIMENTAL SECTION

Materials

Synthesis of 12-ADA. 12-bromododecanoic acid and sodium iodide were purchased from Aldrich. Silica gel 60 was purchased from EMD Chemicals. Sodium azide and all solvents were purchased from VWR.

Cloning. All oligonucleotide primers were ordered from Operon. Polymerase chain reaction (PCR) experiments were carried out in a BioRad DNA Engine Peltier

Thermal Cycler using PfuTurbo DNA Polymerase (Stratagene/Agilent). All restriction enzymes, restriction enzyme buffers, and bovine serum albumin (BSA) were purchased from New England BioLabs (NEB). The pQE60 and pQE80 vectors from Qiagen were used for cloning. NEB DNA Ladders (100 bp and 1 kbp "Quick-Load") were used as markers for all DNA agarose gels, which were visualized with the addition of Plus One ethidium bromide solution from Amersham Biosciences on a UVP UV Transilluminator. Zymo Agarose-Dissolving Buffer (ADB) and Zymo Spin II columns, with their associated buffers, were used to purify DNA out of agarose gels. T4 DNA Ligase from NEB or Roche was used for ligations with equivalent results. All DNA acquisition from cells was completed using the Qiagen Spin Miniprep Kit and columns. All sequencing requests were fulfilled by Laragen.

Protein expression. Plasmids encoding hNMT1 or hNMT2 and methionine-aminopeptidase (Met-AP) were a gift from the laboratory of Professor Richard Kahn at Emory University (Atlanta, GA).[12] *E. coli* BL21(DE3) cells were made chemically competent using the standard Zymo method (Stratagene) and were transformed with either the hNMT1 plasmid or hNMT2 plasmid. M9 minimal medium was composed of M9 salts plus 0.4% dextrose, 100 μM $CaCl_2$, 35 μg/mL thiamine, 1 mM $MgSO_4$, and 4% 20 amino-acid solution (1 g/L each). LB medium was composed of 10 g tryptone (casein hydrolysate), 5 g yeast extract, and 10 g NaCl per liter. 2xYT medium was composed of 16 g tryptone (casein hydrolysate), 10 g yeast extract, and 5 g NaCl per liter. All media were autoclaved before use. Kanamycin (Kan) was used at a working concentration of 35 μg/mL, and ampicillin (Amp) was used at a working concentration of 200 μg/mL. Myristic acid was purchased from Fluka. All optical density (OD) values were measured at 600 nm on a Cary UV-Vis spectrophotometer. All SDS-PAGE gels described in this chapter were 12% acrylamide, Tris-Tricine gels cast in-house or NuPAGE Novex 4-12% Bis-Tris pre-cast gels (Invitrogen). SeeBlue Plus2 Pre-Stained Protein Marker from Invitrogen served as the molecular weight ladder. Gels were stained with Coomassie colloidal blue from Invitrogen.

Protein purification. Nickel-NTA resin manufactured by Qiagen was used for purification of 6xHis-tagged yARF-GFP and Fyn-GFP from lysate. For denaturing Ni-NTA purification, Buffers B, C, D, and E consisted of 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl with pH = 8.0, 6.3, 5.9, or 4.5, respectively. For native Ni-NTA purification, buffers contained 50 mM NaH_2PO_4, 300 mM NaCl, and varying concentrations of imidazole (25–500 mM). B-PER lysis buffer from Pierce was usually used for native Ni-NTA purification. Lysozyme was purchased from Aldrich.

Mass spectrometry. The Pierce BCA Assay Kit was used to measure protein concentration in pure protein fractions prior to MS analysis. Promega porcine trypsin was used in digests. Microcon Centrifugal Devices were used to concentrate and buffer-exchange whole-protein samples for intact LC-MS, while Microcon Centrifugal Devices and C_{18} Zip-Tips were both employed to concentrate and de-salt peptide samples for MALDI-MS (both from Millipore). MALDI-MS data were collected on an Applied Biosystems Voyager DE-PRO MALDI TOF-MS. Intact LC-MS data were collected on an Agilent 1100 MSD quadrupole ESI-MS.

Fluorescence detection. Lysate samples were treated with the reagents and according to the protocols of the Click-IT Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit from Invitrogen. After reaction and precipitation, protein samples were run on Invitrogen NuPAGE Novex 4%–12% Bis-Tris pre-cast gels and imaged on a GE Typhoon laser scanner. Gels were stained with Coomassie colloidal blue from Invitrogen.

Methods

Synthesis of 12-ADA. 12-Azidododecanoic acid (12-ADA) was synthesized according to literature precedent[2] with minor modifications; in particular, the flash chromatography step was found to be unnecessary to obtain pure product. Standard characterization techniques (ESI-MS, ^{1}H NMR, ^{13}C NMR) yielded data that matched published results.[2,4]

Cloning. The pQE80 plasmid possesses an ampicillin resistance gene and a Col E1 Origin of Replication. The GFP gene was PCR-amplified from the vector pQE9_GFP6_lacI_yPheRS_T415G, which was prepared by a former member of the Tirrell Lab, Inchan Kwon. Two rounds of PCR with novel primers yielded a fragment including the GFP gene carrying an EcoRI restriction enzyme site, a ribosome-binding site appropriate for pQE vectors, and base pairs encoding the yeast ARF recognition sequence (MGLFASK, from ATG GGT CTG TTC GCG TCT AAA) or the Fyn recognition sequence (MGCVQCKTK, from ATG GGT TGC GTG CAA TGC AAA ACC AAA) at the 5′ end, and a BglII site at the 3′ end. The PCR product and pQE60 were digested with EcoRI and BglII, and the GFP insert was ligated into pQE60 to allow for the addition of a C-terminal 6xHis tag; each GFP construct was also placed under the control of a T5 promoter, which is inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG). The presence of the insert in the pQE60 construct was confirmed via test digests and sequencing. Then, the entire cassette was digested out of pQE60 using AatII and NheI and ligated into pQE80, which had been digested with the same two enzymes. The final pQE80 plasmids were used for protein expression because pQE80 carries the $lacI^q$ repressor necessary for *cis* regulation of the T5 promoter. Each final construct, pQE80_yARF-GFP or pQE80_Fyn-GFP, was sequenced and transformed into competent cells already harboring an NMT plasmid for co-expression experiments.

Protein expression. Overnight cultures were inoculated in LB supplemented with Kan and Amp and grown in an incubator-shaker (37°C, 250 rpm). The following day, overnight cultures were diluted 1:50 into fresh M9, LB, or 2xYT supplemented with Kan and Amp for expression cultures, which ranged in volume from 5 mL to 250 mL. Cultures were grown in an incubator-shaker (37°C, 250 rpm), and protein expression was induced with IPTG (1 mM, from 1 M stock in water) when the OD_{600} value was between 0.8 and 1.1. Pre-induction samples (1 mL) were collected as needed. The azide fatty acid 12-ADA (500 μM, from 500 mM stock in DMSO) was also added at the time of induction. After 3–4 hr of protein expression, cells were harvested via centrifugation (10 min x 10,000 *g*) and the final OD_{600} value measured. Cell pellets were lysed according to the following formula, regardless of which lysis buffer was used: 50 μL

lysis buffer per mL culture per OD_{600} unit. Crude lysates were centrifuged once more, and the supernatant (clarified lysate, i.e., cytosolic fraction) was saved for further experiments.

Protein purification. Protocols were followed largely as described in *The QIAExpressionist* handbook from Qiagen. For denaturing Ni-NTA purification, cells were lysed in Buffer B. The clarified lysate was incubated with Ni-NTA agarose for 1–2 hr at 4°C with agitation and loaded on an empty column to collect fractions. The protein-agarose mixture was washed with Buffers B, C, and D, and the protein eluted with Buffer E (pH 4.5). Purification fractions were analyzed by SDS-PAGE.

For native Ni-NTA purification, cells were lysed in B-PER buffer or in the standard native Ni-NTA buffer (see "Materials") with 10 mM imidazole. Furthermore, for native Ni-NTA purification, lysozyme was added to the lysis buffer at 1 mg/mL, and the resuspended cell pellet was sonicated to aid in lysis. The clarified lysate was incubated with Ni-NTA agarose for 1–2 hr at 4°C with agitation and loaded on an empty column to collect fractions. The protein-agarose mixture was washed with buffer containing imidazole (25–100 mM) and the protein eluted at 150-200 mM imidazole. Purification fractions were analyzed by SDS-PAGE.

Mass spectrometry. For MALDI-MS experiments, solutions of pure protein were concentrated using Microcon columns (MWCO = 30 kDa). Fyn-GFP samples were reduced and alkylated according to a standard published protocol.[17] Protein samples were digested as follows: 90 μL of 75 mM NH_4CO_3 buffer and 1 μL porcine trypsin were added to 10 μL of a concentrated protein solution, and the mixtures were incubated at 37°C for 2–8 hr, after which they were quenched with 10 μL 10% TFA. C_{18} ZipTips were used to concentrate and de-salt the trypsin digest mixtures in preparation for MALDI-MS (α-cyanohydroxycinnamic acid matrix).

For intact LC-MS experiments, solutions of pure protein were concentrated using Microcon columns (MWCO = 30 kDa) and buffer-exchanged into a 0.1% TFA (trifluoroacetic acid) solution. A final solution of 100 pmol protein in 100 μL was run on the MSD instrument.

Fluorescence detection. Cells were lysed with the buffer recommended in the instructions for the Invitrogen Click-iT kit (1% SDS, 50 mM Tris-HCl, pH 8.0) according to the formula mentioned earlier (50 μL lysis buffer per mL culture per OD_{600} unit). Clarified lysate samples were treated with alkyne-TAMRA and other kit reagents according to the protocols supplied by Invitrogen; the only modification was the use of 15 μL of alkyne-TAMRA dye solution rather than 100 μL. At the conclusion of the 25-min reaction time, samples were precipitated following the methanol-chloroform precipitation protocol described in the same kit instructions; the only modification was the completion of one additional methanol wash of the protein pellet. For SDS-PAGE analysis, protein pellets were resuspended in a denaturing buffer (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl) and loaded on NuPAGE Novex 4-12% Bis-Tris pre-cast gels. To detect TAMRA signal on the Typhoon, the 532 nm laser served as the excitation source (filter set: 580 BP 30). Gels were stained with Coomassie colloidal blue, then imaged again, with the 633 nm laser now serving as the excitation source (no filter).

REFERENCES

1. Kishore, N. S. *et al.* The substrate specificity of Saccharomyces cerevisiae myristoyl-CoA: protein N-myristoyltransferase. *J. Biol. Chem.* **266**, 8835–55 (1991).

2. Devadas, B. *et al.* Substrate specificity of Saccharomyces cerevisiae myristoyl-CoA: protein N-myristoyltransferase. *J. Biol. Chem.* **267**, 7224–39 (1992).

3. Lu, T. *et al.* The substrate specificity of Saccharomyces cerevisiae myristoyl-CoA: protein N-myristoyltransferase. Polar probes of the enzyme's myristoyl-CoA recognition site. *J. Biol. Chem.* **269**, 5346–57 (1994).

4. Hang, H. C. *et al.* Chemical probes for the rapid detection of fatty-acylated proteins in mammalian cells. *J. Am. Chem. Soc.* **129**, 2744–5 (2007).

5. Saxon, E. & Bertozzi, C. R. Cell surface engineering by a modified Staudinger reaction. *Science* **287**, 2007–10 (2000).

6. Rostovtsev, V. V, Green, L. G., Fokin, V. V & Sharpless, K. B. A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed. Engl.* **41**, 2596–9 (2002).

7. Tornøe, C. W., Christensen, C. & Meldal, M. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **67**, 3057–64 (2002).

8. Agard, N. J., Prescher, J. A. & Bertozzi, C. R. A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* **126**, 15046–7 (2004).

9. Agard, N. J., Baskin, J. M., Prescher, J. A., Lo, A. & Bertozzi, C. R. A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol.* **1**, 644–8 (2006).

10. Sletten, E. M. & Bertozzi, C. R. From mechanism to mouse: a tale of two bioorthogonal reactions. *Acc. Chem. Res.* **44**, 666–76 (2011).

11. Pasqualato, S., Renault, L. & Cherfils, J. Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for "front-back" communication. *EMBO Rep.* **3**, 1035–41 (2002).

12. Van Valkenburgh, H. A. & Kahn, R. A. Coexpression of proteins with methionine aminopeptidase and/or N-myristoyltransferase in Escherichia coli to increase acylation and homogeneity of protein preparations. *Methods Enzymol.* **344**, 186–93 (2002).

13. Palacios, E. H. & Weiss, A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23**, 7990–8000 (2004).

14. Giang, D. K. & Cravatt, B. F. A second mammalian N-myristoyltransferase. *J. Biol. Chem.* **273**, 6595–8 (1998).

15. Wang, Q. *et al.* Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* **125**, 3192–3 (2003).

16. Sletten, E. M. & Bertozzi, C. R. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem. Int. Ed. Engl.* **48**, 6974–98 (2009).

17. Kinter, M. & Sherman, N. E. Chapter 6: The Preparation of Protein Digests for Mass Spectrometric Sequencing Experiments. *Protein Sequencing and Identification Using Tandem Mass Spectrometry* 147–165 (2000).