Steps toward peptide polymerization in polyacrylamide gels

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Introduction

In 2003, Trimeris and Roche reported a new treatment for the human immunodeficiency virus (HIV) labeled Enfuvirtide[1]. This drug was formulated to specifically inhibit HIV-1 entry into CD4 lymphocytes, which are known to be acted upon by HIV-1. Enfuvirtide is the 36 amino acid C-terminal sequence of gp41, a protein that forms an alpha-helical trimer that when associated with another protein, gp120, undergoes conformational changes that allows fusion of HIV-1 with the lymphocyte membrane.

Recently, a similar HIV fusion inhibitor was created with an overlapping C-terminal segment from Enfuvirtide. This peptide is 37 amino acids long so will be further referred to as the C37 peptide. This peptide has already been cloned into *E. coli* with a fusion protein (xanthine-guanine-phosphoribosyl transferase, GPRT) for effective purification, and there is a Tobacco Etch Virus NIa protease (TEV protease) cleavage site connecting the fusion protein to the C37 peptide. When cleaved with TEV protease, the C37 peptide will have an N-terminal cysteine that can be subjected to native chemical ligation[2]. Since our lab already had access to this strain of *E. coli*, the C37 peptide was a prime candidate for our studies on peptide polymerization.

Peptide polymerization can play a variety of roles. It may be possible to make a more potent inhibitor by locally concentrating inhibitor peptides by via polymerization. Protein gels have already been established as methods for measuring protein concentration and kinetics[3]. So as to reduce the amount of protein required to make a gel, acrylamide can be copolymerized with the protein. However, in order to polymerize the protein, it needs to be conjugated to a polymerizable species. A novel compound,

2-(4-vinylbenzoylthio)acetic acid (Figure 1b), also known as the benzoic acid vinyl thioester, was utilized as the conjugate species. The vinyl group is that by which polymerization will occur. The protein will be conjugated by native chemical ligation in which an N-terminal cysteine reacts with a carboxylic acid and rearranges to form an amide bond[4].

The goal of this work was to isolate the C37 peptide, ligate it to the benzoic acid vinyl thioester, and polymerize it into a gel. In order to isolate the C37 peptide, it had to be cleaved from its carrier protein via TEV protease, whose cleavage is efficient under reducing and dialyzing conditions[3]. The reducing conditions prevent TEV protease, a cysteine protease, from becoming deactivated via self-disulfide bond formation. Dialysis prevents aldehydes in solution (potentially from glycrerol oxidation) from reacting with N-terminal cysteines and preventing them from ligation in the next step. The C37 peptide was then ligated to the thioester, and the ligation mixture was purified by either high-performance liquid chromatography (HPLC) or Pepclean C-18 spin columns. Analysis of each step was performed by matrix-assisted laser desorption/ionization mass spectrometry via time-of-flight detection (MALDI-TOF MS).

Materials and Methods

Purification of TEV protease

To replenish laboratory stocks, active TEV protease was expressed and purified. TEV protease was obtained via bacterial expression and inclusion body purification. A colony from a plate of *E. coli* transformed with a plasmid containing the TEV protease gene with an N-terminal His_6 tag was inoculated into 5 mL LB liquid media containing 10 mM kanamycin (Kan). This culture was incubated overnight at 37 °C with shaking.

The culture was then transferred to 1 L LB liquid media + Kan and incubated at 37 °C with shaking for approximately 3 h. To induce expression of TEV protease, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to make its final concentration 0.1 mM. Expression proceeded for 4 h at 37 °C with shaking. The culture was then centrifuged at 5000 rpm (JA10 rotor) for 10 min at 4 °C. Supernatant was then removed, and the cell pellets were stored overnight in a -80 °C freezer.

The pellets were then resuspended in 20 mL buffer containing 50 mM potassium phosphate, pH 8.0, and 5 mM β-mercaptoethanol (β-ME). Cells were then chilled on ice for 10 min, and then lysed via sonication (30 s on/1 min off for 7 min total). The lysate was centrifuged at 12000g for 1 h at 4 °C (JA20 rotor). Supernatant was again discarded, and the pellet was resuspended in 20 mL of a denaturing buffer including 7 M guanidine hydrochloride (GuHCl), 5 mM β-ME, and 50 mM potassium phosphate, pH 7.5. The resuspended pellet was then stirred 1 h at room temperature (25 °C) followed by centrifugation (12000g, 40 min, 4 °C).

The supernatant was then loaded onto a pre-equilibrated 1.5 mL Ni^{2+}-NTA column (Qiagen, equilibrated with the denaturing buffer previously described). The column was then washed with 10 column volumes of a buffer containing 300 mM sodium chloride, 5% w/v glycerol, 10 mM imidazole, 6 M urea, 5 mM β-ME, and 50 mM potassium phosphate, pH 7.5. The His-tagged TEV protease was eluted from the Ni^{2+}-NTA column with 200 mM imidazole (in a buffer also including 6 M urea, 5 mM β-ME, and 50 mM potassium phosphate, pH 7.5) in ten 0.5 mL fractions. Samples of each fraction were run on a 15% polyacrylamide gel to determine which fractions to combine. Combined fractions were then diluted with a solution of 6 M urea, 5 mM β-ME, and 50

mM potassium phosphate, pH 7.5, to obtain a final volume of 20 mL. TEV protease was refolded via dialysis in 25 mM potassium phosphate buffer, pH 7.5, with 5 mM β-ME at 4 °C. After 2 h, the dialysis buffer was switched, and dialysis was allowed to proceed for an additional 36 h at 4 °C. Dialyzed TEV protease solution was centrifuged at 4000g for 10 min to pellet precipitated protease. Purified TEV protease was then stored for later use at -20 °C.

Polyacrylamide gel electrophoresis

For protein visualization, 15% polyacrylamide gels were used. The resolving gel was first polymerized for 30 min and contained 15% w/v polyacrylamide, 0.4% BIS-acrylamide, 0.05% w/v ammonium persulfate, 0.1% sodium dodecyl sulfate (SDS), 0.0005% v/v *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 375 mM Tris-HCl, pH 8.8. It was overlaid with N-butanol to create a uniform layer. The butanol was removed from the gel apparatus, and the resolving gel was rinsed with deionized water and dried prior to pouring the stacking gel. The stacking gel was then polymerized for 30 min and consisted of 4% w/v polyacrylamide, 0.11% BIS-acrylamide, 0.07% w/v ammonium persulfate, 0.1% SDS, 0.001% TEMED, and 125 mM Tris-HCl, pH 6.8. All samples were loaded 1:1 with loading dye containing bromophenol blue, 5 mM β-ME, and 0.1% SDS. Protein markers were premade from New England Biolabs (Prestained Protein Markers, Broad Range). Samples were heated at 95 °C for 5 min prior to loading on a gel. Once samples were loaded, the gels were run at 200 V for 45 min – 1 h in 1X SDS buffer (BioRad TGS solution, 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% w/v SDS).

Gels were stained 30 min with 1 mg/mL Coomassie Brilliant Blue R-250 solution containing 45% methanol and 10% acetic acid. The solution was decanted from the gels, and the gels were rinsed with deionized water. Gels were destained 20 min with a solution of 30% methanol and 10% acetic acid with gentle rotation and twisted kimwipes to absorb dye. This solution was decanted, kimwipes were removed, and fresh destain and kimwipes were added to the gels, which were allowed to destain another 30 min. Gels were then dried with Invitrogen Gel-Dry solution in Dry Erase mini cellophane squares.

Cleavage of proteins with TEV protease

Various amounts of either His_6-GPRT or Gly-GPRT-C37-His_6 proteins were cleaved with TEV protease. An arbitrary 100 μL TEV protease was added per 1 mg protein to be cleaved. This was allowed to proceed 24 h under reducing dialysis conditions (1 mM β-ME in 25 mM potassium phosphate buffer, pH 7.5). 5 mg Gly-GPRT-C37-His6 was cleaved by 200 μL TEV protease over three days with dialysis buffer changed daily (same buffer as previously mentioned). A time course of cleavage was analyzed via polyacrylamide gel electrophoresis (as described above).

High-Performance Liquid Chromatography (HPLC)

A Thermo Separation Products HPLC with a C-18 column and UV detector was employed for purification of the cleavage and ligation mixtures. The column was equilibrated with 90% A buffer (0.1% trifluoroacetic acid, TFA) and 10% B buffer (0.1% TFA in acetonitrile, ACN). A 1 mL sample was injected and separated with a gradient of

10-80% B buffer over 30 min. The peak fractions were then pooled and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in positive ion mode.

Labeling of proteins

Without further purification of the cleaved protein, Gly-GPRT-C37-His_6 was ligated to a thioester-containing small molecule via native chemical ligation (N-terminal cysteine available on C37-His_6 peptide). Two molecules were ligated, a biotin thioester and later a benzoic acid vinyl thioester (Figure 1). Ligations were carried out at room temperature in 30 mM 2-mercaptoethanesulfonic acid and 30 mM potassium phosphate buffer, pH 7.5, for 24 h with 4 mM thioester and total volume of the TEV protease cleavage mixture.

To isolate ligated peptide, Pepclean C-18 spin columns were used (Pierce Biochemicals). A portion of ligation mixture (120 μL) was diluted with TFA and ACN to reach final concentrations of 0.5% and 5%, respectively. The column was activated with 50% methanol and equilibrated with 0.5% TFA and 5% ACN. The diluted ligation mixture was loaded onto the spin column and spun 1 min at 1500g. The flow-through was reloaded and column spun again. The spin column was washed four times with 0.5% TFA and 5% ACN. The peptide was eluted with 70% ACN. Ligation success was determined by MALDI-TOF MS. All samples were analyzed within 24 h of experimentation.

Completed Work

Tobacco Etch Virus (TEV) protease was expressed in *E. coli* cells and purified in order to replenish laboratory stocks. As can be seen from column elution fractions in Figure 2, most TEV protease eluted in the first few fractions. Fractions 2-8 were combined, diluted with buffer, and dialyzed to be purified TEV protease as described in the Materials and Methods section. The purification process was successful in that a high amount of TEV protease was collected and concentrated, which is visualized in Figure 3. Uninduced *E. coli* does not show a band corresponding to TEV protease, whereas a small band can be seen in the induced *E. coli*. The post-sonication supernatant has a multitude of proteins. Since this was discarded, TEV protease was purified in the centrifugation step. Purified TEV protease shows one medium-sized band, where in the TEV protease standard, there is one large band in the expected location and several bands likely arising from degradation. Thus, the purified TEV protease is less concentrated than the standard but is purer. TEV protease was analyzed by UV spectrophotometry via its absorbance at 280 nm, and its concentration was determined to be 7.6 μM.

Once TEV protease was purified, it needed to be analyzed for activity. A test cleavage of His_6-GPRT was performed, where the TEV recognition site falls between the His tag and GPRT protein. As can be seen in Figure 4, TEV protease is active and cleaved approximately 50% of the His tags off GPRT over 24 h. Because the C37 peptide is the one of interest, it also needed to be cleaved from its carrier protein. TEV protease was used to cleave Gly-GPRT from the C37 peptide with a C-terminal His tag. A small scale (1 mg) cleavage was performed over 24 h, and approximately 60% of the fusion protein was cleaved.

Sixty percent cleavage was not adequate, so a reaction of the same scale was performed but allowed to proceed for 52 h. This mixture was purified via HPLC, and the peak fraction was submitted for MALDI-TOF MS, which shows that the C37 peptide was successfully cleaved and reasonably pure (Figure 5, 5632 Da). The biotin thioester (Figure 1 a) was then added to the peak fraction in order to ligate to the C37 peptide with an N-terminal cysteine. This mixture was then purified by HPLC, and the peak fraction was submitted for MALDI-TOF MS. Figure 6 shows the spectrum obtained from the peak fraction, which contains successfully ligated C37 peptide to the biotin thioester (5856 Da). However, these compounds were in very low concentrations due to HPLC purification.

To address this problem, Pepclean C-18 columns were employed for purification of the ligated compounds. The C37 peptide was cleaved from Gly-GPRT over 52 h, and the biotin thioester was ligated to C37 over 24 h. A sample was then purified with a Pepclean column and submitted for MALDI MS. In Figure 7, it is apparent that the biotin thioester was successfully ligated to the C37 peptide and is at an adequate concentration.

Once the cleavage and ligation protocol had been optimized, the focus shifted to ligating the C37 peptide to the benzoic acid vinyl thioester (Figure 1 b). The C37 peptide was again cleaved from its carry protein over 52 h under dialyzing and reducing conditions. The benzoic acid vinyl thioester was added to the mixture, and ligation was allowed to proceed 24 h at room temperature in the dark. The benzoic acid vinyl thioester is light-sensitive, so it was kept in the dark to prevent side reactions from occurring. Ligation appears successful in the first attempt, but there are many peaks present that cannot be accounted for resulting from side reactions (Figure 8). In attempt at attaining a

more purified product, C37 was obtained via 52 h cleavage from its carrier protein and ligated 24 h with the benzoic acid vinyl thioester. Prior to Pepclean purification, one sample was pretreated with dithiothreitol (DTT, 99%, Sigma), and the other was left untreated. As can be seen in Figures 9 and 10, DTT made no difference in sample purity. These figures also suggest that ligation was only partially successful in this experiment. It is unlikely that DTT degraded the ligated product as ligation formed an amide bond rather than a disulfide bond or thioester, upon which DTT acts. This also shows that a disulfide bond has not formed between 2-mercaptoethanesulfonic acid and the N-terminal cysteine. However, it remains unclear as to the identity of the other peaks present.

Future Work

Based on these results, it appears that the peptide of interest (C37) can be ligated to thioesters successfully. Thus, it would seem possible that a successfully ligated C37-benzoic acid vinyl thioester could be polymerized into a gel. This could be attempted with acrylamide or simply the ligated species. Once polymerization is demonstrated, protein binding and isolation could be performed for proteins known to bind the C37 peptide. This could allow for easier purification of the binding proteins such that they could be analyzed more closely. There is also potential for the development of more potent inhibitors through polymerization of peptide drugs into larger units with more drugs in a single molecule.

References

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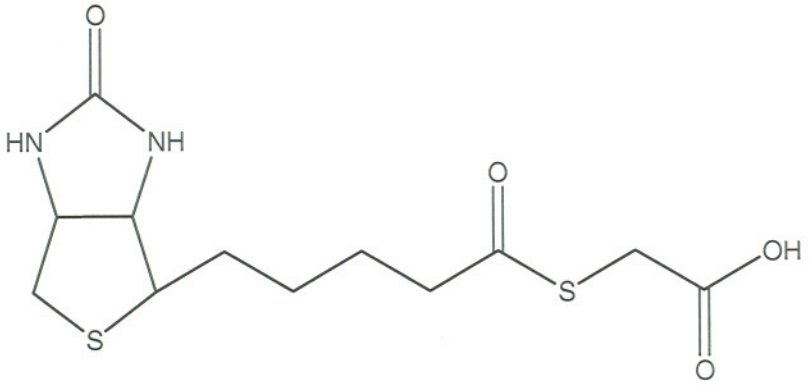
2. Tolbert, T.; Wong, C. *Angew. Chem. Int. Ed.* (2002) **41**: 2171-2174
3. Brueggemeier, S.; Kron, S.; Palecek, S. *Anal. Bioch.* (2004) **329**: 180-189
4. Dawson, P.; Muir, T.; Clark-Lewis, I.; Kent, S. *Science* (1994) **266**: 776-779

Figures

A.



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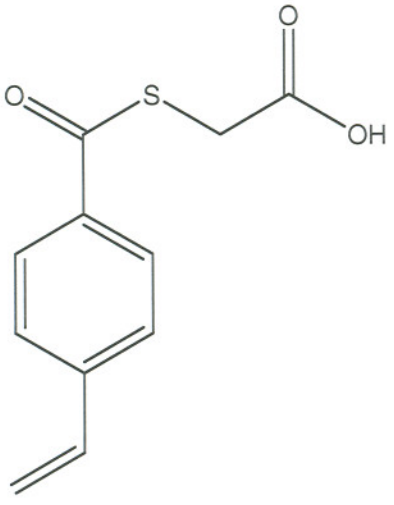


Figure 1. Thioesters ligated to C37-His6 peptide via native chemical ligation. A. Biotin thioester. B. 2-(4-vinylbenzoylthio)acetic acid mentioned as the benzoic acid vinyl thioester.

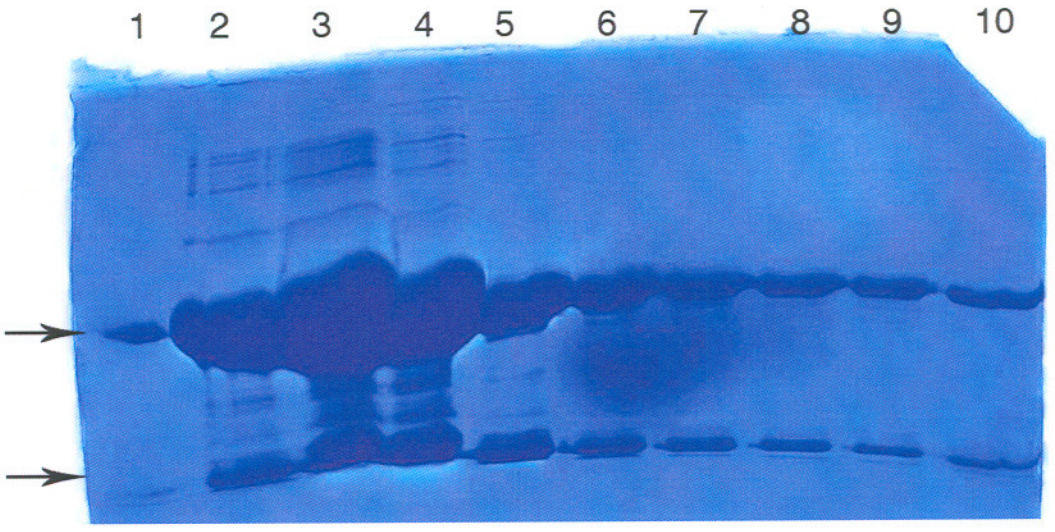
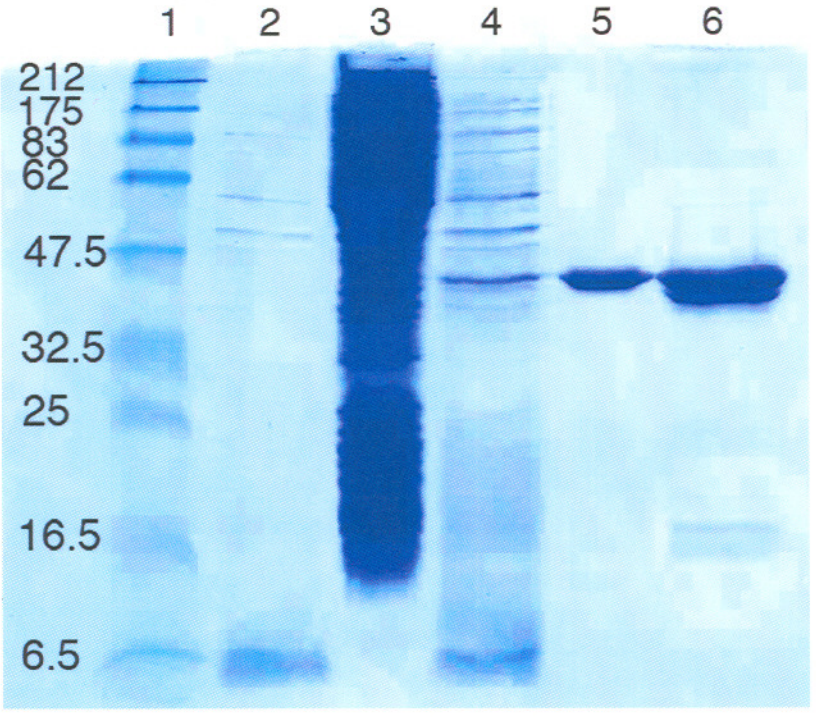


Figure 2. Ni-NTA column fractions of TEV protease. Denatured TEV protease was eluted from a Ni-NTA column with 200 mM imidazole in ten 500 μL fractions. These fractions were run in respectively-labeled lanes on a 15% polyacrylamide gel, stained with Coomassie Brilliant Blue, and destained with a 10% acetic acid/30% methanol solution. As can be seen, there is a high concentration of TEV protease in fractions 2, 3, and 4. There are decreasing amounts of TEV protease in fractions 5-10. Fractions 2-8 were combined, diluted, and dialyzed to obtain folded, active TEV protease. The top arrow denotes intact TEV protease. The lower arrow denotes auto-proteolysis of TEV protease. Due to saturating concentrations of TEV protease in the column, it was expected that a small amount of TEV protease would be degraded.



Lane Identities

1 Promega Prestained Protein Markers

2 Uninduced *E. coli*

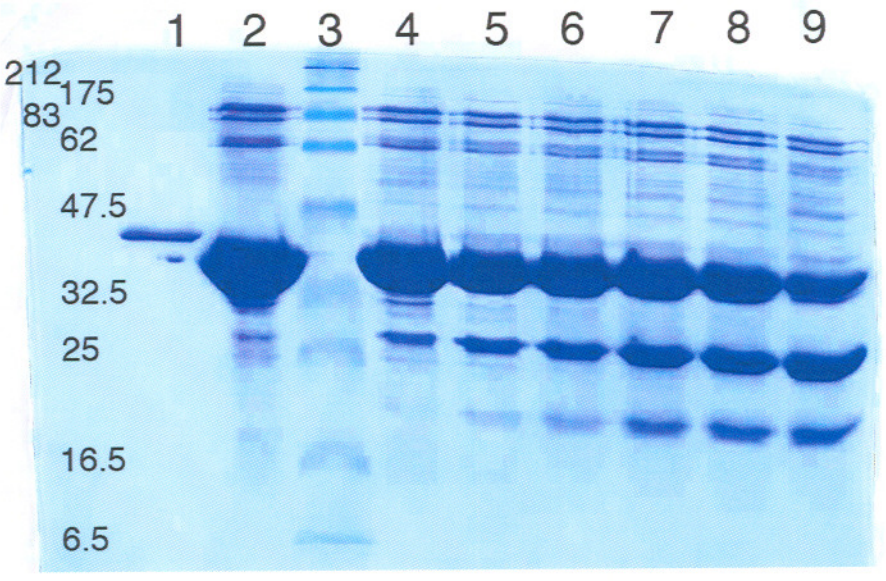
3 Post-sonication supernatant

4 Post-expression *E. coli*

5 Purified TEV protease

6 TEV protease standard

Figure 3. Visualization of TEV protease purification. Steps of the purification process were run on a 15% polyacrylamide gel for qualitative determination. Lane 2 shows that TEV protease is not produced in a noticeable concentration without the induction of *E. coli* to synthesize the protein. Lane 4 is a sample of *E. coli* after it had produced TEV protease for six hours. To see if much TEV protease was lost after *E. coli* was sonicated, the supernatant was analyzed (Lane 3). TEV protease is an insoluble protein, so the gel also shows that this portion greatly enhanced the purification process by removing many proteins. It is likely that some TEV protease remained in the soluble fraction. It can be determined by Lane 5 that purification of TEV protease was successful in that it is the only band that appears. Lane 6 is a TEV protease standard by which comparisons were made. The concentration of TEV protease is not as high as the standard, but no degradation products are observed in the purified TEV protease. Lane 1 holds protein molecular weight markers, denoted by the number of kDa on the vertical axis.



Lane Identities

1 Purified TEV protease

2 His_6-GPRT

3 Promega Prestained Protein Markers

4 0 h

5 0.5 h

6 1 h

7 4 h

8 6 h

9 24 h

Figure 4. Test cleavage of purified TEV protease. A 15% polyacrylamide gel was run for 1 hour at 200 V, stained with Coomassie Brilliant Blue, and destained with a 10% acetic acid/30% methanol solution. Lanes 4-9 follow the time course of TEV protease cleaving a His_6 tag from His_6-GPRT linked by a TEV recognition sequence. Lanes 1 and 2 are samples of TEV protease and His_6-GPRT before being combined. Lane 3 is a protein molecular weight marker, with the molecular weights (in kDa) listed on the x-axis. At time 0 (Lane 4), there is a very large amount of tagged GPRT (densest band). However, after 24 h (Lane 9), this amount has decreased significantly. Two bands below the tagged GPRT show GPRT and the released His_6 tag with its C-terminal TEV recognition sequence.

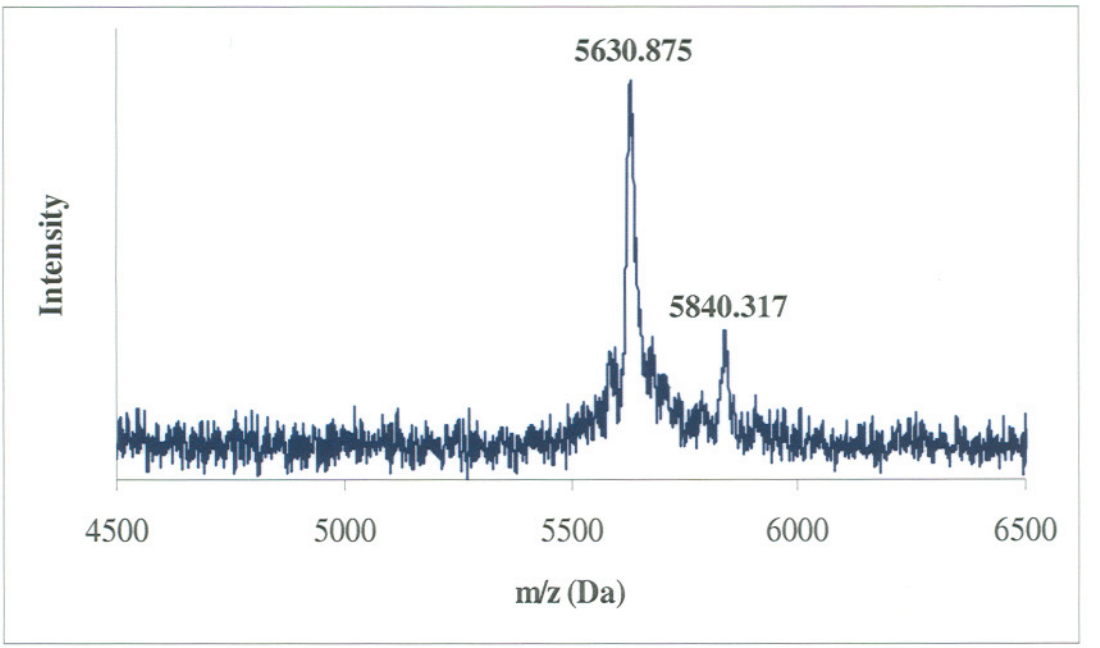


Figure 5: MALDI-TOF MS spectrum of the cleavage mixture including the C37 peptide, GPRT, and TEV protease. GPRT and TEV protease are large proteins (MW > 30 kDa), so they are not seen in the figure. However, there is a peak at 5630 Da, which is within error of the C37 peptide (expected mass 5624 Da).

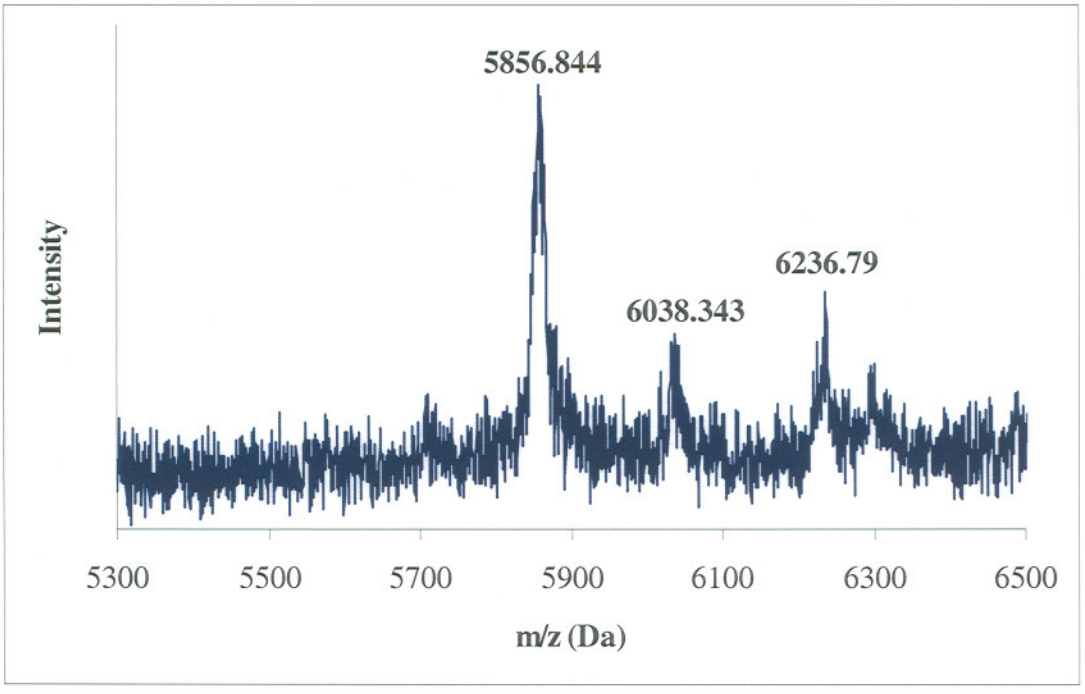


Figure 6: MALDI-TOF MS spectrum of the ligation mixture of the C37 peptide and the biotin thioester. The peak at 5856 Da is the ligated peptide and thioester (expected mass 5854 Da). The other peaks may be noise in the instrument as the signal-to-noise ratio is low in this sample, indicating a low concentration of peptide.

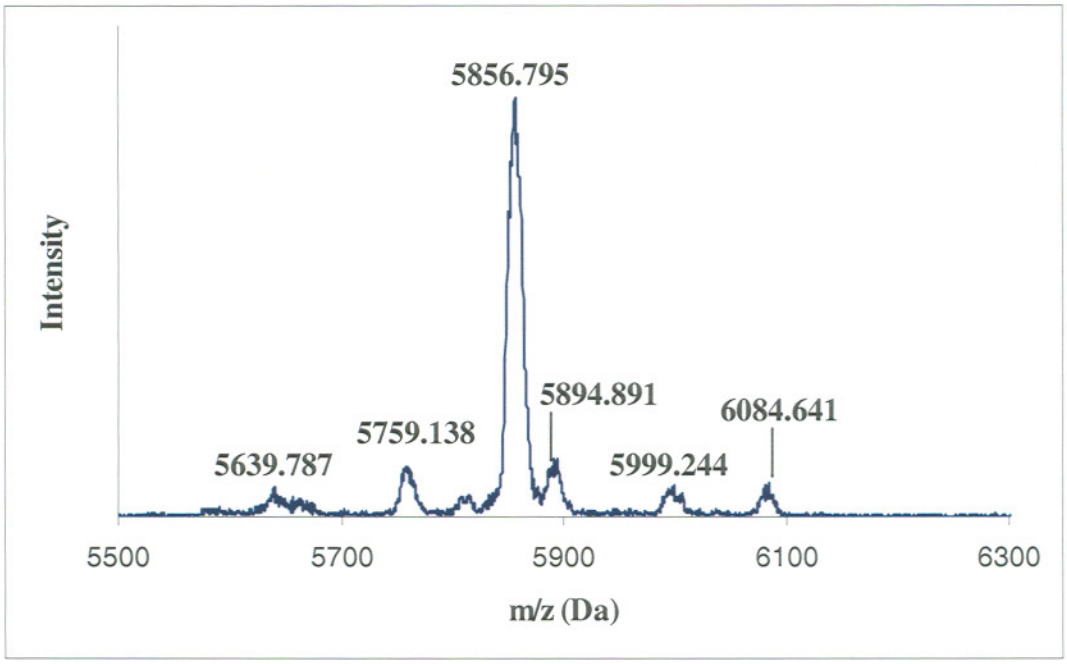


Figure 7: MALDI-TOF MS spectrum of the ligation of the C37 peptide with the biotin thioester after purification via Pepclean C-18 columns. The strong peak at 5856 Da indicates that ligation was successful (expected mass 5854 Da). The peak at 5639 may be the C37 peptide unligated with a sodium adduct. The rest of the peaks may be attributed to artificial reactivity from the MALDI matrix.

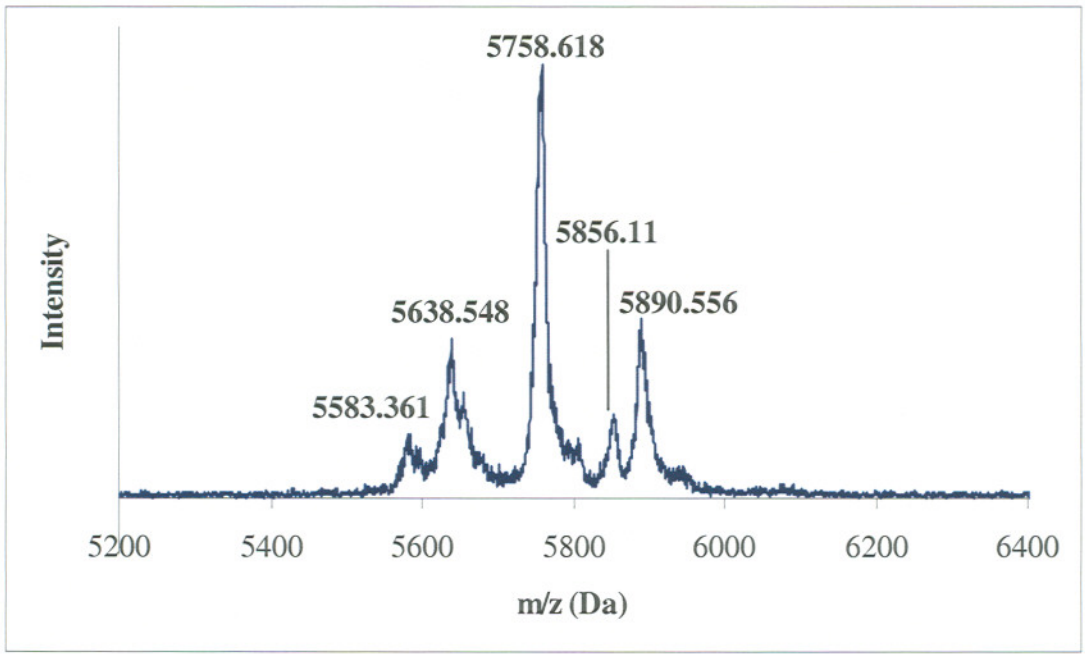


Figure 8: MALDI-TOF MS spectrum of the ligation between the C37 peptide with the benzoic acid vinyl thioester after Pepclean purification. The strong peak at 5758 matches very closely with the expected mass of the ligated product (5757 Da). The 5638 peak may be within error of a sodium adduct to the unligated C37 peptide (expected mass 5647 Da). The peak at 5890 could potentially be the ligated product reacted with another, unligated benzoic acid vinyl thioester via the N-terminal cysteine (expected mass 5889 Da). Other peaks may be attributed to reactivity with the MALDI matrix.

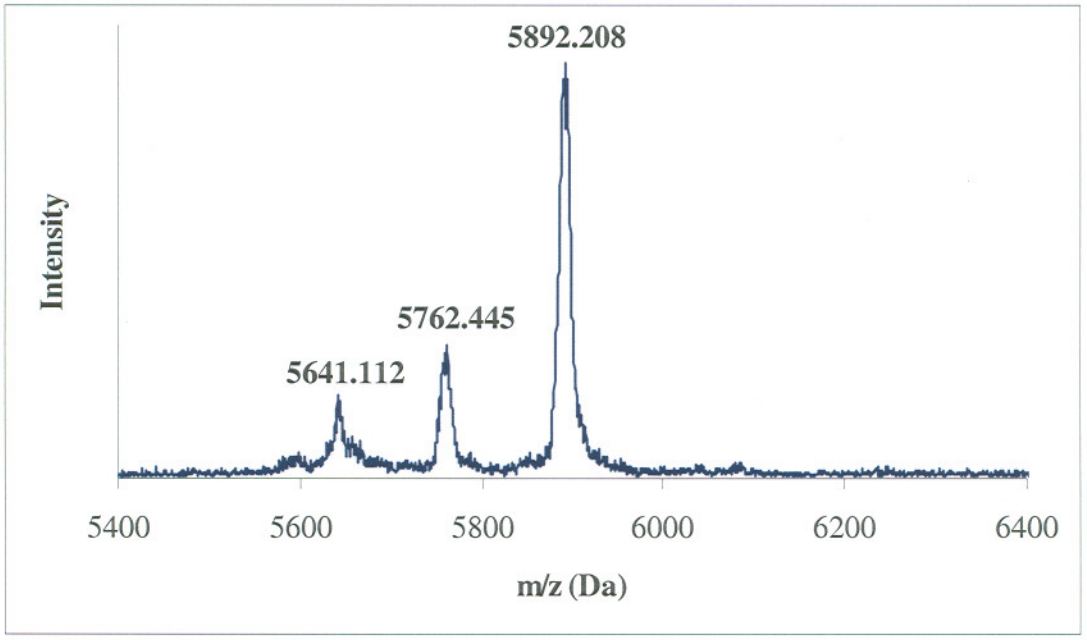


Figure 9: MALDI-TOF MS spectrum of the ligation between the C37 peptide with the benzoic acid vinyl thioester pretreated with DTT before Pepclean purification. The sample appears purer, but the ligated product is not the major product observed (5757 vs. 5892, respectively). It is possible that the 5641 peak is a sodium adduct of the unligated C37 peptide (expected mass 5647). The major peak (5892) correlates with the mass of the C37 peptide ligated to a thioester and the cysteine reacting with another unligated thioester, but this is not likely to occur in the presence of DTT.

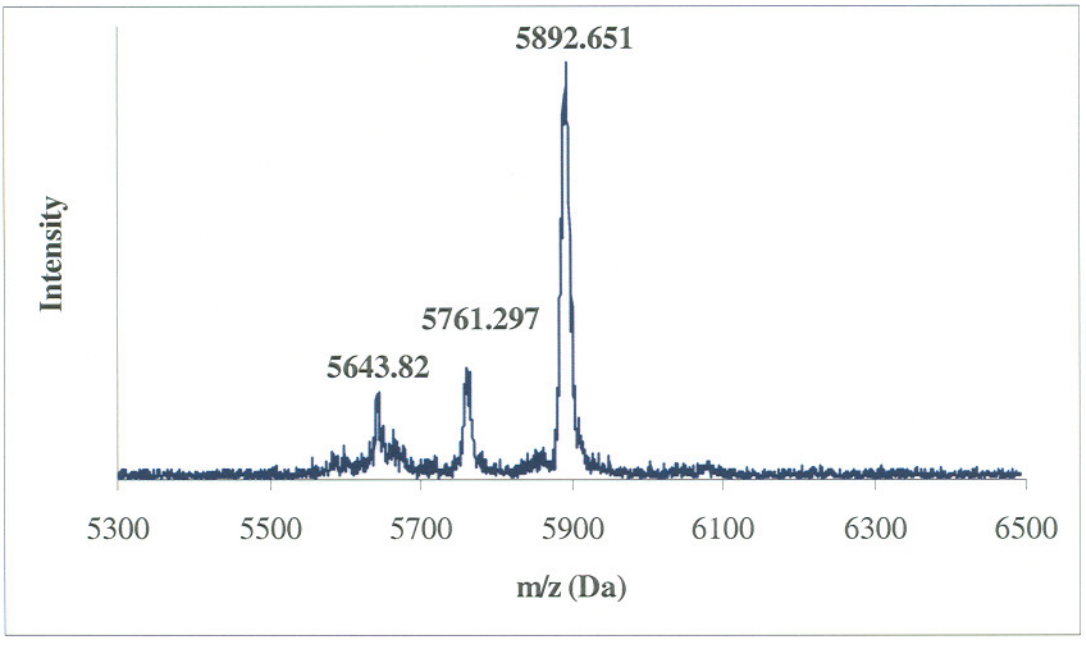


Figure 10: MALDI-TOF MS spectrum of the ligation between the C37 peptide with the benzoic acid vinyl thioester untreated prior to Pepclean purification. The sample appears equivalent to the DTT treated sample. The ligated product is present (5761, expected mass 5757). The 5643 peak is likely to be a sodium adduct of the C37 peptide (expected mass 5647). The mass of the major peak correlates with a ligated C37 peptide with the benzoic acid vinyl thioester and another unligated thioester reacting with the cysteine on the C37 peptide.