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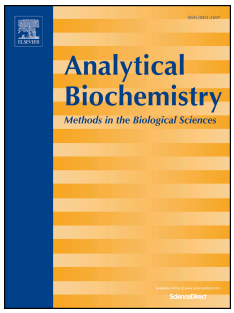

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Examining Ubiquitinated Peptide Enrichment Efficiency through an Epitope Labeled Protein

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Abstract

Ubiquitination is a dynamic process that is responsible for regulation of cellular responses to stimuli in a number of biological systems. Previous efforts to study this post-translational modification have focused on protein enrichment; however, recent research utilizes the presence of the di-glycine (Gly-Gly) remnants following trypsin digestion to immuno-enrich ubiquitinated peptides. Monoclonal antibodies developed to the cleaved ubiquitin modification epitope, (*tert*-butoxycarbonyl) glycylglycine (Boc-Gly-Gly-NHS)[1], are used to identify the Gly-Gly signature. Here, we have successfully generated the Boc-Gly-Gly-NHS modification and showed that when conjugated to a lysine containing protein, such as lysozyme, it can be applied as a standard protein to examine ubiquitinated peptide enrichment within a complex background.

Keywords:

- Ubiquitination
- Gly-Gly Enrichment
- Enrichment Efficiency

1. Introduction

The process of ubiquitination occurs through the E3 ligase conjugation of ubiquitin to ε-amines of lysine, and is known to be a post-translational modification in living organisms that is used to regulate protein activity in response to a stimulus [1, 2]. Progress in the area of ubiquitomics has evolved to include antibody recognition of the K-ε-Gly-Gly group that remains after cleavage with the endoproteinase trypsin [3]. Using the epitope Boc-Gly-Gly-NHS conjugated to histone, researchers were able to generate the Gly-Gly antibody and subsequently use it for Gly-Gly peptide enrichment [3]. Kits that utilize anti-Gly-Gly are now commercially available, and have been shown to be successful in capturing up to thousands of ubiquitinated peptides, providing new information on dimensions of the proteome that were previously untouched by researchers [4, 5]. However, when trying to optimize experimental conditions, resulting changes in the enrichment process cannot always be attributed to be the result of the applied experimental variable, as variability in handling during enrichment is possible. Therefore, changes in the enrichment process due to human error must be ruled out. The use of synthetic peptides can be used to examine enrichment efficiency as well as disassociation bias during LC-MS/MS analysis [6], but an epitope labeled protein, spiked in before digestion, enables researchers to track each step of the sample handling process from digestion to LC-MS/MS.

[1] Abbreviations used: Boc-Gly-Gly-NHS, (*tert*-butoxycarbonyl) glycylglycine; Gly-Gly, di-glycine; T3P®, propylphosphonic anhydride solution; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; LC, liquid chromatography; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; FASP, filter aided sample preparation; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; DMP, dimethylpimelimidate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; IAP, immunoaffinity purification; PBS, phosphate buffered saline; FA, formic acid; ACN, acetonitrile; AGC, automatic gain control; FDR, false discovery rate

To the best of our knowledge, there is no evidence of the application of an epitope labeled protein being used to measure ubiquitinated peptide enrichment efficiency. The need for such a modified protein encouraged us to generate the epitope Boc-Gly-Gly-NHS [3, 7-9] and conjugate this to lysozyme in order to generate an epitope labeled protein standard with the same chemical modification as the peptides of interest. Because the modified protein enters the experimental design at the beginning of the sample workflow, one can examine if experimental procedures hinder the enrichment process by calculating the enrichment efficiency of the protein standard. Here we show that even when spiked in at low concentrations into a complex background, we were able to track a modified peptide through the Gly-Gly enrichment procedure and successfully calculate enrichment efficiency.

2. Materials and Methods

2.1 2,5-diOxopyrrolidin-1-yl-(*tert*-butoxycarbonyl)glycylglycinate synthesis

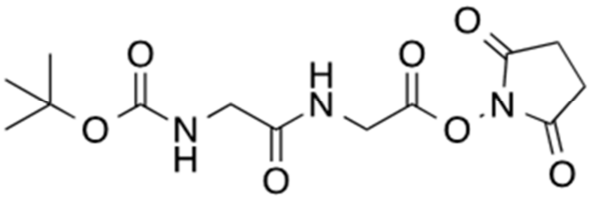


Fig. 1 . Chemical structure of (*tert*-butoxycarbonyl) glycineglycine

To a solution of Gly-Gly (1.00 g, 7.57 mmol) in dioxane:water (30:5 mL) at room temperature was added triethylamine (1.15 g, 11.4 mmol) and di-*tert*-butyl-dicarbonate (1.84 g, 8.33 mmol) consecutively. The mixture was stirred at room temperature overnight, then diluted with water, acidified to approximately pH = 2 via the addition of solid $KHSO_4$, extracted with EtOAc, dried (Na_2SO_4), and concentrated *in vacuo* to afford (*tert*-butoxycarbonyl)glycylglycine [8] as a white solid. To a solution of crude (*tert*-butoxycarbonyl)glycylglycine (0.106 g, 0.451 mmol) in anhydrous dicholoromethane (5.00 mL) was added triethylamine (45.6 mg, 0.451 mmol) and propylphosphonic anhydride solution (T3P®) [9] (0.344 g, 0.541 mmol) at room temperature. The mixture was stirred for 20 min, followed by the addition of *N*-hydroxy succinimide (51.9 mg, 0.451 mmol). The reaction was stirred for 48 h and upon completion, the organic layer was washed with brine (3×), dried (Na_2SO_4), and concentrated *in vacuo* to afford the product as a white solid. The crude solid was triturated from diethyl ether to afford 65.4 mg (44%) of the product as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (brs, 1 H), 5.15 (brs, 1 H), 4.43 (d, 2 H, *J* = 5.7 Hz), 3.87 (d, 2 H, *J* = 5.3 Hz), 2.85 (s, 4 H), 1.45 (s, 9 H); ESIMS *m/z* 330 $[M+H]^+$. Direct infusion ESI-MS on a high resolution accurate mass measurements (Exactive Plus, Thermo Fisher Scientific) in the negative ion mode yielded an elemental composition of the expected product within 5 ppm.

Reactions were monitored by LC-MS (Shimadzu LC-MS 2020 with Kinetex 2.6 mm C18 50 × 2.10 mm). ^{1}H and ^{13}C NMR spectra were obtained on a Varian Mercury-VX 300, a Varian Mercury-VX 400, or a Varian Mercury-Plus 300 instrument in $CDCl_3$ unless otherwise noted. Chemical shifts were reported in parts per million with the residual solvent peak used as an internal standard ($CDCl_3$ ^{1}H δ = 7.26 and ^{13}C δ = 77.23). ^{1}H NMR spectra were run at 300 or 400 MHz and are tabulated as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, qd = quartet of doublets, ddt = doublet of doublet of triplet), number of protons, and coupling constant(s).

All reactions were performed under an Ar atmosphere and all glassware was dried in an oven at 135 ℃ overnight prior to use, unless otherwise not ed. Dichloromethane was purified using an

alumina filtration system. Diglycine, triethylamine, di-*tert*-butyl-dicarbonate, *N*-hydroxy succinimide, and T3P® were purchased from Sigma Aldrich and Fisher Scientific and used as received unless otherwise noted.

2.2 Lysozyme modification

Gly-Gly-modified lysozyme was generated by following the protocol described in Xu et al [3]. In brief, 1 mg of lysozyme (Sigma, CAS# 12650-88-3) was dissolved in 100 mM $NaHCO_3$ buffer (2.5 mL) at pH 10. 125 µL of 50 mM, Boc-Gly-Gly-NHS in dimethyl sulfoxide (DMSO) was added to protein solution and the reaction was carried at 25 ℃ for 1 h by constant shaking (200 rpm) on a plate rotator. This reaction was repeated three times. For de-protection of Boc group from the modified protein, 1.5 mL trifluoroacetic acid (TFA) was added, and the solution was shaken for 2 h at 25 ℃. After neutralized with 10 M NaOH dropwise on ice, the reaction was dialyzed (8-10 MWCO, Spectrum Laboratories) four times (1 h 3 times at 25 ℃ and overnight at 4 ℃) against 20 mM acetic acid (3 L).

2.3 Western blot analysis

To confirm the Gly-Gly modification of lysozyme, we followed the protocols previously published by Xu et al [3]. Briefly, 5 µg of each unmodified lysozyme, Gly-Gly modified, and Boc-Gly-Gly modified lysozyme samples were separated on a 4–20% gradient gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). Blots were blocked in 5% bovine serum albumin and antibody incubations were carried out in 5% skim milk followed by washes. Signals were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce), following the manufacturer's instructions. The following antibodies were used: Gly-Gly lysine hybridoma clone GX41 (1:2000, Lucerna) and Anti-mouse IgG conjugated to horseradish peroxidase (1:3000, Cell Signaling Technology).

2.4 Sample preparation and protein extraction

The complex background proteome was generated from mycelia collected from a *M. oryzae* strain 70-15 culture. Spore harvest and inoculation was performed as in Oh et al [10]. The mycelia were treated with nitrogen limiting minimal media (10 g sucrose, 1 mL *A. nidulans* trace elements, 1 g thiamine, and 5 µg biotin in 1 L). Proteins were extracted using lysis buffer (8M urea, 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 50uM PR-619, Protease Inhibitor cocktail (Roche)) [11]. Protein concentration was measured by Bradford assay.

2.5 Sample digestion

2.5.1 Lysozyme

Lypholized epitope modified lysozyme was re-suspended in 0.001% Zwittergent 3-16 (Calbiochem, La Jolla, CA), and a 1:1 combination of modified to unmodified lysozyme was generated from 0.001% Zwittergent reconstituted epitope modified lysozyme and unmodified lysozyme. The protein standard mixture was spiked in at 0.1%, 1%, and 5% (mg lysozymel/mg *Magnaporthe oryzae* proteins) using total proteins extracted from the fungus *Magnaporthe oryzae* (*M.oryzae*) as the complex protein background. All samples were digested using filter aided sample preparation (FASP) [12]. Each sample was diluted 2-fold with 100 mM dithiothreitol (DTT) in 8 M urea, 50 mM Tris, pH 7.0 and incubated at 56 ℃ for 30 min. Samples

were alkylated with a final concentration of 50 mM chloroacetamide in 8 M urea, 50 mM Tris, pH 7.0 at 37 ℃ for 60 min. The alkylated sample was t hen transferred to a 10 kDa centrifugal filter (Millipore, #UFC501096) and concentrated by centrifuging at 14,000 × g at 20 ℃, 15 min. The filter containing the sample was then washed 3 times with 2 M urea, 10 mM $CaCl_2$ in 50mM Trish pH 7.0. Each sample was trypsin digested at a 1:50 enzyme:protein ratio. The digestion reaction was incubated overnight at 37 ℃. The reac tion was quenched with 400 uL of 1% formic acid in 0.001% Zwittergent, and subsequently centrifuged at 14,000 × g at 20 ℃, 15 min to elute the peptides.

2.5.2 *Magnaporthe oryzae*

Samples prepared for enrichment were digested and enriched as in Porras-Yakushi et al [6], with minor modifications. A total of 10 mg of protein extracted from treated *M. oryzae* was used for Gly-Gly enrichment. The sample was cleared by centrifugation at 16000 × g for 15 min. Before the digestion process, 1:1 ratio of modified: unmodified protein standard was spiked in at 0.1%. The sample was reduced for 45 min at room temperature with a final concentration of 4.5 mM DTT. Following reduction, the sample was alkylated with final concentration of 10 mM *N*-ethylmaleimide (NEM). This was performed for 30 min in the dark at room temperature. LysC digestion was carried out at a ratio of 1:200 at room temperature. Following the first digestion, the sample was diluted to 2 M urea with 100 mM Tris (pH 8.0). $CaCl_2$ was added to the sample for a final concentration of 1 mM, and subsequent trypsin digestion at a ratio of 1:100 was performed at 37 ℃ overnight.

The digestion reaction was quenched with 0.1% TFA in H_2O and concentrated *in vacuo*. Following centrifugation at 16000 × g for 15 min to clear the sample, desalting was performed using the 500 mg SepPak column (Waters). The column was washed with 21 mL of 100% acetonitrile (ACN) and equilibrated with 21 mL of equilibration buffer (0.1% TFA, in H_2O). The dried peptides were re-suspended in equilibration buffer and loaded onto the SepPack column. A 21 mL wash with the same buffer was performed and followed with a wash with 9 mL wash with 0.1% TFA, 5% ACN in H_2O. Peptides were eluted with 6 mL of 0.1% TFA, 40% ACN in H_2O.

2.6 Resin cross-linking and Gly-Gly enrichment

For the chemical cross-linking of antibody to bead, we followed protocols previously published by Udeshi et al and Porras-Yakaushi et al [6, 11]. Briefly, the antibody-bound beads were washed three times with 1 mL of antibody cross-linking wash buffer (100 mM sodium borate, pH 9.0). The antibody beads were re-suspended in 1 mL of antibody cross-linking buffer (20 mM dimethylpimelimidate (DMP) in 100 mM sodium borate, pH 9.0) and incubated in gentle end-over-end rotator for 30 min at room temperature. The cross-linking reaction was stopped by washing the antibody beads twice with 1 mL of antibody blocking buffer (200 mM ethanolamine, pH 8.0). The antibody beads were re-suspended with 1 mL antibody blocking buffer and incubated for 2 h at 4 °C with gentle rotation. The cross-linked antibody was washed three times with 1.5 ml of IAP buffer (50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.2), 10 mM Na_3PO_4 and 50 mM NaCl).

De-salted peptides were dried down with a speed vacuum and subsequently re-suspended in 1 mL 1× immunoaffinity purification (IAP) buffer (50 mM MOPS (pH 7.2), 10 mM sodium phosphate, and 50 mM NaCl). At this point, 100 µg of sample was removed and set aside for a non-enriched sample. After centrifuging at 20000 × g for 5 min, the pH was adjusted to 7.0 by

adding 1 M Tris pH 7.0. Re-suspended peptides were added to the washed cross-linked antibody resin and incubated with end-over-end rotation for 1 h at 4 °C [6, 11]. After incubation, the flow through was removed, and the resin was washed three times with 500 µL 1 × IAP buffer, centrifuging at 2000 × g for 1 min each time. One wash with 1 × phosphate-buffered saline (PBS) followed by one wash with H_2O was performed, centrifuging at 2000 × g for 1 min after each wash. Peptides were eluted with 150 µL 0.15% TFA.

Samples were cleaned up using C18 ZipTips (Millipore). The dried enriched peptides were re-suspended in 15 µL of 0.1% formic acid (FA). The ZipTip was washed with 9 µL of 100% ACN, and equilibrated with 90 µL of 0.1% FA, H_2O. The sample was loaded onto the ZipTip by drawing up the sample and expelling it slowly repeatedly for 2 min. The loaded sample was washed with 100 µL 0.1% FA and subsequently eluted with 25 µL 80% ACN, 20% 0.1% FA. The sample was dried before preparing for LC-MS/MS analysis.

2.7 LC-MS/MS

2.7.1 Lysozyme

All samples were separated using the Easy nanoUPLC (Thermo) and analyzed on a Q Exactive High Field (Thermo). Columns were Self-Pack PicoFrit Columns 360 µm O.D. 75 µm I.D. 15 um tip I.D. with no coating (New Objective Inc. PF360-75-15-N-5). Columns were packed with 2.6 µm C18 100 Å Kinetix packing material (Phenomenex). Mobile phase A consisted of 98% H_2O, 2% ACN, and 0.2% FA; Mobile phase B consisted of 2% H_2O, 98% ACN, and 0.2% FA.

Both the Gly-Gly epitope modified lysozyme and the protein standard 1:1 mixture were loaded onto a 25 cm packed column and eluted at a flow rate of 300 nL/min with a 30 min linear gradient (5-30%). Full MS was acquired with a resolving power of 60,000, an automatic gain control (AGC) target of 1e6 and a scan range 300-2000 m/z. During data dependent MS^2, a 15,000 resolution was applied with an AGC target of 2e4. Maximum injection time was set to 30 ms for both MS^1 and MS^2. A total of 12 sequencing events were allowed per scan. A 2 m/z isolation window was utilized with normalized collision energy of 27and a dynamic exclusion of 30 sec.

2.7.2 Enriched, flow through, and non-enriched samples

The 0.1% spiked in control 1:1 protein in the *M. oryzae* background as well as the enrichment samples (enriched peptides, flow through peptides, and non-enriched peptides) were loaded onto a 20 cm packed column and eluted at a flow rate of 300 nL/min with a 120 min linear gradient. The control sample was analyzed using a 5-30% gradient of mobile phase B, and the enrichment, flow through, and non-enriched samples were analyzed using a 5-32% gradient. Full MS was acquired with a resolving power of 120,000, an AGC target of 3e6, a scan range 300-1600 m/z, and a maximum injection time of 50 ms. During data dependent MS^2, a 15,000 resolution was applied with an AGC target of 1e5 and a maximum injection time of 30 ms. A total of 20 sequencing events were allowed per scan. A 2 m/z isolation window was utilized with normalized collision energy of 27, and a dynamic exclusion of 20 sec.

2.8 Database searching

RAW files were submitted to Proteome Discoverer 1.4 and using SEQUEST HT, searched against a targeted *M. oryzae* database of 13,043 sequences [13]. Trypsin (Full) was selected for the digestion enzyme, and 4 maximum missed cleavage sites were allowed. A precursor mass

tolerance of 5 ppm was utilized along with a fragment mass tolerance of 0.02 Da. Up to 4 dynamic modifications were allowed per peptide. Dynamic modifications included deamidation (0.984 Da, N, Q), oxidation (15.995 Da, M), GlyGly (114.043 Da, K), and LeuArgGlyGly (383.228 Da, K) [14, 15]. For static modifications, all files were searched with carbamidomethyl (57.021 Da, C) except enriched sample files that were searched with *N*-ethylamaleimide (+125.0477, C).The Percolator node was used to calculate the false discovery rate (FDR) with a strict cut off of 0.01 and validation based on q-value.

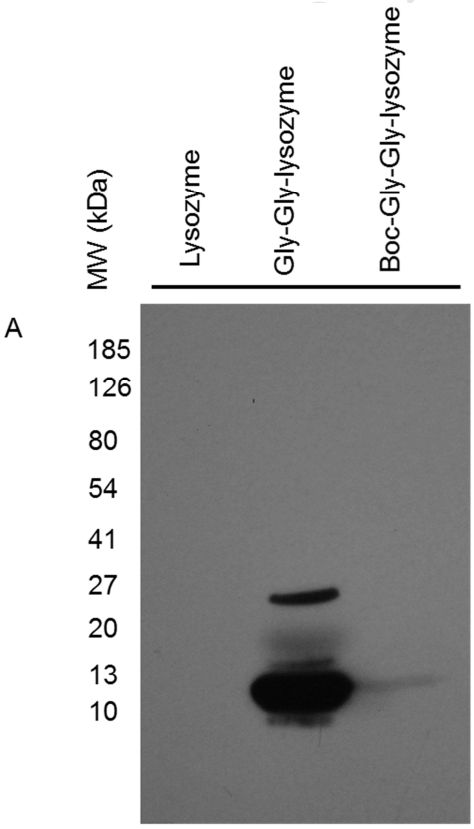
3 Results and Discussion

3.1 Epitope labeled lysozyme Western blot

In order to determine if the Boc-Gly-Gly-NHS modification procedure of lysozyme was successful, a Western blot utilizing the commercially available anti-Gly-Gly was performed [3]. As is demonstrated in Fig. 2A, lysozyme and Boc-Gly-Gly-lysozyme was not probed by the antibody due to the lack of available K-ε-GlyGly groups. The epitope labeled protein was identified by anti-Gly-Gly after Boc removal (Gly-Gly lysozyme). Variable molecular weight shifts are representative of different numbers of modified lysines [3]. In order to further identify how many lysines were modified and what peptides were being modified, LC-MS/MS was performed on the epitope labeled protein.

3.2 Protein standard characterization

Pure modified lysozyme was analyzed by LC-MS/MS using the Thermo Q-Exactive High Field instrument. A sequence coverage of 72.11% was achieved for the protein, Fig. 2B. Of the 6 available lysines, 5 were identified as having the Gly-Gly modification present. Each Gly-Gly shifts the weight of a protein 114.04 Da; therefore Gly-Gly modifications to 5 lysines within lysozyme as well as the additional dynamic (O, D, L) and static modifications (C) should create a shift of 954.171 Da for a total molecular weight of 17.184 kDa, Fig. 2A. These results suggested that not only did the modification procedure successfully label lysozyme, but that almost all lysines had the Gly-Gly modification.



B

MRSLLILVLC FLPLAALGKV
FGR**c**ELAAA**m** **k**RHGLD**n**YRG
YSLG**n**WV**c**AA **k**FES**n**F**n**TQA
TNR**n**TDGSTD YGILQINSRW
WCNDGRTPGS RNL**cn**IP**c**SA
LLSIDITASV **nc**A**kk**IVSDG
nGmnAWVAWR NR**ck**GTDV**q**A
WIRGCRL

Fig. 2. (A) Western blot of Boc-Gly-Gly-NHS modified lysozyme when probed with anti-Gly-Gly. (B) The sequence for Lysozyme (126608) and the identified modified lysines. Identified peptides are underlined.

3.3 Protein standard spike-in concentration optimization

In order to determine the mass percent at which the protein standard should be spiked into a complex sample, a range of concentrations of a 1:1 epitope modified to unmodified protein standard mixture was added to a complex *M. oryzae*

background. Three concentrations, 0.1%, 1%, and 5% of the 1:1 spiked in control were added to the extracted protein. Each sample was subsequently digested and analyzed by LC-MS/MS. The modified protein standard was identified with a sequence coverage of 76.19%, 80.27%, 80.27%, in the 0.1%, 1%, and 5% spiked-in samples, respectively. Both Gly-Gly modified lysine

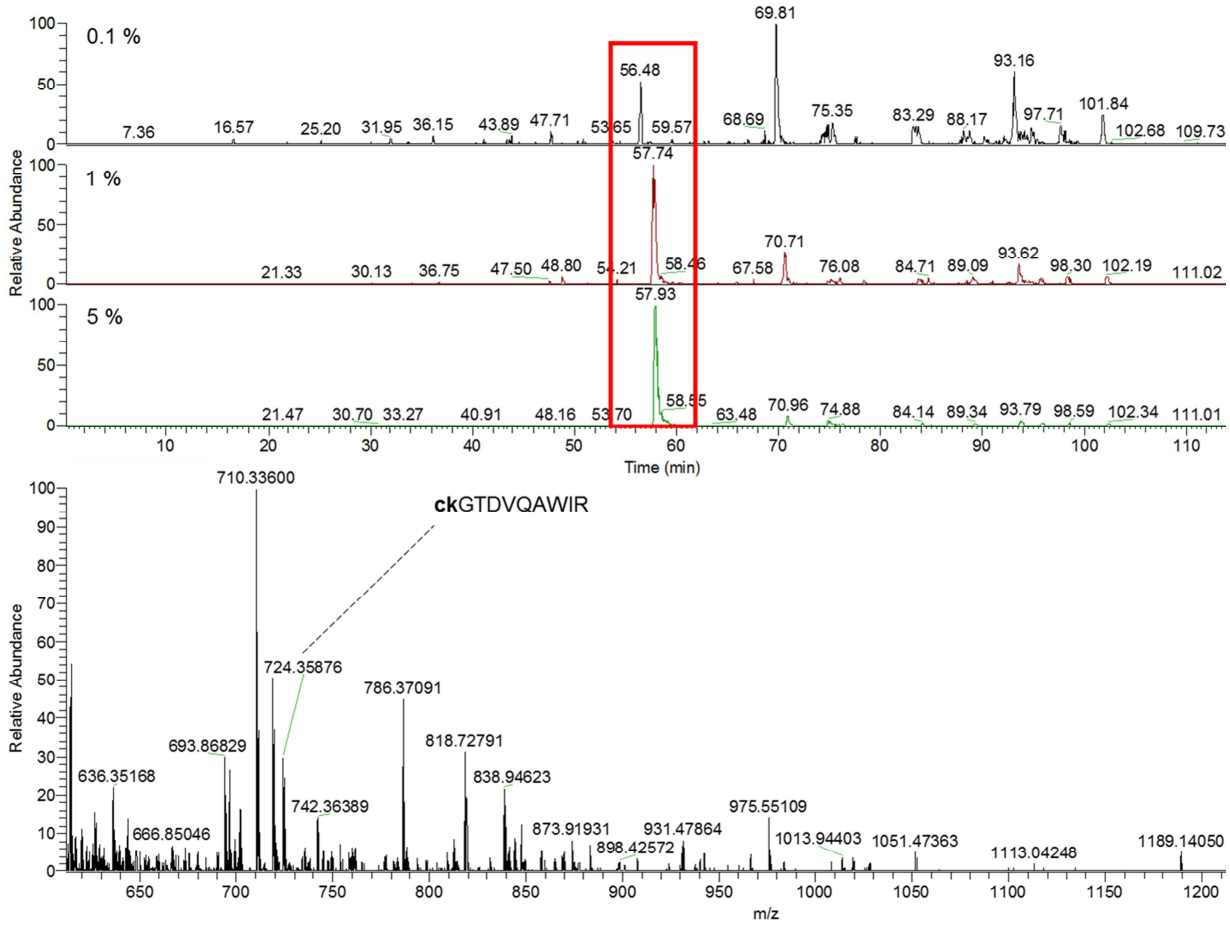


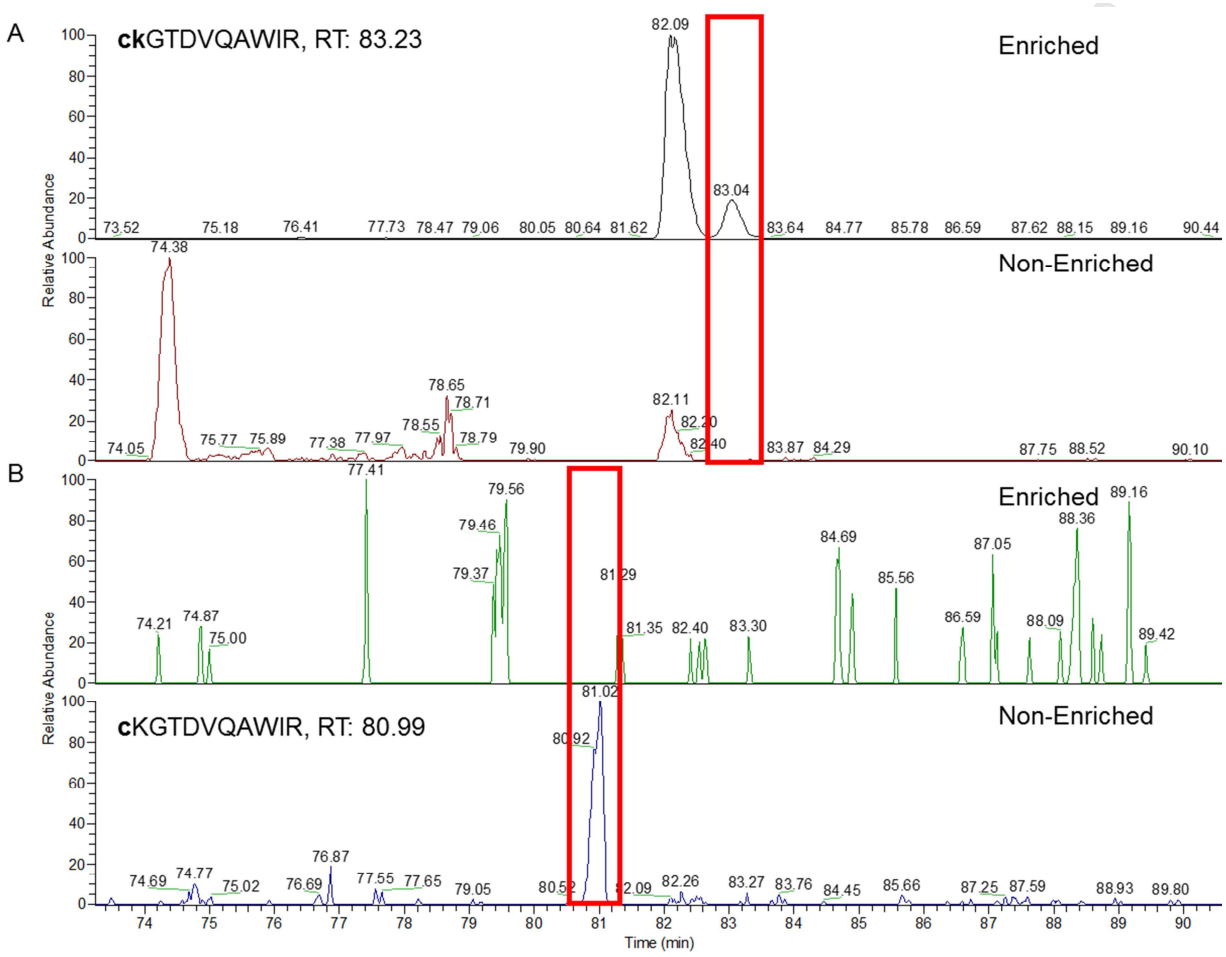
Fig. 3. Protein standard concentration optimization. The peptide **ck**GTDVQAWIR identified in each sample spiked with the protein standard at three different concentrations.

containing peptides (Fig. 3, Table 1, Supplemental Material) and unmodified lysine containing peptides were identified in each tested concentration, suggesting that 0.1% concentrations of the protein standard were capable of not only being identified but would provide information on Gly-Gly containing peptides and resulting information about enrichment efficiency.

3.4 Gly-Gly enrichment efficiency examined

Application of the epitope modified protein standard was performed using the Cell Signaling Enrichment kit. Using the optimized concentration of the protein standard mixture, 0.1% of the protein standard mixture was combined with the *M. oryzae* complex background, and Gly-Gly enrichment using anti-Gly-Gly was performed. The enriched peptides, the flow through, and a non-enriched sample were analyzed by LC-MS/MS. Fig. 4A is representative of an enriched modified peptide, **ck**GTDVQAWIR, identified in the enriched sample; the unmodified counterpart of this peptide was identified in the non-enriched sample Fig. 4B. The percent coverage of the protein standard was 78.91%, 10.88%, and 32.65%, respectively, with 100% (5/5) of identified

protein standard peptides having the Gly-Gly modification in the enriched sample, no Gly-Gly modified peptides in the flow through sample, and 25% (1/4) of peptides with the Gly-Gly modification in the non-enriched sample (Table S2). In order to determine how well the enrichment method was applied, enrichment efficiency was calculated using Equation 1:



Enrichment efficiency = $(\Sigma x'_{Enriched} / (\Sigma x'_{Enriched} + \Sigma x'_{Non\text{-}Enriched} + \Sigma x_{Enriched} + \Sigma x_{Non\text{-}Enriched}))$

Enrichment efficiency, derived from the sum of enriched modified protein peptides peak areas, x', divided by the total of the sum of all enriched peptide peak areas and the sum of the total non-enriched peptide peak areas [16], was calculated to be 99.3% (Table 1). By using the spiked in protein standard, we were able to observe, with the use of a simple calculation, that

Fig. 4. (A) The Gly-Gly modified protein standard peptide **ck**GTDVQAWIR in the enriched sample. (B) The unmodified peptide **c**KGTDVQAWIR in the non-enriched sample.

enrichment was performed as a result of the 99.3% enrichment of the protein standard.

4. Conclusions

Post-translational modification enrichment processes are at the forefront of the field of proteomics, and the development of these methods allows researchers to continue to gather valuable information on dynamic changes occurring throughout the proteome. This information

is then pieced together enabling the creation of a more complete picture used to explain what regulatory mechanisms are controlling responses to applied stimuli. However, with enrichment methods often comes extensive sample handling and introduction of variability that is difficult to account for. We demonstrate that with the use of a spiked in Gly-Gly epitope modified protein standard, peptide peak area of modified peptides can be used to calculate enrichment efficiency. Because the protein standard is spiked in from the beginning of sample handling and is characteristic of a ubiquitinated protein, any introduced variability that results in a change to the enrichment process and outcomes are revealed to the researcher.

Acknowledgements:

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Sequence	Modification	Enriched Peak Area	Non Enriched Peak Area
cELAAA**mk**	C1(Nethylmaleimide); M7(Oxidation); K8(GlyGly)	9.67E+08	0.00E+00
cELAAAMk	C1(Nethylmaleimide); K8(GlyGly)	9.59E+08	0.00E+00
cELAAA**mk**R	C1(Nethylmaleimide); M7(Oxidation); K8(GlyGly)	1.26E+10	1.42E+07
cELAAAMKR	C1(Nethylmaleimide); K8(GlyGly)	1.89E+10	7.62E+06
ckGTDVQAWIR	C1(Nethylmaleimide); K2(GlyGly)	1.65E+10	0.00E+00
ckGTDV**q**AWIR	C1(Nethylmaleimide); K2(GlyGly); Q7(Deamidated)	2.37E+08	0.00E+00
cKGTDVQAWIR	C1(Nethylmaleimide)	0.00E+00	5.18E+07
ckGTDVQAWIRG**c**RL	C1(Nethylmaleimide); K2(GlyGly); C13(Nethylmaleimide)	1.39E+07	0.00E+00
GYSLGNWV**c**AA**k**FESNFNTQATNR	C9(Nethylmaleimide); K12(GlyGly)	1.08E+09	0.00E+00
GYSLGNWV**c**AA**k**FESNFNTQATNRN TDGSTDYGILQINSR	C9(Nethylmaleimide); K12(GlyGly)	0.00E+00	0.00E+00
HGLDNYRGYSLGNWV**c**AA**k**FESNFN TQATNR	C16(Nethylmaleimide); K19(GlyGly)	1.75E+08	0.00E+00
kIVSDGNG**m**NAWVAWR	K1(GlyGly); M9(Oxidation)	2.08E+08	0.00E+00
kIVSDGNG**mn**AWVAWR	K1(GlyGly); N7(Deamidated); M9(Oxidation)	3.62E+07	0.00E+00
kIVSDGNGMNAWVAWR	K1(GlyGly)	1.72E+08	0.00E+00
NL**c**NIPCSALLSSDITASVNCAK**k**	C3(Nethylmaleimide); C7(Nethylmaleimide); C21(Nethylmaleimide); K24(GlyGly)	2.44E+07	0.00E+00
NR**ck**GTDVQAWIR	C3(Nethylmaleimide); K4(GlyGly)	1.49E+08	0.00E+00
NR**c**KGTDVQAWIR	C3(Nethylmaleimide)	5.56E+06	0.00E+00
NTDGSTDYGILQINSR		5.28E+07	1.39E+08
VFGR**c**ELAAA**mk**R	C5(Nethylmaleimide); M11(Oxidation); K12(GlyGly)	4.98E+07	0.00E+00
WW**c**NDGR	C3(Nethylmaleimide)	0.00E+00	2.51E+07
WW**c**NDGRTPGSR	C3(Nethylmaleimide)	0.00E+00	7.28E+07

Table 1. Protein Standard Enrichment Analysis

Note: Please refer to supplementary material for peptide sequence data.