Analytical Biochemistry 512 (2016) 114–119

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Examining ubiquitinated peptide enrichment efficiency through an epitope labeled protein

J. Parker [a, b], Y. Oh [c, d], Y. Moazami [a], J.G. Pierce [a], P.L. Loziuk [a, b], R.A. Dean [c, d], D.C. Muddiman [a, b, *]

[a] Department of Chemistry, North Carolina State University, Raleigh, NC, United States
[b] W. M. Keck FTMS Laboratory for Human Health Research, North Carolina State University, Raleigh, NC, United States
[c] Department of Plant Pathology, North Carolina State University, Raleigh, NC, United States
[d] Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC, United States

ARTICLE INFO

*Article history:*
Received 14 February 2016
Received in revised form
2 July 2016
Accepted 19 August 2016
Available online 22 August 2016

*Keywords:*
Ubiquitination
Gly-Gly enrichment
Enrichment efficiency

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.



## 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.

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

*Abbreviations:* 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.

* Corresponding author. 2620 Yarborough Dr, Raleigh, NC 27695, United States.
*E-mail address:* dcmuddim@ncsu.edu (D.C. Muddiman).

http://dx.doi.org/10.1016/j.ab.2016.08.017
0003-2697/

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

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 dichloromethane (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 (see Fig. 1).

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 °C overnight prior to use, unless otherwise noted. 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 °C for 1 h by constant shaking (200 rpm) on a plate rotator. This reaction was repeated three times. For deprotection 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 °C. 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 °C and overnight at 4 °C) against 20 mM acetic acid (3 L).

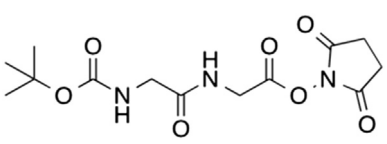

**Fig. 1.** Chemical structure of (*tert*-butoxycarbonyl) glycineglycine.

### 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 (8 M urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM PR-619, Protease Inhibitor cocktail (Roche)) [11]. Protein concentration was measured by Bradford assay.

### 2.5. Sample digestion

#### 2.5.1. Lysozyme

Lyophilized 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 lysozyme/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 °C 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 °C for 60 min. The alkylated sample was then transferred to a 10 kDa centrifugal filter (Millipore, #UFC501096) and concentrated by centrifuging at 14,000*g* at 20 °C, 15 min. The filter containing the sample was then washed 3 times with 2 M urea, 10 mM $CaCl_2$ in 50 mM Tris pH 7.0. Each sample was trypsin digested at a 1:50 enzyme:protein ratio. The digestion reaction was incubated overnight at 37 °C. The reaction was quenched with 400 μL of 1% formic acid in 0.001% Zwittergent, and subsequently centrifuged at 14,000*g* at 20 °C, 15 min to elute the peptides.