

Investigation of Cysteine Modifications in Recombinant Protein Tetanus Toxoid Heavy Chain Fragment C

Cindy X. Cai, Nicole A. Schneck, Taryn Cozine, Vera B. Ivleva, Daniel Ragheb, Deepika Gollapudi, Aakash Patel, Nathan Barefoot, Daniel B. Gowetski, and Q. Paula Lei*



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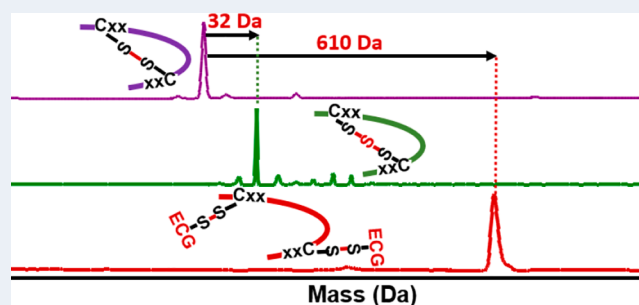


Article Recommendations



Supporting Information

ABSTRACT: For conjugated HIV-1 fusion peptide vaccine development, recombinant Tetanus toxoid heavy chain fragment C (rTTHC) was applied as a carrier protein to boost peptide immunogenicity. Understanding the characteristics of rTTHC is the first step prior to the peptide conjugation. A comprehensive mass spectrometry (MS) characterization was performed on *E. coli* expressed rTTHC during its purification process. Intact mass along with peptide mapping analysis discovered the existence of three cysteine modification forms: glutathionylation, trisulfide bond modification, and disulfide bond shuffling, in correlation to a three-peak profile during a hydrophobic interaction chromatography (HIC) purification step. Coexistence of these multiple oxidative forms indicated that the active thiols underwent redox reaction in the rTTHC material. Identity confirmation of the rTTHC carrier protein by MS analysis provided pivotal guidance to assess the purification step and helped ensure that vaccine development could proceed.



INTRODUCTION

Small peptides used as candidate vaccine antigens are often conjugated with a carrier protein, which improves peptide *in vivo* stability and efficacy for successfully inducing desirable T cell and B cell mediated immune response.¹ Tetanus toxoid is one of the commonly used carrier proteins for polysaccharide-based conjugate vaccines.² An HIV-1 vaccine under development is the HIV-1 fusion peptide covalently conjugated with a carrier protein, which in this case is the nontoxic recombinant tetanus toxoid heavy chain fragment C (rTTHC).³

A three-peak-profile was observed when hydrophobic interaction chromatography (HIC) was applied during rTTHC protein purification (Figure 1a). To evaluate if the HIC peaks were related to in-process impurities or different rTTHC variants, several high-throughput analytical tools were first applied, including size-exclusion chromatography (SEC) and capillary gel electrophoresis (CGE). Both SEC and CGE (Figure S1) analyses showed differences among the three HIC fractions, but all coeluted with the same profile after disulfide bond (DSB) reduction, which indicated different cysteine modifications on rTTHC in the HIC fractions. Based on the crystal structure, rTTHC contains four cysteine residues, two of which are involved in an intramolecular DSB, and the other two existing as free thiols.⁴ This suggested that the three HIC peaks likely correlated to different cysteine-related redox forms of rTTHC.⁵ Mass spectrometry provides mass measurement with high resolution, and a combination of different LC–MS approaches can offer unambiguous peak identification. There-

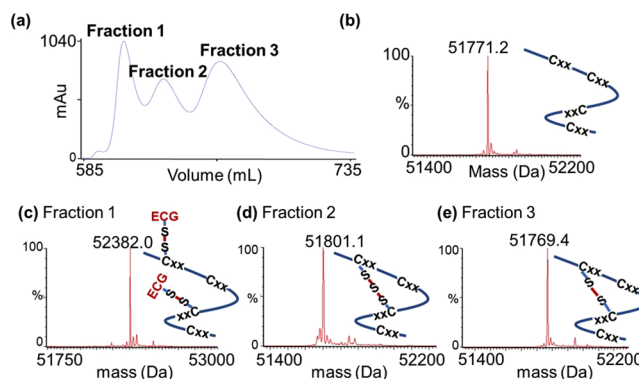


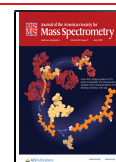
Figure 1. Intact mass analysis of (a) three rTTHC peaks from preparative HIC. (b) Reduced intact mass analysis of three fractions showed the same deconvoluted mass 51771.2 Da, corresponding to rTTHC without DSB. Without reduction, (c) fraction 1 showed mass 52382.0 Da corresponding to rTTHC with two glutathionylation modifications, (b) fraction 2 showed mass 51801.1 Da corresponding to one TSB modification, and (d) fraction 3 showed mass 51769.4 Da corresponding to one DSB linkage.

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fore, with the complementary mass spectrometry approaches such as peptide mapping and intact protein analysis,⁶ the aberrant peaks can be well characterized.

EXPERIMENTAL SECTION

Materials and Reagents. LC–MS grade water and acetonitrile were purchased from J. T. Baker (Center Valley, PA); formic acid and *N*-ethylmaleimide (NEM) were from Thermo Fisher Scientific (Tempe, AZ). Guanidine HCl and dithiothreitol (DTT) were purchased from G Bioscience (St. Louis, MO). Lys-C was purchased from Promega (Madison, WI); frozen *E. coli* cell paste containing rTTHC protein was obtained from Massachusetts General Hospital (MGH) and stored at -70°C for 3 years prior to preparation of test articles.

rTTHC Purification. Cell paste was suspended in a $\sim 20\%$ (v/v) slurry, lysed using a microfluidizer, and purified by a two-column chromatography process. First, capture was performed using Q-XL resin (Cytiva, Marlborough, MA) followed by subsequent elution using 0.8 M ammonium sulfate and 20 mM Tris–HCl (pH 7.5). Second, the conditioned eluate was loaded onto a Butyl HP polishing column (Cytiva, Marlborough, MA) and eluted with 20 mM Tris–HCl and 0.5 M ammonium sulfate (pH 7.5). Butyl HP fractions were collected in accordance with the elution peaks (Figure 1a).

Sample Preparation. For the intact protein mass analysis, each sample was injected before and after DTT reduction. For DSB peptide mapping analysis, each sample was denatured using guanidine HCl, capped with NEM, buffer exchanged to 50 mM ammonium bicarbonate, and then digested with Lys-C protease at 37°C overnight. The mass ratio of protein to enzyme was 20:1. Each sample was then split into two portions for nonreduced and DTT-reduced LC–MS/MS analyses.

LC–MS/MS Analysis. The Acquity H-class Bio UPLC system (Waters, Milford, MA) was operated using MassLynx v4.1 and coupled with MS detection for analysis. Mobile phases A and B consisted of 0.1% (v/v) formic acid in water and acetonitrile, respectively, and were delivered at a flow rate of 0.2 mL/min. The mass spectrometer was operated in positive ionization mode. For intact protein mass analysis, the SYNAPT G2 QToF (Waters) was operated with a detection range of 600–4500 Da. Samples were injected onto a BEH C4 column (300 Å, 1.7 μm , 2.1 mm $\times$ 50 mm) heated at 80°C . The UPLC gradient was (min–%B) 0–25%, 2–25%, 30–37%, 30.1–90%, 35–90%, 35.1–25%, and 45–25%. For peptide mapping analysis, a Q Exactive HF mass spectrometer (Thermo-Fisher Scientific) was operated with full mass scan in the range of 150–2000 Da, followed by 10 MS/MS scans. Samples were injected onto a BEH C18 column (300 Å, 1.7 μm , 2.1 mm $\times$ 150 mm) heated at 65°C . The UPLC system gradient (min–%B) was 0–3%, 1–3%, 43–90%, 100–90%, 102–3%, and 113–3%. BioPharma Finder v.2.0 was applied for data processing (mass error <5 ppm) followed by manual verification.

RESULTS AND DISCUSSION

The intact protein mass results were first compared between the reduced and nonreduced rTTHC fractions using reversed-phase LC–MS analysis. The deconvoluted mass (Figure 1) of 51771.2 Da was observed for all three fractions after reduction, matching the molecular weight (MW) of rTTHC without any DSB linkage (theoretical mass 51770.3 Da). Without

reduction, fraction 1 showed MW 52382.0 Da, 611.7 Da higher than the theoretical reduced MW of rTTHC, matching the MW with the addition of two glutathionylation groups (theoretical mass shift of 610.6 Da with the addition of tripeptide, ECG, components of glutathione).⁷ The nonreduced fraction 2 showed MW at 51801.1 Da, 32.8 Da higher than rTTHC with one DSB linkage (theoretical mass 51768.3 Da). This suggested that either double oxidation or a less uncommon PTM, a trisulfide bond modification (TSB, addition of one sulfur atom),⁸ was present on rTTHC. However, this mass shift disappeared after cysteine reduction, which supported the TSB hypothesis.⁹ Lastly, the nonreduced fraction 3 showed MW 51769.4 Da, corresponding to rTTHC with one pair DSB.

Based on the intact protein mass analysis, these plausible modifications were further verified by peptide mapping experiments. This commonly used bottom-up strategy is more applicable for protein sequence confirmation, post translational modification (PTM) identification and DSB characterization.^{10,11} First, >90% amino acid sequence coverage for all HIC fractions confirmed different rTTHC variants. Second, multiple DSB linkages were only detected in the nonreduced samples in all three fractions. As shown in the extracted ion chromatogram (XIC) of the MS full scan in fraction 3 (Figure 2), a DSB-linked L211–K225/F226–K231 (via C214–230) peptide eluted at 30.1 min (deconvoluted mass 2605.256 Da, Figure S2a). In fraction 1, a glutathionylated N3–K19 peptide (tripeptide ECG added via DSB

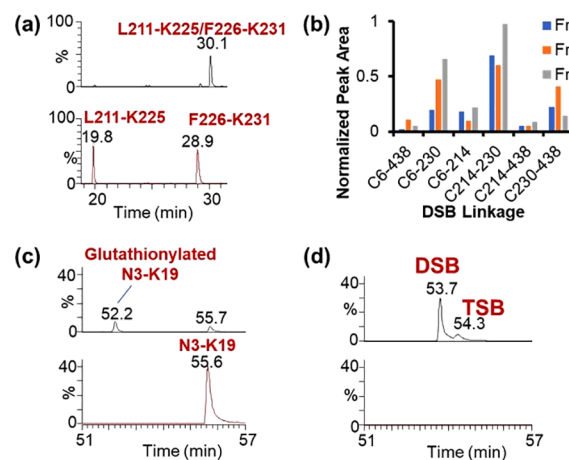


Figure 2. XICs of Lys-C digested HIC fractions, the nonreduced one on the top and reduced one at the bottom for each set of chromatograms. (a) DSB C214–230 linked peptide L211–K225/F226–K231 eluted at 30.1 min, which disappeared after reduction. The peak intensities increased for the individually peptides, L211–K225 (19.8 min) and F226–K231 (28.9 min) (m/z range 407.5–409.5, 652.8–653.8, 898.0–900.0, and 529.5–531.5). (b) Comparison of multiple DSBs among three HIC fractions. (y-axis is each DSB-linked peptide peak area normalized against the area of peptide G¹³⁴NNLIWTLK¹⁴², which does not contain cysteine or coelute with other peptides and showed high signal.) (c) Glutathionylated C6 peptide N3–K19, eluted at 52.2 min, which disappeared after reduction. The peak intensities increased for the unmodified peptides, N3–K19 (55.6 min) (m/z range 1169.3–1171.3, 1016.8–1018.8 and 529.5–531.5). (d) DSB and TSB C6–230-linked peptide N3–K19/F226–K231 eluted at 53.7 and 54.3 min, respectively, both of which disappeared after reduction (m/z range 711.4–713.4, 719.4–721.4 and 529.5–531.5) (y-axis of XICs was normalized against the peptide G134–k142 m/z 529.8).

linkage, mass shift of +305 Da)¹² eluted at 52.2 min (2337.010 Da, Figure S3a). In fraction 2, a DSB C6–230 linked peptide N3–K19/F226–K231 eluted at 53.7 min (2842.364 Da, Figure S4a). A TSB-linked peptide with a mass shift of 32 Da eluted at 54.3 min (2874.339 Da, Figure S4b).

MS/MS further proved the hypothesized cysteine modifications. On the basis of literature studies, residues C6 and C230 are in close distance and likely to form a DSB,¹³ which was confirmed in this study (Figures S5 and S6). However, additional DSBs, not reported before, were also observed in high abundance. Here, peptide F226–K231-linked b5–b10 fragments of L221–K225 via DSB C214–C230 were reported in rTTHC for the first time (Figure 3a). On the basis of the

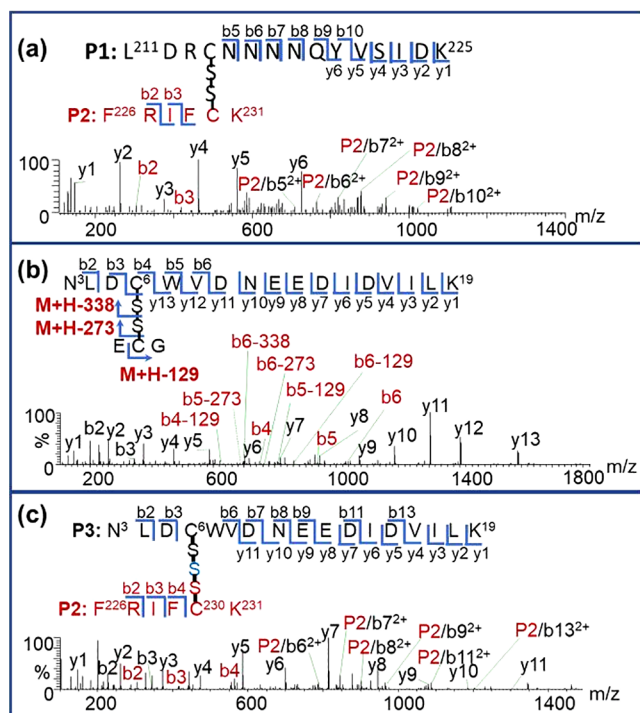


Figure 3. MS/MS spectra for DSB linkages showed the b/y-ions of individual peptide and the DSB-linked characteristic fragment ions for (a) DSB C214–230-linked peptide 1 (P1) L211–K225 and peptide 2 (P2) F226–K231, glutathionylated C6 (peptide N3–K19), and (c) TSB C6–230-linked peptide 3 (P3) N3–K19/ (P2) F226–K231.

normalized peak areas of full scan mass signals (Figure 2b), C214–C230 and C6–C230 were the main DSBs in HIC fraction 3. However, both DSBs were present at a lower level in HIC fraction 1, due to higher abundance of other cysteine modifications. The amount of DSB C214–C230 was also abundant in HIC fraction 2. The three DSBs all contained C230, which was a predominant cysteine residue involving in DSB scrambling. Other DSBs, C6–438 and C214–438, were observed but at lower levels. Thus, DSB shuffling was apparent in all three fractions.

Additionally, a straightforward comparison of b/y-ion series between the corresponding nonmodified (Figure S3c) and glutathionylated peptides (Figure 3b) further provided evidence for glutathionylation. Consistent y-ions (y1–y13) were detected for both peptides, but a +305 Da mass difference for the b-ions (b4–b6) was observed (Table S1). The b-ions retaining the unique DSB structure of a glutathione (GSH) moiety were also detected, including a neutral loss of glutamic

acid (E, –129 Da) and cleavages at either side of the DSB yielding a loss of GSH (–273 Da and –338 Da). Overall, the glutathionylation modifications were confirmed at all four cysteine residues and potentially reduced the formation of DSBs. Two glutathionylation modifications on the rTTHC molecule resulted in a + 610 Da mass shift for the intact protein mass analysis in HIC fraction 1. Finally, a 32 Da mass difference (Table S2) of MS/MS ions was also detected between the corresponding DSB- (Figure S4c) and TSB-linked fragments (Figure 3c), F226–K231-linked b6–b13 of N3–K19. Besides TSB C6–C230, TSBs C214–C230 and C230–C438 were also identified. Overall, the TSBs were demonstrated and correlated to the +32 Da mass shift in the intact mass analysis of HIC fraction 2.

A combination of the intact mass and peptide mapping analyses revealed that HIC fractions 1, 2, and 3 mainly correlated to three cysteine modifications: glutathionylation, TSB, and DSB shuffling for rTTHC, while the protein was stored in cell culture paste. The freeze and thaw cycles were not the reason triggering these protein variants since none of the cysteine modifications were observed for purified rTTHC stored under the same condition. We propose that oxidative stress might play an important role of breaking of native-linked DSB, which then initiated protein conformational change and DSB scrambling even under the low temperature storage conditions (–70 °C).^{14,15} While GSH naturally exists in cell culture media, two active free thiols can conjugate with GSH through DSB interaction and result in glutathionylation.¹⁶ Furthermore, TSB levels can increase while proteins are incubated with hydrogen sulfide, which can be generated via the catabolism of cysteine during the cell culture process.¹⁵ For our case, the in-depth MS characterization elucidated the material quality and provided identification of the aberrant peak phenomena as different oxidative modifications of cysteine residues. Since lysine residues were involved in the linker conjugation reaction, the cysteine modifications on rTTHC posed minimal risk for the conjugation step using this heterogeneous carrier protein. Although cross-linking could occur between cysteine residues within or among rTTHC molecules, this would potentially facilitate the formation of a larger and more immunogenic conjugate, which however was beyond the scope of this study.

CONCLUSIONS

MS characterization successfully elucidated the identities of the recombinant protein rTTHC variants. This tool can be applied to other recombinant protein analysis when free cysteine contributes to protein heterogeneity. Coexistence of three oxidative modification states on cysteine (i.e., glutathionylation, TSB formation, and DSB scrambling) was revealed for the first time, which did not impact the conjugation reaction. To this end, the conjugated vaccine development was able to proceed successfully using this purified rTTHC carrier protein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.1c00075>.

Methods for SEC and CGE, Figures S1–S6, and Tables S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Investigation of Cysteine Modifications in Recombinant Protein Tetanus Toxoid

Heavy Chain Fragment C (rTTHC)

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Methods for SEC and CGE:

For both SEC and CGE analysis, each sample was injected before and after DTT reduction. For SEC method, the Acquity H-class UPLC system (Waters, Milford, MA) coupled with UV detector was operated using Empower 3. Mobile phase is 2x PBS, an isocratic run of 30 minutes was setup at a flow rate of 0.3 mL/min. Samples were injected onto a Tosoh TSKgel UP-Sw3000 (4.6x300mm, 2 μ m, 250Å) at the ambient temperature. For CGE method, lab-on-chip GXII instrument (Perkin Elmer, Waltham, MA) with fluorescent detection was used for protein size analysis under reduced and non-reduced conditions. For GXII analysis, samples were labeled with fluorescent dye and analyzed at the ambient temperature. Data were analyzed using Empower 3.

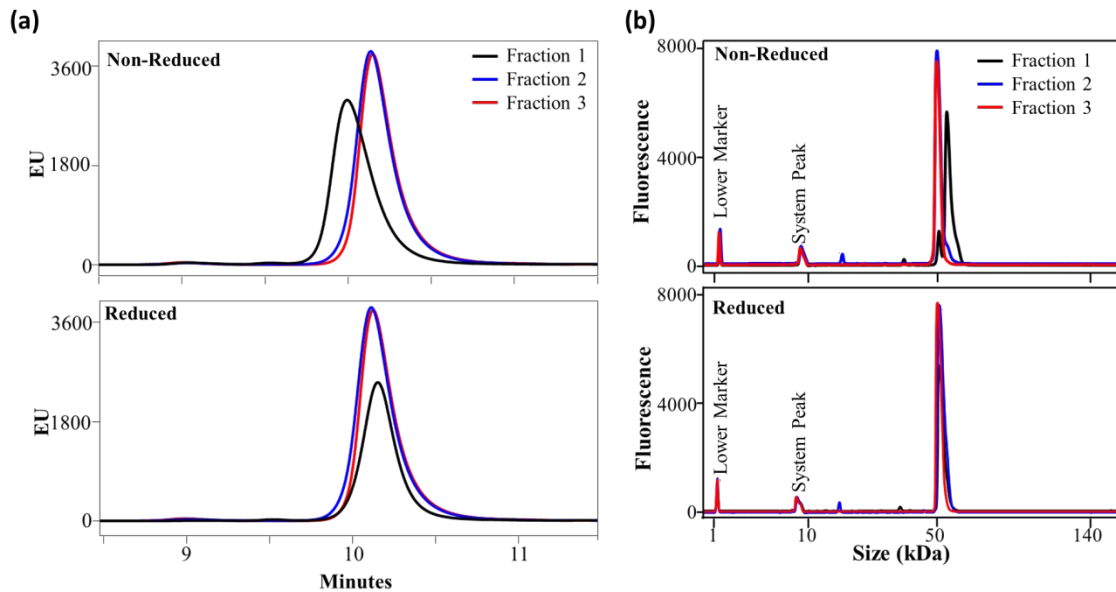


Figure S1. Overlays of (a) size exclusion chromatogram (SEC) and (b) capillary gel electropherogram (CGE) for three HIC rTTHC fractions. Non-reduced samples on the top and the reduced ones at the bottom. Fraction 1 eluted out earlier in SEC but later in the CGE compared to fractions 2 and 3 when injected as the non-reduced form, indicating the differences among the fractions. However, the traces of the three fractions overlapped well after DTT reduction, suggesting the modification could potentially occur on cysteine residues.

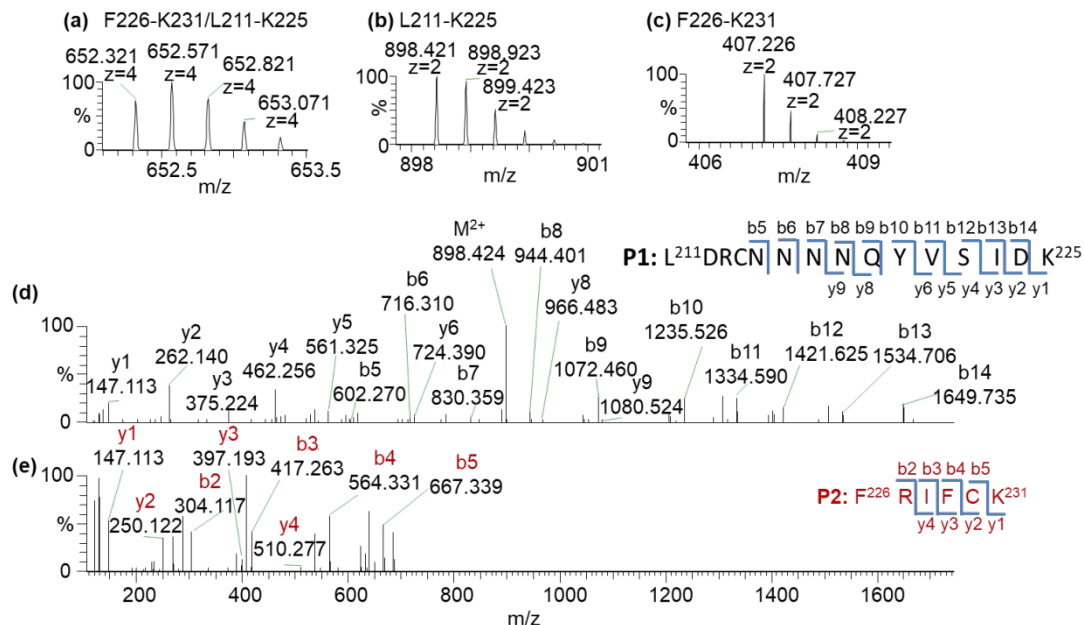


Figure S2 MS analysis of DSB C214-230, peptide L211-K225 and F226-K231 in non-reduced and reduced Lys-C digest of HIC fraction 3 from Figure 2a. (a) The DSB linked peptide L211-K225/F226-K231 eluting at 30.1 min was observed m/z 652.321 (deconvoluted mass 2605.256 Da, mass error 1 ppm). (b) The reduced peptide L211-K225 eluting at 19.8 min was observed m/z 898.421 (mass 1794.826 Da, mass error -3 ppm), and (c) the peptide F226-K231 eluted at 28.9 min was observed m/z 407.226 (mass 812.434 Da, mass error -3 ppm). MS/MS fragments of b/y-ions further proved the peptide identification for (d) L211-K225 and (e) F226-K231.

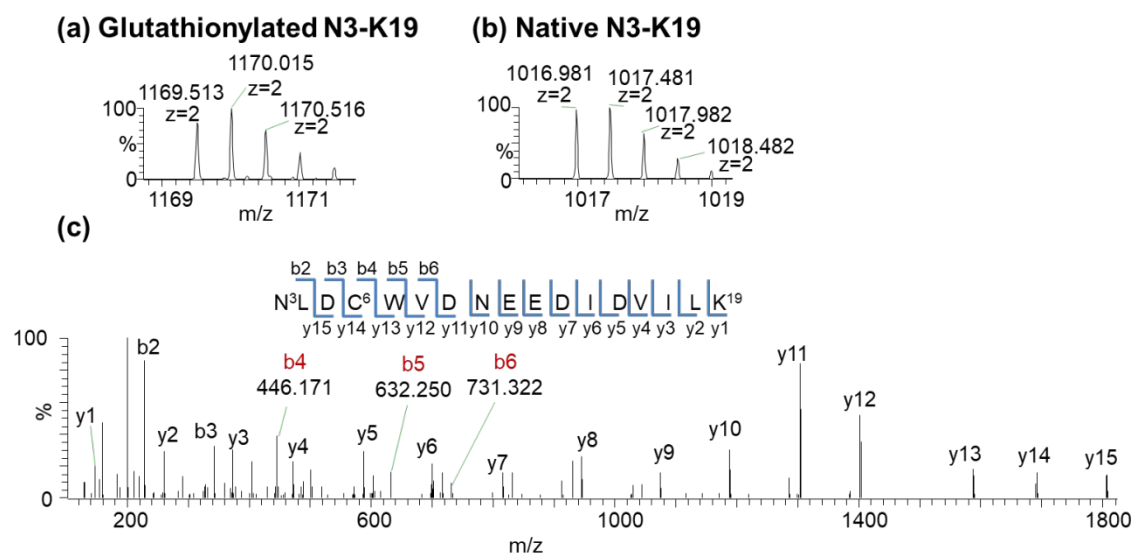


Figure S3 MS analysis of glutathionylated peptide for C6, peptide N3-K19 in non-reduced and reduced Lys-C digest of HIC fraction 1 from Figure 2c. (a) Glutathionylated N3-K19 eluting at 52.2 min was observed m/z 1169.513 (mass 2337.010 Da, mass error - 2 ppm), (b) native N3-K19 eluting at 55.6 min was observed m/z 1016.981 (mass 2031.946 Da, mass error 0 ppm). (c) MS/MS fragments further proved the peptide identification for unmodified peptide N3-K19.

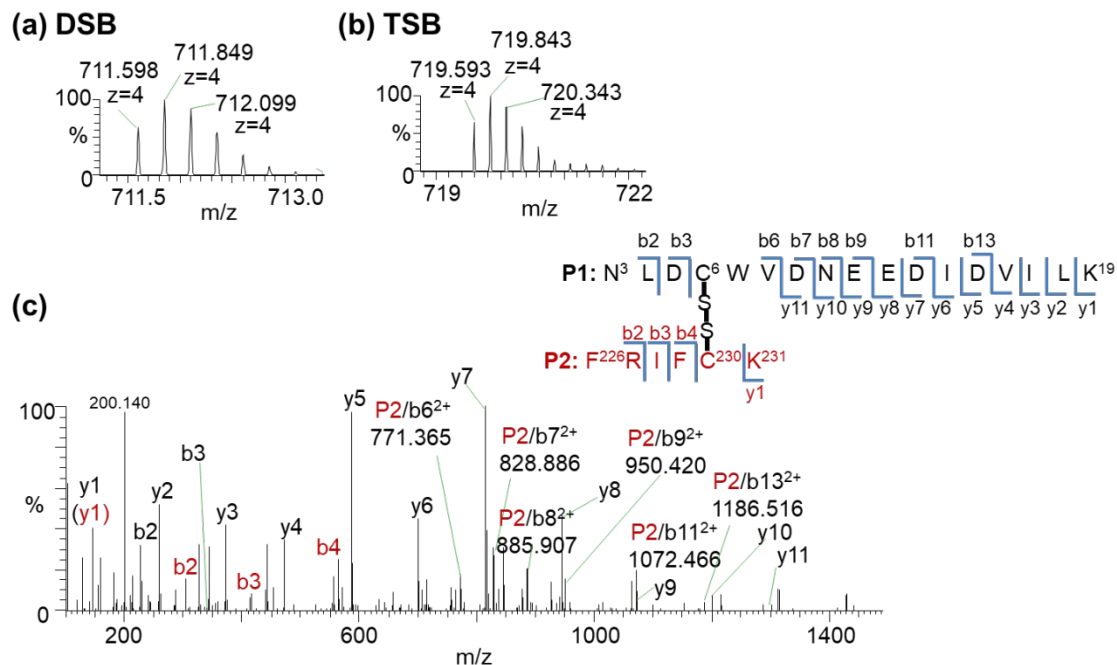


Figure S4 MS analysis for DSB and TSB C6-230, peptide N3-K19/F226-K231 in non-reduced Lys-C digest of HIC fraction 2 from Figure 2d. (a) DSB linked peptide eluting at 53.7 min was observed m/z 711.598 (mass 2842.367 Da, mass error -2 ppm), and (b) the TSB linked peptide eluting at 54.3 min was observed m/z 719.593 (mass 2874.339 Da, mass error 1 ppm). (c) b/y ions for individually peptides as well as F226-K231 linked b6-b13 fragments of N3-K19 were observed and proved the existence of DSB C6-230.

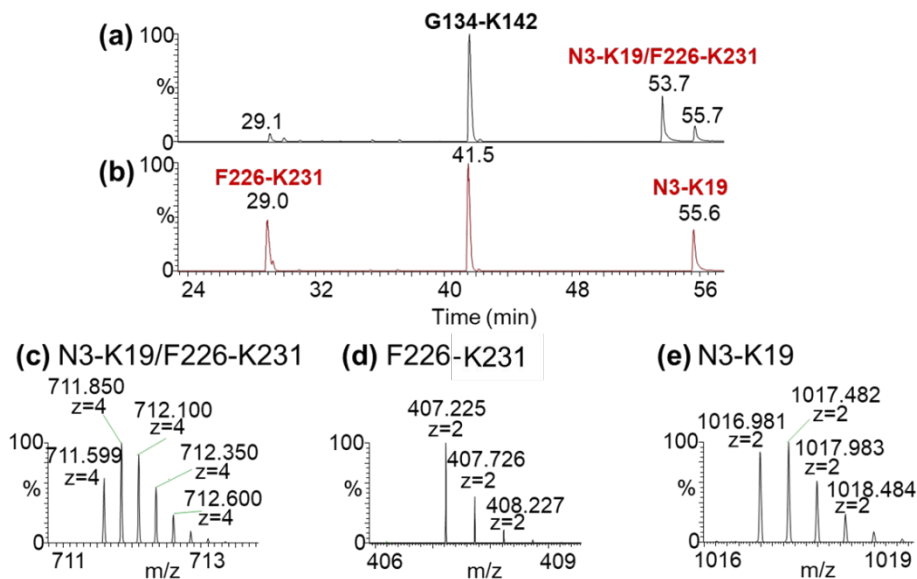


Figure S5 MS full scan analysis of the DSB C6-230, peptide N3-K19 and F226-K231 for Lys-C digested HIC fraction 3. The XICs (m/z 407.5-409.5, 529.5-531.5, 711.0-713.0 and 1016.5-1018.5) of (a) non-reduced sample showed the DSB linked peptide N3-K19/F226-K231 eluted at 53.7 min. (b) After DTT reduction, the peptide F226-K231 (FRIFCK) eluted at 29.0 min and N3-K19 (NLDCWVDNEEDIDVILK) eluted at 55.6 min, but the peptide N3-K19/F226-K231 peak disappeared. (c) The DSB-linked peptide was observed at m/z 711.599 (mass 2842.364 Da, mass error -1 ppm), (d) the peptide F226-K231 was observed at m/z 407.226 (mass 812.434 Da, mass error -4 ppm), and (e) the peptide N3-K19 was observed at m/z 1016.981 (mass 2031.946 Da, mass error 0 ppm). (y-axis of XICs was normalized against peptide G¹³⁴NNLIWTLK¹⁴², 41.5 min, m/z 529.8, which doesn't contain cysteine or coelute with other peptides and showed high signal.)

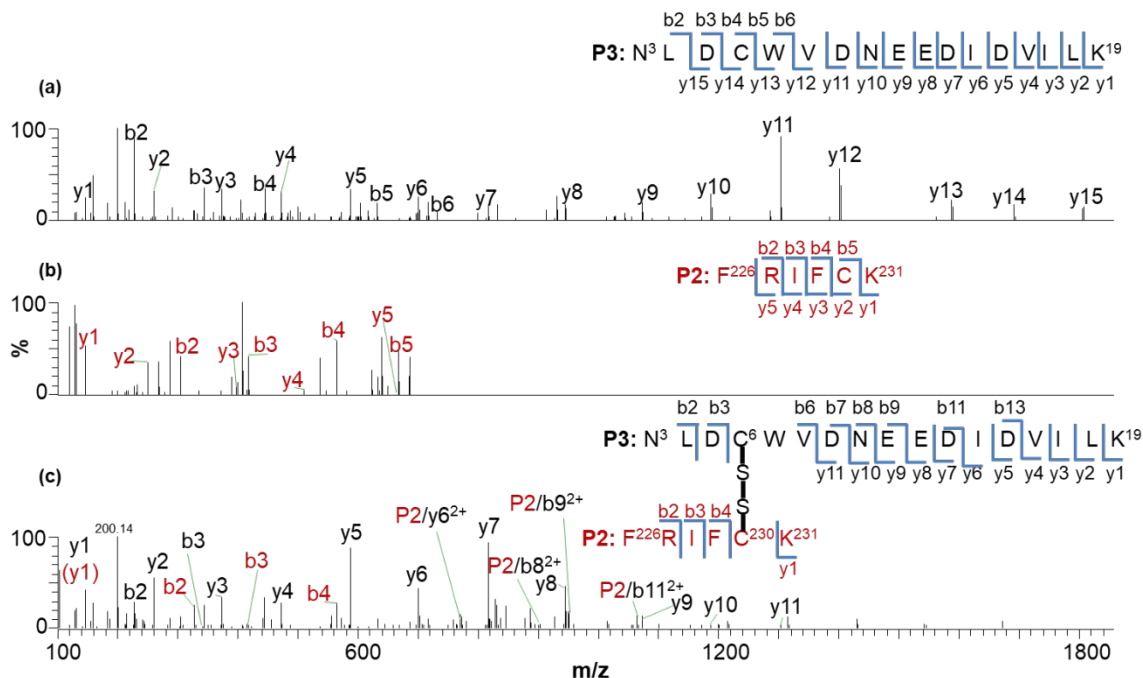


Figure S6 MS/MS analysis of DSB C6-230, individual and DSB-linked peptide 1 (P3) N3-K19 and peptide 2 (P2) F226-K231 of Lys-C digested HIC fraction 3. In reduced digest, b/y-ions were observed for (a) P1 (precursor ion m/z 1017.5) labeled in black color and (b) P2 F226-K231 (precursor ion m/z 407.2) labeled in red color. (c) In the non-reduced sample, some b/y-ions of peptide N3-K19 and F226-K231 were observed. More importantly, the MS/MS fragment ions for the characteristic DSB linkage peptide, F226-K231 linked b6-b11 fragments of N3-K19, were observed and critical to prove the existence of DSB C6-230.

Table S1. The mass shifts for C6 glutathionylated MS/MS ions of peptide N3-K19. (Data were from Figure 2b and Figure S3c.)

Fragment ions of peptide N3-K19	Fragment ion m/z (z=1)		Mass difference (Da)
	Glutathionylated	Unmodified	
b4	751.236	446.171	305.065
b5	937.321	632.250	305.071
b6	1036.371	731.322	305.049

Table S2. The mass differences between TSB and DSB linked MS/MS ions for C6-230, peptide N3-K19/F226-K231. (Data were from Figure 3c and Figure S4c.)

Fragment ions	Fragment ion m/z (z=2)		Fragment ion mass (Da)		Mass difference (Da)
	TSB	DSB	TSB	DSB	
P2/b6	787.355	771.365	1572.694	1540.714	31.980
P2/b7	844.868	828.886	1687.720	1655.756	31.964
P2/b8	901.891	885.907	1801.766	1769.798	31.968
P2/b9	966.412	950.42	1930.808	1898.824	31.984
P2/b11	1088.459	1072.466	2174.902	2142.916	31.986
P2/b13	1202.514	1186.516	2403.012	2371.016	31.996