Supporting Information

Cross-Linking Electrochemical Mass Spectrometry for Probing Protein Three-Dimensional Structures

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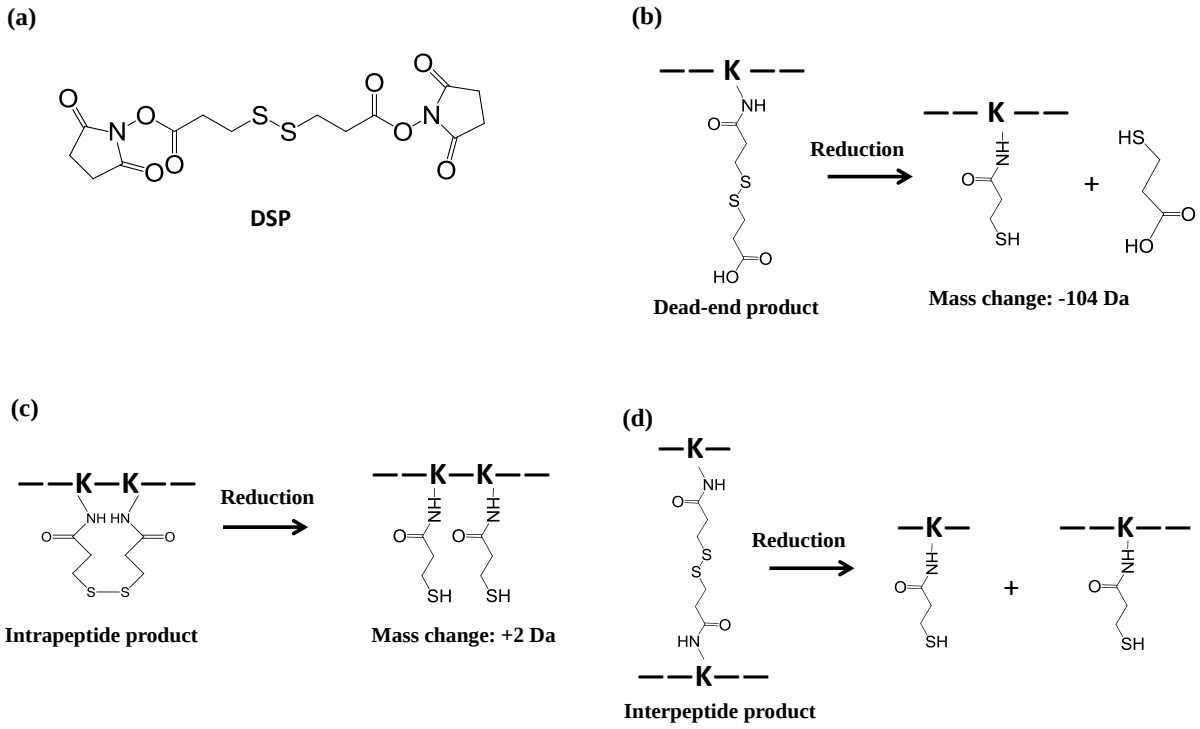
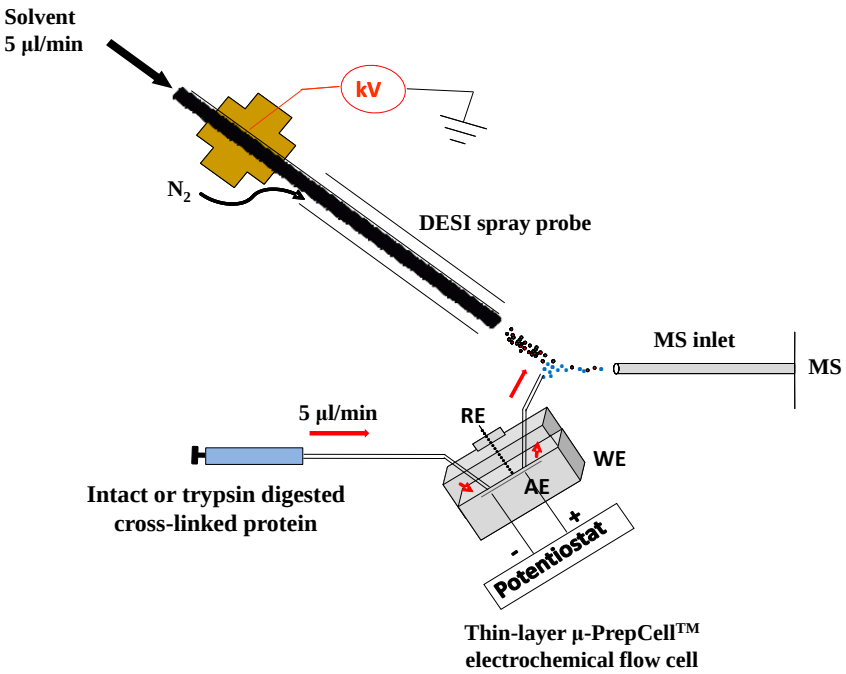


Scheme S-1. (a) Structure of DSP; possible cross-linking reaction products and their conversion upon electrochemical reduction: (b) dead-end cross-linked product, (c) intrapeptide cross-linked product and (d) interpeptide product.



Scheme S-2. Schematic showing the apparatus of online electrolytic reduction and online MS analysis of cross-linked products in mixture

Detailed DSP cross-linking experimental procedure

Peptide HCKFWW

A 100 μL of aqueous peptide solution (100 μM) was mixed with 1 μL of 10 mM DSP in DMSO and reacted for 1 h at room temperature. Then, 250 mM NH_4HCO_3 was used to quench the reaction. C18 Ziptip was used for purifying and enriching the modified peptide which was dissolved in MeOH:H_2O (3:2 by volume) containing 0.1% FA to a final concentration of 20 μM for EC/DESI-MS analysis.

Insulin

A 200 μL of 100 μM insulin in 10 mM PBS (pH 7.4) and 20 μL of 10 mM DSP in DMSO were mixed and reacted for 1 h at room temperature. Then, 250 mM NH_4HCO_3 was used to quench the reaction. The resulting mixture solution was desalted using a 5000 molecular weight cutoff filter. The desalted protein was re-dissolved in MeOH:H_2O (1:1 by volume) containing 1% FA to a final concentration of 20 μM, and was subject to EC/DESI-MS analysis.

To cross-link denatured insulin, 100 μL of 100 μM insulin in 1% formic acid was first mixed with 50 μL of 8 M urea for denaturation. The mixture was desalted via centrifugation to remove access amount of urea by using a 3000 Da molecular weight cutoff filter and re-dissolved in 100 μL of H_2O. A 20 μL of 10 mM DSP in DMSO was added and reacted for 1 h at room temperature. The resulting mixture solution was desalted using a 5000 molecular weight cutoff filter and the desalted protein was re-dissolved in MeOH:H_2O (1:1 by volume) containing 1% FA to a final concentration of 20 μM for EC/DESI-MS analysis.

Ubiquitin

A 600 μL of 200 μM ubiquitin solution in 10 mM PBS buffer (pH 7.4) and 120 μL of 10 mM DSP in DMSO were mixed and reacted for 1 h at room temperature. Then, 250 mM NH_4HCO_3 was used to quench the reaction. The resulting mixture solution was desalted using a

5000 molecular weight cutoff filter and was re-dissolved in 25 mM NH_4HCO_3. A 24 μL of 200 μM trypsin was added (protein:enzyme=25:1) for overnight digestion. The C18 Ziptip was used for further sample desalting. The desalted protein was re-dissolved in MeOH:H_2O (1:1 by volume) containing 1% FA for EC/DESI-MS analysis to a final concentration of 20 μM, before EC/DESI-MS analysis.

Calmodulin-Melittin complex

A 3 mL of 20 μM of calmodulin and melittin in $CaCl_2$ (80 μM in PBS, pH 7.4) and 120 μL of 10 mM DSP in DMSO were mixed and reacted for 1 h at room temperature. Then, 250 mM NH_4HCO_3 was used to quench the reaction. The resulting mixture solution was desalted using a 5000 molecular weight cutoff filter. Then the sample was re-dissolved in 25 mM NH_4HCO_3 and 12 μL of 200 μM trypsin was added (protein:enzyme=25:1) for overnight digestion. C18 Ziptip was used for purifying and the mixture was re-dissolved in MeOH:H_2O (3:2 by volume) containing 0.1% FA to a final concentration of 20 μM for EC/DESI-MS analysis.

Tables

Table S-1. Relative intensity changes of peptide ions after electrolysis (using the peak of the disulfide-free peptide ion $[HCK^{3}FWW+2H]^{2+}$ as the reference)

Peptide	Peptide ions	*m/z*	Relative intensity before reduction (%)	Relative intensity after reduction (%)	Relative change of relative intensity (%)
HCKFWW	$[P1+2H]^{2+}$	540.7	171.80	64.47	-62
	$[P2+2H]^{2+}$	549.7	42.77	26.91	-37

Table S-2. Solvent accessibilities of N termini and lysine residue of insulin*

Residue	Position	Total	Apolar	Backbone	Sidechain	Ratio (%)	In/Out
G	1 (A Chain)	58.3	27.3	58.3	0.0	66.9	Out
F	1 (B Chain)	163.7	132.3	54.3	109.4	60.7	Out
K	29 (B Chain)	16.2	0.0	16.2	0.0	0.0	In

*****Solvent accessibility data was obtained by using online software GETAREA 1.1 for protein surface area calculation and insulin crystal data file ("1APH.pbd") downloaded from Protein Data Bank.

Table S-3. Relative intensity changes of peptide ions after electrolysis (using the peak of the disulfide-free peptide $[LGEDNINVVEGNEQFISASK+2H]^{2+}$ as the reference)

Protein	Peptide ions	*m/z*	Relative intensity before reduction (%)	Relative intensity after reduction (%)	Relative change of relative intensity (%)
Ubiquitin	**1** $[P3+2H]^{2+}$	769.8	3389.7	1175.68	**-65**
	2 $[P4+3H]^{3+}$	775.0	166.2	109.46	**-34**
	3 $[TITLEVEPSDTIENVK+2H]^{2+}$	894.4	214.0	198.65	-7
	4 $[P5+2H]^{2+}$	948.9	160.3	93.24	**-42**
	6 $[P6+3H]^{3+}$	1217.2	14.9	0.00	**-100**

Table S-4. Relative intensity changes of peptide ions after electrolysis (using the peak of the disulfide-free peptide $[VLTTGLPALISWIK+2H]^{2+}$ as the reference)

Complex	Peptide ions	*m/z*	Relative intensity before reduction (%)	Relative intensity after reduction (%)	Relative change of relative intensity (%)
Calmodulin-Melittin	**1** $[P7+3H]^{3+}$	744.7	1.65	1.03	**-38**
	3 $[HVMTNLGEKLTDEEVDEMIR+3H]^{3+}$	787.4	8.72	8.28	-5
	4 $[P8+H]^{+}$	849.4	28.92	14.95	**-48**
	5 $[P9+2H]^{2+}$	930.5	35.76	14.24	**-60**
	6 $[P10+2H]^{2+}$	974.4	50.35	23.20	**-54**
	7 $[EADIDGDGQVNYEEFVQMMTAK+2H]^{2+}$	1246.0	11.67	9.90	-15
	8 $[VLTTGLPALISW+H]^{+}$	1270.7	9.24	9.49	3

Additional DESI-MS and MS/MS data

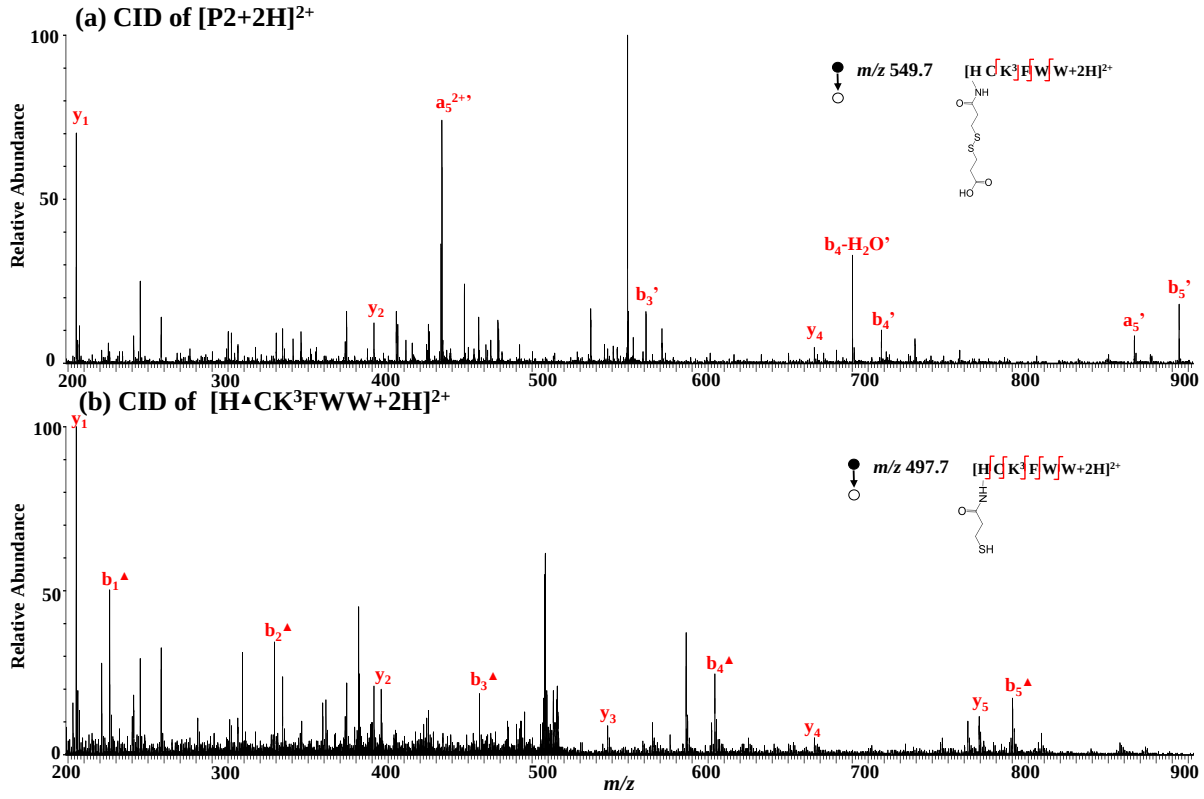


Figure S-1. CID MS/MS spectra of (a) $[P2+2H]^{2+}$ (*m/z* 549.7); and (b) $[H^{▲}CK^{3}FWW+2H]^{2+}$ (*m/z* 497.7). The symbol ' denotes a dead-end tag of $–C(O)CH_2CH_2SSCH_2CH_2COOH$ and ▲ denotes one reduced tag of $–C(O)CH_2CH_2SH$.

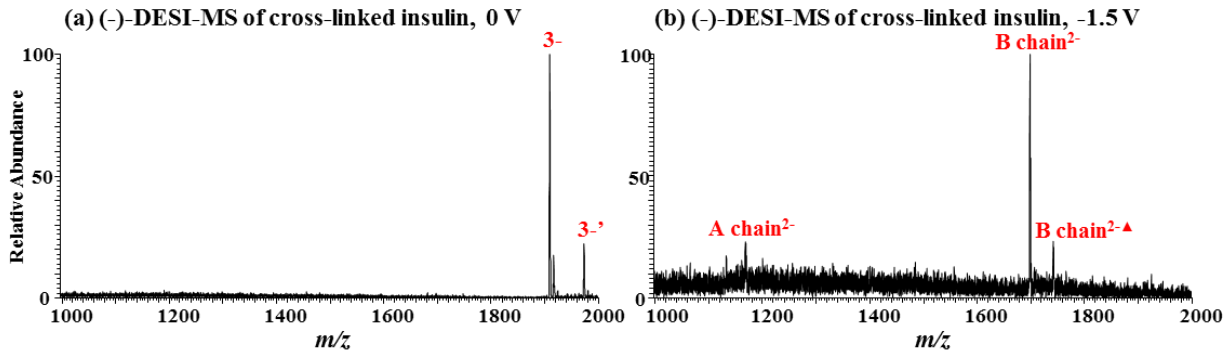


Figure S-2. (-)-DESI-MS spectra of cross-linked insulin (a) before and (b) after electrochemical reduction (applied potential: -1.5 V).

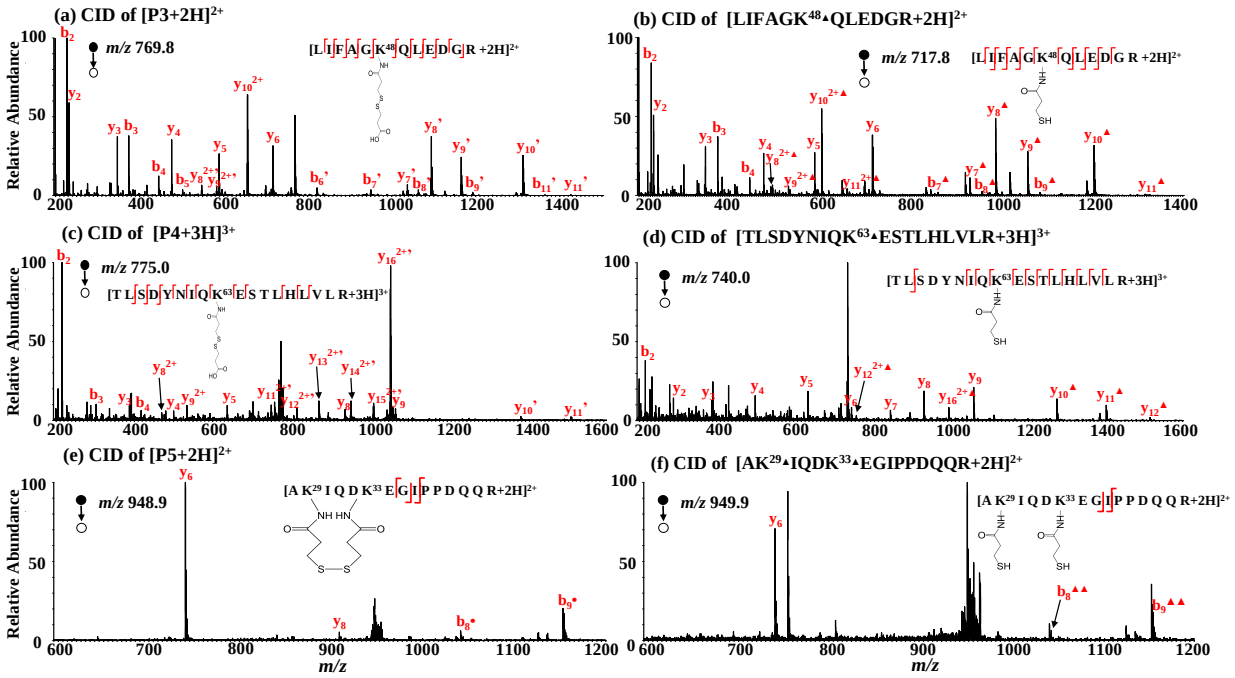


Figure S-3. CID MS/MS spectra of (a) $[P3+2H]^{2+}$ (*m/z* 769.8), (b) $[LIFAGK^{48}▲QLEDGR+2H]^{2+}$ (*m/z* 717.8); (c) $[P4+EH]^{3+}$ (*m/z* 775.0) (d) $[TLSDYNIQK^{63}▲ESTLHLVLR+3H]^{3+}$ (*m/z* 740.0); (e) $[P5+2H]^{2+}$ (*m/z* 948.9); and (f) $[AK^{29}▲IQDK^{33}▲EGIPPDQQR+2H]^{2+}$ (*m/z* 949.9).

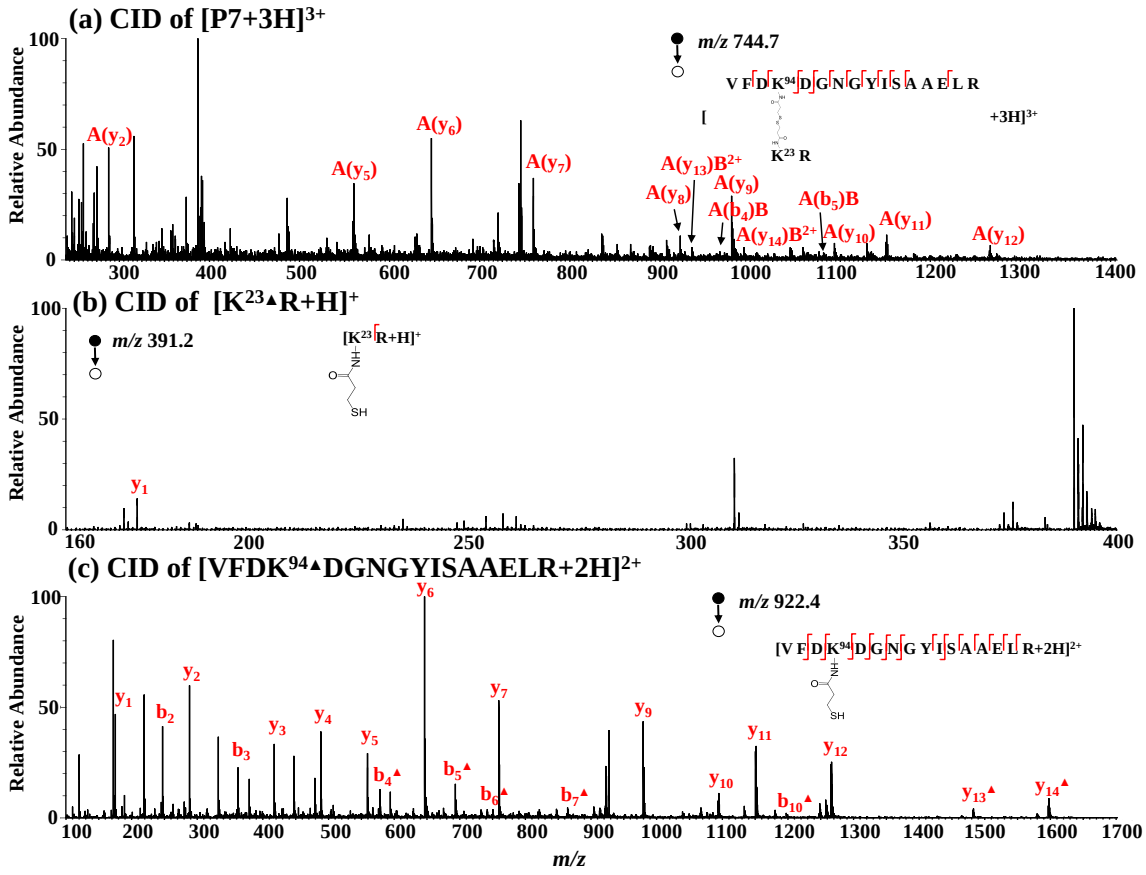


Figure S-4. CID MS/MS spectra of (a) $[P7+3H]^{3+}$ (*m/z* 744.7); (b) $[K^{23}▲R+H]^{+}$ (*m/z* 391.2); (c) $[VFDK^{94}▲DGNGYISAAELR+2H]^{2+}$ (*m/z* 922.4).

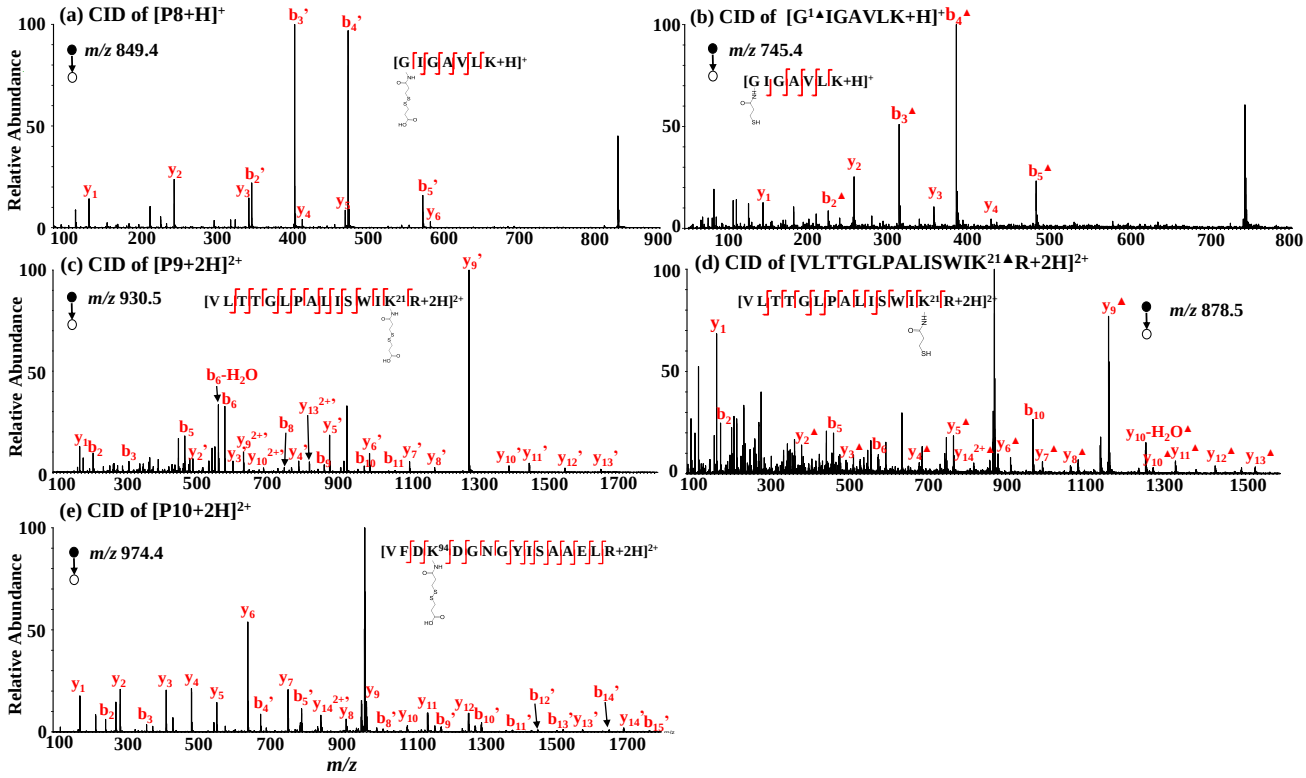


Figure S-5. CID MS/MS spectra of (a) $[P8+H]^{+}$ (*m/z* 849.4); and (b) $[G^{1▲}IGAVLK+H]^{+}$ (*m/z* 745.4), (c) $[P9+2H]^{2+}$ (*m/z* 930.5), (d) $[VLTTGLPALISWIK^{21▲}R+2H]^{2+}$ (*m/z* 878.5), and (e) $[P10+2H]^{2+}$ (*m/z* 974.4).

CID of $[VLTTGLPALISWIK^{21▲}R+2H]^{2+}$ (*m/z* 878.5) (Figure S-5d) produced y_1, $y_2^{▲}$, $y_3^{▲}$, $y_4^{▲}$, $y_5^{▲}$, $y_6^{▲}$, $y_7^{▲}$, $y_8^{▲}$, $y_9^{▲}$, y_{10}-$H_2O^{▲}$, $y_{10}^{▲}$, $y_{11}^{▲}$, $y_{12}^{▲}$, $y_{13}^{▲}$, $y_{14}^{2+▲}$, b_2, b_5, b_6, and b_{10}, covering the majority of backbone cleavages and locating the modification position at K^{21}. P9 (measured mass: 1859.0 Da, see the structure of P9 in Figure 5a inset) is considered as the dead-end cross-link precursor because the mass difference between P9 and its reduced product $VLTTGLPALISWIK^{21▲}R$ (measured mass: 1755.0 Da) is 104.0 Da. The structure P9 is confirmed by CID of $[P9+2H]^{2+}$ (*m/z* 930.5), as shown in Figure S-5c. For P10 (measured mass: 1946.8 Da), it is assigned as the dead-end cross-link of $VFDK^{94}DGNGYISAAELR$ (see the structure of P10 in Figure 5a inset), because of the 104.0 Da mass difference between P10 and

the reduced product VFDK$^{94\blacktriangle}$DGNGYISAAELR (measured mass: 1842.8 Da). Upon CID, $[P10+2H]^{2+}$ (*m/z* 974.4) yielded $y_1, y_2, y_3, y_4, y_5, y_6, y_7, y_8, y_9, y_{10}, y_{11}, y_{12}, y_{13}', y_{14}', y_{14}^{2+\prime}, b_2, b_3, b_4', b_5', b_8', b_9', b_{10}', b_{11}', b_{12}', b_{13}', b_{14}'$, and b_{15}', providing evidence for this assignment (Figure S-5e).