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Development of Novel Free Radical Initiated Peptide Sequencing Reagent: Application to Identification and Characterization of Peptides by Mass Spectrometry

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Abstract

By incorporating a high proton affinity moiety to the charge localized free radical-initiated peptide sequencing (CL-FRIPS) reagent, FRIPS-MS technique has extended the applicability to hydrophobic peptides and peptides without basic amino acid residues (lysine, arginine, and histidine). Herein, the CL-FRIPS reagent has three moieties, 1) pyridine acting as the basic site to locate the proton, 2) 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, a stable free radical) acting as the free radical precursor to generate the nascent free radical in the gas phase, and 3) *N*-hydroxysuccinimide (NHS) activated carboxylic acid acting as the coupling site to derivatize the *N*-terminus of peptides. The CL-FRIPS reagent allows for the characterization of peptides by generating sequencing ions, enzymatic-cleavage-like radical-induced side chain losses, and the loss of TEMPO simultaneously via one-step collisional activation. Further collisional activation of enzymatic-cleavage-like radical-induced side chain loss ions provides more information for the structure determination of peptides. The application of CL-FRIPS reagent to characterize peptides is proved by employing bovine insulin as the model peptide. Both scaffold structure of bovine insulin and sequencing information of each chain are achieved.

GRAPHICAL ABSTRACTS

INTRODUCTION

Gas-phase free radical/electron techniques combined with mass spectrometry has recently gained significant interest in the field of characterization of biological macromolecules,

including proteins,^{1–11} glycans,^{12–21} lipids,^{22–25} and nucleic acids^{26–27}. A free radical is generated by three major methods, collision-induced dissociation (CID) of fragile bonds, photodissociation (PD) of fragile bonds, and electron activated dissociation (ExD including ECD and ETD). CID and PD can localize the generation of the free radical by incorporating a free radical moiety. ECD and ETD require multiply charged precursor ions. As a CID-based technique, free radical-initiated peptide sequencing mass spectrometry (FRIPS-MS) has made a significant advancement and has recently gained popularity in the field of proteomics.^{1–7} FRIPS-MS relies on radical-induced dissociation initiated by the generation of hydrogen-deficient radicals, which is generated mainly via homolytic dissociation of fragile bonds by either photoactivation or collision induced dissociation (CID).^{5, 7–8} In 2004, Porter's group first demonstrated that free radical-induced peptide cleavage can be initiated by collisional activation of the weak peroxide bond that was added to the peptide via site-specific modification of the ϵ -amino group of lysine residues or the *N*-terminus.⁹ Two consecutive applications of CID are needed to achieve the free radical-induced peptide cleavages: the nascent aminyl radical is generated by the first collision activation of peptide-metal complexes, wherein fragmentation of the weak O-O bond is followed by loss of CO₂; then fragmentation of the peptide is generated by the aminyl radical upon the second collision activation. In 2005, Beauchamp's group brought up the concept of free radical initiated peptide sequencing (FRIPS), in which the Vazo-68 free radical initiator was coupled to the *N*-terminus of a peptide (1st generation FRIPS, Scheme 1).¹ However, a three-step gas-phase collisional activation is needed to achieve the free radical induced fragmentation of the peptide. Similarly, several groups reported free radical induced peptide cleavages through the generation of the initial radical at the cysteine and tryptophan residues via the nitrosylation formation, and oxidative dissociation of metal-peptide complexes.^{28–31} Later, 2nd generation FRIPS reagents, employing 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, a stable free radical) as the free radical precursor, were developed for peptide characterization (Scheme 1).^{2–5, 7} Recently, FRIPS has been combined with chemical cross-linking/MS as a powerful tool to elucidate structures of peptides.^{32–36} Sinz and Schaefer incorporated either Azo or TEMPO to a cross-linker as the free radical precursor for structure analysis of peptides and proteins. The TEMPO-based free radical technique brings bright prospects in the fields of proteomics. However, this technique cannot be used on peptides without basic amino acid residues since the proton will remain on the TEMPO in the positive-ion mode, which blocks the generation of the free radical. This is due to the proton affinity of TEMPO being relatively high (209.5 kcal/mol).³⁷ To address the limitations of TEMPO-based FRIPS technique, we report significant progress in the FRIPS technique by developing the CL-FRIPS reagent via incorporating a high proton affinity moiety to the TEMPO radical precursor, as done previously with the characterization of glycans using Me-FRAGS reagent.¹³ By introducing a localized charge on the FRAGS reagent, glycan rearrangement can be significantly eliminated.¹³ Its further application into protein analysis is also confirmed by employing insulin as the model protein.

EXPERIMENTAL SECTION

Materials.

The model peptides YPFVEPI (β -Casomorphin, human), YPFPGPI (β -Casomorphin, bovine), and model protein bovine insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). β -amyloid peptide (33–42) trifluoroacetate salt was purchased from Bachem (Torrance, CA, USA). The HPLC grade solvents were purchased from EMD Merck (Gibbstown, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CL-FRIPS reagent was synthesized according to the procedure reported previously.^{4–5} Detailed procedures for the synthesis and characterization of the reagent are described in the supporting information.

Conjugation.

Approximately 20 μ L of 1 mM model peptide in water, 5 μ L of 10 mM FRIPS reagent in *N,N*-dimethylformamide, and 2 μ L of 0.50 M phosphate buffer (pH 8.0) were mixed and allowed to react for 2 hours at room temperature. After removing the solvents by using a SpeedVac concentrator, the conjugated peptide was redissolved in 0.1% (v/v) trifluoroacetic acid in H₂O followed by desalting using Millipore C18 ZipTips according to the manufacturer's instructions. The 10 μ L elution solvent containing the conjugated peptide was diluted to a final volume of 250 μ L in a 50/50/0.1% (v/v) solution of methanol/water/formic acid and was electrosprayed directly into the mass spectrometer. The derivatization yield is approximately 95%, as shown in Figure S1.

Mass Spectrometry.

A Thermo-Fisher Scientific linear quadrupole ion trap (LTQ-XL) mass spectrometer (Thermo, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source was employed. Derivatized peptide sample solutions were directly infused into the ESI source of the mass spectrometer via a syringe pump at a flow rate of 5 μ L/min. Critical parameters of the mass spectrometer include spray voltage of 5–6 kV, capillary voltage of 30–40 V, capillary temperature of 275 °C, sheath gas (N₂) flow rate of 10 (arbitrary unit), and tube lens voltage of 50–200 V. Other ion optic parameters were optimized by the auto-tune function in the LTQ-XL tune program for maximizing the signal intensity. CID was performed by resonance excitation of the selected ions for 30 ms. The normalized CID energy was 7–35 (arbitrary unit).

RESULTS AND DISCUSSION

The product ions are classified according to proposed nomenclature for peptide ion fragmentation developed by Siu et al.³⁸

To our knowledge, application of FRIPS on peptides without basic residues (lysine, arginine, and histidine) has not been studied in detail. To broaden the application of FRIPS technique to peptides without basic residues, we developed the CL-FRIPS reagent. This reagent has a radical precursor (TEMPO) and a basic site (pyridine) to retain the proton during the electrospray ionization (ESI) process as well as in the gas-phase. It is known that TEMPO

has a proton affinity of 209.5 kcal/mol while pyridine has a proton affinity of 223.8 kcal/mol.³⁹

Peptide YPFVEPI and YPFPGPI.

β -Casomorphin 1–7 human (YPFVEPI) and β -Casomorphin 1–7 bovine (YPFPGPI) were chosen as model peptides without basic amino acid residues. As the control, collisional activation of singly-protonated YPFVEPI generates a, b, and internal ions, mostly resulting from the mobile proton (Figure 1). Similar fragmentation patterns (a and b ions) were obtained by collisional activation of singly-protonated 2nd FRIPS-derivatized YPFVEPI (Figure 1). Interestingly, the generation of the nascent free radical by loss of TEMPO was not observed and therefore no free radical induced peptide fragments were generated. This is rationalized by considering the proton affinity of the TEMPO moiety, which is 209.5 kcal/mol. The proton of singly-protonated 2nd FRIPS-derivatized YPFVEPI is located on the nitrogen atom of the TEMPO moiety, which blocks the pathway to generate the nascent free radical. Similar fragmentation patterns of the CID of singly protonated 2nd FRIPS-derivatized YPFPGPI were obtained, which confirms the protonation occurring at the TEMPO moiety (Figure S2).

CL-FRIPS reagent:

As mentioned above, the CL-FRIPS reagent includes both pyridine and TEMPO moieties, in which pyridine acts as proton holder while TEMPO acts as the free radical precursor. The pyridine moiety has higher proton affinity than TEMPO, and thus precludes the protonation occurring on the TEMPO. The MS² CID of CL-FRIPS-derivatized YPFVEPI generated not only the nascent free radical via loss of TEMPO as expected, but also backbone dissociation and side-chain losses. The backbone fragments mainly include a, b, and y ions.^{40, 41} As reported previously, the enzymatic-cleavage-like radical-induced side chain losses were observed on glutamic acid (–45 Da, –59 Da, –72 Da), isoleucine (–29 Da, –56 Da), and tyrosine (–106 Da).¹⁰ The side chain loss has been proposed to be hydrogen abstraction from the side chain, followed by β -elimination.^{10–11, 33, 42–45} All the FRIPS fragmentation patterns of the MS² CID, including backbone fragments and side chain losses, can be confirmed by the MS³ CID on the ion representing the loss of TEMPO, as shown in Figure 2. Additionally, further CID on the side chain loss ions provided complementary structural information for peptide identification (Figure 2). For instance, the CID of the ion representing the loss of 72 Da generated not only the side chain loss of other residues, but also more sequencing ions, such as [a₃+H]⁺, [a₅]⁺, [b₆]⁺, and [y₄+2H]⁺ that MS² and MS³ CID did not show. As shown in Figure 2b and 2c, the two [b₄]⁺ ions have the same m/z while the two [b₅]⁺ ions have the m/z difference of 72. This provides more structural information and doubly confirms the location of the glutamic acid residue. The MS² CID allows us to obtain peptide sequencing through one-step FRIPS analysis of peptides. The MS³ and MS⁴ CID confirms the fragments generated by the MS² CID and provides more structural information for peptide identification, especially the location of residues with side chain losses. Similar results have been obtained by employing YPFPGPI (β -Casomorphin, bovine) as the model peptide, which are shown in Figure S3.

It is quite common to generate multiple-protonated peptide ions via electrospray ionization (ESI), even though the peptides are hydrophobic or lack basic amino acid residues. Therefore, it is useful to prove that CL-FRIPS reagent works for multiple-protonated peptides. Similarly, sequencing ions, side chain losses and loss of TEMPO were obtained simultaneously through one-step collisional activation (Figure 3). This proves that doubly-protonated CL-FRIPS-derivatized YPFVEPI has two protons, one located on the pyridine ring and the other located on one of the amino acid residues. The protonation of the pyridine moiety prevents the protonation on TEMPO due to the closeness of TEMPO to the pyridine moiety. Compared to the MS² CID of singly-protonated CL-FRIPS-derivatized peptides, more sequencing ions ($[y_2+2H]^+$, $[b_2]^+$, and $[y_5+2H]^+$ ions for YPFVEPI, and $[y_2+2H]^+$, $[y_3+2H]^+$, and $[y_6+2H]^+$ ions for YPFPGPI) were generated by MS² CID of doubly-protonated CL-FRIPS-derivatized peptides. Not only were doubly charged side chain losses generated, singly charged analogues were also formed. Moreover, side chain loss combined with CO₂ or H₂O loss was also observed. Furthermore, more y ions were generated for doubly charged precursor ions than their singly protonated analogues. That can be rationalized by considering the existence of two mobile protons, which catalyze the generation of y ions. Again, both the backbone fragments and side chain losses are confirmed by the MS³ CID on the doubly protonated ion representing the loss of TEMPO.

β-amyloid peptide (33–42)—β-Amyloid peptide (33–42) has the sequence of GLMVGGVVIA, wherein all the amino acid residues have either hydrophobic side chains (such as L, M, V, I, A) or no side chains (such as G). Therefore, β-amyloid peptide (33–42) not only represents a peptide without basic residues, but also represents a hydrophobic peptide. As expected, the MS² CID of CL-FRIPS-derivatized β-amyloid peptide (33–42) generated sequencing ions, side chain losses, and the loss of TEMPO simultaneously via one-step collisional activation. The loss of TEMPO and side chain losses are the major fragments. Fortunately, the sequencing ions can be clearly identified by magnification of the spectrum. Again, the enzymatic-cleavage-like radical-induced side chain losses were observed on the methionine residue (–74 Da, –61 Da, –47 Da), isoleucine residue (–29 Da, –56 Da), and leucine residue (–43 Da, –56 Da), which has been reported previously.^{10, 44–48} As mentioned in the case of YPFVEPI, the side chain loss has been proposed to be hydrogen abstraction from the side chain, followed by β-elimination.^{10–11, 33, 42–45} Interestingly, more b ions with the presence of TEMPO were generated upon the MS² CID, which proves the activation energy for the formation of b ions with TEMPO is similar to that of the loss of TEMPO. All the sequencing ions and side chain losses can be verified by the MS³ CID on the ion representing the loss of TEMPO, as shown in Figure 4. Further CID on side chain loss (–74) generated sequencing ions and side chain loss on other residues, therefore providing supplemental information for the identification of the peptide. Similar to CID on the singly-protonated CL-FRIPS-derivatized β-amyloid peptide (33–42), CID on the $[b_9]^+$ with TEMPO ion generated sequencing ions, side chain losses, loss of TEMPO, and b ions with TEMPO (Figure 4). Doubly-protonated CL-FRIPS-derivatized β-amyloid peptide (33–42) was barely generated, and therefore the collisional activation study on this ion was not reported here.

Bovine insulin:

Bovine insulin is a protein consisting of two polypeptide chains, A chain (21 amino acids including four cysteine residues) and B chain (30 amino acids including two cysteine residues), which are linked by two interchain disulfide bonds. Its A chain also has an intrachain disulfide bond. Bovine insulin has been studied by using TEMPO-acetyl FRIPS reagent (one of the 2nd FRIPS reagent in Scheme 1).⁵ In that study, TEMPO-acetyl FRIPS reagent was reported to couple with the *N*-terminus of B chain. In the MS² study, collisional activation of TEMPO-acetyl FRIPS-derivatized insulin results in highly selective interchain disulfide bond cleavages, generating highly abundant A chain and B chain product ions. This allows the sequencing study of these two chains by the following collision activation in MS³. The loss of protonated 2,2,6,6-tetramethylpiperidine from TEMPO has also been observed in the CID spectrum of tetra-protonated (4+) TEMPO-acetyl FRIPS-derivatized insulin, which significantly decreases the generation of the nascent free radical. This can be rationalized by considering the protonation of TEMPO.

CL-FRIPS-derivatized insulin.

To improve the performance of FRIPS technique on model protein analysis, we investigated the fragmentation pattern of collisional activation upon CL-FRIPS-derivatized insulin. To avoid the unwanted lysine derivatization and disulfide scrambling, the CL-FRIPS reagent reacts with bovine insulin at pH 6.3. As shown in Figure S4, the derivatization of insulin is efficient for the following collisional activation study since single derivatization is the major modification for each charge state. As expected, collisional activation of single CL-FRIPS-derivatized insulin ions generates highly selective disulfide bond cleavages (Figure 5). For instance, the MS² CID on CL-FRIPS-derivatized 3+ insulin at *m/z* 2003 simultaneously produces the +3 charged nascent free radical ion by loss of TEMPO, +2 charged B chain ion (*m/z* 1669), +1 charged A chain ion (*m/z* 2335), and +1 charged A chain with free radical ion (*m/z* 2455), all of which can be verified by the fragmentation patterns of MS³ CID on +3 charged nascent free radical ion as shown in Figure 5. Similarly, MS² CID on CL-FRIPS-derivatized 4+ insulin at *m/z* 1503 mainly produces +4 charged nascent free radical ion by loss of TEMPO, +3 charged B chain ion (*m/z* 1133), +1 charged A chain ion (*m/z* 2335), and +1 charged A chain with free radical ion (*m/z* 2455), all of which can be verified by the fragmentation patterns of MS³ CID on +4 charged nascent free radical ion as shown in Figure 5. The derivatization site is proposed to be the *N*-terminus of A chain since each of the four CIDs resulted in a shared ion, +1 charged A chain with free radical. The tandem disulfide bond cleavages have been proposed to be generated by cascaded free radical reactions occurring at the disulfide bond sites.⁵ Unlike CID of TEMPO-acetyl FRIPS-derivatized insulin, CID of CL-FRIPS-derivatized insulin doesn't generate any loss of protonated 2,2,6,6-tetramethylpiperidine (part of TEMPO), and thus significantly increases the abundance of the nascent free radical by loss of TEMPO.⁵ Subsequent activation of A and B chain ions provides further information for the sequencing of A and B chains, individually (Figure S5 and S6). Therefore, the FRIPS technique provides both scaffold structure of bovine insulin and sequencing information of each chain, indicating its further application to the structure analysis of proteins, especially ones containing disulfide bonds.

CONCLUSION

The capability of CL-FRIPS reagent to characterize peptides without basic amino acid residues and hydrophobic peptides via one-step collisional activation is demonstrated. The CL-FRIPS reagent overcomes the limitation of 2nd FRIPS reagents and therefore broadens the application of the free radical approach for the characterization of peptides. This is rationalized by considering the proton affinity of the pyridine moiety, which is higher than that of the TEMPO moiety. The protonation would first occur at the pyridine, and therefore prevents the protonation of the TEMPO due to the closeness of these two moieties. By incorporating the pyridine moiety into the CL-FRIPS reagent, the loss of TEMPO can be easily generated, which simultaneously generates the sequencing ions (mainly a, b, c, and y ions) and side chain losses through one-step collisional activation (MS²). Meanwhile, mobile proton-catalyzed sequencing ions are obtained. MS³ on the ion representing the loss of TEMPO confirms the peak assignments of the MS² results. The enzymatic-like free radical induced side chain loss provides the structural information to confirm the presence of some specific amino residues, which has been reported thoroughly. Moreover, the further collisional activation of the side chain loss ions generates supplemental fragments for the structural analysis of peptides.

Further application of CL-FRIPS for protein analysis is demonstrated by employing bovine insulin as the model protein. Collisional activation of single CL-FRIPS-derivatized insulin ions selectively cleaves the internal disulfide bonds, generating B chain ions, A chain ions, and A chain ions with a free radical. Further collisional activation on such ions generates sequencing ions. It is known that CID of 2nd FRIPS-derivatized insulin generates the loss of protonated 2,2,6,6-tetramethylpiperidine (part of TEMPO), which significantly decreases the generation of the loss of TEMPO. This is due to the protonation occurring at the nitrogen atom of the TEMPO moiety, which induces the heterolytic cleavage of N-O within the TEMPO moiety. Fortunately, CID of CL-FRIPS-derivatized insulin doesn't generate any loss of protonated 2,2,6,6-tetramethylpiperidine, and thus significantly increases the abundance of the nascent free radical ion by the loss of TEMPO, which can be used for further CID to get the sequence information of the A and B chains. Therefore, the FRIPS technique provides not only the scaffold structure of bovine insulin, but also the sequencing information of each chain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of the CID of singly-protonated model peptide YPFVEPI (a) and CID of singly-protonated 2nd FRIPS-derivatized YPFVEPI (b). Precursor ion refers to the protonated molecular ion.

Figure 2. The MS^2 on singly-protonated CL-FRIPS-derivatized YPFVEPI (a), MS^3 on the ion representing the loss of TEMPO (b), and MS^4 on the ion representing side chain loss (-72E) (c).

Figure 3.

The MS² on doubly-protonated CL-FRIPS-derivatized YPFVEPI (a), MS³ on the ion representing the loss of TEMPO (b), MS² on doubly-protonated CL-FRIPS-derivatized YPFVGPI (c), and MS³ on the ion representing the loss of TEMPO (d).

Figure 4.

The MS² on singly-protonated CL-FRIPS-derivatized β -amyloid peptide (33–42) (a), MS³ on the ion representing the loss of TEMPO (b), MS³ on -74 side chain loss ion (c), and MS² on $[b_9]^+$ with TEMPO ion (d).

Figure 5.

The CID spectrum of tandem mass spectrometry on multiple-charged CL-FRIPS-derivatized insulin. (a) MS² on CL-FRIPS-derivatized 3+ insulin at m/z 2003. (b) MS³ on the ion representing the loss of TEMPO from CL-FRIPS-derivatized 3+ insulin at m/z 1951. (c) MS² on CL-FRIPS-derivatized 4+ insulin at m/z 1503. (d) MS³ on the ion representing the loss of TEMPO from CL-FRIPS-derivatized 4+ insulin at m/z 1463.

a: Hodyss et al., **2005**, *J. Am. Chem. Soc.* 127, 12436-12437 (reference 1)

b: Thomas et al., **2014**, *J. Phys. Chem. A* 118, 8380-8392 (reference 4), and Sohn et al., 2015, *Chem. Sci.* 6, 4550-4560 (reference 5)

c: Lee et al., **2009**, *Analyst*, 134, 1706-1712 (reference 2), Lee et al., **2013**, *Anal. Chem.* 85, 7044-7051 (reference 3), and Jang et al., **2017**, *J. Am. Soc. Mass Spectrom.* 28, 154-163 (reference 7)

Scheme 1.

The development of free radical-initiated peptide sequencing reagents.