Peroxynitrite induced fibrinogen site identification

Yunjing Luo*, Jianlong Shi and Jingjing Li
College of Life Science and Bioengineering, Beijing University of Technology, 100124 P.R. China

Abstract. An increasing number of peroxynitrite-mediated fibrinogen nitrifications have been associated with thrombotic diseases. However, few reports related to priority nitrified fibrinogen injury sites exist. In this paper, an improved method, which simulated the environment in vivo, was used to inspect the structural changes of fibrinogen treated with peroxynitrite and LC-MS/MS in order to investigate the fibrinogen injury sites. The SDS-PAGE results indicated that γ534 subunits of Fg were vulnerable to oxidative/nitrative modifications induced by peroxynitrite. An in-depth analysis of fibrinogen γ chain (Fg I) nitration site identification and susceptibility to peroxynitrite-utilizing LC-MS/MS strategy was performed. Based on a large dataset, the results indicated the priority injury sites during database searching were Y96, Y262, Y274, Y348, and Y363; these results could be applied to biomedical studies.

Keywords: Fibrinogen, LC-MS/MS, SDS-PAGE, peroxynitrite, tyrosine nitration

1. Introduction

Fibrinogen (Fg) is a 340 kDa plasma protein, which is converted into fibrins by thrombin during blood coagulation. It consists of two sets of three polypeptide chains, termed Aα, Bβ, and γ, and its dimer is joined by disulfide bonds to form a central hydrophobic N-terminal E domain connected to two outer hydrophobic C-terminal D domains [1], as shown in Figure 1 [2]. Each D domain is connected to the E domain through a flexible coiled-coil section, allowing the protein to bend. Fg structural changes significantly impact biological activity, thereby playing an essential role in some cardiovascular diseases.

The toxicant Peroxynitrite (PN), derived from a diffusion-controlled interaction between O2·− and NO3, can damage a wide array of plasma albumins in vivo, leading to serious biological consequences [3]. Nitrotyrosine is recognized as an indicator of oxidative injury and inflammation in proteins [4]. As a result of this post-translational modification induced by ONOO−, protein spatial conformation and associated functions are usually altered [5]. In several cases, the identification of specific nitrated proteins has assisted in illuminating the molecular mechanisms of diseases, thus inducing atherosclerosis disorders [6] and coronary artery disease [7].

The effect of nitrification on fibrinogen function has been intensely investigated. Many researchers have concluded that ONOO− would destroy and inactivate fibrinogen [8]. The identification of predominantly nitrated fibrinogen tyrosine sites could contribute to targeted drug development.

*Address for correspondence: Yunjing Luo, College of Life Science and Bioengineering, Beijing University of Technology, 100 Ping Le Yuan, Chaoyang District, Beijing 100124, China. Tel.: +86-10-6739-1667; Fax: +86-10-67391667; E-mail: luoyj@bjut.edu.cn.

0959-2989/15/$35.00 © 2015 – IOS Press and the authors.

This article is published with Open Access and distributed under the terms of the Creative Commons Attribution and Non-Commercial License.
However, since a 70 tyrosine residues are involved in the three fibrinogen subunits, the determination of sites of interest remains enigmatic. In this study, the LC-MS/MS method was successfully utilized to confirm the insulin [9] and p16 protein [10] nitration sites induced by ONOO−. Tang et al. [11] investigated the tryptic digests of nitrated fibrinogen in human biofluids; although 26 different nitrated tyrosine sites were detected, the Tyr-containing sequence coverage was not high enough to identify the sites that were more likely to suffer injury. Since fibrinogen γ chains (Fg I) are most susceptible, they could be a signature for several oxidative stress pathologies.

Considering this information, the selectivity and priority of Fg I nitration sites were characterized using high-resolution liquid chromatography coupled with tandem mass spectrometry analysis, and the MS/MS spectra were compared to the MASCOT database in order to assist in drug design for related diseases.

2. Materials and methods

2.1. Materials

Bovine fibrinogen was purchased from Sigma-Aldrich (USA). Fibrinogen fraction I (γ chains) was obtained from TCI (Shanghai). The protein molecular weight marker for electrophoresis was obtained from Shenzhen Biotechnology (Shanghai). Dithiothreitol and iodoacetamide were obtained from Merck (Darmstadt, Germany). Peroxynitrite was synthesized according to the Uppu et al. operation approach [12]. The prepared stock solution was aliquoted in 0.1 M NaOH, and its concentration was determined by spectrophotometry (ε393 = 1670 M−1·cm−1) immediately before use. All other chemicals were analytical-grade and commercially pure.

2.2. SDS-PAGE analysis

Polyacrylamide gel electrophoresis (12%) in the presence of sodium dodecyl sulfate (SDS) was performed according to the Tetik procedure [13]. Bovine fibrinogen (1 mg/mL) in PBS (pH 7.4) was induced by sequential titration with the obtained peroxynitrite to different final concentrations (0 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, and 2.5 mM) and incubated for 30 min at 37°C. The protein gels were stained with Coomassie brilliant blue.

2.3. Preparation of nitrated Fg I and enzymatic digests

Bovine fibrinogen γ chains (Fg I, 1 mg/mL) in PBS (pH 7.4) were induced by the sequential titration of the obtained peroxynitrite to different final concentrations (0 nM, 0.3 nM, 1 nM, 3.3 nM, 10 nM) with a water bath for 30 min at 37°C. Then, the protein samples were denatured with 50 mM Tris(β-chloroethyl) phosphate (TCEP) in a 25 mM NH4HCO3 buffer (pH 8.0) and incubated for 1 h at 56°C. The reduced peptides were alkylated with iodoacetamide to a 50 mM concentration for 1 h at room temperature in the dark. Subsequently, the fibrinogens were digested with trypsin/Glu-C (chymotrypsin was added to the native Fg I) at a protein ratio of 1:50 mixture (w/w) overnight at 37°C with gentle shaking. The resulting peptides were desalted with 0.1% trifluoroacetic acid (TFA)/2% acetonitrile solution prior to lyophilization, then vacuum centrifuged at 20000 g for 30 min. The supernatant fractions were collected for peptide sequencing.
2.4. Identification of Fg I nitration sites with LC-MS/MS

The site identification was performed using a high-performance capillary LC system (NanoLC-2D, Eksigent Technologies, CA) coupled online with the LTQ ion trap mass spectrometer (Finnigan, Thermo, San Jose, CA) via an in-house prepared needle tip and nanospray source. The column was packed with 5 μm particles with 300 Å diameter pores (PepMap 300, Dionex, USA) into a 75 μm × 15 cm C18-bonded silica capillary. The mobile phases consisted of A (2% acetonitrile, 0.1% TFA, 98% water) and B (20% water, 0.1% TFA, 80% acetonitrile). The peptide separation was conducted as a linear gradient using the following program: from 2% to 40% B over 90 min and to 100% B in 15 min, and lasted it for the same while, finally eluted at 100% A for 30 min at a flow rate of 0.3 μL/min. Peptide ions were acquired in a survey scan across the m/z range (350-1600) followed by the data-dependent MS/MS mode (35% normalized collision energy). The ion spray voltage was operated at 2.2 kV. The data was collected for 130 min at 200°C.

2.5. Data processing

The MS/MS spectra were compared to the MASCOT (version 2.1.0) database [14] which contains all the sequences of fibrinogen subunits. In the presence of peroxynitrite, the following criteria were used for further positioning of the targeted nitrotyrosines: variable modification of methionine (+15.99 Da) corresponding to single oxygen, nitration modification (+44.9851 Da) matching the mono-nitro on tyrosine residues, and an additional mass of 90 Da or 135 Da corresponding to the di-nitro or tri-nitrotyrosine species. The primary error and secondary error were 1.5 Da and 0.8 Da, respectively. The peptide identifications were analyzed as previously described [15].

3. Results

3.1. Analysis of native and nitrated fibrinogen using SDS-PAGE

The fibrinogen exposed to peroxynitrite (0.5-2.5 mM) changed distinctly in its electrophoretic running gel (Figure 2). Not only were the bands of Aα (66 KDa), Bβ (52 KDa), and γ (46 KDa) chains observed, but also some of the new high molecular weight (HMW) bands on the top. As the concentration of peroxynitrite increased, the γ chain weakened and disappeared, followed by the Bβ chains, indicating that the three fibrinogen polypeptide chains were injured in the order of γ, Bβ, and Aα. Furthermore, the HMW aggregate could be associated with the dityrosine crosslink formation between Aα chains [16].

3.2. Analysis of native and nitrated fibrinogen γ chains using precursor-ion scanning (LC-MS/MS)

Trypsin, chymotrypsin, and Glu-C were employed and supplemented during digestion in order to ensure the accuracy of the detection results. The high coverage of the MS/MS spectra easily matched to the peptides even though the tryptic peptides existed up to two non-cleavage sites. The sequence coverage was 95.6% and 97.6% before and after the protein nitration, respectively. Hence, the total peptide sequence amount was large enough to expediently evaluate the 100% tyrosine coverage.

Fibrinogen γ chains (Fg I) were digested into peptides by trypsin under the action of ONOO−. The resulting peptides were separated using liquid chromatography (LC) and tandem electrospray mass
spectrometry with automatic fraction collection. Each fraction was subsequently searched for and analyzed in the database as described previously. As an example, the LC-MS/MS analysis of Fg I nitrated with ONOO\(^{-}\) at a 1 nM solution concentration is shown in Figure 3. Figure 3a presents the total-ion current chromatographic traces collected during LC over a large time scale. The base peak chromatogram was similar to the UV chromatogram (215 nm). The corresponding mass spectra yielded a molecular weight of the precursor ions triggered by the survey scan. Figures 3b and 3c show a single MS/tandem MS spectra at the 31.5 min and \(m/z\) 814.6 analysis point. Numerous peptide ion signals were observed in the \(m/z\) scan data. The precursor-ions of interest for the MS/MS spectrum were extracted in order to acquire mass spectral information on the molecular weight and heterogeneity of the modified proteins.

After acquisition of the MS/MS spectra, a list of possible peptide molecular weights was obtained. The best-matching peptides suffered a rigorous algorithm. The nitration extent was evaluated by integrating the repetitive identification of the same peptide in one or multiple fractions, which were consistent in their theoretical predictions. This dataset provided a unique pointcut that indicated the location of nitrotyrosine sites. The representative data are discussed below. As suggested in Table 1, among the 20 observed tyrosine residues, not only the amount but also the nitration extent of the identified nitrotyrosine sites decreased as the ONOO\(^{-}\) solution concentration decreased. When exposed to 3.3 nM ONOO\(^{-}\), 15 species with mostly di-nitro and tri-nitro tyrosine formed; however, when exposed to the 1 nM inducer, only 9 fewer-nitro tyrosine sites formed. After Fg I was treated with a trace amount of ONOO\(^{-}\) (0.3 nM), three mono-nitro identified sites and two di-nitro identified sites formed. Furthermore, the nitration sensitivity was significantly demonstrated. Y96, Y262, Y274, Y348, and Y363 were corroborated to be five susceptible nitration targets; their locations in the fibrinogen molecule are shown in Figure 4 [17].

In addition, the precursor ion scanning MS/MS of a tryptic digest peptide was compared: 89-108 in the absence and presence of ONOO\(^{-}\), as displayed in Figures 5a and 5b, respectively. A database search for tyrosine-containing peptides, with a shift of 135 Da in the mass (from 1474.7 Da to 1609.7 Da), suggested that three nitro groups were added, which provided adequate evidence for the Y96 identification of tri-nitratated species. In addition, Y96 required a molarity relatively low to ONOO\(^{-}\) modification and was a priority nitration site.

---

**Fig. 1.** The structure of fibrinogen.

**Fig. 2.** SDS-PAGE of native and oxidized fibrinogen induced by different peroxynitrite concentrations.
Fig. 3. LC-MS/MS analysis of digested fibrinogen γ chains (Fg I) nitrated with ONOO⁻ (1 mM solution concentration). (a) Total ion chromatograms of peroxynitrite-induced Fg I. Peptides were selected for MS/MS analysis. (b) The MS survey scan time was 31.5 min. The co-eluting peptide ions were sequentially selected for further sequence identification. (c) MS/MS spectra of precursor ion with m/z 814.6. Some b- and y-ions indicated on the spectra were derived from the peptide ion.

Fig. 4. Primary structure of fibrinogen I with nitration sites (in red and italics) (PDB entry 3ghg).
Fig. 5. MS/MS Fragmentation peaks of peptide, 89-108: MLEEIMKYEASILTDSSIR from the tryptic digestion of fibrinogen γ chains. (a): native peptide, (b): nitrated peptides (final concentration of ONOO$^-$ was 3.3 nM).

Table 1

<table>
<thead>
<tr>
<th>Final concentration of ONOO$^-$</th>
<th>Nitrated Tyr coverage [NY/Y(%) nitration]</th>
<th>Mr. (expt)</th>
<th>Mr. (calc)</th>
<th>Identified peptide sequence</th>
<th>Tyrosine site</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 nM</td>
<td>75%(15/20)</td>
<td>1917.7</td>
<td>1918.8</td>
<td>1 – 14: YVATRDNCICLDER.F</td>
<td>Y1: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2937.2</td>
<td>2938.2</td>
<td>15–38: R.FGYSYCPCTTCGIADFLSTYQTKVDK.D</td>
<td>Y18: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4252.8</td>
<td>4251.8</td>
<td>89 –120: K.MLEEIMKYEASILTDSSIRYQEYNNSNNK.I</td>
<td>Y96*: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1594.7</td>
<td>1593.9</td>
<td>160 – 173: K.GAKQSGLYF1KPLK.A</td>
<td>Y167: Nitro (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3302.6</td>
<td>3301.5</td>
<td>206 –232: K.KNW1QYKEGFGHLSPTGTELFLGNEK.I</td>
<td>Y211: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2390.5</td>
<td>2389.9</td>
<td>257 –275: R.TSTADYAMFKVGEADKRYL</td>
<td>Y262*: Nitro2Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3103.8</td>
<td>3103.1</td>
<td>276 –302: R.LTYAYFAGGGGADG.D FDFGDPSDK.F</td>
<td>Y278: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2102.2</td>
<td>2100.9</td>
<td>339 – 356: K.CHAGHNLGYYQGGYKSYK.A</td>
<td>Y348*: Nitro2Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3071.4</td>
<td>3070.3</td>
<td>357 – 380: K.ASTPENGYDNGIIWATWKTREWSMK.K</td>
<td>Y363*: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2924.6</td>
<td>2923.1</td>
<td>276 – 302: R.LTYAYFAGGGDAGDAFDGDFGDPSDK.F</td>
<td>Y280: Nitro3Y (Y)</td>
</tr>
<tr>
<td>1 nM</td>
<td>45%(9/20)</td>
<td>4027.9</td>
<td>4026.8</td>
<td>89 –120: K.MLEEIMKYEASILTDSSIRYQEYNNSNNK.I</td>
<td>Y96*: Nitro (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3302.7</td>
<td>3301.5</td>
<td>206 – 232: K.KNW1QYKEGFGHLSPTGTETFLGNEK.I</td>
<td>Y211: Nitro3Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2390.3</td>
<td>2389.9</td>
<td>257 – 275: R.TSTADYAMFKVGEADKRYL</td>
<td>Y262*: Nitro2Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2924.6</td>
<td>2923.1</td>
<td>276 – 302: R.LTYAYFAGGGDAGDAFDGDFGDPSDK.F</td>
<td>Y280: Nitro2Y (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2056.8</td>
<td>2055.9</td>
<td>339 – 356: K.CHAGHNLGYYQGGYKSYK.A</td>
<td>Y348*: Nitro (Y)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2980.8</td>
<td>2980.3</td>
<td>357 – 380: K.ASTPENGYDNGIIWATWKTREWSMK.K</td>
<td>Y363*: Nitro2Y (Y)</td>
</tr>
<tr>
<td>0.3 nM</td>
<td>25%(5/20)</td>
<td>3937.7</td>
<td>3936.8</td>
<td>89 –120: K.MLEEIMKYEASILTDSSIR</td>
<td>Y96*: Nitro (Y)</td>
</tr>
</tbody>
</table>
4. Discussion and conclusion

Since fibrinogen (Fg) injury induced by ONOO\(^{-}\) is closely associated with cardiovascular diseases [18], the nitration sites and extents of fibrinogen were identified using LC-MS/MS. As suggested from the SDS-PAGE gel, fibrinogen polypeptide chains were damaged in the order of γ, Bβ, and Aα as the ONOO\(^{-}\) amount increased. The γ chains broke apart and disappeared when the final ONOO\(^{-}\) concentration reached 1.5 mM.

The fibrinogen γ chains were subjected to a series of limited proteolysis and MS analyses in order to identify the preferential nitration sites. Molecular weight measurements could reveal a relatively detailed picture of nitrotyrosine sites by automatically searching the mass spectral data for characteristic m/z differences. Considering the ONOO\(^{-}\) levels in a physiological setting, the potential final concentration of ONOO\(^{-}\) was employed in vivo. The results indicated that the decrease in the amount of nitro added to tyrosine sites was nearly proportional to the decline of the identified sites over incubation time. Even the lowest levels of peroxynitrite (0.3 nM) could cause the cleavage and modification of fibrinogen. Five nitrotyrosines were identified as highly susceptible to nitration attack. This occurrence is completely probably in pathological conditions. The protein cytotoxicity and injury caused by ONOO\(^{-}\) was demonstrated.

Among the five tyrosine sites detected for primarily nitration, Y262, Y274, Y348, and Y363 were located in the domain 2: C-terminal fragment; another site, Y96, was present in the N-terminal within the fibrinogen molecule; and domain 1 (chain A) did not contain the susceptible nitration targets. As indicated by comparing the structural partition of the γ chain, the C domains primarily contact the aqueous solution and undergo the majority of the modifications. The accessible surface around these nitrated tyrosine sites were characteristic in loops, and thereby crucial to the physiological activity of fibrinogen.

Determining the locations and heterogeneity of tyrosine in fibrinogen is valuable to oxidative stress-induced pathological studies. Even though it is only a beginning to structural characterization, the sensitivity of tyrosine sites to peroxynitrile could be as a useful reference in cardiovascular disease drug design.

Acknowledgments

This work was supported by the Beijing Natural Science Foundation (No. 2102005), the General program of science and technology development project of the Beijing Municipal Education Commission (KM201210005032), and the National Natural Science Foundation of China (No. [NY/Y (%)], an estimate of the extent of nitration, is based on the number of nitrated peptides on the observed spectra. Tyrosine sites designated by asterisks were vulnerable to nitration. Mr indicates the molecular weight (Da) of the corresponding peptides. “expt” and “calc” are abbreviations of experiment and calculation, respectively.

| IRYLQEIQNSNQRK | 2344.3 | 257 – 275: R.TSTADYAMFKVGPEADKYR.L | Y262*: Nitro2Y (Y) Y274*: Nitro2Y (Y) |
| 2054.5 | 2055.9 | 339 – 356: K.CHAGHLNGVYYAGGNTYK.S.K | Y348*: Nitro (Y) |
| 2890.1 | 2890.3 | 357 – 380: K.ASTPNGYDNGHIIWATW KTRWYSMK.K | Y363*: Nitro (Y) |

Note: The position of fibrinogen nitrotyrosines (denoted in bold font) in the primary sequence is indicated by their corresponding numbers. [NY/Y (%)], an estimate of the extent of nitration, is based on the number of nitrated peptides on the observed spectra. Tyrosine sites designated by asterisks were vulnerable to nitration. Mr indicates the molecular weight (Da) of the corresponding peptides. “expt” and “calc” are abbreviations of experiment and calculation, respectively.
References

[17] Protein Data Bank (PDB entry 3ghg), Available at:www.rcsb.org, last visit date: 2015-01-07.