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***In vitro* oxidative footprinting provides insight into apolipoprotein B-100 structure in low density lipoprotein**

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Abstract

Low density lipoprotein (LDL) is a major cholesterol carrier in human blood. Oxidations of apolipoprotein B-100 (apo B-100, LDL protein) could be pro-atherogenic and play critical roles in early stages of plaque formation in the arterial wall. The structure of apo B-100 is still poorly understood, partially due to its size (550 KDa, 4563 amino acids). To gain an insight into LDL structure, we mapped the regions of apo B-100 in human LDL which were prone to oxidation using peroxynitrite and hypochlorite as probes. In this study, LDL was incubated with various concentrations of peroxynitrite and sodium hypochlorite in bicarbonate buffer. The LDL protein apo B-100 was delipidated, denatured, alkylated and subjected to tryptic digestion. Tryptic peptides were analyzed employing liquid chromatography – tandem mass spectrometry (LC-MS/MS). Database search was performed against the apo B-100 database (P04114) using “SEQUEST” algorithm to identify peroxynitrite and hypochlorite mediated oxidations markers nitrotyrosine, nitrotryptophan, hydroxy-tryptophan and 3-chlorotyrosine. Several site specific oxidations were identified in apo B-100 after treatment of intact LDL particles with the oxidants. We hypothesize that these regions could be accessible to oxidant and critical for early events in atherosclerotic plaque deposition.

Keywords

Apo B-100; hypochlorite; LC-MS/MS; ox-LDL; peroxynitrite

1 Introduction

Low density lipoprotein (LDL) is a cholesterol transporter in animal circulation. Human LDL particles are comprised of a lipid core and a lipid outermost layer encircled by a single monomeric protein apo B-100 (4563 amino acids) [1]. A wide array of strong oxidants can damage partially exposed apo B-100 at the surface of the LDL to form ox-LDL.

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Pathological implications of ox-LDL in human are severe and could be attributed to atherosclerosis, a disease causing inelastic plaque formation in arterial intima [2-5].

Oxidants can damage LDL via different pathways. Oxidatively and/or enzymatically modified LDL particles are inflammatory which activate cytokines and monocyte adhesion molecules [6, 7]. Direct protein damage yields post translational modifications in apo B-100 which were shown to cause accumulation of oxidized apo B-100 in atherosclerotic lesions [3, 8, 9]. Mechanistically, oxidative modification in apo B-100 in endothelial cells promotes binding and uptake of ox-LDL by scavenger receptor CD-36 in macrophages causing endothelial dysfunction and subsequent initiation of atherosclerotic plaques [10, 11].

Endothelial nitric oxide synthase (eNOS) converts L-arginine to L-citrulline and releases nitric oxide [12], which combines with superoxide to produce peroxynitrite (ONOO⁻). Peroxynitrite damages amino acid residues in proteins via multiple pathways [13]. Among different amino acid modifications, tyrosine nitration was studied extensively as a potential disease biomarker. The mechanism of tyrosine oxidation operates via tyrosyl radical formation aided by bicarbonate [14-16]. Tyrosyl radical reacts with peroxynitrite to form 3-nitrotyrosine (3-NT). Hamilton *et al.* reported some apoB-100 bound tyrosine nitration [17]. Tryptophan residues can also be nitrated or hydroxylated by peroxynitrite [18]. Also, the peroxynitrite fission byproduct hydroxyl radical could modify side chains of other amino acid residues [19].

During respiratory burst, phagocytes release superoxide and hydrogen peroxide [20]. In the presence of the heme enzyme myeloperoxidase (MPO), hydrogen peroxide reacts with physiological concentrations of chloride to form reactive hypochlorous acid (HOCl) which oxidizes intracellular LDL or LDL in circulation [21]. Hypochlorite induced apo B-100 damage could be diverse including side chain oxidation, peptide bond fragmentation and aggregation of proteins [22]. Hazen *et al.* observed LDL-bound chlorotyrosine in atherosclerotic lesions, a specific marker for hypochlorite induced damage [23]. Oxidation of amino acids was kinetically more favored than its lipid counterpart in the presence of hypochlorite [24, 25]. Transient intermediates from amino acid oxidation can further oxidize the accessible lipids to form cholesteryl ester hydroperoxides which further contribute to early events in plaque formation.

Tyrosine chlorination depends on chloramine transfer reactions and is directed by three dimensional protein structure [21]. Bergt *et al.* documented chloramine transfer in HOCl mediated oxidation of HDL protein A-I [26]. Early attack occurs on the ϵ -NH₂ group of Lys resulting in a chloramine transfer to a nearby Tyr residue (+34 Da). ϵ -NH₂ then reverts back to its original state [27]. Histidine can participate in transferring the chloramine by aromatic side chain oxidation [28]. Hypochlorite is prone to damage sulfur containing amino acid residues such as cysteine and methionine, and convert them to corresponding sulfonic acids (cysteic acid, +48 Da) and methionine sulfoxide (+16 Da) [29]. By a complex mechanistic pathway, the tryptophan indole residue side chain could be directly oxidized to 2-oxindole derivative (+16 Da) [21].

Yang *et al.* presented pioneering *in vitro* work in identifying post translational modifications in apo B-100 modified by HOCl and myeloperoxidase. Tryptic peptides were separated and identified by an off-line high performance liquid chromatography (HPLC) followed by electrospray ionization – mass spectrometry (ESI-MS) [30]. Recent developments in LC-tandem mass spectrometry (LC-MS/MS) based proteomics are of paramount importance since this method attenuates analysis time and reduces tedious separation and purification work [31, 32].

Free radical mediated [33, 34] and/or other [35, 36] oxidations often leave permanent footprints on the side chains of acid residues in proteins. By identifying these footprints, it is possible to map the solvent accessible regions of protein surface [37, 38]. Currently available protein structure determination tools are inadequate to reveal the tertiary structure of apo B-100. Molecular modeling, although very useful, becomes speculative for proteins such as apo B-100 and requires additional information to predict the structure (4563 amino acids and 550 KDa) [39-41]. Developments in the area of hyphenated mass spectrometry provided new and powerful tools for protein analysis. Previously we have shown the usefulness of LC-MS/MS in hydroxyl radical based surface mapping of apo B-100 [42]. In this article, we have assessed the site specific oxidations of apo B-100 in LDL with the oxidants peroxynitrite and hypochlorite. The approach employed in this study will help elucidate the oxidant accessible residues in apo B-100 and provide useful information about major early events in atherosclerotic plaque formation.

2 Materials and methods

Human low density lipoprotein (LDL) was purchased from VWR (Radnor, PA) (purity >95%). Each batch of LDL was acquired from a single, healthy human subject. LDL was refrigerated (4 °C) in 150 mM aqueous ethylenediaminetetraacetic acid (EDTA) at pH 7.4. Micro-dialysis tubes and floats were purchased from VWR. Sodium nitrite (NaNO_2), hydrogen peroxide (30%), and all solvents were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hypochlorite (NaOCl) (15% chlorine content), ammonium bicarbonate (ABC), formic acid, dithiothritol (DTT), and iodoacetamide were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). In all experiments, high quality purified water (obtained from distillation, deionization, carbon filtration and UV irradiation) was used.

2.1 Synthesis and characterization of peroxynitrite

Chemical synthesis of peroxynitrite was carried out by modifying a previously published high flow rate mixing method [43, 44]. In short, 0.60 M NaNO_2 solution was mixed with 0.65 M H_2O_2 (in HCl) at 29 mL/min flow rate by two peristaltic pumps. Due to the acid labile nature of peroxynitrite, the mixture was immediately quenched by a cold and constantly stirred three molar NaOH solution. Excess H_2O_2 in the mixture was degraded in a beaker over powdered MnO_2 (Sigma-Aldrich, St. Louis, MO). 2 mL aliquots were transferred into microcentrifuge tubes and stored at -80°C until further use.

Each day a microcentrifuge tube of peroxynitrite was thawed over ice followed by addition of MnO_2 to remove any residual H_2O_2 from the peroxynitrite stock. 25 μL of this

peroxynitrite solution was then diluted to 3 mL in 0.1 M NaOH and quantified via absorbance at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$) [45].

2.2 Reagent hypochlorite characterization

Sodium hypochlorite was stored at 4°C until use. The concentration of NaOCl was calculated in 0.01 M NaOH by absorbance using the molar extinction coefficient ($\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$) at 292 nm [46]. Hypochlorite stock solution was diluted to concentrations of 0.343 mM, 3.43 mM and 34.3 mM with 0.01 M NaOH and stored on ice until addition to LDL.

2.3 LDL oxidation

Stock protein concentration was recorded as 4.10 mg/mL from manufacturer's specifications. For each sample, 12.2 μL LDL stock solution was diluted to 100 μL with ammonium bicarbonate buffer (pH adjusted to 7.4) in microcentrifuge tubes. A total of eight samples were used in each set of experiments. Each exposure was carried out in duplicate. At time zero, three microliters of peroxynitrite (stock concentration 0.273 mM, 2.733 mM or 27.33 mM) was added to exposed samples maintaining the final concentration of peroxynitrite in LDL sample as 10 μM , 100 μM and 1000 μM respectively. At 10 and 20 minutes another spike of oxidants were added. Three microliters of 0.1 M NaOH were added to the blank.

Sodium hypochlorite exposure to LDL was carried out similarly. 12.2 μL LDL was diluted to 100 μL by ammonium bicarbonate (pH 7.4). At time zero, three microliters of NaOCl (concentration 0.343 mM, 3.43 mM and 34.3 mM) was added to each LDL sample to obtain final oxidant concentration 10 μM , 100 μM and 1000 μM respectively. 3 μL 0.01 M NaOH was added to the blank. Each exposure was carried out in duplicate.

The microcentrifuge tubes were shaken in an Eppendorf Thermomixer R at 37 °C and 900 rpm for 10 minutes. An additional 3 μL of oxidant solution (peroxynitrite or NaOCl) was added to the respective tube and shaken for another 10 minutes. Finally, 3 μL of peroxynitrite/NaOCl was added again to the same tubes and shaken. After 10 minutes these reaction mixtures were transferred to previously labeled microdialysis tubes.

The samples were dialyzed against 500 mL methanol:toluene:methylene chloride (3:1:1) (v/v) for two hours at 4 °C for delipidation. The dialysis float was transferred to another 500 mL solution containing 300 mL methanol with 200 mL water for two hours dialysis. A third dialysis was carried out in 300 mL methanol mixed with 200 mL 50 mM ABC buffer. The cloudy protein solutions were collected and transferred to microcentrifuge tubes. BCA assay was used to estimate the protein concentration after oxidation. The protein solution was boiled in water for 10 minutes for further denaturing. Thirty molar excess dithiothritol was added, and samples were incubated for 30 minutes at 37 °C with constant shaking to cleave the disulfide linkages. Next, 30 molar excess iodoacetamide was added and shaken for 90 minutes at 37 °C for alkylation of the cysteinyl residues. Tryptic digestion was carried out overnight following previous methods [42, 47, 48]. Tryptic digestions were quenched with liquid nitrogen, vacuum dried and stored until further use.

2.4 LC-MS/MS of apoB-100 peptides, database search and peptide validation

LC-MS/MS of apo B-100 peptides and database search were similar to our previously published work [42]. Full details are included in the supporting information.

3 Results and discussion

Apo B-100 was digested using trypsin and resultant peptides were analyzed by LCMS/MS. Tryptic peptides were identified and compared between treated and untreated samples. Only modified tryptic peptides that did not appear in the untreated samples were listed in Tables 1 and 2. Conversely, oxidized peptides from the blank samples are listed in Table S1. Differentially modified amino acids such as 3-nitrotyrosine (Y+45), nitro-tryptophan (W+45) [16], chlorotyrosine (Y+34), methionine sulfoxide (M+16), oxidized tryptophan (W+16), cysteic acid (C+48) [21] and hydroxyl radical pathway associated amino acid oxidation products [49], were searched against apo B-100 protein sequence (P04114). Although previous studies provided semi-quantitative information on peroxynitrite oxidation [17], we had difficulty in obtaining quantitative data from LC-MS-MS measurements; perhaps due to the heterogeneous nature of LDL and oxidation conditions. Our attempt to quantify relative oxidation of specific amino acid residues in apo B-100 revealed that this dataset is not optimum for quantitative work due to variations in the retention times of the peptides (Fig. S1) for comparison.

Therefore, our study will only provide information related to qualitative changes associated with peptide post translational modifications due to different oxidation conditions.

3.1 Peroxynitrite mediated modification of apo B-100

We assessed apo B-100 nitration in bicarbonate buffer in an *in vitro* model system [13]. Our results showed low abundance of nitrated tyrosines while nitrated tryptophan residues remained undetected. Amino acid hydroxylation products were observed in peroxynitrite treated samples indicating possible involvement of hydroxyl radical [19].

All oxidations from three different peroxynitrite concentrations were listed in Table 1. Peptide 1830-52 was of particular importance as it showed both Tyr1840 nitration and Tyr1831 hydroxylation (Fig. S2). Four tryptophans and Cys1505 (Fig. S3) were also oxidized.

With intermediate peroxynitrite concentration (100 μ M), no nitrotyrosines were detected using LC – MS/MS (Table 1). Hamilton *et al.* reported modification of Cys1112 to corresponding sulfonic acid by peroxynitrite [17]. We observed oxidation of the same amino acid without peroxynitrite treatment i.e. in untreated controls. We predict that Cys1112 might be exposed to the surface of LDL and were oxidized in the patient or by ambient conditions. Multiple peptides (bearing +2 and +3 charge states) from intermediate exposure confirmed Cys1112 modification (data not shown).

In high peroxynitrite exposure, four tyrosine residues were nitrated, of which Tyr1602 nitration was not reported before. Tyr2524 was nitrated (+45 Da) (Table 1 and Fig. S4) and hydroxylated (+16 Da) (data not shown) in different peptides indicating a possibility of

differential modification of the same amino acid residue. Previously, we reported Tyr2524 hydroxylation in control samples [42]. Now, we confirmed that Tyr2524 is readily oxidizable by peroxynitrite supporting Hamilton *et al.*'s previous observation; which strongly associates this tyrosine residue to the surface of LDL protein [17]. Also, Trp3970 was oxidized under all three peroxynitrite concentrations perhaps indicating it to be a surface accessible amino acid residue.

We performed computational analysis to investigate the abundance of tyrosine residues in apo B-100 sequence using "Protparameter" tool [50]. As a result we obtained a total of 151 Tyr residues in apo B-100. Very few of them were nitrated in our experiments. Hamilton *et al.* also observed a total of ten nitrated tyrosines in LDL with reagent peroxynitrite and 3-morpholininosyndnomine (SIN-1) at a concentration of 1 mM [17]. We used variable oxidant concentrations in our experiments and observed even more limited numbers of nitrotyrosine possibly because of either (1) inaccessibility, (2) competition kinetics and/or (3) due to the presence of bicarbonate buffer as was reviewed by Schoneich *et al.* [51]. Previously we showed higher probability of Tyr3295 oxidation using hydroxyl radical [42]. The fact that the same residue was nitrated by peroxynitrite (Table 1 and Fig. 3) suggests that Tyr3295 is a surface accessible amino acid residue. Notably, LC-MS/MS analysis of all peroxynitrite treated samples had low sequence coverage, which may be related to aggregation and covalent cross-linking in proteins after oxidation [52].

3.2 Hypochlorite mediated oxidations of apo B-100

Phagocytic activity *in vivo* elevates the concentration of hypochlorous acid, which oxidizes LDL in circulation [53]. Alternatively, in vascular endothelial cells, HOCl causes damage to reactive amino acid residues of proteins by side chain oxidations, fragmentation, and cross-linking [54, 55]. It was not yet understood which intermediates cause LDL protein damage in hypochlorite mediated oxidations; however, a physiologically relevant model was proposed to be mediated via MPO catalyzed reaction between peroxide and chloride [56].

All oxidized apo B-100 peptide sequences from hypochlorite induced oxidations are listed in Table 2. In low hypochlorite exposures (10 μ M), Tyr3295 was monochlorinated. Trp3563 (+16 Da) was oxidized to 2-oxindolic derivative in peptide 3558-63. We did not detect any cysteine oxidation at low hypochlorite dose.

At the intermediate hypochlorite concentration (100 μ M), no monochlorinated or dichlorinated tyrosines were detected. Eight tryptophans and three cysteine residues were oxidized (Table 2).

In high hypochlorite dose, eleven Tyr residues were monochlorinated and four Tyr residues were dichlorinated (Table 2). Tyr144 monochlorination and Tyr4451 dichlorination (+68 Da) were observed indicating hypochlorite can attack both N- and C- terminus amino acids. Tyr3295 was monochlorinated (Fig. 1) and dichlorinated (Fig. 2), perhaps indicating partial modification of the Tyr3295 residue. We confirmed Tyr3771 chlorination and Cys3761 oxidation (Fig. S5) from two different peptides (Table 2), possibly representing oxidation prone region of the protein. Furthermore, these positions belong to the amphipathic β 2

motif [39], which is part of the lipid associated domain of LDL and could undergo subsequent secondary lipid oxidation.

All oxidized amino acids in apo B-100 from all oxidants at three concentrations are presented in Fig. 3. Chloramine transfer from Lys and His to a Tyr residue was proposed to be an important mechanism of tyrosine chlorination in proteins [21]. Peptide 3292-3311 belongs to the amphipathic β_2 strand which has a well established barrel structure [39]. We believe tyrosine chlorination was mediated via His3308 chloramine transfer. Interestingly, in peptide 440-54, Tyr443 was selectively chlorinated, possibly due to the spatial proximity of Tyr443 to His447. The same peptide contained Tyr452, which was not chlorinated. Peptide 440-54 belongs to the $\beta\alpha_1$ domain of apo B-100, which assumes an intermediate structure between a helix and barrel [39]. Similar oxidation was observed in peptide 1985-2002, where Lys2002 might be involved in chloramine transfer to Tyr1999.

In some cases inconclusive identification was apparent when amino acid residues were repeated in an observed peptide sequence. Corresponding b- and y- ions series failed to uncover the exact position of the post translational modification in the sequence. MS/MS spectrum for 4499 – 4512 (precursor ion m/z 893.68, +2 charge) at 18.46 min showed either tyrosine (4509/10) could be monochlorinated (X_c : 4.97 and 4.44 respectively) (Fig. S6). Furthermore, MS/MS spectrum of 4499-4512 (precursor ion m/z 909.96, +2 charge) at 19.16 min showed three different matches with very high scores. Based on the mass spectrum we were unable to overrule possibilities of either (1) dichlorination of single Tyr (X_c : 5.16 and 4.17 respectively) or (2) monochlorination of both Tyr (X_c : 4.69) (Fig. S7). From a broad structural mapping perspective this difficulty did not undermine the research findings as the sites of oxidations were adjacent. Model peptide study using LQDFSDQLSDYYEK could reveal mechanistic details about tyrosine chlorination(s) in the said region of apo B-100.

Previously, we have identified 5- and 7-hydroxytryptophan (+16 Da) residues as products of hydroxyl radical attack on the phenyl ring [42]. Hypochlorite mediated tryptophan oxidation occurs at the indole ring to yield 2-hydroxyindole residue (+16 Da), which stays in its resonance form, 2-oxindole [29]. As explained earlier, in peptide 2535-59 either 2553/54Trp residues could be oxidized. This oxidized peptide was observed in both intermediate and high hypochlorite exposure. Intermediate hypochlorite exposure also matched oxidation in peptide 1451-74 (+16 Da) which could be either due to Trp1461 or Met 1465 (Fig. S8). We could not rule out the possibility of either oxidations or perhaps a mixture of peptides. However, common loss for methionine oxidation, CH_3SOH (–64 Da), was not observed in the MS/MS spectra [32].

Cysteine oxidation was observed in both intermediate and high hypochlorite doses. Cys3761 was oxidized to the corresponding sulfonic acid in the presence of intermediate and high concentrations of hypochlorite. The number of observed oxidations at high hypochlorite dose was fewer than that of the intermediate dose, which could be due to extensive damage to the protein at high dose [42].

High hypochlorite concentration (1000 μM) might render various other changes in protein including peptide bond fragmentation [21]. At this point it is beyond the scope of our current

work to address whether apo B-100 is fragmented by hypochlorite. 1-D gel electrophoresis might be useful, although previous attempts by other researchers and our group (unpublished) were unsuccessful. Several issues were discussed elsewhere indicating protein loading to be a key problem in the gel [53].

3.3 Concluding remarks

Comprehensive data visualization was carried out by comparing oxidations from hydroxyl radical [42], peroxynitrite and hypochlorite (Fig. 3). Strong patterns were observed especially in tyrosine oxidation. Tyr2524 and Trp583 were oxidized by both hydroxyl radical and peroxynitrite (Table 1). Tyr144, Tyr276, Tyr4451 and Tyr4509/10 were oxidized by both hydroxyl radical and hypochlorite. Tyr3295 was oxidized by all three oxidants (Fig. 3). Oxidation of the same amino acid residues using different oxidants emphasizes that these residues are likely to be solvent accessible and are damaged readily during oxidative stress. Also, amino acids such as Tyr413, Tyr2524 and Tyr3295 were previously observed to be nitrated with peroxynitrite [17], which indicates that there is a common mechanism of oxidation that may be related to early events in atherosclerosis. Dose dependent oxidations with peroxynitrite and hypochlorite revealed useful information about solvent and/or oxidant accessibility. Although fewer oxidations were observed with peroxynitrite, we identified Tyr1602 nitration which was not reported before. Notably, 583Trp was oxidized by both hydroxyl radical [42] and peroxynitrite (Fig. 3) but not by hypochlorite. Taken together the mechanism of oxidation in apo B-100 is highly dependent upon the chemical nature of the oxidant. At high hypochlorite treatment, two oxidations, 3139Tyr and 3153Trp were observed in the LDL receptor binding region. These oxidations could alter LDL receptor binding and be major players in early events in atherosclerotic plaque formation. To our knowledge, apo B-100 surface mapping using a concentration gradient with hypochlorite was not detailed by any other research group except for the founding work by Yang *et al.* [30, 56]. We are aware of the fact that loss of sequence coverage is of concern as it limits peptide oxidation information. However, this problem is endemic to apo B-100. Even though limited coverage somewhat undermines the complete picture, oxidative footprinting using multiple oxidants none the less provides deeper insight into surface accessible amino acids in apo B-100. We are further investigating other approaches to improve sequence coverage for larger proteins such as apo B- 100. Finally, we believe this study will provide not only valuable structural information of apo B-100 in LDL but also help elucidate mechanistic details of atherosclerotic plaque formation *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

LDL	Low density lipoproteins
apo B-100	Apolipoprotein B-100

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Figure 1. MS/MS spectrum of peptide 3292-2311 depicting Tyr3295 monochlorination (+34 Da) obtained from 1000 μ M hypochlorite exposure. Matching b- and y-ions are highlighted in table S2.

Figure 2. MS/MS spectrum of peptide 3292-3311 indicating Tyr3295 dichlorination (+68 Da) from 1000 μ M hypochlorite exposure. Matching b- and y-ions are highlighted in table S3.

Figure 3.

A schematic representation of all oxidized amino acids in apo B-100. Downward lines (dashed) indicate oxidations from peroxynitrite (solid) and upward lines indicate oxidations from hypochlorite. The colors black, blue and red represent oxidations from 10 μ M, 100 μ M and 1000 μ M oxidants respectively. Line thickness increases with increasing concentrations. Finally, the symbol * indicates amino acids oxidized by hydroxyl radical (compared with our previously published work [42]).

Table 1

All peroxynitrite modified peptides from three different concentrations (10, 100 and 1000 μ M) are summarized in the following table.

AA modification	Peptide	Amino acid sequence for the peptide	Charge State	X _c ^a	C _n ^b
10 μM peroxynitrite					
Y + 45	1830-52	AY ¹⁸³¹ QNNKHIY ¹⁸⁴⁰ AISSAALSASYK ^c	+3	3.53	0.09
	577-90	IVQILPW ⁵⁸³ EQNEQVK	+2	3.32	0.55
W + 16	3953-73	DFSAEYEEDGKFEGQLQEW ³⁹⁷⁰ EGK	+3	3.45	0.76
	1451-74	GLLIFDASSSW ¹⁴⁶¹ GPQMSASVHLDSK	+3	3.54	0.66
C + 48	1499-1513	GTYGLSC ¹⁵⁰⁵ QRDPNTGR	+2	3.08	0.66
100 μM peroxynitrite					
Y + 45		ND ^d			
	577-90	IVQILPW ⁵⁸³ EQNEQVK	+2	3.36	0.49
W + 16	2241-55	SGSSTASW ²²⁴⁸ IQNVDTK	+2	3.62	0.64
	3953-73	DFSAEYEEDGKFEGQLQEW ³⁹⁷⁰ EGK	+3	3.28	0.68
C + 48		ND			
1000 μM peroxynitrite					
	2523-34	M ²⁵²³ Y ²⁵²⁴ QM ²⁵²⁶ DIQQLQR	+2	4.52	0.69
Y + 45	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	6.34	0.61
	401-27	VHANPLLIDVVTY ⁴¹³ LVALIPEPSAQLR	+3	4.29	0.31
	1600-10	SEY ¹⁶⁰² QADYESLR	+2	2.68	0.50
W + 16	3953-73	DFSAEYEEDGKFEGQLQEW ³⁹⁷⁰ EGK	+3	3.28	0.70
C + 48		ND			

^aX_c: X correlation score (a measure of goodness of match of a peptide to the database).

^bDelta C_n score: Specificity of a peptide match.

^c1831Tyr was hydroxylated (+16 Da) and 1840Tyr was nitrated (+ 45 Da)

^dND: Not detected

Table 2

All oxidized peptides from three different hypochlorite concentrations (10, 100, 1000 μ M) are listed.

AA modification	Peptide	AA sequence for the peptide	Charge State	X _c	C _n
10 μM hypochlorite					
Y+34	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	3.26	0.34
W+16	3559-68	IYSLW ³⁵⁶³ EHSTK	+2	3.06	0.38
100 μM hypochlorite					
W+16	2118-33	LPQQANDYLNSEFNW ²¹³¹ ER	+2	4.84	0.61
	3587-3603	ATLESPW ³⁵⁹⁴ QMSALVQVH	+2	4.89	0.54
	4056-71	VNW ⁴⁰⁵⁸ EEEEASGLTSLK	+2	4.40	0.62
	2675-94	TIDQMQNSQLW ²⁶⁸⁶ PVPDIYLR	+2	2.70	0.42
	2489-2509	ITLIINW ²⁴⁹⁵ LQEALSSASLAHMK	+3	3.04	0.17
	2535-59	YLSLVGQVYSTLVYISDW ²⁵⁵³ W ²⁵⁵⁴ TAAK	+2	4.33	0.04
	1451-74	GLLIFDASSW ¹⁴⁶¹ GPQMSASVHLDSK	+3	3.43	0.54
	720-32	YW ⁷²¹ VNGQVPDGVSK	+2	3.33	0.61
C+48	1102-18	ITEVALM ¹¹⁰⁸ GHLSC ¹¹¹² DTKEER	+3	5.22	0.68
	1101-18	KITEVALM ¹¹⁰⁸ GHLSC ¹¹¹² DTKEER	+3	5.05	0.60
	4214-22	EELC ⁴²¹⁷ TM ⁴²¹⁹ FIR	+2	2.51	0.55
	3754-66	TDLQVPSC ³⁷⁶¹ KLDFR	+2	3.01	0.53
1000 μM hypochlorite					
Y+34	276-87	Y ²⁷⁶ GM ²⁷⁸ VAQVTQTLK	+2	3.85	0.48
	440-54	ATLY ⁴⁴³ ALSHAVNNYHK	+3	4.31	0.62
	1985-2002	TQFNNNEYSQDLDAY ¹⁹⁹⁹ NTK	+2	5.44	0.22
	3763-72	LDFREIQIY ³⁷⁷¹ K	+2	2.71	0.25
	4499-4512	LQDFSDQLSDY ⁴⁵⁰⁹ Y ⁴⁵¹⁰ EK ^e	+2	4.97	0.11
	1189-1202	M ¹¹⁸⁹ TSNFPVDLSDY ¹²⁰⁰ PK	+2	3.61	0.65
	3137-48	LPY ³¹³⁹ TIITTPPLK	+2	3.77	0.59
	140-57	QVFLY ¹⁴⁴ PEKDEPTYILNIK	+3	4.13	0.21
	655-69	IEGNLIFDPNNY ⁶⁶⁶ LPK	+2	3.57	0.48
	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+2	4.51	0.58
	4520-40	LIDLSIQNYHTFLIY ⁴⁵³⁴ ITELLK	+3	5.72	0.49
	3137-48	LPY ³¹³⁹ TIITTPPLK	+2	3.95	0.58
Y+68	4499-4512	LQDFSDQLSDY ⁴⁵⁰⁹ Y ⁴⁵¹⁰ EK ^f	+2	5.16	0.09
	140-57	QVFLY ¹⁴⁴ PEKDEPTYILNIK	+3	3.73	0.19
	4447-61	NIQEY ⁴⁴⁵¹ LSILTDPDGK	+2	4.04	0.69
	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	4.85	0.60

AA modification	Peptide	AA sequence for the peptide	Charge State	X _c	C _n
W+16	3137-55	LPYTIITTPPLKDFSLW ³¹⁵³ EK	+3	3.95	0.51
	2535-59	YLSLVGQVYSTLVITYISDW ²⁵⁵³ W ²⁵⁵⁴ TLAAK ^g	+3	4.28	0.25
	4056-66	VNW ⁴⁰⁵⁸ EEEEASGL	+2	3.02	0.65
C+48	3754-66	TDLQVPSC ³⁷⁶¹ KLDFR	+2	3.06	0.44

^e Both Tyr modifications possibilities were accepted.

^f Either Tyr residues could be dichlorinated or both could be monochlorinated.

^g Either Trp could be modified.