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Light-induced Conversion of Trp to Gly and Gly Hydroperoxide in IgG1

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Abstract

The exposure of IgG1 in aqueous solution to light with λ = 254 nm or λ > 295 nm yields products consistent with Trp radical cation formation followed by $^{\alpha}$C-$^{\beta}$C cleavage of the Trp side chain. The resulting glycyl radicals are either reduced to Gly, or add oxygen prior to reduction to Gly hydroperoxide. Photoirradiation at λ = 254 nm targets Trp at positions 191 (light chain), 309 and 377 (heavy chain) while photoirradiation at λ > 295 nm targets Trp at position 309 (heavy chain). Mechanistically, the formation of Trp radical cations likely proceeds via photo-induced electron- or hydrogen-transfer to disulfide bonds, yielding thiyl radicals and thiols, where thiols may serve as reductants for the intermediary glycyl or glycylperoxyl radicals.

Keywords

Immunoglobulin; IgG1; tryptophan; photochemistry; glycine radical; hydroperoxide

Due to its indole side chain, tryptophan (Trp) is the strongest UV-absorbing amino acid and a major target for photochemical degradation.[1] For example, Trp can be oxidized to N-formyl kynurenine (NFK) and kynurenine and sensitize the reduction of peptide and/or disulfide bonds, forming thiyl radicals and thiolate.[2,3] The latter process requires photo-induced electron- or H-atom transfer between Trp and the disulfide bond. Modification of Trp residues in proteins has resulted in conformational changes and loss of biologic activity, and presents a major concern for the production and formulation of biopharmaceuticals.[4-6] Moreover, kynurenines are efficient photosensitizers, inferring that Trp oxidation can lead to additional photosensitivity of a protein formulation.[7]

Gas-phase mass spectrometry studies have indicated the possibility for $^{\alpha}$C-$^{\beta}$C side chain fragmentation reactions of Trp-derived radical cations.[8-10] A side chain fragmentation was also reported as key to the enzyme-catalyzed conversion of Trp to 3-methyl-2-indolic acid, but the mechanism has not been characterized in detail.[11] Considering the potential of protein Trp residues for photoionization,[1] a $^{\alpha}$C-$^{\beta}$C side chain cleavage of Trp may also be expected during photodegradation of protein pharmaceuticals. Here, we show that the exposure of IgG1 to light indeed results in the fragmentation of Trp, resulting in the formation of Gly and/or Gly hydroperoxide. Additionally, photoirradiation of a synthetic

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SUPPORTING INFORMATION
Figures S1-S2 and Scheme S1. This material is available free of charge via the Internet at http://pubs.acs.org

model peptide containing Trp and a disulfide bond shows the Trp to Gly hydroperoxide modification. As a consequence, light exposure not only modifies the original amino acid (Trp) but also leads to the generation of a reactive hydroperoxide. Amino acid hydroperoxides have been shown to exhibit higher reactivity towards some oxidation targets as compared to hydrogen peroxide, implying that products such as Gly hydroperoxide may induce further protein oxidation (and/or fragmentation) upon storage.[12,13]

IgG1 was provided by Amgen Inc. and dialyzed against water prior to use. Aliquots of 500 μL IgG1 were then diluted with water to a concentration of 2.3 mg/ml. The final pH of the non-buffered solution was 5.6. The solutions were saturated through head-space equilibration with oxygen, argon, or air for one hour in a quartz or Pyrex tube capped with a rubber stopper. Next, the solutions were irradiated at $\lambda = 254$ or $\lambda_{max} = 305$ nm for 30 minutes in a Rayonet system (Southern New England, Branford, CT, RMA-500).[a] Photo-irradiations at $\lambda_{max} = 305$ nm involved four phosphor-coated low pressure mercury lamps (RPR-3000Å) which emit predominantly between $\lambda = 285$-315 nm ($\lambda_{max} = 305$ nm) and where wavelengths below 295 nm were filtered out by the use of Pyrex glass vials. Directly after photolysis, IgG1 was denatured by increasing the temperature to 75°C at a rate of 1.4°C/minute. Following denaturation, the disulfide bonds in IgG1 were reduced with 0.6 mM dithiothreitol (DTT) for 30 minutes at 45°C. Free thiols and some amino groups were derivatized with 1.8 mM N-ethylmaleimide (NEM) for one hour at 37°C and one hour at room temperature. The protein was purified by precipitation in cold ethanol and centrifugation for 30 minutes at 5,500 RPM at 4°C. The pellet was reconstituted in ammonium bicarbonate buffer (50 mM, pH 7.8) prior to digestion. Following the addition of 20 μg of trypsin (ratio trypsin:protein = 1:65), the samples were incubated for 2 hours at 45°C. Then, 10 μg of Glu-C (ratio Glu-C:protein = 1:130) were added together with an additional 20 μg of trypsin and the samples were incubated for an additional 5 hours at 37°C, and subsequently stored at -20°C until further analysis. Some aliquots of these samples were reduced with 2 mM sodium borohydride ($NaBH_4$) at room temperature for 1 hour. The peptide digests treated and non-treated with $NaBH_4$ were analyzed by liquid chromatography-mass spectrometry (LC-MS) employing a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR, Thermo-Finnigan, Bremen, Germany) combined with an Acquity chromatographer (Waters Corp., Milford, MA). The analytes were eluted from a reverse-phase C18 column (5 cm, 320 μm ID, CVC Microtech, Fontana, CA) at a flow rate of 20 μL/min. Mobile phases consisted of H_2O/acetonitrile (ACN)/formic acid (FA) at a ratio of 99%:1%:0.1% (v:v:v) for solvent A and a ratio of 1%:99%:0.1% (v:v:v) for solvent B. The column was equilibrated with 1% B for 2 min. Between 2-30 min, the eluent composition was linearly increased to 50% B. Collision-induced dissociation (CID) data for MS/MS analysis were obtained after an attenuation of the parent ion by 35%. The MassMatrix software program[14] was used for peptide mapping using the IgG1 sequence provided by Amgen.

During the exposure of IgG1 to light with $\lambda = 254$ nm, fragmentation of Trp[191, LC] resulted in the formation of Gly and Gly hydroperoxide (Figure 1). Furthermore, fragmentation of Trp[309, HC] and Trp[377, HC] resulted in the formation of Gly[309, HC] hydroperoxide (Figure S1; Supporting Information) and Gly[377, HC] (Figure 2), respectively. The original Trp[191, LC] residue is located on the light chain (LC) sequence near intra- and interchain disulfide bonds and close to the hinge region of IgG1 (Chart 1). The original Trp[309, HC] and Trp[377, HC] residues are located on the heavy chain (HC) sequence near intrachain disulfide bonds. The interchain disulfide bonds located near

[a]Actinometry gave a flux of photons at $\lambda = 254$ nm of 2.96×10^{-4} einstein min^{-1}. In Watt per cm^2, the flux at λ=254 nm and λ=305 nm is ~15 W cm^{-2}

residue 191 have been reported to undergo photochemical cleavage, ultimately forming covalent crosslinks such as dithiohemiacetals and cyclic-thioethers.[15]

The MS/MS data of the tryptic peptide sequence [178-192, LC] containing the Gly[191, LC] hydroperoxide are shown in Figure 1. In the ion series, the annotations -17 and -18 refer to the loss of ammonia and water, respectively. The y2 and b13 ions, as well as the putative loss of 34 Da from the b14 ion provide evidence for the replacement of the original Trp side-chain by a hydroperoxide. Interestingly, there is evidence for derivatization of the C-terminal Lys residue by NEM. In order for trypsin to effectively cleave the C-terminal lysine, the lysine amino group must be protonated;[16] while such protonation is still possible after NEM-derivatization, we believe that Lys derivatization likely occurred after digestion with some NEM left after protein precipitation. The NEM derivatization of the Lys side chain has been reported before.[17] The MS/MS data of the peptide sequence [276-388, HC] containing the Gly[377, HC] product are shown in Figure 2. Here, the y11, b3, and y12-17 ions provide evidence for the replacement of the original Trp side-chain by Gly. The formation of Gly and Gly hydroperoxides was experimentally monitored in an oxygen-saturated solution photolyzed at λ = 254 nm, but Gly[309, HC] hydroperoxide was also detected in an air-saturated solution (ca. 20% lower yield compared to the oxygen-saturated solution) (Table 1).

Importantly, Gly[309, HC] hydroperoxide formed upon photolysis of IgG1 with light of λ_{max} = 305 nm in Pyrex vials (Figure S2; Supporting Information). The reduction of Gly [309, HC] hydroperoxide by $NaBH_4$ converts the hydroperoxide into a hydroxide, –NH-CHOH-, which should release the sequence VVSVLTVLQHD-NH_2, which was experimentally observed (Figure 3, Scheme S1). It is important to note that hydroperoxide groups were detected even after 30 minute DTT reduction prior to tryptic digestion. However, when IgG1 was digested overnight, the yield of the Gly hydroperoxides was significantly reduced, likely due to a prolonged exposure to residual DTT. No Gly hydroperoxides were observed after sodium borohydride reduction, consistent with a reaction of $NaBH_4$ with the hydroperoxides. In an Ar-saturated solution, the photolysis of IgG1 at λ = 254 nm generated Gly[191, LC], but no hydroperoxides were detected. In addition to the formation of Gly and Gly hydroperoxides, we detected the formation of NFK and hydroxytryptophan, together with the respective unmodified peptides containing the native Trp residues. The non-irradiated controls showed no Trp to Gly or Gly hydroperoxide fragmentation products. Previous studies have documented the $^{\alpha}$C-$^{\beta}$C bond cleavage of amino acid radical cations in the gas phase[8-10,18,19] and in solution.[20]

In the gas phase, one proposed $^{\alpha}$C-$^{\beta}$C bond cleavage mechanism of Trp involves electron transfer from the excited state Trp to the amino group, followed by the loss of an H-atom and $^{\alpha}$C-$^{\beta}$C carbon-cleavage.[18] In IgG1, the electron is likely transferred onto a disulfide bond, leading to reductive cleavage. We propose that, in solution, the photolytic cleavage of Trp proceeds through an intermediary radical cation ($TrpNH^{+\bullet}$) and leads to a C-centered radical (Scheme 1). In the case of Trp[191, LC] and Trp[309, HC], the C-centered radical likely adds O_2 followed by reaction with an H-donor to form the hydroperoxyl group. Instead, Trp[377, HC] was transformed into non-oxidized Gly[377, HC]. This Trp residue is located in the same environment as Trp 309, so O_2 should have been available for oxidation, but the C-centered radical intermediate likely reacted faster with an H-atom donor than with O_2. The effect of oxygen on product formation can be rationalized in multiple ways. Certainly, oxygen is important for the formation of the Gly hydroperoxide. However, oxygen may also function as an acceptor of the electrons photoejected from Trp, limiting a potential back reaction to restore the reactants.

To independently confirm the light-induced Trp fragmentation, a model Trp-containing peptide connected by a disulfide bond was synthesized, GGCGGL-GGCWGL. This peptide was subjected to the same photolytic conditions as IgG1 (λ = 254 nm), and we observed the transformation of the disulfide bond into thiol as well as the conversion of the Trp residue into Gly hydroperoxide (Figure 4; the free thiol was derivatized with NEM after photolysis). During MS/MS analysis, y3 and b5 fragments were detected, localizing the Gly-OOH product to the original Trp residue. Additionally, a b4-32 Da ion was observed, potentially indicating the loss of O_2 from the hydroperoxide group on the original Trp residue. We also observed a loss of 34 Da (H_2O_2) from ions in this spectrum, a more common fragmentation.[21] The b3 and y4 ions highlight the Cys derivatized with NEM. The formation of the photoproducts in IgG1 likely results from a multi-step reaction: the photo-transformation of the disulfide bond to thiol as well as the transformation of Trp to Gly hydroperoxide in our model peptide suggest that the photochemistry of the disulfide bond and of the Trp residue need to be understood together to explain the formation of the different photoproducts in IgG1. Briefly, the thiol results either from the one-electron reduction of the disulfide bond following photoionization of the Trp residue, or the homolytic cleavage of the disulfide bond, followed by disproportionation of the thiyl radical pair.[15,22] These thiols represent hydrogen atom donors to intermediary glycyl and/or glycine peroxyl radicals. The final product yield may also be affected by intramolecular electron transfer reactions between tryptophan radical cations and electron donors such as tyrosine.

To quantitate the peptide-bound hydroperoxide (ROOH) for our model peptide, we added catalase to the irradiated solution to remove H_2O_2 and analyzed ROOH with the FOX2 assay, as described previously.[23,24]

The ratio of peak areas (hydroperoxide/native Trp-containing peptide) from the LC-MS and a direct comparison of the ratios of MS signal intensities for the hydroperoxide product versus the native peptide gave an upper limit of 3-6% of transformation of the native peptide into the hydroperoxide product (λ = 254 nm), consistent with the analysis by the FOX2 assay which gave 6.0 ± 0.5 μM ROOH/100 μM irradiated peptide. An estimate for the Gly and Gly hydroperoxide-containing peptides formed during photo-irradiation of the antibody (λ = 254 nm) yielded an upper limit of 10 and 40-100%, respectively, relative to unmodified peptides based on MS signal intensity. Confirmation of the Gly hydroperoxide yield by the FOX2 assay is presently not possible because radical chain reactions and singlet oxygen chemistry[3] may lead to additional hydroperoxides.

The amino acid hydroperoxide represents not only a significant chemical alteration, but could serve as origin for further protein oxidation. For example, amino acid hydroperoxides have been shown to inactivate proteins via oxidation of Cys residues.[12] Our work also demonstrates that light exposure, and subsequent radical formation produces Gly hydroperoxides at λ_{max} = 305 nm, i.e. wavelengths not filtered out by glass. The conversion of Trp to Gly and Gly hydroperoxide converts an aromatic amino acid into a small, highly flexible aliphatic amino acid (or its hydroperoxide derivative). These alterations may in part rationalize the loss of conformational integrity observed during photo-irradiation of the antibody, quantified through biophysical measurements.[25] Further mechanistic experiments with GGCGGL-GGCWGL and other model peptides containing Trp residues and disulfide bonds are underway to characterize the influence of peptide/protein sequence and structure on the fragmentation of Trp to Gly and Gly hydroperoxide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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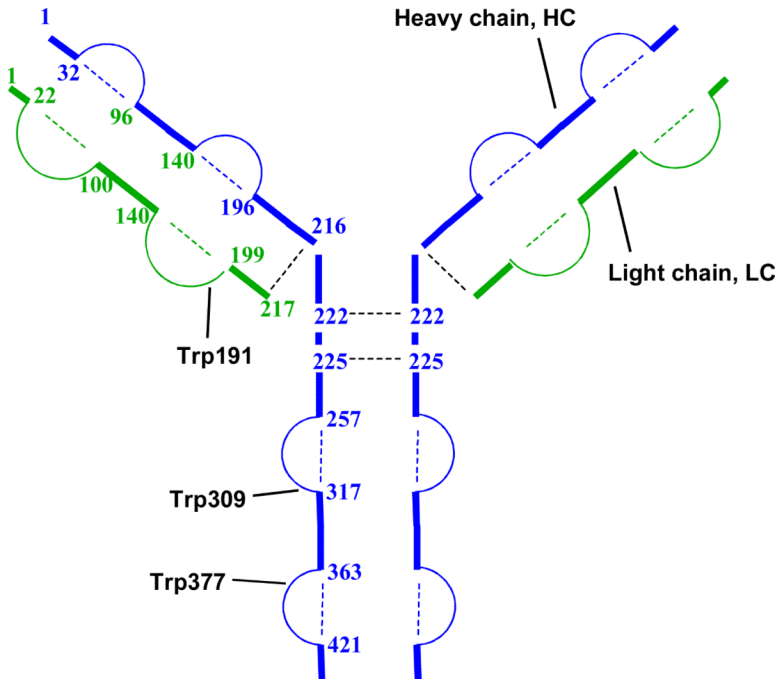


Chart 1.
IgG1 structure, where Trp[191, LC] and Trp[309, HC] are transformed into Gly and Gly hydroperoxide, and Trp[377, HC] is transformed into Gly.

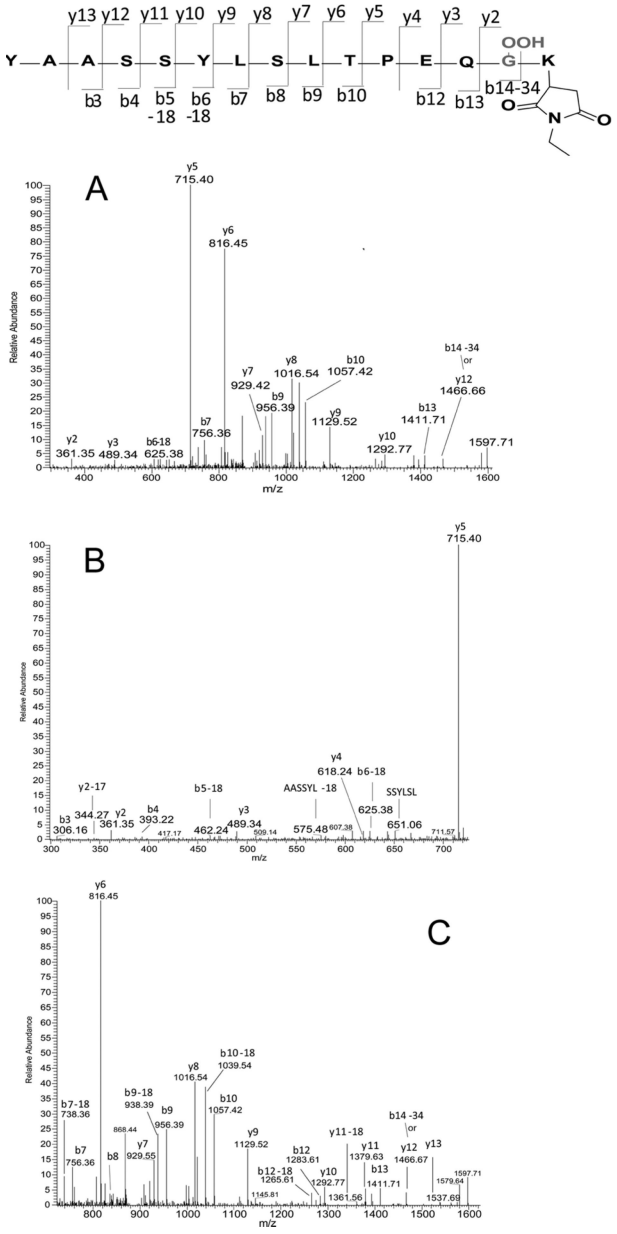


Figure 1.

Full (A) and zoomed (B and C) CID mass spectra of the peptide sequence [178-192, LC] of IgG1 where Trp[191, LC] is transformed into Gly[191, LC] hydroperoxide.

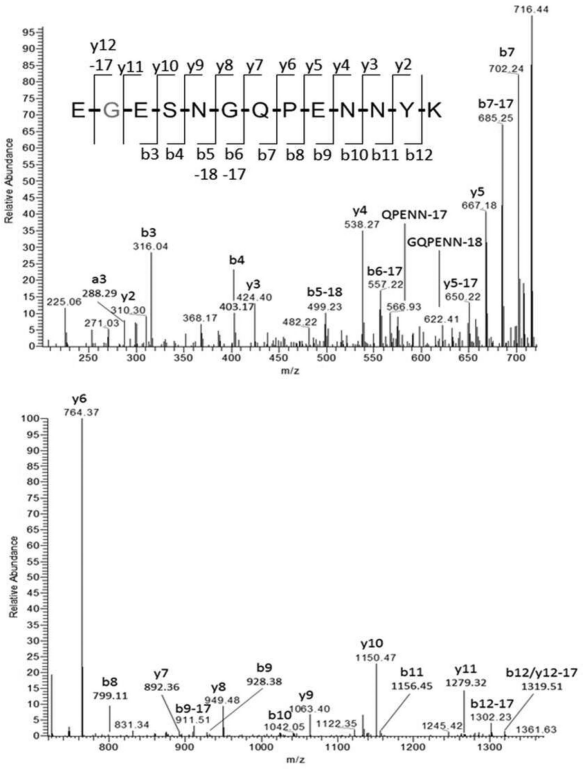


Figure 2.

CID mass spectra of the peptide sequence [376-388, HC] of IgG1 where Trp[377, HC] is transformed into Gly[377, HC] (Top spectrum: m/z 200-750, bottom spectrum: m/z 750-1400).

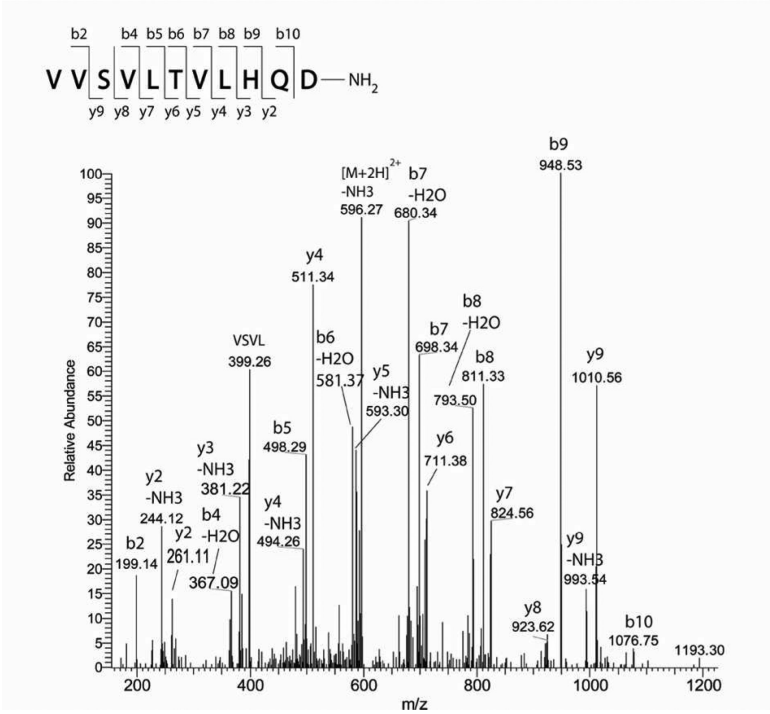


Figure 3.

CID mass spectrum of the peptide sequence [298-308, HC] where the C-terminal position is amidated. Such amidation results from a cleavage between Asp [308, HC] and Gly-OH [309, HC]. A mechanism is provided in the Supplementary Information (Scheme S1).

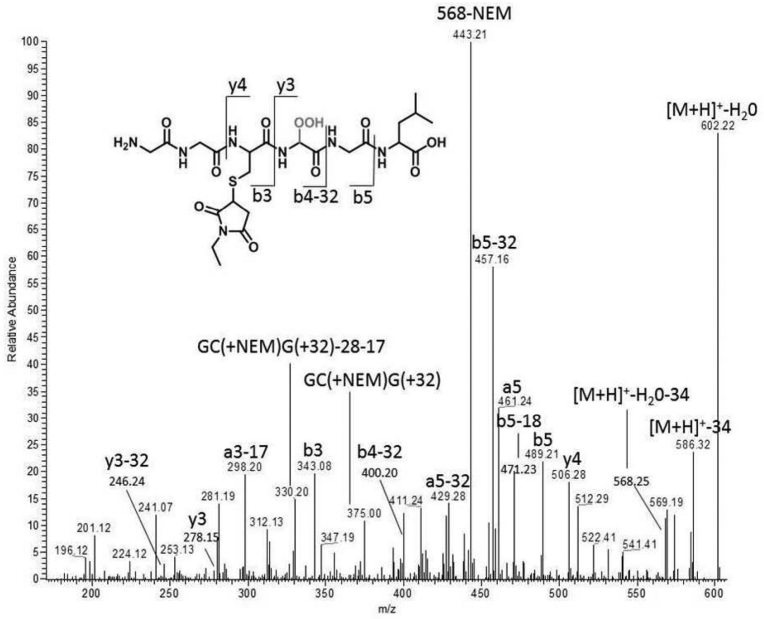
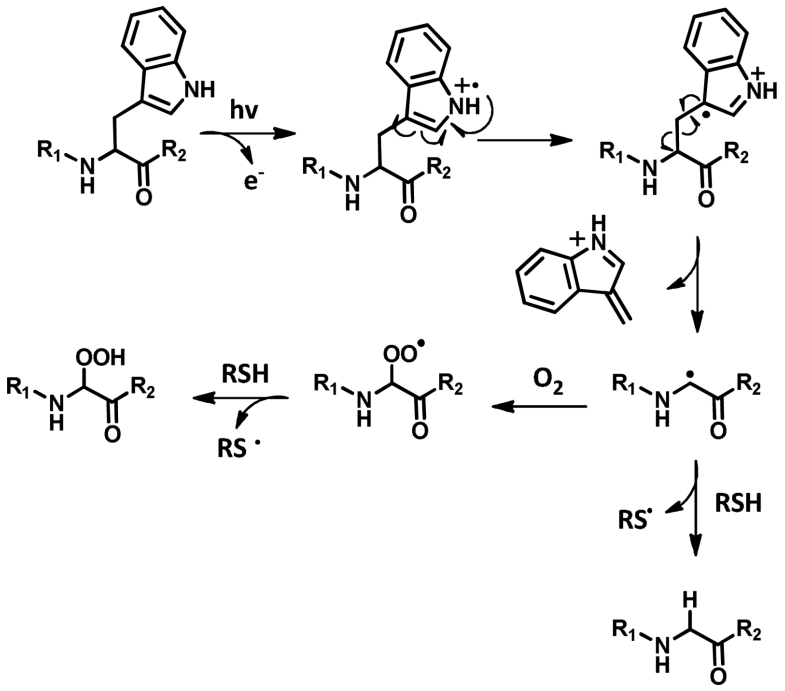


Figure 4.
CID mass spectrum of the model peptide GGCGGL-GGCWGL containing the Trp to Gly hydroperoxide modification.



Scheme 1.

Proposed reaction scheme demonstrating the photolytic cleavage of the Trp side chain and oxidation of the protein backbone.

Table 1

IgG1 photoproducts formed during irradiation at λ = 254 nm under air, O_2, and Ar.

	Product		
Trp residue	**Air**	**O_2**	**Ar**
191, LC	-	Gly-OOH	Gly
309, HC	Gly-OOH	Gly-OOH	-
377, HC	-	Gly	-