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Site-Specific Detection of Radicals on α-Lactalbumin after a Riboflavin-Sensitized Reaction, Detected by Immuno-spin Trapping, ESR, and MS

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Supporting Information

ABSTRACT: Free radicals and other oxidation products were characterized on α-lactalbumin with electron spin resonance (ESR), immuno-spin trapping, and mass spectrometry (MS) after riboflavin-mediated oxidation. Radicals were detected using the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in immuno-spin trapping with both enzyme-linked immunosorbent assay (ELISA) and Western blotting and further characterized with mass spectrometry. A DMPO-trapped radical was identified at His68 and another at one of the tyrosine residues, Tyr50 or Tyr36, respectively, generated by a type II or I mechanism. Not all tyrosyl radicals were trapped, as the secondary oxidation product, 3,4-dihydroxyphenylalanine (DOPA), was detected by mass spectrometry at Tyr18 and Tyr50. A further oxidation of DOPA resulted in the DOPA *o*-semiquinone radical, which was characterized by ESR. Both surface exposure and the neighboring residues in the local environment of the tertiary structure of α-lactalbumin seem to play a role in the generation of DMPO trapped radicals and secondary oxidation products.

KEYWORDS: *riboflavin, α-lactalbumin, immuno-spin trapping, mass spectrometry, oxidation mechanism*

INTRODUCTION

Oxidative changes in milk proteins have been investigated after enzymatically catalyzed reactions,[1−3] metal-catalyzed reactions,[4,5] and photosensitized reactions[6−8] resulting in changes in protein structure,[6] functionality,[2] digestibility,[9] and sensory and nutritional values.[10] The products detected have been primarily secondary oxidation products such as carbonyls, for example, on Trp[2,6,11] His,[6] or Met.[12] Tyrosine-derived secondary oxidation products are, for example, DOPA and dityrosine,[7] and the Met-derived oxidation products are dimethyl disulfide[12] and sulfoxide.[13] All of these products are used as markers for protein oxidation. The level of secondary oxidation products is correlated with the flexibility of the protein structure,[6,7,11] and reactions between two radicals, such as the reaction leading to dityrosine formation, are especially highly dependent on the protein structure.[7,11,14] Reaction between two radicals is a so-called termination reaction and will reduce the level of radicals; this type of reaction has also been referred to as a radical sink reaction,[15] which contributes to the antioxidative potential of proteins.

The photosensitization-mediated reaction can introduce oxidative changes either through type I or type II mechanisms. In the type I mechanism, the triplet excited photosensitizer reacts directly with the substrate by abstraction of a hydrogen or through an electron transfer mechanism creating a radical, whereas in the type II mechanism, the oxidative changes are initiated through an energy transfer between the triplet excited sensitizer and oxygen, generating the highly reactive singlet oxygen, which will react directly with the substrate.[16] To follow the initial oxidation, focus should be shifted toward the primary oxidation products, the radicals, and even though the type II reaction is a nonradical reaction, introduction of hydroperoxides in the protein will subsequently result in radical formation as well.[17] Radicals have been detected on milk proteins by ESR, but only tyrosine-derived radicals have been detected directly on the proteins; the tyrosyl radical has been detected on β-lactoglobulin,[1] and the *o*-semiquione radical derived from DOPA has also been detected directly on β-lactoglobulin and bovine serum albumin.[7] The stability of these radicals has been shown to depend highly on the protein structure.[7] Protein radicals normally have a half-life of minutes,[7,18] which can be a challenge for their characterization. The use of spin traps has been a way to extend the lifetime, and thereby increase the steady-state level, of radicals and facilitate further characterization of specific radicals with ESR by detection of the spin-trapped adducts,[19,20] which can also provide insight into the nature of the radicals. Spin trapping depends on the specific reaction of the radical with a spin trap to form relatively stable spin adducts.

Even though spin trapping may enhance the stability and thereby the detection of radicals, the decay and lifetime of the radical are still a problem, and neither allows for identification of the specific location of the radical in the protein structure. Therefore, this study combines the advances of ESR and an immunochemical detection of DMPO-trapped radicals using

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ELISA and Western blotting to detect radicals or their derivatives on α-lactalbumin, thereby facilitating research on very specific oxidative reaction mechanisms that, so far, have been difficult to decipher. Furthermore, the specific site of the radicals in the protein structure has been determined with MS after riboflavin-mediated oxidation to investigate where the protein became oxidized. This study is the first of its kind to combine the two methods in the evaluation of a food relevant system, in this case, the riboflavin-mediated oxidation of α-lactalbumin.

MATERIALS AND METHODS

Materials. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Dojindo Laboratories (Kumamoto, Japan). The monoclonal mouse anti-DMPO nitrone adduct monoclonal antibody was produced in-house, but this antibody can be obtained commercially. Mouse antiserum (IgG goat-to-mouse coupled to an alkaline phosphate) was obtained from Abcam (Cambridge, MA, USA). Tris-tricine gels (4−12%), membranes, and NuPAGE MES SDS running buffer (×20) were obtained from Invitrogen (Carlsbad, CA, USA). Sodium carbonate−bicarbonate (0.2 M), pH 9.4, and 25 mM Tris-buffered saline (0.150 M sodium chloride, pH 7.2) were purchased from Pierce Protein Research Products, Thermo Scientific (Rockford, IL, USA). Riboflavin (>98%), Tween-20, α-lactalbumin III (calcium-depleted, >85%), guanidine hydrochloride, DL-dithiothreitol (>99%), and iodoacetamide (bioUltra, >99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine kidney superoxide dismutase (SOD) was obtained from Calzyme Laboratories Inc. (San Luis Obispo, CA, USA). Trypsin and chymotrypsin (from bovine pancreas, modified, sequencing grade) and catalase (20 mg/mL, 65000 U/mL) were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Chelex-100 resin was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of analytical grade and were purchased from Sigma or Roche Molecular Biochemicals.

Protein samples were dialyzed in a Slide-A-Lyzer Dialysis Cassette 10000 MWCO from Thermo Scientific for 6 h, followed by overnight dialysis against chelated phosphate buffer to remove trace impurities.

Photooxidation. One milliliter of protein samples (0.2 mM) with 5 μg/mL (∼13 μM) riboflavin and various concentrations of DMPO were exposed to visible light (with cutoff filter ∼ λ > 300 nm) from a 300 W halogen lamp for 30 min at room temperature with gently stirring in 10 mL glass tubes. These photooxidized samples and their respective dark controls were subjected to SDS-PAGE, ELISA, Western blotting, electron spin resonance (ESR), and mass spectrometry (MS). All photo experiments were performed in independent triplicates.

Immunochemical Detection of Protein−DMPO Nitrone Adducts. *Enzyme-Linked Immunosorbent Assay (ELISA).* The radical-derived nitrone adducts were determined on α-lactalbumin using standard ELISA white plates (96-well, Greiner Labortechnik, Solingen, Germany). One microliter of the reaction mixture (∼0.2 pmol of protein) was added to each well together with 300 μL of coating buffer (100 mM sodium bicarbonate, pH 9.4) and incubated at 37 °C for 90 min. The plates were washed with washing buffer (0.05% Tween-20 and 0.2% fish gelatin saline bis-tris (100 mM, pH 7.4) and blocked with blocking buffer (4% of fish gelatin in 100 mM carbonate/bicarbonate, pH 9.4) for 90 min at 37 °C. Monoclonal anti-DMPO nitrone adduct antibody (5 μg/mL) in washing buffer was added and incubated for 60 min and then washed four times. Mouse antiserum IgG goat-to-mouse coupled to an alkaline phosphate (Abcam) was incubated for 60 min, and after four washings, the antigen−antibody complexes were detected using a chemiluminescence system, CDP-Star, from Roche Applied Science (Indianapolis, IN, USA). The emission of light was recorded in arbitrary units using a SpectraFluor Plus instrument equipped with Xfluor software (Tecan, Männedorf, Switzerland). The results obtained are the mean ± standard deviation from three independent photooxidation experiments.

Western Blotting. Coomassie Blue Stain and Western blot reaction mixtures were electrophoresed under reducing conditions through duplicate 4−12% Bis-Tris NuPage acrylamide gels (Invitrogen). After electrophoresis, one gel was stained using Coomassie Blue, and the proteins in the other were blotted to a nitrocellulose membrane. Western blotting was performed as described in Chen et al.[21] with the modification of using monoclonal antiserum (5 μg/mL) for detection of DMPO-trapped radicals. Immunoreactive proteins were measured as the development in light at 800 nm, using an Odyssey Licor from Biosciences (Lincoln, NE, USA).

Electron Spin Resonance (ESR). ESR experiments were conducted at room temperature using a Bruker ELEXSYS E500 spectrometer with a 100 kHz modulation frequency equipped with an ER 4122 SHQ cavity (Bruker BioSpin Corp., Billerica, MA, USA). Samples were placed in a 10 mm flat cell (final volume of 200 μL) from Wilmad Glass Co. (Vineland, NJ, USA). The microwave frequency was 9.60 GHz; microwave power, 1.97 mW; modulation amplitude, 1.0 G; receiver gain, 2×10^5; conversion time, 81.920 ms; and time constant, 81.920 ms; each spectrum was the average of 10 scans.

Protein Digestion. Five microliter aliquots of samples containing 200 μM in protein were treated in a dark room for mass spectrometry analysis. Samples were mixed with 5 M guanidine hydrochloride for 30 min at 50 °C and treated with 4.8 mM DTT for 30 min at 25 °C. Iodoacetamide was added to a final concentration of 14.1 mM, and samples were incubated for 30 min at 25 °C. Samples were then diluted to 7 pmol/μL with 100 mM Tris-HCl, pH 8.5, just prior to digestion with chymotrypsin or trypsin at a protein/enzyme ratio of 20:1, pH 8.0, for 8 h at 37 °C.

Mass Spectrometry. Flow injection electrospray ionization (ESI/MS) analyses were performed with a Micromass Q-TOF Micro (Waters Micromass Corp., Manchester, UK) mass spectrometer. Ten microliters of the protein-containing samples was purified by Millipore C18 ZipTip (Billerica, MA, USA) as described by the manufacturer before elution of the protein-containing fraction with a solution of water/acetonitrile 20:80 (v/v) with 0.1% formic acid. The samples were diluted 1:100 just prior to analysis with a solution of water/acetonitrile 80:20 (v/v) with 0.1% formic acid and infused into the mass spectrometer at 300 nL/min using a pressure injection vessel. The instrumental parameters were as follows: capillary voltage, 3.8 kV; cone voltage, 80 V; collision energy, 10 eV; and source temperature, 80 °C. A Waters Q-Tof Ultima Global hybrid tandem mass spectrometer equipped with a nanoAcquity UPLC system was used for the acquisition of the LC-ESI/MS(/MS) data. Separations were performed using a 3 μm nanoAcquity Atlantis dC_{18} (100 μm × 100 mm) column (Waters Corp., Milford, MA) at a flow rate of 300 nL/min. A 5 μm nanoAcquity Symmetry C18 (100 μm × 20 mm) trapping column (Waters Corp.) was positioned in-line with the analytical column. Injections of 0.7 pmol of α-lactalbumin digests were loaded onto the column. Trapping was performed for 3 min at a 5 μL/min flow rate using an initial solvent composition of 98% solvent A [water/0.1% formic acid (v/v)] and 2% solvent B [acetonitrile/0.1% formic acid (v/v)], and peptides were eluted using a linear gradient to 40% solvent B over 90 min and 95% over 5 min. Mass spectrometer settings for MS analyses were a capillary voltage of 3.5 kV, a cone voltage of 30 V, a source temperature of 80 °C, and a collision energy of 4 eV. The mass spectra were recorded over a scan range of 100−2000 Da using a data-dependent acquisition method (DDA) scanning approach. MS/MS data obtained from DDA were acquired using collision energies based on mass and charge state of the candidate ions. Supplementary ions of peptides identified in MS were also targeted for MS/MS acquisition with collision energy of 25−35 eV. All MS/MS spectra were manually validated. Semiquantitative analyses of oxidized derivatives acquired in the MS mode were performed in duplicate. Extracted ion chromatograms of tryptic digests were used to calculate ratios of the abundances of modified peptides to unmodified peptides. All charge states of peptides detected in MS spectra were added to obtain the total area of unmodified peptides, peptides that contained oxidized residues, and DMPO-containing peptides. Data analyses were

performed using MassLynx version 4.0 (Waters Corp.) and ProteinLynx software supplied by the manufacturer.

RESULTS

Direct ESR Spectroscopy. Direct ESR spectroscopy was used to detect and characterize stable radicals on α-lactalbumin after riboflavin-mediated photooxidation (Figure 1A). A relatively stable radical was observed on α-lactalbumin showing a high degree of hyperfine splitting. The line width of the protein radical was 6.9 G, which is too narrow for a tyrosyl radical. DMPO spin trapping (100 mM) revealed a hydroxyl radical (DMPO/•OH, $a_N = a_H = 14.9$ G), which did not change in intensity in the presence of catalase or superoxide dismutase (data not shown). Although the radical observed at room temperature was not trapped with DMPO, a decrease in the intensity of this radical was observed with increasing DMPO concentration, down to undetectable levels for 100 mM DMPO (Figure 1B).

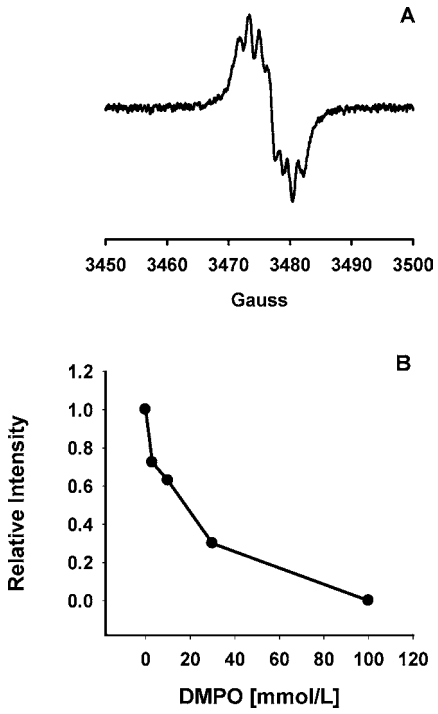

Figure 1. (A) ESR spectra of 0.2 mM α-lactalbumin after 30 min of light exposure in the presence of 13 μM riboflavin. (B) Relative intensities obtained after double integration of the peak shown in (A) when titrated with 0−100 mM DMPO. The exposure to light was conducted at pH 7.4 and room temperature with a 300 W lamp at λ > 300 nm. The spectra were obtained at room temperature with the settings given under Materials and Methods.

Immunochemical Detection. Immunochemical detection of protein−DMPO nitrone adducts was performed with ELISA (Figure 2) and Western blotting (Figure 3) in parallel after riboflavin-mediated photooxidation. The concentration of DMPO (0, 1, 3, 10, 30, 100 mM) was varied in the light exposure experiments. α-Lactalbumin had the highest level of trapped radicals at 30 mM DMPO when detected by ELISA, indicating a scavenging effect of DMPO. Western blotting showed the same intensity at 30−100 mM DMPO in the band corresponding to monomeric α-lactalbumin (Figure 3). However, the Western blot also revealed that riboflavin-mediated oxidation resulted in polymerization of α-lactalbumin.

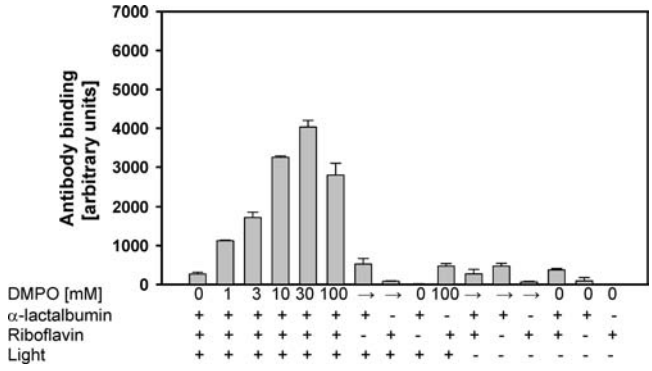

Figure 2. Effect of DMPO concentration on riboflavin-mediated photooxidation of 0.2 mM α-lactalbumin with 13 μM riboflavin. The exposure to light was conducted at pH 7.4 and room temperature with a 300 W lamp at λ > 300 nm, and the DMPO-trapped radicals were detected by the use of monoclonal anti-DMPO as described under Materials and Methods. The arrow (→) is the same as the previous number.

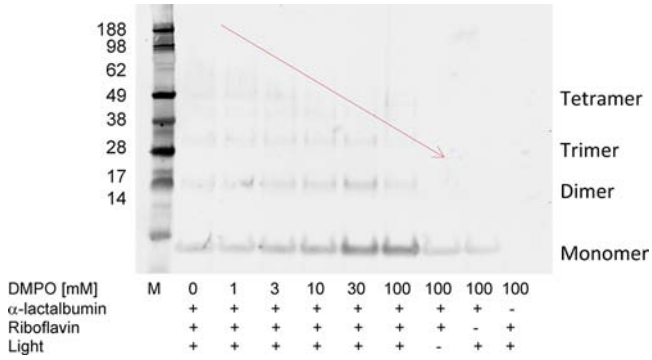

Figure 3. Effect of DMPO concentration on riboflavin-mediated photooxidation of 0.2 mM α-lactalbumin with 13 μM riboflavin measured with anti-DMPO Western blotting. The exposure to light was conducted at pH 7.4 and room temperature with a 300 W lamp at λ > 300 nm. In each lane, 0.4 nmol of α-lactalbumin was loaded.

This polymerization was dependent on the concentration of DMPO, decreasing with increasing DMPO concentration.

Detection of Oxidized Proteins by Flow Rate Mass Spectrometry. The oxidized residues detected by DMPO trapping in α-lactalbumin were identified by mass spectrometry. MS analyses of samples, without preliminary enzymatic digestion, were performed using flow rate mass spectrometry. Figure 4 presents the MS spectra of the α-lactalbumin-containing samples for the ninth charge state, for which the unmodified protein was detected at m/z 1576.4^{9+} (Figure 4A). When the protein was irradiated in the presence of riboflavin, the oxidized products thus formed corresponded to the addition of 16, 32, 48, and 64 Da to the intact protein (Figure 4B). Whereas the presence of the DMPO in the irradiated α-lactalbumin/riboflavin system slightly inhibits the formation of these oxidized products, supplementary peaks were observed for the ninth charge state (Figure 4C). These could be assigned to the addition of DMPO plus an additional 16, 32, 48, and 64 Da compared to the unmodified protein. Comparable analysis of controls did not detect these modifications, indicating that the alterations in the protein were due to irradiation in the presence of riboflavin.

Despite the detection of DMPO derivatives and other oxidative modifications in the α-lactalbumin-containing sam-

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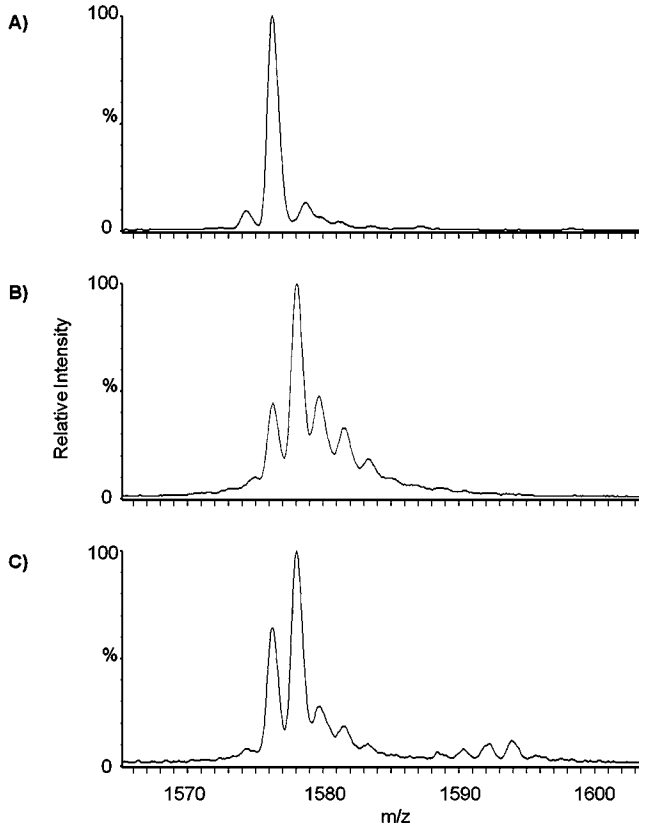

Figure 4. ESI-MS spectra of the ninth charge state of irradiated samples containing (A) α-lactalbumin, 0.2 mM; (B) α-lactalbumin, 0.2 mM plus 13 μM riboflavin; and (C) α-lactalbumin 0.2 mM plus 13 μM riboflavin and 100 mM DMPO. The same spectrum as (A) could be obtained from analyses of protein-containing solutions omitting riboflavin, DMPO, or irradiation.

ples, flow rate mass spectrometry analyses did not allow the identification of target residues of the riboflavin-mediated reaction. Thus, the DMPO−protein adduct ions detected by flow rate MS could correspond to a mixture of several isomers of α-lactalbumin derivatives. Therefore, LC-MS/MS experiments of trypsin digests were performed to disambiguate the locations of both oxidized derivatives and DMPO adducts.

Identification of Oxidation Products by LC-MS/MS. Whereas almost complete sequence coverage was obtained for trypsin digests of the α-lactalbumin, only three peptides contained oxidative modifications after irradiation in the presence of riboflavin. As summarized in Table 1, oxidation was detected for peptides 80−93, 59−79, and 17−58 (α-lactalbumin numbering) when the protein plus riboflavin mixture was analyzed. Ions corresponding to the addition of the spin trap were observed on peptides 59−79 and 17−58 from the LC-MS analysis of DMPO-containing samples (Table 1). These oxidized products were not detected in comparable analyses of control samples omitting riboflavin or DMPO, indicating that their formation was dependent on the photoreaction. MS/MS experiments were then performed to target oxidized products and DMPO-containing peptides detected by MS to disambiguate the locations of modified residues.

Analysis of Peptide 80−93. The ion corresponding in mass to the protonated peptide 80−93 observed at m/z 858.4^{2+} would correspond to the addition of 16 Da to the unmodified peptide. The corresponding MS/MS spectrum indicated that

Table 1. Peptides with Oxidative Modifications of α-Lactalbumin (0.2 mM after 30 min of Light Exposure in the Presence of 13 μM Riboflavin with or without 100 mM DMPO[a]

peptide	modified residue	modification	m/z
80−93	Met 90	+16 Da	858.4^{2+}
59−79	His 68	+14 Da	869.0^{3+}
	His 68	+32 Da	875.0^{3+}
	His 68	+50 Da	881.0^{3+}
	His 68	+111 Da	901.4^{3+}
17−58	Tyr 50 or 32−41	+16 Da	1576.4^{3+}
	Tyr 50 or 32−41	+127 Da	1613.4^{3+}
17−31	Tyr 18	+16 Da	844.9^{2+}
32−50	Tyr 36	+16 Da	1080.0^{2+}
37−53	Tyr 50	+16 Da	965.9^{2+}

[a]The exposure to light was conducted at pH 7.4 and room temperature with a 300 W lamp at λ > 300 nm.

the main oxidation was Met90 oxidized to sulfoxide (data not shown).

Analysis of Peptide 59−79. In the tryptic digest of α-lactalbumin irradiated in the presence of riboflavin, MS/MS detected the formation of protonated peptides 59−79 with additional 14, 32, and 50 Da moieties. Figure 5 shows the MS/MS spectrum of the ion m/z 869.0^{3+}, which was assigned to the protonated peptide 59−79, with an additional 14 Da. Upon collision-induced dissociation (CID), this precursor ion showed a fragmentation pattern similar to that of the corresponding unmodified peptide and gave rise to the formation of mainly y and b ions. More particularly, the series y_{13}−y_{19} and b_{10}−b_{17} corresponded to a shift in mass of an additional 14 Da, whereas the series y_3−y_{11} and b_2−b_8 remained identical to the same series in the MS/MS spectrum of the unmodified peptide. These data allowed the assignment of an oxidized derivative localized on Pro67−His68 residues of α-lactalbumin. LC-MS analyses also allowed the detection of ions m/z 875.0^{3+} and 881.0^{3+}, which would correspond to the protonated peptide 59−79 with an additional 32 and 50 Da, respectively. These ions were detected at similar retention times, suggesting that m/z 875.0^{3+} could be generated during the ionization process following the elimination of H_2O from the precursor ion observed at m/z 881.0^{3+}. The fragmentation pathway of both ions detected at m/z 875.0^{3+} and 881.0^{3+} would also correspond to the oxidation of His68. More particularly, the MS/MS spectra indicated the rapid formation of a daughter ion at m/z 869.0^{3+} (data not shown), which showed a similar fragmentation as previously reported for the peptide 59−79, with the addition of 14 Da localized on the Pro67−His68 sequence (Figure 5). Because the signals corresponding to the addition of 14 and 32/50 Da were detected at different retention times, the formation of these derivatives must have occurred in solution and not during the ionization process.

In the tryptic digest of α-lactalbumin irradiated in the presence of riboflavin and DMPO, DMPO adducts were detected. The ions detected at m/z 901.4^{3+} correspond to the addition of 111 Da (DMPO) compared to the unmodified peptide. Although the signal-to-noise ratio of the corresponding MS/MS spectra was relatively low, the CID of the precursor ion indicated the formation of a DMPO adduct localized on

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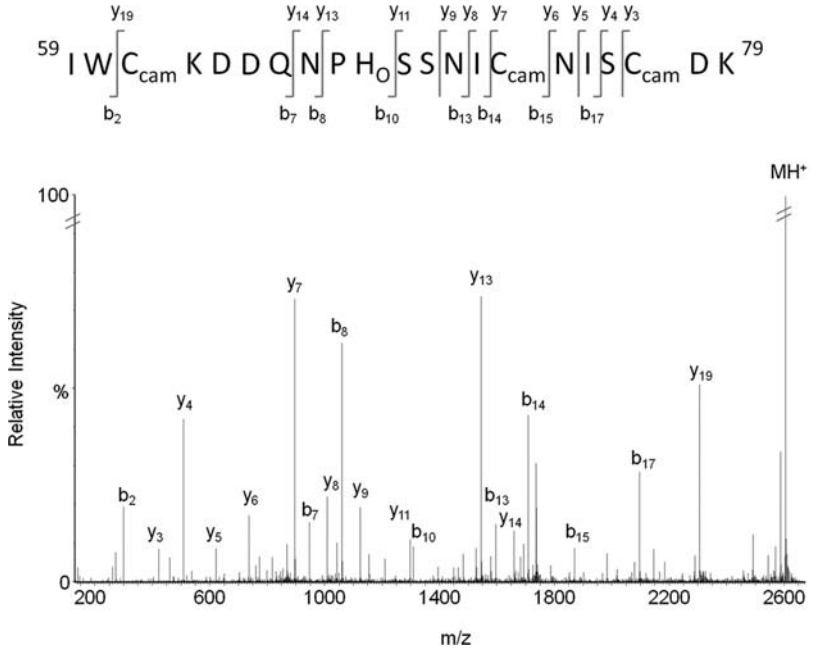

Figure 5. Deconvoluted MS/MS spectrum acquired from the precursor ion of m/z 869.0^{3+}, which corresponds in mass to tryptic peptides 59−79 + 14 Da. For clarity, not all identified fragment ions are labeled in the spectrum. Peptide was obtained from a tryptic digest of sample containing α-lactalbumin plus riboflavin.

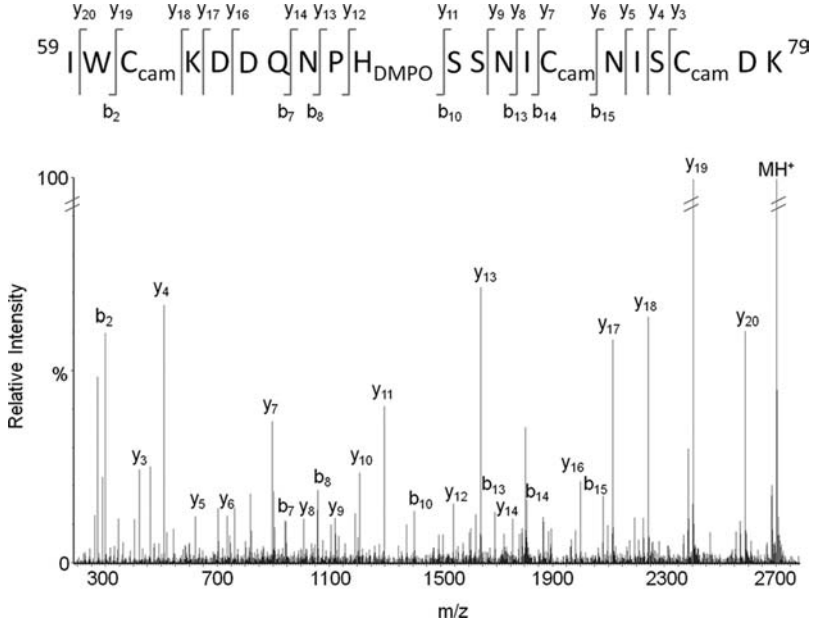

Figure 6. Deconvoluted MS/MS spectrum acquired from the precursor ion of m/z 901.4^{3+}, which corresponds in mass to tryptic peptides 59−79 + 111 Da. For clarity, not all identified fragment ions are labeled in the spectrum. The peptide was obtained from a tryptic digest of sample containing 0.2 mM α-lactalbumin, 13 μM riboflavin, and 100 mM DMPO.

residue 68. More particularly, Figure 6 shows the MS/MS spectrum of the precursor ion m/z 901.4^{3+}, which gave rise mainly to amino-terminal b ions and acid-terminal y ions. The series b_{10}−b_{15} and y_{12}−y_{20} were detected with a shift in mass of 111 Da, whereas the daughter ions y_3−y_{11} and b_2−b_8 were identical to the same series in the MS/MS spectrum of the unmodified peptide. Therefore, these data indicate the formation of the nitrone adduct localized on His68. Moreover, the fragmentation pathways of two other precursor ions, m/z 912.1^{3+} and 917.4^{3+}, also suggest a DMPO adduct plus 16 Da on His68, with supplementary oxidation of Pro67 (Supporting Information, Figures S1 and S2).

Analysis of Peptide 17−58. In the tryptic digest of α-lactalbumin irradiated in the presence of riboflavin and DMPO, the protonated peptide 17−58 was observed with an additional 16 and 127 Da at m/z 1576.4^{3+} and 1613.4^{3+}, respectively. More particularly, the MS/MS spectrum of the ion detected at m/z 1576.4^{3+} would correspond to a mixture of isomers with oxidation of the tyrosine residues in position 18, 36, or 50 (data not shown). Data obtained from the precursor ion m/z 1613.4^{3+} also allowed the assignment of one isomer with an

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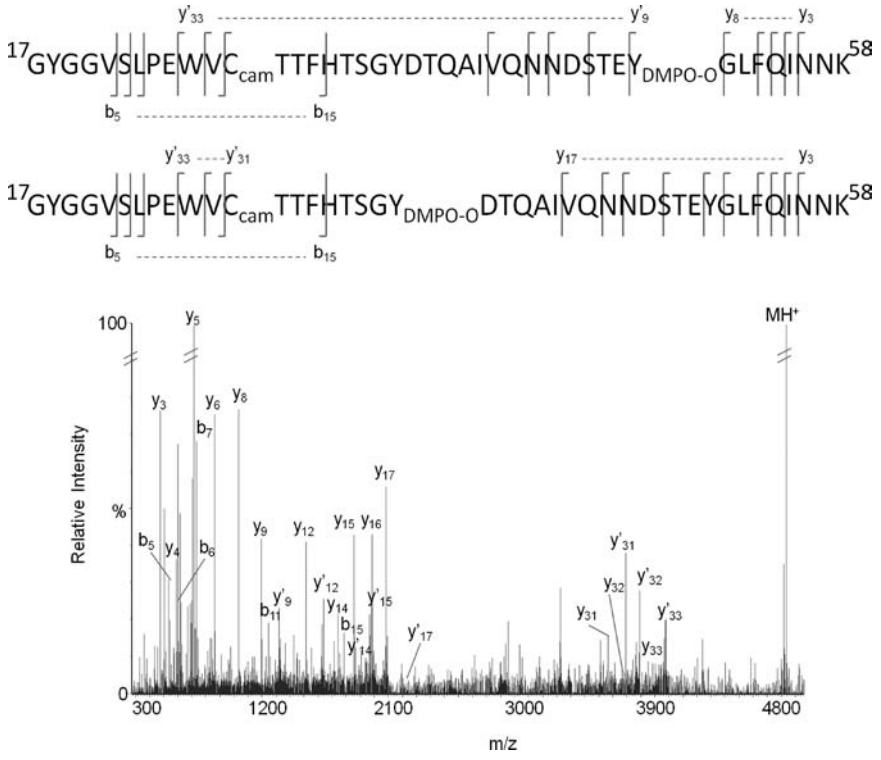

Figure 7. Deconvoluted MS/MS spectrum acquired from the precursor ion of m/z 1613.4^{3+}, which corresponds in mass to tryptic peptides 17−58 + 127 Da. For clarity, not all identified fragment ions are labeled in the spectrum. The peptide was obtained from a tryptic digest of sample containing 0.2 mM α-lactalbumin, 13 μM riboflavin, and 100 mM DMPO.

oxidized derivative localized on one of the tyrosine residues (Figure 7). Modification of sequence 32−41 would leave the series y'_{33}−y'_{31} with a shift in mass of 127 Da and the series y_3−y_{17} unchanged. A second isomer could be attributed to the addition of DMPO plus 16 Da localized on Tyr 50. This would involve the formation of the series y'_9−y'_{33} with an additional 127 Da, whereas daughter ions y_3−y_8 would remain identical when compared to the same series in the MS/MS spectrum of the unmodified peptide. However, despite the detection of oxidized and DMPO derivatives from the analysis of the peptide 17−58, the fragmentation of precursor ions was insufficiently informative to clearly identify the modified residues. The riboflavin-mediated reaction could modify several amino acids on peptide 17−58, including tryptophan, tyrosine, and histidine. Therefore, supplementary experiments of tryptic digested samples further submitted to chymotrypsin digestion were performed to disambiguate the localization of modified residues. The analyses of the obtained samples allowed the detection of the 17−31, 32−50, and 37−53 peptides. Although the signal-to-noise ratio of the MS spectra was low, three oxidative modifications could be observed at m/z 844.9^{2+} (17−31 plus 16 Da), 1080.0^{2+} (32−50 plus 16 Da), and 965.9^{2+} (37−53 plus 16 Da). These modifications were not detected in a comparable analysis of control samples omitting riboflavin and/or DMPO, indicating that their formation was due to irradiation of α-lactalbumin in the presence of riboflavin.

The ion m/z 844.9^{2+} could be assigned to the protonated peptide 17−31 with an additional 16 Da. MS/MS data allowed the assignment of a DOPA derivative localized on Tyr18 because of a shift in mass of the series b_3−b_{13} with an additional 16 Da (Supporting Information, Figure S3). Otherwise, the ion at m/z 1080.0^{2+}, which corresponds to the protonated peptide 32−50 plus 16 Da, showed a fragmentation pattern assigned to the oxidation of Tyr36. Whereas the fragment ion b_2 remained unchanged, the series b_6−b_{15} corresponded to an oxidized daughter ion with a shift in mass of an additional 16 Da (Supporting Information, Figure S4). Finally, a DOPA derivative localized on Tyr50 could also be characterized from the MS/MS spectrum of the ion m/z 965.9^{2+}. Indeed, whereas the series y_1−y_2 and b_2−b_7 remain unchanged compared to the protonated peptide 37−53, the series y_4−y_{11} is detected with a shift in mass of an additional 16 Da (Supporting Information, Figure S5).

Therefore, MS/MS data obtained from the analyses of peptide 17−58 led to the conclusion that tyrosine residues in positions 18, 36, and 50 of the α-lactalbumin were oxidized during irradiation in the presence of riboflavin. Whereas the formation of a DMPO-containing peptide was characterized from the 17−58 trypsin fragments, the conversion of tyrosine residues to DOPA was verified with further chymotrypsin digestion.

DISCUSSION

LC-MS/MS analyses of the α-lactalbumin samples showed that the main modifications by the riboflavin-mediated reaction were traced to five residues, of which three were Tyr residues, one was His, and one was Met. These three amino acid residues have previously been shown to become oxidized by riboflavin-mediated oxidation[6] but have never been investigated in relation to the protein structure. This study was undertaken to determine whether the location of the residues in the protein structure would influence the susceptibility to oxidative modifications and to determine to what extent DMPO was able to trap the protein radicals before they were further oxidized into secondary oxidation products.

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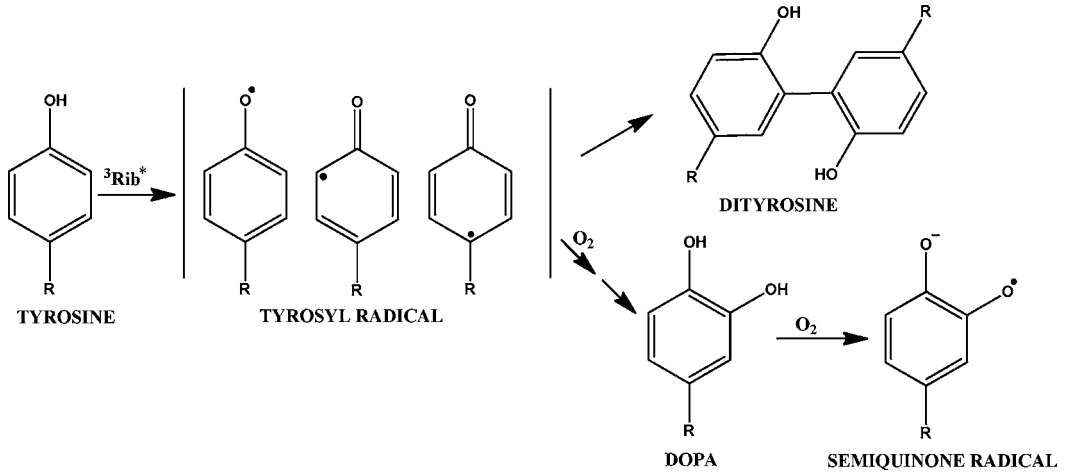

Figure 8. Light-induced oxidation of tyrosine in reaction with triplet excited riboflavin (^{3}Rib*).

α-Lactalbumin holds four Tyr residues (Tyr18, Tyr36, Tyr50, and Tyr103). According to the X-ray crystal structure, Tyr18 is highly exposed to the surface, Tyr103 is exposed, and Tyr36 and Tyr50 are partly exposed to the surface.[22] MS analyses confirmed the reactivity of three of these tyrosine residues (Tyr18, Tyr36, and Tyr50), which are converted to DOPA derivatives following the reaction with riboflavin; a DMPO-trapped tyrosyl radical was also detected. DOPA modification was more frequently observed compared to the DMPO trapped tyrosyl radical, indicating a rapid conversion of tyrosyl radicals into DOPA with less chance of the DMPO competing in trapping the tyrosyl radical. Due to the specific reaction with the triplet excited riboflavin, DOPA and dityrosine are rapidly formed.[7] DOPA is known to cause further oxidative damages to DNA, lipids, and proteins in more complex systems[23] as well as to reduce transition metal ions, which enhance catalytic conversion of hydroperoxides into radicals.[24,25] Although dityrosine formation is a termination reaction,[15] which could suggest proteins and tyrosine-rich peptides to be good antioxidants,[14] triplet excited riboflavin induced damages on tyrosine residues following at least two pathways (Figure 8). In this particular case, the formation of the end-product DOPA seems to be favored over the spin trapping of tyrosyl radicals detected by MS as the DMPO adduct at Tyr36 and Tyr50.

The generation of DOPA correlates well with the radicals detected by ESR, which is due to the narrow line width (<7 G). An identical radical has previously been detected in β-lactoglobulin and BSA after a riboflavin-mediated reaction, where it appeared to be the *o*-semiquione radical[7] as the tyrosyl radical has a broader line width;[1] therefore, the DOPA-derived *o*-semiquione radical detected by Chen,[26] which previously has been detected in other proteins after a riboflavin-sensitized reaction,[7] is suggested. As the *o*-semiquione radical is not trapped by DMPO and because the intensity of this radical decreased with increasing concentration of DMPO (0−100 mM), this indicated a scavenging effect of the spin trap in this system. This result is further supported by a decrease in the degree of polymerization of α-lactalbumin with increasing DMPO concentration, as detected by Western blotting. Fluorescence spectroscopy previously revealed the formation of dityrosine cross-linkages in α-lactalbumin following irradiation in the presence of riboflavin.[6] In agreement with these previously published results, we suggest that the nonreducible cross-links observed in the Western blotting in the present study may be the result of intermolecular dityrosine formation. It may be similar to the intermolecular dityrosine formation between Tyr18 and Tyr50 after a peroxidase-mediated reaction,[3] although our MS results could not confirm the hypothesis as no cross-linking was detected by MS. Oxidative modification could not be detected on Tyr103, even though this particular Tyr residue is rather solvent-exposed.[22] However, the modification is located close to the Trp118 in the tertiary structure of α-lactalbumin (Figure 9), and Trp residues have

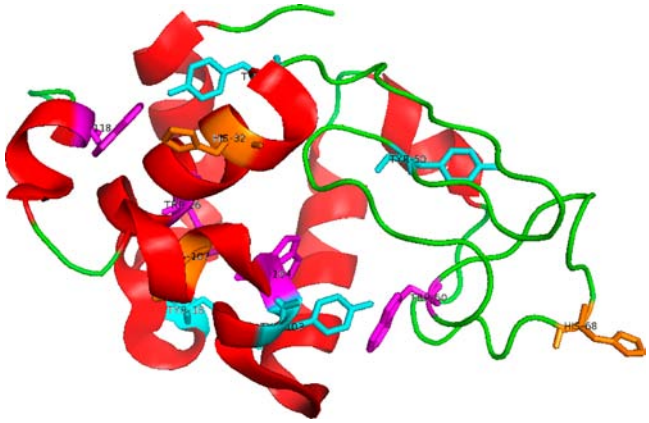

Figure 9. Tertiary structure of monomeric apo-α-lactalbumin III. α-Helical structures are colored in red and loops in green. His residues are colored in orange, Tyr in cyano, and Trp in pink. The structure was obtained from the PDB file IF6R using the software Pymol Molecular Graphic System, 2010 Schrodinger, LLC.

been shown to quench triplet-excited riboflavin with a rate competing with atmospheric oxygen,[27] whereas their reactivity toward singlet oxygen results in the generation of *N*-formylkynurenine.[6,28,29] Hence, quenching by Trp could potentially explain the lack of oxidation on Tyr103, but as the MS analysis did not reveal any Trp modification, this point will require further investigation.

The deconvoluted MS/MS spectra derived from the experiments clearly allow the assignment of a sulfoxide to Met90, which can be generated through a type II mechanism.[30,31] However, sulfoxide can also be generated through an electron transfer reaction and, therefore, the type I mechanism cannot be excluded. MS/MS analysis revealed the

formation of imidazolone and hydrated imidazolone derivatives from the His68 residue. Such unusual oxidation of a histidine residue to +14 and +50 Da products has previously been described as a photooxidative pathway involving $^1O_2$.[32−35] One of the intermediates is a histidine containing a double-bonded oxygen plus two hydroxyl groups (His +50 Da), and the loss of two water molecules from this product would give rise to the His +14 Da product. These species would be formed not during the ionization process, but in solution following the irradiation of the α-lactalbumin in the presence of riboflavin. The reaction of histidine residues to form imidazolone and the hydrated imidazolone derivatives could not be observed with other common reactive oxygen species and, thus, would be specific to the production of singlet oxygen during the riboflavin-mediated reaction. Riboflavin has been suggested to react primarily through a type I reaction,[36,37] but the reaction mechanism is highly dependent on the content of dissolved oxygen, which is lower in aqueous solution than in systems with higher fat content.[37] However, the generation of imidazolone indicates that singlet oxygen in a type II reaction is important as well.

There are two other His residues in the primary structure of α-lactalbumin that were neither modified by DMPO trapping nor identified as other oxidation products. His107 is hidden in the core of the protein structure and is hence less readily accessible to both riboflavin and DMPO, which may explain the lack of oxidative modification on that residue. The other His residue (His32) is highly surface exposed; it is, however, close to Trp118 in the tertiary structure of α-lactalbumin[22] and, as with Tyr103, quenching of triplet-excited riboflavin by Trp118 may cause the lack of oxidation of His32. Trp has previously been shown to compete with oxygen in the reaction with triplet excited riboflavin,[27] and it is the only amino acid that physically quenches singlet oxygen, whereas chemical quenching or scavenging is seen for Tyr, Cys, Met, His, and Trp.[38,39] No chemical changes were observed on any of the Trp residues in the present study; hence, physical quenching could be the explanation, but earlier studies have reported the formation of both *N*-formylkynurenine and kynurenine,[6,28] so further experiments are needed to elucidate the mechanism in detail.

Although α-lactalbumin contains other residues susceptible to oxidation such as histidine, tyrosine, and tryptophan residues, no other oxidation products could be detected. Whereas the signal-to-noise ratio for peptides containing DMPO adducts was low for the corresponding histidine- and tyrosine-containing peptides, MS analyses were sufficiently sensitive to show a correlation between the riboflavin-mediated reaction and the generation of nitrone adducts. However, other modifications could not be excluded.

In conclusion, the present study showed that DMPO trapped protein radicals at one His and one Tyr residue and that both surface exposure and the neighboring residues in the tertiary structure of α-lactalbumin influence the sites of initial radical generation, DMPO trapped radicals, and secondary oxidation products. Both type I and type II mechanisms were involved in the generation of radicals and other oxidation products upon the riboflavin-sensitized reaction. Furthermore, the combination of ESR, immuno-spin trapping, and MS is a promising tool box for the characterization of radical generation in food proteins. The approach of using Western blotting in combination with MS and different separation techniques will also enable us to look into reaction mechanisms in more complex food systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Further modifications on different peptides, peptide 59−79 (Figures S1 and S2), peptide 17−31 (Figure S3), peptide 32−52 (Figure S4), and peptide 37−53 (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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