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Identification of Modified Lysozyme Peptides upon Photo-oxidation by LC-TOF-MS

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ABSTRACT: Protein oxidation can have major implications on the quality and safety of foods, but the majority of methods to evaluate oxidative damage lack specificity. Therefore, this study aimed to identify specific markers for protein oxidation. A well-characterized protein, lysozyme, was modified by photo-oxidation and subsequently hydrolyzed prior to peptide analysis by LC-TOF-MS. A semiquantitative analysis of the peptides indicated that from the seven peptides containing sensitive amino acids, two peptides (HGLDNYR and WWCNDGR) were highly affected upon photo-oxidation and have the potential to serve as markers for protein oxidation. Site-specific modifications enabled the description of the degradation pathway of several lysozyme peptides but also indicated that the surrounding amino acids and the 3D structure of the protein have an impact on the induced modifications. It is therefore advisable to evaluate protein oxidation on the intact protein.

KEYWORDS: *photo-oxidation, lysozyme, peptides, mass spectrometry*

INTRODUCTION

Notwithstanding the fact that the impact of protein oxidation in biological systems has been recognized for many years,[1−3] it was only over the past decade that researchers were focusing on oxidative modification of food proteins.[4−6] The results indicated severe modifications that could affect not only the nutritional quality and the biological activity of the proteins[7] but also the safety by the formation of toxic compounds.[8] As is known, oxidation is generally induced by reactive oxygen species (ROS), which are inevitable in foods.[9] They are formed by different mechanisms such as photo-oxidation,[4] enzyme- and metal-catalyzed oxidation,[10] or the presence of autoxidizing lipids.[11,12] So far, protein oxidation has been mostly evaluated by the analysis of generic markers such as the analysis of protein carbonyls.[13] It is doubtful if this is indeed a good marker due to the fact that not all protein oxidation products are carbonyls and not all carbonyl compounds are a result of protein oxidation as described by Koivumaki et al.[14] Upon comparing hypochlorous and peracetic acid induced oxidation of dairy proteins, Kerkaert et al.[15] moreover concluded that protein carbonyls cannot be used as markers for the detection of the fraudulent use of disinfectants and that more specific markers were required. As an answer to this need a method was developed to analyze α-aminoadipic and γ-glutamic semialdehydes, both specific protein carbonyls formed upon lysine, arginine, and proline degradation.[16] Nevertheless, although this recent method can be applied to evaluate protein oxidation in meat systems, it cannot be used to evaluate photo-oxidation because this mainly affects the aromatic amino acids.[4,17] The amino acids that result in these specific compounds are, moreover, not that prone to oxidation.

Besides the analysis of protein carbonyls, amino acids are often determined after hydrolysis. Whereas this gives an idea of the affected amino acids, it does not give site-specific information, which is required to gain more knowledge about the reaction mechanisms occurring upon oxidation. Schey and Finley[18] were the first to identify sites and structures prone to oxidative modifications by tandem mass spectrometry. A lens protein, α-crystallin, was subjected to metal-catalyzed oxidation, and modified peptides were identified and semiquantified after tryptic digestion, resulting in a better understanding of the oxidation pathway. Equivalent research was recently done for β-lactoglobulin (BLG), except instead of oxidizing the protein, its three tryptic peptides were oxidized after separation and fractionation by preparative liquid chromatography.[14] Several modified peptides were identified, and some of them were suggested to serve as potential indicators for BLG oxidation. Although under mild oxidation conditions, the identification of modified peptides is relatively easy, it becomes more complicated for highly oxidized peptides, especially when they contain several sensitive amino acids. Therefore, Grosvenor et al.[19] evaluated photo-oxidation induced modifications of synthetic peptides that contained only one sensitive amino acid such as tryptophan or tyrosine. As a result, different photo-oxidation pathways of bound tryptophan and tyrosine could be identified. It should be noted that the use of synthetic peptides enabled a full identification of the degradation pathway; it did not take into account the effect of surrounding sensitive amino acids on the tryptophan and tyrosine degradation. Similarly, upon analyzing modifications of peptides that were hydrolyzed prior to oxidation as done for BLG, the effect of some amino acids was included but not the effect of the total protein.[14] Whereas previously mass

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Table 1. Lysozyme Peptides Determined by LC-TOF-MS after Tryptic Digestion[a]

peptide sequence	position	$[M + H]^+$	$[M + 2H]^{2+}$
NLCNIPCSALLSSDITASVNCAK	92−114	2337.1247	1169.06235
NTDGSTDYGILQINSR	64−79	1753.8351	877.41755
IVSDGNGMNAWVAWR	116−130	1675.8009	838.40045
FESNFNTQATNR	52−63	1428.6502	714.8251
GYSLGNWVCAAK	40−51	1268.6092	634.8046
GTDVQAWIR	135−143	1045.5425	523.27125
WWCNDGR	80−86	936.3781	468.68905
HGLDNYR	33−39	874.4166	437.7083
CELAAAMK	24−31	836.4004	418.7002
KVFGR	19−23	606.3722	303.6861

[a]The monoisotopic masses of the single- and double-charged peptides are given.

spectrometry was applied to analyze peptide modifications upon metal-catalyzed[14] or ozone-induced oxidation,[20] this study focused on the identification of modified lysozyme peptides formed after hydrolysis with trypsin, upon photo-oxidation of the intact protein.[17] This study focused on photo-oxidation because it was previously seen that this has a major impact on the peptide profile of dairy proteins.[21,22] Upon photo-oxidation of lysozyme, the modifications were evaluated by the carbonyl content, amino acid, and a semiquantitative peptide analysis, prior to the identification of the modified peptides.

MATERIALS AND METHODS

Chemicals. Hen egg white lysozyme, 2,4-dinitrophenylhydrazine (DNPH), ortho-phthaldialdehyde (OPA), 9-fluorenylmethylchloroformate (FMOC), sodium azide, phenol, amino acid standards, and LC-MS grade acetic acid were delivered by Sigma-Aldrich (Bornem, Belgium). Sequencing grade modified trypsin was obtained from Promega Benelux (Leiden, The Netherlands). Rapigest SF was purchased from Waters (Zellik, Belgium). Potassium phosphate, sodium dihydrogenphosphate, sodium tetraborate, hydrochloric acid, sodium chloride, sodium sulfite, and urea were all of analytical grade and purchased from Chemlab (Zedelgem, Belgium). Mercaptoethanol was delivered from VWR (Leuven, Belgium) and trichloroacetic acid (TCA) from Acros Organics (Geel, Belgium). All solvents were obtained from Fisher Scientific (Aalst, Belgium) and were of LC-MS grade. Water was purified by a Milli-Q system (Millipore, Belgium).

Storage Experiment. Model solutions containing 6 mg/mL lysozyme and 0, 1.5, or 3 μg/mL riboflavin were prepared in 10 mM phosphate buffer saline (pH 6.8). The solutions were sterilized by filtration (CA, 0.45 μm, Novolab, Lokeren, Belgium) and incubated at 4 °C on an orbital shaker (Edmund Bühler, Hechingen, Germany) under homogeneous illumination (1500 lx) obtained in a light cabinet using Philips TL-D 36W/840 fluorescent tubes, fixed 0.5 m above the bottles. The illumination intensity was measured at the level of the bottlenecks using a Lux meter (PAR-cell 532; 400−700 nm, Skye Instruments, Llandrindod Wells, UK). Duran bottles (250 mL) were filled with 150 mL of emulsion. Reference samples, not subjected to illumination, were stored in the dark. Each model solution was prepared and stored in triplicate.

Riboflavin Analysis. The protein solutions were analyzed for their riboflavin content by an HPLC method as previously described.[12] The solutions were injected as such on a LiChrosorb RP-C18 HPLC column (250 mm × 4.6 mm × 10 μm, Varian, Sint-Katelijne-Waver, Belgium) and detected fluorometrically at excitation and emission wavelengths of 450 and 530 nm, respectively. Quantification was performed using an external calibration curve.

Protein-Bound Carbonyls. Protein carbonyls were determined after derivatization with DNPH.[13] Therefore, 0.4 mL of DNPH (10 mM in 2 M HCl) was added to 0.3 mL of protein extract. The extracts were subsequently incubated in the dark for 60 min. Afterward, 0.7 mL of TCA was added (10% final concentration), and the samples were incubated on ice during 10 min and centrifuged (9000*g*, 3 min). The protein pellets were washed three times with 1 mL of ethanol/ethyl acetate (1:1, v/v) to remove the excess DNPH. The final pellet was dissolved in 0.5 mL of 6 M urea, and the absorbance was measured at 370 nm. The protein-bound carbonyl content was determined using a molar absorption coefficient of 22000 M/cm on blank subtracted data.

Tryptophan and *N*-Formylkynurenine (NFK). The tryptophan content was measured fluorometrically (with a Spectramax Gemini XPS fluorometer, Molecular Devices, Brussels, Belgium) on the protein solution using excitation and emission wavelengths of 280 and 330 nm, respectively. For NFK, 330 and 440 nm were used, respectively. For this, 50 μL of protein extract was diluted in 250 μL of 6 M urea to completely unfold the protein structure.[4]

Amino Acid Analysis. Proteins were hydrolyzed to their constituent amino acids, which were then derivatized with OPA and FMOC and separated on HPLC.[23] An acid hydrolysis was performed by adding 2 mL of 12 M HCl containing 0.1% phenol and 0.1% Na_2SO_3 to a 2 mL protein solution in a glass tube with a Teflon screw cap. The samples were vortexed, incubated for 24 h at 105 °C, neutralized, and further diluted to 20 mL. The final hydrolysate was filtered over a 0.45 μM PTFE syringe filter (Grace, Lokeren, Belgium). All of the amino acids were automatically derivatized in the injector of an Agilent 1100 system (Agilent Technologies, Switzerland). The derivatized amino acids were separated on a Zorbax Eclipse AAA Rapid Resolution column (4.6 × 150 mm, 3.5 μm, Agilent Technologies), which operated at 40 °C. A flow rate of 2 mL/min was applied with a gradient of solvent A (45% methanol, 45% acetonitrile, and 10% water) and solvent B (45 mM $NaH_2PO_4 \cdot H_2O$, 0.02% NaN_3, pH 7.8). The OPA and FMOC derivatized amino acids were detected fluorometrically, at excitation and emission wavelengths of, respectively, 340/450 and 266/305 nm. Internal standards norvaline and sarcosine were used for the quantification.

Tryptic Digestion. Prior to hydrolysis, the disulfide bridges were reduced by the addition of 10 μL of mercaptoethanol to 1 mL of diluted lysozyme solution (0.1−0.4 mg/mL) and incubation at 95 °C for 5 min. From this solution 50 μL was taken and 100 μL OF ammonium bicarbonate (100 mM NH_4HCO_3, pH 7.8) was added together with 10 μL OF Rapigest and 10 μL OF sequence grade trypsin, corresponding to a final trypsin/protein ratio of 1:20 (w/w). The solution was subsequently vortexed and incubated in a warm water bath at 37 °C for 8 h. To stop the digestion and inactivate the Rapigest, 20 μL of acetic acid was added, followed by an incubation of 1 h at room temperature. The digests were finally centrifuged for 5 min at 10000 rpm and 1/2 diluted in a mixture of water/acetonitrile/acetic acid (87:10:3) prior to LC-MS analysis.

Semiquantitative Analysis of the Lysozyme Peptides. The tryptic digests were injected (10 μL) on an Ultimate 3000 RSLC (Dionex, Germany) equipped with a Zorbax 300 SB-C8 column (3.5 μm, 2.1 × 150 mm, Agilent Technologies) applying a flow rate of 0.2 mL/min. The column was operated at a constant temperature of 40 °C. A gradient between 0.1% acetic acid in 90% water/10% acetonitrile (solvent A) and 0.1% acetic acid in 10% water/90% acetonitrile (solvent B) was applied: from 0 to 1 min, 10% B; 1−11 min, 10−100%

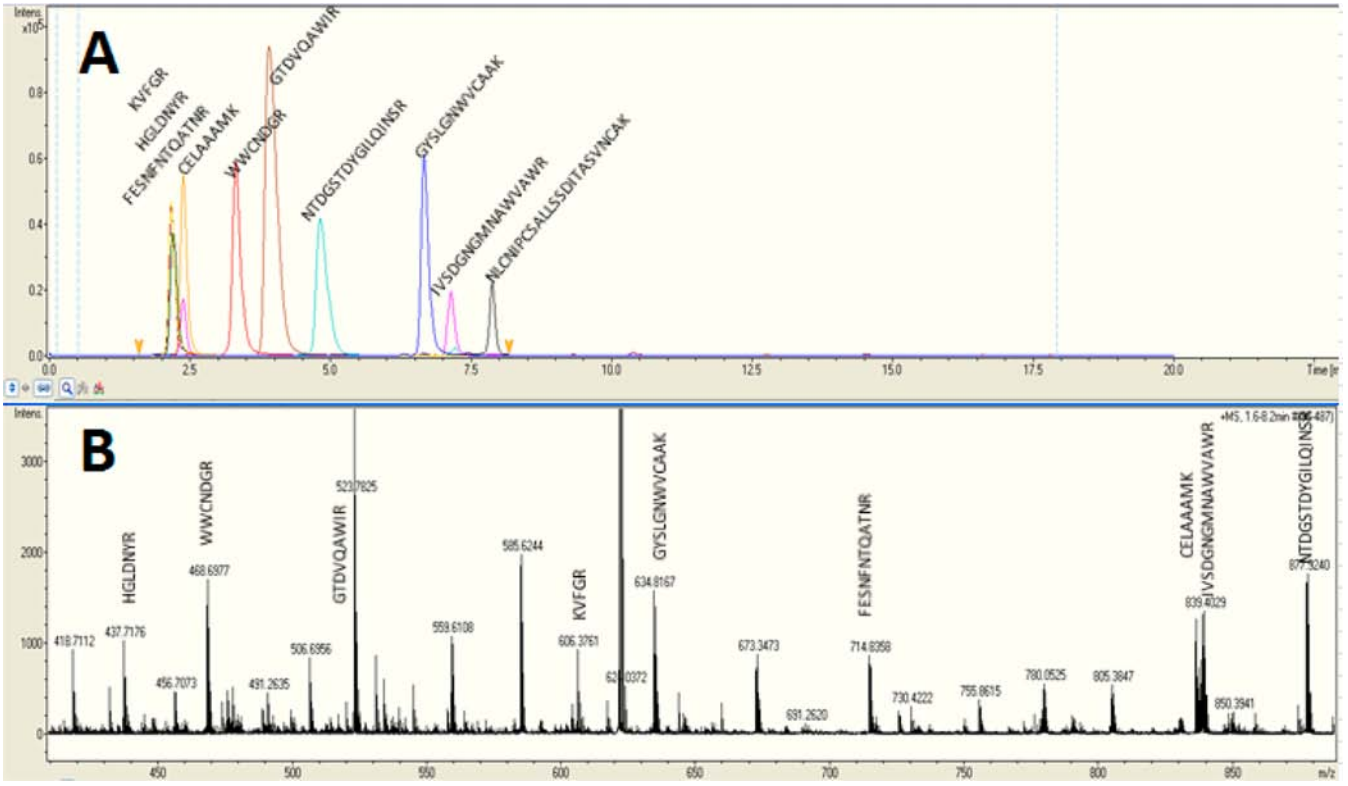

Figure 1. Scan ESI-MS data, m/z range of 100−3000, of the lysozyme peptides that were obtained after an in silico tryptic digestion of lysozyme: extracted ion chromatogram of the peptides detected in native lysozyme (A) and their mass spectrum (B).

Table 2. Photo-oxidation-Induced Degradation of Aromatic Amino Acids in a Peptide or Protein Structure[a]

amino acid	oxidation products and expected mass shifts (ΔDa)				ref
Trp (pathway 1)	OH-Trp +16 (O)	diOH-Trp +32 (2O)	Trp-dione +30 (2O − 2H)	bis Trp-dione +28 (2O − 4H)	19
Trp (pathway 2)	NFK +32 (2O)	Kyn +4 (O − C)	OH-Kyn +20 (2O − C)		19, 28, 33
Trp (pathway 3)	NFK +32 (2O)	OH-NFK +48 (3O)	diOH-NFK +64 (4O)		19
Tyr (pathway 1)	DOPA +16 (O)	TOPA +32 (2O)	TOPA-quinone +30 (2O − 2H)		19
Tyr (pathway 2)	DOPA-quinone +14 (O − 2H)	TOPA-quinone +30 (2O − 2H)			19
His	endoperoxides +32 (2O)	asparagine −23 (−2C − N − H + O)	aspartic acid −22 (−2C − 2N− 2H + 2O)		28, 30, 31
Met	Met sulfoxide +16 (O)	Met sulfon +32 (2O)			28
Cys	cysteic acid +48 (3O)				28

[a]OH-Trp, hydroxytryptophan; diOH-Trp, dihydroxytryptophan; Trp-dione, tryptophandione; bis Trp-dione, unsaturated-2,4-bistryptophandione; NFK, *N*-formylkynurenine; Kyn, kynurenine; OH-Kyn, hydroxykynurenine; OH-NFK, hydroxyformylkynurenine; diOH-NFK, dihydroxyformylkynurenine; DOPA, dihydroxyphenylalanine; TOPA, trihydroxyphenylalanine; DOPA-quinone, dihydroxyphenylalanine quinone; TOPA-quinone, trihydroxyphenylalanine quinone.

B; 11−16 min, 100% B; 16−16.5 min, 100−10% B; and from 16.5 to 21 min, 10% B. The eluted peptides were analyzed by a UV detector (Dionex, Germany) at 214 and 280 nm and by the microTOF II time-of-flight mass spectrometer (Bruker Daltonics, Germany). The electrospray ionization source was operated in the positive mode; the nebulizer (N_2) pressure was set at 2 bar, the nebulizer (N_2) gas flow at 4 L/min, and the dry temperature at 200 °C. The capillary voltage was maintained at 5800 V, and the capillary exit voltage at 150 V; the skimmer potential was 50 V, and the hexapole RF was set at 150 V. Conventional ESI-MS data were recorded using a scan range of m/z from 100 to 3000 and screened for the masses of the lysozyme peptides, which were obtained after an in silico tryptic digestion of

lysozyme (P00698, Swiss-Prot). Because it is known that electrospray ionization of tryptic peptides results in predominantly doubly charged ions, the data were screened for both $[M + H]^+$ and $[M + 2H]^{2+}$. In total, 10 peptides were detected (Table 1; Figure 1), which resulted in a protein sequence coverage of 88% when the signal peptide was not taken into account. An internal mass calibration was performed with the ESI TOF tuning mix (Agilent Technologies). The mass resolution was about 8250 (fwhm), whereas the mass accuracy varied between 8 and 23 ppm with an average of 16 ppm.

A semiquantitative analysis was performed by considering the hydrolysates of the lysozyme solution without riboflavin and stored in the dark as a reference and by assuming that lysozyme was totally digested. The theoretical concentration of the lysozyme peptides in the nonoxidized hydrolyzed lysozyme solution was calculated, and different dilutions of this reference were analyzed to set up a calibration curve for each peptide. Besides, the peptide KVFGR, which does not contain sensitive amino acids, was taken as an internal standard to minimize the signal intensity variation from run to run and to correct for losses during the analysis. All standard curves were based on the relative concentration and relative area of a specific peptide compared to the concentration and area of the internal standard. On the basis of these relative standard curves, the amount of the peptides in the oxidized samples could be calculated and was expressed in micrograms of peptide per milligram of protein.

Identification of Oxidized Lysozyme Peptides. The LC and MS parameters were set as described above. The mass spectra were screened for modified peptides. Therefore, the four most strongly degraded peptides were taken, and all possible modified peptides were considered. To predict which peptides could be formed, the photo-oxidation-induced modifications of the sensitive amino acids, which are described in literature, are summarized in Table 2. As can be observed, photo-oxidation of bound tryptophan can result in eight different mass shifts, whereas only four mass shifts can arise from photo-oxidation of histidine and tyrosine. Methionine can only be oxidized to methionine sulfoxide and methionine sulfone; cysteine, on the other hand, is oxidized to cysteic acid.

For peptides containing one sensitive amino acid, such as GTDQAWIR, it was relatively easy to predict the modified peptides; this became, nevertheless, much more complicated for peptides containing several sensitive amino acids such as WWCNDGR. Upon photo-oxidation of GTDQAWIR, 8 modified peptides could be formed, whereas upon oxidation of WWCNDGR at least 64 (8×8) peptides could be present. The mass spectra of the oxidized and hydrolyzed lysozyme solutions were as such screened on a relatively extended list of monoisotopic single- and double-charged peptides.

Microbial Analysis. During storage, the microbial growth of the samples was controlled by measuring the total psychrotrophic growth on plate count agar (Oxoid, Hampshire, UK).

Statistical Analysis. Nonlinear regression was performed using the SPSS 16 statistics package. Statistical comparison between the amino acid results was done by a one-way ANOVA test, applying a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Riboflavin. Riboflavin remained stable in the lysozyme solutions stored in the dark, whereas it decreased upon illumination. The degradation followed an exponential curve with the following equations, $y = 1.49\ e^{-0.25x}$ solution and $y = 2.948\ \exp^{-0.21x}$ in the solution containing, respectively, 1.5 and 3.0 μg/mL riboflavin (data not shown).

Protein Carbonyls. The protein carbonyl content increased in all lysozyme solutions stored in the light as shown in Figure 2. In the riboflavin-containing solutions, the carbonyl content strongly increased during the first 2 weeks of illumination. Afterward, the formation of protein carbonyls was still observed to a minor extent. The increase in protein carbonyls depended upon the riboflavin concentration with a maximum of 20 and 15 nmol carbonyls/mg protein in, respectively, the 3.0 and 1.5 μg/mL riboflavin-containing lysozyme solutions. In the illuminated solution without riboflavin, no carbonyls were formed during the first 2 weeks of storage, whereas, afterward, a small increase was seen up to 5 nmol carbonyls/mg protein. No carbonyls were formed in the lysozyme solutions stored in the dark (data not shown).

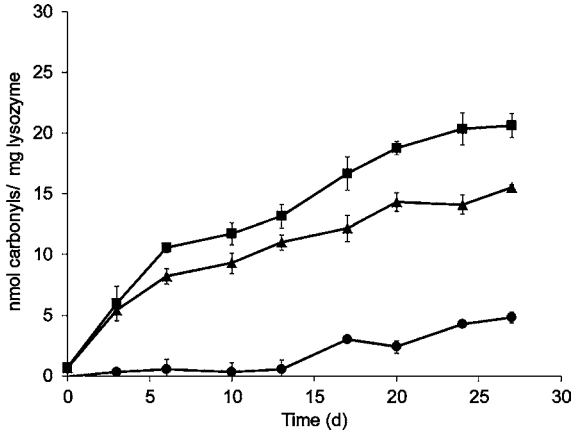

Figure 2. Carbonyl content as a function of the illumination time under homogeneous illumination (1500 lx) obtained in a light cabinet using Philips TL-D 36W/840 fluorescent tubes, fixed 0.5 m above the bottles in lysozyme solutions, with an initial riboflavin concentration of 0 μg/mL (●), 1.5 μg/mL (▲), or 3.0 μg/mL (■). The data points represent mean values ± SD of three independent experiments.

Tryptophan and *N*-Formylkynurenine Determined by Direct Fluorescent Spectroscopy. Upon illumination, the tryptophan content decreased in the riboflavin-containing solutions, whereas it remained stable in the solution without riboflavin (Figure 3A). The degradation followed an exponential curve and depended moreover on the initial riboflavin concentration in solution. The higher this concentration, the higher the reaction rate constant, which was 0.035 and 0.055 in, respectively, the 1.5 and 3.0 μg/mL riboflavin solution. After 27 days of illumination, 64 and 78% of the tryptophan was degraded in, respectively, the 1.5 and 3.0 μg/mL riboflavin-containing solutions. A degradation compound of tryptophan, *N*-formylkynurenine, increased in the riboflavin-containing solutions (Figure 3B). The strongest increase was observed during the first 2 weeks of storage and was significantly more prominent in the 3.0 μg/mL compared to the 1.5 μg/mL riboflavin-containing solution.

Amino Acids. All amino acids were determined by an amino acid analysis after acid hydrolysis except tryptophan and cysteine, which are not stable under strong acidic conditions. From all amino acids only the concentrations of the sensitive amino acids histidine, tyrosine, methionine, and lysine are given in Table 3. Because there was no significant difference ($P < 0.05$) between the 1.5 and 3.0 μg/mL riboflavin solution, only the results of the solution containing 3.0 μg/mL riboflavin are shown. As can be observed, the concentrations of histidine and tyrosine significantly decreased with, respectively, 75 and 11% after 13 days of illumination. Illumination up to 27 days did not result in a further decrease. A decreasing trend was observed in the methionine and lysine concentration upon illumination; however, no significant differences were observed between days 0, 13, and 27.

SDS-PAGE. The electrophoretic pattern of lysozyme under reducing conditions is characterized by one major band of 14.3 kDa (data not shown). Upon illumination, the intensity of the

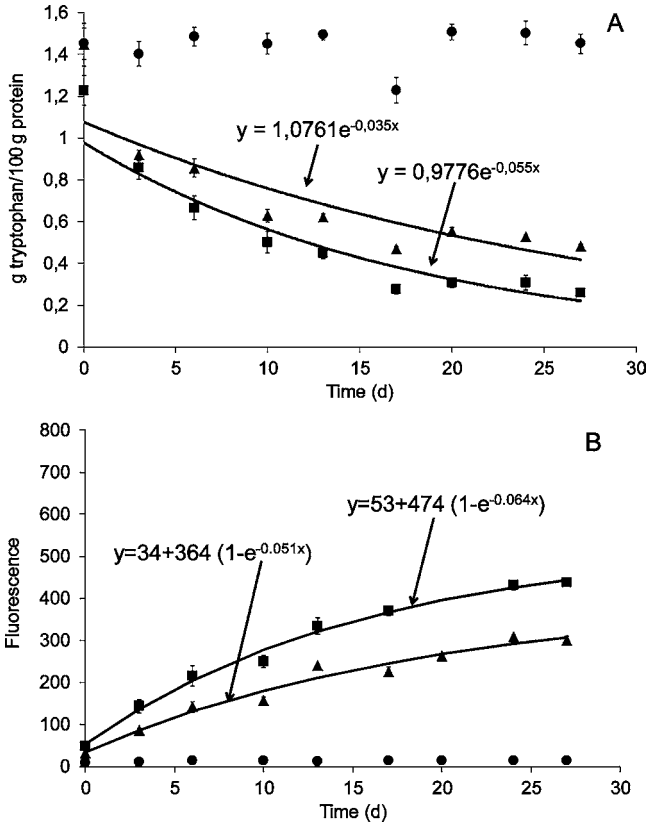

Figure 3. Concentration of tryptophan (A) and fluorescence signal of NFK (B) as a function of the illumination time under homogeneous illumination (1500 lx) obtained in a light cabinet using Philips TL-D 36W/840 fluorescent tubes, fixed 0.5 m above the bottles in lysozyme solutions with an initial riboflavin concentration of 0 μg/mL (●), 1.5 μg/mL (▲), or 3.0 μg/mL (■). The data points represent mean values ± SD of three independent experiments.

Table 3. Concentrations of Histidine, Tyrosine, Methionine, and Lysine in Lysozyme Solution Containing 3.0 μg/mL Riboflavin after 0, 13, and 27 Days of Storage[a]

	concentration (g 100/g protein)		
	day 0	day 13	day 27
histidine	0.85 ± 0.06a	0.22 ± 0.04b	0.18 ± 0.06b
tyrosine	2.97 ± 0.16a	2.64 ± 0.02b	2.67 ± 0.08b
methionine	2.17 ± 0.21a	2.06 ± 0.08a	2.09 ± 0.09a
lysine	5.16 ± 0.45a	4.83 ± 0.12a	4.75 ± 0.16a

[a]The data points represent mean values ± SD of three independent determinations. Values in the same row with a different letter are significantly different (one-way ANOVA, $p < 0.05$).

band remained fairly constant; however, a second band just below 14 kDa appeared. Besides, dimer formation was observed from the band just above 25 kDa, which intensifies with the illumination time. After 27 days of illumination, some smearing toward the top of the gel was observed, which is an indication for the formation of higher aggregates.

Semiquantitative Analysis of Lysozyme Peptides. From the 10 peptides that were determined in the reference solution, 8 peptides were quantified in the illuminated lysozyme solutions that contained 0, 1.5, and 3 μg mL riboflavin. The nonquantified peptides were peptide 19−23 (KVFGR), which was taken as an internal standard, and peptide 92−114 (NLCNIPCSALLSSDITASVN CAK), which had a very low intensity. From the 8 quantified peptides, 7 peptides contained sensitive amino acids. In Figure 4, the concentrations of these

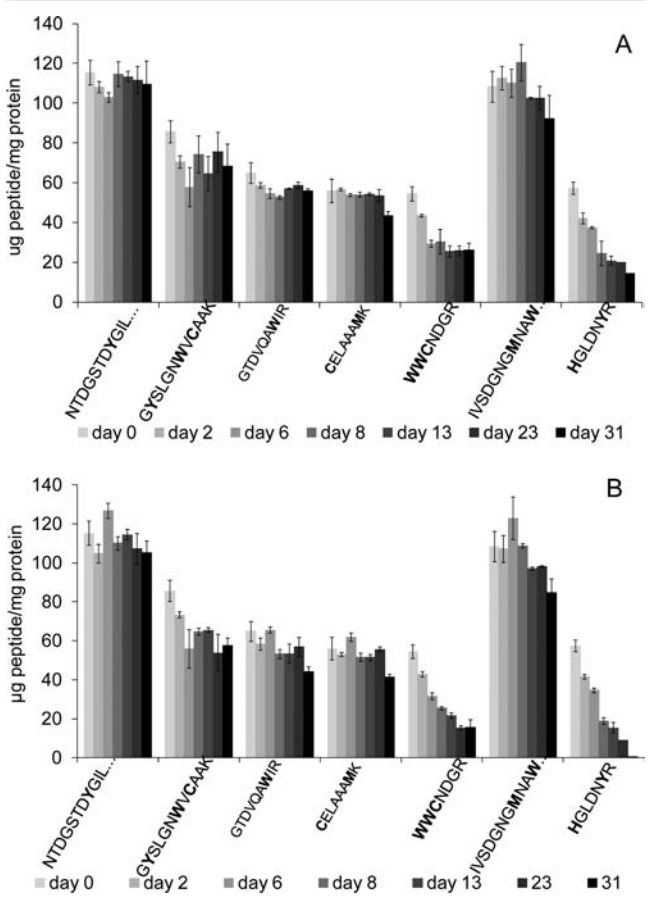

Figure 4. Concentration of lysozyme peptides in the 1.5 (A) and 3.0 (B) μg/mL riboflavin-containing solutions as a function of the illumination time under homogeneous illumination (1500 lx) obtained in a light cabinet using Philips TL-D 36W/840 fluorescent tubes, fixed 0.5 m above the bottles. The peptides were (semi)quantified by a relative calibration curve considering a nonoxidized lysozyme solution as a reference and KVFGR as an internal standard. The data points represent mean values ± SD of two independent experiments.

peptides are given as a function of the illumination time. Whereas the concentration of these 7 peptides remained stable upon illumination in the solution without riboflavin (data not shown), the concentrations of 6 of these decreased significantly in the riboflavin-containing solutions (Figure 4). The peptide NTDGSTDYGILQINSR, containing one tyrosine residue, did not significantly decrease. The peptides GYSLGNWVCAAK and IVSDGNGMNAWVAWR only significantly decreased in the solution with the higher riboflavin concentration of 3.0 μg/mL, in which losses of, respectively, 33 and 22% were observed after 31 days of illumination. The peptides GTDVQAWIR, CELAAAMK, WWCNDGR, and HGLDNYR significantly degraded in both the 1.5 and 3 μg/mL riboflavin-containing solutions. The degradation was stronger in the solution with the higher riboflavin concentration. WWCNDGR and HGLDNYR degraded with, respectively, 52 and 75% in the 1.5 μg/mL riboflavin solution after 31 days of light exposure, whereas losses of, respectively, 71 and 100% were observed in the 3.0 μg/mL solution. The degradation of GTDVQAWIR and CELAAAMK was rather restricted with losses of,

Table 4. Identified Modified Peptides of WWCNDGR, GTDVQAWIR, GYSLGNWVCAAK, and HGLDNYR with the Detected Monoisotopic Mass of the Double-Charged Unmodified and Modified Peptides

	$[M + 2H]^{2+}$	retention time	modification
GTDVQAWIR	523.2713	2.8	
+32	539.2713	3.1	W + 2O
+4	525.2713	3.5	W + O −C
GYSLGNWVCAAK	634.8046	6.8	
+32	650.8046	6.6	W + 2O or Y + 2O
+32 + 48	674.8046	6.9	W + 2O, C + 3O or Y + 2O, C + 3O
+32 + 48 + 20	684.8046	6.9	Y + 2O, C + 3O, W + 2O − C
+32 + 4 or 16 + 20	652.8046	5.3	Y + 2O, W + O − C or Y + O, W + 2O − C
32 + 4 + 48 or 16 + 20 + 48	676.8046	3.9	Y + 2O, W + O − C, C + 3O or Y + O, W + 2O − C, C + 3O
WWCNDGR	468.6891	3.4	
+16	476.6891	2.8	W + O
+32	484.6891	2.9	W + 2O
+4	470.6891	3.2	W + O − C
+48 + 32 + 4	510.6891	2.2	C + 3O, W + 2O, W + O − C
+48 + 20 + 4	504.6891	2.8	C + 3O, W + 2O − C, W + O − C
+48 + 20 + 20	512.6891	5.0	C + 3O, W + 2O − C, W + 2O − C
HGLDNYR	437.7083	2.2	
+16	445.7083	2.4	Y + O
+32	453.7083	7.4	Y + 2O or H + 2O
+14	444.7083	4.1	Y + O − 2H
−22	426.7083		H − 2C − 2N − 2H + 2O
+16 − 22	434.7083		Y + O, H − 2C − 2N − 2H + 2O

respectively, 31 and 26% in the 3.0 μg/mL riboflavin solution after 31 days of illumination.

Identification of Modified Peptides. For the identification of oxidized peptides the study focused on the degradation compounds of peptides GTDVQAWIR, GYSLGNWVCAAK, WWCNDGR, and HGLDNYR, as they suffered the strongest decrease. The observed mass shifts for these peptides are given in Table 4 together with the monoisotopic mass of the double-charged modified peptides because the single-charged modified peptides were not detected. From the eight possible mass shifts that could be expected upon photo-oxidation of GTDVQA**W**IR and which were related to the tryptophan degradation (Table 2), only two shifts were detected. The identified oxidation products were GTDVQAWIR + 2O (+32 Da) and GTDVQAWIR + O − C (+4 Da).

Photo-oxidation of GYSLGN**WV**C**A**AK could result theoretically in 89 modified peptides (8 shifts of W, 4 shifts of Y, and 1 shift of C: $((8 + 4 + 1) + 8 \times 4 + 8 \times 1 + 4 \times 1 + 8 \times 4 \times 1 = 89)$), but only 5 mass shifts were detected (Table 4). One mass shift can have different causes seen from the fact that the observed shift of +32 Da (+2O) can result from photo-oxidation of tryptophan as well as tyrosine. Besides, a mass shift of +48 Da (+3O) could be ascribed to both tryptophan and cysteine oxidation. As this shift occurs together with shifts of +4 Da (O − C) and +20 Da (2O − C), which can only result from tryptophan degradation, it is likely that +48 Da is linked to cysteine oxidation.

As can be observed from Table 4, photo-oxidation of **WWC**NDGR resulted in six mass shifts. The shifts of +16 Da (+O), +32 Da (+2O), and +4 Da(+O − C) could be linked to the degradation of one of the tryptophan residues. In the other shifts, the second tryptophan residue was also oxidized together with the cysteine residue. Upon photo-oxidation of HGLDNYR, five degraded peptides were identified. The mass shifts of +16 Da (+O) and +14 Da (+O − 2H) could only be linked to the tyrosine degradation, whereas the shift of −22 Da (−2C − 2N − 2H + 2O) could only result from histidine oxidation. Both tyrosine and histidine oxidation were possibly responsible for the shift of +32 Da (+2O) (Table 2). The peptides with mass shifts of −22 and +16 − 22 Da were detected only after >2 weeks of illumination, and the intensity of the peaks was very low.

The modified peptides of WWCNDGR and HGLDNYR were determined as a function of the illumination time, and the relative peak areas were calculated as can be observed from Figure 5. Comparison of these relative peak areas was restricted to individual modified peptides because each peptide has its specific sensitivity in ESI-MS. The relative area of the modified peptides of WWCNDGR with mass shifts of +16 and +32 Da increased during the first 8 days of illumination and decreased afterward. The relative area of the peptides with mass shifts of +4 Da (+O) and +48 + 32 + 4 Da (3O + 2O + O − C) increased over the whole illumination period, whereas the relative area of the peptides with a shift of +48 + 20 + 4 Da (3O + 2O + O − C) and +48 + 20 + 20 Da (3O + 2O − C + 2O − C) remained constant over the whole illumination period. The relative peak area of the modified peptides of HGLDNYR with mass shifts of +16, +32, and +14 Da are also given as a function of the illumination time. These relative areas were relatively low, similar for the three peptides, and remained constant over the whole illumination period. The modified peptides of HGLDNYR with mass shifts of −22 and +16 − 22 Da a re not shown in Figure 5 as they had a low intensity and as they appeared only at the end of the illumination period.

Riboflavin, the photosensitizer in the solutions, degraded exponentially and to a slightly slower extent compared to in whey and casein solutions.[17] As riboflavin degraded, lysozyme was oxidized as could be observed from the carbonyl formation and the degradation of the aromatic amino acids. The amount of protein carbonyls formed was significantly higher in the lysozyme solutions compared to the previously studied whey protein solutions (Figure 2).[17] This difference indicates that

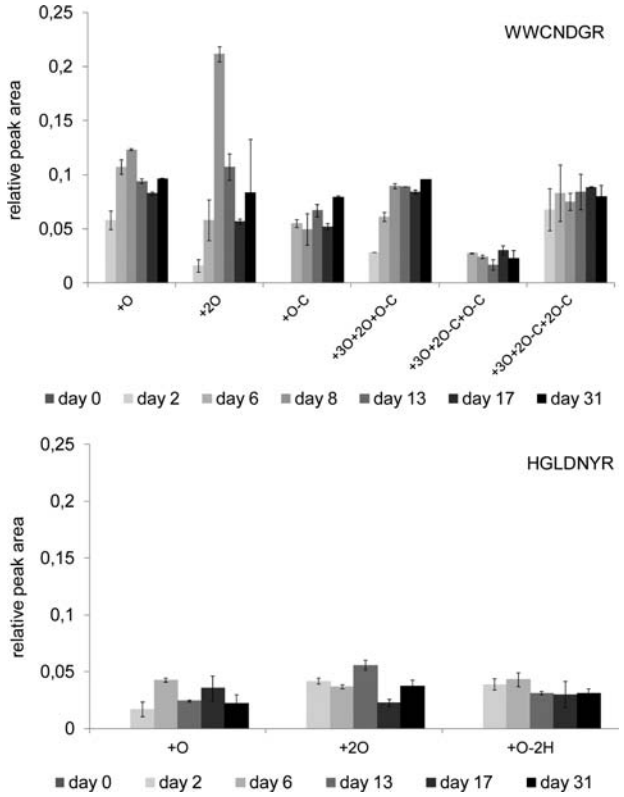

Figure 5. Relative peak area of modified WWCNDGR and HGLDNYR peptides as a function of the illumination time under homogeneous illumination (1500 lx) obtained in a light cabinet using Philips TL-D 36W/840 fluorescent tubes, fixed 0.5 m above the bottles. All areas were relatively compared to the area of the stable peptide KVFGR. The data points represent mean values ± SD of two independent experiments.

ALA, which has a structure similar to that of lysozyme, will contribute to a higher extent in the formation of whey protein carbonyls than BLG. With regard to the homology between ALA and lysozyme, it should be mentioned that although they have a similar native structure, both containing four disulfide bridges, their unfolded structures are different with a sequence similarity of 60%.[24−26] That more carbonyls were formed upon photo-oxidation of lysozyme could be linked to the stronger degradation of tryptophan and histidine in lysozyme compared to in whey proteins. Whereas both amino acids decreased with, respectively, 78 and 75% in lysozyme (Figure 3; Table 3), losses of, respectively, 38 and 30% were observed in the previously studied whey proteins.[17] Lysozyme contains only one histidine residue, but as it is present at the surface of the protein, it is readily oxidized, in contrast to the histidine residues in whey proteins, which are partially protected from photo-oxidation.[27] Tryptophan was oxidized to carbonyl compounds as *N*-formylkynurenine, kynurenine, and tryptophandione,[19,28,29] whereas histidine was degraded to asparagine and aspartic acid, both containing a carbonyl group.[30,31] Besides tryptophan and histidine, also tyrosine was affected upon photo-oxidation of lysozyme. The observed losses were restricted with a maximal loss of 11% after 27 days of illumination, which was similar to the losses observed upon photo-oxidation of whey and casein proteins.[17] Notwithstanding the fact that the sulfur-containing amino acids are known to be sensitive to photo-oxidation,[28,29] methionine did not significantly decrease upon illumination, as was also reported in whey and casein solutions. However, the cysteine residues, present in their oxidized form cystine, were probably oxidized as some specific mass shift could only be explained by the oxidation of cysteine to cysteic acid (Table 4). The photo-oxidation induced amino acid modifications resulted after 27 days of illumination in dimerization of lysozyme and the formation of a restricted amount of higher aggregates. The formation of aggregates upon photo-oxidation was much more enhanced in caseins but was not observed in whey proteins.[17] From this observation and on the basis of the fact that tryptophan and histidine were more severely affected in lysozyme compared to in whey proteins, it can be suggested that it is especially the degradation compounds of tryptophan and histidine which is responsible for the lysozyme dimerization. Kerkaert et al.[17] reported earlier that tryptophan and its degradation products play an important role in the photo-oxidation-induced aggregation of caseins.

Nonetheless, the fact that some important conclusions could be taken from the carbonyl formation, the amino acid degradations, and the electrophoretic pattern, more research was required to better understand the oxidation mechanisms. An LC-ESI-MS method was developed that could determine the unmodified lysozyme peptides and identify modified peptides. Prior to the identification of the oxidized peptides, the unmodified peptides were quantified upon illumination to detect the most sensitive targets (Figure 1). A real quantification was not feasible given the fact that this would require a stable isotope labeled internal standard for each specific peptide. For the tyrosine-containing peptides (NTDGSTDYGILQINSR and HGLDNYR), remarkable differences in their stability were observed (Figure 4). This could partially be explained by the fact that the peptide HGLDNYR not only contained a sensitive tyrosine residue but also the only histidine residue present in lysozyme, which degraded with 75%. The peptide HGLDNYR, however, degraded with 100%, which suggests that the tyrosine residue was possibly also affected. By evaluating the 3D structure of lysozyme (PyMOL), it was observed that this tyrosine residue present in HGLDNYR is allocated at the surface of the protein, where it is susceptible to oxidation, whereas the other nonoxidized tyrosine residue from the peptide NTDGSTDYGILQINSR was present in the inner part of lysozyme. As HGLDNYR was the only peptide that degraded with already >50% after 1 week of storage in the light, this peptide can potentially serve as a marker to evaluate the photo-oxidation of lysozyme. If it would be a good marker for oxidation of lysozyme in general is, however, doubtful because other oxidation mechanisms will affect other amino acids and will probably result in different modifications. Besides HGLDNYR, WWCNDGR was severely affected with a maximum decrease of 71%. The fact that the other peptide containing two tryptophan residues, IVSDGNGMNAWVAWR, decreased with maximally 22% could again be explained by the 3D protein structure from which it became clear that the two residues of WWCNDGR are present at the surface of the protein, whereas one of the tryptophan residues of IVSDGNGMNAWVAWR was allocated at the inner part of lysozyme. This tryptophan residue will as such be less susceptible to oxidation. Besides, the methionine residue present in IVSDGNGMNAWVAWR was not affected because no decrease in the methionine concentration was observed upon illumination (Table 3). This explains why also CELAAAMK was not severely affected; a minor decrease was observed between 27 and 31 days of illumination, which could

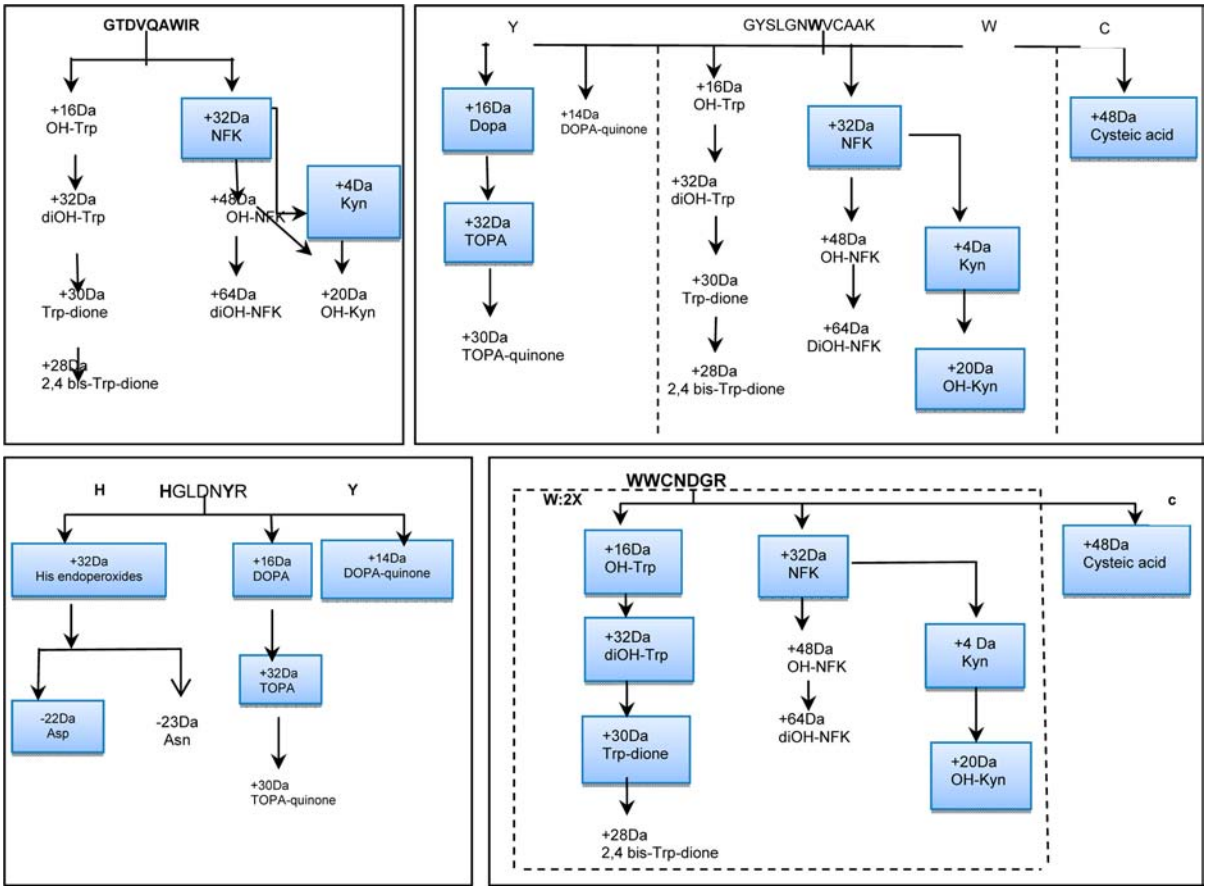

Figure 6. Schematic overview of the degradation of the peptides GTDVQAWIR, GYSLGNWVCAAK, WWCNDGR, and HGLDNYR. The mass shifts that could potentially occur in comparison with the initial peptide mass are given, and the identified shifts are highlighted in gray.

probably be a result of the oxidation of cysteine. The cysteine residues can only be oxidized after reduction of the disulfide bridges, which is enhanced by the presence of photoexcited tryptophan residues as was described by Vanhooren et al.[32] The cysteine residues of CELAAMK and WWCNDGR are surrounded by, respectively, tryptophans W141 and W81, present at the surface of the protein and as such sensitive to photo-oxidation. By electron transfer between the excited tryptophan residue and cystine, a reduction of the disulfide bridge can occur, resulting in free cysteine residues, which can be further oxidized to cysteic acid. The oxidation of cysteine to cysteic acid was indeed confirmed for the peptide WWCNDGR, as will be discussed later (Table 4). The peptide GTDVQAWIR containing only tryptophan as sensitive amino acid degraded to a slightly higher extent (31%) than IVSDNGMNAWVAWR (22%), which contained one extra tryptophan residue. This can be explained by the fact that the tryptophan residue present in GTDVQAWIR is clearly located at the surface, whereas the second tryptophan residue of IVSDNGMNAWVAWR is oriented toward the inner part of the protein. A degradation similar to that of GTDVQAWIR was observed for the peptide GYSLGNWVCAAK, containing one tryptophan, one tyrosine, and one cysteine residue. This peptide contained, just as WWCNDGR and IVSDNGMNAWVAWR, up to three sensitive amino acids, which could result in the formation of a broad range of modified peptides and complicated the identification of the modified peptides.

For the identification, the present study focused on the most affected peptides, HGLDNYR, WWCNDGR, GYSLGNWVCAAK, and GTDVQAWIR. A schematic overview of their degradation is given in Figure 6. Upon photo-oxidation of GTDVQAWIR, two oxidized peptides were identified (Table 4; Figure 6), which could be related to the oxidation of tryptophan to *N*-formylkynurenine (+32 Da) and kynurenine (+4 Da). A mass shift of +32 Da (+2O) could also result from the formation of dihydroxytryptophan, which is, however, doubtful because no further degradation compounds of this unstable compound were detected. Notwithstanding the fact that Grosvenor et al.[19] observed 5 degradation pathways related to 11 oxidation products by photo-oxidation of bound tryptophan, this study confirmed only one pathway and the formation of two oxidized compounds. This oxidation pathway was confirmed upon identification of the oxidized peptides of WWCNDGR and GYSLGNWVCAAK (Figure 6). For both peptides mass shifts of not only +32 Da (+2O) and +4 Da (+O − C) were detected but also a shift of +20 Da (+2O − C) was detected, which could be related to the further degradation of kynurenine to hydroxykynurenine (Table 2, pathway 2). Whereas the identification of the mass shifts of GTDVQAWIR was relatively simple, the interpretation of the mass shift of peptides containing several sensitive amino acids was less straightforward. A mass shift of +32 Da (+2O) of GYSLGNWVCAAK could, for example, result from the oxidation of tryptophan to *N*-formylkynurenine as it could result from the oxidation of tyrosine to trihydroxyphenylalanine. Besides a mass shift of +32 Da (+2O), also a shift of +32 + 48 Da (+2O + 3O) was detected for GYSLGNWVCAAK in which the shift of +48 Da (+3O) could be related to the

oxidation of cysteine to cysteic acid or to the oxidation of *N*-formylkynurenine to hydroxyformylkynurenine. That the cysteine residue in this peptide will be oxidized is plausible given the fact that the residue is surrounded by a tryptophan molecule.[32] Less evidence is, however, found for the oxidation of *N*-formylkynurenine to hydroxyformylkynurenine (Table 2, pathway 3) as the other mass shifts of GYSLGNWVCAAK (Table 4) could only be related to the oxidation of *N*-formylkynurenine (+32 Da) to kynurenine (+4 Da) and hydroxykynurenine (+20 Da) (Table 2, pathway 2). In these other shifts (+32 + 48 + 20 Da, +32 + 4 Da; or +16 + 20 Da, +32 + 4 + 48 Da, or +16 + 20 + 48 Da) tyrosine was possibly oxidized to di- and trihydroxyphenylalanine (+16 Da, +32 Da) and cysteine to cysteic acid (+48 Da). The mass shift of +36 Da could be a result of the oxidation of tyrosine to trihydroxyphenylalanine (+32 Da) and tryptophan to kynurenine (+4 Da) but could also result from the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) (+16 Da) and of tryptophan to hydroxykynurenine (+20 Da). From the five identified modified peptides of GYSLGNWVCAAK (Table 4), it can be observed that the degradation of the peptide was a result of the oxidation of the three amino acids, tyrosine, tryptophan, and cysteine. On the basis of the observed mass shifts, the tyrosine residue was oxidized to di- and trihydroxyphenylalanine but was not further degraded to its quinone form as described by Grosvenor et al.[19] As mentioned, the data also suggest that tryptophan followed the same degradation pathway as in GTDVQAWIR and WWCNDGR.

From the six mass shifts observed for WWCNDGR, it was observed that tryptophan was primarily oxidized to hydroxytryptophan (+O) (Table 2, pathway 1), which could be further degraded to dihydroxytryptophan (+2O). This shift of +32 Da, however, could also be a result of the direct oxidation of tryptophan to *N*-formylkynurenine. Although there was no evidence for the further oxidation of dihydroxytryptophan, the mass shifts of +4 Da (+O − C) and +20 Da (+2O − C) indicated that *N*-formylkynurenine was degraded to kynurenine (O − C) and hydroxykynurenine (2O − C). That no further degradation compounds of dihydroxytryptophan were detected does not imply that they are not formed, as will be discussed further. The other shifts of +84 Da (+48 + 32 + 4 Da), +72 Da (+48 + 20 + 4 Da), and +88 Da (+48 + 20 + 20 Da) all suggest the formation of cysteic acid and the oxidation of kynurenine to hydroxykynurenine as illustrated in Figure 6. Upon comparison of the relative area of these six modified peptides as a function of the illumination time (Figure 5), it was observed that the area of WWCNDGR +16 Da (O) and +32 Da (2O) increased during the first week and decreased afterward. These results indicated that these compounds were degraded, which was in the case of *N*-formylkynurenine (+32 Da) confirmed as the peak of +4 Da (O − C) and +48 + 32 + 4 Da (+3O + 2O + O − C), related to the formation of kynurenine, increased after 1 week of illumination.

Upon oxidation of the most affected peptide, HGLDNYR, five mass shifts were identified. From these shifts it was observed that tyrosine was oxidized to di- and trihydroxyphenylalanine (+16 Da, +32 Da) and to its quinone form (+14 Da). Upon oxidation of histidine, endoperoxides were formed, which degraded as described before by Agon et al.[30] to asparagine and aspartic acid, of which only the last compound was identified in the present study (Figure 6). As the endoperoxides are highly unstable, it is arguable that they are responsible for the mass shift of +32 Da as suggested before.

The fact that only 18 modified peptides were identified from the more than 271 peptides that could be formed upon photo-oxidation of these four peptides could partially be explained by the fact that not all degradation compounds described in the literature were formed. In addition, it could result from a bad ionization of the more polar peptides. As peptides become more oxidized, their polarity increases, which alters the ionization and as such the intensity of the peak. Moreover, the formation of specific peptides can result in ion suppression of other peptides. When the protein is more oxidized, not only the ionization is affected but also the hydrolysis can be altered. This was probably why after 41 days of illumination the signal of the total ion chromatogram was low compared to the total ion chromatogram of hydrolysates, which were photo-oxidized up to 20−30 days of illumination (data not shown). As a result of the altered hydrolysis and the less enhanced ionization, not all oxidized peptides were detected. To be able to detect more photo-oxidized peptides, another approach should be followed in which lysozyme is hydrolyzed and the peptides are fractionated prior to photo-oxidation. By following this approach, the photo-oxidized compounds of each peptide could be analyzed separately from each other as done before for metal-catalyzed β-lactoglobulin peptides.[14,19] The main drawback of this methodology is that by oxidizing the peptides separately, the effect of other surrounding peptides on the oxidation is not taken into account as also the effect of the structure of the protein is not considered. This study showed that this aspect can have a significant impact as the amino acids at the surface of the protein are much more susceptible to oxidation compared to the ones oriented to the inner part of lysozyme. Besides, there can be an electron transfer between tryptophan and cystine, which is not taken into account upon oxidation of the peptides separately.[32]

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