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Changes in Structures of Milk Proteins upon Photo-oxidation

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Changes in protein structures as a result of riboflavin-induced photo-oxidation were studied for six milk proteins: α-casein, β-casein, κ-casein, lactoferrin, α-lactalbumin, and β-lactoglobulin. The milk proteins showed significant variability in sensitivity to photo-oxidation. After photo-oxidation, an increase in carbonyl content because of oxidation of tryptophan, histidine, and methionine, as well as formation of dityrosine, was observed for all proteins studied, although at very different levels. Generally, the increment was highest for α- and β-casein and was lowest for lactoferrin. Loss of tryptophan because of photo-oxidation was well-correlated with the formation of the tryptophan oxidation products, *N*-formylkynurenine and kynurenine. Changes at the tertiary protein structure level were observed after photo-oxidation of the globular proteins, where tryptophan fluorescence emission indicated unfolding of α-lactalbumin and β-lactoglobulin, whereas lactoferrin achieved a more compact tertiary structure. Changes in secondary structure were observed for α-lactalbumin and β-lactoglobulin, whereas the secondary structure of lactoferrin did not change. Polymerization of α- and β-casein and of lactoferrin was observed, whereas κ-casein, α-lactalbumin, and β-lactoglobulin showed little tendency to polymerize after photo-oxidation. Lability toward photo-oxidation is discussed according to the structural stabilities of the globular proteins.

KEYWORDS: Photo-oxidation; milk proteins; riboflavin; protein structure; protein oxidation

INTRODUCTION

Reactive oxidative species are found in all foodstuffs, including milk and milk-derived products. Traditionally, the focus has mainly been on the lipid oxidation products because of their high impact on off-flavor. During the past few years, protein oxidation has been an issue as well, because of off-flavor caused by oxidation of specific amino acids (*1*). Another aspect of high relevance for the dairy industry is the possible effects of protein oxidation on the enzymatic processes essential during many dairy processes, such as fermentations and cheese ripening. Kaye and Jollés (*2*) showed that loss of a histidine residue because of photo-oxidation was responsible for a lack of cleavage of κ-casein at Phe_{105}-Met_{106} by chymosin, whereas a loss of tryptophan and methionine did not affect the cleavage. The loss of a particular residue in the primary structure of a protein may, furthermore, affect the tertiary structure (folding) of a protein. Moreover, conformational changes may result in reduced accessibility for proteases because of less surface exposure of the specific cleavage sites, and thereby, for example, reduce cleavage of the proteins that are necessary for the clotting process in milk.

Protein oxidation in milk can be initiated either enzymatically, for example, by the lactoperoxidase system (*3*), by photo-oxidation (*4*), or by transition metal ions, which can induce protein oxidation in the presence of ascorbic acid by a Fenton reaction (*5*). Protein oxidation leads to different types of modifications, including cross-linkages (*3*), fragmentation of covalent bonds (*6*), and different changes in a wide range of different amino acids. Protein oxidation has hence been identified as changes in methionine (*1*, *7*), histidine (*8*), tryptophan (*8*), and tyrosine (*3*). Amino acid derivatives used as indicators of protein oxidation in milk include introduction of carbonyl groups (*9*) or dityrosine (*3*), where the latter can lead to intra- as well as intermolecular cross-linking. However, these changes in the primary structure have not been correlated with conformational changes of the proteins.

Gilmore and Dimick (*10*) reported that formation of off-flavor in milk upon photo-oxidation required riboflavin and oxygen, which is due to the fact that riboflavin acts as a photosensitizer. Illumination of riboflavin in the presence of oxygen is known to result in the formation of excited-state species, including singlet oxygen (*11*), which is believed to be responsible for the oxidation of methionine to methional (type II reaction) (*7*),

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whereas the formation of dityrosine is primarily a result of the type I mechanism without any significant role of singlet oxygen (*12*). Light-induced oxidation of β-casein resulted in a loss of histidine, tryptophan, and tyrosine (*6*), and longer exposure to light resulted in the cleavage of peptide bonds (*6*). Photo-oxidation of κ-casein resulted in a complete loss of histidine and a 70% loss of tryptophan (*8*). Furthermore, significant changes were observed at the primary structure level of β-lactoglobulin after 72 h of light exposure, where loss of isoleucine and tyrosine was seen, and α-lactalbumin showed a loss of tyrosine residues (*4*).

Changes in the primary structure of milk proteins after light-induced oxidation have been described in the literature, but to the best of our knowledge, the impact of these changes in the primary structure on higher-order protein structures, that is, secondary as well as tertiary structures that are believed to have an impact on accessibility for proteases, has not been described earlier. The present study describes changes in primary, secondary, tertiary, as well as quaternary structures of the milk proteins after light exposure in the presence of the photosensitizer riboflavin. The applied milk proteins constitute three different casein solutions (α_{S1}- and α_{S2}-, β-, and κ-casein), representing random coil proteins, and three globular whey proteins (α-lactalbumin, β-lactoglobulin, and lactoferrin). Because of their random coil structure and lack of ordered secondary structure elements, it is hypothesized that the caseins would be more accessible toward oxidative changes as compared with globular whey proteins, which are much more rigid in their tertiary structures.

MATERIALS AND METHODS

β-casein (C-6905), β-lactoglobulin (L-0130), lactoferrin (L-9507), α-casein, consisting of a mixture of α_{S1}- and α_{S2}-caseins (C-6780), κ-casein (C-0406), α-lactalbumin type III (L-6010), riboflavin (R-4500), guanidine HCl (G-4505), L-tryptophan (T 0254), L-methionine (M 9625), L-histidine (H 8125), and L-kynurenine (K 8625) were purchased from Sigma Aldrich Chemie GmBh (Steinheim, Germany). The tripeptides, Gly Trp-Gly (H-3655), Gly Met-Gly (H3585), and Gly His-Gly (H-3505) were obtained from Bachem, Bubendorf Switzerland. Sodium dihydrogen phosphate was obtained from Merck (Damstadt, Germany). Tris and TFA came from Applichem GmbH (Damstadt, Germany). Sodium monohydrogen phosphate was from Baker Analyzed (Deventer, Holland), and 2,4-Dinitrophenylhydrazine (4291) was from Lancaster Synthesis, (Morecamb, England). Acetonitrile was obtained from Rathburn Chemical (Walkerburn, Scotland). The Whatman Mini-UniPrep vial, with a pore size of 0.2 μm, was from Chromtech GmbH, Idstein, Germany.

Experimental Design of Photo-oxidation Reaction. The protein solutions of α-casein, β-casein, κ-casein, lactoferrin, α-lactalbumin, and β-lactoglobulin were incubated at a concentration of 3 mg/mL at 10 °C with 1.75 μg/mL riboflavin for 0, 4, 8, 24, and 44 h at pH 6.8 in a 10 mM phosphate buffer. The incubation of control samples with equal amounts of riboflavin, but wrapped in silver foil, paralleled the incubation of control samples without riboflavin. All samples were placed in a rotor with a diameter of 30 cm and a rotation angle of approximately 45° to the light source (TL-D 90 de Luxe Pro 18W/965 SLV from Philips, Germany), from which they were illuminated with fluorescent light (400–600 nm), with an intensity of 2200–2600 lx, depending on the position of the rotor. Changes in different levels of protein structure (primary, secondary, tertiary, and quaternary structures) were measured. For investigation of secondary structure changes, additional experiments with increased riboflavin concentration (17.5 μg/mL) were performed as well.

LC-MS Analysis of Carbonyl Groups. Carbonyl groups in tryptophan, histidine, and methionine were detected by reverse phase HPLC after acid hydrolyzis of the studied proteins combined with 2,4-dinitrophenylhydrazine (2,4-DNPH) derivatization. Single amino acid standards and the tripeptides, Gly-Trp-Gly, Gly-Met-Gly, and Gly-His-Gly were used for identification of the carbonyl groups formed in the presence of riboflavin after photo-oxidation. Kynurenine, an oxidation product of tryptophan, was applied as a carbonyl standard as well. Identification of carbonyl groups in tryptophan (209 *m/z*), methionine (298 *m/z*), and histidine (298 *m/z*) was performed according to their mass as well as their retention time (6.5 min, 15.2, and 15.8 min, respectively). After photo-oxidation for 0, 4, 8, 24, and 44 h, 500 μL of oxidized protein solution was acid hydrolyzed in 6 M HCl. After addition of HCl, the samples were flushed with argon and left at 105 °C overnight. The samples were freeze–dried before derivatization with 500 μL of 10 mM 2,4-DNPH according to ref *13*. After incubation for 15 min at room temperature, the samples were transferred to a Whatman Mini-UniPrep vial with a pore size of 0.2 μm. Twenty μL of sample were injected onto a reverse phase C18 column (218TP5215, 15 × 2.1 mm i.d., 5 μm particle size) from Vydac (CA, USA). Chromatographic separation of the samples was performed at a flow rate of 0.20 mL/min, and the samples were eluted from the 20 °C thermostatted column by applying a linear gradient of solvent B (80% acetonitrile and 0.1% TFA) within the time schedule 2–10 min; 40%, 15 min; 50%, 45–50 min; 100% solvent B. The column was equilibrated for 10 min with 0.1% TFA (solvent A) before injection of the next samples. Analyses were performed on an Agilent (Waldbronn, Germany) HPLC series 1100 comprised of a model G1312A binary pump, a model G1379A micro vacuum degasser, a model G1327A thermostated auto sampler, a model G1316A thermostated column compartment, a model G1315B diode-array detector, and a model G2707DA LC/MSD SL detector fitted with a model G1948A electrospray source. The station was controlled, and the results were analyzed with Agilent's ChemStation software (Rev. A.10.02). UV-spectra were recorded at 370 and 280 nm with a bandwidth of 4 nm and the reference fixed at 800 nm. The mass spectra of samples were recorded simultaneously applying SCAN (from *m/z* 50 to 600) and SIM (ions with *m/z* 209 and 298) in positive modes. The acquisition parameters were as follows: fragmentor 100 V, gain 1.0 EMV, step size 0.20. Nitrogen was used as a drying gas at a flow rate of 13 L/min and as a nebulizing gas at a pressure of 60 psig (413.7 kPa) and a temperature of 300 °C. A potential of 3000 V was used on the capillary. Quantification of carbonyl groups in the different amino acid residues was performed according to the area under the chromatographic peak with UV-detection at 370 nm. Retention time as well as ion mass were applied for identification of the different carbonyl groups.

Dityrosine. Dityrosine was detected using acid hydrolysis of the proteins and subsequent detection by HPLC as described by Daneshvar et al. (*14*) and Østdal et al. (*15*). All protein samples were precipitated in 10% TCA and were centrifuged at 4000 rpm for 20 min. The precipitates were washed with 1.33 mL of 1 M HCl and were centrifuged again at 4000 rpm for 10 min The precipitates were solvated in 0.33 mL of 6 M HCl and were flushed with argon before hydrolyzed at 105 °C overnight. The samples were subsequently neutralized with 6 M NaOH. Twenty μL of samples were injected onto a HPLC column (Microsorb 100–5, C-18, 250 × 4.6, Varian, Walnut Creek, CA) equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55). Chromatographic separation was performed at a constant flow of 1 mL/min of 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55) on a HPLC system consisting of a 9012 HPLC pump, connected to a 9100 autosampler and a 9075 fluorescence detector, where excitation and emission were performed at 283 and 410 nm, respectively (Varian Chromatographic Systems, Walnut Creek, CA). The chromatograms were analyzed applying Star chromatography Workstation Varian, ver. 4.51 (Varian associates Inc., Walnut Creek CA).

Secondary Structure. Changes in secondary protein structure upon photo-oxidation were measured by circular dichroism (CD). CD-spectra were obtained from 190 to 250 nm in a 0.1 cm cell length. The step size was 0.2 nm, and the spectra were obtained with a scan speed of 50 nm/min with a response factor of 4. The spectra were accumulated three times each. Protein samples were diluted to 0.2 mg/mL before measurements were performed on a J-810 spectropolarimeter from Jasco (Jasco Spectroscopic Co. Ltd., Hachioji City, Japan), equipped with a JASCO PTC-348WI temperature control unit. Illumination of the samples was performed with a xenon short arc lamp from Osram

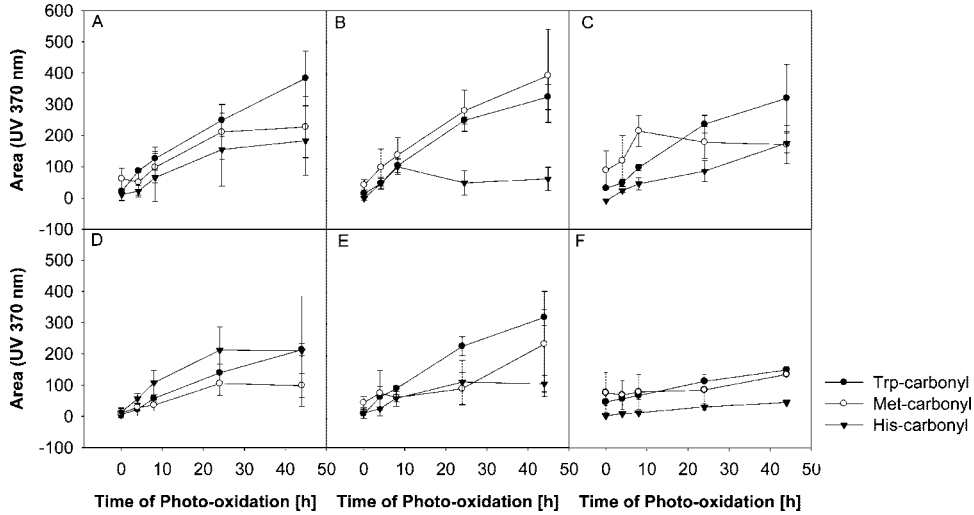

Figure 1. Carbonyl groups in (**A**) α-casein, (**B**) β-casein, (**C**) κ-casein, (**D**) α-lactalbumin, (**E**) β-lactoglobulin and (**F**) lactoferrin. Acid hydrolyzates of photo-oxidized proteins were derivatized with 2,4-DNPH and were measured by LC-MS. According to detection at 370 nm and MS, carbonyl groups were found in tryptophan, methionine, and histidine, with ion 209 *m/z* for tryptophan and 298 *m/z* for methionine and histidine carbonyl groups, respectively. The discrimination between the methionine and histidine carbonyl was performed according to their retention time in the chromatographic reverse phase separation.

(Germany) under a flow of 5.0 mL/min of 99.999% nitrogen. The spectra obtained for the α-lactalbumin and β-lactoglobulin solution were subsequently normalized through 208 and 215 nm for better visualization of the changes in the spectra.

Thermal stability of lactoferrin was determined using CD spectroscopy as well (*16*). Thermal scanning was performed from 20 to 100 °C with at temperature gradient of 0.2 °C/min to follow loss of α-helical structure at 222 nm. The data were fitted to a two-state model, shown below.

$$\mathrm{N} \rightleftarrows \mathrm{U} \qquad K_{\mathrm{eq}} = \frac{[\mathrm{U}]}{[\mathrm{N}]}$$

The use of the spectrophotometric methods comprises the observed parameter Y_{obs}, which constitutes the fraction of native ([N]/([N] + [U])) and the fraction of unfolded proteins ([U]/([U] + [N])), respectively, and the spectroscopic signal of the native (Y_N) and the unfolded (Y_U) state, and is given by the following equation.

$$Y_{\mathrm{obs}} = \frac{[\mathrm{N}]}{[\mathrm{N}] + [\mathrm{U}]} Y_{\mathrm{N}} + \frac{[\mathrm{U}]}{[\mathrm{U}] + [\mathrm{N}]} Y_{\mathrm{U}}$$

The KaleidaGraph version 4.3 software (Synergy Software, Reading, PA) was applied for determination of melting temperature (T_m) by fitting the thermal scan to the following equation.

$$Y_{\mathrm{obs}} = \frac{Y_{\mathrm{N}} + Y_{\mathrm{U}}\left(\mathrm{e}^{\left[\Delta \mathrm{H}_{U-\mathrm{N}} T_{\mathrm{m}}\left(1-\frac{T}{T_{\mathrm{m}}}\right) - C_{\mathrm{p}}\left(T_{\mathrm{m}} - T + T\left(\ln\left(\frac{T}{T_{\mathrm{m}}}\right)\right)\right)\right]/RT}\right)}{1 + \mathrm{e}^{\left[\Delta \mathrm{H}_{U-\mathrm{N}} T_{\mathrm{m}}\left(1-\frac{T}{T_{\mathrm{m}}}\right) - C_{\mathrm{p}}\left(T_{\mathrm{m}} - T + T\left(\ln\left(\frac{T}{T_{\mathrm{m}}}\right)\right)\right)\right]/RT}}$$

Tertiary Structure and Loss of Tryptophan. Fluorescence spectroscopy was used to monitor the loss of tryptophan as well as changes in tertiary protein structure due to photo-oxidation. Excitation of the 0.3 mg/mL protein solution was performed at 298 nm to ensure selective excitation of tryptophan residues, and emission spectra were obtained from 310 to 500 nm in a 1 × 0.1 cm quartz cell using a LS 50B spectrofluorometer from Perkin-Elmer (Beaconsfield, England). Excitation was performed with a Xenon discharge lamp, and a photomultiplier measured the emission. The band width was 5 nm. To take protection afforded by the folded structure into account, the same experiment was performed on protein unfolded in 6 M guanidium chloride. All measurements were performed in triplicate.

N-Formylkynurenine. Formation of the *N*-formylkynurenine, an oxidation product of tryptophan, was also measured by fluorescence spectroscopy. Excitation of the 0.6 mg/mL protein solution was performed according to Pirie (*17*) at 330 nm, and emission spectra were obtained from 350 to 550 nm as relative emission intensities using a 1 × 0.1 cm quartz cell. Subsequently, the intensities at the emission maxima (400 nm) were extracted from the data to follow formation of *N*-formylkynurenine. All measurements were performed in triplicate.

Quaternary Structure. Gel filtration was applied for measuring polymerization of the proteins as a result of photo-oxidation. A 100 μL portion of protein sample was injected onto a Superdex 200 HR 10/30 high-performance gel filtration column connected to an ÄKTA FPLC system from Amersham Pharmacia Biotech (Uppsala, Sweden). The samples were eluted from the column using a 10 mM tris buffer, pH 8.0, and 500 mM NaCl at a flow of 0.25 mL/min. The UV-absorbance was measured at 280 nm. The chromatograms were analyzed by UNICORN version 3.20.16, from Amersham Pharmacia Biotech.

RESULTS

Changes in primary as well as higher order protein structures of the milk proteins α-casein (α_{S1}- and α_{S2}-casein), β-casein, κ-casein, lactoferrin, α-lactalbumin, and β-lactoglobulin were measured after photo-oxidation of the proteins in the presence of riboflavin. The measurements were performed after 0, 4, 8, 24, and 44 h of incubation at 10 °C with and without the presence of 1.75 μg/mL riboflavin and under illumination with fluorescent light with an intensity of 2200–2600 lx.

Changes in primary structure of the proteins, that is, changes in individual amino acid residues, were measured by formation of carbonyl groups in histidine, methionine, tryptophan (kynurenine, *N*-formylkynurenine), and dityrosine.

Different Increase in Protein Carbonyl Groups over Time. A newly developed method applying LC-MS combined with UV detection of 2,4-DNPH derivatives on acid-hydrolyzed, photo-oxidized proteins showed that carbonyl groups were formed over time in tryptophan, methionine, and histidine residues (**Figure 1**). Identification of carbonyl groups in tryptophan (209 *m/z*), methionine (298 *m/z*), and histidine (298 *m/z*) was performed according to their mass as well as their retention time (6.5 min, 15.2, and 15.8 min, respectively). The retention time was decisive for the differentiation between the methionine and histidine carbonyls, because they both showed a *m/z* value of 298. The increase in detected carbonyl groups with time varied considerably for the different substrates. α- and β-casein, which are both random coil proteins, seemed to be the two proteins most susceptible to the formation of carbonyl

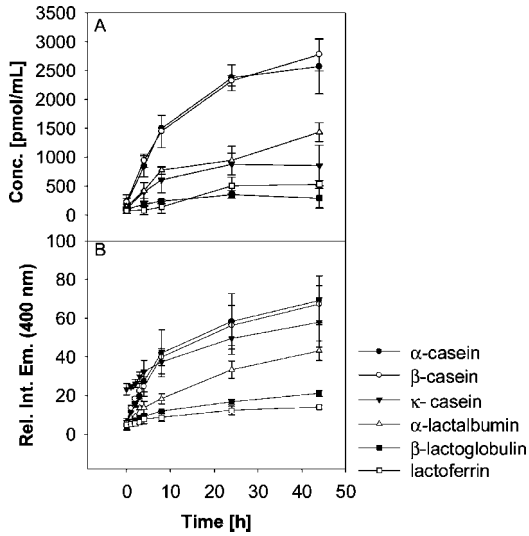

Figure 2. Formation of (**A**) dityrosine and (**B**) *N*-formylkynurenine was followed as a function of time for light exposure in the presence of riboflavin. Three mg/mL solutions of protein were incubated under fluorescent light for 44 h with 1.75 μg/mL riboflavin. For the determination of dityrosine, 0.5 mL of the light-treated samples were hydrolyzed and analyzed on HPLC with a fluorescence detector. Excitation and emission were performed at 283 and 410 nm, respectively. N-formylkynurenine was measured by fluorescence spectroscopy with excitation at 330 nm and emission at 400 nm of 0.6 mg/mL of protein. Standard errors are shown as bars.

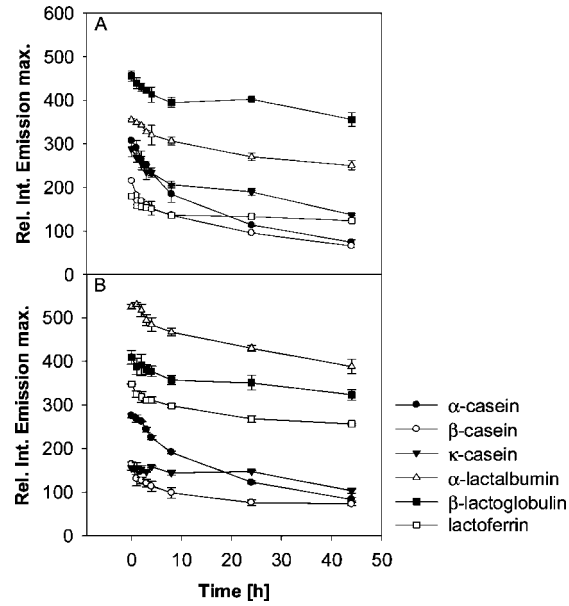

Figure 3. Loss of tryptopans was measured in (**A**) aqueous solution and (**B**) 6 M guanidium chloride. Three mg/mL solutions of protein were exposed to fluorescence light for 44 h in the presence of 1.75 μg/mL riboflavin and were diluted to 0.3 mg/mL of protein before measuring. Selective tryptophan excitation was performed at 298 nm, and the loss of tryptophan was followed over time by extracting the emission maxima found for all proteins. Standard errors are given as bars.

groups, whereas lactoferrin was the globular protein showing the lowest content of carbonyl groups. Formation of the tryptophan oxidation product, kynurenine, was found to be highest in α-casein, followed by β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin, whereas the lowest content of kynurenine was detected in lactoferrin. The carbonyl formation in the histidines after photo-oxidation was found to be highest in α-lactalbumin, followed by κ-casein and α-casein, and lowest in lactoferrin. The level of carbonyl groups introduced as a result of methionine oxidation was highest in β-casein, followed by α-casein and β-lactoglobulin, whereas it was low in α-lactalbumin. The initial level of carbonyl groups due to methionine oxidation was relatively high in κ-casein, but declined during longer incubation.

Different Increase in Dityrosine Content of All Proteins over Time. Cross-linkages can be formed during photo-oxidation between two tyrosine residues, and such dityrosine formation was followed over time by HPLC-fluorescence detection of acid hydrolyzates of photo-oxidized protein–substrates. The two random coil proteins α-casein and β-casein were the two proteins showing the highest content of dityrosine (**Figure 2A**), wherease the two globular proteins β-lactoglobulin and lactoferrin were the two showing the lowest content of dityrosine. The formation of dityrosine was found to be higher in the highly α-helical-structured α-lactalbumin and the random coil protein κ-casein than in β-lactoglobulin and lactoferrin.

Increase in N-Formylkynurenine for All Proteins over Time. *N*-Formylkynurenine is formed during oxidation of tryptophan, and the formation of this compound was followed over time using fluorescence spectroscopy (**Figure 2B**). The increase in *N*-formylkynurenine content during photo-oxidation was highest in the random coil proteins α- and β-casein, followed by α-lactalbumin, being the globular protein with the highest content. κ-Casein showed a remarkably high content of *N*-formylkynurenine at time zero, whereas the increase as a function of time for photo-oxidation was lower than that of α-lactalbumin. However, β-lactoglobulin and lactoferrin showed the lowest content of *N*-formylkynurenine after photo-oxidation.

Loss of Tryptophan in All Proteins Because of Oxidative Degeneration. Loss of tryptophan and changes in the tertiary structures of the proteins as a consequence of oxidation were further studied by selective tryptophan excitation at 298 nm and subsequent determination of the emission maximum. Upon light exposure and in the presence of riboflavin, a decrease in the intensities of the emission maximum for the native protein was observed (**Figure 3A**). The decrease in tryptophan emission could either be because of solvent exposure of the tryptophan residues caused by protein unfolding or because of oxidative degeneration of tryptophan residues. To exclude the contribution of conformational changes to the fluorescence intensity, the proteins were dissolved in 6 M guadinium chloride to follow the loss of tryptophan (**Figure 3B**). Introduction of 6 M guadinium chloride shifted the emission maximum to approximately 355 nm, and the emission intensities for the globular proteins (α-lactalbumin, β-lactoglobulin, and lactoferrin) increased significantly as compared with the aqueous solution, whereas the emission intensities for the caseins were more or less the same in guanidinium chloride and aqueous solution because of the random coil structure with less buried tryptophan. Loss of tryptophan due to oxidative degradation was seen for all six proteins but was highest in α-casein, β-casein, and α-lactalbumin (**Figure 3B**).

Observed Changes in Tertiary Structure for Globular Proteins. Fluorescence spectroscopy was furthermore used to follow changes in the tertiary structures of the proteins after photo-oxidation, primarily due to changes in the fluorescence of tryphophans. After excitation at 298 nm in aqueous solution, the emission maxima were identified at 350 nm for α-casein and β-casein (**Figure 4A–B**), whereas in κ-casein the tryptophan emitted at 340 nm (**Figure 4C**). The globular proteins α-lactalbumin, β-lactoglobulin, and lactoferrin all showed emission

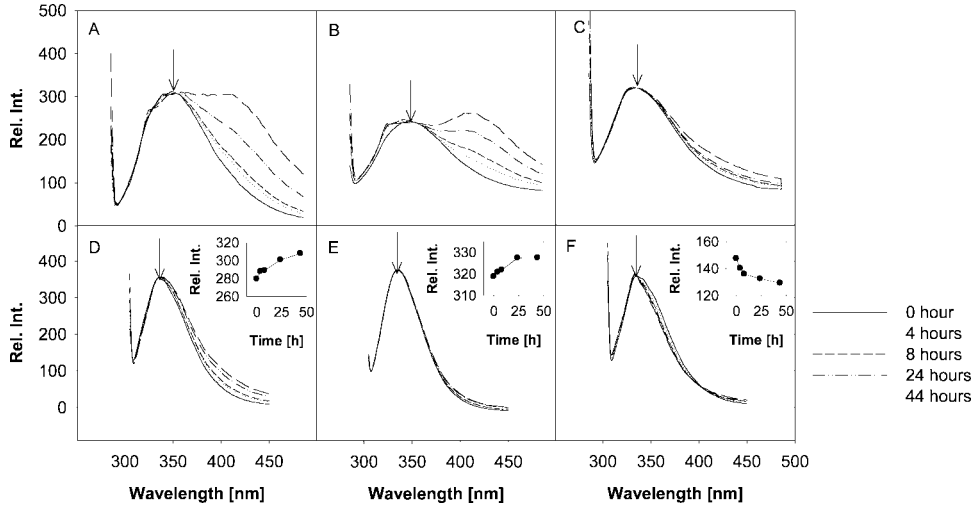

Figure 4. Normalized emission spectra were obtained for (**A**) β-casein, (**B**) α-casein, (**C**) κ-casein, (**D**) α-lactalbumin, (**E**) β-lactoglobulin, and (**F**) lactoferrin. Three mg/mL solutions of each protein were incubated at fluorescence light for 44 h in the presence of 1.75 μg/mL riboflavin and the mixtures were diluted to 0.3 mg/mL of protein before measuring over a period of 44 h. Selective tryptophan excitation was performed at 298 nm, and emission spectra were obtained from 305 to 450 nm. Emission peaks are indicated with an arrow. Unfolding of the globular proteins was followed at 355 nm (shown in insets of panels **D**–**F**)) after normalization of the spectra.

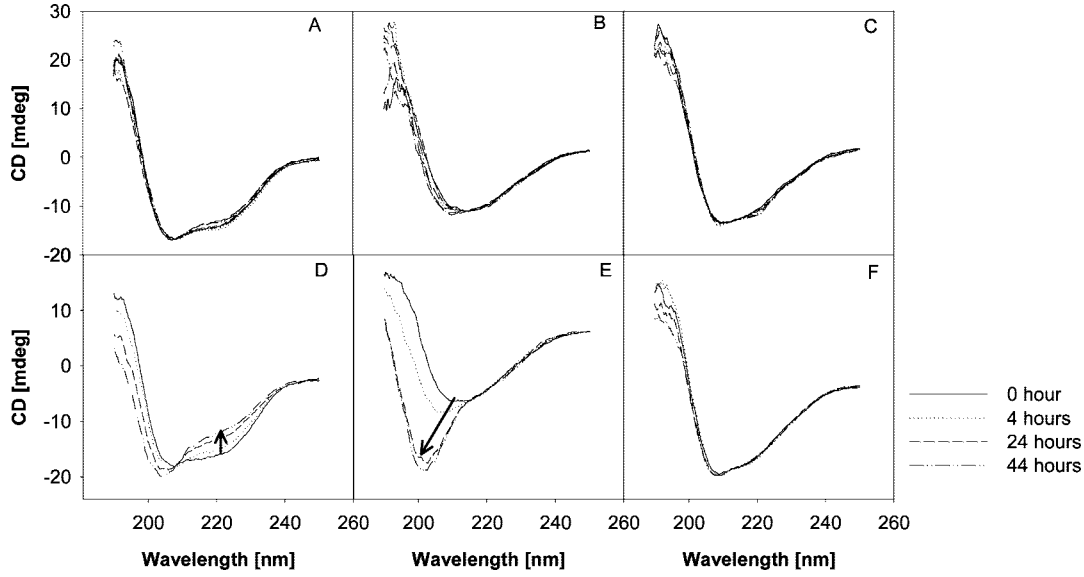

Figure 5. CD spectra obtained for proteins before and after light-induced oxidation with riboflavin used as photosensitizer. Three mg/mL solutions of protein were incubated with 1.75 μg/mL riboflavin in **A**–**C** or with 17.5 μg/mL riboflavin in **D**–**F**. **A** and **D**, α-lactalbumin; **B** and **E**, β-lactoglobulin; and **C** and **F**, lactoferrin. The samples were all diluted to a final concentration of 0.2 mg/mL before the CD measurement.

maxima at 335 nm (**Figure 4A**–**C**), indicating that the tryptophan in the globular protein structures are more shielded from the solvent than the tryptophan in α- and β-casein. Red shifts in the spectra were seen, especially for α- and β-casein, which showed a high degree of red shifts with emission maxima around 410 nm. For visualization of this shift, all of the spectra were normalized through the emission maximum found for the proteins taken at $t = 0$. Further analysis of the changes in tertiary structure of the globular proteins was performed by extracting the emission measured at 355 nm, which was found to be the emission maxima for all of the unfolded proteins in guanidium chloride. The emission intensities measured at 355 nm indicated unfolding of α-lactalbumin as the emission from tryptophan in α-lactalbumin increased, that is, they became more solvent-exposed upon oxidation (**Figure 4D**, inset). The increase in emission intensity after 44 h of photo-oxidation was 10% for α-lactalbumin. The effect on β-lactoglobulin was smaller (**Figure 4E**, inset); the emission intensities only increased by 3%. The tryptophan in lactoferrin seemed to become less solvent exposed upon oxidation, as the extracted emission at 355 nm showed a decreasing curvature (**Figure 4F**, inset). This decrease in emission measured at 355 nm for lactoferrin after 44 h of photo-oxidation was calculated to be 12% of the initial value at 0 h.

Loss of Secondary Structure Observed in Globular Proteins. Changes in secondary structures of photo-oxidized milk proteins were measured by CD. The three casein preparations all showed CD-spectra with high intensities at the region around 200 nm, indicating a high content of random coil structure, both before and after photo-oxidation (data not shown). The CD-spectra obtained for α-lactalbumin indicated a high content of α-helical structure with the double peak at 205–210 nm and around 220 nm (**Figure 5A**). The CD-spectra obtained for β-lactoglobulin showed high intensities in the β-sheet region at 210–215 nm (**Figure 5B**), and the CD-spectra obtained for lactoferrin indicated a high content of α-helical structure (**Figure**

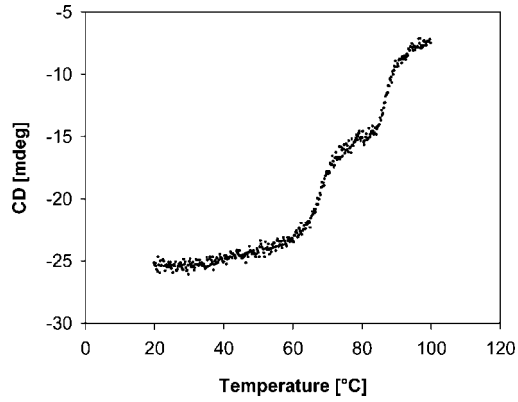

Figure 6. Thermal stability of lactoferrin determined using CD spectroscopy. Thermal scanning was performed from 20 to 100 °C with at temperature gradient of 0.2 °C/min following loss of α-helical structure at 222 nm.

5C). No changes were seen in the CD-spectra when the proteins were incubated with riboflavin but without illumination with fluorescent light (results not shown). Illumination with fluorescent light without the presence of riboflavin also did not affect the secondary structure of any of the proteins (data not shown). No significant changes were seen in the CD-spectra obtained for the random coil proteins α-, *β*-, and *κ*-casein in any of the treatments with riboflavin and light (data not shown). The secondary structure of lactoferrin was not affected by photo-oxidation, and only marginal changes were observed for both α-lactalbumin and *β*-lactoglobulin when incubated with 1.75 *μ*g/mL riboflavin under fluorescent light. Increasing the concentration of riboflavin 10-fold to 17.5 *μ*g/mL, however, induced changes in the secondary structure of α-lactalbumin and *β*-lactoglobulin, and no observable changes were seen in the secondary structure of lactoferrin. Increased intensity in the CD-spectra at 222 nm indicated loss of α-helical structure for α-lactalbumin after 24 h of illumination with fluorescent light in the presence of 17.5 *μ*g/mL riboflavin. After 44 h, almost the same loss of α-helical structure was observed for α-lactalbumin, but the spectra indicated further random coil structure identified by a left shift toward the region around 200 nm. CD-spectra obtained for *β*-lactoglobulin indicated minor changes in the secondary structure after 4 h of photo-oxidation in the presence of 17.5 *μ*g/mL of riboflavin. CD-spectra of *β*-lactoglobulin were shifted slightly toward the random coil region after 4 h of exposure to fluorescent light, and after 24 h illumination with fluorescent light, only random coil was seen in the CD-spectra of *β*-lactoglobulin.

Because the three globular proteins were found to have different sensitivities toward photo-oxidation, a comparison of the structural stability of the proteins was included in the study, using the melting temperature as a simple parameter. Melting temperatures were available for α-lactalbumin (*18*) and *β*-lactoglobulin (*19*) at neutral pH, but not for lactoferrin in low concentration. The melting temperature of α-lactalbumin was determined to be 68 °C (*18*), and *β*-lactoglobulin has been shown to have both a reversible and an irreversible transition at 65 and 77 °C, respectively (*19*). The melting temperature of lactoferrin was determined by applying CD with thermal scan between 20 and 100 °C. Two transitions were observed; the first had a midpoint around 65 °C according to a two-state analysis (**Figure 6**), and the data strongly indicated a second midpoint around 86 °C. The latter midpoint at 86 °C could not be demonstrated by applying the two-state analysis, but calculation of the first derivative supported this hypothesis.

Polymerization of Some of the Proteins after Photo-oxidation. Changes in quaternary structure were studied by gel filtration experiments of photo-oxidized milk proteins after 44 h of photo-oxidation. α- and *β*-Casein accumulated dimers after 4 h of photo-oxidation (**Figure 7A** and **B**), and furthermore, higher polymers of *β*- and α-casein were seen after 24 and 44 h of incubation, respectively. Because α- and *β*-casein do not contain cysteine, the polymer formations were not the result of disulfide bonding. Only slight changes were observed in the quaternary structures of *κ*-casein (**Figure 7C**), α-lactalbumin (**Figure 7D**), or *β*-lactoglobulin (**Figure 7E**), even after 44 h of photo-oxidation. Dimers and further polymerization of lactoferrin (**Figure 7F**) formed during incubation. Polymerization of the oxidized lactoferrin was confirmed by unreducing SDS PAGE, but not in the presence of the reducing agent DTT, which indicates that reducible disulfide bonds were primary responsible for the polymerization of lactoferrin (data not shown).

Correlation between Parameters. Because both intra- and intermolecular cross-linkages are detected when dityrosine links are measured in the assay, it was considered helpful to be able to distinguish between these two outcomes. This was carried out by measuring the loss of monomeric protein molecules using gel filtration. The formation of dityrosine links correlated very well with loss of native protein for the two random coil proteins, α- and *β*-casein ($R^2 = 0.99$ and 0.98, respectively), whereas a weaker correlation was seen for the other proteins ($0.19 < R^2 < 0.77$). These findings reflect the absence of cysteine residues in α- and *β*-caseins, and therefore, cross-linkages in these two protein preparations merely reflect the formation of dityrosine. In contrast, in lactoferrin, where cysteine residues are present, cross-linkages are probably the results of a combination of dityrosine formation and disulfide shifts leading to polymer formation.

Tryptophan emission was believed to be closely related to the formation of *N*-formylkynurenine and kynurenine. The linear regression was performed and, as expected, the two parameters correlated very well with R^2 values ≥ 0.88 and ≥ 0.84, respectively.

The emission spectra obtained for the oxidized proteins showed red shifts with emission maxima around 400–410 nm for α- and *β*-casein (**Figure 4A** and **B**). Because dityrosine formation is measured in that region, the correlation between dityrosine determined by the HPLC-fluorescence method (**Figure 2A–F**) and the emission (410 nm) extracted from the normalized emission spectra (**Figure 4A–F**) was calculated. Correlation ($0.74 < R^2 < 0.97$) was seen for all proteins, with the two random coil proteins, α- and *β*-caseins, being those showing the best correlation.

DISCUSSION

The purpose of this study was to investigate changes in protein structures at different levels (secondary, tertiary, and quaternary) and to compare those findings with changes in primary structure (carbonyl groups in tryptophan, histidine, and methionine, as well as oxidation products of tyrosine). Furthermore, the aim was to evaluate the different accessibility for photo-oxidation occurring in proteins with different secondary structures. Photo-oxidation in the presence of the photosensitizer riboflavin resulted in changes in the primary structures of all the milk protein preparations studied: α-, *β*-, and *κ*-casein, α-lactalbumin, *β*-lactoglobulin, and lactoferrin. Changes at the secondary structure level were only seen for the two globular proteins, α-lactalbumin and *β*-lactoglobulin, whereas no changes were observed in the secondary structures of the third globular

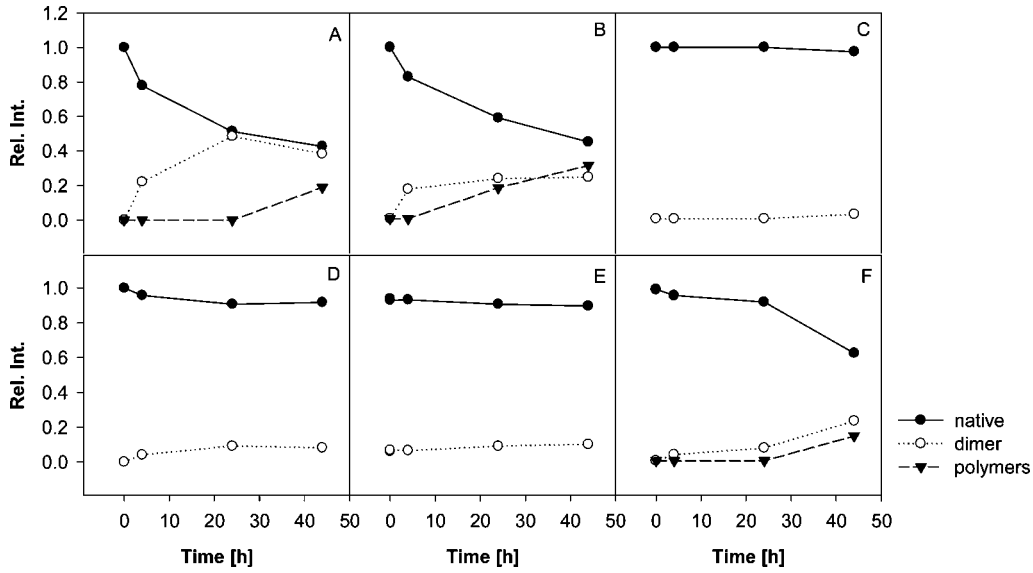

Figure 7. Polymerization of milk proteins was followed by gel filtration as a function of the time of light exposure in the presence of 1.75 μg/mL riboflavin. Three mg/mL solutions of (**A**) α-casein, (**B**) β-casein, (**C**) κ-casein, (**D**) α-lactalbumin, (**E**) β-lactoglobulin, and (**F**) lactoferrin were incubated under fluorescence light for up to 44 h. Light absorbance was monitored at 280 nm.

Table 1. Summary of Changes Introduced in the Protein Structures after Photo-Oxidation of the Six Different Protein Solutions[a]

	α-casein	β-casein	κ-casein	α-lactalbumin	β-lactoglobulin	lactoferrin
carbonyl	high	high	medium	medium	medium	low
dityrosine	high	high	medium	medium	low	low
loss of Trp	high	high	low	medium	low	low
secondary structure	no changes	no changes	no changes	loss of α-helix	loss of β-sheets	no changes
tertiary structure	n.d.	n.d	n.d.	unfolding (10%)	unfolding (3%)	more compact structure (12%)
polymerization	dimers and polymers	dimers and polymers	no changes	no changes	no changes	dimers and polymers

[a] The numbers in parentheses indicate the level of measured decrease or increase in Trp-emission.

protein investigated, lactoferrin, or for the random coil structured caseins. By tryptophan emission, spectroscopy changes were seen at the tertiary structure level for lactoferrin and for α-lactalbumin, whereas only marginal changes were seen in the tertiary structure of β-lactoglobulin. Changes in the tertiary structures of α- and β-caseins could not be evaluated by applying tryptophan emission at 355 nm, because 355 nm was too close to the emission maximum (350 nm) of the native α- and β-caseins. Changes in quaternary structures in terms of dimerization or polymerization were observed for lactoferrin, α- and β-caseins, and α-lactalbumin, whereas only slight changes were observed at this level for κ-casein and β-lactoglobulin.

It has been shown earlier that singlet oxygen only interacts with the amino acids histidine, tryptophan, tyrosine, methionine, and cysteine (*20*). In this study, oxidative changes were observed in tryptophan, histidine, methionine, tyrosine, and indirectly in cysteine (by reducible cross-linkages in lactoferrin). Changes were already observed at the primary structural level after 4 h of photo-oxidation as increments in carbonyl content and dityrosine formation. Within the same period of time, changes in tryptophan emission were observed as well. Tryptophan residues are mostly buried in the core of native globular proteins, and the emission maximum of buried tryptophan lies in the region of 330–335 nm (*21*), as also observed in this study for the three globular whey proteins, α-lactalbumin, β-lactoglobulin, and lactoferrin. The emission maxima obtained for the three caseins were all red-shifted when compared with the globular proteins, which is in agreement with those proteins having a more random coil secondary structures and, therefore, the less buried tryptophan. Because a decrease in tryptophan emission can be caused by oxidative degradation of tryptophan or by changes in the local microenvironments of tryptophan residues induced by changes in the tertiary protein structure, tryptophan excitation was performed both in aqueous solution and in a solution of 6 M guanidinium chloride. Loss of tryptophan was clearly shown to occur in guanidium chloride, where the proteins are expected to be completely unfolded, and the decrease in tryptophan emission can be assigned solely to the loss of tryptophan because of oxidative degradation. The formation of *N*-formylkynurenine has been found to be well-correlated with the loss of tryptophan in a study characterizing photo-oxidation of α-crystallin from the bovine eye lens (*22*), and a similar correlation between loss of tryptophan and formation of either of the two oxidation products, *N*-formylkynurenine or kynurenine, was seen in this study.

The random coil proteins were found to be most sensitive toward oxidation in terms of changes in primary structures (chemical modifications) and quaternary structures (polymerization). We did not observe any changes at the secondary and tertiary structure level, which may reflect the fact that they remain predominantly unstructured in spite of chemical changes and polymerization. Michaeli and Feitelson (*23*) previously showed that free amino acids are more labile toward singlet oxygen oxidation than tripeptides and that peptides are more labile than disulfide-bond containing polypeptides. Thus, oxidative lability is highly dependent on the structure of the protein or peptide. Our observation that the random coil proteins studied were more accessible to oxidation than the globular proteins holding a more compact structure are consistent with these results. The globular whey proteins investigated in this study

showed different susceptibility to oxidation, with lactoferrin being most resistant. α-Lactalbumin showed a remarkably high content of dityrosine, whereas the formation of dityrosine was rather low in β-lactoglobulin. The oxidative stability of the globular proteins may reflect the overall structural stability, which can be evaluated through their melting temperatures. The melting temperature of α-lactalbumin has been determined to be 68 °C at pH 7.7 (*18*), whereas β-lactoglobulin and lactoferrin both have two transitions. The lowest thermal transition of β-lactoglobulin (65 °C) was caused by a dissociation of the naturally occurring dimer, and a further transition to a molten globule-like state was identified at neutral pH at 77 °C (*19*). The first transition state of bovine lactoferrin was, in this study, determined to be 65 °C and was accompanied by a loss of α-helical structure by CD, which is consistent with transition measured for bovine lactoferrin in much higher concentration (36 mg/mL) (*24*). Paulsson et al., (*24*) furthermore identified a second thermal transition for lactoferrin at 92 °C, which is 6 °C higher than the one found in the present study. The reason for this difference in the second thermal transition between the two studies is not known. Although the first thermal transition state of lactoferrin was rather similar to the transition of α-lactalbumin, and also to the first of the transitions (reversible) of β-lactoglobulin, lactoferrin seemed remarkably stable toward oxidative stress. No changes were seen at the secondary structural level of lactoferrin, even when applying high concentrations of riboflavin. The fluorescence spectroscopy data strongly indicated a more compact structure of lactoferrin after photo-oxidation and that tryptophan in lactoferrin became more shielded from the solvent. This change in the local microenvironment of tryptophan residues could be due to dimerization or further polymerization of the oxidized lactoferrin, which was not seen for neither α-lactalbumin nor β-lactoglobulin. Nevertheless, lactoferrin was the most stable protein, in terms of changes at both the primary as well as the secondary structural level. The structural changes observed after photo-oxidation of the six protein solutions are summarized in **Table 1**.

The carbonyl *N*-formylkynurenine has been characterized as a photosensitizer (*25*), and formation of this oxidation product may hence contribute to a chain reaction producing more free radicals, which in turn increases oxidation. Because lactoferrin showed a remarkably low content of this carbonyl compound after photo-oxidation, the explanation for the stability at a higher structural level of this particular protein may actually be a result of the only slight changes introduced by photo-oxidation into the primary structure of lactoferrin. Furthermore, the shielding of tryptophan may protect this residue from further oxidation. In a study performed by Bihel and Birlouez-Aragon (*26*) it was shown that the presence of lactoferrin actually inhibited tryptophan oxidation initiated by the presence of iron and ascorbic acid (Vitamin C). This effect might be primarily due to the chelating effect of lactoferrin, but with iron in excess where only one-fifth was chelated with lactoferrin, asorbate-iron-mediated tryptophan oxidation was still significantly lowered in the presence of lactoferrin (*26*). Recently, tryptophan emission of lactosylated lactoferrin indicated more buried tryptophan in the core of lactoferrin and, therefore, a more compact structure of the protein after lactosylation (*27*). Hence, lactoferrin seems to be special in terms of oxidative stability, irrespective of whether it is initiated by lactosylation, transition metal catalyzed oxidation, or photo-oxidation.

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