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Milk Whey Protein Modification by Coffee-Specific Phenolics: Effect on Structural and Functional Properties

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Supporting Information

ABSTRACT: A suitable vehicle for integration of bioactive plant constituents is proposed. It involves modification of proteins using phenolics and applying these for protection of labile constituents. It dissects the noncovalent and covalent interactions of β-lactoglobulin with coffee-specific phenolics. Alkaline and polyphenol oxidase modulated covalent reactions were compared. Tryptic digestion combined with MALDI-TOF-MS provided tentative allocation of the modification type and site in the protein, and an in silico modeling of modified β-lactoglobulin is proposed. The modification delivers proteins with enhanced antioxidative properties. Changed structural properties and differences in solubility, surface hydrophobicity, and emulsification were observed. The polyphenol oxidase modulated reaction provides a modified β-lactoglobulin with a high antioxidative power, is thermally more stable, requires less energy to unfold, and, when emulsified with lutein esters, exhibits their higher stability against UV light. Thus, adaptation of this modification provides an innovative approach for functionalizing proteins and their uses in the food industry.

KEYWORDS: *coffee phenolic compounds, whey proteins, antioxidants, protein-phenol interactions, modeling, functionalizing proteins*

INTRODUCTION

As new health-promoting ingredients are identified and their efficacy is documented, the next challenge of food and supplement industries is to find appropriate means to deliver them in a matrix that consumers will accept and use repeatedly. In this study, we wanted to combine high-quality nutritional proteins, for example, β-lactoglobulin, with health-promoting substances (e.g., phenolic compounds) into a multifunctional ingredient that could be used to design a wide range of food products and at the same time provide better accessibility, protection, and bioavailability of bioactive easily oxidized lipophilic compounds (in this case, the carotenoids). The initial step of this concept therefore involves the modification of proteins such that they provide an effective antioxidant interface agent that will be applied to simulate a simple food system. A suitable application could be achieved by testing the modified proteins in an oil-in-water emulsion. The easily oxidized lipophilic compounds (in this case, the carotenoids) will be located in the oil droplet.

Interest in phenolic compounds is related to their dual role as substrates for oxidative browning reactions and as antioxidants, emphasizing their impact on sensory and nutritional qualities of fruits and vegetables, their role in plant growth and metabolism, and, more recently, their demonstrated physiological activity in humans.[1] The action of phenolics as antioxidants is viewed as beneficial in both foods (e.g., gallates as supplementary food ingredient) and the body, where phenolics are oxidized in preference to other food constituents or cellular components and tissues.[1−4] Coffee beans are one of the major sources of chlorogenic acid, an ester formed between caffeic and quinic acid, resulting in caffeoylquinic acids (CQA). Therefore, a green coffee extract may provide the necessary antioxidants for the planned study.

Most of the literature sources deal with noncovalent type of interactions between phenolic compounds and proteins.[7] There is only limited experimental data on the covalent bonds between these two reactants.[7,8] The phenolic compounds are susceptible to both enzymatic and nonenzymatic oxidation in the presence of oxygen.[4,7] The resulting reactive *o*-quinones are capable of undergoing a nucleophilic addition to proteins, thereby covalently modifying the proteins.[7] On the basis of this knowledge, further work in this field showed that modified proteins with an increased antioxidative capacity can be developed.[7−9]

Bovine whey proteins (WP) were chosen as protein component for the concept described above. WP are valuable food ingredients due to their ability to aggregate and provide structure to foods, their solubility over a wide pH range, and many other functional properties.[10,11] Among the latter, their excellent emulsification properties make them ideal model proteins.[12,13]

A recent study demonstrates the suitability of applying lutein in a model emulsion, with recommendation to provide a suitable light-sensitive probe for testing the concept described.[13] Lutein, a dihydroxy derivative of β-carotene ((3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol) is a nonprovitamin A carotenoid. Lutein initiates two main mechanisms that could explain its protective role in the eye. The first mechanism is the ability of the macular pigment to absorb blue light, particularly before the light induces a negative effect on the photoreceptor cells, and the second is an antioxidant action. The influence of thermal treatment and light exposure on degradation and

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isomerization of lutein has been assessed in several studies. Lutein studies on stability against UV light demonstrated that the most damaging wavelengths to lutein were identified in the UV range of 200−400 nm and at 463 nm in a lutein-fortified model colloidal beverage.[14]

Thereafter, the objectives of this study were to modify (using enzymatic and alkaline options) the whey proteins (β-lactoglobulin) with an aqueous green coffee extract, to characterize the modified proteins with respect to the site and art of modification and, finally, to determine the effect on the functional properties of such modified proteins. A positive outcome of these studies would open the possibility for incorporation of these multifunctional ingredients into various food products and enable consumers to benefit from a health-promoting effect of phytonutrients found in vegetables.

MATERIALS AND METHODS

Materials. Green coffee beans from Uganda (Rohkaffee-Co., Berlin, Germany) representing *Coffea canephora* var. *robusta* (CR) and from Colombia (Deutsche Extraktkaffee GmbH, Berlin, Germany) representing *Coffea arabica* L. (CA) were employed.[15] The beans were milled (particle size ≤ 0.5 mm) using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany), equipped with a 24 tooth rotor and a ring sieve having 0.5 mm holes. The milling was conducted at a speed of 15000 rpm, and all the powdered samples were frozen at −20 °C. WP (Biopure, lot JE 002-8-415) was obtained from Davisco Foods International, Inc. (Le Sueur, MN, USA). The powder contained 98% of protein, as determined by Kjeldahl analysis (N × 6.38), 0.1% fat, 1.9% ash, and 4.7% moisture, as specified by the supplier. The major whey protein fraction was ca. 93.4% β-lactoglobulin as determined by SDS-PAGE. Skimmed milk powder (Sucofin, TSI GmbH, Zeven, Germany), casein-Na (Carl Roth GmbH, Karlsruhe, Germany), and soy glycinin were applied as reference proteins. The latter was prepared from defatted unheated soy flour (type 1, protein content ca. 52%, Sigma Chemicals Co., St. Louis, MO, USA) according to the method of Thanh and Shibasaki.[16] For polyphenol oxidase (PPO) activity, the juice of commercially available Braeburn apple samples was applied. The activity of PPO and the content of phenolic compounds in the juice was estimated as described below (Supporting Information, Figure S3). After removal of the seeds, apple fruits were processed in juicing equipment (Philips, HR7775, China). After that, the juice was filtered through a cloth sieve and saved at −20 °C until needed. Lutein (20%) was provided by DSM Nutritional Products (Kaiseraugst, Schwitzerland). 5-Caffeoylquinic acid (5-CQA) was used as a standard for the quantitative analysis and was commercially obtained (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). All other chemicals and HPLC solvents were of reagent grade or gradient grade, respectively.

Methods. *Characterization of Interactions between CQA and Proteins.* Four experiment sets were investigated: (1) Binding to reference food proteins (WP, casein-Na, soy glycinin, and skimmed spray dried milk; end concentration = 0.1 mg protein/mL) depended on the concentration of 5-CQA (10−200 μM) in 5 mM phosphate buffer (PBS), pH 7.2−7.4. Corresponding blanks included solutions of protein (100%) or the individual 5-CQA concentration alone in PBS buffer for correction of the measured fluorescence. The samples were excited in a Jasco fluorescence detector FP 920 at 290 nm (slit 18 nm), and the emission was recorded at 320−420 nm (slit = 18 nm). The area under curve (AUC) was used to analyze the exposition. (2) The effect of protein concentration (10−400 μg protein/mL) using whey proteins as model was studied at two constant concentrations of 5-CQA (4 and 40 μM). (3) Maintaining a constant ratio of CQA/protein ratio of 1:7 (14.4:100 mg/L), the binding was further studied for different combinations of the two reactants. (4) Finally, the major hydroxycinnamic acid derivatives were fractionated from green and roasted coffee beans (Colombia) under conditions of HPLC and freeze-dried.[8] Subsequently, their binding to whey proteins (0.1 mg/mL) was studied at a constant concentration of 40 μM, directly after addition of the ligand and after incubation at room temperature for 24 h.

Determination of PPO Activity in Apple Juice. PPO activity was determined on the basis of the formation of a pink proline−catechol adduct recorded at 525 nm (ε = 1550 L mol^{-1} cm^{-1}).[17] PPO activity was calculated from the slope within the initial linear range of the absorbance−time curve. For blank correction, the slopes of a sample blank (0.1 mL of water instead of extract) and a reagent blank (0.2 mL of reaction buffer instead of 25 mM 4-methylcatechol) were subtracted. The enzyme activity was expressed in units per liter of the sample juice.

Covalent Modification of β-Lactoglobulin. Ground green coffee (3g) was extracted for 15 min with 90 mL of boiling distilled water. The extract was allowed to cool to room temperature (25 °C) and centrifuged at 4000*g* for 10 min at 4 °C, and the supernatant was filled up to 250 mL. For PPO modulated reaction, an aliquot (25 mL) was added to 0.25 g of β-lactoglobulin and mixed with 6 mL of apple juice (the resulting pH was 4.7). For the alkaline modulated reaction, the pH of the extracted coffee phenolics was adjusted to pH 9, without any addition of apple juice. A blank control constituted the same amount of β-lactoglobulin (0.25 g) dispersed in distilled water. After 24 h of reaction time under continuous stirring at room temperature (25 °C) with free exposure to air, the samples were dialyzed for 18−20 h against water at room temperature and finally lyophilized. Samples were removed at the beginning and after 24 h to determine the amount of CQA. The modification was repeated three times to confirm the reproducibility of the modification procedure. The samples were designated with the following abbreviations: ULG, unmodified β-lactoglobulin; PPO-LG, PPO-modulated β-lactoglobulin modification; and ALG, alkaline-modulated β-lactoglobulin modification.

Identification of the Major Phenolic Compounds. Extracts were removed during the modification of the proteins, and the chromatographic separation was carried out on a Shimadzu HPLC system (Kyoto, Japan) using a Prontosil 120-3-C18 ACE-EPS column (Bischoff Analysentechnik und -geräte GmbH, Leonberg, Germany; 150 × 4.6 mm; 3 μm) at 40 °C, connected to a C18 precolumn containing the same material with a flow rate of 1 mL/min and with a dual wavelength UV−vis detection at 280 and 325 nm. The eluents were A (2% acetic acid) and B (methanol). The gradient was applied under the following conditions: 20% eluent B, 3 min; 20−35% eluent B, 17 min; 35−68% eluent B, 17 min; 68% eluent B, 3 min; 68−20% eluent B, 3 min; 20% eluent B, 12 min. Run time was 55 min, and the injection volume was 20 μL (lower amounts were used for identification of the phenolic compounds by HPLC-MS; Supporting Information provided in Figure S1).[8] Concentrations of 10−100 μg/mL of 5-CQA were used for calibration. The content was expressed as 5-CQA equivalents in milligrams per 100 g coffee bean material or in milligrams per gram protein.

Characterization of the Modified β-Lactoglobulin. *Free Amino Groups.* The method based on using trinitrobenzenesulfonic acid (TNBS) was applied as previously described.[18]

Thiol Groups. The procedure using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid); DTNB] was applied to determine the thiol (sulfhydryl) group (−SH) content.[19]

Determination of Tryptophan Fluorescence. Protein solutions (1 mg/mL) in 5 mM PBS buffer, pH 7.2, or 8 M urea were measured using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan) as described above and expressed as tryptophan equivalents.[18]

Determination of Antioxidative Capacity. Trolox equivalent antioxidant capacity assay (TEAC)[20] was applied with a few modifications, using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK). TEAC values were obtained by comparison with those obtained from trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid, Sigma-Aldrich) which served as reference. Results are expressed as nanomoles trolox equivalents (TE) per milligram protein.

The antioxidative capacity of proteins was additionally determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described in the

literature.[21] A standard curve was prepared using trolox in the range of 12.5−100 μM. The activity was expressed as nanomoles TE per milligram protein.

Covalent Bound Chlorogenic Acid. To estimate the amount of chlorogenic acid bound to the proteins, 4 mg of modified protein was dissolved in 1 mL of 8 M urea solution followed by precipitation of protein with 20% trichloroacetic acid. After this treatment, the precipitate was redissolved in 1 mL of 8 M urea. RP-HPLC was conducted with a Shimadzu 10A system (Kyoto, Japan) with a Perfectsil C18 300 ODS column, 150 × 4.6 mm, 5 μm, at a temperature of 37 °C. The eluents were 0.1% trifluoroacetic acid (v/v) (A) and acetonitrile (B). The gradient was applied under the following conditions: 10−18% B, 22 min; 18−80% B, 8 min; 80% B, 3 min; 80−10% B, 2 min; 10% B, 7 min. Run time was 42 min. Fifty microliters was injected. Detection and external calibration using 5-CQA was conducted at 325 nm.

Surface Hydrophobicity. Using 1-anilino-8-naphthalensulfonate (ANS),[22] fluorescence was recorded in 5 mM sodium phosphate saline (PBS) buffer, pH 7.2, with a JASCO fluorescence detector using excitation at 390 nm (slit = 18 nm) and emission between 390 -and 900 nm (slit = 40 nm). The initial slope (S_0) of the fluorescence intensity versus soluble protein concentration was used as an index of the protein surface hydrophobicity.

Far-UV and Near-UV Circular Dichroism (CD). The measurements of the samples were conducted in the range of 178−260 nm in PBS buffer, pH 7.2, using a Jasco J 710 spectropolarimeter (Gross-Umstadt, Germany).[12,18]

Differential Scanning Calorimetry (DSC). DSC of 15% protein solutions in 5 mM PBS buffer, pH 7.2, was performed using a Seiko 120 DSC analyzer (equipped with thermal analysis system SSC5200, Seiko Instruments Inc., Japan) as outlined in the literature[18] with a few modifications. Sample solutions (40 μL) were analyzed in airtight 70 μL aluminum containers. Aluminum oxide in PBS buffer served as reference. Thermal curves were established at a heating rate of 3 °C min^{-1} over a temperature range of 20−105 °C. The thermal parameters T_d (denaturation temperature, temperature at maximum heat flux), ΔH (enthalpy of denaturation), and onset temperatures (T_{onset}) were determined from all thermal curves.

SDS-PAGE. The method of Laemmli[18,23] was adapted (T = 14−18%) for determination of the molecular weight of the proteins. The band intensity was estimated using scanning utilities with Quantity one software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate Milano, Italy). Sample buffer was 0.05 M Tris-HCl buffer, pH 6.8, containing 4 g of sodium dodecyl sulfate, 12 g of glycerol, 5 g of 2-mercaptoethanol, and 0.01 g of Coomassie Brilliant blue R 250. The sample treatment consisted of denaturing the proteins (1 mg/mL) by heating at 95 °C for 3 min prior to analysis. A low molecular weight calibration kit for SDS electrophoresis (GE Healthcare Europe GmbH, Freiburg, Germany) was applied.

Matrix-Assisted Laser Desorption/Ionization−Time-of-Flight−Mass Spectrometry (MALDI-TOF-MS) for Modified Intact Proteins. Experiments of the proteins were performed by dissolving 1 mg of the protein sample in 1 mL of 0.1% trifluoroacetic acid/acetonitrile (50%, v/v) containing 2 mg of DTT. Of this solution, 0.5 μL was brought on the target and covered with 0.5 μL of 2,5-dihydroxyactetophenone (DHAP; Bruker Daltonik GmbH, Bremen, Germany) as matrix (7.6 mg of 2,5-DHAP was dissolved in 375 μL of ethanol and 125 μL of a solution containing 18 mg/mL diammonium hydrogen citrate dissolved in water). After crystallization of the sample by air-drying, measurements were carried out on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH). The instrument was internally calibrated using the signals of the positive $[M + H]^+$ monoisotopic ions of a protein 1 calibration standard (Bruker Daltonik GmbH).

Identification of Reaction Sites and Types of Modification. Protein (1 mg) was dissolved in 1 mL of 50 mM ammonium bicarbonate (pH 7.5−8.5) buffer. To 20 μL of this solution were added 77.5 μL of 50 mM ammonium bicarbonate buffer and 2.5 μL of trypsin working solution (Pierce Biotechnology, Inc., Rockford, IL, USA). The digestion was conducted at 37 °C for 24 h and stopped by adding 5 μL of 10% trifluoroacetic acid (v/v). A saturated matrix solution (α-cyano-4-hydroxycinnamic acid (HCCA), Bruker Daltonik GmbH) was prepared in 30:70 (v/v) acetonitrile/0.1% trifluoroacetic acid in water. Equal volumes (for example, 2 μL each) of protein sample solution and matrix solution were premixed. Half a microliter of this mixture was applied onto a steel target for MS analysis. The MALDI-TOF-MS analysis of the samples was performed on AUTOFLEX-III LRF200-CID, in the reflector-mode operation; the acceleration voltage was 20 kV, and the effective flight path was 200 cm. The instrument was internally calibrated using the signals of the positive $[M + H]^+$ monoisotopic ions of a peptide calibration standard II (Bruker Daltonik GmbH). The data analysis was performed using the software packet Bruker Daltonics FlexAnalysis (vers. 3,3; Bruker Daltonik GmbH). Sequence database search using the m/z values of the digested peptides was performed with Bruker Daltonics BioTools (vers. 3,2; Bruker Daltonik GmbH) combined with the MASCOT search program (Matrix Science Ltd., London, UK).[24] The SwissProt 2011_06 (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+top; http://www.uniprot.org/) database was applied. On the basis of the sequence information available, further analysis of the data was performed using the software "Sequence editor" (v 3.2; Bruker Daltonik GmbH). Analysis of optional modifications by CQA was based on the covalent reaction of lysine and thiol amino acid side groups with 5-CQA and those theoretically possible.[8,17] A compilation of these data is supplied (Supporting Information, Table S2).

Molecular Modeling Experiments. Molecular docking and energy minimization experiments were performed on a workstation with dual xenon quad cores using the following molecular modeling software as described in ref 8: Molecular Operating Environment (MOE), 2010.10, Gaussian 09w (structure calculation for small molecules), and Molegro 5.0 (docking experiments). Both MOE 2010.10 and Yasara 12.01 were used for homology modeling. For the in silico experiments the protein structure from bovine β-lactoglobulin (unliganded form, PDB code 3NPO; http://www.rcsb.org/pdb/explore/explore.do?structureId=3npo) was applied. Complementary PDB code 3NQ3 was also considered.

Effect on Functional Properties of β-Lactoglobulin. *Solubility.* Modified and unmodified β-lactoglobulin samples (0.1% w/v) were dissolved in 0.05 M sodium phosphate buffer at different pH values. The samples were centrifuged (10 min, 9300*g*, and 4 °C). Afterward, the protein concentrations of the supernatants were measured at 595 nm with a spectrophotometer (a Novaspec II, Pharmacia Biotech, Cambridge, UK) using a modified Lowry method.[25]

Preparation of Emulsion. Emulsions with 10% medium-chain triglyceride (MCT) oil, containing oleoresin (lutein ester), 4 mg/mL, in 5 mM PBS buffer, pH 7.24, were prepared by mixing the MCT oil and β-lactoglobulin solution (0.2%) at 500 rpm for 10 min to form a pre-emulsion. After that, the emulsion was homogenized by using an ultrasonic homogenizer (Sonopuls HD 2070 with titanium probe, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The sonication time was 5 min in an ice bath at an energy input of 70% of the maximum power. An emulsion without any lutein ester was also prepared as a control sample.

Emulsion Stability. The creaming stability of the emulsion was measured under centrifugal force. After homogenization, 1 mL of emulsion was placed in a UV cuvette and centrifuged at 1500*g* (until 40 min) and at 3000*g* from 40 to 65 min. After every 5 min of centrifugation, the absorbance of emulsions at 500 nm was recorded by a UV−vis spectrophotometer (SPEKOL, Carl Zeiss, Germany).

Oil Droplet Size. Oil droplet size and their distribution in the emulsion were evaluated by laser diffraction using a Mastersizer S (Malvern Instruments GmbH, Herrenberg, Germany). Emulsion samples were diluted in distilled water until the intensity of the laser beam decreased by ∼14% (obscuration). The average size of oil droplets and their size distribution was calculated by the equipment software according to Mie's theory. The following characteristics of the emulsion were measured: surface area diameter D [3.2], width of distribution (span), size of particle for 10th, 50th (as the mass median diameter), and 90th percentiles of the diameter.

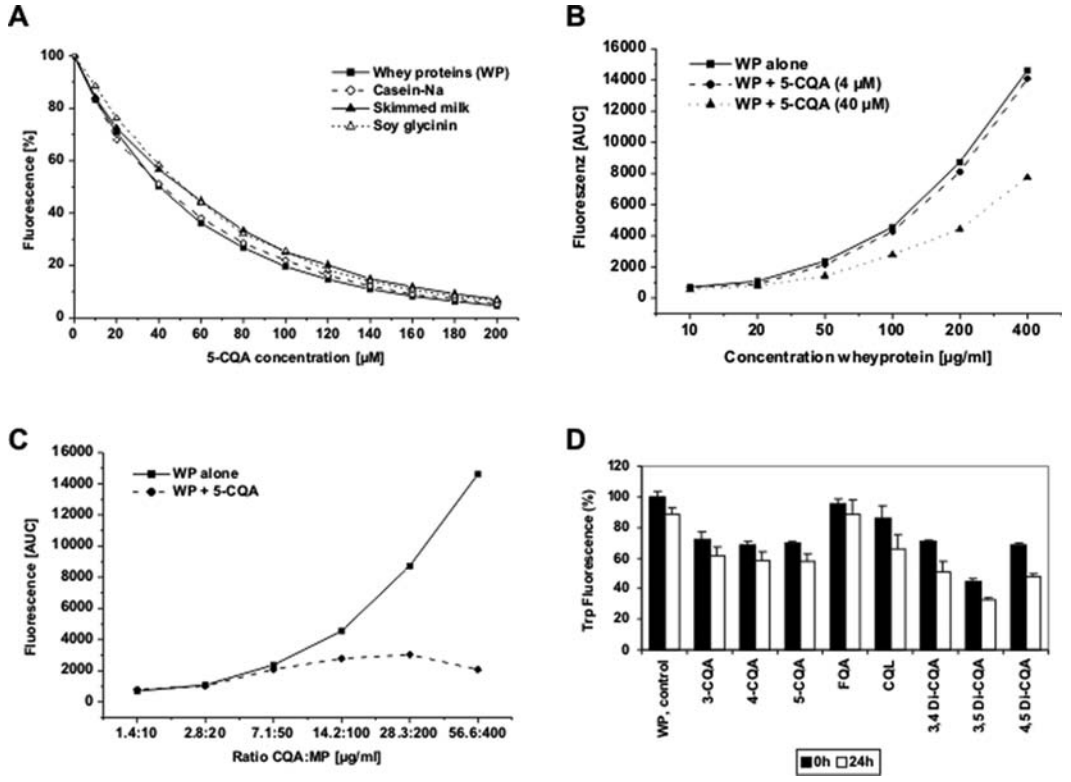

Figure 1. Binding of 5-CQA as determined by the quenching of intrinsic tryptophan fluorescence (A) with different protein-containing model systems, (B) depending on the amount of protein added with 5-CQA concentration remaining constant, (C) depending on different 5-CQA/whey protein concentration ratios, and (D) with different CQA derivatives at a constant concentration of 40 μM directly after addition of the ligand and after incubation at room temperature for 24 h.

Microscopic Imaging of Emulsion. The distribution of particles in emulsions was examined and photographed using a microscope (normal light conditions, model BX50, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a ColorView 12 CCD video camera (SIS, Münster, Germany) with magnification power of 40×. Images were processed using analysis TM 3.0 software (SIS).

Stability of Emulsified Lutein Ester against UV Light. The previously prepared emulsions were exposed to ultraviolet in 1.5 mL glass vials for 0, 24, 48, and 72 h at 365 nm using a UV lamp (VL-6LM 6W-365 nm tube, power 12 W, Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany) at room temperature. Lutein ester was extracted from emulsion by adding 1000 μL of a specially developed hexane-containing extraction solvent (BioAnalyt GmbH, Teltow, Germany) to 150 μL of emulsion, mixing for 10 s with a further shaking for 10 min followed finally by centrifugation, for 5 min at 9300*g*. Alternatively, hexane can also be applied as extraction solvent.[13] The supernatant was transferred into a new tube. The extraction step was repeated once more, and then the volume was filled to 2 mL with extraction solution for injection in the HPLC.

HPLC Analysis for Lutein Content. Lutein ester content was determined using a JASCO HPLC system (JASCO, Japan). The separation was carried out with a C18 analytical Waters-Spherisorb column SC-04 125 × 4.0 mm, ODS2, 3.0 μm (Bischoff Chromatography, Leonberg, Germany). The column temperature was kept at 30 °C. The binary mobile phase consisted of acetonitrile/methanol (9:1, v/v; solvent A) and ethyl acetate (solvent B). Elution was carried out with the following gradient program: from 20 to100% B from 0 to 15 min and from 100 to 20% B from 15 to 30 min. The flow rate was kept at 0.6 mL/min with an injection volume of 20 μL. The run time was 30 min, and lutein ester was detected at 450 nm. Lutein was used for calibration of the lutein ester content based on its molar extinction coefficient ε (L mol^{-1} cm^{-1}) = 2550 at 445 nm in ethanol as described in ref 13.

Statistical Analysis. All experiments were conducted at least three times. The number of parallel samples in the experiments was three or more. All data are expressed as means of their standard deviations. The results were analyzed using SPSS statistical software (SPSS, version 18) when applicable. Values of $P < 0.05$ or $P < 0.01$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of Interactions between CQA and Proteins. To elucidate the interactions of coffee-specific CQAs with proteins, experiments were conducted with different reference food proteins by following the quenching of intrinsic tryptophan fluorescence of proteins by ligand binding.[26] The interaction of 5-CQA with model proteins (milk whey β-lactoglobulin, casein-Na, soy glycinin, and skimmed spray-dried milk) was monitored. The results (Figure 1A) document the concentration-dependent effect of binding of 5-CQA to these proteins, which seems to be relatively independent of the protein applied. The binding depends on the protein concentration applied as illustrated using β-lactoglobulin as model system (Figure 1B). At the concentration of 40 μM 5-CQA, a 50% decrease in tryptophan fluorescence was obtained at a CQA/protein ratio of 1:7 (14.4:100 mg/L); therefore, with this ratio maintained, the binding was further studied for different absolute concentrations of both reactants (Figure 1C). These results show that a certain minimum absolute concentration of both ligands is necessary to provoke the binding. Thereafter, the presence of higher absolute concentrations of both ligands, and with a CQA/protein ratio of 1:7 maintained, may lead to a saturation of the binding sites. Finally, each of the major hydroxycinnamic acid derivative was

Table 1. Characterization of the Modified Whey Proteins

property/sample	ULG (mean ± SD)	PPO-LG (mean ± SD)	ALG (mean ± SD)
1. free amino groups (nmol/mg protein)	861.3 ± 4.3	822.2 ± 43.1	750.1 ± 32.3
2. free thiol groups (nmol/mg protein)	42.9 ± 0.8	23.2 ± 0.8	12.8 ± 0.3
3. covalent bound 5-CQA equivalents (nmol/mg protein)	0.0 ± 0.0	23.0 ± 0.8	29.8 ± 1.7
4. TEAC (nmol TE/mg protein)	48.5 ± 0.5	273.8 ± 5.2	228.8 ± 8.8
5. DPPH (nmol TE/mg protein)	24.7 ± 1.9	173.2 ± 5.8	112.2 ± 11.2
6. ANS			
S_0	244185.0 ± 11380.6	83589.5 ± 8387.2	178227.9 ± 3335.8
S_0 in %	100.0 ± 4.7	34.2 ± 10.0	73.0 ± 1.9
7. tryptophan fluorescence (nmol/mg protein)			
in PBS buffer	49.6 ± 0.7	5.8 ± 0.1	8.0 ± 0.1
in 8 M urea	88.6 ± 0.1	9.7 ± 0.2	14.8 ± 0.1
8. CD			
α-helix (%)	20.5 ± 0.1	7.3 ± 0.1	9.3 ± 0.1
β-strand (%)	39.9 ± 0.1	35.2 ± 0.1	29.5 ± 0.1
β-turn (%)	15.2 ± 0.1	22.5 ± 0.1	17.6 ± 0.1
unordered (%)	24.5 ± 0.1	35.0 ± 0.1	43.5 ± 0.1
9. DSC			
T_{onset}	69.6 ± 0.1	74.1 ± 0.6	67.2 ± 0.1
T_d (°C)	75.0 ± 0.1	80.6 ± 0.3	76.7 ± 0.0
ΔH (mJ/mg protein)	10.8 ± 2.7	7.4 ± 0.3	3.4 ± 0.3
10. modified peptides			
IIAEKT**K**IPAVFKIDALNENK		K77	K77
IDALNEN**K**VLVLDTDYK		K91	
FD**K**ALKALPMHIR		K138	K138
VYVEEL**K**PTPEGDLEILLQK		K47	K47
YLLFCMENSAEPEQSLVCQ**C**LVR		C121	C121

fractionated from green coffee beans (Colombia),[8] and their binding to β-lactoglobulin was studied at a constant concentration of 40 μM, directly after addition of the ligand and after incubation at room temperature for 24 h (Figure 1D). It can be noted that the diCQA are more reactive than CQA isomers, with 3,5 diCQA being the most eligible reaction partner. A further incubation for 24 h (Figure 1D) results in a continued binding of CQA to β-lactoglobulins. Such interaction may have a character of covalent modification in proteins as described previously.[7,27] A slight shift for feruloylquinic acids (FQA) and for protein alone was also observed, which may putatively be attributed to structural changes involving thiol−disulfide interexchange.

Covalent Modification of β-Lactoglobulin. Distribution information on the typical hydroxycinnamic acid derivatives (for structures see Supporting Information, Table S1) found in the applied green coffee beans is provided (Supporting Information, Figures S1 and S2).[8] Recent studies reporting the composition of coffee phenolics have documented more than 50 hydroxycinnamic acid derivatives being present.[5,6] However, the major phenolic compounds present are chlorogenic acid (5-CQA), its isomers (3-CQA and 4-CQA), and their dimeric forms (dicaffeoylquinic acids; 3,4-diCQA, 4,5-diCQA, and 3,5-diCQA), accounting for 70% of these components.[8]

In the next step different commercially available apple samples were tested with respect to their PPO activity and 5-CQA content (Supporting Information, Figure S3). The variety 'Braeburn' was found to be suitable for the planned experiments, having a high PPO activity (ca. 6262 U/L) and low 5-CQA content in its juice (ca. 77 mg/L).

The modification of β-lactoglobulin was conducted with a hot water extract from green coffee powder (*Coffea canephora* var. *robusta*), and the change induced in the composition and content at the beginning and after 24 h modification time is provided (Supporting Information, Figures S4 and S5). It was observed that quantitatively the main phenolic component converted was 5-CQA, whereas the most reactive hydroxycinnamic acid derivatives were the dicaffeoylquinic acids (diCQA).

On the protein side, the reaction proceeds with a decrease in the amount of free amino and thiol groups (Table 1). The *o*-quinones generated from the hydroxycinnamic acid derivatives are capable of undergoing a nucleophilic addition to these amino acid side chains, thereby covalently modifying the proteins. A rough evaluation of the extent of modification (covalent bound 5-CQA equivalents in nmol/mg protein) is possible by applying a RP-HPLC method, which also provides a valuable tool in assessing the quality of the modified β-lactoglobulins (Table 1). The results of these three parameters indicate that the alkaline conditions (ALG, Table 1) enforce a stronger interaction between the two reacting components. If pure 5-CQA is applied under alkaline conditions, it will isomerize more quickly and, with the advanced exposure to air, will produce CQA dimeric forms. If the mechanism proposed by Namiki and colleagues is followed,[28,29] a dimerization prior to the interaction with proteins under alkaline conditions seems to be precedent as documented for chlorogenic acid[27] and recently validated for the adduct formation with the amino group in a model system.[7,17] This modification leads consequently to an increase in the antioxidative capacity of the β-lactoglobulin (Table 1). The PPO modulated reaction provides a sample (PPO-LG) with a higher antioxidative power in this respect, despite the lower reactivity noted with the protein. An explanation of this observation can be hypothesized in the oxidized state of the reaction products in ALG. A covalent modification of proteins preferably takes place at the ε-

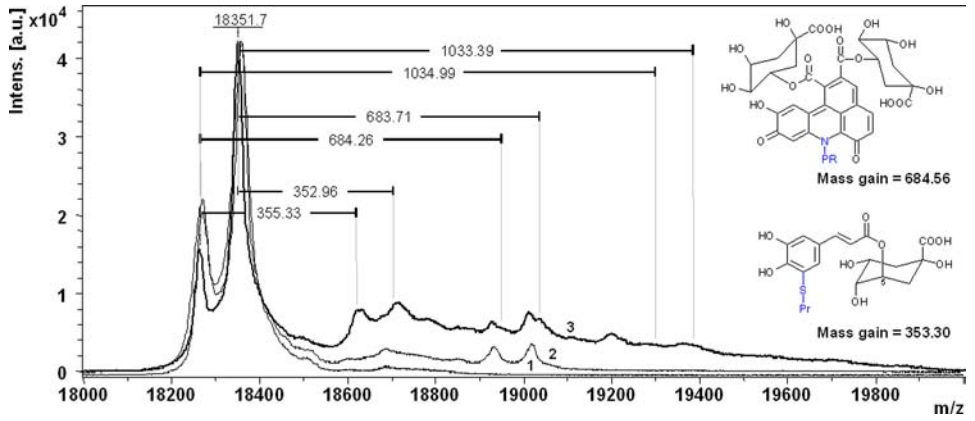

Figure 2. MALDI-TOF-MS spectra of the intact protein molecules and changes induced in the molecular weight distribution upon modification by CQA. Peaks: 1, ULG; 2, PPO-LG; 3, ALG.

amino group of lysine and thiol groups of cysteine with a prior conversion to a quinone as documented for 5-CQA in the presence of tyrosinase.[17,28,29] An adduct with lysine results in a benzacridine derivative as reported[28,29] and confirmed[17] with the aid of HPLC coupled with ESI-MS^n. The reduced form of the benzacridine derivative, containing a trihydroxy structural element, was found to be yellow, being very reactive with oxygen, yielding semiquinone and quinone type of products with characteristic green colors,[28,29] in agreement with observed coloration of the ALG.

The characterization of the molecular properties of β-lactoglobulin by SDS-PAGE (Supporting Information, Figure S7) and by MALDI-TOF-MS analysis (Figure 2) support the observed trend of reactivity, where the ALG appears to be more strongly modified by coffee phenolics. Thus, on the basis of these observations, a tentative list of possible adduct structures was derived (Supporting Information, Table S2), and proposed allocations based on intact protein MALDI-TOF-MS analysis of the CQA derivatives of β-lactoglobulin are provided for the sample ALG (Supporting Information, Figure S8).

To get more information on the type and site of β-lactoglobulin modification, the proteins were digested by trypsin. The peptides thus produced were analyzed with MALDI-TOF-MS to produce a peptide mass fingerprint, which was then submitted to a Mascot MS search in the data bank Swiss-Prot. The corresponding spectrum analysis reports are provided (Supporting Information, Figure S9). On the basis of this preliminary identification and comparison of the derived sequence with the available complete primary structure for β-lactoglobulin-bovine, variant A, in the protein knowledgebase (UniProtKB; http://www.uniprot.org/), the following candidate with the best match was found to be appropriate: P02754 (for sequence details see Supporting Information, Figure S10). Therefore, further analysis with regard to identification of the type and site of modification in the β-lactoglobulin with CQA was performed by applying this P02754 complete model sequence. The tentative classification of the modification art and sites is provided (Supporting Information, Figure S11 and S12, based on the proposed different types of modifications as proposed in Table S2 and Figure S8).

In summary, the peptide analysis shows that a few peptides are liable to modification (Table 1), and the sites of modification are ε-amino groups of lysine (K77, K91, K138, K47) and the thiol group of cysteine (C121). The allocation of the first three modification sites to peptides results in molecular weights that coincide with those that are also released by the tryptic digestion of the unmodified ULG. Therefore, their allocation can be predicted, but further MS/MS analysis is needed to confirm this result. The peptide fingerprints of both the modified β-lactoglobulin showed two new peptide peaks, which could not be allocated as products of tryptic digestion of ULG even when a higher number of missed cleavages (or partials accounting for tolerated internal missed cleavage sites in the matching peptides) was considered. These two peptides contain two sites (K47 and C121), which we may assume as modified, although a high mass tolerance (the peptide mass error) needed to be applied. Consequently, the nature of modification can be predicted to be a benzacridine derivative with lysine after dimerization of 5-CQA or the direct addition of diCQA and that with cysteine resulting in an adduct (Supporting Information, Table S2) as already reported.[17] The possibility that these two peptides may originate from coffee extract or apple juice can be discarded, because they were also found when the modification of β-lactoglobulin with pure 5-CQA was investigated under alkaline conditions (Supporting Information, Figure S12c).

From the sequence of P02754 (Supporting Information), it can be observed that two tryptophan residues, W19 and W61, are present. W19 is in an apolar environment and contributes 80% of total fluorescence, whereas W61 is partly exposed to the aqueous solvent and makes a minor contribution to tryptophan fluorescence.[30] This observation can be confirmed by the results for ULG (Table 1). The modification leads to a significant decrease and can be assumed to come from the interaction between CQA and β-lactoglobulin, because it remains low even in the presence of urea. If tryptophan side chains themselves are involved in the interactions remains unclear, because both peptides containing W19 and W61 were released by tryptic digestion of both modified PPO-LG and ALG (Supporting Information, Figures S11 and S12).

Effect on Structural Properties. ANS, known to bind to hydrophobic pockets on the protein surface, is a much-utilized fluorescent "hydrophobic probe" for examining the nonpolar character of proteins. It is also often used to study conformational changes in a given protein by probing its surface hydrophobic binding sites. The modification leads to a significant decrease of ANS fluorescence depending upon the mode of modification (Table 1; Supporting Information, Figure S14). PPO-LG exhibits a more hydrophilic surface property than both ULG and ALG. This also substantiates the

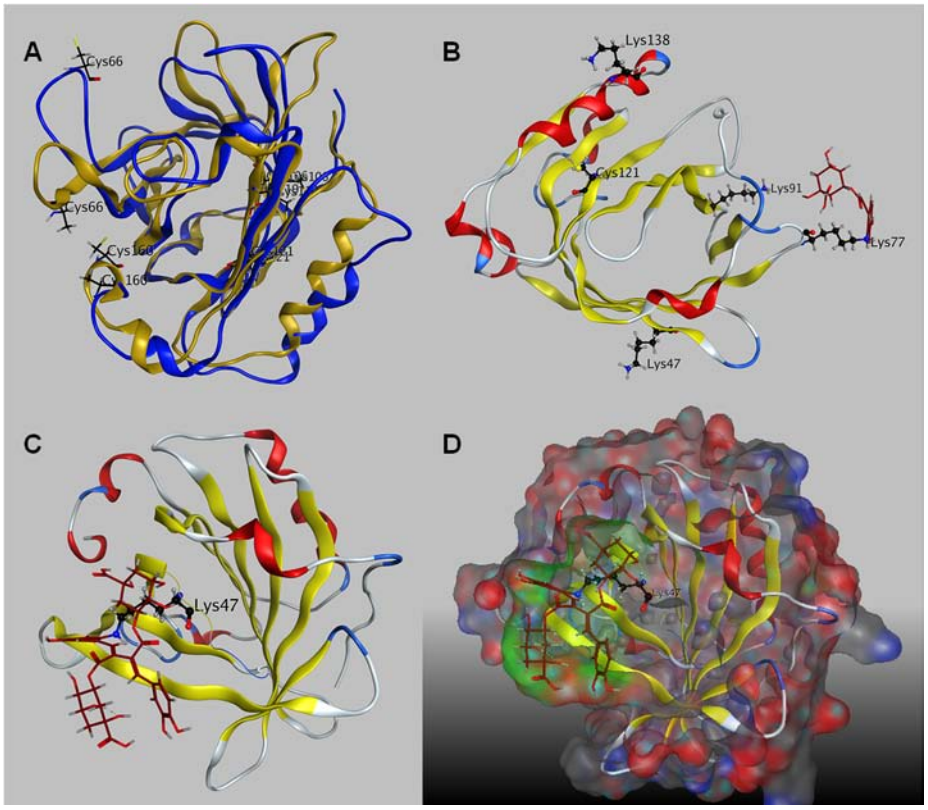

Figure 3. Molecular modeling of β-lactoglobulin: (A) disulfide/thiol exchange (C66−C160) as provoked by the presence of CQA leading to initial enfolding (blue protein conformation) of the initial protein molecule (gold protein conformation); (B) proposed sites of modification of ε-amino groups of lysine (K77, K91, K138, K47) and the thiol group of cysteine (C121) in β-lactoglobulin (the simulation also illustrates the modification at the lysine site K77); (C) modeling of CQA modification at the lysine site 47 in β-lactoglobulin; (D) modeling of CQA modification at the lysine site 47 in β-lactoglobulin (positions of the modifications in the monomer of 11s protein). The simulation shows the exposure of the charged molecular surface of the protein.

hypothesized oxidized state of the reaction products in ALG, the phenolic OH groups of the CQA adducts with β-lactoglobulin side chains not being available, and the hydrophobic vicinity of tryptophan (W19 and W61) being involved in the CQA−protein interactions.

Circular dichroism (CD) spectroscopy is a valuable method for examining the extent of structural changes in the proteins as a result of CQA binding. The estimation of the secondary structures according to CONTIN method showed that ULG contained 20.5% α-helix, 39.9% β-sheet, 15.2% turn, and 24.5% unordered structure elements. The protein conformational analysis based on CD data suggests that free β-lactoglobulin contains 12% α-helical, 58% β-sheet, 10% turn, and 20% random coil.[30] The difference could be explained by different experimental conditions and application of different methods for collection and evaluation of CD data. Additionally, the preparation of whey protein samples (drying conditions) might have also influenced the data produced. The CQA modification leads to significant changes in the secondary structures (Table 1; Supporting Information, Figure S13), the proteins becoming more unordered. It is also evident that interaction of CQA with β-lactoglobulin is accompanied by the corresponding loss of structured elements (α-helix and β-strand) and an increase in the amount allocated to the random coil fractions (Table 1).

The influence of the modification on the tertiary structure of β-lactoglobulin was also evaluated by means of a near-UV study (Supporting Information, Figure S13). The CD of proteins in the near-UV (310−240 nm) derives from transitions in the prosthetic groups. The variation of CD in this region can be used to monitor changes in conformation and local environment. These spectra highlight the conformational changes induced by the reaction with CQA, affecting the tertiary structure and perturbing the initial conformation of the β-lactoglobulin, the effect being stronger in ALG. Summing up, the CD analysis shows that the reaction of CQA with β-lactoglobulin induced microenvironmental and conformational changes.

To further study the thermostability of the modified β-lactoglobulins, DSC was applied. It is well-known that denaturation processes in proteins commonly take place in two steps, the first reversible, corresponding to the unfolding process, and the second irreversible, leading to the degradation of the unfolded protein molecule.[31] The DSC of ULG showed one transition with a peak temperature (T_d) at 75 °C and an enthalpy (ΔH) of 10.8 mJ/mg (Table 1). Upon modification with CQA, there is correspondingly an increase in T_d and a decrease in ΔH, both of these parameters being differently affected depending on reaction conditions during the modification process (Table 1). Intriguingly, whereas the modified β-lactoglobulins are thermally more stable, they required less energy to unfold than ULG. These results suggest that modification may have partially unfolded certain regions of LG, resulting in lowering the ΔH and conferring stability in other regions of the protein resulting in higher T_d.

To understand this structural behavior, it is necessary to consider the structure of β-lactoglobulin in more detail.[32] The

molecule consists of 162 amino acids folded into a compact globular conformation, stabilized by two intramolecular disulfide bonds, one inside the molecule (C106−C119) and one near the C-terminus (C66−C160).[32] Furthermore, it contains one free thiol group (at C121), which in the native molecule is buried in the hydrophobic part, but becomes exposed and more reactive on dissociation of dimers and/or partial unfolding of the molecule, for example, with increasing temperature.[33] We propose that the disulfide−thiol exchange in the C-terminus (C66−C160) may be initiated by the redox conditions provided in the presence of CQA. The protein structure thereupon becomes more disordered as simulated by molecular dynamic calculation (34 ns) results shown in Figure 3A. β-Lactoglobulin properties (Supporting Information, Table S3) computed after this simulation support the previously documented increase in the hydrophilic surface area from experiments applying ANS binding and tryptophan fluorescence (Table 1). This unfolding process may additionally be supported by the reaction of the CQA at the proposed sites of modification of ε-amino groups of lysine (K77, K91, K138, and K47) and the thiol group of cysteine (C121). The data thus correlate with the observed structural changes obtained for PPO-LG and under the progressed reactivity for ALG sample. The results of molecular modeling indicate the accessibility of these reaction sites as illustrated in Figure 3B. Further in the next step, the accessibility of two reaction sites (K47 and K77) was simulated by molecular modeling to emphasize the possibility of these modifications (for example, with the corresponding modification types CQA5 and CQA2 as well as considering the possibility of a further reaction of CQA5 to CQA1 at K47; Supporting Information, Table S2). Thereafter, the pharmacophores, describing the molecular features that are necessary for molecular recognition for a ligand to react in the vicinity of K47 and K77 in the β-lactoglobulin, were defined. On the basis of this definition, the modification of the sites was conducted, such that the docking distance of under 4 Å for different poses could be achieved. The results of these simulations confirm the possibility of the proposed reactions at K77 (Figure 3B) and K47 (Figure 3C). The simulation also shows exemplary the exposure of the charged molecular surface of the β-lactoglobulin after the modification of site K47 (Figure 3D).

Effect on Functional Properties. Solubility is a condition precedent for an ingredient protein and is critically necessary for functional products such as beverages. Whey proteins remain soluble during precipitation of casein at pH 4.4−4.6. Thus, the loss in solubility at this pH is commonly used to access the extent of protein denaturation.[22] The results illustrating the change in the pH-dependent solubility profiles of the CQA modified β-lactoglobulins are provided (Supporting Information, Figure S15). The ALG sample showed a decrease in solubility of ca. 20% at pH 4.4−4.6 documenting again a denaturation due to modification, whereas PPO-LG exhibited diminished solubility between pH 2 and 5.

Oil in water emulsions were prepared with modified β-lactoglobulin samples with the inclusion of lutein ester and their droplet size was measured subsequently. Good emulsifying properties of β-lactoglobulin were not significantly affected by the reaction of CQA, as documented by the characterization of emulsion with regard to the 10th and 50th percentiles of particles size $D(v, 0.1)$ and $D(v, 0.5)$ (Supporting Information, Figure S17). The 90th percentile of the particle size $D(v, 0.9)$ illustrated that considerable increase in size distribution could be observed upon alkaline modification as recorded for ALG (Supporting Information, Figure S17). Flocculation of droplets/aggregation was probably encouraged at the interface as a consequence of CQA modification. These emulsions were also studied by the microscopic imaging of the particles, which showed a normal distribution with a partial aggregation of oil droplets (Supporting Information, Figure S16), thereby providing a suitable vehicle for emulsified lutein esters.

Stability results (Figure 4a) revealed that in the emulsions centrifuged to provoke the formation of the serum layer, both

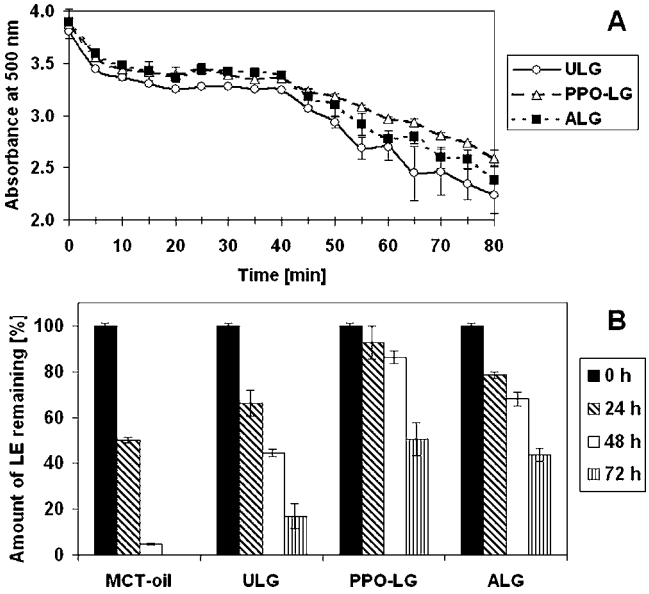

Figure 4. Change in the emulsion stability of the CQA modified proteins (A) and the change in the UV stability of lutein ester in protein emulsions as affected by CQA modification and time of UV exposure (B).

modified samples PPO-LG and ALG were better eligible in improving the quality of thus prepared emulsions. An instant in situ creaming, as most likely caused by flocculation and an instant oiling-off, did not occur in any emulsion, suggesting that strong viscoelastic β-lactoglobulin films were formed around oil droplets.

β-Lactoglobulin emulsified lutein esters with MCT oil exhibited a higher stability against UV light (Figure 4b) compared to lutein esters in MCT oil without emulsification. On the other hand, PPO-LG and ALG emulsified oil exhibited a moderate increase in the amount of lutein ester remaining after 24−72 h of UV exposure, emphasizing the fact that UV stability should also be given for long-term storage and usage of thus fortified food stuffs.

In conclusion, the PPO modulated reaction provides a modified β-lactoglobulin sample (PPO-LG) with a high antioxidative power, thermally more stable as reflected by a higher T_d, requiring less energy to unfold, and, when emulsified with lutein esters, exhibiting higher stability against UV light. Furthermore, MCT oil represents a good medium to protect lutein ester against temperature and emulsion with MCT oil against UV light, both being important factors when fortification issues in developing countries are considered, especially those with tropical and subtropical climates.[13] Proteolytic treatment of such phenol-modified proteins[18] also revealed that they can still be degraded, thus probably enabling

a quick release of the bioactive ingredient after the digestion of the protective/antioxidant mantle of the oil droplet. Furthermore, the derivatization of whey proteins with CQA does not lead to an additional deficiency in specific indispensable amino acids in growing rats fed 10% modified protein.[34] Only at a relatively high level of derivatization may the nutritional quality of whey proteins be affected.[34] Finally, as recently reported, the combined effect of binding of carotenoids to proteins and the process of emulsification may lead to an effective use of selected carotenoids in supplements, provided it is possible to optimize their bioavailability under these conditions.[13] Therefore, the PPO/CQA modulated reaction may also provide a tool to improve the stability and bioavailability of carotenoids while using such modified proteins in emulsions. Further experiments are also in progress to clarify these aspects.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary analysis data are provided on phenolic compounds in coffee beans used, on modification and structural characterization of the β-lactoglobulin, as well as on changes induced in protein functional properties. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ANS, 1-anilino-8-naphthalensulfonate; AUC, area under curve; CA, *Coffea arabica*; CD, circular dichroism; CQA, caffeoylquinic acids; CQA1-6, modifications in β-lactoglobulin, for details see also the Supporting Information; CR, *Coffea canephora* var. *robusta*; diCQA, dicaffeoylquinic acids; DSC, differential scanning calorimetry; FQA, feruloylquinic acids; MALDI-TOF-MS, matrix-assisted laser desorption ionization−time-of-flight−mass spectrometry; MOE, molecular operating environment; PPO, polyphenol oxidase; TEAC, Trolox equivalent antioxidant capacity assay; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; WP, whey proteins

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