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# Characterization and Modeling of the Interactions between Coffee Storage Proteins and Phenolic Compounds

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Supporting Information

**ABSTRACT:** This study addresses the interactions of coffee storage proteins with coffee-specific phenolic compounds. Protein profiles of *Coffea arabica* and *Coffea canephora* (var. *robusta*) were compared. Major phenolic compounds were extracted and analyzed with appropriate methods. The polyphenol−protein interactions during protein extraction have been addressed by different analytical setups [reversed-phase high-performance liquid chromatography (RP-HPLC), sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), matrix-assisted laser desorption ionization−time of flight−mass spectrometry (MALDI−TOF−MS), and Trolox equivalent antioxidant capacity (TEAC) assays], with focus directed toward identification of covalent adduct formation. The results indicate that *C. arabica* proteins are more susceptible to these interactions and the polyphenol oxidase activity seems to be a crucial factor for the formation of these addition products. A tentative allocation of the modification type and site in the protein has been attempted. Thus, the first available *in silico* modeling of modified coffee proteins is reported. The extent of these modifications may contribute to the structure and function of "coffee melanoidins" and are discussed in the context of coffee flavor formation.

**KEYWORDS:** *Coffee beans, storage proteins, phenolic compounds, antioxidants, protein−phenol interactions, modeling*

## INTRODUCTION

The coffee plant belongs to the Rubiaceae family and *Coffea* genus, which comprises more than 70 species. The two most commonly grown species are *Coffea arabica* (CA) and *Coffea canephora* var. *robusta* (CR).[1] Consumer preference is given to CA because of its sensory properties.[2] The crude protein content of green coffee beans ranges from 8.5 to 12% after being corrected for the content of caffeine and trigonelline.[3] The crude protein fractions thus represent proteins/enzymes, peptides, and free amino acids. These nitrogenous compounds contribute to the development of coffee flavor and quality during roasting as a result of the Maillard reaction.[4,5] Despite their obvious role in many chemical reactions during maturation, storage, and roasting, little is known about the coffee seed proteins.[6,7] A few studies have been carried out with CA, these especially with the water-soluble fractions, giving some indications of their importance in the coffee beverage quality.[8,9] The most abundant of these proteins are the legumin-like seed storage proteins of the 11S size class.[10] The typical structure of an 11S storage protein consists of 3−6 monomers, which migrate into storage vacuoles (protein bodies) and generate by hydrophobic interactions the tri- and hexameric quaternary forms, with molecular weights of 150−400 kDa.[11] Acidification of protein bodies appears to start the process of germination by affecting their quaternary structure. In this context, as a result of post-mortem reactions in re-imbibed coffee seeds, a characteristic bluish-green color develops,[12] which may putatively be attributed to some of the specificities of 11S-polyphenol adducts addressed in the present work. The rupture of the disulfide bonds in 11S monomers releases under reducing conditions the $\alpha$ (acidic) and $\beta$ (basic) subunits.[13] In CA, the 11S globulin monomers were identified with a molecular weight of 55 kDa and consisted of 33 kDa ($\alpha$) and 24 kDa ($\beta$) subunits.[10,14] CR contained an abundant protein monomer at 58 kDa, producing the corresponding two subunit fractions with 32−38 kDa ($\alpha$) and 20−22 kDa ($\beta$).[6,15] More detailed studies of the coffee seed legumin suggest the presence of different isoforms.[6,10,14]

Green coffee bean is also a major source of chlorogenic acid, an ester formed between caffeic and quinic acids, resulting in caffeoylquinic acid (CQA) with three isomers (3-CQA, 4-CQA, and 5-CQA), dicaffeoylquinic acid (diCQA) with three isomers (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA), and feruloylquinic acid with three isomers (3-FQA, 4-FQA, and 5-FQA). These are main derivatives of hydroxycinnamic acid (for structures, see Table S1 of the Supporting Information) found in coffee beans. Extensive literature exists on the composition of coffee phenolics, and more than 50 hydroxycinnamic acid derivatives have been recently identified.[16,17]

A series of complex reactions is responsible for the flavor development during the roasting of coffee beans. Some of them may be allocated to Maillard and Strecker reactions, degradation of proteins, polysaccharides, trigonelline, and chlorogenic acids.[5,18] However, the mechanisms leading to defined mixtures of free amino acids and peptides remain

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unknown. Recent work has proposed that the legumin-like seed storage proteins of the 11S size class may undergo fragmentation and contribute to this process.[7] Further, this process seems to be preceded by intensive interactions between the nitrogenous compounds and phenolic compounds (mainly CQAs)[3,7] The development of mass spectrometric methods has recently provided a more detailed biochemical characterization of the interactions of CQA and amino acids.[19,20] Therefore, as a result, it can be assumed that a unique complex combination of enzymatic and non-enzymatic browning processes (including autoxidation reactions to the formation of melanoidins) may lead to the typical distinct coffee flavor profile and antioxidative operating browning products.

Consequently, the aim of this study was to increase the knowledge on the composition and structure of green coffee bean proteins and to report on the changes induced in them especially with regard to their interactions with the phenolic compounds. Further, it was intended to optimize the protein extraction, characterize the modifications by CQA, and conceive the influence of such reactions on protein structure.

## MATERIALS AND METHODS

**Materials.** Different varieties of green coffee beans were represented from five different countries: Brazil (B), Guatemala (G), and Columbia (K) represented *C. arabica* (CA), whereas samples from Uganda (U) and Indonesia (I) represented *C. canephora* var. *robusta* (CR). Coffee beans from Columbia were provided by the kind courtesy of Deutsche Extraktkaffee GmbH (DEK GmbH, Berlin, Germany). All other samples were commercially obtained (Rohkaffee-Company, Berlin, Germany) and stored at −20 °C. Fava beans (FBs), two varieties *Vicia faba* var. Giza 3 and *V. faba* var. Sakha 3, were obtained from the Agriculture Research Center, Institute of Field Crops, Egypt. The frozen beans were milled (particle size ≤ 0.4 mm) using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany), equipped with a 24-tooth rotor and a ring sieve having 0.4 mm holes. The milling was conducted at a speed of 15 000 rpm, and all of the powdered samples were freezed at −20 °C until analysis. 5-CQA was commercially obtained (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). All of the other chemicals and high-performance liquid chromatography (HPLC) solvents were reagent- or gradient-grade, respectively.

**General Parameters.** The moisture content of the samples was determined by a moisture meter (Sartorius MA30, Gottingen, Germany). The total crude protein content (using 5.75 as a factor) was determined using a semimicro Kjeldahl unit (Vapodest 30, Gerhardt, Bonn, Germany). The total phenolic content was estimated using the Folin−Ciocalteau procedure.[21] The quantification was performed with an external calibration with diluted standards using chlorogenic acid (10−80 μg/mL).

**Determination of the Antioxidative Capacity.** Trolox equivalent antioxidant capacity (TEAC) assay[22] was applied with a few modifications, using a fluorescence microplate reader (Fluostar Optima, BMG LABTECH, Ortenberg, Germany). Results were expressed as millimolar 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox, Sigma Aldrich) equivalents (TE).

**Determination of the Polyphenol Oxidase (PPO) Activity.** PPO was extracted,[23] and its activity was determined on basis of the formation of a pink proline−catechol adduct recorded at 525 nm ($\varepsilon$ = 1550 L mol$^{-1}$ cm$^{-1}$).[20] PPO activity was calculated (after appropriate blank corrections) from the slope within the initial linear range of the absorbance−time curve. The enzyme activity was expressed in units/100 g of sample.

**Extraction of Coffee Storage Proteins.** Green coffee proteins and FB proteins (reference proteins) were extracted using different solvents and additives,[24,25] with some modifications: 2 g of frozen GC flour and 1 g of polyamide or polyvinylpolypyrrolidone (PVPP) applied as additives were placed in a 50 mL centrifuge tube, except FB flour, where the extraction without these additives was performed. Then, 20 mL of different solvents [0.04% ascorbic acid and 0.04% acetylcysteine in distilled water and 0.04% ascorbic acid in 0.03 M tris(hydroxymethyl)aminomethane−HCl at pH 8] was added. All samples were shaken at room temperature for 3 h. Extracts were centrifuged at 4000*g* for 20 min, and after that, the clear supernatants were carefully removed, followed by dialysis against distilled water for 24 h at 4 °C, and freeze-dried. Proteins were stored at −20 °C until analysis.

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE).** SDS−PAGE (14% T gel)[3] was applied for molecular weight determination. The band intensity was estimated using densitometer scanning with Quantity One software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy). The sample buffer applied was 0.05 M Tris-HCl buffer at 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 general treatment was to denature the proteins by heating at 95 °C for 3 min prior to analysis.

**Covalent Bound Chlorogenic Acid.** To calculate the amount of chlorogenic acid attached to the proteins, 4 mg of protein was dissolved in 1 mL of 8 M urea solution followed by precipitation of protein with 20% trichloracetic acid. After this treatment, the precipitate was redissolved in 1 mL of 8 M urea. Reversed-phase high-performance liquid chromatography (RP-HPLC) was conducted with a Shimadzu 10A system (Kyoto, Japan) with a column Perfectsil C18 300 ODS, 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; and 10% B, 7 min. The run time was 42 min. A total of 50 μL was injected. External calibration was conducted using 5-CQA at 325 nm.

**Surface Hydrophobicity.** Using 1-anilino-8-napthalensulfonate (ANS),[26] fluorescence was recorded with a JASCO fluorescence detector using excitation at 390 nm (slit of 18 nm) and emission between 390 and 900 nm (slit of 40 nm). The initial slope ($S_o$) of the fluorescence intensity versus soluble protein concentration was used as an index of the protein surface hydrophobicity.

**Identification of Reaction Sites and Types of Modification in Coffee Beans.** Exemplary, the powdered coffee (50 mg) or the partly purified and extracted storage proteins (2 mg) from CA (Brazil) and CR (Indonesia) were dissolved in 1 mL of SDS−PAGE sample buffer, and 10 μL was separated by electrophoresis. SDS−PAGE was preformed with 14% T gel, as described above. The proteins were stained with a colloidal Coomassie Brilliant Blue G 250 (Serva, Heidelberg, Germany) solution. Protein bands of interest were excised and exposed to a series of treatments, destaining buffers, reduction, and alkylation reagents. The treatments were conducted according to producer instructions, as described in the "In-Gel Tryptic Digestion Kit" (Pierce Biotechnology, Inc., Rockford, IL). The digestion was conducted at 30 °C for 16 h (overnight). The proteolytic digests were dissolved in 25 μL of digestion buffer (25 mM ammonium bicarbonate, Pierce Biotechnology, Inc., Rockford, IL). A saturated matrix solution (α-cyano-4-hydroxycinnamic acid, HCCA, Bruker Daltonik GmbH, Bremen, Germany) was prepared in 30:70 (v/v) acetonitrile/0.1% trifluoracetic acid in water. Premix equal volumes (for example, 2 μL each) of sample solution and matrix solution were analyzed. A total of 0.5 μL of this mixture was applied to a steel target for mass spectrometry (MS) analysis. The matrix-assisted laser desorption ionization−time of flight−mass spectrometry (MALDI−TOF−MS) analysis of the samples was performed on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH, Bremen, Germany) 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, Bremen, Germany). The data analysis was performed using the software packet: Bruker Daltonics FlexAnalysis (version 3.3, Bruker Daltonik GmbH, Bremen, Germany). A sequence database search using the $m/z$ values of the digested peptides was

Table 1. Content of Dry Matter, Protein, and Selected Constituents in Coffee Beans: Characterization of Relevant Bioactivities

| | CA | | | | CR | |
|---|---|---|---|---|---|---|
| | Columbia | | Brazil | Guatemala | Uganda | Indonesia |
| parameter | green CA-GB-C | roasted CA-RB-C | green CA-GB-B | green CA-GB-G | green CR-GB-U | green CR-GB-I |
| DM | 92.3 ± 0.5 | 96.7 ± 0.6 | 89.8 ± 1.0 | 88.1 ± 0.5 | 91.2 ± 0.1 | 89.0 ± 0.9 |
| protein N[a] | 11.0 ± 0.2 | 12.0 ± 0.1 | 11.0 ± 0.1 | 9.5 ± 0.1 | 12.0 ± 2.3 | 10.3 ± 0.1 |
| caffeine[a] | 1.2 ± 0.0 | 1.5 ± 0.1 | 0.9 ± 0.0 | 0.8 ± 0.0 | 1.5 ± 0.0 | 1.6 ± 0.1 |
| total phenolics[a] | | | | | | |
| Folin | nd[b] | nd | 4.1 ± 0.1 | 3.6 ± 0.2 | 7.8 ± 0.1 | 6.9 ± 0.5 |
| HPLC | 5.5 ± 0.2 | 2.0 ± 0.1 | 4.3 ± 0.0 | 4.0 ± 0.1 | 7.8 ± 0.2 | 7.0 ± 0.1 |
| antioxidative capacity[c] | | | | | | |
| TEAC | 22.6 ± 1.4 | 34.4 ± 0.7 | 11.4 ± 0.5 | 10.2 ± 0.3 | 18.0 ± 0.7 | 18.5 ± 0.2 |
| PPO activity[d] | nd | nd | 291.8 ± 39.2 | 159.9 ± 5.9 | 104.7 ± 0.9 | 107.3 ± 17.0 |

[a]Composition in g/100 g of DM. [b]nd = not determined. [c]Antioxidative capacity in mmol of TE/100 g of DM. [d]Activity in units/100 g of DM.

performed with Bruker Daltonics BioTools (version 3.2, Bruker Daltonik GmbH, Bremen, Germany) combined with the MASCOT search program (Matrix Science, Ltd., London, U.K.).[27] SwissProt 2011_06 (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+top; http://www.uniprot.org/) and Plants_EST (http://www.plantgdb.org/search/) databases were applied. Dependent upon the database, individual protein scores defined as significant were applicable ($p < 0.05$). On basis of the sequence information available, further analysis of the data was performed using the software "Sequence editor" (version 3.2, Bruker Daltonik GmbH, Bremen, Germany) for coffee storage proteins with sequence retrieved from SwissProt for p93079_cofar 11S storage globulin. The following parameters were considered during the search: fixed modifications, such as reductive disulfide cleavage and carbamidomethylation, i.e., reaction with iodoacetamide, mass tolerance MS (the peptide mass error) of 100 ppm, and number of missed cleavages (or partials accounting for tolerated internal missed cleavage sites in the matching peptides). The last option was according to the best results obtained (generally two partials were applied). Sequence coverage of at least 20% of the digested proteins was set as an internal quality parameter. Analysis of optional modifications by CQA was based on the covalent reaction of lysine with 5-CQA[20] and those theoretically possible. A compilation of this data is supplied (see Figure S17 of the Supporting Information).

**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: Molecular Operating Environment (MOE) 2010.10,[28] Gaussian 09w,[29] and Molegro 5.0.[30] Both MOE 2010.10 and Yasara 12.01[31−33] were used for homology modeling of the 11S storage globulin. The general methodology for the modeling of the 11S storage protein is supplied (see Figure S35 of the Supporting Information).

**Homology Modeling.** Templates applied for the modeling were X-ray structures deposited in the Protein Data Bank (PDB) under the PDB codes 3FZ3-B-D, 2D5F-B, and 3C3V-A (monomer) and 3KSC, 2E9Q, and 1UD1 (trimer). The structures were prepared for homology modeling by adding hydrogen atoms and partial charges to the peptides using the Protonate3D application of MOE 2010.10. In Yasara, the homology modeling macro in the standard configuration was used for the modeling. The consensus models were than finally built with MOE and Yasara by applying the homology macro. This consensus model was refined with a molecular dynamics (MD) simulation. The stereochemistry quality aspects of the resulting model were checked using MOE.

**Optimization of the Ligand Structure.** The initial structures of different CQAs were built using ChemOffice Suite 2010 and transferred to a MOE database. Molecular mechanical energies of the different conformers of each ligand were minimized until a root-mean-square deviation (rmsd) of 0.01 kcal/mol was reached. Energy minimization was performed using the MMFF94 force field option,[38] with the restriction to preserve original chirality of the molecules. The resulting structures were finally optimized with Gaussian09W A02 (Level of Theory HF/6-31).

*Docking.* The docking experiments were performed with MOE and Molegro. These two different docking environments were used to compare the results for validity. With respect to the position of a ligand to a reactive amino acid, rotations of the whole molecule as well as rotations around single bonds were allowed with the restriction that the original configuration would still be preserved. In Molegro, a search space around the reactive amino acid was defined as a sphere having a 15 Å diameter. In the program MOE, a pharmacore (aromatic phenyl reactant) was defined in the vicinity of the reactive amino acid side chain.

**Statistical Analysis.** The experiments were generally conducted at least 3 times. All data are expressed as means of their standard deviations. The results were analyzed using SPSS statistical software (SPSS, version 18), where applicable. Values of $p < 0.05$ or $p < 0.01$ were considered statistically significant.

## RESULTS AND DISCUSSION

**General Parameters.** The general characterization data of green coffee beans is provided in Table 1. The moisture content of the ground green coffee beans ranged from 8.9 to 11.9%. CA and CR species have total protein content in the range of 9−12% on a dry matter (DM) basis. Total phenolic compounds as determined by the Folin−Ciocalteu method using 5-CQA as a standard are 3.6−5.5% (CA) and 6.9−7.8% (CR) on a DM basis. The comparison between CA and CR beans shows typical phenol profiles to be present in each case, with clear differences in their amounts (typical chromatograms, identification parameters, and distribution in percentage are provided in the Supporting Information). The TEAC assay was used to determine the total antioxidative capacity of the coffee bean extracts, and the results are shown in Table 1. The antioxidative capacity for 5-CQA (0.9−1.4 mmol of TE/mmol) agrees with those described elsewhere (1.15 mmol of TE/mmol of CQA).[34] The highest TEAC values were obtained for di-CQA, where 4,5-di-CQA appears to be the most active component. Thus, knowing the individual TEAC values of the analyzed CQA fractions, it was possible to estimate their individual contributions to the total antioxidative capacity determined (see Figure S3 of the Supporting Information). PPO is a copper-containing enzyme responsible for hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. The results (Table 1) show that both CA varieties have higher activities, resulting in significant differences to CR ($p \leq 0.05$).

**Characterization of Interactions of CQA with Model Amino Components.** Recent studies[3,6,7,19] show that coffee

proteins are liable to undergo interactions with CQA during maturation, processing, and storage of coffee beans as well as during their extraction. Principally, noncovalent and covalent types of interactions have been reported. Three potential modes of noncovalent interactions of hydroxycinnamates and proteins have been suggested: hydrogen, hydrophobic, and ionic bonding.[19] The CQAs are also susceptible to both enzymatic and non-enzymatic oxidation in the presence of oxygen. In both cases, the subsequent oxidation of these to reactive and redox-active *o*-quinones appear to be necessary to generate electrophilic species capable of undergoing a nucleophilic addition to proteins.[19]

**Covalent Reaction with Lysine.** An adduct formation of 5-CQA preferably occurs with the $\varepsilon$-amino group of lysine and/or with thiol groups of cysteine.[20,35,36] The reaction of lysine and N-Boc-lysine with 5-CQA in a model system[20] (see Figures S4−S16 of Supporting Information) initially leads to the formation of 3-CQA and 4-CQA, with oxidation giving rise to the formation of a dimer, which subsequently forms an adduct with lysine to finally result in a benzacridine derivative, as reported[35,36] and confirmed[20] with the aid of HPLC coupled with electrospray ionization (ESI)−$MS^n$. Our results show that the reaction can be modulated through the loss of CQA and the amount of free amino groups depending upon the reaction time, pH, incubation temperature, and amount of tyrosinase applied (see Figures S6−S16 of the Supporting Information). The optimal conditions for the conversion as assessed by both the loss of CQA and free amino groups of lysine can be given at pH 7 and 25 °C, with the conversion increasing with the incubation time and also depending upon the amount of tyrosinase present. The maximum loss of CQA occurs at pH 9 (mainly because of non-enzymatic oxidation processes) and pH 7 and 25 °C (mainly because of enzymatic conversion). The reaction has only a slight effect on the antioxidative capacity as exemplarily documented depending upon the reaction time (see Figure S8 of the Supporting Information).

The HPLC−MS analysis data for incubation of lysine and N-Boc-lysine with 5-CQA showed a large number of intermediary substances of low intensities, where among others, a $[M - H]^+$ ion at $m/z$ 826 corresponding to a benzacridine derivative with lysine and a $[M - H]^+$ ion at $m/z$ 947 corresponding to an adduct of a CQA dimer with N-Boc-lysine could be confirmed (see Figure S16 of the Supporting Information). Thus, on the basis of these results and those supplied by other groups,[20,35,36] a tentative list of possible adduct structures was derived (see Figure S17 of the Supporting Information).

**Extraction and Characterization of Coffee Proteins.** The first step involved the optimization of the protein extraction using various solvents/additives with the least possible modification of the proteins. The protein samples thus obtained were evaluated on basis of their color, electrophoresis data, and amount of covalently bound CQA.

**Coloration of the Extracted Proteins.** The color ranged from light yellow/cream white to dark green (see Figures S18−S20 of the Supporting Information). It has been shown that the principle structure of green and yellow pigments resulting from the reaction of CQA with an amino compound under aeration in alkali was a semi-quinone-type radical compound of the trihydroxy benzacridine derivative.[35] On the other hand, brown-colored pigments generally result from enzymatic (polyphenol oxidase-modulated) oxidation of CQA and their reaction with proteins. A treatment of acetylcysteine and ascorbic acid in the presence of polyamide delivered dark brown products, suggesting an extensive oxidation of the phenolic compounds in the extracted proteins. According to the results obtained, the lightest cream-colored products were obtained by combining ascorbic acid with PVPP, instead of polyamide. These results also seem to depend primarily upon the amount of PPO activity in the coffee beans. The protein samples obtained from CR beans were a shade lighter than those from CA (see Figure S20 of the Supporting Information).

**Electrophoresis Data.** Protein bands with an apparent molecular mass of 57 ± 3 kDa were predominant in samples of CA and CR extracted under reducing conditions (see Figure S21 of the Supporting Information). These results are in agreement with previous studies.[9,10] Further, marked differences in the electrophoretic patterns were also noted between the samples of CA and CR. Proteins of CA were more aggregated and delivered high molecular fractions, whereas the extracts of CR showed a higher abundance of low-molecular-weight fractions, with part of the $\alpha$ chain being liberated during the extraction conditions applied (see Figure S21 of the Supporting Information). Differences in the molecular weight distribution between different schemes of protein extraction were also observed under reducing conditions (see Figure S22 of the Supporting Information). Protein bands at lower apparent molecular masses were found (33.9 ± 0.3 and 24 ± 0.2 kDa), in line with previous studies.[13] Using PVPP instead of polyamide improved the quality of the protein obtained; the best conditions were with the use of PVPP in the presence of the antioxidant ascorbic acid. Both PVPP and polyamide are known to bind phenolic compounds, and the presence of ascorbic acid helps in preventing the oxidation of CQA as well as in inhibiting the indigenous PPO activity.[37] Among such type of inhibitors, which principally affect the active site for the phenolic substrate, the aromatic carboxylic acids of the benzoic and cinnamic series have been widely studied.[37]

**Determination of CQA Bound.** A RP-HPLC method was used to estimate the amount of CQA covalently bound to the extracted proteins. The CQA liberated, and the remaining coffee protein adduct showed similar ultraviolet−visible (UV−vis) scans, both absorbing at the same wavelength (325 nm) but eluting at different retention times (panels A and B of Figure 1). The more hydrophobic coffee protein elutes at 30 min, agreeing with the retention data obtained from similar 11S storage proteins from soy bean or FB. The proteins from soy bean or FB did not show any relevant absorption at 325 nm (data not shown). Therefore, using an external 5-CQA calibration curve, this property was used to estimate the amount of CQA attached covalently in the coffee protein adduct. Although this approach does not quantify the exact amount of CQA bound because of the unknown nature and absorbance behavior of the covalently bound CQA moiety/reaction products to proteins, the method still permits a rough evaluation of the extent of modification and provides a valuable tool in assessing the quality of the extracted proteins. The results showed that, although the extracted coffee proteins were exhaustively dialyzed against distilled water, some CQA still remains noncovalently bound to the coffee proteins and is released only after the treatment with urea, precipitation of proteins, redissolving, and consequent chromatographic separation. This, thus liberated CQA, amounted to 0.55−1.2 $\mu$g/mg of protein (Figure 1C), with the value being slightly higher for the proteins extracted from CR. On the other hand, the amount of CQA estimated to be firmly involved in the adduct formation with the proteins was in the range of 1.97−3.56 $\mu$g/mg of

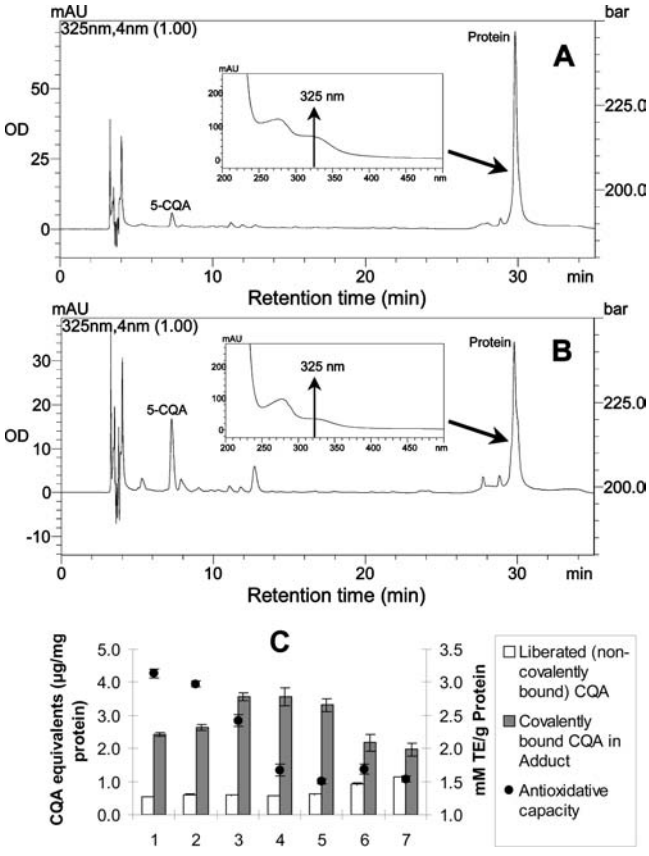



**Figure 1.** RP-HPLC for the extracted coffee proteins and their corresponding UV−vis spectra: (A) coffee beans from Brazil and (B) coffee beans from Indonesia. (C) Amount CQA equivalents and antioxidative capacity of the coffee proteins extracted with different additives and from different coffee types. Legend: 1, CA-B proteins extracted with acetylcysteine in the presence of polyamide; 2, CA-B proteins extracted with ascorbic acid in the presence of polyamide; 3, CA-B proteins extracted with Tris-HCl at pH 8 in the presence of polyamide; 4, CA-B proteins extracted with ascorbic acid in the presence of PVPP; 5, CA-G proteins extracted with ascorbic acid in the presence of PVPP; 6, CR-I proteins extracted with ascorbic acid in the presence of PVPP; and 7, CR-U proteins extracted with ascorbic acid in the presence of PVPP.

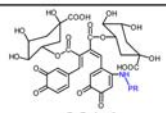
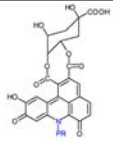
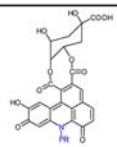
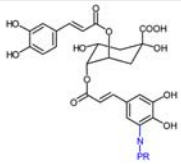
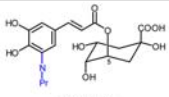
protein (Figure 1C), with the amount being significantly (2 times) higher for the proteins extracted from CA. The data imply that approximately 0.3−0.6 $\mu$mol of CQA/$\mu$mol of the monomer of the coffee 11S protein (on the basis of the molecular weight of 60 kDa) is involved in adduct formation. These results again reflect the role of the PPO activity in the beans for the adduct formation with coffee proteins, with the amount of available CQA playing only a secondary role. The corresponding PPO activity and total CQA determined for the two coffee types are provided (Table 1). Interestingly, the proteins extracted with Tris-HCl at pH 8 in the presence of polyamide gave the same amount of CQA bound, suggesting approximately the same degree of their modification (samples 3 and 4 in Figure 1C). The reduced form of the benzacridine derivative involving a trihydroxy structure derivative was found to be yellow, being very reactive with oxygen, yielding semiquinone and quinone types of products with characteristic green colors.[35,36] The use of acetylcysteine and ascorbic acid in the presence of polyamide showed lower amounts of CQA covalently bound (samples 1 and 2 in Figure 1C) but also delivered dark brown products, suggesting an extensive oxidation of the phenolic compounds contained in the extracted proteins.

The antioxidative capacity of the extracted coffee proteins (mM TE/g) is shown in Figure 1C. There are no significant differences at $p \leq 0.05$ between CR and CA. The protein extraction regime influences the antioxidative capacity considerably, with the treatment of acetylcysteine and ascorbic acid in the presence of polyamide leading to the highest activity documented. Under consideration of the dark pigmentation of these protein products (see Figure S19 of the Supporting Information) and the observed low amount of CQA bound (Figure 1C), it can be proposed that these samples are likely to contain products resulting from progressed PPO-modulated oxidation of CQA. Proteins extracted with Tris-HCl at pH 8 in the presence polyamide were also darkly pigmented and are thus also likely to contain similar potentially antioxidative operating reaction products. It seems that, during the extraction, a series of CQA derivatives and their oxidized products become noncovalently attached[19] to the surface of the coffee protein molecules. To prove this assumption, the extracted coffee proteins were tested with respect to the nature of their surface, following the binding of a hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS). ANS is known to bind to hydrophobic pockets on the protein surface. The enhancement of fluorescence because of the binding of ANS was determined and compared to model 11S proteins obtained from FBs. The data showed (see Figure S23 of the Supporting Information) that the coffee proteins were not capable of binding any ANS, thus exhibiting a very hydrophilic protein surface. The FBs in comparison exhibited the customary high binding of ANS. Therefore, it also remains unclear if this surface blanketing of the coffee proteins (micelle formation) via noncovalent binding of CQA derivatives is a result of the protein extraction or their typical status in the beans. This behavior would also explain our failure to analyze the intact coffee proteins using MALDI−TOF−MS, where the proteins need to be ionized but the blanketing CQA surface probably prevents their ionization.

**Tentative Modeling of the Modification.** To obtain some more information on the type and site of adduct formation in the coffee proteins, the coffee proteins were directly extracted from the coffee meal and, consequently, separated by electrophoresis. The bands of interest ($\alpha$- and $\beta$-polypeptide chains of the coffee 11S proteins) were excised, treated as described in the Materials and Methods, and finally digested by trypsin (see Figures S24−S25 of the Supporting Information). 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 databank Plant_Est. The corresponding spectrum analysis reports are provided (see Figures S26, S28, S30, and S32 of the Supporting Information). On basis of this preliminary identification and comparison of the derived sequence to the available complete primary structure for coffee 11S storage proteins in the protein knowledgebase (UniProtKB; http://www.uniprot.org/), the following candidate with the best match was found to be appropriate: P93079 (P93079_COFAR; for sequence details, see Figures S27, S29, S31, and S33 of the Supporting Information). Therefore, the further analysis with regard to identification of the type and site of modification in the protein with CQA was performed applying this P93079 complete model sequence belonging to the 11S seed storage

protein (globulin) family. The tentative classification of the modification art and sites is given in Table 2 (on the basis of

**Table 2. Proposed Types and Sites of Modification in Coffee 11S Storage Proteins**

| Protein | Modification type | Modification site | Peptide |
|---|---|---|---|
| α-polypeptide chains from coffee beans from Brazil (*C. arabica*) |  CQA-6 | K34<br>K41<br>K204 | 31-50<br>35-50<br>204-217 |
| |  CQA-4 | K217 | 204-228 |
| β-polypeptide chains from coffee beans from Brazil (*C. arabica*) |  CQA-4 | K91 | 89-98 |
| |  CQA-3 | K114 | 114-126 |
| α-polypeptide chains from coffee beans from Indonesia (*C. robusta*) |  CQA-2 | K155 | 153-158 |
| β-polypeptide chains (*C. robusta*) | none | none | none |

the proposed different types of modifications; see Figure S17 of the Supporting Information). The isolation/extraction of proteins may lead to further modification of the proteins depending upon the extraction conditions applied (see Table S3 of the Supporting Information). It appears that the storage proteins from CA beans are more intensively involved in covalent interactions with CQA, agreeing with data on amounts of CQA bound (samples 4 and 5 in Figure 1C). Further, as shown in Table 2, the CQA modification of coffee storage protein also proceeds with a preferred reaction at the $\alpha$ chain, whereas the $\beta$ chain of the 11S proteins remains more or less intact. To understand this behavior, it is necessary to consider the structure of 11S storage proteins in more detail. The 11S storage proteins of legumes are built up of polymorphic subunits encoded by multigene families.[3,38] They form, however, regular quaternary structures, hexameric in the case of legumin-like proteins, whose association−dissociation behavior and three-dimensional and surface structure as well as the conformational stability are most important for understanding the functionality of these proteins.[3,38] It is the non-specific hydrophobic interaction of the 50−60 kDa monomers that allows them to assemble in a legumin-like quaternary structure.[3,38] The two disulfide-bridged polypeptide chains of these subunits fulfill different functions in stabilizing the globular structure.[3,38] The alignment of 11S storage proteins from different sources and the S−S bridge connecting the $\alpha$ chain to the corresponding $\beta$ chain is provided (see Figure S38 of the Supporting Information). However, the most important fact is that the strong hydrophilic C-terminal region of the $\alpha$ chain is located at the surface of the protein molecule and protects the structural domains from the solvent. It is therefore of high importance not only for the solubility and interfacial properties of 11S globulins but also seems to be the preferred reaction site for chlorogenic acid.[3,38] The limited tryptic hydrolysis of 11S globulins results in the splitting of the surface-exposed regions of $\alpha$ chains, while the $\beta$ chains located in the inner part of the protein molecules remain intact,[38] thus supporting the observations documented in Table 1. Therefore, in the next step, the accessibility of two reaction sites (Lys 34 and Lys 139) was simulated by molecular modeling to underline the possibility of these modifications (exemplary with the modification types CQA2 and CQA5 as well as considering the possibility of a further reaction to CQA1; see Table S3 of the Supporting Information).

The general methodology for the modeling of the 11S storage protein, template searching, 3D model building and validation, and model refinement is provided (see Figures S34−S38 of the Supporting Information). Thereafter, the pharmacore in the vicinity of Lys 34 and Lys 139 in the 11S coffee protein was defined (see Figure S39 of the Supporting Information). On 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 (see Figure S40 of the Supporting Information). The results of these simulations confirm the accessibility of the two reaction sites Lys 34 and Lys 139 and are illustrated in panels A and B of Figure 2. The modeling of the two modification sites in the monomer of the coffee 11S protein is given in Figure 2C. Simulations showing the exposure of the charged molecular surface of the protein based on the molecular electrostatic potential and the surface accessibility of the trimer of 11S coffee protein with the corresponding modifications are provided (see Figures S41 and S42 of the Supporting Information).

In conclusion, this study demonstrates the occurrence of covalent reactions between CQA and coffee bean storage proteins. The diversity of the different CQA derivatives present in green coffee beans[16] complicates the series of reactions occurring, providing a broad palette of reaction products. Further work will focus on applying more sensitive $MS^n$ and nuclear magnetic resonance (NMR) techniques to identify these adduct formations. The most reactive and susceptible protein fractions to covalent reactions are the $\alpha$ chains. These reactions are likely to be dependent upon maturation, post-harvest processing, and storage,[3,6,7] as well as the coffee type, as shown in this study. The reactions are more progressed in CA than in CR. The key mechanism appears to be governed by the PPO activity, which modulates these interactions and not the amount of CQA derivatives present. In fact, the PPO activity appears to increase with maturation, while catalase activity decreases.[39] Further, strong differences in catalase activity between CA versus CR have also been identified.[40] These interactions are not unique to coffee or to 11S (e.g., sunflower proteins show similar behavior[19]), and if these adducts are formed during maturation (protein synthesis) or only later

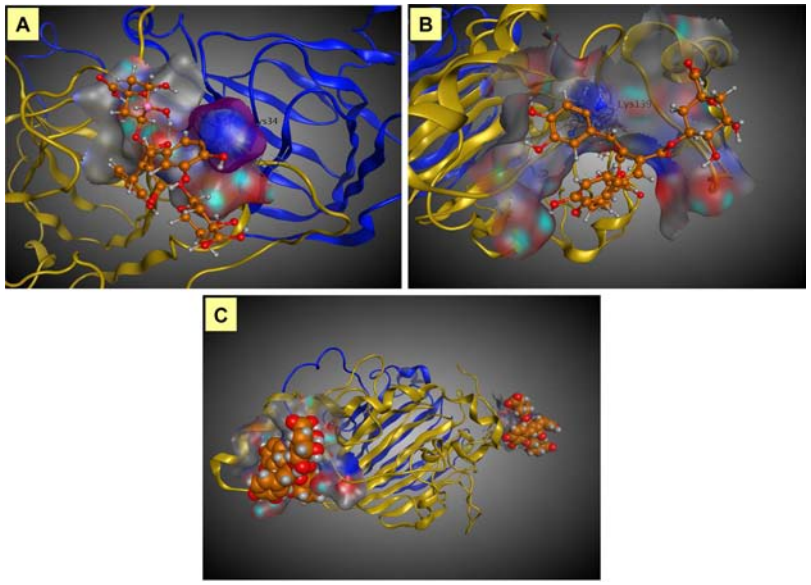



**Figure 2.** Modification of the lysine sites 34/139: (A) modeling of CQA modification at the lysine site 34 in the 11S coffee protein and (B) modeling of CQA modification at the lysine site 139 in the 11S coffee protein. (C) Positions of the modifications in the monomer of 11S coffee protein.

during, e.g., post-harvest processing or storage or extraction (11S are segregated in protein vacuoles, which may contain the specific enzymes required[11,12]), they still need to be clarified. The interactions may be intensified during coffee roasting.[7] Further, a fragmentation cascade involving the 11S coffee storage protein was identified during roasting and incubation of green coffee suspensions at 37 °C.[7] Here again, the most susceptible protein fraction is likely to be the $\alpha$-chain fraction, as shown by the application of the *in vitro* tryptic digestion.[3] Coffee aroma development and coffee browning are essentially considered to be consequences of Maillard reactions, which also involve the contribution by the fragments from 11S coffee storage protein. Further, this would explain a part of unidentified constituents with antioxidative capacity produced during roasting of coffee beans. The presence of such adducts in coffee beverages is likely (partly integrated in the "coffee melanoidines"), and first detailed studies in this context have been initiated.[5] From these observations, it can be deduced that one of the factors for coffee quality may be identified as the extent of the coffee storage protein modifications by the CQA derivatives. Indeed, coffee beans from Kopi luwak are one of the world's most expensive varieties of coffee. They are prepared from the beans of coffee berries that have passed through the digestive tract of the Asian Palm Civet (*Paradoxurus hermaphroditus*) and other related civets. This treatment would not only provoke a partial digestion of the storage proteins but also provide more sites for further reaction of CQA with them, with their roasting yielding a much more aromatic coffee with less bitterness. Further experiments are directed to investigate the proteins from these beans in more detail and to confirm this hypothesis.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supplementary analysis data on phenolic compounds in coffee beans, on interactions with amino acids, on extraction and characterization of coffee proteins, and on adduct formation and modeling strategy. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

ANS, 1-anilino-8-napthalensulfonate; CA, *Coffea arabica*; CQA, caffeoylquinic acid; CQA1−CQA6, modifications in coffee proteins; for details, also see the Supporting Information; CR, *Coffea canephora* var. *robusta*; diCQA, dicaffeoylquinic acid; FQA, feruloylquinic acid; FB, fava bean; MALDI−TOF−MS, matrix-assisted laser desorption ionization−time of flight−mass spectrometry; MD, molecular dynamics; MOE, Molecular Operating Environment; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

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