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Effects of Non-covalent Interactions with 5-*O*-Caffeoylquinic Acid (Chlorogenic Acid) on the Heat Denaturation and Solubility of Globular Proteins

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The non-covalent interactions between the monomeric phenolic compound chlorogenic acid (5-CQA) and bovine serum albumin (BSA), lysozyme, and α-lactalbumin were characterized, and their effect on protein properties was examined. 5-CQA had a low affinity for all three proteins, and these interactions seemed to show a negative cooperativity. 5-CQA−BSA binding decreased with increasing temperature, whereas pH (pH 3.0 compared to pH 7.0) and ionic strength had no pronounced effect. At high 5-CQA/protein molar ratios, both the denaturation enthalpy and temperature of BSA increased; however, covalent bonds were created at high temperatures. The presence of 5-CQA had no effect on the solubility of BSA and α-lactalbumin as a function of pH, whereas it decreased lysozyme solubility at alkaline pH due to covalent interactions. These results indicate that the non-covalent interactions with 5-CQA do not have pronounced effects on the functional properties of globular proteins in food systems.

KEYWORDS: Phenolic compounds; BSA; lysozyme; α-lactalbumin; pH; temperature; ionic strength

INTRODUCTION

Phenolic compounds are secondary plant metabolites with a broad range of chemical structures. Phenolics that are able to precipitate alkaloids, gelatin, and globular proteins from solutions are usually called tannins, as defined by Bate-Smith and Swain in 1962 (*1*). The interaction of phenolics with proteins is also the main cause of undesirable haze in beer, wine, and clear fruit juices (*2*). In potato juice from the potato starch industry, phenolic compounds are considered to be responsible for a decrease in protein solubility during protein recovery (*3*). Phenolics can also have positive aspects by improving foam formation and stability (*4*). The addition of phenolics from green tea results in an increased heat stability of milk, underscoring the possible use of phenolics as food additives (*5*). Controlling the interactions between proteins and phenolics would therefore provide a tool to improve the functionality of plant proteins in foods. This is, however, only possible if the mechanisms underlying these interactions are known.

Plant phenolics may interact covalently or non-covalently with proteins. Both ways can lead to precipitation of proteins, via either multisite interactions (several phenolics bound to one protein molecule) or multidentate interactions (one phenolic bound to several protein sites or protein molecules). Which type of interaction occurs will depend on the molar ratio phenolic/protein (*6*).

The non-covalent interactions between phenolic compounds and proteins have been suggested to be created by hydrophobic association, which may subsequently be stabilized by hydrogen bonding (*6*, *7*). However, whereas quite some research has been devoted to interactions between tannins and proline-rich proteins (*2*, *8*−*15*), the nature and extent of the non-covalent interactions between monomeric phenolics and globular proteins are still unclear. The interactions between tannins and proline-rich proteins may be due to π-bonded complexes formed by the overlapping of the rings of the phenolic and those of aromatic amino acid residues in proteins (*8*). Proanthocyanidins are supposed to interact with bovine serum albumin (BSA) via hydrophobic interactions (*16*), whereas hydrogen bonding seems to be responsible for the interactions between 11S sunflower protein and 5-*O*-caffeoylquinic acid (*17*), caffeic acid, or quinic acid. In contrast to *p*-hydroxybenzoic acid, protocatechuic acid (3,4 dihydroxybenzoic acid) and caffeic acid have been observed

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to interact with BSA, which may reduce their antioxidant properties (*18*).

The monomeric phenolic compound, 5-*O*-caffeoylquinic acid (5-CQA, chlorogenic acid), is the most common individual compound of the cinnamic acid family (*19*) and often represents the majority of the phenolic compounds in fruits (*20*). In this study, 5-CQA was used as a model compound for cinnamic acid derivatives. The interactions of 5-CQA with the globular proteins BSA, α-lactalbumin, and lysozyme were studied. α-Lactalbumin has a chemical structure very similar to that of lysozyme but has a lower isoelectric point and a lower structural stability (*21*).

This paper presents a study of the interactions of 5-CQA with globular proteins concerning binding constants, effects of environmental parameters (ionic strength, pH, temperature) on the interactions between 5-CQA and proteins, and the effects of these interactions on protein properties (thermal denaturation and solubility) using a variety of techniques and methods.

MATERIALS AND METHODS

Materials. BSA, fractionated by cold alcohol precipitation and essentially fatty acid free, α-lactalbumin (type I), lysozyme (from chicken egg white), and 5-*O*-caffeoylquinic acid (5-CQA) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The buffer used for gel filtration chromatography experiments and determination of the binding parameters at low ionic strength (equilibrium dialysis and technique of Hummel and Dreyer) was 0.02 M potassium phosphate buffer [pH 7.0; ionic strength (I) = 0.062 M] containing 0.02 M NaCl. All of the other experiments were performed in 0.1 M potassium phosphate (pH 7.0; I = 0.184 M) to obtain a higher ionic strength, with the exception of solubility experiments for which a very low ionic strength was chosen (I = 0.017 M). Furthermore, a buffer of pH 3.0 (0.2 M phosphate buffer) was used to study the effect of low pH on the interactions, with an ionic strength similar to the 0.1 M potassium phosphate buffer of pH 7.0. The protein concentrations were similar for gel filtration and the techniques used to determine the binding parameters and the effect of pH and temperature (equilibrium dialysis, technique of Hummel and Dreyer, ultrafiltration). For isothermal titration calorimetry, differential scanning calorimetry (DSC), and protein solubility experiments, the protein concentrations were chosen to fit the sensitivity of the technique.

Gel Filtration Chromatography. Samples of 25 μL of 1.2% (w/v) BSA incubated with 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl, for 5 min or 24 h, at room temperature, with or without 0.02% (w/v) 5-CQA, were applied to a Superdex-200 gel filtration column (3.2 × 300 mm; flow = 0.1 mL/min, Amersham Pharmacia Biotech, Uppsala, Sweden) on a SMART system (Amersham Pharmacia Biotech). Protein elution was detected at 280 nm and 5-CQA elution at 324 nm.

Reversibility of 5-CQA Interactions. Samples of 0.01% (w/v) BSA in 0.1 M potassium phosphate buffer (pH 7.0) were incubated during 48 h with 5-CQA at a ratio of 4 mol of 5-CQA per mole of protein and with 0.02% (w/w) of sodium azide. Blanks without 5-CQA were also incubated during the same time. After incubation, all samples were dialyzed for 3 h in Slide-A-Lyzer minidialysis units (Pierce Chemical Co., Rockford, IL) with a molecular weight cutoff of 10 kDa against buffer at room temperature. The removal of 5-CQA was checked by measuring the absorbance at 324 nm in a spectrophotometer. The experiment was performed in duplicate.

Samples of 0.1% (w/v) lysozyme and α-lactalbumin were incubated, in 0.1 M potassium phosphate buffer (pH 7.0), during 15 min with 5-CQA at a ratio of 300 mol of 5-CQA per mole of protein. Blanks without 5-CQA were also incubated during the same time. After incubation, all samples were dialyzed overnight in Slide-A-Lyzer minidialysis units (Pierce Chemical Co.) with a molecular weight cutoff of 10 kDa against distilled water at room temperature. Samples were prepared for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The experiment was performed in duplicate.

MALDI-TOF MS Analysis. MALDI-TOF MS analysis was performed following the drying droplet method using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Sigma) as a matrix dissolved to a concentration of 10 mg/mL in 50% (v/v) acetonitrile containing 0.3% (v/v) trifluoroacetic acid (TFA). After external calibration using insulin (5.735 kDa), thioredoxin (11.674 kDa), and apo-myoglobin (16.953 kDa) (PerSeptive Biosystem, Framingham, MA), 1 μL of the solutions was analyzed on a Voyager DE-RP MALDI-TOF MS (PerSeptive Biosystems). Each sample was analyzed in duplicate.

Equilibrium Dialysis. Equilibrium dialysis experiments were performed at 25 °C during 16 h in an apparatus from Dianorm GmbH with neutral cellulose dialysis membranes (molecular weight cutoff of 5 kDa, Dianorm GmbH, Munich, Germany). One milliliter solutions of various concentrations of 5-CQA, in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl, were dialyzed against the same volume of 1.2% (w/v) BSA in the same buffer. To determine the amount of 5-CQA bound to the membrane, 5-CQA was dialyzed against buffer without BSA. After dialysis, the free 5-CQA concentration (5-CQA_{free}) was measured at 324 nm in a spectrophotometer using a molar extinction coefficient of 18500 M^{-1} cm^{-1} (*17*). After correction for the 5-CQA bound to the membrane, the number of 5-CQA molecules bound per molecule of protein (ν) was calculated. The binding constants, k_i, and the number of binding sites (n_i) were determined using a Scatchard plot representing $\nu/(5\text{-}CQA_{free})$ as a function of ν (*22*). The curve was analyzed with a graphic parameter fitting for two sets of binding sites (*23*). The curve was resolved into two straight lines, each line representing one set of binding sites, and their interceptions with the x and y axis representing n_i, and $n_i \times k_i$, respectively. Each 5-CQA/BSA ratio was studied 10 times.

Hummel and Dreyer Analysis. Binding experiments were performed on an ÄKTA purifier (Amersham Pharmacia Biotech) according to the protocol of Hummel and Dreyer (*24*) with a Superdex-30 PG gel filtration column (0.5 × 9.5 cm; flow = 0.4 mL/min) (Amersham Pharmacia Biotech) equilibrated with the different running buffers containing different concentrations (0−1.7 mM) of 5-CQA. Buffers used were 0.02 M potassium phosphate buffer [pH 7.0; ionic strength (I) = 0.062 M], containing 0.02 M NaCl, and 0.1 M potassium phosphate buffer (pH 7.0; I = 0.184 M). Samples applied were 25 μL of a solution containing 1.2% (w/v) BSA and various 5-CQA concentrations. Hummel−Dreyer experiments were also performed with 1.5% (w/v) lysozyme in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl. The binding of 5-CQA from the elution buffer to protein decreased the concentration of free 5-CQA, creating a trough in the baseline of which the area was quantified, at 324, 380, or 400 nm depending on the optical density. The results from the Hummel−Dreyer method satisfied the criteria necessary to obtain correct binding parameters according to Busch and colleagues (*25*). The binding constants and number of binding sites were determined with graphic parameter fitting as described before. Four different protein/5-CQA ratios were performed for each 5-CQA concentration in the elution buffer.

Isothermal Titration Calorimetry (ITC). The heat effect of successive additions of 5-CQA on BSA was measured with an MCS isothermal titration calorimeter (MicroCal, Inc., Northampton, MA) at 25 °C. Solutions of BSA and 5-CQA in 0.1 M potassium phosphate buffer (pH 7.0) were degassed during 25 min under vacuum. The reference cell contained the degassed buffer. The sample cell contained 0.72% (w/v) BSA, whereas 0.89% (w/v) 5-CQA was in a 250 μL syringe. The initial delay before the first injection was 600 s. Every 300 s, the syringe injected 10 μL of 5-CQA in BSA solution in a span of 13 s. The reference offset was 50%. Samples with only BSA or 5-CQA were used as blanks, and the data points of only 5-CQA were subtracted from data points of BSA with 5-CQA. The heat changes were analyzed with the use of the software Origin (MicroCal Software, Inc.).

Differential Scanning Calorimetry. Samples used for DSC experiments were 0.05% (w/v) BSA, 0.1% (w/v) α-lactalbumin, and 0.1% (w/v) lysozyme in 0.1 M potassium phosphate buffer (pH 7.0). After filtration over a 0.45 μm cellulose acetate filter (<5% of protein or 5-CQA concentration lost), the proteins were incubated with various concentrations of 5-CQA, degassed, and injected in the sample cell of

a VP-DSC calorimeter (MicroCal Inc.). The reference cell contained buffer with the same amount of 5-CQA as the sample cell. The differential heat capacity was measured at a scanning rate of 60 °C per hour, from 25 to 70, 80, and 90 °C for BSA, α-lactalbumin, and lysozyme, respectively. Data were analyzed with the MicroCal Origin software (MicroCal Inc.). The phase transition temperature, T_m, was defined as the temperature at the maximum of the peak. Each experiment was repeated at least three times.

To investigate the presence of covalent modifications due to heating, samples of α-lactalbumin and lysozyme incubated with 5-CQA in a ratio of 50 mol of 5-CQA per mole of protein were heated at 80 and 85 °C, respectively, and subsequently were desalted by centrifugation in Microcon YM-10 centrifugation devices (Amicon, Millipore Corp., Beverly, MA) and then analyzed using MALDI-TOF MS, as described previously. Blanks consisted of protein heated using DSC without 5-CQA and unheated protein.

Effect of Temperature on 5-CQA—Protein Interactions. To determine the effect of temperature on the 5-CQA−BSA interactions, the amount of 5-CQA bound to 1.2% (w/v) BSA was determined according to the technique of Hummel and Dreyer (*24*) at 5, 25, and 60°C in 0.1 M potassium phosphate buffer (pH 7.0). The buffer and the Superdex-30 PG gel filtration column (0.5 × 9.5 cm; flow = 0.4 mL/min) (Amersham Pharmacia Biotech) were thermostated by a water bath. Four different protein/5-CQA ratios were performed for each 5-CQA concentration in the elution buffer.

Effect of pH on 5-CQA—Protein Interactions. Samples of 1.2% (w/v) of BSA were incubated with 5-CQA to different ratios in 0.1 M potassium phosphate buffer (pH 7.0) or in 0.2 M phosphate buffer (pH 3.0). A small part of the unbound 5-CQA was removed by ultrafiltration (5 min, 16*g*, 25°C) through membranes of 3 kDa molecular weight cutoff, with MPS micropartition devices (Amicon, Millipore Corp.). After dilution with buffer, the 5-CQA concentration of the filtrate was quantified at 324 nm in a spectrophotometer. Blanks used to correct for 5-CQA bound to the filtration unit or to the membrane consisted of 5-CQA without protein. Four replicates were performed per 5-CQA/protein ratio.

Effect of Protein Denaturation on 5-CQA—Protein Interactions. Samples of 2.4% (w/v) BSA and α-lactalbumin were denatured by DSC as described before, but without prior filtration, by heating for at least 30 min at 70 and 90 °C, respectively. Heated samples, and a calibration mixture (14.2−2000 kDa) for molecular weight determination, were applied on a Superdex-200 gel filtration column (30 × 0.35 cm; flow = 0.1 mL/min) (Amersham Pharmacia Biotech) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) in order to estimate the amounts and sizes of potentially aggregated proteins. The eluate was monitored for the presence of proteins at 280 nm.

To quantify the amounts of 5-CQA bound to heated proteins [0.13% (w/v)], 10 mol of 5-CQA was added per mole of protein. Subsequently, the unbound 5-CQA was partly removed by ultrafiltration, as described before (four replicates).

Effect on Protein Solubility. The protein concentrations used were 0.1% (w/v) BSA, 0.1% (w/v) α-lactalbumin, and 0.2% (w/v) lysozyme. The proteins were dissolved in 0.01 M potassium phosphate buffer (pH 7.0), with 100 mol of 5-CQA per mole of protein. After filtration of the solutions, the samples were adjusted to various pH values with NaOH or HCl (1 or 6 M). After 2 h, the samples were centrifuged (15 min, 12700*g*, 25°C). The protein concentrations of the supernatants were measured at 595 nm in a spectrophotometer using the quantitative Coomassie Blue assay as modified by Boyes et al. (*26*). Blanks consisted of protein without 5-CQA and 5-CQA without protein. Each pH was studied in triplicate.

The samples at pH 7.0, 8.0, and 9.0 containing lysozyme with 5-CQA were also analyzed by MALDI-TOF MS, as described previously, to detect possible covalent interactions. Blanks were lysozyme at pH 7.0 and 9.0 without 5-CQA.

RESULTS

Binding Affinity of 5-CQA for Proteins at pH 7.0. The binding of 5-CQA to BSA was investigated by three methods (equilibrium dialysis, the Hummel−Dreyer technique, and ultrafiltration) with identical results. The number of molecules of 5-CQA bound per molecule of BSA (ν) at pH 7.0 (25 °C) was measured by the Hummel−Dreyer technique and by equilibrium dialysis, at two different ionic strengths (**Figure 1**). The free 5-CQA constituted >30% of the total 5-CQA. This excess in free 5-CQA was necessary to accurately determine the free ligand concentration, thereby avoiding a systematic error in the Scatchard plots (27). The concave shape of the Scatchard plots, representing the mechanism of 5-CQA−BSA binding, seemed to show a pattern of negative cooperativity: after the binding of one 5-CQA molecule, the binding of another 5-CQA molecule becomes more difficult. The increasing difficulty to bind more and more 5-CQA molecules may be due either to a change in protein tertiary structure by ligand binding, which reveals a negative cooperative mechanism (Koshland−Némethy−Filmer sequential model), or to the initial presence of classes of sites of different affinities (*28*). Circular dichroism (CD) experiments in the 270−310 nm region (no further data given) did not show any clear change in the tertiary structure of BSA in the presence of 5-CQA. Thus, the increasing difficulty to bind more and more 5-CQA molecules is likely due to the initial presence of different affinity classes of non-interacting sites on BSA rather than to a structural change of BSA. However, the absorbance of 5-CQA led to a high background noise in CD spectra. Thus, a cooperative mechanism (resulting from a structural change) cannot be totally excluded, especially for the lower affinity binding sites. The Scatchard plots were analyzed with a graphic parameter fitting for the simplest model of several affinity classes, that is, two classes of binding sites. At an ionic strength of 0.062 M, BSA contained one site of medium affinity with affinity constants of $13 \times 10^3 \pm 2 \times 10^3$ and $16 \times 10^3 \pm 5 \times 10^3$ M^{-1}, according to the results of the Hummel−Dreyer technique and equilibrium dialysis experiments, respectively. The other BSA binding sites had a very low affinity for 5-CQA. Because of this low affinity, the exact number of binding sites for the second set of sites was difficult to determine. At 0.184 M ionic strength, the scattering of the points resulted in a nonprecise estimation of the binding constant. One binding site with an affinity of $52 \times 10^3 \pm 25 \times 10^3$ M^{-1} was present, whereas the other binding sites had a very low affinity for 5-CQA (**Figure 1**).

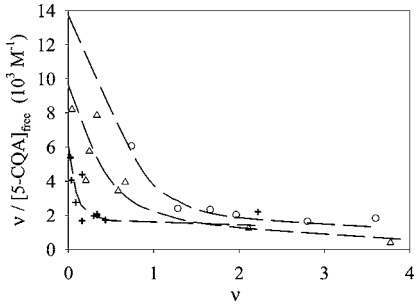

Figure 1. Scatchard plot for the binding of 5-CQA to BSA (pH 7.0, 25 °C), using different techniques: (△) Hummel–Dreyer (I = 0.062 M); (+) Hummel–Dreyer (I = 0.184 M); (○) equilibrium dialysis (I = 0.062 M). The dotted lines were obtained from the fitting models for two sets of binding sites.

The binding of 5-CQA to BSA was also studied by ITC (**Figure 2**). The slope of the curve was not steep, indicating that not all added ligand was bound, thus that the binding was moderately tight (*29*). The ITC data analysis software allowed the testing of three models of interactions: one set of sites, two sets of independent sites, or multiple interacting sites. The best model for fitting the curves (with randomized errors) was that

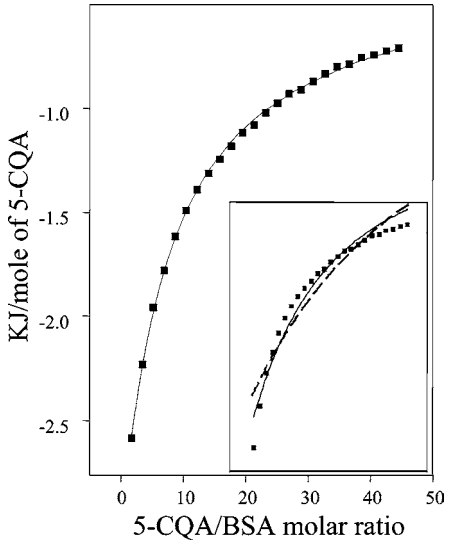

Figure 2. Isothermal titration calorimetry of 5-CQA–BSA interactions (25 °C): (■) data points; (—) fitting model for two sets of interacting sites. Inset: (—) one set of sites; (- - -) two sets of independent sites.

of at least two sets of interacting binding sites (**Figure 2**). However, ITC alone cannot distinguish truly interacting sites from sites with differing binding constants (except if the sites of the protein are known to be identical). Thus, according to the ITC results, the affinity of 5-CQA for BSA was the result of either at least two sets of interacting binding sites or more than two sets of independent sites. No unambiguous value of the binding constant could be extracted from ITC data as the estimation of the binding constants led to several outcomes with similar error.

Reversibility of 5-CQA—Protein Interactions. To determine how quickly the interactions between 5-CQA and protein were occurring and to prove that covalent interactions were absent, gel filtration chromatography and MALDI-TOF MS experiments were performed. After incubation of 3−4 mol of 5-CQA per mole of BSA for 5 min (pH 7.0, $I = 0.062$ M), a 5-CQA−BSA complex could already be detected by gel filtration chromatography, and the amount of complex formed (as estimated from the absorbance of 5-CQA at 324 nm) did not increase upon longer incubation. Thus, the interactions between 5-CQA and BSA occurred within 5 min.

To verify that covalent protein−5-CQA complexes were absent, lysozyme and α-lactalbumin incubated with a high ratio of 5-CQA were analyzed by MALDI-TOF MS. The analysis showed that lysozyme and α-lactalbumin did not form covalent complexes with 5-CQA at pH 7.0 (data not shown). BSA was not analyzed in this way because the lack of sensitivity of MALDI-TOF MS for high molecular weight proteins prevented the detection of covalent modifications of BSA. However, after extensive dialysis of BSA incubated with 5-CQA, no 5-CQA could be detected anymore in the sample according to absorbance measurements, indicating that the interactions were totally reversible.

Effect of Low pH on 5-CQA—Protein Interactions. The binding of 5-CQA to BSA at low pH was investigated to determine which kind of non-covalent interactions play a role in the interactions between 5-CQA and proteins. The amount of 5-CQA bound per BSA molecule was slightly higher at pH 3.0 than at pH 7.0 at intermediate ratios [10−15 mol of 5-CQA per mole of BSA, ultrafiltration experiments (**Figure 3**)].

Effect of Temperature and Protein Denaturation on 5-CQA—Protein Interactions. The amount of 5-CQA bound to BSA at various temperatures (5, 25, and 60 °C) was measured by the Hummel−Dreyer technique (**Table 1**), concerning the higher affinity binding site. The number of molecules of 5-CQA bound per molecule of BSA decreased when the temperature increased (**Table 1**).

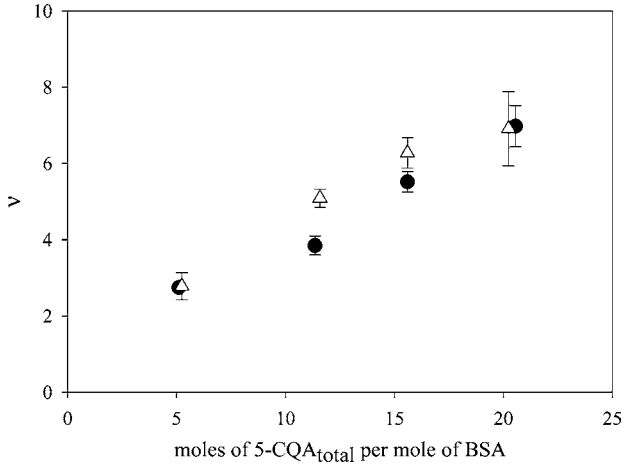

Figure 3. Number of molecules of 5-CQA bound per molecule of BSA as a function of the number of moles of 5-CQA added per mole of BSA at different pH values [$I = 0.184$ M, 25 °C; 5-CQA, from 0.033 to 0.1% (w/v)] as determined using ultrafiltration: (△) pH 3.0; (●) pH 7.0.

Table 1. Number of Molecules of 5-CQA Bound per Molecule of Protein (ν) at pH 7.0 for BSA, α-Lactalbumin (α-La), and Lysozyme, under Various Conditions and at Different 5-CQA/Protein Molar Ratios Using the Hummel–Dreyer Technique (HD) or the Ultrafiltration Technique (UF)

technique	protein	temp (°C)	concn (mM 5-CQA)	molar ratio	ν
HD	BSA	25	0.02[a]	0.12	0.04
HD	BSA	60	0.02[a]	0.01	0.01
HD	BSA	25	0.06[a]	0.52	0.18
HD	BSA	60	0.06[a]	0.37	0.03
HD	BSA	5	0.12[a]	1.00	0.34
HD	BSA	25	0.12[a]	0.87	0.22
HD	BSA	60	0.12[a]	0.74	0.08
HD	BSA	20	0.17[a]	1.65	0.66
HD	lysozyme	20	1.69[a]	1.65	0.10
UF	native BSA	25	0.20[a]	10	0.8 ± 0.1
UF	heat-denatured BSA	25	0.20[a]	10	0.4 ± 0.2
UF	native α-La	25	0.93[a]	10	0.4 ± 0.3
UF	heat-denatured α-La	25	0.93[a]	10	0.45 ± 0.08

[a] For HD, the parameter that can be controlled (and indicated in the table) is the concentration of 5-CQA in the elution buffer (5-CQA$_{free}$), whereas the indicated concentration for UF is the total concentration of 5-CQA.

The amount of 5-CQA bound to heat-denatured BSA and α-lactalbumin was studied using ultrafiltration. Lysozyme was not studied by ultrafiltration because of its low solubility after heat denaturation, whereas BSA and α-lactalbumin remained soluble after denaturation. The efficiency of the denaturation was monitored by DSC. Heated samples were applied on a gel filtration column. Nearly all BSA and α-lactalbumin molecules were aggregated. The aggregates were on average composed of about 14 molecules (900 kDa) and 21 molecules (300 kDa) of BSA and α-lactalbumin, respectively, as estimated by their apparent molecular weight. The number of molecules of 5-CQA bound to BSA decreased by 50% when BSA was denatured (**Table 1**). The number of molecules of 5-CQA bound to α-lactalbumin did not significantly change when α-lactalbumin was denatured (**Table 1**).

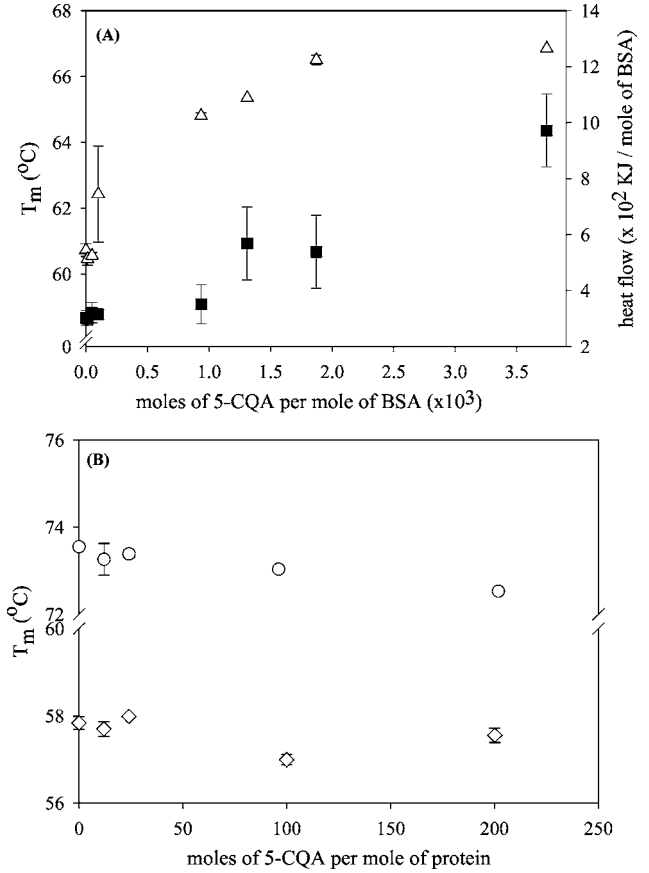

Figure 4. Denaturation temperature and enthalpy as a function of the number of moles of 5-CQA present in solution per mole of protein (pH 7.0), as determined by DSC: (A) (△) denaturation temperature of BSA, (■) heat flow of BSA; (B) (○) lysozyme, (◇) α-lactalbumin.

Effect of 5-CQA on Protein Heat Denaturation. The denaturation temperature of BSA, lysozyme, and α-lactalbumin was measured as a function of 5-CQA concentration using DSC (**Figure 4**). In the presence of 3.7×10^3 mol of 5-CQA/mol of BSA, the denaturation temperature increased by 6 °C, whereas the enthalpy of denaturation of BSA increased by ∼700 kJ/mol (**Figure 4A**). The change in denaturation temperature for lysozyme and α-lactalbumin due to the presence of 5-CQA was not as pronounced as for BSA (**Figure 4B**). A decrease of 1 °C in the denaturation temperature of lysozyme required the presence of 200 mol of 5-CQA/mol of lysozyme (**Figure 4B**), whereas the enthalpy of denaturation (330 kJ/mol of lysozyme) was not significantly changed at this molar ratio. At the same ratio, the denaturation temperature of α-lactalbumin was not significantly different from that without 5-CQA (**Figure 4B**). The enthalpy of denaturation of α-lactalbumin (270 kJ/mol of α-lactalbumin) was also not significantly modified with increasing 5-CQA concentration.

Effect of 5-CQA on Protein Solubility. The solubility, as a function of pH, of BSA, α-lactalbumin, and lysozyme was investigated in the presence of 100 mol of 5-CQA/mol of protein. The solubility of BSA (p*I* 4.7−4.9) and α-lactalbumin (p*I* 4.2−4.5) was not significantly affected by the presence of 5-CQA (**Figure 5**). 5-CQA significantly decreased the solubility of lysozyme (p*I* 10.5−11.3) at pH ≥ 8.0. As at elevated pH values, 5-CQA may be oxidized by autoxidation, leading to formation of reactive radicals or quinones, which may interact covalently with proteins; the presence of covalent interactions was investigated at pH ≥ 7.0. According to MALDI-TOF MS

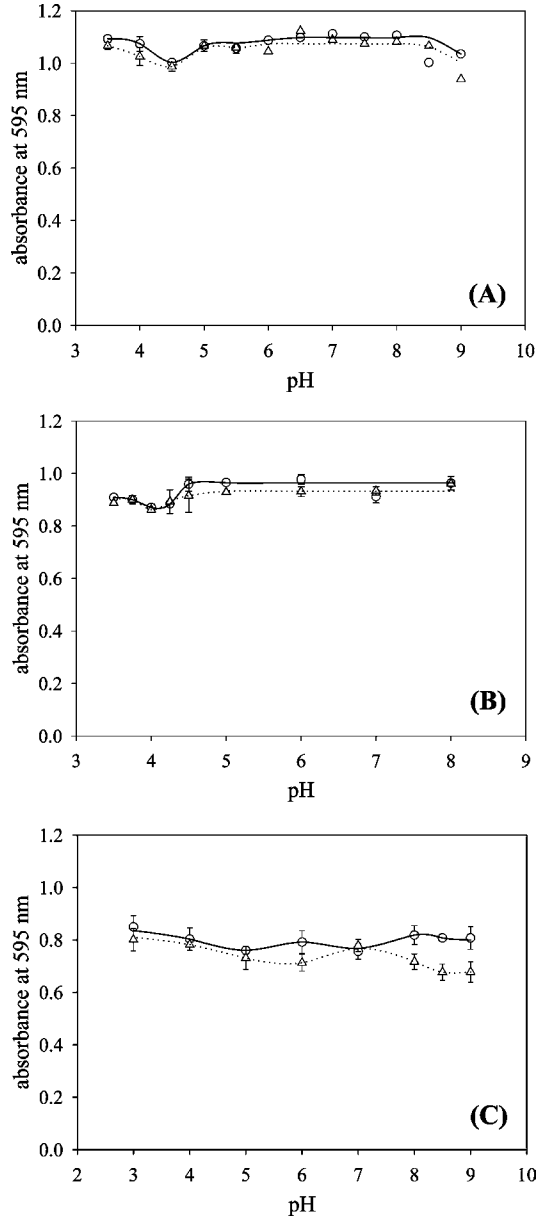

Figure 5. Protein solubility as a function of pH in the absence (○) and in the presence of 100 mol of 5-CQA per mole of protein (△): (A) BSA (1 mg/mL); (B) α-lactalbumin (1 mg/mL); (C) lysozyme (2 mg/mL).

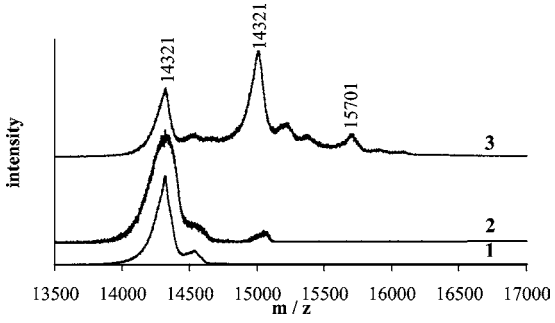

Figure 6. Mass spectra of lysozyme in the presence of 100 mol of 5-CQA per mole of protein, at pH 7.0 (1), 8.0 (2) and 9.0 (3).

(**Figure 6**), the covalent addition corresponded to two molecules of 5-CQA (680−690 Da) per molecule of lysozyme at pH 8.0 and more than two at pH 9.0, whereas no such event occurred at pH 7.0.

DISCUSSION

Characterization of Non-covalent Interactions between 5-CQA and Proteins. Using equilibrium dialysis, the Hummel−Dreyer technique, and ITC, it was shown that at pH 7.0 the affinity of 5-CQA for BSA was the result of one site of medium affinity [$(13-16) \times 10^3$ M^{-1} at $I = 0.062$ M] and various sites of low affinity. These interactions resulted in an increasing difficulty to bind more and more 5-CQA, which was likely due to the initial presence of sites of different affinities rather than to a negative cooperativity. In contrast to this, the interactions observed between 5-CQA and 11S sunflower protein were the result of four binding sites with a binding constant affinity of 4.5×10^3 M^{-1} and showed a positive cooperativity (**17**).

In **Table 1**, the amounts of 5-CQA bound to BSA and lysozyme at pH 7.0 were compared as a function of the free 5-CQA concentration according to the technique of Hummel and Dreyer. The lysozyme concentration used was higher than the BSA concentration used because of the lower affinity of 5-CQA for lysozyme compared to BSA. The number of molecules of 5-CQA bound per molecule of protein at pH 7.0 was clearly smaller for lysozyme than for BSA and seemed slightly lower for α-lactalbumin than for BSA (**Table 1**). Differences among proteins in the amount of binding could be explained by different facts, such as the amino acid composition, for example, the proline residues (*8*), the value of the isoelectric point (*30*), and the difference in average hydrophobicity [BSA > α-lactalbumin > lysozyme according to several spectroscopic methods (*31*)].

Increasing the ionic strength increased the binding strength between 5-CQA and BSA. Lowering the pH from 7.0 to 3.0 slightly increased the binding at some 5-CQA/protein ratios, without pronounced effects on the binding constants. As more than one 5-CQA was bound per molecule of protein, this modification seemed to concern the low-affinity binding sites. Furthermore, as lowering the pH from 7.0 to 3.0 had no effect on the binding constants, electrostatic interactions with amino acids having a pK_a between 7.0 and 3.0 do not seem to play a role in the interactions between 5-CQA and BSA. Shamanthaka Sastry and Narasinga Rao (*17*) observed that increasing the pH from pH 4.0 to 7.0 reduced the total number of 5-CQA binding sites on the sunflower 11S protein without affecting the binding constants.

Increasing the temperature strongly decreased the amount of 5-CQA bound to BSA, even at temperatures at which BSA was not heat denatured. This could indicate that 5-CQA interactions are not dominated by hydrophobic interactions, as these increase upon heating. Hydrophilic interactions (hydrogen bonds, van der Waals interactions, electrostatic interactions) decrease with increasing temperature (*32*). Shamanthaka Sastry and Narasinga Rao (*17*) concluded from the effect of temperature on the binding affinity and the number of binding sites that 5-CQA seemed to interact with 11S sunflower protein by hydrogen bonding rather than by hydrophobic interactions. We can, however, not conclude from our results that hydrophobic interactions do not play a role in the interactions between 5-CQA and BSA.

The denaturation of BSA decreased by 50% the number of moles of 5-CQA bound to BSA, whereas the denaturation of α-lactalbumin did not significantly modify the amount of binding. However, the denaturation produced aggregates of proteins, composed on average of about 14 molecules of BSA and 21 molecules of α-lactalbumin. When the proteins are considered as spheres with a surface $= 4\pi \times \text{radius}^2$ and a volume $= 4\pi \times \text{radius}^3/3$, according to the volume ($Å^3$) = 1.27 × protein molecular weight (*33*), the surfaces of aggregates of 14 molecules of BSA and 21 molecules of α-lactalbumin would be 60 and 65% smaller than the surfaces of 14 monomers of BSA and 21 monomers of α-lactalbumin, respectively. The decrease of the binding of 5-CQA to the denatured BSA compared to the native BSA can thus be explained by the decrease of the total protein surface induced by the aggregation of BSA. In contrast to this, denatured α-lactalbumin seems to bind more 5-CQA than the native α-lactalbumin, which could be explained by a difference in amino acid exposure at the surface of the protein.

Effect of Non-covalent Interactions with 5-CQA on Protein Functional Properties. At high 5-CQA/protein ratios, 5-CQA increased the denaturation temperature of BSA and the denaturation enthalpy of BSA. This may indicate a stabilization of BSA against heat denaturation by binding of 5-CQA due to a stronger binding to the native protein than to the denatured protein. The denaturation temperature, however, continued to increase even when BSA should be considered fully occupied with ligand. Then, according to Cooper (*34*), the stabilization does not result from extra ligand binding but from the additional free energy necessary to remove the ligand before the unfolding of the protein, as well as from the entropy of mixing of 5-CQA (released into the bulk solution) that decreases with increasing concentrations of ligand in the bulk solution.

The presence of 5-CQA slightly decreased the denaturation temperature of lysozyme. If a ligand binds preferentially to the unfolded form of a protein rather than to the native form, then the protein will be destabilized and unfolding will be encouraged (*34*). This may indicate that 5-CQA binds with a slightly higher affinity to unfolded lysozyme than to native lysozyme. 5-CQA did not significantly modify the denaturation temperature of α-lactalbumin.

The presence of 5-CQA with α-lactalbumin and lysozyme during heat denaturation at pH 7.0 induced a covalent addition of 680−690 Da, according to MALDI-TOF MS experiments (data not shown). This modification would be due to the thermal oxidation of phenols into quinones, which may react covalently with proteins. Such reaction was shown to occur between quinones and amino, sulfhydryl, thioether, phenolic, indole, and imidazole groups of proteins (*35*). An addition of 680−690 Da corresponds to the addition of two molecules of 5-CQA modified into a quinone (36). Because only additions of two molecules of 5-CQA, but no addition of monomeric 5-CQA, were detected, it can be expected that two molecules of 5-CQA react by condensation, prior to the interaction with proteins. Such dimers can be formed by coupled oxidation between a quinone and another phenolic molecule (*20*). If the mechanism proposed by Namiki and colleagues (*37*) is followed, the binding of one dimer of 5-CQA will lead to an addition of 683 Da. The observed addition of 680−690 Da during heating and at alkaline pH seems thus to indicate a dimerization of 5-CQA prior to the interaction with proteins.

Also the decrease in solubility of lysozyme at high 5-CQA/protein ratios and basic pH values can be attributed to the oxidation, in alkaline solution, of 5-CQA into quinone. In contrast to the solubility of lysozyme, the solubilities of BSA and α-lactalbumin were not decreased by the presence of 5-CQA at alkaline pH values. Covalent interactions created at alkaline pH with 5-CQA have been observed not to modify the solubility of BSA (*38*) and α-lactalbumin (data not shown) at these pH values. The differences between lysozyme and BSA and α-lactalbumin may be caused by the large differences in isoelectric point between proteins. The non-covalent interactions

with phenolic compounds may precipitate proteins by a multidentate mechanism (by interacting simultaneously with several proteins) or by covering the protein with a less hydrophilic monolayer (*6*). However, 5-CQA is only monodentate and seems to have a too low affinity for BSA, α-lactalbumin, and lysozyme to induce precipitation via a non-covalent way.

This study shows that only at very high molar 5-CQA/protein ratios (≥100) can large effects on protein properties be observed, such as increasing the unfolding temperature by several degrees. In foods these ratios are generally much lower. For defatted sunflower meal, for example, which can be considered one of the richest sources of phenolic compounds, the molar ratio 5-CQA/protein is only 30 (calculated from ref *39*). In conclusion, when it interacts non-covalently, 5-CQA does not have pronounced effects on the functional properties of globular proteins in food systems. However, the quinones from 5-CQA, via their covalent interactions with proteins, are expected to have more pronounced effects on the functional properties of globular proteins.

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