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Binding of Selected Phenolic Compounds to Proteins

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In the context of this study, the noncovalent binding of selected phenolic compounds (chlorogenic, ferulic, and gallic acids, quercetin, rutin, and isoquercetin) to different proteins (human serum albumin, bovine serum albumin, soy glycinin, and lysozyme) was studied with direct (Hummel−Dreyer/size exclusion chromatography) and/or indirect methods (fluorescence absorbance properties of the binding components). In the latter case, the measurement of the phenol binding was achieved by exploiting the intrinsic fluorescence emission properties of quercetin as a probe. From the data obtained, the binding constants and the number of binding sites were calculated. The binding parameters were influenced by different factors, where, e.g., increasing temperature and ionic strength as well as decreasing pH cause a diminished binding. The structures of the proteins as determined by circular dichroism indicate changes in the tertiary structure with the secondary structure remaining intact.

KEYWORDS: Phenol−protein binding; binding constants; binding sites; nonlinear regression; Hummel−Dreyer method; quercetin fluorescence

INTRODUCTION

The structure, classification, and distribution of the phenolic compounds in plant foods, their chemistry, and signification with regard to food processing and storage as well as their physiological effects have been summarized recently in refs *1* and *2*. Dietary phenolic substances have received much attention due to their biological activity. They have been attributed with positive properties such as having antimutagenic and anticancerogenic effects as well as being antioxidants (*2*). Phenolic compounds on the other hand can also display many possible detrimental effects, including complexation of gut enzymes and dietary proteins caused by interactions with proteins (*3*). Physiologically and technologically interesting data from literature in this context suggest that a larger part of these interactions may be governed by noncovalent binding (*3*−*8*).

Most of our former work was focused mainly on such reactions of the phenolic substances with proteins and enzymes that lead to covalent bonds (*2*). The derivatives thus formed were characterized in terms of changes in their physicochemical and structural properties. The effect on the proteolytic in vitro digestion was also illustrated. Further aspects reported included the influence on enzyme activity and kinetic parameters. The nutritional−physiological consequences of such reactions in food and the body, especially considering their significance to food science and technology, are discussed together with a review of our work in this field in ref *2*.

In the context of the present study, the attention was given more to the role of noncovalent interactions, although both covalent and noncovalent binding are likely to take place simultaneously as shown recently for the binding of chlorogenic acid to proteins (*9*). The present work applies two different experimental approaches for determining binding parameters for protein−plant phenolics on the basis of noncovalent interactions: (i) separation of free and protein-bound phenols by Hummel−Dreyer (HD) size exclusion chromatography (direct binding study) and (ii) detection in a change of physicochemical properties of the ligand due to protein binding. Some proteins enhance intrinsic fluorescence of quercetin. This effect may be consulted for determination of binding constants.

Furthermore, possible changes in secondary and tertiary structure of proteins were measured by circular dichroism (CD). Beside quercetin and the quercetin glucosides rutin and isoquercetin, ferulic, chlorogenic, and gallic acids were also applied in this study. Because the intake of phenolic compounds is associated with many beneficial effects, it is also necessary to consider their transport in blood by plasma proteins. Therefore, the main part of the investigation involved bovine serum albumin (BSA) as a model protein. Further investigations were conducted with human serum albumin (HSA), soy glycinin (SG), and lysozyme. Additionally, the effects of pH, temperature, and ionic strength on the binding between plant phenolics and proteins were determined.

MATERIALS AND METHODS

Materials. BSA, α-amylase (Merck, Darmstadt, Germany), HSA, lysozyme (Fluka Chemie AG, Buchs, Switzerland), milk whey proteins (DSE 1591, New Zealand milk products), gelatin (SIHA-Klärgelatine, E. Begerow GmbH & Co., Langenlonsheim, Germany), trypsin from porcine pancreas (EC 3.4.21.4, Sigma Chemicals Co., St. Louis, MO), and human serum (N Protein Standard SL, Dade Behring Marburg GmbH, Marburg, Germany) were applied.

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SG was prepared from defatted unheated soy flour (type 1, protein content ca. 52%, Sigma Chemicals Co.) according to the method of Thanh and Shibasaki (*10*). Kjeldahl determination of the extracted SG gave a protein content of 99.8%. Glycinin purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and was estimated to be about 95% by densitometric analysis of the gel.

The phenolic compounds were applied as follows: quercetin (Riedel-deHaën Laborchemikalien GmbH & Co. KG, Seelze, Germany), isoquercetin (quercetin-3-O-glucoside), rutin (quercetin-3-O-rhamnoglucoside; both from Carl Roth GmbH, Karlsruhe, Germany), and ferulic, chlorogenic, and gallic acids (Fluka Chemie AG).

Methods. *Direct Binding Studies—HD.* The principle of the HD is explained in detail in refs *11* and *12* and given shortly in the Supporting Information I. Gel filtration chromatography was carried out on a Shimadzu 10A system (Duisburg, Germany) using an Econo-Pac size exclusion chromatography cartridge P-6 (size exclusion > 6 kDa, Bio-Rad Laboratories Inc., Munich, Germany) with diode array detection and a column temperature of 25 °C. The eluent A consisted only of a buffer (0.05 M Hepes or 0.05 M Na-acetate buffer, pH 7, 6, 4.8, and 3.5). The pH of Hepes buffer was adjusted with diluted NaOH or HCl whereas that of Na-acetate buffer was adjusted with diluted acetic acid or NaOH. Eluent B consisted of the phenolic compounds (0.5 mM) dissolved in the same corresponding buffer as eluent A. Isocratic chromatography was performed, and the desired phenol concentration (0.03−0.5 mM) was obtained by using a gradient mixer. The number of ligand (phenol) molecules per protein molecule is fixed by the ligand concentration in the buffer and therefore can be considered constant during elution (*11*). The amount of the ligand bound may be quantified from internal calibration using the peak area (*11*). The internal calibration was performed by injecting a series of samples containing a fixed protein concentration (45−181 μM) and increasing ligand concentrations (for details, see Supporting Information II). The flow rate was 0.8−1 mL/min, and the injection volume amounted to 30−50 μL. For each phenolic compound investigated, some adjustments of the chromatographic conditions had to be conducted and the details are given in the Supporting Information II.

The binding of ferulic acid was also studied for a series of proteins [gelatin, milk whey proteins, SG, α-amylase, lysozyme, BSA, HSA, and human serum (N protein standard SL)] in two different buffer systems. For many food systems relevant acidic conditions, a 0.05 M Hepes pH 4.8 buffer was chosen. Whereas for comparable physiological conditions, 0.067 M Na-phosphate buffer, pH 7.4, was applied. The experiments were performed under isocratic high-performance liquid chromatography conditions similar to those described above with only a single ferulic acid concentration (0.24 mM) in the eluent (for details, see the Supporting Information II). The data were evaluated by means of external calibration (*11*).

Indirect Binding Studies Using Quercetin Fluorescence. This method applies the detection of a change in the physicochemical properties of the ligand due to protein binding, e.g., enhancement of the intrinsic fluorescence of quercetin. The method applied was adopted from ref *13*. Binding studies were conducted under the following conditions.

Effect of pH. 0.05 M Hepes buffer, pH 7 (standard), pH 6, and pH 5.

Effect of Ionic Strength: 0.05 M Hepes buffer, pH 7; 50, 250, and 500 mM NaCl.

Effect of Temperature: BSA solutions (in 0.05 M Hepes buffer, pH 7) were incubated at 40, 60, and 90 °C for 10 min and cooled to room temperature before addition of quercetin and measurement.

Protein−Bulk Solutions. Ten grams per liter (150 μM) BSA and HSA, 3534 g/L (150 μM) trypsin, 10 g/L (29.1 μM) SG, and 40 g/L (2.74 mM) lysozyme; all proteins were dissolved in corresponding buffers.

Phenol−Bulk Solutions. One gram per liter (3.31 mM) quercetin, 1 g/L (1.50 mM) rutin, and 1 g/L (2.15 mM) isoquercetin; all phenolic compounds were dissolved in dimethyl sulfoxide (DMSO).

Concentrations of the Proteins Applied. BSA and HSA, 0−90 μM; trypsin, 0−90 μM; lysozyme, 0−1.64 mM; and SG, 0−17.46 μM; altogether, 10−20 concentrations for each protein ($n = 4$ for each concentration) were investigated.

Phenol Concentration Applied. Fifteen micromolar quercetin, 15 μM rutin, and 1, 5, and 15 μM isoquercetin; as a result, the final DMSO concentration was less than 1% in the measured samples.

The fluorescence was estimated using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan). The samples were excited at 370 nm (slit, 18 nm), and the emission was recorded over the wavelength range of 380−900 nm (slit, 18 nm). Preliminary experiments showed that the protein alone could also be excited at 370 nm; as a result, "blank" series of different concentrations of proteins were also conducted, containing the corresponding amount of DMSO (without phenol) for all of the experiments.

In a second experiment, the interactions of rutin and isoquercetin as glucosides of quercetin with proteins were also tested under "standard conditions" (0.05 M Hepes buffer, pH 7; BSA, 0−90 μM). The samples were excited at 354 nm (slit, 18 nm), and the emission was recorded over the wavelength range of 380−900 nm (slit, 18 nm).

To confirm that the protein−phenols interactions are of a noncovalent nature under the applied conditions, experiments were also repeated under standard buffer conditions (0.05 M Hepes buffer, pH 7) with 8 M urea. The concentrations for the proteins tested were 30 μM for BSA and HSA, 6.98 μM for SG, and 0.66 mM for lysozyme. The quercetin concentration was held constant at 15 μM. Fluorescence measurement conditions were as described for quercetin above.

Structural Characterization. Far-ultraviolet absorption (UV)-CD of the samples were recorded in the range of 178−260 nm in 0.1 M sodium phosphate buffer at a protein concentration of 3 μM (0.2 g/L) using a Jasco J 710 spectropolarimeter. A quartz cylindrical cell having a 1 mm path length was used for the measurements. The parameters were set as follows: step resolution, 1 nm; speed, 50 nm/min; bandwidth, 1 nm; response, 4 s; and sensitivity, 200 mdeg. The mean ellipticity was determined using a mean residue molecular weight, calculated from the amino acid sequence of the corresponding proteins (SWISSPROT Protein bank, Data bank source). The CD spectra were analyzed by a curve-fitting software CDPro using CONTIN/LL, SELCON, and CDSSTR methods as described in Sreerama and Woody (*14*, *15*) to obtain the secondary structural contents of the proteins. The estimation was performed using a 48 protein reference set (*14*, *15*).

Bulk solutions of quercetin were prepared in ethanol (3.31 mM, 1 g/L), and those of rutin (1.5 mM, 1 g/L) and isoquercetin (2.15 mM, 1 g/L) were prepared in water/ethanol mixture in ratio of 1:2. The final concentrations of the phenols applied to study their effect on the secondary structure of proteins were 15, 30, 45, 60, and 75 μM. The effects of pH, ionic strength, and temperature were also studied as described under indirect binding studies on the basis of quercetin fluorescence. The only change was that of the buffer system (Na-phosphate buffer), since Hepes buffer was found to interfere with the CD measurements. Blanks (without protein) containing only corresponding amounts of phenols and those containing proteins plus the organic solvents ("controls") were also measured.

CD spectra in near-UV (250−320 nm) were measured in 0.1 M sodium phosphate buffer at a protein concentration of 3 μM (0.2 g/L for BSA and 1.029 g/L for SG) to monitor the changes in tertiary structure after the addition of the phenolic compounds. Parameters were set as follows: path length, 5 mm; step resolution, 1 nm; speed, 50 nm min^{-1}; bandwidth, 1 nm; response, 4 s; and sensitivity, 200 mdeg. Bulk solutions of quercetin (16.55 mM, 5 g/L) and those of rutin (10.53 mM, 7 g/L) and isoquercetin (10.77 mM, 5 g/L) were prepared in DMSO. The final concentrations of the phenols applied to study their effect on the secondary structure of proteins were 15, 30, 45, 60, and 75 μM. The effects of pH, ionic strength, and temperature were also studied as described under indirect binding studies on basis of quercetin fluorescence. Blanks containing corresponding amounts of phenols and those containing proteins plus the organic solvents ("controls") were also measured.

Statistical Analysis. Generally, the analysis was repeated at least four times and evaluated by their means and standard deviations. The averaged data obtained from the binding studies were used for the calculations of the binding parameters (binding constant K_D and number of binding sites n). The best fit values for binding parameters were achieved by applying nonlinear least squares regression using the

software Microcal Origin 6.0 (Microcal Software Inc., Northampton, United States).

RESULTS AND DISCUSSION

Considering the interactions between protein and phenol as a bimolecular association and phenol as the ligand = L, then a single step thermodynamic equilibrium according to the following scheme can be described

$$\text{plant phenol (L)} + \text{protein (P)} \leftrightarrow \text{plant phenol–protein complex (PL)}$$

Binding studies generally involve the determination of the association or dissociation constants (K_A or K_D) for this equilibrium and the number of the phenol molecules that bind to the different classes of binding sites present on the protein molecule. The equilibrium constants are then given by:

$$K_A = \frac{1}{K_D} = \frac{[PL]}{[L] \cdot [P]} \quad (1)$$

In eq 1, attention is drawn to [L] and [P], which means here the free concentration of the plant phenol and protein. If the specific binding B (also termed in the literature as ν, the Greek letter, or r; eq 2) is defined as the number of moles of ligand bound per mole of protein (*9*, *11*, *12*)

$$B = \frac{[PL]}{[P_{total}]} \quad (2)$$

then since $[P_{total}] = [PL] + [P]$, we get the following equation:

$$B = \frac{[PL]}{[P] + [PL]} \quad (3)$$

By substitution of eq 1 in eq 3, we get

$$B = \frac{[L]}{K_D(1 + 1/K_D[L])} = \frac{[L]}{K_D + [L]} \quad (4)$$

Because the plant phenols are small molecules (180−700 Da) and proteins are comparatively very large (14000−350000 Da), it is possible that more than one phenol molecule can bind to one protein molecule. Furthermore, taking into account that the phenol is bound at specific binding sites, a new variable n for the number of such sites is needed

$$B = n \cdot \frac{[L]}{K_D \cdot [L]} \quad (5)$$

Equation 5 applies only under those conditions, when the phenol binds to all of the binding sites with the same affinity. In case more than one class of binding sites is possible, then we have

$$B = \sum_{i=1}^{z} \frac{n_i \cdot [L]}{K_{D_i} + [L]} \quad (6)$$

where variable z is the number of the classes of specific binding sites. For every class, a particular number of binding sites n is possible for which in turn a specific dissociation constant K_D applies.

The best fit values for binding parameters were achieved by applying nonlinear least squares regression using the software Microcal Origin 6.0 according to (eq 7 for one set of binding sites and eq 8 in the case of two sets of binding sites):

$$B = \frac{n \cdot L}{K_D + L} \quad (7)$$

$$B = \frac{n_1 \cdot L}{K_{D1} + L} + \frac{n_2 \cdot L}{K_{D2} + L} \quad (8)$$

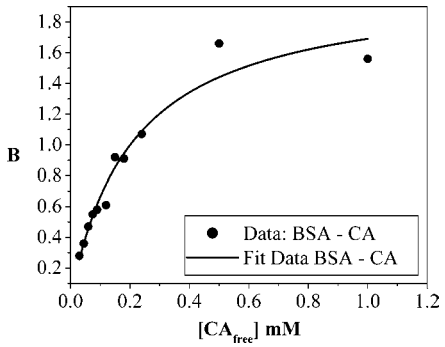

Figure 1. Determination of K_D and n using HD for the binding of chlorogenic acid (CA) to BSA at pH 7; B = mmol chlorogenic acid bound/mmol protein; model = one site binding, $\chi^2 = 0.01$, $R^2 = 0.96$, $n = 2.0 \pm 0.1$, and $K_D \times 10^{-6} = 0.21 \pm 0.03$ M.

Table 1. Dissociation Constants (K_D) and Number of Sites (n) for Binding of Selected Phenolic Compounds as Determined by the HD (Model = One Site Binding)

protein	phenol	buffer	$K_D \times 10^{-6}$ (M)	n
BSA	ferulic acid	0.05 M Hepes, pH 4.8	58 ± 5	2.2 ± 0.1
BSA	ferulic acid	0.05 M Hepes, pH 7.0	249 ± 14	3.6 ± 0.1
BSA	chlorogenic acid	0.05 M Hepes, pH 4.8	48 ± 8	1.0 ± 0.1
BSA	chlorogenic acid	0.05 M Hepes, pH 7.0	210 ± 30	2.0 ± 0.1
BSA	gallic acid	0.05 M Na-Ac, pH 3.5	2300 ± 500	14 ± 3
BSA	gallic acid	0.05 M Na-Ac, pH 4.8	1000 ± 100	5.3 ± 0.4
BSA	gallic acid	0.05 M Na-Ac, pH 6.0	380 ± 80	3.7 ± 0.4

Direct Binding Studies—HD. The specific amount of phenolic compound bound to protein (B, eq 2) was calculated using an internal calibration as described in ref *11*. In the internal calibration, a series of samples with a fixed protein concentration and increasing phenol concentrations were injected. A simple interpolation then allows the calculation of the phenol bound (*11*) (see also Supporting Information I). The value of free concentration of the phenol also needed for the evaluation of the equilibrium can be set equal to the concentrations of the phenolic compounds in the eluent buffer (*11*). The data thus obtained were evaluated by nonlinear least squares regression, and **Figure 1** shows exemplarily the binding of chlorogenic acid to BSA at pH 7. The binding parameters of all of the tested phenolic compounds determined with HD are summarized in **Table 1**. Under the conditions described in **Table 1**, it was not possible to determine the binding of quercetin, rutin, and isoquercetin to BSA due to their low (or hardly any) solubilities. Even the addition of 1% DMSO or 20% ethanol to the buffer did not give any useable results (Supporting Information II). The primary reason for the latter is in the binding of these phenolic compounds to the column material. Because the results of gallic acid in 0.05 M Hepes buffer were not reproducible due to double peaks (most likely the result of binding of gallic acid to the Hepes), an alternative buffer system (Na-acetate) had to be applied. Furthermore, at pH values at 7 and above,

gallic acid was found to oxidize visibly (coloration as a result of nonenzymatic oxidation), even though, as shown in **Table 1**, the values of K_D are not optimal. The K_D standard deviation generally underestimates the real uncertainties of the experiment, and every nonlinear least squares regression therefore actually represents the extent of the goodness of the curve fitting rather than the real error (*16*). All the same, the values of K_D can be improved by applying higher concentrations of gallic acid and by having more data points. However, because gallic acid is very sensitive to oxidation and tends to oligomerize at higher concentrations, its application under such conditions is rather limited. This especially creates problems, because the determination of a single K_D value requires the gallic acid solution in the eluent to remain stable for 24 h. Therefore, a saturation of the protein with gallic acid was tempted by decreasing the protein concentration (90 μM). This was achieved only partly as can be seen in **Table 1**. A further decrease of protein concentration (30 μM) for the conditions at pH 3.5 did not produce a saturation curve during nonlinear regression but rather a linear curve. An even further decrease of protein concentration was not possible due to the detection limit set by the detector. Nevertheless, a distinct trend for gallic acid can be noticed, where K_D values and the number of binding sites decrease with increasing pH (**Table 1**). This means the binding improves with increasing pH. The high K_D value and the high number of binding sites obtained at pH 3.5 are indicative of an unspecific binding. At this pH, the BSA molecule is supposed to attain the fast (F) form (*17*). In F form, the α-helices in domain III of the BSA molecule are unfolded causing this domain to become separated from domain II (*17*). Because there seems to be little or no specific binding of gallic acid at pH 3.5, it can be assumed that the domain III of BSA molecule seems to be the primary binding site for this phenolic compound at higher pH. A further indication is given by quenching experiments, where no change in tryptophan fluorescence of BSA with gallic acid was observed (data not shown) and in the fact that the domain III does not contain any tryptophan residues. On the other hand, at pH 3.5, all of the hydroxyl groups of gallic acid are protonized ($pK_{a1} = 6.89$; $pK_{a2} = 10.16$) (*18*). This and the fact that the binding is low at this pH underline the importance of electrostatic interactions for binding of gallic acid to BSA.

Both ferulic and chlorogenic acids bind with approximately the same affinity to BSA, which decreases with increasing pH (**Table 1**). Prigent et al. (*9*) also found on the basis of ultrafiltration experiments that at certain BSA/chlorogenic acid ratios more chlorogenic acid was bound at lower pH values, although no change was observed in values of the binding constants. The number of binding sites increases with pH and the binding of ferulic acid seems to be favored due to its smaller molecular mass (ferulic acid MW 194 Da; chlorogenic acid MW 336 Da). With approximately the same molecule size of gallic acid (MW 188 Da) as that of ferulic acid, a comparable number of binding sites (3.7) at pH 6 is also achieved (**Table 1**). Of course, besides molecule size of the ligand, other factors may also effect the binding, e.g., the protein structure. The pH-dependent change of the BSA tertiary structure causes the number of binding sites to decrease for both ferulic and chlorogenic acids. The fact that a higher affinity of these two phenolic acids is observed around the isoelectric point of BSA (IP = 5) suggests the importance of hydrophobic interactions during their binding. A further indication to the importance of hydrophobic interactions was also made for the affinity of chlorogenic acid to BSA, which increased with rising ionic strength of the buffering system (*9*). Although in the same study

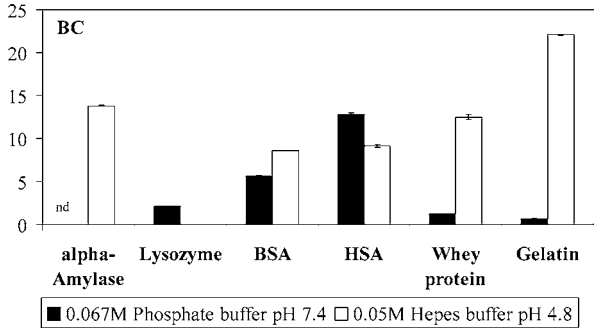

Figure 2. Binding of ferulic acid to different proteins as determined by HD (eluent B = 0.24 mM ferulic acid; protein concentration = 12 g/L; BC in mg ferulic acid bound/g protein; and n.d. = not determined).

(*9*), the affinity was found to decrease with increasing temperature. At increased temperature, the hydrophobic interactions are known to be preferred, but the conformation/tertiary structure of BSA may also be altered. In comparison, the interaction of chlorogenic acid with sunflower 11 S protein was reported to be on the basis of hydrogen bonds.

In a second series of experiments, the binding of ferulic acid to different proteins was investigated and the results are given in **Figure 2**. A buffer system (pH 4.8) corresponding to a "food technological" relevant system (e.g., wine, juices) and a second system consisting of a physiologically relevant phosphate buffer (0.067 M, pH 7.4) were applied. Ferulic acid was chosen because it is known not only to be present in many plant foods (e.g., cereals, prunes) but also to be present in intact form in blood plasma after dietary consumption of foods containing phenolic compounds (*19*, *20*). The specific amount of phenolic compound bound to protein (binding capacity, BC) was calculated using an external calibration as described in ref *11*. Depending on the buffer and protein applied, a BC of 0−22 mg/g protein was determined. α-Amylase was found not to be soluble in phosphate buffer, pH 7.4, at the required concentration, so no value was obtained here. Both food technologically relevant milk whey proteins and gelatin showed higher binding at pH 4.8, and accordingly, gelatin is also known to be applied as a fining agent in wine, fruit, and vegetable juice manufacture. Gelatin is a proline rich protein, and the affinity of such proteins toward polyphenols has already been reported in detail (*3*, *5*−*7*). However, at neutral pH, low binding of ferulic acid to these proteins was noted. Furthermore, ferulic acid also showed a high affinity toward the physiologically important proteins (BSA, HSA, lysozyme) at the corresponding relevant pH. A comparison of blood serum standard and HSA at the same concentration of HSA in both samples (7.7 g/L) gave corresponding binding of 10.3 ± 0.1 and 8.9 ± 0.1 mg ferulic acid/g protein. This confirms that HSA seems to be the major binding protein and may be termed as a "metabolite sponge" in blood serum. A direct transferability of these data to humans is not completely possible since many other factors, which may effect the binding, e.g., cell fractions and plasma proteins, are absent and are not considered. However, serum proteins may bind phenolic compounds (and their metabolites) and eventually transport them to different sites in the body, where they may then display different physiological effects, e.g., antioxidative properties.

Indirect Binding Studies. *Quercetin Fluorescence.* The main criterion in the selection of methods in the presented study was to find an appropriate possibility to evaluate the binding of quercetin and its glycosides to proteins. Quercetin is only slightly soluble in aqueous solutions (solubility < 1 g/L) and is very sensitive to oxidation. Therefore, methods that require high

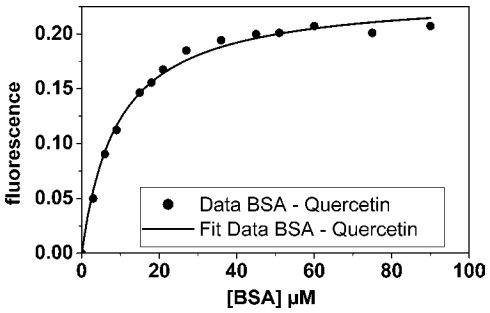

Figure 3. Determination of K_D using enhancement of quercetin fluorescence when bound to BSA. [Quercetin] = 15 μM; [BSA] = 3–90 μM; model = one site binding; $\chi^2 = 0.00001$, $R^2 = 0.99$, $F_{max} = 0.237 \pm 0.004$, and $K_D \times 10^{-6} = 9.1 \pm 0.5$ M.

concentrations or those that have long analysis times, e.g., equilibrium dialysis, are not suitable. Furthermore, it is has been reported that 60−80% of quercetin may bind to separation membranes applied in equilibrium dialysis or ultrafiltration methods (*21*). Preparative ultracentrifugation with radioactively marked quercetin is the only direct binding assay so far successfully applied to study its binding to HSA (*21*).

On the other hand, quercetin has been shown to fluoresce when excited at 370 nm (*22*). This fluorescence emission can be enhanced, when quercetin binds to proteins on the basis of resonance energy transfer. Following the equation according to ref *13* can be than applied to calculate the binding parameters:

$$\frac{1}{\Delta F} = \frac{1}{K_A \cdot \Delta F_{max}} \cdot \frac{1}{[\text{protein}_{total}]} + \frac{1}{\Delta F_{max}} \qquad (9)$$

where ΔF represents the difference between the emission of the protein−quercetin complex and the quercetin alone and ΔF_{max} is the maximum fluorescence noted when the protein becomes saturated with quercetin. Because $K_A = 1/K_D$, the equation was modified to

$$\Delta F = \frac{\Delta F_{max} \cdot [\text{protein}_{total}]}{K_D + [\text{protein}_{total}]} \qquad (10)$$

In contrast to the references mentioned above, a correction of the emission values was also made by subtracting that of protein alone, which was also found to fluoresce when excited at 370 nm (see Supporting Information IV). For calculation of ΔF, the peak height at maximum emission (526 ± 2 nm) was applied. The mean data thus obtained for different concentrations of protein added to a fixed amount of quercetin were evaluated by means of nonlinear least squares regression, and **Figure 3** shows exemplarily the binding of quercetin to BSA at pH 7. Different proteins (HSA, SG, lysozyme, trypsin) were tested, and the effects of different reaction conditions (pH, temperature, ionic strength) on the binding of quercetin to BSA were studied. The results obtained are summarized in **Table 2**. The highest affinity of quercetin was recorded for HSA, followed by SG, BSA, and finally lysozyme. The calculation of the binding constant for lysozyme was only possible after setting $\Delta F_{max} = 0.05$ (see Supporting Information IV); also, the application of other fitting models (two sets of binding sites or cooperative binding mechanism) did not give satisfactory results. The K_D value is rather high, meaning nonspecific binding, probably due to the compact globular structure of the protein and/or the strongly charged nature of the molecule under the applied conditions (pH 7; IP = 10.8). Again, the attention is drawn to the high K_D standard deviation for lysozyme, which generally underestimates the real uncertainties of the experiment as already mentioned above. In the case of trypsin, no enhancement of quercetin fluorescence was observed. Quercetin on the other hand seems to prefer to bind near the tryptophan side chains of HSA and BSA (also measured by tryptophan fluorescence quenching, Supporting Information). HSA has only one tryptophan residue, which is situated in the domain IIa and is also known to be an important part of the binding site Sudlow1 (*23*). This in turn would propose the participation of tryptophan in the necessary resonance energy transfer.

Table 2. Dissociation Constants (K_D) for Binding of Quercetin to Different Proteins Depending on the Reaction Conditions as Determined by the Enhancement of Its Fluorescence Intensity (Model = One Site Binding)

protein	conditions	$K_D \times 10^{-6}$ (M)
BSA	standard[a]	9.1 ± 0.5
BSA	pH 6	14.9 ± 0.4
BSA	pH 5	24 ± 2
BSA	T = 40 °C	10.2 ± 0.7
BSA	T = 60 °C	13 ± 1
BSA	T = 90 °C	15 ± 2
BSA	50 mM NaCl	17.3 ± 0.4
BSA	250 mM NaCl	22 ± 1
BSA	500 mM NaCl	24.0 ± 0.9
HSA	standard	1.9 ± 0.2
lysozym	standard	(2000 ± 800)
sojaglycinin	standard	4.1 ± 0.6
trypsin	standard	

[a] Means standard conditions: 0.05 M Hepes buffer, pH 7, room temperature (25 °C), and 0 mM NaCl.

The heating of BSA solutions prior to binding studies causes an increase in K_D values (**Table 2**), meaning that the affinity of quercetin decreases. Although the heating causes a stronger denaturation as documented by structural analysis with CD, the effect on the binding affinity of quercetin to BSA is more pronounced by a change in pH or ionic strength. This decrease in BC due to thermal denaturation may result from the accompanying polymerization of the BSA molecules and the resulting decrease in the surface area (*9*). The specific binding of chlorogenic acid to thermally treated BSA also showed a similar behavior (*9*). In contrast, thermally treated α-lactalbumin binds more chlorogenic acid per molecule due to exposition of other (different) amino acid residues on the surface of the polymerized protein (*9*).

A decrease in the pH leads to an increase in the K_D values, and correspondingly, the binding becomes diminished (**Table 2**). The isoelectric point of BSA lies at pH 5. The BSA molecule has an overall low negative charge (domain I, −11; domain II, −7; domain III, +1) at pH 7 and has no net charge at pH 5 (*23*). Therefore, generally at the isoelectric point, the hydrophobic interactions may be favored. Because the binding affinity of quercetin decreases with the lowering of the pH, this would mean that the hydrophobic interactions may not play a determining role. The binding studies of chlorogenic acid to BSA and sunflower proteins showed no effect on the binding constants, when the pH was reduced from 7 to 3 or 4 (*9*). The authors concluded that in this case the amino acids with pK_a values between 3 and 7 may not be involved in the BSA−chlorogenic acid interactions (*9*). In contrast, the corresponding interpretation for the BSA−quercetin model would mean that these amino acid side chains (e.g., histidin, pK_a = 5.9) are likely to be involved in their interactions. Attention is also drawn to the fact that protein-bound amino acids may differ in their pK_a values from their corresponding unbound forms and other

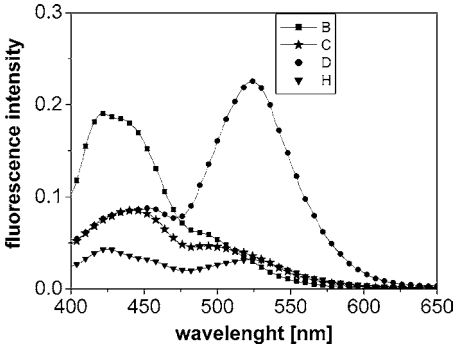

Figure 4. Confirmation of the reversibility of the noncovalent binding of quercetin to BSA by addition of urea to the reaction system; q = quercetin.

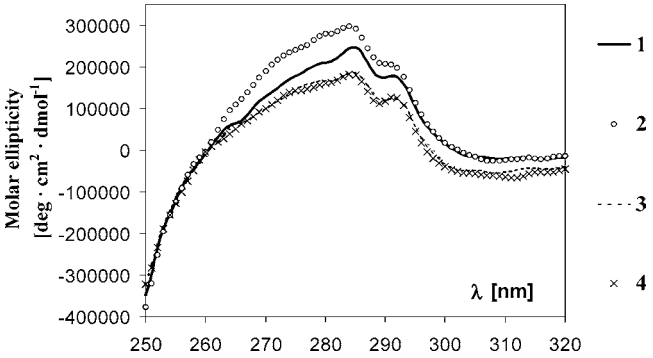

Figure 5. Near-UV-CD spectra of SG (3 μM) in the presence of phenolic compounds (90 μM). Code: 1 = SG, control; 2 = +quercetin; 3 = +rutin; and 4 = +isoquercetin.

charged amino acid residues are also likely to be involved. Because with the lowering of pH the BSA molecules get more and more positively charged, it can be concluded that quercetin has more affinity for a negatively charged molecule. Quercetin is supposed to be more or less neutral in the investigated pH range (pK_{a1} = 7.3, pK_{a2} = 8.4) (*24*). Thus, the electrostatic interactions are more likely to be on the basis of partial charges. Because the formation of hydrogen bonds may not be favored by decreasing pH, they may also be involved in the BSA−quercetin interactions.

The increase in ionic strength also leads to deterioration of the interactions between quercetin and BSA (**Table 2**). Again, the same argument as above applies here as with increasing ionic strength: The electrostatic interactions present at the neutral pH and low ionic conditions are dissolved. As the hydration hull of the protein molecule becomes stronger at higher ionic strength, this may further prevent these interactions. The change in the dissociation constant proceeds to increase with rising ionic strength in a hyperbolic trend, reaching an asymptotic value of 25 μM. The association of BSA with quercetin remains in this condition, despite forcing back of the observed electrostatic interactions. Even at the isoelectric point of BSA (pH 5), a similar K_D value of 24 μM was determined. Because of the phenolic character of quercetin, it is predestined to undergo hydrophobic interactions and to form π-bonds. These properties of quercetin may be responsible for this "residue" binding affinity. Under conditions of high ionic strength, the BSA molecule may also become unfolded, exposing the buried hydrophobic amino acid residues needed for these interactions. Both rutin and isoquercetin did not produce any enhancement of fluorescence when incubated with proteins, as was the case with quercetin.

The focus of the presented study was directed to evaluate the role of noncovalent interactions between proteins and quercetin. The proof of the nature of these interactions was given by adding urea (8 M), which is known to destroy noncovalent interactions. **Figure 4** shows exemplarily the effect produced by urea for the model system BSA−quercetin. The observed enhancement (at 526 nm) of quercetin fluorescence upon binding to BSA is removed, confirming the nature of noncovalent binding. Similar observations were also made for lysozyme, SG, and HSA (Supporting Information IV). Covalent binding is reported to take place at conditions above pH 7 (*25*, *26*). In this context, experiments were also performed to test if covalent-bound quercetin to BSA is capable of increasing fluorescence at 526 nm (Supporting Information IV). Interestingly, we observed that when the same amount of quercetin is present in the system, then the covalent-bound form is capable of producing a stronger effect on the emission as compared to the noncovalent-bound one (Supporting Information IV). So, it seems the nature of the binding (covalent or noncovalent) may affect the enhancement of quercetin fluorescence observed. Furthermore, the involvement or presence of certain protein side chains, e.g., tryptophan, within or in the vicinity of the binding site may play a role but needs to be investigated.

Structural Characterization. *Influence of Binding of Phenolic Compounds on Protein Structure.* A far-UV-CD study was conducted to evaluate the influence of binding of quercetin on the secondary structure of BSA. The far-UV spectrum of the unmodified BSA (results not shown) exhibited two negative minima at 209 and 222 nm and a maximum at 190−195 nm, which is typical of the $\alpha + \beta$ class protein (*27*). In the tested concentrations of quercetin, rutin, and isoquercetin (15−75 μM), no significant changes in the far-UV spectra were observed (results not shown). The CD spectra were analyzed by a curve-fitting program software CDPro using CONTIN, SELCON, and CDSSTR methods (Supporting Information IV) as described in refs *14* and *15*. Exemplarily, the unmodified control BSA contained according to COTIN allocation 65% α-helix, 3% β-strand, 12% β-turn, and 20% unordered structure elements (Supporting Information IV). Even under consideration of these allocations, no significant changes in the structural elements were observed by applying CONTIN and SELCON calculation methods (Supporting Information IV). The CDSSTR method showed an increase in the α-helix content, but this was found to be the effect of the solvent applied to solubilize the phenolic compound (Supporting Information IV).

The influence of the binding of quercetin, rutin, and isoquercetin on the tertiary structure of BSA (Supporting Information IV) and SG (**Figure 5**) was evaluated by means of a near-UV study. Both quercetin and rutin induced significant changes in the near-UV-CD spectra of BSA (Supporting Information IV) and SG (**Figure 5**). Isoquercetin was found to interact only with SG (**Figure 5**). The molar ellipticity was especially influenced in the wavelength range of 250−290 nm, where the changes involved may be attributed in the vicinity of tryptophan, tyrosine, phenylalanine, and disulfide bonds (*28*). However, this does not mean that these groups are directly involved in the binding of the flavonols applied. On the other hand, for BSA, the quenching of tryptophan confirmed (Supporting Information IV) the binding of quercetin in its vicinity. In conclusion, the noncovalent binding of phenolic compounds (e.g., quercetin) has no effect on the secondary structure of the proteins studied but causes significant changes in the tertiary structure. Whereas in comparison, the covalent binding may effect both secondary and tertiary structures as reported elsewhere (*25*, *29*).

Effect of the Reaction Conditions on the Structure of BSA. The effect of the reaction conditions (heating, pH value, and

Table 3. Effect of Reaction Conditions on the Allocation of the Secondary Structure Elements According to Refs *15* and *16* by Applying 48 Protein Reference Set (190–240 nm) and CONTIN Method ([BSA] = 3 μM)

	α-helix	β-strand	β-turn	unordered
		pH value		
pH 5	63.2	3.2	13.4	20.1
pH 6	67.1	3.1	12.6	17.3
pH 7	66.9	2.5	12.0	18.6
		ionic strength		
0 mM NaCl	66.9	2.5	12.0	18.6
50 mM NaCl	61.5	3.2	13.8	21.6
250 mM NaCl	61.6	2.9	14.8	20.8
500 mM NaCl	63.1	2.8	12.0	22.1
		temperature		
25 °C	66.9	2.5	12.0	18.6
40 °C	64.1	3.3	13.3	19.3
60 °C	60.7	3.2	14.4	21.7
90 °C	50.6	6.5	16.3	26.4

ionic strength) as applied during indirect binding studies using quercetin fluorescence (**Table 2**) produced changes both in the secondary as well as in the tertiary structure of BSA (**Table 3**; Supporting Information IV). A thermal denaturation (increase in the unfolded structural elements) of BSA is observed upon heating above 60 °C (**Table 3**). Investigations with differential scanning calorimetry confirm a change in conformation of BSA at 58.1 °C (*30*) and denaturation upon heating above 62 °C (*31*). This partial unfolding of the BSA molecule leads to an exposition of a cystein group (cys-34), which is then in position to undergo cross-linking by reacting with the same cystein group of another molecule (*32*). Therefore, as a result of thermal denaturation, we observe the polymerization of BSA molecules, a corresponding decrease in the exposed molecule surface, and as a consequence a diminished binding of quercetin to BSA as documented in **Table 2**.

Upon decreasing the pH from 7 to 5, a slight change in the secondary (**Table 3**) and the tertiary structure (Supporting Information) is observed. Because the isoelectric point of BSA lies at 5.1, more significant changes were expected. The structure of BSA remains stable in the pH range of 4.3−8 according to ref *32*. Below pH 4.3 (4.3−3.5), there is a transition of the N form (normal) to the F form (fast) together with an unfolding of the domain III of BSA molecule. The CD results obtained for BSA at pH 5 confirm a slight decrease in the amount of α-helix content with a parallel increase in the unfolded elements (**Table 3**). The consequences of these pH-dependent structural changes on binding affinity of quercetin to BSA have been discussed above, and the binding parameters are listed in **Table 2**.

The increase of ionic strength makes the measurement under 200 nm in the near-UV studies difficult due to the interfering effect of the increasing chloride ions. Nevertheless, the CD spectra above 200 nm were evaluated and the allocation of the secondary structure elements is given in **Table 3**. Therefore, these results should be considered with caution. It appears that the BSA molecule tends to unfold with increasing ionic strength. The unfolding of BSA leads to a diminished binding of quercetin as shown in **Table 2**. The corresponding change in tertiary structure was also observed with the first level of increase in ionic strength (50 mM NaCl), and a further increase up to 500 mM NaCl produced no further change in the far UV spectra (Supporting Information IV).

In conclusion, in general, both applied methods are suitable to explore interactions between proteins and plant phenolic compounds. Each method does not necessarily lead to a useable result. So, for example, no protein-bound quercetin, rutin, and isoquercetin could be determined with HD. The extent of binding is determined by parameters such as equilibrium binding constants, which in turn may depend on the method applied. The best agreement of the indirect method was obtained for the binding parameters of quercetin to HSA, agreeing with those reported elsewhere (*21*, *22*) using other methods (quercetin anisotropie and ultracentrifugation). Furthermore, the molecule size and structure of the proteins, but also the evaluation of the raw data obtained, plays an important role.

Table 4. Standard Gibbs Free Energy (ΔG) for Binding of Selected Phenolic Compounds as Determined Using Different Methods

			ΔG (kJ/mol)—method applied	
protein	phenol	pH	quercetin fluorescence	HD method
BSA	quercetin	7.0	–30.1	
lysozym	quercetin	7.0	–16.8	
sojaglycinin	quercetin	7.0	–32.2	
HSA	quercetin	7.0	–34.0	
BSA	ferulic acid	4.8		–25.6
BSA	ferulic acid	7.0		–22.0

On the basis of the binding constants obtained, it is possible to calculate the standard Gibbs free energy as documented in **Table 4** (Supporting Information III, eq 9). The free enthalpy is negative for the studied binding constants, indicating the "freeness" of the interactions taking place between the proteins and the phenolic compounds. The relatively strong binding enthalpy obtained for HSA− and BSA−quercetin complexes underlines the stability of these complexes from the energetic point of view.

Because the intake of phenolic compounds is associated with many beneficial effects, it is also necessary to consider their transport in blood by plasma proteins. In this case, the binding to serum proteins is likely to play an important role. The high affinity binding sites of BSA and HSA for quercetin underline the fact that a high amount of quercetin can be bound in an organism. Our studies show that this binding to BSA and HSA is influenced by a series of factors, where, e.g., increasing temperature and ionic strength as well as decreasing pH may lead to diminishing binding. The structure of the proteins as determined by CD indicates changes in the tertiary structure with the secondary structure remaining intact. Eventually, protein binding may protect against the prooxidative consequences of high phenol doses as found in supplementary plant foods.

ABBREVIATIONS USED

CD, circular dichroism; HD, Hummel−Dreyer method; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SG, soy glycinin; UV, ultraviolet absorption.

Supporting Information Available: Principles of the HD method, binding studies with BSA, determination of ferulic acid bound, theory to the calculation of the binding parameters, fluorescence emission spectra, graphs of determination of K_D, effect of urea on the quercetin fluorescence, resonance energy transfer comparison, quenching of tryptophan fluorescence, assignment of secondary structures, and near-UV-CD spectra of BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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