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Efficacy of Food Proteins as Carriers for Flavonoids

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Supporting Information

ABSTRACT: Enrichment of flavonoids in food is often limited by their off-tastes, which might be counteracted by the use of food proteins as carriers of flavonoids. Various milk proteins, egg proteins, and gelatin hydrolysates were compared for their binding characteristics to two flavan-3-ols. Among the proteins tested for their affinities toward epigallocatechin gallate (EGCG), β-casein and gelatin hydrolysates, in particular fish gelatin, were found to be the most promising carriers with an affinity on the order of 10^4 M^{-1}. A flexible open structure of proteins, as present in random coil proteins, was found to be important. The saturation of binding observed at high flavonoid/protein ratios was used to estimate the maximal binding capacity of each protein. To reach a daily intake of EGCG that has been associated with positive health effects, only 519 mg of gelatin B and 787 mg of β-casein were required to complex EGCG on the basis of their maximal binding capacity. When the absence of turbidity is taken into account, β-casein prevails as carrier. Three selected proteins were further investigated for their binding potential of representative flavonoids differing in their C-ring structure. An increase in hydrophobicity of flavonoids was related to a higher affinity for proteins, and the presence of a gallic acid ester on the C-ring showed an overall higher affinity.

KEYWORDS: *flavonoid−protein interaction, β-casein, gelatin, EGCG, galloylation, ultrafiltration, ITC*

INTRODUCTION

Due to their beneficial health effects, mainly cardioprotective and anticarcinogenic, flavonoids are considered as functional dietary ingredients.[1] Most of these compounds, however, are bitter and/or astringent. Enhancing their contents in foods may result in off-tastes and, therefore, low consumer acceptance.[2] Astringency in foods can be reduced via the use of proteins able to complex flavonoids. A typical example is the addition of milk to tea, which has been shown to result in complexation of milk proteins and tea catechins without impairing the bioavailability of the catechins.[3,4] Milk proteins also have been reported as possible carriers for bioactive compounds.[5]

Interactions between flavonoids and proteins have been extensively reported, but the emphasis was predominantly on binding of flavonoids to serum albumins (e.g., Dufour and Dangles[6] and Ishii et al.[7]). A particular interest has also been on polyphenols and proteins involved in haze formation in beverages (e.g., Siebert[8]). Interaction of flavonoids with common food proteins, however, is less reported, with mainly a few studies on flavanols interacting with ovalbumin, gelatin, α-lactalbumin, and lysozyme[9,10] or milk proteins, such as β-casein[11,12] and β-lactoglobulin.[13,14] To our knowledge, no comparison of common food proteins for their potential binding characteristics with flavonoids has been made within a single study.

Several critical structural features of proteins for binding to flavonoids have been highlighted in the literature. The amino acid composition is a major factor, as prolines are known to be involved in nonspecific interactions primarily via ring stacking with their prolyl residues and with a preference for Pro-Pro repeats.[8,15,16] Other amino acids, such as phenylalanine, tyrosine, arginine, and histidine, have also been suggested to interact with flavonoids.[15,17,18] In addition, the presence of bulky amino acid residues close to potential binding sites can reduce their accessibility.[17] The conformation of the protein is of critical importance as random coil proteins have been shown to display a higher interaction with tannins than globular proteins.[19,20] Proteins were shown to display an increased binding affinity at a pH close to their p*I*.[10]

The use of various food proteins as carriers for flavonoids could help the development of functional foods by targeted delivery of bioactive compounds to the gut without sensory defects. The strength of the interaction should be high enough during the residence time in the mouth so as to limit interaction with bitter taste receptors[21] and with salivary proteins involved in astringency perception.[22] The proteins themselves should not impair the taste of foods. Therefore, animal-derived proteins are preferred over plant-derived proteins because of their generally known bland taste. Interaction of proteins with proanthocyanidins and monomeric flavan-3-ols (e.g., catechin, epigallocatechin gallate (EGCG)) is of particular interest because of their relevance for technological and organoleptic properties in beverages (e.g., tea, wine, or beer). In the present study, EGCG and catechin are used as reference ligands to compare a broad range of food proteins for their binding capacity to flavonoids. The diversity of potential animal-derived food proteins to be used as carriers and the large structural diversity of potential flavonoids to be selected, in addition to the reference ligands, limit the use of laborious methods, such as fluorescence quenching (e.g., Dufour and Dangles[6]) or isothermal titration calorimetry (ITC) (e.g.,

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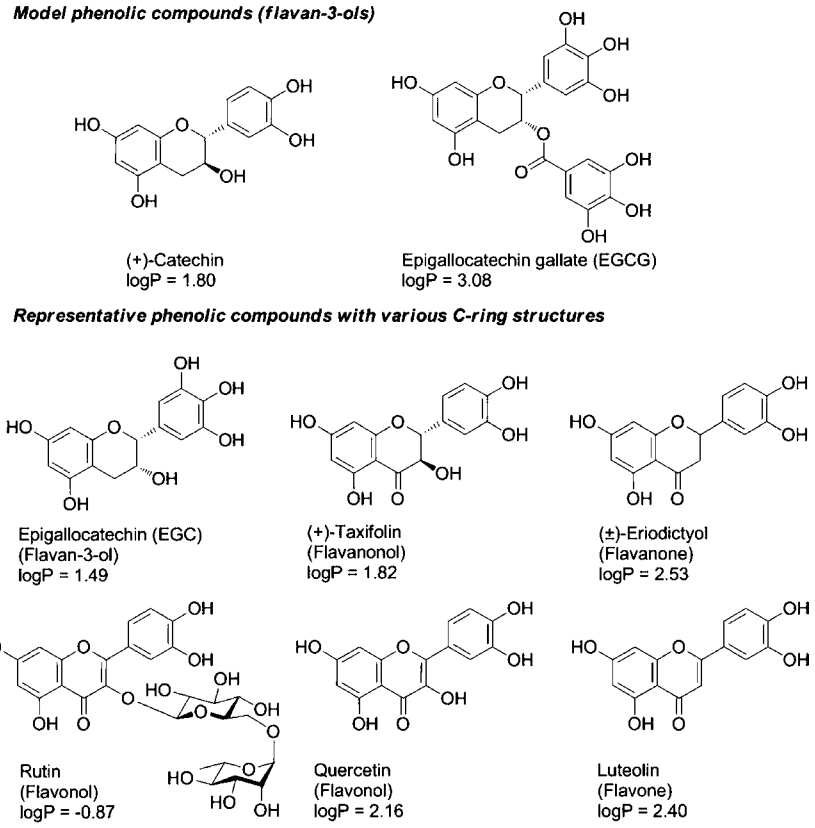

Figure 1. Chemical structures of the flavonoids investigated.

Poncet-Legrand et al.[23]). Among appropriate methods available, ultrafiltration (UF) seems to be the most versatile and simplest method for the comparison of various animal-derived food proteins binding flavonoids. Our objectives were to first investigate which animal-derived food proteins had the most potential as carriers of EGCG and catechin and, next, to study these most promising proteins for their binding potential to representative compounds from several classes of flavonoids.

MATERIALS AND METHODS

Materials. *Phenolic Compounds.* Rutin hydrate (95%), quercetin dihydrate (99%), and (+)-catechin hydrate (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-Taxifolin (>90%) and (±)-eriodictyol (racemic mixture; >90%) were purchased from Extrasynthèse (Genay, France). Luteolin (99%) was purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). EGCG (Teavigo, ≥85%) was kindly provided by DSM Food Specialties (Delft, The Netherlands). All aforementioned phenolic compounds are detailed in Figure 1 regarding their structures and log *P* values. Log *P* values were estimated by the software Marvin (Chemaxon).

Proteins. Calcium-depleted type III bovine α-lactalbumin (≥85%), BSA (Cohn V fraction, ≥96%), bovine β-casein (≥98%), bovine β-lactoglobulin (≥90%), solid fish gelatin (gelatin F) from cold-water fish skin, gelatin type A (Gelatin A) from porcine skin (90−110 bloom), gelatin type B (gelatin B) from bovine skin (75 bloom), lysozyme from chicken egg white (≥90%), ovalbumin from chicken egg white (grade V, ≥98%), and phosvitin from chicken egg yolk were purchased from Sigma-Aldrich. The protein purity of the three gelatins was estimated to be ≥90% on the basis of the sum of amino acid residue weights as detailed later in this section. The protein purity of phosvitin was estimated to be ∼75% on the basis of the ratio between the experimental nitrogen content determined by the Dumas method (%N (w/w) = 9.29) and the theoretical nitrogen content (%N (w/w) = 12.43). The latter was calculated from the amino acid composition of phosvitin given in the UniProtKB entry P0245 (uniprot.org), corrected for the phosphorylation and glycosylation of phosvitin.

Other Chemicals. Tannase (γ-tannase, Gammazyme) was purchased from Gamma Chemie (Darmstadt, Germany). Methanol (MeOH, analytical grade) was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of Epigallocatechin (EGC). EGC was prepared by a tannase treatment of EGCG. EGCG (2 g/L) was dissolved in a 0.1 M sodium acetate buffer, pH 5.0. The tannase was subsequently added (final concentration = 80 mg/L), and the headspace of the flask was flushed with N_2. The reaction flask was incubated in the dark at 30 °C under continuous stirring. After 24 h, a fresh amount of enzyme (final concentration = 80 mg/L) was added. After 48 h, the sample was treated by solid phase extraction (SPE) using a 10 g C18 Sep-Pak column washed and eluted with water and MeOH according to the instructions of the manufacturer (Waters, Milford, MA, USA). The MeOH fraction was evaporated, dissolved in water, and freeze-dried.

The freeze-dried material was dissolved in water/acetonitrile/acetic acid (99:1:0.1, v/v/v) at a concentration of ∼50 mg/mL. Next, it was purified by flash chromatography with a 12 g Reveleris C18 column on a Reveleris flash system (Grace, Deerfield, IL, USA) operated at 30 mL/min. The eluents used were water/acetonitrile/acetic acid (99:1:0.1, v/v/v) (eluent A) and acetonitrile/acetic acid (100:0.1, v/v) (eluent B). The elution profile was 0−1 min, 0% B; 1−11 min, 0−35% B; 11−12 min, 35−100% B; 12−15 min, 100% B. Three fractions were generated and analyzed by RP-UHPLC-MS as described elsewhere.[24] The fractions contained gallic acid, EGC, and possible oxidation products from the sample treatment. The EGC fraction showed a single peak in UV with a *m*/*z* value corresponding to EGC (*m*/*z* 305 in MS in negative mode). Throughout the procedure the sample was kept from light by using aluminum foil.

Amino Acid Analysis and Protein Content of Gelatins. The amino acid analysis of the gelatin samples (A, B, and F) used in this

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study was conducted by Ansynth Service BV (Berkel en Roodenrijs, The Netherlands). Most amino acids were analyzed after acid hydrolysis (6 M HCl, 22 h, 110 °C) by classical ion-exchange liquid chromatography with postcolumn ninhydrin derivatization and detection at 440 or 570 nm using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, U.K.). An oxidation step (performic acid, 16 h, 0−5 °C) was included prior to the acid hydrolysis to separately quantify cysteine and methionine. Tryptophan was analyzed after alkaline hydrolysis (4.2 M NaOH, 22 h, 110 °C) by reversed phase HPLC using a Beckman Gold HPLC system (Beckman Coulter Inc., Brea, CA, USA) with fluorometric detection. The sum of amino acid residue weights (% (w/w)) was calculated to evaluate the protein content of the samples. Amino acid contents reported in the present study were also corrected for the water contents of the gelatin samples estimated by oven-drying (5.1% (w/w) for gelatin A, 5.7% (w/w) for gelatin B, and 6.9% (w/w) for gelatin F).

Circular Dichroism (CD). Gelatins were analyzed by far-UV CD on a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA). A quartz cell (path length = 1 mm) was filled with 0.1 mg/mL solution of each gelatin in 50 mM sodium phosphate buffer, pH 7.0. Spectra were recorded from 190 to 260 nm with a bandwidth of 2 nm, a scanning speed of 100 nm/min, and an accumulation of 10 scans. Each sample was measured at 4, 20, and 70 °C considering that gelatin would be fully structured at 4 °C (reference 100% structure) and totally denatured at 70 °C (reference 0% structure).[25] Spectra were corrected for buffer signal using Jasco Standard Analysis software. The intensities of the signals at 200 nm for samples measured at 4 and 70 °C were used to evaluate the proportion of structure present in gelatins at 20 °C (see the Supporting Information).

Dynamic Light Scattering (DLS). β-Casein was analyzed by DLS on a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm and a back scattering angle of 173°. The protein was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and diluted to a range of concentrations (6.25, 12.5, 25, 50, and 100 μM). Measurements were carried out at 25 °C.

Binding Affinity by Ultrafiltration. All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (50 μM) were prepared freshly before each experiment. Similarly, stock solutions (6 mM, in the same buffer) of catechin and EGCG were used to obtain a range of dilutions between 0 and 6 mM. The stock solution of taxifolin was prepared in MeOH and then diluted to 10% (v/v) MeOH with the above-mentioned buffer to obtain the aforementioned range of concentrations. Other flavonoids tested were dissolved in DMSO prior to use.

Using a microtiter plate, flavonoid solutions were mixed 1:1 with the protein stock solution (final volume = 300 μL) to obtain polyphenol to protein molar ratios ranging from 0 to 120. In the case of flavonoids dissolved in DMSO, these were directly added to the protein solution in a volume ratio leading to the same aforementioned range of concentrations and with a final cosolvent concentration of maximum 4% (v/v). At their final concentrations, the amounts of cosolvent (10% (v/v) MeOH or 4% (v/v) DMSO) have negligible effects on the structure of the proteins as verified for BSA by fluorometry at 280 nm (emission spectrum 290−500 nm) and circular dichroism for 10% MeOH (data not shown). The concentration of each cosolvent was kept as low as possible, but their possible influence on the binding affinities measured cannot be excluded.

The microtiter plate was incubated in the dark for 10 min at 25 °C under continuous shaking at 300 rpm (Thermomixer comfort, Eppendorf AG, Hamburg, Germany). Next, the samples were pipetted into an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) and centrifuged (30 min, 1425g, 25 °C). The filtrates, containing the unbound flavonoid fraction, were subsequently diluted 10−20 times with the buffer and measured at 280 nm. For each set of samples, a calibration curve per flavonoid was made using ultrafiltered blanks. In addition, possible contaminations of the filtrate by proteins were systematically checked with protein controls (no flavonoid added) measured at 280 nm after ultrafiltration.

The protein-bound and free fractions of each flavonoid at each concentration tested were calculated, and plots of the bound fraction against the free fraction were made. For each binding curve obtained, a linear regression was used on the initial linear increase ($R^2 > 0.8$) to estimate the binding affinity (K) of the compounds. A maximal binding capacity (R_{max}) was derived from the plateau value or the highest bound fraction observed at high phenolic compound/protein molar ratios.

Isothermal Titration Calorimetry (ITC). Several samples analyzed by the ultrafiltration assay were further investigated by ITC. All measurements were conducted in duplicate on a MicroCal ITC_{200} microcalorimeter (GE Healthcare, Piscataway, NJ, USA) at 25 °C. EGCG or catechin (8 and 14 mM, respectively) was titrated into the measurement cell (V = 200.1 μL) containing the protein (0.025 mM). Each titration consisted of up to two series of 49 injections of 0.8 μL. The time between injections was set at 4 min, and the sample was continuously stirred at 600 rpm.

Raw data were integrated peak-by-peak to obtain a plot of observed enthalpy change (ΔH) versus the polyphenol to protein molar ratio. Control titrations of EGCG or catechin into buffer were performed for data correction. Experimental data were fitted using the "one set of sites" and "two sets of sites" models provided by the equipment supplier in Origin 7.0 (OriginLab, Northampton, MA, USA). The equations used in both models are available in the Supporting Information. The fitting provided one or two sets of parameters depending on the model used: n (the number of binding sites), K (the binding constant (in M^{-1})), and ΔH (the change in enthalpy (in kJ mol^{-1})). Changes in Gibbs free energy (ΔG) and entropy (ΔS) were calculated for each set of fitting parameters using the standard equation $\Delta G = -RT \ln K = \Delta H - T\Delta S$, where T is the temperature in Kelvin and R = 8.32 J mol^{-1} K^{-1}.

RESULTS

Binding Characteristics of Common Animal-Derived Food Proteins with Catechin and EGCG. A range of animal-derived food proteins commonly used as food ingredients was screened for their binding potential to catechin and EGCG. Typical binding curves obtained by ultrafiltration are shown in Figure 2, and the corresponding affinity values are presented in

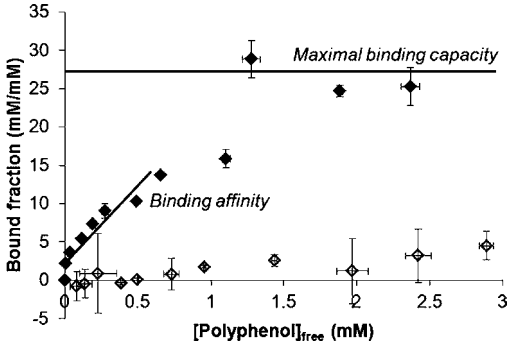

Figure 2. Typical binding curve obtained by ultrafiltration: BSA−EGCG (◆) and BSA−catechin (◇) in 50 mM sodium phosphate buffer, pH 7.0.

Figure 3A and Table 3. It is clear that the affinities of most proteins toward EGCG exceeded those toward catechin. Only in the case of lysozyme, no clear difference in affinity could be observed. Hence, the comparison was further made based on data obtained with EGCG. Proteins displaying the highest affinities were gelatin F and β-casein, followed by gelatin A and gelatin B (Figure 3A).

Four of the proteins tested covering a range of affinities to EGCG were also measured with ITC as summarized in Table 1. β-Lactoglobulin was chosen as a representative of the proteins

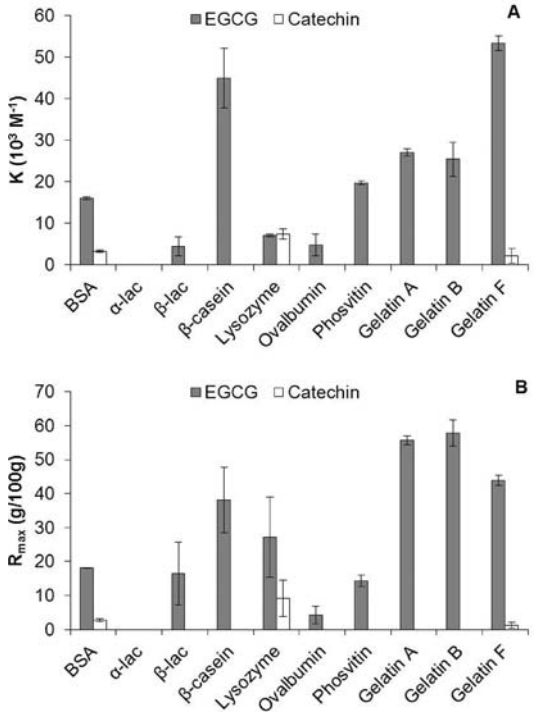

Figure 3. Binding affinity (A) and maximal binding capacity (B) for the interaction of EGCG and catechin with various animal-derived food proteins at pH 7.0 (α-lac, α-lactalbumin; β-lac, β-lactoglobulin).

displaying low binding affinity to EGCG and compared to BSA, gelatin B, and β-casein. The ITC data confirmed the outcome of the UF assay. At the protein concentration used, no clear heat signal related to binding could be observed for all proteins titrated with (+)-catechin, which indicates a low binding affinity. A similar observation was made for β-lactoglobulin titrated with EGCG. The calculated binding affinities by ITC were generally on the same order of magnitude as those obtained by ultrafiltration for BSA and gelatin B. For β-casein, a lower binding affinity to EGCG by ITC was calculated. In terms of stoichiometry, the UF assay (Table 3) and ITC were in good agreement for BSA and β-casein, although different for gelatin B. It is noteworthy that, except for β-lactoglobulin, all binding isotherms for proteins interacting with EGCG could be fitted with a two sets of sites binding model. This model was found to give the best fit, although it was designed for pharmaceutical compounds with a more specific binding in simpler systems. It does not take into account the possibility of nonspecific polyphenol−polyphenol interactions on the surface of the protein or complex structural arrangements of proteins, such as micelles of β-casein. The latter might explain the discrepancy between ITC and the UF assay found in the present study. More complex models (e.g., Buurma and Haq[26]) should be considered for further detailed analysis of ITC data in protein−polyphenol interaction. The thermodynamic parameters calculated for BSA and β-casein indicated a predominance of hydrogen bonding (ΔH_1) in the first binding event, whereas hydrophobic interactions ($-T\Delta S_1$) dominated in the case of the first binding event of gelatin B.

Phosvitin had an affinity for EGCG comparable to that of BSA (Figure 3A). This protein contains around 50% serine in its sequence, of which most, if not all, are phosphorylated. These phosphoserines are organized by blocks of up to 14 residues and make phosvitin a strong metal ion chelating agent, especially for iron.[27] Dialysis of this protein against subsequently EDTA and water was performed to remove the iron. This treatment resulted in a large decrease in binding affinity of EGCG to phosvitin, as observed by UF (from $(19.7 \pm 0.4) \times 10^3$ to $(5.6 \pm 0.9) \times 10^3$ M^{-1}) and ITC (data not shown). The presence of iron on the surface of the protein seemed to be critical to the binding of EGCG, which is considered to be related to the metal ion binding capacity of flavonoids.[28]

Proteins showing the highest affinity did not necessarily bind the most EGCG on a weight basis as can be seen in Figure 3B and Table 3. In this case, gelatin B and gelatin A had the highest binding capacity followed by gelatin F, β-casein, and lysozyme. With the aim to use proteins as carriers for flavan-3-ols, proteins combining a high binding affinity with a high binding capacity seem to be most appropriate, as is the case for β-casein and gelatin F, followed by gelatin A and gelatin B. Turbidity could be clearly observed for the three gelatins at polyphenol/protein molar ratios >20, whereas no precipitation occurred with β-casein. β-Casein was selected as the best carrier for further study and compared with gelatin B, which had a similar average molecular mass and a higher maximal binding capacity despite its lower binding affinity. BSA was used for further studies as a representative globular protein as it had one of the

Table 1. Interaction of EGCG and Catechin with Selected Food Proteins by ITC at pH 7.0 and 25°C[a]

	BSA		gelatin B		β-casein		β-lactoglobulin	
	EGCG[b]	catechin[c]	EGCG[b]	catechin	EGCG[b]	catechin	EGCG	catechin
n_1	2.9(±0.4)	6.6(±1.5)	24.5(±4.7)	nd	2.4(±0.8)	nd	nd	nd
K_1 (10^3 M^{-1})	29.5(±14.1)	0.5(±0.1)	68.6(±10.1)	nd	0.3(±0.1)	nd	nd	nd
ΔG_1 (kJ mol^{-1})	−25.3(±1.2)	−15.5(±0.4)	−27.5(±0.4)	nd	−13.8(±1.1)	nd	nd	nd
ΔH_1 (kJ mol^{-1})	−26.6(±1.1)	−18.1(±1.2)	−1.8(±0.2)	nd	−461.2(±92.1)	nd	nd	nd
$-T\Delta S_1$ (kJ mol^{-1})	1.3(±0.1)	2.6(±0.8)	−25.8(±0.2)	nd	447.4(±91.0)	nd	nd	nd
n_2	107(±7.1)	na	55.4(±18.0)	nd	19.1(±0.4)	nd	nd	nd
K_2 (10^3 M^{-1})	0.8(±0.3)	na	0.8(±0.2)	nd	8.9(±4.3)	nd	nd	nd
ΔG_2 (kJ mol^{-1})	−16.6(±0.8)	na	−16.5(±0.7)	nd	−22.4(±1.2)	nd	nd	nd
ΔH_2 (kJ mol^{-1})	−4.4(±0.5)	na	−22.2(±14.8)	nd	−24.7(±4.1)	nd	nd	nd
$-T\Delta S_2$ (kJ mol^{-1})	−12.2(±1.4)	na	−5.7(±14.1)	nd	2.4(±5.4)	nd	nd	nd

[a] n_i, stoichiometry; K_i, binding constant; ΔG_i, Gibbs free energy; ΔH_i: enthalpy; $-T\Delta S_i$, entropic contribution; nd, not detectable; na, not applicable. [b] ITC data derived from "two sets of sites" model. [c] ITC data derived from "one set of sites" model.

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highest affinities and binding capacities among all globular food proteins tested.

Influence of C-Ring Structure of Flavonoids on Their Interaction with Selected Proteins. The three proteins selected were tested for their affinities toward various flavonoids (Figure 1). These compounds were selected because they had structural variations only on their C-ring compared to catechin and their A- and B-rings carried the same number of hydroxyl groups.

The binding constants for BSA, β-casein, and gelatin B of the flavonoids selected are summarized in Figure 4 and Table 3.

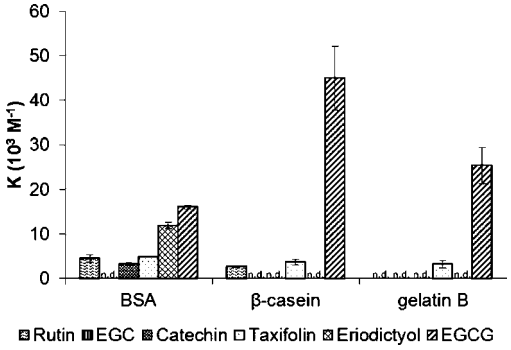

Figure 4. Interaction of flavonoids with various C-ring substitutions with BSA, β-casein, and gelatin B at pH 7.0 measured by ultrafiltration (n.d., not detected).

Only BSA showed binding curves with low standard deviations for all compounds. Contrary to what was observed for EGCG, none of the proteins tested displayed a clearly measurable interaction with epigallocatechin (EGC), catechin, and taxifolin. This emphasizes the importance of the extra gallic acid group in EGCG to enhance the affinity to proteins. In addition, a higher binding affinity was measured in the case of eriodictyol binding to BSA, whereas no interaction was detected for the same compound with β-casein and gelatin B. No binding curve could be obtained by ultrafiltration for quercetin and luteolin because of their low solubility in an aqueous environment despite the use of up to 5% (v/v) DMSO as a cosolvent. Rutin was found to have an affinity similar to that of catechin and taxifolin for BSA and β-casein.

DISCUSSION

Influence of Amino Acid Composition on the Potential of Food Proteins as Carriers. The most potent proteins as carriers for EGCG are also the ones that have the highest content in proline (Table 2). It is commonly accepted that proline residues play a key role in protein−polyphenol interactions.[8,15] However, gelatins A and B have higher total proline contents (sum of proline and hydroxyproline) than β-casein, but displayed a lower affinity to EGCG than the latter. Aromatic amino acid residues have also been found to interact by hydrophobic interaction with phenolic compounds.[15] Histidine residues have also been considered as possible binding sites[18] although contradictory results were later found.[15] β-Casein has a higher content in aromatic amino acids and histidine than the gelatins (Table 2), which might explain our observation. For random coil proteins (i.e., β-casein, phosvitin, and gelatins A, B, and F), only weak correlations were calculated in an attempt to estimate the linear relationship between their binding affinities for EGCG and their relative contents (% mol/mol) in hydrophobic amino acids and prolines. The differences in binding affinities for EGCG of random coil proteins cannot be solely explained by their content in these amino acids. For globular proteins, this analysis was not done, as many amino acids are less freely accessible.

Influence of Protein Structure on Its Potential as Flavonoid Carrier. Among the proteins tested, it appeared that the most potent proteins are the ones having a random coil or random/helical structures (Table 2). This correlates with studies investigating the binding of random coil and/or globular proteins to tannins, also in terms of order of magnitude of the

Table 2. Summary of Protein Properties[a]

protein (UniprotKB entry)	source	general structure	molecular mass (kDa) (no. of amino acid residues)	p*I*	S−S bridges	% proline[b] (no.)	% hydroxyproline[b] (no.)	% histidine[b] (no.)	% aromatic amino acids[b,c] (no.)
α-lactalbumin (P00711)	bovine	globular	14.2 (123)	4.80	4	1.6 (2)		2.4 (3)	9.8 (12)
β-casein (P02666)	bovine	random	23.6 (209)	5.13		16.7 (35)		2.4 (5)	6.7 (14)
β-lactoglobulin (P02754)	bovine	globular	18.3 (162)	4.83	2	4.9 (8)		1.2 (2)	6.2 (10)
BSA (P02769)	bovine	globular	66.4 (583)	5.60	17	4.8 (28)		2.9 (17)	8.4 (49)
gelatin type A (acid-cured)[d]	porcine	random/ helical	20−25 (283)	7−9		11.4 (32)	9.5 (27)	0.6 (2)	1.6 (5)
gelatin type B (lime-cured)[d]	bovine	random/ helical	20−25 (295)	4.7−5.2		11.4 (33)	9.5 (28)	0.4 (1)	1.4 (4)
gelatin F[d]	fish	random/ helical	60 (720)	6		9.4 (68)	5.6 (40)	0.7 (5)	1.5 (11)
lysozyme (P00698)	chicken	globular	14.3 (129)	9.32	4	1.6 (2)		0.8 (1)	9.3 (12)
ovalbumin (P01012)	chicken	globular	44.5 (385)	5.19	1	3.6 (14)		1.8 (7)	8.6 (33)
phosvitin (P02845)	chicken	globular/ random[e]	34.0 (217)	~4[e]		1.4 (3)		6.0 (13)	1.4 (3)

[a]Data are derived from the UniprotKB database (www.uniprot.org). [b]Percent of amino acid residues is given as amino acid residues per 100 residues. [c]Sum of phenylalanine, tryptophan, and tyrosine. [d]Molecular weight and p*I* based on commercial data available at Sigma-Aldrich; amino acid composition determined experimentally. [e]Structure of phosvitin is pH-dependent;[34] p*I* derived from ref 35.

Table 3. Summary of Interactions between Tested Flavonoids and Various Food Proteins by Ultrafiltration at pH 7.0[a]

flavonoid		BSA	α-lactalbumin	β-lactoglobulin	β-casein	lysozyme	ovalbumin	phosvitin	gelatin A	gelatin B	gelatin F
EGCG	K (10^3 M^{-1})	16.0(±0.4)	nd	4.5(±2.3)	45.0(±7.2)	7.1(±0.3)	4.7(±2.6)	19.7(±0.4)	27.1(±0.8)	25.4(±4.1)	53.3(±1.8)
	R_{max} (mol/mol)	26.3(±0.2)	nd	6.6(±3.7)	19.6(±4.9)	8.5(±3.7)	4.1(±2.5)	10.7(±1.3)	30.4(±0.7)	31.6(±2.1)	57.5(±1.8)
	R_{max} (g/100 g)	18.1(±0.1)	nd	16.5(±9.2)	38.1(±9.6)	27.2(±11.8)	4.2(±2.6)	14.4(±1.7)	55.7(±1.3)	57.8(±3.8)	43.9(±1.4)
EGC	K (10^3 M^{-1})	nd			nd					nd	
	R_{max} (mol/mol)	nd			nd					nd	
	R_{max} (g/100 g)	nd			nd					nd	
catechin	K (10^3 M^{-1})	3.2(±0.3)	nd	nd	negligible	7.4(±1.2)	nd	negligible	nd	nd	2.1(±1.7)
	R_{max} (mol/mol)	6.4(±1.0)	nd	nd	negligible	4.6(±2.6)	nd	negligible	nd	nd	2.7(±1.8)
	R_{max} (g/100 g)	2.8(±0.4)	nd	nd	negligible	9.2(±5.3)	nd	negligible	nd	nd	1.3(±0.9)
taxifolin	K (10^3 M^{-1})	4.8(±0.0)	2.3(±0.8)	nd	3.7(±0.7)	4.0(±0.4)	2.4(±1.1)	negligible	1.4(±0.2)	3.1(±0.8)	3.4(±0.3)
	R_{max} (mol/mol)	12.6(±3.2)	5.2(±0.7)	nd	7.3(±1.0)	7.3(±0.8)	5.6(±2.8)	negligible	3.8(±0.4)	7.8(±3.3)	7.7(±0.5)
	R_{max} (g/100 g)	5.8(±1.5)	11.1(±1.5)	nd	9.4(±1.3)	15.4(±1.7)	3.8(±1.9)	negligible	4.6(±0.5)	9.5(±4.0)	3.9(±0.3)
eriodictyol	K (10^3 M^{-1})	11.8(±0.7)			nd					nd	
	R_{max} (mol/mol)	8.8(±0.3)			nd					nd	
	R_{max} (g/100 g)	3.8(±0.1)			nd					nd	
rutin	K (10^3 M^{-1})	4.5(±0.9)			2.7(±0.1)					nd	
	R_{max} (mol/mol)	6.3(±1.1)			4.3(±0.3)					nd	
	R_{max} (g/100 g)	5.8(±1.0)			11.1(±0.7)					nd	

[a]Data are the mean ± SD values of two replicates; nd, not detectable.

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binding affinity.[11,19,20] In globular proteins, the reduced accessibility of amino acid residues often implicated in binding polyphenols is suggested to be responsible for their overall lower affinity observed compared to that of random coil proteins. BSA is an exception in this respect as it is known to have specific polyphenol binding cavities (e.g., Dufour and Dangles[6]).

Although the proline content is frequently reported as a key factor in protein−polyphenol interactions, gelatins A and B displayed a lower binding affinity to EGCG compared to β-casein and gelatin F, despite their higher total proline contents (sum of proline and hydroxyproline). Proline repeats induce a helical structure in the gelatin monomer (polyproline II helix).[16] This structure reduces the flexibility in the protein backbone and, thereby, its capacity to interact with ligands.[17] In addition, hydroxylation of prolines enhances both the formation and the stability of triple-helix structures by hydrogen bonding, thereby possibly lowering ligand binding.[19,29] CD spectra of the gelatin samples showed more residual triple-helix structures at room temperature for gelatins A and B (100 and 68%, respectively) compared to gelatin F (56%, see the Supporting Information), thereby possibly explaining the higher binding affinity observed for gelatin F. This structural difference can be explained by the different critical helix-to-coil temperatures of the gelatins, which are ∼36 °C for mammalian gelatins and between 15 and 20 °C for cold-water fish gelatin.[30] The reduced flexibility of gelatins A and B is thought to hinder the availability of binding sites for EGCG compared to gelatin F. In the case of β-casein, prolines are scattered along its chain and probably do not influence its flexibility.

β-Casein is known to form micelles (hydrodynamic radius ∼12 nm) by reversible association at low concentration (critical micelle concentration of ∼21 μM).[31] In the present study, micelles (hydrodynamic radius ∼ 13 nm) were detected with DLS at concentrations exceeding 25 μM. A shift to monomers with remaining micelles was observed at a concentration of 25 μM, although that concentration was too low for an accurate measurement of the particle sizes (data not shown). The involvement of part of the β-casein in micelles is thought to influence its interaction with EGCG. In fact, it could reduce the accessibility of its amino acid residues, thereby reducing the interaction. On the contrary, it could provide a hydrophobic environment inside the micelles, which could also positively influence the interaction. Nevertheless, β-casein displayed one of the highest affinities to EGCG as measured by the UF assay.

Influence of the C-Ring Structure of Flavonoids on Their Binding to Selected Proteins. The hydrophobicity of the compounds tested (Figure 1) could be related to their binding affinity to BSA (Figure 4), which is in line with observations on binding of various flavan-3-ols to poly(L-proline).[23] Small variations in hydrophobicity resulting from minor changes in the C-ring structure of the phenolics could neither be linked to changes in binding affinity to BSA nor to β-casein and gelatin B. Therefore, at this stage, animal-derived food proteins do not appear to be suitable carriers for monomeric flavonoids.

Variations in the C-ring of flavonoids involve substitutions on the C(3) position, for example, glycosylation or galloylation. Rutin (glycosylated quercetin) is more hydrophilic than its aglycone and displayed a low binding affinity to BSA, consistent with Dufour and Dangles.[6] On the contrary, C(3) galloylation of EGCG increased both the hydrophobicity of the flavonoid and its ability to form hydrogen bonds, which resulted in an overall higher binding affinity to the proteins tested, consistent with previous studies.[11,13,23] Under the experimental conditions of this study, only galloylation seems to be a major factor for enhancing binding affinity of monomeric flavonoids to food proteins.

Use of Animal-Derived Food Proteins as Flavan-3-ol Carriers in Relation with Dietary Intake. With gelatin B and β-casein as examples, these proteins were found to be able to bind a maximum of 57.8 and 38.1 g EGCG/100 g protein, respectively, with affinities $>2 \times 10^4$ M^{-1}. Several intervention studies have shown beneficial health effects after daily consumption of green tea supplements containing 200−300 mg of EGCG.[32] If the highest value is taken as a recommended daily dietary intake, this would mean that 519 mg of gelatin B or 787 mg of β-casein saturated with EGCG would be needed in food to meet this intake. Obviously, other factors must be taken into account when this estimate is extrapolated to a food system, such as the stability of these complexes in a food matrix and in the mouth and their fate during digestion for targeted release. Nevertheless, it appears that the use of animal-derived food proteins as carriers of flavonoids in food is feasible, as supplementation in food would require only gram quantities of proteins as ingredients.

In terms of application in food systems, β-casein and gelatin F seemed to be the most promising carriers as they displayed the highest affinities for EGCG with good maximal binding capacity for EGCG, which was related to their random coil character. Among these two proteins, β-casein is the most suitable as it did not show any turbidity in the concentration range of EGCG used in this study, contrary to gelatin F (also observed for gelatins A and B). A relatively higher affinity is linked to a better stability of the complexes for targeted delivery. Formation of insoluble aggregates could be undesirable in food formulation and could also lower the bioaccessibility of flavonoids. Thus, β-casein seems to be the most promising flavonoid carrier among all food proteins tested in the present study. This conclusion is supported by publications on the use of this protein as a carrier for drugs.[33]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Equations applied in ITC data modeling and CD spectra of gelatin B at various temperatures and determination of the proportion of residual structures in gelatins A, B, and F based on the CD signal intensity at 200 nm. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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