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Binding of Phenolic Compounds and Their Derivatives to Bovine and Reindeer β-Lactoglobulin

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In plant-based food, phenolic compounds usually do not exist in their native form, but as esters, glycosides, or polymers. The native forms, however, require deglycosylation for their intestinal absorption, and aglycone has been considered to be the potential health-protecting/promoting form. The binding of the aglycones of phenolic compounds to bovine and reindeer β-lactoglobulins (βLG) using fluorescence quenching was studied. The effects of pH and storage were also studied. Of the compounds investigated, the majority of flavones, flavonols, flavanones, and isoflavones were bound to βLG. In the pH studies, no significant effects were found. The fact that the phenolic compounds were not released at pH 2 might indicate that they bind to an external part rather than to the central cavity. Studies implicated that βLG could act as a binder or carrier for phenolic compounds in acidic, basic, or neutral conditions and that the ligand/βLG complex can remain stable during storage.

KEYWORDS: β-Lactoglobulins; flavonols; phenolic compounds; binding; fluorescence

INTRODUCTION

Phenolic compounds such as flavonoids and phenolic acids are secondary metabolites present in vegetables, fruits, berries, and beverages. Flavonoids are classified into different classes, including flavanones, flavones, flavonols. and isoflavonols (*1*). All food of plant origin contains flavonoids (*2*). The majority of flavanones are found in citrus fruits and juices, but hesperidin, for example, can also be found in cumin and peppermint. Flavones are mostly found in grains and herbs. Flavonols are found in all plants, but mainly in fruits, berries. and vegetables (*2*). Isoflavones exist in legumes, particularly in soybeans (*2, 3*). Catechins are present in berries, fruits, and fruit juices, in green and black teas, and in red wine (*4*). The characteristics of phenolic compounds that are beneficial for health are widely studied. They are reported to be significant antioxidants, to prevent cancers, osteoporosis, and cardiovascular diseases, and to possess many other useful properties, including anti-inflammatory activity, enzyme inhibition, antimicrobial activity, and antiallergenic activity (see, e.g., refs *5*−*8*). In food, phenolic compounds usually do not exist in native form, but as esters, glycosides, and polymers (*9*). For example, most flavonoids in the diet exist as the aglycone component in glycosides, which must undergo hydrolysis in order for the flavonoid to be effective (*10*). The delivery mechanism and the binding with any biopolymers of phenolic compounds have been poorly investigated to date. During food processing, phenolic compounds will also be exposed to heat, for example, during cooking and baking (*11*). Unfortunately, there is limited information about the fate of, for example, flavonoid glycosides during thermal treatment, such as cooking, frying, and roasting (*12*). Such processing techniques may alter the flavonoid structure, resulting in changes in the bioavailability and activity of the flavonoids. Upon heating at 100 °C in aqueous solution, quercetin and rutin undergo degradation to the point that HPLC-DAD signals of the primary compound become undetectable (*13*). On the other hand, roasting has been shown to lead to beneficial effects, such as degradation of quercetin glycosides to monoglycosides and aglycones, which are most often the bioactive forms (*12*).

β-Lactoglobulin (βLG), the main whey protein in bovine milk, has also been found in the milk of at least 40 other ruminants such as reindeer, sheep, and goat (*14*). βLG occurs usually as a homodimer and has been shown to bind hydrophobic ligands, such as retinol, vitamin D, and fatty acids, in the central cavity of the monomers (*15*−*21*). The central cavity, the main binding site of βLG, is an eight-stranded, antiparallel β-barrel (*22, 23*). A very recent X-ray crystal structure study demonstrated a secondary binding site for vitamin D_3 (*24*). Other binding sites have also been reported (see, e.g., refs *25*−*28*). For example, in computational studies with norfloxacin and levofloxacin, three

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possible binding sites were identified: one in the calyx, one between the α-helix and β-strand, and one on the exterior (*27*). Belgorodsky et al. (*28*) suggested that carboxyfullerene would bind near the α-helices.

βLG also exists in the milk of other ruminants such as reindeer (*29*). The amino acid sequence and the structure of reindeer βLG closely resemble those of bovine βLG, with differences limited to the solvent-exposed residues (*30*). This results in differences in the charge distribution on the protein surface (*30*). Reindeer βLG seems to also exhibit better immunological properties than bovine βLG (*31*).

Because βLG has been shown to bind different ligands, we were interested in studying whether bovine and reindeer milk βLG could be used as a carrier molecule for phenolic compounds and their derivatives in food products in controlled delivery applications or whether these proteins can protect the phenolic compounds and their derivatives in food products, for example, during food processing and storage. Phenolic compounds and their derivatives bound to βLG could be used as a nutritional enrichment in processed dairy products. Milk and milk products containing βLG are also often applied to coffee, tea, and other plant beverages that contain phenolic compounds, which further emphasizes the potential role of βLG as a carrier molecule. Tannins that are considered to be polyphenolic metabolites have the ability to precipitate proteins (*32*). Thus, the tannins should be removed from plant foods enriched with βLG when using extracts or fractions thereof. By using our miniaturized 96-well plate method based on fluorescence quenching (*21*), we screened a set of phenolic compounds and their derivatives for their binding to bovine and reindeer milk βLG and studied the effect of pH and storage conditions on the binding. We used aglycones as model compounds, because they usually serve as the bioactive form of the natural compounds. Furthermore, the binding of the compounds to the central calyx of βLG was evaluated by computational docking experiments.

MATERIALS AND METHODS

Reagents and Standards. The phenolic compounds and their derivatives, presented in **Figure 1**, were purchased from Fluka (Buchs, Switzerland), Extrasynthese (Genay, France), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma (St. Louis, MO). All compounds were of quality for research and development. *all-trans*-Retinol ≥95%, piperine 97%, and bovine βLG 90% (product L0130, 3× crystallized and lyophilized) were purchased from Sigma. Reindeer βLG was isolated at the Sotkamo Biotechnology Laboratory (Kajaani University Consortium, University of Oulu, Finland) from reindeer milk, using a modification of the method of de Jongh et al. (*33*) as described in Heikura et al. (*34*). The purity of reindeer milk βLG was ≥99%. Reindeer milk (*Rangifer tarandus tarandus* L.) was obtained from the Reindeer Research Station (Kaamanen, Finland). Phosphate-buffered saline (PBS) 0.0067 M, pH 7.3−7.5 (PO_4) without Ca^{2+} and Mg^{2+} was purchased from BioWhittaker (Cambrex Bio Science, Verviers, Belgium). Dimethyl sulfoxide (DMSO) was purchased from Merck.

Sample Preparation. *all-trans*-Retinol, piperine, and phenolic compounds and their derivatives were dissolved in DMSO, and the solutions were further dissolved in PBS, pH 7. The final concentration of DMSO was below 2% (v/v) and did not affect fluorescence quenching. Ligand solutions were prepared daily. To determine the effect of pH on the binding of ligands, 5 μL of a 1.0 M HCl solution was pipetted into the well to achieve a pH of 2, and 2 μL of 1.0 M NaOH was pipetted into the well to achieve a pH of 10.

Fluorescence Measurements. Fluorescence measurements were made according to the method of Riihimäki et al. (*21*) using a Varioskan Scanning spectrofluorometer and spectrophotometer (Thermo Electron Corp., Vantaa, Finland). Experiments were performed using a UV 96-well plate (UV Flat Bottom Microtiter, Thermo Labsystems, Franklin, MA). Nine different ligand dilutions were pipetted onto the plate. Two hundred and twenty-five microliters of βLG solution was added to each well [12.5 μM (Sigma) and 13.9 μM (reindeer)] together with 25 μL of ligand solution. Fluorescence emission spectra of βLG were recorded in the absence and in the presence of the added ligands. Before measurement, the plates were shaken for 30 s. The excitation wavelength was 280 nm, and the emission measurement range was 300−450 nm. The concentration of bovine βLG was 11.25 μM, and the concentration of reindeer βLG was 12.5 μM in the well after the ligand addition. All ligand dilutions were made in eight replicates. The intensity of the blank PBS solution was subtracted from that obtained for the sample. Retinol was used as a positive control in all measurements, except the measurements at pH 10 in which piperine was used as a reference compound. Measurements were made directly after the samples had been pipetted. When the effect of the pH was determined, the measurements were made immediately and then every hour over the course of 6 h. In pH studies ligand dilutions were made in four replicates.

Docking Experiments. The docking experiment was performed using the QM-polarized ligand docking protocol implemented in the Glide molecular docking program (*35, 36*). The structures of the ligand molecules were drawn with ChemSketch (Advanced Chemistry Development, Inc., Toronto, ON, Canada) and converted into three-dimensional structure models using CORINA (*37*). The carboxylic acid groups were modeled in the deprotonated state. The βLG structure model was taken from the Protein Data Bank entry 1gx8, a complex structure with retinol (*38*), and prepared for docking using the Protein Preparation tool in Maestro, the graphical user interface to Glide. The preparation algorithm adds missing hydrogen atoms, adjusts the protonation states of amino acid residues, and optimizes the geometry of the added hydrogen atoms to relieve steric clashes. The cocrystallized water molecules within a radius of 5 Å from retinol were included in the protein model. The retinol in the crystal structure was used to determine the binding site volume for the Receptor Grid Generation step using the default settings of the program. The automated docking protocol then docked the ligand molecules into the prepared receptor grid using the Glide SP (standard precision) mode. Partial atomic charges were calculated for the five best positions using the "fast" option and finally, the quantum-polarized ligand structures were redocked using the Glide XP (extra precision) (*39*) accuracy level.

Determination of the Stability of the Ligand/βLG Complex. The stability of the ligand/bovine βLG complex was studied with retinol, myricetin, and daidzein. The storage conditions were 25 ± 2 and 5 ± 3 °C for 3 months and 40 ± 2 °C for 2 weeks. With the 25 ± 2 °C storage conditions, the fluorescence quenching was measured at the beginning and end of the stability studies. With the 40 ± 2 °C storage conditions, the measurements were made daily. In stability studies, six wells were used for each ligand concentration.

Data Analysis. The apparent dissociation constant (K_d) and the binding sites per monomer (n) for ligands were calculated from the fluorescence intensity results using the method of Cogan et al. (*40*) and data analysis programs SkanIt Software 2.4.1 RE for Varioskan Flash and Excel 2003. Results were expressed as means ± SD and statistically confirmed using unpaired t tests using Excel 2003. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Phenolic compounds have been investigated in many studies. Food contains many different polyphenols, many of which are not known (*9*). Polyphenol content also varies between natural plant products. For example, environmental factors, storage, methods of culinary preparation, and industrial food processing affect the content of polyphenols in plant products. All flavonoids, except flavanols, exist in glycosylated form in food. From a nutritional point of view, bioavailability is the most important question in the evaluation of physiological effects. Glycosylation affects absorption (*9*). Daidzein and genistein have been shown to be absorbed in the rat stomach, but their glucosides were not absorbed (*41*). Walgren et al. (*42*) have also shown that quercetin can be absorbed through the Caco-2

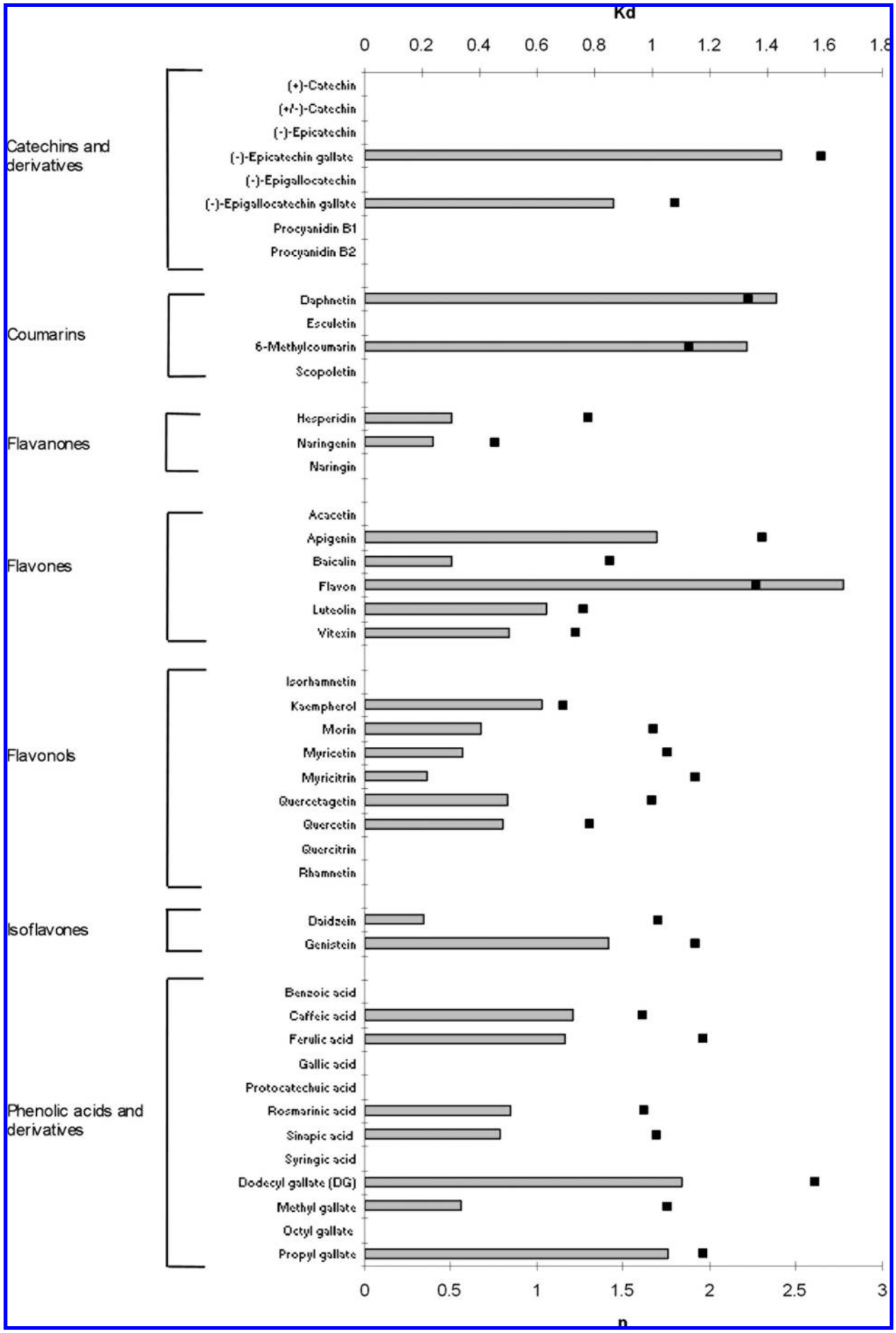

Figure 1. Phenolic compounds and their average ($n = 8$) apparent dissociation constants (K_d, gray bars) and binding sites per monomer (n, ■).

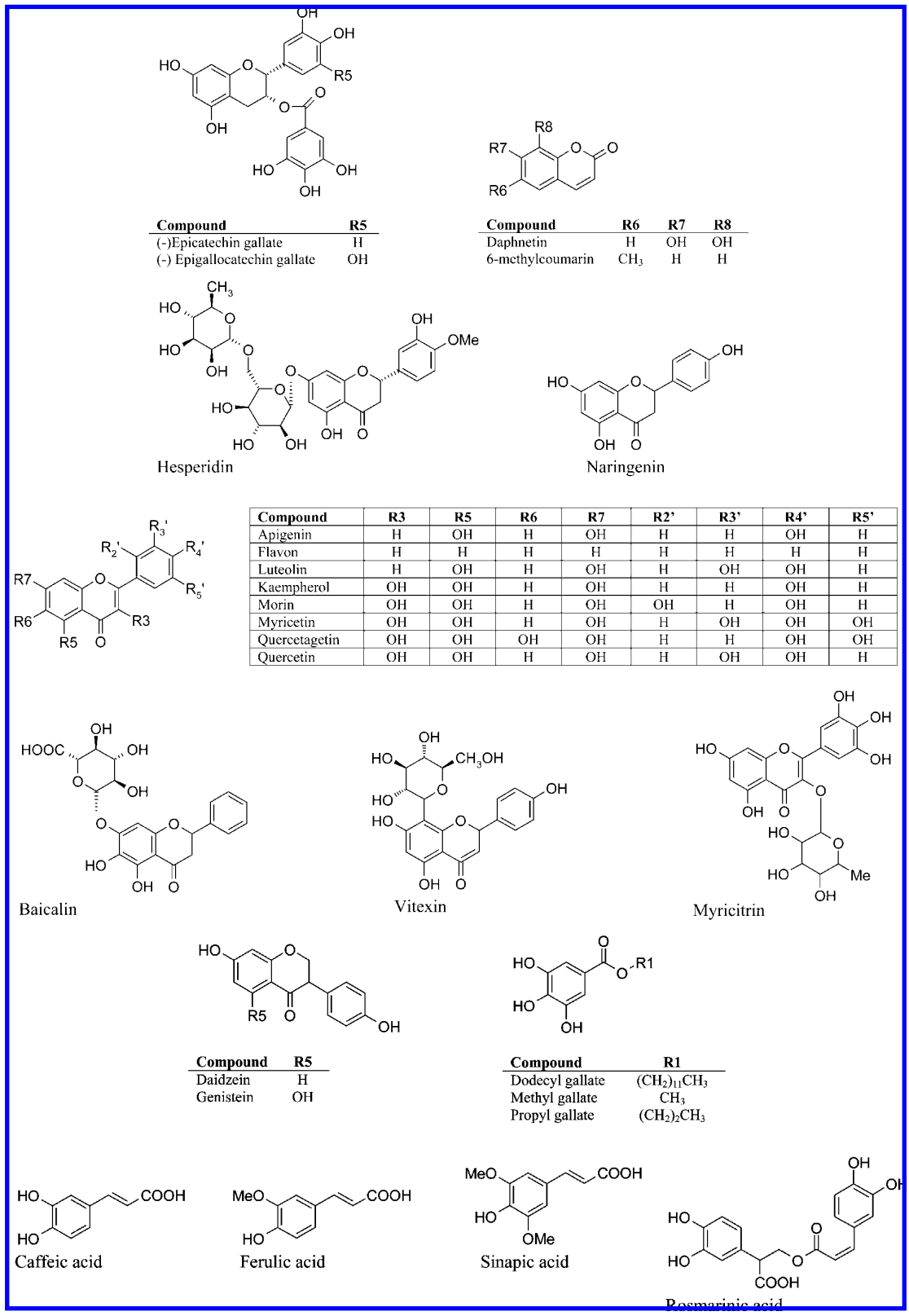

Figure 2. Structural formulas of the phenolic compounds (catechins and derivatives, coumarins, flavanones, flavones, flavonols, isoflavones, phenolic acids, and derivatives) that bound to βLG.

cell line, but its glucosides were not appreciably absorbed. Crespy et al. (*43*) observed similar results when they studied the absorption of quercetin. In that study, no conjugated forms of quercetin were identified in the gastric lumen.

We studied the role of βLG as a binder and carrier protein for the aglycone forms of phenolic compounds and their derivatives. The binding of a set of phenolic compounds and their derivatives to bovine and reindeer βLG was measured using a miniaturized 96-well plate method based on fluorescence quenching that we have developed and validated earlier (*21*). The quenching is caused by the tryptophan (Trp) residues Trp^{19} and Trp^{61} (*38, 44*). Trp^{19} is located at the bottom of the calyx and Trp^{61} near the Cys^{66}−Cys^{160} disulfide bridge (*45*). The apparent dissociation constant (K_d) and the binding sites per

Table 1. Binding Constants of Phenolic Compounds and Their Derivatives to Reindeer βLG versus Bovine βLG[a]

	reindeer βLG				bovine βLG			
	K_d[b] (μM)		n[c] (no./mol)		K_d[b] (μM)		n[c] (no./mol)	
ligand	av	SD	av	SD	av	SD	av	SD
coumarins								
daphnetin	0.58**	0.25	1.29***	0.14	1.43	0.86	2.21	0.53
flavanones								
hesperidin	0.57**	0.24	1.06***	0.13	0.30	0.25	1.28	0.10
flavones								
luteolin	0.65	0.26	1.31	0.21	0.63	0.19	1.27	0.30
vitexin	0.39	0.40	1.47	0.34	0.51	0.25	1.22	0.22
flavonols								
kaempherol	nb[d]				0.62	0.66	1.15	0.39
morin	0.34	0.15	1.15***	0.18	0.41	0.30	1.67	0.26
myricetin	0.57*	0.23	1.38**	0.26	0.34	0.15	1.74	0.17
myricitrin	0.33	0.12	1.29***	0.23	0.22	0.18	1.91	0.29
quercetagetin	0.56	0.37	1.36*	0.21	0.50	0.31	1.66	0.39
quercetin	0.54	0.28	1.26	0.16	0.48	0.20	1.30	0.34
isoflavones								
daidzein	0.44**	0.19	1.42***	0.18	0.20	0.13	1.70	0.13
genistein	0.72	0.24	1.27***	0.12	0.85	0.59	1.91	0.25
phenolic acids and derivatives								
ferulic acid	0.60	0.33	1.78	0.40	0.70	0.56	1.96	0.47
sinapic acid	0.60	0.27	1.16***	0.14	0.47	0.19	1.69	0.11

[a] p values are calculated between bovine and reindeer βLG ($n = 8$): *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$. [b] Apparent dissociation constant. [c] Binding sites per βLG monomer. [d] nb, no binding.

monomer (n) were determined from the measured intensities. A low K_d value indicates high binding affinity. The results of the compounds that were found to bind to bovine βLG are summarized in **Figure 1**, and the chemical structures of these compounds are presented in **Figure 2**. The binding to reindeer βLG and the influence of pH 2 to the binding was studied with those compounds that were found to bind to bovine βLG (**Table 1**). The stability and the influence of pH 10 were studied with myricetin and daidzein, which had high affinity toward bovine βLG ($K_d = 0.34 \times 10^{-6}$ and 0.20×10^{-6} M, respectively). Retinol was included in all studies as a reference ligand. The K_d between retinol and bovine βLG was 0.3×10^{-6} M, and the number of independent binding sites (n) was 1.8.

Binding of Catechins and Their Derivatives or Coumarins to Bovine βLG. Of catechins and their derivatives, only (−)-epigallocatechin gallate bound slightly to βLG ($K_d = 0.86 \times 10^{-6}$ M). Earlier the binding of catechins and some of their derivatives to bovine serum albumin (BSA) and human salinary α-amylase had been studied by fluorescence quenching, and it was shown that in different proteins the same polyphenol exhibited different binding affinities (*46*). None of the studied coumarins were able to bind to βLG. As we do not have a binding of the catechins or the coumarins to βLG, it seems unlikely that βLG would act as a carrier protein for these compound classes.

Binding of Flavanones, Flavones, Flavonols, and Isoflavonols to Bovine βLG. Almost all flavanones, flavones, flavonols, and isoflavonols studied bound at least slightly to βLG. Flavonols and flavanones bound to βLG with higher affinity than flavones and isoflavonols. For example, the K_d values of the flavones hesperidin and naringenin were 0.30×10^{-6} and 0.24×10^{-6} M, respectively. The K_d values of the flavonols morin, myricetin, myricitrin, quercetagetin, and quercetin were between 0.22×10^{-6} and 0.50×10^{-6} M. In the group of isoflavones, daidzein ($K_d = 0.20 \times 10^{-6}$ M) was bound tightly, whereas the K_d value of genistein (0.85×10^{-6} M) was much higher. Of flavones, only baicalin ($K_d = 0.30 \times 10^{-6}$ M) and vitexin ($K_d = 0.51 \times 10^{-6}$ M) were tightly bound, whereas the others were weaker binders ($K_d > 0.63 \times 10^{-6}$ M). The number of binding sites per βLG monomer for those compounds that bound to βLG was between 0.75 and 1.7. These values might indicate either that these compounds bind into a site within the βLG monomer rather than between the monomers of the βLG dimer or that there are two similar sites between the monomers due to symmetry.

Earlier studies have also demonstrated the binding of some flavonols to βLG. The amount of quercetin and rutin covalently bound to βLG has been estimated by their characteristic absorption between 300 and 340 nm (*47*). The results showed that both quercetin and rutin were bound to βLG, but the amount of covalently bound quercetin was significantly higher. Rawel et al. (*47*) also studied the structural effects of the binding of quercetin and rutin on βLG using circular dichroism and intrinsic fluorescence emission of βLG and by measuring changes in surface hydrophobicity using the fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS). These experiments indicated significant changes in the conformation of βLG after a reaction with quercetin and rutin. By comparison of these earlier results to our results with βLG, it seems that some flavonols could bind to βLG with high affinity. For example, in our studies the binding constant with quercetin and βLG was 0.48×10^{-6} M. This could partly be explained by different aggregate formation, which has been shown to exist for quercetin (*48*), but also by the fact that the binding to different proteins should be evaluated separately, as a small change, already in one amino acid, might make a difference in the binding affinity. The binding of genistein to βLG ($K_d = 0.85 \times 10^{-6}$ M) was weaker than with quercetin, but genistein was still significantly bound to βLG.

Binding of Phenolic Acids and Their Derivatives to Bovine βLG. The binding affinities of caffeic acid, ferulic acid, methyl gallate, sinapic acid, and rosmaric acid to βLG were moderate [K_d within $(0.34 - 0.72) \times 10^{-6}$ M]. The other phenolic acids and their derivatives had higher K_d values ($> 1.10 \times 10^{-6}$ M) or they did not bind to βLG.

Binding of Phenolic Compounds and Their Derivatives to Reindeer βLG. The affinity to reindeer βLG was investigated for the same compounds that bound to bovine βLG. The results are presented in **Table 1**. The compounds that bound to bovine βLG bound also to reindeer βLG. In our previous studies, we have shown that bovine and reindeer βLG have similar binding profiles for retinol and its derivatives that bind to the central calyx (*21*). If the phenolic compounds bind to the external part of βLG instead of the calyx, the differences between the binding constants of reindeer and bovine βLG might derive from the fact that the charge distribution on the surface of the reindeer protein is significantly different from that of bovine βLG (*30*). This led us to investigate the effects of pH on the binding of phenolic compounds to βLG.

Influence of pH on the Binding of Phenolic Compounds and Their Derivatives to Bovine βLG. The influence of acidic and basic pH on the binding properties of phenolic compounds and their derivatives was studied. Previous NMR studies have shown that palmitic acid starts to be released at a pH lower than 6, and 80% of the palmitic acid has already been released at pH 2 (*19*). The release occurs because the EF loop that acts as a gate in the central cavity is in a closed conformation below pH 6.5 (*49*). Between pH 6.5 and 8.0, βLG undergoes conformational transition, called the Tanford transition (*22, 50, 51*). Studies with piperine and βLG have demonstrated that the Tanford transition is reversible and that binding is possible again when the pH is raised (*52*). In our binding studies with retinol

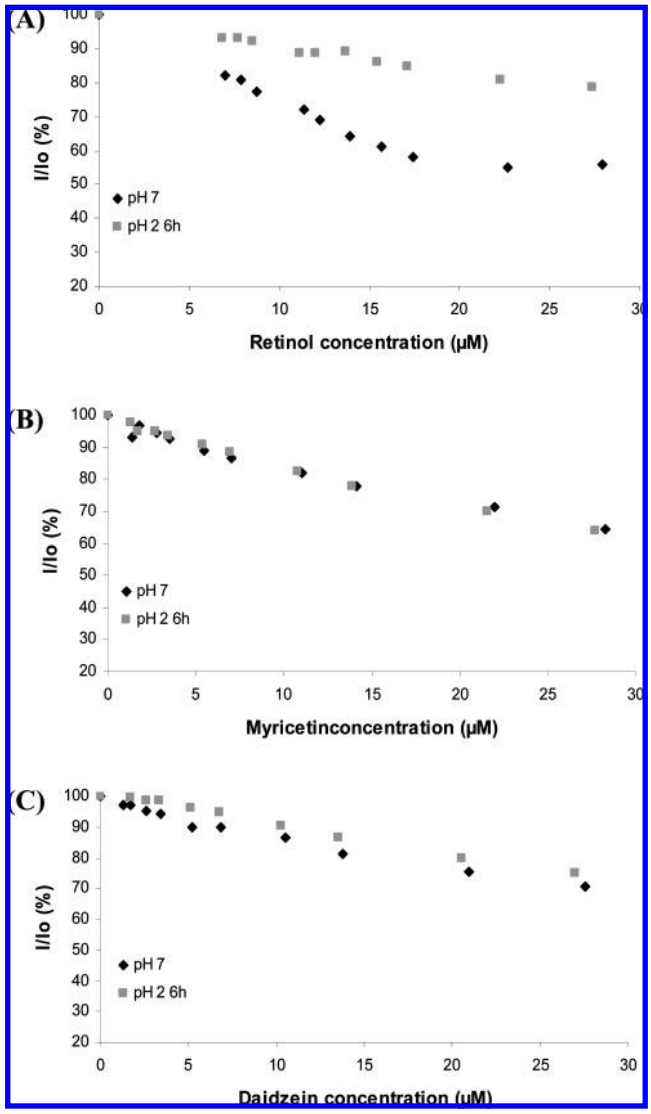

Figure 3. Influence of acidic pH on the binding of retinol (**A**), myricetin (**B**), and daidzein (**C**) to bovine βLG.

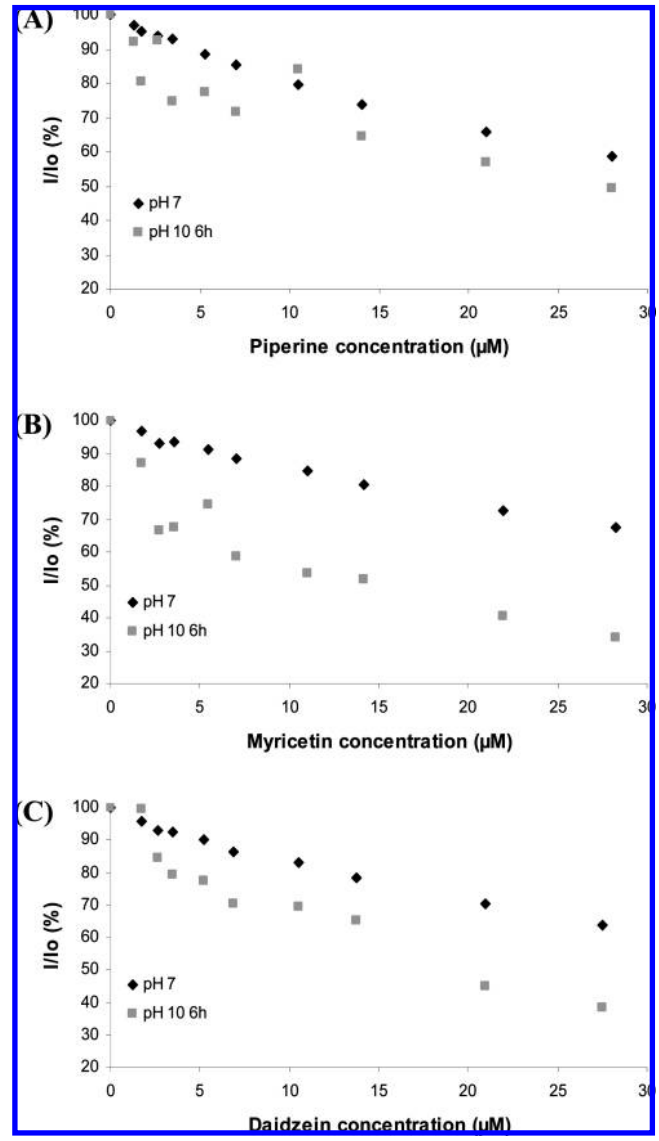

Figure 4. Influence of basic pH on the binding of piperine (**A**), myricetin (**B**), and daidzein (**C**) to bovine βLG.

and βLG, we showed that retinol also starts to release after 3 h at pH 2 (**Figure 3A**) Contrary to retinol, the release of phenolic compounds was not observed at acidic pH (**Figure 3**), which suggests that phenolic compounds and their derivatives do not bind to the central calyx. The results of Belgorodsky et al. (*28*), Yang et al. (*24*), and Liang et al. (*53*) support this hypothesis. Yang et al. (*24*) found a secondary binding site for vitamin D_3 in a crystallization study. Using fluorescence quenching, they also found that vitamin D_3 interacts with βLG at a pH between 2 and 6, but not retinol. The secondary vitamin D_3 binding site is located on the surface near the C terminus of βLG (residues 136−149), including part of the α-helix and β-strand. Belgorodsky et al. (*28*) have studied the binding of carboxyfullerene (a carbonaceous nanomaterial) to βLG and predicted interactions between carboxyfullerene units and the main α-helices of the βLG dimer, located near the dimerization site. They also found that the binding of carboxyfullerene to βLG was unaffected by changes in pH conditions. Liang et al. (*53*) studied the binding of the natural polyphenolic compound resveratrol to bovine βLG using circular dichroism, fluorescence, and UV−vis absorbance. The observed blue shift of the fluorescence emission maxima and the increase in the emission intensity implied that the environment of the polyphenol bound to βLG is not as hydrophobic as the cavity of βLG, suggesting binding on the surface of the protein (*53*). If the compounds bind to some of the binding sites on the surface of βLG, the quenching of fluorescence is due to Trp^{61}, which is located at the end of the β-strand C near the Cys^{66}−Cys^{160} disulfide bridge, which is close to the molecular surface (*22, 54*). Because the results at low pH indicated that the phenolic compounds and their derivatives bind to a site other than the calyx, we became interested in conducting docking experiments.

Piperine was used as a reference compound in the binding studies at pH 10. The results showed that piperine withstands basic pH (**Figure 4A**). The slight differences between the results obtained at pH 7 and 10 indicated that piperine binds to βLG tighter at pH 10 than at pH 7. For myricetin and daidzein, the difference between pH 7 and 10 was even greater, indicating that these flavonoids withstand basic pH very well (**Figure 4B**,**C**). The observation that phenolic compounds in solution with βLG withstand basic conditions demonstrates that βLG might protect the phenolic compounds during food processing when exposed to high pH. It has been shown that some naturally occurring polyphenolic compounds are damaged when exposed to high pH (*11*). Mathias et al. (*55*) studied the effect of pH on

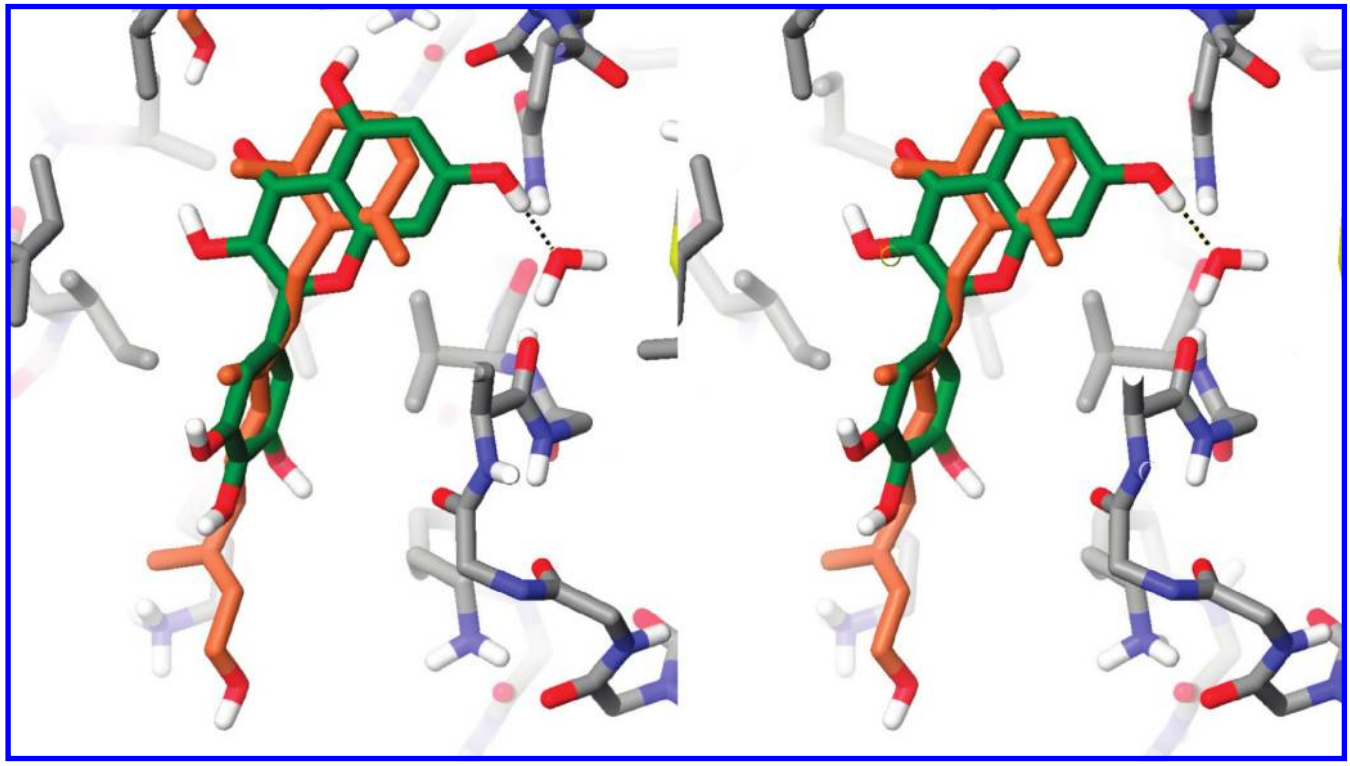

Figure 5. Wall-eyed stereoview of the calyx of βLG showing the cocrystallized retinol (light brown carbon atoms) and the highest scoring docked pose for myricetin (green carbon atoms). Myricetin is able to form only one hydrogen bond (dots) with a cocrystallized water.

the conjugated forms of genistin and daidzin isoflavones and found that a considerable loss in the total amount of known isoflavone derivatives occurred at elevated pH. At pH 8, quercetin could not be detected by HPLC-DAD, and the amount of rutin was shown to be depleted to approximately 20% (*13*).

Docking Experiments. The phenolic compounds, their derivatives, and retinol were docked to the central calyx of the βLG structure model as described above (**Figure 5**). The predicted binding mode of retinol, used as a positive control compound, was close to the crystal structure conformation, with a root mean square deviation of 1.4 Å for the non-hydrogen atoms. Most of the flavonoids were predicted to fill the hydrophobic pocket, but in those poses the hydroxyl groups of the flavonoids reside inside the pocket so that they cannot form hydrogen bonds. The missed opportunity for hydrogen bonding raises suspicions about the correctness of the predicted binding mode. Thus, the qualitative docking results suggest that phenolic compounds and their derivatives would not bind to the central calyx, supporting the results of the pH studies.

Stability Studies of the Ligand/βLG Complex. It has been shown that storage conditions greatly affect the degradation of phenolic compounds. Srivastava et al. (*56*) have demonstrated that prolonged storage of blueberry extract at room temperature reduces the concentration and activity of the phenolic compounds in the extract. The storage conditions may especially affect the concentration of polyphenols that are easily oxidized (*9*). In this study, we explored whether βLG could protect phenolic compounds from degradation during storage at different temperatures. The stability studies are summarized in **Figure 6**. No significant changes in the K_d and n values were observed for retinol, myricetin, and daidzein after storage at 25 ± 2 and 5 ± 3 °C for a period of 3 months. However, at 40 ± 2 °C, changes were already observed after 2−3 days. The stability studies indicate that βLG could be used to protect phenolic

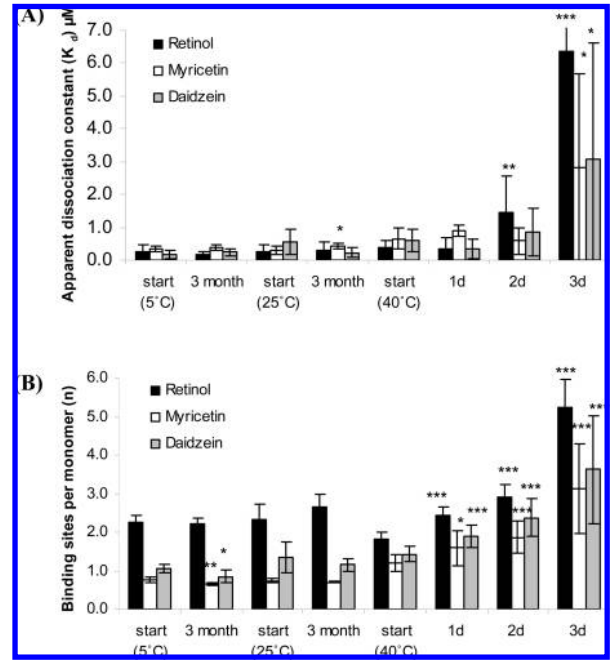

Figure 6. Binding constants of retinol, myricetin, and daidzein during stability studies: apparent dissociation constant (K_d) (**A**); binding sites per monomer (**B**). p values were calculated against initial values ($n = 6$): ∗, $p < 0.1$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.01$.

compounds in food preparations, for example, in milk products, stored at refrigerator temperatures.

We have demonstrated that some phenolic compounds, such as flavonols and isoflavonols, can bind to βLG. We have also shown that the complexes of these phenolic compounds with both bovine and reindeer βLG are stable in acidic pH

conditions, indicating that the phenolic compounds probably bind to the exterior of βLG instead of the calyx. The results of the binding experiment with reindeer βLG and the docking experiments also support this hypothesis. Because the complex of a phenolic compound with βLG is stable at acidic and basic pH, but not during thermal treatment, βLG might be a carrier molecule for phenolic compounds and protect them from degradation in food preparations during nonheating processing. The results highlight the potential role of βLG in mediating the diverse pharmacological activities of flavonols by binding and protecting them in food, such as milk products, which are consumed in considerable amounts in, for example, Finland (about 180 kg per capita per year) (*57*). Furthermore, processed dairy products fortified with phenolic compounds in complex with βLG thus would offer an additional source of phenolic compounds in the diet.

ABBREVIATIONS USED

βLG, bovine β-lactoglobulin; HPLC-DAD, high-performance liquid chromatography−diode array detection; K_d, apparent dissociation constant; *n*, binding sites per βLG monomer.

LITERATURE CITED

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