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Binding Sites of Resveratrol, Genistein, and Curcumin with Milk α- and β-Caseins

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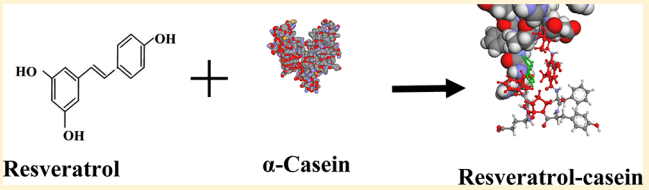
ABSTRACT: The binding sites of antioxidant polyphenols resveratrol, genistein, and curcumin are located with milk α- and β-caseins in aqueous solution. FTIR, CD, and fluorescence spectroscopic methods and molecular modeling were used to analyze polyphenol binding sites, the binding constant, and the effects of complexation on casein stability and conformation. Structural analysis showed that polyphenols bind casein *via* hydrophilic and hydrophobic interactions with the number of bound polyphenol molecules (n) 1.20 for resveratrol, 1.42 for genistein, and 1.43 for curcumin with α-casein and 1.14 for resveratrol, 1.27 for genistein, and 1.27 for curcumin with β-casein. The overall binding constants of the complexes formed are $K_{res-\alpha\text{-casein}}$ = 1.9 (±0.6) × 10^4 M^{-1}, $K_{gen-\alpha\text{-casein}}$ = 1.8 (±0.4) × 10^4 M^{-1}, and $K_{cur-\alpha\text{-casein}}$ = 2.8 (±0.8) × 10^4 M^{-1} with α-casein and $K_{res-\beta\text{-casein}}$ = 2.3 (±0.3) × 10^4 M^{-1}, $K_{gen-\beta\text{-casein}}$ = 3.0 (±0.5) × 10^4 M^{-1}, and $K_{cur-\beta\text{-casein}}$ = 3.1 (±0.5) × 10^4 M^{-1} for β-casein. Molecular modeling showed the participation of several amino acids in polyphenol−protein complexes, which were stabilized by the hydrogen bonding network with the free binding energy of −11.56 (resveratrol−α-casein), −12.35 (resveratrol−β-casein), −9.68 (genistein−α-casein), −9.97 (genistein−β-casein), −8.89 (curcumin−α-casein), and −10.70 kcal/mol (curcumin−β-casein). The binding sites of polyphenols are different with α- and β-caseins. Polyphenol binding altered casein conformation with reduction of α-helix, indicating a partial protein destabilization. Caseins might act as carriers to transport polyphenol *in vitro*.

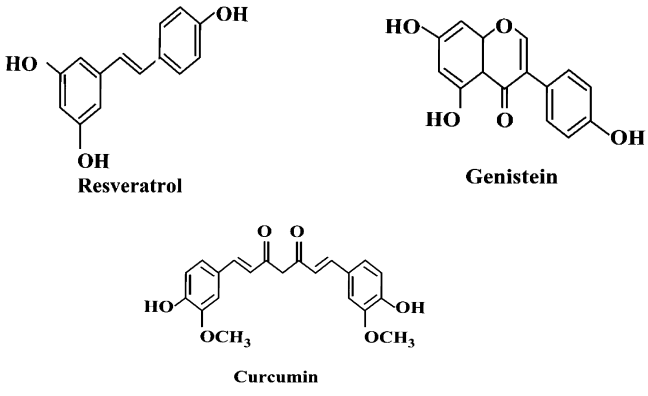


INTRODUCTION

Caseins are the major phosphoproteins of mammalian milk and exist as micelles made of polypeptides known as α-, β-, and κ-caseins.[1,2] The three casein components are almost similar in size, molecular weight (24 kD), and net negative charge but differ in their degree of unfoldedness.[3−5] Caseins belong to the rapidly growing family of unstructured protein that lately attracted much interest due to their unique unfolded structure under native conditions, brought about by a combination of high net charge and low intrinsic hydrophobicity.[2,6] α-Casein contains two tryptophan (Trp) residues, while β- and κ-caseins have one Trp residue.[4] Another unique feature of caseins is the large amount of proline residues, especially in β-casein, which greatly affect the structure of caseins, because the proline residues disrupt the formation of α-helical and β-sheet.[7] In addition, all casein proteins have different hydrophobic and hydrophilic regions along the protein chain. α-Caseins are the major casein proteins containing 8−10 seryl phosphate groups, while β-casein contains about 5 phosphoserine residues, and it is more hydrophobic than α-casein and κ-casein.[6] The structural differences indicate a different affinity for α-, β-, and κ-caseins toward hydrophilic and hydrophobic interactions. In solution, polyphenols can form insoluble complexes with milk proteins including caseins.[8] The binding affinity of polyphenols to protein is size dependent and increases with their molecular size.[9] The binding can affect the electron donation capacity of the polyphenols by reducing the number of hydroxyl groups available in solution and altering the antioxidant activity of polyphenols. Therefore, the structural characterization of the interaction between milk proteins and polyphenol is a right step in elucidating the induced effect of polyphenol on milk protein structure and the possibility of polyphenol transportation by caseins. A recent report showed the use of casein nanoparticles in a drug delivery system.[10]

Resveratrol (3,5,4′-trihydroxystilbene) (Scheme 1) is a natural polyphenolic compound produced in plants (e.g.,

Scheme 1. Chemical Structures of Polyphenols



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grapes, peanuts, mulberries) in response to injury and fungal attack. Resveratrol can also be found in food products and beverages such as peanut butter, red wine, grape juice, and more recently in dark chocolate and cocoa liquor.[11−13] It has been identified as a potential cardioprotective and chemopreventive agent against chemical carcinogens.[14] It is known to arrest cell cycle at the transition phase from S to G2/M in SW480 human colorectal cells.[15] The OH group at the C-4 position in resveratrol has a major role in antioxidant activity (Scheme 1).[16] Genistein (4′,5,7-trihydroxyisoflavone) (Scheme 1), present in soybean and chick peas, has a wide spectrum of physiological and pharmacological functions. It is known to antagonize human melanoma cell growth at G2/M transition[17,18] and found to inhibit H_2O_2/Cu(II) mediated DNA strand breaks acting as a direct scavenger of reactive oxygen species with the OH group at the C-4 position responsible for its antioxidant activity. Curcumin [(1,7-*bis*-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione)] (Scheme 1) is the main yellow pigment of the powdered rhizome (turmeric) of the herb *Curcuma longa* used for centuries as a spice and food coloring agent.[19] It has also been used to treat diseases such as inflammation, skin wounds, and tumors as traditional medicine.[20] Curcumin exhibits antioxidant activity both *in vivo* and *in vitro*.[21] Apart from its anti-inflammatory, antimicrobial, and antiviral properties, curcumin is considered as a cancer chemopreventive agent.[21,22] However, polyphenols can be transported by caseins *in vivo* and thus their interactions with these proteins are of major biological importance.

Fluorescence quenching is considered as a technique for measuring binding affinities. Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule.[23,24] Therefore, it is possible to use quenching of the intrinsic tryptophan fluorescence of Trp-37, Trp-66 in α_{s1}-casein and Trp-193, Trp-109 in α_{s2}-casein as well as Trp-143 of β-casein[4] as a tool to study the interaction of polyphenol with caseins in order to characterize the nature of polyphenol−casein complexation.

In this report, the spectroscopic analysis and molecular modeling of milk casein complexes with resveratrol, genistein, and curcumin in aqueous solution at physiological conditions are presented. Structural information regarding polyphenol binding sites and the effect of polyphenol−casein complexation on the protein stability and secondary structure is reported here.

MATERIALS AND METHODS

Materials. α- and β-caseins (with a purity of 70 and 98%), resveratrol, genistein, and curcumin (99.9%) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO) and used as supplied. Other chemicals were of reagent grade and used without further purification.

Preparation of Stock Solutions. Casein was dissolved in an aqueous solution (8 mg/mL for α-casein and 11.8 mg/mL for β-casein to obtain 0.5 mM protein content) containing 10 mM Tris−HCl buffer (pH 7.4). 1 mM polyphenol was prepared in water/ethanol (75/25%) and diluted to various concentrations (0.5, 0.25, and 0.125 mM) in Tris−HCl. The protein concentration was determined spectrophotometrically using extinction coefficients of 11 000 M^{-1} cm^{-1} (β-casein, MW = 24 000) and 15 000 M^{-1} cm^{-1} (α-casein, MW = 23 600) at 280 nm.[5,25]

FTIR Spectroscopic Measurements. Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model), equipped with a deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, using AgBr windows. A solution of polyphenol was added dropwise to the casein solution with constant stirring to ensure the formation of homogeneous solution and to have ligand concentrations of 0.125, 0.25, and 0.5 mM with a final protein concentration of 0.25 mM. Spectra were collected after 2 h incubation of casein with pigment solution at room temperature, using hydrated films. Interferograms were accumulated over the spectral range 4000−600 cm^{-1} with a nominal resolution of 2 cm^{-1} and 100 scans. The difference spectra [(protein solution + polyphenol solution) − (protein solution)] were generated using water combination mode around 2300 cm^{-1}, as a standard.[26] When producing difference spectra, this band was adjusted to the baseline level, in order to normalize difference spectra.

Analysis of Protein Conformation. Analysis of the secondary structure of casein and their polyphenol complexes was carried out on the basis of the procedure previously reported.[27] The protein secondary structure is determined from the shape of the amide I band, located around 1660−1650 cm^{-1}. The FT-IR spectra were smoothed, and their baselines were corrected automatically using Grams AI software. Thus, the root-mean-square (rms) noise of every spectrum was calculated. By means of the second derivative in the spectral region 1700−1600 cm^{-1}, the major peaks for casein and the complexes were resolved. The above spectral region was deconvoluted by the curve-fitting method with the Levenberg−Marquadt algorithm and the peaks corresponding to α-helix (1658−1656 cm^{-1}), β-sheet (1640−1610 cm^{-1}), turn (1670−1665 cm^{-1}), random coil (1648−1641 cm^{-1}), and β-antiparallel (1692−1680 cm^{-1}) were adjusted and the area was measured with the Gaussian function. The areas of all the component bands assigned to a given conformation were then summed up and divided by the total area.[28] The curve-fitting analysis was performed using the GRAMS/AI version 7.01 software of the Galactic Industries Corporation.

Circular Dichroism. CD spectra of caseins and their polyphenol complexes were recorded with a Jasco J-720 spectropolarimeter. For measurements in the far-UV region (178−260 nm), a quartz cell with a path length of 0.01 cm was used in a nitrogen atmosphere. Casein concentration was kept constant (12.5 μM), while varying each pigment concentration (0.125, 0.25, and 0.5 mM). An accumulation of three scans with a scan speed of 50 nm/min was performed, and data were collected for each nm from 260 to 180 nm. Sample temperature was maintained at 25 °C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvettes. Spectra were corrected for buffer signal, and conversion to the Mol CD ($\Delta\varepsilon$) was performed with the Jasco Standard Analysis software. The protein secondary structure was calculated using CDSSTR, which calculates the different assignments of secondary structures by comparison with CD spectra, measured from different proteins for which high quality X-ray diffraction data are available.[29,30] The program CDSSTR is provided in the CDPro software package, which is available at the Web site http://lamar.colostate.edu/~sreeram/CDPro.

Fluorescence Spectroscopy. Fluorimetric experiments were carried out on a Varian Cary Eclipse. Stock solutions of 1 mM polyphenol in buffer (pH 7.4) were prepared at room temperature (24 ± 1 °C). Various solutions of polyphenol (1−100 μM) were prepared from the above stock solutions by

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successive dilutions also at 24 ± 1 °C. A solution of casein (15 μM) in 10 mM Tris−HCl (pH. 7.4) was also prepared at 24 ± 1 °C. The above solutions were kept in the dark and used soon after. Samples containing 2 mL of the above casein solution and 2 mL of various polyphenol solutions were mixed to obtain final polyphenol concentrations of 1−100 μM with a constant protein content of 7.5 μM. The fluorescence spectra were recorded at λ_{exc} = 280 nm and λ_{em} from 287 to 500 nm. The intensity at 340 nm (tryptophane) was used to calculate the binding constant (K) according to literature reports.[31−36]

On the assumption that there are (n) substantive binding sites for quencher (Q) on protein (B), the quenching reaction can be shown as the following.

$$nQ + B \Leftrightarrow Q_nB \quad (1)$$

The binding constant (K_A) can be calculated as

$$K_A = [Q_nB]/[Q]^n[B] \quad (2)$$

where [Q] and [B] are the quencher and protein concentration, respectively, [Q_nB] is the concentration of non-fluorescent fluorophore−quencher complex, and [B_0] gives the total protein concentration.

$$[Q_nB] = [B_0] - [B] \quad (3)$$

$$K_A = ([B_0] - [B])/[Q]^n[B] \quad (4)$$

The fluorescence intensity is proportional to the protein concentration as follows:

$$[B]/[B_0] \propto F/F_0 \quad (5)$$

Results from fluorescence measurements can be used to estimate the binding constant of the polyphenol−protein complex. From eq 4,

$$\log[(F_0 - F)/F] = \log K_A + n \log[Q] \quad (6)$$

The accessible fluorophore fraction (f) can be calculated by a modified Stern−Volmer equation.

$$F_0/(F_0 - F) = 1/(fK[Q]) + 1/f \quad (7)$$

where F_0 is the initial fluorescence intensity and F is the fluorescence intensity in the presence of quenching agent (or interacting molecule). K is the Stern−Volmer quenching constant, [Q] is the molar concentration of quencher, and f is the fraction of accessible fluorophore to a polar quencher, which indicates the fractional fluorescence contribution of the total emission for an interaction with a hydrophobic quencher.[23,24] The plot of $F_0/(F_0 - F)$ vs $1/[Q]$ yields f^{-1} as the intercept on the y axis and $(fK)^{-1}$ as the slope. Thus, the ratio of the ordinate and the slope gives K.

Molecular Modeling and Docking. The docking studies were performed with ArgusLab 4.0.1 software (Mark A. Thompson, Planaria Software LLC, Seattle, WA, http://www.arguslab.com). The casein structures were obtained as reported,[4] and the polyphenol three-dimensional structure was generated from PM3 semiempirical calculations using Chem3D Ultra 6.0. The whole protein was selected as a potential binding site, since no prior knowledge of such a site was available. The docking runs were performed on the ArgusDock docking engine using regular precision with a maximum of 1000 candidate poses. The conformations were ranked using the Ascore scoring function, which estimates the free binding energy. Upon location of the potential binding sites, the docked complex conformations were optimized using a steepest decent algorithm until convergence, with a maximum of 20 iterations. Amino acid residues within a distance of 3.5 Å relative to the polyphenol were involved in the complexation.

RESULTS AND DISCUSSION

FTIR Spectra of Polyphenol Complexes with α- and β-Caseins. The polyphenol binding with caseins was characterized by infrared spectroscopy and its derivative methods. Both spectral shifting and intensity variations for the casein amide I band at 1656−1652 cm^{-1} (mainly C═O stretch) and the amide II band at 1546−1545 cm^{-1} (C−N stretching coupled with N−H bending modes)[27,28,37] were monitored upon polyphenol interaction, and the results are shown in Figures 1 and 2. Similarly, the infrared self-deconvolution with

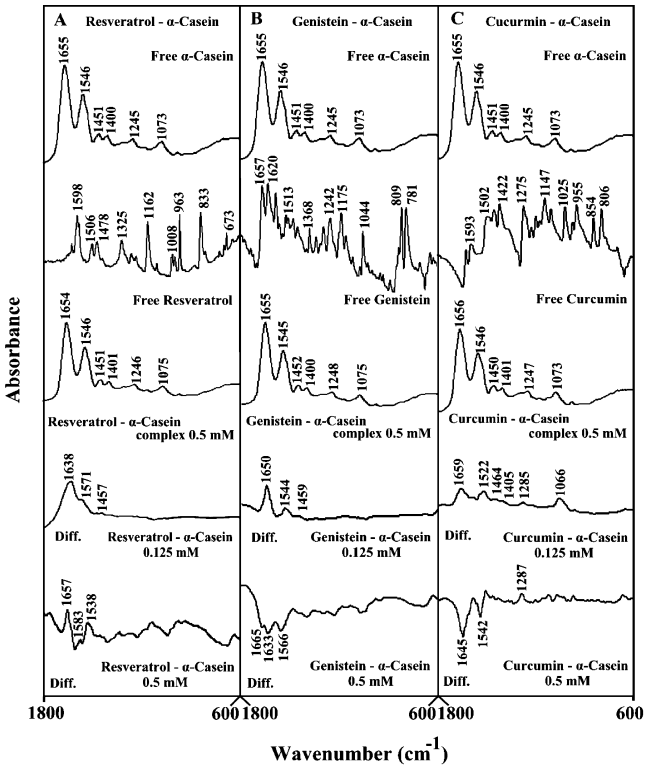

Figure 1. (A) FTIR spectra and difference spectra in the region 1800−600 cm^{-1} for free α-casein (0.5 mM) and its polyphenol complexes obtained at different polyphenol concentrations (indicated on the spectra).

second derivative resolution enhancement and curve-fitting procedures[27] were used to determine the protein secondary structure in the presence of resveratrol, genistein, and curcumin (Figure 3 and Table 1).

At low polyphenol concentration (0.125 mM), an increase of intensity was observed for the protein amide I at 1655−1652 cm^{-1} and amide II at 1546−1545 cm^{-1}, in the difference spectra of the polyphenol−casein complexes. The positive features located in the difference spectra for amide I and II bands at 1638, 1571 cm^{-1} (resveratrol−α-casein), at 1650, 1544 cm^{-1} (genistein−α-casein), at 1659, 1522 cm^{-1} (curcumin−α-casein), at 1644, 1543 cm^{-1} (resveratrol−β-casein), at 1656, 1531 cm^{-1} (genistein−β-casein), and at 1654, 1542 cm^{-1} (curcumin−β-casein) are due to the increase of the intensity of casein amid I and amide II upon polyphenol complexation

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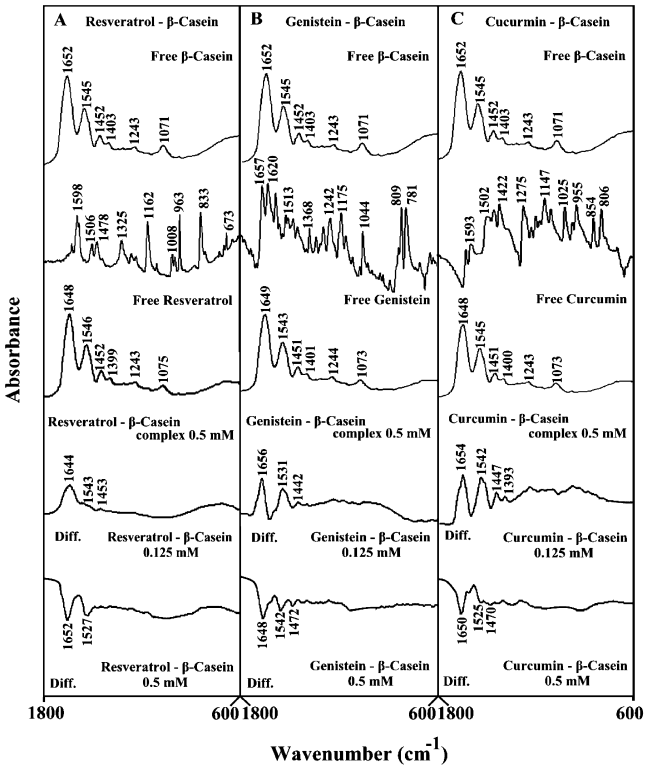

Figure 2. (A) FTIR spectra and difference spectra in the region 1800−600 cm^{-1} for free β-casein (0.5 mM) and its polyphenol complexes obtained at different polyphenol concentrations (indicated on the spectra).

(Figures 1 and 2, diffs., 0.125 mM) The increase in intensity of the amide I and amide II bands is due to polyphenol binding to protein C═O, C—N, and N—H groups (hydrophilic contacts). Additional evidence to support the polyphenol interactions with C—N and N—H groups comes from the shifting of the protein amide A band at 3300 cm^{-1} (N—H stretching) in the free caseins to higher frequency at 3310 cm^{-1} upon polyphenol interaction (spectra not shown). As the polyphenol concentration increased to 0.5 mM, positive features were observed for casein amide I and amide II bands at 1657 and 1538 cm^{-1} for resveratrol−α-casein, while were negative features observed for other polyphenol−casein complexes at 1633, 1566 cm^{-1} (genistein−α-casein), at 1645, 1542 cm^{-1} (curcumin−α-casein), at 1652, 1527 cm^{-1} (resveratrol−β-casein), at 1648, 1542 cm^{-1} (genistein−β-casein), and at 1650, 1525 cm^{-1} (curcumin−β-casein) (Figures 1 and 2, diffs., 0.5 mM). The observed decrease in the intensity of the amide I band at 1656−1654 cm^{-1} and the amide II band at 1546−1545 cm^{-1}, in the spectra of the polyphenol−α-caseins and polyphenol−β-caseins suggests a reduction in the protein α-helical structure, upon polyphenol complexation, while an increase in the intensity of the amide I and amide II bands in the spectra of the resveratrol−α-casein adduct indicates no major alteration of protein α-helix structure (Figures 1 and 2, diffs., 0.5 mM). Similar infrared spectral changes were observed for protein amide I and amide II bands in several drug−protein complexes,[38] where major and minor protein conformational changes occurred, which will be discussed below.

A quantitative analysis of the protein secondary structure for the free α- and β-caseins and their polyphenol adducts in hydrated films has been carried out, and the results are shown in Figure 3 and Table 1. The free α-casein has 35% α-helix (1657 cm^{-1}), 16% β-sheet (1626 and 1614 cm^{-1}), 23% turn structure (1672 cm^{-1}), 3% β-antiparallel (1688 cm^{-1}), and 23% random coil (1641 cm^{-1}) (Figure 2A and Table 1). The free β-casein contains 31% α-helix (1655 cm^{-1}), 23% β-sheet (1628 and 1612 cm^{-1}), 25% turn structure (1673 cm^{-1}), 3% β-antiparallel (1687 cm^{-1}), and 18% random coil (1641 cm^{-1}) (Figure 2E and Table 1). These results are consistent with spectroscopic studies of caseins previously reported.[39,40] Upon polyphenol interaction, a minor reduction of the α-helix occurred for genistein−α-casein and curcumin−α-casein but not for resveratrol−α-casein complexes (Figure 2B, C, and E and Table 1). Similarly, a decrease of α-helix was observed for resveratrol−β-casein, genistein−β-casein, and curcumin−β-casein (Figure 2F−H and Table 1). The reduction of α-helical structure is due to alterations of protein conformation upon polyphenol complexation.

CD Spectroscopy. CD spectroscopy was also used to analyze the protein conformation in the polyphenol−casein complexes, and the results are shown in Table 2. The CD results exhibit marked similarities with infrared data (Tables 1 and 2). The protein conformational analysis based on CD data showed that free α-casein contains 35% α-helical, 12% β-sheet, 20% turn, and 33% random coil, while free β-casein has 33% α-helical, 16% β-sheet, 17% turn, and 34% random coil (Table 2). The CD data for free caseins are consistent with the literature report.[41,42] Upon polyphenol interaction, a decrease of α-helix was observed for both caseins with a minor increase of β-sheet structure, consistent with the infrared data (Tables 1 and 2). The CD results also support minor perturbations of casein secondary structure upon polyphenol interaction.

Fluorescence Spectra and Stability of Polyphenol Complexes with α- and β-Caseins. α-Casein (mixture of α_{s1}-casein and α_{s2}-casein) has two tryptophan residues Trp-66 and Trp-37 (in α_{s1}-casein) and Trp-109 and Trp-193 (in α_{s2}-casein), while β-casein contains one tryptophan Trp-143 with intrinsic fluorescence. These tryptophan residues are located in the protein surfaces.[4] Tryptophan emission dominates casein fluorescence spectra in the UV region. When other molecules interact with casein, tryptophan fluorescence may change depending on the impact of such interaction on the protein conformation.[23,24] The decrease of fluorescence intensity of caseins has been monitored at 347 nm for polyphenol−protein systems (Figure 4A−F) and show representative results for each system. The plot of $F_0/(F_0 - F)$ vs 1/[polyphenol] shows representative results (Figure 4A′−F′). Assuming that the observed changes in fluorescence come from the interaction between polyphenol and caseins, the quenching constant can be taken as the binding constant of the complex formation. The K values given here are averages of four-replicate and six-replicate runs for protein−polyphenol systems, each run involving several different concentrations of polyphenol (Figure 4A−F). The binding constants estimated were $K_{res-\alpha\text{-casein}}$ = 1.9 (±0.6) × 10^4 M^{-1}, $K_{gen-\alpha\text{-casein}}$ = 1.8 (±0.4) × 10^4 M^{-1}, and $K_{cur-\alpha\text{-casein}}$ = 2.8 (±0.8) × 10^4 M^{-1} with α-casein and $K_{res-\beta\text{-casein}}$ = 2.3 (±0.3) × 10^4 M^{-1}, $K_{gen-\beta\text{-casein}}$ = 3.0 (±0.5) × 10^4 M^{-1}, and $K_{cur-\beta\text{-casein}}$ = 3.1 (±0.5) × 10^4 M^{-1} for β-casein (Figure 4A′−F′ and Table 3). The association constants calculated for the polyphenol−protein suggest a moderate affinity of pigment−casein interaction, compared to the other strong ligand−protein complexes.[9,43,44] The larger binding constant obtained for curcumin−casein complexes is due to the more hydrophobic nature of curcumin[45] than resveratrol and

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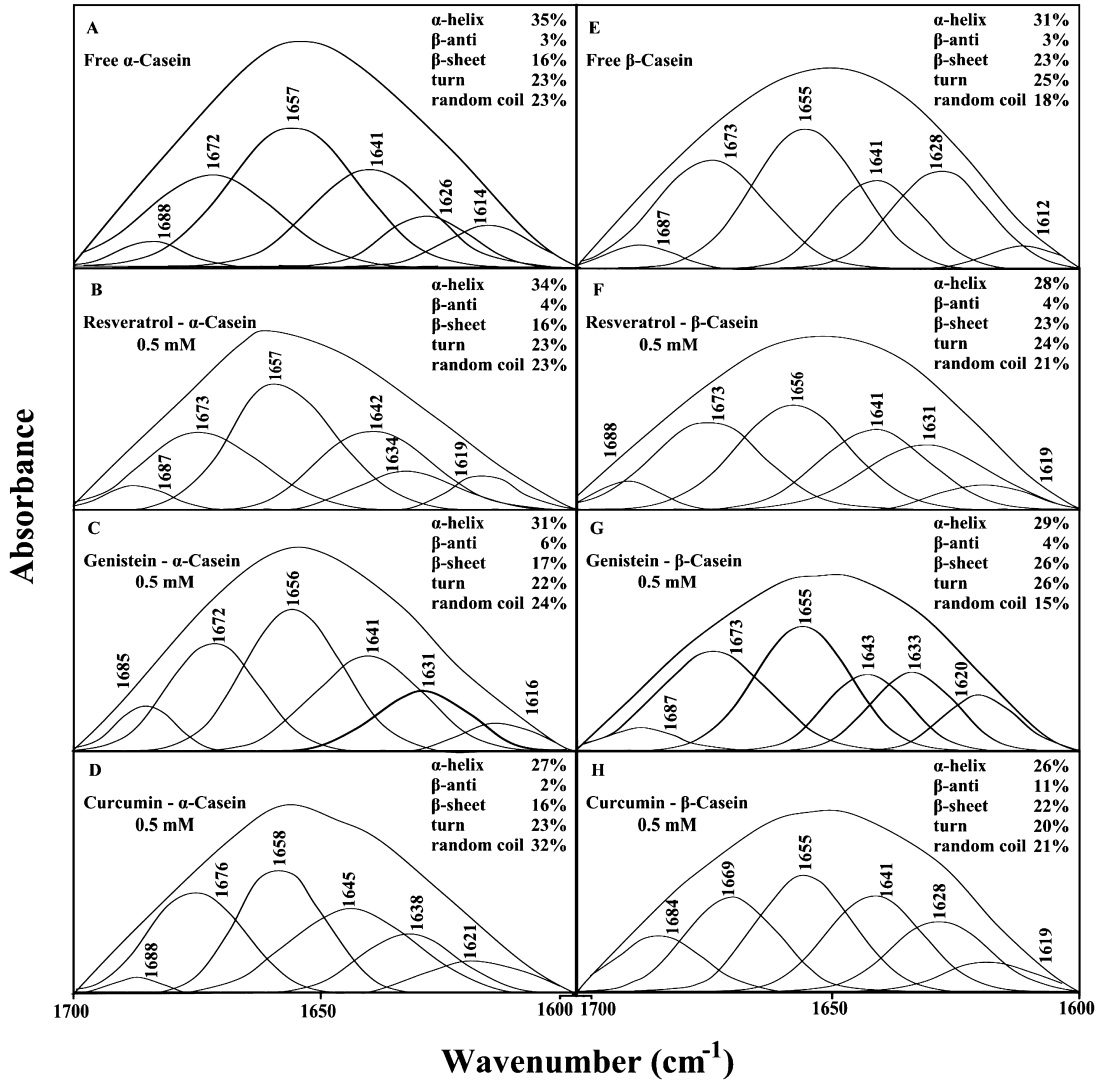

Figure 3. (A) Second derivative resolution enhancement and curve-fitted amide I region (1700−1600 cm^{-1}) of IR spectra for free α- and β-caseins with 0.5 mM polyphenol.

Table 1. Secondary Structure Analysis (FTIR) of Caseins (α and β) and Their Complexes with Polyphenols in a Hydrated Film at pH 7.4

amide I components (cm^{-1})	0.5 mM free casein (%)		0.5 mM resvera-trol−casein (%)		0.5 mM genistein−casein (%)		0.5 mM curcumin−casein (%)	
	α	β	α	β	α	β	α	β
1692−1680, β-anti (±1%)	3	3	4	4	6	4	2	11
1680−1660, turn (±2%)	23	25	23	24	22	26	23	20
1660−1650, α-helix (±2%)	35	31	34	28	31	29	27	26
1648−1641, random coil (±4%)	23	18	23	21	24	15	32	21
1640−1610, β-sheet (±2%)	16	23	16	23	17	26	16	22

Table 2. Secondary Structure Analysis (CD Spectra) from the Free Caseins (α and β) and Their Polyphenol Complexes in Solution at pH 7.4, Calculated by CDSSTR Software

conformational components	0.25 mM free casein (%)		0.5 mM resvera-trol−casein (%)		0.5 mM genistein−casein (%)		0.5 mM curcumin−casein (%)	
	α	β	α	β	α	β	α	β
turn (±2%)	20	17	22	20	23	22	25	24
α-helix (±2%)	35	33	32	30	30	28	26	25
random (±2%)	33	34	32	36	32	36	36	38
β-sheet (±1%)	12	16	14	14	15	14	13	13

genistein, which forms stronger hydrophobic contacts with casein hydrophobic domains. The change in fluorescence intensity of Trp-37, Trp-66 in α_{s1}-casein and Trp-193, Trp-109 in α_{s2}-casein as well as Trp-143 of β-casein in the presence of polyphenol may arise as a direct quenching or as a result of protein conformational changes induced by polyphenol−casein complexation. The results indicate that polyphenol interactions do not change the emission λ_{max} at 340 nm for genistein and curcumin, whereas a major shifting of this band occurred upon resveratrol−casein complexation (Figure 4A−F). The lack of spectral shifting observed for the emission band of tryptophan at 340 nm upon genistein and curcumin interaction is indicative

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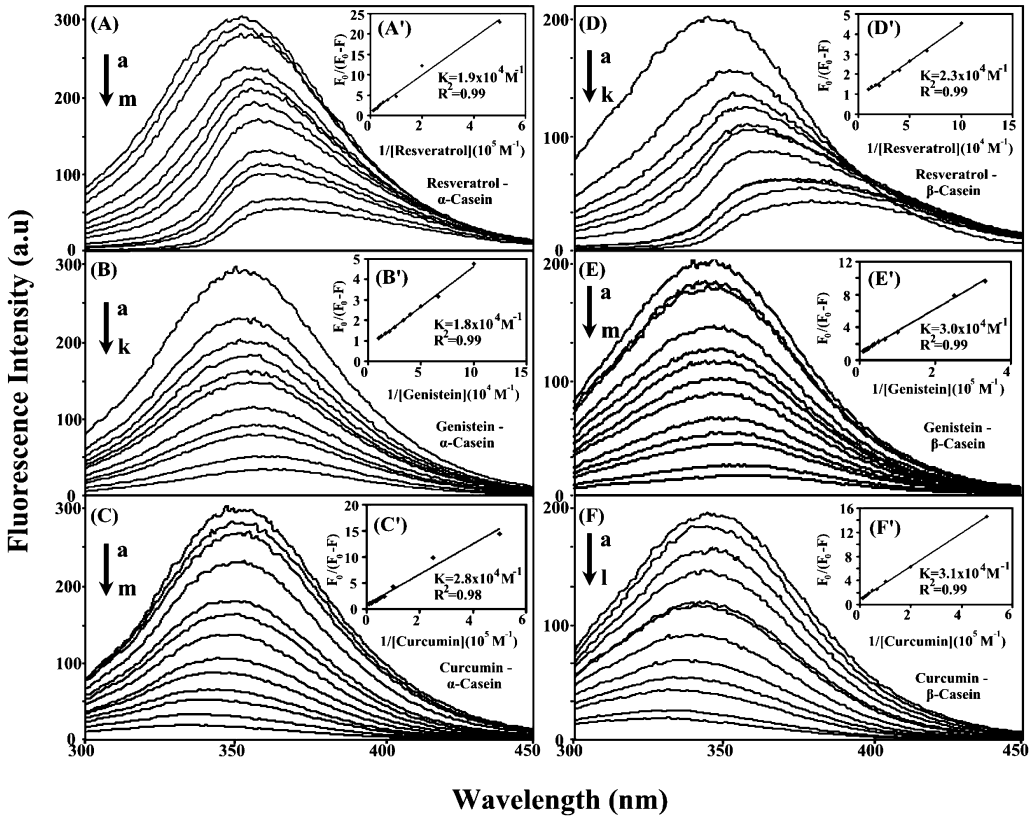

Figure 4. Fluorescence emission spectra of polyphenol−casein systems in 10 mM Tris−HCl buffer pH 7.4 at 25 °C for (A) (a) free α-casein 7.5 μM and (b−m) resveratrol at 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM; (B) (a) free α-casein 7.5 μM and (b−k) genistein at 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM; (C) (a) free α-casein 7.5 μM and (b−m) curcumin at 2, 4, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM; (D) (a) free β-casein 7.5 μM and (b−k) resveratrol at 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM; (E) (a) free β-casein 7.5 μM, (b-m) genistein at 3, 4, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM; (F) (a) free β-casein 7.5 μM and (b−l) curcumin at 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 μM. The binding constant K is the ratio of the intercept and the slope for polyphenol−casein complexes (A′, B′, C′, D′, E′, and F′).

Table 3. Binding Parameters for Polyphenol−Casein Complexes

complexes		K ($\times 10^4$ M^{-1})	n	K_q ($\times M^{-1}$ S^{-1})
resveratrol−casein	α	1.9 ± 0.6	1.20 ± 0.06	7.7×10^{12}
	β	2.3 ± 0.1	1.14 ± 0.05	1.19×10^{13}
genistein−casein	α	1.8 ± 0.1	1.42 ± 0.06	1.08×10^{13}
	β	3.0 ± 0.2	1.27 ± 0.06	1.86×10^{13}
curcumin−casein	α	2.8 ± 0.8	1.43 ± 0.08	1.53×10^{13}
	β	3.1 ± 0.2	1.27 ± 0.07	1.95×10^{13}

that tryptophan molecules are not exposed to any change in polarity. The major shifting of the emission band at 340−360 nm for the resveratrol−casein adduct is due to exposed tryptophan (Figure 4A and D). However, the binding mode of resveratrol is somehow different from genistein and curcumin in these polyphenol−casein complexes.

In order to verify the presence of static or dynamic quenching in polyphenol−casein complexes, we have plotted F_0/F against Q, and the results are shown in Figure 5. The plot of F_0/F versus Q is a straight line at low Q concentrations and nonlinear at high pigment contents, for polyphenol−casein adducts, indicating that the quenching is mainly static in nature with some degree of dynamic quenching at high Q concentrations.[36] The quenching constant was estimated according to the Stern−Volmer equation:

$$F_0/F = 1 + k_Q t_0[Q] = 1 + K_D[Q] \quad (8)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the quencher concentration, and K_D is the Stern−Volmer quenching constant, which can be written as $K_D = k_Q t_0$, where k_Q is the bimolecular quenching rate constant and t_0 is the lifetime of the fluorophore in the absence of quencher, 4.53 ns for α-casein and 3.30 ns for β-casein.[42] The quenching constants (K_Q) are 7.7×10^{12} M^{-1}/s for resveratrol−α-casein, 1.19×10^{13} M^{-1}/s for resveratrol−β-casein, 1.08×10^{13} M^{-1}/s for genistein−α-casein, 1.86×10^{13} M^{-1}/s for genistein−β-casein, 1.53×10^{13} M^{-1}/s for curcumin−α-casein, and 1.95×10^{13} M^{-1}/s for curcumin−β-casein adducts (Figure 5 and Table 3). The quenching constants show that quenching is mainly static for these polyphenol−casein complexes.

The number of polyphenol molecules bound per casein (n) is calculated from $\log[(F_0 - F)/F] = \log K_S + n \log$[polyphenol] for the static quenching.[46−49] The linear plot of $\log[(F_0 - F]/F]$ as a function of log[polyphenol] is shown in Figure 6. The number of bound resveratrol molecules per protein (n) was 1.20 (±0.06) for α-casein, 1.14 (±0.05) for β-casein, genistein 1.42 (±0.07) for α-casein, 1.27 (±0.06) for β-casein and curcumin 1.43 (±0.07) with α-casein, 1.27 (±0.06) with β-casein in these polyphenol−protein adducts (Figure 6 and Table 3).

Docking Studies. Our spectroscopic results were complemented with docking experiments in which polyphenols were docked to α- and β-caseins to determine the preferred binding sites. The docking results are shown in Figure 7 and

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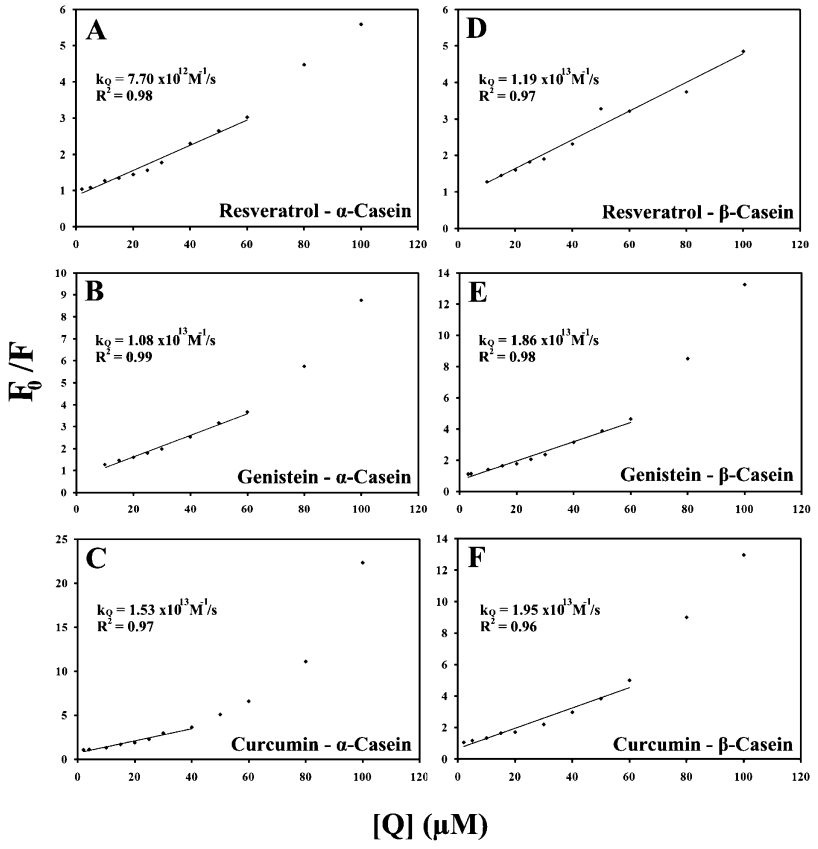

Figure 5. Stern–Volmer plots for fluorescence quenching constant (k_Q) of the polyphenol–casein complexes at different polyphenol concentrations: (A) resveratrol–α-casein, (B) genisteinl–α-casein, (C) curcumin–α-casein, (D) resveratrol–β-casein, (E) genistein–β-casein, and (F) curcumin–β-casein.

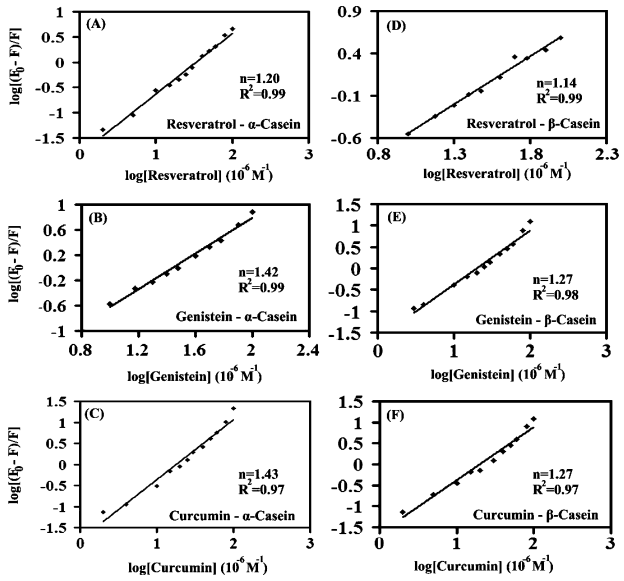

Figure 6. The plot of $\log(F_0 - F)/F$ as a function of log[polyphenol] for calculation of the number of bound polyphenol molecules (n) in polyphenol–casein complexes.

Table 4. In the resveratrol–α-casein adduct, resveratrol is surrounded by Leu-142, Phe-150, Pro-147, and Tyr-144 with average binding distances resveratrol–amino acid) of 1.7 to 2.5 Å and the free binding energy of −11.56 kcal/mol (Figure 7 and Table 4). In the resveratrol–β-casein, resveratrol is located in the vicinity of Asn-7, Leu-3, Leu-88, Phe-87, Phe-119, Pro-115, and Val-116 with average binding distances (resveratrol–amino acid) of 1.5–2.5 Å and a free binding energy of −12.36 kcal/mol (Figure 7 and Table 4). In the genistein–α-casein adduct, genistein is surrounded by Arg-22* (H-bonding), Gln-30, Phe-23, Phe-24* (H-bonding) Phe-28, Phe-32, Pro-29, and Val-31 with average binding distances (genistein–amino acid) of 1.5–2.7 Å and a free binding energy of −9.68 kcal/mol, while, in genistein–β-casein, genistein is located near Gly-203, Ile-208, Leu-191, Leu-192, Leu-198, Phe-190, Tyr-180, Tyr-193, Val-197* (H-bonding), and Val-209* (H-bonding) with average binding distances (genistein–amino acid) of 1.5–2.9 Å and a free binding energy of −9.97 kcal/mol, (Figure 7 and Table 4). In the curcumin–α-casein adduct, curcumin is surrounded by Ala-158, Ile-136, Leu-156, Pro-115, Tyr-159* (H-bonding), and Val-138 with average binding distances (curcumin–amino acid) of 1.5–2.9 Å and a free binding energy of −8.89 kcal/mol, while, in curcumin–β-casein, the pigment is located near Ala-189, Ile-208, Leu-191, Leu-192, Phe-190, Pro-204, Tyr-180* (H-bonding), Tyr-193, Val-178, and Val-209* (H-bonding) with average binding distances (curcumin–amino acid) of 1.5–2.9 Å and a free binding energy of −10.70 kcal/mol (Figure 7 and Table 4). The docking results show the binding of polyphenols is different with α-casein and β-casein (Table 4). It demonstrates that the binding sites involved are different for each polyphenol (Table 4). Curcumin forms more stable complexes with casein than those of genistein and resveratrol curcumin > genisten > resveratrol (Table 4), consistent with fluorescence spectroscopic results (Figure 4 and Table 3).

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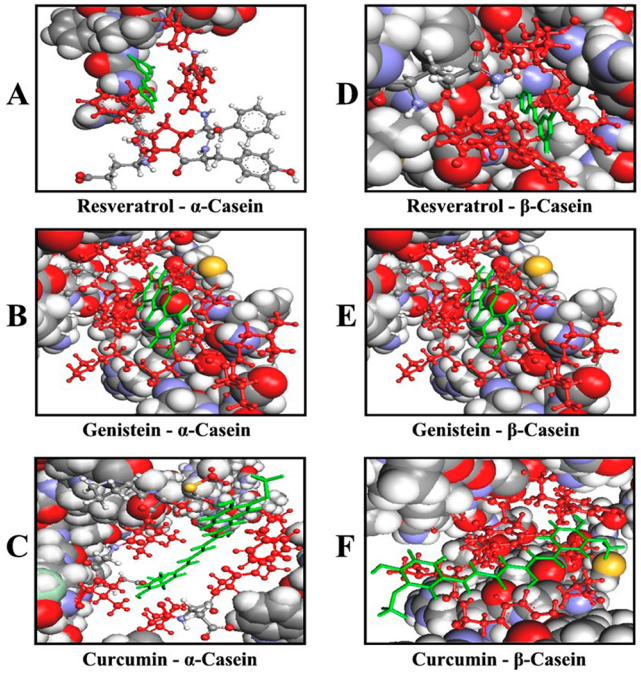

Figure 7. Best docked conformations of polyphenol−casein complexes. Amino acid residues of interest are shown in red color and the polyphenol in green color: (A) resveratrol complexed to α-casein, (B) genistein complexed to α-casein, (C) curcumin complexed to α-casein, (D) resveratrol complexed to β-casein, (E) genistein complexed to β-casein, and (F) curcumin complexed to β-casein.

Table 4. Residues Involved in Polyphenol−Protein Interactions and Free Binding Energies for the Complexes

complexes		residues involved	$\Delta G_{binding}$ (kcal/mol)
resveratrol−casein	α	Leu-142, Phe-150, Pro-147, Tyr-144	−11.56
	β	Asn-7, Leu-3, Leu-88, Phe-87, Phe-119, Pro-115, Val-116	−12.35
genistein−casein	α	Arg-22,[a] Gln-30, Phe-23, Phe-24,[a] Phe-28, Phe-32, Pro-29, Val-31	−9.68
	β	Gly-203, Ile-208, Leu-191, Leu-192, Leu-198, Phe-190, Tyr-180, Tyr-193, Val-197,[a] Val-209[a]	−9.97
curcumin−casein	α	Ala-158, Ile-136, Leu-156, Pro-160, Tyr-159,[a] Val-138	−8.89
	β	Ala-189, Ile-208, Leu-191, Leu-192, Phe-190, Pro-204, Tyr-180,[a] Tyr-193, Val-178, Val-209[a]	−10.70

[a]Hydrogen bonding observed with this amino acid.

CONCLUSIONS

On the basis of our spectroscopic results and modeling data, polyphenols bind caseins *via* hydrophilic and hydrophobic interactions and induce a partial protein destabilization. The protein binding sites involved are different for each polyphenol. The order of affinity of polyphenol−casein complexation is curcumin > genistein > resveratrol with more stable complexes formed with β-casein than α-casein. Caseins might act as carriers to transport polyphenol *in vitro*.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

cas, casein; res, resveratrol; gen, genistein; cur, curcumin; FTIR, Fourier transform infrared spectroscopy; CD, circular dichroism

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