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Interaction between Whole Buttermilk and Resveratrol

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ABSTRACT: The interaction between buttermilk and resveratrol (*trans*-3,5,4′-trihydroxystilbene) was examined using fluorescence spectroscopy. Ultracentrifugation of buttermilk (10% total solids, TS)−resveratrol (100−1600 μM) mixtures yielded three fractions comprising cream (14.79% w/w, 1.12% TS), milk serum (75.94% w/w, 5.56% TS), and a casein-rich precipitate (9.27% w/w, 2.94% TS). The majority of the added resveratrol was partitioned into the casein-rich precipitate (50.5−56.8%), with lesser amounts in the milk serum (35.3−41.2%) and cream layer (7.9−8.7%), demonstrating that most of the resveratrol interacted with the proteins. The interaction of the milk proteins with resveratrol was investigated by measuring the quenching of protein intrinsic fluorescence. Complex formation was spontaneous and exothermic. The apparent binding constants between milk proteins in buttermilk and resveratrol were $\geq 2.47 \times 10^4$, 1.65×10^4, 1.11×10^4, and 0.72×10^4 M^{-1}, respectively, at 278, 288, 298, and 308 K. The increased aqueous solubility of resveratrol by complexation to whole buttermilk makes it useful as a food vehicle for carrying resveratrol.

KEYWORDS: *buttermilk, trans-resveratrol, fluorescence quenching, binding constant*

INTRODUCTION

Buttermilk, the liquid phase byproduct of butter and anhydrous milk fat manufacture, comprises milk proteins, lactose, residual lipids, and milk fat globule membrane (MFGM). The MFGM is composed primarily of proteins, phospholipid, and glycolipid components.[1,2] Buttermilk has been used as a milk powder replacer in a range of food products. Buttermilk has also been fractionated to obtain enriched fractions of the various components to optimize the functionality of the buttermilk components for specific applications.[3−5]

trans-Resveratrol is a phenolic compound found in foods such as red grapes, wines, cranberries, strawberries, and peanuts. It has been associated with many benefits for human health,[6] including its antioxidative, cardioprotective, anticancer, and anti-inflammatory properties.[7−9] However, the low water solubility and poor stability and bioavailability of resveratrol limit its application in medicine and functional foods.[10] Resveratrol is sensitive to oxidation and transforms from the *trans* to the *cis* isomer under exposure to UV.[11]

Resveratrol has low solubility in the aqueous phase (13.6 μg/g in phosphate buffer, pH 7.4) but higher solubility in oils (e.g., 179.8 μg/g in coconut oil).[12,13] The binding of resveratrol to proteins or solubilization in lipids in a bioactive delivery system or formulating resveratrol in oil-in-water emulsions increases the solubility and stability of resveratrol and improves its bioavailability.[12,14,15] Milk proteins (caseins and whey proteins) have been used as vehicles for carrying a range of bioactive phenolic compounds.[16−20] The interaction between resveratrol and β-lactoglobulin, the major component of whey protein, was investigated by fluorescence spectroscopy, which showed that 1:1 complexes were formed with binding constants ranging from 10^4 to 10^6 M^{-1}.[16] Whey protein isolate and its β-lactoglobulin- and α-lactalbumin-rich fractions also formed 1:1 complexes with resveratrol.[18] The number of binding sites of α- and β-caseins for resveratrol were 1.2 and 1.14, respectively, with corresponding binding constants of 1.9×10^4 and 2.3×10^4 M^{-1}.[20] There are controversies about the influence of protein on the bioavailability of phenolic compounds.[21] However, a recent study has shown that in vitro bioaccessibility of polyphenols is not influenced by the interaction of polyphenols with milk proteins.[22] Liposomes and emulsion-based systems have been used for delivering phenolic compounds.[12,23−25] However, the behavior of resveratrol in a complex system in the presence of protein, fat, and milk fat globule membrane is still unclear.

The unique composition of whole buttermilk, which comprises proteins, fat, and milk fat globule membrane, makes it amenable to be used as a carrier for resveratrol, enabling each of the components in the whole buttermilk to contribute to its resveratrol-carrying potential. In this work, mixtures of buttermilk with resveratrol were examined. The relative affinities of resveratrol for different fractions of buttermilk−resveratrol mixtures after separation by ultracentrifugation (i.e., cream layer, milk serum, casein micelles) were investigated. Fluorescence spectroscopy was used to investigate the interaction between resveratrol and the proteins in buttermilk. Apparent binding constants for resveratrol with buttermilk proteins were obtained, and the influence of temperature on the interaction between resveratrol and buttermilk was also examined.

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Table 1. Composition of Fractions upon Ultracentrifugation of Buttermilk (10% TS)−Resveratrol Mixtures at 30000*g* and 20 °C for 1 h

		total solids		protein		
fraction	wt fraction (% w/w)	% w/w	g/100 g	% w/w, dry basis	g/100 g	resveratrol distribution %[a]
cream	14.79 ± 1.04	7.57 ± 0.12	1.12 ± 0.10	10.01 ± 0.52	0.11 ± 0.00	7.88−8.66
milk serum	75.94 ± 1.51	7.32 ± 0.09	5.56 ± 0.18	14.02 ± 0.48	0.78 ± 0.05	35.34−41.17
casein-rich precipitate	9.27 ± 0.47	31.71 ± 0.23	2.94 ± 0.17	78.51 ± 3.42	2.30 ± 0.03	50.50−56.78

[a]% resveratrol partitioned between each fraction of buttermilk (10% TS)−revseratrol (100−1600 μM).

MATERIALS AND METHODS

Materials. Resveratrol (98% purity) was purchased from Shanghai Novanat Bioresources Co., Ltd., China. Buttermilk powder (316 mg/g total protein, 80 mg/g milk fat, 501 mg/g lactose, 971 mg/g total solid) was supplied by Warrnambool Butter and Cheese Factory, Australia. Ethanol and phosphate buffer were purchased from Sigma-Aldrich (Sydney, Australia). Milli-Q water was prepared by using a Continental Water System (Continental Water Systems Pty. Ltd., Australia).

Preparation of Buttermilk−Resveratrol Mixtures. A buttermilk stock dispersion (10% total solids, TS) was prepared by dispersing buttermilk powder in Milli-Q water. Concentrated resveratrol solutions (1, 10, and 500 mM) were prepared daily by dissolution in 100% ethanol. These were further diluted with 100% ethanol for preparation of resveratrol solutions for direct addition to buttermilk at a volume ratio of 1:49 to obtain the final concentration of resveratrol required in the buttermilk (10% TS)−resveratrol (100−1600 μM) mixtures while keeping the ethanol concentration in the final mixture constant at 2% w/w. This procedure was used to enable the introduction of a soluble resveratrol to buttermilk. The ethanol concentration was kept to 2% w/w in the final mixture to minimize effects on the structure of proteins. All resveratrol−buttermilk mixtures were incubated for 30 min at the required temperature prior to fluorescence measurement.

Partitioning of Resveratrol in Buttermilk−Resveratrol Mixtures. *Fractionation of Buttermilk−Resveratrol Mixtures.* Ultracentrifugation of buttermilk (10% TS)−resveratrol (100−1600 μM) mixtures was carried out at 30000*g* and 20 °C for 1 h (Beckman Coulter Optima L-90K ultracentrifuge). Under these conditions, buttermilk separates into a cream layer, a milk serum layer, and a casein-rich precipitate.[26] The milk serum phase is composed primarily of whey proteins and small amounts nonmicellar casein. Each fraction was weighed. The protein content of each fraction was measured using a LECO FP-2000 nitrogen analyzer (LECO Australia Pty Ltd., Castle Hill, NSW, Australia), and the moisture content was determined using a Sartorius moisture analyzer (MA30-000 V2).

Extraction and Quantification of Resveratrol in Fractions. Resveratrol was extracted from each fraction with ethanol. A final concentration of 80% w/w ethanol in the cream layer−ethanol or milk serum layer−ethanol mixtures was used for extraction. This concentration of ethanol precipitated the milk proteins and extracted the resveratrol. The concentration of resveratrol in different fractions was quantified by fluorescence spectroscopy, using the linear calibration curve of resveratrol in 80% ethanol at concentrations of 2−40 μM using an excitation wavelength of 320 nm and emission at 400 nm.[27]

The cream layer−ethanol or milk serum layer−ethanol mixtures were centrifuged at 10000 rpm and 20 °C for 15 min. The supernatant was collected and made up to 1 mL using 80% ethanol and then diluted as required, and fluorescence was measured. Re-extraction of the precipitate obtained after centrifugation with 80% ethanol resulted in an extract with undetectable levels of resveratrol.

For the casein-rich precipitate fraction, 0.2 g of this fraction was added to 1.6 mL of 80% ethanol, and the mixture was shaken at 40 °C for 45 min. After centrifugation at 10000 rpm and 20 °C for 15 min, the supernatant was collected. The precipitate was re-extracted twice with 1.6 mL of 80% w/w ethanol. The supernatants from each sample were combined and diluted as required with 80% w/w ethanol for quantification of resveratrol by fluorescence spectroscopy. Spiking experiments with known quantities of resveratrol were carried out to estimate the recovery of resveratrol from the cream, milk serum, and casein-rich precipitate fractions. These experiments showed that the total recovery of resveratrol from the cream layer, milk serum layer, and casein-rich precipitate were all ∼92%.

Fluorescence Spectroscopy. The fluorescence spectra of neat resveratrol, buttermilk, and resveratrol−buttermilk mixtures were measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific Inc., Denmark). Samples (300 μL) were loaded into a 96-well microplate. The fluorescence emission spectra of resveratrol in 2% ethanol (pH 6.54) were recorded from 340 to 500 nm at λ_{ex} of 320 nm and 298 K.[27] To probe the quenching of the intrinsic fluorescence of proteins in buttermilk, emission spectra were recorded from 300 to 500 nm at an excitation wavelength (λ_{ex}) of 280 nm at various temperatures (278, 288, 298, and 308 K). The spectral bandwidths of the excitation and emission slits were at 2.0 nm with 0.1 nm resolution.

Data Analysis. All analyses were carried out in triplicate. The mean values of the triplicate analysis ± SD are presented.

RESULTS AND DISCUSSION

Distributions of Resveratrol in Ultracentrifuged Fractions of Buttermilk−Resveratrol Mixtures. Upon ultracentrifugation of buttermilk (10% TS)−resveratrol (100−1600 μM) mixtures, three layers were obtained, namely, the cream (14.8% w/w, 7.6% TS), the milk serum (75.9% w/w, 7.3% TS), and the casein-rich precipitate (9.3% w/w, 31.7% TS). The compositions of each fraction of buttermilk are shown in Table 1. Protein was present in all fractions, with highest content (78.5% w/w, dry basis) being in the casein-rich precipitate followed by the milk serum layer (14.0% w/w, dry basis) and the cream layer (10.0% w/w, dry basis). Resveratrol was also present in all three fractions of buttermilk. The majority of the added resveratrol in buttermilk (10% TS)−resveratrol (100−1600 μM) mixtures partitioned into the casein-rich precipitate (50.5−56.8%), with lesser amounts in the milk serum layer (35.3−41.2%) and the cream layer (7.9−8.7%), indicating that all fractions (cream, milk serum, casein-rich precipitate) had resveratrol-carrying potential (Table 1).

Figure 1A shows the distribution of resveratrol (μmol/100 g) in each of the fractions obtained upon ultracentrifugation. Using the total solids data for each fraction (Table 1), it is possible to express the distribution of resveratrol in each fraction on a dry basis for ease of comparison. Figure 1B shows the concentration of resveratrol (μmol/g, dry basis) in each fraction. Both the distribution and concentration increased linearly ($R^2 \geq 0.995$) with increasing concentration of added resveratrol (100−1600 μM). Within these concentrations of added resveratrol, there was 0.78−10.30 μmol/g (dry basis) in the cream layer, 0.64−9.30 μmol/g (dry basis) in the milk serum layer, and 1.65−28.26 μmol/g (dry basis) in the casein-rich precipitate (Figure 1).

The similar partitioning of resveratrol (μmol/g on dry basis) into the cream and milk serum fractions (Figure 1), despite the

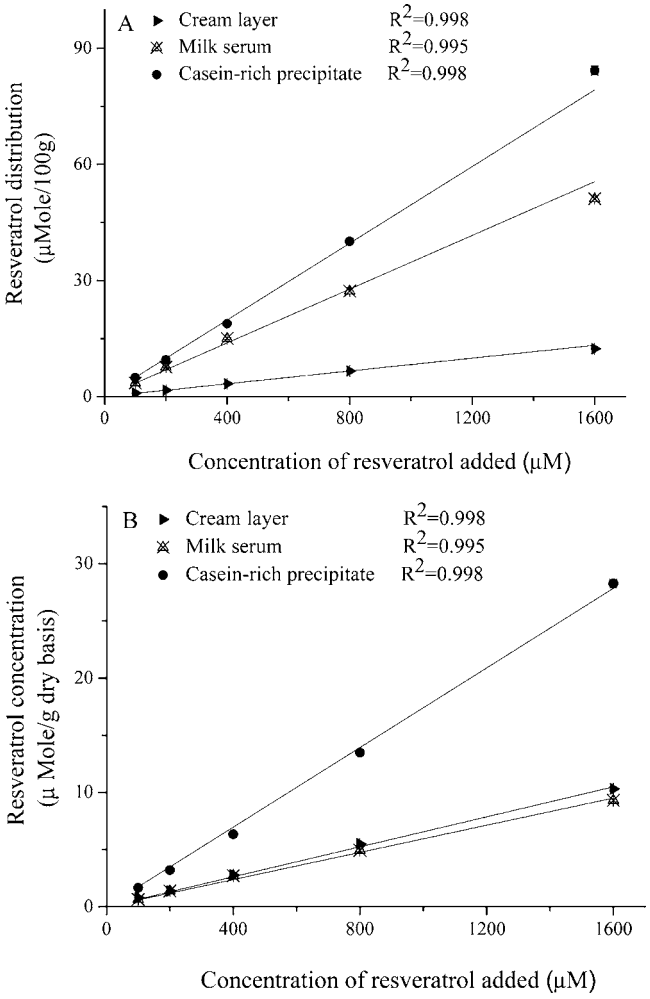

Figure 1. Distribution (A) and concentration (B) of resveratrol in fractions obtained upon ultracentrifugation of buttermilk (10% TS)–resveratrol (100–1600 μM) mixtures.

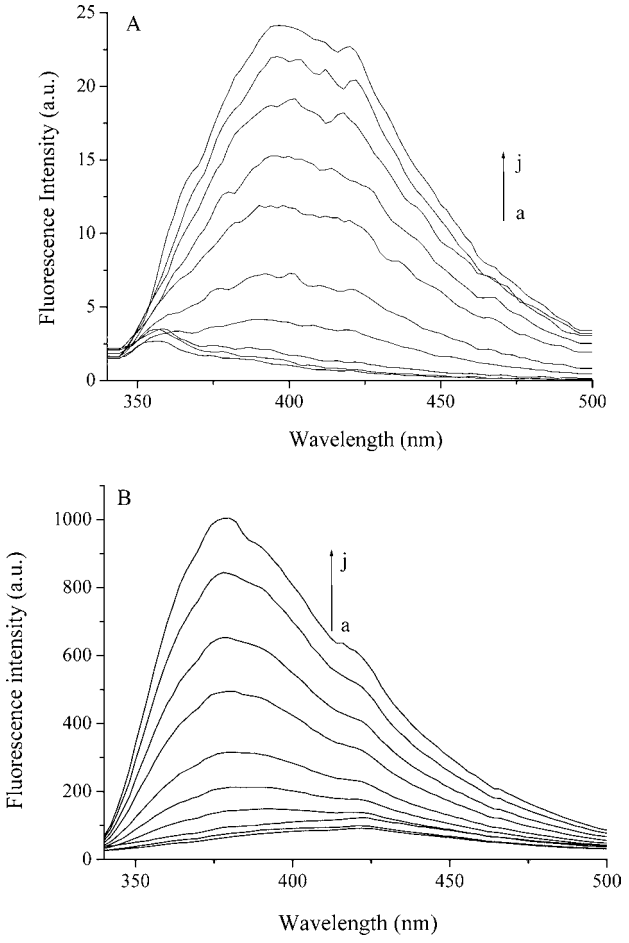

Figure 2. Fluorescence spectra of (A) resveratrol in 2% v/v ethanol and (B) resveratrol–protein complex in 10% TS buttermilk (pH 6.54) at an λ_{ex} of 320 nm and 298 K. a–j represent 0, 2, 6, 10, 15, 20, 25, 30, 35, and 40 μM resveratrol concentrations, respectively.

lower protein content of the cream (0.11 g/100 mL buttermilk–resveratrol mixture) compared to the milk serum fraction (0.78 g/100 mL buttermilk–resveratrol mixture) (Table 1), suggests that resveratrol is not only bound to the proteins in the cream layer but also possibly incorporated into the fat and the milk fat globule components. This result confirmed that resveratrol is also associated with the milk fat globule or inserts at the interface of the milk fat globule. Others have shown that resveratrol can partition more effectively into the oil phase than into the water phase,[12,13] and in addition the amphiphilic nature of resveratrol allows it to assemble at an interface.

When resveratrol was added at levels of 100–1600 μM, there was 4.6–66.4 μmol resveratrol/g protein in the milk serum fraction and 2.2–36.0 μmol/g protein in the casein-rich precipitate fraction. Comparing the results in this way suggests that resveratrol was preferentially bound to the whey proteins because the milk serum layer is rich in whey proteins, whereas the casein-rich precipitate is rich in caseins. Others have shown that resveratrol binds to whey proteins ($K_a \sim 1.2 \times 10^5$)[18] and caseins ($K_a \sim 1.9 \times 10^4$ and 2.3×10^4 M^{-1} for α- and β-caseins).[20] These binding constants suggest that whey proteins bind more strongly to resveratrol than caseins, which is consistent with the result obtained in our study.

Fluorescence Spectra of Resveratrol and Buttermilk–Resveratrol Mixtures. *Excitation at 320 nm.* Figure 2 shows the fluorescence spectra of resveratrol in 2% v/v ethanol solution and resveratrol–buttermilk complexes in 10% TS buttermilk at an λ_{ex} of 320 nm. As the concentration of resveratrol increased, the fluorescence intensities of resveratrol in 2% v/v ethanol solution increased, with the maximum emission band at 400 nm (Figure 2A). Increasing the concentration of added resveratrol in buttermilk also increased fluorescence intensity (Figure 2B). The fluorescence intensities of buttermilk–resveratrol mixtures (Figure 2B) were much higher than those for the same concentrations of resveratrol in 2% v/v ethanol (Figure 2A). The maximum emission band was at 380 nm for buttermilk–resveratrol mixtures (Figure 2B). The results are consistent with the formation of a complex of resveratrol with buttermilk components. A small peak at 424 nm was observed in both spectra (Figure 2). This is possibly related to the π-stacking of the aromatic rings of resveratrol. Others have shown that π-stacking occurs in resveratrol solutions over the concentration range from 2×10^{-2} to 1×10^{-5} M.[28]

Excitation at 280 nm: Fluorescence Quenching Induced by Resveratrol. Fluorescence spectroscopy has been widely used to investigate the structural changes in proteins and the intermolecular interactions between proteins and phenolic compounds.[29–31] Proteins have primarily two intrinsic

fluorophores: tryptophan (Trp) and tyrosine (Tyr) both can be excited at an λ_{ex} of 280 nm, with emission wavelengths at 353 and 304 nm in water at neutral pH, respectively.[32] The fluorescence of these amino acid residues is sensitive to the microenvironment.[18]

The fluorescence spectra of resveratrol-buttermilk mixtures at an λ_{ex} of 280 nm are shown in Figure 3. At the low

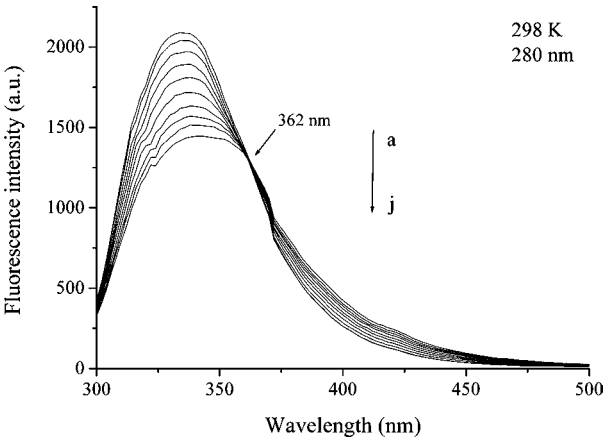

Figure 3. Fluorescence spectra of buttermilk (10% TS)−revseratrol mixtures (pH 6.54) at a λ_{ex} of 280 nm and 298 K. a−j represents 0, 2, 6, 10, 15, 20, 25, 30, 35, and 40 μM resveratrol concentrations in 10% buttermilk, respectively.

concentration of added resveratrol (0−40 μM), the maximum fluorescence intensity of the buttermilk−resveratrol mixture decreased with increasing concentration of resveratrol, and a slight red shift was observed from 334 to 346 nm (Figure 3). This suggested that resveratrol interacted with proteins present in the whole buttermilk. This observation is in line with others who also observed a red shift on binding of phenolics to proteins.[33] The isoemissive point at 362 nm in Figure 3 indicated two emissive species were present in buttermilk−resveratrol mixtures. At higher levels of added resveratrol (1200 μM), the fluorescence spectra of resveratrol−buttermilk systems red-shifted to a longer emission wavelength and reduced intensity, and further increase in resveratrol concentrations red-shifted the spectra more without a significant increase in intensity (data not shown). Similar fluorescence phenomena were observed in bovine serum albumin−resveratrol mixtures.[33]

Interaction between Resveratrol and Proteins in Buttermilk. Fluorescence quenching data were analyzed to investigate the resveratrol−protein interaction. The fluorescence quenching can be binding-related quenching (complex formation) and collisional quenching. The dominant quenching mechanism can be distinguished by examining the temperature dependence of quenching.[34]

The Stern−Volmer equation is used to interpret the quenching process:[35]

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

F_0 and F are the fluorescence emission intensities in the absence and presence of quencher, respectively. K_{SV} is the Stern−Volmer quenching constant, and [Q] is the concentration of free quencher, namely, free resveratrol. K_q is the quenching rate constant, and τ_0 is the average lifetime of the biopolymer fluorophore without quencher.

Figure 4 shows the linear plot of F_0/F as a function of total resveratrol [Q], from which apparent K_{SV} values of 1.46×10^4,

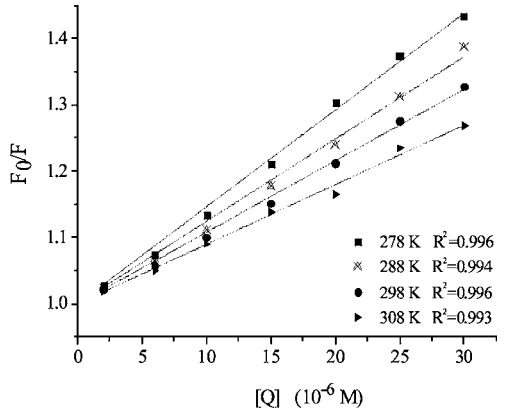

Figure 4. Stern−Volmer plots for the binding between resveratrol and proteins in buttermilk at different temperatures for buttermilk (10% TS) with 0, 2, 6, 10, 15, 20, 25, and 30 μM resveratrol.

1.24×10^4, 1.08×10^4, and 0.90×10^4 M^{-1} for 278, 288, 298, and 308 K were respectively obtained. Plots of F_0/F as a function of total resveratrol [Q] used in this work (Figure 4) have also been used by various authors.[14,16,33] It should be noted that the use of total resveratrol concentration as opposed to the free resveratrol concentration will underestimate the quenching constant.[36] Total added resveratrol concentration was used in these plots as opposed to the free concentration of resveratrol in resveratrol−buttermilk mixtures because of the difficulties associated with the measurement of free resveratrol. Therefore, whereas the K_{SV} values are underestimates of true values, they nevertheless provide evidence of strong binding of resveratrol to proteins.

The apparent K_{SV} values decreased as the temperature increased, indicating that a lower temperature is favorable for complex formation. Collisional quenching depends upon diffusion, and higher temperature increases diffusion coefficients. If collisional quenching is predominant, then an increase in K_{SV} value will be obtained with increase in temperature, but this was not the case in our studies (Figure 4). On the other hand, binding-related quenching is due to the formation of ground-state or excited-state complexes,[36] and high temperature is unfavorable for the formation of the complex, which results in a lower K_{SV} value.[37] The fluorescence lifetime of the biopolymer is 10^{-8} s.[32] The quenching constants K_q calculated from eq 1 were found to be $(0.90-1.46) \times 10^{12}$ L mol^{-1} s^{-1} using total resveratrol concentration. These values were much higher than the maximum value possible for diffusion-limited quenching in solution ($\sim 10^{10}$ L mol^{-1} s^{-1}),[32] suggesting that quenching is not initiated by collision. These data suggest that binding-related quenching was the principal mechanism for the fluorescence quenching of proteins in buttermilk due to the formation of resveratrol−protein complexes.

For binding-related quenching, the apparent binding constant K_A and the number of binding sites n can be calculated using the following equation:[36]

$$\log[(F_0 - F)/F] = \log K_A + n \log[Q] \quad (2)$$

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[Q] is the concentration of free quencher, and F_0 and F are the fluorescence intensities before and after the addition of the quencher. K_A and n values were obtained by the linear plot of $\log[(F_0 - F)/F]$ versus log[Q], with correlation coefficients R^2 above 0.996 (Table 2). As in the case for the Stern–Volmer plots, total resveratrol concentration was used to calculate the minimum K_A values and binding sites n. From the slope and the intercept of the straight line, K_A values were 2.47×10^4, 1.65×10^4, 1.11×10^4, and 0.72×10^4 M^{-1} for 278, 288, 298, and 308 K, respectively, with all binding sites close to 1 (Table 2). As the temperature increased, the binding constants K_A decreased, confirming the above results which showed that low temperature favored the formation of resveratrol–protein complexes. The n values were all close to 1, indicating that a 1:1 complex formed at the Trp/Tyr binding site with resveratrol.

Table 2. Apparent Binding Constants, Binding Sites, and ΔG Values for Resveratrol–Buttermilk Interaction

temperature (K)	K_A (M^{-1})	n	R^2	ΔG (kJ mol^{-1})
278	2.47×10^4	1.05	0.998	−23.37
288	1.65×10^4	1.03	0.997	−23.24
298	1.11×10^4	1.00	0.997	−23.06
308	0.72×10^4	0.98	0.996	−22.73

Thermodynamic Parameters. Hydrogen bonding, van der Waals force, and hydrophobic and electrostatic interactions are the main noncovalent interaction forces between small molecules and biomacromolecules.[38] The Gibbs free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS) were calculated from the data. ΔG was calculated by the following equation:[39]

$$\Delta G = -RT \ln K_A \quad (3)$$

Thermodynamic parameters of ΔH and ΔS were obtained from the van't Hoff equation[39]

$$\ln K_A = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad (4)$$

where K_A is the apparent binding constant, T is the temperature, and R is the gas constant. ΔH is the amount of heat released or absorbed when the reaction occurs at constant pressure. ΔS is a measure for the degree of freedom of the absorbed species or the disorder of a system. ΔH and ΔS were determined from the slope and intercept of the van't Hoff plot of K_A versus $1/T$ (Figure 5). The negative ΔG values (Table 2) indicated that the binding between resveratrol and Trp/Tyr residues on buttermilk occurred spontaneously. The higher negative value of ΔG at lower temperature suggested that low temperature favored the binding interaction. The values of ΔH and ΔS were −29.15 kJ mol^{-1} and −20.65 J mol^{-1} K^{-1} (Figure 5), respectively, suggesting that the binding between resveratrol and buttermilk proteins was exothermic. The negative ΔH and ΔS values are indicative of the involvement of hydrogen bonding and van der Waals forces.[37,38] The ΔH value for van der Waals force ranges from −10 to −4 kJ mol^{-1}, for hydrogen bonding from −40 to −2 kJ mol^{-1}, and for chemical bonding below −60 kJ mol^{-1}.[40] This suggests that hydrogen bonding is an important contributor for the interaction between resveratrol and Trp/Tyr residues on proteins. This result is consistent with previous work relating to resveratrol–α-casein and resveratrol–β-casein complexes, where a molecular docking approach suggested that the complex was stabilized by the hydrogen-bonding network.[20] Others have suggested that the interaction between resveratrol and bovine serum albumin was mainly driven by hydrogen bonding.[37]

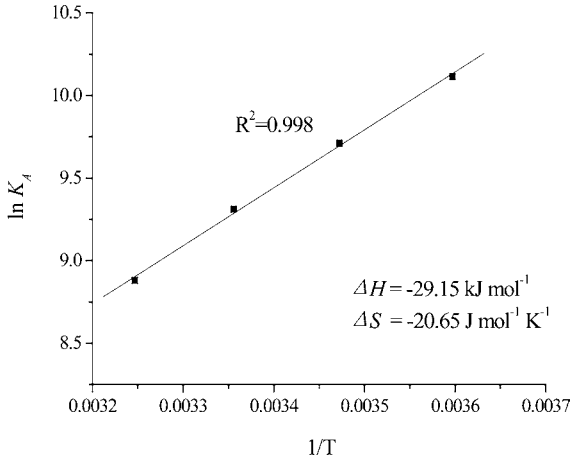

Figure 5. van't Hoff plot for the interaction between resveratrol and binding sites on buttermilk proteins.

This work has shown that buttermilk can be used as an effective carrier for resveratrol. The implication of this work is that a byproduct of the dairy industry, itself with associated health-promoting properties, may be used as an alternative to skim milk proteins or pure protein fractions for the delivery of a bioactive, resveratrol, into functional foods.

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Notes

The authors declare no competing financial interest.

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