JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Binding between Bixin and Whey Protein at pH 7.4 Studied by Spectroscopy and Isothermal Titration Calorimetry

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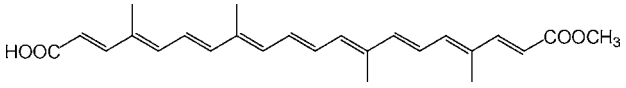
ABSTRACT: Bixin is the major coloring component of annatto used in manufacturing colored cheeses, but its presence in liquid whey causes undesirable quality of the recovered whey protein ingredients. The objective of this work was to study molecular binding between bixin and three major whey proteins (β-lactoglobulin, α-lactalbumin, and bovine serum albumin) at pH 7.4 using UV−vis absorption spectroscopy, fluorescence spectroscopy, isothermal titration calorimetry, and circular dichroism. These complementary techniques illustrated that the binding is a spontaneous complexation process mainly driven by hydrophobic interactions. The complexation is favored at a lower temperature and a higher ionic strength. At a lower temperature, the binding is entropy-driven, while it changes to an enthalpy-driven process at higher temperatures. The binding also increases the percentage of unordered secondary structures of proteins. Findings from this work can be used to develop whey protein recovery processes for minimizing residual annatto content in whey protein ingredients.

KEYWORDS: *bixin, whey protein, isothermal titration calorimetry, UV−vis absorption spectroscopy, fluorescence quenching, circular dichroism*

INTRODUCTION

Proteins recovered from cheese whey have numerous applications because of their excellent functional and nutritional qualities. The liquid whey derived from manufacturing colored cheeses contains residual coloring agent, with annatto being the most popular choice.[1] Annatto coloring is extracted from seeds of the tropical bush *Bixa orellana*, and bixin (Scheme 1), present in the pericarp of annatto seeds, is the major coloring component.[2] To produce neutral-colored whey protein products, the dairy industry employs a bleaching process using hydrogen peroxide or benzoyl peroxide to decolorize whey, but the bleaching process may result in various oxidation products and deteriorate sensory quality.[3] The fate of annatto in cheese manufacturing and the subsequent impact on flavor and functionality of whey proteins were studied.[3] Flavor quality of whey proteins after bleaching by hydrogen peroxide or benzoyl peroxide was compared, with benzoyl peroxide being a more desirable agent.[4] Conversely, information about the binding between annatto and whey protein is needed for the manufacturers to adjust parameters such as solvent conditions (e.g., pH and ionic strength) and temperature so as to minimize the residual content of annatto in protein ingredients, while maintaining whey protein functionality.

Scheme 1. The Molecular Structure of Bixin



The objective of this work was to study molecular binding between bixin and whey protein at neutral pH (pH 7.4) as impacted by salt (ionic strength and types of ions) and temperature. This pH was chosen in our first step of understanding bixin−whey protein binding because it is the physiological pH and, although the pH of sweet whey is 6.35, neutral pH is used in protein separation.[4] In order to understand the binding mechanism, three major whey proteins, i.e., β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), and bovine serum albumin (BSA),[5] were used in the purified form. A commercial whey protein isolate (WPI) product was also used, wherever applicable. Four analytical techniques, i.e., UV−vis absorption spectroscopy, fluorescence spectroscopy, isothermal titration calorimetry (ITC), and circular dichroism (CD), were adopted as they are commonly used to study binding between proteins and small molecules such as metabolites, dyes, and drugs.[6−8] UV−vis absorption spectroscopy provides information about protein structure changes due to complexation and/or reaction with another compound. Since all three whey proteins have tryptophan (Trp) residues that possess intrinsic fluorescence, fluorescence quenching characterizes the accessibility of quencher bixin to fluorophore groups of whey proteins.[9] ITC allows the determination of thermodynamic parameters due to the binding between bixin and whey proteins, while CD spectra illustrate secondary structures of whey proteins as impacted by the binding.

MATERIALS AND METHODS

Materials. WPI was from Hilmar Ingredients (Hilmar, CA). Purified β-Lg, α-Lac, and BSA were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Bixin was obtained from Faltz-Bauer, Inc. (Waterbury, CT). Other chemicals were procured from either Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA). Except for WPI prepared at 5 mg/mL, stock solutions of individual whey proteins were prepared in a 0.01 M phosphate buffer at pH 7.4 (PBS) and kept in the dark at 4 °C before diluting to a final concentration using PBS. Bixin was dissolved in ethanol at a concentration of 0.1 M as a stock solution

Received: December 7, 2011
Revised: January 18, 2012
Accepted: January 23, 2012
Published: January 23, 2012

dx.doi.org/10.1021/jf2050262 | J. Agric. Food Chem. 2012, 60, 1880−1886

that was diluted with PBS to 5 mM before use. The final concentrations of protein and bixin were adjusted for individual analytical instruments, detailed below.

UV Absorption Spectroscopy. The concentration of WPI solution was 0.5 mg/mL, while that of individual whey protein (β-Lg, α-Lac, and BSA) was 10 μM. The UV−vis absorption spectra were acquired between 200 and 800 nm using a UV−vis spectrometer (Unicam, Cambridge, U.K.). All experiments were performed in triplicate at room temperature (21 °C).

Fluorescence Spectroscopy. The fluorescence spectra were recorded using a RF-1501 spectrofluorometer (Shimadzu, Japan). The excitation wavelength was 285 nm. Both the excitation and emission slit widths were set at 10 nm. The emission spectra were recorded between 300 and 450 nm, with the background fluorescence calibrated using PBS. Triplicate samples were measured.

Isothermal Titration Calorimetry. The ITC experiments were carried out with a Microcal VP-ITC microcalorimeter (Microcal Inc., Northampton, MA). The protein solutions were prepared from the above stock solution to contain 5% ethanol and 50 μM protein. The titrant was bixin at a concentration of 5 mM in 5% ethanol, and all solutions were degassed before each titration. The sample cell was loaded with 1.451 mL of a protein solution that was stirred by the injection syringe at 307 rpm, which enabled rapid mixing without causing foaming of the protein solution. The programmable titration was conducted under different temperatures, and the titrant volume of each injection was 4 μL. To correct the thermal effect due to mixing and dilution, a control experiment was performed by injecting the titrant into the PBS without protein.

Circular Dichroism. Far-UV CD spectra were recorded between 200 and 260 nm with an interval of 1 nm using a model 202 CD spectrometer (Aviv Biomedical, Inc., Lakewood, NJ) at 298 K. The protein concentrations were 10 μM for BSA, 50 μM for α-Lac, and 50 μM for β-Lg. A quartz cell with a 0.1 cm path length was used, and a constant nitrogen flush was used during data acquisition. The secondary structure contents of the samples were estimated from the CD spectra using the SELCON 3 method available in the CDPro software package of the instrument. Triplicate samples were measured.

Statistical Analysis. A second-order polynomial regression model was employed to determine the statistical differences of quenching constants at different ionic strengths. A one-way analysis of variance (ANOVA) was used to compare quenching constants of different proteins and effects of ion types. A significance level of 0.05 was used in analyses assisted by the SAS software (version 9.0, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Effects of Bixin Binding on the UV−Vis Absorption Spectra of Whey Protein. The UV−vis absorption spectra of WPI after mixing with 0−250 μM bixin are shown in Figure 1. Without bixin, the absorption spectrum of WPI showed a strong peak centered on about 230 nm and another weaker peak centered on near 279 nm. The 230 nm peak is characteristic of the peptide backbone, while the 279 nm peak is typical for proteins containing aromatic amino acids.[10] With bixin (Figure 1), the absorbance increased with an increase in bixin concentration, and the center of the 279 nm peak shifted slightly to a shorter wavelength (blueshift), while the 230 nm peak shifted to a longer wavelength (redshift).When the binding between bixin and individual whey

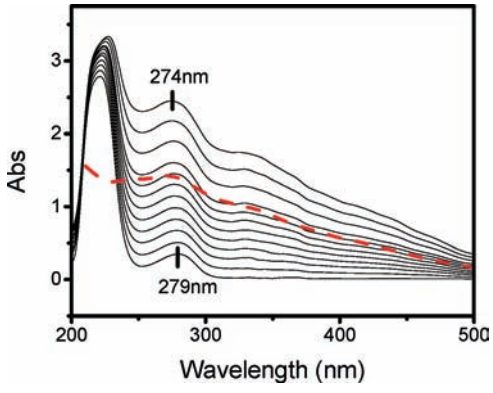


Figure 1. The UV−vis absorption spectra of WPI (0.5 mg/mL) treated with 0, 20, 40, 60, 80, 100, 120, 140, 160, 200, 240, and 280 μM, from bottom to top, of bixin. The dashed red curve represents the spectrum of bixin.

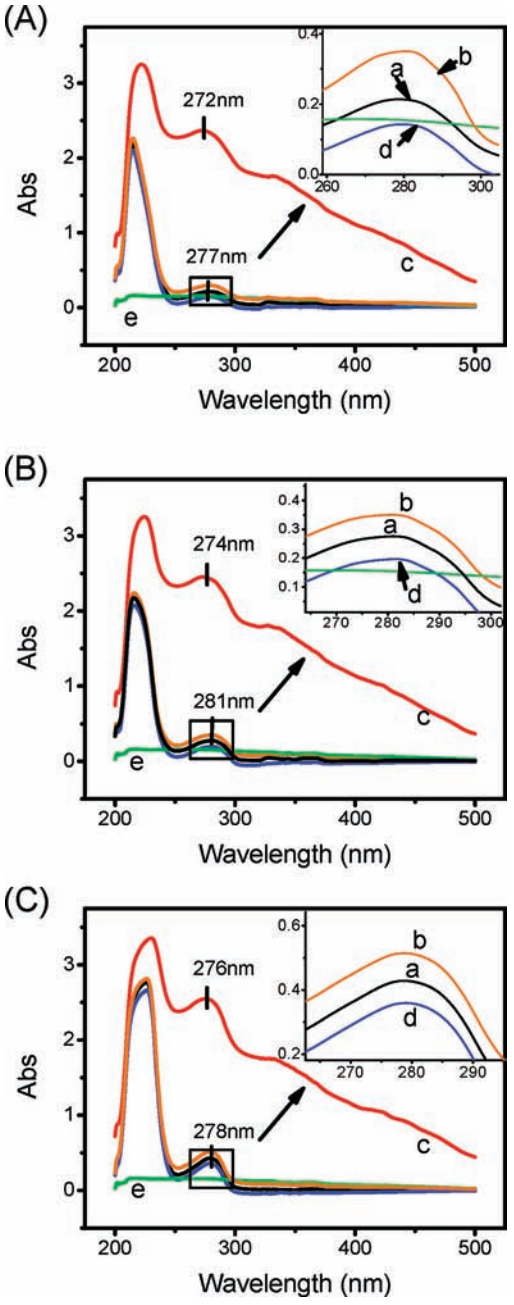


Figure 2. UV−vis absorption spectra of β-Lg (A), α-Lac (B), and BSA (C) in the absence and presence of bixin. Samples contained 10 μM protein and 0 (black curve, a), 10 μM (orange curve, b), and 280 μM (red curve, c) bixin. The blue curve (d) shows the absorption spectrum of the protein sample with 10 μM bixin after subtraction by the spectrum of the bixin only solution. The spectrum of the 10 μM bixin only solution is shown as the green curve (e).

dx.doi.org/10.1021/jf2050262 | J. Agric. Food Chem. 2012, 60, 1880−1886

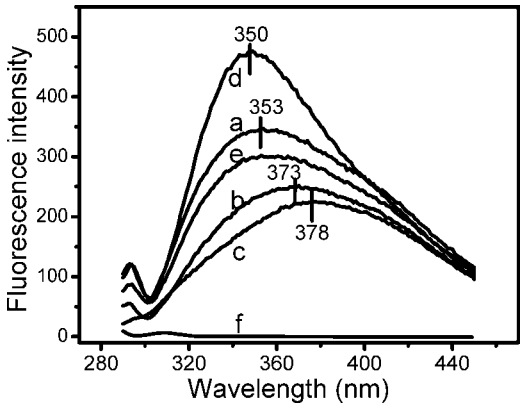


Figure 3. Intrinsic emission spectra of WPI (curve a), β-Lg (curve b), α-Lac (curve c), BSA (curve d), and bixin (curve f). The titration of bixin induces fluorescence quenching, shown for WPI in curve e. The concentration of β-Lg, α-Lac, and BSA was 10 μM, while that of WPI was 0.5 mg/mL. The temperature was 298 K, and the excitation wavelength was 285 nm.

Table 1. Stern–Volmer Quenching Constants K_{SV} and K_a Due to Binding between Bixin and Whey Protein[a] at pH 7.4 and 298 K

protein	K_{SV} ($\times 10^4$ M^{-1})	R	SD	K_a ($\times 10^4$ M^{-1})	R	SD
WPI	2.31 ab	0.965	0.142	1.31 ab	0.995	0.021
β-Lg	1.95 b	0.976	0.126	1.32 ab	0.997	0.014
α-Lac	1.81 b	0.985	0.044	1.18 b	0.999	0.006
BSA	2.75 a	0.969	0.136	1.45 a	0.991	0.017

[a]WPI was used at 0.5 mg/mL, and individual whey proteins were used at 10 μM. The correlation coefficient (R) and standard deviation (SD) of data fitting the model were reported. Means in the same column without a common letter differ significantly ($P < 0.05$).

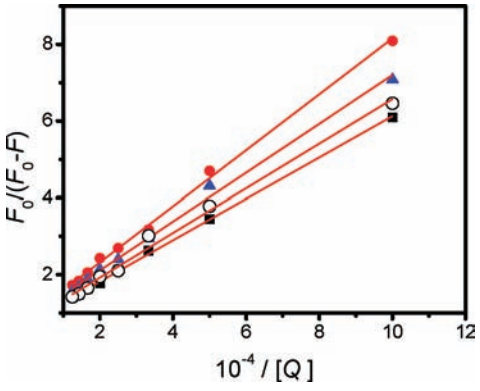


Figure 4. Modified Stern–Volmer plots of WPI (○), β-Lg (▲), α-Lac (●), and BSA (■) fluorescence quenching caused by the addition of bixin at 298 K and pH 7.4.

proteins was studied, the blueshift was observed from 277 to 272 nm for α-Lg, from 281 to 274 nm for α-Lac, and from 278 to 276 nm for BSA (Figure 2). The results suggest that the addition of bixin changes the polarity around protein: some residues such as Trp and Tyr become exposed to a more polar environment, while some external residues become embedded in globular proteins.[11] Figures 1 and 2 also indicate that the extent of these tertiary structure changes due to complexation by bixin differs for each whey protein.

Effects of Bixin Binding on Intrinsic Tryptophan Fluorescence of Whey Protein. The fluorescence quenching method was used to study the binding reaction between bixin and whey protein because β-Lg, α-Lac, and BSA all contain Trp residues that possess intrinsic fluorescence after being excited at 285 nm. Trp residues are present at Trp-19 and Trp-61 for β-Lg; Trp-26, Trp-60, Trp-104, and Trp-118 for α-Lac; and Trp-212 and Trp-134 for BSA.[12−14] The intrinsic emission fluorescence spectra of WPI and each of the three proteins are presented in Figure 3. The differences in intensity and width of emission peak, as well as the wavelength corresponding to the peak center, for each protein sample are due to different amounts of Trp residues and their positions. Residues on or near the surface of a protein show an emission spectrum centered on a longer wavelength.[15] The fluorescence emission of WPI is a combination of the three proteins, and the titration of bixin led to a decrease in the fluorescence intensity (curve a vs e in Figure 3).

Fluorescence quenching can be caused by mechanisms like excited state reaction, molecular rearrangement, energy transfer, ground state complexation, and collision quenching.[16] In order to elucidate the exact mechanism, fluorescence quenching was further studied for different bixin concentrations. The data were fit into the Stern–Volmer model (eq 1),[17] with the estimated quenching constants tabulated in Table 1. The system of bixin–BSA has the highest K_{SV} value, which suggests the most stable complex. The K_{SV} value of bixin–WPI is intermediate and is close to the average of the three proteins.

$$\frac{F_0}{F} = 1 + k_q t_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher. [Q] is the quencher concentration, K_{SV} is the Stern–Volmer quenching constant, k_q is the bimolecular quenching rate constant, and t_0 is the lifetime of fluorescence in absence of a quencher.[17]

Fluorescence quenching can be further classified as dynamic or static quenching. Dynamic quenching refers to a collision between a fluorophore and a quencher during the lifetime of the excited state, while static quenching refers to a complex formation at the ground state.[17,18] Because the collision does not change the UV spectrum of protein,[19] changes in UV–vis absorption spectra after binding between bixin and protein (Figures 1 and 2) indicate the formation of complexes, and thus the fluorescence quenching induced by bixin is static quenching. For the case of static quenching, the quenching data can be analyzed according to the modified Stern–Volmer model (eq 2),[17] with plots shown in Figure 4 and the derived K_a listed in Table 1. The correlation coefficients of data fitting were all greater than 0.99. The K_a of α-Lac is the highest. Like K_{SV}, the magnitude of K_a of WPI is between those of individual proteins. The above analyses suggest that the quenching of whey protein fluorescence by bixin is due to complexation and is caused by Trp residues moving closer to the protein surface.

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \quad (2)$$

where f_a is the fraction of accessible fluorescence and K_a is the effective quenching constant for the accessible fluorophores, which can be considered as an associative binding constant for a quencher–acceptor system. The linear regression between F_0/

Table 2. The Modified Stern−Volmer Quenching Constant K_a ($\times 10^4$ M^{-1}) Due to Binding between Bixin and Whey Protein[a] as Affected by Select Ions at pH 7.4 and 298 K

ion	WPI	β-Lg	α-Lac	BSA
control	1.30 ± 0.19 ab	1.39 ± 0.12 a	1.10 ± 0.08 a	1.55 ± 0.12 b
Cr^{3+}	1.29 ± 0.21 ab	1.28 ± 0.07 bc	1.03 ± 0.02 b	1.73 ± 0.20 ab
K^+	1.27 ± 0.22 ab	1.32 ± 0.15 ab	1.12 ± 0.05 a	1.53 ± 0.11 b
Fe^{3+}	1.16 ± 0.11 b	1.23 ± 0.05 c	0.91 ± 0.10 d	1.78 ± 0.21 a
SO_4^{2-}	1.52 ± 0.20 a	1.26 ± 0.11 c	0.98 ± 0.09 bc	1.80 ± 0.06 a
Ni^{3+}	1.34 ± 0.09 ab	1.14 ± 0.13 d	1.03 ± 0.15 b	1.77 ± 0.15 a
Ca^{2+}	1.38 ± 0.13 ab	1.25 ± 0.10 c	0.93 ± 0.08 cd	1.59 ± 0.13 b

[a]WPI was used at 0.5 mg/mL, and individual whey proteins were used at 10 μM. Concentrations of common ions were 100 μM. Means ± standard deviations from triplicate tests are presented. Values followed by different letters in the same column indicate differences in the mean ($P < 0.05$).

Table 3. Effect of Ionic Strength on the Modified Stern−Volmer Quenching Constant K_a ($\times 10^4$ M^{-1}) Due to Binding between Bixin and Whey Protein[a] at pH 7.4 and 298 K

NaCl[b] (M)	WPI	β-Lg	α-Lac	BSA
0	1.43 ± 0.09	1.39 ± 0.11	1.10 ± 0.08	1.63 ± 0.19
0.01	1.40 ± 0.13	1.53 ± 0.11	1.28 ± 0.15	1.70 ± 0.08
0.05	1.68 ± 0.03	1.57 ± 0.03	1.37 ± 0.05	1.74 ± 0.18
0.1	1.69 ± 0.03	1.62 ± 0.02	1.41 ± 0.41	1.85 ± 0.24
0.5	1.83 ± 0.11	1.90 ± 0.08	2.16 ± 0.30	1.72 ± 0.26

[a]WPI was used at 0.5 mg/mL, and individual whey proteins were used at 10 μM. Means ± standard deviations from triplicate tests are presented. [b]The NaCl concentration described by second order polynomial regression showed a highly significant ($p < 0.05$) effect on K_a.

ΔF and $1/[Q]$ enables the determination of $1/f_aK_a$ (slope) and $1/f_a$ (intercept), and therefore K_a.[17]

Effects of Common Ions and Ionic Strength on the Interaction between Bixin and Whey Protein. The assessment of K_a allows the study of influences of ion types and ionic strength on the interaction between bixin and whey protein. The impacts of several ions present in milk and dairy products[20] on the binding between bixin and whey protein were studied at 100 μM, with K_a listed in Table 2. The K_a for WPI was not significantly affected by the ions at the studied conditions. For β-Lg and α-Lac, the presence of multivalent ions lowered the K_a, while the monovalent potassium ions did not have significant impact on K_a. For BSA, potassium ions also had no influence on K_a, but multivalent ions enhanced the binding.

The effects of ionic strength were studied at 0−0.5 M NaCl (Table 3). The K_a increased monotonically with an increase in NaCl concentration for WPI, β-Lg, and α-Lac, while the highest K_a was observed at 0.1 M for BSA. The ionic strength influenced the K_a of α-Lac-bixin most significantly (from 1.10 to 2.16 × 10^4 M^{-1}). The data shows that the accessibility of bixin to Trp residues of the three proteins becomes easier, especially for α-Lac, at a higher ionic strength. Although the distribution of charges on protein surface is a major factor for ionic strength impacting binding constant,[21] it is likely not the cause of observations in Table 3 because Trp residues in the three proteins are not present at either the −C or −N terminus and are thus not charged. Conversely, the enhanced bixin-protein binding indicates that Trp residues become closer to the protein surface, which in turn increases protein surface hydrophobicity at an increased ionic strength, as expected from protein biochemistry.[22] Therefore, hydrophobic interactions are important for binding between bixin and whey protein.

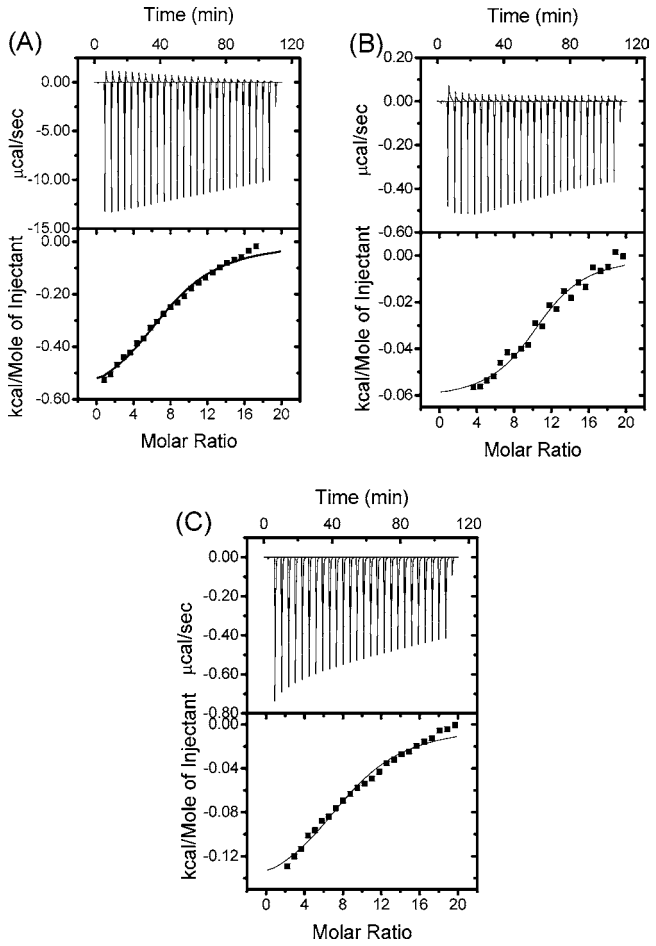


Figure 5. Titration profiles at 298 K when a titrant of 5 mM bixin was mixed at 40 μL per injection with a solution containing 50 μM β-Lg (A), α-Lac (B), and BSA (C) at pH 7.4. Curves in bottom figures show the fitting of data to a one-set model by the nonlinear Levenberg−Marquardt fitting algorithm supplied by the instrument.

Calorimetric Characterization of Binding Energy between Bixin and Whey Protein. The ITC is a powerful technique to study biomolecular interactions because it allows the determination of thermodynamic parameters like enthalpy change (ΔH), entropy change (ΔS), binding stoichiometry (n), and binding constant (K_b) in one experiment.[23] Figure 5 shows representative calorimetric profiles during the titration of bixin into whey protein solutions at 298 K. The binding process is exothermic in all cases. The energy change during titration to incrementing molar ratios of bixin:protein (bottom curves in Figure 5) was fitted to a one-set model by the nonlinear

dx.doi.org/10.1021/jf2050262 | J. Agric. Food Chem. 2012, 60, 1880−1886

Table 4. Thermodynamic Properties for Binding between Bixin and Whey Protein at pH 7.4[a]

protein	T (K)	n	K_b (10^4 M^{-1})	ΔG (kcal mol^{-1})	ΔH (cal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔC_p (cal mol^{-1} K^{-1})
β-Lg	288	9.50 ± 0.30	1.84 ± 0.23	−5.62	−238.5 ± 23.88	18.7	−14.2
	298	7.91 ± 0.21	1.42 ± 0.19	−5.56	−462.4 ± 30.11	17.1	
	308	7.78 ± 0.16	1.08 ± 0.14	−5.65	−506.5 ± 54.11	16.7	
	318	4.92 ± 0.29	0.78 ± 0.13	−5.09	−700.1 ± 66.18	13.8	
α-Lac	288	10.9 ± 0.09	3.03 ± 0.42	−5.89	−44.42 ± 2.30	20.3	−6.36
	298	10.5 ± 0.36	2.50 ± 0.75	−6.02	−62.11 ± 2.40	20.0	
	308	10.0 ± 0.27	2.12 ± 0.92	−6.13	−94.30 ± 2.49	19.6	
	318	9.36 ± 0.29	1.50 ± 0.27	−6.07	−245.6 ± 11.07	18.3	
BSA	288	8.83 ± 0.61	1.75 ± 0.34	−5.55	−103.2 ± 11.00	18.9	−4.65
	298	8.71 ± 0.39	1.22 ± 0.45	−5.58	−157.9 ± 20.13	18.2	
	308	8.03 ± 0.28	1.00 ± 0.27	−5.75	−203.2 ± 8.13	18.0	
	318	7.77 ± 0.39	0.91 ± 0.15	−5.90	−243.4 ± 10.55	17.8	

[a]Averages ± standard deviations from triplicate measurements.

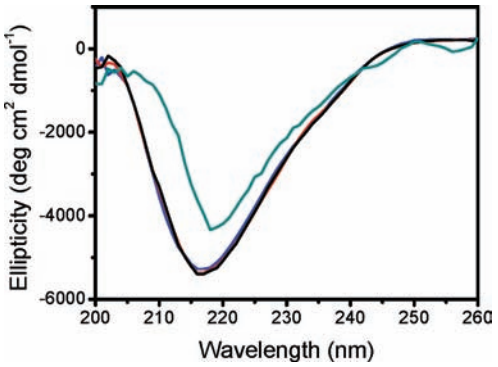


Figure 6. Circular dichroism spectra of virgin (black curves) β-Lg and after treating bixin at protein:bixin molar ratios of 1:1 (red curves), 1:10 (blue curves), and 1:50 (green curves) at pH 7.4 and 298 K.

Levenberg−Marquardt fitting algorithm (supplied by Microcal), and the estimated thermodynamic parameters are summarized in Table 4.

The K_b for β-Lg, α-Lac, and BSA at 298 K was determined to be 1.42×10^4, 2.50×10^4, and 1.22×10^4 M^{-1}, respectively (Table 4). These values have the same magnitude as the binding constants K_a obtained from fluorescence quenching data (Table 1), but there are some differences. Because the fluorescence quenching method is based on quenching of Trp residues when the excitation wavelength is set between 280 and 295 nm, the fluorescence of protein quenched by bixin is due to the binding sites near Trp residues only. Conversely, thermodynamic changes due to bixin binding onto all possible specific and nonspecific sites on proteins are measured in ITC. For example, β-Lg has two specific binding sites. The primary one is located in the central position within the β-barrel combined by eight antiparallel β-strands, while the other potential binding site is external.[24,25] For fluorescence quenching, since Trp-19 is a little far from the primary binding site and Trp-61 is within an extremely mobile loop of the pocket, the measurement only reveals the binding process partially.[26] In ITC, binding to both sites is measured. This may explain the difficulty to directly compare binding constants estimated from the two methods: K_b is bigger than K_a for β-Lg and α-Lac, while it is the opposite for BSA. Nevertheless, the number of binding sites (n) obtained from ITC is commonly treated as a more reliable parameter for biomolecular interactions, and K_b is used to indicate the overall binding properties.[23] The n of α-Lac is bigger than that of β-Lg and BSA, likely because α-Lac has more specific binding sites than the other two proteins.[27]

The values of ΔH are negative, whereas those of ΔS are positive (Table 4), which indicates that both entropy and enthalpy contribute to the binding process. The negative ΔG ($\Delta G = \Delta H - T\Delta S$) indicates the spontaneity of the binding between bixin and whey protein. Among the three proteins, the enthalpy contribution is the smallest for α-Lac. For each protein, the main source of the ΔG value is derived from ΔS, implying the significance of hydrophobic interaction.[28] Since a positive ΔS value and a small negative ΔH value are also characteristics of a specific electrostatic force,[28] the electrostatic interactions may have contributed to the binding of bixin to

Table 5. Secondary Structures of β-Lg, α-Lac, and BSA Affected by Complexation with Bixin at pH 7.4 and 298 K

protein	molar ratio of protein:bixin	α-helix (%)	β-sheet (%)	turn (%)	unordered (%)
β-Lg	1:0	6.7 ± 0.1	34.2 ± 0.5	22.7 ± 0.6	37.2 ± 0.5
	1:1	7.0 ± 0.3	33.5 ± 0.3	22.8 ± 0.5	37.7 ± 1.4
	1:10	7.3 ± 0.2	32.2 ± 0.6	23.6 ± 0.1	40.9 ± 1.1
	1:50	7.6 ± 0.2	27.2 ± 0.1	23.0 ± 0.0	41.6 ± 0.9
α-Lac	1:0	23.1 ± 0.6	16.0 ± 1.0	22.8 ± 0.2	35.9 ± 0.3
	1:1	23.0 ± 0.1	16.0 ± 0.6	21.7 ± 0.6	37.6 ± 0.4
	1:5	21.5 ± 0.2	17.1 ± 0.1	22.0 ± 0.0	36.3 ± 0.5
	1:50	16.6 ± 0.6	25.4 ± 0.7	22.7 ± 0.3	37.5 ± 1.2
BSA	1:0	52.8 ± 1.2	7.1 ± 0.2	17.6 ± 0.8	24.5 ± 0.9
	1:1	51.8 ± 1.7	7.6 ± 0.7	18.5 ± 0.5	24.4 ± 0.9
	1:10	51.5 ± 0.9	9.6 ± 0.6	17.6 ± 1.0	24.7 ± 0.5
	1:50	43.8 ± 1.2	11.0 ± 0.8	17.6 ± 0.9	29.4 ± 1.0

whey protein. At pH 7.4, the three whey proteins are overall negatively charged but carry some positively charged groups that may enable localized binding with bixin.[29,30] However, based on the previous discussion about K_a impacted by ionic strength,[31] electrostatic attraction contributes insignificantly to the binding between bixin and whey protein and hydrophobic interactions are the major force for the binding.

To further investigate the interactions between bixin and whey protein, thermodynamic parameters were estimated at 288−318 K (Table 4). Overall, ΔH is more negative and ΔS is less positive at a higher temperature. The smaller ΔS at a higher temperature is in agreement with smaller n and K_b. When individual whey proteins are compared, temperature affected the n of β-LG more significantly than the other two proteins. Because β-LG is the most abundant whey protein, the data in Table 4 suggests that a higher temperature is recommended in whey protein recovery to reduce the residual content of bixin in whey protein products.

The ΔH acquired at various temperatures also can be used to determine heat capacity (ΔC_p) of binding by eq 3. The negative values of ΔC_p for all proteins (Table 4) strengthen the conclusion of hydrophobic interactions being the major force in binding and indicate that the binding is entropy-driven at a lower temperature and transits to an enthalpy-driven process at higher temperatures.[32,33]

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p \tag{3}$$

Conformation Changes of Whey Protein upon Interaction with Bixin. The far-UV CD spectra of three whey proteins before and after interacting with different concentrations of bixin were also investigated, with those of β-Lg presented in Figure 6. The widened valley around 216 nm is a feature typical for a β-sheet dominant protein.[34] After the titration by bixin, the intensity of the negative band changed significantly, suggesting the impact of binding on secondary structures of the protein. Bixin also affects the CD spectra of α-Lac and BSA (not shown). The algorithm SELCON3 was used to quantify the contents of different types of secondary structures from the CD spectra by referencing to 43 soluble proteins with known precise secondary structures.[35,36] The results are summarized in Table 5. BSA and α-Lac showed a decreased content of α-helical structure and an increased content of β-sheet structure at a higher concentration of bixin, but the trends were opposite for β-Lg. For all proteins, a higher bixin content corresponded to a higher percentage of the unordered structure.

In summary, the results of this study revealed that the interactions between bixin and whey proteins are mainly based on a complex formation process driven by hydrophobic interactions. The complexation is favored at a lower temperature and higher ionic strength, and the binding is entropy-driven and changes to an enthalpy-driven process with an increase in temperature. The binding between bixin and protein is expected to be a function of pH and degree of protein denaturation, which are currently being investigated. Changes in secondary structures of whey proteins because of binding with bixin may also impact protein functionality. These fundamental properties are critical for the dairy industry to manufacture whey protein ingredients with minimal coloring residues and optimal functionality by adopting techniques such as inert adsorbents to selectively remove coloring compounds.

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Funding

The authors are grateful for the financial support from Dairy Research Institute (Rosemont, IL).

Notes

The authors declare no competing financial interest.

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