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Effects of Thermal Denaturation on Binding between Bixin and Whey Protein

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ABSTRACT: Heating is commonly used in the manufacture of dairy products, but impacts of thermal denaturation on binding between whey protein and molecules such as pigments are poorly understood. The objective of this work was to study the impacts of thermal denaturation on binding between bixin, a pigment relevant to colored cheeses, and whey proteins using several complementary techniques. Fluorescence spectroscopy data showed that heat treatment caused tryptophan in β-lactoglobulin and α-lactalbumin to be exposed to a more polar environment, but the opposite was observed for bovine serum albumin. The fluorescence quenching study indicated that the quenching of whey protein fluorescence by bixin was static quenching, and the affinity of binding with bixin was enhanced after thermal denaturation, caused by a higher extent of unordered structures, as revealed by results from circular dichroism and Fourier transform infrared spectra. β-Lactoglobulin was responsible for overall impacts of thermal denaturation on binding between bixin and whey protein isolate.

KEYWORDS: *bixin, whey protein, binding, thermal denaturation, fluorescence quenching, circular dichroism*

INTRODUCTION

Whey proteins have promising applications in various food products because of their excellent functional properties and nutritional quality. Thermal treatment is used not only in the manufacture of most dairy products, including cheeses, to ensure safety and quality but also to modify the functional properties of proteins.[1] Heating alters surface characteristics of proteins that are correlated with emulsifying activity, emulsion stability, foaming capacity, and water-holding capacity.[2] Effects of thermal treatment on the structure of whey proteins have been extensively studied, especially for β-lactoglobulin (β-Lg), the most abundant whey protein. After heat treatment at 80−90 °C, β-Lg partially unfolds by a $\beta \rightarrow \alpha$ transition.[3] There are also several intermediate states caused by various denaturants,[4] but the mechanisms of unfolding and refolding are still not well understood. Moreover, changes of secondary and tertiary structures during thermal denaturation affect the binding affinity of small molecules on proteins, for example, pigments applied to color dairy products.[5]

Particularly, cheese whey is the major source of whey protein recovery, and the dairy industry applies a bleaching step to decolorize liquid whey derived from the production of colored cheeses. Annatto is a major pigment used to color cheeses, and some annatto ends up in cheese whey. Understanding interactions between annatto and whey proteins is important for designing protein purification processes, adopting more benign bleaching conditions, or developing alternative decolorization technologies to manufacture whey protein ingredients with superior quality and functionality. Bixin is the major coloring component in annatto but is water insoluble. After alkaline saponification, the methyl ester group of bixin is converted to a hydroxyl group, and the derived dicarboxylic acid, norbixin, in the form of sodium or potassium salts, is water-soluble at neutral and alkaline conditions.[6] In our previous study, we reported that hydrophobic interactions are the major mechanism for the binding between whey protein and bixin at neutral acidity and that the binding is favored at a lower temperature between 15 and 45 °C and a higher ionic strength.[7] It is well-known that hydrophobicity of whey proteins is affected by pH and thermal denaturation.[8] The latter is the focus of the present work because thermal pasteurization of milk and possibly cheese whey can be achieved above the denaturation temperatures of three major whey proteins,[9] which are 78, 62, and 64 °C for β-Lg, α-lactalbumin (α-Lac), and bovine serum albumin (BSA), respectively.[10] Denaturation causes an increase in the surface hydrophobicity of whey proteins, with dominant contribution from β-Lg and α-Lac.[8,11] The molecular bases of impacts of thermal denaturation on binding between bixin and whey protein are to be studied so that dairy manufacturers can adopt the information to adjust parameters in whey protein recovery.

The objective of this work was thus to study the impacts of thermal denaturation on binding between bixin and whey protein at pH 6.4, corresponding to the pH of Cheddar cheese whey.[12] As in our previous study,[7] common analytical techniques in protein biochemistry were applied, including differential scanning calorimetry (DSC), fluorescence spectroscopy, Fourier transform infrared spectroscopy (FT-IR), and circular dichroism (CD). Three major whey proteins, that is, β-Lg, α-Lac, and BSA, were heated at 80 °C for 15 or 30 min, and the binding affinity between bixin and native and denatured whey proteins was evaluated.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) was from Hilmar Ingredients (Hilmar, CA, USA). Purified β-Lg, α-Lac, and BSA were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Bixin was

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obtained from Faltz-Bauer, Inc. (Waterbury, CT, USA). All other chemicals used in this study were obtained from either Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA, USA). Protein stock solutions were prepared in distilled water, adjusted to pH 6.4, and kept in the dark at 4 °C. Bixin was dissolved in ethanol at a concentration of 0.1 M as a stock solution that was diluted with distilled water to 5 mM before use.

DSC. The thermal denaturation of native and preheated WPI dispersions (20 mg/mL, pH 6.4) was characterized using a VP-DSC (MicroCal Inc., Northampton, MA, USA). The samples were degassed before loading in the calorimeter cell. The reference cell was filled with distilled water. A pressure of 186 kPa was applied during heating samples at a rate of 1.5 °C/min from 25 to 105 °C. The denaturation enthalpy change (ΔH) was obtained by fitting the data in a two-state model (supplied by MicroCal).

Fluorescence Spectroscopy. The fluorescence spectra were recorded using an RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, 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 distilled water. Triplicate samples were measured.

CD. Far-UV CD spectra were recorded between 200 and 260 nm with an interval of 0.5 nm using a model 202 CD spectrometer (Aviv Biomedical, Inc., Lakewood, NJ, USA) 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 by reference to 43 soluble proteins with known precise secondary structures. Triplicate samples were measured.

FT-IR Spectroscopy. The instrument was a Nicolet Nexus 670 FT-IR spectrometer (Thermo Nicolet Corp., Madison, WI, USA) equipped with a germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector, and a KBr beam splitter. All spectra were taken via the ATR method with a resolution of 4 cm^{-1} using 64 scans. WPI was prepared in deuterated water to prevent the interference of hydrogen bonds in the amide region of the protein spectra.[13] For the same reason, bixin was dissolved in dimethyl sulfoxide (DMSO) because the amount of DMSO added in solution has no absorbance in the 1850−1500 cm^{-1} range of the IR spectrum.[14]

UV−Vis Absorbance Measurement. The absorbance of bixin was acquired between 200 and 800 nm using a UV−vis spectrophotometer (Unicam, Cambridge, UK). Triplicate samples were measured at room temperature (21 °C).

RESULTS AND DISCUSSION

Heat Denaturation of WPI. In DSC, the heat absorbed by protein molecules in water can be mainly attributed to protein denaturation. A single endothermic peak centered on 79.6 °C was observed for native WPI (Figure 1A). Because WPI is a mixture of several proteins and each protein unfolds at a different temperature, the peak can be considered as an overall denaturation temperature of WPI. The ΔH of native WPI was estimated to be 60.30 ± 0.17 kcal/mol, which is similar to the value for β-Lg reported by Burgos et al.[15] For WPI heated at 80 °C for 15 min, the intensity of the denaturation peak decreased significantly, the thermal midpoint (T_m) shifted to a higher temperature (85.1 °C), and the ΔH decreased to 0.89 ± 0.38 kcal/mol, showing the strong impact of preheating on the protein. The impacts of preheating on ΔH and T_m indicate that WPI is denatured effectively after heating at 80 °C for 15 min and remains in an unfolded state after cooling to room temperature and 4 h of storage.[16]

The intrinsic emission fluorescence spectra of native and denatured WPI are compared in Figure 1B. After 15 min of

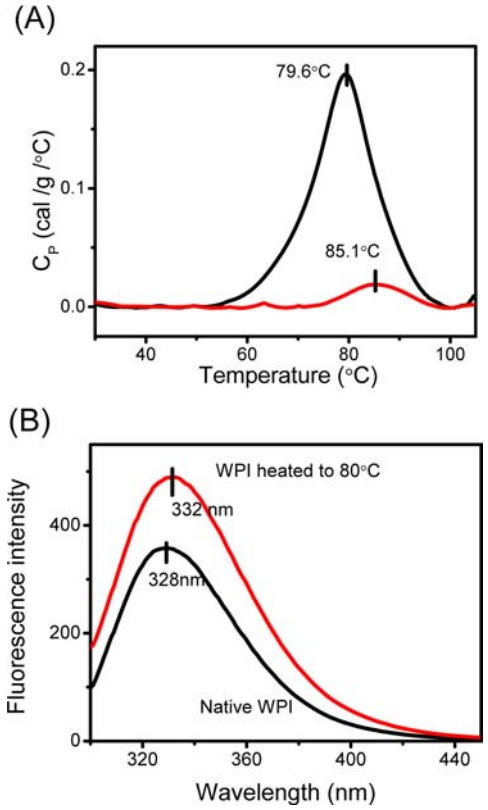

Figure 1. (A) DSC thermogram and (B) intrinsic emission spectra of 20 mg/mL WPI solution (pH 6.4) before (black) and after (red) heating to 80 °C for 15 min.

thermal denaturation at 80 °C, the fluorescence intensity of WPI increased, and the wavelength of the peak maximum shifted from 328 to 332 nm. The red shift indicates that the polarity around the major fluorophore tryptophan (Trp) increased,[17] whereas the increased exposure of Trp residues enhanced the emission intensity.

The fluorescence spectra of three individual whey proteins after heat treatment were also measured (Figure 2) before and after heating at 80 °C for 15 min. The fluorescence intensity of β-Lg and α-Lac increased after heat treatment, whereas that of BSA decreased. The red shift of the emission maximum was observed for β-Lg and α-Lac, showing that Trp was exposed to a more polar environment after heating. Conversely, the blue shift of BSA suggests increased hydrophobicity surrounding the Trp residues. The fluorescence spectra changes of WPI (Figure 1) generally are in agreement with those of β-Lg and α-Lac (Figure 2), which are the two most abundant whey proteins.

Impacts of Denaturation on Binding between Bixin and Whey Protein. In our previous study, the binding reaction between bixin and whey protein was investigated by the fluorescence quenching method.[7] In this work, the emission spectra of denatured WPI after titration with bixin are shown in Figure 3. The impacts of WPI concentration on fluorescence quenching are not presented because the estimated binding constant was similar when β-Lg was used at different concentrations (2.0×10^{-6} and 1.0×10^{-5} M^{-1}) in our preliminary studies. The fluorescence intensity of denatured WPI decreased significantly with an increase of bixin concentration. The fluorescence quenching data were analyzed by the Stern−Volmer equation (demonstrated in Figure 3, inset)

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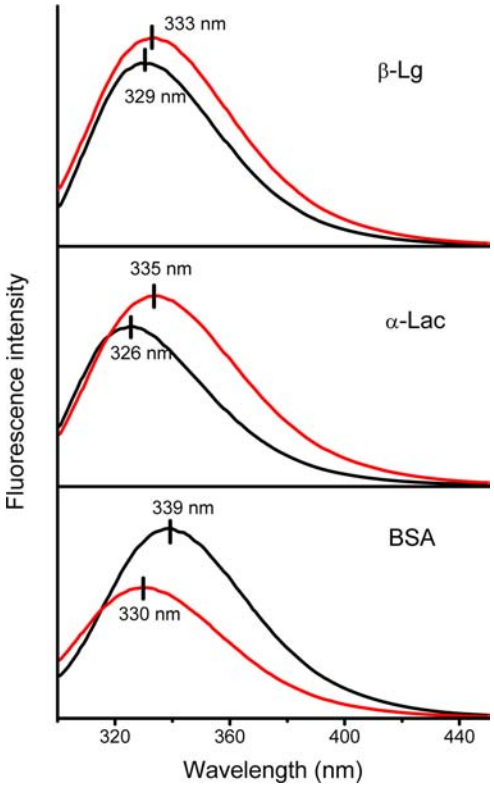

Figure 2. Intrinsic emission spectra of β-Lg, α-Lac, and BSA before (black) and after (red) heating at 80 °C for 15 min.

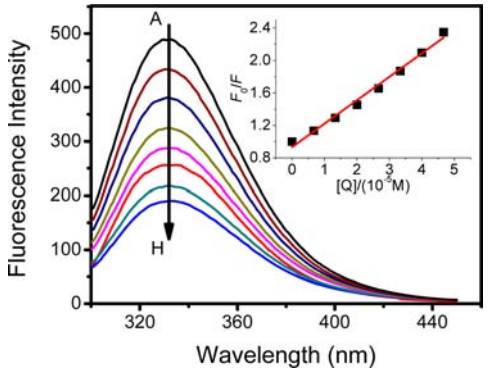

Figure 3. Effect of bixin on the fluorescence spectra of denatured WPI at 298 K and pH 6.4. The concentration of WPI was 0.1 mg/mL, and the concentrations of bixin were 0, 0.67×10^{-5}, 1.3×10^{-5}, 2×10^{-5}, 2.67×10^{-5}, 3.33×10^{-5}, 4×10^{-5}, amd 4.67×10^{-5} M, corresponding to curves A–H. (Inset) Stern–Volmer plot showingF_0/F of WPI as a function of bixin concentration. Denaturation corresponded to heating at 80 °C for 15 min.

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

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, [Q] is the concentration of the quencher, K_{SV} is the Stern–Volmer quenching constant, k_q is the biomolecular quenching constant, and t_0 is the lifetime of fluorescence in the absence of a quencher and equals 10^{-8} s.[18]

Fluorescence quenching can be classified as dynamic quenching or static quenching, and our previous study indicates that binding between bixin and whey protein is mainly driven by a static quenching mechanism.[7] For dynamic quenching, the maximum collisional quenching constant of various quenchers is 2.0×10^{10} M^{-1} s^{-1}.[19] The K_{SV} in this work is 2.89×10^4 M^{-1} (Table 1), and k_q is calculated to be 2.89×10^{12} M^{-1} s^{-1}, which is 2 orders of magnitude larger than 2.0×10^{10} M^{-1} s^{-1}. The results suggest that the quenching process is also static quenching for denatured whey protein.

Table 1. Stern–Volmer Quenching Constant K_{SV} due to Binding between Bixin and Native or Denatured[a] Whey Protein

	native			denatured		
protein	K_{SV} ($\times 10^4$ M^{-1})	R	SD	K_{SV} ($\times 10^4$ M^{-1})	R	SD
WPI	2.25	0.976	0.037	2.89	0.988	0.033
β-Lg	2.41	0.985	0.032	2.81	0.979	0.043
α-Lac	2.66	0.993	0.023	2.72	0.990	0.029
BSA	4.10	0.975	0.034	2.80	0.985	0.018

[a]Heating was conducted at 80 °C for 15 min.

The quenching constants of bixin with individual denatured proteins were calculated and compared with those under native conditions (Table 1). For native proteins, the K_{SV} value was the highest for BSA but decreased significantly after thermal denaturation (from 4.10×10^4 to 2.80×10^4 M^{-1}). For β-Lg and α-Lac, denaturation caused an increase of K_{SV}, which followed the same trend as WPI.

The modified Stern–Volmer model (eq 2) can be used to analyze quenching data for systems identified to be static quenching:[17]

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

f_a is the fraction of accessible fluorescence, and K_a is the effective quenching constant for the accessible fluorophores. K_a can be treated as an associative binding constant between a quencher and an acceptor.

After fitting of the data according to eq 2, the slope and intercept following linear regression between $F_0/\Delta F$ and $1/[Q]$ can be used to determine $1/f_aK_a$ and $1/f_a$, respectively, and therefore K_a.[18] The plots for individual proteins are shown in Figure 4, and the derived K_a values for both WPI and individual whey proteins are listed in Table 2. The correlation coefficients

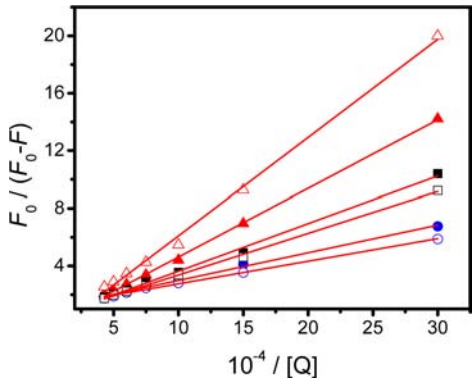

Figure 4. Modified Stern–Volmer plots of native and denatured β-Lg (squares), α-Lac (circles), and BSA (triangles) fluorescence quenching caused by the addition of bixin at 298 K and pH 6.4. Solid and open symbols are for native and denatured proteins, respectively. Denaturation corresponded to heating at 80 °C for 15 min.

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Table 2. Modified Stern–Volmer Quenching Constant K_a due to Binding between Bixin and Native or Denatured[a] Whey Protein

	native			denatured		
protein	K_a ($\times 10^4$ M^{-1})	R	SD	K_a ($\times 10^4$ M^{-1})	R	SD
WPI	0.95	0.997	0.008	1.51	0.997	0.008
β-Lg	0.98	0.996	0.009	1.60	0.999	0.004
α-Lac	1.20	0.996	0.005	1.30	0.997	0.004
BSA	2.32	0.999	0.005	1.52	0.996	0.016

[a]Heating was conducted at 80 °C for 15 min.

of linear regression were all >0.996, demonstrating the excellent applicability of the model. Like K_{SV}, the K_a of β-Lg and α-Lac increased after heat treatment, whereas the opposite was the case for BSA, and the magnitude of K_a was similar for WPI and β-Lg.

Binding Distance between Bixin and Protein As Affected by Thermal Denaturation. To further reveal the binding affinity of bixin on native and denatured whey protein, the energy transfer efficiency and mean distance between amino acid residues (the donor) and bound molecules (the acceptor) were determined according to Förster's nonradiative energy transfer (FRET) theory. On the basis of the theory, the efficiency of energy transfer between a donor and an acceptor (E) is a function of separation distance (r) as in eq 3[18]

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (3)$$

where R_0 is the critical distance corresponding to an E value of 50% and can be calculated by

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \phi J \quad (4)$$

where K^2 is the orientation factor of a dipole and is two-thirds for the random orientation as in a fluid solution, N is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the receptor, defined according to eq 5

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \quad (5)$$

where $F(\lambda)$ and $\varepsilon(\lambda)$ are the corrected fluorescence intensity of the donor and the molar absorption coefficient of the acceptor at wavelength λ, respectively.

As an example, Figure 5 shows an overlap between the emission spectrum of denatured β-Lg and the absorption spectrum of bixin, which was used to determine the J value, 1.2×10^{-15} cm^3 M^{-1}. R_0 was then calculated to be 3.56 nm on the basis of an N value of 1.4, a ϕ value of 0.12, and two-thirds for K^2 in eq 4.[19] According to eq 3, a binding distance (r) was determined to be 4.83 nm on the basis of the E value estimated from the experimental F/F_0 value of 0.14. The estimated r values for the three whey proteins at native and unfolded states are listed in Table 3 and are in the range between 2 and 8 nm expected for a donor fluorophore and an acceptor fluorophore.[20]

The three whey proteins contain different amounts of Trp residues, present at Trp-19 and Trp-61 for β-Lg, at Trp-26, Trp-60, Trp-104, and Trp-118 for α-Lac, and at Trp-212 and

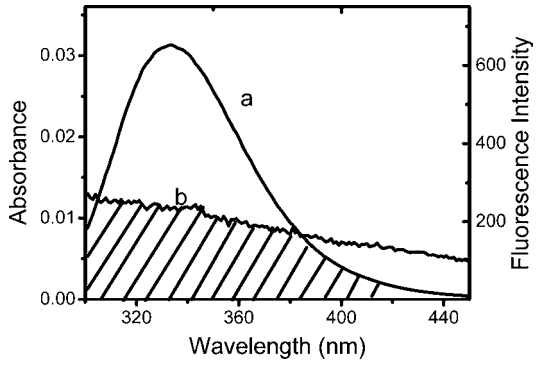

Figure 5. Overlap of fluorescence spectrum of denatured β-Lg (a) and absorption spectrum of bixin (b) at pH 6.4 and 298 K at equal β-Lg and bixin concentrations of 2.0×10^{-6} M^{-1}. Denaturation corresponded to heating at 80 °C for 15 min.

Table 3. Binding Distance between Bixin and Whey Protein As Affected by Heating at 80 °C for 15 min

	binding distance, r (nm)	
protein	native	denatured
β-Lg	4.97	4.83
α-Lac	4.65	4.55
BSA	4.61	4.83

Trp-134 for BSA, all of which have to be taken into account in the calculation of E.[21] The determined r is the average value of bixin binding to all Trp residues in each whey protein. By comparison with the native state, r of β-Lg and α-Lac decreased after heat treatment, indicating higher E and the binding sites for bixin being closer to the Trp residues. The data for BSA are opposite. The overall trend agrees well with the impacts of thermal denaturation on K_{SV} and K_a.

Conformation Changes of Native and Denatured Whey Protein upon Interaction with Bixin. Denaturation of proteins involves the disruption and possible destruction of both secondary and tertiary structures.[22] The far-UV CD method was used to measure secondary structure changes of the three whey proteins before and after heat treatment, with and without bixin. Examples of CD spectra are presented in Figure 6 for BSA. BSA exhibited a wide negative band between

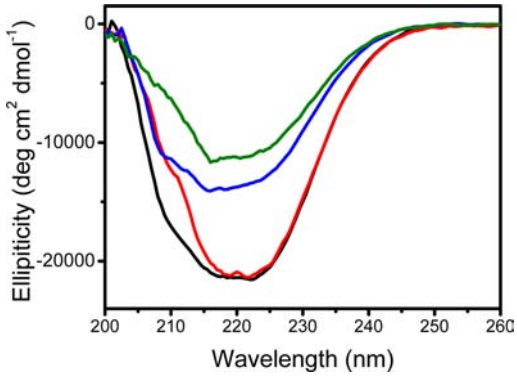

Figure 6. CD spectra of native and denatured BSA before and after interacting with bixin at a protein/bixin molar ratio of 1:16 at pH 6.4 and 298 K: (black curve) native BSA, without bixin; (red curve) denatured BSA, without bixin; (blue curve) native BSA, with bixin; (green curve) denatured BSA, with bixin. Denaturation corresponded to heating at 80 °C for 15 min.

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Table 4. Secondary Structures[a] of Native and Denatured[b] β-Lg, α-Lac, and BSA As Affected by Complexation with Bixin at pH 6.4 and 298 K

sample		molar ratio of protein/bixin	α-helix (%)	β-sheet (%)	turn (%)	unordered (%)
β-Lg	native	1:0	6.6 ± 0.4	33.8 ± 0.2	23.5 ± 0.2	36.5 ± 1.8
		1:16	5.9 ± 0.8	31.1 ± 0.9	23.3 ± 0.1	40.1 ± 1.1
	denatured	1:0	6.9 ± 0.7	29.6 ± 1.5	23.6 ± 0.4	38.4 ± 0.9
		1:16	6.2 ± 1.3	29.1 ± 1.2	24.3 ± 0.4	42.3 ± 1.5
α-Lac	native	1:0	26.7 ± 0.2	17.2 ± 0.5	22.6 ± 0.3	34.9 ± 0.9
		1:16	22.6 ± 0.1	20.3 ± 0.3	22.3 ± 0.2	34.3 ± 0.7
	denatured	1:0	17.9 ± 0.5	17.2 ± 0.4	24.0 ± 0.4	39.8 ± 1.0
		1:16	14.2 ± 0.2	25.0 ± 0.3	23.5 ± 0.1	41.2 ± 1.1
BSA	native	1:0	57.6 ± 0.9	7.5 ± 0.5	14.1 ± 0.4	21.6 ± 1.2
		1:16	50.4 ± 1.2	7.3 ± 0.2	17.0 ± 0.2	28.4 ± 0.8
	denatured	1:0	36.5 ± 1.0	16.6 ± 0.7	19.4 ± 0.3	29.7 ± 0.8
		1:16	24.1 ± 0.7	19.2 ± 1.0	24.8 ± 0.5	36.2 ± 1.1

[a]Percentages are averages ± standard deviations from three replicates. [b]Heating was conducted at 80 °C for 15 min.

210 and 230 nm, which represents the typical α-helix structure of protein. After heat treatment, the intensity of the negative band decreased significantly, indicating that the secondary structure of BSA had been changed. The titration of bixin also affected the CD spectra. The contents of α-helix and β-strand calculated using the algorithm SELCON3 are summarized in Table 4.[23]

BSA showed a decreased content of α-helical structure and an increased content of β-sheet structure after heat treatment. Heat denaturation also reduced the α-helix content of α-Lac but had no obvious influence on the β-sheet structure. For β-Lg, the content of β-sheet decreased while the α-helix content changed slightly. For all proteins, heat treatment increased the percentage of the unordered structure. With the addition of bixin, BSA and α-Lac showed a decrease of α-helix and an increase of β-sheet structure at both native and denatured states, but the trends were opposite for β-Lg. The data indicate that the binding of bixin to all three proteins can further denature whey protein to more unordered structures, regardless of their (folded or unfolded) state.

FT-IR Analysis of Binding between Bixin and Whey Protein As Affected by Thermal Denaturation. The FT-IR spectra in the 1800−1400 cm^{-1} range (Figure 7) were used to directly analyze the effect of bixin on secondary structures of native and denatured WPI. Without bixin, WPI showed no obvious difference for the amide I band, corresponding to the region between 1600 and 1700 cm^{-1}, before and after heating. The peak at 1542 cm^{-1}, mainly due to the amide II band (60% N−H bend and 40% C−N stretch, near 1550 cm^{-1}),[24] decreased and broadened after heat treatment, whereas the peak at 1459 cm^{-1} increased after thermal treatment. On the basis of the literature, the bands at 1692, 1644, and 1459 cm^{-1} are contributed from turns and bends, random coil structure, and characteristics of β-sheet structure, respectively.[25] The FTIR data indicate that thermal denaturation affects the secondary structure of WPI significantly, which is in good agreement with the CD results of β-Lg in the literature and the present study (Table 4).[25b]

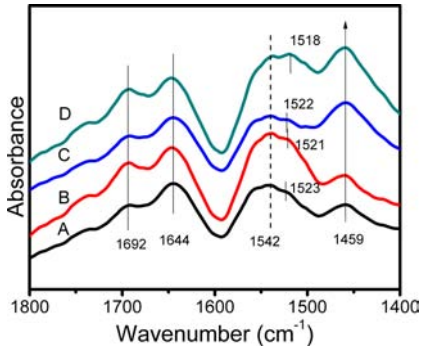

Figure 7. FT-IR spectra of (A) native WPI, (B) a mixture of native WPI and bixin, (C) heat-treated WPI, and (D) a mixture of heat-treated WPI and bixin. The concentration of WPI is 2%, whereas that of bixin is 2 mM. Heat treatment was at 80 °C for 30 min.

With the addition of bixin, a shift to a smaller wavenumber was observed for the shoulder around 1520 cm^{-1}, and the intensity of bands at 1692 and 1542 cm^{-1} increased. The results indicate that the complex formation between bixin and WPI changed secondary structures of both native and unfolded whey protein.

Mechanism of Increased Binding Affinity of Bixin with Thermally Denatured Whey Protein. Because WPI contains approximately 68% β-Lg,[26] the characteristic properties of denatured WPI are mainly contributed by β-Lg. For most protein structures, heat treatment induces the cleavage of disulfide bonds (at ∼70−80 °C), the formation of new intra/intermolecular interactions, and the rearrangement of disulfide bonds (at ca. 80−90 °C).[27] For native β-Lg, the low-emission quantum yield results from the quenching of Trp-61 by the nearby Cys-66−Cys-160 disulfide bond.[28] Heat treatment can break the disulfide bonds and cause the rearrangement of Cys-66 and Cys-160 to form new disulfide bonds, corresponding to a new state. This was demonstrated by the increased fluorescence (Figures 1B and 2 for β-Lg) and the red shift in fluorescence spectra after thermal denaturation that suggest the increased polarity around Trp residues, as discussed above. The CD and DSC results suggest that heat-denatured whey proteins are less compact and more stable than at the native state. The deep hydrophobic pockets of β-Lg become more available for binding with some small molecules after thermal denaturation,[3] which enhances the binding affinity between bixin and β-Lg, as observed in the present study.

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Notes

The authors declare no competing financial interest.

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