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Principal Component Similarity Analysis of Raman Spectra To Study the Effects of pH, Heating, and κ-Carrageenan on Whey Protein Structure

NOOSHIN ALIZADEH-PASDAR, SHURYO NAKAI, AND EUNICE C. Y. LI-CHAN*

Faculty of Agricultural Sciences, Food, Nutrition, and Health, Food Science Building, The University of British Columbia, 6640 N.W. Marine Drive, Vancouver, British Columbia, Canada V6T 1Z4

Raman spectroscopy was used to elucidate structural changes of β-lactoglobulin (BLG), whey protein isolate (WPI), and bovine serum albumin (BSA), at 15% concentration, as a function of pH (5.0, 7.0, and 9.0), heating (80 °C, 30 min), and presence of 0.24% κ-carrageenan. Three data-processing techniques were used to assist in identifying significant changes in Raman spectral data. Analysis of variance showed that of 12 characteristics examined in the Raman spectra, only a few were significantly affected by pH, heating, κ-carrageenan, and their interactions. These included amide I (1658 cm^{-1}) for WPI and BLG, α-helix for BLG and BSA, β-sheet for BSA, CH stretching (2880 cm^{-1}) for BLG and BSA, and CH stretching (2930 cm^{-1}) for BSA. Principal component analysis reduced dimensionality of the characteristics. Heating and its interaction with κ-carrageenan were identified as the most influential in overall structure of the whey proteins, using principal component similarity analysis.

KEYWORDS: Raman spectroscopy; whey proteins; pH; heating; κ-carrageenan; principal component analysis; principal component similarity

INTRODUCTION

Whey protein ingredients such as whey protein isolate (WPI) are often used for their foaming (*1*), emulsifying (*2*), and gelation (*3*) properties. However, factors such as pH and temperature or interaction with polysaccharides such as κ-carrageenan (KCG) can affect the properties of proteins in whey (*4*). KCG, a galactan polysaccharide with anionic character due to its sulfate groups (*5*), is used as a stabilizing, thickening, or gelling agent in dairy and other food products. Due to the importance of incorporation of polysaccharides in protein-containing foods, the control of the interaction between these biopolymers, and therefore understanding its mechanism, is essential in order to achieve desired functionality.

The nature of the interaction between proteins and polysaccharides is determined by the physicochemical properties of each macromolecule, their relative concentration, and the solution conditions such as pH, temperature, and ionic strength (*6*). These variables may cause the protein to precipitate (*7*−*9*) or prevent its precipitation (*10*−*12*). β-Lactoglobulin (BLG), the major protein component in whey, is soluble over a wide range of pH from 2.0 to 10.0 (*13*). It exists as a monomer, containing two disulfide bonds, at extreme pH <3.5 and >8.0 (*14*). Some conformational changes, which are accompanied by the expansion of the molecule, occur at pH 7.5. Increase in the reactivity of the thiol group and dissociation of the dimer occurs at pH >7.0. Unfolding and aggregation of this protein occurs at >55 °C, upon which cysteine and cystine groups and hydrophobic surfaces are exposed (*15*). Bovine serum albumin (BSA) is another major component of the whey proteins, which undergoes acid denaturation at pH 4.0, due to charge repulsion. It maintains its native form up to 42 °C. At 60 °C, significant aggregation of molecules occurs, whereas gelation of aggregates occurs at ∼70 °C (*15*). At neutral pH, both BSA and BLG carry a net negative charge, but electrostatic interactions may take place between negative charges on anionic polysaccharides and positively charged regions on the protein to form soluble ionic complexes (*16*).

Various techniques have been used to study protein−polysaccharide interactions. For example, Hidalgo and Hansen (*17*) used moving boundary electrophoresis and analytical ultracentrifugation to study the interactions between food stabilizers and BLG. Hattori et al. (*18*) applied differential scanning calorimetry (DSC) and polarizing microscopy to study the functional changes of carboxymethyl potato starch by conjugation with whey proteins. Galazka et al. (*19*) used spectrofluorometry, DSC, and size exclusion chromatography to study the effects of pH, ionic strength, and high pressure on the complexes of BSA with sulfated polysaccharides. However, evaluation of the structural properties of the protein molecules as a result of their interaction with polysaccharides was not addressed in any of these studies, probably due to the difficulty of studying the precipitated or gelled systems that are formed

* Author to whom correspondence should be addressed [telephone (604) 822-6182; fax (604) 822-3959; e-mail ecyl@interchange.ubc.ca].

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upon the interaction of proteins and polysaccharides at concentrations typically found in foods.

The effects of pH, heating, and KCG on the surface hydrophobicity of whey proteins were recently investigated by using dilute solutions (0.03% protein and 0.00048−0.025% KCG) (*4*). Under those dilute conditions, most of the whey protein/KCG mixtures did not contain any visible precipitates, thus facilitating measurement of hydrophobicity with a fluorescent probe method. However, the relevance of using results obtained under dilute conditions to predict structural properties of proteins at the higher concentrations existing in actual food systems is unclear.

Changes in chemical structure and molecular environment in precipitated or gelled samples, such as those that are formed upon the interaction of proteins and polysaccharides, can be monitored by Raman spectroscopy, a vibrational spectroscopic technique (*20*, *21*). Raman spectroscopy can be used to detect transitions from ordered to disordered structures during protein denaturation (*22*). For example, it has been used to study the effects of pH (*23*), heating (*24*), and chemical agents (*25*) that may cause protein unfolding and changes in the peptide backbone and/or side chains, including cleavage of disulfide bonds. Raman spectroscopy has also been used to study intermolecular interactions, including ligand (methyl orange)−protein interactions (*26*), complexes of BSA and ionic detergents (*27*), conformational features of lipids and proteins in myelin membrane (*28*), and structural changes in cod myosin after frozen storage or modification with formaldehyde (*29*). However, to our knowledge, Raman spectroscopy has not been previously reported for the study of food protein−polysaccharide interactions.

In this study, we report, for the first time, the use of Raman spectroscopy to study the interaction of whey proteins and KCG as influenced by pH and heat treatment. The objective of this research was to apply Raman spectroscopy to monitor changes in protein secondary structure (α-helix, β-sheet, and random coil) or amino acid side-chain vibrations (disulfide bonds, aromatic rings of tryptophan and tyrosine, CH groups) that may arise from changes in pH, heating, and presence of KCG. Analysis of variance was used to identify the factors and their interactions that significantly affected individual spectral characteristics. Principal component analysis (PCA) was performed to reduce the dimensionality of the Raman spectral data, followed by principal component similarity (PCS) analysis to assess the overall groupings of the treated whey protein samples based on the Raman spectroscopic data.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) was from Foremost Farms USA (Waukon, IA; Daritek NVB 389, lot 21-4080, containing 89.43% protein and 4.26% moisture; obtained as a gift from Canadian Inovatech Inc., Abbotsford, BC, Canada). BLG (L-2506, 80% purity), BSA (A-4503, 96% purity), and KCG (C-1263, type III) were purchased from Sigma (St. Louis, MO). Buffers were prepared according to the method of Alizadeh-Pasdar and Li-Chan (*4*).

Preparation of Samples. Stock protein solutions containing 25% (w/v) protein were prepared in buffers at pH 5.0, 7.0, and 9.0 containing 0.02% sodium azide. These pH conditions were chosen due to their application in food formulations. Protein concentrations of WPI, BLG, and BSA were determined by absorbance at 280 nm of diluted aliquots of the stock solutions, using $E_{1cm}^{1\%}$ of 11.7 (*30*), 9.6 (*31*), and 6.61 (*31*), respectively.

KCG stock solutions (0.6%) were prepared by dispersing the powder in various buffers and continuously stirring for 20 min at 70 °C. KCG concentration was determined according to the methylene blue method of Soedjak (*32*).

Table 1. Assignment of Major Bands in the Raman Spectrum of the Whey Proteins

band assignment	wavenumber (cm^{-1})	ref
S–S stretching	508	*24*
tryptophan	761	*24*
tyrosine doublet	860/830	*24*
α-helix	938	*23*
amide I	1658	*21*
CH_2 bending	1453	*33*
CH_3 symmetrical and R_3C–H stretching bands of aliphatic amino acids	2880	*33*
CH stretching bands of both aromatic and aliphatic amino acids as well as charged amino acids	2930	*33*
=C–H stretching bands of aromatic amino acids	3060	*33*

Samples for Raman spectroscopic analysis were prepared by diluting the stock protein solutions to 15% (w/v) protein concentration with the appropriate buffer. To prepare KCG/protein mixtures, the protein and KCG stock solutions were individually warmed to 35 °C (to avoid gelation), then mixed in a 6:4 (v/v) ratio to yield 15 and 0.24% final concentrations of protein and KCG, respectively. Heated samples were prepared by placing Parafilm-sealed capillary tubes (Nichiden-Rika Glass Co. Ltd., Japan) containing the sample in a glass Petri dish floating in a water bath at 80 °C for 30 min. The heated samples were allowed to cool for an hour at room temperature and then stored in a refrigerator (4 °C), overnight, before analysis. All samples containing protein/KCG mixtures formed gels.

Raman Spectroscopy. Raman spectral data were collected as described by Howell et al. (*33*) on a JASCO NR 1100 spectrometer, with 488 nm excitation from an argon ion laser (Coherent Innova 70C series, Coherent Laser Group, Santa Clara, CA, cooled with the Coherent Laser Pure heat exchanger system). Capillary tubes containing the samples were held horizontally, and the incident laser beam was vertical to the capillary axis. To increase signal to noise ratio, at least 10 scans of each sample were collected to obtain averaged spectral data. Duplicate spectra of two independent samples (each an average of 10 scans) were measured for eight samples and showed similar trends for replicates. A coefficient of variation below 10% has been reported previously in this laboratory for a variety of protein and amino acid samples using the same instrument (*24*, *33*−*35*).

The averaged spectral data from the scans of samples in the Raman spectrophotometer were baseline corrected and smoothed using the five-point Savitsky−Golay function and normalized against the phenylalanine band at 1004 cm^{-1} (i.e., adjusted to 1.0 for the 1004 cm^{-1} band for all spectra) using Grams 386 (Galactic Industries Corp., Salem, NH). The intensity and location of the phenylalanine band at 1004 cm^{-1} band is not sensitive to conformation or microenvironment and, therefore, can be used as an internal standard to normalize the Raman spectrum of proteins (*21*).

The algorithm of Williams (*36*) was used for detailed analysis of the secondary structure composition of samples, based on the averaged scans of the "raw" (not baseline corrected, smoothed, or normalized) Raman spectra in the amide I region, using the Raman spectral analysis package (RSAP) program (version 2.1) of Przybycien and Bailey (*37*). On the basis of this program, the protein secondary structure composition was classified into total α-helix (including ordered and unordered α-helix), total β-sheet (including parallel and antiparallel β-sheet), and total random coil (including unordered structure and β-reverse turn). Although this program tends to overestimate β-sheet content of proteins, it is useful for observing comparative changes in the secondary structure composition of samples as a function of different variables.

Assignments of bands in the Raman spectrum were performed according to available literature, based on specific vibrational modes of amino acid side chains or the polypeptide backbone (**Table 1**).

Data Processing. Data were analyzed by an analysis of variance (ANOVA) procedure applying the General Linear Model, with further

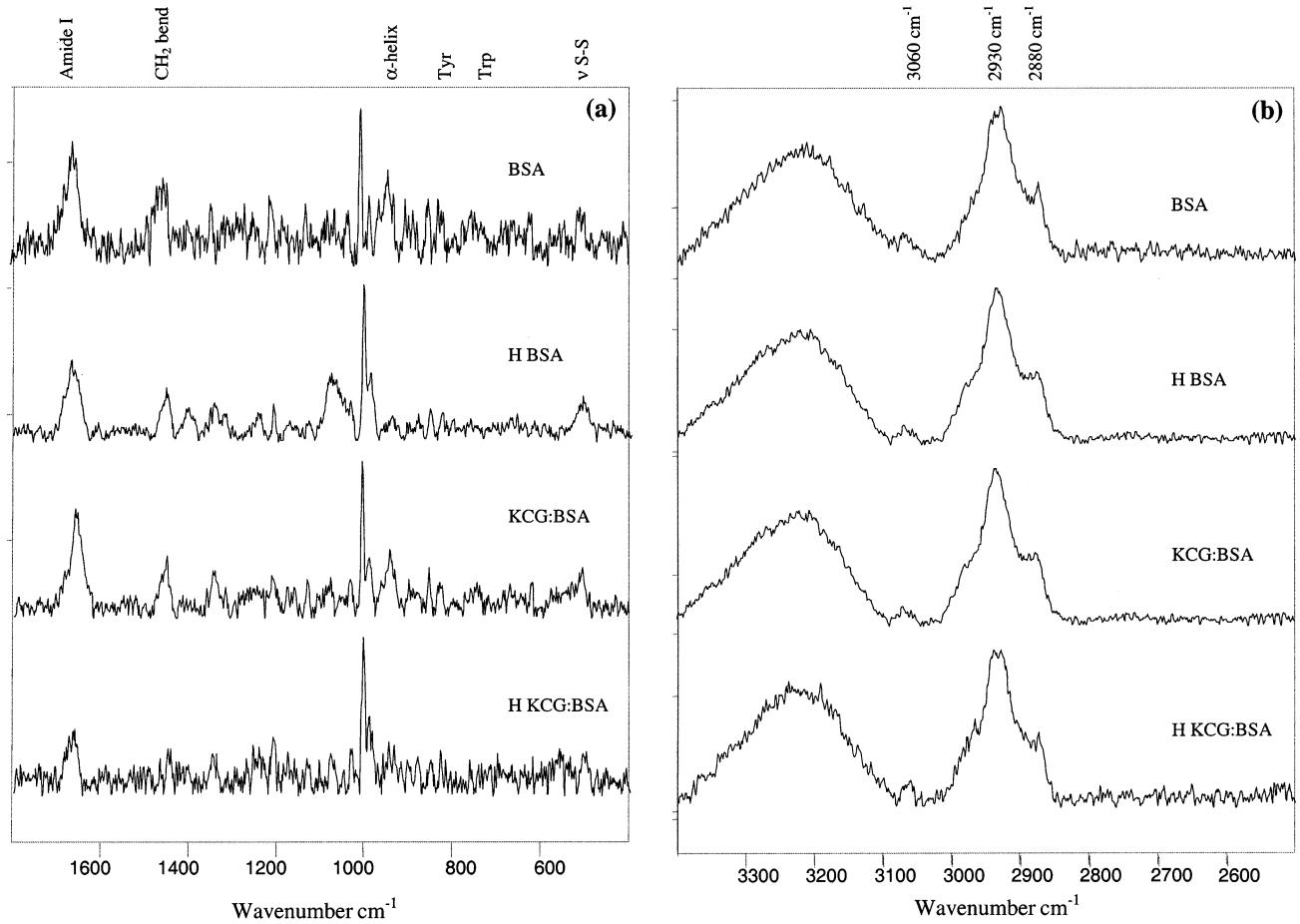

Figure 1. Raman spectra in the (a) 400–1800 cm^{-1} and (b) 2500–3400 cm^{-1} regions of heated and unheated BSA at pH 7.0 in the presence or absence of κ-carrageenan. H BSA, heated BSA; KCG:BSA, mixture of κ-carrageenan and BSA; H KCG:BSA, heated mixture of κ-carrageenan and BSA. Each spectrum is the averaged data from 10 scans, baseline corrected and smoothed using the five-point Savitsky–Golay function, and normalized against the phenylalanine band at 1004 cm^{-1}. Assignment of bands is shown above the spectra.

analysis using Tukey's pairwise comparison test to determine significant differences between treatment means (MINITAB for Windows version 12, MINITAB Inc., State College, PA).

Principal component analysis (PCA) was performed using MINITAB software to reduce the dimensionality of the Raman spectral data, which may include a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. To interpret the results of PCA, only the general pattern of the coefficients is of interest. Jolliffe (*38*) has proposed a method to simplify presentation of the results. If the coefficient is greater than half the maximum coefficient (in absolute value) for the relevant principal component, only the sign of the value is noted. Similarly, a (+) or (−) indicates a coefficient with an absolute value between a fourth and a half of the largest absolute value for that principal component. An empty space is assigned when the value is below a fourth of the largest absolute value for the principal component of interest.

Principal component similarity (PCS) analysis was applied using the PCS package of Nakai (The University of British Columbia) as a means for dimensionality reduction, to identify differences in samples compared to a reference on a single two-dimensional plot (*39*). PCS analysis was developed by combining PCA and pattern similarity computation (*39*). In this method, PCA was first applied to the Raman spectroscopic data to compute PC scores and eigenvalues. Linear regression analysis of the "deviation of the PC scores of the samples from those of reference sample" versus "variability considered for by PC scores" was then performed. Finally, the slope (S) and coefficient of determination (r^2) computed by linear regression analysis were plotted as a PCS scattergram. In this study, the Raman spectral data of each protein at pH 7.0, without heating or KCG, were used as the reference.

RESULTS AND DISCUSSION

Typical Raman spectra of heated or unheated BSA at pH 7.0, and in the presence or absence of KCG, are illustrated in **Figure 1**. Similar spectra were obtained for BSA at the other pH values studied and for WPI and BLG (data not shown). No bands were observed in the Raman spectrum of KCG alone at the concentration used in this study. In addition, no qualitative changes were identified in the spectra obtained by addition of KCG to proteins.

The typical bands assigned to the disulfide bond, tryptophan, tyrosine doublet, α-helix, amide I, and the C−H bending and stretching regions of the Raman spectrum are shown in **Table 1**. In addition to these nine characteristic Raman bands, three other characteristics were obtained from the Raman spectra, representing the percentage of secondary structure parameters (α-helix, β-sheet, and random coil) estimated from the amide I band by the RSAP. The results of estimation of secondary structural parameters are shown in **Table 2**.

Analysis of Variance and Significant Characteristics. Analysis of variance was used to assess whether pH, heating, or the presence of KCG affected the 12 Raman spectral characteristics identified above. **Tables 3**−**5** show the Raman bands in the spectra of BLG, BSA, and WPI, respectively, which were significantly ($p < 0.05$) affected by the three mentioned factors, whereas **Figures 2**−**4** show the detailed effects of pH, heating, and presence of KCG on the significant characteristics. It is interesting to note that all of the significant effects were

Table 2. Effects of pH, Heating (H), and κ-Carrageenan on Protein Secondary Structure Content (Percent) Estimated by the Raman Spectral Analysis Package for the Amide I Band[a]

unheated sample	total α-helix	total β-sheet	total random coil	heated sample	total α-helix	total β-sheet	total random coil
BLG5[b]	55	20	25	H BLG5	70	30	0
BLG7	25	40	35	H BLG7	5	70	25
BLG9	40	30	30	H BLG9	0	100	0
KCG:BLG5	10	60	30	H KCG:BLG5	50	30	20
KCG:BLG7	20	50	30	H KCG:BLG7	20	50	30
KCG:BLG9	25	45	30	H KCG:BLG9	25	45	30
BSA5	65	5	30	HBSA5	25	60	15
BSA7	60	15	25	HBSA7	35	35	30
BSA9	50	20	30	HBSA9	20	50	30
KCG:BSA5	40	30	30	H KCG:BSA5	0	50	50
KCG:BSA7	85	0	15	H KCG:BSA7	30	40	30
KCG:BSA9	65	15	20	H KCG:BSA9	35	35	30
WPI5	45	25	30	H WPI5	20	40	40
WPI7	30	40	30	H WPI7	50	30	20
WPI9	25	55	20	H WPI9	10	65	25
KCG:WPI5	10	55	35	H KCG:WPI5	60	20	20
KCG:WPI7	15	55	30	H KCG:WPI7	5	60	35
KCG:WPI9	20	50	30	H KCG:WPI9	25	45	30

[a] Secondary structure estimation was performed using averaged spectral data from 10 scans. [b] 5, 7, and 9 are pH values of the samples.

Table 3. Significant Raman Bands Identified by Analysis of Variance of the Effects of pH, Heating, and Presence of κ-Carrageenan on β-Lactoglobulin

Raman band	significant factor(s)	*p* value
amide I (1658 cm^{-1})	pH	0.048
	pH × KCG	0.041
α-helix (RSAP)	pH	0.025
	heating	0.049
	pH × heating	0.038
	heating × KCG	0.035
CH stretching (2880 cm^{-1})	heating	0.026
	heating × KCG	0.048

Table 4. Significant Raman Bands Identified by Analysis of Variance of the Effects of pH, Heating, and Presence of κ-Carrageenan on Bovine Serum Albumin

Raman band	significant factor(s)	*p* value
α-helix (938 cm^{-1})	pH	0.015
	heating	0.017
	heating × KCG	0.035
α-helix (RSAP)	heating	0.011
β-sheet (RSAP)	heating	0.028
CH stretching (2880 cm^{-1})	heating	0.010
	heating × KCG	0.035
CH stretching (2930 cm^{-1})	heating	0.042

related to either secondary structural characteristics or the C−H stretching bands. These effects are described below for each of the three proteins

β-Lactoglobulin. The total α-helix/β-sheet content of BLG at pH 7.0 was 25:40 (**Table 2**), which is similar to the values of 15:50 and 10:43 reported by Kinsella et al. (*40*) and Swaisgood (*41*), respectively, based on circular dichroic analysis. Analysis of variance (**Table 3**) determined that the amide I band at 1658 cm^{-1} in the Raman spectrum of BLG was affected by pH and the interaction of pH with KCG (**Figure 2a**), whereas the proportion of α-helix estimated by RSAP was influenced by pH, heating, the interaction of pH with heating, and the

Table 5. Significant Raman Bands Identified by Analysis of Variance of the Effects of pH, Heating, and Presence of κ-Carrageenan on Whey Protein Isolate

Raman band	significant factor(s)	*p* value
amide I (1658 cm^{-1})	pH	0.032
	heating	0.005
	KCG	0.006
	pH × heating	0.015
	heating × KCG	0.006

interaction of heating with KCG (**Figure 2b**). Heating BLG at pH 7.0 resulted in decreased α-helix content from 25 to 5% and increased β-sheet content from 40 to 70% (**Table 2**). Increased β-sheet content also resulted after heating of BLG at pH 9.0, but not at pH 5.0. The presence of KCG resulted in higher β-sheet content of BLG at all three pH conditions studied, but heated KCG/BLG mixtures at pH 7.0 and 9.0 had lower β-sheet contents than BLG heated in the absence of KCG.

The heat treatment (80 °C, 30 min) used in this study was expected to cause denaturation of BLG, because 70 °C has been reported to be the critical temperature for BLG denaturation at pH 7.0 (*42*), and DSC analysis has determined the denaturation temperature of BLG to be 65 °C (*43*). However, studies on the specific influence of pH and/or heating on the molecular structure of BLG are conflicting. Das and Kinsella (*44*) stated that BLG has a rigid conformation around pH 3.0−7.0 and that pH has no effect on its secondary structure. However, using FTIR to monitor structural properties, Casal et al. (*45*) concluded that small structural and conformational changes were observed in BLG as a function of pH (2.0−13.0) and temperature (−100 to 90 °C). An increased intensity of amide I bands at 1636 and 1681 cm^{-1} observed when the pH was increased from 5.0 to 6.0 was attributed to small changes in the β-strands, whereas other changes in band intensity with increasing pH were due to deprotonation of carboxylate groups. Casal et al. (*45*) suggested that alkaline denaturation of BLG is the product of two stages; the first involves unfolding of the α-helices and the exposed β-strands, and the second involves unfolding of the remaining β-strands. Two stages were also proposed for the thermal denaturation of BLG. The intermediate stages of alkaline and thermal denaturation were suggested to be similar, but the final

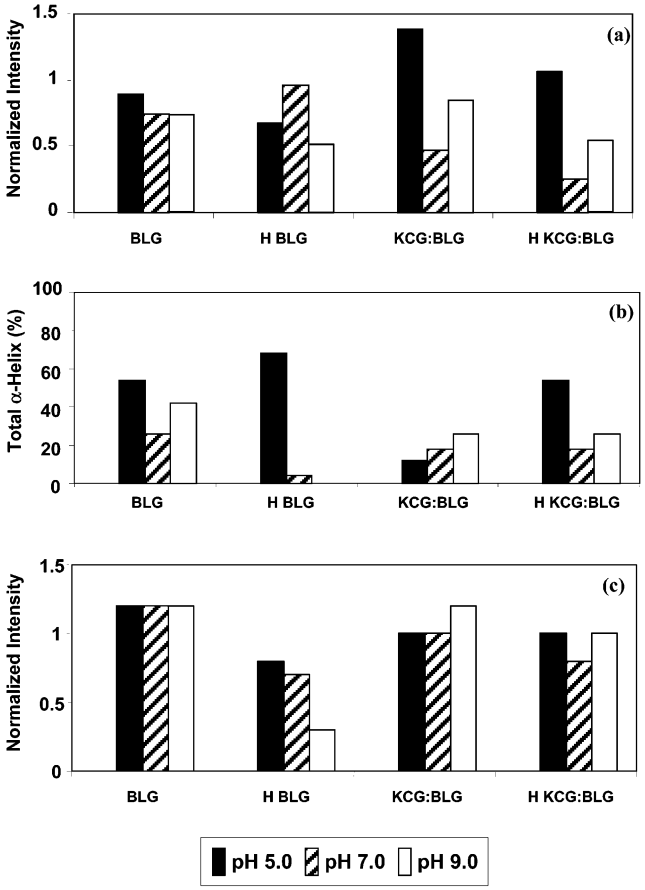

Figure 2. Effects of pH, heating (H), and presence of KCG on (a) the normalized intensity of amide I band at 1658 cm^{-1}, (b) α-helix content (percent, estimated from the amide I band by the RSAP), and (c) normalized intensity of the CH stretching band at 2880 cm^{-1} of the Raman spectrum of BLG.

stage of thermal denaturation is completely different from alkaline denaturation because protein unfolding is more affected by high pH than by high temperature due to repelling forces of the negatively charged functional groups (*45*).

On the basis of qualitative analysis of FTIR spectra, Boye et al. (*46*) suggested that increasing pH from 3.0 to 9.0 as well as heating (26−97 °C) increased the formation of *β*-sheet structure. Increased *β*-sheet content due to heating of 15% whey protein solutions was reported by Nonaka et al. (*24*) and Howell and Li-Chan (*34*), but not by Das and Kinsella (*44*) or Casal et al. (*45*). Some of these conflicting reports may be explained by the observations of Matsuura and Manning (*47*), who noted the influence of varying protein concentration from 0.1 to 100 mg/mL on the secondary and tertiary structures of BLG. At low (0.1−10 mg/mL) concentration, heating resulted in α-helix formation, whereas at higher (70 mg/mL) concentration, heating resulted in clear gels with increased *β*-sheet formation (*47*). Our results, based on samples with 15% protein, further indicate that changes in secondary structure were dependent not only on the particular pH or temperature conditions under investigation but also on the interaction between these two factors as well as the presence of other constituents such as KCG.

In addition to significant changes in secondary structure, **Table 3** also indicates that the CH stretching band at 2880 cm^{-1} in the Raman spectrum of BLG was affected by heating and by the interaction of heating with KCG. Hydrophobic groups of amino acids, peptides, and proteins exhibit C−H stretching vibrational bands in the 2800−3100 cm^{-1} region. Bands found

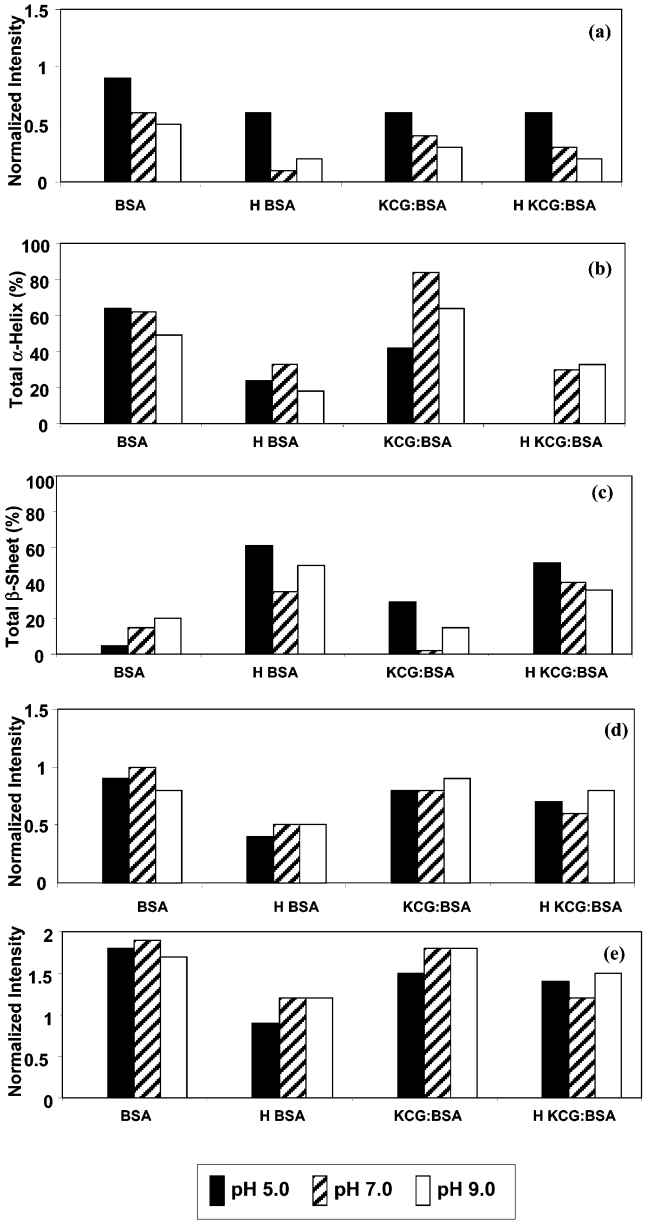

Figure 3. Effects of pH, heating (H), and presence of KCG on (a) the normalized intensity of the α-helix band at 938 cm^{-1}, (b) α-helix content (percent, estimated from the amide I band by the RSAP), (c) *β*-sheet content (percent by RSAP), (d) normalized intensity of the CH stretching band at 2880 cm^{-1}, and (e) normalized intensity of the CH stretching band at 2930 cm^{-1} of the Raman spectrum of BSA.

near 2874−2897 cm^{-1} are assigned to CH_3 symmetrical stretching and R_3C-H stretching bands of aliphatic amino acids, whereas the C−H stretching bands of aromatic amino acids can be found near 3061−3068 cm^{-1} (*33*). A decrease was observed in the 2880 cm^{-1} band intensity of the Raman spectrum of BLG after heating at pH 5.0 and 7.0 and, particularly, at pH 9.0 (**Figure 2c**). The decrease in the 2880 cm^{-1} band intensity was less pronounced when BLG was heated in the presence of KCG (**Figure 2c**), suggesting the influence of KCG on hydrophobic interactions of the protein aliphatic side chains during heating.

The band at 2880 cm^{-1} is related predominantly to exposure of aliphatic amino acids, and the area or intensity of this band in the Raman spectrum of proteins has been reported to be correlated with hydrophobicity values measured by fluorescent probes (*33*). The influence of KCG on the surface hydrophobicity of whey proteins measured by the fluorescent probe

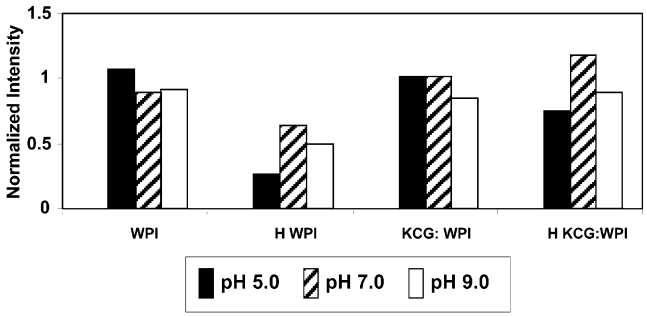

Figure 4. Effects of pH, heating (H), and presence of KCG on the normalized intensity of the amide I band (~1658 cm^{-1}) of the Raman spectrum of WPI.

PRODAN has been reported previously (*4*). Surface hydrophobicity was decreased by addition of KCG alone or after heating of the KCG/whey protein mixtures. Similar trends were thus noted by both fluorescent probe and Raman studies, indicating significant effects of KCG on hydrophobic interactions in heated KCG/protein mixtures compared to proteins heated alone. However, in contrast to the decreased surface hydrophobicity observed by the fluorescent probe method upon addition of KCG to whey proteins, no significant effects were observed by the addition of KCG alone when investigated by Raman analysis of the C−H stretching bands. These differences between the results of the fluorescent probe and Raman studies may be due to the influence of protein concentration on intermolecular interactions.

Bovine Serum Albumin. The secondary structure of BSA reported by Suttiprasit et al. (*48*) (α-helix, 55%; β-sheet, 16%; and unordered, 29%) is very similar to the findings in the present study at pH 7.0 of 60%/15%/25%, respectively (**Table 2**). Analysis of variance (**Table 4**) indicated that the intensity of the α-helix band at 938 cm^{-1} was affected by pH, heating, and interaction of heating with KCG, whereas the contents of α-helix and β-sheet estimated by RSAP were both influenced by heating. The CH stretching band at 2880 cm^{-1} was affected by heating and the interaction of heating with KCG, whereas the CH stretching band at 2930 cm^{-1} was influenced only by heating.

In an earlier study of BSA by Raman spectroscopy, Lin and Koenig (*23*) reported that β-conformation of BSA is formed by heating at temperatures >70 °C due to intermolecular hydrogen bonding after intermolecular disulfide exchanges. Heat denaturation of BSA at 70 °C and pH 8.0 also caused a decrease in band intensity at 938 cm^{-1}, which was attributed to the loss of α-helical content. Similar but reversible changes of decreased α-helical content and increased β-sheet content were also observed by acid and alkaline denaturation of BSA between pH 1.72 and 10.9. The destruction of α-helical structure as well as formation of β-sheet due to heating was also reported by Takeda et al. (*49*), who studied conformational changes in BSA using circular dichroism.

The results of the present study confirm the decrease in helical structure and increase in β-sheet structure of BSA with heating and increasing pH, as monitored by the 938 cm^{-1} band intensity and the results of RSAP analysis of the amide I band (**Figure 3a−c**). Adding KCG to BSA at pH 5.0 also resulted in decreased α-helical content and increased formation of β-sheet structure, but the reverse trends were observed at pH 7.0 and 9.0. Heated KCG/BSA mixtures at pH 5.0 had increased content of disordered or random coil structure and almost no helical structure (**Table 2** and **Figure 3b**).

The intensity of the CH stretching band at 2880 cm^{-1} of BSA was decreased by heating (**Figure 3d**). Although this band was not significantly influenced by the presence of KCG alone, a significant effect was observed for the interaction between heating and KCG. Similar to the trends observed for BLG, the decreased intensity of the 2880 cm^{-1} band for heated BSA was less pronounced in the heated KCG/BSA mixture, suggesting again the influence of protein−polysaccharide interactions on hydrophobic interactions of the aliphatic hydrocarbon side chains of these protein molecules during heating. Heating also significantly decreased the intensity of 2930 cm^{-1} band of the Raman spectrum of BSA, and the magnitude of this decrease was again less for the heated KCG/BSA mixtures (**Figure 3e**). Decreased intensity of the 2930 cm^{-1} band has been suggested to reflect decreased polarity (or increased nonpolarity) of the environment around hydrocarbon chains (*50*).

Table 6. Principal Component Analysis of the Raman Spectral Characteristics for β-Lactoglobulin

principal component	eigenvalue	proportion	cumulative
1	4.7831	0.319	0.319
2	4.2096	0.281	0.600
3	2.0411	0.136	0.736
4	1.3885	0.093	0.826
5	1.1544	0.077	0.905

Whey Protein Isolate. The intensity of the amide I band at 1658 cm^{-1} was the only characteristic feature in the Raman spectra of WPI that was significantly affected by pH, heating, presence of KCG, and interaction between these factors (**Table 5**). As observed for BLG (**Figure 2**), the intensity of the amide I band of WPI at 1658 cm^{-1} was higher at pH 5.0 than at pH 7.0 or 9.0 (**Figure 4**). Heating caused a decrease in the intensity of the amide I band at all three pH values, but this decrease was moderated by the presence of KCG in heated KCG/WPI mixtures. The presence of KCG decreased the helical content of both WPI and BLG at pH 5.0 to ~10%, whereas heating of these KCG/protein mixtures resulted in increased helical content (**Table 2**).

Generally, whey protein isolates contain >90% protein, and the two proteins BLG and BSA constitute approximately 50 and 5% of the total whey proteins, respectively (*51*). Thus, similar structural changes due to treatment conditions may be expected for BLG and WPI. However, except for the similar effects of pH on the intensity of amide I band at 1658 cm^{-1}, no other similarities were noted for significant effects of temperature and KCG on the Raman spectra of BLG and WPI (**Tables 3** and **5**).

Such differences between the behavior of BLG and WPI can be probably attributed to the different processes to which these proteins were exposed during manufacture. Processing conditions including the method of concentration or isolation may cause protein denaturation or otherwise influence protein structure and stability (*52*). Furthermore, proteins in WPI other than BLG may interact with each other during processing and, consequently, affect the structural parameters (*53*). The WPI used in this study was a food grade product, whereas BLG was obtained as an 80% pure reagent grade product.

Principal Component Analysis. The results of PCA of the Raman spectral characteristics for BLG, BSA, and WPI are shown in **Tables 6**, **8**, and **10**, respectively. **Figure 5** shows the scree plots for all principal components of the three whey proteins.

A scree plot shows the eigenvalues assigned for each principal component. However, on the basis of Kaiser's rule of thumb (*54*), it is suggested that only those components with eigenvalues

Table 7. Principal Component Coefficients of the Raman Spectral Characteristics for β-Lactoglobulin, Expressed Using the Method of Joliffe (*38*)

characteristics from Raman spectrum[a]	PC1	PC2	PC3	PC4	PC5
508	–				(–)
761	–				
860/830	(+)	(–)	(+)	–	(–)
1453	–	(–)	+		(+)
2880		+			(+)
2930		+	(+)		(–)
3060	–	(–)	(+)	+	(+)
938	–	(–)			(+)
1658	–	(+)	(+)		-
α-helix	(–)	(+)	–		(+)
β-sheet	(+)	–	+		–
random coil		+	+	+	(+)

[a] The numbered characteristics refer to normalized intensity or intensity ratio values at those Raman wavenumbers, as described in **Table 1**. The last three characteristics are the secondary structure proportions estimated from the amide I band by the RSAP.

Table 8. Principal Component Analysis of the Raman Spectral Characteristics for Bovine Serum Albumin

principal component	eigenvalue	proportion	cumulative
1	5.2375	0.349	0.349
2	4.8925	0.326	0.675
3	1.8136	0.121	0.796
4	1.1392	0.076	0.872

Table 9. Principal Component Coefficients of the Raman Spectral Characteristics for Bovine Serum Albumin, Expressed Using the Method of Joliffe (*38*)

characteristics from Raman spectrum[a]	PC1	PC2	PC3	PC4
508	+	+	(+)	
761	+	(+)		(–)
860/830		–	–	–
1453	+			–
2880	+	–		(+)
2930	+	–		
3060	+	(+)	–	(+)
938	+		+	(+)
1658	+		–	
α-helix		–	+	
β-sheet		+		(–)
random coil		+	–	(+)

[a] The numbered characteristics refer to normalized intensity or intensity ratio values at those Raman wavenumbers, as described in **Table 1**. The last three characteristics are the secondary structure proportions estimated from the amide I band by the RSAP.

>1 need to be considered. PCA of the Raman bands resulted in five, four, and five principal components with eigenvalues >1 for BLG, BSA, and WPI, respectively, accounting for 90.5, 87.2, and 90.2% of the total variation in the data set, respectively. For all three proteins, the first principal component (PC1) accounted for >30% of variability in the data set, and the first two principal components (PC1 and PC2) accounted for ~60% of the total variability.

Tables 7, **9**, and **11** show the PC coefficients of the input variables (i.e., Raman characteristics) for the first five principal components for BLG, BSA, and WPI, respectively, expressed using the simplified methodology of Jolliffe (*38*). For all three

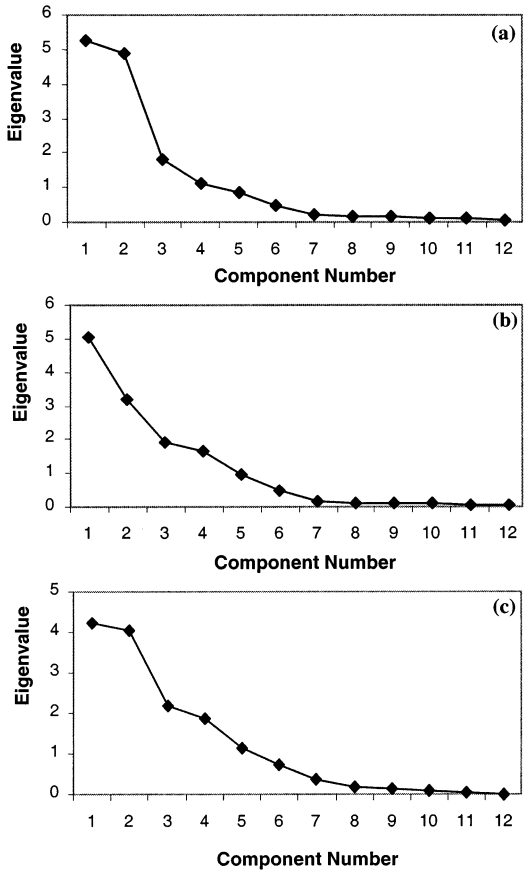

Figure 5. Scree plots for (a) BLG, (b) BSA, and (c) WPI.

Table 10. Principal Component Analysis of the Raman Spectral Characteristics for Whey Protein Isolate

principal component	eigenvalue	proportion	cumulative
1	5.0549	0.337	0.337
2	3.3261	0.222	0.559
3	2.4075	0.160	0.719
4	1.7372	0.116	0.835
5	1.0026	0.067	0.902

proteins, PC1 comprised a large number of Raman characteristics with strong ("+" and "−") PC coefficients, including the intensity of the bands assigned to disulfide bond, tryptophan, C−H stretching, and C−H bending, as well as the 938 cm^{-1} band assigned to α-helical structure. In comparison, PC2 showed strong PC coefficients for the C−H stretching bands and the RSAP estimates of secondary structure. From the large number of Raman characteristics assigned to each principal component, it is evident that complex intercorrelations exist between the original variables. Although PCA successfully reduced the dimensionality of the data from 12 Raman characteristics to 4 or 5 principal components, none of the characteristics could be eliminated and the main trends for the effects of the treatments on characteristics of samples could not be easily visualized. The PCS approach was therefore applied for further data analysis.

Principal Component Similarity Analysis. The PCS plots or scattergrams for the three whey protein samples (BLG, BSA, and WPI) are shown in panels a, b, and c, respectively, of **Figure 6**. In each PCS scattergram, the *x*- and *y*-axis coordinates are the proportion of variation or coefficient of determination (r^2) and the slope (*S*) values, respectively. The scattergrams show the r^2−*S* values for each protein under various conditions of

Table 11. Principal Component Coefficients of the Raman Spectral Characteristics for Whey Protein Isolate, Expressed Using the Method of Joliffe (*38*)

characteristics from Raman spectrum[a]	PC1	PC2	PC3	PC4	PC5
508	–	(+)	(+)		–
761	–	(+)	+	(+)	(+)
860/830	+				–
1453		+	+		(+)
2880	+	+			(+)
2930	+	+			+
3060	(+)	+		–	(+)
938	–				
1658		(–)	+	+	
α-helix	(–)	+	-	+	(–)
β-sheet	(+)	–	(+)	(–)	+
random coil	(+)		+	–	–

[a] The numbered characteristics refer to normalized intensity or intensity ratio values at those Raman wavenumbers, as described in **Table 1**. The last three characteristics are the secondary structure proportions estimated from the amide I band by the RSAP.

pH, heating, and KCG, compared to the reference unheated protein at pH 7, which is located at (r^2, S) coordinates of (1.0, 1.0). The slope S is a parameter that distinguishes negative or positive deviation from the diagonal line denoting the perfect correspondence between the protein under reference and other conditions. However, because the slope is not a formally defined parameter and its value greatly depends on the chosen reference, the coefficient of determination is usually used for comparison (*55*).

Effect of pH. On the basis of the information gathered for all 12 characteristics from the Raman spectra, the PCS plots show no or little difference in the coefficient of determination between pH 5.0, 7.0, and 9.0 for all three whey proteins, although WPI (**Figure 6c**) showed slightly greater spread than BLG (**Figure 6a**) or BSA (**Figure 6b**). Considering both slope and coefficient of determination, pH 5.0 was more different from pH 7.0 and pH 9.0 for BLG and WPI, whereas pH 9.0 was more different from pH 5.0 and pH 7.0 for BSA. These trends were also observed by fluorescent probe measurement of the surface hydrophobicity of the whey proteins as a function of pH (*4*), although, in the latter case, the greatest influence of pH was observed for BLG.

Effect of Heating. In general, a much larger spread in the coefficient of determination values as a function of pH was observed for heated samples compared to unheated samples. Little change was observed by heating BLG at pH 5.0 or 7.0, whereas a moderate change was observed at pH 9.0. Similar trends were observed by fluorescent probe measurement, in which the highest decrease in the surface hydrophobicity of BLG due to heating was observed at pH 9.0, with no significant change at pH 5.0 and 7.0. In the case of BSA, considerable effect of heating was observed under all three pH conditions studied, again similar to the results of our previous study (*4*) where heating significantly decreased the surface hydrophobicity of BSA at all pH values. For WPI, heating caused little effect at pH 5.0 or 9.0, but a large effect at pH 7.0, whereas our previous study showed that heating did not affect the surface hydrophobicity of WPI at pH 5.0 and 7.0, but decreased it slightly at pH 9.0.

Effect of KCG. The presence of KCG caused considerable differences in the coefficients of determination for BLG at pH 7.0 and 9.0 (**Figure 6a**) and for BSA at pH 5.0 (**Figure 6b**), whereas WPI (**Figure 6c**) did not show much effect at any pH.

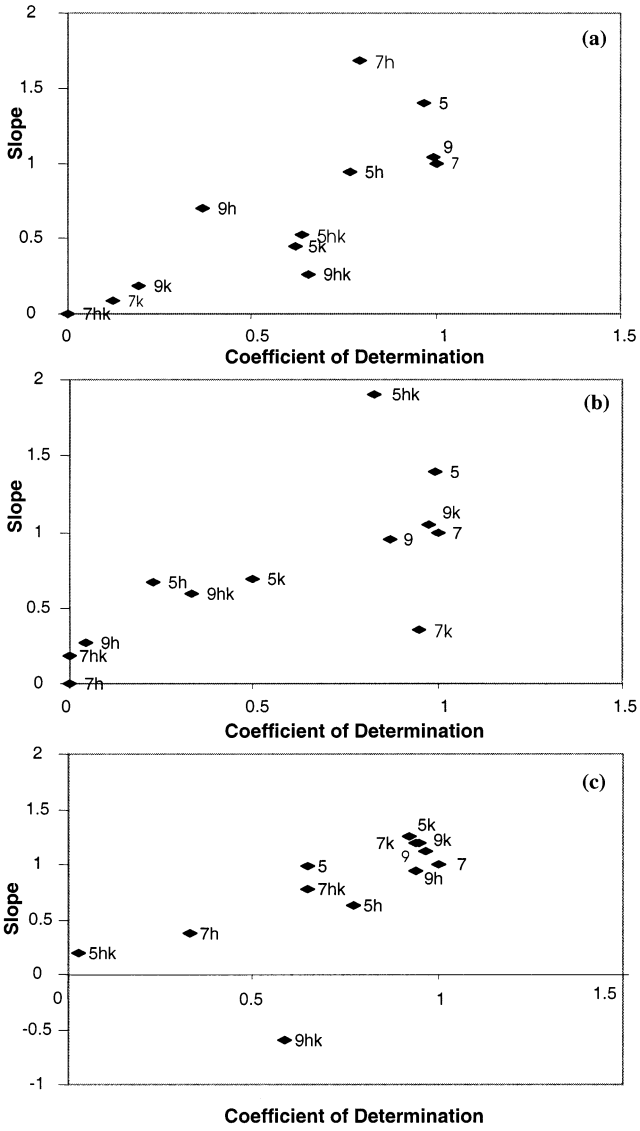

Figure 6. PCS scattergrams of (a) BLG, (b) BSA, and (c) WPI, for samples at various pH values, with or without heating or the presence of KCG. The reference used for PCS analysis was the unheated protein at pH 7.0 ($r^2 = 1$, slope = 1.0 for each protein at pH 7.0). Sample identification: 5, 7, 9, proteins at pH 5.0, 7.0, and 9.0, respectively; 5h, 7h, 9h, heated proteins at these pH values; 5k, 7k, 9k, KCG/protein mixtures at these pH values; 5hk, 7hk, 9hk, heated KCG/protein mixtures at these pH.

In comparison, similar trends were observed in the surface hydrophobicity values when KCG was added to BLG, where the presence of KCG caused a significant decrease in surface hydrophobicity at all pH conditions studied, but the amount of decrease was much larger at pH 7.0 and 9.0 compared to that at pH 5.0 (*4*). The presence of KCG also caused significant decreases in surface hydrophobicity of BSA, whereas the present Raman study indicated an effect of KCG primarily at pH 5.0. No differences in surface hydrophobicity of WPI were observed at any of the pH conditions studied (*4*), similar to the results observed by Raman analysis.

Combined Effects of Heating and KCG. The presence of KCG accompanied by heating caused the largest differences in the Raman spectra for BLG at pH 7.0, for BSA at pH 7.0 and 9.0, and for WPI at pH 5.0, compared to the effect of heating or the presence of KCG alone. In our previous study (*4*), heating BLG in the presence of KCG significantly decreased the surface hydrophobicity at all pH values, compared to unheated BLG

alone. On the other hand, heating BSA in the presence of KCG had no effect on the surface hydrophobicity at pH 5.0 but caused decreases at pH 7.0 and 9.0. For WPI, adding KCG followed by heating did not affect the surface hydrophobicity at any pH (*4*).

Although the results of Raman spectroscopy obtained in the present study were in general agreement with those obtained by the PRODAN fluorescent probe method in our previous study (*4*), some discrepancies were noted, which may be attributed in part to differences in protein concentrations used in these studies. At the low protein concentrations used during measurement with the PRODAN method, molecules are less likely to associate or aggregate even if unfolded. Furthermore, it must be emphasized that the previous study examined only the effects of the factors on surface hydrophobicity of the proteins, whereas the present study using Raman spectroscopy monitored various other structural attributes in addition to characteristics related to hydrophobicity.

It should also be noted that the three techniques used, namely, ANOVA, PCA, and PCS analysis, each have distinct advantages and limitations and were applied for different purposes to aid in the interpretation of the Raman spectral data. In this study, ANOVA was used to determine the factors or their interactions, which significantly influenced specific characteristics in the Raman spectra. The Raman characteristics were considered independently in this analysis, even though in fact they may be inter-related. In contrast, the objective of PCA was to reduce the dimensionality of the complex data set while minimizing the loss of information, through transformation of the original variables (individual Raman characteristics) to a smaller number of latent variables (principal components) that are uncorrelated to each other. Although PCA thus offered the advantage of minimizing errors in interpretation due to multicollinearity of related variables, the trends in terms of individual variables were more difficult to observe in PCA than by simple analysis of variance of the data. PCS analysis, on the other hand, by combining the advantage of PCA with similarity analysis, facilitated grouping of samples and visualization of the influence of specific factors on the samples in comparison to the reference.

Because PCS analysis considered the similarity of the Raman spectra of samples compared to a reference in terms of 5 principal components rather than the 12 individual Raman characteristics, it is not surprising to note some differences in the interpretation of results provided by PCS analysis and ANOVA. For example, the results of ANOVA (**Tables 3** and **4**) showed that KCG alone did not have a significant effect on the individual Raman spectral characteristics of BLG and BSA, whereas PCS analysis determined that the presence of KCG had a considerable effect on the spectra of these two proteins. In contrast, in the case of WPI, KCG was identified as being a significant factor by ANOVA (**Figure 4** and **Table 5**) but not by PCS analysis (**Figure 6c**). Similarly, some Raman characteristics (e.g., the intensities at 508 and 761 cm^{-1} assigned to S−S stretching and tryptophan, respectively, and the intensity ratio of the tyrosine doublet at 860/830 cm^{-1}) were not found to be significant by ANOVA but were included in PCA (**Tables 7**, **9**, and **11**) and the subsequent PCS analysis. Changes in these characteristics were reported when BLG was heated at 90 °C for 30 min in previous studies (*24*, *34*) but not when BLG was heated at 70 °C for 30 min (*24*). It is possible that the heat treatment (80 °C, 30 min) in the present study resulted in only subtle spectral changes that were insignificant by ANOVA but yet were important enough to be included in PCA.

Nevertheless, the results obtained by PCS analysis were in general agreement with those noted by ANOVA. In particular, PCS analysis indicated that the greatest changes in the Raman spectra, when compared to that for the reference protein at pH 7.0, were observed when mixtures of the protein and KCG were heated, followed by heating of proteins alone, addition of KCG to proteins, and finally the effect of pH on unheated proteins. These trends reflect the conclusions inferred from analysis of variance (**Tables 3**−**5**), which also showed heating and heating × KCG as the most prevalent significant factors influencing the individual Raman characteristics. However, it is an advantage of PCS that the combined effects of all influential factors are simultaneously depicted in the two-dimensional scattergrams, so that their effects on samples can be readily evaluated.

Conclusions. Three data-processing techniques were used in this study, namely, analysis of variance (ANOVA), principal component analysis (PCA), and principal component similarity (PCS), to assist in observing trends and identifying significant changes in Raman spectral characteristics of whey proteins as a function of pH, heating, and presence of κ-carrageenan.

ANOVA of 12 characteristics in the Raman spectra of whey proteins indicated significant influences of pH and heating and as well as the interactions between these factors on Raman characteristics related primarily to the secondary structure and C−H stretching vibrations of hydrocarbon side chains. In particular, the characteristics affected included the amide I band at 1658 cm^{-1} for both BLG and WPI, the CH stretching band at 2880 cm^{-1} and the α-helical content estimated from the amide I band for both BLG and BSA, and the CH stretching band at 2930 cm^{-1} and the β-sheet content estimated from the amide I band for BSA.

PCA reduced the dimensionality of the Raman data to four or five principal components. All of the Raman characteristics were found to be important to explain variability of the data. Heating and the interaction of heating with KCG were identified by PCS analysis as the two most important factors producing the greatest variability in the Raman spectral characteristics, reflecting their significant influence on the structure of the whey proteins.

ABBREVIATIONS USED

ANOVA, analysis of variance; BLG, β-lactoglobulin; BSA, bovine serum albumin; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; KCG, κ-carrageenan; PCA, principal component analysis; PCS, principal component similarity; RSAP, Raman spectral analysis package; WPI, whey protein isolate.

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