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Fourier Transform Raman Spectroscopy Study of Heat-Induced Gelation of Plasma Proteins as Influenced by pH

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Fourier transform (FT) Raman spectroscopy was used to elucidate heat-induced structural changes of albumin, globulins, serum, and plasma protein solutions (15% w/w) as affected by pH (4.5, 6.0, and 7.5). Reduction of α-helix and formation of β-sheet, disulfide bond reactions, and exposure and buriedness of hydrophobic groups and amino acid residues were observed. All of these features contributed to the formation of strong, irreversible heat-induced gels. The application of a dimensionality reducing technique such as principal component analysis proved to be useful to determine the most influential qualities of protein samples, as well as the pH-dependent behavior of some of the attributes of both unheated and heated solutions. Analysis of Raman spectra in terms of differences demonstrated the interactions of albumin and globulins in co-occurrence and the significant role of fibrinogen on the gel's attributes.

KEYWORDS: FT-Raman spectroscopy; heat-induced gelation; albumin; globulins; fibrinogen; serum; plasma proteins; principal component analysis

INTRODUCTION

Proteins are widely used in the food industry as functional ingredients because of the interesting physical properties some of them have, such as heat-induced gelation. Naturally occurring protein mixtures can be found, for example, in whey, soy, egg, or blood plasma. Blood plasma is a common byproduct of the meat industry, with a high potential for utilization due to its abundance and its useful functional properties, especially for gel formation upon heating (*1*−*3*). When heat-treated or a concentration threshold is achieved, plasma proteins reorganize into self-assembled aggregates that develop a three-dimensional network, forming consistent gels that entrap water within the protein matrix (*1*). Plasma contains a complex mix of proteins, which can be classified into three major groups, namely, albumin, globulins, and fibrinogen. Albumin, a globular protein with a molecular mass of 69 kDa, is the most abundant, representing up to 60% of the protein content. Globulins comprise a heterogeneous group of globular proteins that include a variety of enzymes and carrier proteins, but mainly the immunoglobulins. Immunoglobulins are subdivided into α, β, and γ fractions, with molecular masses ranging from few to hundreds of kilodaltons; they correspond to more than 40% of the protein content. Fibrinogen is a fibrous protein of 340 kDa formed by three pairs of polypeptide units that represents around 3% of blood plasma proteins (*4*).

During the heat-induced gelation of proteins, structural and molecular environment changes take place. Modifications of the secondary structure, such as β-sheet formation; hydrophobicity due to conformational changes; or covalent bonds, such as disulfide bond reactions, have been described (*5*−*9*). Furthermore, such modifications are influenced by pH, because proteins change their structure and stability as the pH becomes near or further away from their isoelectric point (*5*, *10*, *11*). All of these features can lead to protein gels with different macroscopic properties, which, in the case of plasma, have already been described (*3*, *12*−*14*).

It is notable that the behavior of plasma during thermal treatments depends not only on individual proteins but on the sum of all proteins present. Although the role of each individual fraction in relation to the physical properties of gels has been studied following its isolation or removal (*13*, *14*), the mechanisms leading to the observed different behaviors have not been investigated noninvasively to date. Such changes may be studied by means of techniques providing structural information on proteins, such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, or circular dichroism (CD). However, most of these technique are costly and time-consuming or do not distinguish all types of structures in some conditions. On the other hand, a vibrational spectroscopic technique such as Fourier transform (FT) Raman spectroscopy is particularly suitable for high protein concentrations and solid samples (*15*), and it has been used in the monitoring of heat-induced gelation of proteins (*5*−*9*, *16*−*22*).

A direct spectroscopic method such as FT-Raman spectroscopy is able to examine the role of covalent, disulfide bonds as

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well as electrostatic and hydrophobic bonding in protein−protein interactions (*15*, *17*). The FT-Raman spectra of proteins will also give information regarding the peptide backbone conformation and the molecular environment around the side chains (*15*). Thus, FT-Raman spectroscopy provides a powerful tool to study the transitions and interactions of individual and combined plasma proteins in the formation of gels under different pH conditions. The objective of the present study was to report heat-induced structural changes during the thermal gelation of plasma proteins, isolated or in naturally occurring mixtures, at different pH conditions by means of FT-Raman spectroscopy.

MATERIALS AND METHODS

Materials. Blood was obtained from an industrial slaughterhouse using sterile bleeding recipients containing sodium citrate as anticoagulant (1% w/v). Plasma was separated by centrifuging blood at 2250*g* at 4 °C for 15 min (Sorvall RC 5C Plus, DuPont Co., Newtown, CT) and decanting.

Plasma protein fractions were separated by the salting-out method. All precipitation steps were carried out in an ice bath. A saturated solution of ammonium sulfate in 10 mM Tris-HCl (pH 7.4) at 4 °C was added drop by drop to fresh plasma, and individual protein fractions were progressively precipitated as salt saturation increased. Fibrinogen was first precipitated at 20% saturation and then removed by centrifugation (10000*g* at 4 °C for 15 min). The supernatant containing globulins and albumin, namely, serum, was recovered. Ammonium sulfate was then added to an aliquot of serum up to 60% saturation to precipitate globulins, which were separated by centrifugation. The albumin remaining in the supernatant after centrifugation was also precipitated by increasing the ammonium sulfate concentration up to 75% saturation. Albumin and globulin precipitates were washed once with a saturated solution of ammonium sulfate (75 and 60%, respectively) and recovered again by centrifugation. Protein precipitates were dissolved in 10 mM Tris-HCl (pH 7.4) and, together with serum and plasma aliquots, were exhaustively dialyzed against distilled deionized water at 5 °C with a membrane of 12−14 kDa pore diameter (Medicell International Ltd., London, U.K.) to reduce the content of the precipitating salt. Dialyzed solutions were frozen at −80 °C for 24 h and dried in a Virtis Unitop SQ freeze-dryer (The Virtis Co., Gardiner, NY) at −15 and 15 °C for the primary (sublimation) and secondary (desorption) drying stages, respectively. Purity of final protein powders was confirmed by SDS-PAGE, and the proximate composition for moisture (ISO R-1442), total protein (ISO R-937), and ash content (ISO R-936) was determined. Dried proteins were vacuum-packed and stored under refrigeration temperatures until use.

Preparation of Samples. Protein solutions 15% (w/w) were prepared in distilled deionized water containing 50 mM NaCl. The pH of the solutions was adjusted to 4.5, 6.0, or 7.5 with 0.5 M NaOH or 0.5 M HCl. Aliquots of protein solutions (1 mL) were poured into NMR tubes (5 mm diameter, precision grade, Aldrich Chemical Co., Milwaukee, WI) and heated in a water bath at 80 °C for 45 min to form gels. Gels were immediately cooled to room temperature and stored overnight at 4 °C to age. Aliquots of fresh protein solutions and formed gels were both analyzed by FT-Raman spectroscopy in triplicate.

FT-Raman Spectroscopy. FT-Raman spectra were recorded on a Perkin-Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, U.K.). Spectral resolution was set at 4 cm^{-1} and laser power at 1437 mW, and frequency calibration of the instrument was undertaken using the sulfur line at 217 cm^{-1}. All data presented are based on 128 co-added spectra. The spectra were analyzed using Origin v 7.0 software (OriginLab, Northampton, MA). The original spectra in the 100−3500 cm^{-1} region were baseline corrected and normalized using the phenylalanine peak near 1005 cm^{-1} (*21*). **Table 1** shows the tentative assignment of the major bands in the spectra to vibrational motions of various side chains or peptide backbone, which was based on comparison to FT-Raman spectra reported in the literature (*6*, *15*, *20*, *22*).

A detailed analysis of the amide I region (1600−1700 cm^{-1}) was performed to calculate the secondary structure components of each sample, according to the placement of bands described elsewhere (*9*, *23*−*28*). Spectral data within this region were smoothed with the Savitsky−Golay five-point algorithm and deconvoluted using a nonlinear least-squares curve-fitting subroutine with Gaussian type functions. The percentage of each secondary structure component (α-helix, β-sheet, β-turn, and random coil) was determined as the corresponding fitting peak area contained in the fitting range. Peak intensities and secondary structure components are expressed as the average of the replicate spectra with a typical coefficient of variation of 10%.

Table 1. Tentative Assignment of Major Bands in the FT-Raman Spectra of Plasma Proteins

band assignment	wavenumber (cm^{-1})
S–S stretching	510
tryptophan (Trp)	760
Ttyrosine (Tyr) doublet	850/830
amide III′ (α-helix)	940
amide III′ (random coil)	960
phenylalanine (Phe)	1034
CN or CH stretching	1060
CN stretching	1130
amide III (β-sheet)	1240
amide III (α-helix)	1320
CH deformation, Trp	1345
COO^- aspartic (Asp), glutamic (Glu) acids	1425
CH_2 or CH_3 bend	1450
amide I	1600–1700
CH_3 symmetrical and R_3–CH stretching (aliphatic amino acids)	2880
CH stretching amino acids (aromatic, aliphatic, charged)	2930
═CH stretching (aromatic amino acids)	3060

Data Processing. Descriptive and statistical analyses of data were calculated using SPSS v 13.0 for Windows (SPSS Inc., Chicago, IL). One-way ANOVA and Tukey's post hoc tests were calculated with a significance level of $p < 0.05$. Principal component analysis (PCA) was performed to reduce the dimensionality of the FT-Raman spectral data, retaining as much as possible of the variation present in the data set. The extraction method used the correlation matrix, and an orthogonal rotation with the Varimax method was included. Eigenvalues and variability gathered by principal components (PCs) were calculated, and sample scores in the first four PCs were selected and plotted. To interpret the results of the PCA, a simplified presentation of the PC coefficients of the included variables was used (*29*). If the PC coefficient is greater than half the maximum coefficient in absolute value, a double-positive or a double-negative sign is displayed (depending on the direction toward which the variable contributes in the PC). A single positive or negative sign indicates a coefficient between a fourth and a half of the largest absolute value, and an empty space is assigned when the value is below a fourth of the largest absolute value.

RESULTS AND DISCUSSION

FT-Raman Spectroscopy. Although the study of plasma protein gelation has been carried out by means of general methods, such as DSC and electrophoreses (*13*, *30*), investigation of the changes in structural properties has not been addressed yet, probably due to the difficulty of studying these gelled systems at the high concentrations typically found in foods. In the present study, the use of FT-Raman spectroscopy allowed the direct monitoring of structural changes at a molecular level and, through a detailed spectral analysis, provided a valuable tool for the study of plasma protein gelation.

Typical spectra of heated and unheated plasma, serum, globulins, and albumin samples at pH 7.5 are shown in **Figure 1**. Similar spectra were obtained for all samples at the other pH values (not shown). Relative intensities of bands tentatively assigned to the FT-Raman spectra and percentages of secondary structure components obtained from the deconvolution of the amide I region are shown in **Tables 2**−**5**.

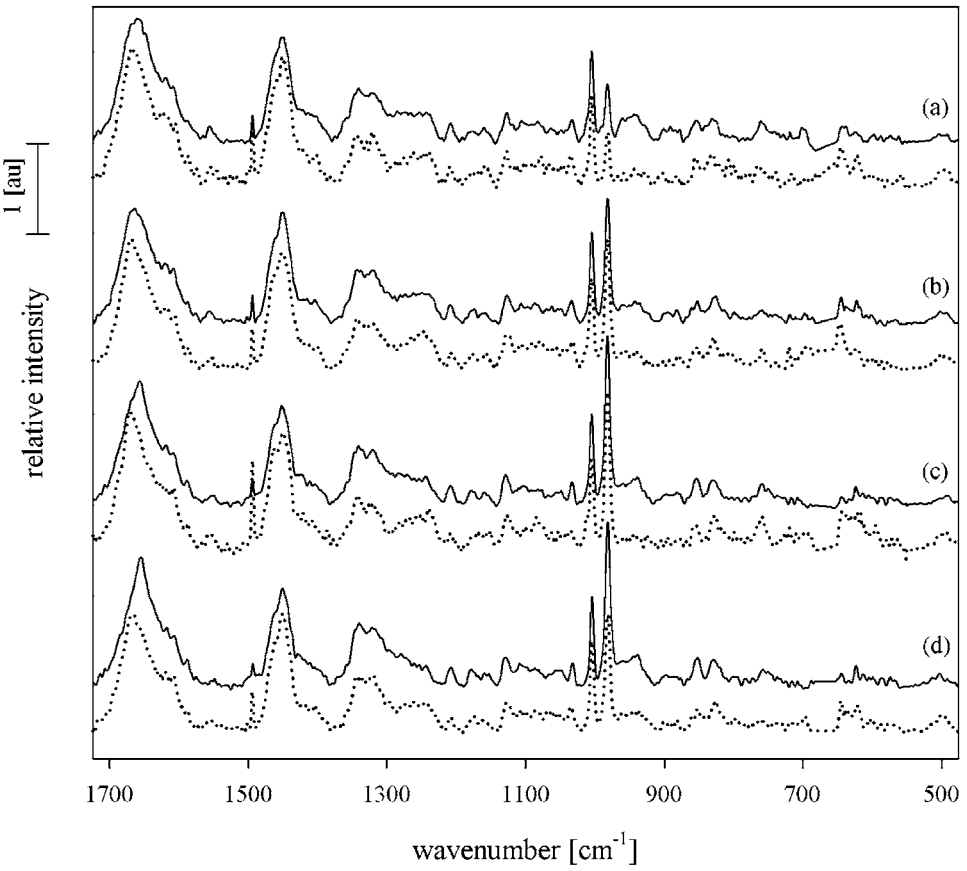

Figure 1. FT-Raman spectra (500–1700 cm^{-1}) for heated (dotted line) and unheated (solid line) protein samples 15% (w/w) of (a) plasma, (b) serum, (c) globulins, and (d) albumin, at pH 7.5. The spectra were baselined and normalized to the phenylalanine peak at 1005 cm^{-1}.

Table 2. Relative Peak Intensity Values at Selected Regions and Percentages of Secondary Structure Components of the FT-Raman Spectra of Unheated (U) and Heated (H) Albumin Samples[a]

		albumin					
		pH 4.5		pH 6.0		pH 7.5	
peak assignment	wavenumber (±2 cm^{-1})	U	H	U	H	U	H
S–S stretching	(492)		0.35	0.07	0.11	0.08	0.16
	(503)	0.25	0.41	0.13	0.16	0.14	0.18
Trp	(759)	0.18	0.15	0.16	0.22	0.20	0.14
Tyr doublet	(828/852)	1.08	0.70	1.16	0.71	1.08	0.74
amide III′	(942)	0.36	0.16	0.42	0.27	0.36	0.24
	(959)	0.30	0.17	0.35	0.25	0.29	0.22
Phe	(1033)	0.25	0.33	0.31	0.30	0.26	0.28
CN, CH stretching	(1061)	0.22	0.26	0.21	0.21	0.16	0.24
CN stretching	(1127)	0.31	0.37	0.34	0.33	0.33	0.34
amide III	(1244)	0.19	0.35	0.21	0.34	0.24	0.33
	(1322)	0.65	0.57	0.64	0.59	0.66	0.63
CH deformation, Trp	(1341)	0.68	0.62	0.69	0.62	0.70	0.62
COO^- Asp, Glu	(1434)	0.42	0.52	0.43	0.52	0.42	0.46
CH_2, CH_3 bend	(1451)	1.09	1.31	1.09	1.34	1.09	1.30
CH_3, R_3CH stretching	(2876)	0.49	0.75	0.48	0.79	0.50	0.79
CH stretching	(2935)	1.21	1.78	1.17	1.87	1.26	1.92
=CH stretching	(3068)	0.60	0.56	0.59	0.60	0.61	0.58
α-helix		50.92	2.16	52.71	3.35	60.73	8.44
β-sheet		22.79	48.85	24.46	90.59	19.26	72.19
β-turns		9.16	11.26	12.23	6.07	8.83	13.99
random coil		17.13	37.74	10.59	0.00	11.18	5.37

[a] Results are expressed as the average of three baseline-corrected and normalized spectra of 128 co-added scans.

Table 3. Relative Peak Intensity Values at Selected Regions and Percentages of Secondary Structure Components of the FT-Raman Spectra of Unheated (U) and Heated (H) Globulins Samples[a]

		globulins					
		pH 4.5		pH 6.0		pH 7.5	
peak assignment	wavenumber (±2 cm^{-1})	U	H	U	H	U	H
S–S stretching	(493)	0.29	0.56	0.08	0.16	0.11	0.20
	(503)	0.26	0.55	0.10	0.17	0.09	0.21
Trp	(760)	0.33	0.33	0.33	0.35	0.20	0.39
Tyr doublet	(828/852)	0.84	0.62	1.09	0.59	1.08	0.69
amide III′	(941)	0.19	0.17	0.18	0.14	0.32	0.21
	(959)	0.19	0.12	0.16	0.19	0.24	0.17
Phe	(1033)	0.23	0.35	0.26	0.43	0.26	0.26
CN, CH stretching	(1061)	0.13	0.26	0.12	0.29	0.14	0.20
CN stretching	(1129)	0.31	0.38	0.33	0.42	0.34	0.40
amide III	(1243)	0.42	0.47	0.46	0.46	0.32	0.50
	(1322)	0.48	0.51	0.50	0.54	0.62	0.57
CH deformation, Trp	(1341)	0.47	0.60	0.54	0.58	0.66	0.63
COO^- Asp, Glu	(1433)	0.42	0.68	0.48	0.64	0.39	0.47
CH_2, CH_3 bend	(1451)	1.12	1.44	1.19	1.44	1.24	1.30
CH_3, R_3CH stretching	(2878)	0.60	1.02	0.57	1.00	0.54	0.99
CH stretching	(2938)	1.37	2.34	1.36	2.23	1.33	2.26
=CH stretching	(3069)	0.71	0.93	0.64	0.84	0.58	0.88
α-helix		3.00	2.09	0.00	0.00	1.70	0.08
β-sheet		51.05	38.32	51.17	54.25	26.43	45.28
β-turns		9.16	19.52	8.12	17.81	16.56	13.47
random coil		36.78	40.07	40.71	27.94	55.31	41.17

[a] Results are expressed as the average of three baseline-corrected and normalized spectra of 128 co-added scans.

Characteristics of Unheated and Heated Proteins. Plasma proteins exhibited several shoulders in the amide I region (1600−1700 cm^{-1}) that pointed out the presence of overlapping bands, which were pH and heat dependent (**Figure 2**, right).

The average percentages of secondary structures of unheated albumin (55, 22, 10, and 13% for α-helix, β-sheet, β-turn, and random coil, respectively) are very similar to the ones found by other workers (*7*, *31*). The α-helix content decreased when

Table 4. Relative Peak Intensity Values at Selected Regions and Percentages of Secondary Structure Components of the FT-Raman Spectra of Unheated (U) and Heated (H) Serum Samples[a]

		serum					
		pH 4.5		pH 6.0		pH 7.5	
peak assignment	wavenumber (±2 cm^{-1})	U	H	U	H	U	H
S–S stretching	(493)	0.15	0.53	0.06	0.17	0.12	0.16
	(503)	0.27	0.52	0.10	0.19	0.11	0.17
Trp	(760)	0.28	0.23	0.27	0.27	0.17	0.23
Tyr doublet	(828/852)	0.85	0.76	1.15	0.73	0.85	0.67
amide III′	(941)	0.25	0.17	0.30	0.20	0.26	0.22
	(959)	0.23	0.17	0.25	0.20	0.22	0.24
Phe	(1035)	0.20	0.35	0.24	0.33	0.27	0.32
CN, CH stretching	(1061)	0.14	0.27	0.15	0.31	0.20	0.27
CN stretching	(1127)	0.37	0.38	0.33	0.47	0.33	0.40
amide III	(1248)	0.34	0.38	0.41	0.41	0.36	0.43
	(1320)	0.61	0.50	0.61	0.57	0.60	0.53
CH deformation, Trp	(1340)	0.63	0.63	0.67	0.62	0.60	0.57
COO^- Asp, Glu	(1434)	0.49	0.60	0.53	0.60	0.45	0.57
CH_2, CH_3 bend	(1451)	1.12	1.44	1.19	1.44	1.24	1.30
CH_3, R_3CH stretching	(2880)	0.61	0.89	0.63	0.91	0.69	0.93
CH stretching	(2937)	1.50	2.03	1.40	2.04	1.71	2.15
═CH stretching	(3069)	0.67	0.72	0.70	0.75	0.55	0.71
α-helix		34.29	8.35	35.11	14.50	32.45	13.24
β-sheet		28.64	35.82	37.96	38.57	35.61	50.81
β-turns		14.44	20.82	4.14	14.76	15.55	10.25
random coil		22.63	35.01	22.79	32.18	16.39	25.70

[a] Results are expressed as the average of three baseline-corrected and normalized spectra of 128 co-added scans.

Table 5. Relative Peak Intensity Values at Selected Regions and Percentages of Secondary Structure Components of the FT-Raman Spectra of Unheated (U) and Heated (H) Plasma Samples[a]

		plasma					
		pH 4.5		pH 6.0		pH 7.5	
peak assignment	wavenumber (±2 cm^{-1})	U	H	U	H	U	H
S–S stretching	(493)	0.25	0.42	0.09	0.13	0.09	0.23
	(503)	0.29	0.52	0.10	0.15	0.11	0.20
Trp	(760)	0.28	0.29	0.25	0.23	0.24	0.27
Tyr doublet	(828/852)	0.86	0.75	1.11	0.73	0.87	0.76
amide III′	(941)	0.31	0.22	0.30	0.22	0.33	0.28
	(959)	0.23	0.16	0.25	0.19	0.27	0.22
Phe	(1035)	0.23	0.35	0.22	0.32	0.30	0.37
CN, CH stretching	(1061)	0.18	0.28	0.14	0.22	0.17	0.29
CN stretching	(1127)	0.29	0.38	0.33	0.40	0.33	0.42
amide III	(1246)	0.37	0.43	0.38	0.42	0.35	0.44
	(1320)	0.55	0.56	0.59	0.61	0.56	0.64
CH deformation, Trp	(1341)	0.63	0.60	0.66	0.64	0.60	0.64
COO^- Asp, Glu	(1434)	0.50	0.69	0.47	0.60	0.48	0.64
CH_2, CH_3 bend	(1451)	1.16	1.57	1.13	1.47	1.18	1.45
CH_3, R_3CH stretching	(2879)	0.68	0.98	0.55	0.94	0.57	1.13
CH stretching	(2938)	1.64	2.29	1.36	2.13	1.37	2.44
═CH stretching	(3069)	0.67	0.77	0.70	0.70	0.90	0.90
α-helix		27.14	9.83	40.55	8.35	36.08	12.45
β-sheet		42.35	58.84	30.77	55.96	33.67	37.38
β-turns		14.38	23.71	2.91	15.81	10.48	19.53
random coil		16.13	7.61	25.76	19.89	19.77	30.64

[a] Results are expressed as the average of three baseline-corrected and normalized spectra of 128 co-added scans.

albumin samples were heated ($p < 0.05$), and it was accompanied by a significant increase of the β-sheet structures, highly pH dependent. This development entails very strong hydrogen bond interactions leading to irreversible aggregation of the protein (*32*).

Analysis of the amide I region of globulins showed predominantly β-sheet and random coil as the secondary structure, in good agreement with the description of Amzel and Poljak (*33*). **Table 3** shows that heating of globulins caused strong pH-dependent changes in the secondary structure components: whereas heating at pH 7.5 entailed an increase in β-sheet content and a decrease in random coil, these features were reversed at pH 4.5. Vermeer and Norde (*34*) and Li et al. (*35*) described a decrease in β-sheet and an increase in random coil when heating IgG; these differences can be explained by the presence of many different globulins within such a complex plasma protein subgroup; thus, the overall thermal behavior can differ considerably from that of a single protein due to cooperative effects.

Secondary structure components in serum and plasma could be explained to a large extent by the naturally occurring mixture of albumin and globulins. However, differences in β-sheet and random coil contents in heated samples were observed ($p < 0.05$), suggesting that fibrinogen may induce the formation of β-sheet in the case of plasma.

Changes in amide III and III′ due to heating agreed with the variations observed in amide I secondary structure components, especially for the weakening of the peak near 940 cm^{-1}, which is related to the α-helix content, and the peak near 1240 cm^{-1}, which reflects the presence of β-sheet (*15*−*17*). However, a sharp band at 990 cm^{-1} that could be assigned to antiparallel β-sheet was observed in unheated samples, the intensity of which decreased when they were heated. Because this was not reasonable, we treated this band as a β-sheet-like structure, present because of some kind of aggregation (*36*).

Two bands near 495 and 505 cm^{-1} were observed in all samples. The location of these peaks corresponded to S−S stretching vibrations of disulfide bonds in the all-gauche conformation (*37*). It is known that serum albumin has one cysteinyl residue at position 34 (Cys-34) and 17 disulfide bridges per molecule, whereas globulins have their subunits paired by disulfide bridges (*4*). Disulfide bond formation or interchange reactions play an important role in nonreversible changes during the heat-induced gelation of globular proteins (*38*), and the observed changes in S−S band intensities demonstrated their contribution to the formation of strong gels. However, the intensity of these changes was lower at pH 6.0 ($p < 0.05$), suggesting that reactive groups may be buried within proteins at this pH due to changes in structural conformation, a possibility that always clouds cross-linking reactions (*39*).

Hydrophobic residues on the outer surface of proteins can be subjected to changes in their buriedness due to protein unfolding (*6*, *17*). The band at 760 cm^{-1} corresponds to the indole-ring vibrations of the tryptophan residue, and a decrease in the intensity indicates exposure of the tryptophan hydrophobic residues. Albumin showed a decrease in the intensity of this band when heated at pH 4.5 and 7.5, but an increase at pH 6.0 ($p < 0.05$). Globulins revealed a marked increase of the tryptophan band only when heated at pH 7.5 ($p < 0.05$). Serum samples performed similarly to albumin at acidic conditions and to globulins at pH 7.5, indicating exposure of the residues in the first case due to protein unfolding and buriedness into a nonpolar environment as a result of intermolecular hydrophobic interactions and disulfide exchange reactions in the latter.

The bands located at 830 and 850 cm^{-1}, corresponding to the tyrosine doublet bands, are useful for monitoring the microenvironment around the tyrosine (*17*, *40*). All protein samples showed a decrease in the I_{855}/I_{830} ratio when they were heated, suggesting that tyrosine residues were in a more buried environment when denatured. Furthermore, there were changes in the intensity of the band at 1450 cm^{-1}, which is assigned to the bending of CH_2 and CH_3 groups and indicates changes in the environment around the aliphatic and hydrocarbon side chains, meaning that hydrophobic residues were buried instead

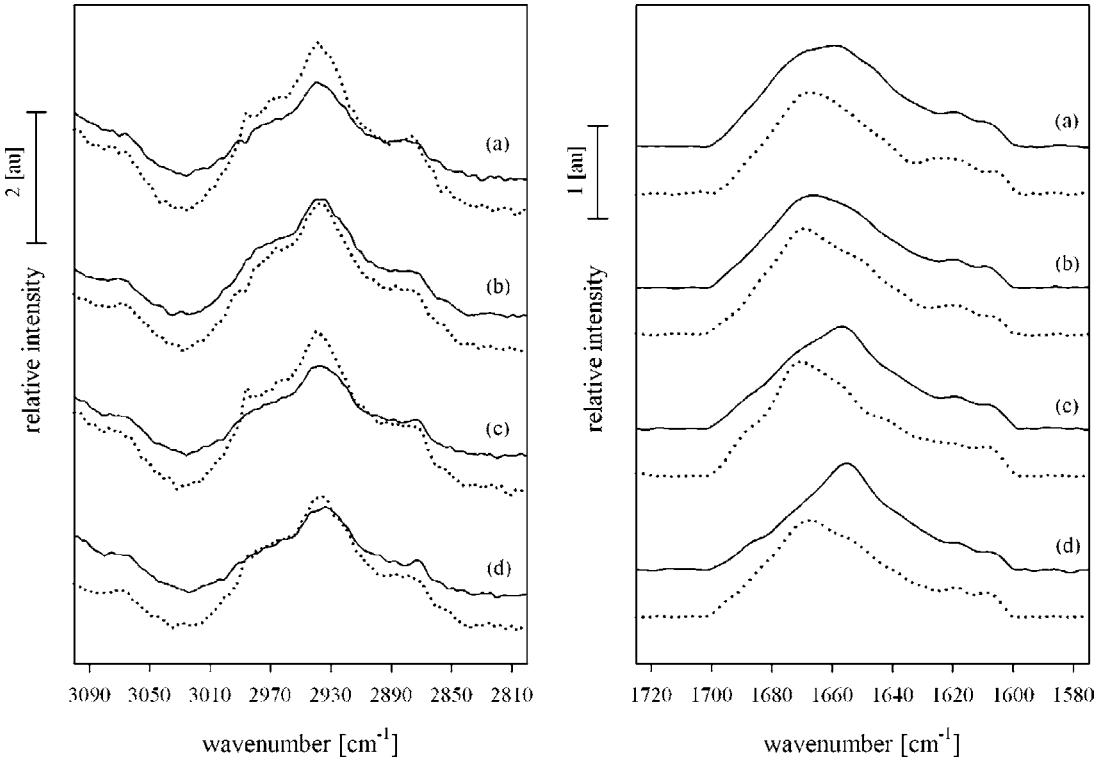

Figure 2. FT-Raman spectra of the CH stretching region (2800–3100 cm^{-1}, left) and the amide I region (1600–1700 cm^{-1}, right) for heated (dotted line) and unheated (solid line) protein samples 15% (w/w) of (a) plasma, (b) serum, (c) globulins, and (d) albumin, at pH 7.5. The spectra were baselined and normalized to the phenylalanine peak at 1005 cm^{-1}, and the amide I region was smoothed with the Savitsky–Golay five-point algorithm.

Table 6. Principal Component (PC) Solutions of the FT-Raman Spectral Data of Heated and Unheated Protein Samples

sample	PC	eigenvalues	% of variance	cumulative %
unheated	1	7.53	37.67	37.67
	2	3.85	19.26	56.93
	3	2.78	13.88	70.80
	4	2.19	10.93	81.73
heated	1	5.52	26.30	26.30
	2	4.45	21.20	47.50
	3	3.22	15.36	62.85
	4	3.05	14.53	77.38

of exposed. This feature has also been observed for egg albumen (*9*) and β-lactoglobulin (*18*).

In heated samples, there was a marked increase ($p < 0.05$) in the intensity of bands assigned to aliphatic amino acids in the CH stretching region (2800−3100 cm^{-1}; **Figure 2**, left) attributed to the diversity of CH, CH_2, and CH_3 groups in the side chains, ionization state, and microenvironment. However, slight changes, or even a decrease, were observed in the band assigned to aromatic amino acids. The above results confirmed that heating of samples leads to conformational rearrangements, which contributes extensively in the development of the protein gels (*16*, *20*).

Principal Component Analysis of FT-Raman Spectra. **Table 6** shows the first four PC solutions of the PCA for unheated and heated samples. The first four axes accounted for 82% of the variability in the first case, whereas 77% was retained in the second. The contribution of each Raman spectral characteristic included in the PCA is shown in **Tables 7** and **8** for unheated and heated samples, respectively. PC coefficients are expressed in a simplified version (*29*) for the sake of clarity. Plotting the obtained PC scores facilitates the rapid comparison between samples and pH conditions, based on the placement of each sample along the components—which are defined by Raman data through PC coefficients. **Figures 3** and **4** display the PCA plots for unheated and heated samples, respectively.

Table 7. Principal Component (PC) Coefficients for the Relevant FT-Raman Spectral Data Included in the Principal Component Analysis for Unheated Samples[a]

		coefficient[b]			
peak assignment		PC1	PC2	PC3	PC4
S–S stretching	a	−		++	+
	b			++	
Trp		−−		+	−
Tyr doublet		+	−−	−−	−
amide III′	a	++	−		
	b	++	−		
Phe		+		−−	+
CN, CH stretching		++			+
CN stretching				+	
amide III	a	−−	++		−
	b	++			
CH deformation, Trp		++			
COO^-			++		−−
CH_2, CH_3 bend			++	−	
CH_3, R_3CH stretching		−	++		
CH stretching			++	+	
α-helix		++			−
β-sheet		−−	+		
β-turns					++
random coil		−−	−		

[a] See **Tables 2**–**5** for corresponding peak wavenumbers. [b] Double signs represent a PC coefficient greater than half the maximum coefficient in absolute value. Single signs indicate a coefficient between a fourth and a half of the largest absolute value. Positive signs mean positive-correlated contributions, whereas negative signs, negative-correlated contributions.

Unheated albumin samples are closely grouped in a cluster defined by a high I_{855}/I_{830} ratio, high α-helix content, and low content of nonpolar groups such as methylene and ethylene bends in the two first components. Unheated globulins are

Table 8. Principal Component (PC) Coefficients for the Relevant FT-Raman Spectral Data Included in the Principal Component Analysis for Heated Samples[a]

peak assignment		coefficient[a] PC1	PC2	PC3	PC4
S–S stretching	a		++		
	b		++		
Trp		++			–
Tyr doublet		–			++
amide III′	a		–		++
	b	–	– –		
Phe		+		++	–
CN, CH stretching				++	
CN stretching		+	–	++	
amide III	a	++			–
	b		– –		++
CH deformation, Trp				–	++
COO^-		++	+	++	
CH_2, CH_3 bend		++	++	++	
CH_3, R_3CH stretching		++		+	
CH stretching		++			
═CH stretching		++			
α-helix				++	++
β-sheet		–	–	–	
β-turns		++	++	+	
random coil		+	+		

[a] See **Tables 2**–**5** for corresponding peak wavenumbers and **Table 7** for symbol explanation.

mostly defined by a high content in tryptophan and β-sheet structures. Unheated serum and plasma samples move along positive PC2 coordinates, through buriedness of nonpolar CH groups. Because both contain a mixture of albumin and globulins, they occupy an intermediate position in PC1, between the two opposite ones of the individual fractions. Moreover, differences between serum and plasma would suggest significant effects caused by the presence of fibrinogen.

The PC3 versus PC4 plot for unheated solutions reveals the differences caused probably by pH conditions, because samples are fairly well grouped according to pH, moving toward high disulfide bands at acidic conditions, a more ionized state of the COO^- group of Asp and Glu carboxyl residues at pH 6.0, and a higher CN stretching and β-turn content at pH 7.5.

Albumin gels were more similar to serum than to plasma ones (**Figure 4**). Plasma and globulin gels were similar in having hydrophobic groups more buried in a nonpolar environment and some aliphatic amino acid residues more exposed, whereas serum and albumin had these features to a lesser extent. Changes in disulfide bonds, as well as in β-turns and the remaining α-helix content, were more evident as the pH decreased.

The PC3 versus PC4 plot of heated samples displayed a larger dispersion in these components. Albumin and globulins were differentiated from plasma and serum by the degree of CN

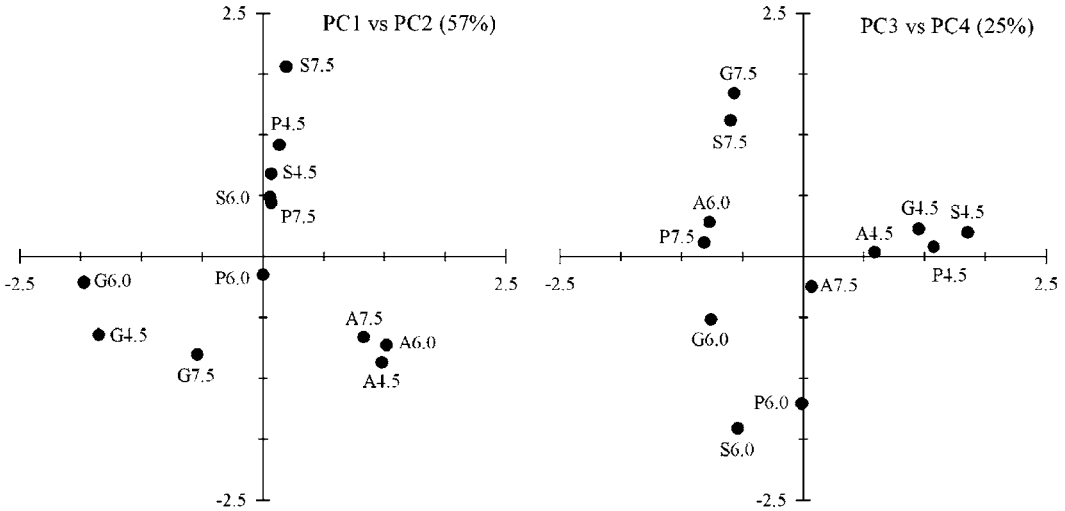

Figure 3. Principal component scores for unheated protein samples in principal components (PC) 1–2 (left) and 3–4 (right). Numbers in parentheses correspond to gathered variability for the selected axes. Letter labels refer to plasma (P), serum (S), globulins (G) and albumin (A) and numbers to pH conditions.

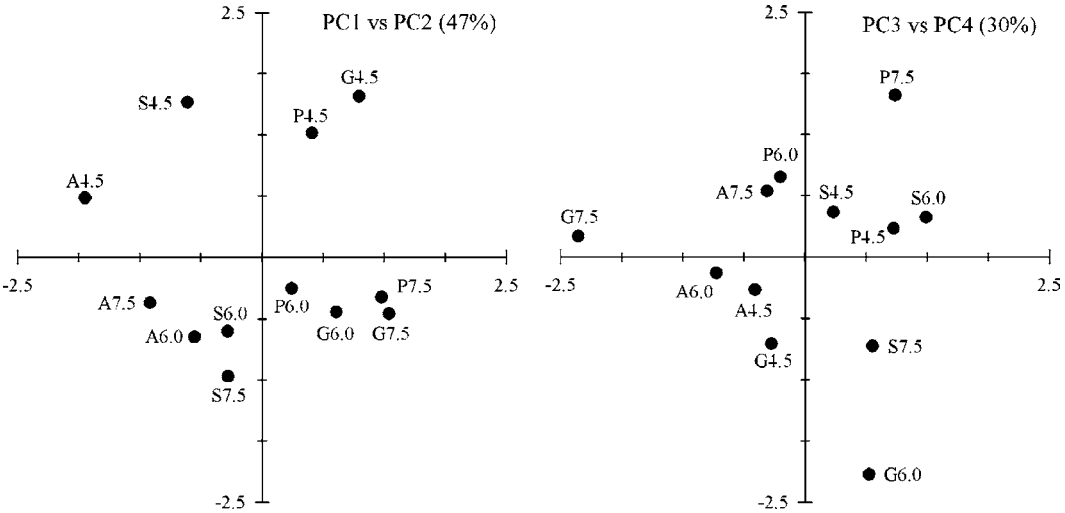

Figure 4. Principal component scores for heated protein samples in principal components (PC) 1–2 (left) and 3–4 (right). Numbers in parentheses correspond to gathered variability for the selected axes. Letter labels refer to plasma (P), serum (S), globulins (G), and albumin (A) and numbers to pH conditions.

stretching, the buriedness of some CH groups, the ionized state of COO^- residues, and the α-helix content.

Conclusions. FT- Raman spectroscopy proved to be a useful tool for the study of specific groups and structural components of plasma proteins in heat-induced gelation, at a molecular level. Changes such as reduction to α-helix and formation of β-sheet, disulfide bond reactions, and exposure and buriedness of hydrophobic groups and amino acid residues were observed between albumin, globulins, serum, and whole plasma. All of these features contributed to the formation of strong, irreversible heat-induced gels. The application of a dimensionality reducing technique such as PCA was useful to show in a few plots the most important qualities in terms of differences between protein samples. As shown by the PCA, some of the attributes of both unheated and heated solutions showed a strong pH-dependent behavior. Additionally, the role of fibrinogen when present was suggested as having a significant effect on the gel's attributes, due to the differences observed between serum and plasma. The information obtained from this study will be useful in the understanding of the physical−chemical properties of heat-induced plasma protein gels.

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