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Effects of Bound Water on FTIR Spectra of Glycinin

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Soy protein is a major food component, but the secondary structure of its major proteins is not well established, especially after heat treatment in the presence of moisture. To study secondary structure changes that take place in glycinin upon hydration using infrared spectroscopy, the effect of bound water must be removed from the protein spectra. This study examined the effects of added water on glycinin infrared spectra. Samples hydrated from 2.6 to 95% water, but not heated, showed significant broadening in the amide I region and changes in the amide I to amide II maximum absorbance ratio as water content increased. A spectral ratio method to derive coefficients for multiplying and subtracting spectra was used to obtain the bound water component spectrum and protein component spectra. The spectrum of dry glycinin could then be regenerated by subtracting the bound water component from glycinin that had added water. Curve-fitting of deconvoluted spectra gave the same secondary structure at all levels of added water after bound water had been subtracted from the spectra (30% β, 24% helical, 35% turns, and 11% unordered). Spectra of glycinin in aqueous buffer were also determined. Side-chain contributions were removed, and the resulting secondary structure was found to be 33% β, 25% helical, 31% turns, and 12% unordered. This compares to 32% β, 21% helical, 34% turns, and 14% unordered before side-chain contributions were subtracted. The data indicate that glycinin has the same structure in solution and in hydrated solids.

Keywords: *Soy protein; glycinin; bound water; FTIR; infrared; secondary structure*

INTRODUCTION

Proteins are affected in their native state by a wide range of aqueous environments. Collagen conformational structure and function, for example, are dependent on the amount of bound water present (Lazarev et al., 1992). To follow these conformational changes, films of proteins cast on inorganic crystals have been exposed to various amounts of hydration, and the shift in infrared absorbances in the amide I spectral region has been determined. Jakobsen et al. (1986) studied the effects of pressure, pH, and nonaqueous solvents on albumin, as a cast film or in saline solution, to show that more ordered helical structures resulted as bound water was removed from the protein environment. Algorithms for subtracting free water from aqueous solutions of proteins have been published (Powell et al., 1986; Dousseau et al., 1989). The absorptivity of β-lactoglobulin and myoglobin amide I (1600−1700 cm^{-1}) and amide II (1500−1600 cm^{-1}) regions was measured in KBr disks, in ground KBr by diffuse reflectance, in solution, and as adsorbed films by attenuated total reflectance (Ishida and Griffiths, 1993). The absorptivity of the amide II region was comparable in KBr disks and solution spectra taken at pH 7 for these proteins, but amide I/II intensity ratios were much higher for aqueous solutions. This increase in amide I relative to amide II intensity was attributed to differences in ordered conformations between the adsorbed or ground solid proteins and proteins in solution. However, the changes described by Ishida and Griffiths are in the direction of aqueous solution causing more intense amide I absorptivity (higher order), whereas according to Jakobsen et al. (1986) more order was observed as bound water was removed. Proteins in adsorbed films are known to have conformations different from those in a crystalline state (Jakobsen and Wasacz, 1987). To determine the effect of bound water on the spectrum of soy glycinin in the solid state and to compare solid state spectra to spectra in solution, we determined the spectral contribution of bound water at several water levels using the general solution of Koenig et al. (1977) for Hirschfield's method of derived coefficients to obtain the water contribution. This study is a necessary step prior to a study of heated samples. The effect of added water which does not lead to gelation must be determined before the contribution from heat can be determined. The primary difference between samples studied in this work and those in previous studies comparing infrared spectra of proteins in solid and solution is that the solid glycinin samples were neither ground nor adsorbed onto a surface.

The secondary structure of glycinin was predicted to be 25% α-helices, 25% β-sheet, 42% turns, and 8% unordered structures on the basis of its amino acid sequence by modeling (Argos et al., 1985). Chen et al. (1990) published spectra from which they derived values of 8% α-helices, 27% β-sheet, and 66% unordered structures. Dev et al. (1988) concluded that the secondary structure of 11S globulin was mainly β-sheet, β-turns, and disordered structures with very little

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α-helix. The purpose of this research was to develop a method for removing bound water contributions from glycinin spectra and to determine glycinin secondary structure in solution and the solid state.

MATERIALS AND METHODS

Soy glycinin was prepared by cryoprecipitation, followed by affinity chromatography and then gel permeation chromatography (Wolf, 1993). Samples were freeze-dried after column separation and dialysis. Because the samples were never heated, intermolecular β-sheets, which have been proposed as a mechanism of gelation, would not have been formed in the preparation procedure. Glycinin prepared in this way should be similar to native glycinin. Water was micropipetted onto accurately weighed glycinin samples (on a Cahn 29 automatic electrobalance) in aluminum pans typically used for analyzing samples in differential scanning calorimetry (DSC). The pans were hermetically sealed and equilibrated overnight at room temperature and then stored at 4 °C until mounting and testing.

Glycinin in Solution. Spectra of glycinin in solution were obtained in a 6.3 μm path length liquid cell with CaF_2 windows. Glycinin (25.9 mg) was accurately weighed to 0.01 mg and dissolved in 173 μL of pH 7.6 phosphate buffer (0.033 M K_2HPO_4, 0.0026 M KH_2PO_4) containing 0.4 N NaCl. The sample was centrifuged to remove insoluble matter. FTIR solution spectra were collected at 2 cm^{-1} resolution by coadding 1000 scans on a Mattson Sirius 100 FTIR spectrophotometer (ATI, Madison, WI) equipped with a DTGS detector. Single-beam spectra of the protein solutions were ratioed to a single-beam spectrum of dry CO_2-free air. A spectrum of aqueous buffer measured in the same cell was subtracted from the protein solution. The subtraction factor was based on the volume fraction of buffer in the solutions. Accurate concentration of protein in solution was determined by measuring the UV absorbance at 280 nm based on an absorbance of 8.4 AU for a 1% solution of glycinin in a 1 cm cell (Wolf, 1993). The weight percent of protein (11.1 g/100 mL of solution, for example), thus determined, was converted to the volume fraction of protein in solution using a partial specific volume of 0.73 for glycinin (Badley et al., 1975). This results in a factor of 0.919, which was used for multiplying the buffer spectrum before subtracting it from an 11.1% glycinin solution spectrum (11.1 × 0.73 = 8.1; 100−8.1 = 91.9). Variations on subtracting the buffer contribution from the solution spectra are described later in the text. The solution spectrum of glycinin was determined three times and structural analysis determined on each.

The absorbance spectrum of side chains in the glycinin amide I region were determined from the amino acid composition determined by Badley et al. (1975) on a VYSIS Protein Analysis Workstation (VYSIS, Inc., Downers Grove, IL), which uses an algorithm based on the work of Venyaminov and Kalnin (1990). Infrared absorbance spectra of glycinin in solution were calculated in units of molar absorptivity per amino acid residue by dividing the absorbance spectrum by path length (6.31 E-4 cm), molar concentration (typically 111 g/L divided by 320 000 for glycinin), and amino acid residues per mole (2785 for glycinin). The side-chain absorbance spectrum (expressed in molar absorptivity) was subtracted from the spectrum of glycinin in solution, and the resulting spectrum was curve-fitted as described below.

Glycinin plus 2.6−50% Water. Solid glycinin containing 2.6−50% water was embedded in paraffin wax and microtomed into 4−8 μm thick slices. Samples were mounted on 3M Disposable IR Cards (3M Co., St. Paul, MN) that had been trimmed to fit an FTIR microscope slide holder. The films were warmed in a 40 °C air oven for 90 s to make the surface of the wax and sample uniformly flat. Spectra were collected in a Spectra-Tech IR-PLAN microscope (Spectra-Tech, Inc., Stanford, CT) attached to the Mattson FTIR. The sampling area (100 μm in diameter, double apertured) was scanned in a single-scan survey mode while the sample was moved by 500 μm steps in the x-direction and 400 μm steps in the y-direction.

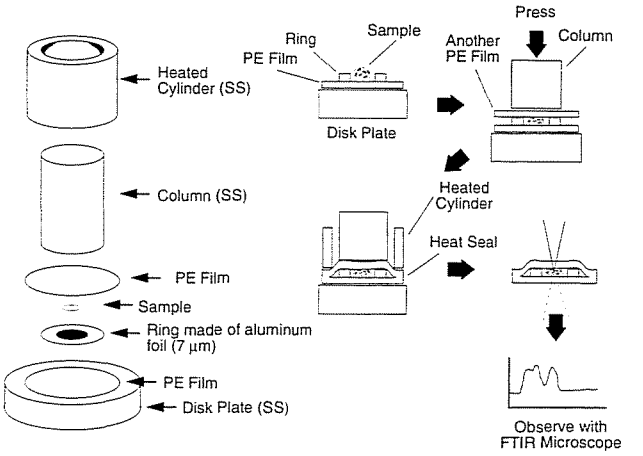

Figure 1. Press and procedure for sealing cryoscopically sectioned glycinin samples. SS, stainless steel; PE, polyethylene.

Areas with protein absorbance maxima of 0.4−0.9 in the frequency range of 1600−1700 cm^{-1} were scanned 1000 times at 2 cm^{-1} resolution, using an MCT detector.

Glycinin plus 50−65% Water. Solid glycinin containing 50−65% water, which were too elastic for sectioning in wax, were embedded in cryoscopic tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and sectioned to about 10 μm thick slices. The sections were mounted by pressing in a laboratory Carver hydraulic press between polyethylene film with an aluminum foil spacer (7 μm thick) and sealed on the edges with wax (Figure 1). Samples in the sealed cell were then mounted in the FTIR microscope and spectra collected according to the same method used for wax-embedded samples.

Glycinin plus 65−95% Water. Protein samples containing 65−95% water by water addition to the DSC pans were pressed without sectioning in the press shown in Figure 1. Spectra were determined in the same way as for other solid samples. Typically, five spectra were collected for a solid protein in different areas of embedded sample in wax as described for solid samples and three for the solution spectrum. Absorbance spectra, with water vapor, bound water, or buffer spectra subtracted, were deconvoluted on a VYSIS Protein Analysis Workstation with a maximum likelihood algorithm (Spectrum Square Associates, Inc., Ithaca, NY). Second-derivative spectra were generated on the same equipment. The centers of underlying component peaks in the amide I and II regions were identified in the deconvoluted and second-derivative spectra. Deconvoluted spectra were fit iteratively as described previously (Abbott et al., 1991). However, the curve fits were performed with GRAMS/386 (Galactic Industries, Inc., Salem, NH) software. Each spectrum was curve-fitted with Gaussian peaks whose centers were determined from enhanced spectra. Initial values of peak centers of component absorbances obtained with "Autofind" in the curve-fitting software agreed well with the values determined from second-derivative spectra except for two small peaks at 1675 and 1644 cm^{-1} that were manually added. Mean values of height, width at half-height, and wavelength were taken at each 10 wavenumber interval for the resulting component peaks from the curve-fitting. Mean values were then used as starting values for the refit of all five spectra. The final fit of all five spectra was used to determine percentage of area under each component peak. Absorbance peaks were assigned to protein structure on the basis of previous studies (Byler et al., 1986; Dong et al., 1990).

RESULTS AND DISCUSSION

The deconvoluted spectra of glycinin at several water levels are shown in Figure 2. Each spectrum in Figure 2 is the average of four to six spectra of different samples at each water level; the spectrum of each sample was from coadded 1000 scans. Using the ratio

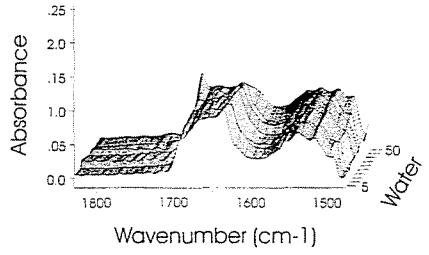

Figure 2. Deconvoluted FTIR absorbance spectra of glycinin with added water (*Z*-axis is water content).

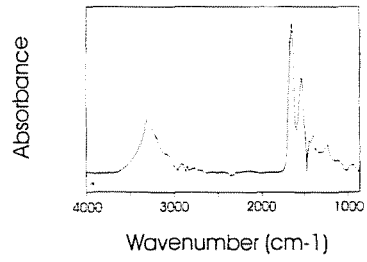

Figure 3. Derived spectrum of glycinin protein from solid samples according to the Koenig ratioing method (see text for details).

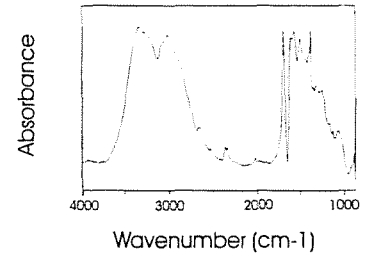

Figure 4. Derived spectrum of bound water according to the Koenig ratioing method (see text for details).

method of Koenig et al. (1977), which gives spectra of individual components with no outside standards, we obtained the spectra shown in Figures 3 and 4 for protein and bound water components. The averaged spectrum for bound water was subtracted from each averaged protein plus water spectrum until the amide I/amide II peak height ratio was 1.5, as exemplified in Figure 5 by glycinin samples containing 11.7, 32.6, and 52.8% added water. Subtracting bound water to a ratio of amide I/II intensity of 1.5 is justified by the fact that the ratio in the driest glycinin (2.6% H_2O) is approximately 1.5, and this value has often been reported for other proteins. The protein spectra resulting from subtraction of added water were curve-fitted using the peak-fitting method described under Materials and Methods. The results are shown in Tables 1 and 2. One might conclude from the spectral data in Figures 4 and 5 that the effect of bound water was to broaden peak areas of various conformations due to the broader distribution of energies of hydrogen bonding with water when compared to intramolecular bonding in the solid between carbonyl oxygens and amide hydrogens. This agrees with the results of Jakobsen et al. (1986) on experiments with albumin.

Another possible explanation of our results is that the protein assumes more energetically favored conformations as the water content is increased because protein segments are more mobile and the bound water fills interstitial voids in the favored conformations. Jackson and Mantsch (1992) observed an increase in 1623 cm^{-1} absorptions in hydrated films of concanavalin A compared to dry protein spectra. In concanavalin A, which

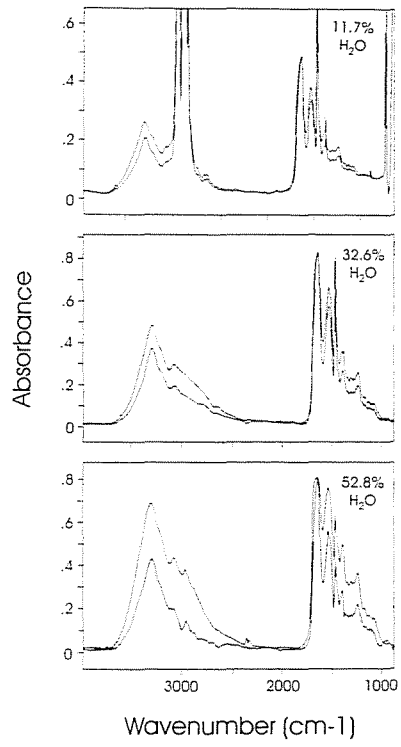

Figure 5. Spectra of glycinin before (upper) and after (lower) bound water subtraction.

Table 1. Structure from Curve-Fitting Solid Glycinin FTIR Spectra after Subtraction of the Bound Water Spectral Contribution

% H_2O	peak area at each wavelength[a]							
	1697T	1691T	1680T	1671T	1658H	1645U	1635B	1622B
2.6	0	6.62	4.53	7.87	13.79	5.9	7.74	8.66
11.7	2.33	4.87	4.32	7.83	12.94	6.57	9.73	8.99
14.4	0	7.85	4.23	7.3	12.41	5.32	9.26	8.79
16.4	0	6.41	5.41	7.72	11.92	5.89	9.77	7.96
28.2	2.45	5.29	3.5	7.59	14.86	6.07	6.56	7.95
32.6	1.84	5.17	4.09	7.52	13.43	5.99	7.38	8.23
37.6	0	6.25	4.41	8.12	14.35	6.37	7.59	8.14
38.5	1.17	5.33	4.53	7.63	12.88	5.83	9.12	8.89
52.8	0	6.51	5.77	7.64	12.71	5.47	6.9	8.24

[a] Abbreviations: T, turns; H, helix structure; U, unordered (unassigned) structure; B, β-structure. Assignments based on results of Byler et al. (1986) and Dong et al. (1990).

Table 2. Glycinin Secondary Structure by FTIR at Various Water Contents

% H_2O	percentage of structure			
	α-helix	β-sheet	turns	unordered
2.6	25.0	29.8	34.5	10.7
11.7	22.5	32.5	33.6	11.4
14.4	22.5	32.7	35.1	9.6
16.4	21.6	32.2	35.5	10.7
28.2	27.4	26.7	34.7	11.2
32.6	25.0	29.1	34.7	11.2
37.6	26.0	28.5	34.0	11.5
38.5	23.3	32.5	33.7	10.5
52.8	23.9	28.4	37.4	10.3

is a predominantly β-sheet structure protein, these authors attribute the 1623 cm^{-1} band to a hydrated form of β-sheet structure and intermolecular β-sheets. When glycinin solutions (15% concentration) are heated to 85 °C and then cooled, they gel. Subtraction of the sol spectrum from the gel spectrum showed increased absorbance at 1618 and 1680 cm^{-1} and decreased absorbance at 1645 cm^{-1} (Nagano et al., 1994). The changes were attributed to increased β structure, but the changes are very similar to the changes that we

Table 3. Amide I/Amide II Ratios of FTIR Spectra of Glycinin and Water

% H_2O	amide I/II ratio	% H_2O	amide I/II ratio
2.6	1.467	38.5	1.159
11.7	1.350	50.0	1.033
14.4	1.409	52.8	1.063
27.9	1.359	62.6	1.285
32.5	1.127	73.0	1.167
32.6	1.267	88.9 (solution)	2.894
37.6	1.268	88.9 (solution − buffer)	1.635

observe for increased water contributions to the infrared spectra of unheated glycinin. If the decrease in absorbance at 1645 cm^{-1} is from a loss of water in water–water associations and the comparable gain in absorbance at 1618 and 1680 cm^{-1} is attributed to broader absorbances in protein–water associations, then the results could be explained by changes in bound water not by increased β structure. The bound water component spectrum observed for glycinin showed increased absorbance at 1612 cm^{-1}. This is not to say that hydration changes are the only explanation for the gelation observed by Nagano et al., but that in order to study the changes which take place with heating and subsequent gelation, the effects of bound water without heating should be taken into account. This was the purpose of our study.

In other tests (data not shown) subtracting a liquid water spectrum from the spectra of hydrated proteins gave no meaningful results. That method did not work because the spectra of dry solid proteins and of proteins in solution (with buffer subtracted) have amide I/amide II ratios greater than the amide I/amide II ratios of hydrated solid. Thus, subtracting a liquid water spectrum which contributes more to the amide I region than the amide II region from hydrated glycinin spectra resulted in a decrease in the amide I/amide II ratio. This did not bring hydrated spectra back to the spectrum equivalent of either dry glycinin or glycinin protein in solution.

An increase in amide I/amide II absorbance ratio in solution compared to solid protein spectra was due primarily to an increase in the amide I intensity according to Ishida and Griffiths (1993). In contrast, we found that adding water up to 73% of the total weight of protein plus water decreased the amide I/amide II ratio of maximum absorbances from changes attributable to the bound water (Table 3). From the derived bound water spectrum in Figure 4 we see a broadening effect and increased absorbance at about 1580 cm^{-1} which contributes more to the intensity of the amide II region than any corresponding contribution to the amide I region from bound water. Thus, a subtraction of the bound water spectrum reduces amide II intensity but has a smaller effect on the amide I region. The subtraction was continued until the amide I/amide II ratio was 1.5, the same as for glycinin, with 2.6% H_2O. However, if we extend the application of the Koenig method to protein solutions, we obtain the spectrum shown in Figure 6 for the derived protein component. In the spectrum of glycinin, obtained by subtraction of a liquid water spectrum, the amide I/amide II ratio is 1.64. By using the derived protein spectrum method, it is 1.67. The amide I/amide II ratio is sensitive both to small errors in the measurement of either amide absorbance maxima and to the baseline, especially if a subjective baseline correction has been made. No baseline corrections were made in the solution spectra in this work except to offset the entire

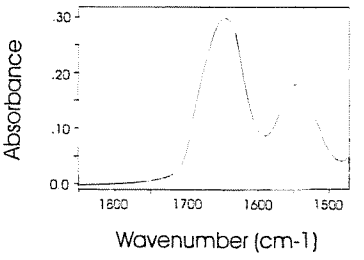

Figure 6. Derived spectrum of glycinin protein from solution samples according to the Koenig method.

Table 4. Glycinin in Solution: Curve-Fit Absorbances, Structure Assigned,[a] and Percent of Total Amide I Absorbance Area

solution minus buffer	Koenig derived protein spectra	after side-chain spectra subtraction
1687T, 12.6%	1687, 12.6%	1689T, 8.01%
1677T, 4.56%	1677T, 4.27%	1678T, 3.79%
1672T, 2.97%	1672T, 2.6%	
1667T, 13.8%	1667T, 14.6%	1667T, 18.9%
1655H, 20.6%	1655H, 20.8%	1653H, 25.1%
1644U, 13.8%	1644U, 14.2%	1643U, 11.6%
1633B, 22.6%	1633B, 21.5%	1633B, 25.5%
1618B, 9.02%	1619B, 9.50%	1619B, 7.02%

[a] Abbreviations as in Table 1.

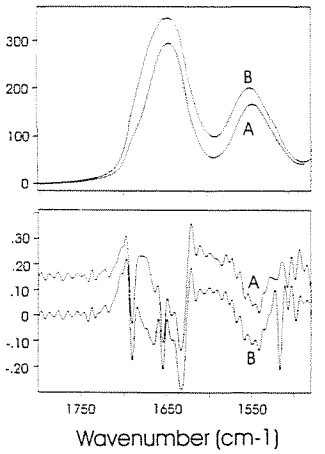

Figure 7. Glycinin in solution: absorbance spectrum before (B) and after (A) side-chain subtraction and second derivatives of both spectra.

spectrum to bring the baseline to zero at 1936 cm^{-1}. Curve-fitting the protein spectra of glycinin in solution, the spectrum obtained using the Koenig method gave essentially the same results as curve-fitting protein spectra derived by subtracting buffer using the known concentration of protein and buffer (Table 4). Ratioed spectra of proteins in solution to buffer showed that the absorbance at 1542 cm^{-1} is most sensitive to protein only. This technique was used to determine the coefficient a_1. The absorbance at 2080 cm^{-1} was most sensitive to water alone and was used to determine the coefficient a_2 necessary to derive spectra of each component according to the Koenig method.

Subtracting side-chain contributions (Figure 7) and curve-fitting the resulting spectra eliminated the absorbance at 1672 cm^{-1} which resulted in 4% lower turns and 4% higher helix content. Subtracting contributions from side chains in the amide I region and then curve-fitting gave a structural analysis that we believe to be the most accurate determination of glycinin secondary structure by FTIR to date. Compared to the secondary structure of glycinin in the solid state and in solution without side-chain contributions subtracted, there was

only a 3–4% increase in helix and unordered structures content. Almost all absorbances shifted from their values in solution spectra by 2–5 cm^{-1} higher in the solids spectra.

We can conclude from these data that glycinin has the same structure in the solid and solution states, although this may not be true for all proteins. The Koenig method appears to be useful for separating the contribution of bound water from solid protein spectra. The structure of unheated glycinin is approximately 33% β structure, 25% α-helix, 31% turns, and 12% unordered in solution and only very slightly different in unheated solid glycinin, in the presence of added water. This is in fairly good agreement with the values predicted by Argos et al. (1985) but radically different from the values reported by Chen et al. (1990). The spectra shown in Chen et al. appeared too noisy for curve-fitting; no method for the curve-fitting was described; and ground proteins pressed into KBr discs may have caused denaturation of the protein structure.

Dev et al. (1988) studied the structure of 11S globulin by infrared, using denaturing urea, heat, and helical-inducing trifluoroethanol to change the protein structure. The resolution of their spectra was 8 cm^{-1}, and they observed only three bands in the amide I region. The 1638 cm^{-1} band was assigned to β-sheet, the 1660 cm^{-1} band to disordered, and the 1687 cm^{-1} band to β-turn and β-sheet structure. On the basis of the urea and thermally denatured 11S protein, there is support for assigning the 1658–1660 cm^{-1} absorbance to disordered structures. If we used this assignment, all of the helical content in Table 2 would be shifted to unordered, increasing the unordered content to 35%. The same assignment of the 1653–1655 cm^{-1} absorbance in solution is unlikely and is indicative of helical structure. Because the wavenumbers of the proposed assignments in Dev et al. for disordered and helical are close and the resolution was 8 cm^{-1}, we prefer the assignments made in Table 2, which agree with the assignments for these absorbances made by Byler et al. (1986) and Dong et al. (1990). There are no quantitative or semiquantitative values for secondary structure in Dev et al. with which to compare our data, but the qualitative agreement depends on assignment of the 1658–1660 cm^{-1} absorbance.

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