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Carbon-13 NMR Studies of Glycinin and β-Conglycinin at Neutral pH

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Carbon-13 NMR spectra have been obtained at 4.7 T for soy β-conglycinin and glycinin in pH 7.0 solutions containing 35 mM phosphate and 0.4 M NaCl. Peak assignments for amino acids in both proteins and for the carbohydrate side chain of β-conglycinin were based on published compositions and chemical shifts and the results of *J*-modulated double-spin-echo experiments. Structural differences between glycinin and β-conglycinin included (a) differences in the apparent areas of spectral envelopes for aliphatic, α-carbon, and carboxyl groups and (b) bandwidth differences in peaks for glutamine and glutamate, (c) differences in backbone and side-chain flexibility, and (d) appearance of the carbohydrate moiety of β-conglycinin. Structural similarities included (1) proline in the trans conformation and (2) a single peak for the guanidino carbon of arginine.

Nuclear magnetic resonance spectroscopy (NMR) is a powerful tool for obtaining both gross structural and microenvironmental information about proteins. NMR studies of food proteins from seeds have emphasized the alcohol-extractable proteins (prolamines) from cereal grains. The NMR characteristics of corn zein (Augustine and Baianu, 1986, 1987), wheat glutenins and gliadins (Baianu, 1981; Baianu et al., 1982), wheat gluten (Belton, et al., 1987), and C hordein from barley (Tatham et al., 1985) have been studied. None of these studies has investigated the aqueous salt-extractable proteins of cereal grains or oilseeds by NMR.

NMR studies of soy proteins have emphasized the nonstorage proteins such as soy leghemoglobin (Trewhella et al., 1986; Mabbutt and Wright, 1983), lipoxygenase (Slappendel et al., 1982), and trypsin inhibitor (Baillargeon et al., 1980) or metabolism of specific amino acids during soybean germination (Coker et al., 1987). While purified soy proteins have been examined previously by NMR (Kakalis and Baianu, 1985; Baianu, 1989), and some peaks tentatively assigned, to our knowledge no effort to fully examine glycinin and β-conglycinin by ^{13}C NMR at neutral pH has been made.

To date, the structural studies of soy storage proteins have focused on gross structural changes observable by either calorimetric (Hermansson, 1978) or spectrophotometric (Dev et al., 1988) methods, particularly CD and ORD (Ishino and Kudo, 1980; Yamauchi et al., 1979; Koshiyama and Fukushima, 1973). Recently, the conformational conclusions made with CD and ORD have been qualitatively confirmed with FT-IR (Dev et al., 1988). In this study, the structures of the soy storage proteins glycinin and β-conglycinin are examined by solution-state ^{13}C NMR, many peaks assigned, relaxation times determined, and some conclusions drawn regarding structure and conformation of these two proteins.

PROCEDURE

Protein Isolation and Purification. Crude glycinin was prepared from Nutrasoy 7B flakes (Archer Daniels Midland Co., Decatur, IL) by a modification of the procedure of Thanh et al. (1975), which is based on the differential solubility of β-conglycinin and glycinin in 0.063 M Tris buffer (pH 6.6) con-

taining 0.010 M 2-mercaptoethanol (2-ME). Soy flakes were dispersed in 0.063 M Tris buffer (pH 7.8) containing 0.010 M 2-ME (1:15 ratio). The suspension was extracted for 2 h at ambient temperature and centrifuged for 30 min at 10000g, and the precipitate was discarded. The resulting supernatant was filtered through a 0.22-μm Millipore filter and then exhaustively dialyzed against water at 4 °C. The retentate was centrifuged for 30 min at 10000g. The precipitate was suspended in deionized water and lyophilized. The crude glycinin (750-mg portions) was dispersed in pH 7.6 0.035 M phosphate buffer containing 0.4 M NaCl and 0.010 M 2-ME and then fractionated on a 2.5 × 80 cm column of Sepharose 6B with a flow rate of 25 mL/min. Purity of the preparation was ascertained by lithium dodecyl sulfate–polyacrylamide gel electrophoresis with the procedures outlined by Laemmli (1970). The remaining column eluate was dialyzed against water and lyophilized. Glycinin prepared in this manner was approximately 95% pure and was used without further purification.

β-Conglycinin was prepared by Walter J. Wolf (Northern Regional Research Center, USDA—ARS, Peoria, IL) using a modification of the procedure of Thanh and Shibasaki (1976). In it, the initial extraction was made with 0.03 M Tris-HCl containing 0.005 M ethylenediaminetetraacetic acid (EDTA). The crude β-conglycinin was purified by dissolving 2 g in 25 mL of pH 7.6 0.5 ionic strength phosphate–NaCl buffer containing 0.01 M 2-ME and 0.01% Thimerosol and pumping it through a 2.6 × 13 cm column of Concanavalin A–Sepharose 4B. The β-conglycinin was eluted with a gradient from 0 to 0.5 M of methyl α-D-glucoside. Samples, assayed for purity by Dr. Wolf by ultracentrifugation, were combined to yield a spectrometric sample containing approximately 93% β-conglycinin and 7% glycinin.

Amino acid analyses were done in duplicate for glycinin and β-conglycinin by Medallion Laboratories, Minneapolis, MN.

NMR Methods. All samples were measured as 100 mg/mL solutions in an aqueous buffer containing 30% D_2O, 0.035 M potassium phosphate, and 0.40 M sodium chloride, adjusted to an apparent pH of 7.0. Solvent was filtered through piggyback 0.45- and 0.22-μm filters to remove fungal and bacterial contaminants. Tubes were washed with 95% ethanol and acetone and dried at 110 °C. In samples treated this way, the obvious results of decomposition (e.g., foul odor) did not appear until after 10 days of storage in solution at room temperature. Acetonitrile (0.25%) was used as an internal reference (chemical shift at pH 7.0, 25 °C, 1.30 and 119.61 ppm). All spectra were obtained with a Varian VXR-200 spectrometer using a 10-mm sample tube containing 3 mL of solution and 0.30 g of protein. Spectra were acquired at 50.309 MHz over a spectral width of 11 148.3 Hz and proton-decoupled with the WALTZ sequence (Shaka et al., 1983) using 256-pulse blocks, and each block was preceded by a four-pulse steady-state sequence. Recycle time was 0.6 s, and tip angle was 62.5°. Fourier transforms were done on 13 376 points, zero-filled to 16 384, and processed with 5-Hz exponential line-broadening and 0.05-s Gaussian apodization ($\exp(-t^2/0.0025)$). To distinguish between close peaks, lines were converted from Lorentzian line shape to Gaussian line shape by combined exponential ($\exp(t/s)$) and Gaussian functions. To emphasize narrow peaks, FIDs were convolution difference weighted with the function $(1 - (0.9 \exp(-t/0.03)))$. To determine peak multiplicity, the Varian-supplied APT sequence (Patt and Shoolery, 1982), a J-modulated double-spin-echo sequence, was used. Spectra used to estimate spin–lattice relaxation times (T_1's) for the dominant peaks in the glycinin spectrum were obtained with the fast inversion–recovery sequence (T–180°–τ–90°; $T \approx 2T_1$, τ varied in 12 increments from 8 ms to 4 s) (Levy et al., 1980) and the T_1's calculated with the Varian-supplied software.

RESULTS AND DISCUSSION

Amino acid analysis data are presented in Table I; chemical shift, peak assignment, and bandwidth data are presented as Table II; spin–lattice relaxation times (T_1) are in Table III. Peak assignments were made based on literature values (Howarth and Lilley, 1978; Wüthrich, 1976; Kakalis and Baianu, 1985), aided by convolution difference and resolution enhancement weighting of the free induction decays (FIDs) as stated in NMR Methods. In several instances, most notably the 10–22 ppm region of β-conglycinin (Figure 1B; Table II), assignment of peaks that could not be observed in the normal NMR spectrum were made with use of the APT sequence (Patt and Shoolery, 1983). Bandwidths were determined with the software available on the NMR instrument. Unreliable bandwidths resulted when accurate base line or half-height could not be determined by the software due to peak overlap. All bandwidths reported by the instrument to be unreliable were rejected.

Table I. Amino Acid Analysis Data (%)

	glycinin		β-conglycinin	
	lit.[a]	exptl[b]	lit.[c]	exptl[b]
Lys	4.4 ± 0.4[d]	4.7 ± 0.1	6.4 ± 0.6[d]	6.1 ± 0.3
His	2.0 ± 0.2	1.9 ± 0.2	2.0 ± 0.3	2.3 ± 0.2
Arg	6.6 ± 1.1	7.2 ± 0.2	7.9 ± 0.8	9.4 ± 0.0
Asn[e]	7.9 ± 0.5	7.7 ± 0.1	8.2 ± 0.8	7.2 ± 0.0
Gln[e]	10.2 ± 0.8	11.5 ± 0.4	9.2 ± 0.3	10.4 ± 0.5
Asp[e]	4.3 ± 0.3	4.1 ± 0.1	4.7 ± 0.3	4.2 ± 0.0
Glu[e]	8.9 ± 0.6	10.1 ± 0.4	10.7 ± 0.3	12.2 ± 0.5
Thr	3.9 ± 0.4	3.6 ± 0.1	2.6 ± 0.2	2.2 ± 0.0
Ser	5.6 ± 1.3	5.0 ± 0.0	7.0 ± 0.4	4.9 ± 0.0
Pro	6.2 ± 0.7	7.8 ± 0.8	5.0 ± 1.2	4.3 ± 0.7
Gly	6.4 ± 2.1	4.4 ± 0.2	4.2 ± 1.2	2.6 ± 0.0
Ala	5.0 ± 1.7	3.7 ± 0.2	4.5 ± 0.7	3.2 ± 0.1
Val	5.2 ± 0.3	4.4 ± 0.3	4.7 ± 0.5	3.7 ± 0.1
Ile	4.5 ± 0.3	4.1 ± 0.0	5.6 ± 0.8	4.6 ± 0.0
Leu	7.0 ± 0.3	7.2 ± 0.1	9.4 ± 0.7	8.6 ± 0.3
Tyr	2.8 ± 1.0	4.1 ± 0.6	2.5 ± 1.1	3.2 ± 0.3
Phe	4.4 ± 1.2	5.0 ± 0.5	6.1 ± 1.1	6.3 ± 0.3
Trp	0.8 ± 0.2	1.6 ± 0.0	0.2 ± 0.2	[f]
Met	1.2 ± 0.5	1.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.1
Cys	1.3 ± 0.2	1.8 ± 0.1	0.3 ± 0.0	0.7 ± 0.0

[a] Unweighted average of values reported in Moreira et al. (1979), Kitamura and Shibasaki (1975), and Badley et al. (1975). [b] Average of two determinations by Medallion Laboratories (see NMR Methods). [c] Unweighted average of values reported in Coates et al. (1985), Thanh and Shibasaki (1977), and Nielsen (1985). [d] Standard deviation. [e] Proportions of Asn, Asp, Gln, and Glu calculated from Wright (1987). [f] Not determined.

Glycinin is a hexameric protein with an approximate molecular weight of 360 000. Each of its six subunits consists of a pair of polypeptide chains, made up of one acidic and one basic polypeptide connected by a single disulfide linkage (Nielsen, 1985a,b). Glycinin's secondary structure is roughly 20% α-helix, 17% β-structure, and 63% random coil (Ishino and Kudo, 1980). β-Conglycinin is a trimeric glycoprotein of molecular weight 180 000. It is reported to be 20% α-helix, 23% β-structure, and 57% random coil (Ishino and Kudo, 1980) and to contain about 5% carbohydrate (Koshiyama, 1968) composed of mannose and N-acetylglucosamine (Yamauchi and Yamagishi, 1979). Amino acid analyses of the glycinin and β-conglycinin used in this study are presented and compared with average published compositions in Table I.

Although glycinin and β-conglycinin have large regions of random-coil structure (Ishino and Kudo, 1980), neither the one previous ^{13}C NMR study (Kakalis and Baianu, 1985) of glycinin or a recent NMR study of soy protein isolates at high pH (Baianu, 1989) led us to expect that their ^{13}C NMR spectra should have as many well-defined, narrow lines at pH 7 as can be seen in Figure 1. Convolution difference (Figure 2) and J-modulated double-spin-echo (APT, Figure 3) spectra confirmed that these peaks were in positions very similar to those found for free or "peptide shifted" amino acids (Howarth and Lilley, 1978; Wüthrich, 1976). Consequently, most of the peaks for both the major and minor components of glycinin and β-conglycinin have been assigned (Table II), and spin–lattice relaxation times (T_1's) have been deter-

Table II. Glycinin and β-Conglycinin Peak Assignments, Peak Positions, and Bandwidths

peak assignment	glycinin ppm[a]	glycinin bw[b]	β-conglycinin ppm	β-conglycinin bw
Glu Cδ	181.6	26.2	181.5	363.1
Gln Cδ	178.0	38.6	178.0	c
C=O + Asn Cγ	173.6	97.1	174.0	89.0
C=O	172.1	215.0	172.4	221.2
C=O	d		172.0	220.8
Arg Cζ	157.2	20.8	157.1	22.9
Tyr Cζ	154.8	50.6	e	
Phe Cγ	136.2	c	136.5	34.4
His Cδ + Cε	135.6	40.5	135.6	c
Tyr 2Cδ	131.1	225.5	131.1	257.9
Phe 2Cδ	129.5	62.4	129.6	111.6
Phe 2Cε	129.1	68.4	129.0	113.0
His$^+$ Cε	128.5	227.3	f	
Phe Cζ + Tyr Cγ + Trp Cδ2 [h]	127.5	247.4	127.3	245.7
Trp Cη [h]	122.3	94.4	e	
His$^+$ Cδ + Trp Cε [h]	118.9	23.9	e	
His Cδ + Trp Cζ2 [h]	117.6	40.4	e	
Tyr 2Cε	115.8	39.0	115.8	23.0
α-Man C1	g		102.6	26.0
GlcNAc(Asn) C1	g		101.2	44.1
GlcNAc(Asn) C2	g		78.7	50.4
α-Man C5	g		73.5	c
α-Man C2, C3, C4	g		70.5	94.2
Thr Cβ + β-Man C4, C6[g]	68.2	45.9	67.2	c
Ser Cβ + α-Man C6[g]	61.4	171.6	61.5	99.5
Pro Cα + GlcNAc(Asn) C2[g]	60.9	c	60.7	383.4
Ser Cα + GlcNAc(β-Man) C2[g]	55.9	c	56.2	118.1
Cα Env	55.0	296.6	f	
Cα Env	53.9	105.0	54.1	85.3
Asn Cα	52.0	307.8	52.1	c
Pro Cδ	48.0	c	48.3	c
Gly Cα	42.9	c	42.9	c
Lys Cε + Arg Cδ	41.0	144.6	41.0	115.4
Asn Cβ + Asp Cβ	39.6	40.2	39.7	36.3
Leu Cβ + Cys 2Cβ [h]	38.9	294.6	d	
Tyr Cβ	37.9[c]	c	d	
Ile Cβ	36.9[i]	c	36.7	c
Phe Cβ + Ile Cβ	36.5	c	d	
Glu Cγ	33.9	28.0	33.9	91.2
Gln Cγ	31.4	41.3	31.4	c
Lys Cβ + Val Cβ + Met Cβ [h]	30.7	c	30.7	c
Pro Cβ + Met Cγ [h]	29.7	c	29.7	c
Val Cβ	29.3[i]	c		
Arg Cβ + Glu Cβ	28.0	115.5	27.9	c
Lys Cδ	27.1	129.6	27.2	c
Gln Cβ	26.7	129.6	26.7	c
Arg Cγ + Pro Cγ + Ile Cγ1	24.8	242.4	24.7	c
Leu Cγ	24.3[i]	c	25.0[i]	
Lys Cγ	22.3	247.0	22.3	c
Leu 2Cδ	21.1	c	21.5[i]	c
Thr Cγ	19.2	c	19.2[i]	c
Val Cγ1	18.7	c	18.8[i]	c
Val Cγ2	18.1	c	d	
Ala Cβ	17.2[i]	c	17.2[i]	c
Ile Cγ2 + Met Cε [h]	15.2	c	f	
Ile Cδ	11.0	c	e	

[a] Chemical shifts in parts per million, acetonitrile as reference at 1.300 ppm. [b] Bandwidth at half-height (Hz). [c] Bandwidth calculation unreliable. [d] Merged with preceding peak. [e] Peak intensity too low to permit assignment. [f] Cannot be assigned separately from surrounding peak envelope. [g] β-Conglycinin only. [h] Glycinin only. [i] Peak assigned via the APT pulse sequence (Patt and Shoolery, 1983).

Table III. Glycinin Carbon-13 Spin-Lattice Relaxation

assignment[b]	shift,[a] ppm	T_1, s
Glu Cδ	181.6	2.26
Gln Cδ + Asp Cγ	178.0	1.85
backbone C=O	173.9	2.18
backbone C=O	172.4	2.24
Arg Cζ	157.2	2.24
Tyr 2Cδ	131.0	1.21
Phe 2Cδ + Phe 2Cε	129.5	0.74
Tyr 2Cγ	128.1	0.74
His Cδ	117.7	0.74
Tyr 2Cε	116.2	0.75
Thr Cβ	66.2	0.93
Ser Cβ	61.3	0.53
Pro Cα	59.8	0.39
backbone Cα	53.9	0.31
Asn Cα	52.0	0.31
Pro Cδ	48.2	0.35
Gly Cα	42.9	0.33
Lys Cε + Arg Cδ	40.9	0.33
Asn Cβ + Asp Cβ	39.7	0.33
Phe Cα + Ile Cβ	36.6	0.27
Glu Cγ	33.8	0.22
Gln Cγ	31.4	0.17
Pro Cβ + Val Cβ	29.7	0.17
Arg Cβ + Glu Cβ	27.9	0.17
Gln Cβ + Lys Cδ	26.9	0.17
Leu Cγ + Arg Cγ + Pro Cγ + Ile Cγ1	24.8	0.17
Lys Cγ	22.4	0.20
Leu 2Cδ	21.3	0.31
Thr Cγ + Val Cγ1&2	19.0	0.54
Ile Cγ + Met Cε	14.5	0.51
Ile Cσ	10.5	0.76
acetonitrile	1.3	10.30

[a] From the spectra used in the T_1 calculations. Does not necessarily agree with Table I. [b] From Table I.

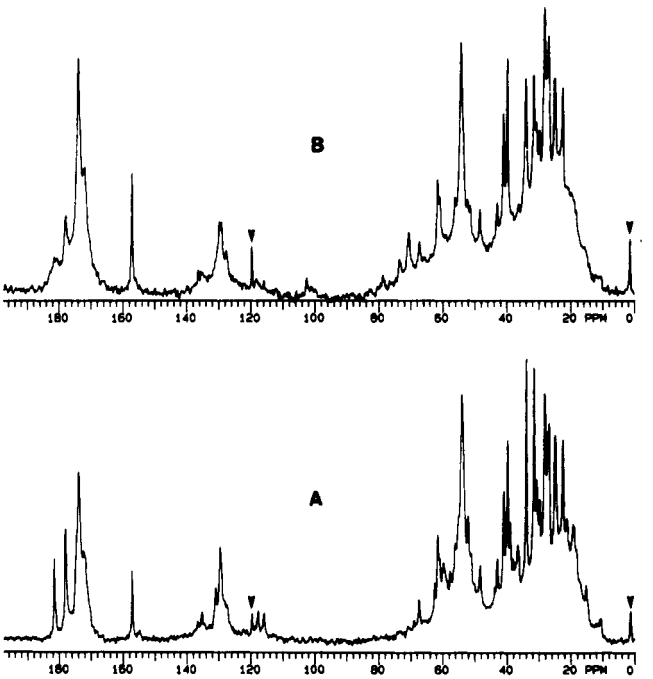

Figure 1. Normal ^{13}C NMR spectra of soy globulins obtained at 25 °C in 0.035 M phosphate buffer, pH 7.0, containing 0.4 M NaCl. Recycle time 0.6 s, 192 000 transients. (A) Glycinin; (B) β-conglycinin. Arrows denote peaks from the internal standard.

mined for many of these peaks in glycinin (Table III). It was found that previous assignments (Kakalis and Baianu, 1985) of peaks were in error by as much as 5 ppm. Due to low concentrations of tryptophan in glycinin (Table I), only a few peaks from it could be assigned (Table II); the even lower concentration in β-conglycinin precluded assignment of any tryptophan peaks. Similar results were observed for methionine and cysteine/cystine. The peak Kakalis and Baianu (1985; Baianu, 1989) report at 160–63 ppm does not appear in the spectra in this study (Figures 1–3). However, peaks at 157 and 155 ppm have been identified as arising from the guanidino carbon (Cζ) of arginine and ζ-carbon of tyrosine, respectively. APT experiments (Figure 3) were instrumental in differentiating between methyl, methylene, and methine carbons in the

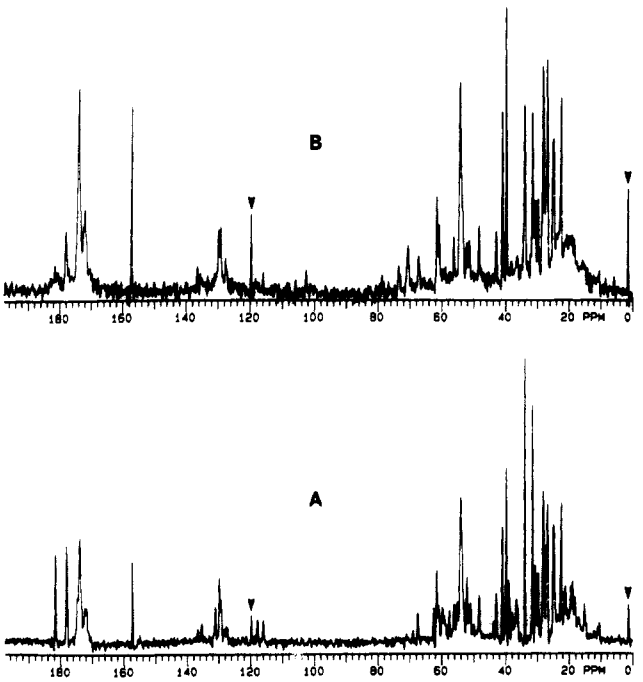

Figure 2. Convolution difference spectra of soy globulins from Figure 1, weighted with the function $(1 - (0.8 \exp(-t/0.08)))$. Obtained at 25 °C in 0.035 M phosphate buffer, pH 7.0, containing 0.4 M NaCl. Recycle time 0.6 s, 192 000 transients. (A) Glycinin; (B) β-conglycinin. Arrows denote peaks from the internal standard.

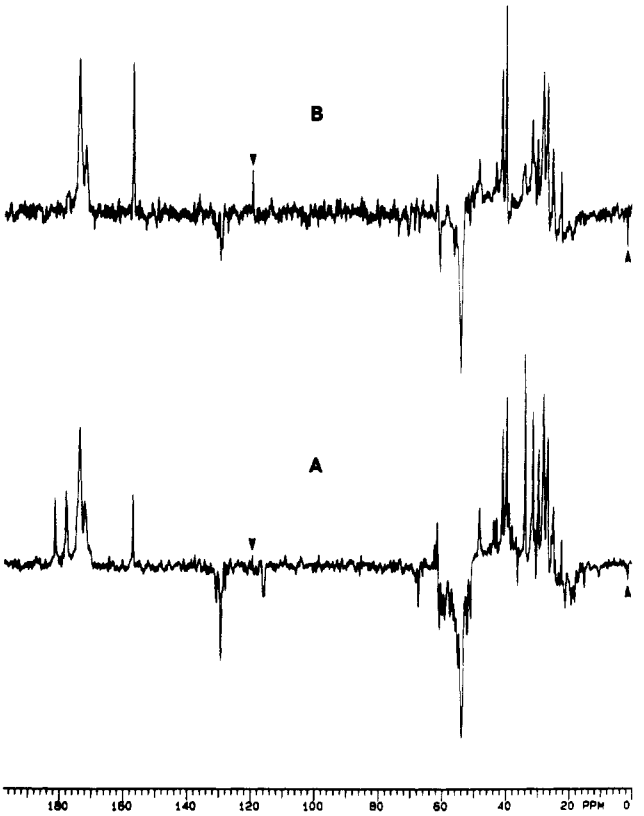

Figure 3. *J*-modulated double-spin-echo (APT; Patt and Shoolery, 1982) spectra of soy globulins obtained at 25 °C in 0.035 M phosphate buffer, pH 7.0, containing 0.4 M NaCl. Recycle time 0.6 s, 192 000 transients. (A) Glycinin; (B) β-conglycinin. Arrows denote peaks from the internal standard. Upward peaks are from methylene and quaternary carbons; downward peaks denote methyl and methine carbons.

10–100 ppm region. In particular, the Cβ carbons of valine and isoleucine were assigned as the only CH peaks between 30 and 40 ppm (see Table II).

This predominance of sharp peaks from a large number of amino acids in glycinin and β-conglycinin (Figure 1) contrasts with spectra obtained for proteins from corn (Augustine and Baianu, 1986), wheat (Baianu et al., 1982), and barley (Tatham et al., 1985) whose primary structures are dominated by only a few amino acids. The broader distribution of different amino acids in glycinin and β-conglycinin (Table II; Nielsen, 1985b) necessitated long data acquisitions in order to detect and identify minor components. Long data accumulation times also made the APT sequence the method of choice to determine peak multiplicity. APT is not dependent on polarization transfer, which can cause base-line distortions and requires T_1-related delays between pulses, which lengthen recycle and data acquisition times. Also unlike polarization transfer techniques, APT does not require an initial 90° pulse, which causes emphasis of regions with short T_1's and deemphasis of regions with long T_1's. Convolution difference weighting, as in Figure 2, accomplishes this task, emphasizing narrow peaks and suppressing broad ones with minimal loss of signal to noise. It can be applied to APT spectra to help in differentiating between close peaks. APT is not limited by decoupler strength or frequency, and T_2 line broadening is reduced with composite 180° pulses.

The ^{13}C NMR spectra of glycinin and β-conglycinin (Figure 1) show areas (envelopes) where the apparent base line deviates significantly from the true spectral base line. These areas contain large numbers of overlapping peaks whose slight differences in chemical shift are due to their location in structured regions (whether helical, β-structure, or random/disordered) within the proteins. This effect is most easily seen in the aliphatic region (10–40 ppm) of β-conglycinin (Figure 1). Structured regions also cause lines to be broadened by spin–spin relaxation (T_2) effects. The T_1 and T_2 effects are the result of restrictions on side-chain and backbone motion imposed by the internal and external interactions referred to collectively as "structure". Under conditions of large amounts of structure, bandwidths become directly related to protein tumbling times in solution (τ_r) rather than side-chain/segmental motions (τ_C). For large proteins such as glycinin and β-conglycinin, the result is broad peaks. Thus, the regions within the protein that are highly structured yield spectra that are difficult to assign, even using pulse sequences such as APT, leaving qualitative gross structural information available from the apparent envelope areas. Conversely, more poorly structured regions, which yield sharp, easily assignable peaks, provide microstructural and compositional information on the differences and similarities between glycinin and β-conglycinin, but only limited amounts of conformational information.

In Figure 1, the aliphatic (10–45 ppm), α-carbon (50–60 ppm), and backbone carbonyl (165–175 ppm) envelopes are all visibly larger for β-conglycinin than for glycinin, denoting regions involving these side chains that are more structured and motion-restricted in β-conglycinin than in glycinin. This can also be seen in the large number of β-conglycinin peak bandwidths (Table II) that are unreliable due to the broadness of the peak and inability of the instrument to find an accurate base line. Convolution difference weighting (Figure 2) provided only incomplete emphasis of these peaks, especially in the aliphatic region of β-conglycinin, emphasizing how wide these lines are. In these regions, peak assignments were only possible through use of the APT pulse sequence (Figure 3).

The APT sequence also gave data sufficient to discrim-

inate between close peaks with differing numbers of hydrogens (Figure 3). Thus, the Ser Cβ CH_2 at 61.4 ppm (Table II) could be differentiated from the Pro Cα CH at 60.9 ppm. Similarly, the two CH's from Ile Cβ and Val Cβ (36.9 and 30.7 ppm, respectively, Table II) can be separated from the surrounding CH_2's, particularly in glycinin (Figure 3A). In addition, the Trp Cε CH at 118 ppm can be seen clearly in glycinin, and not in β-conglycinin, reflecting amino acid composition differences between the two proteins (Table I).

The most noticeable similarity between glycinin and β-conglycinin spectra (Figure 1) is the narrowness (Table II) of the dominant upfield (21–62 ppm) peaks and the backbone carbonyl envelope (174 ppm), emphasized in the convolution difference spectra in Figure 2. The sharpness of these peaks and the similarity of their chemical shifts to those of free amino acids are reflections of the large regions of disordered structure in the polypeptide backbone and evidence of its flexibility. These dominant peaks have been identified as arising from the side-chain carbons of glutamine, glutamate, arginine, and lysine (Table II). The flexibility of the protein chain is underscored by the strong, comparatively narrow peaks encompassing the backbone α-carbons and carbonyls around 54 and 174 ppm, respectively (Figures 1 and 2) and by T_1 values (Table III) intermediate between those observed for free amino acids (Wüthrich, 1976) and highly motion constrained amino acid residues (Komoroski et al., 1976; Jardetzky and Roberts, 1981). This can also be seen in the amount of fine structure around 54 and 174 ppm that emerges in convolution difference weighted spectra (Figure 2). The similarities in these regions of the two proteins are such that the general conclusions drawn from the spin–lattice relaxation (T_1) measurements (Table III) for these peaks in glycinin are applicable to β-conglycinin.

There are fewer emphasized peaks around the primary Cα peak (54 ppm) in the convolution difference spectrum of β-conglycinin (Figure 2B) than in the same region of the glycinin spectrum (Figure 2A). The β-conglycinin peaks between 10 and 22 ppm are poorly resolved even after convolution difference weighting. These two observations point to fewer mobile α-carbons in β-conglycinin and indicate not only a higher proportion of structured regions in β-conglycinin but, more importantly, a higher proportion of flexible regions in glycinin. This goes beyond the spectroscopic observations (Dev et al., 1988; Ishino and Kudo, 1980; Yamauchi et al., 1979; Koshiyama and Fukushima, 1973) of the amounts of helical, β-structure, and random coil in purified soy storage proteins; these methods are unable to distinguish flexible regions.

Of particular note in the spectra of both glycinin and β-conglycinin (Figure 1) is the narrow, solitary peak at 157 ppm from the ζ-carbon of arginine (Table II). Its narrowness (≈20 Hz), solitary nature, and height suggest both exposure to solvent and location in nonrigid regions of both proteins. This is supported by its T_1 (Table III) between the T_1's anticipated for the free amino acid and a motion-constrained residue (Levy et al., 1980; Komoroski et al., 1976; Jardetzky and Roberts, 1981). Side-chain flexibility is also seen in the narrow line widths of the remaining arginine side-chain peaks at 25, 28, and 41 ppm (Figure 1; Table II).

The δ-carbon of glutamate in β-conglycinin (Figure 1B, 182 ppm) is sharply different from the δ-carbon of glutamate in glycinin (Figure 1A, 182 ppm). The δ-carboxyl region in β-conglycinin is unintense and broad (Table II). It remains so after convolution difference weighting (Figure 2), losing intensity and gaining noise. The most likely explanation is that the carboxyl is involved in inter- or intrachain hydrogen bonding at several sites. Furthermore, the β- and γ-carbon peaks from the glutamate side chain (at 28 and 34 ppm) are wider in β-conglycinin than the equivalent peaks in glycinin (see Table II). This is consistent with both the carboxyl group and the side chain being partially motion constrained in β-conglycinin. Glutamine (178 ppm) shows similar effects (Table II). None of these effects are seen for glutamate, aspartate, or glutamine in glycinin. This is particularly important structurally, as glutamate and glutamine are understood to prefer α-helical regions (Levitt, 1978) and not to be buried in proteins (Chothia, 1976).

It can be inferred that glutamate residues contribute to helical and other structure in β-conglycinin, probably via hydrogen bonding to the carbohydrate moiety and/or ionic interactions with positively charged side chains. While peaks from carbons near the positive charge of deprotonated histidine are inseparable from neighboring peaks (see His^+ Cε, 128.5 ppm, Table II) or too low and/or broad (see His^+ Cδ, 118.9 ppm, Table II), the Cε of lysine (41.0 ppm, Table II) appears to be as mobile or more so than its counterpart in glycinin. Similarly, the Cζ and Cδ peaks of arginine (157.1 and 41.0 ppm, Table II) are narrow and well-defined as is the Cβ peak from serine (61.5 ppm). Thus, the interaction of glutamate with positively charged residues is speculative at best, with histidine being the only apparent possibility.

The presence of a carbohydrate side chain in β-conglycinin is readily observed from the peaks between 70 and 110 ppm in Figure 1B (see also Table II). Both carbohydrate and amino acid carbons contribute to peaks between 60 and 70 ppm, as can be seen (Table II) by the presence of these peaks in glycinin. The extension of the α-carbon envelope of β-conglycinin (centered at 54 ppm) to include the carbohydrate peaks (Figure 1, 65–79 ppm; Table II) does indicate that the carbohydrate side chain is locally motion-constrained, consistent with involvement in secondary structure, and makes it the most likely site for interaction with glutamate. It should be noted that β-conglycinin is 5% carbohydrate, consisting of seven to nine mannose residues and two *N*-acetylglucosamines (Yamauchi and Yamagishi, 1979; Koshiyama, 1968), each with two to four hydroxyl groups available for hydrogen bonding. The absence of carbohydrate from glycinin may therefore explain its observed larger proportion of flexible regions and, in part, its larger proportion of disordered structure.

It is obvious from the large aliphatic and aromatic peak envelopes observed for β-conglycinin (Figure 1B) that glutamate is not the sole contributor to the structurally ordered regions of β-conglycinin. The large shoulder on this envelope (15–≈20 ppm), broadness of the peak at 10.5 ppm, and comparison with the equivalent region of glycinin (Figure 1A) make it apparent that alanine, valine, isoleucine, and leucine are also involved. In glycinin, the Arg Cζ peak is accompanied by a small, single peak at 155 ppm for the Tyr Cζ, with other Tyr peaks readily assignable in the aromatic region (120–140 ppm). In β-conglycinin these peaks are either difficult to discern or, as in the case of the Cζ peak, absent (Figure 1B). Since the content of tyrosine in these two proteins is very similar (Table I), this points to the presence of Tyr in motion-constrained regions, most likely in the protein interior. The poorly resolved aromatic peaks around 130 ppm, plus the presence of a base-line-distorting peak envelope and

absence of identifiable tyrosine Cζ peak, imply more involvement of tyrosine and phenylalanine in the structure of β-conglycinin than in that of glycinin (Figure 1A).

From the peaks at 61, 48, 30, and 25 ppm in glycinin and β-conglycinin (Figure 1; Table II), it appears that proline (5–6% of the total amino acid content) occurs in the trans configuration in both proteins (Howarth and Lilley, 1978; Wüthrich, 1976). This may be important to understand the high degree of random-coil structure within these proteins, as proline tends to occur in reverse turns and breaks up α-helical regions (Levitt, 1978). It is also not usually buried in the interior of proteins (Chothia, 1976). This point is supported by Dev et al. (1988) who interpreted their FT-IR results in terms of large amounts of β-sheet, β-turn, and disordered structure.

SUMMARY

Describing glycinin and β-conglycinin strictly in terms of percentage of structured regions is insufficient. Carbon-13 NMR shows that these two proteins differ sharply in their relative amounts of flexible regions, with glycinin having the higher proportion. The involvement of specific amino acids in both proteins and the carbohydrate moiety of β-conglycinin in protein structure can be seen. It seems likely that these differences contribute to differences in other physicochemical properties of these two proteins.

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Carbon-13 NMR Studies of the Effects of Gelation and Heat and Chemical Denaturation at Neutral pH on Soy Glycinin and β-Conglycinin

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Carbon-13 NMR studies have been carried out on soy glycinin and β-conglycinin under conditions of gelation and heat and chemical denaturation in pH 7.0 aqueous solutions containing 0.035 M phosphate and 0.4 M NaCl. With 6 M urea as a chemical denaturant, major changes were seen in the appearance of the aliphatic and aromatic carbon regions of the protein spectra, particularly in that of β-conglycinin. However, carbon chemical shifts underwent little or no change during denaturation, confirming that the sharp peaks observed in the native proteins were largely from mobile groups in random regions. As the temperature of the protein solutions was increased, many additional peaks were observed, especially in β-conglycinin, and existing peaks in both proteins became sharper, consistent with increased molecular motion and protein unfolding. Upon gelation, both protein gels gave spectra consistent with involvement of aliphatic and aromatic amino acids in gel structure. The β-conglycinin gel also showed evidence of the involvement of the carbohydrate and glutamate side chains in gel structure. The data support the concept that gelation of both proteins is a four-step process involving (1) unfolding (different from denaturation), (2) aggregation, (3) strand formation, and (4) strand ordering/gelation.

Although ^{13}C NMR has been used to study a number of food proteins (Baianu, 1989), some at elevated temperature (Belton et al., 1987; Baianu et al., 1982), the effects of heat and chemical denaturation and gel formation of purified soy proteins has hitherto not been studied by NMR. Hermansson (1978) and Koshiyama et al. (1981) have reported the denaturation temperature for soy 7S and 11S proteins and studied turbidity changes during the denaturation-gelation process. Suresh Chandra et al. (1984) used circular dichroism and optical rotatory dispersion to determine the effects of temperature on the secondary and tertiary structures of glycinin. They found no change in the native structure over the temperature range 15-60 °C. However, in the presence of urea or guanidine hydrochloride denaturant, a disordered structure was observed at 15 °C that appeared to become more ordered at higher temperatures. The highest temperature used by Suresh Chandra et al. (1984) was 60 °C, below the denaturation temperature for both glycinin and β-conglycinin (Hermansson, 1978). Dev et al. (1988) have studied changes in glycinin conformation upon urea and heat denaturation with FT-IR.

Both glycinin and β-conglycinin can form self-supporting gels upon heating. When glycinin is heated to 95 °C in the presence of sodium chloride, the gel structure is dominated by ordered strands rather than disordered aggregates; β-conglycinin produces a somewhat less ordered gel matrix after being heated to 85 °C (Hermansson, 1986). Gel formation occurs at concentrations above 2.5% and 7.5% for glycinin and β-conglycinin, respectively (Nakamura et al., 1986a). To date, structural studies of soy storage protein gels have used electron microscopy (Hermansson, 1985, 1986; Mori et al., 1986), electrophoresis, gel immunodiffusion, and other methods (Utsumi and Kinsella, 1985; Nakamura et al., 1986a,b). The methods used by these workers precluded actual observation of protein behavior at particular temperatures. Their methods supply only limited information on specific types of amino acids that may be involved in maintaining gel structure and are limited in their inability to observe pro-