26-9; hexyl 2-methylpropanoate, 2349-07-7; 2-methylpropyl hexanoate, 105-79-3; 2-methylbutyl pentanoate, 55590-83-5; 1(*E*,*Z*),3,5-undecatriene, 19883-27-3; ethyl (*E*)-4-octenoate, 78989-37-4; (*Z*)-3-hexenyl butanoate, 16491-36-4; butyl hexanoate, 626-82-4; hexyl butanoate, 2639-63-6; ethyl octanoate, 106-32-1; β-cyclocitral, 432-25-7; dodecane, 112-40-3; γ-octalactone, 104-50-7; (*Z*)-3-hexenyl 2-methylbutanoate, 53398-85-9; hexyl 2-methylbutanoate, 10032-15-2; 3-methylbutyl hexanoate, 2198-61-0; 2-methylbutyl hexanoate, 24551-95-9; pentyl hexanoate, 540-07-8; propyl octanoate, 624-13-5; tridecane, 629-50-5; 2-methylpropyl octanoate, 5461-06-3; ethyl (*Z*)-4-decenoate, 7367-84-2; hexyl hexanoate, 6378-65-0; butyl octanoate, 589-75-3; ethyl decanoate, 110-38-3; dihydro-β-ionone, 17283-81-7; γ-decalactone, 706-14-9; δ-decalactone, 705-86-2; dihydroactinidiolide, 17092-92-1; pentadecane, 629-62-9; hexyl benzoate, 6789-88-4; hexyl octanoate, 1117-55-1; butyl decanoate, 30673-36-0; ethyl dodecanoate, 106-33-2; tetradecanal, 124-25-4; γ-dodecalactone, 2305-05-7; hexadecanal, 629-80-1; ethyl hexadecanoate, 628-97-7; 2-hexanone, 591-78-6; 3-hexanol, 623-37-0; 2-hexanol, 626-93-7; (*E*)-2-hexenal, 6728-26-3; (*Z*)-3-hexenol, 928-96-1; (*E*)-2-hexenol, 928-95-0; benzaldehyde, 100-52-7; (*Z*)-2-heptanal, 57266-86-1; 6-methyl-5-hepten-2-one, 110-93-0; (*E*)-2-hexenyl acetate, 2497-18-9; phenylacetaldehyde, 122-78-1; 2,2,6-trimethylcyclohexanone, 2408-37-9; 3-nonen-2-one, 14309-57-0; (*E*)-2-nonenal, 18829-56-6; α-terpineol, 10482-56-1; nerol, 106-25-2; geraniol, 106-24-1; (*E*,*E*)-2,4-decadienal, 25152-84-5; 3,4-didehydro-β-ionol, 3293-47-8; geranylacetone, 3796-70-1; epoxy-β-ionone, 36340-49-5; β-ionone, 79-77-6.

Determination of the Protein Activity of Corn Zeins in Alkaline Solutions from ^{1}H Nuclear Spin Relaxation Data as a Function of Concentration and Heat Treatments

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A marked nonlinear concentration dependence was observed for 10-MHz ^{1}H NMR transverse relaxation rates of corn zeins in solution at pH 11.5 for both unheated and heat-treated solutions that were cooled to room temperature. Simplex and Gauss–Newton nonlinear regression analyses of the NMR transverse relaxation data were employed to calculate the average virial expansion coefficients of protein activity. Two different virial expansions were found to fit the entire concentration range of the NMR data. The first model (A) contains three virial coefficients, B_0, $B_{3/2}$, and B_2, for the c_p, $c_p^{3/2}$, and c_p^2 terms, respectively, where c_p is corn protein concentration up to 80% (w/w). The second model (B) included four virial coefficients, B_0, B_2, B_3, and B_4, for the c_p, c_p^2, c_p^3, and c_p^4 terms, respectively, with slightly improved standard deviations over the first model. In the lower concentration range, up to 30%, an improved fit was obtained with only one virial coefficient, $B_0 = 2.46 \pm 0.18$ mL/g, which is significantly lower than the value of 6.9 mL/g obtained from the best fit over the entire concentration range with model B. All three models yielded a decrease in the *average* protein activities and the average transverse relaxation rate ($1/T_2$) of bound water after heat treatments. Since the heated samples were measured at room temperature after cooking, the changes in the average virial coefficients reflect *irreversible* protein conformational changes induced by heating. The alternating signs of the virial coefficients in both models indicate the presence of both repulsive and attractive interactions among corn zeins. A plot of the ratio T_2/T_1 as a function of corn protein concentration indicates that cross-relaxation is much less significant than chemical exchange for these samples. The decreasing T_2/T_1 ratio with increasing protein concentration suggests the presence of certain, relatively slow, water motions that are detected by the T_2 relaxation measurements and not by T_1. For this reason, the T_2 relaxation dependence on concentration is much steeper than the T_1 relaxation dependence.

1. INTRODUCTION

With the current emphasis of corn wet-milling focused on the production of high-fructose corn syrups and corn starch, there is an increasing need to find uses for the corn gluten byproducts, particularly zein. This prolamine protein fraction of corn is currently used as a coating agent in pharmaceuticals and as animal feed due to the low "quality" of the product (Shroder and Heiman, 1970). In order to exploit this byproduct for its possible use in human foods, zein's physicochemical properties must be characterized so that technologists may predict and control its behavior during food processing. Nondestructive, useful techniques for conformation, composition, and hydration analyses of proteins are provided by nuclear magnetic resonance (NMR) relaxation and spectroscopy. High-resolution NMR and relaxation techniques have already been used to study other food systems such as wheat (Baianu et al., 1982), corn (Augustine and Baianu, 1987), and soy proteins (Kakalis and Baianu, 1989).

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Much of the previous work done to characterize corn zeins involved standard biochemical techniques, and most investigations were carried out only on zeins extracted in the laboratory from corn kernels (Neumann et al., 1984; Wilson, 1986); there has been very little work done on the commercially available zeins (Wilson, 1986). There are also two recent NMR studies on zeins from Colorcon Co. (Augustine and Baianu, 1986, 1987). As shown previously, because of the markedly hydrophobic character of corn zeins, adequate solubilization is only obtained either in organic solvent mixtures or at high pH (>11.2), and significant fractions of structured regions are retained in these proteins under such conditions (Augustine and Baianu, 1986, 1987). It is, therefore, difficult at present to estimate directly the degree of corn protein denaturation caused by the solvents that are currently used in corn zein studies. Because of the wide distribution of chemical shift anisotropies, the solid-state ^{13}C NMR (cross-polarization, magic-angle spinning) spectra of most cereal proteins have very limited resolution; such limitations do not allow an estimate of the degree of solvent-induced denaturation to be made directly (Baianu, 1989). The solution ^{13}C NMR spectra of corn zeins do indicate, however, the presence of structured domains in corn zeins (Baianu, 1989) in alkaline solutions (pD 11.5). The degree of structuring present in alkaline solutions of zein is similar to that in organic solvent mixtures routinely used in commercial zein applications and in biochemical analysis.

The scope of our study is to determine quantitatively by ^1H NMR relaxation techniques the extent of protein–protein and protein–solvent interactions for commercially available zeins in aqueous solutions at pH 11.5. We also aim to determine the effect(s) of heat treatments on such interactions in relation to heat processing of proteins from corn.

2. THEORY

According to the two-"state" model with fast exchange of Zimmerman and Brittin (1957), the observed NMR relaxation rate of an ideal solution is a weighted average of the bound (R_B) and free (R_F) relaxation rates of these two water populations; if there are no additional contributions present to relaxation (e.g., from cross-relaxation and paramagnetic relaxation), then

$$R_{obs} = P_B R_B + P_F R_F \quad (1)$$

where P_B and P_F represent the fractions of "bound" and free water, respectively. Derbyshire (1982) proposed that a deviation from linearity of the dependence of NMR relaxation data on concentration could be explained as a change in the hydration of the macromolecule or as a change in the relaxation rate of the bound water. This simple two-state model with fast exchange is a useful approximation, but several other processes that contribute to nuclear spin relaxation need to be considered.

Because proteins are polyampholytes, there are several types of interactions present between proteins in solution; such interactions are caused by long-range electrostatic interactions, charge fluctuations (in the absence of salt), dipole moments, multipoles, and fluctuations in the distribution of the protein protons (changes in both the number and the configuration of protons; Kirkwood and Shumaker, 1952). These interactions will lead to nonideal behavior of protein solutions, even at very low concentrations (Doty and Steiner, 1952; Kronman and Timasheff, 1959). Therefore, a model that includes such protein–protein and protein–solvent interactions (Kirkwood and Shumaker, 1952) present in nonideal solutions is more realistic and can account quantitatively for the nonlinear concentration dependence of the NMR relaxation rates (Kumosinski and Pessen, 1982). Such a model takes into account the chemical activity of the protein

$$a_p = \gamma_p c_p \quad (2)$$

where a_p is the protein activity, γ_p is the activity coefficient, and c_p is the protein concentration. The activity coefficient can be expressed through a virial expansion of the protein concentration

$$d \ln \gamma_p / dc_p = 2B_0 + 3B_2 c_p + ... \quad (3)$$

where the B_i quantities are the virial coefficients of the protein activity. Some of the protein activity coefficients can be determined with this model from light-scattering measurements (Kumosinski and Timasheff, 1966) for very dilute, clear solutions. However, a more complete set of virial coefficients can be obtained from the dependence of the NMR relaxation rates on protein concentration/hydration level. When this virial expansion is applied in conjunction with the two-state model with fast exchange, two possible equations result for single species proteins

$$R_{obs} - R_F = n_H(R_B - R_F) \times c_p \times \exp[2B_0 c_p + 0.667 B_{3/2} c_p^{3/2} + 1.5 B_2 c_p^2 + ...] \quad (4)$$

$$R_{obs} - R_F = n_H(R_B - R_F) \times c_p \times \exp[2B_0 c_p + 1.5 B_2 c_p^2 + B_3 c_p^3 + B_4 c_p^4 + ...] \quad (5)$$

where n_H is the total hydration (Pessen and Kumosinski, 1985; Asbi and Baianu, 1986) and all other variables are as defined above. The B_0 virial coefficient reflects various repulsions related to the *average* net charge on the proteins ($\bar{Z}$), a preferential binding term (β_{22}) and a contribution from the protein-excluded volume ($\bar{v}_p$) (Kumosinski et al., 1987)

$$2B_0 = (\bar{Z})^2/(4m_s M_p) + \bar{v}_p/1000 - (\partial g_s/\partial g_p)^2 \times 1/m_s \quad (6)$$

where m_s is the molarity of NaOH in solution, $\bar{v}_p$ is the average of the partial specific volumes of zeins, and $\beta_{22} = (\partial g_s/\partial g_p)^2 \times 1/m_s$ is the preferential interaction or binding term; this β_{22} term must be included in order to take into account the preferential interactions of Na^+, OH^-, and water at the protein interface (Kumosinski et al, 1987; Arakawa and Timasheff, 1982). In cases where the NMR relaxation rates are linearly dependent on the protein concentration, the protein activity coefficient does not differ significantly from unity. Also, the charge to mass ratios of the protein components are small, resulting in a low B_0 virial coefficient (Pessen and Kumosinski, 1985).

The B_2 virial coefficient represents attractive forces caused by higher fluctuating multipoles that modulate protein–protein interactions. Under salt-free, "isoionic" conditions, it has been shown that the protein light-scattering data exhibit a square root ($c_p^{1/2}$) dependence on the protein concentration (Timasheff et al., 1957; Kronman and Timasheff, 1959). In the latter case, another virial coefficient, $B_{1/2}$, would need to be added to the expansion in order to fit the data. The $B_{1/2}$ virial coefficient of the $c_p^{1/2}$ term is caused by charge fluctuations of residues at the protein surface. Furthermore, charge fluctuations become dominant in the dilute concentration range, *in the absence of salt* or other ionizable species. Addition of salt or base to the solution results in a charge-screening effect that practically eliminates the $B_{1/2}$ term (Kirkwood and Shumaker, 1952; Timasheff et al., 1957). Doty and Steiner (1952) proposed a different model based on the virial coefficients of a Boltzmann expansion (eq 20 in their article) that appeared to fit the light-scattering data for bovine serum albumin (BSA) but suffers from ignoring protein ionization and preferential binding of salts (Timasheff et al., 1957; Arakawa and Timasheff, 1982; Kumosinski and Pessen, 1982).

Kumosinski and Pessen (1982) [also Pessen and Kumosinski (1985)] have applied this *protein activity* model to the analysis of the NMR relaxation data for β-lactoglobulin A in the concentration range 0–12%. They observed a nonlinear dependence of R_2 and R_1 values on protein concentrations. When the calculated protein activities were used instead of concentrations, a linear dependence of the relaxation rates on activity was obtained. Similar results were recently obtained for caseins in D_2O at 15 °C by employing low-field ^1H NMR relaxation

measurements (Kumosinski et al., 1987).

In the case of protein mixtures, such as corn zeins and soy proteins, the fast-exchange process yields equations of the same apparent form as eq 4 and 5. In this case, however, the relaxation and hydration parameters, as well as the apparent virial coefficients, are *weighted averages* over the protein species present in the sample (e.g., $\bar{B}_0$). In the case of corn zeins, the molecular weight distribution appears to include three major components at ~22 000, 44 000, and 62 000, with the latter component dominating the relaxation. The isoelectric points distribution of corn zeins (Myers-Betts and Baianu, unpublished results) as determined by isoelectric focusing (Wilson, 1986) included about 20 zein components; at pH 11.5, however, all components are expected to have similar, net negative charges, therefore simplifying the virial coefficient analysis.

3. MATERIALS AND METHODS

3.1. Materials. The corn zein used in our experiments was quoted as a "special"-grade zein by Freeman Industries, Tuckahoe, NY (Lot No. F5000515C). The moisture content was 6.9% as determined by oven drying at 60 °C for 24 h. Aqueous protein samples were prepared by mixing the appropriate amount of zein in H_2O at pH 10.0 and then adjusting to pH 11.5 by adding small volumes of 6 N NaOH in 20-μL increments. All samples were prepared on ice and were a clear, transparent yellow with no cloudiness. pH measurements of solutions were determined with a pH meter (Hanna Instruments, HI8417) connected to a combination electrode (Fisher Scientific, No. 13-639-285) with a precision of ±0.01 pH unit and were reproducible at that level. Samples above approximately 30% (w/w) were very viscous and required the use of a combination spear-tip pH-electrode (Ross Co., No. 8163BN) to determine the pH. Heated aqueous samples were prepared by placing the sealed tubes in water baths held at 80 or 90 °C for 20 min. Samples were then rapidly chilled on ice.

3.2. NMR Measurements. ^{1}H NMR transverse (T_2) and longitudinal (T_1) relaxation measurements were made with a PC-10 NMR process analyzer (Bruker/IBM Instruments, Danbury, CT) at 10-MHz resonance frequency. The T_2 measurements were obtained with phase-sensitive detection by using the multipulse 90°–τ–180°–2τ–(ECHO) pulse sequence (CPMG) with a 90° phase shift for refocusing pulses (relative to the first 90° pulse) in order to avoid problems with diffusion of the magnetization (Carr and Purcell, 1954; Meiboom and Gill, 1958). The decay of the transverse magnetization was monitored with a 30-MHz bandwidth Tektronix storage oscilloscope (Model 5113, dual beam), and the train of spin echoes was digitized with an 8-bit A/D at 1-MHz ADC rate at the maximum amplitudes of the nuclear spin echoes. All measurements were made in triplicate, and the T_2 values thus obtained varied by less than 2%. The 90° pulse widths were ~2 μs, and the probe ringdown periods (spectrometer deadtime) were about 16 μs; the spectrometer bandwidth employed was 1 MHz. Static, magnetic field inhomogeneity corresponds to about 1.5 Hz, and measured T_1 values for distilled water were 3.2 ± 0.2 s.

The T_1 measurements were obtained with the inversion recovery pulse sequence (180°–τ–90°). Measurements were made in triplicate, and the T_1 values varied by less than 1% for most samples.

3.3. Microcomputer Analysis of Experimental Data. Our treatment of the NMR relaxation data employed Turbo Basic and Quick Basic nonlinear regression programs that utilize a Simplex algorithm (F-Curve, 1985). The programs were run on an IBM PC-AT microcomputer equipped with an 80287 mathematical coprocessor, a professional color graphics monitor, and an IBM Proprinter (IBM Co.). Iteration of the concentration dependence for the NMR data yielded the virial coefficients of the protein activity according to eq 4 and 5 with a minimization of the SD (standard deviation). The SD, which is the average deviation of the calculated curve from the data points, was calculated as follows:

$$\mathrm{SD} = \left[\sum (R_{2\,\mathrm{obs}} - R_{2\,\mathrm{calc}})^2/(\text{no. data pts} - \text{no. parameters})\right]^{1/2} \quad (7)$$

Additional calculations were made on a MODCOMP computer using a Gauss–Newton program (Kumosinski, 1987). The virial coefficients were virtually the same in value and accuracy as those determined on the microcomputer. Further statistical calculations were carried out on a 32-bit Macintosh II microcomputer equipped with a 68020 CPU and a 68881 (16-MHz) mathematical coprocessor using a quasi-Newton algorithm (Systat, v. 3.1) in order to obtain confidence intervals for the iterated parameters (which Simplex algorithms do *not* provide).

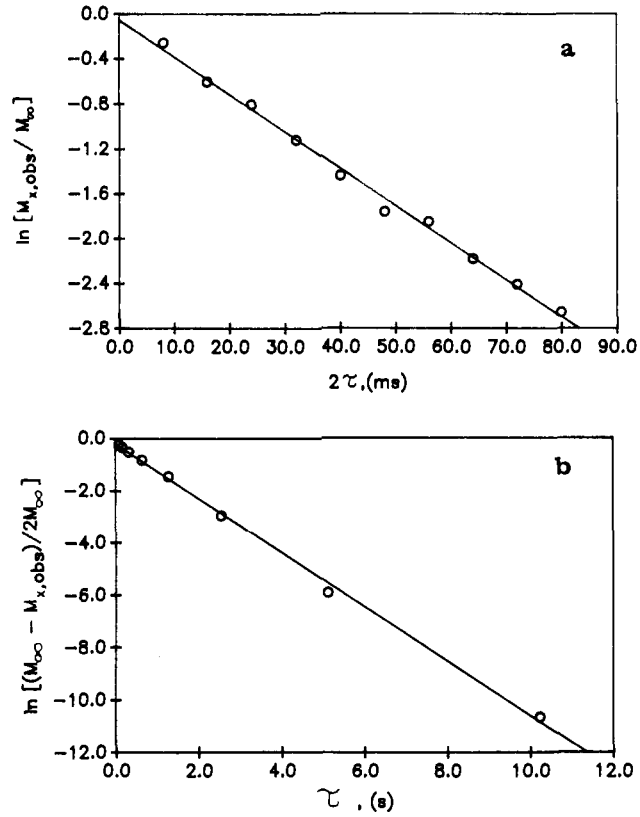

Figure 1. (a) log magnetization plot for determination of the ^{1}H NMR transverse relaxation rate, $1/T_2$, for a 15% (w/w) zein sample at pH 11.5. T_2 was obtained with the CPMG pulse sequence. $M_{x,\mathrm{obs}}$ is the observed magnetization at time 2τ, and M_∞ is the infinite value of the magnetization. (b) log magnetization plot for determination of the ^{1}H NMR longitudinal relaxation rate, $1/T_1$, for a 2% (w/w) zein sample at pH 11.5. T_1 was obtained with the inversion recovery pulse sequence. $M_{x,\mathrm{obs}}$ is the observed magnetization at time τ, and M_∞ is the infinite value of the magnetization.

4. RESULTS AND DISCUSSION

4.1. Dependence of ^{1}H NMR Relaxation Rates on Protein Concentration. Parts a and b of Figure 1 show logarithmic plots of the transverse and longitudinal magnetization time dependences, respectively. The lines in the logarithmic plots of Figure 1a,b correspond to single exponential decays of the transverse and longitudinal magnetizations as a function of time (Farrar and Becker, 1971) and are consistent with a fast exchange between bound and free water populations (Derbyshire, 1982). The slope of the line in Figure 1a is proportional to the T_2^{-1} value, while the slope of the line in Figure 1b is proportional to the T_1^{-1} value. Figure 2 shows the measured concentration dependence of the 10-MHz ^{1}H NMR transverse relaxation rates ($R_{2\,\mathrm{obs}} = 1/T_{2\,\mathrm{obs}}$) for heat-treated and unheated zein solutions at pH 11.5. Our ^{1}H NMR transverse relaxation data for zein solutions in H_2O at pH 11.5 (Figure 2) have a nonlinear dependence on concentration that becomes clearly noticeable above 8% (w/w). Zein samples seemed to gradually gel *without precipitation* above approximately 16–20%. For the heat-treated samples,

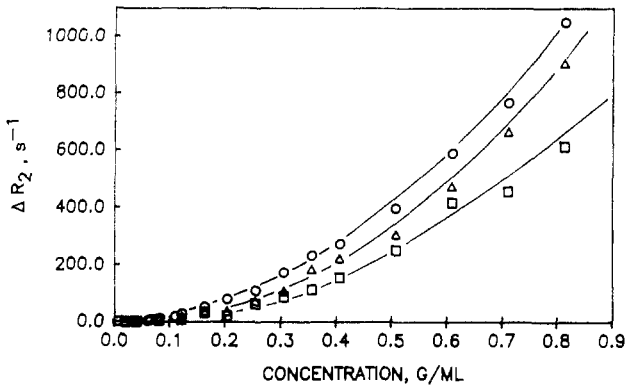

Figure 2. Concentration dependence of 10-MHz ^{1}H NMR transverse relaxation rates ($1/T_2$) for aqueous zein solutions at pH 11.5. T_2 values were obtained with the CPMG pulse sequence: ○, no heat treatment; △, 80 °C for 20 min; □, 90 °C for 20 min.

Table I. Calculated Virial Coefficients from Nonlinear Regression Analyses of ^{1}H NMR Relaxation Data for Zeins at pH 11.5 in the Concentration Range 0–80% (w/w)

parameter	unheated	80 °C for 20 min	90 °C for 20 min
A. Results from Equation 4 Using a Modified Gauss–Newton Algorithm			
$\bar{R}_{2B}$, s^{-1}	57.9 ± 11.5	25.2 ± 8.3	12.1 ± 5.4
$\bar{B}_0$, mL/g	9.86 ± 3.74	12.9 ± 5.6	13.5 ± 4.5
$\bar{B}_{3/2}$, $(mL/g)^{3/2}$	−48.2 ± 19.9	−64.7 ± 29.4	−63.1 ± 23.7
$\bar{B}_2$, mL^2/g^2	10.5 ± 4.4	14.3 ± 6.4	12.9 ± 5.1
SD	5.27	9.32	5.65
B. Results from Equation 5 Using Simplex[a] and Quasi-Newton Algorithms for Nonlinear Regression Analysis			
$\bar{n}_H(R_{2B} - R_{2F})$	62.62 ± 10.78	8.92 ± 3.51	9.86[a]
$\bar{B}_0$, mL/g	6.91 ± 0.76	11.75 ± 1.53	10.05
$\bar{B}_2$, mL^2/g^2	−20.55 ± 3.2	−34.12 ± 6.03	−27.8
$\bar{B}_3$, mL^3/g^3	32.70 ± 6.41	52.77 ± 11.69	40.74
$\bar{B}_4$, mL^4/g^4	−12.36 ± 3.05	−20.14 ± 5.49	−14.71
SD	3.52	5.32	5.66

[a] The Simplex algorithm does *not* calculate confidence intervals.

after cooling to 22 °C, the concentration dependence of $R_{2\,obs}$ appeared to be linear up to approximately 12% (w/w), and there was a general decrease in the values of the $R_{2\,obs}$ in comparison with the unheated samples measured at the same temperature. This general decrease in the R_2 relaxation rates, after the heat treatments, could be caused by conformational changes of zeins. This is likely to involve the partial unfolding of the protein, thus allowing for increased motions of the side chains and a corresponding increase in the mobility of the bound water. Previous ^{13}C and ^{15}N NMR heating studies on wheat gliadins have shown an increase in ^{13}C peak heights and narrowing of the resonances for glutamine and proline residues upon heating to 60 °C followed by cooling to 45 °C. This was attributed to irreversible conformational changes in the protein that increased side chain mobility (Baianu et al., 1982). Such conformational changes could be expected to occur in heated corn zein samples; however, to date, there are no reported studies of heated corn zeins by ^{13}C NMR.

4.2. Calculation of Virial Coefficients and Protein Acivities. Parts A and B of Table I contain the virial coefficients of the two virial expansions in eq 4 and 5 obtained by nonlinear regression analyses for unheated and heat-treated zein samples for the concentration range from 0 to 80%. It can be seen that there is a decrease in the relaxation rate of the bound water with either of the two virial expansions after the heat treatment of zein samples, assuming a hydration of 0.12 g of H_2O/g of dry protein (Bull, 1944; Asbi and Baianu, 1986); this behavior is also illustrated in Figure 2.

Model A (eq 4), which included only the B_0, $B_{3/2}$, and B_2 virial coefficients, yields a $\bar{B}_0$ (average) that increases slightly upon heating of the zein solutions. This could be caused by a small increase in the protein-excluded volumes due to a thermal denaturation and unfolding of the proteins. The $\bar{B}_{3/2}$ term in Table IA is negative and increases upon heating but is virtually the same for the two heat treatments at 80 and 90 °C. The $\bar{B}_2$ virial coefficient also increased upon heating but showed no significant difference between the two heat treatments. This model would therefore suggest that the interactions described by the higher virial coefficients (primarily the long-range fluctuating multipoles) contribute more to the protein activity in the samples that were heated, especially at the higher concentrations, in comparison with the unheated samples.

Model B includes four virial coefficients for the first to the fourth integer powers of protein concentration (eq 5). Again, $\bar{B}_0$ increases slightly with heating. The higher coefficients show a trend with heating that would also indicate that long-range interactions become stronger upon heating or there is an increased tendency toward aggregation. In accordance with general theory of electrolytes, the presence of alternating positive and negative signs of the coefficients in both models indicates the presence of both attractive (negative values) and repulsive interactions (positive values) among corn proteins. These results obtained with either model A or B disagree significantly with the Boltzmann expansion proposed by Doty and Steiner (1952), which has been cited as a "first approach" (Kronmann and Timasheff, 1959), due to a neglect of salt–macroion interactions and an implicit assumption of a small preferential ion binding term.

It should be emphasized that all virial coefficients listed in Table I represent average values over all protein species present in the samples. For heterogeneous protein systems with a very wide distribution of molecular weights (i.e., soy protein isolates), a different approach may be required. For example, thermodynamically linked functions have been successfully employed to describe the salt-induced solubility patterns of native and heat-denatured soy protein mixtures (Kumosinski, 1988) and protein aggregates or mixtures, in general (Wyman, 1964, 1984). Such an approach can also be applied to the analysis of the NMR data for corn zeins under aggregating conditions (lowest pH range or high concentrations, for example). However, since the molecular weight distribution of corn zeins is relatively narrow, the approach used here goes beyond a first approximation and should provide reasonably accurate values for the *average* parameters (in the sense of weighted averaging for the protein fractions present). For a detailed, mathematical derivation of the statistical averaging for protein mixtures, refer to Wyman (1984).

Neither model in part A or B of Table I could be fitted if a nonzero $\bar{B}_{1/2}$ virial coefficient (the charge fluctuation term) was included, presumably due to a screening-out effect from the NaOH that was required to solubilize the zeins at pH 11.5. Both models fit the NMR data and demonstrate the need to include virial coefficients higher than $\bar{B}_0$ even for moderately concentrated protein solutions (>30%).

Both models in Table I show low SD values that appear to be not significantly different from one another. In an attempt to determine which model is a better fit of the

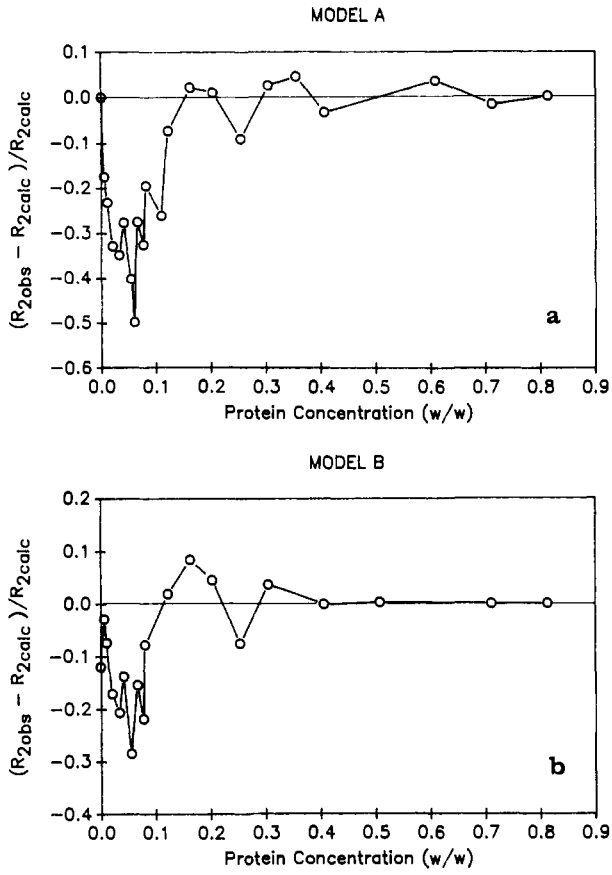

Figure 3. Residual plot for unheated alkaline solutions of corn zeins: (a) model A, SD = 5.27; (b) model B, SD = 3.52.

data, a simple "runs" test with accompanying residual plots was calculated for each set of data using each model separately (Motulsky and Ransnas, 1987). A "run" is a series of consecutive points with a residual of the same sign, positive or negative. Parts a and b of Figure 3 show residual plots for the unheated zein samples using models A and B from Table I, respectively. The heated samples gave similar results. The residual plot is a graphical representation of the $(R_{2\,obs} - R_{2\,calc})/R_{2\,calc}$ vs the c_p values. If the theoretical equation is appropriate for the data, the residuals will represent only experimental error and there should be no systematic relation to the c_p values; the plot will have a random arrangement of positive and negative residuals. Figure 3a, which represents the runs for model A ($\bar{B}_0$, $\bar{B}_{3/2}$, $\bar{B}_2$) appears to have a cluster of 13 points in the concentration range from 0 to 0.12 g/mL, indicating a systematic error of the fit in the dilute range. Figure 3b, which represents the runs for model B ($\bar{B}_0$, $\bar{B}_2$, $\bar{B}_3$, $\bar{B}_4$) contains a clustering of only 10 points in the concentration range from 0 to 0.10 g/mL. Both parts a and b of Figure 3 contain about eight runs in the concentration range from 0 to 0.80 g/mL, but model B resulted in somewhat closer fits above 30% (w/w). For these unheated samples, the SD for model A is 5.27 while the SD for model B is 3.52. From such statistical results, we cannot determine with certainty which model represents more closely the protein–protein interactions for zein in H_2O at pH 11.5 since the fitting differences are within experimental error. Note also that the B_0 virial coefficient values and the hydration parameters obtained for 90 °C heated zeins with either model A or B are within the confidence intervals and are, therefore, not significantly different.

In order to obtain a better understanding of the effects of heat treatments on the corn zein, the data were also

Table II. Results of Nonlinear Regression Analyses of ^1H NMR Relaxation Data for Corn Zeins at pH 11.5 in the Concentration Range 0–30% (w/w)

parameter	unheated	80 °C for 20 min	90 °C for 20 min
$n_H(\bar{R}_{2B} - R_{2F})$	127.97 ± 12.75	82.472 ± 2.30	49.345 ± 8.79
$\bar{B}_0$, mL/g	2.463 ± 0.178	2.380 ± 0.065	2.928 ± 0.32
SD	4.49	4.32	3.32

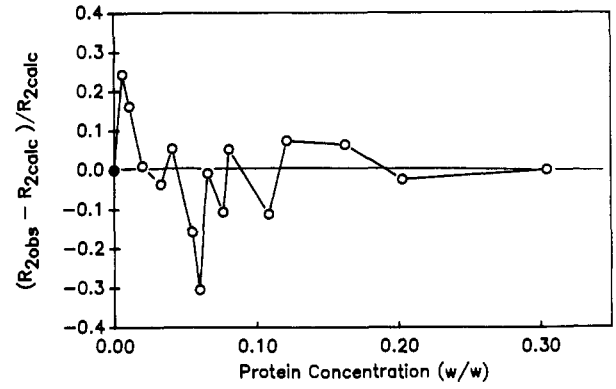

Figure 4. Residual plot for unheated alkaline solutions of corn zeins using the parameters listed in Table II.

evaluated by nonlinear regression in the lower concentration range, 0–30% (Table II). In this range, all three sets of data were found to require only the $\bar{B}_0$ virial coefficient, which is sensitive to the proteins' net average charge, average partial specific volume, and preferential binding of small ions. Table II also shows that the average hydration parameter, which includes the relaxation rate for bound water, decreases with the use of heat treatments whereas the $\bar{B}_0$ virial coefficient remains virtually unchanged. Figure 4 shows the residual plot for the unheated sample in the concentration range from 0 to 30% using only the $\bar{B}_0$ virial coefficient. In contrast to the fits summarized in parts A and B of Table I, this fit of the data does not contain any clustering of data points in the dilute range (0–12%) and contains nine runs in the concentration range from 0 to 30%. Since the $\bar{B}_0$ virial coefficient determines the curvature of the plot in the dilute range, the values listed in Table II are thought to more accurately represent the $\bar{B}_0$ virial coeoefficient than those listed in Table IA,B. The $\bar{B}_0$ values obtained for the concentration range 0–80% (Table I) are larger than those obtained for the concentration range 0–30% (Table II). The differences between these $\bar{B}_0$ estimates can be attributed to two likely effects that are now discussed. First, at higher protein concentrations, where closer packing of protein molecules occurs, increased electrostatic repulsions can be expected to be present resulting in a larger, more positive $\bar{B}_0$. Second, at higher protein concentrations, chemical exchange and cross-relaxation between protein protons and water protons may contribute significantly to the observed relaxation rate. This would result in an excessively large, apparent $\bar{B}_0$ containing a chemical exchange and, perhaps, also a cross-relaxation contribution (see also Discussion). In view of the fact that the residuals of both models in Table I appear to have a clustering of points in the dilute range, the virial coefficients obtained for the concentration range 0–30% (Table II) are thought to represent the repulsive protein–protein interactions more closely than the models for the concentration range 0–80%.

Protein activities were calculated with the *average* virial coefficients obtained by nonlinear regression analysis using eq 4. We found it convenient to scale the pro-

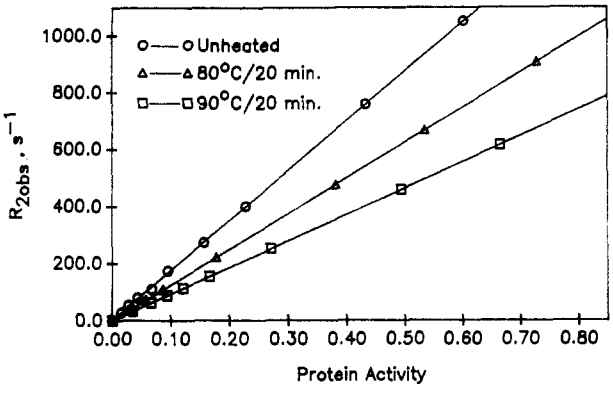

Figure 5. Dependence of the ^1H NMR transverse relaxation rates on protein activities for aqueous zein solutions at pH 11.5. Protein activities were calculated using eq 4 and the parameters in Table IB: ○, no heat treatment; △, 80 °C for 20 min; □, 90 °C for 20 min.

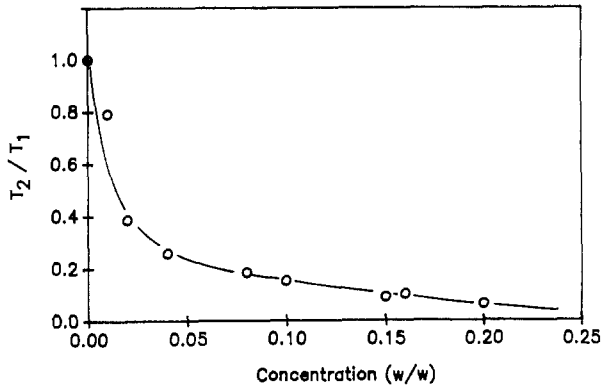

Figure 6. Concentration dependence of the T_2/T_1 ratio of NMR relaxation times for unheated aqueous corn zeins at pH 11.5.

tein activities so that $a_p = 1$ for $c_p = 1$. Figure 5 shows the NMR transverse relaxation data as a function of protein activity with the parameters in Table IB. The relationship is linear for all samples as predicted by the equations proposed by Kumosinski and Pessen (1982); in our case, such equations appear to hold even for concentrated samples, well above 16% (w/w). The decrease in slope in Figure 5 for the heat-treated samples reflects a decreased relaxation rate of the "bound" water and is consistent with a greater mobility of the corn zein side chains after heat treatments.

4.3. Calculation of the Average Net Charge of Corn Proteins. Using $\bar{B}_0 = 9.86$ mL/g obtained from Table IB, we were able to evaluate a net, average charge per protein molecule for the dominant fraction of average molecular weight $\bar{M}_p$ 62 000 (Augustine and Baianu, 1985) using eq 6. We calculated $\bar{v}_p/1000 = 2.3 \times 10^{-3}$ mL/mg for corn zeins of approximately MW 62 000 with a radius of 42 Å and $\beta_{22} = -2.3 \times 10^{-5}$ M^{-1} for 0.058 M NaOH added. With these values we obtained a net average charge of −15.8 esu for zein in H_2O at pH 11.5. This value was derived from the $\bar{B}_0$ virial coefficient obtained from the nonlinear regression analysis over the entire concentration range, 0–80%. However, when we used the values of the $\bar{B}_0$ virial coefficient obtained for the concentration range 0–30% ($\bar{B}_0 = 2.46$ mL/g), we obtained a net average charge of −6.1 esu. In view of the improved fit obtained for the concentration range of 0–30% and the possibility of protein aggregation at high concentrations, the value of −6.1 esu for the average net charge is considered to be a better estimate than the value obtained with the $\bar{B}_0$ for the entire concentration range.

5. DISCUSSION

Several investigators have fitted protein light-scattering data as a function of concentration and attempted to quantitate the extent of protein–protein and protein–solvent interactions in very dilute protein solutions (Doty and Steiner, 1952; Timasheff et al., 1957; Timasheff and Kronman, 1959; Kronman and Timasheff, 1959). However, studies at higher concentrations are not possible by light scattering because of intrinsic limitations of this technique. The theory should, however, apply even at higher concentrations by the appropriate addition of higher virial coefficients (Pessen and Kumosinski, 1985). Note that, in our set of virial coefficients (Table I) for corn zeins, the coefficients alternate in sign, with $\bar{B}_0$ being positive, in sharp disagreement with eq 20 of Doty and Steiner (1952); these observations are, however, in agreement with the Kirkwood–Shumaker–Timasheff–Kumosinski–Pessen (KSTKP) models of protein activity. The observed sign alternation of $\bar{B}_i$'s is caused by the presence of *both* repulsive and attractive interactions among corn proteins. In the concentration range up to 30%, the analysis with only the $\bar{B}_0$ coefficient agrees very well with the protein activity approach to NMR relaxation proposed by Kumosinski and Pessen (1982). We have shown also that the KSTKP models can be used to fit the NMR relaxation data with higher virial coefficients for concentrations up to 80%; however, systematic deviations of such models in the dilute concentration range (0–12%) may lead to anomalously large values of $\bar{B}_0$ if the higher order virial coefficients are included in the fit. Even so, we find it interesting and significant that the higher virial coefficients must be included, as predicted by the KSTKP theory, in order to be able to relate the concentration dependence of our NMR relaxation data for corn proteins to the protein activity at concentrations higher than approximately 30% where protein aggregation may occur. Piecewise regression analysis for the two concentration ranges, low and high, may result in an improved fitting of the high concentration range (>30%).

It should also be pointed out that ^1H NMR relaxation studies using 98% D_2O (data not shown) indicated only a small, or negligible, contribution of the cross-relaxation to the observed relaxation rates measured at 20 MHz for corn zeins. According to Kumosinski and Pessen (1989), the rate of magnetization transfer from water to protein protons is proportional to the protein concentration; in addition, cross-relaxation of protons is primarily exhibited in the longitudinal (T_1) rather than in the transverse (T_2) relaxation data. If cross-relaxation was significant, the T_2/T_1 ratio would be expected to increase with protein concentration. However, Figure 6 shows that the T_2/T_1 ratio for corn zeins in H_2O at pH 11.5 *decreases* with increasing protein concentration. This indicates that cross-relaxation does not make a major contribution to relaxation (at 10 MHz) in the corn protein concentration range from 0 to 20%. However, at higher protein concentrations chemical exchange and cross-relaxation may become significant, thereby increasing the $\bar{B}_0$ virial coefficient as explained in section 4.2. The gradual decrease in the T_2/T_1 ratio with increasing concentration in Figure 6 could be due to contributions from certain relatively slow water motions (or exchangeable protein protons), which would decrease T_2 more than T_1 (T_1's are insensitive to slow motions in the extreme narrowing limit whereas T_2's are shortened by these slow processes). The chemical exchange of protons is the more probable mechanism for this behavior. Recent results from multinuclear ^1H, ^2H, and ^{17}O NMR

relaxation data of several proteins indicated that chemical exchange contributes significantly to both ^1H and ^2H NMR relaxation rates (Kakalis and Baianu, 1988, 1989; Myers-Betts and Baianu, unpublished results).

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